A New Genus of the Order *Actinomycetales*, *Couchioplanes* gen. nov., with Descriptions of *Couchioplanes caeruleus* (Horan and Brodsky 1986) comb. nov. and *Couchioplanes caeruleus* subsp. *azureus* subsp. nov.

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During our taxonomic study of motile actinomycetes, soil isolate RA 335 was found to form a blue substrate mycelium and aerial mycelia with motile arthrospores and to have lysine as the cell wall diamino acid. *Actinoplanes caeruleus* IFO 13939^T (T = type strain) and “*Actinoplanes azureus*” IFO 13993^T are known to have the same characteristics. Therefore, the taxonomic position of these three strains was studied. Aerial mycelia of these strains fragmented during the growth cycle and produced motile spores arranged in chains within the mycelia. Sporangia were not observed. The strains contained menaquinone 9(H₄), had guanine-plus-cytosine contents of 69.9 to 72.1 mol%, and had D-glutamic acid, D- and L-serine, glycine, L-alanine, and L-lysine as cell wall amino acids (type A3 α). The taxonomic characteristics of these strains differ from those of the previously described motile actinomycetes. On the basis of morphological, physiological, and chemotaxonomic data and the results of DNA-DNA hybridization and comparative 16S rRNA studies, we propose a new genus, *Couchioplanes*, for these organisms. The type species is *Couchioplanes caeruleus* comb. nov. (type strain, IFO 13939), which is divided into two subspecies, *Couchioplanes caeruleus* subsp. *caeruleus* subsp. nov. (type strain, IFO 13939) for *A. caeruleus* IFO 13939^T and strain RA 335 and *Couchioplanes caeruleus* subsp. *azureus* (type strain, IFO 13993) for “*A. azureus*” IFO 13993^T.

During our taxonomic study of motile actinomycetes isolated from natural sources, we found that strain RA 335, which was isolated from soil in Japan, produces zoospores, has L-lysine in its cell wall, and forms mature mycelium that changes from orange to dark blue. *Actinoplanes caeruleus*, as described by Horan and Brodsky (9), and “*Actinoplanes azureus*” (2) are known to have the same characteristics. *Actinoplanes* species other than *Actinoplanes caeruleus* contain meso-diaminopimelic acid in their cell walls (29), but *Actinoplanes caeruleus* and “*Actinoplanes azureus*” have lysine instead of meso-diaminopimelic acid in their cell walls (9). Therefore, Stackebrandt and Kroppenstedt (24) and Goodfellow et al. (4) insisted that *Actinoplanes caeruleus* should not be included in the genus *Actinoplanes*. Thus, the taxonomic position of *Actinoplanes caeruleus* and “*Actinoplanes azureus*” has remained uncertain.

We studied the taxonomic position of these two species and isolate RA 335 and found that these organisms are motile arthrospore-bearing actinomycetes that contain menaquinone 9(H₄) [MK-9(H₄)] and have lysine and glycine in their cell walls (wall chemotype VI) and therefore differ from the previously described members of the motile arthrospore-bearing actinomycete genera *Sporichthya*, *Actinosynnema*, *Actinokineospora*, and *Catenuloplanes* (31).

In this paper we describe the characterization and classification of *Actinoplanes caeruleus*, “*Actinoplanes azureus*,” and isolate RA 335, and we propose that these organisms should be included in a new genus, *Couchioplanes*, as members of *Couchioplanes caeruleus* comb. nov., which is divided into two

subspecies, *Couchioplanes caeruleus* subsp. *caeruleus* subsp. nov. and *Couchioplanes caeruleus* subsp. *azureus* subsp. nov.

MATERIALS AND METHODS

Microorganisms and culture conditions. The following strains were used: *Actinoplanes caeruleus* IFO 13939^T (IFO, Institute for Fermentation, Osaka, Japan) (T = type strain), “*Actinoplanes azureus*” IFO 13993^T, and strain RA 335. Strain RA 335 was isolated from soil in Shiga Prefecture, Japan, on medium containing 1.0% soluble starch, 0.1% casein, 0.05% K₂HPO₄, and 1.5% agar (pH 7.0 to 7.5) and supplemented with 25 μ g of cefsulodin per ml and 6.25 μ g of kabicidin per ml. *Catenuloplanes japonicus* IFO 14176^T, IFO 14177, and RA 330 (31) were used as reference strains for the DNA relatedness study and for a comparison of 16S rRNA sequences.

Cultural observations. Cultural characteristics were recorded after 14 days of incubation at 28°C by using the International *Streptomyces* Project (ISP) method (23). Colors were described in common terminology, but exact colors were determined by comparison with color chips from the *Color Harmony Manual* (2a).

Morphological and physiological characterization. Morphological features were observed on HV agar (7), and physiological features were observed on media commonly used for identification of members of the order *Actinomycetales* (23). Motility was observed with a light microscope by using cells in the logarithmic growth phase grown on HV agar or cells in the stationary phase after incubation at 28°C for 1 h in 0.01 M phosphate buffer (pH 7.0) containing 10% soil extract. Flagellation was observed with a model JEM-1200EX transmission electron microscope (JEOL, Ltd., Tokyo, Japan) after shadowing with platinum-palladium. Cultures grown on HV agar for 14 days at 28°C were observed with a scanning electron

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FIG. 1. Light micrograph (A) and scanning electron micrographs (B through D) of *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T grown on HV agar for 14 days at 28°C.

microscope (model JSM-5400; JEOL Ltd.). Samples for scanning electron microscopy were prepared by cutting a block from the agar on which a culture grew, fixing the block in osmium tetroxide vapor at room temperature for 4 h, dehydrating the cells through a graded ethanol series and then in a Hitachi model HCP-2 critical point drying apparatus, and sputter coating the preparation with palladium under a vacuum.

Freeze-dried cells for chemotaxonomic analysis were obtained from cultures grown in yeast extract-glucose broth (containing 10 g of yeast extract and 10 g of D-glucose per 1,000 ml of distilled water; pH 7.0) on a rotary shaker at 28°C.

Peptidoglycan analysis. Cell walls were prepared from ca. 500 mg of dry cells by mechanical disruption with an ultrasonic oscillator and were purified as described by Schleifer and Kandler (22). The amino acid compositions of complete wall hydrolysates were determined by high-performance liquid chromatography (HPLC) with a model LC-6AD apparatus (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a Wakopak WS-PTC column (Wako Pure Chemical Ind., Ltd., Osaka, Japan); the amino acid phenyl thiocarbamoyl derivatives were identified according to the manufacturer's instructions (30).

