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Molecular and phenotypic features for identification of the opportunistic pathogens *Ochrobactrum* spp.

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Among the six species characterized within the genus *Ochrobactrum*, *Ochrobactrum anthropi* and *Ochrobactrum intermedium* are currently reported as opportunistic pathogens in humans. Since the species identification is mainly based on 16S rDNA analysis, the aim of this study was to search for other characteristics useful for *Ochrobactrum* species discrimination. Ribotyping, morphological and biochemical analyses, and antimicrobial susceptibility testing were performed for a panel of 35 clinical isolates, first identified to the species level using 16S rDNA sequencing. Type and reference strains of five *Ochrobactrum* species were comparatively analysed. Commercial identification systems such as API 20NE and VITEK 2 were tested for their ability to identify *Ochrobactrum anthropi* and to detect other members of the genus *Ochrobactrum*. An improved protocol for the identification of *Ochrobactrum* spp. by routine medical microbiology practices is proposed: isolation of a non-fastidious non-fermenting oxidase-positive Gram-negative rod resistant to all β-lactams except imipenem indicates the genus *Ochrobactrum*, and the API 20NE system confirms the genus identification for most strains, whereas the VITEK 2 system using ID-GNB cards was less powerful. Urease activity, the mucoidy of the colonies, growth at 45 °C on tryptic soy agar, and susceptibility to colistin, tobramycin and netilmicin should be considered as differential characteristics for identification of *O. anthropi* and *O. intermedium* to the species level. However, definitive identification depends on genotyping methods.

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INTRODUCTION

The genus *Ochrobactrum* and its type species *Ochrobactrum anthropi* were created in 1988 for the organisms formerly known as CDC group Vd (Holmes *et al.*, 1988). Since this description, five other species, *Ochrobactrum intermedium* (Velasco *et al.*, 1998), *Ochrobactrum tritici*, *Ochrobactrum grignonense* (Lebuhn *et al.*, 2000), *Ochrobactrum gallinifaecis* (Kämpfer *et al.*, 2003) and '*Ochrobactrum lupini*' (Trujillo *et al.*, 2005) have been characterized, mainly on the basis of 16S rDNA sequencing. They are non-fermentative, strictly aerobic, motile, oxidase-positive and indole-negative, Gram-negative rods. On the basis of phenotypic characteristics, the genus *Ochrobactrum* could be related to the genera *Alcaligenes*, *Achromobacter*, or to the members of *Pseudomonadaceae*. However, molecular taxonomy places *Ochrobactrum* in the α-subgroup of proteobacteria, closely related to the genus *Brucella* (Lebuhn *et al.*, 2000; Velasco *et al.*, 1998). Surprisingly, 16S rDNA-based phylogeny, as well as protein profiling (Velasco *et al.*, 1998) and AFLP analysis (Leal-Klevezas *et al.*, 2005), place *O. intermedium* strains closer to *Brucella* spp. than any other members of the genus *Ochrobactrum*.

O. anthropi has been isolated from various clinical specimens and is recognized as an opportunistic pathogen. Nosocomial infections due to *O. anthropi* have been increasingly reported during the last decade, particularly bacteraemia and endocarditis in patients with indwelling central venous catheters (Gill *et al.*, 1997; Mahmood *et al.*, 2000; Stiakaki *et al.*, 2002). Other cases of infections and outbreaks have been described in patients in dialysis (see Daxboeck *et al.*, 2002 for a review), after surgery in ophthalmology (Berman *et al.*, 1997; Greven & Nelson, 2001; Inoue *et al.*, 1999), in neurosurgery (Christenson *et al.*, 1997), after transplantation (Ezzedine

Abbreviations: AFLP, amplified fragment length polymorphism; DIG, digoxigenin.

The GenBank/EMBL/DDBJ accession numbers for the 16s rDNA sequences reported in this paper are AF526518–AF526526, AY917104–AY917119 and AY918295–AY918296.

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et al., 1994), or after valve replacement (Romero Gomez *et al.*, 2004). The virulence of *O. anthropi* is generally considered to be low, but reports suggested a high virulence for some strains involved in pyogenic infections (Brivet *et al.*, 1993; Cieslak *et al.*, 1996; Wheen *et al.*, 2002). *O. anthropi* was described as one of the most antibiotic-resistant Gram-negative rods (Nadjar *et al.*, 2001; Higgins *et al.*, 2001). Indeed, clinical strains of *O. anthropi* are multiresistant to common antibiotics, in particular they are usually resistant to all β-lactams except imipenem. The resistance to β-lactams is explained by the presence of an AmpC β-lactamase described as chromosomal, inducible and resistant to inhibition by clavulanic acid (Nadjar *et al.*, 2001). The majority of *Ochrobactrum* infections in humans have been imputed to the species *O. anthropi*, except for a liver abscess caused by *O. intermedium* (Moller *et al.*, 1999).

The aim of this study was to determine genotypic and phenotypic features allowing discrimination between the *Ochrobactrum* species. We carried out comparative analyses of 35 clinical isolates to type or reference strains of *O. anthropi*, *O. intermedium*, *O. tritici*, *O. grignonense* and *O. gallinifaecis*. '*O. lupini*' was not included in the study due to its very recent description (Trujillo *et al.*, 2005). Comparative analysis with clinically relevant α-proteobacterial genera, such as *Brucella*, *Agrobacterium*, *Sinorhizobium* and *Inquilinus* is also presented. The isolates were first identified by 16S rDNA sequencing and analysed by ribotyping. Then, we studied cell and colony morphology, growth conditions, biochemical traits on commercial identification systems, and antibiotic susceptibility, in order to define characteristics that could be used for species identification in a routine medical microbiology protocol.

