

# Identification of complex composition, strong strain diversity and directional selection in local *Pseudomonas stutzeri* populations from marine sediment and soils

Johannes Sikorski,<sup>1</sup> Martin Möhle<sup>2</sup>  
and Wilfried Wackernagel<sup>1\*</sup>

<sup>1</sup>Genetik, Fachbereich Biologie, Geo- und Umweltwissenschaften, Universität Oldenburg, D-26111 Oldenburg, Germany.

<sup>2</sup>Arbeitsbereich Stochastik, Mathematisches Institut, Eberhard Karls Universität Tübingen, D-72076 Tübingen, Germany.

## Summary

Members of *Pseudomonas stutzeri* have been isolated world-wide from various habitats including aquatic and terrestrial ecosystems. The global population has a clonal structure, is of exceptionally high genetic diversity and has been grouped into eight genomovars. We have analysed four local populations ( $n = 89$ – $125$ ) from three geographically separated habitats (two from a marine sediment and two from different soils) by random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR), restriction fragment length polymorphism (RFLP) of the *rpoB* gene and 16S rDNA sequences in order to quantify the influence of evolutionary forces on closely related groups of proliferating cells *in situ*. All populations consisted of a complex structure of genomic subgroups with variable numbers of members. The analyses revealed that the two populations from marine sediment were rather similar. At least three of the populations were influenced by migrational input as concluded from the presence of members from different genomovars. All populations showed very high strain diversity suggesting strong influence of mutability. Neutrality tests indicated that two or possibly three of the populations were shaped by directional selection. Thus, the local populations of *P. stutzeri* reflect already the high genetic diversity of the global population and are influenced, to different extents, by migration, mutation and directional selection.

## Introduction

The species *Pseudomonas stutzeri* is a non-fluorescent member of the genus *Pseudomonas* ( $\gamma$ -Proteobacteria). On the taxonomic level, members of *P. stutzeri* are grouped by DNA–DNA hybridization into at least eight genomic groups, termed genomovars (Rossello *et al.*, 1991; Ursing *et al.*, 1995; Sepulveda-Torres *et al.*, 2001), which lack differentiating phenotypic characteristics necessary for own species status (Wayne *et al.*, 1987). The genomovar grouping has been confirmed by 16S rRNA gene sequencing (Bennasar *et al.*, 1996; Sikorski *et al.*, 1999), total fatty acid analysis, total protein analysis (Rosselló-Mora *et al.*, 1994), macrorestriction fragment analysis of genomic DNA (Ginard *et al.*, 1997), PCR-based genomic fingerprinting (Sikorski *et al.*, 1999) and 16S-23S ITS sequence analysis (Guasp *et al.*, 2000). Corresponding to the high genomic diversity also high genetic diversity has been found by multilocus enzyme analysis (Rius *et al.*, 2001).

*P. stutzeri* is widely present in the environment including marine, sediment and soil habitats (Rossello *et al.*, 1991). Many strains were also recovered from clinical specimen (Rossello *et al.*, 1991; Noble and Overman, 1994) or have been isolated from bottled water (Papapetropoulou *et al.*, 1994), vertebrate faeces (Hubalek *et al.*, 1998), and paper-making chemicals (kaolin; Vaisanen *et al.*, 1998).

Strains of *P. stutzeri* have often ecologically important features including the potential for denitrification, the ability to degrade xenobiotics or the competence for natural genetic transformability. In a study comprising 12 strains from six genomovars six strains have been shown to be naturally transformable (Lorenz and Sikorski, 2000). The frequency of transformation varied over three orders of magnitude. Studies with strains JM300 and ZoBell showed the potential for genetic transfer by natural genetic transformation in marine and soil environment (Stewart and Sinigalliano, 1991; Paget and Simonet, 1997; Sikorski *et al.*, 1998). Members of *P. stutzeri* are strong denitrifiers. With strain ZoBell the biochemistry and genetics of denitrification has been intensively studied (Zumft, 1997). The high metabolic versatility includes the degradation of environmental pollutants (Baggi *et al.*,

Received 12 April, 2002; accepted 30 May, 2000. \*For correspondence. E-mail: wilfried.wackernagel@uni-oldenburg.de; Tel. (+49) 441 798 3298; Fax (49) 441 798 5606.

1987), naphthalene and methyl-naphthalenes, which are the most abundant aromatic components of crude oil and potentially toxic components of petroleum (Rosselló-Mora *et al.*, 1994), high-molecular-weight polyethylene glycols (Obradors and Aguilar, 1991) and other xenobiotics (Criddle *et al.*, 1990; Chauhan *et al.*, 1998; Coates *et al.*, 1999).

Very little is known about the distribution of the various *P. stutzeri* genomovars in the environment. Moreover, the composition and genetic structure of local environmental *P. stutzeri* populations are not known. So far, mostly single or only few strains have been obtained by chance from environmental samples (Carlson *et al.*, 1983; Garcia-Valdes *et al.*, 1988; Criddle *et al.*, 1990; Anzai *et al.*, 1997; Metcalf and Wolfe, 1998; Coates *et al.*, 1999; Sorokin *et al.*, 1999; Petri and Imhoff, 2000; Rockne *et al.*, 2000; von Wintzingerode *et al.*, 2000), which does not allow to draw any conclusions on the structure of local populations and the diversity of their members. Only one study aimed at the specific isolation of *P. stutzeri* cells from environmental samples utilizing enrichment cultures (Bennasar *et al.*, 1998). However, strains isolated by enrichment procedures do not correctly reflect the size and genetic diversity of the population in the environmental sample.

