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ORIGINAL ARTICLE

Transcription of nitrification genes by the methane-oxidizing bacterium, *Methylococcus capsulatus* strain Bath

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Methylococcus capsulatus **strain Bath, a methane-oxidizing bacterium, and ammonia-oxidizing bacteria (AOB) carry out the first step of nitrification, the oxidation of ammonia to nitrite, through the intermediate hydroxylamine. AOB use hydroxylamine oxidoreductase (HAO) to produce nitrite.** ***M. capsulatus*** **Bath was thought to oxidize hydroxylamine with cytochrome P460 (*cytL*), until the recent discovery of an *hao* gene in its genome. We used quantitative PCR analyses of cDNA from** ***M. capsulatus*** **Bath incubated with CH_4 or CH_4 plus 5 mM $(NH_4)_2SO_4$ to determine whether *cytL* and *hao* transcript levels change in response to ammonia. While mRNA levels for *cytL* were not affected by ammonia, *hao* mRNA levels increased by 14.5- and 31-fold in duplicate samples when a promoter proximal region of the transcript was analyzed, and by sixfold when a region at the distal end of the transcript was analyzed. A conserved open reading frame, *orf2*, located 3′ of *hao* in all known AOB genomes and in *M. capsulatus* Bath, was cotranscribed with *hao* and showed increased mRNA levels in the presence of ammonia. These data led to designating this gene pair as *haoAB*, with the role of *haoB* still undefined. We also determined mRNA levels for additional genes that encode proteins involved in N-oxide detoxification: cytochrome *c*′-β (CytS) and nitric oxide (NO) reductase (NorCB). Whereas *cytS* mRNA levels increased in duplicate samples by 28.5- and 40-fold in response to ammonia, the cotranscribed *norC-norB* mRNA did not increase. Our results strongly suggest that** ***M. capsulatus*** **Bath possesses a functional, ammonia-responsive HAO involved in nitrification.**
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Introduction

Although different in their basic modes of metabolism, methane-oxidizing (methanotrophic) bacteria and obligate lithotrophic ammonia-oxidizing bacteria (AOB) share physiological characteristics that arise mainly from their ability to initiate the oxidation of both methane (CH_4) and ammonia (NH_3) with homologous enzymes, particulate methane monooxygenase (pMMO) and ammonia monooxygenase (AMO), respectively (Holmes *et al.*, 1995; Klotz and Norton, 1998; Norton *et al.*, 2002). On the basis of physiological studies (Jones and Morita, 1983; Ward, 1987, 1990) and genomic inventories (Ward *et al.*, 2004; Arp *et al.*, 2007), methanotrophs and AOB use distinct pathways for the assimilation of carbon and production of CO_2 from CH_4. On the other hand, their pathways for the oxidation of NH_3 to nitrite (NO_2^-) and possibly the production of N-oxides directly or as by-products of NH_3 catabolism appear related in terms of mechanism and genomic inventory (Zahn *et al.*, 1994; Arp and Stein, 2003; Hooper *et al.*, 2005; Klotz and Stein, 2008). An understanding of the latter process is particularly important for the understanding of the global nitrogen cycle, as recent studies indicate that both groups of bacteria may release considerable amounts of N_2O (Klotz and Stein, 2008; and references therein).

Pioneering work by Howard Dalton and associates showed that *Methylococcus capsulatus* Bath, like AOB, produces hydroxylamine (NH_2OH) as a product of NH_3 oxidation, and that this conversion is facilitated by pMMO or the soluble MMO (Dalton, 1977). More than a decade later, a pathway for the oxidation of NH_3 to NO_2^- for *M. capsulatus* Bath was

