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Brucella abortus 16S rRNA and Lipid A Reveal a Phylogenetic Relationship with Members of the Alpha-2 Subdivision of the Class *Proteobacteria*

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On the basis of ribosomal 16S sequence comparison, *Brucella abortus* has been found to be a member of the alpha-2 subdivision of the class *Proteobacteria* (formerly named purple photosynthetic bacteria and their nonphototrophic relatives). Within the alpha-2 subgroup, brucellae are specifically related to rickettsiae, agrobacteria, and rhizobiae, organisms that also have the faculty or the obligation of living in close association to eucaryotic cells. The composition of *Brucella* lipid A suggests a close phylogenetical relationship with members of the alpha-2 group. The chemical analysis of the lipid A fraction revealed that *Brucella* species contain both glucosamine and diaminoglucose, thus suggesting the presence of a so-called mixed lipid A type. The serological analysis with polyclonal and monoclonal antibodies is in agreement with the existence of mixed lipid A type in *B. abortus*. The amide-linked fatty acid present as acyl-oxyacyl residues were 3-O-$C_{(16:0)12:0}$, 3-O-$C_{(16:0)13:0}$, 3-O-$C_{(16:0)14:0}$, and 3-O-$C_{(18:0)14:0}$. The only amide-linked unsubstituted fatty acid detected was 3-OH-$C_{16:0}$. The ester-linked fatty acids are 3-OH-$C_{16:0}$, 3-OH-$C_{18:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$. Significant amounts of the large-chain 27-OH-$C_{28:0}$ were detected together with traces of 25-OH-$C_{26:0}$ and 29-OH-$C_{30:0}$. Comparison of the *Brucella* lipid composition with that of the other *Proteobacteria* also suggests a close phylogenetical relationship with members of the alpha-2 subdivision. The genealogical grouping of *Brucella* species with pericellular and intracellular plant and animal pathogens as well as with intracellular plant symbionts suggests a possible evolution of *Brucella* species from plant-arthropod-associated bacteria.

Members of the genus *Brucella* are gram-negative facultative intracellular pathogens that induce abortion and severe clinical symptoms in mammals (10, 30). The importance of the disease, mainly in developing countries, is recognized by the considerable economic losses due to infection of domestic animals and by the zoonotic problems caused through the ingestion or contact of contaminated secretions and products (30).

Six species of *Brucella* have been described (10, 30): *B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B. ovis*, and *B. canis*. This classification has been based mainly on the animal host specificity, susceptibility to dyes, metabolic patterns, phage typing, and serological testing (10, 30). Recently Verger et al. (72), using DNA similarity, challenged the separation of *Brucella* into different species and proposed a single species only: *B. melitensis*, containing several biovars. In addition, De-Ley et al. (14) established taxonomic affiliations of *Brucella* species with members of the Centers for Disease Control group Vd in the rRNA superfamily IV and close relationship of these organisms to members of the family *Rhizobiaceae*.

In preliminary reports on the *Brucella* 16S rRNA sequence (18) and on lipid A analyses (42, 47; J. W. Cherwonogrodzky, G. Dubray, E. Moreno, and H. Mayer, *in* K. Nielsen and B. Duncan, ed., *Animal Brucellosis*, in press), we have suggested, that *Brucella* species are related to the alpha-2 subdivision of the class *Proteobacteria* (64), formerly named "purple photosynthetic bacteria and their nonphototrophic relatives" (77), which includes phototrophic and chemoorganotrophic organisms (77, 78). In the present study we propose that species of the genus *Brucella* are closely related to gram-negative bacteria, such as agrobacteria, rhizobiae, and rickettsiae, which also form intimate pericellular or intracellular associations with eucaryotic cells.

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MATERIALS AND METHODS

Extraction and purification of LPS. The characteristics and culture conditions of smooth *B. abortus* 1119-3, smooth *B. melitensis* 16M, rough *B. abortus* 45/20, rough *B. melitensis* B115, *B. canis* (strain obtained from R. Dìaz, University of Navarra, Spain), and *B. ovis* REO have been described elsewhere (10, 48, 51, 72). Extraction and partial purification of *Brucella* smooth and rough lipopolysaccharide (LPS) have been described elsewhere (3, 46–51). Removal of small quantities of phospholipids and ornithine-containing lipids, which contaminated the LPS preparations, was performed with chloroform-methanol-water (1:2:0.8, vol/vol/vol) followed by chloroform–methanol–7 M ammonia (65:25:4, vol/vol/vol) as described by Holst et al. (28). Lipid A and degraded polysaccharide O chain (PS) were prepared and purified from *B. abortus* 1119-3 smooth LPS containing 1.2% protein (determined by amino acid analysis) by treating the material with 2% acetic acid at 100°C for 4 h as described elsewhere (43, 46, 49, 51).

