

Transcript profiles of *Nitrosomonas europaea* during growth and upon deprivation of ammonia and carbonate

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Introduction

Ammonia-oxidizing bacteria are important players in the global N cycle because they initiate the transformation of ammonia (NH₃) to nitrate (NO₃⁻). *Nitrosomonas europaea* is one of the best-studied ammonia-oxidizing bacteria at the molecular, biochemical and physiological levels (Hooper, 1989; Arp *et al.*, 2002a, b; Chain *et al.*, 2003). Although predominantly a lithoautotroph, *N. europaea* can also grow lithoheterotrophically (Hommes *et al.*, 2003). In addition to CO₂/carbonate, *N. europaea* can also use fructose or pyruvate as a carbon source for growth although growth rates are slower and final cell densities are lower (Hommes *et al.*, 2003). *Nitrosomonas europaea* converts NH₃ to NO₂⁻ by the successive actions of ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Wood, 1986; Hooper *et al.*, 1997). *Nitrosomonas europaea* fixes carbon via the Calvin–Benson–Bassham (CBB) cycle with a type I ribulose biphosphate carboxylase/oxygenase (RubisCO). The responses of AMO, HAO and RubisCO to the lack or abundance of (NH₄)₂SO₄ and CO₂ have been documented (Sayavedra-Soto *et al.*, 1996; Wei *et al.*, 2004). Actively growing *N. europaea* cells had higher mRNA levels for AMO, HAO and RubisCO than starved cells.

Abstract

The transcriptome of *Nitrosomonas europaea* was analyzed with whole-genome microarrays. Growing cells were compared to cells deprived of (NH₄)₂SO₄ and Na₂CO₃. Hybridization signals were detected for 76% of the genes represented on the array under either or both conditions. Transcript levels for 68% of the genes were at least twofold higher in growing cells than in deprived cells, while only 0.42% of the genes were present at more than twofold higher levels in deprived cells. Transcript levels for the remaining 7% of the genes did not change significantly with the treatments. These trends were confirmed for selected genes by Northern hybridizations and quantitative RT-PCR. Compared to heterotrophic bacteria, *N. europaea* downregulates a greater proportion of its genes and fewer genes appear to be associated with the adaptation to starvation.

Nitrosomonas europaea requires NH₃ for energy and for growth. However, in many soils NH₃ is present at low concentrations or is present intermittently; cells must nonetheless survive in the absence of NH₃. In light of this restrictive substrate range, the transcript profile of *N. europaea* cells incubated under substrate-deprived (starvation) conditions might be expected to be different from that observed in other more metabolically flexible bacteria.

In this work, microarrays were used to compare the mRNA pool levels in growing cells (late exponential phase) to cells deprived of (NH₄)₂SO₄ and Na₂CO₃ (with only air CO₂). Microarrays have been used to describe the responses of *Corynebacterium glutamicum* to the absence of phosphate (Ishige *et al.*, 2003), *Bacillus subtilis* to nitrogen limitation (Jarmer *et al.*, 2002), *Escherichia coli* to stationary phase or growth in minimal medium (Tao *et al.*, 1999; Tani *et al.*, 2002), *Pasturella multocida* to growth in minimal medium (Paustian *et al.*, 2002), and *Mycobacterium tuberculosis* to complete starvation (Betts *et al.*, 2002). To our knowledge, this is the first gene profiling for the response of a non-pathogenic chemolithotrophic microorganism to complete deprivation of the growth substrates. *N. europaea* decreases overall gene expression in response to the lack of its two growth-supporting substrates.

