

Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp.

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The relatedness of *Brucella* spp. and *Ochrobactrum anthropi* was studied by protein profiling, Western blot, immunoelectrophoresis and 16S rRNA analysis. Whole-cell and soluble proteins of brucellae and *O. anthropi* showed serological cross-reactivities quantitatively and qualitatively more intense than those existing with similar extracts of *Agrobacterium* spp. Numerical analysis of Western blot profiles of whole-cell extracts showed that *O. anthropi* LMG 3301 was closer to *Brucella* spp. than to *O. anthropi* LMG 3331^T, a result not obtained by protein profiling. These differences were not observed by Western blot with soluble fractions, and immunoelectrophoretic analyses suggested that this was due to destruction of conformational epitopes in Western blot procedures with the subsequent simplification of antigenic profile. Analysis of the 16S rRNA sequences of strains previously used in the species definition confirmed that strain LMG 3301, and also LMG 3306, were closer to the brucellae, and that LMG 3331^T was in a separate cluster. The LMG 3301 and the LMG 3331^T clusters could also be separated by their different colistin sensitivity and by PCR with 16S rRNA *Brucella* primers, and both methods showed strains of both clusters among clinical isolates classified as *O. anthropi* by conventional tests. These results and those of previous DNA–DNA hybridization studies [Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988). *Int J Syst Bacteriol* 38, 406–416] show that the LMG 3301 cluster and related clinical isolates should be given a new species status for which the name *Ochrobactrum intermedium* sp. nov. is proposed (type strain is LMG 3301^T = NCTC 12171^T = CNS 2-75^T).

Keywords: *Brucella*, *Ochrobactrum*, antigenic relatedness, 16S rRNA

INTRODUCTION

The α -2 subclass of the class *Proteobacteria* includes plant and animal pathogens that are characteristically associated pericellularly or intracellularly with eukaryotic cells (in the case of *Brucella*, *Bartonella* and *Agrobacterium* spp., and the rickettsiae), plant endosymbionts (the rhizobia) and other bacteria, mostly

soil inhabitants (Moreno, 1992; Moreno *et al.*, 1990; Yanagi & Yamasoto, 1993).

Early antigenic studies have shown that the brucellae are a very homogeneous antigenic group (Díaz *et al.*, 1966, 1968) and, consistent with this, DNA–DNA hybridization studies have shown that the classical nominal species (*Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella ovis*, *Brucella canis* and *Brucella neotomae*) can be grouped into a single genomic species (*B. melitensis*) (Verger *et al.*, 1985). Several groups of data (reviewed by Moreno, 1992) show that

Abbreviation: IEP, immunoelectrophoresis.

The GenBank accession number for the sequence of LMG 3301 is U70978.

Ochrobactrum anthropi is the closest known relative of brucellae. Moreover, although a likely member of the microbiota of soil (Aguillera *et al.*, 1993; Holmes *et al.*, 1988), an increasing number of works report the isolation of *O. anthropi* from immunocompromised patients (Bizet & Bizet, 1995; Brivet *et al.*, 1993; Chang *et al.*, 1996; Cieslak *et al.*, 1992; Ezzedine *et al.*, 1994; Grandsden & Eykyn, 1992; Haditsch *et al.*, 1994; Holmes *et al.*, 1988) and preliminary data suggests that some strains can multiply within cultured epithelial cells (E. Moreno, J. Velasco & I. Moriyón, unpublished results). Therefore, *O. anthropi* and *Brucella* spp. show a different degree of interaction with eukaryotic cells and provide a model for comparative studies which could shed light on the adaptations to intracellular life in the α -2 subclass. However, as presently defined, *O. anthropi* is heterogeneous: on the basis of a classical phenotypical characterization, three different biotypes (A, C and D corresponding to the former *Achromobacter* biotypes A, C and D of CDC Vd) have been proposed (Holmes *et al.*, 1988), and DNA-DNA hybridization studies show the existence of two groups of strains (Holmes *et al.*, 1988) (thereafter referred to as hybridization groups 1 and 2) which would grant the definition of a new genomic species. In the present study, we have assessed the relatedness of *Brucella* and *O. anthropi* by whole-cell protein profiling, immunological methods and 16S rRNA sequence analysis. This polyphasic approach (Vandamme *et al.*, 1996) was chosen because, in addition to its taxonomic value, it could lead to identification of antigenically relevant proteins useful in further clinical (Velasco *et al.*, 1997) and comparative molecular work. The results show a heterogeneity for *O. anthropi* that further supports the definition of a new species for hybridization group 2, and demonstrate that the brucellae are closer to this group than to hybridization group 1 which includes the *O. anthropi* type strain.

METHODS

Bacterial strains and growth conditions. The bacterial strains used are presented in Table 1. For cell fractionation, *B. melitensis* 115, *B. abortus* RB51, *O. anthropi* LMG 3301 and 3331^T, and *Escherichia coli* were grown in a peptone-glucose broth supplemented with 0.01% antifoam A/butyl acetate (1:3) (Sigma) in a fermenter (Aragón *et al.*, 1996). *B. ovis* REO 198 and *B. suis* 2 were grown in tryptic soy broth (Difco) with or without 2.5% yeast extract, respectively, in 2 l flasks on an orbital shaker. The agrobacteria were grown in 1.3% tryptone (Merck), 1.0% yeast extract (Merck), 0.1% (NH₄)₂SO₄, 0.03% NaH₂PO₄ and 1.6% glucose in 2 l flasks on an orbital shaker at 26 °C. In each case, cells were collected (7000 g, 15 min) and washed twice with 0.8% NaCl [virulent *Brucella* strains were inactivated with phenol (0.5%, 37 °C, 48 h) before harvesting] for cell fractionation. For immunizations, bacteria were grown in Roux flasks containing the above-described media plus 1.5% agar, collected, washed twice with sterile saline (7000 g, 15 min), and then acetone-dried (Díaz *et al.*, 1966). For protein analysis by SDS-PAGE bacteria were grown on the same solid media in Petri dishes. For DNA extraction, the *O.*

anthropi strains were grown in tryptic soy agar (Difco) for 24 h at 37 °C.

