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Matthias Richard

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Matthias Richard. Mechanisms of regulation involved in resistance to antibiotics in *Mycobacterium abscessus*. Life Sciences [q-bio]. Université de Montpellier, 2019. English. NNT : 2019SORUS640 . tel-04847846

HAL Id: tel-04847846

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Microbiologie

École doctorale CBS2

Unité de recherche : Pathogénie Mycobactérienne et Nouvelles Cibles Thérapeutiques

Mécanismes de régulation impliqués dans la résistance aux antibiotiques chez *Mycobacterium abscessus*

Présentée par Matthias RICHARD
Le 25 novembre 2019

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Remerciements

Je remercie grandement les membres de mon jury,

Le Pr Jean-Luc Mainardi et le Dr Franck Biet,

Pour avoir accepté d'évaluer mon manuscrit de thèse en tant que rapporteurs.

Le Pr Hélène Marchandin et le Dr Ruben Hartkoorn,

Pour avoir accepté d'évaluer mon travail de thèse en tant qu'examineurs.

Le Dr Stéphane Canaan,

De m'avoir fait l'honneur de présider mon jury de thèse.

J'adresse de chaleureux remerciements à toutes les personnes l'équipe de recherche parmi laquelle j'ai fait partie ces 3 ans et demi,

Le Dr Laurent Kremer mon directeur de thèse,

De m'avoir supervisé ces trois, en me faisant confiance et en me permettant de développer un esprit critique et scientifique, à qui j'adresse l'expression de ma profonde gratitude.

Le Dr Mickael Blaise,

De m'avoir enseigné toutes ces techniques de biochimie et de cristallogénèse

Le Dr Albertus Viljoen,

De m'avoir permis d'acquérir autant de connaissance en matière de biologie moléculaire

Mais également à nos collaborateurs,

Le Dr Stéphane Canaan et le Dr Jean-François Cavalier,

D'avoir grandement participer dans la publication de mon premier article en tant que premier auteur

Ainsi qu'aux membres de mon comité de suivi de thèse,

Le Dr Edouard Tuillon et le Dr Alain Baulard,

De m'avoir aiguiller et conseiller les deux premières années de ma thèse

Un grand merci également à tous mes collègues de l'IRIM, dont beaucoup sont devenus des amis par la suite,

De m'avoir fait passer autant de bon moment intra comme hors institut, scientifique ou non

Et bien sûr je remercie infiniment ma famille, mes parents et mes frères, mes amis, d'Ardèche mais aussi de Montpellier et bien plus loin encore, et mes proches, Ana Victoria, de m'avoir soutenu pendant cette aventure.

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Å : angström

AAC(2') : pour aminoglycoside 2'-acétyltransférase

ABC : ATP-binding cassette

ADN : acide désoxyribonucléique

AMK : amikacine

ARN : acide ribonucléique

ARNr : ARN ribosomique

ARNt : ARN de transfert

AT-Hook : domaine riche en adénosine et thymine de l'ADN cible

ATP : adénosine triphosphate

ATS : American Thoracic Society

AZM : azythromycine

BDQ : bédaquiline

BPCO : broncho-pneumopathies chroniques obstructives

CoA : coenzyme A

CLSI : Clinical & Laboratory Standards Institute

CLR : clarithromycine

CFTR : cystic fibrosis transmembrane conductance regulator

CFX : céfoxitine

CFZ : clofazimine

cMBT : carboxymycobactine

CMI : Concentration Minimal Inhibitrice

DAT : diacycle tréhalose

DNA_{op} : ADN opérateur / ADN cible

DTT : dithiothréitol

Eis : enhanced intracellular survival

EMB : éthambutol

Erm : erithromycine resistance methylase

ESAT6 : early secretory antigenic target of 6 kDa

ESX : ESAT6 secretion systems

ETH : éthionamide

ETZ : electron translucent zone

facteur σ : facteur sigma

FAD : flavine adénine dinucléotide

GC% : taux de guanine – cytosine

GDND : glycosyl diacylated nonadecyl diol

GPL : glycopeptidolipide

Gram - : Gram négative

Gram + : Gram positive

GW-rich : domaine riche en glycine – tryptophane

h- α : hélices- α

HTH : helix – turn – helix

IDSA : Infectious Diseases Society of America

IL : interleukine

IFN : interféron

INH : isoniazide

IPM : imipénème

KAN : kanamycine

Kb : mille paires de base

kDa : kilo dalton

LAM : lipoarabinomannane

LBP : ligand binding pocket

LM : lipomannane

LNZ : linézolide

LPS : lipopolysaccharide

MALDI-TOF : matrix-assisted laser desorption/ionisation – time of flight

MarR : multiple antibiotic resistance regulator

MATE : multidrug and toxic compound

Mb : un million de paires de bases

MBT : mycobactine

MEW : mycolic ester wax

MCL : mycobactérie à croissance lente

MCR : mycobactérie à croissance rapide

MDR-Tb : tuberculose multi résistante

MFX : moxifloxacine

MFP : membrane fusion-like protein

MMDAG : monomeromycolyl diacylglycerol

MmpL : mycobacterial membrane protein large

MmpS : mycobacterial membrane protein small

MNC : minocycline

MNT : mycobactérie non-tuberculeuse

MQ : ménaquinone

NAD : nicotinamide adénine dinucléotide

NAG : N-acétylglucosamine

NAM : N-acétylmuramique

NDH-2 : NADH oxydoréductase de type II

NGM : N-glycolylmuramique

OMF : outer-membrane factor

OMS : Organisation Mondiale de la Santé

PAT polyacyle tréhalose

pb : paire de base

PCR : polymerase chain reaction

PDIM : phthiocerol dimycocerosate

PHE : Public Health England

PIM : phosphatidyl-*myo*-inositol mannoside

Pks : polykétide synthase

PL : phospholipide

PMF : force proto-motrice

PMA : pays les moins avancés

qRT-PCR : quantitative real-time PCR

R : rough / rugueux

RGO : reflux gastro-œsophagien

RND : resistance nodulation cell division

ROS : reactive oxygen species

S : smooth / lisse

SARM : *Staphilococcus aureus* résistant à la méthicilline

SCFM : Synthetic Cistyc Fibrosis Sputum

SGL : sulfoglycolipide

SL : sulfolipide

SMR : small multidrug resistance

SNC : système nerveux central

SMART : small molecule aborting resistance

SPM : streptomycine

TAC : thiacétazone

TACa : analogue structuraux du TAC

TBM : tobramycine

TDM : tréhalose dimycolate

TDZ : tédizolide

TetR : tetracycline repressor

TGC : tigécycline

TLR : toll-like receptor

TM : trnsmembranaire

TMM : tréhalose monomycolate

TNF : tumor necrosis factor

TPP : tréhalose polyphléate

Whi : white mutants

WHO : World Health Organisation

ZF : zebrafish

Introduction

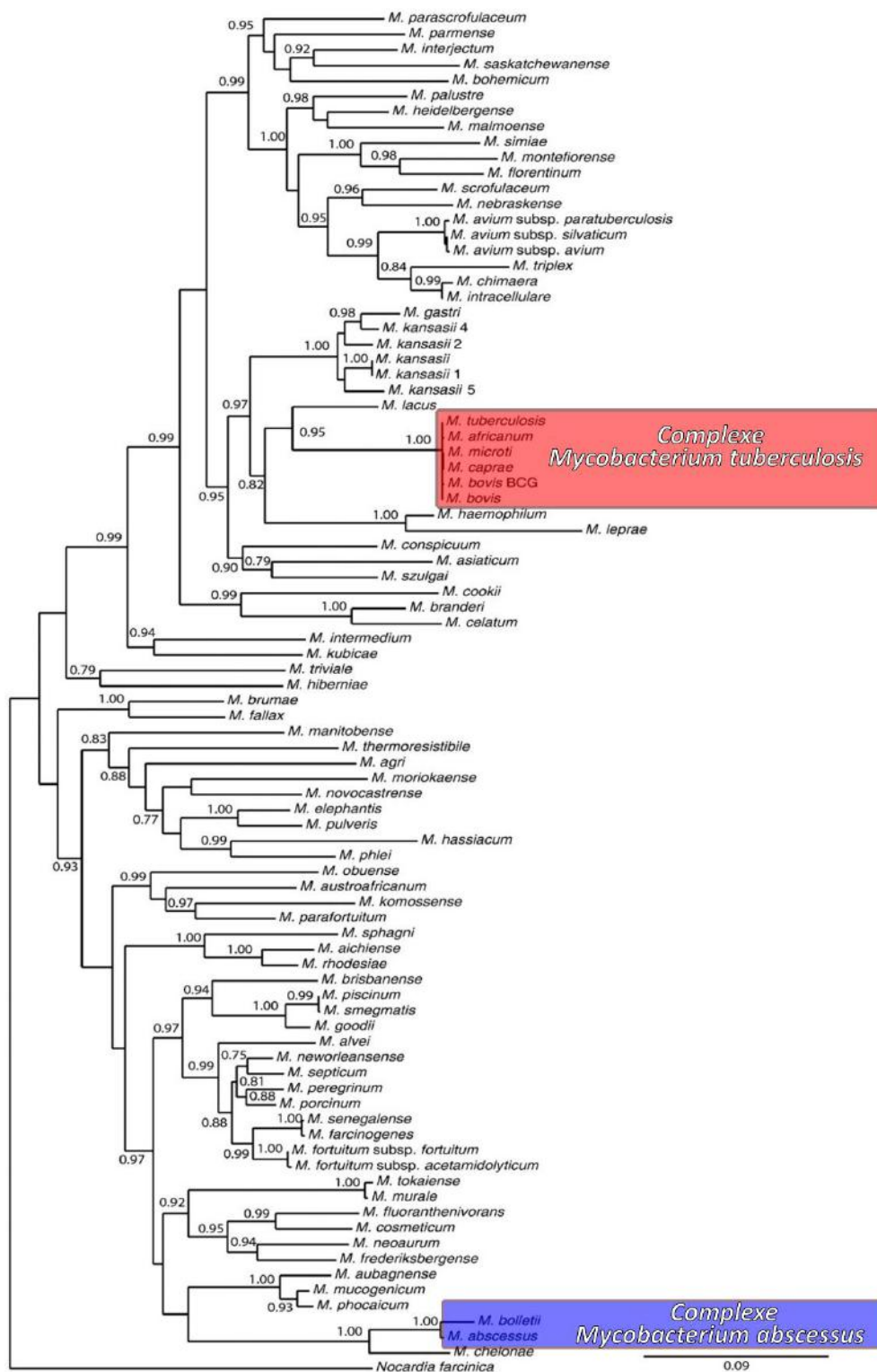


Figure 1. Arbre phylogénétique basé sur l'analyse de la séquence complète de 7 gènes (*ARN 16S*, *hsp65*, *rpoB*, *smpB*, *sodA*, *tmRNA* et *tuf*) sur 87 espèces de mycobactéries. *Nocardia farcinica* a été utilisé comme *outgroup*. La bar représente 0,09 substitution de nucléotide par site (Adapté de Mignard et Flandrois 2008).

1) Phylogénie et niches écologiques :

Les mycobactéries appartiennent au phylum des *actinobacteria*. Ces bactéries, pour la plupart des Gram positives (Gram +), sont des microorganismes saprophytiques que l'on retrouve dans l'eau et le sol sous forme solide ou en aérosol mais également chez toute une variété d'organismes supérieurs. La notoriété de ces organismes leur vient principalement du fait que certains d'entre eux sont de grands producteurs d'antibiotiques de différentes classes comme les macrolides, les aminoglycosides, les rifamycines, les tétracyclines, les β -lactamines et les glycopeptides (Genilloud 2017). Ces antibiotiques ainsi que leurs dérivés sont aujourd'hui largement utilisés en clinique. Les mycobactéries ne produisent aucune classe d'antibiotique et sont connues pour des raisons bien plus funestes, car étant notamment responsables de maladies comme la lèpre et la tuberculose. Elles appartiennent à la famille des *Mycobacteriaceae*, au sein du sous-ordre des *Corynebacterineae* et de l'ordre des *Actinomycetales*. La phylogénie mycobactérienne a évolué ces dernières années grâce à l'ajout de 6 gènes très conservés (*hsp65*, *rpoB*, *smpB*, *sodA*, *tmRNA* et *tuf*) au séquençage de l'ARNr 16S, qui était utilisé seul auparavant (Mignard et Flandrois 2008). Les techniques de séquençage à haut débits permettant une analyse complète du génome y ont aussi contribué. On dénombre à ce jour près de 200 espèces de mycobactéries (<http://www.bacterio.net/mycobacterium.html>) dont la grande majorité sont des bactéries environnementales non-pathogènes. On parle ici de Mycobactéries Non-Tuberculeuses (MNT) qui s'opposent aux bactéries appartenant au complexe de *Mycobacterium tuberculosis* (Figure 1). Cependant, certaines de ces MNT se révèlent capables d'infecter plusieurs espèces animales comme des poissons, des amphibiens, des oiseaux ainsi qu'une grande variété de mammifères incluant des animaux d'élevage (bovins, chèvres et moutons) et également l'homme (Medjahed et al. 2010) (Palmer et al. 2011). Cette grande variété de niches écologiques auxquelles ont été exposées ces bactéries se traduit par une forte capacité d'adaptation. Cette force est le reflet d'un large arsenal génétique pour à la fois compétitionner avec d'autres microorganismes (résistances aux antibiotiques, acquisition de nutriments) et manipuler leurs éventuels hôtes lors du processus infectieux (survie intramacrophagique, manipulation du système immunitaire et latence).

2) Caractéristiques microbiologiques et génétiques :

Une des grandes spécificités des mycobactéries est leur importante masse lipidique, près de 40 % du contenu cellulaire, que l'on retrouve principalement dans la paroi, qui renferme également

une grande variété de sucres et de protéines (Chiaradia et al. 2017). Les mycobactéries sont les seules à répondre à la coloration de Ziehl-Neelsen grâce à leurs propriétés acido-alcoolo-résistantes

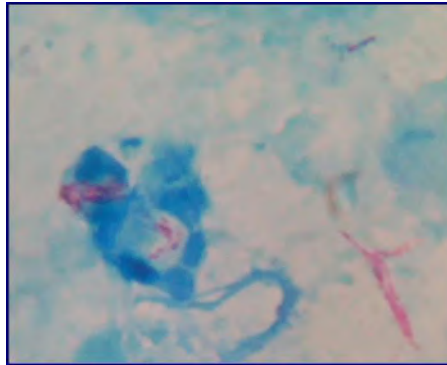


Figure 2. Coloration de mycobactéries par la technique de Ziehl-Neelsen. Les bacilles sont colorés en rose car leurs parois très hydrophobes ont retenu la fuschine phénylée. Les structures bleues peuvent être des polynucléaires ou des cellules épithéliales.

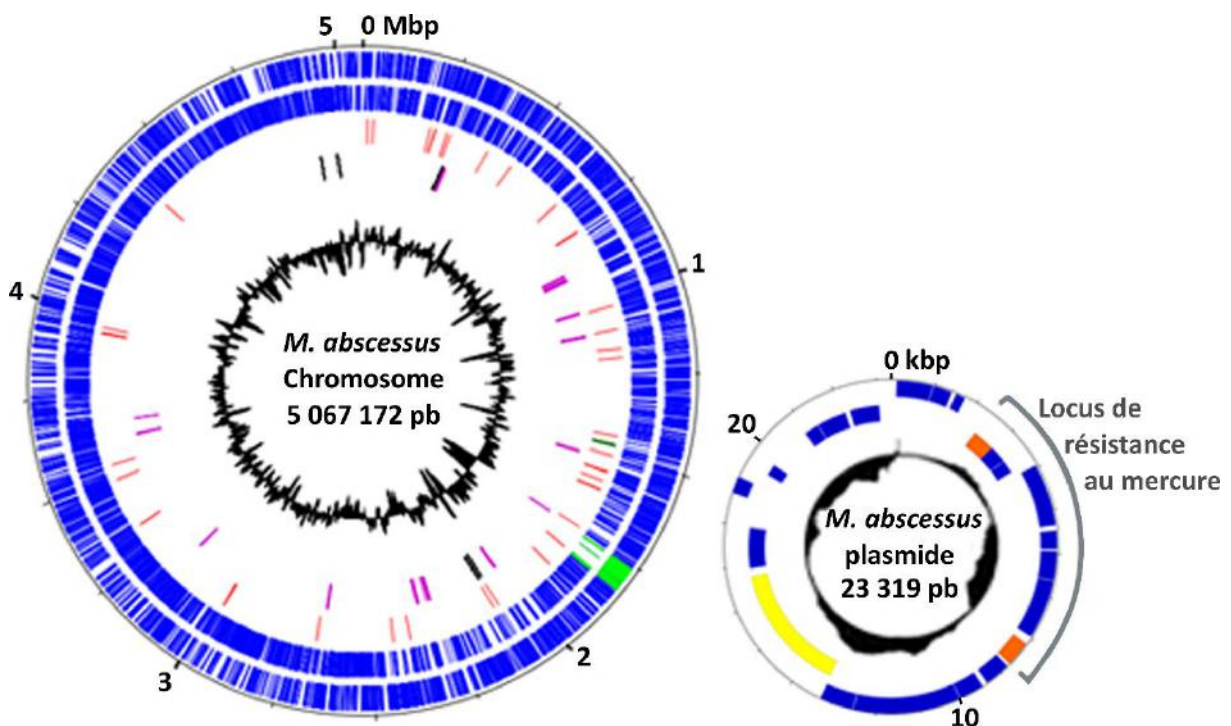


Figure 3. Cartes génétiques du chromosome et du plasmide circulaires de *M. abscessus* CIP104536^T. En ce qui concerne le chromosome, le cercle bleu le plus externe montre les gènes mycobactériens transcrits dans le sens de l'annotation génomique. Le cercle bleu sous-jacent montre les gènes mycobactériens transcrits en anti-sens. Les gènes phagiques sont en vert. En partant de l'extérieur, les troisième et quatrième cercles représentent les ARNt et les ARNr. Le plasmide est annoté de la même façon que le chromosome. Les parties orange et jaune codent pour des recombinaisons et des éléments

nécessaires à la conjugaison du plasmide. Les cercles noirs au centre représentent le GC%. (Adapté de Ripoll et al. 2009)

(Vilchère et Kremer 2017). Cette technique est basée sur la capacité des bactéries à retenir un colorant phéniqué, la fuschine, à la suite d'un lavage avec un mélange acido-alcoolique. La rétention de la fuschine est due à la grande hydrophobicité de la paroi des mycobactéries et leur donnera une couleur rose (Figure 2). Tout autre type de bactéries sera décoloré, étant donné le manque de glycolipides permettant de piéger ce colorant. Une étape supplémentaire consiste à ajouter du bleu de méthylène donnant l'aspect visuel caractéristique de cette coloration, bacilles rouges si l'échantillon est positif, sur fond bleu. Cette coloration s'effectue sur un frottis qui sera ensuite examiné au microscope. Ces mycobactéries sont des bacilles d'une taille comprise entre 1 et 4 µm de long pour un diamètre de 0,3 à 0,5 µm, non-flagellés et non-sporulants avec un métabolisme à aérobie strict (Cook et al. 2009). Parmi les actinomycètes, le genre *Mycobacterium* a le plus haut taux de Guanine et de Cytosines (GC%) au sein de leurs ADN. Egalement appelé coefficient de Chargaff, ce GC% serait synonyme de meilleure stabilité de d'ADN et des structures dérivant de l'ARN, comme les ARNr et les ARNt. L'environnement dans lequel phylum ont évolué est en majeure partie responsable de ce plus ou moins haut GC% (Foerstner et al. 2005). Leurs génomes sont sous formes d'un chromosome circulaire d'une taille comprise entre 3,27 millions de paires de base (Mb) pour *M. leprae* TN et 7,69 Mb pour *M. brisbanense* UM_WWY, appartenant au complexe de *M. fortuitum* (Wee, Dutta, et Choo 2017). La taille du génome est généralement signe de capacité d'adaptation. *M. leprae* a un génome extrêmement réduit qui reflète son mode de vie uniquement intracellulaire. Le génome de *M. abscessus* compte un peu plus de 5 Mb supplémentées par un plasmide de 23 000 paire de bases (Kb), avec un GC% de respectivement 64 et 68 % (Ripoll et al. 2009) (Figure 3). Cet élément génétique mobile, identique à 99,9% à celui de *M. marinum*, contient un locus de résistance au mercure encodant pour des gènes permettant de soit exporter activement les ion Hg^{2+} , soit de les réduire sous leur forme Hg qui diffuseront librement vers l'extérieur du cytoplasme (Barkay et al. 2003) (Schué et al. 2009). La haute conservation de séquence entre ces deux plasmides atteste de la cohabitation dans un environnement commun des deux espèces. Ce type de plasmide confère une capacité accrue à résister aux désinfectants et rend les bactéries plus susceptibles de provoquer des infections nosocomiales *via* la contamination de matériel chirurgical.

3) Les parois bactériennes :

Les procaryotes sont divisés en deux groupes distincts, en en se basant toujours sur la composition de leurs enveloppes. On utilise cette fois-ci encore une coloration réalisée sur frottis, appelée Coloration de Gram. Les étapes en elles-mêmes diffèrent de la coloration de Ziehl-Neelsen mais le principe en très similaire et repose toujours sur le niveau d'hydrophobicité de la paroi.

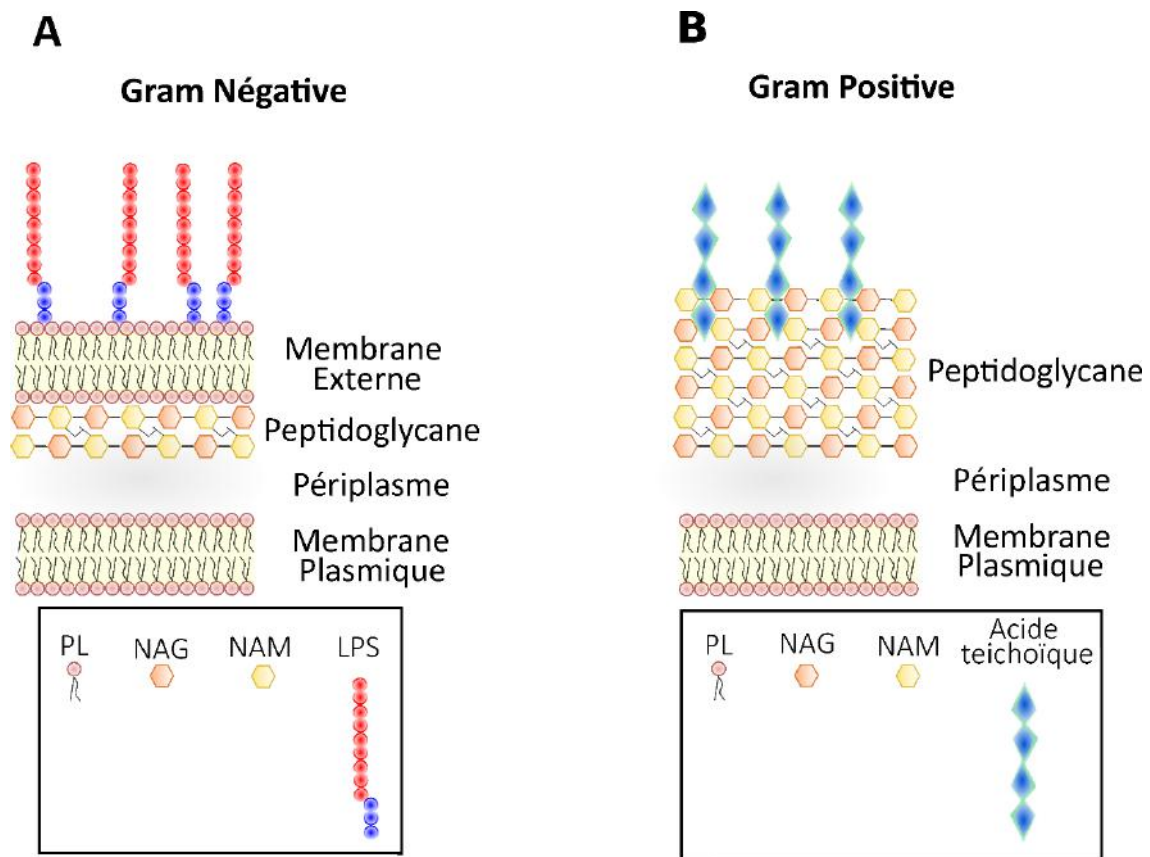


Figure 4. Représentation schématique des parois bactérienne des Gram – et des Gram +. (A) La paroi des bactéries Gram – comporte un fine couche de peptidoglycane entre la membrane plasmique classique et une membrane externe au sein de laquelle sont insérés différents facteurs de virulence comme le LPS. (B) Les bactéries Gram + possèdent uniquement une épaisse couche de peptidoglycane au-dessus de leur membrane plasmique, sont insérés dans ce peptidoglycane des facteurs de virulence comme les acides teichoïques. PL, phospholipide ; NAM, N-acétylmuramique ; NAG, N-acétylglucosamine ; LPS, lipopolysaccharides (Adapté de Gutiérrez et al. 2018).

3a) La paroi des Gram – :

Les bactéries à Gram –, qui apparaissent en roses, possèdent une fine couche de peptidoglycane, également appelée muréine. Il s'agit d'un polymère composé d'une partie glucidique comprenant une molécule d'acide N-acétylmuramique (NAM) rattachée par une liaison β 1 – 4 osidique à une molécule de N-acétylglucosamine (NAG). Cette unité glycosidique est liée à une chaîne de 4 acides aminés dextrogyres (D), une conformation unique aux bactéries, pour former une nouvelle unité glycopeptidique. Deux de ces unités sont liées entre elles *via* leurs chaînes peptidiques et forme l'unique couche de peptidoglycane située dans le périplasme entre la membrane plasmique et une membrane externe. On retrouve dans ce dernier feuillet de nombreuses molécules importantes pour la virulence comme les lipopolysaccharides (LPS) (Beveridge 1999) (Figure 4A).

3b) La paroi des Gram + :

Les bactéries à Gram +, violettes suite à la coloration, ne possèdent pas de membrane externe mais une très épaisse couche de peptidoglycane. La muréine est cette fois-ci constituée de plusieurs couches consécutives toujours liées entre elles par les mêmes liaisons peptidiques, faisant penser à une organisation en maille qui donne une forte rigidité à la paroi de ces bactéries. Cette partie de la paroi est également riches facteurs de virulence mais sont différents de ceux des Gram –, comme par exemple les acides teichoïques (Brown et al. 2013) (Figure 4B).

3c) La paroi mycobactérienne :

L'enveloppe mycobactérienne est unique en son genre de par sa complexité et sa composition excluant donc les mycobactéries des Gram – et +.

Insérés dans la partie extérieure de la bicouche phospholipidique formant la membrane plasmique se trouvent des glycolipides comme le Phosphatidyl-my^o-Inositol Mannoside (PIM) et ses dérivés, du LipoMannane (LM) et du LipoArabinoMannane (LAM).

Au-dessus se dresse un large périplasme suivi d'une couche de peptidoglycane liée de manière covalente à un polysaccharide appelé arabinogalactane, lui-même estérifié par de longues molécules d'acides mycoliques. Le LAM se retrouve jusque dans la couche de la paroi mycobactérienne.

Le peptidoglycane mycobactérien se démarque de celui retrouvé chez les autres bactéries car il ne contient pas uniquement du NAG et du NAM mais aussi de l'acide N-glycolylmuramique (NGM) (Mahapatra et al. 2005). Les unités glycosidiques sont néanmoins toujours reliées par des liaisons β 1 – 4 osidiques. La présence de N-glycolylation dans le peptidoglycane mycobactérien pourrait en réalité le renforcer et le rendre encore plus résistant que celui des Gram + (Alderwick et al. 2015).

M. abscessus

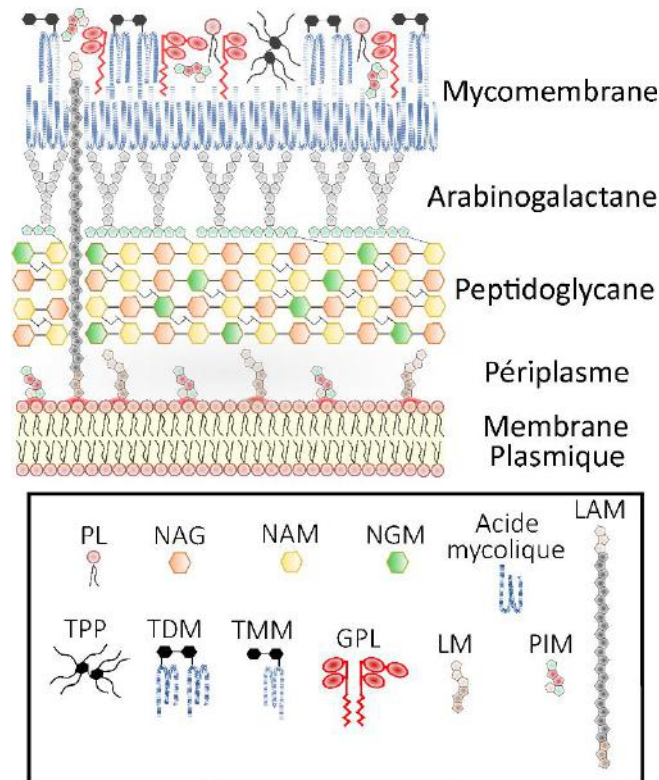


Figure 5. Organisation schématique de la paroi mycobactérienne de *M. abscessus*. On retrouve attaché à la membrane plasmique dans le périplasmique du LM, du PIM et du LAM. Le LAM se retrouvera jusque dans la mycomembrane. Le peptidoglycane est composé de NAG, NAM et de NGM. Le TMM, le TDM, le TPP, les GPL mais également du LIM et du PIM se retrouve insérés entre des molécules d'acides mycolique dans le feuillet externe de la mycomembrane. PL, phospholipide ; NAM, N-acétylmuramique ; NAG, N-acétylglucosamine ; NGM, N-glycolylmuramique ; LM, lipomannane ; LAM, lipoarabinomannane ; PIM, phosphatidyl-*myo*-inositol mannoside ; TPP, tréhalose polyphléate ; TMM, tréhalose monomycolate ; TDM, tréhalose dimycolate ; GPL, glycopeptidolipide (Adapté de Gutiérrez et al. 2018).

L'arabinogalactane est donc un polysaccharide constitué d'arabinose et de galactose. Plus précisément, la partie galactane est une chaîne de 30 molécules de D-galactofuranosyles liée covalamment au peptidoglycane *via* une molécule de L-rhamnose. Cette chaîne galactane est reliée de manière covalente à 3 domaines tricosamériques chacun composés de 23 molécules de D-arabinofuranosyles (Alderwick et al. 2015).

Les acides mycoliques, uniques aux mycobactéries, sont de longues molécules pouvant compter jusqu'à 90 carbones chez *M. tuberculosis*. Ils estérifient entre autre la partie terminale de l'arabinogalactane. On retrouve 3 types d'acides mycoliques, les alphas, méthoxys et ketos-acides mycoliques au sein desquels sont intercalés une immense variété de lipides et glycolipides complexes ayant un rôle structural et participant à la virulence des bacilles. Cet ensemble de composés exotiques forme le feuillet interne d'une structure plus exposée au milieu extérieur appelée mycomembrane. Du tréhalose peut être greffé à ces acides mycoliques par les membres du complexe Antigène 85 et former du Tréhalose MonoMycolate (TMM) et du Tréhalose DiMycolate (TDM) également appelé « *cord factor* ». Le TDM est le glycolipide le plus abondant de la mycomembrane. A la fois très immunogène, il participe aussi dans la survie des bactéries dans l'hôte infecté (Thanna et Sucheck 2016). D'autres molécules sont également présentes comme du DiAcyle Tréhalose (DAT), du PolyAcyle Tréhalose (PAT), des Sulfo/SulfoGlycoLipides (SL/SGL) et Phthiocerol DIMycocerosate (PDIM) (Abrahams et Besra 2018). On y retrouve du PIM mais aussi du LAM qui joueront ici un important rôle dans l'inactivation des macrophages et dans la neutralisation des composés réactifs de l'oxygène (ROS pour Reactive Oxygen Species) (Mishra et al. 2011). Tous ces lipides, glycolipides et dérivés forment le feuillet externe de la mycomembrane chez *M. tuberculosis*. *M. abscessus* ne possède pas de DAT, PAT et SL mais un autre glycolipide, le Tréhalose PolyPhléates (TPP). *M. abscessus*, comme également *M. smegmatis* et *M. avium*, peut posséder des GlycoPeptidoLipides (GPL) impliqués aussi dans la virulence (Figure 5).

Une capsule peut être retrouvée au-dessus de la mycomembrane chez certaines mycobactéries pathogènes, notamment chez *M. tuberculosis* et *M. marinum* (Daffé et Marrakchi 2019). Elle est composée d'un mélange de protéines ainsi que de mannane, glucane et d'arabinomane qui sont respectivement des polymères de mannose, glucose et d'arabinose/mannose.

Cet ensemble varié de molécules complexes font de la paroi mycobactérienne une structure extrêmement hydrophobe et robuste qui représente une première barrière physique à de nombreux antibiotiques, notamment ceux de nature hydrophile qui ne peuvent la pénétrer que s'il y a un transport actif vers l'intérieur du cytoplasme. Cette structure offre également aux mycobactéries la capacité de résister à de forte température et variation de pH.

4) Classification de Runyon :

Dès 1959, une observation visuelle et temporelle a permis à Ernest Runyon de proposer une classification faisant la séparation entre deux grands types de mycobactéries, celles à croissance rapide (MCR) et celles à croissance lente (MCL). Les MCR forment des colonies sur milieu solides en moins de 7 jours correspondant à un temps de génération ; i.e. période pendant laquelle un bacille donne naissance à deux nouvelles bactéries à la suite d'une répliation par fission binaire ; inférieur à 6 heures. Les MCL ont un temps de génération supérieur à 6 heures et mettent donc plus de 7 jours pour former des colonies. Il a ensuite discriminé au sein des MCL trois sous-catégories :

- Les non-chromogènes dont les colonies ne sont pas pigmentées,
- Les scotochromogènes qui développent une pigmentation dans l'obscurité,
- Les photochromogènes qui ont besoin d'une source lumineuse pour devenir pigmentées.

Cette classification a été par la suite appuyée par les nouvelles techniques de génétiques et de séquençages.

5) Mycobactéries à croissance lente :

Les MCL sont généralement des pathogènes dont le plus connu est *M. tuberculosis*. Cette mycobactérie également appelé Bacille de Koch, d'après sa découverte par Robert Koch en 1882, est l'agent étiologique de la tuberculose. Cette maladie, dont les preuves moléculaires les plus anciennes ont été rapportées jusqu'en 600 avant JC (Donoghue et al. 2010), est encore un problème de santé publique majeur à l'échelle mondiale avec près de 10 millions de nouveaux cas annuels et presque 2 millions de décès (WHO 2018). Plus de 250 000 de ces décès sont dus à des souches multi résistantes (MDR-Tb) aux traitements disponibles, incitant au développement de nouveaux antibiotiques et stratégies thérapeutiques (WHO 2017). Cette bactérie provoque généralement une pathologie pulmonaire mais des formes osseuses et des méningites peuvent se produire. D'autres MCL provoquant des maladies pulmonaires existent comme les bactéries du complexe *M. avium* mais elles sont retrouvées généralement chez des personnes avec des problèmes pulmonaires préexistants ou un système immunitaire affaibli (Daley 2017). Certaines de ces MCL provoquent des types d'infection plus variés, notamment dans les régions tropicales d'Afrique, comme *M. ulcerans* responsable de l'ulcère de Buruli qui se caractérise par une importante nécrose d'une plaie infectée (van der Werf et al. 2005).

6) Mycobactéries à croissance rapide :

Les MCR sont des bactéries environnementales qui peuvent parfois se transformer en pathogènes opportunistes si les facteurs environnementaux appropriés sont réunis. Un de ces facteurs est le statut immunitaire/pathologique du patient (Cowman et al. 2019). Mais un prérequis à la mise

en place de l'infection est bien sûr un contact physique avec ces microorganismes. Il se trouve que ces contacts sont en réalité très fréquents car on retrouve des MCR dans les systèmes de plomberies domestiques (Falkinham 2011) ainsi que dans les jardins ou même la terre de rempotage (Thomson et al. 2013) (De Groot et al. 2006). Des études récentes ont d'ailleurs mis en évidence une transmission possible de patient à patient, particulièrement dans les infrastructures de santé (Bryant et al. 2016). Ces MCR cibleront préférentiellement l'appareil pulmonaire mais sont capables d'infecter n'importe quelle partie du corps. Les MCR pathogènes les plus souvent isolées sont *M. fortuitum*, *M. kansasii* ainsi que le complexe *M. abscessus*.

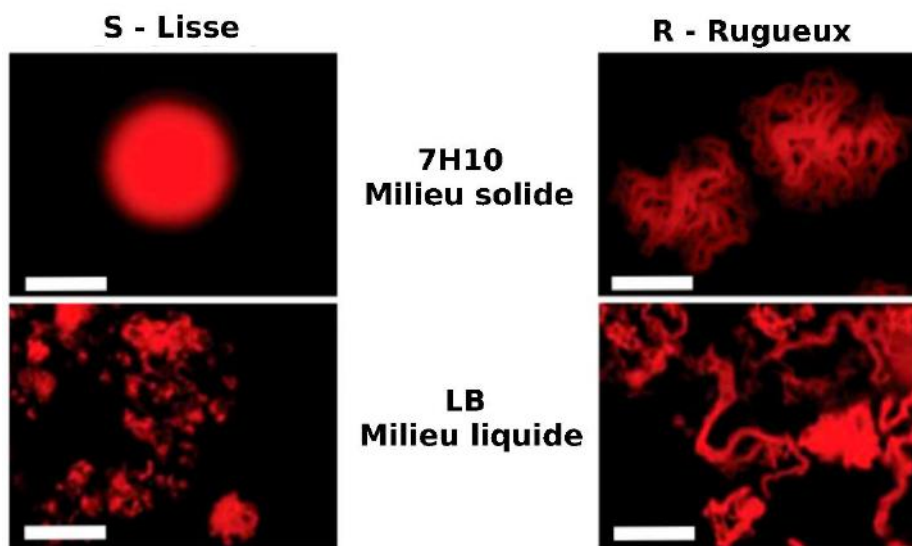


Figure 6. Les différents morphotypes de *M. abscessus* en milieu liquide et en milieu solide. Le morphotype S (colonne de gauche) forme des colonies lisses et rondes sur 7H10 et des petits amas répartis de manière homogène en culture liquide. Le morphotype R (colonne de droite) s'organise en chaînettes sur la périphérie de ses colonies très déshydratées sur 7H10 et forme des agrégats volumineux en culture liquide. L'échelle est ici de 1 mm (Adapté de Bernut et al. 2014).

Chapitre II) Mycobacterium abscessus, généralités

1) Histoire et présentation du complexe :

Isolé pour la toute première fois en 1953 chez une patiente présentant un abcès au genou, *M. abscessus* a d'abord été rattaché à *M. chelonae* formant un groupe au sein du complexe *M. fortuitum* jusqu'en 1970. Il a ensuite fallu attendre 1992 pour que *M. abscessus* soit séparé de *M. chelonae* et qu'il soit considéré comme une espèce bactérienne à part entière. A l'heure actuelle, le complexe *M. abscessus* contient trois sous-espèces, *M. abscessus abscessus*, *M. abscessus massiliense* et *M. abscessus bolletii* (Tortoli et al. 2016) (Adekambi et al. 2017). Cette séparation taxonomique devrait impérativement être prise en compte lors du choix de l'antibiothérapie car ces espèces répondent différemment aux antibiotiques constituant la thérapie (Benwill et Wallace 2014).

2) Les différents morphotypes et leurs répercussions :

M. abscessus présente deux morphotypes qui peuvent être différenciés visuellement sur la simple observation de l'aspect des colonies sur milieu solide. Cette simple différence morphologique a un impact fort sur leur physiologie et physiopathologie respective. Il est donc possible de se retrouver face à des colonies rondes dites « Smooth » (S) ayant un aspect lisse et crémeux. Les autres colonies observables, « Rough » (R), sont très rugueuses, semblent être déshydratées et sont organisées en structures serpentines sur leur périphérie. En milieu liquide, le variant S est présent sous la forme de suspensions relativement homogènes quand on le compare au variant R qui forme de gros agrégats (Bernut et al. 2014) (Figure 6).

La capacité à former des biofilms confère aux bactéries un énorme avantage en termes de survie, transmission et pathogénicité. Un biofilm est défini par l'agrégation de cellules planctoniques dans une matrice auto-sécrétée sur une surface solide. Ce comportement est temporaire et pourrait s'apparenter à un style de vie multicellulaire en réponse à différents signaux environnementaux. Ce biofilm procure aux bactéries la capacité de coloniser des niches écologiques variées. Ce phénomène peut également favoriser la persistance et la transmission à un nouvel hôte, notamment pour les bactéries pathogènes retrouvées dans les conduits d'aération, les robinets, ou même les instruments chirurgicaux (Bryant et al. 2016). La formation de biofilms peut également se produire au sein d'un hôte infecté et confèrera aux bactéries la capacité de contourner les défenses immunitaires (Baker et al. 2017) (Fennelly et al. 2016). Le morphotype R était considéré comme incapable de former des biofilms, mais Clary et al. ont récemment fourni de nouvelles données permettant de réfuter en partie cette observation. En effet, il semblerait que la capacité d'agrégation du variant R donne au biofilm

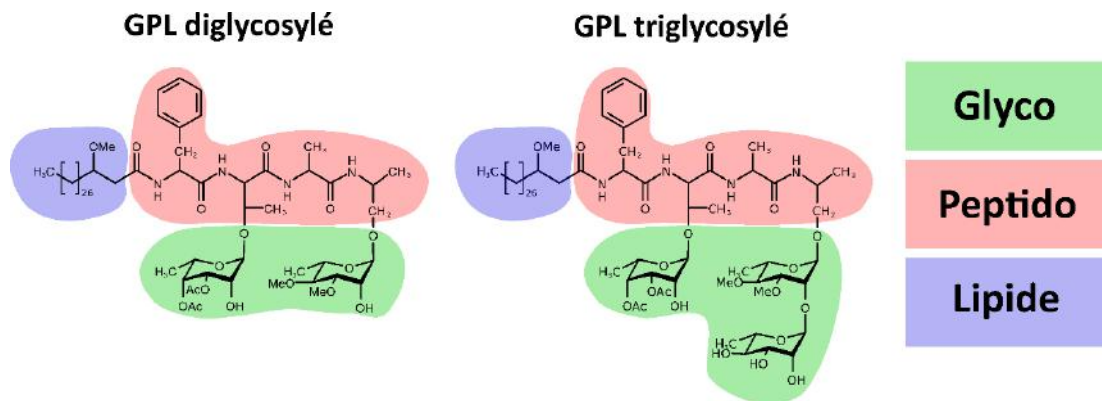


Figure 7. Structures des différents GPL produits par *M. abscessus*. Le GPL triglycosylé est plus soluble que sa version diglycosylé (Adapté de Gutiérrez et al. 2018).

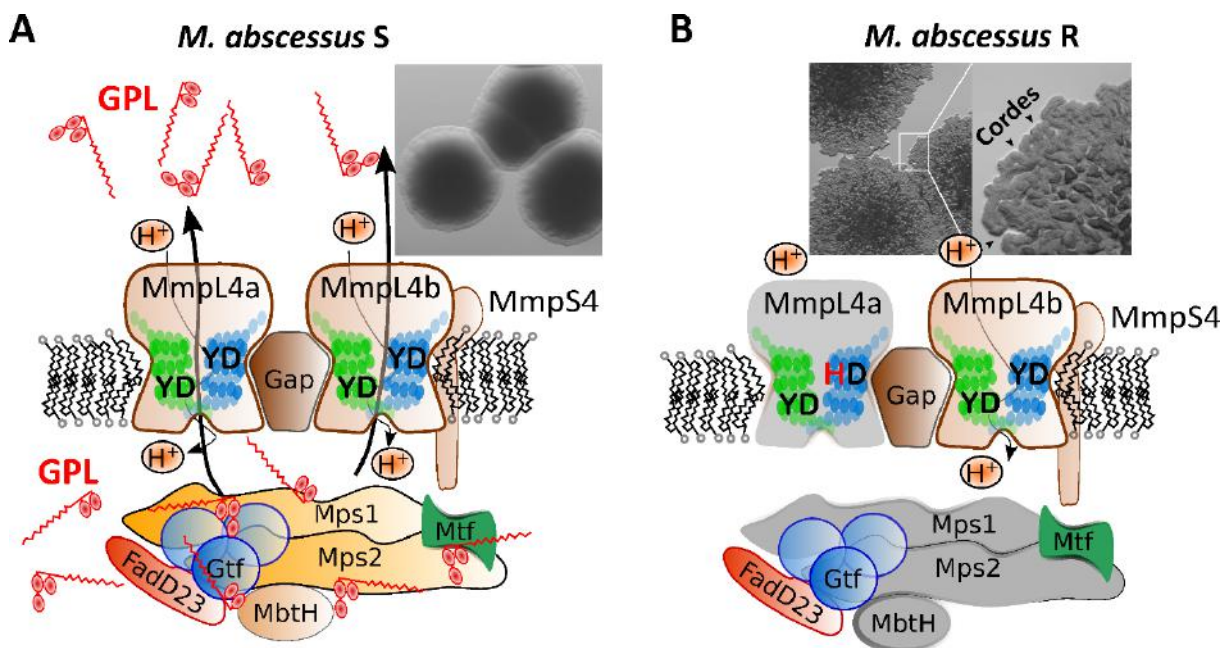


Figure 8. Les GPL influencent le morphotype de *M. abscessus*. Représentation schématique de la voie de biosynthèse et du système de transport des GPL chez le morphotype S (A) et chez le morphotype R (B). Les gènes retrouvés mutés chez le variant R sont en gris. Le résidu tyrosine 842 substitué par une histidine de *mmpL4a* chez *M. abscessus bolletii* est ici représenté en rouge. Cette mutation mène à un arrêt de la force proton motrice et au transport des GPL ayant pour conséquence la transition vers le morphotype R (Adapté de Gutiérrez et al. 2018).

un aspect plus cireux que le variant S, mais les deux morphotypes semblent capables de s'organiser en biofilm de même taille et de même densité.

En revanche, le morphotype S est lui considéré comme mobile car il peut glisser (*sliding motility*) vers les bords d'une boîte de pétrie, alors que le morphotype R en est incapable (Howard et al. 2006). Ces phénomènes d'agrégation et de mobilité seraient en grande partie dus à la présence chez le variant S, et à son absence chez le variant R, de GPL présents dans le feuillet externe de la mycomembrane (Recht et al. 2000). Comme son nom l'indique, cette molécule comprend une partie lipidique, composée d'une mixture d'acide gras de chaîne carbonée allant de 28 à 30 atomes, qui est reliée à un noyau peptidique soit diglycosilé soit triglycosilé, ce dernier exhibant une plus forte polarité (Gutiérrez et al. 2018) (Figure 7). Le morphotype R ne possède pas de GPL car on retrouve dans son génome des mutations dans le locus de biosynthèse de ces molécules (Pawlik et al. 2013) et dans le complexe transmembranaire impliqué dans leur export (Bernut, Viljoen, et al. 2016) (Figure 8).

Ces GPL sont responsables de l'aspect lisse du morphotype S. Leur absence chez le variant R provoque la formation de « cordes », qui sont en réalité les structures serpentes observées en périphérie des colonies sur milieu solide et qui affectent fortement la virulence de ce morphotype (Howard et al. 2006) (Bernut et al. 2014) (Halloum et al. 2016). Ce phénomène de « *cording* » chez *M. abscessus* est également dû à une autre molécule, le TPP (Llorens-Fons et al. 2017). Le *cording* est défini comme une agrégation tridimensionnelle latérale ou au niveau des pôles des bacilles incapables de se séparer après réplication. Ce trait microbiologique confère au morphotype R une hypervirulence retrouvée majoritairement chez les patients souffrant d'infections pulmonaires aiguës (Catherinot et al. 2009) (I. K. Park et al. 2015). A l'opposé, le morphotype S serait plus une forme environnementale colonisante retrouvée dans des plaies infectées après contact direct (Jönsson et al. 2007).

3) Physiopathologie, infection du macrophage par le morphotype S :

Les GPL influencent fortement la réponse phagocytaire des macrophages à *M. abscessus*. Le morphotype S est phagocyté individuellement et les GPL vont permettre de retarder l'importante réponse pro-inflammatoire en empêchant notamment la production initiale d'InterLeukine-8 (IL), (Davidson et al. 2011). Les GPL recouvrent les agonistes des Toll-Like Receptor-2 (TLR) présents à la surface des bacilles et empêchent toutes interactions avec les récepteurs membranaires de l'inflammation (Rhoades et al. 2009). Ceci va limiter le recrutement de nouveaux macrophages et la

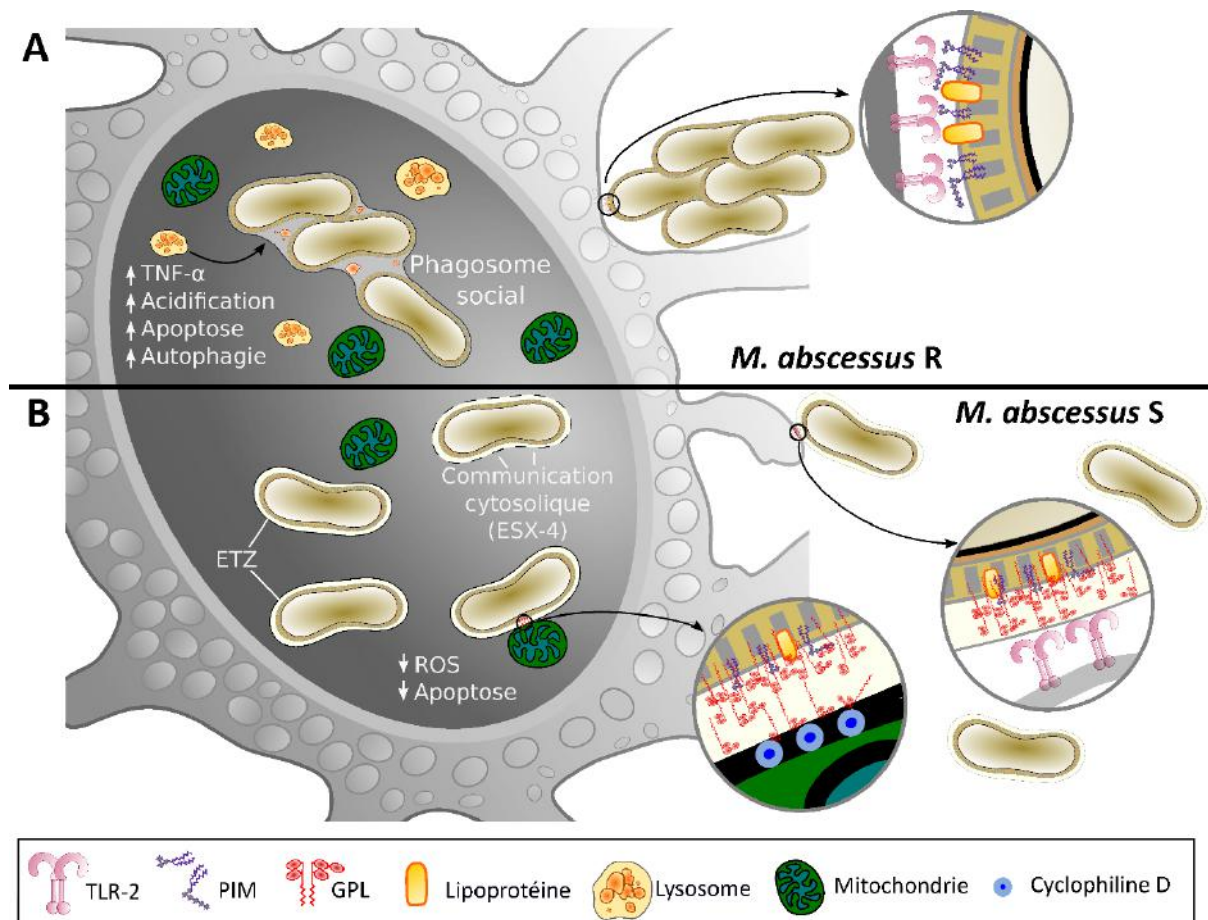


Figure 9. Infection du macrophage par le morphotype R et S. (A) Chez le variant R, l'exposition des agonistes du TLR-2 comme le PIM₂ et des lipoprotéines vont provoquer une forte réponse inflammatoire menant à une infection sévère due une surproduction de ROS et à l'augmentation de l'apoptose et de l'autophagie. (B) Les GPL présents chez le variant S vont recouvrir les agonistes de TLR-2 et vont permettre une communication cellulaire avec le macrophage résultant en un contrôle de l'infection. TLR-2, toll-like receptor 2 ; PIM, phosphatidyl-*myo*-inositol mannoside ; TNF- α , tumor necrosis factor alpha ; ETZ, electron translucent zone ; ROS, reactive oxygen species (Adapté de Gutiérrez et al. 2018).

surproduction d'autres cytokines pro-inflammatoires qui conduiraient à une réponse exacerbée puis à une mort cellulaire. Une fois internalisés, les GPL vont de nouveau former une barrière physique mais cette fois-ci entre la membrane du phagosome et la paroi mycobactérienne. Ce phénomène est visible par microscopie électronique et est caractérisé par une zone non-colorée par les électrons appelée « Electron Translucent Zone » (ETZ) (Roux et al. 2016). Les GPL permettent également de bloquer la fusion avec les lysosomes ainsi que la production de ROS ce qui permet de contrôler l'infection. Les GPL participeront à l'inhibition de l'apoptose en interagissant à travers la membrane phagosomale avec la cyclophiline D de la mitochondrie (Whang et al. 2017). Le bacille va ensuite continuer de moduler la réponse macrophagique en bloquant l'acidification du phagosome grâce à l'établissement d'une communication avec le cytoplasme médiée par le système de sécrétion de type VII ESX-4 (Laencina et al. 2018). Cet ensemble de mécanismes va permettre au variant S d'établir une niche de réplication sécurisée et stable au court du temps (Figure 9A). Mais une fois la charge bactérienne intracellulaire trop importante, les macrophages entrent en apoptose, libérant les bacilles dans le milieu extracellulaire qui seront de nouveau phagocytés et qui entameront alors un nouveau cycle infectieux.

4) Physiopathologie, infection du macrophage par le morphotype R :

Les cordes mycobactériennes exhibées par le variant R sont un véritable défi pour les cellules phagocytaires (Figure 9B). Ces structures sont parfois longues de plus d'une centaine de micromètres et excèdent largement la taille des macrophages qui ne peuvent donc pas phagocyter ces bactéries. Chez le variant R dépourvu de GPL, les agonistes du TLR-2 comme le PIM et certaines lipoprotéines ne sont plus masqués et déclenchent une forte réponse inflammatoire (Roux et al. 2011). Néanmoins, il arrive que de plus petits agrégats bacillaires ne se forment et soient phagocytés, donnant naissance à un phagosome « social » (Roux et al. 2016). Ces bacilles vont être incapables d'empêcher la fusion phagolysosomale et donc son acidification. Il n'y aura également pas d'inhibition de l'autophagie et de l'apoptose comme c'est le cas pour le variant S augmentant ainsi la lyse des macrophages (Whang et al. 2017). Il se produit en parallèle une production exacerbée de cytokines pro-inflammatoires comme le Tumoral Necrosis Factor α (TNF- α), qui se traduira par une activation des autres macrophages à proximité et une escalade de la production de ces molécules qui reflètera le caractère hypervirulent du variant R de *M. abscessus* (Figure 9B).

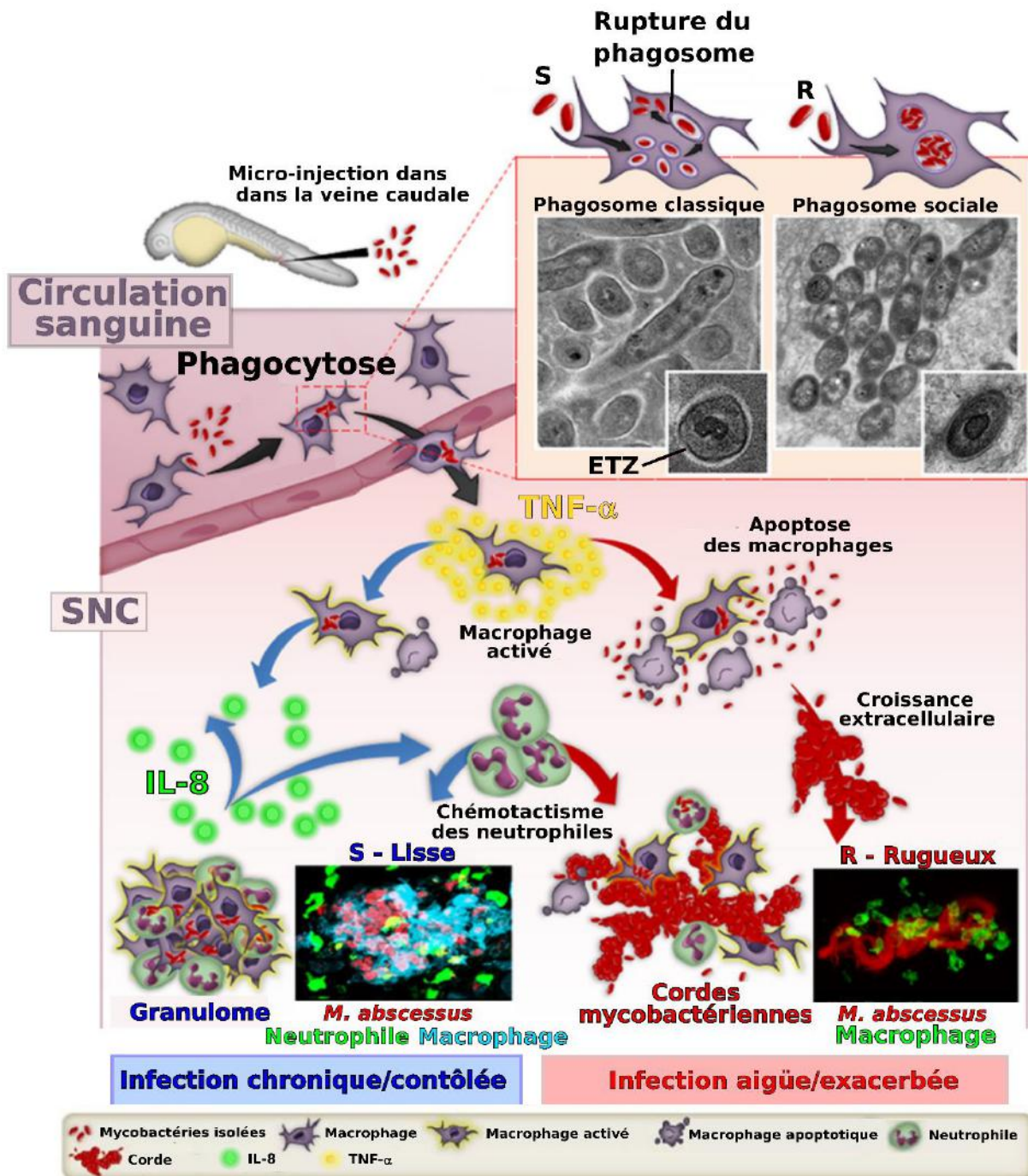


Figure 10. Infection des morphotypes S et R chez le zebrafish. L'infection provoquée par le morphotype S (partie de gauche) est contenue au sein de structures immunologiques appelées granulome ce qui conduit à une infection chronique et contrôlée. Le morphotype R (partie de droite) va lyser les macrophages, se développer extracellulairement en produisant des cordes mycobactériennes et provoquer une infection aigüe conduisant à la mort du zebrafish. (Adapté de Bernut et al. 2017)

5) Physiopathologie, infection chez le poisson-zèbre :

Le *Dario rerio*, poisson-zèbre ou encore zebrafish (ZF) est un excellent modèle pour suivre en temps réel les interactions hôte-pathogène. L'embryon de ce poisson, endémique d'Inde, est un excellent outil pour disséquer les processus infectieux viral et bactérien dû au fait :

- qu'il possède une immunité innée proche de celle de l'homme avec des macrophages et des neutrophiles jusqu'à quatre semaines de développement (Torraca et al. 2014),
- qu'il est aisément modifiable génétiquement ce qui permet la génération de cellules immunitaires fluorescentes (Torraca et Mostowy 2018),
- que sa transparence optique permet l'utilisation de méthodes de microscopie non-invasives offrant la possibilité de suivre l'interaction entre des bactéries et cellules immunitaires exprimant différents fluorophores (Bernut et al. 2017).

Le ZF a été largement utilisé dans le cadre de l'étude de la physiopathologie de *M. abscessus* et a mis en exergue le même phénotype infectieux que chez le macrophage. Mais ce modèle a également permis de découvrir chez les deux morphotypes un tropisme neuronal (Bernut et al. 2014). Après micro-injection de *M. abscessus* dans le système circulatoire *via* la veine caudale, les bacilles vont être phagocytés par des macrophages circulants. Ces cellules immunitaires font offices de cheval de Troie et fournissent aux bactéries un moyen de transport à travers la barrière hémato-encéphalique du Système Nerveux Central (SNC) et se retrouver dans le cerveau et la moelle épinière.

5a) Le morphotype S, infection chronique chez le ZF :

L'infection des macrophages du ZF par le variant S se déroule de la même manière que lors d'une infection *in vitro* (« **Chapitre II) *Mycobacterium abscessus*, généralités, 3) Physiopathologie, infection du macrophage par le morphotype S** »). La différence est qu'ici on retrouve d'autres types cellulaires présents chez le ZF, comme les neutrophiles. Les cycles infectieux ; i.e. phagocytose – apoptose – phagocytose ; vont se produire dans le SNC ou potentiellement toujours au site d'injection. Le relargage du contenu cellulaire et des cytokines du macrophage après son apoptose va provoquer l'activation d'autres macrophages à proximité qui produiront à leurs tours du TNF- α mais aussi désormais de l'IL-8. Les neutrophiles vont être recrutés grâce à cette IL-8 et s'agglutiner aux macrophages. Cette structure immunologique pluricellulaire a pour but de d'emprisonner les bacilles extracellulaires et les phagocytes infectés afin de limiter la dissémination de l'infection. Ces édifices sont appelés granulomes et permettent de contrôler l'infection (Figure 10, partie de gauche). Les phagocytes au centre du granulome vont rentrer en apoptose ce qui permet la création d'un compartiment hermétique empêchant la propagation des bactéries vers le milieu extérieur. On assistera ici à une infection contrôlée, qualifiée de chronique (Bernut, Nguyen-Chi, et al. 2016).

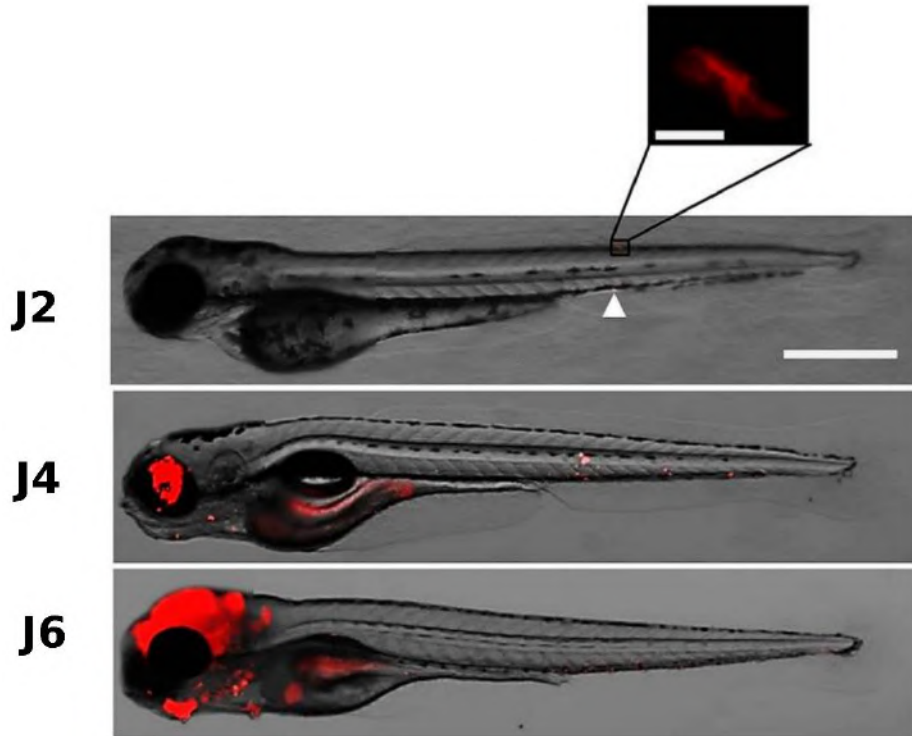


Figure 11. Formation d’abcès dans le système nerveux central d’un embryon de zebrafish. Suivi spatiotemporelle d’une infection non-traitée par le morphotype R de *M. abscessus* montrant l’évolution 2, 4 et 6 jours après injection (J2, J4 et J6 respectivement). A J2, le triangle blanc pointe la présence de petit agrégats de bacille et l’encadrer montre la genèse de cordes mycobactérienne. La barre d’erreur représente 200 μm (Adapté de Dupont et al. 2017).

5b) Le morphotype R, infection aigüe chez le ZF :

Avec le morphotype R, les premiers macrophages infectés entrent rapidement et massivement en apoptose. Les neutrophiles et macrophages établissent de nombreux granulomes dans le SNC et à travers l'ensemble de l'embryon. Cependant les bacilles se répliquent trop rapidement et de manière anarchique dans le milieu extracellulaire formant alors des cordes démesurées empêchant de nouvelles phagocytoses (Bernut et al. 2014) (Figure 10, partie de droite). Ceci a pour conséquence un orage cytokinique qui mènera une dégradation des tissus proches et à la formation d'abcès (Figure 11). Ces événements sont caractérisés comme étant la phase aigüe de l'infection qui entraînera la mort de l'embryon de ZF.

6) Symptômes et épidémiologie des infections extrapulmonaires à *M. abscessus* :

M. abscessus est une des principales MNT à provoquer des infections extrapulmonaires qui se caractériseront par des atteintes cutanées et des tissus mous (Lee et al. 2015). Elles provoquent généralement des nodules pourpres, des abcès et des lésions ulcéraives, qui dans 45% des cas surviennent après un acte opératoire (Chadha et al. 1998).

La contamination de matériels chirurgicaux invasifs, comme les aiguilles, représentent le principal accès à travers la barrière cutanée. La réalisation de tatouage (Bechara et al. 2010) ou les injections de silicone sont également mises en causes (Fox et al. 2004). La localisation de ces infections dépend aussi de la nature des opérations et des instruments contaminés. Des cas à *M. abscessus* ont été répertoriés après des transplantation cardiaques (Richey et al 2013) ou des poses de prothèses (Eid et al. 2007).

Mais le matériel chirurgical n'est pas toujours mis en cause. Plusieurs épidémies ont été provoquées l'injection de solutions contaminées. L'exemple le plus frappant s'est produit en Colombie entre 1992 et 1993, où parmi 2000 personnes injectées avec un anesthésique locale, la lidocaïne, 205 ont développé des abcès cutanés ou des cellulites dus à *M. abscessus* (Villanueva et al. 1997).

Bien qu'étant plus rares, des infections disséminées à *M. abscessus* peuvent se produire chez des personnes souffrant de maladies génétiques immunosuppressives, chez des personnes âgées ou sous traitement immunosupresseurs comme lors d'une chimiothérapie. Les personnes avec une grande partie de l'épiderme endommagée, comme les grands brûlés, sont également sujet à ce genre d'infection nosocomiales (Vaghaiwalla et al. 2014). Dans certains cas, *M. abscessus* est capable d'avoir le même tropisme que chez le zebrafish et va provoquer des infections du SNC s'il parvient à traverser la barrière hémato-encéphalique (Lee et al. 2012) (Giovannenze et al. 2018).

7) Symptômes et épidémiologies des infections pulmonaires à *M. abscessus* :

Comme pour les infections de la peau et des tissus mous, des cas d'infections pulmonaires à *M. abscessus* sont répertoriés sur tous les continents même si l'incidence varie d'une région à une autre. Par exemple, la proportion d'infection à *M. abscessus* parmi les MNT est de seulement 13,3 % en Chine, contre 45, 52 et 56 % aux Etats-Unis, en France et au Royaume-Uni respectivement (Mougari et al. 2016). La plupart des infections pulmonaires à *M. abscessus* sont d'ordres chroniques et caractérisées par une toux récurrente et persistante, une dyspnée, une hémoptysie, une production excessive de mucus, le tout accompagné de fièvre et de perte de poids. La maladie peut ensuite progresser sous la forme de bronchectasie nodulaire ou la forme fibro-cavitaire, qui est le pronostique le plus sévère. En l'absence de traitement, cette dernière forme peut provoquer un déclin des fonctions respiratoires dû à la formation de larges cavités et d'une fibrose qui réduira irrémédiablement le tissu pulmonaire (National Organisation for Rare Disorders, 2015 - 2018). Environ un tiers des personnes souffrant d'infections pulmonaires à *M. abscessus* sont *a priori* en bonne santé (Griffith et al. 1993), sans pathologie pulmonaire sous-jacente, mais il existe plusieurs maladies chroniques qui sont menacées par une surinfection par cette MNT.

8) Facteurs de risques, les troubles pulmonaires chroniques d'origines environnementales :

Les troubles pulmonaires chroniques d'origines environnementales augmentant le risque d'infection à *M. abscessus* sont variés et provoquent tous une inflammation du système respiratoire. Ces facteurs déclenchants incluent par exemple :

- les pneumoconioses, qui sont des inflammations des poumons pouvant mener à une fibrose suite à l'inhalation de particules minérale comme la silice, l'amiante ou le charbon (American Lung Association, 2018).
- les Broncho-Pneumopathies Chroniques Obstructives (BPCO) regroupant deux pathologies, la bronchite chronique et l'emphysème. Ce dernier est caractérisé par une dégradation du septa, qui est la paroi des alvéoles pulmonaires (National Health Service 2017).
- les séquelles pulmonaires liées à une tuberculose antérieure.
- les syndromes de reflux gastro-œsophagien (RGO), où les patients vont régulièrement subir des remontées gastriques voire des vomissements. Le contenu acide de l'estomac favorisera alors une inflammation de l'œsophage (Won-Jung Koh et al. 2007).

9) Facteurs de risque, les troubles pulmonaires chroniques d'origine génétique, cas de la mucoviscidose :

La maladie génétique qui prédispose le plus aux infections pulmonaires à *M. abscessus* est la mucoviscidose. C'est une maladie autosomale récessive avec une prévalence estimée de 1 cas toutes les 2500 naissances dans la population caucasienne globale, même si cette valeur peut osciller d'une région à une autre (Leitch et Rodgers 2013). Elle est due à des mutations du gène *cftr* localisé sur le chromosome 7 encodant pour la protéine Cystic Fibrosis Transmembrane conductance Regulator (CFTR). A ce jour, plus de 1000 altérations génétiques différentes ont été identifiées, donnant naissance à 6 classes de mutations distinctes. Néanmoins, une d'entre elles prédomine la délétion de classe 2 de l'acide aminé phénylalanine en position 508 (Ratjen et Döring 2003). La fonction de cette protéine est le transport des ions chlorures à travers la membrane apicale de cellules épithéliales de plusieurs organes comme le foie, le pancréas, l'appareil génital, les sinus et les poumons. Le système respiratoire représente l'organe le plus débilitant de la maladie. Le non-adressage du canal CFTR à la membrane apicale des cellules épithéliales pulmonaires provoque un épaississement et un changement de composition du mucus présent dans les bronches. Cela empêche la fonction mucociliaire normale conduisant à une accumulation accrue de mucus ce qui aboutira à une forte détresse respiratoire. Ce mucus pathologique représente un environnement propice à la prolifération de *M. abscessus*. Cependant, ce n'est pas l'espèce bactérienne que l'on retrouve le plus fréquemment. Elle est précédée, dans cet ordre-ci, par les bactéries *Pseudomonas aeruginosa*, *Staphylococcus aureus* Résistant à la Méthicilline (SARM), *Haemophilus influenzae* et *Stenotrophomonas maltophilia* (Surette 2014).

Le contenu modifié de ce mucus est primordial pour la mise en place de l'infection bactérienne. Premièrement, ce mucus est très riche en cytokines pro-inflammatoires et déficient en IL-10 (une cytokine anti-inflammatoire) ce qui va promouvoir l'infection de *P. aeruginosa* dans le modèle souris (Chmiel et al. 1999). Deuxièmement, le canal CFTR non-fonctionnel conduit à une augmentation de la concentration en NaCl du mucus, qui a pour effet d'inactiver les défensines et leur pouvoir bactéricide (Smith et al. 1996). Enfin, il a été montré *in vitro* que les propriétés anoxiques de ce mucus perturbent le fonctionnement normal des neutrophiles qui ne peuvent plus combattre efficacement une infection à *P. aeruginosa* (Worlitzsch et al. 2002).

Ces canaux CFTR ont également un rôle à jouer dans l'élimination des pathogènes. Chez une personne saine, les canaux CFTR des cellules épithéliales pulmonaires vont servir de récepteur et permettre l'internalisation de *P. aeruginosa* et son élimination (Pier et al. 1996). Les cellules épithéliales possédant des canaux CFTR défectueux ne sont pas capables d'internaliser ce pathogène et de le détruire. Les bactéries se retrouvent alors libre dans le mucus et continuent de se multiplier (Pier et al. 1996). Plus récemment, il a été démontré que dans le modèle zebrafish, que les

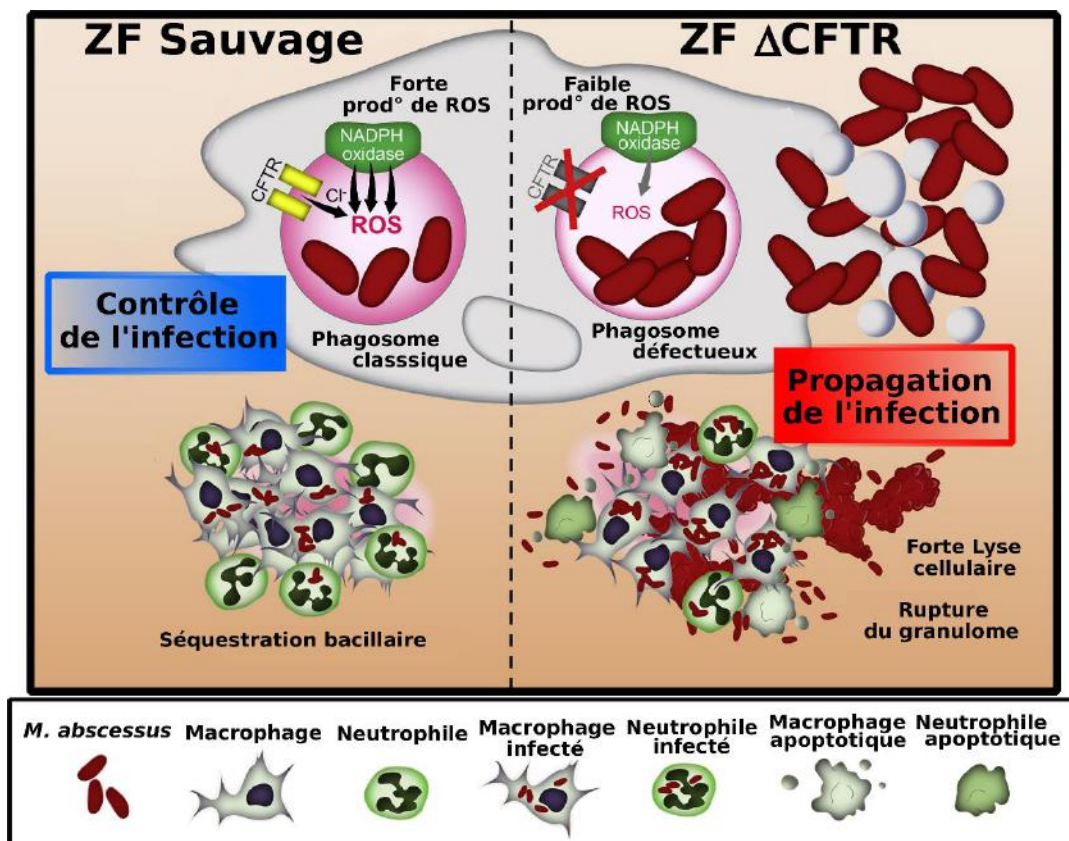


Figure 12. Les macrophages Δcfr ne sont plus en mesure de contrôler l'infection de *M. abscessus*. (Partie de gauche) Forte production de ROS dans le phagosome médiée par la NADPH oxidase et favorisée par l'import de Cl^- menant à la mort des bacilles. Le chimiotactisme des neutrophiles est efficace et conduit à la formation d'un granulome. (Partie de droite) Les canaux CFTR mutés ne sont plus en mesure de favoriser la production de ROS. Les bactéries se divisent jusqu'à la lyse du macrophage. Le chimiotactisme des neutrophiles est altéré et la formation du granulome échoue. Les bacilles se multiplient extracellulairement et provoquent d'important dégât tissulaire menant à la mort du zebrafish. ZF, zebrafish ; ROS, reactive oxygen species (Adapté de Bernut et al. 2019).

transporteurs CFTR jouent un rôle primordial dans l'élimination intracellulaire de *M. abscessus* (Bernut et al. 2019). En effet, les canaux CFTR vont permettre l'entrée des ions chlorures dans les phagosomes permettant une forte production de ROS par la NADPH oxydase ce qui permettra d'éliminer les bactéries internalisées (Figure 12). Cependant, chez des embryons de ZF $\Delta cftr$, les bactéries phagocytées ne sont pas détruites. Elles lysent les cellules infectées et se multiplieront dans le milieu extracellulaire. Dans ce contexte génétique, les deux morphotypes provoqueront une forte mortalité des larves de ZF.

Chez une personne atteinte de mucoviscidose, l'épaisseur du mucus rend l'expectoration très délicate. Les bactéries pathogènes comme *M. abscessus* se retrouvent alors bloquées dans un environnement où tout semble être propice pour qu'elles prolifèrent.

10) Détection des infections à *M. abscessus*, les différentes méthodes de diagnostic :

Les méthodes de diagnostic varient selon les pays qui sont dépendants des moyens financiers à disposition. Un diagnostic rapide et surtout précis est un des éléments clefs pour le traitement efficace d'une infection à *M. abscessus*.

Les « Pays les Moins Avancés » (PMA) ne disposent pas des toutes dernières techniques de séquençage/génotypage et de spectrométrie de masse permettant de reconnaître une infection causée par *M. abscessus* d'une autre mycobactériose. Le personnel de santé peut donc uniquement compter que sur un examen des symptômes cliniques (décrits en section « **Chapitre II) Mycobacterium abscessus, généralités, 7) Symptômes et épidémiologie des infections pulmonaires à *M. abscessus* ») ou une coloration de Ziehl-Neelsen (Sarro et al. 2018). L'utilisation restreinte de ces méthodes de diagnostic soulève un grave problème dans ces PMA. Il s'agit en effet la plupart du temps de région où la tuberculose est endémique, comme en Afrique ou en Inde, où l'on sous-estime l'incidence des infections à *M. abscessus* et aux autres MNT (Maiga et al. 2012). Il est impossible de différencier une tuberculose d'une autre mycobactériose avec ces deux techniques et les médecins vont alors traiter ces patients pour une tuberculose au lieu d'une MNT. Ceci a de graves répercussions envers le patient mais aussi sur le point de vue économique. Les MNT et plus particulièrement *M. abscessus* sont totalement résistantes aux traitements anti-tuberculeux de bases. Les patients sont donc traités avec des thérapies longues, inefficaces et coûteuses. Ils souffrent la plupart du temps des effets secondaires liés aux antibiotiques et l'infection continue de se propager et de causer d'importante lésions engageant le pronostic favorable à une guérison. Un examen radiographique ou un scanner de la cage thoracique peuvent aussi être pratiqués. Cependant, cette méthode ne permet pas de distinguer**

avec certitude *M. abscessus* d'une autre MNT, étant donné les caractéristiques communes observées :

- une bronchiectasie bilatérale.
- la présence d'opacités réticulonodulaires.
- et la présence de cavités dans les parties supérieures des lobes pulmonaires.

Suite à un examen radiographique positif à la présence d'une infection par une MNT, un examen microbiologique (Koh et al 2014) peut être réalisé selon les critères de l'Infectious Diseases Society of America (IDSA) et de l'American Thoracic Society (ATS) qui sont basés sur des cultures positives issues d'un lavage des bronches, de biopsies pulmonaires et d'expectorations couplées à une observation histologique d'une inflammation granulomateuse.

Un séquençage des gènes de ménages *rpoB*, *hsp65* et *rrs* (ARN 16S) permet d'identifier avec certitude une infection par 7 MNT parmi lesquelles font parties les espèces du complexe *M. abscessus* ainsi que *M. avium*, *M. chelonae*, *M. chimera* et *M. intracellulare*. Ce kit, GenoType NTM-DR (Hain Lifescience, Nehren, Allemagne), permet également d'identifier, au sein du complexe *M. abscessus*, le profil de résistance pour : (i) les macrolides en séquençant les gènes *erm(41)* et de l'ARNr 23S, (ii) pour les aminoglycosides via le séquençage de l'ARNr 16S. La discrimination entre les trois sous-espèces *M. abscessus* est possible car la protéine Erm(41) est tronquée chez la sous-espèce *M. massiliense*. Cette enzyme va méthyle de l'A2058 de l'ARNr 23S de la grande sous-unité ribosomique et le protéger des macrolides. Le séquençage d'*erm(41)* permet de détecter un polymorphisme nucléotidique bien particulier chez les sous-espèces *M. abscessus* et *M. bolletii*, une mutation en position 28 (T28C), qui rend Erm(41) non-fonctionnelle (Brown-Elliott et al. 2015). Le séquençage de l'ARNr 23S permet de détecter pour ces 7 MNT un polymorphisme nucléotidique sur l'A2058 ciblée par les macrolides. Les mutations dans l'ARNr 16S comme celle du résidu A1408 rendent ces 7 MNT résistantes aux aminoglycosides et sont donc recherchées par séquençage (Kehrmann, Kurt, et al. 2016) (Mougari et al. 2017). Il est donc primordial d'identifier quelle sous-espèce de *M. abscessus* est responsable de l'infection car chaque membre de complexe répond différemment aux antibiotiques.

