Intrinsic and selected resistance to antibiotics binding the ribosome: analyses of Brucella 23S rrn, L4, L22, EF-Tu1, EF-Tu2, efflux and phylogenetic implications

Shirley M Halling* and Allen E Jensen

Address: Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, 2300 Dayton Avenue, Ames, IA 50010, USA

Email: Shirley M Halling* - shalling@nadc.ars.usda.gov; Allen E Jensen - ajensen@nadc.ars.usda.gov

* Corresponding author

Abstract

Background: Brucella spp. are highly similar, having identical 16S RNA. However, they have important phenotypic differences such as differential susceptibility to antibiotics binding the ribosome. Neither the differential susceptibility nor its basis has been rigorously studied. Differences found among other conserved ribosomal loci could further define the relationships among the classical Brucella spp.

Results: Minimum inhibitory concentration (MIC) values of Brucella reference strains and three marine isolates to antibiotics binding the ribosome ranged from 0.032 to >256 μg/ml for the macrolides erythromycin, clarithromycin, and azithromycin and 2 to >256 μg/ml for the lincosamide, clindamycin. Though sequence polymorphisms were identified among ribosome associated loci 23S rrn, rplV, tuf-1 and tuf-2 but not rplD, they did not correlate with antibiotic resistance phenotypes. When spontaneous erythromycin resistant (eryR) mutants were examined, mutation of the peptidyl transferase center (A2058G Ec) correlated with increased resistance to both erythromycin and clindamycin. Brucella efflux was examined as an alternative antibiotic resistance mechanism by use of the inhibitor L-phenylalanine-L-arginine β-naphthylamide (PAβN). Erythromycin MIC values of reference and all eryR strains, except the B. suis eryR mutants, were lowered variably by PAβN. A phylogenetic tree based on concatenated ribosomal associated loci supported separate evolutionary paths for B. abortus, B. melitensis, and B. suis/B. canis, clustering marine Brucella and B. neotomae with B. melitensis. Though Brucella ovis was clustered with B. abortus, the bootstrap value was low.

Conclusion: Polymorphisms among ribosomal loci from the reference Brucella do not correlate with their highly differential susceptibility to erythromycin. Efflux plays an important role in Brucella sensitivity to erythromycin. Polymorphisms identified among ribosome associated loci construct a robust phylogenetic tree supporting classical Brucella spp. designations.
Background

Brucellosis is a zoonotic disease caused by the Gram-negative bacterium Brucella. It is taxonomically related to plant pathogens and other animal symbionts and is transmitted to humans from infected domestic animals and wildlife through contact during animal husbandry practices, meat production, or by ingestion of unpasteurized milk products. The genus Brucella contains six classical species reflecting host preferences [1,2], and additional species have been proposed to include marine isolates from seal, dolphin, and porpoise [3]. The classical species and their hosts are: B. abortus, bovine; B. melitensis, caprine; B. suis, porcine; B. ovis, ovine; B. canis, canine; and B. neotomae, desert wood rat. However, B. suis and B. canis have similar metabolic profiles [4] and genomic maps [5], supporting their close relationship. Similarly, the metabolic characteristics and phage susceptibility of B. suis biovar 5 are more like that of B. melitensis rather than B. suis [6,7].

The classical Brucella spp. designations are still widely used to emphasize important pathogenicity, virulence, and host preference differences among the Brucella even though similarity among the ribosomal RNA loci led to the designation of Brucella as a monospecific species [8,9]. B. melitensis. Brucella speciation may have arisen as a result of their isolation due to different preferred hosts and to divergence of the host species [10] even though their 16S rRNA loci are identical [11-14]. In any case, discordant genotype/phenotype may require the use of other widely conserved loci to define bacterial species [15,16].

Meyer [17] found differences in sensitivity to erythromycin among the classical species of Brucella and their biovars by measuring inhibition of growth using high and low concentration antibiotic discs. Brucella abortus biovars except biovar 2 were resistant to erythromycin, and B. ovis, B. melitensis, and B. canis were intermediate in resistance between B. abortus and B. suis. Only B. suis strains were sensitive to the high concentration antibiotic discs. Meyer argued that investigating the ribosomal structure could explain these differences in sensitivity and generate critical knowledge "to account for and recapitulate the lineage of species and biotypes of Brucella".

Bacterial susceptibility to macrolide and lincosamide antibiotics results from their binding to 23S rRNA, inhibiting protein synthesis by blocking the peptide exit tunnel [18-20]. Bacteria can become resistant to macrolides and lincosamides by spontaneous mutations of ribosomal associated loci or by increased efflux. Resistance to macrolides and lincosamides is commonly due to (i) mutation of ribosomal proteins L4 or L22 leading to widening the entrance to the peptide exit tunnel allowing access to the tunnel even in the presence of the antibiotics [18,20,23-25], (iii) methylation of ribosomal 23S rRNA [26], or (iv) increased efflux [27,28]. Bacterial resistance to synthetic macrolides or ketolides can be conferred by mutation of ribosome associated factor EF-Tu [29].

There are several families of efflux pumps, though few non-RND (resistance nodulation division) family efflux pumps cause intrinsic or spontaneous resistance of Gram-negative bacteria to common clinical antibiotics [27,28,30,31]. Inability to demonstrate efflux activity however does not necessarily mean a lack of efflux. Efficiency of efflux of antibiotics is variable, being dependent on the structure of the antibiotic. Antibiotic resistance can be complex as observed for Haemophilus influenzae L22 mutant HMC-C [32]. For this mutant, an increase in macrolide MIC values was only shown in the presence of efflux [33]. Here, we show that the large differences in relative intrinsic susceptibilities of reference strains of Brucella and three marine isolates to macrolide antibiotics and a lincosamide do not correlate with ribosomal associated polymorphisms. We establish that antibiotic efflux plays an important role in differential antibiotic susceptibility in Brucella. A robust phylogenetic tree constructed from concatenation of ribosome associated polymorphisms illuminates relationships among the Brucella.

Results

MIC determination by Etest

The relative MIC values of the classical Brucella spp. and three marine isolates (Table 1) to macrolides and a lincosamide were determined by use of the Etest. Log-fold differences in MIC values were found (Fig. 1). The susceptibility of Brucella was similar for the three macrolides erythromycin, azithromycin, and clarithromycin. Only B. abortus, except biovar 2, and B. melitensis had MIC values of ≥16 μg/ml. The pattern of sensitivity of Brucella to the lincosamide clindamycin differed from that of the macrolides. Generally, MIC values were higher for clindamycin than for the macrolides. Brucella abortus, except biovar 2, was the most resistant to clindamycin, having MIC values of ≥128 μg/ml. Only B. melitensis biovars 2 and 3 had lower MIC values for clindamycin than for erythromycin. For B. suis, clindamycin MIC values ranged from a low of 3 μg/ml to a high of 24 μg/ml. The other Brucella with the exception of the seal isolate, ranged from 2 to 64 μg/ml. The seal isolate was resistant to clindamycin.
23S rRNA sequence comparisons

Sequences of two regions of the Brucella 23S rRNA encoding 2498 nts (69 to 1678 and 1920 to 2807), including sites of 23S rRNA mutations known to increase bacterial resistance to macrolides and clindamycin were determined and compared (Table 2). Mixtures of cells or DNA (1:3) with disparate 23S rRNA sequences were amplified to demonstrate that heterogeneity among the three 23S rRNA copies would be detectable (data not shown). Though the distal portion of 23S rRNA C from the genomic B. suis 1330 sequence could not be amplified with either of two primer pairs that were complementary to the published B. suis 23S rRNA genomic sequence, amplification was successful using primers homologous to internal, conserved genomic rRNA sequences from all three Brucella genomes and sequences flanking rRNA C from B. abortus and B. melitensis. The amplified distal portion of rRNA C from B. suis 1330 was identical in sequence to that of rRNA A and rRNA B from B. suis. Among the 23S rRNA sequences from Brucella, three polymorphic and three monomorphic sites were identified. In addition, three monomorphisms were identified in the 23S rRNA intervening sequences.

