***Streptomyces thermocoprophilus* sp. nov., a cellulase-free endo-xylanase-producing streptomycete**

Bongcheol Kim,¹ Amira M. Al-Tai,^{1,2} Seung Bum Kim,¹
Paramaswari Somasundaram² and Michael Goodfellow¹

Author for correspondence: Michael Goodfellow. Tel: +44 191 222 7706. Fax: +44 191 222 5228.
e-mail: m.goodfellow@ncl.ac.uk

¹ Department of
Agricultural and
Environmental Science,
University of Newcastle,
Newcastle upon Tyne
NE1 7RU, UK

² Institute of Postgraduate
Studies and Research,
University of Malaya,
50603, Kuala Lumpur,
Malaysia

The taxonomic position of a thermophilic actinomycete strain isolated from poultry faeces was examined using a polyphasic approach. The isolate, designated B19^T, was assigned to the genus *Streptomyces* on the basis of chemotaxonomic and morphological criteria. An almost complete 16S rRNA gene (rDNA) sequence obtained for the test strain was compared with those of representative streptomycetes, notably thermophilic streptomycetes. 16S rDNA sequence data not only supported the assignment of the strain to the genus *Streptomyces* but also showed that the isolate formed a distinct phyletic line within the evolutionary branch composed of *Streptomyces thermodiastaticus* and related species. The strain was distinguished from related validly described *Streptomyces* species by a number of phenotypic properties. It is, therefore, proposed that strain B19^T be classified in the genus *Streptomyces* as *Streptomyces thermocoprophilus* sp. nov.

Keywords: *Streptomyces thermocoprophilus* sp. nov., thermophilic streptomycetes, polyphasic taxonomy, xylanase production

INTRODUCTION

Most thermophilic streptomycetes grow well at 50 °C and over the temperature range 25–55 °C. Representatives of these organisms have been distinguished from their better known mesophilic counterparts in numerical phenetic (Goodfellow *et al.*, 1987; O'Donnell *et al.*, 1993) and 16S rRNA sequencing studies (D. Kim *et al.*, 1996; S. B. Kim *et al.*, 1998; B. Kim *et al.*, 1999). It is apparent from the sequencing studies that thermophilic streptomycetes form at least two evolutionary clades and hence cannot be considered as a distinct taxon within the genus *Streptomyces* as proposed by Craveri & Pagani (1962). It is also clear that novel streptomycete species should be circumscribed using a judicious combination of genotypic and phenotypic data (Labeda *et al.*, 1997; S. B. Kim *et al.*, 1998; Miyajima *et al.*, 1998; B. Kim *et al.*, 1999).

Streptomycetes are a rich source of bioactive compounds, notably antibiotics, enzymes, enzyme in-

hibitors and pharmacologically active agents (Bérdy, 1995). In a search for xylanase producing bacteria, a streptomycete-like organism, strain B19^T, was isolated from poultry faeces and shown to produce a cellulase-free endo-xylanase. In the present investigation, this organism was the subject of a polyphasic taxonomic study which showed that it formed a new centre of taxonomic variation within the genus *Streptomyces*.

METHODS

Organism and cultural conditions. Strain B19^T was isolated on a yeast extract starch agar plate (Somasundaram, 1995), supplemented with nystatin (20 µg ml⁻¹), which had been inoculated with a suspension of poultry faeces and incubated at 45 °C for 2–3 d; the faeces sample was obtained from the poultry farm at the University of Malaya. The isolate was maintained on inorganic salt-starch agar (ISP medium 4, Difco; Shirling & Gottlieb, 1966) at room temperature and as spore and hyphal suspensions in glycerol (20%, v/v) at –20 °C. Biomass for the chemotaxonomic and molecular systematic analyses was prepared as described by B. Kim *et al.* (1999). The phenotypic tests were carried out at 45 °C for 7 d.

Morphology and pigmentation. Aerial spore mass colour, substrate mycelial pigmentation and the production of soluble pigments were detected on standard media (Table 1)

Abbreviation: A₂pm, diaminopimelic acid.

The EMBL accession number for the 16S rRNA sequence of strain B19^T is AJ007402.

following incubation at 45 °C for 7 d. Spore chain morphology and spore surface ornamentation were examined by scanning electron microscopy, as described previously (O'Donnell *et al.*, 1993).