La spectrométrie de masse de type MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation – Time Of Flight) est une technique très présente en milieu hospitalier qui permet une identification rapide du pathogène responsable de l'infection (Kehrmann, Wessel, et al. 2016) (Sriram et al. 2018). Des marqueurs de résistance peuvent également être ajoutés au panel de détection ainsi que des souchiers d'isolats cliniques permettant une identification très précise et voire même de suivre le déroulement d'une épidémie. Ces caractéristiques techniques permettent de diminuer grandement les risques de diagnostics erronés entraînant des traitements inadéquats.

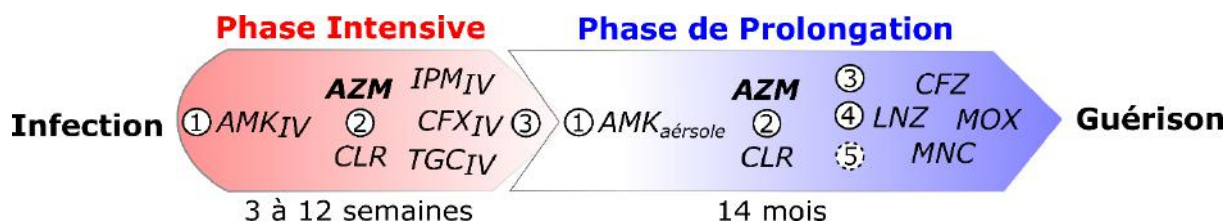


Figure 13. Antibiothérapie dans le cadre d'infections pulmonaire par le Complexe *M. abscessus*.

La phase intensive est composée d'une dose journalière de macrolide comme de la clarithromycine (CLR) ou préférablement de l'azithromycine (AZM), d'amikacine (AMK), et d'un des antibiotiques suivant: céfoxitine (CFX), tigécycline (TGC) ou imipénème (IPM). Cette phase est recommandée pour une durée de 3 à 12 semaines, en tenant compte de la sévérité des symptômes et de la tolérance des effets indésirables. La phase de prolongation comprend toujours une dose journalière d'AZM de préférence et d'AMK administrée cette fois-ci en aérosol. Ajoutés à ce cocktail, on retrouve pris par voie orale 2 à 3 des antibiotiques suivant: clofazimine (CFZ), moxifloxacine (MOX), linézolide (LNZ) et minocycline (MNC). Cette phase de prolongation s'étend sur une période de 14 mois et l'utilisation de ces molécules est relative à la susceptibilité aux antibiotiques de la souche incriminée. Cette susceptibilité est définie par la valeur de la Concentration Minimal Inhibitrice (CMI) de chaque antibiotique. Néanmoins ces recommandations semblent relativement arbitraires et pourraient toujours mener à une utilisation inappropriée d'antibiotique (Citation de Floto et al. 2016 « guided but not dictated by drug susceptibility testing » i.e. l'utilisation des antibiotiques cités ci-dessus). IV, intraveineuse ; voie d'administration non précisée = voie orale (Adapté de Floto et al. 2016).

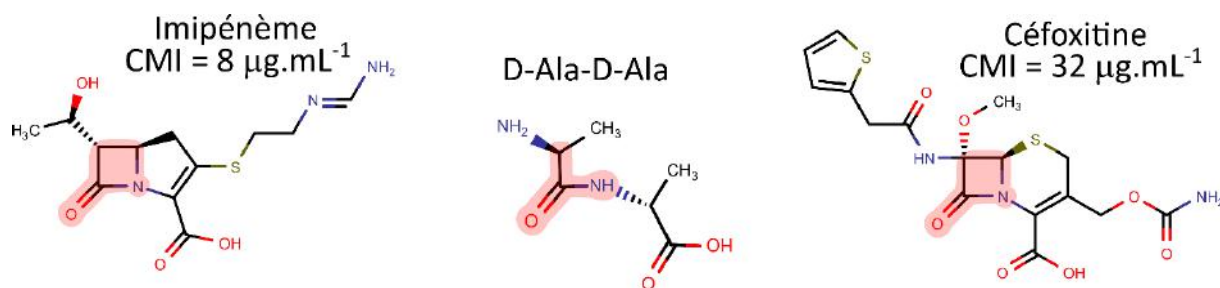


Figure 14. Structures des β -lactamines utilisées dans le traitement de *M. abscessus*. Le D-Ala-D-Ala, substrat des D, D-transpeptidases, est représenté au centre. La partie du substrat imitée par les β -lactamines *via* leurs noyaux β -lactames est encadrée en rouge pour l'imipénème (à gauche) et la céfoxitine (à droite).

1) Présentation de l'antibiothérapie dite « classique » :

Les recommandations pour le traitement d'une infection pulmonaires à *M. abscessus* ont été pendant plusieurs années basées sur une trithérapie s'étalant sur une période de 12 mois composée d'une β -lactamine, d'un aminoglycoside, tous deux administrés par voie parentérale, ainsi que d'un macrolide administré par voie orale. C'est dans l'optique d'optimiser cette trithérapie chez les personnes souffrant de mucoviscidose que s'est réuni en 2016 un consortium entre l'US Cystic Fibrosis Fondation et l'European Cystic Fibrosis Society. Il en a émergé une stratégie thérapeutique préconisant un traitement divisée en deux parties, la phase « intensive » et la phase de « prolongation » comprenant chacune différentes combinaisons d'antibiotiques (Floto et al. 2016) (Figure 13). Un régime antibiotique similaire peut être envisagé chez des personnes ne souffrant pas de mucoviscidose. Ce traitement peut être accompagné d'actes chirurgicaux qui paraissent améliorer l'issue des traitements (Jeon et al. 2009) (Jarand et al. 2011).

1a) Les β -lactamines :

La céfoxitine (CFX) est une céphalosporine semi-synthétique de deuxième génération. Son activité bactéricide est due à l'inhibition du stade final de la biosynthèse du peptidoglycane, un composé essentiel de la paroi mycobactérienne. La CFX rentre en compétition avec le substrat d'une classe d'enzymes appelées « D, D-transpeptidases » (Figure 14). Ces enzymes également désignées Protéines Liant la Pénicilline (PLP) (Sauvage et al. 2008), sont présentes dans la partie externe de la membrane plasmique et tournées vers le périplasme (Luthra et al. 2018). Les β -lactamines ont donc de manière générale structure proche du substrat des PLP, le dipeptide D-alanyl-D-alanine. Leur stratégie d'inhibition est donc basée sur un mimétisme de substrat qui provoquera l'arrêt de la synthèse du peptidoglycane (Kohanski et al. 2010).

L'imipénème (IPM) est également une β -lactamine semi-synthétique mais appartient à la famille des carbapénème. Tout comme la CFX, l'IPM va inhiber ces transpeptidases avec un mécanisme similaire mais avec une efficacité accrue (Dubée et al. 2015).

1b) Les aminoglycosides :

L'amikacine (AMK) est un 2-deoxystreptamine aminoglycoside et également une molécule semi-synthétique dérivée de la kanamycine A (KAN A) (Figure 15A). Cet antibiotique interagit avec

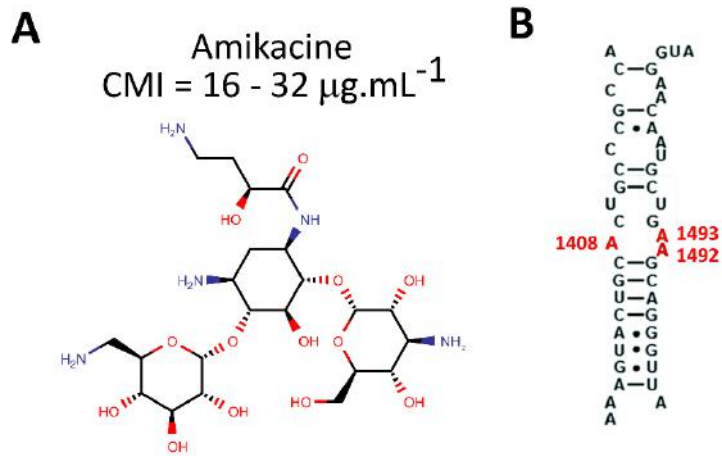


Figure 15. Structures de l'amikacine (A) et de la région qu'elle cible dans l'ARNr 16S (B). Les nucléotides du site-A ciblés par l'amikacine sont en rouge (Adapté de Sarpe et al. 2019)

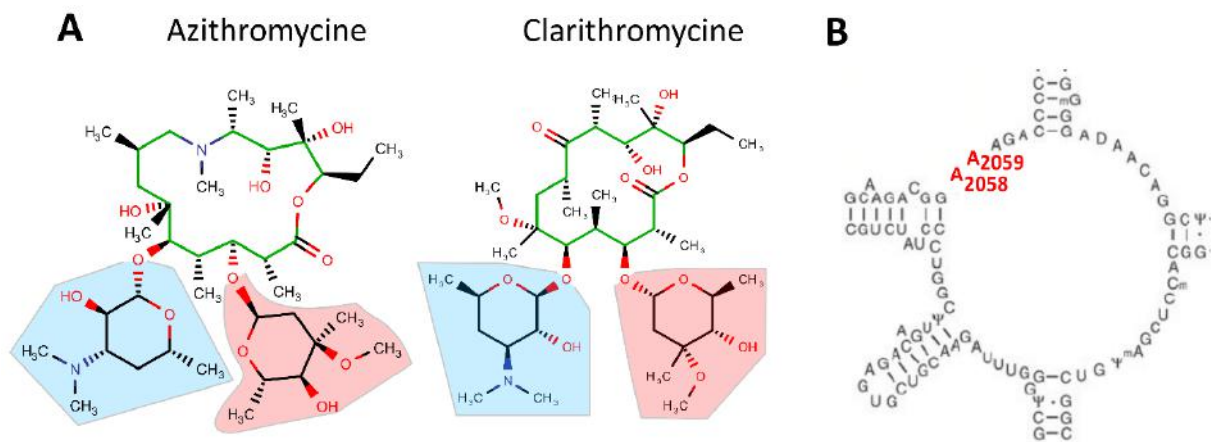


Figure 16. Structure de l'azithromycine et de la clarithromycine (A) ainsi que de la région qu'elles ciblent dans l'ARNr 23S (B). Les cycles lactone sont en vert, les sucres désosamines et cladinoses sont encadrés en bleu et en rouge respectivement. Les adénines de la boucle centrale du domaine V de l'ARNr 23S ciblées par ces macrolides sont marqués en rouge (Adapté de Douthwaite 2001).

l'ARNr 16S de la petite sous-unité du ribosome (30S). Les aminoglycosides étant des antibiotiques polycationiques et l'ARNr 16S chargé négativement (Shakil et al. 2008), l'AMK va former des liaisons électrostatiques avec les adénines en position 1408 (A1408), 1492 (A1492) et 1493 (A1493) responsable de l'activité de *decoding*, perturbant l'entrée du bon ARNt dans le site A (O'Sullivan et al. 2018) (Dudek et al. 2014) (Figure 15B). Ceci provoque l'incorporation d'acides aminés ne correspondant pas à l'ARNm. Ces erreurs de traduction mènent à la synthèse de protéines mal repliées ce qui génère alors un fort stress oxydatif et membranaire (Kohanski et al. 2010). Afin d'éviter les effets secondaires liés à l'injection intraveineuse d'AMK comme la perte de l'ouïe et la toxicité rénale, cette antibiotique peut être administré en aérosol, notamment lors de la phase de continuité (Yagi et al. 2017).

1c) Les macrolides :

Dérivés de l'érythromycine, la clarithromycine (CLR) et l'azithromycine (AZM) sont des antibiotiques d'hémisynthèses qui diffèrent principalement par le nombre d'atomes composant leur cycle lactone, 14 et 15 respectivement (Figure 16A). Cette classe d'antibiotiques cible également la synthèse protéique mais en interagissant cette fois avec l'ARNr 23S de la grande sous-unité ribosomale (50S), plus précisément avec la boucle du centre peptidyltransférase dans le domaine V (Figure 16B). La CLR et l'AZM vont former des liaisons hydrogènes réversibles avec deux nucléotides, l'A2058 et l'A2059 *via* leur sucre désosamine (Garza-Ramos et al. 2001). Ces deux antibiotiques établiront également des interactions électrostatiques avec d'autres nucléotides de cette région du ribosome qui forment la sortie d'un tunnel par lequel sort la chaîne polypeptidique néo-synthétisée. Ceci aura pour conséquence d'empêcher la translocation, c'est-à-dire la formation du pont peptidique entre la protéine en cours de synthèse et le nouvel acide aminé porté par l'ARNt. *M. abscessus abscessus* et *M. abscessus bolletii* sont de manière générale naturellement résistants à ces macrolides (Nash et al. 2009). Néanmoins, ces molécules peuvent s'avérer efficaces lorsqu'elles sont utilisées en combinaison, particulièrement contre la sous-espèce *M. abscessus massiliense* (Zhang et al. 2017). L'AZM possède une demi-vie plus élevée que la CLR et ne provoque pas d'interaction médicamenteuse avec les autres antibiotiques présents dans l'antibiothérapie contre *M. abscessus* (Periti et al. 1992). Cependant, Renna et al. ont tiré la sonnette d'alarme quant à l'utilisation à long terme d'AZM, particulièrement chez les personnes souffrant de mucoviscidose. Tout d'abord, il arrive que l'AZM soit administrée pour ses propriétés immunomodulatrices et non pas pour traiter une infection. Les doses données sont subinhibitrices et peuvent donc permettre la sélection de MNT résistantes aux macrolides (Levy et al. 2008). Ces mêmes propriétés immunomodulatrices vont bloquer l'autophagie, la fusion phagolysosomale et l'élimination des bacilles dépendante de la production d'IFN- γ et de TNF- α par les macrophages. Autant de facteurs combinés qui favorisent la colonisation par les MNT (Renna et al. 2011).

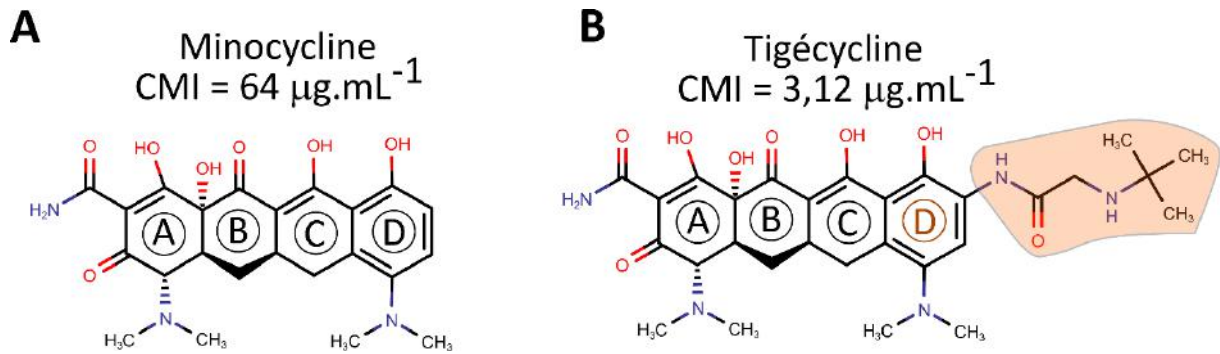


Figure 17. Structures de la minocycline (A) et de la tigécycline (B). La chaîne 2-tert-butylglycylamido en position C9 du cycle aromatique D de la tigécycline est encadrée en orange.

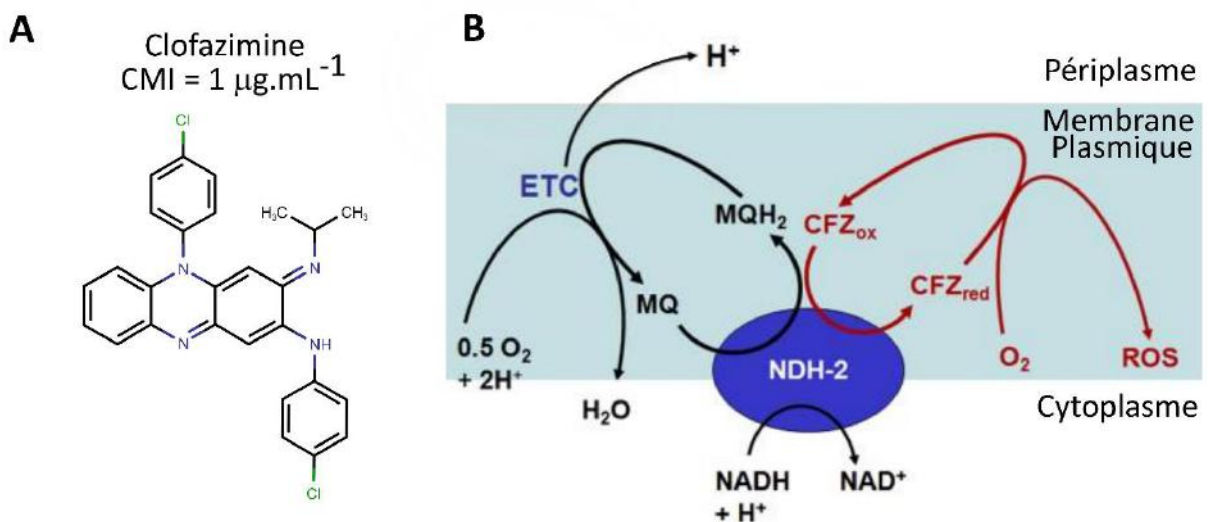


Figure 18. Structure de la clofazimine (A) et représentation schématique de sa voie d'activation (B). La NDH-2 catalyse le transfert d' H^+ du NADH à la ménaquinone (MQ) qui participera ensuite dans le transfert d'électrons vers les autres oxydoréductases de la chaîne respiratoire mycobactérienne avec comme réaction finale la réduction de O_2 en H_2O et une sortie d' H^+ vers le périplasme. Cette étape terminale générera une force proton motrice indispensable à la synthèse d'ATP. La CFZ est activée sous sa forme réduite par la NDH-2 *via* le NADH également. La CFZ_{red} interagira ensuite avec les molécules d' O_2 obtenues en bout de chaîne respiratoire pour conduire à la production de ROS nocifs pour la bactérie. La production de ROS va ré-oxyder de manière non-enzymatique la CFZ qui pourra à nouveau être réactivée par la NDH-2. Ainsi la CFZ subit une réactivation redox cyclique menant à la production croissante de ROS. ETC, electron transport chain ; MQ, ménaquinone ; CFZ, clofazimine ; ROS, reactive oxygen species ; ox, oxydée ; red, réduite (Adapté de Yano et al. 2011).

1d) Les tétracyclines :

La minocycline (MNC) est une tétracycline de deuxième génération qui ne possède généralement pas une très bonne activité *in vitro* contre *M. abscessus*. Son utilisation en deuxième phase de traitement contre les infections pulmonaires ne doit être envisagée que contre des souches sensibles. De plus, les récents travaux de Ruth et al. de 2018 rapporte la même inefficacité seule qu'en combinaison avec d'autres antibiotiques *in vitro*.

La tigécycline (TGC) est cependant elle un des seuls analogues de tétracycline possédant une activité antimicrobienne contre *M. abscessus*. Cette glycylycylcine administrée en intraveineuse possède un très bon taux d'absorption et d'accumulation intracellulaire en faisant donc un bon candidat pour lutter contre les infections mycobactériennes (Ferro et al. 2016). La TGC est la seule de toutes les tétracyclines prescrites à posséder une chaîne 2-tert-butylglycylamido en position C9 de son cycle aromatique D (Figure 17). Cette particularité structurale lui confèrerait la capacité de résister à l'efflux actif et à l'inactivation enzymatique que subie cette classe d'antibiotiques chez *M. abscessus* (Rudra et al. 2018). Comme les autres types de tétracyclines, la MNC et la TGC vont bloquer la traduction en interagissant avec la sous-unité 30S du ribosome et empêcher l'entrée des aminocayl-ARNt dans le site-A du centre peptidyltransférase provoquant l'arrêt de la synthèse protéique.

1e) Les phénazines :

La clofazimine (CFZ), autrefois utilisée pour traiter la lèpre mais faisant partie actuellement du régime thérapeutique anti-MDR-Tb (Mirnejad et al. 2018) est toujours en cours d'évaluation clinique pour son efficacité contre *M. abscessus*. C'est une molécule de synthèse appartenant à la famille des phénazines. Cet antibiotique considéré comme bactériostatique possède aussi de bonnes propriétés anti-inflammatoires ce qui lui donne l'occasion de se retrouver dans le traitement de certaines maladies non-infectieuses comme le lupus et le psoriasis (Sanchez 2000). Le mécanisme d'action de la CFZ contre *M. abscessus* n'est pas décrit. Néanmoins, il a été montré chez *M. smegmatis* que la CFZ est une pro-drogue nécessitant d'être bio-activée par la NADH oxydoréductase de type II (NDH-2) insérée dans le feuillet interne de la membrane plasmique (Yano et al. 2011). Cette bio-activation mène à une importante production de ROS pouvant s'avérer fatale pour le bacille (Figure 18B).

1f) Les fluoroquinolones :

La moxifloxacine (MXF) est une fluoroquinolone de quatrième génération retrouvée en phase de continuité dans le traitement de *M. abscessus*. Cet antibiotique peut être administré par voie orale, parentérale mais aussi de manière locale mais aussi pour un traitement ophtalmologique.

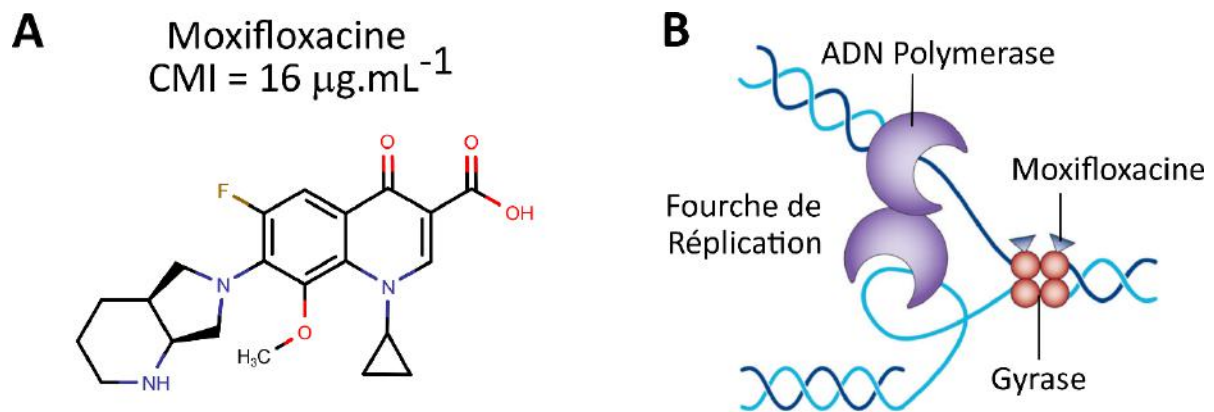


Figure 19. Structure de la moxifloxacin (A) et représentation schématique de son interaction avec l'ADN gyrase lors de la réplication de l'ADN (B). L'ADN gyrase va dénouer l'ADN en générant des coupures temporaires de la double-hélice permettant à l'ADN polymérase de répliquer le matériel génétique. La moxifloxacin va former un complexe avec l'ADN et l'ADN gyrase ce qui provoquera un arrêt de la réplication et laissera l'ADN clivé. Ces cassures de l'ADN généreront un fort stress oxydatif déclenchant la mort cellulaire (Adapté de Kohanski, et al. 2010).

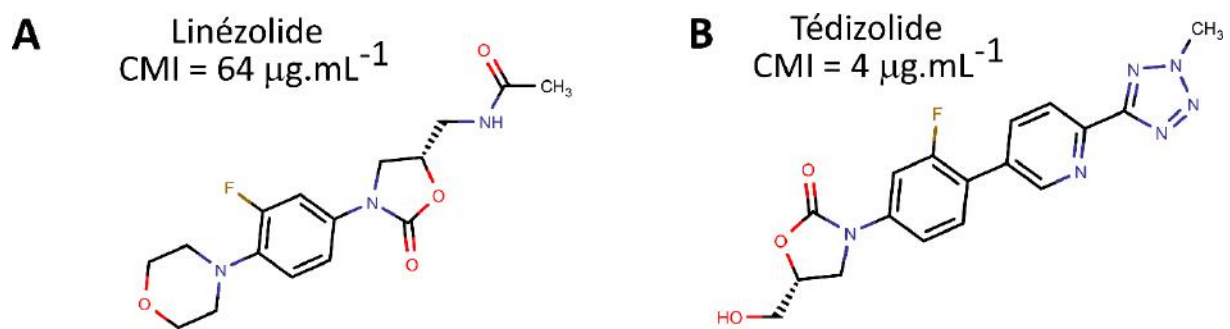


Figure 20. Structure du linézolide (A) et du tédzolide (B)

Cette molécule de synthèse dérive de l'acide nalidixique découvert en 1962 (Figure 19A). Elle va interagir avec les sous-unités α de l'ADN gyrase/Topoisomérase II lorsqu'elle forme un complexe avec l'ADN (Figure 19B). Cette enzyme est essentielle et a pour rôle de « dénouer » les super enroulements du chromosome bactérien causés lors de la réplication de d'ADN et de la progression de la fourche de réplication. Elle agit également sur une autre enzyme structurellement proche de l'ADN gyrase, la topoisomérase IV. Cette enzyme est impliquée dans les derniers stades de la réplication en séparant les deux chromosomes néo-synthétisés avant leurs ségrégations dans les deux cellules filles. L'interaction de la MOX avec ces deux complexes enzyme-ADN aboutira au même résultat : cassure des chromosomes bactériens et lyse du bacille. Malgré l'efficacité des fluoroquinolones, l'Agence Européenne du Médicament recommande de limiter leur utilisation afin de prévenir l'émergence de résistance comme ce fut le cas pour les fluoroquinolones de deuxième et troisième générations (Kowalski et al. 2003) mais aussi à cause de leurs effets secondaires (Francisco 2018).

1g) Les oxazolidinones :

Il s'agit d'une autre classe de molécules intégrées dans la deuxième phase de l'antibiothérapie contre *M. abscessus*, parmi lesquelles fait partie le LNZ. Ce sont des composés organiques de synthèse hétérocycliques qui vont bloquer la synthèse protéique. La LNZ va interagir de manière non-covalente avec les nucléotides uraciles 2504 (U2504), 2506 (U2506), 2585 (U2585) et les résidus A2451 et A2503 de l'ARNr 23S chez la bactérie *Deinococcus radiodurans* (Wilson et al. 2008). Ces nucléotides sont proches de l'interface 50S/30S et l'interaction du LNZ va bloquer formation du complexe d'initiation 70S. Ces antibiotiques peuvent être administrés par voie orale ou par intraveineuse. Le tédizolide (TDZ), un analogue structural du LNZ a une très bonne activité contre un large panel d'isolats cliniques de *M. abscessus in vitro* (Compain et al. 2018) (Figure 20). Le TDZ semble également être une molécule très prometteuse lorsqu'elle est utilisée en combinaison contre *M. abscessus* (Le Run et al. 2019)

2) Présentation des autres possibilités thérapeutiques :

Malgré les associations possibles entre ces différentes classes d'antibiotiques avec des cibles subcellulaires variées, 40 % des traitements de *M. abscessus* aboutissent à un échec thérapeutique (Koh et al. 2014). Les cliniciens ont donc parfois recouru à des antibiotiques peu communs et sans précédents d'utilisations contre les MNT, mais dont l'utilisation est approuvée pour le traitement d'autres bactérioses.

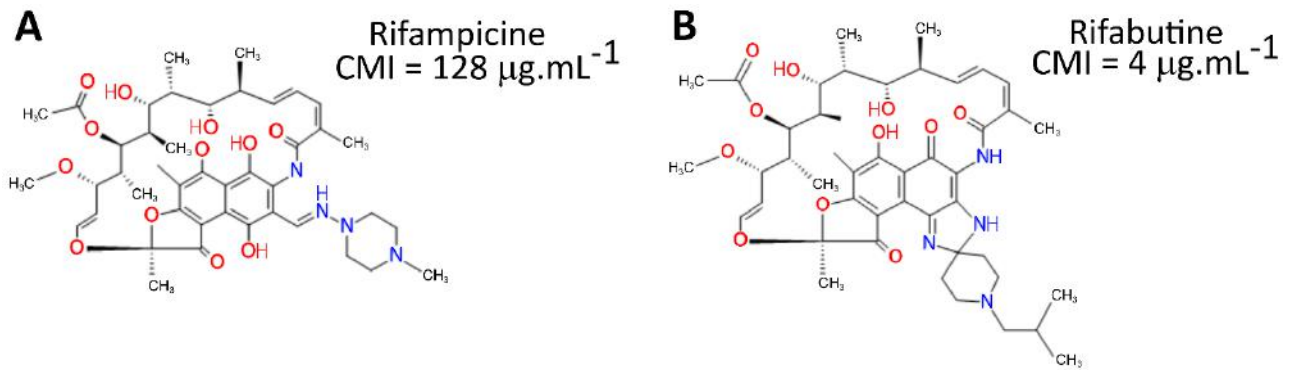


Figure 21. Structure de la rifampicine (A) et de son dérivé la rifabutine (B).

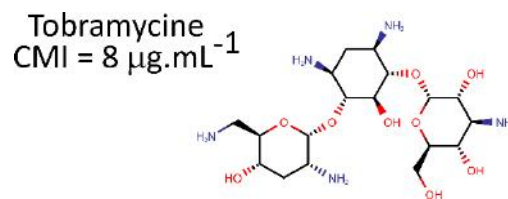


Figure 22. Structure de la tobramycine.

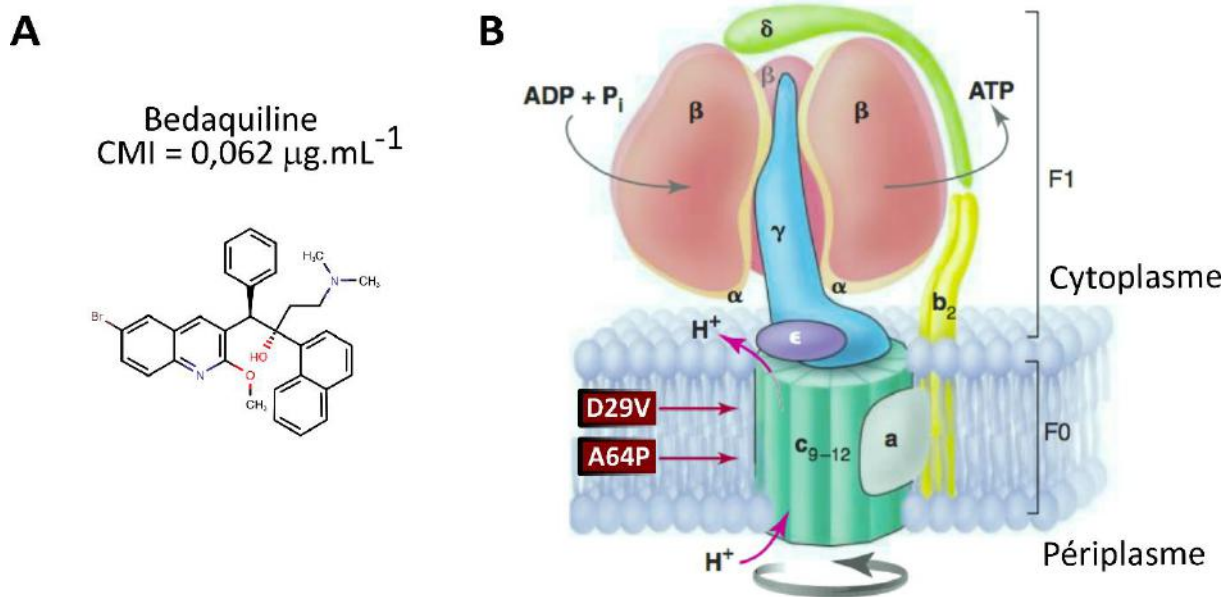


Figure 23. Structure de la bédaquiline (A) et représentation schématique de sa cible, l'ATP synthase (B). L'ATP synthase est constituée d'une partie cytoplasmique F₁ et d'une partie transmembranaire F₀. Les sous-unités c sont arrangées en disques symétriques capables de rentrer en rotation grâce à la force proto-motrice, qui est nécessaire par la suite pour la synthèse d'ATP. Les mutations D29V et A64P confère à *M. abscessus* de hauts niveaux de résistance (Adapté de Cole et Alzari 2005).

2a) Les rifamycines :

La rifabutine est une rifamycine semi-synthétique, une variété d'antibiotiques appartenant à la classe des ansamycines. Cette nouvelle molécule a récemment montré une activité antimicrobienne bien supérieure contre *M. abscessus* que la molécule de laquelle elle dérive, la rifampicine (Rominski, Roditscheff, et al. 2017) (Aziz et al. 2017) (Figure 21). Cet antibiotique, administré par voie orale, va inhiber la synthèse d'ARN catalysée par l'ARN polymérase. Les mécanismes par lesquels la rifabutine interagit avec l'ARN polymérase ne sont pas décrits mais il est probable qu'elle opère de la même manière que la rifampicine. Ces antibiotiques, *via* des interactions de type van der Waals avec la sous-unité β de l'ARN polymérase, vont bloquer le sillon par lequel l'enzyme entre en contact avec l'ADN (Campbell et al. 2001). De plus, la rifabutine semble avoir un fort potentiel synergique *in vitro* avec d'autres antibiotiques utilisés contre *M. abscessus* (Le Run et al. 2018) (Cheng et al. 2019).

2b) Les aminoglycosides non-conventionnels :

La tobramycine (TBM) est un autre 2-deoxystreptamine aminoglycoside qui peut aussi être utilisé contre *M. abscessus*. Comme l'AMK, elle peut être administrée par voie orale et parentérale mais également localement pour traiter des infections oculaires. La TBM agit de la même manière que l'AMK sur la sous-unité 30S du ribosome en se liant aux mêmes résidus A1492 et A1493 (Yang et al. 2006). La TBR est légèrement plus efficace que l'AMK *in vitro* mais sa sensibilité aux enzymes dégradant les aminoglycosides est différente ce qui limite son utilisation (Rominski, Selchow, et al. 2017) (Figure 22).

2c) Les diarylquinolines :

C'est en 2012 que la bédaquiline (BDQ) fait son arrivée dans le traitement des souches de MDR-Tb. Comme la CFZ, son utilisation est envisagée pour traiter *M. abscessus* et son efficacité est en cours d'évaluation. *A contrario*, la BDQ n'a pas un effet bactéricide chez *M. abscessus* mais est extrêmement active *in vitro* (Dupont et al. 2017). La BDQ est un antibiotique de synthèse appartenant à la famille des diarylquinolines qui est administrée par voie orale (Figure 23A). Elle va inhiber le complexe enzymatique membranaire F_0-F_1 ATP synthase. Les cibles de la BDQ sont les sous-unités c, qui forment un anneau transmembranaire par lesquels transitent les protons nécessaires à la production d'ATP. Plus précisément, la BDQ interagirait avec les résidus acide aspartique 32 (D32) et alanine 64 (A64) (Dupont et al. 2017) (Figure 23B). Il en résulte un blocage de la rotation de l'anneau formé par toutes les unités c arrêtant la production d'ATP (Preiss et al. 2015). La BDQ semble également avoir une seconde cible chez *M. tuberculosis*, la sous-unité ϵ de l'ATP synthase (Kundu et al. 2016).

Table 1) Récapitulatif des études menées *in vitro/in vivo* depuis 2005 contre le complexe *M. abscessus*.

| | Combinaison antibiotiques/ <u>inhibiteurs</u> | Type d'étude | Nb de Souches | Résultats obtenus | Réf. |
|-----------------|--|---|-------------------|-----------------------------|---------------------------|
| 1 | Clarithromycine + linézolide | <i>In vitro</i> | 2 | 100% | Cremades et al. 2009 |
| 2 | Clarithromycine + tigécycline | <i>In vitro</i> | 20 | 65% <i>M. massiliense</i> | Zhang et al. 2017 |
| | | | | 25% | |
| | | <i>In vitro</i> | 31 | 80.6% | Huang et al. 2013 |
| 3 | Clarithromycine + vancomycine | <i>In vitro</i> | 12 | 100% | Mukherjee et al. 2017 |
| 4 | Clarithromycine + moxifloxacine | <i>In vitro</i> | 28 | 39.3% <i>M. massiliense</i> | Choi et al. 2012 |
| | | | 26 | 3.8%/65.4% | |
| | | macrophage | 15 | 33.3% <i>M. massiliense</i> | |
| | | | | 6.6%/66.7% | |
| | | souris | 6 | 50% <i>M. massiliense</i> | |
| | | | | 7 | 71.4% <i>M. abscessus</i> |
| <i>In vitro</i> | 20 | 85% <i>M. massiliense</i> 5%/45% <i>M. abscessus</i> | Zhang et al. 2017 | | |
| 5 | Azithromycine + moxifloxacine | <i>In vitro</i> | 28 | 35.7% <i>M. massiliense</i> | Choi et al. 2012 |
| | | | 26 | 3.8%/46.2% | |
| | | macrophage | 15 | 20% <i>M. massiliense</i> | |
| | | | | 6.6%/40.0% | |
| | | souris | 6 | 50% <i>M. massiliense</i> | |
| | | | | 7 | 71.4% <i>M. abscessus</i> |
| 6 | Clarithromycine + linézolide + moxifloxacine/gatifloxacine/lévofloxacine | <i>In vitro</i> | 2 | 100% | Cremades et al. 2009 |
| 7 | Clarithromycine + ciprofloxacine + rifabutine | <i>In vitro</i> | 2 | 100% | Cremades et al. 2009 |
| 8 | Imipénème + clarithromycine | <i>In vitro</i> | 21 | 43% | Miyasaka et al. 2007 |
| 9 | Imipénème + lévofloxacine | <i>In vitro</i> | 21 | 29% | Miyasaka et al. 2007 |
| 10 | Amikacine + clofazimine | <i>In vitro</i> | 40 | 100% | Shen et al. 2010 |
| | | <i>In vitro</i> | 77 | 80.5% | van Ingen et al. 2012 |
| 11 | Amikacine + linézolide | <i>In vitro</i> | 32 | 53.1% <i>M. massiliense</i> | Zhang et al. 2018 |
| | | | | 37.5% | |
| 12 | Tigécycline + clofazimine | <i>In vitro</i> | 19 | 42% | Singh et al. 2014 |

3) Associations d'antibiotiques et synergies pour le traitement de *M. abscessus* :

L'association d'antibiotiques permet l'éradication de germes multi-résistants en élargissant leurs spectres individuels et prévient les poly-infections. Les doses d'antibiotiques administrées lors d'une thérapie ne sont forcément réduites comme c'est le cas *in vitro* et les doses dictées par les autorités sanitaires de chaque antibiotique sont respectées (Auboyer et al. 2000). Ainsi il peut en résulter une cumulation d'effets indésirables très handicapants menant à l'abandon du traitement. Certains antibiotiques vont également avoir une pharmacocinétique différente chez l'homme et perdre en efficacité. Ceci explique pourquoi les données sur les synergies entre différents antibiotiques fluctuent entre les études *in vitro* et les études *in vivo*. Par définition, une synergie entre deux ou plusieurs antibiotiques résulte en un meilleur effet antibactérien que le cumule des effets de chaque antibiotique utilisé séparément. Il est donc préférable d'utiliser des antibiotiques avec des cibles distinctes afin de cumuler le pouvoir antimicrobien de chaque molécule.

Les études de l'efficacité de combinaison antibiotiques sur patients sont peu abondantes. L'une d'elle (Choi et al. 2011) a mis en évidence une synergie visible entre une bithérapie MOX/CLR comparée à une combinaison AMK/CLR dans le cadre d'infections cutanées causées par *M. abscessus*. La convalescence des patients étaient également plus courtes avec l'utilisation de MOX et CLR.

Les informations sur les synergies antibiotiques *in vitro* sont largement plus conséquentes que celles faites *in vivo*. L'ensemble de ces synergies testées contre *M. abscessus* est récapitulé en Table 1. Certaines de ces combinaisons étant plus originales ou novatrices sont décrites ci-dessous.

L'une d'elle inclue l'utilisation de TDZ. Le Run et al. ont récemment démontré *in vitro* ainsi que dans un modèle macrophage une synergie du TZD avec l'IPM et la rifabutine en combinaison avec un inhibiteur de β -lactamases de dernière génération, l'avibactame. De plus, la rifabutine et le TDZ ont été utilisés à des concentrations sériques, ce qui encourage leur évaluation clinique contre *M. abscessus*.

Similaire à la stratégie incluant l'avibactame, le vérapamil (VPM), un inhibiteur de courant calcique membranaire, permet d'optimiser les antibiotiques ciblant les voies métaboliques dépendantes des gradients électrochimiques comme les pompes à efflux ou l'ATP synthase. Le VPM seul est inactif mais en combinaison avec la BDQ présente un fort effet synergique contre des isolats cliniques de *M. abscessus in vitro*. Cet effet est également observé au sein de macrophages infectés, mettant en avant l'intérêt de cette combinaison contre *M. abscessus* (Viljoen et al. 2019).

La résistance inductible aux macrolides chez les sous-espèces *M. abscessus* et *M. bolletii* est un réel frein à l'utilisation de cette classe de molécules qui est un pilier dans le traitement de ces mycobactérioses. Cependant, la vancomycine, un antibiotique glycopeptidique ciblant la synthèse de

la paroi, est en réalité efficace en combinaison avec la CLR. Cette combinaison est même effective contre des souches dont la résistance aux macrolides a été induites antérieurement. (Mukherjee et al. 2017). Chez ces deux sous-espèces, ce système de résistance inductible est en partie responsable du faible taux de succès thérapeutique, avoisinant les 25 % (Jarand et al. 2011). Cette combinaison mériterait également une évaluation clinique, d'autant plus que la vancomycine est un antibiotique approuvée par les administrations sanitaires.

Chapitre IV) L'Antibiorésistance chez *M. abscessus*

1) Polymorphisme nucléotidique, génétique et mutations acquises :

L'antibiorésistance innée de *M. abscessus* est due, en plus de l'imperméabilité de sa paroi, à un fort polymorphisme génétique retrouvé soit sur les cibles de certains antibiotiques soit sur les gènes codants les enzymes responsables de leurs activations. La résistance intrinsèque aux antituberculeux de première intention est un bon exemple de ce polymorphisme génétique comme c'est le cas pour l'éthambutol (EMB). Cet antibiotique cible les arabinosyltransférases produites par l'opéron *embCAB* qui sont impliquées dans l'arabinosylation de l'arabinogalactane et du LAM (Zhang et al. 2003). Deux substitutions d'acides aminés ont un rôle dans la résistance à l'EMB. La présence de résidus glutamine et méthionine en position 303 et 304 de la protéine ciblée cet antibiotique EmbB confère de haut niveau de résistance à *M. abscessus* et à d'autres mycobactéries (Nessar et al. 2012). *M. tuberculosis* possède des résidus isoleucine et leucine à ces positions 303 et 304, le rendant sensible à cet antibiotique (Alcaide et al 1997).

La résistance aux fluoroquinolones de deuxième et troisième génération est aussi dû à un polymorphisme génétique de leurs cibles, les gènes *gyrA* et *gyrB*, encodant les deux sous-unités de l'ADN gyrase (Guillemin et al 1998). Les résidus, selon la numérotation faite chez *E. coli*, alanine en position 83 (A83) dans le gène *gyrA*, ainsi qu'une arginine et qu'une asparagine en position 447 (R447) et 464 (N464) dans le gène *gyrB* respectivement, sont critiques. En effet, la substitution de ces aminoacides par les résidus d'*E. coli* chez *M. tuberculosis*, qui possède le même polymorphisme de résistance que *M. abscessus*, provoquent une sensibilité du bacille de Koch (Matrat et al. 2008).

Un autre exemple de polymorphisme génétique responsable de résistance intrinsèque à un antituberculeux de première ligne est celui de l'isoniazide (INH) et du gène *katG*, codant pour une catalase/péroxydase. L'INH est une pro-drogue inactive qui doit être peroxydée par KatG. Les dérivés de l'INH produits se lient au NADH cytoplasmique. Ce complexe va par la suite inhiber InhA, une énoyle réductase faisant partie du complexe enzymatique FAS-II impliquée dans la synthèse des précurseurs des acides mycoliques (Wiseman et al. 2010). Cette bio-activation de l'INH ne se produit pas chez *M. abscessus*. KatG est présente et fonctionnelle mais elle est incapable de transformer l'INH en métabolite actif (Luthra et al. 2018).

Ce défaut de bio-activation est aussi observé pour un antibiotique utilisé dans le traitement de MDR-TB, l'éthionamide (ETH). Cette molécule est un dérivé structural de l'INH qui va également inhiber la synthèse d'acides mycoliques en ciblant InhA. L'ETH est activé par la monooxygénase EthA chez *M. tuberculosis* (Dover et al. 2007). L'EthA de *M. abscessus* n'est pas capable d'activer l'ETH mais cette

enzyme est fonctionnelle car elle active d'autres antibiotiques de la même classe, le thiacétazone (TAC) et ses dérivés (Halloum et al. 2017). La résistance au TAC chez *M. abscessus* est due à une redondance fonctionnelle de sa cible, la déhydratase HadBC. Ces phénomènes de redondances fonctionnelles transforment les cibles initiales essentielles en cibles secondaires, et l'effet antibiotique est alors perdu. L'autre déhydratase résistante au TAC, MAB_4780, remplit la fonction de HadBC lorsqu'elle est inhibée par le TAC (Halloum et al. 2016).

Les mutations chromosomiques spontanées sur les cibles des antibiotiques après une exposition trop courte ou après des concentrations trop faibles représentent également un mécanisme majeur d'émergence de résistances chez *M. abscessus*. Cependant, il convient également de prendre compte le fait qu'une infection bactérienne est provoquée par une population génétiquement très hétérogène. Si le traitement n'est donc par exemple pas administré de manière régulière et ponctuel, l'irrégularité de la prise d'antibiotique va dans un premier temps éliminer les populations sensibles. Les sous-populations résistantes résiduelles, possédant à la fois des mutations conférant une résistance sans impacter le métabolisme basal vont alors émerger, se multiplier et occuper le vide laissée par l'élimination des populations sensibles. L'émergence de ces souches résistantes mènent éventuellement à l'échec thérapeutique (Blair et al. 2015). Ces altérations génétiques sont rencontrées lors de thérapies basées sur l'utilisation de plusieurs classes d'antibiotiques comme les macrolides, les aminoglycosides et les fluoroquinolones.

En ce qui concerne les macrolides, aucune information n'est disponible sur la différence de pouvoir mutagène entre la CLR et l'AZM. Les mutations semblent toutes se produire sur le gène cible *rll* codant pour l'ARNr 23S. Les mutations apparaissent au niveau des nucléotides responsables des liaisons hydrogènes avec ces antibiotiques, en l'occurrence les nucléotides A2058 et A2059. Ces événements mutagènes sont néanmoins très rares, plus particulièrement sur le résidu A2059 (Carvalho et al. 2018). Il semblerait cependant que le taux de mutations soit plus important chez *M. massiliense*, cette dernière ne possédant pas de système de résistance inductible *via* méthylation de l'ARNr 23S car Erm(41) est tronquée (Liu et al. 2017). Le peu de souches *M. abscessus sensu stricto* ayant mutées possèdent probablement un polymorphisme nucléotidique délétère comme la substitution classique T28C, rendant Erm(41) non-fonctionnelle (Nash et al. 2009). La sous-espèce *M. massiliense* et le séquoar T28 sont donc soumis à une importante pression de sélection pouvant expliquer ces mutations chromosomiques sur le gène *rrl*. Vraisemblablement, en présence d'un système de résistance inductible très efficace, *M. abscessus abscessus* et *M. abscessus bolletii* n'acquerront aucune mutation.

Tous les 2-deoxystreptamines aminoglycosides ne vont pas interagir avec exactement les mêmes nucléotides de l'ARNr 16S. Néanmoins, tous vont avoir ont une forte affinité pour l'A1408, qui est l'un des quatre nucléotides les plus communément muté, avec les A1492, A1493 et la G1494

(Shakil et al. 2008) (Prammananan et al. 1998) (M. Wu et al. 2019). Comme pour le gène *rrl* et la résistance constitutive aux macrolides, cette mutation ne semble pas être un événement fréquent. Ceci est potentiellement dû en partie à des mécanismes de résistances inductibles inhérents à cette classe d'antibiotique. *M. abscessus* possède des enzymes modifiant directement ces antibiotiques et des pompes à efflux les expulsant du cytoplasme (Hurst-Hess et al. 2017) (Pryjma et al. 2017). Un type de mutation original a été identifié chez des isolats cliniques de *M. tuberculosis* et entraîne une résistance à la KAN. Il s'agit cette fois-ci de mutation non pas sur le gène cible mais dans son promoteur. Le gène en question, Rv2416c, code pour une acétyltransférase impliquée dans la survie intramacrophagique (Zaunbrecher et al. 2009). Les mutations dans le promoteur vont entraîner une surexpression de cette protéine qui est également capable de bio-inactiver certains des 2-deoxystreptamines aminoglycosides.

Le polymorphisme génétique responsable de la résistance intrinsèque de *M. abscessus* aux fluoroquinolones de deux- et troisième génération, A83 pour *gyrA* et R447-N463 pour *gyrB*, ne semble pas empêcher l'activité antimycobactérienne de la MXF. Quarante souches appartenant au complexe *M. abscessus* considérées comme résistantes à la MXF ont été séquencées pour les gènes *gyrA* et *gyrB*. Ces séquençages n'ont révélé aucune mutation pour l'ADN gyrase (Kim et al. 2018). Cet antibiotique cible également la topoisomérase IV, il serait donc, *a minima*, nécessaire de séquencer les gènes encodant cette enzyme, *parC* et *parE*, voire d'effectuer un séquençage complet du génome pour incriminer l'apparition de mutation dans ce phénotype de résistance.

A ce jour concernant la BDQ, aucune mutation dans le gène *atpE* chez des isolats cliniques de *M. abscessus* n'a été rapporté dans la littérature. Cependant, des mutations dans ce gène générées au sein de notre laboratoire ont offert une forte résistance *in vitro* rendant *M. abscessus* 250 fois plus résistant (Dupont et al. 2017). Au vu de l'énorme avantage en termes de résistance apportées par ses mutations qui n'ont apparemment ni coût répliatif ni coût métabolique pour les bactéries, on ne peut omettre la possibilité qu'une telle sous-population existe d'ores et déjà ou qu'elle soit susceptible d'apparaître.

2) Les enzymes modifiant / inactivant les antibiotiques :

Les enzymes capables d'inactiver les antibiotiques en les modifiant chimiquement ont une grande place dans l'arsenal enzymatique constituant le résistome de *M. abscessus*. Leurs mécanismes d'action ainsi que leurs cibles sont variés limitant ainsi les options thérapeutiques.

2a) Les β -lactamines :

Une seule β -lactamase a été à ce jour caractérisée chez *M. abscessus*, MAB_2875, également

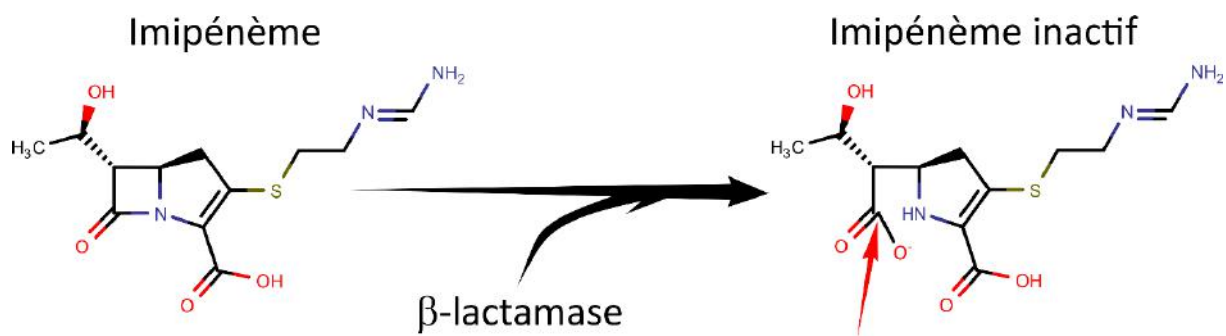


Figure 24. Exemple de dégradation d'une β -lactamine par une β -lactamase. La flèche rouge indique où se situe la dégradation effectuée par une β -lactamase sur l'imipénème (Adapté de Zhai et al. 2012)

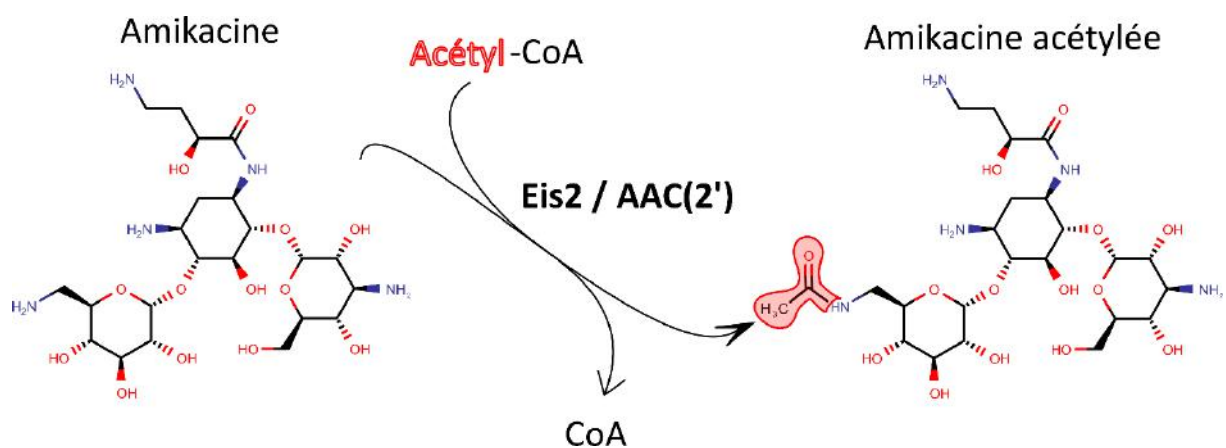


Figure 25. Inactivation enzymatique de l'amikacine par Eis2 et AAC(2'). Ces deux N-acétyltransférases transfèrent un acétyle d'une molécule d'acétyl-CoA sur le groupement amine situé en position 2' du cycle 1 de certains 2-deoxystreptamines aminoglycosides (Adapté de Tran et al. 2018).

appelée *Bla_Mab*, est une β -lactamase de classe A. Il existe quatre classes de β -lactamases (A – B – C – D) séparées en fonction de leurs séquence en acides aminés et par la présence de certains motifs conservés (Bush 2013). Toutes agissent sur le noyau β -lactame des pénicillines, céphalosporines et carbapénèmes (Figure 24). *Bla_Mab* possède une très grande variabilité de substrats, exprimant à la fois une activité pénicillinase, céphalosporinase et carbapénémase, tout en conservant une très bonne efficacité en présence d'inhibiteurs classiques de β -lactamases (Soroka et al. 2014). L'IPM et la CFX sont des substrats de *Bla_Mab* mais il semblerait que ces deux β -lactamines soient en réalité dégradées très lentement, en particulier la CFX, ce qui rend leur utilisation clinique toujours possible. La présence d'un groupement méthoxyle (OCH₃) sur le C7 du noyau β -lactame de la CFX semblerait notamment ralentir encore plus sa dégradation par *Bla_Mab* (Soroka et al. 2014).

2b) Les aminoglycosides :

Chez *M. abscessus*, trois enzymes sont responsables de la résistance aux aminoglycosides. Deux d'entre elles, AAC(2') et Eis2, produits respectifs des gènes *MAB_4395* et *MAB_4532c*, sont des N-acétyltransférases tandis que la troisième est la phosphotransférase *MAB_2385* (Luthra et al. 2018). Ces trois enzymes vont modifier de manière covalente des groupements amines clefs de la structure des aminoglycosides diminuant fortement l'affinité de ces antibiotiques pour l'ARNr 16S de la petite sous-unité du ribosome (Blair et al. 2015).

AAC(2'), pour Aminoglycoside 2'-Acétyltransférase, va transférer un groupement acétyle d'une molécule d'acétyle Coenzyme-A (CoA) sur le groupement amine situé en position 2' du cycle 1 de certains 2-deoxystreptamines aminoglycosides (Maurer et al. 2015). Cette enzyme modifie aussi la gentamycine C, la dibécacine, la tobramycine ainsi que la KAN B. L'inactivation génétique de *MAB_4395* provoque une sensibilité pour les antibiotiques ci-dessus (Rominski, Selchow, et al. 2017). Les protéines Eis (Enanced Interacellulair Survival) sont présentes de manière globale chez les mycobactéries mais aussi chez les Gram +. Eis chez *M. tuberculosis*, en plus d'être aussi impliquée dans la résistance aux aminoglycosides, joue un rôle dans la survie intramacrophagique grâce à son activité acétyltransférase. Eis va bloquer les processus d'autophagie, de maturation phagosomale, de production de cytokines pro-inflammatoires et d'apoptose en inhibant des voies signalétiques dépendantes des ROS (Shin et al. 2010) (K. H. Kim et al. 2012). Même si l'homologie de séquence entre Eis de *M. tuberculosis* et Eis2 de *M. abscessus* est modérée (28 %), la superposition de leurs structures cristallographiques atteste de la similarité des deux protéines (Ung et al. 2019). Des travaux menés avec l'aide d'un mutant *eis2* chez *M. abscessus* a également montré un déclin dans le survie intracellulaire dans des modèles amibiens et macrophagique (Dubois et al. 2019). Il se peut donc qu'Eis2 de *M. abscessus* agit de la même manière qu'Eis de *M. tuberculosis*. Concernant la résistance au 2-

deoxystreptamines aminoglycosides, Eis2 va inactiver, comme AAC(2'), la KAN B, mais aussi la KAN A, l'AMK, l'hygromycine B et la capréomycine en acétylant le premier groupement amine de ces antibiotiques. Un mutant $\Delta eis2$ est largement plus sensible à ces cinq antibiotiques en comparaison avec la souche parentale. Eis1, un autre homologue présent chez *M. abscessus*, ne semble pas jouer un rôle dans la résistance à cet classe d'antibiotiques là (Rominski, Schulthess, et al. 2017). Eis1 est peut-être impliquée dans d'autres processus cellulaire comme la compaction du chromosome bactérien. Cette fonction est rempli par Eis chez *M. tuberculosis*, toujours grâce à son activité acétyltransférase (Ghosh et al. 2016).

La dernière enzyme MAB_2385, est une 3''-O-phosphotransférase responsable de l'inactivation de la streptomycine (SPM), membre de l'autre sous-classe des aminoglycosides, les streptidines. Dal Molin et al. ont identifié MAB_2385 comme étant le plus proche homologue de *aph(3'')-Ic*, une phosphotransférase connue pour être responsable de la résistance à la SPM chez *M. fortuitum* (Ramón-García et al. 2006). Ils ont ensuite montré à l'aide d'une étude génétique basée sur un mutant ΔMAB_2385 et de complémentation fonctionnelle chez *M. smegmatis* que ce gène est en effet responsable de la résistance intrinsèque à la SPM chez *M. abscessus*. Le mutant ΔMAB_2385 était 16 fois plus sensible à la SPM que la souche sauvage. Une analyse phylogénétique a révélé la présence d'autres acétyltransférases (MAB_0247c, MAB_0404c, MAB_0745, MAB_4235c et MAB_4324c) et phosphotransférases (MAB_0163c, MAB_0313c, MAB_0327, MAB_0951 et MAB_1020) assez proches génétiquement pour pouvoir être aussi impliquées dans la résistance aux aminoglycosides et/ou dans la survie intracellulaire (Luthra et al. 2018).

2c) Les ryfamycines :

La rifabutine exhibe une forte activité antibactérienne car elle est capable de contourner les mécanismes de résistance intrinsèque qui touchent les autres rifamycines comme la rifampicine et la rifapentine. Ces antibiorésistances sont conférées par deux types d'inactivations enzymatiques distinctes (Ganapathy et al. 2019).

La rifampicine et la rifapentine sont sujettes à l'auto-oxydation à l'extérieur et l'intérieur de *M. abscessus* car elles possèdent une hydroquinone (groupements hydroxyles en position C1 et C4 du cycle naphthyle) ce qui n'est pas le cas de la rifabutine (Buss et al. 1977) (Figure 26A). La présence de cette partie hydroquinone dans leurs structures les rend également sensibles à la monooxygénase Rox (Rox_{Mab}) qui provoque l'ouverture du cycle naphthoquinone de l'antibiotique et son inactivation (Koteva et al. 2018) (Figure 26B). La meilleure accumulation cytoplasmique, la résistance à l'auto-oxydation et à la monooxygénation par Rox_{Mab} explique le meilleur pouvoir antibactérien de la rifabutine par rapport

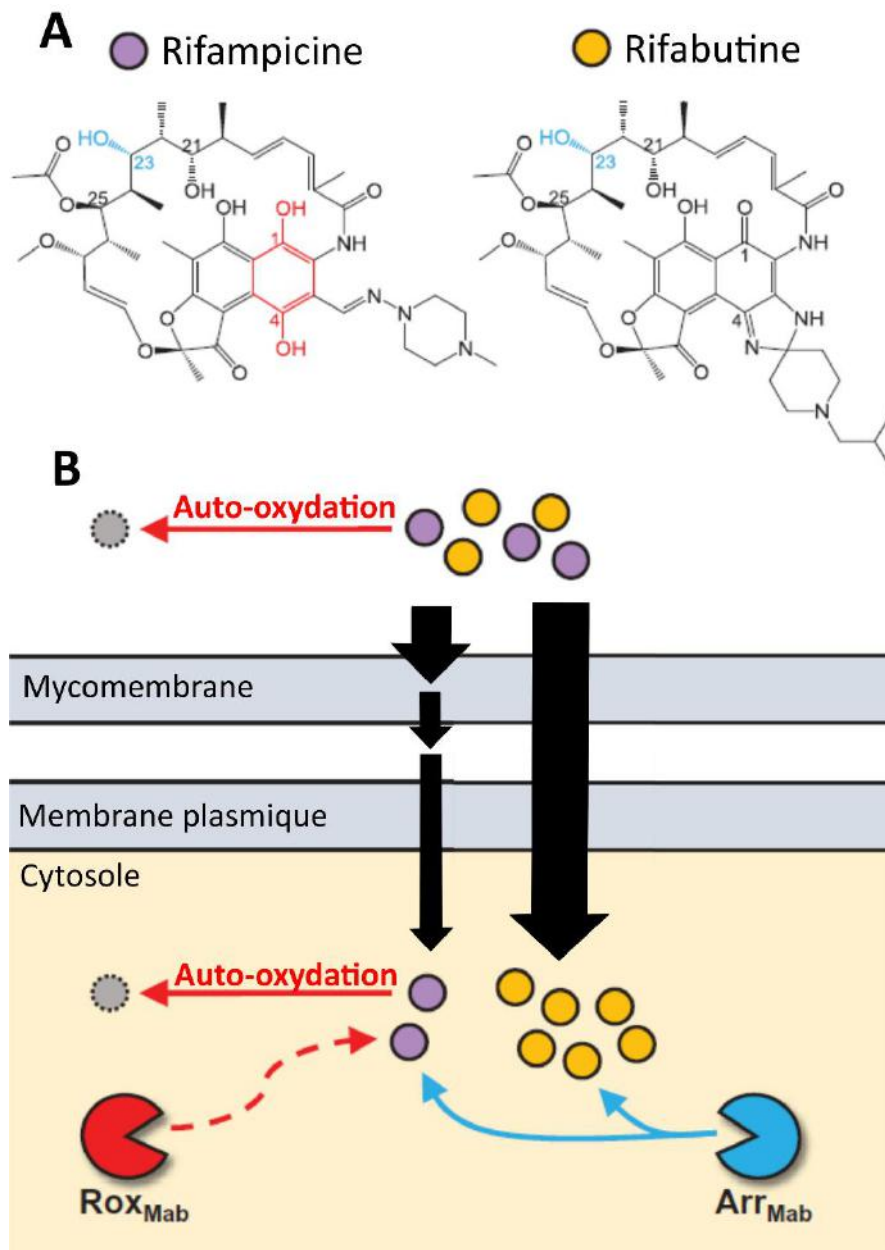


Figure 26. La rifabutine possède des propriétés structurales lui conférant une meilleure absorption ainsi qu'une meilleure résistance à l'auto-oxydation et aux dégradations enzymatiques. (A) Le C23 ADP-ribosylée modifié par Arr_{Mab} est marqué en bleu sur les structures de la rifampicine et de la rifabutine. Le cycle naphtoquinone responsable de l'auto-oxydation et de la dégradation par Rox_{Mab} est marqué en rouge sur la structure de la rifampicine. (B) La rifabutine pénètre de manière plus efficace la paroi mycobactérienne que la rifampicine et possède alors une meilleure pharmacocinétique. La rifabutine n'est pas sensible à l'auto-oxydation de Rox_{Mab} mais ces deux rifamycines sont sensibles à Arr_{Mab} (Adopté de Ganapathy et al 2019).

aux autres rifamycines (Ganapathy et al. 2019). Le mécanisme de résistance majeur concernant toutes les rifamycines, en moindre mesure pour la rifabutine, est leur inactivation enzymatique par l'ADP-ribosyltransférase Arr_{Mab} encodée par le gène *MAB_0591* (Rominski, Roditscheff, et al. 2017) (Figure 26B). La suppression chromosomique d'Arr_{Mab} rend *M. abscessus* extrêmement sensible à la rifampicine et à la rifapentine et son expression hétérologue chez deux organismes naturellement sensibles à ces rifamycines, *E. coli* et *M. tuberculosis*, rend ces deux bactéries très résistante (Rominski, Roditscheff, et al. 2017). Chez *M. smegmatis*, Arr_{Mab} modifie chimiquement le C23 de la rifampicine (Baysarowich et al. 2008). La rifabutine possède le même C23 que les autres rifamycines sensibles mais elle est en réalité moins efficacement ADP-ribosylée par Arr_{Mab}. Il est alors probable que d'autres parties de la molécule ont un rôle dans la résistance à l'inactivation enzymatique, comme pour Rox_{Mab} et le cycle naphthoquinone. En réalité Rominski et al. ont prouvé que des modification du C25 permettent d'augmenter l'activité antimicrobienne des rifamycines. La grande taille de ces molécules ne leurs permet pas d'être activement absorbées par des porines et elles doivent donc traverser la membrane plasmique de manière passive (Lambert 2002). La meilleure accumulation de la rifabutine est possiblement due à des différences structurales qui ne sont pas présentes chez les autres rifamycines et favoriseraient la diffusion à l'intérieur de la bactérie. Ainsi, une optimisation structurale des rifamycines paraît être une option attractive dans le développement de nouveaux antibiotiques.

2d) Les tétracyclines :

La TGC est un autre un bel exemple de modifications structurales conférant une résistance à un mécanisme d'inactivation enzymatique intrinsèque. Cette glycylycylcline possède une chaîne 2-tert-butylglycylamido qui la protège de la FAD-dépendante (Flavine Adénine Dinucléotide) monooxygénase MabTetX (*MAB_1496*) de *M. abscessus* (Rudra et al. 2018). Rudra et al. ont démontré l'hydroxylation de la tétracycline et de la doxycycline chez *M. abscessus* par MabTeTX et sa délétion rend la bactérie 20 fois plus sensible à ces deux antibiotiques, soulignant l'importance de l'activité intrinsèque de cette enzyme. L'expression de MabTetX est régulée par le répresseur de transcription MabTetR_x. Cette protéine codée par le gène *MAB_1497c*, appartient à la superfamille des régulateurs TetR. En l'absence de son ligand, en l'occurrence les antibiotiques de la famille des tétracyclines, MabTetR_x va réprimer sa propre expression ainsi que celle de MabTetX en se liant à une séquence d'ADN cible située en amont de l'opéron *MAB_1497c/MAB_1496c*. Une fois à l'intérieur de la bactérie, les tétracyclines vont interagir avec MabTetR_x, qui libèrera son ADN cible à la suite d'un réarrangement conformationnel ce qui mènera à l'expression de l'opéron *MAB_1497c/MAB_1496c* (Rudra et al. 2018). La TGC est actuellement la seule glycylycylcline utilisée dans la thérapie contre *M. abscessus* mais deux autres composés, l'éravacycline et l'omadacycline sont en cours d'évaluation et exhibent une efficacité *in vitro* similaire à la TGC (Kaushik et al. 2019).

3) Les enzymes protégeant la cible des effecteurs, le cas d'Erm(41) et des macrolides :

La littérature ne recense qu'une seule protéine capable de protéger *M. abscessus* en modifiant biochimiquement la cible d'une classe d'antibiotique. Il s'agit de la méthyltransférase Erm(41) protégeant la boucle du centre peptidyltransférase par méthylation de l'ARNr 23S de la grande sous-unité ribosomale (Maurer et al. 2014). « Erm » est l'acronyme de « Erythromycin Resistance Methylase », ces gènes sont présents chez d'autres genres bactériens, notamment chez les staphylococci et les streptococci ainsi que chez *Campilobacter* mais, contrairement à *M. abscessus*, sont généralement portés sur des éléments génétiques mobiles de types plasmidiques (Schroeder et Stephens 2016) (Bolinger et Kathariou 2017) (Feßler et al. 2018). Ces enzymes catalysent sur l'atome d'azote N6 de l'adénine en position A2058 de l'ARNr 23S le transfert de un ou deux groupements méthyles depuis de la S-adénosyl-L-méthionine. Cette réaction va produire de la S-adénosyl-homocystéine et ces groupements méthyles vont briser la liaison hydrogène entre le C2'-OH du le sucre désosamine des macrolides et l'A2058. La gêne stérique occasionnée par cette simple ou double méthylation va gravement diminuer l'affinité de ces antibiotiques. Il a été rapporté chez plusieurs espèces bactériennes Gram - et Gram +, notamment *S. aureus*, que la diméthylation offre un moyen de résistance plus efficace que la monométhylation contre les macrolides mais aussi contre les lincosamides et la streptogramine B (Weisblum 1995) (Maravić 2004). C'est en 2009 que Nash et al. ont fourni la première, mais aussi la plus complète, des études sur la résistance inductibles aux macrolides *via* Erm(41) chez *M. abscessus*. Ils ont montré que comme *S. aureus* et sa diméthyltransférase ErmC, *M. abscessus* est résistant aux macrolides, aux lincosamides et à la streptogramine B, supportant l'hypothèse d'une activité N, N6-diméthyltransférase d'Erm(41). Les gènes *erm* ne sont pas présents chez toutes les mycobactéries, comme par exemple chez *M. chelonae*, contre qui les macrolides sont théoriquement efficaces en l'absence de mutation chromosomique du gène *rrl*. On retrouve néanmoins ces gènes *erm* chez *M. tuberculosis*, *M. smegmatis*, *M. fortuitum* et *M. mageritense*. En termes de phylogénie, *erm(41)* est plus proche d'*erm(37)* de *M. tuberculosis* et leurs séquences sont très différentes des trois autres espèces. La position d'*erm(41)* sur le chromosome est également unique suggérant que *M. abscessus* aurait acquis ce gène indépendamment des autres mycobactéries, probablement à la suite d'un transfert horizontal de matériel génétique provenant d'une autre bactérie (Nash et al. 2009). Chez la sous-espèce *M. massiliense*, *erm(41)* est tronquée, un segment de 198 nucléotides situés au centre du gène a disparu, réduisant le cadre de lecture de 522 à 324 pb. Une hypothèse est que suite à l'absence de pression de sélection, la bactérie a tout simplement perdu une partie de son patrimoine génétique n'en ayant pas l'utilité, rendant cette protéine non-fonctionnelle. Contrairement à certaines autres bactéries où l'expression des gènes *erm* est constitutive, la résistance de *M. abscessus* aux macrolides est un phénomène inductible et qui augmente de manière quasi-exponentielle au fil du temps (Maravić 2004)

(Maurer et al. 2014). En effet, la CMI de la CLR et de l'AZM doit être évaluée sur 14 jours car, lorsqu'on la détermine de manière classique au bout de 3 ou 4 jours, sa valeur varie entre de 1 et 32 $\mu\text{g.mL}^{-1}$ alors qu'elle est ensuite supérieure à 256 $\mu\text{g.mL}^{-1}$ au bout de 14 jours. Il existe une relation entre pouvoir inductible des macrolides et la présence dans leur structure d'un sucre, le cladinose (Douthwaite 2001). Cependant, la télithromycine, un macrolide de la sous-classe des kétolides ne possède pas de cladinose mais subi quand même ce phénomène de résistance inductible (Nash et al. 2009). Chez *M. tuberculosis* et *M. abscessus*, cette induction est directement liée à la présence de l'activateur de transcription WhiB7 (Burian et al. 2012) (Ramón-García et al. 2013) (Hurst-Hess et al. 2017). Les gènes *whiB* sont propres aux actinomycètes, les autres bactéries résistantes aux macrolides doivent donc posséder d'autres stratégies de régulation. L'absence de ces activateurs de transcription peut également expliquer le caractère constitutif de l'expression des gènes *erm* chez certains de ces organismes.

4) Export actif par des pompes à efflux :

Les pompes à efflux sont des protéines transmembranaires abondantes chez les mycobactéries. Elles sont responsables de l'homéostasie de la cellule bactérienne en réalisant l'export de composés majeurs de la paroi, de facteurs de virulence ainsi que de toxines et de leurs possibles métabolites. Ces protéines ont donc une large variété de substrats, ce qui peut parfois leurs permettre d'expulser hors du cytosol, une ou plusieurs classes d'antibiotiques aux structures éloignées en plus de leur substrat premier. La perméabilité ajustable de la paroi mycobactérienne par ces pompes à efflux représente un premier mécanisme de résistance intrinsèque de plus ou moins grande efficacité (Louw et al. 2009). Ce phénomène d'export n'est pas à l'heure actuelle aussi bien décrit chez *M. abscessus* que chez d'autres bactéries, mais il a déjà été démontré qu'il concerne le LNZ et des dérivés structuraux du TAC. L'export de l'AMK et possiblement d'AZM et de rifamycines serait dû à un seul transporteur transmembranaire MAB_1409c (MAB_{Tap}) appartenant à la famille MFS (Major Facilitator Superfamily), un homologue de la pompe à efflux Tap de *M. tuberculosis*. La fonction première de Tap chez *M. bovis* BCG et vraisemblablement comme chez d'autres mycobactéries n'est pas l'efflux d'antibiotique. Son rôle principal est en réalité de détoxifier le cytoplasme après le début de l'entrée en phase stationnaire (Ramón-García et al. 2012).

4a) L'export de l'AMK :

L'efflux de cet aminoglycoside chez *M. abscessus* implique une fois de plus l'activateur de transcription WhiB7. Hurst-Hess et al. ont identifié par RNA-Seq le régulome complet de WhiB7 et ont généré un mutant $\Delta\text{whiB7}/\text{MAB}_{3508c}$ chez *M. abscessus*. Ils ont alors identifié MAB_{Tap} comme une cible de WhiB7 et la délétion de *MAB_3508c* rend *M. abscessus* 4 fois plus sensible à l'AMK.

L'acétyltransférase Eis2 est aussi sous le contrôle de WhiB7. Des isolats cliniques résistant à l'AMK possédaient à la fois des mutations du gène *rrs* mais également une surexpression des transcrits de *whiB7* et de *MAB_{Tap}* ou une surexpression de *eis2* (Wu et al. 2019). Il semblerait donc que la résistance à l'AMK soit multifactorielle chez *M. abscessus*, combinant à la fois export basal, mutation chromosomique et inactivation enzymatique.

4b) Le cas des rifamycines et de l'AZM :

L'implication de *MAB_{Tap}* dans l'export de ces deux familles de molécules ne repose pour le moment sur aucune donnée expérimentale mais plutôt sur les comparaisons de degrés d'homologie entre protéines. Tap est responsable de l'export de la RFP chez *M. tuberculosis* (Szumowski et al. 2013). D'après sa grande homologie de structure secondaire avec *MAB_{Tap}* (Schmalstieg et al. 2012), Ganapathy et al. suggèrent que *MAB_{Tap}* exporte de la rifampycine et de la rifabutine chez *M. abscessus*. La perméase de *M. avium*, MAV_1406 est impliquée dans l'export d'AZM et partage aussi un bon degré d'homologie avec *MAB_{Tap}* (Schmalstieg et al. 2012). Ces deux phénomènes d'efflux sont possibles mais ne mèneraient probablement qu'à de faibles niveaux de résistances basales, étant donné la présence de systèmes de résistance beaucoup plus performants.

4c) L'export du LNZ :

Classiquement, la résistance au LNZ est associée à la présence de mutations dans sa cible, le gène *rrl* codant l'ARNr 23S. Cependant chez *M. abscessus*, il semblerait que dans 25% des cas, cette résistance ne soit pas due à des mutations du gène cible mais plutôt dans des régulateurs de transcription potentiels (Ye et al. 2019). Toutes ces souches surproduisaient les transcrits de deux pompes à efflux, l'une appartenant aussi à la famille MFS comme *MAB_{Tap}*, LmrS, et l'autre est un transporteur de la famille RND (Resistance Nodulation cell Division), le MmpL *MAB_4746*. *M. abscessus* acquiert potentiellement des mutations dans des répresseurs qui ne sont alors plus en mesure de réguler ces deux pompes à efflux qui extrudent le LNZ.

4d) L'export des analogues structuraux du TAC (TACa) :

L'optimisation structurale du TAC a notamment généré trois analogues désignés D6, D15 et D17 avec une très bonne activité antibactérienne contre à la fois *M. tuberculosis* et *M. abscessus* (Halloum et al. 2017). Cette étude a révélé que la surexpression du transporteur MmpS/MmpL codé par l'opéron *MAB_4383c/MAB_4382c* était due à des mutations dans un régulateur de transcription situé juste en amont. Des mutants spontanés à ces dérivés exhibent de très hauts niveaux de résistances à D6, D15 et D17 parfois supérieur à un facteur 100. Ces phénotypes sont en parfaite corrélation avec l'importante production des transcrits de la pompe à efflux *MAB_4383c/MAB_4382c*.

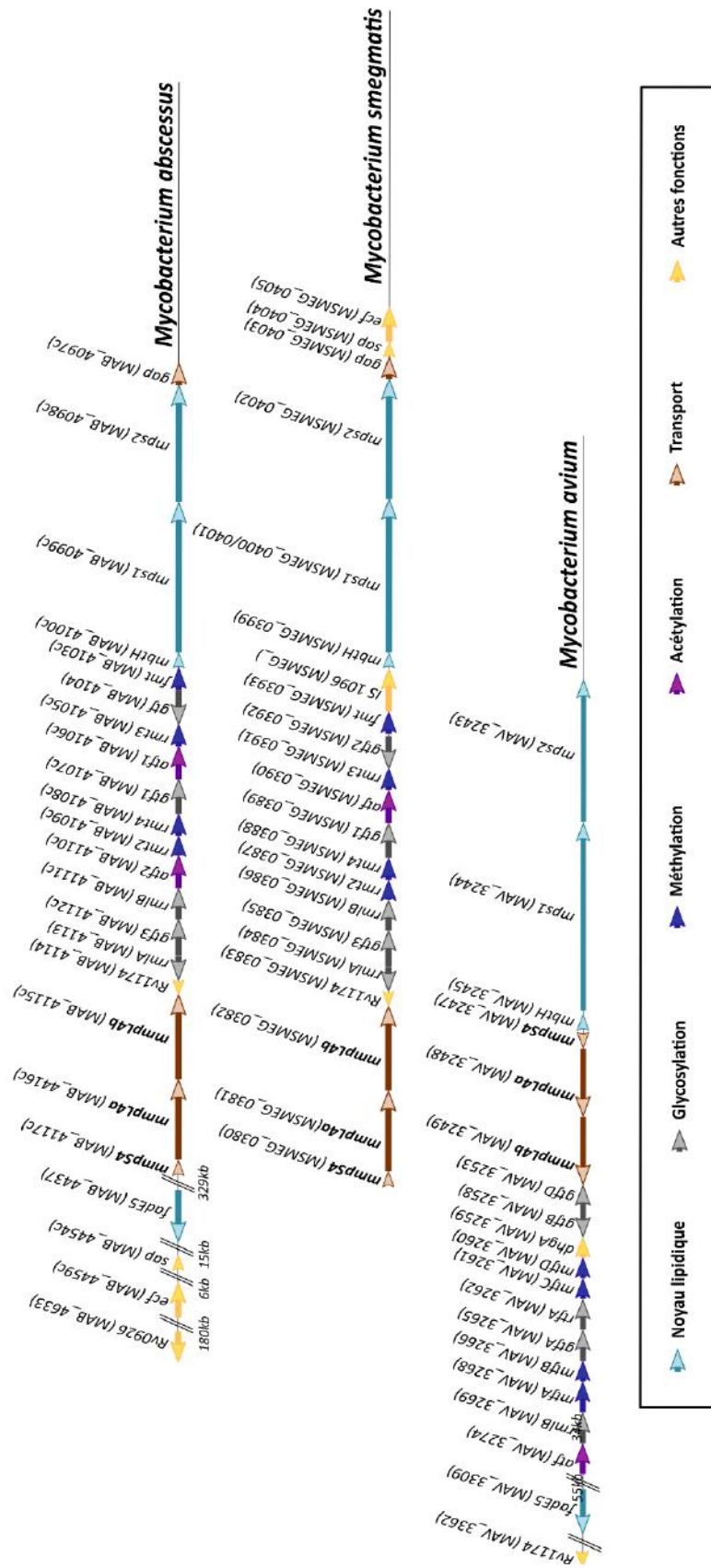


Figure 27. Organisation génomique des locus *gpl* de *M. abscessus*, *M. smegmatis* et *M. avium* (Adapté de Gutiérrez et al. 2018).

