Utility of internally transcribed 16S–23S rDNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species

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Bacteria identified and classified as *Pseudomonas stutzeri*, on the basis of traditional criteria, are recognized to be markedly heterogeneous, such that a systematic phenotypic characterization has not been correlated with genotypic groupings (i.e. genomovars) based upon DNA–DNA similarities. The internally transcribed 16S–23S rDNA spacer (ITS1) regions of *P. stutzeri* were analysed with respect to the ability of these nucleic acid regions to differentiate and identify the genomic groups (i.e. genomovars) of *P. stutzeri*. The ITS1s of 34 strains of *P. stutzeri* were amplified by PCR and the PCR product was subjected to RFLP analysis, which allowed the differentiation and identification of the strains to their respective genomovars. Sequence determination and analysis of ITS1s supported further the results obtained by RFLP, i.e. nucleotide signatures were identified in strains belonging to different genomovars. The ITS1s of all strains of *P. stutzeri* contained the tandem tRNA^{Ile}/tRNA^{Ala} genes and did not exhibit distinct sequence heterogeneity between different operons of a strain. Phylogenetically informative variable sites were located, exclusively, in non-coding regions. The results of the RFLP and sequence analysis of ITS1s supported and correlated with the phylogenetic relationships estimated from 16S rRNA gene sequence comparisons and DNA–DNA hybridizations, offering an alternative tool for genomovar and species differentiation.

Keywords: 16S–23S internally transcribed spacer region, genomovar, DNA–DNA hybridization, 16S rRNA gene sequencing, RFLP fingerprinting

INTRODUCTION

Pseudomonas stutzeri is a motile, non-fluorescent, denitrifying, Gram-negative, rod-shaped bacterium. Characteristically, *P. stutzeri* is able to use maltose and starch as sole carbon and energy sources. *P. stutzeri* is distributed widely in the environment and has been isolated as an opportunistic pathogen from humans (Holmes, 1986). Some strains of the species have received particular attention because of specific metabolic properties, e.g. denitrification under anaerobic growth conditions (Zumft, 1992) and the degradation of aromatic compounds (Rosselló-Mora *et al.*, 1994)

and high-molecular-mass polyethylene glycols (Obradors & Aguilar, 1991). Furthermore, some strains of *P. stutzeri* have been shown to be naturally transformable and have been studied extensively for their capacities for transformation (Carlson *et al.*, 1983; Stewart & Sinigalliano, 1991; Lorenz & Wackernagel, 1994; Sikorski *et al.*, 1998).

Studies have shown that *P. stutzeri*, as a species, possesses a high degree of phenotypic and genotypic heterogeneity (Stanier *et al.*, 1966; Palleroni *et al.*, 1970; Rosselló *et al.*, 1991). Eight genomic groups, based upon DNA–DNA hybridization similarities, termed 'genomovars', have been proposed (Rosselló *et al.*, 1991; Rosselló-Mora *et al.*, 1996). Phenotypic characteristics have not been determined to allow a

The EMBL accession numbers are given in Table 1.

systematic discrimination of the genomic groups for differentiating and proposing new species. Only one of the described *P. stutzeri* genomovars has been reclassified and validly published, as the new species *Pseudomonas balearica* (Bennasar *et al.*, 1996). Thus because of the phenotypic heterogeneity existing among strains of *P. stutzeri* species, DNA similarity, as measured by DNA–DNA reassociation experiments (Johnson, 1973; Johnson & Palleroni, 1989; Wayne *et al.*, 1987), has been the standard genotypic method for assigning a strain to a given genomovar (Rosselló *et al.*, 1991). Although 16S rRNA gene sequence comparisons have provided insight into the natural relationships among the genomovars and have further supported the genomovar concept (the sequence similarities of 16S rRNA genes between members of the same genomovar are 99.9–100%) (Bennasar *et al.*, 1996; Rosselló-Mora *et al.*, 1996), the 16S rRNA gene sequences of *P. stutzeri*, alone, do not provide sufficient resolution of all genomovars (the similarities of 16S rRNA gene sequences between strains of different genomovars range from 98.0 to 99.7%) (Bennasar *et al.*, 1996).

In spite of the high degree of conservation of rRNA (and rRNA genes), the degree of nucleotide variance among rRNA sequences is, generally, large enough to be used for estimating relationships across the phylogenetic spectrum of bacteria (Gutell *et al.*, 1994). However, in some cases, the sequences of 16S rRNA genes have been shown to be very similar between different species within a genus, making it desirable to find alternative specific sequences for closely related genomic groups, such as the case of genomovars of *P. stutzeri* and others (Manceau & Horvais, 1997; Mendoza *et al.*, 1998; Pérez-Luz *et al.*, 1998; Vaneechoutte *et al.*, 1998; Yoon *et al.*, 1998). The intergenic 16S–23S internally transcribed spacer (ITS1) regions are assumed to be less susceptible to selection pressure, because of their non-coding function, and should have accumulated a higher percentage of mutations than the rRNA genes (Tyrrell *et al.*, 1997). The analysis and comparison of sequences of ITS1s from different bacteria indicate that considerable variation in length and primary sequence occurs (Gürtler & Stanisich, 1996) and that they may be good candidates to be used as markers for discriminating between closely related taxa, e.g. genomovars (Barry *et al.*, 1991; Jensen *et al.*, 1993; Gürtler & Stanisich, 1996; Leblond-Bourget *et al.*, 1996; Pérez-Luz *et al.*, 1998; Riffard *et al.*, 1998).

