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Mutagenesis and Expression of *amo*, Which Codes for Ammonia Monooxygenase in *Nitrosomonas europaea*

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Nitrosomonas europaea **has two copies of the operon encoding ammonia monooxygenase (AMO). The nucleotide sequences of the two copies of *amoA* were obtained, and they were found to differ by one nucleotide. To determine if both copies of *amoA* were functional, insertional mutagenesis was performed to inactivate either copy of *amoA* alone. A DNA cassette containing the *lacZ* and *kan* genes inserted into *amoA* was constructed. Mutagenesis was done by using transformation and homologous recombination to mobilize the cassette into the chromosomal copies of *amoA*. Mutations were obtained in both copies of *amoA*. Either copy of *amoA* was sufficient to support growth when the other copy was disrupted. However, inactivation of one copy of *amoA*, but not the other, resulted in slower growth. Measurements of ammonia-dependent O_2 consumption, which depends on AMO, confirmed that the slower-growing mutant had lower activity while the faster-growing mutant had near wild-type levels of activity. Similarly, as measured by [^{14}C]acetylene label incorporation, there was less active AMO present in the slower-growing mutant than in the faster-growing mutant or in the wild type. Northern blot analysis of transcription likewise showed that the slower-growing mutant had less full-sized AMO mRNA.**

Nitrosomonas europaea is a chemolithoautotrophic soil bacterium that derives its carbon for growth from CO_2 and its energy for metabolism by the oxidation of ammonia (NH_3) to nitrite (NO_2^-) in the process of nitrification (40). Nitrification is a bacterial process that affects the availability of NH_4^+-based fertilizers applied to agricultural soils (18) and plays a role in the reclamation of NH_4^+-rich wastewaters (30). The oxidation of NH_3 to NO_2^- by *N. europaea* is carried out in two steps: first, NH_3 is oxidized to hydroxylamine (NH_2OH) by ammonia monooxygenase (AMO), and second, NH_2OH is oxidized to NO_2^- by hydroxylamine oxidoreductase (HAO).

One unusual genetic feature of nitrifiers is that most of the genes involved in nitrification identified to date are present in more than one copy in the genome. In *N. europaea*, the genes encoding AMO (*amoC*, *amoA*, and *amoB*) are adjacent to each other and are present in two copies (24, 35). The putative catalytic polypeptide of AMO is encoded in *amoA* (15). The transcript for AMO includes *amoA* and *amoB* (36). In *N. europaea*, the genes encoding HAO and the genes encoding cytochrome *c*-554 (*hcy* or *cyc*), which are in proximity to HAO, are found in three copies (3, 12, 25, 34). A gene which apparently codes for another *c*-type cytochrome is present as two copies immediately downstream of two of the three copies of the gene coding for cytochrome *c*-554 (3). The reason why *N. europaea* has multiple copies of some genes remains unclear. Each of the three copies of *hao* could be disrupted by insertional mutagenesis with a marker gene, indicating that none of the three copies of *hao* were essential (11). Apparently, the remaining copies compensated for the loss of the mutagenized copy. It is not known if all copies of the duplicated genes are expressed concomitantly or if they are differentially regulated.

Multiple copies of *amoA*, *amoB*, and *amoC* have been identified in other nitrifiers such as *Nitrosospira* sp. strain NpA V, *Nitrosospira briensis*, *Nitrosolobus multiformis*, *Nitrosomonas eutrophus*, and *Nitrosovibrio tenuis* (19, 27, 28). In *Nitrosospira* sp. strain NpA V, *amoA* was present in three copies, with 99.6% DNA similarity among the copies (28). The transcriptional regulation of the multiple gene copies in these nitrifiers has not yet been examined.

Gene duplication in other bacteria appears to be relatively uncommon outside the rRNA and tRNA genes. Nonetheless, a number of cases have been investigated. In some cases, the duplicate genes are silent copies, e.g., the pilin genes in *Neisseria gonorrhoeae* MS11 (10). In other cases, genes may be duplicated and expressed in a similar manner, e.g., the *tuf* genes encoding the elongation factor EF-Tu in *Escherichia coli* and other gram-negative bacteria (37, 39) and the *mer* genes in *Thiobacillus ferrooxidans* encoding mercury resistance (16). Genes may be duplicated but expressed differently, e.g., the genes encoding lysyl-tRNA synthetases, *lysS* and *lysU*, in *E. coli* (17, 32) and the *psb* genes in *Synechocystis* sp. (4, 21, 26). There are cases of duplicated operons, e.g., the *cbb* operon in *Alcaligenes eutrophus* (22) and an operon encoding two multidrug efflux transporters in *Bacillus subtilis* (1).

Gene function and expression studies in other bacteria showing multiple gene copies have often made use of insertional mutagenesis to characterize the function and expression of each gene copy. The insertion of an exogenous DNA fragment containing a genetic marker serves to disrupt the target gene, preventing the translation of a functional enzyme from that locus. This paper describes the insertional inactivation of the two copies of *amoA* in *N. europaea* with cassettes conferring antibiotic resistance.

