Nitrite reductase genes in halobenzoate degrading denitrifying bacteria

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Abstract

Diversity of the functional genes encoding dissimilatory nitrite reductase was investigated for the first time in denitrifying halobenzoate degrading bacteria and in two 4-chlorobenzoate degrading denitrifying consortia. Nitrite reductase genes were PCR-amplified with degenerate primers (specific to the two different types of respiratory nitrite reductase, nirS and nirK), cloned and sequenced to determine which type of nitrite reductase was present in each isolate and consortium. Halobenzoate degrading isolates belonging to the genera Ochrobactrum, Ensifer and Mesorhizobium, as well as Pseudomonas mendocina CH91 were found to have nirK genes, which were closely related to the previously published nirK genes of Ochrobactrum anthropi, Achromobacter cycloclastes, Alcaligenes faecalis and Pseudomonas aureofaciens, respectively. The isolates assigned to the genera Acidovorax, Azoarcus and Thauera as well as all other species in the genera Thauera and Azoarcus contained nirS genes, which were closely related to the nirS genes from Pseudomonas stutzeri with some exceptions. In addition, only nirS genes were found in 4-chlorobenzoate degrading denitrifying consortia. Three different major terminal restriction fragments from the nirS genes were detected by terminal restriction fragment length polymorphism analysis of the consortia, and five different nirS genes were cloned from one consortium. Three nirS gene clones were closely related to nirS genes from Thauera chlorobenzoica, Azoarcus tolulyticus and Pseudomonas aeruginosa, respectively. The phylogeny of nir genes was not entirely congruent with the 16S rRNA phylogeny of the genera nor was it correlated with the ecological and geographical origins or isolation substrates used for isolation and enrichment of consortia.

1. Introduction

Denitrification is defined as dissimilatory transformation of nitrate or nitrite to gas concomitant with energy conservation. This process has been thoroughly examined because of its importance in the global nitrogen cycle, the production of greenhouse gases and the removal of contaminants in the environment. Several enzymes are involved in the complete denitrification process. Nitrate is converted to dinitrogen through the sequential action of the enzymes nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (for reviews see [1-3]).

Nitrite reduction is ecologically important for several reasons. In the environment, nitrite reduction can remove toxic nitrite and prevent the production of carcinogens generated by nitrite and amines. In bacterial metabolism, nitrite reduction is the key process in denitrification, which converts nitrite to the gaseous product, nitric oxide. This reaction serves a respiratory function in denitrifying bacteria and is an effective process for removal of nitrogen from the pool of fixed nitrogen, making it unavailable for most other organisms. Nitrite reductase, the enzyme that catalyzes this process, is located in the periplasmic space [2,3] and occurs in two major types: cytochrome cd1-type nitrite reductase (NirS) and Cu-type nitrite reductase (NirK). Both appear to perform the same physiological reaction producing NO from NO3- (for reviews see [1,2]).

The capability for dissimilatory nitrite reduction cannot be easily detected by relying on taxonomic affinities identified by 16S rRNA gene probes because nitrite reducing bacteria are distributed in many genera of the prokaryotes [4] and many of these genera also contain non-denitrifying
strains. Using functional genes is an alternative approach to investigate denitrifying organisms in the environment. Nitrite reductase genes have been successfully detected by gene probe analysis and direct polymerase chain reaction (PCR) amplification with gene-specific primers [5–12]. More than 200 complete or partial sequences of nitrite reductase genes from pure cultures and environmental samples have already been deposited in the GenBank database.