Analysis of the lipid A backbone sugars. Lipid A-OH was prepared by treatment of the 10 mg of lipid A with 2.5 ml of 0.25 M NaOH; subsequent reduction of the material was

carried out with sodium borohydride (22). Hydrazinolysis of the reduced lipid A-OH was performed with anhydrous hydrazine in a sealed tube for 40 h at 100°C (22). The reduced *Brucella* backbone was N-acetylated by treating 1 mg of the material, dissolved in 0.5 ml of double-distilled water, with 0.005 ml of acetic acid anhydride and NaOH (10%, 0.02 ml) for 3 min at 20°C. The procedure was repeated twice. The pH of the solution was brought to 3 with Dowex 50X8 (H^+) and then taken to dryness. Combined gas-liquid chromatography–mass spectrometry for identification of the backbone sugars as alditol acetate derivatives was done in a Finnigan 3200 gas-liquid chromatograph-mass spectrometer fitted with a 6000 data system (27). High-voltage paper electrophoresis of *Brucella* lipid A was performed with pyridine-acetate buffer (pH 5.3) at 3 kV as described elsewhere (34, 43). Amino sugars in electropherograms were stained with ninhydrin. Amino sugar analysis and ethanolamine determination were carried out with a Kontron (Anacomp) automatic amino acid analyzer. The relative concentration of 2-amino-2-deoxy-D-glucose (glucosamine) in lipid A was estimated by amino acid analysis (43). The relative concentration of 2,3-diamino-2,3-dideoxy-D-glucose (diaminoglucose) in the lipid A moiety was estimated by densitometry of the diamino sugar spot, revealed by Trevelyan staining, in relation the spot of glucosamine in paper electropherograms (62). Determinations of organic and inorganic phosphorus were performed with the ammonium molybdate reagent (34).

Fatty acid analysis. Total fatty acids were determined colorimetrically by the method of Haskins (23) by using tripalmitin (Sigma Chemical Co.) as a standard. The relative quantities of amide- and ester-linked fatty acids from lipid A were determined as described by Mayer et al. (43) and Wollenweber et al. (79, 80). The fatty acids were converted into their methyl esters by reaction with diazomethane in ether and then analyzed by gas-liquid chromatography–mass spectroscopy. Methylation and N-deacylation of free lipid A, for release and determination of 3-acyl-oxyacyl residues, and identification of the fatty acids were performed as described by Wollenweber et al. (79, 80).

Sugar analysis. Neutral sugars were liberated by hydrolysis with 0.1 M HCl at 100°C for 12, 24, and 48 h and determined as alditol acetate derivatives by gas-liquid chromatography with a Varian gas chromatograph (model 1520 B) equipped with a packed column of 3% ECNSS-M on Gas Chrom Q (100-200 mesh) (43). Amino sugar analysis was performed as described above. Determination of 2-keto-3-deoxyoctonate, was performed by a modification of the thiobarbituric acid assay (48, 50), with authentic 2-keto-3-deoxyoctonate (Sigma) as a standard. ^{13}C nuclear magnetic resonance was carried out on a Buker FT spectrometer WM 300 MHz at 75.47 MHz as described previously (7, 49).

Serological analysis. Polyclonal antibodies against *B. abortus* O chain, core oligosaccharide, and lipid A were produced in rabbits as described in detail previously (45, 48–51). Monospecificity against each of the moieties was assured by cross-absorption with rough *B. abortus* 45/20, acid-treated rough *B. abortus* 45/20, and smooth *B. abortus* 1119-3, respectively (45, 48–51). Rabbit serum against outer membrane proteins of *B. abortus* 1119-3 was obtained from I. Moriyòn (University of Navarra, Spain). Monospecificity was assured by cross-absorption with *Yersinia enterocolitica* O9 LPS and protein-free rough LPS from *B. abortus* 45/20 (48) as described earlier (48, 49). Monospecific rabbit antiserum against *Rhodopseudomonas viridis* lipid A was obtained from J. Roppel (Max-Plank-Institut für Immunbiologie). Similarly, monospecific antiserum against *R. viridis* lipid A was obtained after exhaustive absorption with acid-treated rough *Escherichia coli* K-12 stain D21f1 (48, 49). Monoclonal antibody (immunoglobulin M [IgM]) against *Salmonella minnesota* lipid A was obtained from A. Lindberg (Karolinska Institute, Sweden). Rabbit IgG was purified from antiserum by protein A-Sepharose affinity chromatography as described in the manual of Pharmacia (Uppsala, Sweden). All IgG fractions were dialyzed and lyophilized. Direct, indirect, and competitive enzyme-linked immunoassays with alkaline phosphatase-goat IgG conjugates, anti-rabbit IgG (Sigma), alkaline phosphatase-rabbit IgG conjugate, and anti-mouse IgM (Sigma), were performed as reported previously (49, 51).

