Sequence-based differentiation of strains in the *Streptomyces cyaneus* species-group

Ehsan Rashidian[1] and Michael Goodfellow[2]

[1]*Department of Microbiology, School of Veterinary Medicine, Lorestan University, Khorramabad, I.R. Iran.*
[2]*School of Biology, University of Newcastle, Newcastle Upon Tyne, NE1 7RU, United Kingdom.*

Abstract
In an extensive numerical phenetic survey, a number of blue, red and gray spored streptomycete strains were grouped together as Streptomyces cyaneus species-group, a taxon which encompasses strains known to produce antitumor antibiotics, notably anthracyclines. In the present investigation these and related streptomycetes were the subject of morphological and 16S rRNA sequencing studies designed to clarify their taxonomic relationships. It is evident from these results that the Streptomyces cyaneus species-group encompasses misclassified strains and members of several distinct species. However, some of the red-spored strains (e.g. Streptomyces janthinus ISP 5206^T, Streptomyces roseoviolaceus ISP 5277^T and Streptomyces violatus ISP 5209^T) formed a distinct clade. In contrast, most of the blue and gray-spored strains showed much less sequence homology between and with one another and were scattered throughout the 16S rRNA Streptomyces tree. The improved classification of this group of streptomycetes provides an essential basis for establishing their industrial and economical significance.
Keywords: *16S rDNA sequencing; Streptomycetes; Streptomyces cyaneus.*

INTRODUCTION

Traditional streptomycete taxonomy was dependent on morphological characteristics such as aerial spore mass colouration, diffusible pigments and spore chain morphology. The subjective nature of such tests, the high natural morphological diversity and intense economic interest in these organisms led to a massive overspeciation of the genus (Trejo, 1970). The second largest phenon (cluster 18) delineated in the numerical phenetic survey of the genus *Streptomyces* carried out by Williams *et al.* (1983) and encompassed the type strains of 31 validly described species. This taxon was designated the *Streptomyces cyaneus* species-group as the earliest validly described species it contained was *Streptomyces cyaneus* (Krassilnikov, 1941) Waksman 1953. A taxon which encompasses strains known to produce antitumour antibiotics, notably anthracyclines (Brockmann and Bauer, 1950; Oki, 1977; Omura *et al.*, 1978; Yoshimoto *et al*., 1980; Oki *et al*., 1981; Kawai *et al*., 1987; Fedorova *et al*.,1996). Strains assigned to this species group still showed heterogeneity in respect to the coloures the aerial spore mass produced by them. The taxonomic integrity of cluster 18 was further brought into doubt by the results of fatty acid analysis (Saddler *et al*., 1987), total protein pattern (Manchester *et al*., 1990), DNA: DNA relatedness (Labeda and Lyons, 1991), numerical phenetic survey (Goodfellow *et al*., 1992) and the LFRFA (Beyazova and Lechevalier, 1993) studies. It is evident from the results of the studies outlined above that the *Streptomyces cyaneus* species-group recognized by Williams *et al.* (1983) encompasses a taxonomically diverse group of strains. The DNA: DNA relatedness and associated studies show that some of these strains can be considered as synonyms of *Streptomyces coeruleorubidus* and *Streptomyces purpurascens* (Labeda and Lyons, 1991). However, it is difficult in

Correspondence to: **Ehsan Rashidian, Ph.D**
Telefax: +98 661 4203470
E-mail: er117kh@yahoo.co.uk

light of the inconsistences in some of the other datasets to know which of the remaining strains might be sufficiently closely related to be included in any additional DNA: DNA pairing studies. It is evident that the current classification of streptomycetes can be critical in establishing taxonomic relationships between strains which produce commercially significant bioactive compounds, notably antibiotics. This aspect is exemplified in the present study which was designed to establish taxonomic relationships between streptomycetes belonging to the *Streptomyces cyaneus* species-group (Williams *et al.*, 1983) based on 16S rDNA sequencing data.

