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Analysis of Ten *Brucella* Genomes Reveals Evidence for Horizontal Gene Transfer Despite a Preferred Intracellular Lifestyle▽§

Alice R. Wattam,[1]* Kelly P. Williams,[1] Eric E. Snyder,[1] Nalvo F. Almeida, Jr.,[1,6] Maulik Shukla,[1] A. W. Dickerman,[1] O. R. Crasta,[1] R. Kenyon,[1] J. Lu,[1] J. M. Shallom,[1] H. Yoo,[1] T. A. Ficht,[2] R. M. Tsolis,[3] C. Munk,[4] R. Tapia,[4] C. S. Han,[4] J. C. Detter,[4] D. Bruce,[4] T. S. Brettin,[4] Bruno W. Sobral,[1] Stephen M. Boyle,[5] and João C. Setubal[1,7]

Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, Virginia 24061[1]; Department of Veterinary Pathobiology, Texas A&M University and Texas Agricultural Experiment Station, College Station, Texas 77843-4467[2]; Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave., Davis, California 95616-8645[3]; Bioscience Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87544[4]; Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia 24061[5]; Departamento de Computacao e Estatistica/Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil[6]; and Department of Computer Science, Virginia Tech, Blacksburg, Virginia 24061[7]

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The facultative intracellular bacterial pathogen *Brucella* infects a wide range of warm-blooded land and marine vertebrates and causes brucellosis. Currently, there are nine recognized *Brucella* species based on host preferences and phenotypic differences. The availability of 10 different genomes consisting of two chromosomes and representing six of the species allowed for a detailed comparison among themselves and relatives in the order *Rhizobiales*. Phylogenomic analysis of ortholog families shows limited divergence but distinct radiations, producing four clades as follows: *Brucella abortus-Brucella melitensis*, *Brucella suis-Brucella canis*, *Brucella ovis*, and *Brucella ceti*. In addition, *Brucella* phylogeny does not appear to reflect the phylogeny of *Brucella* species' preferred hosts. About 4.6% of protein-coding genes seem to be pseudogenes, which is a relatively large fraction. Only *B. suis* 1330 appears to have an intact β-ketoadipate pathway, responsible for utilization of plant-derived compounds. In contrast, this pathway in the other species is highly pseudogenized and consistent with the "domino theory" of gene death. There are distinct shared anomalous regions (SARs) found in both chromosomes as the result of horizontal gene transfer unique to *Brucella* and not shared with its closest relative *Ochrobactrum*, a soil bacterium, suggesting their acquisition occurred in spite of a predominantly intracellular lifestyle. In particular, SAR 2-5 appears to have been acquired by *Brucella* after it became intracellular. The SARs contain many genes, including those involved in O-polysaccharide synthesis and type IV secretion, which if mutated or absent significantly affect the ability of *Brucella* to survive intracellularly in the infected host.

Brucellosis is a disease caused by bacteria of the genus *Brucella*. This disease is zoonotic and endemic in many areas throughout the world, causing chronic infections with common outcomes being abortion and sterility in infected animals. In humans, it is a severe acute febrile disease, producing focal lesions in bones, joints, the genitourinary tract, and other organs. Complications may include arthritis, sacroiliitis, spondylitis, and central nervous system effects. *Brucella* can cause abortions in women (as can other bacteria), mostly in the first and second trimesters of pregnancy (21, 27), and men can exhibit epididymo-orchitis (37).

Currently, there are nine recognized species of *Brucella*, based on host preferences and phenotypic differences. Six classically recognized species are *Brucella abortus* (cattle), *Brucella canis* (dogs), *Brucella melitensis* (sheep and goats), *Brucella neotomae* (desert wood rats), *Brucella ovis* (sheep), and *Brucella suis* (pigs, reindeer, and hares). These six species have been subdivided into 18 biovars based on a panel of culture and biochemical characteristics (41). Recently, three additional species have been identified, namely *Brucella microti* from voles (49), "*Brucella pinnipediae*" from pinnipeds, and *Brucella ceti* from cetaceans (20).

The genome from *B. melitensis* was the first to be sequenced (16), followed by those from strains of *B. suis* and *B. abortus* (9, 11, 24, 44). New genome sequences for *B. canis*, *B. ceti*, *B. melitensis*, and *B. suis*, as well as the recent release of the *B. ovis* genome, allow a more detailed look into this group. Furthermore, the increasing number of genomes for *Brucella* relatives from the order *Rhizobiales* allows examination of this genus in a broader context.

The main objectives of this study were to examine the phylogeny of *Brucella*, to examine differences among the different genomes and clades, and to do a detailed comparison between the *Brucella* genomes and those of their closest relatives in *Rhizobiales*. Techniques used to examine these differences included structural analysis of the *Brucella* chromosomes, an in-depth study of areas of possible horizontal transfer into the

* Corresponding author. Mailing address: Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, VA 24061. Phone: (540) 231-1263. Fax: (540) 231-2606. E-mail: wattam@vbi.vt.edu.

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TABLE 1. *Rhizobiales* genomes used in OG analysis

Genome	Strain	GenBank accession number(s)	Family
Agrobacterium tumefaciens	C58 Cereon	NC_003062, NC_003063, NC_003064, NC_003065	*Rhizobiaceae*
Azorhizobium caulinodans	ORS 571	NC_009937	*Xanthobacteraceae*
Bartonella bacilliformis	KC583	NC_008783	*Bartonellaceae*
Bartonella henselae	Houston-1	NC_005956	*Bartonellaceae*
Bartonella quintana	Toulouse	NC_005955	*Bartonellaceae*
Bartonella tribocorum	CIP 105476	NC_010161 NC_010160	*Bartonellaceae*
Bradyrhizobium	BTAi1	NC_009475, NC_009485	*Bradyrhizobiaceae*
Bradyrhizobium	ORS278	NC_009445	*Bradyrhizobiaceae*
Bradyrhizobium japonicum	USDA 110	NC_004463	*Bradyrhizobiaceae*
Brucella abortus	9–941	NC_006932, NC_006933	*Brucellaceae*
Brucella abortus	S19	NC_010742, NC_010740	*Brucellaceae*
Brucella canis	ATCC 23365	NC_010103, NC_010104	*Brucellaceae*
Brucella ceti	Cudo	NZ_ACJD00000000	*Brucellaceae*
Brucella melitensis	16 M	NC_003317, NC_003318	*Brucellaceae*
Brucella melitensis	ATCC 23457	NC_012441, NC_012442	*Brucellaceae*
Brucella abortus	2308	NC_007618, NC_007624	*Brucellaceae*
Brucella ovis	ATCC 25840	NC_009504, NC_009505	*Brucellaceae*
Brucella suis	1330	NC_004310, NC_004311	*Brucellaceae*
Brucella suis	ATCC 23445	NC_010169, NC_010167	*Brucellaceae*
Fulvimarina pelagi	HTCC2506	NZ_AATP00000000	"*Aurantimonadaceae*"
Hoeflea phototrophica	DFL-43	NZ_ABIA00000000	*Phyllobacteriaceae*
Mesorhizobium	BNC1	NC_008254, NC_008242, NC_008243, NC_008244	*Phyllobacteriaceae*
Mesorhizobium loti	MAFF303099	NC_002678, NC_002679, NC_002682	*Phyllobacteriaceae*
Methylobacterium extorquens	PA1	NC_010172	*Methylobacteriaceae*
Nitrobacter hamburgensis	X14	NC_007959, NC_007960, NC_007961, NC_007964	*Bradyrhizobiaceae*
Nitrobacter winogradskyi	Nb-255	NC_007406	*Bradyrhizobiaceae*
Ochrobactrum anthropi	ATCC 49188	NC_009667, NC_009668, NC_009669, NC_009670, NC_009671, NC_009672	*Brucellaceae*
Rhizobium etli	CFN 42	NC_007761, NC_007762, NC_007763, NC_007764, NC_007765, NC_007766, NC_004041	*Rhizobiaceae*
Rhizobium leguminosarum bv. viciae	3841	NC_008380, NC_008381, NC_008382, NC_008383, NC_008384, NC_008378, NC_008379	*Rhizobiaceae*
Rhodopseudomonas palustris	BisA53	NC_008435	*Bradyrhizobiaceae*
Rhodopseudomonas palustris	BisB18	NC_007925	*Bradyrhizobiaceae*
Rhodopseudomonas palustris	BisB5	NC_007958	*Bradyrhizobiaceae*
Rhodopseudomonas palustris	CGA009	NC_005297, NC_005296	*Bradyrhizobiaceae*
Rhodopseudomonas palustris	HaA2	NC_007778	*Bradyrhizobiaceae*
Sinorhizobium medicae	WSM419	NC_009620, NC_009621, NC_009622, NC_009636	*Rhizobiaceae*
Sinorhizobium meliloti	1021	NC_003047, NC_003037, NC_003078	*Rhizobiaceae*
Xanthobacter autotrophicus	Py2	NC_009717, NC_009720	*Xanthobacteraceae*

