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MINI-REVIEW

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Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*

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Abstract *Nitrosomonas europaea* uses only NH_3, CO_2 and mineral salts for growth and as such it is an obligate chemo-lithoautotroph. The oxidation of NH_3 is a two-step process catalyzed by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). AMO catalyzes the oxidation of NH_3 to NH_2OH and HAO catalyzes the oxidation of NH_2OH to NO_2^-. AMO is a membrane-bound enzyme composed of three subunits. HAO is located in the periplasm and is a homotrimer with each subunit containing eight *c*-type hemes. The electron flow from HAO is channeled through cytochrome c_{554} to cytochrome c_{m552}, where it is partitioned for further utilization. Among the ammonia-oxidizing bacteria, the genes for AMO, these cytochromes, and HAO are present in up to three highly similar copies. Mutants with mutations in the copies of *amoCAB* and *hao* in *N. europaea* have been isolated. All of the *amoCAB* and *hao* gene copies are functional. *N. europaea* was selected by the United States Department of Energy for a whole-genome sequencing project. In this article, we review recent research on the molecular biology and biochemistry of NH_3 oxidation in nitrifiers.

Keywords *Nitrosomonas europaea* · Nitrification · Ammonia oxidation · Obligate chemo-lithoautotrophy · AMO mutant strains · HAO mutant strains · Bioremediation · Cytochromes · Electron flow

Introduction

The ammonia (NH_3)-oxidizing bacterium *Nitrosomonas europaea* is an obligate chemolithotroph that derives all of the reductant required for energy and biosynthesis from the oxidation of NH_3 to nitrite (NO_2^-). *N. europaea* is also an obligate autotroph that derives all of its carbon for growth from CO_2. The NO_2^- produced by ammonia-oxidizing bacteria is oxidized subsequently to nitrate (NO_3^-) by NO_2^--oxidizing bacteria (*Nitrobacter* sp.). This sequential transformation of NH_3 to NO_3^- is the process of nitrification, a part of the biogeochemical N cycle. In croplands fertilized with NH_3, ammonium (NH_4^+) salts, or urea-based compounds (which are hydrolyzed to NH_3), nitrification leads to the production of NO_3^-. NH_3 remains bound to typical soils primarily in the form of NH_4^+ where it is available for crop utilization. NO_3^- is very mobile and can readily leach into ground and surface waters before crops can utilize the added N. NO_3^- leaching can cause eutrophication of surface waters and contamination of ground waters intended for human consumption. NO_3^- can also be reduced to N_2 in the subsequent step in the biogeochemical N cycle, denitrification, with the result that the NH_3 fertilizer is lost to the atmosphere as N_2. Trace amounts of NO and N_2O, both greenhouse gases, are also released in the processes of nitrification and denitrification (Macdonald 1986). On the other hand, active nitrification is highly desirable in the reclamation of water in raw sewage (an NH_4^+-rich environment). In this environment, ammonia-oxidizing bacteria play an important role by initiating the conversion of NH_3 to N_2. Ammonia-oxidizing bacteria also have potential applications in the bioremediation of polluted soils and waters through the indiscriminate action of the monooxygenase that initiates nitrification (see below).

N. europaea, like most ammonia-oxidizing bacteria, is in the β-subdivision of the proteobacteria (Head et al. 1993; Woese et al. 1984). Most of the breakthroughs in our understanding of the biochemistry and molecular biology of ammonia-oxidizing bacteria have been achieved using *N. europaea*. Much attention has been focused on *N. europaea* simply because it is so amenable to culture relative to many other ammonia-oxidizing bacteria. Furthermore, the first genetic system in an ammonia-oxidizing bacterium was developed in *N. europaea* and the first mutants were engineered in this bacterium. Today it is possible to manipulate *N. europaea* genetically using electro-