1) Propriétés génétiques :

Les *mmpL* sont exclusifs aux mycobactéries, mais on retrouve des orthologues relativement proches de ces gènes chez d'autres actinomycètes, notamment chez le genre *Corynebacterium*, *Rhodococcus* et *Nocardia*. Ces trois genres bactériens possèdent des lipides ayant une structure proche de celle des acides mycoliques mycobactériens (Nataraj et al. 2015). Chez les mycobactéries, les *mmpL* sont des gènes de grande taille, près de 3000 pb. On retrouve également en opéron avec certains *mmpL* des gènes de plus petites taille, d'environ 500 pb, appelés Mycobacterial Membrane Protein Small (*mmpS*). L'hypothèse est que ces protéines épaulent les MmpL dans leur rôle de transport mais ne sont pas indispensables étant donné qu'ils ne sont pas retrouvés à proximité de tous les *mmpL*. En effet, *M. tuberculosis* possède 13 *mmpL*, mais seulement 4 *mmpS*, supposément co-transcrits avec *mmpL1*, *mmpL2*, *mmpL4* et *mmpL5* (Chalut 2016). La même observation est faite pour *M. abscessus*, qui possède 24 *mmpS* et 31 *mmpL* (Viljoen et al. 2017). Une caractéristique frappante liée à l'environnement génomique des *mmpL* est de la présence de gènes impliqués dans la biosynthèse des molécules que ces protéines exportent. Chez *M. tuberculosis*, les MmpL connus pour leurs implications dans le transport de lipides retrouvés dans la paroi sont plus ou moins proches de gènes codants des Polyketides Synthases (*pks*), indispensables à la synthèse de lipides complexes, à l'exception de MmpL3 et 11 (Chalut 2016). Le locus impliqué dans la production et l'export des GPL est le mieux caractérisé chez *M. abscessus* et reflète avec fidélité ce qui est observé chez *M. tuberculosis* (Gutiérrez et al. 2018). L'opéron composé de *mmpS4–mmpL4a–mmpL4b* responsable de l'export de ces molécules est entouré de gènes impliqués dans la biosynthèse et l'assemblage des parties glucidique, peptidique et lipidique composant les GPL. Il a été démontré chez *M. smegmatis* qu'une autre protéine accessoire est impliquée dans la translocation des GPL au travers de la membrane plasmique (Sondén et al. 2005). Il s'agit de la protéine Gap, dont le gène est situé à l'extrémité du locus *gpl* chez *M. abscessus*. Il est fort probable que d'autres protéines accessoires jouent un rôle primordial d'assistance dans le transport effectué par les MmpL. Il existe une certaine synténie dans la répartition des *mmpL* au sein du génome des mycobactéries. Des exemples convaincants montrent que si ces loci sont conservés entre deux espèces, alors la fonction du transporteur et les molécules qu'il exporte sont présentes chez ces dernières. Ceci est le cas pour le locus de *mmpL3* qui est strictement conservé parmi les espèces *M. tuberculosis*, *M. leprae*, *M. avium*, *M. marinum* et *M. abscessus* (Viljoen et al. 2017). Ces observations étaient anticipées étant donné que *mmpL3* est le seul MmpL essentiel chez les mycobactéries, ce qui en fait donc une cible thérapeutique extrêmement intéressante. Le locus GPL est très conservé entre *M. abscessus*, *M. avium* et *M. smegmatis*, trois espèces productrices de GPL (Gutiérrez et al. 2018) (Figure 27). A l'opposé, la non-conservation entre espèces de ces blocs

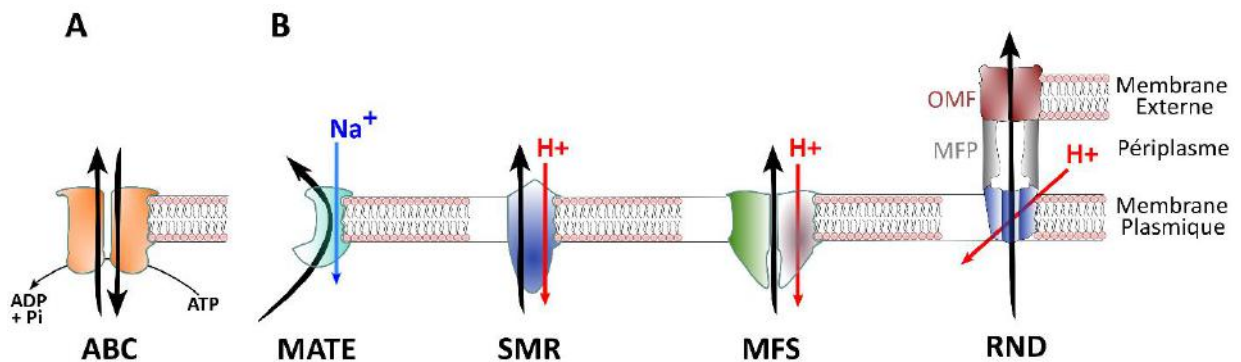


Figure 28. Représentations schématiques des deux superfamilles de transporteurs retrouvées chez les mycobactéries. (A) Les transporteurs primaires ABC utilisent l'hydrolyse de l'ATP pour transporter à travers la membrane plasmique leurs substrats et sont des homodimères ou des hétérodimères. (B) Les transporteurs MATE sont les seuls à utiliser un gradient de sodium afin d'expulser leurs substrats du cytoplasme et sont monomériques. Les transporteurs SMR, MFS et RND utilisent la PMF pour extruder leurs substrats vers le milieu extérieur et sont respectivement des monomères, des homodimères ou des hétérodimères et des systèmes tripartites. ABC, ATP-binding cassette ; MATE, multidrug and toxic compound ; SMR, small multidrug resistance ; RND, resistance nodulation cell division ; MFP, membrane fusion-like protein ; OMF, outer-membrane factor, PMF, force protonotrice (Adapté de Lubelski et al. 2007).

synténiques résultent en une perte de fonction de ces *mmpL*. Par exemple ce locus *gpl* est éparsé chez les espèces où ces molécules sont absentes, comme chez *M. tuberculosis* ou encore *M. leprae* (Viljoen et al. 2017). La fonction et la nature des molécules exportées par les MmpL sont très peu connues chez *M. abscessus*. Cependant, il est possible d'acquérir des informations préliminaires, voire même de prédire la fonction de certains de ces transporteurs, à l'aide d'études phylogénétiques et bio-informatiques. Un exemple de ce type d'approche est la recherche de séquences d'acides aminés bien précises parmi les protéines Pks. Ces séquences d'acides aminés sont caractéristiques du lipide que les Pks synthétisent (Yadav et al. 2003).

2) Propriétés biochimiques et structurales :

Les pompes à efflux bactériennes peuvent être séparées en deux superfamilles présentes chez tous les procaryotes. Cette différenciation est basée sur la façon dont elles se procurent l'énergie nécessaire au transport actif de leurs substrats.

La première est la superfamille des transporteurs ABC (ATP-Binding Cassette), également appelée transporteur primaire (Figure 28A). Ces protéines transmembranaires vont obtenir de l'énergie en fixant et hydrolysant l'ATP *via* leur domaine cytoplasmique (Locher 2009). Ces transporteurs vont généralement importer à l'intérieur du cytosol des nutriments hydrophiles comme des aminoacides ou des sucres mais aussi des ions ainsi que des sidérophores. Ces transporteurs expulsent également des molécules toxiques ou antigéniques.

La seconde, celle des transporteurs secondaires, regroupe 4 familles de protéines qui tirent leur énergie d'un gradient électrochimique dû à différente concentration de sodium ou de proton de part et d'autre de la membrane expulsent tous type de substrats hors du cytoplasme (Lubelski et al. 2007) (Figure 28B). Un seul type de transporteur utilise un gradient de sodium, il s'agit des MATE (Multidrug And Toxic compound Extrusion). Les trois autres type de transporteurs secondaires utilisent tous les trois la force proton-motrice (PMF) entre le milieu extérieur et le cytoplasme pour expulser leurs substrats et sont donc séparés par rapport à leurs caractéristiques structurales. On retrouve donc les transporteurs MFS, comme MAB_{Tap}, les transporteurs SMR (Small multiDrug Resistance) et les transporteurs RND dont les MmpL font partis, L'organisation des RND est plus complexe et la PMF s'effectue grâce à un gradient de proton entre le périplasma et le cytosol. Ce sont des systèmes tripartites, composé d'une protéine périplasmique MFP (Membrane Fusion-like Protein) qui relierait le transporteur transmembranaire homotrimerique à une protéine insérée dans la membrane externe appelée OMF (Outer-Membrane Factor). Ils ne sont pas présents chez les Gram +. Les connaissances sur la présence avérée de ces protéines MFP et OMF pour les MmpL n'est pas démontrée

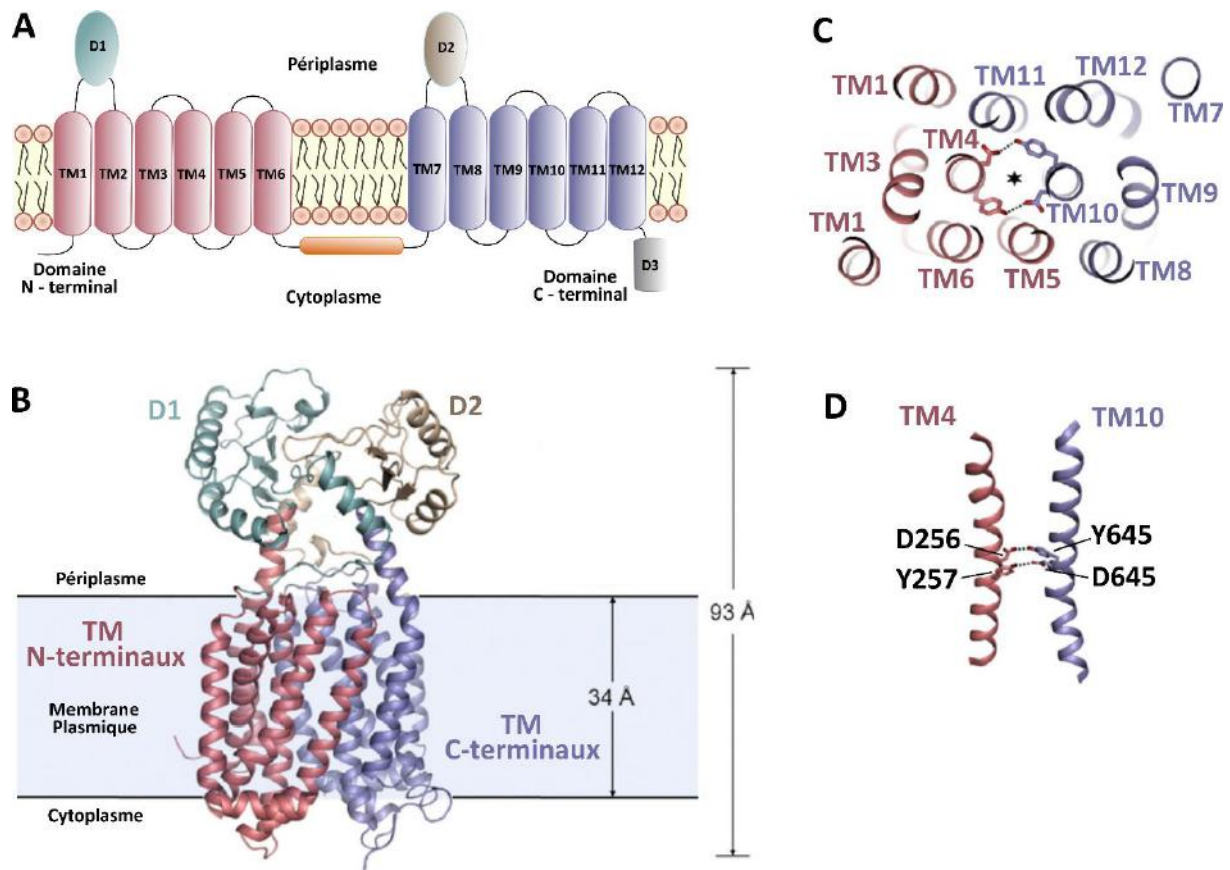


Figure 29. Organisation structurale des MmpL. Schéma représentant les 6 domaines transmembranaires N-terminaux en rouge (TM1-6) et les 6 domaines transmembranaires C-terminaux en bleu (TM7-12). Les domaines périplasmiques D1 et D2 sont représentés en turquoise et beige respectivement. L'hélice cytoplasmique reliant les domaines N- et C-termini est en orange et le domaine cytoplasmique D3 en gris. (B) Structure 3D de MmpL de *M. smegmatis* reprenant le même code couleur que décrit en (A). (C) Organisation spatiale des 12 domaines transmembranaires avec le TM4 et le TM10 formant un canal. (D) Positionnement des résidus critiques D256 et Y257 pour le TM4 et Y645 et D645 pour le TM10 responsables du transport des électrons (Adapté de Viljoen et al. 2017 et de Zhang et al. 2019)

expérimentalement mais est cependant très probable et pourrait inclure certaines lipoprotéines (Melly et Purdy 2019). Les prédictions faites après analyses de la séquence des MmpL et de leurs homologies avec d'autres transporteurs RND proposent qu'ils soient composés de 11 ou 12 hélices transmembranaires (TM1 à TM12) (Figure 29A). On retrouve deux domaines périplasmiques (D1 et D2) situés entre les TM1 et 2 et les TM7 et 8. Il a été proposé que ces régions solubles aient un rôle dans la liaison et le transport des substrats (Chim et al. 2015). Le domaine N-terminal contient entre 5 et 6 TM et la partie C-terminale est composée de 6 TM. La jonction entre les domaines N et C terminaux est due à la présence d'une hélice cytoplasmique positionnée parallèlement à la membrane plasmique. La partie C-terminale est cytoplasmique (D3) (Viljoen et al. 2017). L'ensemble de ces hypothèses a été fortement appuyé récemment grâce à l'obtention de la structure 3D à très haute résolution de MmpL3 de *M. smegmatis* (Zhang et al. 2019) (Figure 29B). Le passage des protons créant la PMF nécessaire au transport des substrats hors du cytoplasme est relié par des résidus aspartate et tyrosine extrêmement conservés parmi les TM4 et TM10 des MmpL (Figure 29C). C'est grâce à une combinaison d'étude génétique et bio-informatique que Bernut et al. ont pu mettre en avant l'importance des résidus D265 du TM4 et de la paire D625 – Y626 du TM10 dans le relais des protons à travers MmpL4a pour le transport des GPL chez *M. boletii*. Ils ont aussi démontré l'importance des résidus D251 du TM4 et de la paire D640 – Y641 du TM10 de MmpL3 de *M. tuberculosis* à l'aide d'expérience de complémentation fonctionnelle. Chez *M. smegmatis*, la cristallographie aux rayons X de MmpL3 révèle deux paires aspartate – tyrosine. Il s'agit des paires D256 – Y257 et D645 – Y646 situées respectivement dans les TM4 et TM10 (Zhang et al. 2019) (Figure 29D).

3) Rôles et fonctions des MmpLs :

Ces données restent éparées concernant *M. abscessus*, mais seront affinées prochainement compte tenu de l'évolution des techniques de lipidomiques et de génétiques appliquées spécialement à ces mycobactéries. C'est chez *M. tuberculosis* que les informations à ce sujet sont les plus importantes, avec les fonctions de 6 protéines MmpL identifiées sur un total de 13 transporteurs. Les rôles les plus connus mais aussi le plus étudiés concernent en général le transport de lipides majeurs à travers la paroi mycobactérienne, l'import passif de fer, l'export de sidérophores et d'antibiotiques.

3a) Export des lipides :

En ce qui concerne le transport de lipides chez *M. abscessus*, l'implication de trois MmpL a été confirmée avec certitude.

MmpL3, pour le transport du TMM, est encodée par le gène *MAB_4508*. L'utilisation d'une nouvelle classe d'inhibiteur et l'étude du profil lipidique en découlant a montré une accumulation du

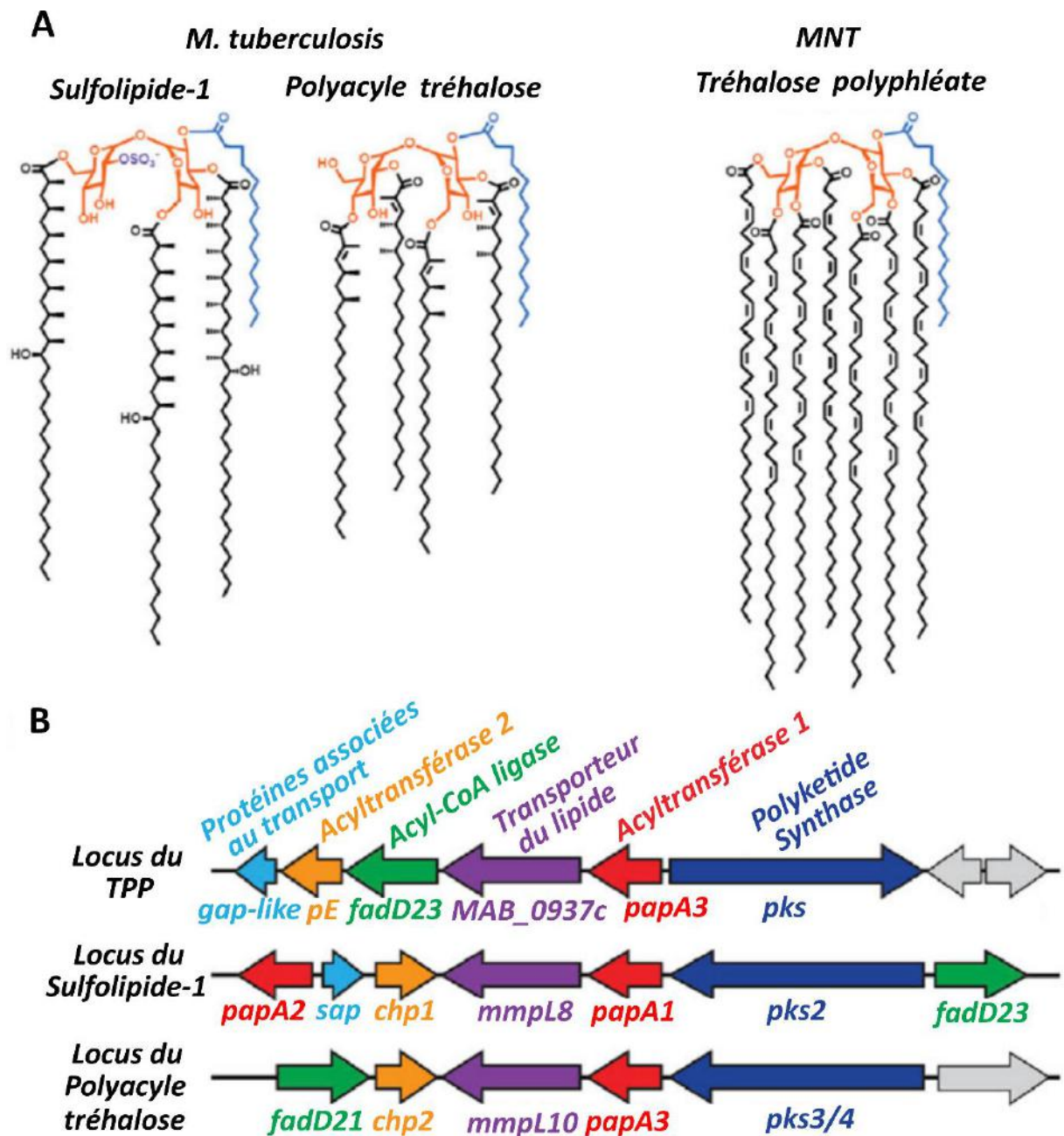


Figure 30. Le tréhalose polyphléate de MNT semble être une chimère structurale (A) et génétique (B) entre le sulfolipide-1 et le polyacyl tréhalose de *M. tuberculosis*. MAB_0937c est un homologue proche de gène de MmpL10 qui transporte les polyacyles tréhaloses (Adapté de Seeliger et Moody 2016).

TMM cytoplasmique ainsi qu'une disparition concomitante de TDM et de la mycolylation de l'arabinogalactane, confirmant le rôle de « MmpL3 » du gène *MAB_4508* (Dupont et al. 2016).

L'opéron *MAB_4117c – MAB_4116c – MAB_4115c* responsable de la formation du complexe MmpS4 – MmpL4a – MmpL4b transportant les GPL hors du cytosol a également été bien caractérisé. Ce rôle de transporteur des GPL est également élucidé chez *M. smegmatis* et *M. avium* (Gutiérrez et al. 2018).

Le troisième est le MmpL encodé par le gène *MAB_0855* qui est responsable du transport d'un lipide exclusivement retrouvé chez *M. chelonae* et chez le complexe *M. abscessus*. Un mutant Δ *MAB_0855* perd en grande partie sa capacité de survie intracellulaire dans un modèle amibien, macrophagique et *in vivo* chez le zebrafish. Ceci est dû à l'absence d'un glycolipide unique, le GDND (Glycosyl Diacylated Nonadecyl Diol), sans qui une adhésion optimale au macrophage et une dégradation de la membrane phagolysosomale n'est possible. Cette dégradation n'ayant pas lieu, la communication avec le cytoplasme est impossible et il n'y a donc pas inhibition de la réponse immunitaire, ce qui réduit fortement la capacité de survie intramacrophagique (Dubois et al. 2018).

Le TPP est un glycolipide composé d'une molécule de tréhalose sulfatée et multi-acylée de très grande taille que l'on retrouve uniquement chez les MNT. Ces lipides sont synthétisés par un opéron « *fadE5-gap-like* » conservé parmi les MNT (Seeliger et Moody 2016). Chez *M. smegmatis*, les délétions du *mmpL* de l'opéron et de l'acyltransférase *pE* provoquent une disparition du TPP de la mycomembrane (Burbaud et al. 2016). Ce même groupe a également montré que chez *M. abscessus* la délétion de *pE* a le même effet. L'organisation génomique de ces deux loci *fadE5-gap-like* est très conservée entre ces deux MNT, il est donc très probable que le MmpL *MAB_0937c* de ce locus chez *M. abscessus* soit responsable de l'export des TPP. La génération d'un mutant *MAB_0937c* permettrait d'affirmer son rôle avec certitude. La fonction des TPP, étant donnée leur taille et leur hydrophobicité, est avant tout structurale mais pourrait aussi être impliqué dans le *cording* du morphotype R et donc son hypervirulence (Llorens-Fons et al. 2017). Le TPP pourrait être décrit comme un hybride structural de SL-1 et de PAT (PentAcyl Trehalose), une sorte d'araignée à 8 pattes (*eight-legged spider*) comme aiment à l'appeler Seeliger et Moody (Figure 30A). C'est en effet comme si ces deux lipides complexes avaient fusionnés pour produire cet imposant glycolipide. L'opéron *fadE5-gap-like* des TPP possèdent un mélange de gènes que l'on retrouve seulement dans le locus de SL-1 et celui de PAT, comme par exemple *fadD23* et *papA3* respectivement (Figure 30B). En plus de MmpL3 chez *M. tuberculosis*, MmpL7 transporte les PDIM vers la mycomembrane. Le PDIM a un rôle important dans la virulence et l'imperméabilité de la paroi mycobactérienne mais aussi dans la résistance à des détergents. (Camacho et al. 2001).

Le SL-1 fait partie de la même famille de glycolipides que le TPP mais est lui organisé autour d'une molécule de tréhalose sulfatée et tetra-acylée. Ce glycolipide est capable de moduler la réponse immunitaire de l'hôte en altérant la maturation phagolysosomale, la production de ROS et de cytokines et serait vraisemblablement transporté par MmpL8 (Converse et al. 2003) (Seeliger et al. 2012).

Belardinelli et al. ont démontré que le DAT est transporté par MmpL10 et se retrouvera alors comme le PAT dans la mycomembrane. Ils proposent que le PAT soit formé dans le périplasme à partir de trois molécules de DAT par une réaction de transestérification réalisée par l'acyltransférase Chp2 qui produira également trois molécules de monoacyl-tréhalose. Le PAT n'est donc pas exporté par MmpL10 mais résulte d'un produit de la translocation de DAT par ce transporteur. Le DAT et le PAT modulent aussi la réponse immunitaire orchestrée par le macrophage en agissant sur le phagosome en bloquant son acidification (Passemar et al. 2014). Grâce à un mécanisme original de recyclage, ces tréhaloses polyacylés vont indirectement détoxifier le cytoplasme de leur hôte et limiter son stress métabolique (Lee et al. 2013). Une fois phagocyté, les principaux nutriments accessibles à *M. tuberculosis* sont les acides gras et le cholestérol. Leurs dégradations par ces mycobactéries vont générer du propionyl-CoA, dont les intermédiaires métaboliques peuvent se révéler toxiques à long terme. Les bacilles vont alors incorporer ce propionyl-CoA dans la chaîne de biosynthèse du DAT en l'utilisant pour estérifier le tréhalose. Ces molécules participent donc indirectement à un autre mécanisme de survie intramacrophagique.

Pacheco et al. ont mis en évidence chez *M. smegmatis* l'importance de MmpL11 de *M. tuberculosis* dans le transport des MMDAG (MonoMeromycolyl DiAcylGlycerol) et MEW (Mycolic Ester Wax) grâce à des expériences de complémentation fonctionnelle. Ce MmpL11 est impliqué dans la formation de biofilms.

3b) Système d'acquisition du fer

Le fer est un nutriment essentiel à la survie des mycobactéries comme de tout être vivant. Au cours de l'infection, le système immunitaire de l'hôte va tenter de priver au maximum l'accès au fer des pathogènes. Mais les mycobactéries ont su s'adapter en devenant capables de récupérer ce fer libre dans la lumière phagosomale ou associé à des structures de stockage, comme des gouttelettes lipidiques. La stratégie pour palier à cette privation de fer est axée autour de l'import/export de sidérophores : la MycoBacTine (MBT) et la CarboxyMycoBacTine (cMBT), qui vont chélater, séquestrer les cations à leur proximité. (Sandhu et Akhter 2017). Les MBT et cMBT sont transportées par les systèmes MmpS4-MmpL4 et MmpS5-MmpL5 chez *M. tuberculosis*. Wells et al. en sont parvenus à ces conclusions grâce à un travail colossal comprenant de multiples inactivations géniques ainsi que des études biochimiques, structurales et *in vivo* chez la souris. Ils ont pu également déduire que *mmpS4* et *mmpS5* sont impliqués dans la synthèse de ces sidérophores et seraient également des adaptateurs

périplasmiques pour l'ancrage de leurs MmpL respectifs. Les bacilles mutés présentait des défauts de croissance sur milieu limité en fer et la mortalité des souris infectées avec ces mutants était nulle même au bout de 180 jours. Le système par lequel les sidérophores retournent jusqu'aux mycobactéries n'a pas encore été découvert. Il a été montré que MmpL3 et MmpL11, avec l'aide de la protéine Rv0203, ont la capacité d'importer le fer endogène lié à des cofacteurs de l'hôte jusqu'à l'intérieur du cytoplasme (Owens et al. 2013). Les sidérophores ainsi que leurs transporteurs semblent tous comme MmpL3 être des candidats de choix dans le développement de nouveaux pharmacophores.

Le ou lesquels des *mmpS* et/ou *mmpL* impliqués dans l'homéostasie du fer n'ont pas encore été identifiés chez *M. abscessus*. Cependant quatre *mmpL*, dont les rôles n'ont pas encore été définis, organisés en deux clusters *MAB_4116c – MAB_4115c* et *MAB_4704c – MAB_4703c* sont phylogénétiquement proches de *mmpL4* de *M. tuberculosis* et pourraient être alors remplir la fonction d'exporter les sidérophores.

3c) Export d'antibiotiques :

A l'exception des travaux publiés faisant corps avec cette thèse, peu d'informations sont disponibles sur le rôle des MmpL dans l'export d'antibiotiques chez *M. abscessus*. L'une de ces études a été le pilier de l'article 1 présent dans ces travaux de thèse. Il s'agit de l'étude réalisée par Halloum et al. impliquant un homologue de *mmpS5/mmpL5* de *M. tuberculosis*, la pompe à efflux *MAB_4383c/MAB_4382c* conférant une résistance TACa. (Partie détaillée en section « **Chapitre IV) L'Antibiorésistance chez *M. abscessus*, 4d) Export actif des analogues structuraux du TAC (TACa) »** »)

L'autre étude impliquant un MmpL dans l'export d'antibiotique est celle de Ye et al. qui ont mis en avant l'implication du MmpL MAB_4746 dans la résistance au LNZ (discutée dans la section « **Chapitre IV) L'Antibiorésistance chez *M. abscessus*, 4e) Export du LNZ »** »)

Concernant *M. tuberculosis*, deux MmpL sont impliqués dans l'efflux d'antibiotiques, parmi lesquels font partis MmpS5/MmpL5, responsable de l'export d'azole, de CFZ et de BDQ ainsi que le transporteur des PDIM, MmpL7, exportant hors de la bactérie l'INH.

Historiquement, le premier MmpL impliqué dans la résistance à un antibiotique est MmpL7. Pasca et al. en 2005 ont attribué ce rôle à ce transporteur en introduisant le MmpL7 de *M. tuberculosis* chez *M. smegmatis*, ce qui provoqua une forte augmentation de sa résistance à l'INH.

C'est ensuite en 2009 que MmpS5/MmpL5 a été incriminé dans la résistance aux azoles, des molécules de petites tailles généralement utilisées dans le traitement d'infections fongiques (Milano et al. 2009). Les mutants spontanés qu'ils ont généré exprimaient des niveaux de résistance modérés, 4 et 8 fois supérieur à la souche sauvage. Leurs analyses ont révélé un haut taux d'expression des transcrits de MmpS5/MmpL5, liés à des mutations dans le régulateur de transcription juxtaposé

Rv0678, appartenant à la famille MarR (Multiple antibiotic resistance Regulator). En 2014, Hartkoorn et al. ont mis en avant la résistance croisée à deux antibiotiques d'intérêts thérapeutiques majeurs dans le traitement de MDR-Tb, la CFZ et la BDQ. Ils ont utilisé la même stratégie en générant des mutants spontanés qui possédaient encore une fois tous des mutations dans *Rv0678*. Les niveaux de résistance observés étaient similaires. MmpS5/MmpL5 de *M. tuberculosis* et MAB_4383c/MAB_4382c de *M. abscessus* sont des homologues mais ne partagent apparemment pas la même spécificité de substrats. La force respective de leurs promoteurs peut jouer un rôle déterminant dans le contraste observé entre les deux niveaux de résistance.

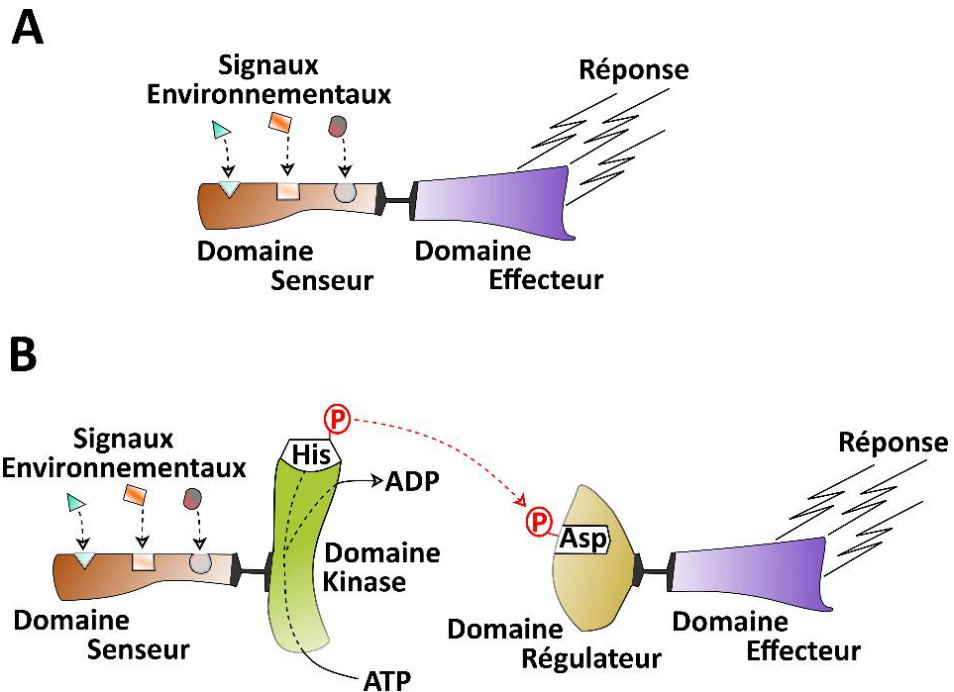


Figure 31. Représentation schématique des deux principaux types de système de régulation procaryotique. (A) Système à un composant. Une seule protéine comporte à la fois un domaine senseur et un domaine effecteur. Chaque domaine peut être en N- ou C-terminal. (B) Système à deux composants. La première protéine du système est composée d'un domaine senseur et d'un domaine kinase qui peut s'auto-phosphoryler à partir d'ATP sur un résidu histidine de son sous-domaine phosphotransférase. Ce phosphate sera ensuite transféré sur un résidu aspartate de la deuxième protéine du système aux propriétés régulatrices (Adapté de Ulrich et al 2005).

Chapitre VI) La Régulation des Mécanismes de Résistance aux Antibiotiques

Le profil d'expression génique de n'importe quelle forme de vie varie régulièrement en fonction du stade de croissance et ceci grâce à des systèmes de régulation fins et complexes, y compris pour des organismes pouvant paraître moins évolués comme les procaryotes. Ces phénomènes de régulation se produisent en réponse à un stress environnemental et à un besoin métabolique. Ils se matérialisent par une transduction de ces signaux exogènes ou endogènes et se manifestent soit par une activation, soit par une inactivation de la transcription de gènes clefs. Ces mécanismes sont le résultat d'une longue optimisation évolutive qui procure une réponse rapide et adaptée. Chez une bactérie et particulièrement en présence d'un antibiotique, la mise en place rapide du mécanisme de résistance approprié est une condition *sine qua non* à la survie du microorganisme, avant que ces molécules antimicrobiennes ne causent trop de dégâts. La mise en place de ce processus a un coût énergétique, plus spécialement dans le cas de la synthèse de grosses molécules comme les MmpL, et doit donc être finement contrôlé.

1) La superfamille des régulateurs de transcription TetR :

Les bactéries répondent aux signaux environnementaux qu'elles rencontrent grâce à deux grands groupes de régulateurs, les systèmes à un composant et les systèmes à deux composants (Figure 31). Comme l'indique leur nom, les systèmes à un composant sont constitués d'une seule protéine, qui contient à la fois une partie effectrice et une partie senseur. La partie effectrice interagit avec l'ADN cible du régulateur et la partie senseur reconnaît un ligand spécifique. Il existe une relation entre localisation de la partie effectrice et l'action du régulateur. En effet, il semblerait que les protéines avec le site de liaison à l'ADN situé en N-terminal seraient des répresseurs de transcription. A l'inverse, un site de liaison à l'ADN à l'extrémité C-terminale serait le reflet d'une activité activatrice (Cuthbertson et Nodwell 2013). Ces fonctions « senseur & effecteur » sont attribuées à deux protéines chez les systèmes à deux composants. Le signal adéquat déclenche un transfert de phosphate entre la protéine histidine kinase senseur au niveau d'un résidu aspartate situé dans la région régulatrice de la protéine effectrice. Ce phosphotransfert a pour conséquence de lever l'inhibition de la protéine effectrice qui pourra assurer sa fonction régulatrice (Hoch 2000). Les informations sont plus nombreuses concernant les systèmes à deux composants, cependant, ce sont les systèmes à un composant qui sont les plus abondants parmi les bactéries. Ces derniers sont constitués de plusieurs superfamilles, dont celle des TetR.

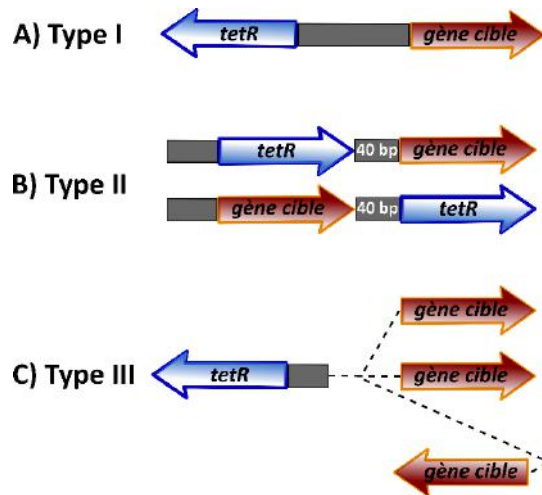


Figure 32. Organisation génomiques des *tetR* et de leurs gènes cibles. (A) Les TetR de type I. Le *tetR* et le gène cible sont voisins et orientés de manière opposée sur le génome. (B) Les TetR de type II. Le *tetR* est soit en aval soit en amont son gène cible dans la même orientation génomique, avec une région intergénique les séparant de moins de 40 bp. (C) Les TetR de type III. Le *tetR* est situé soit juste à côté soit à distance de son gène cible sur le chromosome (Adapté de Cuthbertson et Nodwell 2013).

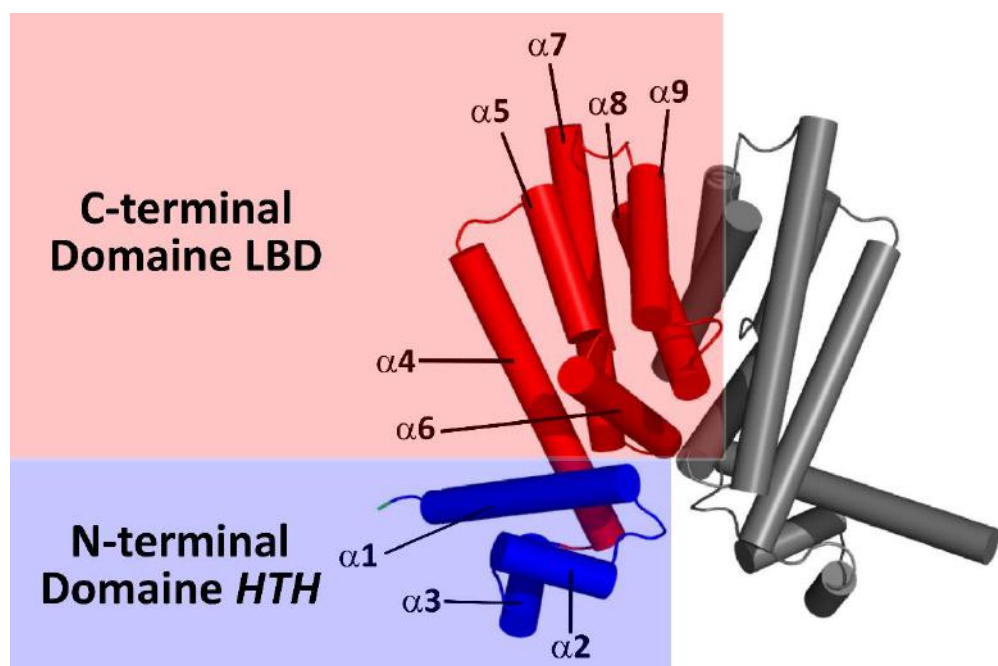


Figure 33. Schéma de l'organisation structurale d'un régulateur TetR. Structure du TetR Rha06780 de *Rhodococcus sp.* RHA1. Les hélices- α sont représentées par des cylindres. En rouge, les hélices 1, 2 et 3 formant le domaine de liaison à l'ADN. En bleu, on retrouve les hélices 4, 5, 6, 7, 8 et 9 qui forment le domaine de liaison au ligand et de dimérisation. Le deuxième monomère est totalement gris. HTH : Helix – Turn – Helix ; LBD : Ligand Binding Domain (Adapté de Yu et al. 2010).

1a) Propriétés génétiques :

Ces protéines sont en quasi-totalité des répresseurs de transcription. Le premier du genre a été identifié en 1966 chez *E. coli*, et contrôle l'expression de la pompe à efflux TetA, impliquée dans la résistance intrinsèque à la tétracycline, d'où le nom de TetR (Tétracycline Repressor) (Izaki et al. 1966). Les TetR peuvent être classifiés en fonction de leur orientation génomique et de celle du ou des gènes qu'ils contrôlent (Cuthbertson et Nodwell 2013). On retrouve alors trois types de régulateur TetR. Ceux de type I, où le régulateur et le gène cible sont voisins, séparés par région intergénique non-codante, et qui possède une orientation génomique opposée (Figure 32A). Les TetR de type II, qui possèdent la même orientation génomique que le gène dont ils contrôlent l'expression. Le TetR peut être soit en amont soit en aval de son gène cible. La région intergénique située entre eux deux doit être de moins de 40 pb, la taille minimum d'un promoteur mycobactérien (Newton-Foot et Gey van Pittius 2013) (Figure 32B). Il est possible pour les TetR de type I et II de se baser sur ces critères génomiques pour prédire la relation régulateur – gène cible. Cependant, pour les TetR de type III, il est impossible de se baser sur les mêmes paramètres génétique. Ces TetR peuvent réguler des gènes voisins comme des gènes dispersés sur le chromosome (Figure 32C). Le nombre de ces régulateurs est extrêmement variable d'une espèce à l'autre, en particulier chez les mycobactéries. La quantité de TetR présents sur le génome est, comme pour le nombre de MmpL, le reflet de la capacité d'adaptation et de mode de vie intra/extracellulaire. Par exemple *M. leprae*, pathogène intracellulaire strict aux nombres de gènes réduit, et *M. tuberculosis*, possèdent 13 et 49 régulateurs TetR respectivement. *M. abscessus* ainsi que *M. smegmatis*, tous deux des MNT environnementales, disposent respectivement de 138 et 137 gènes *tetR* sur leurs génomes. Les gènes *tetR* mesurent environ 660 pb codant donc pour des protéines d'approximativement 220 aminoacides.

1b) Propriétés biochimiques et structurales :

Les membres de la superfamille de régulateurs TetR sont des homodimères, chacun composés de 9 à 10 hélices- α ($h-\alpha$) (Figure 33). Le domaine N-terminus régit les interactions avec l'ADN cible tandis que le domaine C-terminus est responsable de la reconnaissance du ou des ligands ainsi que des interactions monomère – monomère. Le domaine N-terminal est très conservé et est composé de trois $h-\alpha$ d'une longueur totale moyenne de 41 acides aminés (h_1 , h_2 , et h_3) (Yu et al. 2010). L' h_1 a un rôle d'interaction avec le petit sillon de l'ADN mais sert principalement de connexion entre les domaines N/C-termini en liant les h_4 et les h_6 (Le et al. 2011). Les h_2 et h_3 vont former le domaine structural typique de liaison à l'ADN des systèmes à un composant, le motif hélice – coude – hélice (HTH pour Helix – Turn – Helix). Ce motif va se comporter comme une pince ou une tenaille et aller se verrouiller sur l'ADN cible. L' h_3 ainsi que l' h_3' , appartenant au deuxième monomère, vont participer à la majorité

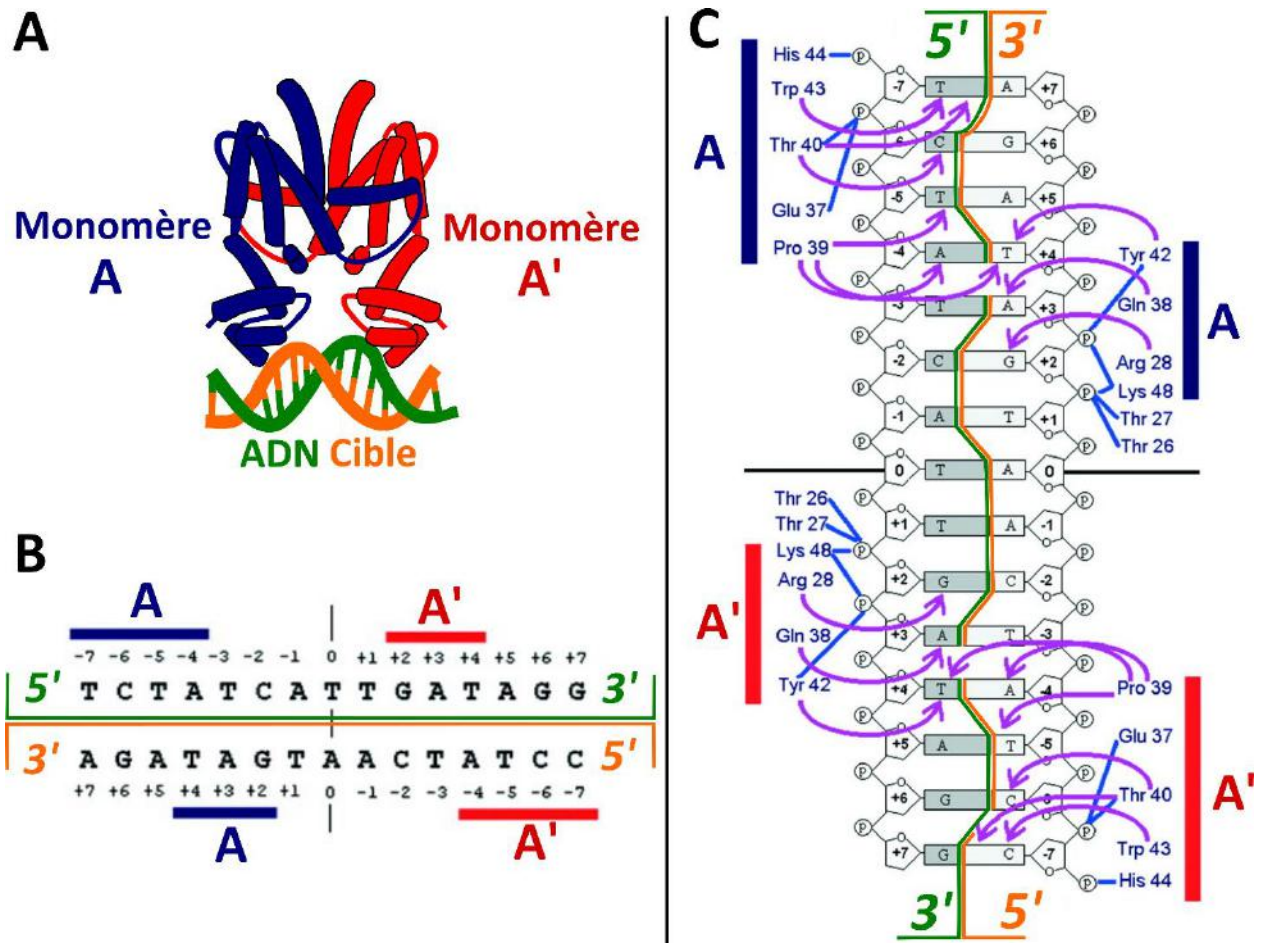


Figure 34. Interaction d'un dimère du TetR d'*E. coli* avec son ADN cible. (A) Représentation schématique du monomère A en bleu et du monomère A' en rouge du même dimère interagissant avec l'ADN cible. (B) Site d'interaction des domaines HTH des monomères A et A' avec le DNA_{op}. (C) Spécificité et nature des liaisons des aminoacides des domaines HTH avec les bases nucléotidiques (flèches violettes) ou avec les phosphates du squelette d'ADN (flèches bleues) (Adapté de Ramos et al. 2005).

des contacts avec l'ADN en s'insérant dans les grands sillons adjacents (Schumacher et al. 2002). Ramos et al. proposent grâce à un alignement d'acides aminés de plusieurs TetR que les résidus 37, 39 et 43 (annotation du TetR d'*E. coli*) de cette h3 sont primordiaux pour les contacts régulateurs – DNA car ils sont conservés parmi les séquences analysées. Les h2 déforment la structure en double-hélice et permettent aux h3 de s'orienter parallèlement aux grands sillons de l'ADN pour maximiser les contacts spécifiques (Bhukya et Anand 2017). Les contacts TetR – ADN peuvent être deux types. Les acides aminés chargés de domaine HTH, comme les résidus arginines, vont interagir avec les phosphates chargés négativement de l'ADN (PO_4^{3-}) par des liaisons hydrogènes par exemple. D'autres aminoacides comme des tyrosines présentes dans le domaine HTH vont former les liaisons dites « base-spécifique » via des interactions hydrophobes ou électrostatiques (Ramos et al. 2005). Cependant, les acides aminés n'ont pas de rôle arbitraire pour l'un ou l'autre type d'interaction. La composition, l'organisation du domaine HTH et la séquence d'ADN vont guider le mode d'interaction et non pas forcément la nature de l'acide aminé. Un aminoacide peut alors établir soit des liaisons avec les PO_4^{3-} du squelette de l'ADN soit avec une base nucléotidique. Ceci est très bien illustré par les données cristallographiques du complexe régulateur – ADN du TetR d'*E. coli* (Orth et al. 2000) (Figure 34).

Le domaine C-terminal est quant à lui beaucoup moins conservé et reflète la diversité des ligands reconnus par chaque TetR. Les h5 et h7 ainsi que les boucles les reliant vont former un triangle qui sera la poche de reconnaissance du ligand du TetR (LBP pour Ligand Binding Pocket). La nature de ces ligands est aussi variée que les voies métaboliques et les fonctions que ces TetR remplissent. Ils peuvent être des substrats de molécules toxiques comme des antibiotiques et mais aussi d'ions, de sucres, de protéines, de lipides ainsi que de leurs dérivés potentiels. La nature des aminoacides présents dans cette poche va déterminer le type de ligands qui ira s'y nicher. Néanmoins, il n'est pas possible d'affirmer que telle ou telle molécule est le ligand d'un TetR juste en se basant simplement sur la composition de sa LBP même s'il existe cependant une corrélation. QacR contrôle la pompe à efflux de type MFS QacA responsable de multi-résistance antibiotique chez *S. aureus*. Plusieurs co-structures de ce régulateur ont été obtenues avec des antibiotiques cationiques, où les interactions avec les résidus chargés négativement de la LBP étaient prédominants (Cuthbertson et Nodwell 2013). La stoechiométrie des substrats au sein de la LBD est également variable, comme c'est le cas pour LfrR de *M. smegmatis* qui accommode une molécule de proflavine ou de fluoroquinolone (Bellinzoni et al. 2009) alors que ActR de *S. coelicolor* accueille soit deux molécules d'actinorhodine, soit quatre molécules du précurseur biosynthétique de cet antibiotique (Willems et al. 2008).

Les h8, h9 et éventuellement l'h10, vont former l'interface de dimérisation connectant les deux monomères. Ces h5, 7, 8, 9 voire 10 forment une grappe au sein de laquelle les substrats peuvent pénétrer la LBP de manière latérale, apicale ou frontale (Cuthbertson et Nodwell 2013).

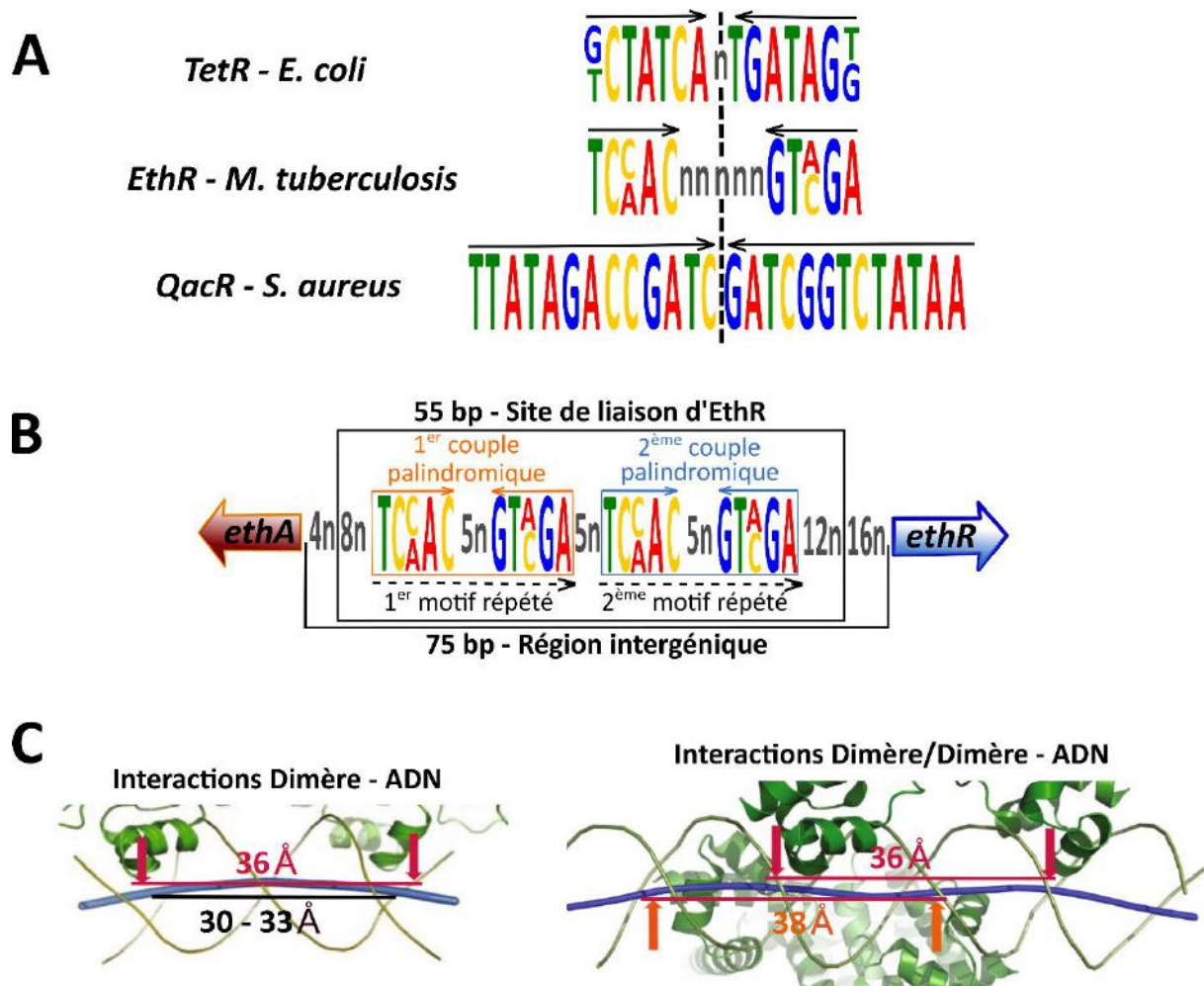


Figure 35. Structure de l'ADN cible d'un TetR. (A) Exemple de palindromes et de leurs architectures. Le DNA_{op} de TetR d'*E. coli* est composé d'un palindrome dégénéré d'une base sur ses extrémités. Il est composé de 15 pb avec un spacer de un nucléotide. Le DNA_{op} d'EthR de *M. tuberculosis* est composé de deux couples de palindromes dégénérés de 15 pb avec des spacers de 5 nucléotides. Le DNA_{op} de QacR de *S. aureus* est un palindrome parfait de 26 pb sans aucun spacer. (B) Représentation schématique de la région intergénique d'EthR. Les deux couples de palindrome sont indiqués en orange et bleu clair. Ils sont séparés par 5 nucléotides et forment deux motifs répétés orientés dans le même sens. Ces motifs sont indiqués en pointillé. La région de 55 pb protégée par EthR suite à la digestion à la DNase I est encadrée. (C) Représentation 3D des déformations que subi la double-hélice d'ADN suite à l'interaction avec un TetR (Adapté de Bhukya et Anand 2017).

1c) Structure de l'ADN cible et réarrangements conformationnels :

L'ADN cible ou ADN opérateur (DNA_{op}) est de taille et de composition très variable. De manière générale, les TetR reconnaissent des séquences palindromiques ou des motifs répétés inversés, qui sont en réalité des palindromes dégénérés. Ces régulateurs se lient la plupart du temps à des séquences d'ADN d'environ 15 nucléotides, où les motifs palindromiques sont séparés ou non par un ou plusieurs nucléotides (Figure 35A). Ces nucléotides entre les palindromes sont appelés *spacers*, et peuvent être particulièrement longs. EthR de *M. tuberculosis* est impliqué dans la résistance à l'ETH et au TAC en contrôlant l'expression de monooxygénase EthA qui transforme ces deux pro-drogues en métabolites actifs. Une analyse par digestion à la DNase I de la région intergénique située entre *ethA* et *ethR* a révélé que la liaison d'EthR sur ce fragment d'ADN protégeait une région de 55 pb (Engohang-Ndong et al. 2004). Ce DNA_{op} contient deux couples de palindromes dégénérés de 15 nucléotides séparés par un *spacer* de 5 pb. Ces deux couples de palindromes forment deux séquences répétées orientées dans la même direction (Figure 35B). Les TetR vont également influencer la morphologie de la double hélice d'ADN et l'impacter différemment selon la stœchiométrie avec leurs DNA_{op} . Lorsqu'un seul dimère interagit avec le DNA_{op} , ces h2 vont modifier la courbure de la double hélice en induisant un léger arc de cercle. Cette déformation permet d'optimiser l'orientation des h3 dans les grands sillons. Cette courbure unidirectionnelle provoque le passage d'un pas entre deux grands sillons de 36 Å à 30-33 Å et empêche l'appareillement d'un autre dimère sur le brin d'ADN opposé (Bhukya et Anand 2017). Lorsque deux TetR se lient à leurs motifs cibles, ils se retrouvent de part et d'autre de l'ADN et exercent donc un effet de force opposée. Les h2 remplissent toujours le même rôle ce qui mène à une courbure bidirectionnelle moins prononcée, allant de 36 à 38 Å (Bhukya et al. 2014). L'un comme l'autre vont modifier dans leur intérêt la conformation optimale de l'ADN-B (Figure 35C). Le mode de liaison dimère – dimère est généralement synonymes de grande spécificité du régulateur pour son DNA_{op} alors que l'autre type d'interaction mettant en jeu un seul dimère suggérerait une certaine plasticité du régulateur pour sa cible et donc une implication possible dans la régulation de plusieurs gènes.

Les protéines TetR elles-mêmes subissent un réarrangement structural quand les apoprotéines sont en conformation avec l'ADN ou quand elles sont sous formes libres liées au substrat. Cette allostérie est le résultat d'un mouvement dit de « balancier » ou de « pendule » des trois premières h- α vers l'extérieur lorsqu'elles relâchent le DNA_{op} suite à l'arrivée du ligand dans la LBP. On parle alors d'état relaxé, où les domaines HTH sont éloignés les uns des autres résultant en une perte de complémentarité de structure avec la double hélice d'ADN. Le mouvement est déclenché principalement par l'h4 interagissant avec l'h1 mais aussi par l'h6, qui sont responsables

de la transduction du signal entre les domaines LBP et HTH. Ce balancier des HTH est réalisé suite à la disparition et formation de liaison hydrogène entre les h8, 9 et 10 mais aussi suite à des déroulements de la structure hélicoïdale des h4, h5 et h6 (Cuthbertson et Nodwell 2013) (Bhukya et Anand 2017).

1d) Rôles et fonctions :

La quasi-totalité des voies métaboliques chez les bactéries peuvent contenir une étape régulée par une protéine de la superfamille des TetR. Ces régulateurs sont donc impliqués dans l'antibiorésistance, les métabolismes lipidique, protéique, de cofacteurs, du sucre et de l'azote ainsi que dans le *quorum sensing* (Cuthbertson et Nodwell 2013).

Le rôle des TetR dans l'antibiorésistance peut être séparé en deux classes. La première classe constitue les TetR impliqués dans l'antibiorésistance chez les bactéries productrices d'antibiotiques. La deuxième classe est composée des TetR impliqués dans l'antibiorésistance chez les bactéries non-productrices d'antibiotiques.

La première classe regroupe principalement des actinomycètes appartenant au genre *Streptomyces*. L'un d'entre eux est ActR qui contrôle la pompe à efflux ActA sous le contrôle du produit final, l'actinorhobine, et de certains de ses précurseurs métaboliques (Tahlan et al. 2007). Le contrôle de la pompe à efflux par les produits intermédiaires de la chaîne de biosynthèse permet à la bactérie de s'assurer que le mécanisme de résistance sera en place une fois le produit final obtenu. Ce genre de mécanismes permet à ces bactéries productrices résistantes d'éliminer les bactéries sensibles voisines ce qui leur confère un fort avantage adaptatif. Certains de ces TetR, en plus de réguler une protéine transmembranaire impliquée dans l'efflux de l'antibiotique produit, régule des enzymes impliquées dans la biosynthèse et la maturation des précurseurs métaboliques des produits finaux. C'est le cas de LanK chez la bactérie *S. cyanogenus* S136 qui produit la landomycine A. En plus de la pompe à efflux LanJ, ce TetR contrôle l'expression du gène *lanZ1*, codant pour une épimérase impliquée dans les stades tardifs de glycosylation de la landomycine A (Ostash et al. 2008).

Les TetR impliqués dans l'antibiorésistance de classe 2 sont également très répandus et peuvent être le fruit de transfert horizontal entre bactéries productrices résistantes et bactéries non-productrices sensibles. Chez ces dernières devenues résistantes, on retrouve la plupart du temps plus de multi-résistances que de mono-résistance. Le couple TetR contrôlant TetA chez *E. coli* est uniquement responsable de l'export de la tétracycline. Mais toujours chez *E. coli*, un régulateur impliqué dans des multi-résistances est AcrR contrôlant la pompe à efflux multi-substrat AcrAB, un transporteur RND, la famille dont font partis les MmpL. Des mutations ponctuelles situées dans le

gène d'*acrR* provoquent la surexpression de la pompe à efflux AcrAB chez des isolats cliniques d'*E. coli* résistants aux fluoroquinolones (Gibson et al. 2010). A l'inverse, la délétion du gène *ethR* mène à une surexpression de la monooxygénase EthA chez *M. bovis* BCG ce qui conduit à une hypersensibilité à l'ETH (Baulard et al. 2000). Cela laisse à penser que des molécules capables de déréguler les TetR impliqués dans l'activation de pro-drogue seraient de nouveaux pharmacophores originaux. Ce type de molécules, appelées « boosters », ont d'ores et déjà prouvé leurs efficacité *in vitro* et *in vivo* contre des isolats cliniques MDR-Tb en combinaison avec l'ETH (Blondiaux et al. 2017). Des mutations dans *ethA* provoquent une résistance accrue à l'ETH chez *M. tuberculosis* car cette enzyme n'est plus en mesure d'activer cet antibiotique le laissant sous sa forme de pro-drogue. Cependant, la combinaison d'ETH et d'une molécule de synthèse de la famille des spiroisoxazoline désignée SMART-420 (Small Molecule Aborting Resistance) augmente la sensibilité des souches résistantes à l'ETH. Cette combinaison a complètement éradiqué les bacilles chez un modèle murin. Ceci est fait dû à la présence d'un système auxiliaire *ethA2 – ethR2* dans la même conformation génomique qu'*ethA – ethR*. EthR2 est un régulateur de la famille TetR et EthA2 est ici oxydoreductase, elle aussi capable de bio-activer l'ETH. Une étude biochimique a révélé que ce deuxième système est indépendant de EthA – EthR, que les deux régulateurs TetR se lient uniquement à leurs ADN cibles respectifs. SMART-420 va se positionner dans la LBP d'EthR2 qui dérégulera EthA2 activant alors l'ETH. La présence d'un système homologue à celui-ci n'est pas connue chez *M. abscessus*, mais ces travaux ouvrent des perspectives sur de nouvelles stratégies thérapeutiques originales.

2) La famille de régulateur WhiB :

Cette famille de protéines est restreinte aux actinobactéries. Comme la superfamille des TetR, ces protéines sont en charges de la régulation de nombreuses voies métaboliques impliquées dans la réponse à des stress environnementaux, des changements morphologiques et au cours des différentes phases de croissance. Ces gènes ont été identifiés il y a presque 50 ans grâce à l'observation microscopique de colonies blanches (*whi* pour « white mutants ») ou grises de *S. coelicolor* capables ou incapables de sporuler respectivement (Chater 1972). C'est donc la fonction de sporulation chez cette bactérie gouvernée par le gène *whiB* qui a d'abord été étudiée dans le détail.

2a) Propriétés génétiques :

Des paralogues de ces gènes *whiB* ont ensuite été identifiés au sein d'autres genres bactériens, comme chez les mycobactéries. Que ce soit chez les streptomycètes ou les mycobactéries, les

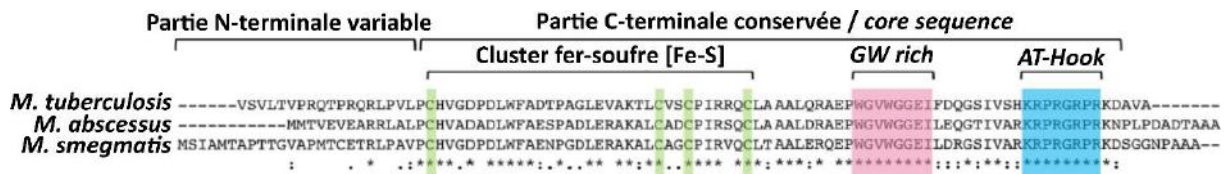


Figure 36. Alignement des séquences d'acides aminés de WhiB7 de mycobactéries. Alignements protéiques de WhiB7 de *M. tuberculosis*, *M. abscessus* et *M. smegmatis* mettant en avant le caractère conservé de la partie C-terminale. Les résidus marqués d'un astérisque (*) sont ultra-conservés, comme les 4 cystéines du cluster fer-soufre, les glycines, tryptophanes et autres acides aminés composant le domaine *GW rich* ainsi que les arginines et lysines de l'*AT-Hook* (Adapté de (Hurst-Hess et al 2017)).

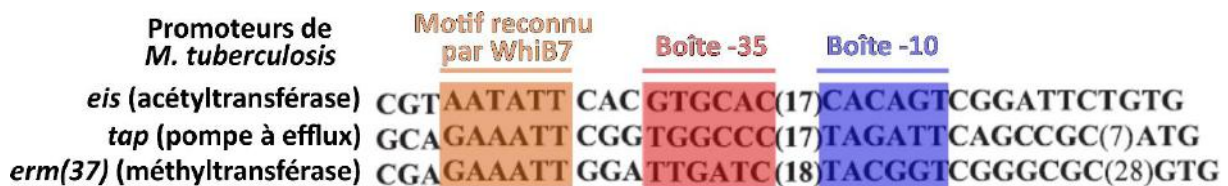


Figure 37. Alignement nucléotidique des séquences promotrices contrôlées par WhiB7 chez *M. tuberculosis*. L'alignement des promoteurs d'*eis*, de *tap* et d'*erm(37)* responsable, respectivement, des résistances aux aminoglycosides, aux rifamycines et aux macrolides révèle l'organisation typique des gènes contrôlés par WhiB7. Un motif riche en AT en orange, situé à 3 nucléotides de la boîte -35 en rouge. La boîte -10 est colorée en bleu, et est située à 17-18 nucléotides de la boîte -35 (Adapté de Burian et al. 2012).

gènes *whiB* ont une taille comprise entre 250 et 400 pb. Chez *M. tuberculosis*, 7 gènes de type *whiB* ont été identifiés contre 6 chez *M. abscessus*. A l'inverse des *mmpL* et des *tetR*, le nombre de gènes *whiB* ne paraît pas être relié à la capacité d'adaptation, étant donné que *M. leprae* possède 5 homologues potentiels sur son génome (Morris et al. 2005).

2b) Propriétés biochimiques et structurales :

Ces gènes de tailles réduites produisent des polypeptides de petite taille, 94 acides aminés pour le WhiB7 de *M. abscessus*. Ces protéines sont partiellement très conservées parmi les mycobactéries mais également au sein d'autres genres comme *Rhodococcus* et *Streptomyces* (Hurst-Hesset et al 2017). La région N-terminale est moins conservée et de taille variable que le reste de la protéine qui est appelée « *core sequence* » (Figure 36). Cette *core sequence* est composée d'un cluster fer-soufre où quatre résidus cystéines sont absolument conservés dans toutes les séquences. Ces cystéines sont capables de lier deux ou quatre atomes de fer et deux ou quatre de soufre ([2/4Fe – 2/4S]) en fonction de leur état oxydatif (Ramón-García et al. 2013). Si ce cluster fer-soufre ne lie aucun de ces deux atomes, les cystéines vont former des ponts intramoléculaires provoquant un réarrangement structural avec un impact sur la fonction de régulation. Les formes [2/4Fe – 2/4S] ne sont pas forcément les configurations protéiques capables de lier l'ADN et inversement. On retrouve ensuite une partie riche en glycine – tryptophane (GW-rich) et la partie responsable de l'interaction, de l'hameçonnage de domaine riche en adénosine et thymine de l'ADN cible (AT-Hook). Ces motifs sont tous les deux hautement conservés. Le domaine GW-rich forme un feuillet bêta qui sera en contact avec les acides aminés chargés positivement composant l'AT-Hook, des arginines et des lysines. Ce domaine de liaison en C-terminal attribue classiquement une fonction d'activateur au système à un composant. Cependant, chez *M. tuberculosis*, les protéines WhiB peuvent être des répresseurs comme par exemple WhiB1 ou des activateurs comme WhiB2, 3, 4 et 7 (Burian et al. 2012). Les protéines WhiB activatrices vont alors interagir avec différents facteurs sigmas (σ). La préférence pour tel ou tel facteur σ est liée à l'architecture du promoteur en amont du gène (Newton-Foot et Gey van Pittius 2013). Ces protéines WhiB se lieraient aux parties C-terminales de leurs facteurs σ respectifs et catalyseraient la transcription des régulons par l'ARN polymérase. La taille et la séquence de ces motifs riches en AT sont variables d'un gène et d'une espèce à l'autre. En règle générale, ils sont composés de 5-6 pb situés à 3 nucléotides en amont de la boîte hexamérique -35 du promoteur, comme illustré pour les gènes *erm(37)*, *eis* et *tap* de *M. tuberculosis* (Burian et al 2012) (Figure 37). Chez les mycobactéries, la distance en la boîte -35 et la boîte -10 détermine le type de facteur σ qui interagira avec ce promoteur (Newton-Foot et Gey van Pittius 2013).

2c) Rôles et fonctions :

Chez *M. abscessus*, c'est le gène *whiB7* qui est principalement étudié étant donné son implication dans les processus de multi-résistances intrinsèques aux antibiotiques. Deux études récentes rapportent l'enrôlement de WhiB7 dans la résistance de *M. abscessus* à l'AMK, à la CLR, à la TGC mais aussi à la TBM, à la gentamycine et au chloramphénicol (Hurst-Hess et al 2017) (Pryjma et al. 2017). Des études de RNA-seq comparant l'expression des gènes entre une souche sauvage et un mutant $\Delta whiB7$ ont révélé que le régulon de *whiB7* s'étend à plus de 120 gènes, suggérant un rôle global de WhiB7 dans l'homéostasie de cette MNT (Hurst-Hess et al 2017). Pryjma et al. proposent un antagonisme *in vitro* entre de CLR avec l'AMK à des doses subinhibitrices. En effet une faible concentration de CLR induit une résistance croisée à l'AMK.

En revanche chez *M. tuberculosis*, les fonctions des 7 gènes *whiB* sont connues. Plusieurs de ces protéines partagent des rôles similaires comme dans virulence au sein du macrophage ainsi que les différentes phases de croissance et la persistance. C'est le cas par exemple de WhiB1, 2 et 3 qui sont surexprimés lors de la phase stationnaire.

WhiB1 est également impliqué dans la survie intramacrophagique et la possible réactivation des bacilles après une infection latente (Smith et al. 2010). En effet, WhiB1 contrôle l'expression du gène *rpfA*, un facteur de croissance nécessaire à la sortie de la phase de latence (Kana et al. 2008).

WhiB2 est aussi surexprimé en phase stationnaire ainsi qu'en carence de nutriments et en présence de molécules ciblant la paroi mycobactérienne comme l'EMB (Geiman et al. 2006).

WhiB3 s'active également lors d'un stress nutritif et oxydatif que l'on retrouve en phase de latence (Singh et al. 2007). Il catalyse aussi l'anabolisme de lipides nécessaires à la pathogénicité de *M. tuberculosis* dans le macrophage *via* l'utilisation du propionate pour la synthèse de certains glycolipides comme le DAT (Singh et al. 2009).

WhiB4 est lui exprimé tout au long des phases de croissance et joue un rôle dans la virulence mais aussi dans la dissémination de l'infection. Un mutant $\Delta whiB4$ montre une hypervirulence dans le système pulmonaire mais un défaut de dissémination des bactéries jusqu'à la rate chez un modèle murin (Chawla et al. 2012).

WhiB5 est quant à lui seulement exprimé en phase exponentielle de croissance mais semble répondre à des agents provoquant un stress membranaire (Geiman et al. 2006). Comme WhiB1, cette protéine aurait un rôle dans la réactivation et la reprise de croissance après une infection latente (Casonato et al. 2012). Le régulon de WhiB5 est important puisqu'il contient au moins 58 gènes identifiés. Parmi ses candidats, on retrouve les gènes codants les systèmes de sécrétion de

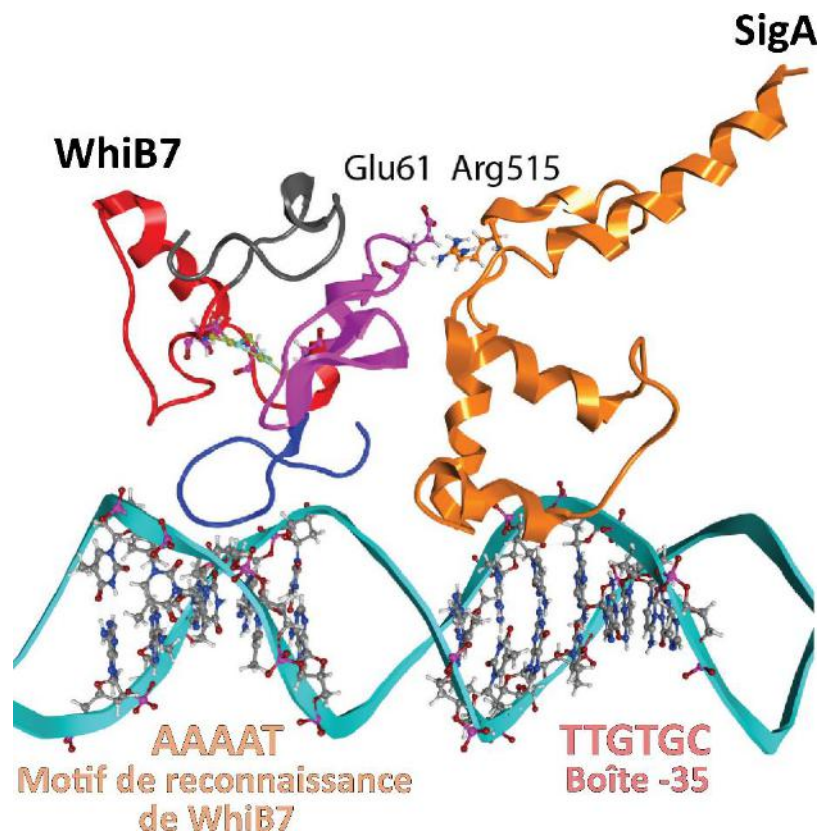


Figure 38. Modèle de prédiction d'interaction du complexe WhiB7 – SigA lié à l'ADN cible. L'interaction du résidu Glu61 de WhiB7 avec le résidu Arg515 de la région 4,2 C-terminale de SigA a été déterminée expérimentalement. La région N-terminale, le cluster fer-soufre, l'AT-Hook et la région intermédiaire située en ces deux derniers domaines sont colorés en gris, rouges, bleu et rose respectivement. La région 4 du facteur SigA d'*E. coli* a été utilisée dans ce modèle et est coloré en doré (PDB : 4IGC_X) (Adapté de Burian et al. 2013).

type VII ESX-2 et ESX-4, des protéines nécessaires à la survie au sein de cellules dendritiques et de macrophages (Mendum et al. 2015).

WhiB6 est aussi surexprimé en conditions de stress membranaire par exemple après exposition au SDS, à de l'éthanol et après un choc thermique (Geiman et al. 2006). Cette protéine semble aussi activer l'expression d'un système de sécrétion de type VII, cette fois-ci ESX-1 (Wiseman et al. 2010). ESX-1 est responsable de la sécrétion d'antigènes majeurs liés à la virulence de *M. tuberculosis*. Les pertes de virulence des souches de *M. bovis* BCG et *M. microti* utilisées dans les vaccins anti-tuberculeux sont liées à des mutations dans locus d'*esx-1* (Pym et al. 2002).

Le rôle de WhiB7 est identique chez *M. abscessus* et chez *M. tuberculosis*. Une approche biochimique a permis de montrer que WhiB7 interagit avec la partie C-terminal du facteur végétatif SigA pour améliorer le niveau de transcription des gènes cibles (Burian et al 2013) (Figure 38). Cette interaction serait due à la région située entre le cluster fer-soufre et l'AT-Hook. L'activation n'est pas uniquement dépendante de la présence d'antibiotique ciblant le ribosome. Ce régulateur s'active au moment de l'entrée en phase stationnaire ou en absence de fer, comme par exemple à l'intérieur d'un phagosome (Homolka et al. 2010). Des conditions entraînant une réduction du potentiel redox du cytoplasme suivie d'un stress oxydatif peut provoquer en théorie une activation de WhiB7. Burian et al. le démontrèrent simplement en mesurant le niveau d'expression de l'ARNm par qRT-PCR de *whiB7* en présence d'antibiotiques couplé soit à un agent réducteur soit à un agent oxydatif, respectivement du DiThioThréitol (DTT) ou du diamide. Le DTT couplé à une très faible concentration d'érythromycine augmente fortement le niveau de transcrits de *whiB7* alors que le diamide en stoppe la production. Ceci reflète expérimentalement le changement de la balance redox du mycothiol dans le cytoplasme de la bactérie. Le mycothiol est aux actinomycètes ce qu'est le glutathion chez les Gram - et les eucaryotes. C'est un agent réducteur qui permet entre autre de se débarrasser des ROS lorsqu'il est retrouvé en grande quantité sous sa forme réduite. En plus des gènes de résistance présents dans le régulon de *whiB7*, ce mécanisme d'oxydoréduction participe à la lutte contre les antibiotiques en inactivant la production de radicaux libres que ces antimicrobiens engendrent indirectement (Rawat et al. 2002).

Objectifs

Améliorer la compréhension des mécanismes qui régissent l'antibiorésistance intrinsèque chez *M. abscessus* est primordial pour :

- le développement de nouveaux pharmacophores biologiquement actifs,
- la découverte de nouvelles cibles médicamenteuses,
- l'élaboration de nouvelles stratégies thérapeutiques utilisant les antibiotiques dont l'utilisation est déjà approuvée.

Mes travaux de thèse s'inscrivent dans le domaine de la recherche fondamentale. Ces recherches se sont, dans un premier temps, axées sur des transporteurs membranaires impliqués dans l'efflux d'antibiotiques, dont les principaux objectifs ont consisté à :

- identifier les antibiotiques potentiellement expulsés par *M. abscessus*,
- identifier le ou les transporteurs MmpL impliqués dans ce processus,
- décrire les mécanismes de régulation de ces pompes à efflux,
- proposer de nouveaux marqueurs de résistance à certains antibiotiques.

Trois études comprenant des approches biochimique, génétique et structurale ont permis de disséquer les mécanismes de résistance basale de trois couple TetR – MmpS/MmpL impliqués dans la résistance à trois classes d'antibiotiques différentes. Nous avons tout d'abord identifié le système MAB_4384 – MAB_4383c/MAB_4382c, responsable de l'export des analogues du TAC (**Article 1**). Nous avons également décrit et attribué la résistance croisée à la CFZ et à la BDQ à deux couples MmpS/MmpL, les paires MAB_2300/MAB_2301 (**Article 2**) et MAB_1135c/MAB_1134c (**Article 3**), toutes deux régulées par le même TetR MAB_2299c.

La deuxième partie de ma thèse s'est portée sur la résistance inductible aux macrolides chez *M. abscessus*. Ce phénomène, encore très mal compris, est d'autant plus problématique que les macrolides sont la pierre angulaire de la thérapie anti – *M. abscessus*. Les objectifs ont consisté à :

- identifier les éléments nécessaires à l'activation de cet événement inductible,
- comparer l'activité antimicrobienne et la cinétique de l'induction de l'AZM et de la CLR,
- mettre en évidence et observer ce phénomène de résistance induite dans un modèle animal, l'embryon de zebrafish.

Nous avons confirmé *via* une étude génétique et biochimique l'implication de WhiB7 dans l'activation de cette résistance et nous avons également clôturé le débat sur les effets respectifs de l'AZM et de la CLR en démontrant que l'AZM n'est pas un meilleur antibiotique que la CLR *in vitro*. Ces deux macrolides induisent également la résistance *in vivo* chez le zebrafish. Ces travaux ont aussi souligné la complexité de cette cascade de régulation qui a pour répercussion un antagonisme entre l'AMK et ces deux macrolides.

Résultats

« Mechanistic and Structural Insights Into the Unique TetR-Dependant Regulation of a Drug Efflux Pump in *Mycobacterium abscessus*. » Richard M, Gutiérrez AV, Viljoen A, Ghigo E, Blaise M, Kremer L. Front Microbiol. 2018 Apr 5; Vol 9 Article 649.