The amino acid compositions were also examined by developing preparations on cellulose thin-layer chromatography plates (Tokyo Kasei Co., Ltd., Tokyo, Japan), using two-dimensional descending chromatography and the method of Harper and Davis (5). Amino acid configurations were determined by measuring the amino acid contents of the hydrolysates before and after incubation with D- and L-amino acid oxidases (alanine and serine), L-lysine decarboxylase (lysine), and L-glutamic acid decarboxylase (glutamic acid), using the method of Kandler and König (11).

Cell wall sugar analysis. Cell walls were hydrolyzed with 2 N HCl at 100°C for 2 h, dried in vacuo, and then analyzed by the method of Mikami and Ishida (16) by using a Shimadzu model LC-5A HPLC apparatus equipped with a Shim-pack ISA 07/S2504 column (250 by 4 mm) and a Shimadzu model RE-530 spectrofluorometer.

Glycolyl analysis. The glycolyl test was performed by the method of Uchida and Aida (28).

Analysis of cellular fatty acids. Fatty acids were extracted from 50 mg of dry cells by acid methanolysis and were examined by using a gas-liquid chromatograph (model GC-9A; Shimadzu) equipped with a glass column (2 mm by 5 m)

FIG. 2. Scanning electron micrograph of *Couchioplanes caeruleus* subsp. *azureus* IFO 13993^T grown on HV agar for 14 days at 28°C.

containing 10% diethyleneglycol succinate on Chromosorb W at 180°C (26).

Analysis of polar lipids. Free lipids were extracted from 100 mg of dry cells, purified by the method of Minnikin et al. (18), and examined by two-dimensional thin-layer chromatography, using Kiesel gel 60 F₂₅₄ plates. Lipids were visualized by spraying preparations with 10% molybdophosphoric acid in ethanol and then heating them at 140°C for 10 min. Specific spray reagents for lipid phosphate, α -naphthol (sugar), and ninhydrin (amino groups) were also used.

Analysis of mycolic acids. Mycolic acids were analyzed by the method of Minnikin et al. (17).

Analysis of isoprenoid quinones. Menaquinones were extracted from 200 mg of dry cells with chloroform-methanol (2:1, vol/vol), purified by thin-layer chromatography in which hexane-diethyl ether (1:1, vol/vol) was used as the solvent, extracted with diethyl ether, dried with a stream of nitrogen, and then analyzed by HPLC by using a Shimadzu model LC-5A apparatus equipped with a Zorbax octyldecyl silane column (4.6 by 150 mm). Methanol-isopropyl ether (7:1) was used as the mobile phase.

DNA base composition. DNA was obtained by the method of

Saito and Miura (20). The guanine-plus-cytosine (G+C) content of DNA was determined by the method of Mesbah et al. (15) after treatment with P1 nuclease and alkaline phosphatase by using a Shimadzu model LC-6AD HPLC apparatus equipped with a Cosmosil 5C₁₈-AR column (4.6 by 150 mm; Nacalai Tesque, Inc., Kyoto, Japan); 0.2 M ammonium phosphate-acetonitrile (40:1) was used as the mobile phase.

DNA-DNA hybridization. DNA-DNA relatedness was measured fluorometrically by the method of Ezaki et al. (3), using biotinylated DNA.

PCR and sequencing of the products. In order to generate sequence templates, a PCR was performed twice. First, 16S rRNA genes were amplified by a PCR by using a standard protocol (19), with some modifications (each 100 μ l of reaction mixture contained each of the primers at a concentration of 20 nM, 50 to 100 ng of total DNA, and 2.5 U of AmpliTaq DNA polymerase [Cetus Inc.]). The sequences of the primers used for the PCR were identical to the sequence at positions 10 to 25 (5'-AGTTTGATCCTGGCTC-3'; primer 9F) in the *Escherichia coli* numbering system of Brosius et al. (1), identical to the sequence at positions 343 to 357 (5'-TACGGGAGGCA GCAG-3'; primer 340F), complementary to the sequence at

FIG. 3. Flagellation of *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T.

positions 512 to 529 with bases complementary to primer M13 (5'-TGTAACGACGCGCCAGT-3'; primer M) (5'-[M]GAATTACGCGGCTGCTG-3'; primer M519R), complementary to the sequence at the positions 905 to 924 with the sequence of primer M (5'-[M]CCGTCAATTCATTTAAGT TT-3'; primer M907R), and complementary to the sequence at positions 1406 to 1392 with the sequence of primer M (5'-[M]ACGGGCGGTGTGTAC-3'; primer M1392R). Using a primer with bases added to the 5' end gave products which had the same sequence as the dye-labelled primer. The temperature program (30 s at 98°C, 30 s at 50 or 55°C, and 2 min at 72°C; 25 cycles after 2 min at 98°C) was carried out with a Perkin-Elmer Cetus DNA thermal cycler.

Nucleotide sequences were determined by using a model 373A DNA sequencer and a dye primer cycle sequencing kit (catalog no. 21M13; Applied Biosystems, Foster City, Calif.) according to the manufacturer's directions.

Analysis of nucleotide sequences and nucleotide sequence accession numbers. DNA sequences were aligned by using the ODN system (10). Evolutionary distances were represented by K_{nuc} values as described by Kimura (12), and a phylogenetic tree was constructed by the neighbor-joining method (21) from K_{nuc} values derived from the sequences determined in this study and sequences available from the EMBL and GenBank nucleotide sequence data bases under the following accession numbers: *Actinomyces viscosus* DSM 43027, M53225; *Actinoplanes philippinensis*, X72864; *Aeromicrobium erythreum* NRRL B-3381^T, M37200; *Amycolatopsis azurea* NRRL 11412^T, X53199; "*Amycolata petrophila*" IFAM 78, X55608; *Arthrobacter globiformis*, M23411; *Clavibacter xyli*, M60935; *Corynebacterium xerosis*, M59058; *Dactylosporangium aurantia-cum*, X72779; *Frankia* sp., M55343; *Gordonia terrae* DSM 43249^T, X53202; *Kibdelosporangium aridum*, X53191; *Kurthia zopfii*, M58800; *Micrococcus luteus*, M38242; *Mycobacterium intracellulare* ATCC 15985, X52927; *Nocardia asteroides* ATCC 3306, X57949; *Nocardioides simplex* ATCC 6946^T, X53213; *Nocardioides albus* DSM 43109^T, X53211; *Propionibacterium freudenreichii* DSM 20271^T, X53217; *Pseudonocardia ther-*

