Antonie van Leeuwenhoek (2012) 102:621–628
DOI 10.1007/s10482-012-9757-4

ORIGINAL PAPER

Kineococcus endophytica sp. nov., a novel endophytic actinomycete isolated from a coastal halophyte in Jiangsu, China

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Received: 20 April 2012 / Accepted: 23 May 2012 / Published online: 6 June 2012

Abstract A novel Gram-positive, motile, non-spore-forming coccus-shaped bacterial strain, designated KLBMP 1274^T, was isolated from a halophytic plant (*Limonium sinense*) collected from the coastal region of Nantong, Jiangsu Province, in east China. Phylogenetic analyses based on the 16S rRNA gene sequence showed that strain KLBMP 1274^T belongs to the genus *Kineococcus* and is closely related to *Kineococcus rhizosphaerae* RP-B16^T (98.72 %), *Kineococcus aurantiacus* IFO 15268^T (98.71 %), *Kineococcus radiotolerans* SRS30216^T (98.69 %) and *Kineococcus gynurae* KKD096^T (97.33 %). The 16S rRNA gene sequence similarity to other species of the genus *Kineococcus* was <97 %. The cell wall contained *meso*-diaminopimelic acid as the diagnostic diamino acid, with arabinose and galactose as the characteristic sugars. The predominant menaquinone was MK-9(H_2). The polar lipids were found to be diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, an unknown phospholipid, an unknown glycolipid, and three unknown lipids. Major cellular fatty acids were found to be anteiso-$C_{15:0}$ and iso-$C_{14:0}$. The chemotaxonomic data for strain KLBMP 1274^T were typical of the genus *Kineococcus*. The total DNA G+C content was 73.4 mol %. DNA–DNA relatedness and differential phenotypic data demonstrated that strain KLBMP 1274^T was clearly distinguished from all closely related species of the genus *Kineococcus*. Thus, strain KLBMP 1274^T represents a novel species of the genus *Kineococcus*, for which the name *Kineococcus endophytica* sp. nov. is proposed. The type strain is KLBMP 1274^T (=KCTC 19886 ^{T} = NBRC 108674^T).

Keywords *Kineococcus endophytica* sp. nov. · 16S rRNA gene · Endophytic actinomycete · Polyphasic taxonomy

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Electronic supplementary material The online version of this article (doi:10.1007/s10482-012-9757-4) contains supplementary material, which is available to authorized users.

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Introduction

The genus *Kineococcus* was proposed by Yokota et al. (1993) to accommodate an aerobic, Gram-positive, motile, coccus-shaped bacterium with *meso*-diaminopimelic acid in the whole-cell hydrolysate, arabinose, and galactose as the characteristic cell wall sugars and MK-9(H_2) as the predominant menaquinone. At the time of writing, the genus *Kineococcus* has five species with validly published names: *Kineococcus aurantiacus* (Yokota et al. 1993), *Kineococcus radiotolerans* (Phillips et al. 2002), *Kineococcus gynurae* (Duangmal et al. 2008), *Kineococcus xinjiangensis* (Liu et al. 2009), and *Kineococcus rhizosphaerae* (Lee 2009). Recently, another new species awaiting validation, *Kineococcus glutineturens* (Nie et al. 2012), was found from a dry-hot river valley soil sample, in Yunnan, south-west China. During a search for novel endophytic rare actinobacteria from coastal halophytes, Jiangsu Province, east China, a *Kineococcus*-like strain was isolated. Comparative 16S rRNA gene sequence analysis indicated that strain KLBMP 1274^T was phylogenetically most closely affiliated to the genus *Kineococcus*. In this study, we report the taxonomic characterization of strain KLBMP 1274^T by using a polyphasic approach. The data obtained demonstrate that this strain represents a novel species of the genus *Kineococcus*, for which the name *Kineococcus endophytica* sp. nov. is proposed.

