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Journal

ISSN

Authors

Allen, Andrew E
Booth, Melissa G
Frischer, Marc E
et al.

Publication Date

DOI

Copyright Information

Peer reviewed

Diversity and Detection of Nitrate Assimilation Genes in Marine Bacteria

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A PCR approach was used to construct a database of *nasA* genes (called *narB* genes in cyanobacteria) and to detect the genetic potential for heterotrophic bacterial nitrate utilization in marine environments. A *nasA*-specific PCR primer set that could be used to selectively amplify the *nasA* gene from heterotrophic bacteria was designed. Using seawater DNA extracts obtained from microbial communities in the South Atlantic Bight, the Barents Sea, and the North Pacific Gyre, we PCR amplified and sequenced *nasA* genes. Our results indicate that several groups of heterotrophic bacterial *nasA* genes are common and widely distributed in oceanic environments.

The importance of inorganic N (NH_4^+ or NO_3^-) for the nutrition and growth of marine phytoplankton has long been recognized (5, 7, 8), while the utilization of inorganic N by bacteria has historically received less attention (11, 13, 15, 17, 43). The primary role of heterotrophic bacteria is classically considered to be the decomposition and mineralization of dissolved and particulate organic nitrogen (27). Bacterial NO_3^- assimilation is not a pathway currently considered in pelagic carbon and nitrogen cycle models (1, 6, 10). A recent review of freshwater and marine studies, however, reported that bacteria may rely on both NH_4^+ and NO_3^- for growth and biomass synthesis, and overall they may be significant consumers of inorganic N; mean consumption values of 30 and 40% have been reported for NH_4^+ and NO_3^-, respectively (14). Under certain conditions, such as in the presence of high concentrations of dissolved organic carbon relative to the concentration of dissolved organic nitrogen, bacteria may be responsible for most, if not all, of the observed NO_3^- uptake and disappearance (16, 24, 25). Significant heterotrophic bacterial utilization of dissolved inorganic nitrogen likely would have profound effects on the fluxes of N and C in the water column.

Bacterial nitrate utilization in aquatic communities, however, is difficult to study by conventional tracer approaches. Within the bacterial size class, autotrophic cyanobacteria (picoplankton) are often abundant (4, 41) and are likely to complicate conclusions regarding the total flux of labeled nitrogen into the heterotrophic fraction of the bacterial community. Also, size fractionation does not allow for examination of nitrate uptake by attached bacteria or large cells caught in filters.

It is known that some, but not all, heterotrophic bacteria are capable of growth on NO_3^- as a sole N source (28). In *Klebsiella pneumoniae*, the structural genes for nitrate assimilation form an operon, *nasFEDCBA* (19–21). The NASC protein is thought to mediate electron transfer from NADH to NASA, which contains the active site for nitrate reduction (19, 20). The NASA protein has also been purified from a phototrophic member of the alpha subclass of the class *Proteobacteria*, *Rhodobacter capsulatus*, and characterized (2, 22). Examination of currently available prokaryotic genome sequences suggests that *nasA* is present in a wide diversity of organisms, although these observations need verification (28).

Molecular techniques can be employed to illuminate factors which control the rates of fluxes and transformations in nitrogen-cycling processes (40, 46, 47, 51). Molecular approaches have been successfully used to detect and characterize bacteria and the genes that are important in several aspects of the nitrogen cycle, including nitrification, dentrification, and nitrogen fixation (18, 29, 30, 37–39, 45, 48–50).

Here we describe the design and optimization of a series of nested heterotrophic bacterium-specific *nasA* PCR primers. The detection of *nasA* genes in a variety of marine environments provided a basis for the hypothesis that the potential for NO_3^- utilization by heterotrophic bacteria is significant. Phylogenetic analysis of *nasA* genes cloned from diverse samples indicated that there are several distinct clades and suggests that there is a clear genetic distinction between *nasA* genes from heterotrophic bacteria and *nasA* genes from autotrophic cyanobacteria.

