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Physiological and Molecular Biological Characterization of Ammonia Oxidation of the Heterotrophic Nitrifier *Pseudomonas putida*

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Abstract. The heterotrophic nitrifier *Pseudomonas putida* aerobically oxidized ammonia to hydroxylamine, nitrite, and nitrate. Product formation was accompanied by a small but significant release of NO, whereas N_2O evolution could not be detected under the assay conditions employed. The isolate reduced nitrate to nitrite and partially further to NO under anaerobic conditions. Aerobically grown cells utilized γ-aminobutyrate as a carbon source and as a N-source by ammonification. The physiological experiments, in particular the inhibition pattern by C_2H_2, indicated that *P. putida* expressed an ammonia monooxigenase. DNA-hybridization with an *amoA* gene probe coding for the smaller subunit of the ammonia monooxigenase of *Nitrosomonas europaea* allowed us to identify, to clone, and to sequence a region with an open reading frame showing distinct sequence similarities to the *amoA* gene of autotrophic ammonia oxidizers.

In nitrification, bacteria oxidize ammonia to nitrite and nitrate. These end products are easily drained out to the ground water, assimilated by plants and bacteria, or converted to gaseous N-compounds by denitrification. Classically, nitrifying bacteria are chemoautotrophs [24]. Relatively few of these bacteria can be grown under laboratory conditions, and their growth rates are usually low. In soil, nitrifying bacteria may represent an essential part of the unculturable population. Scattered in the literature are reports that many soil nitrifiers are not restricted to an autotrophic mode of life but can catabolize organic soil compounds. Such heterotrophic nitrifiers have been characterized only poorly, although some are culturable [18, 19, 25].

In the present study, nitrification of the heterotrophic nitrifier *Pseudomonas putida* was investigated. This bacterium was isolated from the upper soil layer of a Norway spruce forest (''Höglwald'' between Munich and Augsburg). The present communication will report that this bacterium oxidizes NH_4Cl in the presence of diverse organic compounds. Product formation of ammonia oxidation will also be described. The isolate also reduces nitrate anaerobically. DNA isolated from *P. putida* hybridized with an *amoA* probe coding for the smaller subunit of the ammonia monooxigenase from *Nitrosomonas europaea*. The physical map of the region and the sequence data of the gene from *P. putida* will be presented.

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Materials and Methods

Origin of *Pseudomonas putida* isolate. Bacteria from the organic soil layer (upper 5 cm) of a Norway spruce (*Picea abies*) forest (Höglwald, 40 km northwest of Munich) were separated aerobically on peptone meat extract (5 g peptone + 3 g meat extract per L, pH 7.0) with agar plates. Single colonies were transferred into liquid media of the same composition and selected for their capability to form nitrite aerobically [18]. The heterotrophically nitrifying isolate chosen for the present study was classified as *Pseudomonas putida* DSMZ-1088-260 by the Deutsche Sammlung für Mikroorganismen (Braunschweig, Germany) because of its morphologic and physiological properties (e.g., growth on acetate, caprate, citrate, phenylacetate, L-arabinose, D-galactose, fructose, glucose, gluconate, D-alanine, L-serine, aerobic acid production from glucose, fructose, and xylose, nitrite formation from nitrate) and its missing production of a fluorescent dye on *Pseudomonas*-F-Agar.

Growth conditions. The *P. putida* isolate was grown aerobically with shaking at 25°C in peptone meat extract (5 g peptone + 3 g meat extract per L, pH 7.0) or on mineral medium containing the following salts, in mM: 4.6 NH_4Cl, 0.16 $MgSO_4$, 0.5 $CaCl_2$, 0.036 $FeSO_4$ as EDTA complex, 0.0001 $CuSO_4$, 2.0 K-phosphate buffer pH 7. Glucose, glutamine, citrate, malate or γ-aminobutyrate (20 mM), respectively,

Table 1. Aerobic nitrite and nitrate production by *Pseudomonas putida* grown in the presence of different carbon sources[a]

	Carbon source					
	D-Glucose	Malate	Citrate	γ-Amino-butyrate	L-Glutamate	Without C-source
$O.D._{680\ nm}$	0.8	1.0	0.6	1.4	1.0	0.05
Nitrite (μM)	1.0	0.7	0.7	1.7	1.3	0.3
Nitrate (μM)	<0.2	<0.2	<0.2	0.5	0.8	<0.2

[a] Cells were grown aerobically on mineral medium (4.6 mM NH_4Cl) at 25°C with the carbon sources given in the table. The optical density (680 nm) of the culture and the nitrate and nitrite concentrations in the medium were determined after 24 h.

were added as filter-sterilized solutions after autoclaving the basal medium.

