Genomovars 11 to 18 of *Pseudomonas stutzeri*, identified among isolates from soil and marine sediment

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Amongst 440 strains of *Pseudomonas stutzeri* isolated from soil and marine sediment for a population genetic study, eight strains were each presumed to represent a novel genomic group and were compared with each other and to reference strains of *P. stutzeri* genomovars 1 to 10 and other *Pseudomonas* species by DNA–DNA hybridization, 16S rRNA and internally transcribed 16S–23S rRNA spacer region (ITS1) sequences and basic physiological properties defining the species. While 16S rRNA and ITS1 gene sequences positioned the eight strains within the phylogenetic branch of *P. stutzeri*, the DNA–DNA hybridizations with reference strains of the 10 described genomovars and among the novel strains were generally below 70 %, which is the threshold for species and genomovar differentiation. Since the physiological properties studied in the eight strains fitted the profile of *P. stutzeri*, eight new genomovars of *P. stutzeri*, numbered 11 to 18, are proposed, with strains 28a50, 28a39, 28a22, 28a3, 4C29, 24a13, 24a75 and MT-1 being the reference strains. The highly transformable reference strain 28a3 of genomovar 14 had a localized 16S rRNA gene sequence tag characteristic of genomovar strains 2 and 3, suggesting a possible horizontal gene transfer event involving part of the 16S rRNA gene.

The species *Pseudomonas stutzeri* is a non-fluorescent member of the genus *Pseudomonas* (γ -Proteobacteria) displaying high genetic (Rius *et al.*, 2001; Cladera *et al.*, 2004) and physiological diversity (Rosselló *et al.*, 1991). Strains of *P. stutzeri* have been isolated from a variety of environmental and clinical habitats (Sikorski *et al.*, 2002a and references therein). Some strains received attention as model organisms because of their specific metabolic properties (Musarrat & Hashsham, 2003; Obradors & Aguilar, 1991; Rosselló-Mora *et al.*, 1994; Zumft, 1997) and their ability for natural genetic transformation (Berndt *et al.*, 2003; Meier & Wackernagel, 2003; Sikorski *et al.*, 1998, 2002b).

Taxonomically, *P. stutzeri* strains have been grouped into 10 genomovars by DNA–DNA hybridization (García-Valdés *et al.*, 2003; Rosselló *et al.*, 1991; Rosselló-Mora *et al.*, 1996; Sepúlveda-Torres *et al.*, 2001; Ursing *et al.*, 1995). In a recent population-genetic study, approximately 440 strains from soil and marine environments were studied by random amplified polymorphic DNA-PCR (RAPD-PCR) and the 16S rRNA gene sequence was determined (>1450 bp) (Sikorski *et al.*, 2002a) for 34 of the strains (several being representatives of the main RAPD groups). The 16S rRNA gene sequences suggested that seven representative strains (28a50, 28a39, 28a22 and 28a3, from soil close to Tel Aviv airport, Israel; 4C29, from marine sediment on the shore of the North Sea coast, Germany; 24a13 and 24a75, from a soil contaminated with mineral oil, Germany) and strain MT-1 (from Mariana Trench, Japan; Tamegai *et al.*, 1997) were members of new genomovars. Based on their 16S rRNA gene sequence dissimilarity values, these eight strains were as different from each other and from reference strains of the established genomovars 1 to 10 as the genomovar reference strains differed from each other (Table 1). Moreover, the lowest dissimilarity value of any of these strains to each other or to a reference strain of an established genomovar (0.41 %; Table 1) was larger than the maximum dissimilarity value observed among nine strains within three established genomovars (0.31 %; Table 1). In the following,

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Abbreviation: RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the ITS1 rRNA gene sequences of genomovars 11 to 18 of *Pseudomonas stutzeri* are AY850023–AY850031.

Two phylogenetic trees based on 16S rRNA and ITS1 gene sequences and tables detailing DNA–DNA relatedness values and physiological properties of the genomovars are available as supplementary material in IJSEM Online.

