

Comparative sequence analysis of the internal transcribed spacer 1 of *Ochrobactrum* species

Michael Lebuhn^{a,b,*}, Stephan Bathe^b, Wafa Achouak^c, Anton Hartmann^a,
Thierry Heulin^c, Michael Schloter^a

^aGSF-National Center for Environment and Health, Institute of Soil Ecology, Ingolstaedter Landstr. 1, 85764 Neuherberg, Germany

^bInstitute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, 85748 Garching, Germany

^cDSV-DEVN, Laboratoire d'Ecologie Microbienne de la Rhizosphère, UMR 6191 CNRS-CEA-Université de la Méditerranée, CEA Cadarache, 13108 Saint-Paul-lez-Durance, France

Received 19 October 2005

Abstract

The internal 16S/23S rDNA (*rrs/rrl*) internal spacer region 1 (ITS1) of 54 *Ochrobactrum* strains and close relatives was analysed. Separation of ITS1 containing PCR products by gel-electrophoresis, DGGE, cloning and sequencing revealed ITS1 length and sequence heterogeneity. We found up to 5 different allelic ITS1 stretches within a single strain (*Ochrobactrum intermedium* LMG 3301^T), and 2–3 different ITS1 alleles in *O. tritici*. Within ITS1, ITS1c, being part of the conserved double-stranded *rrn* processing stem dsPS1, produced the most reliable segment tree. The overall ITS1, ITS1c and *rrs* phylogenetic tree topologies were generally consistent, but there was evidence for horizontal *rrn* (segment) transfer in *O. tritici* LMG 2134 (formerly *O. anthropi*). Good correlations were found between ITS1, ITS1c and *rrs* sequence similarity and DNA–DNA hybridization values indicating that phylogenetic analysis of ITS1 and ITS1c both can be used to preliminarily deduce the phylogenetic affiliation if HGT was excluded. Strains sharing >96.19% ITS1c (>95.11% ITS1) similarity fell within a species, and ≤68.42% ITS1c (≤70.33% ITS1) similarity outside a genus. Both ITS1 and ITS1c analysis resolved microdiversity more profoundly than *rrs* analysis and revealed clades (genomovars) within *O. anthropi* that were also produced in rep cluster analysis. There was no evidence for habitat-specific ITS1 genomovars within *Ochrobactrum* species. Diversity of *Ochrobactrum* was higher in soil than at the rhizoplane below and at the species level. Isolates from soil contained only 1 *rrn* type whereas isolates from human clinical, animal and rhizoplane specimens could contain more.

© 2005 Elsevier GmbH. All rights reserved.

Keywords: *Ochrobactrum*; Internal transcribed spacer 1; 16S rDNA; DNA–DNA hybridization; Microdiversity; Phylogeny

Introduction

The genus *Ochrobactrum* belongs to the family Brucellaceae within the alphaproteobacterial order Rhizobiales, and currently comprises five described

*Corresponding author. Institute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, 85748 Garching, Germany. Tel.: +49 89 289 13717; fax: +49 89 289 13718.

E-mail address: m.lebuhn@bv.tum.de (M. Lebuhn).

species, *Ochrobactrum anthropi*, *O. intermedium*, *O. tritici*, *O. grignonense* and *O. gallinifaecis*. The closest phylogenetic neighbour of *Ochrobactrum* is the genus *Brucella* which contains highly pathogenic strains. Paraphyly of *Ochrobactrum* with *Brucella* was reported with *O. intermedium* being more closely related to *Brucella* than to the other *Ochrobactrum* species [17,33].

Ochrobactrum strains were recovered from diverse habitats including soil, plants and their rhizosphere, (waste)water, animals and humans. *O. anthropi* and *O. intermedium* have a wide habitat range. Both species contain opportunistic pathogenic strains [10,23]. *O. tritici* was originally isolated from the wheat rhizoplane [17], and recently *O. tritici* strains were recovered from wastewater [5]. *O. grignonense* and *O. gallinifaecis* both currently comprise only very few described strains that were isolated from soil and chicken faeces, respectively [13,17].

In soil, *Ochrobactrum* strains were found to constitute approximately 2% of the bacteria cultivable on 1/10 strength tryptone-soy agar, and on the wheat rhizoplane, this fraction was about 0.3% [2,17], indicating that *Ochrobactrum* is a substantial part of the currently cultivable soil and rhizosphere microbial communities. Its importance may have been neglected due to the inconspicuous appearance of the colonies and misidentification of strains. From a biotechnological point of view, *Ochrobactrum* strains are of particular interest for bioremediation. Various isolates have been reported to be potent degraders of pollutants [1,19,24,27,28].

Although a clear differentiation of the species within the *Brucellaceae* complex and the separate identification of pathogenic and non-pathogenic strains is essential, this is hampered on the 16S rDNA (*rrs*) level by their high similarity. For example, *O. anthropi* and *O. tritici* share over 99% and the genera *Ochrobactrum* and *Brucella* over 96% (up to 99.28%) *rrs* sequence identity [17]. A better discrimination of species may be achieved by sequence comparison of the more variable internal transcribed spacer (ITS) within the *rrn* operon [6,7]. The ITS region is under functional selective pressure, displays extensive secondary structure, and within-strain differences between ITS alleles did not exceed the differences to the most closely related species [6,25,29]. The ITS region may therefore be used for phylogenetic

comparison. It has successfully been used to subtype bacterial populations and is a promising candidate for a better differentiation between species eventually down to the strain level [6,26].

Information on the various *Rhizobiales* *rrn* sequences accessible at EMBL-EBI suggests that the organization of the *rrn* elements is highly conserved within this order. From the 5' transcription start to the 3' end, the external transcribed spacer 1 (ETS1) is followed by *rrs*, the first part of the internal transcribed spacer 1 (ITS1a), the tRNA^{ile} gene, ITS1b, the tRNA^{ala} gene, ITS1c, the 23S rDNA (*rrl*), ITS2, the 5S rDNA (*rff*), the first part of ETS2 (ETS2a), the tRNA^{met} gene, and ETS2b (Fig. 1). In this study, we focus on the stretch between *rrs* and *rrl*, containing ITS1a and ITS1c which are involved in conserved double stranded processing stems in various bacteria (dsPS1 and dsPS2 stems, respectively) [25,29], and can have a role in rRNA processing, transcription and ribosome assembly [21].

Very little is known on the microdiversity of *Ochrobactrum* species although this assessment is of particular interest to answer the following questions that we address in this paper: (i) is the ITS1 region useful to differentiate species and genomovar (gv.) clusters within *Ochrobactrum*, (ii) is the phylogeny deduced from comparative ITS1 (segment) sequence analysis consistent with the valid taxonomy based on DNA–DNA hybridization, (iii) is there a relationship between occurrence in a particular environment (habitat-preference) and the ITS1 genotype of *Ochrobactrum* species, and (iv) is the (micro)diversity of *Ochrobactrum* strains higher in soil than at the wheat rhizoplane?