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finally described (Zahn *et al.*, 1994; Bergmann *et al.*, 1998). However, AOB and *M. capsulatus* Bath were thought to differ in the enzymes used for the intermediate conversion of NH_2OH to NO_2^- (DiSpirito *et al.*, 2005; Hooper *et al.*, 2005; and references therein). Known AOB genomes encode two enzymes that oxidize NH_2OH, hydroxylamine oxidoreductase (HAO) and cytochrome P460 (Arp and Stein, 2003; Hooper *et al.*, 2005; Arp *et al.*, 2007). HAO oxidation of NH_2OH provides electrons for both AMO activity and the generation of proton motive force; HAO exhibits much higher NH_2OH-oxidizing activity than cytochrome P460 (Hooper *et al.*, 2005; and references therein). Because AOB cannot grow without functional HAO (Hommes *et al.*, 2002), the lower activity of cytochrome P460 is consistent with a physiological role as an ancillary N-oxide detoxification enzyme. However, the role of cytochrome P460 might differ in *M. capsulatus* Bath, as it is not an obligate lithotrophic ammonia oxidizer (Klotz and Stein, 2008).

Before its genome sequence was known, *M. capsulatus* Bath was assumed to lack HAO and to use a cytochrome P460 in its nitrification pathway (Zahn *et al.*, 1994). The *hao* gene is transcribed in response to NH_3 in the AOB *Nitrosomonas europaea* (Sayavedra-Soto *et al.*, 1996), whereas in *M. capsulatus* Bath, cytochrome P460 expression is low in either the presence or the absence of NH_3 (Bergmann *et al.*, 1998, 2000). Determination of the genome sequence of *M. capsulatus* Bath revealed a putative homolog of the HAO protein (Ward *et al.*, 2004; Bergmann *et al.*, 2005). This protein not only shares more than 65% sequence similarity with Hao from AOB, but also contains the heme and ligand-coordinating residues (Tyr467, Aps267 and His268) that are critical to the function of the trimeric HAO complex (Bergmann *et al.*, 2005). Moreover, a conserved open reading frame (*orf2*) of unknown function is located 3′ of the *hao* gene in *M. capsulatus* Bath and all known AOB (Bergmann *et al.*, 2005; Arp *et al.*, 2007). This conservation of primary and secondary Hao structure as well as observed synteny suggest that *M. capsulatus* Bath could produce an active HAO trimer. If *M. capsulatus* Bath transcribes *hao* and uses HAO for NH_2OH oxidation, then the nitrification pathways of methanotrophs and AOB could be more similar than has been appreciated previously.

The objectives of this study then were (1) to determine whether *M. capsulatus* Bath transcribes *hao* and *orf2*, (2) to assess the production of these gene transcripts in response to NH_3 and (3) to quantify transcription of mRNAs for other genes encoding products (cytochrome P460, cytochrome *c*′-β (CytS) and nitric oxide (NO) reductase cNOR) that may function in the detoxification of N-oxides produced during NH_3 catabolism.

Results and discussion

Use of quantitative PCR (qPCR) for the analysis of M. capsulatus *Bath gene expression*

As we did not find any prior descriptions of the use of qPCR to study *M. capsulatus* Bath gene expression, we first determined the expression of transcript levels for *pmoB* (encoding the catalytic subunit of pMMO (Prior and Dalton, 1985; Martinho *et al.*, 2007)), *cytL* (cytochrome P460) and *cytS* for comparison with previous determinations by northern blots (Nielsen *et al.*, 1997; Bergmann *et al.*, 2000). We used the Relative Expression Software Tool (REST v.2005) (Pfaffl *et al.*, 2002) to calculate expression ratios, which represent the n-fold differences in the levels of a target sequence in the CH_4 plus 5 mM $(NH_4)_2SO_4$ ($CH_4 + NH_3$) treatment compared to the CH_4-only treatment for all genes tested. As expected, *pmoB* was expressed at constant basal levels in both the CH_4-only and $CH_4 + NH_3$ treatments when expression ratios were normalized to 16S rRNA (*rrsA*) in independent experiments. Because *pmoB* transcript levels did not vary between treatments, we used this mRNA along with *rrsA* as an internal standard in comparing transcript levels for other genes. Also as expected, we found that *cytL* transcription did not respond to NH_3, but observed a strong increase (28.5- and 40-fold) in *cytS* transcript levels in the presence of NH_3 (Figure 1b). Both results are consistent with previous reports (Bergmann *et al.*, 2000). Because qPCR yielded expected results for previously studied genes with different predicted expression patterns, we deemed it suitable as a method to study the transcript levels for genes whose expression had not been described previously (*hao*, *orf2*, *norC* and *norB*).

