Phycococcus jejuensis gen. nov., sp. nov., an actinomycete isolated from seaweed

Soon Dong Lee

Correspondence

Soon Dong Lee
sdlee@cheju.ac.kr

Department of Science Education, Cheju National University, Jeju 690-756, Republic of Korea

A marine actinomycete strain, designated KSW2-15^T, was isolated from a dried seaweed sample collected from a sandy beach on the coast of Jeju in the Republic of Korea. The organism produced non-motile, non-endospore-forming, Gram-positive, coccoid cells. The colonies were circular, translucent and yellow in colour with entire margins. *meso*-Diaminopimelic acid was present as the diamino acid of the peptidoglycan. The acyl type of the muramic acid was acetyl. Mycolic acids were not present. The predominant menaquinone was MK-8(H₄). The polar lipids were phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol. The major cellular fatty acids were of the saturated, unsaturated and iso-branched methyl types. The DNA G + C content was 74 mol%. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain KSW2-15^T formed a loose association with '*Candidatus Nostocoida limicola*', within the radiation of the family *Intrasporangiaceae* of the suborder *Micrococcineae*. The organism showed the highest levels of sequence similarity with '*Candidatus Nostocoida limicola*' (96.1%), *Terrabacter tumescens* (96.1%) and *Terrabacter terrae* (96.0%). The levels of 16S rRNA gene sequence similarity between the isolate and members of other genera of the family *Intrasporangiaceae* were in the range 92.1–95.5%. On the basis of the polyphasic evidence, the isolate should be classified within a novel genus and species, for which the name *Phycococcus jejuensis* gen. nov., sp. nov. is proposed. The type strain of *Phycococcus jejuensis* is strain KSW2-15^T (=KCCM 42315^T =NRRL B-24460^T).

The family *Intrasporangiaceae* Rainey *et al.* 1997 emend. Stackebrandt & Schumann 2000 (Stackebrandt *et al.*, 1997; Stackebrandt & Schumann, 2000) currently includes 11 genera with validly published names and can be divided into three groups according to the isomer and the kind of diamino acid in the peptidoglycan. The group possessing LL-diaminopimelic acid (DAP) in the peptidoglycan contains four genera: *Arsenicococcus* (Collins *et al.*, 2004), *Intrasporangium* (Kalakoutskii *et al.*, 1967), *Terrabacter* (Collins *et al.*, 1989) and *Terracoccus* (Prauser *et al.*, 1997). Members of the second group have L-ornithine in the cell wall; this group contains three genera, namely *Ornithinicoccus* (Groth *et al.*, 1999), *Ornithinimicrobium* (Groth *et al.*, 2001) and *Serinicoccus* (Yi *et al.*, 2004). Only four genera in the family, *Janibacter* (Martin *et al.*, 1997), *Knoellia* (Groth *et al.*, 2002), *Oryzihumus* (Kageyama *et al.*, 2005) and *Tetrasphaera* (Maszenan *et al.*, 2000), have both *meso*-DAP in the peptidoglycan and MK-8(H₄) as the major menaquinone, but these genera can be readily distinguished on the basis of other phenotypic characteristics (Martin

et al., 1997; Maszenan *et al.*, 2000; Hanada *et al.*, 2002; Groth *et al.*, 2002; Kageyama *et al.*, 2005).

During the course of studying marine bacteria sampled from the coast of Jeju in the Republic of Korea, a yellow-pigmented actinomycete, strain KSW2-15^T, was isolated from a dried seaweed sample collected at Gwakji beach and was subjected to polyphasic taxonomic characterization. For bacterial isolation, a piece of dried seaweed was directly transferred onto WAT agar plates (Li *et al.*, 2002) supplemented with 60% (v/v) sterilized natural seawater. This isolation medium (WAT-SW agar) consisted of 0.05% MgSO₄·7H₂O, 0.05% CaCl₂·2H₂O and 1.5% agar in 60% natural seawater and 40% distilled water (pH 7.3). A colony on the plate, incubated at 30 °C for 14 days, was subcultured on YE-SW medium (0.4% yeast extract, 1.0% malt extract, 0.4% glucose, 1.8% agar, 60% natural seawater and 40% distilled water). The pure culture was maintained at –20 and –80 °C in a 20% glycerol suspension supplemented with 60% natural seawater.

