Chromobacterium piscinae sp. nov. and *Chromobacterium pseudoviolaceum* sp. nov., from environmental samples

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Two strains (LMG 3947 and LMG 3953) of motile, Gram-negative, violet-pigmented bacteria, respectively isolated in 1972 and before 1953 from environmental samples and previously assigned to *Chromobacterium violaceum*, were studied again in detail for their taxonomic position. 16S rRNA gene sequence analysis showed that both strains clustered together with the type strain of *Chromobacterium violaceum* (99.8% sequence similarity for strain LMG 3953^T and 98.9% sequence similarity for LMG 3947^T). *recA* sequence similarities were significantly lower (95–96%), and fatty acid profiles were also different from those reported for *C. violaceum* and other *Chromobacterium* species. Both strains exhibit polar-lipid profiles consisting of the major compound phosphatidylethanolamine and moderate amounts of phosphatidylglycerol in addition to some minor lipids. The quinone systems consist of the major compound ubiquinone Q-8 and moderate amounts of Q-7. Polyamine patterns are composed of the major compounds putrescine and 2-hydroxyputrescine, moderate amounts of 1,3-diaminopropane and variable amounts of cadaverine and spermidine. The results of DNA–DNA hybridizations and physiological as well as biochemical tests allowed both genotypic and phenotypic differentiation of the two strains from described *Chromobacterium* species. It is evident from the genotypic and phenotypic data that both strains represent novel species in the genus *Chromobacterium*, for which we propose the names *Chromobacterium piscinae* sp. nov. (type strain LMG 3947^T = CCM 3329^T) and *Chromobacterium pseudoviolaceum* sp. nov. (type strain LMG 3953^T = CCM 2076^T = NCTC 8182^T).

Two Gram-negative, violet-pigmented strains, previously assigned to *Chromobacterium violaceum*, were studied again in detail for their taxonomic position because they were found to be markedly different from the type strain of *C. violaceum* as determined by *recA*-PCR RFLP analysis (Scholz *et al.*, 2005). Strain LMG 3947 was isolated from pond water in 1972 at Sungai Buloh, Malaysia; the origin of strain LMG 3953 (=strain Lewitus) is unclear, but the strain was included in the study of Gilman (1953). Both strains were maintained on nutrient agar (NA; Oxoid) after incubation at 32 °C for 48 h. Subcultivation was done on NA at 30 °C for between 48 h and 3 days.

Cell morphology was observed under a Zeiss light microscope at ×1000, using cells that had been grown for 3 days at 30 °C on NA; results are given in the species descriptions. Gram-staining was performed as described by Gerhardt *et al.* (1994). Results of physiological characterization are given in the species descriptions and in Table 1 (differential characters); experiments used methods described previously (Kämpfer *et al.*, 1991). Tests were read after 48 h of incubation. Both strains showed few differences from *C. violaceum* NCTC 9757^T.

The 16S rRNA gene was analysed as described by Kämpfer *et al.* (2003). Phylogenetic analysis was performed using the software package MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura-2 model) were calculated and clustering with the neighbour-joining, maximum-parsimony and maximum-likelihood methods was performed by using bootstrap

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 3947^T and LMG 3953^T are AJ871127 and AJ871128, and those for the *recA* sequences of strains LMG 3947^T and LMG 3953^T are respectively AJ871123 and AJ871120.

Table 1. Physiological characteristics of the type strains of *Chromobacterium* species

Strains: 1, LMG 3953; 2, LMG 3947; 3, *C. violaceum* ATCC 12472^T; 4, *C. aquaticum* SEYA-1^T; 5, *C. subtsugae* DSM 17043^T; 6, *C. haemolyticum* CCUG 53230^T. Data were obtained in this study. All strains were positive for acid formation from D-glucose and trehalose. All strains were negative for acid formation from cellobiose, lactose, sucrose, D-mannitol, methyl D-glucoside, dulcitol, salicin, adonitol, erythritol, melibiose, D-arabitol, D-mannose, inositol, sorbitol, L-arabinose, raffinose and L-rhamnose. All strains were positive for the hydrolysis of L-alanine *p*-nitroanilide (pNA), bis-*p*-nitrophenyl (pNP) phosphate, pNP phosphorylcholine, pNP phenylphosphonate and 2-deoxythymidine-5'-pNP phosphate. All strains were negative for hydrolysis of aesculin, pNP β-D-galactopyranoside, pNP β-D-glucuronide and pNP β-D-xylopyranoside. All strains were also positive for assimilation of *N*-acetyl-D-glucosamine, D-fructose, D-glucose, gluconate, D-ribose, trehalose, fumarate, acetate, DL-lactate, L-malate, pyruvate, L-alanine, β-alanine, L-histidine and L-serine. All strains were negative for assimilation of *N*-acetyl-D-galactosamine, *p*-arbutin, cellobiose, D-galactose, D-mannose, melibiose, L-rhamnose, sucrose, salicin, D-xylose, adonitol, maltitol, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, glutarate, itaconate, mesaconate, suberate, L-ornithine, L-phenylalanine, L-tryptophan, phenylacetate and 3- and 4-hydroxybenzoate.

