The Journal of Antibiotics (2014) 67, 465–470

www.nature.com/ja

ORIGINAL ARTICLE

Streptomyces hokutonensis sp. nov., a novel actinomycete isolated from the strawberry root rhizosphere

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A polyphasic approach was used to determine the taxonomic position of actinomycete strain R1-NS-10^T, which was isolated from a sample of strawberry root rhizosphere obtained from Hokuto, Yamanashi, Japan. Strain R1-NS-10^T was Gram-staining-positive and aerobic, and formed brownish-white aerial mycelia and grayish-brown substrate mycelia on ISP-2 medium. The strain grew in the presence of 0–5% (w/v) NaCl and optimally grew without NaCl. The strain grew at pH 5–8, and the optimum for growth was pH 7. The optimal growth temperature was 30 °C, but the strain grew at 5–37 °C. Whole-cell hydrolysates of strain R1-NS-10^T contained A_2pm, galactose, mannose and rhamnose. The predominant menaquinones were MK-9(H_6) and MK-9(H_8). The major cellular fatty acids were anteiso-$C_{15:0}$ and iso-$C_{16:0}$. Comparative 16S rRNA gene sequence analysis revealed that strain R1-NS-10^T was most closely related to *Streptomyces prunicolor* NBRC 13075^T (99.4%). The draft genome sequences of both strains were determined for characterization of genome sequence-related parameters such as average nucleotide identity (ANI) and the diversity of secondary metabolite biosynthetic gene clusters. DNA–DNA hybridization (DDH) and ANI values for both strains were below the species delineation cutoff, and differences in physiological and biochemical characteristics differentiated strain R1-NS-10^T from its closest phylogenetic relative. On the basis of these data, we propose that strain R1-NS-10^T (= NBRC 108812^T = KCTC 29186^T) should be classified as the type strain of a novel *Streptomyces* species named *Streptomyces hokutonensis* sp. nov.
The Journal of Antibiotics (2014) **67,** 465–470; doi:10.1038/ja.2014.20; published online 2 April 2014

Keywords: polyphasic taxonomy; strawberry root rhizosphere; *Streptomyces*

INTRODUCTION

The genus *Streptomyces* is characterized by the presence of LL-diaminopimelic acid (A_2pm) in the cell-wall peptidoglycan, a large amount of saturated iso- and anteiso-branched fatty acids, and nine isoprene units as the predominant menaquinones.[1] As of this writing, the genus *Streptomyces* consists of 640 species with validly published names.[2] Since the discovery of streptomycin from *Streptomyces griseus*, various pharmaceutically important drugs have been discovered from the genus *Streptomyces*.[3–6] Given the importance of the *Streptomyces* as a source of pharmaceuticals, exploration of the natural environment with the aim of discovering novel species in this genus is important. In addition, characterization of the physiological and genotypic features of members of this genus will broaden our understanding of the behavior of these organisms in various ecosystems.

Recent progress in genome sequencing methods has led to the discovery that the *Streptomyces* have the potential to produce a diverse array of secondary metabolites.[7–9] Furthermore, genomic data have given rise to new taxonomic parameters that can be used for species classification, such as the average nucleotide identity (ANI) of common genes and the percentage of conserved DNA. Comparison of DNA–DNA hybridization (DDH) and ANI values has shown that an ANI of 95–96% correlates well with the current bacterial species boundary of 70% DDH similarity.[10–12]

Streptomyces spp. are distributed in a variety of habitats, such as soil, freshwater and marine environments, as well as in association with lichens.[13,14] *Streptomyces* spp. are particularly abundant in the soil and rhizosphere. *Streptomyces* spp. known as plant-growth-promoting rhizobacteria capable of producing auxin and/or siderophores have been isolated from the rhizosphere.[15,16]

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Received 12 April 2013; revised 5 February 2014; accepted 18 February 2014; published online 2 April 2014

While screening for plant-growth-promoting actinomycetes, we discovered strain R1-NS-10^T associated with healthy strawberry roots obtained from Hokuto City, Yamanashi Prefecture, Japan. The aim of the present study was to determine the taxonomic position of strain R1-NS-10^T using a polyphasic taxonomic approach involving chemotaxonomic, morphological, physiological, molecular and genomic characterizations as well as prediction of secondary metabolite biosynthetic gene clusters.

