RESEARCH ARTICLE

Comparative Genomics of Early-Diverging *Brucella* Strains Reveals a Novel Lipopolysaccharide Biosynthesis Pathway

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ABSTRACT ***Brucella*** **species are Gram-negative bacteria that infect mammals. Recently, two unusual strains (*Brucella inopinata* BO1^T and *B. inopinata*-like BO2) have been isolated from human patients, and their similarity to some atypical brucellae isolated from Australian native rodent species was noted. Here we present a phylogenomic analysis of the draft genome sequences of BO1^T and BO2 and of the Australian rodent strains 83-13 and NF2653 that shows that they form two groups well separated from the other sequenced *Brucella* spp. Several important differences were noted. Both BO1^T and BO2 did not agglutinate significantly when live or inactivated cells were exposed to monospecific A and M antisera against O-side chain sugars composed of *N*-formyl-perosamine. While BO1^T maintained the genes required to synthesize a typical *Brucella* O-antigen, BO2 lacked many of these genes but still produced a smooth LPS (lipopolysaccharide). Most missing genes were found in the *wbk* region involved in O-antigen synthesis in classic smooth *Brucella* spp. In their place, BO2 carries four genes that other bacteria use for making a rhamnose-based O-antigen. Electrophoretic, immunoblot, and chemical analyses showed that BO2 carries an antigenically different O-antigen made of repeating hexose-rich oligosaccharide units that made the LPS water-soluble, which contrasts with the homopolymeric O-antigen of other smooth brucellae that have a phenol-soluble LPS. The results demonstrate the existence of a group of early-diverging brucellae with traits that depart significantly from those of the *Brucella* species described thus far.**

IMPORTANCE **This report examines differences between genomes from four new *Brucella* strains and those from the classic *Brucella* spp. Our results show that the four new strains are outliers with respect to the previously known *Brucella* strains and yet are part of the genus, forming two new clades. The analysis revealed important information about the evolution and survival mechanisms of *Brucella* species, helping reshape our knowledge of this important zoonotic pathogen. One discovery of special importance is that one of the strains, BO2, produces an O-antigen distinct from any that has been seen in any other *Brucella* isolates to date.**

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Brucellosis is a disease caused by Gram-negative bacteria in the genus *Brucella*. This disease is zoonotic and endemic in many areas throughout the world, causing chronic infections with common outcomes of abortion and sterility in infected animals. In humans, it can cause a severe acute febrile state, producing focal lesions in bones, joints, the genitourinary tract, and other organs. The official classification of *Brucella* is based solely on phenotypic characterization using a range of bacteriological, serological, and biochemical tests (1, 56); classically, six nomenspecies (*Brucella abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*) have been described. Human infections, usually associated with contact with infected animals and abortion materials or with consumption of unpasteurized dairy products from infected animals (3, 4), are most often caused by *B. abortus*, *B. melitensis*, or *B. suis* (5, 6).

The *Brucella* genus is expanding. Over the last 20 years, new strains have been identified from marine mammals (7, 8), leading to the designation of two new species (*B. ceti* and *B. pinnipedialis*). Recently, strains showing atypical fast growth and the ability to cause lethal infections in rodents (9) have been isolated from common voles (*Microtus arvalis*) and designated *B. microti* (10, 11). This has led to the addition of at least three new branches to the

TABLE 1 General features of the BO1^T, BO2, NF2653, 83-13, and *B. suis* 1330 genomes[a]

	Value for indicated genome				
	B. inopinata		*Brucella* sp.		*B. suis*
Genome feature	BO1	BO2	NF2653	83-13	1330
Size (bp)	3,366,774	3,305,941	3,110,281	3,153,851	3,315,175
No. of contigs	55	174	113	20	2
%GC	57	56.8	57	56.5	57.2
Sequencing coverage	17.4×	25.7×	29.1×	28.5×	Complete
Protein-coding genes					
Total	3,404	3,350	3,250	3,167	3,432
No. (%) with functional assignment	2,624 (77.0)	2,583 (77.1)	2,522 (77.6)	2,508 (79.1)	2,699 (78.6)
No. (%) hypothetical	780 (22.1)	767 (22.8)	728 (22.4)	659 (20.8)	733 (21.3)
No. (%) of proteins with EC no.	886 (26.0)	875 (26.1)	869 (26.7)	823 (25.9)	884 (25.7)
RNA					
No. of rRNA operons	3	3	3	3	3
No. of tRNAs	49	49	49	49	55

[a] For the first four strains, the numbers are totals for the presumed two chromosomes. *B. suis* 1330 is included as a reference. Sequence assembly identified only one *rrn* operon, as the sequences are identical. However, Southern blotting and digestion with I-CeuI confirmed the presence of 3 *rrn* operons (G. Bourg, B. Saadeh, and D. O'Callaghan, unpublished data).

traditional phylogeny of *Brucella* (10, 12–14). In the middle of the first decade of the 21st century, two novel strains were isolated from atypical human infections (15, 16). The first one described was *B. inopinata* BO1^T, isolated from an infected breast implant in a 71-year-old patient from Oregon in the United States. BO1^T is very different from the classic species, showing very rapid growth similar to that of *B. microti*. Although DNA-DNA hybridization experiments and the presence of the *Brucella*-specific IS*711* element showed that the BO1^T strain was a member of the *Brucella* genus (15), BO1^T has a unique biochemical profile and antimicrobial susceptibility, and 16S rRNA sequence and multilocus sequence typing (MLST) analysis shows that this strain is markedly different from classic *Brucella* species (15). Thus, a new species, *B. inopinata* sp. nov. type strain BO1^T, was proposed (17). A second unusual strain, BO2, was isolated from a 52-year-old Australian man with a history of chronic destructive pneumonia (16). Standard biochemical profiles identified the strain as a member of the *Brucella* genus, with the 16S rRNA sequence showing 100% identity to that of BO1^T (16). However, comparison of *omp2a* and *omp2b* genes showed strong similarity between BO2 and the atypical *B. suis* strain 83-210 (16, 18).

A reexamination of seven strains isolated from native rat species in Queensland, Australia, in 1964 (19, 20) and originally classified as *B. suis* biovar 3 (19, 20) has further changed the picture of *Brucella* phylogeny. Both MLST and multiple-locus variable-number tandem-repeat analyses have shown that these strains exhibit distinct profiles, and phylogenetic analysis based on 16S rRNA, *recA*, and *rpoB* genes demonstrated that these form a distinct clade separated from both the classic *Brucella* species and BO2 (20). The primary hosts of the BO1^T and BO2 strains are unknown. Their similarity to two atypical *Brucella* strains isolated from the Australian rodents suggested that they, too, might have a rodent reservoir (16).

