Streptomyces heilongjiangensis sp. nov., a novel actinomycete that produces borrelidin isolated from the root surface of soybean [*Glycine max* (L.) Merr]

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A borrelidin-producing actinomycete, designated strain NEAU-W2^T, was isolated from the root surface of soybean [*Glycine max* (L.) Merr] and characterized using a polyphasic approach. The organism was found to have morphological and chemotaxonomic characteristics typical of streptomycetes. The G + C content of the DNA was 66.12 mol%. Analysis of the 16S rRNA gene sequence of strain NEAU-W2^T revealed that the strain formed a distinct clade within the 16S rRNA gene sequence phylogenetic tree and showed highest similarity (99.61 %) to *Streptomyces neyagawaensis* ATCC 27449^T. However, the DNA–DNA relatedness between strain NEAU-W2^T and *S. neyagawaensis* ATCC 27449^T was 58.51 %. Strain NEAU-W2^T could also be differentiated from *S. neyagawaensis* ATCC 27449^T and other *Streptomyces* species showing high 16S rRNA gene sequence similarity (98–99 %), as well as other borrelidin-producing strains, based on morphological and physiological characteristics. On the basis of its physiological and molecular properties, it is proposed that strain NEAU-W2^T represents a novel *Streptomyces* species, *Streptomyces heilongjiangensis* sp. nov. The type strain is NEAU-W2^T (=CGMCC 4.7004^T =ATCC BAA-2424^T =DSM 42073^T).

Endophytic actinobacteria have been isolated from many crops in recent years, such as maize (de Araujo *et al.*, 2000), wheat (Coombs & Franco, 2003), Chinese cabbage (Lee *et al.*, 2008) and tomato plants (de Oliveira *et al.*, 2010), and some of these isolates are known to possess the ability to inhibit a wide variety of harmful fungi. Thus, there is great application value to develop antifungal agents from endophytic actinobacteria. In an effort to discover antifungal compounds with novel chemical structures or modes of action against *Phytophthora sojae*, which is a destructive oomycete affecting soybean production throughout the world, a potential endophytic antagonist, strain NEAU-W2^T effective against the growth of *P. sojae*, was isolated from healthy soybean root, and the active compound borrelidin was finally obtained. The isolation and identification of borrelidin from another endophytic *Streptomyces* with strong antifungal activity against *P. sojae* has been reported by ourselves (Liu *et al.*, 2012). Based on the present polyphasic taxonomic study, we propose that strain NEAU-W2^T represents a novel species of the genus *Streptomyces*.

Strain NEAU-W2^T was isolated from the root surface of soybean collected from Hulin, Heilongjiang province, northern China (46° 16' N 133° 36' E). In the field, the plant was tagged and stored in a clean plastic bag until used (approx. 24 h). The root sample was air-dried for 24 h at room temperature and then washed with an ultrasonic step (160 W, 15 min) to remove surface soil and adherent epiphytes completely. After drying, the sample was cut into pieces of 5–10 mm in length. Fifty milligrams of root sample was placed in 10 ml sterile tap water in a sterile beaker and vigorously stirred for 60 s using a magnetic stirrer. The washing process was repeated using fresh 10 ml sterile tap water, and the root sample was then rinsed in sterile tap water containing cycloheximide (100 mg ml⁻¹) and nalidixic acid (20 mg ml⁻¹) for 60 s. The rinsed root samples were transferred temporarily onto sterile filter paper to eliminate excess moisture and then placed on a plate of humic acid-vitamin agar (Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹). After 21 days of aerobic incubation at 28 °C, colonies were transferred and purified on oatmeal agar (ISP medium 3) and incubated at 28 °C for 2–3 weeks.

The morphology of the spore chain and the spore surface ornamentation of strain NEAU-W2^T were observed by light and scanning electron microscopy using cultures

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NEAU-W2^T is JF431459.

