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Isolation and Characterization of Thermotolerant Bacterium Utilizing Ammonium and Nitrate Ions under Aerobic Condition

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Keywords Ammonium nitrate · Thermotolerant bacterium · *Bacillus licheniformis* · Denitrification · Ferrous ion · Aerobic condition

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Abstract A thermotolerant bacterium, strain T-7-2, was isolated from a rice field and identified as *Bacillus licheniformis*. This strain completely utilized NH_4^+ and NO_3^- at 30 and 50°C under aerobic condition at controlled Fe^{2+} concentration. The conversion of total nitrogen was analyzed in the cells and culture supernatant of strain T-7-2. Twenty-four percent of the total nitrogen originally provided was observed in the cells, 20% of which was in culture supernatant at 50°C. Residual nitrogen (56% of total nitrogen) was removed from the culture. The cell extract contained enzymes involved in denitrification. GC-MS demonstrated that $\text{NH}_4^{15}\text{NO}_3$ was converted to $^{15}\text{N}_2\text{O}$. These results indicate that strain T-7-2 has denitrification ability under aerobic condition.

Introduction

As countermeasures against eutrophication in lakes, wetlands, and enclosed sea areas, the removal of nitrogen compounds from industrial and domestic wastewaters has become an urgent subject. Environmental Quality Standards for water pollutants were established to maintain the quality of environmental waters and to prevent eutrophication under the fundamental environment law (Ministry of the Environment 2005). A fundamental law concerning nitrogen compounds was enacted with the aim of controlling of BOD, COD, and DOES.

Two processes are thought to be involved in the microbial removal of NH_4^+ and NO_3^- : NH_4^+ oxidation to NO_3^- by ammonia-oxidizing bacteria under aerobic condition and NO_3^- denitrification by denitrifying bacteria under anaerobic condition (Bock et al. 1992; Zumft 1992). In this removal processes, we have to prepare two batch processes under aerobic and anaerobic conditions and set number of bacteria. However, recent research has shown that some bacteria can simultaneously perform heterotrophic nitrification and aerobic denitrification, and convert nitrogen compounds to denitrified products such as N_2O or N_2 under aerobic condition (Frette et al. 1997; Joo et al. 2005; Lukow and Diekmann 1997; Wehrfritz et al. 1993). Such heterotrophic bacteria would be useful and manageable in removing NH_4^+ and NO_3^- under aerobic condition in a wastewater treatment system. They could be utilized to reduce the cost of maintaining an anoxic tank or reduce its size. We have attempted to isolate heterotrophic microorganisms that utilize both NH_4^+ and NO_3^- as nitrogen sources in a medium containing NH_4NO_3 . *Klebsiella pneumoniae* strain F-5-2 actually performs heterotrophic nitrification and aerobic denitrification (Kim et al. 2002). However, mesophilic bacteria including strain F-5-2 cannot grow well at more than 40°C. Different types of strain that can grow well and remove NH_4^+ and NO_3^- at 30 to 50°C are also required for application.

Here, we report the isolation and identification of a soil bacterium that can utilize NH_4NO_3

at 30 and 50°C. The features of the removal of nitrogen compounds and denitrification by the isolate are described.

Materials and methods

Isolation of microorganism that can utilize NH_4^+ and NO_3^-

The screening medium (ammonium nitrate medium) containing 3.0% (w/v) D-glucose, 0.1% (w/v) NH_4NO_3 (12.5 mM), 0.1 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 8 pM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ was prepared as reported previously (Kim et al. 2002). Soil samples from rice fields and farms in Hyogo and Osaka, Japan and in Chiang Mai, Thailand were used as isolation sources. One gram of soil was suspended in 0.8% (w/v) NaCl solution, and then one ml of the supernatant was transferred to 0.1% (w/v) ammonium nitrate medium. Incubation was performed in a tube (18×180 mm) capped with a butyl rubber stopper at 50°C without shaking. From the culture, that showed a loss of NH_4^+ and NO_3^- without NO_2^- accumulation, one drop was taken and spread on an ammonium nitrate medium plate. The plate was incubated at 50°C. Organisms that grew were transferred to ammonium nitrate solid medium in a tube capped with a butyl rubber stopper.

