Comparative Whole-Genome Hybridization Reveals Genomic Islands in *Brucella* Species†

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† Supplemental material for this article may be found at http://jb.asm.org/.

Brucellosis is a zoonotic disease endemic in many areas of the world and is characterized by chronic infections, abortion, and sterility (7). In humans, brucellosis is a systemic, febrile illness resulting in osteoarthitis, endocarditis, and several neurological disorders (7, 53). Brucellosis is caused by many species belonging to the genus *Brucella* that are aerobic facultative intracellular bacteria. *Brucella* species are closely related to intracellular symbionts and pathogens of plants and animals and are classified as α-proteobacteria based on rRNA sequence comparison (36).

The genus *Brucella* consists of six species, designated on the basis of host preference, antigenic and biochemical characteristics as *Brucella melitensis* (goats and sheep), *B. abortus* (cattle), *B. suis* (pigs), *B. canis* (dogs), *B. ovis* (sheep), and *B. neotomae* (wood rats) (6). *B. abortus, B. melitensis,* and *B. suis* can all infect humans with similar serious disease consequences (7). *B. melitensis,* originally isolated as a pathogen of goats and sheep, is highly pathogenic and a frequent cause of human brucellosis. In contrast, human infections of *B. ovis* and *B. neotomae* have not been reported, and *B. canis* rarely causes infection in humans. Human brucellosis occurs via contact with infected animals or animal products and is common in countries where the disease is endemic in domestic animals (13). *Brucella* also can be readily dispersed through aerosol (13), making it a potential biowarfare threat (22, 26).

Although six species are recognized in the genus *Brucella,* DNA-DNA hybridization and multilocus enzyme electrophoresis studies have revealed very limited genetic diversity among *Brucella* species (16, 50). Further, Verger et al. have proposed, based on DNA-DNA hybridization studies, that *B. melitensis* is the only species in the genus *Brucella,* with other *Brucella* species being biovars of *B. melitensis* (50). Genomes of *Brucella* species are stable and have similar genomic organizations (23, 34). Comparison of the complete genome sequences of *B. suis* and *B. melitensis* has revealed extensive similarity in genetic content (>90% of the genes share 98 to 100% identity at the nucleotide level) and gene order (40). In addition, single-nucleotide polymorphisms between these two species are very low (40). These findings strengthen the notion of *Brucella* as a monospecific genus (16, 50) and suggest a relatively small number of differences are responsible for the host preference and virulence restriction of *Brucella* species.

Of serious concern in *Brucella* research is the identification of genetic factors that permit these species to multiply within the host and cause disease. Well-characterized virulence factors of many pathogenic bacteria such as cytolsins, capsules, exotoxins, secreted proteases, pili and/or fimbriae, flagella, phage-encoded toxins, and virulence plasmids are absent in *Brucella* (8). Whole-genome comparison, at greater resolution, of closely related *Brucella* spp. expressing different pathogenicities may provide insights into their virulence determinants. DNA microarrays have been used to investigate genetic content among closely related bacteria (3, 9, 10, 12, 42, 45). The complete genome sequence of the *Brucella* species most pathogenic to humans, *B. melitensis* 16M, permits high-throughput whole-genome comparison of *Brucella* species by using microarrays. Here we have used the genomic sequence of *B. melitensis* 16M to assemble a whole-genome high-density oligonucleotide DNA microarray to compare 16M with other *Brucella* species genomes.
TABLE 1. Brucella strains used in this study* 

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<th>Source</th>
<th>Species</th>
<th>Strain/biotype</th>
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a. Brucella strains used for genomic comparison using B. melitensis 16M microarray are indicated in boldface.
b. Human isolates are indicated with an asterisk.
c. NADC, National Animal Disease Center, Ames, Iowa; LC, Splitter’s Laboratory Brucella culture collection; CAH, Cooper Animal Health, Kansas City, Kans.; WSLH, Wisconsin State Laboratory of Hygiene, Madison, Wis.; ATCC, American Type Culture Collection, Manassas, Va.; WVDL, Wisconsin Veterinary Diagnostic Laboratory, Madison, Wis.; LSU, Philip H. Elzer, Louisiana State University, Baton Rouge, La.

