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Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov.

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A large collection of bacterial strains, immunotrapped from soil and from the wheat rhizoplane, was subjected to polyphasic taxonomy by examining various pheno- and genotypic parameters. Strains were grouped on (inter) repetitive extragenic palindromic DNA (REP) PCR profiles at the intraspecies level. Pheno- and genotypic characters were assessed for representatives from 13 different REP groups. Strains of nine REP groups constituting two physiological BIOLOG clusters fell in the coherent DNA–DNA reassociation group of *Ochrobactrum anthropi*. Strains of two REP groups constituting a separate BIOLOG cluster fell in the coherent DNA–DNA reassociation group of *Ochrobactrum intermedium*. Additional phenotypic characters differentiating *O. anthropi* and *O. intermedium* were found. REP group K strains constituted a different BIOLOG cluster, a separate DNA–DNA reassociation group and a distinct phylogenetic lineage in 16S rDNA homology analysis, indicating that REP group K strains represent a new species. Diagnostic phenotypic characters were found. Closest relatives were *Ochrobactrum* species. The name *Ochrobactrum grignonense* sp. nov. is proposed (type strain OgA9a^T = LMG 18954^T = DSM 13338^T). REP group J strains again constituted a different BIOLOG cluster, a separate DNA–DNA reassociation group and showed, as a biological particularity, a strict preference for the rhizoplane as habitat. Diagnostic phenotypic characters were found. This indicated that REP group J strains represent a further new species, although phylogenetic analyses using 16S rDNA homology were not able to separate the cluster of REP group J sequences significantly from 16S rDNA sequences of *Ochrobactrum anthropi*. The name *Ochrobactrum tritici* sp. nov. is proposed (type strain SCII24^T = LMG 18957^T = DSM 13340^T).

Keywords: *Ochrobactrum*, immunotrapping from bulk soil and wheat rhizoplane, polyphasic taxonomy, geno- and phenotyping, repetitive extragenic palindromic DNA

INTRODUCTION

The genus *Ochrobactrum* was described first by Holmes *et al*. (1988) and belongs to the α-2 subclass of the *Proteobacteria* (De Ley, 1992). The phylogenetic position of *Ochrobactrum* sp. was defined by De Ley (1992) and Yanagi & Yamasato (1993) on the basis of DNA–rRNA hybridization and 16S rDNA homology studies. Swings *et al*. (1992) described the genus *Ochrobactrum*. Its closest known relative is *Brucella* (De Ley, 1992; Moreno, 1992; Yanagi & Yamasato, 1993; Velasco *et al*., 1998). Moyer & Hausler (1992) provide an overview of the genus *Brucella*.

Holmes *et al*. (1988) proposed *Ochrobactrum anthropi*

Abbreviations: REP, repetitive extragenic palindromic DNA; T_m, melting temperature.

The GenBank/EMBL accession numbers for the 16S rDNA sequences of strains CLM18, CLM14, isolate 1a, LAIII106, *O. anthropi* LMG 5140, OgA9a^T, OiC8-6, OiC8a and SCII24^T are AJ242576–AJ242584, respectively.

Table 1. Bacterial strains investigated in this study, their origin and relevant references

Strain* (currently valid or proposed affiliation)	Origin	Reference
Reference strains:		
LMG 3331^T = CIP 149-70^T (*Ochrobactrum anthropi* type strain)	Unknown	Holmes *et al.* (1988)
LMG 5140 = LMG 2134 = LMG 2320(t1) = NCIB 8688 (*Ochrobactrum anthropi*)	Arsenite cattle-dip trays	Holmes *et al.* (1988)
LMG 3301^T = CNS 2-75^T (*Ochrobactrum intermedium* type strain)	Human blood	Holmes *et al.* (1988), Velasco *et al.* (1998)
Isolate 1a (*Ochrobactrum anthropi*)	Soil, Germany	Schloter *et al.* (1996)
Isolates originating from this study:		
ALM4–ALM21, ALM23–ALM32	Grignon soil A, France	This study
CLM5–CLM17, CLM18 (= *Ochrobactrum anthropi* LMG 18953), CLM20–CLM28	Grignon soil C, France	This study
LAI4, LAI8, LAI16, LAI20, LAI24, LAI101, LAI104–LAI110, LAI114, LAI116; LAII1, LAII4, LAII7, LAII10, LAII12, LAII104, LAII108, LAII110, LAII114, LAII118, LAII120; LAIII2, LAIII8, LAIII10, LAIII14, LAIII101, LAIII102, LAIII104, LAIII106 (= *Ochrobactrum tritici* sp. nov. LMG 18958 = DSM 13341), LAIII108, LAIII109, LAIII111, LAIII113, LAIII115, LAIII116	Rhizoplane of wheat (cultivar Lloyd) grown in Grignon soil A, France	This study
LMA1–LMA9	Grignon soil A, France	This study
LMC1, LMC2	Grignon soil C, France	This study
OaA14-1–OaA14-7, OaA19-1, OaA19-2, OaA20-1–OaA20-6	Grignon soil A, France	This study
OaC6-1, OaC6-2, OaC13a, OaC13-1–OaC13-4, OaC17-1–OaC17-4	Grignon soil C, France	This study
OgA9a^T (= *Ochrobactrum grignonense* sp. nov. type strain LMG 18954^T = DSM 13338), OgA9c (= *Ochrobactrum grignonense* sp. nov. LMG 18955 = DSM 13339)	Grignon soil A, France	This study
OiC8a, OiC8b, OiC8-1–OiC8-5, OiC8-6 (= *Ochrobactrum intermedium* LMG 18956)	Grignon soil C, France	This study
RPSCII9	Rhizoplane of wheat (cultivar Soissons) grown in Grignon soil C	This study
SAI1, SAI2, SAI6, SAI8, SAI12, SAI13, SAI15, SAI101, SAI104, SAI105, SAI107, SAI108, SAI110, SAI113, SAI116; SAII1, SAII8, SAII12, SAII16, SAII101 (= *Ochrobactrum anthropi* LMG 18952), SAII102, SAII105, SAII107, SAII110; SAIII4, SAIII5, SAIII8, SAIII16, SAIII20, SAIII101, SAIII104, SAIII106, SAIII108	Rhizoplane of wheat (cultivar Soissons) grown in Grignon soil A	This study
SCII4, SCII8, SCII10, SCII16, SCII20, SCII22, SCII24^T (= *Ochrobactrum tritici* sp. nov. type strain LMG 18957^T = DSM 13340^T), SCII102, SCII103, SCII105, SCII108, SCII110	Rhizoplane of wheat (cultivar Soissons) grown in Grignon soil C	This study

*CIP, Collection de l'Institut Pasteur, Paris, France; CNS, Centre National des Salmonella, Paris, France; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; LMG, Laboratorium Microbiologie Gent Culture Collection, Universiteit Gent, Gent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, UK.

as the sole and type species of *Ochrobactrum*, but they observed heterogeneities in geno- or phenotypic characters within the tested *O. anthropi* collection. The authors described three biovariants (A, C, D) of *O. anthropi* based on phenotypic differences. The strains of biovariant C and three strains of biovariant A (including strain LMG 3301) constituted a homogeneous DNA–DNA hybridization group, and the three biovariant A strains showed DNA–DNA hybridization values of only 51 % or less to *O. anthropi* type strain LMG 3331^T (Holmes *et al.*, 1988). Although this argues for the presence of different species, the authors left the respective strains with *O. anthropi* because the geno- and phenotypic groupings did not lead to consistent results.

In a recent study, Velasco *et al.* (1998) described a new *Ochrobactrum* species, *Ochrobactrum intermedium*, and transferred the former *O. anthropi* reference strain LMG 3301 to *O. intermedium* as the type strain. They

renamed the former *O. anthropi* biotype A strain LMG 3306 as *O. intermedium* LMG 3306 and added three novel clinical isolates. Factors discriminating between *O. anthropi* and *O. intermedium* were their low DNA–DNA hybridization (Holmes *et al.*, 1988), different Western blot profiles of SDS-PAGE-separated whole-cell protein antigens and resistance of *O. intermedium* to colistin and polymyxin B (Velasco *et al.*, 1998). Interestingly, the 16S rDNA similarity of *O. anthropi* and *O. intermedium* was above 98·0 % and 16S rDNA similarity between both *Ochrobactrum* species and *Brucella* spp. was even higher, with values up to 99·28 % between the two genera (Velasco *et al.*, 1998).

O. anthropi strains have been isolated from samples originating from different continents. Most available *O. anthropi* isolates are from human clinical specimens, particularly from immunocompromised persons. *O. anthropi* LMG 5140 has been isolated from arsenical cattle dipping fluid (Holmes *et al.*, 1988) and is described as identical to *O. anthropi* strains LMG 2134 and LMG 2320(t1). There are some reports on the presence of *O. anthropi* in soil, on wheat roots and in internal root tissues of different plants (Aguillera *et al.*, 1993; McInroy & Kloepper, 1994; Sato & Jiang, 1996), but identification was based only on phenotypic characters. *O. intermedium* LMG 3306 has been isolated from a French soil (Holmes *et al.*, 1988) and other *O. intermedium* strains are from human blood (Velasco *et al.*, 1998).

Different mAbs against outer membrane epitopes of *O. anthropi* isolate 1a (Table 1) from a German agricultural soil (Scheyern Experimental Station of the Forschungsverbund Agrarökosysteme München; Schloter *et al.*, 1996) have been produced. Results from quantitative ELISA indicated that the reactive bacterial serotype was present in high numbers [10^4–10^6 bacteria (g dry soil)$^{-1}$] in soil from Scheyern (Schloter *et al.*, 1996) and in two different French soils (Lebuhn *et al.*, 1997) from the Grignon Experimental Station (Barriuso & Houot, 1996). The abundance of *O. anthropi* in soils makes them an ideal tool for ecological studies on microbial diversity at different scales of taxonomic resolution and for an evaluation of exogenous influences on microbial diversity in soil. It is, however, an essential prerequisite for such studies to unambiguously identify and characterize the investigated strains at and below the species level.

The objective of the present study was to examine the identity of bacteria which were immunotrapped from Grignon bulk soil samples and from the rhizoplane of wheat plants grown in the Grignon soils, at different scales of taxonomic resolution. For this purpose, a polyphasic taxonomic approach was used, testing various phenotypic and genotypic characters of the immunotrapped strain collection. Strains belonging to two novel *Ochrobactrum* species were immunotrapped, *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov., and are described here.

