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Comparative Phylogenomics and Evolution of the Brucellae Reveal a Path to Virulence

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***Brucella* species include important zoonotic pathogens that have a substantial impact on both agriculture and human health throughout the world. Brucellae are thought of as "stealth pathogens" that escape recognition by the host innate immune response, modulate the acquired immune response, and evade intracellular destruction. We analyzed the genome sequences of members of the family *Brucellaceae* to assess its evolutionary history from likely free-living soil-based progenitors into highly successful intracellular pathogens. Phylogenetic analysis split the genus into two groups: recently identified and early-dividing "atypical" strains and a highly conserved "classical" core clade containing the major pathogenic species. Lateral gene transfer events brought unique genomic regions into *Brucella* that differentiated them from *Ochrobactrum* and allowed the stepwise acquisition of virulence factors that include a type IV secretion system, a perosamine-based O antigen, and systems for sequestering metal ions that are absent in progenitors. Subsequent radiation within the core *Brucella* resulted in lineages that appear to have evolved within their preferred mammalian hosts, restricting their virulence to become stealth pathogens capable of causing long-term chronic infections.**

The *Alphaproteobacteria* are an ecologically diverse group of Gram-negative bacteria among which several lineages evolved from niches in the environment toward obligate intracellular parasitism of diverse eukaryotic hosts. The adaptation of certain *Alphaproteobacteria* to intracellular life within a host has been associated with genome reduction, resulting in the loss of genes no longer necessary in this specialized environment (1, 2). Free-living bacteria in water or soil must exploit diverse conditions and compete with other organisms in these environments, while bacteria that reside within host cells encounter less competition but are exposed to different stresses (3, 4). As facultative intracellular pathogens, *Brucella* species establish long-term, often chronic, interactions with higher eukaryotes (1) but also must survive outside the host. This genus includes species considered among the world's most important zoonotic pathogens (5) with a major impact in the poorer, rural areas of the world that lack the resources to establish surveillance and eradication programs for livestock. Brucellae use virulence factors, including a type IV secretion system (T4SS), to modulate host cell biology to create a novel intracellular replication niche in both professional and nonprofessional phagocytes (6), causing infectious abortion and sterility in infected animals and a debilitating disease known as Malta fever in humans.

For many years the genus *Brucella* comprised six "classical" species differentiated by a preferential mammalian host and a set of antigenic and metabolic phenotypes. Since the early 1990s, new *Brucella* strains have been isolated from marine mammals, rodents, and atypical human infections, raising the number of recognized species to 10 (5), with additional species likely to be described. A recent study has reported the isolation of *Brucella*-like strains from frogs, pushing the boundaries of natural hosts into amphibians (7). Brucellae grow poorly in the environment, and each species infects a preferential host. It has been proposed that each species represents a clonal lineage selected by this restricted virulence and evolution within the vertebrate host (8–10). The Brucellae provide a unique opportunity to examine a facultative pathogen, exploring the potential parallel genome reduction and genomic divergence resulting in unique species. The genomes of prominent strains have recently been sequenced, and here we detail the comparative genomics of 40 *Brucella* genomes with reference to their closest relative, *Ochrobactrum*, a soil bacterium.

MATERIALS AND METHODS

Isolate preparation. *Brucella* isolates were revived from freeze-dried stocks in the Animal Health & Veterinary Laboratories Agency (AHVLA) collection by subculture onto serum dextrose agar (SDA) containing 10% equine serum and incubation at 37°C for 3 days in the presence of additional 10% CO_2. DNA was extracted by standard phenol-chloroform procedures using growth from two spread plates, as described previously (11). After extraction, DNA was resuspended in an average of 400 μl Tris-EDTA buffer, resulting in an average DNA concentration of ~3,000 μg/ml (minimum, 300 μg/ml), which was stored at 4°C prior to shipping to the Broad Institute.

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Sequencing, assembly, and annotation. A list of all genomes used in the present study is provided in Table S1 in the supplemental material. Genome sequencing and assembly for 25 of the genomes were performed at the Broad Institute. These genomes were sequenced to at least 15-fold coverage using 454 FLX pyrosequencing (Roche) with DNA fragment libraries and 3-kb paired-end reads according to the manufacturer's recommendations and assembled using Newbler. Before assembly, the quality of the 454 sequencing data was analyzed, and suspect libraries were removed. The runAssembly script was then used to assemble reads into contigs and scaffolds using the parameters −ar −rip −g. Final assemblies were used in BLAST searches of the NCBI nonredundant (NR) database, UniVecCore, and a reasonable mitochondrial database to remove any contaminating sequence. The other 17 genomes were sequenced and annotated by different sources and were downloaded from the Pathosystems Resource Integration Center (PATRIC) (www.patricbrc.org) (12, 13). All genomes used in this analysis were annotated consistently at PATRIC using RAST (14). In addition, PATRIC's Protein Family Sorter tool (13) was used to visualize shared homology and recognize areas of horizontal transfer.

A comparison of metabolic functionality across the genomes relied on FIGfams, a collection of over 100,000 protein families that are isofunctional homologs (15) and are available at PATRIC. Although all genomes were compared in the functional analysis, for simplicity only differences between *Brucella suis* 1330 and *Ochrobactrum anthropi* were displayed.

OGs. OrthoMCL (16) was used to delineate groups of orthologous proteins. To create a representative set of ortholog groups (OGs) for *Brucella* and its closest relatives, genomes from *Ochrobactrum anthropi*, *O. intermedium*, *Bartonella quintana*, *Mesorhizobium loti*, and *Agrobacterium tumefaciens* were included. Multiple-sequence alignments were generated for each OG and were visually inspected.

