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Urease-Encoding Genes in Ammonia-Oxidizing Bacteria†

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Many but not all ammonia-oxidizing bacteria (AOB) produce urease (urea amidohydrolase, EC 3.5.1.5) and are capable of using urea for chemolithotrophic growth. We sequenced the urease operons from two AOB, the β-proteobacterium *Nitrosospira* sp. strain NpAV and the γ-proteobacterium *Nitrosococcus oceani*. In both organisms, all seven urease genes were contiguous: the three structural urease genes *ureABC* were preceded and succeeded by the accessory genes *ureD* and *ureEFG*, respectively. Green fluorescent protein reporter gene fusions revealed that the *ure* genes were under control of a single operon promoter upstream of the *ureD* gene in *Nitrosococcus oceani*. Southern analyses revealed two copies of *ureC* in the *Nitrosospira* sp. strain NpAV genome, while a single copy of the *ure* operon was detected in the genome of *Nitrosococcus oceani*. The *ureC* gene encodes the alpha subunit protein containing the active site and conserved nickel binding ligands; these conserved regions were suitable primer targets for obtaining further *ureC* sequences from additional AOB. In order to develop molecular tools for detecting the ureolytic ecotype of AOB, *ureC* genes were sequenced from several β-proteobacterial AOB. Pairwise identity values ranged from 80 to 90% for the UreC peptides of AOB within a subdivision. UreC sequences deduced from AOB urease genes and available UreC sequences in the public databases were used to construct alignments and make phylogenetic inferences. The UreC proteins from β-proteobacterial AOB formed a distinct monophyletic group. Unexpectedly, the peptides from AOB did not group most closely with the UreC proteins from other β-proteobacteria. Instead, it appears that urease in β-proteobacterial autotrophic ammonia oxidizers is the product of divergent evolution in the common ancestor of γ- and β-proteobacteria that was initiated before their divergence during speciation. Sequence motifs conserved for the proteobacteria and variable regions possibly discriminatory for *ureC* from β-proteobacterial AOB were identified for future use in environmental analysis of ureolytic AOB. These gene sequences are the first publicly available for *ure* genes from autotrophic AOB.

We are interested in the metabolic diversity of ammonia-oxidizing bacteria (AOB) and the selective advantage that individual traits may afford these specialized bacteria. AOB obtain usable energy and reductant solely from ammonia and fix carbon autotrophically (5, 19). The abilities to hydrolyze urea as a source of ammonia and carbon dioxide and to use the products of ureolysis for modification of the pH in the vicinity of the cell (4) appear to be important ecologically selected traits provided by the urease enzyme. AOB isolated from acidic soils are generally ureolytic (6), and ureolytic AOB can grow at lower pH with urea as an ammonia source (4). Ureolytic AOB may also have an advantage in soils receiving animal wastes or urea fertilizers. Molecular tools for examining functional genes involved in N metabolism in AOB have previously been developed for the genes encoding ammonia monooxygenase (17, 20, 21) and hydroxylamine oxidoreductase (3). In order to develop tools to examine the functional diversity of the AOB and to explore how the ammonia monooxygenase (*amo*) and urease (*ure*) genes relate to each other, a survey of the urease genes of AOB was required.

Bacterial urease is a trimer of three subunits (encoded by *ureA, ureB,* and *ureC*) and requires up to four accessory proteins for activation and Ni^{+} incorporation (most commonly encoded by *ureD*, *ureE*, *ureF*, and *ureG*) (7). The urease peptides have highly conserved active sites and Ni^{+} binding residues (15). In this study, we examined the urease (*ure*) operon in cultured AOB from the *Nitrosospira*, *Nitrosomonas*, and *Nitrosococcus* genera. These gene sequences are the first publicly available for *ure* genes from autotrophic AOB.

MATERIALS AND METHODS

Bacterial strains, culture maintenance, and DNA isolation. The bacterial strains used in this study are listed with references and source information in Table 1. Standard growth conditions and media have been described previously (17). Production of nitrite from urea was assessed on standard growth media with filter-sterilized urea replacing ammonium as the substrate at a final concentration of 2.0 mM. The production of nitrite from urea was monitored colorimetrically with the Griess-Ilosvay reaction (10) over a 4-week period. DNA was isolated from late-stationary-phase cultures by the procedures of McTavish et al. (14). Genomic DNA was stored at −20°C in deionized water until used. In the case of *Nitrosospira* sp. strain NpAV, after determining the sequences of all *ure* genes from genomic DNA, a copy-specific template was prepared by isolation of a 13-kb HindIII DNA fragment from an agarose gel as previously described for individual *amo* operon copies (18). This procedure was not necessary for *Nitrosococcus oceani*.

Primers, PCR amplification, and sequencing. Degenerate primers for *ureC* genes were designed from conserved regions within the *ureC* gene from *Ralstonia eutropha* (AEY13732) and other ureolytic proteobacteria. These and other primers were commercially prepared by Genemed Synthesis Inc. (San Francisco, Calif.) or Biosynthesis Inc. (Lewisville, Tex.). The 50-μl PCRs contained 0.5 μM each primer, 2.0 mM $MgCl_2$, 0.20 mM deoxynucleoside triphosphate mix, 1×

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TABLE 1. Bacterial strains used in this study and GenBank accession numbers for the *ure* genes[a]