METHODS

Soils and plants, immunotrapping and bacterial strains. Soil samples (1 g fresh wt) from two agricultural soils (soils A and C, Grignon Experimental Station, France; Barriuso & Houot, 1996) were suspended in sodium cholate and subjected to a Retsch mill treatment. Bacteria were further removed from soil particles by stirring with Chelex 100, PEG 6000 and Na^+/Amberlite resin IR-120. After centrifugation of 5 μm filtrates, pellets were resuspended in PBS solution (0·145 M NaCl, 0·15 M sodium phosphate). Immunotrapping was carried out as outlined below using aliquots of these suspensions.

Root samples were from two wheat cultivars (*Triticum aestivum* L. cultivars Lloyd and Soissons) which were grown in Grignon soils A and C. Seed surfaces of the wheat cultivars were sterilized, rinsed with sterile distilled water, checked for sterility (on nutrient agar; NA) and aseptically germinated in sterile tapwater in Petri dishes. Three seedlings per cultivar were grown in two plastic tubes filled up with Grignon soils A and C and under controlled growth chamber conditions for 7 d. The harvested root systems (except cultivar Lloyd which did not grow in soil C) were liberated from adherent soil particles by washing gently in sterile tapwater, ground four times in a mortar and suspended in 0·85 % KCl solution. Immunotrapping was carried out as described below using aliquots of the suspensions.

Immunotrapping was performed as previously described (Biebel *et al.*, 1995; Schloter *et al.*, 1995) using mAb 2.11. This antibody showed highest affinity to *O. anthropi* strains and has surface lipopolysaccharides as antigenic epitopes (M. Schloter, personal communication). Briefly, mAb 2.11 was bound to protein A-coated microtitre plates. After washing, aliquots of the soil (or root) suspensions were incubated in the wells, washed and treated with glycine/HCl for antibody disruption. Three repetitions were performed in two (soil samples) or four (root samples) parallels per variant. Parallels were pooled and serial dilutions of the repetitions plated on NA Petri dishes. About 700 isolated colonies were purified on NA. A random selection of these colonies (Table 1) was examined by polyphasic taxonomy.

Table 1 shows a list of strains used in this study and their origin. Several strains that were isolated and described in this study were deposited in the LMG and DSMZ culture collections (Table 1).

Genotypic characters

(i) REP-PCR. Whole cell REP-PCR [PCR amplification of highly variable genomic regions with primers matching repetitive extragenic palindromic DNA (REP) sequences] was carried out using primers REP1R-I and REP2-I (Versalovic *et al.*, 1994) as described by Louws *et al.* (1996), except that 1·5 U *Taq* polymerase (GoldStar; Eurogentec) was added after the initial denaturation step. After gel electrophoresis [in 0·75 × TAE buffer (0·75 × TAE is 30 mM Tris.HCl, 7·5 mM sodium acetate, 0·75 mM sodium EDTA)] of ethidium bromide-stained 1·5 % agarose gels that had been loaded with the PCR products, REP profiles were UV-visualized, digitalized and grouped on similarity using ADOBE photoshop 4 software. REP groups were defined as distinct when they differed by the presence of at least one strong band (signature band). Subgroups of REP groups were defined when respective REP profiles differed only by the presence of weak bands and/or by band strength.

(ii) DNA base composition. Molar G+C contents of strains OgA9a^T, OgA9c, OiC8-6, LAIII106, SAI12 and SCII24^T

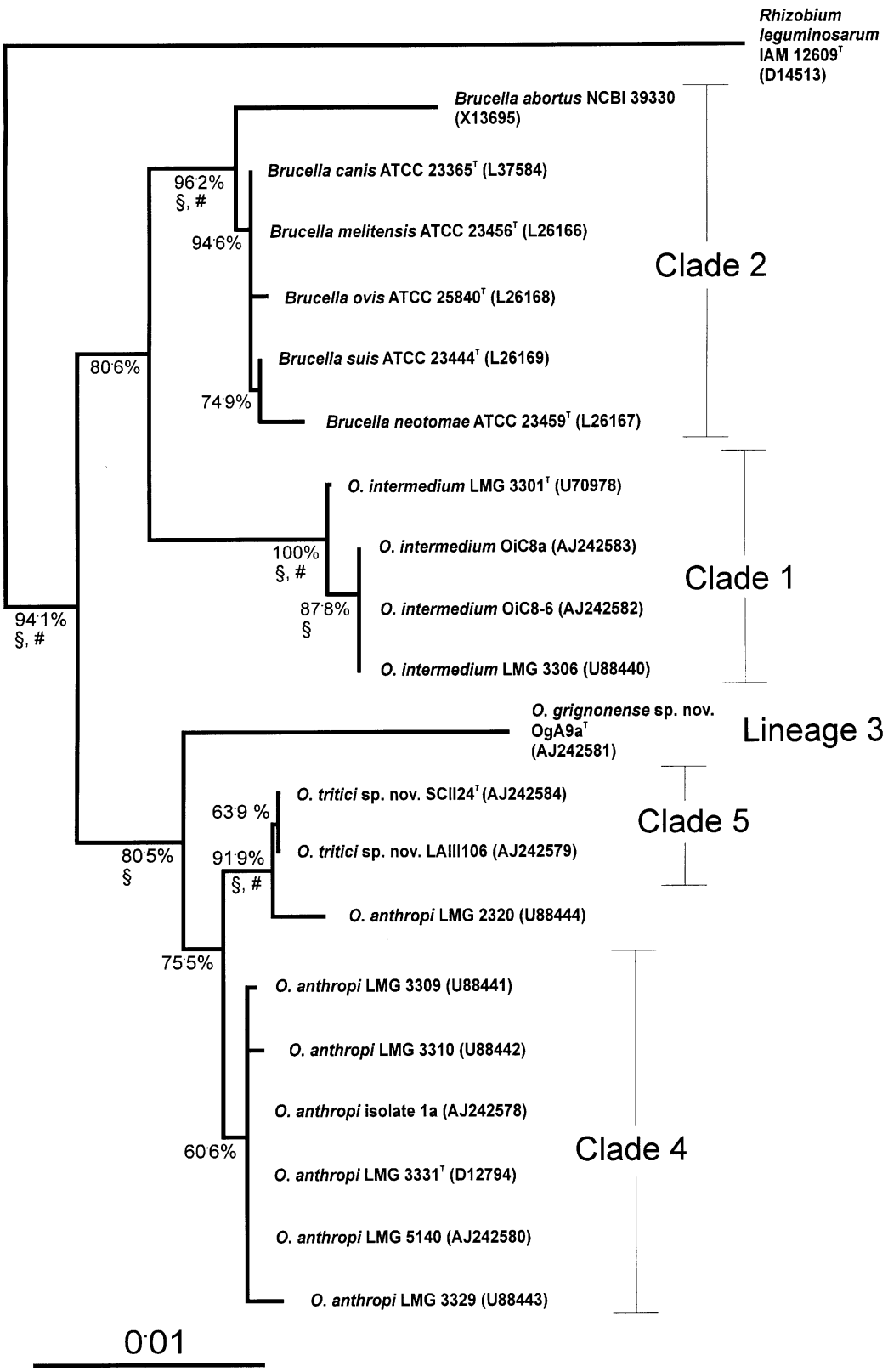

Fig. 1. Phylogenetic tree of 16S rDNA sequences (1292 nt) obtained using CLUSTREE neighbour-joining analysis and the Kimura two-parameter model. Bootstrap probabilities are shown in percentages of 1000 replicates. Scale bar, 0·01 divergent residues per site. Congruent topologies ($P < 25\,\%$) obtained using PUZZLE maximum-likelihood and ARB maximum-parsimony analyses are shown by § and #, respectively. The tree was rooted by outgrouping sequence D14501.

were determined in three replications by custom service at the DSMZ, Braunschweig, Germany. In brief, DNA of French press-treated strains was purified on hydroxylapatite (Cashion *et al.*, 1977). DNA was hydrolysed by P1 nuclease and nucleotides were dephosphorylated by alkaline phosphatase (Mesbah *et al.*, 1989). Deoxyribonucleosides were analysed by HPLC and molar G+C contents were calculated by the ratio dA/dG (Mesbah *et al.*, 1989).

(iii) DNA–DNA hybridization and ΔT_m determination. DNA was extracted and purified in large scale preparation according to Brenner *et al.* (1982) from representative strains of the different REP groups (Table 3), until absorbance ratios 260/280 nm and 260/230 nm were at least 1·75 and 2·1, respectively. DNA–DNA reassociation studies were performed for at least two replicates following the S_1 nuclease TCA (trichloroacetic acid) precipitation method (Crosa *et al.*, 1973; Grimont *et al.*, 1980). A brief description is given as follows. Purified DNA of strains LMG 3331^T, LMG 5140, SCII24^T and OiC8-6 was microdialysed and labelled with ^{3}H-dGTP and ^{3}H-dCTP by nick-translation. After chloroform-isoamylalcohol extraction and dialysis, ssDNA was removed by optimized S_1 nuclease activity. dsDNA was extracted with chloroform-isoamylalcohol, dialysed and the radiolabel of the probes was determined. Unlabelled DNA (including a herring DNA sample to determine unspecific background label) was fragmented on ice by repeated ultrasonication pulses (Ultrasonic 250 TO; Ultrasons) to 0·4 kb and dialysed. Hybridization of labelled probes and unlabelled DNA was performed under stringent conditions (68 °C, 0·42 M NaCl). ssDNA was removed by S_1 nuclease activity (S_1+) or not (S_1-). After TCA precipitation of DNA, the samples were briefly frozen and filtered immediately after melting. Radioactivity of the S_1+ and S_1- reassociates (at least two parallels) was counted (at least six repetitions) and the specific radiolabel of dsDNA was calculated. Values for heterologous reassociation are expressed as percentages of the specific radiolabel of the corresponding homologous reassociates.

The difference between half-maximum melting temperatures of homologous and heterologous DNA reassociates (ΔT_m) was determined according to Grimont *et al.* (1980) for certain strains showing heterologous reassociation values of 50–70 % (Table 3). In brief, reassociated homologous and heterologous DNA was melted at six temperatures between 68 and 100 °C. The samples were treated by S_1 nuclease as described above, precipitated by TCA, filtered and the radiolabel was counted. ΔT_m is the difference between half-maximum homologous and heterologous T_m values.