Determination of hao and orf2 transcript levels

To determine whether *M. capsulatus* Bath expresses *hao* in response to NH_3 and cotranscribes *hao* and the adjacent downstream *orf2*, we performed qPCR on cDNA synthesized from total RNA of duplicate cultures exposed either to CH_4 or to CH_4 plus 5 mM $(NH_4)_2SO_4$. Primer sets (Table 1; Figure 1a) were designed to amplify the 5′ and 3′ ends of *hao* (q1hao and q2hao, respectively), as well as to target the overlap between these genes (qhaoorf2) and two regions within *orf2* (q1orf2 and q2orf2). The fluorescence versus cycle number curves and electrophoretic verification of product size (data not shown) indicated that all primer sets amplified the expected target sequences from cDNA. No products were obtained from the corresponding nonreverse-transcribed RNA samples (negative control), indicating that *M. capsulatus* Bath transcribed *hao* and *orf2* in the presence of either CH_4 or $CH_4 + NH_3$.

Transcription of *hao* by *M. capsulatus* Bath exposed to $CH_4 + NH_3$ was 14.5- and 31-fold ($P < 0.05$) higher than under CH_4-only conditions based on the relative expression ratios obtained for the two independent trials, when calculated with

the cycle threshold (C_t) values of the q1hao amplicon located at the 5′ end of the transcript (Figure 1a). The q2hao primer set targeting the 3′ end of *hao* showed that transcription increased only sixfold ($P<0.05$) in response to NH_3, which reflects an apparent decrease in steady-state levels of *hao* and *orf2* mRNA around a putative stem-loop structure (Figure 1a). The qhaoorf2 primer set targeting a region that spans the 3′ end of *hao* and 5′ end of *orf2* (Figure 1a) yielded expression ratios consistent with those obtained from qPCR with the q2hao primer set (Figure 1b). On the basis of both primer pairs targeting *orf2* (q1orf2, q2orf2), cDNA from one trial showed that *M. capsulatus* Bath exposed to $CH_4 + NH_3$ increased the expression of *orf2* by 9-fold and 12-fold ($P<0.05$), whereas the other cDNA set yielded expression ratios of 3.2 for both amplified regions of *orf2*, but with marginally nonsignificant *P*-values of 0.097 (q1orf2) and 0.083 (q2orf2) for the randomization test performed by the REST program (Figure 1b). The C_t values of *hao* and *orf2* targets amplified from cDNA were higher in the CH_4-only cultures than in $CH_4 + NH_3$ cultures (Supplementary Table S1; note that C_t values are inversely proportional to the expression levels).

A comparison of C_t values obtained with each primer set on cDNA derived from both CH_4-only and $CH_4 + NH_3$ cultures as described above showed variation in the steady-state mRNA levels in different regions along the *hao* and *orf2* transcript, with primers designed to detect the upstream *hao* yielding smaller C_t values (higher steady-state levels) than those targeting *orf2* and the overlapping region between the two ORFs. There are several possible explanations for differences seen when targeting different regions with the same transcript. Given the higher G + C content of *orf2* (70%) compared with *hao* (62%), the results could be a methodological artifact due to different efficiencies of *in vitro* reverse transcription of RNA. On the other hand, there is a putative stem-loop structure ($\Delta G = -25.3\,kcal\,mol^{-1}$) centered approximately 29 base pairs downstream from the putative translational start of *orf2* (Figure 1a) possibly capable of forming a Rho-independent terminator, and resulting in reduced *orf2* transcript levels relative to *hao*. Although other factors (that is, alternate secondary structures arising during elongation or degradation,