Materials and methods

Bacterial strains and culture conditions

Healthy leaf samples of a coastal halophytic medicinal plant, *Limonium sinense* (Girard) Kuntze, collected from the coastal region of Nantong, Jiangsu Province, east China were used as sources for the isolation of endophytic actinomycetes. The samples were firstly surface sterilized using previously described procedures (Qin et al. 2009). Subsequently, the surface sterilized leaves were aseptically crumbled into smaller fragments using a commercial blender (Joyoung) and then spread onto glycerol-asparagine agar (ISP 5; Shirling and Gottlieb 1966). The pure culture obtained was preserved on yeast extract-malt extract agar (ISP 2; Shirling and Gottlieb 1966) and maintained as a glycerol suspension (20 %, w/v) at −80 °C. This strain was deposited in the NITE Biological Resource Center (NBRC) as strain NBRC 108674^T and in the Korean Collection for Type Cultures (KCTC) as strain KCTC 19886^T.

Morphological, physiological, and biochemical characterization

Cell morphology and mobility were examined using light microscopy (SA3300-PL), scanning electron microscopy (Hitachi; S-3400 N) and transmission electron microscopy (H-7650; Hitachi) with cells grown on ISP 2 medium for 7 days and on LB agar for 3 days at 28 °C. Gram staining was carried out by the standard Gram reaction in parallel with the KOH lysis test (Gregersen 1978). Morphological characteristics were determined after incubation for 14 days at 28 °C on various media described by Shirling and Gottlieb (1966) including yeast extract-malt extract (ISP 2), oatmeal (ISP 3), inorganic salt-starch (ISP 4), glycerol-asparagine (ISP 5), potato-dextrose agar, Czapek's agar, and nutrient agar (Waksman 1967). The ISCC-NBS color charts were used to determine the designations of colony colors (Kelly 1964). Growth was tested on ISP 2 agar at 4, 10, 15, 20, 28, 32, 37, 40, 45, and 50 °C, and with 0–15 % NaCl (at intervals of 1 % NaCl). The pH range (pH 4.0–12.0, at intervals of 1.0 pH units) for growth was determined using ISP 2 broth medium adjusted with 1 M HCl, 20 % (w/v) Na_2CO_3 and 1 M NaOH solution after sterilization and incubated at 28 °C for 14 days. Carbon-source utilization was tested by using ISP 9 medium (Shirling and Gottlieb, 1966) supplemented with 1 % (final concentration) carbon sources. Nitrogen source utilization was examined using the basal medium recommended by Williams et al. (1983) supplemented with a final concentration of 0.1 % of the nitrogen sources. Production of acid and other physiological and biochemical characteristics were tested by using well established procedures (Gordon et al. 1974). Four phylogenetic nearest reference *Kineococcus* strains (*K. rhizosphaerae* RP-B16^T, *K. aurantiacus* IFO 15268^T, *K. radiotolerans* SRS30216^T and *K. gynurae* KKD096^T) were used for comparison under the same conditions.

Molecular studies

To determine the taxonomic status of the isolate, extraction of genomic DNA, amplification and sequencing of the 16S rRNA gene were carried out using the methodology described by Li et al. (2007). A nearly

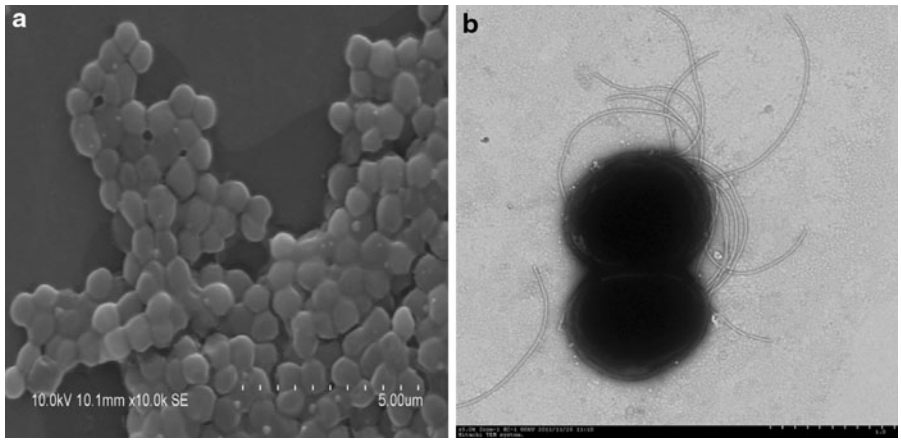

Fig. 1 Scanning electron micrograph of strain KLBMP 1274^T grown on ISP 2 agar for 7 days at 28 °C (**a**) and transmission electron micrograph of cells grown on LB agar for 3 days at 28 °C (**b**). *Bar*, 5 μm (**a**) and 1 μm (**b**)