MATERIALS AND METHODS

Strains and cell cultures. Strains of *N. europaea* and *E. coli* used are described in Table 1. *E. coli* cells were grown in Luria-Bertani medium as described previously (33). *N. europaea* cells were grown in liquid medium (6) and on solid medium (11) containing 50 mM NH_4^+. The solid medium for *N. europaea* was

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
E. coli		
DH5α	F′ ϕ80d*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169	9
InvαF′	F′ *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169	Invitrogen
N. europaea		
ATCC 19178		American Type Culture Collection
A141	Insertion of *lacZ/kan* into the *Bam*HI site of $amoA_1$	This study
A142	Insertion of *lacZ/kan* into the *Bam*HI site of $amoA_2$	This study
A1	Insertion of *kan* into the *Bam*HI site of $amoA_1$	This study
A2	Insertion of *kan* into the *Bam*HI site of $amoA_2$	This study
Plasmids		
pNHA10	842-bp PCR fragment containing most of *amoA* cloned into pCRII vector	This study
pNHA11	pNHA10 with a mutation in *amoA* at position 715 (24), creating a *Bam*HI site	This study
pNHA12	*amoA* insert cut out of pNHA11 with *Eco*RI and cloned into *Eco*RI site of pRL139	This study
pNHA14	*lacZ/kan* fragment from pKOK6.1 inserted into *Bam*HI site of pNHA12	This study
pNHA15	*kan* from pUC4 pKSAC inserted into *Bam*HI site of pNHA12	This study
pUC4 KSAC	Plasmid containing the *kan* cassette from Tn*903*	Pharmacia
pKOK6.1	Plasmid containing a promoterless *lacZ* and *kan* with its own promoter	20
pCRII	TA PCR cloning vector (Kan^r Amp^r *lacZa* f1*ori* ColE1 *ori*)	Invitrogen
pRL139	pUC19 with a symmetrical multiple cloning site	5

liquid medium containing 1% Bacto Agar (Difco Laboratories, Detroit, Mich.). The growth plates were prepared by placing an autoclaved Nytran membrane (6 by 6 cm) (Schleicher & Schuell, Keene, N.H.) on the solid medium. The *N. europaea* cells were then spread on the membrane and incubated at 30°C. The membrane was transferred to fresh plates weekly. Individual colonies were transferred to liquid culture after about 14 days.

The NO_2^- formation in cultures, which can be correlated to growth, was measured colorimetrically (8). NO_2^- formation, rather than NH_3 consumption, was used as a measure of metabolic activity since it is a simpler assay. In *N. europaea*, the vast majority of NH_3 consumed is oxidized to NO_2^- rather than incorporated into cell mass such that the rate of NH_3 consumption is indistinguishable from that of NO_2^- formation. Growth was measured as cell density by light scattering at 600 nm in a spectrophotometer (model DU7; Beckman, Palo Alto, Calif.) as well as by protein determinations. The protein content of the cell suspensions was estimated by the biuret assay (7), after the cells were solubilized in 3 N NaOH for 30 min at 65°C. Bovine serum albumin was used as a standard. Protein content was proportional to optical density throughout the course of cell growth. NH_3-dependent O_2 uptake, which depends on AMO activity, was measured with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) as described previously (13). [U-^{14}C]acetylene labeling of AMO was done as described previously (14).

DNA manipulation. Genomic and plasmid DNA preparations, DNA restriction digestions, Southern hybridizations, and other standard DNA manipulations were done as described previously (2, 33). DNA probes were labeled by random priming using the Prime-a-Gene kit from Promega Corporation (Madison, Wis.) and with [α-^{32}P]dCTP (either 3,000 or 6,000 Ci/mmol; DuPont NEN Products, Wilmington, Del.). The hybridization signals were visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale Calif.) and analyzed by densitometry by using the ImageQuant (Molecular Dynamics) software. DNA sequencing and oligonucleotide synthesis were done by the Center for Gene Research Central Laboratory, Oregon State University. The PCRs were performed on an Easycycler (Ericomp, San Diego, Calif.) instrument by using either *Taq* DNA polymerase (Perkin-Elmer, Branchburg, N.J.) or *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) in a 25-μl reaction volume with the following program: 1 repetition of cycle A (2 min at 94°C) and then 40 repetitions of cycle B (1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C).

Total RNA was isolated as described previously (31) with the following modifications. RNA was prepared by inhibiting RNase activity with vanadyl ribonucleoside (Life Technologies, Rockville, Md.) before lysis followed by acid-phenol chloroform extraction. RNA samples were resuspended in diethyl pyrocarbonate-treated H_2O and resolved in a denaturing 1.2% agarose gel. Prior to electrophoresis, the RNA was prestained with ≤5 μg of ethidium bromide per ml of loading buffer. The RNA was blotted into Nytran membranes by using a vacuum Hoefer TE70 blotter (Pharmacia Biotech, Piscataway, N.J.), leaving high-molecular-weight DNA in the gel. This technique allowed RNA to be harvested from relatively small volumes (≤200 ml) of low-density cultures (optical density at 600 nm [OD_{600}] of ≤0.03) without the need for CsCl step-gradient centrifugation.

***amoA* gene nomenclature.** *N. europaea* contains two copies of *amoA* which are distinguishable by Southern hybridization (25). An *Eco*RI digest of *N. europaea* genomic DNA probed with an *amoA* probe detects two DNA fragments (6.5 and 5 kb [Fig. 1c]). In this paper, the *amo* copies contained on the lower and upper *Eco*RI fragments are referred to as $amoA_1$ and $amoA_2$, respectively. This nomenclature parallels that used for the multiple copies of *amoA* and *amoB* in *Nitrosospira* sp. strain NpA V (28).

