**Streptomyces avermectinius sp. nov., an avermectin-producing strain**

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We propose the establishment of a new species, *Streptomyces avermectinius*, based on characterization of strain MA-4680T and morphological and phylogenetic comparisons with closely related members of the genus *Streptomyces*. The 16S rDNA sequence was obtained from this strain and used to place it among *Streptomyces* species using the variable \( \alpha \) region and the nearly complete 16S rDNA sequence. Four *Streptomyces* species were selected as related species from phenotypic data, three species from phylogenetic databases on \( \alpha \) region sequences and two species from phylogenetic data using nearly complete 16S rDNA sequences. Analysis of DNA–DNA hybridization tests distinguished strain MA-4680T from these eight *Streptomyces* species. The type strain is strain MA-4680T (= ATCC 31267T = NRRL 8165T).

**Keywords:** *Streptomyces avermectinius*, avermectins, 16S rDNA sequencing, polyphasic taxonomy

**INTRODUCTION**

In this paper, we present morphological, physiological, cellular biochemical and phylogenetic evidence for the establishment of species *Streptomyces avermectinius* (formerly ‘*Streptomyces avermitilis*’). Taxonomic characteristics of the strain were reported by Burg et al. (1979), but its scientific name had not been validated at the time of writing. Strain ‘*S. avermitilis*’ MA-4680T was isolated from soil and produces a polyketide antibiotic, avermectin, which is widely used as an anthelmintic and insecticidal agent. Avermectin is also an important anthelmintic in veterinary science and for controlling onchocerciasis in humans. The biosynthetic gene cluster responsible for avermectin production has been described in detail (Ikeda et al., 1999) and further genetic characterization of the strain has been carried out by this group (Omura et al., 2001).

Conventional taxonomy based on morphological characteristics and physiological properties, as proposed by Shirling & Gottlieb (1966), and numerical taxonomy based on the 139 phenotypic characteristics proposed by Williams et al. (1983) are widely known, but species classifications obtained using these two methods are not necessarily consistent. Although more than 500 species of the genus *Streptomyces* are validly described at present, a standard method for identifying *Streptomyces* species has not yet been developed. Recent progress in molecular biology has dramatically increased the amount of sequence data useful for species classification. In particular, sequences of 16S rDNA have been successfully used to determine phylogenetic relationships in bacteria (Woese, 1987). However, the database of complete 16S rDNA sequences is insufficient for phylogenetic analysis within the genus *Streptomyces*. Kataoka et al. (1997) reported that the variable \( \alpha \) region (120 bp, nucleotide positions 158–277) of the 16S rDNA gene is useful for the identification of *Streptomyces* species.

Since the *Streptomyces* species whose complete 16S rDNA sequences data are known is limited, it is not currently possible to make a phylogenetic tree of the strain MA-4680T with all species. We used phylogenetic analysis of the \( \alpha \) region and available data of the nearly complete 16S rDNA sequences in addition to comparison of phenotypic characteristics to select species closely related to strain MA-4680T. Phenotypic and genotypic data show that this strain should be formally recognized as a new species of the genus *Streptomyces*, for which the name *Streptomyces avermectinius* sp. nov. is proposed.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain MA-4680T is AB078897.
**METHODS**

**Micro-organisms.** Strain MA-4680\(^T\) (= ATCC 31267\(^T\) = NRRL 8165\(^\)\(T\)) was isolated in 1978 from soil samples collected at Kawana, Ito City, Shizuoka Prefecture, Japan. The strain was stored at 5 °C under lyophilization and cultured on inorganic salts-starch agar (ISP medium 4; Nihon Pharmaceutical).

**Morphology.** The morphological characteristics of the strain were observed using a JEOL JSM-5600 scanning electron microscope after incubation on glycerol-asparagine agar (ISP medium 5) for 14 days at 27 °C. Samples were prepared by fixing the agar block in osmium tetroxide vapour for 12 h.

**Physiology.** Decomposition of adenine, casein, cellulose, hypoxanthine and xanthine were tested following the methods of Gordon et al. (1974). Tolerance to 5% (w/v) sodium chloride, streptomycin (20 µg ml\(^{-1}\), Sigma) and novobiocin (20 µg ml\(^{-1}\), Sigma) were examined on yeast extract-glucose agar (1.5% agar, 1% yeast extract, 1% d-glucose, pH 7.2).