(iv) Sequencing of 16S rDNA and phylogenetic studies. 16S rDNA from isolate 1a, SCII24^T, OgA9a^T, OiC8a, OiC8-6, LMG 5140, CLM14 and CLM18 was amplified by PCR using primers rD1 and fD1 (Weisburg *et al.*, 1991). Amplificates of isolate 1a, SCII24^T, OgA9a^T, OiC8a and OiC8-6 were purified by low-melting agarose gel electrophoresis and sequenced following the dideoxy termination protocol of Anderson *et al.* (1992) using primers S3, S6, S8, S10, S12, S14, S15 and S17 (Ruimy *et al.*, 1994). Electrophoresis was done in a Beckman Genomyx DNA sequencer with 6 % polyacrylamide sequencing gels as described by Achouak *et al.* (1999). Amplificates of strains LMG 5140, CLM14 and CLM18 were purified using QIAquick PCR purification kit (Qiagen) and double strand sequencing was performed by custom service of TopLab using BigDye Terminator technology (Perkin Elmer Applied BioSystems).

16S rDNA from strain LAIII106, isolate 1a and OiC8a was additionally PCR-amplified using primers 63f and 1387r (Marchesi *et al.*, 1998). DNA purification on QIAquick columns and double strand sequencing (BigDye Terminator technology; Perkin Elmer Applied BioSystems) was done by custom DNA sequencing service at Eurogentec.

EMBL/GenBank 16S rDNA sequences that were used for phylogenetic analyses are listed in Fig. 1. In addition to these, sequences D14501 of *Agrobacterium rhizogenes* IFO 13257^T and X67223 of *Agrobacterium tumefaciens* LMG 196 were used.

The following bases of EMBL/GenBank sequences, for which results of secondary structure analysis (Gutell *et al.*, 1994) indicated that they may not have been correctly determined, were replaced by N in our alignments (*Escherichia coli* numbering for EMBL/GenBank sequence A14565): 770, 771 (U88443); 860, 861 (U70978, U88440, U88441, U88442, U88443, U88444, X13695); 1042 (U88442); 1271 (D12794); 1343 (D12794); 1490 (D12794).

Alignments (CLUSTALW), similarity studies (FASTA) and phylogenetic analyses (CLUSTREE neighbour-joining, PUZZLE maximum-likelihood, PAUP and EDNAPARS maximum-parsimony) of 1292 nt were performed at DKFZ Heidelberg on the GENIUSnet HUSAR computer (http://genome.dkfz-heidelberg.de/biounit/) using Wisconsin Package Version 10.0 [Genetics Computer Group (GCG), Madison, Wisconsin]. Evolutionary distances were corrected for multiple substitutions using the implemented Kimura two-parameter model. Positions with gaps were excluded or treated as a fifth nucleotide state. Probability of nodes were calculated from 1000 bootstrap replications. Additional neighbour-joining, maximum-likelihood and parsimony trees were reconstructed using the same alignment and ARB software (http:\\www.mikro.biologie.tu-muenchen.de; Strunk & Ludwig, 1996).

Phenotypic characters

(i) Colony morphology, growth, microscopy and aminopeptidase test. The immunotrapped strains were checked for colony morphology and development at 20, 30 and 37 °C on NA, tryptone-yeast extract agar (TYA), Luria–Bertani agar (LBA) and 1/10 diluted LBA, 1/10 tryptic soy agar (1/10 TSA) (Atlas, 1995) and on MacConkey agar (bioMérieux 51036). Microbial growth was tested in nutrient bouillon (NB; Prolabo), LB and 1/10 TS (Difco) broth at the same temperatures.

Cell shape and motility were checked using a Zeiss Axioplan 2 epifluorescence microscope at 1000 × magnification. The presence of flagella was visualized by SEM of 1 % glutaraldehyde-fixed bacterial suspensions.

Aminopeptidase activity (Bactident; Merck) was tested according to the manufacturer's recommendations.

(ii) Substrate utilization, catalase and oxidase tests. BIOLOG-GN microtitre plates (Garland & Mills, 1991) containing 95 different carbon sources were incubated with suspensions (in 0·85 % KCl) of 61 randomly chosen representative strains (Fig. 2) from the different REP groups (see REP-PCR section). Strains were grown overnight at 30 °C in NB, washed twice in 0·85 % KCl and adjusted to OD 0·25 at 590 nm (5×10^8 c.f.u. ml^{-1}). After adding 150 µl suspension to each microtitre plate well, plates were incubated for 24 h at 30 °C (two repetitions). Three substrate utilization levels (as a percentage of the water control) of the sole C sources were defined: positive (+, > 160 %), borderline (±,

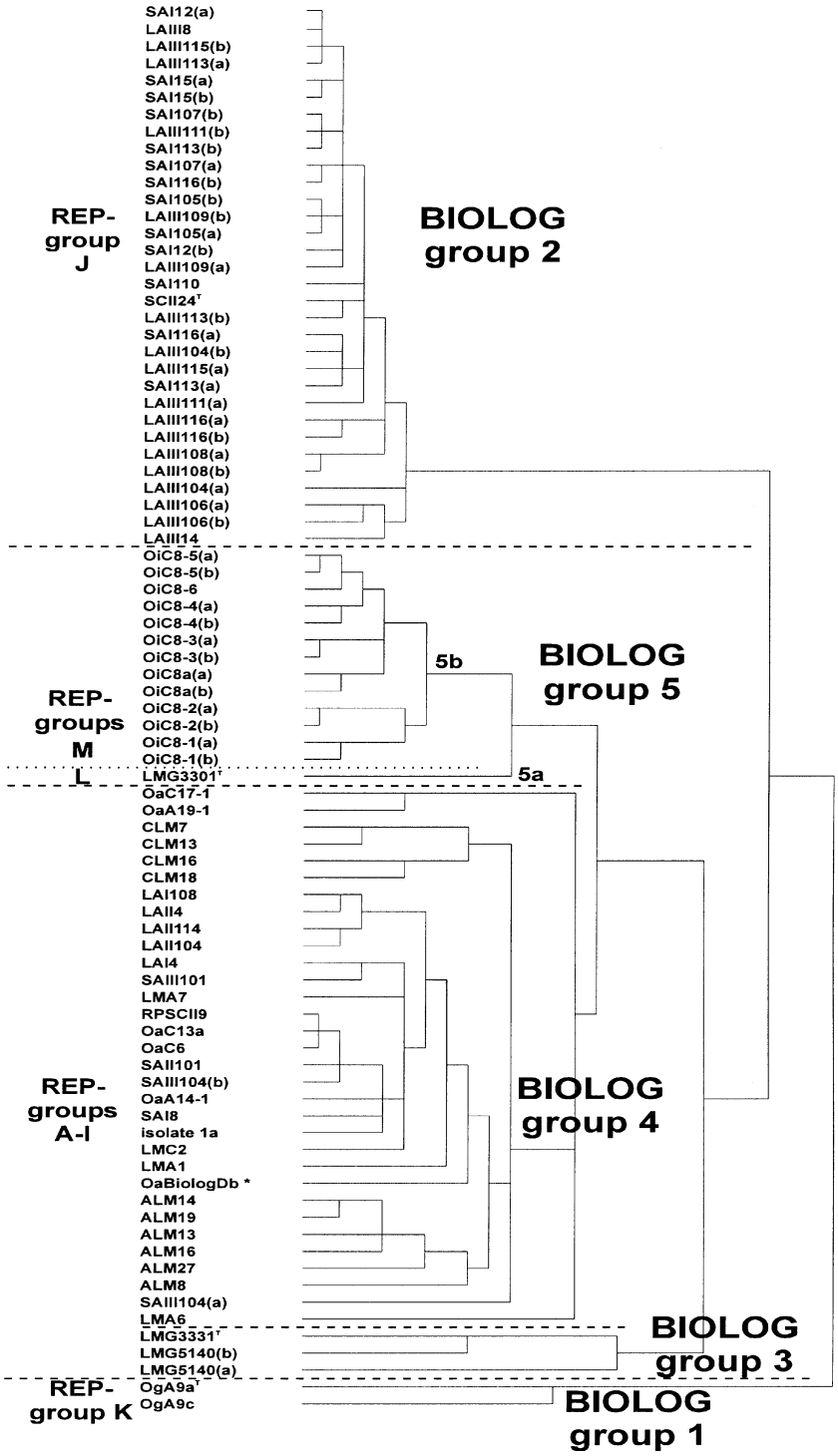

Fig. 2. Hierarchical cluster analysis of BIOLOG-GN substrate utilization profiles. Different profiles obtained for repetitions are marked by (a, b). *OaBiologDb, MICROLOG 2 release 3.50 database profile of *O. anthropi*.

130–160%) and negative (−, < 130%). Substrate utilization profiles were compared to BIOLOG database profiles using MICROLOG 2 release 3.50 software and grouped on similarity by hierarchical cluster analysis using SPSS 6.1 for Windows. Carbon sources of diagnostic value for differentiation between species were identified.

Representative strains from the different BIOLOG clusters (BIOLOG group 1: strains OgA9a^T, OgA9c; BIOLOG group 2: strains SAI12, SCII24^T, LAIII106; BIOLOG group 3: strains LMG 3331^T, LMG 5140; BIOLOG group 4: strains 1a, LAI4, SAII101; BIOLOG group 5: strains LMG 3301^T, OiC8-6) were additionally analysed for substrate

conversion and oxidase activity using API 20NE and API 20E systems (bioMérieux). Tests and evaluations were performed as recommended by the manufacturer. Reactions were categorized as follows: positive, 100–60 %; borderline, 60–50 %; negative, 50–0 %.

Catalase activity of the strains was tested by addition of 3 % H_2O_2 to colonies of bacteria on rigid black paper. Fast development of gas bubbles indicated a positive reaction.

(iii) Antibiotic tests. The above-mentioned representative strains from the different BIOLOG clusters were checked for sensitivity to the antibiotics chloramphenicol (30 μg; bioMérieux 54072), polymyxin B (300 units; bioMérieux 54522), gentamicin (10 μg; bioMérieux 54262) and colistin (10 μg; bioMérieux 54102). Kirby–Bauer agar-diffusion tests (Mueller–Hinton 2 agar; bioMérieux 51075, at least six repetitions) and evaluation of sensitivity or resistance were performed according to the recommendations of the National Committee for Clinical Laboratory Standards, Bauer *et al.* (1966) and Velasco *et al.* (1998).