METHODS

Bacterial strains. Thirty-five clinical isolates were obtained during a 5 year period from patients hospitalized in the academic hospitals of Montpellier, Nîmes and Clermont-Ferrand (France). The type strains of *O. anthropi* (ATCC 49188^T) and *O. intermedium* (LMG 3301^T, deposited as *O. anthropi* but now transferred to *O. intermedium* as the type strain; Velasco *et al.*, 1998), were obtained from the American Type Culture Collection (ATCC) and the Collection Française des Bactéries Phytopathogènes, respectively. The reference strains of *O. tritici* (DSM 13340^T and DSM 13341), *O. grignonense* (DSM 13338^T and DSM 13339) and *O. gallinifaecis* (DSM 15295^T) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Agrobacterium tumefaciens* C58 and *Sinorhizobium meliloti* 1021 were gifts from X. Nesmes and M. Fernandez (Laboratoire d'Écologie Microbienne du Sol, Université Claude Bernard Lyon I, Villeurbanne, France) (Jumas-Bilak *et al.*, 1998). *Inquilinus limosus* LMG 20952^T was obtained from Laboratorium voor Microbiologie, Universiteit Gent, Belgium (LMG).

16S rRNA gene analysis. Bacteria were grown on tryptic soy agar (TSA) for 24 h at 37 °C. One colony was suspended in 50 µl sterile water, and the DNA was liberated by a boiling-freezing method (Teyssier *et al.*, 2003). The 16S rRNA gene was selectively amplified from this crude lysate by PCR using universal primers 27f and 1492r, as previously described (Teyssier *et al.*, 2003). PCR products of about 1400 bp were sequenced directly on an Applied Biosystems Automatic Sequencer (Genome Express). Partial 16S rDNA sequences were compared with sequences deposited in databases using the standard nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/blast/). The genetic distances between strains were calculated using the DNADIST program (option similarity table) in the PHYLIP package (www.pasteur.fr) after sequence alignment by the DIALIGN software (www.expasy.org).

Ribotyping. Intact genomic DNAs were prepared in agarose plugs for enzymic digestion as previously described (Teyssier *et al.*, 2003). DNAs were digested with 40 U *Hin*dIII or *Eco*RI (New England Biolabs), and then electrophoresed for 3 h at 80 V in a 0·8 % agarose gel in 0·5× TBE. Gels were transferred onto nylon membrane by vacuum blotting (vacuum blotter; Bio-Rad) in 20× SSC. The 16S rDNA digoxigenin (DIG)-labelled probe was obtained by PCR using the 27f/1492r pair of primers with a dNTPs mixture containing 0·1 mM DIG-dUTP (Roche), and with the DNA of *O. intermedium* LMG 3301^T as a template. The hybridization of the probe was detected by the CSPD chemiluminescent system (Roche).

Phenotypic analysis. Cultures were grown at 30, 37 and 45 °C on TSA, blood Columbia agar (bioMérieux), cetrimide agar, Drigalski medium and MacConkey medium (Difco BRL) for 24 h. Cell morphology was observed by photonic microscopy after Gram staining. Identification systems API 20E, API 20NE and VITEK 2, ID-GNB card version WSVT2-R03.01 (bioMérieux), were used according to the supplier's recommendations, and particular care was taken to obtain the recommended turbidity for the bacterial suspensions. Urease activity was detected after bacterial growth on urea-indole medium (bioMérieux). The strain's susceptibility to antibiotics was determined by the disk-diffusion assay on Mueller–Hinton agar, according to the guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie (Members of the SFM Antibiogram Committee, 2003). The antibiotic disks (Bio-Rad) used were as follows: amoxycillin (25 µg), amoxycillin/clavulanic acid (20 µg/10 µg), ticarcillin (75 µg), ticarcillin/clavulanic acid (75 µg/10 µg), piperacillin (75 µg), piperacillin/tazobactam (75 µg/10 µg), imipenem (10 µg), cefalotine (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefpirome (30 µg), cefepime (30 µg), latamoxef (30 µg), aztreonam (30 µg), gentamicin (15 µg), tobramycin (10 µg), netilmicin (30 µg), nalidixic acid (30 µg), pefloxacin (5 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), rifampicin (30 µg), colistin (50 µg), chloramphenicol (30 µg) and trimethoprim/sulfamethoxazole (1·25 µg/23·75 µg).

RESULTS AND DISCUSSION

Genotyping

Sequence accession numbers and 16S rDNA-based identifications are given in Tables 1 and 2 for *Ochrobactrum* spp., and for type or reference strains of the genera *Brucella*, *Agrobacterium*, *Sinorhizobium* and *Inquilinus*. Sequencing of 16S rDNA clearly differentiated members of the genus *Ochrobactrum* from related genera since the sequence similarity was always below 95·5 %, except for *Brucella* spp. and *Ochrobactrum* spp., which displayed a sequence similarity of over 97 % for most of the strains. The clinical isolates of *Ochrobactrum* spp. were identified to the species level by 16S rRNA gene sequencing. All 16S rDNA sequences obtained matched with sequences deposited either for *O. anthropi* or *O. intermedium* after BLAST analysis. No isolates were affiliated to the species *O. tritici*, *O. grignonense* or *O. gallinifaecis*. The genetic distance calculated on a 534 bp

Table 1. Genotypic and phenotypic identification of *O. anthropi* strains, including the type strain, based on 16S rDNA sequencing, ribotyping, and API 20NE and VITEK 2 system analyses

NFGNB, non-fermenting Gram-negative bacilli; EI, excellent identification; VGI, very good identification; GI, good identification; AI, acceptable identification; LD, low discrimination.