For monitoring growth, substrate utilization, and product formation during heterotrophic nitrification, 1-L, gas-tight glass vessels with 250 ml of mineral medium (containing 20 mM γ-aminobutyrate, 4.6 mM NH_4Cl) were inoculated with a *P. putida* preculture grown in the same medium (cells from the late logarithmic growth phase) to start with an initial optical density of 0.05 at 680 nm (equivalent to 6.7 μg protein ml^{-1}). The vessels were flushed with sterile synthetic air prior to the beginning of the experiment. For analytical determinations, aliquots of 11 ml from the cultures and 31 ml from the head space above the cultures were removed each hour. To ensure aerobic growth conditions, the gas-tight vessels were supplemented with 42 ml O_2 (99.995% purity) at each sampling, resulting in an O_2-content of the gas phase between 18% and 30% during the entire incubation period. The acetylene (type 2.6, Messer Griesheim, Munich), which was added to block ammonia oxidation, was purified by flushing through a vessel of concentrated H_2SO_4 and subsequent flushing through distilled water.

For assaying anaerobic nitrate reduction, cells were aerobically grown on mineral medium with γ-aminobutyrate and NH_4Cl as described above for 7 h, centrifuged twice (5000 *g*, 10 min) and resuspended in mineral medium (containing 20 mM γ-aminobutyrate, 5 mM nitrate). Cells were transferred into 300-ml, gas-tight glass vessels in which the suspension had an optical density (680 nm) of 1.5 at the start. To establish anaerobic conditions, oxygen was removed from the flasks by repeatedly evacuating and flushing with nitrogen for 15 min and shaking vigorously. The residual oxygen content of the gas phase in the vessels was less than 0.01% vol/vol.

Analytical determinations. NO was quantified by injecting aliquots of the gas phase of the cultures into a chemiluminescence NO_x-analyzer (CLD 760 AL ppt, Tecan AG, Munich) as described [19]. Concentrations of N_2O and O_2 were determined by gas chromatography (Shimadzu GC 14A, ^{63}Ni electron capture detector, injector and oven temperature 50°C; detector temperature 320°C; N_2 as carrier gas, flow rate 16 ml min^{-1}, a stainless steel column 3 m, 1/8″ filled with Porapak N 60-80 mesh). Protein content of intact cells was assayed by a modified Lowry method [6]. The following parameters were determined in the supernatant after centrifugation (15,000 *g*, 10 min): nitrite by the α-naphthylamine/sulfanilic acid reaction [23]; hydroxylamine photometrically after coupling to 8-hydroxychinoline [4]; ammonium and nitrate by ion chromatography (Dionex DX 500, Sunnyville, CA, USA). In the nitrate reduction assays, nitrate was determined with salicylic acid [2]. For γ-aminobutyrate measurements, the amino group was derivatized with o-phthaldialdehyde prior to separation by reversed phase HPLC (Beckman, 5 μm C18 column, 1 ml/min flow, 70% 50 mM potassium phosphate buffer, pH 6.3:30% methanol) and fluorometric detection (excitation at 340 nm, emission at 455 nm).

Screening for the occurrence of *amoA* in *P. putida* by heterologous hybridization. Isolated genomic DNA [12] from *P. putida* was digested by restriction enzymes (see Fig. 3) and separated electrophoretically on 0.5% agarose gels. The DNA was transferred onto Nylon$^+$ filters (Qiagen GmbH, D-Hilden). Hybridizations were performed at 68°C overnight without formamide (hybridization buffer: 0.75 M NaCl, 0.075 M Na-citrate, sodium dodecylsulfate 0.02% wt/vol, N-lauroylsarcosine 0.1% wt/vol, blocking reagent 1% wt/vol, Boehringer Mannheim, D-Mannheim) using a segment of *amoA* of *N. europaea* [12] labeled with digoxygenin. The hybridized Nylon filters were stained with a nonradioactive DNA labeling and detection kit (Boehringer Mannheim) for 2 h in the dark.

Cloning and sequencing. For mapping and sequencing of the hybridizing genomic region, total DNA of *P. putida* was completely cut by *Eco*RI and *Sst*I. The digest was separated on a 0.7% agarose gel. DNA segments of about 3 kb were isolated from the gel by electroelution and cloned into M13mp18. The resulting partial genomic library was screened with the *amoA* probe from *N. europaea*. One identified clone harboring a 1783 bp *Hinc*II–*Hinc*II–*Eco*RI segment in the vector hybridized with the *amoA* probe. This segment was subcloned and sequenced on both strands by the dideoxy chain-termination technique [20]. The sequence was deposited to EMBL, GenBank, and DDBJ Nucleotide Sequence Databases (accession number Y14338).