SNP phylogeny. Single nucleotide polymorphism (SNP) discovery was performed using MUMmer (17, 18) and In Silico Genotyper (TGen; unpublished program). MUMmer was run on the complete genomes to produce pairwise sequence alignments against the reference (*B. suis* 1330) utilizing a sliding window with each potential SNP flanked by 100 bases on each side. SNPs from the resulting binary alignment map (BAM) alignments were determined using SolSNP (http://sourceforge.net/projects/solsnp/). Taxa were then grouped by SNP loci shared across all taxa using In Silico Genotyper. Loci missing from one or more genomes (no read or sequence coverage in the alignment) were excluded from the analysis, as were repeated regions and paralogous genes. We defined homologous SNPs as those found in all genomes and paralogous SNPs as those that came from a region that had been duplicated. Orthologous SNPs were those homologous SNPs that remained after the paralogous SNPs were removed.

When using only shared orthologous SNPs in a phylogeny, the number of SNPs will vary depending on which taxa are included in the comparisons. For example, when *Ochrobactrum* species are included in SNP discovery, fewer SNPs are found within a clade such as *Brucella abortus* due to the requirement that the locus containing the SNP occur in all genomes, including even more distantly related ones with higher levels of genomic changes. We resolved this issue by rerunning SNP discovery in a stepwise fashion down through the phylogeny. First we included both *Ochrobactrum* species and all *Brucella* species, then just the *Brucella* species, then just the core *Brucella* species, and finally strains within each species containing more than one taxon. Within-species comparisons used the most complete genome sequence of that species as the reference. None of these analyses changed the overall tree topology, but they did provide for more-detailed depictions of the SNPs relevant to each clade. Trees were constructed using maximum parsimony in the program PAUP* 4.0b10 using a matrix of SNP calls (19). We employed a full heuristic search and tested nodal support with 1,000 bootstrap repetitions.

Protein family phylogeny. Of the 6,991 protein families (OGs) for the 40 *Brucella* and 5 outgroup genomes, 1,844 were found to have one and only one representative of each *Brucella* genome, and these were used for the phylogenetic analysis (20, 21). At each of these loci, outgroup sequences were included if present as a single locus but excluded if paralogous. Of the 1,844 protein families used in the analysis, *O. anthropi* was represented in 1,695 protein families, *O. intermedium* in 1,670, *B. quintana* in 794, *M. loti* in 1,484, and *A. tumefaciens* in 1,462. The protein sequences of each family were aligned using MUSCLE (22), and ambiguous portions of the alignment were removed using Gblocks (23). The concatenation of these trimmed alignments contained 521,801 amino acid positions. RAxML 7.2.3 (24) was used with the PROTGAMMALG model to prepare a maximum-likelihood tree and in its quick mode to prepare 100 bootstrap trees.

RESULTS AND DISCUSSION

Sequencing and annotation. Twenty-five strains largely representing type strains or groups not previously characterized by whole-genome sequencing were chosen for sequencing and were compared with 17 previously sequenced genomes (see Table S1 in the supplemental material). The new genomes were finished but not closed (25), ranging from 11 (*Brucella neotomae* 5K33) to 89 (*Brucella pinnipedialis* M163/99/10) contigs. The *Brucella* genomes have an average of 3,463 genes annotated, ranging from 3,167 (*Brucella* sp. strains 83/13 and NF2653) to 3,610 genes (*Brucella ceti* Cudo).

Molecular phylogeny of the *Brucellaceae*. Phylogenetic trees were built from over 190,000 SNPs and separately from over 1,800 concatenated protein sequences (20, 21). These robust phylogenetic analyses of the *Brucella* and outgroup genomes shows that all *Brucella* species are monophyletic and distinct from their *Ochrobactrum* relatives (Fig. 1), confirming earlier studies (26). There is also a clear separation between the "classical" *Brucella* species that are united in a core clade and a second group that diverged earlier. This early diverging basal group contains strains from Australian rodents (83/13 and NF2653) and two recent atypical human isolates, BO1 and BO2, where the natural host is unknown (27, 28). Of the 193,760 shared SNPs within the overall phylogeny, *Brucella* species are separated from the two *Ochrobactrum* species by 158,016 SNPs. The basal *Brucella* species are clearly differentiated from the core *Brucella* species, with 2,672 SNPs uniting the core *Brucella* species. The relative diversity is substantially higher in the early-diverging *Brucella* species than in the classical species, suggesting that this group is undersampled.

The core clade experienced a radiation that resulted in six to eight lineages, with *Brucella microti* being the most basal and the remaining clades (*Brucella neotomae*, *Brucella canis*-*Brucella suis*, *Brucella ceti*-*Brucella pinnipedialis*, *Brucella ovis*, *Brucella* sp. strain NVSL07-0026, and *Brucella abortus*-*Brucella melitensis*) separated by such short internal branch lengths that they appear to have diverged nearly simultaneously (Fig. 2). In the phylogeny comparing all *Brucella* species to *Ochrobactrum* and in the core clade phylogeny, *B. ovis* and *Brucella* sp. strain NVSL07-0026 are united by an extremely shallow branch, indicating a shared common ancestor, but the lengths of the branches that separate these two indicate significant divergence since that time. This is also seen in the phylogeny based on protein families (Fig. 3). The phylogenetic placement of *B. suis* bv. 5 in the tree containing only the core *Brucella* species (Fig. 2) is within the *B. suis*-*B. canis* clade with an extremely shallow branch, but in the phylogeny of the whole genus it falls in its own separate lineage (Fig. 1).

Changes at the genus level: emergence of *Brucella* as a pathogen. The virulence of *Brucella* is dependent on its ability to survive and multiply within host cells, including macrophages (6, 29, 30).