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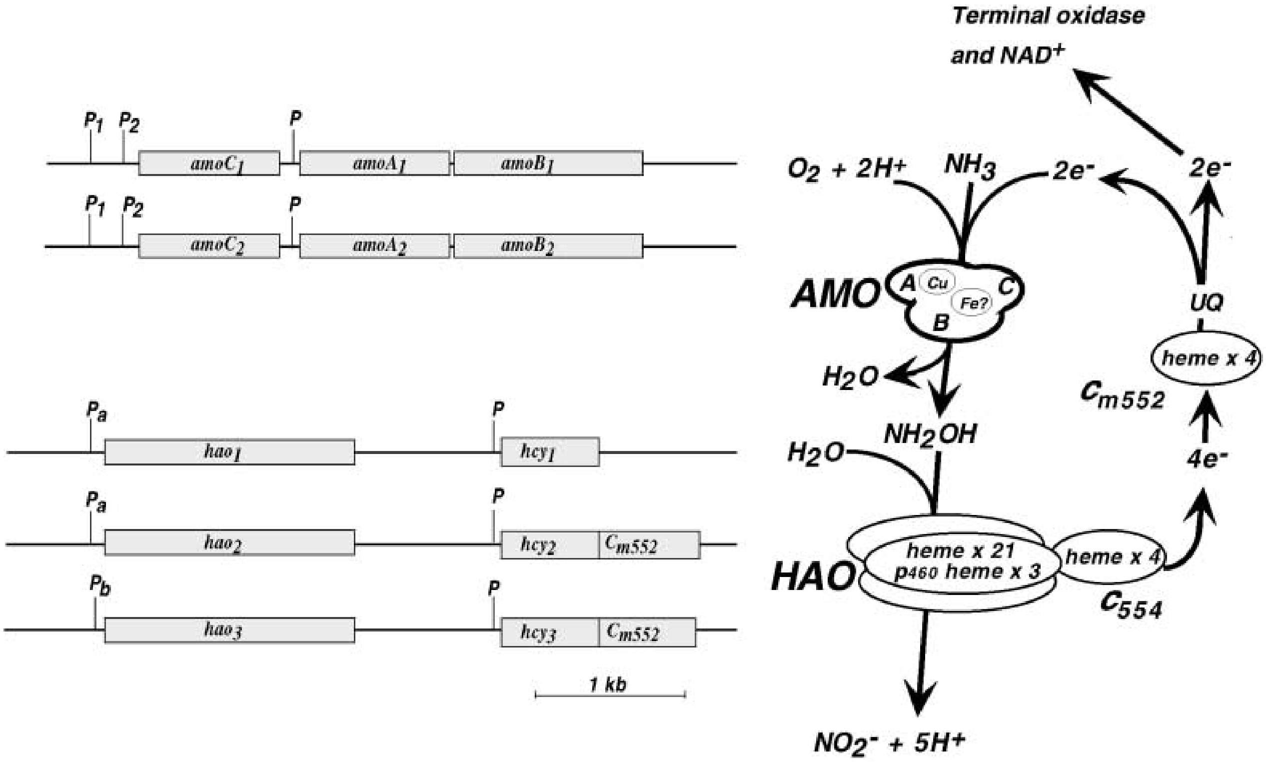

Fig. 1 Map of the loci of the genes for ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO) and cytochromes [c_{554} (encoded by *hcy*) and c_{m552}] and the ammonia-oxidizing pathway. The involvement of the ubiquinone pool (UQ) is also depicted in the pathway

poration and recombination to insert DNA constructs into its genome (Hommes et al. 1996, 1998; Iizumi and Nakamura 1997). Other *Nitrosomonas* species, as well as ammonia-oxidizing proteobacteria of the genera *Nitrosospira* (β-subdivision) and *Nitrosococcus* (γ-subdivision), have also received considerable attention (Kowalchuk and Stephen 2001), especially at the level of nucleotide sequence comparisons and phylogenetic studies.

The purpose of this review is to highlight the advances in the biochemistry and molecular biology of NH_3 oxidation in *N. europaea* and related NH_3 oxidizers. The recent developments in the genetics of the two key enzymes involved in NH_3 oxidation, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), in this proteobacterium are discussed.

Biochemistry

The oxidation of NH_3 to NO_2^- is a two-step process. AMO catalyzes the oxidation of NH_3 to NH_2OH, and HAO catalyzes the oxidation of NH_2OH to NO_2^- (Fig. 1). In vivo and in vitro studies have yielded considerable information regarding the structure and activities of AMO and HAO.