RESULTS AND DISCUSSION

The whole-cell hydrolysate of strain R1-NS-10^T contained LL-A_2pm, galactose, mannose and rhamnose. The major menaquinones were MK-9 (H_6) (39.6%) and MK-9 (H_8) (60.4%). The major fatty acids (>10% of the total) detected in strain R1-NS-10^T were anteiso-$C_{15:0}$ (24.6%) and iso-$C_{16:0}$ (22.3%; Supplementary Table S1). The DNA G+C content of strain R1-NS-10^T was 71.2 mol%. On the basis of the phylogenetic and chemotaxonomic findings, strain R1-NS-10^T was identified as a member of the genus *Streptomyces*.[1]

The nearly complete 16S rRNA gene sequence (1493 nt) of strain R1-NS-10^T was compared with sequences of known bacterial species using the EzTaxon server.[17] The results of these comparisons showed that this strain had the highest sequence similarity (99.4%) to *S. prunicolor* NRRL B-12281^T, followed by *S. resistomycificus* NBRC 12814^T (98.6%), *S. phaeoluteigriseus* NRRL ISP-5182^T (98.6%) and *S. bobili* JCM 4624^T (98.6%). The phylogenetic tree constructed with 16S rRNA gene sequence data using the neighbor-joining method showed that strain R1-NS-10^T formed a monophyletic clade with *S. prunicolor*, and this result was in agreement with those obtained using the maximum-parsimony and maximum-likelihood methods (Figure 1). Further characterizations of strain R1-NS-10^T were conducted using *S. prunicolor* as the closest phylogenetic relative.

Strain R1-NS-10^T formed extensively branched substrate mycelia, and the aerial mycelia formed straight spore chains. Scanning electron microscopy showed that the spore surface was smooth and elliptical in shape and about 1.5 μm long (Figure 2). The growth characteristics of strain R1-NS-10^T cultured on different media are shown in Table 1. The differential growth characteristics of strains R1-NS-10^T and *S. prunicolor* NBRC 13075^T are shown in Table 2 and Supplementary Table S2. Soluble pigment was observed when cells were cultured on ISP-3 and ISP-5 but not when cultured on TSA, ISP-2, ISP-4, ISP-6

