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ORIGINAL ARTICLE

Streptosporangium oxazolinicum sp. nov., a novel endophytic actinomycete producing new antitrypanosomal antibiotics, spoxazomicins

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An actinomycete strain K07-0460^T producing new antitrypanosomal antibiotics, spoxazomicins, was isolated from the roots of a variety of orchid collected in the subtropical Okinawa prefecture. The 16S ribosomal RNA gene sequence analysis indicated that the strain belonged to the genus *Streptosporangium* and showed high similarities with *S. amethystogenes* subsp. *amethystogenes* DSM 43179^T (99.4%), *S. amethystogenes* subsp. *fukuiense* IFO 15365^T (99.2%) and *S. longisporum* DSM 43180^T (98.7%). The DNA–DNA hybridization relatedness values between strain K07-0460^T and the three strains were below 70%. On the basis of phylogenetic analysis, DNA–DNA hybridization relatedness and physiological characteristics, the strain should be classified as a new species *Streptosporangium oxazolinicum* sp. nov. in the genus *Streptosporangium.* The type strain of *S. oxazolinicum* is K07-0460^T (=JCM 17388^T).
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Keywords: antitrypanosome; endophytic actinomycete; plant root; spoxazomicins; *Streptosporangium oxazolinicum*

INTRODUCTION

Numerous actinomycetes have been isolated from soils, and these have contributed significantly to the discovery of many useful bioactive compounds, such as antibiotics, antitumor agents and immunosuppressive agents.[1] Over recent years, it has become more and more difficult to find novel bioactive compounds derived from actinomycete strains isolated from soils, so the focus of attention has switched to find novel actinomycetes from other sources. The majority of actinomycetes isolated from soils belong to the genus *Streptomyces*,[2] although the microbial flora in plants is different and many rare actinomycetes are being isolated from plants.[3,4] We searched for actinomycetes from plants for the purpose of finding new microbial resources to use in routine screening for novel bioactive compounds. We proposed novel genera, *Phytohabitans suffuscus* K07-0523^T and *Actinophytocola oryzae* GMKU 367^T, obtained through our research.[5,6] It is clear that plant roots are a useful source for new actinomycetes. Furthermore, we also discovered new antitrypanosomal compounds, the spoxazomicins, from the cultured broth of actinomycete strain K07-0460^T. This research will be reported in the accompanying paper.[7] The strain K07-0460^T was phylogenetically identified as being a member of the genus *Streptosporangium* described by Couch.[8] At present, the genus *Streptosporangium* comprises 15 species and 2 subspecies: *Streptosporangium roseum*,[8] *S. amethystogenes* subsp. *amethystogenes*, *S. album*, *S. vulgare*,[9] *S. longisporum*,[10] *S. nondiastaticum*, *S. pseudovulgare*,[11] *S. violaceochromogenes*,[12] *S. fragile*,[13] *S. carneum*,[14] *S. amethystogenes* subsp. *fukuiense*,[15] *S. claviforme*,[16] *S. subroseum*,[17] *S. purpuratum*, *S. yunnanense*[18] and *S. canum*.[19] We describe taxonomy of the strain K07-0460^T in this paper.

MATERIALS AND METHODS

Strain K07-0460^T was isolated from the root of a plant, a variety of orchid collected in Okinawa prefecture in March 2007. It was cultured on water-proline agar (proline 1.0%, tap water, pH 7.0) using the method previously

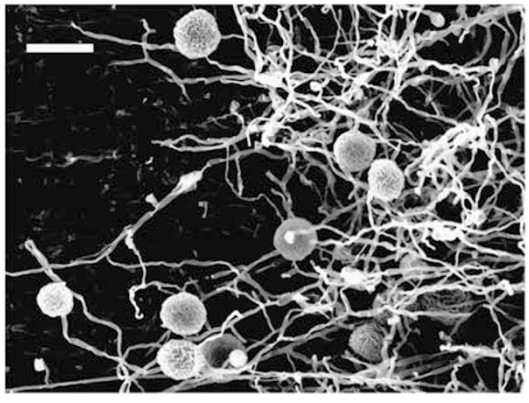

Figure 1 Scanning electron micrograph of strain K07-0460^T grown on International *Streptomyces* Project (ISP) medium 3 for 4 weeks at 27 °C. Bar=10 μm.

