Heterologous expression of heterotrophic nitrification genes

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***Paracoccus denitrificans* is a heterotrophic organism capable of oxidizing ammonia to nitrite during growth on an organic carbon and energy source. This pathway, termed heterotrophic nitrification, requires the concerted action of an ammonia monooxygenase (AMO) and hydroxylamine oxidase (HAO). The genes required for heterotrophic nitrification have been isolated by introducing a *Pa. denitrificans* genomic library into *Pseudomonas putida* and screening for the accumulation of nitrite. In contrast to the situation in chemolithoautotrophic ammonia oxidizers, the genes encoding AMO and HAO are present in single linked copies in the genome of *Pa. denitrificans*. AMO from *Pa. denitrificans* expressed in *Ps. putida* is capable of oxidizing ethene (ethylene) to epoxyethane (ethylene oxide), which is indicative of a relaxed substrate specificity. Further, when expressed in the methylotroph *Methylobacterium extorquens* AM1, the AMO endows on this organism the ability to grow on ethene and methane. Thus, the *Pa. denitrificans* AMO is capable of oxidizing methane to methanol, as is the case for the AMO from *Nitrosomonas europaea*. The heterotrophic nitrification genes are moderately toxic in *M. extorquens*, more toxic in *Ps. putida*, and non-toxic in *Escherichia coli*. Toxicity is due to the activity of the gene products in *M. extorquens*, and both expression and activity in *Ps. putida*. This is the first time that the genes encoding an active AMO have been expressed in a heterologous host.**

Keywords: heterotrophic nitrification, ammonia monooxygenase, hydroxylamine oxidoreductase, *Paracoccus denitrificans*, *Methylobacterium extorquens*

INTRODUCTION

Chemolithoautotrophic nitrifying bacteria such as *Nitrosomonas europaea* derive energy for growth from the oxidation of ammonia to nitrite (Bock *et al.*, 1991). Ammonia is first oxidized to hydroxylamine in a reaction catalysed by ammonia monooxygenase (AMO), and hydroxylamine is subsequently oxidized to nitrite by hydroxylamine oxidase (also known as hydroxylamine oxidoreductase; HAO). Some heterotrophic bacteria are also capable of nitrification, in an analogous process which is less well understood. The

physiological role of heterotrophic nitrification is somewhat obscure, but it is believed to be involved in the dissipation of excess reductant (Robertson & Kuenen, 1988; Wehrfritz *et al.*, 1993). Heterotrophic nitrification may be linked to aerobic denitrification, such that the nitrite produced by nitrification is converted through nitric oxide and nitrous oxide to dinitrogen (Robertson & Kuenen, 1990a, b; Wehrfritz *et al.*, 1993). The α -proteobacterium *Paracoccus denitrificans* GB17 (formerly known as *Thiosphaera pantotropha*) has previously been reported to be capable of heterotrophic nitrification (Robertson & Kuenen, 1983, 1988).

The biochemistry of nitrification has been extensively studied in chemolithoautotrophs, especially *N. europaea*. The AMO of *N. europaea* is a membrane-bound enzyme which has extensive sequence similarity to the particulate methane monooxygenases (pMMOs) of methanotrophic bacteria (Semrau *et al.*, 1995; Holmes *et al.*, 1995). Both enzymes contain copper at the

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Abbreviations: AMO, ammonia monooxygenase; HAO, hydroxylamine oxidase; pMMO, particulate methane monooxygenase.

active site, which may be arranged as a trinuclear centre (Ensign *et al.*, 1993; Nguyen *et al.*, 1994, 1996). The pMMO has recently been purified from *Methylococcus capsulatus* (Bath) and reported to be a three-subunit enzyme that also contains iron (Zahn & DiSpirito, 1996). Genes designated *pmoA* and *pmoB* have been characterized, and the nucleotide sequence of a third gene, *pmoC*, is described in the sequence databases (Semrau *et al.*, 1995; Zahn & DiSpirito, 1996; accession numbers L40804 and U94337). The AMO is also believed to be encoded by three genes, *amoA*, *amoB* and *amoC*, of which there are two copies each in the *N. europaea* genome (McTavish *et al.*, 1993a, b; Klotz *et al.*, 1997). The AMOs and pMMOs have broad substrate specificities; they are capable of oxidizing ammonia, methane, short-chain alkanes, alkenes and chlorinated hydrocarbons, as well as catalysing the hydroxylation of aromatic compounds (Burrows *et al.*, 1984; Hyman & Wood, 1984; Keener & Arp, 1994).