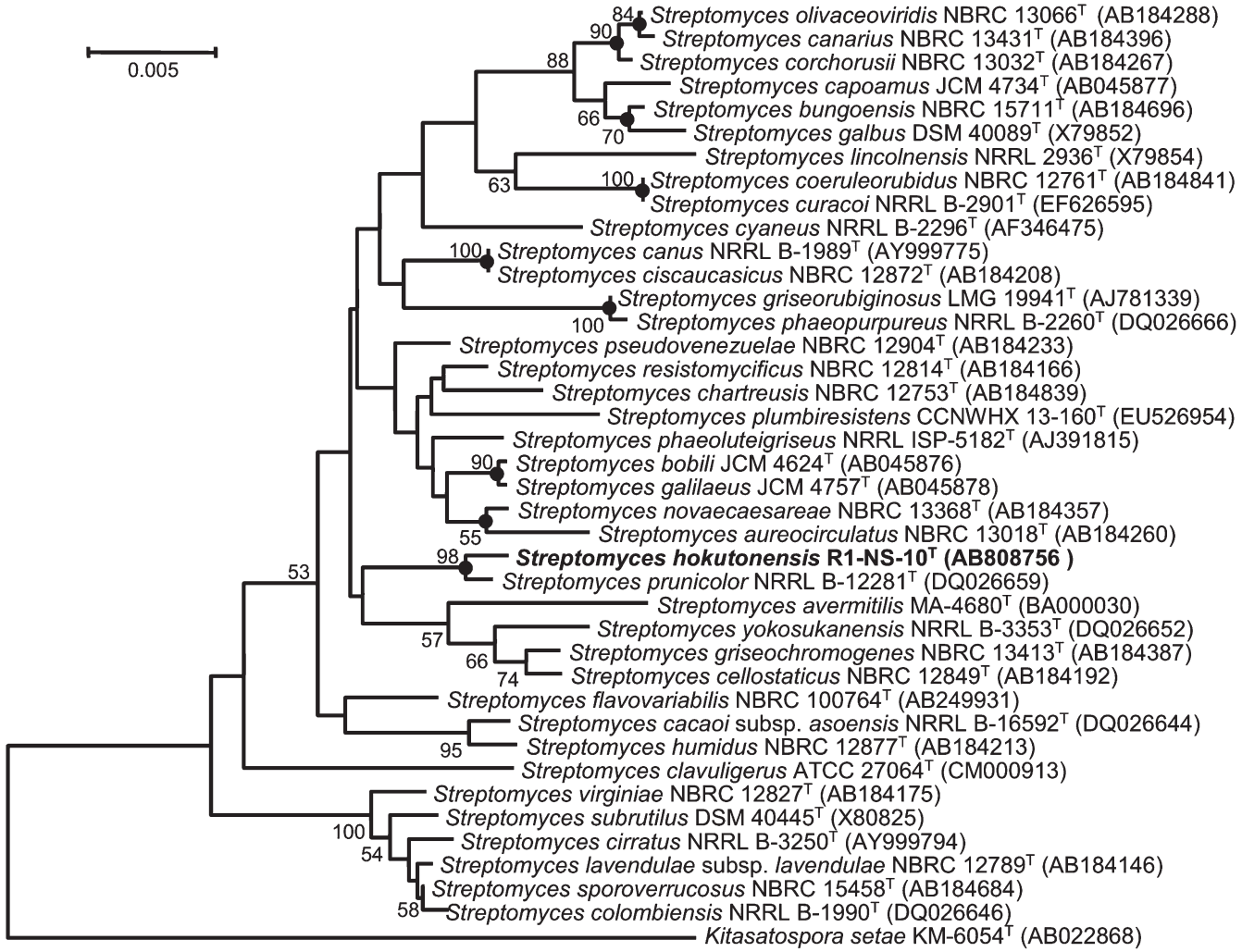

Figure 1 Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between strain R1-NS-10^T and its phylogenetic relatives. The root position of the neighbor-joining tree was determined using *Kitasatospora setae* KM-6054^T (AB022868) as the outgroup. The tree was constructed using the neighbor-joining method and K_{nuc} values.[43] Only bootstrap values above 50% are shown (1000 resamplings) at the branching points. Solid circles indicate that corresponding nodes were also recovered in analyses using the maximum-parsimony and maximum-likelihood algorithms.[44] Bar, 0.005 K_{nuc}.

and ISP-7. Melanin production was negative on ISP-6 and ISP-7 media. Strain R1-NS-10^T grew in the pH range 5–9 and in the presence of 0–5% NaCl (w/v), with optimal growth occurring at pH 7 and 0% NaCl (w/v). The temperature range for growth was 5–37 °C, with an optimum of 30 °C. Strain R1-NS-10^T was easily differentiated from *S. prunicolor* by its growth characteristics on ISP media, its pH-, temperature- and NaCl tolerance, β-glucosidase activity and utilization of D-mannitol, sucrose and inositol (Table 2 and Supplementary Table S3). Before the emergence of molecular taxonomy approaches, simple diagnostic keys such as morphology and phenotypic characterizations were used for streptomycete systematics. However, the use of simple identification keys alone cannot provide adequate identification compared with polyphasic taxonomy. Kämpfer *et al.*[18] suggested that descriptions of *Streptomyces* species should be based on a combination of genotypic and phenotypic data.

In addition to the biologically interesting aspects of strain R1-NS-10^T as a plant-control agent (evidenced by the production of indole-3-acetic acid), the strain also exhibited antimicrobial activity against to *Aspergillus niger* ATCC 9642, *Bacillus subtilis* NBRC 3134, *Saccharomyces cerevisiae* NBRC 10217^T, *Staphylococcus aureus* NBRC 3061 and *Pythium aphanidermatum* NBRC 32440. Comprehensive genome mining employing the antiSMASH secondary metabolite identification pipeline identified 19 candidate gene clusters in strain R1-NS-10^T and *S. prunicolor* NBRC 13075^T (Table 3). Although it is difficult to accurately determine the structure of a metabolite using only genomic information, the above bioassay data suggest that strain R1-NS-10^T produces antibacterial and antifungal molecules.

