Applied and Environmental Microbiology, June 2007, p. 3547–3555 Vol. 73, No. 11
0099-2240/07/$08.00+0 doi:10.1128/AEM.02275-06
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Ammonia-Oxidizing β-Proteobacteria from the Oxygen Minimum Zone off Northern Chile▽

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Received 26 September 2006/Accepted 28 March 2007

The composition of ammonia-oxidizing bacteria from the β-*Proteobacteria* subclass (βAOB) was studied in the surface and upper-oxycline oxic waters (2- to 50-m depth, ~200 to 44 μM O_2) and within the oxygen minimum zone (OMZ) suboxic waters (50- to 400-m depth, ≤10 μM O_2) of the eastern South Pacific off northern Chile. This study was carried out through cloning and sequencing of genes coding for 16S rRNA and the ammonia monooxygenase enzyme active subunit (*amoA*). Sequences affiliated with *Nitrosospira*-like cluster 1 dominated the 16S rRNA gene clone libraries constructed from both oxic and suboxic waters. Cluster 1 consists exclusively of yet-uncultivated βAOB from marine environments. However, a single clone, out of 224 obtained from the OMZ, was found to belong to *Nitrosospira* lineage cluster 0. To our knowledge, cluster 0 sequences have been derived from βAOB isolated only from sand, soil, and freshwater environments. Sequences in clone libraries of the *amoA* gene from the surface and upper oxycline could be grouped in a marine subcluster, also containing no cultured representatives. In contrast, all 74 *amoA* sequences originating from the OMZ were either closely affiliated with cultured *Nitrosospira* spp. from clusters 0 and 2 or with other yet-uncultured βAOB from soil and an aerated-anoxic Orbal process waste treatment plant. Our results reveal the presence of *Nitrosospira*-like βAOB in both oxic and suboxic waters associated with the OMZ but with a clear community shift at the functional level (*amoA*) along the strong oxygen gradient.

Oxygen minimum zones (OMZs) are permanent areas of the ocean with dissolved O_2 concentrations of ≤22 μM at intermediate depths (15, 18) caused by low rates of ventilation and high biological O_2 demand. The main zones are found in the eastern tropical Pacific and Arabian Sea. Associated with a sharp decrease of dissolved O_2 concentration with depth is an intense nitrogen cycle that generates characteristic vertical distributions of nitrogen compounds within the OMZ: i.e., high NO_3^- deficit, a secondary nitrite maximum, and low NH_4^+ concentrations are found within the core, as well as N_2O maxima towards the OMZ boundaries (8, 26, 30, 41). Such strong chemical gradients produced by OMZs could be considered as gene flow barriers and their oxygen-deficient waters as suitable environments for innovation, where organisms develop specific adaptations to exploit these unique conditions (9, 30, 42). In fact, novel and yet-uncultivated groups of denitrifiers have been found in the OMZ of the Arabian Sea (17a) and the eastern South Pacific (6a) by cloning and sequencing of the *nirS* genes. However, the diversity of most of the OMZ microbial world remains unexplored.

Ammonia-oxidizing bacteria (AOB) are considered to play a key role in the nitrogen cycling of these OMZs, contributing to NH_4^+ removal and NO_2^- and N_2O production (26, 33, 52). AOB consist of a group of chemolithoautotrophic microorganisms able to oxidize aerobically NH_4^+ to NO_2^-, via NH_2OH (hydroxylamine), a dissimilative metabolic process that also produces N_2O as a by-product (20). Known AOB are found in three genera of the *Proteobacteria* phylum: *Nitrosomonas* and *Nitrosospira*, in the β class (βAOB), and *Nitrosococcus*, in the γ class (γAOB) (40, 50). In marine systems, the study of genes encoding the active site-containing subunit of ammonia monooxygenase (*amoA*), which is involved in the oxidation of NH_4^+ to NH_2OH, has indicated that βAOB are predominant over γAOB (35). In addition, within the β subdivision, 16S rRNA genes libraries of AOB indicate that in planktonic marine samples sequences from *Nitrosospira*-like organisms are more frequent than those from *Nitrosomonas*-like bacteria, which in turn are mainly found associated with particles (3, 13, 16, 37, 39). However, in the OMZ off Peru, Ward et al. (52) found high chemoautotrophic activity related to *Nitrosomonas* sp. (marine) and *Nitrosococcus oceanus* by combining autoradiography with immunofluorescence methods. Thus, what the main AOB in OMZs are and what their contribution to the nitrogen cycle is remain unknown, since other NH_4^+ oxidizers could also be influencing the NH_4^+ removal in OMZs, such as the anammox bacteria (23, 49) and perhaps even the recently-reported ammonia-oxidizing archaea (11, 56).

Herein, we address the first question by exploring the community structure of βAOB in an O_2 gradient associated with the OMZ off northern Chile (~20°S) using phylogenetic analysis of the genes coding for the 16S rRNA and the ammonia monooxygenase enzyme active subunit (*amoA*).

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▽ Published ahead of print on 6 April 2007.