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Table 1 Cultural characteristics of strain K07-0460^T and type strains of related species

Medium	*1*	*2*	*3*	*4*
ISP medium 2				
Growth	Good	Good	Good	Poor
	Light tan (3gc) to burgundy (8pl)	Bamboo (2gc) to golden brown (3pi)	Mustard (2le) to dark brown (2pn)	Light ivory (2ca)
Reverse	Light tan (3gc) to burgundy (8pl)	Bamboo (2gc) to golden brown (3pi)	Light mustard tan (2ie) to dark brown (2pn)	Light ivory (2ca)
Aerial mycelium	None	None	None	None
Soluble pigment	Rose wine (8nc)	None	None	None
ISP medium 3				
Growth	Good	Good	Good	Good
	Pale pink (7ca) to dark rose brown (7pn)	Pearl pink (3ca) to camel (3ie)	Camel (3ie) to dark brown (3pn)	Copper (6lc)
Reverse	Pale pink (7ca) to dark rose brown (7pn)	Pearl pink (3ca) to camel (3ie)	Bamboo (2gc) to deep brown (3pl)	Copper tan (6ic)
Aerial mycelium	Poor, white (a)	Poor, white (a)	None	None
Soluble pigment	Cherry wine (7pe)	None	None	None
ISP medium 4				
Growth	Good	Good	Good	Poor
	Light wheat (2ea) to dark rose brown (7pn)	Bamboo (2gc)	Pearl pink (3ca) to camel (3ie)	Light ivory (2ca)
Reverse	Colonial yellow (2gc) to taupe brown (6ni)	Bamboo (2gc)	Pearl pink (3ca)	Light ivory (2ca)
Aerial mycelium	None	None	None	None
Soluble pigment	Wine (7pg)	None	None	None
ISP medium 5				
Growth	Good	Good	Poor	Poor
	Pale pink (7ca)	White (a)	White (a)	White (a)
Reverse	Pale pink (7ca)	White (a)	White (a)	White (a)
Aerial mycelium	None	Poor, white (a)	None	None
Soluble pigment	Light rose ($7_{1/2}$ga)	None	None	None
ISP medium 6				
Growth	Good	Good	Good	None
	Light tan (3gc) to deep brown mahogany (6pl)	Yellow maple (3ng)	Light mustard tan (2ie) to light brown (3lg)	
Reverse	Light tan (3gc) to deep brown mahogany (6pl)	Yellow maple (3ng)	Light mustard tan (2ie) to light brown (3lg)	
Aerial mycelium	None	Poor, white (a)	None	
Soluble pigment	None	None	None	
ISP medium 7				
Growth	Good	Good	Poor	Poor
	Pale pink (7ca) to cherry (7nc)	White (a)	White (a)	White (a)
Reverse	Pale pink (7ca) to cherry (7nc)	White (a)	White (a)	White (a)
Aerial mycelium	None	Poor, white (a)	None	None
Soluble pigment	Light rose ($7_{1/2}$ ga)	None	None	None
Glucose–asparagine agar				
Growth	Good	Poor	Poor	Poor
	White (a) to dark rose brown (7pn)	White (a)	Light ivory (2ca)	White (a)
Reverse	White (a) to burgundy (8pl)	White (a)	Light ivory (2ca)	White (a)
Aerial mycelium	None	None	None	None
Soluble pigment	Wine (7pg)	None	None	None
Sucrose–nitrate agar				
Growth	Good	Good	Poor	Good
	Pale pink (7ca)	White (a)	White (a)	Pearl pink (3ca)

