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Stenotrophomonas rhizophila sp. nov., a novel plant-associated bacterium with antifungal properties

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A polyphasic taxonomic study was performed on 16 *Stenotrophomonas* strains from environmental and clinical sources. A group of three plant-associated isolates were shown to be phenotypically different from the other strains. This group formed a separate physiological cluster (B1) with 42% heterogeneity to the other isolates. The defining characteristics of the new species were as follows: growth at 4 °C and the absence of growth at 40 °C; the utilization of xylose as a carbon source; lower osmolytic tolerance (< 4·5% NaCl, w/v), although the isolates can produce trehalose and glucosylglycerol as osmoprotective substances; the absence of lipase and β-glucosidase production; and antifungal activity against plant-pathogenic fungi. The whole-cell fatty acid profile of this group was different and characterized by the main fatty acids iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Numerical analysis of the fatty acid profiles of the strains examined supports the differentiation of the physiological B1 group. By 16S rDNA analysis, three clusters were distinguished. The three strains of the B1 group formed a separate environmental cluster (E1). They showed a mean similarity of 99·5% within the cluster, and differed from strains of a second environmental cluster (E2) by 2·2% and from the clinical cluster (C) by about 3·0%. DNA–DNA hybridization data supported the taxonomic differentiation. All results led to the proposal of a new species, *Stenotrophomonas rhizophila* sp. nov., with strain e-p10^T (= DSM 14405^T = ATCC BAA-473^T) as the type strain.

Keywords: *Stenotrophomonas rhizophila* sp. nov., plant-associated, antifungal, osmolytes, 16S rDNA

INTRODUCTION

The genus *Stenotrophomonas* is phylogenetically placed in the γ-subclass of the *Proteobacteria* (Moore *et al*., 1997). The *Stenotrophomonas* genus was described with the species *Stenotrophomonas maltophilia* (Palleroni & Bradbury, 1993), previously called *Pseudomonas maltophilia* (Hugh & Ryschenko, 1961) and later changed to *Xanthomonas maltophilia* (Swings *et al*., 1983). Recently, Drancourt *et al*. (1997) proposed a new species; *Stenotrophomonas africana*, which is biochemically identical to *S. maltophilia* except for its ability to assimilate *cis*-aconitate. Genotypic analysis, however, revealed only 35% DNA homology between the two species. Finkmann *et al*. (2000) characterized *Stenotrophomonas* strains with an unusual denitrification reaction as new species *Stenotrophomonas nitritireducens*.

Stenotrophomonas species have an important ecological role in the element cycle in nature (Ikemoto *et al*., 1980). The biotechnological importance of *S. maltophilia* is partly due to its potential plant growth-promoting effects and applications in the biological control of fungal diseases in plants (Berg *et al*., 1994; Kobayashi *et al*., 1995; Nakayama *et al*., 1999). Recent interest has also focused on the use of the species in decontamination of soil (bioremediation) because of its capacity to degrade xenobiotic compounds (Binks

Abbreviation: DGGE, denaturing-gradient gel electrophoresis.

The EMBL accession number for the 16S rDNA sequence of the isolate e-p10^T is AJ293463.

et al., 1995). In the last decade, *S. maltophilia* has also become important as a nosocomial multidrug-resistant pathogen associated with significant case/fatality ratios in certain patient populations, particularly in those who are severely debilitated or immunosuppressed (for a review, see Denton & Kerr, 1998). *Stenotrophomonas africana* is also associated with human infections (Drancourt *et al*., 1997). Nothing is known about the pathogenic potential of the *S. nitritireducens* strains for humans or plants.

S. maltophilia was found in a wide variety of environments and geographical regions, and occupies ecological niches both inside and outside hospitals (Denton & Kerr, 1998). *S. maltophilia* has been isolated not only from typical environments such as the rhizospheres of various plants (Juhnke & Des Jardins, 1989; Berg *et al*., 1996) and from aquatic habitats (Minkwitz & Berg, 2001), but also from the extreme environment of a soda lake and from the faeces of an arthropod (Denton & Kerr, 1998). The variety of isolation sources has attracted scientific investigations concerning the epidemiology and differentiation of the strains. A certain heterogeneity in the physiological parameters has already been shown by Swings *et al*. (1983), Van den Mooter & Swings (1990) and also by Palleroni & Bradbury (1993) in the type description of *S. maltophilia*. Heterogeneity has been confirmed by genotypic studies (Gerner-Smidt *et al*., 1995; Chatelut *et al*., 1995; Nesme *et al*., 1995; Hauben *et al*., 1999; Berg *et al*., 1999). From the 16S rDNA sequencing analysis, 50 *S. maltophilia* isolates could be separated into three genomovars: two comprised isolates (especially rhizosphere isolates) originating from the environment, and one comprised clinical and aquatic strains (Minkwitz & Berg, 2001).