MATERIALS AND METHODS

Organisms and growth conditions: Thirty seven strains of Streptomysetes were obtained from different sources (Table 1). Working stock cultures were maintained on yeast extract-malt extract agar slants at 4°C. Biomass used for the extraction of DNA was grown on non-sporulating agar plates for 3-5 days at 28°C. Approximately 50 mg (one loopful or rise grain size of biomass) was needed for DNA extraction.

Colour grouping: The strains were examined for aerial spore mass colour, substrate mycelium colour and soluble pigment production following incubation on oatmeal agar at 28ºC for up to 21 days. Peptone-yeast extract- iron agar plates were used to detect the formation of melanin pigments following incubation at 28ºC for up to 4 days.

Morphology: The Spore chain morphology was observed by light and in some cases by scanning electron microscopy (SEM) of 21-day-old cultures grown at 28ºC on oatmeal agar plates.

Isolation of genomic DNA: Chromosomal DNA was extracted and purified using Genomic G2 Kits (Helena BioScience, Sunderland, UK) with some minor modifications in the recommended procedure; like lyses by lysozyme.

PCR amplification of 16S rDNA: The 16S rRNA genes of the test strains were amplified using conserved primers (Lane, 1991). PCR was performed for 30 cycles at 95°C for 45 seconds, 45°C for 45 seconds, and 72°C for 1.5 minutes.

16S rRNA sequencing: Approximately 10 µl of each of the purified PCR product alongwith 10 µl of each of the oligonucleotide primers specifically designed for hybridising to conserved sites in the 16S rRNA molecule (Lane, 1991) were sequenced using the dideoxy method. Sequencing reactions were run on an ABI Prism 377 automated sequencer.

Data analysis: The 16S rRNA sequences were aligned automatically or manually by using the CLUSTAL X (Thompson *et al*., 1997) and PHYDIT (Chun, 1995) programs against those of reference streptomycete nucleotide sequences retrieved from the RDP (Maidak *et al*., 2001) and EMBL-GenBank (Benson *et al*., 2000, Stoesser *et al*., 2001) databases. Phylogenetic trees were inferred by using the neighbour-joining (Saitou and Nei, 1987), maximum-parsimony, least-squares algorithms and generated by either the PHYLIP software package (Felsenstein, 1993) or the TREECON program (Van de Peer and De Wachter, 1994).

RESULTS

The tested strains were divided into four groups on the basis of the production of aerial spore mass pigments on oatmeal agar (Table 2). Nearly all of the organisms assigned to these groups can be distinguished from one another on the basis of diffusible pigment and substrate mycelial colour and by their ability to produce melanin pigments on peptone yeast iron agar. The tested strains showed a range of spore chain morphologies and spore surface ornamentations when grown on oatmeal agar plates at 28°C for 21 days. Many of the strains formed chains of spiral, spiny spores (Fig. 1).

Almost complete 16S rDNA sequences (>1,400 nucleotides) were obtained for all of the strains. The data was compared with 27 corresponding and almost complete nucleotide sequences of representatives of the genus *Streptomyces* retrieved from the RDP database. The accession numbers of all of the strains are given in figure 2. The strains can be assigned to four loose multimembered aggregate groups, clades A to D, based on the results of the neighbour-joining analysis. Generally the 16S rDNA sequencing data support the grouping of the red-spored strains viz, *S. violatus, S. roseoviolaceus, S. janthinus, S. violarus, S. fumanus* and *S. pallidus* in one group. On the other hand, the blue spored strains had much less 16S sequences homology with each other and are widely dispersed on