mophila ATCC 19285^T, X53195; *Renibacterium salmoninarum* ATCC 33209^T, X51601; *Rhodococcus erythropolis* DSM 43188, X53203; *Saccharomonospora viridis* ATCC 15386^T, X54286; *Saccharopolyspora erythraea* NRRL 2338^T, X53198; *Saccharopolyspora hordei*, X53197; *Saccharopolyspora rectivirgula*, X53194; *Saccharothrix australiensis*, X53192; *Streptomyces griseus* KCTC 9080, M76388; *Streptomyces coelicolor* A3, Y00411; *Streptomyces setae*, M55220; *Terrabacter tumescens* NCIB 8914^T, X53215; and *Tsukamurella paurometabola* DSM 20162^T, X53206. The sequences of 16S rRNAs determined for *Catenuloplanes japonicus* IFO 14176, IFO 14177, and RA 330 and *A. caeruleus* IFO 13939^T were deposited in the DNA Data Bank of Japan Data Library under accession numbers D14642, D14643, D14644, and D14645, respectively. The topology of the trees was evaluated by bootstrap analysis of the sequence data by using the Clustal V program (8).

RESULTS AND DISCUSSION

Morphological observations. *Actinoplanes caeruleus* IFO 13939^T, "*Actinoplanes azureus*" IFO 13993^T, and isolate RA 335 developed branched aerial mycelia. Spores were formed in chains. Sporangia were not observed. The spore chains and aerial mycelia often aggregated into clusters resembling sporangia, but these clusters were not true sporangia because they were not covered with a sheath (Fig. 1A and B and 2). Morphological observation of a 14-day-old culture grown on HV agar revealed the presence of an aerial mycelium with short spore chains arranged in irregular spirals, which might have arisen from the substrate mycelium. Several spores per spore chain were observed, and the spores were oval to short rods (0.5 to 0.9 by 1.0 to 1.5 μm) and smooth, as revealed by scanning electron microscopy (Fig. 1C and D).

After incubation at 28°C for 1 h in 0.01 M phosphate buffer (pH 7.0) containing 10% soil extract, many spores exhibited active motility. The zoospores had polar or nearly polar flagella (Fig. 3).

Growth characteristics. As shown in Table 1, the three

TABLE 1. Cultural characteristics of *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T, strain RA 335, and *Couchioplanes caeruleus* subsp. *azureus* IFO 13993^T

Medium	Characteristic	Strain IFO 13939 ^T	Strain IFO 13993 ^T	Strain RA 335
Yeast extract-malt extract agar (ISP medium 2)	Growth	Good, yellow (2ga) to deep teal blue (17pe) ^a	Good, yellow (3ga) to dark blue (13pi to 14pi)	Good, yellow (2ga) to 17 pe
	Aerial mycelium	Absent	Absent	Absent
	Reverse color	Yellow (3ia)	Yellow (3ic)	Yellow (3ia)
	Soluble pigment	Absent	Pale brown (3pl)	Absent
Oatmeal agar (ISP medium 3)	Growth	Moderate, shell (2ca) to dark gray blue (14li)	Moderate, yellow (3ea) to ivy (24ml)	Moderate, shell (2ca) to 14 pi
	Aerial mycelium	Good, white	Absent	Moderate, white
	Reverse color	Wheat (2ea) to shadow blue (16lg)	Shell (3ca) to dark ivy (24po)	Wheat (2ea) to shadow blue (16lg)
	Soluble pigment	Absent	Absent	Absent
Inorganic salts-starch agar (ISP medium 4)	Growth	Good, shell (2ca) to dark gray blue (16li)	Good, shell (3ca) to dusty jade (21ec)	Good, shell (2ca) to dark gray blue (16li)
	Aerial mycelium	Good, white	Absent	Good, white
	Reverse color	Shell (2ca) to shadow blue (16lg)	Shell (3ca to 20ca)	Shell (2ca) to shadow blue (16lg)
	Soluble pigment	Absent	Absent	Absent
Glycerol-asparagine agar (ISP medium 5)	Growth	Good, yellow (3ga) to dusty (20ie)	Good, yellow (3la) to 14pi	Good, yellow (3ga) to surf green (22ie)
	Aerial mycelium	Absent	Absent	Absent
	Reverse color	Wheat (2ea) to jade green (21le)	Yellow (3ga) to medium blue (16ng)	Wheat (2ea) to jade green (21le)
	Soluble pigment	Pale brown	Absent	Pale brown
Peptone-yeast extract-iron agar (ISP medium 6)	Growth	Moderate, brown (3lg) to tan (3ie)	Moderate, brown (3lg) to tan (3ie)	Moderate, tan (3ie)
	Aerial mycelium	Absent	Absent	Absent
	Reverse color	Brown (3lg) to tan (3ie)	Brown (3lg) to tan (3ie)	Tan (3ie) to yellow (3ic)
	Soluble pigment	Pale brown (3nl to 3pi)	Pale brown (3nl to 3pi)	Absent or pale brown
Tyrosine agar (ISP medium 7)	Growth	Moderate, yellow (2ga)	Moderate, yellow (3ea)	Fair, yellow (2ga)
	Aerial mycelium	Absent	Absent	Absent
	Reverse color	Yellow (2ia)	Wheat (2ea)	Yellow (2ia)
	Soluble pigment	Absent	Absent or dark brown	Absent
Nutrient agar	Growth	Moderate, wheat (2ea)	Moderate, tan (3gc)	Moderate, wheat (2ea)
	Aerial mycelium	Fair	Absent	Absent
	Reverse color	Yellow (3ea)	Yellow (3ic)	Yellow (3ea)
	Soluble pigment	Absent	Absent	Absent
Bennett's agar	Growth	Good, yellow (3nc)	Good, yellow (3ga)	Good, wheat (2ea)
	Aerial mycelium	Absent	Absent	Absent
	Reverse color	Gold (2ne)	Yellow (3ic)	Gold (2ne)
	Soluble pigment	Absent	Absent	Absent
Glucose-asparagine agar	Growth	Moderate, wheat (2ea) to dark blue (17pi)	Fair, wheat (3ea) to 18li	Moderate, yellow (2ga) to dark blue (17pi)
	Aerial mycelium	Absent	Absent	Absent
	Reverse color	Wheat (2ga) to dark blue (17pi)	Wheat (3ea) to 18li	Wheat (2ga) to dark blue (17pi)
	Soluble pigment	Yellow (2ia)	Absent	Yellow (2ia)
Water agar	Growth	Fair, dull white	Fair, shell (3ba)	Fair, dull white, colorless
	Aerial mycelium	Good	Absent	Moderate, white
	Reverse color	Dull white	Shell (2ba)	Dull white, colorless
	Soluble pigment	Absent	Absent	Absent

^a The color codes correspond to the color codes in the *Color Harmony Manual*, 4th ed.

