Phylogenetic analysis and siderotyping as useful tools in the taxonomy of *Pseudomonas stutzeri*: description of a novel genomovar

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An examination of the results of phylogenetic analyses based on the sequences of fragments of the 16S rRNA, *gyrB* and *rpoD* genes, and the discrimination of genomovars based on siderophore diversity within the genus *Pseudomonas*, has added important taxonomic tools in the characterization of *Pseudomonas stutzeri*. Eighteen reference strains, nine newly identified hydrocarbon-degrading strains and three strains showing relevant physiological characteristics of *P. stutzeri*, together with the type strains of four related species, were included in the study. A novel genomovar within the species is described. A summary of the methodology used in these studies and the results of our attempts to define a solid internal subdivision of this important species within the genus *Pseudomonas* are presented and discussed.

Pseudomonas stutzeri is a species of extremely broad phenotypic and genotypic diversity which has been subjected to intensive studies including substrate assimilation tests, determination of cellular component profiles (whole-cell and outer-membrane proteins, lipopolysaccharides, fatty acids), multilocus enzyme electrophoresis studies as well as nucleic acid sequence similarity studies and multilocus sequence analysis (MLSA). Integration of these studies with the results of DNA–DNA hybridization justifies the recognition of 17 genomic groupings (genomovars) within the species (Lalucat *et al.*, 2006). In recent times, taxonomic studies based on the diversity of siderophores produced by members of the various groups of pseudomonads have resulted in the development of powerful determinative techniques at the species level (Meyer *et al.*, 2002, 2007; Meyer, 2007). Zawadzka *et al.* (2006) studied siderophore production among members of

nine genomovars of *P. stutzeri* and also the presence of ferrioxamine outer-membrane receptor genes in these strains. Consequently, siderophore production has been examined in all 17 reference strains and in nine novel hydrocarbon-degrading isolates included in the present study. The studies reported here are extended to (i) the results of Cladera *et al.* (2004) demonstrating the monophyletic origin of eight genomovars described by Sikorski *et al.* (2005), as well as the agreement of phylogenetic affiliation through DNA–DNA relatedness analysis, and (ii) the determination and validation of siderotyping and DNA relatedness studies as practical approaches for assigning new isolates of *P. stutzeri* to their corresponding genomovars within the species.

The strains of *P. stutzeri* studied are representative of widely separated geographical locations and were isolated by different procedures and from very different habitats (Table 1). Two strains of *Pseudomonas balearica* (former genomovar 6) and the type strain of *Pseudomonas xanthomarina* have been included in the study as representatives of closely related species. The outgroup was represented by the type strains of *Pseudomonas mendocina* and *Pseudomonas aeruginosa*. Alternative designations of strains studied are indicated in Supplementary Table S1 (available in IJSEM Online).

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are AM905851–AM905859 (16S rRNA gene), AM905860–AM905873 and AM939370–AM939377 (*rpoD*) and AM905825–AM905838 and AM939378–AM939385 (*gyrB*).

Details of strains, primers and sequence accession numbers and consensus similarity indices are available as supplementary material with the online version of this paper.

Table 1. Bacterial strains used in this study

Ref, Reference strain of genomovar. Siderophore types are indicated as A (catecholate type) and B (desferri-ferrioxamine E). ND, Not detected; –, not done. Additional strain designations are detailed in Supplementary Table S1.