TABLE 1. Composition of PS and lipid A of *B. abortus* 1119-3[a]

Compound	% of dry weight	
	PS	Lipid A
Yield from the LPS	68	23
Glucosamine	ND[b]	8
Diaminoglucose	ND	11
Fatty acids	ND	47
Protein	ND	14
Phosphorus[c]	ND	0.3
Heptose	ND	ND
KDO[d]	0.1	ND
N-Formyl perosamine	93	ND
Quinovosamine	2	ND
Mannose	2	ND
Glucose	3	ND
Ethanolamine	ND	ND

[a] The composition of *B. melitensis* 16M lipid A is very similar to that of *B. abortus* 1119-3 (42, 47; Cherwonogrodzky et al., in press).
[b] ND, Not detected.
[c] The quantity of phosphorus is reported tentatively, since analyses of other samples from the same strain gave deviating results.
[d] KDO, 2-Keto-3-deoxyoctonate.

Phylogenetic analysis. The 16S rRNA sequence of *B. abortus* (18) was aligned to homologous regions of sequences from several reference bacteria and plant mitochondria. The published sequences are from the following bacterial groups: alpha (*Agrobacterium tumefaciens* [12, 81], *Rochalimaea quintana* [12, 74], *Hyphomicrobium vulgare* [63], and plant mitochondria of *Glycine max* [12] and *Triticum aestivum* [12]); beta (*Comamonas testosteroni* [12, 81], *Neisseria gonorrhoeae* [56]), gamma (*E. coli* [12], *Ruminobacter amylophylus* [12], and *Pseudomonas aeruginosa* [69]), and delta (*Desulfovibrio desulfuricans* [12, 53]) subdivisions of *Proteobacteria* as well as *Anacystis nidulans* (12), the plastid of *Euglena gracilis* (12), and *Heliobacterium chlorum* (12) as an outgroup. The phylogenetic analysis was carried out by using the neighborliness method (19, 60). The algorithm was implemented as part of the program package SAGE (Technoma GmbH, Heidelberg, Federal Republic of Germany) designed for the IBM XT/AT and compatible computers.

RESULTS

Hydrolysis of LPS from *B. abortus* 1119-3 with 2% acetic acid gave lipid A and PS. The precipitated lipid A, after mild hydrolysis, contained approximately 10-fold more protein (14%) than the starting smooth LPS (1.2%). The PS remained in the supernatant and was free of protein (Table 1). The analysis of the PS was in agreement with published data (7, 47, 49). Serological detection confirmed the association of outer membrane proteins to *Brucella* LPS and lipid A (see below). The presence of protein strongly bound to lipid A of

TABLE 2. Fatty acids in *B. abortus* 1119-3 lipid A

Fatty acid	Type of linkage	Fatty acid (μmol/μg of lipid A)
$C_{16:0}$	E	0.679
$C_{17:0}$	E	0.033
$C_{18:0}$	E	0.079
3-OH-$C_{12:0}$[a]	A	0.169
3-OH-$C_{13:0}$[a]	A	0.038
3-OH-$C_{14:0}$[a]	A	0.291
3-OH-$C_{16:0}$	E, A	0.395[b]
3-OH-$C_{18:0}$	E	0.060
25-OH-$C_{26:0}$	?[c]	t[d]
27-OH-$C_{28:0}$	?	++[e]
30-OH-$C_{28:0}$	?	t

[a] Found as acyl-oxyacyl residues (expressed as percentages of the total acyl-oxyacyl residues): 3-O-$C_{(16:0)12:0}$ (25%), 3-O-$C_{(16:0)13:0}$ (4%), 3-O-$C_{(16:0)14:0}$ (64%), 3-O-$C_{(18:0)14:0}$ (7%). E, Ester linked; A, amide linked. The moderate quantities of 3-OH-$C_{14:0}$ in *Brucella* LPS as compared with the amount in *E. coli* LPS might account for equivocal results in the past (36, 50).

[b] The 3-OH-$C_{16:0}$ is in approximately equal amounts both ester (0.181 μmol/μg of lipid A) and amide (0.214 μmol/μg of lipid A) linked.

[c] The linkage has not been determined.

[d] t, Present in trace amounts.

[e] Present in significant amount but not quantified.