The aim of the present work was to examine the ITS1 regions and their value for differentiating and identifying *P. stutzeri* genomovars, specifically, and, in general, their potential for defining bacterial species.

METHODS

Bacterial strains. The strains used in this study and their sources of isolation are presented in Table 1. Environmental isolates from samples obtained at different times and places

were used. The environmental strains were identified previously, using conventional phenotypic characterization (Palleroni, 1984) and specific PCR amplification (Bennasar *et al.*, 1998a). A total of seven *P. stutzeri* clinical strains, isolated from patients at different times from geographically diverse sources, were examined. The clinical isolates had been classified previously as presumptive *P. stutzeri* by routine testing using API 20NE (bioMérieux), and specific PCR. All strains were grown on LB agar medium at 30 °C.

Approximately one-third (i.e. the 5'-end) of the PCR-amplified 16S rRNA genes from the different strains were sequenced, using the primer 16R518 (5'-CGTATTACCG-CGGCTGCTGG-3') and the protocols described by Abraham *et al.* (1999), in order to confirm the preliminary identifications (Table 2).

DNA–DNA hybridizations. Genomic DNA from bacterial strains was isolated following the method of Marmur (1961). DNA–DNA hybridizations were performed using a modification of the hydroxyapatite method described previously (Ziemke *et al.*, 1998). Reference DNAs were double-labelled with DIG-11-dUTP and Biotin-16-dUTP, using the Nick-Translation kit (Boehringer Mannheim) and instructions of the manufacturer.

PCR amplification of ITS1. A single bacterial colony from a fresh culture was resuspended in 100 µl sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The cell suspension was boiled for 10 min, centrifuged for 5 min at 14000 *g* and 1 µl of the resulting supernatant was used for PCR (Moore *et al.*, 1999). The ITS1 was amplified by PCR with oligonucleotide primers 16F945 and 23R458 (Lane *et al.*, 1985), designed to anneal to conserved positions in the 3' and 5' regions of the bacterial 16S rRNA and 23S rRNA genes, respectively. Primers 16F945 (5'-GGG CCC GCA CAA GCG GTG G-3') and 23R458 (5'-CTT TCC CTC ACG GTA C-3') targeted positions 927–945 of the *Escherichia coli* 16S rRNA gene (Brosius *et al.*, 1978) and positions 458–473 of the *E. coli* 23S rRNA gene (Brosius *et al.*, 1980), respectively.

PCRs were carried out in 100 µl reaction volumes containing 10 mM Tris/HCl pH 9.0, 1.5 mM MgCl₂, 50 mM KCl, 200 µM of each of the four deoxyribonucleoside triphosphates (Pharmacia Biotech), 0.5 µM forward and reverse primers, 1 µl template DNA and 2.5 U Amplitaq DNA polymerase (Perkin-Elmer Cetus). The reactions were performed in a PE9600 (Perkin-Elmer Cetus GeneAmp) thermocycler, with an initial denaturation of 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. Following amplifications, samples were incubated at 72 °C for 10 min and then cooled to 4 °C. PCR products were analysed by electrophoresis in 1.5% agarose and stained with ethidium bromide.

RFLP of ITS1. The ITS1 amplicons were treated with 1 U *TaqI*, *AluI*, *HaeIII*, *DraI* or *BclI* restriction endonucleases (Boehringer Mannheim), following the instructions of the manufacturer, to determine the characteristic RFLP for each strain. After incubation, the resulting fragments were separated by electrophoresis in a 3% agarose (NuSieve 3:1 agarose; FMC BioProducts) gel in TBE (89 µM Tris, 88 µM boric acid, 2 µM EDTA) buffer and stained with ethidium bromide.

RFLP cluster analysis of ITS1. An image analysis system (Whole Band Analyser; Bioimage) was used for calculating similarities between all possible RFLP restriction fragment pair comparisons and for cluster analysis from the generated