Bacterial fractions

(i) Whole-cell protein extracts. Cells were resuspended in 1% SDS, 0.7 M 2-mercaptoethanol, 10% glycerol, 10 mM Tris/HCl (pH 6.8), extracted at 100 °C for 10 min and cell debris removed by centrifugation (12000 g, 10 min). Duplicate extracts of two independently grown batches of cells were prepared to check the reproducibility of the results.

(ii) Soluble fractions. Cells were disintegrated in the presence of nucleases in a 40K French Pressure Cell Press (SLM Instruments), and the soluble fraction was obtained by ultracentrifugation (supernatant) and characterized on the basis of the distribution of cytoplasmic and cell envelope markers as described previously (Moriyón & Berman, 1982). For SDS-PAGE and Western blot (see below), the soluble fractions were resuspended in 1% SDS, 0.7 M 2-mercaptoethanol, 10% glycerol, 10 mM Tris/HCl (pH 6.8). Duplicates of the fractions were prepared to assess reproducibility.

(iii) Genomic bacterial DNA. DNA was obtained as described previously with minor modifications (Romero *et al.*, 1995). Briefly, cells were suspended in 50 mM NaCl, 125 mM EDTA, 50 mM Tris/HCl (pH 7.6), heat-killed, and then disrupted by incubation with SDS and proteinase K. After precipitation of unwanted materials with 5 M NaCl and hexadecyltrimethylammonium bromide (CTAB)/NaCl, the DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated with 2-propanol, washed with 70% ethanol and dissolved in sterile distilled water.

SDS-PAGE and numerical analysis. Whole-cell extracts and soluble fractions were analysed by SDS-PAGE (Pot *et al.*, 1994), and gels were stained with Coomassie Blue and scanned using a ScanJet IICx scanner (Hewlett Packard). The normalized densitometric traces of the electrophoretic patterns were grouped by performing a numerical analysis using the GelCompar 3.1 software package (Vauterin & Vauterin, 1992). The level of similarity between each pair of traces was expressed as the Pearson product-moment correlation coefficient (*r*), which for convenience was converted to a percentage (Pot *et al.*, 1994). The levels of similarity between duplicate protein patterns were at least 90%.

Serological analyses

(i) Immunoelectrophoresis (IEP). IEP was carried out in 1.8% Indubiose (IBF-Biotechnics, Villeneuve la Garenne, France), 0.05% NaN₃, 0.04 M sodium diethylbarbiturate/HCl (pH 8.6) at 6 V cm⁻¹ for 2 h (Díaz *et al.*, 1966). After diffusion of the sera and immunoprecipitation (18 h at 25 °C), the plates were soaked for 24 h in saline, washed with distilled water for 24 h, air-dried and then stained with 1% Coomassie R-Blue in water/ethanol/acetic acid (9:2:9, by vol.).

(ii) Western blot. After SDS-PAGE, proteins were electroblotted on Immobilon P (Millipore) using a Trans-Blot SD apparatus (Bio-Rad) according to the instructions of the manufacturers. After overnight incubation in 0.15% Tween 20/10 mM PBS (pH 7.2) (PBS-Tween) supplemented with 3% skimmed milk, the blots were washed four times with PBS-Tween and incubated at room temperature with the appropriate serum diluted in PBS-Tween. After a new series of PBS-Tween washings, IgG was detected with peroxidase-conjugated Protein G (Pierce) (2 µg ml⁻¹ in PBS-Tween) and

Table 1. Bacterial strains

Strain*	Relevant details	Reference
<i>A. radiobacter</i> ICMP 10854	Isolated from osier	This work
<i>A. rhizogenes</i> IVIA 020-AP	Isolated from peachtree	This work
<i>A. vitis</i> IVIA 565-5	Isolated from vine	This work
<i>B. abortus</i> US19	Attenuated vaccine strain	Alton <i>et al.</i> (1988)
<i>B. abortus</i> 2308	Virulent challenge strain, biotype 1, serotype A	García-Carillo & Casa-Olascoaga (1977)
<i>B. abortus</i> RB51	Rough mutant of <i>B. abortus</i> 2308	Schurig <i>et al.</i> (1991)
<i>B. abortus</i> 45/20	Rough mutant of <i>B. abortus</i> 45/0	McEwen (1940)
<i>B. melitensis</i> 115	Rough mutant directly isolated from goat	Alton <i>et al.</i> (1988)
<i>B. ovis</i> Reo 198	Naturally rough <i>Brucella</i> species, CO ₂ -independent strain	Alton <i>et al.</i> (1988)
<i>B. suis</i> 2	Attenuated vaccine strain, biovar 1	Xin (1986)
<i>B. suis</i> 1330	Virulent, reference strain of biotype 1, serotype A	Alton <i>et al.</i> (1988)
<i>E. coli</i> NCTC 8007	O111 K58H2, enteropathogenic	Griffiths & Humphreys (1978)
<i>O. anthropi</i> LMG 3331 ^T , CIP 149-70	Type strain, DNA-DNA hybridization group 1, unknown origin	Holmes <i>et al.</i> (1988)
<i>O. anthropi</i> LMG 3310, CNS 4-78	DNA-DNA hybridization group 1, isolated from faeces	Holmes <i>et al.</i> (1988)
<i>O. anthropi</i> LMG 3329, CIP 81-74	DNA-DNA hybridization group 1, isolated from abscess	Holmes <i>et al.</i> (1988)
<i>O. anthropi</i> LMG 2320, NCIB 8688	DNA-DNA hybridization group 1, isolated from arsenic-containing cattle-dipping fluid	Holmes <i>et al.</i> (1988)
<i>O. anthropi</i> LMG 3309, CNS 7-77	DNA-DNA hybridization group 1, isolated from human ear discharge	Holmes <i>et al.</i> (1988)
<i>O. anthropi</i> LMG 3301, CNS 2-75	DNA-DNA hybridization group 2, isolated from human blood	Holmes <i>et al.</i> (1988)
<i>O. anthropi</i> LMG 3306, CNS 23-76	DNA-DNA hybridization group 2, isolated from soil	Holmes <i>et al.</i> (1988)
<i>O. anthropi</i> CUN 559	Clinical isolate from human blood	This work
<i>O. anthropi</i> CUN 2944	Clinical isolate from human blood	This work
<i>O. anthropi</i> CUN 6130	Clinical isolate from human blood	This work
<i>O. anthropi</i> CUN 9446	Clinical isolate from human blood	This work
<i>O. anthropi</i> CUN 11040	Clinical isolate from human blood	This work