Initially, three nested universal degenerate *nasA* primers were designed based on five previously determined sequences from cyanobacteria and one sequence from a heterotrophic bacterium (3, 12, 20, 23, 34). The sequences from cyanobacterial strains were from *Oscillatoria chalybea*, *Anabaena* sp. strain PCC7120, *Synechocystis* sp. strain PCC6803, a *Synechococcus* sp., and *Synechococcus* sp. strain 7942, and the sequence from a heterotrophic strain was from *Klebsiella oxytoca*. The GenBank accession numbers for these sequences are X89445, L49163, BAA17488, CAA52675, P39458, and L06800, respectively. An alignment of the inferred amino acid sequences encoded by *nasA* indicated that there were conserved regions suitable for targeting by PCR oligonucleotide primers. Such

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)	Amino acid sequence	Application
nas22	TGYCCNTAYTGYGGNGT	CPYCG	nasA/narB PCR amplification
nas964	CARCCNAAYGCNATGGG	QPNAM	nasA/narB PCR amplification
nasA1735	ATNGTRTGCCAYTGRTC	DQWHT	nasA PCR amplification
nas1933	CARTGCATNGGNAYRAA	F/L V/I/M PMH	nasA/narB PCR amplification
fd1	AGAGTTTGATCCTGGCTCAG		16S rRNA forward primer
rp2	ACGGCTACCTTGTTACGACTT		16S rRNA reverse primer
M13F	GTAAAACGACGGCCAG		Forward sequencing primer, M13 vector
522F	CAGCCGCGGTAATAC		Forward sequencing primer, 16S rRNA
1056F	TGGCTGTCGTCAGCTCGTGT		Forward sequencing primer, 16S rRNA
M13R	CAGGAAACAGCTATGAC		Reverse sequencing primer, M13 vector
1056R	ACACGAGCTGACGACAGCCA		Reverse sequencing primer, 16S rRNA
522R	GTATTACCGCGGCTG		Reverse sequencing primer, 16S rRNA

primers have been used to amplify nasA sequences in other heterotrophic bacteria, and a group-specific degenerate primer was designed to specifically amplify the nasA gene from heterotrophic bacteria. All of the primers used in this study are listed in Table 1.

Surface water samples were collected during two cruises in the South Atlantic Bight (SAB) off the Georgia coast aboard the R/V *Bluefin* during October 1998 and aboard the R/V *Cape Hatteras* during March 1999 (31 to 33°N, 78 to 81°W). SAB samples were also collected from docks located on the Skidaway River (March 1999) and the Wilmington River (July 1998). Additional water samples used in this study were collected at depths of 5, 30, and 80 m in the Barents Sea (70 to 78°N, 30°E) aboard the R/V *Jan Mayen* during July 1999 and from the surface of the North Pacific Gyre at Hawaii Ocean Time Series stations (22°45′N, 158°W) during May 1997 and aboard the R/V *Melville* during June 1999. For DNA extrac-

tion, bacteria were collected from 40 liters of water. To remove eukaryotic plankton, the water was prefiltered under a vacuum through a 3-μm-pore-size polycarbonate cartridge filter (Gelman Sciences, Inc., Ann Arbor, Mich.) and then through a 142-mm-diameter, 0.8-μm-pore-size polycarbonate Supor filter (Gelman) with a custom-manufactured acrylic filter holder. Bacterial cells in the filtrate were collected on a 142-mm-diameter, 0.2-μm-pore-size polycarbonate Supor filter (Gelman) and stored at −20°C aboard ship and then transferred to storage at −80°C in the lab. DNA in the SAB samples collected in October 1998 was extracted as described by Gonzalez et al. (9), and DNA in all other samples was extracted with an Ultra-Clean Mega Prep soil DNA kit (Mo Bio Laboratories, Inc., Solana Beach, Calif.). For the latter procedure, frozen filters were crushed inside Whirl-Pak bags (Nasco, Fort Atkinson, Wis.) and put directly into the lysing matrix used for step one of the soil DNA extraction procedure. Visualization of purified