Brucella genomes, and a comparison of known genes and pseudogenes present in other *Brucella* genomes that correspond to them.

MATERIALS AND METHODS

Genome sequences and annotation. Ten different strains from six of the *Brucella* species were used in this comparison. Three strains with complete genomes (*B. canis* ATCC 23365, *B. melitensis* ATCC 23457 [bv. 2], and *B. suis* ATCC 23445 [bv. 2]) were sequenced by Los Alamos National Labs and the Joint Genome Institute. They also sequenced *B. ceti*, which has an incomplete genome with seven contigs. All were given their primary annotation by PATRIC, which is the NIAID/PathoSystems Resource Integration Center, a major repository for *Brucella* genomic data (51). Six additional strains that had been annotated previously (*B. abortus* S19, *B. abortus* bv. 1 strain 9-941, *B. melitensis* 16 M, *B. abortus* 2308, *B. ovis* ATCC 25840, and *B. suis* 1330) were reannotated by PATRIC prior to the comparison to ensure uniformity.

Genome alignment. Chromosomal DNA sequences from nine *Brucella* species (all except *B. ceti*) were aligned using Mauve 2.2.0 (14).

OG identification. We used OrthoMCL (32) to create groups of orthologous proteins. To create a representative set of ortholog groups (OGs) for the order *Rhizobiales*, 37 complete or nearly complete genomes were used (Table 1), incorporating 8 of the 11 families in the order.

Pseudogenes. In this study, a pseudogene is defined as a gene containing one or more in-frame stop codons and/or frameshifts (FS) compared to those of its orthologs. Three methods were used to identify potential pseudogenes within *Brucella*. The first method was based on the program GenVar, an analytical pipeline used to examine closely related species or strains and identify missed gene calls as well as split genes or indels (62). The second method aligns neighboring pairs of protein predictions using BLASTP (3) against the National Center for Biotechnology Information (NCBI) nonredundant protein database. Neighbors with alignments to the same target sequence with an E value of $<10^{-5}$ were further evaluated by manual curation. If the pseudogene prediction from either method proved to be correct upon manual examination, the original gene and coding sequence (CDS) features were deleted, and a new gene feature spanning both gene predictions was created and marked with the pseudogene qualifier.

Once a first set of pseudogenes was identified by the above-described two methods, a third method was used to identify additional pseudogenes based on the first set. The DNA sequences of pseudogenes in the first set were first aligned to the bacterial subdivision of NCBI's nonredundant protein database using BLASTX and subjected to cutoffs of 165 bits and an E value of 10^{-9} or, to ensure alignments to very short pseudogenes are not missed, greater than 85% identity (at the protein level) for 50% of the query length. For each pseudogene, the protein sequence with the highest-scoring alignment (by bit score) was retrieved for use in the next step. These retrieved protein sequences were used as queries in a TBLASTN search of the nine *Brucella* genomes to identify new genes or pseudogenes by orthology. The resulting alignments were processed to merge overlapping or nearby (within 30 bp) high-scoring segment pairs to form meta-alignments to determine the approximate coordinates of the new (pseudo)gene. To identify its endpoints more precisely and determine the number of FS and in-frame stop (nonsense) codons relative to the functional homolog used as a

query, the program estwise from the Wise2.0 package (6) was used to generate an alignment spanning the FS and nonsense features. The command line option "-alg 333" was used to select the simplest FS-tolerant alignment algorithm instead of using the hidden Markov model, with states for intron identification (which is enabled by default and intended for processing eukaryotic sequences). Note that this third method in effect also computes groups of genes related by similarity. While our primary method for computing OGs was OrthoMCL, as noted above, we used the method described here to identify pseudogenes that are "genome specific." A pseudogene is genome specific if it is the only pseudogene in a gene similarity group containing at least one other member.

Identification of anomalous regions and lateral transfer candidates. We employed Alien Hunter (AH) (59), a program that identifies regions that may have been laterally transferred. These are regions that have unusual sequence composition in terms of *k*-mers for various values of *k* (called interpolated variable order motifs in the terminology used in reference 59). An anomalous region is one whose AH score is above a genome-dependent and automatically calculated threshold that takes into account the sequence composition of the whole genome (termed background composition). AH was run on all 10 *Brucella* genomes. We called the regions identified by AH anomalous regions.

Because AH has been noted to have low specificity (29), we applied additional filters to the regions detected by AH. Anomalous regions that contained syntenic protein-coding genes in different *Brucella* genomes as given by OrthoMCL ortholog data and double checked by BLAST2seq (56) were labeled shared anomalous regions (SARs). We then compared the SARs obtained to those of the *Ochrobactrum anthropi* genome using MUMmer, option PROmer, which compares translated nucleotide sequences in all six frames (28). Using a SAR as the query and the whole genome of *O. anthropi* as the subject, we computed the coverage of that SAR in the *O. anthropi* genome by adding up the total length of all matches found by PROmer, regardless of their location in the *O. anthropi* genome, and dividing the result by the SAR length. Note that this approach is conservative, because matches found by PROmer may be disjointed and therefore may not correspond to a contiguous region in the *O. anthropi* genome (as would be expected if *O. anthropi* did in fact share that region). SARs that were absent or less than 50% complete in *O. anthropi* were selected for further analysis. Finally, SARs were cross-referenced with previously published studies. Several of these interrupt a tRNA gene and were originally named (36) to designate the size of the region in kilobases and the tRNA identity (e.g., 8T is an 8-kb region that interrupts a tRNA that codes for a threonine). SARs are labeled by chromosome and region order within the chromosome (e.g., SAR 1-8 is the eighth shared anomalous region on chromosome 1).

