Nakamurella intestinalis sp. nov., isolated from the faeces of Pseudorhynchus japonicus

Soo-Jin Kim,¹ Hayyoung Cho,¹ Jae-Ho Joa,² Moriyuki Hamada,³ Jae-Hyung Ahn,¹ Hang-Yeon Weon¹ and Soon-Wo Kwon¹,*

Abstract

One strain, designated 63MJ-1ᵀ, was isolated from fresh faeces of broad-winged katydids collected in Jinan-gun, Jeollabuk-do, the Republic of Korea. The organism stained Gram-positive and was an aerobic, non-flagellated and short-rod-shaped bacterium. The organism grew in the range of 4–35°C (optimum, 28–30°C) and pH 6.0–9.0 (optimum, pH 7.0), and in the presence of 5% NaCl (w/v), but not in media containing 7% NaCl. According to the 16S rRNA gene sequence analysis, strain 63MJ-1ᵀ showed the highest sequence similarities with Nakamurella panacisegetis P4-7ᵀ (95.9%), Nakamurella endophytica 2Q3S-4-2ᵀ (95.8%) and Nakamurella multipartita DSM 44233ᵀ (95.7%). Phylogenetic trees also indicated that strain 63MJ-1ᵀ formed one robust cluster with members of the genus Nakamurella. The predominant quinone of strain 63MJ-1ᵀ was MK-8(H₄). Polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, an unidentified aminophospholipid and two unidentified lipids. The major fatty acids were C₁₆:0, anteiso-C₁₅:0 and iso-C₁₅:0. The peptidoglycan type was A₁γ with meso-diaminopimelic acid as the diagnostic amino acid. The DNA G+C content was 66.4 mol %.

Based on the phylogenetic, physiological and chemotaxonomic data, it was demonstrated that strain 63MJ-1ᵀ represents a novel species of the genus Nakamurella, for which the name Nakamurella intestinalis sp. nov. is proposed. The type strain is 63MJ-1ᵀ (=KACC 18662ᵀ=NBRC 111844ᵀ).

Originally, the genus Microsphaera was proposed for a strain isolated from activated sludge [1]. The new genus name Nakamurella was proposed as a substitute for Microsphaera Yoshimi et al. 1996, and then the new family name Nakamurellaceae substituting the former name Microsphaeraceae Rainey et al. 1997 was also proposed [2]. Members of the genus Nakamurella are characterized as aerobic, Gram-stain-positive, spherical, non-motile and non-spore-forming bacteria [1]. They contain meso-diaminopimelic acid in cell-wall peptidoglycan, MK-8(H₄) as the major quinone, and iso-C₁₆:0, iso-C₁₅:0 and C₁₈:1 as the major fatty acids [1, 2]. At present, the genus Nakamurella includes six species, Nakamurella multipartita as type species of the genus, Nakamurella endophytica [3], Nakamurella flavida, Nakamurella lactea, Nakamurella panacisegetis [4] and Nakamurella silvestris [5]. Members of the genus Nakamurella have been isolated from soil, rock, sludge and plant bark [3–7].

Broad-winged katydids were collected in Jinan-gun, Jeollabuk-do, the Republic of Korea (35° 46’ 19” N 127° 25’ 10” E). Samples of fresh faeces from the insects were diluted serially in 0.85% (w/v) saline solution, spread on R2A agar (Difco) and incubated for 7 days at 28°C. Single colonies were selected and subcultured onto R2A agar plates to obtain pure cultures. One isolate, designated 63MJ-1ᵀ, was preserved in 20% (v/v) glycerol at −70°C.

The amplification of the 16S rRNA gene of strain 63MJ-1ᵀ was conducted using two universal primers, 9F and 1512R [8], and the PCR products were sequenced by Solgent (Daejeon, Republic of Korea). The nearly full length of the sequence (1457 bp) was obtained. The sequence was blasted using EzBioCloud [9], and the sequence similarity values were calculated. The sequence of strain 63MJ-1ᵀ represents the closely related 16S rRNA gene sequences were retrieved. The 16S rRNA gene sequences were aligned using the ARB software package [10]. Phylogenetic trees were reconstructed by the MEGA 6 program [11] with the neighbour joining [12], maximum parsimony [13] and maximum-likelihood [14] methods. The bootstrap values were calculated.
based on 1000 replications [15]. The 16S rRNA gene sequence of strain 63MJ-1\textsuperscript{T} showed the highest sequence similarity to \textit{N. panacisegetis} P4-7\textsuperscript{2} (95.9\%), \textit{N. endophytica} 2Q3S-4-2\textsuperscript{T} (95.8\%) and \textit{N. multipartita} DSM 44233\textsuperscript{T} (95.7\%), and revealed similarity values of 94.8–95.4\% with other members of the genus \textit{Nakamurella}. According to the neighbour-joining phylogenetic tree (Fig. 1), strain 63MJ-1\textsuperscript{T} formed one robust cluster with members of the genus \textit{Nakamurella}, which was also supported by the maximum-parsimony and maximum-likelihood phylogenetic trees.