Strain	Relevant characteristics	Nitrite production from urea[a]	Accession no.	Source or reference
Nitrosospira sp. strain NpAV	From agricultural soil in Minnesota	++	AF411008	E. Schmidt (University of Minnesota) via M. Bruns (17)
Nitrosospira briensis C-128	Isolated from soil: same as ATCC 25971	+	AY158897	F. Valois (Woods Hole, Mass.)
Nitrosospira sp. strain 39-19	From agricultural soil in Minnesota	++	Partial	E. Schmidt
Nitrosospira tenuis NV-12[b]	Isolated from soil	+	AY438637	F. Valois (Woods Hole, Mass.)
Nitrosospira multiformis C-71[c]	Similar to ATCC 25196	++	AY168418	F. Valois (Woods Hole, Mass.)
Nitrosospira multiformis 24C[c]	From agricultural soil in Minnesota	++	AY267385	E. Schmidt
Nitrosomonas europaea ATCC 19178	Isolated from soil	+	No *ure* genes detected	ATCC[d]
Nitrosomonas eutropha C-91	Type strain from sewage	+	No *ure* genes detected	F. Valois (Woods Hole, Mass.)
Nitrosomonas cryotolerans ATCC 49181	Type strain, marine	+	AY184498	ATCC
Nitrosococcus oceani C-107	Marine strain, same as ATCC 19707	++	AF417006	F. Valois (Woods Hole, Mass.)
Nitrosococcus sp. strain C-113	Marine strain isolated from Red Sea	−	No *ure* genes detected	F. Valois (Woods Hole, Mass.)

[a] Nitrite production from urea: −, not observed; +, moderate; ++, rapid.
[b] Also known as *Nitrosovibrio tenuis* (8).
[c] Also known as *Nitrosolobus multiformis* (8, 23).
[d] ATCC, American Type Culture Collection.

Taq polymerase buffer B (20 mM Tris-HCl [pH 8.0 at 25°C], 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol, 0.5% Tween 20, and 0.5% Nonidet P-40), approximately 100 ng of template DNA, and 2.5 U of *Taq* polymerase (Promega, Madison, Wis.).

To obtain the desired products, the PCR conditions were as follows: an initial 10-min denaturation step at 94°C, followed by 30 cycles of a 1-min denaturation step (94°C), a 1-min annealing step (55 to 65°C), and a 2-min extension step (72°C). A final extension step of 10 min at 72°C completed the reaction. PCR products were screened on 0.7 to 1% agarose gels, and amplicons of the proper size were gel purified. Selected amplicons were either cloned (pCR2.1-TOPO; Invitrogen, Carlsbad, Calif.) for sequencing or sequenced directly. Once a region of *ureC* was sequenced, specific primers were made from within these known regions that extended toward unknown regions, for which additional degenerate primers were designed. The resulting PCR products were then reamplified for direct sequencing or cloned before sequencing. Correct extension clones were identified by Southern hybridization with probes generated from known sequence (see below).

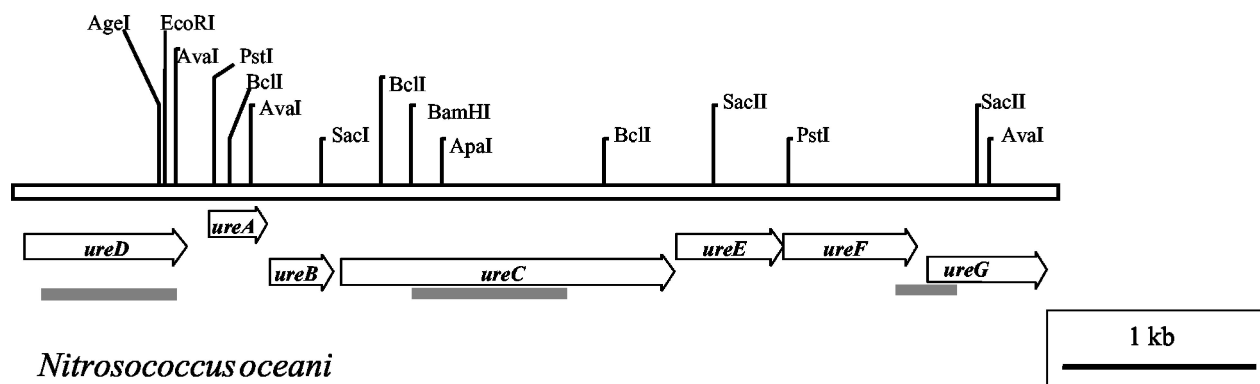
Sequencing was performed on an ABI Prism 3100 (Utah State University Biotechnology Center) or ABI Prism 310 (University of Louisville Biology) genetic analyzers with *Taq*FS terminator chemistry. Sequences were edited and aligned with Sequencher 4.1.1 (Gene Codes Corporation, Madison, Wis.). The sequences obtained were extended upstream and downstream until the entire *ureC* gene or *ure* operon was sequenced. All regions were sequenced in both directions for minimum coverage. When the design of degenerate primers was insufficient to extend the sequence, inverse PCR (also known as chromosome crawling), adapter ligation, or random primer techniques were employed to obtain the sequences at the 5′ and 3′ ends of the operon. For *Nitrosospira* sp. strain NpAV, copy-specific PCR products were obtained with the 13-kb HindIII fragment and sequenced with methods similar to those described for multiple copies of the *amo* operons in these bacteria (17).

Hybridization analysis for the *ure* operon. Hybridization techniques were similar to those of Norton et al. (17). Probes were developed from the *ureC, ureD,* and *ureG* regions of *Nitrosospira* sp. strain NpAV to examine Southern blots of *Nitrosospira* sp. strain NpAV and other closely related organisms within the β-proteobacterial AOB. Separate probes were developed from *Nitrosococcus oceani* for the γ-proteobacterial AOB. Important probe regions are shown in Fig. 1. DNA probes were prepared by PCR-mediated random incorporation of digoxigenin-UTP and chemiluminescent detection (Roche Applied Science). Blots were hybridized and washed under medium- to high-stringency conditions as recommended by the manufacturer and adjusted as necessary depending on target similarity. The molecular mass of the hybridizing fragments was determined with the RFLPscan program (Scanalytics/CSPI, Billerica, Mass.).