Table 1. Strains used in this study

Species or isolate*	Strain designation†	ITS1 EMBL accession no.	Source
<i>Pseudomonas stutzeri</i> gv. 1‡	CCUG 11256 ^T	AJ251910	Clinical isolate (Copenhagen, Denmark, before 1966)
<i>Pseudomonas stutzeri</i> gv. 1	ATCC 17594	AJ251900	Clinical isolate
<i>Pseudomonas stutzeri</i> gv. 2‡	ATCC 17591	AJ251901	Clinical isolate (Copenhagen, Denmark, 1956)
<i>Pseudomonas stutzeri</i> gv. 2	ATCC 17587	AJ251902	Clinical isolate (Copenhagen, Denmark, 1956)
<i>Pseudomonas stutzeri</i> gv. 3‡	DSM 50227	AJ251903	Clinical isolate
<i>Pseudomonas stutzeri</i> gv. 3	AN 10	AJ251904	Marine isolate (Barcelona, Spain, 1982)
<i>Pseudomonas stutzeri</i> gv. 3	AN 11	AJ251905	Marine isolate (Barcelona, Spain, 1982)
<i>Pseudomonas stutzeri</i> gv. 4‡	19SMN4	AJ251906	Marine isolate (Barcelona, Spain, 1988)
<i>Pseudomonas stutzeri</i> gv. 4	ST27MN3	AJ251907	Marine isolate (Barcelona, Spain, 1988)
<i>Pseudomonas stutzeri</i> gv. 5‡	DNSP21	AJ251908	Wastewater isolate (Mallorca, Spain, 1988)
<i>Pseudomonas balearica</i> gv. 6‡§	DSM 6083 ^T	AJ279238	Wastewater isolate (Mallorca, Spain, 1988)
<i>Pseudomonas balearica</i> gv. 6§	LS401	AJ279239	Marine isolate (Barcelona, Spain, 1988)
<i>Pseudomonas stutzeri</i> gv. 7‡	DSM 50238	AJ251909	Soil isolate (Berkeley, California, before 1966)
<i>Pseudomonas stutzeri</i> gv. 8‡	JM 300	AJ390581	Soil isolate (California, before 1982)
<i>Pseudomonas aeruginosa</i>	CCM 1960 ^T	L28148	
<i>Pseudomonas putida</i>	DSM 50202 ^T		Lactate enrichment
<i>Pseudomonas fragi</i>	CECT 446 ^T	AJ279241	
<i>Pseudomonas chlororaphis</i>	DSM 50083 ^T	AJ279240	Plate contaminant
<i>Pseudomonas cichorii</i>	DSM 50259 ^T	AJ279242	
<i>Pseudomonas syringae</i>	LMG 1247 ^T	D86356	
<i>Pseudomonas agarici</i>	LMG 2112 ^T	AJ279243	Cultivated mushroom
<i>Pseudomonas tolaasii</i>	LMG 2342 ^T	AJ279244	Cultivated mushroom
<i>Pseudomonas asplenii</i>	LMG 2137 ^T		
<i>Pseudomonas mendocina</i>	ATCC 25411 ^T	L28159	Soil
<i>Pseudomonas pseudoalcaligenes</i>	ATCC 17440 ^T	AJ279245	Sinus drainage
<i>Pseudomonas alcaligenes</i>	LMG 1224 ^T		
<i>Pseudomonas stutzeri</i>	SADN 19	AJ390582	Brackish water (Mallorca, Spain, 1993)
<i>Pseudomonas stutzeri</i>	SD55473	AJ390583	Clinical isolate
<i>Pseudomonas stutzeri</i>	SD93936	AJ390584	Clinical isolate
<i>Pseudomonas stutzeri</i>	AER 2.5	AJ390585	Aircraft-oil-contaminated soil (Mallorca, Spain, 1995)
<i>Pseudomonas stutzeri</i>	AER 2.7	AJ390586	Aircraft-oil-contaminated soil (Mallorca, Spain, 1995)
<i>Pseudomonas stutzeri</i>	AER 5.1	AJ390587	Aircraft-oil-contaminated soil (Mallorca, Spain, 1995)
<i>Pseudomonas stutzeri</i>	JD4	AJ390588	Garden soil (Mallorca, Spain, 1995)
<i>Pseudomonas stutzeri</i>	PII1	AJ390589	Wastewater (Mallorca, Spain, 1996)
<i>Pseudomonas stutzeri</i> gv. 2	ATCC 14405 ZoBell	AJ390590	Marine

* gv., genomovar (Rosselló *et al.*, 1991).

† CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; ATCC, American Type Culture Collection, Manassas, VA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; LMG, Laboratorium Microbiologie Rijksuniversiteit, Gent, Belgium.

‡ Type strain of genomovar.

§ Genomovar reclassified as *P. balearica* (Bennasar *et al.*, 1996).

matrix of similarity values. Dendrograms of relationships were deduced by the unweighted pair group method with averages (UPGMA) cluster algorithm.

Nucleotide sequence determination of ITS1. PCR-amplified ITS1 regions were purified using Microcon-100 concentrators (Amicon) and sequenced directly, using an Applied

Table 2. Nucleotide signatures identified for each genomovar, on the basis that each nucleotide signature position is conserved only for those strains belonging to the same genomovar

Signature positions for the corresponding genomovars are indicated in bold. gv., genomovar; —, gaps in the alignment of ITS1 sequences.