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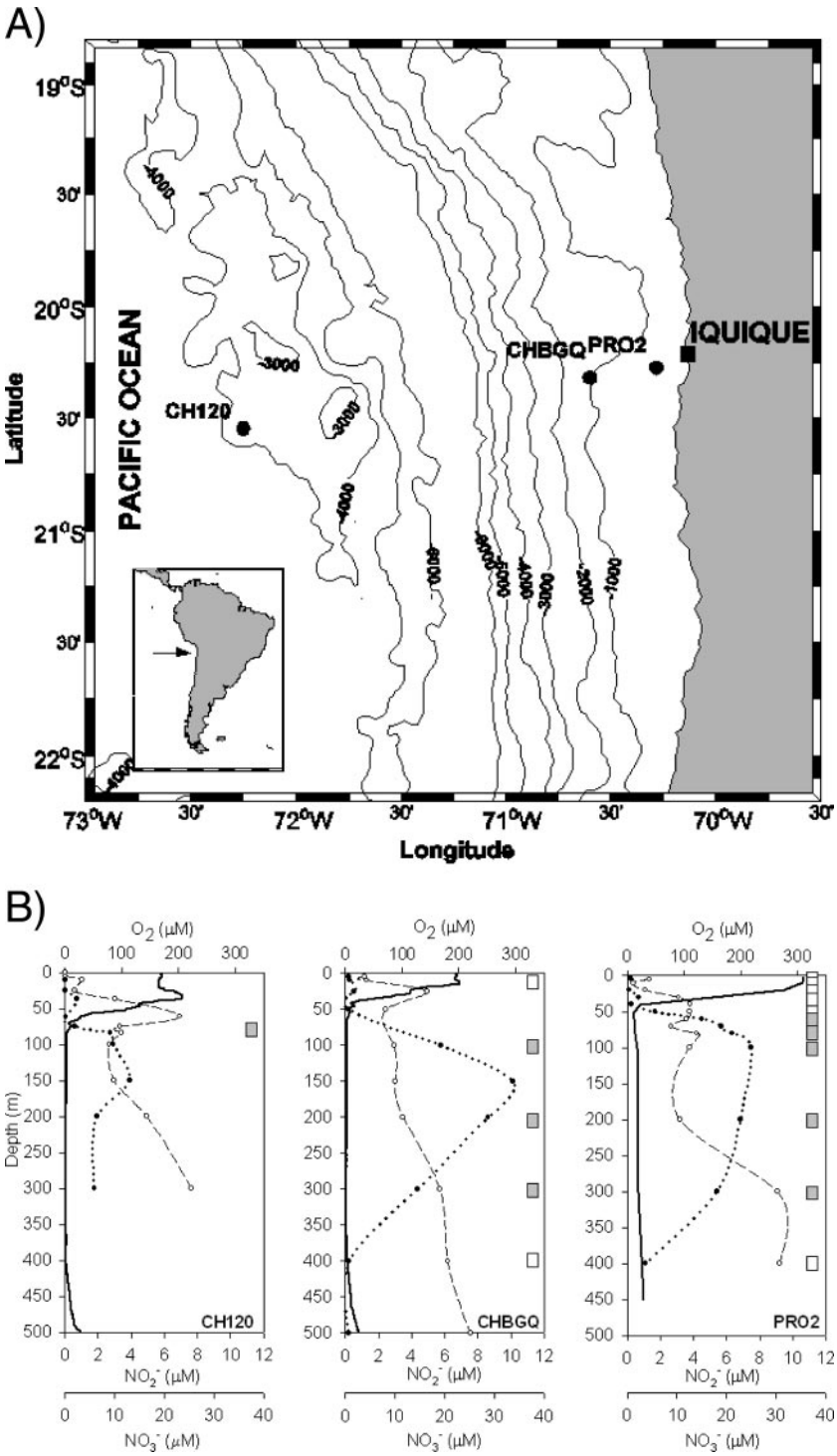

FIG. 1. (A) Map of the study area showing the sampling stations off the northern Chile coast (~20°S, Iquique): CH120 and CHBGQ (CHUPS cruise, March 2003) and PRO2 (PRODEPLOY cruise, July 2004). (B) Distribution of dissolved oxygen (solid line), nitrite (dotted line with black circles), and nitrate (dashed line with white circles) and the sampling depths from which DNA was extracted. White and gray boxes indicate the samples derived from the more-oxygenated and oxygen-deficient waters, respectively.

MATERIALS AND METHODS

Sample collection and chemical analyses. The study area (Fig. 1) was visited during March 2003 (stations CH120 and CHBGQ, CHUPS cruise onboard R/V *Abate Molina*) and July 2004 (station PRO2, PRODEPLOY cruise onboard R/V *Carlos Porter*). The physical and chemical characteristics of the water column were measured using a conductivity-temperature-depth profiler with an O_2 sensor (Seabird). Profiles of dissolved O_2 concentrations were examined to select the depths for collecting water samples for DNA extraction. The O_2 gradient sampled was between ~2 and 250 μM O_2. Seawater (~10 liters) was collected for environmental DNA isolation at each station using Niskin bot-

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TABLE 1. Primers used to amplify 16S RNA and ammonia monooxygenase active subunit (*amoA*) genes, specific target groups, nucleotide positions, and particular annealing temperature for PCR

Primer set	Base sequence (5′ to 3′)	Nucleotide position	Target group	Annealing temp (°C)	Reference
EUB 1	GAGTTTGATCCTGGCTCAG	9[a]	General eubacteria	40	25
EUB 2	AGAAAGGAGGTGATCCAGCC	1542[a]			
NIT A	CTTAAGTGGGGAATAACGCATCG	137[a]	βAOB	57	51
NIT B	TTACGTGTGAAGCCCTACCCA	1234[a]			
βAMO f	TGGGGRATAACGCAYCGAAAG	141[a]	βAOB	57	28
βAMO r	AGACTCCGATCCGGACTACG	1320[a]			
amoC305f[d]	GTGGTTTGGAACRGICARAGCAAA	1012[c]	*amoC*	57	36
amoA1f	GGGGTTTCTACTGGTGGT	332[b]	*amoA*	50	44
amoA2r	CCCCTCKGSAAAGCCTTCTTC	822[b]			
amoA34f[d]	GCGGCRAAAATGCCGCCGGAAGCG	28[b]	*amoA*	57	This study

[a] *E. coli* 16S rRNA genes.
[b] *Nitrosomona europaea* ammonia monooxygenase gene (*amoA*).
[c] *Nitrosospira* sp. strain NpAV.
[d] Combined with amoA2r.

tles attached to a rosette at the following nominal depths: CH120 (82 m), CHBGQ (10, 100, 200, 300, and 400 m), and PRO2 (2, 10, 20, 40, 50, 60, 80, 100, 200, 300, and 450 m).