Phylogenetic analysis. Protein sequences for the 10 *Brucella* genomes and four outgroup species (*Ochrobactrum intermedium* [57], *O. anthropi* ATCC 49188, *Bartonella quintana* Toulouse, and *Mesorhizobium loti* MAFF 303099) were clustered by applying OrthoMCL (32) to all-versus-all BLAST data, yielding 2,246 protein families with one and only one representative from each *Brucella* genome. Each protein family was made representative for the outgroup strains by excluding strains with more than one member in the family, leaving *O. anthropi* represented in 1,970 families, *O. intermedium* in 1,924, *B. quintana* in 851, and *M. loti* in 1,699. The protein sequences from each family were aligned using MUSCLE (18), and ambiguous portions of the alignment were removed using Gblocks (8). The concatenation of these alignments contained 671,030 amino acid characters, though only 8,004 were *Brucella* informative (for which at least two *Brucella* genomes differed from the others or one *Brucella* genome differed from the others and an outgroup was present). RAxML (53) was used with the PROTGAMMAWAGF model to prepare a maximum likelihood tree and in its quick mode to prepare 100 bootstrap trees.

RESULTS

General features of the genomes. All nine *Brucella* genomes studied have two circular chromosomes. Chromosome 1 is the larger chromosome, with a median length of 2.1 Mb, and chromosome 2 has a median length of 1.2 Mb. Both have similar G+C content, averaging 57.1% for chromosome 1 and 57.3% for chromosome 2. The total number of genes per genome (about 3,460) is very similar among the nine complete genomes studied, as is the number of protein-coding genes (about 3,180). These results are summarized in Table 2 on a per-genome and per-chromosome basis.

Genome alignment. Multiple replicon alignments were done for 9 of the 10 genomes. (*B. ceti* was excluded because it is an unfinished genome.) Chromosome 1 is similarly arranged among all nine genomes, with the only major difference being the *B. suis* ATCC 23445 genome (Fig. 1). Examination of both chromosomes of this species indicated that a 210-kb segment of chromosome 1 has been translocated to chromosome 2. Chromosome 2 appears to be more plastic than chromosome 1, with more internal rearrangements. A segment of approximately 700 kb in chromosome 2 is a shared inversion among the three *B. abortus* genomes (Fig. 1), with respect to the others.

Phylogenetic analysis. The results of a maximum likelihood phylogenetic analysis of the 10 *Brucella* strains plus four outgroup species are shown in Fig. 2. This tree sorts the *Brucella* genomes studied here into four clades, as follows: (i) the *B. melitensis-B. abortus* clade; (ii) the *B. ovis* clade; (iii) the *B. suis-B. canis* clade; and (iv) the *B. ceti* clade. Each node received 100% bootstrap support except for two extremely short internal branches. Although the tree is nominally bifurcating, the shortness and suboptimal support of those two branches suggest caution in assigning a strict evolutionary branching order to the four *Brucella* clades; they appear to have radiated explosively. The generated tree (Fig. 2A) also shows, as expected, that *Ochrobactrum* is the closest relative to *Brucella* (48).

Anomalous regions, OGs, and lateral transfer analysis. We identified an average of 40 anomalous regions in the *Brucella* strains (range, 32 to 51 regions). Chromosome 1 had an average of 17.4 regions (range, 13 to 21 regions), and chromosome 2 had an average of 23 (range, 14 to 38 regions). This variation is explained in part by variation in the genome-specific threshold score determined by AH, which was the main reason that led us to adopt the concept of the SAR. Seventeen SARs were absent or nearly absent in *O. anthropi* and were examined further (Table 3). These SARs ranged in size from 2 to 19 kb, with SAR 1-12 being the smallest and SAR 1-17 being the largest (see Table S1 in the supplemental material). Four of the 17 SARs showed the hallmark pattern of genomic islands, flanked on one side by an intact tRNA gene and on the other side by a fragment of that tRNA gene. Three of these, 8T (SAR 1-2), 15G (SAR 1-7), and 2I (SAR 1-12), have been described previously in chromosome 1 (36), and we identified a novel genomic island; SAR 2-10 is found in chromosome 2 and is 14 kb in length. This island is integrated into a tRNA-Thr and contains a type I restriction-modification system. For several additional SARs with tRNA gene neighbors, no tRNA fragment was identified at the other end (SARs 1-3, 1-5, 1-6, 1-8, 1-14, 1-16, 2-7, and 2-11). These may be older genomic islands that have lost the tRNA fragment, or the association with a tRNA gene may be accidental. It was more difficult to assign the endpoints of these SARs; the end of the sequence of the last shared ortholog identified by AH was used. Complete information on the 17 SARs examined, including the genes carried by them, is provided (see Table S1 in the supplemental material).

The translocation of a 210-kb segment from chromosome 1 to chromosome 2 in *B. suis* ATCC 23445 also moved SAR 1-16, and the inversion on chromosome 2 in the *B. abortus* genomes inverted SARs 2-8 and 2-10 (Fig. 1). Other SARs of interest

TABLE 2. Summary of annotation information for 10 *Brucella* genomes

Characteristic[a]	Annotation information for:										Total characteristics			
	B. abortus strain			*B. canis*	*B. ceti*[b]	*B. melitensis* strain		*B. ovis*	*B. suis* strain		Minimum	Maximum	Average	Median
	9-941	2308	S19			16 M	23457		1330	23445				
Chromosome 1														
Length	2,124	2,121	2,122	2,105	1,905	2,117	2,125	2,111	2,107	1,923	1,923	2,125	2,095	2,117
G+C%	57.2	57.2	57.2	57.2	NA[c]	57.2	57.2	57.2	57.2	57.1	57.1	57.2	57.2	57.2
Genes	2,310	2,343	2,263	2,199	NA	2,268	2,236	2,289	2,276	2,006	2,006	2,343	2,243	2,268
CDSs	2,127	2,098	2,090	2,060	NA	2,049	2,078	2,093	2,126	1,876	1,876	2,127	2,066	2,090
Pseudogenes	81	81	79	83	NA	66	68	134	80	83	66	134	84	81
P'gene fraction	3.70%	3.70%	3.60%	3.90%	NA	3.10%	3.20%	6.00%	3.60%	4.20%	3.10%	6.00%	3.90%	3.70%
GS pseudogenes	1	2	2	13	NA	7	4	59	8	18	1	59	13	7
Chromosome 2														
Length	1,162	1,156	1,161	1,206	1,483	1,177	1,185	1,164	1,207	1,400	1,156	1,400	1,202	1,177
G+C%	57.3	57.4	57.3	57.3	NA	57.3	57.4	57.2	57.3	57.3	57.2	57.4	57.3	57.3
Genes	1,222	1,232	1,171	1,224	NA	1,191	1,189	1,195	1,224	1,417	1,171	1,417	1,229	1,222
CDSs	1,108	1,083	1,063	1,133	NA	1,056	1,077	1,053	1,139	1,300	1,053	1,300	1,112	1,083
Pseudogenes	62	59	60	69	NA	59	65	112	70	85	59	112	71	65
P'gene fraction	5.30%	5.20%	5.30%	5.70%	NA	5.30%	5.70%	9.60%	5.80%	6.10%	5.20%	9.60%	6.00%	5.70%
GS pseudogenes	0	1	3	9	NA	8	13	48	10	16	0	48	12	9
Genome														
Length	3,286	3,277	3,283	3,311	3,388	3,294	3,310	3,275	3,314	3,323	3,275	3,323	3,297	3,294
G+C%	57.2	57.2	57.2	57.2	57.2	57.2	57.2	57.2	57.3	57.2	57.2	57.3	57.2	57.2
Genes	3,459	3,425	3,484	3,500	3,501	3,459	3,425	3,484	3,500	3,423	3,423	3,500	3,462	3,459
CDSs	3,235	3,181	3,153	3,193	3,293	3,105	3,155	3,146	3,265	3,176	3,105	3,265	3,179	3,176
Pseudogenes	143	140	139	152	NA	125	133	246	150	168	125	246	155	143
P'gene fraction	4.20%	4.20%	4.20%	4.50%	NA	3.90%	4.00%	7.30%	4.40%	5.00%	3.90%	7.30%	4.60%	4.20%
GS pseudogenes	1	3	5	22	NA	15	17	107	18	34	1	107	25	17
OGs	3,118	3,068	3,024	3,086	3,125	3,089	3,039	3,098	3,117	3,060	3,024	3,118	3,078	3,086