23S rRNA polymorphisms were detected at nts 1085 (934, Ec), 1564 (1423, Ec), and 2632 (2610, Ec), clustering the Brucella into three groups: (1) B. abortus, (2) B. canis and B. suis, except biovar 5, and (3) B. melitensis, B. ovis, B. neotomae, B. suis biovar 5, and the dolphin, seal and porpoise isolates (Table 2). Note that all the 23S rRNA Brucella positions are numbered based on B. abortus 23S rRNA, including the intervening sequence. The only polymorphism that occurred in the peptidyl transferase center was nt 2632 (2610, Ec). No correlation could be made between the polymorphisms and relative antibiotic susceptibility. Other sites known to affect susceptibility to macrolides and clindamycin were not polymorphic.

Monomorphisms were found in both 23S rRNA and in the 23S rRNA intervening sequences. Monomorphisms were identified in 23S rRNAs from dolphin (A955G); B. neotomae (insertion of a C between nt 1002–1006); and B. suis biovar 5 (T2090C). Several intervening sequences of the Brucella 23S rRNA loci varied from the consensus sequence reported by Bricker [34]. The C indel in the intervening sequences of B. melitensis 16 M, forming a string of six Cs instead of five beginning at nt 222, reported by Bricker was confirmed. The other two monomorphisms occurred in B. suis biovar 5 (C219T) and B. melitensis biovar 3 (C206T).

L4 analyses

Though the GenBank Brucella genomic rplD sequences encoding ribosomal protein L4, differed due to an indel in rplD found only in the B. melitensis 16 M genomic sequence, we did not observe this indel in our sequence of rplD from B. melitensis 16 M. We found the three rplD genes from the genomes were identical. Though no polymorphisms were identified among the rplD sequences, three monomorphisms were discovered among their amino termini. Two sequence transitions, B. neotomae

---

Table 1: Strains of Brucella used in this study.

| Species          | Biovar | Strain | Host     | Origin | Reference |
|------------------|--------|--------|----------|--------|-----------|
| B. abortus       | 1      | 544    | Cattle   | England| ATCC* 23448|
|                  | 1      | 9–941  | Cattle   | USA    | 11        |
| 2                | 86/859 | Cattle | England  |         | ATCC 23449|
| 3                | Tulya  | Cattle | Uganda   |         | ATCC 23450|
| 4                | 292    | Cattle | England  |         | ATCC 23451|
| 5                | B3196  | Cattle | England  |         | ATCC 23452|
| 6                | 870    | Cattle | Africa   |         | ATCC 23453|
| 9                | C68    | Cattle | England  |         | ATCC 23455|
| B. canis         | RM6/66 | Dog    | USA      |         | ATCC 23365|
| B. melitensis    | 1      | 16 M   | Goat     | USA    | ATCC 23456|
|                  | 2      | 63/9   | Goat     | Turkey | ATCC 23457|
|                  | 3      | Ether  | Goat**   | Italy  | ATCC 23458|
| B. neotomae      | 5K33   | Wood rat | USA |         | ATCC 23459|
| B. ovis          | 63/290 | Sheep  | Africa   |         | ATCC 25840|
| B. suis          | 1      | 1330   | Pig      | USA    | ATCC 23444|
|                  | 2      | Thomsen| Hare     | Denmark| ATCC 23445|
|                  | 3      | 686    | Pig**    | USA    | ATCC 23446|
|                  | 4      | 40     | Reindeer | USSR   | ATCC 23447|
|                  | 5      | 513    | Mouse    | USSR   | ***       |
| Brucella spp.    | 2/94   | Seal   | Scotland | 6      |
| ("maris")       | 1/94   | Porpoise | Scotland | 6      |
|                  | 14/94  | Dolphin | Scotland | 6      |

*ATCC, American Type Culture Collection, Beltsville, MD; **Isolate from human; ***Reference strain 513 not deposited in ATCC.
Minimal inhibitory concentration (MIC) of antibiotics to Brucella reference strains and marine isolates. MICs of three macrolides, azithromycin, clarithromycin, erythromycin, and the lincosamide, clindamycin, were determined by Etest. Maximum MIC measurable by the Etest is 256 μg/ml for each of the antibiotics. *Brucella* strains as listed in Table 1. *Ba* = *B. abortus*; *Bs* = *B. suis*; *Bm* = *B. melitensis*; *Bc* = *B. canis*; *Bn* = *B. neotomae*; *Bo* = *B. ovis*; numbers following species designate biovar.
(G108A) and B. suis biovar 2 (C213T), were found. Both of which were silent. A transversion identified in the porpoise isolate (G314T) would replace an Arg, a charged amino acid (aa), with Leu, a noncharged one.

**L22 polymorphisms**

Putative L22 sequences from the *Brucella* reference strains and three marine isolates (Fig. 2) were determined and their tertiary structures predicted and compared by using Swiss-Pdb Viewer (Fig. 3). Among the *Brucella* putative L22 sequences, all variations except one occurred in the \(\beta\)-hairpin loops or near the carboxy termini. *Brucella suis* biovar 5 alone had an alternate Ala codon at aa 44. The *Brucella* \(\beta\)-hairpin loops were polymorphic and variable in length due to variable copy numbers of a two aa motif, Gly-Arg. The lengths of all the \(\beta\)-hairpin loops of putative L22 peptides except those from *B. neotomae* and *B. suis* biovars 2 and 3 were equal, 11 aa. The \(\beta\)-hairpin loops of L22 from *B. suis* biovars 2 and 3 were shorter due to a net two aa (Gly-Arg) deletion, while the \(\beta\)-hairpin loop from *B. neotomae* was longer due to a net two aa (Gly-Arg) insertion. Within the loop of the \(\beta\)-hairpin at aa 101, there was either a Gly, Val, or an Asp. While the variation of sequence at aa 101 of L22 did not greatly affect the predicted tertiary structures, the indels did (Fig. 3). A polymorphism was also identified very near the 3'-end of rplV. L22 polymorphic sites grouped the *Brucella* differently than other loci in this study. Putative L22 sequences from *B. abortus* and *B. melitensis* were identical. *Brucella suis* biovars 1, 4 and 5, and the marine isolates were identical. Putative L22 sequences from *B. suis* biovars 2 and 3 differed from those of biovars 1, 4 and 5 in having one rather than two Gly-Arg motifs. Putative L22 sequences from both *B. ovis* and *B. neotomae* were unique. No correlation could be made between relative antibiotic susceptibilities of the *Brucella* strains and their L22 sequences.