Results

Aerobic growth of the *P. putida* strain was dependent on the presence of an organic carbon source (Table 1). After 24 h, optical density was highest in cultures grown with γ-aminobutyrate, followed by those grown with glutamate and malate. Glucose and citrate stimulated growth to a lower extent (Table 1). During heterotrophic growth on glucose, malate, and citrate, the cells had oxidized the initial ammonium (4.6 mM) partly to nitrite (Table 1). Remarkably, highest concentrations of nitrite were found in cultures grown in the presence of both ammonium and amino group bound N (γ-aminobutyrate, glutamate). These cultures had also formed nitrate, whereas the concentration of nitrate in the cultures grown with the other carbon substrates was lower than the detection limit (0.2 μM). In the absence of any added organic C-source, cells did not grow and nitrite concentration in the medium remained at the inoculation level of 0.3 μM

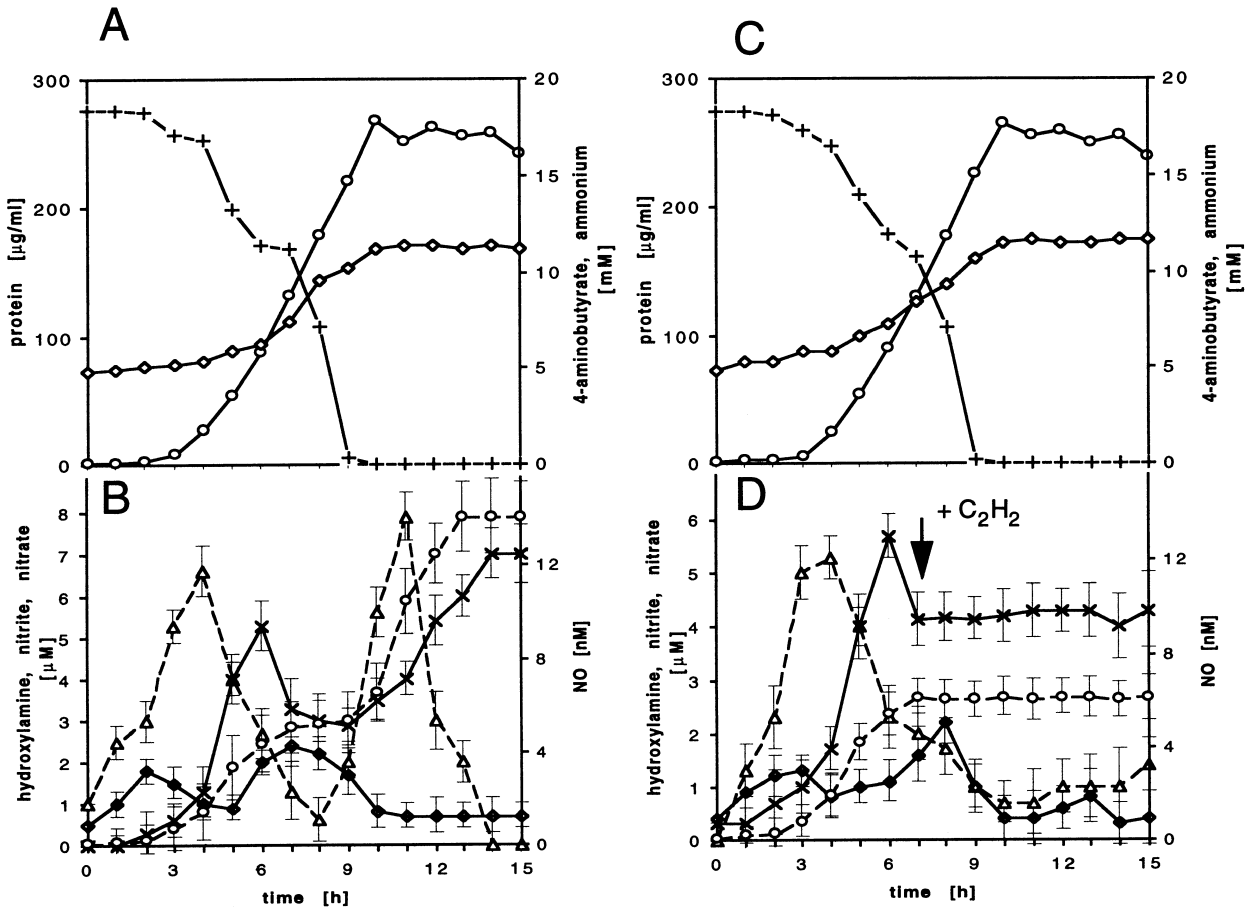

Fig. 1. Aerobic growth and heterotrophic nitrification by *Pseudomonas putida* (A, B) and effect of C_2H_2 addition on product formation (C, D). 250 ml of mineral medium was inoculated ($O.D._{660nm}$ = 0.05) by an overnight culture grown in the same medium containing γ-aminobutyrate as carbon source. Determinations: (A, C) protein (o-o), γ-aminobutyrate (+-+), ammonium (◇-◇); (B, D) hydroxylamine (△-△), nitrite (x-x), nitrate (◆-◆), the concentrations of NO referred to the culture medium (o-o). The culture conditions were identical for cultures (A, B) and (C, D). C_2H_2 (final concentration: 5 vol %) was added to the culture after 7 h (C, D).