[a] P'gene fraction, pseudogene fraction. P'gene fractions are calculated as follows: pseudogene count/(pseudogene count + CDS count). GS pseudogenes, genome-specific pseudogenes. The number of GS pseudogenes indicates the number of cases in which the genome of interest contains a nonfunctional gene, but the gene is functional in all other genomes.

[b] *Brucella ceti* assembly is incomplete; thus, all numbers are approximate.

[c] NA, not applicable.

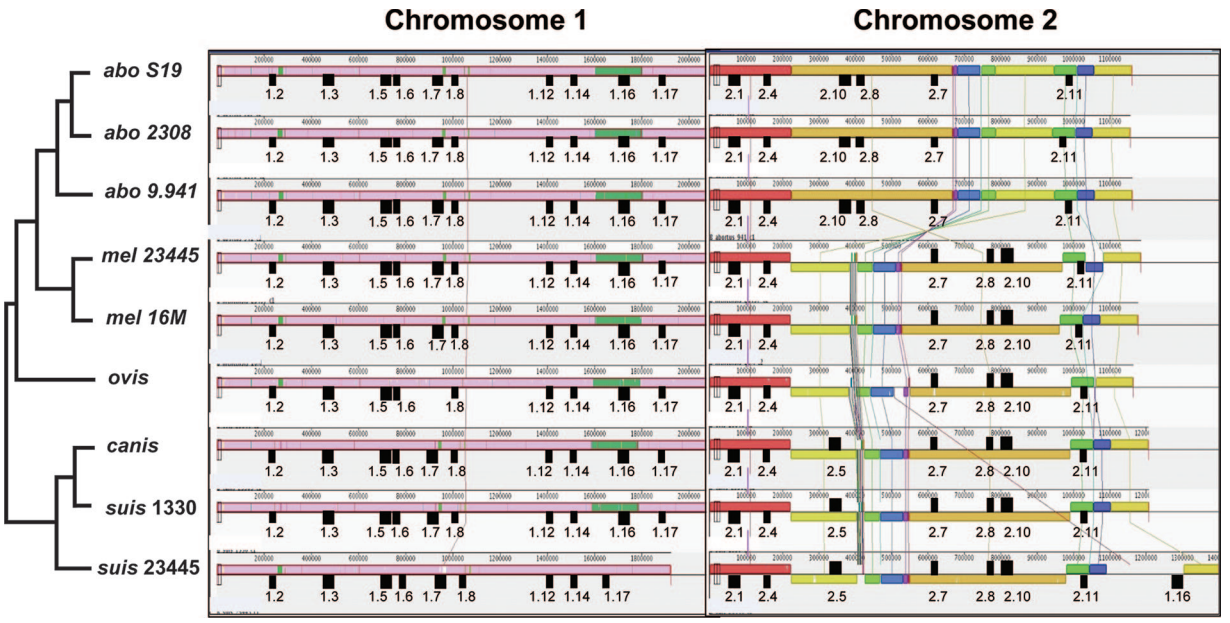


FIG. 1. Mauve alignment of both chromosomes from the nine complete *Brucella* genomes. A phylogenetic map of the strains derived from the tree shown in Fig. 2 (topology only, not branch lengths) is on the left side (*abo*, *B. abortus*; *mel*, *B. melitensis*; *ovis*, *B. ovis*; *canis*, *B. canis*; *suis*, *B. suis*). SARs of interest are noted by filled boxes, with the names of those regions directly below them.

that were not near tRNA genes include 1-17, 2-1, 2-4, 2-8, and the previously described IncP island (30), corresponding to SAR 2-5. Interestingly, SAR 1-2 (8T) contains a three-gene segment that is also found in SAR 1-8, including a resolvase family site-specific recombinase. Either these genes entered the *Brucella* twice independently or there was an insertion into one of the sites from either SAR 1-2 or SAR 1-8. SAR 2-5 is also interesting, as it had been noted previously that this region, the IncP island, was found only in *B. suis*, *B. canis*, *B. neotomae*, and in some of the marine strains (30). In this study, we found SAR 2-5 in *B. ceti*, but as noted previously, it is missing from *B. ovis* and from all the *B. abortus* and *B. melitensis* genomes.

SAR 1-7, first identified as 15G by Mantri and Williams (36) and later examined experimentally (45, 46), contains 15 genes, 2 of which (*wboA* and *wboB*) are of particular interest, since they help determine the smooth phenotype (see below).

We obtained 15,986 OGs from 37 *Rhizobiales* genomes (this number does not include singleton proteins that failed to group with others). Within the genus *Brucella*, there were 747 OGs

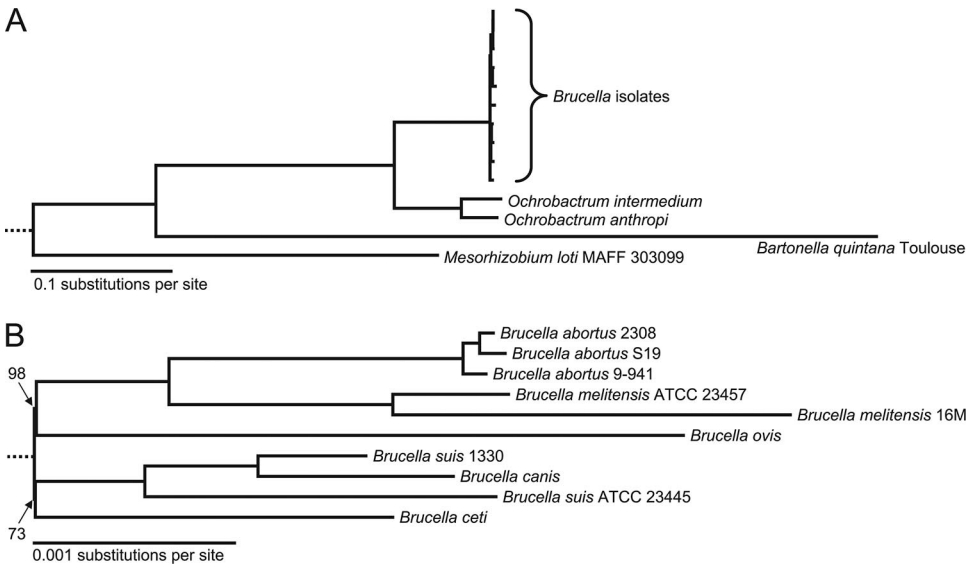


FIG. 2. Phylogenetic trees of 10 *Brucella* genomes with outgroups (A) and without outgroups (B). The maximum likelihood tree is based on a concatenated alignment of 2,246 protein families. (A) Full tree with outgroup species. (B) *Brucella* portion, only at a smaller scale. All nodes received 100% bootstrap support except the two very short ones indicated in panel B.