Phenotypic tests. Strain B19^T was examined for a set of phenotypic properties using procedures described in a previous study (Al-Tai *et al.*, 1999). Xylanase production was detected using Czapek Dox agar (Oxoid) supplemented with remazol brilliant blue R-xylan (Sigma) after incubation for 7 d following the procedure of Biely *et al.* (1985).

Chemotaxonomy. The isomeric form of diaminopimelic acid (A₂pm) was determined by TLC of whole-organism hydrolysates after Stanek & Roberts (1974). Standard procedures were also used for the extraction and analysis of menaquinones and polar lipids (Minnikin *et al.*, 1984), as described by Chun *et al.* (1997a). The presence of mycolic acids was sought using the method described by Minnikin *et al.* (1980).

Sequencing of 16S rDNA. Isolation of chromosomal DNA and PCR amplification of 16S rDNA were carried out after Chun & Goodfellow (1995). The amplified fragment was purified by gel electrophoresis and directly sequenced by using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and previously described primers (Chun & Goodfellow, 1995). Sequencing gel electrophoresis was carried out and the nucleotide sequences automatically obtained by using an Applied Biosystems DNA sequencer (model 373A) and software provided by the manufacturer.

Analysis of sequence data. The 16S rDNA sequences were aligned manually with available streptomycete nucleotide sequences retrieved from the Ribosomal Database Project (Maidak *et al.*, 1997) and the EMBL/GenBank databases by using the AL16S program (Chun, 1995). Evolutionary trees were inferred by using three treeing algorithms, namely, the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the least-squares and maximum-likelihood methods were prepared as described by Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used with all three algorithms. The resultant tree topologies were evaluated by carrying out bootstrap analyses (Felsenstein, 1985) of the neighbour-joining data based on 1000 re-samplings using the SEQBOOT and CONSENSE programs in the PHYLIP package (Felsenstein, 1993). The root position of the tree based on the least-squares method was estimated using the 16S rRNA sequence of *Arthrobacter globiformis* (DSM 20214^T) as outgroup.

DNA base composition. The base composition of the genomic DNA of the test strain was determined as described by S. B. Kim *et al.* (1998).

RESULTS AND DISCUSSION

The chemical and morphological properties of strain B19^T are consistent with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995). The organism forms an highly branched substrate mycelium, aerial hyphae which carry smooth surfaced spores (1.1 ~ 1.7 × 0.5 µm) in straight chains, contains LL-A₂pm in the peptidoglycan, lacks characteristic sugars and mycolic acids, has tetra-, hexa- and octahydrogenated (the most abundant) menaquinones

with nine isoprene units, contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides as typical polar lipids, and has a DNA base composition of 68.6 mol % G + C. The assignment of the strain to the genus *Streptomyces* is also supported by 16S rDNA sequence data.

Comparison of the almost complete 16S rDNA sequence (1492 nucleotides) of the test strain with corresponding streptomycete sequences showed that strain B19^T lies at the periphery of the evolutionary clade occupied by *Streptomyces thermodiastaticus* and allied taxa in the trees generated using the least-squares, maximum-likelihood and neighbour-joining algorithms (Fig. 1). The mean 16S rDNA similarity value found between the test strain and members of the *S. thermodiastaticus* clade was 97.9%. Strain B19^T shares particularly high percentage nucleotide sequence similarities with *Streptomyces thermoviolaceus* subsp. *apingens* (98.6%) and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* (98.2%); these values correspond to 21 and 27 nucleotide differences out of 1475 nucleotide positions. Nucleotide similarities within this range have been reported for several validly described species belonging to the *S. thermodiastaticus* clade, for instance, between *Streptomyces thermocarboxydovorans* and *Streptomyces thermoviolaceus* (98.8%; S. B. Kim *et al.*, 1998). Members of these taxa form distinct genomic species and can be separated using a set of phenotypic properties (Goodfellow *et al.*, 1987; B. Kim *et al.*, 1999).

Strain B19^T forms a brown substrate mycelium, a grey aerial spore mass and brown diffusible pigments on several standard media (Table 1). It also produces melanin pigments on peptone iron and tyrosine agars. The organism can be distinguished from members of the *S. thermodiastaticus* clade using a combination of phenotypic properties (Table 2). This latter observation is in line with recent studies which show that members of streptomycete species found to be closely related on the basis of genotypic data can also be distinguished using phenotypic properties, notably morphological and pigmentation features (Labeda & Lyons, 1991; Chun *et al.*, 1997b; Labeda *et al.*, 1997; S. B. Kim *et al.*, 1998).

