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ORIGINAL ARTICLE

Isolation and characterization of a heterotrophic nitrifier *Proteus mirabilis* strain V7 and its potential application in NH_4^+-N removal

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Abstract Ammonia (referring to both NH_3 and NH_4^+), is one of the most toxic nitrogen forms, and is toxic to many aquatic organisms. The present study identified a heterotrophic nitrifier *Proteus mirabilis* strain V7, isolated from the coastal seawater. Almost 100 % of 82 mg L^{-1} NH_4^+-N was removed within 48 h, while 51.2 % of total inorganic nitrogen was removed within 96 h after inoculation of *Proteus mirabilis* strain V7. Moreover, *Proteus mirabilis* strain V7 could remove NH_4^+-N in a seawater matrix, but with much lower efficiency. Three water samples containing NH_4^+-N at concentrations of 64.40, 18.00 and 9.82 mg L^{-1} were chosen to further test the NH_4^+-N removal ability of *Proteus mirabilis* strain V7. All of the NH_4^+-N was removed, with only a trace production of NO_2^- and NO_3^-. A putative subunit of ammonia monooxygenase from *Proteus mirabilis* strain V7, *amoA*$_{Pm}$, was cloned and its mRNA level in the presence of NH_4^+ was characterized by real time reverse transcriptase PCR (RT-PCR). A 1.8-fold increase in the mRNA level of *amoA*$_{Pm}$ was observed, which indicated that AmoA may be involved in NH_4^+ oxidization. Our results indicated that *Proteus mirabilis* strain V7 could potentially be used as new resource for the treatment of NH_4^+-N contaminated environments.

Keywords Ammonia · *Proteus mirabilis* · NH_4^+-N removal · Ammonia monooxygenase · mRNA level

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Introduction

Ammonia in the form of un-ionized ammonia (NH_3) or ionized ammonia (NH_4^+) is an inorganic and dissolved nitrogen compound that can be detected frequently in the aquatic environment, especially where dissolved oxygen is lacking (Alcaraz et al. 1999). In solution, the acid ionization of NH_3 is realized via the equation described by Whitfield (1974). The relative proportion of NH_4^+ and NH_3 depends mainly on environmental factors such as temperature, salinity and pH of the aqueous solution (Alcaraz et al. 1999). As an important nitrogen compound, ammonia within the range of 2–10 mg L^{-1} has been proved to be severely toxic to most aquatic organisms. The acceptable ammonia concentration in drinking water has been designated as 1.5 mg L^{-1} by the US Environmental Protection Agency (US EPA) (WHO 1990; US EPA 2009).

Considering the hazardous effects of ammonia, methods to efficiently remove ammonia from aquatic environments have been urgently recommended (US EPA 1993; Taylor et al.

2009). Among the methods developed, biological transformation through the metabolism of nitrifying bacteria is a superior approach due to its advantages of higher efficiency and cost-effectiveness over the alternative physical and chemical processes such as ion exchange and adsorption (US EPA 1993; Rostron et al. 2001; Jorgensen and Weatherley 2003; Khardenavis et al. 2007; Andrade do Canto et al. 2008). Traditionally, NH_4^+ was subsequently oxidized to NO_3^- by two kinds of ubiquitous chemoautotrophic nitrifying bacteria, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), via the intermediate of NO_2^- (Ward 1996; Schaechter 2009). Recently, studies on the NH_4^+-N removal depending on NH_4^+ oxidization by heterotrophic nitrifying bacteria, which also possess the ability to oxidize NH_4^+ but catabolize organic compounds in a life mode different from that of traditional chemoautotrophic nitrifying bacteria, are increasing (Winogradsky 1890; Witzel and Overbeck 1979; Papen and von Berg 1998; Taylor et al. 2009; Zhao et al. 2010; Yang et al. 2011; Sarioglu et al. 2012). Compared with traditional chemoautotrophic nitrifying bacteria, heterotrophic nitrifying bacteria not only possess relatively high growth rate and nutrient utilization efficiency but also have the advantage of higher tolerance to NH_4^+ and organic matter (Zhao et al. 2010). Many heterotrophic nitrifying bacteria, including *Alcaligenes faecalis*, *Acinetobacter calcoaceticus*, *Thiosphaera pantotropha*, *Bacillus* sp., *Microvirgula aerodenitrificans*, *Pseudomonas stutzeri*, *Pseudomonas putida*, *Comamonas* sp. and *Diaphorobacter* sp., have been characterized for their potential application in bioremediation of NH_4^+ or other nitrogenous pollutants (Otte et al. 1996; Patureau et al. 1997; Daum et al. 1998; Su et al. 2001; Kim et al. 2005; Khardenavis et al. 2007; Taylor et al. 2009; Zhao et al. 2010; Yang et al. 2011).

