

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).

Acknowledgements

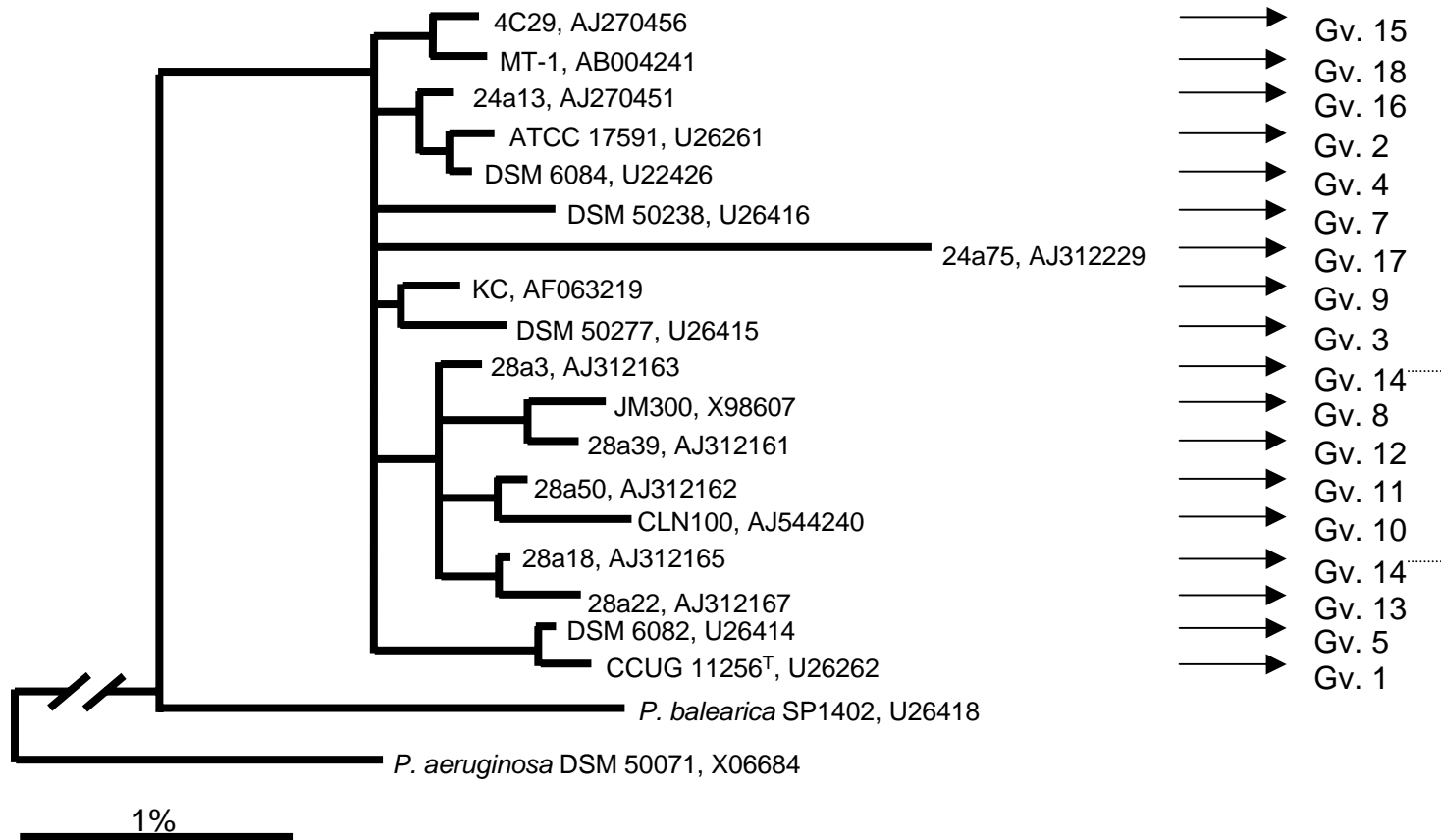
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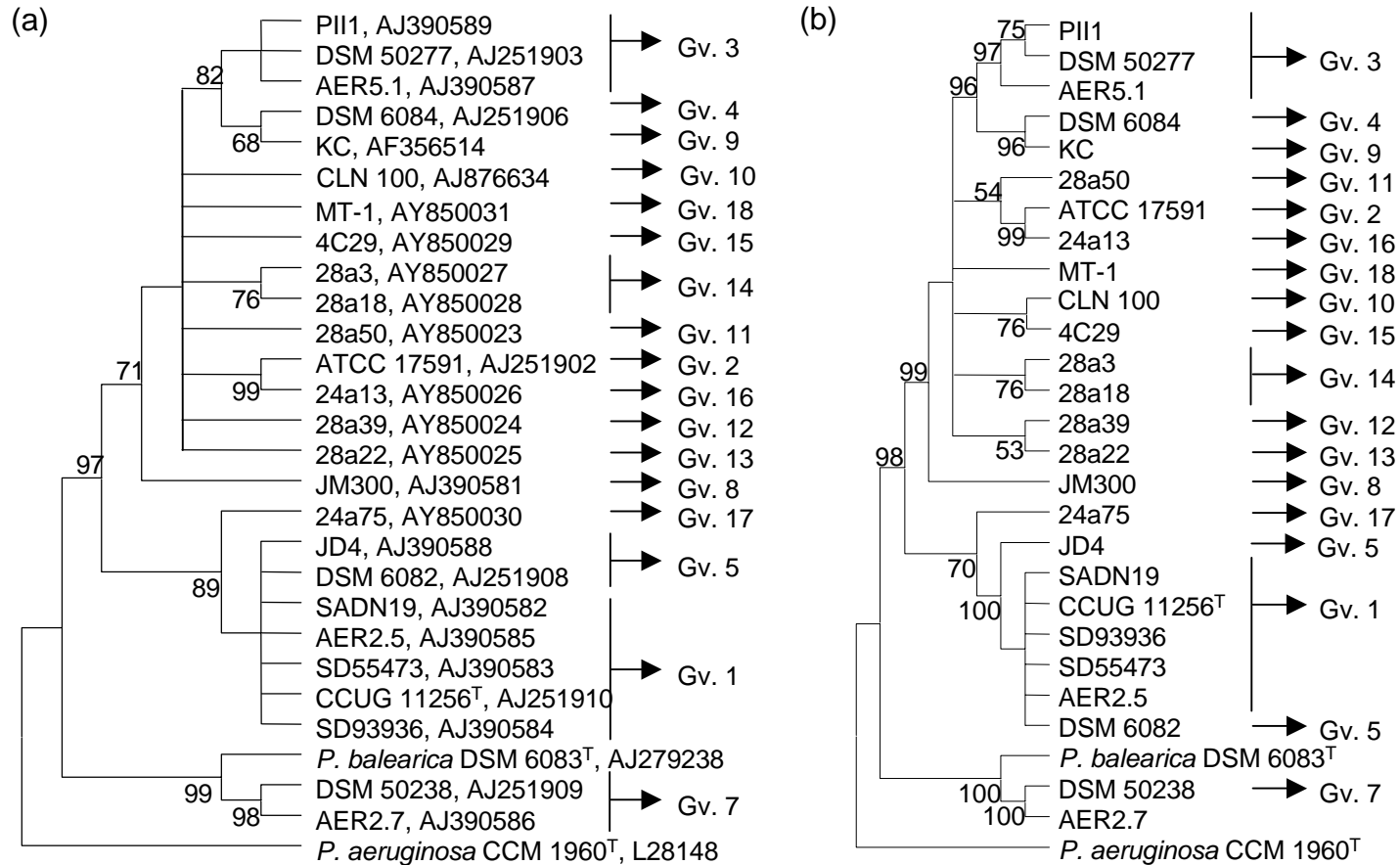
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Supplementary Fig. S1. Phylogenetic 16S rRNA gene sequence-based tree reflecting the relationships of strains belonging to *P. stutzeri* genovars and reference strains of *P. stutzeri*, as well as strains of *Pseudomonas balearica* (former genovovar 6) and *Pseudomonas aeruginosa*. The tree is based on maximum-parsimony analysis using complete or almost complete 16S rRNA gene sequences from 94 *P. stutzeri* strains and from representative bacteria of other phylogenetic branches. The topology of the tree was evaluated and corrected according to the results of distance matrix, maximum-parsimony and maximum-likelihood analyses of various datasets by the ARB package. Multifurcations indicate topologies that could not be resolved unambiguously. The dotted bracket indicates strains belonging to the same genovovar. The sequence of strain 28a18 is identical to that of strain 28a42, for which the accession number is given (Sikorski *et al.*, 2002a). Bar, 1 % estimated sequence divergence.