**EF-Tu sequence comparisons**

Though the nt sequences of EF-Tu loci, *tuf*-1 and *tuf*-2, were polymorphic (Table 3), the putative peptide sequences of EF-Tu1 and -2 were conserved. In most *Brucella* strains, *tuf*-1 and *tuf*-2 sequences were identical. In the cases where they were not identical, they varied by a single nt near either the 5' or 3' termini of the genes, namely nt 12 and nt 1158. Unlike the 23S *rrn* sequences, the sequences of *tuf*-1 and *tuf*-2 from *B. abortus* strains 9–941 and 2308 was identical to those from *B. abortus* biovars 2 and 4 but differed from the other *B. abortus* biovars. Whereas a

---

Table 2: *Brucella* 23S *rrn* polymorphisms.

| *Brucella strains* | *Nt 1085 934 (Ec)* | *Nt 1564 1423 (Ec)* | *Nt 2632 2610 (Ec)* |
|--------------------|-------------------|-------------------|-------------------|
|                    | A     | G             | A      | G             | T     | C     |
| Ba b1              | X     | X             | X      | X             |       |       |
| Ba b2              | X     | X             | X      | X             |       |       |
| Ba b3              | X     | X             | X      | X             |       |       |
| Ba b4              | X     | X             | X      | X             |       |       |
| Ba b5              | X     | X             | X      | X             |       |       |
| Ba b6              | X     | X             | X      | X             |       |       |
| Ba b9              | X     | X             | X      | X             |       |       |
| Bc                 |       |               | X      | X             |       |       |
| Bs b1              | X     | X             | X      | X             |       |       |
| Bs b2              | X     | X             | X      | X             |       |       |
| Bs b3              | X     | X             | X      | X             |       |       |
| Bs b4              |       |               | X      | X             |       |       |
| Bs b5              |       |               | X      | X             |       |       |
| Bo                 | X     | X             | X      | X             |       |       |
| Bn                 | X     | X             | X      | X             |       |       |
| Bm b1              | X     | X             | X      | X             |       |       |
| Bm b2              | X     | X             | X      | X             |       |       |
| Bm b3              | X     | X             | X      | X             |       |       |
| Dolphin            | X     | X             | X      | X             |       |       |
| Porpoise           | X     | X             | X      | X             |       |       |
| Seal               | X     | X             | X      | X             |       |       |

* GenBank accession numbers for each *Brucella* 23S *rrn* sequences are listed in Methods. *Brucella* strains are as listed in Table 1. *Nt* positions as per *B. abortus* 23S *rrn* [GenBank:AE017222]. (Ec) denotes position in *Escherichia coli* 23S *rrn* [GenBank:U00096].

---

**Table 2:** *Brucella* 23S *rrn* polymorphisms.

*Brucella* strains

| *Brucella strains* | *Nt 1085 934 (Ec)* | *Nt 1564 1423 (Ec)* | *Nt 2632 2610 (Ec)* |
|--------------------|-------------------|-------------------|-------------------|
|                    | A     | G             | A      | G             | T     | C     |
| Ba b1              | X     | X             | X      | X             |       |       |
| Ba b2              | X     | X             | X      | X             |       |       |
| Ba b3              | X     | X             | X      | X             |       |       |
| Ba b4              | X     | X             | X      | X             |       |       |
| Ba b5              | X     | X             | X      | X             |       |       |
| Ba b6              | X     | X             | X      | X             |       |       |
| Ba b9              | X     | X             | X      | X             |       |       |
| Bc                 |       |               | X      | X             |       |       |
| Bs b1              | X     | X             | X      | X             |       |       |
| Bs b2              | X     | X             | X      | X             |       |       |
| Bs b3              | X     | X             | X      | X             |       |       |
| Bs b4              |       |               | X      | X             |       |       |
| Bs b5              |       |               | X      | X             |       |       |
| Bo                 | X     | X             | X      | X             |       |       |
| Bn                 | X     | X             | X      | X             |       |       |
| Bm b1              | X     | X             | X      | X             |       |       |
| Bm b2              | X     | X             | X      | X             |       |       |
| Bm b3              | X     | X             | X      | X             |       |       |
| Dolphin            | X     | X             | X      | X             |       |       |
| Porpoise           | X     | X             | X      | X             |       |       |
| Seal               | X     | X             | X      | X             |       |       |

* GenBank accession numbers for each *Brucella* 23S *rrn* sequences are listed in Methods. *Brucella* strains are as listed in Table 1. *Nt* positions as per *B. abortus* 23S *rrn* [GenBank:AE017222]. (Ec) denotes position in *Escherichia coli* 23S *rrn* [GenBank:U00096].

---

**Table 2:** *Brucella* 23S *rrn* polymorphisms.

*Brucella* strains

| *Brucella strains* | *Nt 1085 934 (Ec)* | *Nt 1564 1423 (Ec)* | *Nt 2632 2610 (Ec)* |
|--------------------|-------------------|-------------------|-------------------|
|                    | A     | G             | A      | G             | T     | C     |
| Ba b1              | X     | X             | X      | X             |       |       |
| Ba b2              | X     | X             | X      | X             |       |       |
| Ba b3              | X     | X             | X      | X             |       |       |
| Ba b4              | X     | X             | X      | X             |       |       |
| Ba b5              | X     | X             | X      | X             |       |       |
| Ba b6              | X     | X             | X      | X             |       |       |
| Ba b9              | X     | X             | X      | X             |       |       |
| Bc                 |       |               | X      | X             |       |       |
| Bs b1              | X     | X             | X      | X             |       |       |
| Bs b2              | X     | X             | X      | X             |       |       |
| Bs b3              | X     | X             | X      | X             |       |       |
| Bs b4              |       |               | X      | X             |       |       |
| Bs b5              |       |               | X      | X             |       |       |
| Bo                 | X     | X             | X      | X             |       |       |
| Bn                 | X     | X             | X      | X             |       |       |
| Bm b1              | X     | X             | X      | X             |       |       |
| Bm b2              | X     | X             | X      | X             |       |       |
| Bm b3              | X     | X             | X      | X             |       |       |
| Dolphin            | X     | X             | X      | X             |       |       |
| Porpoise           | X     | X             | X      | X             |       |       |
| Seal               | X     | X             | X      | X             |       |       |
MGKAKAPROL KDNEAKAVAR TLRVSPQKLN LVASMIRGKK VNAALADLTF
SRKRIAGTVK KTLESAIANA ENNHDLDVDA LIVA EAYVGK STVMKRFHVR

| (G/V/D)R | GR | GR |
|----------|----|----|
| **B. suis** b 2, 3 | - | - |
| **B. suis** b 1, 4, 5 | **B. suis** b 1, 4, 5 | - |
| **B. canis** | **B. canis** | - |
| **B. neotomae** | **B. neotomae** | **B. neotomae** |
| Marine isolates | Marine isolates | - |

**VR**

| **B. ovis** | **B. ovis** | - |

**DR**

| **B. abortus** b 1-6, 9 | **B. abortus** b 1-6, 9 | - |
| **B. melitensis** b 1-3 | **B. melitensis** b 1-3 | - |

**ASRIEK PFSHLTIVVR EVAEKGE (K/E) AA**

| **K** | **B. abortus** b 1-6, 9 | **B. melitensis** b 1-3 |
| **E** | **B. suis** b 1-5 | **B. ovis**, **B. canis**, **B. neotomae** | Marine isolates |

Figure 2

Ribosomal protein L22 polymorphisms among *Brucella* reference strains and three marine isolates. The putative peptide sequence of L22 is underlined, parentheses bracket polymorphic sites and list amino acids found among L22 peptides at that site. In regions where sequence was variable, the sequence for each putative *Brucella* L22 is given below. Amino acids occurring in the stalk of the β-hairpin appear in **bold-italics** and amino acids occurring in the loop of the β-hairpin are in **bold**. Beneath amino acids that are double underlined is a list of *Brucella* strains containing those aa. The single letter code is used to denote the putative aa sequence of the peptides; b = biovar and numbers following "b" designate biovar numbers. Accession numbers of *rplV* sequences for each strain are deposited in GenBank and are listed in Materials.

single nt varied among copies of *tuf*-1 and *tuf*-2 from *B. abortus* biovar 1 strain 9–941 and *B. suis* 1330, eight nt varied between *B. suis* 1330 and *B. melitensis* 16 M. The other classical spp. and the marine *Brucella* were intermediate between *B. abortus/B. suis* and *B. melitensis* (Table 3).