Methods

Bacterial culture and treatments

Nitrosomonas europaea (ATCC 19178) was grown in batch cultures with 25 mM $(\text{NH}_4)_2\text{SO}_4$ (50 mM NH_4^+) and 3.8 mM Na_2CO_3 as described (Ensign *et al.*, 1993; Stein & Arp, 1998). Cells were harvested from mid- to late-exponential phase cultures by centrifugation. For the ammonia/carbonate deprivation treatment (starvation), the cells were washed in $(\text{NH}_4)_2\text{SO}_4$ - and Na_2CO_3 -free medium to remove residual growth substrates and to avoid the effects of accumulated metabolites (Stein & Arp, 1998). The washed cells were then resuspended to the density at the time of harvest ($\text{OD}_{600} \sim 0.05$) in the $(\text{NH}_4)_2\text{SO}_4$ - and Na_2CO_3 -free medium and incubated for 16 h on a rotary shaker at 30 °C while exposed to the atmosphere. *Nitrosomonas europaea* cells during this starvation treatment do not change in OD_{600} significantly, and upon transfer to growth medium, grow readily. The harvested cells (growing or substrate deprived) were subjected to either immediate total RNA isolation, or suspended in an RNA stabilizer solution (RNAlater, Ambion, Austin, TX) and stored at -70 °C until RNA isolation.

Nucleic acid manipulation and hybridization

DNA preparation, restriction digestions and agarose gel electrophoresis were performed following standard protocols (Sambrook *et al.*, 1989). The recovery of probe DNA fragments was done with a commercial kit (Qiagen, Valencia, CA). PCR was performed with *Taq* DNA polymerase (Promega Co., Madison, WI) as directed by the manufacturer.

Nitrosomonas europaea cells were lysed with TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was prepared using a commercial kit (RNeasy Mini kit; Qiagen). DNA was removed from RNA samples with DNase (RNase-Free DNase set, Qiagen). The same total RNA preparations were used for microarray hybridizations and for quantitative reverse transcriptase-PCR (QRT-PCR). The concentration of RNA for microarrays was estimated on a Nanodrop spectrophotometer (NanoDrop Technologies, Rockland, DE). The QRT-PCR was performed using a kit and as directed by the manufacturer (Bio-Rad, Hercules, CA). Total RNA for Northern hybridization was isolated by phenol-chloroform extraction as described (Reddy & Gilman, 1993; Sayavedra-Soto *et al.*, 1998; Vangnai *et al.*, 2002; Wei *et al.*, 2004). Northern hybridization and transcript quantification were done as described (Wei *et al.*, 2004).

Microarray construction and analyses

Whole-genome cDNA microarrays of *N. europaea* were constructed with gene-specific DNA fragments representing duplicate spots of all putative ORFs. Construction of arrays

was done as described previously for *Shewanella oneidensis* MR-1 (Gao *et al.*, 2004). Briefly, gene-specific fragments (< 75% homology) were generated by PCR using individual primer sets designed by PRIMEGENS software (Xu *et al.*, 2002). Each gene was amplified (8–16 × 100 µl each) in 96 well plates and purified using an automated workstation (Biomek F/X; Beckman Coulter, Fullerton, CA). Of the 2461 total predicted genes, 2318 ORFs were correctly amplified (94.2% of the genome). For the 74 genes that were not amplified, specific 50-mer oligonucleotide probes were synthesized. For the remaining 69 genes unique probes could not be made because identical or nearly identical copies of the genes are present in the genome. In those cases, a single probe interrogated all the copies. In total, the PCR amplicons and oligo probes represented 97.2% of the total predicted gene content of *N. europaea*.