We analysed the ITS1 of a large collection of *Ochrobactrum* strains that were immunotrapped from soil and the rhizoplane [17] and included isolates from other environments, strains associated with human or animal disease and *Rhizobiales* ITS1 sequences from EMBL-EBI.

Material and methods

Strains

The investigated *Ochrobactrum* strains (Table 1) are from a large strain collection that was immunotrapped

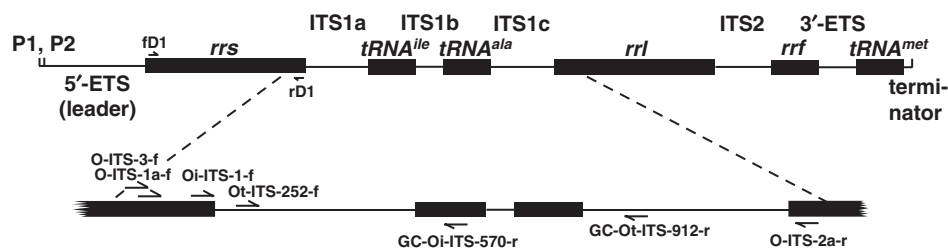


Fig. 1. Schematic organisation of the typical *rrn* operon of *Rhizobiales* and location of primer annealing. P1, P2, promoters; fD1, rD1, annealing location of primers used for amplification of *rrs* as described in [17]. For other abbreviations, see in text.

either from 2 different French bulk soils or from the rhizoplane of wheat cultivated in soil from these plots [17]. The isolates investigated in the present study represent the whole rep-profile-based genetic diversity identified in the collection [17]. Additionally, strains from culture collection BCCMTM-LMG, particularly from clinical specimens, and from other sources were included in the analyses. Cultivation and strain preservation was as reported previously [17].

Sequence generation

Whole cell PCR of ITS1 was performed with primers O-ITS-3-f (5'-TAATCGCGGATCAGCATGCCG-3' (or O-ITS-1a-f (5'-TGCCGCGGTGAATACGTTCCC-3')) and O-ITS-2a-r (5'-GCCAAGGCATCCACCAAATGCC-3'), annealing at *rrs* positions 1372–1392 (1388–1408) and *rrl* positions 19–40 (Fig. 1) of *Sinorhizobium meliloti* 1021 (AL591782). Primers were designed to exclude potential amplification of *Escherichia coli* DNA that is present in recombinant *Taq* polymerases [18]. PCR of *rrs* was as reported earlier [17]. The PCR master mix contained 1 × buffer, 4.5 mM MgCl₂, 200 μM dNTPs, 0.6 μM of each primer and 2.5 μM of Qiagen hot star *Taq* polymerase. Ca. 10⁴ template cells and water were added to a PCR volume of 100 μL. PCR conditions (Applied Biosystems 9600 thermal cycler) were: (i) 15 min 95 °C for activation/denaturation; (ii) 35 cycles of 30 s 95 °C for denaturation, 1 min 63.5 °C for stringent annealing, 3 min 72 °C for extension; (iii) a final 15 min 72 °C step for extension.

PCR products (7 μL) were separated in 1.5% agarose gels at 100 V for ca. 2 h in 1 × TAE, stained with SybrGreen, and visualized on a UV transilluminator. PCR products giving single bands were directly custom sequenced (Medigenomix, Sequiserve, Germany) after silica spin column purification (Qiagen). Intermediary sequencing primers were designed from the obtained sequences. Multiple bands were cut from the gel, and the DNA purified (QiaExII, Qiagen) and sequenced.

In case of overlaying peaks resulting from different sequences (obtained for *O. tritici* and for some *O. intermedium* strains, Table 1), PCR products were separated by PCR-DGGE (denaturing gradient gel electrophoresis) in a Bio-Rad DCodeTM system. For PCR-DGGE, we used primers Ot-ITS-252-f (5'-CAGGCCAGTCAGCCTGACG-3', annealing in the proximal still relatively conserved ITS1a segment at AL591782 ITS1a positions 73–94) and GC-Ot-ITS-912-r (5'-36mer-GC-clamp-CACGATTCATACAGAACAGGCAGAC-3', annealing in a relatively conserved ITS1c segment at AL591782 ITS1c positions 73–97) for *O. tritici*, and Oi-ITS-1-f (5'-CTGGCTGGATCACCTCC-3', annealing at the 3' *rrs* end at AL591782 *rrs* positions 1464–1482) and GC-Oi-ITS-570-r (5'-36mer-GC-clamp-CCCACGCTTATCAAGC-

GTGTGC-3', annealing within the tRNA^{ile} gene at AL591782 tRNA^{ile} positions 25–46) for the *O. intermedium* strains (Fig. 1). PCR conditions were as described above. The denaturing gradient for the 6% polyacrylamide (PAA) gel was 60–95%. PAA gels were run at 80 V and 55 °C for 18 h, and stained with SybrGreen. Homoduplex bands were cut, and the DNA purified and sequenced. Sequencing primers were designed specifically to close the sequence gaps to *rrs* and *rrl*. In parallel, heteromorphous O-ITS-1a-f/O-ITS-2a-r amplicons were cloned using the Topo-TA cloning kit (Invitrogen). At least 10 insert-containing plasmids were sequenced from each reaction applying the above described strategy to obtain full-length sequences.

Sequence analyses

All of the different sequences identified in the PCR-DGGE approach were retrieved in the cloning strategy, suggesting that all polymorphous alleles of the investigated *O. tritici*, and *O. intermedium* strains were detected. Sequences were analysed at the german EMBL-node DKFZ (Deutsches Krebsforschungszentrum) Heidelberg using the online accessible bioinformatics tools of HUSAR (<http://genius.embnnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz/>). *rrs* sequences were aligned using ClustalW, and ITS sequences using DiAlign and MUSCLE. Relevant closely related sequences identified by BLASTN2 and FASTA searches were integrated in the alignments. ITS alignments were adjusted manually according to [15] using secondary structure information from MFOLD free energy minimization.

Phylogenetic trees (Fig. 2) were constructed from the alignments using CLUSTREE and the implemented Kimura-2-parameter model. Gaps were treated as a fifth nucleotide state. Thousand bootstrap resamplings were performed. *Pseudoaminobacter salicylatoxidans* KTC001 (AJ294416) was used as outgroup for the *rrs* and *Pseudoaminobacter* sp. LCSAOTU18 (AY119681) for the ITS1c and ITS1 trees. Nodes with less than 50% bootstrap support were collapsed, and bootstrap probabilities higher than 75% are shown. Almost identical topologies were retrieved in PUZZLE maximum likelihood trees (not shown), suggesting that topologies are stable.