MATERIALS AND METHODS

Strains and culture conditions. The Brucella strains used in the present study are listed in Table 1. These strains were from diverse sources isolated over the last 40 to 50 years and include several strains from human infections. All Brucella strains were grown to stationary phase at 37°C in brucella broth or brain heart infusion broth (Becton Dickinson, Sparks, Md.) with or without 5% CO2. For microarray studies, B. abortus S2308, B. canis RM6/66, B. melitensis 16M, B. neotomae SK33, B. ovis RE0198, and B. suis S100 were used.

B. melitensis 16M DNA microarray construction. A high-density oligonucleotide array was designed by using the published DNA sequence of B. melitensis 16M genome (GenBank accession numbers AE008917 [chromosome I] and AE008918 [chromosome II]) (8). The genome has 3,294,931 total bases and encodes a predicted 3198 open reading frames (ORFs). We designed a high-density oligonucleotide array for the 16M genome by using several criteria for oligonucleotide probe selection. A general description of the array layout and methods for high-density in situ oligonucleotide microarray synthesis are previously described (46). Each oligonucleotide probe is 24 nucleotides, and probes are organized into probe pairs consisting of a perfect-match (PM) probe and a mismatch (MM) probe. The array has a total of 191,834 oligonucleotide probes that are organized into 95,917 probe pairs. The MM probe has two single-base substitutions relative to the corresponding PM probe sequence at positions 6 and 12 (bases were changed as follows: A→T, C→G, G→C, and T→A). Using the programs ProbeAnalyzer and CheckUnique (NimbleGen), potential probe sequences were scanned for high-quality probes and for minimal sequence redundancy by checking each probe against a database of the entire 16M genome and a collection of 8,500 mouse sequences from RefSeq (45). We screened probes against the mouse sequences so that the chip design could be used for in vivo expression profiling of Brucella in future experiments. A total of 17 probe pairs were selected from the coding strand of each of the 3,198 ORFs. For 3,113 ORFs, 17 probe pairs were selected that showed no redundancy within the 16M and mouse sequences. For the remaining 85 ORFs, probes were selected ignoring the fact that many probes for these ORFs had significant matches to mouse or 16M sequences. The array also consists of probes specific to non-ORF encoding regions of the genome, which include the untranslated RNA encoding genes. Probes for these regions were selected by using the same criteria as for the ORF probes. The probe pairs were synthesized at randomized locations on the chip by using a maskless array synthesizer (NimbleGen [46]).

Genomic DNA extraction and labeling. The genomic DNA (gDNA) was prepared from Brucella strains grown to stationary phase in brucella broth by using MasterPure genomic DNA extraction kit (Epicentre, Madison, Wis.), and 10 to 15 μg of gDNA was fragmented to 50 to 150 bp by partial digestion with DNase I (0.015 U/μg of DNA; Roche, Indianapolis, Ind.) at 37°C for 10 min. DNase I was heat inactivated at 95°C for 15 min. Fragmentation was confirmed by agarose gel electrophoresis. Fragmented gDNA was purified through a Microcon YM-10 column (Millipore) and 10 μl of labeled DNA was hybridized to the 16M microarray in 300 μl of hybridization solution containing 50 mM morpholinomethanesulfonic acid (MES), 0.5 M NaCl, 10 mM EDTA, 0.005% (vol/vol) Tween 20, 1 μm CPE oligo, 0.2 mg of acetylated bovine serum albumin, and 0.04 mg of herring sperm DNA for 16 h at 42°C. After hybridization, arrays were washed three times over a 5-min period at room temperature with nonstringent wash buffer (6× SSPE [1× SSPE is 0.18 M NaCl, 10 mM Na2HPO4, and 1 mM EDTA; pH 7.7], 0.01% [vol/vol] Tween 20), followed by stringent wash buffer (100 mM MES, 0.1 M NaCl, 0.01% Tween 40).
results

Microarray analysis. To identify genetic determinants of 16M that contribute to its virulence and pathogenicity, we performed comparative genomic analysis of *Brucella* species. This microarray comparison only allows the detection of regions that are missing in other *Brucella* species relative to the 16M genome and will not detect regions that are unique to other *Brucella* strains. Hybridization results revealed that major-ity of the 16M ORFs are likely present across all five *Brucella* genomes examined as illustrated by the majority of ORFs with a signal intensity ratio close to 1 relative to 16M (Fig. 1). This finding is consistent with the notion that *Brucella* is a monospecific genus with limited genetic diversity (16, 50).

