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 monoxygenase (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 oceanii (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 monoxygenase (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 c₅₅₄, in two-electron steps. These electrons are thought to be transferred to a membrane-associated, tetraheme protein, cytochrome c₅₅₂, 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 aa₃ 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-ccycA-cycB, present in two identical copies, and the cluster hao-ORF2-ccycA, present in a single copy (Bergmann et al. 1994; Sayavedra-Soto et al. 1994; Chain et al. 2003). The genes ccycA and ccycB gene code for cytochrome c₅₅₄ and cytochrome c₅₅₂, 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 *Nitrosonomas 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 Qiaquick 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 μg/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-ACA-ATA-TGA-GAA-TAG-GGG-AGT-GGA-3’) and reverse primer HAOR1 (5’-CAA-CAA-CTC-GAG-TCA-AGC-TGG-GGT-CTG-CTT-3’). The gene (ORF2) for NE0961 was amplified by PCR using forward primer ORF2F1 (5’-GAA-GAA-CCA-TGG-CGC-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 Phuson High-Fidelity PCR Kit (Finnzymes, Inc., MA, USA) containing 500 nmoles of primer, 2 ng of template, 0.2 mM dNTPs, 5X Phuson HF buffer, and 1.5 mM MgCl₂. The PCR program used an initial denaturation at 9 °C for 30 s; 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 Ndel/Xho1 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). \textit{NdeI}/\textit{XhoI} digested \textit{hao} PCR product was ligated to \textit{NdeI}/\textit{XhoI} digested pETDuet-1 to produce the plasmid pHAO. \textit{NcoI}/\textit{SalI} digested \textit{hao} PCR product was ligated into \textit{NcoI}/\textit{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 \textit{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 \(\mu\)g/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. \textit{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 \(\mu\)g/mL chloroamphinecol and 50 \(\mu\)g/mL ampicillin for the screening of the single colonies with two plasmids (pEC86 and pHAO, pEC86 and pORF2, and pEC86 and pHAO-ORF2).

\textbf{Cloning of \textit{hao} into an arabinose-inducible expression plasmid and ORF2 into a separate IPTG inducible plasmid}—The gene \textit{hao} was amplified using 2.0 \(\mu\)M primers HAOFA and HAOR3 (5'-GTC-TCT-AGA-CAT-TGC-CAG-TGG-TTA-CCT-GT-3'), 60 mM Tris-SO\(_4\) (pH 8.9), 18 mM (NH\(_4\))\_2SO\(_4\), 4 mM MgSO\(_4\), 20 ng template DNA, 0.2 mM dNTPs, and 5 Units of Platinum Taq Polymerase (Life Technologies) in a volume of 50 \(\mu\)L. 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 \textit{NdeI} and \textit{XbaI} and ligated into the plasmid pUCPNDE (Cronin and McIntyre 1999) (a gift of Ciaran Cronin) which had been digested with \textit{NdeI} and \textit{XbaI}. The recombinant plasmid (pUHAOF2) was transformed into \textit{E. coli} strain DH5\(\alpha\)FIQ (Life Technologies) as described by Chung et al. (1989), and transformants grown on LB media with 100 \(\mu\)g/mL ampicillin. pUHAOF2 plasmid DNA was purified and digested with \textit{NdeI} and \textit{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 \textit{NdeI} and \textit{XbaI}) to produce the plasmid pIHAO, which has \textit{hao} downstream of the arabinose-inducible \textit{ara} promoter. The purified ORF2 PCR product, digested with \textit{NcoI} and \textit{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 \textit{E. coli} BL21(de3) cells (Novagen) by heat-shock and grown on LB media with the appropriate antibiotics (50 \(\mu\)g/mL for pEC86, 30 \(\mu\)g/mL Kanamycin for pRSF-ORF2, and 50 \(\mu\)g/mL ampicillin for pIHAO).
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 OD$_{600}$ 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 CuCl$_2$ 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-
PAGE gels stained with Coomassie Blue (not shown) nor were c-cytochromes detected from heme staining of SDS-PAGE gels of cytoplasmic and periplasmic fractions of these cells (not shown). Membrane extracts of cells with pHAO and pHAO-ORF2 showed large amounts of an apparently 60 kDa polypeptide when induced with 1 mM IPTG, and cells with pORF2 and pHAO-ORF2 showed production of small amounts of a 39 kDa polypeptide when induced by IPTG (Figure 1). In-gel trypsin digestion and MALDI-TOF mass spectrometry of the 60 kDa polypeptide yielded thirteen peptides (with masses of 916.57, 983.52,
1131.61, 1145.65, 1230.69, 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, 1826.50, 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 2a (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 Indicible 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 ml. 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 x 10^-12 moles of heme c. If one assumes that approximately half of the protein in the membrane extracts was
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

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<th>Plasmids</th>
<th>pISC2+pRSF1b</th>
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A. Gel stained with Coomassie Blue. (B) Gel stained for heme. The positions of the HAO and NE0961 polypeptides are indicated.
50 µM IPTG, and higher, but still limited, amounts of NE0961 were produced in cells induced with 100-1000 mM 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 ε-cytochromes were present in the periplasm (not shown).

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**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 CuCl₂. 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.
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 alone 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