* CIP, Collection de l'Institut Pasteur, Paris, France; CNS, Centre National des Salmonella, Paris, France; CUN, Clínica Universitaria, Universidad de Navarra, Pamplona, Spain; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; IVIA, Instituto Valenciano de Investigación Agrarias, Valencia, Spain; LMG, Culture Collection of the Laboratory for Microbiology, Ghent, Belgium; NCIB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, UK; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK.

blots developed with 4-chloro-1-naphthol/H₂O₂ (Hawkes *et al.*, 1982). The normalized densitometric traces of the blots were obtained and analysed as described above for the SDS-PAGE protein patterns. For a given extract or fraction, the sera used in the analysis were those showing the maximal reactivity with the homologous antigen.

(iii) Immune sera. White New Zealand rabbits were hyper-immunized with acetone-dried cells of *B. abortus* RB51, *B. melitensis* 115, *B. suis* 1330, *B. ovis* REO 198, *A. radiobacter* ICPB 10854, *A. rhizogenes* IVIA 020-AP, *A. vitis* IVIA 565-5, *O. anthropi* LMG 3331^T and 3301, and *E. coli* O111 as described previously (Díaz *et al.*, 1966). Controls performed with sera taken before immunizations showed no reactivity in any of the immunological tests.

16S rRNA analysis

(i) PCR with *Brucella* primers. The oligonucleotide primers, selected from the 16S rRNA sequence of *B. abortus* previously published (Dorsch *et al.*, 1989), were F4 (5'-TCGAGCGCCCGCAAGGG-3') and R2 (5'-AACCATA-GTGTCTCCACTAA-3'), and the PCR assay was performed as described in previous works (Romero *et al.*, 1995). Positive controls contained 80 ng *B. abortus* 2308 DNA as template, and negative controls consisted of sterile water instead of DNA template. Generally recommended procedures were used to avoid contamination (Rolfs *et al.*, 1992), and each sample was tested at least in triplicate.

(ii) PCR amplification, cloning and sequencing of the 16S rDNA genes. Approximately 0.1 µg bacterial DNA was used as a PCR

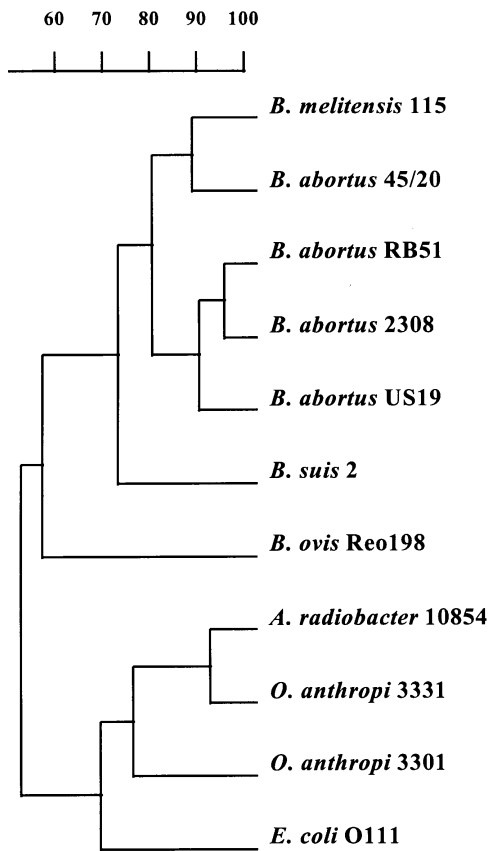


Fig. 1. Dendrogram derived from unweighted pair group average linkage of correlation coefficients for the protein patterns of the indicated strains.

template with primers fD1 and rD1 to amplify the entire 16S rRNA gene (Weisburg *et al.*, 1991). Amplifications were performed in a 100 µl reaction mixture containing each of the dNTPs at a concentration of 200 µM, each of the primers at a concentration of 0.5 µM, and 5 U *Taq* DNA polymerase (Promega). The Mg²⁺ concentration was optimized at 1.5 mM MgCl₂. Other PCR conditions were: 35 cycles consisting of 1 min at 95 °C, 2 min at 60 °C, and 4 min at 72 °C, plus an additional cycle with a 10 min chain-elongation step. Amplifications were performed with a GeneAmp Amplification System 2400 kit (Perkin-Elmer).

Amplified DNA was analysed by electrophoresis on a 0.8% agarose gel (100 V for 30 min) and visualized by ethidium bromide staining. The amplified DNA was re-electrophoresed on a 0.7% low-melting-point agarose gel (Hispanlab) and purified with Wizard PCR Preps DNA Purification System (Promega). This DNA was then cloned in the pMOSBlue T-vector (Amersham) following the manufacturer's instructions. The resulting recombinant plasmids were transformed and amplified in competent *E. coli* MOSBlue, subsequently purified with the Wizard Minipreps DNA Purification System (Promega) and sequenced by using a Thermo Sequenase fluorescent cycle sequencing kit (Amersham). The sequencing primers were the following forward and reverse internal primers (Edwards *et al.*, 1989): pC (positions 342–361; *E. coli* 16S rRNA sequence numbering), pE (positions 907–926), pD (positions

536–519), and pF (positions 1030–1054). In addition, M13 forward and reverse primers were used to determine the sequences of both ends of the cloned 16S rDNA gene. The sequencing reactions were analysed with an ALF DNA sequencer (Pharmacia).

