

EXPRESSION OF TWO *NITROSOMONAS EUROPAEA* PROTEINS, HYDROXYLAMINE OXIDOREDUCTASE AND NE0961, IN *ESCHERICHIA COLI*

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ABSTRACT

We describe the heterologous expression of the *Nitrosomonas europaea* genes for hydroxylamine oxidoreductase (HAO) and a membrane protein, NE0961, in *Escherichia coli* strain BL21(de3), which also constitutively expressed the *E. coli* *ccmA-H* genes for *c*-cytochrome maturation and transport. Both HAO and NE0961 were expressed only in the membrane fraction of cells; only slight insertion of heme into HAO was observed. Co-expression of the genes for HAO and NE0961 was not sufficient for HAO transport to the periplasm or for complete heme insertion.

INTRODUCTION

Nitrosomonas europaea is a well-studied, obligatory, chemoautotrophic, ammonia oxidizing bacterium (AOB) (Wood 1986). Because of the presence of such enzymes as ammonia monooxygenase (AMO), it has been proposed for use in bioremediation of a variety of halogenated organic compounds, such as trichloroethylene (Arciero et al. 1989). *N. europaea* and other AOB play a vital role in the nitrogen cycle by oxidizing ammonia (NH₃) to nitrite (HNO₂), through which they obtain energy for growth and survival. However, in some aerobic environments, chemoautotrophic, ammonia-oxidizing Archaea are the predominant organisms oxidizing ammonia to nitrite (Francis et al. 2007). AOB are found in two phylogenetic lineages of the *Proteobacteria*: the closely related genera *Nitrosomonas* and *Nitrosospira* within the *Betaproteobacteria* and several strains in the gammaproteobacterial genus *Nitrosococcus*, including *Nitrosococcus oceani* (Head et al. 1993; Teske et al. 1994; Purkhold et al. 2000).

Ammonia oxidation to nitrite by AOB occurs in two enzyme-catalyzed steps. Ammonia is first oxidized to hydroxylamine (NH₂OH) by a membrane bound, hetero-trimeric copper enzyme, ammonia monooxygenase (AMO) (Arp et al. 2002; Norton et al. 2002; Hooper et al. 2005). The resulting hydroxylamine is

further oxidized to nitrite by a periplasmic enzyme, hydroxylamine oxidoreductase (HAO) (Hooper et al. 1978; Whittaker et al. 2000). HAO contains seven *c*-type hemes and an active-site heme, known as heme P460, which contains a novel, covalent link between the heme and Tyr 467 (Arciero et al. 1993). A periplasmic, monoheme enzyme, cytochrome P460, may oxidize some of the hydroxylamine not oxidized by HAO, and has a unique active site *c*-heme which is connected covalently to a lysine side-chain (Erickson et al. 1972; Numata et al. 1990). The four electrons produced by hydroxylamine oxidation are accepted by a periplasmic, tetraheme, protein, cytochrome c_{554} , in two-electron steps. These electrons are thought to be transferred to a membrane-associated, tetraheme protein, cytochrome c_{m552} , before the electrons are accepted by membrane ubiquinone (Hooper et al. 2005; Hooper et al. 1978). Two electrons are used in the AMO reaction and the other two are designated for an oxidative electron transfer chain and the cytochrome aa_3 terminal oxidase (DiSpirito et al. 1986).

The gene encoding HAO (*hao*) is part of a cluster of three or four genes present in three copies in *N. europaea* genome: the gene cluster *hao*-ORF2-*cycA*-*cycB*, present in two identical copies, and the cluster *hao*-ORF2-*cycA*, present in a single copy (Bergmann et al. 1994; Sayavedra-Soto et al. 1994; Chain et al. 2003). The genes *cycA* and *cycB* gene code for cytochrome c_{554} and cytochrome c_{m552} , respectively, which transfer electrons from HAO into the electron transport chain. ORF2 of the *hao* gene cluster encodes a putative integral membrane protein (NE2338 or NE0961) (Bergmann et al. 2005). NE0961 of *N. europaea* has sequence homology with no other proteins, except for those in the *hao* gene cluster of other Bacteria and Archaea.

Apart from AOB, a number of other Bacteria and some Archaea are known to have genes encoding HAO in their genome (Bergmann et al. 2005). In all cases, the gene encoding HAO was present as a tandem with the gene encoding NE0961. This suggests possible roles for NE0961, either as an HAO export/processing protein, or perhaps mediating interactions between HAO and the cytoplasmic membrane.

In most gram-negative bacteria, the polypeptides for periplasmic *c*-cytochromes are transported from the cytoplasm through the cytoplasmic membrane into the periplasm via the SecYEG export system, and hemes are covalently inserted onto cysteine side-chains of the polypeptide through the action of the CcmA-H gene products (Thoeny-Meyer 2002). Despite the modification of its *c*-heme with an additional heme-polypeptide crosslink, cytochrome P460 of *N. europaea* does not require any unique heme processing system, and can be readily expressed in *Pseudomonas aeruginosa* (Bergmann et al. 2003) and in an *E. coli* strain which constitutively expressed the *ccmA*-H genes (Elmore et al. 2006). While it is likely that the SecYEG export system and the CcmA-H heme insertion system are involved in HAO export and heme insertion, it is not known if additional proteins are required for this process.

