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Isolation and characterization of novel halotolerant and/or halophilic denitrifying bacteria with versatile metabolic pathways for the degradation of trimethylamine

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Abstract

Four denitrifying bacteria capable of degrading trimethylamine under both aerobic and denitrifying conditions were newly isolated from coastal sediments and wastewater contaminated by marine water. All strains were in α-*Proteobacteria*. Strain GP43 was classified as a member of genus *Paracoccus*, and strain PH32, PH34 and GRP21 were novel organisms with remote phylogenetic position from other genus α-*Proteobacteria*. Among these four strains were the halophilic strains PH32, PH34 and GRP21, which did not grow in the absence of sodium chloride in culture medium. Cells grown under denitrifying conditions possessed trimethylamine dehydrogenase while cells grown aerobically possessed two different enzymes for oxidation of trimethylamine, trimethylamine dehydrogenase and trimethylamine monooxygenase. The newly isolated strain PH32, PH34 and GRP21 may be the first halophilic bacteria to degrade trimethylamine under denitrifying conditions.

Keywords: Trimethylamine; Denitrifying bacteria; Anaerobic degradation

1. Introduction

Trimethylamine is a malodorous pollutant frequently found in effluents from fish-meal manufacturing processes [1]. This compound has been known to inhibit the synthesis of macromolecules such as DNA, RNA and proteins, and has teratogenic effects on animal embryos [2]. Due to the malodorous and hazardous properties, an intensive attention has been paid to microbial degradation of trimethylamine, which may be an attractive strategy for the removal of pollutants from contaminated environments.

Many microorganisms have been reported to degrade trimethylamine, including strict aerobic [3–8] and anaerobic bacteria [9–11]. Some microorganisms, named denitrifying bacteria, have the ability to utilize not only molecular oxygen but also nitrogen oxides in absence of molecular oxygen for their respiration. Due to the facultative nature, those denitrifying bacteria have advantages over strict aerobic and anaerobic microorganisms in the removal of organic pollutants. Up to now, only two species of denitrifying bacteria, *Hyphomicrobium* sp. [12] and *Paracoccus* sp. T231 [13], have been reported as trimethylamine degraders. Here, we report four denitrifying trimethylamine-degrading bacteria that have been newly isolated from marine and other environments characterized with high salinity. The phylogenetic and physiological characteristics indicate that the isolates, which have not been reported previously, are halotolerant and/or halophilic trimethylamine-degrading microorganisms. They have versatile metabolic pathways for trimethylamine degradation, which has not observed in other denitrifying bacteria.

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2. Materials and methods

2.1. Medium and culture conditions

Mineral salts medium (MSM) was used both for enrichment and pure cultures of trimethylamine-degrading organisms. The medium contained 1 g K_2HPO_4, 2.6 g KH_2PO_4, 1.4 g $MgCl_2{\cdot}6H_2O$, 0.2 g NH_4Cl, 0.25 g KCl, 2.5 g $NaNO_3$, 30 g NaCl and 1 ml trace element mixture [14]. The pH of the medium was adjusted to 7.0–7.2. Strict anaerobic techniques were used for preparation of the anaerobic medium, aseptic handling, and sampling of denitrifying cultures as described in our previous report [13]. The anaerobic cultures were performed in 125-ml serum bottles containing 100 ml of liquid medium. Aerobic cultures were performed in 500 ml-Erlenmeyer flasks containing 100 ml of nitrate-free MSM in a shaker incubator at 150 rpm. All cultures were incubated in the dark at 30°C.

2.2. Isolation of trimethylamine-degrading bacteria

Coastal sediment samples were collected from two geologically different sites, Guryonpo and Pohang, Korea, and a wastewater sample was collected from an effluent of marine-fish market in Gampo, Korea. Each sample (10 g of sediment or 10 ml of wastewater) was anaerobically incubated in a serum bottle containing 90 ml of MSM with 10 mM of trimethylamine and 30 mM of nitrate. When the complete degradation of trimethylamine was observed, aliquot of culture (10 ml) was transferred and incubated in freshly prepared MSM (90 ml) with 10 mM trimethylamine. The serially diluted enrichment culture was streaked on MSM agar plates containing 10 mM of trimethylamine and incubated in an anaerobic jar with a CO_2–H_2 gas-generating system and palladium catalyst. After 2 weeks, each colony on the plates was transferred to new plates, and incubated them once again in the anaerobic jar to purify single colonies. Single colonies formed on the agar plates were transferred in serum bottles containing liquid medium with trimethylamine (10 mM) to confirm the trimethylamine-degrading ability under denitrifying conditions.