(derived from the preculture). Therefore, the *P. putida* strain is, indeed, a heterotrophic nitrifier. Cells plated onto agar with Luria-Bertani medium after the experiments formed uniform *P. putida* colonies.

To identify the intermediate and end-product formation of nitrification during heterotrophic growth of *P. putida*, we inoculated γ-aminobutyrate-grown cells of a culture in the late exponential phase into fresh medium (Fig. 1A). *P. putida* started to utilize γ-aminobutyrate and to increase protein content in the culture after a lag phase of 2–3 h. The ratio between protein content and optical density remained constant during the entire incubation period, indicating that the cells did not store poly-β-hydroxybutyrate. Generation time was fast (T = 1.4 h) during exponential growth. The concentration of ammonium steadily increased up to 12 mM, indicating that the cells had catabolized γ-aminobutyrate by ammonification. After 10 h, *P. putida* had completely utilized γ-aminobutyrate and the stationary phase commenced (Fig. 1A).

The pH value of the culture steadily increased from pH 7.2 to 8.4, most likely owing to the utilization of γ-aminobutyric acid and/or the release of ammonium from this substrate into the medium by ammonification activity of the cells. *P. putida* produced the same spectrum of intermediates (NH_2OH, NO) and end products (NO_2^-, NO_3^-) during heterotrophic nitrification as classical autotrophic ammonia and nitrite oxidizers together [8, 24]. However, the kinetics of production, accumulation, and consumption of each of the oxidized nitrogen compounds mentioned were complex (Fig. 1B).

Hydroxylamine concentration in the medium started to increase immediately after inoculation and accumulated until the early exponential growth phase commenced. During exponential growth most of the hydroxylamine was consumed. A second increase in hydroxylamine con-

centration was observed at the onset of the stationary phase, where a concentration of 8 μM was reached (Fig. 1B). This second production of hydroxylamine must have been generated exclusively by the oxidation of ammonium, since γ-aminobutyrate had already been consumed completely at the end of exponential growth. The cells reconsumed the hydroxylamine completely during the stationary growth phase (Fig. 1B). Nitrite formation started with the beginning of the exponential growth phase and showed a second increase during the stationary phase (Fig. 1B). The increase in nitrite concentration was always accompanied by a parallel decrease in the amount of NH_2OH, indicating that this compound was the source of nitrite formation. The cells also formed small but clearly detectable amounts of nitrate (up to 2 μM) with a broad maximum during the exponential growth phase (Fig. 1B). The two maxima in the formation of NH_2OH were accompanied by a release of NO, though in an approximately 400-fold lower concentration (Fig. 1B). N_2O was not formed by the cultures during the experiments. This complex pattern of product formation and consumption during heterotrophic nitrification of *P. putida* was observed in three independently performed experiments. Plating on agar ensured that the cultures were not contaminated.

In autotrophic ammonia oxidizers, C_2H_2 is known to effectively block ammonia monooxigenase at low (10 Pa) partial pressures [9]. However, in heterotrophic nitrifiers so far studied, a higher concentration of C_2H_2 is needed to inhibit ammonia oxidation [16]. Also, in *P. putida*, 10 Pa C_2H_2 did not significantly inhibit hydroxylamine production (data not shown). Therefore, this inhibitor was added at a concentration of 5% 7 h after start of a second set of experiments (Fig. 1C and 1D). The γ-aminobutyrate utilization, the increases in cell protein, and ammonia releases of these cultures were not changed compared with cells not incubated with C_2H_2 (Fig. 1C). However, in contrast to the untreated control culture (Fig. 1A and B), *P. putida* did not produce NH_2OH, nitrite, NO, and nitrate any more after the addition of the inhibitor (Fig. 1C and D). Thus, the apparent blockage of ammonia monooxigenase by C_2H_2 prevented the formation of these compounds in this specific heterotrophic nitrifier.

As nitrite accumulated in the medium, NO formation could have been generated by dissimilatory nitrite reductase, and nitrate formation could have been catalyzed by dissimilatory nitrate reductase in reverse direction, since this enzyme is closely related to nitrite oxidoreductase in *Nitrobacter* [11]. To show the presence of these enzymes, we assayed cells of *P. putida* for the capability to reduce nitrate to nitrite and NO anaerobically. In these experiments, *P. putida* reduced nitrate to nitrite (Fig. 2), indicating the presence of a dissimilatory nitrate reductase, which could well be involved in the nitrate production observed during aerobic heterotrophic nitrification of this strain. Under the anaerobic conditions NO was also formed, but in concentrations at least three orders of magnitude lower than nitrite (Fig. 2). NO-formation could be due to presence of a dissimilatory nitrite reductase, which may also be responsible for the observed NO-production in the aerobic growth experiment (Fig. 1). The lack of N_2O formation by the cultures in the presence of 10% C_2H_2—known to inhibit N_2O-reductase [1, 26]—indicates that the cells did not possess a dissimilatory NO-reductase (data not shown). Further experiments (not documented) indicated that the cells also did not reduce N_2O even when added at such high concentrations where *E. coli* is able to reduce N_2O to N_2 [10].