As BO1^T and BO2 are unique and different from other *Brucella* spp., they merit closer genomic scrutiny. We have performed whole-genome sequencing of the genomes of *B. inopinata* strains BO1^T and BO2 and one representative of the rodent species, NF2653. In this study, we present an analysis of these three genomes and compare them to other publicly available genomes in the genus *Brucella* and to genomes of species of *Ochrobactrum*, a genus that contains the closest known relatives of *Brucella* (21). As the genome of rodent strain *Brucella* sp. 83-13 has been noted as being particularly close to the genome of NF2653, its genome sequence was also included in our detailed analysis. Based on these results, and given the importance of lipopolysaccharide (LPS) in *Brucella* virulence (22, 43), a biochemical analysis of the unique LPS of BO2 was also carried out.

RESULTS AND DISCUSSION

General features of the genomes. Table 1 presents the general features of the nearly complete BO1^T, BO2, NF2653, and 83-13 genomes compared to the complete *B. suis* 1330 genome (23). Values for the percentages of the genes annotated with functional assignments or with hypothetical proteins or those assigned enzyme commission (EC) numbers are similar across all five genomes. Although none of the four new genome sequences is complete, there is good evidence that, like all sequenced *Brucella* strains, they have two chromosomes. All four genomes have the *oriC* chromosomal replication initiation gene (24) on the large chromosome. They also have the plasmid-like replication genes *repA*, *repB*, and *repC* associated with the smaller chromosome (23). In addition, sequence comparison to the genome sequence of *Brucella suis* 1330 shows that contigs from all four genome sequences map either to the main chromosome or to the second chromosome (data not shown).

Phylogenetic analysis. A maximum-likelihood phylogenetic analysis was performed based on 1,681 single-copy protein families conserved over 17 *Brucella* and 5 closely related outgroup bacterial genomes that were used in this study (Table 2). The resulting tree (Fig. 1A) shows that *Ochrobactrum* is the outgroup closest to *Brucella*, as has been shown previously (25, 26). The *Brucella* region of this tree (Fig. 1B and 1C) basally branches into three clades: a clade that contains 13 of the 17 *Brucella* nomenspecies that we define as the classic clade; the N8 clade (containing NF2653 and 83-13); and the BO clade (BO1^T and BO2). There is some uncertainty (83% bootstrap support) regarding the placement of the N8 clade as a sister to the BO clade, rather than as a

TABLE 2 Genomes used in analysis[a]

Species	Strain	No. of contigs	Accession no.	Size (nt)
Brucella abortus	S19	2	NC_010742, NC_010740	3,283,936
Brucella abortus	2308	2	NC_007618, NC_007624	3,278,307
Brucella abortus	2308A	9	NZ_ACOR01000001-NZ_ACOR01000009	3,277,197
Brucella abortus	9-941	2	NC_006933, NC_006932	3,286,445
Brucella melitensis	16 M	2	NC_003317, NC_003318	3,294,931
Brucella melitensis	ATCC 23457	2	NC_012441, NC_012442	3,311,219
Brucella ovis	ATCC 25840	2	NC_009504, NC_009505	3,275,590
Brucella ceti	Cudo	7	NZ_ACJD01000001-NZ_ACJD01000007	3,389,269
Brucella neotomae	5K33	11	NZ_EQ999575-NZ_EQ999585	3,329,623
Brucella canis	ATCC 23365	2	NC_010104, NC_010103	3,312,769
Brucella suis	1330	2	NC_004310, NC_004311	3,315,175
Brucella suis	ATCC 23445	2	NC_010169, NC_010167	3,324,607
Brucella microti	CCM 4915	2	NC_013119, NC_013118	3,337,369
Brucella sp.	83-13	20	NZ_DS999649-NZ_DS999668	3,153,851
Brucella sp.	NF2653	113	ADFB01000000	3,106,792
Brucella inopinata	BO1	55	ADEZ00000000	3,366,774
Brucella inopinata	BO2	174	ADFA00000000	3,305,941
Ochrobactrum anthropi	ATCC 49188	6	NC_009667, NC_009668, NC_009669, NC_009670, NC_009671, NC_009672	5,114,435
Ochrobactrum intermedium	LMG 3301	4	NZ_ACQA00000000	4,725,392
Bartonella quintana	Toulouse	1	NC005955	1,581,384
Mesorhizobium loti	MAFF303099	3	NC_002678, NC_002679, NC_002682	7,596,297
Agrobacterium tumefaciens	C58	5	NC003064, NC003065, NC003062, NC003063, NC004972	5,682,546

[a] Accession numbers of unclosed genomes are abbreviated to the first and last of the sequential numbers to save space where applicable.

sister to the classic clade, but both the N8 and BO clades are considered early-diverging relatives of the classic clade.

Regions specific to the classic *Brucella* species are missing in new *Brucella* strains. We have identified 20 genomic regions that are present in the classic *Brucella* genomes and absent in BO1^T, BO2, NF2653, and 83-13 (Table 3). A complete list of the missing genes found in these regions is provided (see Table S1 in the supplemental material). Genes identified in region 1 were based on *B. abortus* 2308, as the genes that it contains are missing from *B. suis* 1330. Genes from all other regions were based on the *B. suis* 1330 genome. The regions specific to the classic *Brucella* species range in size from the smallest (region 3, at 706 nucleotides [nt]) to the largest (region 20, at 47,259 nt). The proportion of hypothetical genes annotated in these regions is 42%, with the average across the entire *B. suis* 1330 genome being 21% (Table 1).

Both strains 83-13 and NF2653 are missing regions present in the classic and BO clades. Combined, these sections total more than 146.4 kb, corresponding to 152 genes, with 34.8 kb missing from what is the first chromosome in *B. suis* 1330 and 108.3 kb from the second (see Table S2 in the supplemental material). Some of the genes in these regions that are missing in the N8 clade have been annotated with specific functionality. Among these is region 10, a 26.9-kb section of *B. suis* 1330 containing five syntenic genes that are involved in tyrosine metabolism. In *Escherichia coli*, these genes are defined as part of the *hpc* operon, a meta-cleavage pathway involved in the degradation of homoprotocatechuate (3,4-dihydroxyphenlacetate), an aromatic compound used as a source of carbon or nitrogen (27). All other *Brucella* genomes maintain this capability, while strains 83-13 and NF2653 apparently function without it.

The genomes in the BO clade are also uniquely missing regions that are conserved among the other *Brucella* strains (see Table S3 in the supplemental material). Many of the genes in the Wbk region (region 2; see Table S3 in the supplemental material), containing several of the genes important in LPS synthesis and maintained by BO1^T, have been lost from BO2. In BO2, this region carries the unique genes encoding dTDP-4-dehydrorhamnose reductase (*rmlD*), dTDP-glucose 4,6-dehydratase (*rmlB*), dTDP-4-dehydrorhamnose 3,5-epimerase (*rmlC*), and glucose-1-phosphate thymidylyltransferase (*rmlA*). These four genes are involved in the formation of the O-antigen component of the LPS in many Gram-negative bacteria (28) but were not previously known to be present in *Brucella* and are discussed in more detail below.

