Stenotrophomonas terrae sp. nov. and *Stenotrophomonas humi* sp. nov., two nitrate-reducing bacteria isolated from soil

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Three Gram-negative, rod-shaped, non-spore-forming, nitrate-reducing isolates (R-32746, R-32768^T and R-32729^T) were obtained from soil. Analysis of repetitive sequence-based PCR showed that the three isolates represented two different strains. 16S rRNA gene sequence analysis and DNA–DNA hybridization placed them within the genus *Stenotrophomonas* and revealed that they were genotypically different from each other and from all recognized *Stenotrophomonas* species. Analysis of the fatty acid composition and physiological and biochemical tests allowed differentiation from their closest phylogenetic neighbours. They are therefore considered to represent two novel species, for which the names *Stenotrophomonas terrae* sp. nov. and *Stenotrophomonas humi* sp. nov. are proposed, with strains R-32768^T (=LMG 23958^T=DSM 18941^T) and R-32729^T (=LMG 23959^T=DSM 18929^T), respectively, as the type strains.

In the large-scale phenotypic study of Stanier *et al.* (1966), *Pseudomonas maltophilia* was regarded as an authentic member of the genus *Pseudomonas*. Subsequent allocation of this species to the genus *Xanthomonas* as *Xanthomonas maltophilia* was supported by rRNA hybridization data (Swings *et al.*, 1983), but resulted in a reclassification at the genus level into *Stenotrophomonas* Palleroni and Bradbury 1993, the genus being differentiated from both *Pseudomonas* and *Xanthomonas* based on various taxonomic methods. At the time of writing, the genus *Stenotrophomonas* comprises six recognized species, *Stenotrophomonas maltophilia* (Palleroni & Bradbury, 1993), *S. nitritireducens* (Finkmann *et al.*, 2000), *S. acidaminiphila* (Assih *et al.*, 2002), *S. rhizophila* (Wolf *et al.*, 2002), *S. korensis* (Yang *et al.*, 2006) and *S. dokdonensis* (Yoon *et al.*, 2006).

A cultivation-dependent study on soil used selective isolation conditions to focus on the microbial diversity involved in nitrogen removal. Within the dominant gammaproteobacterial nitrate reducers, seven isolates were retrieved on the defined isolation medium G3M12 – with ethanol as carbon source and nitrate as nitrogen source

(Heylen *et al.*, 2006) – that could be assigned to *Stenotrophomonas* based on partial 16S rRNA gene sequences. Three of these isolates, R-32746, R-32768^T and R-32729^T, could possibly represent novel species based on partial 16S rRNA gene sequence similarities and were analysed further in a polyphasic study. With all of the techniques employed the strains were identified as not representing *S. maltophilia* (recommended comparison). The type strains of all recognized *Stenotrophomonas* species, except that for the phylogenetically most distant *S. dokdonensis* (Fig. 1), were re-examined for phenotyping, chemotaxonomy and biochemical analysis to guarantee comparable data. In addition, *S. maltophilia* LMG 22072, which was the proposed type strain of *Stenotrophomonas africana*, was included [*Stenotrophomonas africana* was found to be a later heterotypic synonym of *Stenotrophomonas maltophilia* (Coenye *et al.*, 2004a)].

In order to avoid studying duplicate isolates of the same strain, genotyping by random amplified polymorphic DNA PCR analysis (Coenye *et al.*, 2002) and repetitive sequence-based PCR analysis with REP and BOX primers (Heyrman *et al.*, 2005) were carried out. The three fingerprint methods generated identical patterns for isolates R-32746 and R-32768^T, but showed clear genetic differences for isolate R-32729^T (data not shown). As R-32746 and R-32768^T are most probably members of a single strain, only R-32768^T was subject to further study. The DNA G+C contents of strains R-32768^T and R-32729^T, based on single HPLC determinations (Mesbah *et al.*, 1989), were 65 and

Abbreviation: RFLP, restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains R-32768^T and R-32729^T are AM403589 and AM403587, respectively.

A table giving the cellular fatty acid compositions of strains R-32768^T and R-32729^T and their closest phylogenetic neighbours is available as supplementary material with the online version of this paper.

Fig. 1. Phylogenetic dendrogram of 16S rRNA gene sequences showing the position of strains R-32729^T and R-32768^T among the type strains of *Stenotrophomonas* species. Neighbour-joining clustering was performed with BioNumerics version 4.6. Bootstrap values (expressed as percentages of 1000 replicates) are shown at branch points. EMBL accession numbers are shown in parentheses. '*Stenotrophomonas daejeonensis*' is also included, but this name has not been validly published and only the 16S rRNA gene sequence is available.