The hydroxylamine cytochrome *c* oxidoreductase of *N. europaea* is a complex trimer of 63 kDa subunits each containing eight haems, including an unusual P460 haem (Arciero & Hooper, 1993; Arciero *et al.*, 1993; Igarashi *et al.*, 1997). *N. europaea* also expresses another, distinct, cytochrome P460 with HAO activity, analogous to that of *Methylococcus capsulatus* (Bergmann & Hooper, 1994a; Zahn *et al.*, 1994). In contrast, the HAO from *Pa. denitrificans* is a monomeric protein of approximately 18.5 kDa containing non-haem iron (Wehrfritz *et al.*, 1993; Moir *et al.*, 1996a). The *hao* gene of *N. europaea* has been characterized and is closely linked to the *hcy* gene encoding cytochrome *c*₅₅₄, which may be the primary electron acceptor for HAO (Hommes *et al.*, 1994; Sayavedra-Soto *et al.*, 1994; Arciero *et al.*, 1991). Three copies each of the *hao* and *hcy* genes are present in the *N. europaea* genome (McTavish *et al.*, 1993a).

It is of considerable interest to understand the biochemistry of heterotrophic nitrification, which appears to be different to that of autotrophic nitrification. Ultimately, it is also important to estimate the impact of heterotrophic nitrification on the global nitrogen cycle. The present communication reports the cloning of the heterotrophic nitrification genes from *Pa. denitrificans* and their expression in the heterologous hosts *Pseudomonas putida* and *Methylobacterium extorquens* AM1.

METHODS

Bacterial strains and plasmids. *Pa. denitrificans* Pd1222 was obtained from N. Harms (Vrije Universiteit, Amsterdam), and *M. extorquens* AM1 ATCC 14718 from M. D. Page (University of Oxford). Other strains used were *Ps. putida* ATCC 12633, and *E. coli* strains HB101 [*supE44 hsdS20* (*r*_b⁻*m*_b⁻) *recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1*], JM101 [*supE thi Δ(lac-proAB) F'* [*traD36 proAB⁺ lacI^a lacZ ΔM15*]], 803 [*met*], DH5α [*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi1 relA1*] and S17-1 [*thi pro hsdR recA RP4-2 integrated Tc::Mu Km::Tn7*]. Plasmids used were pUC18 (Yanisch-Perron *et al.*, 1985), the broad-host-range cosmid pLAFR3 and pLAFR5 (Staskawicz *et al.*, 1987; Keen

et al., 1988), pRK2013 (Figurski & Helinski, 1979) and pGRPd1 (van Spanning *et al.*, 1991).

Pa. denitrificans was grown at 30 °C either in LB medium (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 10 g NaCl l⁻¹) or on the minimal medium of Harms *et al.* (1985). *Ps. putida* and *M. extorquens* were grown at 30 °C in LB, or on minimal phosphate medium (Robertson & Kuenen, 1988). *E. coli* strains were grown in LB at 37 °C. Liquid cultures were grown in 50 ml medium in 250 ml flasks shaken at 200 r.p.m. Growth on methane was on solid medium in a gas jar containing a 50% (v/v) mix of methane and air. Growth on ethene was in 20 ml bottles fitted with gas-tight lids and shaken at 200 r.p.m., and growth on methanol was in minimal phosphate medium containing 1% (v/v) methanol. Antibiotics were added to growth media as appropriate: kanamycin (200 µg ml⁻¹), ampicillin (200 µg ml⁻¹ for *E. coli*, 50 µg ml⁻¹ for *Ps. putida*) and tetracycline (12.5 µg ml⁻¹ for *E. coli*, *M. extorquens* and *Ps. putida*, 1 µg ml⁻¹ for *Pa. denitrificans*).