TABLE 3. ELISA serological reactivity of *B. abortus* preparations[a]

Antibody against[b]:	Lipid A[c]	PS[d]	Rough LPS[c]
R. viridis lipid A	++++[e]	−	+
S. minnesota lipid A[f]	++++	−	−
E. coli lipid A	++++	−	+
B. abortus lipid A	++++	−	+
B. abortus PS	−	++++	−
B. abortus core	−	−	++++
B. abortus OMPs[g]	++	−	−

[a] Lipid A and PS were from *B. abortus* 1119-3. Protein-free rough LPS was from *B. abortus* 45/20 (48).

[b] All rabbit antibodies were protein A-purified IgG fractions (49).

[c] A direct enzyme-linked immunosorbent assay was performed adsorbing lipid A onto the plastic wells.

[d] An indirect enzyme-linked immunosorbent assay was performed by adsorbing *Y. Enterocolitica* O9 LPS onto the plastic wells and competing with free *B. abortus* PS (49).

[e] ++++, Optical density more than 2.00 after 30 min; ++, optical density between 1.00 and 2.00 after 30 min; +, optical density between 0.100 and 0.200 after 30 min; −, optical density below 0.100. The background level was an optical density of 0.02 after 30 min.

[f] Monoclonal antibody, IgM isotype.

[g] OMPs, Outer membrane proteins.

B. abortus 1119-3 but not to the O chain has been extensively documented (3, 37, 45, 46–48, 50, 51).

In addition to glucosamine, an amino sugar with a high mobility ($M_{glucosamine}$ = 1.26), stained with Trevelyan reagent, was detected in the high-voltage paper electropherograms. The migration of this amino sugar was identical to that of the authentic standard of diaminoglucose. Densitometry of the electropherogram suggested that glucosamine and diaminoglucose were present in a ratio of about 1:1. Glucosamine and diaminoglucose were also identified by their characteristic elutions in the amino acid analyzer, where diaminoglucose was traced at 570 nm. The mass spectra of the amino alditol acetates of the lipid A diamino sugar and the authentic diaminoglucose were identical and in accordance with the published spectra (43, 82). The presence of primary fragments at *m/z* 288, 215, and 144, together with a number of secondary fragments confirmed that the amino sugar was diaminoglucose. None of the analyses performed with the isolated lipid A indicated the presence of neutral sugars, heptoses, 2-keto-3-deoxyoctonate, glucosamine 1-phosphate, or ethanolamine (Table 1).

The fatty acids in the lipid A of *B. abortus* 1119-3 are shown in Table 2. Significant amounts of an unusual hydroxylated long-chain fatty acid, 27-hydroxy-octacosanoic acid (27-OH-$C_{28:0}$), and traces of 25-OH-$C_{26:0}$ and 29-OH-$C_{30:0}$ were detected in the lipid A preparations. The acyl-oxyacyl fatty acids identified are the following (with percentages of the total acyl-oxyacyl residues): 3-O-$C_{(16:0)12:0}$ (25%), 3-O-$C_{(16:0)13:0}$ (4%), 3-O-$C_{(16:0)14:0}$ (64%), and 3-O-$C_{(18:0)14:0}$ (7%). The only amide-linked unsubstituted fatty acid detected was 3-OH-$C_{16:0}$. The very low quantities of free $C_{18:0}$ detected during the analysis of the acyl-oxyacyl residues strongly suggest that this fatty acid is mainly (or totally) present as a substituent of 3-OH-$C_{14:0}$, whereas the relatively large quantities of free $C_{16:0}$ detected in the assay indicate that this fatty acid is also present in ester linkage to the lipid A backbone. The other ester-linked fatty acids were 3-OH-$C_{16:0}$, 3-OH-$C_{18:0}$, and $C_{17:0}$. The same fatty acids, as revealed in the lipid A of *B. abortus* 1119-3 and *B. melitensis* 16M, were also detected in the rough LPS from *B. abortus* 45/20, *B. melitensis* 115, *B. canis*, and *B. ovis* REO; however, only qualitative analyses of these LPS preparations were performed.

Table 3 presents the serological analysis of the different fractions of *B. abortus* 1119-3. The serological data are in agreement with the proposition that *Brucella* lipid A contains both glucosamine and diaminoglucose as well as strongly bound outer membrane proteins, whereas the PS does not contain these sugars or associated proteins.

Table 4 shows the percent similarity between 16S rRNAs of *B. abortus* and other published reference organisms calculated from a multisequence alignment with the total length of 1,208 positions. *B. abortus* 1119-3 showed 95.7 and 95.1% similarity to *R. quintana* and *A. tumefaciens*, respectively. These values are of the same magnitude as that found between the latter two species, namely, 95.4%. The other values ranged between 78.7 and 91.9%. Assuming a constant rate of evolution, an association of this facultative intracellular pathogen with the alpha subdivision of *Proteobacteria* can be concluded. This finding is supported by certain features in the variable regions of its 16S rRNA secondary structure (77) that have been omitted for the calculation of the phylogenetic tree. As compared with other *Proteobacteria*, all members of the alpha subdivision lack a long stalk between positions 200 and 220 (International Union of Biochemistry [IUB] nomenclature) and also lack a long stalk and loop between positions 455 and 480.