AMO is a membrane-bound enzyme and is similar to particulate methane monooxygenase (pMMO) in the methanotrophs in putative subunit composition, inhibitor profiles, broad (though not identical) substrate range, and nucleotide sequences of the genes coding for the proteins (Murrell and Holmes 1996; Semrau et al. 1995). Recently, a third member of this class has been recognized in butane-grown *Nocardioides* sp. CF8. The monooxygenase that oxidizes butane shares many properties with AMO and pMMO (Hamamura et al. 2001). AMO has not yet been purified to homogeneity with activity. The enzyme likely consists of three polypeptides. When cells of *N. europaea* are incubated with $^{14}C_2H_2$, AMO activity is lost and a 27-kDa polypeptide (AmoA) is labeled (Hyman and Wood 1985). AmoA is thought to have the catalytic site for NH_3 oxidation. A second polypeptide (AmoB; 38 kDa) copurified with AmoA (McTavish et al. 1993a). The evidence for a third polypeptide for AMO (AmoC; 31.4 kDa) is indirect: the genes of AmoC, AmoA, and AmoB are cotranscribed into a single mRNA (Sayavedra-Soto et al. 1998). AMO readily loses activity upon cell breakage; still, it is possible to test for activity in vitro (Ensign et al. 1993). Two lines of evidence suggest that Cu is a cofactor for AMO: Cu-selective chelators inactivate AMO in whole cells (Bedard and Knowles 1989), and the addition of Cu activates AMO in cell-free extracts (Ensign et al. 1993). An iron center involved in NH_3 oxidation has also been suggested (Zahn et al. 1996). Further characterization of the protein and metal composition, as well as details of the catalytic mechanism of this recalcitrant enzyme, await purification with good activity.

AMO can oxidize a broad range of substrates in addition to NH_3, including the oxidation of C-H bonds to alcohols, C=C bonds to epoxides (Hyman and Wood 1984), and sulfides to sulfoxides (Juliette et al. 1993). Among the substrates for these reactions are alkyl and aryl hydrocarbons, halogenated hydrocarbons (Rasche et al. 1991), aromatic molecules (Keener and Arp 1994) and other compounds. This conspicuously broad substrate range offers potential applications for bioremediation of sites contaminated with chlorinated aliphatic hydrocarbons.

Nitrite, a product of ammonia-oxidizing metabolism, is toxic to *N. europaea* by a unique mechanism specific for AMO (Stein and Arp 1998b). The cell may overcome some of the negative effects of NO_2^- by means of a periplasmic copper-type nitrite reductase (NirK) (Beaumont et al. 2002). Interestingly, NO_2^- was also found to stimulate NH_3 oxidation in *N. europaea* cells recovering from starvation (Laanbroek et al. 2002). Ammonia-limited *N. europaea* loses AMO activity although other cell functions, such as HAO activity, remain unaffected (Stein and Arp 1998a).

HAO is located in the periplasm and is a homotrimer of 64-kDa subunits with each subunit containing eight *c*-type hemes (Hendrich et al. 2001; Hooper et al. 1997; Igarashi et al. 1997). Seven of the hemes are each covalently bound to the protein by two thioether linkages typical of *c*-type hemes. The eighth heme, designated P460, has an additional covalent bond to the protein through a tyrosine residue and is at the active site of NH_2OH oxidation. The crystal structure of HAO has revealed the orientation of the hemes in each subunit and suggested potential paths of electron flow through the enzyme (Igarashi et al. 1997). The eight hemes group into four clusters. Four hemes are involved in two diheme clusters; two hemes and the P460 heme form a triheme cluster; and one heme is separate (Hendrich et al. 2001). The heme pairs function as two-electron redox centers in the electron transfer process. Although mid-point potentials have not been assigned to specific hemes, the potentials are known to vary from +288 to –412 mV (Collins et al. 1993; Hendrich et al. 2001).

The electron flow from HAO is channeled through cytochrome c_{554} to cytochrome c_{m552}, where it is partitioned to AMO and to the terminal oxidase through the ubiquinone pool (Whittaker et al. 2000). In the reaction catalyzed by AMO, one O from O_2 is inserted into NH_3 while the second O is reduced to H_2O. This reaction requires two additional electrons. Because NH_3 is the only source of reductant for these bacteria, the electrons required for the formation of H_2O must come from the subsequent oxidation of NH_2OH (Fig. 1). Of the four electrons released in the oxidation of NH_2OH by HAO, two must be directed towards the oxidation of NH_3 and the remaining two are used for other reductant-requiring cellular processes such as biosynthesis and ATP generation (Wood 1986).