Analytical methods. Nitrite was assayed colorimetrically using the overlay technique of Glaser & DeMoss (1971) on solid medium and the method of Coleman *et al.* (1978) in liquid medium. Gas chromatography was carried out on a Unicam gas chromatograph fitted with a Hayesep Q column (Supelco) maintained at 120 °C; the carrier gas was N₂ at a flow rate of 30 ml min⁻¹. The injector was maintained at 200 °C and detection was by flame ionization at 325 °C in H₂ (33 ml min⁻¹) and air (300 ml min⁻¹). The system was calibrated by injection of a known amount of epoxyethane, and epoxyethane in samples was estimated by integration. Cultures were incubated overnight in the presence of ethene in sealed vessels shaken at 200 r.p.m. in the dark at 30 °C, and were then heated to 80 °C for 10 min prior to sampling 100 µl headspace gas. The corresponding concentration of epoxyethane in the solution phase was calculated on the assumption that it is a standard gas at room temperature and pressure. Nitrate reductase activity was assayed and cells were fractionated as described by Bell *et al.* (1990). Protein concentrations were estimated using the Bradford (1976) method.

Immunological methods. To raise antibodies to HAO, the protein was purified according to Wehrfritz *et al.* (1993) and conjugated to bovine serum albumin. Antibodies were raised in sheep by Immunogen International. Western blotting was carried out following a modification of the method of Burnette (1981), using a Pharmacia Novoblot apparatus. The secondary antibody was donkey anti-sheep conjugated to alkaline phosphatase (Sigma). Blots were developed in 10 ml 100 mM Tris/HCl, 100 mM NaCl and 50 mM MgCl₂ (pH 9.5) containing 40 µl (75 mg ml⁻¹) nitro blue tetrazolium and 40 µl (50 mg ml⁻¹) 5-bromo-4-chloro-3-indolyl phosphate.

DNA manipulation: library construction and transfer to *Ps. putida*. Routine DNA manipulations were performed according to Sambrook *et al.* (1989). A genomic library of *Pa. denitrificans* Pd1222 was constructed in the broad-host-range cosmid vector pLAFR3. Genomic DNA was subjected to partial digestion with *Mbo*I and digestion products of approximately 20–25 kb were purified by sucrose gradient centrifugation (Sambrook *et al.*, 1989) and ligated into *Bam*HI-digested and dephosphorylated pLAFR3. The ligation reaction was packaged *in vitro* using a Gigapack packaging extract (Stratagene) and infected into HB101. The *Pa. denitrificans* library was then introduced en masse into *Ps. putida* by a triparental mating utilizing the helper strain *E. coli* 803/pRK2013. Overnight cultures (50 ml) of HB101 carrying the library, 803/pRK2013 and *Ps. putida* were harvested,

washed in LB, resuspended in LB, mixed, placed onto a sterile nitrocellulose filter on LB agar, and incubated overnight at 30 °C. The filter was then washed in LB and the mating mixture was diluted and plated for single colonies on LB agar containing tetracycline and ampicillin (*Ps. putida* is naturally ampicillin resistant, allowing counter-selection against the *E. coli* donors). Colonies were picked with sterile toothpicks and plated in duplicate on minimal media with succinate and ammonia as carbon and nitrogen sources, respectively. After growth for 3 d at 30 °C, colonies were tested with the colorimetric nitrite plate overlay assay. Positives for nitrite accumulation were picked from the stock plate for further analysis.

DNA:DNA hybridization. Using the published *amoAB* sequence (McTavish *et al.*, 1993b), primers were designed for the amplification of part of the coding region of *amoB* (from nucleotides 1080 to 1659, corresponding to an N-terminal fragment ending at Gly-95). These primers were used in PCRs with *N. europaea* genomic DNA as the template, and the amplification product was cloned into pUC18. Restriction digests of *Pa. denitrificans* DNA were transferred to filters by Southern blotting, and were hybridized with the ³²P-labelled *amoB* probe. Hybridization was at 30 °C overnight, and filters were washed for 2 × 5 min in 6 × SSC at room temperature, and developed by autoradiography (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