One supplementary table and four supplementary figures are available with the online version of this paper.

grown on ISP3 agar for 14 days. Cultural characteristics were observed on a number of standard media (Table 1) after 2 weeks at 28 °C. The utilization of sole carbon sources, decomposition of urea and cellulose, hydrolysis of starch, aesculin and hippurate, utilization of calcium malate, sodium oxalate, sodium citrate and sodium succinate, reduction of nitrate, liquefaction of gelatin and production of H₂S were examined as described previously (Gordon *et al.*, 1974; Yokota *et al.*, 1993). Grown over a range of temperatures, pH and NaCl concentrations were tested using modified Bennett's agar (Williams *et al.*, 1983). Resistance to antibiotics was examined according to Goodfellow & Orchard (1974). Biomass for chemical studies was prepared by growing the strain in trypticase soy broth (TSB: tryptone, 15 g; soya peptone, 5 g; NaCl, 0.5 g; distilled water, 1 litre; pH 7.0–7.4) in Erlenmeyer flasks for 3 days at 28 °C. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diaminopimelic acid in the whole-cell hydrolysates were analysed by HPLC using an Agilent TC-C₁₈ column (250 × 4.6 mm, i.d. 5 µm) with a mobile phase consisting of acetonitrile/0.05 mol phosphate buffer l⁻¹, pH 7.2 (15:85) at a flow rate of 0.5 ml min⁻¹. Peak detection used an Agilent G1321A fluorescence detector with a 365 nm excitation filter and 455 nm longpass emission filters (McKerrow *et al.*, 2000). The whole-organism sugars were analysed according to the procedures developed by Lechevalier & Lechevalier (1980). Polar lipids were examined by two-dimensional TLC and identified using the method of Minnikin *et al.* (1984). Menaquinones were extracted from freeze-dried biomass and purified according to Collins (1985). Extracts were analysed by HPLC-UV using an Agilent Extend-C₁₈ column (150 × 4.6 mm, i.d. 5 µm), typically at 270 nm. The mobile phase was acetonitrile/propyl alcohol (60:40, v/v) and the flow rate was set to 1.0 ml min⁻¹ and the run time was 60 min. The injection volume was 20 µl, and the chromatographic column was controlled at 40 °C (Wu *et al.*, 1989). Mycolic acids were checked by the acid methanolysis method as described previously (Minnikin *et al.*, 1980). Biomass for fatty acid analysis was prepared by scraping a culture from ISP3 agar. Cellular fatty acids were analysed using the method of Xiang *et al.* (2011).

Strain NEAU-W2^T and *Streptomyces neyagawaensis* ATCC 27449^T were grown in 50 ml fermentation medium (glucose 1 %, soluble amylum 4 %, yeast extract 0.5 %, soybean powder 2.5 %, peptone 0.5 %, CaCO₃ 0.2 %, MgSO₄ · 7H₂O 0.8 %, FeSO₄ · 7H₂O 0.6 %, ZnSO₄ · 7H₂O 0.2 %, MnSO₄ · H₂O 0.2 %, CoCl₂ · 6H₂O 0.05 %, Na₂MoO₄ · 2H₂O 0.2 %; pH 7.0) in a 250 ml Erlenmeyer flask and incubated at 28 °C on a rotary shaker at 250 r.p.m. After 5 days of cultivation, a total of 50 ml fermentation broth was filtered and the mycelial cakes were extracted for about 10 h with 50 ml methanol. The methanol extracts were then analysed by HPLC-UV (acetonitrile/0.1 % acetic acid, 30:70–100:0; Agilent 1100, TC-C₁₈, 5 µm, 250 × 4.6 mm i.d.; 1.0 ml min⁻¹; 257 nm; Agilent).

Table 1. Growth and cultural characteristics of strain NEAU-W2^T and *Streptomyces neyagawaensis* ATCC 27449^T

Agar medium	NEAU-W2 ^T			<i>S. neyagawaensis</i> ATCC 27449 ^T		
	Aerial mycelium	Substrate mycelium	Diffusible pigment	Aerial mycelium	Substrate mycelium	Diffusible pigment
Yeast extract/malt extract (ISP2)	White; good	Yellowish-brown; good	None	Yellow; good	Yellowish-brown; good	None
Oatmeal (ISP3)	Grey; good	Colourless; good	None	Brownish-grey; good	Grey; good	None
Inorganic salts/starch (ISP4)	Grey; good	Yellowish-grey; good	None	Grey; good	Bluish-grey; good	None
Glycerol/asparagine (ISP5)	White; good	Colourless; good	None	Yellow; good	Yellow; good	None
Peptone/yeast extract/iron (ISP6)	Grey; good	Black; good	Black	Black; good	Black; good	Black
Tyrosine (ISP7)	Grey; good	Reddish-brown; good	Brown	Yellowish-brown; good	Black; good	Brown

