Arch Microbiol (1995) 163 : 167–175

ORIGINAL PAPER

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Diversity of culturable denitrifying bacteria

Limits of rDNA RFLP analysis and probes for the functional gene, nitrite reductase

Received: 28 March 1994 / Accepted: 26 September 1994

Abstract Sequence divergence in the ribosomal genes of known strains and isolates of aquatic denitrifying bacteria was investigated using restriction fragment length polymorphism (RFLP) analysis. The same cultures were characterized for their homology with antibody and gene probes for nitrite reductase (NiR), a key enzyme in the denitrification pathway, and for amplification with a set of polymerase chain reaction primers designed to amplify a portion of the NiR gene. The NiR probes were developed from *Pseudomonas stutzeri* (ATCC 14405) and several *P. stutzeri* strains were included in the analyses. The RFLP analysis clustered most of the *P. stutzeri* strains together, but detected considerable diversity within this group. Isolates from three aquatic environments exhibited within – and among – habitat diversity by RFLP. Hybridization with the NiR probes and amplification with the NiR primers were not correlated with the clustering of strains by rDNA RFLP analysis. The relationships among strains deduced from ribosomal DNA RFLP reflect heterogeneity within the *P. stutzeri* group and among other pseudomonads, and the patterns differ from those inferred from specificity of the NiR probes.

Key words Denitrifying bacteria · Nitrite reductase · Restriction fragment length polymorphism · Diversity

Abbreviations *NiR* Nitrite reductase · *PCR* polymerase chain reaction · *RFLP* restriction fragment length polymorphism

Introduction

Denitrification is the major loss term for fixed nitrogen in the environment and is therefore important in nitrogen budgets on ecosystem to global scales. In respiratory denitrification, nitrogen oxides are reduced in stepwise fashion from nitrate through nitrite, nitric oxide, nitrous oxide to dinitrogen. Each enzyme in the sequential reduction from nitrate to dinitrogen exhibits some degree of oxygen sensitivity and inducibility, and some organisms do not possess the complete pathway. The key step in the process is the reduction of nitrite to nitric oxide, which results in the release of gaseous products. The ability to respire nitrate to nitrite is quite widespread in the bacterial kingdoms, but the ability to respire nitrite is somewhat more restricted. Therefore, the enzyme that mediates this reaction, nitrite reductase, has been the focus of physiological and biochemical studies on denitrification.

Bacteria capable of denitrification are easily isolated from aquatic, sediment, and soil environments. Using phenotypic characters, Gamble et al. (1977) identified *Pseudomonas* as the most common denitrifying genus isolated from terrestrial environments. Baumann et al. (1972) characterized aerobic eubacteria isolated from seawater and revealed that *Pseudomonas* was also a commonly isolated genus in the marine environment. The ability to denitrify, however, is present in phylogenetically diverse groups of eubacteria (Woese 1987). While they share aspects of the basic pathway, the pathway itself is really a collection of somewhat independent processes (Zumft 1992). The genetics and regulation of the pathways differ widely among denitrifying strains, but key enzymes appear to be somewhat conserved. Two forms of nitrite reductase are common in denitrifiers (strains generally have one or the other, not both), and each is conserved across a range of denitrifying bacteria (Bryan 1981). Nitrite reductase (NiR) is therefore a prime candidate for development of functional probes for the identification and quantification of bacteria capable of denitrification, regardless of the genus or species affiliation of the bacteria.

In addition to the identification or classification of bacterial strains, immunological assays for NiR could be useful in ecological studies. Enrichment and isolation studies have shown that the genetic ability for denitrification is widespread, but the expression of the inducible pathway

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depends on environmental conditions. Thus, detection of the protein in natural samples would indicate that conditions that induce denitrification were present at the time of sampling. This would be useful for studying environmental regulation and the flux of nitrogen due to denitrification in the environment.

Both immunological (Körner et al. 1987; Coyne et al. 1991; Ward et al. 1993) and DNA probes (Smith and Tiedje 1992; Ward et al. 1993) have been developed to investigate nitrite reductase in bacteria. Körner et al. (1987) and Ward et al. (1993) reported that an antiserum produced against NiR (heme cd_1 type) purified from *P. stutzeri* (ATCC 14405) was essentially strain-specific for *P. stutzeri*, cross-reacting with some other strains of *P. stutzeri* but not with all *P. stutzeri* strains tested. Coyne et al. (1989) produced antisera for the heme cd_1 NiRs purified from a different strain of *P. stutzeri* (JM300), and from *P. aeruginosa*. The *P. aeruginosa* antiserum reacted with denitrifiers in other genera as well as with other *Pseudomonas* species, including *P. stutzeri* and *P. fluorescens*, while the *P. stutzeri* antiserum apparently did not react with *P. aeruginosa*. Even proteins purified from different strains, but of very similar size and composition and with demonstrable in vivo and in vitro nitrite reduction capability, were immunologically distinct (Ward et al. 1993). The immunological characterizations implied that even a conserved key enzyme such as NiR might not be useful as a general probe for the identification of the functionally defined group of denitrifying bacteria.

DNA probes for fragments including portions of the NiR structural gene detected homology in a wider selection of denitrifying strains than did antibody probes (Smith and Tiedje 1992; Ward et al. 1993). The unusual antigenic behavior of the NiR protein remains to be resolved, but even the DNA homologies implied relatively great variability in phyenotypically related strains. Instead of providing general probes for the identification of functional groups of bacteria, the DNA and antibody probes implied large variability within those groups. To reconcile the contradictions raised by the immunological and molecular data for nitrite reductase with the characterization of strains as closely related *Pseudomonas* species and strains, a study of the genetic diversity of culturable aquatic denitrifying bacteria based on variability of their ribosomal RNA genes, measured by restriction fragment length polymorphism (RFLP) analysis, was undertaken. Ribosomal RNA sequence information is a very powerful discriminant for bacterial phylogeny, and its use has imposed order on hitherto chaotic bacterial classifications (Woese 1987). Short of complete phylogenetic analysis, RFLP analysis of ribosomal genes can provide a basis for identification of groups within bacterial genera or species. Unlike genes encoding functional inducible enzymes such as nitrite reductase, the ribosomal genes are assumed to be stable. Thus, similarity patterns among ribosomal genes and other less stable genes might be expected to differ. In this paper, the similarity among denitrifying strains as estimated from RFLP of ribosomal genes is compared with that inferred from antibody and DNA homology studies of the NiR enzyme and gene. A simple polymerase chain reaction (PCR) assay is also used to differentiate strains according to homology of the NiR gene. The different methods exhibit different degrees of specificity and sensitivity and allow the comparison of interpretations derived from ribosomal relationships with those derived from the functional gene.