RESULTS

Genotypic characters

(i) REP-PCR. Table 2 shows the affiliation of immunotrapped, type and reference strains to REP groups and subgroups.

(ii) DNA base composition. Molar G+C percentages of strains LAIII106, SAI12 and SCII24^T from REP group J (Table 2) were 58·8±0·5, 57·4±0·4 and 56·2±0·5 mol %, respectively. G+C contents of REP group K strains OgA9a^T and OgA9c (Table 2) were 57·8±0·3 and 55·3±0·5 mol %, respectively, and that of REP group M strain OiC8-6 (Table 2) was 59·1±0·2 mol %. These values correspond to the description of the genus *Ochrobactrum* (Holmes *et al.*, 1988).

(iii) DNA–DNA hybridization and ΔT_m determination. Results from the DNA–DNA hybridization experiments are presented in Table 3. The tested strains fell into four hybridization groups (Table 3): group 1, consisting of strains of REP groups A–I (including *O. anthropi* LMG 3331^T and LMG 5140); group 2, consisting of strains of REP group J; group 3, consisting of REP group K strain OgA9a^T; and group 4, consisting of strains of REP groups L and M (including *O. intermedium* LMG 3301^T).

(iv) Phylogenetic studies on 16S rDNA and sequence accession numbers. The phylogenetic 16S rDNA trees (1292 nt) that were reconstructed by the different neighbour-joining, maximum-likelihood and maximum-parsimony methods showed identical topologies. CLUSTREE neighbour-joining trees presented the highest resolution of internal branching. The use of sequences X67223 (*A. tumefaciens* LMG 196) or D14501 (*A. rhizogenes* IFO 13257^T) as alternative or additive outgroups did not affect tree topology.

Fig. 1 shows a CLUSTREE neighbour-joining tree (Saitou & Nei, 1987) using the Kimura two-parameter model (Kimura, 1980) and 1000 bootstrap resamplings of 16S rDNA sequences. Congruent topologies with PUZZLE maximum-likelihood (Strimmer & Von Haeseler, 1996) and ARB (Strunk & Ludwig, 1996) maximum-parsimony trees are indicated. The sequences fell into five clades or lineages and the outgroups. Clade 1 comprised the sequences of *O. intermedium* LMG 3301^T and LMG 3306 and of REP group M strains OiC8a and OiC8-6. Clade 2 was composed of *Brucella* sequences. The sequence of REP group K strain OgA9a^T constituted genealogically independent cluster 3 (lineage 3) which branched deeper than clades 4 and 5 (see below). In clade 4, the core *O. anthropi* sequences [*O. anthropi* sequences except U88444 of *O. anthropi* LMG 2320(t1)] were found. The partial sequences AJ242577 and AJ242576 of REP group B strain CLM14 and REP group F1 strain CLM18, respectively, were identical with core *O. anthropi* clade 4 sequences AJ242578 and AJ242580. Clade 5 consisted of sequences AJ242584 and AJ242579 of REP group J strains SCII24^T and LAIII106, respectively. Sequence U88444 of *O. anthropi* LMG 2320(t1) clustered most closely with clade 5 sequences and was separated from clade 5 by a node in CLUSTREE neighbour-joining analysis (Fig. 1). The short branch to clade 5 sequences was collapsed in our maximum-likelihood and parsimony trees, yielding polytomy (not shown). The node separating the cluster of clade 5 plus sequence U88444 from *O. anthropi* clade 4 and the collective of clades/clusters 1–3 was supported by bootstrap probability of 76 % in the CLUSTREE neighbour-joining tree (Fig. 1), and of 65 % and 72 % in PUZZLE maximum-likelihood and ARB maximum-parsimony analyses, respectively. The branch leading from this node to *O. anthropi* clade 4 was collapsed in maximum-likelihood and parsimony analyses, yielding polytomy (not shown).

The genus *Ochrobactrum* showed paraphyly: *O. intermedium* clade 1 was separated from the (core) *Ochrobactrum* clusters/clades 3–5 (containing the type species *O. anthropi*) by a node leading to *Brucella* clade 2, and sequences of *O. intermedium* clade 1 were more homologous to those of the *Brucella* than to those of the (core) *Ochrobactrum* clade (Fig. 1).

Phenotypic characters

(i) Motility, cell shape, growth, colony morphology and aminopeptidase reaction. The studied bacteria were all short rods and highly motile. The rods belonging to REP groups A–I were 0·4–0·8 × 0·3–0·4 μm, rods of REP group K were 0·6–1·2 × 0·4 μm, and rods of REP group J, L and M were 0·6–1·4 × 0·4–0·6 μm. SEM showed that variable numbers of flagella can be present: two subpolar or several flagella with peritrichous insertion.

The tested strains grew slowly in/on diluted TS and LB, better in/on LB, TY and on MacConkey, and best in/on NB media/agar plates. The strains were able to

Table 2. Affiliation of immunotrapped and reference strains to REP groups and subgroups

REP group…	A	B	C				D	E		F		G		H	I	J		K	L	M
Subgroup…			C1	C2	C3	C4		E1	E2	F1	F2	G1	G2			J1	J2			
Reference strains	LMG 3331^T	LMG 5140	Isolate 1a																LMG 3301^T	
Isolates from bulk soil		ALM25, ALM26, ALM28, ALM30, ALM32, CLM5, CLM8–CLM11, CLM14–CLM16, CLM27, CLM28	OaA14-1–OaA14-7, OaA19-1, OaA19-2, LMA1–LMA9 OaC6-1, OaC6-2, OaC17-1–OaC17-4, LMC1, LMC2				CLM7, CLM12, CLM13, CLM17, CLM25	CLM26	ALM14, ALM16–ALM21, ALM27, ALM31	CLM6, CLM18, CLM20–CLM24, OaA20-3, OaA20-4, OaA20-5	ALM13, ALM15, ALM24, ALM29, ALM13, OaA20-1, OaA20-2, OaA20-6	OaC13-1, OaC13-2, OaC13-3, OaC13-4, OaC13-5, OaC13a, ALM4–ALM12							OgA9a^T, OgA9c		OiC8a OiC8b, OiC8-1–OiC8-6
Isolates from wheat rhizoplane			LAI4, LAI8, LAI16, LAI20, LAI24, LAI101, LAI104, LAI109, LAII10, LAIII2, LAIII102, RPSCII9, SAI1, SAI2, SAI13, SAI108, SAII2, SAII8, SAII12, SAII16, SAII102, SAII105, SAII107, SAII110, SAIII4, SAIII5, SAIII8, SAIII12, SAIII16, SAIII20, SAIII106, SAIII108, SCII4, SCII8, SCII16, SCII20, SCII102, SCII103, SCII105, SCII108	SCII10, SCII22, SCII110	SAII101	SAIII101						LAI12, LAI105, LAI106, LAI107, LAI116, LAII1, LAII4, LAII7, LAII108, LAII118, LAII120, LAIII10, LAIII101, SAI6, SAI104, SAII1	LAI108, LAI110, LAI114, LAII104, LAII110, LAII114, SAI101	SAI8	SAIII104	LAIII8, LAIII14, LAIII104, LAIII106, LAIII108, LAIII109, LAIII111, LAIII113, LAIII115, LAIII1116, SAI12, SAI15, SAI105, SAI107, SAI110, SAI113, SAI116	SCII24^T			

Table 3. DNA–DNA reassociation values and ΔT_m

DNA of strain (REP group)	Reassociation with DNA from strain LMG 3331^T as the probe (%)	ΔT_m to DNA probe from strain LMG 3331^T (°C)	Reassociation with DNA from strain LMG 5140 as the probe (%)	Reassociation with DNA from strain OiC8-6 as the probe (%)	Reassociation with DNA from strain SCII24^T as the probe (%)	Hybridization group
LMG 3331^T (A)	100	0		45·3±9·2	50·4±4·8	1
LMG 5140 (B)	91·3±9·2		100	50·2±2·3	53·8±8·0	1
CLM14 (B)			97·8±9·7	43·9±4·3		1
Isolate 1a (C1)	69·6±5·2		84·5±2·2	44·2±5·3	57·8±12·4	1
SAII16 (C1/2)	74·8±12·8					1
SAIII4 (C1/2)	75·5±11·0					1
SAIII12 (C1/2)	78·4±5·2		73·3±4·1	46·0±5·0	52·8±8·8	1
SCII20 (C1/2)	78·4±15·1		75·7±14·2	40·0±11·8	51·3±9·4	1
SCII22 (C2)			87·7±11·7	48·0±5·3		1
CLM7 (D)					54·7±11·2	1
CLM12 (D)	81·0±10·5		76·4±5·8	54·6±8·1		1
ALM19 (E2)	90·1±21·2		88·5±8·7	42·3±5·8	54·5±8·0	1
CLM18 (F1)	68·7±8·9					1
CLM21 (F1)			76·5±16·9	41·2±6·0		1
ALM15 (F2)	76·8±5·2		73·4±5·0	44·3±4·0	47·4±5·5	1
ALM8 (G1)	71·1±12·7		89·6±15·0	46·6±4·7	47·7±3·5	1
LAI105 (G2)	68·6±13·4					1
LAII4 (G2)			84·4±7·7	49·2±7·0		1
LAII7 (G2)	74·5±22·8		65·9±8·2	42·5±5·6	49·0±2·6	1
SAI8 (H)	87·1±0·7			58·1±0·7		1
SAIII104 (I)	71·8±3·1	4·4±0·3		50·6±5·5	63·8±7·3	1
SCII24^T (J2)	56·1±0·7	8·5±0·2		46·9±5·0	100	2
LAIII106 (J1)	51·0±0·3	8·4±0·3		40·6±9·4	98·4±9·6	2
SAI110 (J1)	59·8±0·3		49·3±8·6	42·9±7·0	100·3±10·1	2
OgA9a^T (K)	17·3±2·1		20·4±5·1	19·2±1·3	17·1±5·6	3
OiC8-6 (M)	38·6±5·6			100	40·4±7·7	4
LMG 3301^T (L)	49·5±0·6			88·2±5·5	43·8±8·5	4
OiC8a (M)	48·8±0·5		39·3±3·5		33·7±11·6	4
OiC8b (M)	40·7±6·6			95·1±6·9	38·3±11·9	4
OiC8-2 (M)	42·2±5·9			99·1±5·6	44·4±9·7	4

grow at 4 and 40 °C, and at pH values of 3–9. Optimum growth temperature was 30 °C and the pH optimum was pH 6–7.