Table 1. 16S rRNA gene sequence dissimilarities

Strains: 1, Genomovar 1 reference strains CCUG 11256^T, ATCC 17589, ATCC 17593; 2, genomovar 2 reference strains ATCC 17591, ATCC 14405, ATCC 17587; 3, genomovar 3 reference strains DSM 50227, AN10, AN11; 4, genomovar type strain CCUG 11256^T (gv. 1) and reference strains ATCC 17591 (gv. 2), DSM 50227 (gv. 3), DSM 6084 (gv. 4), DSM 6082 (gv. 5), DSM 50238 (gv. 7), JM 300 (gv. 8), KC (gv. 9), CLN 100 (gv. 10).

Comparison	Dissimilarity measures (%)			
	Mean	Median	Min.	Max.
Within established genomovars				
1*	0.20	0.30	0.00	0.30
2*	0.09	0.07	0.07	0.14
3*	0.25	0.31	0.14	0.31
Among established genomovars 1 to 10†				
4	1.34	1.38	0.07	2.23
Comparison of reference strains of new genomovars 11 to 18 to reference strains of established genomovars 1 to 10†				
Gv. 11 28a50	0.99	0.75	0.42	2.75
Gv. 12 28a39	1.31	1.19	0.63	3.11
Gv. 13 28a22	1.23	1.01	0.69	3.06
Gv. 14 28a3	1.07	0.83	0.42	2.86
Gv. 15 4C29	1.22	1.09	0.41	3.00
Gv. 16 24a13	1.25	1.22	0.69	2.58
Gv. 17 24a75	2.89	2.93	2.58	3.11
Gv. 18 MT-1	1.29	1.18	0.41	2.74
Among new genomovars 11 to 18	1.42	1.13	0.41	3.11

*Affiliation of three members per genomovar was previously shown by DNA–DNA hybridizations (Rosselló *et al.*, 1991).

†Genomovar 6 was reclassified as *Pseudomonas balearica* (Bennasar *et al.*, 1996).

we present further data to support the identification of eight new genomovars including the results of DNA–DNA hybridization studies, sequence analyses of 16S–23S rRNA internally transcribed spacer regions (ITS1) and comparisons of physiological properties. Additionally, two strains (28a18 and 28a69) which were suggested by RAPD-PCR to belong to the new genomovars represented by strains 28a3 (genomovar 14) and 28a22 (genomovar 13), respectively (Sikorski *et al.*, 2002a), were included in part of the studies.

Genomic DNA from the eight new genomovar reference strains and from strains 28a18 and 28a69 was isolated using the Genomic DNA kit from QIAGEN and from the reference strains of genomovars 1–10 by the method of Marmur (1961). DNA–DNA hybridizations were performed using a modification of the hydroxyapatite method as described by Ziemke *et al.* (1998). Reference DNAs were double-labelled with DIG-11-dUTP and biotin-16-dUTP, using a nick-translation kit (Boehringer Mannheim). Sequences of the 16S rRNA and ITS1 genes were determined as described by Guasp *et al.* (2000) and Sikorski *et al.* (2002a). Phylogenetic analysis was performed using the ARB package for 16S rRNA gene sequences (Ludwig *et al.*, 2004) and MEGA version 2.1

(Kumar *et al.*, 2001) for ITS1 sequences. Physiological tests were done according to Smibert & Krieg (1994).

The high DNA–DNA relatedness values of strain 28a18 with 28a3 (91 %) and 28a69 with 28a22 (93 %) indicate membership of the respective genomovar, as suggested by RAPD-PCR (Sikorski *et al.*, 2002a). In contrast, the DNA–DNA relatedness values of the eight representative strains of the new genomovars to each other and to the reference strains of the genomovars 1 to 10 were at or below the threshold value of 70 % for species delineation (Table 2) except for two pairs (77 %, MT-1/28a22; 82 %, 28a3/24a13; Supplementary Table S1 available in IJSEM Online). However, the 16S rRNA and ITS1 gene sequence analyses group all of them into the same phylogenetic branch as the previously described strains of *P. stutzeri* (Supplementary Figs S1 and S2 available in IJSEM Online), indicating their phylogenetic affiliation with the *P. stutzeri* group. Additionally, physiological traits characteristic of the species *P. stutzeri* were present in the eight strains (see Supplementary Table S2 in IJSEM Online). Motility, denitrification and starch hydrolysis are characteristic of most *P. stutzeri* strains, but exceptions have been described (strain JM300 is amylase-negative, strain ZoBell is described as non-motile). New