Previous research on denitrification enzymes has focused on denitrifiers classified in the genus *Pseudomonas* because the isolation studies cited above had identified *Pseudomonas* as a numerically important culturable genus in aquatic and sediment environments. Because most of our present knowledge about the biochemistry and regulation of the inducible denitrification pathway is based on pseudomonads, it is useful to investigate to what extent this knowledge is applicable to marine denitrifying bacteria whose phylogenetic affiliations are unknown. This study therefore included known denitrifying strains and recent isolates from marine, sediment, and lake environments. The present analysis is limited to experiments with culturable strains. This is a limitation in that we cannot estimate the extent to which the culturable strains represent the natural assemblage of denitrifiers. Evidence is presented, however, that even strains isolated using identical enrichment methods may represent considerable diversity of denitrifiers from seawater and other aquatic environments.

Materials and methods

Strains and isolates

Pseudomonas stutzeri (PST14405) was obtained from the ATCC and used as our primary strain throughout the investigation. It was maintained in CP medium (Carlucci and Pramer 1957) containing 1–10 g peptone/l of aged natural seawater. Several other strains were purchased from ATCC (Table 1) and maintained in LB medium (5 g yeast extract, 10 g tryptone, 10 g NaCl at pH 7 per liter distilled water). All strains were stored in 15 % glycerol at –70° C. *P. stutzeri* strains are referred to by the abbreviations used in Table 1.

For isolation of denitrifying strains from seawater and sediment, we used two standard enrichment media containing nitrate plus either a complex carbon source (i.e., peptone: CP medium amended with 1 mM KNO_3) or succinate (per liter of aged seawater: 10 g KNO_3, 0.05 g K_2HPO_4, 0.2 g $MgSO_4$ $7H_20$, 0.1 g $CaCl_2$ $2H_20$, 4 g Na succinate, 1 ml trace metals solution (Biebl and Pfennig 1978)). Samples of seawater (1 ml) or sediment (approximately 1 g) were inoculated into 10 ml of enrichment medium in screw-cap tubes containing inverted Durham tubes. Following growth, isolates were streaked to purity on CP plates. In order to encourage the growth of strains adapted to the natural environment, enrichments were maintained as closely as possible to the original ambient temperature until pure cultures were obtained (14–15° C for the Tomales Bay and SCB (Southern California Bight) isolates; 4° C for the Antarctic isolate). The isolates were then frozen until required for experiments. Subsequent growth of isolates for DNA extractions or screening took place at higher temperatures in order to obtain more rapid growth (approximately 25° C except for the Antarctic isolate, ELB17, which was grown at 12° C). Ability of isolates to denitrify was ascertained by sequential growth in CP medium on oxygen, then nitrate, then nitrite as electron acceptors. Ability to grow on NO_2^- (0.01 to 0.25% w/v) and detection of N_2O (by electron capture detection gas chromatography; see below) in the growth vessel were taken as evidence of ability to denitrify completely to nitrogen gas.

Table 1 List of strains and results of assays for nitrite reductase homology (*PCR amplification EUB* amplification with eubacterial universal primers, *PCR amplification NiR* amplification with NiR primers, *NiR ab reaction* detection of 66 kDa subunit with NiR antiserum in Western blot analysis of total cell protein from anaerobically grown cells, *NiR DNA hybridization* detection of hybridization with 721 bp NiR probe at 65° C in slot blots of total genomic preparation)

Strain (abbreviation)	PCR amplification		NiR ab reaction	NiR DNA hybridization	Source[a]
	EUB	NiR			
Denitrifying strains					
Pseudomonas stutzeri ATCC 14405 (PST14405)	+	+	+	+	a
MK202 (MK202)	+	+	+	+	b
P. stutzeri ATCC 11607 (PST11607)	+	+	+	+	a
P. stutzeri ATCC 17588 (PST17588)	+	+	+	+	a
P. stutzeri JM300 (PSTJM300)	+	+	+	+	c
P. aureofaciens ATCC 13985 (PAUREF)	+	–	–	–	d
P. denitrificans ATCC 13867 (PDENIT)	+	+	–	+	d
P. atlantica (PATL)	+	+	–	–	e
P. fluorescens ATCC 33512 (PFLUOR)	+	–	–	+	a
P. aeruginosa (PAER)	+	+	–	+	e
Alcaligenes faecalis ATCC 8750 (ALFAEC)	+	–	–	+	d
Paracoccus denitrificans ATCC 19367 (PARDENIT)	+	–	–	–	d
Thiosphaera pantotropha (THPANT)	+	–	–	–	d
Non-denitrifying comparison strain					
E. coli LE392 (ECOLI)	+	–	–	–	
Isolates from bottom water in Southern California Bight					
SCB-4	+	–	–	+	f
SCB-6	+	–	–	+	f
SCB-14	+	–	–	–	f
SCB-16	+	–	–	+	f
SCB-18	+	–	–	–	f
SCB-20	+	+	–	+	f
Isolates from intertidal sediments in Tomales Bay					
TBD-8B	+	–	+	+	g
TBD-8D	+	–	+	+	g
TBD-8Q	+	–	–	+	g
TBD-8H	+	–	+	+	g
TBD-8I	+	–	+	+	g
TBD-8M	+	–	–	+	g
TBD-8W	+	–	+	+	g
Isolate from Lake Bonney, Antarctica					
ELB 17	+	–	–	+	h