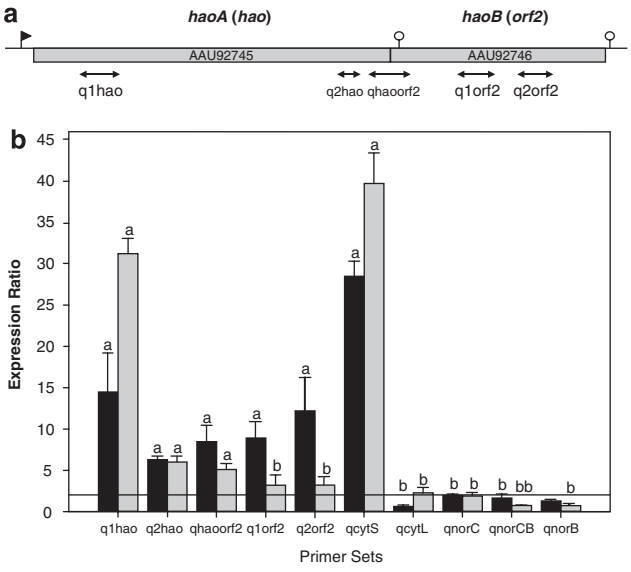

Figure 1 Map of the *haoAB* (formerly *hao–orf2*, Bergmann *et al.*, 2005) gene cluster from *Methylococcus capsulatus* Bath (**a**) and expression ratios calculated from C_t values of products amplified with primers indicated in the figure and listed in Table 1 (**b**). (**a**) Map of the *haoAB* gene cluster with locations of the putative promoter (flag) for *haoA*, stem-loop structures and amplicon positions. The translational start codon (TTG) of *haoA* (locus tag: MCA0956) beginning at position 1000257 (complement strand) in the NCBI annotation (AE017282) has been relocated to position 1000182 based on the presence of σ^{70} promoter consensus sequences and a ribosomal binding site (RBS). The first and second stem-loop structures have ΔG-values of −25.3 and $-16.5\,kcal\,mol^{-1}$, respectively, but only the second terminator is succeeded by a stretch of thymidines. (**b**) Expression ratios were calculated with the REST program using C_t values of products amplified with primers listed in Table 1. Black and gray bars represent independent trials 1 and 2, respectively, for each primer set. Error bars represent standard errors calculated for triplicate qPCR reactions. The horizontal line demarcates an expression ratio of 2. Letters designate significance (a = $P<0.05$; b = nonsignificant) of the increased expression ratio as tested through the randomization of the data set. C_t, cycle threshold; REST, relative expression software tool; qPCR, quantitative PCR.

Table 1 Primer sets used in qPCR of cDNA from *M. capsulatus* Bath

Primer	*Forward primer*	*Reverse primer*
qmca16S	GCACCTCAGCGTCAGTGTT	CGTAGGCGGTTTGATAAGTC
qpmoB	GGAGAACTACAACGAAGGCAAC	CAGCAGACGGGGGATGAA
q1hao	CGCTTTGCTGATGTTCGTT	CCGTGGGCGGTTGATAGA
q2hao	GCTCTACAAGGGGCTGGTC	GCAAACGGGTGTTCTCGTC
qhaoorf2	CCGGAAAGGATGACTCGAAC	GGAACCGGTCGTACAGCA
q1orf2	TGCGGCACGACACATGGAA	TACGCTCCGCTCCTCATC
q2orf2	TACCTGCTCGTCTGTCTGGA	AGATAGCTGGCCTGATCGAC
qcytL	ATCCTCGGCAACGACATC	GGTGCTCTTTTTCCACGACA
qcytS	CAAGGTGAAATACCCCGATG	TAGTTGCCGCCCCTCAGT
qnorC	AGTCCTACAGCGTCATCAACC	AGCAGGGTGTGGCAGTTC
qnorCB	TTCCCACCAATTTCAAGACC	CAGACCGAACAGCATCTGAA
qnorB	TGGTCTACCTGTGGGTTCAG	TAGAAAAACACGCCCAGCA

Abbreviations: *M. capsulatus*, *Methylococcus capsulatus*; qPCR, quantitative PCR.

the presence of ribosomal complexes, proteins and other RNA species that interact with nascent RNA chains and so on) can also influence the steady-state levels of mRNA *in vivo* (Carrier and Keasling, 1997), our data are consistent with a post-transcriptional regulatory mechanism in synthesis of the *hao–orf2* transcript. A similar situation was found for transcription of the gene cluster encoding AMO in the AOB *Nitrosococcus oceani*. This transcriptional unit of 5 genes (*amoRCABD*) was reported to contain leaky terminators (between *amoC* and *amoA* as well as *amoB* and *amoD*) and premature termination at these leaky terminators accounted for the difference in transcript concentrations as measured by northern analysis (El Sheikh and Klotz, 2008; El Sheikh *et al.*, 2008).

