Gene expression profiles of *Nitrosomonas europaea*, an obligate chemolitotroph.

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Nitrosomonas europaea is an aerobic lithoautotrophic bacterium that uses ammonia (NH_3) as its energy source. As a nitrifier, it is an important participant in the nitrogen cycle, which can also influence the carbon cycle. The focus of this work was to explore the genetic structure and mechanisms underlying the lithoautotrophic growth style of *N. europaea*.

N. europaea converts NH_3 to nitrite (NO_2^-) by the successive action of ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) in a reaction:

$$NH_3 + O_2 + 2e^- \xrightarrow{AMO} NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + 5H^+ + 4e^-;$$

of the resulting four electrons, two return to the AMO reaction and two either provide reductant for biosynthesis or pass to a terminal electron acceptor generating energy.

The genome of *N. europaea*, determined through the DOE Microbial Genome Program, made possible the studies here reported at the levels of whole genome and of single gene categories. The *N. europaea* genome consists of a single circular chromosome of 2,812,094 base pairs. Genes are distributed evenly around the genome, with ~47% transcribed from one strand and ~53% from the complementary strand. A total of 2460 protein-encoding genes emerged from the modeling effort, averaging 1011 bp in length, with intergenic regions averaging 117 bp.

Whole genome gene expression. The gene expression profile of cells in exponential growth and during starvation was analyzed using microarrays. During growth, 98% of the genes increased in expression at least two fold compared to starvation conditions. In growing cells, approximately 30% of the genes were expressed eight fold higher including genes encoding cytochrome c oxidase subunit I, cytochrome c, HAO, fatty acid desaturase and other energy harvesting genes. Approximately 10% were expressed more than 15 fold higher. Approximately 3% (91 genes) were expressed to more than 20 fold of their levels in starved cells including the gene encoding multicopper oxidase type 1. Interestingly, the expression of the genes for AMO increased approximately two fold during growth. During starvation, the bulk of the genes were down-regulated with approximately 60% conserving low levels of expression (mRNAs) compared to cells in exponential growth. Fewer than 2% of the genes were expressed more than two fold higher in starved cells. Genes expressed during starvation include those encoding NUDIX hydrolase, tyrosinase, multicopper oxidase, lipoxygenase, cycloxygenase-2, a putative transmembrane protein and other oxidative stress genes (reported at DOE Genomics: GTL. Contractor-Grantee Workshop II. Washington, D.C. February 29 - March 2, 2004.).

Carbon fixation gene expression. *N. europaea* fixes carbon via the Calvin-Benson-Bassham (CBB) cycle via a type I ribulose bisphosphate carboxylase/oxygenase (RubisCO). The RubisCO operon is composed of five genes, *cbbLSQON*. This gene organization is similar to the operon for "green-like" type I RubisCOs in other organisms. The *cbbR* gene coding for the putative regulatory protein for RubisCO transcription was identified upstream of *cbbL*. This study showed that transcription of *cbb* genes was up-regulated when the carbon source was limited, while *amo*, *hao* and other energy harvesting related genes were down-regulated. *N. europaea* responds to carbon limitation by prioritizing resources towards key components for carbon assimilation. Unlike the *amo* genes, NH_3 was not required for the transcription of the *cbb* genes. All five *cbb* genes were only transcribed when an external energy source was provided. In actively growing cells, mRNAs from the five genes in the RubisCO operon were present at different

levels, probably due to premature termination of transcription, rapid mRNA processing and mRNA degradation (results reported in Wei et al. 2004, Microbiology **150:**1869-1879).

Iron related gene expression. Because *N. europaea* has a relatively high content of hemes, sufficient Fe must be available in the medium for it to grow. The genome revealed that approximately 5% of the coding genes in *N. europaea* are dedicated to Fe transport and assimilation. Nonetheless, with the exception of citrate biosynthesis genes, *N. europaea* lacks genes for siderophore production. The Fe requirements for growth and the expression of the putative membrane siderophore receptors were determined.