Scatter plots and regression analyses were performed with data from previously performed DNA–DNA-hybridization experiments [9,13,17], *rrs*, ITS1 and ITS1c similarity values, in order to determine the degree of correlation between these parameters for the analysed data sets.

Accession numbers and sequence annotation

Sequences were submitted to the DDBJ/EMBL/GenBank database (at EMBL-EBI, European Bioinformatics

Table 1. Compilation of *Ochrobactrum* strains and features

Species	Strain	Isolated from	Reference	REP-group [1]	BOX-cluster [1,2]	ITS1 length (bp)	ITS1c-gv./rank	(Updated) Accession number
<i>O. anthropi</i>	CLM5; CLM14	Soil	[17]	B	3.1.2.1.2	708	1a	AJ867291; AJ242577
<i>O. anthropi</i>	LMG 34; LMG 3329	Clinical source	LMG	n.a.	3.1.2.1.2	708	1a	AJ867293; AJ867294
<i>O. anthropi</i>	LMG 3331 ^T	Unknown	LMG	A	3.1.2.1.2	708	1a	AJ867295
<i>O. anthropi</i>	LMG 5140	Animal	LMG	B	3.1.2.1.2	708	1a	AJ242580
<i>O. anthropi</i>	LMG 5440	Unknown	LMG	n.a.	3.1.2.1.2	708	1a	AJ867296
<i>O. anthropi</i>	ALM4	Soil	[17]	G	3.1.2.1.1	708	1b	AJ867297
<i>O. anthropi</i>	LAI12; LAI114	Rhizoplane	[17]	G	3.1.2.1.1	708	1b	AJ867298; AJ867299
<i>O. anthropi</i>	LAI11; LAI14; LAI118	Rhizoplane	[17]	G	n.a.	708	1b	AJ867300; AJ867301; AJ867302
<i>O. anthropi</i>	LMG 7991	Wastewater	LMG	n.a.	n.a.	708	1b	AJ867304
<i>O. anthropi</i>	SAI8	Rhizoplane	[17]	H*	3.1.2.1.1	708	1b	AJ867303
<i>O. anthropi</i>	ALM13; CLM6; CLM18	Soil	[17]	F	3.1.2.2.1.1	756	2	AJ867305; AJ867292; AJ242576
<i>O. anthropi</i>	ALM14; CLM26	Soil	[17]	E	3.1.2.2.2	756	2	AJ867306; AJ867307
<i>O. anthropi</i>	CLM7; CLM12	Soil	[17]	D	3.1.2.2.1.1	756	2	AJ867308; AJ867309
<i>O. anthropi</i>	DSM 14396; LMA1	Soil	[17]	C	3.1.2.2.1.1	756	2	AJ242578; AJ867289
<i>O. anthropi</i>	LMG 3305	Clinical source	LMG	n.a.	3.1.2.2.2	756	2	AJ867311
<i>O. anthropi</i>	LMG 3330	Clinical source	LMG	n.a.	3.1.2.2.1.3*	756	2	AJ867312
<i>O. anthropi</i>	LMG 3333	Clinical source	LMG	n.a.	3.1.2.2.1.1	756	2	AJ867313
<i>O. anthropi</i>	OaC13a	Soil	[17]	G*	3.1.2.2.1.1	756	2	AJ867310
<i>O. anthropi</i>	SAI2; SAII101; SAIII101; SAIII108; SCII10	Rhizoplane	[17]	C	3.1.2.2.1.1	756	2	AJ867314; AJ867315; AJ867316; AJ867317; AJ867318
<i>O. anthropi</i>	SAIII104	Rhizoplane	[17]	I*	3.1.2.2.1.1	756	2	AJ867290
<i>O. anthropi</i>	LMG 35	Clinical source	LMG	n.a.	3.1.2.2.2*	756	3	AJ867320
<i>O. anthropi</i>	LMG 3300	Clinical source	LMG	n.a.	3.1.2.2.1.2	756	3	AJ867321
<i>O. anthropi</i>	LMG 5444	Clinical source	LMG	n.a.	3.1.1.2	756	3	AJ867319

<i>O. tritici</i>	LAIII106	Rhizoplane	[17]	J	1.1	A: 743; B: 782	<i>O. tritici</i> clade	AJ242579, AJ867327, AJ867328
<i>O. tritici</i>	LMG 2134 [†]	Animal source	LMG	n.a.	1.1	A: 708; B: 743; C: 782		AJ864999, AJ867331, AJ867332, AJ867333
<i>O. tritici</i>	LMG 2320(t1) [†]	Animal source	LMG	n.a.	1.2	A: 743; B: 743; C: 782		AJ865000, AJ867336, AJ867335, AJ867334
<i>O. tritici</i>	SCII24 [†]	Rhizoplane	[17]	J	1.1	A: 743; B: 782		AJ242584, AJ867330, AJ867329
<i>O. intermedium</i>	LMG 379	Clinical source	LMG	n.a.	2.2.1	A: 735; B: 735; C: 767	<i>O. intermedium</i> clade	AJ867337, AJ867338, AJ867339
<i>O. intermedium</i>	LMG 3301 [†]	Clinical source	LMG	L	2.1.1	A: 734; B: 736; C: 736; D: 757; E: 806		AJ867340, AJ867341, AJ867342, AJ867343, AJ867344
<i>O. intermedium</i>	LMG 3306	Soil	LMG	n.a.	2.1.2	767		AJ867322
<i>O. intermedium</i>	LMG 5425	Clinical source	LMG	n.a.	2.2.2	A: 735; B: 767		AJ867345, AJ867346
<i>O. intermedium</i>	LMG 5426	Clinical source	LMG	n.a.	2.2.2	A: 735; B: 783		AJ867347, AJ867348
<i>O. intermedium</i>	LMG 5443 [†]	Clinical source	LMG	n.a.	n.a.	735		AJ867323
<i>O. intermedium</i>	OiC8a; OiC8-2	Soil	[17]	M	n.a.	767		AJ242583; AJ867325
<i>O. intermedium</i>	OiC8-6	Soil	[17]	M	2.1.2	767		AJ242582
<i>O. intermedium</i>	OspIndien (DWR)	Soil	AK Tripathi	M	2.2.1	735		AJ867324
<i>O. grignonense</i>	OgA9a [†]	Soil	[17]	K	3.2	859	<i>O. grignonense</i> cl	AJ242581
<i>O. grignonense</i>	OgA9c	Soil	[17]	K	n.a.	859		AJ867326
<i>Ochrobactr.</i> sp.	TK14	Wastewater	[3]	n.a.	n.a.	719	<i>O.</i> lineage	AJ550273

gv., genomvar; LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCMTM-LMG); n.a., not analysed.