Characteristic	1	2	3	4	5	6
Acid formation from D-mannose	+	+	+	–	–	–
Utilization of:						
<i>myo</i> -Inositol	–	–	–	–	–	+
D-Mannitol	–	–	–	–	–	+
D-Sorbitol	–	–	–	–	–	+
Putrescine	–	–	–	+	–	+
<i>cis</i> -Aconitate	+	–	–	+	+	–
Propionate	+	–	–	+	+	–
Citrate	–	–	–	+	–	+
DL-3-Hydroxybutyrate	+	–	+	–	–	–
Oxoglutarate	–	–	–	+	–	+
L-Aspartate	+	+	–	+	+	+
L-Leucine	–	–	–	+	–	–
L-Proline	+	+	–	+	+	+

values based on 1000 replications. The almost-complete 16S rRNA sequences of all strains were compared by sequence similarity calculations after a neighbour-joining analysis. A tree is shown in Fig. 1. The branching pattern was confirmed by maximum-parsimony and maximum-likelihood analyses (not shown).

Sequence similarity calculations indicated that both strains showed the highest similarity to *C. violaceum* ATCC 12472^T (99.9 and 98.9%). Lower sequence similarities (<98.5%) were found with all other *Chromobacterium* species.

The partial *recA* genes (860 nt) of strains LMG 3947 and LMG 3953 were analysed previously together with other *C. violaceum* strains (Scholz *et al.*, 2005). Whereas most *C. violaceum* strains differed in only three or four nucleotide positions, the *recA* sequences of strains LMG 3947 and LMG 3953 were markedly different, with similarities of 95–96% (>40 nt differences) when compared with the *C. violaceum* type strain. Deduced RecA amino acid sequences differed in 9 (LMG 3947) and 5 (LMG 3953) amino acid positions from the *C. violaceum* type strain. Consequently, in phylogenetic analysis, the two strains grouped separately from *C. violaceum*, forming separate lineages (not shown). Lower *recA* sequence similarities (84%) were found with *Ralstonia*, *Bordetella* and *Burkholderia* strains.

DNA–DNA hybridization experiments were performed with *C. violaceum* NCTC 9757^T, *Chromobacterium subtsugae* DSM 10743^T, *Chromobacterium aquaticum* SEYA-1^T and *Chromobacterium haemolyticum* CCUG 53230^T using the method described by Ziemke *et al.* (1998) except that, for nick translation, 2 µg DNA was labelled during a 3 h incubation at 15 °C. Strain LMG 3947 showed DNA–DNA relatedness of 51.9% to strain LMG 3953^T, 36.9% to *C. violaceum* NCTC 9757^T, 48.3% to *C. subtsugae* DSM 10743^T, 30.6% to *C. aquaticum* SEYA-1^T and 31.8% to *C. haemolyticum* CCUG 53230^T. Strain LMG 3953 showed DNA–DNA relatedness of 47.5% to *C. violaceum* NCTC 9757^T, 35.1% to *C. subtsugae* DSM 10743^T, 43.4% to *C.*

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences using the neighbour-joining method. Bootstrap values based on 1000 replications are listed as percentages at branching points. Bar, 0.01 nucleotide substitutions per nucleotide position.

aquaticum SEYA-1^T and 32.7 % to *C. haemolyticum* CCUG 53230^T.

Fatty acid methyl esters were prepared, separated and identified according to the instructions of Microbial Identification System (MIDI; Microbial ID). Analyses were performed as described by Kämpfer & Kroppenstedt (1996). The fatty acid profiles of both strains were similar to those given for *Chromobacterium* species, but also showed some minor differences (Table 2). Biomass to be subjected to analyses of polar lipids, quinones and polyamines was grown in PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2). Polar lipids were extracted and analysed as described by Tindall (1990a) and Altenburger *et al.* (1996) and quinones as described by Tindall (1990b). Biomass of strain LMG 3953 was harvested at approximately 70 % of the maximum optical density as reported by Busse & Auling (1988). Strain LMG 3947 exhibited flocculent growth, preventing reliable measurement of optical density. In order to allow harvest of exponentially growing biomass, two flasks with the same volume of PYE medium were inoculated from the same overnight culture, one with three times the inoculum volume of the other. The two cultures were incubated under identical conditions and the culture inoculated with

Table 2. Fatty acid compositions of type strains of the genus *Chromobacterium*

Strains: 1, LMG 3953; 2, LMG 3947; 3, *C. violaceum* ATCC 12472^T; 4, *C. aquaticum* SEYA-1^T; 5, *C. subsugae* DSM 17043^T; 6, *C. haemolyticum* CCUG 53230^T. Data were obtained in this study. Values are percentages of total fatty acids; —, not detected. The position of the double bond in unsaturated fatty acids is obtained by counting from the methyl (ω) end of the molecule.

