ORIGINAL ARTICLE

Flexivirga alba gen. nov., sp. nov., an actinobacterial taxon in the family *Dermacoccaceae*

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A novel actinobacterial strain ST13^T isolated from soil near wastewater treatment facilities of an electroplating plant was subjected to a polyphasic taxonomic study. Cells of this organism were non-sporulating, and were irregular coccoid to comma shaped. The peptidoglycan of strain ST13^T contained glutamic acid, serine, alanine, glycine and lysine, and represented the peptidoglycan type A4 α . The whole-cell sugars contained ribose, glucose, galactose, rhamnose and mannose. The predominant menaquinone was MK-8(H₄). The major fatty acid was iso-C_{16:0}. The polar lipid contained phosphatidylglycerol. The DNA G+C content was 67.4 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain ST13^T fell within the radius of the family *Dermacoccaceae*, and its closest neighbor was *Luteipulveratus mongoliensis* MN07-A0370^T (95.1%). However, strain ST13^T did not make a coherent clade with members of the recognized organisms. On the basis of the phylogenetic and phenotypic characteristics of this actinobacterium, a novel genus and species, *Flexivirga alba* gen. nov., sp. nov., is proposed. The type strain of *F. alba* is ST13^T (=NBRC 107580^T=DSM 24460^T).

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INTRODUCTION

The family *Dermacoccaceae* was first proposed by Stackebrandt and Schumann,¹ and its pattern of 16S rRNA signature nucleotides was emended by Zhi *et al.*² At the time of writing, the family *Dermacoccaceae* comprised of six recognized genera: *Dermacoccus*,³ *Demetria*,⁴ *Kytococcus*,³ *Luteipulveratus*,⁵ *Yimella*⁶ and *Branchiibius*.⁷ The organisms belonging to these genera are Gram-positive, aerobic, non-halophilic, coccoid actinobacteria, and their peptidoglycan type is variation A4 α . Isolates assigned to this family are typically associated with terrestrial habitats, notably, cured meat products, skin, human blood and soil.^{8–11} The recently described *Branchiibius hedensis* was isolated from the branchia of a codfish.⁷ During a study of aerobic bacteria from soil samples, we isolated a novel actinobacterium, designated ST13^T, from soil near wastewater treatment facilities of an electroplating plant in Seki, Gifu prefecture, Japan. Phylogenetic analysis based on 16S rRNA gene sequence of strain ST13^T revealed that the isolate was a member of the suborder *Micrococccineae*. The aim of this study was to determine the taxonomic position of strain ST13^T by using a polyphasic approach.

MATERIALS AND METHODS

Isolation and cultivation

The soil sample was collected from a depth of ~2 cm from the surface near the wastewater treatment facilities of an electroplating plant in Seki, Gifu prefecture, Japan. The sample was dried at room temperature for several days and suspended in 10 ml sterile water. The sample was vortexed, allowed to settle for 1 min, and 100 μ l of the resultant solution was further diluted and spread onto International *Streptomyces* Project (ISP) medium 2.¹² The plates were

incubated at 28°C for 1 week. The strain ST13^T was obtained after three successive transfers on yeast extract-soluble starch medium (YS medium; 2 g yeast extract, 10 g soluble starch and 15 g l⁻¹ agar; pH 7.3) and maintained as pure culture at room temperature until used. For long-term preservation, strain ST13^T was suspended in 10% glycerol solution and stored at –80°C.

Morphological and physiological characteristics

The cultural characteristics of strain ST13^T were observed on ISP media at 28°C for 5 days. Morphological features were observed under a scanning electron microscope (JSM-6060; JEOL, Tokyo, Japan).¹³ Cell motility was tested under a light microscope by observing cells suspended in phosphate buffer (1 mM; pH 7.0). Gram staining was performed using Hucker's modification.¹⁴ Growth under anaerobic conditions was determined by incubating in an anaerobic chamber with an O₂-absorbing and CO₂-generating agent (Anaero-Pack; Mitsubishi Gas Chemical Company, INC., Tokyo, Japan). Growth tests for pH range were carried out by using media adjusted to pH 3–12 with 4 M HCl or 5 M KOH after sterilization, and NaCl tolerance was examined on YS medium supplemented with 1–10% NaCl (w/v) after 1–5 days of incubation at 28°C. The optimum temperature and temperature range for growth were determined by incubating at 5, 10, 15, 20, 25, 28, 37, 45 and 50°C. ISP medium 8 was used to test for nitrate reduction.¹⁵ Decomposition of urea was determined on Christensen urea agar containing 2.0% urea.¹⁶ Degradation of casein and other compounds (final concentration 0.5%) was determined using nutrient agar as the basal medium.¹⁶ Catalase activity was determined by production of bubbles after the addition of a drop of 3.0% H₂O₂. Oxidase activity was examined using cytochrome oxidase paper (Nissui Pharmaceutical CO., Tokyo, Japan). Other physiological and biochemical characteristics were determined by using API ZYM, API Coryne and API 50CH systems (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

