Streptomyces tateyamensis sp. nov., *Streptomyces marinus* sp. nov. and *Streptomyces haliclonae* sp. nov., isolated from the marine sponge *Haliclona* sp.

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Three Gram-positive, NaCl-requiring actinobacteria were isolated from a marine sponge, *Haliclona* sp., collected from the coast of Tateyama City, Japan. Comparison of 16S rRNA gene sequences indicated that these strains represent novel members of the genus *Streptomyces*, exhibiting low 16S rRNA gene sequence similarities of 98.3–97.4 % with recognized members of the genus. The cell hydrolysates contained the LL-isomer of diaminopimelic acid and the predominant quinones were MK-9 (H_6 and/or H_8). The DNA G+C contents were in the range 72–75 mol%. A polyphasic study of the strains and comparison of the characters with related species of the genus show that these strains represent three novel species of the genus *Streptomyces*. Therefore, the names *Streptomyces tateyamensis* sp. nov., *Streptomyces haliclonae* sp. nov. and *Streptomyces marinus* sp. nov. are proposed for strains Sp080513SC-30^T (=NBRC 105048^T =DSM 41969^T), Sp080513SC-31^T (=NBRC 105049^T =DSM 41970^T) and Sp080513GE-26^T (=NBRC 105047^T =DSM 41968^T), respectively.

Marine sponges are an attractive source of novel chemicals and bioactive compounds (Zhang *et al.*, 2005). These members of the phylum Porifera are filter feeders and can accumulate as much as 2.76×10^6 bacteria (g sponge wet weight)$^{-1}$ (Wehrl *et al.*, 2007), therefore concentrating bacteria found otherwise diluted in the seawater. A number of reports are available on the isolation of actinobacteria from marine sponges (Mincer *et al.*, 2002; Ward & Bora, 2006; Khan *et al.*, 2010). Bacteria, especially the members of the genera *Streptomyces* and *Micromonospora*, produce a number of novel compounds (Lam, 2006), and are therefore a promising source of novel compounds for therapeutic use.

In our studies on the isolation of actinobacteria from a marine sponge, *Haliclona* sp., three strains, Sp080513SC-30^T, Sp080513SC-31^T and Sp080513GE-26^T, were isolated. A polyphasic phylogenetic study shows that these strains represent three novel species of the genus *Streptomyces*, for which the names *S. tateyamensis* sp. nov., *S. haliclonae* sp. nov., and *S. marinus* sp. nov. are proposed, respectively.

A sample of the marine sponge *Haliclona* sp. was collected from the pacific coastline of Tateyama City, Chiba prefecture, Japan, and was transferred to the laboratory within 3 h of collection at room temperature. The sponge sample was then rinsed with sterile seawater, cut into tiny pieces with sterile scissors and resuspended in sterile seawater. An aliquot of 100 µl from this suspension was spread on Jewfish (*Ruditapes philippinarum*) extract agar (Khan *et al.*, 2010) and starch casein nitrate agar plates prepared with 50 % (v/v) seawater and supplemented with 35 µg nalidixic acid ml^{-1} and 50 µg cycloheximide ml^{-1}. Strains Sp080513SC-30^T and Sp080513SC-31^T were isolated from starch casein nitrate agar and Sp080513GE-26^T was isolated from Jewfish extract agar. ISP2 (International *Streptomyces* project; Shirling & Gottlieb, 1966) medium prepared with 50 % (v/v) artificial seawater (Naigai Chemicals; ISP2 M) was used for further purification and maintenance of the strains. For long-term preservation,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strains Sp080513SC-30^T, Sp080513SC-31^T and Sp080513GE-26^T are AB473555, AB473556 and AB473554, respectively.

Scanning electron micrographs showing spore chain morphology, cellular fatty acid profiles, and a comparison of 16S rRNA gene sequences with closely related species are available with the online version of this paper.

strains were stored at −80 °C in 50 % (v/v) artificial seawater supplemented with 15 % glycerol (v/v).

Prepman Ultra (Applied Biosystems) was used to prepare template DNA for 16S rRNA gene amplification. The genes were amplified by using a universal primer set (9f and 1492r; Brosius *et al.*, 1978) and sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). In BLAST searches (Altschul *et al.*, 1990) against the sequences available in the DDBJ and EzTaxon server (Chun *et al.*, 2007), strains shared a maximum of 98.35 % 16S rRNA gene sequence similarity with both recognized and unpublished members of the genus *Streptomyces*. Results of the sequence comparison are summarized in Supplementary Table S1, available in IJSEM Online. Closely related sequences with validly published names were downloaded from DDBJ and pairwise sequence similarities were checked using the Needleman-Wunsch alignment algorithm (Needleman & Wunsch, 1970; http://www.ebi.ac.uk/emboss/align/). Strain Sp080513SC-30^T shared a maximum similarity of 98.35 % with *Streptomyces sioyaensis* NRRL B-5408^T (DQ026654) amongst the close relatives with validly published names. Strains Sp080513SC-31^T and Sp080513GE-26^T shared maximum similarities of 97.66 % and 97.4 % with *Streptomyces cacaoi* subsp. *cacaoi* NBRC 12748^T (AB184115) and *Streptomyces albiaxialis* NBRC 101002^T (AY999901), respectively. These two novel strains share 98.02 % sequence similarity with each other and share low sequence similarities (96.4–96.9 %) with strain Sp080513SC-30^T.