It is apparent from the genotypic and phenotypic data that strain B19^T forms a distinct centre of taxonomic variation within the genus *Streptomyces*. It is proposed, therefore, that this organism be recognized as a new species of the genus *Streptomyces*, namely, *Streptomyces thermocoprophilus* sp. nov.

Description of *Streptomyces thermocoprophilus* sp. nov.

Streptomyces thermocoprophilus (ther.mo.co.pro.phi'lus. Gr. n. *therme* heat; Gr. n. *copro* dung; Gr. adj. *philus* loving; M.L. adj. *thermocoprophilus* dung-loving thermophile).

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rDNA sequences of 44 streptomycetes. Asterisks indicate branches of the tree that were also formed using the maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) methods. Numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values more than 70% are given. Arrow indicates the estimated position of the root of the tree. Scale bar indicates 0.01 nucleotide substitutions per nucleotide position. T, type strain.

Table 1. Growth and cultural characteristics of strain B19^T

Agar medium	Growth	Aerial hyphae	Substrate mycelium	Soluble pigment
Modified Bennett's	Moderate	None	Pale brown	None
Czapek Dox	Abundant	None	Brown	Brown
Glycerol-asparagine (ISP medium 5)	Abundant	Grey	Light yellow	None
Inorganic salts-starch (ISP medium 4)	Abundant	Grey	Light yellow	None
Oatmeal (ISP medium 3)	Abundant	Grey	Not distinctive	None
Peptone-yeast extract-iron (ISP medium 6)	Abundant	None	Pale brown	Brown
Starch-casein	Abundant	Grey	Not distinctive	None
Yeast extract-malt extract (ISP medium 2)	Abundant	Grey	Brown	Brown

Aerobic, Gram-positive, moderately thermophilic actinomycete which forms a highly branched substrate mycelium and aerial hyphae which differentiate into

long chains of straight spores which are cylindrical and have smooth surfaces (1.1 ~ 1.7 × 0.5 µm). The aerial spore mass colour is grey. Diffusible pigments are

Table 2. Phenotypic properties separating the test strain from related *Streptomyces* species

Strains: 1, *Streptomyces* strain B19^T; 2, *S. thermoalcalitolerans* DSM 41741^T; 3, *S. thermocarboxydovorans* DSM 44296^T; 4, *S. thermocarboxydus* DSM 44293^T; 5, *S. thermodiastaticus* DSM 40573^T; 6, *S. thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T; 7, *S. thermoviolaceus* subsp. *apingens* DSM 41392^T; 8, *S. thermovulgaris*. Abbreviations: RA, *retinaculiaperti*; RF, *rectiflexibiles*; SP, *spirales*. Symbols: +, positive or more than 90% of strains positive in the case of the *S. thermocarboxydovorans*, *S. thermogriseus* and *S. thermovulgaris* strains; –, negative or more than 90% of strains negative in the case of the *S. thermocarboxydovorans*, *S. thermogriseus* and *S. thermovulgaris* strains. ND, Not determined; v, variable. All strains had grey aerial spore mass; results were ‘+’ for starch degradation and growth at 25 and 50 °C; results were ‘–’ for growth at 10, 15 and 60 °C.

Property	Strain:							
	1	2	3*	4	5*	6*	7*	8
Pigmentation of substrate mycelium on oatmeal agar	Not distinctive	Not distinctive	Not distinctive	Not distinctive	Yellowish brown	Yellow/purple†	Yellow	Not distinctive
Spore chain	RF	SP	RF	RA	SP	SP	SP	SP
Spore surface	Smooth	Warty	Smooth	Warty	Tuberculate	Tuberculate	Tuberculate	Smooth
Melanin production	+	–	–	–	–	–	–	–
Nitrate reduction	ND	+	+	+	–	–	–	+
Degradation of:								
Adenine	–	–	+	ND	+	+	+	v
Casein	+	+	ND	ND	+	+	ND	+
Elastin	–	–	+	+	+	+	+	+
Gelatin	+	+	–	+	+	+	+	+
Hypoxanthine	+	–	+	+	–	–	+	–
Testosterone	–	+	+	+	+	–	–	v
L-Tyrosine	+	+	+	–	+	+	+	+
Xanthine	+	–	+	+	–	–	–	–
Xylan	+	+	+	–	–	–	–	–
Growth on sole carbon sources:								
L-Arabinose	+	+	–	ND	–	–	+	–
meso-Inositol	+	+	–	+	+	+	+	+
Mannitol	+	+	–	+	+	+	+	+
Raffinose	–	–	–	–	+	–	–	–
Sucrose	–	+	–	–	–	–	+	+
Growth at:								
20 °C	+	–	+	+	–	+	+	–
55 °C	–	+	+	+	+	+	+	+