Punctuating this pattern of conservation, the data suggest a number of regions likely to be deleted or duplicated in one or more of the *Brucella* species (Fig. 1). Many of the putative deleted regions were clustered within the 16M genome. We identified nine regions containing at least three contiguous ORFs, designated genomic islands (GIs), that were absent among five *Brucella* species. The GIs were numbered 1 through 9 based on the gene order in 16M.

Putative deletions identified by microarray-based comparisions were examined by PCR amplification and sequencing. Table 3 lists the ORFs altered and the precise boundaries of deleted regions with respect to the 16M genome. The predicted product of each altered ORF and its distribution across *Brucella* species can be seen in Fig. 2. Amplification and sequencing of the predicted deletion junction confirmed nearly all of the predicted differences. ORFs that were present in multiple copies in the 16M genome or partially missing were scored as present by microarray hybridization and later confirmed to be absent from a GI by PCR and sequencing. Although the number of probe pairs for each ORF was constant (17 probe pairs), their spacing within ORFs was variable. Since we averaged probe pair values across ORFs, detection of partially deleted ORF was dependent on probe location in an ORF and on ORF length. Only two ORFs, BMEI0728 and BMEI1314, although called deleted by the microarray, were present by PCR and sequencing. ORF BMEI11071 was identified as absent by microarray analysis in *B. canis*, *B. neotomae*, and *B. suis*, even though a DNA fragment of size similar to 16M was amplified from each species. Sequencing revealed that the BMEI11071 region from these three species was highly divergent compared to 16M.

16M ORFs absent in *Brucella* species: comparison with *B. ovis* RE0198. *B. ovis* causes contagious epididymitis in rams and, rarely, abortion in ewes. *B. melitensis*, although originally isolated as a pathogen of goats and sheep, is pathogenic to humans, whereas *B. ovis* does not infect humans and the ovine brucellosis due to *B. ovis* is not zoonotic. Comparison of *B. ovis* and *B. melitensis* genomes may identify the factors responsible for these changes in host specificity. Array hybridization, followed by PCR and sequencing, revealed 84 ORFs distributed in nine chromosomal loci were either partially or completely absent (Table 3 and Fig. 2). All but four deleted ORFs were clustered in five islands relative to the 16M genome (GI-1, GI-2, GI-5, GI-7, and GI-9).

GI-1 (~8.1 kb; nine ORFs) contains mostly ORFs encoding hypothetical proteins (HPs) and phage-related genes, including a resolvase similar to phage Mu DNA inverase.

GI-2 (~15.1 kb; 20 ORFs) includes ORFs encoding HPs, transposases, and a phage family integrase. Interestingly, this island also contains two ORFs (BMEI0997 and BMEI0998) involved in lipopolysaccharide (LPS) biosynthesis and their absence may contribute to the rough phenotype associated with *B. ovis*. In addition, an ORF encoding a 25-kDa outer membrane protein precursor, Omp25, is also included in this cluster.

GI-5 (~44.1 kb; 42 ORFs) includes ORFs encoding peptide ABC transporters such as spermidine-putrescine (Pot), oligopeptide (Opp), and dipeptide (Dpp); transcriptional regulators similar to an AtrA regulator that is required for attachment and virulence of *Agrobacterium tumefaciens* (2); and two ORFs similar to cephaplorin acylases. *B. suis* and *B. abortus* genome sequences contain these two cephaplorin acylases encoding ORFs from 16M (317 and 466 amino acids) fused as a single ORF encoding a 761-amino-acid protein 47% identical to 774-amino-acid protein from *Pseudomonas* species (30). Cephaplorin acylases are involved in the activation of cephalosporin antibiotics, and the eightfold-higher MIC observed for *B. ovis* relative to *B. abortus* (49) may be due to lack of acylase from *B. ovis*.

GI-7 (~4.4 kb; five ORFs) and GI-9 (~4.9 kb; four ORFs) consist of AraC and GntR family transcriptional regulators, a hydrolase, and an ORF involved in amino acid metabolism.