Downloaded sequences were aligned using the CLUSTAL X program (Thompson *et al.*, 1997), and phylogenetic trees were reconstructed by using the neighbour-joining algorithm (Saitou & Nei, 1987). The robustness of the tree topology was evaluated by bootstrap analysis using 1000 resamplings of the sequences (Felsenstein, 1985). Strain Sp080513SC-30^T shared maximum 16S rRNA gene sequence similarity with *S. sioyaensis*, but formed an independent clade (Fig. 1). Strains Sp080513GE-26^T and Sp080513SC-31^T formed an independent clade supported by a high bootstrap value of 97.5 % in a neighbour-joining phylogenetic tree (Fig. 2). Almost similar results were obtained with maximum-parsimony analysis (data not shown).

As the phylogenetic trees for the novel strains were inconclusive, phylogenetic neighbours identified from the BLAST searches (EzTaxon server) were compared in the detailed polyphasic study.

The arrangement of hyphae, spore chains and spore surface were observed under light (Olympus CX41LF) and scanning electron (JEOL JSM-6060) microscopes after growing the cells on ISP2 M or on water agar [1.6 % agar in 50 % (v/v) seawater] at 28 °C for 15–20 days. Growth of standard strains used in this study (*S. cacaoi* subsp. *cacaoi* NBRC 12748^T, *S. albiaxialis* NBRC 101002^T, and *S. sioyaensis* NBRC 12820^T) and that of novel strains was checked on ISP2 and ISP2 M media. Standard strains grew well on both media, while the novel strains isolated during this study showed poor growth on ISP2 medium. Therefore, media (Shirling & Gottlieb, 1966) prepared with 50 % (v/v) seawater were used to determine the cultural and physiological characteristics. DNA was extracted from cells grown to late exponential growth phase using the protocol of Minamisawa (1990), and the method described by Mesbah *et al.* (1989) was used to determine the G+C content of the genomic DNA. Commercially available API ZYM and API Coryne systems (bioMérieux) were used following the instructions of the manufacturer for the biochemical characterization of the strains. Menaquinones, cellular fatty acids and the diaminopimelic acid isomer in whole cell hydrolysates were determined as described previously (Tamura *et al.*, 1994).

Phenotypic and biochemical features of the novel strains are listed in Tables 1 and 2 and in the species descriptions. Comparison of the characteristics with previously described relatives shows that these strains represent novel species of the genus *Streptomyces*. Strain Sp080513SC-30^T shares 98.35 % 16S rRNA gene sequence similarity with its closest phylogenetic neighbour, *S. sioyaensis* NBRC 12820^T, but differs in a number of phenotypic characteristics (Table 1), such as the production of soluble pigment on ISP media, growth at 40 °C, and the presence of α-mannosidase, α-chymotrypsin and urease. Based on the significant distance in 16S rRNA gene sequence (Stackebrandt & Ebers, 2006) and the phenotypic differences, we propose that strain Sp080513SC-30^T should be classified as a new

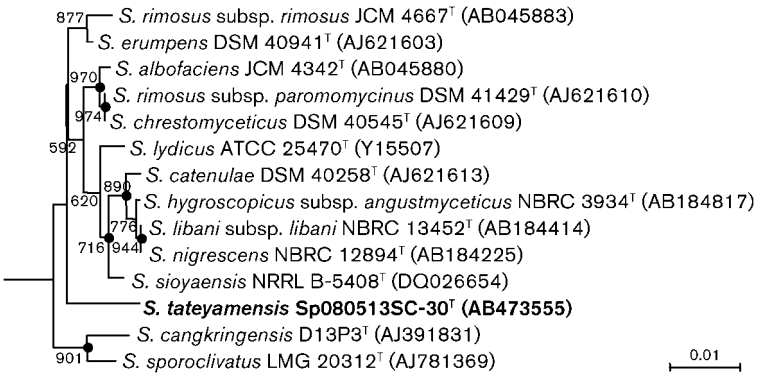

Fig. 1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the phylogenetic position of strain Sp080513SC-30^T and related species. Bootstrap values from 1000 replications are indicated at branches when the values are significant (>500). Nodes recovered in maximum-parsimony analysis are marked with closed circles. Accession numbers of sequences downloaded from DDBJ are shown in parentheses. The sequence of *Paraoerskovia marina* (AB445007) was used as an outgroup. Bar, 0.01 K_{nuc}.