Sequence comparison and analysis. Initially, sequence similarities were investigated with the NCBI BLAST program (1). To summarize all available sequence information, full-length UreC protein sequences were aligned with ClustalX version 1.81 (24) with the Gonnet 250 protein weight matrix and gap opening and gap extension penalties of 35/15 and 0.75/0.35, respectively, in the pairwise and multiple sequence alignments. A total of 68 available UreC protein sequences were included in the alignment, and a distance neighbor-joining tree was constructed with the BioNJ function in PAUP* version 4.10b (22) and used as a guide tree for manual refinement of the ClustalX alignment.

The GenBank accession numbers for *ureC* and deduced UreC sequences from the AOB used are given in Table 1. Sources and abbreviations for the other organisms whose *ure* sequences were used in alignments and for phylogenetic inference (shown in Fig. 3) were as follows: *Deinococcus radiodurans* strain R1 (AE001863.1); the cyanobacteria *Nostoc* sp. strain PCC 7120 (NPCC7120; AP003593.1), *Prochlorococcus marinus* PCC 9511 (Pmar; AF242489.1), *Synechocystis* sp. strain PCC 6803 (PCC6803; NC_000911.1), *Synechococcus* sp. strain WH 7805 (WH7805; AF056189.1), *Thermosynechococcus elongatus* BP-1 (Telo; AP005369.1); the α-proteobacteria *Agrobacterium tumefaciens* C58 (Atum; AE009188.1), *Brucella melitensis* biovar abortus strain 2308 (Babo; AF361941.1), *Mesorhizobium loti* strain MAFF303099 (Mlot; AP003005.2), *Rhodobacter capsulatus* B100 (Rcap; AB006984.1), *Sinorhizobium meliloti* (Smel; S69145.1), *Sinorhizobium leguminosarum* biovar viciae UPM791 (Sleg; AF347070.1); the β-proteobacteria *Bordetella bronchiseptica* BB7866 (Bbro; AF000579.1), *Burkholderia fungorum* (Bfung; ZP_00033670.1), *Ralstonia solanacearum* GMI1000 (Rsol; AL646067.1), *Ralstonia metallidurans* (*R. eutropha*) H16 (Rmet; Y13732.1); and the γ-proteobacteria *Azotobacter vinelandii* (Avin; ZP_00088492.1), *Escherichia coli* O157:H7 EDL933, O-island 43 (Ecol933_43; AE005272.1), *Escherichia coli* O157:H7 EDL933, O-island 48 (Ecol933_48; AE005308.1), *Klebsiella aerogenes* CG253 (Kaer; M36068.1 (16), *Microbulbifer degradans* 2-40 (Mdeg; ZP_00066423.1), *Proteus mirabilis* (Pmir; M31834.1), *Proteus vulgaris* DSM 30118 (Pvul; X51816.1), *Pseudomonas aeruginosa* PAO1 (Paer; AE004091), *Pseudomonas fluorescens* PfO-1 (Pfluo; ZP_00083320.1), *Pseudomonas putida* KT2440 (Pputi; AE016784.1), *Pseudomonas syringae* pv. tomato DC3000 (Psyr; AE016873), and *Vibrio parahaemolyticus* TH3996 (Vpar; AB038238.1).

Additional sequences used in alignments (Fig. 4 and collapsed branches of Fig. 3) included the actinobacteria *Actinomyces naeslundii* WVU45 (Anae; AF056321.1), *Corynebacterium glutamicum* ATCC 13032 (Cglu; AJ251883.1), *Mycobacterium tuberculosis* CDC 1551 (Mtub; AE007047.1), *Streptomyces coelicolor* A3(2) (Scoe; AL391014.1); the low-G+C gram-positive bacteria *Bacillus* sp. strain TB-90 (Btb90; AF056321.1), *Bacillus halodurans* (Bhal; AP001507.1), *Clostridium perfringens* CP76 (Cper; Y10356.1), *Lactobacillus fermentum* JCM5869 (Lfer; D10605.1), *Sporosarcina (Bacillus) pasteurii* DSM 33 (= ATCC



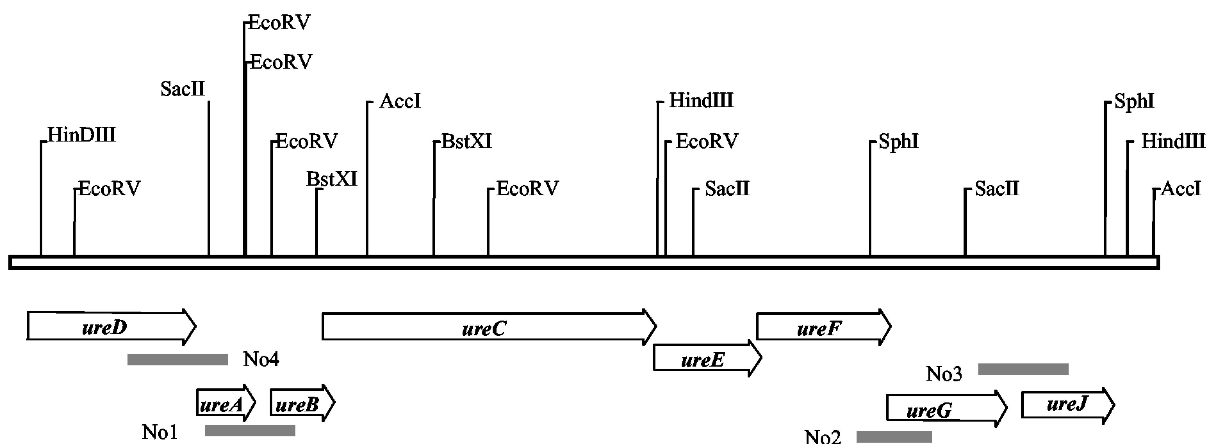


FIG. 1. Urease operon maps of *Nitrosospira* sp. strain NpAV (top) and *Nitrosococcus oceani* (bottom). Structural genes include *ureA*, *ureB,* and *ureC*; the accessory genes are *ureD*, *ureE,* and *ureF*. *Nitrosococcus oceani* primers to make the probes were A101-M104 (No1), M105-R12 (No2), GF1-JR1 (No3), and DF2-AR1 (No4). Probes No1 and No2 were used for the blots shown in Fig. 2. Probes No3 and No4 were used to isolate, clone, and select the operon extension clones.