Within several genomovars high strain diversities were observed (Sikorski *et al.*, 1999; Rius *et al.*, 2001). High diversity between strains must result from evolutionary processes such as the diversification of descendants from a single strain by mutation and perhaps recombination. These processes therefore must occur in local populations. One aim of this study was to examine whether the high genomic diversity of the species *P. stutzeri* observed by looking at world-wide collected single isolates would be seen in local populations where species diversity evolves. Another aim was to determine whether structure and composition of local populations could have been influenced by additional evolutive forces as migration and directional selection. For these studies, local populations were obtained by a recently developed method for the specific isolation of culturable *P. stutzeri* cells from environmental samples (Sikorski *et al.*, 2002). With this method four such populations of about 100 strains each from three different environmental samples (one marine sediment and two soils) were available for analysis by high and low resolution methods (RAPD-PCR, 16S rDNA sequencing, RFLP of a PCR product of a part of the *rpoB* gene). In this way we characterized the genetic diversity of the populations of a single species as *P. stutzeri* from geographically closely restricted areas. The results of our analysis indicate a complex composition and high strain diversity of local populations which is the result of combined influence of *in situ* diversification, migrational input and natural selection.

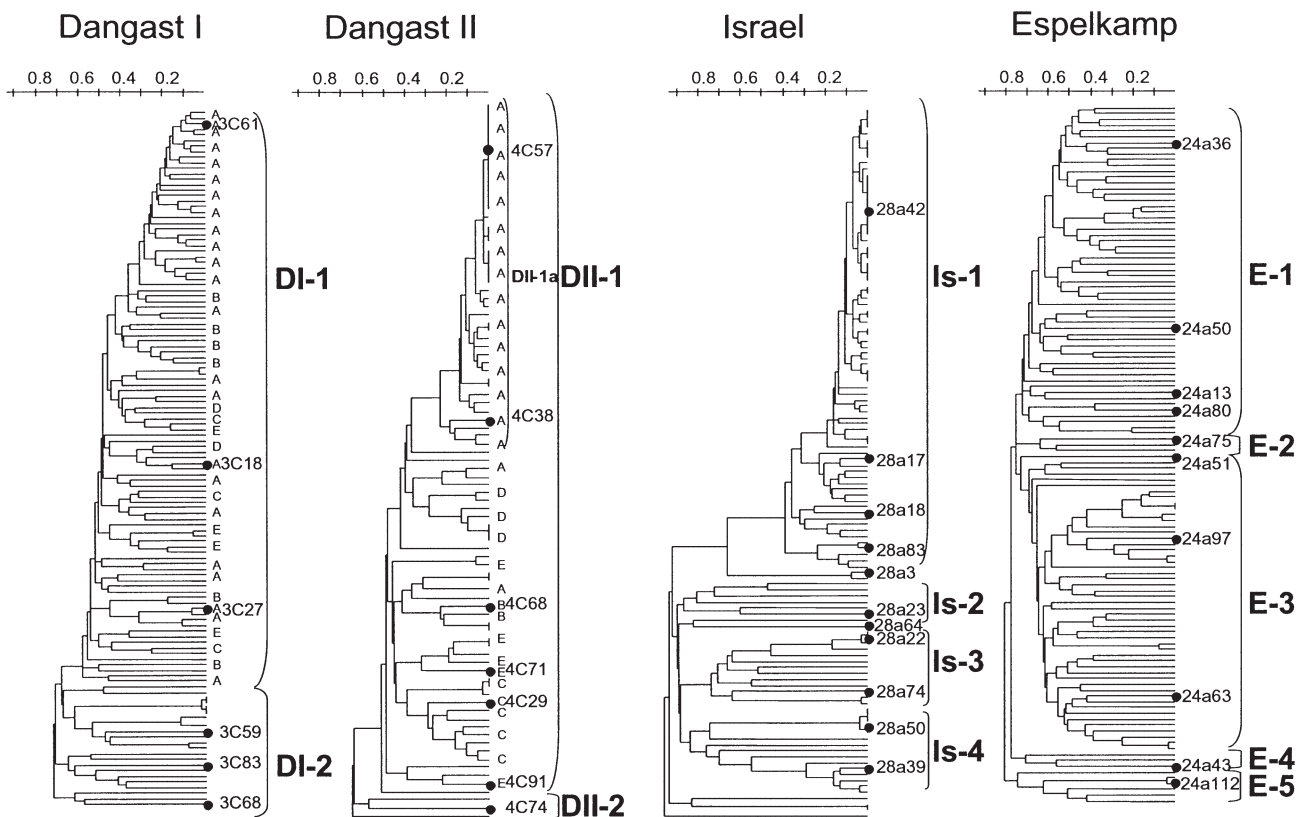
## Results

### *Genetic relationship of strains of populations Dangast I and II*

The procedure used here for the specific isolation of *P. stutzeri* strains from environmental samples depends on their utilization of ethylene glycol, starch or maltose as sole carbon source (Sikorski *et al.*, 2002) and was applied to samples which gave a positive signal with a highly specific PCR-based monitoring of 16S rRNA genes of *P. stutzeri* in total extracted DNA. Two populations of *P. stutzeri* were isolated from North Sea sediment samples (400 mg each) taken about 2 m apart close to the coast of Dangast (North Germany). The sediment contained about 600 colony forming units (CFU) of *P. stutzeri* per gram (Sikorski *et al.*, 2002). The genetic relationships of strains of the populations Dangast I ( $n = 125$ ) and Dangast II ( $n = 89$ ) were identified by phenetic cluster analyses on the basis of RAPD patterns of all strains (UPGMA; Fig. 1) and 16S sequences (neighbour-joining; Fig. 2) of strains representing major RAPD clusters.

The RAPD patterns were generated with three primers and produced a total of 164 and 126 band positions (loci), and an average number of  $32 \pm 4$  and  $31 \pm 3$  bands per strain for Dangast I and Dangast II respectively. In the bush-like tree topology of the dendrograms of Dangast I and II (Fig. 1) we marked the major groups of strains (DI-1 and DII-1) which were clearly separated from the remaining groups (DI-2 and DII-2). In both populations the haplotype diversity and haplotype divergence were high (Table 1).