64 mol%, respectively. Nearly complete 16S rRNA gene sequences of strains R-32768^T and R-32729^T were determined as described by Vanparrys *et al.* (2005). Phylogenetic analysis was performed by using BioNumerics software version 4.6 after multiple alignment with CLUSTAL_X (Thompson *et al.*, 1997). Cluster analysis via the neighbour-joining algorithm with or without corrections for evolutionary distances as described by Jukes & Cantor (1969) and Kimura (1980) were in agreement with the maximum-parsimony and maximum-likelihood methods. Strains R-32729^T and R-32768^T clustered together with *S. nitritireducens* LMG 22074^T and *S. acidaminiphila* LMG 22073^T, supported by high bootstrap values (Fig. 1). Therefore, DNA–DNA hybridization experiments were performed within this cluster, by using a modification of the microplate method of Ezaki *et al.* (1989) as described by Willems *et al.* (2001). A hybridization temperature of 45 °C (calculated with correction for the presence of 50 % formamide) was used. Strains R-32768^T and R-32729^T showed a mean DNA–DNA relatedness of 44.2 ± 2.8 % ($n=2$ for all determinations) with each other. Strain R-32768^T showed a mean DNA–DNA relatedness of 41.3 ± 7 and 35.8 ± 4.7 % with *S. nitritireducens* LMG 22074^T and *S. acidaminiphila* LMG 22073^T, respectively. Strain R-32729^T showed a mean DNA–DNA relatedness of 37.2 ± 6.6 and 38.1 ± 5.1 % with *S. nitritireducens* LMG 22074^T and *S. acidaminiphila* LMG 22073^T, respectively. These results confirmed that strains R-32729^T and R-32768^T represent two novel genotypic species.

Cell morphology, motility and possible sporulation were investigated using phase-contrast microscopy at a magnification of 1000 $\times$ with cells grown on tryptone soy agar (TSA; Oxoid) for 48 h at 28 °C. Cells were Gram stained and examined for catalase and oxidase activity. Utilization of carbon sources and enzyme production were tested with

the API 20NE (48 h, 28 °C), API ZYM (4 h, 28 °C) and API 50 CH (inoculated with AUX medium, 48 h, 28 °C) systems (bioMérieux), according to the manufacturer's instruction. Strains R-32729^T and R-32768^T were identified as not representing *S. maltophilia* with these conventional phenotypic taxonomic tests. The temperature range (at 4, 15, 28, 37 and 52 °C), pH range (4.5–10.5 at 28 °C) and salinity range (0.5–5 % w/v, at 28 °C) for growth were recorded after incubation for 72 h on TSA. The ability to reduce nitrate was tested, as described by Smibert & Krieg (1994), after growth for 2 weeks on tryptone soy broth (TSB; Oxoid) supplemented with 10 mM potassium nitrate at 37 °C and on liquid G3M11 medium containing 18 mM potassium nitrate and 22.5 mM ethanol (Heylen *et al.*, 2006) at 20 °C – the solid variant of the latter was used as isolation medium for the *Stenotrophomonas* strains. For all strains, these results were in agreement with the nitrate reduction test in the API 20NE system. Lipolytic activity was tested via hydrolysis of Tween 80, as described by Sierra (1957). The phenotypic and biochemical characteristics of all strains tested are given in Table 1.

Cells of all strains were incubated under identical conditions for 24 h at 28 °C on TSA. One loopful of cells was harvested, fatty acid methyl esters were prepared and these were then extracted according to the standardized protocol of the Microbial Identification System (MIS; Microbial ID Inc.). The MIDI with the TSBA50 database was used for identification. All test strains contained the fatty acids iso-C_{11:0}, iso-C_{11:0} 3-OH and iso-C_{13:0} 3-OH characteristic for the genus *Stenotrophomonas* (Assih *et al.*, 2002; Yang *et al.*, 1993), and iso-C_{15:0} as the dominant fatty acid. Strains R-32768^T and R-32729^T had highly similar fatty acid profiles, only differing in the amount of specific components, and mostly containing iso-branched compounds. Characteristic fatty acids for strains R-32768^T

Table 1. Physiological characteristics of strains R-32768^T and R-32729^T and their closest phylogenetic neighbours in the genus *Stenotrophomonas*

Strains: 1, R-32768^T (*Stenotrophomonas terrae* sp. nov.); 2, R-32729^T (*Stenotrophomonas humi* sp. nov.); 3, *S. nitritireducens* LMG 22074^T; 4, *S. acidaminiphila* LMG 22073^T; 5, *S. koreensis* LMG 23369^T; 6, *S. maltophilia* LMG 958^T; 7, *S. maltophilia* LMG 22072; 8, *S. rhizophila* CCUG 47042^T. The type strain of *S. dokdonensis* is not included. Data are from this study unless indicated. +, Positive; w, weakly positive; –, negative; v, variable. All strains characterized in this study are positive for catalase, alkaline phosphatase, esterase, esterase lipase, trypsin, acid phosphatase and glucose fermentation. All are negative for lipase, cystine arylamidase, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, α -mannosidase, α -fucosidase, indole production, arginine dihydrolase, urease, β -galactosidase, assimilation of caprate, adipate, phenyl acetate, glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, inulin, D-melezitose, D-raffinose, starch, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate.