Table 1 (Continued)

Medium	*1*	*2*	*3*	*4*
Reverse	Pale pink (7ca)	White (a)	White (a)	Pearl pink (3ca)
Aerial mycelium	Poor, white (a)	Poor, white (a)	None	None
Soluble pigment	Dark rose (8lc)	None	None	None
Glucose–nitrate agar				
Growth	Poor Light melon yellow (3ea) to tile red (5ne)	None	None	None
Reverse	Light melon yellow (3ea) to tile red (5ne)			
Aerial mycelium	None			
Soluble pigment	None			
Glycerol–calcium malate agar				
Growth	Good	Good	Poor	Poor
	Pale pink (7ca)	White (a)	White (a)	White (a)
Reverse	Pale pink (7ca)	White (a)	White (a)	White (a)
Aerial mycelium	None	Poor, white (a)	None	None
Soluble pigment	Dark rose (8lc)	None	None	None
Peptone–beef extract agar				
Growth	Good	Good	Good	Poor
	Light amber (3ic) to light wine ($7_{1/2}$ne)	Bamboo (2gc)	Mustard (2le) to light brown (3lg)	Orange (6la)
Reverse	Light amber (3ic) to light wine ($7_{1/2}$ne)	Bamboo (2gc)	Mustard (2le) to light brown (3lg)	Orange (6la)
Aerial mycelium	None	Poor, white (a)	None	None
Soluble pigment	Light wine ($7_{1/2}$ne)	None	None	None

Strains: 1, K07-0460^T; 2, *Streptosporangium amethystogenes* subsp. *amethystogenes* DSM 43179^T; 3, *S. amethystogenes* subsp. *fukuiese* IFO 15365^T; 4, *S. longisporum* DSM 43180^T.
Color designation based on Color Harmony Manual.[22]