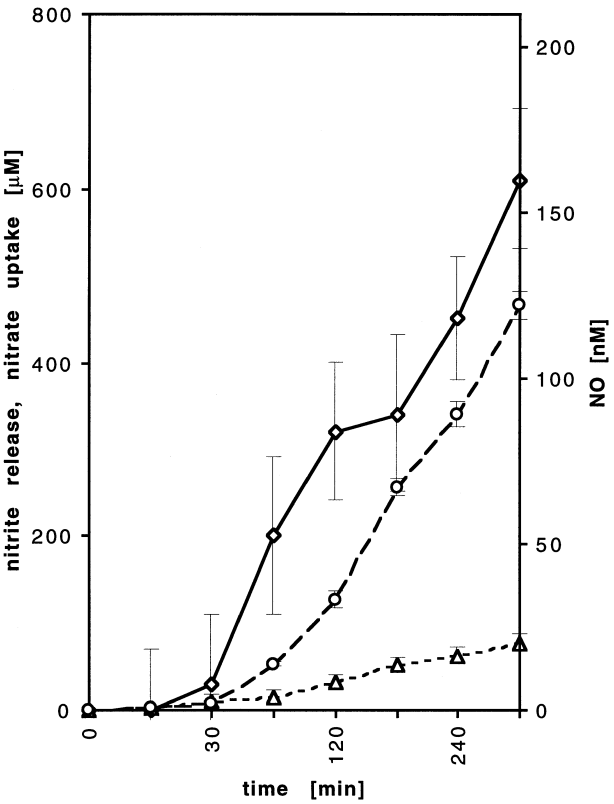

Fig. 2. Anaerobic nitrate reduction by *Pseudomonas putida*. Cells of an aerobic preculture (OD_{680nm} = 0.8) were pelleted, resuspended in mineral medium (containing 20 mM γ-aminobutyrate, 5 mM nitrate, without ammonia) to a final OD_{680nm} of 1.5, and oxygen was removed from the vessels. The consumption of nitrate (◇-◇), the release of nitrite (△-△) and NO (o-o) in the medium were determined as described in Materials and Methods.

The apoprotein of ammonia monooxigenase in autotrophic nitrifiers consists of two subunits [15]. Since a gene probe coding for the smaller subunit (*amoA*) of *N. europaea* was available [12], hybridization was per-