11859) (Bpas; X78411.1), *Staphylococcus xylosus* (Sxyl; X74600.1), *Streptococcus salivarius* 57.I (Ssal; U35248.1), and *Ureaplasma urealyticum* (Uure; NC_002162.1); the proteobacteria *Actinobacillus pleuropneumoniae* CM5 (Aple; U89957.1), *Brucella melitensis* 16 M (Bmel; AE009506.1), *Helicobacter heilmannii* isolate 2 (L25079.1), *Helicobacter felis* ATCC 49179 (Hfe; X69080.1), *Helicobacter pylori* J99 (HpylJ99; NC_000921.1), *Helicobacter hepaticus* (Hhep; AF332656.1), *Yersinia enterocolitica* 6471/76 (serotype O:3) (Yent; Z18865.1); the crenarchaeon *Sulfolobus tokodaii* (Stok; AP000984.1); and the plant *Oryzae sativa* (indica cultivar group) cultivar IR36 (AB075476.2).

The refined alignment of 68 full-length UreC sequences was used for the inference of phylogeny. Phylogenetic relationships were investigated by character-based tree-searching methods with maximum-parsimony or maximum-likelihood object functions. A maximum-parsimony tree was built from the ClustalX alignment with the PAUP* program with the following in effect: 50% majority consensus; random-order taxon addition replicates, and tree bisection-reconnection branch-swapping, mulpars, and steepest descent functions. The quality of the branching patterns was assessed by bootstrap resampling of the data sets with 100 replications.

Because inclusion or exclusion of a few characters can greatly affect the bootstrap proportions of maximum-parsimony trees derived from limited data sets, we also conducted a maximum-likelihood inference by subjecting the alignment to a Bayesian inference of phylogeny with the program MrBayes (version 3.0b4; written by Huelsenbeck and Ronquist; available at http://morphbank.ebc.uu.se/mrbayes/). In this analysis, the protein sequence alignment was subjected to Metropolis-Coupled Monte Carlo Markov chain sampling over 100,000 generations. Four equally heated Markov chains were used to build a sufficient number of reliable trees after the likelihoods of the trees have converged on a stable value and to allow successful swapping between chains. Three independent runs led to convergence on stable likelihood values after 30,000 generations (data not shown). The searches were conducted by assuming an equal or a gamma distribution of rates across sites and with the JTT empirical amino acid substitution model (9). In a postrun analysis, MrBayes summarized the results concerning tree topology and branch lengths. By ignoring the trees generated before the search converged on stable likelihood values (removed as burn-in), a 50% majority rule consensus phylogram was constructed that displayed the mean branch lengths and posterior probability values of the observed clades. These probability values were comparable to the bootstrap proportions calculated for the branches in the maximum-parsimony consensus tree. To emphasize the positions of the AOB UreC proteins in the tree, the posibacterial clade of the tree was pruned.

Genomic sequence data for *Nitrosomonas europaea* were obtained before publication (5) from the DOE Joint Genome Institute at http://spider.jgi-psf.org/JGI_microbial/html/.

Nucleotide sequence accession numbers. The sequence data available from this project have been submitted to the GenBank database and are available under the accession numbers listed in Table 1.

RESULTS AND DISCUSSION

Initial *ure* gene sequences. We used a preliminary alignment of *ureC* genes and conserved regions previously identified as Ni^{+} binding sites in several proteobacteria (15) to design degenerate primers (ure1F and ure2R; Table 2 and Fig. 4). These primers were used to amplify approximately 300-bp fragments

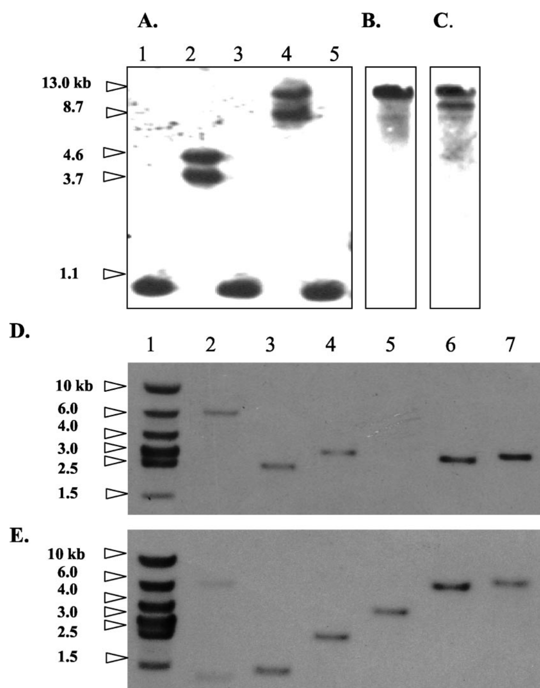


FIG. 2. Southern blots with *ure* probes. (A to C) *Nitrosospira* sp. strain NpAV genomic DNA hybridized with an internal *ureC* probe (A) (lane 1, BclI; lane 2, EcoRI; lane 3, EcoRI plus BclI; lane 4, HindIII; lane 5, HindIII plus BclI), hybridized with an internal *ureD* probe (B) (HindIII digest), or hybridized with a *ureFG* probe (C) (HindIII digest). (D and E) *Nitrosococcus oceani* genomic DNA hybridized with the No1 (*ureAB*) probe (D) and *Nitrosococcus oceani* genomic DNA hybridized with the No2 (*ureFG*) probe (E). Lane 1, molecular size markers; lane 2, SphI; lane 3, SacII; lane 4, HindIII; lane 5, EcoRV; lane 6, BstXI; and lane 7, AccI. The locations of probe sequences and restriction sites important in the interpretation of Fig. 2 are shown in Fig. 1.