Removal of halogenated aromatic contaminants has also been linked to denitrification [13–15] and halobenzoate degrading denitrifying bacteria have been isolated from various geographic sites with differing ecological site characteristics [16]. Taxonomic diversity and functional diversity of denitrifying halobenzoate degrading bacteria were described previously from studies in pure cultures and bacterial consortia [16–18]. The isolates were classified into nine genera belonging to the α-, β-, and γ-subdivisions of the Proteobacteria [16]. The isolates belonging in the genera Azorarcus and Thauera were further characterized in terms of their taxonomic and metabolic diversity related to aromatic compound degradation [17]. Denitrifying bacterial consortia capable of degrading 4-chlorobenzoate were characterized with terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes and the nucleotide sequence responsible for each terminal restriction fragment (T-RF) was determined by 16S rRNA gene cloning and sequencing [18]. Two major populations, one assigned to the genus Thauera, and another related to the genera Raistonia and Limnobacter, were associated with 4-chlorobenzoate degradation under denitrifying conditions [18]. However, the genetic diversity of functional genes in these halobenzoate degrading denitrifying bacteria and their relationship to other denitrifying groups has not been investigated previously. The genes encoding nitrite reductase were thus investigated in this study using direct PCR amplification methods with gene-specific primers for the first time to determine the type of nitrite reductase present and to examine the diversity of nitrite reductase genes in halobenzoate degrading denitrifying bacteria and some related species.

### 2. Materials and methods

#### 2.1. Bacterial strains and growth conditions

Twenty halobenzoate degrading denitrifying strains and 15 additional denitrifying strains in the Proteobacteria, as well as two denitrifying bacterial consortia capable of degrading 4-chlorobenzoate were examined in this study (Table 1). All the strains in the genus Thauera were cultivated anaerobically in a minimal salts medium [19] with succinate as a carbon source and nitrate as an electron acceptor, with the exception of Thauera mechenichensis TL1T (DSM 12266), which was cultured aerobically in Luria–Bertani broth. All other strains were grown on M-R2A medium as described previously [20]. Stable bacterial consortia were obtained as described previously [18]. All the cultures growing in denitrifying liquid media [19] were monitored periodically for the loss of nitrate and nitrite using an ion chromatography system ( Dionex DX-100, Sunnyvale, CA, USA) with conductivity detector as previously described [15].

#### 2.2. Genomic bacterial DNA isolation

Chromosomal DNA of the strains in the genera Azorarcus and Thauera was isolated as previously described [21]. DNA from other strains and bacterial consortia was extracted using a modified phenol:chloroform method [22]. The purity of the DNA was determined by measuring absorbance at 230, 260 and 280 nm.

#### 2.3. PCR amplification of nitrite reductase genes

The primers for nitrite reductase genes (nirS and nirK) have been described previously [11,12,23]. For direct PCR amplification of the Cu-type nitrite reductase gene (nirK), primers Cunir3–4 were used with a touchdown PCR program as described previously [12]. Three different sets of primers were used for PCR amplification of cytochrome cd1-type nitrite reductase gene (nirS). Primers Nir1–2, specific for a central region (721 bp) of the nirS gene of Pseudomonas stutzeri [11], and degenerate primers nirS1F and nirS6R [7] were used for nirS gene amplification from halobenzoate degrading isolates and bacterial consortia following the previously described conditions [7,11]. In addition, degenerate primers Nir3–4 (Nir3-AAYGT-NAARGARACBGG and Nir4-ACRTTRAAYTTNCC-NGT) designed by Kriststein and Ward [23] were used to amplify the 3’ end sequences of nirS genes (730 bp). PCR amplification was performed with Nir3–4 primers in a total volume of 50 μl containing 5 μl of 10× PCR buffer (500 mM KCl, 200 mM Tris–HCl [pH 8.4]), 1.5 mM MgCl2, 20 μM of each deoxyribonucleoside triphosphate, 1 μM of each primer, 1 U Taq polymerase, and ~ 100 ng of genomic DNA. The PCR cycle was started with a 5 min denaturation step at 95°C, followed by 30 cycles of denaturation of 30 s at 95°C, primer annealing for 2 min at 47°C, and followed by 2 min extension at 72°C. The amplified products were examined on 1.0% agarose gels by electrophoresis and then were purified using a Qiagen, Bothell, WA, USA) gel extraction kit (Qiagen, Bothell, WA, USA) according to the manufacturer’s instructions.