After growth on NA for 24 h, strains of REP groups A–G and I developed circular, low convex, smooth, shining, entire colonies of about 1 mm in diameter, with the exception that some rhizoplane isolates of REP groups C and G (strains LAI106, LAI108, LAI109, LAII1, LAII110, LAIII101, SAI1, SAI2, SAI6, SAII1, SAIII108, SCII105 and SCII108), as well as strain SAI8 (REP group H), soon became mucoid and opaque. Colonies of REP group J were mucoid-opaque and quickly became highly confluent. Colonies of REP group K strains were circular, entire, low convex, smooth, milky-opaque and about 1 mm in diameter. Colonies of REP group L and M strains were mucoid-opaque and tended to confluency, with the exception of strain LMG 3301^T, which grew first as circular, low convex, smooth, shining, entire colonies that later became mucoid-opaque.

All tested strains were aminopeptidase-positive or effected borderline coloration, suggesting Gram-negative reaction. No pigment development was observed.

(ii) Substrate utilization, catalase and oxidase tests. All tested strains were catalase- and cytochrome oxidase-positive, suggesting aerobic metabolism. Fig. 2 shows a similarity dendrogram of BIOLOG-GN substrate utilization profiles of representative strains from the different REP groups (Table 2). Five distinct physiological BIOLOG clusters were obtained: BIOLOG group 1, consisting exclusively of REP group K strains; BIOLOG group 2, consisting only of REP group J strains; BIOLOG group 3, consisting of *O. anthropi* strains LMG 3331^T and LMG 5140; BIOLOG group 4 (being most closely related to group 3), comprising REP group B–I strains (except strain LMG 5140) as well as the BIOLOG database profile of *O. anthropi*; and BIOLOG group 5, consisting of REP group L *O. intermedium* strain LMG 3301^T (constituting BIOLOG subgroup 5a) and the subcluster of

Table 4. Diagnostic phenotypic characters for the differentiation between *Ochrobactrum* pheno- and genotypes

Phenotyping test	Parameter	REP group/BIOLOG group (*Ochrobactrum* species)*			
		A–I/3, 4 (*O. anthropi*)	J/2 (*O. tritici*)	K/1 (*O. grignonense*)	L, M/5 (*O. intermedium*)
BIOLOG-GN	γ-Hydroxybutyric acid	+	−	+	+
	Sebacic acid	− to ±	+	− to ±	−
	Adonitol	+	+	−	+
	D-Glucosaminic acid	+	+	−	+
	Malonic acid	−	−	+	−
	D-Trehalose	+†	+	−	+
	Quinic acid	+‡	−	−	−
	Xylitol	− to ±§	−	±	+‖
	Cellobiose	+	−	− to ±	+
	Gentiobiose	+	−	− to ±	+
	Glycerol	± to +¶	−	+	−
	DL-α-Glycerol phosphate	± to +#	− to ±	± to +	−
	DL-Carnitine	+**	− to ±††	− to ±	+
	Uridine	± to +‡‡	− to ±	+	− to ±
API 20E	Melibiose (acid production after 48 h)	−	−	+	−
	Arabinose (acid production after 48 h)	−	−	± to +	−
	Urease (after 24 h)	+	+	−	−
	Urease (after 48 h)	+	+	−	±
API 20NE	Mannose (assimilation after 24 h)	−	−	+	−
	Maltose (assimilation after 24 h)	− to ±	− to ±	−	+
	Maltose (assimilation after 48 h)	+	+	−	+
	Gluconate (assimilation after 48 h)	− to ±	+	− to ±	− to ±
	Citrate (assimilation after 24 h)	−	− to ±	+	+
	Citrate (assimilation after 48 h)	− to ±	± to +	+	+
	Urease (after 24 h)	+	+	−	−
	Urease (after 48 h)	+	+	−	−
Sensitivity to antibiotics	Polymyxin B	Susceptible	Susceptible	Resistant	Resistant
	Colistin	Susceptible–intermediate	Susceptible	Resistant	Resistant
	Chloramphenicol	Resistant	Intermediate	Susceptible–intermediate	Resistant
Colony morphology	On nutrient agar (after 24 h)	Shiny/opaque, isolated/mucoid	Opaque, highly confluent	Opaque, isolated	Opaque, mucoid/confluent§§

* For values corresponding to symbols +, ± and −, see Methods, phenotypic characters.

† Isolate 1a, LMA1, ALM27: − to ±.

‡ Strains LMG 3331^T, LMG 5140: − to ±.

§ Strains CLM7, CLM13: +.

‖ Strain LMG 3301^T: −.

¶ Strains LAII114, LMA1, LMA6, OaA14-1, OaA19-1, OaC17-1, SAI8: −.

Strains LAI4, LAII104, LAII114, LMA1, LMA6, LMC2, OaA14-1, OaA19-1, OaC17-1, SAI8, SAII101, SAIII101: −.

** Strains CLM7, CLM13, LMG 3331^T, LMG 5140: − to ±.

†† Strains LAIII111, LAIII115, SCII24^T: +.

‡‡ Strains LAI108, LMG 3331^T: −.

§§ Strain LMG 3301^T: shiny, isolated/mucoid.

REP group M isolates (constituting BIOLOG subgroup 5b). MICROLOG 2 release 3.50 database profiles of *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella neotomae*, *Brucella ovis* and *Brucella suis* formed a separate branch that was again different from the cluster collective of BIOLOG group 1–5 profiles (not shown).

MICROLOG database searching significantly identified BIOLOG group 3 and 4 strains (similarity > 75%) as *O. anthropi*, except strains LMG 5140, LMA6 and OaA19-1, which were not identified significantly (similarities between 50 and 75%) as *O. anthropi*. For BIOLOG group 5 strains, similarities to the *O. anthropi* database profile ranged between 71 and 86%; for isolates of BIOLOG group 1, similarities were between 40 and 76% and for BIOLOG group 2, between 53 and 72%.

All tested strains converted (+) the following sole sources of carbon (BIOLOG-GN): Tween 40, Tween 80, L-arabinose, D-arabitol, i-erythritol, D-fructose, L-fucose, D-galactose, α-D-glucose, m-inositol, maltose, D-mannose, psicose, L-rhamnose, turanose, monomethyl succinate, β-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, succinic acid, bromosuccinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-serine, L-threonine, γ-aminobutyric acid and inosine. All representative strains

from the five BIOLOG groups (including subgroups 5a and 5b; Fig. 2) gave positive (+) reactions in the following API 20NE and API 20E tests: denitrification (gas production from NO_3^-/NO_2^-, reduction of NO_3^- to NO_2^-), glucose assimilation (48 h), arabinose assimilation (48 h) and citrate utilization (48 h).

None of the tested strains utilized (−) L-phenylalanine or phenylethylamine as sole C source (BIOLOG-GN). All tested strains gave negative (−) reactions in the following API 20NE and API 20E tests: indole production, glucose acidification (fermentation), arginine dihydrolase, gelatin hydrolysis (protease), β-galactosidase (PNPG), mannitol assimilation (24 h), adipate assimilation, phenylacetate assimilation, β-galactosidase (ONPG), lysine decarboxylase, ornithine decarboxylase, citrate utilization (24 h), H_2S production, tryptophan deaminase, gelatinase, and acid production from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose and amygdalin.

Physiological characters of diagnostic value for the identification of *Ochrobactrum* species (within BIOLOG groups 1–5) are listed in Table 4. Other individual physiological responses of strains are listed in Table 5.

In contrast to BIOLOG group 1–5 (*Ochrobactrum* spp.) strains, *Brucella* spp. did not utilize the following BIOLOG-GN substrates (data from MICROLOG 2 release 3.50 database): D-arabitol, m-inositol, psicose, L-rhamnose, bromosuccinic acid, L-alanine, L-alanylglycine, L-aspartic acid, glycyl-L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-threonine, γ-aminobutyric acid, sucrose, D-gluconic acid, D-glucuronic acid, *N*-acetyl-D-glucosamine, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, L-ornithine (*Brucella canis*, ±) and D-serine. Due to the high dissimilarity of *Ochrobactrum* spp. and *Brucella* spp. profiles, these genera were clearly separated in hierarchical cluster analysis (not shown).

(iii) Antibiotic tests. All tested representative strains from the five BIOLOG groups (including subgroups 5a and 5b; Fig. 2) were sensitive to gentamicin (strain LMG 3301^T, intermediate). Sensitivity reactions of the strains against polymyxin B, colistin and chloramphenicol were of diagnostic value for differentiation between BIOLOG groups 1–5. The differential responses are given in Table 4.

DISCUSSION

The aim of the present study was to assess the taxonomic affiliation of bacterial strains which were immunotrapped from soil and from the rhizoplane of wheat. For this purpose, a polyphasic approach (Vandamme *et al*., 1996) was used, assessing various geno- and phenotypic characters. Decision priority was given to the results of DNA–DNA hybridization because the phylogenetic approach using 16S rDNA homology has some limitations for species definition (Stackebrandt & Goebel, 1994).

For the immunotrapping, mAbs raised against isolate 1a were used. This strain has been characterized as *O. anthropi* by phenotypic features (Schloter *et al*., 1996). DNA–DNA reassociation values of 70% with DNA from *O. anthropi* LMG 3331^T and 85% with DNA from *O. anthropi* LMG 5140 (Table 3), phenotypic affiliation of isolate 1a to BIOLOG group 4 (which clustered most closely with BIOLOG group 3 consisting of *O. anthropi* LMG 3331^T and *O. anthropi* LMG 5140; Fig. 2), and 16S rDNA sequence identity with *O. anthropi* LMG 3331^T and *O. anthropi* LMG 5140 (Fig. 1) prove that isolate 1a is *O. anthropi*.