M. capsulatus Bath cotranscribes *hao* and *orf2* in response to ammonia and no sequences similar to known promoter elements upstream of *orf2* were identified. As there is conservation of *hao–orf2* synteny in all known AOB and *M. capsulatus* Bath, we propose that these genes be named *haoA* and *haoB*, respectively, and we refer to them as such in the remainder of this paper.

Transcription of norC and norB and implications for NO reduction

M. capsulatus Bath produces N_2O when supplied with either NH_3 or NH_2OH (Sutka *et al.*, 2003, 2006), although the pathway is not well characterized. While NO production has not been detected in *M. capsulatus* Bath, presumably it is a precursor of N_2O production, and both NO and N_2O are presumed to be generated as by-products of NH_2OH oxidation by cytochrome P460 (Sutka *et al.*, 2006). The presence of putative NO reductase genes (*norC* and *norB*) in the genome of *M. capsulatus* Bath raises the possibility that N_2O production is catalyzed by a dedicated enzyme. In many bacteria, genes for NO reductase are expressed in response to NO and nitrosating agents (sodium nitroprusside and *S*-nitrosothiols and so on) (Poole, 2005). Hence, we designed primer pairs (Table 1, qnorC, qnorCB and qnorB) for the contiguous genes encoding NO reductase subunits C and B (*norC* and *norB*) of *M. capsulatus* Bath to test whether these genes are expressed in the presence of ammonia. All primer sets amplified products from CH_4-only and $CH_4 + NH_3$ cDNA (data not shown). These genes were cotranscribed and produced similar steady-state levels of mRNA in the CH_4-only and $CH_4 + NH_3$ treatments (Figure 1b). Hence, *norCB* transcription did not respond to the presence of ammonia.

Cytochrome *c* NO reductase (cNOR) and CytS (Elmore *et al.*, 2007) are candidate enzymes for mediating NO reduction to N_2O in *M. capsulatus* Bath; however, the genomic inventory involved in NO production remains elusive. The typical mechanism for NO reduction involves nitrite reductases (Ferguson and Richardson, 2005), yet there are neither known NO-producing (NirK and NirS) nor ammonium-producing (NrfA and NirB) nitrite reductases in the genome of *M. capsulatus* Bath (Ward *et al.*, 2004). In addition to copper-dependent nitrite reductase (NirK), HAO has also been implicated in NO production by AOB (Hooper and Terry, 1979), suggesting that an active HAO in *M. capsulatus* Bath could also produce NO. We found that the steady-state level of *haoA* mRNA was lower at the 3′ end of the transcript than at the 5′ end, which may result in translation of a truncated HaoA (Hao') protein that lacks the tyrosine (Y467) needed for the intersubunit crosslink to catalytic heme 4 and a hydrophobic transmembrane spanning domain for anchoring HAO to the plasma membrane (Figure 2). Recently, the ability to form this crosslink was described as the critical acquisition during the evolution of HAO from an N-oxide-reducing octaheme cytochrome *c* nitrite reductase to an N-oxide oxidase (for a general discussion of HAO evolution, see (Klotz *et al.*, 2008; Klotz and Stein, 2008)). The potential involvement of a C-terminal truncated Hao in the process of NO_2^- reduction to N-oxides is of particular interest as an additional mechanism by which *M. capsulatus* Bath may produce NO.