Strain	Genomovar	Siderophore type	Origin and/or physiological characteristics	Geographical location	Reference
<i>P. stutzeri</i>					
CCUG 11256 ^T	Ref gv. 1	B	Clinical	Copenhagen, Denmark	Lalucat <i>et al.</i> (2006)
ATCC 17591	Ref gv. 2	B	Clinical	Copenhagen, Denmark	Lalucat <i>et al.</i> (2006)
DSM 50227	Ref gv. 3	B	Clinical	Unknown	Lalucat <i>et al.</i> (2006)
19SMN4	Ref gv. 4	B	Marine	Barcelona, Spain	Lalucat <i>et al.</i> (2006)
DNSP21	Ref gv. 5	B	Wastewater	Mallorca, Spain	Lalucat <i>et al.</i> (2006)
DSM 50238	Ref gv. 7	A	Soil	California, USA	Lalucat <i>et al.</i> (2006)
JM300	Ref gv. 8	A	Soil	California, USA	Rosselló-Mora <i>et al.</i> (1996)
KC	Ref gv. 9	B	Aquifer	California, USA	Sepúlveda-Torres <i>et al.</i> (2001)
CLN100	Ref gv. 10	ND	Deposit of chemical production plant	Germany	Lalucat <i>et al.</i> (2006)
28a50	Ref gv. 11	ND	Soil	Israel	Sikorski <i>et al.</i> (2005)
28a39	Ref gv. 12	ND	Soil	Israel	Sikorski <i>et al.</i> (2005)
28a22	Ref gv. 13	ND	Soil	Israel	Sikorski <i>et al.</i> (2005)
28a3	Ref gv. 14	ND	Soil	Israel	Sikorski <i>et al.</i> (2005)
4c29	Ref gv. 15	ND	Marine sediment	Germany	Sikorski <i>et al.</i> (2005)
24a13	Ref gv. 16	ND	Soil contaminated with mineral oil	Germany	Sikorski <i>et al.</i> (2005)
24a75	Ref gv. 17	ND	Soil contaminated with mineral oil	Germany	Sikorski <i>et al.</i> (2005)
MT-1	Ref gv. 18	ND	Mariana Trench	Japan	Sikorski <i>et al.</i> (2005)
CCUG 46542	Ref gv. 19*	ND	<i>Didemnum</i> sp. (marine ascidian)	Maldives	Romanenko <i>et al.</i> (2005)
ATCC 27951	gv. 1	B	Yogurt	Algeria	Lalucat <i>et al.</i> (2006)
st103	gv. 5*	B	<i>Phragmites australis</i> rhizosphere	Unknown	This study
st104	gv. 5*	B	<i>Spartina patens</i> rhizosphere	Unknown	This study
2FA	gv. 3*	B	Culture collection, Dept of Biochemistry and Microbiology, Rutgers University	Unknown	This study
st106A	gv. 3*	B	<i>Spartina alterniflora</i> rhizosphere; growth with naphthalene and hexadecane	USA	This study
st107A	gv. 3*	B	Soil; growth with naphthalene and hexadecane	USA	This study
st108A	gv. 3*	B	<i>S. alterniflora</i> rhizosphere; growth with naphthalene and hexadecane	USA	This study
CCUG 36651	gv. 3*	–	Water, borehole; anaerobic growth with ferric citrate without nitrate	Sweden	This study
WM88	gv. 3*	–	Soil enriched with phosphite; hypophosphite and phosphite oxidizer	Urbana, IL, USA	Metcalf & Wolfe (1998)
2FB7A	gv. 7*	A	River sediment; growth with 2-fluorobenzoate	Kyungan, Korea	Song <i>et al.</i> (2000)
4FB3	gv. 7*	A	Estuarine sediment; growth with 4-fluorobenzoate	Arthur Kill, NJ, USA	Song <i>et al.</i> (2000)
Other species					
<i>P. xanthomarina</i> CCUG 46543 ^T		ND	<i>Halocynthia aurantium</i> (marine ascidian)	Sea of Japan	Romanenko <i>et al.</i> (2005)
<i>P. balearica</i> DSM 6083 ^T		A	Wastewater	Mallorca, Spain	Lalucat <i>et al.</i> (2006)
<i>P. balearica</i> LS401		A	Marine	Barcelona, Spain	Lalucat <i>et al.</i> (2006)
<i>P. balearica</i> st101*		A	<i>S. patens</i> rhizosphere	Unknown	This study
<i>P. mendocina</i> ATCC 25411 ^T		–	Soil	Argentina	Lalucat <i>et al.</i> (2006)
<i>P. aeruginosa</i> CCM 1960 ^T		–	Unknown	Unknown	–

*Assignment from this study.

Culture media (Luria–Bertani medium; Miller, 1972) and growth conditions (30 °C), procedures for DNA extraction and PCR amplification and DNA sequencing conditions have been described previously by Cladera *et al.* (2004). Primers used for 16S rRNA (1080 nt), *gyrB* (B subunit of gyrase, 849 nt) and *rpoD* (D subunit of RNA polymerase, 786 nt) gene amplifications are indicated in Supplementary Table S2. The nucleotide sequences determined in this study have been deposited in the EMBL database. Other sequences used in the analysis have been deposited previously in public databases (strains and accession numbers are indicated in Supplementary Table S3).

