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ARTICLE

Streptomyces pacificus sp. nov., a novel spongiicolazolicin-producing actinomycete isolated from a coastal sediment

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Abstract

A polyphasic approach was used to determine the taxonomic position of a marine actinomycete, designated isolate CWH03^T, which we previously reported to produce new linear azole-containing peptides spongiicolazolicins A and B. Strain CWH03^T is mesophilic, neutrophilic, and halotolerant streptomycete that forms spiral spore chains on aerial mycelium. Comparative 16S rRNA gene sequencing showed that CWH03^T was most closely related to *Streptomyces tirandamycinicus* HNM0039^T (99.7%), *Streptomyces spongiicola* HNM0071^T (99.4%), '*Streptomyces marianii*' ICN19^T (99.1%) and *Streptomyces wuyuanensis* CGMCC4.7042^T (99.0%). The phylogenetic tree prepared using the 16S rRNA gene, as well as the phylogenomic tree using the genome BLAST distance phylogeny method and 81 core housekeeping genes, respectively, showed that the closest relative of strain CWH03^T was *S. spongiicola* HNM0071^T. The average nucleotide identity and digital DNA-DNA hybridization values between strains CWH03^T and *S. spongiicola* HNM0071^T were 91.46% and 44.2%, respectively, which were below the thresholds of 96% and 70% for prokaryotic conspecific assignation. The G+C content of the genomic DNA of strain CWH03^T was 72.3%. Whole-cell hydrolysates of strain CWH03^T contained LL-diaminopimelic acid. The predominant menaquinone was MK-9(H_8) (88.3%), and the major fatty acids were iso-$C_{16:0}$ (28.4%), anteiso-$C_{15:0}$ (15.0%) and iso-$C_{15:0}$ (12.9%). The major phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unidentified phospholipid. Based on data obtained from phenotypic, phylogenetic, genomic, and chemotaxonomic analyses, strain CWH03^T represents a novel species of the genus *Streptomyces*, for which the proposed name is *Streptomyces pacificus* sp. nov. The type strain is CWH03^T (= NBRC 114659^T = TBRC 15780^T).

Introduction

The early studies of soil actinomycetes conducted by Waksman and his colleagues led to the discovery of antibiotics such as actinomycin and streptomycin, of which streptomycin subsequently became medicine for tuberculosis [1]. Since streptomycin was discovered from *Streptomyces griseus* in 1944, multiple types of antibiotics have been isolated from actinomycetes, primarily from organisms of the genus *Streptomyces*; the early years of this process are now considered the "Golden Age" of antibiotic development. Classically, soil-derived *Streptomyces* has been used as a resource in screening for novel antibiotics [2, 3]. To date, the number

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of bioactive secondary metabolites produced by microorganisms, including those from actinomycetes, is thought to exceed 50,000, and those with bioactivity are thought to number some 22,000 to 23,000 [2]. However, only a tiny fraction (<1%) of such bioactive microbial secondary metabolites are used directly in our day-to-day life [2]. Most of these compounds are derived from actinomycetes, such that about 50–55% of known bioactive compounds, are produced by members of the genus *Streptomyces* [4].

Since the late 1980s, the number of novel compounds isolated from terrestrial microorganisms has steadily decreased, causing a decline in the identification of new antibiotics [5, 6]. Therefore, many researchers have been interested in exploring actinomycetes from under-investigated (non-soil) habitats [7, 8]. In recent studies, many actinomycetes have been found in marine environments such as coastal sediments, deep-sea sediments, marine sponges, seaweeds, etc. [9–13]. For example, *Streptomyces tirandamycinicus* [14] was isolated from a marine sponge and *Streptomyces xinghaiensis* [10] was isolated from marine sediment. Notably, such marine-derived *Streptomyces* have been the sources of novel compounds, including respectively the antibacterial tirandamycin [14], desertomycin G [15] and the antimicrobial tunicamycin E [16]. Thus, the screening of samples obtained from marine environments is expected to lead to the discovery of novel actinomycetes and antibiotics [17]. Therefore, we decided to focus on screening marine environments, such as coastal sediments in Japan, for both novel actinomycetes and metabolites.