[†]Probably misassigned (see text).

[‡]Formerly *O. anthropi*.

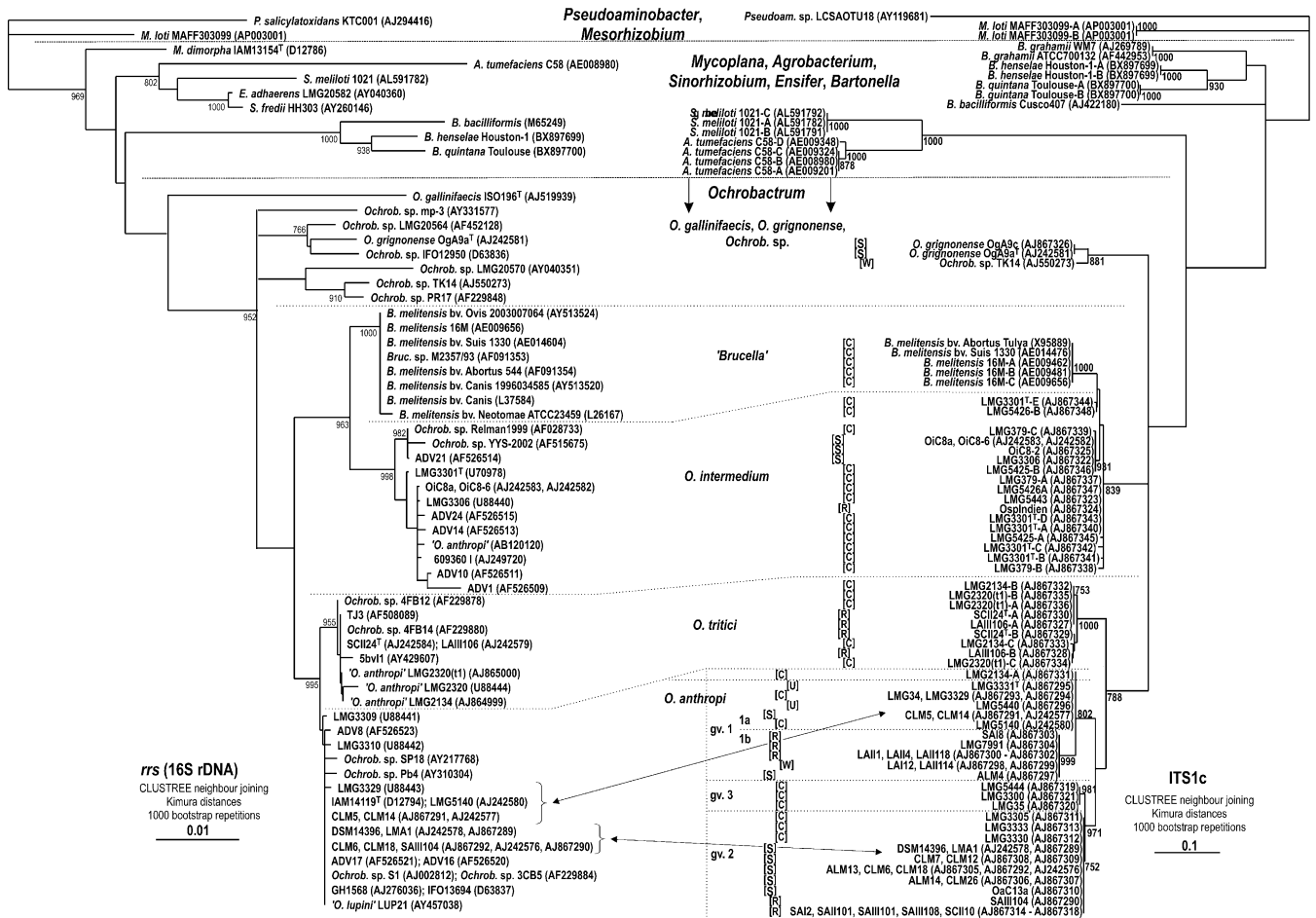


Fig. 2. Comparative phylogenetic neighbour-joining analyses of *Ochrobactrum* *rrs* (16S rDNA, 1394 characters (gaps included), left part) and ITS1c (complete, 835 characters, right part) sequences. Nodes with less than 50% bootstrap support were collapsed, and bootstrap probabilities > 75% are shown. Affiliation to genera, *Ochrobactrum* species and *O. anthropi* genomovars (gv.) is indicated. Accession numbers are in parentheses. Taxonomic names in 'quotation marks' refer to currently misassigned strains or sequences (cf. Table 1, see text). Different ITS1 alleles are denoted -A to -E after the strain name. Source of isolation of *Ochrobactrum* isolates: [C], clinical/animal; [R], rhizoplane; [S], soil; [U], unknown; [W], wastewater.

Institute, <http://www.ebi.ac.uk/embl/index.html>). The following accession numbers of sequences that had previously been submitted by us, were updated for the additionally sequenced loci: AJ242576–AJ242584 and AJ550273. The following accession numbers were obtained for newly entered sequences: AJ864999–AJ865000, and AJ867289–AJ867348.

Annotations are based on *rrs* end and *rrl* start points as defined in *S. meliloti* 1021 accession numbers AL591782, AL591791 and AL591792. tRNA genes were identified by tRNAscan-SE [22].

Results and discussion

Heterogeneity of *Ochrobactrum* ITS1 and number of different *rrn* types

The ITS1 region of *Ochrobactrum* strains (*O. gallinifacis* was not analyzed) was found to be variable in

length, similarly as described in numerous papers for other genera, and ranged between 708 (some *O. anthropi* strains) and 858 bp (*O. grignonense*) (Table 1). Both *O. grignonense* strains showed 100% identity of the sequenced ITS1a and ITS1b. The higher length of *O. grignonense* ITS1 was due to an insertion of 103 bp in ITS1b which was predicted to form four consecutive RNA hairpins (not shown). Since ITS1b nts. 5–113 were more similar (ca. 70%) to *O. grignonense* ITS1a nts. 155–269 than to any other accessible sequence, the ITS1b insert was probably generated by within-strain duplication, heterologous recombination and divergence. Some other *Rhizobiales* members also have a prolonged ITS1b (not shown). Homologous recombination at *rrn* loci has similarly been found in *Ochrobactrum* and *Brucella* strains [11,31]. Genome rearrangements apparently frequently occurred within the *Brucellaceae*.

One type of ITS1 per strain was found in *O. anthropi*, *O. grignonense*, *Ochrobactrum* sp. TK14 and few

O. intermedium strains (Table 1). For these, a single PCR band and no sequence overlays were observed (not shown). However, their exact *rrn* copy number per genome remains unclear. Engel [4] found 1 *rrn* copy in *O. anthropi* LMG 3305, but Jumas-Bilak et al. [12] 2 megareplicons hybridizing with an *rrs* probe in *O. anthropi* ATCC 49188^T.