On the basis of their 16S sequences 13 strains representing characteristic clusters (marked with a filled circle in Fig. 1) were integrated into a dendrogram of all 16S sequences presently available of a total of 86 *P. stutzeri* strains representing the established genomovars 1–9 (Fig. 2). The former genomovar 6 has been reclassified and now constitutes the species *Pseudomonas balearica* [not included in Fig. 2; (Bennasar *et al.*, 1996)]. The tree is consistent with previously published trees (Moore *et al.*, 1996; Rosselló-Mora *et al.*, 1996; Sikorski *et al.*, 1999). The identical 16S sequences of the nine strains from clusters DI-1 and DII-1 (only strain 4C57 had a single A to G transition at *E. coli* position 1036) grouped these isolates outside the established genomovars. The four strains from clusters DI-2 and DII-2 affiliate closely with the genomovar 3 reference strains (0.07–0.22% dissimilarity). Thus, the clustering according to the 16S sequences corroborates the grouping obtained by the RAPD patterns (Fig. 1) and shows that the Dangast populations are made up of two different taxonomic groups of which one is the genomovar 3 and the other is perhaps a new genomovar.



**Fig. 1.** Phenetic analysis (UPGMA) of the relationship of the isolates within the populations Dangast I, Dangast II, Israel, and Espelkamp based on RAPD analysis. From the strains marked with (●) the 16S sequence (approx. 1450 nucleotides) was determined. The scale bars denote dissimilarity values. Capital letters A to E in the populations Dangast I and Dangast II mark strains of lineages A to E in Fig. 3.

#### *Dangast I and Dangast II are two samples of one population*

In a cluster analysis of RAPD-PCR data with the combined 214 strains from Dangast I and II the strains of DI-1 clustered with the strains of DII-1 and the strains of DI-2 clustered with the strains of DII-2 (data not shown) suggesting high similarity of at least large parts of the populations. An exact test of population differentiation (Raymond and Rousset, 1995) on the combined data set of the two populations indicated differentiation of these ( $P < 0.0001$ ). However, the differentiation results from the representation of some haplotypes by several isolates in

DII-1 (in particular in the cluster DII-1a; Fig. 1). When only the haplotypes were considered in the test, the populations were no longer differentiated ( $P = 0.228$ ). We conclude that Dangast I and Dangast II are two samples from one population, but that they differ by the stronger representation of some haplotypes in Dangast II.

#### *The subpopulation of Dangast clusters 1 was influenced by directional selection pressure*

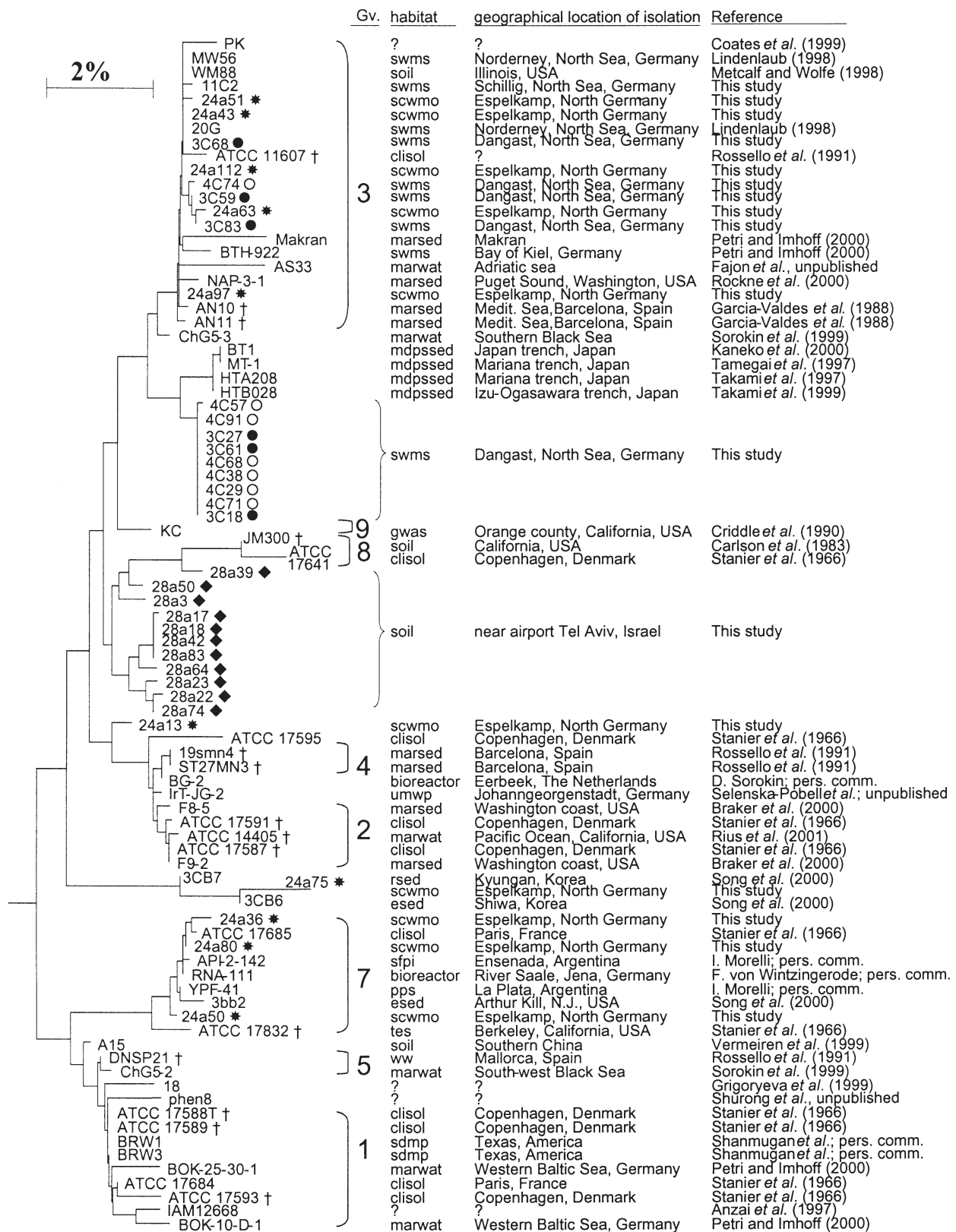
Based on the 16S sequence identity in DI-1 and DII-1 isolates we assumed a direct genealogical relationship of