**Erythromycin mutants**

EryR mutants of several reference *Brucella* strains having MIC values less than 2 μg/ml and the three marine *Brucella* were selected. Mutant strains were not recovered from *B. ovis*, *B. abortus* biovar 2, or *B. suis* biovar 5. Three ribosomal associated loci, 23S rm, *rplV*, and *rplD*, were analyzed from eryR mutants of *B. suis* biovar 1, *B. canis*, *B. neotomae*, and the three marine *Brucella* (Table 4). The spontaneous eryR rate among the classical *Brucella* spp. varied by 100-fold. Rates of mutation to eryR and highest concentration of erythromycin allowing growth for each parental strains of the classical *Brucella* spp. were: *B. suis* 3.7 × 10⁻⁷ (5 μg/ml erythromycin); *B. canis* 1.5 × 10⁻⁸ (20 μg/ml erythromycin), and *B. neotomae* 6.6 × 10⁻⁸ (5 μg/ml erythromycin). All the marine eryR isolates were selected from plates containing 20 μg/ml erythromycin, and mutational rates were 1.9 × 10⁻⁶, 5.1 × 10⁻⁷, and 2.7 × 10⁻⁶ for porpoise, seal, and dolphin, respectively.

Though MIC values of the eryR mutants of the classical strains *B. suis*, *B. canis*, and *B. neotomae* increased, they did not increase as much as those of the marine isolates (Table...
4). While MIC values increased for the eryR mutants of the classical Brucella strains, the increases were only 2 to 6-fold compared to 15 to > 256-fold for the marine Brucella. Over half of the marine eryR mutants had MIC values of 128 μg/ml or higher, and all the dolphin mutants had MIC values greater than 256 μg/ml. The clindamycin MIC values of the eryR mutants were similar to those of the parental strains except for porpoise b which had a mutation in 23S rrm and the seal isolates a and c.

Mutations among the marine eryR mutants were found in two ribosomal associated loci, 23S rrm and rplD. Only a single mutation was identified in 23S rrm. Porpoise isolate b had a mutation within the peptidyl transferase center of 23S rrm, nt 2058 (Ec), but all three 23S rrm copies were not mutated, as the signal was mixed. This mutant was resistant to both erythromycin and clindamycin, MIC values >256 μg/ml. Most ribosomal associated mutations occurred in rplD (Table 4), and these were only found among the marine isolates. The mutations were not random. Several eryR isolates had mutations at nt 209 or nt 217. At nt 209, porpoise isolates a and d and dolphin isolates a and c had an A instead of a G, substituting an Asp for a Gly. Dolphin eryR mutants isolates b and d had a T instead of a C at nt 217 of rplD, resulting in the incorporation of a Cys rather than an Arg. Seal eryR isolate c had an A instead of a C at nt 217 which resulted in the incorporation of a Ser rather than an Arg. Two of the porpoise eryR isolates c and e had deletions in rplD, resulting in the loss of 18 or 30 codons. The deletion of 18 aa in L4 of porpoise c is consistent with recombination between two copies of 5′GGG-CCG-CGC-3′ occurring between nt 153–161 and 207–215.

Only one ribosomal associated loci mutation was identified among the B. suis, B. canis, and B. neotomae eryR mutants by analyzes of 23S rrm, rplD, and rplV. A duplication of a six-bp repeat in the β-hairpin loop of L22 of B. neotomae isolate d expanded the number of Gly-Arg aa repeats from three to four (Fig. 2, Table 4). This mutant had a slightly higher MIC value for erythromycin.

**Efflux**

Erythromycin and clindamycin MIC values of the reference strains and the eryR mutants were analyzed in the presence of the efflux inhibitor PAβN (Tables 4 and 5). Using Etest strips, a decrease in MIC values could only be detected if the MIC values >256 μg/ml fell to or below 256 μg/ml. MIC values of the reference strains decreased variably in the presence of the inhibitor (Table 5). Though PAβN affected the erythromycin MIC value for B. suis biovar 1, reducing it two-fold or by two dilutions as per the Etest, the MIC values for the B. suis biovar 1 eryR mutants were not affected. In the case of B. abortus biovar 5, even though its erythromycin MIC value was lowered in the presence of PAβN, the clindamycin MIC value was unaffected.

Efflux inhibition among the eryR mutants by PAβN (Table 4) was variable among the strains. The B. canis and B.
neotomae eryR mutants had decreases in their erythromycin and clindamycin MIC values in the presence of PAβN. The seal eryR mutants had increased erythromycin MIC values that were variably reduced in the presence of PAβN. For example, isolate a had an erythromycin MIC value of 24 μg/ml which was reduced to 12 μg/ml by PAβN, but isolate d had a MIC value of 128 μg/ml which was reduced to 16 μg/ml. Only the seal eryR clindamycin MIC values were either identical to (>256 μg/ml) or lower than (96 and 16 μg/ml) that of the parental strain, and none were affected by PAβN. The dolphin eryR mutants differed from all the other eryR mutants in that they had uniform erythromycin MIC increases and the highest MIC increases of any of the other groups. Though all the dolphin eryR mutants' erythromycin MIC values increased from 3 μg/ml to >256 μg/ml, their erythromycin MIC values were differentially affected by PAβN. In the presence of PAβN, two dolphin eryR mutants had MIC values equal to or greater than 128 μg/ml while the rest had MIC values of 8 μg/ml or less. Like the porpoise isolates, except isolate b, all the dolphin isolates had lower clindamycin MIC values than the parental strain and the clindamycin MIC values were only slightly affected by PAβN.

**Phylogenetic tree**

A phylogenetic tree was constructed using concatenated 23S rRN, rplV, tuf-1, and tuf-2 (Fig. 4). Brucella formed a node with the closest clades being other α-Proteobacteria, Agrobacterium, Mesorhizobium, and Cardiobacter followed by Lepiosira and γ-Proteobacteria, Xylella, Acinetobacter, and the facultative intracellular animal pathogen Legionella. The cluster containing the Brucella species is robustly formed (high bootstrap values) into a distinct clade separate from the outgroups and forming four nodes subclustering: (1) B. abortus and B. ovis; (2) B. suis and B. canis; (3) B. melitensis, B. neotomae and the marine Brucella; and (4) B. suis biovar 5.

The tree constructed from a concatenated sequence, i.e. a supergene or supermatrix, was consistent with a concatenated tree calculated from individual loci (data not shown). Both trees supported classical classification, clustered the marine isolates with B. melitensis, and indicated intrinsic differences among marine Brucella. Bootstrap numbers (Fig. 4) were robust for all nodes (99 or 100) except B. ovis, which clustered with B. abortus; in the additive tree, B. ovis formed a unique branch. Though B. suis

### Table 3: Polymorphic sites among tuf-1 and tuf-2 from Brucella.

| Nt position | 12 | 36 | 141 | 183 | 198 | 219 | 345 | 378 | 511 | 609 | 936 | 1158 |
|-------------|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Bab 9–941   | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | 2   |
| Bab b1      | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bab b2      | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bab b3      | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bab b4      | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bab b5      | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bab b6      | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bab b9      | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| B. suis b1  | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| B. suis b2  | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| B. suis b3  | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| B. suis b4  | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| B. suis b5  | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bmel b1     | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bmel b2     | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bmel b3     | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Bneo        | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| B. ovis     | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| B. canis    | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Seal        | 1  | 2  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Dolphin     | 1  | 2  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |
| Porpoise    | X  | X  | X   | X   | X   | X   | X   | X   | X   | X   | X   | X   |

Nt = nucleotide; Brucella strains, see Table 1; X = tuf-1 and tuf-2 have identical sequences; 1 = tuf-1 sequence only, 2 = tuf-2 sequence only. Polymorphic sites are in **bold**. tuf-1 and tuf-2 GenBank accession numbers are listed in Materials and methods.
and *B. canis* composed a node, *B. suis* biovars 1 and 4 were on one branch and *B. suis* biovars 2 and 3 on another branch with *B. canis*. Shared 23S rrn polymorphisms divided the *Brucella* into three groups, placing *B. melitensis* between *B. abortus* and *B. suis* (Table 2). The *tuf*-1 and *tuf*-2 sequences (Table 3) separated *B. abortus* and *B. suis* and placed *B. neotomae* and the marine isolates intermediate between *B. abortus* and *B. suis* and *B. melitensis*. The rplV from *B. abortus* and *B. melitensis* were identical. Indels in rplV split *B. suis* biovars into two groups.

