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Mami Yamamoto · Akihiro Ishii · Yuichi Nogi
Akira Inoue · Masahiro Ito

Isolation and characterization of novel denitrifying alkalithermophiles, AT-1 and AT-2

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Abstract Two novel denitrifying alkalithermophilic bacteria, AT-1 and AT-2, were isolated from manure-amended soil. The isolates grew at 35–65°C with an optimum temperature at 50–60°C, and pH 6.5–10.0 with an optimum pH at 9.5. Both isolates were Gram-positive, facultative anaerobic, non-motile rod-shaped bacteria. A phylogenetic analysis based on 16S rRNA sequence data indicated that both AT-1 and AT-2 are members of the genus *Anoxybacillus*. DNA-DNA hybridization revealed moderate relatedness between AT-1 and AT-2 and one phylogenetically related strain, *A. pushchinensis* K1 (69.5 and 69.1%, respectively). Comparative analysis of morphology and biochemical characteristics of the two isolates also showed similarity to *A. pushchinensis* K1. Based on these results, we identified AT-1 and AT-2 as *A. pushchinensis*. To our knowledge, this is the first report of denitrifying bacterium isolated from alkalithermophilic *Anoxybacillus* spp.

Keywords Alkalithermophiles · Denitrification · *Anoxybacillus pushchinensis* · Extremophiles · Thermophiles · Alkaliphiles

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M. Yamamoto · A. Ishii
REDS Group (Saitama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST), Saitama Small Enterprise Promotion Corporation, 3-12-18 Kamiaoki, Kawaguchi, Saitama 333-0844, Japan

M. Yamamoto · A. Ishii
Faculty of Life Sciences, Toyo University, Oura-gun, Gunma 374-0193, Japan

Y. Nogi
Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka 237-0061, Japan

A. Inoue · M. Ito (✉)
Faculty of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura-machi, Oura-gun, Gunma 374-0193, Japan
e-mail: ito1107@toyonet.toyo.ac.jp
Tel.: +81-276-829202
Fax: +81-276-829202

Abbreviations HPLC: High performance liquid chromatography · GP: Genome profiling · NED: N-(1-naphthyl)-ethylenediamine · PCR: Polymerase chain reaction

Introduction

Denitrification is reduction of nitrates to nitrites, nitrous oxides or dinitrogen (N_2) catalyzed by facultative aerobic soil bacteria under anaerobic conditions. Denitrification can be an ecologically important drain of nitrogen from the soil. Denitrification occurs in the soil when conditions become anaerobic (e.g., in waterlogged soil), and during composting and sludge digestion (Casella and Payne 1996). Denitrification is greatly influenced by environmental factors, such as pH, temperature, nutrition, and dissolved oxygen, and it is considered that the optimal condition is generally neutral pH and mesophilic temperature. Moreover, it is also known that the rate of denitrification is dependent on temperature. Denitrification by *Pseudomonas aerugionsa* and *Paracoccus denitrificans* has been well studied, espceially in the former (Carlson and Ingraham 1983). These bacteria are neutralophilic and mesophilic. On the other hand, novel denitrifying bacteria that can grow at high temperature or in an alkaline environment have been reported in recent years. Nazina et al. reported that facultative aerobic thermophiles *Geobacillus subterraneus* and *Bacillus thermoleovorans* isolated from soils are capable of growth from 45 to 70°C at pH 6.2–7.8 and show anaerobic denitrifying phenomena (Nazina et al. 2001). Moreover, a large number of mesophilic *Halomonas* species perform denitrification under anaerobic conditions between neutral to alkaline pH (Berendes et al.1996; Mormile et al. 1999; Mata et al. 2002; Martinez-Canovas et al. 2004). In recent years, alkalithermophiles that are adapted to the two extreme conditions of high temperature and high pH were isolated (Pikuta et al. 2000; Belduz et al. 2003; Pikuta et al. 2003; Clerck

et al. 2004; Dugler et al. 2004; Yumoto et al. 2004; Wiegel and Kevbrin 2004). However, these alkalithermophiles showed no denitrifying phenomena.

Denitrifying bacteria that belong to the genus *Pseudomonas* and *Alkaligenus* are already utilized for sewage disposal or waste treatment (Casella and Payne 1996). It is a process in which the carbon and nitrogen ingredients are contained abundantly, and composts stock raising waste producing alkaline pH and high temperature is known (Pedro et al. 2001). This process includes denitrification. To isolate novel denitrifying bacteria from such extreme conditions would extend the application of biological denitrification into other fields. Therefore, we tried to screen novel alkalithermophilic bacteria from various locations that contain composting plants and domestic animal dung.