TABLE 2. Srains used in this study and 16S rRNA accession numbers

Strain	Closest relative	nasA PCR amplification	16S rRNA accession no.
Known cultures	NA[c]	+	
Fischerella sp.[a]	NA	+	
Plectonema boryanum[a]	NA	+	
Trichodesmium sp. strain IMS101[a]	NA	+	
Klebsiella pneumoniae ATCC 13883[a]	NA	+	
Clostridium oceanica[a]	NA	+	
Pseudomonas sp.[a]	NA	+	
Vibrio diazotrophicus[a]	NA	+	
Bacillus sp.	NA	−	
Micrococcus luteus	NA	−	
Vibrio sp. strain S-14	NA	−	
Pseudomonas stutzeri	NA	−	
Sagittula stellata E37	NA	−	
Strains isolated in this study		−	
South Atlantic Bight A[b]	*Cytophaga* sp.	+	AF300973
South Atlantic Bight B[b]	*Aerococcus viridans*	−	AF300974
South Atlantic Bight C[b]	*Alteromonas* sp. strain MS23	+	AF300975
Barents Sea isolate 4[b]	*Pseudoalteromonas* sp. strain ANG.ro2	+	AF300976
Barents Sea isolate 10[b]	*Marinobacter* sp. strain DS40M8	+	AF300977
Barents Sea isolate 23	*Pseudoalteromonas haloplanktis*	−	AF300978
Barents Sea isolate 25[b]	*Pseudoalteromonas citrea*	+	AF300979
Barents Sea isolate 32	*Erythrobacter citreus*	−	AF300980
Skidaway River 1[b]	*Vibrio carchariae*	+	AF300981
Skidaway River 2[b]	*Vibrio furnissii* ATCC 35016[T]	+	AF300982
Skidaway River 3	*Micrococcus luteus* FO-084a	−	AF300983

[a] Sequence amplified in this study by using oligonucleotide primers nas964 and nas1934.
[b] Sequence amplified in this study by using oligonucleotide primers nas964 and nas1735.
[c] NA, not applicable.

DNA by gel electrophoresis revealed the presence of high-molecular-weight DNA with little shearing and no RNA contamination. From 40 liters of seawater, this method typically yielded an average of 100 to 110 μg of DNA. If it was assumed that the average concentration bacterial cells was 10^6 cells per ml of seawater and the average DNA content was 3 fg/cell (31), the approximate extraction efficiency of this method was between 80 and 90%.

PCR was performed by using a nested format to improve specificity and sensitivity. The PCR products obtained with the outermost degenerate *nasA/narB* universal primers (*nas*22, *nas*1933) were subsequently used as templates in PCR with the heterotroph-specific internal primer set (*nas*964, *nasA*1735). Amplification was accomplished by using the Qiagen *Taq* PCR Master Mix System and the standard protocol recommended by the vendor (Qiagen, Valencia, Calif.); a hot start at 94°C for 5 min was followed by 35 cycles consisting of 94°C for 5 s, 55.5°C for 10 s, and 72°C for 1 min, with a 7-min final extension step at 72°C. DNA template (10 to 100 ng of community DNA or 0.1 to 10 ng of genomic DNA from a pure culture) was added to each 25-μl PCR mixture. First-round reaction mixtures contained 1 μM primer *nas*22, 1 μM primer *nas*1933, and 3.5 mM $MgCl_2$. Second-round nested PCR mixtures contained 1 to 2 μl of product from the first round, 2.5 mM $MgCl_2$, 1 μM primer *nas*964, and 1 μM primer *nasA*1735, and the extension time in each cycle was decreased to 30 s. The *nasA*-specific primers yielded a PCR product that was 750 to 800 bp long. 16S rRNA amplification of the nearly complete 16S rRNA gene was facilitated by using eubacterial primers fd1 and rp2 (Table 1) (42) (100 nM each). Thermal cycling was performed with a model 2400 or 9700 thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.).

Although *nasA* PCR could be optimized for specific community DNA samples by raising the annealing temperature to 57 to 60°C, a somewhat less stringent annealing temperature, 55°C, was used during the initial construction of clone libraries to increase the yield and the likelihood of amplification with most primer-template combinations.

The PCR product of the desired size was excised from the gel and purified by using GenElute agarose spin columns (Supelco, Bellefonte, Pa.). PCR products were ligated and cloned by using either a TOPO TA Cloning kit (for pure cultures) or an Original TA Cloning kit (for community DNA samples). In both cases, the PCR product was ligated into a pCR 2.1 plasmid vector and cloned into TOP10 One Shot competent *Escherichia coli* cells (Invitrogen, Carlsbad, Calif.). Plasmid DNA was extracted and purified by using the Wizard Plus Minipreps DNA purification system (Promega, Madison, Wis.).