### Discussion

The Etest was used to determine MIC values of the classical reference *Brucella* spp., their biovars, and three marine isolates to macrolides and a lincosamide. Our results differed somewhat from those reported by Meyer [17] using antibiotic discs containing low or high concentrations of erythromycin. Meyer found *B. ovis* and *B. canis* more resistant than *B. suis* to erythromycin, but Etest MIC values for *B. ovis* and *B. canis* were less than those of the reference strains of *B. suis* and *B. melitensis*.

#### Table 4: Determination and characterization of erythromycin and clindamycin MICs and molecular characterization of *Brucella* eryR mutants.

| *Brucella* eryR strains | ERY | ERY + PAβIN | CL | CL + PAβIN | Loci | Mutation |
|-------------------------|-----|-------------|----|------------|------|----------|
| *B. suis* WT            | 1.5 | 0.75        | 8  | 4          | NA   |          |
| a                       | 6   | 6           | 12 | 12         | ND   |          |
| b                       | 6   | 6           | 12 | 12         | ND   |          |
| c                       | 4   | 6           | 8  | 8          | -    |          |
| d                       | 6   | 6           | 12 | 12         | ND   |          |
| e                       | 6   | 6           | 8  | 8          | ND   |          |
| *B. canis* WT           | 0.5 | 0.094       | 8  | 0.5        | NA   |          |
| a                       | 2   | 0.32        | 8  | 1.0        | -    |          |
| b                       | 2   | 0.047       | 6  | <0.016     | -    |          |
| c                       | 3   | 0.047       | 8  | 0.38       | -    |          |
| d                       | 3   | 0.047       | 4  | 0.125      | -    |          |
| e                       | 2   | 0.19        | 8  | 3          | -    |          |
| *B. neotomae* WT        | 0.75| 0.047       | 3  | 0.75       | NA   |          |
| a                       | 4   | 0.125       | 8  | 0.75       | -    |          |
| b                       | 3   | 0.125       | 6  | 2          | ND   |          |
| c                       | 2   | 0.064       | 6  | 1.5        | -    |          |
| d                       | 6   | 0.25        | 6  | 1.0        | L22  | 6 bp indel, β-loop |
| e                       | 2   | 0.38        | 4  | 2          | -    |          |
| Propoise WT             | 1.5 | 0.25        | 16 | 3          | NA   |          |
| a                       | 16  | 0.75        | 4  | 3          | L4   | G209A; Gly70Asp |
| b                       | >256| >256        | >256|>256       | 23S  | A2058G (Ec) |
| c                       | 192 | 1.5         | 4  | 3          | L4   | Δ18aa (54–71) |
| d                       | 48  | 0.38        | 6  | 3          | L4   | G209A; Gly70Asp |
| e                       | 128 | 1.5         | 4  | 3          | L4   | Δ30aa (54–81) |
| Seal WT                 | 6   | 1.5         | >256|48         | NA   |          |
| a                       | 24  | 12          | 96 | 96         | -    |          |
| b                       | 32  | 24          | >256|>256       | -    |          |
| c                       | >256| >256        | 16 | 16         | L4   | C217A; Arg73Ser |
| d                       | 128 | 16          | >256|>256       | -    |          |
| e                       | 128 | 24          | >256|>256       | ND   |          |
| Dolphin WT              | 3   | 0.38        | 2  | 0.75       | NA   |          |
| a                       | >256| 128         | 1.0| 1.5        | L4   | G209A; Gly70Asp |
| b                       | >256| 6           | 1.5| 1.0        | L4   | C217T; Arg73Cys |
| c                       | >256| 192         | 1.5| 1.5        | L4   | G209A; Gly70Asp |
| d                       | >256| 8           | 2.0| 1.0        | L4   | C217T; Arg73Cys |
| e                       | >256| 8           | 1.5| 1.0        | ND   |          |

Mutants examined were isolates of *B. suis* biovar 1, *B. canis*, *B. neotomae*, and marine porpoise, seal and dolphin strains as per Table 1; WT, wild type; Ery, erythromycin; CL, clindamycin, PAβIN, efflux inhibitor L-phenylalanine-L-arginine-β-naphthylamide; -, no mutation identified in any of three ribosomal loci examined; NA, not applicable; ND, sequence not determined.
were low and more similar to those of \textit{B. suis} than to those of either \textit{B. melitensis} or \textit{B. abortus}. The patterns of relative sensitivity to macrolides of the reference \textit{Brucella} were similar for erythromycin, clarithromycin, and azithromycin but differed from that for clindamycin. The susceptibility of \textit{B. suis} to relatively low concentrations of the macrolide azithromycin suggests that this antibiotic may be a beneficial treatment for \textit{B. suis} infections as it has a long \textit{in vivo} half-life (50 hours), concentrates in macrophages, and lacks uptake saturation [35].

Ribosomal associated loci 23S \textit{rrn}, \textit{rplD}, \textit{rplV}, \textit{tuf}-1, and \textit{tuf}-2 were analyzed for polymorphisms. Three monomorphisms were identified among \textit{rplD} loci, but only one of them resulted in a difference among the putative L4 sequences. Although polymorphism was high among the \textit{tuf}-1 and \textit{tuf}-2 loci, all were silent. Sequences among 23S \textit{rrn} and \textit{rplV} loci were polymorphic.

The three polymorphic sites identified among the \textit{Brucella} 23S \textit{rrn} loci separated them into three groups (Table 2). The only sequence difference among the 23S \textit{rrn} peptidyl transferase centers of the reference \textit{Brucella} strains was at nt 2610 (Ec), where there was either a T or a C. Many nucleotides in the peptidyl transferase center are conserved among bacteria and other organisms, but nt 2610 (Ec) is not. Either a T or C is common in bacteria. In any case, a T2610C (Ec) mutation in 23S \textit{rrn} from \textit{S. pneumoniae} only slightly affected its MIC values for macrolides and clindamycin [36]. Mutation of the peptidyl transferase center of 23S RNA (A2058G, Ec) of porpoise \textit{eryR} mutant isolate b increased the erythromycin and clindamycin MIC values from 1.5 and 16 μg/ml, respectively, to >256 μg/ml. These MIC values were unaffected by the presence of efflux inhibitor PAβN. Concurrent appearance of resistance to erythromycin and clindamycin by mutation of nt 2058 (Ec) is observed in other bacteria [23]. Methylation of either nt 2059 or 2058 (Ec) of the peptidyl transferase center reduces the sensitivities of bacteria to macrolides and lincosamides [26]. We were unable to identify homologs of any 23S \textit{erm} methylation genes by BLAST [37], but, then, methylation of ribosomal rRNA is much more widely described in Gram-positive clinical isolates [26].