Strain and origin	16S rDNA GenBank accession no.	16S rDNA-based identification (*Hin*dIII ribotype)	API 20NE† (quality of identification)	VITEK 2 ID-GNB card (quality of identification)
ATCC49188^T, unknown, Holmes *et al.* (1988)	D12794	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (EI)
ADV8*, rectum	AF526518	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (LD), *P. aeruginosa*
ADV15*, respiratory tract	AF526519	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (LD), *P. aeruginosa*
ADV16*, rectum	AF526520	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (VGI)
ADV17*, rectum	AF526521	*O. anthropi* (B)	*O. anthropi* (EI)	Various NFGNB (GI)
CLF18*, throat	AF526522	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (EI)
CLF19*, throat	AF526523	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (LD), various NFGNB, *Brucella* spp.
CLF20*, throat	AF526524	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (LD), *P. aeruginosa*
Nimes22*, respiratory tract	AF526525	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (LD), *Yersinia pseudotuberculosis*
ADV23*, bone marrow	AF526526	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (VGI)
Nimes25*, blood	AY917104	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (LD), various NFGNB, *Brucella* spp.
Nimes27*, blood	AY917105	*O. anthropi* (B)	*O. anthropi* (EI)	*O. anthropi* (LD), various NFGNB
Nimes28*, wound	AY917106	*O. anthropi* (B)	*O. anthropi* (VGI)	*O. anthropi* (LD), various NFGNB, *Brucella* spp.
ADV29*, urine	AY917107	*O. anthropi* (B)	*O. anthropi* (VGI)	*O. anthropi* (LD), *P. aeruginosa*
ADV34*, rectum	AY917108	*O. anthropi* (B)	*O. anthropi* (EI)	Non-identified
ADV37*, rectum	AY917109	*O. anthropi* (B)	*O. anthropi* (EI)	Various NFGNB (LD), *O. anthropi*
ADV38*, rectum	AY917110	*O. anthropi* (B)	*O. anthropi* (VGI)	Various NFGNB
ADV39*, rectum	AY917111	*O. anthropi* (B)	*O. anthropi* (VGI)	Non-identified
ADV40*, rectum	AY917112	*O. anthropi* (B)	*O. anthropi* (VGI)	Various NFGNB
ADV45*, rectum	AY917113	*O. anthropi* (B)	*O. anthropi* (VGI)	*O. anthropi* (AI)
ADV48*, rectum	AY917114	*O. anthropi* (B)	Non-identified	*O. anthropi* (LD), *P. aeruginosa*

*This work and human origin.

†Reading after 24 h of incubation.

alignment showed that the 20 strains affiliated to *O. anthropi* displayed strictly identical 16S rDNA sequences. Sequences of isolates affiliated to *O. intermedium* ($n = 15$) were more polymorphic, with identity ranging from 99·4 to 100 %. These results suggested a higher genetic divergence in the species *O. intermedium* than that observed in *O. anthropi*, and contrasted with AFLP results recently reported on a very small number of *O. intermedium* strains (Leal-Klevezas *et al.*, 2005). Sequence similarity between *O. anthropi* and *O. intermedium* ranged from 97·9 to 98·7 % according to the strains. Despite the fact that there is no generally accepted cut-off value for the bacterial species delineation, a 97 % similarity level in 16S rDNA has been proposed (Stackebrandt & Goebel, 1994). According to this value, *O. anthropi* and *O. intermedium* were not separated as well as *O. intermedium* and *Brucella melitensis*. As a consequence, the efficiency of 16S rDNA sequencing for the identification of *Ochrobactrum* to the species level could be questioned. In order to confirm the splitting of our collection of isolates into two clusters corresponding to *O. anthropi* and *O. intermedium*, we tested ribotyping as an alternative genotyping method. Although ribotyping has been frequently used to investigate the intra-species level, it is known that in the genus *Brucella*, which is phylogenetically related to *Ochrobactrum*, ribotyping patterns are species-specific and not strain-specific (Verger *et al.*, 2000). Ribotyping has also been shown to be efficient for the identification of species belonging to the genus *Agrobacterium* (Clermont *et al.*, 2001). We tested ribotyping as a genotyping method to discriminate between *Ochrobactrum* species, and between *Ochrobactrum* and related genera. The clinical isolates showed two groups of *Hin*dIII ribotypes named A and B (Fig. 1), clearly differentiated on the basis of their hybridization patterns. Ribogroup A grouped the *O. intermedium* type strain and all the strains affiliated to *O. intermedium* by 16S rDNA sequencing, whereas ribotype B grouped the *O. anthropi* type strain and all *O. anthropi* clinical isolates (Fig. 1). Three other ribogroups named C, D and E corresponded to the type and reference strains of *O. grignonense*, *O. tritici* and *O. gallinifaecis*, respectively (Fig. 1). *I. limosus* and *A. tumefaciens* displayed poor *Hin*dIII ribotype patterns (F and G) with only one hybridizing band of about 20 and 4·8 kb, respectively, whereas we did not obtain any exploitable pattern for *Sinorhizobium meliloti*.

Table 2. Genotypic and phenotypic identification of strains of *O. intermedium*, other *Ochrobactrum* species and related genera, including reference and type strains based on 16S rDNA sequencing, ribotyping, and API 20NE and VITEK 2 system analyses

NFGNB, non-fermenting Gram-negative bacilli; EI, excellent identification; VGI, very good identification; GI, good identification; AI, acceptable identification; LD, low discrimination.

