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Accepted Manuscript

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PII: S0378-1135(07)00303-3
DOI: doi:10.1016/j.vetmic.2007.06.002
Reference: VETMIC 3726

To appear in: *VETMIC*

Received date: 25-4-2007
Revised date: 30-5-2007
Accepted date: 4-6-2007

Please cite this article as: Bourg, G., O'Callaghan, D., Boschiroli, M.L., The genomic structure of *Brucella* strains isolated from marine mammals gives clues to evolutionary history within the genus, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.06.002

The genomic structure of *Brucella* strains isolated from marine mammals gives clues to evolutionary history within the genus.

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Abstract

The genomic structure and the restriction maps were studied in 24 *Brucella* strains isolated from marine mammals. From *Spe*I restriction profiles, the strains could be ascribed to three clonal groups, each corresponding to a specific host. Cross contamination between exclusively terrestrial and exclusively marine hosts is unlikely suggesting the divergence of the different species of the genus *Brucella* may have taken place 60 million years ago, concomitant with the radiation of their mammalian hosts (Artiodactyla) from other mammalian orders.

Key words: *Brucella*; genomic organisation; physical map; evolution; marine mammal; PFGE

1. Introduction

Brucella is a small Gram negative bacterium usually isolated from ruminants, pigs and rodents. Classically the genus is divided into six species which are further subdivided into several biovars, each infecting a preferential but not exclusive host (Boschiroli *et al.*, 2001). From the mid 1990s *Brucella*-like bacteria were isolated from carcasses of marine mammals, such as seals, dolphins, porpoises and whales. These bacteria have been found in a wide range of tissues and have been shown to cause both abortion and meningoencephalitis (Foster *et al.*, 2002; Ohishi *et al.*, 2003; Miller *et al.*, 1999; Gonzalez *et al.*, 2002) however very few human infections have been reported (Sohn *et al.*, 2003; Brew *et al.*, 1999; McDonald *et al.*, 2006). These strains isolated form marine mammals could not be ascribed to the known six species based on classical typing methods but could, however, be subdivided into three groups based on CO_2 dependence, galactose oxidation, dominant antigen and animal host (Jahans *et al.*, 1997).

Over the past few years, molecular analysis using DNA/DNA hybridization and ribotyping (Verger *et al.*, 2000), genomic fingerprinting of *Xba*1 profiles using PFGE (Jensen *et al.*, 1999), analysis of insertion sequence profiles (Cloeckaert *et al.*, 2000) and DNA polymorphism in outer membrane protein genes (Cloeckaert *et al.*, 2001a; Vizcaino *et al.*, 2004), has confirmed that the marine strains are genetically distinct from the terrestrial strains and, therefore, two new species, *Brucella pinnipediae* (group I strains) and *Brucella cetaceae* (group II and III strains) have been proposed (Cloeckaert *et al.*, 2001b). However, these names have not yet been validly published and there remains some debate about the genetic relationships within this group. Here we present the physical maps of the genomes of three representative strains of marine *Brucella* which suggest that the group II and III strains represent two distinct species.

2 Materials and Methods

2.1 Bacterial Strains

The *Brucella* strains used in this study and their hosts are listed in Table 1. Isolates were from seals, dolphins, porpoises, an otter and a minke whale (Jahans *et al.*, 1997; Clavareau *et al.*, 1998).

2.2 Pulsed Field Gel Electrophoresis

PFGE was performed as previously described (Jumas-Bilak *et al.*, 1995; Michaux-Charachon *et al.*, 1997). Briefly, intact genomic DNA was prepared from bacterial embedded in agarose plugs and subjected to PFGE (BioRad CHEF DRII), either undigested or after digestion with *Pac*I or *Spe*I. Pulse conditions are described in figure legends.

2.3 Phylogenetic Analysis

Similarity between strains was calculated as the Dice coefficient as described by Grothues and Tummler (Grothues and Tummler, 1991) and clustered using the Phylip programme.