In this study we attempted the co-expression of the genes for HAO and NE0961, alone and together, in the *Escherichia coli* strain BL21(de3). We demonstrate that the NE0961 polypeptide can be expressed in the membrane fraction of *E. coli*, although at low levels. HAO apoprotein can be produced at high levels in the membrane (insoluble) fraction of *E. coli* cells expressing the HAO

gene and constitutively expressing the *ccmA*-H cytochrome processing genes; however, little heme insertion and no transport to the periplasm was observed, even if the gene for NE0961 is co-expressed. This indicates that the transport and processing of HAO may require proteins in addition to the SecYEG transporter, the Ccm heme processing system, and NE0961.

METHODS

Source of *N. europaea* DNA, DNA purification, and DNA modifying enzyme—Genomic DNA from *Nitrosomonas europaea* (Schmidt strain) was prepared as described by McTavish et al. (1993) and was provided by Dr. Alan B. Hooper at University of Minnesota. Restriction endonuclease digestions and ligation with T4 DNA ligase were performed as recommended by the manufacturer (Promega). Purification of plasmid DNA from *E. coli* cells, restriction fragments from agarose gels, and PCR products were performed using Qiaprep Spin Miniprep, Qiaquick Gel Extraction, and Qiaquick PCR Cleanup kits, respectively, as recommended by the manufacturer (Qiagen).

Construction of *E. coli* Expression Host Strain—Expression studies were conducted in *E. coli* strain BL21(de3) (Novagen, Inc.). Plasmid pEC86 (Arslan et al. 1998), a gift of Dr. Linda Thoeny-Meyer, constitutively expresses the *E. coli ccm* genes for cytochrome processing. pEC86 was transformed to *E. coli* BL21(de3) competent cells by heat-shock as recommended by the manufacturer (Novagen), and the transformed colonies were grown on LB agar with 30 µg/mL chloroamphenicol. Additional plasmids containing the *hao* and/or ORF2 genes were also introduced by heat-shock transformation and the transformants were cultured on LB agar with the appropriate antibiotics (30 µg/mL chloroamphenicol with 50 mg/mL ampicillin or 30 µg/mL chloroamphenicol with 50 µg/mL ampicillin and 30 µg/mL kanamycin).

Cloning of *hao* and ORF2 into an IPTG-Inducible, Dual-Promoter Plasmid Vector—The gene *hao* was amplified by PCR from the genomic DNA by using forward primer HAOFA (5'-GCT-AAC-ATA-TGA-GAA-TAG-GGG-AGT-GGA-3') and reverse primer HAOR1 (5'-CAA-CAA-CTC-GAG-TCA-AGC-TCG-GGT-CTG-CTT-3'). The gene (ORF2) for NE0961 was amplified by PCR using forward primer ORF2F1 (5'-GAA-GAA-CCA-TGG-CCG-CAC-TGA-CAA-CCG-ACC-GG-3') and reverse primer ORF2R1 (5'-CAA-CAA-GTC-GAC-TCA-TTG-TAC-CTG-ATC-GAC-C-3'). The total volumes of the PCR reactions were 50 µL and used the Phusion High-Fidelity PCR Kit (Finnzymes, Inc., MA, USA) containing 500 nmoles of primer, 2 ng of template, 0.2 mM dNTPs, 5X Phusion HF buffer, and 1.5 mM MgCl₂. The PCR program used an initial denaturation at 9 °C for 30s; 30 cycles of denaturation at 98 °C for 10 s, annealing at 58.1 °C for (for *hao*) or 59.1 °C (for ORF2) for 30 s, and extension at 72 °C for 60 s; and a final extension at 72 °C for 10 min. The purified PCR products of the HAO gene and ORF2 gene and also the expression vector pETDuet-1 (Novagen®, Madison, WA, USA) were digested with restriction enzymes *NdeI/XhoI* and *NcoI/SalI* overnight at 35 °C. The *NcoI/SalI* digested ORF2 PCR product and *NcoI/SalI* digested pETDuet-1 vector

were ligated to produce the plasmid pETORF2 (Table 1). *NdeI/XhoI* digested *hao* PCR product was ligated to *NdeI/XhoI* digested pETDuet-1 to produce the plasmid pHAO. pHAO-ORF2 was made by ligation of the *NcoI/SalI* digested *hao* PCR product into *NcoI/SalI* digested pORF2. Refer to Table 1 for a summary of the plasmids used in this study. Transformation of the pHAO, pORF2, pHAO-ORF2 and pETDuet-1 vector into *E. coli* BL21(DE3) competent cells was performed by heat-shock as recommended by the manufacturer (Novagen Inc). The transformed colonies were plated on solid LB agar with 50 µg/mL ampicillin and incubated overnight at 37 °C. Characterization of recombinant plasmids was confirmed by restriction digestion of the recombinant plasmid with the appropriate restriction enzymes, and by dideoxy-chain-termination sequencing using ABI Big Dye version 3.0 (Applied Biosystems) at the Center for Conservation of Genetic Resources at Black Hills State University, Spearfish, SD USA. *E. coli* BL21 cells previously transformed with the plasmid pEC86 were also transformed with plasmid pETDuet-1, pHAO, pETORF2 and pHAO-ORF2. The transformed colonies were grown on solid LB (agar 1.5%) with 30 µg/mL chloroamphenicol and 50 µg/mL ampicillin for the screening of the single colonies with two plasmids (pEC86 and pHAO, pEC86 and pORF2, and pEC86 and pHAO-ORF2).