(iii) Phylogenetic analysis of 16S rRNA sequences. The 16S rRNA sequences of appropriate members of the α-2 subclass of the *Proteobacteria* [obtained from the GenBank (see below)] and those obtained for *O. anthropi* LMG strains 2320, 3301, 3306, 3309, 3310, 3329 and 3331^T were aligned by using the GENEWORK program (version 2.45n). The PHYLIP package (Felsenstein, 1993) was used for the evolutionary tree inference. Positions of sequence and alignment uncertainty were omitted from the analysis, and the pairwise evolutionary distances for 1250 nucleotides were computed by using the Jukes–Cantor correction calculated with the DNADIST program. Phylogenetic relationships were inferred by using the neighbour-joining (NEIGHBOR), Fitch–Margoliash (FITCH), maximum-parsimony (DNAPARS) and maximum-likelihood (DNAML) algorithms. The resulting unrooted tree topologies were evaluated by bootstrapping of the neighbour-joining method data based on 500 resamplings and using the following programs: SEQBOOT, DNADIST, NEIGHBOR-JOINING and CONSENSE.

Nucleotide sequence accession numbers. The accession numbers for the nucleotide sequences (taken from GenBank) are: D12794 for *O. anthropi* LMG 3331^T; U70978 for *O. anthropi* LMG 3301; U88440 for *O. anthropi* LMG 3306; U88441 for *O. anthropi* LMG 3309; U88442 for *O. anthropi* LMG 3310; U88443 for *O. anthropi* LMG 3329; and U88444 for *O. anthropi* LMG 2320; D12788 for *Agrobacterium rhizogenes* IAM 13570^T; D12784 for *Agrobacterium tumefaciens* IAM 13129^T; D12795 for *Agrobacterium vitis* IAM 14140; X13695 for *Brucella abortus* NCBI 39330; L37584 for *Brucella canis* ATCC 23365^T; L26166 for *Brucella melitensis* ATCC^T 23456; L26169 for *Brucella neotomae* ATCC 23459^T; L26168 for *Brucella ovis* ATCC 25840^T; L26169 for *Brucella suis* ATCC 23444^T; D12786 for *Mycoplana dimorpha* IAM 13154; D12789 for *Phyllobacterium myrsinacearum* IAM 13584; D12790 for *Phyllobacterium rubiacearum* IAM 13587; Z70003 for *Bartonella bacilliformis* LA6.3; M73229 for *Bartonella (Rochalimaea) henselae* ATCC 49882^T; M73228 for *Bartonella (Rochalimaea) quintana* ATCC VR-358^T; and M73230 for *Bartonella (Rochalimaea) vinsonii* ATCC VR-152^T.

Conventional phenotypical characterization. The API 20 NE (bioMérieux), GNI (bioMérieux Vitek) and BBL Crystal E/NF (BBL Microbiology Systems) systems were used to investigate the response of the *O. anthropi* strains in conventional phenotypical tests. The presence and position of flagella was determined by electron microscopy. Susceptibility to amoxicillin, ampicillin/clavulanic acid, aztreonam, ceftazidime, ceftriaxone, cephalotin, chloramphenicol, piperacillin, gentamicin, ofloxacin and trimethoprim/sulfamethoxazole was assessed on Mueller–Hinton agar using the standardized disk diffusion test (Bauer *et al.*, 1966) with antibiotic disks of commercial origin (Difco). Interpretative criteria for zone diameters for the above antimicrobial agents were those recommended by the NCCLS (National Committee for Clinical Laboratory Standards, 1997). Susceptibility to polymyxin B and colistin was also tested by the disk diffusion method with either commercial disks (10 µg per disk; Difco) or sterile disks (Difco) loaded with 10–50 µg polymyxin B (Sigma; 7000 U mg⁻¹). Since there is no correlation between sensitivity and

size diameter for these antibiotics (Bauer *et al.*, 1966), the results were interpreted as sensitivity or resistance on the basis of the presence or complete absence of an inhibition halo.

RESULTS

SDS-PAGE of whole-cell protein extracts

The dendrogram obtained after numerical analysis of the whole-cell protein patterns of the bacteria studied (Fig. 1) shows that two separate clusters were differentiated. One cluster comprised all *Brucella* strains, with *B. abortus* in a position closer to *B. melitensis* than to *B. suis* and *B. ovis*. A second cluster grouped the two *O. anthropi* strains representative of hybridization groups 1 (LMG 3331^T) and 2 (LMG 3301) with *A. radiobacter* ICPB 10854. According to this analysis, *O. anthropi* LMG 3331^T was closer to *A. radiobacter* 10854 than to *O. anthropi* LMG 3301.

Serological cross-reactivity

(i) **Whole-cell proteins.** Representative results of the Western-blot analyses of whole-cell protein extracts are presented in Fig. 2. As expected, a strong reactivity was observed with the sera and the homologous antigens (Fig. 2a, lane 6, and Fig. 2b, lanes 1–4). Although a marked heterologous cross-reactivity was observed with the serum to *O. anthropi* LMG 3301 and *Brucella* whole-cell extracts (Fig. 2a, lanes 1–4), this serum showed only a weak reactivity with the extracts of *O. anthropi* LMG 3331^T (Fig. 2a, lane 5) and *A. radiobacter* ICPB 10854 (Fig. 2a, lane 7) of intensity comparable to that obtained with *E. coli* extracts (Fig. 2a, lane 8). Moreover, numerical analysis of the banding patterns grouped strain LMG 3301 with the brucellae, and strain LMG 3331^T with *A. radiobacter* (Fig. 2a). Western blots with the antiserum to *B. suis* and the corresponding numerical analysis (Fig. 2b) confirmed the above results with respect to the in-