According to the *rrs*, ITS1, ITS1c and genetic fingerprinting analyses (Figs. 1 and 2; Table 1; [3]), ‘*O. anthropi*’ strain LMG 5443 and the strain corresponding to accession number AB120120 should be reclassified as *O. intermedium*, and ‘*O. anthropi*’ strains LMG 2134 and LMG 2320(t1), showing in addition the typical confluent colony morphology on NB agar of *O. tritici*, as *O. tritici*. Results for these strains are consequently discussed with *O. intermedium* and *O. tritici*. ‘*O. lupini*’ *rrs* (strain LUP21, acc. no. AY457038) was 100% identical with *O. anthropi* (Fig. 2). It should be checked if this strain belongs to *O. anthropi*.

O. tritici and many *O. intermedium* strains were found to be heterogeneous in *rrn* composition. We identified 1–5 different types of ITS1 per strain in *O. intermedium* and 2–3 in *O. tritici* (Table 1). Teyssier et al. [31] identified 4 *rrn* operons in *O. intermedium* LMG 3301^T, but we found 5 different ITS1 alleles suggesting the presence of at least 5 *rrn* operons in this strain. Superimposed bands may have obscured the presence of a higher copy number in the analysis of [31]. Interestingly, one of the three ITS1 alleles of *O. tritici* (formerly *O. anthropi*) LMG 2134 was 100% identical to the ITS1 of *O. anthropi*, whereas the other two alleles clustered with *O. tritici* (Fig. 2). We assume that an *rrn* operon was recently transferred from *O. anthropi* to the ancestor of this hybrid strain, and that either *rrs* heterogeneity was not detected due to preferential PCR amplification of the major *O. tritici* *rrs* type or that *rrs* was quickly homogenized [8,20]. Alternatively, only the ITS1 region (including the tRNA^{ile} and tRNA^{ala} genes) may have been recombined and was not yet homogenized. Similarly, horizontal transfer (HGT) of *rrn* segments was identified in *Rhizobiaceae* strains [32]. Our data suggest that this mechanism of evolution giving rise to reticulate phylogeny also occurs in *Ochrobactrum*.

Except for ITS1 allele A of *O. tritici* LMG 2134, within-strain ITS1 and ITS1c (Fig. 2) heterogeneity did not exceed the species limits (also true for uncorrected distances, not shown). Since this feature has also been noticed for ITS of other genera (Guertler, 1999), and ITS is under selective pressure (see above), ITS can be used for phylogenetic classification.

ITS1 (segment) phylogenies and their value for taxonomy

We compared ITS1, ITS1 segment and *rrs* phylogenies of *Ochrobactrum* strains and close relatives, and evaluated the similarity values with DNA–DNA hybridization data. ITS1 alignments needed thorough manual adjustment due to locally very high sequence variation, and was guided by secondary structure information [15]. The tRNA^{ile} and tRNA^{ala} genes were found to be too conserved for species-level delineation (Table 2). ITS1b was typically too short to infer phylogeny (Table 2) and prone to recombination (see above). In the ITS1a tree (not shown), *O. tritici* was split in 2 clades. One of them was found with *Brucella*, and the other was polyphyletic with *O. grignonense* and 2 *O. anthropi* clades. Except for polyphyly of *O. tritici* in the ITS1 (containing ITS1a, tRNA^{ile}, ITS1b, tRNA^{ala}, ITS1c) tree (not shown), topologies in ITS1, ITS1c and *rrs* trees were much more consistent. In the *rrs* and ITS1c trees (Fig. 2), *O. tritici* sequences formed a monophyletic branch, and in *rrs*, ITS1 and ITS1c trees, *O. anthropi* was monophyletic and *O. grignonense* branched off much closer to the *Ochrobactrum* root. Some informative ITS1a characters apparently provided ambiguous information for phylogeny, although ITS1a provided the highest percentage of parsimony-informative characters within ITS1 (Table 2). Particular plasticity of the region between *rrs* and tRNA^{ala} (corresponding to ITS1a in our study) was shown for enterococci [25].

A high percentage of informative characters (Table 2) and the most reliable phylogenetic signal was derived from segment ITS1c. ITS1c and *rrs* topologies were generally concordant, except for the closer relation of

Table 2. Discriminatory features of analysed *rrs* and ITS1 (segment) sequences

Sequence (segment)	<i>rrs</i>	ITS1	ITS1a	tRNA ^{ile}	ITS1b	tRNA ^{ala}	ITS1c
Total characters	1394	1925	599	77	338	76	835
Parsimony-constant characters (%)	1134 (81.35)	630 (32.73)	161 (26.88)	62 (80.52)	77 (22.78)	64 (84.21)	353 (31.86)
Parsimony-informative characters (%)	154 (11.05)	852 (44.26)	319 (53.26)	9 (11.69)	115 (33.92)	4 (5.26)	346 (41.44)
Non-redundant <i>Ochrobactrum</i> sequences* (%)	41 (100)	35 (100)	29 (82.86)	3 (8.57)	7 [†] (20.00)	4 (11.43)	25 (71.43)

*Number of non-redundant *Ochrobactrum* sequences in the alignment of the respective sequence segment.

[†]6 of these sequences contain less than 15 nucleotides.

the *Sinorhizobium/Agrobacterium* clade with *Ochrobactrum* and the (non-significant) rooting of *Brucella* in an *O. intermedium* subcluster in the ITS1c tree (Fig. 2). In contrast, *O. intermedium* and *Brucella* were significantly monophyletic in the ITS1 and ITS1a trees (not shown). *Brucella* fell within the *Ochrobactrum* clade and was found inside the intervals for affiliation to *Ochrobactrum* at the genus level in each of the phylogenetic and regression (see below) analyses (Figs. 1 and 2) [17,33]. *Brucella* must hence be regarded as an *Ochrobactrum* species from the scientific point of view and was consequently treated as an *Ochrobactrum* species in this article. However, to avoid confusion [30], *Brucella* as medically important organism with defined clinical symptoms may maintain its name.

