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TAXONOMIC DESCRIPTION

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Naasia lichenicola sp. nov., an actinobacterium isolated from lichen

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Abstract

A Gram-stain-positive, yellow-pigmented, catalase-positive and oxidase-negative, strictly aerobic actinobacterium, designated strain YIM 131853^T, was isolated from lichen collected from the South Bank of the Baltic Sea. The novel strain was non-spore-forming, short rod-shaped and motile with a single polar flagellum. The strain could grow at 4–37 °C (optimum, 28 °C), at pH 4.0–12.0 (pH 6.0) and at 0–3% (w/v) NaCl (1%). The DNA G+C content of strain YIM 131853^T based on the draft genome sequence was 68.3 mol%. Predominant cellular fatty acids (>10%) were identified as anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The polar lipid profile included diphosphatidylglycerol, dimannosyldiacylglycerol, three unknown glycolipids, two unknown phospholipids and one unknown lipid. Strain YIM 131853^T had 2,4-diaminobutyric acid as the diagnostic cell-wall diamino acid, galactose and glucose as whole-cell sugars, and MK-10, MK-14, MK-13 and MK-12 as the major menaquinones. Although strain YIM 131853^T exhibited a highest 16S rRNA gene sequence similarity (96.6%) to *Amnibacterium kyonggiense* NBRC 109360^T, phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain formed a tight lineage with *Naasia aerilata* NBRC 108725^T (96.5% 16S rRNA gene sequence similarity), which was the only species of genus *Naasia*. Based on the results of phenotypic, chemotaxonomic and phylogenetic analyses, strain YIM 131853^T should belong to the genus *Naasia* and represents a novel species of the genus *Naasia*, for which the name *Naasia lichenicola* sp. nov. is proposed. The type strain is YIM 131853^T (=CGMCC 4.7565^T=NBRC 113605^T).

The genus *Naasia* of the family *Microbacteriaceae* was proposed by Weon *et al.* [1], which currently comprises a single species, *Naasia aerilata* NBRC 108725^T, isolated from an air sample. The characteristics of the genus *Naasia* were aerobic, Gram-stain-positive, non-spore forming rods and motile with one flagellum. Peptidoglycan in the cell wall was of the type B1 with 2,4-diaminobutyric acid (DAB) as the diamino acid. The predominant menaquinones were MK-10, MK-14, MK-13 and MK-12. Dimannosyldiacylglycerol (DMG) was the predominant polar lipid. The cellular fatty acid profile were dominated by anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$ [1]. In the course of investigation of the actinobacterial diversity in a lichen symbiotic system, a novel actinobacterium (YIM 131853^T) was isolated from *Candelaria* species lichen.

The lichen sample was collected from the South Bank of the Baltic Sea (10° 12′ E 54° 31′ N), Germany, and transferred to sterile paper bag immediately and air-dried at 28 °C for 7 days. Strain YIM 131853^T was isolated using a standard dilution plate method on humic acid–vitamin (HV) agar [10.0 g glycerol, 1.0 g asparagine, 1.0 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.3 g $CaCO_3$, 10.0 mg B vitamin, 1 ml trace salt (0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $MnCl{\cdot}2H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 100 ml water), 15.0 g agar; pH 7.2) [2]. The isolated colony was selected and further purified on YIM 38 medium (4.0 g yeast extract, 4.0 g glucose, 2.5 g malt extract, 1.0 mg B vitamin, 12.0 g agar, pH 7.2) [3], the pure culture was maintained in 20% (v/v) glycerol suspensions at −80 °C. The isolation procedure was performed as described by Liu *et al.* [4]. The reference strains, *N. aerilata*

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Abbreviations: DMG, dimannosyldiacylglycerol; DPG, diphosphatidylglycerol; GL, glycolipid; L, lipid; LB, Luria–Bertani; NA, nutrient agar; PL, phospholipid; R2A, Reasoner's 2A; TSA, tryptic soy agar.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 131853^T is MK603523 and the genome sequence is SSSM00000000.

Three supplementary figures are available with the online version of this article.

