Diversity of Nitrite Reductase Genes in *Accumulibacter phosphatis*

**Dominant Cultures Enriched by Flow Cytometric Sorting**

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Running title: NITRITE REDUCTASE GENE OF *A. PHOSPHATIS*

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**Key words:** enhanced biological phosphorus removal (EBPR), denitrifying polyphosphate-accumulating organisms (DNPAOs), nitrite reductase gene (*nirS, nirK*), fluorescence in situ hybridization (FISH), flow cytometric sorting

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AEM accepts, published online ahead of print on 18 May 2007

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Accumulibacter phosphatis is considered a polyphosphate accumulating organism (PAO) though it has not been isolated yet. To reveal the denitrification ability of this organism, we first concentrated this organism by flow cytometric sorting following fluorescence in situ hybridization (FISH) using specific probes for this organism. The purity of the target cells was about 97% of total cell count in the sorted sample. The PCR amplification of the nitrite reductase gene (nirK and nirS) from unsorted and sorted cells was performed. Although nirK and nirS were amplified from unsorted cells, only nirS was detected from sorted cells, indicating that A. phosphatis has nirS. Furthermore, nirS fragments were cloned from unsorted (Ba) and sorted (Bd) cells and classified by RFLP analysis. The most dominant clone in clone library Ba, which represented 62% of the total number of clones, was not found in clone library Bd. In contrast, the most dominant clone in clone library Bd, which represented 59% of the total number of clones, represented only 2% of total number of clones in clone library Ba, indicating that this clone could be that of A. phosphatis. The sequence of this nirS clone exhibited less than 90% similarities to those of known denitrifying bacteria in the database. The recovery of the nirS genes makes it likely that A. phosphatis behaves as a denitrifying polyphosphate-accumulating organism (DNPAO) capable of utilizing nitrite instead of oxygen as an electron acceptor for phosphorus uptake.
INTRODUCTION

Enhanced biological phosphorus removal (EBPR) processes for wastewater have been widely used over the past several decades because of their low cost compared with chemical treatment methods. EBPR is characterized by the use of polyphosphate-accumulating organisms (PAOs) capable of accumulating polyphosphate as an intracellular storage compound using oxygen as an electron acceptor. However, EBPR processes combined with biological nitrogen removal processes have a drawback in that the organic substrate used is a limiting factor for the activities of both PAOs and denitrifying bacteria. Recently, the occurrence of denitrifying polyphosphate-accumulating organisms (DNPAOs) capable of utilizing nitrite or nitrate instead of oxygen as an electron acceptor for phosphorus uptake has been reported (2, 10, 13). The use of DNPAOs in biological nutrient removal processes is advantageous because identical organic substrates such as acetate can be efficiently used as the energy source for both nitrogen and phosphorus removals. Other advantages associated with DNPAO activity include a reduction in surplus sludge production (14).

However, DNPAOs as well as PAOs have not been isolated yet. Or none of the isolated DNPAOs and PAOs could assimilate acetate and synthesize poly-hydroxy-alcanoates (PHA) anaerobically, concomitant with phosphorus release, which were different from EBPR sludge behavior (25). Thus, the biochemistry and genetics of PAOs remain to be...
elucidated. Recently, molecular techniques such as cloning and sequencing of rRNA genes have revealed that *Accumulibacter phosphatis*, a member of the order Rhodocyclales (proposed name, *Candidatus* ‘Accumulibacter phosphatis’ [7]) is abundant in acetate-fed laboratory-scale reactors under cyclic anaerobic/aerobic or anaerobic/anoxic conditions, particularly when efficient phosphorus removal is achieved (2, 31).

However, rRNA-targeted molecular analyses are unable to reveal the ecophysiological traits of *A. phosphatis* as DNPAOs in an anaerobic/anoxic sequencing batch reactor (SBR). Therefore, metabolic analyses targeting *A. phosphatis* were performed using fluorescence in situ hybridization (FISH) combined with microautoradiography (11, 12), polyphosphate staining or PHA staining (5, 7, 15, 17, 31, 32). These studies revealed that *A. phosphatis* could take up acetate, form PHA anaerobically, and accumulate polyphosphate under aerobic or anoxic condition.

However, it was not revealed by these metabolic analyses whether *A. phosphatis* can denitrify or not. An attempt to obtain a better understanding of the population dynamics of denitrifying bacteria in anaerobic/aerobic SBR was made in our previous study (29), but the investigation of *A. phosphatis*’s denitrification ability was not successful.