Characteristic	1	2	3	4	5	6	7	8
Enzymic activities								
Hydrolysis of Tween 80 (lipolytic activity)	+	–	–, +*	–, +†	+	+	–	+
Nitrate reduction	+	+	–	v‡	–	+	–	+
Denitrification	–	–	v§	–, +†	–	–	–	–
Oxidase	+	+	+	+	–, +	–	–	+
Leucine arylamidase	+	–	–	–	–	–	+	+
Valine arylamidase	–	–	–	–	–	–	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	–	+	+	+
β -Glucosidase	–	–	–	–	–	w	+	+
N-Acetyl- β -glucosaminidase	–	+	+	+	–	–	–	–
β -Glucosidase (aesculin hydrolysis)	w	–	–	+	–	+	+	+
Protease (gelatin hydrolysis)	+	w	–	–	+	+	+	+
Assimilation of:								
D-Cellobiose	–	–	–	–	–	+	+	+
D-Fructose	+	+	+	+	–	+	+	+
Gentiobiose	–	–	–	–	–	+	+	–, +¶
D-Glucose	+	–	–	+	–	+	+	+
D-Lactose	–	–	–	–	–	–, +#	w, +#	–
Maltose	+	+	–	+	–	+	+	+
D-Mannose	+	+	–	+	–	+	+	+
D-Melibiose	–	–	–	–	–	–	+	–
Sucrose	–	–	–	–	–	+	+	–
Trehalose	–	–	–	–	–	–, +#	+	–, +¶
D-Turanose	–	–	–	–	–	–	+	–, +¶
D-Xylose	–	–	–	–	–	–	–	–, +¶
Citrate	+	+	+	–	–	+	+	+
Malate	–	+	–	–	–	+	+	+
Inositol	w	–	–	–	–	–	–	–
N-Acetylglucosamine	+	+	+	+	–	+	+	+
Amygdalin	–	–	–	–	–	+	+	–
Arbutin	–	–	–	–	–	+	+	–
Aesculin	–	–	–	–	–	+	+	+
Salicin	–	–	–	–	–	+	–	–
Glycogen	–	–	–	–	–	–	–	–, +¶

*Data from Finkmann *et al.* (2000).

†Data from Assih *et al.* (2002).

‡LMG 22073^T reduces nitrate when grown on TSA supplemented with 10 mM nitrate, but does not reduce nitrate when grown on defined growth medium G3M11.

§LMG 22074^T cannot denitrify starting from nitrate, only from nitrite.

||Data from Yang *et al.* (2006).

¶Data from Wolf *et al.* (2002).

#Data from Drancourt *et al.* (1997).

and R-32729^T were iso-C_{14:0} (14.2 and 15.7 %, respectively), iso-C_{15:1} (4.6 and 2 %), iso-C_{16:0} (8 and 12.7 %) and iso-C_{17:1ω9c} (7.2 and 4.6 %). The complete fatty acid profiles of all the test strains are available as Supplementary Table S1 in IJSEM Online. Numerical analysis of the fatty acid profiles (Fig. 2) revealed that strains R-32768^T and R-32729^T formed a distinct cluster, supported with a high co-phenetic correlation, and grouping closely with their phylogenetically nearest neighbours *S. nitritireducens* LMG 22074^T and *S. acidaminiphila* LMG 22073^T. The MIDI fatty acid identification system showed no relevant matches for strains R-32768^T and R-32729^T. However, the position of these strains within the group *Stenotrophomonas*–*Xanthomonas* was further confirmed by comparing the fatty acid profiles qualitatively and quantitatively with an in-house database containing over 60 000 fatty acid profiles. The failure of the MIDI identification system to allocate these strains within the genus *Stenotrophomonas* was also observed for several highly similar profiles of members of the genus *Xanthomonas* (K. Heylen and P. De Vos, unpublished data) and reported previously for *S. acidaminiphila* by Assih *et al.* (2002).