Comparison with *B. neotomae* SK33. *B. neotomae* only infects desert wood rats under natural conditions and is not
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<td>BMEII1015F</td>
<td>CCAAGTTGCAAACGTTGTAAGCA</td>
</tr>
<tr>
<td>BMEII1017</td>
<td>BMEII1017R</td>
<td>TCTGGGAAATGGAACAGTAC</td>
</tr>
<tr>
<td>BMEII1071</td>
<td>BMEII1071F</td>
<td>CTGCAAGGAGCTGCAAGA</td>
</tr>
<tr>
<td>BMEII1072R</td>
<td>GCTGTCAGGCGGAGGGAATA</td>
<td></td>
</tr>
</tbody>
</table>

*ID, identification designation.
associated with disease. Whole-genome comparison of *B. neotomae* might reveal a defined set of genes critical for 16M pathogenesis. Hybridization of *B. neotomae* gDNA to the 16M array revealed a very similar gene content, a finding consistent with previous work (34), suggesting a close phylogenetic relationship between these two species compared to other Brucella species. Only 17 ORFs were identified as altered, including a region containing 10 contiguous ORFs (~7.5 kb, GI-6) (Table 3 and Fig. 2). ORFs in GI-6 include those encoding transposases with significant similarity to ORFs on plasmid pNGR234a from *Rhizobium* species (15). A 2.2-kb region in chromosome II containing ORFs involved in denitrification, including a transcriptional regulator of the CRP/FNR family (BMEII0986), was also missing.

**Comparison with *B. canis* RM6/66.** *B. canis* is a pathogen of dogs that causes epididymo-orchitis in males and abortion in females. Although *B. canis* infects humans, clinical disease is rarely reported. Comparison of *B. canis* genome to 16M may reveal determinants responsible for pathogenicity in humans. A total of 38 ORFs distributed in seven chromosomal loci were found either partially or completely deleted (Table 3 and Fig. 2). Most *B. canis* deletions were identical to *B. suis* deletions except for three ORFs, a finding consistent with the presumed close phylogenetic relationship between these two species (16, 34). Thirty ORFs clustered within a 21-kb region in chromosome I (GI-3), mostly encoding proteins of unknown functions were missing in *B. canis* as well as *B. suis*. There are three ORFs absent from *B. canis* but present in all other
Brucella species, including B. suis. Particularly interesting is the absence of a polysaccharide deacetylase that is similar to chito oligosaccharide deacetylase NodB of Rhizobium species, since this factor is involved in establishing a symbiotic interaction between bacteria and host (44).

Comparison with B. suis S100. A total of 39 ORFs were absent either completely or partially (Table 3 and Fig. 2), and these alterations were nearly identical to the set identified in B. canis. A B. suis genome has been sequenced, and the differences that are described by whole-genome sequence comparison (40) are almost entirely consistent with the microarray results except for three small regions (<200 bp) that were not identified by microarray analysis. Closser analysis of these ORFs led to the conclusion that these regions were not identified as deleted. Interestingly, we also identified a 2-kb region involving two ORFs missing in B. suis 100 (BMEI1746 and BMEI1747) but present in the sequenced B. suis 1330 (biotype 1). B. suis 100 is also biotype 1, isolated from swine with abortion, and is virulent in the IRF-1−/− mouse model similar to B. suis 1330 and B. melitensis 16M (G. Rajashekara and G. Splitter, unpublished data), suggesting that this 2-kb region is dispensable for virulence and pathogenicity.

Comparison with B. abortus S2308. Forty ORFs were absent from the B. abortus genome, including two clusters involving more than three contiguous ORFs, one in chromosome I (5 ORFs, ~3.8 kb, GI-4) and another in chromosome II (25 ORFs, ~25.1, GI-8) (Table 3 and Fig. 2). GI-8 primarily contains ORFs encoding proteins involved in sugar metabolism and LPS biosynthesis, whereas GI-4 has ORFs encoding butanoate metabolism. Other ORFs include a response regulator (BMEI0292) and a diguanylate cyclase (BMEI0929). Most of these regions were absent from the genome sequence of B. abortus (University of Minnesota [http://www.cbc.umn.edu/ResearchProjects/AGAC/Pub.Brucella/Brucellahome.html]), except for GI-4, suggesting that the strain we used, similar to B. suis, was not identical to the sequenced strain.