To clarify *A. phosphatis*’s denitrification ability, it is necessary to separate *A. phosphatis* from microbial consortia. Several techniques such as flow cytometry (30) and density gradient centrifugation (8, 23) can be used to separate microbial cells. Recently, cells in complex microbial consortia stained by FISH with rRNA-targeted oligonucleotide probes...
have been successfully sorted by flow cytometry (24, 30).

On the other hand, PCR primer sets specific for functional genes involved in
denitrification, namely, nirS and nirK, which encode cytochrome cd$_{1}$- and copper-
containing nitrite reductases, respectively (4, 6), and nosZ, which encodes nitrous oxide
reductase (21), have been developed.

In this study, bacteria hybridized with specific probes for A. phosphatis were highly
concentrated from sludge samples by flow cytometric sorting, and molecular analysis of the
nitrite reductase gene was subsequently performed to obtain a better understanding of A.
phosphatis’s ability to denitrify.

MATERIALS AND METHODS

Sequencing batch reactor operation. A sequencing batch reactor (SBR) with a 2 l
working volume was operated using the anaerobic/aerobic cycle at room temperature.
Separable flask (cylinder type, flat bottom, 2000 ml) (Sibata, Japan) caped with acrylic was
used as the reactor. Seed sludge was originally taken from a local municipal wastewater
treatment plant (Ariake WWTP, Tokyo, Japan) where anaerobic/anoxic/oxic (A$_{2}$O) process
was adopted. Then, this seed sludge was cultivated in a laboratory-scale reactor under
anaerobic/aerobic cycle for one year. The reactor was operated with an 8 h cycle that
consisted of a 15 min filling time, a 90 min anaerobic condition, a 285 min aerobic condition, a 65 min settling time and a 25 min withdrawing time. Since both influent and effluent volumes were 1 l, 16 h of hydraulic retention was maintained. Once per day, 250 ml of mixed liquor was removed at the end of the aerobic condition so that the 8 days of sludge retention was maintained. The SBR was mixed constantly with a magnetic stirrer (300 rpm) in anaerobic and aerobic phases. Synthetic wastewater of the following composition was used as the feeding solution: 512 mg of CH₃COONa, 99.5 mg of KH₂PO₄, 90 mg of MgSO₄, 107 mg of NH₄Cl, 14 mg of CaCl₂·2H₂O, 1 mg of yeast extract, and 0.3 ml of nutrient solution (26) per liter. Soluble P, N, TOC and pH were measured according to Tsuneda et al. (29). Acetate has been commonly used for enrichment of PAOs responsible for an enhanced biological phosphorus removal in wastewater treatment plants (25). An acetate-fed reactor containing phosphorus is basic and conventional to research an enhanced biological phosphorus removal.

FISH and DNA staining. After the reactor was operated at least 6 months with more than 540 cycles, about 11 ml of sludge was withdrawn from the SBR and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) solution (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄; pH 7.2) for 2 h at 4°C. The fixed sludge samples were washed twice in PBS, resuspended in PBS-ethanol solution (1:1, vol/vol), and then stored at -20°C. The following oligonucleotide probes specific for A. phosphatis were used for hybridization: PAO462 (5’-CCg TCATCTACWCAgggTATTAAC-3’),
PAO651 (5’-CCCTCTgCCAAACTCCAG-3’), and PAO846 (5’-gTTAgCTACggCACTAAAAgg -3’) (5). These probes were labeled with 6-carboxyfluorescein at the 5’ end. After centrifugation and discarding the supernatant, approximately 10^8 cells were resuspended by vortexing and sonication in a hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate, 35% formamide (pH 7.2), and PAO462, PAO651 and PAO846 (2 ng/µl each), and the suspension was incubated for 3 h at 46°C (30). Then, the cells were centrifuged and washed in a hybridization buffer for 15 min at 46°C. The supernatant was discarded, and 200 µl of PBS and 100 µl of DAPI (4’,6-diamidino-2-phenylindole; Molecular probes, Eugene, OR) solution (50 µg/ml) were added to the cells. The cells were incubated for 10 min at room temperature to stain DNA. Afterwards, the cells were resuspended in PBS and stored at 4°C for the subsequent flow cytometric sorting.