In our continuing efforts to explore marine-derived actinomycetes, more than 200 strains were isolated, and tentative identification based on the 16S rRNA gene sequences revealed that they belong to 18 genera. One of those, strain CWH03^T was isolated from coastal sediments surrounding Ishigaki Island, Okinawa, Japan. As reported previously, this strain produces spongiicolazolicins, a novel class of linear-azole-containing peptides [18]. Furthermore, strain CWH03^T was considered to be a novel species closely related to *S. spongiicola* based on average nucleotide identities (ANI) using blast search [19] and multilocus sequence analysis (MLSA) with a concatenated sequence of *atp*D, *gyr*B, *rec*A, *rpo*B, and *trp*B. The aim of the present study was to clarify the taxonomic position of strain CWH03^T using a polyphasic taxonomic approach based on genomic, molecular, physiological, chemotaxonomic, and morphological characterizations.

Materials and Methods

Isolation and maintenance of organism

The isolation and maintenance of strain CWH03^T was previously described by Suzuki et al. [18]. Briefly, strain CWH03^T was isolated from a marine sediment sample collected from the coast of Ishigaki Island, Okinawa, Japan, and maintained on ISP (International *Streptomyces* Project) Medium No. 2 (ISP 2) [20] agar plates.

Molecular analyses

Colonies grown on a solid medium were processed using PrepMan® Ultra Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions; the resulting genomic DNA was used as a template for PCR amplification. Reactions consisted of 25 µL of 2 × Quick Taq® HS DyeMix (TOYOBO, Japan), 1 µL of each primer, and 1 µL of template DNA, brought to a total volume of 50 µL with ddH_2O. The 16S rRNA gene was amplified from the template DNA using a pair of primers: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') [21]. The PCR cycle program was as follows: initial denaturation at 95 °C for 5 min; followed by 30 cycles at 95 °C for 60 s (denaturation), 55 °C for 60 s (annealing), and 72 °C for 65 s (extension). The amplified PCR products were purified using a MonoFas DNA Purification Kit (GL Sciences, Japan) and then sequenced by a commercial sequencing service (FASMAC, Japan) using the following primers: 9F, 1541R, 785F (5'-GGATTAGATACCCTGGTAGTC-3'), and 802R (5'-TACCAGGGTATCTAATCC-3'). Nucleotide sequence assembly and editing were performed using GENETYX ATGC software, version 7.0 (GENETYX Co., Tokyo, Japan). The calculation of 16S rRNA gene sequence similarities was performed using the EzBioCloud database [22]. The phylogenetic tree was reconstructed using the neighbor-joining [23], maximum-parsimony [24], and maximum-likelihood (ML) [25] tree-making algorithms in Molecular Evolutionary Genetics Analysis (MEGA) software, version 10 [26]. Evolutionary distances were evaluated using the Kimura's two-parameter model, and tree topologies in all algorithms were estimated by bootstrap analysis with 1000 replicates [27].

Genomic DNA sequences of strain CWH03^T were determined as described by Suzuki et al. [18] and are deposited in DDBJ/ENA/GenBank under registration number BLLG00000000. The genomic sequence of strain CWH03^T was annotated using the DDBJ Fast Annotation and Submission Tool [28]. A genome-based ML tree with bootstrap values (1000 replications) of strain CWH03^T and the closely related taxa based on the concatenated nucleotide sequences of 81 core housekeeping genes was constructed using the UBCG2 pipeline (http://leb.snu.ac.kr/ubcg2) [29]. A genome-based phylogenomic tree was constructed using TYGS web server (https://tygs.dsmz.de/) [30]. Average nucleotide identity (ANI) analysis was performed using the OrthoANIu (OrthoANI using USEARCH) algorithm [31]. Digital DNA–DNA hybridization (dDDH)

values were calculated with the server-based Genome-to-Genome Distance Calculator, version 3.0 (http://ggdc.dsmz.de/distcalc2.php) [32] using the recommended Formula 2. Genome sequences of closely related species were collected from EzBioCloud and NCBI databases.