2.3. Characterization of isolates

Cell morphology was observed using a transmission electron microscopy after staining cells negatively with 1% (w/v) phosphotungstic acid. Gram stain, catalase and oxidase tests were performed in accordance with the procedures outlined in "Manual of Methods for General Bacteriology" [15]. For the phylogenetic analysis, chromosomal DNA was extracted and purified as described by Yoon et al. [16]. The 16S rDNA was amplified and sequenced according to the methods described by Yoon et al. [17]. Related sequences were obtained from the GenBank database by using the BLAST search program. Alignment of sequences was carried out with the CLUSTAL W software [18]. Gaps at 5′- and 3′-ends of the alignment were omitted from further analyses. Evolutionary distance matrices were calculated by using the algorithm of Jukes and Cantor [19] with the DNADIST program within the PHYLIP package [20]. A phylogenetic tree was constructed by using the neighbor-joining method [21] as implemented within the NEIGHBOR program of the same package.

2.4. Enzyme activity assay

Cells grown on a trimethylamine or acetate were washed

Table 1
Basic characteristics of marine trimethylamine-degrading isolates

Characteristics	Strain				
	GP43	PH32	PH34	GRP21	T231[a]
Morphology					
Size (μm)	1.7×1.1	1.5×1.2	1.5×1.0	1.6×1.0	1.5×0.8
Flagellation	no	no	no	no	no
Gram stain	−	−	−	−	−
Catalase/oxidase	−/+	+/+	+/+	+/+	−/+
Growth on[b]:					
Trimethylamine	+/+	+/+	+/+	+/+	+/+
Dimethylamine	+/+	+/+	+/+	+/+	+/+
Methylamine	+/+	+/+	+/+	+/+	+/+
Tetramethylammonium	+/+	+/+	+/+	+/+	−/−
N,*N*-Dimethylformamide	−/+	−/−	−/−	−/−	−/−*
Dichloromethane	−/−	−/±	−/±	−/−	−/−*
Thiocyanate	−/−	−/±	−/±	−/−	−/−*
Methanethiol	−/−	−/−	−/±	−/−	−/−*
Dimethyl sulfide	−/−	−/−	±/±	−/−	−/−*
Dimethyl disulfide	−/−	−/−	−/±	−/−	−/−*

+: growth in 1 week; ±: growth in 2 weeks; −: no growth in 2 weeks.
[a]Strain T231 was isolated in our previous study [13]. Substrate utility results determined in this study are marked with asterisk.
[b]The growth was determined under aerobic/anaerobic conditions after the incubation of isolates with a substrate (5 mM).

twice with 50 mM sodium phosphate buffer (pH 7.0), and re-suspended in 1 ml of the same buffer containing 1 mM of dithiothreitol. The cells were disrupted by sonication (Braun Biotech Int., France) at 20 kHz in an ice bath. After centrifuging at 25 000 × *g* for 20 min, clear supernatant was used as a crude enzyme. Trimethylamine-, dimethylamine- and methylamine monooxygenase activities were determined spectrophotometrically by measuring the oxidation of NADPH at 340 nm as described by Boulton et al. [4]. Trimethylamine *N*-oxide dimethylase activity was determined as described previously [13]. Trimethylamine-, dimethylamine- and methylamine dehydrogenase activities were determined spectrophotometrically by measuring the reduction of 2,6-chlorophenol-indophenol at 600 nm as described by Colby and Zatman [3].

2.5. Analytical methods

Methylated amines were analyzed in a gas chromatograph as described previously [13]. Ammonium concentration was determined by an enzyme test kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Nitrate and nitrite were analyzed by ion chromatography (model 790 personal IC, Metrohm, Switzerland) as described previously [13]. Nitrogen gas produced in the headspace of serum bottles was analyzed with the gas chromatograph equipped with a thermal conductivity detector and an ATTM-mole sieve plot GC column (30 m × 0.53 mm ID, Alltech, IL, USA). Protein concentration was determined by the method of Lowry et al. [22] with crystalline bovine serum albumin as standard.