complete 16S rRNA gene sequence was determined for strain KLBMP 1274^T (1,453 bp) by using an ABI PRISM 3730XL automatic sequencer. The determined sequence was compared with available 16S rRNA gene sequences in GenBank databases using the BLAST program (http://blast.ncbi.nlm.nih.gov/). Identification of phylogenetic neighbors and calculation of levels of pairwise 16S rRNA gene sequence similarity were achieved by using the EzTaxon-e database (Kim et al. 2012). Multiple alignments with sequences from closely related species were performed by using the Clustal W program in MEGA version 5 (Tamura et al. 2011). Evolutionary distances were calculated using Kimura's two-parameter (Kimura 1980). Phylogenetic trees were inferred using neighbor-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971) and the maximum-likelihood (Felsenstein 1981) tree making algorithms from the MEGA version 5 program (Tamura et al. 2011). The topologies of the resultant trees were evaluated in a bootstrap analysis (Felsenstein 1985) based on 1,000 resamplings.

Levels of DNA–DNA relatedness were determined according to the fluorometric micro-well method (Ezaki et al. 1989; He et al. 2005). The 16S rRNA gene sequence of strain KLBMP 1274^T determined in this study has been deposited in GenBank under the accession no: JQ819257.

Chemotaxonomic characterization

Biomass for most chemotaxonomic studies was prepared by growing the strain in ISP 2 broth at 150 rpm for 7 days at 28 °C. Cells were harvested by centrifugation, washed in distilled water, re-centrifuged and freeze-dried. The isomer of diaminopimelic acid and sugar analysis of whole-cell hydrolysates were performed according to the procedures described by Hasegawa et al. (1983) and Lechevalier and Lechevalier (1970). Analysis of mycolic acids was performed using the previously described method by Minnikin et al. (1980). Polar lipids were extracted with chloroform/methanol (1:2) and identified by two-dimensional TLC followed by spraying with the appropriate detection reagent (Minnikin et al. 1984). Menaquinones were extracted and purified as described by Collins et al. (1977) and analyzed by HPLC (Groth et al. 1997). Fatty acids were analyzed using the standard MIDI (Microbial Identification, Sherlock version 6.0) procedure (Sasser 1990) and a Agilent GC 6850 gas chromatograph, with cells grown on ISP 2 agar plates for 5 days at 28 °C. The resulting profiles were identified using the database library TSBA6 version 6.0. The DNA G+C content was determined by the method of Mesbah et al. (1989).

Results and discussion

Morphological, cultural, physiological, and biochemical characteristics

Cells of strain KLBMP 1274^T were observed to be aerobic, Gram positive, non-spore-forming, and coccoid-shaped. No hyphae were present. The cells

Table 1 Differential phenotypic characteristics between strain KLBMP 1274^T and the type strains of related species of the genus *Kineococcus*

Characteristic	1	2	3	4	5
Assimilation of sole carbon sources:					
D-Arabinose	−	−	−	−	+
Cellobiose	+	+	+	−	+
Inositol	+	−	−	−	−
Lactose	−	−	−	+	+
Maltose	+	−	+	+	+
D-Mannose	−	+	−	−	−
Raffinose	−	+	+	−	−
Sorbitol	−	−	−	−	+
Xylitol	−	−	−	−	+
Acid produced from:					
Galactose	+	+	+	−	−
Fructose	+	−	−	−	−
Maltose	+	−	+	−	−
Glucose	+	−	+	+	+
Inositol	+	−	−	−	−
Assimilation of sole nitrogen sources:					
L-Arginine	−	−	+	−	−
L-Asparagine	+	−	−	+	+
L-Cysteine	−	−	−	+	−
L-Histidine	+	+	−	−	−
L-Alanine	+	−	+	+	−
L-Serine	+	+	−	−	−
L-Tyrosine	−	+	+	−	−
L-Valine	−	+	−	−	+
Growth at 4 °C	+	−	−	−	−
Growth at 37 °C	+	+	+	+	−
Growth on 7 % NaCl	−	−	−	−	+
Polar lipids	DPG, PG, PI, PIM, PL, GL, 3L	DPG, PG, PI, 2PL*	DPG, PG, GL*	DPG, PG, PI, 3PL*	ND
DNA G+C content (mol %)	73.4	73.8*	73.9*	74.3*	73.3*