New *Brucella* strains have unique regions not found in the classic Brucella genomes. The four new strains had unique regions in their genomes that are not present in the classic *Brucella* species (Table 4). None of these are shared between the N8 and BO clades but are specific to each clade. Details of each of these regions, including the size, the number of genes, and their locations on contigs, are available in Table S4 in the supplemental material.

(i) Shared regions: BO1^T and BO2. The BO1^T and BO2 genomes had 11 regions they share that are not present in the other *Brucella* genomes. They ranged in size from 4.7 to 74.6 kb (Table 4). These unique regions had a significantly higher number of hypothetical proteins, with 56% in BO1^T and 47% in BO2 (Table 4), than is seen across the genome at large (22% or 23%; Table 1), and several had flanking tRNA genes, a hallmark of mobile genomic islands.

Of particular interest is region B-7 (10.8 kb), which had a number of genes coding for proteins associated with L-rhamnose utilization. These genes included an isomerase, an aldolase, a transcriptional regulator, and proteins associated with an ABC transporter (see Table S4 in the supplemental material), which were all conserved in both strains in this clade. This region was present in both *Ochrobactrum* genomes as well as in the genomes of several *Agrobacterium* and *Rhizobium* species (occurring there in secondary chromosomes or in large plasmids).

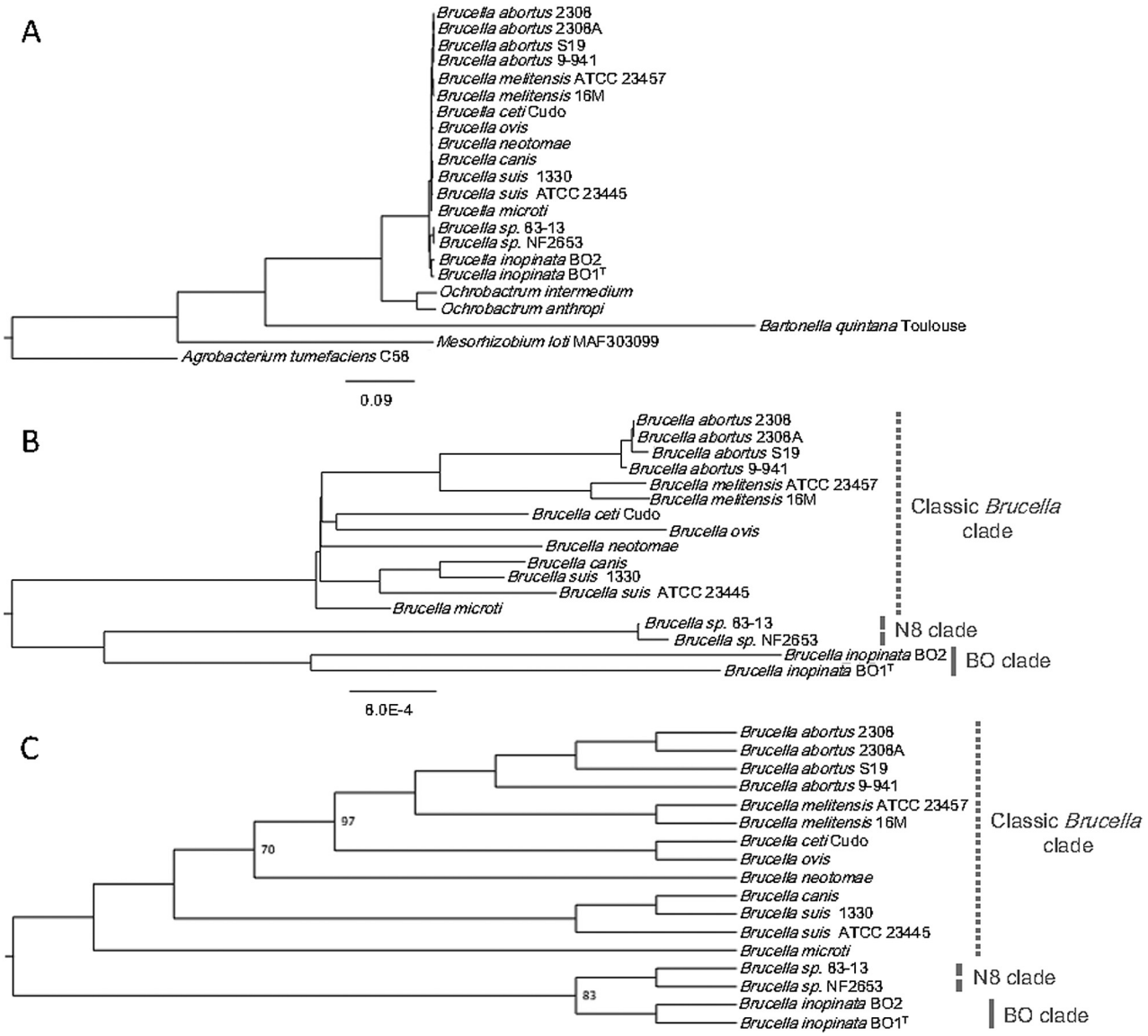

FIG 1 Maximum-likelihood tree for 17 *Brucella* and 5 outgroup species. All nodes received 100% bootstrap support except for the three that are otherwise marked. (A) Full tree. (B) *Brucella* region only. (C) *Brucella* region in cladogram form.

BLASTN analysis of region B-9 (9.2 kb) shows a good match (94% coverage, 72% nt identity) to a region in plasmid pRLG201 of *Rhizobium leguminosarum* bv. *trifolii* WSM2304; parts of this region also appear (total coverage, between 43% and 50%, with 66% nucleotide identity in the largest matched section) in the genomes of several *Burkholderia* species. Further investigation of this region showed that it was part of a larger match (27.9 kb) between pRLG201 and contig NZ_ADEZ01000028 in BO1^T (22.6 kb of this region was also present in the BO2 genome but on three separate contigs). A BLASTN analysis of GenBank's NR database using the extended BO1^T region as the query showed that the genomes of several other *Brucella* species had good matches to the first part of this region (see Fig. S1 in the supplemental material); the section corresponding to B-9 was clearly absent from the alignment. These results and the phylogenetic relationship between *Brucella* and *Rhizobium* suggested that the extended matched region in the *R. leguminosarum* and BO1^T genome sequences represents the ancestral condition and that region B-9 was lost by the other sequenced *Brucella* genomes as well as by all other sequenced members of the *Rhizobiales* order. While not particularly parsimonious, this explanation seems to fit better with the absence of any horizontal gene transfer evidence. The protein-coding gene content of the extended region is shown in Table S5 in the supplemental material.