Gene sequences were aligned with the closest relatives retrieved from the GenBank nucleotide sequence database. The alignment was done using a hierarchical method for multiple alignments implemented in the CLUSTAL_X computer program (Thompson *et al.*, 1997). Automatically aligned sequences were checked manually. Similarities and evolutionary distances were calculated with programs implemented in PHYLIP (Felsenstein, 1989). Gene distances were calculated from nucleotide sequences by the Jukes–Cantor method (Jukes & Cantor, 1969). Dendrograms were generated by neighbour-joining, maximum-likelihood and bootstrap analyses (1000 replications), algorithms included in programs of the PHYLIP package. Topologies of the trees were visualized with the TreeView program (Page, 1996). In addition to individual trees of the 16S rRNA, *gyrB* and *rpoD* gene partial sequences for each bacterial strain (total length 2445 nt), a concatenated and a consensus analysis of the three genes were performed (Cladera *et al.*, 2004) to represent the combined molecular evolutionary relationships. In brief, for the consensus analysis, the evolutionary analysis was performed by calculating the corresponding distance matrices for each gene, using the algorithm of Jukes & Cantor (1969). Finally, a unique matrix of distances was obtained by averaging the resulting sets and used as an additional measure of divergence between strains. The consensus multilocus tree was calculated from the consensus multilocus matrix by the neighbour-joining method.

Phylogenetic Bayesian analyses were also conducted in a parallel version of MrBayes 3.1.2 available online at <http://cbsuapps.tc.cornell.edu/mrbayes.aspx>. Each Bayesian search performed two independent runs of four chains, where each chain was run in a single node, giving eight in total. We assumed a GTR+I+ Γ model, which was the optimal model to explain our data as estimated with MODELTEST, which compares 56 models of DNA sequence evolution, as described by Posada & Crandall (1998).

Individual trees for the three genes considered in this study, as well as the concatenated and consensus trees, were constructed for the strains studied, together with other 16 strains studied previously (Cladera *et al.*, 2004). Fig. 1 shows the dendrogram corresponding to the consensus analysis. All tree topologies were very similar, maintaining the groupings of strains. Individual trees are not shown. In

the present study, three housekeeping genes have been selected from the seven analysed previously for MLSA studies: the 16S rRNA gene, necessary to affiliate the species phylogenetically, and two other housekeeping genes, *rpoD* and *gyrB*. They yield appropriately sized amplicons and low d_N/d_S ratios (non-synonymous versus synonymous nucleotide substitutions) and have good discrimination power for different genomovars. Very good correlation was found between the similarity indexes determined for six genes and for three genes ($r^2=0.88$), being almost equally discriminative in the consensus analysis (Fig. 2), indicating that analysis of the 16S rRNA, *gyrB* and *rpoD* genes is sufficient for proper phylogenetic discrimination. The consensus tree obtained corroborates the monophyletic origin of the genomovars within the *Pseudomonas* branch. A phylogenetic tree including the nine species of the *P. aeruginosa* complex, as defined by Yamamoto *et al.* (2000), also corroborated these results (not shown).

Reference strains of eight recently described genomovars (gv. 11 to gv. 18) were affiliated in independent sub-branches as shown in Fig. 1. Six of the novel strains included in this study (CCUG 36651, WM88, st106A, st107A, st108A and 2FA) clustered clearly within genomovar 3. Strain st104 clustered with DNSP21 (reference strain of gv. 5) in all phylogenetic trees analysed. The closest genomovar reference strain for 2FB7A and 4FB3 was DSM 50238 (reference of gv. 7), close to AER 2.7, a recognized member of gv. 7. Strain st101 was affiliated with the *P. balearica* phylogenetic branch in all trees with bootstrap values of 1000. Strain CCUG 46542 did not cluster clearly in any described genomovar. *P. xanthomarina* was the only species included within the *P. stutzeri* consensus phylogenetic branch.