from several AOB. Some nonspecific products were also amplified, possibly because other metal binding sites may be targets for these degenerate primers. We focused our efforts on *Nitrosospira* sp. strain NpAV and *Nitrosococcus oceani* as representatives of AOB from the β- and γ-proteobacteria, respectively.

By using the urease gene arrangement of the *Ralstonia* species as a guide (*ureDABCEFG*), random or degenerate primers targeting *ureA* and *ureF* upstream and downstream, respectively, of *ureC* were paired with specific *ureC* primers to acquire flanking sequences. An iterative process allowed us to obtain the entire sequence (extending from *ureD* to *ureG*) of the *ure* operons for *Nitrosospira* sp. strain NpAV and *Nitrosococcus oceani*. The maps of both operons are shown in Fig. 1. While the codon usage was significantly different in *Nitrosospira* sp. strain NpAV and *Nitrosococcus oceani*, both *ureC* genes encoded a putative peptide of 568 amino acids with a theoretical size of 61 kDa and pI of 5.7. Conserved Ni^{+} binding motifs and active-site residues were as predicted from comparative studies (15).

Gene arrangement and copy number of the *ure* operon. The urease structural genes *ureA, ureB,* and *ureC* as well as the accessory genes *ureD*, *ureE, ureF*, and *ureG* were sequenced for *Nitrosospira* sp. strain NpAV (AF411008) and *Nitrosococcus oceani* (AF417006) (Fig. 1). In *Nitrosospira* sp. strain NpAV and *Nitrosococcus oceani*, the arrangement of urease genes within the operon was similar to that in the *Ralstonia eutropha* and *Klebsiella aerogenes* urease operons, respectively. The *Nitrosococcus oceani ureBC* and *ureEF* genes overlapped by 4 bp (ATGA). The intergenic regions in the urease operon and several of the urease subunit proteins were slightly smaller in *Nitrosococcus oceani*.

Southern analysis with NpAV genomic DNA restricted with both EcoRI and HindIII and an internal *ureC* probe (Fig. 2) revealed two bands, even though these enzymes did not cut within the probe sequence. Southern analysis of BclI-digested genomic DNA with the same probe revealed only one band of the predicted size of 1.1 kb. Hybridization of the *ureC* probe with genomic DNA cut with a combination of EcoRI or HindIII and BclI produced single bands of 1.1 kb, indicating that the BclI restriction sites are conserved in both *ureC* gene copies from NpAV. These observations were supported by our sequence data. Figures 2B and C show that the 13-kb HindIII fragment hybridized to both the *ureD* and *ureG* probes. These results indicated that the 13-kb HindIII fragment from NpAV genomic DNA contained almost the entire *ure* operon, and this fragment was thus chosen as a copy-specific template for sequencing and further characterization. We conclude that *Nitrosospira* sp. strain NpAV has two copies of *ureC* in its genome and that they are nearly identical. Further work is needed to characterize the extent of this operon duplication. *Nitrosospira* sp. strain NpAV has been shown to have three nearly identical copies of the *amo* operon (18) that are believed to be the result of gene duplications and the operation of a rectification mechanism (11).

Genomic DNA of *Nitrosococcus oceani* restricted with a variety of different endonucleases was subjected to Southern analyses with probes based on the sequences of the *ureAB* and *ureFG* (Fig. 2D and E) and *ureC* genes. All these *Nitrosococcus oceani* blots generated single bands except in the case of SphI, which cut within the *ureFG* probe sequence and therefore showed two hybridizing bands (Fig. 2E, lane 2). To study whether the urease genes constituted a single or multiple transcriptional units in *Nitrosococcus oceani*, reporter gene fusions were generated. The intergenic spacers upstream of the *ureBC* and *ureEF* genes (Fig. 1) that were ligated upstream of a promoterless *gfp* gene (pGLOW-TOPO; Invitrogen) did not reveal any promoter activities in *Escherichia coli* (data not shown). In contrast, flanking sequence upstream of the *ureD* gene ligated upstream of a promoterless *lacZ* gene (pBlue-TOPO; Invitrogen) revealed urea-inducible promoter activity in *Escherichia coli* (S. P. Clifford, A. F. El-Sheikh, and M. G. Klotz, unpublished data). These results indicate that the genome of *Nitrosococcus oceani* contains only a single *ure* operon copy, just as it has a single copy of the *amo* operon (2).

Southern analyses with genomic DNA from both *Nitrosospira* sp. strain NpAV and *Nitrosococcus oceani* did not reveal close proximity of the *ure* and *amo* operons in their genomes, but further investigations into genome organization and possible coordinate regulation are needed.