In the present study, a *Proteus mirabilis* strain V7 isolated from a coastal seawater sample was newly identified as a heterotrophic nitrifying bacterium. The products of NH_4^+ oxidization by this heterotrophic nitrifying bacterium *P. mirabilis* strain V7 were primarily determined. The gene encoding a putative subunit of ammonia monooxyegnase (AMO), *amoA*$_{\text{Pm}}$, was cloned and its relative mRNA level in the presence and absence of NH_4^+ was analyzed.

Materials and methods

Strains, medium, growth conditions and chemicals

Escherichia coli strains Top10 and BL21(DE3) used in this study were grown in Luria-Bertani (LB) medium at 37 °C with appropriate antibiotics. The antibiotics ampicillin (Ap) and kanamycin (Kn) were used at working concentrations of 100 mg L^{-1} and 50 mg L^{-1}, respectively. Five environmental bacteria, i.e., *Proteus mirabilis* strain V7, *Bacillus cereus* strain KF8, *Bacillus pumilus* strain Bb, *Candida boidinii* strain Cb and *Pseudomonas putida* strain SP1 isolated from coastal seawater, were grown in specific medium at 30 °C. LB medium consisted of 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L ddH_2O. Synthetic succinate medium contained 6.0 g K_2HPO_4, 3.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 4.0 g succinic acid in 1 L ddH_2O. Phosphate succinate medium without $(NH_4)_2SO_4$ consisted of 6.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, and 4.0 g succinic acid in 1 L ddH_2O. 2216E medium consisted of 5 g tryptone, 1 g yeast extract, and 0.01 g $FePO_4$ in 1 L aged seawater.

Plasmid pBS-T was purchased from Transgen Biotech (Beijing, China). Restriction endonucleases, alkaline phosphatase, T4 DNA ligase and *Taq* DNA polymerase were purchased from Fermentas (MBI, Beijing, China). Plasmid preparation, the extraction of DNA fragments from agarose gels, and the purification of PCR products were performed using the respective kits from Omega Bio-Tek (Norcross, GA) according to the manufacturer's instructions. Most of the other chemicals used in this study were purchased from Sangon at analytical grade (Sangon, Shanghai, China).

Bacteria selection by semi-quantitative ammonia detection

Selection of a bacterium capable of decreasing the concentration of NH_4^+-N was carried out using semi-quantitative ammonia detection kits (Luheng Biotech, Hangzhou, China). Briefly, supernatants from bacterial cultures of *Proteus mirabilis* strain V7, *B. cereus* strain KF8, *B. pumilus* strain Bb, *C. boidinii* strain Cb and *Pseudomonas putida* strain SP1 grown in synthetic succinate medium, were diluted ten times into 15 mL tubes. One pack of reagent I was added to each tube, followed by gently shaking and standing at room temperature for 2 min. Then a pack of reagent II was added, followed by mixing uniformly and standing at room temperature for another 15 min. The developed color of each tube was compared with the standard board to determine the remaining NH_4^+-N semi-quantitatively.

DNA techniques

One pair of primers PmamoF1 (5′-GACGCATATGTCATTT TTAAAAAAACTACC-3′) and PmamoR1 (5′-CTCGAGCT CACGTTTTCTTTTCTG-3′) was designed based on the nucleotide sequence of AM942759 to amplify the putative *amoA* gene from *Proteus mirabilis* (*amoA*$_{\text{Pm}}$). Another pair of primers PmamoF2 (5′-TACACTGACTTTTATCCAACTG ACCT-3′) and PmamoR2 (5′-ACAGCACCTAACATAGCC TCTTCT-3′), was designed for real time reverse transcriptase PCR (RT-PCR) to analyze relative mRNA levels of *amoA*$_{\text{Pm}}$. All primers used in this study were synthesized by Sunny

Biotech (Shanghai, China). PCR amplifications were carried out in a Tpersonal thermocycler (Biometra, Goettingen, Germany). PCR products were separated on 0.8 % agarose gel, and DNA fragments were purified and ligated into specific vectors. The ligation mixture was transformed into the competent cells of *E. coli* strains Top10, and the transformants were selected on LB plates supplemented with appropriate antibiotic. DNA sequencing was carried out by Sunny Biotech (Shanghai, China). To obtain the purified $AmoA_{Pm}$, a plasmid $pETamoA_{Pm}$ was constructed, in which a PCR product amplified with primers PmamoF1 and PmamoF1, was digested simultaneously with *Nde*I and *Xho*I, and then inserted into pET258, constructed by Zhang and Sun (2007), between *Nde*I/*Xho*I sites.