The tested strain collection fell into 13 different REP groups (A–M; Table 2). Most strains of these 13 REP groups were tested by BIOLOG profiling (Fig. 2). Since only representative strains from the five BIOLOG groups were characterized by additional phenotyping tests (API 20E, API 20NE and sensitivity to antibiotics; Table 4), further phenotyping studies should be performed with a larger set of strains to confirm the differentiating value of the respective diagnostic characters presented in Table 4. Selecting representatives from the different REP groups for DNA–DNA reassociation and 16S rDNA homology studies (Table 3, Fig. 1) is justified because REP profiling resolves below the species level (Louws *et al*., 1996; Vandamme *et al*., 1996), which is currently defined by values for reassociation of heterologous DNA of $\geqslant 70\%$ and ΔT_m values of less than 5 °C (Stackebrandt & Goebel, 1994). Taxonomy using 16S rDNA homology provides less resolution. There is no report that two strains sharing the same REP profile belong to different species.

BIOLOG group 3 profiles (*O. anthropi* LMG 3331^T of REP group A and *O. anthropi* LMG 5140 of REP group B) were most similar to the cluster of BIOLOG group 4 profiles (REP group B–I strains) (Fig. 2). Since no difference between the REP profiles of *O. anthropi* LMG 5140 and REP group B strains was found (Table 2), BIOLOG group 4 strains constitute a phenotype which is different from BIOLOG group 3 strains. However, these two phenotypes should be held as one species because they were almost identical in their genotypic character (see below). Representatives of BIOLOG group 3 and 4 (REP group A–I) strains were clearly distinguishable by several diagnostic characters from BIOLOG group 1, 2 and 5 (REP group J–M) strains (Table 4). Representatives from REP group A–I (BIOLOG groups 3 and 4) strains formed the coherent DNA–DNA reassociation group 1 containing *O. anthropi* type and reference strains (Table 3). It is therefore concluded, and phylogenetic analyses did not contradict this (see below), that REP group A–I strains are *O. anthropi*.

In our phylogenetic analysis of 16S rDNA sequences using CLUSTREE neighbour-joining, monophyly of clade 4, consisting of core *O. anthropi* sequences [excluding sequence U88444 of *O. anthropi* LMG 2320(t1)], was weakly supported (probability of 61%

Table 5. Physiological characters of *Ochrobactrum* sp. strains

Physiological character	Strains giving positive (+) results*	Strains giving borderline (±) results*	Strains giving negative (−) results*
α-Cyclodextrin†	92 %	LMA6, CLM16, CLM7, CLM13, OgA9c	
Dextrin†		OgA9a^T	
Glycogen†	75 %	ALM13, isolate 1a, LAI4, LAI108, LAII104, LAII114, LMA6, OaA14-1, OaC17-1, OgA9c, SAIII101	ALM14, ALM19, ALM27, OaA19-1
N-Acetyl-D-galactosamine†	84 %	LAIII111, LAIII116(a), LMG 5140, OiC8a, OiC8-2, OiC8-3, OiC8-4(a), OiC8-5, OiC8-6	ALM13, LAIII116(b), OiC8-4(b)
N-Acetyl-D-glucosamine†	98 %		LMA1
α-Lactose†		OaA19-1, LMA7	97 %
α-D-Lactose lactulose†		Isolate 1a	98 %
D-Mannitol†	87 %	LAIII14, LAIII104, LAIII106(b), LAIII108, LAIII109, LAIII111(b), LMG 3301^T, LMG 5140(b), SAI105, SAI107, SAI110	
D-Melibiose†	ALM8	ALM13, CLM7, CLM13, CLM16, LMA1, LMA7, LMG 3301^T, OaA14-1, OgA9a^T	84 %
Methyl β-D-glucoside†	ALM8, ALM14, ALM16, ALM19	ALM13, CLM13, LMA6, LMA7, LMG 3301^T, LMG 3331^T, OaA14-1, OgA9a^T, OgA9c,	79 %
D-Raffinose†		ALM8	98 %
D-Sorbitol†	98 %	LAIII106(b)	OgA9c
Sucrose†	98 %	OgA9a^T	
Methyl pyruvate†	95 %	LMG 3301^T, OiC8-5	LMA6
Acetic acid†	95 %	OgA9a^T	LAIII108, LMA6
cis-Aconitic acid†	95 %	LMG 3331^T, SAI113, SAI116(b)	LMG 5140
Citric acid†		LMG 5140(b)	LMG 3331^T
Formic acid†	89 %	LMG 5140, SAI107(a), SAI110, SAI116(b), OiC8-5, OiC8-6	OiC8a, OiC8-3, OiC8-4
D-Galactonic acid lactone†	97 %	OiC8-2	LMG 5140(b), OgA9c
D-Galacturonic acid†	67 %	ALM13, ALM14, ALM16, CLM7, LAIII104(b), LMC2, LMG 3301^T, LMG 5140(a), OaC17-1, OiC8-3, OiC8-4(a), OiC8-5(b), OiC8-6, SAIII104	ALM19, ALM27, LAI4, LAIII104(a), LMA7, LMG 3331^T, LMG 5140(b), OaA19-1, OiC8-4(b), SAIII101
D-Gluconic acid†	95 %	LMG 5140(a), OiC8-4, OiC8-5, OiC8-6	
D-Glucuronic acid†	97 %	LMG 3301^T, LMG 3331^T	
α-Hydroxybutyric acid†	98 %	OaA19-1	LMG 5140(a)
p-Hydroxyphenylacetic acid†		CLM7, CLM13, LAIII113(b), LMA7, LMG 3301^T, LMG 3331^T, OgA9c, OiC8-1, SAI12(b), SAI105(a), SAI110, SAI113(a), SAIII104(a), SCII24^T	85 %
Itaconic acid†	LMC2	CLM7, CLM16, OgA9c	93 %
α-Ketovaleric acid†	77 %	LAI4, LAI108, LAII4, LAII104, LAII114, LMC2, LMG 3301^T, LMG 5140(a), OaC17-1, OiC8a(a), OiC8-4, OiC8-5, OiC8-6, SAI8,	OaA19-1, OiC8-2
Propionic acid†	90 %	Isolate 1a, OgA9a^T	LAIII14, LMA6, OaA19-1, OaC17-1
D-Saccharic acid†		LMA7, OgA9a^T	97 %
Alaninamide†	92 %	OaA19-1	LAI108, LAII4, LAII104, LAII114

Table 5 (*cont.*)

Physiological character	Strains giving positive (+) results*	Strains giving borderline (±) results*	Strains giving negative (−) results*
L-Pyroglutamic acid†	CLM18	ALM16, CLM7, CLM13	93 %
D-Serine†	97 %	LMG 3331^T	LMG 5140
Urocanic acid†	98 %	SAI15	
Thymidine†		CLM7, CLM16, CLM13, CLM18, LMG 5140(a), OgA9c	92 %
Putrescine†		CLM7, isolate 1a, LMG 5140(a), OgA9c	95 %
2-Aminoethanol†		CLM16, LAII4, LMG 5140(a)	97 %
2,3-Butanediol†		ALM16, CLM7, LAIII104(b), LAIII106(a), LAIII113(b), LAIII115(a), LAIII116, LMG 5140, OgA9c, SAI8, SAI15, SAI113(a), SAI116(a), SAIII104(a)	89 %
Glucose 1-phosphate†		LAIII104(b), LAIII108(b), LAIII111(a), LAIII115(a), LMG 5140(a), SAI110, SAI113(a), SAI116(a), SAIII104	97 %
Glucose 6-phosphate†	CLM13, CLM16	ALM8, ALM19, CLM7, CLM18, LAI108, LAII4, LAIII104(b), LAIII106(a), LAIII108, LAIII111, LAIII113(b), LAIII115, LMA7, LMG 3331^T, LMG 5140, OaC13a, OgA9c, OiC8-5(a), OiC8-1(b), OiC8-2, OiC8-3(b), RPSCII9, SAI15, SAI107(b), SAI110, SAI113, SAI116(a), SAIII101, SAIII104, SCII24^T	61 %
Aesculin hydrolysis (24 h)‡		SCII24^T	92 %
Aesculin hydrolysis (48 h)‡	LMG 3301^T, SAI12, SCII24^T		75 %
Glucose assimilation (24 h)‡	LMG 3331^T, OgA9a^T, OgA9c	Isolate 1a, SAI12, SCII24^T	50 %
Arabinose assimilation (24 h)‡		67 %	Isolate 1a, LAIII106, LMG 5140, SAII101
Mannose assimilation (48 h)‡	50 %	LAIII106, LMG 3331^T, SCII24^T	LAI4, LMG 5140, SAII101
Mannitol assimilation (48 h)‡	SAI12	Isolate 1a, LMG 3301^T, SCII24^T	67 %
N-Acetylglucosamine assimilation (24 h)‡	LMG 3301^T, OgA9a^T, OgA9c	LAIII106, SAI12, SCII24^T	42 %
N-Acetylglucosamine assimilation (48 h)‡	83 %	LAI4, SAII101	
Gluconate assimilation (24 h)‡		LAIII106, OgA9a^T, SCII24^T	75 %
Caprate assimilation (24 h)‡		SAI12, SCII24^T	83 %
Caprate assimilation (48 h)‡	58 %	LAI4, LMG 5140, SAII101, OgA9a^T	LMG 3331^T
Malate assimilation‡	92 %		SAI12
Acetoin production‡		LMG 3331^T, OgA9a^T, OgA9c	75 %

* (a) and (b) denote different responses of a strain in repeated experiments. For values corresponding to symbols +, ± and − (see Methods, phenotypic characters section).

† Utilization, using BIOLOG-GN system.

‡ Using API 20NE or API 20E systems.

for the respective node; Fig. 1) and separated from clade 5 (sequences AJ242584 and 242579 of REP group J strains SCII24^T and LAIII106), which represents a novel *Ochrobactrum* species (*Ochrobactrum tritici* sp. nov., see below). The branch of clade 4 was collapsed to the subterminal node in maximum-likelihood and maximum-parsimony trees, yielding polytomy. This may be due to the very high 16S rDNA similarity of *O. anthropi* and *O. tritici* sp. nov. strains of about 99 %. Stackebrandt & Goebel (1994) showed

that the phylogenetic 16S rDNA approach possesses almost no resolution for species delineation at similarity values above 97·5%, as compared to DNA–DNA hybridization approaches. Accordingly, our results show that phylogeny of 16S rDNA is not suitable for delineation of these two species (see also below).