**Table 1.** Diversity indices derived from the RAPD-PCR patterns of the four populations.

	Dangast I	Dangast II		Israel			Espelkamp
		All isolates	Cluster DII-1a	All isolates	Cluster Is-1	All isolates except cluster Is-1	
No. of isolates (n)	125	89	43	120	78	42	120
No. of haplotypes (k)	123	62	22	89	52	37	119
Haplotype diversity <sup>a</sup>	0.98	0.70	0.51	0.74	0.67	0.89	0.99
Haplotype divergence <sup>b</sup>	35.5	23.8	8.6	20.4	6.5	30.8	43.1

**a.** Ratio of all haplotypes among all isolates.

**b.** Mean number of pairwise differences between all pairs of haplotypes.



the strains due to proliferation and diversification in the habitat. This prompted us to analyse the recent evolutionary history of DI-1 and DII-1 (Fig. 1) by neutrality tests. These tests are based on the infinite-alleles and infinite-sites models of mutation and calculate on the basis of a given data set the probability that a neutral evolution of the population has occurred, i.e. only under the influence of mutation and drift, but not under selection (Hartl and Clark, 1997). The tests, however, have not been sufficiently adjusted for the binary kind of data produced by RAPD-PCR. Additionally, the applied models do not allow testing of data for selection which have been influenced by recombination (Hartl and Clark, 1997). Since several strains of the cluster 1 subpopulations have been shown to be natural transformable (Sikorski *et al.*, 2002) and RAPD patterns are based on a total genome scanning approach, the RAPD patterns may have been influenced by recombination. In order to have data suitable for the neutrality tests which are most likely not influenced by recombination, we used restriction fragment length polymorphisms of a single gene. A PCR product (1.5 kb) of part of the *rpoB* gene from every third strain of DI-1 and DII-1 including those strains with known 16S sequence (plus 3C83, 3C59, 3C68, and 4C74) was analysed by four restriction enzymes. The dendrogram based on the RFLP patterns (Fig. 3) shows (i) that the cluster 1 strains differ strongly from cluster 2 strains (ii) that each subcluster A to E contains strains from Dangast I and II, and (iii) that the dendrogram obtained from *rpoB* polymorphisms (Fig. 3) largely matches that derived from the RAPD data.

We applied neutrality tests to the RFLP data. Based on the haplotype configuration (infinite-alleles model), the observed *F*-value for homozygosity (0.2548; Table 2) is significantly higher ( $P \geq 0.989$ ; the 95% confidence interval for *F* is 0.1877; Table 2) than the expected *F*-value (0.1072; Table 2). This indicates influence of directional selection pressure (visible in the representation of one haplotype by 35 strains). The Tajima test, which compares the number of polymorphic bands to the mean number of pairwise differences (infinite-sites model), does not show deviation from neutrality ( $P = 0.786$ ; Table 2).

**Table 2.** Ewens-Watterson-Slatkin's tests and Tajima's test on selective neutrality on RFLP data of the *rpoB* gene of strains of Dangast subclusters 1.

Test	
<i>Ewens-Watterson-Slatkin's tests</i>	
Sample size ( <i>n</i> )	70
No. of haplotypes in sample	20
Observed <i>F</i> -value	0.25476
Expected <i>F</i> -value	0.10717
95% confidence interval for expected <i>F</i>	0.1877
No. of simulated samples	100 000
Watterson's homozygosity test	$P = 0.998$
Slatkin's exact test	$P = 0.989$
<i>Tajima's test</i>	
Sample size ( <i>n</i> )	70
No. of segregating sites ( <i>S</i> )/ $\theta_s$	19/3.967
Mean no. of pairwise distances ( <i>Pi</i> ) = $\theta_\pi$	4.7902
Tajima's <i>D</i>	0.6278
No. of simulations	10 000
$P (D_{\text{simul}} < D_{\text{observation}})$	0.786

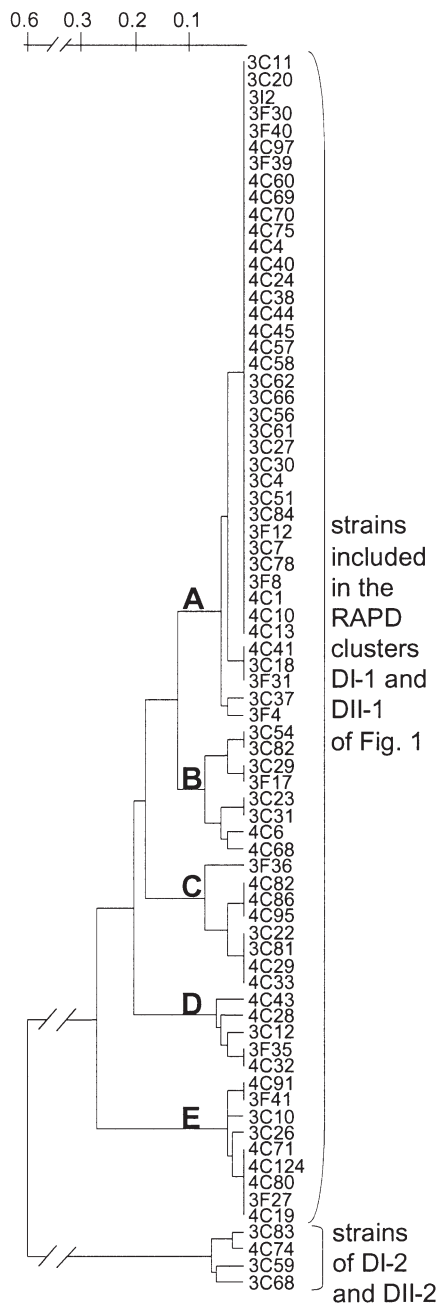
However, the Tajima *D*-value is positive ( $D = 0.6278$ ; Table 2), which is characteristic for balancing selection (visible by the fairly long internal branches of the five lineages A to E).