Deletions in Brucella species are present in multiple strains. Since microarray hybridizations were performed with only one strain of each species, it is possible that the deletions observed are specific to the strain that was used in the microarray experiment. To confirm that the microarray data comparing single strains is in fact representative of the species, we examined 47 strains representing B. abortus, B. canis, B. ovis, and B. suis by PCR. PCR was performed with primers specific to ORFs flanking the predicted edges of each deletion. As expected, the deletions observed in these four species were present in multiple strains of the same species (Table 4). Only two regions in B. abortus (BMEI0929 and BMEI1919-1923; GI-4), one region in B. ovis (BMEI0405), and two regions in B. suis (BMEI0899-
FIG. 2. Genomic comparison of five Brucella species. ORFs, deleted either partially or completely in five Brucella species relative to 16M, are shown. Putative deletions were selected if the average hybridization signal ratio was 4 SD below the mean. Each putative deletion was confirmed by PCR, followed by sequencing. ORFs in red had ratios lower than the average and were confirmed missing. ORFs in green and blue were...
confirmed missing by PCR and sequencing but did not have low ratios since the sequences were either present in multiple copies in the 16M genome (green) or were partially deleted (blue). ORFs that were unaltered relative to 16M are gray. An asterisk indicates ORFs in predicted islands for which the ratio was only 3 SD below the mean.
or 0900 and BMEI1746-1747) were not deleted in some strains (see Tables S1A to D in the supplemental material for the PCR results for each strain). For *B. neotomae*, however, more strains could not be tested since only two strains have been described, including the one used here. Since deletions identified by microarray are conserved in multiple strains of other *Brucella* species, it is more likely that the changes in *B. neotomae* are species specific. In contrast, except for the two regions (BMEI0899-0900 and BMEI0926), none of the 15 *B. melitensis* isolates had alterations in regions that were identified by microarray as deleted in other *Brucella* species (Table 4) (see Tables S2A and B in the supplemental material for the PCR results for each strain). Interestingly, the GIs missing in *Brucella* species that are nonpathogenic to humans are present in all other *Brucella* species, including 15 *B. melitensis* strains examined (Table 4) and in at least 3 *B. abortus* and 2 *B. suis* strains (data not shown), suggesting that these GIs may contribute to human infection.

### 16M GI acquisition

Analysis of sequences surrounding the GIs in 16M suggests that many of the GIs were acquired through horizontal transfer. Figure 3 shows the schematic representation of five GIs with hallmarks of horizontal transfer. Typical of pathogenicity islands (PIs) of many pathogenic bacteria (17, 19), these islands were integrated adjacent to or within tRNA genes with an integrase or insertion sequence (IS) flanking the ends. The presence of direct or inverted repeats at the island boundaries may indicate the sites of recombination (Fig. 3). In addition, these regions have variable GC content compared to the remaining genome and most ORFs present in GI-1, -2, and -3 have no significant similar sequence in GenBank. ORFs on GI-5 have an atypical trinucleotide composition, whereas ORFs in GI-6 are similar to ORFs on *Rhizobium* plasmid pNGR234a (15). These features suggest that these GIs were acquired by lateral transfer of DNA.

**Duplication of a GI in *B. ovis***

Microarray analysis revealed 30 ORFs with ratios significantly >1, suggesting that these ORFs are present in multiple copies (Table 5). In the *B. ovis* genome, a cluster of 22 contiguous ORFs relative to 16M (~25.5 kb; duplicated region [DR]) mostly encoding HPs and t-amino acid transport genes, had ratios of ~2, suggesting duplication of the entire region. One ORF (BMEI1214) in the cluster, however, had a ratio of ~3, suggesting a higher copy number of this ORF. In addition, seven ORFs, five encoding ISs were duplicated across four species (Table 5), including IS6501 ORFs that are known to be present in more copies in *B. ovis* than *B. melitensis* (37).

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**TABLE 4. PCR analysis of *Brucella* strains to detect deletions identified by microarray**