O. anthropi strains LMG 2320(t1), LMG 5140 and LMG 2134 are considered to be identical in the BCCM/LMG bacteria collection. However, sequence U88444 of *O. anthropi* LMG 2320(t1) clustered most closely with clade 5 sequences (REP group J strains, *O. tritici* sp. nov., see below), whereas sequence AJ242580 of *O. anthropi* LMG 5140 clustered with clade 4 core *O. anthropi* sequences in our reconstructed 16S rDNA trees (e.g. Fig. 1). Only four consecutive base pairs in the hypervariable helix 10 differentiated clade 5 from clade 4 sequences (not shown). *O. anthropi* LMG 2320(t1) (and *O. intermedium* strains) shared the typical sequence of clade 5 strains (GAAA), but *O. anthropi* LMG 5140 had the typical sequence of *O. anthropi* clade 4 (TTCG). Jumas-Bilak *et al.* (1998) showed that *O. anthropi* and *O. intermedium* have two chromosomes and two *rrn* genes. Assuming that this is also the case for clade 5 strains (*O. tritici* sp. nov.), an explanation for the observed heterogeneity may be that 16S rDNA operons are different in the respective regions, and that either one or the other 16S rDNA was preferentially amplified during PCR. Genetic crossing-over in ribosomal sequences (Sneath, 1993) also cannot be excluded. Again, this suggests that phylogeny of 16S rDNA is not suited to delineate clade 4 (*O. anthropi*) and clade 5 strains (*O. tritici* sp. nov.). However, the taxonomic position of *O. anthropi* LMG 2320(t1) is hence uncertain and should be re-examined.

REP group L (*O. intermedium* LMG 3301^T) and REP group M strains formed the coherent phenotypic cluster of BIOLOG group 5 (Table 2, Fig. 2). Several phenotypic parameters were found which differentiated representatives of this group from BIOLOG group 1–4 strains (Table 4). Representatives from BIOLOG group 5 constituted DNA–DNA reassociation group 4 containing *O. intermedium* type strain LMG 3301^T (Table 3), and 16S rDNAs of strains OiC8a and OiC8-6 were most homologous to those of *O. intermedium* strains (Fig. 1). The G+C content of 59·1 mol% for strain OiC8-6 corresponds to the value given for the genus *Ochrobactrum* (Holmes *et al.*, 1988). It is concluded that REP group M strains are *O. intermedium*.

In our phylogenetic trees of 16S rDNA sequences (e.g. Fig. 1), clade 1 of *O. intermedium* sequences was found to be more closely related to clade 2 of *Brucella* sequences than to clade 4 sequences of the type species *Ochrobactrum anthropi*. Velasco *et al.* (1998) also presented this paraphyly of *Ochrobactrum*. Paraphyly is not rare in the α-2-subclass of the *Proteobacteria* and is the source of taxonomic debates (Yanagi & Yamasato, 1993; Moreno, 1997). However, results from rRNA–DNA hybridization (De Ley *et al.*, 1987) suggested a position for *O. intermedium* between and equally distant from *Brucella* spp. and *O. anthropi*. DNA–DNA hybridization studies indicated an even closer relationship of *O. intermedium* to *O. anthropi* than to *Brucella* spp. (Holmes *et al.*, 1988). The genera *Brucella* and *Ochrobactrum* were clearly separated in the dendrogram on various phenotypic characters presented by Holmes *et al.* (1988) as well as in our cluster analysis of BIOLOG profiles (not shown). Since the phylogenetic 16S rRNA approach possesses low resolution at the terminal branches as compared to DNA–DNA hybridization when 16S rDNA similarity values are high (Stackebrandt & Goebel, 1994), and 16S rDNA similarity between the genera *Ochrobactrum* and *Brucella* show even values of over 96% (Fig. 1; Velasco *et al.*, 1998), it seems to be reasonable to base polyphasic taxonomy of the *Ochrobactrum–Brucella* group predominantly on the results of DNA–DNA hybridization and phenotyping. Concerning the paraphyly of *Ochrobactrum*, a taxonomic revision of the genus is strongly recommended. A possible solution, yielding monophyletic lineages which correspond to the results from genotyping, would be to give *O. intermedium* the status of a new genus. This will be a matter for a subsequent paper.

Very few phenotypic features differentiate *O. intermedium* from *O. anthropi* (Holmes *et al.*, 1988; Velasco *et al.*, 1998). Using API 20E and API 20NE systems, strong assimilation of maltose (after 24 h) and citrate, as well as no or very low urease activity of *O. intermedium*, were found to be additional diagnostic characters to distinguish these two species (Table 4). However, Velasco *et al.* (1998) reported urease activity for *O. intermedium* and citrate utilization was reported by Velasco *et al.* (1998) for *O. anthropi* and by Holmes *et al.* (1988) for *O. anthropi* CIP 14970^T (= LMG 3331^T). In contrast to our results, Velasco *et al.* (1998) report no H_2S production for *O. anthropi* and *O. intermedium*. In agreement with Velasco *et al.* (1998), no arabinose utilization (acid production) was obtained for *O. anthropi* and *O. intermedium*, whereas Holmes *et al.* (1988) reported positive D- and L-arabinose utilization for all tested *Ochrobactrum* strains. D-Mannose assimilation was negative and gluconate assimilation was very low for *O. anthropi* and *O. intermedium* in our experiments, but D-mannose and gluconate utilization were positive for both species in the tests of Holmes *et al.* (1988). These differences may be explained in part by the use of different test systems. Holmes *et al.* (1988) used API 50AA, API 50AO and API 50CH galleries, and Velasco *et al.* (1998) API 20NE, GNI and BBL Crystal E/NF systems. Results for substrate assimilation (increase in turbidity) may also be different from respective results for substrate utilization (acidification) and metabolic differences may be due to different culture conditions. A coherent study with a larger set of strains may resolve these contradictions.

REP group K strains OgA9a^T and OgA9c constituted BIOLOG group 1 (Table 2, Fig. 2). Their profiles were

most closely related to BIOLOG group 3 and 4 profiles of *O. anthropi* (Fig. 2), and quite different from BIOLOG profiles of *Brucella* spp. strains (not shown). BIOLOG group 1 strains were clearly separated by several diagnostic phenotypic characters from BIOLOG group 2–5 strains of *O. tritici* sp. nov. (see below), *O. anthropi*, and *O. intermedium* (Table 4). DNA–DNA reassociation values for strain OgA9a^T with *O. anthropi*, *O. intermedium* and hybridization group 2 (REP group J, *O. tritici* sp. nov., see below) references were as low as 20 % (Table 3). In each of our phylogenetic 16S rDNA trees (e.g. Fig. 1), strain OgA9a^T represented a genealogically independent branch exhibiting high bootstrap probabilities (74–98 %). A reason for the variability of bootstrap support between the different applied methods might be that 16S rDNA similarity to *O. anthropi* and *O. tritici* sp. nov. (REP group J) sequences, the closest relatives, was as high as about 98 %, a value that is at the limit of the delineation capacity of phylogenetic 16S rDNA approaches (Stackebrandt & Goebel, 1994). According to our congruent results from genotypic, phenotypic and phylogenetic analyses, REP group K strains must be given the status of a novel species within the genus *Ochrobactrum*. REP group K strains fulfilled the criteria given for the genus *Ochrobactrum* by Holmes *et al.* (1988). *Ochrobactrum grignonense* sp. nov. is proposed for REP group K strains OgA9a^T and OgA9c because both strains were isolated from soil of the Grignon Experimental Station. Strain OgA9a^T is the type strain of *Ochrobactrum grignonense* sp. nov. The description of *O. grignonense* is given below.

EMBL sequences D63836 (*Ochrobactrum* sp.) and U71004 (*O. anthropi*) clustered most closely with the sequence of *O. grignonense* OgA9a^T (not shown). It is therefore suggested that the taxonomic position of the respective strains is re-examined.

REP group J strains constituted the coherent cluster of BIOLOG group 2 (Table 2, Fig. 2). Their BIOLOG profiles were most closely related to those of *O. anthropi* (Fig. 2) and quite different from those of *Brucella* spp. (not shown). Several diagnostic phenotypic characters differentiating BIOLOG group 2 from the other BIOLOG groups were found (Table 4). BIOLOG group 2 representatives constituted the coherent DNA–DNA reassociation group 2 (Table 3). This group showed DNA–DNA reassociation values of less than 60 % with both *O. anthropi* probes and ΔT_m values with *O. anthropi* LMG 3331^T DNA were higher than 8 °C. Reassociation values with *O. intermedium* and *O. grignonense* sp. nov. DNA were even less (50 and 20 %, respectively; Table 3).

In our different phylogenetic 16S rDNA trees (e.g. Fig. 1), sequence U88444 of *O. anthropi* LMG 2320(t1) was most closely related to clade 5 (REP group J sequences, see above). Since the taxonomic position of *O. anthropi* LMG 2320(t1) is uncertain (see above), sequence U88444 was not included in clade 5. Bootstrap support for the delineation of REP group J and *O. anthropi* sequences was low. The phylogenetic approach using 16S rDNA similarity was hence not able to differentiate clearly between REP group J strains and *O. anthropi*. However, our results on DNA–DNA reassociation and the presence of diagnostic phenotypic characters (see above) suggest that REP group J strains represent a species that is different from *O. anthropi*. This position is reinforced by a biological particularity of REP group J strains: their activity apparently depends strongly on the presence of plant roots because they were immunotrapped exclusively from the rhizoplane and not from soil (Tables 1 and 3). This suggests adaptation of REP group J strains to the specific rhizoplane habitat. This preference was not observed for other *Ochrobactrum* species.

The differences at the geno- and phenotype level and the ecological particularity suggest that REP group J strains must be given the status of a new species. REP group J strains correspond to the description of the genus *Ochrobactrum* (Holmes *et al.*, 1988) and are most closely related to the type species *O. anthropi*, as shown by phenotyping and 16S rDNA homology (Figs 1 and 2) and by the relatively strong DNA–DNA reassociation of about 50 % (Table 3). Hence, they affiliate with *Ochrobactrum* at the generic level. The name *Ochrobactrum tritici* sp. nov. is proposed for REP group J strains since these strains were isolated from the rhizoplane of wheat (*Triticum aestivum*). Strain SCII24^T is the type strain of *Ochrobactrum tritici* sp. nov. Characteristic features of *O. tritici* are described below.