#### 2.4. Cloning and sequencing PCR products

The cleaned PCR products were used for direct cloning with the TOPO-TA® cloning system (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instruction. The ligated plasmids were transformed in high transform-
ing efficiency *Escherichia coli* TOP10F™ (Invitrogen) following the manufacturer’s instructions. The transformed cells were plated on Luria–Bertani agar plates containing 50 μg ml⁻¹ kanamycin. One strand of the inserts was initially sequenced with the primers M13 reverse or T7 using Big-dye® terminator chemistry (Applied Biosystems, Foster City, CA, USA) and ABI model 310 automated DNA sequencer (Applied Biosystems). In addition, the inserts were sequenced again with the PCR primers for nirS or nirK genes.

2.5. Phylogenetic analysis of nitrite reductase genes

The amino acid sequences of the nirS and nirK genes in the reference species were obtained from the GenBank database. The GenBank accession numbers of reference nirS and nirK genes used in this study are given in Figs. 1 and 2. The partial amino acid sequences of NirS (240 amino acids) and NirK (140 amino acids) were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/). The phylogenetic analyses were performed with the PHYLIP 3.5 program [24] according to the Dayhoff PAM matrix method [25]. Phylogenetic trees were reconstructed using the neighbor-joining method [26]. The SEQBOOT program was used to obtain the confidence level for neighbor-joining analysis using 100 bootstrapped data sets [27].

2.6. T-RFLP analysis of nitrite reductase genes

For T-RFLP analysis, primer nirS1F 5’ end-labeled with 6-FAM (5-[6]-carboxy-fluorescein, Operon Technology, Alameda, CA, USA) and unlabeled nirS6R primers were used to amplify nirS genes from the extracted DNA samples of bacterial consortia. PCR products were purified using a Qiaquick® gel extraction kit (Qiagen). The concentration of purified PCR products were determined by image analysis of the agarose gel and quantified by comparison to the concentration of standards. The purified PCR products (40 ng) were digested with 1 U of MspI restriction endonuclease (New England Biolabs, Beverly, MA, USA) for 6 h at 37°C. The digested samples were analyzed on an ABI 310 automated sequencer. The sizes of fragments were compared with internal standards and determined by the GeneScan software (Applied Biosystems).

3. Results

3.1. PCR amplification of nirS and nirK genes

Twenty halobenzoate degrading denitrifying isolates and 15 additional denitrifying bacterial strains were examined for the presence of nir genes with specific primers for amplification of nirS and nirK genes. Twenty-seven strains yielded the amplified nir gene products (Table 1) although all 35 strains were capable of nitrite reduction, which was verified by the loss of nitrite during anaerobic growth (data not shown). Complete denitrification was not assayed, but disappearance of nitrite from the medium was taken as evidence for the capability of nitrite reduction. nirK genes were found in eight isolates of the genera *Ochrobactrum*, *Ensifer* and *Mesorhizobium*, as well as *Pseudomonas mendocina* CH91. nirS genes were successfully amplified from seven halobenzoate degrading isolates and 11 additional strains of the genera *Acidovorax*, *Azoarcus* and *Thauera* with the various nirS primer sets (Nir1–2, Nir3–4 and NirS1F–6R). Interestingly, nirS genes could be amplified from most of the *Thauera* strains (except *Thauera terpenica* and *Thauera mechinichensis*) and *Azoarcus evansii* with the Nir1–2 primers, even though the Nir1–2 primers were designed with 100% identity to the nirS gene of *P. stutzeri*. The nirS genes were also amplified with the degenerate Nir3–4 and NirS1F–6R primers except from *Thauera aromatica* AR-1, which was amplified only with the Nir1–2 primers. In addition, two denitrifying bacterial consortia were determined to contain nirS genes by successful PCR amplification with the NirS1F–6R primers, but nirK genes were not detected in the consortia by PCR amplification with NirS1–3 primers.