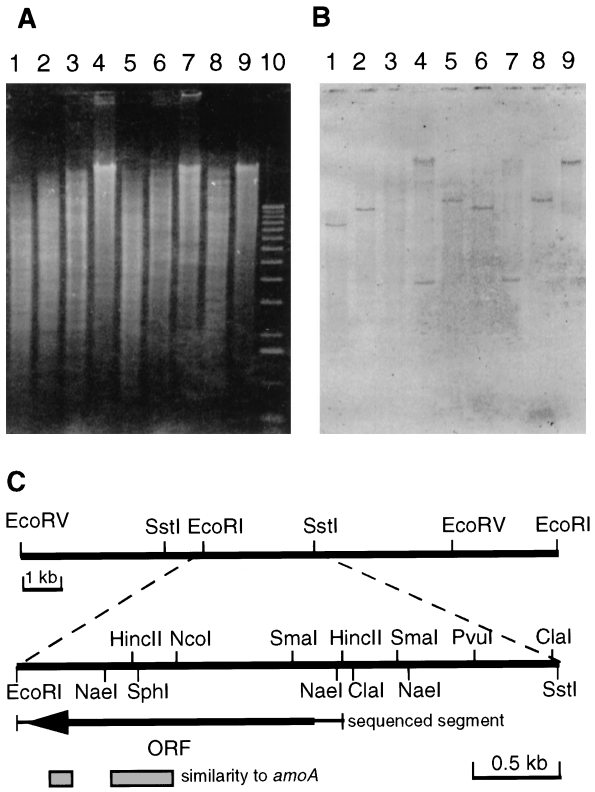

Fig. 3. Identification and mapping of the genomic region of *Pseudomonas putida* hybridizing with *Nitrosomonas europaea amoA*. Total DNA (1 µg DNA for each assay) of *P. putida* was restricted by *Eco*RI/*Eco*RV (lane 1), *Eco*RI/*Kpn*I (lane 2), *Sst*I/*Eco*RV (lane 3), *Sst*I/*Kpn*I (lane 4), *Eco*RV/*Kpn*I (lane 5), *Eco*RI (lane 6), *Sst*I (lane 7), *Eco*RV (lane 8), and *Kpn*I (lane 9) and separated on an 0.5% agarose gel (A). The DNA was transferred onto positively charged Nylon membrane and hybridized with the *amoA*-probe of *N. europaea* at 68°C without formamide (B). A 1-kb ladder (Gibco BRL) was used as size marker (lane 10). The physical genomic map of the hybridizing region of *P. putida* is based on the hybridization restriction pattern of the *amoA* probe of *N. europaea* (C) (e.g., see (B)). The detailed map of the insert subcloned in M13mp18/19 is given below the genomic map. The location of the identified ORF and the regions similar to *amoA* are indicated.

formed with DNA isolated from *P. putida*. The DNA restricted by several enzymes (Fig. 3A) hybridized distinctly with the probe at high stringency conditions (Fig. 3B). The hybridization pattern allowed construction of a physical map of the hybridizing genomic region (Fig. 3C).

For subcloning, genomic DNA was digested with *Eco*RI and *Sst*I, separated on agarose gel, and segments of about 3.0 kb were cloned into M13mp18. The resulting clones were screened with the *amoA* probe. One identified positive clone was characterized by restriction analysis (Fig. 3C). From this clone an 1783-bp *Hinc*II–*Hinc*II–*Eco*RI segment was subcloned and sequenced on both strands.

An open reading frame of 1530 bp (=510 amino acids) was identified on this segment, preceded by a putative ribosome-binding site [21] GAGG at position −17 to −14 upstream of the ATG start codon. A computer-aided gene bank search indicated that the deduced amino acid sequence of the ORF possessed two regions (amino acids 226–325, 392–434) with similarities of 46.0% and 32.6%, respectively, to AmoA of the autotrophic ammonia oxidizers *Nitrosospira briensis* and *Nitrosovibrio tenuis* (Fig. 4A). The similarities to the corresponding *N. europaea* AmoA regions were slightly lower (39.0% and 27.9%). The similarity to the smaller subunit of hitherto sequenced methane monooxigenases was less than 30% (alignments not shown). Furthermore, under the different culture conditions tested, *P. putida* was not able to oxidize methane at atmospheric or higher concentrations (data not shown).

The predicted hydropathy blot of the ORF indicated that the whole ORF probably coded for an integral membrane protein, as it contains several hydrophobic regions (Fig. 4B). In comparison with AmoA from *N. europaea*, the deduced ORF of *P. putida* was 234 amino acid residues longer. Remarkably, the two regions of the deduced amino acid sequence of the ORF, which showed the similarity to the *amoA* gene product of *N. europaea*, had a similar hydrophobicity profile (calculated according to [14]) as AmoA of the autotrophic ammonia oxidizers (Fig. 4B).

Discussion

The *P. putida* strain is capable of catalyzing diverse N-transformations within the N-cycle. It performs ammonification from γ-aminobutyric acid and reduces nitrate anaerobically to nitrite and partially further to NO. Under aerobic conditions, it oxidizes ammonia to hydroxylamine, nitrite, and nitrate in the presence of diverse carbon sources and is, therefore, a heterotrophic nitrifier. In contrast to autotrophic nitrifiers that do not release NH_2OH and form nitrate only by the concerted action of ammonium-oxidizing bacteria and nitrite oxidizers, the *P. putida* strain excretes hydroxylamine into the medium and produces nitrate. Also in contrast to autotrophic ammonia oxidizers, there was no evidence for any nitrous oxide formation at any step of the N-transformations investigated for *P. putida*.

The physiological role of the ammonia oxidation in *P. putida* and other heterotrophic bacteria [18] is not yet understood. *P. putida* cells performed ammonia oxidation, possibly owing to a demand for reductants for the generation of maintenance energy and/or for the synthesis of enzymes of carbon substrate catabolism as long as either the enzymes for carbon substrate utilization were