Cultural condition

B. licheniformis strain T-7-2 was cultivated on 0.1% (w/v) ammonium nitrate medium containing 0.5 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ under aerobic condition, in order to examine the effects of various factors on the removal of NH_4^+ and NO_3^- and to study denitrification. TAITEC bio-shaker BR-41FL (Tokyo, Japan) was used throughout this study. Shaking speed was fixed at 140 rpm (swing of 25 mm), unless otherwise specified. Ten milliliter-vials (Maruemu Corporation, Tokyo, Japan) capped with a butyl rubber stopper and an aluminum seal, test tubes (18×180 mm), and 500-ml flasks were used. Preculture was performed for 36 h using a test tube; 1 ml of preculture was inoculated into 100 ml of fresh 0.1% (w/v) ammonium nitrate medium.

Morphological and phenotypic characterization

Strain T-7-2 was identified on the basis of morphological and biochemical characteristics using methods described previously (Komagata 1985) and by analysis of the 16S rRNA gene, amplified following the method of Edwards et al. (1989).

Analytical methods

The NH_4^+ , NO_3^- , and NO_2^- concentrations in the culture were measured by the indophenol

blue method (Weatherburn 1967), micro-salicylate method (Bhanadari 1989), and diazo-coupling reaction method (Aoki et al. 19881), respectively. Kjeldahl digestion and steam distillation were performed according to the attached manual by BÜCHI Labortechnik AG to analyze total nitrogen in the culture supernatant and cells. The amino nitrogen of organic materials and free ammonia is converted to NH_4^+ by using the Kjeldahl digestion unit K-424 (BÜCHI Labortechnik AG, Zürich, Switzerland). The obtained NH_4^+ is distilled from the digested solution and dissolved in boric acid solution using the distillation unit K-342 (BÜCHI Labortechnik AG). NH_4^+ was potentiometrically measured using the pH and conductivity combination meter HM-25R (DKK-TOA Corporation, Tokyo, Japan). N_2O gas production was monitored using a Hitachi G-3900 gas chromatograph equipped with a photon ion detector. A stainless steel column (3 mm $\times$ 2 m) packed with Porapak Q 50/80 mesh (GL Science, Tokyo, Japan) was used at 40°C. $^{15}\text{N}_2\text{O}$ and $^{15}\text{N}_2$ were measured on a Hitachi M-2500 mass spectrometer equipped with a Hitachi G-3000 gas chromatograph (Kim et al. 2002). Nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase were assayed according to published procedures (Aoki et al. 1981; Kakutani et al. 1981; Heiss et al. 1989; Kristjansson and Hollocher 1980).

Chemicals

$\text{NH}_4^{15}\text{NO}_3$ and $^{15}\text{NH}_4\text{NO}_3$ (98.0 atom %) were purchased from Sigma-Aldrich. $^{15}\text{N}_2$ (99.6 atom %) was from Shoko Co. (Tokyo, Japan); N_2O (99.5 atom %) was from GL science.

Results and discussion

Isolation and identification of strain T-7-2

Of 8 isolated NH_4NO_3 -removing bacteria, strain T-7-2 grew well on 0.1% (w/v) ammonium nitrate medium and could utilize NH_4^+ and NO_3^- at 30 and 50°C (Table 1). The maximum NO_2^- concentration was 3 mM after 1 day of cultivation; NO_2^- disappeared completely with the growth of strain T-7-2. The isolate could remove nitrogen compounds at 30 and 50°C. This indicates that it is a thermotolerant bacterium that can utilize and remove NH_4^+ and NO_3^- .