The \textit{rplV} sequences of the reference \textit{Brucella} strains and marine \textit{Brucella} were polymorphic, resulting in the differences among their putative L22 peptide sequences and lengths of the L22 β-hairpin loops. This was unexpected because L22 peptide sequence is conserved within a bacterial species [18,38] and the length of the L22 β-hairpin loop is highly conserved across biological kingdoms [38]. Differences in β-hairpin loop lengths among the \textit{Brucella} L22 peptides were due to variable numbers of Gly-Arg repeats (Fig. 2). Though \textit{B. neotomae} \textit{eryR} isolate d had four Gly-Arg repeats, due to a six base insertion, the mutant’s

| Brucella strains | MIC | MIC | MIC | MIC |
|------------------|-----|-----|-----|-----|
| B. \textit{ab} b1 | >256 | 96  | >256 | 96  |
| B. \textit{ab} b2 | 0.19 | 0.0275 | 3   | 0.25 |
| B. \textit{ab} b3 | 128  | 64  | 192  | 128  |
| B. \textit{ab} b4 | 128  | 64  | 192  | 64   |
| B. \textit{ab} b5 | >256 | 192 | >256 | 128  |
| B. \textit{ab} b6 | >256 | 256 | >256 | 96   |
| B. \textit{ab} b9 | >256 | >256| >256 | >256 |
| B. \textit{mel} b1 | 16   | 4   | 24   | 16   |
| B. \textit{mel} b2 | >256 | 48  | 64   | 12   |
| B. \textit{mel} b3 | 256  | 48  | 64   | 32   |
| B. \textit{suit} b1 | 1.5  | 0.75| 8    | 4    |
| B. \textit{suit} b2 | 0.125| 0.032|3| 0.047|
| B. \textit{suit} b3 | 1.5  | 1.0 | 24   | 48   |
| B. \textit{suit} b4 | 2    | 1.5 | 24   | 32   |
| B. \textit{suit} b5 | 0.094| 0.047|6| 2    |
| B. \textit{canis} | 0.50 | 0.094|8| 0.50 |
| B. \textit{avis} | 0.064| <0.016|3| <0.016|
| B. \textit{neo} | 0.75 | 0.047|3| 0.75 |
| Porpoise | 1.5 | 0.25|16| 3    |
| Seal | 6 | 1.5 | >256 | 48 |
| Dolphin | 3 | 0.38 | 2 | 0.75 |

\textit{Brucella} strains are as listed in Table 1; MIC, minimum inhibitory concentration; Ery, erythromycin; CL, clindamycin; PAβN, efflux inhibitor L-phenylalanine-L-arginine β-naphtylamide; b, biovar; ab, abortus; mel, melitensis; neo, neotomae.
Phylogeny of *Brucella* calculated using highly conserved ribosomal associated loci. Shown is the single optimization alignment tree based on *rplV*, *tuf*-1, *tuf*-2, and 23S rRNA sequences from 28 taxa consisting of the 21 *Brucella* strains (see Table 1), which included the 18 classical *Brucella* reference strains and three marine *Brucella*, and seven outgroups of known genomic sequences. Branch lengths (mean number of differences per residue along each branch) are given as well as bootstrap values (percentage of bootstrap support based on 100 replicates). *Legionella pneumophila* subspecies Pneumophila strain Philadelphia [GenBank:NC_002942] was used to root the tree. Other bacterial outgroups include: Acinetobacter species ADP1 [GenBank:NC_005966], Caulobacter crescentus CB15 [GenBank:NC_002696], Leptospira interrogans serovar Copenhagen strain Fiocruz L1-130 [GenBank:NC_005823], Mesorhizobium loti MAFF303099 [GenBank:BA000012], Agrobacterium tumefaciens C58 circular [GenBank:NC_003062] and linear chromosomes [GenBank:NC_003063], and Xylella fastidosa 9a5c [GenBank:NC_002488].
erythromycin and clindamycin MIC values were only slightly increased.

The single amino acid difference found among the putative L4 sequences of the reference and marine strains could not be correlated with a difference in MIC values. Among the eryR mutants, all but two of the mutations were identified in rplD, and, interestingly, they only occurred among the marine eryR isolates. All erythromycin MIC values that increased among the eryR marine isolates were lowered by the efflux inhibitor PAβN. Nevertheless, some of the MIC values remained relatively high in the presence of PAβN. The L4 peptides of these mutants may work in conjunction with or be dependent on specific efflux RND pumps as shown for Haemophilus influenzae HMC-C [32,33].

The tuf-1 and tuf-2 loci were the most polymorphic of the ribosomal associated loci examined, yet their putative peptide sequences were identical. Strain sequence differences between tuf-1 and tuf-2 were confined to the borders. This is consistent with gene conversion occurring more efficiently within conserved sequences rather than near the borders. Given that B. melitensis and B. abortus genomes have fewer single nucleotide polymorphisms (SNP) between them than either has with B. suis, tuf-1 and tuf-2 from B. abortus and B. melitensis were expected to be highly similar. This was not the case. Brucella abortus and B. suis tuf-1 and tuf-2 had few sequence differences (Table 3). The tuf-1 and tuf-2 sequences from the marine isolates were intermediate between B. abortus/B. suis and B. melitensis/B. neotomae. The tuf-1 and tuf-2 genes encode a core metabolic product and the apparent selective pressure on conserving EF-Tu sequences in the face of tuf-1 and tuf-2 polymorphism supports different evolutionary paths [39] for B. abortus and B. melitensis.

MIC values and sequences of ribosomal related loci did not correlate with antibiotic susceptibility. To determine if efflux played a part in Brucella differential antibiotic resistance, we studied the effect of an RDF efflux inhibitor on MIC values. With the possible exception of B. abortus biovar 9, erythromycin MIC values of all the reference strains were reduced by the inhibitor PAβN though MIC values decreases were variable. Even low erythromycin MIC values decreased further in the presence of PAβN, demonstrating that efflux afforded the Brucella a low level of intrinsic antibiotic resistance similar to that reported for Campylobacter [40].

Many clinical isolates are resistant to antibiotics due to increased efflux as a result of mutations of efflux promoters and global and physically linked regulator genes or mobilization of insertion sequences (for a review see [28]). Most of the eryR strains had increased antibiotic efflux, though the marine eryR strains had larger increases in efflux than those of the classical reference strains of B. suis biovar 1, B. canis, and B. neotomae (Table 4). This suggests a fundamental biological difference between these groups. It is known that the marine Brucella have a high copy number [41] of the insertion sequence IS711 [42]. IS711 has been shown to mobilize in Brucella under stress or selective pressure [43,44] and could be a source of instability [42] in marine Brucella.

Brucella phylogenetic trees and dendrograms have been constructed based on genomics maps [3,5,6], amplified fragment length polymorphisms (AFLP) [45], multilocus enzyme electrophoresis (MLEE) [6], and outer membrane proteins omp2a/omp2b [3]. Now, other universally conserved loci, especially 23S rrn, EF-Tu, rpoB, and gyrA, are increasingly being used to establish relationships among highly similar bacteria with important phenotypic differences to determine their relationships [16]. We constructed a phylogenetic tree based on concatenated sequences of ribosomal associated loci. Most phylogenetic trees and dendrograms, including ours, place B. abortus, B. suis/B. canis, and B. melitensis on separate branches, supporting alternative evolutionary paths. Recently, it was shown that Brucella isolates could be identified at the species level using 21 variable number tandem repeats (VNTR) [46]. The neighbor joining tree based on VNTR data produced major clusters that encompassed the classical Brucella spp. On this tree, the reference B. suis biovar 5 strain, which appears as a unique branch on our tree, was shown to be only distantly related to all other reference strains and isolates by VNTR analyses [46]. Though B. ovis formed a single cluster by VNTR analyses, it clustered, albeit with a low bootstrap value, with B. abortus on our tree. Significant sequence differences have been reported between B. ovis and other classical Brucella spp. reference strains [47,48]. Brucella neotomae grouped with B. melitensis here but was on a separate node. Based on VNTR data, B. neotomae occurs on a unique branch but groups with B. abortus on a AFLP generated dendrogram [45]. Marine isolates are not found on many Brucella phylogenetic trees. Ours grouped the marine Brucella and B. neotomae with B. melitensis but on separate branches. This is in agreement with the genetic diversity observed among the marine isolates and proposals that marine isolates may comprise more than one species [3,41].