Coenye *et al.* (2004b) and Hauben *et al.* (1999) suggested that, prior to their description, the relationship of novel species within the genus *Stenotrophomonas* with the heterogeneous species *S. maltophilia* should be investigated. *gyrB*-restriction fragment length polymorphism (RFLP) analysis of *S. maltophilia* strains has proven to be in accordance with DNA–DNA hybridization results (Coenye *et al.*, 2004b). Therefore, the *gyrB*-RFLP profiles of strains R-32768^T and R-32729^T were analysed as described by Coenye *et al.* (2004b), together with an additional set of *Stenotrophomonas* strains representing *gyrB*-RFLP clusters A, B, C, E, F, G and I. Strains R-32768^T and R-32729^T did not render a *gyrB* amplicon, even after several repeats. The same observation was made for two *Stenotrophomonas* spp. strains (T. Coenye, personal communication). It was concluded that this approach was not suitable for the novel strains, which, therefore, must differ from the strains that did render *gyrB* amplicons.

To substantiate further that strains R-32729^T and R-32768^T do not represent *S. maltophilia*, SDS-PAGE analysis of whole-cell proteins was performed on all strains included

in the *gyrB*-RFLP trials. Aerobically grown cells were harvested after incubation at 28 °C for 24 h on phosphate-buffered nutrient agar (pH 6.8). An SDS-PAGE banding pattern for all strains was generated according to the standardized protocol of Pot *et al.* (1994). Pearson's correlation similarity coefficients were clustered with UPGMA and analysed with the co-phenetic correlation method in BioNumerics version 4.6 (Fig. 3). The grouping of the whole-cell protein profiles was supported by high co-phenetic correlation values but did not correlate with the *gyrB*-RFLP grouping, except for *gyrB*-RFLP cluster G. Strains R-32729^T and R-32768^T did not group together. Their phylogenetically closest neighbours, *S. nitritireducens* LMG 22074^T and *S. acidaminiphila* LMG 22073^T, together with strain R-12772, grouped separately from all other *Stenotrophomonas* strains.

Based on the data presented from the polyphasic study, strains R-32768^T and R-32729^T are considered to represent two novel species of the genus *Stenotrophomonas*, for which the names *Stenotrophomonas terrae* sp. nov. and *Stenotrophomonas humi* sp. nov. are proposed.

Description of *Stenotrophomonas terrae* sp. nov.

Stenotrophomonas terrae (ter'rae. L. gen. n. *terrae* of/from soil).

After 24 h incubation at 28 °C on TSA, colonies are irregular in shape and light yellow. Cells are motile, non-spore-forming, Gram-negative rods. Catalase- and oxidase-positive. Growth is observed at 15–37 °C (but not at 4 or 52 °C), at pH 5–10.5 (but not at pH 4.5) and at salinity of 0.5–5 %. Anaerobic growth is possible through nitrate reduction. Enzyme activities and carbon utilization results are given in Table 1. Can be differentiated from the type strains of its closest phylogenetic neighbours, *S. humi*, *S. nitritireducens* and *S. acidaminiphila*, by SDS-PAGE analysis and the presence of leucine arylamidase and protease, the assimilation of glucose and the absence of *N*-acetyl-β-glucosaminidase.

The type strain is R-32768^T (=LMG 23958^T=DSM 18941^T), which has a DNA G+C content of 65 mol%, and was isolated from soil from a university test field in Ghent, Belgium.

Fig. 2. Numerical comparison of the fatty acid profiles obtained. UPGMA clustering with Pearson's correlation similarity coefficients was performed by using BioNumerics version 4.6. The co-phenetic correlation tool, which distinguishes reliable from unreliable subclusters, was used for cluster significance analysis.

Fig. 3. Grouping of normalized digitized SDS-PAGE patterns. UPGMA clustering with Pearson's correlation similarity coefficients was performed by using BioNumerics version 4.6. The co-phenetic correlation tool, which distinguishes reliable from unreliable subclusters, was used for cluster significance analysis. The identification of non-type strains and the assignment to *gyrB*-RFLP clusters were taken from Coenye *et al.* (2004b).

Description of *Stenotrophomonas humi* sp. nov.

Stenotrophomonas humi (hu'mi. L. gen. n. *humi* of/from soil).

After 24 h incubation at 28 °C on TSA, colonies are round, smooth and beige. Cells are motile, non-spore-forming, Gram-negative rods. Catalase- and oxidase-positive. Growth is observed at 15–37 °C (but not at 4 or 52 °C), at pH 5–10.5 (but not at pH 4.5) and at salinity of 0.5–4 % (but not at 5 %). Anaerobic growth is possible through nitrate reduction. Enzyme activities and carbon utilization results are given in Table 1. Can be differentiated from the type strains of its closest phylogenetic neighbours, *S. terrae*, *S. nitritireducens* and *S. acidaminiphila*, by SDS-PAGE analysis and the assimilation of malate.

The type strain is R-32729^T (=LMG 23959^T=DSM 18929^T), which has a DNA G+C content of 64 mol%, and was isolated from soil from a university test field in Ghent, Belgium.

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