Fig. 1. Scanning electron micrograph of cells of strain NEAU-W2^T grown on ISP3 medium for 14 days at 28 °C. Bar, 1 µm.

Genomic DNA of strain NEAU-W2^T was extracted as described previously by Lee *et al.* (2003) and PCR amplification of the 16S rRNA gene was carried out according to the procedures described by Loqman *et al.* (2009). The 16S rRNA gene sequence of strain NEAU-W2^T was aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using CLUSTAL X 1.83 software. The alignment was manually verified and adjusted prior to the construction of phylogenetic trees. Phylogenetic trees were generated with the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Tamura *et al.*, 2011) algorithms using MEGA 5.05 (Tamura *et al.*, 2011). The stability of the clades in the trees was appraised using a bootstrap procedure with 1000 repeats (Felsenstein, 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated

from the dataset (complete deletion option). DNA–DNA relatedness experiments were performed using the optical renaturation method as described by De Ley *et al.* (1970). The DNA G+C content of the genomic DNA was determined by the thermal denaturation method as described by Mandel & Marmur (1968), and *Escherichia coli* JM109 was used as the reference strain.

Morphological observation of a 14 day culture of strain NEAU-W2^T grown on ISP3 medium revealed that strain NEAU-W2^T had characteristics typical of the genus *Streptomyces*. Aerial and substrate mycelium were well developed without fragmentation. Long, straight spore chains were observed (Fig. S4 available in IJSEM Online), and spores were smooth and non-motile (Fig. 1). Cultural characteristics of strain NEAU-W2^T are shown in Table 1. Aerial mycelium of strain NEAU-W2^T was abundant, well developed and varied from white to grey on different tested media. The substrate hyphae varied from yellowish-brown to reddish-brown. Melanoid pigment was observed on peptone/yeast extract/iron medium (ISP6). Strain NEAU-W2^T grew well at pH 5–11, with an optimum pH of 7.0. The range of temperature for growth was 15–40 °C, with the optimum growth temperature being 30 °C. Strain NEAU-W2^T grew in the presence of 0–5 % NaCl (w/v). Detailed physiological characteristics are presented in the species description.

Strain NEAU-W2^T contained LL-diaminopimelic acid as the diamino acid. Whole-cell hydrolysates comprised ribose and glucose (Fig. S3). Mycolic acids were absent.

Fig. 2. Neighbour-joining tree showing the phylogenetic position of strain NEAU-W2^T and related taxa based on 16S rRNA gene sequences. Asterisks indicate branches that were also found using the maximum-likelihood (Tamura *et al.*, 2011) method. Numbers at branch points indicate bootstrap percentages (based on 1000 replicates); only values >50 % are indicated. Bar, 0.005 substitutions per nucleotide position.

Table 2. Differential physiological characteristics between strain NEAU-W2^T and *Streptomyces neyagawaensis* ATCC 27449^T

+, Positive; –, negative.

Characteristic	NEAU-W2 ^T	<i>S. neyagawaensis</i> ATCC 27449 ^T
Utilization of sodium oxalate	+	–
Decomposition of urea	–	+
Hydrolysis of starch	+	–
Liquefaction of gelatin	–	+
Reduction of nitrate	+	–
Nitrogen utilization:		
Creatine	+	–
Threonine	+	–
Tolerance to:		
Carbenicillin (100 mg l ^{–1})	+	–
Chloramphenicol (30 mg l ^{–1})	+	–
Kanamycin (10 mg l ^{–1})	–	+
Rifamycin (50 mg l ^{–1})	+	–