In this study, we characterized the molecular and physiological attributes of clinical and environmental *S. maltophilia* isolates, and defined isolates of one biovar and genomovar as a new species, *Stenotrophomonas rhizophila* sp. nov.

METHODS

Isolates and reference strains. A total of 16 clinical and environmental isolates of *Stenotrophomonas* were investigated (Table 1). The clinical strains were isolated in the Rigshospitalet, Copenhagen, Denmark, from various sites (tracheal aspirates, sputa, blood, throat, wounds, skin, ulcers, drainage fluids and aspirates, catheters, urine, etc.; Gerner-Smidt *et al*., 1995). *S. maltophilia* DSM 50170^T (= ATCC 13637^T, a type strain isolated from the pleural fluid of a patient with oral carcinoma; Hugh & Ryschenko, 1961), *S. nitritireducens* DSM 12575^T (a type strain isolated from an ammonia-supplied biofilter; Finkmann *et al*., 2000) and *Xanthomonas campestris* DSM 3586^T were used as reference strains. All *S. maltophilia* isolates were identified using the API system (BioMérieux) and the Biolog identification system (Biolog) as *S. maltophilia* (Berg *et al*., 1999). Unless otherwise stated, isolates were routinely grown in nutrient broth 2 (Sifin) and stored in broth containing 15% glycerol at −70 °C.

Physiological tests. The ability to use various sugars and derivatives was tested by using the API 50C system (bioMérieux). The cells were suspended in AUX-medium according to the manufacturer's manual, and were added to 50 different carbon sources. The ability to grow at 30 °C was judged visually from the turbidity of the cultures. Significant reactions, i.e. those showing differences in carbon-source utilization between the strains, were repeated in 50 ml MM8 medium at 180 r.p.m. and 30 °C. The ability to grow at 4, 37 and 41 °C was tested in Luria–Bertani medium. Growth at various salinities was investigated at 30 °C in 5 ml medium according to Palleroni & Doudoroff (1972), with the addition of 3·0, 4·0, 4·5, 5·0, 5·5 and 6·0% NaCl (w/v). In all cases, the optical density was measured at 600 nm. For analysing the production of osmolytes, isolates were grown in the medium suggested by Palleroni & Doudoroff (1972), supplemented with DL-methionine (40 mg l^{-1}) and NaCl to obtain the indicated salinity. Cells were harvested by centrifugation and washed with growth medium in which all organic constituents had been replaced by NaCl. Low-molecular-mass compounds were extracted with 80% ethanol, and the extracts were desalted using ion-exchange resins. HPLC analysis was performed according to Mikkat *et al*. (2000). Antifungal activity was determined by using a dual-culture *in vitro* assay on Waksman agar containing 5 g proteose-peptone (Merck), 10 g glucose (Merck), 3 g meat extract (Chemex), 5 g NaCl (Merck), 20 g agar (Difco) and distilled water (to 1 l), pH 6·8. Zones of inhibition were measured after 5 days incubation at 20 °C according to Minkwitz & Berg (2001). All strains were tested in three independent replicates. Fungi used in this bioassay include *Rhizoctonia solani*, *Verticillium dahliae*, *Sclerotinia sclerotiorum* and *Candida albicans*. The fungal strain *R. solani* DSM 63010 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The other pathogenic fungi were obtained from the strain collection of the University of Rostock (Department of Microbiology). These fungi were routinely grown on Sabouraud medium (Gibco) and stored in broth containing 15% glycerol at −70 °C.

Fatty acid analysis. Isolates were cultivated for 3 days on trypticase soy agar at 25 °C. Harvesting, saponification, methylation and extraction of fatty acid methyl esters were performed according to Sasser (1990). Identification of the fatty acid methyl esters was performed by GC-MS as described by McInroy & Kloepper (1995). Data on the fatty acids from *S. nitritireducens* strains were analysed by Finkmann *et al*. (2000).