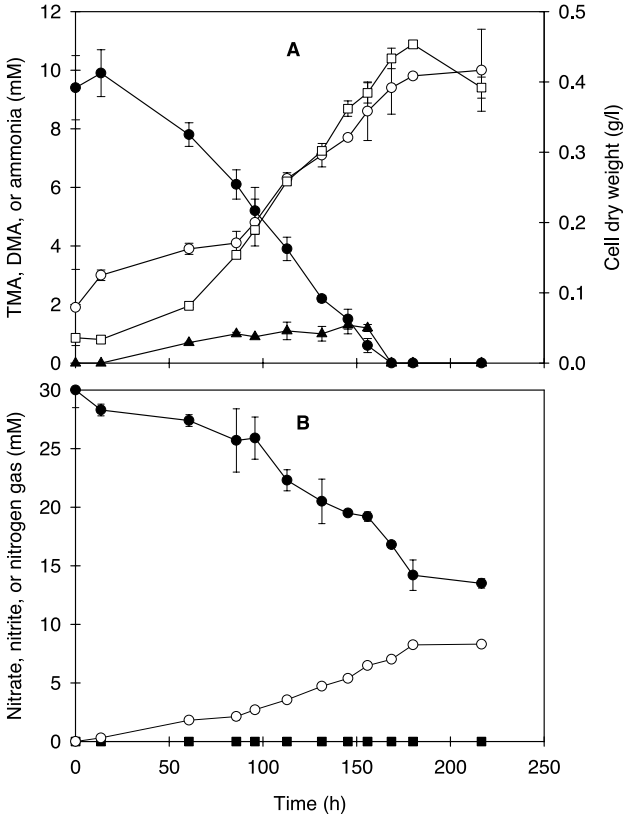

Fig. 1. Anaerobic degradation of trimethylamine by strain PH32 under denitrifying conditions. Error bars represent the standard error from duplicate cultures. Symbols in (A): ●, trimethylamine; ▲, dimethylamine; ○, ammonia; □, cell dry weight, and in (B): ●, nitrate; ○, molecular nitrogen; ■, nitrite. The concentration of molecular nitrogen is theoretical values estimated from the amount of molecular nitrogen in headspace of culture bottle.

3. Results and discussion

3.1. Isolation and characterization of trimethylamine-degrading isolates

Four trimethylamine-degrading strains were isolated from denitrifying enrichments established with three different environmental samples. Strains PH32 and PH34 were isolated from the coastal sediment in Pohang, Korea. Strains GRP21 and GP43 were isolated from the coastal sediment in Guryongpo, Korea and from the wastewater of marine-fish market wastewater in Gampo, Korea, respectively.

The basic characteristics of isolates were summarized in Table 1. All the isolates were rod, Gram-negative and non-motile organisms. No flagellum was found in their electron microscopy. All strains were oxidase positive and, except for strain GP43, all strains were also catalase positive. They grew well in pH 6.5–8.0. All strains grew on trimethylamine, dimethylamine, methylamine and tetramethylammonium under both aerobic and denitrifying conditions. Strain PH34 exhibited the broadest substrate utilization capability. To be specific, this strain grew on all the compounds tested except *N*,*N*-dimethylformamide under aerobic conditions. Strain PH34 was the only isolate grown on dimethyl sulfide under denitrifying conditions. GRP21 showed the narrowest substrate utilization ability among the strains. Those results indicate the physiological diversity of the isolates. The basic characteristics of the new isolates were compared with those of the previously reported trimethylamine-degrading bacterium, strain T231, which had been isolated from an activated sludge sample (Daejeon, Korea) [13]. The substrate utility property of strain T231 is distinct from those of the newly isolated strains. For example, it did not degrade tetramethylammonium while the newly isolated organisms degraded this compound under both aerobic and denitrifying conditions.

3.2. Anaerobic degradation of trimethylamine coupled with nitrate reduction

Fig. 1 shows the anaerobic degradation of trimethylamine by strain PH32 under denitrifying conditions. The strain completely degraded 10 mM trimethylamine in 170 h (Fig. 1A). Dimethylamine transiently appeared and then