In this paper, we present the first report of denitrifying bacteria isolated from alkalithermophilic *Anoxybacillus* spp.

Materials and methods

Culture media, enrichment and isolation procedure

Samples of compost and soil were collected at various regions in Japan. Isolation was performed using a nitrate medium, which contained (per liter) Polypepton-S (Nippon Pharmaceutical, Fukuoka, Japan), 3.0 g; yeast extract (Difco), 0.2 g; KNO_3, 0.1 g; the trace element solution SL10 (without H_3BO_3, $NiCl_2$) (Widdel et al. 1983), 1 ml. After the medium was autoclaved for 15 min at 121°C, the pH of media was adjusted to ten with sterile 10% Na_2CO_3. 8 ml aliquots of the medium in 12 ml screw-cap test tubes with an inverted Durham tube were inoculated with 0.5 g samples. The enrichment culture was incubated for 3 days at 60°C. After incubation, test tubes showed positive reactions as indicated by the turbidity due to bacterial growth and gas formation in the inverted Durham tubes. 10% (v/v) transfers inoculated from tubes considered exhibiting positive reactions were used in the subcultures. After three subcultures under the same conditions, the strains were purified by repeatedly isolating single colonies in agar plates containing the same medium plus 2.0% agar (Nacalai Tesuque, Inc., Kyoto, Japan). The plates were incubated for 1–2 days at 50°C.

Anoxybacillus flavithermus NBRC 15317(=DSM 2641) and *A. pushchinensis* ATCC 700785(=DSM 12423) were used as reference strains for DNA-DNA relatedness.

Genome profiling

Genome profiling was performed by the method of Nishigaki et al. (2000). Random PCR was carried out with pfM12 primer (dAGAACGCGCCTG). Micro TGGE was conducted in a μTG (Taitec, Saitama, Japan).

Biochemical characterization

The temperature range and pH for growth were determined in PSY medium that contained Polypepton-S (Nihon Pharmaceutical, Tokyo, Japan), 3.0 g; yeast extract (Difco), 0.2 g. The medium was adjusted to the desired pH values (pH 6.0–10.0) by adding 10% Na_2CO_3 sterile stock solution. The strains were subcultured once under the same conditions prior to determination of growth rates. The temperature range for growth at pH 9.5 was determined in PSY medium in which the temperature ranged from 30 to 70°C.

Carbohydrate assimilations were performed with an API 50 CH (bioMérieuzsa, Marcy-l'Etolile, France) system using nutrient medium adjusting the pH to 8.2. The presence of oxidase, catalase, and the production of endospores were determined by following the methods in Barrow and Feltham (1993). Anaerobic growth was tested on a nitrate medium by using the AnaeroPack/AnaeroPouch System (Mitsubishi Gas Chemical, Tokyo, Japan).

When denitrification studies were performed, the isolate was cultivated in the nitrate medium. The presence of nitrate and nitrite were tested using the modified Griess reagent (Doane and Horwath 2003). Nitrate is reduced to nitrite in the presence of Vanadium (III) trichloride. The nitrite concentration is determined by diazotizing with sulfanilamide and coupling with NED dihydrochloride to form a colored azo dye. Measurements were taked at 540 nm.

Gas analysis

Gas samples were withdrawn from the headspace of denitrifying cultures. Gas samples were analyzed using a gas chromatograph (SimazuGC-14AT) equipped with a thermal conductivity detector (100 mA). Samples (0.25 ml) were injected onto a Molecular Sieves 5A (60–80 mesh) column and eluted with helium. Temperatures were as follows; injector, 100°C; column, 50°C.

Extraction of genomic DNA and determination of G+C content

DNA was extracted and purified by the method of Marmur (1961). The DNA was enzymatically digested, and G+C content was determined by separating the nucleotides by HPLC.

DNA-DNA hybridization method

DNA-DNA hybridization was performed using a modification of the microplate method of Ezaki et al. (1989) with photobiotin-labeled DNA probes and microplates. A hybridization temperature of 42°C (calculated with correction for the presence of 50% formamide) was used.