Chemotaxonomy

The biomass of strain CWH03^T used for chemotaxonomic analyses was obtained by culturing in ISP 2 broth for 7 days at 30 °C in a shake flask using a reciprocating shaker. The cultured cells were harvested by centrifugation, washed twice with physiological saline solution, and lyophilized. Diaminopimelic acid (A_2pm) isomers and sugars in whole-cell hydrolysates were analyzed by the methods of Hasegawa et al. [33] and Tamura et al. [34], respectively. Cellular fatty acids were processed and analyzed as methyl esters following the protocol for the MIDI Sherlock Microbial Identification System [35]. Menaquinones and phospholipids were extracted and analyzed according to standard procedures [36]. Menaquinone content was determined using liquid chromatography/mass spectrometry (LC/MS), as described by Hamada et al. [37]. Phospholipids were identified by two-dimensional thin-layer chromatography, followed by spraying with appropriate detection reagents, according to the method of Yassin et al. [38].

Cultural and physiological characterizations

Strain CWH03^T was grown on humic acid-vitamin (HV) [39] agar for 12 days at 30 °C, and morphological characterization was observed using both light microscopy (OLYMPUS CX41) and scanning electron microscopy (JEOL JSM-6010LV). The aerial mycelium, substrate mycelium, and pigmentation colors of strain CWH03^T were recorded for cells cultured on ISP media (No. 2–7), Czapek's agar, Nutrient agar, Marine agar (Difco), and ISP 2 agar supplemented with artificial seawater (Daigo Artificial Sea Water SP). The Guide to Color Standards (Japan Color Research Institute 1954 [40]) was used for color determination. Gram staining was performed using the standard Gram stain method [41]. To determine the range of growth temperature, strain CWH03^T was incubated for 2 weeks on ISP 2 agar at temperatures of 4, 10, 15, 20, 25, 30, 37, 40, 45, and 50 °C. In addition, growth at 4, 10, and 15 °C was observed after 3 weeks of incubation. Growth at pH values ranging from 4 to 12 (at intervals of 1.0 pH unit) and in the presence of various concentrations of NaCl (0–10% [w/v], at intervals of 1% NaCl) was evaluated after 2 weeks of incubation on ISP 2 agar at 30 °C. Melanin production was assessed after 4 days of growth on ISP 1, ISP 6, and ISP 7 plates. Carbon-source utilization was examined using ISP 9 as a basal medium. Gelatin liquefaction was assayed after 2 weeks of incubation on a glucose-peptone-gelatin medium at 20 °C [42]. Decomposition of adenine, xanthine, hypoxanthine, tyrosine, casein, aesculin, urea, and starch was evaluated by using the media of Gordon et al. [43]. Enzyme activity was tested using the API ZYM Kit (bioMérieux Japan, Ltd.) according to the manufacturer's protocol. Production of oxidase was assessed using the cytochrome-oxidase paper test (Nissui, Japan).

Results and Discussion

Molecular analysis

The almost-complete 16S rRNA gene sequence (1452 nt) of strain CWH03^T was compared with those of known bacterial species using the EzBioCloud database. This similarity search indicated that the strain was the most closely-related to *S. tirandamycinicus* HNM0039^T (99.6%), followed by *Streptomyces spongiicola* HNM0071^T (99.4%), '*Streptomyces marianii*' ICN19^T (99.1%), and *Streptomyces wuyuanensis* CGMCC4.7042^T (99.0%). The phylogenetic tree reconstructed with the 16S rRNA gene sequences (using the neighbor-joining method) showed that strain CWH03^T formed an evolutionary lineage with *S. spongiicola* and other closely related species, an observation confirmed using other tree-making algorithms (Figs. 1, S1, S2).