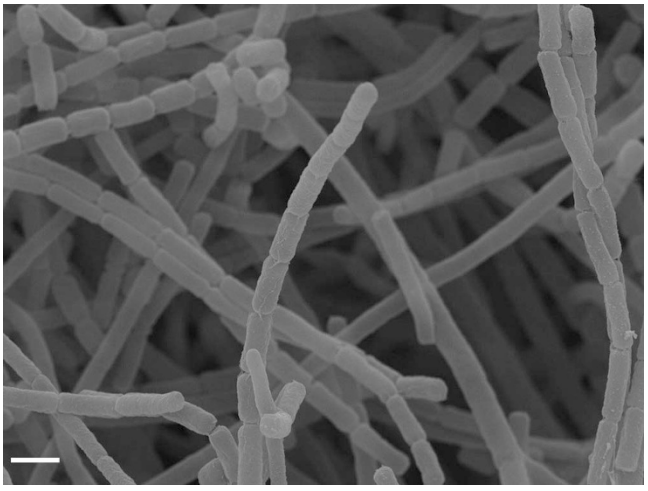

Figure 2 Scanning electron micrograph of strain R1-NS-10^T cultured on HA agar for 2 weeks at 30 °C. Bar indicates 1 μm.

The results of phylogenetic analysis of the 16S rRNA gene in the present study indicate that strain R1-NS-10^T is closely related to *S. prunicolor* NBRC 13075^T. Sequencing of the 16S rRNA gene is widely used for primary molecular identification of prokaryotes.[1,13] However, in the case of *Streptomyces*, the resolving power of 16S rRNA gene sequencing is not sufficient for discrimination at the species level. Several researchers have therefore proposed the use of multilocus sequence analysis,[19–21] which provides 'intermediate resolution' of 16S rRNA gene sequences, and the use of genome-based approaches such as DDH and ANI. Here, we demonstrated the utility of genomic analyses for species-level identification by employing DDH analysis and calculation of ANI values. The DNA–DNA relatedness value between strain R1-NS-10^T and *S. prunicolor* NBRC 13075^T was 52.9 ± 3.1% (reciprocal reaction = 41.2 ± 2.6%), which is below the 70% cutoff point recommended for the assignment of bacterial strains to the same genomic species.[22] Table 4 shows the ANIb values for strains R1-NS-10^T, *S. prunicolor* NBRC 13075^T and other *Streptomyces* species. These data were well below the ANI species threshold (95–96% ANI value).[11]

On the basis of its phenotypic and genotypic characteristics, strain R1-NS-10^T represents a novel species within the genus *Streptomyces*, for which the name *Streptomyces hokutonensis* sp. nov. is proposed.

Description of *Streptomyces hokutonensis* sp. nov.

Streptomyces hokutonensis (ho.ku.to.nen'sis, N.L. masc. adj. *hokutonensis*, pertaining to Hokuto City, Yamanashi Prefecture, Japan, where the organism was originally isolated).

Cells are aerobic and Gram-positive. The substrate mycelia are well-branched, and aerial mycelia fragment into long straight chains of smooth-surfaced cylindrical spores. The spores and aerial mycelia are brownish-white in color, and substrate mycelia are grayish-brown when cells are cultured on ISP-2 medium. Soluble pigment is observed on ISP-3 and ISP-5; however, the strain does not produce melanin on ISP-6 and ISP-7 media. Whole-cell hydrolysates contain A_2pm, galactose, mannose and rhamnose. The major fatty acids (> 10% of total) are anteiso-$C_{15:0}$ and iso-$C_{16:0}$. MK-9(H_6) and MK-9(H_8) are the major menaquinones. The organism grows at 5–37 °C (but not at 45 °C), in the presence of 0–5% NaCl (w/v), and at an initial pH of 5–9. Optimal growth conditions are 30 °C, pH 7 and 0% NaCl. Nitrate is not reduced and the catalase reaction is positive. Starch is hydrolyzed. D-Glucose, D-fructose, D-galactose, D-maltose, D-mannose, D-trehalose, D-raffinose, D-xylose, L-arabinose, glycerol and L-rhamnose are utilized as sole carbon sources; however,