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strains Sp080513SC-31^T and Sp080513GE-26^T and related species. Bootstrap values from 1000 replications are indicated at branches when the values are significant (>500). Nodes recovered in maximum-parsimony analysis are marked with closed circles. Accession numbers of sequences downloaded from DDBJ are shown in parentheses. The sequence of *Paraoerskovia marina* (AB445007) was used as an outgroup. Bar, 0.01 K_{nuc}.

species of the genus *Streptomyces* for which the name *Streptomyces tateyamensis* sp. nov. is proposed.

Similarly, strains Sp080513GE-26^T and Sp080513SC-31^T also differ from their closest relatives and from each other in a number of characters summarized in Table 2. Although the two strains cluster together in a clade supported by a high bootstrap value (97.5 %), they can be distinguished based on colony morphology, the production of soluble pigment, aerial mass, and the presence or absence of naphthol-AS-BI-phosphohydrolase and α-mannosidase. These novel strains can be differentiated from their phylogenetic neighbours by growth temperature range, nitrate reduction, soluble pigment production and absence of urease (Table 2). Significantly low 16S rRNA gene sequence similarities (Stackebrandt & Ebers, 2006) and the differences in a number of phenotypic characteristics support the classification of these strains as novel species of the genus *Streptomyces*. Therefore, the names *Streptomyces haliclonae* sp. nov. and *Streptomyces marinus* sp. nov. are proposed for strains Sp080513SC-31^T and Sp080513GE-26^T, respectively.

Table 1. Characteristics that differentiate strain Sp080513SC-30^T from *Streptomyces sioyaensis* NBRC 12820^T

Strains: 1, Sp080513SC-30^T; 2, *S. sioyaensis* NBRC 12820^T. Both strains produced white and grey aerial mycelium on ISP2 medium. Data from this study. +, Positive; w, weakly positive; −, negative.

Characteristic	1	2
Growth without sea salts (50 %, v/v)	Poor	Good
Soluble pigment on ISP media	Brownish black to golden yellow (on ISP1-6)	None
Growth at 40 °C	−	+
Enzyme activity (API ZYM)		
Trypsin	+	w
α-Chymotrypsin	+	−
β-Galactosidase	−	+
α-Mannosidase	+	−
Urease (API Coryne)	+	−

Description of *Streptomyces tateyamensis* sp. nov.

Streptomyces tateyamensis (ta.te.ya.men′sis. N.L. masc. adj. *tateyamensis* pertaining to Tateyama, the place from where the type strain was isolated).

Aerobic, Gram-positive and catalase-positive actinomycete. Spore chains are spiral with multiple turns and the spore surface is smooth. Predominant fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. Detailed fatty acid profile is given in Supplementary Table S2. The major respiratory quinones are MK-9 (H_6) and MK-9 (H_8). MK-9 (H_2) and MK-9 (H_4) are also present. Grows at 15–37 °C and optimally at 25–30 °C and at pH 6.0–8.0 (weakly at pH 9.0). Growth occurs with 2–7 % (w/v) NaCl. Weak growth is observed with 1 and 10 % (w/v) NaCl. Growth is good on all ISP media tested [tryptone yeast extract agar (ISP1), yeast extract malt agar (ISP2), oatmeal agar (ISP3), inorganic salts–starch agar (ISP4), glycerol–asparagine agar (ISP5), peptone–yeast extract iron agar (ISP6) and tyrosine agar (ISP7)]. Spores are grey and aerial mycelium is white, except on ISP1 where aerial mass is not produced. Melanin pigments are not produced on ISP6 and ISP7 media. Light brown soluble pigments are produced on most of the ISP media (except on ISP7). In API ZYM and API Coryne tests, positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, gelatin hydrolysis, α-glucosidase, β-glucosidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, pyrazinamidase, pyrolidonyl arylamidase, trypsin and urease. Negative activity was observed for esterase, esterase lipase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase and lipase. Starch is not degraded and nitrate is not reduced. D-Mannitol, sucrose, D-glucose, sorbitol and *myo*-inositol are utilized as carbon sources. D-Fructose, L-arabinose, raffinose, L-rhamnose and D-xylose are not utilized. The DNA G+C content of the type strain is 74 mol%.

The type strain is Sp080513SC-30^T (=NBRC 105048^T =DSM 41969^T), isolated from the marine sponge *Haliclona* sp.

Description of *Streptomyces haliclonae* sp. nov.

Streptomyces haliclonae (ha.li.clo′nae. N.L. gen. n. *haliclonae* of *Haliclona*, isolated from the marine sponge *Haliclona* sp.).