Region B-5 (74.6 kb in BO1^T and 35.7 kb in BO2) is another region not seen in either *Ochrobactrum* genome. It had many phage and hypothetical genes, but there was no large-scale similarity to regions in other completed or draft genomes available from GenBank.

(ii) Shared regions: 83-13 and NF2653. There were eight regions in strains 83-13 and NF2653 that none of the other *Brucella* genomes share (Table 4). Few genes in these areas had functional

TABLE 3 Summary of regions present in the genomes of the classic *Brucella* clade and missing in strains BO1^T, BO2, NF2653, and 83-13[a]

Chromosome	Region	Size (nt)	No. (%) of genes			Presence of tRNA
			Total	Hypothetical	Named	
1	1	21,256	29	23	6	
	2	4,270	7	2	5	√
	3	706	3	2	1	
	4	16,174	16	9	7	√
	5	2,012	4	4	0	√
	6	7,242	16	10	6	
	7	1,378	5	3	2	
	8	6,489	9	7	2	√
	9	2,800	6	4	2	
	10	5,140	6	3	3	√
	11	2,069	5	1	4	
	12	9,203	12	2	10	√
2	13	1,193	3	3	0	
	14	5,923	7	2	5	
	15	17,696	17	10	7	
	16	10,704	11	1	10	
	17	16,645	19	3	16	√
	18	7,384	8	0	8	
	19	3,764	6	6	0	√
	20	47,259	46	3	43	√
Total		189,307	235	98 (42)	137 (59)	

[a] Regions 1 and 2 are from *B. abortus* 2308, and all others are from *B. suis* 1330.

assignments, the vast majority (80% in 83-13 and 82% in NF2653) of the genes being annotated as hypothetical. This differs from what is seen more broadly across these genomes, where the majority of the genes have been annotated with functional assignments (Table 1).

(iii) Unique regions. The BO1^T genome had five unique regions that total 39.7 kb (Table 4), and most of the genes associated with these regions were annotated as hypothetical (69%). Specific genes and their locations are provided (see Table S4 in the supplemental material).

BO2 also had five unique regions, totaling 52.4 kb (Table 4), with many of the genes annotated as hypothetical (48%). One of these regions (BO2-2) was unique in having a number of genes involved in rhamnose-based LPS O-antigen synthesis (see Table S4 in the supplemental material).

LPS synthesis genes. Nineteen genes essential to the synthesis of LPS and necessary to produce the smooth phenotype have been identified in *B. melitensis* (29, 30). The presence or absence of all known LPS genes in the four new genomes is shown in Table 5. As these genomes are not closed, it is difficult to make absolute statements about specific genes that appeared to be missing, and a BLASTN search of the genome was carried out for genes not annotated in any of the four new genomes. There are several differences found in the new genomes compared to those of smooth members of the classic *Brucella* clade. The *wbkD* gene in the NF2653 strain has a single nucleotide deletion that results in a frameshift that truncates the gene. As NF2653 is smooth and mutation of *wbkD* has been shown to result in a rough phenotype (30), which is present in the classic rough *Brucella* species (26, 31), we believe that the deletion is a sequencing error. There are two copies of the *manB* gene in the classic *Brucella* genomes: $manB_{core}$ (BRA0348 in 1330) is thought to play a role in synthesis of the LPS core, and $manB_{O\text{-}Ag}$ is part of the Wbk region (BR0537 in 1330), but its role in O-antigen synthesis is unclear. While all four new genomes have copies of $manB_{core}$, no gene is annotated as $manB_{O\text{-}Ag}$ in the genome of either 83-13 or NF2653. BLASTN searches show that $manB_{O\text{-}Ag}$ in both 83-13 and NF2653 is missing 837 of 1,404 nt compared to its homolog (BR0537) in *B. suis* 1330; what remains of the gene has many deletions. NF2653 and 83-13 also share a conserved insertion, resulting in a frameshift mutation that extends the normally 284-nt *wbkB* gene to 575 nt. Early evidence indicated that $ManB_{O\text{-}Ag}$ was necessary for converting mannose-6-phosphate to mannose-1-phosphate (32), but mutation of this gene in *B. melitensis* 16M did not produce a rough phenotype (30). As the $manB_{O\text{-}Ag}$ gene in all known *B. ceti* strains is truncated by a naturally occurring transposon and yet these strains are smooth (26, 31), this could be considered further evidence that $ManB_{O\text{-}Ag}$ is not required for LPS synthesis.

The Wbk region in strains NF2653 and 83-13 has a few differences with respect to the other *Brucella* strains. Whereas, in all other *Brucella* strains, the *wbkB* gene has 855 nt, in NF2653 and 83-13 the gene is twice as long (1,728 nt). Mutations in this gene in other *Brucella* strains have not resulted in an altered LPS (29), making it seem unlikely that this mutated version has any impact on LPS synthesis. There is also a hypothetical protein annotated between *wbkC* and *wbkB* (Fig. 2) that has strong similarity (e-value, 10^{-63}) to *wboA*, making this a potential paralog. The presence of this second *wboA*-type gene in the members of the N8 clade prompted a closer look at that gene and its neighbor *wboB*. The *wboA* and *wboB* genes from the classic *Brucella* strains have almost 100% identity (by BLASTN) to each other, but those from 83-13 and NF2653 have considerably reduced percentages of identity (82% for *wboA* and 80% for *wboB*), indicating that they either have diverged significantly or had different origins.

BO2 produces an atypical smooth LPS. The BO1^T genome appears to carry the full complement of LPS genes seen in the classic *Brucella* (Table 5). Although gene *wbkA* is not present in the BO1^T annotation, BLASTN shows that most of this gene (1,110 of 1,119 total nucleotides) is present, and its sequence runs to the end of contig NZ_ADEZ01000023. Presumably, the remaining 9 nt are

TABLE 4 Summary of the size, number, and presence or absence of tRNA-associated genes across regions unique or shared in the four *Brucella* strain genomes studied here[a]

