<table>
<thead>
<tr>
<th>Title</th>
<th>Isolation and characterization of thermotolerant bacterium utilizing ammonium and nitrate ions under aerobic conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Takenaka, Shinji / Zhou, Qi / Kuntiya, Ampin / Seesuriyachan, Phisit / Murakami, Shuichiro / Aoki, Kenji</td>
</tr>
<tr>
<td>Citation</td>
<td>Biotechnology Letters, 29(3): 385-390</td>
</tr>
<tr>
<td>Issue date</td>
<td>2007-03</td>
</tr>
<tr>
<td>Resource Type</td>
<td>Journal Article / 学術雑誌論文</td>
</tr>
<tr>
<td>Resource Version</td>
<td>author</td>
</tr>
<tr>
<td>DOI</td>
<td>10.1007/s10529-006-9255-8</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/90000423">http://www.lib.kobe-u.ac.jp/handle_kernel/90000423</a></td>
</tr>
</tbody>
</table>

PDF issue: 2018-12-27
Isolation and Characterization of Thermotolerant Bacterium Utilizing Ammonium and Nitrate Ions under Aerobic Condition

Shinji Takenaka¹, Qi Zhou², Ampin Kuntiya³, Phisit Seesuriyachan³, Shuichiro Murakami¹, and Kenji Aoki¹*

Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Rokko, Kobe 657-8501, Japan¹; Department of Biosystems Science, Division of Biosystems Chemistry Graduate School of Science and Technology, Kobe University, Rokko, Kobe 657-8501, Japan²; Department of Biotechnology, Faculty of Agro-industry, Chiang Mai University, Chiang Mai 50100, Thailand

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

*Corresponding author (Kenji Aoki)

Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Rokko, Kobe 657-8501, Japan Fax +81-78-882-0481; e-mail: kaoki@kobe-u.ac.jp
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 NH$_4^{15}$NO$_3$ was converted to $^{15}$N$_2$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$_2$O 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$_4$NO$_3$. 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$_4$NO$_3$
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$_4$NO$_3$ (12.5 mM), 0.1 mM FeSO$_4$·7H$_2$O, and 8 pM Na$_2$MoO$_4$·2H$_2$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 FeSO₄·7H₂O under aerobic condition, in order to examine the effects of various factors on the removal of NH₄⁺ and NO₃⁻ and to study denitrification. TAITIIEC 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 NH₄⁺, NO₃⁻, and NO₂⁻ concentrations in the culture were measured by the indophenol
blue method (Weatherburn 1967), micro-salytilate method (Bhanadari 1989), and diazo-coupling reaction method (Aoki et al. 1981), 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$_2$O gas production was monitored using a Hitachi G-3900 gas chromatograph equipped with a photon ion detector. A stainless steel column (3 mm × 2 m) packed with Porapak Q 50/80 mesh (GL Science, Tokyo, Japan) was used at 40°C. $^{15}$N$_2$O and $^{15}$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

NH$_4^{15}$NO$_3$ and NH$_4^+$ NO$_3$ (98.0 atom %) were purchased from Sigma-Aldrich. $15^N_2$ (99.6 atom %) was from Shoko Co. (Tokyo, Japan); N$_2$O (99.5 atom %) was from GL science.

Results and discussion

Isolation and identification of strain T-7-2

Of 8 isolated NH$_4$NO$_3$-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 0.64×1.6–3.2 μm 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 NH₄NO₃ utilization by strain T-7-2**