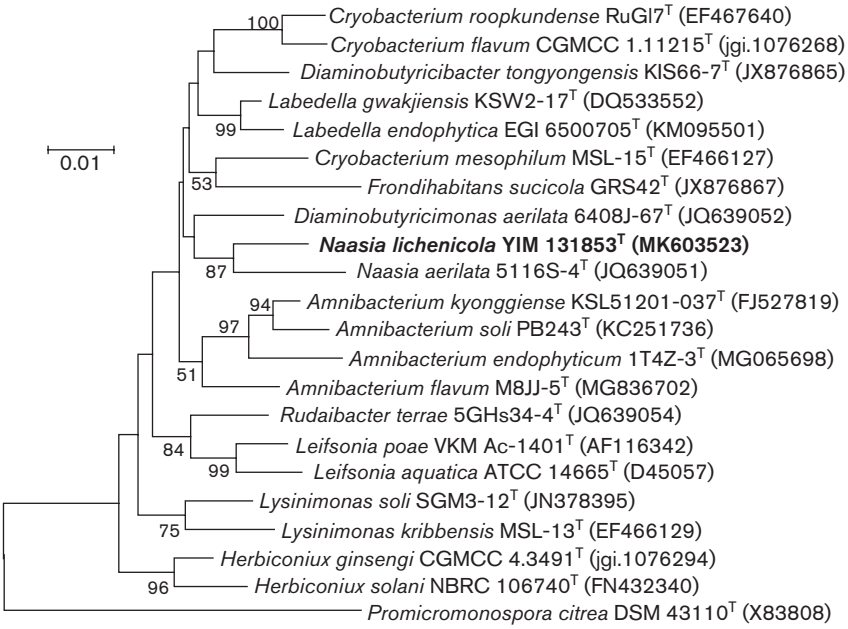

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain YIM 131853^T in relation to its nearest phylogenetic neighbours. Numbers at nodes indicate the level of bootstrap support (>50%) based on 1000 resamplings. *Promicromonospora citrea* DSM 43110^T (X83808) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

NBRC 108725^T (=5116 S-4^T) and *Amnibacterium kyonggiense* NBRC 109360^T (=KSL51201-037^T) were obtained from the NITE Biological Resource Centre (NBRC), Japan.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described by Li *et al.* [5] and Lane *et al.* [6]. The purified product was cloned by using the *p*EASY-T1 sample cloning kit. The almost-complete 16S rRNA gene sequence (1530 bp) of strain YIM 131853^T was obtained and deposited in the GenBank database with the accession number MK603523. The relative phylogenetic neighbours were identified using the EzBioCloud (www.ezbiocloud.net/) [7]. Multiple alignments with corresponding sequences of the closely related strains were aligned using CLUSTAL_X version 1.83 [8]. The phylogenetic tree was reconstructed by using three tree-making algorithms (neighbour-joining [9], maximum-likelihood [10] and maximum-parsimony [11]) using MEGA version 7.0 [12]. Bootstrap values were calculated based on 1000 replications [13]. Strain YIM 131853^T showed high levels of 16S rRNA gene sequence similarity to *A. kyonggiense* NBRC 109360^T (96.6 %) and *N. aerilata* NBRC 108725^T (96.5 %). However, the neighbour-joining phylogenetic tree showed that strain YIM 131853^T formed a distinct lineage in the genus *Naasia*, with 87% bootstrap support with *N. aerilata* NBRC 108725^T (Fig. 1). Similar topologies were also recovered by the maximum-likelihood (Fig. S1, available in the online version of this article) and maximum-parsimony algorithms (Fig. S2).

The whole-genome sequence was determined using the Illumina HiSeq 4000 sequencing platform. The draft genome was assembled using SOAPdenovo version 2.04 and the short oligonucleotide of the assembled result was further polished using SOAPaligner 2.21 [14, 15]. The draft genome of strain YIM 131853^T (4.0 M) included 3762 genes, 3664 coding sequences (CDSs), three complete rRNA genes, 47 tRNA genes and had a G+C content of 68.3 mol%. The final assembly contained 13 contigs with an N50 contig length of 683442 bp.

