Blastococcus jejuensis sp. nov., an actinomycete from beach sediment, and emended description of the genus *Blastococcus* Ahrens and Moll 1970

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A novel actinomycete, strain KST3-10^T, was isolated from sand sediment of a beach in Jeju, Korea, and was subjected to polyphasic taxonomic characterization. The organism produced circular, smooth, translucent, apricot-coloured colonies comprising coccoid- or rod-shaped cells. Phylogenetic analyses based on 16S rRNA gene sequences showed that the organism belonged to the family *Geodermatophilaceae* and consistently formed a distinct sub-branch outside the radiation of the genus *Blastococcus*. The organism showed 16S rRNA gene sequence similarity values of 98.2 % with respect to *Blastococcus aggregatus* DSM 4725^T and 98.1 % with respect to *Blastococcus saxosidens* BC444^T. The type strains of the two *Blastococcus* species shared 98.2 % sequence similarity with respect to each other, whereas the levels of sequence similarity between the novel organism and the type strains of the less closely related neighbours, *Modestobacter multiseptatus* and *Geodermatophilus obscurus*, were in the range 96.2–96.9 %. The physiological, biochemical and chemotaxonomic data revealed that the novel organism can be readily differentiated from members of the genus *Blastococcus* and that it merits separate species status. On the basis of the phenotypic and genotypic evidence, strain KST3-10^T represents a novel species of the genus *Blastococcus*, for which the name *Blastococcus jejuensis* sp. nov. is proposed. The type strain is KST3-10^T (=NRRL B-24440^T=KCCM 42251^T).

The genus *Blastococcus* was first proposed by Ahrens & Moll (1970) to accommodate aerobic, Gram-positive, coccus-shaped bacteria, which often reproduce by budding and multiple fission. The only (and type) species of the genus *Blastococcus*, *Blastococcus aggregatus* (Ahrens & Moll, 1970), was isolated from the Baltic Sea. Although the genus name was included on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980), it has not been described in detail taxonomically for many years. Its description was recently emended after a detailed taxonomic investigation that included morphological, chemotaxonomic and phylogenetic analyses; this resulted in the addition of the second species, *Blastococcus saxosidens* (Urzì *et al.*, 2004). The genus is characterized chemotaxonomically by the following features: the presence of *meso*-diaminopimelic acid in the cell wall; the presence of MK-9(H₄) as the major menaquinone; cellular fatty acids consisting mainly of unsaturated and iso-branched acids; a phospholipid profile that includes diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine; and a DNA G + C content of 74 mol%. Phylogenetically, members of this

genus are related to members of the family *Geodermatophilaceae* and were isolated from somewhat restricted environments, such as the Baltic Sea and rock surfaces.

During the study of marine bacteria recovered from the coast of Jeju in the Republic of Korea, strain KST3-10^T was isolated from sand sediment from Gwakji beach; the taxonomic status of this organism was investigated by means of polyphasic characterization. Sediment samples were taken, at a depth of 1 m, from surface water of the beach and were placed into sterilized 50 ml Falcon tubes containing seawater. For bacterial isolation, 1 g sand sediment was placed into a sterile plastic tube containing 9 ml sterile distilled water and then mixed in a tube rotator for 30 min at a moderate speed. Aliquots (100 µl) of the serial diluent of the samples were transferred onto SC-SW agar plates supplemented with 60 % (v/v) sterilized natural seawater. The isolation medium (SC-SW agar) consisted of 1 % soluble starch, 0.03 % casein, 0.2 % KNO₃, 0.2 % NaCl, 0.002 % CaCO₃, 0.005 % MgSO₄·7H₂O, 0.001 % FeSO₄·7H₂O and 1.8 % agar in 60 % sterilized natural seawater and 40 % distilled water (pH 7.2). The isolate was maintained on ISP 2 medium (Shirling & Gottlieb, 1966) supplemented with 60 % sterilized natural seawater (YE-SW agar), and in a 20 % (v/v) glycerol suspension supplemented with 60 % (v/v) sterilized natural seawater, at –20 and –80 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KST3-10^T is DQ200983.