Real time RT-PCR

Real time RT-PCR was carried out according to the description of Zhang et al. (2008). Briefly, total RNA was extracted from cells grown in the presence and absence of NH_4^+. Real time RT-PCR was carried out in an ABI 7500 real-time detection system using a Sybr ExScript RT-PCR kit (Takara Biotech, Dalian, China). The primers of $amoA_{Pm}$ used for RT-PCR were PmamoF2 and PmamoR2. 16S rRNA was used as the reference gene. The primers of 16S rRNA used for the RT-PCR were 933 F (5′-GCACAAGCGGTGGAGCATGTGG-3′) and 16SRTR1 (5′-CGTGTGTAGCCCTGGTCGTA-3′). Dissociation analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified. The comparative threshold cycle method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative mRNA level of $amoA_{Pm}$.

Expression of recombinant $AmoA_{Pm}$

Escherichia coli strain BL21(DE3) harboring the plasmid $pETamoA_{Pm}$ was cultured to an OD_{600} of 0.5, and expression of $AmoA_{Pm}$ was expected to be induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After grown at 30 °C for additional 6 h, bacterial cells were harvested by centrifugation and the cell pellet was resuspended in protein denaturing buffer consisted of 100 mM NaH_2PO_4, 10 mM Tris-Cl and 8 M urea. Then the cell lysate was centrifuged at 13,000 *g* for 20 min, and supernatant containing the whole cell proteins was applied for SDS-PAGE analysis.

NH_4^+-N removal by *Proteus mirabilis* strain V7

To investigate the effect of initial NH_4^+-N concentration on the removal efficiency of *Proteus mirabilis* strain V7, cells were inoculated into synthetic succinate medium containing initial NH_4^+-N concentrations of 20, 50, 100, 200, 500, 1000 and 2000 mg L^{-1}. After culturing at 30 °C for 64 h, supernatants were collected and analyzed for the remaining NH_4^+-N. The supernatant from the medium containing the same concentration of NH_4^+-N without inoculation of bacterial cell was used as a control. To investigate whether *Proteus mirabilis* strain V7 could remove NH_4^+-N from a seawater matrix, 1.0×10^7 CFU mL^{-1} bacterial cells were inoculated into 2216E medium containing initial concentrations of NH_4^+ at 20, 50, 100, 200, 500, 1000 and 2000 mg L^{-1}, and the remaining NH_4^+-N in the supernatant was measured at different time points. To determine the products of NH_4^+ oxidization by *Proteus mirabilis* strain V7, experiments were also conducted in a 1-L flask inoculated with 1.0×10^7 CFU mL^{-1} bacterial cells in synthetic succinate medium containing 80 mg L^{-1} NH_4^+-N. Aliquots were collected at different time points and supernatants were analyzed for the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N. Several water samples were collected around the mouths of several rivers around Yantai, and three samples containing the highest levels of NH_4^+-N were chosen as the matrices for NH_4^+-N removal by *Proteus mirabilis* strain V7. After incubated at 30 °C for 48 h, the supernatants were collected and analyzed for concentrations of NH_4^+-N, NO_2^--N and NO_3^--N. Measurement of cell density and concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N was carried out as follows. The OD_{600} of the bacterial culture was measured with a spectrophotometer (Thermo NanoDrop, http://www.nanodrop.com). NH_4^+-N was determined by Nessler's reagent photometry (APHA