DNA characterization and sequence of 16S rRNA genes

The 16S rRNA was amplified by PCR with the following forward and reverse primers: pA, 5'-AGAGTTTGATCCTGGCTCAG (corresponding to positions 49–69 of *Escherichia coli* 16S rRNA) and pH, 5'-AAGGAGGTGATCCAGCCGCA (positions 926–907) (Edward et al. 1989). The 16S rRNA genes were amplified with KOD plus DNA polymerase (TOYOBO, Osaka, Japan). The PCR products were separated by agarose gel electrophoresis and were purified using the Qiagen PCR purification kit (Qiagen, Valencia, California, USA). Purified PCR products were sequenced with 16S primers: pA; pH; 350f, 5'-CCTACGGGAGGCAGCAGT (positions 329–350) (Hou and Dutta 2000); 907r, 5'-CCGTCAATTCMTTTRAGT TT (positions 927–907) (Muyzer et al. 1995). The 16S rRNA sequences were performed by Hokkaido System Sciences (Hokkaido, Japan), using an ABI-100 model 377 sequencer. The resulting sequences were assembled to produce a 1487 base contiguous 16S rRNA sequence. The assembled 16S rRNA sequences were aligned with equivalent 16S sequences of all closely related strains found in the GenBank database via a BLAST search and aligned using Clustal W. *B. subtilis* 168 16S rRNA sequence (GenBank accession number AF008220) as outgroup. The phylogenetic tree was calculated with the neighbor-joining method in the PHYLIP package (Felsenstein 1993), and rendered as graphics using TreeView (Page 1996). To evaluate the robustness of the inferred tree, the bootstrap resampling method of Junkes–Cantor was used with 100 replicates.

Results and discussion

Isolation

Several isolates that could grow at 60°C and pH10 were obtained from samples of composts in Itakura, Gunma, Japan and were tested for their ability to reduce nitrate and production of gas. Only one could reduce nitrate and produce gas under high temperature and alkaline conditions. More than 50 strains of alkalithermophiles were isolated from the sample. Ten strains were chosen at random and these were used for the next experiment. Seven out of ten strains showed the ability to reduce nitrate and produce gas. These seven strains were analyzed by the Genome profiling (GP) method. This is a method that identifies genomic DNA fragments common to closely related species without prior knowledge of the DNA sequence (Nishizaki et al. 2000; Naimuddin et al. 2002). The seven strains were categorized into two groups according to results of gel patterns of the GP method (Fig. 1). One strain was selected from each group and named AT-1 and AT-2, respectively. These strains were used in future experiments.

Morphology

Cells of each strain were rods with a length of 2.0–3.0 μm and a width of about 0.7–0.8 μm for AT-1, and with a length of 1.5–2.0 μm and a width of about 0.7–0.8 μm for AT-2. Colonies were white to cream colored. Cells formed endospores.

16S rRNA sequence analysis

The 1455 bp 16S rRNA sequences of strain AT-1 and AT-2 were determined. The 16S rRNA sequences of the two strains were 100% similar to each other. Based on the evolutionary distances, strain AT-1 and AT-2 belong to the diverse genus *Anoxybacillus*. The 16S rRNA sequences of the two strains were above 99% similar to *A. pushchinensis* and *A. flavithermus* (Fig. 2). The nucleotide sequences of strain AT-1 and AT-2 have been deposited in the GenBank database under the accession number AB234213 and AB234214, respectively.

Comparison of the phylogenic and phenotypic properties

The results of comparison of the phylogenic and phenotypic properties of strains AT-1, AT-2, *A. pushchinensis* type stain NBRC 15317 and *A. flavithermus* type

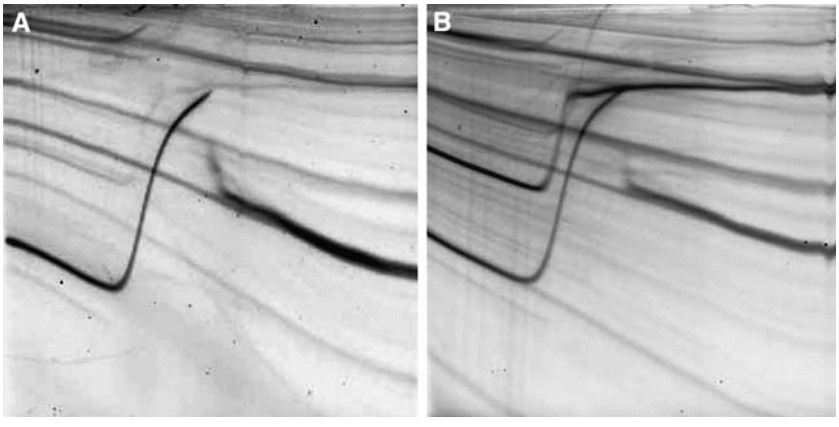

Fig. 1 Genome Profiling of strains AT-1 and AT2. Two typical gel patterns of seven strains were obtained using primers pfM12. a AT-1 type gel pattern of three strains. b AT-2 type gel pattern of four strains