The DAP isomer, the cell-wall acyl type and the presence of mycolic acids were determined as described by Stanek & Roberts (1974), Uchida & Aida (1984) and Minnikin *et al.* (1980), respectively. Polar lipids and menaquinones were extracted by using the integrated procedure of Minnikin

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KSW2-15^T is DQ345443.

et al. (1984). Polar lipid profiles were determined by two-dimensional TLC (Minnikin *et al.*, 1977). Menaquinones were analysed by using HPLC (Kroppenstedt, 1985). For the above analyses, the organism was cultivated on YE-SW broth for 3 days at 30 °C and harvested by centrifugation at 3000 r.p.m. for 20 min. Extraction of the cellular fatty acids and determination of the fatty acid composition were performed by gas chromatography performed according to the instructions of the Microbial Identification System (MIDI), using cells grown on trypticase soy agar (Difco) for 3 days at 30 °C. The DNA G + C content was determined by HPLC (Mesbah *et al.*, 1989). The results of the chemotaxonomic analyses are given in the genus description. The fatty acid profile of the organism was characterized by the predominance of C_{17:1}ω8c (19.4 %), iso-C_{16:0} (15.1 %), iso-C_{15:0} (13.9 %), C_{15:0} (9.5 %) and C_{17:0} (8.8 %). Other fatty acids present at levels above at least 1 % were iso-C_{14:0} (5.3 %), anteiso-C_{15:0} (4.8 %), C_{17:0} 3-OH (4.5 %), C_{17:0} 10-methyl (3.1 %), C_{16:0} (2.7 %), C_{12:0} (2.2 %), C_{18:1}ω8c (1.9 %), anteiso-C_{17:0} (1.8 %) and C_{18:0} (1.1 %).

The 16S rRNA gene sequence was determined as described elsewhere (Lee *et al.*, 2000; Lee & Jeong, 2006). Phylogenetic analyses were performed by using three tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods on a multiple alignment matrix (Thompson *et al.*, 1997). A neighbour-joining tree was reconstructed from evolutionary distances calculated using the Jukes–Cantor coefficient (Jukes & Cantor, 1969). The tree topology was evaluated by means of a bootstrap analysis (Felsenstein, 1985) of 1000 replicated datasets. An almost-complete 16S rRNA gene sequence for strain KSW2-15^T, containing a continuous stretch of 1413 nt, was compared with those of related organisms from the family *Intrasporangiaceae*. A total of 1259 unambiguous aligned positions present in all strains were used for phylogenetic analyses. A neighbour-joining tree (Fig. 1) based on 16S rRNA gene sequences revealed that strain KSW2-15^T was loosely associated with '*Candidatus Nostocoida limicola*' within the radiation encompassing representatives of the family *Intrasporangiaceae*. This clustering, albeit with moderate bootstrap support (56 %), was also found in the trees obtained using maximum-likelihood and maximum-parsimony methods. The highest levels of sequence similarity for the isolate occurred with '*Candidatus N. limicola*' (96.1 %), *Terrabacter tumescens* (96.1 %) and *Terrabacter terrae* (96.0 %). The levels of 16S rRNA gene sequence similarity between the isolate and other representatives of the family *Intrasporangiaceae* were in the range 92.1–95.5 %.

Colony pigmentation was observed visually and recorded after 7 days growth at 30 °C on YE-SW agar. Colony morphology was observed using a stereoscan microscope. Cell morphology and motility were determined by using phase-contrast and transmission electron microscopy. Cells were grown on YE-SW medium, with/without agar supplementation, at 30 °C. Cells were harvested after 15,

Fig. 1. Neighbour-joining tree showing the phylogenetic position of strain KSW2-15^T within the radiation encompassing related genera of the family *Intrasporangiaceae*. The tree was reconstructed from evolutionary distances calculated using the Jukes–Cantor coefficient. Asterisks indicate branches replicated with both the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets (only values greater than 40 % are indicated). Bar, 1 substitution per 100 nucleotides. '*Candidatus N. limicola*' Ver 1 is described elsewhere in this issue as the type strain of *Tetrasphaera veronensis* (McKenzie *et al.*, 2006).