[a] a, ATCC; b, W. G. Zumft, Lehrstuhl für Mikrobiologie, Universität Karlsruhe, Karlsruhe, Germany; c, G. J. Stewart, Department of Biology, University of South Florida, Tampa, Fla., USA; d, D. Castignetti, Department of Biology, Loylola University, Chicago, Ill., USA; e, M. Pontius-Brewer, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, Calif., USA; f, sediment/water interface, 900 m, Santa Monica Basin; g, intertidal sediments, Tomales Bay, California; h, water column, permanently ice covered lake, Antarctica

Denitrifying strains from an intertidal marine sediment were obtained from Tomales Bay, California, a fault line inlet north of San Francisco. Samples were collected by syringe core from an area of mudflat/cyanobacterial mat near the seaward end of the bay. To investigate the diversity of isolates that could be obtained from a single enrichment sample from Tomales Bay microbial mats, inocula of the original enrichment (after 10 days' incubation in the enrichment tube) were spread onto CP plates containing 1 mM NO_3^- and incubated in a nitrogen atmosphere at the original ambient temperature of the mat (15° C). Twenty-five individual colonies from the spread plates were picked and transferred to multiwell plates containing CP-NO_3^- medium and allowed to grow under a nitrogen atmosphere. Small cores from the individual wells were removed with a Pasteur pipette and assayed for the presence of nitrite using the colorimetric assay of Bendschneider and Robinson (1952). When nitrite was detected, the colonies were streaked to purity by 3 passes on CP-NO_3^- plates. Pure isolates were transferred to gas-tight tubes containing liquid CP medium including 0.01 to 0.25% NO_2^- and monitored for growth and gas production. Presence of nitrous oxide in the headspace of the tube was verified by gas chromatography.

Marine isolates were obtained from sediment collected by box core from approximately 900 m depth in the Santa Monica Basin, a partially enclosed basin in the California borderland. A single strain was obtained from each sample, but all isolates originated in bottom sediments at the same station location. The Antarctic lake sample was obtained by Niskin bottle sampling through a hole in the 4-m-thick surface ice from Lake Bonney, a permanently ice-covered lake in the Taylor Valley, Antarctica.

Restriction fragment length polymorphism (RFLP) analysis

The plasmid pNO1301, containing the *rrn*b gene (one of the ribosomal RNA operons) from *Escherichia coli*, is a derivative of the plasmid pKK3535 (Brosius et al. 1981) and was a gift from Dr. Richard Gourse (University of Georgia). It was purified from 400-ml cultures of *E. coli* LE392 following standard protocols

(Ausubel et al. 1987). The 3.5 kb *Sac*II fragment was purified after digestion with *Sac*II by gene cleaning (Bio 101, San Diego, Calif., USA) the fragment from 1.75% low melting point agarose gels electrophoresed at 80 V for 2 h. The fragment was labelled by random priming with digoxygenin using the Genius (Boehringer Mannheim, Mannheim, Germany) protocol, purified from unincorporated nucleotides by gene cleaning (Bio 101) and stored at –20° C.

Genomic DNA was isolated from bacterial cultures following standard protocols (Ausubel et al. 1987). DNA concentration was estimated by absorption at 260 nm. Genomic DNA was digested with six restriction enzymes (*Hind*III, *Eco*RI, *Bgl*I, *Sac*II, *Sau*3A, *Hae*III) according to the protocols recommended by the manufacturer (Boehringer Mannheim). Digested DNA was electrophoresed at 80 V for 2 h in a Hoeffer mini-gel apparatus and transferred to Nytran (Schleicher and Schuell, Keene, NH, USA) or Immobilon P (Millipore, Marlborough, Mass., USA) membranes by Southern blotting (Southern 1975). Blots were hybridized to the labelled 3.5 kb *Sac*II pNO1301 fragment in 5–7 ml hybridization solution (0.3% SDS, 5×SSC (20×SSC = 175 g NaCl and 88 g Na-citrate l^{-1}), 3% powdered milk, 0.5 mg ml^{-1} sheared salmon sperm DNA) overnight at 65°C. Blots were washed and developed according to the Genius protocol using a colorimetric substrate for alkaline phosphatase.

Sizes of labelled fragments were determined by comparison with either *Hind*III digested lambda DNA or the 1 kb ladder (Bethesda Research Laboratories, GIBCO BRL, Gaithersburg, Md., USA) using linear regression. In order to assure comparability among replicate blots, a digest of *P. stutzeri* (ATCC 14405) was included in every set of blots. Each digest was repeated and analyzed by hybridization 3–4 times to reduce error and to provide an estimate of experimental variability among blots. Each digest usually produced 4–8 fragments (except *Sac*II), indicating the presence of several rRNA operons. More fragments were generally obtained with the 4 bp restriction enzymes, but the uncertainty increased with larger numbers of smaller fragments (i.e., small bands were not always reproducibly detected on the blots). The smallest fragment size reproducibly detected was 300 bp. Similarity indices based on the number of fragments of the same size that were shared between strains were computed using the equation $F = 2n_{xy}/(n_x + n_y)$ (Nei and Li 1979) where n_x is the total number of fragments in one strain, n_y the number of fragments in the second strain and n_{xy} the number of fragments appearing in both strains. Pairwise similarity indices were computed from the total number of bands from all digests observed for each isolate. The restriction sites were not mapped, so the possibility of overlapping fragments or sequence variation outside the rDNA gene has not been discounted. An estimate of the genetic distance (d, substitutions per site) was obtained from F as described by Navarro et al. (1992). When zero common bands were observed, d equals 1.0. Genetic distance values were used in a cluster analysis (SYSTAT; Euclidean distance, average linkage) to detect groupings among the entire suite of strains and isolates.