* Data taken from previous studies (Shirling & Gottlieb, 1969, 1972; Williams *et al.*, 1983; Goodfellow *et al.*, 1987; S. B. Kim *et al.*, 1998; B. Kim *et al.*, 1999; Xu *et al.*, 1998).

† Substrate mycelium is initially yellow but becomes purple after 5 d due to the formation of a diffusible purple pigment.

formed on some standard media such as inorganic salts starch agar. Melanin pigments are produced on peptone iron and tyrosine agars. Casein, starch, xanthine and xylan are degraded but not adenine. L-Arabinose, D-fructose, D-galactose, D-glucose, *meso*-inositol, maltose, D-mannitol, D-mannose and D-xylose are used as sole carbon sources for energy and growth but not carboxymethylcellulose, D-raffinose, starch or sucrose. Growth occurs between 20 and 50 °C and in the presence of ampicillin (10 µg ml^{–1}), erythromycin (15 µg ml^{–1}) and sodium chloride (7%, w/v) but not at 10 or 55 °C. Growth is inhibited by chloramphenicol (30 µg ml^{–1}), gentamicin sulphate (15 µg ml^{–1}), kanamycin sulphate (30 µg ml^{–1}), neomycin sulphate (30 µg ml^{–1}), streptomycin sulphate (10 µg ml^{–1}) and tetracycline hydrochloride (30 µg ml^{–1}). Antimicrobial activity is shown against *Bacillus subtilis* NCIB 3610 but not towards *Escherichia coli* NCIB 9132 or *Staphylococcus aureus* ATCC 12600, or against representative strains of *Candida albicans*, *Curvularia lunata*, *Pestalotiopsis gnepini*, *Pyricularia oryzae* and *Trichoderma viride*. The G+C content of the genomic DNA is

68.6 mol%. The organism was isolated from a sample of poultry faeces collected from the poultry farm at the University of Malaya. The type strain is B19^T (= DSM 41700^T).

It is also evident from the phylogenetic tree that *S. thermogriseus* Y-14046^T and *S. thermovulgaris* DSM 40444^T are closely related. These strains show a similarity of 99.5%, which corresponds to seven differences out of 1445 nucleotides. However, all of the differences occur in the conserved regions of the 16S rDNA. Further comparative studies are needed to determine the relationship between these two strains.

ACKNOWLEDGEMENTS

B.K. was supported by an Overseas Research Student Award and S.B.K. by a Chevening–Most Scholarship. A.A.T. gratefully acknowledges receipt of a Postdoctoral Grant from the Ministry of Science and Technology, Malaysia and is indebted to Professor Dato Osman Baker (University of Malaya) for supporting her visit to the University of Newcastle. The work was also supported by BBSRC grant

R185/05688/01. The authors are also grateful to Professor V. G. Kumar Das (University of Malaya) for the gift of fungal cultures and to Professor D. E. Minnikin for help with the polar lipid analysis.