Diversity of the *ure* genes in AOB. We obtained the complete sequences of the *ureC* genes from *Nitrosospira* sp. strain NpAV, *Nitrosococcus oceani, Nitrosospira briensis, Nitrosospira (Nitrosolobus) multiformis* C71, *Nitrosospira (Nitrosolobus)*

FIG. 3. Phylogenetic analysis of 68 full-length UreC (alpha subunit) protein sequences in the urease structural gene family (aligned with ClustalX): 50% majority consensus tree of all credible topologies sampled by MrBayes over 100,000 generations with a maximum-likelihood approach with empirical substitution frequencies (JTT) and assuming equal rates across sites. Posterior probability values for the clades are shown at the branch points. A full circle indicates the division of the tree into two parts, a posibacterial and a negibacterial subclade, which reflects an early gene duplication event, after which the two genes evolved independently in either the same or different host cells or taxa. Shading is used to highlight the classification of the UreC source organisms. Ammonia-oxidizing bacteria are in bold. AOB UreC accession numbers are given in Table 1, and GenBank accession numbers and other organisms used are given in Materials and Methods.

multiformis 24C, *Nitrosospira tenuis* NV12, and *Nitrosomonas cryotolerans*. In addition, a partial *ureC* gene sequence was obtained for *Nitrosospira* sp. strain 39-19. Accession numbers and strain designations are given in Table 1. BLAST searches with portions of or the complete *ureC* gene sequences from *Nitrosospira* sp. strain NpAV and *Nitrosococcus oceani* against the *Nitrosomonas europaea* genome sequence (http://genome.ornl.gov/microbial/neur/embl/) were negative, indicating that no silent copies of the *ure* operon were present in the genome of *Nitrosomonas europaea* ATCC 19718.

Our probes developed from the *ure* operon of *Nitrosococcus oceani* were also used to investigate the residence of urease genes in other γ-proteobacterial ammonia oxidizers. A Southern blot prepared with genomic DNA isolated from *Nitrosococcus* sp. strain C-113 (2), which failed to grow on 25 mM urea as the sole source of N, energy, and reductant (data not shown), yielded no bands when probed for the presence of *ureAB*, *ureC,* and *ureG* genes. Similarly, genomic DNA from *Nitrosococcus halophilus* Nc-4, which has been described as a nonureolytic marine ammonia oxidizer (11), did not hybridize with any of the urease probes. While *N. halophilus* and *Nitrosococcus oceani* C-107 are different species and appear to be uniquely distributed in the world's oceans (25), the lack of an urease operon in *Nitrosococcus* sp. strain C-113 indicated that it is more genetically distinct from *Nitrosococcus oceani* than was previously indicated by the high sequence identity of their 16S rRNA genes (>99%) and of the intergenic noncoding regions in the *amo* operons (>78%) (2).

Our phylogenetic analysis revealed that the structural urease protein sequences from β-proteobacterial ammonia oxidizers form a monophyletic clade that did not group most closely with urease sequences from other β-proteobacteria. This is significant, as this grouping is incongruent with the small-subunit rRNA-based species phylogeny of AOB (17, 20, 23). We con-