Amplified PCR DNA fragments were spotted in duplicate onto SuperAmine (TeleChem, Sunnyvale, CA) coated microarray slides using MicroGridII (BioRobotics, Bath, UK). Prehybridization and cDNA synthesis and labeling were performed as described using 10 µg of total RNA, random primers (Invitrogen), and a 2:3 ratio of 5-(3-aminoallyl)-dUTP (Cy3- or Cy5-dUTP) to dTTP (Amersham Biosciences UK Ltd, Bucks, UK). Six hybridizations (three biological replicates and two technical replicates with dye swap incubated at 50 °C for 14 h) for each treatment were performed. After hybridization of the arrays with labeled cDNAs, slides were washed, dried and laser scanned on a Scan Array 5000 (Parkard BioChip Technologies, Billerica, MA). The grid and quantification of all microarray data was carried out using ImaGene 5.0 software (BioDiscovery, El Segundo, CA). Data were normalized both per chip and per gene (dye-swap normalization method Workman *et al.*, 2002). The signal-to-noise ratio (SNR) was computed for each spot to establish the threshold to identify statistically significant hybridization signals (He *et al.*, 2005). The SNR was calculated as follows: $\text{SNR} = (\text{signal mean} - \text{background mean}) / (\text{background standard deviation})$; values > 3.0 were considered statistically significant (He *et al.*, 2005). Supplemental data for all SNR values, normalized relative fluorescent intensities and for the genes for which 50-mers were used in these whole genome microarrays are available at <http://www.science.oregonstate.edu/bpp/Labs/arpd/links.htm>.

Results

Overall transcription

Whole genome microarrays were used to analyze the *Nitrosomonas europaea* mRNA pools from actively growing cells and following growth substrate deprivation (no $(\text{NH}_4)_2\text{SO}_4$ or Na_2CO_3) for 16 h. Approximately 76% of the ORFs

showed a hybridization signal in the microarray slides above SNR = 3 (fluorescence intensities) as follows: 1363 ORFs associated with the growing condition only, seven ORFs associated with the starving condition only, and 439 ORFs with both conditions. For the remaining ORFs, the hybridization signal was either absent or below SNR = 3 (Table 1). Of the 583 ORFs that did not show significant hybridization, 54 (9%) are pseudogenes (parts of ORFs interrupted by IS elements or frame shifts), 276 (48%) are annotated as hypothetical proteins, 46 (7%) are genes for pili and flagellar biogenesis and chemotactic motility, 46 (8%) code for ECF σ factor and regulatory proteins, and 28 (4.8%) code for transporters (mostly TonB-dependent receptors, putative spermidine/putrescine transporters, and mercury transporters). Only 120 genes (20%) are annotated with functions other than those mentioned above and are also known in other organisms (supplemental data) (Chain *et al.*, 2003). Of the 74 genes for which 50-mers were used, 69 failed to pass SNR test and were mostly genes for small hypothetical proteins or pseudogenes. Among the 1809 ORFs showing significant hybridization signals, only 168 ORFs (including 45 encoding hypothetical proteins) had similar transcript levels (less than twofold difference) in the two treatments. The remaining ORFs differed more than twofold in transcript levels between growing and substrate-deprived conditions.

Growth vs. starvation gene expression

Two general observations become evident by comparing transcript levels of growing cells and substrate-deprived

cells: (a) during exponential growth, the number of mRNAs detected and their overall amounts were greater than during substrate deprivation (Fig. 1), and (b) only 10 genes were transcribed to twofold higher levels in the substrate deprivation treatment (Table 1).

Of the 1809 transcripts detected under one or both conditions, 1631 (90%) were present at more than twofold greater levels in growing cells relative to deprived cells (Table 1 and Fig. 1). Genes in every metabolic category were expressed at higher levels during growth than under starvation. The transcript levels of 319 genes (18%) were at least five-fold higher (Table 1). These included genes encoding ribosomal proteins, fatty acid desaturase, NADH dehydrogenase subunit I (*nuoH*), and HAO. The mRNA levels for 38 (2%) of these genes were more than 10-fold higher including cytochrome *c* oxidase subunits II and III (*coxB*, *coxC*), cytochrome *c* (NE0925/6), cytochrome *b/b₆* (NE0810), multicopper oxidase type I (NE0924), and some genes for carbon assimilation (CBB cycle) such as *cbbQ* (NE1919) and *cbbG* (NE0327). The growth/ammonia-carbonate deprivation (*G/S*) mRNA ratios for NirK (*aniA*; NE0924), and its putative associated cytochromes (NE0925/0926), were also greater than 10, but gene *pan1* (NE0927), the first gene in this putative operon, did not give a significant signal. This result is puzzling since NE0927 has been linked to NirK-dependent tolerance to NO₂⁻ (Beaumont *et al.*, 2005), and NO₂⁻ readily accumulates to toxic levels in growing cells (Stein & Arp, 1998). Transcript levels for most of the genes classified as energy production and conversion; translation, ribosomal structure and biogenesis; and nucleotide