Cell morphology was examined by transmission electron microscope (JEM-2100, JEOL) with cells grown on YIM 38 medium at 28 °C for 5 days. Motility was assessed in YIM 38 containing 0.4% agar. Gram-staining was performed according to the procedure described by Doetsch [16]. Growth was assessed on various media, including YIM 38 medium, Reasoner's 2A (R2A; MB cell), tryptone soya agar (TSA; Difco), nutrient agar (NA; Difco) and Luria–Bertani (LB; Difco) agar. Growth at various temperatures in the range 4–45 °C (4, 10, 15, 20, 25, 28, 30, 37, 40 and 45 °C) was determined on YIM 38 medium for 5 days. The pH range for growth (pH 4.0–13.0 at intervals of 1.0 pH unit) was tested in trypticase soy broth (TSB; Difco) at 28 °C using the buffer system described by Xu *et al.* [17]. Tolerance to NaCl for growth was examined by cultivation in YIM 38 agar containing 0–8% NaCl (w/v, at 1% intervals). Antibiotic susceptibility tests were performed by the disc-diffusion method after plating with cell suspensions on YIM 38 agar (28 °C, 5 days) equivalent to 0.5 McFarland standards. Catalase activity was determined as the production of bubbles after the addition of 3% (v/v) hydrogen peroxide. Oxidase activity was determined using 1% (w/v) tetramethyl-*p*-phenylenediamine. Anaerobic growth was determined after incubation on YIM 38 medium for 14 days

at 28 °C using the GasPak EZ Anerobe Pouch System (BD). Hydrolysis of starch and cellulose, Tweens (20, 40, 60 and 80), coagulation and peptonization of milk, H_2S production, and nitrate reduction were observed using the methods described by Tindall *et al.* [18]. Enzyme activity, utilization of various carbon sources and acid production from substrates were tested with commercial API ZYM, API 20NE and API 50CH kits (bioMérieux) and Biolog GEN III Microplates according to the manufacturers' protocols. The reference strains (*N. aerilata* NBRC 108725^T and *A. kyonggiense* NBRC 109360^T) for phenotypic comparison were grown and tested under identical conditions.

Cells of strain YIM 131853^T were aerobic, Gram-stain-positive, short rod-shaped (0.4–0.8×0.9–1.2 µm) and motile with a single polar flagellum. Colonies of strain YIM 131853^T on YIM 38 agar were yellow, circular and moist after incubation at 28 °C for 5 days. Strain YIM 131853^T showed good growth on YIM 38 medium and R2A, weak growth on TSA and NA and no growth occurred on LB agar. Strain YIM 131853^T grew at 4–37 °C (optimum, 28 °C) and pH 4.0–12.0 (pH 6.0), the NaCl concentration range for growth was 0–3% (w/v; 1%). The strain was found to be sensitive to gentamicin (10 mg), rifampicin (5 mg), streptomycin (10 mg), nalidixic acid (30 mg), penicillin (10 mg), polymyxin (300 IU) and vancomycin (30 mg). Catalase activity and hydrolysis of Tweens 20, 40 and 60 were positive, while oxidase activity, milk coagulation and peptonization, nitrate reduction, and hydrolysis of gelatin, cellulose, starch and Tween 80 were negative. The detailed physiological and biochemical characteristics of strain YIM 131853^T are presented in Table 1 and in the species description.

For chemotaxonomic analysis, cell biomass of strain YIM 131853^T was harvested after cultivation on YIM 38 at 28 °C for 5 days. Cell-wall amino acids and whole-cell sugars were extracted, detected and analysed according to procedures described by Schleifer and Kandler [19] and Tang *et al.* [20]. Polar lipids were extracted and analysed by two-dimensional TLC according to Minnikin *et al.* [21]. The cellular fatty acids were extracted and analysed according to the standard protocol of the Microbial Identification System (MIDI) [22, 23]. Menaquinones were extracted and analysed as described previously using reversed-phase HPLC [24, 25].