Since *R. quintana* and *A. tumefaciens* both belong to the alpha-2 subdivision, the allocation of *B. abortus* to the alpha-2 subdivision is indicated, although complete sequences from representatives of the other subdivisions have not yet been published. To verify the approximate position of *B. abortus*, its putative RNase T-resistant oligonucleotides of the 16S rRNA sequence were compared with the set of signature oligonucleotides indicated to separate the three alpha subdivisions (20). None of the alpha-1 and alpha-3 signature oligonucleotides was present in the *B. abortus* sequence; however, three alpha-2 signature oligomers (AA ACUUG, AUUUACUG, and UUUUACCCG) were actually present. This number falls in the range of two to six signature oligomers found in the catalogs of the other organisms of the alpha-2 subdivision. As judged from the lack of oligonucleotide signatures indicated to define several highly

TABLE 4. Percent similarity between 16S rRNA sequences of *B. abortus* and other members of *Proteobacteria* calculated from multisequence alignment with total length of 1208 positions (lightface type) and equally weighted (Hamming) distances between 16S rRNA sequence pairs of *B. abortus* and other members of *Proteobacteria* (boldface type)[a]

Organism	% Similarity or Hamming distance[b]											
	1	2	3	4	5	6	7	8	9	10	11	12
1. *Brucella abortus*		95.7	95.1	91.9	78.7	79.7	82.9	82.0	83.4	81.8	83.8	83.4
2. *Rochalimaea quintana*	**52**		95.4	91.9	80.1	80.2	83.1	81.8	83.2	82.2	83.2	83.5
3. *Agrobacterium tumefaciens*	**58**	**55**		91.5	79.8	79.7	83.4	81.0	83.0	82.2	83.4	84.4
4. *Hyphomicrobium vulgare*	**98**	**98**	**103**		78.5	78.8	81.5	82.2	83.4	81.8	82.8	82.9
5. *Glycine max*[c]	**257**	**241**	**245**	**260**		99.2	74.0	74.8	74.0	74.4	74.8	73.5
6. *Triticum aestivum*[c]	**253**	**239**	**243**	**256**	**10**		74.0	74.8	74.0	74.4	74.8	73.7
7. *Neisseria gonorrhoeae*	**206**	**204**	**200**	**224**	**314**	**313**		87.7	84.4	83.7	85.2	80.4
8. *Comomonas testosteroni*	**217**	**220**	**230**	**215**	**304**	**305**	**148**		84.0	82.8	85.9	80.4
9. *Escherichia coli*	**199**	**203**	**205**	**199**	**313**	**314**	**189**	**195**		89.9	87.3	82.5
10. *Ruminobacter amylophilus*	**219**	**214**	**215**	**219**	**309**	**309**	**197**	**208**	**122**		87.3	82.1
11. *Pseudomonas aeruginosa*	**201**	**204**	**201**	**208**	**305**	**305**	**179**	**174**	**154**	**154**		79.8
12. *Desulfovibrio desulfuricans*	**205**	**199**	**200**	**206**	**320**	**319**	**237**	**237**	**211**	**216**	**224**	

[a] Unsequenced regions of *H. vulgare* and *B. abortus* (positions 1 through 45 and 1490 through 1542, IUB nomenclature) and regions of alignment uncertainties due to length variation (positions 64 through 103, 182 through 220, 410 through 432, 447 through 487, 838 through 848, 997 through 1042, 1130 through 1144, and 1436 through 1465) were omitted from the analysis.

[b] Numbers in subheadings correspond to the species listed in the lefthand column.

[c] Mitochondria from *G. max* and *T. aestivum* were used.

related pairs of the species of the alpha-2 subdivision (20, 78), *B. abortus* possesses an isolated position within this subdivision, but a more precise placement cannot be given at present.

To detect various rates of evolution in these eucaryote-associated eubacteria and to relate the origin of mitochondria, the phylogenetic analysis was carried out by employing the neighborliness method (19, 59, 60). In simulation studies this method has been shown to be one of the best for recovering a given tree topology from distance data under a varied rate of nucleotide substitution (59). A phylogenetic tree has been constructed from equally weighted (Hamming) distances between sequence pairs of *Proteobacteria* (Table 4) by using *A. nidulans*, the plastid of *E. gracilis*, and *H. chlorum* as the outgroup (Fig. 1). The tree shows a clear distinction between the four subclasses of *Proteobacteria*, with the delta subdivision branching off earliest and the beta and gamma subdivisions being more closely related to one another than to the alpha subdivision. As expected, *B. abortus* clearly falls within the alpha subdivision. As far as the limited data base allows conclusions, *B. abortus* is specifically related to members of the alpha-2 subgroup, *R. quintana* and *A. tumefaciens*. Its closest relative is the animal pathogen *R. quintana*, but this picture may change as more organisms are added. Plant mitochondrial sequences, the only mitochondrial sequences without major deletions, clearly show an affiliation with the alpha subdivision. Their origin might not lie within the alpha-2 subdivision, but this problem might also be solved when data from more representatives are made available.