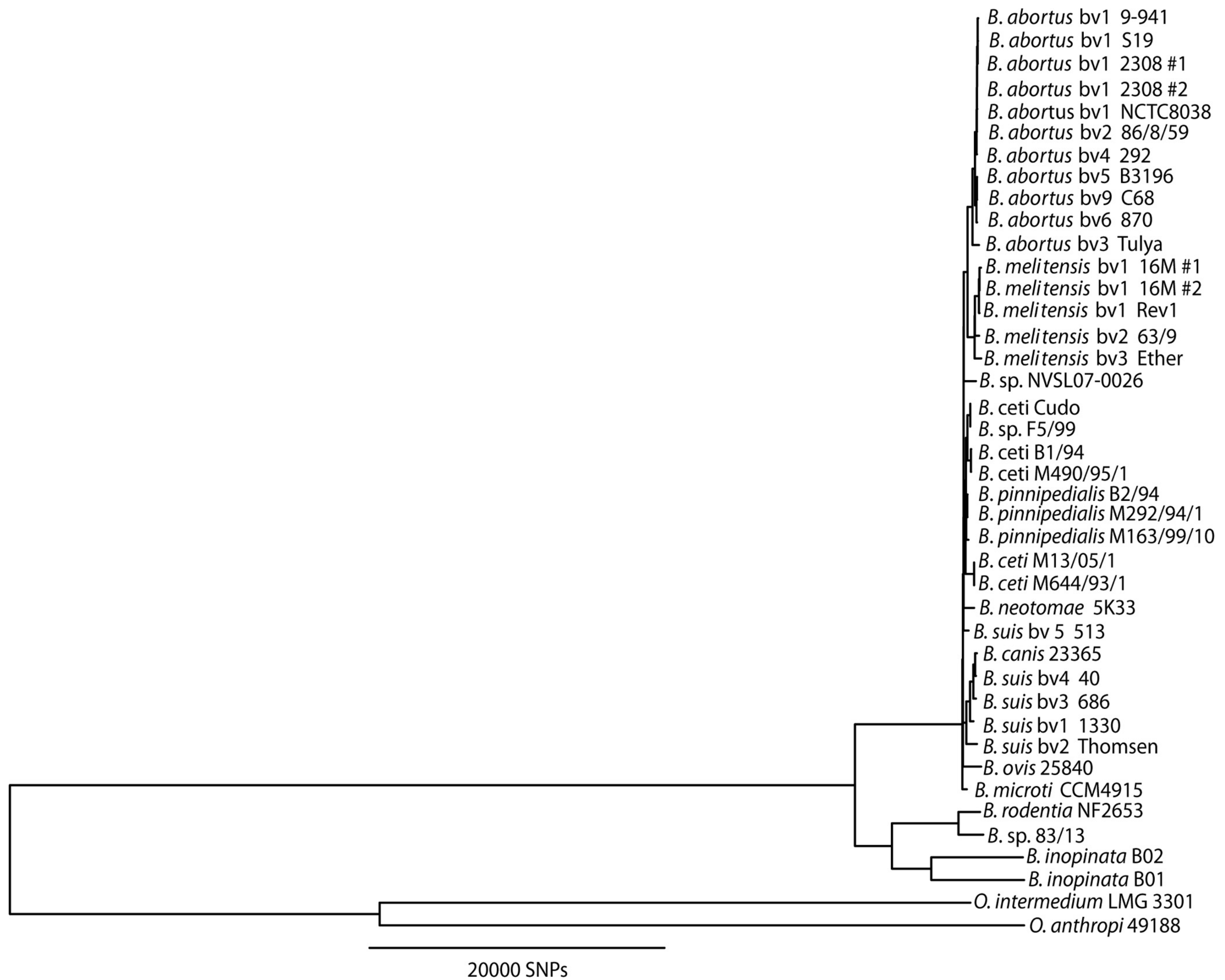

FIG 1 Phylogenetic analysis of 42 *Brucellaceae* genomes. The maximum-parsimony tree is based on 193,760 SNPs. The early-diverging *Brucella* strains are clearly differentiated from the classic *Brucella* strains, with 2,672 SNPs unique to the classic *Brucella* strains and 1,172 SNPs unique to the outer clade (strains NF2653, 83/13, BO1, and BO2). The tree was rooted with *Ochrobactrum* spp. as outgroups. All branches have 100% support unless otherwise noted.

Its closest known relatives, *O. anthropi* and *O. intermedium*, are soil bacteria that can cause opportunistic infections in immunocompromised hosts but are not known to replicate intracellularly within them. Specific events must have led to the evolution of the ability to survive and multiply within a vertebrate cell.

(i) Genome reduction. Adaptation to eukaryotic hosts in the *Alphaproteobacteria* has been associated with genome reduction (1), and the typical *Brucella* genome (~3.3 Mb) is at least 30% smaller than that of *Ochrobactrum* (4.77 Mb). Further evidence of genome reduction can be seen in the analysis of protein families defined by OrthoMCL. The two *Ochrobactrum* genomes share more than 900 OGs that are not found in any *Brucella* genome (see Table S2 in the supplemental material). Our data cannot rule out the possibility that *Ochrobactrum* species have larger genomes due to acquisition by lateral gene transfer, but only 24 of these OGs were found on the plasmids associated with the *O. anthropi* genome, which would rule out plasmids as the source of lateral transfer.

We calculated a pan-genome for *Brucella*, defined as the superset of proteins encoded across all 40 genomes. There were 5,920 OGs in the pan-genome and 2,285 OGs in the core genome. A representation of the differences between *Brucella* and *Ochrobactrum* showing the presence or absence of protein families is depicted in Fig. 4, organized in the order that the genes occur in either *B. microti* (Fig. 4A) or *O. anthropi* (Fig. 4B). Black areas indicate regions that are missing, and the areas of loss are especially prevalent when *Brucella* is oriented according to *Ochrobactrum* (Fig. 4B). The *Brucella* orientation confirms what previous studies across fewer strains have found: there is high conservation of genes across *Brucella* (31) and extensive similarity in genetic content and gene order (32).