The whole-genome sequence data suggested a genome size of 6.7 Mbp with 72.3% G + C content, which consisted of 120 contigs and 5798 coding sequences (CDSs) [18]. The distribution of the genes into COGs functional categories is shown in Table S1. A phylogenomic tree based on 81 core orthologous housekeeping genes revealed that strain CWH03^T was clustered with *S. spongiicola* (Fig. 2) and following phylogenetic relatives are the same as 16S rRNA gene phylogeny. Similarly, a genome-based phylogeny using the Type (Strain) Genome Server (Fig. 3) showed good agreement with the phylogeny of 16S rRNA genes and concatenated housekeeping genes. The results of genome-based comparisons with closely related species, evaluated using average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH), are provided in Table 1. The highest values of ANI and dDDH between strain CWH03^T and related species were observed with *S. spongiicola*, which showed values of 91.46% and 44.2%, respectively. The other phylogenetically closely related species (*S. tirandamycinicus*, '*S. marianii*', *S. wuyuanensis*) showed lower values of OrthoANIu and dDDH. These data indicated that the comparisons to strain CWH03^T yielded values below the species cutoff of 95–96% for ANI and 70% for dDDH, indicating species distinction [44].

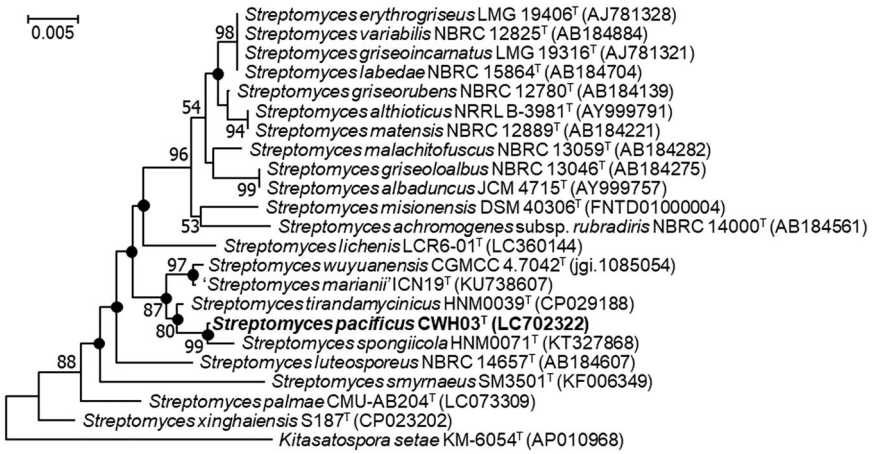

Fig. 1 Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between strain CWH03^T and its phylogenetic relatives. The root position of the neighbor-joining tree was determined using *Kitasatospora setae* KM-6054^T (AP010968) as the outgroup. The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Only bootstrap values above 50% are shown (1000 resamplings) at the branching points. Solid circles indicate that corresponding nodes also were recovered in analyses using the maximum-parsimony and maximum-likelihood algorithms. Scale bar, 0.005 K_{nuc}

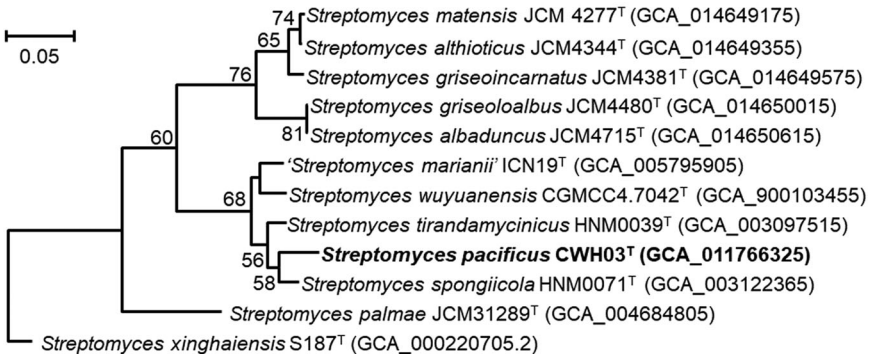

Fig. 2 Phylogenomic tree of strain CWH03^T and its relatives based on 81 core housekeeping genes; this tree was generated using the UBCG2 pipeline. The assembly accession numbers for genome sequences are shown in parentheses. Values at the nodes show the number of bootstrap values of 1000 replications; only values over 50% are shown. Scale bar, 0.05 K_{nuc}