Strain T-7-2 was a motile rod of $0.64 \times 1.6\text{--}3.2\ \mu\text{m}$ with peritrichous flagella. It was Gram-positive, spore-forming, catalase-positive, and oxidase-negative. It produced acid fermentative from D-glucose, D-fructose, D-mannose, lactose, and sucrose. Nitrate reduction is positive; Reactions for hydrogen sulfide production and the Voges–Proskauer test were positive. Reactions for Methyl red test, indole production, and urease were negative. The strain can grow at 25–55°C on a nutrient medium. The nucleotide sequence of the 16S rRNA

gene of strain T-7-2 (1501 bp, accession no. AB275356) was 99.8 and 99.6 % identical to those of *Bacillus licheniformis* strain KL-185 (AY030337) and *Bacillus licheniformis* strain ATCC 14580 (CP000002), respectively. Thus, strain T-7-2 was identified as a species of *Bacillus licheniformis*.

Effects of metal ions on growth of strain T-7-2 and NH_4NO_3 utilization by strain T-7-2

In a previous study, *K. pneumoniae* strain F-5-2 grew well on 0.4% (w/v) NH_4NO_3 medium and could remove NH_4^+ and NO_3^- under aerobic condition, when 0.1 mM Fe^{2+} and 8 pM MoO_4^{2-} were added to the medium (Kim et al. 2002). The effects of metal ions on the cell growth of strain T-7-2 and utilization of NH_4NO_3 were examined by culturing strain T-7-2 on 0.1% (w/v) ammonium nitrate medium containing 0.1 mM metal ions (i.e., Mn^{2+} , Zn^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , and Ni^{2+}). When 0.1 mM Fe^{2+} was added to the test medium, the strain can effectively remove NH_4^+ and NO_3^- at 30 and 50°C. Subsequently, the effects of metal ions were examined using 0.2% (w/v) ammonium nitrate medium containing 0.1 mM metal ions. Although the growth of strain T-7-2 reached on OD_{660} values of 4.6 (at 30°C) and 3.5 (at 50°C) in the test medium containing Mn^{2+} , NO_2^- accumulated. Glutamine synthetase from *Bacillus licheniformis* strain A5 requires Mn^{2+} to show high activity (Donohue and Bernlohr 1981). Mn^{2+} also enhances glutamine synthetase and glutamyl transferase activities in crude extract from *Bacillus licheniformis* (Leonard et al. 1962). In strain T-7-2, the supplementation

of Mn^{2+} to the test medium promoted effective ammonium assimilation and resulted in good growth. Zn^{2+} , Co^{2+} , Cu^{2+} , and Ni^{2+} markedly inhibited the growth of strain T-7-2 and NH_4^+ and NO_3^- remained in the test medium. Among the tested metal ions, Fe^{2+} was the most effective for strain T-7-2 to utilize nitrogen compounds at 30 and 50°C. Figure 2 shows the removal of NH_4^+ and NO_3^- at 50°C in the presence of 0.5 mM Fe^{2+} . This effect indicates the same tendency for the removal of NH_4^+ and NO_3^- at 30°C. The cell growth of *Bacillus licheniformis* strain 40-2 at 37°C is markedly enhanced in a medium containing Fe^{2+} under aerobic condition (Konohana et al. 2000).

The presence of a small amount of MoO_4^{2-} in the growth medium appears to have a marked dramatic effect on the removal of NH_4NO_3 by strain F-5-2 (Kim et al. 2002). However, strain T-7-2 did not require MoO_4^{2-} to utilize NH_4NO_3 . The lack of molybdenum (VI) in the growth medium appears to have no effect on the kinetics of the denitrification and growth of *Bacillus licheniformis* (Juszczak et al 1996).