**Dispersion of cells for single-cell sorting.** First, cells were dispersed by ultrasonic treatment (Sonifier II, model 150; Branson, Danbury, CT), and then filtered successively through a gauze filter (pore size: 35 µm; Falcon-type 2235 tube with strainer cap; Becton Dickinson, Franklin Lakes, NJ) and a filter paper (pore size: 8 µm; Millipore, Billerica, MA) to remove large cell aggregates. The samples obtained were diluted and sonicated immediately before flow cytometric analysis and sorting.

**Flow cytometric analysis and cell sorting.** Flow cytometric analysis and cell sorting were performed using FACS Aria (Becton Dickinson) equipped with 488 nm, 633 nm and
407 nm lasers. The 488 nm laser was used for measuring forward scatter, side scatter (488 nm band-pass filter for detection) and probe-conferred fluorescence (530 nm band-pass filter). The 407 nm laser was used for measuring DAPI-DNA fluorescence (450 nm band-pass filter). Data analysis and instrument control were carried out using FACS DiVa software (Becton Dickinson). All analyses were performed at low-flow-rate settings (about 10 µl/min) so that cells would pass through the laser beam in a single-file stream. Data were collected from 10,000 cells per sample.

Cell sorting was carried out in a “single-cell” mode to obtain the highest purity. For the sorting, autoclaved sodium chloride solution (0.1%) was used as the sheath fluid so as not to affect the subsequent microscopic and molecular analyses (30). Before the sorting, the internal sheath path was decontaminated with ethanol. Sorted cells were collected in sterile 1.5-ml tubes.

**Cell counting.** The FISH/DAPI double-stained cells before and after sorting were sonicated (Sonifier II, model 150; Branson) and placed on microscopic slides. The relative abundance of hybridized cells in the sludge samples before and after sorting were estimated as the number ratio of hybridized cells to DAPI-stained cells using epifluorescence microscopy (Zeiss Axioskop 2 plus, Zeiss, Hallbergmoos, Germany). For this cell counting, 10 images for unsorted cells (1338 cells) and 24 images for sorted cells (1086 cells) were arbitrarily selected and cells were counted manually.

**Detection of nirS and nirK gene.** DNAs were extracted from unsorted and sorted samples
(designated as samples Ba and Bd, respectively) using Isoplant (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. For sample Bd, a coprecipitant (Ethachinmate; Nippon Gene) was used to recover DNA successfully during the ethanol precipitation step. The fragments of nirS genes (approximately 890 bp) were amplified using the primers nirS1F (5’-CCTA(C/T)TGCGCCGCA(A/G)CA(A/G)T-3’) and nirS6R (5’-CGTTGAACCT(A/G)CCGT-3’) (4). The fragments of nirK genes (approximately 514 bp) were amplified using the primers nirK1F (5’-GG(A/C)ATG(T/G)CCG(T/G)TGGCA -3’) and nirK5R (5’-GCCTGATCAG(A/G)TT(A/G)TGG -3’) (4). PCR amplification was conducted in an automated thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, Calif.) using the following protocol: initial denaturation for 5 min at 94°C and 30 cycles (for nirS from sample Ba) or 35 cycles (for the other reactions) of denaturation for 1 min at 94°C, annealing for 1 min at 54°C, extension for 1 min at 72°C, followed by a final extension for 7 min at 72°C. The PCR mixture had a final volume of 50 µl, which contained 5 µl of 10 × PCR buffer (containing 20 mM Mg²⁺), 4 µl of dNTP mixture (2.5 mM each), 1.25 U of TaKaRa Ex Taq polymerase (Takara Bio, Otsu, Japan), 0.5 mM of each primer, and 0.5 µl of purified DNA from sample Ba or 7 µl from sample Bd. PCR products were detected by agarose gel electrophoresis with ethidium bromide staining. Bands were visualized by UV excitation.

**Cloning of PCR products.** NirS PCR products of the expected size (890 bp) were excised from a gel and purified using a Wizard SV gel and a PCR cleanup system (Promega,
Madison, WI). Purified PCR products were cloned using a Qiagen PCR cloning plus kit (Qiagen, Valencia, Calif.) and inserts were amplified using PCR mix (Insert Check-Ready-; Toyobo, Osaka, Japan) according to Tsuneda et al. (29).