Conclusion

Ribosomal associated polymorphisms among the reference Brucella spp. did not correlate with differential intrinsic antibiotic resistance to erythromycin or clindamycin. Efflux is an important mechanism of resistance to macrolides and the lincosamide clindamycin in Brucella and can be inhibited by the RND efflux inhibitor PAβN. A phylogenetic tree constructed based on concatenated
ribosomal associated loci supports alternative evolutionary pathways for *B. melitensis*, *B. abortus*, and *B. suis*, and clustered the marine *Brucella* with *B. melitensis*, and *B. canis* with *B. suis*. It also supports the doubtful close relationship of *B. suis* biovar 5 with *B. suis*.

**Methods**

**Bacterial strains and growth conditions**

Bacterial strains (Table 1) were obtained from our laboratory collection for this study. Bacteria were grown at 37°C on tryptose agar (DIFCO Laboratories, Detroit, MI) containing 5% bovine serum in the presence of 7.5% CO₂. Results were read after 48 h. Etest strips applied. Plates were incubated (37°C) in 7.5% CO₂. Selection of erythromycin mutants

*Brucella* strains having erythromycin MIC values <5 μg/ml were suspended in saline and plated (10⁸ cfu) in triplicate onto Difco™ Mueller Hinton agar (Becton, Dickinson and Company, Sparks, MD) in the presence or absence of 25 μg/ml of erythromycin (Sigma Chemical Co., St. Louis, MO) using a cotton swab, and the Etest strips applied. Plates were incubated (37°C) in 7.5% CO₂. Results were read after 48 h.

**PCR amplification**

Master mixes for PCR reactions were prepared by use of the Fast Start Taq DNA polymerase kit (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's instructions. Methanol treated cells were diluted 1/10 in water and used immediately or stored at 4°C up to 2 months. One μl was added per 25 μl of reaction mixture. Reactions were 50 or 100 μl. Cells were disrupted and amplification initiated by heating the reactions to 95°C for 5 min. Melting, annealing, and elongation temperatures and times were 95°C for 15 sec, 60°C for 30 sec, and 72°C for 90 sec, respectively. After amplification for 35 or 40 cycles, elongation was extended by 4 min.

**Primers**

The *rrn* genes were annealed to *rrnA* from *B. abortus* 9–941 [GenBank:AEl7223, BruAb1_rRNA_0005]: nt 37–62 (CAT-GCA-CAG-GGC-ATG-AAG-GAC-GTG-AT) and nt 340–518 (CGA-ITT-CAC-GTG-TCC-GGC-CCT-ACT-GA) (note that this set amplified intervening sequence); nt 455–481 (AGT-TGG-AAA-CTG-AAG-GTG-GTG-GGC-GAC-TGA) and nt 1153–1127 (CCT-TAT-AGG-GTT-AGG-ATT-GTT-GCC); nt 1013–1039 (GAG-CAC-TGG-ATG-GGC-TAT-GGG-GAC-TCA) and nt 1732–1707 (GTT-CAT-TGG-AGA-TGG-TCT-CAT-CAA-CTG); nt 1868–1894 (CGC-GTG-CTG-GAA-GGT-TAA-GAG-GAG); and nt 2605–2579 (CCC-ACC-TCA-GTG-ACC-GCT-TTA-AAT-GCC) and nt 2505–2529 (CCG-GGT-TGA-TGC-CAT-CGC-TAT-C) and nt 2850–2825 (CCC-GGC-CTA-TCA-AGC-TGG-TGG-TGT-CTC). Primers used in PCR reactions to amplify the 3’ end based on the genomic sequence of *rrnC* from *B. suis* [GenBank:NC_004311, Bb23SC] were forward (GGT-TTC-CCT-CTG-ATAG-TGG-CAT-GA) and reverse 1 (CTT-CAG-AGA-TTA-CTC-CTG-CTG-TAT-ATM-TAC). Primers based on 23S *B. suis* rRNA unique sequences and *B. suis* sequences flanking *rrnC*, respectively, were: (GGG-TTC-ACC-AGG-GTT-TAT-GTG-GGC-TAT) and (CTT-CCA-TTG-AGA-GGA-GCC-GAA-GGA-GAT-G). Primers for amplification of L4, L22, EF-Tu1, and EF-Tu2 were: L4 forward 1, (AGC-ACC-AGG-ATG-TGC-GTA-ACC-AGG); reverse, (GCC-AGC-TGG-ATA-CAG-GGC-GAG-TCC) and reverse 2, (GTG-CAT-CGT-GAC-ATC-GCG-AGG-GGA-ACC-ACC); and EF-Tu1, forward, (TAC-CGA-ATG-CCG-GAA-GCA-AGA-AAG); reverse, (GGA-TTC-AGT-GGC-CTG-GAG-CAG). Amplified products were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and sequenced at the Genomics Center at the National Animal Disease Center, Ames, IA (ABI Prism 3700 DNA Analyzer) using primers as listed above. For large products, internal primers were synthesized as needed to obtain coding sequences. Sequences were assembled and aligned, and polymorphisms were identified by use of Sequencer 3.1.2 (Genes...
Gene ID: [Gene ID], and AE014292 and AE008918.

Protein folding

The Swiss-Pdb viewer software version 3.7 [50,51] was used to predict folding of L22 based on the coordinates determined for L22 from Thermus thermophilus [52].

23S rrn base numbering

The numbering for Brucella 23S rrn is based on rrnA from B. abortus 9–941 [GenBank:NC_006932, Gene ID: 3339965]. When the nts refer to E. coli 23S rRNA rntG [GenBank:U00096, GI:48994873], the nts are followed by (Ec).

Accession numbers

Genome and genic sequences referred to in this study: B. suis 1330 [GenBank:AE014291 and AE014292], B. melitensis 16 M [GenBank:AE008917 and AE008918], B. abortus 9–941 [GenBank:AE017223 and AE017224], B. abortus 2308 [GenBank:AM040264], and B. abortus rrnA sequence [GenBank:NC_006932, Gene ID: 3339965]. Genomic sequences determined in this study: L4 (rplD) [GenBank:DQ289557 to DQ289572], L22 (rplV) [GenBank:DQ227901 to DQ227921], EF-Tu1 (tuf-1) [GenBank:DQ227922 to DQ227942], EF-Tu2 (tuf-2) [DQ227943 to DQ227963], 23S rrn region 1 (nt 69 to 1678) [GenBank:DQ287886–DQ287906], and 23S rrn region 2 (nt 1920–2807) [GenBank:DQ287865–DQ287885]. Ery* mutant strain ribosomal associated sequences: B. neotomae (rplV) [GenBank:DQ659536], porpoise b (23S rrn) [GenBank:DQ659537], dolphin a-d (rplD) [GenBank:DQ660399–DQ660402], porpoise a, c-e (rplD) [GenBank:DQ6600403–DQ6600406], and seal c (rplD) [GenBank:DQ660407].

Dendrogram

Data sets consisted of concatenated genes (rplV, tuf-1, tuf-2) and sequences from 23S rrn from 28 taxa consisting of the 18 Brucella reference strains, three marine isolates, and seven outgroups of known genomic sequences (see Table 1 and Fig. 4). 23S rrn intervening sequences were eliminated from the compared. Assembled sequences were aligned using ClustalW v1.83 [53]. Each nucleotide data set was then analyzed under the optimal criteria of maximum likelihood using MrBayes v3.1.2 Bayesian analysis and Markov Chain Monte Carlo methods to search tree space and infer posterior distribution of topologies [54]. Settings for MrBayes were the general time reversible substitution model with sites drawn from a gamma distribution. The outgroup for MrBayes was Legionella pneumophila subspecies Pneumophila strain Philadelphia [GenBank:NC_002942]. The number of generations was set at 1,000,000, number of chains at 4, print frequencies at 10,000, and sample frequencies at 100. Branch lengths were saved and all other settings were default. The evolutionary tree was displayed using Tree Explorer [55] based on options used to compute or display the phylogeny.