Effect of various factors on growth of strain T-7-2 under aerobic condition

Strain F-5-2 grew well under aerobic condition (shaking at 140 rpm) and could remove 1.29% (w/v) NaNO_3 at maximum after 96 h of cultivation (Kim et al. 2002). In contrast, it grew poor in the test medium containing 0.1% (w/v) NaNO_3 instead of NH_4NO_3 and could hardly

remove NaNO_3 at the same shaking speed. Shaking speed was gradually reduced to 100 rpm. Thereafter, strain T-7-2 could remove NO_3^- at 30 and 50°C. Then, shaking speed was fixed at 140 rpm and the effect of NH_4Cl on NO_2^- utilization was examined. The strain could completely remove NO_3^- containing 0.1% (w/v) NaNO_3 and 0.01% (w/v) NH_4Cl at 30 and 50°C.

Analysis of total nitrogen concentration in cultural supernatant and cells

Figures 2 (A)-(D) show that strain T-7-2 grew at 30 and 50°C and that the total nitrogen in the culture decreased with an increase of that in the cells of strain T-7-2. After 72 h of cultivation, NH_4^+ and NO_3^- were removed completely without NO_2^- accumulation (Figs. 2 (A) and (C)). Twenty-five percent of the total nitrogen originally provided was observed in the cells and 44 % of that was in the cultural supernatant at 30°C. Residual nitrogen (31% of total nitrogen) was removed from the culture. Twenty percent of the total nitrogen originally provided was observed in the cells and 24% of that was in the cultural supernatant at 50°C. Residual nitrogen (56% of total nitrogen) would probably be removed into the atmosphere. The noninoculated control samples were incubated at 30 and 50°C similarly; no notable amount of ammonia (less than 5%) dispersed into the air during incubation.

Analysis of enzymes involved in denitrification

Strain T-7-2 was cultivated using a 10-ml vial capped with a butyl rubber stopper and aluminum seal to identify and estimate the amount of the gases from $^{15}\text{NH}_4\text{NO}_3$ and $\text{NH}_4^{15}\text{NO}_3$. The strain grew well and completely removed NH_4^+ and NO_3^- in 50 h at 30°C. The growth reached on OD_{660} of 2.3 at 30 h. It grew faster at 50°C; it completely removed NH_4^+ and NO_3^- in 35 h (Fig. 3). Accumulated gas was analyzed every 2 h after 5 h of cultivation. After NO_2^- accumulation of nitrite in the culture, $^{15}\text{N}_2\text{O}$ gas also accumulated in each vial.

Crude extracts of strain T-7-2 showed a high nitrite reductase activity, when NO_2^- or NO_3^- was present in the medium. Cells of strain T-7-2 were harvested as long as NO_2^- and NO_3^- still remained; the activities of respiratory nitrate reductase, nitrite reductase, and NO reductase in the crude extracts prepared from the cells were determined. The specific activities of these enzymes were 6.47, 6.70, and 2.49 nmol of production min^{-1} mg of protein $^{-1}$, respectively. These results indicate that strain T-7-2 synthesizes enzymes involved in denitrification ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$) under aerobic condition.

Potential application for removal of nitrogen compounds by thermotolerant bacteria

Pichinoty et al. (1978) reported gas production by 15 strains of *Bacillus licheniformis* from peptone medium containing nitrate. Although some of the strains showed denitrification, gas

production was irregular and quite slow. Strain T-7-2 produced $^{15}\text{N}_2\text{O}$ from the culture containing $\text{NH}_4^{15}\text{NO}_3$; however, the amount of released $^{15}\text{N}_2\text{O}$ gas always decreased during shaking (Fig. 3). N_2O returned into the medium was probably converted to N_2 gas by strain T-7-2. To our knowledge, denitrification by *B. licheniformis* under aerobic condition in a medium containing NH_4^+ and NO_3^- has not yet been observed. Although *K. pneumoniae* strain F-5-2 is a manageable bacterium for removal of NH_4NO_3 , *K. pneumoniae* is a notorious pathogen. *B. licheniformis* is generally considered non pathogenic to humans (de Boer et al. 1994). Strain T-7-2 grows well and utilizes NH_4NO_3 at both low and high temperatures, and is an aerobic denitrifier. These indicate that thermotolerant strain T-7-2 has potential for application in wastewater treatment.