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Chemotaxonomic analysis

Strain ST13^T was subjected to chemotaxonomic analysis for its classification in the family *Dermacoccaceae*, including menaquinones,¹³ polar lipids,¹⁷ whole-cell sugars¹⁸ and DNA G+C content.¹⁹ Analysis of cellular fatty acids was carried out using the Microbial Identification System version 4.02 (MIDI, Inc., Newark, DE, USA),²⁰ and the results were compared with the ACTINO library in the Microbial Identification System. Cell-wall amino acids were analyzed by using the method described by Tamura *et al.*,¹³ and the amino acid isomers in cell-wall peptidoglycan were analyzed using the method described by Nozawa *et al.*²¹ Biomass for chemotaxonomic studies was obtained by cultivation in shaken flasks containing YG broth (10 g glucose, 10 g yeast extract and 15 g l⁻¹ agar; pH 7.3) for 24–72 h at 28 °C.²²

Phylogenetic analysis

DNA extraction from strain ST13^T and PCR amplification of the 16S rRNA gene were performed as described by Tamura and Hatano.²³ PCR product was directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an automatic DNA sequencer (ABI Prism 3730 Genetic Analyzer; Applied Biosystems).

The CLUSTAL_X program²⁴ was used to align the 16S rRNA gene sequences with related sequences (available in the GenBank/EMBL/DDBJ databases) from all of the type strains of the family *Dermacoccaceae*. Phylogenetic trees were constructed by the neighbor-joining²⁵ and maximum-likelihood²⁶ methods using MEGA (Molecular Evolutionary Genetics Analysis) version 3.1.²⁷ The topology of the tree was evaluated by means of bootstrap analysis based on 1000 replicates.²⁸

Figure 1 Scanning electron micrographs of irregular coccoid or comma-shaped cells from 1-week-old cultures of strain ST13^T grown on 1/2 ISP2 (a) and ISP2 (b).

Figure 2 Phylogenetic tree based on 16S rRNA gene sequences constructed with the neighbor-joining algorithm showing the relationships between strain ST13^T and members of the family *Dermacoccaceae*. Numbers at branch points are confidence limits estimated with bootstrap analysis based on 1000 replicates; only values of 50% are presented. Solid circles indicated that the corresponding nodes were also recovered in maximum-likelihood algorithms. Bar, 0.01 K_{nuc} in nucleotide sequences.

RESULTS AND DISCUSSION

Morphological and physiological characteristics

Strain ST13^T formed moist, smooth and white colonies on ISP medium 2 and YS medium. The cells were aerobic, Gram-positive, catalase-negative, oxidase-negative, non-sporulating and non-motile. Mycelia had not developed. Strain ST13^T exhibited good growth in NaCl concentrations of up to 5.0% and weak growth at 6.0%. Good growth was observed at 15–37 °C, and no growth at 5 °C and 50 °C. The optimum temperature for growth was 25 °C. The pH range for growth was 5.0–9.0 and the optimum was 7.0–8.0. The strain grew well on ISP medium 2, 3, 5, 6 and 7, but grew weakly on ISP medium 4. Scanning electron microscopy revealed near-coccoid cells (0.6–1.2 µm in diameter) with buds on 1/2 ISP medium 2 (2 g yeast extract, 5 g malt extract, 2 g dextrose and 15 g l⁻¹ agar; pH 7.3) (Figure 1a). However, there seemed to be near-comma shaped cells that varied in size (0.5–0.8 × 1.2–1.8 µm) on ISP medium 2 without budding cells (Figure 1b). From the initial stage of cultures, comma-shaped cells were observed on ISP medium 2. It revealed that the cell morphology did not depend on culture stage. Other physiological and biochemical characteristics are shown in the species description.