Cell morphology was examined by phase-contrast microscopy (AX10; Carl Zeiss) and transmission electron microscopy (LEO model 912AB; Carl Zeiss) with cells grown at 28 °C on R2A for 3 days. Gram staining was tested with the Difco Gram staining kit according to the manufacturer instructions. Temperature and pH range for growth were checked at various temperatures (4, 10, 15, 20, 28, 30, 37 and 42 °C) and pH (pH 5.0–10.0 in increments of 1.0 pH units) on R2A medium. pH was adjusted using citrate/phosphate buffer (pH 4.0–7.0), Tris/hydrochloride buffer (pH 7.0–9.0) and carbonate/bicarbonate buffer (10.0). Growth with added salt was tested in R2A broth supplemented with NaCl (0, 1, 2, 3, 5, 7 and 10 %). Oxidase activity was tested by oxidation of 1 % \textit{p}-aminodimethylaniline oxalate, and catalase activity was determined by measurements of bubble production after the application of 3 % (v/v) hydrogen peroxide solution. Hydrolysis of various substrates was tested using R2A agar medium supplemented with the following substrates: casein (1 %, w/v), chitin (1 %, w/v), carboxymethyl cellulose (1 %, w/v), hypoxanthine (0.5 %, w/v), starch (1 %, w/v), Tween 80 (1 %, w/v), tyrosine (0.1 %, w/v) or xanthine (0.5 %, w/v). A DNase test was conducted on DNase test agar (Difco). API 20NE, API ID 32GN and API ZYM kits (bioMérieux) were used to determine biochemical properties, assimilation of carbohydrates, acid production from carbohydrates and enzymic activities, according to the manufacturer’s instructions. Cells of strain 63MJ-1\textsuperscript{T} were aerobic, Gram-stain-positive, non-flagellated, short rods (1.0–1.2 µm in length and 0.8–1.0 µm in width) (Fig. S1, available in the online Supplementary Material). Strain 63MJ-1\textsuperscript{T} could be differentiated from the closely related species of the genus \textit{Nakamurella} on the basis of habitat, growth conditions (temperature, pH and NaCl range for growth), substrate hydrolysis, assimilation of carbon sources and enzymic activities. Especially, strain 63MJ-1\textsuperscript{T} assimilated a broad range of carbon sources (Table 1). The phenotypic comparison among strain 63MJ-1\textsuperscript{T} and members of the genus \textit{Nakamurella} is shown in Table 1.
Table 1. Phenotypic differences among strain 63MJ-1<sup>T</sup> and some members of the genus Nakamurella

Strains: 1, *Nakamurella intestinalis* sp. nov. 63MJ-1<sup>T</sup>; 2, *N. endophytica* KACC 18872<sup>T</sup>; 3, *N. flavida* KACC 15065<sup>T</sup>; 4, *N. lactea* KACC 14982<sup>T</sup>; 5, *N. multipartita* KACC 20258<sup>T</sup>; 6, *N. panacisegetis* KACC 18707<sup>T</sup>. Data were obtained in this study unless mentioned otherwise. +, Positive; −, negative; nd, not determined.

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<td>Rock&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup>Data from: <sup>a</sup>Tuo et al. [3]; <sup>b</sup>Yoon et al. [7]; <sup>c</sup>Lee et al. [6]; <sup>d</sup>Yoshimi et al. [1]; <sup>e</sup>Kim et al. [4].

For the fatty acid analysis, strain 63MJ-1<sup>T</sup> and the closely related type strains *N. endophytica* KACC 18872<sup>T</sup>, *N. flavida* KACC 15065<sup>T</sup>, *N. lactea* KACC 14982<sup>T</sup>, *N. multipartita* KACC 20258<sup>T</sup> and *N. panacisegetis* KACC 18707<sup>T</sup> were grown on R2A agar medium at 28°C for 2.5 or 3.5 days to the exponential phase depending on the strains [16]. Fatty acid methyl esters were prepared and analysed according to the standard protocol of MIDI (Sherlock Microbial Identification System, version 5.0) and identified by the TSBA 50 database of the Microbial Identification System. Menaquiones and polar lipids were extracted and purified according to the protocol of Minnikin et al. [17]. Individual polar lipids were separated by two-dimensional TLC and visualized by using the following reagents: 5% ethanolic...
molybdophosphoric acid was used for detection of all lipids, ninhydrin reagent for lipids containing free amino groups, Zinzadze reagent for phosphorus-containing lipids and α-naphthol reagent for glycolipids. The DNA G+C content was determined by the fluorometric method [18] using SYBR Green 1 and a real-time PCR thermocycler (Bio-Rad). Biomass for peptidoglycan analysis was obtained by cultivation using trypticase soy broth (Difco) at 28 °C for 3 days. Amino acids and their isomers in cell-wall hydrolysates were analysed as described by Hamada et al. [19].