Strain and origin	16S rDNA GenBank accession no.	16S rDNA-based identification (*Hin*dIII ribotype)	API 20NE† (quality of identification)	VITEK 2 ID-GNB card (quality of identification)
LMG3301^T, blood, Holmes *et al.* (1988); Velasco *et al.* (1998)	U70978	*O. intermedium* (A)	*O. anthropi* (GI)	*O. anthropi* (AI)
ADV1*, respiratory tract	AF526509	*O. intermedium* (A)	*O. anthropi* (EI)	*O. anthropi* (LD), *P. aeruginosa*
ADV3*, respiratory tract	AF526516	*O. intermedium* (A)	*O. anthropi* (VGI)	*P. aeruginosa* (VGI)
ADV9*, ear	AF526510	*O. intermedium* (A)	*O. anthropi* (EI)	*P. aeruginosa* (VGI)
ADV10*, finger wound	AF526511	*O. intermedium* (A)	*O. anthropi* (EI)	*P. aeruginosa* (LD), *O. anthropi*
ADV11*, rectum	AF526512	*O. intermedium* (A)	*O. anthropi* (VGI)	*O. anthropi* (LD), *P. aeruginosa*
ADV14*, axilla	AF526513	*O. intermedium* (A)	*O. anthropi* (VGI)	*O. anthropi* (LD), *P. aeruginosa*
ADV21*, rectum	AF526514	*O. intermedium* (A)	*O. anthropi* (EI)	*P. aeruginosa* (VGI)
ADV24*, axilla	AF526515	*O. intermedium* (A)	*O. anthropi* (GI)	Non-identified
ADV32*, rectum	AY918296	*O. intermedium* (A)	*O. anthropi* (EI)	Non-identified
ADV33*, axilla	AY918295	*O. intermedium* (A)	Non-identified	Non-identified
ADV35*, blood	AY917115	*O. intermedium* (A)	*O. anthropi* (VGI)	Non-identified
ADV36*, blood	AY917116	*O. intermedium* (A)	*O. anthropi* (GI)	*O. anthropi* (GI)
ADV42*, rectum	AY917117	*O. intermedium* (A)	*O. anthropi* (VGI)	Non-identified
ADV44*, rectum	AY917118	*O. intermedium* (A)	Non-identified	Various NFGNB (LD), *P. aeruginosa*, *Methylobacterium* sp.
ADV46*, drain fluid	AY917119	*O. intermedium* (A)	*O. anthropi* (GI)	Non-identified
O. grignonense DSM13338^T, soil, Lebuhn *et al.* (2000)	AJ242581	*O. grignonense* (C)	*P. fluorescens* (LD) *O. anthropi*	Non-identified
O. grignonense DSM13339, soil, Lebuhn *et al.* (2000)		*O. grignonense* (C)	Non-identified	Non-identified
O. tritici DSM13340^T, rhizosphere, Lebuhn *et al.* (2000)	AJ242584	*O. tritici* (D)	*O. anthropi* (VGI)	*O. anthropi* (LD), various NFGNB, *Brucella* spp.
O. tritici DSM13341, rhizosphere, Lebuhn *et al.* (2000)	AJ242579	*O. tritici* (D)	*O. anthropi* (EI)	*O. anthropi* (VGI)
O. gallinifaecis DSM15295^T, chicken faeces, Kämpfer *et al.* (2003)	AJ519939	*O. gallinifaecis* (E)	Non-identified	Non-identified
I. limosus LMG 20952^T, human, Coenye *et al.* (2002); Chiron *et al.* (2005)	AY043374	*I. limosus* LMG 20952^T (F)	*Sphingomonas. paucimobilis* (VGI)	*P. fluorescens* (LD), *Sphingomonas paucimobilis*
Sinorhizobium meliloti 1021, plant, Jumas-Bilak *et al.* (1998)	NC_003047	*Sinorhizobium meliloti* 1021 (ND)	*A. radiobacter* (GI)	*Rhizobium radiobacter* (EI)
A. tumefaciens C58, plant, Jumas-Bilak *et al.* (1998)	NC_003062	*A. tumefaciens* C58 (G)	*A. radiobacter* (EI)	*Rhizobium radiobacter* (EI)
B. melitensis 16M, human Jumas-Bilak *et al.* (1998)	NC_003317	*B. melitensis* 16M (H)	ND	ND

ND, Not determined.

*This work and human origin.

†Reading after 24 h of incubation.

The *Brucella* pattern (H, a schematic representation) determined from the complete genome sequence (DelVecchio *et al.*, 2002) clearly differed from those of all *Ochrobactrum* strains and other genera. The partition of the clinical strains into two ribogroups, named A′ and B′, was also observed after *Eco*RI restriction (Fig. 2). The two types of *Eco*RI pattern corresponded to the patterns of *O. anthropi* and *O. intermedium* type strains. Therefore, the distribution of the strains according to their ribotype was consistent with 16S rDNA-based identification (Tables 1 and 2). The other

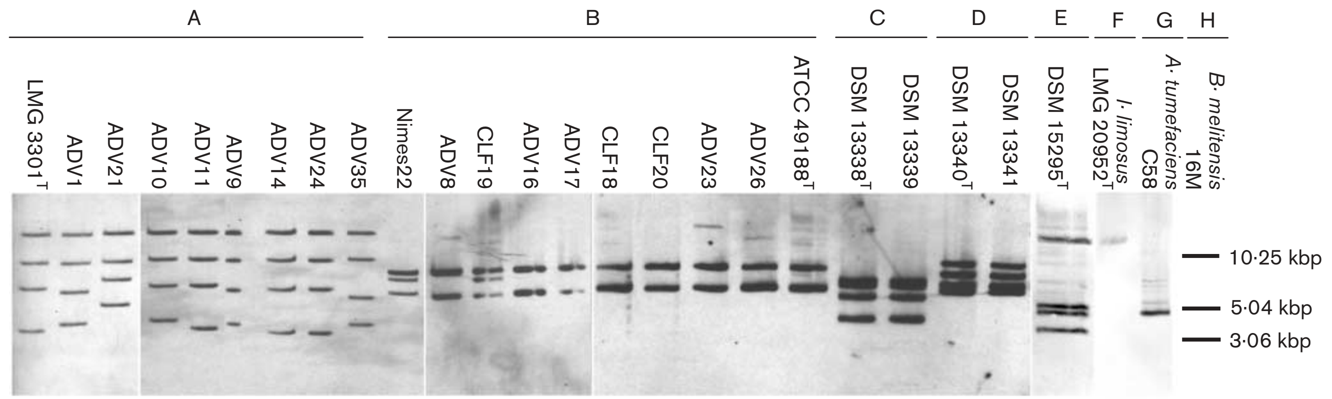

Fig. 1. Hybridization patterns of DIG-labelled 16S rRNA gene probe with Southern blotted *Hin*dIII restriction fragments from type strains and selected isolates of *Ochrobactrum* spp. and related genera. A, B, C, D and E correspond to the five *Ochrobactrum* ribogroups. F, G and H correspond to *I. limosus* LMG 20952T, *A. tumefaciens* C58 and *B. melitensis* 16M ribogroups, respectively. H is a schematic representation deduced from the complete genome sequence. Molecular mass marker: bacteriophage λ digested by *Hin*dIII (data not shown).