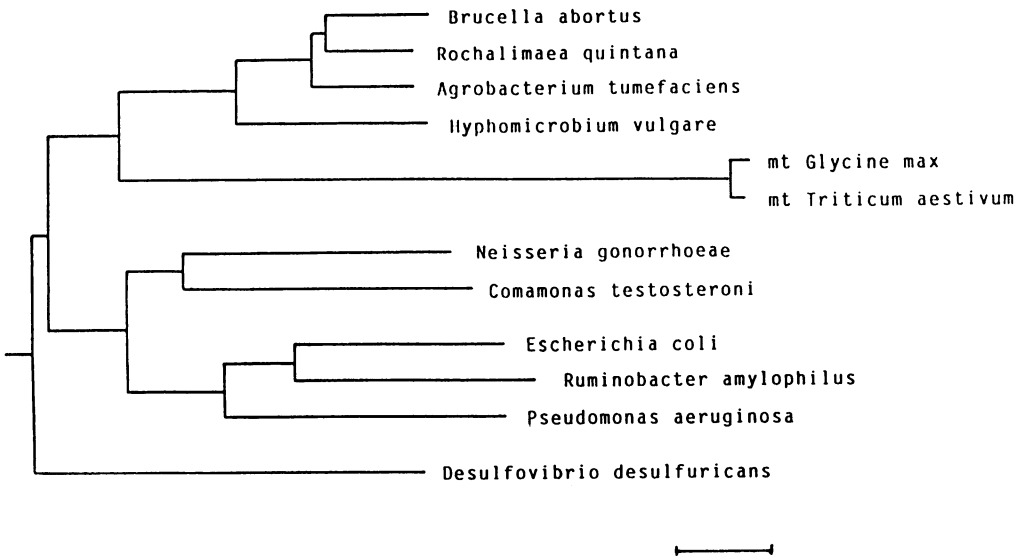

FIG. 1. Phylogenetic tree showing the position of *B. abortus* among the *Proteobacteria*. The tree was constructed by using the neighborliness method (19, 59, 60). The root was defined by using *Anacysis nidulans*, the plastid of *Euglena gracilis*, and *Heliobacterium chlorum* as the outgroup. Bar, 20-nucleotide substitution.

TABLE 5. Membrane lipids of members of the alpha-2 subdivision and other *Proteobacteria*[a]

Species	Lipid A						Heptose	Ubiquinone	OCL	PC	Phospholipid fatty acids[b]		Subdivision	Reference(s)
	Backbone		Fatty acids[c]											
	GlcN	DAG	A 14OH	A 16OH	E 16:0	28OH					$C_{18:1}$	$C_{19:0}$ cyclic		
Rhodospirillum rubrum	?[d]	?	?	?	?	?	+	Q9	−	−	++	−	Alpha-1[e]	9, 21, 24, 29, 52
Rhodomicrobium vanielli	+	−	+	+	+	?	−	Q10	+	+	+++	−	Alpha-2[e]	9, 24, 28, 29, 52
Rhodopseudomonas acidophila	+	−	−	+	−	+	−	Q10	+	+	?	?	Alpha-2[e]	9, 29, 73
Rochalimaea quintana	?	?	+	?	?	?	+	?	?	+[f]	+++	+	Alpha-2[e]	26, 44, 70, 76
Agrobacterium tumefaciens	+	−	+	+	+	+	±[g]	Q10	+	+	+++	+++	Alpha-2[e]	1, 9, 40, 58, 61, 68
Rhizobium leguminosarum	+	−	+	+	+	+	±[g]	Q10	+	+	+++	+++	Alpha-2[e]	9, 5, 57, 61, 73
Rhizobium meliloti	+	−	+	+	+	+	−	Q10	+	+	+++	+++	Alpha-2[h]	9, 21, 57, 68, 71
Brucella abortus	+	+	+	+	+	+	−	Q10	+	+	+++	+++	Alpha-2[e]	9, 42, 61, 66, 73
Brucella melitensis	+	+	+	+	+	+	−	Q10	+	+	+++	+++	Alpha-2[h]	9, 42, 61, 66, 73
Thiobacillus novellus	+	+	+	−	+	+	−	Q10	?	+	+++	+++	Alpha-2[e]	2, 33, 82
Bradyrhizobium japonicum	+	+	+	+	+	+	−	Q10	+	+	+++	+++	Alpha-2[e]	5, 9, 21, 41, 73
Rhodopseudomonas palustris	−	+	+	+	+	+	+	Q10	+	+	+++	−	Alpha-2[e]	9, 29, 42, 73
Rhodopseudomonas viridis	−	+	+	−	−	+	−	Q9	?	?	?	?	Alpha-2[e]	9, 24, 29, 42, 52
Nitrobacter winogradskyi	−	+	+	−	+	+	+	?	?	?	?	?	Alpha-2[e]	42, 73
Phenylobacterium immobile	−	+	−	+	+	?	+	?	?	?	?	?	Alpha-2[e]	42, 73
Achromobacter sp. strain CDC Vd	?	?	?	?	?	?	?	?	+	+	+++	+++	Alpha-2[h]	16, 67
Mitochondria[i]								Q10	−	+[f]	+++	−	Alpha-?[e]	13
Rhodobacter capsulatus	+	−	±[j]	−	−	?	−	Q10	+	+	+++	−	Alpha-3[e]	9, 21, 24, 43, 61
Rhodocyclus gelatinosus	+	−	−	−	−	−	+	Q8	+	−	+	−	Beta-1[e]	21, 29, 52, 73
Escherichia coli	+	−	+	−	−	−	+	Q8	−	−	−	−	Gamma-3[e]	9, 21, 25, 73
Desulfovibrio desulfuricans	?	?	?	?	?	?	?	MK6	+	−	++	−	Delta[e]	9, 38, 39