**Cellular biochemistry.** Isomers of diaminopimelic acid in whole-organism hydrolysates (Becker et al., 1965) were identified by thin-layer chromatography (TLC) (Hasegawa et al., 1983). The N-acyl muramic acid concentrations were determined using the colorimetric method of Uchida & Aida (1977). Menaquinones were extracted and purified according to Collins et al. (1977) and then analysed by HPLC using a JASCO 802-SC chromatograph equipped with a Shiseido CAPCELL PAK C18 column (Tamaoka et al., 1983). Mycolic acid composition was determined by TLC (Tomiyasu, 1982). Cellular fatty acids were transmethyllated with methanolic HCl and analysed on a Shimadzu GC-14A GLC equipped with a fused-silica capillary column coated with methyl silicon (1:22 mm by 25 m; Shimadzu) (Suzuki & Komagata, 1983). The G+C content (mol%) of DNA, isolated according to the method of Marmur (1961), was determined by HPLC (Tamaoka & Komagata, 1984).

**Phylogeny.** DNA was isolated following the method of Marmur (1961). The 16S rDNA was amplified using primers described by Tajima et al. (2001). Amplifications were carried out in a TakaRa thermal cycler with an initial incubation of 1 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by a 2-min final extension at 72 °C. The PCR products were purified using a QIAquick Gel Extraction kit (QIAGEN) and were sequenced directly on a 377A automatic DNA sequencer (Applied Biosystems) using PRISM Ready Reaction Dye Primer cycle sequencing kits (Applied Biosystems).

The variable region (positions 158–277) of 16S rDNA from 363 known streptomycete species obtained from the DDBJ database and strain MA-4680\(^T\) were aligned. The nearly complete 16S rDNA sequence was manually aligned with corresponding sequences of representative Streptomyces species retrieved from DDBJ by using the BLAST homology search (Altschul et al., 1997). CLUSTALW (Thompson et al., 1994) was used to estimate evolutionary distances (the K\(_{\text{sp}}\) value of Kimura, 1980) and similarity values used to construct the phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) using 1000 resamplings. The phylogenetic tree was drawn using TREEVIEW software.

**DNA–DNA hybridization.** DNA–DNA hybridization experiments using strain MA-4680\(^T\) comparative strains *Strepto- myces bottropensis* ISP 5262\(^T\) (= IFO 13023\(^T\)), *Streptomyces cinambarinus* ISP 5467\(^T\) (= IFO 13028\(^T\)), *Streptomyces galbus* ISP 5089\(^T\) (= IFO 12864\(^T\)), *Streptomyces griseochromogenes* ISP 5499 (= IFO 13413\(^T\)), *Streptomyces luteo-griseus* ISP 5483\(^T\) (= IFO 13402\(^T\)), *Streptomyces mirabilis* ISP 5553 \(^T\) (= IFO 13450\(^T\)), *Streptomyces olivochromogenes* ISP 5451\(^T\) (= IFO 13067\(^T\)) and *Streptomyces phaeochromogenes* ISP 5073\(^T\) (= IFO 12898\(^T\)) were carried out according to the methods of Ezaki et al. (1989).

**RESULTS AND DISCUSSION**

**Morphology and physiology**

The spore chains were spiral and contained more than 20 spores per chain. The spores were oval in shape and were 0.8 × 1.2 µm in size with a smooth surface (Fig. 1). No whirs, sclerotic granules, sporangia or flagellate spores were observed. Burg et al. (1979) reported the

![Fig. 1. Scanning electron micrographs showing the spore-chain morphology of strain MA-4680\(^T\) grown on glycerol-asparagine agar (ISP medium 5) for 14 days at 27 °C. Bar, 2 µm.](image)

**Table 1. Fatty acid composition of strain MA-4680\(^T\)**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Percentage of total fatty acids</th>
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<tbody>
<tr>
<td>iso-14:0</td>
<td>2.8</td>
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<tr>
<td>14:0</td>
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<tr>
<td>iso-15:0</td>
<td>6.9</td>
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<tr>
<td>anteiso-15:0</td>
<td>13.6</td>
</tr>
<tr>
<td>15:0</td>
<td>0.9</td>
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<tr>
<td>16:1</td>
<td>16.4</td>
</tr>
<tr>
<td>iso-16:0</td>
<td>26.0</td>
</tr>
<tr>
<td>16:0</td>
<td>4.3</td>
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<tr>
<td>17:1</td>
<td>14.7</td>
</tr>
<tr>
<td>iso-17:0</td>
<td>3.4</td>
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<tr>
<td>anteiso-17:0</td>
<td>8.3</td>
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<td>17:0</td>
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<tr>
<td>18:1</td>
<td>2.1</td>
</tr>
<tr>
<td>18:0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Abbreviations for fatty acids are as follows: anteiso-15:0, 12-methyltetradecanoic acid; 16:1, hexadecenoic acid; iso-16:0, 14-methylpentadecanoic acid; 17:1, heptadecenoic acid.
Streptomyces avermectinius sp. nov.