The peptidoglycan of strain YIM 131853^T contained glutamic acid (Glu), glycine (Gly), alanine (Ala) and 2,4-diaminobutyric acid (DAB) as cell-wall amino acids, which was consistent with *N. aerilata* NBRC 108725^T [1] and different from *A. kyonggiense* NBRC 109360^T [26]. Whole-cell sugars consisted largely of glucose and galactose. The polar lipid profile of strain YIM 131853^T were diphosphatidylglycerol, DMG, three unknown glycolipids, two unknown phospholipids and one unknown lipid (Fig. S3) which was similar to *N. aerilata* NBRC 108725^T, but phosphatidylglycerol was absent in strain YIM 131853^T. It differed from *A. kyonggiense* NBRC 109360^T based on the difference of DMG as the predominant polar lipid [27]. The major cellular fatty acids (>10%) were anteiso-$C_{15:0}$ (68.4%), anteiso-$C_{17:0}$ (16.0%) and iso-$C_{16:0}$ (12.0%) which were similar

Table 1. Phenotypic characteristics that differentiate strain YIM 131853^T from its closely related reference strains

Strains: 1, YIM 131853^T; 2, *Naasia aerilata* NBRC 108725^T; 3, *Amnibacterium kyonggiense* NBRC 109360^T. +, Positive; –, negative. All data were obtained from this study except where indicated. All strains were negative for hydrolysis of gelatin, cellulose, starch and Tween 80. In API 20NE tests, all strains were positive for PNPG and hydrolysis of aesculin, D-glucose and D-mannitol; negative for production of indole, fermentation of glucose, L-arginine, urease, gelatin hydrolysis, *N*-acetylglucosamine, potassium gluconate, citrate and adipic acid. In the API ZYM kits, all strains were positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase. In the API 50CH kits, all strains were positive for acid production from L-arabinose D-ribose, D-xylose and trehalose.

Characteristic	1	2	3
Isolation source	Lichen	Air	Water
Colony colour	Yellow	Light yellow	Yellow
Morphology	Short rod	Rod*[a]	Short rod*[b]
Motility	+	+	–
Flagella	One	One*[a]	Non*[b]
Growth at (°C)	4–37	10–37	10–37
pH range for growth	4.0–12.0	6.0–10.0	5.0–10.0
Tolerance of NaCl (%, w/v)	0–3	0–1	0–3
Grow on YIM 38 medium	+	–	+
Catalase/ Oxidase	+/–	–/+	+/–
Nitrate reduction	–	+	–
Hydrolysis of:			
Tweens 20, 40, 60	+	–	+
Assimilation of:			
L-Arabinose	+	–	–
D-Mannose	+	–	–
Maltose	+	–	+
Capric acid	+	–	–
Malic acid	+	+	–
Phenylacetic acid	+	–	–
Enzyme activity:			
Alkaline phosphatase	–	–	+
Trypsin	+	+	–
α-Galactosidase	–	–	+
β-Galactosidase	–	+	+
N-acetyl-β-Glucosaminidase	+	–	–
α-Mannosidase	+	–	+
α-Fucosidase	+	–	–

Continued

Table 1. Continued

Characteristic	1	2	3
Acid production from：			
D-Glucose	–	+	+
D-Mannose	–	+	+
D-Fructose	–	–	+
L-Sorbose	–	+	–
Dulcitol	–	+	–
D-Mannitol	+	–	+
Amygdalin	+	–	–
Arbutin	+	–	+
Aesculin ferric citrate	+	–	+
Gentiobiose	+	–	+
D-Tagatose	–	–	+
Potassium 5-ketogluconate	–	–	+
Menaquinones (MK)	10,14,13,12	10,14,13,12	11,12
DNA G+C content (mol%)	68.3	70.0*[a]	72.7*[b]

*Data from other studies indicated as: a, Weon *et al.* [1]; b, Kim and Lee [26].

to the related reference strains. Strain YIM 131853^T differed from *N. aerilata* NBRC 108725^T for the existence of minor anteiso-$C_{15:1}$ A and the proportion of major cellular fatty acids, and the absence of antesio-$C_{19:0}$ and summed feature 4 comprising anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I differentiated it from *A. kyonggiense* NBRC 109360^T. The detailed fatty acid profiles of strain YIM 131853^T and its related reference strains are shown in Table 2. The menaquinones were MK-10 (38%), MK-14 (27%), MK-13 (23%) and MK-12 (12%), which were the typical menaquinones of the genus *Naasia*.

In summary, the major phenotypic characteristics of strain YIM 131853^T were consistent with descriptions of the genus *Naasia* with regard to morphological, biochemical and chemotaxonomic properties. Meanwhile, the result of G+C content and the phenotypic differences between strain YIM 131853^T and its closest phylogenetic neighbours indicated that strain YIM 131853^T should be assigned to the genus *Naasia* as a novel species, for which the name *Naasia lichenicola* sp. nov. is proposed.