RFLP analysis of nirS clones. Restriction fragment length polymorphism (RFLP) analysis was conducted to screen PCR products from clones. The products were digested in separate two reactions using the restriction enzymes HhaI and MspI at 37°C overnight. The digested products were electrophoresed on 3.2% (wt/vol) Metaphor agarose gels (Takara Bio) in freshly prepared, chilled 1 × Tris-borate-EDTA buffer for approximately 90 min at 50 V. After electrophoresis, the gels were stained with SYBR Gold or SYBR green I (Wako Pure Chemical Industries, Osaka, Japan) for 30 min, and then visualized on an UV transilluminator. The RFLP patterns were compared visually, and clones showing identical RFLP patterns were grouped into operational taxonomic units (OTUs).

Sequencing of clones and phylogenetic analysis. Representatives of each OTU were selected for sequencing. When an OTU was composed of two or more clones, at least two clones in each OTU were sequenced. DNA fragments were sequenced with nirS1F and nirS6R using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems) according to the manufacturer’s instructions. The sequences and some nirS sequence fragments obtained from the DNA Data Bank of Japan (DDBJ) Database were aligned using the CLUSTAL W program (28), and a phylogenetic tree was constructed by the
neighbor-joining method (20).

**Nucleotide sequence accession number.** The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers from AB208081 to AB208105.

**RESULTS**

**EBPR Reactor.** An anaerobic-aerobic reactor was operated over 6 months. Good phosphate removal was observed with a rapid carbon consumption and phosphorus release under the anaerobic conditions and phosphorus over-accumulation under the subsequent aerobic conditions (Fig. 1). Percentage of phosphorus weight in the sludge at the end of aerobic phase was 21%. This sludge was taken for subsequent molecular analysis and flow cytometry.

**Cell sorting following FISH.** The sludge sample was hybridized with specific probes for *A. phosphatis* and stained with DAPI. Probe-positive cells were 39% of DAPI-positive cells in the sample. Even after the successive filtration of stained cells using 35- and 8-µm-pore size filters, the intensities of their fluorescence signals were almost the same as those before filtration. Thus, this sample was used for the flow sorting of *A. phosphatis* cells with a high purity. Prior to the flow sorting, region 1 (Fig. 2A) in bivariate plots of side scatter versus forward scatter was set to separate cells from noise. Next, in bivariate plots of...
probe-conferred fluorescence versus DAPI-DNA fluorescence in which events of population 1 were only plotted, two groups of population were detected (Fig. 2B). Region 2 was set to distinguish probe-positive cells from other cells and $3.4 \times 10^5$ objects were sorted from region 2. The direct cell counting using epifluorescence microscopy revealed that the purity of sorted cells that hybridized with the probes was about 97% for DAPI-stained cells (Fig. 3). Among the sorted cells, relatively small, round cells that did not hybridize with the probes were slightly observed. This contamination was probably caused by their binding to hybridized cells in the droplets.

**PCR amplification of nirK and nirS genes.** NirS gene fragments were successfully amplified by PCR from both unsorted and sorted sludge samples (referred to as samples Ba and Bd, respectively). These results indicate that *A. phosphatis* may have the nirS gene and an ability to reduce nitrite. NirK gene fragments were successfully amplified from sample Ba, though nonspecific bands were also detected by gel electrophoresis. On the other hand, specific nirK gene fragments were not detected from sample Bd and only thin smears were observed (Fig. 4). From these results, it seems likely that *A. phosphatis* lacks the nirK gene.

**Classification and sequencing of nirS clones.** Since no specific nirK PCR products were detected, only nirS PCR products were cloned. Ninety-one and 65 clones were produced from samples Ba and Bd, respectively. These clones were classified by RFLP analysis and found to fall into 18 OTUs for sample Ba and 14 OTUs for sample Bd. Forty-three clones for sample Ba and 12 clones for sample Bd including representatives of all different RFLP
patterns were sequenced. The sequences of 9 clones for sample Ba and 5 clones for sample Bd could not be determined probably because of the contamination of some other clones or the lack of nirS data in the DDBJ database using BLAST search. We found that some OTUs had similar sequences (>98%), so they were treated as one OTU. As a result, OTUs in sample Ba decreased to 7 consisting of 82 clones and OTUs in sample Bd decreased to 4 consisting of 51 clones.