Ammonia monooxygenase genes

The three putative polypeptides of AMO are encoded by three contiguous genes: *amoC*, *amoA*, and *amoB* (Fig. 1) (Bergmann and Hooper 1994; Klotz et al. 1997; McTavish et al. 1993a; Sayavedra-Soto et al. 1998). The genes for AMO are in two nearly identical (>99%) copies in the genome of *N. europaea* (McTavish et al. 1993a, b). A third copy of *amoC* (60% identity) is also present (Sayavedra-Soto et al. 1998). Other NH_3-oxidizing bacteria (e.g. *Nitrosomonas cryotolerans*, *Nitrosococcus oceanus*, *Nitrosospira* sp. NpAV) possess *amo* genes highly similar to those of *N. europaea* (Klotz and Norton 1995). Among the different ammonia-oxidizing bacteria, the gene cluster *amoCAB* is present in up to three copies (Klotz and Norton 1995; McTavish et al. 1993a, 1993b; Norton et al. 2002; Sayavedra-Soto et al. 1998). The strikingly high level of identity among individual *amo* gene copies within a species led Klotz and Norton to propose the existence of a rectification mechanism to maintain this identity over time (Klotz and Norton 1998).

Three AMO transcripts are detected in growing *N. europaea* cells: one corresponding to *amoC*, another corresponding to *amoAB* and another corresponding to *amoCAB* (Sayavedra-Soto et al. 1996, 1998). The reason for three AMO mRNAs is unknown; they may originate from *amoCAB* mRNA processing or from transcription from *amoC* and *amoA* (Sayavedra-Soto et al. 1998). Potential transcription start sites have been identified 166 and 103 bp upstream of the *amoC* start codon and 114 bp upstream of the *amoA* start codon in the intergenic region between *amoC* and *amoA* (Hommes et al. 2001). All three transcript start sites have putative σ^{70} promoter sequences associated with them. Both copies of *amoCAB* appear to have identical promoters since the nucleotide sequence of the regions upstream of *amoC* and *amoA* are identical in the two copies (Hommes et al. 2001).

Mutants of *N. europaea* with either copy of *amoA* (*amoA*$_1$ or *amoA*$_2$) inactivated suggest that both copies are functional and differentially expressed (Hommes et al. 1998; Stein et al. 2000). Mutants in *amoA*$_1$ grow about 25% more slowly than wild-type cells, while mutants in *amoA*$_2$ grow at rates similar to wild-type (Hommes et al. 1998). The *amoA* transcript levels in the mutants show a pattern similar to their growth rates (Hommes et al. 1998). Strains carrying a mutation in either the *amoA*$_2$ or *amoB*$_2$ genes respond similarly to wild-type cells, but the strains carrying mutations in the *amoA*$_1$ or *amoB*$_1$ genes respond differently from the wild-type and from each other in induction experiments (Stein et al. 2000). Because the *amo* promoters are identical, we must seek a different explanation for the different responses of the mutants; perhaps their location in the genome is important.

In *N. europaea*, NH_3 appears to induce a global transcription response. A broad range of labeled mRNA is detected by gel electrophoresis of total RNA prepared from cells treated with NH_4^+ and $^{14}CO_2$ (Sayavedra-Soto et al. 1996). In contrast, only a few labeled proteins are detected in these treatments (Hyman and Arp 1995). Transcription of the genes for *amoCAB* and *amoAB* is only induced in the presence of NH_4^+, even in the presence of AMO inhibitors that prevent NH_3 from serving as an energy source. These results suggest dual roles for NH_3, that of a signal for gene expression and that of an energy source (Hyman and Arp 1995; Sayavedra-Soto et al.

1996, 1998). Unlike *amoCAB* and *amoAB* mRNAs, a very stable *amoC* mRNA can be found for at least 72 h after NH_3 is removed (Sayavedra-Soto et al. 1998). Analysis of the two identified transcription start sites for *amoC* show that they respond differently to the addition of NH_4^+. In the absence of NH_4^+, transcripts starting at both potential promoters are found (i.e. derived from the stable *amoC* mRNA); in the presence of NH_4^+, transcripts from the distal promoter greatly predominate (i.e. derived from *amoCAB* and new *amoC* mRNAs) (Hommes et al. 2001).