TABLE 3. Seventeen specifically examined SARs and their presence/absence among the *Brucella* genomes and *Ochrobactrum anthropi*[a]

SAR	Other name	tRNA	Size (kb)	Presence/absence of SARs in:										
				B. suis strain		*B. canis*	*B. ceti*	*B. ovis*	*B. melitensis* strain		*B. abortus* strain			*O. anthropi* (%)
				23445	1330				16 M	23457	9941	2308	S19	
Chromosome 1														
SAR 1-2	8T	tRNA-Thr	8,193	√	√	√	√	√	√	√	√	√	√	28
SAR 1-3	GI-1	tRNA-Thr	18,298	√	√	√	√	√	√	√	√	√	√	15
SAR 1-5		tRNA-Arg	13,859	√	√	√	√	√	√	√	√	√	√	14
SAR 1-6		tRNA-Val and tRNA-Asp	4,038	√	√	√	√	√	√	√	√	√	√	49
SAR 1-7	15G	tRNA-Gly	15,116	√	√	√	√	abs	√	√	√	√	√	35
SAR 1-8		tRNA-Leu and tRNA-Leu	7,213	√	√	√	√	part	√	√	√	√	√	24
SAR 1-12	2I	tRNA-Ile	2,007	√	√	√	√	√	√	√	√	√	√	0
SAR 1-14		tRNA-Gly	4,199	√	√	√	√	√	√	√	√	√	√	37
SAR 1-16		tRNA-Ser	10,483	√	√	√	√	part	√	√	√	√	√	36
SAR 1-17			19,446	√	√	√	√	√	√	√	√	√	√	44
Chromosome 2														
SAR 2-1			11,103	√	√	√	√	√	√	√	√	√	√	15
SAR 2-4			1,852	√	√	√	√	√	√	√	√	√	√	14
SAR 2-5	IncP		19,305	√	√	√	√	abs	abs	abs	abs	abs	abs	4
SAR 2-7		tRNA-Cys	4,437	√	√	√	√	√	√	√	√	√	√	17
SAR 2-8			4,856	√	√	√	√	√	√	√	√	√	√	6
SAR 2-10	14T	tRNA-Thr	14,312	√	√	√	√	√	√	√	√	√	√	10
SAR 2-11		tRNA-Leu and tRNA-Ser	3,864	√	√	√	√	√	√	√	√	√	√	39

[a] In the *Brucella* genomes, if the SAR is completely present, this is noted by a check mark; if it is only partially present, this is noted by part; and if it is completely absent, this is noted by abs. All of these SARs are at best only partially present in *O. anthropi*, and the last column provides the estimated coverage.

that contained any combination of the 10 *Brucella* genomes but none of the other *Rhizobiales* genomes. Of these, 140 OGs had at least one representative from each of the 10 *Brucella* genomes (see Table S2 in the supplemental material). Using this set of 747 OGs, we identified a region that is found in all the *Brucella* genomes except for the three *B. abortus* genomes. This 23-kb segment contains a number of important genes, including those encoding glycosyl transferase and glycerol kinase (Table 4); this region was not identified as anomalous. It should be noted that the glycosyl transferase and glycerol kinases are the second copies of these genes. The *B. abortus* genomes have only a single copy of each gene.

A single protein representative from each of the 747 OGs was used to query the NCBI nonredundant protein database. Of these, 688 OGs had no BLASTP hits to any genome other than *Brucella*, accounting for 21.5% of all *Brucella* proteins (see Table S3 in the supplemental material). The majority of these *Brucella*-specific OGs are annotated as hypothetical pro-

TABLE 4. Genes found in a 23-kb segment in *Brucella suis* 1330 and in all others, except for the three *B. abortus* genomes in which all genes are missing[a]

RefSeq ID	PATRIC ID	Start (kb no.)	End (kb no.)	Size (bp)	Strand	Gene symbol	Description
BRA0418	VBI0007BS2_0414	402846	403826	981	−		GDP-*L*-fucose synthase 1
BRA0419	VBI0007BS2_0415	403810	404880	1,071	−	*gmd*	GDP-mannose 4,6-dehydratase
BRA0421	VBI0007BS2_0417	406415	407650	1,236	+		Hypothetical protein
BRA0422	VBI0007BS2_0418	407647	408843	1,197	+		Hypothetical protein
BRA0423	VBI0007BS2_0419	408914	409636	723	−		Hypothetical protein
BRA0424	VBI0007BS2_0420	410033	410647	615	−	*nodL*	Nodulation protein L
BRA0426	VBI0007BS2_0422	411918	412535	618	−		Hypothetical protein
BRA0427	VBI0007BS2_0423	412532	413413	882	−	*waaE*	LPS core biosynthesis glycosyl transferase *waaE*
BRA0428	VBI0007BS2_0424	413410	414537	1,128	−	*rfe*	Putative undecaprenyl-phosphate alpha-*N*-acetylglucosaminyl 1-phosphate transferase
BRA0429	VBI0007BS2_0425	414830	416323	1,494	+		Hypothetical protein
BRA0430	VBI0007BS2_0426	416339	417352	1,014	+	*hyaD*	Hyaluronan synthase
BRA0431	VBI0007BS2_0427	417308	418549	1,242	−		Hypothetical protein
BRA0432	VBI0007BS2_0428	418816	420045	1,230	+		Hypothetical protein
BRA0433	VBI0007BS2_0429	420083	421444	1,362	+	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase
BRA0434	VBI0007BS2_0430	421423	422757	1,335	+		Hypothetical protein
BRA0435	VBI0007BS2_0431	422878	423939	1,062	+	*exoB*	UDP-glucose 4-epimerase
BRA0436	VBI0007BS2_0432	423978	425291	1,314	+		Hypothetical protein
BRA0437	VBI0007BS2_0433	425254	425778	525	−	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase
BRA0438	VBI0007BS2_0434	426099	427400	1,302	+		Hypothetical protein
BRA0439	VBI0007BS2_0435	427403	428212	810	+	*mpg1*	Mannose-1-phosphate guanyltransferase

[a] RefSeq, reference sequence; ID, identification.

