Difficulty in the identification and differentiation of clinically relevant *Ochrobactrum* species

The genus *Ochrobactrum* currently comprises nine species, *Ochrobactrum anthropi* (Holmes *et al.*, 1988), *Ochrobactrum intermedium* (Velasco *et al.*, 1998), *Ochrobactrum lupini* (Trujillo *et al.*, 2005), *Ochrobactrum tritici* and *Ochrobactrum grignonense* (Lebuhn *et al.*, 2006), *Ochrobactrum gallinifaecis* (Kämpfer *et al.*, 2003), *Ochrobactrum oryzae* (Tripathi *et al.*, 2006), *Ochrobactrum pseudintermedium* (Teyssier *et al.*, 2007) and *Ochrobactrum cytisi* (Zurdo-Piñeiro *et al.*, 2007). Only three species, *O. anthropi*, *O. intermedium* and *O. pseudintermedium*, have been reported to occur in clinical material (Alnor *et al.*, 1994; Appelbaum & Campbell, 1980; Barson *et al.*, 1987; Braun *et al.*, 1996; Brivet *et al.*, 1993; Cieslak *et al.*, 1992, 1996; Earhart *et al.*, 1997; Greven & Nelson, 2001; Inoue *et al.*, 1999; Jelveh & Cunha, 1999; Kettaneh *et al.*, 2003; Mahmood *et al.*, 2000; Perez-Blanco *et al.*, 2005; Romero-Gomez *et al.*, 2004; Vaidya *et al.*, 2006; Wheen *et al.*, 2002; Teyssier *et al.*, 2007).

O. anthropi was described by Holmes *et al.* (1988) to accommodate oxidase-positive, Gram-negative, non-fermentative bacilli previously known as 'Achromobacter group Vd', occasionally infecting humans. Today *O. anthropi* is recognized as an emerging pathogen of low virulence, predominantly isolated from immunocompromised patients, but there are also 19 cases of it being isolated from immunocompetent hosts (Vaidya *et al.*, 2006). Only two cases of *O. intermedium* infection have been reported. In 1999, *O. intermedium* was isolated from blood and a liver abscess in a patient after liver transplantation (Möller *et al.*, 1999), and in 2005, a presumptive systemic *O. intermedium* infection in a patient with bladder cancer was reported (Apisarnthanarak *et al.*, 2005). Recently a

novel species, *O. pseudintermedium*, was described that was isolated from patients hospitalized in intensive-care units (Teyssier *et al.*, 2007). The differentiation of *Ochrobactrum* species by physiological tests is quite difficult, because of their high phenotypic similarity (Table 1). *O. anthropi* is often the only *Ochrobactrum* species present in the databases of commercially available identification systems (including the API 20NE kit) (Teyssier *et al.*, 2005a) and as a consequence *O. intermedium* may be misidentified as *O. anthropi* as occurred in a recently published case report describing *O. anthropi* as the causative agent of a pelvic abscess in an immunocompetent host (Vaidya *et al.*, 2006). The initial identification of that isolate (RMA 16449) as *O. anthropi* (Vaidya *et al.*, 2006) was performed with the API 20E kit (bioMérieux) and by partial 16S rRNA gene sequencing. Strain RMA 16449 was later included in comparative analysis to study the genetic diversity among 112 *Ochrobactrum* strains by applying a polyphasic approach using extended 16S rRNA gene analysis, *recA* gene sequencing, fatty acid analysis and DNA–DNA hybridization studies. Of the 16S rRNA gene, 1390 bp were sequenced as described by Kämpfer *et al.* (2003). In the BLASTN search using the sequence information from the entire 1390 bp fragment (GenBank accession no. AM409326), the highest similarity of 100 % was indeed detected with '*O. anthropi*' strain CCUG 39736 (GenBank accession no. AM114408). However, to add to the confusion, this reference strain, initially identified as *O. anthropi*, was recently transferred to *O. intermedium* (see the CCUG Homepage: www.ccug.se). An identity of 99 % was detected with the type strain of *O. intermedium*, CCUG 24694^T (GenBank accession no. AM114411), and only 97.8 % to the type strain of *O. anthropi*, LMG 3331^T (GenBank accession no. AM114398). Hence isolate RMA 16994