HAO and cytochromes genes

The gene coding for HAO, *hao*, is 1,710 bp in length and is expressed as a monocistronic transcript (Sayavedra-Soto et al. 1994). The gene also encodes an 18–24 amino acid leader sequence, typical of periplasmic proteins, which is removed during translocation and maturation of HAO. The genome of *N. europaea* contains three widely separated copies of the gene for HAO (McTavish et al. 1993b). The coding regions for the three copies of *hao* are identical except for one nucleotide difference in one gene copy (Hommes et al. 2001).

The amino acid sequence of HAO appears to be unique to nitrifiers since, to date, there is no known similarity to any other sequence deposited in the data banks. The nucleotide sequences of two *hao* gene copies, hao_1 and hao_2, are nearly identical for 160 bp upstream, whereas the sequence of the third copy, hao_3, diverges from the other two 15 bp upstream of the start codon. Transcript analysis identified putative transcript start sites for hao_1 and hao_2 71 bp upstream of the start codon, and 54 bp upstream of the start codon for hao_3 (Hommes et al. 2001). All three transcript start sites had σ^{70} promoter sequences associated with them. Similar to AMO, HAO mRNA was induced by the addition of NH_4^+, although to a lesser extent (Sayavedra-Soto et al. 1996). However, it is not yet known whether the three copies are expressed differently. Mutants with any one copy of *hao* disrupted grew with no discernible difference from the wild-type strain (Hommes et al. 1996).

The genes for AMO and HAO have been examined in the closely related strain *Nitrosomonas* sp. ENI-11 (Hirota et al. 2000). Gene mapping revealed that hao_1 was located about 23 kb upstream of $amoCAB_1$; hao_2 was located about 15 kb downstream from $amoCAB_2$ and hao_3 was located about 87 kb upstream of $amoCAB_2$. The two copies of *amoCAB* were separated by 388 kb in *Nitrosomonas* sp. ENI-11 compared to *N. europaea* where the clusters were separated by 1,626 kb. Unlike *N. europaea*, three single *hao* mutants were created in *Nitrosomonas* sp. ENI-11 which had 68–75% of wild-type growth rates and 58–89% wild-type HAO activity (NH_2OH-dependent NO_2^- formation) (Yamagata et al. 2000).

Ammonia-oxidizing bacteria possess several unique cytochromes involved in electron transport from HAO (Hooper et al. 1997). The direct electron acceptor from HAO is cytochrome c_{554}, a periplasmic *c*-type tetraheme cytochrome. There is a copy of the gene that codes for this cytochrome (*hcy* or *cyc*) located 1,162-bp downstream of each copy of *hao* (Bergmann et al. 1994; Hommes et al. 1994; Sayavedra-Soto et al. 1996). The 1,162-bp intervening sequence contains no identifiable genes. Two of the three copies of the gene for c_{554} have been sequenced and were identical (Bergmann et al. 1994; Hommes et al. 1994). As with *amo* and *hao*, a σ^{70} promoter sequence was found associated with the putative transcription start site located 97 bp upstream of the start codon (Hommes et al. 2001). Another membrane-bound tetraheme *c*-type cytochrome, cytochrome c_{m552}, is encoded by genes contiguous with two of the three copies of *cyc* (Bergmann et al. 1994). The third copy of *hcy* does not appear to have a gene in this location. It is likely that the cytochrome c_{m552} is co-transcribed with cytochrome c_{554} since it is separated by only two nucleotides. Cytochrome c_{m552} is also likely to be involved in electron transfer from HAO mediated through cytochrome c_{554} (Hooper et al. 1997). A soluble cytochrome c_{552} involved with NH_3 oxidation has also been identified. The amino acid sequence and solution structure of this c_{552} have been determined (Fujiwara et al. 1995; Timkovich et al. 1998).