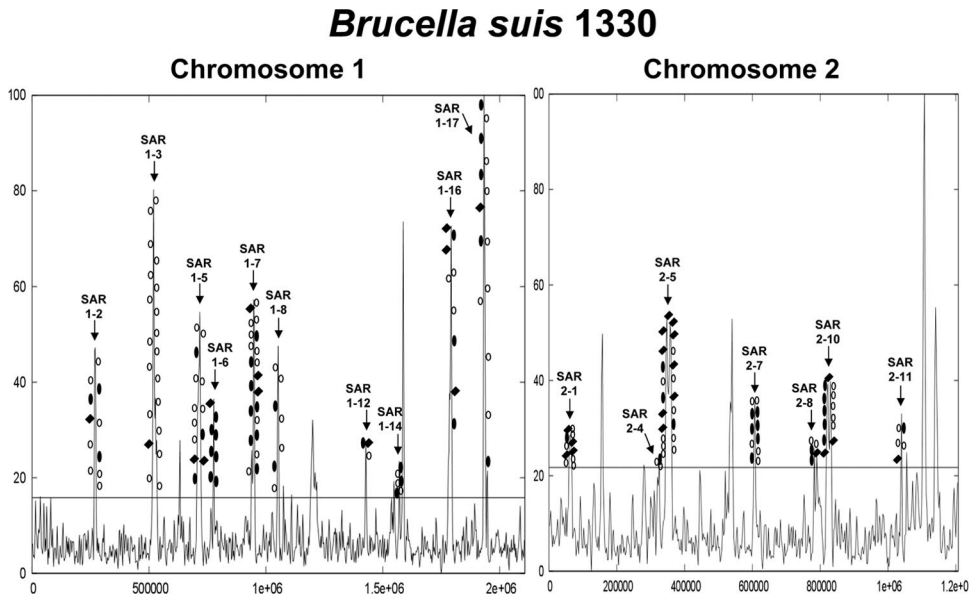


FIG. 3. AH analysis of *B. suis* 1330 chromosomes. The 17 shared SARs are annotated in this plot. Genes that were shared among the majority of the OGs are depicted on these peaks, with open circles indicating genes that are present in *Brucella* and among other members of *Rhizobiales*. Filled circles indicate genes that are found only among *Brucella* and share no homology outside this genus. Filled diamonds represent genes that are present in *Brucella*, absent among other *Rhizobiales*, but have significant BLASTP hits to genomes that are not members of the *Rhizobiales* order. The *x* axis represents the length of the chromosome, and the *y* axis represents the score range provided by the AH program.

teins; over 50% have either 9 or all 10 of the genomes represented. There were 59 OGs with BLASTP hits (E value cutoff, $>10^{-10}$) for *Brucella* and for genomes outside of *Rhizobiales* (see Table S4 in the supplemental material), indicating that the proteins with the nearest homology are not present in the closest relatives of *Brucella*; this is a small percentage (1.7%) of the *Brucella* proteins.

Notable among these 59 OGs are the components of the type IV secretion system (maps to SAR 2-1), *tra* genes (map to SAR 2-5), and the *wbk* gene cluster (maps to SAR 1-3), which was previously identified (22).

In a comparison of the AH and ortholog/BLASTP data, the observation that one of the regions contained a housekeeping gene led to the identification of SAR 1-17 as a composite. It contains a five-gene region shared with *O. anthropi* but flanked on both sides by genes unique to *Brucella*. Figure 3 shows an annotation of the 17 SARs in the *B. suis* 1330 genome.

Pseudogenes. A total of 1,396 pseudogenes were identified (this analysis excludes the unfinished *B. ceti* genome). Of these, 222 were found to be genome specific. Many such genome-specific pseudogenes may simply be the result of a sequencing error. The other identified pseudogenes are members of 522 OGs (see Table S5 in the supplemental material). The ratio of pseudogenes to total genes carried by a genome (the pseudogene fraction) was used as a benchmark for comparison between organisms. Values ranged from a low of 3.9% for *B. melitensis* 16 M to a high of 7.3% for *B. ovis*. The average value for the nine complete *Brucella* genomes was 4.6%. The highest number of genome-specific pseudogenes is found in *B. ovis*, with 107 (Table 2). The next highest number is found in *B. suis* ATCC 23445, which has 34, followed by *B. canis*, with 22. Pseudogene fractions were also calculated on a per-chromosome basis. Chromosome 2 had a higher percentage of pseudogenes than chromosome 1 for all nine genomes studied; on average, the pseudogene fraction was 3.9% for chromosome 1 and 6.0% for chromosome 2.

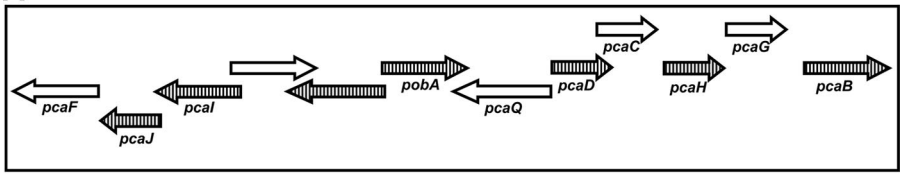
β-Ketoadipate pathway. In the initial analysis of the *B. suis* 1330 genome sequence, Paulsen et al. (44) noted an unexpected capacity of this organism to use plant-derived compounds as an energy source. The β-ketoadipate pathway takes two aromatic compounds, protocatechuate and catechol, which are produced by the degradation of plant-derived molecules, and metabolizes them to intermediates that can enter the tricarboxylic acid cycle (34). There are 12 protein-coding genes that have been identified as being part of this pathway in *B. suis* 1330 (44); all of them are found on chromosome 2. In the case of *Agrobacterium tumefaciens* C58, the enzymes involved in this pathway are organized into two distinct operons (43); *Brucella* seems to have a similar arrangement, as do both *Ochrobactrum* genomes. Examination of all 10 *Brucella* genomes showed that at least 1 of the 12 genes carried by every genome except *B. suis* 1330 has become a pseudogene and that both of these operons are completely missing in *B. suis* ATCC 23445 (Fig. 4).

DISCUSSION

The 10 different *Brucella* genomes examined here are quite similar in genome size and the numbers of genes and proteins. They are also similar in the structural organization of the chromosomes, with the exceptions being a 210-kb translocation seen in *B. suis* ATCC 23445 and a 700-kb inversion in chromosome 2 shared by the *B. abortus* genomes (Fig. 1).

The combined phylogenomic analysis of 2,377 ortholog families shows that the depth of divergence for these 10 *Brucella* strains is quite shallow (Fig. 2B). Despite this low level of divergence, with few characters differing among the genomes,

A



B

RefSeq	Name	Gene	*abortus*			*melitensis*		ovis	ceti	canis	*suis*		*O. anthropi*
			9941	2308	S19	16M	23457				1330	23445	
BRA0636	β-ketoadipyl CoA thiolase	*pcaF*	✓	✓	✓	✓	✓	✓	✓	✓	✓	o	✓
BRA0637	3-oxoacid CoA-transferase, B subunit	*pcaJ*	✓	✓	✓	✓	✓	ψ	✓	✓	✓	o	✓
BRA0638	3-oxoacid CoA-transferase, A subunit	*pcaI*	✓	✓	✓	ψ	✓	✓	✓	ψ	✓	o	✓
BRA0639	β-ketoadipate pathway transcriptional reg		✓	✓	✓	✓	✓	✓	✓	✓	✓	o	✓
BRA0640	Putative HTH-type transcriptional reg		✓	✓	✓	✓	✓	ψ	✓	✓	✓	o	✓
BRA0641	4-hydroxybenzoate 3-monooxygenase	*pobA*	ψ	ψ	ψ	✓	✓	✓	✓	✓	✓	o	✓
BRA0642	pca operon transcription factor PcaQ	*pcaQ*	✓	✓	✓	✓	✓	✓	✓	✓	✓	o	✓
BRA0643	3-oxoadipate enol-lactonase	*pcaD*	✓	✓	✓	✓	✓	ψ	✓	✓	✓	o	✓
BRA0644	4-carboxymuconolactone decarboxylase	*pcaC*	✓	✓	✓	✓	✓	✓	✓	✓	✓	o	✓
BRA0645	protocatechuate 3,4-dioxygenase, β subunit	*pcaH*	✓	✓	✓	ψ	ψ	ψ	✓	ψ	✓	o	✓
BRA0646	protocatechuate 3,4-dioxygenase, α subunit	*pcaG*	✓	✓	✓	✓	✓	✓	✓	✓	✓	o	✓
BRA0647	3-carboxy-cis,cis-muconate cycloisomerase	*pcaB*	✓	✓	✓	✓	✓	ψ	ψ	✓	✓	o	✓