Position*	gv. 1	gv. 2	gv. 3	gv. 4	gv. 5	gv. 7	gv. 8
26	T	T	T	T	T	T	G
31	C	T	C	C	C	C	C
33	T	C	T	T	T	T	T
34	A	A	A	A	A	A	G
38	T	T	T	T	T	T	G
63	T	A	A	A	T	T	G
66	G	G	G	G	G	G	T
314	T	A	T	T	T	T	T
329	T	C	G	G	T	T	T
332	C	G	T	T	C	A	T
334	G	G	C	G	G	G	A
339	A	G	C	C	A	C	—
343	T	—	T	C	T	G	—
351	C	—	G	T	C	—	—
356	T	—	—	—	A	—	—
358	G	G	G	G	G	A	G
380	C	C	C	C	C	—	T
400	A	A	A	A	C	A	A
431	A	G	A	A	A	T	A
432	T	G	G	G	T	T	A
433	G	T	T	T	G	C	T
434	T	A	G	G	T	G	G
437	T	T	C	C	T	C	G
438	G	A	A	A	G	A	T
439	C	C	C	C	C	T	C
448	G	G	G	G	G	A	G
449	C	T	T	T	C	T	G
450	A	A	G	G	A	G	C
456	G	T	T	T	G	A	T
458	C	C	C	C	C	T	C
460	C	A	A	A	C	G	C
481	G	G	G	G	G	A	G
483	T	T	T	A	T	T	T
495	—	C	—	A	—	C	C
498	—	—	—	T	—	G	A

* Numbering with respect to the ribosome-binding site (3'-end of the 16S rRNA gene).

Biosystems 373A DNA sequencer and the protocols of the manufacturer (Perkin-Elmer, Applied Biosystems Division) for 'Taq cycle-sequencing' with fluorescent-dye-labelled dideoxynucleotides. The primers used for sequencing were rrn16S (5'-GAA GTC GTA ACA AGG-3') and rrn23S (5'-CAA GGC ATC CAC C-3') (Jensen *et al.*, 1993), targeting the 3'- and 5'-ends of the 16S and 23S rDNAs, positions 1491–1505 and 21–35, respectively. All oligonucleotides were purchased from Pharmacia Biotech. Nucleotide sequence accession numbers are included in Table 1.

In cases where more than one amplified ITS1 product for a strain was detected, the individual ITS1s were PCR-amplified with rrn16S and rrn23S primers, and separated by electrophoresis in pre-cast non-denaturing polyacrylamide gels (CleanGel DNA Analysis kit; Pharmacia Biotech) on a horizontal Multiphor II electrophoresis unit, with a pre-run at 200 V for 10 min, followed by 45 min at 600 V. After electrophoresis, the gels were stained using the Plus One Silver Staining kit (Pharmacia Biotech) and following the protocols of the manufacturer. Separated bands were excised from the gel and the DNA was eluted from the acrylamide, to be used as template for the regeneration of the ITS1 by PCR, using primers rrn16S and rrn23S. Amplicons from these PCRs were sequenced directly after purification as described above.

Nucleotide sequence analysis of ITS1. ITS1 nucleotide sequences were aligned using the computer program CLUSTAL W (Thompson *et al.*, 1994) with a final manual adjustment (Rambaut, 1996). Percentage similarities between the ITS1 regions of different strains were determined after the corresponding 16S and 23S rRNA and tRNA gene sequence regions were omitted. Evolutionary distances (Jukes & Cantor, 1969) were calculated from pairwise sequence similarities and estimations of relationships were generated using the Fitch program within the Phylogeny Inference Package (PHYLP) (Felsenstein, 1989).

RESULTS AND DISCUSSION

PCR amplification of ITS1

In the present study, the ITS1s of 34 strains, including representative strains of each described genomovar, as well as new isolates of *P. stutzeri* and type strains of other *Pseudomonas* species, were successfully amplified by PCR. Amplicons of nearly 1550 bp in length were calculated to comprise approximately 550 bp of the 3' portion of the 16S rRNA gene, the ITS1 and 455 bp of the 5' portion of the 23S rRNA gene. A single band of the same size, as seen after agarose gel electrophoresis, was obtained from all strains of *P. stutzeri* and from *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes* and *Pseudomonas tolaasii*. The PCR-ITS1 amplification products of *Pseudomonas putida* (3), *Pseudomonas fluorescens* (3), *Pseudomonas agarici* (3), *Pseudomonas fragi* (3), *Pseudomonas mendocina* (3), *Pseudomonas syringae* (3) and *Pseudomonas chlororaphis* (2) comprised 2 or 3 bands (number in parentheses) of lengths of 1500–1600 bp (data not shown). Although differences were observed in the number and size of the PCR-ITS1 products obtained from different species, these characteristics alone could not be used for a general differentiation of all *P. stutzeri* genomovars or *Pseudomonas* species.

Single-strand conformational polymorphism electrophoresis, in comparison with simple agarose gel electrophoretic analysis, has been shown previously to resolve some PCR-ITS1 products of *P. stutzeri* strains on the basis of their sequence heterogeneities (Bennasar *et al.*, 1998b). However, in order to differentiate each genomovar of *P. stutzeri*, higher-resolution methods are needed.