Cloning of heterotrophic nitrification genes in *Ps. putida*

Nitrite accumulation was chosen as an indicator of heterotrophic nitrification because there is a sensitive colorimetric assay for nitrite which can be carried out both in liquid culture and on solid media. *Ps. putida* ATCC 12633 was used as the host organism for cloning, since it does not express nitrification or denitrification pathways (unpublished results). Therefore, it was anticipated that the introduction of an ability to nitrify would cause nitrite to accumulate to a detectable level. A *Pa. denitrificans* genomic library was introduced into *Ps. putida* and exconjugants capable of nitrite accumulation were selected. Altogether, 25 cosmids which fell into three classes were independently isolated by this procedure (Table 1). The first class, exemplified by pB110, conferred on *Ps. putida* reduced methyl viologen

dependent nitrate and chlorate reductase activities (Table 1), which localized to the membrane fraction and were sensitive to inhibition by azide (data not shown). This suggests that pB110 carries the genes for the membrane-bound respiratory nitrate reductase of *Pa. denitrificans*, an enzyme which characteristically also reduces chlorate and is sensitive to azide (Berks *et al.*, 1995). This was confirmed by demonstrating hybridization between pB110 and the *narH* gene encoding the β -subunit of the enzyme. The second class of cosmids, exemplified by pL110, also conferred a reduced methyl viologen dependent nitrate reductase activity on *Ps. putida* but no detectable chlorate reductase activity (Table 1). In this case, nitrate reductase activity was localized in the periplasmic fraction and was insensitive to inhibition by 20 μ M azide (data not shown). This is consistent with pL110 carrying the genes for the soluble periplasmic nitrate reductase of *Pa. denitrificans*, which does not reduce chlorate and is insensitive to azide (Berks *et al.*, 1995). This was confirmed by hybridization with the *napA* gene encoding the catalytic subunit of the enzyme. Although no nitrate was added to the media used for the isolation of the pB110-type cosmids, it is likely that the ability of the membrane-bound nitrate reductase to reduce trace amounts of nitrate in the media accounts for the isolation of these cosmids using the nitrite accumulation assay. By contrast, cosmids of the pL110 class were only isolated in subsequent experiments in which nitrate was added to growth media. This procedure may be generally applicable as a means of cloning nitrate reductase genes. The 24 cosmids of the pB110 and pL110 classes were isolated from a total of approximately 15000 exconjugants.

Approximately 5000 exconjugants were incubated for 3 d prior to the nitrite overlay assay (rather than the overnight incubation used in the isolation of nitrate reductase clones). A single cosmid belonging to a third class, designated pLCC5, was isolated by this procedure, and it conferred on *Ps. putida* the ability to accumulate nitrite slowly and to low levels. The 3 d incubation period was necessary for nitrite to accumulate to detectable levels in the *Ps. putida*/pLCC5 exconjugant.

Table 1. Properties of cosmids isolated from *Ps. putida* exconjugants which accumulate nitrite

Cosmid	Nitrate reductase (nmol min ⁻¹ mg ⁻¹)*	Chlorate reductase (nmol min ⁻¹ mg ⁻¹)*	Nitrate induction of nitrite production	Nitrite accumulation in absence of ammonia	Hybridization†		Cross- reaction to anti-HAO
					<i>narH</i>	<i>napA</i>	
pB110	1270	4780	+	+	+	—	—
pL110	3300	ND	+	+	—	+	—
pLCC5	ND	ND	—	—	—	—	+

* Cells were disrupted by sonication; reduced methyl viologen dependent nitrate and chlorate reductase activities were determined in duplicate as described by Bell *et al.* (1990) and are expressed as nmol methyl viologen oxidized min⁻¹ (mg protein)⁻¹. ND, Not detectable. Data are from assays of two independent cultures.

† Hybridization experiments were performed using probes for the structural genes for the *Pa. denitrificans* membrane-bound (*narH*) and periplasmic (*napA*) nitrate reductases.

Fig. 1. Immunological detection of *Pa. denitrificans* HAO expressed in *Ps. putida*. Pure HAO and cell-free extracts from the strains indicated were separated by SDS-PAGE, transferred to a nitrocellulose membrane and analysed with an anti-HAO antiserum. Lanes: 1, pure HAO; 2, *Pa. denitrificans*; 3, *Ps. putida*; 4, *Ps. putida*/pLCC5.