DNA amplifications and constructs. The DNA constructs are described in Table 1. The *lacZ/kan* cassette used in these experiments was isolated from the plasmid pKOK6.1 (20), which was a gift from W. Lotz and T. Bauer (Friedrich-Alexander-Universität). The *lacZ/kan* cassette contains a promoterless *lacZ*

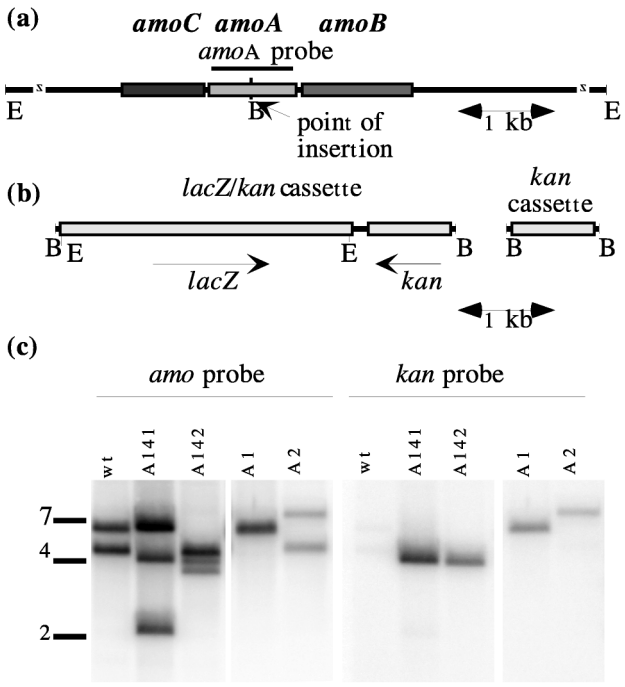

FIG. 1. Physical map of the genes coding for AMO, the DNA cassettes used, and Southern hybridization to a probe for *amoA* and for the *kan* cassette. (a) The three genes coding for known and putative AMO peptides and the probe used to detect *amoA* are shown. The *Bam*HI (B) restriction site created for the insertion of the cassettes is shown. The sizes of the *Eco*RI (E) fragments for the two gene copies are approximately 5 and 6.5 kb. (b) The *lacZ/kan* and *kan* cassettes that were inserted into the *Bam*HI site. (c) Southern hybridization to the *amo* probe and the *kan* probe. wt, *N. europaea* wild-type strain.

TABLE 2. Oligonucleotide primer sequences

Oligonucleotide primer	Sequence[a]	Reference
ANT	5′-GCGGCCAAGATGCCGCCG	24
ACT	5′-GATCCCCTCTGGAAAGCC	24
AM5	5′-CTCTGGTTGGAGGTGGAT**C**CTTCGGTC	24
AM4	5′-ACCGAAG**G**ATCCACCTCCAACCAGAGCCG	24
NPT1	5′-ATGAGCCATATTCAACGGGAAACG	29
NPT2	5′-CTGCAGGGGGGGGGGGCGCTGAG	29
S2301	5′-TTCTGCAGTACCGTGAGGGAAAGG	37
S23R01	5′-GGCTGCTTCTAAGCCAAC	37

[a] The changed nucleotide is shown in bold type.

gene, and in the opposite orientation it contains a *kan* gene with its own promoter (Fig. 1b). There is a transcriptional terminator between the two genes. Although the insertion of the cassette into *amoA* allowed the *amo* promoter to drive the expression of *lacZ*, in this study the cassette was used only for purposes of insertional mutagenesis. A second cassette containing the *kan* gene with its promoter and without *lacZ* (from pUC4 KSAC; Pharmacia Biotech) was also used (Fig. 1b).

The following manipulations were made to produce the DNA fragments containing *amoA* and the cassettes that were to be used in the transformation experiments. First, a 0.8-kb DNA fragment containing *amoA* was amplified by using the oligonucleotide primers ANT and ACT (Table 2). This fragment was cloned into the pCRII (Stratagene) vector to form pNHA10. In order to use the *Bam*HI sites flanking the *lacZ*/*kan* cassette, a *Bam*HI insertion site had to be created in *amoA*. The *Bam*HI site was created by site-directed mutagenesis in which a thymine nucleotide at position 338 of the *amoA* coding region was changed to cytosine, creating a *Bam*HI site at that location. The *Dpn*I-PCR mutagenesis method (ExSitePCR; Stratagene) uses two overlapping oligonucleotide primers containing the mutant sequence which span the mutation site in opposite directions. PCR was done on a circular plasmid containing wild-type *amoA* as a template (pNHA10), with the mutagenic primers, AM5 and AM4 (Table 2), and the high-fidelity *Pfu* DNA polymerase. The PCR parameters were as follows: 12 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 50°C, and 15 min at 72°C. The template DNA was removed by digestion with *Dpn*I, which requires methylated DNA for activity. The newly synthesized PCR product, containing the mutated site (pNHA11), was transformed into the *E. coli* DH5α. To ensure that the newly created *Bam*HI site in *amoA* would be unique, the *Eco*RI fragment containing *amoA* was subcloned out of pNHA11 and inserted between the *Eco*RI sites of the vector pRL139 (5) (pNHA12). The *lacZ*/*kan* cassette, excised from pKOK6.1 with *Bam*HI, was inserted into the *Bam*HI site in *amoA* on plasmid pNHA12 to form pNHA14. Transformants were selected for kanamycin resistance. Colonies were screened by restriction digestion for clones containing the *lacZ*/*kan* cassette inserted into *amoA* in the correct orientation. In a similar manner, a cassette containing *kan* alone was excised from pUC4 KSAC with *Bam*HI and cloned into the *Bam*HI site of *amoA* to form pNHA15.