PCR amplification of NiR fragment

Using PCR primers previously described (Ward et al. 1993), strains and isolates were screened for amplification of a 721 bp region homologous with the NiR gene. The primers were designed to amplify a central region of the structural gene for nitrite reductase from *P. stutzeri* (PST14405) (Jungst et al. 1991) that was reported to be highly homologous with NiR from *P. aeruginosa* (Sylvestrini et al. 1989) and *P. stutzeri* PSTJM300 (Smith and Tiedje 1992). The fragment represents the section between 628 and 1349 bp

Table 2 Estimates of genetic distances from RFLP band patterns. *Lower half:* Number of bands used in calculation of similarity index *(F)*, leading to calculation of *d*. The number of restriction fragments shared by pairs of isolates *(before the slash)* and the number of total fragments observed for the two isolates in each pair *(after the slash)*. *Upper half:* Genetic distances (*d*, substitutions per site) for each pairwise strain comparison (calculated as described by Navarro et al. 1992). F (the fraction of shared fragments) = $2n_{xy}/$

	PST14405	MK202	PSTJM300	PST11607	PST17588	PFLUOR	PDENIT	PAUREF	PATL	PAER	THPANT	ECOLI
PST14405		0.021	0.136	0.121	0.084	0.127	0.105	0.107	0.207	0.124	0.144	0.116
MK202	32/41		0.139	0.102	0.057	0.090	0.108	0.071	1.000	0.094	0.147	0.188
PSTJM300	10/46	14/47		0.117	0.097	0.110	0.122	0.161	0.181	0.191	0.159	0.242
PST11607	8/30	10/31	6/34		0.085	0.122	0.091	0.146	1.000	0.117	0.142	0.200
PST17588	16/41	22/42	16/45	12/31		1.000	0.108	0.071	1.000	0.082	0.124	0.159
PFLUOR	12/48	18/49	16/52	10/38	24/49		0.109	0.059	0.228	0.110	0.141	0.174
PDENIT	10/32	10/33	10/36	8/22	10/33	12/40		0.124	0.252	0.152	0.099	0.136
PAUREF	12/39	18/40	8/43	6/29	18/40	24/47	8/31		0.204	0.066	0.119	0.154
PATL	4/35	0/36	6/39	0/25	0/36	4/43	2/27	4/34		0.277	0.200	0.173
PAER	10/39	14/40	6/43	8/29	16/40	14/47	6/31	18/38	2/34		0.119	0.226
THPANT	8/38	8/39	8/42	6/28	10/39	10/46	10/30	10/37	4/33	10/37		0.152
ECOLI	12/43	6/44	4/47	4/33	8/44	8/51	8/35	8/42	6/38	4/42	8/41	
PARDENIT	4/31	4/32	6/35	0/21	6/32	4/39	4/23	4/30	6/26	2/30	8/29	4/34
SCB4	16/34	12/35	4/38	6/24	10/35	10/42	8/26	10/33	2/29	6/33	4/32	8/37
SCB6	14/31	12/32	4/35	6/24	10/32	10/39	8/23	10/30	0/26	6/30	4/29	4/34
SCB14	14/45	14/46	8/49	4/35	12/46	18/53	4/37	12/44	4/40	8/44	8/43	12/48
SCB18	10/41	4/42	2/45	2/31	4/42	8/49	4/33	10/40	12/36	4/40	6/39	12/44
SCB20	10/42	4/43	8/46	4/32	4/43	8/50	8/34	6/41	10/37	6/41	12/40	10/45
TBD 8B	2/37	4/38	6/41	2/27	8/38	6/45	2/29	6/36	6/32	4/36	6/35	6/40
TBD 8H	2/40	4/41	8/44	0/30	6/41	4/48	2/32	8/39	6/35	6/39	6/38	6/43
TBD 8M	6/33	4/34	2/37	2/23	4/34	4/41	0/25	4/32	6/28	0/32	2/31	10/36
TBD 8Q	2/35	4/36	6/39	2/25	6/36	6/43	0/27	4/34	6/30	2/34	4/33	6/38
TBD 8W	0/36	2/37	4/40	0/26	4/37	2/44	0/28	2/35	6/31	2/35	4/34	6/39
ELB 17	2/34	0/35	0/38	2/24	2/35	4/42	2/26	2/33	2/29	2/33	4/32	2/37

from the N terminus of the gene sequence in *P. stutzeri* (PST14 405) (Jungst et al. 1991). We used Taq polymerase (Promega, Madison, Wisc., USA) with the manufacturer's buffer at a Mg^{++} concentration of 1.5 mM. Amplification (35 cycles of 1 min at 94° C, 1 min at 55° C, 2 min at 72° C) resulted in production of one approximately 700 bp fragment from *P. stutzeri* (PST14405).

Universal eubacterial rRNA primers (Liesack et al. 1991) were used as controls to verify the existence of amplifiable DNA in all the genomic DNA extracts tested for amplification with the NiR primers. These primers (5′ positions 9–27 and 3′ positions 1525–1542 in the *E. coli* sequence) amplified a region of approximately 1530 bp. Successful amplification with the eubacterial primers was obtained for every DNA preparation.

Antibody and DNA probes for nitrite reductase

A polyclonal antiserum produced in rabbits by immunizing with the purified nitrite reductase from *Pseudomonas stutzeri* (PST14 405) has been described previously (Ward et al. 1993). It was used to test denitrifying strains and isolates for homology with the *P. stutzeri* PST14405 NiR protein in Western blots of total cell lysates as previously described. Lysates were obtained from cells grown under denitrifying conditions and denitrification was verified as described above. The antibody recognized a 66 kd subunit in denaturing gels.

The DNA probe for a 721 bp fragment corresponding to a section of the NiR structural gene has also been described (Ward et al. 1993). This probe was used to screen total genomic extracts of denitrifying strains and isolates for homology using slot blots and the Genius detection system (see above). Hybridization when screening isolates was done at 65° C. Some of these data have been presented previously (Ward et al. 1993), but are included here for comparison with the RFLP data and PCR results.

Ancillary chemical measurements and sources of materials

Nitrite was assayed by the spectrophotometric method of Bendschneider and Robinson (1952) using a Hitachi double-beam spectrophotometer. Nitrous oxide was detected using a Shimadzu Mini-2 gas chromatograph equipped with an electron capture detector and a 2 m Poropak Q column run at an injection temperature of 300° C and a column temperature of 45° C. Gas standards were obtained from Scott Specialty Gases (San Bernardino, Calif.).