16S rDNA sequencing. Bacterial DNA was prepared by following the protocol of Andersen & McKay (1983), modified for genomic DNA. 16S rDNA was amplified using the prokaryote-specific forward primer 16F27 and reverse primer 16R1525 (Lane, 1991) as described by Minkwitz & Berg (2001). For amplification, the PCR SuperMix High Fidelity (Gibco), with a mixture of *Taq* DNA polymerase and DNA polymerase from *Pyrococcus* sp. GB-D, was used. The amplified bands were eluted from the agarose and purified by using the GFX PCR DNA and Gel band purification kit (Amersham Pharmacia Biotech). The 16S rDNA fragments were cloned in the pGEM-T vector (Promega) and transformed into *Escherichia coli*. Plasmid isolation was carried out using the GFX *Micro* Plasmid Prep kit (Amersham Pharmacia Biotech), and sequencing reactions were done with the SequiTherm EXCEL *II* Long Read DNA Sequencing kit-LC (BIOzym), both according to the manufacturer's instruction. The gel run was performed

Table 1. Bacterial strains investigated in this study, their origins, relevant references, and clustering by 16S rDNA sequencing and fatty acid methyl esters

Strain	Currently valid or proposed name	Origin	Reference	Cluster biovar	Cluster fatty acid	Cluster genomovar
e-p10	*S. rhizophila*	Rhizosphere of rape; Rostock, 1993	Minkwitz & Berg (2000)	B1	F1	E1
e-p14	*S. rhizophila*	Rhizosphere of potato; Lüsewitz, 1996	Minkwitz & Berg (2000)	B1	F1	E1
e-p17	*S. rhizophila*	Geocaulosphere of potato; Lüsewitz, 1996	Minkwitz & Berg (2000)	B1	F1	E1
e-p3	*S. maltophilia*	Rhizosphere of rape; Poel, 1989	Minkwitz & Berg (2000)	B2	F2	E2
e-p19	*S. maltophilia*	Geocaulosphere of rape; Lüsewitz, 1996	Minkwitz & Berg (2000)	B2	F2	E2
e-p20	*S. maltophilia*	Rhizosphere of potato; Braunschweig, 1998	Berg *et al.* (2001)	B2	F2	E2
c5	*S. maltophilia*	Human; Copenhagen, 1995	Gerner Smidt *et al.* (1995)	B4	F2	C
c6	*S. maltophilia*	Human; Copenhagen, 1995	Gerner Smidt *et al.* (1995)	B4	F3	C
c20	*S. maltophilia*	Human; Copenhagen, 1995	Gerner Smidt *et al.* (1995)	B4	F2	C
e-a1	*S. maltophilia*	Brackish water; Zingst, 1996	Minkwitz & Berg (2000)	B5	F4	C
e-a22	*S. maltophilia*	Sewage; Braunschweig, 1999	Minkwitz & Berg (2000)	B4	F4	C
e-a21	*S. maltophilia*	Sewage; Braunschweig, 1999	Minkwitz & Berg (2000)	B4	F4	C
e-a23	*S. maltophilia*	Eye-care solution; Munich, 1999	Baader (1999)	B4	F4	C
e-p13	*S. maltophilia*	Rhizosphere of rape; Rostock, 1995	Minkwitz & Berg (2000)	B3	F2	C
DSM 50170^T	*S. maltophilia*	Human; 1961	Hugh (1961)	B4	ND	C
DSM 12575^T	*S. nitritireducens*	Ammonia-supplied biofilters	Finkmann *et al.* (2000)			
DSM 3586^T	*X. campestris*	*Brassica oleracea*	Skerman *et al.* (1980)			

using a LI-COR automated DNA sequencing machine (MWG Biotech). The sequence data were collected and analysed by the MWG-Biotech software package Base-ImagIR, version 4.1. The 16S rDNA sequence of each strain was aligned with reference 16S rRNA gene sequences from the GenBank, EMBL and DBJ databases by using the BLAST algorithm (Altschul *et al.*, 1997). Sequence similarities were calculated for the complete sequence by using unambiguously determined nucleotide positions with the sequence alignment program ALIGN PLUS, version 2.0 (Scientific and Educational Software). Distance and bootstrap analyses were performed with the ARB program (Ludwig & Strunk; http://www.mikro.biologie.tu-muenchen.de), using the neighbour-joining method (Saitou & Nei, 1987), maximum parsimony and maximum likelihood (Felsenstein, 1992).