La récente optimisation chimique du TAC a permis d'obtenir une nouvelle génération d'analogues structuraux (TACa) à la fois efficaces contre *M. abscessus* et *M. tuberculosis* (Coxon et al. 2013). Une étude antérieure menée dans l'équipe a mis en avant un lien entre la résistance aux TACa et la présence de mutations dans le régulateur TetR MAB_4384 qui s'accompagnent d'une augmentation des transcrits de la pompe à efflux de type MmpS/MmpL MAB_4383c/MAB_4382c (Halloum et al. 2017). Sur la base d'une approche biochimique incluant la technique d'EMSA, nos travaux ont permis de prouver l'interaction physique du TetR MAB_4384 avec un motif palindromique situé en amont de l'opéron MAB_4383c/MAB_4382c. Des EMSA réalisés avec des protéines mutées sur des résidus spécifiques ont permis de démontrer le rôle de ces acides aminés dans la capacité du régulateur TetR à se lier à son ADN cible. Par ailleurs, des substitutions nucléotidiques dans le motif palindromique ont permis de démontrer la spécificité de MAB_4384 pour sa séquence cible. Une étude génétique basée sur l'utilisation du gène rapporteur *lacZ* sous le contrôle de la séquence promotrice de MAB_4383c/MAB_4382c a mis en lumière la force de ce promoteur, permettant ainsi d'expliquer les hauts niveaux de résistance observés dans les souches de *M. abscessus* devenues spontanément résistantes aux TACa. Les expériences menées avec cette souche rapportrice suggèrent également un export basal et intrinsèque des TACa via l'activité de pompe à efflux de MAB_4383c/MAB_4382c. La résolution de la structure cristallographique de MAB_4384 combinée à des expériences de *docking in silico* des TACa au sein de son domaine LBP suggèrent fortement la possibilité d'une expression accrue de la pompe à efflux lorsque ces molécules se fixent sur le régulateur TetR. L'ensemble de ces résultats mettent en avant, pour la première fois, un mécanisme de régulation et d'expression basal de pompes à efflux de la famille MmpL impliqué dans la résistance aux antibiotiques chez *M. abscessus*.



Mechanistic and Structural Insights Into the Unique TetR-Dependent Regulation of a Drug Efflux Pump in *Mycobacterium abscessus*

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Antimicrobials, Resistance
and Chemotherapy,
a section of the journal
Frontiers in Microbiology

Received: 13 February 2018

Accepted: 20 March 2018

Published: 05 April 2018

Citation:

Richard M, Gutiérrez AV, Viljoen AJ,
Ghigo E, Blaise M and Kremer L
(2018) Mechanistic and Structural
Insights Into the Unique
TetR-Dependent Regulation of a Drug
Efflux Pump in *Mycobacterium
abscessus*. *Front. Microbiol.* 9:649.
doi: 10.3389/fmicb.2018.00649

Mycobacterium abscessus is an emerging human pathogen causing severe pulmonary infections and is refractory to standard antibiotherapy, yet few drug resistance mechanisms have been reported in this organism. Recently, mutations in *MAB_4384* leading to up-regulation of the MmpS5/MmpL5 efflux pump were linked to increased resistance to thiacetazone derivatives. Herein, the DNA-binding activity of *MAB_4384* was investigated by electrophoretic mobility shift assays using the palindromic sequence *IR_{S5/L5}* located upstream of *mmpS5/mmpL5*. Introduction of point mutations within *IR_{S5/L5}* identified the sequence requirements for optimal binding of the regulator. Moreover, formation of the protein/*IR_{S5/L5}* complex was severely impaired for *MAB_4384* harboring D14N or F57L substitutions. *IR_{S5/L5}/lacZ* reporter fusions in *M. abscessus* demonstrated increased β -galactosidase activity either in strains lacking a functional *MAB_4384* or in cultures treated with the TAC analogs. In addition, X-ray crystallography confirmed a typical TetR homodimeric structure of *MAB_4384* and unraveled a putative ligand binding site in which the analogs could be docked. Overall, these results support drug recognition of the *MAB_4384* TetR regulator, alleviating its binding to *IR_{S5/L5}* and steering up-regulation of MmpS5/MmpL5. This study provides new mechanistic and structural details of TetR-dependent regulatory mechanisms of efflux pumps and drug resistance in mycobacteria.

Keywords: *Mycobacterium abscessus*, TetR regulator, MmpL, efflux pump, structure, thiacetazone analogs, EMSA

INTRODUCTION

Mycobacterium abscessus is a rapid growing mycobacterium (RGM) that has recently become an important health problem (Mougari et al., 2016). This non-tuberculous mycobacterial (NTM) pathogen can cause serious cutaneous, disseminated or pulmonary infections, particularly in cystic fibrosis (CF) patients. In CF patients, infection with *M. abscessus* is correlated with a decline in lung function and poses important challenges during last-resort lung transplantation (Esther et al., 2010; Smibert et al., 2016). An epidemiological study has recently documented the prevalence

and transmission of *M. abscessus* between hospital settings throughout the world, presumably *via* fomites and aerosols and uncovered the emergence of dominant circulating clones that have spread globally (Bryant et al., 2016). In addition, *M. abscessus* exhibits innate resistance to many different classes of antimicrobial agents, rendering infections with this microorganism extremely difficult to treat (Nessar et al., 2012; van Dorn, 2017). Recent studies have started to unveil the basis of the multi-drug resistance characterizing *M. abscessus*, uncovering a wide diversity of mechanisms or regulatory networks. These involve, for example, the induction of the *erm(41)* encoded 23S rRNA methyltransferase and mutations in the 23S rRNA that lead to clarithromycin resistance (Nash et al., 2009), the presence of a broad spectrum β -lactamase that limits the use of imipenem (Dub e et al., 2015; Lefebvre et al., 2016, 2017) or the presence of *eis2*, encoding an acetyltransferase that modifies aminoglycosides, specifically induced by *whiB7*, which contributes to the intrinsic resistance to amikacin (Hurst-Hess et al., 2017; Rominski et al., 2017b). Other studies reported the role of the ADP-ribosyltransferase MAB_0591 as a major contributor to rifamycin resistance (Rominski et al., 2017a) whereas MAB_2385 was identified as an important determinant in innate resistance to streptomycin (Dal Molin et al., 2018).

Recently, we reported the activity of a library of thiacetazone (TAC) derivatives against *M. abscessus* and identified several compounds exhibiting potent activity against a vast panel of clinical strains isolated from CF and non-CF patients (Halloum et al., 2017). High resistance levels to these compounds were linked to mutations in a putative transcriptional repressor MAB_4384, together with a strong up-regulation of the divergently oriented adjacent locus encoding a putative MmpS5/MmpL5 transporter system. That ectopic overexpression of MmpS5/MmpL5 in *M. abscessus* also increased the minimal inhibitory concentration (MIC) to analogs of TAC further suggested that these two proteins may act as an active efflux pump which was sufficient to confer drug resistance (Halloum et al., 2017). In addition to uncovering new leads for future drug developments, this study also highlighted a novel mechanism of drug resistance in *M. abscessus*. Unexpectedly, an important difference relies on the fact that, in *M. tuberculosis*, MmpS5/MmpL5 acts as a multi-substrate efflux pump causing low resistance levels to antitubercular compounds such as clofazimine, bedaquiline, and azoles (Milano et al., 2009; Andries et al., 2014; Hartkoorn et al., 2014) whereas the *M. abscessus* strains overexpressing MmpS5/MmpL5 are very resistant to the TAC analogs but fail to show cross-resistance against clofazimine or bedaquiline (Halloum et al., 2017). This implies that, despite their high primary sequence identity, the MmpS5/MmpL5 orthologs from *M. tuberculosis* and *M. abscessus* do not share the same substrate specificity. Moreover, whereas Rv0678, the cognate regulator of MmpS5/MmpL5 in *M. tuberculosis* belongs to the MarR family (Radhakrishnan et al., 2014), MAB_4384 is part of the TetR family of regulators and the change in the transcriptional level of *mmpS5/mmpL5* was much more pronounced in the *M. abscessus* mutants than in the

M. tuberculosis mutants (Milano et al., 2009; Hartkoorn et al., 2014).

The TetR transcriptional regulatory factors are common single component signal transduction systems found in bacteria. These proteins possess a conserved helix-turn-helix (HTH) signature at the N-terminal of the DNA-binding domain as well as a ligand binding domain (LBD) located at the C-terminal part (Cuthbertson and Nodwell, 2013). They often act as repressors and interact with a specific DNA target to prevent or abolish transcription in the absence of an effector. In contrast, the binding of a specific ligand to the LBD induces structural changes, conducting the dissociation of the repressor from the target DNA, and the subsequent transcription of the TetR-regulated genes. Being largely associated with resistance to antibiotics and regulation of genes coding for small molecule exporters, TetR regulators also govern expression of antibiotic biosynthesis genes, quorum sensing and in distinct aspects in bacterial physiology/virulence (Cuthbertson and Nodwell, 2013). A recent global analysis indicated that the TetR regulators represent the most abundant class of regulators in mycobacteria, the vast majority remaining uncharacterized (Balhana et al., 2015). In order to provide new insight into the mechanism of gene regulation by TetR regulators in mycobacteria and to describe a new and specific drug resistance mechanism in *M. abscessus*, we focused our efforts on the molecular and structural characterization of the MAB_4384-dependent regulation of MmpS5/MmpL5.

In this study, a combination of genetic and biochemical analyses was applied to determine the specificity of this regulatory system in *M. abscessus* and to describe the contribution of key residues that are important in driving the DNA-binding of MAB_4384 to its operator. We report also the crystal structure of the MAB_4384 TetR regulator. Overall, the results provide new insights into the regulation of members of the MmpL family and on a novel mechanism of drug resistance in *M. abscessus*.

MATERIALS AND METHODS

Plasmids, Strains, Growth Conditions, and Reagents

The *Mycobacterium abscessus* subsp. *abscessus* CIP104536^T reference strain and all derived mutant strains are listed in Supplementary Table S1. Strains were grown in Middlebrook 7H9 broth (BD Difco) supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% oleic acid, albumin, dextrose, catalase (OADC enrichment; BD Difco) (7H9^{T/OADC}) at 30°C or in Sauton's medium in the presence of antibiotics, when required. On plates, colonies were selected either on Middlebrook 7H10 agar (BD Difco) supplemented with 10% OADC enrichment (7H10^{OADC}) or on LB agar. For drug susceptibility testing, cultures were grown in Cation-Adjusted Mueller-Hinton Broth (CaMHB; Sigma-Aldrich). The TAC analogs D6, D15, and D17 were synthesized as reported previously (Coxon et al., 2013) and dissolved in DMSO. Other antibiotics were purchased from Sigma-Aldrich.

Cloning of Wild-Type and Mutated MAB_4384 and Site-Directed Mutagenesis

MAB_4384 was PCR-amplified from *M. abscessus* CIP104536^T purified genomic DNA using the *MAB_4384_full* primers (Supplementary Table S2) and Phusion polymerase (Thermo Fisher Scientific). The amplicon was cloned into pET32a restricted with KpnI and HindIII (New England Biolabs), enabling the introduction of *MAB_4384* in frame with the thioredoxin and poly-histidine tags as well as a Tobacco Etch Virus protease (TEV) cleavage site between the N-terminus of *MAB_4384* and the tags. The *MAB_4384* alleles harboring the g40a (D14N) and t169c (F57L) mutations were PCR-amplified using the primers described above and using the purified genomic DNA of the two spontaneous resistant *M. abscessus* strains to TAC analogs reported previously (Halloum et al., 2017). The double mutant carrying both g40a and t169c mutations was obtained from the *MAB_4384* (g40a) allele using the PCR-driven primer overlap extension method (Aiyar et al., 1996). Briefly, two separate PCR reactions were set up using Phusion polymerase. The first was generated using the forward primer *MAB_4384_full* Fw and a reverse internal primer *MAB_4384_DM* Rev harboring the nucleotide substitution responsible for the mutation. The second PCR was set up with an internal forward primer *MAB_4384_DM* Fw overlapping the internal reverse primer *MAB_4384_DM* Rev and with the original reverse primer *MAB_4384_full* Rev. PCR products were purified, annealed and amplified by a last PCR amplification with the *MAB_4384_full* primers. All mutated genes were cloned into pET32a, as described for wild-type *MAB_4384*.

Expression and Purification of MAB_4384 Variants

The various pET32a-derived constructs containing either the wild-type or the mutated *MAB_4384* gene alleles were used to transform *Escherichia coli* strain BL21 Rosetta 2 (DE3) (Novagen). Cultures were grown in Luria-Bertani (LB) medium containing 200 µg/mL ampicillin and 30 µg/mL chloramphenicol until an optical density at 600 nm (OD₆₀₀) of between 0.6 and 1.0 was reached. Liquid cultures were then placed on icy water for 30 min prior to the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation for an additional 20 h at 16°C. Bacteria were then collected by centrifugation (6,000 × g, 4°C, 60 min) and the pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8, 200 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 1 mM benzamidine). Cells were opened by sonication and the lysate clarified by centrifugation (28,000 × g, 4°C, 45 min) and subjected to a first step of nickel affinity chromatography (IMAC) (Ni-NTA Sepharose, GE Healthcare Life Sciences). After elution, the protein was dialyzed overnight at 4°C in a buffer containing 50 mM Tris-HCl pH 8, 200 mM NaCl, 5 mM β-mercaptoethanol and TEV protease (1 mg of protease/50 mg of total protein) to cleave the thioredoxin and histidine tags from the recombinant proteins. The dialyzed preparations were purified again by IMAC, followed by an anion exchange chromatography step

(HiTrap Q Fast Flow, GE Healthcare Life Sciences) as well as a final polishing step using size exclusion chromatography (SEC) (SephadexTM 75 10/300 GL, GE Healthcare Life Sciences) and a buffer containing 50 mM Tris-HCl pH 8, 200 mM NaCl and 5 mM β-mercaptoethanol.

The selenomethionine-substituted protein was expressed in the methionine auxotroph *E. coli* strain B834 (DE3) (Novagen). A 1L culture was grown very densely in LB medium containing 200 µg/mL ampicillin for 36 h at 37°C. Bacteria were harvested by centrifugation and the pellets resuspended in minimal medium A without antibiotic and methionine traces (M9 medium, trace elements, 0.4% glucose, 1 µM MgSO₄, 0.3 mM CaCl₂, biotin and thiamine at 1 µg/mL). After an additional wash in medium A, the bacterial pellet was resuspended in 6L of medium A containing 200 µg/mL ampicillin and incubated for 2 h at 37°C. Finally, S/L selenomethionine was added at a final concentration of 100 µg/mL. After 30 min of incubation, expression of the protein was induced with 1 mM IPTG for 5 h at 37°C. The protein was purified using a protocol similar to the one used for the proteins expressed in the *E. coli* strain BL21 Rosetta 2(DE3).

Determination of Oligomeric States of MAB_4384 by Size Exclusion Chromatography

The oligomeric state of *MAB_4384* and *MAB_4384*:DNA complex in solution were assessed on an ENrichTM SEC 650 size exclusion column (Bio-Rad) run on an ÄKTA pure 25M chromatography system (GE Healthcare Life Science). The protein, DNA or protein:DNA complex were eluted with 50 mM Tris-HCl pH 8, 200 mM NaCl and 5 mM β-mercaptoethanol at a flow rate of 0.4 mL/min at 4°C. *MAB_4384* (dimer) was concentrated to 3.9 mg/mL, while DNA was at 2.8 mg/mL and complexes were formed at different protein(dimer)/DNA molar ratios of 1:1, 2:1, 3:1. The molecular weights were determined based on a calibration curve generated using the Gel Filtration Markers Kit (Sigma-Aldrich) for proteins ranging from 12,400 to 200,000 Da. The column void volume was assessed with the elution peak of dextran blue. The apparent mass was obtained by plotting the partition coefficient K_{av} against the log values of the molecular weights of the standard proteins.

Disruption of MAB_4384 and mmpL5 in M. abscessus

To generate *MAB_4384* and *mmpL5* knock-out mutants, internal fragments of the genes were PCR-amplified using Phusion polymerase and the specific oligonucleotide sets: *MAB_4384*::pUX1 Fw with *MAB_4384*::pUX1 Rev and *mmpL5*::pUX1 Fw with *mmpL5*::pUX1 Rev, respectively, digested with NheI and BamHI and ligated to NheI-BamHI-linearized pUX1 (Supplementary Table S1), a suicide vector specifically designed to perform gene inactivation in *M. abscessus* (Viljoen et al., 2018). Electrocompetent *M. abscessus* was transformed with the plasmids pUX1-*MAB_4384* and pUX1-*mmpL5* and plated on 250 µg/mL kanamycin LB plates. After 5 days of incubation at 37°C, red fluorescent colonies were selected and gene disruption resulting from homologous recombination

between the plasmid DNA and the target genes was confirmed by PCR and sequencing with appropriate primers (Supplementary Table S2).

Quantitative Real-Time PCR

Isolation of RNA, reverse transcription and qRT-PCR were done as reported earlier (Halloum et al., 2017) using the primers listed in Supplementary Table S2.

Drug Susceptibility Assessment

The MICs were determined according to the CLSI guidelines (Woods et al., 2011), as reported earlier (Halloum et al., 2017).

Electrophoretic Mobility Shift Assays

First, a typical DNA binding motif recognized by the TetR regulator, often composed of palindromic sequences or inverted repeats, was identified *in silico* within the intergenic region located between *MAB_4384* and the *MAB_4383c* (*mmpS5_{Mabs}*)/*MAB_4382c* (*mmpL5_{Mabs}*) gene cluster, hereafter referred to as IR_{S5/L5}, using the MEME Suite 4.20.0 online tool¹ (Bailey et al., 2009). A 45 bp double stranded DNA fragment (Probe 1) containing the 27 bp palindromic sequence was labeled with fluorescein at their 5' ends (Sigma-Aldrich). Increasing amounts of purified MAB_4384 protein were co-incubated with 280 nM of the fluorescein-labeled probes in 1X Tris Base/acetic acid/EDTA (TAE) buffer for 1 h at room temperature. The samples were then subjected to 6% native polyacrylamide gel electrophoresis for 30 min at 100 V in 1X TAE buffer. Gel shifts were visualized by fluorescence using an Amersham Imager 600 (GE Healthcare Life Sciences). All additional modified probes listed Supplementary Table S2 and used in this study were synthesized and used in electrophoretic mobility shift assay (EMSA) assays, as described above.

Construction of β -Galactosidase Reporter Strains and β -Gal Assays

The *lacZ* reporter gene encoding the β -galactosidase was amplified from the *E. coli* HB101 using primers listed in Supplementary Table S2. The amplicon was cloned into pMV261 cut with BamHI and HindIII, thus yielding pMV261_P_{hsp60}-*lacZ*. The 208 bp intergenic region IR_{S5/L5} was amplified by PCR using *M. abscessus* CIP104536^T genomic DNA and the *MAB_4384*_P_{S5/L5} primers (Supplementary Table S2) and subsequently cut with XbaI and BamHI. The *hsp60* promoter was removed from pMV261_P_{hsp60}-*lacZ* construct by restriction using XbaI and BamHI and replaced with IR_{S5/L5}, thus creating pMV261_P_{S5/L5}-*lacZ*. A promoterless pMV261-*lacZ* construct was also generated by removing the *hsp60* promoter from the pMV261_P_{hsp60}-*lacZ* with BamHI and XbaI, blunting the overhang extremities using the T4 DNA Polymerase and self-religation.

The β -galactosidase activity of the *M. abscessus* strains carrying either wild-type or mutated *MAB_4384* alleles and the various β -gal reporter constructs was monitored streaking the

strains directly on 7H10^{OADC} agar plates supplemented with 100 μ g/mL kanamycin and 50 μ g/mL X-gal (Sigma-Aldrich). The quantification of the β -gal activity was also assayed in liquid medium using a protocol adapted from Miller's method. Briefly, a 10 mL culture in Sauton's medium supplemented with 0.025% tyloxapol was grown until the OD₆₀₀ reached 0.6–1. Cultures were collected by centrifugation (4,000 \times g for 10 min at 4°C) and the bacterial pellets were resuspended in 700 μ L 1X phosphate-buffered saline (PBS) prior to mechanical lysis by bead beating (3 min treatment, 30 Hz). Lysates were finally centrifuged at 16,000 \times g for 10 min at 4°C. 10 μ L of clarified lysate were co-incubated 30 min at 37°C with 100 μ L of reaction buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol) in 96-well plates. Enzymatic reactions were initiated by adding 35 μ L of 2-Nitrophenyl β -D-galactopyranoside (ONPG, Sigma-Aldrich) at 4 mg/mL and absorbance was recorded at 420 nm at 34°C using a Multimode Microplate Reader POLARstar Omega (BMG Labtech). The β -galactosidase specific activity (SA $_{\beta-Gal}$) was calculated using the following formula: SA $_{\beta-Gal}$ = (Absorbance_{420nm} \times min⁻¹)/(OD_{280nm} \times liter of culture). To test the β -gal-induction by the TAC analogs, the drugs were added directly to the cultures grown in Sauton's medium (OD₆₀₀ = 0.6–1) and incubated with slow shaking for 96 h at 37°C. The β -gal activity was determined as described above.

Crystallization, Data Collection, and Refinement

The MAB_4384 crystals were grown in sitting drops in MR Crystallization Plates (Hampton Research) at 18°C by mixing 1.5 μ l of protein solution concentrated to 4.7 mg/mL with 1.5 μ l of reservoir solution made of 100 mM sodium cacodylate pH 6.5, 200 mM MgCl₂, 16% PEG 8000 and 5% DMSO. Crystals were briefly soaked in 100 mM Cacodylate buffer pH 6.5, 200 mM MgCl₂, 16% PEG 8000, 5% DMSO and 10% PEG 400 prior to being cryo-cooled in liquid nitrogen. The selenomethionine-substituted MAB_4384 crystals were obtained in sitting drops in 96-well SWISSCI MRC plates (Molecular Dimension) at 18°C by mixing 0.8 μ l of protein solution concentrated to 2.5 mg/mL with 0.8 μ l of reservoir solution consisting of 35% (v/v) 1,4 dioxane. Crystals were cryo-cooled without any additional cryo-protection. Data were processed with XDS and scaled and merged with XSCALE (Kabsch, 2010). Data collection statistics are presented in **Table 1**. The MAB_4384 structure was solved by the single wavelength anomalous dispersion method. *AutoSol* from the *Phenix* package was used to solve the structure (Adams et al., 2010). Twelve of the fourteen potential selenium sites in the asymmetric unit were found using a resolution cutoff of 3.4 Å for the search of the Se atoms. After density modification, a clear electron density map for the two TetR monomers allowed initial model building. The resulting partial model was used to perform molecular replacement with the 1.9 Å native dataset using *Phaser* (McCoy et al., 2007) from the *Phenix* package (Adams et al., 2010). *Coot* (Emsley et al., 2010) was used for manual rebuilding while structure refinement and validation were performed with

¹<http://meme-suite.org>

TABLE 1 | Data collection and refinement statistics.

| | MAB_4384 native | Selenium peak |
|------------------------------------|--------------------------------|-------------------------------|
| Data collection statistics | | |
| Beamline | ESRF-ID30B | ESRF-ID30B |
| Wavelength (Å) | 0.979 | 0.979 |
| Resolution range (Å) | 36.5–1.9 (1.96–1.9) | 47–2.3 (2.38–2.3) |
| Space group | P 1 2 1 1 | P 1 2 1 1 |
| Unit cell (Å, °) | 40.8 100.8 56.0 90 105.8 90 | 41.4 99.3 55.7 90 106.9 90 |
| Total reflections | 73028 (7378) | 126590 (12013) |
| Unique reflections | 31705 (3193) | 18983 (1849) |
| Multiplicity | 2.3 (2.3) | 6.7 (6.5) |
| Completeness (%) | 92.1 (93.4) | 98.6 (98.1) |
| Mean I/sigma (I) | 11.09 (1.06) | 11.66 (1.36) |
| Wilson B-factor (Å ²) | 33.9 | 46.02 |
| R-meas | 0.06 (0.92) | 0.13 (1.21) |
| CC1/2 | 0.99 (0.51) | 0.97 (0.67) |
| CC* | 1 (0.82) | 0.99 (0.89) |
| Data refinement statistics | | |
| Reflections used in refinement | 31695 (3193) | |
| Reflections used for R-free | 2000 (201) | |
| R-work | 0.184 (0.312) | |
| R-free | 0.213 (0.351) | |
| Number of non-H atoms | 3460 | |
| Macromolecules | 3178 | |
| Solvent | 282 | |
| Protein residues | 402 | |
| RMS bonds (Å) | 0.002 | |
| RMS angles (°) | 0.45 | |
| Ramachandran favored (%) | 98.99 | |
| Ramachandran allowed (%) | 1.01 | |
| Ramachandran outliers (%) | 0.00 | |
| Rotamer outliers (%) | 0.95 | |
| Average B-factor (Å ²) | 43.6 | |
| Macromolecules | 43.2 | |
| Solvent | 47.6 | |
| PDB accession number | 5OVY | |

The values in parenthesis are for the last resolution shell.

the *Phenix* package (Adams et al., 2010). The statistics for data collection and structure refinement are displayed in **Table 1**. Figures were prepared with PyMOL². The atomic coordinates and the structure factors for the reported MAB_4384 crystal structure has been deposited at the Protein Data bank (accession number 5OVY).

Docking of TAC Analogs Into the Ligand Binding Site

Docking studies was performed with *PyRx* (Dallakyan and Olson, 2015) running *AutoDock Vina* (Trott and Olson, 2010). Search was done with grid dimensions of 39.45, 39.05, 29.25 Å and origin coordinates at $x = -17.8$, $y = 6.9$, $z = 0.63$. The search

²www.pymol.org

was performed on chain B of the crystal structure of MAB_4384 without any additional model modification.

RESULTS

MAB_4384 Specifically Regulates Susceptibility to TAC Analogs in *M. abscessus*

We recently showed that mutations in the MAB_4384 regulator were associated with the transcriptional induction of the divergently oriented adjacent genes coding for an MmpS5/MmpL5 efflux pump and accounting for high resistance levels toward various TAC analogs (Halloum et al., 2017). To gain more insight into this drug resistance mechanism in *M. abscessus*, detailed genetic, functional and structural characterizations of the MAB_4384 regulator were undertaken. First, the expression profile of 19 *mmpL* genes was analyzed by qRT-PCR using the *M. abscessus* D15_S4 strain which possesses an early stop codon in MAB_4384 resulting in high resistance levels to the TAC analogs D6, D15 and D17 (MIC > 200 µg/mL), presumably due to derepression of the MmpS5/MmpL5 efflux pump machinery (Halloum et al., 2017). The results clearly showed a pronounced increase in the expression level of MAB_4382c (*mmpL5*) mRNA in D15_S4 in comparison to the wild-type strain as reported previously, while no marked effect on the remaining *mmpL* genes was detected (**Figure 1A**). The expression levels of *tgs1*, encoding the primary triacylglycerol synthase responsible for the accumulation of triglycerides in *M. abscessus* (Viljoen et al., 2016) was included as unrelated gene control. As expected, expression of *tgs1* stayed unchanged (**Figure 1A**). To further confirm these results, the MAB_4384 and MAB_4382c genes were inactivated by homologous recombination using the recently developed genetic tool dedicated to facilitate gene disruption in *M. abscessus* (Viljoen et al., 2018), as illustrated in Supplementary Figures S1A,B. The mutant strain, designated MAB_4384::pUX1, failed to show morphological changes (Supplementary Figure S1C) and grew similarly to its parental strain and the MAB_4382c::pUX1 mutant (Supplementary Figure S1D), suggesting that MAB_4384 does not play a significant role under normal *in vitro* conditions. However, this mutant exhibited high resistance levels to D6, D15, and D17 (MIC > 200 µg/mL, corresponding to >8-, >32-, and >16-fold-increases in MIC levels, respectively) (**Table 2**) similarly to our previous results for D15_S4 (Halloum et al., 2017), thus validating the expected phenotype of the strain. Analysis of the transcriptional profile of all 19 *mmpL* genes in MAB_4384::pUX1 confirmed the results obtained in the D15_S4 strain (**Figure 1B**). Interestingly, expression of MAB_4384 itself was significantly induced in MAB_4384::pUX1, (**Figure 1C**), albeit lower than the expression level of *mmpL5*, thus indicating that MAB_4384 is self-regulated.

Overall, these results suggest that MAB_4384 is a unique and highly specific regulator controlling expression of *mmpL5* in *M. abscessus*, which was strongly up-regulated in the absence of MAB_4384.

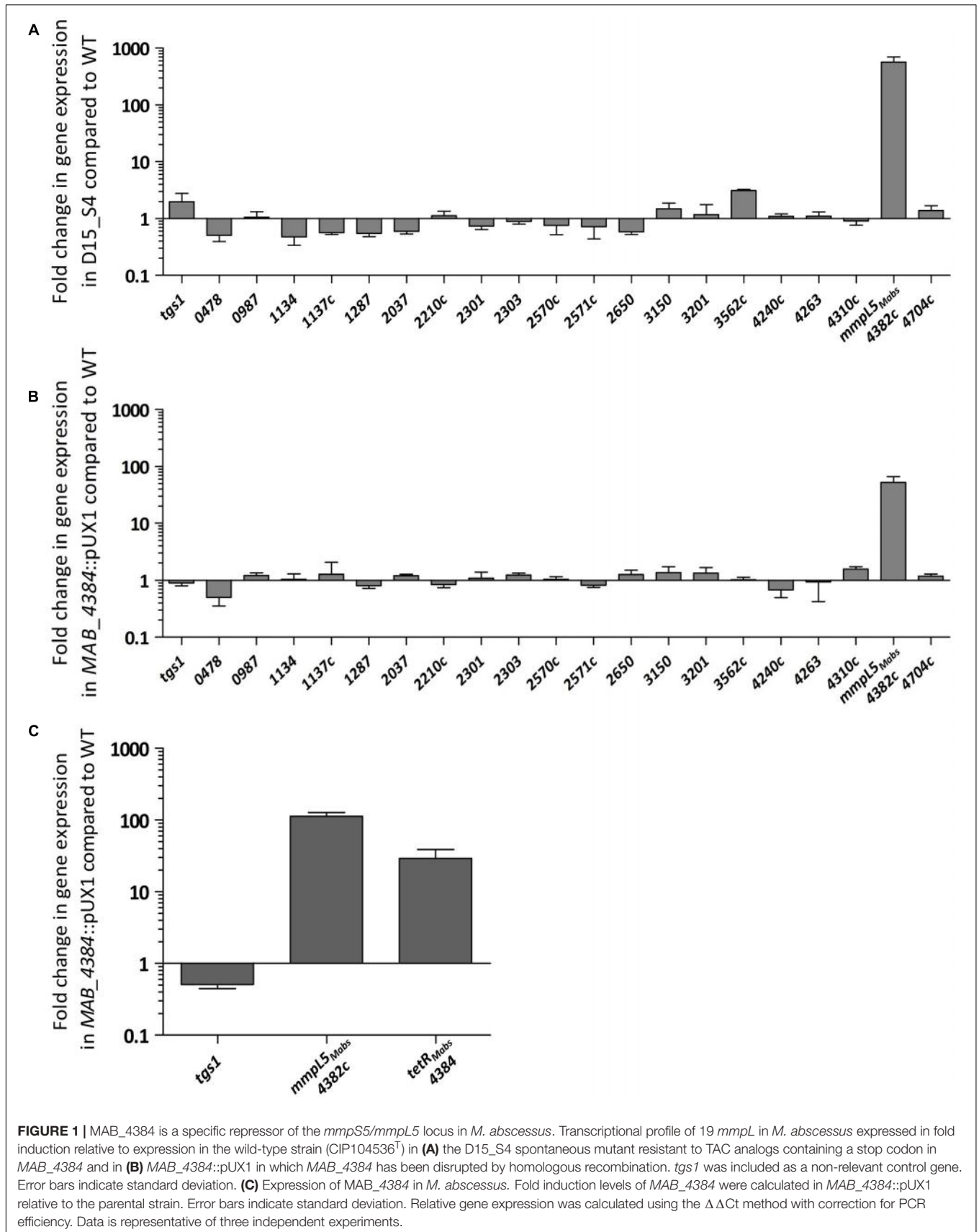


TABLE 2 | Drug susceptibility profile of *M. abscessus* S strains inactivated in either *MAB_4384* (*tetR* gene) or *MAB_4382c* (*mmpL5* gene).

| Strain | MIC ($\mu\text{g/mL}$) | | | | |
|-------------------------|--------------------------|------|------|-----|------|
| | D6 | D15 | D17 | CFZ | BDQ |
| CIP104536 ^T | 25 | 6.2 | 12.5 | 1.6 | 0.05 |
| <i>MAB_4384</i> ::pUX1 | >200 | >200 | >200 | 1.6 | 0.05 |
| <i>MAB_4382c</i> ::pUX1 | 12.5 | 6.2 | 6.2 | 1.6 | 0.05 |

The MIC ($\mu\text{g/mL}$) was determined in CaMH medium. Data are representative of three independent experiments. CFZ, clofazimine; BDQ, bedaquiline.

MAB_4384 Negatively Regulates Expression of *mmpS5/mmpL5*

The pMV261_P_{S5/L5}_lacZ plasmid was constructed, containing the β -galactosidase gene as a reporter in *M. abscessus* to further confirm the negative regulation of MAB_4384 on the target gene (*mmpS5/mmpL5* locus) expression. To do this, the 208 bp intergenic region located between *MAB_4384* and *mmpS5/mmpL5* (Figure 2A), designated IR_{S5/L5}, was cloned upstream of *lacZ*. pMV261_P_{S5/L5}_lacZ was subsequently introduced in parental smooth (S) and rough (R) variants of *M. abscessus* as well as in three different strains carrying single point mutations in MAB_4384 (M1A, F57L, and D14N), previously selected for their high resistance phenotype to TAC analogs (Halloum et al., 2017). In addition, pMV261_P_{hsp60}_lacZ allowing constitutive expression of *lacZ* under the control of the strong *hsp60* promoter was produced. As expected, pMV261_P_{hsp60}_lacZ led to high expression of *lacZ* in the wild-type strain and in the mutants compared to the promoter-less plasmid, as evidenced by the production of intense blue colonies and a strong β -Gal activity (Figure 2B). In contrast, whereas IR_{S5/L5} resulted in very low expression of LacZ in the wild-type strains, characterized by a pale blue color on plates and low β -Gal activity in liquid-grown cultures, a pronounced *lacZ* induction was detected in all three mutant strains (Figure 2B). Strikingly, the *lacZ* expression levels in these strains was almost comparable to the one observed in the pMV261_P_{hsp60}_lacZ-containing strains.

Overall, these results indicate that expression of *lacZ* is strongly repressed in the presence of an intact MAB_4384 regulator and that under derepressed conditions, the promoter driving expression of *mmpS5/mmpL5* appears almost as strong as the *hsp60* promoter.

MAB_4384 Binds to a Palindromic Sequence Within IR_{S5/L5}

Motif-based sequence analysis using MEME (Bailey et al., 2009) revealed the presence of a 27 bp segment within the divergently oriented IR_{S5/L5} intergenic region and harboring a palindromic sequence (Figure 3A). To test whether this motif represents a DNA binding site for MAB_4384, EMSA was first performed using increasing concentrations of purified MAB_4384 expressed in *E. coli* in the presence of a 45 bp fragment of IR_{S5/L5} (Probe 1; Figure 3B) carrying extra nucleotides flanking the palindromic sequence. Under these conditions, a DNA-protein

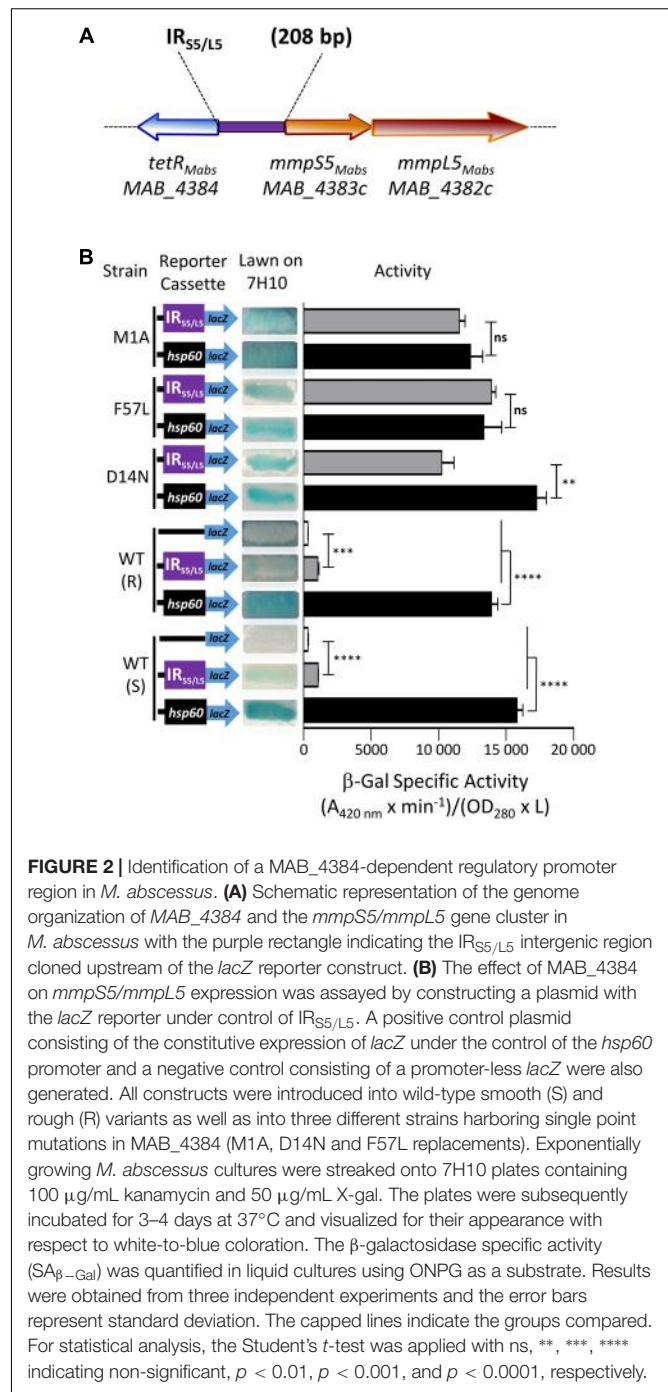


FIGURE 2 | Identification of a MAB_4384-dependent regulatory promoter region in *M. abscessus*. **(A)** Schematic representation of the genome organization of *MAB_4384* and the *mmpS5/mmpL5* gene cluster in *M. abscessus* with the purple rectangle indicating the IR_{S5/L5} intergenic region cloned upstream of the *lacZ* reporter construct. **(B)** The effect of MAB_4384 on *mmpS5/mmpL5* expression was assayed by constructing a plasmid with the *lacZ* reporter under control of IR_{S5/L5}. A positive control plasmid consisting of the constitutive expression of *lacZ* under the control of the *hsp60* promoter and a negative control consisting of a promoter-less *lacZ* were also generated. All constructs were introduced into wild-type smooth (S) and rough (R) variants as well as into three different strains harboring single point mutations in MAB_4384 (M1A, D14N and F57L replacements). Exponentially growing *M. abscessus* cultures were streaked onto 7H10 plates containing 100 $\mu\text{g/mL}$ kanamycin and 50 $\mu\text{g/mL}$ X-gal. The plates were subsequently incubated for 3–4 days at 37°C and visualized for their appearance with respect to white-to-blue coloration. The β -galactosidase specific activity (SA _{β -Gal}) was quantified in liquid cultures using ONPG as a substrate. Results were obtained from three independent experiments and the error bars represent standard deviation. The capped lines indicate the groups compared. For statistical analysis, the Student's *t*-test was applied with ns, **, ***, **** indicating non-significant, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

complex was seen (Figure 3C). To confirm the specificity of the binding, a competition assay with increasing concentrations of the corresponding unlabeled probe (cold probe) was carried out, leading to a dose-dependent decrease of the DNA-protein complex (Figure 3C). In addition, in the presence of an excess of a non-related labeled probe, the shift was maintained, thus indicating that a specific protein-DNA complex was seen only when MAB_4384 was incubated with DNA containing the specific inverted repeat sequence. To better define the

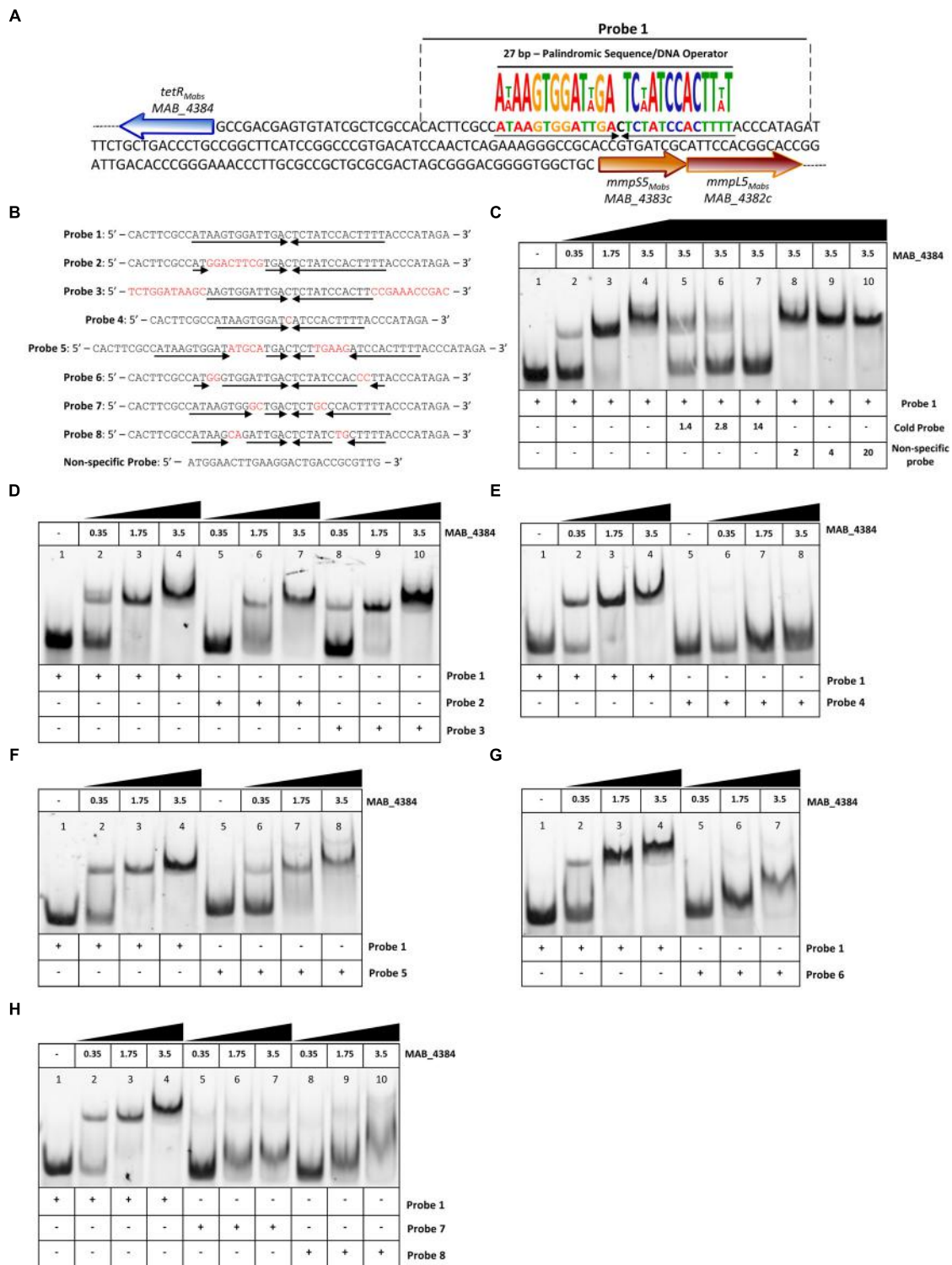


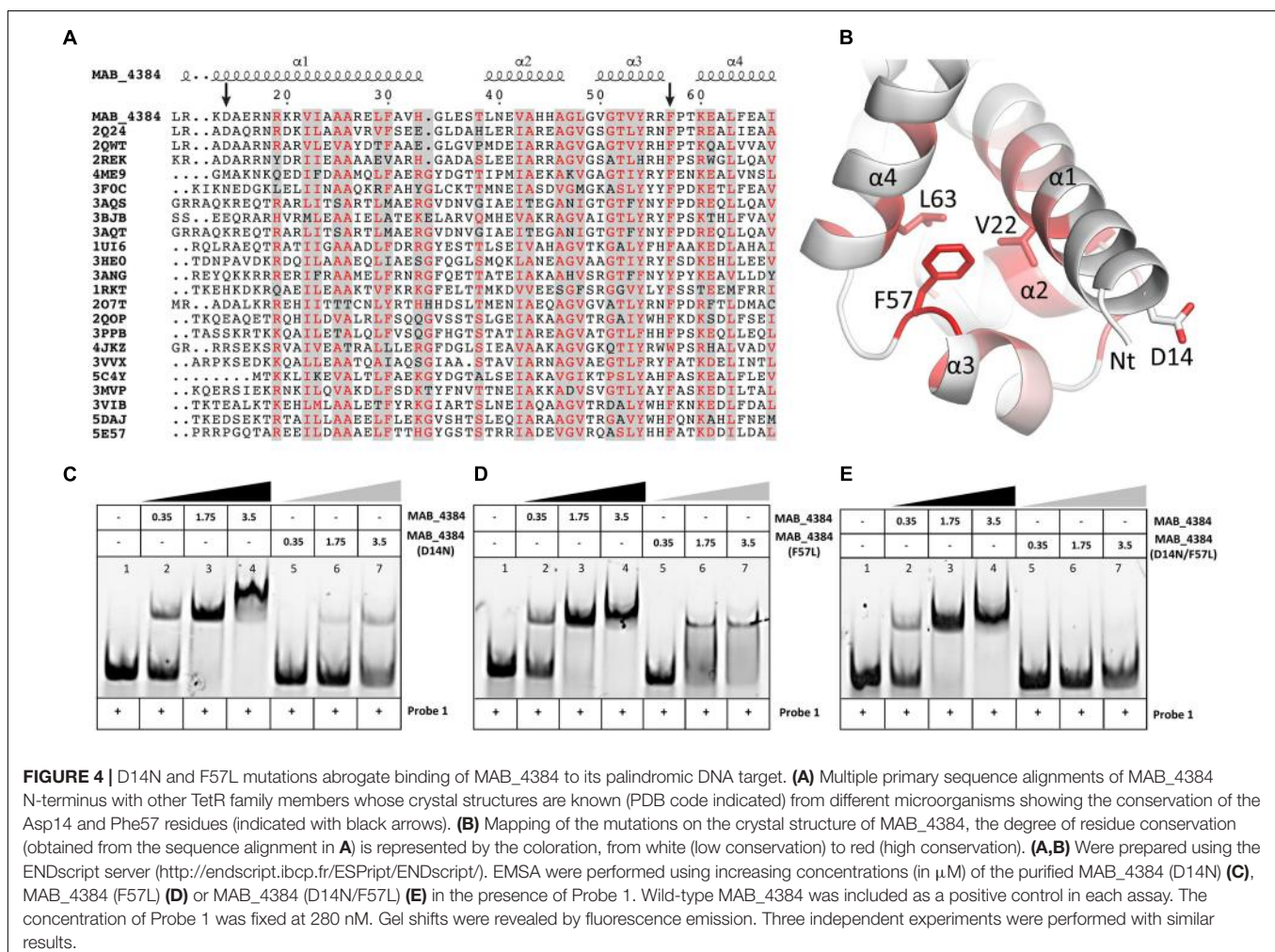
FIGURE 3 | Binding activity of MAB_4384 to a palindromic region within *IR*_{S5/L5}. **(A)** DNA sequence of *IR*_{S5/L5} and representation of the operator composed of a 27 bp region containing two degenerated inverted sequences of 13 nucleotides each (black arrows) and separated by a one nucleotide spacer. The probe used to perform the EMSA is delimited by dotted lines (Probe 1). **(B)** DNA sequences of all the various 5' fluorescein-labeled probes used in this study. **(C)** EMSA and competition assay using probe 1. Protein and DNA concentrations are expressed in μ M. In competition assays, the concentration of Probe 1 was fixed at 280 nM. Gel shifts were revealed by fluorescence emission. **(D–H)** EMSA using Probes 2 to 8, each time compared to the shift profile obtained with Probe 1. Experiments were reproduced three times with similar results.

minimal motif and the importance of the nucleotides involved in recognition and binding of the protein, a large set of fluorescein-labeled probes differing in their size and/or sequence were next assayed (**Figure 3B**). In the presence of Probe 2, in which only the right inverted sequence was conserved, a delay in the DNA shift was observed (partial in the presence of 1.75 μM of protein as compared to the reaction in the presence of Probe 1). Shifts using Probe 3, where the extra nucleotides surrounding the palindromic sequence were changed randomly were comparable to those obtained with Probe 1 (**Figure 3D**), indicating that the extra-palindromic sequence does not influence protein binding. With Probe 4, where the inverted repeats were shortened by six nucleotides, the formation of the DNA-protein complex was severely impeded even with the highest concentration of protein tested (3.5 μM) (**Figure 3E**). Increasing the spacer between the two inverted repeats by 10 nucleotides (Probe 5) negatively impacted the shift (**Figure 3F**). Substitutions of two nucleotides at the extremities in each repeat sequence (Probe 6) was accompanied by a pronounced shift alteration (**Figure 3G**) and similar results were obtained when di-nucleotide substitutions occurred at other positions within the conserved palindromic sequence (Probes 7 and 8) (**Figure 3H**).

Overall, these results confirm that a strict preservation of this inverted sequence and space separating these two motifs are crucial for binding of MAB_4384 to its target.

Asp14 and Phe57 Are Critical for Optimal DNA-Binding Activity of MAB_4384

Multiple primary sequence alignments of the MAB_4384 N-terminus with other TetR regulators with known three-dimensional structures indicate that the N-terminus Asp14 residue is well conserved in several other Tet regulators (**Figure 4A**). Similarly, Phe57 was also found to be part of a highly conserved stretch of amino acids in these proteins, although, in some instances, Phe was replaced by bulky/hydrophobic residues (**Figure 4A**). The importance of the conservation of these two residues for the function of MAB_4384 and presumably also for that of the other TetR regulators, was next assessed by EMSA using the purified MAB_4384 mutated variants. As compared to the shift profile with wild-type MAB_4384, the production of the DNA-protein complex was severely impaired in the presence of either MAB_4384 (D14N) (**Figure 4C**) or MAB_4384 (F57L) (**Figure 4D**) and fully abrogated in the presence of the double mutant (D14N/F57L) (**Figure 4E**).



Overall, these results support the importance of both residues in the DNA-binding capacity of MAB_4384 and the impaired ability of the mutants to bind to the operator is in agreement with the derepression of *lacZ* transcription in the *M. abscessus* strains carrying the D14N or F57L mutations (Figure 2B).

Oligomeric States of MAB_4384 and MAB_4384:DNA in Solution

To further characterize the MAB_4384:DNA complex formation, we next assessed its stability in solution by SEC (Figure 5). The oligomeric state of MAB_4384 in solution has an apparent 42.6 kDa molecular weight as compared to its 24.7 kDa theoretical molecular mass calculated from its primary sequence, thus highlighting the dimeric state of MAB_4384 in solution. MAB_4384 (dimer) was next incubated with the non-fluorescent DNA Probe 1 (Figure 3A) in a 1:1 molar ratio. A stable complex elution peak at 12.6 mL clearly shifted from the elution peak of MAB_4384 and DNA alone (Figure 5), allowing deduction of the molecular mass of the protein:DNA complex at 102 kDa. As the DNA alone in solution appeared on SEC as a 24.5 kDa molecule, these results strongly suggest the existence of two MAB_4384 dimers bound to one DNA molecule as such a complex would possess a molecular mass of 109.7 kDa ($2 \times 42.6 \text{ kDa} + 24.5 \text{ kDa}$). This 2-to-1 binding mode was further corroborated by the fact that an elution peak corresponding to free DNA can be seen at 14.4 mL when we mixed the MAB_4384 dimer and DNA in a 1:1 ratio. Moreover, increasing the molar ratio of the MAB_4384 dimer:DNA complex (2:1 and 3:1) did not yield larger protein:DNA complexes (data not shown), suggesting that, at a 1:1 molar ratio, the operator is already saturated by the protein. This observation is not unique as two TetR dimers have been shown to bind their DNA targets in other microorganisms, such as in *Staphylococcus aureus* (Grkovic et al., 2001) or in *Thermus thermophilus* (Agari et al., 2012).

Crystal Structure of MAB_4384

To understand, at a molecular basis, how the D14N or F57L mutations generate resistance to TAC analogs, we first crystallized and determined the X-ray structure of MAB_4384. Although the structure of the protein could not be solved by molecular replacement, the phase problem was overcome with the SAD method using crystals of selenomethionine-substituted MAB_4384 (Table 1). The crystal structure of the native protein was subsequently solved with the partial model obtained from the SAD data and refined to a resolution of 1.9 Å. The asymmetric unit contains two subunits. Chain A was modeled from residues Asp14 to Thr213, indicating that the first thirteen residues, one residual Gly residue from the tag and the last eight residues in the C-terminus were not visible in the electron density. Chain B showed also disordered regions as the first ten residues, one Gly residue from the tag in N-terminus as well as the last nine residues in the C-terminus, could not be modeled. Analysis of the crystal packing using the PISA server (Krissinel and Henrick, 2007) predicted the existence of a stable homodimer formed within the crystal, consistent with other TetR regulators (Cuthbertson

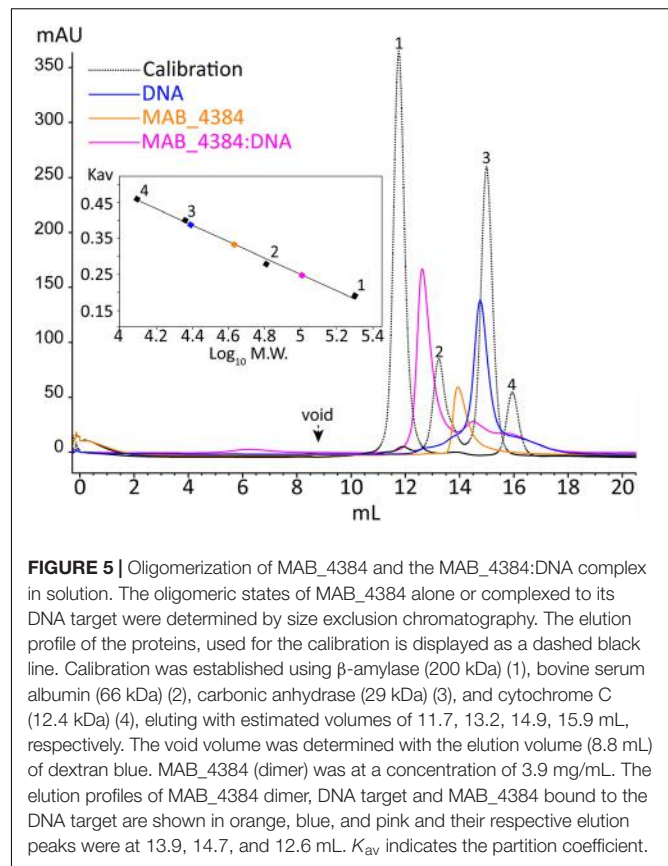
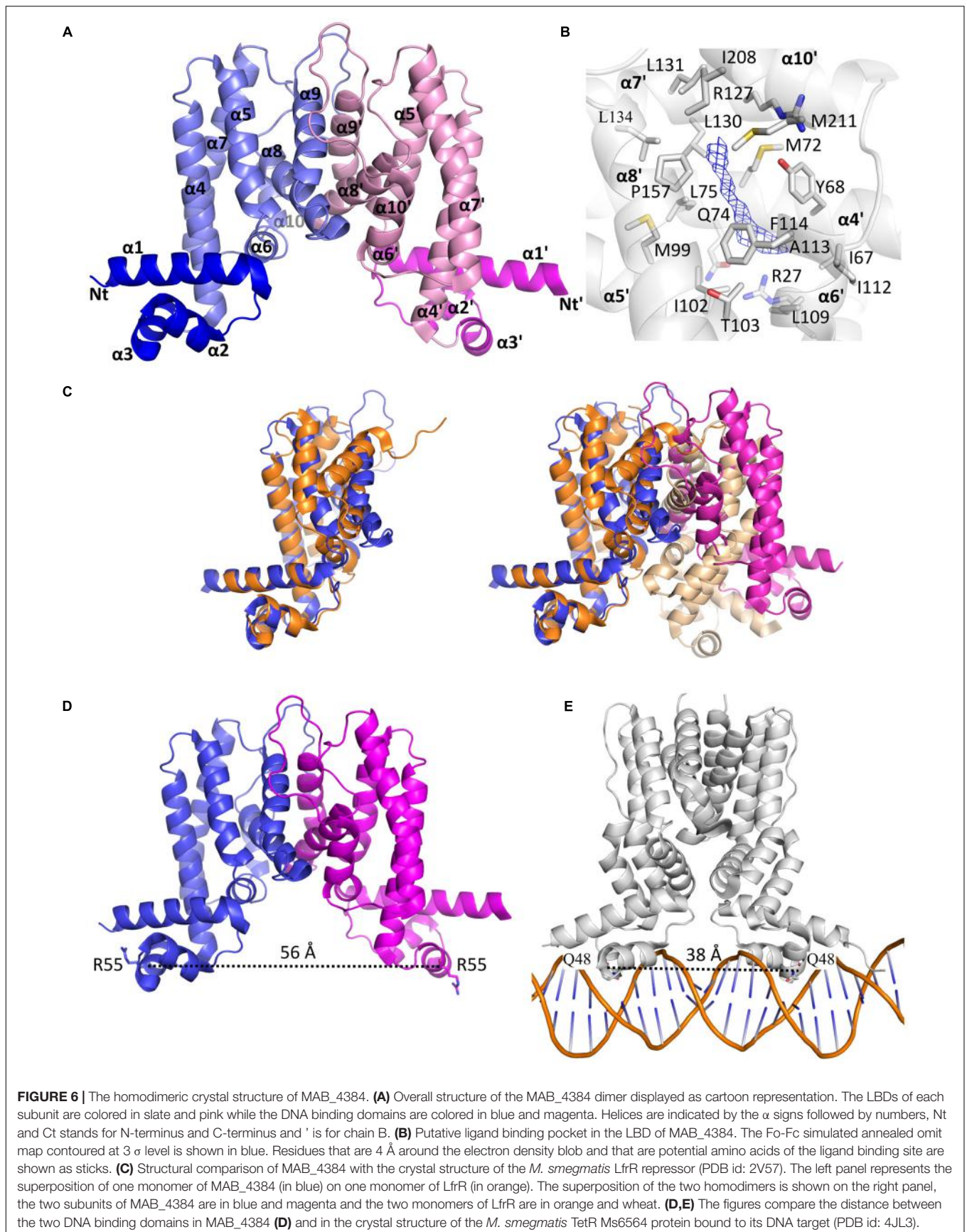


FIGURE 5 | Oligomerization of MAB_4384 and the MAB_4384:DNA complex in solution. The oligomeric states of MAB_4384 alone or complexed to its DNA target were determined by size exclusion chromatography. The elution profile of the proteins, used for the calibration is displayed as a dashed black line. Calibration was established using β -amylase (200 kDa) (1), bovine serum albumin (66 kDa) (2), carbonic anhydrase (29 kDa) (3), and cytochrome C (12.4 kDa) (4), eluting with estimated volumes of 11.7, 13.2, 14.9, 15.9 mL, respectively. The void volume was determined with the elution volume (8.8 mL) of dextran blue. MAB_4384 (dimer) was at a concentration of 3.9 mg/mL. The elution profiles of MAB_4384 dimer, DNA target and MAB_4384 bound to the DNA target are shown in orange, blue, and pink and their respective elution peaks were at 13.9, 14.7, and 12.6 mL. K_{av} indicates the partition coefficient.

and Nodwell, 2013) and with the SEC profile of MAB_4384 in solution (Figure 5).

The two subunits are very similar as their superposition leads to a root mean square deviation (r.m.s.d.) of 0.53 Å over 198 aligned residues. The N-terminus comprises the DNA binding domain (DBD), followed by the LBD. The DBD is composed of three α -helices α 1: residues 12–33, α 2: 39–46, and α 3: 50–56, where helices 2 and 3 form a helix-turn-helix (HTH) motif. The LBD is made of seven α -helices, α 4: 60–82, α 5: 88–104, α 6: 107–114, α 7: 121–143, α 8: 155–170, α 9: 178–188, and α 10: 205–211 (Figure 6A). The surface of dimerization of about 1,700 Å² is mediated by 31 residues mainly from helices α 8 and α 9 of each subunit and involves numerous interactions notably five salt bridges, fourteen hydrogen bonds and van der Waals interactions.

Interestingly, we noticed the presence of extra electron density in the LBD of chain B in a rather hydrophobic pocket (Figure 6B). Although we could not interpret this density, we hypothesize that it may correspond to a compound present in the crystallization solution, such as PEG. Search for structural homologs in the PDBFold server indicated that the closest structure to MAB_4384 corresponds to the LfrR TetR transcriptional regulator from *Mycobacterium smegmatis* bound to proflavin (PDB id : 2V57) (Bellinzoni et al., 2009) with an r.m.s.d. of 2.6 Å and sharing 16% primary sequence identity with MAB_4384. However, only one subunit of each structure could



be superposed as the overall dimers differed largely (**Figure 6C**). LfrR represses the expression of the LfrA efflux pump (Buroni et al., 2006) and mediates resistance to ethidium bromide, acriflavine, and fluoroquinolones (Takiff et al., 1996).

Due to the occurrence of an extra electron density within the LBD of MAB_4384 and that the closest structure of MAB_4384 is LfrR in its ligand bound form, it is very likely that MAB_4384 was crystallized in its open conformation, i.e., its derepressed form that is not able to interact with DNA. This was assessed by determining the distance between two residues from the DBD susceptible to interact with DNA. Residues Arg55 from chains A and B are about 56 Å apart (**Figure 6D**). In comparison, the distances between the equivalent residues in various TetR:DNA complexes are largely reduced. In the TetR:DNA complex (PDB id: 4PXI) from *Streptomyces coelicolor* this distance is 45 Å (Bhukya et al., 2014), in the TetR:DNA complex from *M. smegmatis* (PDB id: 4JL3) (Yang et al., 2013) (**Figure 6E**), *E. coli* (PDB id: 1QPI) (Orth et al., 2000), *Corynebacterium glutamicum* (PDB id: 2YVH) (Itou et al., 2010) or *Staphylococcus aureus* (PDB id: 1JT0) (Schumacher et al., 2002) the distances are 38 Å, 30 Å, 42 Å, and 37 Å, respectively. From these results it can be inferred that the DBDs of MAB_4384 are too far from each other to bind to the DNA groove. These observations combined with the presence of an unidentified ligand in the LBD strongly suggest that the MAB_4384 structure is in an open conformation.

Structural Basis of the Resistant Phenotype of the Mutants

To determine the impact of the mutations in the spontaneous resistant *M. abscessus* mutants, the D14N and F57L residues were mapped on the crystal structure of MAB_4384. Asp14 is located at the beginning of helix $\alpha 1$ and is conserved in several TetR protein members (**Figures 4A,B**). Residues from helix $\alpha 1$ are often found in contact with DNA as seen in several TetR:DNA crystal structures. Nonetheless, due to the acidic nature of Asp, it is more likely that this residue repulses DNA. We, therefore, hypothesize that it may instead contribute to the correct positioning of other residues located in its close vicinity. Alternatively, repulsion may promote important interactions of DNA with other residues. Indeed, in other collected datasets at lower resolution (data not shown), Asp14 was found to establish a salt bridge interaction, thereby stabilizing the side chain of Arg17 that could interact with DNA. In the absence of a crystal structure of MAB_4384 bound to DNA it is, however, difficult to convincingly affirm the impact of the D14N substitution. However, neither the repulsion of DNA nor the establishment of a salt bridge would be possible if Asn is present instead of Asp, presumably explaining the loss of DNA binding activity of the TetR D14N mutant.

The role of Phe57 situated on helix $\alpha 3$ is more obvious as this position appears always occupied by bulky residues (Phe, Tyr, or Trp) in numerous TetR proteins (**Figure 4A**). The side chain of Phe57 contacts the side chains of Val22 from helix $\alpha 1$ and Leu63 from helix $\alpha 4$ (**Figure 4B**). Phe57 is very likely to perform an important structural role in stabilizing the DBD. Replacement with a less bulky side chain such as Leu would abolish these

contacts with helices $\alpha 1$ and $\alpha 4$ residues, thus perturbing the overall structural fold of this domain and suppressing the DNA-binding capacity of MAB_4384.

Drug Recognition of MAB_4384 Induces Expression of MmpS5/MmpL5

TetR regulators can respond to small molecules and the best characterized member of this family of regulators is *E. coli* TetR itself. It confers resistance to tetracycline by regulating the expression of the tetracycline TetA efflux pump (Hillen and Berens, 1994). When tetracycline binds to TetR, the regulator loses affinity for the operators, conducting derepression of *tetA* and extrusion of tetracycline out of the bacteria (Lederer et al., 1995). To investigate whether TAC derivatives could bind to the LBD of MAB_4384, *in silico* docking was performed. Despite using a large grid box covering the entire LBD, all three compounds seem to be accommodated by the same binding pocket (**Figure 7A**). Interestingly, this pocket positioned exactly where the extra electron density was seen in the LBD (**Figure 6B**). All the compounds bind with similar energies in the aforementioned hydrophobic binding pocket. A slightly stronger interaction for the most hydrophobic derivative D17 was nonetheless observed. D17 and D6 that seem to bind stronger are more hydrophobic and in their best docking poses their thiosemicarbazide group is differently oriented as compared to D15.

Next, we determined whether expression of *mmpS5/mmpL5* can be conditionally induced by the substrates that are extruded by the efflux pump system. This was achieved by determining the effect of the D6, D15, and D17 analogs on LacZ production using the pMV261_P_{S5/L5}_lacZ reporter strain in *M. abscessus* incubated in Sauton's medium with various drug concentrations consisting of 1X, 2.5X, and 5X the MIC for D15 and of 0.5X, 1X, and 2.5X for D6 and D17. Kinetic studies indicated that optimal expression was obtained after 96 h of treatment (data not shown). The LacZ assay showed that transcription was induced by the TAC analogs in a dose-dependent manner whereas non-related drugs such as amikacin or the DMSO control had no effects (**Figure 7B**). In comparison with the basal transcriptional level in Sauton's medium (no drug control), the addition of TAC derivatives in the cultures resulted in a reproducible 2.5- to 5-fold increase in the detection of β -Gal activity with D17 being the most potent inducer at 2.5X MIC. However, ethionamide, an antitubercular drug that, like the TAC and TAC analogs, requires to be activated by the EthA monooxygenase (Baulard et al., 2000; DeBarber et al., 2000; Dover et al., 2007; Halloum et al., 2017) failed to induce lacZ at 5X MIC (previously determined at 16 μ g/ml). Induction of lacZ by D17 treatment was further confirmed at a transcriptional level from the pMV261_P_{S5/L5}_lacZ cultures treated with 2.5 \times MIC of D17 for 8 h (**Figure 7C**, left). This effect was specific to D17 as no gene induction was observed in the DMSO-treated cultures. Consistently, transcription profiling of *mmpS5* and *mmpL5* in the D17-exposed cultures clearly showed a marked induction level as compared to the DMSO-treated cultures and no effect on *tgsl* expression (**Figure 7C**, right).

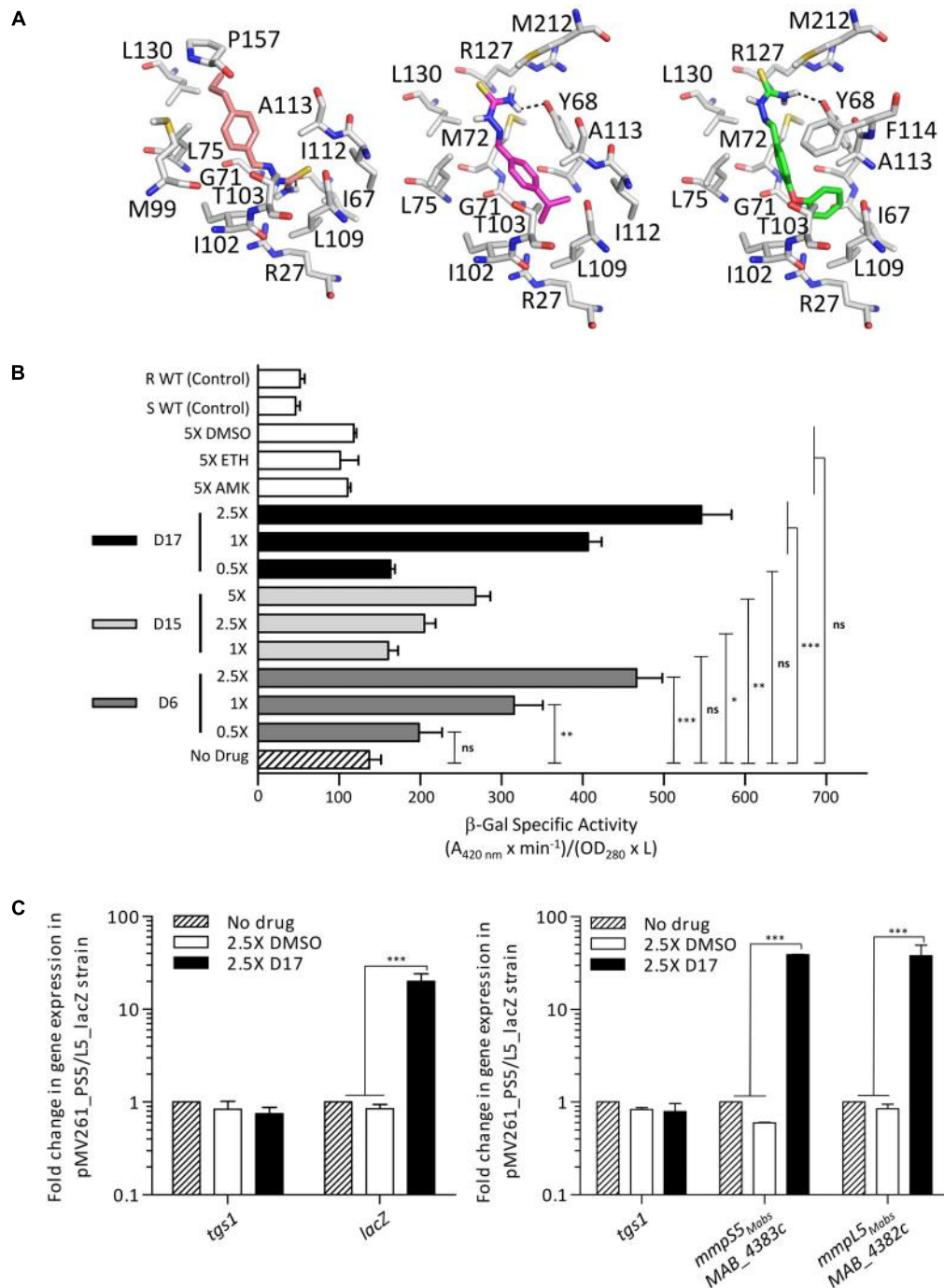
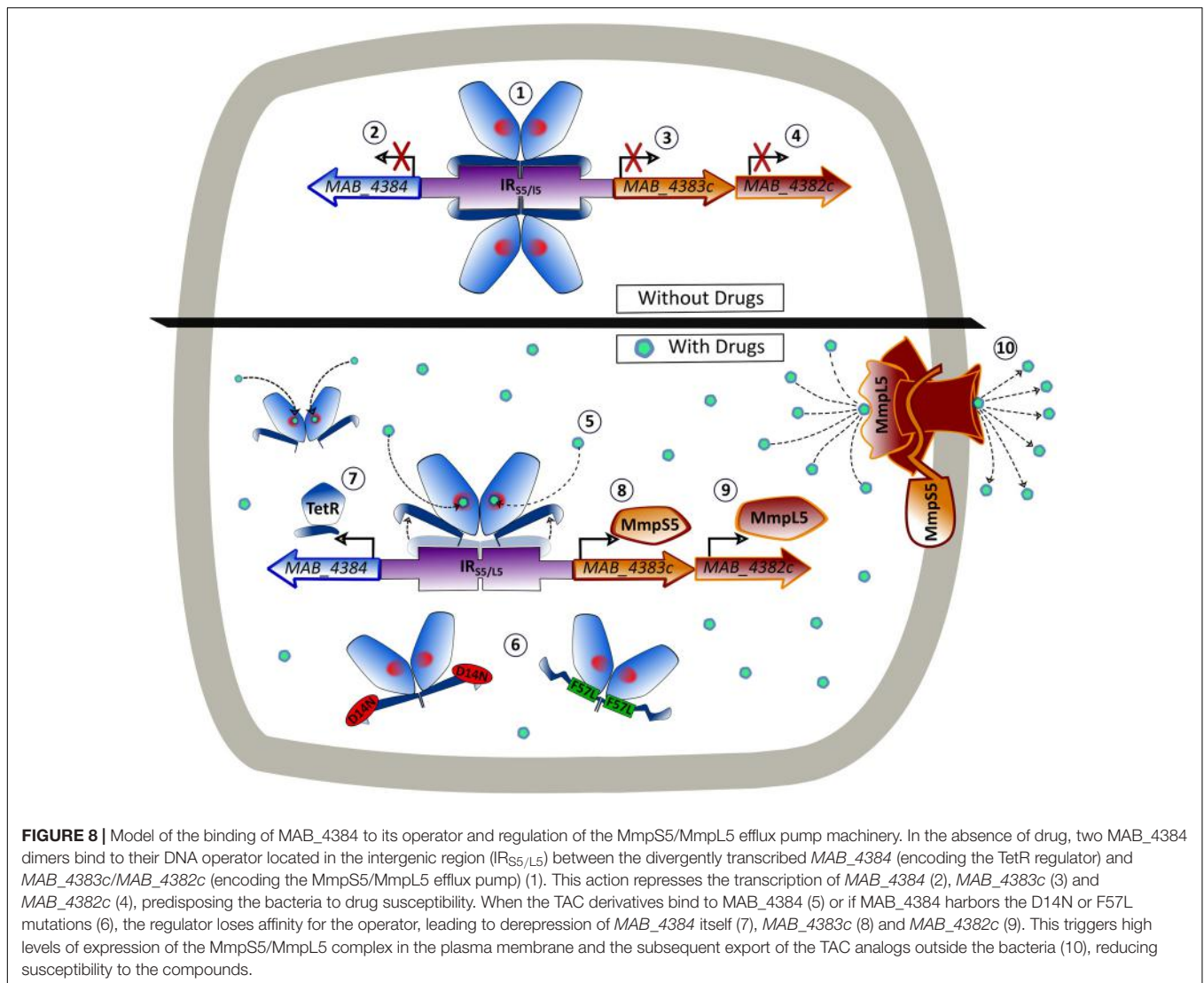


FIGURE 7 | IR_{S5/L5} can be induced by structural analogs of thiacetazone. **(A)** Docking of TAC derivatives in the ligand binding site of MAB_4384. All the residues involved in van der Waals, hydrophobic bonds or hydrogen bonds (in black dashes) are displayed as sticks. D15 in salmon has a binding energy of $\Delta G = -6.6$ kcal/mol, D6 in magenta has a $\Delta G = -7.3$ kcal/mol, and D17 seems to bind slightly stronger with a $\Delta G = -8.3$ kcal/mol. **(B)** Conditional induction of *lacZ* by structural analogs of TAC in *M. abscessus*. Induction of β -Gal activity in wild-type *M. abscessus* S carrying pMV261_PSS/L5_*lacZ* was assayed using mid-log phase cultures incubated with increasing drug concentrations varying from 1X to 5X the MIC for D15 and varying from 0.5X to 2.5X the MIC for D6 and D17. Inductions were performed for 96 h at 37°C. The β -galactosidase specific activity (SA _{β -Gal}) was quantified in liquid cultures using ONPG as a substrate. Amikacin (AMK) and ethionamide (ETH) were included as unrelated drug controls. **(C)** Transcriptional profile of *lacZ* in the *M. abscessus* pMV261_PSS/L5_*lacZ* reporter strain exposed to 2.5X the MIC of D17 for 8 h (left). *tgs1* was included as a non-relevant control. Replacing D17 by an equal volume of DMSO had no effect on *lacZ* transcription. Transcriptional induction of *mmpS5_{Mabs}* and *mmpL5_{Mabs}* following exposure to 2.5X the MIC of D17 for 8 h (right). Results were obtained from three independent experiments and the error bars represent standard deviation. For statistical analysis the Student's *t*-test was applied with ns, *, **, *** indicating non-significant, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.



Together, these results support the view that TAC analogs, which are substrates of MmpS5/mmpL5, are also effectors of MAB_4384-induced transcription of *mmpS5/mmpL5*.

DISCUSSION

Herein, a combination of genetic, biochemical and structural studies was used to demonstrate that MAB_4384 is part of the TetR family of regulators, which represses the transcriptional expression of the MmpS5/MmpL5 efflux pump. MAB_4384 belongs to the type I class TetR family of regulators, characterized by a divergent orientation to one of the adjacent target genes (Cuthbertson and Nodwell, 2013). In *M. tuberculosis*, MmpS5/MmpL5 is under the control of the MarR repressor Rv0678 (Radhakrishnan et al., 2014) and mutations in this regulator leads to drug resistance (Andries et al., 2014; Hartkoorn et al., 2014; Zhang et al., 2015). EMSA indicated a direct binding of Rv0678 to the intergenic region located between *mmpS5* and

Rv0678. However, shifts were also found using the promoter regions of *mmpS2-mmpL2*, *mmpS4-mmpL4*, and *Rv0991-Rv0992* (Radhakrishnan et al., 2014), suggesting that a single regulator can control expression of several *mmpS/mmpL* loci. Our analysis indicates that, despite the fact that *M. abscessus* possesses the highest number of *mmpL* genes among all mycobacterial species studied (Viljoen et al., 2017), the MAB_4384 regulator is highly specific to the *mmpS5/mmpL5* pair as demonstrated by the lack of transcriptional regulation of a large set of *mmpL* genes and the presence of a unique inverted DNA sequence target that was not found elsewhere in the chromosome. This unique trait might also be reflected by the modest structural homology of MAB_4384 with other TetR crystal structures. The tight regulation and the high specificity of interaction with its target DNA, however, cannot be solely explained on the basis of the MAB_4384 crystal structure and the structure of the MAB_4384:DNA complex is, therefore, greatly warranted to dissect these underlying mechanisms. Nevertheless, our structural analysis underscores the strategy employed by *M. abscessus* to acquire mutations

impacting the DNA-binding capacity or the folding/stability of the DBD of MAB_4384 to become resistant.

EMSA and *lacZ* reporter fusions confirmed that D14N and F57L mutations, alleviating the DNA-binding activity of MAB_4384, cause a strong up-regulation of *mmpS5/mmpL5* gene expression, in agreement with our previous qRT-PCR analyses (Halloum et al., 2017). This leads to extrusion of the TAC derivatives out of the cells, contributing to the high MIC values for TAC derivatives against these mutants, as illustrated in **Figure 8**. Expression of multi-drug resistant efflux pumps can also be conditionally induced using structurally diverse substrates (Kaatz and Seo, 1995; Rosenberg et al., 2003; Buroni et al., 2006). This induction is caused by the direct interaction of these substrates with the repressors, interfering with binding of the repressors to their target operators and resulting in increased expression of the pumps. Here, we show inducible β -galactosidase activity following treatment with D6, D15, or D17, a mechanism that is very likely to be mediated by MAB_4384. This view is reinforced by the fact that docking studies highlighted the possibility that all three analogs could be accommodated in the LBD of the protein, which perfectly coincided with the extra electron density observed. Since, the LBD are remote from the DBD, the derepression of TetR family regulators involves allosteric mechanisms that include conformational changes transmitted largely within the same subunit (Ramos et al., 2005). The interaction of ligands with the LBD captures a conformational state where the DBD is repositioned relative to the LBD in a way that the dimer is prevented from binding to its target DNA. However, definitive proof of this mechanism awaits the elucidation of the crystal structure of the D17-bound form of MAB_4384, as reported for instance with the hexadecyl octanoate-bound EthR repressor (Frénois et al., 2004) or the LfrR regulator complexed with proflavine (Bellinzoni et al., 2009). Lack of inducible *lacZ* expression in *M. abscessus* cultures exposed to amikacin, for which mutations in 16S rRNA represent a major mechanism of resistance (Prammananan et al., 1998), indicates that MmpL5-mediated efflux cannot mediate resistance toward this antibiotic in line with the lack of cross-resistance toward amikacin observed for TAC derivative-spontaneous resistant mutants (Halloum et al., 2017). The specificity of the MAB_4384-driven resistance mechanism described herein is further supported by the lack of *lacZ* induction during exposure to ETH, that similarly to TAC and TAC analogs, requires bio-transformation by EthA, whose expression is also dependent on the EthR regulator belonging to the TetR family (Baulard et al., 2000; Engohang-Ndong et al., 2004; Halloum et al., 2017). Together, these findings strongly suggest that when TAC analogs bind to MAB_4384, the regulator loses affinity for its DNA target, resulting in up-regulation of *mmpS5/mmpL5* and export of the drugs from the cells (**Figure 8**). These results also point out the selectivity of this efflux-based mechanism. Indeed, no change in the MIC of clofazimine or bedaquiline were noticed in a *MAB_4384*-disrupted strain, which appears intriguing as MmpL5 has been reported as a multi-substrate efflux pump responsible for low-level resistance to both of these drugs in *M. tuberculosis* (Hartkoorn et al., 2014). The LBD of MAB_4384 potentially can accommodate bulky

molecules and might thus indicate that MAB_4384 is involved in efflux of other types of compounds in addition to TAC analogs. However, we could neither dock clofazimine nor bedaquiline in the LBD of MAB_4384 (not shown). Several reasons can be put forth to explain these species-specific variations. In *M. tuberculosis*, expression of MmpL5 is under the control of a MarR regulator rather than a TetR regulator. Alternatively, we have previously reported the occurrence in *M. abscessus* of three *mmpS5/mmpL5* paralogs (Halloum et al., 2017), thus, it remains possible that either of the two remaining genes may participate in co-resistance to these drugs in *M. abscessus*.

