Isolation and Characterization of a Thermotolerant Ammonia-Oxidizing Bacterium *Nitrosomonas* sp. JPCCT2 from a Thermal Power Station

YuhiKane ItoH1, KeiKo Sagakam1, YosHiHito UChin2, Chanita Boonmak1, TetsuoRiYama1, FuyuMi Tojo1, MitsuFumi Matsumoto1, and MasakiMorikawa*

1Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita 10 Nishi 5, Kita-ku, Sapporo 060–0810, Japan; 2NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2–5–8 Kasetakamatari, Kisarazu, Chiba 292–0818, Japan; and 3Wakamatsu Research Institute, Technology Development Center, Electric Power Development Co., Ltd., Yanagisaki, Wakamatsu, Kitakyushu, Fukuoka 808–0111, Japan

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A thermotolerant ammonia-oxidizing bacterium strain JPCCT2 was isolated from activated sludge in a thermal power station. Cells of JPCCT2 are short non-motile rods or ellipsoidal. Molecular phylogenetic analysis of 16S rRNA gene sequences demonstrated that JPCCT2 belongs to the genus *Nitrosomonas* with the highest similarity to *Nitrosomonas nitrosa* Nm90 (100%), *Nitrosomonas* sp. Nm148 (99.7%), and *Nitrosomonas communis* Nm2 (97.7%). However, G+C content of JPCCT2 DNA was 49.1 mol% and clearly different from *N. nitrosa* Nm90 and *N. communis* Nm2 could not grow at 42°C. Moreover, JPCCT2 grew similarly at concentrations of carbonate 0 and 5 g L⁻¹. This is the first report that *Nitrosomonas* bacterium is capable of growing at temperatures higher than 37°C.

Key words: *Nitrosomonas*, thermotolerant ammonia-oxidizing bacterium, activated sludge

Chemolithoautotrophic ammonia-oxidizing bacteria (AOB), which convert ammonium to nitrite, play an important role in the global cycling of nitrogen (19, 22). Isolation of AOB was first reported in 1890 (2, 28), and since then a considerable number of AOB within the Betaproteobacteria and Gammaproteobacteria have been obtained from various environments (6, 10, 23, 26, 29). In particular, members of the betaproteobacterial genera, *Nitrosomonas* and *Nitrosospira*, are considered as the most dominant AOB in activated sludge (4, 14, 15, 20). Most strains of *Nitrosomonas* and *Nitrosospira* preferably grow in a relatively narrow range of moderate temperatures between 25 and 30°C (3).

It has been recently recognized that geothermal environments are also favorable habitats for AOB and ammonia-oxidizing archaea (AOA) (31). There are several reports on isolation and characterization of thermophilic AOA (5, 18); however, AOB cultures are unstable at high temperatures and no successful isolation has been reported (13). Here, we report for the first time the isolation of a thermotolerant AOB from activated sludge in a wastewater treatment plant continuously operated at 37–45°C.

Materials and Methods

Isolation of and physiological characterization of JPCCT2

A JPCC (J-Power Culture Collection) T2 (water treatment tank) bacterium sample was isolated from activated sludge in a thermal power station of the Electric Power Development Co., Ltd (J-Power) (Fukuoka, Japan). Enrichment cultures were repeated several times at intervals of 7 days in modified Alexander (MA) medium containing, per liter, 2 g of (NH₄)₂SO₄ (30 mM ammonium) as the sole source of nitrogen, 0.5 g NaHCO₃ (6 mM carbonate) as the sole source of carbon, 0.5 g KH₂PO₄, 50 mg MgSO₄·7H₂O, 5 g CaCl₂·2H₂O, 2 mg MnSO₄·4H₂O, 5 g Fe-EDTA (III), 0.1 mg CuSO₄·5H₂O, 0.05 mg NaMoO₂·2H₂O, 0.001 mg CoCl₂·6H₂O, 0.1 mg ZnSO₄·7H₂O, and 50 mM HEPES (pH 7.8) (30) at 28°C with rotary shaking at 130 rpm. MA solid medium containing 1% gellan gum in MA medium (25) was used for single colony isolation of AOB after sub-culturing for two months. Consumption of ammonium and production of nitrite was confirmed in every culture using the Ammonia-test (Wako, Osaka, Japan) and naphthylethylene-diamine spectrophotometric analysis (8), respectively. Sucrose density gradient centrifugation was further applied for isolation of JPCCT2. Culture purity was confirmed by the non-growth test in LB medium (1% NaCl, 0.5% yeast extract, and 1% peptone, pH 7.2) and also by no multiple peaks at single base positions in the 16S rRNA gene sequence raw data (ABI3130; Applied Biosystems, Carlsbad, CA, USA) for JPCCT2.