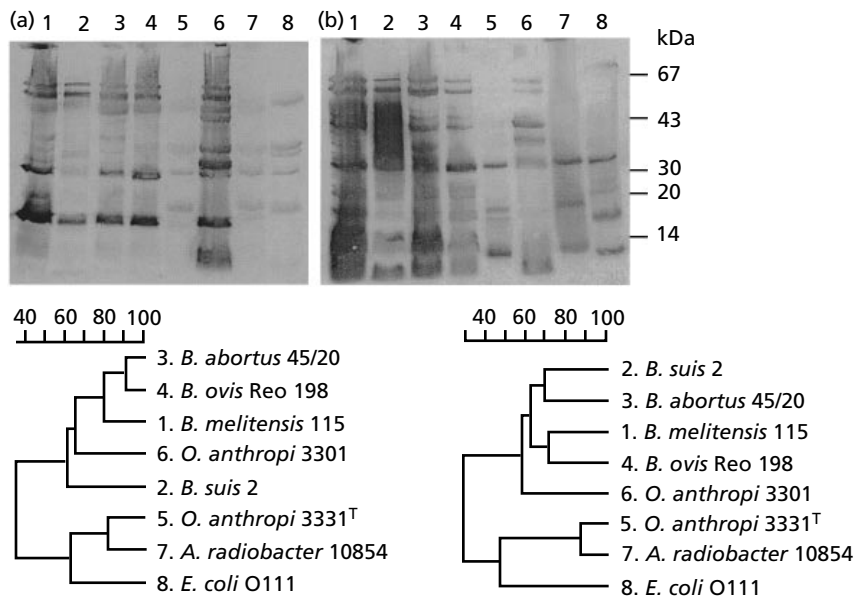


Fig. 2. Western-blot analyses of whole-cell protein antigens detected with sera from rabbits hyperimmunized with *O. anthropi* 3301 (a) and *B. suis* 1330 (b). The dendrograms derived from unweighted pair group average linkage of correlation for the corresponding banding patterns are shown below. Numbers given in the dendrogram for the bacteria analysed correspond to those of the Western blot lanes.

Table 2. Number of precipitation lines by IEP with cytosols and hyperimmune sera

Cytosol of:	Sera to:							
	2	3	5	6	7	8	9	10
1 <i>B. ovis</i> Reo 198	> 10	> 10	6	8	1	3	3	0
2 <i>B. abortus</i> RB51	> 10	> 10	6	6	0	2	2	0
3 <i>B. melitensis</i> 115	> 10	> 10	> 10	> 10	2	1	3	0
4 <i>B. suis</i> 1330	> 10	> 10	> 10	> 10	2	2	3	0
5 <i>O. anthropi</i> LMG 3301	6	> 10	> 10	> 10	6	3	5	0
6 <i>O. anthropi</i> LMG 3331 ^T	5	> 10	> 10	> 10	> 10	5	4	0
7 <i>A. radiobacter</i> 10854	0	1	0	0	> 10	4	5	0
8 <i>A. rhizogenes</i> 020-AP	0	1	0	0	6	> 10	5	0
9 <i>A. vitis</i> 565-5	0	2	1	3	> 10	> 10	> 10	0
10 <i>E. coli</i> O111	0	0	0	1	0	0	0	> 10

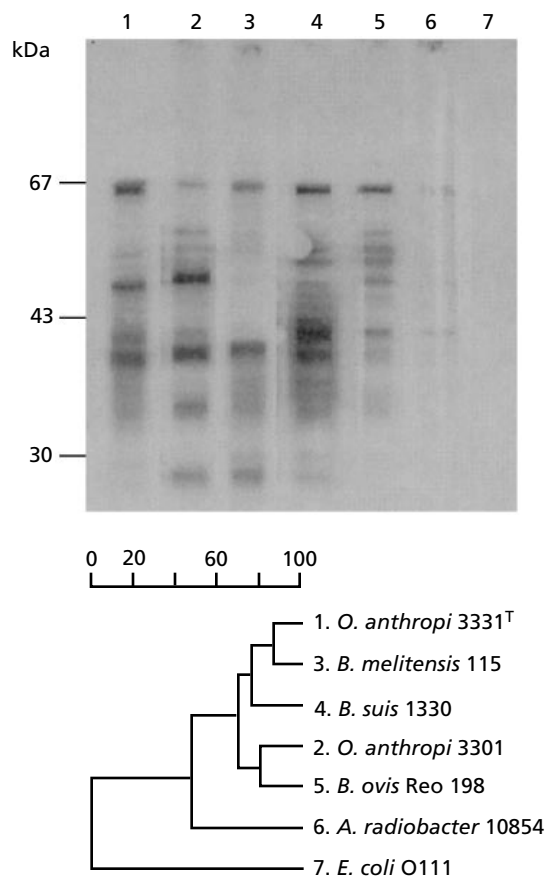


Fig. 3. Western-blot analyses of soluble fraction antigens detected with sera from rabbits hyperimmunized with *O. anthropi* 3301. The dendrogram derived from unweighted pair group average linkage of correlation for the corresponding banding patterns is shown below. Numbers given in the dendrogram for the bacteria analysed correspond to those of the Western blot lanes.

tensity of the cross-reactivities and the similarities and differences in banding patterns.