3.2. Phylogenetic analysis of Cu-type nitrite reductase sequences (nirK) in halobenzoate degrading denitrifying isolates

The nirK gene fragments obtained by PCR amplification with the Cinir3–4 primers were sequenced and translated to amino acid sequences for phylogenetic analysis. The NirK sequences from halobenzoate degrading isolates in the genus *Ochrobactrum* clustered with the NirK sequence of the type strain of *Ochrobactrum anthropi* (Fig. 1), which was consistent with the 16S rRNA gene phylogeny [16]. The NirK sequence of *Mesorhizobium* sp. 4FB11 was more closely related to the NirK sequences of *Alcaligenes faecalis* ATCC 8750 and *Nitrosomonas* sp. TA-921i-NH4 than those of *Rhizobium, Sinorhizobium* and *Bradyrhizobium* strains (Fig. 1), although strain 4FB11 was closely related to the genera *Bradyrhizobium* and *Sinorhizobium* on the basis of 16S rRNA sequence analysis [16]. The NirK sequences from halobenzoate degrading isolates in the genus *Ensifer* had more than 98.5% identity to NirK sequences from *Achromobacter cycloclastes* (Fig. 1). *P. mendocina* CH91 had a NirK, which is closely related to those of *Pseudomonas aureofaciens*, *Achromobacter xylosoxidans* and *Alcaligenes* spp. with about 81% identities (Fig. 1).

3.3. Phylogenetic analysis of cytochrome cd₁-type nitrite reductase sequences (nirS) in denitrifying isolates

The nirS gene fragments amplified with the Nir1–2,
Nir3–4 or NirS1F–6R primers were sequenced and translated to amino acid sequences to compare phylogenies. Phylogenetic analysis of the NirS sequences obtained with the Nir1–2 primers showed that all the NirS sequences from the *Thauera* strains and *A. evansii* were closely related to the NirS sequence of *P. stutzeri* with more than 80% identity, as expected based on the specificity of the primers (data not shown).

The translated amino acid sequences of the *nirS* genes obtained with the Nir3–4 and NirS1F–6R primers were used here to illustrate the phylogenetic relationships among the strains because most of the *nirS* genes from the genera *Acidovorax*, *Azoarcus* and *Thauera* were amplified with the Nir3–4 and NirS1F–6R primers and because more NirS sequences covering this region are available in the GenBank database. The NirS sequences in all the *Acidovorax*, *Azoarcus* and *Thauera* strains, except *Azoarcus* toluutorans and *T. mechernichensis*, clustered with the NirS sequences of *P. stutzeri* and *A. faecalis* with more than 85% amino acid sequence identities (Fig. 2).

### Table 1

<table>
<thead>
<tr>
<th>Genus and species</th>
<th>Strain</th>
<th>PCR amplification</th>
<th>Ecological source</th>
<th>Geographical source</th>
<th>GenBank number of nir gene</th>
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<td></td>
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<td></td>
<td>2FB6</td>
<td>+ + +</td>
<td>Aquifer sediment</td>
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<tr>
<td></td>
<td>4FB10</td>
<td>+ + +</td>
<td>Aquifer sediment</td>
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<tr>
<td><em>Thauera</em> terpenica</td>
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<td>Estuarine sediment</td>
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<tr>
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<td>River sediment</td>
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</tbody>
</table>

### α subdivision of the Proteobacteria

| *Ochrobactrum* sp. | 3CB4 | + + + | Estuarine sediment | Arthur Kill, New Jersey | AY078250 |
|                   | 3CB5 | + + + | Agricultural soil | Wyoming | AY078251 |
|                   | 2FB10| + + + | Agricultural soil | Väiki, Finland | AY078249 |
|                   | 4FB13| + + + | Agricultural soil | Väiki, Finland | AY078252 |
|                   | 4FB14| + + + | River sediment | Kyungan, Korea | AY078253 |
| *Ensifer* sp. | 2FB8  | + + + | River sediment | Kyungan, Korea | AY078247 |
|                   | 4FB6  | + + + | Agricultural soil | Wyoming | AY078248 |
| *Mesorhizobium* sp. | 4FB11| + + + | Marine sediment | Hopewell Rock, Canada | AY078254 |
|                   | 2FB3  | + + + | Pond sediment | New Brunswick, New Jersey | n/a |