	Size (nt)	Total Genes	Hypothetical	Known	tRNA	Size (nt)	Total Genes	Hypothetical	Known	flanking tRNA	*Ochrobactrum* homology
ID	83/13					NF2653					
N8-1	23141	32	28	4	√	23121	35	31	4	√	no
N8-2	20465	15	14	1	√	20466	17	16	1	√	no
N8-3	7540	7	5	2	√	7540	7	5	2	√	no
N8-4	1630	4	2	2		1630	4	2	2		partial
N8-5	5064	4	3	1	√	5064	4	3	1	√	no
N8-6	4297	4	2	2	√	4297	4	2	2	√	no
N8-7	1160	3	3	0		1099	3	3	0		no
N8-8	6000	2	0	2		6000	2	0	2		no
Totals	69297	71	57	14		63217	76	62	14		
% Total			80%	20%				82%	18%		
ID	BO1[T]					BO2					
B-1	22643	31	25	6		49763	55	40	17		partial
B-2	10856	11	8	3	√	8364	10	7	3	√	partial
B-3	5031	6	4	2	√	4781	5	2	3	√	no
B-4	5089	7	4	3		5245	7	4	3		partial
B-5	74611	78	52	26		35770	23	10	13	√	no
B-6	12550	11	5	6		7432	6	2	4		no
B-7	10796	10	0	10		11806	10	0	10		yes
B-8	6795	5	0	5		7682	6	0	6		no
B-9	9273	10	1	9		9121	10	1	9		no
B-10	9763	8	0	8		9763	8	0	8		no
B-11	11881	3	1	2		11811	3	1	2		partial
Totals	179288	180	100	80		161538	143	67	78		
% Total			56%	44%				47%	55%		
BO-1	1163	3	3	0							partial
BO-2	1981	4	3	1							partial
BO-3	1661	4	2	2	√						no
BO-4	13387	9	7	2							no
BO-5	21597	25	16	9							no
Totals	39789	45	31	14							
% Total			69%	31%							
BO2-1						11597	16	7	9		no
BO2-2						5353	5	0	5		yes
BO2-3						8030	6	3	3		no
BO2-4						17570	16	8	8		no
BO2-5						9891	11	8	3	√	yes
Totals						52441	54	26	28		
% Total								48%	52%		

[a] For the regions shared by strains NF2653 and 83-13, each of the areas is identified by the prefix N8 (N for NF2653 and 8 for 83-13) followed by the number of the region. For the regions shared by strains BO1[T] and BO2, each of the areas is identified by the prefix B.

TABLE 5 Summary of presence, absence, or alteration of sequence of genes involved in LPS synthesis in *Brucella*[a]

Strain	Presence, absence, or alteration of sequence of indicated gene																								
	wbkD	*wbkF*	*wbkC*	*wbkB*	*wzt*	*wzm*	*per*	*gmd*	*wbkA*	*manB*	*manC*	*manA*	*wbkE*	*wboA*	*wboB*	*wadA*	*wadB*	*wadC*	*waaA*	*pgm*	*manB* core	*manC* core	*rmlD*	*rmlB*	*rmlC*	*rmlA*
Smooth *Brucella*	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	O	O	O	O
83-13	√	√	√	#	√	√	√	√	√	#	√	√	√	√	√	√	√	√	√	√	√	√	O	O	O	O
NF2653	#	√	√	#	√	√	√	√	√	#	√	√	√	√	√	√	√	√	√	√	√	√	O	O	O	O
BO1[T]	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	O	O	O	O
BO2	√	√	O	O	O	O	O	O	O	O	O	O	O	O	O	√	√	√	√	√	√	√	√	√	√	√

[a] √, presence; O, absence; #, alteration of sequence. Data are partly based on identifications by González et al. (30), as visualized among the smooth *Brucella* members, the known rough species, and the four new strains.

present in the genome and this gene is functional in BO1[T]. Analysis of the BO2 genome shows that 13 LPS genes are not present in the annotation and cannot be found by BLASTN (Table 5). The *wboA* and *wboB* genes, not present in BO2, have been discussed above. The other missing genes (*wbkC*, *wbkB*, *wzt*, *wzm*, *per*, *gmd*, *wbkA*, *manB*, *manC*, *manA*, and *wbkE*) are in the Wbk region, leaving only *wbkD* and *wbkF* still present. This should mean that BO2 is unable to make the *N*-formyl-perosamine-based O-antigen found in the classic smooth *Brucella* strains. This would explain why it does not agglutinate with the classic A-and M-specific antisera used to type *Brucella* strains and why it is not sensitive to the classic *Brucella* phage (16).

As BO2 appears to be missing most of the genes that the other *Brucella* strains require to produce O-antigen, it is possible that, like *B. ovis* and *B. canis*, BO2 is naturally rough. However, the absence of autoagglutination in acriflavine (data not shown) argues against this. Interestingly, the BO2 Wbk region carries four unique genes not seen in any other *Brucella* strains: *rmlA*, *rmlB*, *rmlC*, and *rmlD* (Fig. 2 and Table 5; see also Table S4 in the supplemental material). These four genes also exist in an operon in other bacteria (28) and catalyze the conversion of glucose-1-phosphate to dTDP-l-rhamnose (33). Rhamnose is an important residue in the O-antigen in many Gram-negative bacteria, and dTDP-l-rhamnose is an immediate precursor of the rhamnose moiety in the O-antigen (34). Both *Ochrobactrum* genomes also contain these four genes in an operon associated with other LPS genes (data not shown). Further evidence that this operon is active in LPS synthesis is the presence of rhamnitol and glucosamitol as the main sugar components of the O-antigen of *O. anthropi* (35). This suggests that BO2 uses these genes to produce a novel O-side chain. As noted above, both BO1[T] and BO2 carry genes not found in other *Brucella* strains that encode enzymes involved in rhamnose uptake and metabolism. In addition, the absence of *wzm* and *wzt* is significant, since they encode the ABC transporters involved in the export of all known homopolymeric O-antigens (36), including that of the classic smooth *Brucella* species. Also significant is the conservation of *wbkD* and *wbkF*: they encode proteins putatively related to bactoprenol priming for O-antigen polymerization, a function essential in the generation of smooth-type LPS. All of these genetic features are consistent with the presence of a smooth LPS in BO2 carrying a heteropolymeric, possibly rhamnose-based, O-antigen. Concerning the LPS core oligosaccharide, the gene encoding the 2-keto-3-deoxy-*D*-manno-octulosonic acid (Kdo) transferase (*waaA*) was present as expected from the constant presence of this sugar in all Gram-negative LPSs. In the classic *Brucella* species, three additional glycosyltransferase genes, *wadA* (previously called *wa***), *wadB*, and *wadC* (22, 37, 38), have been identified thus far, and homologues of these three genes were identified in all the newly sequenced genomes. This finding is in keeping with the fact that core oligosaccharide genes are usually conserved among closely related bacteria, but does not necessarily mean a total structural identity at the core level with the classic *Brucella* spp. In fact, Zygmunt et al. (39) have recently reported that sodium dodecyl sulfate (SDS)-proteinase K-extracted LPSs of BO1[T] and BO2 fail to react with a monoclonal antibody specific for the lipid A-core region of the classic *Brucella* species, suggesting there exist some differences in this region of the molecule.