Samples were prefiltered serially through a 20-μm mesh and a 5-μm filter and then filtered onto a 47-mm-diameter 0.22-μm membrane Nuclepore filter under a gentle vacuum flow. After filtration, each filter was soaked with 1 ml of DNA buffer (50 mM Tris-HCl [pH 9.0], 0.75 M sucrose, 400 mM NaCl) and stored in liquid nitrogen during transport and then at −80°C until DNA extraction in the laboratory.

In addition, discrete water samples were collected for determination of N_2O, NH_4^+, NO_3^-, and NO_2^- concentrations. To avoid oxygenation, the headspace of the Niskin bottles was filled with N_2 under atmospheric pressure. Samples for N_2O analyses were processed according to Castro-González and Farías (7). NH_4^+ (40 ml, triplicate) was determined by a fluorometric method (17) using a Turner Designs 10-AU fluorometer. Samples for NO_3^- and NO_2^- determination were filtered (GF/F; Whatman) and stored frozen until later analysis with an automatic analyzer (ALPKEM, Flow Solution IV).

Hydrographic data associated with the CHUPS cruise have been published by Castro-González and Farías (7) and have been submitted for publication by V. Molina and L. Farías.

DNA extraction. The procedure used to extract environmental DNA was modified from that described by West and Scanlan (55). Briefly, filters were cut into small pieces and placed in 3 ml of lysis solution (45 mM glucose, 23 mM Tris [pH 8.0], 59 mM EDTA) containing lysozyme at a concentration of 1 mg ml^{-1}. This mixture was incubated for 1 h at 37°C, frozen at −20°C (15 min), and then thawed at 50°C. DNA was extracted by adding 350 μl of 10% sodium dodecyl sulfate and 33.5 μl of a proteinase K solution (10 mg ml^{-1}), followed by incubation at 37°C (30 min) and at 55°C (10 min). This solution was mixed gently with the pipette, frozen at −20°C (15 min), and then thawed at 50°C. The thawed mixture was extracted with water-saturated phenol (neutralized with 0.5 M Tris-HCl buffer [pH 7.8 to 8.5])–chloroform–isoamyl alcohol (25:24:1) and centrifuged at 4,000 rpm (5 min). After centrifugation, the aqueous phase was carefully removed and again the DNA was extracted with chloroform-isoamyl alcohol (24:1) and centrifuged at 4,000 rpm (5 min). DNA was precipitated by adding 0.4 volume of sodium acetate (7.5 M) and 2 volumes of ice-cold ethanol (95%). The mixture was kept at −80°C for 20 min and then centrifuged at 13,000 rpm (10 min). The DNA pellet was washed with 70% ethanol, left to dry for 10 to 15 min, resuspended in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and stored at −80°C. DNA was examined in an agarose gel. For PRODEPLOY cruise samples, the DNA extraction procedure was modified by the introduction of a physical breakage step according to Stevens et al. (48).

PCR. The primers used to amplify 16S rRNA genes (CHUPS and PRODEPLOY cruise samples) and *amoA* genes (PRODEPLOY cruise samples), as well as their target groups, positions, particular annealing temperatures, and references are shown in Table 1. The forward primer amoA34f was designed to target a position close to the 5′ region of the *amoA* gene, in order to retrieve a longer *amoA* fragment than if the amoA1f primer was used. This new primer was generated by visual inspection of an alignment of 27 full-length *amoA* gene sequences from 14 AOB species of the following genera: *Nitrosomonas* (7 species), *Nitrosospira* (5 species, including *Nitrosolobus multiformis* and *Nitrosovibrio tenuis*), and *Nitrosococcus* (2 species). The primer targets a conserved region in βAOB and does not match γAOB. PCR amplification using amoA34f in combination with reverse primers, such as amoA2r (44), produces a fragment of approximately 800 bp. The primer specificity was evaluated experimentally with several cultures of known βAOB (e.g., *Nitrosomonas eutropha*, *Nitrosomonas europaea*, *Nitrosospira briensis*, *Nitrosospira multiformis*, and *Nitrosovibrio tenuis*) and also non-βAOB available in the laboratory (e.g., *Mesorhizobium loti* and *Rhizobium tropici*).

Direct and nested PCRs, using genomic DNA and PCR-EUB 1–2 and *amoC-amoA* products as templates, respectively, were used in this study, due to the low abundance of AOB in environmental samples (53, 54). PCRs used to obtain 16S rRNA genes from known βAOB were conducted employing the nested PCR approach, using EUB 1–2 PCR products as template (with NIT A-B and βAMO f-r primer sets) and directly using genomic DNA as template (NIT A-B primers). For all of the 16S rRNA gene amplifications, a 50-μl PCR was carried out using a master mix containing 1 μl template DNA (~10 ng) or PCR product (in the case of nested PCR) and a final concentration of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1% Triton X-100, 1.25 mM $MgCl_2$; Roche), 200 μM each deoxynucleotide triphosphate, 0.1 μM each primer (forward and reverse), and 1 U of *Taq* DNA polymerase (Roche). For the 16S rRNA gene amplification, a thermocycler program consisting of the following steps was used: 5 min at 94°C and then 35 cycles of 30 s at 94°C, 45 s at annealing temperature according to the primer (see Table 1), and 1.5 min at 72°C. PCRs used to obtain the *amoA* gene from known βAOB were done by a direct (amoA1f-2r primers) and nested (amoA1f-2r and amoA34f-2r) approach using amoC305f-amoA2r PCR products as a template (36, 44). For all of the *amoA* gene amplifications, a 25-μl PCR mixture containing 1 μl template DNA (~10 ng) or PCR product (in the case of nested PCR) and a final concentration of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl; Roche), 4 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 0.1 μM each primer (forward and reverse), 100 mg/ml bovine serum albumin (Sigma), 50% formamide, and 1 U of *Taq* DNA polymerase (Roche) was used. A thermocycler program consisting of 5 min at 94°C, then 35 cycles of 1 min at 94°C, with 1 min at annealing temperature according to the primer (see Table 1) and 2 min at 72°C, and with a final extension of 7 min at 72°C was used for all *amo* amplifications. All PCRs were carried out with positive (*Nitrosomonas europaea*) and blank controls. PCR products were run together with appropriate ladders (1,000 or 100 bp) and visualized on agarose gels using standard electrophoresis procedures.