Fatty acid	1	2	3	4	5	6
C _{10:0}	—	—	—	0.2	—	—
C _{10:0} 3-OH	5.1	3.0	5.2	4.6	3.2	2.8
C _{12:0}	4.9	3.2	5.6	8.8	3.3	4.5
C _{11:0} 3-OH	0.4	—	—	—	—	—
C _{13:0}	0.4	—	—	—	—	—
C _{12:0} 2-OH	3.3	1.9	3.0	0.2	1.9	1.2
C _{12:0} 3-OH	4.8	2.8	5.0	4.4	2.9	2.3
C _{14:1} ω 5c	—	—	—	—	—	0.5
C _{14:0}	3.5	3.1	1.7	2.6	2.0	1.6
C _{15:0}	3.0	2.3	—	0.6	1.0	0.7
C _{16:1} ω 5c	—	—	—	0.3	0.3	1.4
C _{16:1} ω 7c and/or iso-C _{16:1} ω 7c 2-OH*	27.5	28.7	35.8	33.4	38.9	41.7
C _{16:0}	26.6	32.0	23.9	25.8	30.2	27.6
C _{17:0} cyclo	4.3	13.2	1.7	—	—	—
C _{17:1} ω 6c	0.4	—	—	—	0.2	—
C _{18:1} ω 7c	14.8	8.7	15.0	18.8	15.7	14.9
C _{18:0}	0.3	0.6	—	0.5	0.5	—

*Summed fatty acids that cannot be separated by GLC with the MIDI System.

the smaller volume was harvested when, compared with the other culture, lower turbidity was still apparent. HPLC analyses of quinones and polyamines were carried out using the equipment described by Stolz *et al.* (2007). Polyamines were extracted and analysed as described by Busse & Auling (1988) and Stolz *et al.* (2007). Strains LMG 3947 and LMG 3953 exhibited similar quinone systems, consisting of ubiquinones Q-8 (62 and 74 %, respectively) and Q-7 (38 and 26 %). Polar lipid profiles of the two strains were rather similar, and consisted of the major compound phosphatidylethanolamine, moderate amounts of phosphatidylglycerol and minor to trace amounts of diphosphatidylglycerol, an unknown phospholipid and three unknown aminolipids. In contrast to strain LMG 3947, minor amounts of two unknown polar lipids and an additional aminolipid could be detected in the polar lipid profile of LMG 3953 (Fig. 2). However, we do not want to attribute too much importance to this observation, because the distinguishing lipids were found only in very small amounts and may be present in LMG 3947 in even smaller, undetectable amounts. Hence, the distinguishing lipids should only be used as distinguishing traits between the two species proposed in this paper. Both strains lack a characteristic phospholipid reported for *Janthinobacterium lividum*, another violet-pigment-producing species, and the close relative *Janthinobacterium agaricidamnosum* (Lincoln *et al.*, 1999; Kämpfer *et al.*, 2008). The polyamine pattern of strain LMG 3947 consisted of the predominant compounds putrescine [78.2 μ mol (g dry weight)⁻¹] and 2-hydroxyputrescine [72.2 μ mol (g dry weight)⁻¹], a moderate amount of 1,3-diaminopropane [9.6 μ mol (g dry weight)⁻¹] and a

Fig. 2. Total polar lipid profile of strain LMG 3953 after two-dimensional TLC and detection with molybdatophosphoric acid. PE, Phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; AL1–4, unknown aminolipids; PL1, unknown phospholipid; L1–2, unknown polar lipids. The first dimension is represented horizontally.

small amount of cadaverine [0.8 μmol (g dry weight)⁻¹]. The polyamine pattern of strain LMG 3953 consisted of the predominant compounds putrescine [84.3 μmol (g dry weight)⁻¹] and 2-hydroxyputrescine [37.0 μmol (g dry weight)⁻¹] and moderate amounts of 1,3-diaminopropane [9.9 μmol (g dry weight)⁻¹] and cadaverine [6.4 μmol (g dry weight)⁻¹]. Trace amounts of spermidine [0.1 μmol (g dry weight)⁻¹] were also detected. Based on our experience, the differences observed should not be considered useful for discrimination between the two strains. However, the results support the affiliation of the two strains with the *Betaproteobacteria* and the conclusion that they are most similar to *C. violaceum* (Busse & Auling, 1988). Emphasis has to be given to our finding that, like *C. violaceum* DSM 30191^T, the strains contain a relatively large amount of 1,3-diaminopropane [>5 μmol (g dry weight)⁻¹]. To the best of our knowledge, no other betaproteobacterium has so far been reported to contain comparable amounts of 1,3-diaminopropane (Busse & Auling, 1988; Auling *et al.*, 1991; Hamana & Takeuchi, 1998; Hamana *et al.*, 2000, 2007).