### γ subdivision of the Proteobacteria

| *Pseudomonas* mendocina | ATCC 25411T | + + + | Estuarine sediment | Arthur Kill, New Jersey | AY078274 |
|                         | CH91   | + + + | Agricultural soil | Mendoca, Argentina | AY078255 |

### Bacterial consortium

| 4-chlorobenzoate degrading denitrifying consortium cultures | + + + | Estuarine sediment | Arthur Kill, New Jersey | AY078274 |

+: amplified and verified by sequencing; −: no amplification; n/a: not available.
ever, the NirS sequences from *A. tolulyticus* Tol4T and *T. mechnichensis* TL1T clustered together regardless of their taxonomy and were distantly related to the NirS sequences of *Pseudomonas fluorescens* and *P. aeruginosa*. The NirS sequences of these two species shared 97.8% amino acid sequence identity and about 66% identity to the NirS sequences from *P. fluorescens* and *P. aeruginosa*, respectively. Interestingly, the NirS sequence from *A. tolulyticus* 2FB6 did not cluster with that of *A. tolulyticus* Tol4T, although they were designated as the same species. The NirS sequences from *T. aromatica*, *Thauer chlorobenzoica* and *T. terpenica* clustered according to the taxonomic classification of the strains [17]. The NirS sequences from the species *Azovarcus toluovorans* and *A. evansi* were grouped together and separated from the cluster of *Thauer* NirS sequences, although NirS sequences from two genera share about 86% identity. However, the NirS sequence from *Acidovorax* sp. 2FB7 was closely related to those of *T. chlorobenzoica* regardless of their taxonomic difference.

### 3.4. Diversity of nirS genes in bacterial consortium cultures

Diversity of nirS genes in two different bacterial consortia capable of degrading 4-chlorobenzoate under denitrifying conditions was examined with T-RFLP analysis and cloning of nirS genes from PCR-amplified products using the NirS1F–6R primers. T-RFLP analysis detected relatively few peaks in both consortia, but showed that the consortium culture established with Arthur Kill sediments had greater nirS gene diversity than that from Wyoming soils (Fig. 3). Increasing the T-RFLP signal strength by using more template DNA did not yield a greater number of fragments. Further effort for cloning of nirS genes was focused on the Arthur Kill consortium culture. RFLP analysis with *HaeIII* endonuclease digestion was performed to find distinct RF patterns from the nirS gene clones. Five distinct RFs (clones nirSAK1 to nirSAK5) were found in 96 clones (data not shown). Clones corresponding to five different RFs were sequenced and translated to amino acid sequences to compare phylogenies with known NirS sequences. Phylogenetic analysis showed that clones nirSAK1, nirSAK2 and nirSAK3 were closely related to the NirS sequences from *T. chlorobenzoica*, *A. tolulyticus* and *P. aeruginosa*, respectively (Fig. 2). However, NirS sequences of clones nirSAK4 and nirSAK5 were not associated with any known NirS sequences (Fig. 2). T-RFLP analysis of nirS genes in the Arthur Kill consortium showed that at least three major T-RFs were
present of 97 bp, 136 bp and 166 bp length (Fig. 3). The T-RFs of 97 bp and 136 bp length were matched with clones nirSAK5 and nirSAK1, respectively. The T-RF of 166 bp length was matched with clones nirSAK2 and nirSAK3, which were not differentiated with MspI restriction endonuclease digestion (Fig. 3) but were differentiated with RFLP analysis with HaeIII endonuclease digestion (data not shown). Clone nirSAK4 yielded a 367-bp T-RF, which was observed as a small peak on T-RFLP analysis (Fig. 3).