Table 2. DNA–DNA relatedness values (%)

The strains used for comparisons were the same as in Table 1.

Dissimilarity measures	Mean	Median	Max.	Min.
Among new genomovars 11 to 18	52	56	82	28
Comparison of reference strains of new genomovars 11 to 18 to reference strains of established genomovars 1 to 10	38	45	68	9
Comparison of reference strains of new genomovars 11 to 18 to <i>Pseudomonas balearica</i> DSM 6083 ^T (former gv. 6)	31	22.5	60	10

genomic species, as indicated here by DNA–DNA hybridization for the eight strains, should not be classified as novel species unless differentiating phenotypes are found (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002; Ursing *et al.*, 1995), which is not the case here. Thus, we propose eight new genomovars, 11 to 18, of *P. stutzeri*, with strains 28a50, 28a39, 28a22, 28a3, 4C29, 24a13, 24a75 and strain MT-1, respectively, as the reference strains for each genomovar. The strains have been deposited as CCUG 50538–50545 (= DSM 17082–17089).

It is remarkable that strain 28a18 (Sikorski *et al.*, 2002a) was found to be highly similar to the genomovar 14 representative 28a3, with a DNA–DNA relatedness value of 91 % (Supplementary Table S1 in IJSEM Online), yet this strain had a position in the 16S rRNA tree different from that of 28a3 (Supplementary Fig. S1 available in IJSEM Online). The two strains were isolated from the same soil sample and are nearly identical in their ITS1 sequences (Supplementary Fig. S2 available in IJSEM Online), their RAPD patterns (Sikorski *et al.*, 2002a) and their partial *rpoB* sequences [as determined by restriction enzyme profiling of 1.5 kb PCR products (nucleotides 532 to 2034 of the *Escherichia coli* *rpoB* sequence); J. Sikorski and W. Wackernagel, unpublished]. The divergence of their 16S rRNA gene sequences results from five nucleotide changes within a stretch of 15 nucleotides (*E. coli* positions 74 to 92; *E. coli* has an insert of four nucleotides compared with all *P. stutzeri* strains), which makes the 16S rRNA gene sequence of strain 28a3 in this region identical to that of genomovars 2 and 3 strains. This rare specific local sequence identity between a single member of one genomovar and the members of two far-distant genomovars may be explained by a horizontal gene transfer event involving part of the 16S rRNA gene. Strain 28a3, as the putative recipient, was shown to be highly transformable, which is generally not the case for strains of the RAPD group of which 28a3 is the representative (Sikorski *et al.*, 2002b).