Fig. 1. Agarose gel showing the *TaqI* restriction products of PCR-ITS1. (a) *P. stutzeri* genomovars (including *P. balearica*): 1, molecular mass marker 1 kb (Gibco-BRL); 2, CCUG 11256^T gv. 1; 3, ATCC 17591 gv. 2; 4, DSM 50227 gv. 3; 5, 19SMN4 gv. 4; 6, DNSP21 gv. 5; 7, SP1402^T = DSM 6083^T gv. 6; 8, DSM 50238 gv. 7; 9, JM300 gv. 8; 10, AER2.5; 11, AER2.7; 12, JD4; 13, AER5.1. (b) *Pseudomonas* species: 1, molecular mass marker 1 kb (Gibco-BRL); 2, *P. aeruginosa* CCM 1960^T; 3, *P. alcaligenes* LMG 1224^T; 4, *P. mendocina* ATCC 25411^T; 5, *P. fluorescens* DSM 50090^T; 6, *P. tolaasii* LMG 2342^T; 7, *P. chlororaphis* DSM 50083^T; 8, *Pseudomonas cichorii* DSM 50259^T; 9, *P. putida* DSM 50202^T; 10, *P. agarici* LMG 2112^T; 11, *P. fragi* CECT^T; 12, *Pseudomonas pseudoalcaligenes* ATCC 17440^T; 13, *Pseudomonas asplenii* LMG 2137^T gv., genomovar.

RFLP and cluster analysis of ITS1

Three enzymes recognizing four-nucleotide restriction sites, with expected high restriction frequencies, *TaqI*, *AluI* and *HaeIII*, were selected and applied for RFLP analysis. The enzymes *TaqI* and *AluI* produced as many as six different restriction profiles for the *P. stutzeri* strains tested. However, *HaeIII* was able to distinguish only genomovar 6 (*P. balearica*) from the other genomovars. The *TaqI* restriction profiles of the ITS1 amplicons, using the PCR primers 16F945 and 23R458, comprised four to seven bands of sizes ranging from 75 bp to 650 bp (Fig. 1). In all cases, identical *TaqI* digestion profiles were obtained for strains belonging to a given genomovar (as defined by genomic DNA similarities). All *P. stutzeri* genomovars pre-

Fig. 2. UPGMA clustering of *P. stutzeri* strains and other species of *Pseudomonas* based on *TaqI* RFLP of PCR-ITS1. *P. stutzeri* is indicated by strain numbers.

sented two characteristic bands of 360 and 230 bp in their *TaqI* restriction patterns, while other bands of the restriction patterns allowed differentiation of genomovars (Fig. 1). Strain JD4 of genomovar 5 displayed an identical *TaqI* restriction pattern to that of strains of genomovar 1, as also the strains of genomovars 3 and 4 exhibited an identical RFLP pattern.

The ITS1 restriction patterns of strains of *P. stutzeri* and other species of *Pseudomonas*, generated by *TaqI* digestion, were clustered by UPGMA (Fig. 2). Branching dichotomies, due to ITS1 polymorphisms, resulted in clusters of strains at the species level (Fig. 2). Interestingly, *P. mendocina* was observed to cluster within the spectrum of *P. stutzeri* genomovars. *P. stutzeri* strains belonging to the same genomovar were observed to group within distinct, robust clusters, with the exception of strain JD4, which clustered with the strains of genomovar 1, and strains of genomovars 3 and 4, which were not resolved.

In order to obtain profiles of higher resolving power, due to the discrepancies observed with respect to strain

Fig. 3. Organization of the ITS1 region of *P. stutzeri* genomovars and *P. balearica* DSM 6083^T. tRNA^{Ile} and tRNA^{Ala} genes (black) are arranged in tandem. Regions V1, V2 and V3, between the 16S rRNA-tRNA^{Ile}, tRNA^{Ile}-tRNA^{Ala} and tRNA^{Ala}-23S rRNA genes, respectively, are highly variable (white). RBS, ribosome-binding site (Shine & Dalgarno, 1974).

JD4 and genomovar 1 and genomovars 3 and 4, additional restriction enzymes were tested. The PCR-ITS1 amplicons of genomovars 3 and 4 (which could not be distinguished by *TaqI* restriction patterns) were digested and resolved by *BclI* digestion. A pattern of two bands of approximately 1000 bp and 550 bp was generated for genomovar 4. The PCR-amplified ITS1s of the other genomovars were not cut by *BclI* (data not shown). Of other species of *Pseudomonas* analysed, only the ITS1 of *P. aeruginosa* presented the same *BclI* band pattern as that observed for genomovar 4.

Nucleotide sequence determination and analysis of ITS1

While *TaqI* RFLP analysis of *P. stutzeri* strains provided, in most cases, differentiation at the genomovar level and differentiation of *Pseudomonas* at the species level, the observed cases which were not resolved indicated that an even finer resolution may be possible to attain, e.g. through sequence determinations.