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Table 1 Utilization of NH_4^+ and NO_3^- by isolated strains under aerobic condition

Each isolated strain was incubated in the screening medium containing 0.1% (w/v) NH_4NO_3 at 30 and 50°C with shaking at 140 rpm. After 96 h of cultivation, cell growth and remaining NH_4^+ and NO_3^- were measured.

Isolate	30°C			50°C		
	Remaining		Growth (OD ₆₆₀)	Remaining		Growth (OD ₆₆₀)
	NH_4^+	NO_3^-		NH_4^+	NO_3^-	
S-2-1	2.4%	53%	1.7	3.2%	66%	1.7
T-1-1	2.4	53	3.8	2.4	62	4.6
T-7-2	0.8	6.4	4.3	4.0	16	3.8
T-12-1	1.6	48	3.7	2.4	58	3.7
T-18-1	1.6	53	4.4	4.8	80	4.4
C-6-1	2.4	47	2.8	2.4	77	2.8
F-2-3	0.8	20	2.3	2.4	54	2.3
F-17-2	1.6	38	4.7	4.0	45	4.7

Figure legends

Fig. 1 Effect of Fe^{2+} concentration on NH_4NO_3 utilization by *B. licheniformis* strain T-7-2 at 50°C. The bacterium was incubated in a test tube (18×180 mm) under aerobic condition (shaking at 140 rpm) in 0.2% (w/v) [25 mM] NH_4NO_3 medium (7 ml/tube) containing 0.01, 0.05, 0.1, 0.3, 0.5, 0.7, or 1.0 mM Fe^{2+} . As a control, the strain was cultivated without Fe^{2+} . After 72 h of cultivation, residual nitrate ($\square$) and ammonium ($\blacktriangle$) concentration and the OD_{660} of the culture (hatched bar) were measured.

Fig. 2 Utilization of ammonium nitrate by *B. licheniformis* strain T-7-2 at 30°C (A) and 50°C (C) and total nitrogen concentration in the culture at 30°C (B) and 50°C (D). (A and C) The bacterium was incubated in a 500-ml flask under aerobic condition in 0.1% (w/v) [12.5 mM] NH_4NO_3 medium (70 ml/flask) containing 0.5 mM Fe^{2+} at 30°C and 50°C. Residual nitrate ($\square$) and ammonia ($\blacktriangle$) concentrations, amount of accumulated nitrite ($\diamond$) and the growth ($\circ$) were measured. Total nitrogen concentrations in the cells (solid bar) and cultural supernatant (open bar) were converted to nitrogen per 10 ml of culture.

Fig. 3 Utilization of ammonium nitrate by *B. licheniformis* strain T-7-2 and gas analysis from the culture. The bacterium was incubated in a 10-ml vial with shaking at 140 rpm in 0.1% (w/v) [12.5 mM] $\text{NH}_4^{15}\text{NO}_3$ medium (1.0 ml/vial) containing 0.5 mM Fe^{2+} at 50°C. Residual nitrate ($\square$) and ammonia ($\blacktriangle$) concentrations, amount of accumulated nitrite ($\diamond$) and the growth ($\circ$) were measured. Amount of $^{15}\text{N}_2\text{O}$ gas (hatched bar) was measured and converted to μmol per vial.

Fig. 1., S. Takenaka et al.