To confirm that BO2 synthesizes a smooth LPS, we isolated LPSs from strains 2308, BO1[T], and BO2 and examined them by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Fig. 3). As expected, 2308 and BO1[T] LPS were present only in the phenol phase (lanes A and B, respectively) and not the aqueous phase (lanes D and C, respectively) following hot aqueous phenol extraction. In contrast, the O-antigen of BO2 displayed the discontinuous ladder-like pattern typical of those LPSs, with O-antigens made of repeated oligosaccharide units (2). Interestingly, the BO2 LPS partitioned almost equally into the water (lane E) and phenol (lane F) phases. The only minor difference was that the phenol-phase LPS was more enriched in small-molecule-size components. This partition pattern is consistent with both the absence of *N*-formyl-perosamine (phenol solubility is a trait of *N*-formyl) or *N*-acetyl-perosamine O-antigens (40, 41) and the highly hydrophobic structure of *Brucella* lipid A (38). The monoclonal antibody Bru-38, recognizing the *N*-formyl-perosamine O-antigen, reacted with *B. abortus* 2308 and S19 LPSs (both A-dominant O-antigens) but did not react with either the aqueous-phase or the phenol-phase LPS from BO2 by immunoblotting. However, BO1[T] LPS (from the phenol phase only) also reacted with Bru-38 (data not shown). These results are consistent with those recently reported by Zygmunt et al. (39), who found that BO1[T], although reacting weakly with a polyclonal M-specific antiserum used in routine typing, was recognized by some M-specific monoclonal antibodies. However, BO1[T] has an anomalously high reactivity with a monoclonal antibody of C/Y (A > M) specificity, which is in agreement with reactivity to antibody Bru-38. Moreover, those same authors also reported that BO2 LPS is smooth but not reactive to monoclonal antibodies to either A or M epitopes. Compositional analysis of the BO2 LPS by gas chromatography-mass spectrometry (GC-MS) confirmed that, unlike LPS from the classic *Brucella* spp., rhamnose, as well as galactose, is a major component of the carbohydrate region (Fig. 4A). Glucose, glucosamine, and Kdo are also present and are components of the core region of classic *Brucella* LPS (32). Of interest was that only a small amount of mannose, which is a component of the core oligosaccharide of most smooth *Brucella*

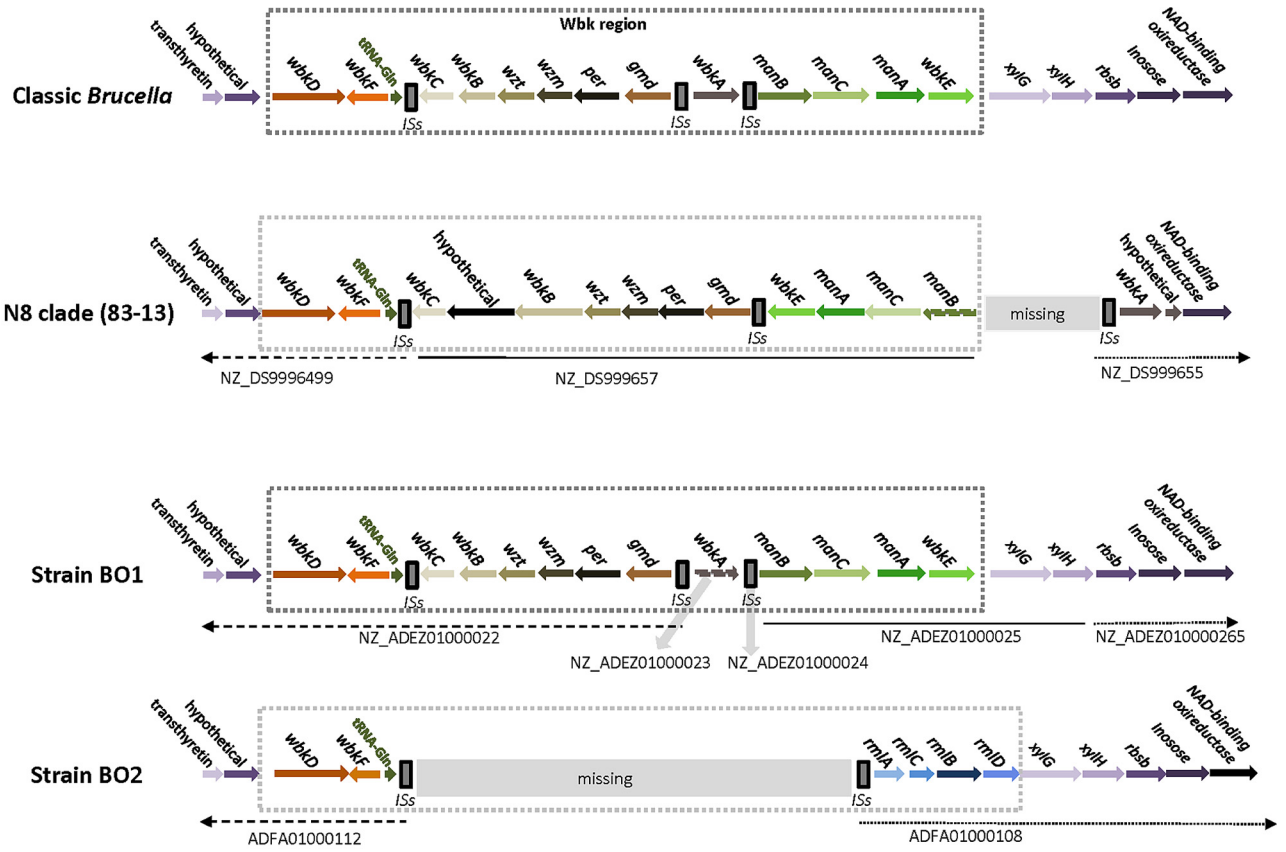

FIG 2 Comparison of the *wbk* regions across *Brucella* strains, with orientation based on *B. suis* 1330. Flanking genes are noted outside the dashed boxes, which represent the *wbk* regions. The contigs on which the genes occur are noted.

strains, was detected in BO2 LPS (Fig. 4A [the small peak to the left of the galactose peak]). This result is consistent with the small amount of mannose observed in rough *Brucella* LPS (42). A possible explanation is that the mannose is phosphorylated or bears an acid-resistant residue, which would make it not subject to cleavage by methanolysis. In addition, 2,6-dideoxy-2-aminohexose, which occurs in classic *Brucella* LPS as quinovosamine, was present (37). A variety of fatty acids, including C14:0, C15:0, C14:03-OH, C16:03-OH, C17:03-OH, C18:03-OH, and some C28:027-OH, were identified in BO2 LPS. If confirmed, the C15:0 and C17:03-OH fatty acids would be considered novel in *Brucella* LPS.