Molecular typing by denaturing gradient gel electrophoresis. Total DNA was extracted from the bacterial pellet as described above. PCR amplification of the bacterial 16S rDNA fragment was carried out using primers V3341F (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and V3534R (5′-ATT ACC GCG GCT GCT GG-3′) spanning the 16S rDNA from positions 341 to 534, including the V3 region. Separation of the PCR products was done by denaturing-gradient gel electrophoresis (DGGE) analysis as previously described (Heuer *et al.*, 1997). The procedure was replicated three times.

DNA–DNA hybridization. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Huss *et al.* (1983) and Escara & Hutton (1980). Analysis was done using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER : BAS program of Jahnke (1992).

Statistical data analysis. Interisolate relationships were measured by the Euclidian metric, unweighted pair group arithmetic average-linkage algorithm method using the program STATISTICA (StatSoft). Thus, physiological data and fatty acid methyl ester data were converted to a binary code.

RESULTS

Physiological tests

A total of 16 *Stenotrophomonas* strains from different sources (listed in Table 1) were characterized by their physiological profiles including the following: growth at different temperatures and on different carbon sources; osmotolerance and production of osmoprotective substances; and enzymic and antifungal activity. These profiles were compared using numerical methods, and the resultant dendrogram based on percentage similarity between the isolates showed the diversity. Three different clusters (B1, B2 and B4) and two single strains (e-p13 = B3 and e-a1 = B5) could be separated on the basis of 85% similarity (Table 1). Three plant-associated isolates (e-p10^T, e-p14 and e-p17) formed the separate cluster B1. They showed 42% heterogeneity with respect to the other *Stenotrophomonas* isolates. Two other clusters (B2 and B3) were formed by plant-associated isolates with 20% heterogeneity to other B4-group strains. Cluster B4 contained human-associated strains as well as aquatic and sewage strains.

Table 2 shows a number of physiological characteristics that separate the B1-group isolates from other *S. maltophilia* isolates. Differences were found in the growth at different temperatures. While isolates of the B1 group grew at 4 °C but not at 40 °C, in contrast, the majority of *S. maltophilia* isolates grew at 40 °C but not at 4 °C. Furthermore, the B1 strains demonstrated the ability to use xylose as a carbon source, whereas the majority of *S. maltophilia* isolates were unable to use xylose. Other differences between B1 isolates and *S. maltophilia* included tolerance to 5% NaCl (w/v), and the production of osmoprotective substances. Most *S. maltophilia* isolates were able to tolerate this high (5% NaCl) salinity even though they produced only one osmoprotective substance (trehalose). The growth of the three isolates of the B1 group was inhibited by 5% NaCl (w/v). However, at a salinity of

Table 2. Physiological characteristics that differentiate *S. rhizophila* from *S. maltophilia*

Character	Positive strains (%)	
	B1 isolates (*S. rhizophila*) ($n = 3$)	*S. maltophilia* ($n = 13$)
Growth at:		
4 °C	100	16
37 °C	100	100
41 °C	0	72
Growth on xylose	100	18
Growth in the presence of 5% NaCl	0	100
Production of osmolytes		
Trehalose	100	100
Glucosylglycerol	100	0
Production of specific enzymes		
Lipase	0	82
β-Glucosidase	0	100
Antifungal activity towards:		
Candida albicans	100	0
Rhizoctonia solani	100	0
Sclerotinia sclerotiorum	100	0
Verticillium dahliae	100	0

Table 3. Physiological characteristics of the *Stenotrophomonas* and *X. campestris* isolates and reference strains

Strains: 1, *S. maltophilia* DSM 50170^T; 2, *X. campestris* DSM 3586^T; 3, *S. nitritireducens* DSM 12575^T; 4, *S. rhizophilia* ($n = 3$).

Characteristic	1	2	3	4
Nitrate reduction to nitrite	+	−	−	100
Aesculin hydrolysis	+	−	−	100
Lecithinase	+	+	−	0
Growth at:				
4 °C	−	+	−	100
37 °C	+	−	+	100
Growth in the presence of:				
Erythromycin	+	+	−	67
Kanamycin	−	−	−	0
Gentamicin	+	+		100
Tetracycline	−	−	−	0
Novobiocin	+	−	−	67
4% NaCl	+	−		100
Production of xanthomonadins	−	+	−	−
Plant pathogenicity	−	+	−	−

3%, the strains of B1 group produced two different osmolytes: trehalose and glucosylglycerol. At salinities higher than 5% NaCl (w/v), no growth could be detected for all strains tested (data not shown). Using APIZym, differences in enzyme production were found between both groups. Lipase and β-glucosidase activity was found for most *S. maltophilia* isolates but was absent among the B1 group strains. Antifungal activity against phytopathogenic and human-pathogenic fungi was tested using dual-culture assay. All of the B1 isolates possessed antagonistic activity, whereas the other isolates gave a negative reaction. Physiological properties of the strains of B1 group in comparison with other *Stenotrophomonas* and *X. campestris* strains are shown in Table 3.