Sequences were determined by automated sequencing at the Molecular Genetics Facility (University of Georgia) with ABI automated sequencers (models 373 and 377). Sequencing reactions were facilitated by using an ABI Big Dye Prism dideoxy sequencing dye terminator kit as recommended by the manufacturer. Sequence analysis was accomplished by using ABI software, version 3.3 (ABI, Foster City, Calif.). The sequencing primers used are listed in Table 1.

Bacteria were isolated from seawater samples collected from the SAB continental shelf during March and June 1999 (31 to 33°N, 78 to 81°W). Bacteria were also isolated from Barents Sea water samples collected during July 1999 (70 to 80°N,

TABLE 3. Abilities of bacterial isolates to grow on nitrate media and detection of *nasA* PCR gene product

Isolate	Growth on NO_3^- as sole nitrogen source	*nasA* PCR amplification
Barents Sea isolates		
Barents Sea isolate 4[a]	Y	+
Barents Sea isolate 10[a]	Y	+
Barents Sea isolate 23	N	−
Barents Sea isolate 25[a]	Y	+
Barents Sea isolate 32	N	−
Barents Sea isolate 1	N	−
Barents Sea isolate 2	Y	+
Barents Sea isolate 3	N	−
Barents Sea isolate 5	N	−
Barents Sea isolate 6	Y	+
Barents Sea isolate 7	N	−
Barents Sea isolate 8	Y	+
Barents Sea isolate 9	Y	+
Barents Sea isolate 12	Y	+
Barents Sea isolate 13	Y	+
Barents Sea isolate 14	Y	+
Barents Sea isolate 15	Y	+
Barents Sea isolate 16	Y	+
Barents Sea isolate 18	Y	+
Barents Sea isolate 19	N	−
Barents Sea isolate 20	N	−
Barents Sea isolate 21	N	−
Barents Sea isolate 22	N	−
Barents Sea isolate 24	Y	+
Barents Sea isolate 26	N	−
Barents Sea isolate 27	Y	+
Barents Sea isolate 28	N	−
Barents Sea isolate 29	Y	+
Barents Sea isolate 30	Y	+
Barents Sea isolate 31	N	−
SAB isolates		
South Atlantic Bight A	N	+
South Atlantic Bight B	N	−
South Atlantic Bight C	N	+
Skidaway River 1	Y	+
Skidaway River 2	Y	+
Skidaway River 3	N	−

[a] The doubling times of Barents Sea isolates 4, 10, and 25 on 10 mM NO_3^- as the sole N source were 3.78, 5.48, and 5.16 h, respectively.

30°E). Bacteria were isolated by using either organic nitrogen or nitrate as the sole nitrogen source. Selected colonies were axenically transferred to new plates twice to ensure that pure cultures were obtained. For long-term storage each isolate was maintained in a 15% (vol/vol) glycerol freezer stock preparation at −80°C.

To screen isolated strains for the presence of *nasA*, PCR-amenable DNA was extracted from each of the isolates by using the FastDNA spin protocol and a Fast Prep instrument (both from BIO 101, Vista, Calif.). In all PCRs, appropriate negative controls without DNA and positive controls were included.

To test isolates for the ability to grow on nitrate as the sole N source, two tubes containing 5 ml of NFG medium (33) were prepared. To one of the tubes a sterile $NaNO_3^-$ solution was added to obtain a final NO_3^- concentration of 10 mM. The second tube did not receive such an addition and served as a negative control. The two NFG medium tubes were then inoculated 1:100 with a stationary-phase culture grown in peptone- and yeast-enriched artificial seawater (26). After 72 h,

the optical densities of the two tubes were compared to the optical density of a NFG medium tube that had not been inoculated. Additionally, several isolates were selected for batch culture growth assays. These experiments were conducted in axenic 100-ml NFG medium cultures containing 80 μM or 10 mM NO_3^- as the sole N source. Doubling rates were determined by estimating cell density at a minimum of four time points during the exponential growth phase. Cell densities were determined by direct epifluorescent microscopy after staining with DAPI (4′,6′-diamidino-2-phenylindole) (44).