Table 1. Number of ORFs that were expressed or not expressed, and the changes in expression level (RNA ratios) and their percentage under growing and substrate-deprived conditions

Category	Ratio range*	Number of genes	Percent of total (2392)	Percent of expressed (1809)
Genes not expressed [†]	–	583	24.37	–
Genes expressed under at least one condition [‡]	–	1809	75.63	–
No significant change [§]	–	168	7.02	9.29
(1) small ratio change	0.5 to <2	156	6.52	8.62
(2) by $P < 0.05$	–	66	2.76	3.65
Growing/starved [¶]	≤ 0.5	10	0.42	0.55
	≥ 2	1631	68.27	90.2
	2 to <5	1271	53.14	70.3
	5 to <10	319	13.34	17.6
	10 to <20	38	1.59	2.1
	≥ 20	3	0.126	0.17

*Fold changes between the two experimental conditions, including 44 ORFs using threshold (fluorescence) value (20) for the NH₄⁺/carbonate-deprived cells.

[†]Signal to noise ratio (SNR) for fluorescence intensity did not pass the threshold (3) for either treatment.

[‡]At least one treatment's fluorescence intensity passes SNR test (≥ 3).

[§]Including 12 genes whose ratios are greater than two but failed $P < 0.05$ test, and 102 genes whose ratios are smaller than two but are statistically significant at $P < 0.05$ or 0.01 (i.e. significantly different between the two treatments). RNA ratios of one treatment to the other was calculated as follows: $G/S = (\text{signal mean of } G - \text{background mean of } G) / (\text{signal mean of } S - \text{background mean of } S)$.

[¶] G/S ratio ≤ 0.5 (S/G ratio ≥ 2) and ≥ 2 ; significant at $P < 0.05$ or $P < 0.01$.

G, growing cells; S, starved, i.e. (NH₄)₂SO₄- and Na₂CO₃-deprived cells.