There was no detectable nitrate reductase activity in whole cells or cellular fractions of *Ps. putida*/pLCC5. This strain did not accumulate nitrite in the absence of ammonia, and the addition of nitrate to the medium did not affect levels of nitrite production. These observations suggest that nitrite accumulation by *Ps. putida*/pLCC5 was a consequence of ammonia oxidation rather than nitrate reduction. Cell-free extracts of *Ps. putida*/pLAFR3 and *Ps. putida*/pLCC5 were separated by SDS-PAGE, transferred to nitrocellulose membranes and analysed with a polyclonal antiserum raised against the *Pa. denitrificans* hydroxylamine oxidoreductase (Fig. 1). An 18.5 kDa polypeptide which reacted with anti-HAO was clearly detectable in extracts of *Ps. putida*/pLCC5 but not in *Ps. putida*/pLAFR3. Thus pLCC5 carries the gene(s) encoding HAO, and since it confers the ability to oxidize ammonia to nitrite, it is very likely that it also carries the genes for AMO. This was confirmed by demonstrating that the introduction of pLCC5 into *Pa. denitrificans* caused a marked increase in AMO activity; indeed this strain provided a suitable source for the purification of the enzyme (Moir *et al.*, 1996b). Thus there is strong evidence that pLCC5 carries all of the *Pa. denitrificans* genes required for the oxidation of ammonia to nitrite. The reason why only a single cosmid expressing nitrification genes could be isolated is not clear at the present time, but may relate to the toxicity that is associated with some nitrification clones (see below).

Ethene oxidation

The AMO of *N. europaea* and the pMMO of *Methylococcus capsulatus* (Bath) both have a broad substrate range and are capable of oxidizing ammonia, methane, short-chain alkanes and alkenes, dehalogenating short-chain halogenated hydrocarbons and catalysing the hydroxylation of aromatic compounds (Burrows *et al.*, 1984; Hyman & Wood, 1984; Keener & Arp, 1994). The pMMO of *Methylococcus capsulatus* (Bath) is routinely assayed by following the production of propene oxide from propene (Colby *et al.*, 1977;

Fig. 2. (a) Oxidation of ethene to epoxyethane by *Ps. putida*/pLCC5. Washed cell suspensions were incubated in the presence of ethene, and the headspace was analysed by gas chromatography. The peak with a retention time of approximately 6 min is epoxyethane. Upper trace, *Ps. putida*/pLCC5; lower trace, *Ps. putida*/pLAFR3. The peak at approximately 2 min in the upper trace is an additional, unidentified, product of ethene oxidation (and is absent from control chromatograms of pure epoxyethane). Both this peak and the epoxyethane peak were absent from a control reaction containing ethene but no cells. (b) Time-course of epoxyethane accumulation by *Ps. putida*/pLCC5. Cell suspensions from two independent cultures incubated in the presence of ethene were sampled at time intervals and analysed as in (a).

Stirling & Dalton, 1979). Ethene oxidation can be detected in *Pa. denitrificans* by monitoring the production of epoxyethane (see below). To determine whether ethene oxidation is due to the activity of AMO, the AMO of *Pa. denitrificans* expressed in *Ps. putida* was tested for the ability to oxidize ethene. Washed cells of *Ps. putida*/pLCC5 and *Ps. putida*/pLAFR3 were incubated overnight in sealed vessels in phosphate buffer containing approximately 500 μ M ethene. The headspace was then sampled and analysed by gas chromato-

Fig. 3. Partial restriction map of the insert in pB8 which confers AMO activity in *Ps. putida*. The *EcoRI*–*PstI* fragment which hybridizes weakly to the *N. europaea amoB* gene is indicated, as is the *SalI* site at which the *Pa. denitrificans* chromosome was disrupted. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I.

graphy (Fig. 2a). Epoxyethane was produced from ethene by *Ps. putida*/pLCC5 but not by *Ps. putida*/pLAFR3. In time-course experiments, washed cells of *Ps. putida*/pLCC5 showed an initial rapid burst of epoxyethane production, followed by a linear accumulation, such that 20 μ M epoxyethane accumulated over a period of 15 h (Fig. 2b). Disrupted cells of *Ps. putida*/pLCC5 prepared by sonication and incubated with ethene produced epoxyethane at broadly similar rates as did whole cells. However, in the presence of the copper chelator diethyldithiocarbamate (1 mM), epoxyethane was not produced from ethene by disrupted cells. This suggests that the AMO of *Pa. denitrificans* is a copper-containing enzyme capable of oxidizing alkenes, as are the AMOs of the autotrophic nitrifiers and the pMMOs of the methanotrophs. The behaviour of pLCC5 with regard to ethene oxidation provides further evidence that this plasmid contains genes involved in ammonia oxidation.