Chromosomal DNA was extracted and purified by using the Wizard genomic DNA purification kit (Promega) according to the instructions of the manufacturer. The 16S rRNA gene of strain KST3-10^T was amplified by a PCR (Lee *et al.*, 2000a) and was subjected to direct sequence determination using the ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). The sequence determined in this study was aligned with corresponding sequences (retrieved from GenBank) by using the CLUSTAL_X program (Thompson *et al.*, 1997) and then manually optimized according to the secondary structure of bacterial 16S rRNA. Phylogenetic analyses were performed using three treeing algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was reconstructed, using the neighbour-joining method, from evolutionary distances calculated by the method described by Jukes & Cantor (1969). The reliability of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of 1000 replicated datasets.

An almost-complete 16S rRNA gene sequence for strain KST3-10^T was determined in this study (1404 nt) and was used for an initial BLAST search against GenBank: the organism was found to belong to the family *Geodermatophilaceae*. The sequence was aligned with the corresponding sequences of related taxa in the family *Geodermatophilaceae*. A total of 1387 unambiguously aligned positions present in all strains between positions 50 and 1477 (*Escherichia coli* numbering) were used for phylogenetic analyses. *Glycomyces harbinensis* was used as an outgroup for tree construction. A neighbour-joining tree (Fig. 1) showed that strain KST3-10^T occupied a unique position outside the *Blastococcus* cluster (74% bootstrap support). Members of the genus *Blastococcus* formed a monophyletic clade with 84% bootstrap support. This relationship was supported by all tree-making methods used in this study. The organism revealed 16S rRNA gene sequence similarity values of 98.1 and 98.2% with respect to the type strains of *B. aggregatus* and *B. saxobsidens*, respectively. The type strains of *B. aggregatus* and *B. saxobsidens* shared 98.2% sequence similarity with respect to each other, whereas the levels of 16S rRNA gene sequence similarity between the novel isolate and the type strains of the loosely associated relatives *Modestobacter multiseptatus* and *Geodermatophilus obscurus* were 96.9 and 96.2%, respectively.

Determination of the diaminopimelic acid isomer (Staneck & Roberts, 1974), peptidoglycan acyl type (Uchida & Aida, 1984), whole-cell sugars (Saddler *et al.*, 1991), mycolic acids (Minnikin *et al.*, 1980), polar lipids (Minnikin *et al.*, 1977) and lipoquinones (Kroppenstedt, 1985) were performed as described previously (Lee *et al.*, 2000b). The cellular fatty acid methyl esters were prepared and analysed according to the instructions of the Microbial Identification System (MIDI), using cells grown on TSA for 3 days at 30 °C. The diaminopimelic acid isomer of the peptidoglycan was

Fig. 1. Phylogenetic tree showing the relationship between strain KST3-10^T and representatives of the family *Geodermatophilaceae* based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method from evolutionary distances calculated using the Jukes–Cantor coefficient. *Micromonospora chalcea* DSM 43026^T (GenBank accession no. X92594) was used as an outgroup (not shown). Asterisks indicate branches that are also present in the maximum-likelihood and maximum-parsimony trees. Numbers at nodes indicate bootstrap values (%) based on a neighbour-joining analysis of 1000 replicated datasets (only values greater than 40% are shown). Bar, 1 substitution per 100 nucleotides. Note that GenBank accession no. AJ316571 lists *B. saxobsidens* reference strain BC448 as the source, not strain BC444^T as indicated by Urzì *et al.* (2004).