Table 1 Characteristics of strain R1-NS-10^T cultured on various media

			Color of mycelium	
Medium	*Growth*	*Soluble pigment*	*Aerial*	*Substrate*
Yeast extract-malt extract agar (ISP 2)	+ + +	None	Brownish-white	Grayish-brown
Oatmeal agar (ISP 3)	+ + +	Yellow	Pale brown	Light brown
Inorganic salts-starch agar (ISP 4)	+ + +	None	Light brownish gray	Pale yellowish brown
Glycerol/asparagine agar (ISP 5)	+ + +	Brown	Pale orange	Reddish-brown
Peptone-yeast extract-iron agar (ISP 6)	+	None	None	Pale yellow
Tyrosine agar (ISP 7)	+ + +	None	Pale orange	Reddish-brown
Trypticase soy agar (TSA)	+	None	None	Pale yellow

Abbreviations: ISP, International *Streptomyces* Project; +, poor growth; + + +, abundant growth.
All media were adjusted to pH 7.0.

Table 2 Differential characteristics of strain R1-NS-10^T and *S. prunicolor* NBRC 13075^T

Characteristic	*1*	*2*
Characteristics on ISP3		
Soluble pigment production	+	−
Aerial mycelium	Pale brown	Grayish-white
Color of substrate mycelium	Light brown	Pale yellow
pH range	5–9	5–8
Temperature range for growth (°C)	5–37	20–37
NaCl tolerance (%, w/v)	5	3
API ZYM		
β-glucosidase	+	−
Utilization of sole C-source		
D-mannitol	−	+
Sucrose	−	+
Inositol	−	+

Strains: *1*, *S. hokutonensis* R1-NS-10^T; *2*, *S. prunicolor* NBRC 13075^T; (+), positive; (−), negative.
All data were generated in the present study.
Both strains were positive for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and hydrolysis of starch, and utilization of D-glucose, D-galactose, D-mannose, D-raffinose, D-xylose, L-arabinose, glycerol and L-rhamnose. Both strains were negative for lipase (C14), cystine arylamidase, α-chymotrypsin, β-glucuronidase, α-fucosidase and utilization of D-sorbitol, D-turanose and L-arabitol.

Table 3 Number of predicted secondary metabolite biosynthetic gene clusters in the genome of strain R1-NS-10^T and *S. prunicolor* NBRC 13075^T, as determined using the antiSMASH 2.0 software[46]

Type of gene or cluster	*1*	*2*
Polyketides (type I)	3	3
Polyketides (type II)	1	1
Polyketides (type III)	1	1
Polyketides (type IV)	0	2
Nonribosomal peptides	8	6
Terpenes	4	6
Lantibiotics	0	0
Lantipeptide	1	1
Bacteriocins	3	6
Siderophores	3	3
Ectoines	1	1
Butyrolactones	1	1
Nucleosides	0	1
Melanins	1	0
Transatpks-t1pks-t2pks	0	1
T4pks-t1pks	0	1
Phosphonate	1	0
Oligosaccharide-t1pks-t4pks	1	0
Nrps-melanin	1	0
Other	4	3
Total	19	19

Strains: *1*, *S. hokutonensis* R1-NS-10^T; *2*, *S. prunicolor* NBRC 13075^T.

Table 4 ANIb values calculated using the JSpecies software.[11]

	Target genome				
Query genome	*1*	*2*	*3*	*4*	*5*
S. hokutonensis R1-NS-10^T	—	82.4	80.1	81.4	76.5
S. prunicolor NBRC 13075^T	82.6	—	80.0	81.4	76.2

Strains: *1*, *S. hokutonensis* R1-NS-10^T (BARG01000001-BARG01000178); *2*, *S. prunicolor* NBRC 13075^T (BARF01000001-BARF01000202); *3*, *S. coelicolor* A3(2) (uid57801); *4*, *S. avermitilis* MA 4680 (uid57739); *5*, *S. griseus* NBRC 13350 (uid58983).