Supplementary Fig. S2. Phylogenetic reconstruction based on ITS1 sequences of strains of *P. stutzeri*, *P. balearica* and *P. aeruginosa* based on (a) maximum-parsimony and (b) neighbour-joining calculations with 1000 bootstrap replications each (percentages are shown at the nodes). The trees are condensed to multifurcations at nodes with less than 50 % bootstrap support. Sequences of strains of genomovars 11 to 18 were added to the alignment of strains presented by Guasp *et al.* (2000). Accession numbers of ITS sequences are given in (a)

Supplementary Table S1. DNA–DNA relatedness values

DNA–DNA relatedness values (%). Strains: 1, Gv. 11 strain 28a50; 2, Gv. 12 strain 28a39; 3, Gv. 13 strain 28a22; 4, Gv. 14 strain 28a3; 5, Gv. 15 strain 4C29; 6, Gv. 16 strain 24a13; 7, Gv. 17 strain 24a75; 8, Gv. 18 strain MT-1. ND , Not determined.

Strain	Genomovar	1	2	3	4	5	6	7	8
<i>P. stutzeri</i> 28a50	11	100	67	56	50	33	54	30	62
<i>P. stutzeri</i> 28a39	12	60	100	57	47	36	68	33	58
<i>P. stutzeri</i> 28a22/28a69	13	56	70	100/93	43	31	46	33	68
<i>P. stutzeri</i> 28a3/28a18	14	66	69	57	100/91	38	82	39	57
<i>P. stutzeri</i> 4C29	15	60	65	54	45	100	65	31	68
<i>P. stutzeri</i> 24a13	16	59	63	54	40	30	100	36	60
<i>P. stutzeri</i> 24a75	17	57	67	67	46	29	63	100	34
<i>P. stutzeri</i> MT-1	18	62	68	77	41	53	68	28	100
<i>P. stutzeri</i> CCUG 11256 ^T	1	59	62	15	9	10	59	22	29
<i>P. stutzeri</i> ATCC 17591	2	23	62	46	15	12	68	24	49
<i>P. stutzeri</i> DSM 50227	3	63	67	18	11	10	63	14	45
<i>P. stutzeri</i> DSM 6084	4	58	67	16	15	12	65	16	53
<i>P. stutzeri</i> DSM 6082	5	62	63	58	15	9	67	18	45
<i>P. stutzeri</i> DSM 50238	7	59	65	54	9	11	39	16	43
<i>P. stutzeri</i> JM300	8	58	63	52	9	10	60	14	65
<i>P. stutzeri</i> KC	9	57	61	20	10	10	63	20	50
<i>P. stutzeri</i> CLN 100	10	59	60	58	10	13	63	12	64
<i>P. balearica</i> DSM 6083 (former Gv. 6)		57	60	19	10	12	26	14	49
<i>P. mendocina</i> ATCC 45411		ND	ND	ND	8	10	ND	10	ND
<i>P. aeruginosa</i> CCM 1960 ^T		ND	ND	ND	7	8	ND	15	ND

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Supplementary Table S2. Physiological properties of strains of genomovars 11 to 18

Strains: 1, Gv. 11 strain 28a50; 2, Gv. 12 strain 28a39; 3, Gv. 13 strain 28a22; 4, Gv. 14 strain 28a3; 5, Gv. 15 strain 4C29; 6, Gv. 16 strain 24a13; 7, Gv. 17 strain 24a75; 8, Gv. 18 strain MT-1. +, Positive; negative; (+), weakly positive; ND, not determined.

Strain	1	2*	3*	4*	5	6	7	8
Motility	+	+	+	+	+	+	+	-
Amylase (iodine test)	(+)	+	+	+	(+)	+	-	-
Oxidation test with glucose (after 6 days)	+†	+	+†	+	ND	+	+†	+
Fermentation test with glucose (after 6 days)	-	-	-	-	ND	-	-	-
Growth on maltose minimal agar plates (after 2 days at 30 °C)	+	+	+	+	+	+	(+)	+
Growth on maltose minimal agar plates (single colonies after 5 days at 30 °C)	+	+	+	+	+	+	(+)	+
Denitrification (gas production after 2 days at 30 °C)	-	-	-	+	-	-	+	+‡
Oxidase test	+	+	+	+	ND	+	+	+
Catalase test	+	+	+	+	ND	+	+	+

*For motility, amylase and growth on maltose minimal agar, three to four additional strains of the same genomovar (as determined by RAPD-PCR, Sikorski *et al.*, 2002a) were tested and gave identical results.

†Alkalinized medium.

‡Data from Tamegai *et al.*, 1997.

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