3.5. Comparison of Nir sequences with environmental clones

All the nir genes cloned from bacterial strains and consortium in this study were compared with nir genes cloned from environmental samples in other studies [10,28]. Pairwise comparison of NirK sequences from this study and 56 different environmental NirK sequences from marine sediments [10] and forested upland and marsh soils [28] showed low relatedness with less than 68% identity, although one clone (U14) shared 85% identity with the NirK sequence of Mesorhizobium sp. 4FB11 (data not shown). The NirS sequences from Acidovorax, Azoarcus and Thauera, as well as from denitrifying bacterial consortium culture did not show high affinities to any of the environmental clones from the previous studies [10,28] (data not shown).

4. Discussion

4.1. Distribution of nir genes in the halobenzoate degrading denitrifying bacteria and related strains

Halobenzoate degrading denitrifying isolates were studied here for the first time to examine functional gene diversity related to dissimilatory nitrite reduction. Both types of nir genes were found in these isolates although
they were originally enriched on the basis of their capabilities for halobenzoate degradation under denitrifying conditions. Different halobenzoates, such as 2-fluorobenzoate, 4-fluorobenzoate and 3-chlorobenzoate, used as a sole energy and carbon source did not appear to be related to the presence of different types of \textit{nir} genes. In addition, neither gene type was correlated with a particular kind of ecosystem or geographical distribution. Halobenzoate degrading denitrifying isolates and related strains were derived from sources ranging from agricultural soils to marine sediments and from North America to Asia and Scandinavia (Table 1).

\textit{nirS} genes were retrieved from strains reflecting this entire range of ecological settings and geographical origins. Although \textit{nirK} was detected in fewer total strains, it was represented in more different genera, in isolates from around the globe.

An interesting correlation was observed between the type of nitrite reductase gene and 16S rRNA gene based taxonomy of the strains in this study. The \textit{nirS} genes were found only in the closely related genera \textit{Acidovorax}, \textit{Azoarcus} and \textit{Thauera}. \textit{nirK} genes, however, were detected in the more phylogenetically diverse genera \textit{Ensifer}, \textit{Ochrobactrum}, \textit{Pseudomonas} and \textit{Mesorhizobium}. The \textit{nirS} and \textit{nirK} genes were not both detected within a single genus in this study. However, some large genera, such as \textit{Alcaligenes} and \textit{Pseudomonas}, with many denitrifying members, are reported to have both types of \textit{nir} gene. Further comparisons between functional gene and 16S rRNA phylogenies will be necessary to evaluate the congruence in the genera investigated here. Failure to detect \textit{nir} genes in denitrifying strains closely related to those in which \textit{nir} genes were detected may imply greater diversity of \textit{nir} genes in denitrifying bacteria. Pairwise comparison of NirK sequences used in this study and a new class of copper-containing nitrite reductase (AniA and related NirK) showed high variation in their amino acid sequences [29]. The \textit{nirK} genes belonging to the new class would not have been detected with the primers used in this study. Thus it is possible that some genes in the isolates went undetected and that the \textit{nir} gene diversity in the consortia could have been underestimated. In addition, nitrite reduction by the isolates cannot be considered as proof of capacity for complete denitrification to N\textsubscript{2}. Their capabilities for complete denitrification can only be verified with the production of N\textsubscript{2}O or N\textsubscript{2} during anaerobic growth. The isolates might not contain \textit{nirK} or \textit{nirS} genes although they reduced nitrite; nitrite reduction to ammonium, for example, involves non-homologous genes. Thus, further characterization of their denitrification potential and further primer development for \textit{nir} genes will both be necessary in order to investigate the genetic diversity of nitrite reductase genes in both pure cultures and environmental samples.