Clone library construction, sequencing analyses, and phylogenetic affiliation within βAOB. The DNA samples obtained during the CHUPS cruise were used to characterize βAOB through 16S rRNA gene analyses. In addition, DNA samples from a second cruise (PRODEPLOY) were used to study the *amoA* genes and to corroborate 16S rRNA genes results from the CHUPS cruise. βAMO f-r products were chosen at the beginning of this study because they are less specific than NIT A-B, thus reducing the risk of missing closely related sequences of βAOB (50) and avoiding potential biases against *Nitrosospira*-like sequences with NIT A-B (19). NIT A-B products were chosen during the second survey because a higher abundance of βAOB-like sequences were obtained in the NIT A-B clone libraries than with βAMO f-r, and the sequences that originated with NIT A-B and βAMO f-r were almost identical. The 16S rRNA gene clone libraries constructed during this study and specific labels are listed in Table 2. *amoA* gene clone libraries were constructed using a nested approach as

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TABLE 2. Number of βAOB-like sequences in the 16S rRNA gene clone libraries generated during this study

Cruise	Depth (m)[a]	Primer or template used for library construction and PCR amplification		Library size (no. of clones)	No. of βAOB-like clones
		Primer	Template		
CH120	82*	AMO f-r	EUB 1–2	130	37
		NIT A-B	Genomic DNA	36	36
CHBGQ	10	βAMO f-r	EUB 1–2	46	12
		NIT A-B	Genomic DNA	44	44
	100*	βAMO f-r	EUB 1–2	91	1
PRO2	40	NIT A-B	Genomic DNA	76	76
	80*	NIT A-B	Genomic DNA	54	18
		NIT A-B	EUB 1–2	30	0
	300*	NIT A-B	EUB 1–2	46	0

[a] *, depth within the OMZ according to the presence of the secondary nitrite maximum (Fig. 1).

follows. amoC305f-amoA2r products were used as templates (36, 44), and the following inner primers were used: amoA34f-amoA2r primers were used for samples PRO2-40 m, PRO2-80 m, and PRO2-300 m, and amoA1f-2r primers were used for samples PRO2-2 m, PRO2-10 m, PRO2-40 m, PRO2-80 m, and PRO2-300 m. A single clone library was also obtained using amoA1f-2r products originating directly from the genomic DNA obtained from sample PRO2-40 m. All of these products were gel purified by using QIAquick PCR purification kit (QIAGEN).

Cloning was performed using the TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. Briefly, the PCR products were ligated into a plasmid vector (pCR4-TOPO vector with genes coding for kanamycin resistance). Competent *Escherichia coli* cells (provided by the manufacturer) were transformed with the ligate and incubated at 37°C (~24 to 48 h) on agar plates containing LB medium with kanamycin (50 μg ml^{-1}) and spread with 40 μl (40 mg ml^{-1}) of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White or light-blue colonies were picked, and inserts of the correct size were evaluated by PCRs using M13 f-r vector-specific primers and agarose electrophoresis. For sequencing, M13 f-r PCR products were purified using multiscreen plates (Millipore). All cycle sequencing reactions were carried out using the Big Dye Terminator v3.1 cycle sequencing kit (ABI PRISM) and analyzed using a 3100 genetic analyzer (ABI, Applied Biosystems). 16S rRNA clone libraries were prescreened by sequencing using primer 341 f 16S rRNA genes of *E. coli* (32). The sequences were analyzed by generating multiple alignments and by comparison with the NCBI database using BLAST. Based on this screening, clones were selected for complete sequencing. Full-length sequencing was performed using five additional primers: M13 f, 926 f, M13 r, 534 r, and 907 r (24, 32). For *amoA* gene products, all clones were sequenced with M13 f and M13 r. Forward and reverse sequences were manually inspected using the Autoassembler (ABI Biosystems) and SeqMan (DNASTAR-Lasergene v6) software packages, and consensus sequences were constructed. These sequences were checked for chimeras (using CHECK-CHIMERA at the Ribosomal Database Project II and Bellerophon chimera detection programs, available from http://35.8.164.52/cgis/chimera.cgi?su = SSU). Consensus sequences constructed by full overlap of forward and reverse sequences were used for further analyses. The phylogenetic analyses were conducted using the Phylip software package (10), available from http://evolution.genetics.washington.edu/phylip/software.html, and the MEGA version 3.0 software package (22), available from http://www.megasoftware.net/. The phylogenetic analyses were based on maximum likelihood (ML) and distance matrix methods (neighbor joining [NJ]). Trees were drawn using the program Bosque for Linux (http://www.profc.udec.cl/bosque). Nucleotide and amino-acid sequences were used for phylogenetic analyses of the *amoA* gene. Amino acid sequences were translated from nucleotide sequences using MEGA and corroborated by the translate tool in ExPASy (http://www.expasy.org/tools/dna.html). The different phylogenetic analyses were compared visually, and a consensus tree was created based on ML topology. Multifurcations were generated when inconsistencies between ML and NJ results were found according to Ludwig et al. (27) and Purkhold et al. (40).

Nucleotide sequence accession numbers. The full sequences of the 16S rRNA gene clones have been deposited in GenBank under accession no. DQ009933 to DQ009964 (CHUPS) and DQ335526 to DQ335537 and DQ813659 (PRODEPLOY). The *amoA* clone sequences have been deposited in GenBank under accession no. DQ519036 to DQ519048.