identified as the *meso*- form. Whole-cell hydrolysates contained arabinose and galactose as the characteristic sugars. The glycan moiety of the murein structure was acetylated. Mycolic acids were not detected. The predominant menaquinone was MK-9(H₄). The polar lipid profile contained significant amounts of phosphatidylcholine, diphosphatidylglycerol and phosphatidylinositol. Small amounts of phosphatidylethanolamine and phosphatidylmethylethanolamine were also detected. Strain KST3-10^T could be readily differentiated from the type strains of *B. aggregatus* and *B. saxobsidens* by the absence of phosphatidylglycerol and the presence of phosphatidylcholine and phosphatidylmethylethanolamine (Urzì *et al.*, 2004). It was shown that the presence of phosphatidylcholine, in particular, could be a chemotaxonomic marker useful for species identification. The fatty acid profile was characterized by significant amounts of iso-C_{16:0} (32.59%), C_{17:1ω8c} (11.15%) and iso-C_{15:0} (10.58%) acids. Small amounts (> 1% of total) of iso-C_{16:1} H (7.20%), C_{18:0} (5.09%), C_{16:0} (4.35%), C_{17:1ω6c} (3.89%), C_{15:0} (2.68%), C_{17:0} (2.63%), iso-C_{14:0} (2.46%), C_{15:1ω6c} (1.98%), anteiso-C_{15:0} (1.78%), C_{18:1ω9c} (1.63%), anteiso-C_{17:0} (1.48%), iso-C_{17:0} (1.38%), C_{12:0} (1.28%) and iso-C_{17:1ω9c} (1.05%) were present, along with summed feature 4 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH, 2.64%). Strain KST3-10^T differed from *B. saxobsidens* in possessing C_{18:0} and C_{12:0}, and in terms of the relative amounts of C_{16:0}, C_{17:1ω8c} and C_{18:1ω9c}, and could be distinguished from *B. aggregatus* in terms of the relative amounts of iso-C_{16:1}, C_{17:1ω8c}, C_{18:1ω9c} and C_{18:0} acids (Urzì *et al.*, 2004).

Colony pigmentation was observed visually and recorded after 7 days growth at 30 °C on YM-SW agar. Cell morphology and motility were observed using an Olympus light microscope equipped with phase-contrast optics (magnification $\times 400$) and a transmission electron microscope. Cells were grown for 3 days at 30 °C on YM-SW agar; cell suspensions for microscopic examination were made using sterile saline. The colonies were circular, translucent, smooth-surfaced and had entire margins. The colour of the colonies ranged from cream to apricot depending on the incubation time. The cells of strain KST3-10^T were coccoid and occurred in pairs or were rod-shaped, flagellated and motile (Fig. 2a). Only the rod-shaped cells showed bud formation (Fig. 2b).

The utilization of a variety of substrates as sole carbon sources was tested using GP2 microplates from the Microlog system (Biolog), which contained 95 substrates. Cells were grown for 3 days at 30 °C on YE-SW agar and suspended in a 2 % (w/v) sea-salts solution (Sigma). An aliquot (150 μ l) of the suspension was transferred to each well and the plates were incubated for 48 h at 30 °C. Reduction of the tetrazolium dye was determined by reading the absorbance of the microtitre plates at 595 nm using a microplate reader. Growth at various temperatures was tested (4, 10, 20, 30, 37, 40 and 42 °C). NaCl tolerance was studied on ISP 2 medium (yeast extract, 0.4 %; malt extract, 1.0 %; glucose, 0.4 %; agar, 1.8 %; pH 7.2) with NaCl added at final concentrations in the range 0–9 % (w/v). Gram staining and the hydrolysis of casein, elastin and starch were examined as described previously (MacFaddin, 1980). The decomposition of hypoxanthine, tyrosine and xanthine was tested using the method described by Gordon *et al.* (1974). The following tests were performed with API 20NE strips (bioMérieux): nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, aesculin degradation, gelatin hydrolysis and β -galactosidase. Catalase activity was determined with a 3 % (v/v) H₂O₂ solution. Oxidase activity was tested by assessing the oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Other physiological and biochemical properties were tested with API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Strain KST3-10^T utilized Tween 80 as a sole source of carbon and energy for growth, but did not use the following substrates: α - and β -cyclodextrin, dextrin, glycogen, inulin, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, arbutin, L-fucose, D-gluconic acid, α -D-glucose, α -D-lactose, lactulose, maltotriose, D-melezitose, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, D-psicose, D-raffinose, salicin, stachyose, D-trehalose, D-xylose, α - and β -hydroxybutyric acids, *p*-hydroxyphenylacetic acid, lactamide, L-lactic acid, methylpyruvate, propionic acid, pyruvic acid, succinamic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D- and L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-glutamic acid, L-pyrogutamic acid, L-serine, glycerol, 2'-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine

Fig. 2. Transmission electron micrographs of strain KST3-10^T grown on YE-SW agar for 3 days at 30 °C. (a) Rod-shaped cell with flagella. (b) Coccoid cells in pairs and rod-shaped cells showing bud formation. Bars, 1.0 μ m.