<table>
<thead>
<tr>
<th>ORFs in the deleted region (GI no.)</th>
<th><em>B. abortus</em></th>
<th><em>B. suis</em></th>
<th><em>B. canis</em></th>
<th><em>B. neotomae</em></th>
<th><em>B. ovis</em></th>
<th><em>B. melitensis</em></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Chromosome I</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>BMEI0284</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1/1</td>
<td>–</td>
<td>0/15</td>
</tr>
<tr>
<td>BMEI0801</td>
<td>–</td>
<td>12/12</td>
<td>7/7</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
</tr>
<tr>
<td>BMEI0899-0900&lt;sup&gt;p&lt;/sup&gt;</td>
<td>–</td>
<td>10/12</td>
<td>7/7*</td>
<td>–</td>
<td>–</td>
<td>2/15</td>
</tr>
<tr>
<td>BMEI0926</td>
<td>15/15</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
</tr>
<tr>
<td>BMEI0929</td>
<td>–</td>
<td>14/15</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
</tr>
<tr>
<td>BMEI0899-0907 (1)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>13/13</td>
<td>0/15</td>
<td></td>
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<tr>
<td>BMEI0993-1012 (2)</td>
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<td>–</td>
<td>–</td>
<td>13/13</td>
<td>0/15</td>
<td></td>
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<tr>
<td>BMEI1435&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
<td>0/12</td>
<td>7/7</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
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<tr>
<td>BMEI1657</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1/1</td>
<td>–</td>
<td>0/15</td>
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<tr>
<td>BMEI1661-1662</td>
<td>15/15</td>
<td>–</td>
<td>7/7</td>
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<td>0/15</td>
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<tr>
<td>BMEI1746-1747</td>
<td>–</td>
<td>12/12</td>
<td>7/7</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
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<tr>
<td>BMEI1746-1747</td>
<td>–</td>
<td>1/12</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
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<tr>
<td>BMEI1819</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>0/15</td>
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<tr>
<td>BMEI1873</td>
<td>–</td>
<td>12/12</td>
<td>7/7</td>
<td>–</td>
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<tr>
<td>BMEI1896</td>
<td>15/15</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>BMEI1919-1923 (4)</td>
<td>4/15</td>
<td>–</td>
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<td>–</td>
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<td>BMEI0129</td>
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<td>–</td>
<td>13/13</td>
<td>0/15</td>
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<td>BMEI0185-0226 (5)</td>
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<td>–</td>
<td>–</td>
<td>13/13</td>
<td>0/15</td>
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<tr>
<td>BMEI0291-0292</td>
<td>15/15</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
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<tr>
<td>BMEI0405</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2/13</td>
<td>0/15</td>
<td></td>
</tr>
<tr>
<td>BMEI0635-0636&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>2/12*</td>
<td>7/7</td>
<td>–</td>
<td>–</td>
<td>0/15</td>
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<tr>
<td>BMEI0708</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>13/13</td>
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<td>BMEI0717</td>
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<td>BMEI0710-0719 (6)</td>
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<td>–</td>
<td>–</td>
<td>1/1</td>
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<td>0/15</td>
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<td>BMEI0811-0815 (7)</td>
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<td>–</td>
<td>–</td>
<td>13/13</td>
<td>0/15</td>
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<tr>
<td>BMEI0826-0850 (8)</td>
<td>15/15</td>
<td>–</td>
<td>–</td>
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<td>0/15</td>
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<tr>
<td>BMEI0875-0878 (9)</td>
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<td>–</td>
<td>–</td>
<td>13/13</td>
<td>0/15</td>
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<tr>
<td>BMEI0986-0988</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1/1</td>
<td>–</td>
<td>0/15</td>
</tr>
<tr>
<td>BMEI1016</td>
<td>15/15</td>
<td>–</td>
<td>–</td>
<td>13/13</td>
<td>0/15</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> PCR was performed on several strains from each species for those regions that were identified as deleted in five *Brucella* spp. compared to 16M by microarray (–, not tested by PCR).

<sup>b</sup> *B. suis*-specific deletion (except in two type 4 isolates from dogs) not present in *B. canis* strains (marked by asterisks).

<sup>c</sup> *B. canis*-specific deletion, not present in *B. suis* strains.

<sup>d</sup> *B. canis*-specific deletion, not present in *B. suis* strains except for the same two type 4 isolates from dogs (marked by asterisks.)