RESULTS AND DISCUSSION

Biogeochemical characteristics of the water column. During this study, we identified some of the typical geochemical features that delineate the presence of an OMZ (Fig. 1). Vertical distributions of NO_2^- and NO_3^- for the CHUPS cruise data have been published by Castro-González and Farías (7). The water column was characterized by a steep oxycline (300 to ≤10 μM O_2) situated within the top 50 and 70 m at the coastal stations CHBGQ and PRO2 and oceanic station CH120, respectively. Below the oxycline, oxygen-deficient waters (~2 to 10 μM O_2) were detected at all stations from the base of the oxycline until ~400 m in depth. The oxygen-deficient waters presented a high accumulation of NO_2^- (up to 8.7 μM) in a prominent secondary nitrite maximum. This secondary nitrite maximum also coincided with NO_3^- concentrations (9.8 to 13.9 μM) lower than those expected from the Redfield stoichiometry (Fig. 1). Nitrogen deficits between 10 and 21 μM were estimated within the OMZ using the N* approach (14; data not shown), suggesting important nitrogen removal from this layer. The OMZ was also characterized by low NH_4^+ (≤0.14 μM) and N_2O (≤32 nM) concentrations (data not shown). In contrast, the upper oxycline not only presented lower NO_2^- and higher NO_3^- concentrations (Fig. 1) but also showed higher NH_4^+ and N_2O concentrations (up to 0.81 μM and up to 69.2 nM, respectively [data not shown]). N_2O and NH_4^+ vertical distributions for the CHUPS cruise had been published by Castro-González and Farías (7) and have been submitted for publication by Molina and Farias, respectively.

These features reflect the strong nitrogen cycling associated with the OMZ off northern Chile, which appears to be influenced by denitrification and nitrification (7, 9a). In fact, during the CHUPS cruise, high rates of NO_3^- reduction (up to 9.1 μM day^{-1}), denitrification (~3.2 μM day^{-1}), and the potential contribution of denitrifiers and nitrifiers to the local N_2O production (92 and 8%, respectively) were measured (7). During the same cruise, high potential aerobic NH_4^+ oxidation and carbon fixation activity believed to be caused by the presence of AOB were found, with respective rates of up to 1.0 μM NH_4^+ day^{-1} and up to 0.22 μg C day^{-1} (Molina and Farias, submitted). These rates suggest that an AOB community is present and may contribute significantly to nitrogen and carbon cycles within the oxyclines and OMZ off northern Chile.

βAOB potential presence in the water column. Positive PCR amplifications of the 16S rRNA and *amoA* genes related to βAOB were obtained in all instances when the nested PCR approach was used (see Materials and Methods). However, direct PCR on genomic DNA using NIT A-B, amoC305f-amoA2r, and amoA1f-2r primers generated positive results only in some of the samples. NIT A-B and amoA1f-2r products showed positive and more intense bands mainly in the samples retrieved from the upper part of the water column (2- to 100-m depth [data not shown]). PCR amplifications of 16S rRNA genes (using NITA-B and βAMO f-r primers) and *amoA* genes in the study of βAOB have been used as indicators of the presence of this group in environmental samples (20, 53, 54),

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and therefore the direct PCR amplification from genomic DNA obtained at some depths and with a higher intensity of the PCR product could indicate variability in the AOB target abundance in the water column. Indeed, Ward et al. (52) found vertical variation in the abundance of AOB in the water column off Peru by immunofluorescence staining against *Nitrosomonas* sp. (marine) and a *Nitrosococcus oceanus* strain, where the highest abundances were concentrated in the upper boundary of the OMZ, ~55-m depth (1.8×10^5 cells liter^{-1}). One must be cautious to infer the presence of βAOB using NIT A-B direct PCR results, as they could include non-βAOB sequences (see cloning results below). However, NIT A-B combined with amoA1f-2r direct PCR may provide further evidence that βAOB are more abundant in the upper part of the water column off northern Chile, including the surface oxic, hypoxic, and suboxic waters that comprise the upper part of the OMZ. Nevertheless, to determine the actual abundance and distribution of this group in the water column, the application of other techniques, such as fluorescence in situ hybridization or quantitative PCR, would be required.

16S rRNA gene cloning. The number of βAOB in the 16S rRNA genes clone libraries derived from 341 f primer sequences analyses are listed in Table 2. In general, nested clone libraries constructed with βAMO f-r and NIT A-B PCR products showed a lower βAOB recovery compared to NIT A-B PCR products that originated directly from genomic DNA (i.e., ≤26% and ≥33%, respectively). All the βAOB-like sequences retrieved from βAMO f-r and NIT A-B clone libraries presented a sequence similarity after BLAST of ≥95% with *Nitrosospira*-like sequences. Most of the non-βAOB-like sequences were related to uncultured β- and γ-proteobacteria (data not shown). The low number of clones and sometimes absence of βAOB clone recovery within nested 16S rRNA genes clone libraries have been previously reported for planktonic samples and were potentially related to the abundance of this group in the water column (39). The results of our 16S rRNA clone libraries (Table 2) indicate the need to obtain PCR products directly from genomic DNA and also to generate larger clone libraries to study βAOB in environments where only a nested PCR approach can be used.

Except for some sequences retrieved from the OMZ (DQ009934, DQ009935, DQ009939, DQ009953, and DQ813659), the βAOB-like sequences obtained from the upper oxycline and OMZ were ≥99% similar (≤5 substitutions). The sequences DQ009934, DQ009935, DQ009939, and DQ009953 presented an unexpectedly high number of nucleotide substitutions at the beginning of the sequence compared with the rest of the βAOB-like sequences. These differences could be attributed to methodological biases during PCR and were excluded from further phylogenetic analyses. No chimeras were detected in the βAOB-like sequences analyzed. The sequence DQ813659 from the OMZ (PRO2-80 m) showed ~95% similarity (~50 substitutions) compared with other *Nitrosospira*-like sequences from our clone libraries.