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References

- Bennasar, A., Rosselló-Mora, R., Lalucat, J. & Moore, E. R. (1996). 16S rRNA gene sequence analysis relative to genomovars of *Pseudomonas stutzeri* and proposal of *Pseudomonas balearica* sp. nov. *Int J Syst Bacteriol* **46**, 200–205.
- Berndt, C., Meier, P. & Wackernagel, W. (2003). DNA restriction is a barrier to natural transformation in *Pseudomonas stutzeri* JM300. *Microbiology* **149**, 895–901.
- Cladera, A., Bennasar, A., Barceló, M., Lalucat, J. & García-Valdés, E. (2004). Comparative genetic diversity of *Pseudomonas stutzeri* genomovars, clonal structure, and phylogeny of the species. *J Bacteriol* **186**, 5239–5248.
- García-Valdés, E., Castillo, M. M., Bennasar, A., Guasp, C., Cladera, A. M., Bosch, R., Engesser, K. H. & Lalucat, J. (2003). Polyphasic characterization of *Pseudomonas stutzeri* CLN100 which simultaneously degrades chloro- and methylaromatics: a new genomovar within the species. *Syst Appl Microbiol* **26**, 390–403.
- Guasp, C., Moore, E. R., Lalucat, J. & Bennasar, A. (2000). Utility of internally transcribed 16S–23S rDNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. *Int J Syst Evol Microbiol* **50**, 1629–1639.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Ludwig, W., Strunk, O., Westram, R. & 29 other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Meier, P. & Wackernagel, W. (2003). Mechanisms of homology-facilitated illegitimate recombination for foreign DNA acquisition in transformable *Pseudomonas stutzeri*. *Mol Microbiol* **48**, 1107–1118.
- Musarrat, J. & Hashsham, S. A. (2003). Customized cDNA microarray for expression profiling of environmentally important genes of *Pseudomonas stutzeri* strain KC. *Teratog Carcinog Mutagen* (Suppl. 1), 283–294.
- Obradors, N. & Aguilar, J. (1991). Efficient biodegradation of high-molecular-weight polyethylene glycols by pure cultures of *Pseudomonas stutzeri*. *Appl Environ Microbiol* **57**, 2383–2388.
- Rius, N., Fusté, M. C., Guasp, C., Lalucat, J. & Loren, J. G. (2001). Clonal population structure of *Pseudomonas stutzeri*, a species with exceptional genetic diversity. *J Bacteriol* **183**, 736–744.

- Rosselló, R. A., García-Valdés, E., Lalucat, J. & Ursing, J. (1991).** Genotypic and phenotypic diversity of *Pseudomonas stutzeri*. *Syst Appl Microbiol* **14**, 150–157.
- Rosselló-Mora, R. & Amann, R. (2001).** The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.
- Rosselló-Mora, R. A., Lalucat, J. & García-Valdés, E. (1994).** Comparative biochemical and genetic analysis of naphthalene degradation among *Pseudomonas stutzeri* strains. *Appl Environ Microbiol* **60**, 966–972.
- Rosselló-Mora, R. A., Lalucat, J. & Moore, E. R. B. (1996).** Strain JM300 represents a new genomovar within *Pseudomonas stutzeri*. *Syst Appl Microbiol* **19**, 596–599.
- Sepúlveda-Torres, L. C., Zhou, J., Guasp, C., Lalucat, J., Knaebel, D., Plank, J. L. & Criddle, C. S. (2001).** *Pseudomonas* sp. strain KC represents a new genomovar within *Pseudomonas stutzeri*. *Int J Syst Evol Microbiol* **51**, 2013–2019.
- Sikorski, J., Graupner, S., Lorenz, M. G. & Wackernagel, W. (1998).** Natural genetic transformation of *Pseudomonas stutzeri* in a non-sterile soil. *Microbiology* **144**, 569–576.
- Sikorski, J., Moehle, M. & Wackernagel, W. (2002a).** Identification of complex composition, strong strain diversity and directional selection in local *Pseudomonas stutzeri* populations from marine sediment and soils. *Environ Microbiol* **4**, 465–476.
- Sikorski, J., Teschner, N. & Wackernagel, W. (2002b).** Highly different levels of natural transformation are associated with genomic subgroups within a local population of *Pseudomonas stutzeri* from soil. *Appl Environ Microbiol* **68**, 865–873.
- Smibert, R. & Krieg, N. R. (1994).** Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002).** Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.
- Tamegai, H., Li, L., Masui, N. & Kato, C. (1997).** A denitrifying bacterium from the deep sea at 11,000-m depth. *Extremophiles* **1**, 207–211.
- Ursing, J. B., Rosselló-Mora, R. A., García-Valdés, E. & Lalucat, J. (1995).** Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups. *Int J Syst Bacteriol* **45**, 604.
- Ziemke, F., Hofle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.
- Zumft, W. G. (1997).** Cell biology and molecular basis of denitrification. *Microbiol Rev* **61**, 533–616.