A comparison of metabolic function between *Brucella* and *Ochrobactrum* using the subsystem approach (33) was performed and highlights the likely process of genome reduction (see Fig. S1 in the supplemental material). In almost all categories, *Brucella* has fewer genes than *Ochrobactrum*; marked losses are seen in the

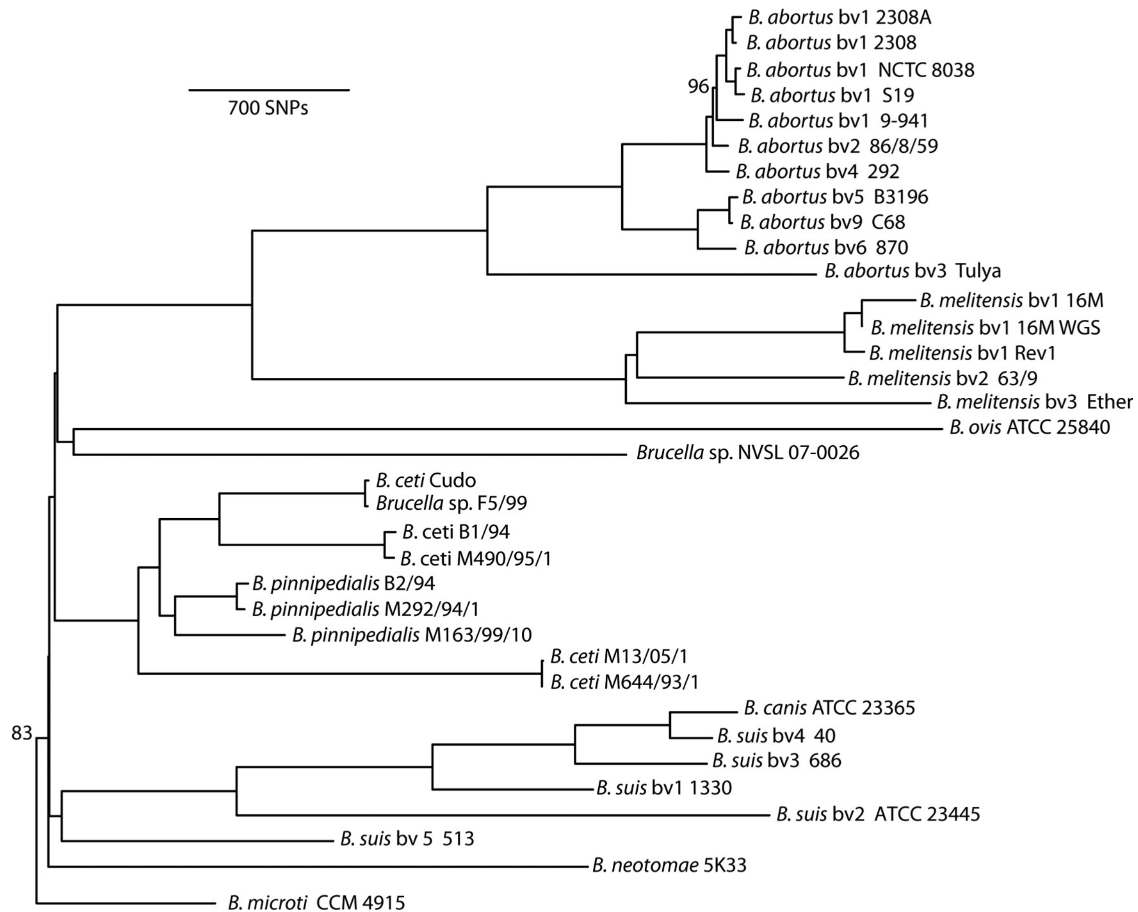

FIG 2 Phylogenetic analysis, based on maximum parsimony of the core *Brucella* genomes, showing a rapid radiation following the divergence of *B. microti*, resulting in six separate clades, with *Brucella* sp. strain NVSL07-0026 and *B. suis* bv. 5 as possible separate clades. The tree was rooted with *B. microti* as the outgroup based on results from Fig. 1. All branches have 100% support unless otherwise noted.

number of genes encoding proteins involved in metabolism and in utilization, degradation, or biosynthesis of both carbohydrates and amino acids. There are two exceptions. *Brucella* has seven genes involved in aromatic amino acid degradation that *Ochrobactrum* lacks, and it also has more genes involved in membrane transport, specifically in the genes used to transport nickel. All genes included in the functional analysis are listed in Table S3 in the supplemental material.

(ii) Gene acquisition. In addition to the nickel transport genes, *Brucella* also has a small number of genes that are not found *Ochrobactrum*, many of which are found clustered in 15 genomic regions that are depicted visually in Fig. 3 and detailed in Table S4 in the supplemental material. Many of these regions have been previously identified as genomic islands and in most cases are completely missing in *Ochrobactrum* (Table 1). In *wbk* and region 14, *Ochrobactrum* has a small number of the total genes, indicating that these could have been present in the common ancestor and have since been lost in *Ochrobactrum*. The presence or absence of these regions, showing their locations on the two chromosomes of a typical *Brucella* genome, is shown in Fig. 5.

(iii) Acquisition of virulence determinants. These 15 regions have many genes that encode virulence factors. Region 15 contains the VirB type 4 secretion system (T4SS), a key virulence factor for *Brucella* (6, 34). *Ochrobactrum* has a T4SS of completely different origin that contains a relaxosome component and a coupling protein, both essential for DNA translocation, which are absent in *Brucella*. The T4SS most similar to that of *Brucella* is found on a broad-host-range plasmid isolated from an unidentified bacterium in the rhizosphere of alfalfa (35).

Three other regions unique to *Brucella* could encode virulence factors. Region 4 (shared anomalous region 1–14 [SAR 1–14]) contains genes encoding enzymes involved in the synthesis of polysaccharides in other bacteria (36) and also the *omp31* gene, encoding a phospholipase with homology to eukaryotic patatin. Some animal and plant pathogens contain a high number of genes with patatin domains, and it has been suggested that these proteins may help them in competition, adaptation to new environments, and interactions with a host (37). Region 7 contains two proteins with homology to eukaryotic proteins (BRA0135 and BRA0136) and a protein with homology to a phospholipase secreted by *Legionella* (BRA0131). Region 14 carries several genes found in pathogenicity islands described in plant pathogens, including *hpaE* (BRA1161) (38).