appeared to be more closely related to *O. intermedium* than to *O. anthropi*. Identification of isolate RMA 16994 as *O. intermedium* was confirmed by the presence of the *O. intermedium*-specific sequence motifs ggctaata and ggtagtaggagacataatcc at positions nt 487–493 and nt 905–924 of the 1390 bp 16S rRNA gene fragment as described by Scholz *et al.* (2006). The second motif is also shared with *Brucella* spp. However, both motifs are absent in *O. anthropi* and are instead represented by the sequences gactttt and ggacacagagatgtgtct at these positions, respectively.

Using *recA* as a highly discriminatory marker to study the phylogeny of *Ochrobactrum* spp. at the inter- and intraspecies level (Scholz *et al.*, 2006) confirmed isolate RMA 16449 as *O. intermedium* (results not shown).

Fatty acid analysis using the Sherlock System (MIDI; Kämpfer & Kroppenstedt, 1996) and comparison with the Sherlock database provided the identification result *O. anthropi* with an ID score of 0.556, indicating that fatty acid analysis is not sufficient for differentiation at the species level (results not shown). Selected results of the physiological characterization (according to Kämpfer *et al.*, 1991) compared with those from *O. anthropi* CIP 14970^T and *O. intermedium* LMG 3301^T are given in Table 1. Only very few biochemical tests are able to differentiate these species. For final species confirmation, DNA–DNA hybridization experiments were performed with RMA 16449 and *O. intermedium* LMG 3301^T using the method described by Ziemke *et al.* (1998) modified as described by Kämpfer *et al.* (2003). Strain RMA 16449 showed 79 % DNA–DNA similarity (mean value of two hybridizations) to *O. intermedium* LMG 3301^T, confirming that RMA 16449 is member of this species, since the homology demonstrated was >70 %.

The DDBJ/EMBL/GenBank accession number for the 16S rRNA gene sequence of isolate RMA 16449 is AM409326.

Table 1. Physiological characteristics of selected type strains of *Ochrobactrum* species tested with the methods according to Kämpfer *et al.* (1991)

Taxa: 1, *O. anthropi* CIP 14970^T; 2, *O. intermedium* LMG 3301^T; 3, *O. intermedium* RMA 16449. +, Positive; –, negative; (+), weakly positive. All strains were positive for hydrolysis of L-alanine pNA* and L-proline pNA‡ and weak hydrolysis of bis-pNP* phosphate and L-glutamate-γ-3-carboxy-pNA. All strains were negative for hydrolysis of aesculin†, pNP β-D-galactopyranoside†, pNP β-D-glucuronide, pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP phenyl phosphonate, pNP phosphorylcholine and 2-deoxythymidine-5'-pNP phosphate. All strains were also positive for assimilation of L-arabinose†, D-cellobiose†, D-galactose†, gluconate, D-glucose, D-mannose, D-maltose†‡, N-acetyl-D-glucosamine†, N-acetyl-D-galactosamine, D-ribose†, D-xylose, D-fructose, adonitol†, i-inositol†, L-rhamnose†, D-sorbitol†, cis-aconitate, citrate†‡, DL-3-hydroxybutyrate†, acetate, 4-aminobutyrate, β-alanine†, propionate†, fumarate†, glutarate, DL-lactate, L-malate†, oxoglutarate†, pyruvate†, L-alanine, L-aspartate†, L-histidine†, L-leucine†, L-proline, L-serine†, ornithine and †4-hydroxybenzoate. All strains were negative for assimilation of p-arbutin, salicin, suberate†, putrescine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, adipate†, itaconate, mesaconate, phenylacetate†, α-D-melibiose† and azelate.

Test	1	2	3
Susceptibility to:			
Colistin, polymyxin	+	–	–
Assimilation of:			
Sucrose†‡, D-trehalose	+	–	–
Maltitol	+	–	–
trans-Aconitate†	–	(+)	–

*pNP, para-nitrophenyl; pNA, para-nitroanilide.

†Test (based on a different method) was also performed by Holmes *et al.* (1988) with *O. anthropi* and gave congruent results.