Figure 5 shows the distribution of RFLP patterns of the nirS clones obtained from each sample. In clone library Ba, clone Ba8 was most dominant (62% of the total number of clones). In clone library Bd, clone Bd69 was most dominant, (59% of the total number of clones). The dendrogram of nucleotides in Fig. 6 shows the relationships between the cloned sequences and the nirS sequences in the DDBJ database. The nirN sequence (accession no. D84475) from Pseudomonas aeruginosa was used as an outgroup for the phylogenetic distance analysis of the nirS sequences. The sequence data and phylogenetic analysis revealed that representative of clones Ba120 (2 clones) and Bd69 (5 clones) had considerably high similarities with each other. The similarities determined by pairwise comparisons of these sequences were higher than 99%. Hence these clones were considered to be identical. Clone Bd30 had 98% similarity to the nirS gene of Ralstonia eutropha. However, all the clones except clone Bd30 had less than 90% nucleotide sequence similarities to nirS genes of known denitrifying bacteria in the database.
DISCUSSION

In this study, the flow cytometric sorting of cells hybridized with specific probes for *A. phosphatis* was performed, followed by molecular analysis of nitrite reductase genes (*nirS* and *nirK*), which are functional genes involved in denitrification. Such flow cytometric sorting following FISH and the subsequent molecular analysis have been reported previously (24, 30). In these studies, 16S rDNAs of sorted cells were analyzed but functional genes were not analyzed.

In our study, FISH-positive cells were clearly discriminated from the other cells on dot plots of flow cytometric analysis (Fig. 2). This successful separation might be attributed to the (i) enhancement in the signal intensity of hybridized cells using three probes specific for *A. phosphatis*, (ii) the removal of aggregated cells by filtration, and (iii) the sonication of cells immediately before flow cytometric analysis. In previous studies (3, 22, 24), CARD-FISH which could improve the sensitivity of FISH compared with probes with a single fluorochrome was adopted for flow cytometric analysis of marine bacterioplanktons. In our study, however, the signal intensity was sufficiently strong to analyze with a flow cytometer without conducting CARD-FISH.

Since *A. phosphatis* was present in samples Ba and Bd, *nirS* clones derived from *A. phosphatis* should be present in both clone libraries Ba and Bd. Clones identical to clone
Ba120 or Bd69 existed in both clone libraries Ba and Bd. However, all the other clones were included in either clone library. Moreover, when the proportion of *A. phosphatis* increased from 39% (sample Ba, before sorting) to 97% (sample Bd, after sorting), the proportion of clones identical to Ba120 or Bd69 to all *nirS* clones increased from 2% (sample Ba) to 59% (sample Bd). These observations indicate that the *nirS* sequences of these clones were that of *A. phosphatis*.

Unexpectedly, the proportions of this *nirS* clone group to all clones were much lower than the proportions of *A. phosphatis* cells determined by microscopic analysis in samples Ba and Bd. This phenomenon was also reported by Sekar et al. (24).

The first possible explanation of this discrepancy is effect of bias caused by PCR (9, 27). Possibly, the primers used in the present study were not perfectly suitable for the PCR amplification of *A. phosphatis*’s *nirS* gene and they tended to amplify some other *nirS* genes. In sample Bd (after sorting), few microorganisms other than *A. phosphatis* were included (3%). There might have existed some *nirS* clones derived from these microorganisms if they are easy to be amplified under the PCR conditions used in this study. Moreover, there is a possibility that none of the observed clones is actually from *A. phosphatis*, but all are from the 3% contaminants in the sorted sample. To confirm that obtained clones include *A. phosphatis*’s *nirS* sequences, additional experiments were performed. Samples were artificially prepared. One consisted of 97% *nirS* positive cells (*Marinobacter*) and 3% *nirS* negative cells (*Alcaligenes xylosoxidans*); the other consisted
of 3% nirS positive and 97% nirS negative. Number of total cells of each sample was
adjusted to be identical, and then DNA extraction and PCR amplification were conducted
under the same condition as we did for sorted cells. As a result, PCR products from 97%
nirS positive cells were detected but PCR products from 3% nirS positive cells were not
detected (Fig. 6). These results supported that DNAs extracted from A. phosphatis
included in sorted cells were actually amplified and cloned.