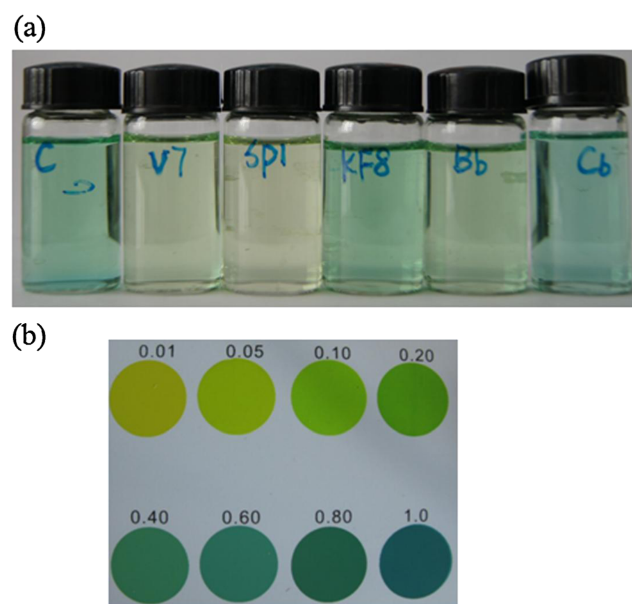

Fig. 1 Selection of bacterial strains capable of removing NH_4^+-N using a semi-quantitative ammonia nitrogen detection kit. The five strains used were *Proteus mirabilis* strain V7, *Pseudomonas putida* strain SP1, *Bacillus cereus* strain KF8, *B. pumilus* strain Bb and *Candida boidinii* strain Cb. **a** Supernatants from bacterial cultures were reacted with reagents I and II to develop the color. **b** The standard color board used for semi-quantitative detection of NH_4^+-N; units of concentration on the board in mg L^{-1}

Table 1 Conversion of nitrogen by consumption of NH_4^+ by *Proteus mirabilis* strain V7. *TIN* Total inorganic nitrogen

Different nitrogen form	Concentrations of each nitrogen form at different time points					
	0 h	12 h	24 h	48 h	72 h	96 h
NH_4^+-N [a]	81.97±6.3	54.62±4.9	10.68±0.9	0	0	0
NO_2^--N [b]	2.55±0.06	5.60±0.5	10.66±0.4	13.16±0.3	16.32±0.2	33.11±1.7
NO_3^--N [a]	0	26.94±1.1	70.34±6.9	79.47±2.7	73.811±7.5	39.98±3.4
TIN [a]	81.97	81.56	81.02	79.47	73.81	39.98
Lost NH_4^+-N	0	27.35	71.29	81.97	81.97	81.97
Lost TIN	0	0.41	0.95	2.50	8.16	41.99

[a] Unit of concentrations of NH_4^+, NO_3^- and TIN = mg L^{-1}

[b] Unit of concentration of NO_2^- = μg L^{-1}

1995). NO_2^--N was determined by the N-(1-naphthalene)-diaminoethane photometry method. NO_3^--N was analyzed by the phenol disulfonic acid method.

Nucleotide sequence accession number and strain accession numbers

The nucleotide sequence of *amoA* $_{Pm}$ has been deposited in the GenBank database with accession number KC775377. The isolate of *Proteus mirabilis* strain V7 was deposited in the China General Microbiological Culture Collection (CGMCC, Beijing, China) with accession number CGMCC 4312.

Results and discussion

Isolation of a bacterial strain capable of decreasing the concentrations of NH_4^+-N

Proteus mirabilis strain V7, *B. cereus* strain KF8, *B. pumilus* strain Bb, *C. boidinii* strain Cb, and *Pseudomonas putida* strain SP1 were cultured to detect whether they have the ability to decrease the concentration of NH_4^+-N in the culture medium. *Pseudomonas putida* strain SP1 was used as a positive control according to the study of Daum et al. (1998), while none of the other strains has been reported to be able to oxidize NH_4^+. After cultured at 30 °C for 5 days, the supernatants were collected and used for the semi-quantitative analysis of the remaining NH_4^+-N. As shown in Fig. 1, the color of supernatants from cultures of *Proteus mirabilis* strain V7 and *Pseudomonas putida* strain SP1 turned yellow-green, which meant that the concentrations of NH_4^+-N in the two supernatants decreased below 4.0 mg L^{-1}, while the color of supernatants from cultures of *B. cereus* strain KF8, *B. pumilus* strain Bb and *C. boidinii* strain Cb remained the same as, or showed little difference from, the color of the supernatant from the control medium. Although inoculation of *Pseudomonas putida* strain SP1 could also lead to a decrease in the concentration of NH_4^+-N, the growth of *Pseudomonas putida* strain SP1 was poor due to the cell self-lysis. Thus, *Proteus mirabilis* strain V7 was selected for further study on NH_4^+ oxidization. In the absence of any organic carbon source, *Proteus mirabilis* strain V7 was defective both in cell growth and NH_4^+-N removal activity (data not shown). However, in the presence of succinic acid as the organic carbon source, both growth of *Proteus mirabilis* strain V7 and decreased concentration of NH_4^+-N were observed. According to the results obtained, *Proteus mirabilis* strain