‡Test (based on a different method) was also performed by Velasco *et al.* (1998) with *O. intermedium* and gave congruent results.

The majority of *Ochrobactrum* infections have been attributed to *O. anthropi* whereas only two cases of *O. intermedium*

infection, one in a patient with bladder cancer (Apisarnthanarak *et al.*, 2005) and another case after liver transplantation (Möller *et al.*, 1999), have been reported. In both cases, the authors emphasized that correct identification and differentiation of *O. anthropi* and *O. intermedium* by biochemical test systems that are routinely used in clinical diagnosis is difficult and even impossible since *O. intermedium* is not present in the databases of these test systems. Furthermore, misidentification of *Brucella* as *O. anthropi* using API 20NE has been reported (Elsaghir & James, 2003).

This finding is of particular clinical importance since correct identification is the prerequisite for applying an adequate therapy. In their article, Elsaghir & James (2003) reviewed the literature on *O. anthropi* infections of which in 32 cases *O. anthropi* was identified by API 20NE. Some of these cases presented with a clinical picture similar to brucellosis requiring multiple antibiotics for therapy. The authors therefore speculated that at least some of these cases might have been caused by *Brucella* that had been misidentified as *O. anthropi*.

At present, 16S rRNA gene sequencing is used for the identification and differentiation of *O. anthropi*, *O. intermedium* and *Brucella* spp. However, this approach, in particular partial sequencing, is prone to lead to misidentification of these pathogens, because of high sequence similarities of >98%. As an example, in the case of the *O. intermedium* infection described by Apisarnthanarak *et al.* (2005), 16S rRNA gene sequencing was not suitable for identifying the disease-causing agent as *O. intermedium* since sequence similarities of 99% were obtained with both *O. anthropi* and *O. intermedium*. The high similarity to *O. anthropi* reported in this article might have resulted from sequences of misidentified strains deposited in the EMBL database, as described above for *O. anthropi* CCUG 39736. We therefore suggest that the obtained BLAST results are carefully checked for their accuracy by the presence of *O. anthropi*- or *O. intermedium*-specific sequence motifs as described by Scholz *et al.* (2006). Vaidya *et al.* (2006) identified '*O. anthropi*' using biochemical testing and partial sequencing of the 16S rRNA gene that we clearly

identified in this study as *O. intermedium* using extended 16S rRNA and *recA* gene analysis as well as DNA–DNA-hybridization studies, emphasizing the need for additional molecular typing approaches in order to correctly identify *O. anthropi* and *O. intermedium*.

In this context, *recA* analysis was shown to serve (Scholz *et al.*, 2006) as a suitable tool for differentiating *O. anthropi*, *O. intermedium* and *Brucella* species. We therefore recommend *recA* gene analysis for accurate typing of these organisms. *RecA* can also be used to correctly identify *O. pseudintermedium* (H. C. Scholz, personal communication), which was recently isolated from clinical specimens.

In addition to molecular typing, colistin (polymyxin E) and polymyxin B are recommended for differentiating between *O. anthropi* and *O. intermedium* in routine diagnosis (Velasco *et al.*, 1998; Teyssier *et al.*, 2005a), whereby *O. anthropi* is sensitive and *O. intermedium* is resistant to both antibiotics. However, in a screening assay, 5 out of 65 confirmed *O. anthropi* strains were resistant and 3 out of 17 tested *O. intermedium* strains were sensitive to both antibiotics (H. C. Scholz, personal communication).

In summary, commercially available test systems used in routine diagnosis are not suitable for species discrimination within the genus *Ochrobactrum*. We therefore recommend molecular typing (Lebuhn *et al.*, 2006; Scholz *et al.*, 2006; Teyssier *et al.*, 2003a, b, 2005a, b) in addition to physiological testing for a precise species identification.

Besides sequencing of the 16S rRNA gene, analysis of *recA* (Scholz *et al.*, 2006) allows accurate identification and differentiation of *O. anthropi*, *O. intermedium* and *O. pseudintermedium*. However, there is still a need to develop differentiating PCR assays for the rapid and correct identification of these organisms.

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