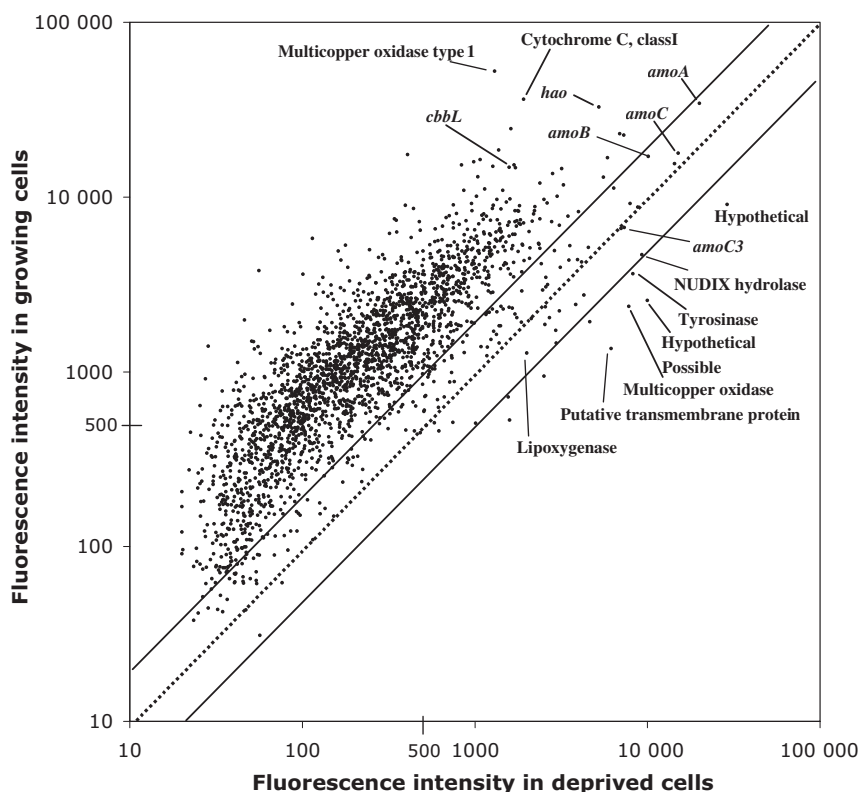


Fig. 1. Scatter plot of the relative signal intensities for the genes expressed under growing and deprived conditions. The diagonal dotted line indicates no change in expression for both conditions (ratio of relative fluorescent intensities = 1). The area between the upper and lower diagonal lines indicates no significant (< twofold) change under both conditions. The area above the upper diagonal line indicates genes expressed significantly higher (\geq twofold) under growing condition, and the area below the lower diagonal line indicates genes expressed significantly higher under deprived condition. Genes with unique expression levels or interesting function are identified in the figure.

transport and metabolism were higher in growing cells than in deprived cells.

During substrate deprivation, the transcripts of only 0.55% of the genes (10 ORFs) increased more than two-fold (Table 1 and Fig. 1). Genes with higher mRNA levels during substrate deprivation encode tyrosinase (NE1241), a putative transmembrane protein (NE1179), glutaredoxin-related protein (NE1911), NUDIX hydrolase (NE2215), two proteins of unknown function (NE2440, NE2441), and four other hypothetical proteins (NE0314, 1236, 1243 and 2439). NE2440, NE2441 are in a single transcriptional unit, and both have transmembrane domains. Six additional genes were expressed to significantly higher levels ($P < 0.05$ or 0.01) under starvation than in growing condition, although the ammonia-carbonate deprivation/growth (S/G) mRNA ratios are below two (1.36–1.93). These include chorismate lyase (NE1837), which is involved in the biosynthesis of ubiquinone (CoQ), lipoxygenase (NE1239), and cyclooxygenase (NE1240). Gene NE0448, encoding a putative ammonia permease, was expressed nearly two-fold higher ($S/G = 1.93$, $P < 0.01$) under ammonia starvation conditions. The S/G mRNA ratios for genes NE0315 (encoding a multicopper oxidase) and NE1244 (hypothetical protein) were greater than 2 but were not statistically significant (at $P < 0.05$) between the two treatments. Overall, following ammonia-carbonate deprivation, 73% of the transcripts

were present at fluorescent intensity levels below 500 (with 26% no longer having a detectable signal above background), compared to 23% for growing cells. In contrast, only 21% of transcripts had fluorescence intensity levels >500 in deprived cells compared to 71% in growing cells (Fig. 1; supplemental data). The decrease in transcript levels in the substrate deprivation treatment reflects both mRNA degradation and decreased rates of mRNA synthesis.

About 7% of the total number of genes (9% of the expressed) did not show significant changes in expression under growth or starved conditions (Table 1). A few of these genes produced high intensity signals in both growing and starved cells. For example, the relative fluorescence intensity data show that significant *amoCAB* mRNA (fluorescence intensity >7000) was conserved in the substrate-deprived cells, agreeing with previous reports (Wei *et al.*, 2004). The G/S values for *amoCAB* and *amoC3* fell between 1.1 and 1.7. G/S values for triosephosphate isomerase (NE1779), and several peptidases also fell in this range (<http://www.science.oregonstate.edu/bpp/Labs/arpd/links.htm>).