The *amo* probe used in the hybridizations was a 0.8-kb PCR fragment containing *amoA* (Fig. 1a). The *amoA* probe was amplified by using primers ANT and ACT from genomic DNA or from pNHA10. The *kan* probe was amplified from the plasmid pUC4 KSAC by using primers NPT1 and NPT2 (Table 2). The probe for the 23S rRNA was produced by DNA amplification of *N. europaea* genomic DNA by using primers S2301 and S23R01 (Table 2) (38).

The DNA sequence of the two copies of *amoA* was obtained from at least two PCR clones for each copy. Genomic DNA obtained from mutagenized strains of *N. europaea* with an insertion of the mutagenic cassette into one of the copies of *amoA* was used as a template for the PCR reactions. Copy-specific amplification of *amoA* fragments was obtained by using one primer site within the *lacZ*/*kan* cassette and a second primer site outside the coding region for *amoA*.

Cell transformation by electroporation. *N. europaea* cells (0.5 liter) from a liquid culture in early stationary growth phase (OD_{600} = 0.1) were harvested by centrifugation and washed three times with sterile H_2O. The sedimented cells were resuspended in 1.5 ml of H_2O and kept on ice until use. Cell transformation with plasmids pNHA14 and pNHA15 was done by electroporation in an ElectroPorator (Invitrogen, Carlsbad, Calif.) in 1-mm-gap cuvettes (Invitrogen). Electroporation was done at 1,200 V, 25 μF, and ∞ resistance. In a prechilled cuvette, 120 μl of cells were mixed with 1 μg (1 μl) of pNHA14 or pNHA15 DNA and pulsed. The cells were transferred to 0.5 liter of fresh medium and allowed to grow for 15 h under nonselective conditions at 30°C while shaking. Cells were then plated on solid nutrient medium as described above containing kanamycin sulfate (10 μg/ml).

Nucleotide sequence accession numbers. The nucleotide sequences for $amoA_1$ and $amoA_2$ from *N. europaea* have been submitted to the GenBank database under accession numbers AF058691 and AF058692, respectively.

RESULTS

DNA sequencing, mutagenesis, and corroboration of the recombination events. The DNA sequences obtained for the coding regions of $amoA_1$ and $amoA_2$ revealed that they differed by only a single nucleotide (position 65 of the *amoA* coding region, C for $amoA_1$ and T for $amoA_2$), which resulted in an amino acid change (Thr for $amoA_1$; Met for $amoA_2$). The nucleotide sequence of $amoA_2$ was identical to the *amoA* sequence determined by McTavish et al. (24). In addition, the 165-bp intergenic region between the end of *amoC* and the beginning of *amoA* was identical in $amoA_1$ and $amoA_2$.

DNA fragments containing either *lacZ*/*kan* or *kan* were inserted into copies of *amoA* in *N. europaea* by electroporation and recombination by using plasmids pNHA14 and pNHA15 (Table 1). Although the fragments were not targeted to a particular copy of *amoA*, we found roughly equal numbers of recombination events in the copies. The transformation efficiency was calculated to be 2×10^{-6} mutant colonies per cell. Kanamycin-resistant colonies grew sufficiently in 14 days to transfer into liquid medium.

Southern hybridizations were used to locate the point of insertion of the mutagenic cassette in the *N. europaea* genome. Genomic DNA from kanamycin-resistant strains was digested with the endonuclease *Eco*RI and blotted onto Nytran Plus nylon membranes. The DNA blots were hybridized to either the *amoA* or *kan* probe. In wild-type cells, two fragments (6.5 and 5 kb) were detected with the *amo* probe after *Eco*RI digestion of genomic DNA (Fig. 1c). In the mutant *N. europaea* A141, one wild-type fragment (6.5 kb) was detected, but the second wild-type fragment was replaced by two new fragments (2.4 and 4 kb). An insertion of the *lacZ*/*kan* cassette into a copy of *amoA* would increase the size of the highlighted fragment. However, because two internal *Eco*RI sites flanking the *lacZ* gene in the *lacZ*/*kan* cassette results in the excision of *lacZ* by *Eco*RI, the expected result would be the conversion of the *amoA* hybridizing fragment into two new smaller hybridizing fragments. Similarly, in strain A142, the 5-kb wild-type fragment is retained, while the 6.5-kb fragment was replaced by 3.8- and 4-kb fragments.

In mutagenesis experiments done with the *kan* cassette, a different result was expected than that obtained with the *lacZ*/*kan* cassette. The *kan* cassette has no internal *Eco*RI sites, and therefore an insertion of the cassette into *amoA* should simply increase the size of the *amoA* hybridizing fragment. Indeed, when genomic DNA from mutant strain A1 was probed with *amoA*, the 5-kb fragment had been shifted to about 6.2 kb, which appeared as a doublet with the wild-type 6.5-kb fragment. In strain A2, the wild-type 5-kb fragment was highlighted by the *amoA* probe, as was a 7.7-kb fragment resulting from an insertion of the *kan* cassette into the larger *amoA* fragment. In all cases, the hybridization patterns detected with the *amo* probe showed that the kanamycin-resistant strains had only one of the two *amoA* gene copies disrupted, and the pattern was consistent with the insertion of a cassette into one of the copies of *amoA*.