In a previous study, *K. pneumoniae* strain F-5-2 grew well on 0.4% (w/v) NH₄NO₃ medium and could remove NH₄⁺ and NO₃⁻ under aerobic condition, when 0.1 mM Fe²⁺ and 8 pM MoO₄²⁻ 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 NH₄NO₃ were examined by culturing strain T-7-2 on 0.1% (w/v) ammonium nitrate medium containing 0.1 mM metal ions (i.e., Mn²⁺, Zn²⁺, Co²⁺, Cu²⁺, Fe²⁺, and Ni²⁺). When 0.1 mM Fe²⁺ was added to the test medium, the strain can effectively remove NH₄⁺ and NO₃⁻ 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 OD₆₆₀ values of 4.6 (at 30°C) and 3.5 (at 50°C) in the test medium containing Mn²⁺, NO₃⁻ accumulated. Glutamine synthetase from *Bacillus licheniformis* strain A5 requires Mn²⁺ to show high activity (Donohue and Bernlohr 1981). Mn²⁺ 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$_4$NO$_3$ by strain F-5-2 (Kim et al. 2002). However, strain T-7-2 did not require MoO$_4^{2-}$ to utilize NH$_4$NO$_3$. 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$_4$NO$_3$ and could hardly
remove NaNO₃ at the same shaking speed. Shaking speed was gradually reduced to 100 rpm. Thereafter, strain T-7-2 could remove NO₃⁻ at 30 and 50°C. Then, shaking speed was fixed at 140 rpm and the effect of NH₄Cl on NO₂⁻ utilization was examined. The strain could completely remove NO₃⁻ containing 0.1% (w/v) NaNO₃ and 0.01% (w/v) NH₄Cl 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, NH₄⁺ and NO₃⁻ were removed completely without NO₂⁻ 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 $\text{NH}_4^+$ and $\text{NO}_3^-$ in 50 h at 30°C. The growth reached on $\text{OD}_{660}$ of 2.3 at 30 h. It grew faster at 50°C; it completely removed $\text{NH}_4^+$ and $\text{NO}_3^-$ in 35 h (Fig. 3). Accumulated gas was analyzed every 2 h after 5 h of cultivation. After $\text{NO}_2^-$ 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 $\text{NO}_2^-$ or $\text{NO}_3^-$ was present in the medium. Cells of strain T-7-2 were harvested as long as $\text{NO}_2^-$ and $\text{NO}_3^-$ 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). $\text{N}_2\text{O}$ returned into the medium was probably converted to $\text{N}_2$ gas by strain T-7-2. To our knowledge, denitrification by *B. licheniformis* under aerobic condition in a medium containing $\text{NH}_4^+$ and $\text{NO}_3^-$ has not yet been observed. Although *K. pneumoniae* strain F-5-2 is a manageable bacterium for removal of $\text{NH}_4\text{NO}_3$, *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 $\text{NH}_4\text{NO}_3$ 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.

**Acknowledgements**

Part of this work was carried out through collaboration in a Core University and supported by the Japan Society for the Promotion of Science (JSPS) and the National Research Council of Thailand (NRCT).

**References**


Leonard CG Housewright RD et al. (1962) Effect of metal ions on the optical specificity of glutamine synthetase and glutamyl transferase of *Bacillus licheniformis*. Biochim Biophys Acta 62:432-434


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$_4$NO$_3$ 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.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>30°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Remaining</td>
<td>Growth (OD$_{660}$)</td>
</tr>
<tr>
<td></td>
<td>NH$_4^+$</td>
<td>NO$_3^-$</td>
</tr>
<tr>
<td>S-2-1</td>
<td>2.4%</td>
<td>53%</td>
</tr>
<tr>
<td>T-1-1</td>
<td>2.4</td>
<td>53</td>
</tr>
<tr>
<td>T-7-2</td>
<td>0.8</td>
<td>6.4</td>
</tr>
<tr>
<td>T-12-1</td>
<td>1.6</td>
<td>48</td>
</tr>
<tr>
<td>T-18-1</td>
<td>1.6</td>
<td>53</td>
</tr>
<tr>
<td>C-6-1</td>
<td>2.4</td>
<td>47</td>
</tr>
<tr>
<td>F-2-3</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>F-17-2</td>
<td>1.6</td>
<td>38</td>
</tr>
</tbody>
</table>
Figure legends

**Fig. 1** Effect of Fe$^{2+}$ concentration on NH$_4$NO$_3$ 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$_4$NO$_3$ 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 (□) and ammonium (▲) 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$_4$NO$_3$ medium (70 ml/flask) containing 0.5 mM Fe$^{2+}$ at 30°C and 50°C. Residual nitrate (□) and ammonia (▲) concentrations, amount of accumulated nitrite (◊) and the growth (○) 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] NH$_4^{15}$NO$_3$ medium (1.0 ml/vial) containing 0.5 mM Fe$^{2+}$ at 50°C. Residual nitrate (□) and ammonia (▲) concentrations, amount of accumulated nitrite (◊) and the growth (○) were measured. Amount of $^{15}$N$_2$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.