#### Genetic relationship of strains of the population Israel

A third population ( $n = 120$ ) was isolated from a sample of soil (400 mg) collected near the airport of Tel Aviv (Israel) and was termed Israel. The sample contained about 1800 CFU of *P. stutzeri* per gram. The RAPD patterns generated with the same three primers as for the Dangast populations produced a total of 190 band positions with an average number of  $15 \pm 4$  bands per strain.

On the basis of RAPD data four major groups of strains were marked (Fig. 1) of which cluster Is-1 (78 strains) dominated the population. The haplotype divergence and haplotype diversity are rather low (Table 1). The 16S sequences of 11 representative strains from the clusters (marked with a filled circle in Fig. 1) indicate relatively close relation between them and perhaps a weak affiliation with the genomovar 8 strains (Fig. 2). The 16S

**Fig. 2.** The phylogenetic tree of *P. stutzeri* based on the 16S rDNA sequence (Jukes and Cantor correction, neighbour-joining). The environmental isolates from this study were marked to identify them as members of populations Dangast I (●), Dangast II (○), Israel (◆), and Espelkamp (\*). Additionally, the genomovar reference strains from Rossello *et al.* (1991) were marked (†). The tree reconstruction calculation included 16S sequences from *Pseudomonas balearica* (U26417, U26418, AJ006107, AF054936), *Pseudomonas citronellolis*<sup>T</sup> (Z76659), *Pseudomonas resinovorans*<sup>T</sup> (Z76668), and *Pseudomonas aeruginosa*<sup>T</sup> (Z76651). The tree was rooted with *E. coli* (J01659). The abbreviations for the column 'habitat' are as follows: clisol, clinical isolate; esed, estuarine sediment; gwas, groundwater aquifer solids; marsed, marine sediment; marwat, marine water; mdpsed, marine deep-sea sediment; pps, petrochemically polluted soil; rsed, river sediment; scwmo, soil contaminated with mineral oil; sdmp, soil from the waste water retention plant of a dicamba manufacturing plant; sfpi, sludge from petrochemical industry; soil, soil; swms, shallow-water marine sediment; tes, tartrate enriched soil; umwp, uranium mining waste pile; ww, waste water; ?, unknown. Strain NAP-3-1 was isolated from a marine sediment contaminated with polycyclic aromatic hydrocarbon. The starter community for the bioreactor from which strain BG-2 was isolated was from an anaerobic waste water paper mill treatment in Eerbeek, the Netherlands. The starter community for the anaerobic, trichlorobenzene transforming bioreactor consortium from which strain RNA-111 was isolated was from River Saale sediment contaminated with chloric industrial waste.



**Fig. 3.** Phenetic analysis (UPGMA) of the relationship of isolates of the Dangast clusters DI-1 and DII-1 and of isolates 3C83, 3C59, 3C68, and 4C74 based on RFLP patterns of a PCR product (1.5 kb) of a part of the *rpoB* gene. The scale bars denote dissimilarity values. Strain numbers of Dangast I begin with a 3 and strain numbers of Dangast II begin with a 4. The capital letters A to E denote the different *rpoB*-RFLP lineages. A total of 41 different band positions was obtained from the 74 strains; of the 28 band positions of the cluster 1 strains 19 were polymorphic.

dendrogram largely conforms with the RAPD dendrogram (Fig. 1). To determine whether the population represents a new genomovar or perhaps more than one requires further taxonomic studies. RFLP analysis of *rpoB* indi-

cated no polymorphism in 45 strains out of Is-1 but many differences between members from Is-1, Is-2, Is-3, and Is-4.

#### *Genetic relationship of strains of population Espelkamp*

The fourth population ( $n = 120$ ) was isolated from a soil sample (400 mg) taken from a filling station in the city of Espelkamp (Germany). In this sample the concentration of culturable *P. stutzeri* strains was about 1400 per gram (Sikorski *et al.*, 2002). The RAPD patterns were generated with the same three primers as for the Dangast and Israel populations and produced a total of 195 band positions with an average number of  $30 \pm 6$  bands per strain. We marked five distinct clusters (E-1 to E-5) in the resulting dendrogram (Fig. 1). Espelkamp has the highest values of haplotype diversity and divergence of the examined populations (Table 1).

On the basis of 16S sequences members of E-1 affiliate with genomovar 7 and members of clusters E-3, E-4, and E-5 affiliate with genomovar 3 (Fig. 2). The representative strain of E-2 (24a75) clusters with two isolates from aquatic habitats in Korea recently described (Song *et al.*, 2000) and together with them apparently constitutes a new phylogenetic branch of *P. stutzeri* (Fig. 2). Strain 24a13 affiliates with strains of genomovars 2 and 4. Again the 16S dendrogram matches largely with the RAPD data. We conclude that Espelkamp consists of taxonomically very different groups of strains which include strains from at least two established genomovars (3 and 7) and possibly one or two new genomovars.