Hybridizations with the *kan* probe confirmed that the *lacZ*/*kan* and *kan* cassettes were inserted into the DNA fragments containing *amoA*. Strains A141 and A142 both had single fragments of about 4 kb highlighted by the *kan* probe. The *kan* probe highlighted 6.2- and 7.7-kb fragments in strains A1 and A2, respectively. These results were consistent with insertions of either the *lacZ*/*kan* or *kan* cassette into the two copies of *amoA*. In all clones examined, no insertions of the cassettes into other locations in the genome were detected. No hybridization to the *kan* cassette in the wild type was observed.

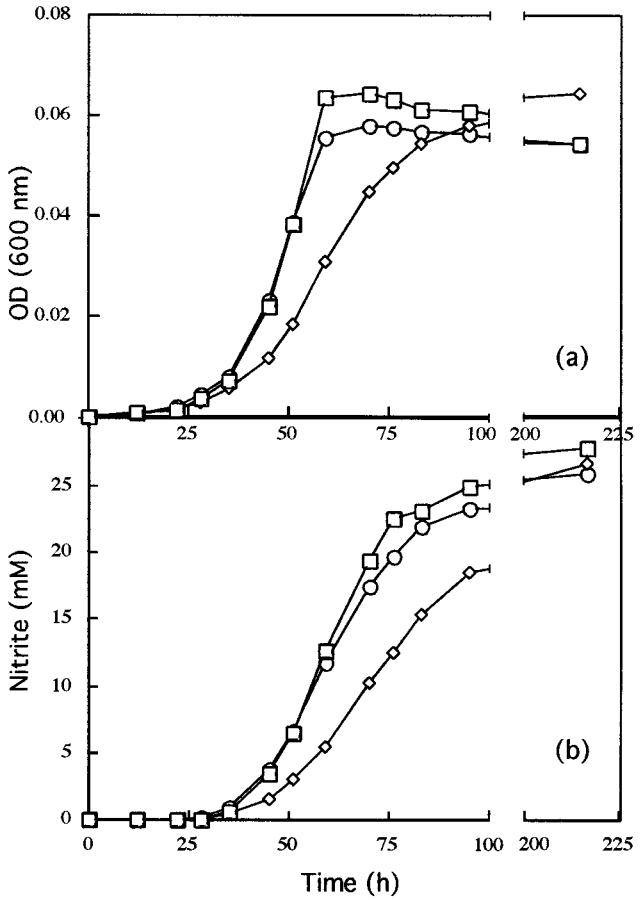

FIG. 2. Growth of the wild-type, A141, and A142 strains of *N. europaea*. (a) OD_{600} during a 215-h growth comparison experiment (see text). Symbols: □, *N. europaea* (wild type) showing a growth rate of 0.089 h^{-1}; ◇, *N. europaea* A141 showing a growth rate of 0.062 h^{-1}; ○, *N. europaea* A142 showing a growth rate of 0.087 h^{-1}. (b) NO_2^- accumulation of the growth experiment depicted in panel a.

Thus, clonal cell lines were obtained with insertions of either *lacZ*/*kan* (*N. europaea* A141 and A142) or *kan* alone (*N. europaea* A1 and A2) into both copies of *amoA* alone. The *amoA*::*lacZ*/*kan* mutations in *N. europaea* have been stable in culture for over a year. The *amoA*::*kan* mutations appeared to be as stable as the insertion of the *lacZ*/*kan* cassette. The ability to get insertions into both copies of *amoA* singly indicated that neither copy was essential and both were sufficient to support growth under the conditions used.

Phenotypical differences in the *N. europaea* mutant strains. Since *N. europaea* requires NH_3 for rapid growth, AMO plays a critical role in the energetics and metabolism of this organism. We therefore considered whether the mutations in *amoA* influenced growth rates of the mutant strains. To test this possibility, cells from early-stationary-phase cultures of mutant and wild-type strains of similar optical densities were inoculated into fresh medium to identical optical densities (OD_{600} = 0.005) and their growth was monitored by their OD_{600} and by NO_2^- formation. *N. europaea* A141, with a *lacZ*/*kan* insertion in $amoA_1$ (5-kb DNA fragment [Fig. 1c]) grew slower than wild-type cells, although it eventually reached the same cell density as wild-type cultures did (Fig. 2a). *N. europaea* A142, with a *lacZ*/*kan* insertion in $amoA_2$ (6.5-kb DNA fragment [Fig. 1c]) had a growth curve more similar to that of wild-type cells. *N. europaea* A1 and A2 exhibited growth patterns similar to *N. europaea* A141 and A142, respectively, confirming the observed copy-specific phenotypes (data not shown). The growth rates of wild-type and mutant strains were calculated from OD_{600} measurements of actively growing cultures during exponential growth from several growth curves. Although special attention was paid to consistency while performing replicate growth experiments, variations in the growth rates of the wild-type and mutant strains were observed. In 6 replicate experiments, the mean growth rate for the wild-type strain was 0.093 ± 0.015 h^{-1}. In 8 replicate experiments, the mean growth rate of $amoA_1$ mutants (A141 and A1) was 0.067 ± 0.009 h^{-1}. In 10 replicate experiments, the mean growth rate of $amoA_2$ mutants (A142 and A2) was 0.084 ± 0.017 h^{-1}. The differences in growth rates between strains was more apparent when the growth rates of the mutant strains were expressed as a percentage of the wild-type growth rate in each experiment. When the growth rates were compared this way, the averaged results from all the replicate experiments showed that strains with $amoA_1$ inactivated generally had slower growth rates than did wild-type cells (about 75% of the wild type rate, while strains with $amoA_2$ inactivated had growth rates similar to that of wild-type cells (about 97% of the wild-type growth rate [Table 3]). When metabolic activity was monitored by NO_2^- formation, curves similar to those based on optical density measurements were obtained (Fig. 2b). All mutants eventually reached NO_2^- levels similar to that of the wild-type strain. In these experiments, the cultures consumed about half the available NH_3.

