This proposal was designed to investigate the expression of the genes required for the oxidation of ammonia (NH₃) by the nitrifying bacterium *Nitrosomonas europaea*. Previously, our laboratory made progress towards the complete physical characterization of the genes for AMO and HAO, which are present in highly similar copies. In this project we conducted experiments to characterize the regulation of the genes involved in nitrification. Our work in the current project period has produced the following communications:

**Refereed articles:**


**Published Conference proceedings:**


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In addition to the published reports, results were presented at several scientific conferences (e.g. Gordon Conferences, ASM meetings) through posters and invited talks.

Summary of results:

*amoC and amoAB mRNAs:* Primer extensions identified two transcription start sites 166 and 103 bp upstream of the amoC start codon which had \( ^{70}p \) type promoter sequences associated with them. The two promoters responded differently to the addition of NH\(_4^+\). Thus it appears that the amo operon may have tandem promoters and the distal promoter is the amoC promoter primarily responsible for new transcription in response to the addition of NH\(_4^+\).

The results of the RNase protection assays and the primer extensions of the amoAB RNA revealed one predominant fragment associated with a potential transcription start site 114 bp upstream of amoA and which had a putative \( ^{70}p \) promoter sequence associated with it. This promoter was able to drive expression of a lacZ promoterless reporter gene in E. coli. However, primer extensions and RNase protection assays also identified several other minor 5’—ends, not associated with an identifiable promoter, which were more consistent with RNA degradation of a larger precursor RNA, perhaps encoding the complete amoCAB transcript. Thus, the amoAB RNA fragments which are observed in the cell appear to be a complex result of both transcription and degradation processes. The primer extension work was published (Manuscript #2).

*Copy specific effects:* Previous work in this laboratory showed that compared to wild-type cells, N. europaea cells with amoA\(_1\) inactivated had about 25% lower growth rates, AMO specific activities and amoA mRNA levels, while cells with amoA\(_2\) inactivated had wild-type levels of AMO activity, amoA mRNA, and growth. Under this grant, this work was extended to situations where N. europaea cells were recovering either from NH\(_4^+\) starvation or acetylene or light inactivation of AMO. The regulation of expression of the two copies of amoA and amoB was examined using four mutant strains with copies of each of the four genes inactivated singly. Expression was measured by NH\(_4^+\)-dependent O\(_2\) uptake rates and the amount of new AmoA peptide synthesis (measured by \(^{14}CO_2\) incorporation) in response to either the addition of NH\(_4^+\) to starved cells, or to recovery from AMO inactivation with acetylene or light. The results indicate that there are differences in expression of the copies of the amoA and amoB genes. The regulation of expression appears complex, depending both on the gene, the copy inactivated, and the experimental conditions used. The expression of these genes appears to involve both translational and post-translational regulation. This work was published (Manuscript #1)

*hao:* In this objective we focused on the genes encoding HAO which are present in three copies in the genome (hao\(_1\), hao\(_2\), and hao\(_3\)). In order to study the expression of each copy alone, the other two copies were inactivated. Three hao mutant strains (hao\(_{12}\), hao\(_{13}\), and hao\(_{23}\)) were isolated representing the three possible double mutant combinations. Single mutant strains with only one copy of hao inactivated, which had been previously constructed, were also tested.
Wild-type cells and the single or double mutant strains did not show any significant differences in growth rates, or NH$_2$OH-dependent O$_2$ uptake rates. However, assays for in vitro HAO activity and hao mRNA levels showed that while the single mutants had activities close to that of wild type cells the double mutants strains had specific-activities averaging about 50% lower than wild-type. The two double mutant strains hao$_{13}$ and hao$_{23}$ were indistinguishable from each other by every measure indicating that hao$_1$ and hao$_2$ are expressed and regulated similarly in *N. europaea*. The genetic construct which forced the cells to rely on the expression of hao$_3$ was unstable. This together with the fact that hao$_3$ has a different promoter sequence than hao$_1$ and hao$_2$ suggests that the expression of hao$_3$ is different than the other copies of hao. The work with the hao mutants is published (Manuscript #5).

**Genome sequence:** We have been involved in the *Nitrosomonas europaea* genomic sequencing project. We prepared the genomic DNA to be sequenced. We also assisted with sequence analysis and annotation and the preparation of the manuscript. The genomic sequence has been published (Manuscript #6).

**Nitrite reductase:** During the current grant period we have participated in two collaborations. Our collaboration with B. Beaumont and R.J. van Spanning (Vrije Universiteit, Amsterdam), Netherlands concerned the function of the copper-type nitrite reductase in *N. europaea*. *Nitrosomonas europaea* is normally sensitive to elevated NO$_2^-$ concentrations and the inactivation of the gene (nirK) for nitrite reductase resulted in increased sensitivity of *N. europaea* to NO$_2^-$. Surprisingly, cells with nirK inactivated still produced nitric oxide and nitrous oxide, the latter in greater amounts than in wild-type cells. The inactivation of the oxygen sensitive transcriptional activator Fnr had no effect on nirK expression. Mr. Beaumont spent several weeks in our laboratory where he learned to grow and assay *N. europaea* and produce mutants. He left the laboratory with a nirK mutant in hand. This work was published (Manuscript #3).

**Electron flow:** In a second collaboration, Andrew Shiemke (Dept. of Biochemistry and Molecular Pharmacology, University of West Virginia) has been characterizing electron flow in *N. europaea* while on sabbatical in our laboratory. Shiemke found that diphenyliodonium chloride (DPI) inhibits AMO activity in whole cells and cell-free extracts of *N. europaea*. In other bacteria, the site of DPI inhibition appears to be the type-2 NADH:quinone oxidoreductase (NDH-2) that mediates reduction of the quinone pool by NADH (Cook, S.A., *et al.*, *Arch Biochem Biophys.* (2002) 398: 32-40). Pre-incubation of *N. europaea* with DPI inhibits ammonia oxidation and ethylene oxidation activity of AMO with either hydrazine or duroquinol as the exogenous reductant. This inhibition is irreversible but does not require AMO turnover or O$_2$, and is not prevented by alternative AMO substrates such as methane, ethylene, toluene or phenol. Thus DPI appears to block electron transfer from the quinone pool to AMO. DPI may covalently modify an AMO-reductase protein or a redox-active cofactor on AMO itself. Allylthiourea (a copper chelator) protects AMO from inhibition by DPI, suggesting that copper is required for reduction of the site of DPI inhibition.