Cloning of hao into an arabinose-inducible expression plasmid and ORF2 into a separate IPTG inducible plasmid—The gene *hao* was amplified using 2.0 µM primers HAOFA and HAOR3 (5'-GTC-TCT-AGA-CAT-TGC-CAG-TGG-TTA-CCT-GT-3'), 60 mM Tris-SO₄ (pH 8.9), 18 mM (NH₄)₂SO₄, 4 mM MgSO₄, 20 ng template DNA, 0.2 mM dNTPs, and 5 Units of Platinum Taq Polymerase (Life Technologies) in a volume of 50 µL. PCR was performed with a GeneAmp 2400 thermal cycler (Perkin-Elmer) using a standard program: initial denaturation for 5 min at 94 °C and 25 cycles consisting of 30 s denaturation at 94 °C, 30 s annealing at 45 °C, and 60 s extension at 68 °C, followed by a final 7 min extension step at 7 °C. The PCR product was digested with *NdeI* and *XbaI* and ligated into the plasmid pUCPNDE (Cronin and McIntyre 1999) (a gift of Ciaran Cronin) which had been digested with *NdeI* and *XbaI*. The recombinant plasmid (pUHAOF2) was transformed into *E. coli* strain DH5αFIQ (Life Technologies) as described by Chung et al. (1989), and transformants grown on LB media with 100 µg/mL ampicillin. pUHAOF2 plasmid DNA was purified and digested with *NdeI* and *XbaI*, and the approximately 2 kBP restriction fragment containing HAO was purified by preparative agarose gel electrophoresis and ligated into the plasmid pISC2 (Thoeny-Meyer et al. 1998) (which had also been digested with *NdeI* and *XbaI*) to produce the plasmid pIHAO, which has *hao* downstream of the arabinose-inducible *ara* promoter. The purified ORF2 PCR product, digested with *NcoI* and *SalI* (see above), was ligated into the plasmid vector pRSF1b (Novagen) after it had been digested with the same restriction endonucleases to produce the recombinant plasmid pRSF-ORF2, which had ORF2 cloned downstream of the IPTG inducible promoter on the vector.

The plasmids pEC86 and pIHAO and/or pRSF-ORF2 were transformed into competent *E. coli* BL21(de3) cells (Novagen) by heat-shock and grown on LB media with the appropriate antibiotics (50 µg/mL for pEC86, 30 µg/mL Kanamycin for pRSF-ORF2, and 50 µg/mL ampicillin for pIHAO).

Table 1. Plasmids used in this study. The source of the plasmid, antibiotic resistance genes present, substance used to induce transcription from the promoter(s) upstream from the cloning site, and *N. europaea* genes expressed (if any) are given. Abbreviations used: cm = chloroamphenicol, amp = ampicillin, IPTG = isopropyl β -D-1-thiogalactopyranoside, and kan = kanamycin.

NAME OF PLASMID	SOURCE	ANTIBIOTIC RESISTANCE	INDUCTION OF PROMOTER	GENES EXPRESSED
pEC86	Linda Thoeny-Meyer	cm	constitutive	<i>E. coli</i> ccmA-H
pETDuet-1	Novagen	amp	IPTG	-
pHAO	this study	amp	IPTG	<i>N. europaea</i> hao
pORF2	this study	amp	IPTG	<i>N. europaea</i> ORF2
pHAO-ORF2	this study	amp	IPTG	<i>N. europaea</i> hao and ORF2
pISC2	Linda Thoeny-Meyer	amp	arabinose	-
pIHAO	this study	amp	arabinose	<i>N. europaea</i> hao
pRSF1b	Novagen	kan	IPTG	-
pRSFORF2	this study	kan	IPTG	<i>N. europaea</i> ORF2

Production of HAO and NE0961 in *E. coli* cells containing recombinant plasmids—The resulting cells containing recombinant plasmids (Table 1) were grown in 3 mL and 15 mL cultures of LB media with the appropriate antibiotics at 30 °C until an OD₆₀₀ of 0.6-0.7 was attained, and the inducer (IPTG or l-arabinose) was added to induce transcription from genes cloned in expression plasmids. After 3-6 h, membrane (insoluble), whole cell and periplasm extracts were prepared using a commercial periplasting kit as directed by the manufacturer (Epicentre, Inc). Proteins in the cell fractions were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Garfin 1990). Proteins in SDS-PAGE gels were visualized by Coomassie Blue or 0.3 M CuCl₂ staining and the *c*-cytochromes by heme-staining (Goodhew et al. 1986). The protein concentration of cell extracts was determined using the method of Bradford (1976). Trypsin digestion of selected protein bands on SDS-PAGE gels, Nano-LC-EST mass spectrometry, and searches of peptide databases using Mascot (Matrix Science, Inc., Boston, MA) were performed at the Proteomics Core Facility at the University of South Dakota, Vermillion, SD.