Disruption of the *Pa. denitrificans* nitrification genes

Restriction fragments derived from the insert of pLCC5 were ligated into pLAFR5, transformed into *E. coli* and then introduced directly into *Ps. putida* by conjugation. In this manner, an 8 kb *Bam*HI fragment was identified (in a pLAFR5 derivative designated pB8; Fig. 3) which conferred AMO activity, as judged by ethene oxidation and the accumulation of nitrite during growth. Furthermore, *Ps. putida*/pB8 showed an ammonia-dependent oxygen uptake activity ($3.7 \text{ nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$) which was sensitive to diethyldithiocarbamate and was not detectable in the wild-type strain. However, cell-free extracts prepared from *Ps. putida*/pB8 showed no immunological cross-reaction with the anti-HAO antibody, suggesting that none or not all of the gene(s) encoding HAO are present on the insert in pB8. No other subclones were identified which conferred any nitrification activity, which may be because there are toxicity problems associated with the cloned nitrification genes (see below). The accumulation of nitrite in *Ps. putida*/pB8, which does not appear to synthesize HAO, may result from chemical oxidation of hydroxylamine to nitrite. Iron(III) ions oxidize hydroxylamine to NOH, which under aerobic con-

ditions reacts with oxygen to form nitrite (Bengtsson, 1973).

Fragments of the *amoB* gene from the chemolitho-autotrophic nitrifier *N. europaea* were amplified by PCR using primers designed to the published sequence (McTavish *et al.*, 1993b; Bergmann & Hooper, 1994b). The amplification product was cloned into pUC18, and then used in hybridization experiments against pB8. Under conditions of extremely low stringency, a very weak hybridization was observed between the *amoB* probe and a 1.7 kb *Eco*RI–*Pst*I fragment derived from pB8 (data not shown; Fig. 3). For this reason, the corresponding region of the *Pa. denitrificans* chromosome was disrupted to confirm that pB8 contains genes required for ammonia oxidation. The 1.7 kb *Eco*RI–*Pst*I fragment from pB8 was cloned into pUC18 and the unique *Sal*I site within this fragment was disrupted with the Ω cartridge, which contains genes for streptomycin and spectinomycin resistance, flanked by transcriptional terminators (Prentki & Krisch, 1984). The disrupted fragment was cloned into the suicide vector pGRPd1 as an *Eco*RI–*Sph*I fragment (using the *Sph*I site in pUC18), generating a plasmid designated pSAD43. This plasmid was introduced into *Pa. denitrificans* by triparental conjugation, and streptomycin-resistant exconjugants were selected. Exconjugants which had acquired the Ω cartridge by a double-crossover event were initially identified on the basis of their failure to hybridize to labelled pGRPd1 DNA in a colony hybridization. Genomic DNAs were isolated from strains identified in this manner, and hybridization using the 1.7 kb *Eco*RI–*Pst*I fragment as a probe confirmed that the genomic DNA had been disrupted by a single copy of Ω at the expected location (data not shown). Of a total of 100 exconjugants screened, three had acquired the Ω cartridge by a double-crossover event. The putative *amo* mutants were tested for their ability to oxidize ethene and were found to have completely lost this activity (Fig. 4). This confirms that the DNA cloned in pLCC5 does indeed contain nitrification genes. Interestingly, the *amo* mutant expresses wild-type levels of HAO, as judged by Western blot analysis (data not shown). This implies that the genes encoding AMO and HAO are not in a single transcription unit, or that if they are, the gene(s) for HAO is promoter proximal. The nitrification mutant had no apparent phenotype when grown in aerobic batch cultures with succinate and ammonia as the carbon and nitrogen sources. A more detailed characterization of this mutant in chemostat cultures is now in progress and will allow ideas about the physiological role of heterotrophic nitrification to be tested, for the first time.