ITS1c and ITS1 provided a much higher percentage of informative sites than *rrs* (Table 2), and in the ITS1c and ITS1 trees, *O. anthropi* consisted of 3 significant genomovar (gv.) clades (gv. 1 subdivided in gvs. 1a, 1b; Fig. 2, Table 1), whereas the respective sequences did not diverge (100% identity) in the *rrs* tree (Fig. 2). ITS1 length difference was the major factor for the separation of the gv. 1a/1b clade and the gv. 2/3 clade (Table 1, Fig. 2). The *O. anthropi* ITS1 genomovars showed nearly perfect conformity with previously performed, even higher resolving rep-fingerprint profile clusters (Table 1; REP-groups are defined in [17]; BOX-groups are defined in [3]). *O. anthropi* gv. 1a comprised REP-groups A and B, and BOX-group 3.1.2.1.2. *O. anthropi* gv. 1b comprised REP-groups G and H, and BOX-group 3.1.2.1.1. *O. anthropi* gv. 2 comprised REP-groups C–F and J, and BOX-groups 3.1.2.2.1.1, 3.1.2.2.1.3 and 3.1.2.2.2 (gv. 2 strain OaC13a, correctly belonging to BOX-group 3.1.2.2.1.1, may have been misassigned in [17] to REP-group G). *O. anthropi* gv. 3 comprised BOX-groups 3.1.1.2 and 3.1.2.2.1.2 (strain LMG 35 appears to be misplaced in [3] in BOX-group 3.1.2.2.2 and probably belongs to cluster 3.1.2.2.1.2). ITS1 analysis is hence useful to differentiate below the species level and provides significant phylogenetic information. However, topologies can need further proof (e.g. by *rrs* analysis), since ITS1a/b plasticity can cause bias and ITS1c is relatively short (about 300 bp). There was evidence that neither ITS1 profiling nor rep-fingerprinting resolves microbial genetic microdiversity down to the strain level: *O. anthropi* DSM 14396 was isolated from German, but strain LMA1, belonging to the same rep- and ITS-groups, from French agricultural soil (Table 1) [17]. Due to the geographical distance, we assume that a clonal origin of these two strains is excluded, and that mutations should have occurred in very rapidly evolving regions. However, neither ITS1- nor rep-differences were detected.

Fig. 3 shows regression analyses between DNA–DNA hybridization data, *rrs*, ITS1c and ITS1 divergence for *Ochrobactrum* and related outgroup strains or se-

quences. The atypical position of the short ITS1 allele of *O. tritici* LMG 2134 (formerly *O. anthropi*) (allele -A, 100% identity with *O. anthropi* LMG 3331^T) in Figs. 2 and 3 was most probably caused by HGT (see above). Data distribution (Fig. 3) was best explained and with high coefficients of determination (R^2) by linear relationships between *rrs*, ITS1 and ITS1c, and by exponential relations between DNA and DNA hybridization vs. *rrs*, ITS1 and ITS1c similarities. Table 3 shows the similarity intervals (uncorrected distances) for the definition of affiliation to *Ochrobactrum* species and to the genus *Ochrobactrum*. According to the results, both ITS1 and ITS1c similarity distances can be used to confirm and delineate species within *Ochrobactrum*, and *Ochrobactrum* from related genera. However, the case of LMG 2134 ITS1-A shows that sequences must be checked for eventual HGT. ITS1 (segment) analysis is therefore a suitable candidate to assess species affiliation, e.g. in a Multi Locus Sequence Typing approach as a potential surrogate for the laborious DNA–DNA hybridization [30]. The advantage of ITS1 and ITS1c over *rrs* analysis lies in the higher resolution down to the genomovar level (Fig. 2) [26], whereas *rrs* topology analysis appears to be more robust in assessing affiliation above/at the species level.

Correlation of ITS1(c) microdiversity and source of isolation

The correlation of source of isolation and ITS1(c) microdiversity (Fig. 2; Table 1) was best studied inside *Ochrobactrum* for *O. anthropi*, for which strains and sequences from various environments are available. *O. anthropi* forms significant ITS1 genomovars (see above). In almost each of these gvs., isolates from different environments were intermingled (Fig. 2; Table 1): *O. anthropi* gv. 1a consisted of strains from soil, clinical, animal and unknown sources but not from the rhizosphere. *O. anthropi* gv. 1b comprised strains from wastewater, soil and the rhizosphere, but no clinical or animal strains. *O. anthropi* gv. 2 consisted of strains from clinical sources, soil and the rhizosphere. *O. anthropi* gv. 3 was the only group that consisted of isolates from one source of isolation (clinical sources) exclusively. However, since this group comprised only 3 sequences, more isolates must be sequenced to corroborate an idiosyncrasy. Presently, there was no clear evidence for an influence of the source of isolation on the *Ochrobactrum* ITS1 microdiversity. Similarly, Bathe et al. [3] found no clear influence of the source of isolation on the genotype (rep-profiles). There are several reports that the genotype can be influenced by specific habitat traits, but results on this topic are controversial (reviewed in [26]). The source of isolation did not influence ITS1 length. Clinical, soil