The phylogenetic relationships of the full βAOB-like 16S rRNA gene sequences from this study area are presented in a consensus tree in Fig. 2. *Nitrosomonas* and *Nitrosospira* lineages were clearly separated into the expected topology by application of the phylogenetic approaches used in the study by Purkhold et al. (40). Independent of the primer used (βAMOf-r or NIT A-B), most of the βAOB-like sequences retrieved from the OMZ and all the sequences from the upper oxycline (hypoxic/oxic waters) fell within cluster 1 of *Nitrosospira*. This affiliation was congruent using neighbor-joining and maximum likelihood phylogenetic methods with bootstrap values of ≥72%. Cluster 1 exclusively contained sequences derived from uncultured representatives derived from 16S rRNA gene-based environmental and metagenomic surveys (e.g., clone AY458645 from a bacterial artificial chromosome library from Monterey Bay). All of the sequences of this cluster have been retrieved from different marine and marine-associated environments with variable oxygen conditions: i.e., sand dunes (21), anoxic sediments and aggregates (12, 13, 31, 39, 47), planktonic samples from polar oceans (3, 16), the northwestern Mediterranean sea (39), the Loch Duich estuary (13), and Monterey Bay (37).

The sequence DQ813659 retrieved from the OMZ clone library (PRO2-80 m) was affiliated with cultured *Nitrosospira* sp. from cluster 0 (bootstrap values of ≥64%). Cultured *Nitrosospira* sequences from cluster 0 are mainly derived from sand, soil, and freshwater environments (20). To our knowledge, this is the first report of the presence of a sequence related to this cluster cloned from seawater. Our finding was also supported by sequences from *amoA* gene clone libraries from the OMZ (see below).

The dominance of *Nitrosospira*-like sequences in planktonic samples has been found previously (39). This result is not expected to be PCR bias against *Nitrosomonas* spp. because βAMO f-r and NIT A-B primer pairs have been designed to amplify 16S rRNA genes of all known βAOB (28, 50, 51). Indeed, environmental studies show that both primer sets could amplify βAOB representatives of *Nitrosomonas* spp. and *Nitrosospira* spp.: e.g., βAMO f-r (39) and NIT A-B (37). However, in an area further north of our sampling site (~7 to 15°S), Ward et al. (52) detected *Nitrosomonas*-like βAOB by means of immunofluorescence assays using antiserum prepared with *Nitrosomonas* sp. (marine) strains. Unfortunately, the cross-reactivity of *Nitrosospira* spp. with *Nitrosomonas*-derived antibodies is not known (52). Therefore, it is possible that the *Nitrosomonas*-like counts obtained by Ward et al. could have also included *Nitrosospira*-like βAOB. On the other hand, Ward et al. (52) carried out immunofluorescence assays using whole-water samples, potentially including "particle-associated" βAOB, where *Nitrosomonas*-like bacteria have been found to be the predominant group (39). However, in the present study DNA was extracted from prefiltered seawater (see Materials and Methods).

***amoA* gene cloning.** Contrary to the results of 16S rRNA genes, the primers used for the *amoA* gene showed a high specificity. NCBI database screening of clone sequences originating from samples obtained at 40, 80, and 300 m using amoA34f-2r primers (35 sequences) and at 2, 10, 40, 80, and 300 m using amoA1f-2r (41 sequences) showed that all the *amoA* gene clones presented βAOB *amoA*-like sequences as the closest match. No differences related to the primer pair used for preparation of the clone libraries were found: i.e., amoA34f-2r versus amoA1f-2r from 40, 80, and 300 m.

The sequences retrieved from the OMZ (80 and 300 m) and from the surface and upper oxycline (2, 10, and 40 m) presented a low similarity: ~73% and ~87% for both nucleotide and amino acid analyses, respectively.

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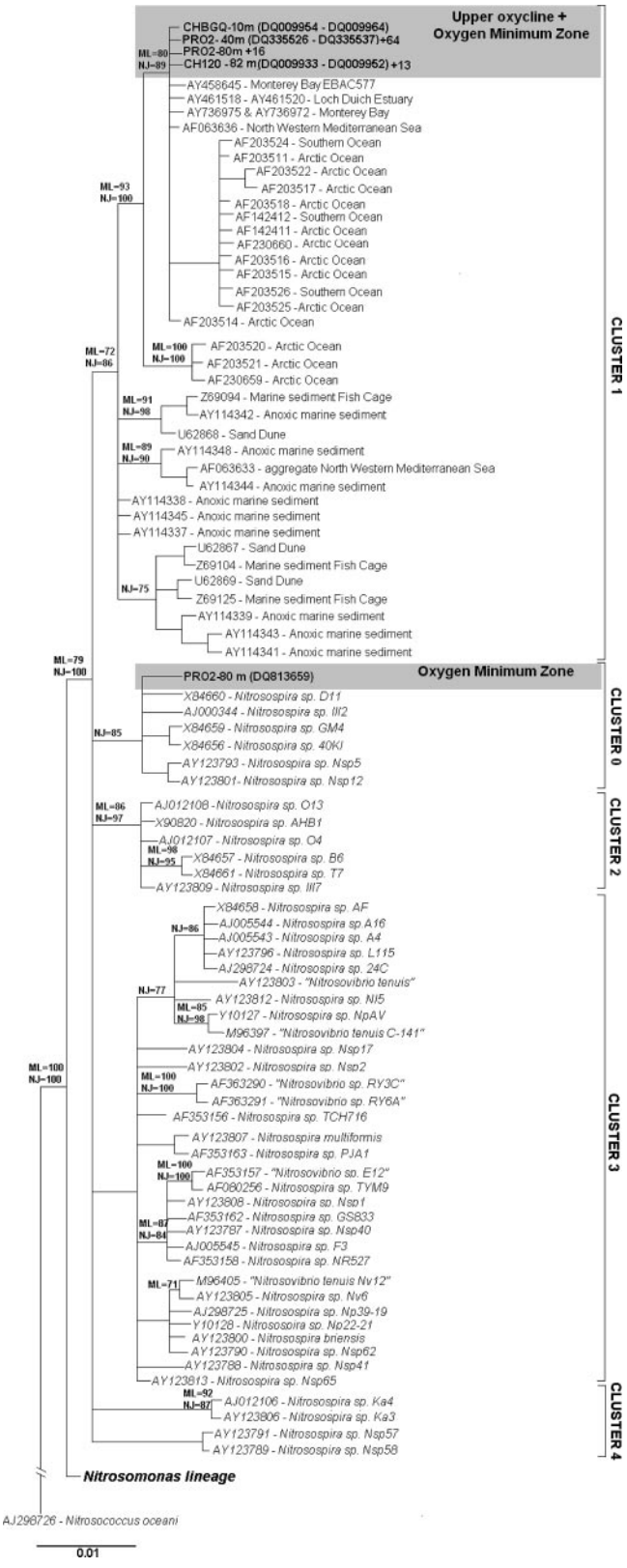