3. Results and Discussion

3.1 Restriction fragment length polymorphism (RFLP) within marine *Brucella* strains.

The genomes of *Brucella* marine strains contain two chromosomes with sizes of about 2.1 Mb and 1.15 Mb, similar to that of the reference strain of the genus, *B. melitensis* 16M (data not shown). The macrorestriction profiles obtained for genomic DNA digested by *Spe*I are shown in Fig. 1. The restriction patterns fell into three groups that were identical to the groups defined by Jahans *et al* (Jahans *et al*., 1997) based on classical typing methods. The group I pattern was found in strains isolated from common, grey and hooded seals, and an otter, group II pattern in strains isolated from common and striped dolphins and group III pattern in strains isolated from porpoises, a white-sided dolphin and a mink whale. One 182 kb fragment was missing from two group I strains (39-94 and 44-94; isolated from common seals). Strain 56-94 (isolated from a hooded seal) shows the most divergence from the group as it lacks the 182 kb fragment, a 62 kb and has other minor differences in the 20-30 kb region. The 62 kb fragment missing from strain 56-94 is specific to group I strains, since it failed to hybridize with genomic DNA from any of the classical *Brucella* species or other marine mammal strains (not shown). Comparison of the published *Brucella* genome sequences (Paulsen *et al.*, 2002; Halling *et al*., 2005; DelVecchio *et al*., 2002) suggests that such species specific regions are rare in the genus, however, we have previously reported a 34kb *Spe*1 fragment specific to *B. ovis* (Michaux-Charachon *et al.*, 1997). Digestion of the genomic DNA of the same strains by *Xba*I gives a similar strain grouping (Jensen *et al*.,

1999). In group II strains, an inversion in the large chromosome (see below) has increased the size of the 330kb fragment to 390 kb in strain 5-94.

3.2 Restriction mapping of the *Brucella* marine isolates. The *Pac*I and *Spe*I restriction maps and localization of *rrn* loci and insertion sequences were determined as described previously (Michaux-Charachon *et al.*, 1997; Jumas-Bilak *et al.*, 1998). The maps are shown in Fig. 2, together with the previously published map of *B. melitensis* 16M (Michaux-Charachon *et al.*, 1997). The location of the restriction sites on the large chromosome of the marine strain shows few modifications when compared to that of *B. melitensis*. In a dolphin isolate, strain 5-94, there is a large inversion of at least 550 kb in the large chromosome. This rearrangement was first suggested by our observation that the 305 kb and 420 kb *Spe*I fragments from *B. melitensis* both hybridized with the 390 kb and 260 kb *Spe*I fragments of strain 5-94. The two fragments flanking the inversion contain copies of IS*6501*IS*711*, suggesting a possible role for the insertion sequence in the rearrangement. On the contrary, the maps of the small chromosome differed greatly from one strain to another, with numerous indels (including the 62 kb *Spe*I fragment unique to the seal isolates) and/or with creation or loss of restriction sites. The reason for this observation is unknown. All the strains contained three *rrn* loci, two in the large chromosome and one in the small.

3.3 Phylogenetic analysis of the marine *Brucella* isolates. A phylogenetic tree (Fig. 3) was obtained from the restriction data to complete the tree proposed previously (Michaux-Charachon *et al.*, 1997). The tree is unrooted, since the macrorestriction patterns are very specific for *Brucella* and definition of a related extra group to place the root is artificial. The marine isolates form a new group, which originates very near the *B. ovis* branch. Within this group, the three clones are separated from each other by branches that are deeper than those

between members of the *melitensis-abortus* group or the *suis* group. This suggest a probable ancient divergence of the three restriction groups described within the marine isolates.

3.4 Evolutionary history of the *Brucella* genus The genus *Brucella* was proposed to be monospecific based on the high levels of homology revealed by DNA-DNA hybridization (Verger *et al.*, 1985). This, however, does not reflect the 'classical' classification based on phenotypic characteristics and host specificity and recently the 'classical' system has been readopted. Since *Brucella* grow poorly in the environment and since each species infects a preferential host, we and others proposed that each species is an evolutionary line adapted to a particular mammalian host (Michaux-Charachon *et al.*, 1997; Moreno *et al.*, 2002; Michaux-Charachon *et al.*, 2002). It has been proposed that *B. suis* is the closest to the common ancestor of the genus (Moreno *et al.*, 2002; Jumas-Bilak *et al.*, 1998). Recent investigation of the distribution of a 18.3 kb genomic island inserted downstream of *guaA* supports this theory, being present in the genomes of *B. suis, B. neotomae* and the marine strains, but not *B. ovis, B. abortus* and *B. melitensis* (Lavigne *et al.*, 2005). Interestingly, three point mutations in the 23S *rrn* gene group the marine strains with *B. melitensis, B. ovis* and *B. neotomae* (Halling and Jensen, 2006), giving rise to the question of whether the loss of the incP island occurred once or at different times during the evolution of the genus. The divergence could be concomitant either with the radiation of mammalian species, since each natural host harbors a particular clone, or with the emergence of domestication, allowing cross-contamination by close contact of different animal species. The phylogeny of the marine strains supports arguments for a divergence of the *Brucella* strains concomitant with the radiation of mammals, since each of the different hosts is infected by a specific clone and cross contamination between exclusively terrestrial and exclusively marine hosts is unlikely. Moreno et al (Moreno *et al.*, 2002) have suggested that *B. abortus* and *B. melitensis* became