Experimental and bioinformatic evidence indicates that strain BO2 synthesizes a polysaccharide containing the sugars rhamnose and galactose, which are distinct from the glycoses present in other *Brucella* isolates. Furthermore, the genes in the *rml* operon, together with *wbkD* and *wbkF* (see above), appear to form an O-antigen that is united with the core oligosaccharide. It is unknown how BO2 transports its O-antigen to the periplasmic side of the cell where it would be ligated to the lipid A core (38). The *wzm/wzt* ABC transporter genes are missing in BO2, but their presence would be in conflict with the observed ladder-like pattern, because, as noted above, these ABC transporters are involved only in the export of homopolymeric O-antigens. O-antigens made of repeating oligosaccharide units are synthesized through the so-called *wzy*-dependent pathway, but no clear *wzy* homologue could be identified in the BO2 genome. A search for the genes involved would require the construction of rough mutants. However, to date BO2 has been recalcitrant to all attempts at genetic manipulation (B. Saadeh and D. O'Callaghan, unpublished data).

Since LPS is a critical virulence factor of *Brucella* (43), the presence of an alternative O-polysaccharide structure is striking. The typical *N*-formyl-perosamine O-polysaccharide of the three classic smooth *Brucella* species is known to contribute to resistance to complement-mediated killing and bactericidal peptides. Moreover, evidence obtained with *B. suis* strongly suggests that the *N*-formyl-perosamine O-polysaccharide also plays a role in the interaction with lipid rafts of the cell membrane, permitting the bacterium to enter cells through a pathway that makes it possible to reach the replicative intracellular niche (44). Although further research is necessary, it seems that the alternative O-polysaccharide structure present in BO2 can perform at least some of these roles. This hypothesis is in keeping with previous interpretations that the *Brucella* O-polysaccharide sterically protects outer membrane targets from complement and bactericidal peptides and that it also prevents the unspecific binding to cells manifested by rough mutants, thereby allowing effective selection of the port of entry (30). The finding of a virulent *Brucella* strain with an alternative O-polysaccharide supports the interpretation that, in spite of a different chemical composition, the O-antigen allows the species to retain its ability to infect and cause disease.

In conclusion, the analysis of the draft genomes of the four new

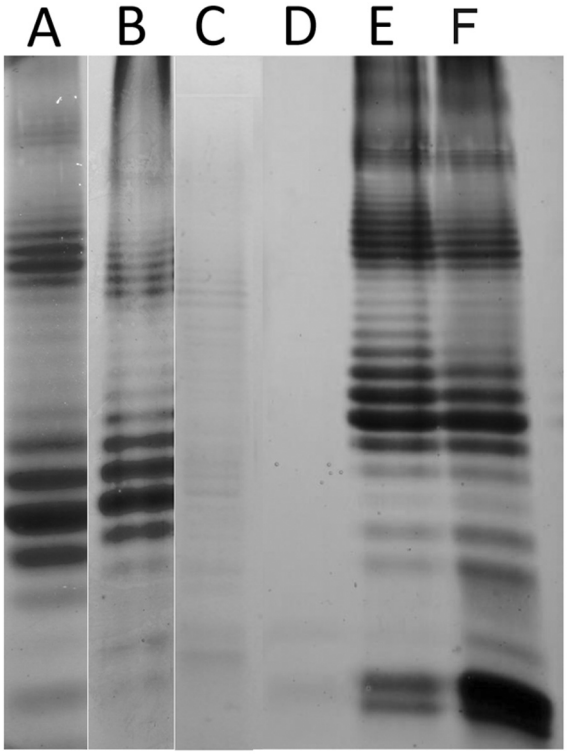

FIG 3 Electrophoretic profiles of the LPS produced by *B. abortus* 2308 and *B. inopinata*-like strains BO1 and BO2. Lanes: A, *B. abortus* 2308 LPS isolated from the phenol phase; B, BO1 LPS isolated from the phenol phase; C, BO1 LPS isolated from the aqueous phase; D, 2308 LPS isolated from the aqueous phase; E, strain BO2 LPS isolated from the aqueous phase; F, BO2 LPS isolated from the phenol phase.

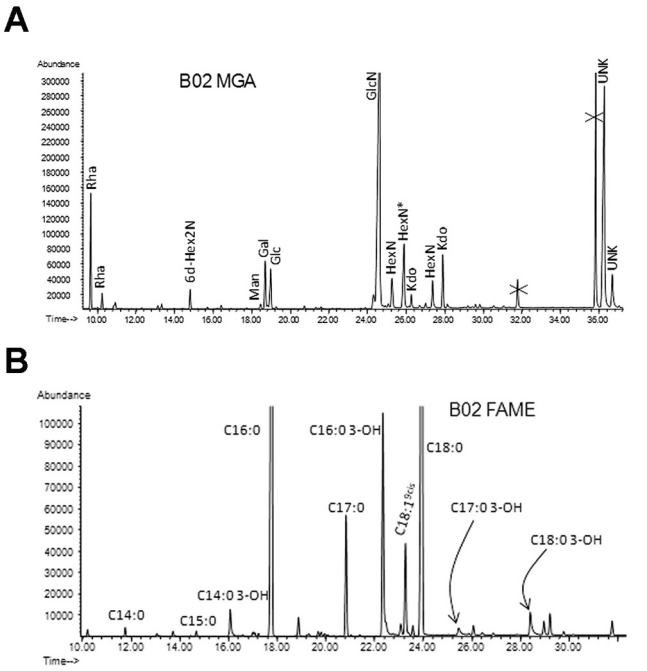

FIG 4 GC-MS (gas chromatography-mass spectrometry) analysis of glycoses and fatty acids in the LPS of *Brucella* BO2. (A) Acetylated *O*-methyl glycoside (MGA) profiles. Rhamnose and galactose are unique glycose components of the BO2 LPS. (B) Fatty acid methyl esters (FAME) of BO2 LPS. C17:03-OH and C15:0 have not been described in other *Brucella* LPSs. Abbreviations used: Rha, rhamnose; 6 d-Hex2N, 2-amino-2,6-dideoxy-hexose; Man, mannose; Gal, galactose; Glc, glucose; GlcN, glucosamine; Kdo, 2-keto-3-deoxy-*D*-manno-octulosonic acid; UNK, unknown component. HexN* should represent a minor peak derived from GlcN.

strains demonstrates clearly that they are true *Brucella* species. It also shows that the genus is far more diverse than previously imagined and suggests that many *Brucella* strains do not fit into the phenotypic definitions drawn up in the 1970s based on the classic *Brucella* strains (45).