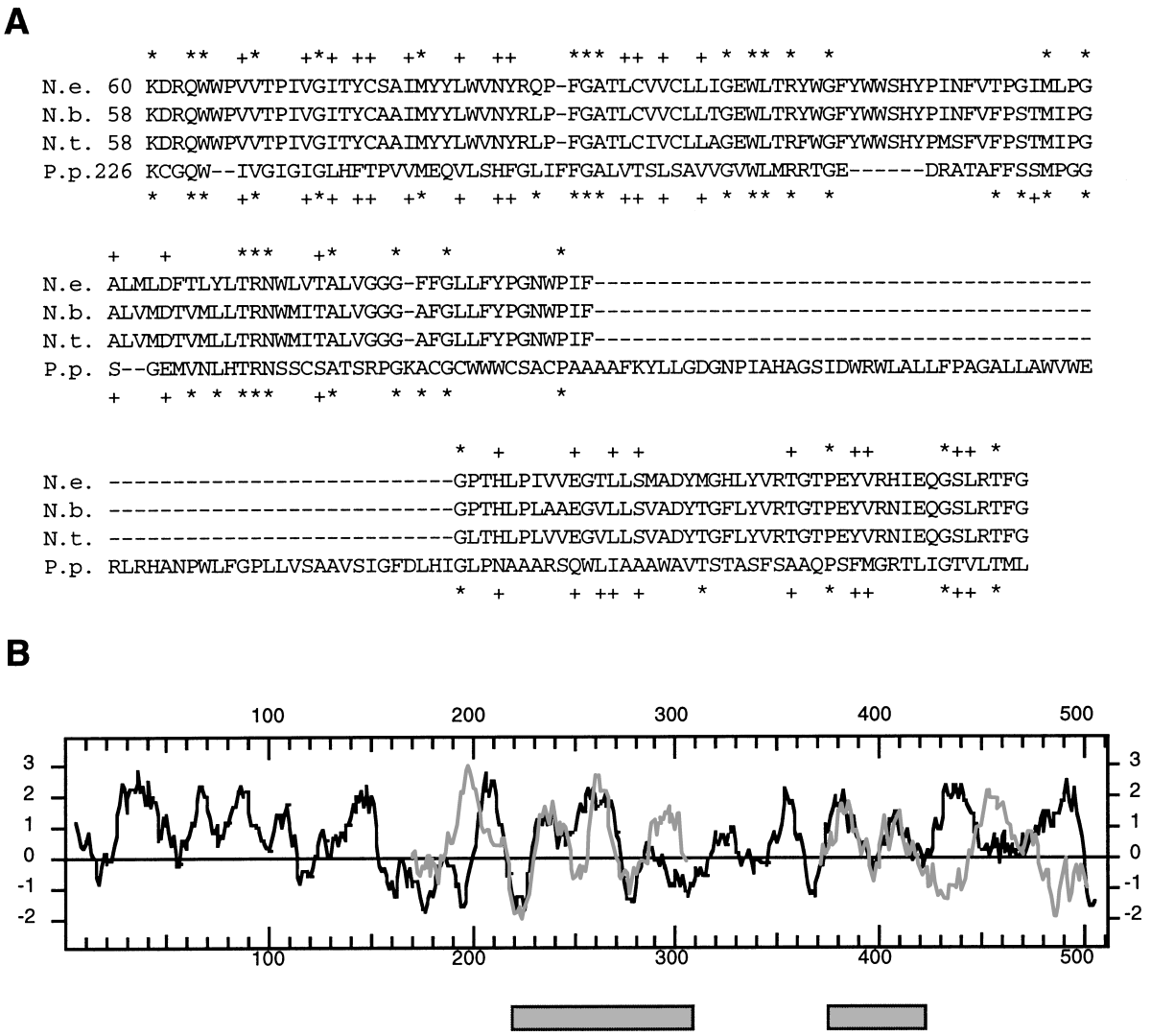

Fig. 4. Comparison of the deduced amino acid sequence of *Pseudomonas putida* ORF and AmoA sequences of autotrophic ammonia oxidizers. Sequences shown in the alignment (A) are from *Nitrosomonas europaea* (N.e., deduced from L08050), *Nitrosospira briensis* (N.b., deduced from U76553), *Nitrosovibrio tenuis* (N.t., deduced from U76552) and *Pseudomonas putida* (P.p., deduced from ORF, this work). The calculated (ClustalW vers. 1.5) identities (*) and conservative exchanges (+) in all four sequences are shown above the alignment; the homology for *N. briensis*, *N. tenuis*, and *P. putida* is given below the alignment. The predicted hydropathy blots (B) for AmoA of *N. europaea* (grey line) and the deduced amino acid sequence of *P. putida* (black line) were determined with the Kyte-Doolittle algorithm [14]. Positive values indicate hydrophobic residues.

not induced or the carbon substrate was exhausted. Under these conditions hydroxylamine formation and nitrite production were observed especially (Fig. 1). The oxidation of hydroxylamine to nitrite could represent a detoxification mechanism for hydroxylamine. However, maximal concentration of hydroxylamine was quite low and, obviously, did not affect growth (Fig. 1).

Nitrate formation during heterotrophic nitrification of *P. putida* could be owing to an oxidation of nitrite catalyzed by the reverse reaction of the dissimilatory nitrate reductase shown to be present in *P. putida* (Fig. 2). Nitrate could also result from an enzymatic NO oxidation, which has been described for a possibly closely related *Pseudomonas* isolate [13]. It could also be a product of a chemical degradation of the produced NO, since NO is rapidly oxidized to NO_2 in aerobic water solutions and NO_2 is changed into NO_2^- and NO_3^- [22, 27].