The highly pronounced expression of *lacZ* under derepressed conditions found in the M1A, F57L, or D14N mutant strains, almost at levels similar to those driven by the strong and constitutive *hsp60* promoter, confirmed the very high expression levels of *mmpS5* and *mmpL5* detected by qRT-PCR and probably explains the very high level of resistance of the mutants (MIC > 200 μ g/mL). This contrasts also with findings where MmpL5 mediates only low-levels of resistance in *M. tuberculosis* (Andries et al., 2014; Hartkoorn et al., 2014), presumably because expression of *mmpL5* is driven by a weaker promoter than in *M. abscessus*. That *mmpS5/mmpL5* expression is tightly controlled suggests that the MmpS5/MmpL5 machinery may exert an important function in the assembly and/or maintenance of the cell wall by exporting a yet unidentified lipid, as already reported for several MmpL transporters in *M. tuberculosis* (Chalut, 2016; Viljoen et al., 2017). Alternatively, they may participate in adaptation during the infection process. However, the growth curves of the wild-type or the strain constitutively expressing high levels of MmpL5 (due to the M1A mutation in MAB_4384) were comparable *in vitro*. In addition, microinjections of the different strains were done in the zebrafish embryo, an animal model previously developed to study the early events of the *M. abscessus* infection (Bernut et al., 2014, 2015). No differences in virulence were noticed between the wild-type and MAB_4384 (M1A) strains (Supplementary Figure S2). Interestingly, the *mmpS5/mmpL5* locus was found to be induced when *M. abscessus* was exposed to a defined, synthetic medium that mimics the composition of CF sputum (Miranda-CasoLuengo et al., 2016). This may be part of a complex adaptive transcriptional response to the mucus layer of the CF airways that leads to the chronic infections of *M. abscessus*.

In summary, this study provides new functional and structural insights into TetR-dependent regulation of MmpL efflux pumps in mycobacteria. Considering the exceptionally high abundance of TetR transcriptional regulators (more than 130) as well as the important MmpL repertoire (around 30) in *M. abscessus*, one can anticipate that mechanisms similar to the one described here are exploited by this pathogen to express its intrinsic resistance level to other antibiotics.

ETHICS STATEMENT

Zebrafish experiments were done at IRIM, according to European Union guidelines for handling of laboratory animals

(http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and approved by the Direction Sanitaire et Vétérinaire de l'Hérault and Comité d'Ethique pour l'Expérimentation Animale de la Région Languedoc Roussillon (CEEA-LR) under the reference CEEA-LR-1145.

AUTHOR CONTRIBUTIONS

MR, AVG, AV, MB, and LK acquired and analyzed the data. EG, MB, and LK wrote the manuscript. LK conceived and designed the study.

FUNDING

This work was supported by the Fondation pour la Recherche Médicale (FRM) (grant number DEQ20150331719 to LK; grant

number ECO20160736031 to MR) and by the InfectioPôle Sud for funding the Ph.D. Fellowship of AVG.

ACKNOWLEDGMENTS

The authors wish to thank G. S. Coxon for the generous gift of the TAC analogs. The crystallographic data were collected on beamline ID30B at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The authors are grateful to Local Contact at the ESRF for providing assistance in using beamline ID30B.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.00649/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article2

« **Mutations in the MAB_2299c TetR Regulator Confer Cross-Resistance to Clofazimine and Bedaquiline in *Mycobacterium abscessus*.** » Richard M*, Gutiérrez AV*, Viljoen A, Rodriguez-Rincon D, Roquet-Baneres F, Blaise M, Everall I, Parkhill J, Floto RA, Kremer L (* = Contribution équivalente) Antimicrob Agents Chemother. 2018 December 21; Volume 63 Issue 1.

La bédaquiline (BDQ) et la clofazimine (CFZ) sont actuellement utilisées dans le traitement de tuberculoses multi-résistantes et sont évaluées cliniquement pour leurs utilisations dans la thérapie contre *M. abscessus*. La sélection de mutants spontanés résistants à la CFZ a révélé la présence de mutations dans le régulateur TetR codé par *MAB_2299c*. De façon surprenante, ces mêmes souches étaient également co-résistantes à la BDQ et toutes présentaient une augmentation modérée des ARNm de *MAB_2300/MAB_2301* codant pour une potentielle pompe à efflux de type MmpS/MmpL. De la même façon que précédemment pour le régulateur TetR MAB_4384 (voir **Article 1**), l'utilisation d'EMSA a permis d'identifier une longue séquence palindromique située en amont de *MAB_2300/MAB_2301* et de prouver l'interaction physique de ce régulateur TetR avec ce segment d'ADN cible. La présence d'un complexe TetR/ADN stable isolé par gel filtration a confirmé la liaison spécifique de cette protéine avec ce motif palindromique. Des substitutions nucléotidiques au sein du motif provoquent un échec dans la formation de ce complexe. Par ailleurs, un variant de MAB_2299c présentant une mutation dans le domaine HTH n'est également plus capable de former un complexe avec l'ADN cible, démontrant ainsi l'importance de ce résidu dans la liaison du régulateur à l'ADN. Le développement d'une nouvelle technique de délétion génique basée sur un évènement de double recombinaison homologue, combinant une première étape de sélection de colonies fluorescentes et d'une seconde étape de contre-sélection en présence d'isoniazide a permis de générer les mutants non-marqués ΔMAB_2299c et $\Delta MAB_2299c - \Delta MAB_2300/2301$. Ces souches mutées ont permis de confirmer le rôle de MAB_2300/MAB_2301 dans la résistance intrinsèque de *M. abscessus* à la CFZ et la BDQ. Cette nouvelle technique de génétique représente un outil très performant ouvrant la possibilité de déléter simultanément de nombreux gènes et d'étudier leur fonction biologique ainsi que leur contribution dans la virulence et/ou résistance aux antibiotiques chez *M. abscessus*.

Article 3

« **The TetR Family Transcription Factor MAB_2299c Regulates the Expression of Two Distinct MmpS-MmpL Efflux Pumps Involved in Cross-Resistance to Clofazimine and Bedaquiline in *Mycobacterium abscessus*.** ». Gutiérrez AV*, Richard M*, Roquet-Banères F, Viljoen A, Kremer L (* = contribution égale) Antimicrob Agents Chemother. 2019 September 23 ; Volume 63 Issue 10.

L'abondance à la fois des régulateurs TetR et des protéines de type MmpL chez *M. abscessus* suggère fortement la possibilité que d'autres couples régulateur/MmpL soient impliqués dans l'export des antibiotiques. Une étude phylogénétique des protéines MmpL chez *M. abscessus* a révélé que la paire MmpS/MmpL codée par *MAB_1135c/MAB_1134c* résulterait d'une duplication génique de *MAB_2300/MAB_2301*. L'analyse *in silico* de la région intergénique située en amont de *MAB_1135c/MAB_1134c* a révélé la présence d'une séquence palindromique identique mais de taille totale plus courte que celle reconnue par MAB_2299c retrouvée en amont de *MAB_2300/MAB_2301* (voir **Article 2**). Des expériences d'EMSA entre MAB_2299c et cette nouvelle région palindromique ont montré que ce régulateur est également capable de se lier à la séquence d'ADN située en amont de *MAB_1135c/MAB_1134c*. Une analyse des transcrits de tous les *mmpL* issue du mutant ΔMAB_2299c a révélé un niveau plus élevé des ARNm de *MAB_2300/MAB_2301* sans surprise, mais aussi de *MAB_1135c/MAB_1134c* par rapport à la souche sauvage, suggérant que MAB_2299c agisse également comme répresseur sur la pompe à efflux *MAB_1135c/MAB_1134c*. Nous avons généré, grâce à notre nouvelle technique de mutagenèse pUX1-*katG* (**Article 2**) un quintuple mutant $\Delta MAB_2299c - \Delta MAB_2300/2301 - \Delta MAB_1135c/1134c$ qui présente une hypersusceptibilité à la CFZ et à la BDQ. Ce phénotype a également été mis en évidence dans des macrophages humains infectés. L'ensemble de cette étude démontre que le régulome *MAB_2299c - MAB_2300/2301 - MAB_1135c/1134c* joue un rôle dans la multi-résistance intrinsèque de *M. abscessus* à deux antibiotiques qui sont structuralement différents et actuellement en cours d'évaluation clinique dans le traitement des infections à *M. abscessus*. MAB_2299c pourrait donc représenter un marqueur de résistance à prendre en considération dans la détermination du profil de susceptibilité/résistance d'isolats cliniques issus de patients traités avec la BDQ et la CFZ.



The TetR Family Transcription Factor MAB_2299c Regulates the Expression of Two Distinct MmpS-MmpL Efflux Pumps Involved in Cross-Resistance to Clofazimine and Bedaquiline in *Mycobacterium abscessus*

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ABSTRACT *Mycobacterium abscessus* is a human pathogen responsible for severe respiratory infections, particularly in patients with underlying lung disorders. Notorious for being highly resistant to most antimicrobials, new therapeutic approaches are needed to successfully treat *M. abscessus*-infected patients. Clofazimine (CFZ) and bedaquiline (BDQ) are two antibiotics used for the treatment of multidrug-resistant tuberculosis and are considered alternatives for the treatment of *M. abscessus* pulmonary disease. To get insights into their mechanisms of resistance in *M. abscessus*, we previously characterized the TetR transcriptional regulator MAB_2299c, which controls expression of the *MAB_2300-MAB_2301* genes, encoding an MmpS-MmpL efflux pump. Here, *in silico* studies identified a second *mmpS-mmpL* (*MAB_1135c-MAB_1134c*) target of MAB_2299c. A palindromic DNA sequence upstream of *MAB_1135c*, sharing strong homology with the one located upstream of *MAB_2300*, was found to form a complex with the MAB_2299c regulator in electrophoretic mobility shift assays. Deletion of *MAB_1135c-1134c* in a wild-type strain led to increased susceptibility to both CFZ and BDQ. In addition, deletion of these genes in a CFZ/BDQ-susceptible mutant lacking MAB_2299c as well as *MAB_2300-MAB_2301* further exacerbated the sensitivity of this strain to both drugs *in vitro* and inside macrophages. Overall, these results indicate that *MAB_1135c-1134c* encodes a new MmpS-MmpL efflux pump system involved in the intrinsic resistance to CFZ and BDQ. They also support the view that MAB_2299c controls the expression of two separate MmpS-MmpL efflux pumps, substantiating the importance of MAB_2299c as a marker of resistance to be considered when assessing drug susceptibility in clinical isolates.

KEYWORDS EMSA, MmpL, *Mycobacterium abscessus*, TetR regulator, bedaquiline, clofazimine, drug resistance mechanisms, efflux pumps

Mycobacterium abscessus is a rapidly growing nontuberculous mycobacterium (NTM) and an emerging pathogen that causes sporadic outbreaks worldwide (1). The *M. abscessus* complex comprises three subspecies, *M. abscessus sensu stricto*, *Mycobacterium bolletii*, and *Mycobacterium massiliense* (2, 3), differing in their drug susceptibility profile and evolution (4). Genomic analyses indicated that, in addition to specific genes linked to mycobacterial virulence, other virulence genes found in *Pseudomonas aeruginosa* and *Burkholderia cepacia*, pathogens commonly isolated from the lungs of cystic fibrosis (CF) patients and acquired by horizontal transfer, are also present in *M. abscessus* (5, 6). Likewise, the *M. abscessus* complex represents the most important cause of pulmonary infections by rapidly growing NTM in patients with chronic lung diseases, such as bronchiectasis and CF (7, 8), which can result in nodular and cavitary granulomas and persistent lung infection (7–10). Patients with chronic *M. abscessus*

Citation Gutiérrez AV, Richard M, Roquet-Banères F, Viljoen A, Kremer L. 2019. The TetR family transcription factor MAB_2299c regulates the expression of two distinct MmpS-MmpL efflux pumps involved in cross-resistance to clofazimine and bedaquiline in *Mycobacterium abscessus*. Antimicrob Agents Chemother 63:e01000-19. <https://doi.org/10.1128/AAC.01000-19>.

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Received 14 May 2019

Returned for modification 23 June 2019

Accepted 19 July 2019

Accepted manuscript posted online 22 July 2019

Published 23 September 2019

infections present higher rates of pulmonary function decline than those without NTM infections (11). Recent epidemiological studies also documented the transmission of dominant circulating *M. abscessus* clones that have spread globally between hospitals (12), highlighting *M. abscessus* as a major threat in many CF centers worldwide (8).

A recent meta-analysis reported that the treatment success rate across all patients with *M. abscessus* pulmonary disease was 45.6%, reaffirming that treatment outcomes are unsatisfactory (13). *M. abscessus* treatment remains very challenging. The American Thoracic Society (ATS)/Infectious Disease Society of America recommend multidrug therapy that includes a macrolide and one or more parenteral drugs (amikacin plus ceftazidime or imipenem) (7). *M. abscessus* has developed a large panel of drug resistance mechanisms that considerably restrict the available antimicrobials to treat these infections (14–16). They include the acquisition of mutations in the target genes, intrinsic resistance to drugs attributed to low permeability of the *M. abscessus* cell envelope, defective drug-activating systems that convert prodrugs into metabolically active compounds, induction of drug export systems, and the expression of numerous enzymes that can modify either the drug target or the drug itself (15, 17). Overall, this leads to the phenomenal resistance level of *M. abscessus* to most classes of antibiotics used for treatment of other infectious diseases, such as macrolides, aminoglycosides, rifamycins, β -lactams, quinolones, and tetracyclines.

Fueling a drug pipeline to discover and develop improved and safer treatments for *M. abscessus* infections relies on *de novo* drug discovery to identify new pharmacophores as well as repositioning of antimicrobials acting against validated targets for other infectious diseases (18). In this context, the repositioning of clofazimine (CFZ) and bedaquiline (BDQ) is gaining increasing interest for the treatment of *M. abscessus* pulmonary disease. CFZ is an orally administered drug, originally approved for the treatment of leprosy, that is reduced by the type 2 NADH:quinone oxidoreductase, a key component in the electron transport chain (19). *In vitro* studies highlighted its advantages in multidrug regimens against *M. abscessus*, acting in synergism with amikacin (AMK) (20) or tigecycline (TIG) (21) and in preventing bacterial regrowth observed with clarithromycin or AMK alone (22). BDQ, approved by the Food and Drug Administration and the European Medicines Agency for the treatment of multidrug-resistant tuberculosis, is a diarylquinoline that targets the ATP synthase in *M. tuberculosis* (23). We and others showed that BDQ exhibits very low MICs against NTM, including clinical *M. abscessus* strains from CF and non-CF patients (24–26). However, despite being a potent growth inhibitor at low concentrations, BDQ lacks bactericidal activity against *M. abscessus* (26, 27). In the *M. abscessus* zebrafish model of infection, BDQ acts very efficiently by reducing the number and size of abscesses, which represent a marker of disease severity and uncontrolled infection (28), protecting the infected larvae from killing by *M. abscessus* (26). The mode of action of BDQ in *M. abscessus* relies on the rapid depletion of ATP, and the transfer of single point mutations into the chromosomal *atpE* locus conferred high resistance levels to BDQ (26). In addition to *atpE*, mutations associated with cross-resistance to CFZ and BDQ were recently uncovered in *MAB_2299c*, encoding a TetR-family transcription factor that controls the expression level of the MmpS-MmpL (*MAB_2300-2301*) efflux pump (29).

Considering the extremely high number of TetR regulators and MmpL proteins in *M. abscessus* (30), it remains possible that additional TetR-dependent MmpS-MmpL efflux pump systems contribute to the drug resistance profile of *M. abscessus* to CFZ and BDQ. In this study, we identified a new *mmpS-mmpL* gene cluster (*MAB_1135c-MAB_1134c*; termed *MAB_1135c-1134c*) whose expression is also dependent on the *MAB_2299c* TetR repressor. Genetic approaches were used to demonstrate that this gene cluster participates in cross-resistance to both CFZ and BDQ. These findings expand our current understanding about transcriptional regulation of the MmpS-MmpL efflux pumps in mycobacteria and mechanisms of drug resistance in one of the most difficult-to-treat mycobacterial species.

RESULTS

Identification of MAB_1135c-1134c as a close paralog of MAB_2300-2301. We recently demonstrated that mutations in the TetR regulator MAB_2299c lead to up-regulation of the MAB_2300-2301 gene cluster, encoding the MmpS-MmpL efflux pump membrane proteins accounting for cross-resistance to CFZ and BDQ in *M. abscessus* (29). With the objective to identify other potential MmpS-MmpL couples that are functionally homologous to MAB_2300-2301 and participate in CFZ/BDQ resistance, we performed a homology analysis of all *M. abscessus* MmpS and MmpL paralogs as well as all of the TetR family transcription factors present in close genomic vicinity to these MmpS-MmpL couples. This analysis identified MAB_1135c-1134c as the closest paralogous MmpS-MmpL couple to MAB_2300-2301 (Fig. 1A). In addition, the TetR protein MAB_1136, whose gene is located upstream of MAB_1135c, was uncovered as the closest homologue of MAB_2299c. Indeed, a comparison of the two loci that contain the *mmpS-mmpL* genes as well as their adjacent upstream (containing the *tetR* genes and their nucleotide target sequences) and downstream nucleotide sequences suggests that one of the two *tetR-mmpS-mmpL* clusters arose as a duplication of the other (Fig. 1B), representing these genes as potential candidates in CFZ/BDQ cross-resistance.

Expression of MAB_1135c-1134c is not controlled by the TetR regulator MAB_1136. Careful inspection of the genomic organization around the MAB_1135c-1134c locus indicated that MAB_1136 is an adjacent and divergently orientated gene (Fig. 2A). To test the hypothesis that this potential regulator controls the expression of the MAB_1135c-1134c gene cluster, a MAB_1136 deletion mutant was produced. This was achieved by using a recently developed strategy based on a new suicide vector, pUX1-*katG*, allowing easy and rapid generation of unmarked genetic deletions in the *M. abscessus* chromosome, facilitated by the presence of the brightly red fluorescent tdTomato positive selectable marker and the *katG* counterselectable marker (29, 31, 32). The selected INH-resistant, KAN-sensitive, and nonfluorescent clones were subsequently genotyped by PCR/sequencing analysis to confirm unmarked deletion of MAB_1136 (Fig. 2A).

The drug susceptibility pattern of the Δ MAB_1136 strain and its parental progenitor to imipenem (IPM; control drug), CFZ, and BDQ was next assessed by comparing growth on agar plates containing the corresponding drugs (Fig. 2B). The two strains displayed similar growth on agar supplemented with 16 μ g/ml IPM, 0.125 μ g/ml BDQ, or 1 μ g/ml CFZ, indicating that the absence of MAB_1136 does not influence the sensitivity of the strain to these compounds. These results were subsequently confirmed by the failure of the Δ MAB_1136 strain to show an upshift in the MIC levels (Table 1). Hypothesizing that MAB_1136 represses transcriptional expression of MAB_1135c-1134c, quantitative reverse transcription-PCR (qRT-PCR) analysis was performed, which failed to show increased expression of MAB_1134c or MAB_1135c in the Δ MAB_1136 strain (data not shown). Together, these results suggest that expression of MAB_1135c-1134c is not controlled by MAB_1136.

The TetR regulator MAB_2299c represses expression of MAB_1135c-1134c. As mentioned above, our *in silico* study revealed a high level of primary protein sequence identity between MAB_1136 and MAB_2299c (Fig. 1A), which might point to a role for the latter in transcriptional repression of MAB_1135c-1134c in the absence of such a function of the former. Interestingly, we recently identified MAB_2299c as a TetR transcriptional regulator that represses its *mmpS-mmpL* (MAB_2300-2301) target genes and found that different single-point mutations in MAB_2299c were associated with upregulation of the MAB_2300-2301 efflux pump system, leading to cross-resistance to CFZ and BDQ (29). Therefore, to interrogate whether MAB_2299c regulates other target genes belonging to the MmpS-MmpL systems, qRT-PCR was performed on 30 *mmpL* genes identified in the *M. abscessus* chromosome (30) in a Δ MAB_2299c deletion mutant (29). This systematic analysis clearly indicated that, in addition to MAB_2301, identified previously (29), only one other *mmpL* gene (MAB_1134c) was upregulated in the Δ 2299c strain (Fig. 3A).

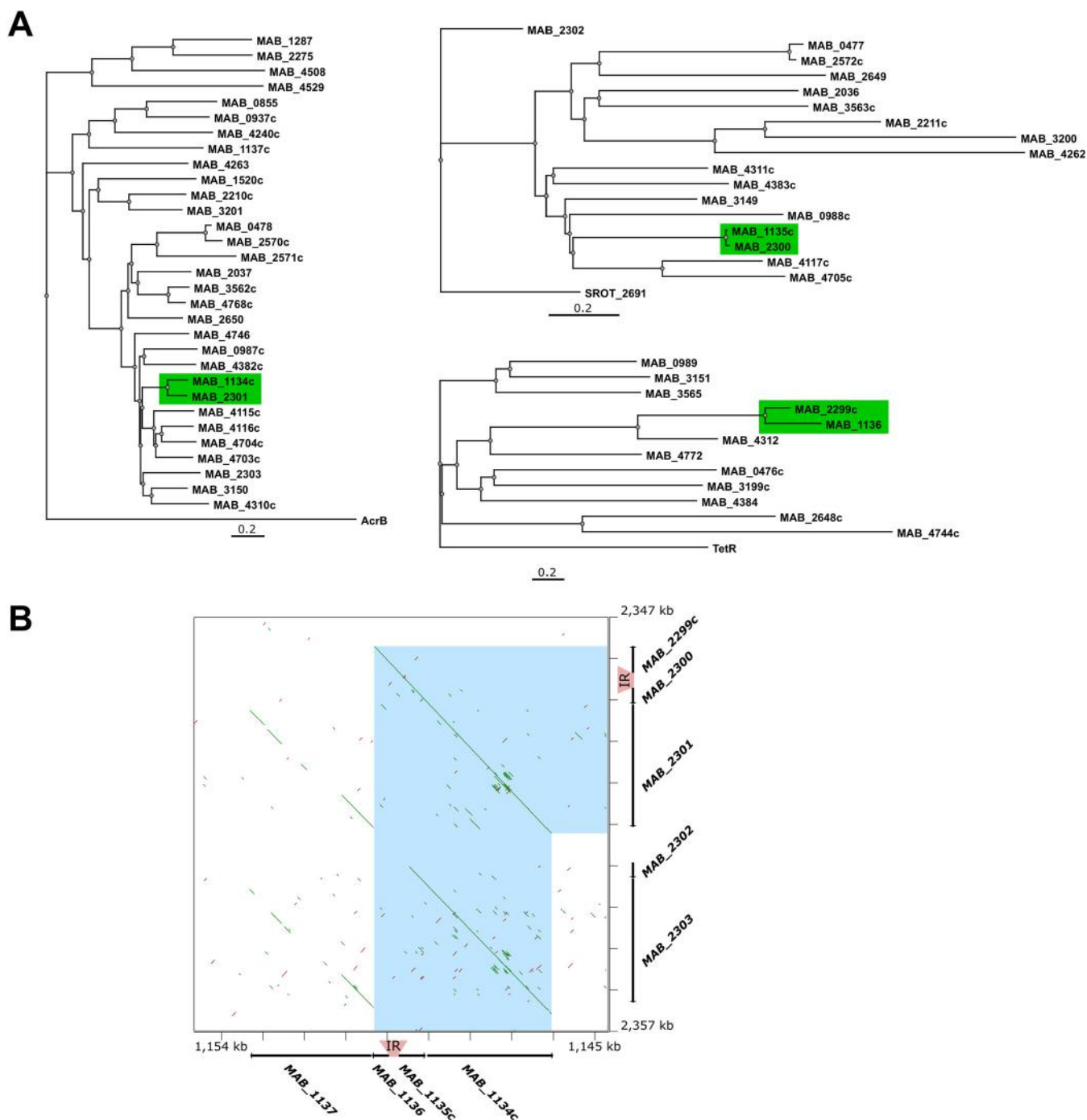


FIG 1 Closest paralogs of the MmpS-MmpL couple MAB_2300-2301, MAB_1135c-1134c, arose as a result of a gene duplication event. (A) Homology trees of all *M. abscessus* MmpL (left), all *M. abscessus* MmpS (top right), and several *M. abscessus* TetR/MarR family transcriptional regulators in close proximity to MmpS-MmpL couples (bottom right). The scale bar below each tree indicates 0.2 amino acid substitutions per site. The trees were rooted to *Escherichia coli* AcrB (MmpL) and TetR (transcriptional regulator) and *Segniliparus rotundus* MmpS ortholog SROT_2691 (MmpS). (B) Dot plot generated using the YASS genomic similarity tool (<http://bioinfo.lifl.fr/yass/yass.php>), showing nucleotide similarity between the MAB_2299c-2300-2301 and the MAB_1136-1135c-1134c loci (highlighted by a light blue inverted-L-shaped box). Green lines indicate forward matches, and red lines indicate reverse matches. The intergenic regions (IR) between the *tetR* genes (MAB_2299c and MAB_1136) and the *mmpS* genes (MAB_2300 and MAB_1135c) are highlighted by light gray trapezoidal boxes.

Increased transcription of both *mmpS-mmpL* gene clusters (MAB_1135c-1134c and MAB_2300-2301) was next confirmed by additional qRT-PCR analysis in both the parental *M. abscessus* smooth (S) strain and in the Δ MAB_2299c strain (Fig. 3B). Complementation of the Δ MAB_2299c strain by introducing the pMV261-MAB_2299c construct (Δ MAB_2299c.C) led to reduced transcriptional levels of both *mmpS-mmpL*

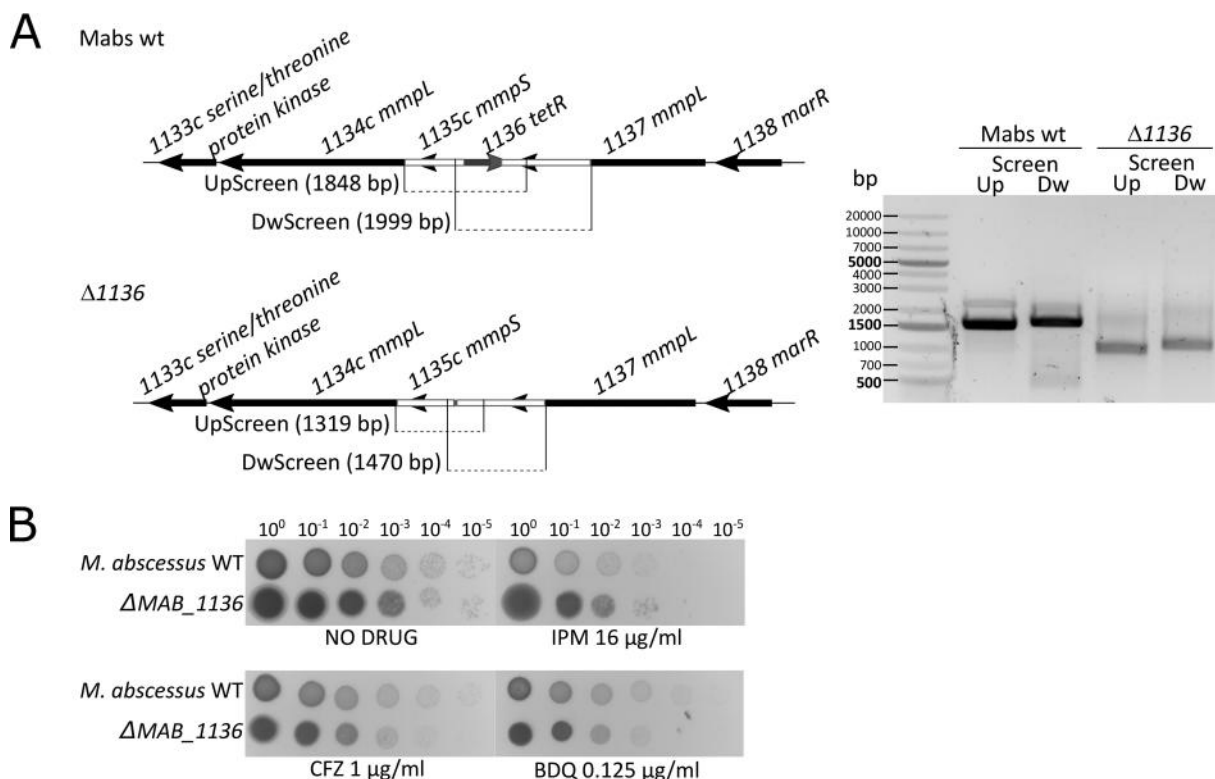


FIG 2 Deletion of *MAB_1136* does not confer resistance to CFZ or BDQ. (A) Genome organization of *MAB_1136* in *M. abscessus* and the screening approach used to confirm deletion of *MAB_1136*. White lines in the drawing represent the left and right arms flanking the region of *MAB_1136* targeted for deletion and exploited in the two-step homologous recombination procedure to generate the Δ *MAB_1136* strain. (Left) Dotted lines represent the size of the expected PCR products in *M. abscessus* WT and Δ *MAB_1136* strains. (Right) Gel migration profile of the amplicons in *M. abscessus* WT and Δ *MAB_1136* strains. All PCR products were purified and sequenced to confirm the *MAB_1136* deletion genotype. Up stands for upstream and Dw stands for downstream amplicons. (B) Susceptibility/resistance profiles of *M. abscessus* WT and Δ *MAB_1136* strains to various drugs. Five microliters of a 10-fold serially diluted bacterial suspension was spotted on 7H10^{ADC} alone or supplemented with 16 μ g/ml imipenem (IPM), 1 μ g/ml clofazimine (CFZ), or 0.125 μ g/ml bedaquiline (BDQ). Growth was observed after 4 days of incubation at 37°C. All experiments were done on three independent occasions.

pairs, as anticipated. The expression levels of the unrelated control gene *tgs1*, encoding the triacylglycerol synthase involved in the synthesis and accumulation of triglycerides in *M. abscessus* (33), remained unchanged in the various strains tested. As anticipated, the 6 CFZ-resistant mutants harboring mutations in *MAB_2299c* and associated with upregulation of the *MAB_2300-2301* efflux pump system (29) also expressed higher transcriptional levels of *MAB_1135c* and *MAB_1134c* (see Fig. S1 in the supplemental material). Together, these results indicate that expression of both *MAB_2300-2301* and *MAB_1135c-1134c* gene couples is repressed by *MAB_2299c* under normal growth conditions.

TABLE 1 Drug susceptibility/resistance profile of WT CIP104536, Δ *MAB_2299c*, Δ *MAB_1135c-1134c*, Δ *MAB_2299c-2300-2301*, Δ *MAB_2299c-2300-2301-1135c-1134c*, and Δ *MAB_1136* strains to various drugs

| Strain | MIC ₉₉ (μ g/ml) | | | | | | | |
|---|---------------------------------|-----|-----|------|-----|-----|-----|--------------|
| | IPM ^a | AMK | EMB | CFZ | TIG | LNZ | MOX | BDQ |
| CIP104536 | 8 | 16 | 16 | 2 | 16 | 64 | 32 | 0.25 |
| Δ <i>MAB_2299c</i> | 8 | 16 | 16 | 4 | 16 | 64 | 32 | 0.5 |
| Δ <i>MAB_1135c-1134c</i> | 8 | 16 | 16 | 0.5 | 16 | 64 | 32 | 0.125–0.06 |
| Δ <i>MAB_2299c-2300-2301</i> | 8 | 16 | 16 | 0.5 | 16 | 64 | 32 | 0.03–0.015 |
| Δ <i>MAB_2299c-2300-2301-1135c-1134c</i> | 8 | 16 | 16 | 0.25 | 16 | 64 | 32 | 0.015–0.0075 |
| Δ <i>MAB_1136</i> | 8 | 16 | 16 | 1 | ND | ND | ND | 0.125 |

^aMIC₉₉ values were visually determined on Middlebrook 7H10 agar after 4 days at 37°C. IPM, imipenem; AMK, amikacin; BDQ, bedaquiline; CFZ, clofazimine; EMB, ethambutol; TIG, tigecycline; LNZ, linezolid; MOX, moxifloxacin; ND, not determined.

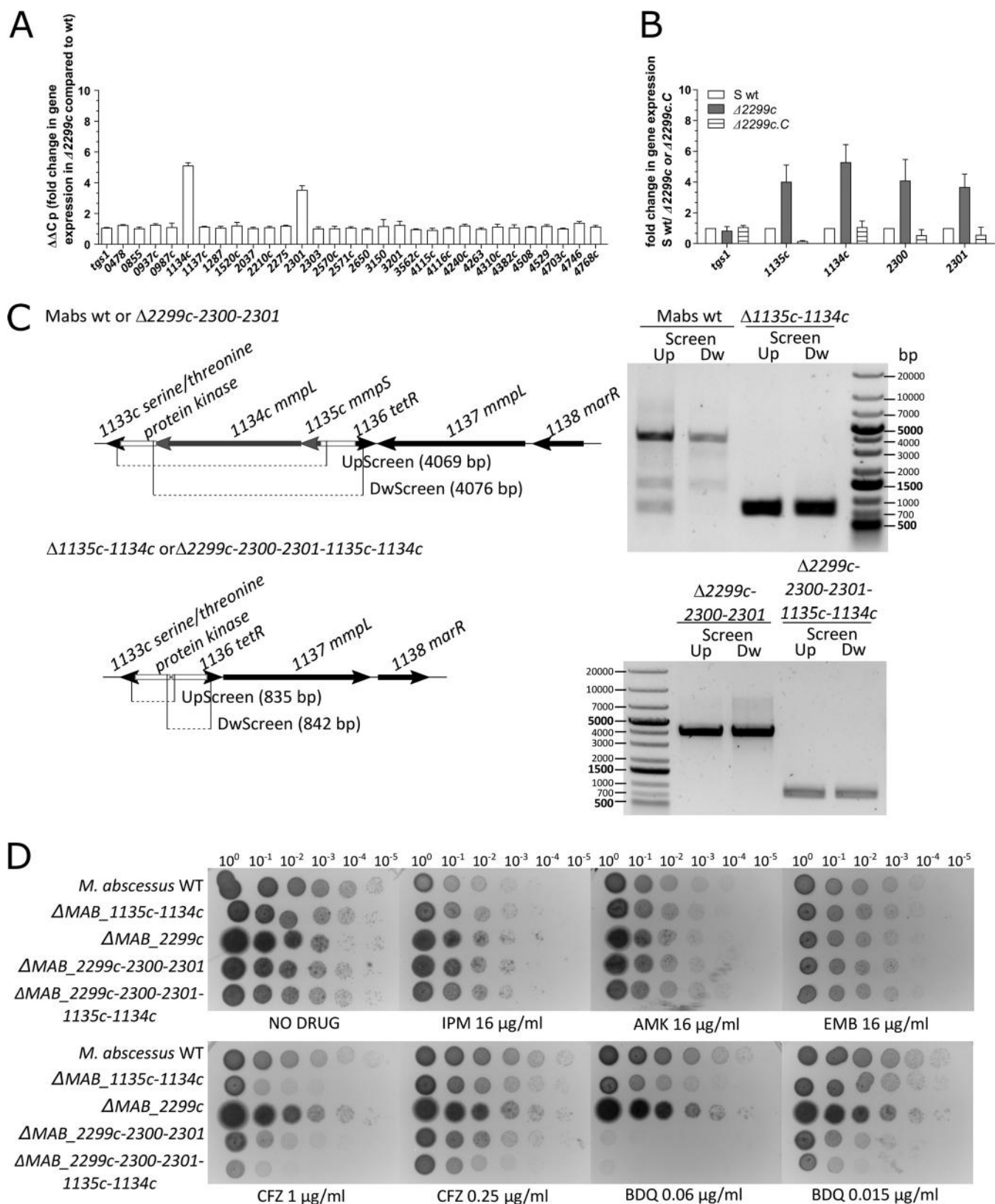


FIG 3 MAB_2299c represses expression of two distinct MmpS-MmpL efflux pump systems. (A) qRT-PCR analysis showing the transcriptional expression profile of 30 *mmpL* genes in the ΔMAB_2299c strain. (B) qRT-PCR analysis showing the transcriptional profiles of the *mmpS-mmpL* couples MAB_1135c-1134c and MAB_2300-2301 in *M. abscessus* WT, the ΔMAB_2299c strain, and its ΔMAB_2299c.C complemented derivative. Results are expressed as fold induction levels in the mutant strains relative to levels of the parental strain. Error bars indicate standard deviations. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method with E correction. Data are representative of two independent experiments. (C) Genome organization of the *mmpS-mmpL* couple MAB_1135c-1134c in

(Continued on next page)

Unmarked deletion of the *MAB_1135c-1134c* efflux pump leads to increased susceptibility to CFZ and BDQ. Using the same strategy as that used to produce the ΔMAB_1136 strain, a two-step homologous recombination procedure was first applied to generate a scarless deletion of *MAB_1135c-1134c* in the *M. abscessus* wild type (WT) (Fig. 3C, left). This unmarked deletion mutant, designated the $\Delta MAB_1135c-1134c$ strain, was subjected to PCR and sequencing analysis to confirm gene deletion (Fig. 3C, upper right). Although WT and $\Delta MAB_1135c-1134c$ strains displayed similar growth on agar without drugs or agar supplemented with 16 $\mu\text{g/ml}$ IPM, AMK, or ethambutol (EMB), the mutant strain showed reduced growth in the presence of 1 $\mu\text{g/ml}$ CFZ or 0.06 $\mu\text{g/ml}$ BDQ (Fig. 3D). These results were confirmed by determination of the MIC values of CFZ and BDQ against the $\Delta MAB_1135c-1134c$ strain, which were almost 4 times lower than those of the parental strain (Table 1). Overall, this increased susceptibility to both drugs strongly suggests that *MAB_1135c-1134c* plays a role in intrinsic resistance to these compounds. That no differences in MIC values were observed with the unrelated drugs IPM, AMK, and EMB indicates that the drug efflux mechanism mediated by *MAB_1135c-1134c* is specific to CFZ and BDQ.

Since we previously demonstrated that a mutant lacking *MAB_2299c* and *MAB_2300-2301* was more sensitive to CFZ/BDQ, we next addressed whether the additional deletion of *MAB_1135c-1134c* in this mutant strain would further increase the susceptibility to these drugs. This was achieved by generating an unmarked deletion of *MAB_1135c-1134c* in the $\Delta MAB_2299c-2300-2301$ background (Fig. 3C). PCR analyses confirmed deletion of the genes, yielding a strain missing 5 genes: *MAB_2299c*, encoding the central transcriptional regulator, and its two pairs of *mmpS-mmpL* target genes. Whereas this quintuple mutant exhibited growth similar to that of the WT strain or the parental $\Delta MAB_2299c-2300-MAB_2301$ triple mutant in the absence of drugs (control plates) or in the presence of IPM, AMK, or EMB, it showed increased sensitivity to both CFZ and BDQ (Fig. 3D). The near absence of growth of the quintuple mutant in the presence of 0.06 $\mu\text{g/ml}$ BDQ indicates that deletion of the second MmpS-MmpL couple further enhances the level of susceptibility of the strain. The MIC values of the quintuple mutant were 8-fold lower for CFZ and 16- to 32-fold lower for BDQ than those of the WT strain and 2-fold lower for both drugs compared to levels for the triple mutant lacking only one MmpS-MmpL pair (Table 1). The multiple deletion had no effect on the MIC of other drugs, including IPM, AMK, EMB, moxifloxacin (MOX), linezolid (LNZ), and tigecycline (TIG) (Table 1). As expected, the ΔMAB_2299c strain, in which both *MAB_2300-2301* and *MAB_1135c-1134c* are induced (Fig. 3B), failed to show growth inhibition in the presence of CFZ or BDQ (Fig. 3D) and displayed a 2-fold increased resistance to both drugs compared to that of the WT strain, as reported previously (29).

Collectively, these results indicate that both the *MAB_2300-2301* and the *MAB_1135c-1134c* efflux pump systems operate simultaneously in the resistance mechanism in *M. abscessus* against CFZ and BDQ.

MAB_2299c binds to a palindromic sequence upstream of *MAB_1135c*. We previously demonstrated that *MAB_2299c* binds to a palindromic sequence upstream of *MAB_2300*. That *MAB_2299c* also represses transcription of *MAB_1135c-1134c* suggested that it recognizes a conserved DNA-binding site located upstream of *MAB_1135c*. Using the MEME Suite software, we identified in the intergenic region upstream of *MAB_1135c* two palindromic sequences comprised of 7 nucleotides that

FIG 3 Legend (Continued)

M. abscessus and the screening approach for identification of $\Delta MAB_1135c-1134c$ in the *M. abscessus* WT and $\Delta MAB_2299c-2300-2301$ genetic backgrounds. White lines in the drawing represent the left and right arms of homology involved in two-step homologous recombination with the pUX1-*katG-MAB_1135c-1134c* suicide vector. Dotted lines represent the size of the expected PCR products in the WT and $\Delta MAB_1135c-1134c$ strains (left), along with the corresponding gel migration profile (right). All PCR products were purified and sequenced to confirm the *MAB_1135c-1134c* deletion genotype. (D) Susceptibility/resistance profiles of *M. abscessus* WT, ΔMAB_2299c , $\Delta MAB_1135c-1134c$, $\Delta MAB_2299c-2300-2301$, and $\Delta MAB_2299c-2300-2301-1135c-1134c$ strains to various drugs. Tenfold serially diluted bacterial suspensions were spotted (5 μl) on 7H10^{ADC} alone or supplemented with 16 $\mu\text{g/ml}$ imipenem (IPM), 16 $\mu\text{g/ml}$ amikacin (AMK), 16 $\mu\text{g/ml}$ ethambutol (EMB), 0.25 $\mu\text{g/ml}$ or 1 $\mu\text{g/ml}$ clofazimine (CFZ), or 0.06 $\mu\text{g/ml}$ or 0.015 $\mu\text{g/ml}$ bedaquiline (BDQ). Growth was observed after 4 days of incubation at 37°C. All experiments were done on two separate occasions.

are fully conserved with those found in the DNA-binding site upstream of *MAB_2300* (Fig. 4A). However, a major difference between the two sequences relies on a short spacer between the two palindromic sequences upstream of *MAB_1135c* and a longer spacer between the two palindromic sequences upstream of *MAB_2300*.

To confirm binding of the regulator to this palindromic sequence, electrophoretic mobility shift assays (EMSAs) were done by incubating increasing concentrations of purified MAB_2299c with a 64-bp DNA segment containing the two palindromes identified upstream of *MAB_1135c* and labeled at its 5' end with fluorescein (probe A). Under these conditions, a DNA-protein complex was detected and specificity of binding was demonstrated in a competition assay by adding increasing concentrations of the corresponding unlabeled DNA fragment (cold probe), which led to a dose-dependent decrease in DNA-protein complex formation (Fig. 4B). In the presence of an excess of unlabeled probe B, corresponding to a 64-bp DNA fragment containing the palindromic sequence found upstream of *MAB_2300*, previously reported to bind to MAB_2299c (29), a dose-dependent decrease in DNA-protein complex formation, similar to the one found with probe A, was noticed (Fig. 4B). This indicates that binding to probe A can be outcompeted by probe B, emphasizing the fact that MAB_2299c has the capacity to bind to both probes. However, in the presence of an excess of a nonrelated probe the shift was maintained, further indicating that a specific protein-DNA complex was formed only when the TetR regulator was incubated with DNA containing its specific target (Fig. 4B). In addition, a pronounced shift impairment was observed when the regulator was incubated with fluorescent probe C, a variant of probe A harboring multiple DNA substitutions within the palindromic sequence (Fig. 4C). This indicates that the TAACGCA/TGCGTTA motif in the palindrome (conserved in probes A and B) is required for optimal binding of MAB_2299c to its operator region.

***M. abscessus* lacking both MmpS-MmpL pairs is highly susceptible to CFZ or BDQ treatment in macrophages.** In the absence of drug treatment, all *M. abscessus* strains (WT, Δ MAB_2299c, Δ MAB_1135c-1134c, Δ MAB_2299c-2300-2301, and Δ MAB_2299c-2300-2301-1135c-1134c strains) grew similarly in 7H9^{T/OADC} (Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and 10% oleic acid, albumin, dextrose, catalase [OADC enrichment]) at 30°C (Fig. 5A), indicating that the multiple-gene deletion of the TetR regulator and its target genes does not influence mycobacterial growth. Exposure of WT cultures to 0.25 μ g/ml BDQ (corresponding to 2 \times MIC) showed an important growth inhibition over the 4-day period of treatment with CFU numbers remaining constant over time, confirming the bacteriostatic effect of BDQ reported earlier (26) (Fig. 5A). As anticipated, growth of the Δ MAB_2299c strain was only partially inhibited, confirming the BDQ-resistant phenotype of the strain. However, deletion of either one or both MmpS-MmpL pairs in Δ MAB_2299c fully abrogated the BDQ resistance phenotype. Moreover, after 4 days of treatment, the CFU numbers of the MmpS-MmpL mutants were approximately 1 to 2 logs lower than those of the WT strain and the original inoculum, indicating that these strains in effect became susceptible to killing by BDQ (Fig. 5A). Similar growth profiles were obtained when the different strains were exposed to a lower concentration of BDQ (0.06 μ g/ml) (data not shown).

The hypersusceptibility of the quintuple mutant to CFZ and BDQ *in vitro* prompted us to investigate whether this phenotype was maintained when bacteria reside inside the macrophage. However, this required us to first examine the potential cytotoxicity induced by the two drugs over a 48-h period of treatment in THP-1 macrophages. The cytotoxicity profiles of CFZ and BDQ were very different, with BDQ showing extremely poor cytotoxicity (no macrophage killing at concentrations up to 50 μ g/ml), while CFZ exhibited toxicity with a 50% inhibitory concentration of 2.6 μ g/ml (Fig. 5B). Macrophages next were infected with various *M. abscessus* strains at a multiplicity of infection (MOI) of 5. Following extensive washing and treatment with AMK to remove the extracellular bacilli, infected cells were either left untreated or were treated with low concentrations of drugs (1 μ g/ml CFZ or 0.25 μ g/ml BDQ) for 48 h. In the absence of drug treatment, all mycobacterial strains replicated in a comparable manner, with a 2-log increase in the intracellular CFU, except for Δ MAB_2299c and the quintuple

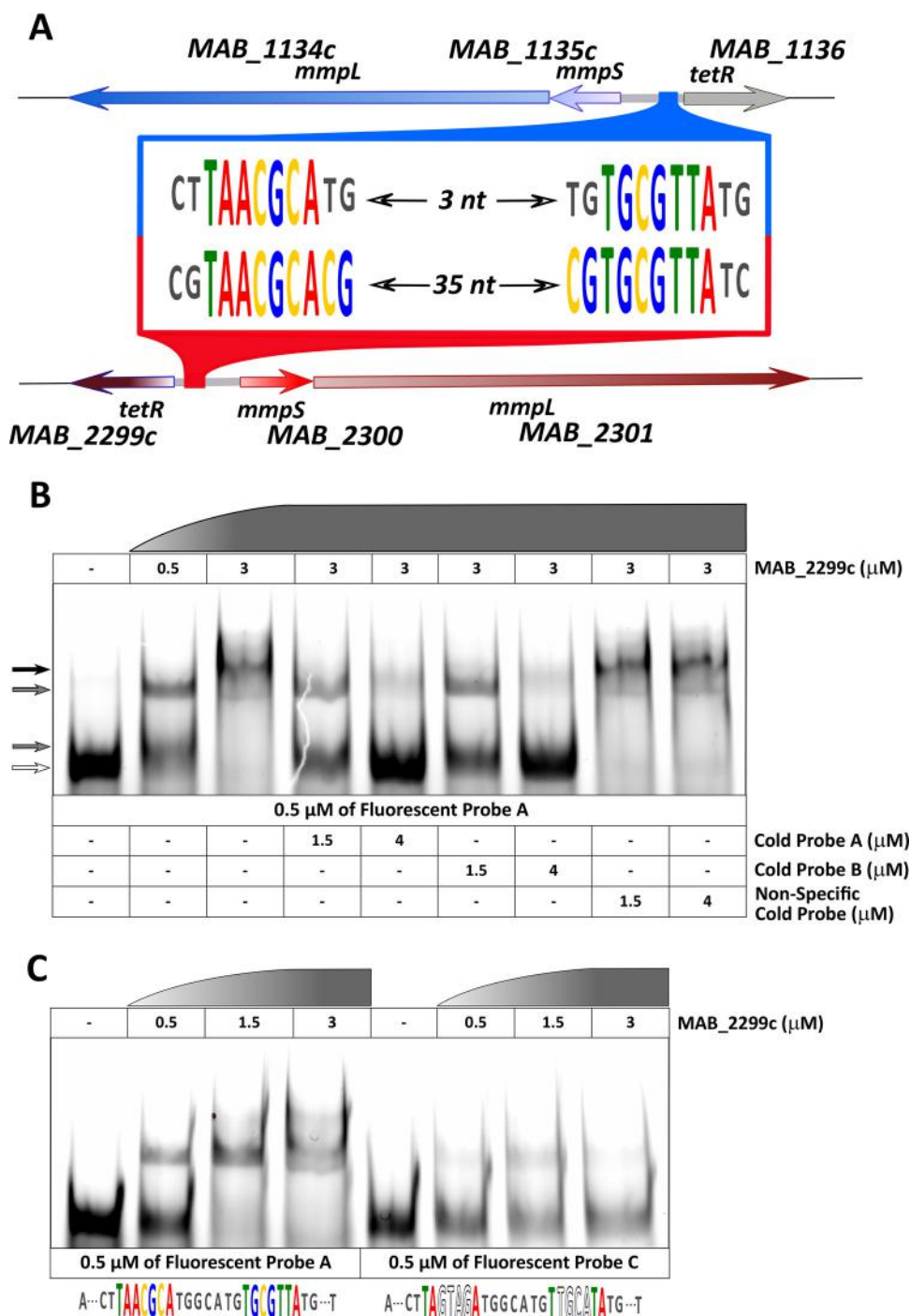


FIG 4 TetR regulator MAB_2299c binds to the intergenic region upstream of *MAB_1135c-1134c*. (A) Schematic representation of the DNA operators located upstream of *MAB_1135c-1134c* (comprising probe A) and *MAB_2300-2301* (comprising probe B). The palindromic sequences of 7 nucleotides in probe A are separated by a spacer and are identical to those found in probe B, in which they are separated by 35 nucleotides. (B) EMSA and competition assays using purified MAB_2299c and incubated with 5' fluorescein-labeled probe A in the absence or presence of an excess of unlabeled probe A or probe B. A nonrelated probe was also included as a control of specificity of the TetR/DNA complex formed. Detection of the unbound or the complexed probe A was revealed by fluorescence emission. The white arrow indicates the DNA alone, gray arrows the formation of intermediate TetR/DNA complexes, and the black arrow the formation of a complete TetR/DNA complex. (C) Comparison of the EMSA pattern of MAB_2299c incubated with 5' fluorescein-labeled probe A and probe C in which 4 of the central nucleotides forming the palindromic sequences of probe A were replaced by a random sequence, resulting in a clear defect in the formation of the TetR/DNA complex. The corresponding unbound and complexed probes were revealed by fluorescence emission.

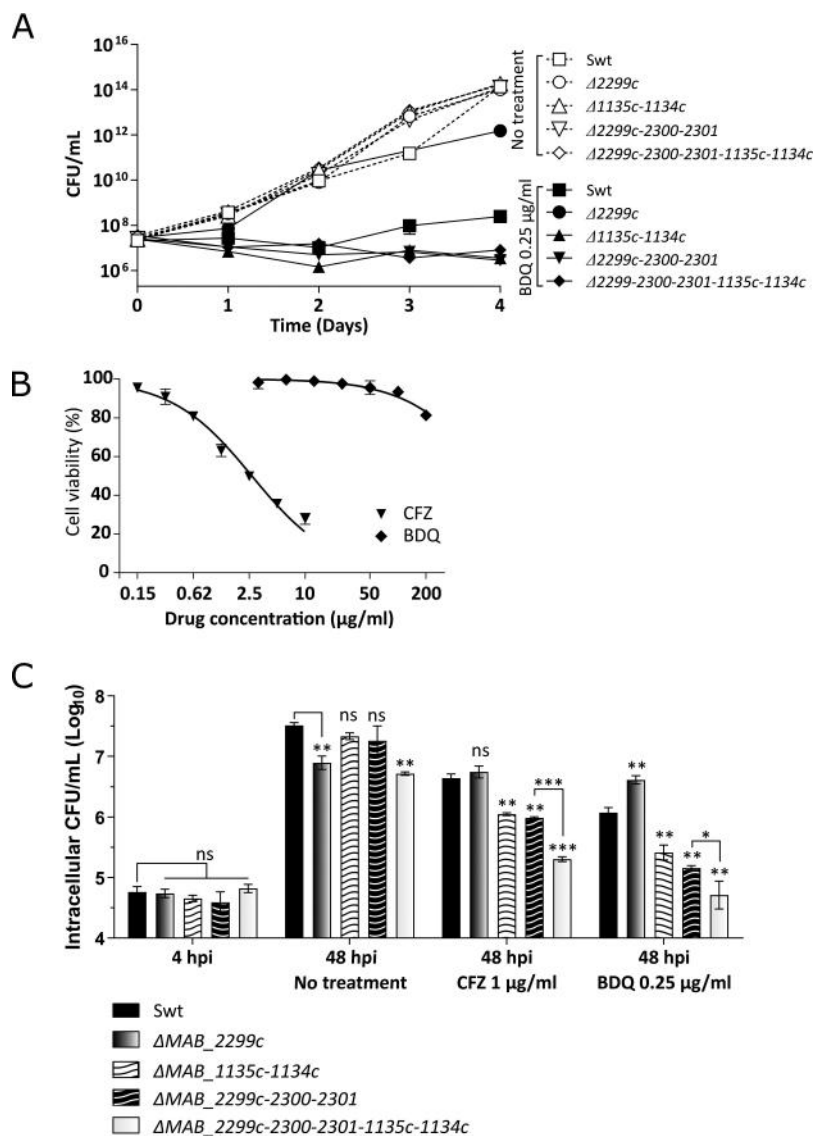


FIG 5 Activity of BDQ *in vitro* and in infected macrophages. (A) *M. abscessus* CIP104536^T WT and its mutant derivatives were either left untreated or exposed to 0.25 $\mu\text{g/ml}$ BDQ in 7H9^{ODC} at 30°C. Bacterial suspensions were collected on a daily basis, diluted, plated on LB agar, and further incubated at 37°C for 4 days prior to CFU determination. Results are expressed as the means \pm standard errors of the means from triplicates and are representative of two independent experiments. Ctrl, untreated cultures; Swt, smooth wild type. (B) Cytotoxicity assay of CFZ and BDQ toward THP-1 macrophages. Cells were treated for 48 h with increasing concentrations of CFZ or BDQ, and viability was determined using the CellTiter-Blue reagent. The percentage of cell survival was determined based on control cells incubated in DMSO-containing medium. (C) *M. abscessus*-infected THP-1 macrophages were exposed to either 1 $\mu\text{g/ml}$ CFZ or 0.25 $\mu\text{g/ml}$ BDQ for 2 days before they were lysed and plated for subsequent CFU enumeration. Histograms and error bars represent means and standard errors of the means calculated from three independent experiments. CFU numbers at 4 h postinfection reflect the sizes of the different inocula. For statistical analysis, Student's *t* test was applied. ns, *, **, and *** stand for nonsignificant, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

mutant, both showing a slight reduction in the number of intracellular CFU (Fig. 5C). The ΔMAB_2299c mutant, however, exhibited slightly higher CFU numbers in macrophages after 48 h of treatment with BDQ than with untreated macrophages, in agreement with its increased resistance phenotype to BDQ *in vitro*. Importantly, the *in vitro* CFZ/BDQ-susceptible $\Delta MAB_1135c-1134c$ and $\Delta MAB_2299c-2300-2301$ mutants were also more susceptible to CFZ and BDQ inside macrophages than the wild-type strain. Moreover, the quintuple mutant (lacking both efflux pump systems) exhibited an even

further increased sensitivity to CFZ and BDQ in macrophages compared to that of the triple mutant (lacking one efflux pump system only). Together, these results indicate that abrogating the functionality of multiple efflux pumps translates into additive effects, resulting in higher sensitivity to drugs *in vitro* and in infected host cells.

DISCUSSION

M. abscessus is intrinsically resistant to a wide range of antimicrobials, including most antitubercular drugs, posing a serious challenge for drug discovery. Hit rates in primary screens for *M. abscessus* are low, primarily because of the low level of susceptibility due to its expansive drug resistome (18). As environmental bacteria, NTM reside essentially in soil and water, and it is plausible that the selection pressure from antimicrobial-producing bacteria sharing the same biological niches have forced NTM to develop a wide diversity of resistance mechanisms, allowing them to survive in hostile environments. The antimicrobial resistome of *M. abscessus* results from mutations in antibiotic target genes or other related genes, mostly occurring during the prolonged course of treatment, as well as genes involved in intrinsic drug resistance mechanisms. Among the latter, efflux pump mechanisms, protecting bacteria against toxic compounds and promoting bacterial homeostasis by transporting toxins or metabolites to the extracellular environment (34), have drawn attention as important elements preventing the intracellular accumulation of drugs.

Resistance mechanisms associated with MmpL-derived efflux pump systems were recently identified in *M. abscessus*. In the case of thiacetazone analogs, mutations in the TetR repressor MAB_4384 led to upregulation of the adjacent genes encoding an MmpS-MmpL efflux pump system (35, 36). Increased expression of MAB_4746, encoding a member of the MmpL family, was also reported recently in LNZ-resistant isolates (37). In the case of CFZ, where CFZ-resistant strains demonstrated cross-resistance to BDQ (29), whole-genome sequencing analyses of the *in vitro*-selected resistors uncovered point mutations in MAB_2299c (29), a member of the TetR family of transcriptional regulators, representing the most abundant family of regulators in mycobacteria. These findings are supported by an independent study that also identified mutations in MAB_2299c, among other genes, in CFZ-resistant strain selected *in vitro* (38). It was originally shown that MAB_2299c regulates transcription of the MAB_2300-2301 target genes, found in an orientation opposite that of MAB_2299c (29). Point mutations (L40W, L151P, and G215S) identified in the spontaneous resistant strains were correlated with a loss of DNA-binding activity of MAB_2299c, resulting in derepression of MAB_2300-2301 (29) and MAB_1135c-1134c (this study) gene expression, leading to increased drug efflux and cross-resistance to CFZ and BDQ (29). In contrast to MAB_2300-2301, adjacent to MAB_2299c, MAB_1135c-1134c is located far from the regulator (Fig. 6). This indicates that MAB_2299c can act simultaneously on closely and distantly located target genes on the chromosome. That both efflux pump systems participate in CFZ and BDQ export strongly suggests that they are structurally and functionally related. This hypothesis was confirmed by the phylogenetic analysis that allowed us to identify MAB_1135c-1134c, along with MAB_2300-2301, products of a possible gene duplication event. That both MmpS-MmpL systems operate simultaneously was verified using multiple gene deletions thanks to the recent development of a suicide vector-based approach, which allows easy and rapid unmarked genetic alteration of the *M. abscessus* chromosome (29). While the triple mutant lacking the regulator and one efflux pump system (Δ MAB_2299c-2300-2301) was more sensitive to CFZ/BDQ than the WT progenitor, the quintuple mutant lacking the regulator and both efflux pump systems further gained in susceptibility to CFZ and BDQ treatment. To verify whether the two efflux pumps and MAB_2299c are widely distributed among *M. abscessus* clinical isolates or restricted to the CIP104536 type strain, a BLAST search for MAB_2299c-2300-2301 and MAB_1135c-1134c was performed (see Table S3 in the supplemental material). This revealed that both loci as well as their DNA operators are conserved in *M. bolletii*, *M. massiliense*, and *M. chelonae* and in all clinical isolates analyzed, suggesting that both efflux pump systems are present and presumably operating in all of these strains.

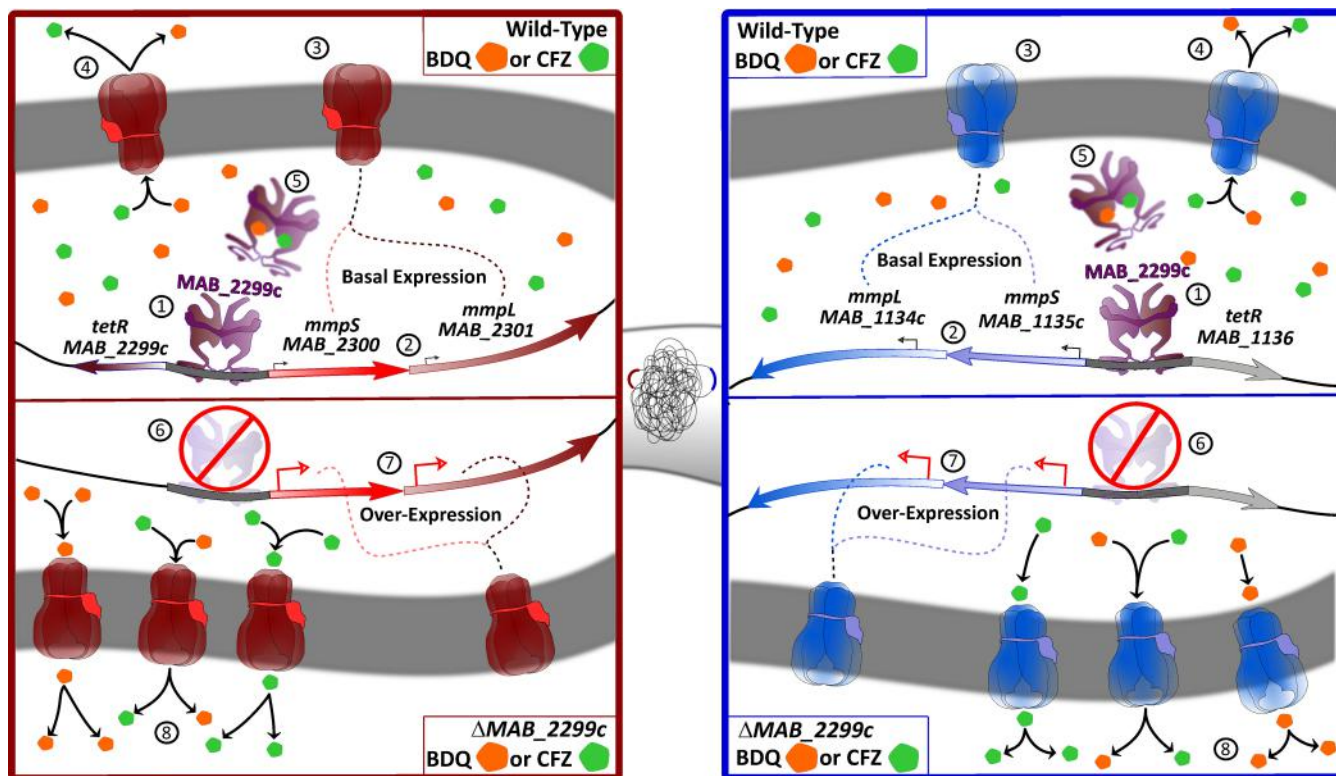


FIG 6 General schematic representing the mechanism of CFZ/BDQ resistance mediated by MAB_2299c-dependent MmpS-MmpL efflux pumps in *M. abscessus*. (1) Binding of MAB_2299c to the intergenic regions of *MAB_2300-2301* (left) and *MAB_1135c-1134c* (right). (2) Transcriptional repression of *MAB_2300-2301* (left) and *MAB_1135c-1134c* (right) allows only basal expression of the two efflux pumps. (3) Insertion of the two MmpS-MmpL efflux pumps into the plasma membrane. (4) Extrusion of CFZ and BDQ conferring intrinsic resistance to both drugs. (5) Possible interaction of the drugs with the TetR regulator, triggering its dissociation from the DNA operator and transcriptional derepression. (6) Lack of binding of the regulator to the intergenic regions either due to point mutations acquired in *MAB_2299c* under CFZ selective pressure *in vitro* or when *MAB_2299c* was inactivated through targeted deletion by homologous recombination. (7) Enhanced expression of the two drug efflux pumps and extrusion of CFZ and BDQ, leading to increased resistance levels to the drugs.

These results indicate that mutations in *MAB_2299c*, which can be easily selected on CFZ *in vitro* (29), affect the expression of two efflux pump systems, both contributing to the resistance mechanism. However, while our transcriptomic studies showed that only these two MmpS/MmpL pairs are regulated by MAB_2299c, one cannot exclude the possibility that additional MAB_2299c-independent MmpL proteins or unrelated efflux pump systems, such as members of the major facilitator superfamily or ATP-binding cassette (ABC) transporters, mediate cross-resistance to CFZ/BDQ. It also remains possible that MAB_2299c regulates other gene targets involved in various metabolic pathways and/or in intracellular survival of *M. abscessus* in macrophages. This hypothesis may be supported by a slightly reduced burden of the Δ *MAB_2299c* strain than the WT at 48 h postinfection in THP-1 cells in the absence of drug treatment (Fig. 5C).

We recently showed that verapamil improves the efficacy of BDQ activity against *M. abscessus* clinical isolates both *in vitro* and in macrophages (39). Interestingly, verapamil kept its capacity to potentiate the activity of BDQ even in *M. abscessus* mutants resistant to CFZ/BDQ (overexpressing both the *MAB_2300-2301* and *MAB_1135c-1134c* efflux pumps), highlighting the value of the verapamil-BDQ combination in cases of low-level acquired resistance to BDQ in this mycobacterium. Importantly, the Δ *MAB_2299c-2300-2301* strain, in which this BDQ efflux pump was deleted, leading to increased BDQ sensitivity compared to that of WT *M. abscessus*, exhibited a further increase in sensitivity to BDQ in the presence of verapamil, suggesting that the mechanism whereby verapamil exerts its potentiating effect on BDQ activity is not dependent on this efflux pump, apparently excluding this efflux pump as the major target of verapamil. In fact, it was recently reported that verapamil targets membrane energetics in mycobacteria (40).

The inhibition by BDQ of the ATP synthase in mycobacteria was also previously shown to impact membrane energetics, an effect which is probably compounded when verapamil is also present (41). Our genetics data suggest that if a specific inhibitor of the two highly related CFZ/BDQ efflux pumps (MAB_2300-2301 and MAB_1135c-1134c) could be developed, such a compound given in combination with BDQ/CFZ and a molecule targeting membrane potential would provide an extremely potent therapeutic cocktail against *M. abscessus*.

We previously speculated that, given the large *mmpL* repertoire (30) and high abundance of TetR transcriptional regulators (42) found in *M. abscessus*, efflux-based mechanisms represent a general mechanism to express its natural resistance pattern to antimicrobial agents. The present study confirms this to be true, at least regarding CFZ and BDQ resistance. In summary, we provide here additional mechanistic insights into the TetR-dependent regulation of MmpL efflux pumps responsible for cross-resistance to CFZ and BDQ in *M. abscessus*, which may have important clinical implications. Overall, this study underpins MAB_2299c as a marker of resistance to be considered when assessing the drug susceptibility profile of *M. abscessus* clinical isolates. However, future studies should also be dedicated to investigating whether mutations in MAB_2299c occur in clinical isolates from patients treated with CFZ or BDQ.

MATERIALS AND METHODS

Strains, growth conditions, and reagents. Bacterial strains used in this study are listed in Table S1 in the supplemental material. *M. abscessus* was grown in Middlebrook 7H9 broth (BD Difco) supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% oleic acid, albumin, dextrose, catalase (OADC enrichment; BD Difco) (7H9^{T/OADC}) at 30°C (unless otherwise stated) in the presence of antibiotics, when required. On plates, colonies were selected either on Middlebrook 7H10 agar (BD Difco) supplemented with 10% OADC enrichment (7H10^{OADC}) or on LB agar. All drugs were purchased from Sigma-Aldrich.

Drug susceptibility testing. The MIC, defined as the minimal drug concentration required to inhibit 99% growth (MIC₉₉), was recorded on Middlebrook 7H10^{OADC} agar after 3 to 4 days of incubation at 30°C. All experiments were done on three independent occasions.

Quantitative real-time PCR. Isolation of RNA, reverse transcription, and qRT-PCR were done as reported earlier (35) using the primers listed in Table S2 in the supplemental material.

Expression and purification of MAB_2299c variants. The *E. coli* BL21(DE3) strain (New England Biolabs) possessing pRARE2 was transformed with a pET32a derivative containing the wild-type MAB_2299c gene. Expression in *E. coli* and purification of MAB_2299c was performed as described earlier (29).

Electrophoretic mobility shift assays (EMSAs). Thanks to the MEME Suite 4.20.0 online tool, we identified a perfect pair of palindromes comprised of 7 nucleotides each and separated by a 7-nucleotide spacer within the intergenic region upstream of MAB_1135c. These palindromes are identical to the ones located between MAB_2299c and MAB_2300. A double-stranded DNA fragment of 64 bp containing the pair of palindromes labeled at their 5' ends with fluorescein (probe A) was purchased from Sigma-Aldrich. The 5' fluorescein probes (500 nM) were coincubated for 1 h at room temperature in 1× Tris base-acetic acid-EDTA (TAE) buffer with increasing amounts of purified MAB_2299c protein with or without excess concentrations of cold probes (unlabeled DNA fragments). Electrophoresis was performed for 30 min at 100 V in 1× TAE buffer, and fluorescence was revealed using a ChemiDoc Imaging System (Bio-Rad). Sequences of the probes are listed in Table S2.

Generation of the MAB_1136c and MAB_1135c-1134c recombination plasmids. The suicide vector pUX1-*katG* (29) was used to generate pUX1-*katG*-MAB_1136. To produce pUX1-*katG*-MAB_1136, genomic DNA from *M. abscessus*, Q5 polymerase (New England Biolabs), and the primers MAB_1136_LA_Fw and MAB_1136_LA_Rev were used to PCR amplify a left arm (LA). A similar PCR but with the primers MAB_1136_RA_Fw and MAB_1136_RA_Rev generated a right arm (RA). The purified LA and RA amplicons were added to a new PCR mix without any primers or genomic DNA. This reaction consisted of two cycles of 95°C for 30 s, 55°C for 2 min, and 72°C for 3 min prior to the addition of primers MAB_1136_RA_Fw and MAB_1136_LA_Rev and 25 additional cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 2 min. The 1.7-kb primer-overlap extension PCR product was restricted with NheI and MfeI and ligated to the NheI-MfeI-linearized pUX1-*katG*, yielding pUX1-*katG*-MAB_1136, designed to delete 530 bp (80%) of the MAB_1136 open reading frame (Table S1).

The same approach was applied to generate pUX1-*katG*-MAB_1135c-1134c, using the primers MAB_1135c-1134c_LA_Fw and MAB_1135c-1134c_LA_Rev to produce the LA and the primers MAB_1135c-1134c_RA_Fw and MAB_1135c-1134c_RA_Rev to produce the RA. The ~1.4-kb primer overlap extension PCR product was restricted with NheI and MfeI and ligated to the NheI-MfeI-linearized pUX1-*katG*, yielding pUX1-*katG*-MAB_1135c-1134c, designed to delete 3,234 bp (96%) of the MAB_1135c-1134c gene cluster (Table S1).

Unmarked deletion in *M. abscessus*. Highly electrocompetent *M. abscessus* smooth (S) WT and S ΔMAB_2299c-2300-2301 strains were prepared as reported earlier (31) and subsequently transformed with pUX1-*katG*-MAB_1136 or pUX1-*katG*-MAB_1135c-1134c. Selection of bacteria having undergone the

first homologous recombination was done by screening for red fluorescent colonies on 7H10^{OADC} supplemented with 200 µg/ml kanamycin. To promote the second homologous recombination event, the selected clones were subcultured overnight in 7H9^{OADC} in the absence of kanamycin. Cultures were 10-fold serially diluted and plated onto 7H10^{OADC} containing 50 µg/ml isoniazid (INH) to select for INH-resistant, KAN-sensitive, and nonfluorescent colonies. Following genomic DNA preparation from such colonies, PCR screening was set up using primer sets binding upstream of the LA and inside the RA or binding inside the LA and downstream of the RA. Amplicons were subsequently sequenced to confirm the junction between LA and RA and the correct unmarked deletion of *MAB_1136* and *MAB_1135c-1134c*.

Macrophage infections. THP-1 macrophages were grown at 37°C in a 5% CO₂ incubator in RPMI 1640 supplemented with 10% FBS (RPMI^{FBS}) and differentiated for 48 h prior to infection with 20 ng/ml phorbol myristate acetate (PMA). *M. abscessus* strains were cultured at 37°C in 7H9^{OADC} until they reach the exponential growth phase and were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS). Single-cell preparations were prepared by passing the bacterial suspension through a 26.5-gauge needle (~20 times) and then through a 5.0-µm filter (Merck Millipore). THP-1 cells (5 × 10⁵ cells/ml) were then infected at an MOI of 5 and incubated at 37°C in the presence of 5% CO₂. After 2 to 3 h of incubation, cells were washed three times with PBS and refed with RPMI^{FBS} supplemented with 250 µg/ml AMK for 1 h in order to kill extracellular bacilli. Cells were washed three times with PBS and incubated for an additional 48 h with either RPMI^{FBS} alone (no treatment; control of intracellular growth), RPMI^{FBS} containing 1 µg/ml CFZ, or RPMI^{FBS} containing 0.25 µg/ml BDQ. For intracellular CFU determination, cells were washed three times with PBS, and 100 µl of 1% PBS-Triton X-100 was added to lyse the cells at day 0 and day 2. Lysis was stopped by adding 900 µl of PBS. Tenfold dilutions were plated onto LB agar, and colonies were counted after 5 days of incubation at 37°C.

Cytotoxicity assay. THP-1 macrophages (5 × 10⁵ cells/ml) in 96-well flat-bottom culture microtiter plates were differentiated for 48 h with PMA, after which RPMI^{FBS} was replaced with 180 µl of fresh medium containing increasing concentrations of either CFZ (ranging from 0.15 to 10 µg/ml) or BDQ (ranging from 3.1 to 200 µg/ml). After 48 h of incubation at 37°C with 5% CO₂, 20 µl of CellTiter-Blue reagent (Promega) was added and cells were incubated for an additional 2 h. The optical density at 560 nm (OD₅₆₀) was recorded using a microplate reader. The percentage of cell survival was determined considering the control wells (cells incubated in dimethyl sulfoxide-containing medium).

Multiple alignments and synteny analysis. Primary protein sequences were retrieved from the KEGG website (<https://www.genome.jp/kegg/>), and multiple alignments of the sequences were done with the MUSCLE algorithm using its default parameters in the UniProt UGENE suite (43). Phylogenetic trees were constructed from the multiple-alignment data using the PHYLIP neighbor-joining tree-building method with default parameters of the UGENE suite. Nucleotide sequences were also retrieved from the KEGG website, and syntenic comparisons of these sequences were done on the YASS genomic similarity tool web server (<http://bioinfo.lifl.fr/yass/yass.php>).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5 (version 5.03; GraphPad Software). Student's *t* test was used for all comparisons. A *P* value of less than 0.05 was considered significant (ns, nonsignificant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01000-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

This work was supported by the Fondation pour la Recherche Médicale (FRM) (grant number DEQ20150331719 to L.K.; grant number ECO20160736031 to M.R.) and the InfectioPôle Sud for funding the Ph.D. fellowship of A.V.G.

We have no conflicts of interest to declare.

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Article 4

« **Dissecting *erm(41)* – Mediated Macrolide Inducible Resistance in *Mycobacterium abscessus*** ». Richard M, Gutiérrez AV, Kremer L. Soumis.

La résistance inductible aux macrolides est particulièrement problématique car elle contribue grandement aux échecs thérapeutiques observés dans les traitements à *M. abscessus*. Les données sur l'utilisation de l'azithromycine (AZM) en faveur de la clarithromycine (CLR) sont discutables et varient considérablement en fonction des travaux réalisés précédemment par diverses équipes. Dans notre étude, nous avons démontré que l'AZM induisait une résistance médiée par Erm(41) plus forte que celle provoquée par la CLR *in vitro*. Ces résultats ont été confirmés en mesurant l'activité β -galactosidase exprimée par le gène *lacZ* sous le contrôle du promoteur d'*erm(41)*. La troncation progressive du promoteur d'*erm(41)* a permis d'identifier précisément les éléments génétiques responsables de l'induction de cette résistance et d'en confirmer le principal protagoniste, WhiB7, connu comme un activateur transcriptionnel central chez les mycobactéries. Le remplacement de *lacZ* par le marqueur de fluorescence *tdTomato* sous le contrôle du promoteur complet d'*erm(41)* a montré que l'induction de la résistance aux macrolides se produit également *in vivo* chez le modèle d'infection du zebrafish. Ces observations pourraient refléter un mécanisme ayant lieu chez un patient infecté par *M. abscessus* et sous traitement aux macrolides. Nous avons finalement mis en évidence la complexité de cette régulation en observant une activation plus rapide d'Erm(41) conduisant ainsi à une résistance plus intense chez des souches traitées au préalable avec de la CLR et de l'AZM. Ces souches prétraitées aux macrolides exhibent par ailleurs un antagonisme avec l'amikacine *in vitro*, ce qui pourrait en partie expliquer les nombreux échecs thérapeutiques, car les macrolides et l'amikacine sont utilisés en association dans l'antibiothérapie classique contre *M. abscessus*. Les souches rapportrices *lacZ* et/ou *tdTomato* générées au cours de ces travaux pourraient constituer des outils de choix pour la mise en place d'une nouvelle approche de criblage de molécules actives contre *M. abscessus* et capables de contourner la résistance induite médiée par Erm(41).

1 **Dissecting *erm(41)*-mediated macrolide inducible resistance**
2 **in *Mycobacterium abscessus***

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4
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20 **Running title:** Dissecting macrolide induced resistance in *M. abscessus*

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27 **Keywords:** *Mycobacterium abscessus*, macrolide, therapeutic activity, *erm(41)*, inducible
28 resistance, β -galactosidase, WhiB7, zebrafish.

31 **ABSTRACT**

32

33 Macrolides are the cornerstone of *Mycobacterium abscessus* multidrug therapy although most
34 patients respond poorly to macrolide-based chemotherapy, notably because of the inducible
35 resistance phenotype occurring during drug treatment. This mechanism is driven by the macrolide
36 inducible ribosomal methylase encoded by *erm(41)*, whose expression is activated by the
37 transcriptional regulator WhiB7. However, there has been a debate regarding whether
38 clarithromycin and azithromycin differ in the extent to which they induce *erm(41)*-mediated
39 macrolide resistance. Herein, we show that macrolide resistance is more rapidly induced in
40 various *M. abscessus* isolates upon exposure to azithromycin than to clarithromycin, based on
41 minimal inhibitory concentration determination. Macrolide-induced expression of *erm(41)* was
42 assessed *in vivo* using a strain carrying *tdTomato* placed under the control of the *erm(41)*
43 promoter. The visualization of fluorescent bacilli in infected zebrafish demonstrates that
44 azithromycin or clarithromycin activate *erm(41)* expression *in vivo*. Consistently with previous work
45 regarding the antagonism between clarithromycin and amikacin, we show that azithromycin
46 antagonizes also the activity of amikacin. That azithromycin induces a more rapid expression of
47 *erm(41)* was confirmed by measuring the β -galactosidase activity of a reporter strain in which *lacZ*
48 was placed under the control of the *erm(41)* promoter. Shortening the promoter region in the *lacZ*
49 transcriptional fusions identified important DNA elements involved in regulating *erm(41)*
50 expression, particularly an AT-rich motif showing partial conservation with the WhiB7-binding site.
51 Mutating this motif abrogated the macrolide-induced and WhiB7-dependent expression of *erm(41)*.
52 Overall, this study provides new mechanistic information regarding the adaptive response to
53 macrolide treatment in *M. abscessus*.