Brucella encounters acid stress during the infection process: the strong acid in the stomach if transmitted via ingestion and the acid conditions in the phagosome (39), which are known to induce expression of several virulence factors (40). Genes involved in acid resistance are located on region 9, which encodes the Hfq-regu-

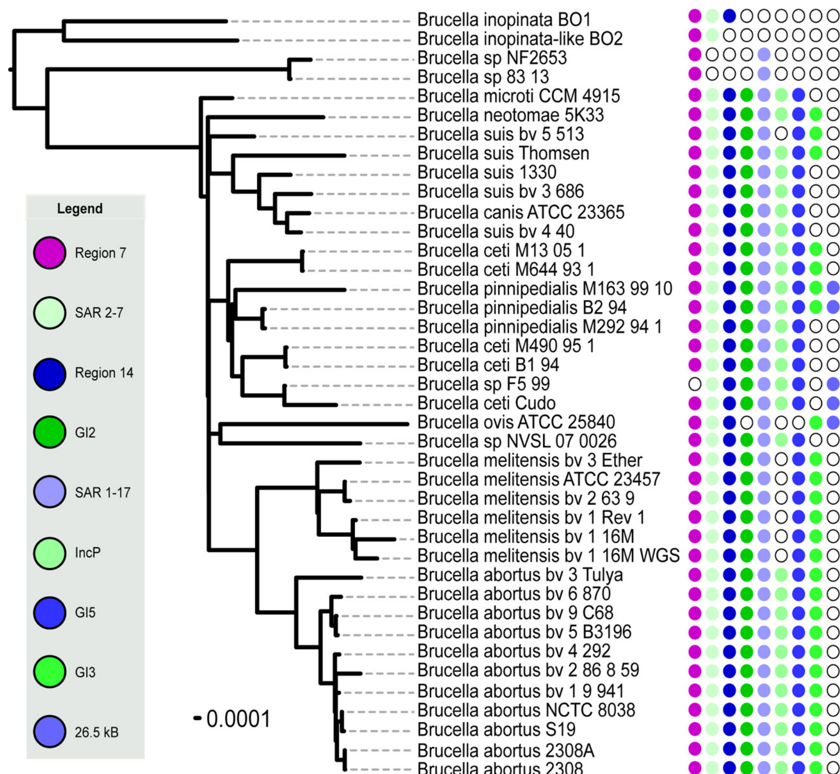

FIG 3 Phylogenetic analysis based on shared protein families in *Brucella*, showing the presence (colored circles) or absence (open circles) of specific genomic islands that are not universally shared across all 40 genomes (as in Table 1).

lated gene *hdeA* that has been shown to be required for resistance to mild acid shock (41). The *gad* operon, which produces the nonproteinogenic amino acid γ-aminobutyric acid (GABA), conferring resistance to strong acid (<pH 3) in *B. microti* (42), is also encoded here (VBIBruSui107850_2552 and VBIBruSui107850_2553). This region seems to be undergoing degradation in the classical species, because several genes are nonfunctional due to frameshifts or stop codons.

Most brucellae produce a smooth lipopolysaccharide (LPS) with an O antigen composed as a homopolymer of *N*-formylperosamine with α(1,3) and α(1,2) linkages. The majority of the genes involved in O-antigen biosynthesis are found in the *wbk* region (region 16 [see Table S4 in the supplemental material]) and have been hypothesized to have been acquired by horizontal transfer (43). Two of the "classical" *Brucella* species, *B. canis* and *B. ovis*, are naturally rough, reflecting distinct mutations. In *B. canis* this is due to truncations in both *wbkD* and *wbkF* (44, 45), while *B. ovis* has a deletion of the *wbo* region (45–47), as well as a mutation that truncates *wzt* (45). Our analysis has also identified additional mutations that truncate *per* and *wbkE* in *B. ovis* (data not shown). Other than these mutations, the LPS biosynthesis genes in "classical" *Brucella* strains are very conserved (44). Examination of the genes encoding the LPS of the atypical *Brucella* strains show considerable diversity (48), although all have a smooth phenotype. The major difference is seen in BO2, which produces a smooth LPS with an O antigen different from that of all other brucellae, using four genes encoding enzymes that synthesize a rhamnose-based LPS that it shares with *Ochrobactrum* (48). Recent data suggest that unlike BO1, strain BO2 is unable to replicate in mammalian cells (B. Saadeh and D. O'Callaghan, unpublished data). This suggests that the loss of the ancestral rhamnose-based LPS and acquisition of the genes encoding the perosamine-based LPS were key steps in the path to intracellular parasitism.

(iv) Adaptation to a limited-metal environment. Bacteria require metal ions for crucial enzymatic functions, including virulence, and intracellular bacteria live in an environment where there is limited availability (48). Many of the regions that are unique to *Brucella* carry genes involved in the transport or acquisition of metal ions, specifically, iron, magnesium, and nickel. Details on these genes are provided in Table S4 in the supplemental material.

Brucellae produce the monocatechol siderophore 2,3-dihydroxybenzoic acid (2,3-DHBA) in response to iron limitation, and the genes that encode this siderophore (49) are in an operon (BRA0013 to BRA0016) found in region 5. Production of this siderophore appears to be an adaptation to the environment found in the ruminant placenta (50–52). Another gene involved in iron regulation is *dhbR*. This gene, BRA1192 (in region 13), encodes an AraC-type transcriptional regulator that provides a second level of regulation of siderophore biosynthesis genes in response to the availability of Fe^{3+} encountered in the external environment (53).