TABLE 2. Phenotypic characteristics of *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T and RA 335 and *Couchioplanes caeruleus* subsp. *azureus* IFO 13993^T

Characteristic	Strains IFO 13939 ^T and RA 335	Strain IFO 13993 ^T
Utilization of:		
Glucose	++ ^a	++
D-Xylose	—	—
Inositol	++	++
Sucrose	++	++
Raffinose	—	—
Rhamnose	++	—
D-Mannitol	++	±
D-Fructose	++	+
D-Arabinose	—	—
Pigmentation in ^b :		
Tryptone-yeast extract broth (ISP medium 1)	P	P
Yeast extract-malt extract agar (ISP medium 2)	A	P
Glycerol-asparagine agar (ISP medium 5)	P	A
Glucose-asparagine agar	P	A
Gelatin liquefaction	P	P
Peptonization of milk	A	A
Starch hydrolysis	P	P
Calcium malate hydrolysis	A	A
Reduction of nitrate	P	P
H ₂ S production	P	P
Growth in 1% NaCl	P	P
Growth in 2% NaCl	A	P
Growth in 3% NaCl	A	A

^a Abbreviations: ++, good; +, moderate; ±, poor; —, none; P, present; A, absent.

^b For pigment colors on yeast extract-malt extract agar, glycerol-asparagine agar, and glucose-asparagine agar, see Table 1. In tryptone-yeast extract broth the pigment was pale brown for all organisms.

strains had nearly the same cultural characteristics. Young colonies were yellow to orange, and later the colony color changed to dark green to dark blue. All of the strains grew well on yeast extract-malt extract agar (ISP medium 2), inorganic salts-starch agar (ISP medium 4), glycerol-asparagine agar (ISP medium 5), and Bennett's agar and produced a pale brown soluble pigment on peptone-yeast extract-iron agar (ISP medium 6). However, *Actinoplanes caeruleus* IFO 13939^T and isolate RA 335 differed from "*Actinoplanes azureus*" IFO 13993^T in that the former two strains developed aerial mycelia on oatmeal agar (ISP medium 3) and inorganic salts-starch agar (ISP medium 4), produced a soluble pigment on glycerol-asparagine agar (ISP medium 5) and glucose-asparagine agar, and did not produce a soluble pigment on yeast extract-malt extract agar (ISP medium 2).

TABLE 4. Phospholipid type and menaquinone compositions of *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T and RA 335 and *Couchioplanes caeruleus* subsp. *azureus* IFO 13993^T

Strain	Phospholipid type ^a	Menaquinone composition			
		MK-9(H ₂)	MK-9(H ₄)	MK-9(H ₆)	MK-9(H ₈)
IFO 13939 ^T	PII	+ ^b	+++	+	—
RA 335	PII	+++	+++	++	+
IFO 13993 ^T	PII	+	+++	++	+

^a Phosphatidylglycerol and phosphatidylethanolamine were detected, but phosphatidylcholine was not detected.

^b +++, >50%; ++, >10%; +, ≤10%.

Physiological and biochemical characteristics. Biochemical properties of the three strains are shown in Table 2. All of the strains were positive for gelatin liquefaction, hydrolysis of starch, and production of H₂S and were negative for decomposition of calcium malate and coagulation and clearing of milk. *Actinoplanes caeruleus* IFO 13939^T and isolate RA 335 differed from "*Actinoplanes azureus*" IFO 13993^T in that the former two strains utilized rhamnose and mannitol.

Chemotaxonomic characteristics. The whole-cell sugar patterns, cell wall amino acid compositions, phospholipid types, menaquinone compositions, and cellular fatty acid compositions of the organisms are shown in Tables 3 through 5. The cell walls contained L-lysine, D- plus L-serine, glycine, D-glutamate, and L-alanine (molar ratio, ca. 1:1:1:1:1), indicating that the wall chemotype of these strains is type VI according to the classification of Lechevalier and Lechevalier (14) and the peptidoglycan type is type A3α according to the classification of Schleifer and Kandler (22). Strain IFO 13939^T contained xylose, glucose, and mannose as the major cell wall sugars in addition to small amounts of arabinose and galactose. Strains IFO 13993^T and RA 335 contained xylose, glucose, arabinose, galactose, and mannose as the major cell wall sugars. The major menaquinone was MK-9(H₄); small amounts of MK-9(H₆), MK-9(H₈), and MK-9(H₂) were also present (Table 4). All strains contained iso-C_{16:0} and anteiso-C_{17:0} as the major cellular fatty acids (Table 5). Mycolic acids were absent. The diagnostic phospholipids were phosphatidylglycerol and phosphatidylethanolamine, but phosphatidylcholine was not detected (phospholipid type II of Hasegawa et al. [6]). The G+C contents of the DNAs were 69.9 to 72.1 mol% (Table 6).

DNA-DNA hybridization. As shown in Table 6, the results of DNA-DNA hybridization studies indicated that the three strains could be divided into two DNA homology groups. The first group contained two strains, *Actinoplanes caeruleus* IFO 13939^T and RA 335, and the other group was a single-member group containing "*Actinoplanes azureus*" IFO 13993^T. The levels of relatedness between members of the two groups ranged from 52 to 62%.