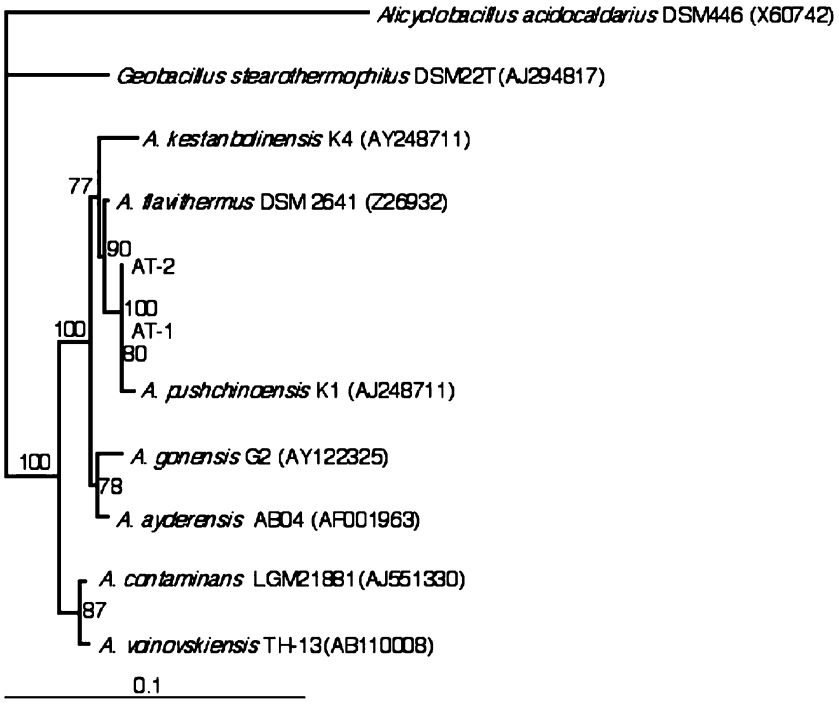

Fig. 2 Phylogenetic tree of representative *Anoxybacillus* species inferred from 16S rRNA gene sequences, using the neighbor-joining method. The *scale bar* indicates 0.1 substitutions per nucleotide position. Numbers on the branches are bootstrap values (expressed as percentages of 100 replications) estimated by a bootstrap analysis performed with 100 replicates

strain ATCC 700785 are shown in Table 1. Growth of strains AT-1 and AT-2 occurred within the temperature range of 35–65°C, with an optimum at 50–60°C. The optimum pH for growth was 9.5 with growth occurring between pH 6.5 and 10.0 (Table 1). Strains AT-1 and AT-2 are alkalithermophiles similar to *A. pushchinensis* and *A. flavithermus*.

There are many phenotypic characteristics common to *A. pushchinensis*. These characteristics include rod-shaped cells; colony color ranging from white to yellow; catalase negative; utilization of carbon sources (D-glucose, sucrose and starch); the ability to reduce nitrate to nitrite; and negativity for the test of hydrolysis of gelatin. Strains AT-1 and AT-2 were found to possess all these characteristics. Other characteristics (tolerance to 3% NaCl and hydrolysis of casein) of the two strains were different from *A. pushchinensis*.

Both G + C contents of genomes of strains AT-1 and AT-2 were 41.6 mol%. This value was close to the values of *A. pushchinensis* and *A. flavithermus*. DNA-DNA hybridization was performed to determine whether strains AT-1 and AT-2 were members of the taxon *A. pushchinensis*. Using DNA of strain ATCC 700785 or NBRC 15317 as reference, similarity values of strains AT-1 and AT-2 to ATCC 700785 were 69.5 and 69.8%, while lower relatedness was found with the *A. flavi-*

Table 1 Physiological and biochemical properties of strains AT-1, AT-2 and other *Anoxybacillus* sp

Characteristic	1	2	3[a]	4[a]
Cell morphology	Rod	Rod	Rod	Rod
Spore formation	+	+	+	+
DNA G-C (mol%)	41.6	41.6	42.2	42.3
Temperature (°C):				
Range	30–65	30–65	37–66	30–72
Optimum	50–60	50–60	62	60–65
pH:				
Range	6.5–10.0	6.5–10.0	8.0–10.5	5.5–9.0
Optimum	9.5	9.5	9.5–9.7	7
Tolerance to 3% NaCl	-	-	+	-
Substrate:				
Glucose	+	+	+	-
Sucrose	+	+	+	-
Starch	+	+	+	-
Lactate	-	-	-	+
Catalase activity	-	-	-	+
Oxidase activity	+	-	ND	ND
Nitrate reduction	+	+	+	-
Hydrolysis of gelatin	-	-	-	+
Hydrolysis of casein	+	+	-	-

Strains: 1 AT-1, 2 AT-2, 3 *Anoxybacillus pushchinensis* strain DSM 12423^T, 4 *Anoxybacillus flavithermus* DSM 264^T + Positive reaction or growth,– No reaction or growth, ND No data available[a]Data from literature: Pikuta et al. (2000).