FIG. 2. Consensus phylogenetic tree based on 16S rRNA gene sequences related to ammonia-oxidizing bacteria retrieved from the study area and other cultured and yet-uncultured representatives of this group. The treeing methods were carried out with ≥1 kb of sequence, except for sequences that were generated with primer 341 f (~700 bp), to confirm and compare βAMO f-r primer performance with NITA-B from CHUPS (82- and 10-m depths) and PRODEPLOY (80- and 300-m depths). The clones' origins and accession numbers from this study are depicted in boldface. A plus sign indicates the number of identical sequences represented by each sequence in the corresponding clone library. The numbers in the branches indicate the bootstrap values (100 resamplings) associated with each treeing method used in the analyses (ML and NJ). The scale bar indicates the percentage of substitutions per site.

The relationships among the βAOB-like *amoA* amino-acid sequences from this study are presented in a consensus tree in Fig. 3. The monophyly of the *Nitrosospira* lineage could only be recovered with low bootstrap values (i.e., 62% and 33% by neighbor joining and maximum likelihood, respectively), whereas the *Nitrosomonas* lineage monophyly was only obtained with the maximum likelihood approach with a bootstrap value of 40%. These results were expected for the *amoA* gene marker phylogeny, since it generally shows a lower bootstrap support of *Nitrosospira* and *Nitrosomonas* lineages compared to 16S rRNA genes (40). The *amoA* gene sequences retrieved from the OMZ were affiliated within the *Nitrosospira* lineage, with bootstrap values of 60% and 29% (NJ and ML, respectively). The OMZ *amoA* gene sequence subcluster also contains sequences from other yet-uncultured βAOB from soil (1; M. Grimm, unpublished data [GenBank]) and from an aerated-anoxic Orbal process waste treatment plant (38), as well as sequences from cultured *Nitrosospira* strains isolated from soil, *Nitrosospira* sp. strain AHB1 (43), and *Nitrosospira* sp. strain Nsp12 (40). Within the cultured representatives, the OMZ *amoA* gene sequences presented a higher similarity (~91% and ~95%, nucleotide and amino acid, respectively) with *Nitrosospira* sp. strain Nsp12. According to the 16S rRNA gene phylogeny nomination of Purkhold et al. (51), Nsp12 affiliates with *Nitrosospira* cluster 0.

The surface and upper-oxycline *amoA* gene sequences grouped in a different subcluster within the *Nitrosospira* lineage, supported by bootstrap values of ≥71% (NJ and ML). This subcluster consists exclusively of yet-uncultured sequences retrieved from different marine environments: i.e., Monterey Bay (37), South Chesapeake Bay (10), Plum Island (4), Kysing Fjord (34), Guaymas Basin (P. Lam, unpublished data [GenBank]).

Environmental covariates, such as salinity, pH, temperature, and fertilizers, have been found to correlate with richness and diversity of *amoA* genes in other ecosystems (2, 4). Therefore, the differences observed by the *amoA* gene marker within the surface and upper oxycline and OMZ off northern Chile could indicate that oxygen and/or other variables linked with this environmental factor in the water column (e.g., NH_4^+ and NO_2^-) might control vertical variability in the βAOB community structure.

16S rRNA and *amoA* gene phylogeny inference. Similarities in the topology of the *amoA* and 16S rRNA gene phylogenies have been found in other marine environments (5, 37). In our study, the *amoA* gene sequences from the surface and upper oxycline were grouped in a subcluster together with sequences from marine uncultured organisms, analogous to sequences from 16S RNA genes into *Nitrosospira* cluster 1. A similar 16S

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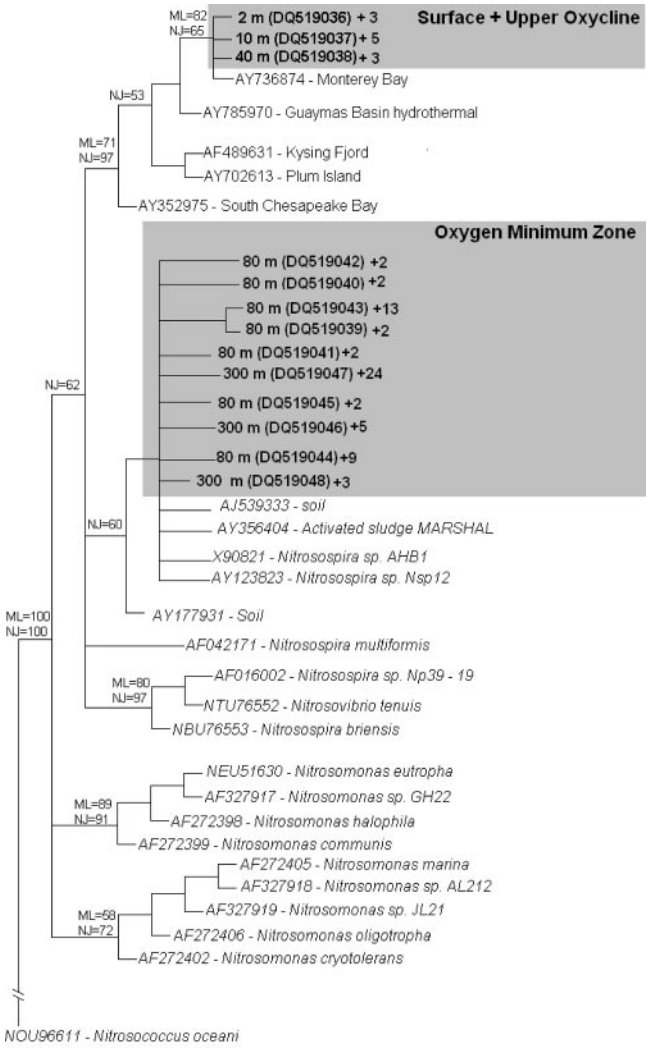