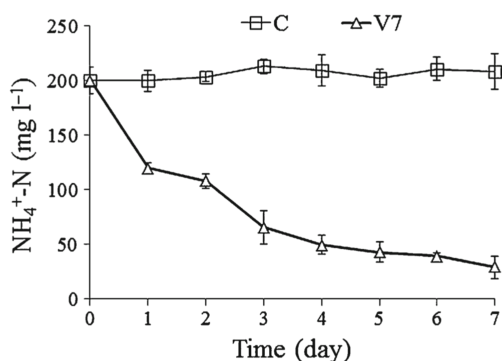

Fig. 2 NH_4^+-N removal by *Proteus mirabilis* strain V7 over time. The remaining NH_4^+-N in the supernatants from cultures of *Proteus mirabilis* strain V7 was analyzed. The NH_4^+-N in the supernatant from medium without inoculation of bacterial cells was used as a control. Data are the means of three independent experiments and are presented as means±SE

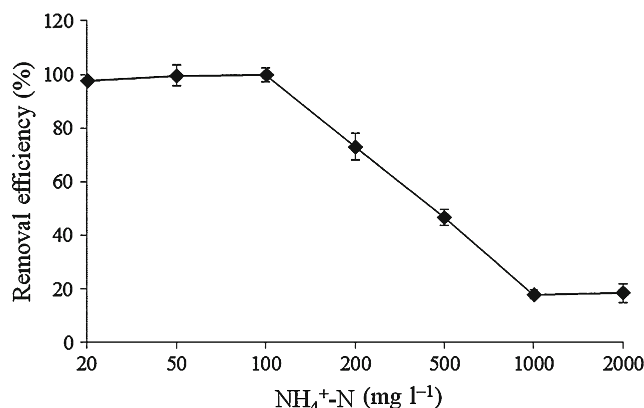

Fig. 3 NH_4^+-N removal efficiency of *Proteus mirabilis* strain V7 under different initial NH_4^+-N concentrations. The NH_4^+-N remaining after inoculation of *Proteus mirabilis* strain V7 was determined and removal efficiency was defined as the percentage of NH_4^+-N that was initially added to the medium. Data are the means of three independent experiments and are presented as means±SE

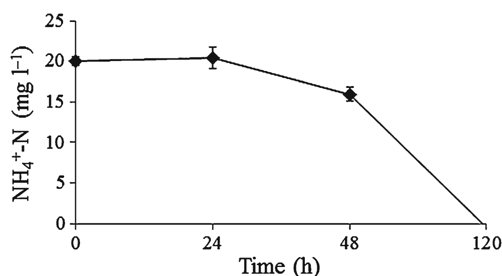

Fig. 4 The NH_4^+-N remaining in 2216E medium after inoculation of *Proteus mirabilis* strain V7. The initial concentration of NH_4^+-N was 20 mg L^{-1}. The remaining NH_4^+-N in the supernatant was determined after inoculation at 30 °C for 64 h. Data are the means of three independent experiments and are presented as means±SE

V7 was defined as a heterotrophic nitrifying bacterium in the light of the previous references (Alexander 1977; Papen et al. 1989). Moreover, this was the first time that *Proteus mirabilis* strain has been characterized as a heterotrophic nitrifier, and it was expected that *Proteus mirabilis* strain V7 could be potentially applied in NH_4^+-N removal.

Products of NH_4^+ oxidization by *Proteus mirabilis* strain V7

The concentrations of NH_4^+-N, NO_2^--N and NO_3^--N in the supernatants of culture were measured during NH_4^+ oxidization at different time points. As shown in Table 1, the NO_2^--N concentration in the medium increased to trace levels (μg L^{-1}) as the culture time increased, whereas the NO_3^--N concentration increased to 79.47 mg L^{-1} as the culture time rose to 48 h. In total, all the NH_4^+-N was removed within 48 h, while 51.2 % of the total inorganic nitrogen (TIN) was removed within 96 h of inoculation with cells of *Proteus mirabilis* strain V7. Thus, it could be concluded that *Proteus mirabilis* strain V7 possessed higher NH_4^+-N removal efficiency but lower TIN removal efficiency. It is generally accepted that the heterotrophic nitrification and aerobic denitrification can take place simultaneously in one heterotrophic nitrifier through subsequent oxidization of NH_4^+ to NO_2^- and NO_3^-, and then reduction of NO_3^-, especially NO_2^-, to gaseous nitrogen (Castignetti and Hollocher 1982, 1984; Robertson and Gijs Kuenen 1984; Robertson et al. 1988; Wehrfritz et al. 1993; Jetten et al. 1997; Richardson et al. 1998). It thus can be suggested that the trace production of NO_2^- may be due to the rapid transformation of NO_2^- to NO_3^-, or due to the reduction of NO_2^- to gaseous nitrogen.