NO production is also observed in autotrophic nitrifiers [8]. The gas is probably a side product in the oxidation of NH_2OH to nitrite by hydroxylamine oxidoreductase [17]. Alternatively, the observed NO formation of *P. putida* could also come from the reaction of a dissimilatory nitrite reductase (Fig. 2). On a global scale, NO formation by autotrophic and heterotrophic nitrifiers

appears to essentially contribute to the release of NO from soil to the atmosphere [3]. In fact, most of the variation of the NO emission rate from the forest soil correlates with the fluctuation of the actual nitrification rate [5] catalyzed by heterotrophic nitrifiers in the soil from which *P. putida* had been isolated [18]. With respect to the heterotrophic nitrifier *P. putida*, NO production during nitrification is fairly low (about 0.1-1 nmol $h^{-1}/10^8$ cells). Assuming all heterotrophic nitrifiers present in this spruce forest soil would produce NO at this low rate, the rate would be high enough to account for most of the observed mean NO emission rate in the field (0.1 nmol g^{-1} soil h^{-1} from the upper 5 cm of the organic layer) [5], since in this forest soil heterotrophic nitrifiers occur in high cell numbers (about 10^8 cells g^{-1} soil dry weight; see [18]).

Both the physiological and molecular biological evidences suggest that the heterotrophic nitrifier *P. putida* possesses an ammonia monooxigenase. The organism oxidizes ammonia (added either as NH_4Cl or γ-aminobutyrate) to NH_2OH, and this production was inhibited by 5% C_2H_2 (Fig. 1C, 1D), but not by low C_2H_2 concentrations (10 Pa) known to inhibit ammonia oxidation in *N. europaea*. Other authors [16] report no or only marginal inhibition of a putative ammonia monooxigenase activity in extracts from the heterotrophic nitrifying *Paracoccus denitrificans* 2621 in the presence of 1 mM C_2H_2 (corresponding to about 2.5% C_2H_2 in the gas phase). However, the authors determined ammonia monooxigenase activity only indirectly by the ammonium-dependent stimulation of oxygen consumption, but not directly by the formation of hydroxylamine as in the present study. High concentrations of C_2H_2 (5%) totally block hydroxylamine production and, in consequence, the formation of the following oxidation products, NO and nitrite, during heterotrophic nitrification of *P. putida* (Fig. 1C and D). Both the hydroxylamine production from ammonia and the C_2H_2 inhibition pattern indicate the presence of an ammonia monooxigenase in *P. putida*.

As a further hint for presence of this enzyme, restricted DNA from *P. putida* consistently hybridized with the *amoA* probe from *N. europaea*. The deduced amino acid sequence of the identified ORF possessed two regions that showed distinct sequence similarities to the corresponding regions of AmoA of autotrophic ammonia oxidizers. In addition, the hydrophobicity profile in these regions was similar to corresponding regions of AmoA of *N. europaea*, *N. briensis*, and *N. tenuis* (Fig. 4). However, the deduced amino acid sequence of the ORF was 232–234 amino acid residues longer than published sequences of the mentioned ammonia oxidizers. This is in agreement with the obtained higher molecular weight of the partially purified putative ammonia monooxigenase of the heterotrophic nitrifier *P. denitrificans* 2621 [16]. The deduced amino acid sequence of the ORF from *P. putida* was more related to AmoA than to the smaller subunit of methane monooxigenases, which are presumed to be related enzymes [7]. The N-terminus of the deduced protein of the *P. putida* ORF possessed a high percentage of hydrophobic amino acids, suggesting a membrane association or integration of the polypeptide. In addition, hydrophobic regions present in the C-terminal part of the protein were also very similar to AmoA of *N. europaea* known to possess a membrane-bound ammonia monooxigenase. In particular, most of the conserved regions of AmoA polypeptides, e.g., the Cu-binding sites [7], were also present in the deduced ORF of *P. putida*. Therefore, the ORF most likely encoded a membrane-bound ammonia monooxigenase.

In conclusion, the physiological and molecular biological data presented in this study indicate the occurrence of an ammonia monooxigenase in the heterotrophic nitrifier *P. putida*. Strict proof of the occurrence of AmoA can come only from the analysis of a constructed mutant defective in this gene. Future work will show whether the heterotrophic nitrifier *P. putida* is readily accessible to site-directed mutagenesis. If, indeed, an *amoA*-related gene exists in *P. putida*, oligonucleotide primers could be developed to identify the homologous gene in other heterotrophic nitrifiers. Such an approach would give access to identify and characterize this physiological group of microorganisms which apparently have considerable impact in the N-cycle of soils.

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