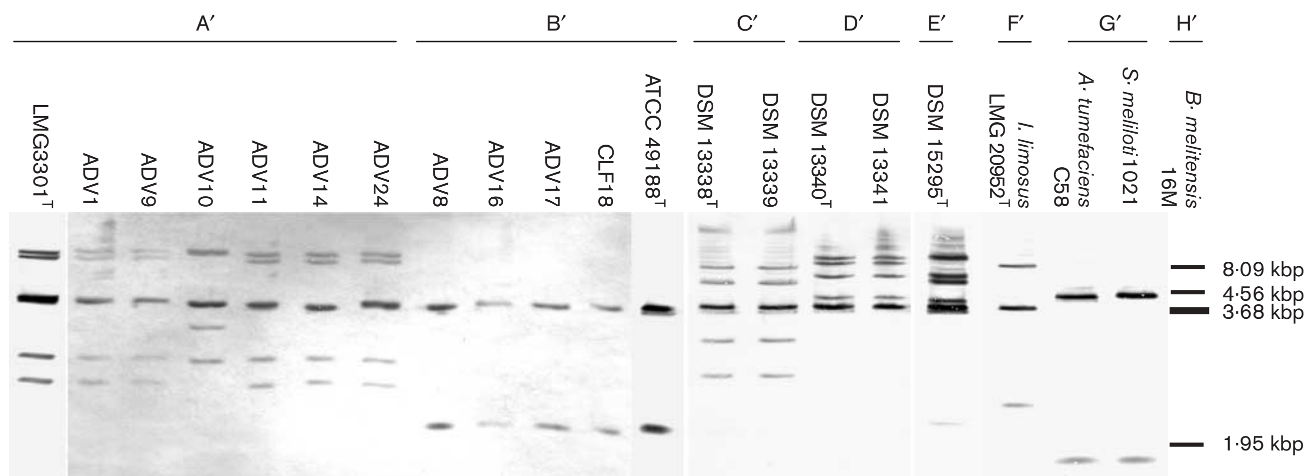

Fig. 2. Hybridization patterns of DIG-labelled 16S rRNA gene probe with Southern blotted *Eco*RI restriction fragments from type strains and selected isolates of *Ochrobactrum* spp. and related genera A′, B′, C′, D′ and E′ correspond to the five *Ochrobactrum* ribogroups. F′, G′ and H′ correspond to *I. limosus* LMG 20952T, *A. tumefaciens* C58 and *Sinorhizobium meliloti* 1021, and *B. melitensis* 16M ribogroups, respectively. H′ is a schematic representation deduced from the complete genome sequence. Molecular mass marker: bacteriophage λ digested by *Hin*dIII (data not shown).

species of the genus *Ochrobactrum* and the related genera displayed different and specific *Eco*RI patterns, except for *Agrobacterium* and *Sinorhizobium* (Fig. 2).

Phenotypic traits of *Ochrobactrum* spp.

All the strains were Gram-negative short rods, straight or slightly curved with one end flame shaped. Wet-mount microscopy examination showed that the cells were highly motile. After 24 h incubation, colony aspect at 30 and 37 °C on TSA, Drigalski medium and MacConkey medium clearly differed among species. *O. anthropi* and *O. grignonense* developed circular, smooth, shiny colonies, whereas the colonies of *O. intermedium* and *O. tritici* were mucoid, opaque and quickly became confluent. The strains of *O. intermedium* were the sole strains able to grow at 45 °C on TSA. No strain grew on cetrimide agar. Haemolysis was never observed on blood Columbia agar. The brown pigment classically described for the genus *Ochrobactrum* was hardly visible on TSA and blood Columbia agar, even when a colony was harvested on a swab.

Using the API 20E and API 20NE systems, all the strains were negative for indole, H_2S and acetoin production, carbohydrate fermentation, utilization of citrate, and assimilation of adipate and phenylacetate. Moreover, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, β-galactosidase and gelatinase activities were not detected. All

the strains were positive for the assimilation of glucose, arabinose, mannose, *N*-acetylglucosamine, maltose and malate. Other characteristics varied among strains of a same species except for urease activity. Positive urease activity, detected either by urea-indole medium (bioMérieux) or on API strips was shown for all *O. anthropi* except strain ADV 45, and for *O. tritici* and *O. gallinifaecis* strains. In contrast, none of the *O. intermedium* and *O. grignonense* strains displayed urease activity, whatever the method used to detect it (Table 3). However, an inoculum heavier than that recommended by manufacturers led to positive reactions for most of these strains. The VITEK 2 ID-GNB card gave urease results consistent with the urea-indole medium and the API systems, except for the results for three strains of *O. intermedium* (strains ADV1, ADV14 and ADV36), which showed urease activity only when analysed on the VITEK 2 apparatus. All these discrepancies suggested that *O. intermedium* possessed a faint urease activity as previously underlined by Lebuhn *et al.* (2000). This could explain the discrepancy observed between the urease activity results reported for *O. intermedium* in previous studies (Lebuhn *et al.*, 2000; Moller *et al.*, 1999; Velasco *et al.*, 1998). Urease activity should no longer be considered as a criterion for genus identification, but rather as a biochemical characteristic useful for the discrimination of the two *Ochrobactrum* species of medical interest, *O. anthropi* and *O. intermedium*.

Only a few of the 41 characteristics tested by the ID-GNB card of the VITEK 2 system were positive for *Ochrobactrum* spp. Pyrrolidonyl arylamidase (PyrA), L-proline arylamidase (ProA) and alkaline phosphatase (PHOS) had been previously described as very useful tests for the identification of non-fermenting Gram-negative rods (Laffineur *et al.*, 2002). The authors found that 100 % of the *Ochrobactrum* spp. strains were positive for PyrA and ProA, and negative for PHOS. Our results were in accordance for PyrA (42+/42) and PHOS (42−/42), but ProA was not positive for all strains (28+/42). The results for γ-glutamyl transferase (17+/42), adonitol (17+/42), D-maltose (12+/42), palatinose (11+/42), sucrose (9+/42) and L-lysine arylamidase (5+/42) were variable between strains, without correlation with species identification. The glu-gly-arg arylamidase (GGAA) was positive for *O. grignonense* strains only, but additional strains of this species need to be tested to determine if it is a species-specific characteristic.