#### *The global distribution of the species P. stutzeri and its genomovars*

We have compiled data of all *P. stutzeri* isolates presently identified by their 16S rDNA sequence ( $n = 86$ ) with respect to the geographical location of the isolation place and the type of habitat if known (Fig. 2). Strains of genomovars 1, 2, 3, and 7 were isolated from soil as well as aquatic habitats. The few members of genomovars 4 and 5 came from aquatic habitats and the one member of genomovar 9 is from ground water aquifer material. Perhaps the genomovar 3 members preferentially exist in marine and the genomovar 7 members mainly in soil habitats contaminated by petrochemicals or other pollutants. Members of genomovars 1, 2, 3, 7 and 8 were isolated on two or three continents. Clinical isolates fell into most genomovars with some preferences for genomovar 1 (Rius *et al.*, 2001) but the sites from where the strains were taken up by their hosts are not known. As a general picture, a clear association of genomovars with particular habitats or geographical locations was not apparent.

## Discussion

This is the first study on the genetic structure of local populations of *P. stutzeri*. It is based on the genetic analysis of strains with methods of different resolutive power. Relatively large numbers of cultivable strains were obtained from 400 mg samples from each environmental site and these were sufficient to determine local species structures. In contrast to studies applying exclusively molecular methods to DNA isolated from environmental samples (which relies on an uncharacterizable mixture of strains), the cultivation-based approach used here allows to assess the informations from individual cells. Furthermore, the availability of the living cells allows to apply any kind of molecular or physiological analyses. For example, the members of the Israel population have been studied in detail for their ability to undergo natural genetic transformation (Sikorski *et al.*, 2002). Even techniques that are not available now can be applied to the strains in the future.

### *Complex composition of the populations*

A major finding was that at least three or even all four of the populations consisted of strains of two or more genomovars. Additionally, each population contained members of genomic groups that can be suspected to represent new genomovars. Further taxonomic studies are necessary to clarify this point. In addition, each population was composed of a unique combination of genomovars and genomic groups which were mostly not present in the other populations. As members of established genomovars found in our populations have previously been identified in geographically strongly separated places, it is concluded that migrational input has contributed considerably to the genetic diversity in each local population.

The complex composition of our populations, together with data compiled in Fig. 2, suggests that strains of genomovar are apparently not limited to exist in a given habitat. This can be explained in two ways which do not exclude each other. First, cells of some genomovars may be physiologically flexible which allows them to successfully occupy different habitats (e.g. genomovar 3). Second, the physical and chemical complexity of soils and sediments may provide various different microhabitats allowing for the presence of strains of different genomovars each with a preference for a specific microhabitat.

### *High haplotype diversity within each population*

The very high haplotype diversity in all of our four populations derived from 400 mg of environmental material each

was remarkable. In other environmental populations of *Bacillus subtilis* (Istock *et al.*, 1992), *Bacillus cereus* – *Bacillus thuringiensis* (Helgason *et al.*, 1998); *Burkholderia cepacia* (Wise *et al.*, 1995; Wise *et al.*, 1996; Dalmastri *et al.*, 1999) and *Rhizobium meliloti* (Paffetti *et al.*, 1996) also considerable haplotype diversity was observed although the soil samples were mostly much larger (approx. 200 g; Istock *et al.*, 1992) or recovered from the rhizospheres of several plants. This would reduce the chance of finding genetically identical cells which are descendants from the same ancestor strain. We propose that high haplotype diversity in environmental populations results from the specific stress situation of cells in the natural habitat. This stress can result from abiotic factors (e.g. limitation of nutrients) or community-related conditions (e.g. presence of growth inhibiting metabolites). Under starvation or during stationary phases point mutation frequencies can dramatically increase (Finkel and Kolter, 1999) due to activation of mutagenic responses [including formation of error-prone DNA-polymerases during SOS induction; (Radman, 1999; Tang *et al.*, 1999; 2000)] or/and suppression of anti-mutagenic activities [such as the mismatch repair; (Taddei *et al.*, 1997b)]. Strong selective challenge, e.g. as imposed by antibiotics can even select mutator strains (Oliver *et al.*, 2000). The effect of deleterious mutations may be masked by a remaining wild-type copy of the mutated gene in stationary cells with several chromosome equivalents (Akerlund *et al.*, 1995; Kogoma, 1997). After cell division, a deleterious mutation may knock out one of the daughter cells which would increase the haplotype diversity. Also, the transposition of mobile genetic elements may increase under stress (Thompson and Landy, 1989), this can lead to chromosomal rearrangements. In a laboratory population of *E. coli* evolving for 10 000 generations most polymorphisms reflected rearrangements (including transpositions) rather than point mutations (Papadopoulos *et al.*, 1999). *Pseudomonas stutzeri* may be susceptible to this kind of genetic variation mechanisms as it is regarded as a species with highly rearranged chromosomes and no long-range conservation of genetic maps (Ginard *et al.*, 1997). In addition, many members of *P. stutzeri* including members of the four populations analysed here are capable of natural transformation which would provide a means of further genomic diversification by recombination (Carlson *et al.*, 1983; Lorenz and Sikorski, 2000; Sikorski *et al.*, 2002). On the basis of laboratory experiments with pure cultures (Bridges, 1997; Bridges and Timms, 1997; Taddei *et al.*, 1997a; Finkel and Kolter, 1999; Foster and Rosche, 1999) relatively high mutability of bacteria in natural habitats was predicted as was seen here. Interestingly, local environmental populations of the cyanobacterium *Nostoc linckia* showed higher mutation rates and higher genetic diversity

in a more stressful environment (Satish *et al.*, 2001; Dvornyk *et al.*, 2002). The higher genetic diversity of populations under environmental stress may be a general phenomenon in biology as it was also observed with various plant and animal species (Nevo, 2001).