FIG. 4. Pseudogenization of the β-ketoadipate pathway among the 10 different *Brucella* genomes and in *Ochrobactrum anthropi* in illustrated form (A) and tabular form (B). (A) Genes involved in this pathway are in two operons on opposite strands, and the individual genes are represented by arrows. Gene symbols, where available, are provided below the arrows. Hatched arrows indicate genes that are pseudogenized in one or more genomes. (B) Table showing the identification, name, gene symbol, and presence, absence, or pseudogenization of individual genes among the genomes. Checkmarks indicate a normal gene. Open circles denote the absence of this gene, and ψ indicates an apparent pseudogene.

the branching order seems to be clear and well supported, as reflected by support values. The major structure is a radiation producing a *B. abortus-B. melitensis* clade, a *B. suis-B. canis* clade, and *B. ovis* and *B. ceti* clades. A recent phylogenetic analysis (7) shows the same four subgroups of *Brucella* observed here, but our use of an outgroup further shows that these are four clades that radiated explosively. The *B. abortus-B. melitensis* clade segregated into two branches, one containing the *B. abortus* genomes and the other containing the *B. melitensis* genomes. *B. canis* nests within the *B. suis* clade, suggesting that there may have been a host switch. Genome sequences for *B. neotomae* and *B. pinnipediae* are not currently available, but previously presented evidence (7) indicates that a similar host switch may have occurred in these two species.

Assuming current knowledge of host preference is accurate, we can ask whether *Brucella* phylogeny reflects the phylogeny of their hosts. The mammalian taxa that have been identified as the preferred *Brucella* hosts belong to three distinct groups, all at the level of order in mammals. *Neotoma* is a genus of cricetid rodent found in the order Rodentia. The genus *Canis* (dogs, wolves, and coyotes) and the family Phocidae (seals) are in the order Carnivora. *Bos* (cattle and oxen), *Ovis* (sheep), *Capra* (goats), *Sus* (pigs), and the cetacean group (whales and dolphins) are all united in Cetartiodactyla. Humans (Primates) have also been infected but are not preferred hosts. These three mammalian orders representing the hosts are all well separated phylogenetically (42). The host and pathogen phylogenies are distinct and not similar. Although our phylogenetic data closely reflect the data found previously (7), our conclusions differ. The phylogeny of the *Brucella* isolates does not match that of their nominal mammalian hosts. This is especially clear from the inclusion of *B. canis* in our study. Considering the fact that most of the *Brucella* isolates have been identified in cetartiodactylid hosts, one could speculate that the ancestor of *Brucella* species infected a member of early cetartiodactylids and radiated within this group, with host switches to Carnivora and Rodentia occurring later.

The species concept in bacteria is a subject of debate (10, 52), as is the definition of different species within *Brucella* (40). The high degree of similarity of all these genomes, in comparison to other bacterial groups, suggests a close phylogenetic relationship. However, clear differences in host preference might still justify the separate species designations as they presently exist. For example, cattle have been described as the natural or primary hosts for *B. abortus*, and yet it has also been found in horses, pigs, sheep, goats, Bactrian camels, dromedary camels, water buffalo, yaks, elk, dogs (12), and humans (5, 55). It has also been isolated from rodents on occasion, although it was noted that these infections seem to be from areas where there was a large number of infected cattle (15). This list alone represents five different orders of mammalian hosts. A survey of the literature shows that the host range exhibited by *B. abortus* strains also extends to different degrees in the other *Brucella* clades. However, the isolation frequencies of different *Brucella* species from infected hosts are consistent with some type of host preference (58).

Genome reduction, or reductive evolution, involves gene loss through mutational inactivation and deletion (4, 19). It has been noted in a number of intracellular pathogenic bacteria, including *Rickettsia prowazekii* (4), *Mycobacterium leprae* (19), *Shigella flexneri*, and *Salmonella enterica* serovar Typhi (13). All of these bacteria are obligate intracellular pathogens, whereas *Brucella* is a facultative intracellular pathogen that can survive outside the host under certain conditions (12). Are the *Brucella* genomes undergoing reductive evolution? Based on genome size alone, the answer seems to be yes. *Brucella* genomes are all similar in size, with an average size of 3.29 Mb. Their nearest sequenced relatives are *O. anthropi* (5.22 Mb) and *O. intermedium* (4.6 Mb), which are both markedly larger. Pseudogene fractions can also be an indication of a genome reduction

process. Excluding genome-specific pseudogenes, the average fraction determined here was 4.6%. This is low compared to the 50% estimate for *Mycobacterium leprae* (19), 24% for *Rickettsia prowazekii* (4), 15% for *Shigella flexneri* (13), 14% for *Bartonella quintana* (2), and 9% for *Bartonella henselae* (2). Of these, only the species of the *Bartonella* genus are in the order *Rhizobiales*. On the other hand, in three free-living *Agrobacterium* species, also in the order *Rhizobiales*, the fraction is less than 2% (50). Because the pseudogene fractions in these other studies were obtained using different methodologies, it is difficult to compare these numbers. However, using the general estimate that bacterial genomes have between 1 and 5% pseudogenes (33), the 4.6% fraction observed in *Brucella* can be considered relatively high and suggestive of genome degradation. Moreover, we did note more pseudogenes on chromosome 2 than on chromosome 1. Together with the higher degree of rearrangements observed on chromosome 2, this supports the conclusion that chromosome 2 is more dynamic, perhaps owing to its hypothesized origin as a plasmid (50).

The presence of many pseudogenes in the β-ketoadipate pathway is striking and reminiscent of the proposed "domino theory" of gene death (13), where after a crucial gene within a complex pathway becomes nonfunctional, a mass gene extinction is triggered. The *B. suis* genome is anomalous in retaining this gene cluster intact, as the cluster is entirely absent in the *B. suis* ATCC 23445 genome, and one or more of its genes have become pseudogenes in other genomes. It is likely that in its adaptation to an intracellular milieu, *Brucella* no longer requires this pathway that allows soil bacteria to break down plant compounds. We suspect that the preservation of this gene cluster in *B. suis* 1330 is anomalous and that over time it will succumb to pseudogenization; however, it is also possible that this particular strain (unlike other members of the *B. canis-B. suis* clade) makes use of these genes during periods of existence outside an animal host.