	Amino Acid #	217	315	389
	Translation	K L H E D W G	M L M V C H H L	Q E D T S K - - - - S D
		URE1F 5'-3'	URE2R 3'-5'	Potential discriminatory region
	primers	AAGMTSCACGAGGACTGGGG	tacgastaccasacggtggtrga 5'	
A.	Mub	AAACTCCACGAAGACTGGGG	ATGCTGATGGTGTGCCACCACCT	GAAGGTGACCCGTCTGGTAGCCAAGCC---GCCGAC
	Scoe	AAGATCCACGAGGACTGGGG	ATGCTGATGGTGTGCCACCACCT	CCCGGCGACACCCGC--------------GCGGAC
	Cglu	AAAATTCACGAGGACTGGGG	ATGGTGATGGTTGCCCACCACCT	ACGGGAGATGCTCCATAC-----------AACGAC
	Anae	AAGATCCACGAGGACTGGGG	ATGCTCATGGTGTGCCACCACCT	GCCGGGGACCCGGAGGAC-----------GGCGAC
B.	Btb90	AAGCTTCACGAAGATTGGGG	ATGTTAATGGTTTGCCACCACCT	CAAGAAGACAATGGTGTG-----------GGAGAC
	Ssal	AAAACACACGAGGACTGGGG	ATGGTTATGGTTTGCCACCACTT	GAAGGGGATTCAGAGTTT-----------AACGAT
	Lfer	AAGACCCACGAAGACTGGGG	ATGACAATGGTTTGCCACAACCT	GACGGTGATTCCAAGTAC-----------GATGAC
	Cper	AAAGTTCATGAAGATTGGGG	ATGCTTATGGTTTGTCATCATTT	GAAGGAGATTCAGAATAT-----------ATAGAT
	Uure	AAAATCCATGAAGACTGAGG	ATGTTAATGGTATGTCACCACTT	AAAGGTGATAGTGAATTC-----------AGTGAT
	Sxyl	AAAGTACATGAAGACTGGGG	ATGGTTATGATTACTCACCATTT	GATGGTGACAGCGAATAT-----------AATGAC
	Bhal	AAGCTTCATGAGGATTGGGG	ATGCTGATGGTTTGCCACCACCT	GCTGAAGATCAAGGAAAAGGA--------AATGAT
	Bpas	AAAATTCACGAAGACTGGGG	ATGCTTATGGTTTGTCACCACTT	GCTGAGGAAAAAAACGGT-----------TCAGAT
C.	Pmar	AAATTACATGAAGACTGGGG	ATGTTAATGGTTTGTCATCATTT	CCTGAAGATTCTGATAGA-----------AATGAT
	PCC6803	AAACTCCATGAAGATTGGGG	ATGTTAATGGTGTGCCATCACCT	CCGGGGGAAACAGGCAAT-----------AATGAT
	WH7805	AAGCTGCATGAAGACTGGGG	ATGTTGATGGTGTGTCACCACCT	CCGCAAGACTCCAGCCGC-----------AACGAC
D.	Hfe	AAAATCCACGAAGACTGGGG	ATGTTAATGGTGTGCCACCACTT	AAAGAGGAAAAAGGCGAT-----------AACGAC
	HpylJ99	AAAATCCACGAAGATTGGGG	ATGCTTATGGTGTGCCACCACTT	AAAGAAGAAAAAGGCGAT-----------AACGAC
	Hhep	AAGGTTCACGAAGATTGGGG	ATGCTTATGGTATGTCATCACTT	AAAGAAGAATGCGGCGAA-----------AATGAT
E.	Smel	AAGCTGCACGAGGATTGGGG	ATGCTGATGGTATGCCATCACCT	AAGGAGGAGACCGGCGAC-----------AACGAC
	Atum	AAGCTGCATGAGGACTGGGG	ATGCTGATGGTGTGTCATCACCT	AAGGAGGAGACGGGCGAA-----------AACGAC
	Babo	AAACTGCATGAGGACTGGGG	ATGCTGATGGTCTGCCATCACCT	CCTGACGATAGGCCGGGC-----------AACGAC
	Bmel	AAGGTCCATGAAGACTATGG	ATGATTATGGTATGCCATAATCT	CCGGAGGATGCGCCCGGA-----------AACGAC
	Rcap	AAATTGCACGAGGATTGGGG	ATGCTGATGGTCTGCCACCACCT	GCCGAGGAGACGGGCGCG-----------AACGAC
F.	Rsol	AAGCTGCACGAAGACTGGGG	ATGCTGATGGTGTGCCACCATCT	GCCGGCGACCCGAACGACGCGCGCGGCGGGCACGAC
	Rmet	AAGCTGCACGAGGACTGGGG	ATGCTGATGGTGTGCCACCACCT	CCGGGCGATCCCAACGATGCGCGCGGCGGGCACGAC
	Bbro	AAGATCCACGAGGACTGGGC	ATGGTCATGGTGTGCCATCACCT	CAAGGCGACAGCGAGCGC-----------TCGGAC
	Bfung	AAGCTGCACGAGGACTGGGG	ATGCTGATGGTGTGCCACCACCT	CCCGAAGACAACGCGCGG-----------CACGAC
G.	***N. briensis***	AAGTTGCACGAAGACTGGGG	ATGTTGATGGTATGTCATCACCT	CAGGAAGACAATTCCAGG-----------AACGAT
	N. tenuis	AAGCTGCACGAAGACTGGGG	ATGGTCATGATCTGTCATCACCT	CAGCAAGATTCATCCAGG-----------AATGAT
	N. multiformis	AAGCTGCACGAAGACTGGGG	ATGCTGATGGTATGCCATCACCT	CAGGAAGATACATCGAAG-----------AATGAT
	N. cryotolerans	AAACTACATGAGGACTGGGG	ATGTTGATGGTTTGCCATCATCT	CAGGAAGATTCTTCTAGA-----------AACGAC
	N. sp. 24C	AAGCTGCACGAAGACTGGGG	ATGCTGTTGGTATGTCACCACCT	CAGGAAGATAATTCCAAA-----------AACGAT
	N. sp. AV	AAGCTGCATGAGGACTGGGG	ATGCTGATGGTATGCCATCACCT	CAGGAAGACACCTCGAAG-----------AACGAC
		--*--**-********	***-*--****-**-**-**-**	***-****----**-*-- **-**-
H.	Noce	AAGTTGCACGAAGACTGGGG	ATGTTGATGGTTTGCCATCACCT	CCGGAGGATAAGGGGGAT-----------CATGAT
	Paer	AAGCTGCACGAGGACTGGGG	ATGCTGATGGTCTGCCACCACCT	GACGGCGACGGCGCGCGC-----------AACGAC
	Psyr	AAACTGCACGAAGACTGGGG	ATGCTCATGGTCTGCCACCACCT	CCGCAGGATGGCCCAGGC-----------AACGAT
	Pputi	AAGCTGCATGAAGACTGGGG	ATGCTCATGGTCTGTCACCACCT	GCACCGGACACCCCGTAC-----------AGCGAC
	Pfluo	AAGCTGCACGAAGACTGGGG	ATGCTGATGGTCTGCCACCACCT	CCTCAGGACGGTGAAGGC-----------AACGAT
	Avine	AAGCTGCACGAGGACTGGGG	ATGCTGATGGTCTGCCACCACCT	CCCGAGGACGTCGCCGGC-----------CACGAC
	Mleg	AAACTGCACGAAGATTGGGG	ATGCTTATGGTATGCCACCACCT	CCCGAGGATGAAGGCACAGGC--------GCAGAT
	Aple	AAAATCCACGAAGACTGGGG	ATGCTAATGGTTTGTCACCACTT	GGCGATGAAGGT-----------------AACGAC
	Kaer	AAGATCCATGAGGACTGGGG	ATGCTGATGGTCTGCCACCATCT	GCGGAGGAGACCGGGGAT-----------AACGAC
	Pvul	AAAATTCATGAGGATTGGGG	ATGTTGATGGTTTGCCATCATCT	GCTGGCGATACAGCTGAA-----------AATGAT
	Pmir	AAAATACATGAAGACTGGGG	ATGTTGATGGTCTGTCATCATCT	GCGGGTGATAGCGCAGAT-----------AATGAT
	Vpar	AAAATCCATGAAGACTGGGG	ATGTTGATGGTCTGCCATCACTT	GAAGGGGATAGTGTATTC-----------AACGAC
	Yent	AAAGTCCACGAAGACTGGGG	ATGATCATGGTGTGTCATAACCT	CCAGAAGATGCGCCGGGT-----------AACGAT
I.	Drad	AAACTGCACGAGGACTGGGG	ATGCTGATGGTCTGCCACCACCT	GCGCCCGATGGCCGC--------------GCCGAC
J.	Stok	AAGATTCATGAAGATTGGGG	ATGTTAATGGCCGTGCACCATTT	ATCCAAATA--------------------AATGAT
		--*--**-**-**-**	***-*-****-----**--*--*	--------------------------------**o