The *P. stutzeri* genome contains four rRNA transcriptional units (*rrnA*, *B*, *C*, *D*) at different chromosomal locations (Ginard *et al.*, 1997). The rRNA operon of *P. stutzeri* strain ZoBell (ATCC 14405) was cloned and characterized by sequence analysis (Kerkhof, 1997) and it was observed to exhibit the typical bacterial organization of the following components in the order: 5'-16S rRNA gene-ITS1-23S rRNA gene-ITS2-5S rRNA gene-3'. The PCR-amplified ITS1s of this study were sequenced directly using the ITS1 flanking primers *rrn16S* and *rrn23S* (Jensen *et al.*, 1993). The lengths of the ITS1 regions of the strains of *P. stutzeri* studied ranged from 495 to 548 bp (Fig. 3), from the 3'-end of the ribosome-binding site (Shine & Dalgarno, 1974) to the identified 5'-end of the 23S rRNA gene (Kerkhof, 1997). Strains of a genomovar were observed to possess nearly identical ITS1 sequences, with two or three observed nucleotide

differences, suggesting that only random micro-heterogeneities exist between strains of a genomovar of *P. stutzeri*, as has been described for other species (Gürtler & Stanisich, 1996). Eight distinct sequence types with respect to length and nucleotide composition were observed, i.e. one for each genomovar of *P. stutzeri*, although genomovars 1 and 5 are very similar, and ITS1 signature positions for each genomovar have been identified (Table 2).

All analysed strains of *Pseudomonas* species shared identical nucleotide sequences immediately adjacent to the 3'- and 5'-ends of the 16S and 23S rRNA genes, respectively. The ITS1 of all strains of *P. stutzeri*, as well as strains of the other species of *Pseudomonas* analysed (except one strain of *P. putida*), contained tRNA (i.e. tRNA^{Ile} and tRNA^{Ala}) genes, as has been reported for most Gram-negative bacteria. The tRNA genes within the ITS1 were observed to be arranged in tandem and located at nucleotide positions (counting from the ribosome-binding site) 76–104 to 187–211, depending on the genomovar (Fig. 3). Comparison of the nucleotide sequences of the tRNA^{Ile}/tRNA^{Ala} genes revealed 100% identity between the respective genes for all *P. stutzeri* strains analysed. Putative secondary structures of the tRNAs, derived from the primary sequences, suggested that the tRNAs formed the characteristic 'cloverleaf' structure (Fuller & Hodgson, 1967; Sprinzl *et al.*, 1998). The trinucleotide 3'-end acceptor sequence CCA, normally present, as well, in mature tRNAs, was observed in both tRNA genes (Berg *et al.*, 1989; Srivastava & Schlessinger, 1990).

Comparisons of aligned ITS1 sequences revealed that rRNA processing motifs are highly conserved within the ITS1 regions of all *P. stutzeri* genomovars, with hypervariable sequence regions located within the non-coding regions. In the sequence region between the tRNA genes, the number of nucleotide positions varied from 7 (in genomovars 1 and 5) to 31 (genomovars 2,

Fig. 4. Dendrogram, derived from calculated evolutionary distances, depicting the estimated evolutionary relationships between the ITS1 sequences of genomovars of *P. stutzeri*. Sequences for tRNA genes were removed from the calculations. The branch scale bar indicates a mean of 10 substitutions at any given nucleotide position per 100 positions.

3, 4 and 8) and, also, downstream from the tRNA^{Ala} gene a high degree of sequence heterogeneity was observed. Within these hypervariable regions, insertions and deletions of blocks of nucleotides were observed, as has been described previously for other species (Gürtler & Stanisich, 1996). Theoretically, if no, or limited, selective pressure is being exerted on these regions, a high degree of variation should be

expected. Presumably, the observed conservation in nucleotide sequence of some smaller segments within the variable regions is due to the conserved secondary structures which the ITS regions must assume for proper processing by RNase III of the primary transcript (Pérez-Luz *et al.*, 1998; García-Martínez *et al.*, 1996). A highly conserved sequence domain of 10 nucleotides, located at nucleotide positions 392–402, within the highly variable region between the tRNA^{Ala} and the 23S gene, containing a putative box-A-like gene sequence (Berg *et al.*, 1989; Srivastava & Schlessinger, 1990), was identified in all the strains analysed. Additionally, this conserved sequence domain was preceded by a region (nucleotides 285–300) which has the potential to form a hairpin loop structure and to act as a box-B-like structure for ribosomal processing genes (Berg *et al.*, 1989; Srivastava & Schlessinger, 1990). This domain was observed to possess a primary sequence unique for each genomovar, although the secondary structure is conserved. The conserved primary sequence regions and secondary structure formed by ITS1 during rRNA processing were considered for generating alignments.

The aligned ITS1 sequences were used to generate a dendrogram of putative relationships (Fig. 4). The inclusion or exclusion of tRNA genes did not affect the topology of the tree. In the ITS1-sequence-based tree, three primary clusters were observed: one comprising genomovars 1 and 5; one comprising genomovars 2, 3, 4 and 8; and the most divergent comprising genomovars 6 (*P. balearica*) and 7. This overall branching order differed from that obtained by 16S rRNA gene sequence comparisons (Bennasar *et al.*, 1996; Rosselló-Mora *et al.*, 1996), and the respective branch lengths derived from the comparison of the ITS1 sequences of different genomovars are generally greater than those generated from the 16S rRNA gene sequences (Table 3). The similarities between ITS1 sequences of strains of a genomovar, in all cases, were

Table 3. Identification of *P. stutzeri* isolates based on DNA–DNA similarity, 16S rRNA gene partial sequence similarity, ITS1 sequence similarity and PCR-ITS RFLP *TaqI* results for *P. stutzeri* isolates