Phylogenetic relationships based on *nasA* gene sequences were determined. Nucleotide sequences were translated into approximately 264 unambiguous amino acids. All of the available *narB*/*nasA* amino acid sequences were then aligned by using the CLUSTAL W (version 1.7) multiple-sequence-alignment algorithm (32). Phylogenetic trees were inferred and drawn by using the TREECON software package (version 1.3b) (35, 36) and the Kimura two-parameter model for inferring evolutionary distances. Bootstrap estimates (100 replicates) of confidence intervals were also made by using the algorithms available in the TREECON package.

For 16S rRNA analysis, 464 unambiguously alignable nucleotide positions were used. The nucleotide sequences were compared to 16S rRNA gene sequences available in the GenBank database by using the Blast program to determine the degrees of sequence similarity to known organisms. All of the *nasA* and 16S rRNA sequences determined in this study (Table 2) have been deposited in the GenBank database.

Results. Using the universal *nasA* nested primer set, we amplified, cloned, and sequenced a 1,000-bp fragment from a group of phylogenetically diverse bacteria, including *Clostridium oceanica*, *Vibrio diazotrophicus*, a *Pseudomonas* sp., *Trichodesmium* sp. strain IMS101, a *Fischerella* sp., and *Plectonema boryanum*, as well as from DNA extracted from the bacterial size fraction of seawater collected at a Hawaii Ocean Times Series station near Hawaii. Also, we attempted to amplify the 1,000-bp *nasA* fragment from *Bacillus* sp., *Micrococcus luteus*, *Vibrio* sp. strain S-14, and *Pseudomonas stutzeri*. These templates, however, did not yield a PCR product, and we concluded that they were *nasA* negative (Table 2).

Using the expanded database of *nasA* sequences, we targeted an additional reverse primer, at amino acid position 579, for heterotrophic organisms. The heterotroph-specific primer was *nasA*1735 (Table 1) and was approximately 200 bp downstream from the universal *nasA* reverse primer.

Using a collection of isolates obtained during a cruise in the Barents Sea, we examined the relationship between the presence of the *nasA* gene and the ability to utilize NO_3^- as a sole N source during aerobic growth. Of the 30 isolates screened, 17 were able to grow by using NO_3^- as a sole N source. All of these strains were PCR positive for the *nasA* gene fragment. Thirteen of the isolates screened could not grow on NO_3^- alone, and none of these strains contained a *nasA* gene fragment. Three isolates from SAB water and three isolates from the Skidaway River estuary in Georgia were also screened. Two of these six isolates were NO_3^- growth positive and *nasA* PCR positive, two were NO_3^- growth negative and *nasA* PCR negative, and two were NO_3^- growth negative and *nasA* PCR positive. Therefore, of 36 isolates examined, 19 were PCR positive for *nasA* and had the ability to utilize NO_3^- as a sole
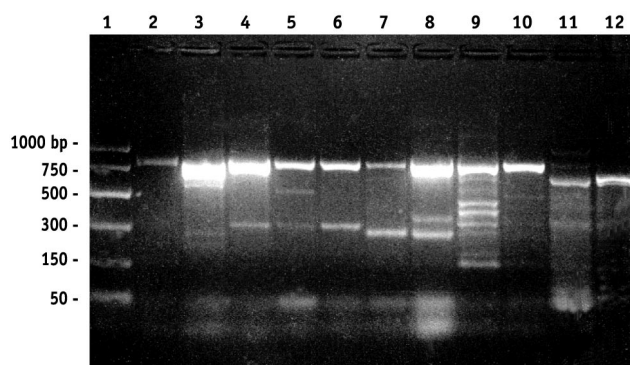

FIG. 1. PCR amplification of the expected 750- to 800-bp *nasA* gene fragment from various marine samples. Lane 2 contained a PCR product from a sample that was prepared by amending filtered seawater with 10^3 *K. pneumoniae* cells/ml (final concentration). *nasA* PCR products were amplified from samples collected from a Norwegian fjord (lane 3), the Barents Sea (lanes 4 to 6), the Skidaway River (lanes 7 and 8), the SAB (lanes 9 and 10), and the North Pacific Gyre (lanes 11 and 12). Lane 1 contained a molecular weight standard PCR marker (Promega).