described by Inahashi *et al.*[5] The strain K07-0460^T, *S. amethystogenes* subsp. *amethystogenes* DSM 43179^T, *S. amethystogenes* subsp. *fukuiense* IFO 15365^T and *S. longisporum* DSM 43180^T were cultured for 2 weeks at 27 °C to observe cultural characteristics on ISP (International *Streptomyces* Project) media 2, 3, 4, 5, 6 and 7 (ref. 20), glucose–asparagine agar, sucrose–nitrate agar, glycerol–calcium malate agar, peptone–beef extract agar[21] and glucose–nitrate agar (glucose 3.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, KCl 0.05%, $FeSO_4{\cdot}7H_2O$ 0.001%, agar 1.5%, pH 7.0). The color of aerial and substrate mycelia and soluble pigments were determined using the Color Harmony Manual.[22] The morphological characteristics were observed by light microscopy and scanning electron microscopy (JEOL JSM-5600, JEOL, Tokyo, Japan), of 28-day-old culture grown on ISP medium 3. The temperature range, the pH range and the NaCl tolerance for growth were determined on nutrient agar (Difco, Detroit, MI, USA). Utilization of carbohydrates as the sole carbon source was tested using ISP medium 9 supplemented with B-vitamins.[20,23,24] ISP medium 4 for starch hydrolysis, ISP medium 8 for nitrate reduction,[20] glucose–peptone–gelatin medium (glucose 2.0%, peptone 0.5%, gelatin 20%, pH 7.0) for gelatin liquefaction, 10% skim milk for coagulation and peptonization of milk, ISP medium 6 for H_2S production and skim-milk agar for casein hydrolysis were used.[25] Tyrosinase activity was determined using ISP medium 7 and other enzyme activities were determined using the API ZYM system (bioMérieux, Lyon, France), according to the manufacturer's instructions. Biomass for the molecular systematics and chemotaxonomic studies was obtained after cultivation in yeast extract–glucose broth (yeast extract 1.0%, glucose 1.0%, pH 7.0) for 1 week at 27 °C. After extraction of isoprenoid quinones, as described by Collins *et al.*,[26] analysis was done by HPLC (Agilent 1100, Agilent, Santa Clara, CA, USA) and mass spectrometer (JEOL JMS-T 100LP) using a Pegasil ODS column (Senshu, Tokyo, Japan), according to Tamaoka *et al.*[27] Isomers of diaminopimelic acid were determined by TLC using whole-cell hydrolysates.[28] The *N*-acyl types of muramic acid were determined by the method of Uchida and Aida.[29] Phospholipids in cells were extracted and identified by the method of Minnikin *et al.*[30] The presence of mycolic acids was examined by TLC following Tomiyasu (1982).[31] Whole-cell sugar composition was analyzed according to the methods of Becker *et al.*[28] Methyl esters of cellular fatty acids were prepared by direct transmethylation with methanolic hydrochloride, and analyzed on a GLC system (HP 6890; Hewlett Packard, Palo Alto, CA, USA). Identification and quantification of the fatty-acid methyl esters, as well as the numerical analysis of the fatty acid profiles, were performed according to the instructions for the Microbial Identification System (MIDI, Newark, NJ, USA). Chromosomal DNA was prepared following the procedure of Saito and Miura,[32] and the DNA G+C content was determined by HPLC, according to Tamaoka and Komagata.[33] DNA–DNA hybridization was performed by the photobiotin-labelling method of Ezaki *et al.*[34] After the 16S ribosomal RNA (rRNA) gene was amplified using PCR, the sequence was analyzed according to the method of Inahashi *et al.*[5] The clustalw2 program was used for multiple alignments with selected sequences for calculating evolutionary distances[35] by Sea View version 4.2 (ref. 36). The phylogenetic tree was constructed based on the neighbor-joining method,[37] maximum-likelihood method[38] and the maximum-parsimony method.[39] Data were resampled with 1000 bootstrap replications.[40] The values of sequence similarities among the closest strains were determined using the EzTaxon server.[41]

RESULTS AND DISCUSSION

Morphological, cultural and physiological characteristics

Strain K07-0460^T grew well on ISP medium 2, 3 and other media, but globose sporangia on aerial mycelia were produced only on ISP medium 3 (Figure 1) and sucrose-nitrate agar. Vegetative mycelia were branched but not fragmented. The colony color of strain K07-0460^T was red, and red soluble pigment was produced in various agar

Table 2 Comparison of physiological characteristics of strain K07-0460^T and type strains of related species

	1	*2*	*3*	*4*
Utilization of				
D-glucose	+	+	+	−
L-arabinose	−	+	+	−
D-xylose	+	+	w	−
Raffinose	−	+	−	−
Melibiose	−	w	−	−
D-mannitol	+	+	+	−
D-fructose	+	+	+	−
L-rhamnose	−	w	+	−
myo-Inositol	−	w	−	−
Sucrose	−	w	w	−
Reduction of nitrate	−	+	−	−
Degradation of casein	+	−	+	+
Liquefaction of gelatin	w	−	−	−
Enzyme activity of				
Esterase (C4)	+	+	+	−
Esterase lipase (C8)	w	+	+	−
Valine allylamidase	+	+	+	−
Cystine allylamidase	+	w	−	−
Trypsin	+	w	−	−
α-Chymotrypsin	w	−	−	−
Acid phosphatase	+	−	−	−
Naphthol-AS-BI-phosphohydrase	+	w	w	w
β-Galactosidase	w	−	−	−
β-Glucuronidase	−	−	−	−
α-Glucosidase	+	+	+	−
β-Glucosidase	+	+	+	−
N-acetyl-β-glucosaminidase	+	w	w	−
α-Mannosidase	+	−	−	−

+, positive; w, weakly positive; −, negative.
All strains were positive for hydrolysis of starch and enzyme activities of alkaline phosphatase and leucine allylamidase, but negative for utilization of cellulose, peptonization of milk, coagulation of milk, production of melanin, production of hydrogen sulfide and enzyme activities of tyrosinase, lipase (C14), α-galactosidase and α-fukosidase.
Strains: 1, K07-0460^T; 2, *Streptosporangium amethystogenes* subsp. *amethystogenes* DSM 43179^T; 3, *S. amethystogenes* subsp. *fukuiense* IFO 15365^T; 4, *S. longisporum* DSM 43180^T.