The major menaquinones were MK-9(H₄) (8.55%), MK-9(H₆) (74.66%) and MK-9(H₈) (16.79%). The phospholipid profile consisted of phosphatidylethanolamine and phosphatidylinositol (Fig. S2). The cellular fatty acid profile comprised C_{14:0} iso (1.27%), C_{14:0} (2.77%), C_{15:0} iso (3.41%), C_{15:0} (11.00%), C_{16:0} (42.76%), C_{16:1} (11.61%), C_{17:0} anteiso (6.56%), C_{17:0} cyclo (1.16%), C_{17:0} iso (1.44%), C_{18:1} (1.47%) and C_{18:0} (15.88%) (fatty acids representing <1.00% of the total were not reported) (Table S1). All the morphological characteristics

and chemotaxonomic data indicated that strain NEAU-W2^T should be assigned to the genus *Streptomyces*.

The almost-complete 16S rRNA gene sequence (1520 nt) of strain NEAU-W2^T was aligned manually with its closest phylogenetic neighbours and two other borrelidin-producing strains (*Streptomyces rochei* NRRL B-1559^T and *Streptomyces* sp. neau-D50) using the procedures described above. It was evident from the phylogenetic tree thus constructed (Fig. 2) that the organism formed a distinct phyletic line with its closest neighbour *S. neyagawaensis*

Table 3. Morphological, physiological and biochemical characteristics of strain NEAU-W2^T and phylogenetically related *Streptomyces* species

Strains: 1, NEAU-W2^T; 2, *S. torulosus* LMG 20305^T; 3, *S. hygroscopicus* subsp. *ossamyceticus* NBRC 13983^T; 4, '*S. ogaensis*' NBRC 13817^T; 5, *S. ipomoeae* DSM 40383^T. Data for reference strains are taken from earlier studies (Lyons & Pridham, 1971; Price & Schmitz, 1966; Nagatsu & Suzuki, 1963; Shirling & Gottlieb, 1969). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Spore surface	Smooth	Knobby	Smooth	ND	Smooth
Spore-chain morphology	Straight	Spiral	Spiral	Spiral	Spiral
Melanoid production	+	+	+	+	–
Colony colour on:					
ISP2	White	ND	Dark brown	Grey	Blue
ISP3	Grey	ND	ND	Grey	Blue
ISP4	Grey	Grey	ND	Grey	Blue
ISP7	Grey	ND	ND	White	ND
Utilization of:					
Fructose	+	+	+	–	+
Arabinose	+	+	+	–	+
Xylose	+	+	+	–	+
Sucrose	+	ND	+	–	ND
Sorbitol	+	ND	–	ND	ND
Decomposition of cellulose	–	ND	+	ND	ND
Antibiotic produced	Borrelidin	ND	Ossamycin	Cervicarin	ND

Table 4. Selected differential characteristics between strain NEAU-W2^T and borrelidin-producing *Streptomyces* species

Strains: 1, NEAU-W2^T; 2, *S. candidus* Y21007-2^T; 3, *S. californicus* G16^T; 4, *S. griseus* BS1325^T; 5, *S. albobovineus* 275^T; 6, *S. rochei* NRRL B-1559^T; 7, *Streptomyces* sp. neu-D50. Data for reference strains are taken from previous studies (Shiang *et al.*, 2001; Saisivam *et al.*, 2008; Dorgrrloh *et al.*, 1988; Singh *et al.*, 1985; Shirling & Gottlieb, 1968; Liu *et al.*, 2012). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Spore-chain morphology	Straight	Straight	Rectiflexible	Straight	Flexuous	Flexuous	Straight
Melanoid production	+	–	ND	–	–	–	–
Utilization of:							
Arabinose	+	+	–	+	+	+	+
Inositol	+	–	–	–	–	+	+
Mannitol	+	+	–	+	+	+	+
Raffinose	+	–	–	+	–	–	–
Sucrose	+	–	–	+	+	–	–
Xylose	+	+	–	+	+	+	+