Chemotaxonomic characteristics

Strain ST13^T contained ribose, glucose, galactose, rhamnose and mannose as whole-cell sugars. The predominant menaquinone was MK-8(H₄), whereas the minor component was MK-8(H₆). Purified peptidoglycan of strain ST13^T contained glutamic acid, serine, glycine, alanine and lysine in a molar ratio of 2.0:1.8:1.1:1.8:0.7, respectively. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of D-Ala, L-Ala, D-Glu, L-Ser and L-Lys. These data indicated that the cell-wall peptidoglycan of strain ST13^T was of the A4α type,²⁹ with L-Lys as the diagnostic cell-wall diamino acid, and the interpeptide bridge of the peptidoglycan contained D-Glu, glycine and L-Ser (two molecules). Although the interpeptide bridge structure is similar to that of the genus *Dermacoccus*, strain ST13^T differs from the genus *Dermacoccus* based on the presence of a glycine residue. The major cellular fatty acid was iso-C_{16:0} (58.5%), and minor components were iso-C_{16:1} H (9.4%), C_{16:0} 10-methyl (4.8%), anteiso-C_{17:0} (4.3%), iso-C_{16:0} 2OH (4.2%), C_{16:1} cis9 (3.2%), anteiso-C_{17:1} C (2.9%), C_{17:0} 10-methyl (2.7%), iso-C_{14:0} (1.8%), iso-C_{18:0} (1.6%), C_{16:0} (1.6%), iso-C_{17:0} (1.1%) and iso-C_{15:0} (1.1%). The polar lipid detected was phosphatidylglycerol, and the DNA G+C content was 67.4 mol%.

Phylogenetic characteristics

An almost-complete 16S rRNA gene sequence (1483 nt) was determined for strain ST13^T. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ST13^T is AB539735. Comparative 16S rRNA gene sequence analysis revealed that the strain ST13^T belonged to the suborder *Micrococccineae* and its closest phylogenetic neighbors were members of the genera within the family *Dermacoccaceae* (Figure 2). The similarity values of the 16S rRNA gene sequences between strain ST13^T and members of the genera *Demetria*, *Dermacoccus*, *Kytococcus*, *Luteipulveratus*, *Yimella* and *Branchiibius* ranged from 93.1 to 95.1%.

On the basis of phylogenetic analysis of 16S rRNA gene sequences, strain ST13^T was closely related to members of the genera in the family *Dermacoccaceae*, and formed a monophyletic branch within the radius occupied by the family. Its closest phylogenetic neighbor was *Luteipulveratus mongoliensis* MN07-A0370^T (95.1%), which differed from the isolate by amino acids, polar lipids and cell morphology (Table 1). Obvious differences in chemotaxonomic, morphological and physiological properties were also found between ST13^T and members of

Table 1 Differential phenotypic and chemotaxonomic characteristics of the genus *Flexivirga alba* nov. and related genera of the family *Dermacoccaceae*

Characteristics	ST13 ^T	Demetria	Kytococcus	Dermacoccus	Luteipulveratus	Yimella	Branchiibius
Cell size (µm)	0.6 × 1.2 or 0.5–0.8 × 1.2–1.8	0.8 × 1.2 or 0.8 × 3.0	0.8 × 1.1	0.9 × 1.6	0.5 × 1.0–0.8 × 1.2	0.7–0.9 × 1.3–1.8	0.7 × 0.9
Cell morphology	Coccoid to comma shaped	Irregular coccoid rod shaped	Coccoid	Coccoid	Coccoid to short-rod shaped	Coccoid	Coccoid
Colony pigmentation	White	White to pale yellow	Deep buttercup yellow or cream-white, muddy yellow	Bright orange, pale, light, brilliant yellow	Cream to bright yellow	Orange	Pale yellow
NaCl range (% w/v)	0–6	0–12	0–10	0–7	0–2	0–8	0–7
Catalase	–	+	+	+	+	+	ND
DNA G+C content (mol%)	67.4	66	68–69	66.0–71.0	68.2	65.8	68
Major menaquinone(s)	MK-8(H ₄)	MK-8(H ₄)	MK-7, MK-8, MK-9, MK-10	MK-8(H ₂)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₂), MK-8(H ₄)
Amino acids composition of peptidoglycan	Ala, Glu, Gly, Lys, Ser	Ala, Asp, Glu, Lys, Ser	Ala, Glu, Gly, Lys	Ala, Glu, Lys, Ser	Ala, Asp, Glu, Lys, Ser	Ala, Asp, Glu, Gly, Lys, Ser	Ala, Glu, Gly, Lys, Ser
Cell-wall sugar(s) or whole-cell sugar(s)	Gal, Rib, Glc	Gal	ND	Gal	Gal, Glc, Man, Rham, Rib	Fuc, Gal	Ara, Gal, Glc, Man, Rham, Rib,
Major fatty acid(s) (>10%)	iso-C _{16:0}	C _{18:1} C _{17:0} C _{18:0}	C _{15:0} , C _{17:0} , anteiso-C _{17:0} , iso-C _{17:1} , iso-C _{15:0} , iso-C _{17:0}	iso-C _{17:0} iso-C _{17:1} iso-C _{15:0} , iso-C _{16:0}	iso-C _{16:0}	iso-C _{15:0} anteiso-C _{15:0} anteiso-C _{17:0}	C _{17:1} c9 iso-C _{16:0} C _{18:1} c9
Polar lipid(s)	PG	DPG, PG, PE, PI, PL	DPG, PG, PI	DPG, PG, PI	DPG, PG, PI	DPG, PI, GlcN-PL, PL	PI, PG, DPG, PL