FIG. 3. Consensus phylogenetic tree based on ≥149-amino-acid-residue sequences of the ammonia monooxygenase active subunit gene (*amoA*) related to ammonia-oxidizing bacterial sequences retrieved from the study area and other cultured and yet-uncultured representatives of this group. The clone origins and accession numbers from this study are depicted in boldface. A plus sign indicates the number of identical sequences represented by each sequence in the corresponding clone library. The numbers in the branches indicate the bootstrap values (100 resamplings) associated with each treeing method used in the analyses (ML and NJ). The scale bar indicates the percentage of sequence divergence.

rRNA and *amoA* gene phylogeny agreement was found by O′Mullan and Ward (37) in Monterey Bay. The combined results support a potential connection between the widespread *Nitrosospira*-like cluster 1 16S rRNA gene sequences and the functional gene *amoA* subcluster.

All of the *amoA* gene sequences that originated from the OMZ were closely affiliated with culture *Nitrosospira* from clusters 0 and 2. Also, a single 16S rRNA gene sequence affiliated with cluster 0 was also found in the OMZ, a coincident result with OMZ *amoA* gene sequences. Furthermore, no *amoA* gene sequences related to uncultured marine *Nitrosospira* were found in the OMZ, despite *Nitrosospira*-like cluster 1 being dominant in 16S rRNA gene libraries retrieved from this layer. One explanation for this discrepancy would be a methodological bias related to the PCR. It is possible that the nested approach used to obtain *amoA* gene products from the OMZ may have favored one target over another. However, in the case of the upper-oxycline DNA samples, the direct and nested *amoA* gene PCR products generated the same βAOB group, which differed from the ones found in the OMZ. Hence, a bias in the PCR does not seem to be a likely explanation.

Another possibility could be that the *amoA* marker in the clone libraries reflects the βAOB abundance in the water column and that the βAOB group within the OMZ presented a higher number of *amoA* copies. Cultured βAOB have between two and three *amoA* copies in their genome (20). Therefore, in the OMZ a higher number of *amoA* gene targets might be present, potentially related to the single 16S RNA gene type affiliated to *Nitrosospira* cluster 0 rather than *Nitrosospira*-like cluster 1. Moreover, the *amoA* found in the OMZ might be related to the 16S RNA genes of *Nitrospira*-like cluster 1. In this case, a potential divergence at the functional level in the OMZ might be an indication of an evolutionary pressure, resulting in the presence of distinct ecotypes of *Nitrosospira*-like βAOB. Further studies must be conducted in order to establish the connection between 16S RNA and functional genes of βAOB in this and other marine environments, for example, by using metagenomic approaches.

Both of the latter explanations about the discrepancy between 16S RNA and *amoA* genes are linked to the presence of a "functional community shift," based on the clear separation between the βAOB communities from the upper oxycline and the OMZ layers when using the *amoA* gene, a marker of functional diversity according to Castro et al. (6a). The upper-oxycline βAOB community should be able to cope with the strong physicochemical gradients and high NH_4^+ availability characteristic of this layer. For example, a strong NH_4^+ cycling by bacterioplankton and flagellate communities has been found in the upper oxycline off northern Chile (29). In contrast, the OMZ βAOB community should be adapted to the more stable but oxygen-NH_4^+-deficient conditions. The presence of a *Nitrosospira*-like cluster 0 sequence in the OMZ suggests also an additional biogeochemical role for the βAOB. For example, members of *Nitrosospira* cluster 0 showed high rates of nitrifier denitrification (45). However, this process has not yet been measured in OMZs.

In summary, analyses of the functional *amoA* gene showed that the OMZ was clearly differentiable from the oxycline and oxic surface layer, but this was not the case when the 16S rRNA gene approach was used. On the other hand, the 16S rRNA and *amoA* genes showed the presence of βAOB sequences closely affiliated with *Nitrosospira* cluster 0 in the OMZ. These OMZ sequences of *amoA* were not novel, as was, for example, the case for the *nirS* sequences of denitrifiers (6a), but until now they have not been reported in marine environments. Finally, the 16S rRNA gene analyses confirm the dominant presence of the still-uncultured *Nitrosospira*-like cluster 1 in clone libraries of βAOB in different marine environments, which now include OMZs. The ubiquity and apparent metabolic plasticity of *Nitrosospira*-like cluster 1 point to the need to determine their ecology and biogeochemical role.

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ACKNOWLEDGMENTS

We acknowledge G. Alarcón, C. Burghardt, M. Castro-González, K. M. Fuentes, F. González, O.-S. Kim, J. F. Santibañez, and K. Wiedenhoeft for their help in the laboratory and in the field and the crews of the *Abate Molina* and *Purihaalar*. We thank H. Stevens and H. Bouman for comments that improved the manuscript and W. Rojas for providing the map of the study area.

This research was financed by the Comisión Nacional de Investigaciones Científicas y Tecnológicas (CONICYT) through FONDECYT grant no. 1030741 and FONDAP grant no. 150100007 and was carried out partly during DAAD (2004) and by an MPI (2005) short-stay visitor's grant in the MPI für Limnologie. V.M. was supported by Fundación Andes and MECESUP UCO002 Ph.D. grants and by a Marine Genomics postdoctoral fellowship (PBCT RUE 004).

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