NH_3-dependent O_2 consumption, which requires AMO, was determined for wild-type and mutant strains. To compare cells in a similar physiological state, cells were harvested at the same optical density rather than at the same culture age. These cultures generally had accumulated similar amounts of NO_2^-. However, the NH_3-dependent O_2 consumption differed in the wild-type and mutant cells. For example, by using data from a typical experiment where the cells were harvested at about 0.05 OD_{600}, the NH_3-dependent O_2 uptake values (in nanomoles of

TABLE 3. Growth rates, NH_3-dependent O_2 consumption, [U-^{14}C]acetylene incorporation, and AMO mRNA

Strain[a]	Growth rate[b] (*n*)[c]	O_2 uptake[d] (*n*)	[^{14}C] incorp.[e] (*n*)	AMO mRNA[f] (*n*)
Wild type	100 (6)	100 (5)	100 (4)	100 (12)
$amoA_1$ mutants	75.2 ± 10.0 (8)	85.2 ± 5.76 (6)	80.7 ± 10.7 (4)	63 ± 14 (12)
$amoA_2$ mutants	96.9 ± 6.7 (10)	104.0 ± 6.9 (14)	92.4 ± 6.6 (4)	97 ± 23 (12)

[a] $amoA_1$ mutants include strains A141 and A1, and $amoA_2$ mutants include strains A142 and A2.
[b] Mean growth rate ($hour^{-1}$) as a percentage of the wild-type activity ± 1 standard deviation. The rate was calculated from an exponential curve fit from growth curves monitored by OD_{600}.
[c] *n* is the number of replicates used to calculate the mean and variance.
[d] Mean activity as a percentage of the wild-type activity ± 1 standard deviation.
[e] Mean amount of [^{14}C] incorporated into the 27-kDa peptide of AMO as a percentage of the wild-type amount ± 1 standard deviation.
[f] Mean amount of *amo* mRNA as a percentage of the wild-type *amo* mRNA amount ± 1 standard deviation.

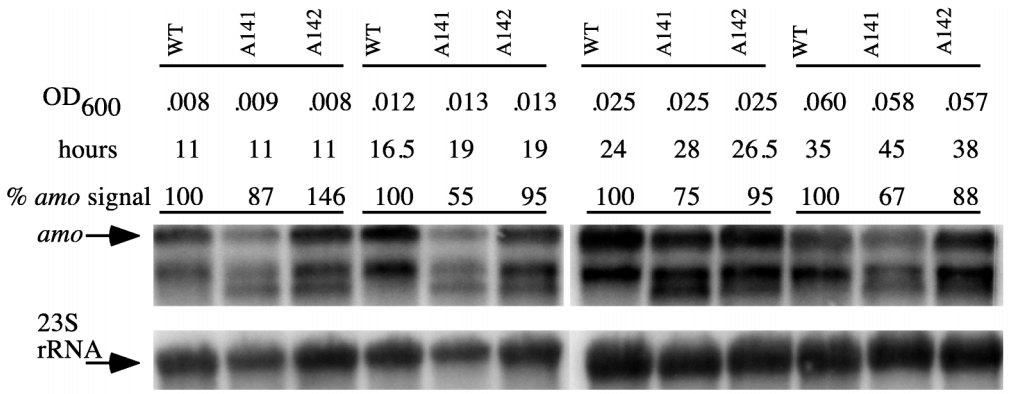

FIG. 3. AMO mRNA levels during growth of the wild-type, A141, and A142 strains. The optical densities and the time elapsed at the sampling time are shown. The position for the full-sized AMO mRNA (about 3.5 kb) is indicated by an arrow. The hybridization to the rRNA after stripping the same blot is shown below the *amo* panel. The *amo* levels are given as a percent of the wild-type level for each optical density group. These levels were calculated by dividing the intensity observed in the *amo* signal by the intensity observed in the rRNA signal. The relative intensity for each time point was divided by the wild-type intensity and this value was multiplied by 100. The truncated *amo* transcript can be observed as an extra band in the *amo* hybridization and corresponds to approximately 2.1 kb. WT, wild type.