5'-monophosphate, D-fructose 6-phosphate, α -D-glucose-1-phosphate or DL- α -glycerol phosphate. Other physiological characteristics are given in Table 1 and in the species description.

The distinct nature of its phylogenetic position and the presence of additional phospholipids suggest that the isolate is a member of novel genus; however, the high 16S rRNA gene sequence similarities (above 97 % – the threshold recognized as delineating a genospecies; Stackebrandt & Goebel, 1994) together with other phenotypic features (e.g. cell morphology, respiratory quinone and cellular fatty acid profile) indicate that the isolate should be assigned to the genus *Blastococcus*. Phospholipid composition has been used as a key chemotaxonomic marker in the delineation of

Table 1. Differentiating characteristics of strain KST3-10^T and species of the genus *Blastococcus*

Taxa: 1, strain KST3-10^T; 2, *B. aggregatus*; 3, *B. saxobsidens*. Data are from Urzì *et al.* (2004) and this study. +, Positive; –, negative; W, weak; V, variable depending on strain; ND, not determined.

Characteristic	1	2	3
Cell shape	Cocci, rods	Cocci, rods, vibrios	Cocci
Bud formation	+	+	–
Germ tube	–	+	–
Motility	+	+	V
Colony colour	Apricot	Pink	Pink to orange
Casein hydrolysis	+	ND	–
API ZYM tests			
Alkaline phosphatase	–	–	+
Esterase (C4)	–	+*	+*
Valine arylamidase	W	–	–
Cystine arylamidase	W	–	–
Naphthol-AS-BI-phosphohydrolase	–	–	V
β-Glucuronidase	–	–	+
β-Glucosidase	–	–	W
API 20NE tests†			
Arginine dihydrolase	–	–	+
β-Galactosidase	–	–	+
Assimilation of:			
D-Arabinose	–	–	+
Gluconate	–	–	W
Malate	–	–	V
Biolog GP2 tests (utilization of):‡‡			
Acetic acid	+	+	–
Adenosine	–	+	+
α-Ketoglutaric acid	–	+	–
Methylsuccinate	–	+	+
D-Ribose	–	+	+
Tween 40	–	+	+
Phosphatidylcholine in polar lipids	+	–	–

*Recorded as weak or positive by Urzì *et al.* (2004).

†Strain KST3-10^T showed conflicting results for assimilation of D-mannitol, D-mannose, D-maltose and malate in the API 20NE and Biolog GP2 systems.

‡Biolog GP2 microplates gave positive results for strain KST3-10^T and negative results for the type strains of *B. aggregatus* and *B. saxobsidens* for utilization of mannan, *N*-acetyl-β-D-mannosamine, amygdalin, D-cellobiose, D-fructose, D-galactose, D-galacturonic acid, gentiobiose, *myo*-inositol, maltose, D-mannitol, D-mannose, D-melibiose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl-D-glucoside, palatinose, L-rhamnose, sedoheptulosan, D-sorbitol, sucrose, D-tagatose, turanose, xylitol, γ-hydroxybutyric acid, α-ketovaleric acid, D-lactic acid methyl ester, L-malic acid, succinic acid, L-asparagine, putrescine, 2,3-butanediol and D-glucose 6-phosphate.

actinomycete genera or genus groups (Lechevalier *et al.*, 1977). However, there are reports of phospholipid profiles varying according to species (Xu *et al.*, 1999; Lee *et al.*, 2001). The differentiating characteristics of the isolate and the type species of the genus *Blastococcus* are given in Table 1. On the basis of the phenotypic and genotypic data, the novel organism represents a novel species of the genus *Blastococcus*, for which the name *Blastococcus jejuensis* sp. nov. is proposed.