24 and 48 h and subjected to microscopic examination. Oxidase activity was assessed from the oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Catalase activity was determined with a 3 % (v/v) H₂O₂ solution. Urease activity was determined from a colour change in Bacto urea broth (Difco). The temperature range for growth was determined by using YE-SW agar at 4–42 °C. The pH range for growth was determined on YE-SW agar adjusted to pH 4.1–10.1 (at intervals of 1.0 pH unit). NaCl tolerance for growth was studied on ISP 2 medium (Shirling & Gottlieb, 1966) that contained NaCl at final concentrations of 0–9 % (w/v). The degradation of elastin was determined on ISP 2 medium supplemented with 0.4 % (w/v) elastin. Nitrate reduction, the fermentation of glucose and the hydrolysis of aesculin, casein, gelatin and starch were examined by using methods described previously (MacFaddin, 1980). To test for DNA hydrolysis, cells were grown for 3 days at 30 °C on DNase test agar (Difco) supplemented with methyl green. The ability of the isolate to utilize a broad range of substrates as sole carbon sources was tested using GP2 microplates (Microlog system; Biolog)

Fig. 2. Transmission electron micrograph of strain KSW2-15^T grown on YE-SW agar for 48 h at 30 °C. Bar, 1.0 µm.

with 95 carbon-containing compounds. The cells were grown for 48 h at 30 °C on tryptic soy broth agar and suspended in 2% (w/v) sea salts solution (Sigma). After adjustment to 20% transmittance, 150 µl suspension was transferred to each well, and the plates were incubated for 48 h at 30 °C. Reduction of tetrazolium violet was determined by reading the absorbance of the microtitre plates at 595 nm using a microplate reader. API ZYM strips (bioMérieux) were used according to the manufacturer's instructions, the inoculum having been prepared with cells grown on YE-SW agar at 30 °C for 3 days. Strain KSW2-15^T was found to be aerobic, non-motile, non-endospore-forming and Gram-positive. The cells were always cocci at all growth stages, irrespective of the physical condition of the medium, and occurred singly, in pairs, in short chains or in clusters (Fig. 2). Colonies were circular, smooth, convex and moderately yellow in colour with entire margins. The results of the physiological and biochemical tests are given in the species description.

In all of the phylogenetic trees, strain KSW2-15^T consistently formed a clade with strains of '*Candidatus N. limicola*' (described as a filamentous bacterium isolated from activated sludge; Blackall *et al.*, 2000). Because of the practical difficulty in obtaining biomass for polyphasic characterization, a validly published name was not given for this filamentous bacterium by Blackall *et al.* (2000) [note: three novel species of *Tetrasphaera* are proposed in this issue by McKenzie *et al.* (2006) for strains of '*Candidatus N. limicola*']. Strain KSW2-15^T can be readily differentiated from '*Candidatus N. limicola*' by the comparison of limited features, namely 16S rRNA gene sequence similarity and morphological characteristics (Blackall *et al.*, 2000). Of the

genera in the family *Intrasporangiaceae*, members of the genus *Terrabacter* showed high levels of 16S rRNA gene sequence similarity (96.0–96.1%) with respect to strain KSW2-15^T, albeit showing a loose association in the phylogenetic trees. It was evident, however, that they were chemotaxonomically different from our isolate in having LL-DAP as the principal diamino acid of the peptidoglycan and in the absence of unsaturated fatty acids (Collins *et al.*, 1989; Martin *et al.*, 1997; Montero-Barrientos *et al.*, 2005). Four genera of the family *Intrasporangiaceae* – *Janibacter* (Martin *et al.*, 1997), *Knoellia* (Groth *et al.*, 2002), *Oryzihumus* (Kageyama *et al.*, 2005) and *Tetrasphaera* (Maszenan *et al.*, 2000) – contain *meso*-DAP as the principal cell-wall diamino acid and contain MK-8(H₄) as the major menaquinone, but they could be distinguished from our isolate with reference to a combination of morphological and chemotaxonomic characteristics (Table 1) as well as phylogenetic distinctness.

On the basis of the phenotypic and phylogenetic data presented in this study, a novel genus and species are described for the isolate, for which the name *Phycoccus jejuensis* gen. nov., sp. nov. is proposed.

Description of *Phycoccus* gen. nov.

Phycoccus (Phy.ci.coc'cus. L. n. *phycos* -i from Gr. n. *phukos* seaweed; N.L. masc. n. *coccus* from Gr. n. *kokkos* a grain or berry; N.L. masc. n. *Phycoccus coccus* from seaweed).

Gram-positive, oxidase-negative and catalase-positive. Cells are non-endospore-forming, non-motile and coccoid. Cells occur singly, in pairs, in short chains or in clusters. The diamino acid of the peptidoglycan is *meso*-DAP. The acyl type of the muramic acid is acetyl. Mycolic acids are not present. The predominant menaquinone is MK-8(H₄). The phospholipid profile contains phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol (phospholipid type PII pattern). The predominant cellular fatty acids are C_{17:1}ω8c, iso-C_{16:0}, iso-C_{15:0}, C_{15:0} and C_{17:0}. The DNA G + C content is 74 mol%. Phylogenetically, the genus is loosely associated with genera of the family *Intrasporangiaceae*, suborder *Micrococcineae*. The type species of the genus is *Phycoccus jejuensis*.