N. europaea changes its heme content when Fe is at a relatively low concentration. Biochemical analyses showed that cytochrome and heme contents of cells grown in Fe-limited medium were 4 fold lower than those from Fe-rich medium. Cellular Fe contents (in both membrane and soluble fractions) showed the same trend. The activity of hydroxylamine oxidoreductase was over three fold lower in cells grown in Fe-limited medium than that in full medium. The growth yields at 0.1 μM Fe and at 0.2 μM Fe were about 35% and 65% respectively of that observed at 10 μM Fe (full Fe medium). *N. europaea* has the mechanisms to cope and grow under Fe limitation.

The *N. europaea* genome has 26 sets of genes that are organized similarly to the genes in a *fecI/fecR* system. Through similarity searches, we have identified possible TonB-dependent Fe receptor genes up- or downstream of these sets. Some of these are similar to genes encoding the siderophore receptors for desferrioxamine (desferal), ferrichrome, and coprogen (TABLE 1). In addition to those listed in Table 1, another 20 Fe/siderophore outer membrane receptor genes are identified that are not immediately adjacent to σ-factor/sensor genes. *N. europaea* also has genes for Fe storage, for Fe ABC transporter, and for other components necessary for Fe uptake.

The addition of desferal (a siderophore commonly produced by soil bacteria) to Fe-limiting medium promoted the growth of *N. europaea*, though with a longer lag phase, suggesting a necessary induction period of the corresponding receptor. A gene for the putative desferal outer membrane receptor was identified by similarity searches (NE1097, a *foxA* homologue). NE1097 was expressed at a higher level (>10 fold) in Fe-limiting, desferal-containing cultures than in Fe-sufficient cultures. The expression of NE1097 required the presence of desferal, since typical lag phases were observed when inoculants from desferal cultures were used. Several membrane proteins expressed at much higher levels in the cells grown in Fe-limited medium may be involved in Fe transport. For example, a membrane peptide with the calculated MW of the putative desferal receptor was observed only in the cells grown in desferal-containing medium. Ferric citrate had an effect similar to that of desferal on *N. europaea* growth in Fe-limiting medium, i.e. with a longer lag phase and a higher final cell density than that in the full medium. Ferrichrome, on the other hand, did not prolong the lag phase, yet increased total cell growth, suggesting that the genes for the ferrichrome receptors were expressed constitutively.

Consistent with the genome sequence data, no siderophores were detected in *N. europaea* culture filtrates under either Fe-limiting or Fe-sufficient conditions using a standard siderophore assay. We considered the possibility that citrate serves as a Fe chelator/siderophore, since *N. europaea* has the necessary genes to produce it. Citrate was detected (2 to 5 μM) in cell-free filtrates from both, low- and full Fe cultures. Surprisingly, cell-free filtrates from full Fe cultures had relatively higher concentrations (5 μM) of citrate than in low Fe cultures (2 to 3 μM). The role of citrate in Fe acquisition, if any, is yet to be determined. *N. europaea* apparently expresses siderophore receptors (e.g. NE0731, NE1097) under low Fe conditions to scavenge Fe more efficiently. These results reinforce the notion that *N. europaea* uses siderophores produced by other organisms in natural habitats.

Membrane fractions of cells grown in low and full Fe media were isolated for comparison of the protein composition. Several proteins were expressed much higher in cells grown in Fe-limited media. Analysis of the proteins highly expressed under Fe limiting condition by LC/MS/MS showed that a

number of TonB-dependent Fe-siderophore outer membrane receptors were the proteins in the predominant bands (Table 2). These proteins include at least six TonB-dependent outer membrane Fe/siderophore receptors that can be potentially involved in Fe uptake. All these six Fe/siderophore receptor genes are those that are not associated with ECF σ-factor and sensor genes. As shown in Table 2, some of them are similar to ferrichrome receptor (NE1089), or catechol/catecholate (enterobactin, NE1540) receptor, but four out of six are unidentified siderophore receptors. This result provides direct evidence for the essential role of the genes and their protein products in Fe acquisition and hence the growth and survival of *N. europaea* under Fe-limiting conditions. Interestingly, an outer membrane protein OmpC (a general diffusion Gram-negative porin) and a type II secretion pathway protein were also identified in the protein bands. These proteins have molecular weights around 78 and 82 kDa. *N. europaea* can adapt to low Fe growth environment. Higher expression of these Fe acquisition related proteins in cells grown in low Fe medium provides further biochemical evidence for the adaptation of *N. europaea* to Fe limiting conditions.