Magnesium is another metal found in limiting quantities in mammalian hosts. *Brucella* appears to only have one system, the MgtBC transporter, to acquire magnesium from the environment. MgtC has been shown to be a key virulence factor required for the survival and growth in low concentrations of Mg^{2+} and within

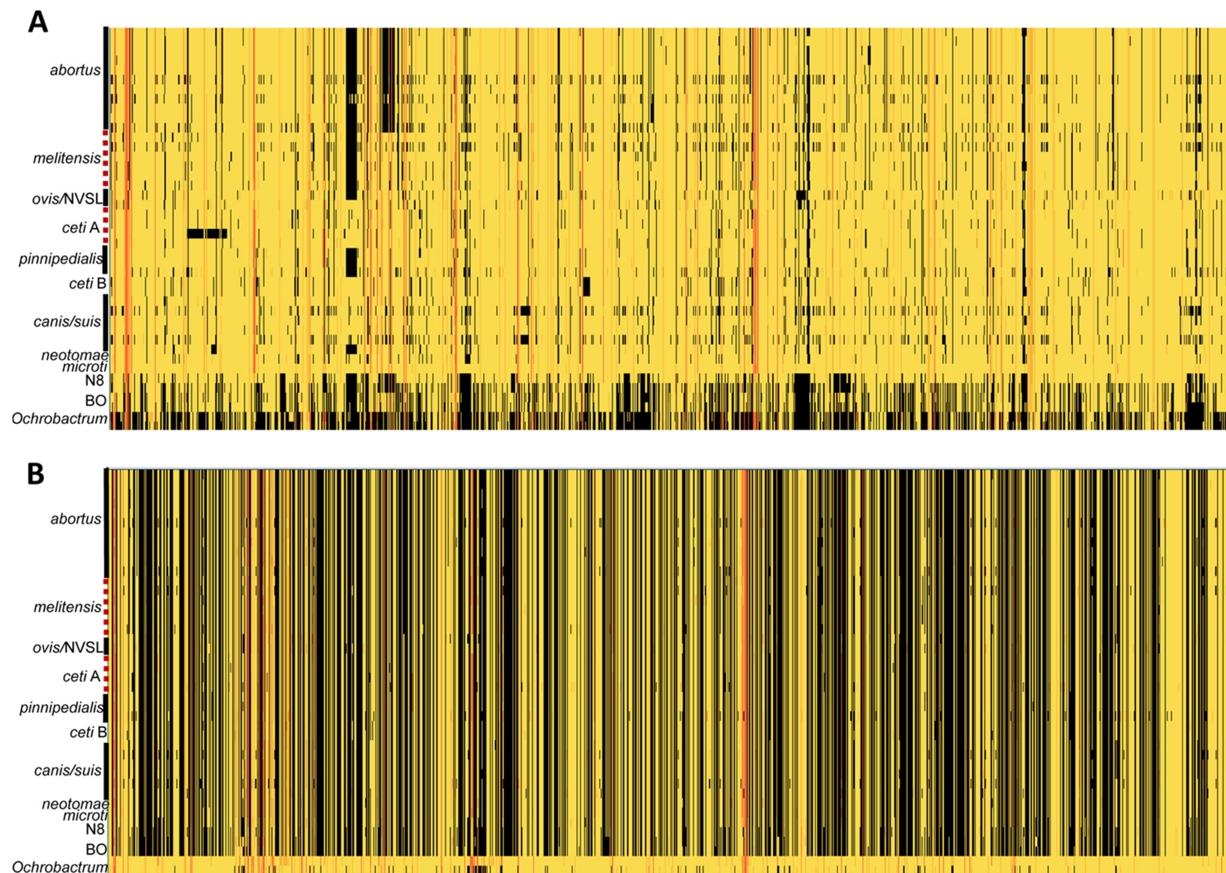

FIG 4 The first half of the *Brucellaceae* pan-proteome with protein families oriented by either *B. microti* (A) or *O. anthropi* (B), generated using the Protein Family Sorter tool at PATRIC. Black cells (columns, protein families; rows, genomes) indicate no annotated proteins, yellow indicates a single protein, and orange to red indicate increasing numbers of proteins annotated in that specific genome.

macrophages of several bacterial pathogens, including *Brucella* (44, 45). In *Brucella*, *mgtC* (BRA0040) is found in region 6 (54).

Many bacteria, including *Ochrobactrum*, use a single protein (HupE/UreJ) to acquire nickel. Brucellae are unusual in that they have two distinct operons (*nikABCDE* and *nikKMLQO*) for nickel transport. They also have two known metalloenzymes that require nickel (carbon monoxide dehydrogenase and urease) and two urease operons. The transporter encoded by *nikKLMQO* (region 3) is the most common transporter found in both *Archaea* and *Bacteria* (55) and has been identified as the primary transporter of nickel in *B. abortus* 2308 (56). One of the urease operons, also in region 3, is located upstream of this transporter. The *nikABCDE* operon (region 12) is not common and is sporadically found across the known bacterial genomes (55). The genes in this operon are among the first to be upregulated during intracellular growth of *Brucella*, suggesting that its niche in the host cell is poor in nickel but that nickel is required by the bacterium, necessitating expression of high-affinity uptake systems (57). Yet the role in virulence of the *nikABCDE* operon is not clear. Mutation of the *nikA* gene in *B. suis* drastically reduces urease activity but does not affect virulence in macrophage infection models (57). Further, many virulent strains of *B. abortus* have a frameshift in *nikA* (56), which may explain the low levels of urease activity reported for many field isolates (58).

It is tempting to speculate that the urease system is undergoing the early events of genome reduction. This is suggested by the lack of urease activity in *B. ovis* and the frequent mutations seen in *nikA* in *B. abortus*. It could be hypothesized that the first pathogenic *Brucella* strains were transmitted by the oral route (perhaps via infected milk), but this has now shifted to include other mucosal routes. Oral infection is still possible, especially when animals consume heavily contaminated infected placentas or abortion material, which can contain up to 10^{10} viable bacteria per gram. It is interesting to note that NVSL07-0026, the smooth strain most closely related to *B. ovis* and recently isolated from a baboon stillbirth, also shares the mutations in the urease system (data not shown). It may be that this strain, like *B. ovis*, is transmitted by sexual contact.