Validation of the microarray data

To corroborate the extent to which the microarray data reflected the mRNA levels of the cells, we proceeded to analyze selected genes using northern hybridization and

Table 2. Comparison of ratios of indicator genes determined by northern hybridization, by microarray analyses and by QRT-PCR

Gene name	NE gene no.	Ratio of mRNAs (G/S)		
		Northern*	Microarray	QRT-PCR*
<i>amoA</i>	NE2063	3.5	1.35	3.02
<i>amoB</i>	NE2062	–	1.53	1.74
<i>amoC</i>	NE2064	–	1.12	1.70
<i>hao</i>	NE2339	35.8	3.48	–
<i>cbbL</i> (RuBisCO large subunit)	NE1921	26.0	8.46	113.64
<i>cbbS</i> (RuBisCO small subunit)	NE1920	15.6	3.30	15.04
<i>cbbQ</i> (NorQ like protein)	NE1919	84.3	14.0	–
<i>cbbO</i> (activation protein)	NE1918	26.5	3.84	–
<i>cbbN</i> (RubisCO ORF5)	NE1917	22.3	2.86	–
<i>sdhC</i> (succinate DH)	NE1046	>100	6.98	–
<i>gdhA</i> (glutamate DH)	NE1616	63.6	3.45	–
<i>gltB</i> (glutamate synthase)	NE2123	17.4	3.40	–
<i>cbbG/gapA</i> (GAPD)	NE0327	23.2	10.10	–
CDS (anion transporter/carbonic anhydrase)	NE1927	98.3	4.10	–
CDS (hypothetical protein UPF0337)	NE2439	–	0.49	0.09
CDS (transmembrane protein)	NE1179	–	0.24	0.09
<i>cmk</i> (cytidylate kinase)	NE1963	–	3.82	0.65
CDS (ABC transporter)	NE2383	–	3.49	17.7
<i>coxB</i> (cytochrome oxidase)	NE1017	–	10.73	161.8
CDS (conserved hypothetical protein)	NE1657	–	1.14	40.7
<i>hoIC</i> (DNA pol subunit III)	NE0442	–	3.13	91.7
<i>amtB</i> (ammonia transporter)	NE0448	–	0.52	0.40

*The correlation coefficients (*r*) from linear regression analysis are 0.395 for Northern/microarray and 0.878 for QRT-PCR/microarray.

G, growing cells; S, starved, i.e. (NH₄)₂SO₄- and Na₂CO₃-deprived cells; DH, dehydrogenase; GAPD, glyceraldehyde-3-phosphate dehydrogenase; CDS, GenBank entry; QRT-PCR, quantitative reverse transcriptase-PCR.

QRT-PCR as alternative methods. With the exception of NE1963, the overall trends were the same with the three methods (Table 2). However, the G/S ratios determined with the microarray experiments fell in a narrower range (0.24–0.7) than those determined with the alternative methods (0.09–62). For example, *amoA* in northern hybridizations and QRT-PCR yielded similar results (G/S~3.5), but the microarrays gave a smaller ratio (G/S~1.35). Whereas our results suggest that microarrays are reliable indicators of trends in the levels of transcription between growing and substrate-deprived cells, they also indicate the need for corroboration by more quantitative methods.

Discussion

When nutrients are limited, bacteria undergo physiological changes to enhance their chances for survival. Many of these physiological changes are initiated by changes in mRNA levels. Examples of responses of the transcriptome in heterotrophic bacteria to the limitation of single essential substrates supplying N (Jarmer *et al.*, 2002), C (Matin, 1991), or P (Ishige *et al.*, 2003) exist in the literature. In a heterotroph, such as *Escherichia coli*, starvation for C, P and N triggered distinctly the pathway to stationary phase depending on the missing substrate (Peterson *et al.*, 2005).

Changes in transcriptomes of chemolithoautotrophs in response to nutrient starvation have not been well studied. As a chemolithoautotroph, *Nitrosomonas europaea* obtains energy and reductant solely from NH₃ oxidation. We examined the starvation response of this bacterium under NH₃⁻ and HCO₃⁻-deprivation, and it appears to respond to famine differently from the heterotrophs.