Genes encoding the putative outer membrane desferal receptor (NE1097 and NE1088, *foxA* homologues) have been cloned, insertional mutant constructs made, and mutant strains obtained through homologous recombination. We have already confirmed that desferal receptor gene is essential for the bacterium to utilize Fe-loaded desferal. Further physiological and genetic characterization of these mutants is in progress (unpublished and reported at DOE Genomics: GTL. Contractor-Grantee Workshop III. Washington, D.C. February 6-9, 2005).

Table 1. Inventory of genes coding for outer membrane σ-factor-sensor-receptor systems involved in iron acquisition. (Data from paper under review; Wei et al 2005, J. Bacteriol.)

σ-factor	**Sensor**	**Receptor (homologue)***	Putative siderophore**
NE0128	NE0127	NE0124/0125/0126 (*fecA*)	[ferric dicitrate]
NE0533	NE0534	NE0535 (*fhuA*)	Ferrichrome
NE0541	NE0542	NE0546 (*fhuA*)	Ferrichrome
NE0547	NE0548	NE0549 (*fhuA*)	Ferrichrome
NE0554	NE0555	NE0556 (*fhuA*)	Ferrichrome
NE0557	NE0558	NE0559 (*fhuA*)	Ferrichrome
NE0818	NE0817	NE0816	U
NE0980	NE0979	NE0978	Ferrichrome
NE1041	NE1039/1040	NE1038/1037	[u]
NE1071	NE1070	NE1063/1062	[u]
NE1079	NE1078	NE1072	U
NE1086	NE1085	NE1087 (*fhuE, fpvA*)	ferric coprogen, pyoverdin
NE1096	NE1095	NE1094/1092 (*fhuE*)	[hydroxamate]
NE1099	NE1098	NE1097 (*foxA*)	Ferrioxamine
NE1101	NE1102	NE1105/1108	[u]
NE1192	NE1191	NE1190	ferric alcaligin, ferripyoverdin
NE1217	NE1218	NE1220	[u]
NE1617	NE1618/1619	NE1620/1621	[u]
NE1992	NE1989	NE1988/1087	[u]
NE2138	NE2137	NE2140	U
NE2435	NE2434	NE2433 (*fhuA*)	Ferrichrome
NE2486	NE2485	NE2484/2482 (*fhuE*)	[hydroxamate]

**fecA* homologues are genes for putative iron siderophore outer membrane receptors, many of them are adjacent to *fecIR* genes.

** The far right column gives putative siderophore types. **u**, unidentified outer membrane ferric siderophore receptor, and square parentheses indicate that the ORF is probably truncated, interrupted by an IS element, or it is a pseudogene due to a reading frame shift.

Table 2. Major proteins identified by tandem mass spectrometry that are highly expressed in cells grown in low Fe media. For mass spectrometry peptide identification, selected bands from PAGE were excised and trypsin digested. The digested peptides were run on a Waters Q-TOF (Time of Flight) Ultima Global (Milford, MA). Mascot software (Matrix Science, London, UK) was used to assign the identities (data from paper under review; Wei et al 2005, J. Bacteriol.).

Gene	Protein product and putative function
NE0731*	Fiu, outer membrane receptor for monomeric catechols
NE2124*	Fiu, outer membrane receptor for monomeric catechols
NE1540*	FepA, outer membrane receptor for ferrienterochelin and colicins
NE1089*	FhuA, ferrichrome receptor, also homologous to FhuE, outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid
NE1531*	CirA, outer membrane receptor proteins mostly for Fe transport
NE1205*	CirA, outer membrane receptor proteins mostly for Fe transport
NE2563	OmpC, outer membrane protein, general diffusion Gram-negative porins
NE1604	General (type II) secretion pathway (GSP) D
NE1548	CaiA, Acyl-CoA dehydrogenases
NE2206	*ppiD*, PpiC-type peptidyl-prolyl cis-trans isomerase, also annotated as SurA, parvulin-like peptidyl-prolyl isomerase

* These are OM Fe/siderophore receptor genes that are not adjacent to sensor/sigma factor genes.