Fatty acid analysis

By the analysis of fatty acid methyl esters of the total cellular fatty acids, significant differences between the isolates could be detected. The fatty acid profiles were compared by using numerical methods; the resultant grouping, founded on the basis of 80% similarity, is shown in Table 1. Three different clusters (F1, F2 and F4) and one single strain (c6 = F3) could be detected. Cluster F1 comprised the same isolates as the physiological B1 group. Iso-branched $C_{15:0}$ was the major fatty acid component of the B1 group and constituted 26–27% of the total content. Other characteristic fatty acids were iso-branched: $C_{14:0}$ (2–3%), $C_{17:1}\omega_{9c}$ (5–6%) and $C_{17:1}\omega_{8c}$ (0·5–0·7%). For the F1 cluster, the absence of the cyclic fatty acid $C_{17:0}$ (cyclopropane), which is typical for *S. maltophilia*, was a defining characteristic. Groups F2 and F4 contained plant- and human-associated isolates. The human isolate c6 formed its own group and was characterized by a very specific fatty acid profile. Detailed results on the fatty acid compositions of the isolates are available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org).

16S rDNA sequencing and phylogenetic analyses

By using primers annealing at positions 27 and 1525, respectively, of the 16S rDNA, nearly the complete gene could be amplified, cloned and finally sequenced. About 1500 bases were determined, corresponding to 99% of the gene. By cloning the amplified fragments and using plasmid- and prokaryote-specific primers, exact sequences of 700 to 800 bp could be obtained. Each gene sequence was confirmed by determining contiguous overlapping sequences in both directions.

A similarity of 98–99% meant that the strains could be determined as *S. maltophilia* by the BLAST algorithm. The most similar were *S. maltophilia* strains LMG 958^T and LMG 957 (with respect to the clinical strains) and LMG 10857 (with respect to the environmental E1 strains).

Fig. 1 shows the inferred phylogenetic relationship between the investigated strains, *S. maltophilia* DSM 50170^T, *S. nitridireducens* DSM 12575^T, *S. africana* and *X. campestris* DSM 3586^T. Three genetic clusters could be distinguished among the *S. maltophilia* isolates. Two of them, E1 and E2, consist of only

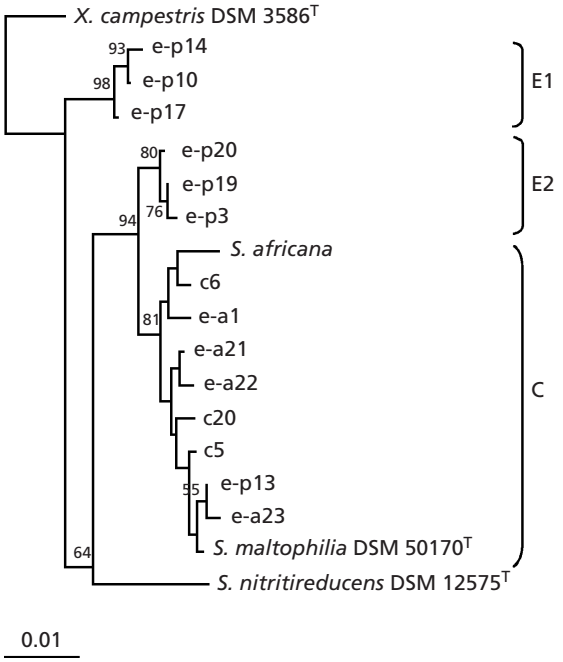

Fig. 1. Phylogenetic tree obtained by neighbour joining without the filter distance matrix, using the ARB program. The reliability of branching was assessed using bootstrapping analysis. Only values of more than 50% are shown. *X. campestris* DSM 3586^T is used as an outgroup for showing the root of the tree. Parsimony and maximum-likelihood estimations, using the PHYLIP program, resulted in the same tree, reflecting the differentiation of the E1 cluster as a separate species. Bar, 1% sequence difference.