Description of *Phycoccus jejuensis* sp. nov.

Phycoccus jejuensis (je.ju.en'sis. N.L. masc. adj. *jejuensis* of Jeju, Republic of Korea, the site at which the type strain was isolated).

Aerobic, Gram-positive, non-motile, non-endospore-forming, oxidase-negative, catalase-positive. Cells are cocci that occur singly, in pairs, in short chains or in clusters. Colonies are circular, smooth, translucent and moderately yellow in colour. The chemotaxonomic characteristics are the same as those given in the genus description. Urease-negative. Nitrate is reduced to nitrite. Gelatin liquefaction is observed. Aesculin, casein, DNA and starch are hydrolysed. Elastin is

Table 1. Phenotypic characteristics of strain KSW2-15^T and meso-DAP-containing members of the family *Intrasporangiaceae*

Data for reference genera were taken from Martin *et al.* (1997) (*Janibacter*), Groth *et al.* (2002) (*Knoellia*), Kageyama *et al.* (2005) (*Oryzihumus*) and Maszenan *et al.* (2000) and Hanada *et al.* (2002) (*Tetrasphaera*). The major menaquinone in all taxa is MK-8(H₄).

Characteristic	Strain KSW2-15 ^T	<i>Janibacter</i>	<i>Knoellia</i>	<i>Oryzihumus</i>	<i>Tetrasphaera</i>
Cell morphology	Cocci	Coccoid to rod-shaped	Coccoid to rod-shaped	Irregular rods	Cocci or irregular rods
Polar lipids*	DPG, PE, PI	DPG, PG, PI	DPG, PE, PI, PG, PL	ND	DPG, PG, PI, PE†, APL†, PL†
Fatty acid types‡	S, A, I, U	S, I, U	S, I, A	S, I, A	S, I, A, U†
DNA G+C content (mol%)	74	70	68–69	72–73	68–71
Isolation source(s)	Dried seaweed	Sludge, sewage waste	Soil from cave	Paddy soil	Sludge, sewage waste

*APL, Unknown aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unknown phospholipid(s); ND, not determined.

†Component detected in only some representatives of the genus (Maszenan *et al.*, 2000; Hanada *et al.*, 2002).

‡A, Anteiso-methyl-branched; I, iso-methyl-branched; S, straight-chain saturated; U, monounsaturated.

degraded. Glucose fermentation does not occur. The temperature range for growth is 4–37 °C, with an optimum at 30 °C. No growth occurs at 40 °C. The pH for growth is in the range pH 5.1–10.1, with an optimum of pH 7.1. Growth occurs in the presence of 0–7 % NaCl. The following substrates are utilized as sole carbon and energy sources: dextrin, glycogen, mannan, Tweens 40 and 80, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, amygdalin, L-arabinose, D-arabitol, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, *myo*-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melezitose, D-melibiose, methyl α-D-galactoside, methyl β-D-galactoside, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylose, acetic acid, α-, β- and γ-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, lactamide, L-lactic acid, L-malic acid, propionic acid, succinamic acid, succinic acid, L-alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, thymidine 5'-monophosphate, uridine 5'-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. Utilization of the following substrates is weakly positive: inulin, arbutin, D-cellobiose, D-fructose, L-fucose, D-galactose, maltotriose, 3-methyl D-glucoside, D-lactic acid methyl ester, monomethyl succinate, *N*-acetyl-L-glutamic acid, L-pyrogutamic acid and adenosine 5'-monophosphate. α-Cyclodextrin, β-cyclodextrin, methylpyruvate and D-malic acid are not used as sole carbon and energy sources. Of the tests in the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase,

naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase are positive. Tests for β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. The predominant cellular fatty acids are C_{17:1}ω8c, iso-C_{16:0}, iso-C_{15:0}, C_{15:0} and C_{17:0}. The DNA G+C content is 74 mol%.

The type strain, KSW2-15^T (=KCCM 42315^T=NRRL B-24460^T), was isolated from dried seaweed on Gwakji beach on Jeju Island, Republic of Korea.

Note added in proof

Another new genus has been described in the *Intrasporangiaceae* (*Kribbia dieselivorans* gen. nov., sp. nov.; Jung *et al.*, 2006), with the same diagnostic diamino acid, after this paper was accepted for publication.

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