	DNA–DNA similarity (%)*				Partial (first 518 bp) 16S rDNA sequence similarity (%) with:				ITS1	
	gv. I ATCC 17589	gv. III DSM 50227	gv. V DN5P21	gv. VII DSM 50238	CCUG 11256 ^T	DSM 50227	DN5P21	DSM 50238	Sequence similarity with CCUG 11256 ^T	RFLP type
ATCC 17589	100	43	45	40	100-00	95-39	99-78	98-42	100-00	I
DSM 50227	37	100	42	55	95-39	100-00	95-62	96-57	89-20	III
DN5P21	55	46	100	58	99-78	95-62	100-00	98-65	99-23	V
DSM 50238	31	33	35	100	98-42	96-57	98-65	100-00	84-38	VII
SADN19	88	NT	NT	NT	100-00	95-39	99-78	98-42	99-62	I
SD55473	99	42	47	35	100-00	95-39	99-78	98-42	99-42	I
SD93936	73	48	NT	45	100-00	95-39	99-78	98-42	99-81	I
AER2.5	83	NT	NT	NT	100-00	95-38	99-78	98-41	99-62	I
AER2.7	26	21	27	95	99-32	95-37	99-55	98-87	84-91	VII
AER5.1	41	80	NT	NT	95-15	99-55	95-39	96-33	88-75	III
JD4	53	NT	76	NT	99-78	95-62	100-00	98-65	98-84	I
PII1	NT	93	NT	NT	99-78	100-00	95-62	96-57	88-98	III
Pooled standard deviation	0.84	0.32	0.42	1.52						

* NT, Not tested; gv., genomovar.

observed to be greater than 99%, while sequence similarities of strains from different genomovars ranged from 81 to 96%, except for the strains of genomovars 1 and 5, which possessed sequence similarities of 98.8–99.6%. The close taxonomic proximity, expressed by ITS1 sequence similarities, between genomovars 1 and 5 is consistent with the observed DNA–DNA reassociation values (Rosselló *et al.*, 1991) and 16S rRNA gene sequence similarities (Bennasar *et al.*, 1996; Rosselló-Mora *et al.*, 1996). Genomovar 7 was observed to possess an ITS1 sequence with many differences to those of all other genomovars (Table 2). In the ITS1-based tree (Fig. 4), genomovar 7 forms a phylogenetic branch, together with *P. balearica*, clearly separated from the branches comprising other *P. stutzeri* genomovars. In fact, sequence similarities between the ITS1 of genomovar 7 and the ITS1s of all other genomovars of *P. stutzeri* are lower (from 81 to 84%) than the ITS1 similarities between *P. aeruginosa* and other strains of *P. stutzeri* (from 85 to 87%). Genomovar 7 is phenotypically (Rosselló *et al.*, 1991) and genotypically (Bennasar *et al.*, 1996) the most atypical of the currently recognized *P. stutzeri* genomovars and, justifiably, should be renamed as a new species of *Pseudomonas* if systematically differential phenotypic characteristics could be determined.

When the ITS1 sequences of different species of *Pseudomonas* (*sensu stricto*) were analysed by clustering, the branching order was observed to differ from the estimated phylogenetic branching order obtained when 16S rRNA sequences were compared (Moore *et al.*, 1996). Within the *Pseudomonas* species studied, the ITS1 sequences are, as expected, relatively similar, although sequence differences increased when other pseudomonad rRNA homology group II species (Palleroni, 1984), e.g. *Burkholderia* species, were included in comparisons. Sequence similarities of the ITS1 of *P. stutzeri* (CCUG 11256^T) with those of other species of *Pseudomonas* ranged from 65% (*P. stutzeri* versus *P. putida* and *P. fragi*), to 86% (*P. stutzeri* versus *P. alcaligenes*).

Assignment of new strains to *P. stutzeri* genomovars

Isolates of *P. stutzeri* are often not easily identified, as has been reported by Holmes (1986). Many strains of *P. stutzeri*, other than those belonging to genomovar 1, will not be classified properly because of the discrepancies observed between the phenotypic and genotypic analyses. Although phenotypic characterization might indicate that a strain of interest is most probably a *P. stutzeri*, in the case of strains belonging to genomovars other than genomovar 1, low levels of DNA similarity with the type strain of the species (CCUG 11256^T, genomovar 1) would contradict such a conclusion (Table 3). When carrying out DNA–DNA reassociation analyses of strains suspected to be *P. stutzeri*, it is important to keep in mind that *P. stutzeri sensu lato* is, in reality, composed of at least

seven genomic groups, and comprehensive DNA–DNA hybridization experiments should include the reference strain of each genomovar (Table 3).

The establishment of reliable, high-resolution, molecular-genetic-based methods would facilitate the systematic identification of *P. stutzeri* strains. However, although specific PCR primers and probes for the detection and identification of *P. stutzeri* have been described (Bennasar *et al.*, 1998a), as the 16S rRNA gene sequences of increasing numbers of new organisms are introduced into the databases the limitations of the resolution or discriminatory abilities of these detection and identification tools are being recognized.