Strains: *1* KLBMP 1274^T; *2* *Kineococcus rhizosphaerae* RP-B16^T; *3* *Kineococcus aurantiacus* IFO 15268^T; *4* *Kineococcus radiotolerans* SRS30216^T; *5* *Kineococcus gynurae* KKD096^T

* Data from Lee (2009), Duangmal et al. (2008), Phillips et al. (2002), and Yokota et al. (1993)

\+ Positive

− Negative

ND no data available

occurred in pairs or in clusters and agglutinated strongly with each other and were motile by means of forming flagella (Fig. 1). Colonies were circular, convex, and orange in color on ISP 2 medium after 5 days at 28 °C. Strain KLBMP 1274^T grew well on ISP 2, ISP 4, ISP 5, nutrient agar, and potato-dextrose agar media, and moderately on ISP 3 and Czapek's medium agar. No diffusible pigments were produced on the tested media. Growth occurred at 4–37 °C (optimum 28 °C), pH 6–11 (optimum pH 7.0) and at up to 5 % NaCl. Strain KLBMP 1274^T hydrolyzed aesculin, Tweens 20 and 40. H_2S was not produced. Other physiological characteristics are given in the type strain description and Table 1.

Phylogenetic analysis

The almost complete sequence of the 16S rRNA gene of strain KLBMP 1274^T (GenBank accession no: JQ819257) was used for phylogenetic analysis. Phylogenetic analyses of 16S rRNA gene sequences showed that strain KLBMP 1274^T is a member of the genus *Kineococcus* (Fig. 2). It is evident from the 16S rRNA gene neighbor-joining tree that strain KLBMP

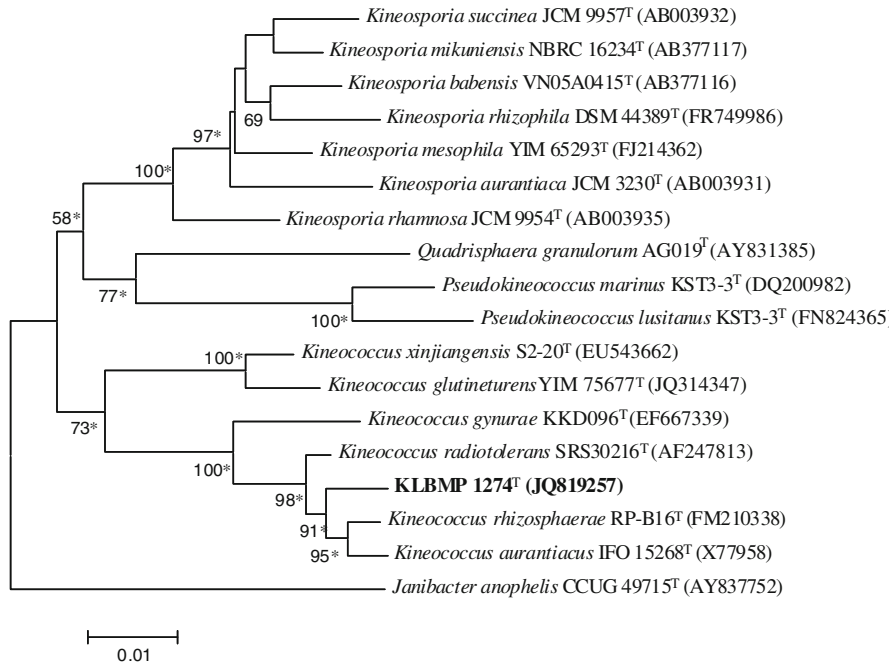

Fig. 2 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between strain KLBMP 1274^T and other species of the genera *Kineococcus*, *Kineosporia*, *Pseudokineococcus,* and *Quadrisphaera*. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50 % are given at nodes. *Asterisks* indicate branches of the tree that were recovered using maximum parsimony and maximum-likelihood analyses. The sequence of *Janibacter anophelis* CCUG 49715^T was used as an outgroup. *Bar* 0.01 sequence variation