Table 3 Cellular fatty-acid compositions (%) of strain K07-0460^T and type strains of related species

	1	*2*	*3*	*4*
iso-$C_{11:0}$ 3-OH	ND	ND	ND	0.3
$C_{13:0}$	1.7	0.7	0.6	2.2
iso-$C_{14:0}$	6.7	5.9	5.1	7.4
$C_{14:0}$	1.3	0.5	1.8	3.5
iso-$C_{15:0}$	1.0	0.8	1.5	0.6
anteiso-$C_{15:0}$	ND	0.3	0.4	0.3
$C_{15:0}$	4.5	3.1	1.4	3.4
iso-$C_{16:1}$	ND	0.6	ND	1.2
iso-$C_{16:0}$	6.0	11.1	8.7	2.7
Summed feature 3*	4.7	3.5	4.3	15.5
$C_{16:0}$	8.0	7.3	17.1	4.7
$C_{16:0}$ 10-methyl	2.0	1.0	2.6	3.4
iso-$C_{17:0}$	ND	0.63	1.1	ND
anteiso-$C_{17:0}$	ND	ND	0.4	ND
$C_{17:1}$ $\omega 8c$	17.0	26.5	6.5	24.1
$C_{17:0}$	10.5	11.6	7.9	3.2
$C_{17:0}$ 10-methyl	24.6	14.4	10.5	15.8
$C_{18:1}$ $\omega 9c$	4.1	7.3	11.2	6.8
$C_{18:1}$ $\omega 7c$	0.6	0.5	1.1	0.9
$C_{18:0}$	1.8	2.3	4.9	1.6
$C_{18:0}$ 10-methyl	5.3	2.0	13.1	2.6

Abbreviation: ND, not detected.
Strains: 1, K07-0460^T; 2, *Streptosporangium amethystogenes* subsp. *amethystogenes* DSM 43179^T; 3, *S. amethystogenes* subsp. *fukuiense* IFO 15365^T; 4, *S. longisporum* DSM 43180^T.
*Summed feature 3 comprises $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH.

media (Table 1). The temperature and pH range for growth were 13–36 °C and pH 6–11, with optimum growth at 21–32 °C and pH 7–10. Strain K07-0460^T did not grow on 3% NaCl medium. Casein was degraded. Starch was hydrolyzed. Gelatin was liquefied weakly. Milk was not peptonized and coagulated. Nitrate was not reduced to nitrite. H_2S and melanin were not produced. The strain K07-0460^T utilized D-glucose, D-xylose, D-mannitol and D-fructose, and did not utilize L-arabinose, raffinose, melibiose, L-rhamnose, *myo*-inositol, sucrose and cellulose. The strain K07-0460^T did not produce tyrosinase. Enzyme activities of the API ZYM system were positive for alkaline phosphatase, esterase (C4), leucine allylamidase, valine allylamidase, cystine allylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase, weakly positive for esterase lipase (C8), α-chymotrypsin and β-galactosidase and negative for lipase (C14), α-galactosidase, β-glucuronidase and α-fukosidase (Table 2).

