FINAL REPORT (08/1/99-8/14/03)
Regulation of the genes involved in nitrification
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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:

- *1. Stein, Lisa Y.; Sayavedra-Soto, Luis A.; Hommes, Norman G.; Arp, Daniel J. (2000) Differential regulation of *amoA* and *amoB* gene copies in *Nitrosomonas europaea*. FEMS Microbiology Letters, **192**:163-168.
- *2. Hommes, Norman G.; Sayavedra-Soto, Luis A.; Arp, Daniel J. (2001). Transcript analysis of multiple copies of *amo* (encoding ammonia monooxygenase) and *hao* (encoding hydroxylamine oxidoreductase) in *Nitrosomonas europaea*. Journal of Bacteriology, **183**:1096-1100.
3. Beaumont, Hubertus J. E.; Hommes, Norman G.; Sayavedra-Soto, Luis A.; Arp, Daniel J.; Arciero, David M.; Hooper, Alan B.; Westerhoff, Hans V.; Van Spanning, Rob J. M. (2002) Nitrite reductase of *Nitrosomonas europaea* is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. Journal of Bacteriology, **184**:2557-2560.
- *4. Arp D.J., Sayavedra-Soto L.A and Hommes N.G. (2002) Molecular biology of ammonia oxidation in *Nitrosomonas europaea*. Mini-Review. Archives of Microbiology. 2002 Oct; 178(4): 250-5.
- *5. Hommes, Norman G.; Sayavedra-Soto, Luis A.; Arp, Daniel J. The roles of the three gene copies of hydroxylamine oxidoreductase in *Nitrosomonas europaea*. Archives of Microbiology. 2002 Dec; 178(6): 471-6.
6. Jane Lamerdin, Frank Larimer, Patrick Chain, Warren Regala, Miriam Land, Loren Hauser, Alan Hooper, Martin Klotz, Jeanette Norton, Luis Sayavedra-Soto, Dave Arciero, Norman Hommes, Mark Whittaker, Daniel Arp, Complete genome sequence of the ammonia oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. Journal of Bacteriology. 2003 May; 185(9): 2759-73.

Published Conference proceedings:

- *1.- Sayavedra-Soto L.A., Hommes N.G. and Arp D.J. Mapping of mRNAs for genes encoding ammonia monooxygenase in the nitrifier *Nitrosomonas europaea*. Abstract and poster. ASM 99th General Meeting. Atlanta, GA. May 30-June 3, 1999.
- 2.- Lamerdin J., Larimer F., Arp D., Hooper A., Norton J., Klotz M., Sayavedra-Soto L., Arciero D., Hommes N., Whittaker M. The Genome of *Nitrosomonas*: Specialization for Growth Only on Ammonia and CO₂. Abstract and poster. ASM 100th General Meeting. Los Angeles, CA. May 21-25, 2000.
- *3.- Hommes N.G., Sayavedra-Soto L.A. and Arp D.J. Characterization and mutagenesis of hydroxylamine oxidoreductase genes in the nitrifier *Nitrosomonas europaea*. Abstract and poster. ASM 100th General Meeting. Los Angeles, CA. May 21-25, 2000.
- 4.- Arp D.J., Wei A., Sayavedra-Soto L. A., Klotz M.G., Zhou J. and Yan T. Gene expression profiles in *Nitrosomonas europaea*, an obligate chemolithoautotroph. Genomes to Life. U.S Department of Energy. Contractor-Grantee Workshop I. Arlington VI. February 9-12, 2003.

*5. Arp D.J., Sayavedra-Soto L.A. and Hommes N.G. (2002). Nitrogen Fixation: Global Perspectives. Proceedings of the 13th International Conference on Nitrogen Fixation. Molecular biology of ammonia oxidation by *Nitrosomonas europaea*. Hamilton, Ontario, Canada. July 2-7, Finan, T., O'Brien, M., Layzell, K., Newton, W. eds., CABI Publishing, New York, p.299-304.

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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 type promoter sequences associated with them. The two promoters responded differently to the addition of NH₄⁺. 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 NH₄⁺.

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 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 NH₄⁺ 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 NH₄⁺-dependent O₂ uptake rates and the amount of new AmoA peptide synthesis (measured by ¹⁴CO₂ incorporation) in response to either the addition of NH₄⁺ 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_2OH -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 mutant 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, SA, *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.