Abbreviations: Ala, alanine; Asp, aspartate; DPG, diphosphatidylglycerol; gal, galactose; Glc, glucose; GlcN-PL, glucosamine-containing phospholipid; Glu, glutamic acid; Gly, glycine; Lys, lysine; Man, mannose; ND, no data available; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unknown phospholipid; Rib, ribose; Rham, rhamnose; Ser, serine; –, negative; +, positive. ST13^T, data from this study; *Demetria*, data from reference;⁴ *Kytococcus*, data from reference;^{3,30} *Dermacoccus*, data from reference;^{3,31,32} *Luteipulveratus*, data from reference;⁵ *Yimella*, data from reference;⁶ *Branchiibius*, data from reference.⁷

other genera in the family *Dermacoccaceae*. On the basis of its chemotaxonomic characteristics and phylogenetic analysis, strain ST13^T represents a novel genus and species in the family *Dermococcaceae*, for which the name *Flexivirga alba* gen. nov., sp. nov. is proposed.

Description of *Flexivirga* gen. nov.

Flexivirga (fle.xi.vir'ga. L. adj. *flexus*, bent; L. fem. n. *virga*, a rod; N.L. fem. n. *Flexivirga*, a bent rod). Cells are Gram-positive, irregular coccoid or comma-shaped, non-motile, aerobic and non-sporulating. The cell-wall peptidoglycan is of the A4 α type with L-lysine as the diamino acid. The predominant menaquinone is MK-8(H₄) and the major polar lipid is phosphatidylglycerol. Whole cells contain ribose, glucose and galactose as major components, with rhamnose and mannose as minor components. The major cellular fatty acid is iso-C_{16:0}. The G+C content of the type species is ~67 mol%. Phylogenetically, the genus *Flexivirga* belongs to the family *Dermacoccaceae*. The type species is *F. alba*.

Description of *F. alba* sp. nov.

F. alba (al'ba. L. fem. adj. *alba*, white, referring to the color of the colonies) displays the following properties in addition to those given for the genus: catalase-negative, oxidase-negative, growth occurs with NaCl concentrations of 0–6%, grows at 10–45 °C (optimum temperature, 25 °C) and pH 5.0–9.0 (optimum pH, 7.0–8.0), xanthine and hypoxanthine are decomposed, but not casein, calcium malate, tyrosine or adenine. According to the API 50CH test, acid is produced from *N*-acetylglucosamine, D-cellobiose, D-glucose, D-arabinose, D-ribose, D-xylose, D-arabinose, glycerol, L-rhamnose, D-mannitol, D-sucrose, D-trehalose, D-turanose, D-lyxose, D-fucose, D-arabitol and 5-ketogluconate, but all very weakly. According to the API ZYM enzyme assay, the strain is positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, *N*-acetyl- β -glucosaminidase, naphthol AS-BI phosphohydrolase, cystine arylamidase, trypsin and acid phosphatase. It is also weakly positive for α -fucosidase, β -galactosidase, and negative for esterase (C4), esterase lipase (C8), lipase (C14), α -chymotrypsin, α -galactosidase, α -mannosidase, β -glucuronidase and β -glucosidase. According to the API Coryne enzyme assay, it is positive for alkaline phosphatase, pyrazin amidase, β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase, and negative for pyrrolidonyl arylamidase and β -glucuronidase. Nitrate is reduced. Aesculin and gelatin are hydrolyzed, but urea is not. The minor component of fatty acids are iso-C_{16:1} H, C_{16:0} 10-methyl, anteiso-C_{17:0}, iso-C_{16:0} 2OH, C_{16:1} *cis*9, anteiso-C_{17:1} C, C_{17:0} 10-methyl, iso-C_{14:0}, iso-C_{18:0}, C_{16:0}, iso-C_{17:0}. The DNA G+C content of the type strain is 67.4 mol%. The type strain ST13^T (=NBRC 107580^T=DSM 24460^T) was isolated from soil in Japan.

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