Results

The strains and isolates used in the analysis are listed in Table 1. Of the 28 strains, 24 were included in the RFLP analysis, while all 28 were screened with the antibody and DNA probes. The genetic distance estimates derived from the RFLP analysis for all pairwise comparisons are presented in Table 2. *P. stutzeri* PST14405 and MK202 were very similar, reflecting the fact that MK202 is a mutant of PST14405 (Körner et al. 1987). The other strains of *P. stutzeri*, PST11607 and PST17588, have distances of 12.1 and 8.4% respectively, from *P. stutzeri* PST14405, while PSTJM 300 is more distant at 13.6% from PST14

$(n_x + n_y)$ (Nei and Li 1979; see text). $d = -(2/r)\ln(\hat{G})$, where r is the number of bases in the restriction site and $\hat{G}$ is the probability that an original restriction site remains unchanged. $\hat{G}$ was computed from 10 iterations of the formula $\hat{G} = \{F(3 - 2G_1)\}^{1/4}$ using $\hat{G}$ for the first cycle as $F^{1/4}$ (Nei and Li 1979). Abbreviated strain names are given in Table 1

PARDENIT	SCB4	SCB6	SCB14	SCB18	SCB20	TBD 8B	TBD 8H	TBD 8M	TBD 8Q	TBD 8W	ELB 17	
0.194	0.066	0.070	0.106	0.129	0.132	0.286	0.295	0.159	0.280	1.000	0.277	PST14405
0.197	0.096	0.088	0.108	0.226	0.228	0.215	0.223	0.204	0.209	0.286	1.000	MK202
0.170	0.221	0.212	0.174	0.312	0.168	0.186	0.163	0.292	0.181	0.226	1.000	PSTJM300
1.000	0.127	0.114	0.207	0.267	0.197	0.252	1.000	0.235	0.244	1.000	0.240	PST11607
0.156	0.114	0.105	0.123	0.226	0.228	0.144	0.181	0.204	0.168	0.212	0.280	PST17588
0.218	0.132	0.124	0.097	0.170	0.172	0.191	0.240	0.223	0.186	0.305	0.226	PFLUOR
0.163	0.107	0.095	0.212	0.200	0.133	0.260	0.271	1.000	1.000	1.000	0.248	PDENIT
0.191	0.108	0.099	0.118	0.127	0.181	0.168	0.147	0.197	0.204	0.280	0.274	PAUREF
1.000	0.260	1.000	0.221	0.099	0.119	0.156	0.165	0.142	0.149	0.152	0.260	PATL
0.264	0.159	0.149	0.159	0.221	0.181	0.209	0.176	1.000	0.277	0.280	0.274	PAER
0.117	0.197	0.187	0.156	0.176	0.109	0.165	0.173	0.267	0.200	0.204	0.197	THPANT
0.204	0.141	0.204	0.127	0.118	0.139	0.178	0.186	0.117	0.173	0.176	0.286	ECOLI
	0.244	0.231	0.283	1.000	0.274	0.256	0.267	1.000	1.000	1.000	1.000	PARDENIT
2/25		0.011	0.176	0.136	0.283	0.194	0.204	0.139	0.146	0.191	1.000	SCB4
2/22	22/35		0.209	0.197	1.000	0.256	0.267	0.168	0.176	0.252	1.000	SCB6
2/36	6/39	4/36		0.108	0.125	0.226	0.233	0.144	0.221	0.298	1.000	SCB14
6/32	8/35	4/32	14/46		0.116	0.144	0.129	0.093	0.168	0.170	0.280	SCB18
2/33	2/36	0/33	12/47	12/43		0.176	0.183	1.000	0.212	0.215	0.283	SCB20
2/28	4/31	2/28	4/42	8/38	6/39		0.045	0.055	0.032	0.064	0.152	TBD 8B
2/31	4/34	2/31	4/45	10/41	6/42	22/37		0.076	0.069	0.072	0.277	TBD 8H
0/24	6/27	4/24	8/38	12/34	0/35	16/30	14/33		0.061	0.096	0.139	TBD 8M
2/26	6/29	4/26	4/40	6/36	4/37	22/32	16/35	14/28		0.029	0.146	TBD 8Q
0/27	4/30	2/27	2/41	6/37	7/38	16/33	16/36	10/29	22/31		0.191	TBD 8W
2/25	0/28	0/25	0/39	2/35	2/36	6/31	2/34	6/27	6/29	4/30		ELB 17