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*RAJASHEKARA ET AL.*

*J. BACTERIOL.*

*November 10, 2017*
active IS element in *B. ovis*. ORFs in GI-2 and GI-5 encode factors involved in *Brucella* virulence. Smooth LPS is one of the key virulence factors in *Brucella* pathogenesis (41); rough LPS mutants of *Brucella* are attenuated (35). Smooth LPS O chain is involved in inhibition of early fusion between *Brucella* containing phagosomes and lysosomes in murine macrophages and mutations that inactivate the LPS biosynthetic gene, *wbdA* (BMEI0997), in *B. suis* attenuate its ability to multiply within macrophages (25). Likewise, Omp25 is implicated in virulence of *B. melitensis* and *B. abortus*, and inactivation of Omp25 in *Brucella* results in attenuation in BALB/c mice (11). Omp25 is an Omp3 homologue found in many pathogens or symbionts of α-proteobacteria, where it plays a role in bacterial surface control and host cell interactions (18). GI-5 has 19 ORFs encoding peptide ABC-type transporters such as Dpp, Opp, and Pot systems. Homologs of these transporters in other bacteria are important for root colonization, intracellular survival, attachment to host cell, and virulence (4, 27, 32). *B. ovis* is highly sensitive to cationic antimicrobial peptides in contrast to other *Brucella* species, including the rough mutants of *B. abortus* (14). Similarly, *Salmonella enterica* serovar Typhimurium Opp mutants exhibit increased susceptibility to antimicrobial peptides and reduced virulence (39). Absence of the Opp system in *B. ovis* may cause increased uptake of peptides due to disregulation of rate of peptide uptake, influencing their intracellular survival. In fact, *B. ovis* is more readily destroyed within nonprofessional phagocytes compared to other *Brucella* (14). Further studies are required to determine whether GI-2 and GI-5 play important roles in *Brucella* pathogenesis and may constitute novel PIs. Despite lacking these potential virulence factors, *B. ovis* is virulent in sheep, suggesting that unknown genes in *B. ovis* that are absent in 16M may contribute to pathogenicity in sheep.

In *B. neotomae*, denitrification genes were altered. BMEI0986 encodes a CRP/FNR family transcriptional regulator with significant homology to the denitrification regulator *nnrR* of other α-proteobacteria that is required for anaerobic growth in the presence of nitrite or nitrate and regulates nitrite reductase activity (1, 28, 52). In fact, *nnrR* (BMEI0986) of *B. melitensis* 16M restores denitrification in NmrR-deficient *A. tumefaciens*, suggesting that denitrification is regulated similarly in α-proteobacteria (Seung-Hun Baek, G. Rajashekara, G. A. Splettier, and J. P. Shapleigh, unpublished data). The nitrate, nitrous oxide, and nitric oxide reductase genes of *B. melitensis*

### DISCUSSION

Microarray analysis revealed extensive similarities among *Brucella* species in their gene content. Only 217 ORFs were absent either completely or partially compared to 16M. We also discovered that >3,110 ORFs of the 3,198 ORFs represented on the microarray were present in any given *Brucella* species. Our results suggest that genomes of *Brucella* species are similar and imply that a relatively small number of genetic changes may be responsible for differences in host preference and virulence among *Brucella* species. In nature, *Brucella* are found predominantly associated with macrophages and are not known to harbor extragenetic material such as plasmids, and there is no evidence of natural transfer of genetic material. Therefore, stability of *Brucella* genomes may be largely due to a lifestyle that keeps these bacteria genetically isolated similar to obligate intracellular symbionts of *Buchnera* species (48).

The large number of changes seen in the *B. ovis* genome (five GIs) may have resulted from more active ISs in this species compared to other *Brucella* spp. *B. ovis* has more IS6501 copies (~30) than other *Brucella* species (4 to 10 copies), and IS6501 hybridization could distinguish strains of *B. ovis* but not strains of *B. melitensis* (37), suggesting a more

### TABLE 5. Duplication of ORFs in *Brucella* spp.

<table>
<thead>
<tr>
<th>BME_ID*</th>
<th>Species</th>
<th>Ratio(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMEI0200</td>
<td><em>B. neotomae</em></td>
<td>1.59</td>
</tr>
<tr>
<td>BMEI0901</td>
<td><em>B. canis</em></td>
<td>1.65</td>
</tr>
<tr>
<td>BMEI192</td>
<td><em>B. ovis</em>, <em>B. suis</em></td>
<td>2.30, 2.24</td>
</tr>
<tr>
<td>BMEI1210-1221</td>
<td><em>B. ovis</em></td>
<td>1.82–2.81</td>
</tr>
<tr>
<td>BMEI1815</td>
<td><em>B. ovis</em></td>
<td>1.83</td>
</tr>
</tbody>
</table>

* B. melitensis ORF designation.
are predicted to be highly expressed (24), suggesting that Brucella can efficiently use alternative energy sources under anaerobic conditions. The lack of nitrite reductase activity suggests that B. neotomae, unlike other Brucella spp., cannot reduce nitrite and is likely not able to use nitrogen as an alternative energy source in oxygen deprivation and be more sensitive to toxic effects of NO encountered within the macrophage.