O_2 consumed minute^{-1} $OD_{600}{}^{-1}$) were 2.62 for the wild-type cells, 2.39 for strain A141, 2.45 for strain A1, 3.09 for strain A142, and 2.97 for strain A2. The activity determinations we observed in a given experiment varied, depending on the optical density at the time of harvest. To allow a comparison of experiments performed as described above but at optical densities other than 0.05, the activities were expressed as a percentage of wild-type activity in each particular experiment. We compared NH_3-dependent O_2 consumption of the wild type and mutants from cultures harvested at optical densities from 0.028 to 0.050. *N. europaea* strains with inactivated $amoA_2$ had a mean NH_3-dependent O_2 consumption of about 104% of the wild-type level (Table 3). *N. europaea* strains with inactivated $amoA_1$ had a mean NH_3-dependent O_2 consumption of about 85% of the wild-type level. The same trends were observed when rates were normalized to protein content. Thus, as with the growth rates, the NH_3-dependent O_2 consumption data showed differences between the mutant strains and wild-type cells. The $amoA_1$ mutant strains had lower rates than wild-type cells, while $amoA_2$ mutants had activities closer to that of wild-type cells.

Given the observed differences between the strains regarding growth rates and NH_3-dependent O_2 consumption, we were interested to know if these differences were also reflected in the amount of active AMO enzyme. Cells were harvested at the same optical density (OD_{600} = 0.03), washed, resuspended in fresh medium without $NH_4{}^+$ but containing 10 mM hydrazine sulfate as a reductant source, and incubated with [^{14}C]acetylene (5×10^6 cpm [14]) for 45 min (which is sufficient to reach completion) at 30°C. [^{14}C]acetylene is a suicide substrate, which can be used to label the 27-kDa polypeptide of AMO (14). When the reaction is allowed to go to completion, the amount of label in the 27-kDa polypeptide is proportional to the amount of active AMO present when the acetylene was introduced. Protein extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the labeled peptides analyzed by densitometry on the PhosphorImage of the gel. The mutant strain A141 had less label incorporated (80.7% of the wild-type level) than either wild-type *N. europaea* (taken as 100%) or strain A142 (92.4% of the wild-type level) (Table 3). These values were based on four replicate samples.

Transcriptional levels in the *N. europaea* mutant strains. Because one of the copies of *amo* was affected in each mutant strain, there was the possibility that the strains would show different transcription levels of the *amo* operon. To test if there were any differences in the mRNA levels transcribed from the two copies of *amoA*, total RNA was isolated from the mutant (A141 and A142) and wild-type strains and blotted for analysis by Northern hybridization. Cells were harvested at different points on their growth curve starting from early growth ($<$0.03 OD_{600}) to early stationary growth ($\leq$0.08 OD_{600}). As described above, the different strains were harvested at similar optical densities, albeit at different times, for purposes of comparison (Fig. 3). Total RNA was extracted, blotted, and probed with a probe for *amoA* or for the 23S rRNA. The amount of RNA loaded onto the gel was approximately the same based on the intensity observed by ethidium bromide staining. The Northern blots with RNA from wild-type *N. europaea* cells and an *amoA* probe (Fig. 3) revealed both the full-sized *amo* transcript (3.5 kb) as well as a second fragment (about 2.4 kb). Both fragments are also highlighted by an *amoB* probe, but neither fragment has yet been fully characterized (35). The 2.4-kb fragment may be derived from the 3.5-kb transcript or may represent a separate transcript. Northern blots of *N. europaea* A141 and A142 probed with *amoA* highlighted an additional smaller transcript (approximately 2.1 kb) not present in wild-type cells (Fig. 3). Since the *lacZ*/*kan* cassette had been inserted into *amoA* as a transcriptional fusion, we expected to find a 4.5-kb fragment highlighted in the Northern blots when probed with *amoA* or *lacZ*. Instead, the *amoA* probe highlighted the 2.1-kb fragment, and no fragment hybridized to the *lacZ* probe. The *amoB* probe did not hybridize to the 2.1-kb fragment. However, the Southern hybridization data clearly show that the *lacZ*/*kan* cassette was inserted into *amoA* (Fig. 1). Thus, the transcript containing *lacZ* is apparently rapidly degraded. The 2.1-kb band would be consistent with a truncated transcript encoding part of *amoA* and possibly part of another gene. No other labeled fragments were detected.

The intensities of the signals detected after hybridizing the *amoA* probe and, after stripping the blot, hybridizing to the 23S rRNA probe, were quantified by densitometry. The hybridization to the 23S rRNA probe was used to normalize the differences in the amounts of RNA in the blot. These intensities were used to calculate the ratio of AMO mRNA/rRNA (Fig. 3). At early and exponential stages of growth, *N. europaea* A141 had 20 to 40% less AMO mRNA than the wild-type strain (Fig. 3). *N. europaea* A142 had a greater amount than

the wild type during early growth but 5 to 12% less AMO mRNA during exponential growth. When the results of 12 replicate AMO mRNA level determinations at several time points were averaged, they showed the same trend (Table 3). The *N. europaea* strains with inactive $amoA_2$ averaged 97% of the AMO mRNA of the wild type. *N. europaea* strains with inactivated $amoA_1$ averaged 63% of the AMO mRNA level of the wild-type cells during growth (Table 3).