JPCCT2 and three related *Nitrosomonas* strains, *N. nitrosa* Nm90, *N. europaea* IFO14298 (= NBRC14298, ATCC19718) and *N. communis* Nm2, were pre-grown for 3 days at 28°C in a rotary shaker (130 rpm) or standing (for Nm90). *N. europaea* IFO14298 was obtained from NBRC, *N. nitrosa* Nm90 and *N. communis* Nm2 were kind gifts from Dr. Andreas Pommerening-Röser (University of Hamburg). MA medium was used to culture *N. europaea* and *N. communis*, and Medium la or lb was used for *N. nitrosa* and *N. nitrosa* was sensitive to strong aeration and was mostly grown under standing culture conditions. Medium la contained, per liter, 535 mg NH₄Cl (10 mM ammonium) as the sole source of nitrogen, 54.4 mg KH₂PO₄, 74.4 mg KCl, 147 mg CaCl₂·2H₂O, 49.3 mg MgSO₄·7H₂O, 1 ml trace element solution, and 1 ml of 0.05% Cresol Red solution, and pH was maintained at 7.8 by 5 g CaCO₃ (Medium lb) or appropriate addition of 10% NaHCO₃. Trace elements solution contained, per liter, 3.5 mM FeSO₄·0.8 mM H₂BO₃, 0.15 mM ZnSO₄·0.1 mM CuSO₄·0.03 mM (NH₄)₂MoO₄·6H₂O, 0.02 mM MnSO₄·0.025 N HCl. After washing with fresh medium, the cells of each strain were inoculated at the appropriate OD₅₇₀ into each medium for growth tests under different conditions of temperature (28, 37, 43, 45 and 48°C), ammonium (7.5, 11.25, 15.0 and 30.0
mM), sodium bicarbonate (0 or 5.0 g L⁻¹), and sodium chloride (100, 300 and 500 mM). Temperature for cultivation was generally 28°C unless otherwise denoted.

**Biochemical characterization**

G+C content (mol%) of DNA was directly determined by complete hydrolysis of the genomic DNA followed by quantification of each nucleoside by HPLC according to the protocols for the DNA GC Kit (Seikagaku Biobusiness, City, Country). The score was calculated as the average of three independent experiments. Genomic DNA was purified according to the protocol for the GenElute Bacterial Genomic Kit (Sigma-Aldrich, St. Louis, MO, USA).

Respiratory quinones were extracted from the cells in the stationary phase of JPCCT2 culture, 3–7 days, according to the protocol of (16) and analyzed with an LCMS-8030 spectrometer (Shimadzu, Kyoto, Japan).

Fatty acid methyl esters were prepared according to the standard protocol described in the MIDI microbial identification system (Microbial ID; Agilent Technologies, City, Country) and analyzed by GC-MS (GC system model 6890 and MSD model 5973; Agilent (Microbial ID; Agilent Technologies, City, Country) and analyzed with 1,000 samples.