TABLE 3. Cell wall amino acid and whole-cell sugar compositions of *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T and RA 335 and *Couchioplanes caeruleus* subsp. *azureus* IFO 13993^{Ta}

Strain	Amino acid composition of peptidoglycan (molar ratio) ^b					Sugar composition of whole cells						
	Glu	Ser	Gly	Ala	Lys	Ara	Xyl	Gal	Glc	Man	Mad	Rib
IFO 13939 ^T	0.66	1.09	1.07	0.62	1.00	tr	+	tr	+	+	—	tr
RA 335	1.00	1.25	1.10	0.80	1.00	+	+	+	+	+	—	tr
IFO 13993 ^T	0.65	1.21	1.15	0.72	1.00	+	+	+	+	+	—	tr

^a Abbreviations: Glu, glutamic acid; Ser, serine; Gly, glycine; Ala, alanine; Lys, lysine; Ara, arabinose; Gal, galactose; Glc, glucose; Man, mannose; Mad, madurose; Rib, ribose; Xyl, xylose. +, present; —, absent; tr, trace amount present.

^b Calculated by defining the amount of lysine as 1.0.

TABLE 5. Cellular fatty acid compositions of *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T and RA 335 and *Couchioplanes caeruleus* subsp. *azureus* IFO 13993^T

Strain	Fatty acid composition (%)											
	anteiso-branched fatty acids		iso-branched fatty acids				Saturated fatty acids			Unsaturated fatty acids		
	anteiso-C _{15:0}	anteiso-C _{17:0}	iso-C _{14:0}	iso-C _{15:0}	iso-C _{16:0}	iso-C _{18:0}	C _{16:0}	C _{17:0}	C _{18:0}	C _{16:1}	C _{17:1}	C _{18:1}
IFO 13939 ^T	16	14			47	1	10	2	1	2	1	3
RA 335		16	1	12	45	2	7	4	2	1	2	3
IFO 13993 ^T	19	20	1		22		8	2	8	14	1	14

Phylogenetic analysis. The percentages of similarity obtained after pairwise alignment of the sequences from *Actinoplanes caeruleus* IFO 13939^T and representative strains of the genera *Actinoplanes*, *Dactylosporangium*, and *Catenuloplanes* and other actinomycete genera are shown in Table 7, and a phylogenetic tree derived from the sequences is shown in Fig. 4. *Catenuloplanes japonicus* IFO 14176^T, IFO 14177, and RA 330 were phylogenetically coherent. *Actinoplanes caeruleus* IFO 13939^T could be separated from its taxonomic neighbors, members of the genera *Actinoplanes*, *Dactylosporangium*, and *Catenuloplanes*, and other actinomycete taxa by phylogenetic analysis (Fig. 4).

Although *Actinoplanes caeruleus* differs from other species of the genus *Actinoplanes* by forming a deep blue vegetative mycelial pigment, by the absence of diaminopimelic acid in its cell wall, by its ability to hydrolyze adenine and hypoxanthine, by its resistance to lysozyme, and by its inability to utilize L-arabinose, D-xylose, and succinate as sole carbon sources, Horan and Brodsky (9) included this organism in the genus *Actinoplanes* for the following reasons: it formed irregular to globose sporangia which upon wetting released spherical to oval, partially flagellated, motile spores and contained arabinose and xylose as diagnostic whole-cell sugars. However, Stackebrandt and Kroppestedt (24) reported that this organism should not be included in this genus because of its peptidoglycan type; the results of numerical taxonomy studies of the genus *Actinoplanes* performed by Goodfellow et al. (4) support this conclusion. Thus, the taxonomic status of this organism has remained uncertain.

In this study, we observed aerial mycelia and motile arthrospores in *Actinoplanes caeruleus*, "*Actinoplanes azureus*," and RA 335 preparations, but no sporangia were observed. The aerial mycelia and arthrospores were intertwined and often developed sporangium-like structures, and during observation with a light microscope these structures often looked like sporangia. Horan and Brodsky (9) used a phase-contrast microscope when they observed "sporangia," but our observations with a scanning electron microscope indicated that these

structures are not sporangia but are intertwined aerial mycelia and arthrospores. Strains IFO 13939^T, IFO 13993^T, and RA 335 differ from strains belonging to the genera *Actinoplanes*, *Dactylosporangium*, *Micromonospora*, and *Pilimelia* of the family *Micromonosporaceae* (4) in that they contain L-lysine instead of meso-diaminopimelic acid in their cell walls and produce spores within the mycelia arranged in chains instead of enclosed in sporangial walls (Table 8). These strains resemble strains belonging to the genus *Catenuloplanes* (31) in that they have motile arthrospores and the same cell wall peptidoglycan type (wall chemotype VI and murein type A3 α) but differ in their menaquinone system [MK-9(H₄)], phospholipid type (type II), and cellular fatty acids (iso-C_{16:0} and anteiso-C_{17:0}) (Tables 5 and 8). Whereas the arthrospores of strains IFO 13939^T, IFO 13993^T, and RA 335 are oval to short rods with polar flagella, *Catenuloplanes* arthrospores are rods with peritrichous flagella.

Thus, on the basis of morphological, biochemical, chemical, and phylogenetical criteria, *Actinoplanes caeruleus*, "*Actinoplanes azureus*," and isolate RA 335 can be distinguished readily from the previously described motile actinomycetes and warrant a new taxon. No assimilation of rhamnose, production of a soluble pigment on yeast extract-malt extract agar (ISP medium 2), no production of a soluble pigment on glycerol-asparagine agar (ISP medium 5) and glucose-asparagine agar, and DNA-DNA similarity values suggest that "*Actinoplanes azureus*" should be classified as a subspecies of *Actinoplanes caeruleus*. The subspecies can be differentiated on the basis of characteristics summarized in Table 9.

Therefore, we propose that strains IFO 13939^T and RA 335 should be placed in a new genus, *Couchioplanes*, with *Couchioplanes caeruleus* comb. nov. (type strain, IFO 13939) as the type species and that strain IFO 13993 should be placed in a new subspecies, *Couchioplanes caeruleus* subsp. *azureus* (type strain, IFO 13993).

Tille et al. (27) described strain IMET 9075, which produced a blue substrate mycelium, did not utilize xylose, and contained lysine instead of meso-diaminopimelic acid in its cell wall. Our results suggest that strain IMET 9075 may also belong to the new genus *Couchioplanes*.