From examining the regions of potential lateral transfer, we note that many regions are unique to *Brucella* and not shared with *Ochrobactrum*. It is likely that these regions (Table 3; see also Table S1 in the supplemental material) entered *Brucella* after diverging from the ancestor it shared with *Ochrobactrum*, indicating that lateral transfer does happen despite intracellular preferences. Dobrindt et al. (17) suggest that horizontal transmission is more likely to occur in niches that contain diverse bacterial species and not as likely to occur in sparsely populated environments, which include intracellular niches like the host macrophage, the ultimate destination of *Brucella* (35). Of course, it is possible that these regions entered the genome at some point before *Brucella* committed to an intracellular preference. But when one considers that the journey to the macrophage takes *Brucella* through a complex series of environments that are inhabited by a wide variety of organisms with which they might interact, it seems plausible that *Brucella* has the opportunity to experience lateral transfer. The mammalian gut has been recognized as one of the most densely populated ecosystems on earth (38), and it has been documented that one of the most common means of transmission of *Brucella* involves ingestion of forage or water contaminated with genital discharge (54) or ingestion of raw milk or milk products (47). Passage through the gut would provide ample opportunity for different species of bacteria to interact, and it is plausible that *Brucella* experienced lateral transfer in this environment. In addition, *Brucella* might also interact with bacteria in the soil on which the blood, tissues, and aborted fetus of the host lies. Crawford et al. (12) report that *Brucella* can survive for up to 66 days in moist soil and up to 185 days in cold soil.

Our study contains strong indications that *Brucella* has acquired genes by lateral transfer. In particular, SAR 2-5, the IncP island, appears to have entered *Brucella* after it diverged from *Ochrobactrum* and after the individual species began to separate. This SAR contains the Tra proteins, known to be a type IV secretion system (31), and it is found in *B. suis*, *B. canis*, and *B. neotomae* (30) and is here identified in *B. ceti*. The fact that the phylogenetic tree places the *B. suis-B. canis* clade and the *B. ceti* clade cluster together makes it seem likely that SAR 2-5 was acquired by their common ancestor. A complete genome from *B. neotomae* is not yet available, but a previous study shows that this species is phylogenetically close to *B. suis* and the marine *Brucella* spp. Thus, we hypothesize that there was a common ancestor to these three clades and that SAR 2-5 was laterally transferred into it. The fact that it is in the same location in the genomes studied here gives further weight to a single, ancestral acquisition (Fig. 1). Because it is shared only among some of the *Brucella* genomes, it could be argued that it was acquired after the ancestor had begun living intracellularly, as it is unlikely that this type of lifestyle developed twice independently. However, it is also possible that the ancestor that gave rise to the *B. ovis* and the *B. melitensis-B. abortus* clades lost this region or that each of the clades lost it independently.

Some of the genes indicated as having been acquired by lateral transfer play an important role in the survival of this pathogen in its host. These include the enzymes involved in producing the smooth phenotype in *Brucella* (26, 45). Lipopolysaccharide (LPS) is the major structural component of the outer membrane of gram-negative bacteria. It is composed of a lipid core, a core oligosaccharide, and a distal O-polysaccharide (O-PS) side chain (22). A phenotypic characteristic used to distinguish between *Brucella* species is the presence of the O-PS. Isolates of *B. abortus*, *B. suis*, and *B. melitensis* have a smooth morphology with the O-PS intact, while *B. canis* and *B. ovis* are rough, as they have the lipid core and the core oligosaccharide but lack O-PS. The O-PS is a major contributor to the antigenic variation of the bacterial envelope as well as the ability of *Brucella* to survive in macrophages (26). Several studies have indicated specific genes as being important for the development of the smooth phenotype in *Brucella* (1, 22, 23, 25, 39, 61). Recently, Gonzalez et al. (23) looked at 19 genes that had been indicated as being important in producing smoothness and found that disruption of 13 genes (*wboA*, *wboB*, *wa***, *wbhE*, *manB*, *wbkA*, *gmd*, *per*, *wzm*, *wbkF*, *wbkD*, *prm*, and $manB_{core}$) resulted in a rough phenotype in *B. melitensis*, with an additional 6 genes indentified as playing roles that were not fully determined. Rajashekara et al. (45) demonstrated that mutations of two genes, BMEI0997 (*wboB*) and BMEI0998 (*wboA*), resulted in a rough phenotype. Furthermore, they showed that BMEI0999, a hypothetical protein whose function is unknown, was necessary to restore a smooth LPS in rough strains. However, we have found that other

smooth strains (*B. abortus* 2308 and *B. melitensis* ATCC 23457) are completely missing this hypothetical gene.

There are two well-established species of *Brucella*, *B. canis* and *B. ovis*, that are naturally rough and yet fully infective, and these 19 LPS-associated genes were specifically examined in these two genomes. *B. ovis* is missing two genes, *wboA* and *wboB*, that encode enzymes that polymerize *N*-formylperosamine (23), and without them, *B. ovis* is unable to complete the distal O-PS. Both of these genes reside in SAR 1-7 (15G) of *B. suis* 1330; their loss in *B. ovis* has been previously reported (46, 60). The *B. ovis* genome also has a truncated *wzt*; this gene encodes a protein that functions as a part of an ABC transporter, with its partner encoded by *wzm*. This specific enzyme (Wzt) is found in SAR 1-3 of *B. suis* 1330 and most likely entered *Brucella* by lateral transfer. This enzyme could be functional even if truncated. However, it could also indicate that the genes involved in LPS synthesis in SAR 1-3 are in a process of decay because the pathway is no longer complete in *B. ovis*. Only direct experimental evidence will determine if these genes are functional in *B. ovis*.

B. canis has truncations in 2 of the 19 LPS synthesis genes, *wbkF*, an undecaprenyl-glycosyltransferase, and *wbkD*, an epimerase/dehydratase. A truncated gene could still be functional, but the fact that *B. canis* is rough and that all other genes appear normal indicates that at least one of these genes is responsible for producing the rough phenotype. It is interesting that the rough phenotype results from different mutations in these two genomes, as follows: *B. canis* with mutations in *wbkF* and *wbkD* and *B. ovis* missing *wboA* and *wboB* and having a truncated *wzt*. It appears that roughness independently developed twice.

All known isolates of *B. ceti* are smooth (A. Whatmore, personal communication), and yet when the enzymes involved in LPS synthesis were examined in this species, *manB*, a phosphomannomutase whose function has not yet been determined (23), was found to be truncated due to a naturally occurring transposon insertion. Apparently, this does not affect the smooth phenotype of this organism. However, a rough phenotype was produced in *B. melitensis* when *manB* was experimentally mutated by a transposon (23).

Many of the 19 genes considered necessary for complete LPS synthesis are in SARs. Eighteen genes are located on chromosome 1, and one is found on chromosome 2. Of the genes found on chromosome 1, 12 are located on SAR 1-3 (Fig. 3), with one additional gene, *wbkD* (VBI0007BS1_0525), directly adjacent to this region. Two additional genes are found in SAR 1-7 (Fig. 3). These SARs are not adjacent on chromosome 1, with 402 kb between them, making it likely that these SARs represent genomic islands laterally transferred into *Brucella* genomes in separate events.

Genes of particular interest that we hypothesize to have entered the genome horizontally include the type IV secretion system, the *tra* genes, and the enzymes responsible for LPS synthesis that give *Brucella* a smooth phenotype. Mutations or the absence of these LPS genes is responsible for the rough phenotype of both *B. ovis* and *B. canis*. All these observations lead us to believe that *Brucella*, despite its preference for an intracellular milieu (e.g., phagocytic cells), has the ability and opportunity to interact with other bacteria in their environment and has acquired useful genes that facilitate its intracellular lifestyle.

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