Comparison of API 20NE strip and VITEK 2 ID-GNB card for *Ochrobactrum* identification

It should be noted that *O. anthropi* is the sole species of the genus *Ochrobactrum* included in the databases of the API and VITEK 2 systems. Using the API 20NE system, nearly all the strains were identified as *O. anthropi* at 24 h, with the mention 'very good identification' or 'excellent identification' (Tables 1 and 2). The two *O. grignonense* strains were unidentified, or misidentified as *Pseudomonas fluorescens* with low discrimination. Moreover, the *O. gallinifaecis* type strain remained unidentified (Table 2). The reading of the strips after 48 h gave the same identification, but associated with the mentions 'good identification' or 'acceptable identification'. Thus, despite the supplier's recommendations, the more confident identification of the genus was obtained after 24 h incubation of the strips. The absence of urease in *O. intermedium* strains did not alter the confidence level of identification, since urease was presented as an inconstant characteristic for *O. anthropi* (84 %) on the API 20NE technical sheet. It is likely that the original calibration of the system used *O. anthropi* strain samples that were actually a mix of *O. anthropi* and *O. intermedium* strains. Thus, the API 20NE system gave satisfactory identification of the genus *Ochrobactrum*, except for *O. grignonense* and *O. gallinifaecis* strains.

The identification reports given by the VITEK 2 system after analysis of the ID-GNB card are shown in Tables 1 and 2. In 8 cases out of 42, the identification of *O. anthropi* was assessed with the following confidence levels: 'excellent identification' ($n = 2$), 'very good identification' ($n = 3$), 'good identification' ($n = 1$), and 'acceptable identification' ($n = 2$). The identification of *O. anthropi* was proposed with a low discrimination for 16 strains; the identification of *Pseudomonas aeruginosa* is proposed as an alternative choice for

Table 3. Differential phenotypic features of *Ochrobactrum* species

The number of strains with the indicated characteristic is given in parentheses. ND, not determined; TSA, tryptic soy agar; R, resistant; S, susceptible.

Species	Urease activity			Antibiotic susceptibility		Mucoid colony (TSA, 37 °C)	Growth (TSA, 45 °C)
	Urea-indole medium	API system	VITEK 2 GNB card	Colistin	Netilmicin, tobramycin		
O. intermedium ($n = 16$)	− ($n = 16$)	− ($n = 16$)	− ($n = 12$), + ($n = 3$), ND ($n = 1$)	R ($n = 16$)	R ($n = 16$)	+ ($n = 15$)	+ ($n = 16$)
O. anthropi ($n = 21$)	+ ($n = 20$)	+ ($n = 20$)	+ ($n = 20$)	S ($n = 21$)	S ($n = 21$)	− ($n = 20$)	− ($n = 21$)
O. grignonense ($n = 2$)	− ($n = 2$)	− ($n = 2$)	− ($n = 2$)	R ($n = 2$)	S ($n = 2$)	− ($n = 2$)	− ($n = 2$)
O. tritici ($n = 2$)	+ ($n = 2$)	+ ($n = 2$)	+ ($n = 2$)	S ($n = 2$)	S ($n = 2$)	++ ($n = 2$)	− ($n = 2$)
O. gallinifaecis ($n = 1$)	+	+	+	R	S	−	−

nine of these. To sum up, the VITEK 2 gave or suggested the identification of *O. anthropi* for 25 strains among the 42 tested. This identification system appeared to be less powerful than the API 20NE system for the identification of the genus *Ochrobactrum*. Particularly, the VITEK 2 ID-GNB card gave the identification of *Pseudomonas aeruginosa* with the mention 'very good identification' for 3 strains and did not achieve identification for 11 strains. The three misidentified isolates (ADV3, ADV9, ADV21) were readily identified, at least to the genus level, by using the API 20NE system (Table 2). The VITEK 2 proposed the name *O. anthropi* for 16 out of 21 *O. anthropi* strains, but for only 6 out of 16 *O. intermedium* strains. The two *O. tritici* strains were identified as *O. anthropi*, but *O. grignonense* and *O. gallinifaecis* strains were not identified. The results suggested that the VITEK 2 system placed urease as a major characteristic for identification. As a consequence, the species without detectable urease activity were not fully identified as members of the genus *Ochrobactrum*.

Antibiotic susceptibility testing

All the *O. anthropi* clinical isolates and the type strains were highly resistant to all β-lactams except imipenem (no growth inhibition was observed for most of the β-lactams). This resistance profile is consistent with the expression of the AmpC β-lactamase characterized in *O. anthropi* (Higgins *et al.*, 2001; Nadjar *et al.*, 2001). Identical susceptibility patterns were obtained for the strains of *O. intermedium* and *O. tritici*, suggesting that a β-lactamase of the same class, or the same enzyme, could be expressed in these two species. The two *O. grignonense* strains were also resistant to all β-lactams except imipenem, but in contrast to the strains of *O. anthropi*, *O. intermedium* and *O. tritici*, growth inhibition was observed around amoxycillin (12 mm diameter), cefotaxime (20 mm diameter), cefpirome (15 mm diameter), cefepime (19 mm diameter) and latamoxef (20 mm diameter) disks. Moreover, the partial restoration of the amoxycillin and ticarcillin activities by clavulanic acid was not in agreement with the presence of a class 1 AmpC β-lactamase. These observations suggested that the mechanism of resistance of *O. grignonense* differed from that of the three other species, but this needs further investigation. In contrast with all the other members of the genus, the type strain of *O. gallinifaecis* was susceptible to all β-lactams except aztreonam.

We observed a general susceptibility of the strains to gentamicin, rifampicin and fluoroquinolones. Susceptibility to netilmicin and tobramycin varied according to the species. All the *O. anthropi* strains were susceptible to netilmicin and tobramycin, whereas all the *O. intermedium* strains were resistant to these aminoglycosides (Table 3). Moreover, we confirmed in our collection of 35 clinical isolates the susceptibility of *O. anthropi* to colistin, and the resistance of *O. intermedium* to this antibiotic, as reported by Velasco *et al.* (1998) (Table 3). Susceptibility to trimethoprim/sulfamethoxazole was observed for all strains of *O. anthropi*, *O. intermedium*, *O. tritici* and *O. gallinifaecis*, whereas the two *O. grignonense* strains were resistant to this association. Moreover, the *O. gallinifaecis* type strain was the sole strain found to be susceptible to chloramphenicol.