Changes within the genus: differentiation within the *Brucella* clades. Brucella has undergone differentiation following its divergence from *Ochrobactrum*. The core *Brucella* clade is distinct from the branch with the two Australian rodent strains and the genomes from the recent atypical human infections (Fig. 1). These genomic comparisons and the genetic relationships among isolates seen here have a relative consistency with a variety of tree topologies from early restriction mapping and multilocus sequence analysis through recent whole-genome analyses (10, 28, 45, 59–63). Phenotypically, the Australian rodent strains are very similar to the core *Brucella* strains, with slow growth and phage sensitivity. *B. inopinata* strain BO1 and *B. inopinata*-like strain

TABLE 1 Presence and absence of genomic regions across the genus *Brucella* and close relatives

Region	Other identifier	Presence[a] in: *B. abortus*	*B. melitensis*	*B. ovis*	*Brucella* sp. strain NVSL	*B. ceti* A	*B. pinnipedialis*	*B. ceti* B	*B. canis*-*B. suis*	*B. suis* bv. 5	*B. neotomae*	*B. microti*	*Brucella* sp. strain N8	*Brucella* sp. strain BO1	*Brucella* sp. strain BO2	*O. anthropi*	*O. intermedium*	*B. quintana*	*M. loti*	*A. tumefaciens*
1	GIBs2	✓	✓[b]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
2		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
3		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
4	SAR 1–14	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
5		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
6	*mgtC*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
7		✓	✓	✓	✓	✓[c]	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
8		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
9		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
10		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
11	SAR 2–7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0[k]	✓	✓	0	0	0	0	0
12	SAR 2–8	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0
13		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0[l]	0	0	0	0
14		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	✓	✓[d]	0	0	0	0	0
15	*virB*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	#	#	#	#	#
16	*wbk*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0[m]	0[n]	✓[e]	0	0	0
Classic 1	SAR 1–2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 2	SAR 1–5	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 3	SAR 1–6	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 4	GI2	✓	✓	0[o]	✓	✓	✓	✓	✓	✓	✓	✓	0	0[p]	0	0	0	0	0	0
Classic 5	GI1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 6	SAR 1–12	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 7	SAR 1–16	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 8	SAR 1–17	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓[f]	0	0	0	0	0	0	0
Classic 9	IncP	✓[g]	0	0	✓	✓	✓	✓	✓	0	✓	✓	0	0	0	0	0	0	0	0
Classic 10	SAR 2–10	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 11	GI5	✓	✓	0	✓[h]	✓	✓	✓	✓	✓	✓	✓	0	0	0	0	0	0	0	0
Classic 12	GI3	✓	✓	✓	0	0	✓[i]	✓	0[q]	✓	✓	0	0	0	0	0	0	0	0	0
Classic 13	26.5 kb	0	0	✓	0	✓[j]	0	0	0	0	0	0	0	0	0	0	0	0	0	

[a] ✓, region is present; 0, region is absent; #, genes of this type are present but are not homologs to those found in *Brucella*.
[b] *Brucella melitensis* 16M, 16M WGS, and Rev1 are missing 5 of the 9 genes in this region.
[c] *Brucella* sp. strain F5/99 is missing all genes in this region.
[d] *Brucella inopinata* BO2 is missing 5 of the total 24 genes.
[e] Of the 12 genes in this area important for forming the LPS in *Brucella*, *Ochrobactrum intermedium* has 7.
[f] *Brucella* sp. strains 83/13 and NF2653 have all genes in this region.
[g] *Brucella abortus* bv. 5 strain B3196 has 9 of the 16 genes in this region.
[h] *Brucella* sp. strain NVSL has 22 of the 37 genes in this region.
[i] Present in both *Brucella pinnipedialis* B2/94 and M163/99/10, but all 27 genes are missing in M292/94/1.
[j] All genes missing *Brucella ceti* M490/95/1 and B1/94, but all are present in *B. ceti* Cudo and *Brucella* sp. strain F5/99. Similarly, all genes are missing in *B. pinnipedialis* M163/99/10, and all present in strains B2/94 and M292/94/1. *B. ceti* A includes *B. ceti* strains Cudo, F5/99, M490/95/1, and B1/94, while *B. ceti* B includes strains M13/05/1 and M644/93/1.
[k] *Brucella* sp. strains 83/13 and NF2653 have 2 of the 8 genes in this region.
[l] *Ochrobactrum anthropi* has only 2 of the 11 genes in this region.
[m] *Brucella inopinata* BO2 has only 2 of the 12 genes in this region that other brucellae have. It has four genes in the *rml* operon in the same place.
[n] *Ochrobactrum anthropi* has only 2 of the 12 genes in this region that other brucellae have. It has four genes in the *rml* operon in the same place.
[o] *Brucella ovis* is missing 21 of the 22 genes in this region.
[p] *Brucella inopinata* BO1 has only 5 of the 22 genes in this region.
[q] *Brucella suis* Thomsen (ATCC 23445) is the only member of this clade that has any of these genes, and it has all 28 of them.