N source, 15 were PCR negative for *nasA* and were not able to utilize NO_3^-, and two displayed somewhat contradictory results because they were PCR positive for *nasA* and apparently not able to grow on NO_3^- as a sole N source (Table 3). The results of the batch growth assays indicated that there was some variability between isolates in terms of their affinity for NO_3^- (Table 3). Data are reported here only for experiments conducted with 10 mM NO_3^-. Experiments conducted with 80 μM NO_3^- generated similar doubling times for the different strains tested, but the final cell yields were lower.

nasA was detected in all of the environments examined, including the SAB, the North Pacific Gyre, a Norwegian fjord, and the Barents Sea (Fig. 1). The sensitivity of *nasA* detection by PCR was initially estimated by amending filtered seawater with 10^3 and 10^2 *K. pneumoniae* cells per ml and detecting *nasA* in the samples (Fig. 1). This approach did not establish a minimum level of detection but demonstrated that a concentration of at least 10^2 cells positive for the *nasA* gene per ml could be detected. Since a strong signal was detected in a wide range of marine samples, heterotrophic bacteria with the capacity for NO_3^- assimilation appear to be very common and well distributed.

In general, *nasA* genes from uncultured organisms do not form clades separate from the clades of cultured bacteria. Also, *nasA* genes in taxonomically related bacteria are not necessarily similar, except in the case of *Vibrio* representatives. *V. diazotrophicus* and two *Vibrio* isolates form a separate clade which includes seven clones from samples collected in the Skidaway River and the SAB midshelf (35 miles offshore). *K. pneumoniae* ATCC 13883 and a *Pseudoalteromonas* isolate typify another clade, which includes three clones from SAB midshelf samples. A third discrete cluster includes *C. oceanica*, *K. oxytoca*, a *Pseudomonas* sp., and a *Marinobacter* sp. isolate. Also in this cluster are clones from the midshelf (25 and 35 miles offshore), the Skidaway River, and the adjacent Wilmington River (Fig. 2).

Among the nitrate-assimilating (*nasA*-positive) and non-nitrate-assimilating (*nasA*-negative) isolates whose 16S rRNA
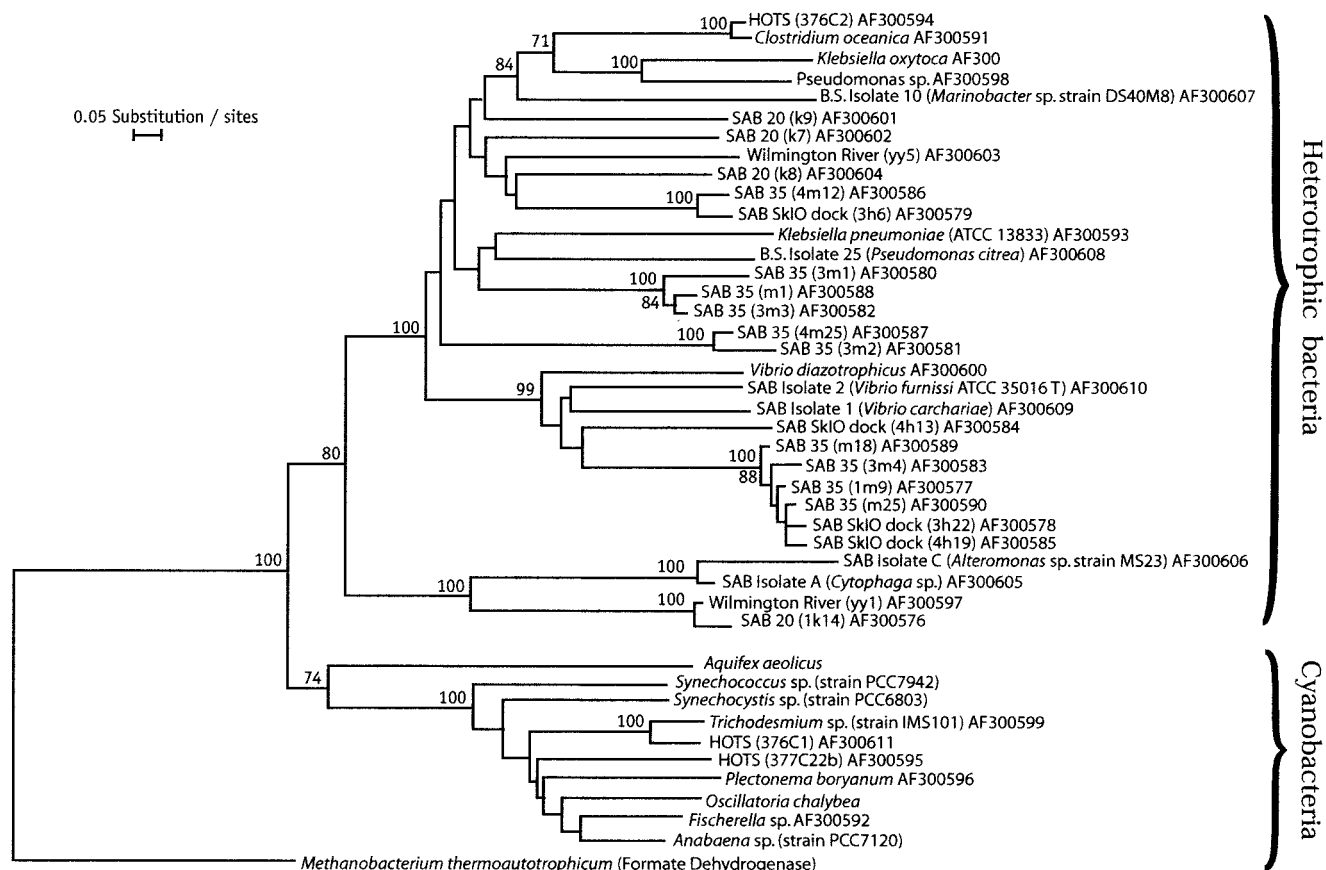
FIG. 2. Inferred phylogenetic relationships of *nasA*- and *narB*-encoded amino acid sequences from heterotrophic bacteria and cyanobacteria, respectively. The scale bar indicates 0.05 fixed amino acid substitution per site. The numbers at the nodes are bootstrap values. Bootstrap values less than 70 (of 100) are not shown. The amino acid sequence of formate dehydrogenase from *Methanobacterium thermoautotrophicum* (GenBank accession number U52681), a putative evolutionary ancestor of the proteins encoded by the *nasA* and *narB* genes, was used to root the tree. HOTS, Hawaii Ocean Time Series.