#### *Development of the local population Dangast*

Isolates from a geographically restricted area being classified as members of the same species are a local population. Local populations are the actual evolving units of a species and the fundamental elements to which population genetics can be applied (Hartl and Clark, 1997). In prokaryotes, the classification of an isolate to a species is based on an arbitrary species concept which is universally applicable (Wayne *et al.*, 1987) but which does not take into account how bacterial species originate, that is, an understanding of the evolutionary processes that generate taxonomic diversity (Goodfellow *et al.*, 1997). The nomenclature *P. stutzeri* contains several genomic species referred to as genomovars (Rossello *et al.*, 1991; Ursing *et al.*, 1995). Recently, Palys *et al.* (1997) pointed out that closely related groups of bacteria from the same geographical area falling into distinct clusters based on sequence similarity must represent different ecological subpopulations inhabiting own ecological niches. Therefore, members of different genomovars of *P. stutzeri* from the same geographical area belong to such different ecological populations and thus constitute different evolving units. We consider the Dangast clusters DI-1 plus DII-1 and the Israel cluster Is-1 to be evolving units within their populations. For DI-1 plus DII-1 the neutrality tests based on the infinite-alleles model (Ewens–Watterson–Slatkin tests) significantly indicated directional selection visible as an over-representation of a specific *rpoB* gene RFLP haplotype. This over-representation is not due to the outburst of a single clone by rapid proliferation in one of the sampling places. Rather, it is inherent to the total Dangast population because strains of this specific *rpoB* haplotype are present and dominant in both Dangast I and Dangast II. In contrast, the Tajima test did not indicate deviation from neutrality. Its positive *D*-value may reflect influence of balancing selection.

We interpret these apparently conflicting results from the neutrality tests to indicate that the Dangast population is passing through a transition state. In the previous situation the subpopulations (DI-1 and DII-1) evolved into different lineages (*rpoB*-RFLP lineages A to E) through selection pressure favouring the various lineages in alternating successive selection phases. This led to the notion of balancing selection, which was not yet strong enough to show deviation from neutrality. At the current stage the population is in the phase of a specific haplotype of lineage A being favoured by directional selection.

This haplotype has already further diverged, as indicated by the diverse RAPD patterns. In contrast, the strains from Israel cluster Is-1 did not yet show *rpoB* gene polymorphisms, probably because selection pressure has not acted for long enough to be identifiable by the limited resolution capacity of the single gene-RFLP.

#### **Conclusion**

Complex composition and high strain diversity was observed for each of the local populations. The presence of strains in a local population belonging to different world-wide occurring genomovars must be the result of migrational input of strains having evolved separately in different local populations. The diversification *in situ* by mutation and possibly recombination can result in the relatively high diversity of strains of the same genomovar. The diversity pattern may be specifically shaped by directional selection in a local population, as observed in the Dangast population.

On a global level *P. stutzeri* was shown to be clonal (Rius *et al.*, 2001). This is in accord with the results of Lorenz and Sikorski (2000) showing that gene transfer between genomovars by transformation is restricted at least by sequence divergence. Nevertheless, high frequencies of recombination may be possible among strains of an ecological unit of *P. stutzeri*. This has been observed in *Rhizobium* species (Souza *et al.*, 1992). Determination of the linkage disequilibrium in local populations and subpopulations of *P. stutzeri* may clarify whether these are clonal or whether *P. stutzeri* is a species with locally recombining evolutionary units (Istock *et al.*, 1996).

#### **Experimental procedures**

##### *Strains and media*

*Pseudomonas stutzeri* strains were isolated from environmental samples by a very efficient combined plating procedure based on the growth of *P. stutzeri* strains on rich media and on artificial sea water medium with ethyleneglycol as the sole carbon source (Sikorski *et al.*, 2002). Four populations were obtained from four samples taken at three different sites. Two samples were taken in September 1999 from the upper two centimetres of the oxic zone of a marine sediment at the North Sea coast of Germany (population Dangast I and Dangast II) about two metres apart from each other. A third sample was taken in January 2000 from soil at a filling station contaminated by mineral oil in northern Germany (population Espelkamp) and the fourth sample from soil in the vicinity of the Tel Aviv Airport in Israel taken in February 2000 (population Israel) (Sikorski *et al.*, 2002). Generally, strains were grown in SW-LB medium [10 g l<sup>-1</sup> Bacto tryptone, 5 g l<sup>-1</sup> yeast extract, 15 g l<sup>-1</sup> agar (all Difco, Detroit, USA) in artificial sea water (24 g l<sup>-1</sup> NaCl, 10.5 g l<sup>-1</sup> MgSO<sub>4</sub> × 7 H<sub>2</sub>O, and 0.11 g l<sup>-1</sup> HNaCO<sub>3</sub>)] at 28°C.

### Genomic fingerprinting by RAPD-PCR

Random amplified polymorphic DNA-polymerase chain reaction was carried out as described previously (Sikorski *et al.*, 2001) using the primers CGAGTTCGCGTACCACCCC, GTTTCGCTCGATGCGCTACC, and CGGCACACTGTTCTCGACG. For each of the four populations a single master mix including all PCR components except for DNA polymerase and template DNA was employed. We performed several control experiments including separate RAPD-PCR runs on the same and/or on separate overnight cultures of the same strains and analyses of RAPD-PCR products in separate agarose gels to ensure the reproducibility of the RAPD patterns. The RAPD patterns were analysed with the software Gene ImagIR ver. 3.52 (Scanalytics, Fairfax, VA, USA) and exported for phenetic analysis into the TREECON software (Van de Peer and De Wachter, 1994) and into ARLEQUIN 2.000 software package for statistical analysis (Schneider *et al.*, 2000).

### Sequencing of the 16S rDNA and phylogenetic analysis

Amplification of 16S rRNA genes by PCR and subsequent sequencing was carried out as described previously (Sikorski *et al.*, 2001). The DNA sequences were aligned using ClustalX 1.8 (Thompson *et al.*, 1997). The alignment was corrected manually. For phylogenetic analysis (neighbour-joining) the sequences (1327 nucleotides *E. coli* position 61–1387) were imported into TREECON software (Van de Peer and De Wachter, 1994).