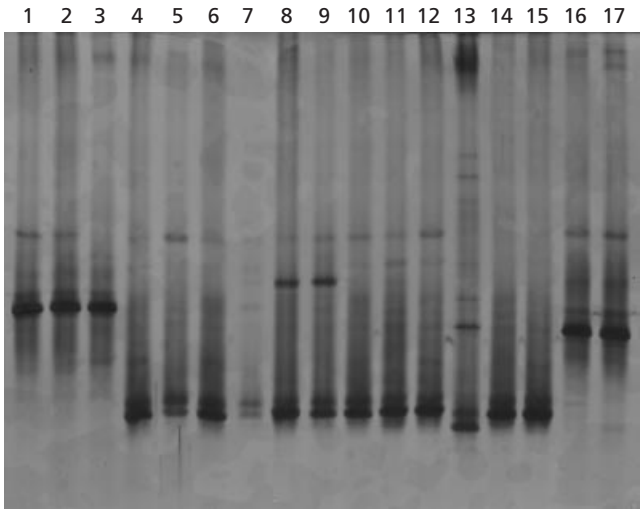

Fig. 2. DGGE profiles of the *S. rhizophila* isolates, *S. maltophilia* and reference organisms, based on their V3-16S rDNA sequence. Lanes: 1, e-p10^T; 2, e-p14; 3, e-p17; 4, e-p3; 5, e-p19; 6, e-p20; 7, c5; 8, c6; 9, c20; 10, e-a1; 11, e-a22; 12, e-a21; 13, e-a23; 14, e-p13; 15, *S. maltophilia* DSM 50170^T; 16, *S. nitritireducens* DSM 12575^T; 17, *X. campestris* DSM 3586^T.

plant-associated strains. The third cluster (C) included clinical and aquatic strains with a strong relationship to strain DSM 50170^T, as well as one plant-associated strain, e-p13. By all treeing methods, strains e-p10^T, e-p14 and e-p17 of the physiological B1 group formed the separate cluster E1 with about 99·5% similarity within the cluster and a difference of 2·5% from the clinical cluster, whereas the strains of the E2 cluster (physiological B2 cluster) showed a mean difference of only 1% from the clinical cluster and 1·5% from cluster E1. The small differences between clusters E2 and C seems not to point to the differentiation of a new species. The results emphasized the separate position of the E1 strains now named *S. rhizophila*. While *S. africana* clearly clustered into the C group, *S. nitritireducens* showed about 3% difference from all analysed sequences. The similarities to *X. campestris* strain DSM 3586^T were 96·5, 96·8 and 97·4 for the clinical, E2 and E1 clusters, respectively.

The main variable regions were located near the 5′ end of the 16S rDNA. Key sequences of the strains differentiated between the clinical and the environmental clusters as well as between the two environmental clusters. The most important area of difference is the V3 variable region (*E. coli* positions 447–487). Here, the clinical and cluster E2 strains were similar, whereas the cluster E1 strains showed the highest variability with nine base substitutions. This is reflected in the DGGE profiles of this region of the investigated and the reference strains (Fig. 2). It was possible to separate strains of E1 group from the other *Stenotrophomonas* strains according to their band positions on the DGGE gels. In the V8 variable region, which is regularly used for DGGE, only the cluster E1 strains showed divergent base compositions, and this was within four positions.

DNA–DNA hybridization experiments

The phylogenetic relationship between strain e-p10^T (the only representative of the E1 group) and the type strains of *S. maltophilia* and *S. nitritireducens* was resolved by DNA–DNA reassociation experiments. The DNA similarities were 48·1 and 52·3% (with *S. maltophilia*) and 30·1% (with *S. nitritireducens*). These values clearly indicate that strains of the E1 group represent a separate species, i.e. *S. rhizophila*.

DISCUSSION

Physiological aspects and fatty acid composition

The strains investigated were clearly members of the genus *Stenotrophomonas*, as seen from the numerous biochemical properties studied. Although the properties showed a high degree of heterogeneity, investigations confirmed that the strains belong to *S. maltophilia* (Berg *et al.*, 1999). We observed that plant-associated strains which belong to the physiological B1 group had the ability to grow at lower temperatures. As the environmental strains had a preference for lower temperatures, it is not surprising that they are able to survive in the rhizosphere (Sørensen, 1997). In

contrast to the other *S. maltophilia* isolates investigated, only members of the B1 group were able to utilize xylose as a carbon source. Xylose is a substantial component of the plant root. The ability to metabolize xylose is an advantage for plant-associated bacteria. Many such bacteria, for example *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas syringae* and *X. campestris*, are able to use this carbon source for both symbiotic and pathogenic interactions.