DISCUSSION

N. europaea has two copies of the genes coding for AMO, an enzyme essential for nitrification. The degree of similarity in the DNA sequences of the copies of *amoA* is striking, but the three copies of *amoA* in *Nitrosospira* sp. strain NpA V also have a similar degree of similarity, differing by only one or two nucleotides (28). The sequence of *amoA* in *N. europaea* is about 75% similar at the DNA level to that of *amoA* in *Nitrosospira* sp. strain NpA V and about 88% similar at the amino acid level.

The existence of almost identical functional copies of genes in the genomes coding for peptide components of a key enzyme in these lithoautothrophic bacteria is intriguing. Basic questions about these genes, whether they are both functional and if either is essential, were addressed by insertional inactivation of the two copies of *amoA*. This study shows that a DNA cassette can be inserted into either of the two copies of *amoA*. Presumably (although we do not show this directly), the insertion of the markers precluded the synthesis of a functional AMO enzyme from the affected locus. Because the complete inactivation of AMO will be lethal to a nitrifier and because the inactivation of either copy could be obtained, we conclude that neither copy of *amoA* is essential to the cell and that both copies are functional.

The growth curves, NO_2^- production, and activity measurements were different for the two mutants of *amo*. The mutations in $amoA_2$ resulted in a near wild-type phenotype with regard to growth and NH_3-dependent O_2 uptake rates. The mutations in $amoA_1$ resulted in slower growth and reduced NH_3-dependent O_2 consumption (Table 3). Differences in the mutant strains were also revealed at the transcriptional level. An analysis of AMO mRNA levels by Northern hybridizations showed that AMO mRNA levels in the $amoA_2$ mutant were nearly the same as in wild-type cells, while the $amoA_1$ mutant strain consistently showed reduced levels of AMO mRNA (with a mean decrease of 37%). The pattern of mRNA expression in the wild-type strain and strains with either an $amoA_1$ or $amoA_2$ mutation paralleled the results seen for NH_3-dependent O_2 consumption (Table 3). These results suggested that *N. europaea* was able to compensate almost entirely for the disruption in the $amoA_2$ gene copy but not the disruption of the $amoA_1$ gene copy under the conditions tested. This observation leads to the conclusion that $amoA_1$ and $amoA_2$ are regulated differently.

A survey of the literature on duplicated genes (see above) shows that these duplications may serve a variety of purposes in the bacterium. Perhaps the two copies of *amo* provide a mechanism to amplify the rate of transcription. In natural environments, *N. europaea* seems unlikely to find itself in a situation where NH_3 is in an abundant and constant supply. Nonetheless, flushes of ammonia are possible within the local environment of the bacteria. The presence of two copies of the *amo* operon might allow more-rapid generation of AMO mRNA during a flush of ammonia. In this model, both genes are regulated similarly and both are used to meet the demand for increased transcription. A precedent for an additive gene expression response for optimal growth exists in *Salmonella typhimurium* where the EF-Tu is encoded by two highly similar genes (37). When either copy is inactivated by insertional mutagenesis, levels of EF-Tu in the cell were reduced by about 65% (39). However, unlike EF-Tu in *S. typhimurium*, when $amoA_1$ is inactivated in *N. europaea*, the cell can partially compensate for the loss of that gene copy, and in the case of an inactivated $amoA_2$, near wild-type AMO activity is observed during optimal growth conditions.

Alternatively, differential expression of two highly similar genes is expected when each copy has a primary role associated with particular conditions where the bacterium must survive. In *N. europaea*, a likely system would be to have a gene copy specialized to suboptimal substrate levels and another specialized to optimal substrate levels where each copy is differentially regulated rather than expressed in an additive form. This system would also allow *N. europaea* to synthesize a large amount of AMO in a short time when the substrate is abundant and to survive during low substrate levels. Maintaining a basal expression by using one gene copy during suboptimal conditions would be a more efficient use of the vital endogenous energy of the cell for this bacterium with a generation time of 8 h. Differential regulation of expression of either copy of *amo* in *N. europaea* in response to environmental conditions would be similar to the case of the lysyl-tRNA synthetase genes of *E. coli* and other members of the family *Enterobacteriaceae*. The lysyl-tRNA synthetase genes of *E. coli* are encoded by two genes, *lysS* and *lysU*, which are differentially expressed, depending on the growth conditions of the cell. The nucleotide and deduced amino acid sequences of these genes are highly similar (23, 32). Both genes are expressed, and *lysU* can functionally replace an inactivated *lysS* (17). The *lysS* gene is expressed constitutively, while *lysU* gene is expressed at low levels but is induced by several factors, including heat shock, low pH, anaerobiosis, or the addition of small hydrophobic leucine peptides or L-alanine or L-leucine to minimal medium.

The differences in growth of the *amoA* mutants are apparently in contrast to the situation with *hao* in *N. europaea*. Insertional mutagenesis of *hao* showed that none of the three copies was essential, and there was no observed growth phenotype associated with the mutations (11). However, since there are three copies of *hao*, double mutations might be required to observe differences in their regulation.

Whether the above similarities to other bacteria are relevant to the *amo* genes of *N. europaea* is unknown, but they provide models which can be explored. The molecular mechanism by which the expression of the two copies of *amo* are regulated remains to be elucidated. Also, we have yet to determine if the two transcripts differ in their stability and turnover rate. The purpose for this differential transcription rate between the copies of *amo* also needs to be determined. Further investigation into the transcription patterns under different growth conditions is needed to characterize the regulation of *amo* expression.

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