Improving the identification of *Ochrobactrum* species in medical microbiology

An efficient procedure for identifying *Ochrobactrum* to the species level is necessary to evaluate the role of each species in human infections, as well as for epidemiological investigations. The results we obtained for isolates and type strains of *O. anthropi* and *O. intermedium* allowed us to propose a routine protocol for identifying these two *Ochrobactrum* species, which were currently the only ones involved in human pathology. Firstly, the isolation of a non-fastidious non-fermenting Gram-negative rod, which is oxidase-positive and resistant to all β-lactams except for imipenem should indicate both the genera *Ochrobactrum* and *Inquilinus* (Coenye *et al.*, 2002). However, *Inquilinus* spp. displays a remarkably huge inhibition diameter (40–60 mm) around an imipenem disk (Chiron *et al.*, 2005). In addition, *I. limosus* can be differentiated from *Ochrobactrum* spp. by its growth on Drigalski agar but not on MacConkey agar plates. Although *Brucella* spp. and *Ochrobactrum* spp. are genetically very closely related, they show clear differences in their phenotypes, i.e. *Brucella* spp. are fastidious and slow-growing Gram-negative cocco-bacilli.

Secondly, commercial identification systems can confirm the genus identification. Members of the genera *Agrobacterium* and *Sinorhizobium* were both identified by the API 20NE system as *Agrobacterium radiobacter* with a high level of confidence (Table 2). The use of the API 20NE system gave, for most of the strains tested, the identification of *O. anthropi*. Since *O. intermedium* is not included in the commercial identification systems' databases, the identification of *O. anthropi* ought to be considered as genus identification. The VITEK 2 system appeared to be less efficient than the API 20NE system at identifying the genus *Ochrobactrum*. However, the newly available VITEK 2 detection system (VITEK 2 Advanced Colorimetry) needs to be evaluated. For routine identification to the species level, the urease test should be taken into account as a good indication (Table 3). Then, the aspect of the colonies, their growth at 45 °C on TSA and their susceptibility to colistin, tobramycin and netilmicin should be considered as additional characteristics allowing the species identification (Table 3).

We cannot propose a robust methodology for the identification of *O. tritici*, *O. grignonense* and *O. gallinifaecis* due to the low number of isolates characterized to date for these species. However, the preliminary results suggest that *O. tritici* strains are readily identified as members of the genus *Ochrobactrum* by the use of an API 20NE strip. The two strains are highly related to *O. anthropi*, with a positive urease test and susceptibility to colistin, netilmicin and tobramycin, but they differ from this species by their highly mucoid colonies on TSA. The criteria for differentiating *O. grignonense* and

O. gallinifaecis from other members of the genus are given in Table 3. However, their genus affiliation is hard to assign since identification systems fail to class them as members of the genus *Ochrobactrum*, and their β-lactam resistance patterns are atypical. Therefore, *O. grignonense* and *O. gallinifaecis* can only be readily identified by molecular means.

Conclusion

To our knowledge, we have presented here the largest collection of *Ochrobactrum* spp. clinical isolates identified to the species level by genotyping methods. The literature included only one case of human infection caused by *O. intermedium* (Moller *et al.*, 1999), suggesting that amongst *Ochrobactrum* species *O. anthropi* has the predominant role in human disease. However, most of the infections involving *O. anthropi* were reported before the description of the other *Ochrobactrum* species, and/or by the use of non-discriminating methods. Thus, the role of each species in human infections needs to be revisited. Our collection showed a nearly equivalent distribution of clinical isolates between *O. anthropi* and *O. intermedium*. This indicates that the role of *O. intermedium* in human infections is probably underestimated due to the lack of a convenient identification system and should be further evaluated.

The phenotypic study of the collection allowed us to determine some characteristics useful for species identification using routine medical microbiology. However, further large-scale studies including more isolates are clearly required to confirm the efficiency of our approach for the identification of *Ochrobactrum* spp. More generally, this study could be considered as an improvement of the identification of Gram-negative non-fermenting rods by conventional methods. Indeed, their identification is often difficult, and the commercial systems are not always reliable, especially for some genera and species (Laffineur *et al.*, 2002; VanPelt *et al.*, 1999). Moreover, recent taxonomic studies resulted in the description of an increasing number of new taxa involved in nosocomial infections, and requiring additional tests for identification. This was particularly true for the genus *Ochrobactrum*.

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REFERENCES

Berman, A. J., Del Priore, L. V. & Fischer, C. K. (1997). Endogenous *Ochrobactrum anthropi* endophthalmitis. *Am J Ophthalmol* **123**, 560–562.

Brivet, F., Guibert, M., Kiredjian, M. & Dormont, J. (1993). Necrotizing fasciitis, bacteremia, and multiorgan failure caused by *Ochrobactrum anthropi*. *Clin Infect Dis* **17**, 516–518.

Chiron, R., Marchandin, H., Counil, F. & 8 other authors (2005). Clinical and microbiological features of *Inquilinus* sp. isolates from five patients with cystic fibrosis. *J Clin Microbiol* **43**, 3938–3943.

Christenson, J. C., Pavia, A. T., Seskin, K., Brockmeyer, D., Korgenski, E. K., Jenkins, E., Pierce, J. & Daly, J. A. (1997). Meningitis due to *Ochrobactrum anthropi*: an emerging nosocomial pathogen. A report of 3 cases. *Pediatr Neurosurg* **27**, 218–221.

Cieslak, T. J., Drabick, C. J. & Robb, M. L. (1996). Pyogenic infections due to *Ochrobactrum anthropi*. *Clin Infect Dis* **22**, 845–847.

Clermont, D., Harmant, C. & Bizet, C. (2001). Identification of strains of *Alcaligenes* and *Agrobacterium* by a polyphasic approach. *J Clin Microbiol* **39**, 3104–3109.