The predominant cellular fatty acids of strain 63MJ-1T were C16:0 (30.0 %), anteiso-C15:0 (19.3 %) and iso-C15:0 (16.0 %) (Table 2). Compared with other closely related species, strain 63MJ-1T contained high amounts of C16:0, iso-C15:0 and summed feature 3, and a lower amount of anteiso-C17:0. Strain 63MJ-1T had MK-8(H4) as the predominant menaquinone, which was consistent with other species of the genus Nakamurella. Polar lipids consisted of diphasatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), an unidentified aminophospholipid (APL) and two unidentified lipids (Fig. S2). Members of the genus Nakamurella contain the polar lipids DPG, PE, APL and PI in common, and the presence and absence of polar lipids such as unidentified phospholipids, unidentified glycolipids, unidentified phosphoglycolipids or unidentified polar lipids is variable depending on species of the genus Nakamurella [3–5]. Strain 63MJ-1T also had DPG, PE, APL and PI, with DPG and PE as the major polar lipids. And strain 63MJ-1T also contained two unidentified polar lipids, which were minor but specific for strain 63MJ-1T. The DNA G+C content of strain 63MJ-1T was 64.6 mol %, which was a little low compared with DNA G+C content values (67.5–74.3 mol %) of the genus Nakamurella. The peptidoglycan samples of strain 63MJ-1T contained alanine (Ala), glutamic acid (Glu) and diaminopimelic acid (A2-pm) in a molar ratio of 1:4.1:0.4. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of D-Ala, L-Ala, D-Glu and meso-A2-pm. These data suggested that the peptidoglycan type of strain 63MJ-1T was A2-pymeso-A2-pm as a diagnostic amino acid. The peptidoglycan structure of all the members of the genus Nakamurella has been reported to contain meso-diaminopimelic acid as the diagnostic amino acid [1, 3–6], which was consistent with that shown for strain 63MJ-1T.

On the basis of phylogenetic analysis, phenotypic characteristics and chemotaxonomic properties, strain 63MJ-1T is considered to represent a novel species of the genus Nakamurella, for which the name Nakamurella intestinalis sp. nov. is proposed.

**DESCRIPTION OF NAKAMURELLA INTESTINALIS SP. NOV.**

**Nakamurella intestinalis** (in. tes.ti. na´lis. N.L. fem. adj. intesti-nalis pertaining to the intestine).

Cells are aerobic, Gram-stain-positive, non-flagellated, short rods (0.8–1.0 µm in width and 1.2–1.8 µm in length). Colonies are white-coloured and round after incubation for 3 days on R2A agar at 28 °C. Catalase-positive and oxidase-negative. Hydrolyses aesculin, casein and gelatin, but does not hydrolyse chitin, carboxymethylcellulose, DNA, hypoxanthine, starch, Tween 80, tyrosine, urea or xanthine. Grows in the range of 4–35 °C (optimum, 28–30 °C) and pH 6.0–9.0 (optimum, pH 7.0). Growth occurs in media with NaCl up to 5 % (w/v); no growth occurs with 7 % NaCl. Negative for nitrate reduction, indole production, glucose fermentation and arginine dihydrolase. Assimilates D-glucose, L-arabinose, D-mannose, D-mannitol, maltose, potassium gluconate, malic acid, trisodium citrate, L-rhamnose, D-ribose, inositol, sucrose, lactic acid, L-alanine, potassium 5-ketogluconate, glycerogen, L-serine, salicin, melibiose, L-fucose, D-sorbitol, L-histidine, potassium 2-ketogluconate, 3-hydroxybutyric acid and L-proline, but does not assimilate N-acetylglucosamine, capric acid, adipic acid, phenylacetic acid, itaconic acid, suberic acid, sodium malonate, sodium acetate, 3-hydroxybenzoic acid, propionic acid, valeric acid or 4-hydroxybenzoic acid. Positive enzymic activity for alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phospho-hydrolase, α-glucosidase and β-glucosidase, but negative activity for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-
glucosaminidase, α-mannosidase and α-fucosidase. The predominant quinone is MK-8(H4). Polar lipids are diphas-
phtidyldiglycerol, phosphatidylethanolamine, phosphatidyli-
nositol, an unidentified aminophospholipid and two unidentified lipids. The major fatty acids are C16:0, anteiso-
C15:0 and iso-C15:0. The peptidoglycan type is A1γ with
meso-diaminopimelic acid as diagnostic amino acid.

The type strain, 63MJ-T (=KACC 18662T=NBRC 111844T), was isolated from fresh faeces of broad-winged
tkatydids collected in Jinan-gun, Jeolabuk-do, the Republic
of Korea. The DNA G+C content of the type strain is
64.6 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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