genes have been sequenced, there are more different types of taxa associated with the *nasA*-negative strains. For example, *nasA*-negative isolates include organisms such as a *Micrococcus* sp. (gram-positive phylum, high-G+C-content subdivision), an *Erythrobacter* sp. (alpha subclass of the *Proteobacteria*), an *Aerococcus* sp. (gram-positive phylum, low-G+C-content subdivision), *Sagittula stellata* E37 (alpha subclass of the *Proteobacteria*), and a *Pseudoalteromonas* sp. (gamma subclass of the *Proteobacteria*) (Table 2). By contrast, the majority of the *nasA*-positive strains whose 16S rRNA have been sequenced are members of the gamma subclass of the *Proteobacteria*. In particular, members of a *Pseudoalteromonas* sp. and a *Vibrio* sp. account for five of seven of the *nasA*-positive strains that we isolated.

Conclusions. The correlation between the presence of *nasA* and nitrate utilization assay results for individual isolates (34 of 36 isolates tested) supports the hypothesis that the *nas*A-specific primer sets developed in this study provide a reliable assay for functional assimilatory nitrate reductase genes. Sequences derived from isolates that were unable to utilize NO_3^- in culture (Table 3) are more closely related to dehydrogenases and proteins encoded by members of other gene families and can be distinguished phylogenetically from functional assimilatory nitrate reductases (Fig. 2). This illustrates the fact that

although degenerate primers are powerful and able to retrieve gene fragments from very diverse organisms, it is important to sequence and phylogenetically analyze PCR products from as many different types of organisms as possible in order to identify potential nonspecific PCR products.

We demonstrated that genetic probes that recognize the functional assimilatory nitrate reductase genes of specific groups of bacteria can be constructed. Heterotrophic *nasA* genes have been detected in every marine sample tested thus far, indicating that bacteria capable of assimilating nitrate are ubiquitous in diverse ocean margins and open water. These observations suggest that heterotrophic bacteria are potentially important consumers of NO_3^- in marine environments.

Nucleotide sequence accession numbers. The *nasA* and 16S rRNA sequences determined in this study have been deposited in the GenBank database under the accession numbers shown in Table 2 and Fig. 2.

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