FIG. 4. Alignment slices showing two conserved regions useful as internal *ureC* primer targets and a variable region potentially discriminatory for AOB *ureC*. Amino acids translated from *Nitrosospira* sp. strain NpAV are positioned on the first base of the codon. Sequences were from the sources given in Materials and Methods. Shaded blocks indicated by letters are groupings of organisms as follows: A, actinomycetes; B, posibacteria; C, cyanobacteria; D, ε-proteobacteria; E, α-proteobacteria; F, β-proteobacteria; G, ammonia-oxidizing β-proteobacteria; H, γ-proteobacteria (including one marine AOB); I, deinococci; and J, crenarchaeota. For the ammonia-oxidizing β-proteobacteria only, * indicates conserved and − indicates variable bases; the symbols at the bottom refer to all the organisms in the alignment.

clude that the urease in extant β-proteobacteria that are capable of ammonia oxidation diverged from that of the main lineage of the β/γ-proteobacterial urease ancestry before orthologous divergence of the α- and β/γ-proteobacteria. It is interesting that many of the α-proteobacteria in the analysis are plant symbionts from soil environments. We propose that the evolution of ureases may be under different functional selective pressures in these plant symbiotic organisms and the chemolithoautotrophic AOB versus the heterotrophic and animal host-associated bacteria. The evolutionary origin of the urease metabolic unit in the AOB is a topic under current investigation (M. G. Klotz, unpublished data).

Based on the alignments of *ureC* DNA and peptide sequences, we conclude that the alpha subunit of urease (UreC) is strongly conserved throughout the eubacteria. Our analysis shows that β-proteobacterial AOB UreC sequences form a distinct monophyletic clade and are therefore distinguishable from those of other closely related proteobacteria (Fig. 3 and 4). Although the *Nitrosospira* strain and *Nitrosococcus oceani* are both AOB, their *ureC* nucleotide sequences are more closely related to those of bacteria within their own subdivision. We identified conserved regions that can serve as potential diagnostic primers for *ureC* (Fig. 4). One variable region that may be discriminatory for the ureolytic AOB from the β subdivision was identified (Fig. 4). A primer or probe target specific to ureolytic AOB would be helpful to differentiate the

TABLE 2. Primer sequences for amplification of *ure* genes from strains and environmental samples[a]

Primer	Target organisms	Target gene	Sequence (all 5′-3′)[b]
ureC1F	Proteobacteria	Internal *ureC* conserved	AAGMTSCACGAGGACTGGGG
ureC2R	Proteobacteria	Internal *ureC* conserved	AGRTGGTGGCASACCATSAGCAT
ureG10R	β-Proteobacteria AOB	Downstream *ureG* primer	TATGGCGCCAGGTCCGTCTTGTTG
ureC12R	*Nitrosospira* sp. strain NpAV	Upstream end of *ureC*	TGGCCCATGCCGTCGCGGATCAC
ureE24R	β-Proteobacteria AOB	Downstream of *ureC* in *ureE*	CGTGGTGCGCAGGCGGCTGTTCTCG
ureC23F	*Nitrosospira* sp. strain NpAV	Internal *ureC* for probe	CATTGCCGGCGAAGGATCCATTCTCA
ureC22R	*Nitrosospira* sp. strain NpAV	Internal *ureC* for probe	CCCGGAAGTTGTCGCTCTTCGAGG
ureD27F	*Nitrosospira* sp. strain NpAV	Upstream primer, in *ureD*	CGGCTSGAYTGGCTGCCSCASGAG
ureB33F	β-Proteobacteria AOB	Upstream of *ureC,* in *ureB*	GGCGACCGGCCGATACAGATCGGTTCG
ureA101	*Nitrosococcus oceani* C-107	Degenerate forward primer for probe No1	AATTATCCNGARGCNGTRGC
ureM104	*Nitrosococcus oceani* C-107	Reverse primer for probe No1	CCGGGATATTTAAGCGCATCCCCC
ureM105	*Nitrosococcus oceani* C-107	Forward primer for probe No2	CCAACGGTACGTTGGGCCTCTC
ureR12	*Nitrosococcus oceani* C-107	Reverse primer for probe No2	TCCGCTCTTGCGCAAGCGCCT
ureGF1	*Nitrosococcus oceani* C-107	Forward primer for probe No3	TCCGCTCTTGCGCAAGCGCCT
ureJR1	*Nitrosococcus oceani* C-107	Reverse primer for probe No3	ATAGTCCCACGGCAAACATTG
ureDF2	*Nitrosococcus oceani* C-107	Forward primer for probe No4	ACTGGCGAGCTAAACGATCA
ureAR1	*Nitrosococcus oceani* C-107	Reverse primer for probe No4	CTCATACCCTCCCGTGCTT

[a] Target gene from *Nitrosospira* sp. strain NpAV (AF411008) or *Nitrosococcus oceani* (AF417006). F, forward primer; R, reverse primer.
[b] The following degenerate nucleotides were used: N = A, C, T, or G; R = A or G; M = A or C; S = C or G; and Y = C or T.

soil ammonia oxidizers from other proteobacteria. Primers designed from the multiple alignment of *ureC* DNA sequences are being assessed for their selectivity and inclusiveness of soil AOB versus other proteobacteria. Our assessment of the diversity of *ure* genes in cultured AOB is an initial step towards linking the ureolytic physiological function to the community structure of the soil AOB.

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