**Results and Discussion**

**Chemotaxonomic properties**

Ubiquinone-8 was the sole detectable respiratory quinone in JPCCT2. Ubiquinone-8 is a common form of ubiquinones among most Gram-negative bacteria, including *Nitrosomonas* bacteria (7). It was also found that JPCCT2 possessed a simple fatty acid composition mainly composed of C16:0 (42.8%) and C16:1ω9c (53.3%). It might be worth noting that fatty acids in *N. europaea* are C16:0 (25.0%), C16:1ω9c (61.6%), and C16:1ω9c (13.1%), while psychrotrophic *Nitrosomonas* sp. W30 are C16:0 (73 and 45%) at 5°C and 25°C, respectively (9, 21).
Table 1. Major characteristics of JPCCT2, N. nitrosa Nm90, N. communis Nm2, N. europaea IFO14298, and N. europaea ATCC25978T. Growth was denoted by OD<sub>600</sub> after 3 days and (1 day) for JPCCT2, Nm2, IFO14298, and 5 days and (3 days) for Nm90<sup>T</sup>. NG, <0.003 (no growth), ND, not determined, NA, not available. Initial OD<sub>600</sub> of culture after inoculation was 0.01 for JPCCT2, Nm2, IFO14298, and 0.002 for Nm90.

| Source of strain | *Nitrosomonas* sp. | *N. nitrosa* Nm90 | *N. communis* Nm2 | *N. europaea* IFO14298 | *N. europaea* ATCC25978<sup>T</sup> |
|------------------|--------------------|------------------|------------------|------------------------|---------------------------|
| Cell morphology  | Activated sludge in a thermal power station | Industrial Sewage | Soil | Soil | Soil |
| Cell dimensions (μm) | 0.5–0.7 × 0.9–1.6 | 1.3–1.5 × 1.4–2.2 | 1.0–1.4 × 1.7–2.2 | 46.1 | 50.4 |
| DNA G + C content (mol%) | 49.1 | 47.9 (12) | 45.8 (12) | 50.7 (1) | 50.5 (27) |

Growth dependence on temperature

| Temperature | Growth | Cell morphology | Cell dimensions (μm) | DNA G + C content (mol%) | Growth dependence on sodium bicarbonate |
|-------------|--------|-----------------|----------------------|-------------------------|----------------------------------------|
| 28°C        | 0.078 [0.046] | Short rods or ellipsoidal with round ends | 0.5–0.7 × 0.9–1.6 | 49.1 | 0.062 [0.024] |
| 37°C        | 0.071 [0.059] | Spheres or short rods with round ends | 1.3–1.5 × 1.4–2.2 | 47.9 (12) | 0.078 [0.046] |
| 42°C        | 0.063 [0.060] | Short rods or ellipsoidal with round ends | 1.0–1.4 × 1.7–2.2 | 45.8 (12) | 0.079 [0.027] |
| 45°C        | 0.045 [0.043] | Short rods or ellipsoidal with round ends or point ends | 0.8–1.1 × 1.0–1.7 | 0.045 [0.043] | NG |
| 48°C        | 0.031 [NG] | Short rods or ellipsoidal with round ends | 0.8–1.1 × 1.0–1.7 | 0.031 [NG] | NG |

Growth dependence on sodium bicarbonate

| Sodium Bicarbonate | Growth | Cell morphology | Cell dimensions (μm) | DNA G + C content (mol%) | Growth dependence on sodium bicarbonate |
|--------------------|--------|-----------------|----------------------|-------------------------|----------------------------------------|
| 0 g L<sup>-1</sup> (pH 4.7) | 0.062 [0.024] | Short rods or ellipsoidal with round ends | 0.5–0.7 × 0.9–1.6 | 49.1 | 0.057 [0.018] |
| 0.5 g L<sup>-1</sup> (pH 7.8) | 0.078 [0.046] | Short rods or ellipsoidal with round ends | 1.3–1.5 × 1.4–2.2 | 47.9 (12) | 0.079 [0.027] |
| 5.0 g L<sup>-1</sup> (pH 8.3) | 0.079 [0.027] | Short rods or ellipsoidal with round ends | 1.0–1.4 × 1.7–2.2 | 45.8 (12) | NG |

Use of urea

| Urea | Growth | Cell morphology | Cell dimensions (μm) | DNA G + C content (mol%) | Growth dependence on sodium bicarbonate |
|------|--------|-----------------|----------------------|-------------------------|----------------------------------------|
| +    | 0.078 [0.046] | Short rods or ellipsoidal with round ends | 1.3–1.5 × 1.4–2.2 | 47.9 (12) | 0.079 [0.027] |
| + (12) | 0.079 [0.027] | Short rods or ellipsoidal with round ends | 1.0–1.4 × 1.7–2.2 | 45.8 (12) | NG |