Morphological and physiological characteristics

Strain CWH03^T formed no aerial mycelia on any of the tested media, with the exception of HV agar, on which white aerial mycelia with spiral spore chains were observed after 13 days of culturing. Scanning electron microscopy showed that the spore surface was smooth and rod-like in shape, with dimensions of approximately 0.7 µm in length and 0.5 µm in width (Fig. 4). These morphological observations revealed that strain CWH03^T has morphological characteristics typical of the genus *Streptomyces*. Strain CWH03^T exhibited good growth on all ISP media. Soluble pigments were observed when grown on ISP 2, marine agar, and ISP 2 supplemented with artificial seawater. No melanoid pigments were produced in the tested cultures, in contrast to the melanoid pigments seen upon culture of the closest relative, *S. spongiicola*. Strain CWH03^T exhibited growth across a temperature range of 15–40 °C (optimum 30 °C), a pH range of 7–10 (optimum 7.0), and a NaCl concentration range of 0–7% (w/v) (optimum 0%). Furthermore, strain CWH03^T showed differential properties from the most closely related *S. spongiicola* in terms of D-cellobiose and D-galactose utilization, alkaline phosphatase activity of API ZYM, hypoxanthine and tyrosine degradation, and H_2S production. Details of the physiological and biochemical characteristics of strain CWH03^T are shown in Tables S2 and S3, and characteristics that are distinct from phylogenetically related species are shown in Table 2.

Chemotaxonomy

The chemotaxonomic characteristics of strain CWH03^T were consistent with assignment to the genus *Streptomyces*. Strain CWH03^T composed of LL-diaminopimelic acid and contained glucose, in the whole-cell hydrolysate. The predominant menaquinone of strain CWH03^T was MK-9(H_8) (88.3%); MK-9(H_6) (the predominant menaquinone of *S. spongiicola* [12]) was also detected as a minor menaquinone. The major polar lipids of strain CWH03^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and an