Characterization of NH_4^+-N removal by *Proteus mirabilis* strain V7

Removal of NH_4^+-N by *Proteus mirabilis* strain V7 grown in synthetic succinate medium at 30 °C in the dark was investigated. As shown in Fig. 2, concentrations of NH_4^+-N in the supernatant of control medium at different selected time points remained almost the same for a 7-day observation, suggesting that NH_4^+-N was relatively steady under the conditions tested; however, inoculation of cells of *Proteus mirabilis* strain V7 caused a significant decrease in the level of NH_4^+-N with increasing time. After incubating for 4 days, only 25 % of the NH_4^+-N still remained in the culture. The removal efficiency under different initial concentrations of NH_4^+-N also showed significant differences. As shown in Fig. 3, NH_4^+-N at concentrations lower than 100 mg L^{-1} could be removed completely. Once the NH_4^+-N concentration rose to above 200 mg L^{-1}, the removal efficiency of *Proteus mirabilis* strain V7 decreased sharply. The removal efficiency of *Proteus mirabilis* strain V7 was below 20 % when the concentration of NH_4^+-N in the medium was higher than 1,000 mg L^{-1}. This result coincided with the fact that higher concentrations of NH_4^+-N also showed a negative effect on NH_4^+-N removal by *Bacillus cereus* strain I6 and *Acinetobacter calcoaceticus* strain STB1, i.e., the removal efficiency of *B. cereus* strain I6 dropped from 62 % to 24.8 % as concentrations of NH_4^+-N increased from 5 to 25 mg L^{-1}, and the removal efficiency of *A. calcoaceticus* strain STB1 also dropped from 93 % to 45 % as concentrations of NH_4^+-N increased from 100 to 210 mg L^{-1} (Hasan et al. 2012; Sarioglu et al. 2012).

NH_4^+-N removal in seawater matrix and water samples

Removal of NH_4^+-N by *Proteus mirabilis* strain V7 grown in 2216E medium containing NH_4^+-N at concentrations of 20, 50, 100, 200, 500, 1000 and 2000 mg L^{-1}, was investigated. After culturing at 30 °C for 64 h, supernatants were collected and analyzed for remaining NH_4^+-N. When the initial concentration of NH_4^+-N in 2216E medium was higher than 50 mg L^{-1}, hardly NH_4^+-N removal by *Proteus mirabilis* strain V7 was observed. Only 20 mg L^{-1} NH_4^+-N in 2216E medium was removed completely by *Proteus mirabilis* strain V7 within 100 h (Fig. 4). Compared with NH_4^+-N removal in synthetic succinate medium, it could be concluded that

Table 2 NH_4^+-N removal by *Proteus mirabilis* V7 in water samples

Sample ID	NH_4^+-N (mg L^{-1})		NO_2^--N (mg L^{-1})		NO_3^--N (mg L^{-1})	
	Initial	Final	Initial	Final	Initial	Final
1	64.40±3.6	1.30±0.06	0.35±0.03	0.03±0.002	30.80±0.002	31.44±3.4
2	18.00±0.4	0.15±0.01	0.00±0.01	0.04±0.009	0.74±0.09	1.70±0.3
3	9.82±1.1	0.86±0.13	0.00±0.01	0.06±0.006	0.00±0.001	0.05±0.003