MATERIALS AND METHODS

Genome sequencing and annotation. Genomes of the three newly identified strains, *B. inopinata* type strain BO1^T, *B.* inopinata-like strain BO2, and *Brucella* spp. NF2653, were sequenced using Roche 454 technology to draft status (coverage values are given in Table 1) and assembled by the Centers for Disease Control and Prevention (CDC). Genomes were annotated by the PAThosystems Resource Integration Center (PATRIC; http://www.patricbrc.org) using the RAST pipeline (46). The gene annotations for the 3 newly sequenced genomes and 14 additional *Brucella* genomes were acquired from PATRIC (57, 58), where they were annotated consistently using RAST (46). All genome sequences and annotations described here are available at PATRIC (http://www.patricbrc.org). Details about the annotation statistics of these newly sequenced genomes are given in Table 1, and a complete list of the genomes from *Brucella* and those from species used as outgroups that were used in this study is given in Table 2.

OGs and synteny analysis. OrthoMCL (59) was used to create groups of orthologous proteins. To create a representative set of ortholog groups (OGs) for the *Brucella* strains and their closest relatives, genomes from *Ochrobactrum anthropi*, *O. intermedium*, *Bartonella quintana*, *Mesorhizobium loti*, and *Agrobacterium tumefaciens* were included (Table 2). The new *Brucella* genomes are not closed and are in multiple contigs, complicating traditional analyses that look for genomic specificities. Syntenic strings of singleton or low-membership OGs were recognized by consecutive locus tags and verified by manual inspection of the genome. Following identification of syntenic areas of interest, the nucleotide sequence for the entire region was used in a BLASTN search (47) of all *Brucella* genomes in PATRIC. Regions that had lacked BLAST hits in specific genomes, or across large groups of genomes, were noted as especially interesting. These areas were also checked to see if they were associated with tRNA genes, as these are a hallmark of genomic islands.

Phylogenetic analysis. Of all the protein families for the *Brucella* and outgroup genomes, 1,681 were found to have one and only one representative in each *Brucella* genome and these were used for the phylogenetic analysis. Each of these families was made representative of the outgroup strains by excluding strains with two or more members in the family, leaving *O. anthropi* represented in 1,564 families, *O. intermedium* in 1,527, *B. quintana* in 730, *M. loti* in 1,360, and *A. tumefaciens* in 1,352. The protein sequences of each family were aligned using MUSCLE (60), and ambiguous portions of the alignment were removed using Gblocks (48). The concatenation of these alignments contained 494,836 amino acid characters. RAxML 7.2.3 (49) was used with the PROTGAMMALG model to prepare a maximum-likelihood tree and in its quick mode to prepare 100 bootstrap trees.

Unique regions. Regions found in any of the four new genomes and not found in the classic *Brucella* genomes by BLASTN (47) were identified as being "unique." These unique syntenic protein signatures were expanded in both the 5′ and 3′ directions along the specific contigs to find the endpoints where conservation with the other *Brucella* genomes continued and genes that were broadly shared across *Brucella* strains began.

LPS purification and analysis. The LPS was extracted as previously described (42, 50) with minor modification. Briefly, acetone-dried cells

were suspended in 50 ml of sterile, distilled water, an equal volume of 45% phenol was added, and the mixture was stirred at 65 to 70°C for 15 min. The sample was then cooled to 15°C, diluted with distilled water, and dialyzed under running tap water until no phenol odor remained (about 2 days). The solid brown, proteinaceous precipitate was discarded, and the soluble and white insoluble material was subjected to centrifugation at 3,000 × *g* at 15°C. Sodium acetate (30 mM final concentration) was added to the supernatant, three volumes of cold 95% ethanol was added, and the mixture was held at −20°C for 2 h. The sample was centrifuged at 10,000 × *g* for 30 min at 4°C. The pellets from the initial centrifugation and from the ethanol precipitation were suspended in 50 mM sodium phosphate buffer containing 20 mM $MgCl_2$ and 5 mM EDTA (pH 7.0). DNase (Qiagen RNase-Free DNase Set; Qiagen, Valencia, CA) and RNase (riboshredder RNase blend; Epicenter, Madison, WI) were sequentially added to achieve a final concentration of 2 μg/ml, and the mixtures were incubated at 37°C for 1 h each. The temperature was then raised to 60°C, proteinase K (Sigma-Aldrich, St. Louis, MO) was added to achieve a final concentration of 20 μg/ml, and the mixtures were incubated overnight. The extraction with phenol was repeated as described above, but the phenol and aqueous phases were separated by centrifugation. The aqueous phase was removed, and the phenol phase and interface were passed through Whatman grade 40 filter paper. Three volumes of cold (−20°C) methanol reagent (1 part methanol saturated with sodium acetate to 99 parts methanol) was added to the aqueous and clarified phenol phases and incubated overnight at −20°C. The samples were subjected to centrifugation at 10,000 × *g* for 30 min at 4°C, and the pellets were suspended in distilled water and lyophilized. LPS samples (20 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with ammoniacal silver, as previously described (51, 52). Some samples were further analyzed by immunoblotting (53), using a 1:100 dilution of Bru-38 monoclonal antibody to *B. abortus* LPS (54).

Fatty acid and monosaccharide composition was obtained by treating LPS (about 1 mg) with methanolic HCl (1 ml) at 80°C for 18 h. The solution was extracted twice with equal volumes of *n*-hexane, the two top layers (*n*-hexane) were combined and dried, and the fatty acid methyl esters were analyzed directly by GC-MS. The bottom layer (methanol) was dried with a stream of air and the resulting methyl glycosides were acetylated as reported elsewhere (55). All GC-MS analyses were performed on a Hewlett-Packard 5890 instrument equipped with an SPB-5 capillary column (Supelco) (30 m by 0.25 inner diameter [i.d.]; flow rate, 0.8 ml/min; He as carrier gas), with the following temperature program: 150°C for 3 min, a gradient of 150 to 300°C with increases at 10°C/min, and 300°C for 18 min. Electron impact mass spectra were recorded with an ionization energy of 70 eV and an ionizing current of 0.2 mA.

Nucleotide sequence accession numbers. The whole-genome shotgun project and the annotations for *B. inopinata* strain BO1^T have been deposited under accession no. ADEZ00000000 at DDBJ/EMBL/GenBank, and the version described in this paper is the first version, ADEZ01000000. Similarly, for the other strains, the deposit accession number and the accession number of the version described in this paper, respectively, are as follows: for BO2, ADFA00000000 and ADFA01000000; and for NF2653, ADFB00000000 and ADFB01000000. The genome of strain 83-13 was sequenced by the Broad Institute and is available from GenBank under accession number ACBQ00000000.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00246-12/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.
Table S4, PDF file, 0.1 MB.
Table S5, DOC file, 0.1 MB.

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