Methane oxidation

The α -proteobacterium *M. extorquens* AM1 (ATCC 14718) is a pink-pigmented facultative methylotroph capable of growth on methanol but not methane (Peel & Quayle, 1961). The cosmid pLCC5 was introduced into this strain by conjugation and exconjugants were

Fig. 4. Ethene oxidation by wild-type *Pa. denitrificans* and a nitrification mutant. Ethene oxidation was analysed by gas chromatography as in Fig. 2. Upper trace, *Pa. denitrificans* Pd1222; lower trace, *Pa. denitrificans* *amo* mutant.

selected on minimal medium containing methanol as the sole carbon source and ammonia as the nitrogen source. The presence of an active AMO in these exconjugants was verified by following the oxidation of ethene in the same manner as described above for *Ps. putida*/pLCC5. Wild-type *M. extorquens* was incapable of oxidizing ethene. In contrast, *M. extorquens*/pLCC5 was found to accumulate epoxyethane to approximately 35 μ M in as little as 10 min, and epoxyethane disappeared equally rapidly (data not shown). This suggests that *M. extorquens* has a pathway for the further metabolism of epoxyethane.

Since AMOs are capable of oxidizing methane, the ability of *M. extorquens*/pLCC5 to oxidize methane to methanol, and therefore to grow on methane, was tested. *M. extorquens* and *M. extorquens*/pLCC5 were plated onto minimal media with nitrate as the nitrogen source and no added carbon source. Plates were incubated in a 50% methane/air atmosphere at 30 °C. After 9 d incubation, *M. extorquens*/pLCC5 formed colonies whereas *M. extorquens* did not. Growth of *M. extorquens*/pLCC5 was methane-dependent since colonies did not form on plates incubated in air alone. This suggests that the AMO of *Pa. denitrificans* supplied by pLCC5 is able to oxidize methane and provide methanol which is utilizable for growth by *M. extorquens*. The ability of the AMO from *Pa. denitrificans* to oxidize methane is an additional biochemical similarity to the AMO of autotrophic organisms.

Gas chromatography data showed that *M. extorquens*/pLCC5 was capable of a rapid oxidation of

Table 2. Expression of AMO in *M. extorquens* allows significant growth on ethene

Growth was in liquid culture for 8 d. Because of the poor growth and clumping of cultures of strains containing pLCC5, growth is expressed in terms of protein concentration. Data are from assays of two independent cultures.

Strain	Growth conditions	Protein (μ g ml ⁻¹)
<i>M. extorquens</i>	Methanol	140.0
<i>M. extorquens</i>	Ethene	1.4
<i>M. extorquens</i> /pLCC5	Ethene (dark)	9.8
<i>M. extorquens</i> /pLCC5	Ethene (light)	4.8
<i>M. extorquens</i> /pLCC5	Methanol	24.8

ethene to epoxyethane, and also subsequent metabolism of epoxyethane. Therefore, experiments were performed to determine whether *M. extorquens*/pLCC5 could grow on ethene, using the plasmid-encoded AMO for the initial step of ethene oxidation. *M. extorquens*/pLCC5 and *M. extorquens* were grown in liquid medium containing nitrate as a nitrogen source and ethene as a carbon source (ethene is soluble in water to approximately 5 mM). *M. extorquens* was incapable of growth on ethene, whereas introduction of pLCC5 permitted significant growth (Table 2). Growth was inhibited to a large extent by normal laboratory lighting, which is consistent with the fact that the purified AMO is light-sensitive (Moir *et al.*, 1996b).

Toxicity of AMO

The increased rate of ethene oxidation by *M. extorquens*/pLCC5 as compared to *Ps. putida*/pLCC5 might suggest that the AMO is more highly expressed and/or more active in *M. extorquens*. Accordingly, 800 nmol nitrite (mg protein)⁻¹ was found to accumulate in cultures of *M. extorquens*/pLCC5 after 3 d growth, compared with 50 nmol mg⁻¹ in *Ps. putida*/pLCC5 and 30 nmol mg⁻¹ in a strain of *Pa. denitrificans* containing pLCC5 and deficient in nitrite reductase activity (in all cases growth was in media containing ammonia as the sole nitrogen source). Introduction of pLCC5 into *M. extorquens* caused a severe defect in growth on media containing ammonia, with cultures clumping and reaching very low yields. There was also a dramatic change in the appearance of cells in electron micrographs, which is consistent with the production of internal membranes (Fig. 5). Extensive internal membranes are seen in the autotrophic ammonia oxidizers and in methanotrophs expressing pMMO (Bock *et al.*, 1991; Hanson & Hanson, 1996). The cosmid was also toxic to *Ps. putida*, causing a threefold reduction in growth rate and yield, but there was no effect on the appearance of cells in electron micrographs (not shown). In the case of *M. extorquens*, the toxicity of pLCC5 was partially alleviated by growth in the light (an inhibitor of