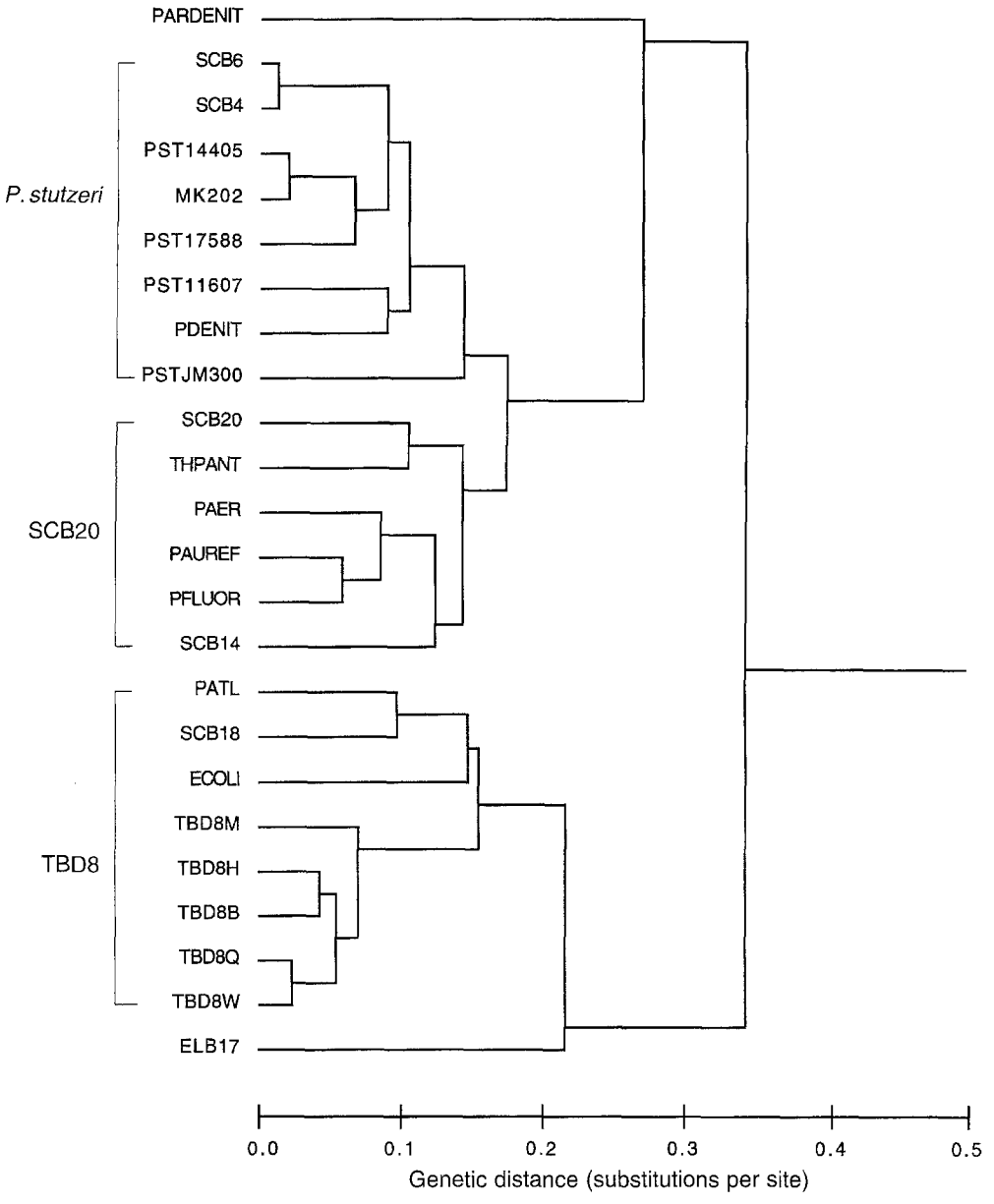

Fig. 1 Cluster analysis on the basis of genetic distance values in Table 2. Abbreviated strain names are given in Table 1

405. Relative to the five *P. stutzeri* strains, *P. aeruginosa* was most different from *P. stutzeri* PSTJM300 and closer to the other *P. stutzeri* strains than was PSTJM300.

A very small genetic distance was found between SCB-4 and SCB-6. These two isolates had *d* values with *P. stutzeri* PST14405 similar to those between the various strains of *P. stutzeri* and were as close to the other *Pseudomonas* strains as these *Pseudomonas* strains are to the *P. stutzeri* strains. The other four SCB isolates had intermediate similarities compared to the collection of pseudomonads. The Tomales Bay sediment strains were not very close to the *P. stutzeri* strains, but appeared most closely related to *P. atlantica*, which was quite different from the other *Pseudomonas* strains. The isolate from the Antarctic lake does not appear to be very similar to any of the known strains.

When the *d* values were used in cluster analysis to obtain a dendrogram (Fig. 1), these relationships were illustrated in the formation of three main clusters, two of which form a large supercluster. The clusters are referred to for convenience by the names *P. stutzeri*, TBD8 and SCB-20. The *P. stutzeri* cluster contains all the *P. stutzeri* strains analyzed and, in addition, contains *P. denitrificans* and two SCB isolates. *P. stutzeri* PSTJM300 fell at the periphery of the *P. stutzeri* cluster with the deepest branch. The SCB-20 cluster included the remaining pseudomonads, two of the SCB isolates and *Thiosphaera pantotropha*. The TBD8 cluster includes a tight subgroup of only the Tomales Bay sediment isolates and, at greater distances, *E. coli*, *P. atlantica*, and one SCB isolate. *Paracoccus denitrificans* and the Antarctic lake isolate, ELB17, fell at the periphery of the main clusters.

The PCR primers designed from the NiR gene of *P. stutzeri* (ATCC 14405) amplified several of the *Pseudomonas* strains, but only one of the unidentified isolates

(Table 1). All of the *P. stutzeri* strains were successfully amplified, as were two other Pseudomonads. Six of the strains that amplified with the NiR primers fell into the *P. stutzeri* cluster, two were in the SCB-20 cluster and one, *P. atlantica,* was in the TBD8 cluster. Within the *P. stutzeri* cluster, only two strains did not amplify.

The only strains in which homologous proteins were detected by Western blot were *P. stutzeri;* many fewer strains and isolates showed cross-reactivity with the NiR antiserum than possessed homology with the NiR gene fragment. Most of the known strains and most of the isolates in Table 1 apparently possess homology with the 721 bp NiR gene probe. All clusters include members that hybridized with the NiR gene probe. *P. aureofaciens* and *Th. pantotropha* possess a non cd_1 heme type NiR and are not expected to have homology with this NiR probe (Zumft et al. 1987).

Discussion

Nonidentity of strains called *P. stutzeri*

The similarity indices (and genetic distance estimates) derived from RFLP analysis of ribosomal sequences are in agreement with previous reports on the heterogeneity of the *P. stutzeri* species group (Palleroni et al. 1970; Rosello et al. 1991). For example, Rosello et al. (1991, 1993) used a suite of genotypic and phenotypic characteristics to identify genomevars (subspecies level groups) of *P. stutzeri;* they found that PST14405, PST17588 and PST11607 each fell into a different genomevar. When *P. perfectomarina* PST14405 was reclassified as a *P. stutzeri*, Döhler et al. (1987) reported that *P. stutzeri* PST14405 had less than 60% DNA homology with *P. stutzeri* PST17588. In the present analysis, *P. stutzeri* JM300 fell at the periphery of the *P. stutzeri* cluster. The other two ATCC strains, *P. stutzeri* PST17588 and PST11607, clustered with the primary strain, PST14405, but were nearly as similar to other *Pseudomonas* strains. Thus, although the use of more or different restriction enzymes might change the absolute values of similarity and distance computed here, the consistency with previous reports and with other comparisons in the present analysis imply at the very least that the currently named *P. stutzeri* group is not homogeneous.