Although there are many differences between the genus *Couchioplanes* and other genera belonging to the family *Micromonosporaceae* with regard to morphological and chemotaxonomic characteristics, the genus *Couchioplanes*, together with the recently described genus *Catenuloplanes* (31), should be placed in the family *Micromonosporaceae* Krassil'nikov 1938 emend. Goodfellow et al. 1990 (4) on the basis of the results of the comparative analysis of 16S rRNA sequences described in this paper and the comparative 16S rRNA cataloging results reported by Stackebrandt et al. (25).

Description of *Couchioplanes* gen. nov. *Couchioplanes* (Couchi.o.pla.nes. N.L. adj. *Couchio*, referring to J. N. Couch [1896

TABLE 6. G+C contents and levels of DNA relatedness among *Couchioplanes caeruleus* subsp. *caeruleus* IFO 13939^T and RA 335, *Couchioplanes caeruleus* subsp. *azureus* IFO 13993^T, and *Catenuloplanes japonicus* IFO 14176^T

Strain	G+C content (mol%)	% of DNA complementary to labelled DNA from strain:			
		IFO 13939 ^T	RA 335	IFO 13993 ^T	IFO 14176 ^T
IFO 13939 ^T	69.9	100	101	53	8
RA 335	70.5	103	100	62	7
IFO 13993 ^T	72.1	52	57	100	10
IFO 14176 ^T	71.0	2	3	2	100

TABLE 7. Nucleotide substitution rates (K_{nuc} values) for the 16S rRNA sequences of members of the genera *Couchioplanes* and *Catenuloplanes* and related actinomycete genera

Organism	K_{nuc} value with:															
	<i>Couchioplanes caeruleus</i> IFO 13939 ^T	<i>Catenuloplanes japonicus</i> IFO 14176 ^T	<i>Catenuloplanes japonicus</i> IFO 14177	<i>Catenuloplanes japonicus</i> RA 330	<i>Actinoplanes philippinensis</i>	<i>Dactylosporangium aurantiacum</i>	<i>Actinomyces viscosus</i>	<i>Aeromicrobium erythreum</i>	<i>Arthrobacter globiformis</i>	<i>Saccharopolyspora rectivirgula</i>	<i>Amycolata petrophila</i>	<i>Amycolatopsis azurae</i>	<i>Saccharothrix australiensis</i>	<i>Pseudonocardia thermophila</i>	<i>Nocardia asteroides</i>	
<i>Couchioplanes caeruleus</i> IFO 13939 ^T																
<i>Catenuloplanes japonicus</i> IFO 14176 ^T	0.020															
<i>Catenuloplanes japonicus</i> IFO 14177	0.020	0.008														
<i>Catenuloplanes japonicus</i> RA 330	0.023	0.010	0.010													
<i>Actinoplanes philippinensis</i>	0.031	0.035	0.035	0.037												
<i>Dactylosporangium aurantiacum</i>	0.041	0.038	0.037	0.039	0.039											
<i>Actinomyces viscosus</i>	0.097	0.089	0.089	0.092	0.096	0.087										
<i>Aeromicrobium erythreum</i>	0.071	0.070	0.073	0.074	0.067	0.072	0.110									
<i>Arthrobacter globiformis</i>	0.067	0.077	0.077	0.078	0.073	0.077	0.085	0.065								
<i>Saccharopolyspora rectivirgula</i>	0.074	0.075	0.075	0.079	0.069	0.070	0.107	0.088	0.099							
<i>Amycolata petrophila</i>	0.078	0.079	0.082	0.081	0.074	0.081	0.095	0.083	0.092	0.084						
<i>Amycolatopsis azurae</i>	0.059	0.063	0.060	0.064	0.059	0.058	0.100	0.079	0.084	0.063	0.084					
<i>Saccharothrix australiensis</i>	0.055	0.056	0.059	0.060	0.054	0.060	0.100	0.072	0.090	0.066	0.071	0.054				
<i>Pseudonocardia thermophila</i>	0.065	0.063	0.063	0.067	0.062	0.054	0.095	0.079	0.092	0.048	0.067	0.060	0.055			
<i>Nocardia asteroides</i>	0.050	0.054	0.054	0.055	0.044	0.055	0.088	0.060	0.061	0.077	0.070	0.060	0.055	0.064		
<i>Streptomyces (Kitasatospora) setae</i>	0.070	0.071	0.071	0.073	0.067	0.073	0.097	0.064	0.068	0.098	0.093	0.077	0.075	0.085	0.061	
<i>Kibdelosporangium aridum</i>	0.053	0.051	0.054	0.058	0.045	0.060	0.097	0.064	0.074	0.067	0.067	0.055	0.051	0.058	0.051	
<i>Tsukamurella paurometabola</i>	0.060	0.067	0.064	0.068	0.066	0.073	0.104	0.073	0.065	0.089	0.070	0.060	0.064	0.075	0.038	
<i>Saccharomonospora viridis</i>	0.073	0.074	0.070	0.078	0.071	0.075	0.113	0.090	0.094	0.074	0.088	0.068	0.068	0.068	0.070	
<i>Saccharopolyspora hordei</i>	0.072	0.071	0.071	0.075	0.063	0.073	0.107	0.083	0.090	0.051	0.087	0.056	0.071	0.062	0.074	
<i>Saccharopolyspora erythraea</i>	0.082	0.079	0.081	0.086	0.077	0.081	0.119	0.100	0.109	0.070	0.089	0.063	0.081	0.078	0.085	
<i>Streptomyces griseus</i>	0.073	0.069	0.071	0.073	0.071	0.071	0.099	0.070	0.072	0.096	0.098	0.078	0.079	0.086	0.065	
<i>Nocardioides albus</i>	0.077	0.079	0.079	0.081	0.075	0.082	0.114	0.051	0.075	0.093	0.096	0.092	0.085	0.095	0.073	
<i>Nocardioides simplex</i>	0.075	0.077	0.074	0.078	0.077	0.081	0.107	0.051	0.069	0.088	0.084	0.079	0.079	0.082	0.068	
<i>Micrococcus luteus</i>	0.067	0.077	0.076	0.078	0.073	0.078	0.085	0.070	0.017	0.097	0.091	0.088	0.084	0.089	0.061	
<i>Gordona terrae</i>	0.061	0.063	0.063	0.067	0.063	0.063	0.093	0.063	0.062	0.073	0.074	0.060	0.060	0.060	0.028	
<i>Corynebacterium xerosis</i>	0.077	0.084	0.082	0.081	0.077	0.081	0.099	0.086	0.076	0.086	0.081	0.086	0.079	0.084	0.059	
<i>Rhodococcus erythropolis</i>	0.063	0.064	0.062	0.066	0.056	0.056	0.099	0.064	0.074	0.067	0.074	0.056	0.060	0.052	0.030	
<i>Clavibacter xyli</i>	0.078	0.084	0.082	0.085	0.080	0.088	0.106	0.075	0.053	0.103	0.098	0.088	0.085	0.098	0.078	
<i>Renibacterium salmoninarum</i>	0.087	0.096	0.098	0.099	0.093	0.098	0.108	0.088	0.030	0.112	0.112	0.105	0.103	0.105	0.081	
<i>Propionibacterium freudenreichii</i>	0.111	0.112	0.111	0.117	0.115	0.113	0.113	0.089	0.099	0.121	0.103	0.115	0.113	0.116	0.091	
<i>Terrabacter tumescens</i>	0.070	0.078	0.078	0.079	0.072	0.080	0.106	0.078	0.050	0.111	0.093	0.087	0.088	0.096	0.066	
<i>Mycobacterium intracellulare</i>	0.070	0.073	0.075	0.073	0.074	0.070	0.096	0.070	0.075	0.075	0.070	0.073	0.066	0.068	0.043	
<i>Streptomyces coelicolor</i>	0.070	0.071	0.071	0.073	0.067	0.070	0.105	0.077	0.069	0.093	0.095	0.077	0.079	0.086	0.063	
<i>Nocardioides fastidiosa</i>	0.088	0.085	0.084	0.089	0.083	0.083	0.118	0.026	0.078	0.093	0.097	0.090	0.086	0.090	0.079	
<i>Nocardioides luteus</i>	0.075	0.079	0.079	0.081	0.074	0.081	0.116	0.050	0.074	0.095	0.095	0.090	0.083	0.093	0.071	