54 INTRODUCTION

55

56 Members of the *Mycobacterium abscessus* complex are rapidly growing nontuberculous
57 mycobacteria (NTM) of increasing clinical significance due to the rising burden of associated
58 pulmonary disease, particularly in cystic fibrosis (CF) patients (1). In CF patients, infection with *M.*
59 *abscessus* correlates with a more rapid decline in lung function and can represent an obstacle for
60 subsequent lung transplantation (2–4). The possible person-to-person transmission of virulent
61 clones among the CF population makes this situation even more worrisome (5, 6). From a
62 taxonomical view, the complex currently comprises three subspecies: *M. abscessus* subsp.
63 *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* (7), which exhibit
64 different clinical outcomes and drug susceptible profiles to antibiotic treatments. *M. abscessus* is
65 notoriously acknowledged as the most drug resistant mycobacterial species, characterized by a
66 wide panel of acquired and innate drug resistance mechanisms against nearly all antitubercular
67 drugs as well as to most classes of antibiotics (8). These mechanisms rely largely on the
68 expression of enzymes able to modify, and therefore inactivate, rifamycins (9), aminoglycosides
69 (10), tetracyclines (11) or β -lactams (12) or the presence of multiple efflux pumps of the MmpL
70 family that participate to low-resistance levels to clofazimine and bedaquiline (13, 14).
71 Therapeutic treatments require prolonged courses of multiple antibiotics, usually combining a
72 macrolide, and β -lactam (cefoxitin or imipenem) and an aminoglycoside (15). Macrolides
73 represent the cornerstone of *M. abscessus* multidrug therapy (15) but treatment success rates
74 are poor, particularly in macrolide-resistance strains. Acquired resistance to macrolides occurs
75 usually at low rates through point mutations at positions 2058 or 2059 of the 23S rRNA *rrl* gene
76 (16). In addition, *M. abscessus* harbors an inducible ribosomal methylase encoded by *erm(41)*,
77 representing the primary intrinsic resistance mechanism against macrolides (17). This adaptive
78 resistance mechanisms can be manifested by prolonging incubation of the bacilli up to 14 days in
79 drug susceptible testing assays instead of the usually 3-4 days of exposure to other drugs. A
80 single nucleotide exchange (T28C resulting in a Trp10Arg replacement) in *erm(41)* is associated
81 with a loss of methylase activity in *M. abscessus*, abrogating the inducible resistance mechanism,
82 thus resulting in a macrolide susceptible phenotype (18, 19). This T28C polymorphism is found
83 in 15-20% of *M. abscessus* isolates (20, 21). In contrast to *M. abscessus* or *M. bolletii*, *M.*
84 *massiliense* has been associated with clarithromycin (CLR) susceptibility due to deletions (bases
85 64–65 and 159–432) within *erm(41)* (17, 22), explaining the more favorable clinical outcomes
86 upon CLR treatment (23). However, the role and contribution of the Erm(41)-dependent
87 mechanism in azithromycin (AZM) is less clear and has been the subject of discrepancies

88 between studies. In accordance with an early report of Brown *et al.* (24), it was subsequently
89 reported that MICs of AZM for *M. abscessus* complex tend to be higher than those of CLR (18).
90 Contrasting with these studies, Choi *et al.* reported that AZM is a poor inducer of *erm(41)* gene
91 expression as compared to CLR and should therefore be preferred in antibiotic therapy of *M.*
92 *abscessus* infections (25). However this work was inconsistent with another study in which both
93 molecules provoked a comparable resistance phenotype in *M. abscessus* (19). A recent study
94 also showed that *erm(41)* expression was increased following exposure to either macrolide for 7
95 days and it was proposed that this should not be an argument in choosing between CLR and
96 AZM for therapy (26). However, most studies are based on the *in vitro* determination of the MIC
97 to cultures exposed to macrolides and data reporting the Erm(41)-dependent inducible
98 mechanisms in the infected host are scarce. Except one study reporting the increased expression
99 of *erm(41)* transcripts in *M. abscessus*-infected mice treated with CLR, but not with AZM (25), it
100 remains to fully establish whether macrolide inducible resistance occurs *in vivo* and if induction
101 occurs rapidly upon drug treatment in the infected host.

102 In the context of this long-lasting debate, the present study was aimed at revisiting the
103 *erm(41)* inducible mechanisms through the i) evaluation of the *in vitro* drug susceptibility of
104 clinical isolates belonging to the *M. abscessus* complex to CLR and AZM; ii) visualization of the
105 *erm(41)* induced expression in zebrafish infected with a *M. abscessus* reporter strain in which a
106 fluorescent marker has been placed under the control of the *erm(41)* promoter region; and iii)
107 dissection of the *erm(41)* promoter to uncover important DNA regions that are participate directly
108 in this regulatory process.

109 **MATERIALS AND METHODS**

110
111 **Strains and growth conditions.** *M. abscessus* subsp. *abscessus* CIP104536^T reference strain
112 and clinical isolates are listed in **Table S1**. Strains were grown either in Middlebrook 7H9 broth
113 (BD Difco) supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% oleic acid, albumin,
114 dextrose, catalase (OADC enrichment; BD Difco) (7H9^{T/OADC}), Sauton's medium or Cation-
115 Adjusted Mueller-Hinton Broth (CaMHB; Sigma-Aldrich) at 30°C. On plates, colonies were
116 selected either on Middlebrook 7H10 agar (BD Difco) supplemented with 10% OADC enrichment
117 (7H10^{OADC}) or on LB agar. Antibiotics were purchased from Sigma-Aldrich.

118
119 **Drug susceptibility assessment.** The minimal inhibitory concentrations (MIC) were determined
120 according to the CLSI guidelines (27).

121
122 **Construction of the *lacZ* and *tdTomato* reporter strains.** The full-length intergenic region (IR)
123 (designated P₁₂₃) as well as its truncated versions (designated P₉₂, P₆₁ and P₃₈) were PCR-
124 amplified from the *M. abscessus* CIP104536^T genomic DNA using the Phusion polymerase
125 (ThermoFischer Scientific) with the primers listed in **Table S2**. To generate the mutated IR
126 (P_{61_Mut(B7)}, P_{61_Mut(-35)} and P_{61_Mut(B7/-35)}), complementary single stranded DNA segments containing
127 the XbaI and BamHI sites (**Table S2**) were first denatured for 15 min at 95°C and then annealed
128 together. All DNA fragments were restricted with XbaI and BamHI and ligated to the linearized
129 pMV261_ *lacZ* (28) digested with the same restriction enzymes, leading to a panel of *lacZ* reporter
130 constructs, as listed in **Table S3**. To generate pM261_P₁₂₃_ *tdTomato*, pMV261_P₁₂₃_ *lacZ* was
131 digested with BamHI and HindIII to replace *lacZ* by a linearized BamHI/HindIII-restricted *tdTomato*
132 gene which was amplified by PCR from pTEC27 using the primers listed in **Table S2**. The
133 pMV361 containing an apramycin resistance cassette was linearized with the XmnI blunt cutter
134 and ligated to the double-blunt *whiB7* PCR product to generate the pMV361_ *whiB7* construct,
135 allowing constitutive overexpression of *whiB7*. Plasmids were electroporated in electrocompetent
136 *M. abscessus* S for the *tdTomato* reporter construct and *M. abscessus* R strains for the *lacZ*
137 constructs.

138
139 **β-galactosidase assay.** The quantification in liquid medium of the β-Gal activity was performed as
140 previously reported (28). Briefly, bacterial cultures were adjusted to an OD₆₀₀ of 0.8 in Sauton's
141 medium supplemented with 0.025% tyloxapol containing the appropriate antibiotics to maintain the
142 plasmids. The β-Gal activity was determined at various time points after the initial addition of CLR

143 or AZM (t=0). At each time point, 1 ml cultures were centrifuged (4,000 × g for 10 min at room
144 temperature) and the pellets were resuspended in 700 µl 1X phosphate-buffered saline (PBS).
145 Bacterial suspensions were lysed by beads beating for 3 min at 30 Hz. Lysates were centrifuged
146 (16,000 × g for 10 min at room temperature) and the supernatant collected for measuring the OD₂₈₀
147 with a NanoDrop 2000c. Four µg of the total protein amount present in the supernatant were co-
148 incubated with 100 µl of a reaction buffer containing 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM
149 KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol in a 96-well plates at 37°C for 30 min. 35 µl of a 4
150 mg/ml solution of 2-Nitrophenyl β-D-galactopyranoside (ONPG, Sigma-Aldrich) were added and
151 the absorbance at 420 nm was measured at 30-34°C for 30 min using a Multimode Microplate
152 Reader POLARstar Omega (BMG Labtech). The specific β-Gal activity (SA_{β-Gal}) was defined as
153 follow: (Absorbance_{420nm} × min)/(4 µg of total protein × liter of culture). To discard macrolides from
154 bacterial cultures after 14 days of treatment, bacteria were centrifuged at 4000 x g for 10 min at
155 room temperature and washed three times in PBS. The pellets were finally resuspended in
156 Sauton's medium supplemented with 0.025% tyloxapol containing the appropriate antibiotics to
157 maintain the plasmids. β-Gal activity on solid medium was directly observed by spotting 2.5 µl of
158 cultures at an OD₆₀₀ of 0.8 onto 7H10^{OADC} and following incubation of the plates at 37°C for 3 days.

159
160 **Unmarked deletion of *erm(41)* in *M. abscessus*.** The pUX1-*katG-erm(41)* was generated
161 following the same strategy as previously described (13, 14) using the primers listed in **Table S2**
162 and introduced in *M. abscessus* S. Selection of bacteria having undergone the first homologous
163 recombination was done by screening for red fluorescent colonies on 7H10^{OADC} supplemented with
164 200 µg/ml kanamycin. To promote the second homologous recombination event, the selected
165 clones were subcultured overnight in 7H9^{OADC} in the absence of kanamycin. Cultures were 10-fold-
166 serially diluted and plated onto 7H10^{OADC} containing 50 µg/ml INH to select for isoniazid-resistant,
167 kanamycin-sensitive and non-fluorescent colonies. Following genomic DNA preparation of selected
168 colonies, a PCR screening was setup using primer sets binding upstream of the left arm (LA) and
169 inside the right arm (RA) or binding inside the LA and downstream of the RA. Amplicons were
170 subsequently sequenced to confirm the junction between LA and RA and proper deletion of
171 *erm(41)*.

172
173 **Zebrafish infections and visualization of macrolide inducible resistance.** All zebrafish
174 experiments were conducted in accordance with the guidelines from the European Union for
175 handling of laboratory animals
176 (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and approved by the

177 Direction Sanitaire et Vétérinaire de l'Hérault et Comité d'Ethique pour l'Expérimentation Animale
178 de la région Languedoc Roussillon under the reference CEEA-LR-1145. Experiments were
179 conducted using the transgenic Tg(*mpeg1:GFP-CAAX*) zebrafish line carrying green fluorescent
180 macrophages (29). Ages of embryos are expressed as hours post fertilization (hpf). *M. abscessus*
181 *S* left untransformed (negative control) or carrying either pTEC27 (constitutive expression of
182 *Tdtomato*) or pMV261- P_{123} -*tdTomato* (macrolide inducible expression of *TdTomato*) were injected
183 in zebrafish embryos and monitored for fluorescence expression, according to protocols reported
184 previously (30). Briefly, infections were performed by intravenous microinjection of 900-1000 CFU
185 in dechorionated and anesthetized 30 hpf embryos. At 24 hpi, infected embryos were exposed to
186 either 32 µg/ml CLR or AZM in osmotic water and this treatment was renewed on a daily basis.
187 Red fluorescence was assessed by immobilizing anesthetized embryos in 1% low melting point
188 agarose and performing life imaging by confocal microscopy (LSM 880 Airyscan) at day 0, day 2
189 and day 6 post treatment (dpt). 2D reconstructions of image stacks were done with the ZEN 2
190 software.

191 **RESULTS**

192

193 **Macrolide inducible resistance of smooth and rough *M. abscessus* CIP104536^T is medium-**
194 **independent.** The current debate regarding the level of induced resistance by CLR or AZM has
195 been hypothesized to be dependent on the medium composition used for the determination of the
196 the MICs of the two drugs. In particular, Choi *et al.* (25) used Middlebrook 7H9 which contains a
197 large number of inorganic salts and has a slightly acidic pH (pH6.8) whereas Bastian *et al.* (18),
198 following the CLSI recommendations, used Cation-adjusted Mueller Hinton broth (CaMHB) which
199 has a higher pH (pH7.4) than 7H9. Because macrolide activity is sensitive to pH (31), it was
200 hypothesized that the different media used in various studies may influence the MIC values.
201 Herein, we evaluated and compared macrolide-induced resistance to both drugs at various time
202 points (3, 5, 7, 10 and 14 day post-treatment (dpt)) in three different media (CaMHB, 7H9 and
203 Sauton's). When assaying the smooth (S) morphotype of the reference strain 104536^T, we
204 consistently found that AZM induced a more rapid up-shift of the MIC than CLR in all three media
205 (**Fig. 1A**). The increase in MIC of AZM started after 3 dpt and rised progressively to reach a MIC
206 of 256 µg/ml at 10 dpt. The same MIC value was reached at 14 dpt with CLR, indicating that,
207 although the intensity of induced resistance is comparable for both macrolides, the kinetic of
208 resistance was delayed with CLR as compared to AZM *in vitro*. As expected, amikacin (AMK),
209 used as a non-relevant control drug, failed to show a similar drug-induced phenotype upon a
210 prolonged exposure time. To address whether this phenomenon was restricted to the S form,
211 similar experiments were conducted using the rough (R) variant of *M. abscessus*. **Fig. 1B** clearly
212 indicates that, as observed with the S variant, the kinetic of resistance to AZM was more rapid
213 than the one with CLR and that macrolide-induced resistance was independent of the medium
214 used to determine the MICs. Overall, these results indicate that both S and R morphotypes
215 respond similarly to macrolide treatment regardless to the medium used.

216

217 **Resistance to macrolides is rapidly induced in the *M. abscessus* complex strains upon**
218 **exposure to CLR and AZM.** We next investigated the kinetic of macrolide inducible resistance in
219 several clinical strains, from CF and non-CF patients, in CaMHB. Clinical *M. abscessus* strains
220 2069 and 3022, from non-CF, were characterized by a more rapid increase in the MIC values to
221 AZM than to CLR, similarly to the reference strain CIP104536^T (**Fig. 2A** and **2B**). However, in the
222 case of 2069, the resistance to CLR reached a plateau at 128 µg/ml at 10 dpt, whereas for 3022
223 the MIC continues to rise up to 256 µg/ml at 14 dpt, sharing a similar profile with the CF strain
224 2648. In contrast, strain 1298 failed to show inducible resistance to either macrolide (**Fig. 2A**).

225 Due to the important contribution of the polymorphism at the 28th nucleotide of *erm(41)*, the T28
226 sequevar (Trp10) being responsible for inducible resistance while the C28 sequevar (Arg10)
227 remains susceptible to macrolides (18), we subsequently sequenced *erm(41)* from strains *M.*
228 *abscessus* 1298, 2069, 2648, 3022, *M. bolletii* and *M. massiliense*. All these strains possess an
229 *erm(41)* gene identical to the *M. abscessus* CIP104536 reference strain, characterized with a Trp
230 in position 10, except for strain 1298 possessing a Leu residue instead of a Phe in position 34
231 (data not shown). To our knowledge, this point mutation, presumably responsible for an inactive
232 Erm(41) enzyme, thereby conferring susceptibility to macrolides, has not been reported yet (20).
233 The *M. bolletii* S strain carrying the T28 sequevar, showed also inducible resistance to
234 macrolides, similarly to *M. abscessus* (**Fig. 2C**). As anticipated, *M. massiliense* was found to be
235 intrinsically susceptible to CLR and AZM due to a mutation in *erm(41)* which renders the enzyme
236 non-functional (18) (**Fig. 2D**). We next constructed an unmarked deletion mutant of *erm(41)* using
237 a recently developed strategy based on a new suicide vector, pUX1-*katG*, allowing easy and
238 rapid generation of genetic deletions in the *M. abscessus* chromosome, facilitated by the
239 presence of the brightly red fluorescent tdTomato positive selectable marker and the *katG*
240 counter selectable marker (10, 32, 33) (**Fig. S1**). As expected, and in agreement with a previous
241 report (25), this strain remained fully susceptible to CLR and AZM, confirming the direct
242 involvement of Erm(41) in macrolide-inducible resistance (**Fig. 2E**).

243 Together, these results confirm that *M. abscessus* and *M. bolletii* clinical isolates carrying a
244 functional Erm(41) methylase undergo a faster increase in resistance to AZM than to CLR and
245 that the newly discovered F34L substitution in Erm(41) abrogates the macrolide-induced
246 resistance.

247
248 **Inducible resistance to macrolides occurs in infected zebrafish.** The vast majority of studies
249 related to macrolide inducible resistance are based on MIC determination and/or determination
250 by qRT-PCR of the level of *erm(41)* transcripts *in vitro* (18, 19, 26). Despite of their clinical
251 importance, reports describing whether macrolide inducible resistance occurs during patient
252 therapy are scarce. One study, however, has reported the increased expression of *erm(41)*
253 transcripts in *M. abscessus*-infected mice treated with CLR but not with AZM (25). We thus took
254 advantage of using the transparency of zebrafish embryos infected with *M. abscessus* to
255 visualize and monitor the induced expression of *erm(41)* during treatment of the infected larvae
256 with macrolides. Indeed, this recently developed animal model allows to easily assess the
257 suitability and sensitivity of clinically relevant drugs and to visualize in a dose- and time-
258 dependent manner, the dynamics of infection in the presence of an active compound (12, 34–36).

259 To do so, a fluorescent reporter *M. abscessus* strain was designed, carrying the
260 pMV261_P₁₂₃_tdTomato in which the *tdTomato* gene was cloned under the control of the 123 bp
261 promoter region of *erm(41)* (**Fig. 3A**). To verify the usefulness of this construct, the recombinant
262 strain was first plated on Middlebrook 7H10 containing 100 µg/ml kanamycin supplemented or not
263 with 2 µg/ml CLR or AZM. Examination of the plates under a fluorescence microscope revealed
264 the presence of red fluorescent colonies in the presence of either macrolide while no
265 fluorescence was detected on plates lacking these drugs (**Fig. 3B**). As a positive control of
266 fluorescence, a strain harboring pTEC27, which allows constitutive expression of *tdTomato* in *M.*
267 *abscessus* (37), was also included in these experiments (**Fig. 3B**). These results clearly highlight
268 the potential of this reporter strain to monitor macrolide inducible resistance in an infected host.
269 Whether red fluorescent bacilli can be visualized directly in *M. abscessus*-infected embryos upon
270 treatment with macrolides was next investigated. Around 1000 CFU of the reporter strain were
271 microinjected in the caudal vein of the *mpeg::GFP-CAAX* transgenic embryos harboring green
272 fluorescent macrophages (29) which, after 24 hrs, were exposed to 32 µg/ml CLR or AZM
273 (directly added into the water). Fish were observed under the fluorescence microscope at 0, 2
274 and 6 dpt. Red fluorescent bacilli could be detected at 2 dpt with either macrolide, while no signal
275 was detected in control embryos (*M. abscessus* with no plasmid) or embryos treated with AMK as
276 a non-relevant drug, indicating that expression of *tdTomato* occurred very rapidly during infection
277 in a living animal and was specifically induced by macrolides (**Fig. 3C**). At later time points (6
278 dpt), red-fluorescent bacilli were detected inside individualized macrophages or inside
279 aggregated macrophages forming granulomas. Red bacilli could also be observed outside of
280 macrophages, although they may reside inside neutrophils (which cannot be visualized in this
281 transgenic zebrafish line harbouring only fluorescent macrophages), which have previously been
282 shown to rapidly engulf *M. abscessus* (38). It remains, however, difficult to explain at this stage
283 whether already fluorescent bacteria are phagocytosed by macrophages or whether macrolides
284 penetrate macrophages/granulomas to induce *tdTomato* expression in the intracellular bacilli.
285 Overall, these results indicate that macrolide inducible resistance occurs in an infected host and,
286 in contrast to a previous work in mouse model (25), AZM induces *erm(41)* expression in the
287 zebrafish model.

288
289 **Low macrolide concentrations induce a more rapid expression of *erm(41)* than high**
290 **concentrations in *M. abscessus*.** The pMV261_P₁₂₃_lacZ containing the *lacZ* gene coding for
291 the β-galactosidase under the control of the *erm(41)* promoter was designed to tackle the
292 fluctuations in *erm(41)* gene expression during exposure of the culture to various drug treatments

293 **(Fig. 4A)**. This strain was exposed to 2, 8, 64 and 128 µg/ml of either CLR or AZM and
294 expression of LacZ was determined by measured the β-Gal activity using ONPG at different time
295 points over a 14-days period **(Fig. 4B)**. Exposure to 2 µg/ml of CLR induced a rapid β-Gal activity
296 during the first 2 days of treatment, which plateaued thereafter. By increasing the drug
297 concentration, similar but delayed specific activity curves were obtained. At the highest
298 concentration tested, the lag phase lasted for 7 days prior to the induction of *lacZ* expression.
299 This is unlikely to be linked to the impaired growth of the cultures treated with high concentrations
300 of macrolide as the specific activity is expressed relative to the total amount of soluble proteins.
301 The maximum specific activity was, however, lower than the β-Gal activity produced when *lacZ*
302 was placed under the control of the strong constitutive *hsp60* promoter **(Fig. 4C, left panel)**. As
303 expected, when the promoterless construct was introduced in *M. abscessus*, no β-Gal activity
304 was detected. Moreover, in another strain in which the *erm(41)* promoter region was substituted
305 by the promoter region of *MAB_4384*, encoding a TetR protein previously characterized (28), no
306 activity was detected. This further supports that the CLR-dependent expression of *lacZ* was
307 specific to the *erm(41)* promoter region. When the strain was treated with increasing
308 concentration of AZM, slightly different trends were observed, characterized by a faster induction
309 of *lacZ* expression at low concentrations (2 and 8 µg/ml) than at higher concentrations (64 and
310 128 µg/ml) and a significantly shorter lag phase for each concentration tested with AZM than with
311 CLR **(Fig. 4C, right panel)**. The cultures treated with 64 µg/ml of AZM reached a β-Gal activity of
312 approximately 8000 units in 3 days only, whereas 10 days were needed for cultures treated with
313 the same CLR concentration to reach this level of activity. Together, these results suggest that
314 transcriptional expression *erm(41)* is more rapidly induced with AZM than with CLR, in agreement
315 with MIC results **(Fig. 1 and 2)**.

316 To interrogate whether the rapid increase in AZM-dependent expression of *lacZ* was only
317 restricted to the reference strain, we next assessed the influence of 8 µg/ml of both macrolides
318 on *lacZ* expression in several clinical strains (*M. abscessus* 2069, 2648, 3022 and *M. bolletii*)
319 transformed with pMV261_P₁₂₃-*lacZ*. In all cases, treatment with 8 µg/ml AZM induced a more
320 rapid induction of *lacZ* expression than treatment with 8 µg/ml CLR, although the intensity of the
321 response varied from one strain to another and was always below the activity associated with the
322 *hsp60* promoter **(Fig. 4D)**. For instance, *lacZ* induction in strain 3022 was significantly slower
323 than in strain 2069. Overall, these results confirm that, although both macrolides induce a potent
324 transcriptional expression of *erm(41)*, AZM induces a more rapid response than CLR,
325 consistently with the MIC kinetics.

326

327 **Both CLR and AZM antagonize the efficacy of AMK in *M. abscessus*.** Drug inducible
328 resistance is a mechanism of adaptive response to antibiotic treatment that is not genetically
329 programmed. As such, this reversible phenomenon is expected to disappear after stopping the
330 drug treatment. Our reporter strain allows to verify if *erm(41)* expression reverses back after
331 removal of the drugs. In our experimental protocol, bacteria were treated for 14 days with 8 µg/ml
332 CLR (**Fig. 4C, left panel**) or with 8 µg/ml AZM (**Fig. 4C, right panel**), after which the cultures
333 were extensively washed with PBS and the β-Gal activity further monitored for 7 days in the
334 absence of drugs (**Fig. 4B**). Rapidly after washing, *lacZ* expression dropped down during the next
335 2 days and then remained unchanged till the end of the experiment (7 days post washes) at
336 levels that were significantly higher (around 4000 units) than prior to drug addition. To assess the
337 possibility that macrolide pre-treated cultures are more tolerant than unexposed cultures, the
338 pMV261_P₁₂₃_lacZ containing bacteria were first pre-exposed or not with 8 µg/ml of CLR or
339 AZM for 4 days. Once the optimal β-Gal activity was reached, the cultures were carefully washed
340 with 1X PBS and bacterial pellets were resuspended and incubated in macrolide-free Sauton's
341 medium for two more days, a time point at which a β-Gal activity of 4000 units was measured
342 (data not shown). From this stage (considered as day 0), the MIC of CLR, AZM and AMK were
343 determined in Sauton's medium at 3, 5, 7, 10 and 14 days (**Table 1**). At day 3, the untreated
344 culture exhibited MIC values of 1, 4 and 16 µg/ml for CLR, AZM and AMK, respectively, while the
345 macrolide-pretreated cultures exhibited higher MIC values. The CLR-treated culture displayed
346 MIC of 64, 128 and 16 µg/ml against CLR, AZM and AMK, respectively. This effect was even
347 more pronounced with the AZM-treated culture (128, 256 and 32 for CLR, AZM and AMK,
348 respectively). Importantly, this phenotypic resistance further increased at day 5 and day 7, with
349 MIC values exceeding 256 µg/ml. While the MIC of AMK remained unchanged for the untreated
350 culture (16-32 µg/ml), pre-treatment with CLR induces cross-resistance to AMK (64 g/ml), as
351 previously reported (39). We found that this was also true with AZM (**Table 1**), indicating that, like
352 CLR, exposure to AZM antagonizes the activity of AMK.

353
354 **Identification of a *whiB7* box upstream of *erm(41)* required for macrolide-induced**
355 **expression.** Careful examination of the promoter region segment using the MEME software
356 identified two pairs of conserved repetitive sequences. To gain further insights into the regulatory
357 DNA elements responsible for macrolide induction, the P₁₂₃ promoter region in pMV261_
358 P₁₂₃_lacZ was replaced by DNA fragments of 92 bp, 61 bp and 38 bp derived from the original
359 P₁₂₃. The segment sizes were chosen to progressively shorten the degenerated inverted repeats
360 (**Fig. 5A-B**). These DNA motifs may eventually represent target of transcriptional factors that

361 contribute the *erm(41)* transcriptional regulation. Cultures were exposed to various
362 concentrations of AZM or CLR and *lacZ* expression was monitored over time by measuring the β -
363 Gal activity. In the presence of 2 $\mu\text{g/ml}$ of either macrolide, the optimal specific activity of the full
364 length construct (123 bp) plateaued around 7000 units, while the 92 bp construct was associated
365 with reduced the activity, around 5000 units (**Fig. 5C, left panel**). This indicates that this
366 construct is lacking important regulatory element that are required for normal transcriptional
367 levels. Unexpectedly, further shortening the promoter region down to 61 bp led to an opposite
368 effect with a much more pronounced β -Gal activity than with P_{124} , reaching the levels of the
369 strong *hsp60* promoter. As shown earlier, these effects were always more rapid when the various
370 strains were exposed to AZM than to CLR. Similar profiles were obtained when treating the
371 various strains with 8 $\mu\text{g/ml}$ (**Fig. 5C, middle panel**) or 64 $\mu\text{g/ml}$ (**Fig. 5C, right panel**) of
372 macrolides. In sharp contrast, the strain carrying $\text{pMV261_}P_{38}\text{-}lacZ$ failed to express any β -Gal
373 activity, indicating that P_{38} is missing important regulatory informations. As expected, when either
374 the promoterless construct (ΔP) or the $\text{pMV261_}P_{MAB_4384}\text{-}lacZ$ were introduced in *M. abscessus*,
375 no β -Gal activity was detected (**Fig. 5C**). Overall, these results suggest the presence of two
376 important regulatory sequences in P_{123} : one contained between the 5'-end of the P_{61} and the 5'-
377 end of the P_{38} that positively regulates expression and a second one present at the 5'-end of the
378 92 bp segment that possibly participates in the downregulation of *erm(41)* expression.

379 By comparing the common sequences of P_{61} and P_{38} , we identified a putative -35 box (**Fig.**
380 **6A**). In addition, WhiB7 is known as a master regulator that activates transcription of many
381 different gene targets involved in the resistance to macrolides and amikacin (39, 40), but its direct
382 implication in this process remains yet to be defined. We, therefore, scrutinized more thoroughly
383 the DNA sequence of P_{61} and found a sequence closed to the typical AT-rich DNA sequence
384 recognized by WhiB7. This sequence displays partial conservation with a motif identified in the
385 region upstream of *whiB7* in various mycobacterial species (41) and which is missing in P_{38} (**Fig.**
386 **6A**). This putative 5 bp WhiB7-binding site (GAAAC) is located 3 nucleotides upstream of a
387 potential -35 box, similarly to the sites and sequences defining the *whiB7* promoter (41, 42).
388 However, the optimal distance of 16-19 nucleotides between the -35 and -10 hexamers is not
389 conserved as they are separated by only 7 nucleotides (**Fig. 6A**). Mycobacterial promoters have
390 been identified with hexamer distances of between 6 and 35 bp (43). To test whether WhiB7
391 influences *erm(41)* expression by binding to this motif, the integrative $\text{pMV361_}whiB7$ was
392 introduced in *M. abscessus* carrying $\text{pMV261_}P_{123}\text{-}lacZ$ and β -Gal activity was followed for 7
393 days in the absence of drug treatment. **Fig. 6B** clearly shows that *lacZ* was expressed
394 constitutively at much higher levels than in the strain missing $\text{pMV361_}whiB7$, indicating that

395 WhiB7 is a positive regulator of *erm(41)* expression, supporting previous findings (39, 40). To
396 further incriminate the contribution of the GAAAC motif as a potential WhiB7-binding site, this
397 sequence was mutated in pMV261_P₆₁_lacZ, (designated P₆₁_Mut(B7)), as illustrated in **Fig. 6A**.
398 This abrogated *lacZ* expression both in liquid cultures (**Fig. 6C, upper panel**) and on agar plates
399 (**Fig. 6C, lower panel**). In addition, mutating the putative -35 box was accompanied by a sharp
400 drop in the β -Gal activity and mutations in both the *whiB7* and the -35 boxes abrogated *lacZ*
401 expression, suggesting that both regions are required to regulation and transcriptional expression
402 of *erm(41)* (**Fig. 6C**). To conclusively demonstrate the importance of the GAAAC motif for
403 macrolide inducible resistance, we treated the three mutated strains - pMV261_P₆₁_Mut(B7)_lacZ,
404 pMV261_P₆₁_Mut(-35)_lacZ and pMV261_P₆₁_Mut(B7/-35)_lacZ - with either 8 μ g/ml CLR or AZM for 7
405 days. This led to complete loss of β -Gal activity upon treatment with ether drugs, as compared to
406 the pMV261_P₆₁_lacZ control strain carrying the intact motifs (**Fig. 6D**).

407 Collectively, these results indicate that the 61bp promoter region comprises a critical WhiB7
408 binding motif that is necessary for macrolide-inducible *erm(41)* expression.

409 **DISCUSSION**

410
411 *M. abscessus* is a challenging pathogen causing severe respiratory infections in patients with
412 underlying lung disorders and these infections have become more common recently, which is of
413 particular concern due to its natural resistant to most conventional antibiotics (8). Macrolides are
414 important components of the multidrug therapy against *M. abscessus sensu lato* and the
415 American Thoracic Society/Infectious Diseases Society of America guidelines for treatment of
416 NTM recommend use of one of these agents as part of a multidrug regimen, with no stated
417 preference for one macrolide or the other (15). In a previous study it was shown that CLR was a
418 stronger inducer of Erm(41), leading to higher mRNA expression and a more rapid increase in
419 MICs during prolonged incubation compared with AZM (25), which led to the conclusion that AZM
420 may be preferred to CLR for the treatment of *M. abscessus* infections. It was subsequently
421 reported that the median MICs of AZM for *M. abscessus sensu lato* were markedly higher than
422 those of CLR (44) while both macrolides induce resistance similarly in a another study (26). Our
423 results confirm that *M. bolletii* and *M. abscessus* clinical isolates carrying a functional Erm(41)
424 methylase undergo a more rapid increase in the MIC to AZM than to CLR. This phenomenon was
425 neither linked to the medium composition since similar kinetics of resistance were obtained in
426 three different media nor to the morphotype of the strains. Based on these results, induction of
427 resistance cannot be a criterion in choosing between CLR and AZM for *M. abscessus* drug
428 treatment.

429 In one case only, macrolide induced resistance was abrogated (clinical strain 1298) due to a
430 F34L substitution in Erm(41), a mutation not reported earlier to our knowledge, presumably
431 inactivating the enzymatic activity. The MIC data were also corroborated with transcriptional data
432 through the development of a *lacZ* reporter strain exposed to various doses of either macrolide
433 and supporting the view that expression of *erm(41)* is more rapidly induced with AZM than with
434 CLR. As expected, our results also support previous findings regarding the effect *erm(41)* gene
435 disruption in *M. abscessus* in eliminating inducible macrolide resistance. That *M. massiliense*,
436 known as a natural *erm(41)* mutant, was associated with durable susceptibility to macrolide
437 treatment explains why patients with *M. abscessus* infection have significantly lower rates of
438 sputum culture conversion in response to CLR-based therapy than patients with *M. massiliense*
439 (45). Importantly, through the development of a *tdTomato* reporter strain, we were able to image
440 macrolide induced resistance in living animals. Thanks to the optical transparency of zebrafish
441 embryos, individual bacilli expressing tdTomato could be detected very early after exposing the
442 embryos to either CLR or AZM (2 dpt). Although qualitative, these results indicate that CLR and

443 AZM inducible resistance occurs very rapidly in the infected host, likely explaining the poor
444 effectiveness of macrolide therapy in patients infected with *M. abscessus* or *M. bolletii* (45, 46).
445 We propose this reporter strain to be particularly attractive to assay next-generation macrolide
446 antibiotics that are unable to induce the *erm(41)*-based resistance mechanism while retaining
447 activity against *M. abscessus*. It would be conceivable to develop in a 96-well plate format a
448 microbiological assay based on the pMV261_P₁₂₃-tdTomato-containing *M. abscessus* strain that
449 couples both MIC determination as well as fluorimetric readings, enabling to simultaneously
450 assess the *in vitro* activity of a compound and macrolide-induced resistance. Such an approach
451 would be adapted to the high throughput screening of large libraries of macrolide scaffolds (47) in
452 a short timeframe.

453 Penetration of drugs into the bacterial cytoplasmic compartment induces an important
454 transcriptional reprogramming resulting in changes in the growth rate, metabolism and induction
455 of genes involved in drug resistance, such as enzymes that modify either the antibiotic or its
456 target as well as efflux pumps. Interestingly, *erm(41)*, also known as *MAB_2297*, is located at the
457 very near proximity of a gene cluster participating in the intrinsic resistance to clofazimine and
458 bedaquiline in *M. abscessus*, comprising a TetR transcriptional repressor (*MAB_2299c*) that
459 regulates the transcription of a MmpS/MmpL efflux pump system encoded by
460 *MAB_2300/MAB_2301* (32). This gene cluster possesses also a second MmpS/MmpL pair
461 (*MAB_2302/MAB_2303*) whose eventual role in resistance to antibiotics has yet to be
462 established. Overall, these observations point out to a multi-drug resistance island participating in
463 the intrinsic resistance to different classes of antibiotics.

464 WhiB7 represents one of the best studied transcriptional activator of the reprogramming
465 circuit that belongs to the WhiB family of regulators conserved in actinomycetes (42, 48). The
466 *whiB7* gene is induced upon treatment of mycobacteria with several structurally unrelated
467 antibiotics as well as compounds that can perturb respiration, redox balance and iron starvation
468 (41, 42, 49, 50). Recently, *MAB_3508c*, which is 75% identical to the *Mycobacterium smegmatis*
469 and *Mycobacterium tuberculosis whiB7*, has been shown to be involved in intrinsic resistance of
470 *M. abscessus* to various antibiotics (40). Induced expression occurred in the presence of
471 erythromycin, clarithromycin, streptomycin, spectinomycin, amikacin and tetracycline and deletion
472 of this gene caused hypersusceptibility to these ribosome-targeting antibiotics in *M. abscessus*
473 (40). In addition, RNAseq data to determine the *whiB7* regulon of *M. abscessus* identified 128
474 genes, including *erm(41)* and *eis2* (40). In another study, it was shown that WhiB7 was induced
475 by CLR and was needed for *erm(41)* induction in *M. abscessus* (39). This is supported by our
476 study where constitutive overexpression of WhiB7 led to increased *lacZ* expression when cloned

477 under the control of the *erm(41)* promoter. In addition, we identified a putative WhiB7 binding site
478 which, when mutated, prevented macrolide induced resistance, thus proving that a direct binding
479 of WhiB7 to the specific AT-rich motif is necessary to induce expression of *erm(41)*. Overall, our
480 results confirm WhiB7 as a primary determinant in transcriptional reprogramming in macrolide
481 inducible resistance in *M. abscessus*.

482 As a none genetically programmed mechanism, macrolide inducible resistance reflects an
483 adaptive response to antibiotic treatment, thought to be reversible upon drug removal. We found
484 that the β -Gal activity, reflecting *erm(41)* transcriptional expression, rapidly decreases during the
485 next 2 days following removal of CLR or AZM from the cultures and then remained constant at
486 levels that were significantly higher than prior to drug addition and until the end of the experiment.
487 These results suggest that induced macrolide resistance is only partially reversible, at least under
488 the conditions used, since the specific activity at 7 days post-washes remained significantly
489 higher than basal level of *lacZ* expression prior to drug treatment. This implies that, even after
490 several rounds of division, the bacteria remain in an activated metabolic state conferring an
491 enduring resistance phenotype. In this particular physiological stage, *M. abscessus* exhibits very
492 high resistance levels to both CLR and AZM and to a lesser extent to AMK when compared to
493 previously unexposed bacteria at days 3, 5, 7 and 10. At day 14, the macrolide resistance profile
494 was similar in all three conditions whilst the MIC of AMK remains higher for the pre-treated
495 bacteria. Therefore, it can be inferred that pre-exposition to CLR or AZM sensitizes the cultures
496 which will respond more efficiently upon re-exposure to the drugs. This may confer the bacilli a
497 protective advantage during subsequent drug therapy. This scenario appears to involve the
498 transfer of signaling events through the subsequent generations and may possibly implicate
499 epigenetic regulation by providing to the newly formed bacteria the opportunity to stay in an on-
500 state macrolide inducible resistance stage. One simple explanation is that during treatment, the
501 bacilli have adapted their metabolism in a way that they continued to produce *erm(41)* transcripts
502 at higher basal levels. That bacilli being previously exposed to either CLR or AZM express higher
503 basal levels of *erm(41)* transcripts (and higher resistance levels to macrolides and amikacin) may
504 have important clinical consequences. Indeed, recent surveys have identified *M. abscessus* as a
505 major threat in many CF centers worldwide (51) and epidemiological studies are documenting the
506 transmission from patient-to-patient of dominant circulating *M. abscessus* clones that have
507 spread globally between hospitals (6). If this happens, it remains possible that patients infected
508 with previously macrolide-exposed bacilli may be more refractory to subsequent macrolide and
509 AMK treatment, thereby further complicating future antibiotic-based therapy.

510 **ACKNOWLEDGMENTS**

511

512 This work was supported by the Fondation pour la Recherche Médicale (FRM) [grant number
513 DEQ20150331719 to LK; grant number ECO20160736031 to MR] and the InfectioPôle Sud for
514 funding the PhD fellowship of AVG.

515 The authors have no conflict of interest to declare.

516

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700 **Table 1. Enduring resistance levels to macrolides and amikacin following pre-exposure of**
 701 ***M. abscessus* to CLR or AZM.** *M. abscessus* CIP104536^T (rough variant) was pre-treated or not
 702 with 8 µg/ml of either CLR or AZM for 4 days, washed and incubated in macrolide-free medium
 703 for 2 additional days prior to monitoring the MIC determination of CLR, AZM and AMK according

| Growth Conditions | Day 3 | | | Day 5 | | | Day 7 | | | Day 10 | | | Day 14 | | |
|-------------------|-------|-----|-----|-------|------|-----|-------|------|-----|--------|------|-----|--------|------|-----|
| | CLR | AZM | AMK | CLR | AZM | AMK | CLR | AZM | AMK | CLR | AZM | AMK | CLR | AZM | AMK |
| Untreated | 1 | 4 | 16 | 16 | 64 | 16 | 64 | 128 | 16 | 128 | 256 | 32 | 256 | >256 | 32 |
| CLR pre-treated | 64 | 128 | 16 | 256 | >256 | 32 | >256 | >256 | 32 | >256 | >256 | 64 | >256 | >256 | 64 |
| AZM pre-treated | 128 | 256 | 32 | >256 | >256 | 64 | >256 | >256 | 64 | >256 | >256 | 64 | >256 | >256 | 64 |

704 to the CLSI guidelines at different time points. The antibiotics concentration are given in µg/ml.

705

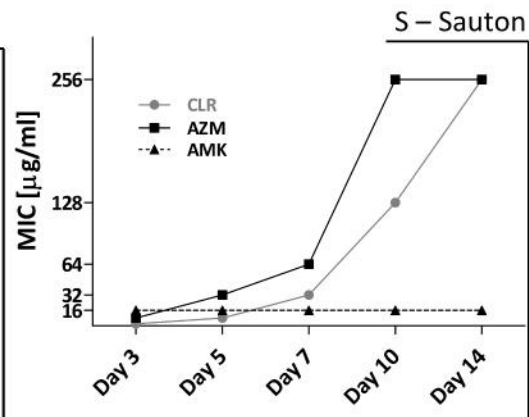
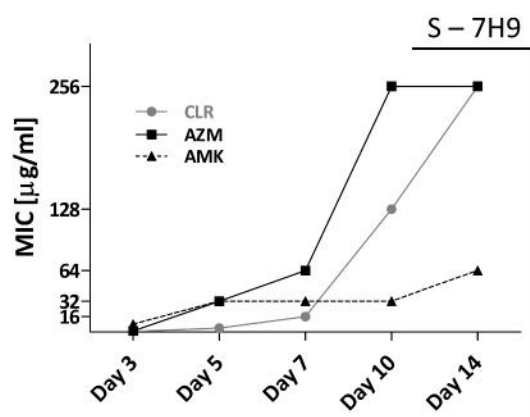
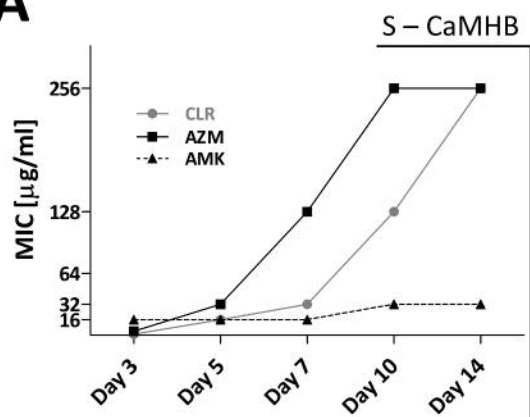
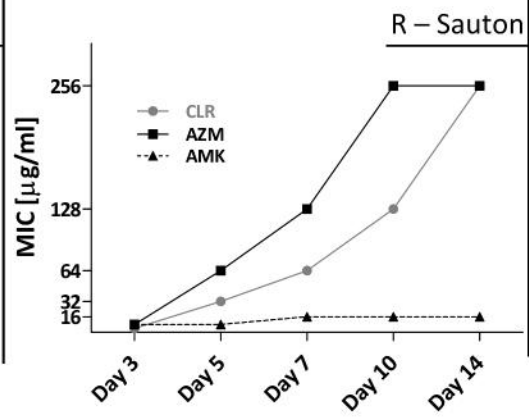
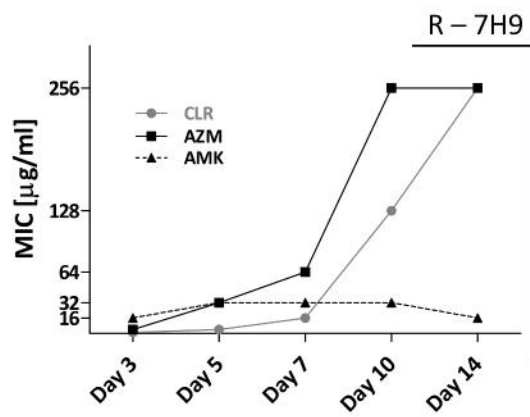
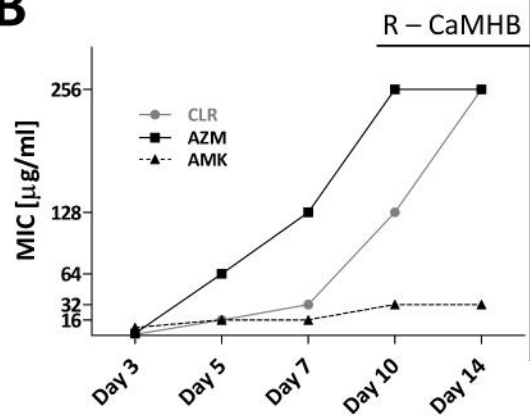
706 **FIGURE LEGENDS**

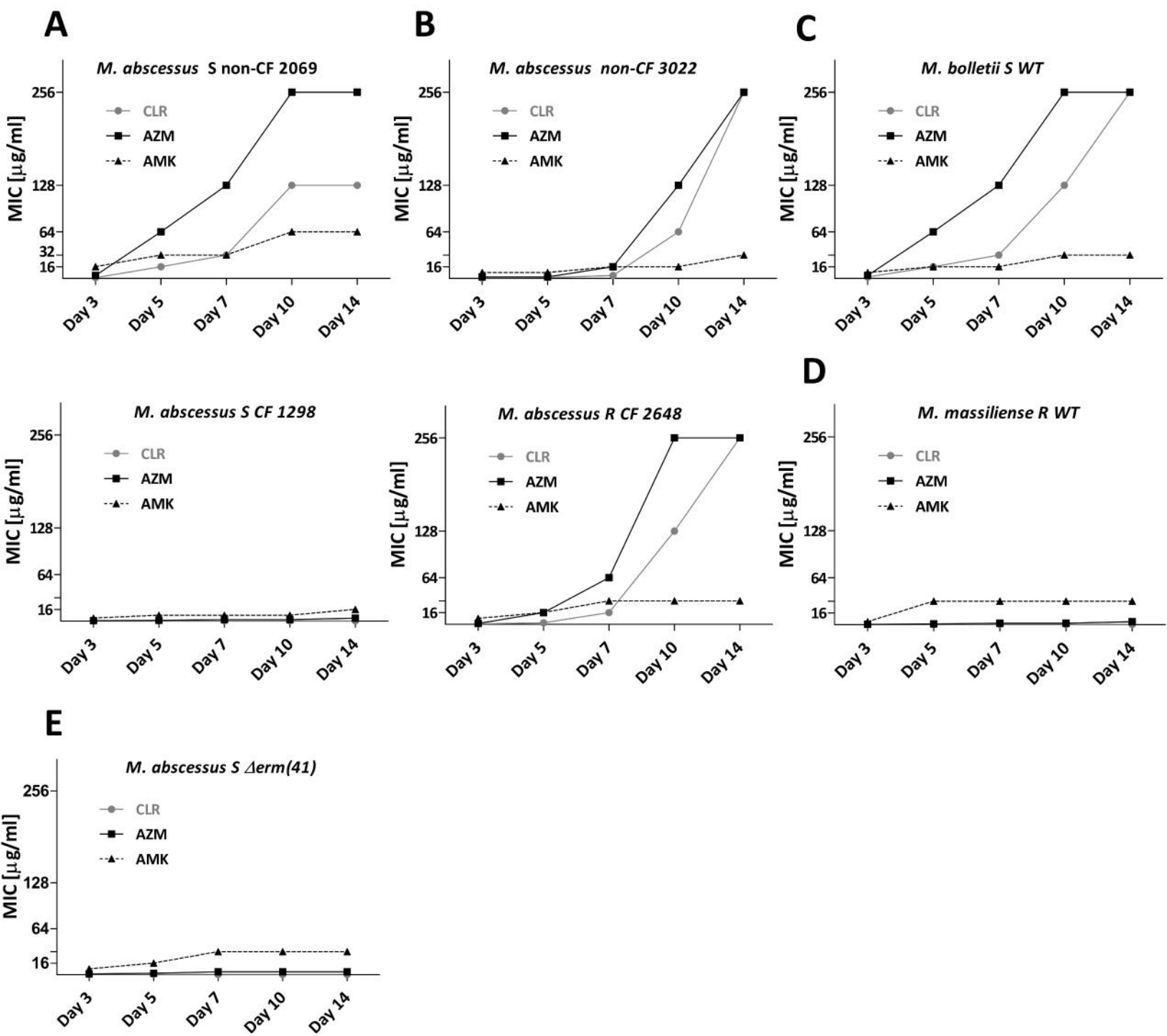
707
708 **Figure 1. Macrolide-induced resistance profile of *M. abscessus* CIP104536^T smooth (A) and**
709 **rough (B) variants in different broth media.** MICs of the two morphotypes were assessed over
710 a period of 14 days in CaMHB, Sauton's medium and Middlebrook 7H9-Glycerol broth. AMK was
711 included as a non-related control drug.

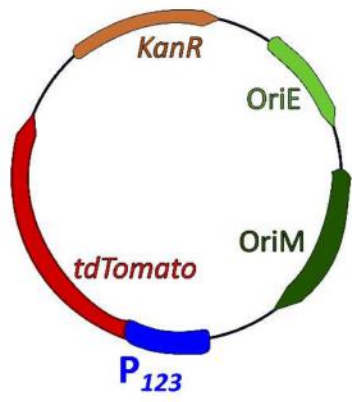
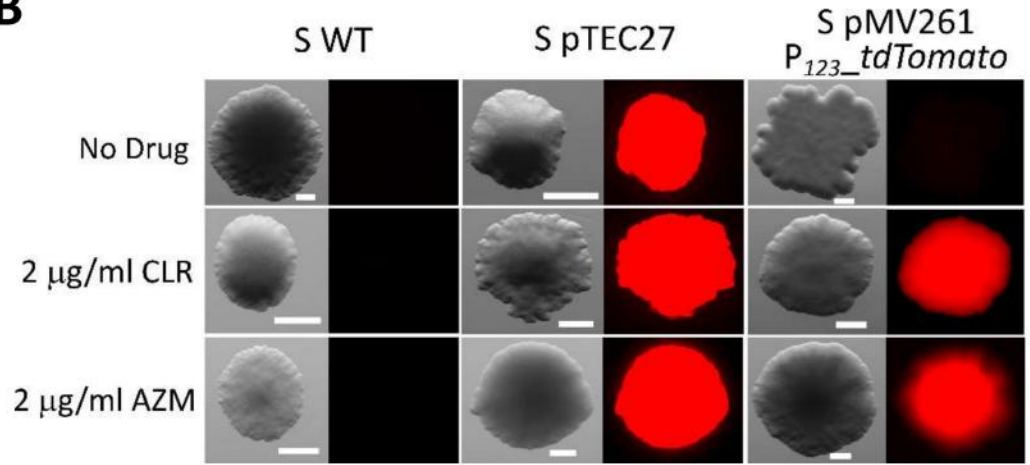
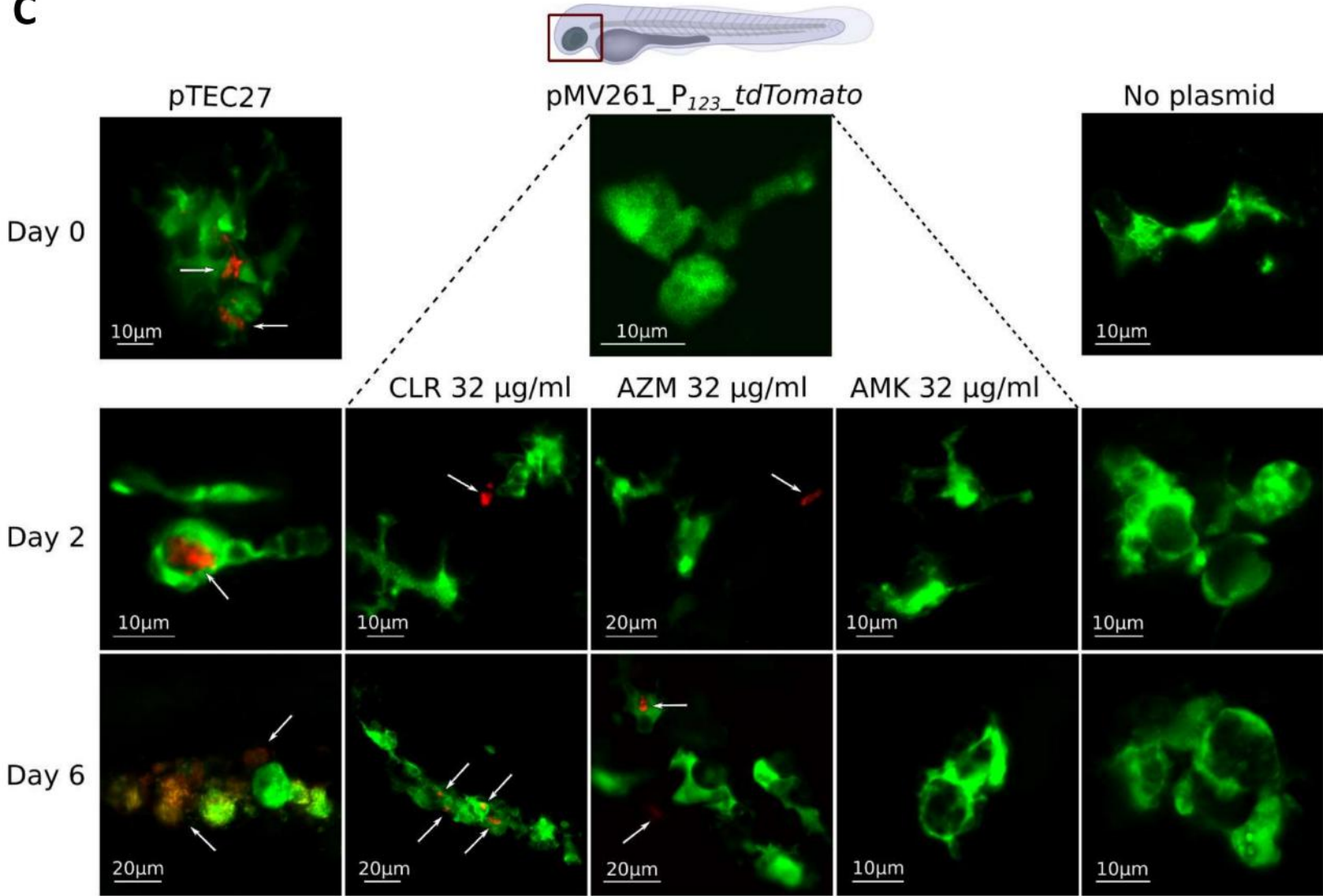
712
713 **Figure. 2. Macrolide-induced resistance profile of clinical isolates in CaMHB. (A)** Smooth *M.*
714 *abscessus* clinical isolates. **(B)** Rough *M. abscessus* clinical isolates. **(C)** Smooth *M. bolletii*
715 CIP108541^T. **(D)** Rough *M. massiliense* CIP108297^T. **(E)** Smooth *M. abscessus* CIP104536^T in
716 which the *erm(41)* gene has been deleted by double homologous recombination using the suicide-
717 vector pUX1-*katG*. AMK was included as a non-related control drug.

718
719 **Figure 3. *In vivo* induction of macrolide resistance in *M. abscessus*-infected zebrafish**
720 **embryos. (A)** Schematic representation of the pMV261_P₁₂₃_tdTomato used to transform *M.*
721 *abscessus* smooth CIP104536^T, where P₁₂₃ represents the full-length promoter region of *erm(41)*.
722 **(B)** Bacteria with no plasmid (WT), harboring either the pTEC27 which constitutively expresses the
723 red fluorescent protein tdTomato, or the pMV261_P₁₂₃_tdTomato for monitoring macrolide
724 inducible expression of tdTomato were plated on 7H10^{OADC} supplemented or not with 2 µg/ml CLR
725 or AZM. Plates were incubated at 37°C for 5 days and observed under a fluorescence
726 microscope. Scale bars represent 0.200 µm. **(C)** Macrolide inducible resistance by clarithromycin
727 and azithromycin occurs *in vivo*. Tg(*mpeg1:GFP-CAAX*) zebrafish embryos, harboring green
728 fluorescent macrophages were injected in the caudal vein with either 900-1000 CFU of the three
729 *M. abscessus* strains described in (B). At 24 hpi, embryos were exposed to osmotic water
730 supplemented with 32 µg/ml CLR, AZM or AMK (day 0 of treatment) and fluorescence was
731 monitored at 2- and 6-days post-treatment. Representative confocal live imaging. White arrows
732 indicate red fluorescent bacteria.

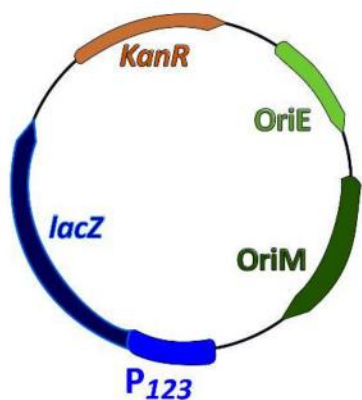
733
734 **Figure 4. Kinetic of macrolide-induced expression of *erm(41)* using a *lacZ* reporter strains.**
735 **(A)** Schematic representation of the pMV261_P₁₂₃_lacZ used to transform the *M. abscessus*
736 CIP104536^T rough variant. **(B)** Schematic protocol of the β-Gal assay to evaluate the kinetic of
737 macrolide-induced resistance. **(C)** The *M. abscessus* strain carrying the pMV261_P₁₂₃_lacZ was
738 first exposed to various concentrations of CLR and AZM for 14 days. Antibiotics were then
739 removed by washing the bacterial pellets with times with PBS prior to β-Gal activity measurements

A**B**

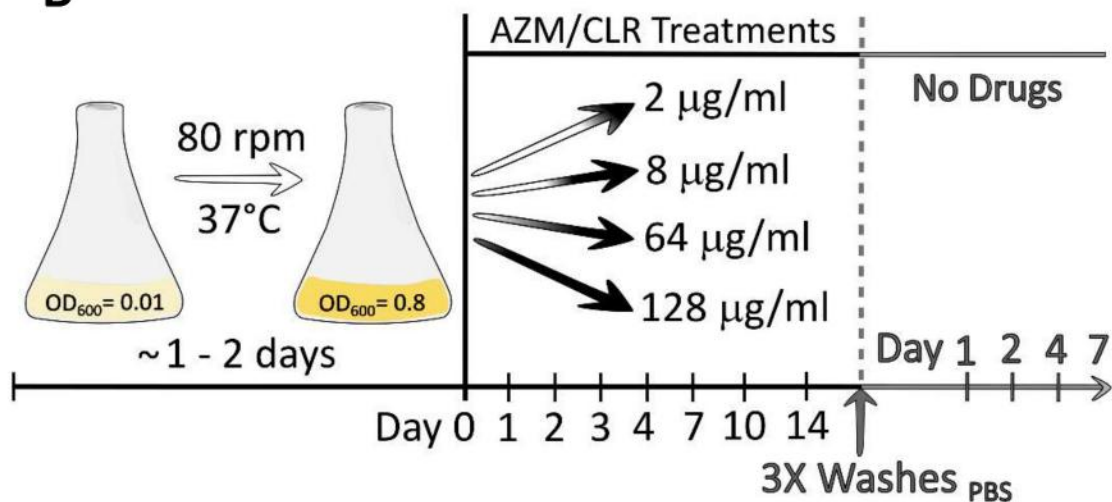


A**B****C**

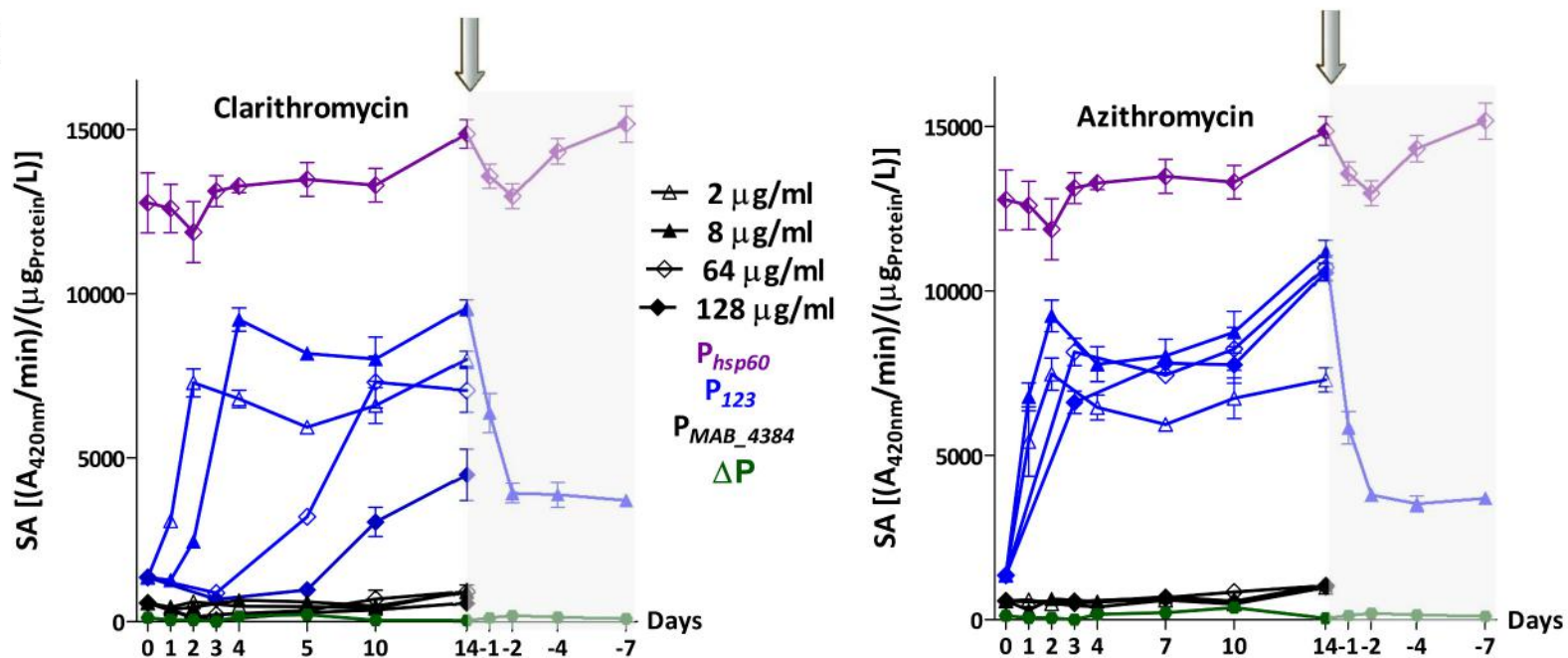
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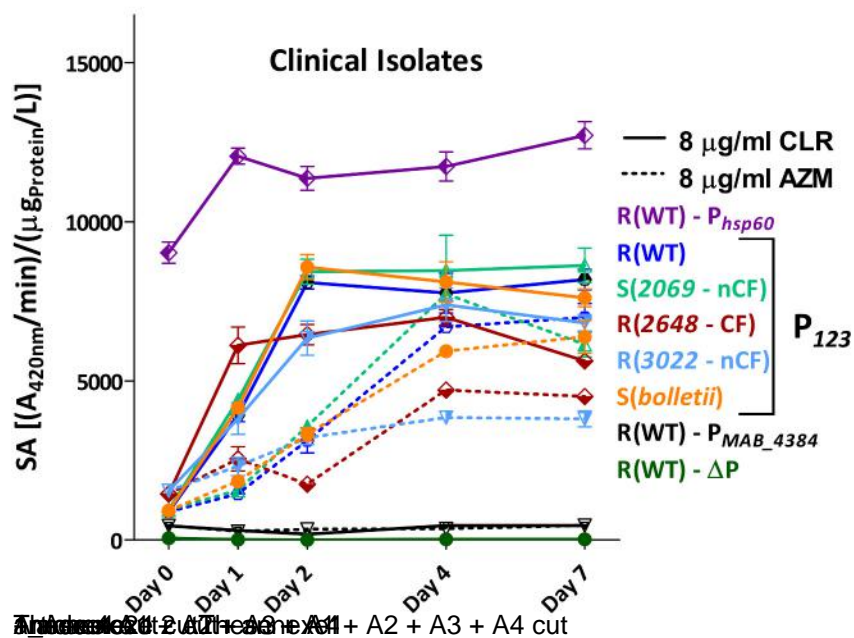
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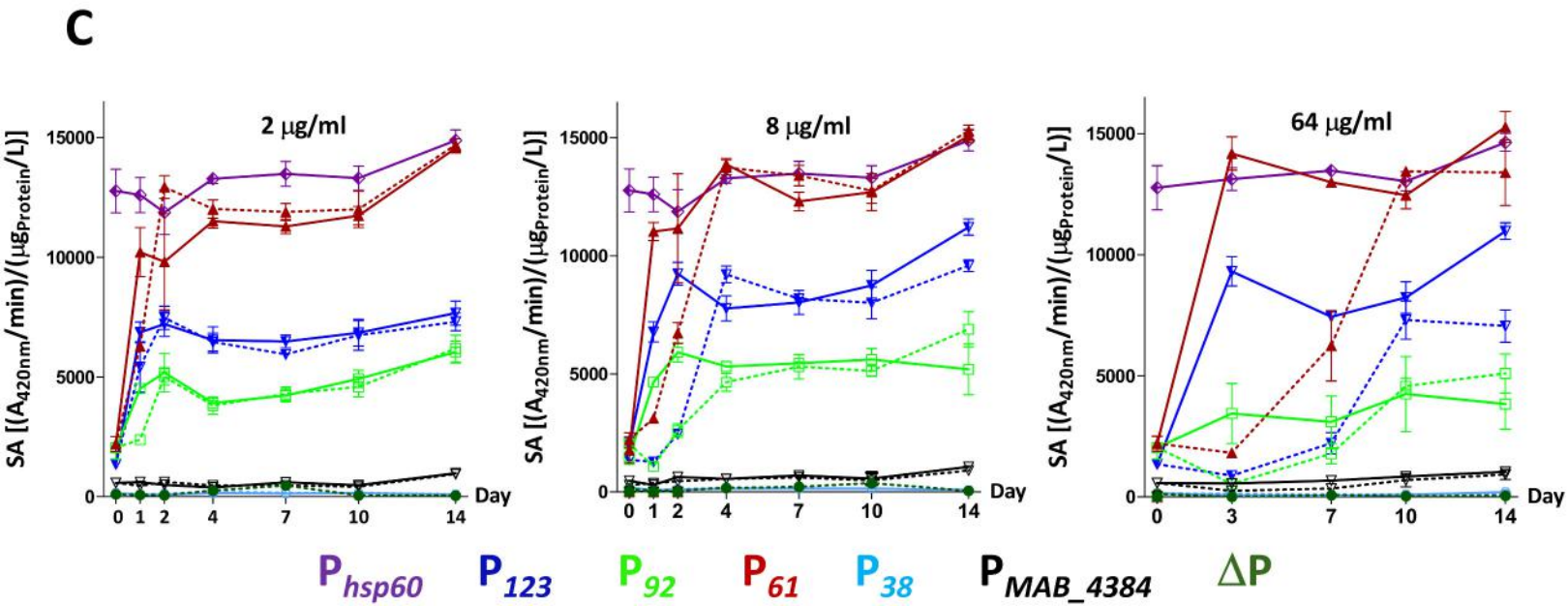
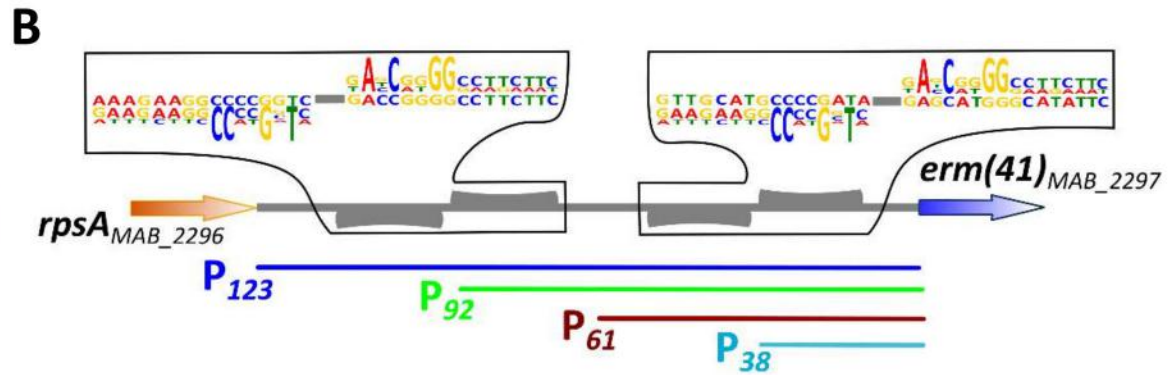
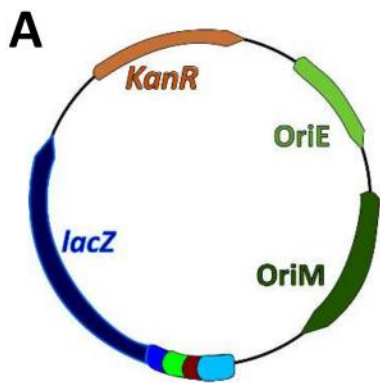
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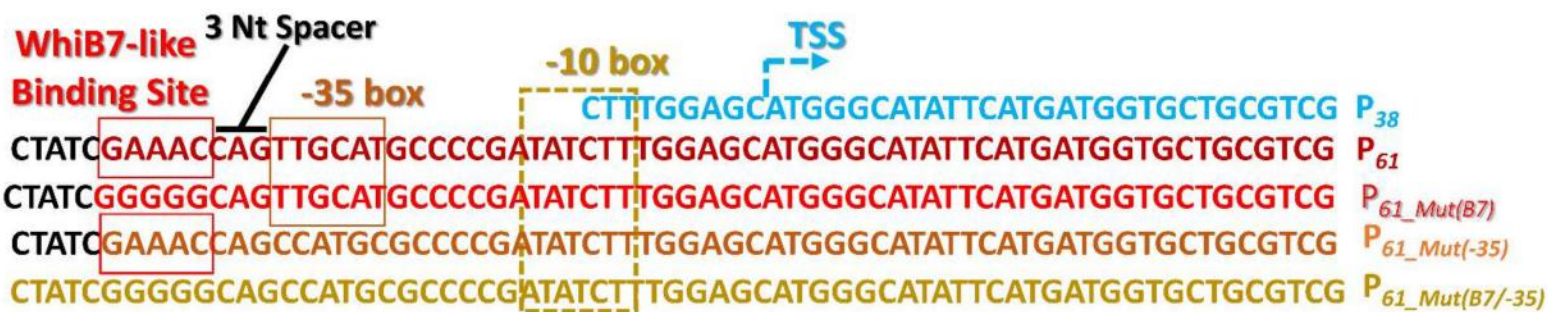
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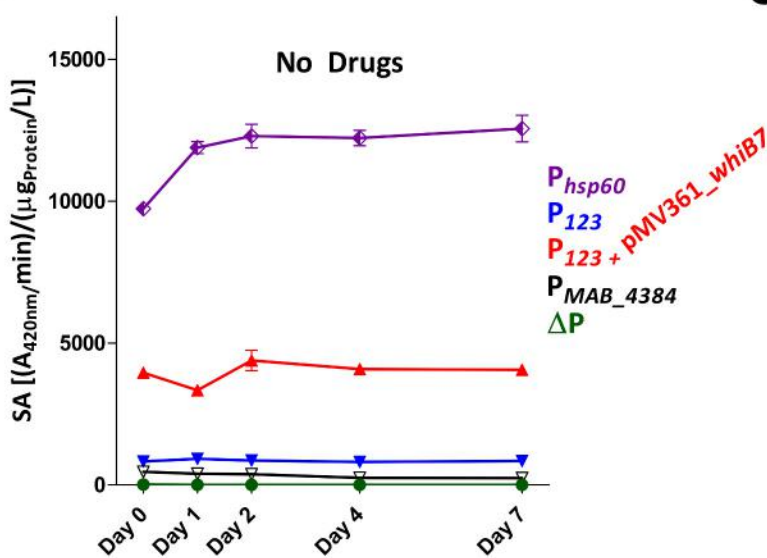
Antibiotic resistance in *A. baumannii* + A2 + A3 + A4 cut



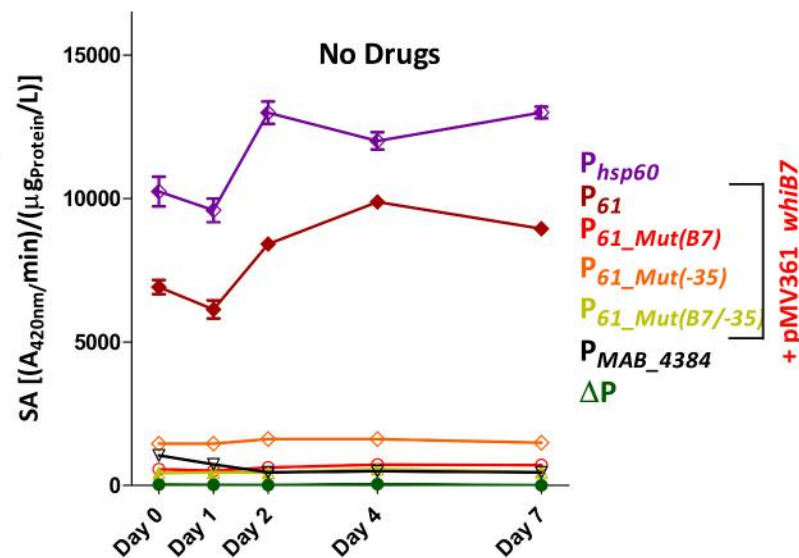
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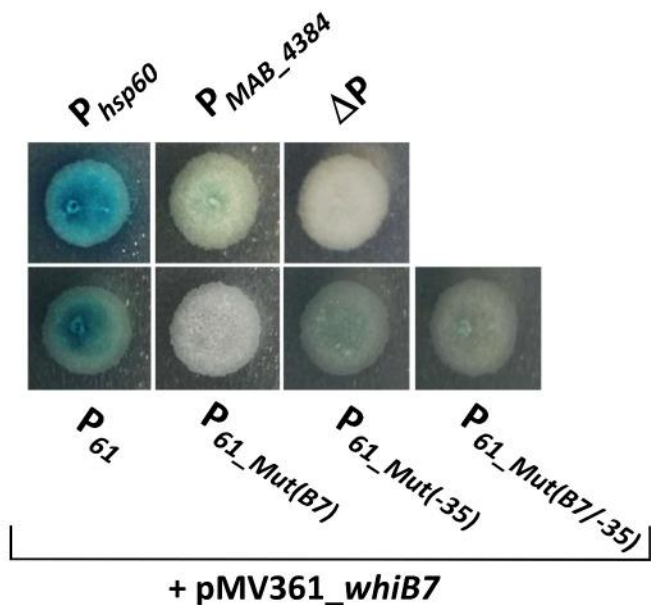
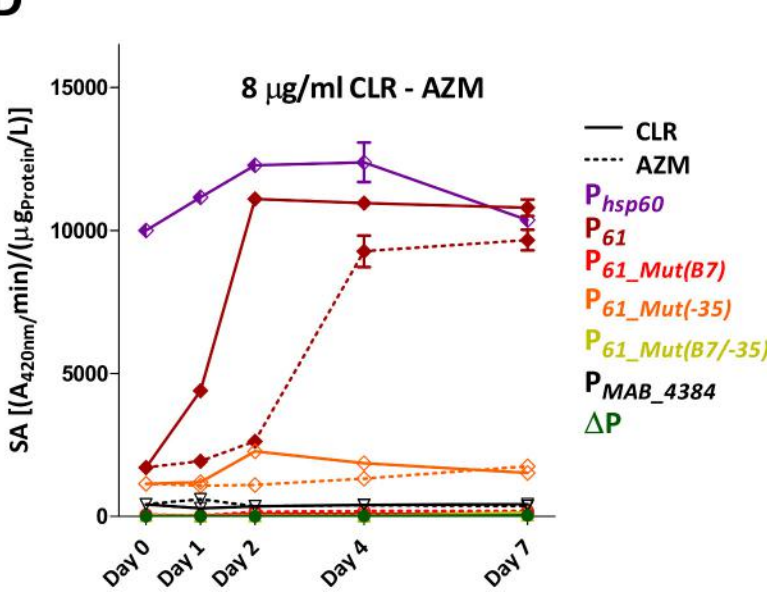
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Discussion

L'apparition et l'expansion de résistances à la plupart des classes d'antibiotiques n'ont cessé d'accroître la morbidité et la mortalité causées par les infections bactériennes. Certaines autorités sanitaires comme l'OMS (Organisation Mondiale de la Santé) ou le PHE (Public Health England) estiment que, d'ici 2050, le nombre global de décès lié à des infections causées par des bactéries multi-résistantes sera de 10 millions chaque année (PHE 2015). Ce constat s'applique également aux mycobactéries. Concernant *M. tuberculosis* par exemple, la moitié des 580 000 personnes souffrant de tuberculose multi-résistante ont trouvé la mort en 2017 (WHO 2017). Tout comme *M. tuberculosis*, certaines MNT provoquent également de plus en plus d'infections multi-résistantes, comme par exemple *M. avium* chez les personnes séropositives aux VIH (Daley 2017). Des observations alarmantes concernent aussi *M. abscessus*. Bryant et al. ont mis en évidence la transmission à l'échelle mondiale d'un clone dominant de *M. abscessus* avec une virulence accrue dans les modèles macrophagique et murin. L'émergence de ce clone dans les centres hospitaliers pourrait sérieusement engager le pronostic des patients infectés et souffrant de mucoviscidose. L'apparition de résistance est en soit un phénomène naturel chez une bactérie. Cependant, une étude récente souligne la plasticité du génome de *M. abscessus*, permettant une évolution rapide et une acquisition aisée de matériel génétique exogène (Choo et al. 2014). Ceci suggère que cette bactérie peut potentiellement hériter de nouveaux facteurs de virulences et/ou de gènes de résistance aux antibiotiques. Deux exemples faisant références à ces transferts génétiques horizontaux sont *MAB_0555* et *MAB_3593* (Ripoll et al. 2009). *MAB_0555* code pour la Phospholipase C, une enzyme capable de moduler des voies signalétiques de l'hôte favorisant la survie intracellulaire et capable aussi de lyser les cellules eucaryotes ainsi que leurs sécrétions (Schmiel et Miller 1999). *MAB_0855* pourrait jouer un rôle dans la dégradation du mucus et l'établissement d'une infection aigüe chez les personnes saines comme celles atteintes de mucoviscidose (Bakala N'Goma et al. 2015). *MAB_3593* code pour la protéine MgtC, décrite pour la première fois chez *Salmonella typhimurium* mais présente chez de nombreux pathogènes intracellulaires (Moncrief et Maguire 1998). Cette protéine fait partie d'un système d'import d'ion Mg^{2+} au sein du phagosome favorisant la survie intramacrophagique des bactéries (Alix et Blanc-Potard 2007). Ces deux gènes non-mycobactériens ne sont pas répandus chez les MNT et offrent à *M. abscessus* un avantage en termes de virulence.

Les travaux de cette thèse ont montré que le génome de *M. abscessus* renferme l'information génétique jouant un rôle déterminant dans son antibiorésistance intrinsèque, en l'occurrence des gènes qui codent les pompes à efflux de type MmpL. Ce génome est également très riche en régulateur de la famille TetR et près de la moitié des MmpL (13/31) sont couplés à ce type de répresseur, offrant un éventuel contrôle de l'expression des protéines MmpL. Ces transporteurs participent indirectement à l'antibiorésistance en exportant à travers la membrane plasmique des lipides qui confèrent à la paroi

mycobactérienne son extrême hydrophobicité (Viljoen et al. 2017). Cette propriété biochimique empêche l'entrée d'un grand nombre de molécules hydrophiles et de certains antibiotiques. Ces mêmes MmpL peuvent aussi exporter des antibiotiques ayant pénétrés la bactérie et deviennent alors des acteurs à part entière du résistome de *M. abscessus*.

Partie I – Régulation

C'est dans le but d'identifier des pompes à efflux participant à la résistance aux antibiotiques que nous avons initié trois études combinant à la fois des approches biochimiques et génétiques. Ainsi, ces stratégies nous ont permis d'identifier le TetR MAB_4384 contrôlant la pompe à efflux MAB_4383c/MAB_4382c impliquée dans de hauts niveaux de résistance aux TACa. Nous avons également identifié MAB_2299c comme étant un TetR contrôlant l'expression de deux paires MmpS/MmpL distinctes et indépendantes, MAB_1135c/MAB_1134c et MAB_2300/MAB_2301, lesquelles participent dans la résistance croisée à la BDQ et à la CFZ. Afin d'identifier ces MmpL, nous nous sommes basés sur la capacité de *M. abscessus* à acquérir des mutations chromosomiques capables de conférer une résistance. Plus particulièrement, des mutations situées dans des gènes codant des régulateurs transcriptionnels qui induiront une perte de leur fonction de répresseur et devenant incapables de se lier à leurs ADN cibles. Les mutations étudiées dans le cas présent englobent des substitutions d'acides aminés impliquées soit dans la reconnaissance de la séquence palindromique du gène cible (dans le domaine HTH, D14N et F57L pour MAB_4384 et L40W pour MAB_2299c) soit dans l'organisation structurale de la protéine (dans le domaine C-terminal, L151P et G215W pour MAB_2299c). Ces altérations conduisent à une surexpression du régulome, et dans ce cas précis des paires MmpS/MmpL.