Fig. 5. Electron micrographs of *M. extorquens* (a) and *M. extorquens*/pLCC5 (b, c). Cultures were grown in media containing succinate as the carbon and energy source and ammonia as the nitrogen source. The white areas are likely to be poly- β -hydroxybutyrate. Bars, 1 μ m.

AMO; Moir *et al.*, 1996b) or by the use of glutamate rather than ammonia as a nitrogen source. This suggests that AMO activity is responsible for the toxicity of pLCC5, perhaps because it causes the accumulation of hydroxylamine. In *Ps. putida*, pLCC5 was only slightly less toxic in the presence of light, suggesting that AMO expression, rather than activity, was the major reason for toxicity in this case. This may be because *Ps. putida* is incapable of making the internal membranes required to accommodate the AMO.

The *amo* genes of *N. europaea* are highly toxic to *E. coli* and have never been cloned on the same fragment of DNA or heterologously expressed (Bergmann & Hooper, 1994b). However, pLCC5 is not toxic to *E. coli*. Cell-free extracts of *E. coli*/pLCC5 and *E. coli*/pLAFR3 were separated on SDS-PAGE and transferred to nitrocellulose membranes which were probed with an anti-

HAO antiserum. No immunologically cross-reacting polypeptide of 18.5 kDa was detectable in either extract, indicating that the *hao* gene(s) are not expressed from pLCC5 in *E. coli*, which is probably because their promoters are not active in *E. coli*. This being the case, it is likely that the *amo* genes are also not expressed from pLCC5. Failure to express the nitrification genes probably accounts for the non-toxicity of pLCC5 in *E. coli*. However, pB8 was toxic to *E. coli*, causing cells to clump and grow at a much reduced rate. Furthermore, subclones from the left end of the insert in pB8 (Fig. 3) could not be isolated, which may indicate that this region of DNA is especially toxic. The reasons for the increased toxicity of some subclones are not clear at this time, but copy number effects and expression from vector promoters are possible explanations.

Concluding remarks

The isolation of heterotrophic nitrification genes and the recent purification of AMO and HAO (Moir *et al.*, 1996b; Wehrfritz *et al.*, 1993) represent the first steps in the molecular and biochemical characterization of this process. The genes encoding an AMO have now been expressed in a heterologous host for the first time. This has not been possible for either AMO from the autotrophic nitrifiers or pMMO from the methanotrophs, since their genes are toxic and have not been cloned on a single piece of DNA (Bergmann & Hooper, 1994b; Semrau *et al.*, 1995). The *amo* genes from *Pa. denitrificans* and *N. europaea* appear to be rather different since only a very weak cross-hybridization is evident. Thus the *amo* genes of *N. europaea* are more closely related to the *pmo* genes, to which they hybridize at high stringency (Holmes *et al.*, 1995; Semrau *et al.*, 1995), than they are to the *amo* genes of *Pa. denitrificans*. Also, degenerate PCR primers which amplify *amo* sequences from autotrophic nitrifiers and *pmo* sequences from methanotrophs do not amplify *amo* from *Pa. denitrificans* (Holmes *et al.*, 1995). An additional distinction is that the *amo* and *hao* genes of *Pa. denitrificans* are present in the genome in single linked copies (hybridization experiments have indicated no evidence of gene duplication), whereas the *N. europaea* genes are unlinked and are present in multiple copies. Nevertheless, the AMO of *Pa. denitrificans* has some notable similarities to that of the autotrophic ammonia oxidizers, particularly the ability to oxidize alkenes, the apparent requirement for copper, and inhibition by light, diethyldithiocarbamate and allylthiourea (Moir *et al.*, 1996b). In the light of these similarities and differences, determination of the primary structure of the *Pa. denitrificans* AMO will be especially interesting.

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