Diversity of aquatic environmental isolates

The inclusion of recently isolated, unidentified marine denitrifiers in the RFLP analysis provides information on the diversity of these isolates and their similarity to known strains. For example, it can be seen that SCB-4 and SCB-6 are more similar to each other than are the two *P. stutzeri* strains PST11607 and PST17588. These two SCB strains fall within the *P. stutzeri* cluster and are closer to PST14405 than any of the other *P. stutzeri* strains. Although purified NiR from these two strains did not react with our NiR antiserum, their DNA did hybridize with the 721 bp probe previously described (Ward et al. 1993), and it seems likely that they are in fact *P. stutzeri* isolates.

Three of the SCB isolates fell outside the *P. stutzeri* cluster and did not cluster together, indicating that diverse denitrifying strains can be isolated from similar habitats using the same enrichment medium. The SCB isolates were all obtained from the same site, but were collected in different samples at different times over a period of a week. The Tomales Bay sediment isolates represent a different situation: they all clustered together, but were not closely related to any other strains. The five TBD isolates included in the RFLP analysis were all isolated from the same 1-g sample of surface sediment. Their morphology, determined from fluorescence microscopy with DAPI staining (data not shown), divides them into two groups: small straight rods and highly curved rods. While they cluster together, they are clearly not identical. These findings indicate that the culturable assemblage of aquatic denitrifiers is diverse both within and among habitats, and it seems likely that the natural population is even more diverse.

In a similar study, Navarro et al. (1992) investigated the relatedness of nitrifying bacterial strains isolated from aquatic and soil habitats. Nitrifiers are a very restricted group in that relatively few nitrifying species are known, and the ability to nitrify is usually associated with nearly obligate autotrophy, which constrains the entire metabolic makeup of the organism. By contrast, the ability to denitrify is widespread within the Eubacterial kingdom and involves a small suite of inducible enzymes that are not dependent upon the other metabolic characteristics of the cell. Not surprisingly, the diversity observed among dentrifying strains by RFLP analysis exceeded the diversity observed among isolates of nitrite oxidizing bacteria. Navarro et al. (1992) reported that essentially identical isolates of nitrite oxidizers could be obtained from different locations, while the magnitude of within-location diversity varied on the same scale as between-location diversity. The greatest genetic distances observed by Navarro et al. (1992) were on the order of 8%, in contrast to the result that some of the denitrifiers tested here did not share any restriction fragments in common. The clustering of nitrite oxidizers obtained from RFLP analysis did not always coincide with serogrouping (Navarro et al. 1992), whereas for denitrifiers, cell surface antibodies are frequently strain specific.

NiR antibody specificity in relation to the RFLP clusters

Efforts to produce an antibody to nitrite reductase that would be broadly reactive with all or most of the cd_1 heme type nitrite reductase proteins have failed: antisera produced in response to immunization with purified NiR from various strains of *P. stutzeri* are unexpectedly specific, reacting only with the immunizing strain and a very limited suite of other known strains (Körner et al. 1987;

Ward et al. 1993). In the antibody tests reported here, only *P. stutzeri* strains produced proteins that reacted with the NiR antibody, and not even strains which clustered closely to *P. stutzeri* PST14405 reacted with the antibody (e.g., SCB-4 and SCB-6). The ecological implications of these findings are that strains and enzymes that are biochemically quite similar may in fact not be very similar, at least in immunological terms, and antibody probes are generally too specific to be of use in identifying unknown denitrifiers as a functional group.

Although the antibody reactions are noted as simply presence/absence in Table 1, we have previously reported that the immunological reactions between the NiR antiserum produced from NiR purified from *P. stutzeri* PST14405 and NiR purified from *P. stutzeri* PST11607, PST17588 and PSTJM300 was very weak compared to the homologous reaction. In the case of PSTJM300, the antibody did not inhibit activity of the purified enzyme (Ward et al. 1993). It was concluded that the most strongly antigenic determinants in the nitrite reductase protein were not shared among even closely related strains. Because NiR is an inducible enzyme and thus subject to short-term selective effects, the sequence of this gene might not necessarily reflect evolutionary relationships of bacterial strains. The RFLP analysis of ribosomal genes indicates that this is indeed the case: PSTJM300 is classified as a *P. stutzeri* strain on the basis of its classically defined physiology, but it clusters only loosely with other *P. stutzeri* strains. Two of the SCB isolates show very high similarities with *P. stutzeri* (PST14405), but their nitrite reductases are apparently not immunologically very similar.

Comparison between NiR probes (amplification vs hybridization) and between NiR probes and RFLP analysis

Fewer isolates amplified with the NiR primers than hybridized to the 721 bp NiR probe. Only in one case, *P. atlantica*, was amplification obtained but hybridization not detected. More commonly, amplification was not successful, but hybridization was detected. Successful amplification depends on a rather high degree of homology in two 20-bp regions, while hybridization could be detected if any moderately sized fraction of the 721 bp region possessed sufficient homology, suggesting that amplification could be a more specific test than hybridization. The primers were designed from the three NiR sequences then available, and it is not known whether the primer regions are conserved among other strains that possess the cd_1 heme type NiR gene.

In the case of the Tomales Bay sediment isolates, no amplification with the NiR primers was obtained, although both antibody and DNA probes indicated that these strains possess the cd_1 heme type enzyme with analogy to the *P. stutzeri* PST14405 enzyme. We have previously reported that *P. stutzeri* PST14405 was not very abundant in this sediment environment (enumerated by species-specific immunofluorescence, Ward and Cockcroft 1993) and show here that commonly isolated denitrifiers from this environment are genetically not very similar to *P. stutzeri* PST14405. However, these isolates do possess denitrification enzymes very similar to the NiR enzyme of *P. stutzeri* PST14405, which means that gene and antibody probes derived from cultured strains may be useful in natural systems. Similarly, Coyne et al. (1989) have reported that a large number of unidentified soil isolates reacted with a NiR antiserum for the cd_1 heme type enzyme of *P. aeruginosa* or *P. stutzeri* (PSTJM300). Although not broadly function specific, the gene and antibody probes should be useful in studying genetic potential and gene expression, respectively, in some denitrifiers.