When cells of *N. europaea* were deprived of (NH₄)₂SO₄ and Na₂CO₃ for 16 h, the mRNA levels for 68% of the 2392 *N. europaea* genes decreased while the mRNA levels for only 0.42% of the genes increased (Fig. 1; Table 1). In one of few reports examining the response to complete deprivation of C, N and energy sources using microarrays, *Mycobacterium tuberculosis*, after 24 h of substrate deprivation, downregulated the mRNA levels of only 8.2% of its genes and upregulated as many as 6.9% (Betts *et al.*, 2002). Even after 96 h in complete deprivation, when internal reserves would likely be even lower, *M. tuberculosis* down regulated only 8.9% of its genes and up regulated 7.6%. As a heterotroph, *M. tuberculosis* probably maintained or expressed metabolic pathways for using organic compounds or recycling cellular components during starvation, as suggested by the fact that the majority of its genes did not change expression levels significantly during starvation compared to during growth, and that 6.9% genes were up regulated. Unlike

M. tuberculosis, the chemolithoautotroph *N. europaea* has no alternative energy source, nor does it have pathways for the recycling of most endogenous compounds (Chain *et al.*, 2003). In other heterotrophs the number of genes up regulated by substrate deprivation is also higher than in *N. europaea*. In *Escherichia coli*, transcripts for 4.6% of its genes increased upon adaptation to famine in stationary phase (Tani *et al.*, 2002). When *B. subtilis* was subjected to N-limitation, about 5% of the genes were significantly up regulated (Jarmer *et al.*, 2002).

Although signal intensities in our microarrays are not strictly proportional to mRNA levels, they can still be used as an approximation for message abundance. An $\text{SNR} \geq 3$ could thus be used to judge whether a gene was expressed or not (Verdick *et al.*, 2002; He *et al.*, 2005). In this way, it is possible to compare the percentages of the genes expressed during growth under a favorable condition (in a regular laboratory medium) among different species. The proportion of genes expressed (with an $\text{SNR} \geq 3$ fluorescence signal) in *N. europaea* was higher than those reported for *E. coli* (Corbin *et al.*, 2003) and *Pseudomonas aeruginosa* (Guina *et al.*, 2003), though this interpretation may be the result of the differences in methodology among laboratories. In *N. europaea* growing in full ammonia medium, 75% of the genes (1802) were expressed, compared to 40% (2250) of the genes in *P. aeruginosa* growing in regular and low Mg medium and to 65% (2800) of the genes in *E. coli* growing in LB medium. These differences may reflect the different ranges of usable growth substrates and metabolic diversities between heterotrophs and autotrophs. It appears that *N. europaea*, with a smaller genome and lower metabolic versatility than *P. aeruginosa* and *E. coli*, uses a higher percentage of genes for growth on ammonia, its sole energy and reductant source.

Although 68% of the *N. europaea* genes were down regulated at least twofold during starvation, the microarrays indicated that fluorescent intensities for 21% of these transcripts remained relatively high in the $(\text{NH}_4)_2\text{SO}_4^-$ and Na_2CO_3 -deprived cells (>500 ; Fig. 1). The survival strategy of *N. europaea* to cope with the lack of growth substrate while maintaining viability appears to include downregulation of most genes while at the same time maintenance of some mRNAs. This strategy may allow *N. europaea* to economize resources during starvation and to respond quickly when substrate again becomes available. For instance, conservation of *amoCAB* mRNA would allow a fast recovery when ammonia becomes available. *N. europaea* cells decreased their mRNA levels upon starvation but readily re-synthesized the mRNA pool when transferred to fresh growth medium (Sayavedra-Soto *et al.*, 1996, 1998; Wei *et al.*, 2004). During starvation, transcripts could be maintained by slowed decay, *de novo* synthesis, or both. Some of the 168 *N. europaea* genes whose mRNA levels did

not change significantly could be involved in the maintenance of cell viability during starvation. Although the current study involved only a short-term starvation response, a previous study showed that *N. europaea* could survive under starvation conditions for up to 42 days (Batchelor *et al.*, 1997). *M. tuberculosis* also maintained long-term viability in the absence of nutrients (Betts *et al.*, 2002).