(ii) Soluble fraction proteins. More than 10 antigenic components were clearly resolved in the homologous native soluble fractions by IEP, with electrophoretic mobilities varying from markedly anodic to slightly cathodic (not shown), and heterologous cross-reactivities of various degrees were also observed. Table 2 presents a quantitative summary of these results, and according to this simple criterion *O. anthropi* LMG 3331^T and LMG 3301 were closer to the brucellae than to the agrobacteria. As reported before (Díaz *et al.*, 1966, 1968), cross-absorptions did not allow a clear identification of *Brucella* species-specific antigens (not shown) and similar cross-absorptions did not allow the identification of strain-specific antigens for *O. anthropi* LMG 3331^T and LMG 3301 (not shown). Consistent with similar absorptions performed with sera from *B. melitensis*-infected goats (Velasco *et al.*, 1997), *Brucella*–*Ochrobactrum* cross-absorption experiments with the soluble fractions

showed genus-specific antigens for both (not shown). Western blots with the same sera and fractions revealed fewer antigenic components than the IEP in both the homologous and heterologous tests (results with the *O. anthropi* LMG 3301 antiserum are presented in Fig. 3), but they showed clear differences between *O. anthropi* LMG 3331^T and LMG 3301 (Fig. 3, lanes 1 and 2), and also differences and similarities with the brucellae (lanes 3, 4 and 5). However, numerical analysis of the banding patterns did not resolve the LMG 3331^T and LMG 3301 strains and the *Brucella* spp. (Fig. 3). By both IEP (Table 2) and Western blot (Fig. 3, lane 6), the cross-reactivity with *A. radiobacter* soluble fraction was weak, and no reactivity was observed with similar *E. coli* fractions (Table 2 and Fig. 3, lane 7).

16S rRNA analysis

The above results showed the heterogeneity of two strains presently included in *O. anthropi* and suggested a closer relationship between strain LMG 3301 and the brucellae closer than that existing with *O. anthropi* LMG 3331^T. To confirm and extend these results, the 16S rRNA genes of several of the strains (LMG 3301, 3306, 3310, 3329 and 2320) used to define *O. anthropi* (Holmes *et al.*, 1988) were amplified, cloned and sequenced. These sequences plus those of *O. anthropi* LMG 3331^T and representative species of the α -2 subclass (all taken from GenBank) were analysed after omitting hypervariable regions and uncertain or ambiguous nucleotide positions of the complete 16S rDNA sequences [1250 nucleotides between *E. coli* positions 29 and 1539 (Brosius *et al.*, 1978)]. The sequence similarity matrix obtained by the Jukes–Cantor correction showed that distances between the *Brucella* spp. and strains LMG 3301 and 3306 (from 98.96 for *B. neotomae* to 99.28 for *B. canis* and *B. suis*) were always below distances found between *Brucella* spp. and the *O. anthropi* LMG 3331^T cluster (LMG 3309, 3310, 3329 and 2320) (from 98.40 for *B. ovis* to 98.64 for *B. canis* and *B. suis*). Moreover, the *Brucella*–*O. anthropi* LMG 3301 and 3306 distance (see above) was shorter than that existing between the *O. anthropi* LMG 3331^T and LMG 3301 clusters (from 98.08 to 98.56). The evolutionary trees obtained with all four algorithms (neighbour-joining, Fitch–Margoliash, maximum-parsimony and maximum-likelihood) were similar. Fig. 4 shows the neighbour-joining tree based on nearly complete 16S rDNA sequences. The confidence limit for each group, as determined by the bootstrap method with 500 resamplings, was more than 50% for most of the nodes, except for the internode connecting the tree with branches of the group containing *M. dimorpha* and the members of the genera *Bartonella* and *Phyllobacterium*, which was 37%. Bootstrap probabilities for the *Brucella*–*Ochrobactrum* groups were more than 95% indicating that the location of the resulting three clusters (Fig. 4) was consistent. The confidence limits of the terminal nodes within each of these groups varied between 27

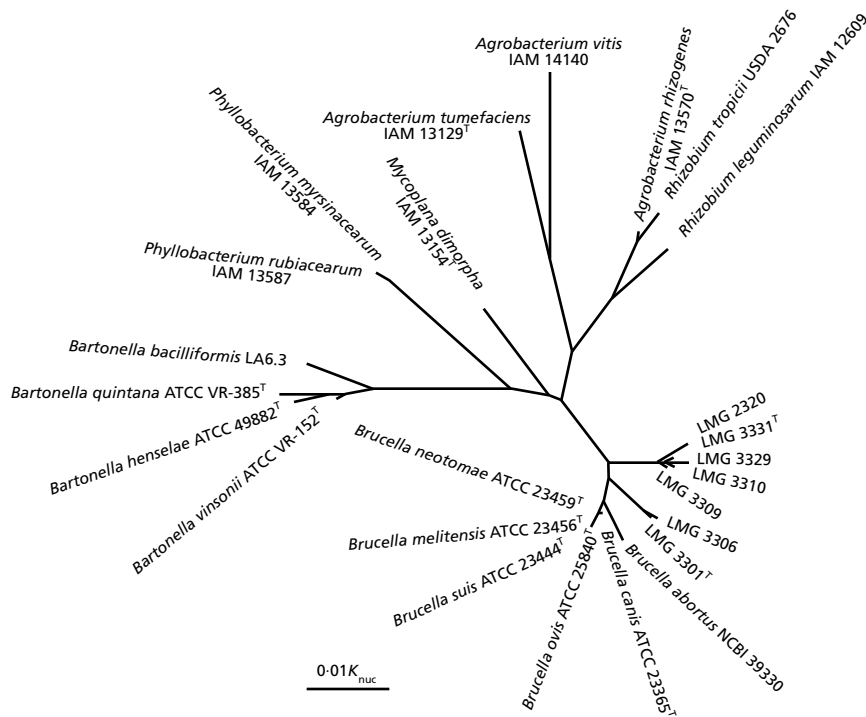


Fig. 4. Unrooted evolutionary tree showing the relationships of *Brucella*, *Ochrobactrum* and several members of the α -2 subclass of the class *Proteobacteria*. The tree was inferred from 1250 nucleotide positions by using the Jukes–Cantor distance and neighbour-joining methods. Bootstrap probabilities for the *Brucella*–*Ochrobactrum* cluster were more than 95%. The scale bar represents 0.01 substitution per nucleotide position.

Table 3. Sequence signatures in the 16S rDNAs of *Brucella* spp. and *O. intermedium* sp. nov.

Species	Nucleotide position*				
	1021	1022	1024	1031	1037
<i>Brucella</i> spp.	A	C	A	A	C
<i>O. intermedium</i> sp. nov.†	A	C	A	C	T

* *E. coli* numbering (Brosius *et al.*, 1978); due to alignment gaps, nucleotides indicated for *E. coli* position 1031 could also correspond to position 1032.