isolated about 20 million years ago with the radiation of the artiodactyls. This isolation could be more ancient, since seals and otters belong to the order of Carnivora, which diverged from the Artiodactyla more than 60 million years ago, and since wild rodents, another anciently isolated order, are natural hosts for *B. suis*. However, a cross-contamination cannot be excluded in this last case, rodents and wild pigs having similar territories. Finally, our results further highlight how the results of the classical typing methods reflect the groups defined by genome organization. From the tree in Fig 3 (where the length of a branch is represents evolutionary distance), we divergence between the three marine groups is far greater than that between, for example, *B. melitensis* and *B. abortus*, suggesting that the three groups could each be classified as a separate species. Further, recent data from both VNTR and MLST typing also suggest that the strains can be divided into three groups (A Whatmore, personal communication). These data are compatible with the proposition in the latest Bergey's Manual of three new species; *B. phocae* (seals), *B. phoecoenae* (porpoises) and *B. delphini* (dolphins) (Corbel and Banai, 2005). Determination of the complete genome sequences of these, and more *Brucella* strains is the next step in deciphering their evolutionary history.

4. Acknowledgments

We thank Alistair Macmillan, Bruno Garin-Bastuji and Jacques Godfroid for providing *Brucella* strains. We thank Michel Ramuz for guidance and encouragement throughout this work. Maria Laura Boschiroli was supported by fellowships from, CONICET and the UNU-BIOLAC. Work in our laboratory is supported by INSERM, the European Community (QLK2-CT-2001-01200), Université de Montpellier 1 (BQR), La Region Languedoc Roussillon and La Ville de Nimes.

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Table 1. *Brucella* strains included in this study.

Brucella strain	Host Vernacular name	Scientific name
Group I*		
2-94	Common seal	*Phoca vitulina*
40-94	Common seal	*Phoca vitulina*
41-94	Common seal	*Phoca vitulina*
48-94	Common seal	*Phoca vitulina*
46-94	Common seal	*Phoca vitulina*
54-94	Common seal	*Phoca vitulina*
61-94	Grey seal	*Halicherus grypus*
55-94	Otter	*Lutra lutra*
39-94	Common seal	*Phoca vitulina*
44-94	Common seal	*Phoca vitulina*
56-94	Hooded seal**	*Cystophora cristata*
Group II		
47-94	Common dolphin	*Delphinus delphis*
14-94	Common dolphin	*Delphinus delphis*
59-94	Striped dolphin	*Stenella coeruleoalba*
5-94	Striped dolphin	*Stenella coeruleoalba*
Group III		
202-R	Mink whale	*Balaenoptera acutorostrata*
49-94	White-sided dolphin	*Lagenorhynchus actus*
1-94	Harbour porpoise	*Phocoena phocoena*
34-94	Harbour porpoise	*Phocoena phocoena*
35-94	Harbour porpoise	*Phocoena phocoena*
36-94	Harbour porpoise	*Phocoena phocoena*
45-94	Harbour porpoise	*Phocoena phocoena*
52-94	Harbour porpoise	*Phocoena phocoena*
57-94	Harbour porpoise	*Phocoena phocoena*