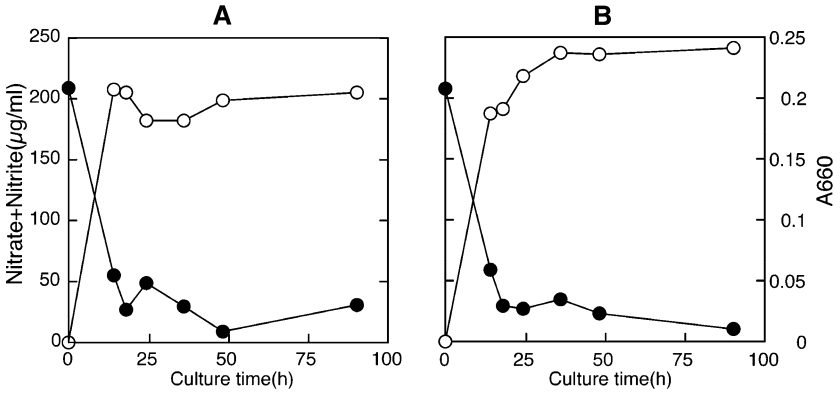

Fig. 3 Changes in cell density and nitrate plus nitrite contenst in batch cultures. Changes over time in cultures of strain AT-1 (*panel A*) and AT-2 (*panel B*). Experiments were carried out as described in Materials and Methods at 60°C and pH10.0. The headspace was filled with air. *Symbols* indicate profiles of cell density (*open circule*), nitrate + nitrite (*filled circle*)

thermus NBRC 15317 (52.0 and 47.4% similarity). In addition, AT-1 and AT-2 showed 100% similarity in DNA-DNA hybridization. Based on their morphological, physiological, biochemical profiles, 16S rRNA sequences, and DNA-DNA hybridization, we identified them as *A. pushchinensis*, even though the percent hybridization of them with *A. pushchinensis* is borderline low for confidently describing these strains as members of that species.

The ability to denitrify

Results from the analysis of the gas produced by strains AT-1 and AT-2 indicated that more than 99% of the composition of the gases was nitrogen gas. The nitrate and nitrite concentration in the culture medium was reduced rapidly at the initial incubation by AT-1 and AT-2 from 200 μM to 26–28 μM after18 h (Fig. 3). These results demonstrate that AT-1 and AT-2 are denitrifying bacteria that can produce nitrogen gas from nitrate.

The newly isolated alkalithermophilic bacteria AT-1 and AT-2 are identified as *A. pushchinensis* and denitrifying bacteria. Anaerobic denitrifying thermophiles (Manachini et al. 2000; Mevel and Prieur 2000) and a denitrifying alkaliphile (Mormile et al. 1999) have been reported. However, this is the first report of a denitrifying bacterium isolated as an alkalithermophile. The occurrence of denitrification to nitrogen gas in *Anoxybacillus* species has never been reported before the present study. We tested *A. pushchinensis* K1 for the ability to perform denitrification in the same culture conditions as AT-1 and AT-2 and did not observe gas bubbles (via denitrification). AT-1 and AT-2 are the first example of a denitrification phenotype of *Anoxybacillus* spp.

AT-1 and AT-2 were isolated from manure-amended soil and *A. pushchinensis* K1 was isolated from manure. Many alkalithermophiles have been isolated from sewage sledges (Wiegel and Kevbrin 2004). Some studies have reported the presence of thermophilic bacteria in hot compost (Blanc et al. 1997). Thermophilic *Anoxybacillus* spp. may be important for denitrification of manure and composts.

The genes concerning denitrification by mesophiles such as *Pseudomonas* or *Paracoccus* sp. have been well studied. While a recent review (Philippot et al. 2002) shows that there are few denitrification genes identified in the genome of *Bacillus* sp. and a nitric oxide reductase similar to *Psudomonas* sp. has not been found yet in the genome of *Bacillus* sp. The denitrification system of *Bacillus* sp. may be different from Gram-negative bacteria. Zumft (2005) showed a relationship between Nitric oxidase (NOR) and stress response. The denitrification system of AT-1 or AT-2 may be related to the growth at high temperature and alkali pH. Furthermore, analysis of the denitrification of *Anoxybacillus* is also important to the application of nitrate removal systems.

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