Emended description of *Blastococcus* Ahrens and Moll 1970

The description is taken from Ahrens & Moll (1970), Urzì *et al.* (2004) and this study. The diagnostic diamino acid and sugars of peptidoglycan are *meso*-diaminopimelic acid and arabinose and galactose, respectively. The predominant menaquinone is MK-9(H₄). MK-9 occurs in variable amounts depending on the strain. The polar lipid profiles

are characterized by the common basis comprising diphosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine. The presence of phosphatidylglycerol, phosphatidylcholine and phosphatidylmethylethanolamine is variable among the species. The major cellular fatty acids consist of saturated, unsaturated and iso-branched fatty acids. The G+C contents of the DNA are in the range 72.3–74 mol%. Gram-positive, oxidase-negative, catalase-positive and aerobic. Some cells may be microaerophilic. Cells are coccoid or rod-shaped and reproduce by budding and binary or multiple fission. Vibrioid or non-motile cocci occur in pairs or tend to form aggregates, whereas rod-shaped cells occur singly and show motility by means of flagella. Bud formation varies according to the species. Most strains utilize a broad spectrum of organic compounds. Phylogenetically, the genus belongs to the family *Geodermatophilaceae*. The type species is *Blastococcus aggregatus*.

Description of *Blastococcus jejuensis* sp. nov.

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

Aerobic, motile, non-spore-forming, oxidase-negative, catalase-positive, Gram-positive. Cells are cocci that occur in pairs or rods. Bud formation is observed for rod-shaped cells. Colonies are circular, smooth, transparent and apricot in colour. Starch and casein are hydrolysed, but elastin is not. Hypoxanthine, tyrosine and xanthine are not decomposed. In API 20NE tests, glucose fermentation and indole production from tryptophan are not observed. Activities of arginine dihydrolase, urease and β -galactosidase are not present. Nitrate is not reduced to nitrite. Aesculin degradation and gelatin hydrolysis are not detected. Caprate, adipate, citrate and phenylacetate are not assimilated. In API ZYM tests, results for leucine arylamidase and α -glucosidase are positive and weakly positive, respectively, whilst results for esterase lipase (C8), lipase (C14), trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are negative. The temperature range for growth is 10–37 °C, with the optimum at 30 °C. Growth occurs at pH values in the range 6.1–10.1, with the optimum at pH 7.1. Growth was observed in the presence of 0–1 % NaCl but not 2 % NaCl. The following substrates are used as sole carbon and energy sources: mannan, *N*-acetyl- β -D-mannosamine, amygdalin, D-cellobiose, D-fructose, D-galactose, D-galacturonic acid, gentiobiose, *myo*-inositol, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, 3-methyl-D-glucoside, palatinose, L-rhamnose, sedoheptulosan, D-sorbitol, sucrose, D-tagatose, turanose, xylitol, acetic acid, γ -hydroxybutyric acid, α -ketovaleric acid, D-lactic acid methyl ester, succinic acid, L-asparagine, putrescine, 2,3-butanediol, adenosine and D-glucose 6-phosphate, but not Tween 40, D-ribose, α -ketoglutaric acid or methylsuccinate. The polar lipid profile contains phosphatidylcholine, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine and

phosphatidylinositol. Mycolic acids are not present. The major cellular fatty acids are iso-C_{16:0}, C_{17:1} ω 8c and iso-C_{15:0}. The predominant menaquinone is MK-9(H₄). Whole-cell hydrolysates contain *meso*-diaminopimelic acid as the diagnostic diamino acid and arabinose and galactose as diagnostic sugars. The DNA G+C content is 72.3 mol%.

The type strain, KST3-10^T (=NRRL B-24440^T=KCCM 42251^T), was isolated from sand sediment from Gwakji beach on Jeju Island, Republic of Korea.

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