RESULTS

Expression of hao and ORF2 from Dual T7 IPTG-Inducible Promoter Plasmid—*E. coli* BL21(de2) cells, all containing the plasmid pEC86, also containing a dual T7-promoter plasmid (either pETDuet-1, pHAO, pORF2, or pHAO-ORF2) were grown in 3 mL LB cultures, either with or without induction with 1 mM IPTG. Cytoplasmic and periplasmic fractions of cells with all of the different plasmids, with or without IPTG induction, appeared identical in the sizes of and relative abundance of polypeptides noted in SDS-

1131.61, 1145.65, 1230.69, 1299.76, 1357.71, 1388.83, 1753.02, 1890.95, 2016.03, 2522.33, and 3348.73 Da) which were identified as fragments of HAO. The 39 kDa polypeptide yielded nine tryptic fragments (with masses of 870.52, 1025.57, 1258.67, 1309.71, 1469.78, 1815.91, 1826.90, 1920.93, and 2422.05 Da) and was identified as NE0961. IPTG-induced cells with pHAO and pHAO-ORF2 also overproduced an apparently 36 kDa polypeptide; mass spectrometry of tryptic digests of this polypeptide yielded 21 peptides (with masses of 2593.60, 2437.48, 2851.70, 1107.57, 1020.57, 1762.75, 1764.77, 3692.64, 1248.57, 2202.22, 1368.76, 1497.85, 1130.64, 1002.55, 1846.89, 1085.57, 2772.53, 2925.48, 2132.04, 2148.03, and 1738.95 Da) identified as the *E. coli* outer membrane porin 1a (OmpF, NP_415449). The sizes of the HAO, NE0961, and OmpF polypeptides predicted by SDS-PAGE (60, 39, and 36 kDa, respectively) are somewhat smaller than their sizes predicted from gene sequences (62.52, 41.84, and 39.31 kDa). Westerhuis et al. (2000) noted that SDS-PAGE tends to underestimate the size of integral membrane proteins, which may bind excessive amounts of SDS. The Heme-staining of SDS-PAGE gels of membrane proteins indicated that a small portion of membrane-bound HAO had heme attached (Figure 1).

Expression of hao on an ara Promoter Arabinose Inducible Promoter Plasmid and ORF2 on a T7 IPTG-Inducible Promoter Plasmid—To independently regulate the amount of HAO and NE0961 produced, *hao* and ORF2 were cloned into separate expression vector plasmids, inducible by arabinose (*hao*) and IPTG (ORF2) and then transformed into cells of *E. coli* BL21(de3) along with the plasmid pEC86. *E. coli* BL21(de3) cells with pEC86 and either pISC2 and pRSFORF2 (negative control), pRSFORF2, pIHAO, or pIHAO and pRSF-ORF2 together were grown in 3 ml cultures and left uninduced, induced with 0.05% arabinose, induced with 1 mM IPTG, or induced with 0.05% arabinose and 1 mM IPTG at mid-log phase. Coomassie Blue stained SDS-PAGE gels of the cytoplasmic and periplasmic fractions of cells with all plasmids, induced and uninduced, showed an identical distribution of polypeptides (data not shown). Also, heme staining indicated no *c*-cytochromes were present in the cytoplasmic or periplasmic fractions of these cells (not shown). SDS-PAGE gels of the membrane fraction of cells (Figure 2) indicated that cells with the pRSF-ORF2 plasmid produced a 36 kDa polypeptide when induced with 1 mM IPTG, while those with the pIHAO plasmid produced a 63 kDa polypeptide when induced with 0.05% arabinose. In-gel trypsin digestion and MALDI-TOF mass spectrometry confirmed that the 63 kDa polypeptide was HAO and the 36 kDa polypeptide was NE0961. Heme-staining of the SDS-PAGE gel of membrane proteins indicated that a small amount of the HAO polypeptide had attached heme (Figure 2). Membrane (insoluble) extracts of these cells with the three plasmids pEC86, pRSF-ORF2, and pIHAO, induced with both arabinose and IPTG, contained 3.7 mg protein per μl . Twenty microliters of the membrane extracts, containing 74 μg of protein, was loaded on an SDS-PAGE gel along with dilutions of known quantities of horse-heart cytochrome *c*, and the gel stained to detect heme (not shown). The intensity of heme-staining of HAO in the membrane extracts was equivalent to 5.0×10^{-12} moles of heme *c*. If one assumes that approximately half of the protein in the membrane extracts was