From sequence analysis of the 16S rRNA gene, it is obvious that both strains belong to the genus *Chromobacterium* and are most closely related to *C. violaceum*. Based on our results, we propose that the strains LMG 3947 and LMG 3953 represent separate novel species of the genus *Chromobacterium*, for which we propose the names *Chromobacterium piscinae* sp. nov. and *Chromobacterium pseudoviolaceum* sp. nov., respectively.

Description of *Chromobacterium piscinae* sp. nov.

Chromobacterium piscinae (pis.ci'nae. L. gen. n. *piscinae* of a pond).

Cells are Gram-negative, aerobic, rod-shaped and motile, 0.3–0.5 μm long and 1.5–2 μm wide. Good growth occurs after 48 h incubation on tryptone soy agar (TSA) and NA at 32 °C. Colonies on complex standard media at 32 °C are smooth, shiny and convex with spreading edges, 1.0–2.0 mm in diameter, and are non-fluorescent. A violet pigment is produced. The optimal temperature for growth is 32 °C. The optimal pH for growth is 6.0; growth occurs at pH 5 and 9.5, but not at pH 4.0 or 10. Growth occurs in the presence of 0–3.5 % (w/v) NaCl; optimal growth occurs in the presence of 0–0.1 % (w/v) NaCl. No anaerobic growth on plain NA or TSA supplemented with nitrate. Shows aerobic metabolism. Oxidase-positive and catalase-negative. Nitrate is reduced to nitrite. Utilizes L-aspartate and L-proline as sole sources of carbon. Results of further physiological and biochemical tests including differentiating characters are shown in Table 1. The fatty acid profile of the type strain is shown in Table 2. The polar lipid profile consists of the major compound phosphatidylethanolamine, moderate amounts of phosphatidylglycerol and minor to trace amounts of diphosphatidylglycerol, three unknown aminolipids and an unknown phospholipid. The quinone system consists of

the major compound ubiquinone Q-8 and moderate amounts of Q-7. The polyamine pattern is composed of the major compounds putrescine and 2-hydroxyputrescine, moderate amounts of 1,3-diaminopropane and small amounts of cadaverine.

The type strain is LMG 3947^T (=CCM 3329^T).

Description of *Chromobacterium pseudoviolaceum* sp. nov.

Chromobacterium pseudoviolaceum (pseu.do.vi.o.la'ce.um. Gr. adj. *pseudes* false; L. neut. adj. *violaceum* violet-coloured and also a bacterial specific epithet; N.L. neut. adj. *pseudoviolaceum* not the true *Chromobacterium violaceum*).

Cells are Gram-negative, aerobic, rod-shaped and motile, 0.3–0.5 μm long and 1.5–2 μm wide. Good growth occurs after 48 h incubation on TSA and NA at 32 °C. Colonies on complex standard media at 32 °C are smooth, shiny and convex with spreading edges, 1.0–2.0 mm in diameter, and are non-fluorescent. A violet pigment is produced. The optimal temperature for growth is 32 °C. The optimal pH for growth is 6.0; growth occurs at pH 5 and 9.5, but not at pH 4.0 or 10. Growth occurs in the presence of 0–3.5 % (w/v) NaCl; optimal growth occurs in the presence of 0–0.1 % (w/v) NaCl. No anaerobic growth on plain NA or TSA supplemented with nitrate. Shows aerobic metabolism. Oxidase-positive and catalase-negative. Nitrate is reduced to nitrite. Utilizes *cis*-aconitate and propionate as sole sources of carbon. Results of physiological and biochemical tests including differentiating characters are shown in Table 1. The fatty acid profile of the type strain is shown in Table 2. The polar lipid profile consists of the major compound phosphatidylethanolamine, moderate amounts of phosphatidylglycerol and minor to trace amounts of diphosphatidylglycerol, four unknown aminolipids, an unknown phospholipid and two unknown polar lipids. The quinone system consists of the major compound ubiquinone Q-8 and moderate amounts of Q-7. The polyamine pattern is composed of the major compounds putrescine and 2-hydroxyputrescine, moderate amounts of 1,3-diaminopropane and cadaverine and trace amounts of spermidine.

The type strain is LMG 3953^T (=CCM 2076^T =NCTC 8182^T).

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