Other genes

In contrast to the relative abundance of studies regarding *amo*, *hao* and the genes coding for cytochromes, studies regarding other genes in *N. europaea* are scant. For example, *N. europaea* assimilates CO_2 via ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). Utaker et al. (2002) isolated and compared RubisCO sequences from 13 ammonia-oxidizing bacteria including *N. europaea*. All 13 strains were found to have type I RubisCO genes. Surprisingly, while all the other ammonia oxidizers in the β-subgroup of the Proteobacteria that were examined had “red-like” RubisCOs (similar to RubisCO genes found in red algae), *N. europaea* Nm50 had a “green-like enzyme” (similar to RubisCO genes found in green algae). The three genes flanking the *cbbLS* genes for RubisCO were likewise of the green type. The G+C content of the five-gene *cbb* gene cluster was determined to be 47 mol% as compared to 51.4 mol% for the *N. europaea* genome as a whole. The authors took these results as evidence of lateral gene transfer of the *cbb* gene cluster into *N. europaea*.

The *nirK* gene encodes a copper-containing dissimilatory nitrite reductase (NirK). The inactivation of *nirK* in *N. europaea* made the cell more sensitive to NO_2^- (Beaumont et al. 2002). Oligonucleotide primers used in PCR to amplify *nirK* from *Nitrosomonas marina* and five other isolates of β-subdivision ammonia-oxidizing bacteria failed to amplify *nirK* from *N. europaea* and three other ammonia-oxidizing bacteria (*Nitrosococcus oceani*, *Nitrosospira briensis*, and *Nitrosomonas eutropha*) (Casciotti and Ward 2001). The nucleotide sequence of the *N. europaea nirK* showed a very different sequence from that of any other known *nirK*.

A gene encoding a periplasmic red cupredoxin-like protein called nitrosocyanin was isolated and sequenced (Arciero et al. 2002). Nitrosocyanin was found to be unique among cupredoxins regarding its spectroscopic, oligomeric and redox properties. The genes encoding enolase (*eno*) and CTP synthase (*pyr*G) are adjacent in *N. europaea*, as they are in *Escherichia coli*, albeit in contrast to most other bacteria examined (Mahony and Miller 1998). A *dnaK* gene from *N. europaea* was identified and sequenced (Iizumi and Nakamura 1997). The transcription start site was mapped to 16 nucleotides upstream of the translational start codon and was preceded by a consensus promoter sequence for σ^{32}-dependent heat shock promoters of gram-negative bacteria. The gene showed an eight-fold increase in expression upon heat-shocking at 37 °C. The extent of the involvement of the above genes in the expression or regulation of NH_3 oxidation remains to be established.

N. europaea was selected by the United States Department of Energy for a whole-genome sequencing project. The sequencing effort is being carried out at the Lawrence Livermore National Laboratory under the auspices of the Joint Genome Institute. The sequences are publicly released as they become available and can be viewed, along with computational annotation of the sequences, at http://bahama.jgi-psf.org/prod/bin/microbes/neur/home.neur.cgi. The discovery of genes involved in the expression and regulation of NH_3 oxidation has now the potential to accelerate rapidly with the complete nucleotide sequence of the genome of *N.* europaea.

Summary

Nitrosomonas europaea has served as a model for our understanding of the biochemistry and molecular biology of NH_3 oxidation. Not surprisingly, much attention has focused on the proteins and genes responsible for the oxidation of NH_3. HAO is well-studied, including a crystal structure of this heme-rich enzyme. Nonetheless, details of the catalytic cycle and the role of all eight hemes in each subunit remain to be determined. In contrast, AMO has yet to be purified with activity so that even basic questions of subunit composition and metal composition can be confirmed. In this organism, as in other ammonia-oxidizing bacteria, two rather unique sets of genes are involved in NH_3 oxidation. The genes coding for AMO, while similar to the genes coding for pMMO, are otherwise unique. To date, the genes coding for HAO are also unique, though a similarity to the hydrazine-oxidizing enzyme found in anaerobic ammonia-oxidizing bacteria might be anticipated based on similar biochemical properties (Schalk et al. 2000). An interesting feature of both of these sets of genes, *amo* and *hao*, is that they are present in multiple and nearly identical copies. The mechanism for maintaining these copies has not been identified. Mutational studies have not provided a clear rationale for the existence of multiple copies of these genes. In all cases, individual copies are dispensable. In some cases, loss of a particular copy leads to decreased growth rates, and there are some indications of independent regulation of the individual copies. The availability of the complete genomic sequence for *N. europaea* is expected to facilitate new developments in our understanding of this nutritionally limited bacterium. Of particular interest will be the discovery of the basis of the obligate nature of this bacterium's chemolithoautotrophic lifestyle.

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