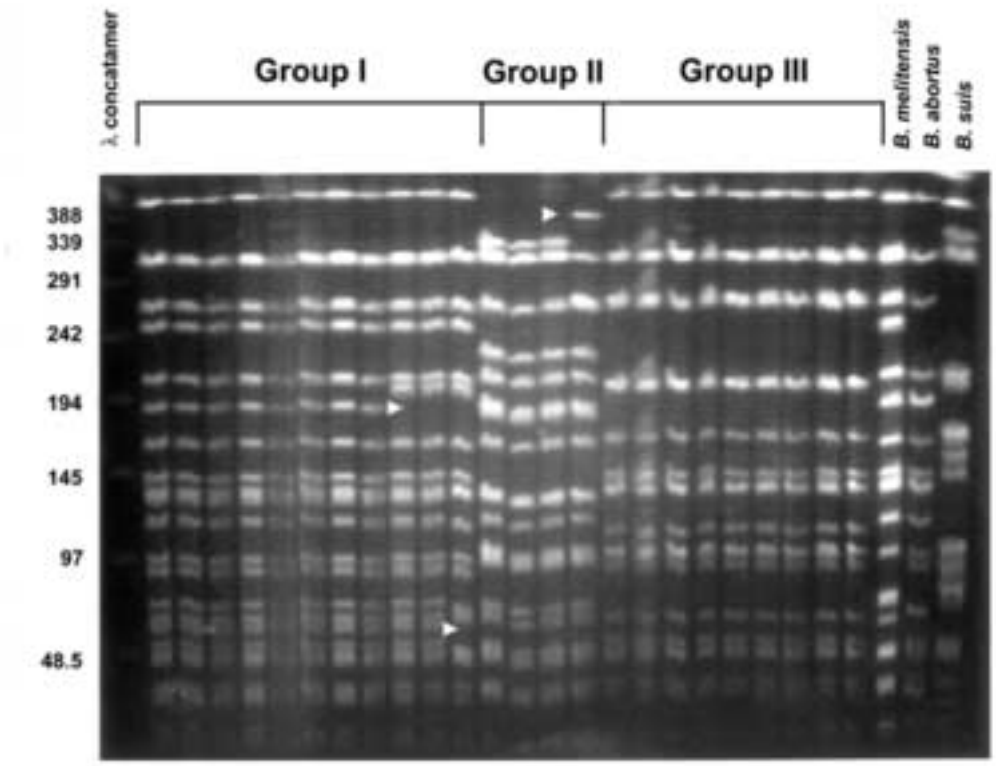
* Groups I, II and III contain strains with a similar *Spe*I-restriction pattern .
** The *Spe*I profile of this isolate differs from the other strains included in this group by the absence of a 62 kb fragment.

Figure Legends

Figure 1. Pulsed field gel electrophoresis of *Spe*I-digested DNA from *Brucella* marine isolates. *Spe*I fragments were separated in a contour clamped electric field apparatus (CHEF-DRII Biorad) with pulse ramps of 35 s - 4 s for 40 h at 180 V. The strains are organized as described in Table 1. λ concatamer size markers are on the left of the figure. Patterns of *Spe*I-digests of *B. melitensis* 16M, *B. abortus* 544 and *B. suis* 1330 DNA are shown at the right. Arrowheads indicate bands migrating differently or absent.

Figure 2. *Spe*I-*Pac*I restriction maps of the chromosomes of *B. melitensis* 16M, strains 59-94 and 5-94 (isolated from dolphins), 34-94 (isolated from a porpoise) and 2-94 (isolated from a seal). The two circular chromosomes are represented in linear form, each one starting from a conserved *Spe*I fragment. A: large chromosomes; B: small chromosomes. For each chromosome map, *Spe*I sites are located above, and *Pac*I sites below. Sizes are given in kilobases for all the restriction fragments of the *B. melitensis* chromosomes, and only for those different from *B. melitensis* for the other species. Fragments carrying *rrn* loci are represented by open squares, and fragments carrying IS*6501*/IS*711* copies by stars. The inversion in the large chromosome of strain 5-94 is shown by two broken lines.

Figure 3. Phylogenetic tree of the marine isolates and the different species and biovars of the genus *Brucella* (modified from (Boschiroli *et al.*, 2001). The terrestrial strains are represented by the reference strains for each species and/or biovar using data from (Michaux-Charachon *et al.*, 1997). *B. abortus* contains two major groups, represented by Bv 1 and 5, due to the 600kb inversion in the small chromosome in certain strains.



Figure