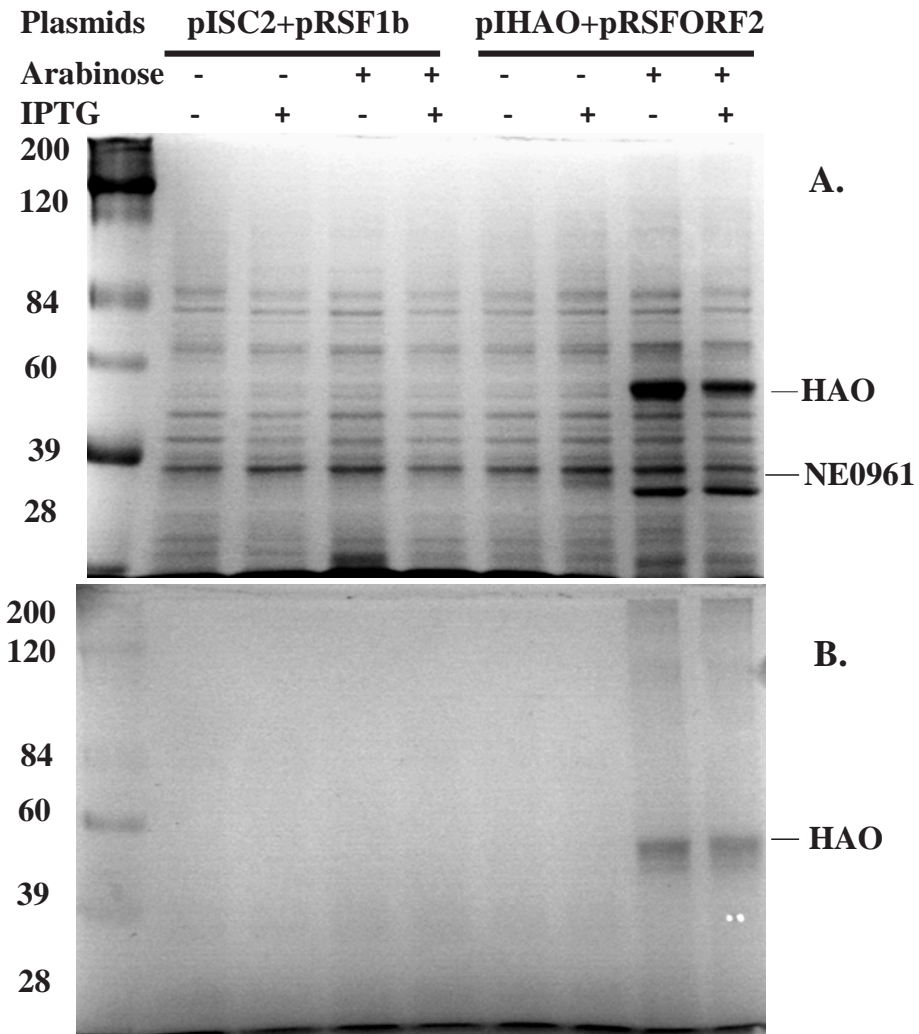


Figure 2. 12% acrylamide 0.8% bisacrylamide SDS-PAGE gel of membrane extracts from *E. coli* BL21(de3) cells containing the plasmid pEC86 and a pISC2 derived plasmid (pISC2 or pIHAO) and a pRSF1b derived plasmid (pRSF1b or pRSF-ORF2) under three different growth conditions (uninduced, induced with 1 mM IPTG, induced with 0.02% arabinose, or induced with 1 mM IPTG and 0.02% arabinose). The first lane contains molecular mass markers. (A) Gel stained with Coomassie Blue. (B) Gel stained for heme. The positions of the HAO and NE0961 polypeptides are indicated.

HAO, then this would indicate that only about 0.1% of the possible hemes had been inserted into HAO.

An experiment with *E. coli* BL21(de3) cells with pEC86, pIHAO, and pRSF-ORF2 was performed by adding varying amounts of IPTG to mid-log phase cells in order to determine the optimal concentration of IPTG required to produce NE0961. NE0961 was detected in the membrane fraction of cells induced with

50 μM IPTG, and higher, but still limited, amounts of NE0961 were produced in cells induced with 100-1000 μM IPTG (Figure 3).

Cells of *E. coli* BL21(de3) with plasmids pIHAO and pRSF-ORF2 were then induced with 100 μM IPTG and varying amounts of arabinose to regulate the amount of HAO produced relative to NE0961. A small amount of HAO polypeptide was visible in the membrane fraction of cells induced with 0.0005% arabinose, and larger amounts in cells induced with 0.001%-0.05% arabinose (Figure 4). A small quantity of the membrane-bound HAO polypeptide had attached heme (Figure 4). However, Coomassie Blue staining of SDS-PAGE gels of periplasmic extracts indicated that none of the cells had polypeptides the size of HAO monomers or homotrimers in the periplasm and heme-staining of these gels indicated no *c*-cytochromes were present in the periplasm (not shown).