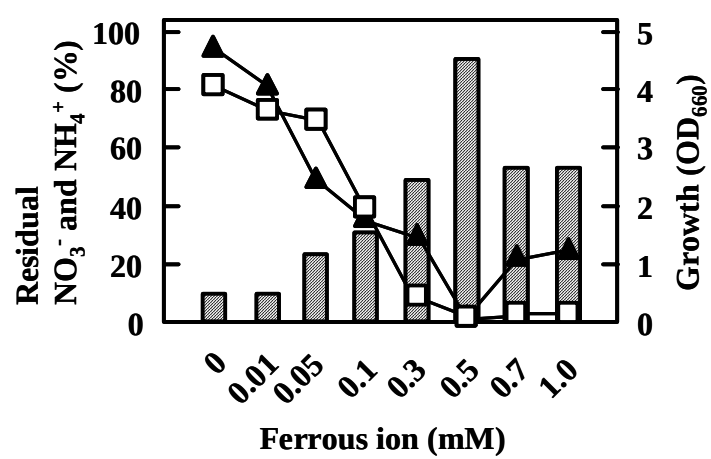


Fig. 2., S. Takenaka et al.

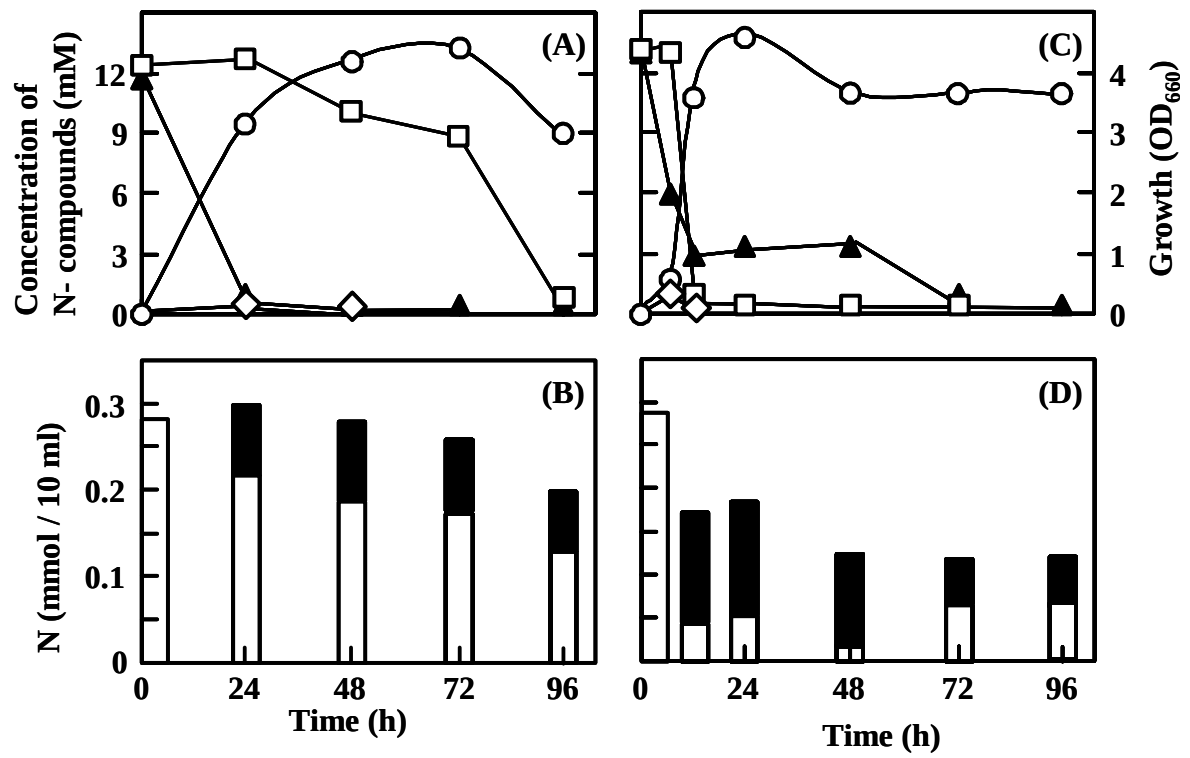


Fig. 3., S. Takenaka et al.