to 1986], a mycologist who contributed to the taxonomy of the family *Actinoplanaceae*; Gr. n. *planes*, a wanderer; M.L.masc.n. *Couchioplanes*, Couch wanderer) strains are gram-positive, non-acid-fast, aerobic organisms with branching hyphae. Non-fragmenting, yellowish orange to blue mycelia are present. Spores are formed in chains and are motile. The spore chains and aerial mycelia often aggregate into clusters resembling sporangia, but true sporangia are not observed. In 14-day-old cultures grown on oatmeal agar aerial mycelia with short spore chains arranged in spirals that have one to five turns and are hooked or rarely flexuous are present. Several spores are present per spore chain, and the spores are oval to short rods (diameter, 1.3 to 2.0 μm) and exhibit motility when they are suspended in sterile distilled water.

Strictly aerobic. Good growth occurs at temperatures between 22 and 28°C. The organisms grow well on yeast extract-malt extract agar, inorganic salts-starch agar, glycerol-asparagine agar, and Bennett's agar. In general, the vegetative mycelia of the strains are pale orange to blue, and the aerial mycelia are white to gray.

Cell walls contain D-glutamate, D- and L-serine, glycine, L-alanine, and L-lysine (molar ratio, ca. 1:1:1:1:1). The wall chemotype is type VI according to the classification of Lechevalier and Lechevalier (14), and the peptidoglycan type is type A (most probably type A3 α) according to the classification of Schleifer and Kandler (22). Xylose, arabinose, and galactose are the characteristic cell wall sugars; iso-pentadecanoic acid (iso-C_{15:0}) and anteiso-heptadecanoic acid (anteiso-C_{17:0}) are the major cellular fatty acids. The G+C contents of DNAs range from 69 to 73 mol%; the G+C contents of the type species range from 69.9 to 70.5 mol%. The major menaquinone is MK-9(H₄); in addition, small amounts of MK-9(H₂), MK-9(H₈), and MK-9(H₂) are also present. Phosphatidylglycerol and phosphatidylethanolamine are the diagnostic phospholipids, but phosphatidylcholine is absent (type PII). Habitat: soil.

The type species is *Couchioplanes caeruleus*.

Description of *Couchioplanes caeruleus* (Horan and Brodsky 1986) comb. nov. *Couchioplanes caeruleus* (cac. ru'le. us. L. masc. adj. *caeruleus*, dark blue, referring to the blue vegetative

TABLE 7—Continued

K_{nuc} value with:																			
<i>Streptomyces</i> (<i>Kitosatosporia</i>) <i>setae</i>	<i>Kibdelosporangium</i> <i>avidum</i>	<i>Tsukamurella</i> <i>paurometabola</i>	<i>Saccharomonospora</i> <i>viridis</i>	<i>Saccharopolyspora</i> <i>hordai</i>	<i>Saccharopolyspora</i> <i>erythraea</i>	<i>Streptomyces</i> <i>griseus</i>	<i>Nocardioides</i> <i>albus</i>	<i>Nocardioides</i> <i>simplex</i>	<i>Micrococcus</i> <i>luteus</i>	<i>Gordonia</i> <i>terrae</i>	<i>Corynebacterium</i> <i>xerosis</i>	<i>Rhodococcus</i> <i>erythropolis</i>	<i>Clavibacter</i> <i>xyli</i>	<i>Renibacterium</i> <i>salmoninarum</i>	<i>Propionibacterium</i> <i>freudenreichii</i>	<i>Terrabacter</i> <i>tumescens</i>	<i>Mycobacterium</i> <i>intracellulare</i>	<i>Streptomyces</i> <i>coelicolor</i>	<i>Nocardioides</i> <i>fastidiosa</i>
0.066																			
0.074	0.056																		
0.096	0.060	0.075																	
0.086	0.062	0.084	0.077																
0.098	0.073	0.078	0.083	0.064															
0.028	0.067	0.077	0.098	0.091	0.099														
0.082	0.077	0.086	0.109	0.097	0.110	0.091													
0.069	0.067	0.074	0.099	0.085	0.095	0.080	0.031												
0.065	0.073	0.067	0.090	0.094	0.105	0.074	0.073	0.070											
0.073	0.055	0.039	0.073	0.075	0.091	0.073	0.078	0.073	0.067										
0.092	0.078	0.066	0.088	0.089	0.085	0.095	0.090	0.089	0.074	0.063									
0.080	0.052	0.050	0.067	0.070	0.085	0.084	0.081	0.071	0.077	0.030	0.066								
0.074	0.078	0.084	0.092	0.096	0.103	0.087	0.081	0.074	0.047	0.084	0.102	0.087							
0.088	0.094	0.089	0.114	0.112	0.127	0.097	0.093	0.089	0.038	0.082	0.092	0.092	0.073						
0.106	0.113	0.097	0.123	0.128	0.138	0.099	0.096	0.089	0.103	0.095	0.109	0.105	0.127	0.124					
0.076	0.080	0.075	0.102	0.100	0.115	0.081	0.084	0.084	0.050	0.071	0.102	0.081	0.063	0.073	0.116				
0.075	0.063	0.054	0.081	0.085	0.091	0.075	0.077	0.078	0.075	0.037	0.054	0.051	0.094	0.093	0.096	0.075			
0.042	0.067	0.075	0.096	0.093	0.102	0.034	0.083	0.081	0.063	0.076	0.101	0.082	0.076	0.091	0.102	0.081	0.080		
0.077	0.074	0.089	0.102	0.095	0.109	0.082	0.046	0.046	0.078	0.079	0.105	0.079	0.082	0.102	0.098	0.081	0.089	0.082	
0.081	0.075	0.085	0.107	0.096	0.109	0.089	0.001	0.030	0.074	0.077	0.089	0.079	0.079	0.092	0.097	0.082	0.075	0.085	0.047