[a] Representative members of the alpha-1, alpha-3, beta, gamma and delta subdivisions are included for comparative purposes. GlcN, Glucosamine; DAG, 2,3-diaminoglucose; A, amide linked; E, ester linked; 14OH, hydroxymyristic; 16OH hydroxypalmitic; 16:0 palmitic; 28OH, 27-hydroxy-octacosanoic; Heptose, L-glycero or D-glyceromanoheptose; OCL, ornithine-containing lipid; $C_{18:1}$, *cis*-vaccenic acid; $C_{19:0}$ cyclic, lactobacillic acid.

[b] For additional phospholipid fatty acids see references 2, 5, 13, 16, 21, 29, 33, 38, 39, 40, 52, 61, 66, 67, and 76. $C_{18:1}$ is precursor of cyclic $C_{19:0}$ (25).

[c] For additional fatty acids linked to lipid A, see references 1, 22, 27, 28, 41, 42, 43, 54, 58, 71, and 76. Data on the presence of 27-OH-$C_{28:0}$ were obtained by R. U. Bhat, M. Busch, and H. Mayer (unpublished data) and from reference 71.

[d] ?, Not known; +, present; −, not detected or in trace amounts.

[e] Assigned by 16S rRNA sequencing or catalogs (12, 18, 20, 74, 81; Oyaizu et al., in press).

[f] Phosphatydilcholine is synthesized by the host cell (13, 76).

[g] Several investigators have not detected heptose in the LPS of these bacteria (1, 54, 71).

[h] Hybridization values of 16S rRNA-23S rRNA and DNA-DNA indicates very close phylogenetical relationship with *B. abortus* and other members of the alpha-2 subdivision (14).

[i] Lipopolysaccharide is absent in mitochondria. In mitochondria, $C_{18:1}$ is oleic acid (13).

[j] Mainly found as 3-oxo-$C_{14:0}$ (43).

DISCUSSION

For many years the taxonomic and phylogenetic position of the genus *Brucella* has been controversial (10). According to the results presented here, *Brucella* species are closer to eucaryotic cell-associated *R. quintana*, *A. tumefaciens*, and *Rhizobium leguminosarum* than to phototrophic and other free-living chemoorganotrophic bacteria of the alpha-2 subdivision. The 16S rRNA sequence of *B. melitensis* 16M is not known, although the DNA-DNA and DNA-rRNA hybridization values (14, 72) and the lipid composition (Table 5) indicate that this bacterium and other *Brucella* species belong to the alpha-2 subdivision.

The presence of either glucosamine or the unusual diaminoglucose in the lipid A is shared by several bacteria of the alpha-2 subdivision; however, so far, *Bradyrhizobium japonicum*, *B. lupini*, and *Thiobacillus novellus* are the only members of the alpha-2 subdivision (41, 82; H. Oyaizu, Y. Oyaizu-Masuchi, A. Yakota, and S. Takakuwa, *Abstract of the International Symposium on Bacterial Ecology*, in press) that, like *Brucella* species, may possess a mixed lipid A (Table 5). It is not known whether the diaminoglucose and the glucosamine are covalently linked to form a mixed disaccharide or whether they represent two different entities of the lipid A backbones. According to recent data (73), lipid A synthase accepts the 2,3-diamino structural analog of lipid X as a substrate, making formation of mixed lipid A backbones feasible.