Members of the same genomovar clustered in the same phylogenetic sub-branch in all trees with only a few exceptions: in the 16S rRNA gene tree, strains st103 and st108A did not cluster with any known genomovar, but, in the *gyrB*, *rpoD*, consensus and concatenated trees, they were clearly affiliated to gv. 5 and gv. 3, respectively. Two other exceptions were strain JD4 (a recognized member of gv. 5) in the *rpoD* and consensus trees and DSM 50238 (reference of gv. 7) in the *gyrB* tree, confirming a previous report (Cladera *et al.*, 2004).

Supplementary Table S4 indicates the similarity indices obtained in the consensus matrix. It shows that all reference strains from eight recently described genomovars (gv. 11–18) have similarity values to their closest genomovar (88.6–94.9 %) in the same range found between the other ten genomovars (gv. 1–10, 92.1–95.2 %). Similarity values of *P. stutzeri* strains to the closest species are 83.3–87.3 % (*P. balearica*), 78.0–82.6 % (*P. mendocina*), 78.4–83.9 % (*P. aeruginosa*) and 84.3–91.5 % (*P. xanthomarina*). Minimal intragenomovar similarities were calculated for those genomovars in which more than one strain was studied (Supplementary Table S4) giving values of 98.8 % (gv. 1), 99.7 % (gv. 2), 97.7 % (gv. 3), 100 % (gv. 4),

Fig. 1. Consensus dendrogram of *P. stutzeri* strains based on phylogenetic analysis of partial sequences of the 16S rRNA, *gyrB* and *rpoD* genes. Bar, 5% sequence divergence. Sequence accession numbers for some strains are detailed in Supplementary Table S3.

92.5 % (gv. 5) and 91.5 % (gv. 7). The similarity values for the 11 novel strains (not previously assigned to genomovars) to the closest genomovar reference strain were higher

Fig. 2. Correlation between phylogenetic similarities (%) between *P. stutzeri* strains considering six or three genes in the consensus analysis.

than 98.1 %, except strains 2FB7A and CCUG 46542 (95.3 and 92.5 %, respectively). Neither strain could be assigned to any known genomovar and both were suspected to represent novel genomovars.

Strain 2FB7A has a minimal similarity to the closest genomovar of 95.3 %, in the range expected for a novel genomovar, although, in the topology of the 16S rRNA, *rpoD* and *gyrB* gene trees, the closest strains located in the same branch are DSM 50238 (gv. 7), AER 2.7 (gv. 7) and the novel strain 4FB3. For that reason, DNA–DNA hybridization experiments were done with the reference strain of gv. 7 and with the type strain of the species, strain CCUG 11256^T, to demonstrate that strain 2FB7A is a member of gv. 7. DNA–DNA relatedness values were calculated in duplicate using a non-radioactive method as described by Ziemke *et al.* (1998). DNA was isolated by the method of Marmur (1961). Reference DNAs were double-labelled with DIG-11-dUTP and biotin-16dUTP using a nick translation kit (Boehringer Mannheim). Pooled standard deviations of the different experiments were between 0.8 and 1.2 %. Results are indicated in Table 2. The four strains affiliated with the same sub-branch as the

Table 2. DNA–DNA relatedness values (%)

Source of unlabelled DNA	Source of labelled DNA				
	1	2	3	4	5
1. 2FB7A	100	82.8	100	–	–
4FB3	88.5	91.5	99.4	–	–
2. AER2.7	85.7	100	79.8	–	–
3. DSM 50238 (gv. 7)	85.6	82.1	100	33*	–
CCUG 11256 ^T (gv. 1)	63.7	56.6	61.1	52.4	50.4
4. DSM 50227 (gv. 3)	–	–	55.1*	100	61.7
5. CCUG 46542 (gv. 19)	–	–	–	64.2	100

–, Not done.

*Data from Guasp (1999).

reference strain of gv. 7 showed interstrain DNA–DNA relatedness values greater than 79 %, and relatedness below 64 % with the *P. stutzeri* type strain, confirming that they represent a homogeneous genomic group.