Bacteria are able to adapt within a certain range of changes in external osmolarity by the accumulation of osmoprotective compounds. The *Stenotrophomonas* strains showed different ranges of osmotolerance. *S. maltophilia* isolates could grow well at 5% NaCl, whereas for members of the B1 group, 4·5% was the highest salinity allowing growth. Tolerance to high and changing salinities is important for adaptation in both microenvironments (the rhizosphere and the human body) (Miller & Wood, 1996). All strains of *Stenotrophomonas* synthesized trehalose, but only strains of the B1 group accumulated glucosylglycerol as an additional substance. The composition of osmoprotective substances provided also valuable taxonomic information to characterize pseudomonads (Mikkat *et al.*, 2000).

Differences between the B1 group and the other *S. maltophilia* strains were found in enzyme production. In contrast to *S. maltophilia*, the B1-group showed no lipolytic activity or β-glucosidase production. *S. maltophilia* strains were strongly lipolytic (Denton & Kerr, 1998). The presence of glycoside hydrolases, e.g. β-glucosidases, is also typical for *S. maltophilia* and plant-pathogenic *Xanthomonas* strains (Van Zyl & Steyn, 1992). The three strains of the B1 group showed remarkable antifungal activity against plant-pathogenic fungi. At present, *S. maltophilia* is not regarded as a plant-pathogenic bacterium; on the contrary, it is associated with human infections. In fact, interaction with eukaryotic cells is typical for the *Stenotrophomonas–Xanthomonas* group. The grouping according to the physiological parameters was confirmed by the data on the fatty acid compositions. The isolates of the physiological B1 group formed a separate cluster (F1) with regard to fatty acid composition.

On the basis of these significant characteristics, we propose the new species *S. rhizophila*. The defining characteristics of the new species are as follows: growth at 4 °C and the absence of growth at 40 °C; the utilization of xylose as a carbon source; lower osmotic tolerance (< 4·5% NaCl, w/v), although they can produce glucosylglycerol in addition to trehalose; the absence of lipase and β-glucosidase production; and antifungal activity.

Analysis of the 16S rDNA sequence

The pairwise comparison of all 16S rDNA sequences resulted in three separate clusters corresponding well with the clustering obtained after analysis of physiological features. Two clusters with environmental rhizosphere isolates (E1 and E2) and one with clinical, aquatic and (one) environmental strains (C) were obtained. The aquatic strains were derived from a brackish lagoon (e-a1), sewage (e-a21, e-a22) and eye-care solution (e-a23). Since the brackish lagoon can be characterized as a prefilter basin of the Baltic Sea, with a high input of sewage from households and hospitals (Schlungbaum *et al.*, 1994), and since the strains isolated from the eye-care solution caused endophthalmitis (Bader *et al.*, 1999), classification of these strains as clinical can be concluded. Strain e-p13 represented an exception as it originated from the rhizosphere of oilseed rape. This complicates the exact differentiation between clinical and environmental isolates by 16S rDNA sequence analysis, but illustrates the possibility that strains may be able to cross the clinical–environmental divide. Interestingly, strain e-p13 was clustered according to its fatty acid profile into group F2 (comprising only plant-associated strains). According to the physiological data, the strain formed a separate cluster (B3), indicating its atypical behaviour.

Upon analysing the sequence data in more detail, we noted that the cluster E1 strains differ in 32 bases (2·1%) when compared with the clinical isolates, most of the differences occurring in the V2, V3 and V8 variable regions. Twenty-three of these were similar to those of *X. campestris* strain DSM 3585^T. The separation of the E1 cluster from the other strains tested was emphasized by all the treeing methods used with high bootstrap values. The base changes can be considered as 'signature positions' and are characteristic for the cluster. The strains of cluster E2 differed from the clinical isolates in 13 bases (0·8%). Seven positions were the same as those of strains in cluster E1 and *X. campestris* DSM 3585^T. The clinical strains varied in 10 bases, corresponding to 0·6% of the whole 16S rRNA operon.