D-mannitol, D-sorbitol, D-sucrose, inositol, D-turanose and L-arabitol are not utilized as sole carbon sources. API ZYM tests for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase activity are positive. The test for esterase lipase (C8) is weakly positive. Tests for lipase (C14), cystine arylamidase, α-chymotrypsin, β-glucuronidase and α-fucosidase activity are negative. The DNA G + C content of the type strain is 71.2 mol%.

The type strain is R1-NS-10^T (= NBRC 108812^T = KCTC 29186^T) and was isolated from a sample of strawberry roots obtained from Hokuto City, Yamanashi, Japan.

MATERIALS AND METHODS

Isolation and maintenance of the organism

A sample of healthy strawberry root was collected in an agricultural field in Hokuto City, Yamanashi, Japan. Although the root sample (1 g) was washed with sterilized water (500 ml) to remove most of the associated soil particles, a small amount of soil remained attached. The root was then homogenized in sterilized distilled water using a mortar and pestle. The resulting suspension (0.2 ml) was spread onto humic acid-vitamin (HV) agar[23] containing nalidixic acid (20 mg l^{-1}) and cycloheximide (50 mg l^{-1}) and then incubated at 30 °C for 2 weeks. Following the incubation period, strain R1-NS-10^T was isolated and transferred to oatmeal-YGG agar.[24] The organism was also preserved in 20% (v/v) glycerol at −80 °C.

Phenotypic characterization

Strain R1-NS-10^T was grown on HA agar[25] for 14 days at 28 °C, and its morphological features were analyzed using both light microscopy and scanning electron microscopy (JEOL, JSM-6500F). The aerial mycelium, substrate mycelium and pigmentation colors of strain R1-NS-10^T were recorded for cells cultured on ISP (International *Streptomyces* Project) media[26] and tryptic soy agar (TSA) (Bacto). The Guide to Color Standard (Japan Color Research Institute 1954) was used for color determination. Cells were Gram-stained according to the method of Hucker.[27] To determine the optimal growth temperature, strain R1-NS-10^T was incubated for 7 and 14 days on oatmeal-YGG agar at temperatures of 5, 10, 20, 30, 37, 40 and 45 °C. Growth at 5 and 10 °C was assayed after 6 weeks of incubation. Growth at pH values ranging from 5 to 11 (in 1 pH unit increments) and in the presence of various concentrations of NaCl (0–8% (w/v), in 1% increments) was evaluated after 14 and 21 days of incubation on ISP-2 medium.[26] Melanin production was assessed after 1–4 days of growth on ISP-6 and ISP-7 media.[26] Carbon-source utilization was examined using ISP-9 as a basal medium. Antimicrobial activity was assayed using an overlay method[28] against 19 microorganisms: *Aspergillus niger* ATCC 9642, *Botryotinia fuckeliana* NBRC 30915, *Bacillus subtilis* NBRC 3134, *Candida albicans* NBRC 1385^T, *Cercospora kikuchii* NBRC 6711, *Clavibacter michiganensis* subsp. *michiganensis* NBRC 13762, *Colletotrichum orbiculare* NBRC 33130, *Escherichia coli* NBRC 3044, *Fusarium oxysporum* NBRC 31213, *Fusarium solani* NBRC 9955, *Pythium aphanidermatum* NBRC 32440, *Pythium helicoids* NBRC 100107, *Rhizobium rhizogenes* NBRC 13257^T, *Rhizobium rubi* NBRC 13261^T, *Saccharomyces cerevisiae* NBRC 10217^T, *Staphylococcus aureus* NBRC 3061, *Streptomyces scabiei* NBRC 12914, *Streptomyces turgidiscabies* NBRC 16080^T and *Thanatephorus cucumeris* NBRC 30455. Briefly, spot-inoculated, 10-day-old colonies of strain R1-NS-10^T cultured on nutrient agar plates were overlaid with 5 ml of sloppy nutrient agar /YEPD agar inoculated with the test organism. The size of the zone of inhibition around each colony was recorded after incubation for 24 h at 30 °C. Production of indole-3-acetic

acid by strain R1-NS-10^T was determined according to the method of Matsukawa *et al.*[29,30] and use of Salkowski reagent.