1221^T formed a distinct subclade with 91 % bootstrap support within the cluster of *K. rhizosphaerae* RP-B16^T, *K. aurantiacus* IFO 15268^T, and *K. radiotolerans* SRS30216^T. The relationship between strain KLBMP 1274^T and *K. rhizosphaerae* RP-B16^T, *K. aurantiacus* IFO 15268^T, and *K. radiotolerans* SRS30216^T was also maintained in the trees constructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 2). Strain KLBMP 1274^T exhibited 16S rRNA gene sequence similarity values of 98.72, 98.71, 98.69, and 97.33 % with its nearest neighbors *K. rhizosphaerae* RP-B16^T, *K. aurantiacus* IFO 15268^T, *K. radiotolerans* SRS30216^T, and *K. gynurae* KKD096^T, respectively. Levels of 16S rRNA gene sequence similarity between strain KLBMP 1274^T and other *Kineococcus* species were less than 97 %.

DNA–DNA relatedness

DNA–DNA relatedness values among strain KLBMP 1274^T and the nearest type strains *K. rhizosphaerae* RP-B16^T, *K. aurantiacus* IFO 15268^T, *K. radiotolerans* SRS30216^T, and *K. gynurae* KKD096^T were 45 ± 2.1, 43.4 ± 1.6, 38.6 ± 2.8, and 41.7 ± 4.0 %, respectively, when their DNAs were used individually as labeled DNA probes for cross-hybridization.

Chemotaxonomic characteristics

The results of chemical analysis indicated that strain KLBMP 1274^T had chemotaxonomic markers typical of members of the genus *Kineococcus* (Yokota et al. 1993). Strain KLBMP 1274^T contained *meso*-diaminopimelic acid in the peptidoglycan and MK-9(H_2) as the predominant menaquinone. No mycolic acids were detected. Cellular fatty acid profiles of strain KLBMP 1274^T and three type strains of the genus *Kineococcus* grown and analyzed under identical conditions in this study are compared in Table 2. The major fatty acids (>5 % of the total fatty acids) found in strain KLBMP 1274^T were anteiso-$C_{15:0}$ (74.7 %) and iso-$C_{14:0}$ (9.6 %). The fatty acid profiles of strain KLBMP 1274^T and related type strains were basically similar, even though there were differences in the proportions of some fatty acids. The polar lipid profile was found to consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, an unknown phospholipid, an unknown glycolipid, and three unknown lipids (see Online Supplementary Fig. S1). The G+C content of the DNA was 73.4 mol %, a value in the range reported for *Kineococcus* species (Table 1).

Table 2 Fatty acid profiles (%) of strain KLBMP 1274^T and related *Kineococcus* species. All the data are from this study. Values are percentages of total fatty acids; fatty acids amounting to less than 0.5 % in all species are not shown

Fatty acid	1	2	3	4
iso-$C_{14:0}$	9.6	14.8	15.1	16.5
iso-$C_{15:0}$	0.5	0.6	0.9	–
iso-$C_{16:0}$	–	0.9	1.1	1.1
$C_{14:0}$	1.3	2.5	1.0	–
$C_{16:0}$	–	1.0	1.1	0.8
anteiso-$C_{15:0}$	74.7	71.8	65.0	70.2
anteiso-$C_{15:1}$ A	0.9	2.3	3.5	5.9
$C_{14:0}$ 2 OH	2.6	1.9	1.7	–
$C_{17:0}$ 2 OH	1.8	0.9	–	–
$C_{17:0}$ 3 OH	1.4	1.0	2.2	1.3
$C_{18:0}$ 3 OH	–	0.6	1.2	–
$C_{16:0}$ N alcohol	–	1.1	1.5	1.0
$C_{17:1}\omega 7c$	–	–	0.5	0.7
$C_{18:1}\omega 9c$	–	–	1.8	–
Summed feature 1*	1.7	–	–	–
Summed feature 2*	0.9	–	0.8	–
Summed feature 5*	–	–	0.9	–

Strains: *1* KLBMP 1274^T; *2 Kineococcus rhizosphaerae* RP-B16^T; *3 Kineococcus aurantiacus* IFO 15268^T; *4 Kineococcus radiotolerans* SRS30216^T