Strain CCUG 46542 (=KMM 235) was isolated by Romanenko *et al.* (2005) and was suspected to represent a novel genomovar. It appeared in an isolated branch in all individual analyses of each gene, with distance values similar to those found for strains of different genomovars. Strain CCUG 46542 showed 61.7 % DNA–DNA relatedness with the reference strain of the phylogenetically closest genomovar (gv. 3), strain DSM 50227 (92.5 % similarity in the consensus analysis), and 50.4 % DNA–DNA relatedness with the *P. stutzeri* type strain CCUG 11256^T. These results demonstrated that strain CCUG 46542 represents a novel genomovar within the species, and it is proposed as the reference strain for genomovar 19.

All genomovars described so far have been described on the basis of DNA–DNA hybridization values. In this paper, we show that phylogenetic analysis is a valuable tool to assign novel strains of *P. stutzeri* to already-known genomovars or to define novel ones. Of the 46 strains analysed, only one, JD4 (gv. 5), could not be assigned clearly to a genomovar by analysing only the three genes selected; however, it fits within gv. 5 when four additional housekeeping genes are

included (Cladera *et al.*, 2004). A good correspondence was found between the DNA–DNA relatedness values obtained in previous studies and the consensus phylogenetic distance. Results of 77 pairwise comparisons of 14 strains are shown in Fig. 3. A gap between ΔT_m values of 1 and 5 °C was observed when strains of the same genomovar or from different genomovars were compared, corresponding to phylogenetic similarity of between 94 and 99 %.

Intergenomovar similarity values were 88.6–95.2 %. The closest *Pseudomonas* species are *P. balearica* (83.3–87.3 %), *P. mendocina* (78.0–82.6 %) and *P. aeruginosa* (78.4–83.9 %). *P. xanthomarina* (84.3–91.5 %) has been described recently and is affiliated within the *P. stutzeri* phylogenetic branch. It shares with *P. stutzeri* the basic phenotypic traits of the species, and could be considered a genomovar of *P. stutzeri*, but additional biochemical tests (inability to hydrolyse starch) and physiological (ability to grow at 4 °C and in the presence of 8 % NaCl) and chemotaxonomic (fatty acid composition) properties justified the proposal of a novel species (Romanenko *et al.*, 2005).

The inclusion of novel strains reinforces and clarifies the genomovar structure of *P. stutzeri*. For instance, strains st103 and st104 clustered together with strain DNSP21, reference strain of gv. 5, in the phylogenetic trees analysed. Similarly, strains 2FB7A and 4FB3 clustered together with AER 2.7, a recognized member of gv. 7. Adding novel strains gives robustness to our knowledge of the phylogenetic relationships between genomovars.

To identify a strain as *P. stutzeri*, it has to have the basic phenotypic traits of the species and it has to show similarity values to a recognized strain of the species of more than 88 % in concatenated or consensus analysis of the 16S rRNA, *gyrB* and *rpoD* genes. This value shall be higher than 96 % for assignment to a given genomovar. Intermediate values have to be studied carefully, including other essential genes or the ITS1 region, or performing DNA–DNA hybridizations to the phylogenetically closest genomovar reference strain. Even though DNA–DNA hybridization cannot be excluded in studies on the taxonomy of *P. stutzeri*, there is currently a tendency in bacterial taxonomy to replace it by multigenic sequencing

Fig. 3. Relationships between phylogenetic similarities calculated for three genes and DNA–DNA relatedness expressed as ΔT_m (°C). Values for ΔT_m were taken from Rosselló *et al.* (1991).

analysis, due to the latter's simplicity, portability and accuracy (Stackebrandt *et al.*, 2002). In the present study, a good correlation has been found between phylogenetic distances and DNA–DNA relatedness values (as shown in Fig. 3) that can help in establishing objective phylogenetic values for species and genomovar discrimination in the *P. stutzeri* complex, as was shown previously in the *Pseudomonas corrugata* group (Cladera *et al.*, 2006).

In a recent study presented by Goris *et al.* (2007), DNA–DNA hybridization values have been compared with the average nucleotide identity (ANI) of common genes in the analysis of complete genomes. The recommended cut-off point of 70 % DNA–DNA hybridization for bacterial species delineation corresponded to an ANI of 95 ± 0.5 %. In our study, the cut-off point established for genomovar discrimination is also 70 % (or 5 °C difference in thermal denaturation temperature, ΔT_m) (Ursing *et al.*, 1995) and it corresponded to a similarity value in the consensus matrix of 95.2 %. The analysis of only three housekeeping genes seems to be sufficiently discriminative for the delineation of genomovars in *P. stutzeri*, and also to differentiate *P. stutzeri* from the closest-related species.