Of the *N. europaea* genes that were expressed at significantly higher levels ($P < 0.05$) in substrate-deprived cells than in growing cells, most of those that could be identified by Blast search are involved in dealing with oxidative stress (see above). Tyrosinase may play a role in the adaptation of bacteria to environmental stresses (Lucas-Elio *et al.*, 2002). Glutaredoxin is an antioxidant that also confers resistance to oxidative stress (Porras *et al.*, 2002; Potamitou *et al.*, 2002). Nudix hydrolase (nucleoside polyphosphate hydrolase) plays a role in the survival of bacteria exposed to metabolic and oxidative stress (Lundin *et al.*, 2003). CoQ acts as an antioxidant to protect cells from oxidative damage by polyunsaturated fatty acids (Do *et al.*, 1996), and lipoxygenase also acts on polyunsaturated fatty acids. These genes, although already induced at significant amounts during normal growth of *N. europaea*, may need to be induced to even higher levels during starvation to cope with oxidative stress when reductant becomes limiting. Aerobic metabolism usually generates superoxide that can cause the release of free iron from iron-sulfur clusters and promotes hydroxyl-radical formation thereby damaging the cell (Keyer & Imlay, 1996). *Nitrosomonas europaea* is known to have a high content of Fe-containing proteins (Whittaker *et al.*, 2000). Unlike heterotrophs, *N. europaea* apparently cannot draw heavily on endogenous reductant sources to mitigate the effect of oxidative stress. In contrast to *N. europaea*, many of the genes expressed in *M. tuberculosis* during substrate deprivation were related to regulation, transport and the metabolism of organic compounds (Betts *et al.*, 2002).

The conservation of relatively high levels of *amoCAB* mRNAs during starvation observed in this study is in agreement with previous results with *amoCAB* mRNAs (up to 16 h in NH_3 -free medium, Wei *et al.*, 2004) and with *amoC* mRNA (up to 72 h, Sayavedra-Soto *et al.*, 1998). However, the *G/S* mRNA ratios determined by the microarrays for these genes were smaller than those obtained by Northern hybridizations (Sayavedra-Soto *et al.*, 1996). Discrepancy between microarrays and Northern hybridizations in the quantification of mRNA levels is not uncommon (Weber & Jung, 2002). Microarrays tend to underestimate increases in transcription levels while Northern hybridizations are not very sensitive to low levels of mRNA. When the transcript level for one treatment is very low, the calculated ratio is easily over-estimated, as was the case with the ratios

for NE1046 and NE1927 by Northern analysis (Table 2). QRT-PCR also detected a greater difference between the two treatments than microarrays did, but the difference was more consistent ($r=0.878$, Table 2). In analyzing the data of each method, it is also evident that transcripts within an operon (or that encode for the subunits of an enzyme) will not necessarily be maintained or produced to the same level (e.g. the operons encoding AMO, RubisCO, and NirK). The significance of the differences of expression within an operon merits corroboration through further investigation such as gene inactivation (e.g. inactivation of NE0924 decreased NirK-dependent tolerance to NO_2^- Beaumont *et al.*, 2005) and by proteomic studies.

This study offers insights to the stress response of a bacterium with a limited number of growth substrates. Few genes appear to be involved in the adaptation of *N. europaea* to the lack of NH_3 and limitation of CO_2 . Downregulation of gene expression at a nearly whole genome level during starvation could be part of the chemolithoautotrophic strategy to save resources and preserve viability for extended periods of time.

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