La structure cristallographique de MAB_4384 et les prédictions des structures secondaires de MAB_2299c ont révélé que leurs domaines HTH sortent de l'ordinaire. En effet, les hélices h1 à h3 sont composées de 56 et 58 acides aminés pour MAB_4384 et MAB_2299c, respectivement. Une autre particularité de MAB_4384, de MAB_2299c et d'EthR (impliqué dans la résistance de *M. tuberculosis* à l'ETH) réside dans la longueur de leur hélice h1. En moyenne, les HTH comprennent 41 résidus, les h1 sont constituées de 11 aminoacides et les TetR reconnaissent des ADN_{op} de 15 pb (Yu et al. 2010). MAB_4384 (h1 = 21 résidus) reconnaît un palindrome de 27 pb mais requiert des nucléotides supplémentaires aspécifiques de part et d'autre de son DNA_{op} pour une fixation optimale. Le motif palindromique atypique d'EthR (h1 = 25 résidus) mesure 35 pb mais le *footprinting* de sa région intergénique révèle que ce TetR recouvre en réalité une séquence de 55 pb (Engohang-Ndong et al. 2004) (Figure 35B). Le motif reconnu en amont de MAB_2300/2301 par MAB_2299c (h1 = 22-25) est composé d'un couple de palindrome de 7 pb séparé par un long *spacer* de 39 nucléotides menant à un

surexprimé dans un milieu mimétique recréant les conditions nutritives du mucus d'une personne atteinte de mucoviscidose, le milieu SCFM (Miranda-CasoLuengo et al. 2016). La forte surexpression de MAB_4383c/4382c dans le milieu SCFM pourrait augmenter le taux de croissance et la capacité à former des biofilms. Si ces caractéristiques phénotypiques sont en effet dépendantes de l'environnement, la surexpression de MAB_4383c/4382c pourrait conférer un avantage en termes de virulence dans le mucus pulmonaire pathologique. Dans ce contexte, MAB_4384 pourrait représenter un marqueur de résistance et/ou de sévérité de l'infection utilisable en diagnostic clinique. De la même façon, en présence d'un isolat clinique résistant à la CFZ et/ou à la BDQ, MAB_2299c est un marqueur de résistance à prendre en considération. Cependant, contrairement aux mutants MAB_4384, le variant Δ MAB_2299c possède un léger défaut de survie au sein du macrophage selon un mécanisme qui reste à définir. Un des scénarios possibles est que la surexpression de MAB_1135c/1134c et de MAB_2300/2301 mène à l'export trop important d'une molécule ou d'un lipide qui se retrouverait piégé à la surface de la paroi bactérienne interagissant avec la cellule hôte. La fonction de modulation normale de la réponse immunitaire exercée par les GPL serait altérée. Il est également possible que MAB_2299c régule d'autres gènes que ces paires *mmpS/mmpL* et que ces derniers participent directement dans la survie intracellulaire de *M. abscessus*. L'utilisation du gène rapporteur *lacZ* ainsi que d'une nouvelle technique de délétion génétique non-marquée nous ont permis de découvrir que ces couples *MmpS/MmpL* sont responsables de résistances basales chez *M. abscessus*.

La régulation, l'intensité et la spécificité de la résistance induite par les macrolides chez *M. abscessus* ont été investigués *in vitro* et *in vivo* à l'aide d'autres souches rapportrices. Ce phénomène nuit grandement à la prise en charge des patients et la sensibilité aux macrolides est ainsi un facteur déterminant dans le pronostic en faveur d'une guérison. Une caractéristique frappante est que le pourcentage de succès thérapeutique est étroitement lié à la proportion d'infections causées par la sous-espèce *M. massiliense* pour laquelle Erm(41) est non-fonctionnelle (Won-Jung Koh et al. 2011) (J. Park et al. 2017). Cette corrélation fait de cette enzyme une cible thérapeutique particulièrement attractive. Notre étude a permis de réfuter les conclusions de travaux antérieurs indiquant que l'AZM n'est pas un inducteur de la résistance médiée par Erm(41). L'AZM semble en réalité déclencher une résistance plus rapide et plus intense que la CLR. L'activité de *lacZ* sous le contrôle du promoteur d'*erm(41)* complet et tronqués en plusieurs segments a révélé que WhiB7 exerce une régulation complexe et atypique. L'architecture du motif de liaison de WhiB7 sur le promoteur d'*erm(41)* de *M. abscessus* est unique. Chez d'autres mycobactéries, les motifs de reconnaissance de WhiB7 pour son propre gène et pour *erm(41)* semblent beaucoup plus conservés. D'après les distances entre les boîtes -35 et -10, ces autres protéines WhiB7 interagiraient avec le facteur de transcription végétatif SigA. Cet écart est beaucoup plus faible chez *M. abscessus* et ne correspond à aucune distance typique de

nouveaux dérivés de ces inhibiteurs. Ces co-structures protéine/inhibiteur permettent l'optimisation de composés en fonction de leurs structures chimiques et de leurs activités biologiques. Ces inhibiteurs de MmpL3 sont bactéricides *in vitro* et réduisent la charge bactérienne *ex vivo* dans le macrophage. PIPD1 réduit significativement la mortalité des embryons de zebrafish infectés par *M. abscessus*, les symptômes liés à l'infection ainsi que la charge bactérienne. Néanmoins, d'autres études sont nécessaires pour intégrer ces molécules dans une multi-thérapie excluant les macrolides et les aminoglycosides. En effet, l'IPM et la CFX ont aussi un effet bactéricide *in vitro* mais sont peu efficaces en monothérapie. Une combinaison d'antibiotiques optimale comprendrait des molécules : (i) ciblant des enzymes ou des compartiments subcellulaires distincts, (ii) qui ne soient pas inactivées des enzymes du régulome de WhiB7. Une trithérapie inhibiteur de MmpL3 – MOX – TGC/LNZ s'avérerait intéressante à étudier, car même si les tétracyclines et le LNZ induisent l'expression de *whiB7*, aucune de ces molécules n'est *a priori* sensibles à une enzyme activée par WhiB7. Un antibiotique visant plusieurs MmpL autre que MmpL3 ciblerait des voies métaboliques non-essentiels mais requises pour une infection efficace ou un export basal d'antibiotique faisant de cette molécule un pharmacophore très efficace. Les acteurs du métabolisme dérivant des acides mycoliques transportés par MmpL3 sont aussi de bonnes cibles antibiotiques. Ainsi, le complexe Antigène 85 est un complexe enzymatique particulièrement attractif non seulement en tant que cible thérapeutique mais également pour le développement de vaccin et de nouveaux tests diagnostiques (Babaki et al 2017) . Il catalyse la formation du TDM et la mycolylation de l'arabinogalactane (Belisle et al. 1997). Dans ce contexte, l'article en **Annexe 2** rapporte la caractérisation d'une nouvelle activité enzymatique pour trois des membres de ce complexe ainsi que de leurs inhibitions *in vitro* et *in vivo* par des analogues de cyclipostines et cyclophostines chez *M. tuberculosis* (Viljoen, Richard et al. 2018). Cette étude est étayée par des travaux plus récents démontrant que les cyclipostines et cyclophostines sont des inhibiteurs très efficaces contre *M. abscessus* (Madani et al. 2019). Ces composés sont prometteurs car ils ciblent un résidu ultra-conservé du site actif des trois enzymes, diminuant de manière considérable les chances d'apparition d'une résistance croisée. La liaison covalente établie avec la sérine du site catalytique est irréversible et ne pourrait donc pas être détruite par une enzyme modifiant la cible d'antibiotiques, comme Erm(41).

L'utilisation de kétolides au lieu de la CLR et l'AZM pourrait permettre de palier à la résistance inductible aux macrolides. L'une des particularités des kétolides est l'absence de cladinose dans leurs structures. Ce sucre est remplacé par un groupement cétone, ce qui fait normalement de ces macrolides des faibles inducteurs des résistances médiées par des méthyltransférases (Douthwaite 2001). Malheureusement cette particularité ne semble pas se vérifier chez *M. abscessus*. Nash et al. ont montré une forte augmentation des niveaux de transcrits d'*erm(41)* suite à une exposition à la

télithromycine, le seul kétolide actuellement approuvé pour un usage clinique. Une autre particularité des kétolides est la présence d'un groupement carbamate permettant à ces molécules d'interagir avec d'autres nucléotides de l'ARNr 23S comme la paire A752 – U2609 (Chellat et al. 2016). Un nouveau kétolide est en cours d'essai clinique pour le traitement de pneumopathies communautaires bactériennes, la solithromycine (Zhanel et al. 2016). Une de ces particularité est qu'un atome de fluor est présent en position 2 de son cycle lactone lui offrant un troisième site de liaison à l'ARNr 23S (Fernandes et al. 2016). Comme la télithromycine, la solithromycine pourrait induire l'expression d'Erm(41) mais son troisième site de fixation ribosomique pourrait lui procurer une meilleure pharmacocinétique et un pouvoir antimicrobien plus prononcé. Néanmoins, on ne peut exclure la possibilité qu'Erm(41) ne soit capable de modifier d'autres nucléotides de l'ARNr 23S. En l'absence de donnée cristallographique et d'un test d'activité enzymatique, une manière de répondre à cette question consisterait à générer des mutants résistants spontanés contre la télithromycine et la solithromycine chez une souche de *M. abscessus* Δ *erm(41)* ou chez *M. massiliense* soumis à des doses croissantes d'antibiotique et de séquencer leur génome. (Martinez et Baquero 2000). La découverte de nouveaux dérivés de macrolide n'induisant pas Erm(41) représenterait une avancée majeure dans la thérapie contre *M. abscessus* mais aussi contre d'autres MNT possédant un système de résistance inductible aux macrolides. La recherche de telles molécules peut s'avérer longue et fastidieuse en l'absence d'outils appropriés. Dans ce contexte, la souche rapportrice exprimant le marqueur de fluorescence *tdTomato* sous le contrôle du promoteur d'*erm(41)* pourrait être décisive dans le criblage de ce type d'antibiotiques. En effet, il est techniquement possible de coupler la détermination de la CMI en plaque 96 puits en respectant les directives du CLSI avec une mesure de l'émission de fluorescence due à l'activation transcriptionnelle d'*erm(41)* reflétée par l'expression de *tdTomato*. Ainsi, il apparait tout à fait envisageable d'évaluer simultanément le pouvoir bactéricide et l'incapacité à déclencher le système *erm(41)* de molécules en criblant une banque de dérivés de macrolides en un temps réduit.

Une autre alternative au développement de nouveaux antibiotiques serait de générer des *boosters* qui augmenteraient l'activité de ces antibiotiques en déstabilisant les mécanismes de résistances intrinsèques et inductibles. Certains composés de ce type ont déjà été testés sur des MmpL *in vitro* et *in vivo*. Ces molécules seules n'ont pas ou peu d'effet antimicrobien mais sont capables d'améliorer fortement l'activité de certains antibiotiques, parmi lesquels font partis la réserpine, le CCCP (CarbonylCyanure m-ChloroPhénylhydrazone) et le vérapamil (VPM). La réserpine est utilisée pour traiter l'hypertension ou certains troubles psychiatriques et comme le VPM, est utilisée dans le traitement de l'hypertension. Ces molécules perturbent le potentiel de membrane en inhibant les canaux calciques. Le CCCP est un protonophore qui va directement cibler la force proto-motrice en

combinaison avec un antibiotique très sensible à WhiB7 comme l'AZM. Ces antibiotiques très inducteurs permettent un fort contraste lorsque l'inhibiteur est actif contre WhiB7. Ces inhibiteurs de WhiB7 peuvent présenter *a minima* deux mécanismes d'action : (i) un contact direct avec WhiB7, (ii) un contact avec la région promotrice du régulome. La région entre les domaines fer – soufre et l'AT-Hook est conservée et relativement unique aux protéines WhiB et serait alors un bon candidat. La protéine WhiBTM4 produite par le mycobactériophage TM4 est très proche structurellement de WhiB2 de *M. tuberculosis*. Elle se lie au motif reconnu par WhiB2 et de ce fait inhibe sa transcription et celles des gènes de son régulome (Rybniker et al. 2010). Imiter ce bactériophage en produisant une protéine recombinante structurellement proche de WhiB7 permettrait d'inhiber sa transcription et celle de son régulome. Un groupe brésilien a récemment rapporté *via* deux études la découverte de nouveaux inhibiteurs de pompes à efflux dérivés de tétrahydropyridines (Vianna et al. 2019a) (Vianna et al. 2019b). Ces molécules bloquent effectivement l'efflux chez *M. abscessus* mais les baisses des CMI de la CLR et d'AMK en combinaison avec leurs composés laissent à penser que ces molécules pourraient en fait inhiber WhiB7. En effet, la résistance à ces deux composés est majoritairement due à Eis2 et Erm(41), l'efflux d'AMK par MAB_{tap} étant un mécanisme de résistance basale de faible intensité et celui de la CLR n'est pas prouvé expérimentalement. Nos lignées rapportrices représenteraient une application rapide afin d'en avoir le cœur net.

La découverte de nouvelles molécules qu'elles soient des antibiotiques ou des inhibiteurs de facteurs de résistance n'est probablement pas à elle seule la solution contre *M. abscessus* et à l'émergence d'épidémies en milieu hospitalier. Un nouveau concept apparu ces dernières années fait permet une prise de recul entre de la résistance antibiotique et la persistance antibiotique (Balaban et al. 2019). Ce dernier phénomène n'est pas pleinement appréhendé mais il pourrait être responsable de récives lors d'un traitement à *M. abscessus*. La persistance antibiotique est caractérisée principalement par la présence d'une sous-population au sein d'un même isolat qui n'est pas tuée à la même vitesse par le même antibiotique que le reste de la population. Ceci s'oppose à la tolérance qui est caractérisée par un changement métabolique de toute la population bactérienne menant à une résistance temporaire ou à un retard dans l'élimination par un antibiotique. Il est possible qu'au terme d'une infection à *M. abscessus*, cet infime nombre de bactéries persistantes soit toujours présent mais non-délectable. Le traitement sera alors arrêté en l'absence de symptômes ou de preuves microbiologique de la présence de ces bactéries. Ces « *persistors* » perdureront pour finalement à nouveau se multiplier et générer une rechute. Ces récives sont communes et très problématiques lorsqu'elles se produisent, notamment chez une personne atteinte de mucoviscidose et/ou ayant subi une greffe pulmonaire (Smibert et al. 2016) (Osmani et al. 2018). Un suivi de ces patients et

l'élaboration d'une thérapie supportable à long terme sont primordiaux pour anticiper ces réinfections récalcitrantes.

En conclusion, les travaux de cette thèse ont fourni des informations inédites concernant les mécanismes de résistance intrinsèques à deux molécules actuellement évaluées cliniquement, la BDQ et la CFZ, et sur la résistance inductible aux macrolides. Ces études proposent également de nouveaux marqueurs de résistance ainsi que des outils génétiques originaux pour étudier la virulence/résistance de *M. abscessus*. La voie semble toute tracée pour le développement de méthodes de criblage à haut débit de nouveaux macrolides et de molécules bloquant les mécanismes de la multi-résistance intrinsèque de *M. abscessus*.

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Targeting Mycolic Acid Transport by Indole-2-carboxamides for the Treatment of *Mycobacterium abscessus* Infections

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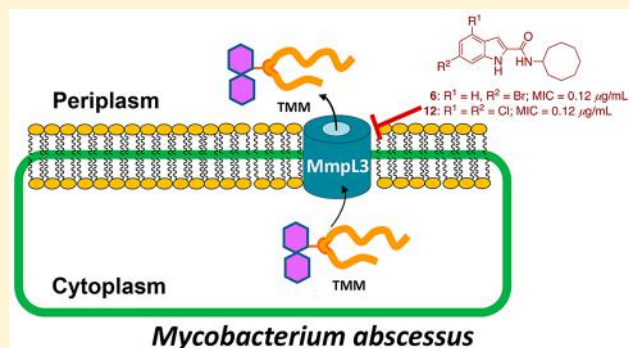
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Supporting Information

ABSTRACT: *Mycobacterium abscessus* is a fast-growing, multi-drug-resistant organism that has emerged as a clinically significant pathogen in cystic fibrosis (CF) patients. The intrinsic resistance of *M. abscessus* to most commonly available antibiotics seriously restricts chemotherapeutic options. Herein, we report the potent activity of a series of indolecarboxamides against *M. abscessus*. The lead compounds, **6** and **12**, exhibited strong activity in vitro against a wide panel of *M. abscessus* isolates and in infected macrophages. High resistance levels to the indolecarboxamides appear to be associated with an A309P mutation in the mycolic acid transporter MmpL3. Biochemical analyses demonstrated that while de novo mycolic acid synthesis remained unaffected, the indolecarboxamides strongly inhibited the transport of trehalose monomycolate, resulting in the loss of trehalose dimycolate production and abrogating mycolylation of arabinogalactan. Our data introduce a hereto unexploited chemical structure class active against *M. abscessus* infections with promising translational development possibilities for the treatment of CF patients.



INTRODUCTION

Fighting multidrug-resistant bacteria requires repurposing of existing drugs or the development of new more rapidly acting and cost-effective antibiotics. Chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) are associated with unresolved therapeutic needs resulting from severe and even fatal infections by multidrug-resistant bacteria. Lung infections in CF patients represent the most frequent and most serious

manifestations because they are responsible for more than 90% of CF patient deaths.¹ In the context of CF and COPD, *Mycobacterium abscessus*, a fast-growing mycobacterial species, has emerged in recent years as an important opportunistic pathogen increasingly responsible for mortality.² The incidence

Received: April 14, 2017

Published: June 2, 2017

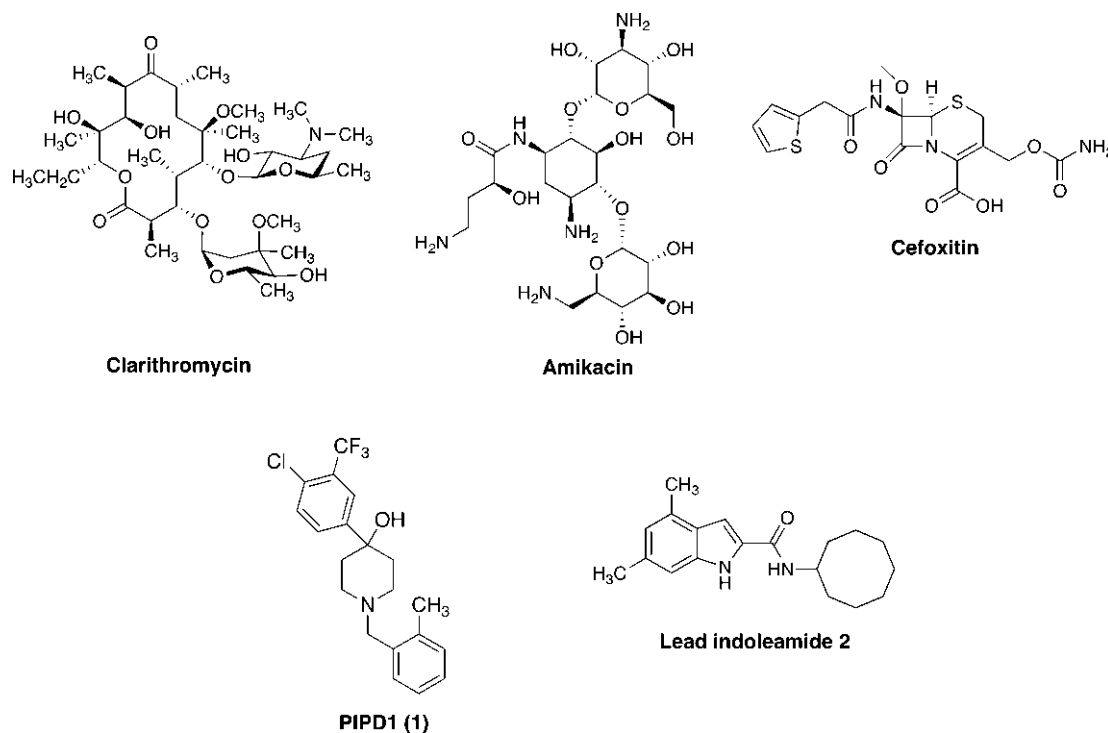


Figure 1. Structures of clarithromycin, amikacin, cefoxitin, PIPD1 (1), and our lead indolecarboxamide 2.

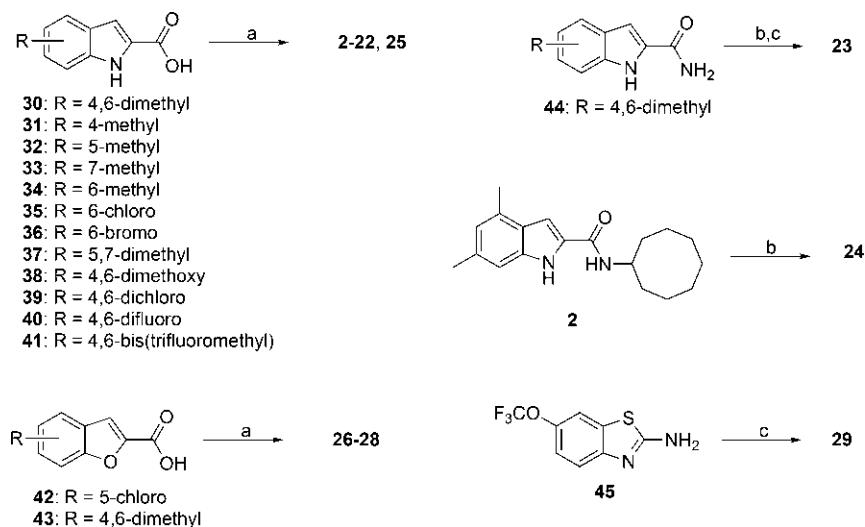
of *M. abscessus* infection in the CF population rises, suggesting a potential link between a genetic defect in the cystic fibrosis transmembrane conductance regulator (CFTR) and *M. abscessus* lung infection. In this population, *M. abscessus* accelerates inflammatory lung damage, leading to increased morbidity and mortality.³ A recent genomic study of a global collection of clinical isolates indicated that the majority of *M. abscessus* infections are acquired through aerosol transmission of recently emerged dominant circulating clones.⁴

Although a rapid grower, *M. abscessus* shares important physiopathologic features with pathogenic slow-growing mycobacteria such as the ability to persist silently for years and even decades⁵ in the human host and to induce lung disease associated with caseous lesions and granuloma formation in lung parenchyma.^{6,7} Infections caused by *M. abscessus* are difficult to treat and to eradicate because *M. abscessus* is naturally resistant to most antibiotics, including nearly all current antitubercular drugs.⁸ The recommended treatment for pulmonary infections includes a combination of a macrolide (clarithromycin or azithromycin), an aminoglycoside (amikacin), and an intravenous β -lactam (cefepime or imipenem) to be taken for at least 12 months^{9,10} (Figure 1). The cure rate is estimated to only 25–40% in the case of resistance to macrolides, which appears in 40–60% of the isolates.¹¹ In addition, unsuccessful eradication of *M. abscessus* represents a contraindication for lung transplantation by several CF centers, leaving patients without therapeutic options. Therefore, more specific, active, and at the same time less toxic antimicrobial agents are urgently needed.

Despite being a formidable respiratory mycobacterial pathogen, little is known with respect to the molecular and cellular mechanisms leading to immunopathogenesis and infection. However, recent genomic data highlighted the considerable sequence similarity between *Mycobacterium tuberculosis* and *M. abscessus*,^{12,13} suggesting that these species may

share common biochemical pathways. From this, it can be inferred that compounds inhibiting biosynthetic pathways in *M. tuberculosis* may also be active against *M. abscessus* and that already existing data generated during previous tuberculosis (TB) drug discovery programs would be useful to identify new chemotypes with strong activity against *M. abscessus*, an approach that would obviate the need to initiate chemical screens de novo. Indeed, we have recently validated this approach and undertaken an *M. abscessus* “cross-screen” with a confirmed chemical series arising from a known set of potent nontoxic antitubercular hits.¹⁴ This led to the discovery of a new piperidinol-based compound 1 (PIP1) (Figure 1), showing selective activity against *M. abscessus* both in vitro and in vivo.¹⁵ This success prompted us to further explore this strategy by testing a small library of indole-2-carboxamide derivatives, previously shown to exhibit strong activity against *M. tuberculosis*.^{16–18}

In the recent past, we have designed a series of indolecarboxamides with potent activity against both drug-susceptible and drug-resistant strains of *M. tuberculosis* by targeting the putative mycolic acid transporter MmpL3.¹⁸ We identified a single point mutation in MmpL3, which confers high resistance to the indolecarboxamide class while remaining susceptible to currently used first- and second-line TB drugs, signifying a lack of cross-resistance. The lead compound 2 (Figure 1) was found to be active against *M. abscessus*, thus prompting further investigation of other indolecarboxamide analogues. The bioavailability of the indolecarboxamides, combined with their ability to kill tubercle bacilli as well as nontubercular mycobacteria (NTM), indicates great potential for the translational development of this structural class for the treatment of *M. abscessus* infections in CF patients. Herein, we describe SAR studies of a series of indolecarboxamide analogues against *M. abscessus* and provide biological evidence

Scheme 1. Synthesis of Compounds 2–29^a

^aReagents and conditions: (a) EDC·HCl, corresponding amines, Et₃N, CH₂Cl₂, rt, 12–16 h; (b) LiAlH₄, THF, reflux, overnight; (c) cycloheptanecarbonyl chloride, CH₂Cl₂, rt, overnight.

that they are working through inhibition of mycolic acid transport.

RESULTS

The synthesis of compounds 2–29 was carried out by following procedures reported previously.^{16–18} Briefly, starting from the appropriate indole-2-carboxylic acid, a standard amide coupling protocol (Scheme 1) was performed to furnish the amides 2–23 and 25–28. Compound 24 was obtained by LiAlH₄ reduction of the amide 2, whereas compound 29 was prepared by coupling intermediate 45 with cycloheptanecarbonyl chloride.

Structure–Activity Relationship of New Indole-2-carboxamides against *M. abscessus*. The indoles 2–29 were initially screened in vitro against the *M. tuberculosis*^{16,17} and *M. abscessus* strains to obtain their respective MIC values (Table 1). The lead compound 2, with a cyclooctyl group, displayed excellent activity against both *M. tuberculosis* and *M. abscessus*. Presence of only a single methyl at positions 4, 5, or 6 of the indole ring (compounds 3–5) resulted in a 2-fold decrease in activity against *M. abscessus* in comparison to 2. A similar trend in activity was observed when these compounds were screened against *M. tuberculosis*,¹⁶ thus indicating that the presence of two nonpolar substituents in the indole ring helps to improve activity. Likewise, compound 6 that possesses a lipophilic bromine atom at position 6 displayed a 2-fold increase in activity when compared to its 6-methyl counterpart, compound 5. Compound 7, bearing an *N*-adamantyl group, was nearly 130-fold less active than compounds 6 and 8. These findings further support the notion that the activity of these compounds is driven by their lipophilicity and that the tolerance for steric bulk at the amide N is fairly high but limited. Additionally, the effect of a single methyl group at position 7, as in compound 9, was evaluated. Compound 9 showed a large loss in activity against *M. tuberculosis* and *M. abscessus* as compared to other monosubstituted compounds with methyl groups at positions 4, 5, or 6 (3–5), indicating that substitution at this position is unfavorable. Next, we investigated the effect of disubstitution at other positions of the indole ring. The 5,7-dimethylindole 10 showed 16- and 2-

fold loss of activity against the *M. tuberculosis* and *M. abscessus* strains, respectively, as compared to its 4,6-dimethyl counterpart 2. In this case, both molecules have equal CLogP values, and the differential activities observed are thus a consequence of the substitution pattern in the indole ring.

After finding that substituents at the 4- and 6-positions of the indole ring were optimal for activity, other 4,6-disubstituted derivatives, such as the 4,6-dimethoxy, 4,6-dichloro, 4,6-difluoro, and 4,6-bis(trifluoro)methyl analogues, were investigated for their activity against *M. abscessus*. Compound 11 bearing the 4,6-dimethoxyindole moiety displayed an 8-fold loss of activity compared to compound 2 (4,6-dimethyl substitution), suggesting that the more polar methoxy substituents are less favorable compared to methyl due to their reduced lipophilicity. Next, we replaced the two methyl groups of 2 with more lipophilic and metabolically stable halogen atoms to afford compounds 12 (4,6-dichloroindole analogue; CLogP = 6.16) and 13 (4,6-difluoroindole analogue; CLogP = 5.02). Both of these compounds showed activities identical (0.12 μg/mL) to that of the lead compound 2 against *M. abscessus*. In the case of *M. tuberculosis*, compound 12 displayed the same activity as did the lead 2, while compound 13 was approximately 8-fold less active than compound 2. Of interest is the fact that while compound 12 is more lipophilic than 13, this property did not enhance its activity in the *M. abscessus* screening assay in contrast to the result that was observed in the case of the *M. tuberculosis* assay. Both compounds 14 and 15 were inactive against *M. abscessus* while retaining activity against *M. tuberculosis*.

Next, the activities of the 4,6-dichloroindole analogues 16–22 bearing different substituents on the amide nitrogen were explored. Compounds 16 (containing a *N*-adamantyl group) and 17 (containing a *N*-(*R*)-(+)-bornyl group) showed poor activities (MIC > 32 μg/mL) against *M. abscessus*. These modifications thus resulted in an at least 256-fold decrease in activity of 16 and 17 as compared to lead compound 2. Introduction of a simple *n*-octyl group or a *trans*-geranyl group on the amide nitrogen yielded the compounds 18 and 19. Again, both of these compounds were found to be inactive (MIC > 32 μg/mL) against *M. abscessus*, suggesting that these

Table 1. MIC Values of Indole-2-carboxamides against *M. tuberculosis* and the *M. abscessus* CIP 104536 Reference Strain, and the Compounds' CLogP Values (Data for Compounds 6 and 12 Are in Bold)

| Compd | X | R | MIC _{M. th.} ^a | MIC _{M. abs.} ^b | IC ₅₀ VERO | SI _{M. th.} ^c | SI _{M. abs.} ^d | CLogP ^e |
|-----------|---------------------|---|------------------------------------|-------------------------------------|-----------------------|-----------------------------------|------------------------------------|--------------------|
| | | | ($\mu\text{g/mL}$) | ($\mu\text{g/mL}$) | ($\mu\text{g/mL}$) | | | |
| 2 | 4,6-dimethyl | | 0.0039 | 0.125 | 16 | 4100 | 128 | 5.59 |
| 3 | 4-methyl | | 0.0313 | 0.25 | | | | 5.10 |
| 4 | 5-methyl | | 0.250 | 0.25 | | | | 5.10 |
| 5 | 6-methyl | | 0.0313 | 0.25 | | | | 5.10 |
| 6 | 6-Br | | 0.0313 | 0.125 | ≥ 64 | ≥ 2050 | ≥ 512 | 5.56 |
| 7 | 6-Br | | 0.0156 | 16.0 | ≥ 64 | ≥ 4100 | ≥ 4 | 5.07 |
| 8 | 6-Br | | 0.0039 | 0.125 | | | | 6.28 |
| 9 | 7-methyl | | 1.00 | 16.0 | | | | 5.10 |
| 10 | 5,7-dimethyl | | 0.0625 | 0.250 | | | | 5.59 |
| 11 | 4,6-dimethoxy | | 0.0625 | 1.00 | | | | 4.57 |
| 12 | 4,6-dichloro | | 0.0039 | 0.125 | 8-16 | 2050-4100 | 64-128 | 6.16 |
| 13 | 4,6-difluoro | | 0.031 | 0.125 | | | | 5.03 |
| 14 | 4,6-dichloro | | 0.0078 | 128 | 16 | 2050 | 0.125 | 6.88 |
| 15 | 4,6-difluoro | | 0.0039 | 32.0 | ≥ 64 | ≥ 16400 | ≥ 2 | 5.74 |
| 16 | 4,6-dichloro | | 0.0039 | >32.0 | ≥ 32 | ≥ 8210 | ≥ 1 | 5.67 |

Table 1. continued

| Compd | X | R | MIC _{M. tb.} ^a ($\mu\text{g/mL}$) | MIC _{M. abs.} ^b ($\mu\text{g/mL}$) | IC ₅₀ VERO ($\mu\text{g/mL}$) | SI _{M. tb.} ^c | SI _{M. abs.} ^d | CLogP ^e |
|-------|--------------------------|---|--|---|---|-----------------------------------|------------------------------------|--------------------|
| 17 | 4,6-dichloro | | 0.0156 | >32.0 | 8 | 513 | 0.25 | 6.88 |
| 18 | 4,6-dichloro | | 128 | >32.0 | | | | 6.72 |
| 19 | 4,6-dichloro | | 4.00 | >32.0 | | | | 6.75 |
| 20 | 4,6-dichloro | | 2.00 | 0.50 | | | | 5.97 |
| 21 | 4,6-dichloro | | 0.250 | >32.0 | | | | 5.00 |
| 22 | 4,6-dichloro | | 0.001953 | >32.0 | ≥ 64 | ≥ 32800 | ≥ 2 | 7.04 |
| 23 | 4,6-dimethyl | | 16.0 | >32.0 | | | | 4.53 |
| 24 | 4,6-dimethyl | | 0.125 | 0.50 | | | | 5.65 |
| 25 | 4,6-bis(trifluoromethyl) | | 0.0156 | 0.50 | | | | 6.64 |
| 26 | 5-chloro | | 8.00 | 0.50 | | | | 5.43 |
| 27 | 4,6-dimethyl | | 8.00 | 0.50 | | | | 5.57 |
| 28 | 4,6-dimethyl | | 1.00 | 1.00 | | | | 5.54 |
| 29 | 5-trifluoromethoxy | | 128 | >32.0 | | | | 5.99 |
| INH | | | 0.04 | | >256 | >6400 | | |
| RIF | | | 0.125 | | >256 | >2050 | | |

^aThe lowest concentration of drug leading to at least a 90% reduction of bacterial growth signal by the microplate AlamarBlue assay (MABA), as previously reported.^{16,17} MIC values are reported as an average of three individual measurements. ^bMIC values ($\mu\text{g/mL}$) were determined using the microdilution method and cation-adjusted Mueller–Hinton broth according to the Clinical and Laboratory Standards Institute guidelines.³⁹ ^cSI = IC₅₀ VERO/MIC_{M. tb.}. ^dSI = IC₅₀ VERO/MIC_{M. abs.}. ^eCalculated using ChemBioDraw Ultra 13.0. INH, isoniazid; RIF, rifampicin.

lipophilic alkyl and alkenyl chains are likely unable to bind to the target site. The analogue **20** bearing an α -branched cyclohexylmethyl group (MIC = 0.5 $\mu\text{g/mL}$) was moderately active, whereas compound **21** bearing the indanyl group proved to be inactive against *M. abscessus*. The spiro compound **22** (CLogP = 7.04) proved to be the most active compound against *M. tuberculosis* (MIC = 0.00098 $\mu\text{g/mL}$) in the in vitro assay; disappointingly, this excellent activity was not duplicated in the *M. abscessus* screening (MIC > 32 $\mu\text{g/mL}$). Furthermore, the reverse amide derivative **23** was inactive against *M. abscessus*, while the amine **24** exhibited a 4-fold drop in activity compared to **2**. Compound **25**, containing a 4,6-bis-

(trifluoromethyl)indole, and the benzofuran derivatives **26–28**¹⁷ showed MIC values between 0.5 and 1.0 $\mu\text{g/mL}$, while the benzothiazole derivative **29** was inactive against the susceptible strain of *M. abscessus*.

Overall, the SAR of these indole-2-carboxamides reveals some parallels in their activity against *M. tuberculosis* and *M. abscessus*, but this is not strictly true for all of the analogues that were studied. In most cases, the indole analogues reported herein are more active against *M. tuberculosis* than against *M. abscessus*. Also, it is apparent that, on the basis of the limited number of compounds studied so far, the cyclooctyl group is preferred for activity against *M. abscessus* and that in a few cases

these cyclooctyl analogues are more active against *M. abscessus* than *M. tuberculosis*. We believe that the present data provide fertile ground to inspire the generation of a host of improved analogues that may show even better activity against *M. abscessus*.

Activity of Compounds 12 and 6 against *M. abscessus* Clinical Isolates. The two lead compounds, 12 and 6, were further evaluated in in vitro and in vivo assays. Both exhibited an MIC of 0.125 $\mu\text{g}/\text{mL}$ against the smooth (S) as well as the rough (R) variants of the reference *M. abscessus* strain CIP104536 (Table 2). Notably, both compounds were similarly

Table 2. MICs ($\mu\text{g}/\text{mL}$) of Compounds 12 and 6 in Cation-Adjusted Mueller–Hinton Broth for 30 Clinical Isolates from CF and Non-CF Patients Belonging to the *M. abscessus* Complex

| strain | morphotype | source | 12 | 6 | AMK ^a |
|---|------------|-------------------|-------|--------|------------------|
| <i>M. abscessus</i> subsp. <i>abscessus</i> | | | | | |
| CIP104536 | R | non-CF | 0.125 | 0.0625 | 12.5 |
| 2524 | R | CF | 0.125 | 0.125 | 25 |
| 2648 | R | CF | 0.125 | 0.125 | 12.5 |
| 3022 | R | non-CF | 0.125 | 0.125 | 12.5 |
| CF | R | CF | 0.125 | 0.125 | 12.5 |
| CIP104536 | S | non-CF | 0.125 | 0.125 | 25 |
| 3321 | S | non-CF | 0.125 | 0.125 | 25 |
| 1298 | S | CF | 0.125 | 0.125 | 12.5 |
| 2587 | S | CF | 0.125 | 0.25 | 25 |
| 2069 | S | non-CF | 0.125 | 0.125 | 25 |
| CF | S | CF | 0.125 | 0.125 | 12.5 |
| <i>M. abscessus</i> subsp. <i>massiliense</i> | | | | | |
| CIP108297 | R | Addison's disease | 0.125 | 0.125 | 12.5 |
| 210 | R | CF | 0.125 | 0.125 | 12.5 |
| CIP108297 | S | Addison's disease | 0.125 | 0.25 | 25 |
| 111 | S | CF | 0.125 | 0.125 | 25 |
| 212 | S | CF | 0.125 | 0.125 | 25 |
| 185 | S | CF | 0.125 | 0.125 | 25 |
| 140 | S | CF | 0.125 | 0.125 | 25 |
| 100 | S | CF | 0.125 | 0.125 | >100 |
| 107 | S | CF | 0.25 | 0.25 | 12.5 |
| 122 | S | CF | 0.125 | 0.125 | 12.5 |
| 120 | S | CF | 0.125 | 0.125 | 12.5 |
| <i>M. abscessus</i> subsp. <i>bolletii</i> | | | | | |
| 19 | R | non-CF | 0.125 | 0.125 | 50 |
| 108 | R | CF | 0.25 | 0.5 | 12.8 |
| 112 | R | CF | 0.125 | 0.0625 | >100 |
| CIP108541 | S | not reported | 0.125 | 0.125 | 25 |
| 17 | S | non-CF | 0.125 | 0.125 | 12.5 |
| 97 | S | CF | 0.125 | 0.25 | 12.5 |
| 114 | S | CF | 0.125 | 0.125 | 12.5 |
| 116 | S | CF | 0.25 | 1 | 12.5 |

^aAMK, amikacin; MIC values are from the literature.²⁷

active against nine different clinical strains isolated from CF and non-CF patients. The *M. abscessus* complex is classified into three subspecies: *Mycobacterium abscessus* subsp. *abscessus*, *Mycobacterium abscessus* subsp. *bolletii*, and *Mycobacterium abscessus* subsp. *massiliense*,^{19,20} and the distinction between these subspecies is clinically relevant because they can respond differently to antibiotics.²⁰ We found that 30 clinical isolates comprising 11 isolates of *M. abscessus* subsp. *abscessus*, 11 of *M. abscessus* subsp. *massiliense*, and 8 of *M. abscessus* subsp. *bolletii*

with different susceptibility profiles to amikacin were equally sensitive to 12 and 6, generally exhibiting MICs of 0.125 $\mu\text{g}/\text{mL}$ (Table 2). The lack of cross-resistance between the indolecarboxamides and amikacin suggests that these compounds inhibit a different biological function, which is not targeted by amikacin.

To further understand the properties of the indolecarboxamides, exposure of *M. abscessus* 104536 (S) in exponential growth phase to 0.125 $\mu\text{g}/\text{mL}$ of compound 12, corresponding to 1 \times MIC, resulted in a notable reduction in viable colony-forming units (CFU) of about 2 orders of magnitude in 3 days as compared to the initial inoculum. This in vitro killing effect of compound 12 was even more pronounced than the one obtained when exposing the cultures to 8 $\mu\text{g}/\text{mL}$ imipenem (2 \times MIC) (Figure 2A). A comparable killing effect was obtained when cultures were treated with the same dose of compound 6 (Figure 2A).

Activity of Compound 12 against Intracellular *M. abscessus*. The intracellular killing activity of 12 was next assessed after infection of PMA-activated THP-1 macrophages. To avoid growth of extracellular mycobacteria, cells were extensively washed and treated with amikacin. When left untreated for 4 days, the number of intracellular *M. abscessus* was increased by more than 2 orders of magnitude while exposure to compound 12 (3 $\mu\text{g}/\text{mL}$, 24 \times MIC) prevented any multiplication within the macrophages (Figure 2B). Treatment with imipenem (96 $\mu\text{g}/\text{mL}$, 24 \times MIC) also significantly reduced the intracellular *M. abscessus* load, similar to compound 12. The intracellular activity of both compounds was further confirmed by counting the number of macrophages infected with *M. abscessus* expressing tdTomato at day 4 post-treatment. A significant decrease in the percentage of infected cells treated with either imipenem or compound 12 was found (Figure 2C). Overall, these results clearly indicate that compound 12 is able to enter human macrophages and to stop bacterial replication.

The A309P Mutation in *MAB_4508* Confers High Resistance Levels to Compound 12. To search for the molecular mechanism of action of the inhibitor, we first selected a spontaneous mutant of *M. abscessus* resistant to compound 12, designated CIP_OK4–10^R, which was subsequently found to exhibit high resistance levels to both compounds 12 and 6 (MIC values of 32 $\mu\text{g}/\text{mL}$) (Table 3). Gene sequencing identified a single g925c single nucleotide polymorphism in *MAB_4508*, encoding MmpL3, which results in an amino acid replacement at position 309 (A309P). Interestingly, we had previously identified the same mutation in a compound 1-resistant mutant (CIP_PIPD1^R),¹⁵ which showed cross-resistance with compounds 12 and 6. As anticipated, the A309P mutant selected on compound 12 was found to be resistant to compound 1 (Table 3).

To further validate the A309P mutation as the mechanistic basis for resistance to the indolecarboxamides, both the wild-type *MAB_4508* gene and the *MAB_4508* allele harboring the A309P mutation were cloned in pMV261 under the control of the constitutive *hsp60* promoter to allow overexpression of the wild-type and mutated versions of the protein in *M. abscessus*. Whereas overproduction of the wild-type protein failed to result in increased resistance to compounds 6 or 12 as compared to the control drug-susceptible strain, overexpression of the mutated protein resulted in high resistance levels when plated on 7H10^{OADC} media (Figure 3). This indicates that transferring the single point mutation identified in the

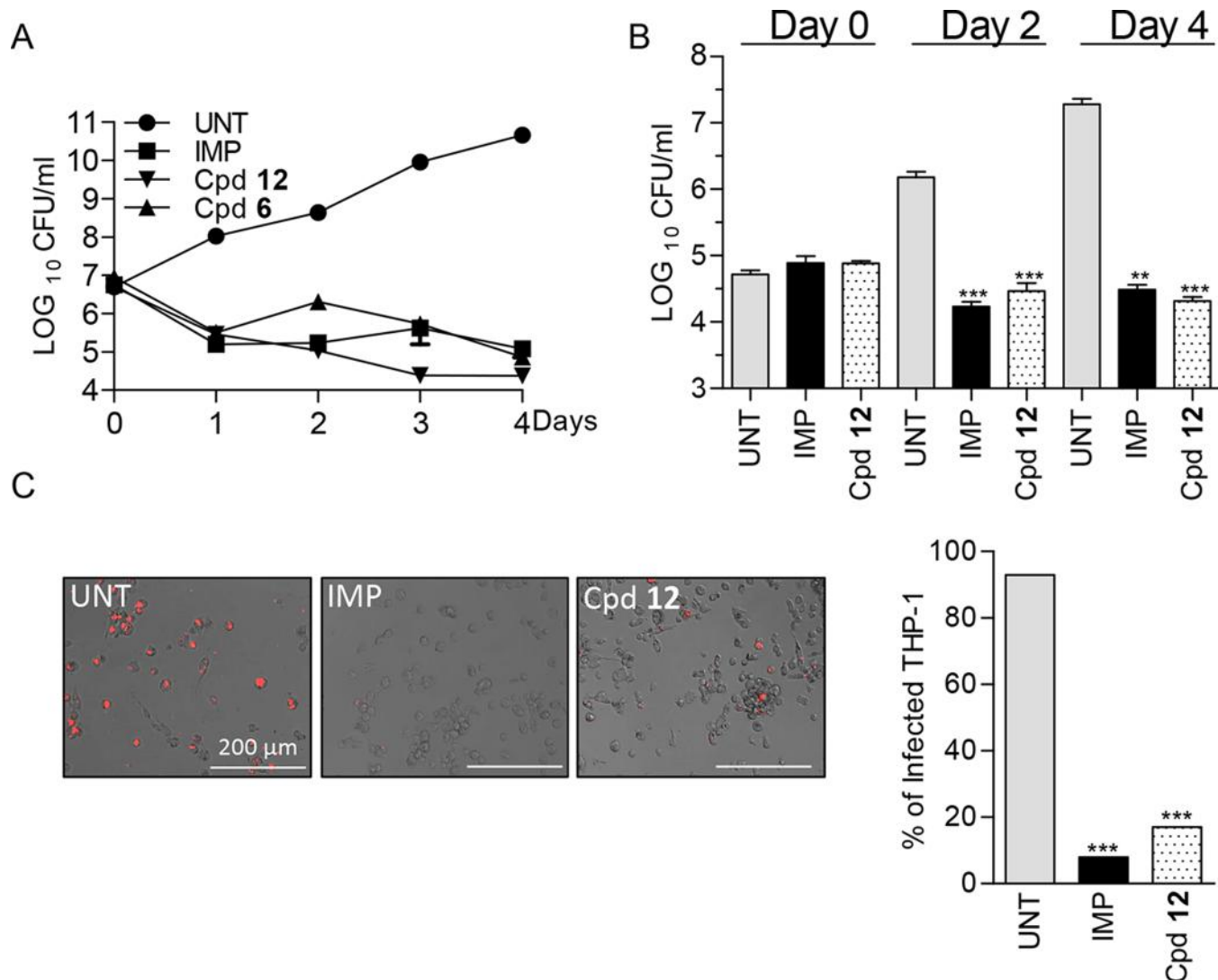


Figure 2. In vitro and intracellular activity of indole-2-carboxamides. (A) In vitro killing curves of *M. abscessus* CIP 104536 (smooth strain) exposed to 8 $\mu\text{g}/\text{mL}$ imipenem (IMP) ($2 \times \text{MIC}$), 0.125 $\mu\text{g}/\text{mL}$ compound 12 ($1 \times \text{MIC}$), or 0.125 $\mu\text{g}/\text{mL}$ compound 6 ($1 \times \text{MIC}$) in CaMH broth at 37 $^{\circ}\text{C}$. Results are expressed as mean of triplicate Log₁₀ values \pm SEM. UNT, untreated cultures. The results for each drug concentration are representative of three independent experiments. (B) THP-1 macrophages were infected with *M. abscessus* CIP 104536. After removal of the extracellular bacteria by extensive washing and treatment with amikacin, cells were treated with IMP at 96 $\mu\text{g}/\text{mL}$ ($24 \times \text{MIC}$) or compound 12 at 3 $\mu\text{g}/\text{mL}$ ($24 \times \text{MIC}$). Cells were washed with PBS, and fresh medium containing the different drugs was renewed every 24 h. CFUs were determined at 0, 2, and 4 days post-treatment. Results are expressed as mean of triplicates from two independent experiments, and error bars represent the SEM. One-tailed Mann–Whitney’s *t* test comparing the values with the day 0 was applied with $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. (C) Following infection with *M. abscessus* expressing tdTomato (red fluorescence), macrophages were treated with either IMP or compound 12 (each at $24 \times \text{MIC}$) and observed under the microscope at day 4 post-treatment (4297 total cells were counted) to determine the percentage of infected cells. Corresponding microscopy captures are also shown. Fisher exact test, $***P < 0.001$.

Table 3. MIC of a Spontaneous Resistant Strain Carrying the A309P Mutation in MAB_4508^a

| strain | MIC 1 | MIC 12 | MIC 6 | mutation in MAB_4508 | |
|-------------------------|-------|--------|-------|----------------------|-----------|
| | | | | SNP | AA change |
| CIP104536 (S) | 0.125 | 0.125 | 0.125 | | |
| CIP_OK4-10 ^R | 8 | 32 | 32 | g925c | A309P |
| CIP_PIPD1 ^R | 8 | 32 | 32 | g925c | A309P |

^aMIC values ($\mu\text{g}/\text{mL}$) were determined in cation-adjusted Mueller–Hinton broth. The resistant strain, designated CIP_OK4-10^R, was derived from the *M. abscessus* CIP104536 (S) parental strain. The CIP_PIPD1^R mutant was reported previously.¹⁵

spontaneously resistant mutant to a susceptible strain is sufficient to confer resistance to compounds 6 and 12.

Together, these results implicate the A309P mutation as critical in conferring high resistance levels to indole-2-carboxamides in *M. abscessus* and present evidence for the involvement of MmpL3 in the mode of action of this family of inhibitors.

Lead Compound 12 Inhibits Mycolic Acid Transport in *M. abscessus*. Indolecarboxamides have previously been proposed to target the mycolic acid transporter MmpL3 in *M. tuberculosis*,¹⁸ and it has been reported that a mutation in MmpL3 correlates with resistance in *M. abscessus*. To explore whether compound 12 may alter this pathway in *M. abscessus*, untreated and 12-treated cultures were metabolically labeled

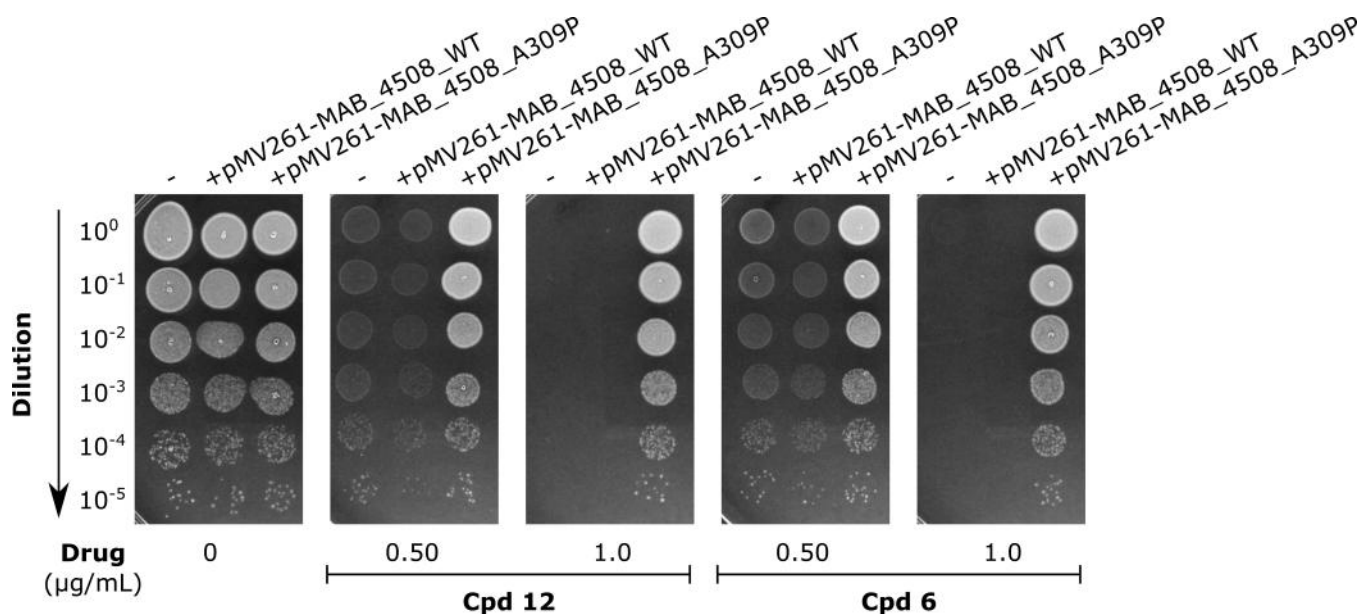


Figure 3. Overexpression of the *MAB_4508* A309P mutated allele in *M. abscessus* renders the bacterium resistant to compounds 12 and 6. Exponentially growing wild-type *M. abscessus*, or *M. abscessus* carrying either the pMV261_MAB_4508_WT or the pMV261_MAB_4508_A309P constructs, were grown in 7H9^{OADC} and serially diluted and spotted (5 μ L) on 7H10^{OADC} media containing different concentrations of compounds 12 and 6. Plates were incubated at 30 °C for 3 days prior to growth inspection.

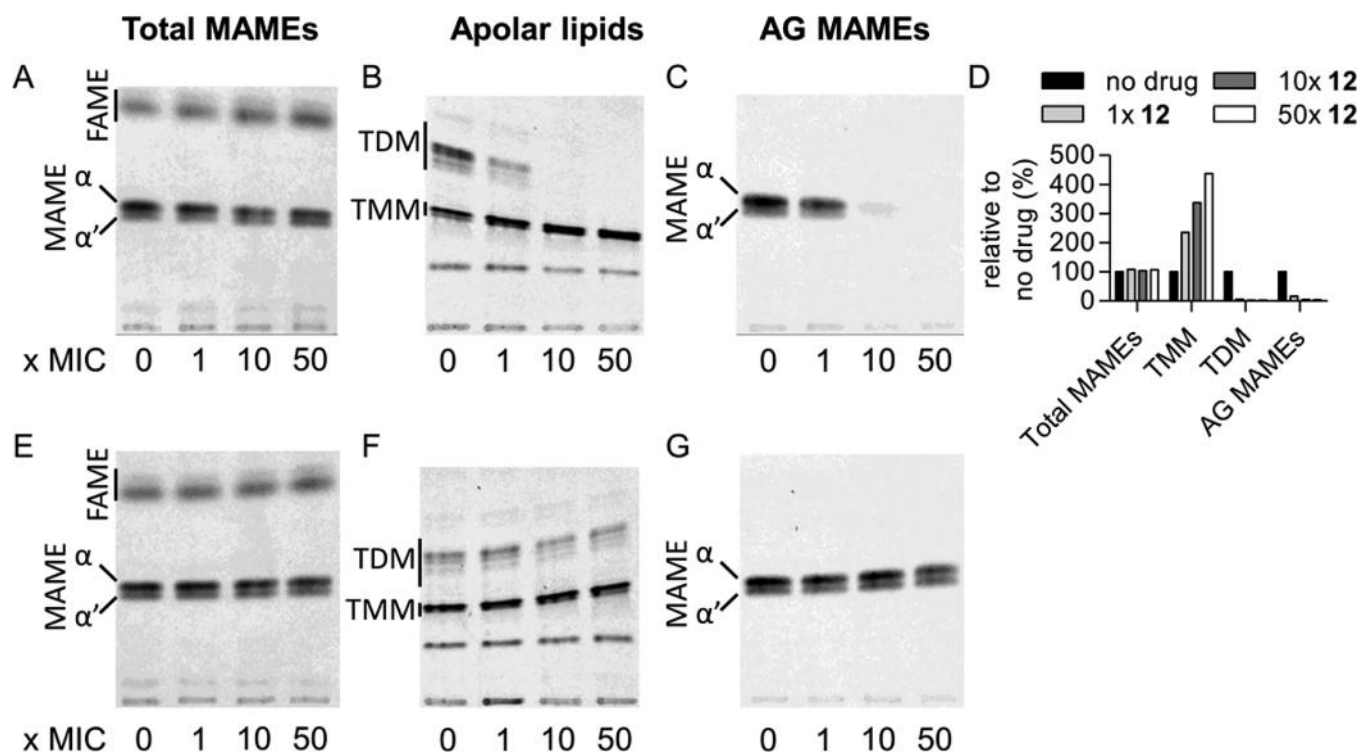


Figure 4. Treatment with compound 12 prevents TMM transport in *M. abscessus* and involves the Ala309 residue in MmpL3. Exponentially growing *M. abscessus*, wild-type (A–D) or carrying the A309P mutation in MmpL3 (E–G), were incubated with increasing concentrations of compound 12 in 7H9^{OADC} at 37 °C with agitation for 1 h. Subsequently, bacteria were labeled with [¹⁴C]acetate for 2 h at 37 °C with agitation. The cultures were split and from the first volume were extracted the total methyl esters of mycolates (MAME) and methyl esters of fatty acids (FAME). From the second volume, apolar and polar fractions were obtained prior to derivatization of arabinogalactan (AG) mycolate methyl esters. (A,E) Equal counts (50000 cpm) of the MAME and FAME fraction were loaded on a TLC plate and resolved once using the solvent system petroleum ether/acetone (95:5, v/v). (B,F) The apolar fraction was loaded (50000 cpm), and TMM/TDM were visualized on a 1D TLC plate using the solvent system chloroform/methanol/water (40:8:1, v/v/v). (C,G) Equal volumes of the arabinogalactan MAME fractions were loaded, and α - and α' -mycolic acids were visualized on a 1D TLC plate using the solvent system petroleum ether/acetone (95:5, v/v). (D) Densitometric analysis of the TLC plates shown in A–C.

with [^{14}C]acetic acid to monitor (glyco)lipid synthesis. When applied at concentrations of up to $50 \times \text{MIC}$ ($6.2 \mu\text{g/mL}$), compound **12** had no effect on de novo mycolic acid biosynthesis (Figure 4A). However, following apolar lipid extraction and their subsequent separation by thin layer chromatography (TLC), a dramatic inhibition of trehalose dimycolate (TDM) synthesis was observed concomitantly with an accumulation of trehalose monomycolate (TMM) (Figure 4B,D), the latter being the substrate of MmpL3 for the transport of mycolic acids onto the mycobacterial surface where TDM is formed. To test whether treatment with compound **12** also impacts mycolylation of arabinogalactan (AG), radio-labeled mycolic acids were extracted from delipidated bacteria.^{15,21} The dose-dependent inhibition of [^{14}C]acetic acid incorporation suggests that compound **12** inhibits AG mycolylation with a pronounced decrease of cell wall-bound α - and α' -mycolic acids (Figure 4C,D). The two types of mycolic acids (i.e., α and α') correspond to long-chain (C_{77-79}) and short-chain (C_{62-64}) mycolic acids, respectively, as reported earlier.²² Indeed, compound **12** behaved similarly to compound **1**, an unrelated compound that inhibits the transport of mycolic acid transport in *M. abscessus* by targeting MmpL3.¹⁵ Importantly, the mutant strain carrying the MAB_4508(A309P) allele was found to be totally refractory to the inhibition of TMM transport and to obstruction of AG mycolylation as compared to the parental strain, even in the presence of high concentrations of compound **12** (Figure 4E–G). In addition, very similar TLC profiles were obtained when treating *M. abscessus* cultures with increasing doses of compound **6** (Supporting Information, Figure S1), indicating that both analogues target the same biosynthetic pathway. To exclude the possibility that compounds **12** and **6** may inhibit the synthesis of TDM and mycolylated arabinogalactan directly rather than the mycolic acid transport by targeting the mycolyltransferase activity of the antigen 85 complex,²³ both compounds were tested for their potential inhibitory activity in an in vitro enzymatic assay using purified Ag85C.²⁴ This assay is based on a fluorescent probe (resorufin butyrate) that produces a direct measurement of the Ag85C enzymatic activity. Concentrations ranging from 2.5 to 50 μM of compounds **6** or **12** failed to inhibit the Ag85C activity (Supporting Information, Figure S2). In sharp contrast, eblesen inhibited the activity of Ag85C in a dose-dependent manner, as reported previously.²⁴

Taken collectively, these biochemical data underline a mode of action that results in the abolition of mycolic acid translocation from the cytoplasm where they are synthesized to the periplasmic side of the plasma membrane for subsequent use in the biogenesis of TDM and transfer onto the essential AG component of the mycobacterial cell wall.

Preliminary ADME Studies on Compound 12. On the basis of the encouraging biological results described above, selected ADME studies were conducted on compound **12** to assess its drug-like properties (Table 4). The cell permeability of compound **12** was measured in the PAMPA cell line assay system. Compound **12** showed a low permeability with $0.2 \times 10^{-6} \text{ cm/s}$ at pH 7.4 and $0.3 \times 10^{-6} \text{ cm/s}$ at pH 5.0, respectively. In mouse and rat liver microsomes, compound **12** showed a high intrinsic clearance of 4.8 mL/min/g liver and 6.3 mL/min/g liver, respectively. Compound **12** showed a low propensity to inhibit CYP 1A2, 2C8 2C9, 2C19, 2D6, and 3A4 with an IC_{50} value of $\leq 20 \mu\text{M}$. The compound exhibited high plasma protein binding in mouse (98.9%) and rat (99.2%). On

Table 4. ADME Data for Compound 12

| assay | values |
|--|--|
| permeability (PAMPA) | low ($0.2 \times 10^{-6} \text{ cm/s}$ and $0.3 \times 10^{-6} \text{ cm/s}$ at pH 7.4 and 5.0, respectively) |
| plasma protein binding (%), mouse/rat | 98.9/99.2 |
| CYP inhibition (% inhibition at 10 μM) | |
| CYP1A2 | 19 |
| CYP2C8 | 20 |
| CYP2C9 | 6 |
| CYP2C19 | 4 |
| CYP2D6 | 10 |
| CYP3A4 | 1 |
| CL_{int} in LM ^a , mL/min/g liver (mouse/rat) | high (4.8/6.3) |
| Ames | negative |

^aLM: Liver microsomes.

the basis of the AMES mutagenicity assay, compound **12** showed no mutagenic potential in the histidine auxotrophic strains of *Salmonella typhimurium* TA98 and TA100 at 0.24–500 $\mu\text{g/well}$. Because the compound showed less than a 2-fold increase in the number of revertant colonies compared to the negative controls in either the presence or absence of S9, it can be inferred that it has no mutagenic potential under the test conditions. The compound showed precipitation at 500 and 250 $\mu\text{g/well}$.

DISCUSSION AND CONCLUSIONS

M. abscessus is an emerging causative agent of chronic lung disease, particularly in patients with altered host defenses or disrupted airway clearance mechanisms, such as in CF. *M. abscessus* infection represents a threat to patients with CF with increased prevalence in recent years.^{25,26} Among mycobacterial species, *M. abscessus* is regarded as one of the most drug-resistant species, and chemotherapeutic options are very limited.⁸ The fact that most available treatments are often unsuccessful or poorly tolerated by patients emphasizes the urgent need for developing more active and better-tolerated drugs. As originally conjectured, the notion of screening drugs that had previously demonstrated activity against *M. tuberculosis* for possible efficacy against *M. abscessus* has been shown to provide good lead candidates for combating *M. abscessus* infections.^{15,27} Following this cross-screen approach, we have screened a library of indole-2-carboxamides, reported to be highly active against *M. tuberculosis*.^{16,17} This allowed us to define a new structural class of compounds exhibiting promising activity against *M. abscessus*, further validating the value of utilizing data obtained from prior TB screens to directly identify new chemotypes with strong activity against *M. abscessus*. Our lead compounds **6** and **12** display very low MIC values against a vast panel of clinical strains with different susceptibility profiles to antibiotics, regardless of whether they were isolated from CF or non-CF patients. Moreover, the efficacy of compounds **6** and **12** was not dependent on the smooth or rough morphotype of the *M. abscessus* subspecies, which is of particular interest because *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* infections can present various drug sensitivity test results, responses to antibiotics, and clinical symptoms.^{28,29} Additionally, compound **12** exhibited appreciable activity in infected macrophages.

To pinpoint the possible molecular mechanism of action of these inhibitors, we identified a spontaneous mutant which exhibited a high resistance phenotype to both lead compounds. Gene sequencing identified a single g925c single nucleotide polymorphism in MAB_4508, encoding MmpL3. This mutation resulted in an A309P amino acid replacement also previously identified in a compound 1-resistant mutant,¹⁵ which itself showed cross-resistance to compounds 6 and 12. Consistent with these findings, compounds 6 and 12 inhibited the translocation of TMM, resulting in the abolition of both TDM formation and mycolylation of AG. Our analyses also revealed that compounds 6 and 12 were not capable of inhibiting the activity of Ag85C in vitro. Together, our combined genetic and biochemical data reflect the inaccessibility of the intracellularly generated TMM to the Ag85 family of mycolyltransferases,²³ similarly to other MmpL3 inhibitors, such as compound 1,¹⁵ the adamantyl-urea AU1235,²¹ or *N*-geranyl-*N'*-(2-adamantyl)ethane-1,2-diamine (SQ109).³⁰

Furthermore, sequence analysis indicates that the MmpL3 protein of *M. abscessus* (MAB_4508) and its counterpart in *M. tuberculosis* (Rv0206c)^{18,31} show high similarity in multiple aspects. In addition to similar gene sizes, 3006 and 2835 base pairs, respectively, amino acid sequence blasting also revealed 56% identity and 69% similarity. Bioinformatic analysis indicates that both MAB_4508 and Rv0206c are transmembrane proteins comprising 12 transmembrane helices. Currently, MmpL3 appears to be one of the most promising antimycobacterial pharmacological targets as multiple chemical entities have recently been shown to inhibit MmpL3 activity not only in *M. tuberculosis* but also in *M. abscessus*, thus opening a new field centered on the inhibition of mycolic acid transport.^{15,18,21,30–34} Interestingly, recent findings indicate that MmpL3 inhibitors can also act synergistically with other antitubercular drugs, which is of interest to efforts aiming at reducing the length of treatments.³⁵ Whether indole-2-carboxamides may also act in synergy with drugs used for the treatment of *M. abscessus* infections remains to be investigated.

Tetrahydropyrazo[1,5-*a*]pyrimidine-3-carboxamide (THPP) has recently been reported to inhibit the biogenesis of mycolic acids by targeting the enoyl-coenzyme A (CoA) hydratase EchA6 rather than the previously assumed MmpL3 target,³⁶ even though several mutations identified in *mmpL3* had been proposed to confer resistance to THPP.³⁷ It was subsequently demonstrated that THPP competes with CoA-binding so as to arrest mycolic acid production, leading to the conclusion that spontaneous resistance-conferring mutations in *mmpL3* can potentially obscure the actual target identification of small inhibitors. Our results failed to show an inhibition of mycolic acid biosynthesis, even in the presence of high concentrations of compounds 6 or 12, and clearly demonstrated a dose-dependent inhibition of TDM synthesis with a concomitant accumulation of TMM. In addition, overexpression of the MmpL3 (A309P) variant in a genetically susceptible strain correlated with high resistance levels to compounds 6 and 12 and rendered the strain refractory to TMM transport inhibition. Together, these results extend the results obtained previously in *M. tuberculosis* that indole-2-carboxamides act by altering the transport of TMM across the membrane in pathogenic, fast-growing nontubercular mycobacteria.

How compound 1 and our indole-2-carboxamides inhibit MmpL3, and whether they have access to the same or different binding cavities in MmpL3, remains to be established. The design of further improved indolecarboxamides targeting

mycolic acid transport would also greatly benefit from future structural and functional studies on MmpL3. Moreover, details as to whether the activity of the indolecarboxamides results from specific inhibition of the MmpL3 transporter functions or through indirect mechanisms, for example, involving the dissipation of the proton-motive force as recently suggested,³⁸ require additional investigations. In summary, our indole-2-carboxamides represent a very promising chemotype that is able to alter the mycolic acid profile in *M. abscessus*, a species that is naturally resistant to most mycolic acid biosynthesis inhibitors. Of particular interest for medicinal chemistry and drug development, these indole analogues are easy to prepare and generally show reasonably good ADME properties as reported earlier¹⁶ and as further investigated herein for compound 12. This work, along with previous findings using compound 1, support the view that targeting the transport of mycolic acids rather than their biosynthesis, has significant translational potential for development of these chemotypes into real drugs for *M. abscessus* treatment and control. Research is currently underway to assess their efficacy in *M. abscessus* animal models.

■ EXPERIMENTAL SECTION

Chemistry: General Information. The tested 28 compounds were synthesized and characterized as reported in literature.^{16,17} Purities of final compounds were confirmed to be $\geq 95\%$ by analytical HPLC, which was carried out using an Agilent 1100 HPLC system with a Synergi 4 μm Hydro-RP 80A column and a variable wavelength detector G1314A; method 1, flow rate 1.4 mL/min, gradient elution over 20 min, from 30% MeOH–H₂O to 100% MeOH with 0.05% TFA added to both solvents; method 2, flow rate 1.4 mL/min, gradient elution over 20 min, from 50% MeOH–H₂O to 70% MeOH–H₂O with 0.05% TFA added to both solvents.

Bacterial Strains. *M. abscessus* subsp. *abscessus* CIP104536^T, *M. abscessus* subsp. *bolletii* CIP108541^T, and *M. abscessus* subsp. *massiliense* CIP108297^T reference strains were used along with a series of clinical isolates as reported previously.²⁷ Strains were routinely grown and maintained at 30 °C in Middlebrook 7H9 broth (BD Difco) supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% oleic acid, albumin, dextrose, and catalase (OADC enrichment; BD Difco) (7H9^{T/OADC}), or on Middlebrook 7H10 agar (BD Difco) containing 10% OADC enrichment (7H10^{OADC}), and in the presence of antibiotics when required. For drug susceptibility testing, bacteria were grown in cation-adjusted Mueller–Hinton broth (CaMHB; Sigma-Aldrich).

Drug Susceptibility Testing. The minimal inhibitory concentrations (MIC) were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.³⁹ The broth microdilution method was used in CaMHB with an inoculum of 5×10^6 CFU/mL in the exponential growth phase. Briefly, the bacterial suspension was seeded in 100 μL volumes in all of the wells of a 96-well plate, except for the first column to which 198 μL of the bacterial suspension was added to each well. In the first column, 2 μL of compound at its highest concentration was added in six wells (the solvent used to dissolve the compound was added to the two outermost wells as a control). Two-fold serial dilutions were then carried out by transferring 100 μL from the wells in the first column to the next column and repeating this for each successive column. Plates were subsequently incubated at 30 °C for 3–5 days. MICs were recorded by visual inspection and by absorbance at 560 nm to confirm visual recordings. Experiments were done in triplicate on three independent occasions.

Time-Kill Assay. Microtiter plates were set up as for the MIC determination. Serial dilutions of the bacterial suspensions from these microtiter plates were plated after 0, 24, 48, 72, and 96 h of exposure to different drug concentrations. Colony-forming units (CFU) were counted after 4 days of incubation at 30 °C.

Intramacrophage Killing Assay. THP-1 cells were grown in RPMI medium containing 10% fetal bovine serum (RPMI^{FBS}) and differentiated with 20 ng/mL phorbol myristate acetate (PMA) in 12-well flat-bottom tissue culture microplates (1×10^5 cells/well) and incubated for 48 h at 37 °C. Cells were infected with a multiplicity of infection (MOI) of 1:2 and incubated at 37 °C in the presence of 5% CO₂ for 2 h. Wells were carefully washed three times with 1× PBS, re-fed with RPMI^{FBS} supplemented with 200 μg/mL amikacin, reincubated at 37 °C in the presence of 5% CO₂ for 1 h, and washed again three times with PBS prior to the addition of 1 mL of RPMI^{FBS} supplemented with either 3 μg/mL compound **12** or 96 μg/mL imipenem. Media were changed on a daily basis with fresh drug preparations. At various time points (0, 48, and 96 h), cells were washed three times with PBS and lysed by adding 100 μL of 1% Triton X100. Cell lysis was stopped after 2 min by adding 900 μL of PBS, and serial dilutions were plated to monitor the intracellular bacterial counts. Experiments were done two times independently. For determining the percentage of infected macrophages, THP-1 cells were prepared and infected with *M. abscessus* expressing tdTomato and treated with drugs, as described above. Untreated and drug-treated infected cells (96 h time point) were observed under a fluorescence microscope to count the number of bacteria-containing macrophages, as previously described.¹⁵

Selection of a Compound 12-Resistant Mutant. Exponentially growing *M. abscessus* cultures were plated on 7H10^{OADC} containing 6 μg/mL of compound **12**. After 1 week of incubation at 30 °C, a single colony was selected and grown in liquid medium, assessed by MIC determination, and subsequently scored for resistance to compound **12**. Identification of putative SNPs in the *MAB_4508* (*mmpL3*) gene was carried out by PCR amplification using the *MAB_4508seq1* (5'-GGCCACCGTCTTACCAATA-3') and *MAB_4508seq7* (5'-GTGCCGTACCTGAATGTCCT-3') primers to produce a 3549 bp amplicon to gain full coverage sequencing of *MAB_4508*, as done previously.¹⁵ The SNP was confirmed by at least two independent sequencing reactions. To overexpress the *MAB_4508* (*A309P*) allele, the *MAB_4508* open reading frame was PCR-amplified using as template purified genomic DNA of CIP_OK4-10^S, Phusion DNA polymerase (Finnzymes, Finland) and the primers *MAB_4508f* and *MAB_4508r*, as previously described.¹⁵ The obtained PCR product was subsequently digested with *Hind*III and ligated to *MscI*/*Hind*III linearized pMV261.

Whole-Cell Radiolabeling Experiments and Lipid Analysis. For visualizing drug-induced changes in the lipid profile, increasing concentrations of compound **12** were added to exponentially growing cultures for 1 h, and metabolic labeling of lipids was performed by adding 1 μCi/mL of [¹⁴C]acetate (56 mCi/mmol) for an additional 2 h at 37 °C. Cells were harvested and either delipidated or used immediately to extract mycolic acids, as previously described.^{22,40} The apolar lipid fraction containing trehalose monomycolate (TMM) and trehalose dimycolate (TDM) was separated on a one-dimensional thin layer chromatography (TLC) plate using chloroform/methanol/water (40:8:1, v/v/v) and revealed after exposure to a film. Delipidated cells were further processed to extract the mycolic acids,⁴¹ which were analyzed by TLC/autoradiography using petroleum ether/acetone (95:5, v/v) and exposure to a film to reveal ¹⁴C-labeled mycolic acid methyl esters (MAME).

Cell Permeability. The permeability of compound **12** was measured at two pH values (pH 7.4 and pH 5.0) in Prisma HT aqueous buffer (pION Inc.). The pH was adjusted with 0.5 N NaOH. The test compound in buffer solutions (25 μM) was added to the PAMPA donor plate (in triplicate). The acceptor plate was painted with 4 μL of GIT-0 lipid solution (pION Inc.), and ASB (acceptor sink buffer, pION Inc.) was added. The acceptor and donor plates were stacked, sealed with a rubber plate, and incubated for 4 h at 25 ± 2 °C. The donor and acceptor plate solutions were analyzed by an LC-MS (UPLC) system.

In Vitro Study: CYP Inhibition. Inhibition of CYP by compound **12** was evaluated using pooled human liver microsomes (catalogue no. 452118, BD-Biosciences). Ten μM compound **12** or the positive control was preincubated with human liver microsomes for 5 min at 37

°C. The reaction was initiated by addition of an NADPH regenerating system and incubated at 37 °C with gentle agitation for the specified time recommended for each of the CYP's (5 min for CYP3A4, 10 min for CYP1A2, 2C8, 2C9, and 2D6, and 40 min for CYP2C19). The samples were vacuum-filtered using Captiva 96-well plates followed by LC-MS/MS analysis. The formation of standard metabolite (concentration/peak area ratio) was estimated. The percentage of inhibition of the test compound and the positive control in comparison to a negative control (DMSO) were determined using the equation:

$$\% \text{inhibition} = 100 - \left\{ \left[\frac{\text{average metabolite concentration of test compound/positive control}}{\text{average metabolite concentration of no inhibitor control}} \right] \times 100 \right\}$$

Plasma Protein Binding. Plasma protein binding studies were performed using an ultracentrifuge method. A 5 μM solution of compound **12** was prepared in mouse or rat plasma using 500 μM stock solution (in DMSO) and incubated for 10 min at 37 °C. For determination of total plasma concentration, 5 μL of incubated plasma was mixed thoroughly with 50 μL of saline, 50 μL of acetonitrile, and 100 μL of 1 μM niflumic acid. For determination of unbound concentrations, the incubated plasma sample was centrifuged at 200000g for 4.5 h, and 50 μL of supernatant from the middle layer was taken after removing the upper layer with a Beckmann Coulter slicer. To this solution, 5 μL of blank plasma, 50 μL of acetonitrile, and 100 μL of 1 μM niflumic acid were added. The samples were filtered into a 96-deep well storage plate with a Captiva 0.45 μm 96-well filter plate, and appropriate volumes of all samples were analyzed by LC-MS/MS.

Metabolic Stability. Compound **12** (0.5 μM) was incubated in a reaction mixture consisting of mouse or rat liver microsomes and an NADPH regenerating system. Aliquots were withdrawn at 3 min intervals for 30 min and analyzed for the parent compound by LC-MS/MS. The loss of parent was expressed as the percentage of the test compound remaining, and the rate of decay was estimated by monoexponential decay kinetics. The rate of decay was normalized to microsomal protein expressed as mL/min/g liver.

AMES Mutagenicity Assay. Compound **12** was evaluated for its mutagenic potential in the presence and absence of the S9 fraction by investigating its ability to induce reverse mutations at the histidine locus in the genome of *Salmonella typhimurium* strains. Two histidine auxotrophic strains of *Salmonella typhimurium* were used, namely the TA98 strain carrying a frame shift mutation and the TA100 strain carrying a base-pair substitution. Plates were incubated at 37 ± 2 °C for 72 h, and the experiments were carried out in triplicates. DMSO was used as vehicle control. Sodium azide, 2-nitrofluorene (2NF), and 2-aminoanthracene (2AA) were used as positive controls. A compound was considered positive if the number of revertants increased by ≥2-fold over the negative control in a dose-dependent manner.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00582.

Mycolic acid profile is affected by treatment with compound **6**; effect of inhibitors on Ag85C activity (PDF)

Molecular formula strings and some data (CSV)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank F. Roquet-Banères for technical assistance. We are indebted to the Howard Hughes Foundation for providing funds that allowed us to initiate this work. O.K.O. acknowledges Roosevelt University for generous financial support. J.S. acknowledges Chicago State University (CSU) College of Pharmacy and CSU Center for Teaching and Research Excellence (Faculty Seed Grant) for financial support. A Faculty Development Stipend (J.S.) from the Marshall B. Ketchum University College of Pharmacy is also acknowledged. We thank the Association Gregory Lemarchal and Vaincre La Mucoviscidose (RF20130500835) for funding C.D. L.K. also acknowledges the support by the Fondation pour la Recherche Médicale (FRM) (DEQ20150331719). This work was funded in whole or in part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, AIO99534-02, NIH/NIAID services under contracts HHSN272201000009I/HHSN27200001 and HHSN272201000009I/HHSN27200005, principal investigator Dr. A.J. Lenaerts, coprincipal investigator, D.O.

ABBREVIATIONS USED

CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; CFTR, cystic fibrosis transmembrane conductance regulator; TB, tuberculosis; NTM, nontubercular mycobacteria; CaMHB, cation-adjusted Mueller–Hinton broth; MIC, minimal inhibitory concentration; CLSI, Clinical Laboratory Standards Institute; CFU, colony-forming units; PMA, phorbol myristate acetate; SAR, structural–activity relationship; TMM, trehalose monomycolate; TDM, trehalose dimycolate; TLC, thin layer chromatography; MAME, mycolic acid methyl ester; DMSO, dimethyl sulfoxide; 2NF, 2-nitrofluorene; 2AA, 2-aminoanthracene; AG, arabinogalactan; THPP, tetrahydropyrazo[1,5-*a*]pyrimidine-3-carboxamide

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Annexe 2



Cyclipostins and cyclophostin analogs inhibit the antigen 85C from *Mycobacterium tuberculosis* both *in vitro* and *in vivo*

Received for publication, November 2, 2017, and in revised form, December 5, 2017. Published, Papers in Press, January 4, 2018, DOI 10.1074/jbc.RA117.000760

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Edited by Chris Whitfield

An increasing prevalence of cases of drug-resistant tuberculosis requires the development of more efficacious chemotherapies. We previously reported the discovery of a new class of cyclipostins and cyclophostin (CyC) analogs exhibiting potent activity against *Mycobacterium tuberculosis* both *in vitro* and in infected macrophages. Competitive labeling/enrichment assays combined with MS have identified several serine or cysteine enzymes in lipid and cell wall metabolism as putative targets of these CyC compounds. These targets included members of the antigen 85 (Ag85) complex (*i.e.* Ag85A, Ag85B, and Ag85C), responsible for biosynthesis of trehalose dimycolate and mycolylation of arabinogalactan. Herein, we used biochemical and structural approaches to validate the Ag85 complex as a pharmacological target of the CyC analogs. We found that CyC_{7β}, CyC_{8β}, and CyC₁₇ bind covalently to the catalytic Ser¹²⁴ residue in Ag85C; inhibit mycolyltransferase activity (*i.e.* the transfer of a fatty acid molecule onto trehalose); and reduce triacylglycerol synthase activity, a property previously attributed to Ag85A. Supporting these results, an X-ray structure of Ag85C in complex with CyC_{8β} disclosed that this inhibitor occupies Ag85C's substrate-binding pocket. Importantly, metabolic labeling of *M. tuberculosis* cultures revealed that the CyC compounds impair both trehalose dimycolate synthesis and mycolylation of arabinogalactan. Overall, our study provides compelling evidence that CyC analogs can inhibit the activity of the Ag85 complex *in vitro* and in mycobacteria, opening the door to a new strategy for inhibiting Ag85. The high-resolution crystal structure obtained will further guide the rational optimization of new CyC scaffolds with greater specificity and potency against *M. tuberculosis*.

With 10.4 million new cases and 1.8 million deaths in 2016, tuberculosis (TB)⁵ continues to be a major global health problem. TB is caused by *Mycobacterium tuberculosis*, a resilient microorganism that persists through long courses of antibiotics and years of dormancy within the host. The emergence of multidrug-resistant and extensively drug-resistant TB has contributed to the difficulties in treating this bacterial infection (1). Chemotherapeutic treatments against TB remain very challenging and complicated, essentially because of the slow rate of growth of the bacilli and the presence of a thick, greasy, and relatively drug-impermeable cell wall (2). This mycobacterial cell wall consists of a complex skeleton comprising covalently linked macromolecules, such as peptidoglycan, arabinogalactan, and mycolic acids, in which non-covalently associated glycolipids are interspersed (3). The mycolic acid portion of the envelope is composed of very long fatty acids (C70–90) that are either covalently attached to the arabinan moiety of the arabinogalactan (AG) polymer or found esterified to trehalose as trehalose monomycolate (TMM) or trehalose dimycolate (TDM). Because several key antitubercular drugs, such as isoniazid, SQ109, delamanid, or ethambutol, target different aspects of the biosynthetic steps responsible for the cell wall attachment of mycolic acids (4–7), this pathway is of particular interest from a drug discovery perspective.