Table 2. Characteristics that differentiate strains Sp080513SC-31^T and Sp080513GE-26^T and related species

Strains: 1, Sp080513SC-31^T; 2, Sp080513GE-26^T; 3, *S. albiaxialis* NBRC 101002^T; 4, *S. cacaoi* subsp. *cacaoi* NBRC 12748^T. Data from this study. +, Positive; w, weakly positive; −, negative.

Characteristics	1	2	3	4
Growth without sea salts (50 %, v/v)	Poor	Poor	Good	Good
Growth at 40 °C	−	−	+	+
Production of soluble pigment (on ISP1–7)	None	Vinaceous (violet red)	None	None
Aerial mass (on ISP1)	None	None	White	White
Aerial mass (on ISP2–7)	White	None	White	White
Enzyme activity (API ZYM)				
Naphthol-AS-BI-phosphohydrolase	+	−	+	w
α-Mannosidase	−	+	+	+
API Coryne tests				
Nitrate reduction	−	−	+	+
Pyrazinamidase	+	+	+	−
Urease	−	−	+	+

Cells are Gram-positive, aerobic and catalase-positive. Spores are smooth-surfaced and borne in spiral chains. The predominant respiratory quinone is MK-9 (H_8). Predominant cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. Detailed fatty acid profile is given in Supplementary Table S2. Grows at 15–37 °C (optimally at 25–30 °C) and at pH 6.0−8.0 (weakly at pH 9.0). Growth occurs with 2–7 % (w/v) NaCl. Weak growth is observed with 1 and 10 % (w/v) NaCl. Grows well on ISP1–ISP7 media except on ISP6 medium where growth was poor. Diffusible pigments are not produced. The spore mass is white and the reverse-side colour is buff to dull white on most media. Melanin pigments are not produced on ISP6 or ISP7 media. Starch is not hydrolysed. Positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, β-galactosidase, α-glucosidase, β-glucosidase, gelatin hydrolysis, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and pyrazinamidase in API ZYM and API Coryne test systems, and negative for α-chymotrypsin, α-fucosidase, α-galactosidase, β-glucuronidase, lipase, α-mannosidase, pyrolidonyl arylamidase, trypsin and urease. Nitrate is not reduced. Starch is not degraded. D-Fructose, D-mannitol, sucrose, D-glucose, raffinose, sorbitol and *myo*-inositol are used as carbon sources. L-Arabinose, L-rhamnose, D-xylose and lactose are not utilized. The DNA G+C content of the type strain is 73 mol%.

The type strain is Sp080513SC-31^T (=NBRC 105049^T =DSM 41970^T), isolated from the marine sponge *Haliclona* sp.

Description of *Streptomyces marinus* sp. nov.

Streptomyces marinus (ma.ri′nus. L. masc. adj. *marinus* of the sea, marine).

Aerobic, Gram-positive and non-motile actinomycete. Spore chains are spiral with multiple turns and spore surface is smooth. Predominant fatty acids are iso-$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{14:0}$. Detailed fatty acid profile is given in Supplementary Table S2. Major respiratory quinone is MK-9 (H_8); MK-9 (H_6) and MK-9 (H_{10}) are also present. Grows at 20–30 °C (optimally at 25–28 °C); grows weakly at 15 and 37 °C. Grows well at pH 6.0–9.0, while no growth is observed at pH 5.0. Growth occurs with 2–7 % (w/v) NaCl. Weak growth is observed with 1 and 10 % (w/v) NaCl. Nitrate is not reduced. Starch is not hydrolysed. Growth is good on ISP1 and ISP2 media and fair on other ISP media (ISP3–6). Grows poorly on ISP7 medium. Produces soluble vinaceous (violet red) pigment on most of the ISP media. Vegetative growth is dark brick-red and spores are not produced on most of the ISP media tested. Melanin pigments are not produced on ISP6 or ISP7 media. Spores are produced only on water agar. In API ZYM and API Coryne test systems, positive for *N*-acetyl-β-glucosaminidase, alkaline phosphatase, acid phosphatase, β-galactosidase, α-glucosidase, β-glucosidase, gelatin hydrolysis, leucine arylamidase, α-mannosidase and pyrazinamidase; negative for α-chymotrypsin, α-fucosidase, α-galactosidase, β-glucuronidase, naphthol-AS-BI-phosphohydrolase, lipase (C14), pyrolidonyl arylamidase, trypsin and urease. D-Fructose, D-mannitol, sucrose, D-glucose, L-arabinose and D-xylose are utilized as sole carbon sources; L-rhamnose, raffinose, sorbitol and *myo*-inositol are not utilized. The DNA G+C content of the type strain is 72 mol%.

The type strain is Sp080513GE-26^T (=NBRC 105047^T =DSM 41968^T), isolated from the marine sponge *Haliclona* sp.

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