The second possible explanation may be existence of another strain of A. phosphatis. In
clon library Bd, second dominant clone represents 27% of total clone population. It can
be expected that this clone is derived from A. phosphatis whose strain is different from the
strain having a sequence of most dominant clone. The total of most dominant and second
dominant clones in the clone library Bd is 86%. This ratio is comparable to the ratio
determined by microscopic analysis. Recently, metagenomic analysis of A. phosphatis
dominated sludge communities was performed (16). According to our search of the
metagenomic data, two types of nirS genes classified to A. phosphatis were found. One
shows high similarity to clone Bd69 (85%) which is most dominant in the clone library of
sorted cells, the other shows high similarity to clone Bd86 (86%) which is second
dominant in the clone library of sorted cells. These observations may support the second
explanation.

In our previous study (29), the cloning of the nirS gene fragment with the same primer
set used in the present study was also performed for similar sludge samples without flow
cytometric sorting. As a result, one of the nirS clones occupied approximately 70% of all nirS clones. As shown in Fig. 6, this clone (28-8, accession number: AB185906) is identical to clone Ba8 which accounted for about 62% of clone library Ba. However, A. phosphatis’s putative clone was not found in the clone library in our previous study (29). This result is not in conflict with the present study because A. phosphatis’s putative clone represented only 2% of clone library Ba.

Combination of flow sorting and molecular analysis described in this study could be adapted to other uncultured bacteria. In addition, single cell amplification of total DNA through multiple displacement amplification (MDA) is a powerful tool for analyzing gene in the uncultured bacterial cell especially from small scale sample (1, 19). Thus, the combined use of MDA will advance our method and contribute to revealing characteristics of uncultured bacteria in natural environments.

REFERENCES


phosphate-accumulating organisms cultivated under different electron acceptor
conditions using polymerase chain reaction-denaturing gradient gel electrophoresis

picoeukaryotes in the natural environment by using taxon-specific oligonucleotide
probes in association with tyramide signal amplification-fluorescence in situ

for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying

L. Blackall. 2000. Identification of polyphosphate-accumulating organisms and design
of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ.
Microbiol. 66:1175-1182.


Zehender. 1999. Enrichment, phylogenetic analysis and detection of a bacterium that


Figure Legends


FIG. 2. Flow cytometric analysis and sorting of bacteria hybridized with specific probes for A. phosphatis from sludge taken from sequencing batch reactor under anaerobic/aerobic condition. (A) Region 1 was set to separate cells from noise. (B) Only the cells dotted within region 1 were dotted. Region 2 was defined in a bivariate dot plot of probe fluorescence versus DAPI-DNA fluorescence and was used for cell sorting.

FIG. 3. Cells sorted from region 2 (Fig. 2B). Probe-conferred fluorescence and DAPI-DNA fluorescence are shown in (a) and (b), respectively. The composite image of micrographs (a) and (b) is shown in (c). Cells indicated by arrows did not hybridize with the probes.

FIG. 4. PCR amplification of nirS (890 bp) and nirK (514 bp) DNAs extracted from sorted cells. Lanes 1 and 4, 100-bp DNA ladder markers; lane 2, nirS PCR product; lane 5, nirK PCR product; lanes 3 and 6, negative control.
FIG. 5. Distribution of RFLP patterns of nirS clones obtained from DNAs in sludge sample before (a) and after (b) sorting. Clones having the same RFLP pattern are represented by the same color.

FIG. 6. Neighbor-joining analysis of partial nucleotide sequences from cloned nirS PCR products. Evolutionary distance and parsimonious analyses were carried out using CLUSTAL W with 1000 bootstrap resamplings. The scale indicates 0.1 nucleotide substitution per nucleotide position. The tree was rooted with the nirN sequence of Pseudomonas aeruginosa as an outgroup. Clone Ba was obtained from the sludge sample before sorting. Clone Bd was obtained from the sorted cells. Clone 28-8 is from an anaerobic/aerobic SBR (29). Sequences of Sludge Oz, Jazz and Phrap included in the tree were metagenomic sequences of nirS binned as A.phosphatis (16). GOI means Gene object

FIG. 7. PCR amplification of nirS (890 bp) DNAs. Lane 1, PCR product from the sample consisting of 97% nirS positive cells and 3% nirS negative cells; lane 2, PCR product from the sample consisting of 3% nirS positive cells and 97% nirS negative cells; lane 3, negative control; lane 4, 200-bp DNA ladder markers. Marinobacter was used as nirS positive cells, and Alcaligenes xylosoxidans as nirS negative.
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