An unusual long-chain 27-OH-$C_{28:0}$ was recently found in the lipid A of *Rhizobium* species (71). Later it was found to be widely distributed among the LPSs of species belonging to the alpha-2 subdivision of *Proteobacteria* (R. U. Bhat, M. Busch, and H. Mayer, unpublished data). The fact that also *Brucella* species contain significant amounts of 27-OH-$C_{28:0}$ is in perfect agreement with the phylogenetic position as revealed by 16S rRNA.

Weisburg et al. (74) were the first to notice that members of *Rickettsiaseae* have common origin with those of *Rhizobiaceae*. Interestingly, the alpha-2 subdivision includes gram-negative bacteria such as *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, *Rochalimaea*, and *Brucella* species (18, 74), which live in facultative or obligate association with eucaryotic cells (10, 11, 31, 32, 44, 54, 75). In addition to several membrane characteristics shared by most members of the alpha-2 subdivision (Table 5), there are additional features that relate brucellae to *Rhizobiaceae* and *Rickettsiae*. Similarly to *Rhizobiaceae*, brucellae contain in their lipid A amide-linked but not ester-linked acyl-oxyacyl residues (80), possess a cytoplasmic cycloglucan (4, 15). The major hydroxy fatty acids present in the lipid A moieties are 27-OH-$C_{28:0}$, 3-OH-$C_{18:0}$, 3-OH-$C_{16:0}$, and 3-OH-$C_{14:0}$ (1, 54,

58, 80), and the guanine-plus-cytosine contents range between 58 and 61 mol% with a genome size ranging between 2.4×10^9 and 2.6×10^9 daltons (10, 14, 31, 72). Under certain circumstances agrobacterium specimens might have the potential to infect mammals (55). Other members of *Rhizobiaceae*, such as *Phyllobacterium* species, which induce leaf nodules in tropical plants (35), and the closely related *Mycoplana* species, have been shown to be taxonomically related to *Brucella* species (14). Similarly to some rickettsiae (75), *Brucella* species share the property of living and reproducing inside eucaryotic cells (32), as well as having the potential of being transovarically transmitted by vector ticks (6, 11). To these properties we have to add the absence of plasmids and flagella in the animal parasitic rickettsiae and brucellae (10, 75), in contrast to the large plasmids and motility of the plant-associated rhizobiae and agrobacteria (31). The preferential habitat of *Achromobacter* sp. strain CDC Vd, which seems to be closely related to *Brucella* species (14), is not known; however, most of the isolates have been obtained from human clinical cases (8).

Sequencing of ribosomal nucleic acids has greatly helped to construct phylogenetical trees of eubacteria and to follow the radiation of the groups during evolution (77, 78). However, without other molecular and phenotypic markers, functional parameters, and preferential habitats, the understanding of the mechanisms of dispersion is limited. The conserved lipid composition and structure of the membranes of the obligate and facultative pericellular or intracellular *Proteobacteria* of the alpha-2 subdivision might contribute in the understanding of the tendency of these organisms to associate with eucaryotic cells and to trace some of the characteristics of the common ancestor of this group. For instance, taking into account the characteristics of the subdivision, it might be suggested that the ancestor of brucellae, rickettsiae, agrobacteria, and rhizobiae was a gram-negative, chemoorganothrophic, motile, aerobic organism (10, 31, 75) containing in the inner membrane ubiquinone Q10 as a respiratory coenzyme, a high concentration of medium-sized cytochrome *c* as one of the components of the respiratory chain of aerobically grown cells (17, 65), and a cytoplasmic cycloglucan (4, 15). The outer and inner membranes, in addition to other phospholipids, would have had relatively large quantities of phosphatidylcholine, substituted by $C_{18:1}$ and cyclic $C_{19:0}$ fatty acids. In addition, the outer membrane would have ornithine-containing lipids and mixed lipid A substituted with amide-linked 3-OH-$C_{14:0}$ fatty acid and the long-chain 27-OH-$C_{28:0}$ fatty acid (71). This ancestor could be initially associated pericellularly with plant cells (31, 74) and eventually with arthropods (6, 11, 44), which then could transmit the organism to vertebrate hosts (6, 11). In this respect the capability of brucellae, rickettsiae, agrobacteria, and rhizobia of evading cytoplasmic digestion by the eucaryotic cell may have been an important property shared by these linage of the *Proteobacteria*.

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