Chemotaxonomy

Cell biomass was hervested for chemotaxonomic studies by incubating strain R1-NS-10^T in yeast extract-glucose (YG) broth for 5–7 days at 30 °C with shaking.[31] Cells were harvested by centrifugation and the resulting pellet was washed twice with distilled water. A_2pm isomers and sugars in whole-cell hydrolysates were analyzed based on the methods described by Hasegawa *et al.*[32] and Tamura *et al.*,[33] respectively. Cellular fatty acids were processed and analyzed as methyl esters following the protocol for the MIDI Sherlock Microbial Identification System.[34] Isoprenoid quinones were extracted and isolated using standard procedures,[35] with the results compared to those of appropriate controls. The isoprenoid quinone content was determined using liquid chromatography/mass spectrometry (LC/MS), as described by Hamada *et al.*[36] The DNA G + C content of strain R1-NS-10^T was determined by HPLC as described by Tamaoka and Komagata.[37]

Molecular analysis

Chromosomal DNA was isolated from strain R1-NS-10^T and purified as described by Saito and Miura,[38] with a minor modification.[39] The 16S rRNA gene from strain R1-NS-10^T was amplified by PCR as described by Tamura and Hatano,[40] and the PCR product was purified using a MonoFas DNA Purification Kit (GL Sciences, Tokyo, Japan). The purified PCR product was directly sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a Model 3730 Genetic Analyzer automated DNA sequencer (Applied Biosystems). The 16S rRNA gene sequence was compared with published 16S rRNA gene sequences of bacterial type strains using the EzTaxon server (http://www.eztaxon.org/).[17] For phylogenetic analyses, 16S rRNA gene sequences were collected from the EMBL/GenBank/DDBJ databases and aligned using the CLUSTAL_X program.[41] Phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis software, version 5.1,[42] with the neighbor-joining, maximum parsimony and maximum-likelihood methods.[43,44] The topologies of the constructed trees were evaluated by bootstrap analysis with 1000 resamplings.[45] The online web server antiSMASH 2.0 (antibiotics & Secondary Metabolites Analysis SHell) was used to predict secondary metabolite biosynthetic gene clusters in the genomes of strain R1-NS-10^T and *S. prunicolor* NBRC 13075^T.[46]

DDH and ANIb calculation

DDH analyses were carried out as described by Kusunoki *et al.*[47] using biotinylated DNA, with five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the mean of the remaining three values was reported as the DNA–DNA relatedness value. Random partial genome pyrosequencing analyses were performed using strains R1-NS-10^T and *S. prunicolor* NBRC 13075^T. The genome sequence was examined using a combined strategy involving GS FLX Titanium and HiSeq 1000 technologies. Two different libraries were constructed for sequencing: a standard library (600–900 bp) for the GS FLX Titanium strategy and a paired ends (200–500 bp insert) library for the HiSeq 1000 strategy. Sequences were assembled using the Newbler v2.6 software (Roche Applied Science, Branford, CT, USA) with the default parameters. The ANI by BLAST value was calculated using the JSpecies program with default settings.[10,11] JSpecies was primarily designed to analyze and compare innerspecies boundaries between genomes, draft genomes or partial random genome sequences.

Nucleotide and genome sequence accession numbers

The 16S rRNA gene sequence of strain R1-NS-10^T determined in this study has been deposited in the DDBJ database under the accession number AB808756. The draft genome sequences of strain R1-NS-10^T and *S. prunicolor* NBRC 13075^T were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers BARG01000001-BARG01000178 and BARF01000001-BARF01000202, respectively.

ACKNOWLEDGEMENTS

This work was conducted and supported under the joint research project 'Locally produced and Consumed' between the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Hokuto City and the University of Yamanashi. This study was supported in part by a research grant from the Institute for Fermentation, Osaka (IFO), Japan. We are grateful to Dr Jean P Euzéby for assistance with nomenclature.

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