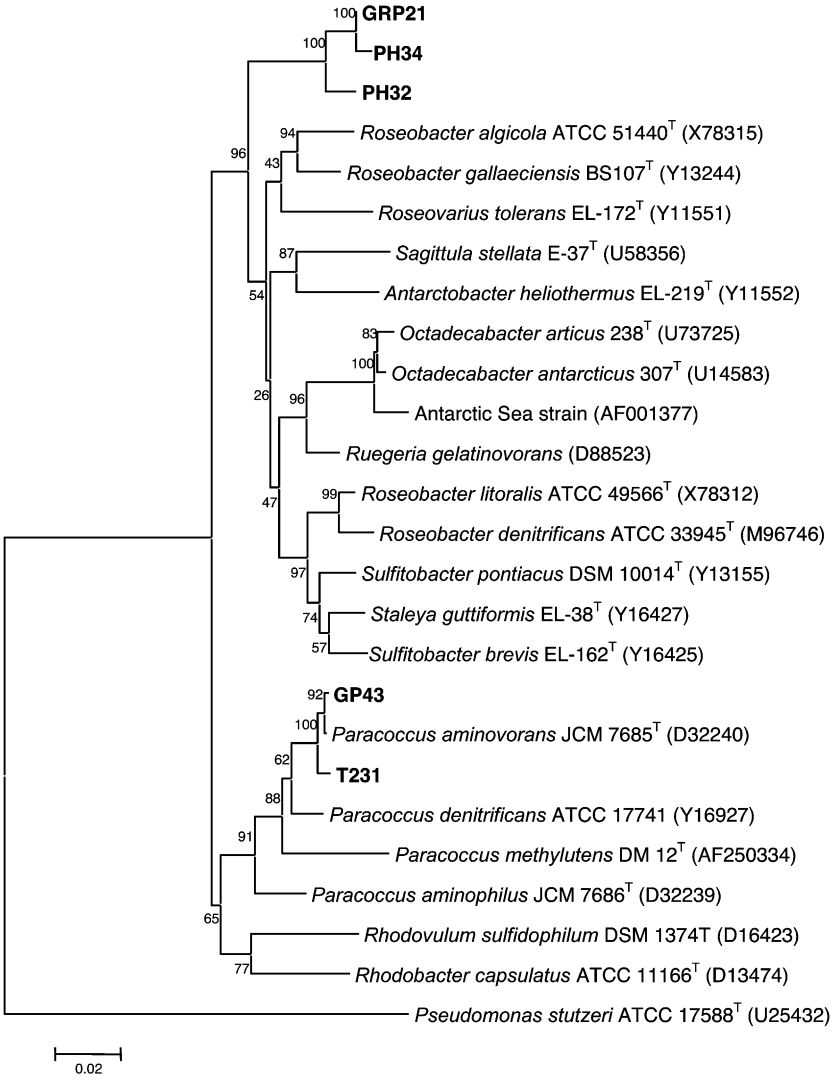

Fig. 2. Phylogenetic tree based on 16S rDNA sequence analysis data using a neighbor-joining method and a 1000 bootstrap for the confidence level. The bar represents 0.02 substitutions per nucleotide.

disappeared whereas ammonia was accumulated during the degradation of trimethylamine. This observation suggests that trimethylamine was dimethylated via dimethylamine to ammonia in the trimethylamine metabolism. On the other hand, the nitrate was concomitantly consumed during the degradation of trimethylamine, and molecular nitrogen (N_2) was accumulated (Fig. 1B). The emission of molecular nitrogen provides the direct evidence that the anaerobic degradation of trimethylamine was coupled with respiratory nitrate reduction. Nitrite, an intermediate of nitrate reduction, was not detected. By the complete degradation of trimethylamine (10 mM), 17 mM of nitrate was consumed and 8 mM of molecular nitrogen was accumulated. Considering the stoichiometric conversion of nitrate to molecular nitrogen, the gaseous intermediates, NO and N_2O, was not likely to be accumulated quantitatively. The anaerobic degradation coupled with nitrate reduction was also found for other isolates. Strains GP43, GRP21 and PH34 degraded 10 mM trimethylamine, reducing 16–20 mM of nitrate to 7.6–9.4 mM of molecular nitrogen.