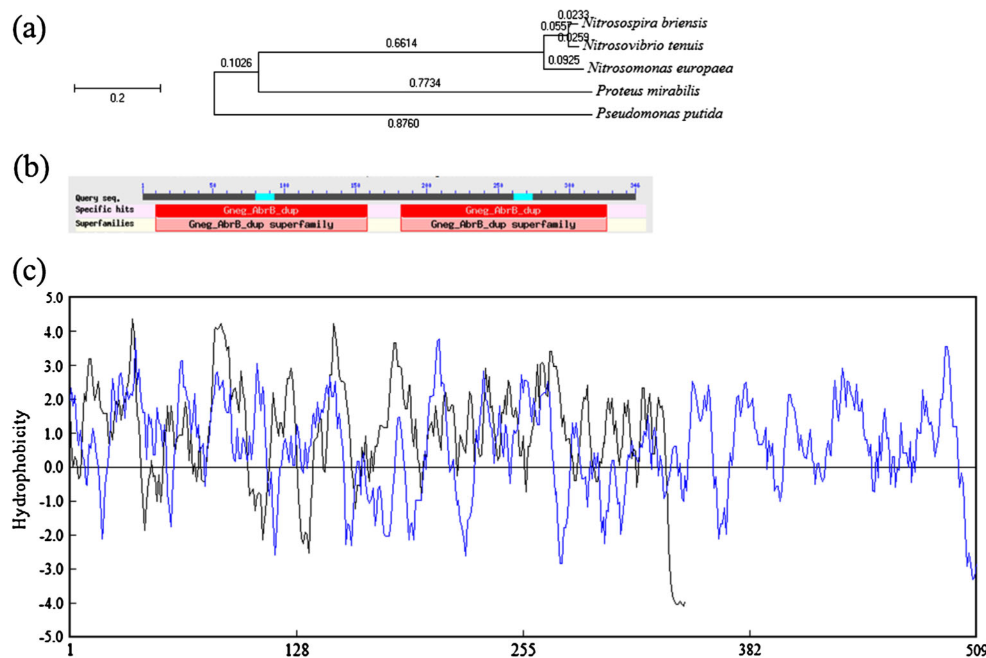

Fig. 5 **a** Evolutionary relationship between $AmoA_{Pm}$ from *Proteus mirabilis* and AmoAs from other bacterial strains. Multiple sequence alignment was produced using CLUSTAL W2 (Larkin et al. 2007). The unrooted phylogenetic trees were constructed by the neighbour-joining method, based on the distances derived from the Dayhoff matrix, with MEGA 4.0 software (Tamura et al. 2007). The robustness of the branches was assessed by the bootstrap method with 1,000 replications. The *scale bar* represents a distance of 0.2 substitutions per site. The sequences have the following NCBI accession numbers: *Nitrosomonas europaea* (AAA66194), *Nitrosospira briensis* (AAB38709), *Nitrosovibrio tenuis* (AAB38710) and *Pseudomonas putida* (CAA74724). **b** Schematic representation of the domains organized in the putative $AmoA_{Pm}$ from *Proteus mirabilis*. **c** The predicted hydropathy blots for AmoA from *Proteus mirabilis* (*short black line*) and AmoA from *Pseudomonas putida* (*long blue line*). Positive values indicate hydrophobic residues

Proteus mirabilis strain V7 possessed a much lower NH_4^+-N removal efficiency in the seawater matrix. According to a previous study by Wehrfritz et al. (1993), the coenzyme Q involved in NH_4^+ oxidization acquired an electron through oxidization of the supplied reducing organic carbon source during the heterotrophic nitrification process, and thus the organic carbon source exerted effects not only on the growth state but also on the nitrification activity of heterotrophic nitrifiers (Mevel and Prieur 2000). Thus, the low nitrification efficiency of *Proteus mirabilis* strain V7 in the 2216E medium may be due to the oligotrophic characteristic of the medium.

Aquatic samples were collected from several estuaries around Yantai, and the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were analyzed. Three water samples containing 64.40, 18.00, and 9.82 mg L^{-1} NH_4^+-N was chosen for the NH_4^+-N removal experiment. Moreover, sample 1 also contained the highest concentrations of NO_2^--N and NO_3^--N. As shown in Table 2, almost all the NH_4^+-N was removed within 48 h after inoculation with cells of *Proteus mirabilis* strain V7. In sample 1, which originally contained 0.35 mg L^{-1} NO_2^--N, the concentrations of NO_2^--N were reduced 10-fold, while in samples 2 and 3, a trace increase in NO_2^--N was detected. Compared with the initial concentrations of NO_3^--N in the samples, a slight increase in the concentrations of NO_3^--N among all the three samples was observed, that might be removed with longer incubated time. The increased production of NO_2^--N and NO_3^--N was postulated to be intermediates of NH_4^+ oxidization by *Proteus mirabilis* strain V7.

Analysis of the $AmoA_{Pm}$ sequence and its mRNA level in the presence of NH_4^+

The key enzyme that catalyzes the first step of NH_4^+ oxidization is AMO (EC 1.14.99.39), which contains three subunits AmoA, AmoB and AmoC, with its active site located in AmoA (McTavish et al. 1993; Klotz et al. 1997; Hirota et al. 2000). *amoA* $_{Pm}$, encoding a putative AmoA from *Proteus mirabilis*, was cloned and submitted for DNA sequencing.