Abbreviations

Standard three letter code was used for amino acids and standard one letter code for DNA bases.

Authors’ contributions

SH identified the loci for study; designed primers, amplified loci, prepared templates for sequencing, analyzed sequence data and drafted the manuscript. AJ cultured the Brucella, determined MIC values, prepared L22 predicted structures, and edited the manuscript. SH and AJ prepared graphs, tables, dendograms, and analyzed data. Both authors have read the manuscript and approved the final version.

Acknowledgements

We thank Brooke Petersen-Burch for his assistance in implementation of protein folding software and Kari Loehndorf for advice in the construction of phylogenetic trees. We thank David Alt and Karen Halloun for assistance in DNA sequencing.

References

1. Meyer ME: The epizootiology of brucellosis and its relationship to the identification of Brucella organisms. Am J Vet Res 1964, 25:553-557.
2. Meyer ME: Host-parasite relationships in brucellosis I. reservoirs of infection and interhost transmissibility of the parasite. 70th Proc Annu Meet U S Livestock Sanit Assoc 1966:129-136.
3. Cloeckaert A, Verger JM, Grayon M, Paquet YJ, Garin-Bastuji B, Foster G, Godfroid J: Characterization of Brucella spp. isolated from marine mammals by DNA polymorphism at the emp2 locus. Microbes Infect 2001, 3(9):729-738.
4. Akton GG, Jones LM, Angus RD, Verger JM: Techniques for the Brucellosis Laboratory. Nouzilly, France, Institut National de la Recherche 1988.
5. Michaux-Charchon S, Bourg G, Jumas-Bilka E, Guigue-Talet P, Allardet-Servent A, O’Callaghan D, Ramuz M: Genome structure and phylogeny in the genus Brucella. J Bacterial 1997, 179(10):3244-3249.
6. Gandara B, Marino AL, Rogel MA, Martinez-Romero E: Limited genetic diversity of Brucella spp. J Clin Microbiol 2001, 39(1):235-240.
7. Jahans KL, Foster G, Broughton ES: The characterisation of Brucella strains isolated from marine mammals. Vet Microbiol 1997, 57(4):373-382.
8. Verger JM, Grimont F, Grimont PAD, Grayon M: Brucella, a monospecies genus as shown by deoxyribonucleic acid hybridization. Int J Syst Bacteriol 1985, 35:292-293.
9. Woese CR, Fox GE: Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 1977, 74:5088-5090.
10. Moreno E, Cloeckaert A, Moriyon I: Brucella evolution and taxonomy. Vet Microbiol 2002, 90(1-4):209-227.
11. Halling SM, Peterson-Burch BD, Bricker BJ, Eue HN, Schaal A, Zwiener RL, Zing Q, Li L, Kapur V, Alt DP, Olsen SC: Completion of the genome sequence of Brucella abortus and comparison to the highly similar genomes of Brucella melitensis and Brucella suis. J Bacteriol 2005, 187(8):2715-2726.
12. Gee JF, De BK, Levett PN, Whitney AM, Novak RT, Popovic T: Use of 16S rRNA gene sequencing for rapid confirmatory identification of Brucella isolates. J Clin Microbiol 2004, 42(8):3649-3654.
13. DelVecchio VG, Kapralov V, Redkar RJ, Patra G, Mujer C, Los T, Ivanova N, Anderson I, Bhattacharya A, Lykidis A, Reznik G, Jablonski L, Larsen N, D’Souza M, Bernal A, Mazur M, Goltsman E, Selkow E,
Elzer PH, Hagus S, O’Callaghan D, Letessier JN, Haselkorn R, Kyridpes N, Overbeek R: The genome sequence of the facultative intracellular pathogen Brucella melitensis. Proc Natl Acad Sci USA 2002, 99(13):7948-51.

Paulsen IT, Seshadri R, Nelson KE, Eisen JA, Heidelberg JF, Read TD, Dodson RJ, Umaylam L, Brinkac LM, Benajam MJ, Daugherty SC, Deboy RT, Durkin AS, Kolonay JF, Madupu R, Nelson WC, Ayodeji B, Kraul M, Sherry J, Malek J, Van Aken SE, Riedmuller S, Tettelin H, Gill SR, White O, Salzberg SL, Hoover DL, Lindler LE, Halling SM, Boyle SM, Fraser CM: The Brucella suis genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc Natl Acad Sci USA 2002, 99(20):13148-13153.

Dewhirst FE, Sogin ML, Scimeca MS, Stokes LN, Bourouina T, Chen T, Paster BJ, Fox JG: Discordant 16S and 23S rRNA gene phylogenies for the genus Helicobacter: implications for phylogenetic inference and systematics. J Bacteriol 2005, 187(17):6106-6118.

Vavra MJ: Evolutionary development and taxonomy of the genus Brucella. In Advances in Brucellosis Research Edited by: Adams LG. College Station, TX , Texas Experimental Station; 1990:12-35.

Berisio R, Schluenzen F, Harms J, Bashan A, Yonath A: Structural insight into the role of the ribosomal tunnel in cell regulation. Nat Struct Biol 2003, 10(6):434-439.

Nissen P, Hansen J, Bashan A, Tocilj A, Albrecht R, Schluenzen F, Zarivach R, Harms J, Bashan A, Tocilj A, Albrecht R, Schurig GG, Roop RM, Bagchi T, Boyle S, Buhrman D, Sriranganathan N: Identification and characterization of an insertion sequence, IS711, from Brucella ovis. Gene 1993, 133(1):123-127.

Chaturvedi YP, Snider SE, Suleiman HH, Khairallah AA, El-Assal Y, Vakil NA, Meis PJ, Siegman-Igra Y, Barlet BL: The crystal structure of ribosome: Elongator of the ribosome. Cell 2005, 121(7):1303-1316.

Peric M, Bozdogan B, Galderisi C, Krissinger D, Rager T, Appelbaum NV: Identification of novel agents for combination therapy. Curr Opin Chem Biol 2006, 10(1):21-27.

Meyer M, Berisio R, Baram D, Harms J, Bashan A, Yonath A: A crevice adjoining the ribosome tunnel: hints for cotranslational folding. FEBS Lett 2005, 579(15):3207-3213.

Chittum HS, Champney WS: Macrolide resistance conferred by base substitutions in 23S rRNA. Proc Natl Acad Sci USA 2003, 100(10):5400-5405.

Paulsen IT, Seshadri R, Nelson KE, Eisen JA, Heidelberg JF, Read TD, Dodson RJ, Umaylam L, Brinkac LM, Benajam MJ, Daugherty SC, Deboy RT, Durkin AS, Kolonay JF, Madupu R, Nelson WC, Ayodeji B, Kraul M, Sherry J, Malek J, Van Aken SE, Riedmuller S, Tettelin H, Gill SR, White O, Salzberg SL, Hoover DL, Lindler LE, Halling SM, Boyle SM, Fraser CM: The Brucella suis genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc Natl Acad Sci USA 2002, 99(20):13148-13153.
penalties and weight matrix choice. Nucleic Acids Res 1994, 22:4673–4680.
54. Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP: Bayesian inference of phylogeny and its impact on evolutionary biology. Science 2001, 294(5550):2310-2314.
55. Kumar S, Tamura K, Nei M: MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004, 5(2):150-163.