Fig. 2. Neighbour-joining phylogenetic tree of 363 Streptomyces species and strain MA-4680T based on the variable region (158–277) of 16S rDNA sequences with Escherichia coli as outgroup. Bootstrap values greater than 50% are indicated at the nodes (1000 replications). One quarter of the entire phylogenetic tree including related sections (boxed) to strain MA-4680T are shown in this figure. The strains used in species appeared in this square are type strains except S. flubrisinus JCM 4238. Bar, 0.1 substitutions per nucleotide position.
following morphological and physiological characteristics: brown vegetative mycelia, grey aerial mass colour, formation of soluble pigment on various agar media, melanin formation, \( \text{H}_2\text{S} \) production, hydrolysis of starch, liquefaction of gelatin and peptonization of milk. We determined the following physiological characteristics in this study: decomposition of adenine, casein, hypoxanthin and xanthin, all positive; decomposition of cellulose, negative; tolerance to 5% NaCl, positive; and tolerance to streptomycin (20 \( \mu \text{gml}^{-1} \)) and novobiocin (20 \( \mu \text{gml}^{-1} \)), both negative.

**Cellular biochemistry**

Strain MA-4680\(^T\) contained L-\( \text{l}-\)diaminopimelic acid and \( \text{N}-\)acetylmyramic acid in the peptidoglycan, and MK-9 (\( \text{H}_\text{c} \)) and MK-9 (\( \text{H}_\text{a} \)) as the predominant menaquinones. No mycolic acid was detected. The predominant cellular fatty acids were 14-methylpentadecanoic acid (i-16:0), hexadecenoic acid (16:1), heptadecenoic acid (17:1) and 12-methyltetradecanoic acid (anteiso-15:0) (Table 1). The \( \text{G} + \text{C} \) content of the DNA was 70.3 mol%.

It is evident from the taxonomic and morphological characteristics that strain MA-4680\(^T\) belongs to the genus *Streptomyces* Waksman and Henrici 1943. Species *S. bottropensis*, *S. galbus*, *S. luteogriseus* and *S. olivochromogenes* were selected in a literature search as being closely related to strain MA-4680\(^T\) based on the phenotypic criteria of possessing grey aerial mass colour, spiral spore chains, smooth spore surface, and forming melanoid and other soluble pigments. One species, *Streptomyces scabiei*, described by Lambert & Loria (1989), is similar to the strain MA-4680\(^T\) in aerial mass colour, spore chain morphology, spore
Table 2. Phenotypic characteristics of strain MA-4680T and related species

<table>
<thead>
<tr>
<th>Character</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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</thead>
<tbody>
<tr>
<td>Morphology</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Spore chain</td>
<td>Spiral</td>
<td>Smooth</td>
<td>Spiral</td>
<td>Smooth</td>
<td>Flexuous or hooked</td>
<td>Smooth</td>
<td>Spiral or hooked</td>
<td>Smooth</td>
<td>Spore or hooked</td>
</tr>
<tr>
<td>Aerial mass colour</td>
<td>Grey</td>
<td>Grey</td>
<td>Red</td>
<td>+</td>
<td>Grey</td>
<td>+</td>
<td>Grey</td>
<td>+</td>
<td>Red</td>
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<tr>
<td>Melanin formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Formation of soluble pigment</td>
<td>Yellow</td>
<td>Brown</td>
<td>Red</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
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<td>Growth on sole carbon sources</td>
<td></td>
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<td></td>
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<tr>
<td>L-Arabinose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Raffinose</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>Rhhamnose</td>
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<td>Sucrose</td>
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<td>+</td>
<td>- or W</td>
<td>- or W</td>
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<td>D-Xylose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Is not/may not be produced on tyrosine agar.