mycelial pigment). The morphological, chemotaxonomic, and general characteristics of this species are the same as those given above for the genus. A yellow to pale brownish soluble pigment is produced on peptone-yeast extract-iron agar. Hydrogen sulfide is produced. Reduces nitrate to nitrite. Gelatin liquefaction is positive. Hydrolyzes starch. Does not decompose calcium malate. Does not coagulate milk. Fructose, glucose, inositol, and sucrose are utilized as carbon sources, but arabinose, raffinose, and xylose are not. The G+C contents of the DNAs range from 69 to 73 mol%.

The type strain is IFO 13939 (= ATCC 33937).

Actinoplanes caeruleus (9) is the basonym of this species.

Description of *Couchioplanes caeruleus* subsp. *caeruleus* subsp. nov. The morphological, chemotaxonomic, and general characteristics of *Couchioplanes caeruleus* subsp. *caeruleus* are the same as those given above for the species. In addition, a yellow to pale brownish soluble pigment is produced on glycerol-asparagine agar and glucose-asparagine agar, but this pigment is not produced on yeast extract-malt extract agar.

Rhamnose and mannitol are utilized as carbon sources. No growth occurs in the presence of 2% NaCl. The G+C content of the DNA of the type strain is 70 mol%. Habitat: soil.

The type strain is IFO 13939.

Description of *Couchioplanes caeruleus* subsp. *azureus* subsp. nov. *Couchioplanes caeruleus* subsp. *azureus* (a.zur'e. us. M. L. masc. adj. *azureus*, azure blue, referring to the blue vegetative mycelial pigment). The morphological, chemotaxonomic, and general characteristics of this subspecies are the same as those given above for the species. In addition, a yellow to pale brownish soluble pigment is produced on yeast extract-malt extract agar, but this pigment is not produced glycerol-asparagine agar and glucose-asparagine agar. Growth occurs in the presence of 2% NaCl. Rhamnose is not utilized as a carbon source, and mannitol is weakly utilized. The G+C content of the DNA of the type strain is 72 mol%. Habitat: soil.

The type strain is IFO 13993 (= ATCC 31157).

"*Actinoplanes azureus*" (2) is the basonym of this subspecies.

FIG. 4. Unrooted phylogenetic tree showing the relationships among *Couchioplanes*, *Actinoplanes*, *Dactylosporangium*, and *Catenuloplanes* species and related actinomycetes.

TABLE 8. Differential characteristics of the genus *Couchioplanes* and related actinomycete genera^a

Genus	Aerial mycelia	Motility of arthrospores	Sporangium formation	Cell wall type ^b	Peptidoglycan type ^c	Glycolyl test	Major menaquinone(s)	Phospholipid type ^d	Fatty acid type ^e	Whole-cell sugar(s)
<i>Couchioplanes</i>	+	+	—	VI	ND ^g	Glycolyl	MK-9(H ₄)	PII	2c	Xyl, Ara, Gal
<i>Actinoplanes</i>	—	—	+	II	A1γ	Glycolyl	MK-9(H ₄), MK-9(H ₆)	PII	2c	Xyl, Ara, Gal
<i>Catenuloplanes</i>	+	+	—	VI	ND	Glycolyl	MK-9(H ₈), MK-10(H ₈)	PIII	1a	Xyl
<i>Dactylosporangium</i>	—	—	+	II	A1γ	Glycolyl	MK-9(H ₈), MK-9(H ₆)	PII	2d	Xyl, Ara
<i>Pilimelia</i>	—	—	+	II	A1γ	Acetyl	MK-9(H ₂), MK-9(H ₄)	PII	2b	Xyl, Ara
<i>Micromonospora</i>	—	—	—	II	A1γ	Glycolyl	MK-10(H ₄), MK-10(H ₆), MK-9(H ₄), MK-9(H ₆)	PII	3b	Xyl, Ara
<i>Cellulomonas</i>	—	—	—	VI		Acetyl	MK-9(H ₄)	PV	2b	Gal
<i>Promicromonospora</i>	+	—	—	VI		ND	MK-9(H ₄)	PV	2b	Gal
<i>Jonesia</i>	—	—	—	VI		ND	MK-9	PV	2b	Gal

^a Data from references 17, 22, 24, 26, 28, 29, and 31.

^b According to the classification of Lechevalier and Lechevalier (14).

^c According to the classification of Schleifer and Kandler (22).

^d According to the classification of Hasegawa et al. (6).

^e According to the classification of Kroppenstedt (13).

^f +, present; —, absent.

^g ND, not determined.

TABLE 9. Differential characteristics of *Couchioplanes caeruleus* subspecies

Organism	Utilization of:		Production of pigments on:			Growth in 2% NaCl
	Rhamnose	Mannitol	Yeast extract-malt extract agar	Glycerol-asparagine agar	Glucose-asparagine agar	
<i>Couchioplanes caeruleus</i> subsp. <i>caeruleus</i>	+	+	—	+	+	—
<i>Couchioplanes caeruleus</i> subsp. <i>azureus</i>	—	±	+	—	—	+

^a +, positive; —, negative; ±, weakly positive.

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