Denitrifying isolates that hybridize with the 721 bp probe or amplify with the NiR primers are scattered throughout all clusters in the RFLP analysis. Some of the diversity in reactivity with the NiR antibody and DNA probes may be explained by lack of relatedness in the strains, but obviously the distribution of NiR homology and the similarity groupings implied by cluster analysis of the RFLP data do not correspond exactly. This difference may be due to the different nature of the genes being investigated. Genes that encode inducible enzymes are subject to selective pressures that are different than the ribosomal genes and are likely to be much more variable and have different evolutionary histories. Denamur et al. (1993) found that allozyme patterns and rRNA RFLP patterns for *Pseudomonas aeruginosa* were uncorrelated, presumably due to the different history of evolution and horizontal gene transfer for the two kinds of genes represented by the two analyses.

These findings have implications for both phylogenetic/evolutionary studies of bacterial relationships and for investigation of bacterially mediated transformations in the environment. Most apparent, relationships deduced from ribosomal genes are not necessarily the same as those between functional genes in the same organisms. The probe and antibody methods used here detected only presence/absence of the NiR gene and gene product. Further analysis of the gene sequences would make it possible to compare genetic similarities between functional and ribosomal genes. Within the suite of organisms called *P. stutzeri*, the RFLP data imply rather large differences among strains identified by phenotypic characters as the same strain. From the specificity and lack of homology demonstrated by the antibody and DNA probes for NiR, it is evident that the functional gene, nitrite reductase, is variable enough to provide a basis for species-specific identification of functional genes and enzymes, but that to define a broadly general probe for the function will require a different approach.

Acknowledgements A. R. Cockcroft did the Western blots for NiR homology screening. A. R. Cockcroft and M. T. Geissler provided technical assistance. This research was supported by NSF and ONR grants to B. B. W.

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Sideman JG, Struhl K (eds) (1987) Current protocols in molecular biology. Wiley, New York

Baumann L, Baumann P, Mandel M, Allen RD (1972) Taxonomy of aerobic marine eubacteria. J Bacteriol 110:402–429

Bendschneider K, Robinson RJ (1952) A new spectrophotometric method for the determination of nitrite in seawater. J Marine Res 11:87–96

Biebl H, Pfennig N (1978) Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch Microbiol 117:9–16

Brosius J, Ullrich A, Raker MA, Gray A, Dull TJ, Gutell RR, Noller HF (1981) Construction and fine mapping of recombinant plasmids containing the *rrn*B ribosomal RNA operon of *E. coli*. Plasmid 6:112–118

Bryan BA (1981) Physiology and biochemistry of denitrification. In: Delwhich CC (ed) Denitrification, nitrification and atmospheric nitrous oxide. Wiley, New York, pp 67–84

Carlucci AF, Pramer D (1957) Factors influencing the plate method for determining abundance of bacteria in sea water. Proc Soc Exp Biol Med 96:392–394

Coyne MS, Arunakumari A, Averill BA, Tiedje JM (1989) Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductases in denitrifying bacteria. Appl Environ Microbiol 55:2924–2931

Denamur E, Picard B, Decoux G, Denis J-B, Elion J (1993) The absence of correlation between allozyme and *rrn* RFLP analysis indicates a high gene flow rate within human clinical *Pseudomonas aeruginosa* isolates. FEMS Microbiol Lett 110: 271–280

Döhler K, Huss VAR, Zumft WG (1987) Transfer of *Pseudomonas perfectomarina* Baumann, Bowditch, Baumann, and Beaman 1983 to *Pseudomonas stutzeri* (Lehmann and Neumann 1896) Sijderius 1946. Int J Syst Bacteriol 37:1–3

Gamble N, Betlach M, Tiedje JM (1977) Numerically dominant denitrifying bacteria from world soils. Appl Environ Microbiol 33:926–939

Jungst A, Wakabayashi S, Matsubara H, Zumft WG (1991) The *nir*STBM region coding for cytochrome cd_1 dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di- and tetraheme proteins. FEBS Letters 279, 205–209

Körner H, Frunzke K, Dohler K, Zumft WG (1987) Immunochemical patterns of distribution of nitrous oxide reductase and nitrite reductase (cytochrome cd_1) among denitrifying pseudomonads. Arch Microbiol 148:21–24

Liesack W, Weyland H, Stackebrandt E (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microbial Ecol 21:191–198

Navarro E, Simonet P, Normand P, Bardin R (1992) Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. Arch Microbiol 157:107–115

Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Palleroni NJ, Doudoroff M, Stanier RY, Solanes RE, Mandel M (1970) Taxonomy of the aerobic Pseudomonads: the properties of the *Pseudomonas stutzeri* group. J Gen Microbiol 60: 215–231

Rosello R, Garcia-Valdes E, Lalucat J, Ursing J (1991) Genotypic and phenotypic diversity of *Pseudomonas stutzeri*. System Appl Microbiol 14:150–157

Rosello R, Garcia-Valdes E, Lalucat J, Ursing J (1993) Taxonomic relationship between *Pseudomonas perfectomarina* ZoBell and *Pseudomonas stutzeri*. Int J Syst Bacteriol 43:852–854

Smith GB, Tiedje JM (1992) Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl Environ Microbiol 58:376–384

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Sylvestrini MC, Galeotti CL, Gervais M, Schinina E, Barra D, Bossa F, Brunori M (1989) Nitrite reductase from *Pseudonomas aeruginosa:* sequence of the gene and the protein. FEBS Lett 254:33–38

Ward BB, Cockcroft AR (1993) Immunofluorescence detection of denitrifying bacteria in seawater and intertidal sediment environments. Microbial Ecol 25:233–246

Ward BB, Cockcroft AR, Kilpatrick KA (1993) Antibody and DNA probes for detection of nitrite reductase in seawater. J Gen Microbiol 139:2285–2293

Woese CJ (1987) Bacterial evolution. Microbiol Rev 51:221–271

Zumft WG (1992) The denitrifying prokaryotes. In: Albert Ballows et al. (eds) The prokaryotes, 2nd edn. Springer, New York, pp 554–582

Zumft WG, Gotzmann DJ, Kroneck PMH (1987) Type 1, blue copper proteins constitute a respiratory nitrite-reducing system in *Pseudonomas aureofaciens*. Eur J Biochem 168:301–307