Our results do not clearly identify deletions that might explain the phenotypic differences, such as oxidase activity, CO₂ requirement, H₂S production, growth on basic fuchsin- or thionin-containing medium, that are conventionally used to distinguish Brucella species. These phenotypic differences may be related to some of the genes whose functions are unknown or due to mutations such as single base changes that are unlikely to be detected by using microarray hybridization.

Examination of sequences within or flanking several 16M GIs revealed striking similarities in genomic organization to PIs from other bacteria. PIs are mostly found adjacent to or integrated into tRNA genes and flanked by ISs. Integrate genes encoded on plagues or plasmids often mediate insertion at tRNA genes, suggesting that PI acquisition is a phage- or plasmid-mediated process (5). Five of the 16M GIs were found either within or adjacent to tRNA genes with ISs flanking one or both ends and have direct or inverted repeats at their ends (Fig. 3), suggesting that these GIs were acquired by lateral gene transfer. Insertions have resulted in either duplication of the entire tRNA gene (GI-1) or reconstitution with a portion of tRNA genes present as a repeat at the opposite end (GI-2 and GI-3). Strikingly, many of the sites of GI integration in Brucella are known to be sites of the integration of islands in other bacteria. Insertion of GI-2 into a Gly-tRNA gene is similar to island insertion in Pseudomonas and Xanthomonas species (29). A symbiosis island in one other member of the α₂-proteobacteria, Mesorhizobium loti, also integrates into a Phe-tRNA gene (47) similar to GI-3. Integration into Gly- or Phe-tRNA genes is likely phage mediated, particularly, insertion of GI-3 seems to be mediated by a P4 family integrase by using the terminal CCA of the tRNA gene as an attachment site similar to the symbiosis island of M. loti. Although mechanisms of GI-3 insertion appear similar, the gene contents are different as ORFs in GI-3 have no similar sequence in M. loti or other members of the α₂-proteobacteria.

Ser-tRNA genes are also frequent targets for horizontal transfer of genetic materials (20, 51), and GI-5 and GI-6 are associated with this locus. Unlike GI-1, -2, or -3, incorporation of GI-5 and GI-6 is likely not phage mediated. GI-5 is present upstream of a Ser-tRNA gene and is flanked by identical copies of an IS similar to S. sonnei IS600 (31). ORFs on GI-5 are conserved in broad range of species and have an atypical trinucleotide composition, suggesting a foreign origin. Whereas, GI-6, is located downstream of a second Ser-tRNA gene and encodes several ISs similar to ORFs on a plasmid pNGR234a of Rhizobium species (15).

In conclusion, our study has identified a defined set of genes missing from Brucella species that are not pathogenic to humans. Our findings now facilitate additional studies to understand the relevance of these genes or gene clusters in host adaptation and virulence restriction of certain Brucella species to humans. Furthermore, our results revealed several genomic islands are likely acquired in Brucella, and many islands have large numbers of ORFs encoding proteins with unknown functions. Brucella lacks well-characterized virulence factors of many pathogenic bacteria such as cytolsins, capsules, exotoxins, secreted proteases, pili and/or fimbriae, flagella, phage-encoded toxins, and virulence plasmids (8, 40). The GIs missing in B. ovis are present in other Brucella species that are pathogenic to humans. However, B. neotomae, a species that is not pathogenic to humans and domestic animals, also possesses these islands. In addition, B. canis and B. suis, although differing in virulence to humans, are genetically very similar. These findings imply that, in addition to the loss or gain of genetic content in Brucella species, mechanisms involving gene inactivation or altered expression of virulence traits as seen in Bordetella species (38) may contribute to differences in host range and virulence of Brucella species for humans. However, we cannot rule out the possibility of genetic contents that are unique to Brucella species other than 16M contributing to these differences. Future studies are necessary to understand the relevance of these GIIs to the host adaptation and virulence of Brucella species.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grant R01AI048490, NIH/NIAID Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (RCE) Program grant 1-U54-AI-057153, and grants BARD-US 2968-98C (to G.A.S.) and R44HG002193 to Roland Green, NimbleGen Systems.

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