3.3. Phylogenetic position of isolates

To analyze the phylogenetic position, the 16S rDNA sequences of isolates (more than 1200 bp) were determined. Fig. 2 showed the phylogenetic relationship between the isolates and other related microorganisms found in the GenBank database. The homology assay result indicated that all the isolates were in the phylogenetic branch of the α-*Proteobacteria*. Strain GP43 was affiliated with the genus *Paracoccus*, the closest database relative of which was *Paracoccus aminovorans* JCM 7685^T with 99.9% sequence similarity. This strain also showed a high sequence similarity (99.3%) with strain T231 [13]; thereby strains GP43, T231 and JCM 7685^T were placed in a single cluster. In spite of the high sequence similarity, strain GP43 is distinguished from strain T231 in some

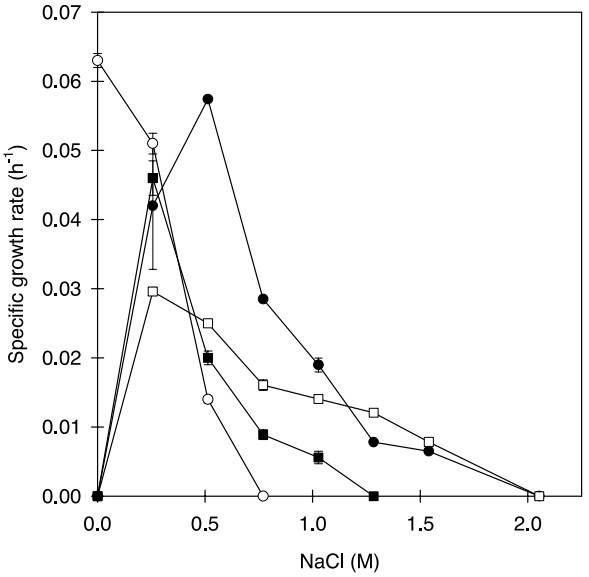

Fig. 3. Specific growth rate of isolates at various concentrations of sodium chloride under denitrifying conditions. Trimethylamine (10 mM) was used as the sole carbon source for the cultures. Error bars represent standard error from duplicate cultures. Symbols: ○, strain GP43; ●, strain GRP21; □, strain PH32; ■, strain PH34.

phenotypic characteristics as indicated by the results in Table 1 and in halotolerance as discussed in the next section. In this point, it is not certain if strains GP43 and T231 can be classified into the same species because of insufficient data such as other genotypic and chemotaxonomic characteristics.

On the other hand, strains GRP21, PH32 and PH34 were clustered on a branch that was quite remote from other genera in the α-*Proteobacteria*. No species in the α-*Proteobacteria* were higher than 95% in sequence similarity with the three isolates. The separated phylogenetic relationship and the low sequence homology suggest that the three isolates would be novel organisms that may be deposited into a novel genus. The 16S rDNA sequences of strains GP43, PH32, PH34 and GRP21 were deposited in the GenBank database under accession number AJ505785, AJ505786, AJ505787 and AJ505788, respectively.

3.4. Halotolerance of isolates

One particular characteristic of the isolates is that they could grow on a medium containing 3% sodium chloride (w/v). In order to determine their halotolerant properties, the specific growth rate of each strain was estimated at different concentrations of sodium chloride under denitrifying conditions using 10 mM of trimethylamine as the growth substrate. Strains GRP21, PH32 and PH34 did not grow in the absence of sodium chloride and exhibited the maximum growth rate at 0.25–0.50 M of sodium chloride (Fig. 3). The results indicated that they are halophilic organisms that strictly require sodium chloride for the growth. Their growth was sustained in 1.0–1.5 M of sodium chloride, but not in higher concentrations (Fig. 3). On the other hand, strain GP43 showed the highest growth rate in the absence of sodium chloride, and its growth rate decreased gradually as the concentration of sodium chloride increased (Fig. 3). This result indicated that strain GP43 is a halotolerant organism that is capable of growing in a saline environment, but it does not strictly require sodium chloride for its growth. It is valuable to compare the halotolerance and growth rate of strain GP43 with those of strain T231, which is closely related to strain GP43 phylogenetically (Fig. 2). Strain T231 grew well in the absence or in the presence of 0.12 M sodium chloride, but did not grow in the presence of 0.5 M of sodium chloride (data not shown). In this aspect, strain T231 is less halotolerant than strain GP43. In the absence of sodium chloride, the specific growth rate of strain T231 was

Table 2
Enzyme activities in crude enzymes from trimethylamine-degrading isolates grown on trimethylamine under aerobic- or anaerobic conditions