Implications of haoAB expression

The presence of an NH_3-inducible *haoA* transcript encoding Hao suggests that *M. capsulatus* Bath could express a functional trimeric HAO complex. The possibility that *M. capsulatus* Bath possesses a functional HAO is supported by the previous work documenting the conservation of ligand-binding residues, the position of heme-binding motifs and an interprotein subunit tyrosine crosslink needed to establish the trimeric structure of the functional HAO complex, all of which aligned well with the structural features of HAO from AOB (Bergmann *et al.*, 2005; Klotz *et al.*, 2008). Although other laboratories have observed NO_2^- production by *M. capsulatus* Bath in the absence of NH_3, the $CH_4 + NH_3$ cultures in this study produced approximately 10 μM NO_2^-, whereas CH_4-only cultures produced no detectable NO_2^-. These facts combined with our observation that *haoA* transcription was enhanced in the presence of NH_3 suggest that *M. capsulatus* Bath has an active HAO. Additionally, cytochrome P460 expression did not respond to NH_3 in this or previous studies (Bergmann *et al.*, 1998, 2000), which further suggests that HAO could be the primary enzyme for the oxidation of NH_2OH produced by pMMO or soluble MMO in *M. capsulatus* Bath.

The NH_3 responsiveness of both *cytS* and *haoAB* transcription suggests that these genes could belong to a common regulatory network for the expression of NH_3-inducible genes. Both genes are preceded by putative σ^{70} promoters (*cytS*, -35: TTGACg and -10: TATgAT; *haoAB*, -35: TTGACg and -10 TActtT),

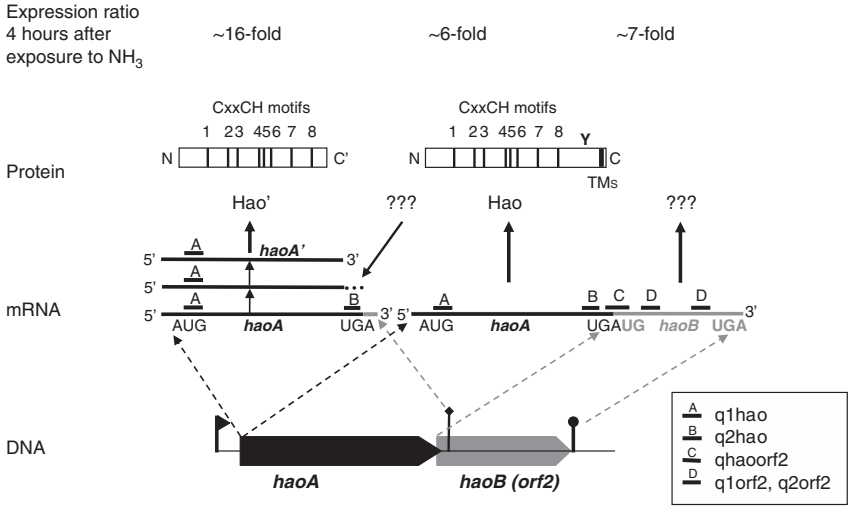

Figure 2 Flow chart showing the proposed consequences of NH_3-responsive differential transcription of the *haoAB* operon in *Methylococcus capsulatus* Bath. Question marks indicate yet unknown protein inventory; flag indicates promoter site; diamond, leaky terminator and full circle indicates terminator.

which indicates that the observed regulation of *cytS* and *haoAB* transcription by NH_3 is likely not mediated by alternate sigma factors (Wosten, 1998). Instead, regulation by either small RNAs (Wassarman, 2002) or trans-acting DNA-binding proteins (Babu and Teichmann, 2003) could be involved. While candidate regulatory proteins have not yet been identified, the genome of *M. capsulatus* Bath encodes over 50 transcriptional regulators, many belonging to two component systems (Ward *et al.*, 2004).