EMBL sequence X54743 of '*Solomonas fluorantheni*' (not shown) was placed within the cluster collection of core *Ochrobactrum* spp. (excluding *O. intermedium*). It is suggested therefore that the taxonomic position of this organism is re-examined.

Description of *Ochrobactrum grignonense* sp. nov.

Ochrobactrum grignonense (gri.gno.nen′se. French n. *Grignon* a location in France; L. neut. suffix *-ense* indicating provenance; N.L. neut. adj. *grignonense* pertaining to Grignon, region from which the strains were isolated).

Cells are Gram-negative (aminopeptidase-positive), aerobic (oxidase- and catalase-positive), pleomorphic short rods (0·6–1·2 × 0·4 μm) and highly motile by subpolar or peritrichous flagella. Grows between 4 and 40 °C (optimum 30 °C) and between pH values of 3 and 9 (optimum pH 6–7). Colonies are circular, isolated, entire, low convex, smooth, milky-opaque and about 1 mm in diameter after incubation for 24 h on NA. No colony pigment development was observed. Able to utilize (oxidize) 68 of 95 carbon sources of BIOLOG-GN microtitre plates and showed positive reactions in 6 of 22 tests and in 8 of 20 tests in the API 20E and API 20NE systems, respectively. Resistant to polymyxin B and colistin. Within the genus *Ochrobactrum*, the following characters were

of unique diagnostic value for *O. grignonense*: (i) BIOLOG-GN: utilization of malonic acid and no utilization of adonitol or D-glucosaminic acid; (ii) API 20E: acid production from melibiose and arabinose (48 h); (iii) API 20NE: assimilation of mannose (24 h) and no assimilation of maltose (48 h). In addition, 23 other phenotypic characters were identified by which *O. grignonense* can be differentiated from *O. anthropi*, *O. intermedium* and *O. tritici*. At the genome level, *O. grignonense* is clearly different from other *Ochrobactrum* species in composition of total DNA and 16S rDNA. *O. grignonense* DNA has a G+C content of 58 mol%. The type strain of *O. grignonense*, strain OgA9a^T, was deposited in BCCM/LMG and DSMZ culture collections as LMG 18954^T and DSM 13338^T, respectively; similarly the *O. grignonense* reference strain OgA9c was deposited as LMG 18955 and DSM 13339.

Description of *Ochrobactrum tritici* sp. nov.

Ochrobactrum tritici (tri′ti.ci. M.L. gen. n. *tritici* from *Triticum*, generic name for wheat, from which the strains were isolated).

Cells are Gram-negative (aminopeptidase-positive), aerobic (oxidase- and catalase-positive), pleomorphic short rods (0·6–1·4 × 0·4–0·6 μm) and highly motile by subpolar or peritrichous flagella. Grows between 4 and 40 °C (optimum 30 °C) and between pH values of 3 and 9 (optimum pH 6–pH 7). Colonies are opaque and mucoid and quickly become confluent during incubation on NA. No colony pigment development was observed. Able to utilize (oxidize) 62 of 95 carbon sources of BIOLOG-GN microtitre plates and showed positive reactions in 4 of 22 tests and in 10 of 20 tests of API 20E and API 20NE systems, respectively. Susceptible to polymyxin B and colistin. Within the genus *Ochrobactrum*, the following characters were of unique diagnostic value for *O. tritici*: (i) BIOLOG-GN: utilization of sebacic acid and no utilization of γ-hydroxybutyric acid; (ii) API 20NE: assimilation of gluconate (48 h). In addition, 24 other phenotypic characters were identified by which *O. tritici* can be differentiated from *O. anthropi*, *O. intermedium* and *O. grignonense*. At the genome level, *O. tritici* is clearly different from other *Ochrobactrum* species in total DNA composition. *O. tritici* is separated from *O. intermedium* and *O. grignonense* by sequence heterogeneity of 16S rDNA, but delineation from *O. anthropi* by 16S rDNA heterogeneity is not significant. *O. tritici* DNA has a G+C content of 59 mol%. The type strain of *O. tritici*, strain SCII24^T, was deposited in BCCM/LMG and DSMZ culture collections as LMG 18957^T and DSM 13340^T, respectively; similarly the *O. tritici* reference strain LAIII106 was deposited as LMG 18958 and LMG 13341.

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REFERENCES

Achouak, W., Christen, R., Barakat, M., Martel, M.-H. & Heulin, T. (1999). *Burkholderia caribensis* sp. nov., an exopolysaccharide-producing bacterium isolated from vertisol microaggregates in Martinique. *Int J Syst Bacteriol* **49**, 787–794.

Aguillera, M. M., Hodge, N. C., Stall, R. E. & Smart, G. C., Jr (1993). Bacterial symbionts of *Steinernema scapterisci*. *J Invertebr Pathol* **68**, 68–72.

Anderson, R. D., Bao, C.-Y., Minnick, D. T., Veigel, M. & Sedwick, W. D. (1992). Optimization of double-stranded DNA sequencing for polymerase chain reaction products. *USB Editorial Comments* **19**, 39–40, 57–58.

Atlas, R. M. (1995). *Handbook of Media for Environmental Microbiology*. Boca Raton, FL: CRC Press.

Barriuso, E. & Houot, S. (1996). Rapid mineralization of the S-triazine ring of atrazine in soils in relation to soil management. *Soil Biol Biochem* **28**, 1341–1348.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**, 493–496.

Biebel, A., Schloter, M., Kirchhof, G. & Hartmann, A. (1995). Enrichment of soil bacteria with magnetic and nonmagnetic immunological methods and identification using Inter-LINE PCR and genomic fingerprinting. In *Proceedings of the International Symposium* '*Exploration of Microbial Diversity, Ecological Basis and Biotechnical Utility*', June 12–15, 1995, Goslar, Germany, p. 25.

Brenner, D. J., McWorter, A. C., Leete Knutson, J. K. & Steigerwalt, A. G. (1982). *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J Clin Microbiol* **15**, 1133–1140.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Crosa, J. M., Brenner, D. J. & Falkow, S. (1973). Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and hetero-duplexes. *J Bacteriol* **115**, 904–911.

De Ley, J. (1992). The proteobacteria: ribosomal RNA cistron similarities and bacterial taxonomy. In *The Prokaryotes*, 2nd edn, pp. 2111–2140. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

De Ley, J., Mannheim, W., Segers, P., Lievens, A., Denjin, M., Vanhoucke, M. & Gillis, M. (1987). Ribosomal ribonucleic acid cistron similarities and taxonomic neighborhood of *Brucella* and CDC group Vd. *Int J Syst Bacteriol* **37**, 35–42.

Garland, J. L. & Mills, A. L. (1991). Classification and characterisation of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl Environ Microbiol* **57**, 2351–2359.

Grimont, P. A. D., Popoff, M. Y., Grimont, F., Coynault, C. & Lemelin, M. (1980). Reproducibility and correlation study of three deoxyribonucleic acid hybridisation procedures. *Curr Microbiol* **4**, 325–330.

Gutell, R. R., Larsen, N. & Woese, C. R. (1994). Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol Rev* **58**, 10–26.

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**, 406–416.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

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

Lebuhn, M., Schloter, M., Hartmann, A., Achouak, W., Berge, O. & Heulin, T. (1997). Investigations on *Ochrobactrum* species in soil and in the wheat rhizosphere. Colloque Rhizosphère Aix '97, p. 63. *SFM Phytopathologie*.

Louws, F. J., Schneider, M. & de Bruijn, F. J. (1996). Assessing genetic diversity of microbes using repetitive sequence-based PCR (rep-PCR). In *Nucleic Acid Amplification Methods for the Analysis of Environmental Samples*, pp. 63–94. Edited by G. Toranzos. Lancaster, PA: Technomic Publishing.

McInroy, J. A. & Kloepper, J. W. (1994). Novel bacterial taxa inhabiting internal tissues of sweet corn and cotton. In *Improving Plant Productivity with Rhizosphere Bacteria*, p. 190. Edited by M. H. Ryder, P. M. Stephens & G. D. Bowen. Adelaide: CSIRO.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. & Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**, 795–799.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Moreno, E. (1992). Evolution of *Brucella*. In *Advances in Brucellosis Research*, pp. 198–218. Edited by M. Plommet. Wageningen: Pudoc Scientific Publishers.

Moreno, E. (1997). In search of a bacterial species definition. *Rev Biol Trop* **45**, 753–771.

Moyer, N. P. & Hausler, W. J., Jr (1992). The genus *Brucella*. In *The Prokaryotes*, 2nd edn, pp. 2384–2400. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Ruimy, R., Breittmayer, V., Elbaze, P., Lafaye, B., Boussemart, O., Gauthier, M. & Christen, R. (1994). Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int J Syst Bacteriol* **44**, 416–426.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sato, K. & Jiang, J.-Y. (1996). Gram-negative bacterial flora on the root surface of wheat (*Triticum aestivum*) grown under different soil conditions. *Biol Fertil Soils* **23**, 273–281.

Schloter, M., Aßmus, B. & Hartmann, A. (1995). The use of immunological methods to detect and identify bacteria in the environment. *Biotechnol Adv* **13**, 75–90.

Schloter, M., Melzl, H., Alber, T. & Hartmann, A. (1996). Diversität von *Ochrobactrum anthropi*–Populationen in landwirtschaftlichen Böden. *Mitt Dtsch Bodenkundl Ges* **81**, 57–60.

Sneath, P. H. A. (1993). Evidence from *Aeromonas* for genetic crossing-over in ribosomal sequences. *Int J Syst Bacteriol* **43**, 626–629.

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.

Strimmer, K. & Von Haeseler, A. (1996). Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Mol Biol Evol* **13**, 964–969.

Strunk, O. & Ludwig, W. (1996). ARB: a software environment for sequence data. Technische Universität München, Munich, Germany.

Swings, J., Lambert, B., Kersters, K. & Holmes, B. (1992). The genera *Phyllobacterium* and *Ochrobactrum*. In *The Prokaryotes*, 2nd edn, pp. 2601–2604. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.

Velasco, J., Romero, C., López-Goñi, I., Leiva, J., Díaz, R. & Moriyòn, 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.

Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**, 25–40.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal amplification for phylogenic study. *J Bacteriol* **173**, 697–703.

Yanagi, M. & Yamasato, K. (1993). Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiol Lett* **107**, 115–120.