† *O. intermedium* sp. nov. corresponds to the LMG 3301 cluster (see text for details).

and 100% and, because of this, not all the differences among species or strains were included in Fig. 4. Sequence signatures for *Brucella* spp. and for the LMG 3301 cluster are shown in Table 3. No sequence signature existed at the same positions for the *O. anthropi* LMG 3331^T cluster.

To ascertain whether the heterogeneity described above for the collection strains was also reflected in recent clinical isolates classified as *O. anthropi* (Holmes *et al.*, 1988), the DNA of five such isolates was extracted and tested by PCR with primers amplifying a 905 kb fragment common to *Brucella* and *O. anthropi* LMG 3301 but absent from *O. anthropi* LMG 3331^T (Romero *et al.*, 1995). The results showed that three isolates (CUN 559, CUN 2944 and CUN 6130 strains, Table 1) belonged to the LMG 3301 cluster.

Conventional phenotypical characterization

Regardless of the hybridization group (Table 1), serological cross-reactivity and 16S rRNA cluster (see above), all *Ochrobactrum* strains tested had the following positive characteristics: growth at 37 °C and at room temperature (18–22 °C); growth on MacConkey agar; presence of 1–3 polar flagella, urease, catalase and cytochrome C oxidase activities; nitrate reduction; H₂S production; utilization of citrate, glycerol, and maltose; and enzymic hydrolysis of *p*-nitrophenyl α - β -glucoside, *p*-nitrophenyl phosphate, and γ -L-glutamyl *p*-nitroanilide.

All strains gave negative responses in the following tests: indole production; gelatinase, lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase; utilization of arabinose, glucose, lactose, maltose, melibiose, mannitol, raffinose, sorbitol, saccharose, inositol, rhamnose and paracoumaric acid; hydrolysis of proline nitroanilide, *o*-nitrophenyl- β -D-galactoside, *p*-nitrophenyl- β -galactoside, *p*-nitrophenylbisphosphate, *p*-nitrophenylxyloside, *p*-nitrophenyl- β -glucuronide, *p*-nitrophenyl-phosphorylcholine, *p*-nitrophenyl-*N*-acetyl glucosaminide, *p*-nitrophenyl- α -arabinoside, *p*-nitro-DL-phenylalanine; and assimilation of adipate, phenyl-acetate, 2,4,4'-trichloro-2'-hydroxydiphenylether, acetamide and malonate.

Variable responses depending on the particular strain were observed in tetrazolium reduction, aesculin hydrolysis, caproate assimilation, polymyxin B susceptibility, and utilization of mannose, xylose and adonitol.

All strains tested were resistant to amoxicillin,

ampicillin/clavulanic acid, aztreonam, ceftazidime, ceftriaxone, cephalotin, chloramphenicol and piperacillin. All were susceptible to gentamicin, ofloxacin and trimethoprim/sulfamethoxazole. The four strains clustering with *O. anthropi* LMG 3331^T plus strains CUN 9446 and CUN 11040 (which were PCR-negative; see above) were clearly sensitive to colistin and polymyxin B with inhibition haloes greater than 20 mm in diameter. In contrast, strains LMG 3301 and 3306 and the related three clinical isolates (CUN 559, CUN 2944 and CUN 6130) were not inhibited by the same antibiotics at the highest concentration tested.

DISCUSSION

The results of this research showed an intense serological cross-reactivity between *Brucella* spp. and strains included in *O. anthropi*, and cross-reactivity with the agrobacteria was also observed. Moreover, coincidence between the analyses of the Western blots of whole-cell extracts and the 16S rRNA sequences was observed. Those results strengthen the position of the brucellae in a close relationship with soil bacteria and plant symbionts which had been shown by rRNA and lipid A analyses (De Ley *et al.*, 1987; Moreno *et al.*, 1990; Yanagi & Yamasoto 1993), further support the definition of a new *Ochrobactrum* species [which was implicit in previous DNA hybridization studies (Holmes *et al.*, 1988)], and show that the proposed new species is closer to the brucellae than to the *O. anthropi* type strain cluster.

Computer-assisted protein profiling has often been used as a complementary method for DNA and RNA taxonomic analysis (Vandamme *et al.*, 1996). The usefulness of this approach is illustrated by the fact that, although only representative strains were tested, it suggested a differentiation of the classical *Brucella* spp. and a separation of *B. suis* and *B. ovis* from the *B. melitensis*-*B. abortus* subcluster in perfect agreement with restriction patterns (Allardet-Servent *et al.*, 1988; Michaux-Charachon *et al.*, 1997), ribotyping (Grimont *et al.*, 1992), sequence analysis of the *omp2* gene (Ficht *et al.*, 1996) and numerical analysis of fatty acid profiles (Tanaka *et al.*, 1977). A logical step forward is the combination of numerical analysis of protein patterns with the detection of the shared antigens by immunoenzymic methods. In the present study, this method allowed a more refined analysis of whole-cell extracts which yielded results consistent with those of the 16S rRNA and other analyses. Previous studies (Holmes *et al.*, 1988) have shown that *B. abortus* and *O. anthropi* hybridization groups 1 (strain LMG 3331^T) and 2 (strain LMG 3301) show 20–30% and 14–28% DNA homology, respectively, a result that does not allow assessment of the relative distances. However, perusal of the literature reveals that some of the strains used to define *O. anthropi* (Holmes *et al.*, 1988) had also been used in rRNA–DNA hybridization studies (De Ley *et al.*, 1987), and that these studies had already suggested a closer relationship of the strains included in hybridization

group 2 and the brucellae. Obviously, these data are confirmed and extended by the results of the present study.