Potential physiological role of HAO in M. capsulatus Bath

In AOB, HAO is a key enzyme in the dissimilatory pathway that extracts electrons from NH_3 and couples them to the generation of proton motive force. Hence, if *M. capsulatus* Bath possesses an active HAO, then it begs the question of whether this methanotroph can acquire metabolically useful electrons from NH_3; the present literature lacks evidence that it can. The respiratory chain components of *M. capsulatus* Bath and AOB bear only partial resemblance: both contain cytochrome bc_1 complex III and cytochrome aa_3 terminal oxidase complex IV, both of which contribute to generation of proton motive force (DiSpirito *et al.*, 2005; Hooper *et al.*, 2005). On the other hand, *M. capsulatus* Bath lacks homologs of cytochromes *c*554 and c_M552 of AOB, which in AOB are proposed to relay electrons from HAO to the quinone pool and hence contribute to proton motive force and usable reductant (Hooper *et al.*, 2005; Klotz *et al.*, 2008; and references therein). Methanotrophs use pMMO to convert CH_4 to methanol, which is then oxidized to formaldehyde by a periplasmic methanol dehydrogenase. The extracted electrons are then relayed through two cytochromes (*c*555 and *c*553) to complex IV where they contribute to proton motive force and the reduction of oxygen (DiSpirito *et al.*, 2005). Further, a dye-linked formaldehyde dehydrogenase donates a pair of electrons gained from the oxidation of formaldehyde in the cytoplasm directly to the cytochrome bc_1 complex III, which contributes to proton motive force and serves pMMO with reductant needed for CH_4 oxidation (DiSpirito *et al.*, 2005).

If *M. capsulatus* Bath possesses an active HAO, it must have an as yet unidentified electron acceptor as a redox partner to continuously turn over substrate, and safely relay the extracted electrons to a terminal electron acceptor. If electrons extracted from NH_2OH through oxidation by HAO were funneled into the quinone pool upstream of complex III, as in the case of the membrane-bound hydrogenase of *M. capsulatus* Bath (Hanczar *et al.*, 2002) or relayed directly to cytochrome bc_1, then NH_2OH oxidation could contribute to the formation of proton motive force and/or serve pMMO with needed reductant, thereby supplementing the energy store of the cell. The *M. capsulatus* Bath genome encodes over 20 cytochromes *c* genes with unknown functions (Bergmann *et al.*, 1999; Ward *et al.*, 2004), offering a pool of potential redox partners to interact with HAO and link the oxidation and detoxification of NH_2OH to the generation of metabolically useful electrons. Comparisons of the *M. capsulatus* Bath genome with genomes from lithotrophs that employ cytochromes *c* in their electron relays have identified the putative monoheme cytochrome *c* AAU915124 (COG4654) and the putative diheme cytochromes *c* AAU91311 (COG2863) and AAU91545 (COG2863) as candidates for a redox link to complexes III or IV (Klotz *et al.*, 2006; Scott *et al.*, 2006; Sievert *et al.*, 2008).

The gene encoding AAU91545 is clustered with *cytS* (AAU91546) as well as genes encoding a complex IV in the A-family of heme copper oxidases (AAU91541-AUU91544), offering candidates that might redox partner with either electron sink.

Conclusions

Our results suggest that *M. capsulatus* Bath possesses an active, NH_3-responsive HAO. As the cytochrome P460 gene was expressed only at low levels regardless of whether ammonia was present, HAO appears to be a better candidate for the primary enzyme that oxidizes NH_2OH to NO_2^-. A more complete understanding of the role of methanotroph metabolism in the N-cycle now requires further biochemical confirmation, as well as identification of a suitable redox partner for HAO and pathway for final disposal of NH_3-derived electrons in *M. capsulatus* Bath. Also of particular interest is the potential involvement of a truncated Hao in a process in which NO_2^- is immediately reduced to N-oxides. Ongoing studies of *M. capsulatus* Bath genes involved in nitrification and processing of N-oxides and their regulation will identify pathways used by methanotrophs in the global nitrogen cycle, and define the roles of reduced inorganic N-species in the physiology and growth of methanotrophs.

Experimental procedures

Culture conditions
M. capsulatus strain Bath was grown at 45 °C in nitrate mineral salts medium containing 10 μM $CuSO_4$ (Whittenbury *et al.*, 1970) under an initial headspace mixing ratio of 47:3:50 (CH_4 to CO_2 to air).