As the nucleotide variability observed in the ITS1 regions is seen to be of value for the identification of genomovars of *P. stutzeri*, in this study analysis of ITS1 regions, as well as DNA–DNA hybridizations and partial 16S rRNA gene sequencing, was applied to the identification of new isolates identified as *P. stutzeri* (Table 3). All new isolates were assigned to a known genomovar by DNA–DNA hybridization experiments (as a general rule, strains are considered to be in the same genomovar when percentages of DNA–DNA similarities are higher than 70%, which is also the level of discrimination for bacterial species; Johnson, 1973, 1984). The results obtained by *TaqI* ITS1 RFLP and analysis of ITS1 sequences correlated with the assignment of each new strain to a known genomovar. Identifications determined by both methods, ITS1 RFLP and ITS1 sequence comparisons, were complementary, although ITS1 sequencing is more discriminative. These results agreed with the DNA similarity results obtained (Table 3).

An example of a discrepancy was observed with respect to the classification of the recently isolated strain JD4. JD4 was assigned to genomovar 5 by the results of DNA–DNA hybridizations (Table 3), as well as nearly full-length 16S rRNA gene sequence analysis. However, ITS1 RFLP analysis using restriction enzymes *TaqI* and *DraI* clustered strain JD4 with strains of genomovar 1, although ITS1 sequence similarities and cluster analysis clearly show JD4 to be most closely related to the reference strain of genomovar 5 (DNSP21). While the DNA relatedness results allocated JD4 to genomovar 5 at the lower limits (76% DNA similarity) recognized for allocating a strain to a genomovar (approx. 70% DNA similarity), the degree of DNA relatedness between JD4 and the reference strain of genomovar 1 (CCUG 11256^T) was significantly lower (53% DNA similarity) (Table 3). Thus it is evident that, although *TaqI* and *DraI* ITS1 RFLP analyses were not able to identify strain JD4 to the genomovar corresponding to genomic DNA similarities, ITS1 sequence determination and analysis allocated this strain to the correct genomovar. Furthermore, in spite of the high similarities between the ITS1 sequences of strains of genomovars 1 and 5, comparative analysis identified signature nucleotide

positions for each *P. stutzeri* genomovar (Table 2), including genomovars 1 and 5, which are recognized to be adequately specific to allow the design of PCR primers that react only with the respective target genomovar (Guasp, 1999).

General conclusions

Sequencing of ITS1 regions is a relatively fast (only one PCR and one sequencing reaction are required) and reliable method for the differentiation of *P. stutzeri* genomovars and the identification of new strains, and may be seen as a useful alternative to DNA–DNA hybridization. The PCR amplicon size variation, together with the nucleotide sequence divergence, of ITS1 also can be applied for identifying strains belonging to the same genomovar and for discriminating species belonging to the same genus. From the data presented, ITS1 nucleotide sequence differentiation of the *P. stutzeri* genomovars correlates with the DNA–DNA hybridization results. Furthermore, it is evident that if two strains have the same ITS1 sequence, or if only punctual differences are detected, they are likely to be members of the same species, i.e. the same genomovar in the case of *P. stutzeri*. This conclusion is in accordance as well with the results obtained by Yoon *et al.* (1998) for strains of the genus *Nocardioide*s. The differences observed in dendrogram branching order and branch lengths obtained from ITS1 sequences compared with those obtained from 16S rRNA gene sequences reflect the respective different rates of evolution. Whereas comparisons of 16S rRNA gene sequences are limited in their ability to resolve closely related species of a genus (Ash *et al.*, 1991; Fox *et al.*, 1992; Martinez-Murcia *et al.*, 1992; Hauben *et al.*, 1997, 1998), the less conserved ITS1 regions are able to be used as a high-resolution indicator of the evolutionary divergence between species. Thus ITS1 regions may be good targets of genomovar- and species-specific probes for environmental monitoring.

A rapid characterization scheme at the genomovar, i.e. genomic species, level is possible based on PCR amplification and subsequent RFLP of ITS1. *TaqI* RFLP fingerprinting of the ITS1 is a rapid approach for characterizing, typing and identifying *P. stutzeri* strains at the genomovar level, in most cases, without the necessity of ITS1 sequencing or DNA–DNA hybridization. The combination of three ITS1 RFLP tests (*TaqI*, *BclI* and *DraI*) allowed a relatively conclusive genomic group differentiation of *P. stutzeri* strains, in accordance with the differentiation observed by DNA–DNA similarities. Although at least one exception was observed, and more might be expected as more strains are analysed, ultimately careful analysis of ITS1 sequence data should positively feed back to optimize additional confirmative rapid RFLP tests for confidently assigning new *P. stutzeri* strains to genomovars. The ITS PCR-RFLP method is the most rapid and least expensive way to routinely identify the

phenotypically and genotypically heterogeneous strains and isolates of *P. stutzeri* (*sensu lato*) at the genomovar level.

Despite the observation that every species of *Pseudomonas* analysed presented a unique restriction pattern, more strains of each species will need to be analysed (studies in progress) before arriving at general conclusions about the utility of ITS1 restrictions for identification of strains at the species level.

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