The phylogenetic, phenotypic and biochemical diversity detected in *P. stutzeri* was also reflected when studying siderophore production. For that purpose, 17 *P. stutzeri* genomovar reference strains and nine novel strains were grown in the iron-poor Casamino acid medium (CAA) which is routinely used for the study of siderophore production by *Pseudomonas* strains. Medium composition and culture conditions were as described previously (Meyer *et al.*, 1997). Detection of siderophores in culture supernatants was done by the chrome azurol S assay (CAS assay; Schwyn & Neilands, 1987). Rapid siderophore characterization by pH_i value was obtained through isoelectrophoresis of 1 µl aliquots of 20-fold-concentrated CAA culture supernatants, with the siderophore spots revealed by a CAS-overlay method, as described previously (Meyer *et al.*, 2002). Cross-incorporation experiments of ^{59}Fe chelated by a siderophore produced by another strain and compared to the homologous system were performed as described previously (Meyer *et al.*, 2002). Following growth on CAA medium, two types of siderophores were produced depending on the strains, allowing the delineation of two siderovars in *P. stutzeri*, as confirmed by cross-incorporation studies. Strains producing a siderophore with a spot at acidic pH ($pH_i=4.6$) were grouped in type A (a catechol type): strains DSM 50238 (gv. 7), 4FB3 (gv. 7), 2FB7A (gv. 7), JM300 (gv. 8), LS401 and st101 (gv. 6, *P. balearica*). Strains of type B siderovar produced a siderophore with a pH_i value (6.6) identical to that of pure desferri-ferrioxamine E (dffE): strains CCUG 11256 (gv. 1), ATCC 27951 (gv. 1), ATCC 17591 (gv. 2), DSM 50227 (gv. 3), 2FA (gv. 3), st106A (gv. 3), st107A (gv. 3), st108A (gv. 3), 19SMN4 (gv. 4), st104 (gv. 5), DNSP21 (gv. 5), st103 (gv. 5) and KC (gv. 9). The other strains studied either did not produce siderophores or they did not grow,

or growth was too weak, which rendered the CAS assay and isoelectrofocusing problematic: the gv. 10 and gv. 13 reference strains did not grow in CAA medium, CCUG 46542 and the gv. 11, 14, 15, 16, 17 and 18 reference strains were CAS negative, and the reference strain of gv. 12 gave an unusual reaction, with an apparent lack of iron uptake specificity due very probably to the production of the two types of siderophore and/or siderophore receptors. *P. xanthomarina* CCUG 46543^T was CAS negative.

Siderophores detected in this study are in accordance with the results obtained by Zawadzka *et al.* (2006), in which two types of siderophores were detected among the nine *P. stutzeri* genomovars studied: a catechol type (amonabactins), which could correspond to our type A siderophore, and a proferrioxamine type, corresponding to our type B siderophore. Although it appears that siderotyping is much less discriminative within the *P. stutzeri* complex compared with the fluorescent *Pseudomonas* group, it is still possible to conclude that *P. stutzeri* strains belonging to the same genomovar by DNA–DNA hybridization also belong to the same siderophore group, and also that members of different genomovars can share the same siderophore. Thus, siderotyping is a good tool to differentiate species within the genus *Pseudomonas* and is also an argument in favour of not splitting the species *P. stutzeri* into several species, one for each genomovar.

Acknowledgements

We thank E. Falsen and W. Metcalf for their generous exchange of strains and J. Pons for advice on phylogenetic analysis. Special thanks are due to J. Arlinger and others, who isolated strain CCUG 36651, and L. A. Romanenko and others, isolators of strain CCUG 46542. A. Cladera collaborated in the first steps of this investigation. This work was supported by grants CGL2004-00838/BOS and VEM-2003-20565 from the CICYT (Spain). M.M. was the recipient of a predoctoral fellowship from the Pla Balear de Recerca i Desenvolupament Tecnològic de les Illes Balears (PRIB).

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