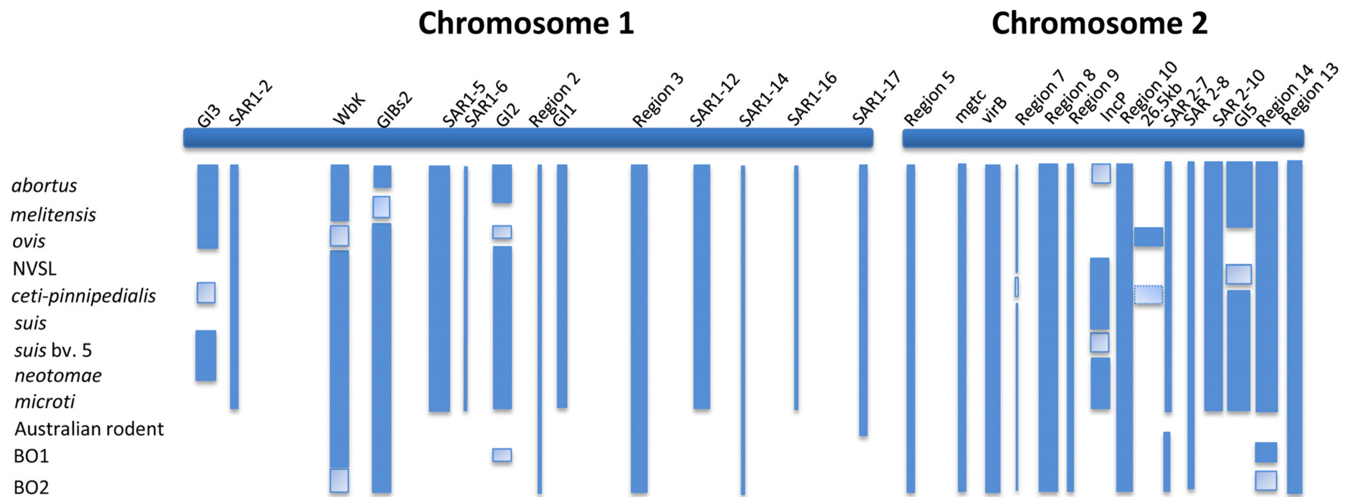

FIG 5 Location of genomic regions of interest across a "typical" *Brucella* strain of two chromosomes. Dark blue bands indicate that a genome or clade has all the genes in the region present. A lighter band indicates that a genome or clade is missing some or all of the genes in a region. Absence of a band shows that the genome or clade does not have this region. These data should be cross-referenced with column 2 of Table 1.

BO2 are different in that they have very fast growth on bacteriological media, are not sensitive to the known *Brucella* phage, and have very different antigenic characteristics (27). However, fast growth is not a distinguishing criterion for the "atypical" strains, since *B. microti* (basal to the divergence of the strains in the core clade) also shows rapid growth. Audic et al. (64) have suggested that the rapid growth of *B. microti* is linked to the presence of an unusual spacer region in the 23S rRNA gene; this spacer is also found in BO1 and BO2.

There are 13 regions that are present in the core strains that are absent in the atypical strains, and a list of genes in these regions is provided in Table S5 in the supplemental material. Most appear to encode proteins that have been acquired horizontally (phage/plasmid or flanked by tRNA genes), and only a few contain proteins with known function. A more detailed analysis of the genomes of the early-diverging strains is addressed in a separate study (48). There are two proteins found in the core clade, BR0735 (GI13, region 2) and BAB1_0279 (region 12), which have eukaryotic Toll interleukin receptor (TIR) domains and have been shown to play a role in the modulation of host innate immune responses (65–71). It is unclear exactly how the acquisition of these TIR domain proteins affected the core *Brucella*, but it is tempting to speculate that this ability to modulate host immunity was a key step in the dramatic spread of *Brucella* through a wide range of mammalian hosts.

A path to the evolution of virulence. Our analysis has identified certain steps that we hypothesize as important developments in the path to pathogenesis (Fig. 6). The first crucial step was the

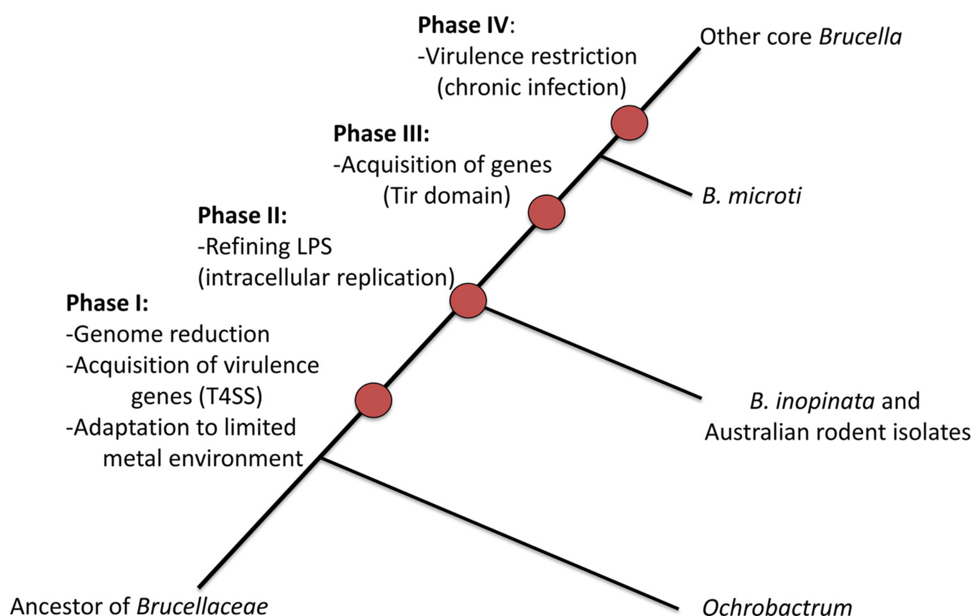

FIG 6 A model for the evolution of virulence in the genus *Brucella*. The phylogenetic tree is not drawn to scale.

acquisition of the VirB T4SS, a key element that has allowed *Brucella* to adapt to a pathogenic niche. This was accompanied by the adaptation of a small set of other factors that have allowed *Brucella* to survive within host cells, and these include genes encoding systems important for sequestering metal ions. A second stage involved the change to a perosamine-based O antigen that seems to be associated with intracellular replication. There is a third step in the path toward virulence; development of the ability to modulate the host immune system using the TIR domain proteins could have been a key step in *Brucella* becoming a stealth pathogen before the radiation to the currently recognized species. As with many intracellular *Alphaproteobacteria*, another key factor appears to be loss or restriction of virulence, allowing the pathogen to avoid a rapid fatal infection and instead keeping the host alive and establishing a chronic infection (1). As more atypical brucellae, such as the recently described strains from frogs (7), are identified and analyzed, we will test our scenario of evolution toward intracellular pathogenicity with more precision.

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