The three functionally and structurally related members of the antigen 85 complex, designated Ag85A, -B, and -C, are among the most abundantly secreted proteins in *M. tuberculosis* (8). These enzymes are responsible for the biosynthesis of TMM and TDM as well as the covalent attachment of mycolic acids to AG (9–11). Deletion of *fbpC2*, encoding Ag85C, resulted in a 40% decrease in the AG-bound mycolic acids but failed to affect the production of non-covalently linked myco-

This work was supported by Fondation pour la Recherche Médicale (FRM) Grants DEQ20150331719 (to L. K.) and ECO20160736031 (to M. R.) and by CNRS and INSERM. The authors declare that they have no conflicts of interest with the contents of this article.

The atomic coordinates and structure factors (code 5OCJ) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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² Supported by the Ph.D. Training program of the University of Science and Technology of Hanoi.

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⁵ The abbreviations used are: TB, tuberculosis; AG, arabinogalactan; CyC, cyclipostins and cyclophostin; DGAT, diacylglycerol acyltransferase; FAME, fatty acid methyl ester; MAME, mycolic acid methyl ester(s); MIC, minimal inhibitory concentration; TAG, triacylglycerol; TDM, trehalose dimycolate; TLC, thin layer chromatography; TMM, trehalose monomycolate; χ_{150} , inhibitor molar excess leading to 50% inhibition; FP, fluorophosphonate; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); DEP, *p*-nitrophenyl phosphate; TAMRA, carboxytetramethylrhodamine; ILI, intracellular lipid inclusion; r.m.s., root mean square; PDB, Protein Data Bank.

Inhibition of Ag85C by cyclipostins and cyclophostin

lates (10), whereas deletion of *fbpA* or *fbpB*, encoding Ag85A and Ag85B, respectively, led to reduced TDM levels (12–14), implying that although a level of functional redundancy exists *in vivo* between the three members, the contribution of each member is significant. The lack of double and triple knockout mutants might indicate that the loss of two or more Ag85 enzymes is detrimental to *M. tuberculosis* viability. An additional isoform, designated Ag85D or MPT51, has been characterized but found to be inactive due to the lack of catalytic elements required for mycolyltransferase activity (11, 15, 16). Ag85A/B/C share the same mycolic acid donor TMM, and their crystal structures present a highly conserved catalytic site, which further supports their similar enzymatic role (17–19).

Due to their importance in mycolic acid metabolism, the Ag85 enzymes have often been proposed as attractive targets for future chemotherapeutic developments against TB (9, 20–22). Because of their high structural conservation, it can be inferred that a single compound may inhibit all three enzymes of the complex at the same time and would make improbable the development of resistance to inhibitors, because resistant mutants would require the simultaneous acquisition of mutations in at least two *fbp* genes. In addition, because these proteins are secreted, targeting the Ag85 complex will minimize the effect of efflux mechanisms that may result in resistance phenotypes. Early inhibitors, such as trehalose analogs, were first designed as Ag85 inhibitors but were found to exhibit relatively poor activity on whole mycobacterial cells (9, 23). Another potentially selective fluorophosphonate α,α -D-trehalose inhibitor of the three antigen 85 enzymes has been reported to form a stable, covalent complex with the Ag85 enzyme following nucleophilic attack on the phosphorus atom of the catalytic Ser¹²⁴ (24). In the same manner, the 2-amino-6-propyl-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile, designated I3-AG85, inhibits Ag85C, and exposure of *M. tuberculosis* to this compound was associated with reduced survival rates in broth medium and in infected primary macrophages. Moreover, I3-AG85 was active against a panel of multidrug-resistant/extensively drug-resistant strains, although it exhibited an MIC of 100 μ M (25). By combining fragment-based drug discovery with early whole cell antibacterial screening, tetrahydro-1-benzothiophene analogs were discovered as potent Ag85C inhibitory molecules against drug-susceptible and drug-resistant *M. tuberculosis* strains (26). The selenazole compound ebselen (2-phenyl-1,2-benzisoxaselenazol-3(2H)-one) was found to inhibit the activity of Ag85C through an original mechanism by reacting with the conserved Cys²⁰⁹ residue located near the active site of the enzyme but not involved in the catalytic activity (27, 28). Ebselen was shown to directly impede the production of TDM and mycolylation of AG (27).

Recently, cyclipostins and cyclophostin (CyC), representing a new class of monocyclic enolphosph(on)ate compounds, have been discovered to act as powerful antitubercular agents affecting growth of *M. tuberculosis* both *in vitro* and in infected macrophages (29). Among the set of 27 CyC analogs previously evaluated against *M. tuberculosis* H37Rv, eight compounds exhibited potent anti-tubercular activities, particularly the cyclophostin analogs CyC_{7 β} and CyC_{8 β} as well as the cyclipostins-related molecule CyC₁₇. Whereas CyC_{7 β} exhibited a

strong activity against extracellular and intracellular mycobacteria (MIC₅₀ of 16.6 and 3.1 μ M, respectively), CyC_{8 β} was mostly found to be active against intracellular bacteria (MIC₅₀ \sim 11.7 μ M). In contrast, CyC₁₇ was a potent inhibitor of *in vitro* growth (MIC₅₀ \sim 0.5 μ M) but failed to show activity against intracellular bacilli (29). To identify the putative target(s) of the CyC inhibitors, an activity-based protein profiling approach was used based on TAMRA-FP and desthiobiotin-FP probes and mass spectrometry analyses. This led to the capture of several active serine/cysteine enzymes in a complex proteome before mass spectrometry identification, among which Ag85A (Rv3804c) and Ag85C (Rv0129c) were identified.

The present study was undertaken to further explore and validate, through a combination of biochemical and structural approaches, the specificity of inhibition of the Ag85 activity by the CyC analogs, to determine their mode of action and to describe how they affect the mycolic acid profile in *M. tuberculosis*.

Results

CyC analogs inhibit TDM biosynthesis and transfer of mycolic acids to arabinogalactan in *M. tuberculosis*

CyCs are a new class of compounds demonstrating potent antitubercular activity, presumably involving inhibition of the Ag85 activity (29). The chemical structures of the cyclophostin analogs CyC_{7 β} and CyC_{8 β} and the cyclipostins CyC₁₇ used in this study are provided in Fig. 1A. To test whether treatment with these CyCs alters the mycolic acid composition of *M. tuberculosis* mc²6230, cultures were exposed to increasing concentrations of CyC₁₇ or CyC_{7 β} , the two inhibitors most active against extracellularly replicating *M. tuberculosis* (29), followed by metabolic labeling with sodium [2-¹⁴C]acetate and lipid analysis. Extraction and separation of the total mycolic acid methyl esters (MAME) by thin layer chromatography (TLC) revealed that neither CyC₁₇ nor CyC_{7 β} altered the *de novo* biosynthesis of mycolic acid (Fig. 1 (B and C), left). In contrast, separation of the apolar lipid fraction by TLC showed a dose-dependent decrease in TDM levels associated with a concomitant increase in the production of TMM, which is the natural substrate of the Ag85 proteins (Fig. 1 (B and C), middle). To address whether CyC treatment also impacts the cell wall-bound mycolic acids, radiolabeled mycolic acids were extracted from delipidated bacteria (30). The autoradiography/TLC analysis confirmed a dose-dependent inhibition of [2-¹⁴C]acetate incorporation into all three forms of the AG-attached mycolic acids (α , methoxy, and keto), suggesting that treatment with CyC₁₇ or CyC_{7 β} inhibits AG mycolylation at low concentrations (Fig. 1 (B and C), right). A quantitative analysis of these effects is provided in the corresponding graphs (Fig. 1, B and C).

Overall, this suggests that *in vivo* inhibition of the mycolyltransferase activity by the CyC compounds results in decreased formation of the virulence-associated TDM and reduced transfer of mycolic acids onto the essential cell wall AG.

Covalent inhibition of the Ag85C mycolyltransferase activity

All three members of the Ag85 complex, sharing between 65 and 75% sequence identity, have been shown to possess a serine

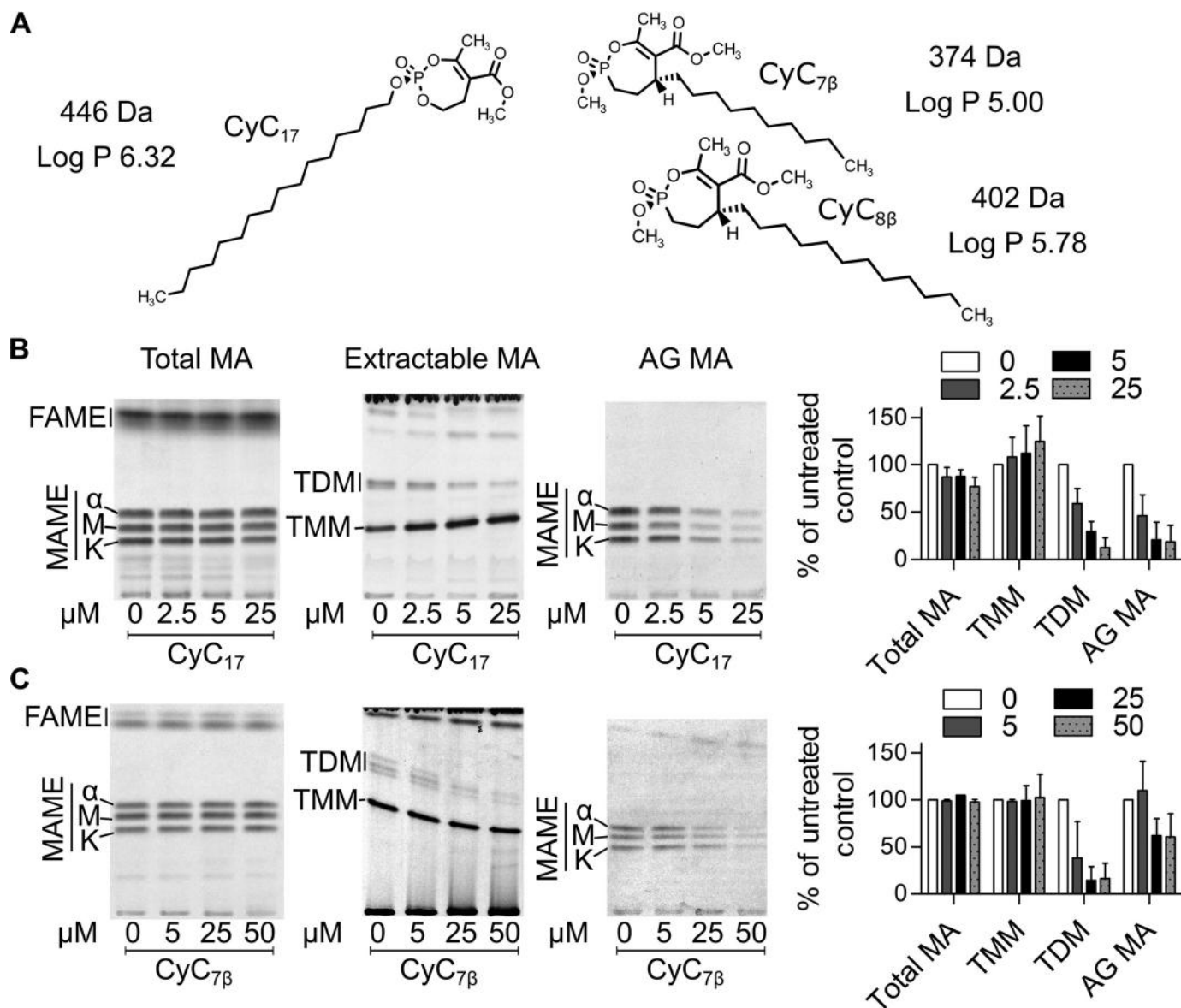


Figure 1. Ag85 complex mycolyltransferase activity is inhibited by CyC analogs in vivo. Exponentially growing *M. tuberculosis* mc²6230 was incubated with increasing concentrations of CyC₁₇ or CyC_{7β} in 7H9^{OADC}/Tween⁸⁰ at 37 °C with agitation for 1 h. Subsequently, bacteria were labeled with sodium [2-¹⁴C]acetate for 6 h at 37 °C with agitation. The cultures were split, and from the first volume were extracted the total methyl esters of mycolates (MAME) and fatty acids (FAME). From the second volume, apolar and polar lipid fractions were obtained before derivatization of arabinogalactan MAME. **A**, chemical structures of the CyC analogs used in this study. **B** and **C**, effect of CyC₁₇ (**B**) or CyC_{7β} (**C**) treatment on the mycolic acid profiles of *M. tuberculosis* mc²6230. Equal counts (50,000 cpm) of MAME + FAME fraction were loaded on a TLC plate and resolved once using the solvent system hexane/ethyl acetate (95:5, v/v) run twice (*far left*). The apolar fraction was loaded (50,000 cpm), and TMM and TDM were visualized on a 1D TLC plate using the solvent system chloroform/methanol/water (40:8:1, v/v/v) (*middle left*). Equal volumes of arabinogalactan MAME fraction were loaded, and α, methoxy, and keto mycolic acids were visualized on a 1D TLC plate using the solvent system hexane/ethyl acetate (95:5, v/v) run twice (*middle right*). Densitometric analysis (*far right*) was performed on the TLCs shown in the left panels. *Histograms* and *error bars*, means and S.D. values calculated from at least two independent experiments.

hydrolase mycolyl esterase/transferase activity (17–19). To test the hypothesis that CyC analogs inhibit the activity of the three Ag85 members, we first cloned Ag85C (*fbpC2*) into pET23b, and the recombinant protein was produced in *Escherichia coli*. The protein was then purified from lysates of *E. coli* by successive nickel-affinity, anion-exchange, and size-exclusion chromatography steps, leading to 3 mg of pure protein/liter of culture. Using a recently developed fluorescent assay based on resorufin butyrate as the acyl donor for Ag85C and trehalose as the acyl acceptor (27), we investigated whether CyC_{7β}, CyC_{8β}, and CyC₁₇ inhibit the acyltransferase activity onto trehalose. In each case, a dose-dependent inhibition was observed with all

three compounds, with CyC_{8β} being the most efficient inhibitor (IC₅₀ of 15 ± 5 μM), followed by CyC_{7β} (IC₅₀ of 43 ± 3 μM) and CyC₁₇ (IC₅₀ of 98 ± 6 μM) (Fig. 2A). Moreover, in terms of molar excess of inhibitor ($x_{150} = IC_{50}/[Ag85C]$) (31), all three CyCs react almost in stoichiometry with pure Ag85C, as judged by their respective x_{150} values of around 0.3, 0.8, and 1.8, respectively.

To address the inhibitory effect on the mycolyltransferase assay, Ag85C (55 μM) was next incubated for 30 min in its native form with 500 μM (*i.e.* enzyme/inhibitor molar ratio of 1:9) of each CyC compound. As expected, the complete loss of activity was confirmed by comparing the pretreated *versus* non-treated

Inhibition of Ag85C by cyclipostins and cyclophostin

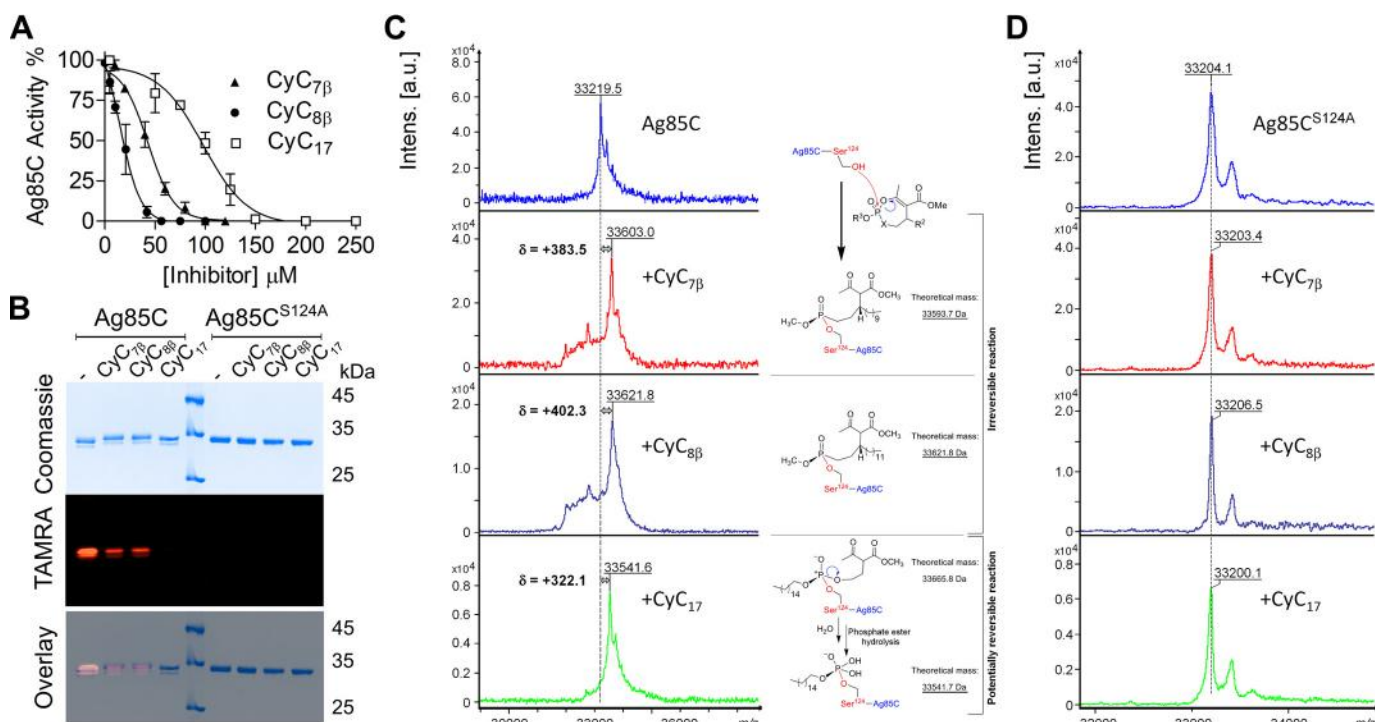


Figure 2. Inhibition of the Ag85C mycolyltransferase activity is mediated by the covalent binding of CyC analogs. *A*, the enzymatic activity of Ag85C was tested using a fluorescence-based assay in the presence of different concentrations of CyC_{7β}, CyC_{8β}, and CyC₁₇. The inhibitory effect was determined at the maximum rate of the reaction. *Error bars*, S.D. calculated from three independent experiments. *Curves* for CyC_{7β}, CyC_{8β}, and CyC₁₇ were fitted using the EC₅₀ shift non-linear regression model in GraphPad Prism with *R*² values of 0.9675, 0.9508, and 0.9415, respectively. *B*, equal amounts of either Ag85C or Ag85C^{S124A} were pretreated with CyC_{7β}, CyC_{8β}, and CyC₁₇; incubated with TAMRA-FP, separated by SDS-PAGE; and visualized by Coomassie Blue staining (*top*) or in-gel fluorescence visualization (*middle*). The *merged image* is shown at the *bottom*. TAMRA labeling of Ag85C is prevented by the covalent binding of the CyC analogs to the catalytic Ser¹²⁴. No TAMRA-FP labeling is seen for the Ag85C^{S124A} variant, confirming Ser¹²⁴ as the TAMRA-binding site. *C* and *D*, global mass modification of Ag85C (*C*) and Ag85C^{S124A} (*D*) preincubated with CyC_{7β}, CyC_{8β}, and CyC₁₇ as determined using an Ultraflex III mass spectrometer (Bruker Daltonics) in linear mode with the LP₆₆ kDa method. The mechanism of action of the phosphonates CyC_{7β} and CyC_{8β} and of the phosphate analog CyC₁₇ based on mass spectrometry analyses is illustrated in *C*. *a.u.*, arbitrary units.

Ag85C. All three Ag85C-CyC adducts were treated with 10 μM TAMRA-FP fluorescent probe, known to bind to serine enzymes (32), for 1 h, and equal amounts of proteins were separated by SDS-PAGE and visualized by Coomassie staining (Fig. 2*B*, *top*) or in-gel fluorescence for TAMRA detection (Fig. 2*B*, *middle*). Pretreatment with either CyC_{7β} or CyC_{8β} resulted in a significant loss in fluorescence intensity (about 75%) as compared with the non-treated protein, whereas incubation with CyC₁₇ abrogated TAMRA labeling. This suggests that reaction with the TAMRA probe is strongly impaired in the Ag85C-CyC adducts, resulting in a decrease/loss of fluorescence emission. To determine the implication of the conserved catalytic Ser¹²⁴ in Ag85C in TAMRA labeling, this residue was replaced by an Ala residue, and the mutated protein was purified (Fig. 2*B*, *top*). Exposure of TAMRA to Ag85C^{S124A} failed to produce a fluorescence signal (Fig. 2*B*, *middle*), indicating that the catalytic Ser¹²⁴ is required for binding of the probe. As expected, no fluorescence emission was observed when pretreating the mutated protein with the CyC analogs (Fig. 2*B*, *bottom*).

MALDI-TOF mass spectrometry was further used to study the (covalent) nature of the inhibition. Mass increments of +383.5 and +402.3 Da in the presence of CyC_{7β} and CyC_{8β}, respectively, were observed within the global mass of treated Ag85C as compared with the global mass of untreated Ag85C (Fig. 2*C*). In contrast, no changes in the global mass were observed with the inactive Ag85C^{S124A} protein (Fig. 2*D*). These data thus support the formation of a covalent Ag85C-CyC com-

plex, as the reaction between the catalytic Ser¹²⁴ and either CyC_{7β} or CyC_{8β} is expected to yield mass increases of +374.2 or +402.25 Da, respectively. Moreover, such results are consistent with the known and irreversible classical mechanism of action of phosphonate compounds, as demonstrated using pure mycobacterial lipolytic enzymes (31).

With respect to CyC₁₇, the observed 322.1-Da mass shift increment was 124.18 Da lower than its expected theoretical molecular mass of 446.28 Da (Fig. 2*C*). This size difference may arise from the specific chemical properties of phosphate (*i.e.* CyC₁₇) versus phosphonate (*i.e.* CyC_{7β} and CyC_{8β}) chemical groups. In all cases, the nucleophilic attack of catalytic Ser¹²⁴ at the phosphorus center induces ring opening. However, the reaction with CyC₁₇ is very likely to form a new phosphate triester, which in turn becomes susceptible to hydrolysis. From these findings, it can be inferred that once the CyC₁₇-Ser¹²⁴ adduct is formed, it becomes rapidly hydrolyzed in the presence of water, resulting in the cleavage and release of the methyl 2-acetyl-4-hydroxybutyrate (*i.e.* 124.1 Da), accounting exactly for the molecular mass discrepancy observed experimentally (Fig. 2*C*).

Taken together, these findings conclusively indicate that Ag85C is covalently modified by CyC analogs, leading to the inhibition of the mycolyltransferase activity and thus supporting the *in vivo* alteration of the mycolic acid pattern by these compounds.

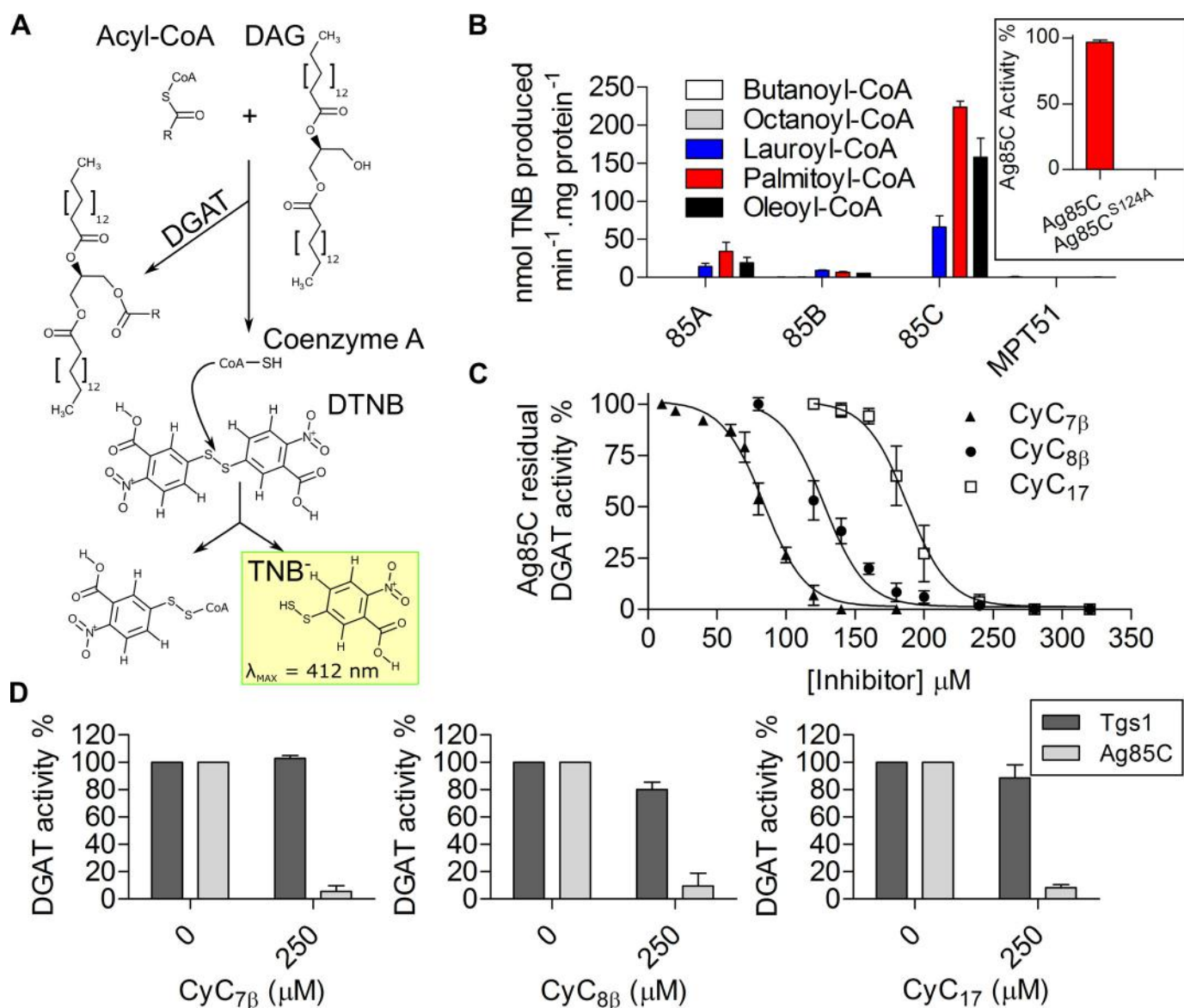


Figure 3. DGAT activity of the antigen 85 complex and inhibition by CyC analogs. A, chemical reaction occurring while determining the DGAT activity. DTNB reacts with the free-thiol group coming from the release of SH-CoA during the formation of TAG from 1,2-dipalmitoylglycerol (DAG) and a molecule of acyl-CoA. B, comparison of the DGAT activity of Ag85A, Ag85B, Ag85C, and MPT51. Enzymatic activity was determined by the colorimetry-based assay illustrated in A. Inset, activity of the wildtype and S124A Ag85C proteins using palmitoyl-CoA (C16) as acyl donor molecule. Error bars, S.D. calculated from three independent experiments. C, inhibitory effect of CyC analogs on Ag85C DGAT activity. Inhibition was performed with increasing concentrations of CyC_{7β}, CyC_{8β}, and CyC₁₇ using the colorimetry-based assay illustrated in A. The inhibitory effect was determined after 1 h of reaction. Error bars, S.D. calculated from three independent experiments. Curves for CyC_{7β}, CyC_{8β}, and CyC₁₇ were fitted using the EC₅₀ shift non-linear regression model on GraphPad with R² values of 0.9755, 0.9641, and 0.9422, respectively. D, comparison of the DGAT activity of Ag85C and Tgs1 in the absence or presence of CyC_{7β}, CyC_{8β}, and CyC₁₇.

Ag85A, -85B, and -85C express DGAT activity

Although the mycolyltransferase activity of the Ag85 complex has been established for a long time (8, 9), more recent work suggested that Ag85A mediates the transesterification of diacylglycerol using long-chain acyl-CoA to produce triglycerides (TAG), which act as storage compounds for energy and carbon (33). Ag85A contains the same catalytic triad as Ag85C or Ag85B, formed by residues Ser¹²⁶, His²⁶², and Glu²³⁰, and possesses a deep substrate-binding groove near the active-site serine, suggesting that Ag85B and Ag85C, similarly to Ag85A, may also express diacylglycerol acyltransferase (DGAT) activity. To test this hypothesis, all of the genes were cloned into pET23b, and the recombinant proteins were produced in *E. coli*

and purified from lysates by successive nickel-affinity, anion-exchange, and size-exclusion chromatography steps. Because Ag85B was poorly expressed in *E. coli*, a synthetic gene was produced by replacing low-usage codons with high-usage codons, as reported previously (34) and subsequently cloned into pET23a. All three proteins were assayed for DGAT activity in the presence of acyl-CoA with various chain lengths (from C4 to C18) as acyl donors and 1,2-dipalmitoyl-*sn*-glycerol (1,2-dipalmitin) as the acyl acceptor, as illustrated in Fig. 3A. Transesterification in the presence of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) leads to the formation of TNB, which can readily be measured at 412 nm (33, 35). In agreement with previous findings, Ag85A was found to express DGAT activity, but such

Inhibition of Ag85C by cyclopostins and cyclophostin

an activity was only detected with C12-C18 acyl-CoAs (Fig. 3B). Whereas Ag85B demonstrated lower activity than Ag85A, Ag85C showed the highest activity, which was optimal in the presence of C16-CoA. No activity was detected with the C4- or C8-containing acyl chains. We also expressed and purified the Ag85 complex-related MPT51 (FbpC1) protein, which possesses an overall structure similar to that of the Ag85 complex members but is defective in the catalytic elements required for mycolyltransferase activity (11, 16). As anticipated, MPT51 failed to express any DGAT activity, suggesting that residues important for mycolyltransferase activity are also key players in the DGAT activity, as proposed earlier for the Ser¹²⁶ in Ag85A (33). Purified Ag85C^{S124A} was next assayed with various acyl-CoA substrates, and, as shown in Fig. 3B (inset), the DGAT activity was abrogated in the mutant protein, implying that Ser¹²⁴ plays a critical role in the enzymatic reaction.

Overall, these data extend insights from previous findings and indicate that all three members of the Ag85 complex express DGAT activity, with Ag85C exhibiting the most pronounced activity. This suggests that Ag85C may also make an important contribution in TAG synthesis in *M. tuberculosis*.

CyC analogs inhibit the *in vitro* DGAT activity of Ag85C but not of Tgs1

The above-mentioned results prompted us to investigate whether CyC analogs alter the DGAT activity of Ag85C. This was achieved by incubating the purified enzyme in the presence of increasing concentrations of CyC_{7β}, CyC_{8β}, and CyC₁₇ using the colorimetric activity assay described in the legend to Fig. 3A. As expected from the previous results on the inhibition of mycolyltransferase activity, a dose-dependent inhibition of the DGAT activity with all three compounds was also observed (Fig. 3C). CyC_{7β} appeared as the most potent inhibitor, with an IC₅₀ value of $85 \pm 2 \mu\text{M}$ (*i.e.* $x_{150} = 2.8$), followed by CyC_{8β} and CyC₁₇ exhibiting IC₅₀ values of $121 \pm 4 \mu\text{M}$ (*i.e.* $x_{150} = 4.0$) and $187 \pm 3 \mu\text{M}$ (*i.e.* $x_{150} = 6.2$), respectively.

Because the TAG synthase *tgs1* in *M. tuberculosis* and *Mycobacterium abscessus* has been reported as the major contributor of TAG accumulation in the form of intracellular lipid inclusions (ILIs) in these two species (35, 36), we addressed whether the DGAT activity of Tgs1 may also be targeted by the CyC analogs. Tgs1 from *M. tuberculosis* (Rv3130c) was expressed and purified from *E. coli* and subsequently used in a DGAT assay in the absence or presence of either CyC_{7β}, CyC_{8β}, or CyC₁₇ (Fig. 3D). Whereas the activity of Tgs1 remained intact even in the presence of a 250 μM concentration of each compound, the DGAT activity of Ag85C assayed in the same conditions was almost abrogated, suggesting that Tgs1 activity is not impacted by CyC treatment.

These results indicate that CyC analogs specifically inhibit the DGAT activity of Ag85C but not of Tgs1 *in vitro*, in agreement with the fact that members of the Tgs family were not identified in our original proteomic profiling study (29).

Overexpressing Ag85C in *M. tuberculosis* is associated with reduced inhibition of TAG production by CyC₁₇

That Ag85C expresses the highest DGAT activity among the three members of the Ag85 complex prompted us to address

whether overexpression of Ag85C in *M. tuberculosis* affects the TAG content. *M. tuberculosis* was first transformed with either pMV261-Ag85C or pMV261-Ag85C^{S124A}. Overexpression of either the wildtype or the catalytically dead proteins was checked by quantitative real-time PCR (Fig. 4A, left) and by immunoblotting using two different monoclonal antibodies and purified Ag85A, -B, and -C as positive controls (Fig. 4A, right). The 17/4 monoclonal antibody recognizes a well-conserved epitope present in Ag85A and Ag85B but not in Ag85C (37). In contrast, the 32/15 antibody revealed all three antigens and the presence of more pronounced bands in the pMV261-Ag85C and pMV261-Ag85C^{S124A} lysates, which, by comparison with the 17/4 blot, could clearly be attributed to Ag85C (Fig. 4B, right). This indicates that both Ag85C variants were overproduced at comparable transcriptional and translational levels and allowed us to investigate whether this may affect the intracellular TAG content of *M. tuberculosis* (38). TAGs are often stored in the form of ILIs, which can be visualized by staining with Nile Red (39, 40). As shown in Fig. 4B (left), although a punctiform labeling corresponding to ILIs is observed in the control strain carrying the empty pMV261, Nile Red staining was much more pronounced in the strain overproducing Ag85C, and the effect returned to control levels in the strain harboring pMV261-Ag85C^{S124A}. Quantification of the fluorescence intensity over the entire length of the individual bacilli from each strain clearly indicates that large and numerous ILIs were present in the Ag85C-overexpressing strain, as compared with the control and Ag85C^{S124A} strains (Fig. 4B, right).

To check whether enhanced ILI formation coincided with increased *de novo* biosynthesis of TAG, metabolic labeling of *M. tuberculosis* cultures with sodium [2-¹⁴C]acetate was performed, followed by extraction and separation of the apolar lipid fraction by TLC. In the absence of CyC treatment, the strain carrying pMV261-Ag85C produced moderately higher amounts of TAGs than the control strain containing the empty pMV261 or the strain overexpressing Ag85C^{S124A} (Fig. 4C, left), supporting the *in vivo* contribution of Ag85C in TAG production. Importantly, exposure to CyC₁₇ inhibited TAG biosynthesis in a dose-dependent manner in the control strain, thus implying that the *de novo* biosynthesis of TAG is also targeted by CyC₁₇ (Fig. 4C). In addition, a less pronounced decrease in TAG production occurred in the strain overexpressing Ag85C as compared with the control strain, presumably because of the inherent capacity of this strain to synthesize more TAG that partially overcomes CyC₁₇ inhibition (Fig. 4C). Collectively, these results suggest that TAG production in *M. tuberculosis* is inhibited by CyC₁₇ and that this is dependent upon Ag85C DGAT activity.

Crystal structure of the CyC_{8β}-bound Ag85C

To gain insight into the mode of action of the CyC compounds, crystallization studies of Ag85C were undertaken in the presence of the three CyC inhibitors. However, diffracting crystals were only obtained with CyC_{8β} for which the X-ray structure of Ag85C bound to CyC_{8β} was solved at a resolution of 1.8 Å (Table 1). The asymmetric unit contains two molecules of Ag85C (Fig. 5A). Residues 6–282 and 8–282 for each subunit

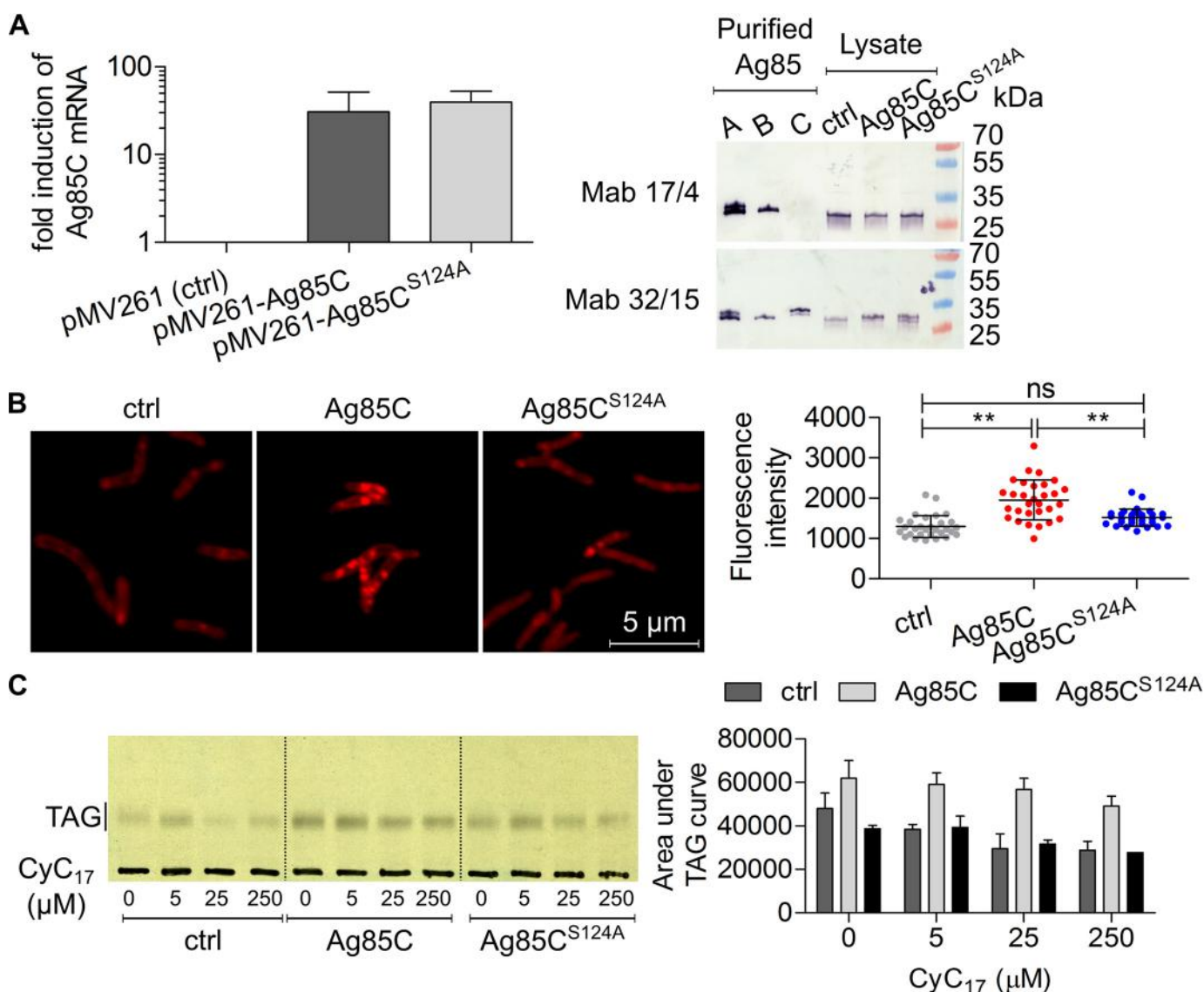


Figure 4. Biosynthesis of TAG in *M. tuberculosis* is inhibited by CyC₁₇, and is dependent upon Ag85C expression. *A*, quantitative real-time PCR analysis showing the fold increase in the Ag85C transcripts in *M. tuberculosis* mc²6230 containing either pMV261 (ctrl), pMV261-Ag85C, or pMV261-Ag85C^{S124A} (left). Western blotting using the 32/15 and 17/4 monoclonal antibodies probed against purified Ag85A/B/C and crude lysates of *M. tuberculosis* mc²6230 containing either pMV261, pMV261-Ag85C, or pMV261-Ag85C^{S124A} (right). *B*, Nile Red staining of *M. tuberculosis* strains growing exponentially (left) with the corresponding fluorescence quantification (right). Fluorescence quantification was performed on 30 bacilli of each group. Shown are the mean fluorescence and S.D. values. Means were compared by the two-tailed Mann-Whitney test. ns, non-significant; **, $p < 0.01$. Results shown are representative of two independent experiments. *C*, cultures were exposed to increasing concentrations of CyC₁₇ in 7H9^{OADC/Tween 80} and labeled with sodium [2-¹⁴C]acetate for 4 h at 37 °C with agitation. The apolar fraction was extracted to analyze *de novo* synthesis of TAG. Equal counts (50,000 cpm) of apolar fraction were loaded, and TAG was visualized on a 1D TLC plate using the solvent system petroleum ether/diethyl ether (90:10, v/v) (left). Right, densitometric analysis of TLCs. Histograms and error bars, means and S.D. values calculated from four independent experiments.

could be built, implying that the last 14 residues as well as the polyhistidine tag in the C terminus were not modeled. The structure of Ag85C has been extensively reported (17). In brief, the protein adopts a typical α/β hydrolase fold made of a central β -sheet surrounded by α -helices. The two monomers are nearly identical, as their superposition over 274 residues gives an r.m.s. deviation of 0.24 Å. However, whereas a clear electron density could be seen for the entire structure of CyC_{8 β} in one monomer, this was only the case for the headgroup of the second molecule (Fig. 5B). It is noteworthy that the extra but non-interpretable electron density (Fig. 5B) in the vicinity of CyC_{8 β} , observed in all data sets collected from either co-crystallization or soaking experiments and in various crystallization condi-

tions (data not shown), appears as a possible molecule interacting with Phe¹⁵⁰ and could be seen in both monomers. As Phe¹⁵⁰ was shown to be involved in stacking of the lipid chain of octylglucoside in the Ag85C-octylglucoside crystal structure (PDB entry 1VA5 (19)), we tried to place the acyl chain of CyC_{8 β} in this extra electron density, but refinement of this alternate conformation of CyC_{8 β} did not converge. Therefore, further modeling of this electron density blob was not pursued. Although CyC_{8 β} has clearly reacted, as evidenced by the presence of an opened ring and the MALDI-TOF data, no covalent bond between the catalytic Ser¹²⁴ residue and the phosphonate group of CyC_{8 β} was observed (Fig. 5, B and D). Therefore, CyC_{8 β} was modeled in an opened conformation (Fig. 5, B and D). CyC_{8 β}

Table 1
Data collection and refinement statistics

| | |
|---|---|
| Data collection statistics | |
| Beamline | ESRF-ID23.1 |
| Wavelength (Å) | 0.972 |
| Resolution range (Å) | 48.09–1.8 (1.86–1.8) ^a |
| Space group | <i>P</i> 2 ₁ 2 ₁ 2 ₁ |
| Unit cell | |
| Å | 67.39, 75.77, 137.32 |
| Degrees | 90, 90, 90 |
| Total reflections | 528,844 (50,342) |
| Unique reflections | 65,871 (6486) |
| Completeness (%) | 99.92 (99.85) |
| Mean <i>I</i> / σ (<i>I</i>) | 16.66 (2.24) |
| Wilson <i>B</i> -factor (Å ²) | 24.34 |
| <i>R</i> _{meas} | 0.0968 (1.049) |
| Refinement statistics | |
| Reflections used in refinement | 65,862 (6486) |
| <i>R</i> _{work} | 0.151 (0.232) |
| <i>R</i> _{free} | 0.175 (0.269) |
| No. of non-hydrogen atoms | 4895 |
| Macromolecules | 4308 |
| Ligands | 54 |
| Solvent | 533 |
| No. of r.m.s. deviations | |
| Bonds (Å) | 0.006 |
| Angles (degrees) | 0.84 |
| Ramachandran favored (%) | 96.7 |
| Ramachandran allowed (%) | 3.3 |
| Ramachandran outliers (%) | 0.00 |
| Rotamer outliers (%) | 1.12 |
| Clashscore | 1.07 |
| Average <i>B</i> -factor | 29.13 |
| Macromolecules | 27.22 |
| Ligands | 50.05 |
| Solvent | 42.43 |
| PDB accession number | 5OCJ |

^a The values in parenthesis are for the highest-resolution shell.

interacts through residues at the entrance of the Ag85C active site (Fig. 5, C and D). The polar head of CyC_{8β} is recognized through hydrogen bonds with the catalytic Ser¹²⁴ side chain as well as with the main chain of Leu⁴⁰ and the Asp³⁸ side chain via two water molecules. The Arg⁴¹ side chain completes the interaction with the headgroup of CyC_{8β} by van der Waals interaction (Fig. 5D). The long aliphatic chain of CyC_{8β} is stabilized by hydrophobic interactions involving the Ile²²², Pro²²³, Phe²²⁶, and Leu²²⁷ side chains (Fig. 5D). The distance between the phosphate of CyC_{8β} and Ser¹²⁴ of 3.6 Å clearly attests that in this crystal, the ligand is not covalently bound. Importantly, this loss of covalent binding was observed in multiple data sets collected, obtained either by soaking or co-crystallization experiments. However, the lack of covalent binding in the crystal structure does not rule out the well-known covalent inhibitory mechanism of the CyC analogs supported by MALDI-TOF mass spectrometry analyses (Fig. 2, C and D).

Furthermore, the polar headgroup of CyC_{8β} is located where trehalose, the natural substrate of the Ag85 proteins, binds, as seen in the crystal of the trehalose-bound structure of Ag85B (PDB entry 1F0P (18)) (Fig. 6A). Interestingly, the fatty acyl chain of CyC_{8β} is placed in a very hydrophobic cavity that was proposed to be part of the TDM/TMM fatty chain recognition site (17). In addition, structural comparison indicated that the important residues in Ag85C interacting with CyC_{8β} are fully conserved in Ag85B and Ag85A (Fig. 6B), strongly suggesting that CyC_{8β}, and presumably all of the other CyC analogs, may inhibit the three members of the Ag85 complex.

Discussion

Toward the generation of new lead compounds with unexplored modes of action in *M. tuberculosis*, the CyC analogs were initially designed to inhibit mycobacterial lipases (31). In particular, by covalently binding to the catalytic serine, they fully inactivated the monoacylglycerol lipase Rv0183 and the triacylglycerol lipase LipY from *M. tuberculosis* but not the mammalian gastric and pancreatic lipases (31). Subsequent biochemical studies involving the selective labeling and enrichment of captured enzymes using appropriate fluorophosphate probes in combination with CyC₁₇ resulted in the identification of 23 potential target lipolytic enzymes, all of which comprise catalytic serine or cysteine residues (29). Because they are multitarget-inhibitory compounds in mycobacteria, the use of CyC analogs could prevent the selection of drug resistance mechanisms. In addition, the lack of cytotoxicity in human cells (29) makes them attractive hits to be further evaluated.

Herein, we provide compelling evidence that at least some of the CyC analogs primarily act by inhibition of the Ag85 complex, resulting in decreased TDM formation and reduced mycolylation of AG, an essential polymer of the mycobacterial cell wall. Although one cannot rule out the possibility that the killing effect of the CyC on *M. tuberculosis* results from the simultaneous and net effect on multiple physiological targets, the inhibition of TMM and AG mycolylation is very likely to represent the major cause of growth inhibition of *M. tuberculosis*, at least in *in vitro* growing cultures. We demonstrate here that all three Ag85 members express DGAT activity *in vitro*, with Ag85C being the most active, thereby extending previous work reporting the DGAT activity of Ag85A (33). Importantly, the S124A site-directed mutation of the active site of Ag85C proved that this residue is involved in the DGAT activity of this enzyme and TAG synthesis. Although the synthesis of TAG relies on the presence of multiple TAG synthases, such as the well-characterized Tgs1 (Rv3130c) (36), our work extends the growing list of enzymes displaying DGAT activity in *M. tuberculosis*. The Ag85 proteins do not belong to the known DGAT families and do not possess the characteristic conserved heptapeptide acyltransferase motif of the mycobacterial Tgs enzymes involved in TAG biosynthesis (35, 41). Nevertheless, the DGAT activity of Ag85C, similarly to Ag85A (33), includes two consecutive reactions, the fatty acyl-CoA hydrolysis (thioesterification) and the subsequent transfer of the acyl chain to the diacylglycerol (transesterification). Overexpressing Ag85C in *M. tuberculosis* was correlated with an increase in *de novo* TAG production and formation of lipid storage inclusions. These findings establish for the first time a connection between cell wall and TAG biosynthesis by Ag85C and expand our understanding of this important enzyme in the physiology of *M. tuberculosis*. However, a direct implication of the DGAT activity of Ag85C in pathogenesis and persistence of *M. tuberculosis* requires further studies. In addition, under conditions where Ag85C is overexpressed, *M. tuberculosis* was more refractory to TAG inhibition by CyC₁₇, further emphasizing the yet unexpected contribution of Ag85C as a player in TAG biosynthesis. Inhibition of the DGAT activity of Ag85C, and therefore TAG inhibition, by the CyC compounds is very unlikely to

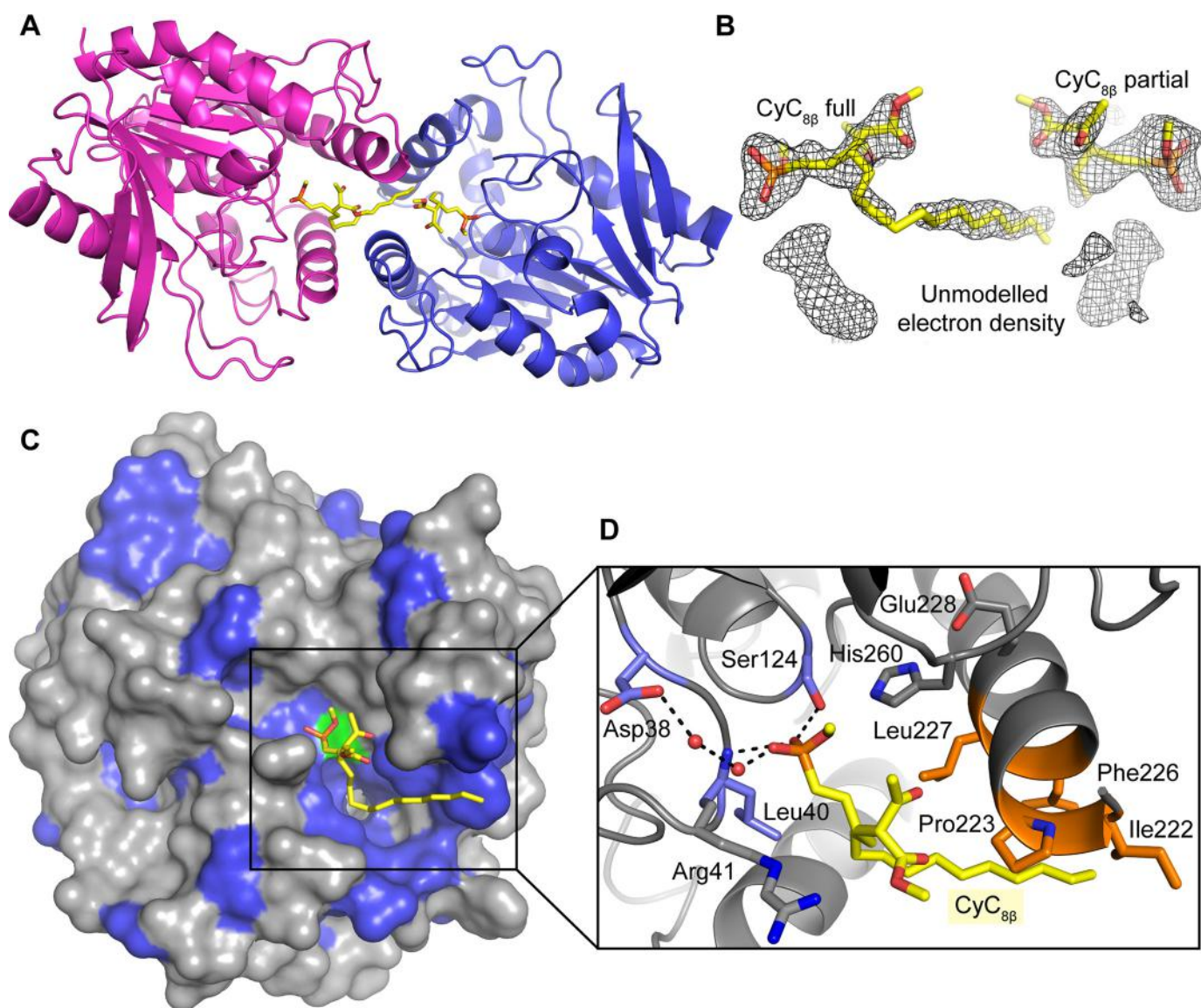


Figure 5. Structural basis for Ag85C inhibition by CyC_{8β}. *A*, crystal structure of Ag85C in complex with CyC_{8β}. The figure displays the overall asymmetric unit with the two monomers represented as *blue* and *magenta* schematics. CyC_{8β} is shown as *sticks* and *colored in yellow*. *B*, simulated annealing $F_o - F_c$ OMIT map contoured at 3σ attesting to the presence of two CyC_{8β} that could be entirely modeled for one molecule and partially for the second one. The map also reveals the presence of an extra, but non-interpretable, electron density in the vicinity of the CyC_{8β} molecule. *C*, surface representation of the Ag85C structure bound to CyC_{8β}. The hydrophobic residues are *colored in blue*, and the catalytic Ser¹²⁴ is shown in *green*. *D*, CyC_{8β} binding site. Ag85C residues involved in CyC_{8β} recognition are displayed as *blue sticks* for those involved in hydrogen bond (*black dashes*) formation. Residues in *orange* are involved in hydrophobic interactions with the acyl chain of CyC_{8β}, and Arg⁴¹ in *gray* contributes to the recognition of the CyC_{8β} headgroup by van der Waals interaction. Ser¹²⁴, Glu²²⁸, and His²⁶⁰ form the catalytic triad. *Red spheres*, water molecules.

participate in growth inhibition of *M. tuberculosis* *in vitro*, but it may have important consequences for *in vivo* survival and/or for maintaining the bacilli in a non-replicating growth phase, such as in foamy macrophages in which *M. tuberculosis* is able to hydrolyze the host-derived TAGs from lipid bodies to fatty acids, which are then reprocessed as TAGs and stored within ILIs (42, 43). In these subcellular structures, TAGs represent the primary storage source of carbon and energy, allowing the bacteria to survive in a non-replicating state and to persist inside these foamy cells, which usually line the necrotic centers of tubercle granulomas and have been proposed to be the intracellular niche of *M. tuberculosis* during latent infection (42). Although this requires further exploration, inhibiting the DGAT activity of Ag85C may help in designing new classes of

molecules that restrict entry of *M. tuberculosis* into dormancy, a strategy that would overcome mycobacterial persistence and prolonged chronic infections.

Biochemical studies involving the TAMRA-FP probe that binds to serine hydrolases along with mass spectrometry and structural analyses indicate that, in addition to covalently binding to the catalytic Ser¹²⁴, the CyC analogs could also be competing with the binding of Ag85 substrate (*i.e.* the trehalose and the acyl chain moieties of TMM). As the Ag85 complex members share similar substrate specificities, our results suggest that CyC analogs could target not only Ag85C but also Ag85B and Ag85A, an assertion reinforced by the fact that Ag85A was also identified as a potential target in the original proteomic screen approach (29). Comparison of the three structures

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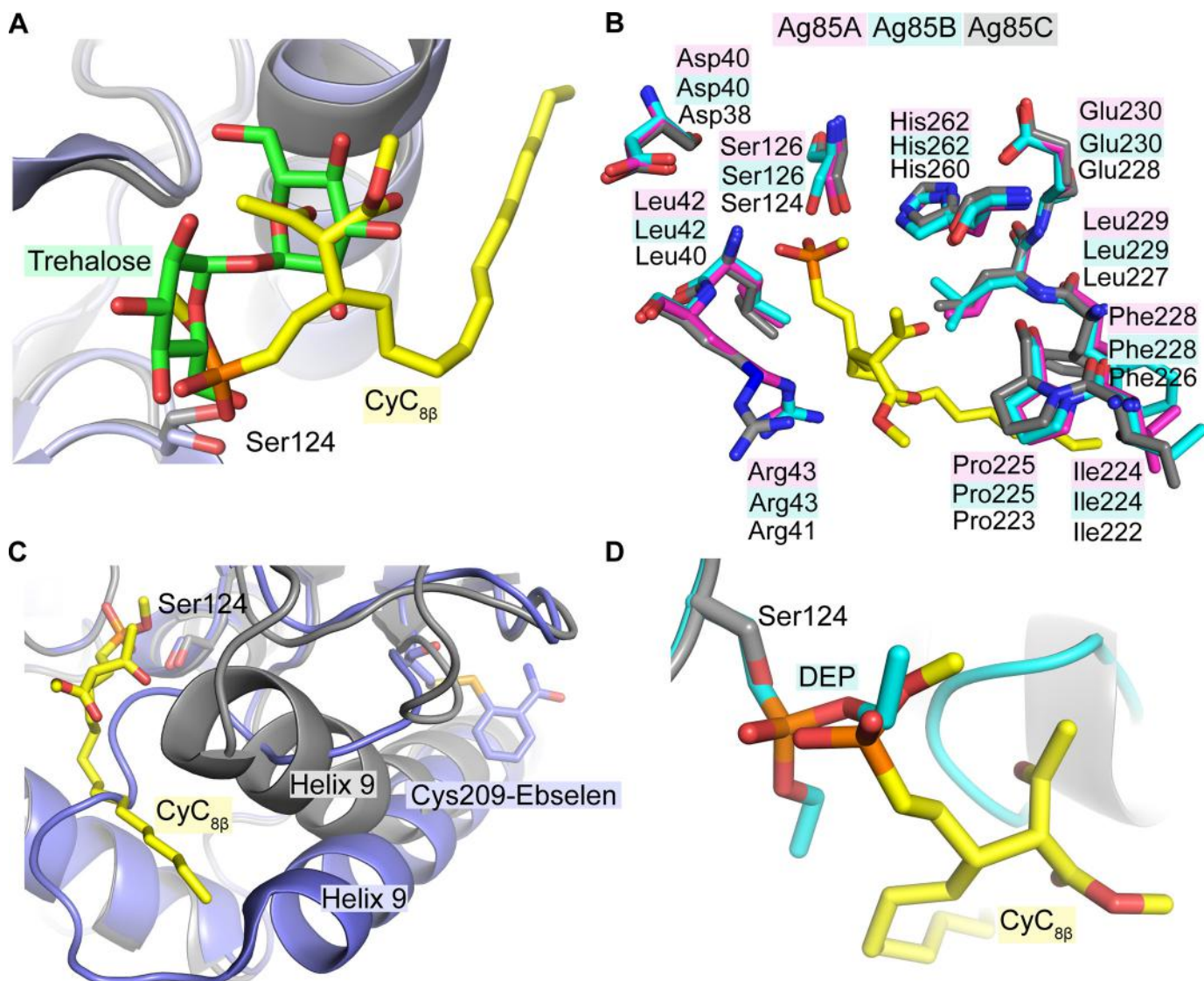


Figure 6. Mode of inhibition of the Ag85 complex by $CyC_{8\beta}$. A, superposition of the Ag85B-trehalose (PDB code 1F0P; blue) and Ag85C- $CyC_{8\beta}$ (gray) crystal structures. The headgroup of $CyC_{8\beta}$ (yellow) occupies the same site as trehalose (green). B, Ag85C residues (gray) involved in the recognition of $CyC_{8\beta}$ are all strictly conserved in Ag85B (cyan) and Ag85A (magenta). C, superposition of the Ag85C-ebiselen (PDB code 4QDU; blue) and Ag85C- $CyC_{8\beta}$ (gray) crystal structures. $CyC_{8\beta}$ binds far away from the ebiselen-binding site and does not trigger structural rearrangement of helix α_9 . D, superposition of the Ag85C-DEP (PDB code 1DQY; cyan) and Ag85C- $CyC_{8\beta}$ (gray) crystal structures. $CyC_{8\beta}$ presents a similar mode of inhibition as DEP (cyan stick), a nonspecific α/β hydrolase inhibitor.

strongly supports this hypothesis, as residues contacting $CyC_{8\beta}$ in Ag85C are strictly conserved in Ag85A and Ag85B. This is of interest, as the inhibitor I3-AG85 binding to the active site of Ag85C exhibits only strict specificity toward Ag85C and does not bind Ag85A and -B (25). Moreover, given their low α_{150} values, the three CyC compounds are able to act in near stoichiometry and alter both the mycolyltransferase and DGAT activities of Ag85C. It is noteworthy that, among the three CyCs investigated, the phosphate CyC_{17} , which appears as the best inhibitor against extracellular *M. tuberculosis*, was the least efficient when assayed on pure recombinant enzyme. However, when assayed on living bacteria, CyC_{17} clearly affected TDM synthesis and mycolylation of AG. The differences in activity with $CyC_{7\beta}$ and $CyC_{8\beta}$ may be related to the chemical properties of the phosphate *versus* phosphonate chemical groups. On the other hand, despite their high activity, phosphate inhibitors

can be subjected to hydrolysis, rendering their covalent binding potentially reversible, as shown here in the case of the CyC_{17} -Ser¹²⁴ adduct (29). Interestingly, using a chemical proteomic approach, the EZ120 β -lactone compound exhibiting strong antitubercular activity and resembling an electrophilic mimic of mycolic acids was recently found to block several serine hydrolases essential for the mycomembrane biosynthesis (44). The polyketide synthase Pks13, whose β -keto mycolate is transferred onto trehalose and reduced to yield TMM, as well as Ag85A were identified as primary targets of EZ120. However, whether this β -lactone acts similarly to the CyC inhibitors in Ag85 awaits structural determination.

Comparison of the Ag85C- $CyC_{8\beta}$ structure with that of Ag85C-ebiselen (PDB entry 4QDU (27)) shows that the mode of inhibition triggered by $CyC_{8\beta}$ is different. Ebiselen indeed covalently modifies Cys²⁰⁹, which is 13 Å away from the catalytic

Ser¹²⁴ (Fig. 6C). Inhibition by ebselen and its derivatives (azido ebselen and adamantyl ebselen) is mediated by inducing structural rearrangements of helix α 9 and the loop between helices 9 and 10 that ends in destabilizing the hydrogen bond network of the active site (27, 28, 45). Comparison of the crystal structures of Ag85C-CyC_{8 β} and the Ag85C native structure (PDB entry 3HRH) possessing the same space group and crystallized in similar conditions shows that the two structures are identical. The superposition of the two structures yields an overall r.m.s. deviation over 251 residues of about 0.19 Å. Furthermore, no local structural rearrangement was observed (data not shown). As expected, the mode of inhibition of CyC_{8 β} consists of blocking the active site (31) and not of destabilizing the overall structure and stability of the protein as reported for ebselen and its analogs (27, 45). Furthermore, the mode of action of CyC_{8 β} is more related to that of the diethyl *p*-nitrophenyl phosphate (DEP), a nonspecific α/β hydrolase inhibitor that covalently modifies the Ser¹²⁴ catalytic residue (17). Superposition of the Ag85C-DEP (PDB entry 1DQY) and Ag85C-CyC_{8 β} structures highlights the similar positioning of the phosphonate groups of the two inhibitors (Fig. 6D).

In summary, the data reported here offer a first look at the potent inhibition of the *M. tuberculosis* Ag85C by cyclopostins and cyclophostin analogs, compounds that effectively inhibit growth of extracellularly and intracellularly replicating *M. tuberculosis* and their mechanism of action. Interestingly, a recent study indicated that these compounds were also effective against clinical isolates of the *M. abscessus* complex (46), mostly encountered in cystic fibrosis patients, and known to be intrinsically resistant to most antitubercular drugs. We anticipate that the high-resolution crystal structure of Ag85C-CyC_{8 β} will now open the way to the development, through structure-based drug design, of improved inhibitors that target the Ag85 complex in various pathogenic mycobacteria.

Experimental procedures

Mycobacterial strains and growth conditions

M. tuberculosis mc²6230 (47) was grown on Middlebrook 7H10 agar plates containing OADC (oleic acid, albumin, dextrose, catalase) enrichment (Difco) and supplemented with 24 μ g/ml pantothenic acid. Liquid cultures were obtained by growing mycobacteria in Middlebrook 7H9 (Difco) supplemented with 10% OADC enrichment, 0.2% (v/v) glycerol, 0.05% (v/v), Tween 80 (Sigma), 24 μ g/ml pantothenic acid, and 25 μ g/ml kanamycin when required.

Plasmids and DNA manipulations

The *fbpC2* gene, encoding Ag85C, was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the forward primer 5'-CTA CTT CAT ATG TTC TCT AGG CCC GGT CTT CCA G-3' (NdeI site in boldface type) and the reverse primer 5'-GAG ATT CTC GAG AGC AGC AGG CGC AGC AGG GG-3' (XhoI site in boldface type). The PCR product was cloned into pET23b cut with NdeI and XhoI (New England Biolabs), enabling the incorporation of a polyhistidine tag in the C terminus of the Ag85C protein. The pET23b-*fbpA* and pET23b-*fbpC1* constructs carrying the genes encoding Ag85A and MPT51, respectively, were described previously (11). A

codon-optimized version of the *fbpB* gene, encoding Ag85B, was synthesized (GenScript) and introduced within the pET23a plasmid thanks to the NdeI and XhoI restriction sites, enabling also the incorporation of a polyhistidine tag in the C terminus of the Ag85B protein. The Ag85C^{S124A} mutant was obtained by using the PCR-driven overlap extension method (48). Briefly, two separate PCRs were set up with the Phusion[®] DNA polymerase (Thermo Fisher Scientific). The first one was set up with the forward primer used to amplify the wildtype *fbpC2* gene and the reverse internal primer 5'-AAG ACC CAC CGC CGC GTT-3'. The second one was set up with a forward internal primer, 5'-AAC GCG GCG GTG GGT CTT GCG ATG TCG GGC GGT TCC G-3', overlapping the internal reverse primer and containing the nucleotide substitution (changed nucleotide in boldface type) with the reverse primer used to amplify the wildtype *fbpC2* gene. The purified PCR products were heterodimerized by heating to 95 °C for 1 min, followed by cooling to 60 °C for 10 min in the presence of Phusion[®] DNA polymerase and dNTPs to generate a double-stranded hybrid. A last step of PCR was performed with the primers used to amplify the wildtype *fbpC2* gene with the hybrid product obtained in the previous step as template. The mutated *fbpC2* gene was finally cloned like the wildtype gene into pET23b and subjected to DNA sequencing to confirm the proper introduction of the mutation. The coding sequence of the gene *Rv3130c*, which encodes Tgs1 from *M. tuberculosis*, was PCR-amplified using the forward primer 5'-GAG GAG CCA TGG aga atc tgta ctt cca ggg AAT GAA TCA CCT AAC GAC ACT TGA CGC-3' (NcoI site in boldface type, tobacco Etch virus protease cleavage site in lowercase type) and the reverse primer 5'-ACG AGG AAG CTT TCA CAC AAC CAG CGA TAG CGC T-3' (HindIII site in boldface type). The PCR amplicon was treated with NcoI and HindIII and ligated to NcoI-HindIII-linearized pET32a. This plasmid containing the polyhistidine and thioredoxin as fusion tags in the N-terminal position was used to produce soluble recombinant Tgs1.

Expression and purification of the individual Ag85 antigens and MPT51

All four plasmids harboring the *fbpA*, *fbpB*, *fbpC2*, and *fbpC1* genes were used to transform the *E. coli* C41 (DE3) expression strain. Transformed bacteria were grown in Luria-Bertani medium containing ampicillin (200 μ g/ml) until the A₆₀₀ reached 0.6. Bacterial cultures were then placed on icy water for 30 min before induction with 1 mM isopropyl β -D-1-thiogalactopyranoside and further incubated at 16 °C for 20 h. Bacterial pellets were collected by centrifugation (6,000 \times g, 4 °C, 1 h) and resuspended in lysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 20 mM imidazole, 5 mM β -mercaptoethanol, 1 mM benzamide). Lysates were sonicated and clarified by centrifugation (27,000 \times g, 4 °C, 45 min) before purification by nickel-affinity chromatography with nickel-nitrilotriacetic acid-Sepharose beads and elution with lysis buffer containing 250 mM imidazole without benzamide (GE Healthcare). Proteins were next dialyzed against 50 mM Tris-HCl, pH 8.0, and 5 mM β -mercaptoethanol buffer and loaded on an anion-exchange HiTrap[®] Q Fast Flow column (GE Healthcare). The protein was eluted with a linear NaCl gradient. The final step of purification was by

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size-exclusion chromatography using a SuperdexTM 75 10/300 GL column (GE Healthcare). Proteins were eluted in potassium phosphate buffer (50 mM KH₂PO₄/K₂HPO₄, pH 7.6) for DGAT activity assessments. Ag85C was eluted in a sodium phosphate buffer (50 mM NaH₂PO₄/Na₂HPO₄, pH 6.0) for mycolyltransferase activity assessments and in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl for crystallization experiments and stored at 4 °C.

Expression and purification of Tgs1

The *M. tuberculosis* Tgs1 was overproduced in *E. coli* and purified. Briefly, *E. coli* BL21 RosettaTM 2 was freshly transformed with pET32a-*tgs1*. Exponentially growing bacteria cultured in 2 liters of NYZ Broth (BD Biosciences) were cooled on icy water for 30 min, and 1 mM isopropyl β-D-1-thiogalactopyranoside was added before incubation at 16 °C for 16 h with agitation (200 rpm). Bacteria were then collected by centrifugation, the medium was discarded, and the pellet was resuspended in lysis buffer containing 10% glycerol, which was maintained for all subsequent buffers used. Lysates were produced and subjected to purification via nickel affinity chromatography. His-tagged tobacco etch virus protease was added to the eluted protein solution at a 1:50 (w/w) ratio, and the mixture was dialyzed overnight before again being subjected to nickel-affinity chromatography. The fraction that flowed through the nickel-nitrilotriacetic acid column, containing tagless Tgs1, was concentrated and subjected to size-exclusion chromatography using a Bio-rad ENrich SEC 650 (Bio-rad) and as buffer 100 mM K₂HPO₄/KH₂PO₄, pH 7.5, supplemented with 400 mM NaCl and 10% glycerol. The fractions containing active Tgs1 were pooled and concentrated to 0.1 mg/ml.

RNA extraction, cDNA production, and quantitative real-time PCR

Mycobacterial RNA was purified using the Nucleospin RNA kit (Macherey Nagel) and assessed for purity on a NanoDrop spectrometer and for integrity using a BioAnalyzer (Agilent). Subsequently, RNA was treated by DNase I (Life Technologies) and converted to cDNA using the SuperScript V reverse transcriptase kit (Life Technologies). Quantitative real-time PCR was performed using the LightCycler 480 SYBR Green master mix (Roche Applied Science) and primers specific to the house-keeping control gene *sigA* (forward, 5'-TGT ACT CGT GCG CAG TAA AG-3'; reverse, 5'-GTC GAA TGT CGG CGT TGA TA-3') and *fbpC2* (forward, 5'-CAG TTT CTA CAC CGA CTG GTA TC-3'; reverse, 5'-TCT CTC TGG TAA GGA AGG TCT C-3'). Triplicate data were analyzed by the ΔΔC_p method with correction for PCR efficiency.

Western blotting

Lysates of *M. tuberculosis* mc²6230 wildtype or overexpressing Ag85C were prepared and subjected to Western blot analysis as described previously (49).

DGAT and mycolyltransferase assays

The DGAT activity assay was performed for 1 h at 37 °C using a protocol reported earlier (33). Briefly, the reaction mixture was composed of 400 μM 1,2-dipalmitoyl-*sn*-glycerol and a 500 μM concentration of the different acyl donor molecules tested

(butanoyl-CoA, octanoyl-CoA, lauroyl-CoA, palmitoyl-CoA, and oleoyl-CoA (Sigma-Aldrich)) in 50 mM potassium phosphate buffer, pH 7.6, containing 2% DMSO. The enzyme concentration in the reaction was 3 μM (0.5 μM in the case of Tgs1). At the end of the assay, an equal volume of DTNB (360 μg/ml) was added to the reaction, and the absorbance was measured at 412 nm with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), enabling the calculation of the specific activity of the enzymes (nmol of TNB produced × min⁻¹ × mg of protein⁻¹).

The mycolyltransferase activity assay was performed for 15 min at 35 °C based on a procedure described previously (27). Measurements were taken every 15 s using a Multimode Microplate Reader POLARstar[®] Omega (BMG Labtech), and the activity of Ag85C was calculated at the maximum rate of the reaction. The reaction mixture was composed of 50 mM sodium phosphate (pH 6.0) containing 2% DMSO, 4 mM trehalose, and 12.5 μM resorufin butyrate (Sigma-Aldrich). The resorufin butyrate was dissolved in DMSO and diluted 100-fold in the reactions. The enzyme concentration in each reaction was 5.5 μM. Data presented were obtained from three independent experiments and analyzed by non-linear regression using GraphPad Prism version 5 software.

Inhibition of the DGAT and mycolyltransferase activity

CyC_{7β}, CyC_{8β}, and CyC₁₇ were synthesized as described previously (31, 50). To study the inhibitory effect on DGAT activity, a 30 μM concentration of either Ag85A, Ag85B, Ag85C, or MPT51 was co-incubated with increasing concentrations of CyC_{7β}, CyC_{8β}, and CyC₁₇ for 1 h at room temperature in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.6), 10% DMSO, and 0.5 times the critical micelle concentration of *n*-dodecyl β-D-maltoside. Inhibition of the mycolyltransferase activity was determined using 55 μM of Ag85C co-incubated with increasing concentrations of CyC_{7β}, CyC_{8β}, and CyC₁₇ for 30 min at room temperature in 50 mM sodium phosphate buffer (pH 6.0), 10% DMSO, and 0.5 times the critical micelle concentration of *n*-dodecyl β-D-maltoside. Ag85C and Ag85C^{S124A} pretreated or not with the CyC analogs were further incubated with 10 μM ActivX TAMRA-FP probe (Thermo Fisher Scientific) for 1 h at room temperature in the darkness. The reaction was stopped by adding 5× Laemmli reducing buffer followed by boiling, and proteins were separated by 12% SDS-PAGE. Subsequently, TAMRA FP-labeled proteins were detected by fluorescent gel scanning (TAMRA: λ_{ex} 557 nm, λ_{em} 583 nm) using the Cy[®]3 filter of a ChemiDoc MP Imager (Bio-Rad) before staining of the gels with Coomassie Brilliant Blue dye.

Overexpression of Ag85C variants in *M. tuberculosis*

The *Rv0129c* gene was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the forward primer 5'-CCC AGC TTG TTG ACA GGG TTC GTG-3' and the reverse primer 5'-ACC ATG GAT CCC TAG GCG CCC TGG GCG-3' (BamHI site in boldface type). After amplification, the PCR product was digested with BamHI (Promega) and cloned into MscI/BamHI-digested pMV261, thus placing the *Rv0129c* open reading frame under control of the *hsp60* promoter to yield pMV261-Ag85C. The pMV261-Ag85C^{S124A} mutant plasmid was constructed by the QuikChange method using

pMV261-Ag85C as template, Phusion[®] DNA polymerase (Thermo Fisher Scientific), the forward primer 5'-GCG GCG GTG GGT CTT **GCG** ATG TCG GGC GGT TCC-3', and the reverse primer 5'-GGA ACC GCC CGA CAT **CGC** AAG ACC CAC CGC CG-3' (Ser → Ala mutation in boldface type). The DNA sequence was confirmed by DNA sequencing. *M. tuberculosis* mc²6230 was subsequently electrotransformed with pMV261 as a control, pMV261-Ag85C, or pMV261-Ag85C^{S124A}.

Whole-cell radiolabeling experiments and lipid analysis

To investigate the CyC-induced changes in the lipid profile, increasing drug concentrations were added to exponentially growing *M. tuberculosis* mc²6230 cultures grown in Middlebrook 7H9 supplemented with OADC enrichment and Tween 80 and 20 μg/ml pantothenate for 1 h. Subsequently, metabolic labeling of lipids was performed by adding 1 μCi/ml sodium [2-¹⁴C]acetate (56 mCi/mmol; American Radio Chemicals) for an additional 6 h at 37 °C. Cells were harvested and delipidated, as described previously (51). The apolar lipid fraction containing TMM and TDM was separated on a 1D TLC plate using the solvent system chloroform/methanol/water (40:8:1, v/v/v) and revealed after exposure to a film. Similarly, the apolar lipid fraction, which also contains TAG, was separated on a 1D TLC plate using the solvent system petroleum ether/diethyl ether (90:10, v/v) and revealed after exposure to film. Delipidated cells were further processed to extract the arabinogalactan-bound mycolic acids (52) and analyzed by TLC/autoradiography using hexane/ethyl acetate (95:5, v/v) run twice in the first dimension followed by exposure to a film to reveal ¹⁴C-labeled mycolic acid methyl esters.

Fluorescent microscopy experiments

Wildtype *M. tuberculosis* mc²6230 or strains harboring either the pMV261-Ag85C or its variant pMV261-Ag85C^{S124A} were stained with Nile Red fluorescent probe (Interchim), as described previously (40). Approximately 7.5×10^7 cells (OD 1.5) were collected at $9,000 \times g$ for 3 min, washed twice with 500 μl of PBS-Tween 0.05%, and resuspended in 300 μl of PBS. Nile Red (15 μl of a solution at 0.5 mg/ml solubilized in ethanol) was added to the bacterial suspension, which was further incubated for 30 min at 37 °C in the darkness. Cells were then centrifuged, washed twice with PBS-Tween 0.05%, and resuspended in 300 μl of PBS. Bacteria were spotted between a 170-μm-thick coverslip and a 1.5% agarose-PBS pad. Image acquisition was performed with an OLYMPUS FV1000 confocal microscope at $\lambda_{\text{ex}} \lambda_{\text{em}} = 530/590 \pm 10$ nm, and images were processed and analyzed using ImageJ.

Mass spectrometry

Mass analyses were performed on a MALDI-TOF-TOF Bruker Ultraflex III spectrometer (Bruker Daltonics, Wissembourg, France) controlled by the Flexcontrol version 3.0 package (Build 51). This instrument was used at a maximum accelerating potential of 25 kV and was operated in linear mode using the *m/z* range from 20,000 to 100,000 (LP_66 kDa method). Five external standards (Protein Calibration Standard II, Bruker Daltonics) were used to calibrate each spectrum to a mass accuracy within 200 ppm. Peak picking was performed

with Flexanalysis version 3.0 software (Bruker) with an adapted analysis method. To eliminate salts from the samples, 10 μl of each preparation was submitted to a desalting step on a C4 Zip-Tip μcolumn (Millipore). 1 μl of desalted sample was mixed with 1 μl of α-cyano-4-hydroxycinnamic acid matrix in a 50% acetonitrile, 0.3% TFA mixture (1:1, v/v). 1 μl was spotted on the target, dried, and analyzed with the LP_66 kDa method. Peak picking was performed with Flexanalysis version 3.0 software (Bruker) with an adapted analysis method. Parameters used were as follows: SNAP peak detection algorithm, S/N threshold fixed to 6, and a quality factor threshold of 30.

Crystallization, data collection, structure determination, and refinement

Crystals were grown in sitting drops at 18 °C by mixing 0.8 μl of protein (in 50 mM Tris-HCl, pH 8.0, and 200 mM NaCl) at a concentration of 8 mg/ml with 0.8 μl of reservoir solution consisting of 0.2 M magnesium chloride hexahydrate, 0.1 M sodium citrate tribasic dihydrate, pH 5.0, and 10% (w/v) polyethylene glycol 20,000. 1-month-old crystals were then soaked for 24 h with a final concentration in the drop of 5 mM CyC_{8β}. Crystals were fished with a litholoop and flash-cooled in liquid nitrogen without any cryoprotection. Data collection was performed at the ID-23.1 beamline at the ESRF synchrotron (Grenoble, France). Data were processed with XDS (53), and the structure was solved by molecular replacement with the structure of Ag85C as search model (PDB code 3HRH (54)) and using Phaser from the PHENIX software suite (55). Manual adjustments of the model were performed with Coot (56), and the structure was refined to 1.8 Å with PHENIX. PDB coordinates and structure factors were deposited in the Protein Data Bank under accession number 5OCJ. Data collection and refinement statistics are displayed in Table 1.

Author contributions—A.V., J.-F.C., S.C., M.B., and L.K. conceptualization; A.V., M.R., P.F., and L.C. data curation; A.V., M.R., P.C.N., P.F., L.C., R.R.P., G.R.G., J.-F.C., S.C., and M.B. investigation; A.V., M.R., P.C.N., P.F., L.C., R.R.P., G.R.G., J.-F.C., S.C., and M.B. methodology; A.V., M.R., P.F., L.C., C.D.S., J.-F.C., S.C., M.B., and L.K. writing-review and editing; P.C.N. visualization; C.D.S. and S.C. resources; M.B. and L.K. supervision; L.K. funding acquisition; L.K. validation; L.K. writing-original draft; L.K. project administration.

Acknowledgments—We thank K. Huygen for kindly providing the 17/4 and 32/15 monoclonal antibodies, W. R. Jacobs, Jr., for *M. tuberculosis* mc²6230, and P. Santucci for help in fluorescent microscopy experiments. This work benefited from the facilities and expertise of the Platform for Microscopy of IMM. We thank the ESRF and SLS beamline staffs for support during data collection. Mass spectrometry analyses were done using the mass spectrometry facility of Marseille Proteomics, supported by IBISA (Infrastructures Biologie Santé et Agronomie), the Cancéropôle PACA, the Provence-Alpes-Côte d'Azur Region, the Institut Paoli-Calmettes, and the Centre de Recherche en Cancérologie de Marseille.

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