Phylogeny

In a phylogenetic tree of strain MA-4680T and 36 Streptomyces species using the variable z region of 16S rDNA (Fig. 2), strain MA-4680T clustered with S. mirabilis, S. olivochromogenes and S. phaeochromogenes. We selected these three species as related species. S. olivochromogenes was also selected as a related species based on phenotypic characteristics.

The phylogenetic tree using nearly complete 16S rDNA sequences of strain MA-4680T with sequences of representative strains of the genus Streptomyces placed strain MA-4680T in a clade with S. cinnabarinus and S. griseochromogenes (Fig. 3). In base pair similarity analysis, S. cinnabarinus and S. griseochromogenes had 23 and 17 nucleotide differences, respectively, out of 1430 sites of the 16S rDNA sequences giving similarity values of 98.4 and 98.8%, respectively. Strain MA-4680T can be readily distinguished from S. scabiei (similarity value of 96.7%; 48 nucleotide differences in 1468 sites).

Phenotypic characteristics of related species

Among the four species (S. bottropensis, S. galbus, S. luteoegriseus and S. olivochromogenes) selected in a literature search on the basis of phenotypic criteria, S. luteoegriseus has the same characteristics as strain MA-4680T (Table 2). S. olivochromogenes, which was selected on both phenotypic criteria and z region phylogeny, has similar carbon utilization. S. mirabilis, selected based on the z region of 16S rDNA sequences, is similar in morphology, aerial mass colour and melanin formation, but differs on formation of soluble pigments and carbon utilization. S. phaeochromogenes and S. cinnabarinus selected according to phylogeny of z region and complete sequences of 16S rDNA, respectively, are clearly distinguished from strain MA-4680T by the morphology and aerial mass colour. S. griseochromogenes selected by phylogeny of 16S rDNA, is similar in morphological and physiological characteristics except for not utilizing rhamnose or forming a soluble pigment.

DNA–DNA hybridization

DNA–DNA hybridization tests were performed between strain MA-4680T and the type strains of eight related species. Strain MA-4680T exhibited levels of DNA–DNA hybridization of 15, 21, 33, 7, 22, 16, 26 and 20% to S. bottropensis, S. cinnabarinus, S. galbus, S. griseochromogenes, S. luteoegriseus, S. mirabilis, S. olivochromogenes and S. phaeochromogenes, respectively. Although S. olivochromogenes was similar in phenotypic and phylogenetic data, it had a low DNA relatedness value (26%). It is clear from the DNA–DNA relatedness study that strain MA-4680T and the eight other species belong to separate species (Wayne et al., 1987).

Strain MA-4680T is clearly differentiated from all of the other validly described species of genus Streptomyces based on a combination of phenotypic characteristics and genotypic data. We therefore propose the name Streptomyces avermectinii sp. nov. based on the morphological, biochemical and phylogenetic characteristics of strain MA-4680T (= ATCC 31267T = NRRL 8165T).

Description of Streptomyces avermectinii sp. nov.

Streptomyces avermectinii (a.ver.mec.ti.ii.us. N.L. adj. avermectinii pertaining to avermectin, an antibiotic produced by the organism).
Aerobic actinomycete isolated from soil. Vegetative mycelia are brown. Aerial mass colour is grey. Forms spiral spore chains. Spores are oval in shape (0.8 × 1.2 μm) and have a smooth surface. Soluble pigment is produced. Arabinose, fructose, glucose, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, sucrose and xylene are decomposed. Adenine, casein, hypoxanthine and xanthine are decomposed but cellulose is not decomposed. Melanin and H₂S production, hydrolysis of starch and casein, liquefaction of gelatin and peptonization of milk are positive. Grows in the presence of 5% (w/v) NaCl, but is sensitive to streptomycin (20 μg ml⁻¹) and novobiocin (20 μg ml⁻¹). Cell wall contains l,l-diaminopimelic acid. Phosphatidylethanolamine appears in the polar lipid fraction. MK-9 (H₄) and MK-9 (H₄) are predominant menaquinones. Major cellular fatty acids are 14-methylpentadecanoic acid (16:1ω0), hexadecenoic acid (16:1ω1), 12-methyltetradecanoic acid (anteiso-15:0) and heptadecenoic acids (17:1ω1). The G+C content of DNA is 70.3 mol%. The type strain is MA-4680T (ATCC 31267T = NRRL 8165T).

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