For RNA extraction, two independent cultures of *M. capsulatus* Bath in early exponential phase were harvested by centrifugation (6000 × *g*, 10 min, 25 °C) and washed twice with phosphate buffer (5.4 g Na_2HPO_4. $7H_2O$ and 2.6 g KH_2PO_4 per liter distilled H_2O). Pellets were resuspended in 1 ml of phosphate buffer and distributed to 0.2 l of nitrate mineral salts medium only (CH_4-only treatment) or nitrate mineral salt medium supplemented with 5 mM $(NH_4)_2SO_4$ ($CH_4 + NH_3$ treatment). Cultures were incubated for 4 h under the above-described growth conditions. Following the incubation, cells were harvested for RNA extraction. The amount of NO_2^- in the medium was measured by a standard colorimetric method (Nicholas and Nason, 1957).

RNA extraction and cDNA synthesis
RNA was extracted using the FastRNA Pro Blue kit (Qbiogene, Irvine, CA, USA) according to the manufacturer's protocol. RNA pellets were resuspended in 100 μl of nuclease-free 0.1 mM EDTA (Ambion, Austin, TX, USA). Resuspended RNA was checked for integrity by visualization of ribosomal bands on an ethidium bromide-stained agarose gel and quantified by absorbance at 260 nm on a spectrophotometer (Beckman DU 640, Fullerton, CA, USA). RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's recommended protocol, ethanol precipitated and resuspended in 20 μl of 0.1 mM EDTA and then reexamined for degradation as described above. A portion of the RNA was used in cDNA synthesis and the remainder was stored at −20 °C and used later as the non-reverse-transcribed control in qPCR.

RNA was converted to first-strand cDNA using 200 ng of random nonamer primer with Superscript III (Invitrogen, Carlsbad, CA, USA) reverse transcriptase according to the manufacturer's recommended protocol at an extension temperature of 55 °C for 60 min. The 20 μl reaction was diluted 1:5 with sterile H_2O and stored at −20 °C for later analysis by qPCR.

Quantitative PCR
Primer sets (Table 1; Figure 1a) were designed with Primer3 software (http://frodo.wi.mit.edu; Rozen and Skaletsky, 2000) to target the following genes: 16S rRNA (*rrsA*; MCA_Mc16SA), pMMO subunit B (*pmoB*; MCA1796), hydroxylamine oxidoreductase (*hao*; MCA0956), *orf2* (MCA0955), cytochrome P460 (*cytL*; MCA0524), cytochrome *c*′-β (*cytS*; MCA2394), NO reductase subunit C (*norC*; MCA2401) and NO reductase subunit B (*norB*; MCA2400). Commercially manufactured primers were resuspended to 5 μM and tested on *M. capsulatus* Bath genomic DNA (gDNA) for specificity and efficiency before use with cDNA.

Quantitative PCR of cDNA was performed in 20 μl reactions prepared in triplicate for each primer set using DyNAmo SYBR Green qPCR (New England Biolabs, Beverly, MA, USA) master mix according to the manufacturer's protocol. qPCR was also performed on nonreverse-transcribed RNA to check for carryover of gDNA. The fluorescence of the accumulated product was measured on a MJ Research thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) at each extension step using the following PCR parameters: initial denaturation at 94 °C for 5 min, 40–50 cycles consisting of 96 °C for 10 s, 55 °C for 10 s and 72 °C for 15 s. A melting curve was generated over a temperature range from 70 °C to 92 °C following PCR.

After log linearization of fluorescence versus cycle number curves and background subtraction of fluorescence, C_t values were obtained at a specific fluorescence level and used to compare the relative expression of *haoA*, *orf2*, *cytL*, *cytS*, *norC* and *norB* between CH_4-only and NH_3 treatments with *rrsA* and *pmoB* as reference genes. Data analysis was performed with the Relative Expression Software

Tool (REST v.2005) (Pfaffl *et al*., 2002). Expression data are reported as expression ratios that represent the n-fold increase in the levels of the gene transcript in the NH_3 treatment compared to the CH_4-only treatment. Transcription of a gene was considered higher in the NH_3 treatment if data analysis yielded an expression ratio >2 and a significant *P*-value (<0.05) for the randomization test performed by the REST program, which indicates the probability that the expression ratio obtained was due to a treatment effect, not chance.

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