DESCRIPTION OF *NAASIA LICHENICOLA* SP. NOV.

Naasia lichenicola (li.che.ni'co.la. L. masc. n. *lichen* a lichen; L. suff. *-cola* (from L. masc. or fem. n. *incola*) inhabitant, dweller; N.L. fem. n. *lichenicola* a dweller of lichens).

Cells are Gram-stain-positive, aerobic, catalase-positive, oxidase-negative, motile with one flagellum and

Table 2. Cellular fatty acid profile of strain YIM 131853^T and its closely related reference strains

Strains: 1, YIM 131853^T; 2, *Naasia aerilata* NBRC 108725^T; 3, *Amnibacterium kyonggiense* NBRC 109360^T. All data were obtained from this study. Values are % of total fatty acids. The major fatty acids (greater than 10%) are shown bold. –, Not detected.

Fatty acid	1	2	3
Saturated:			
$C_{16:0}$	0.3	2.5	0.9
Branched saturated:			
anteiso-$C_{15:0}$	**68.4**	**43.6**	**53.0**
anteiso-$C_{17:0}$	**16.0**	**21.1**	6.6
anteiso-$C_{19:0}$	–	–	1.3
anteiso-$C_{15:1}$ A	1.9	–	–
iso-$C_{14:0}$	0.6	1.8	6.1
iso-$C_{15:0}$	0.5	0.7	1.9
iso-$C_{16:0}$	**12.0**	**30.2**	**24.2**
Summed feature 4*	–	–	1.5

*Summed feature 4 contains anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I.

short-rod-shaped (0.4–0.8×0.9–1.2 μm). Colonies on YIM 38 medium are yellow, moist and circular. Grows well on YIM 38 and R2A media, weakly on TSA and NA, but not at all on LB agar. Growth occurs at 4–37 °C (optimum, 28 °C), at pH 4.0–12.0 (pH 6.0) and at 1–3 NaCl (1%). Hydrolysis of Tweens 20, 40 and 60 are positive, but milk coagulation and peptonization, nitrate reduction, and hydrolysis of gelatin, cellulose, starch and Tween 80 are negative. In the API 20NE tests, hydrolysis of aesculin and 4-nitrophenyl-*β*-D-glucopyranoside and assimilation of D-glucose, L-arabinose, D-mannitol, D-mannose, maltose, capric acid, malic acid and phenylacetic acid are positive. Production of indole, fermentation of glucose, urease and L-arginine and assimilation of potassium gluconate, *N*-acetylglucosamine, citrate and adipic acid are negative. Shows the following enzyme activities: positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, *α*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase and *α*-fucosidase; weakly positive for naphthol-AS-BI-phosphohydrolase and *α*-mannosidase; and negative for alkaline phosphatase, lipase (C14), *α*-chymotrypsin, *α*-galactosidase, *β*-galactosidase and *β*-glucuronidase. In the Biolog GEN III system tests, the following substrates are used as a source of energy: dextrin, maltose, trehalose, cellobiose, sucrose, lactose, methyl *β*-D-glucoside, D-salicin, *N*-acetyl-*β*-D-mannosamine, *α*-D-glucose, D-mannose, D-fructose, D-galactose, 3-methyl-glucose, L-rhamnose, sodium lactate, D-mannitol, glycerol, pectin, D-gluconic acid, methyl, L-lactic acid, D-malic acid, Tween 40, *α*-hydroxy-butyric acid, *β*-hydroxy-D,L-butyric acid, *α*-keto-butyric acid and acetic acid are positive, while others are not used.

The cell-wall peptidoglycan contains 2,4-diaminobutyric acid as the diamino acid. Whole-cell sugars consists largely of glucose and galactose. The major cellular fatty acids (>10%) are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The polar lipid profile comprises diphosphatidylglycerol, DMG, three unknown glycolipids, two unknown phospholipids and one unknown lipid. The predominant menaquinones are MK-10, MK-14, MK-13 and MK-12.

The type strain, YIM 131853^T (=CGMCC 4.7565^T=NBRC 113605^T), was isolated from lichen collected from the South Bank of the Baltic Sea. The DNA G+C content based on the draft genome sequence is 68.3 mol%.

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

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