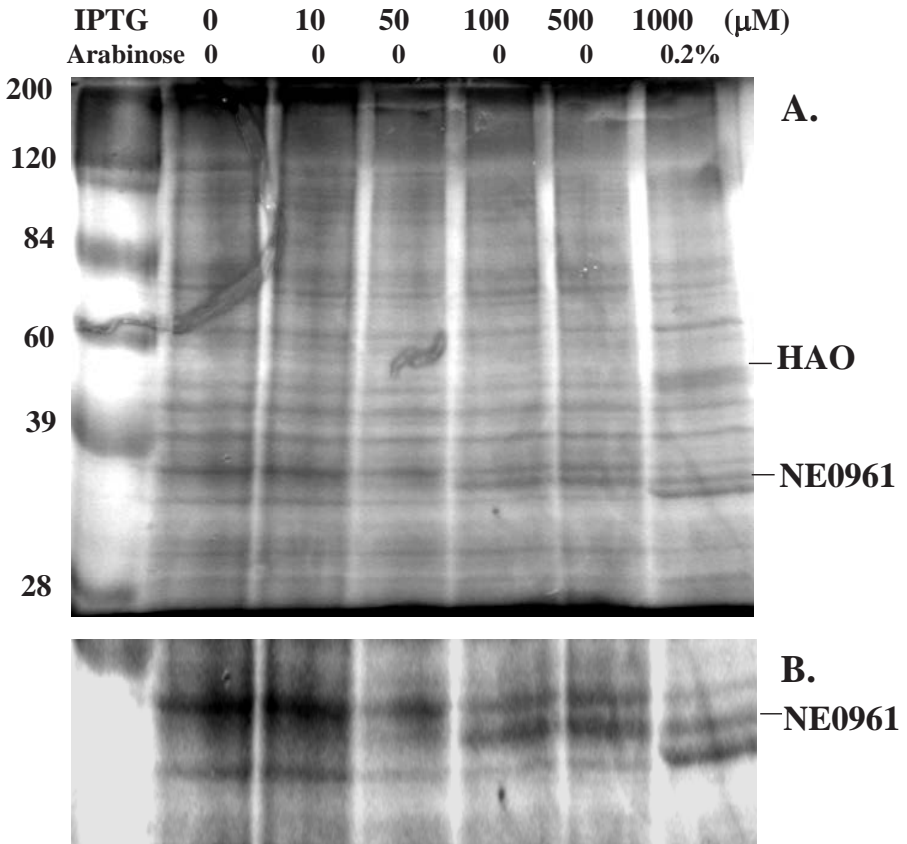


Figure 3. A. 12% acrylamide 0.8% bisacrylamide SDS-PAGE gel of membrane extracts from *E. coli* BL21(de3) cells containing the plasmids pEC86, pIHAO, and pRSFORF2, induced with varying amounts of IPTG (0-1000 μM) and Arabinose (0.0% or 0.5%). The gel was negatively stained with 0.3 M CuCl_2 . The first lane contains molecular mass markers. The positions of the HAO and NE0961 polypeptides are indicated. B. A portion of the SDS-PAGE gel image containing the NE0961 polypeptide, vertically expanded for clarity.