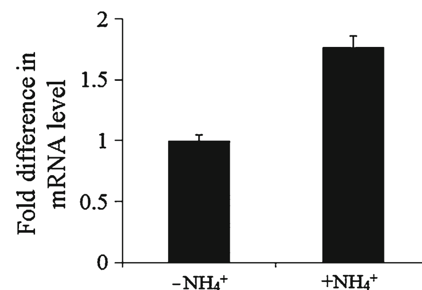

Fig. 6 mRNA analysis of the putative *amoA* $_{Pm}$ gene. The mRNA level of the putative *amoA* $_{Pm}$ was normalized to that of 16S rRNA and all data are presented in terms of relative mRNA. Data are the means for three independent experiments and are presented as the means±SE

Analysis of the nucleotide sequence obtained revealed it was an open reading frame (ORF) with a length of 1,041 bp. The encoded protein had an estimated molecular mass of approximately 38 kDa and pI of 9.96, and shared 99 % overall sequence similarity with the membrane-associated ammonia monooxygenases from three *Proteus mirabilis* strains with accession numbers WP_004245042, YP_008396899 and YP_002149941, respectively. A phylogenetic analysis of $AmoA_{Pm}$ using the other known AmoAs is shown in Fig. 5a. Compared with AmoA from *Pseudomonas putida*, the putative AmoA from *Proteus mirabilis* showed a higher homology to the AmoAs from autotrophic ammonia oxidizers. The deduced amino acid sequence of the identified ORF contained two regions that belonged to Gneg_AbrB_dup superfamily, which showed distinct sequence similarities to the corresponding motif of AmoAs from autotrophic ammonia oxidizers (Fig. 5b). In addition, the hydrophobicity profile of the $AmoA_{Pm}$ was similar to the corresponding regions of AmoA from *Pseudomonas putida*, which was also similar to the AmoAs from *Nitrosomonas europaea*, *Nitrosospira briensis* and *Nitrosovibrio tenuis* (Daum et al. 1998) (Fig. 5c). After being induced by IPTG, no recombinant protein was detected in BL21(DE3)/pETamoA_{Pm} after SDS-PAGE analysis. This phenomenon was consistent with the fact that the expression of *amo* genes was toxic to the *E. coli* strain, thus none of the corresponding gene fragments has been cloned and heterogeneously expressed successfully in *E. coli* combined with the pET system (Bergmann and Hooper 1994; Gupta and Khare 2006). That may account for why only two AmoAs from heterotrophic nitrifiers, i.e., the AmoA from *Pseudomonas putida* and *Paracoccus denitrificans*, have been identified, and AmoAs from heterotrophic nitrifying bacteria exhibited significant differences in strain specificity (Huertas and Duque 1998; Sardessai and Bhosle 2002; Gupta and Khare 2006). In order to investigate whether the transcription of $amoA_{Pm}$ was affected by NH_4^+, *Proteus mirabilis* strain V7 was grown in phosphate salt medium and synthetic succinate medium at 30 °C for 12 h, respectively. Total RNA was extracted and used for real-time RT-PCR to analyze the relative mRNA level of $amoA_{Pm}$. The result showed that a 1.8-fold increase in the mRNA level of $amoA_{Pm}$ was observed in the presence of NH_4^+ (Fig. 6). This result was coincided with the fact that the mRNA levels of *amo* and enzyme activity were positively correlated with concentration of NH_4^+ rose up to 1 mM after incubation for 3 h (Sayavedra-Soto et al. 1996)

Conclusions

In this study, a heterotrophic nitrifying bacterium *Proteus mirabilis* strain V7 was identified and characterized. *Proteus mirabilis* strain V7 removed 100 % of NH_4^+-N at concentrations below 100 mg L^{-1} and 51 % of TIN in the culture medium within 96 h, which indicated that *Proteus mirabilis* strain V7 was a heterotrophic nitrifying bacterium that may possess the ability of both heterotrophic nitrification and aerobic denitrification. *Proteus mirabilis* strain V7 could also remove NH_4^+-N in seawater matrix but with lower removal efficiency. In water samples contaminated by NH_4^+-N, *Proteus mirabilis* strain V7 could remove all the NH_4^+-N with a small increase in the levels of NO_2^--N and NO_3^--N. The mRNA level of $amoA_{Pm}$ was increased in the presence of NH_4^+, which indicated that $AmoA_{Pm}$ from *Proteus mirabilis* strain V7 may be involved in NH_4^+ oxidization.

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