Coenye, T., Goris, J., Spilker, T., Vandamme, P. & LiPuma, J. J. (2002). Characterization of unusual bacteria isolated from respiratory secretions of cystic fibrosis patients and description of *Inquilinus limosus* gen. nov., sp. nov. *J Clin Microbiol* **40**, 2062–2069.

Daxboeck, F., Zitta, S., Assadian, O., Krause, R., Wenisch, C. & Kovarik, J. (2002). *Ochrobactrum anthropi* bloodstream infection complicating hemodialysis. *Am J Kidney Dis* **40**, E17.

DelVecchio, V. G., Kapatral, V., Redkar, R. J. & 22 other authors (2002). The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proc Natl Acad Sci U S A* **99**, 443–448.

Ezzedine, H., Mourad, M., Van Ossel, C. & 7 other authors (1994). An outbreak of *Ochrobactrum anthropi* bacteraemia in five organ transplant patients. *J Hosp Infect* **27**, 35–42.

Gill, M. V., Ly, H., Mueenuddin, M., Schoch, P. E. & Cunha, B. A. (1997). Intravenous line infection due to *Ochrobactrum anthropi* (CDC group Vd) in a normal host. *Heart Lung* **26**, 335–336.

Greven, C. M. & Nelson, K. C. (2001). Chronic postoperative endophtalmitis secondary to *Ochrobactrum anthropi*. *Retina* **21**, 279–280.

Higgins, C. S., Avison, M. B., Jamieson, L., Simm, A. M., Bennett, P. M. & Walsh, T. R. (2001). Characterization, cloning and sequence analysis of the inducible *Ochrobactrum anthropi* AmpC β-lactamase. *J Antimicrob Chemother* **47**, 745–754.

Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988). *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd. *Int J Syst Bacteriol* **38**, 408–416.

Inoue, K., Numaga, J., Nagata, Y., Sakurai, M., Aso, N. & Fujino, Y. (1999). *Ochrobactrum anthropi* endophthalmitis after vitreous surgery. *Br J Ophthalmol* **83**, 501.

Jumas-Bilak, E., Michaux-Charachon, S., Bourg, G., Ramuz, M. & Allardet-Servent, A. (1998). Unconventional genomic organization in the alpha subgroup of the *Proteobacteria*. *J Bacteriol* **180**, 2749–2755.

Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H. J. & Stackebrandt, E. (2003). Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *Int J Syst Evol Microbiol* **53**, 893–896.

Laffineur, K., Janssens, M., Charlier, J., Avesani, V., Wauters, G. & Delmée, M. (2002). Biochemical and susceptibility tests useful for identification of nonfermenting gram-negative rods. *J Clin Microbiol* **40**, 1085–1087.

Leal-Klevezas, D. S., Martinez-de-la-Vega, O., Ramirez-Barba, E. J., Osterman, B., Martinez-Soriano, J. P. & Simpson, J. (2005). Genotyping of *Ochrobactrum* spp. by AFLP analysis. *J Bacteriol* **187**, 2537–2539.

Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000). Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* **50**, 2207–2223.

Mahmood, M. S., Sarwari, A. R., Khan, M. A., Sophie, Z., Khan, E. &

Sami, S. (2000). Infective endocarditis and septic embolization with *Ochrobactrum anthropi*: case report and review of literature. *J Infect* **40**, 287–290.

Members of the SFM Antibiogram Committee (2003). Comité de l'Antibiogramme de la Société Française de Microbiologie Report 2003. *Int J Antimicrob Agents* **21**, 364–391.

Moller, L. V., Arends, J. P., Harmsen, H. J., Talens, A., Terpstra, P. & Slooff, M. J. (1999). *Ochrobactrum intermedium* infection after liver transplantation. *J Clin Microbiol* **37**, 241–244.

Nadjar, D., Labia, R., Cerceau, C., Bizet, C., Philippon, A. & Arlet, G. (2001). Molecular characterization of chromosomal class C β-lactamase and its regulatory gene in *Ochrobactrum anthropi*. *Antimicrob Agents Chemother* **45**, 2324–2330.

Romero Gomez, M. P., Peinado Esteban, A. M., Sobrino Daza, J. A., Saez Nieto, J. A., Alvarez, D. & Pena Garcia, P. (2004). Prosthetic mitral valve endocarditis due to *Ochrobactrum anthropi*: case report. *J Clin Microbiol* **42**, 3371–3373.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stiakaki, E., Galanakis, E., Samonis, G., Christidou, A., Maraka, S., Tselentis, Y. & Kalmanti, M. (2002). *Ochrobactrum anthropi* bacteremia in pediatric oncology patients. *Pediatr Infect Dis J* **21**, 72–74.

Teyssier, C., Marchandin, H., Siméon de Buochberg, M., Ramuz, M. & Jumas-Bilak, E. (2003). Atypical 16S rRNA gene copies in *Ochrobactrum intermedium* strains reveal a large genomic rearrangement by recombination between *rrn* copies. *J Bacteriol* **185**, 2901–2909.

Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludena, D., Mateos, P. F., Martinez-Molina, E. & Velazquez, E. (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* **71**, 1318–1327.

VanPelt, C., Verduin, C. M., Goessens, W. H. F., Vos, M. C., Tummler, B., Segonds, C., Reubsaet, F., Verbrugh, H. & VanBelkum, A. (1999). Identification of *Burkholderia* spp. in the clinical microbiology laboratory: comparison of conventional and molecular methods. *J Clin Microbiol* **37**, 2158–2164.

Velasco, J., Romero, C., Lopez-Goni, I., Leiva, J., Diaz, R. & Moriyon, I. (1998). 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. *Int J Syst Bacteriol* **48**, 759–768.

Verger, J. M., Grayon, M., Cloeckaert, A., Lefevre, M., Ageron, E. & Grimont, F. (2000). Classification of *Brucella* strains isolated from marine mammals using DNA-DNA hybridization and ribotyping. *Res Microbiol* **151**, 797–799.

Wheen, L., Taylor, S. & Godfrey, K. (2002). Vertebral osteomyelitis due to *Ochrobactrum anthropi*. *Intern Med J* **32**, 426–428.