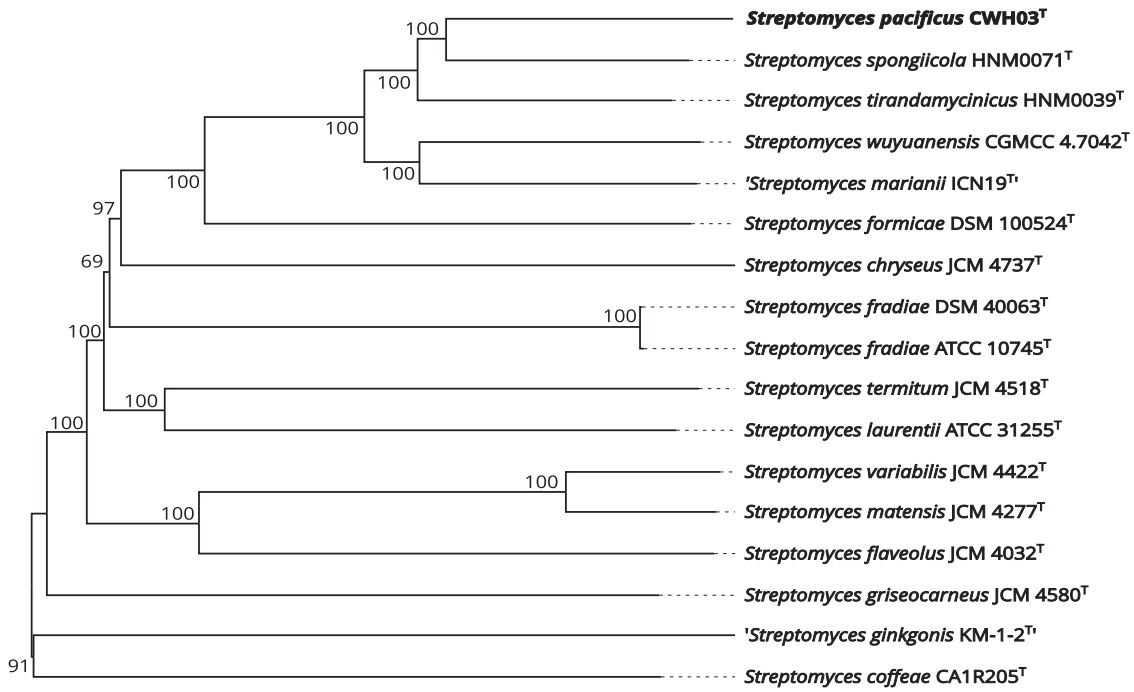

Fig. 3 The phylogenomic tree of strain CWH03^T and its related type strains of the genus *Streptomyces* available on the TYGS database. Tree inferred with FastME 2.1.6.1 from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers below the branches are GBDP pseudo-bootstrap support values from 100 replications, with average branch support of 96.9%

Table 1 Digital DDH and OrthoANIu values between strain CWH03^T and its closely related species

Strains	1	2	3	4	5
1	–	44.2	41.9	36.3	36.2
2	91.46	–	45.1	38.0	38.0
3	90.48	91.45	–	39.7	39.5
4	88.16	89.02	89.65	–	42.8
5	87.97	88.77	89.60	90.97	–

1, *S. pacificus* sp. nov. CWH03^T; 2, *S. spongiicola* HNM0071^T; 3, *S. tirandamycinicus* HNM 0039^T; 4, *S. wuyuanensis* CGMCC 4.7042^T; 5, '*S. marianii*' ICN19^T. OrthoANIu values are in the bottom-left and digital DDH values are in the top-right.

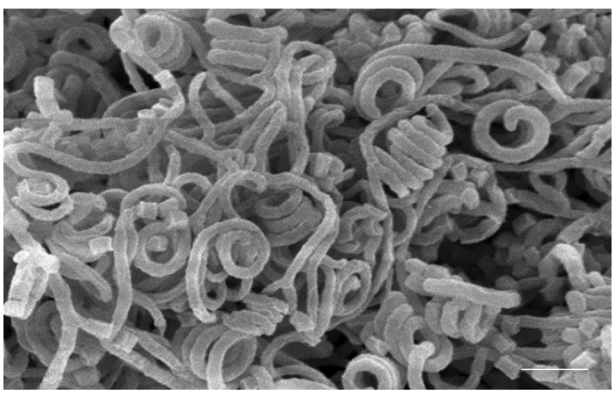

Fig. 4 Scanning electron micrograph of strain CWH03^T cultured on HV agar for 13 days at 30 °C. Scale bar, 2 µm

unidentified ninhydrin-positive phospholipid, along with trace amounts of additional unidentified phospholipids (Fig. S3). The major fatty acids (>10% of the total) of strain CWH03^T were iso-$C_{16:0}$ (28.4%), anteiso-$C_{15:0}$ (15.0%), and iso-$C_{15:0}$ (12.9%) (Table S4).

It is interesting to note that the three type species and an unrecognized species that form a taxonomic cluster with strain CWH03^T were all isolated from the sea [9, 12, 14] or saline samples [45]. Strain CWH03^T is known to produce spongiicolazolicins, while the closely related species *S. tirandamycinicus* HNM0039^T produces tirandamycins, and '*S. marianii*' ICN19^T produces Ala-geninthiocin [46]. In addition, genome mining results for *S. spongiicola* HNM0071^T suggest that it may produce staurosporine and echinomycin [47]. These findings suggest that marine-derived *Streptomyces* spp. are an attractive resource for the discovery of novel antibiotics.

Based upon these genotypic, chemotaxonomic, and phenotypic data, we infer that strain CWH03^T represents a novel species within the genus *Streptomyces*; the name proposed for this strain is *Streptomyces pacificus* sp. nov.