4.2. Genetic diversity of Cu-type nitrite reductase

The copper-type nitrite reductase has been found in various denitrifying bacteria, nitrifying bacteria [12], and archaea [30]. Most \textit{nirK} genes have been described in three subdivisions (\textit{K}, \textit{L}, and \textit{Q}) of the Proteobacteria [7,9,10,12]. For the most part, the phylogeny of NirK sequences in this study correlated with the phylogeny of 16S rRNA genes. The NirK sequences from \textit{Ochrobactrum} strains were grouped together with the NirK sequence of the type strain. In addition, the NirK sequence from \textit{P. mendocina} grouped with the one of \textit{P. aureofaciens}, again, consistent with their 16S rRNA gene-based taxonomy. However, the NirK sequences from \textit{Ensifer} and \textit{Mesorhizobium} strains did not correlate with 16S rRNA gene-based taxonomy but were more closely related to the NirK sequences from the genera \textit{Alcaligenes} and \textit{Achromobacter} in the \textit{L} subdivision of the Proteobacteria.

4.3. Genetic diversity of cytochrome \textit{cd}\textsubscript{1}-type nitrite reductase

Cytochrome \textit{cd}\textsubscript{1}-type nitrite reductase genes (\textit{nirS}) have been sequenced from various bacteria in the \textit{\alpha}, \textit{\beta} and \textit{\gamma
subdivisions of the Proteobacteria [7–10]. Most of the known nirS genes were sequenced from members of the α and γ subdivisions of the Proteobacteria, except the ones from *A. faecalis* and *Ralstonia eutropha*. This study extended the diversity of nirS genes in denitrifying bacteria in the β subdivision of the Proteobacteria. The genera *Acidovorax*, *Azoarcus* and *Thauera* were of special interest because of their involvement in halobenzoate and aromatic compound degradation under denitrifying conditions. Most of the NirS sequences from these genera were closely related to the NirS sequences of *P. stutzeri*, in the γ-Proteobacteria, although the strains are very different in taxonomy. This may provide evidence for horizontal gene transfer of nirS among the genera *Pseudomonas*, *Acidovorax*, *Azoarcus* and *Thauera*. However, novel NirS sequences were found in *A. tolulyticus* Tol4T and *T. mechernichensis* TL1T, which are closely related to each other in spite of their speciation, and cluster separately from the genera *Pseudomonas* and *Ralstonia* (Fig. 2). These clusters may reflect different physiological characteristics for nitrite reduction from the other strains, such as aerobic denitrification, which has been reported in *T. mechernichensis* [31]. In addition, *A. tolulyticus* 2FB6 has a different nirS gene from *A. tolulyticus* Tol4T although they share 79.9% similarity of whole genomic DNAs [17].

The difference between NirS sequence phylogeny and speciation of *Acidovorax*, *Azoarcus* and *Thauera* strains (Fig. 2) implies the independent evolution of genes involved in metabolism. It is desirable to examine the genetic diversity of other metabolic genes, such as the genes encoding aromatic compound degradation under denitrifying conditions, in order to understand the evolutionary history of metabolic pathways.

The denitrifying consortia examined in this study have been characterized for 4-chlorobenzoate degradation and taxonomic diversity previously [18]. Two major 16S RNA gene clones were found to be involved in 4-chlorobenzoate degradation. One 16S rRNA gene clone (4CB1) was closely related to the genus *Thauera* and the other (4CB2) was distantly related to the genera *Ralstonia* and *Limnobacter* [18]. Phylogenetic analysis of NirS sequences from the bacterial consortium derived from the Arthur Kill sediment sample showed that clone nirSAK1 was very similar to the NirS sequences from the genus *Thauera*. Thus, clone nirSAK1 is probably derived from the same organism represented by the 16S rRNA gene clone 4CB1 in this consortium, although isolation of a pure culture is required to verify the connection between nirS and 16S rRNA genes. In addition, clone nirSAK5, generating a T-RF of 97 bp length, could be derived from the same organism containing the 16S rRNA gene clone 4CB2 on the basis of comparison of T-RFLP patterns of 16S rRNA genes and nirS genes (data not shown). Furthermore, even though T-RFLP may still underestimate the number of different sequences present in a sample, the presence of the other nirS gene clones (nirSAK2, nirSAK3 and nirSAK4) showed that functional gene diversity is higher than taxonomic diversity in this bacterial consortium culture.

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References