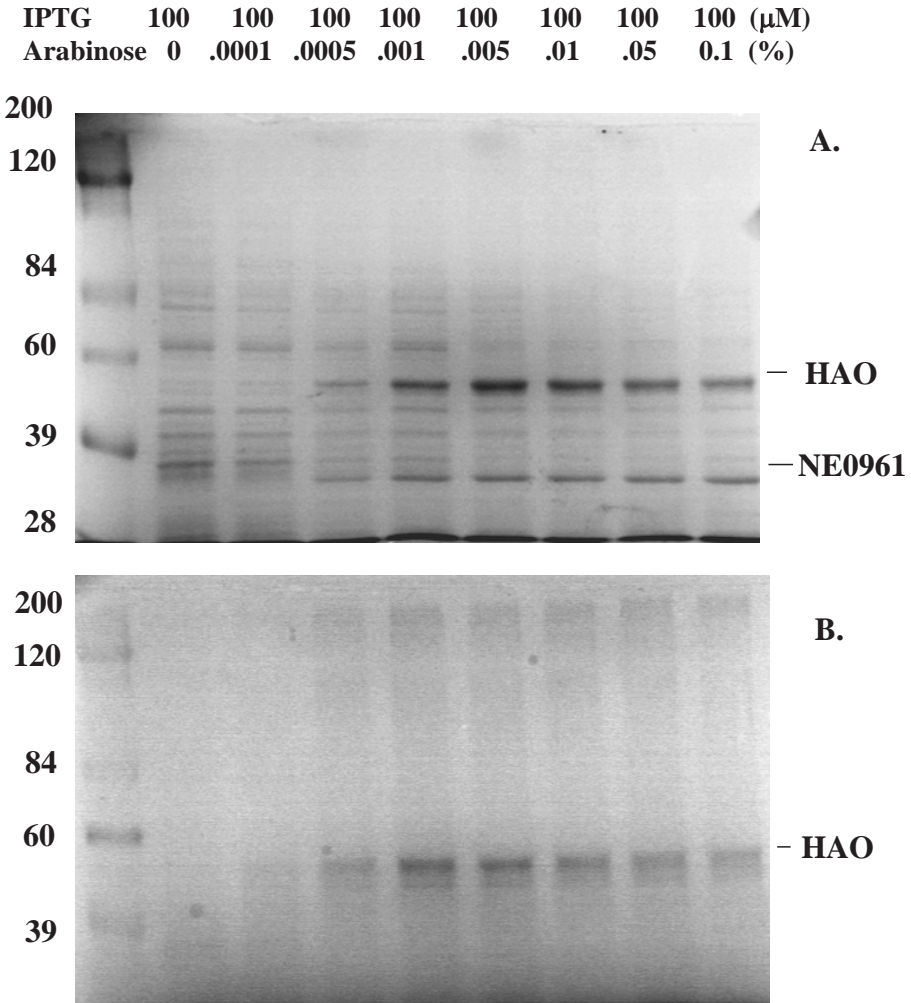


Figure 4. 12% acrylamide 0.8% bisacrylamide SDS-PAGE gel of cell extracts from *E. coli* BL21(de3) cells containing the plasmids pEC86, pHAO, and pRSFORF2, induced with 100 μM IPTG and varying amounts (0-0.1%) of arabinose. The first lane contains molecular mass markers. (A) Gel stained with Coomassie Blue. (B) Gel stained for heme. The positions of the HAO and NE0961 polypeptides are indicated.

DISCUSSION

In an attempt to examine the production of the HAO enzyme of *N. europaea*, which has a unique active site heme, we established two plasmid expression systems in which the HAO polypeptide of *N. europaea* could be over-expressed in the host *E. coli*, either alone or together with another *N. europaea* polypeptide, the integral membrane protein NE0961 (which we hypothesized might be involved in the processing of HAO). When both the genes for HAO and NE0961

were expressed from the T7 promoter, production of HAO was much greater than that of NE0961. HAO was found entirely in the insoluble (membrane) fraction of cells, not in the periplasmic fraction where the correctly exported HAO holoenzyme should be located. It appears that the HAO polypeptide, rather than being correctly exported through the SecYEG export system into the periplasm, instead accumulated as insoluble cytoplasmic inclusion bodies. Although they are usually found in the cytoplasm of bacteria, inclusion bodies, which consist of overproduced, miss-folded polypeptides, are not soluble, and often contain membrane proteins, such as OmpA and OmpF, as well as the elongation factor protein EF-Tu (Hart et al. 1990). OmpF was especially abundant in the insoluble fraction of cells expressing HAO. Because heme is inserted into the polypeptides of gram-negative bacteria during export of cytochromes into the periplasm, it is not surprising that little heme insertion into HAO occurred.

Expression of NE0961 along with HAO did not result in periplasmic export of HAO, even when expression of HAO relative to NE0961 was varied to make the amounts of HAO and NE0961 produced closer to equivalence. If NE0961 is involved in the processing and/or transport of the HAO polypeptide, it, along with the usual systems for periplasmic export and heme insertion in gram-negative bacteria, is not sufficient for production of the HAO holoenzyme in the periplasm. Other gene products, specific to microbes producing HAO, may also be required.

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LITERATURE CITED

- Arciero D, T. Vannelli, M. Logan , and A.B. Hooper. 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Biochem. Biophys. Res. Commun.* 1989 Mar 15; 159(2):640-3.
- Arciero, D.M., Hooper, A.B., Cai, M., and R. Timkovitch. 1993. Evidence for the structure of the active site heme P460 in hydroxylamine oxidoreductase of *Nitrosomonas*. *Biochemistry* 32: 9370-9378.
- Arp, D. J., L.A. Sayavedra-Soto, and N.G. Hommes. 2002. Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch. Microbiol.* 178:250–255.