The V3 variable region was the only one likely to classify strains of cluster E1 from those of clusters E2 and C within a narrow range of nucleotides (Minkwitz & Berg, 2001). The environmental strains of E1 varied in nine independent positions in V3, which therefore makes this region suitable for oligonucleotide probe design (Amann *et al.*, 1995). A larger part of this region has been used in DGGE (Muyzer *et al.*, 1993). As shown in Fig. 2, with suitable primers we could differentiate between strains of cluster E1 and the human-associated and aquatic strains. At the V8 region, which is regularly used for DGGE analysis, the strains of cluster E1 differed in only four bases from the clinical, as well as from the E2, cluster strains. This difference has proved to be too small to allow differentiation by DGGE analysis (Berg *et al.*, 1999).

Additionally, the E1 strains were different in two positions (989 and 1216) of the U6 universal region. Comparison of this with the secondary structure of the 16S rRNA molecule (Woese *et al.*, 1983) indicated that these two positions are complementary and have no influence on the molecular structure.

Phylogenetic analysis

The strains of cluster E1 showed significant differentiation when comparisons were made not only with the physiological profiles but also with the 16S rDNA sequence data. On the basis of 16S rDNA, Moore *et al.* (1997) mentioned a 3% difference between the genera *Stenotrophomonas* and *Xanthomonas*, which is equivalent to 45–68 nucleotide positions. Here, we obtained 44 nucleotide differences between strains *S. maltophilia* DSM 50170^T and *X. campestris* DSM 3586^T, which is exactly 2·96%. Normally, this level of sequence difference suggests strains of different species. If 3% is used as the 'yardstick' between the two genera *Stenotrophomonas* and *Xanthomonas*, and after the transfer of the high 16S rDNA similarity values among various *Xanthomonas* species of 98·9–100% (Hauben *et al.*, 1997) to *S. maltophilia*, the isolates of cluster E1 must be regarded as a different species.

The bacterial species definition should be based on both DNA–DNA hybridization and 16S rDNA sequence analysis (Stackebrandt & Goebel, 1994). In this study, the DNA–DNA hybridization resulted in strong differences between the representative strain of the B1 group (e-p10^T) and the type strains of *S. maltophilia* and *S. nitritireducens*. According to Wayne *et al.* (1987), differences of more than 30% in DNA–DNA hybridization indicates a new species. Because of the clear physiological and phylogenetic clustering of the E1 strains, it did not appear to be necessary to do this analysis within these strains. On the basis of these results, we propose a new bacterial species, *S. rhizophila*.

Description of *Stenotrophomonas rhizophila* sp. nov.

Stenotrophomonas rhizophila (rhi.zo′phi.la. Gr. n. *rhizo* root; Gr. adj. *philos* loving; *rhizophila* root-loving).

Straight or slightly curved rods. Colonies are yellowish; the colour is not due to carotenoid pigments or to xanthomonadins. Growth takes place at 4–37 °C but not at 40 °C. Neither lipolytic nor β-glucosidase activity occurs. The strains use xylose as a carbon source. Strains are able to utilize (oxidize) the following carbon sources: dextrin, glycogen, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-fructose, gentiobiose, α-D-glucose, maltose, D-mannose, methyl β-D-glucoside, D-trehalose, turanose, methylpyruvate, mono-methyl succinate, acetic acid, *cis*-aconitic acid, citric acid, α-hydroxy butyric acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, α-keto-butyric acid, α-keto glutaric acid, DL-lactic acid, malonic acid, propionic acid, succinic acid, bromo succinic acid, succinamic acid, alaninamide, D-alanine, L-alanine, L-alanine, L-alanylgyline, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, L-serine, L-threonine, DL-carnitine, urocanic acid, inosine and uridine. The predominant fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Growth occurs in the presence of 4·5% NaCl but is inhibited at 5% NaCl and above. Produces the osmoprotective substances trehalose and glucosylglycerol at 3% NaCl. Resistant to many antibiotics, e.g. penicillin, tobramycin, imipenem, and ceftazidime, but susceptible to chloramphenicol, kanamycin and trimethoprim/sulfamethoxazole. Strains were plant-associated and isolated from the rhizosphere of oilseed rape and from the the rhizosphere and geocaulsophere (tuber) of potato. Endophytic colonization was found. Antagonistic activity was shown against plant-pathogenic fungi, e.g. *Verticillium dahliae*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and the human-pathogenic fungus *Candida albicans*. They are not active against bacteria. The type strain is *Stenotrophomonas rhizophila* e-p10^T (= DSM 14405^T = ATCC BAA-473^T).

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