Strain	Enzyme	Enzyme activity (mU mg^{-1} protein) in cells grown on TMA	
		Anaerobically	Aerobically
GRP21	TMA monooxygenase	–	41.2 ± 12.7[a]
	TMA *N*-oxide dimethylase	–	72.4 ± 3.8
	DMA monooxygenase	–	53.9 ± 12.7
	MA monooxygenase	–	47.6 ± 9.5
	TMA dehydrogenase	22.0 ± 9.0	96.7 ± 27.0
	DMA dehydrogenase	12.8 ± 3.6	–
	MA dehydrogenase	118.5 ± 12.0	–
PH32	TMA monooxygenase	–	72.3 ± 2.5
	TMA *N*-oxide dimethylase	–	21.7 ± 6.5
	DMA monooxygenase	–	39.9 ± 1.2
	MA monooxygenase	–	–
	TMA dehydrogenase	180.0 ± 12.0	43.0 ± 4.4
	DMA dehydrogenase	26.0 ± 1.2	73.6 ± 2.5
	MA dehydrogenase	–	–
PH34	TMA monooxygenase	–	26.7 ± 2.1
	TMA *N*-oxide dimethylase	–	123 ± 18
	DMA monooxygenase	–	32.8 ± 2.1
	MA monooxygenase	–	24.6 ± 2.1
	TMA dehydrogenase	96.4 ± 1.6	131.3 ± 0
	DMA dehydrogenase	34.9 ± 5.3	–
	MA dehydrogenase	–	–
GP43	TMA monooxygenase	–	10.1 ± 0
	TMA *N*-oxide dimethylase	–	47.2 ± 8.4
	DMA monooxygenase	–	10.8 ± 0.7
	MA monooxygenase	–	11.2 ± 0.4
	TMA dehydrogenase	106.6 ± 15.2	118.6 ± 5.8
	DMA dehydrogenase	49.2 ± 2.0	11.6 ± 5.8
	MA dehydrogenase	9.0 ± 2.5	–
T231[b]	TMA monooxygenase	–	48
	TMA *N*-oxide dimethylase	ND[c]	183
	DMA monooxygenase	ND	221
	MA monooxygenase	ND	35
	TMA dehydrogenase	23	–
	DMA dehydrogenase	6.3	–
	MA dehydrogenase	ND	ND

[a]Mean ± standard deviation.
[b]The results were adopted from [13].
[c]ND: not determined.

0.12 h^{-1}, which is two-fold higher than that of strain GP43 (0.06 h^{-1}).

3.5. Enzymes involved in the metabolism of trimethylamine

In order to examine if there is diversity in the trimethylamine metabolism of isolates, some key enzymes implicated in microbial trimethylamine metabolism [23] were assayed in crude enzymes obtained from cells grown on trimethylamine under aerobic and denitrifying conditions. To examine whether these enzymes are inducible by trimethylamine, their activities were also assayed in the cells grown on acetate under aerobic and denitrifying conditions as a control experiment. The enzymes catalyzing trimethylamine metabolism were detected in cells grown on trimethylamine (Table 2), but were not in cells grown on acetate, suggesting that these enzymes were induced by trimethylamine. Both trimethylamine and dimethylamine dehydrogenase activities were observed in all strains grown under denitrifying conditions, but methylamine dehydrogenase activity was found only in strain GRP21 and GP43. The results suggest that strain GRP21 and GP43 oxidize methylamine, the resulting product from trimethylamine by two successive activities of trimethylamine and dimethylamine dehydrogenase, to formaldehyde and ammonia using methylamine dehydrogenase while strains PH32 and PH34 oxidize the methylamine via other pathways that were not examined in this study.

On the other hand, the results indicate that more versatile enzymes were induced in aerobically grown cells. All the strains possessed two different enzymes for the oxidation of trimethylamine to dimethylamine, namely trimethylamine monooxygenase and dehydrogenase. Strains PH32 and GP43 have both dimethylamine dehydrogenase and dimethylamine monooxygenase, which oxidize dimethylamine, to methylamine, whereas strain PH34 and GRP21 possess only dimethylamine monooxygenase. Methylamine monooxygenase activity was found in strains GRP21, PH34 and GP43, but not in PH32. Previously, the aerobic trimethylamine metabolism in denitrifying bacteria was elucidated only in two strains, *Hyphomicrobium* sp. X [12] and *Paracoccus* sp. T231 [13]. Strain X possessed trimethylamine dehydrogenase and strain T231 had trimethylamine monooxygenase for aerobic degradation of trimethylamine. Therefore, it is a quite novel observation that our isolates possessed both trimethylamine dehydrogenase and trimethylamine monooxygenase for the oxidation of trimethylamine under aerobic conditions.

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