- Arslan, E.H. Schulz, R. Zufferey, P. Kunzler, and L. Thoeny-Meyer. 1998. Overproduction of *Bradyrhizobium japonicum* *c*-type cytochrome subunits of the *cbb*₃ oxidase in *Escherichia coli*. *Biochem. Biophys. Res. Comm.* 251: 744-747.
- Bergmann, D.J., D.A. Arciero, and A.B. Hooper. 1994. Organization of the *hao* gene cluster of *Nitrosomonas europaea*: genes for two tetraheme *c* cytochromes. *J. Bacteriol.* 176:3148-3153.
- Bergmann, D.J., and A.B. Hooper. 2003. Cytochrome P460 of *Nitrosomonas europaea*: Formation of the heme-lysine cross-link in a heterologous host and mutagenic conversion to a non-cross-linked cytochrome *c*. *Eur. J. Biochem.* 270:1935-1941.
- Bergmann, D.J., Hooper, A.B., and M.G. Klotz. 2005. Structure and sequence conservation of *hao* cluster genes of autotrophic ammonia-oxidizing bacteria: evidence for their evolutionary history. *Appl. Environ. Microbiol.* 71: p. 5371-5382.
- Bradford, M.M. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Chain, P., J. Lamerdin, F. Larimer, W. Regala, V. Lao, M. Land, L. Hauser, A. Hooper, M. Klotz, J. Norton, L. Sayavedra-Soto, D. Arciero, N. Hommes, M. Whittaker, and D. Arp. 2003. Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *J. Bacteriol.* 185:2759-2773.
- Chung, C.T., S.L. Niemela, and R.H. Miller. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Nat. Acad. Sci. USA* 86: 2172-2175.
- Cronin, C.N. and W.S. McIntire. 1999. pUCP-Nco and pUCPNde: *Escherichia-Pseudomonas* shuttle vectors for recombinant protein expression in *Pseudomonas*. *Anal. Biochem.* 272: 112-115.
- DiSpirito, A.A., J.D. Lipscomb, and A.B. Hooper. 1986. Cytochrome *aa*₃ from *Nitrosomonas europaea*. *J. Biol. Chem.* 261:17048-17056.
- Elmore, B.O., Pearson, A.R., Wilmot, C.M., and A.B. Hooper. 2006. Expression, purification, crystallization and preliminary X-ray diffraction of a novel *Nitrosomonas europaea* cytochrome, cytochrome P460. *Acta Cryst. F62*, 395-398.
- Erickson, R.H., and A.B. Hooper. 1972. Preliminary characterization of a variant *C*-binding heme protein from *Nitrosomonas*. *Biochim. Biophys. Acta* 275:231-244.
- Francis, C.A., M.J Bemon, and M.M.M. Kuypers. 2007. New Processes and Players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. *ISME Jour.* 1: 19-27.
- Garfin, D.E. 1990. One-dimensional gel electrophoresis. In M.P. Deutscher (ed.) *Guide to Protein Purification. Methods in Enzymology* vol. 182. Academic Press, Inc., San Diego, CA.
- Goodhew, C.F., K.R. Brown, and G.W. Pettigrew. 1986. Haem staining in gels, a useful tool in the study of bacterial *c*-type cytochromes. *Biochim Biophys Acta* 852: 288-294.

- Hart, R.A., U. Rinas, and J.E. Bailey. 1990. Protein composition of *Vitreoscilla* hemoglobin inclusion bodies produced in *Escherichia coli*. J. Biol. Chem. 265: 12728-12733.
- Head, I. M., W. Hiorns, T. Embley, A. McCarthy, and J. Saunders. 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. 13:1147-1153.
- Hooper, A.B., P.C. Maxwell, and K.R. Terry. 1978. Hydroxylamine oxidoreductase from *Nitrosomonas*: absorption spectra and content of heme and metal. Biochemistry 17:2984-2989.
- Hooper, A.B., D.M. Arciero, D. Bergmann, and M.P. Hendrich. 2005. The oxidation of ammonia as an energy source in bacteria in respiration, vol. 2. Springer, Dordrecht, the Netherlands.
- McTavish, H, J.A. Fuchs, and A.B. Hooper. 1993. Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. 175: 2436-2444.
- Norton, J.M., J.J. Alzerreca, Y. Suwa, and M.G. Klotz. 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Arch. Microbiol. 177:139-149.
- Numata, M., T. Saito, T. Yamazaki, Y. Dukumori, and T. Yamanaka. 1990. Cytochrome P-460 of *Nitrosomonas europaea*: further purification and further characterization. J. Biochem. 108:1016-1023.
- Purkhold, U., A. Pommerening-Roser, S. Juretschko, M.C. Schmid, H.-P. Koops, and M. Wagner. 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. 66: 5368-5382.
- Sayavedra-Soto, L.A., N.G. Hommes, and D.J. Arp. 1994. Characterization of the gene encoding hydroxylamine oxidoreductase in *Nitrosomonas europaea*. J Bacteriol. 176: 504-510.
- Teske, A., E. Alm, J. Regan, S. Toze, B. Rittmann, and D. Stahl. 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. 176:6623-6630.
- Thoeny-Meyer L, P. Kuenzler , and H. Hennecke. 1998. Requirements for maturation of *Bradyrhizobium japonicum* cytochrome *c550* in *Escherichia coli*. Eur. J. Biochem. 235:754-761.
- Thoeny-Meyer, L. 2002. Cytochrome *c* maturation: a complex pathway for a simple task? Biochemical Society Transactions 30(4):633-638.
- Westerhuis, W.H.J., J.N. Sturgis, and R.A. Niederman. 2000. Reevaluation of the electrophoretic migration behavior of soluble globular proteins in the native and detergent-denatured states in polyacrylamide gels. Anal. Biochem. 284:143-152.
- Whittaker, M., D. Bergmann, D. Arciero, and A.B. Hooper. 2000. Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium *Nitrosomonas europaea*. Biochim. Biophys. Acta 1459:346-355.
- Wood, P.M. 1986. Nitrification as a bacterial energy source. Nitrification, Special Publications of the Society for General Microbiology Oxford: IRL Press, J.I. Prosser (editor) 20:39-62.