Maximum tolerance to NaCl (mM)

| NaCl (mM) | Growth | Cell morphology | Cell dimensions (μm) | DNA G + C content (mol%) | Growth dependence on sodium bicarbonate |
|-----------|--------|-----------------|----------------------|-------------------------|----------------------------------------|
| 300       | 0.062 [0.024] | Short rods or ellipsoidal with round ends | 0.5–0.7 × 0.9–1.6 | 49.1 | 0.057 [0.018] |
| 300       | 0.078 [0.046] | Short rods or ellipsoidal with round ends | 1.3–1.5 × 1.4–2.2 | 47.9 (12) | 0.079 [0.027] |
| 500       | 0.079 [0.027] | Short rods or ellipsoidal with round ends | 1.0–1.4 × 1.7–2.2 | 45.8 (12) | NG |
| 500 (3)   | 0.079 [0.027] | Short rods or ellipsoidal with round ends | 1.0–1.4 × 1.7–2.2 | 45.8 (12) | NG |

Physiological and morphological characteristics

Table 1 summarizes the comparative characteristics of JPCCT2, *N. nitrosa* Nm90, *N. communis* Nm2, *N. europaea* IFO14298 and ATCC25978<sup>T</sup>. JPCCT2 cells were non-motile short rods or ellipsoidal with round ends whose size is relatively smaller than other *Nitrosomonas* bacteria. JPCCT2 showed moderate thermostability and grew at temperatures up to 48°C. In contrast to JPCCT2, *N. communis* and *N. nitrosa* were rather thermostable when compared with other strains and could only slightly grow at 37°C. It is worth noting that the wastewater treatment tank T2, from which JPCCT2 was isolated, was continuously operated under 37–45°C conditions. More interestingly, the addition of sodium bicarbonate did not significantly affect the growth of strain JPCCT2 in the range of 0–5 g L<sup>-1</sup>, which also shows clear difference from *N. nitrosa* Nm90. Five grams per liter of sodium bicarbonate clearly inhibited the growth of *N. communis* Nm2 and led to cell lysis, and *N. europaea* IFO14298 grew normally at 5 g L<sup>-1</sup> but could not grow without carbon in the medium. *N. nitrosa* Nm90 could grow under neither of these extreme carbonate conditions. In contrast to these three strains, JPCCT2 grew similarly at both 0 and 5 g L<sup>-1</sup> sodium bicarbonate at 28°C. pHs of the media were 4.7 and 8.3 with 0 g L<sup>-1</sup> and 5 g L<sup>-1</sup> sodium bicarbonate, respectively. This indicates that JPCCT2 and Nm2 could grow using a trace amount of naturally dissolved atmospheric carbon dioxide (mostly in the non-dissociated H<sub>2</sub>CO<sub>3</sub> form at pH 4.7) in the medium with no addition of sodium bicarbonate. JPCCT2 utilized urea as a source of nitrogen and showed the maximum growth of 0.14 OD<sub>600</sub> after 3 days’ cultivation at 28°C upon addition of 10 mM urea.

In conclusion, although the 16S rRNA gene sequence was very close to known *Nitrosomonas* species, the characteristics of G+C content, chemotaxonomy and physiological uniqueness, including thermostolerance and carbonate requirements, indicate that JPCCT2 might be a novel species in the genus *Nitrosomonas*.

Description of *Nitrosomonas* sp. JPCCT2

Strict aerobe. Cells are Gram-negative rather small short rods or ellipsoidal with rounded ends, 0.5–0.7 μm wide and 0.9–1.6 μm long and exist mostly as singles. Motility is not observed. Cells pellets are slightly reddish in color. G+C content of the DNA is 49.1 mol%. Quinone type is ubiquinone-8. Utilize both ammonium and urea as sole nitrogen sources. Optimum (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> concentration for growth is between 11.25 and 15.0 mM and additional 10 mM urea further stimulated growth. Optimum growth pH is between 7.5 and 8.0. Grew similarly at 0 and 5 g L<sup>-1</sup> sodium bicarbonate. The range of growth temperature is wide, 28–48°C. JPCCT2 has been registered in culture collections (= JCM17640, = NBRC108559).

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