* Summed features represent groups of two or three fatty acids that cannot be separated by GC with the MIDI system; Summed feature 1 contains one or more of the fatty acids iso-$C_{15:1}$ H and $C_{13:0}$ 3OH; Summed feature 2 contains one or more of the fatty acids $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I); Summed feature 5 contains one or more of the fatty acids $C_{18:2}$ $\omega 6,9c$ and/or anteiso-$C_{18:0}$

– not detected

Conclusion

Strain KLBMP 1274^T was differentiated from the closest type *Kineococcus* species by differences in several phenotypic characteristics, including the different growth characteristics at 4 and 37 °C, on 7 % NaCl, differences in utilization of carbon sources and nitrogen sources and differences of acid production from some substrates obtained by using the same methods under the same conditions in this study. DNA–DNA related data revealed that strain KLBMP 1274^T differs genetically from the type strains of *K. rhizosphaerae* RP-B16^T, *K. aurantiacus* IFO 15268^T, *K. radiotolerans* SRS30216^T, and *K. gynurae* KKD096^T due to the relatively low hybridization values. These values are well below the 70 % cut-off point for assigning strains to the same species (Wayne et al. 1987). Thus, from the unique phenotypic, chemotaxonomic, and genotypic characteristics of strain KLBMP 1274^T, it can be distinguished from previously described *Kineococcus* species, which supports its classification as a new species of the genus *Kineococcus*, for which the name *Kineococcus endophytica* is proposed. The type strain is strain KLBMP 1274^T (=KCTC 19886 ^{T} = NBRC 108674^T).

Description of *Kineococcus endophytica* sp. nov

Kineococcus endophytica (en.do.phy′ti.ca. Gr. pref. *endo*, within; Gr. n. *phyton*, plant; L. fem. suff. -ica, adjectival suffix used with the sense of belonging to; N.L. fem. adj. *endophytica*, within plant, *endophytic*, pertaining to the original isolation from plant tissues).

Cells are aerobic, Gram-positive-staining, non-spore-forming, oxidase-negative, and catalase-positive, motile cocci (about 0.6–1.0 μm in diameter). Colonies are circular, smooth, and orange. Growth is observed at 4–37 °C and pH 6–11, with optimum growth at 28 °C and pH 7.0. The NaCl range for growth is 0–5 % (w/v). Hydrolyses aesculin, Tweens 20 and 40 but not casein, chitin, cellulose, elastin, Tween 80, or starch. Utilizes cellobiose, dextrin, D-fructose, D-galactose, D-glucose, inositol, maltose, rhamnose, trehalose, and D-xylose as sole carbon sources but D-arabinose, D-lactose, mannose, D-raffinose, D-ribose, sorbitol, and xylitol are not. Acid is produced from D-fructose, D-galactose, D-glucose, inositol, maltose, and D-xylose. Uses L-alanine, L-asparagine, L-histidine, L-proline, and L-serine as sole nitrogen sources. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. Whole-cell sugars contain arabinose and galactose. Mycolic acids are not present. The predominant menaquinone is MK-9(H_2). The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, an unknown phospholipid, an unknown glycolipid, and three unknown lipids. The major fatty acids are anteiso-$C_{15:\ 0}$ and iso-$C_{14:\ 0}$. The G+C content of the DNA is 73.4 mol%. The 16S rRNA gene sequence of strain KLBMP 1274^T has been deposited in GenBank under the accession no: JQ819257.

The type strain, KLBMP 1274^T (=KCTC 19886 ^{T} = NBRC 108674^T) was isolated from surface-sterilized leaves of the coastal halophyte *Limonium sinense*

(Girard) Kuntze collected from the coastal region of Nantong, Jiangsu Province, east of China.

Acknowledgments The authors are grateful to Prof. Tomohiko Tamura (NITE Biological Resource Center, NBRC) for kindly providing the type strains and Prof. Iain C. Sutcliffe for his valuable comments on the manuscript. This research was partially supported by National Natural Science Foundation of China (31000005, 31101502), the Program of Natural Science Foundation of the Jiangsu Higher Education Institutions (10KJB180008, 11KJD210002), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and Natural Science Foundations by Xuzhou City (No. XZZD1004).

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