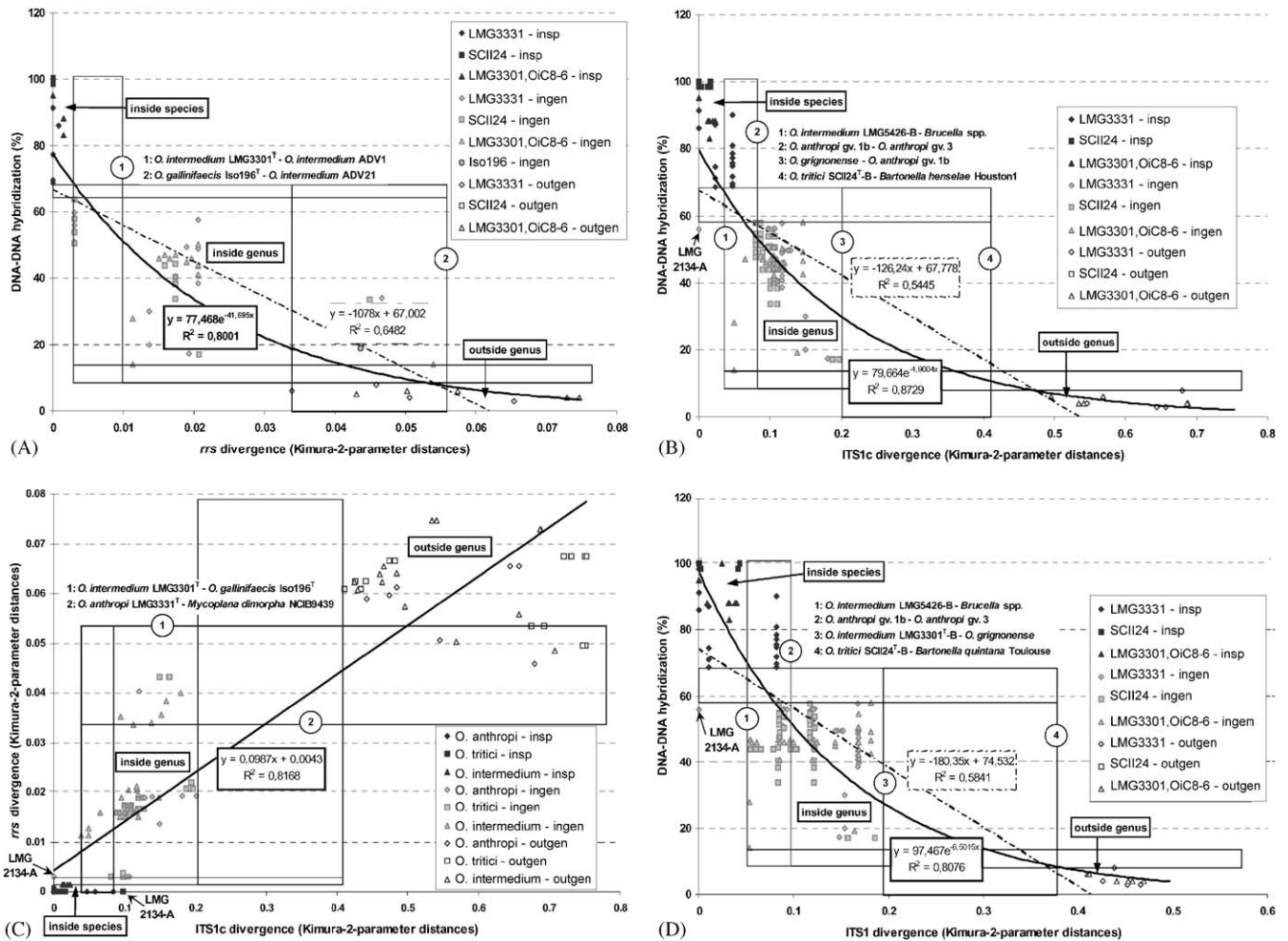


Fig. 3. Regression analyses of DNA–DNA hybridization versus (A) *rrs*, (B) ITS1c and (D) ITS1 divergence, and (C) *rrs* versus ITS1c divergence. DNA–DNA-hybridization data are from [9,13,17]. LMG3331, SCII24, LMG3301, OiC8-6, Iso196: data from *O. anthropi* LMG 3331^T, *O. tritici* SCII24^T, *O. intermedium* strains LMG 3301^T and OiC8-6, and *O. gallinifacis* Iso 196^T as the hybridization probes, respectively. insp, ingen, outgen denote intraspecies, intragenus and intergenus comparisons of the probe or the indicated taxon, respectively. Horizontal and vertical lines denote minimum and maximum values for affiliation at the indicated taxonomic levels. Numbers for strain or taxonomic names show their position if data for 1 parameter are not available. *Brucella* was treated as an *Ochrobactrum* species (see text). LMG2134-A: position of ITS1 allele A of *O. tritici* LMG 2134 (formerly *O. anthropi*).

Table 3. Similarity intervals for the definition of affiliation to *Ochrobactrum* species and to the genus *Ochrobactrum*

Taxonomic/phylo-genetic position	DNA–DNA reassociation* (%)	<i>rrs</i> similarity (% [†])	ITS1c similarity (% [†])	ITS1 similarity (% [†])
Within species	≥ 68.6	> 99.48	> 96.19	> 95.11
Borderline, confirmation needed	< 68.6, > 63.8	≤ 99.92, ≥ 96.94	≤ 96.19, ≥ 92.05	≤ 95.11, ≥ 90.8
Within genus, outside species ^{††}	≤ 63.8, ≥ 14	< 96.94, > 96.42	< 92.05, ≥ 82.81	> 90.8, ≥ 82.97
Borderline, confirmation needed	< 14, > 8	≤ 96.42, ≥ 92.61	< 82.81, > 68.42	< 82.97, > 70.33
Outside genus	≤ 8	< 92.61	≤ 68.42	≤ 70.33

*DNA–DNA-reassociation data are from [9,13,17].

[†]Uncorrected distances.

^{††}*Brucella* was regarded as an *Ochrobactrum* species (see text).

and rhizoplane strains could have either the long (756 bp) or the short (708 bp) ITS1 version (Table 1).

However, there was evidence for higher genetic diversity in soil than on the rhizoplane at different

scales of resolution (Fig. 2, Table 1). Within *O. anthropi*, soil strains were distributed on 3 ITS1 clusters (gvs. 1a, 1b, 2), whereas rhizoplane strains were found only in 2 ITS1 clusters (gv. 1b, 2). Soil strains were found in 3

Ochrobactrum species (*O. anthropi*, *O. intermedium* and *O. grignonense*) but rhizoplane strains only in 2 (*O. anthropi* and *O. tritici*). Bathe et al. [3] similarly found lower microdiversity on the rhizoplane than in bulk soil analysing genetic fingerprints (rep-profiles). Rhizoplane conditions apparently select not only for biovars that can cope with this particular environment [3] but also for distinct genomovars, resulting in reduced microdiversity on the rhizoplane.

Interestingly, *Ochrobactrum* isolates that contained more than one different *rrn* types were from rhizoplane, human clinical or animal specimens, whereas soil isolates contained only a single *rrn* type (Table 1, Fig. 2). Although we did not determine exact copy numbers, this suggests that human clinical, animal and rhizoplane strains in means contain a higher number of *rrn* operons than soil strains. The presence of multiple *rrn* copies can contribute to ecological fitness of microorganisms that switch between poor (e.g. soil, water) and fertile (but potentially hostile, e.g. animal host, rhizoplane) environments. The ability to react rapidly in opened fertile niches by intense ribosome synthesis [14], activity and massive proliferation (*r*-strategy) can allow survival of the population in the presence of concurrents or predators. In contrast, *K*-strategists can better cope with poor soil or water conditions and difficult nutrients and do not need to upregulate metabolism rapidly by multiple *rrn* copies. Additional mechanisms can contribute to the development of *K*- and *r*-strategies [16].

Acknowledgements

We thank Dr. A.K. Tripathi (School of Biotechnology, Banaras Hindu University, Varanasi-221005, India) for consigning strain OspIndien (DWR). Parts of this study were supported by grants from the OECD to ML and from the Deutsche Forschungsgemeinschaft, Project Ha1708/2.