Chemotaxonomy

Strain K07-0460^T contained *meso*-isomer of diaminopimelic acid as the diagnostic diamino acid. Galactose, glucose, mannose, madurose, ribose and xylose were detected as whole-cell sugars. The *N*-acyl type of muramic acid was acetyl. Phosphatidylethanolamine and unknown glucosamine-containing phospholipids were detected. Phosphatidylcholine and phosphatidylglycerol were not detected. Phospholipid pattern corresponded to type IV.[42] Mycolic acids were not detected. The predominant menaquinones were MK-9 (H_2) (50%) and MK-9 (H_4) (41%) and the minor menaquinones were MK-9 (H_0) (7%) and MK-9 (H_6) (2%). The major cellular fatty acids were $C_{17:0}$ 10-methyl (24.6%), $C_{17:1}$ $\omega 8c$ (17.0%), $C_{17:0}$ (10.5%) (Table 3). The G+C content of the genomic DNA was 72 mol%. The chemotaxonomic properties of strain K07-0460^T were consistent with those of members of the genus *Streptosporangium*.

Phylogenetic analysis

The 16S rRNA gene sequence of strain K07-0460^T showed a close relationship with members of the genus *Streptosporangium*, and the similarity values between strain K07-0460^T and the type strains in the genus *Streptosporangium* were 95.3–99.4%. The species showing the highest similarity values to strain K07-0460^T were *S. amethystogenes* subsp. *amethystogenes* DSM 43179^T (99.4%), *S. amethystogenes* subsp. *fukuiense* IFO 15365^T (99.2%) and *S. longisporum* DSM 43180^T (98.7%). The phylogenetic analysis based on the 16S rRNA gene sequences also indicated that strain K07-0460^T formed a cluster with *S. amethystogenes* subsp. *amethystogenes* DSM 43179^T and *S. amethystogenes* subsp. *fukuiense* IFO 15365^T (Figure 2). The DDBJ accession number of the 16S rRNA gene sequences of strain K07-0460^T is AB594818.

DNA–DNA hybridization

DNA–DNA relatedness values with strain K07-0460^T among *S. amethystogenes* subsp. *amethystogenes* DSM 43179^T, *S. amethystogenes*

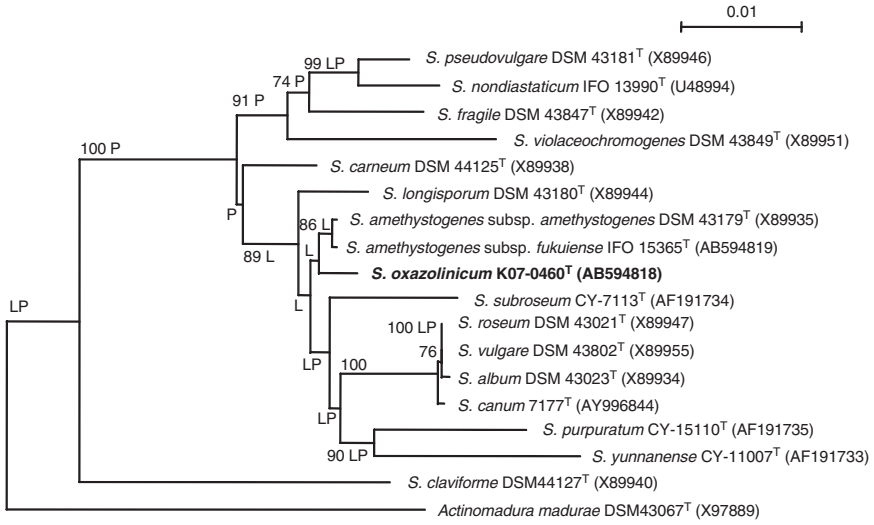

Figure 2 Neighbor-joining tree based on 16S ribosomal RNA gene sequences showing relationship between K07-0460^T and members of the genus *Streptosporangium*. Only bootstrap values above 50% (percentages of 1000 replications) are indicated. L, branch also recovered in the maximum-likelihood tree; P, branch also recovered in the maximum-parsimony tree; Bar, 0.01 nucleotide substitutions per site.