The accession numbers of the 16S sequences of the environmental isolates reported here are AJ312156-158, AJ312169-176, AJ270452-458, AJ410871-872 (EMBL database). The accession numbers of the 16S sequences of the reference strains are AF170358, AJ006108, AF038653, U26415, AJ288148, AJ288147, AJ391194, AF064636, U22427, U25280, AF054933, AB031277, AB004241, AB002660, AB010854, AF063219, X98607, AJ006106, AJ006105, AJ005167, U26419, AF054934, AJ295681, U26261, U26420, U25431, AF229886, AF229885, AJ006103, AJ387903, AF229888, U26416, Y18006, U26414, AF054935, AF152596, AF284764, U26262, U25432, AF025349, AF025351, AJ288150, U58660, AJ006104, D84024, and AJ288149. The sequences for strains F8-2 and F9-2 are available from J. Tiedje (Iowa, Michigan).

### PCR amplification of a part of the *rpoB* gene and determination of restriction fragment length polymorphisms

Polymerase chain reaction amplification of a part of the *rpoB* gene was carried out in 50  $\mu$ l volumes containing 2  $\mu$ l of DNA prepared with GeneReleaser<sup>TM</sup> (Eurogentec, Seraing, Belgium) from a fresh overnight culture. The reaction mixtures [(2% dimethylsulphoxide (DMSO), 1  $\mu$ M of each primer *rpoB*-for2 CTTCGAGTTCGATCCGAAGG and *rpoB*-R<sub>int</sub> CGGTTGGCGTCGTCGTGCTC (Lorenz and Sikorski, 2000), 50  $\mu$ M of each dNTP (Pharmacia)] contained 0.5 U of Taq DNA Polymerase (Eppendorf) in the reaction buffer supplied by the manufacturer. Primer *rpoB*-for2 was chosen from a set

of different primers matching to highly conserved regions of the *rpoB* gene which were identified from an alignment of both nucleotide and amino acid sequence from *Coxiella burnetii* (Acc. no. U86688), *Salmonella typhimurium* (X04642), *Legionella pneumophila* (AF087812) and *Pseudomonas putida* (X15849). The cycling programme in a Robocycler (Stratagene) included an initial incubation at 94°C for 5 min, 40 cycles each at 94°C for 1 min, at 63°C for 1 min, and at 72°C for 1.5 min, and a final extension cycle at 72°C for 10 min. The PCR products (1.5 kb) were treated with restriction enzyme (*Hae*III, *Alu*I, *Hind*III or *Bst*UI) in the supplied reaction buffer in 30  $\mu$ l reaction volumes containing 10  $\mu$ l of the PCR reaction mixture and 5 units of enzyme. The restriction fragments were resolved by electrophoresis on agarose gels (3%). A size marker (Ladder Mix, MBI Fermentas, St Leon-Rot, Germany) was used as a reference in all gels. The restriction patterns were analysed with the software Gene ImagIR ver. 3.52 (Scanalytics, Fairfax, VA, USA) and exported for phenetic analysis into the TREECON software (Van de Peer and De Wachter, 1994) and into ARLEQUIN 2.000 software package for statistical analysis (Schneider *et al.*, 2000).

### Statistical analysis and neutrality tests

The diversity indices, the exact test of population differentiation (Raymond and Rousset, 1995) and the neutrality tests were calculated using ARLEQUIN 2.000 (Schneider *et al.*, 2000).

A haplotype is a unique combination of genetic markers present in a chromosome. The haplotype diversity is the ratio of all haplotypes among all isolates. The haplotype divergence is the mean number of pairwise differences between all pairs of haplotypes. The haplotype configuration describes the numbers and frequencies of different haplotypes. For example, in the configuration '6,4,2,1,1,1,1' one haplotype occurs six times, another four times, a third twice, and all other haplotypes are unique. The exact test of population differentiation is based on the classical exact Fisher-Yates test for  $r \times c$  contingency tables (Fisher, 1935; Yates, 1984) and is performed using the Markov chain Monte Carlo method described by Raymond and Rousset (1995). Based on the infinite-alleles model the Watterson's homozygosity test (Watterson, 1978) and the Ewens-Watterson-Slatkin's exact test (EWS-test) (Slatkin, 1994; 1996) calculate whether the observed haplotype configuration of a population conforms to neutrality or not. From the haplotype configuration based on the numbers of sampled isolates ( $n$ ) and haplotypes ( $k$ ) the homozygosity ( $F$ ) is calculated. Both tests compare the observed configuration to simulated configurations ( $n$  and  $k$  are the same as in the observed data set) using Ewens sampling formula (Ewens, 1972) but apply different summary statistics for calculating the  $P$ -values for the homozygosity test and the exact test (Slatkin, 1994; 1996). The 95% confidence interval of the  $F$ -value expected under neutrality was calculated by the program FTEST which was kindly provided by Dr Thomas Whittam. The Tajima test (Tajima, 1989) of the infinite-sites model compares two different estimators  $\theta_\pi$  and  $\theta_S$  of the population genetic parameter  $\theta = 2 N_e \mu$  (where  $N_e$  is the effective population size and  $\mu$  is the mutation rate per sequence per generation).  $\theta_S$  is calculated from the number of polymorphic alignment positions ( $S$ ) and  $\theta_\pi$  from the mean

number of pairwise differences (Pi). At neutral development Tajima's *D*-value is zero ( $\theta_\pi - \theta_S \approx 0$ ). A positive *D* indicates balancing selection whereas a negative *D* indicates directional selection.

### Acknowledgements

We are grateful to Thomas Höpner, Ralf Petri, James Tiedje and Friedrich von Wintzingerode for supplying 16S sequences of *P. stutzeri* strains prior to their publication, to Nicole Teschner for help with some of the experiments, and to Irma Morelli for *P. stutzeri* strains YPF-41 and API-2–142.

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