References

- [1] S.H. Baek, K.H. Kim, C.R. Yin, C.O. Jeon, W.T. Im, K.K. Kim, S.T. Lee, Isolation and characterization of bacteria capable of degrading phenol and reducing nitrate under low-oxygen conditions, *Curr. Microbiol.* 47 (2003) 462–466.
- [2] S. Bathe, M. Lebuhn, J.W. Ellwart, S. Wuertz, M. Hausner, High phylogenetic diversity of transconjugants carrying plasmid pJP4 in an activated sludge-derived microbial community, *FEMS Microbiol. Lett.* 235 (2004) 215–219.
- [3] S. Bathe, W. Achouak, A. Hartmann, T. Heulin, M. Schloter, M. Lebuhn, Genetic and phenotypic microdiversity of *Ochrobactrum* spp., *FEMS Microbiol. Ecol.* (2005), in press.
- [4] M. Engel, Untersuchungen zur Sequenzheterogenität multipler rRNS-Operone bei Vertretern verschiedener Entwicklungslinien der *Bacteria*, Dissertation, Lehrstuhl fuer Mikrobiologie der Technischen Universitaet Muenchen, 1999.
- [5] J. Goris, N. Boon, L. Lebbe, W. Verstraete, P. De Vos, Diversity of activated sludge bacteria receiving the 3-chloroaniline-degradative plasmid pC1gfp, *FEMS Microbiol. Ecol.* 46 (2003) 221–230.
- [6] V. Guertler, V.A. Stanisich, New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region, *Microbiology* 142 (1996) 3–16.
- [7] V. Guertler, The role of recombination and mutation in 16S–23S rDNA spacer rearrangements, *Gene* 238 (1999) 241–252.
- [8] J.G. Hashimoto, B.S. Stevenson, T.M. Schmidt, Rates and consequences of recombination between rRNA operons, *J. Bacteriol.* 185 (2003) 966–972.
- [9] B. Holmes, M. Popoff, M. Kiredjian, K. Kersters, *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd, *Int. J. Syst. Bacteriol.* 38 (1998) 406–416.
- [10] N. Jelveh, B.A. Cunha, *Ochrobactrum anthropi* bacteremia, *Heart Lung* 28 (1999) 145–146.
- [11] E. Jumas-Bilak, S. Michaux-Charachon, G. Bourg, D. O’Callaghan, M. Ramuz, Differences in chromosome number and genome rearrangements in the genus *Brucella*, *Mol. Microbiol.* 27 (1998) 99–106.
- [12] E. Jumas-Bilak, S. Michaux-Charachon, G. Bourg, M. Ramuz, A. Allardet-Servent, Unconventional genomic organization in the alpha subgroup of the Proteobacteria, *J. Bacteriol.* 180 (1998) 2749–2755.
- [13] P. Kaempfer, S. Buczolits, A. Albrecht, H.J. Busse, E. Stackebrandt, 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 (2003) 893–896.
- [14] J.A. Klappenbach, J.M. Dunbar, T.M. Schmidt, rRNA operon copy number reflects ecological strategies of bacteria, *Appl. Environ. Microbiol.* 66 (2000) 1328–1333.
- [15] K.M. Kjer, Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs, *Mol. Phylogenet. Evol.* 4 (1995) 314–330.
- [16] M. Lebuhn, T. Heulin, A. Hartmann, Production of auxin and other indolic and phenolic compounds by *Paenibacillus polymyxa* strains isolated from different proximity to plant roots, *FEMS Microbiol. Ecol.* 22 (1997) 325–334.
- [17] M. Lebuhn, W. Achouak, M. Schloter, O. Berge, H. Meier, M. Barakat, A. Hartmann, T. Heulin, 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 (2000) 2207–2223.
- [18] M. Lebuhn, M. Effenberger, A. Gronauer, P. Wilderer, Using quantitative real-time PCR to determine the

- hygienic status of cattle manure, *Water Sci. Technol.* 48 (2003) 97–103.
- [19] U. Lechner, R. Baumbach, D. Becker, V. Kitunen, G. Auling, M. Salkinoja-Salonen, Degradation of 4-chloro-2-methylphenol by an activated sludge isolate and its taxonomic description, *Biodegradation* 6 (1995) 83–92.
- [20] D. Liao, Gene conversion drives within genic sequences: concerted evolution of ribosomal RNA genes in bacteria and archaea, *J. Mol. Evol.* 51 (2000) 305–317.
- [21] A. Liiv, T. Tenson, T. Margus, J. Remme, Multiple functions of the transcribed spacers in ribosomal RNA operons, *Biol. Chem.* 379 (1998) 783–793.
- [22] T.M. Lowe, S.R. Eddy, tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence, *Nucl. Acids Res.* 25 (1997) 955–964.
- [23] L.V. Moller, J.P. Arends, H.J. Harmsen, A. Talens, P. Terpstra, M.J. Slooff, *Ochrobactrum intermedium* infection after liver transplantation, *J. Clin. Microbiol.* 37 (1999) 241–244.
- [24] R.H. Muller, S. Jorks, S. Kleinstueber, W. Babel, Degradation of various chlorophenols under alkaline conditions by gram-negative bacteria closely related to *Ochrobactrum anthropi*, *J. Basic Microbiol.* 38 (1998) 269–281.
- [25] A. Naimi, G. Beck, C. Branlant, Primary and secondary structures of rRNA spacer regions in enterococci, *Microbiology* 143 (1997) 823–834.
- [26] M. Schloter, M. Lebuhn, T. Heulin, A. Hartmann, Ecology and evolution of bacterial microdiversity, *FEMS Microbiol. Rev.* 24 (2000) 647–660.
- [27] C.W. Smejkal, F.A. Seymour, S.K. Burton, H.M. Lappin-Scott, Characterisation of bacterial cultures enriched on the chlorophenoxyalkanoic acid herbicides 4-(2,4-dichlorophenoxy) butyric acid and 4-(4-chloro-2-methylphenoxy) butyric acid, *J. Ind. Microbiol. Biotechnol.* 30 (2003) 561–567.
- [28] B. Song, N.J. Palleroni, M.M. Haggblom, Isolation and characterization of diverse halobenzoate-degrading denitrifying bacteria from soils and sediments, *Appl. Environ. Microbiol.* 66 (2000) 3446–3453.
- [29] A.K. Srivastava, D. Schlessinger, Mechanism and regulation of bacterial ribosomal RNA processing, *Annu. Rev. Microbiol.* 44 (1990) 105–129.
- [30] E. Stackebrandt, W. Frederiksen, G.M. Garrity, P.A. Grimont, P. Kampf, M.C. Maiden, X. Nesme, R. Rossello-Mora, J. Swings, H.G. Trueper, L. Vauterin, A.C. Ward, W.B. Whitman, Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology, *Int. J. Syst. Evol. Microbiol.* 52 (2002) 1043–1047.
- [31] C. Teyssier, H. Marchandin, M. Simeon De Buochberg, M. Ramuz, E. Jumas-Bilak, Atypical 16S rRNA gene copies in *Ochrobactrum intermedium* strains reveal a large genomic rearrangement by recombination between *rrn* copies, *J. Bacteriol.* 185 (2003) 2901–2909.
- [32] P. van Berkum, Z. Terefework, L. Paulin, S. Suomalainen, K. Lindstrom, B.D. Eardly, Discordant phylogenies within the *rrn* loci of rhizobia, *J. Bacteriol.* 185 (2003) 2988–2998.
- [33] J. Velasco, C. Romero, I. Lopez-Goni, J. Leiva, R. Diaz, I. Moriyon, 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 (1998) 759–768.