Description of *Streptomyces pacificus* sp. nov

Streptomyces pacificus sp. nov. (pa.ci'fi.cus. L. masc. adj. *pacificus*, peaceful, pertaining to the Pacific Ocean, the origin of the type strain)

Table 2 Physiological and biochemical characteristics differentiating strain CWH03^T from phylogenetically closest members of the genus *Streptomyces*

Characteristic	1	2	3	4
Temperature range for growth(°C)	15–40	15–37	15–40	10–40
NaCl tolerance (%, w/v)	0–7	0–6	0–6	0–7
pH range	7–10	6–12	6–11	6–12
API ZYM				
Alkaline phosphatase	−	+	−	+
α-chymotrypsin	−	−	−	+
Esterase lipase	+	w	−	w
α–mannosidase	+	+	−	w
Utilization of sole C-source				
D-cellobiose	−	+	+	−
Dextrin	+	w	+	−
D-galactose	−	+	+	w
myo–inositol	−	−	+	w
Lactose	−	w	+	w
D-ribose	+	w	+	−
D-xylose	−	w	−	+
Decomposition of:				
Xanthine	−	−	w	+
Hypoxanthine	−	+	+	w
Tyrosine	−	+	w	+
Degradation of:				
Gelatin	−	−	−	+
Reduction of:				
Nitrate	+	+	−	−
Production of:				
H_2S	+	−	−	−
Melanoid pigments	−	+	+	+

All data were generated in the present study. +, positive; w, weakly positive; –, negative. Strain: 1, *S. pacificus* sp. nov. CWH03^T; 2, *S. spongiicola* KCTC 39604^T; 3, *S. tirandamycinicus* KCTC 49236^T; 4, *S. wuyuanensis* KCTC 29112^T.

Gram-stain-positive, aerobic, non-motile actinomycete that forms branching substrate mycelia. White-colored aerial mycelia are produced on HV agar with long, spiral, and smooth-surfaced spores. Grows well on ISP 2, ISP 4, ISP 5, and ISP 6 media. No melanoid pigments are formed on ISP 1, ISP 6, and ISP 7 media. The temperature range for growth is 15–40 °C with optimal growth at 30 °C. The pH range is 7–10 with optimal growth at pH 7.0. The maximum NaCl concentration for growth is 7% (w/v), with optimal growth at 0%. Shows positive for degradation of starch, esculin, and casein; but negative for gelatin liquefaction and decomposition of adenine, xanthine, hypoxanthine, and tyrosine. Esterase, esterase lipase, leucine aryl amidase, valine allyl amidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, and α-mannosidase are present, while urease, alkaline phosphatase, lipase, cystine allyl amidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, and α-fucosidase are not. Catalase reaction is positive, but not oxidase reaction. Production of H_2S and nitrate reduction are positive. Utilizes dextrin, D-fructose, D-glucose, glycerol, D-maltose, D-mannose, melibiose, α-methyl-D-glucoside, D-raffinose, D-ribose, salicin, and trehalose as sole carbon sources, but not adonitol, L-arabinose, D-cellobiose, dulcitol, *meso*-erythritol, D-galactose, *myo*-inositol, lactose, D-mannitol, D-melezitose, α-L-rhamnose, D-sorbitol, sucrose, or D-xylose. The whole-cell hydrolysates contain LL-diaminopimelic acid and glucose. The predominant menaquinone is MK-9(H_8), while MK-9(H_6) presents as a minor component. The phospholipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unidentified phospholipid. The major fatty acids are iso-$C_{16:0}$, anteiso-$C_{15:0}$, and iso-$C_{15:0}$.

The type strain is CWH03^T (= NBRC 114659^T = TBRC 15780^T), isolated from a coastal sediment sample collected near Ishigaki Island, Okinawa, Japan. The DNA G+C content of the type strain is 72.3%. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the draft genome sequence of strain CWH03^T are LC702322 and BLLG00000000, respectively.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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