REFERENCES

- Al-Tai, A. M., Kim, B., Kim, S. B., Manfio, G. P. & Goodfellow, M. (1999). *Streptomyces malaysiensis* sp. nov., a new streptomycete species with rugose ornamented spores. *Int J Syst Bacteriol* **49**, 1395–1402.
- Bérdy, J. (1995). Are actinomycetes exhausted as a source of secondary metabolites? *Biotechnologia* **7–8**, 13–34.
- Biely, P., Mislovicova, D. & Toman, R. (1985). Soluble chromogenic substrates for assay of endo-1,4-xylanases and endo-1,4-glucanases. *Anal Biochem* **144**, 142–146.
- Chun, J. (1995). *Computer-assisted classification and identification of actinomycetes*. PhD thesis, University of Newcastle upon Tyne.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int J Syst Bacteriol* **45**, 240–245.
- Chun, J., Blackall, L., Kang, S.-O., Hah, Y. C. & Goodfellow, M. (1997a). A proposal to reclassify *Nocardia pinensis* Blackall et al. as *Skermania piniformis* gen. nov., comb. nov. *Int J Syst Bacteriol* **47**, 127–131.
- Chun, J., Youn, H.-D., Yim, Y.-I., Lee, H., Kim, M. Y., Hah, Y. C. & Kang, S.-O. (1997b). *Streptomyces seoulensis* sp. nov. *Int J Syst Bacteriol* **47**, 492–498.
- Craveri, R. & Pagani, H. (1962). Thermophilic micro-organisms among actinomycetes in the soil. *Ann Microbiol* **12**, 115–130.
- Felsenstein, J. (1981). Evolutionary tree from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogeny: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package), version 3.5c. Seattle: Department of Genetics, University of Washington, USA.
- Fitch, W. M. & Margoliash, E. (1967). Construction of phylogenetic trees: a method based on mutation distances as estimated from cytochrome *c* sequences is of general applicability. *Science* **155**, 279–284.
- Goodfellow, M., Lacey, J. & Todd, C. (1987). Numerical classification of thermophilic streptomycetes. *J Gen Microbiol* **133**, 3135–3149.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kim, B., Sahin, N., Minnikin, D. E., Zakrzewska-Czerwinska, J., Mordarski, M. & Goodfellow, M. (1999). Classification of thermophilic streptomycetes including the description of *Streptomyces thermoalcalitolerans*. *Int J Syst Bacteriol* **49**, 7–17.
- Kim, D., Chun, J., Sahin, N., Hah, Y.-C. & Goodfellow, M. (1996). Analysis of thermophilic clades within the genus *Streptomyces* by 16S ribosomal DNA sequence comparisons. *Int J Syst Bacteriol* **46**, 581–587.
- Kim, S. B., Falconer, C., Williams, E. & Goodfellow, M. (1998). *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydophilic species from soil. *Int J Syst Bacteriol* **48**, 59–68.
- Labeda, D. P. & Lyons, A. J. (1991). Deoxyribonucleic acid relatedness among species of the *Streptomyces cyaneus* cluster. *Syst Appl Microbiol* **14**, 158–164.
- Labeda, D. P., Lechevalier, M. P. & Testa, R. T. (1997). *Streptomyces stramineus* sp. nov. a new species of the verticillate streptomycetes. *Int J Syst Bacteriol* **47**, 747–753.
- Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1997). The Ribosomal Database Project (RDP). *Nucleic Acids Res* **5**, 109–111.
- Manfio, G. P., Zakrzewska-Czerwinska, J., Atalan, E. & Goodfellow, M. (1995). Towards minimal standards for the description of *Streptomyces* species. *Biotechnologia* **7–8**, 242–253.
- Minnikin, D. E., Hutchinson, I. G., Caldicott, A. B. & Goodfellow, M. (1980). Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. *J Chromatogr* **188**, 221–233.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athayle, M., Schaal, A. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Miyajima, K., Tanaka, F., Takeuchi, T. & Kuninaga, S. (1998). *Streptomyces turgidiscabies* sp. nov. *Int J Syst Bacteriol* **48**, 495–502.
- O'Donnell, A. G., Falconer, C., Goodfellow, M., Ward, A. C. & Williams, E. (1993). Biosystematics and diversity amongst novel carboxydophilic actinomycetes. *Antonie Leeuwenhoek* **64**, 325–340.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Shirling, E. B. & Gottlieb, D. (1969). Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. *Int J Syst Bacteriol* **19**, 391–512.
- Shirling, E. B. & Gottlieb, D. (1972). Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. *Int J Syst Bacteriol* **22**, 265–394.
- Somasundaram, P. (1995). *Studies on xylanase from Streptomyces noboritoensis and Myceliophthora thermophila*. PhD thesis, University of Malaya.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* **129**, 1743–1813.
- Williams, S. T., Goodfellow, M. & Alderson, G. (1989). Genus *Streptomyces* Waksman and Henrici, 1943, 339^{AL}. In *Bergey's Manual of Systematic Bacteriology*, volume 4, pp. 2452–2492. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Xu, L.-H., Tiang, Y.-Q., Zhang, Y.-F., Zhou, L.-X. & Jiang, C.-L. (1998). *Streptomyces thermogriseus*, a new species of the genus *Streptomyces* from soil, lake and hot spring. *Int J Syst Bacteriol* **48**, 1089–1093.