Table 4 DNA–DNA re-association between strain K07-0460^T and type strains of related species

	DNA–DNA re-association (%)			
Strain	*1*	*2*	*3*	*4*
1	100	58	47	41
2	38	100	57	44
3	59	87	100	52
4	63	48	41	100

Strains: 1, K07-0460^T; 2, *Streptosporangium amethystogenes* subsp. *amethystogenes* DSM 43179^T; 3, *S. amethystogenes* subsp. *fukuiense* IFO 15365^T; 4, *S. longisporum* DSM 43180^T.

subsp. *fukuiense* IFO 15365^T and *S. longisporum* DSM 43180^T were below the value of 70% recommended by Wayne *et al.*[43] for the assignment of strains to the same species (Table 4).

Conclusion

The phylogenetical, morphological and chemotaxonomical properties indicated that strain K07-0460^T belongs to the genus *Streptosporangium*. However, DNA–DNA relatedness values between strain K07-0460^T and the related strains were below the value of 70% (Table 4). Furthermore, colonies of strain K07-0460^T were red in color and produced red soluble pigment, whereas those of related strains were white to pale pink and did not produce soluble pigment (Table 1). The strain K07-0460^T is also distinguished from related strains by differences in the utilization of D-glucose, L-arabinose, D-xylose, raffinose, D-mannitol, D-fructose and *myo*-inositol, reduction of nitrate, degradation of casein and enzyme activities of esterase (C4), valine allylamidase, cystine allylamidase, trypsin, acid phosphatase and α-mannosidase (Table 2). The above results support that strain K07-0460^T represents a novel species in the genus *Streptosporangium* for which the name *Streptosporangium oxazolinicum* sp. nov. is proposed.

Description of *S. oxazolinicum* sp. nov.

Streptosporangium oxazolinicum (o.xa.zo.li'ni.cum. N.L. n. *oxazolinum*, oxazoline; L. neut. suff. *-icum*, suffix used with the sense of pertaining to; N.L. neut. adj. *oxazolinicum*, pertaining to oxazoline, referring to the production of oxazoline compounds).

Aerobic, Gram-positive and mesophilic actinomycete. Colonies are red in color. Vegetative mycelia are branched and not fragmented. Red soluble pigment is produced. Exhibits good growth and forms globose sporangia on aerial mycelia. Diagnostic diamino acid is *meso*-diaminopimelic acid. Whole-cell sugars are galactose, glucose, mannose, madurose, ribose and xylose. The acyl type of the peptidoglycan is acetyl. Phosphatidylethanolamine and unknown glucosamine-containing phospholipids are detected. Phospholipid pattern corresponds to type IV. Mycolic acids are not detected. The predominant menaquinones are MK-9 (H_2) and MK-9 (H_4). Major fatty acids are $C_{17:0}$ 10-methyl, $C_{17:1}\omega 8c$ and $C_{17:0}$. Growth occurs at 13–36 °C (optimum 21–32 °C) and pH 6–11 (optimum pH 7–10). No growth in the presence of 3% NaCl. Casein is degraded. Starch is hydrolyzed. Gelatin is liquefied weakly. Cellulose is not degraded. Milk is not peptonized or coagulated. Nitrate is not reduced to nitrite. H_2S and melanin are not produced. D-Glucose, D-xylose, D-mannitol and D-fructose are utilized, but L-Arabinose, raffinose, melibiose, maltose, L-rhamnose, *myo*-inositol and sucrose are not utilized. Tyrosinase is not produced. According to the API ZYM system, alkaline phosphatase, esterase (C4), leucine allylamidase, valine allylamidase, cystine allylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase are positive, esterase lipase (C8), α-chymotrypsin and β-galactosidase are weakly positive and lipase (C14), α-galactosidase, β-glucuronidase and α-fukosidase are negative. The G+C content of the genomic DNA is 72 mol%. The type strain is *S. oxazolinicum* K07-0460^T (=JCM 17388^T).

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