The results obtained in the numerical analysis of Western blots of whole-cell extracts were not reproduced when the same procedure was applied to the soluble fractions. In particular, the brucellae and the *O. anthropi* strains were not resolved numerically. However, since genus-specific proteins were conspicuous by Western blot, it could be that the profiles were too simple. [It is not illogical that numerical analysis should be more meaningful with more complex extracts (i.e. whole-cell extracts) than with subcellular (i.e. soluble) fractions.] Interestingly, the number of common components detected by IEP with soluble fractions was consistently higher than that obtained by Western blot. Since gel immunoprecipitations and Western blots preferentially detect antibodies to conformational and linear epitopes (Butler, 1991), respectively, the results show that a greater number of epitopes of the former type are shared by soluble proteins of closely related bacteria. This same conclusion is reached when the results obtained with the agrobacteria are compared: whereas cross-reactivity with either *Brucella* or *Ochrobactrum* was negligible in Western blot, IEP detected several conspicuous common components. Thus, it seems likely that numerical analysis of Western blot profiles is most useful when closely related bacteria are studied, whereas methods detecting conformational epitopes show relatedness over a wider taxonomic range.

Finally, the results of this and previous works (De Ley *et al.*, 1987; Holmes *et al.*, 1988; Romero *et al.*, 1995) show that the taxonomic status of bacteria routinely classified as *O. anthropi* on the basis of conventional tests (Bizet & Bizet, 1995; Brivet *et al.*, 1993; Chang *et al.*, 1996; Cieslak *et al.*, 1992; Ezzedine *et al.*, 1994; Grandsden & Eykyn, 1992; Haditsch *et al.*, 1994) should be revised. According to currently accepted criteria (Stackebrandt & Goebel, 1994), the degree of internal DNA–DNA homology of hybridization group 2 (relative binding ratio from 73 to 96%) (Homes *et al.*, 1988), the relatively low cross-hybridization with group 1 (from 41 to 51%), and the closer similarity (16S rRNA, and Western blot of whole-cell proteins) of hybridization group 2 to the *Brucella* spp. all show that the LMG 3301 cluster should be given a separate species status for which the name of *O. intermedium* (of intermediate position between *O. anthropi* and *Brucella* spp.) is proposed (see description below). Furthermore, in the light of the new methods of analysis, the biotypes (A, C and D) proposed for *O. anthropi* (Holmes *et al.*, 1988) have no taxonomic value because biotype A would include this new species and most of the classical strains. Unfortunately, the classical morphological and physiological tests examined in this work and in the work of Holmes *et al.* (1988) do not show a phenotypic pattern useful to discriminate the two *Ochrobactrum* groups. However, the LMG 3301 cluster was readily

discriminated by PCR with the primers described. In addition, although this should be confirmed with a larger number of strains, colistin resistance and sensitivity correctly grouped, respectively, the 12 strains examined as either *O. intermedium* (5 strains) or *O. anthropi*. Colistin (polymyxin E) is a polycationic lipopeptide and it is interesting that a trait of the brucellae is their comparative resistance to polycationic peptides including polymyxins (Freer *et al.*, 1996; Martínez de Tejada *et al.*, 1995). In Gram-negative bacteria, this resistance occurs by reduced or no binding of the peptides to the lipopolysaccharide (the target in susceptible bacteria) caused by peculiarities in the lipid A-core chemical structure (Vaara, 1992). Thus, consistent with the results discussed above, the resistance to colistin is likely to mark a structural difference between *O. anthropi* and *O. intermedium* which would also indicate similarity of the latter to the brucellae.

Description of *Ochrobactrum intermedium* sp. nov.

Ochrobactrum intermedium (in'ter.me'dium. L. neut. adj. *intermedium* of intermediate position).

The description is taken from the results of this work and that of Holmes *et al.* (1988), and is based on the data obtained with five strains (LMG 3301, LMG 3306, CUN 559, CUN 2944 and CUN 6130, Table 1). The cells are rod-shaped, usually occur singly, and are motile by 1–3 polar or subpolar flagella. After growth on nutrient agar for 24 h at 37 °C, colonies are typically circular, low convex, about 1 mm in diameter, smooth, shining and entire. A total of 47 characteristics are common to the five strains tested (either positive or negative). The cells have the following positive characteristics: growth at 37 °C; growth at room temperature (18–22 °C); growth on MacConkey agar; and cytochrome-*c* oxidase activities; nitrate reduction, SH₂ production; utilization of citrate, glycerol and maltose; and enzymic hydrolysis of *p*-nitrophenyl α - β -glucoside, *p*-nitrophenyl phosphate and γ -L-glutamyl *p*-nitroanilide. All the strains gave negative responses in the following tests: indole production; gelatinase, lysine and ornithine decarboxylase, and arginine dihydrolase activities; utilization of arabinose, glucose, lactose, maltose, melibiose, mannitol, raffinose, sorbitol, saccharose, inositol, rhamnose and paracoumaric acid; enzymic hydrolysis of proline nitroanilide, *o*-nitrophenyl-galactoside, *p*-nitrophenyl- β -galactoside, *p*-nitrophenyl-bisphosphate, *p*-nitrophenyl-xyloxyde, *p*-nitrophenyl- β -glucuronide, *p*-nitrophenyl-phosphoryl-choline, *p*-nitrophenyl- β -*N*-acetylglucosaminide, *p*-nitrophenyl- α -arabinoside, *p*-nitro-DL-phenylalanine and esculine; and assimilation of adipate, phenyl-acetate, 2,4,4'-trichloro-2'-hidroxydiphenylether, acetamide and malonate. In contrast to seven *O. anthropi* strains tested, five strains of the proposed new species were resistant to colistin. Moreover, PCR test with primers F4 (5'-TCGAGCGCCC-GCAAGGG-3') and R2 (5'-AACCATAGTGCTC-CACTAA-3') are positive with *O. intermedium* and

negative with *O. anthropi* (Romero *et al.*, 1995). The G+C content of the DNAs of strains LMG 3301 and 3306 are between 57.7 and 58.3 mol%. The type strain is LMG 3301^T (= NCTC 12171^T = CNS 2-75^T).

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