ATCC 27449^T (99.61 % gene sequence similarity), an association that was supported by the maximum-likelihood algorithm and by a 86 % bootstrap value in the neighbour-joining analysis. However, DNA–DNA hybridization revealed 58.51 % relatedness between strain NEAU-W2^T and *S. neyagawaensis* ATCC 27449^T, which is well below the 70 % value considered to be the threshold for the delineation of genomic species (Wayne *et al.*, 1987). Morphological and physiological comparisons between strain NEAU-W2^T and *S. neyagawaensis* ATCC 27449^T were performed to differentiate them (Tables 1 and 2). In addition, strain NEAU-W2^T produced the antifungal compound borrelidin in cell cultures (Fig. S1), readily differentiating it from *S. neyagawaensis* ATCC 27449^T. The two tree-making algorithms also supported the position that the strain NEAU-W2^T–*S. neyagawaensis* line was consistently in the same clade along with *Streptomyces hygroscopicus* subsp. *ossamyceticus* NBRC 13983^T, '*Streptomyces ogaensis*' NBRC 13817^T, *Streptomyces torulosus* LMG 20305^T and *Streptomyces ipomoeae* DSM 40383^T. 16S rRNA gene sequence similarities between the novel isolate and these type strains were between 98.46 and 99.11 %. However, a comparative study between strain NEAU-W2^T and the above four related *Streptomyces* type strains revealed that it differed from them in morphological, physiological and biochemical characteristics, as summarized in Table 3. Similarly, strain NEAU-W2^T could be differentiated from six other borrelidin-producing species based on spore-chain morphology, melanoid production and carbon source utilization (Table 4). Among all borrelidin-producing strains, only two 16S rRNA gene sequences (*Streptomyces rochei* NRRL B-1559^T and *Streptomyces* sp. neu-D50) could be retrieved from the GenBank/EMBL/DDJB databases. The low sequence similarity values (<95.57 %) between strain NEAU-W2^T and the two borrelidin-producing strains also confirmed that they represent different genomic species.

In conclusion, it is evident from the genotypic, chemotaxonomic and phenotypic data that strain NEAU-W2^T

represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces heilongjiangensis* sp. nov. is proposed.

Description of *Streptomyces heilongjiangensis* sp. nov.

Streptomyces heilongjiangensis (hei.long.ji.ang.en'sis. N.L. masc. adj. *heilongjiangensis* of or belonging to Heilongjiang, a province of north-east China)

Gram-positive and aerobic. Grows well on all test media. Aerial mass colour is in the grey series and melanin pigment is observed on ISP6 medium. Both aerial and vegetative hyphae are well developed without fragmentation. Long, straight spore (0.7 × 0.9 µm) chains appear, and spores are smooth and non-motile. Negative for decomposition of cellulose and urea, hydrolysis of hippurate and liquefaction of gelatin and positive for reduction of nitrate, hydrolysis of aesculin and starch, production of H₂S, and utilization of calcium malate, sodium succinate, sodium oxalate and sodium citrate. Resistant to (mg l^{–1}) ampicillin (100), carbenicillin (100), chloramphenicol (30), nystatin (50) and rifamycin (50), but sensitive to apramycin (30), gentamicin sulfate (10), kanamycin (10) and thiostrepton (30). Arabinose, fructose, galactose, glucose, inositol, lactose, maltose, mannose, mannitol, raffinose, rhamnose, ribose, sorbitol, sucrose and xylose are utilized as sole carbon sources. Alanine, arginine, asparagine, creatine, glutamine, threonine and tyrosine are utilized as sole nitrogen sources, but aspartic acid and glutamic acid are not. Tolerates up to 5 % NaCl and grows at temperatures between 15 and 40 °C, with an optimum temperature of 30 °C. Growth occurs at initial pH values between 5 and 11, the optimum being pH 7.0. The cell-wall chemotype is I. The major menaquinones are MK-9(H₄), MK-9(H₆) and MK-9(H₈). The phospholipid profile comprises phosphatidylethanolamine and phosphatidylinositol. Major fatty acids are C_{15:0}, C_{16:0}, C_{16:1} and C_{18:0}.

The type strain, NEAU-W2^T (=CGMCC 4.7004^T =ATCC BAA-2424^T =DSM 42073^T), was isolated from the root surface of soybean. The G+C content of the DNA of the type strain is 66.12 mol%.

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