Table 1. Names and strain histories

ISP or code number	Name	Source and strain history
5228^T	*Streptomyces afghaniensis*	DSM 40212< ISP 5228< M. Shimo, 772; soil
5293^T	*Streptomyces arenae*	DSM 40293< ISP 5293< T. J. Oliver, Abbott Laboratories., NA 269-M2; soil
5106^T	*Streptomyces azureus*	DSM 40106
5185^T	*Streptomyces bellus*	S.T. Williams, A106
5084^T	*Streptomyces caelestis*	DSM 40084< ISP 5084< NRRL 2418< A. Dietz, Upjohn Co. UC 2011; soil
5085^T	*Streptomyces chartreusis*	H. J. Kutzner, DSM 40085
5467^T	*Streptomyces cinnabarinus*	DSM 40467< ISP 5467< G. F. Gauze, INA 1242; soil
5144^T	*Streptomyces coeruleofuscus*	DSM 40144< ISP 5144< T. P. Preobrazhenskaya, INA 2922/57; soil
5145^T	*Streptomyces coeruleorubidus*	DSM 40145< ISP 5145< T.P. Preobrazhenskaya, INA 12531/54; soil
5146^T	*Streptomyces coerulescens*	NCIMB 9616
5129^T	*Streptomyces collinus*	DSM 40129< ATCC 19743 < ISP 5129< P. Wilde, Ist 301; soil
5107^T	*Streptomyces curacoi*	DSM 40107
5108^T	*Streptomyces cyaneus*	S. T. Williams, A13
5013^T	*Streptomyces echinatus*	S. T. Williams, A77
5154^T	*Streptomyces fumanus*	A. Seino, KCC S-0497
5499^T	*Streptomyces griseochromogenes*	DSM 40499< ISP 5499< ATCC 14511< Fukunaga 2A-327; soil
5469^T	*Streptomyces griseorubiginosus*	DSM 40469< ISP 5469< G. F. Gauze, INA 7712; soil
5042^T	*Streptomyces hawaiiensis*	DSM 40042< ISP 5042< K. E. Crook, Bristol Laboratories, 678 506; soil
5206^T	*Streptomyces janthinus*	A. Seino, KCC S-0387
5090^T	*Streptomyces lanatus*	DSM 40090< ISP 5090< NRRL B-2291< W. Frommer, SV 1944; soil
5166^T	*Streptomyces longisporus*	DSM 40166< ISP 5166< T. P. Preobrazhenskaya, INA 4417/56; soil

Table 1. (continued)

ISP or code number	Name	Source and strain history
5482[T]	*Streptomyces iakyrus*	A. Seino, KCC S-0773
5483[T]	*Streptomyces luteogriseus*	A. Seino, KCC S-0796
5588[T]	*Streptomyces neyagawaensis*	DSM 40588< ISP 5588< IFO 13477, IFO 3784; soil
MAV[T]	*Streptomyces paradoxus*	DSM 43350< S. T. Williams< KCC A-0052< V. D. Kutznetsov, RIA 655< INMI 3180; soil
5212[T]	*Streptomyces pseudovenezuelae*	DSM 40212< ISP 5212< N. A. Krassilnikov, RIA 1158, RIA 742
5310[T]	*Streptomyces purpurascens*	DSM 40310< ISP 5310< P. Wilde, Maria 515; soil
5133	*Streptomyces resistomycificus*	DSM 40133< ISP 5133< P. Wilde, Pürk 262. [ETH 23893; ETH 32680]; soil
5277[T]	*Streptomyces roseoviolaceus*	A. Seino, KCC S-0513
5205[T]	*Streptomyces violarus*	DSM 40205< ISP 5205< N.A. Krassilnikov, INMI 1221. [NIHJ 493]; soil
5209[T]	*Streptomyces violatus*	A. Seino, KCC S-0851
5140	*Streptomyces sp.*	DSM 40140< ISP 5140 ('*Streptomyces bicolor*')< T.P. Preobrazhenskaya, INA 5140; soil
5207	*Streptomyces sp.*	S. Kurylowicz, ATCC 15893
5227	*Streptomyces sp.*	A. Seino; KCC S-0519
5432	*Streptomyces sp.*	DSM 40432< ISP 5432 ('*Streptomyces indigocolor*')< N.A. Krassilnikov, INMI 206
5531	*Streptomyces sp.*	A. Seino, KCC S-0810 ('*Streptomyces pallidus*')
5592	*Streptomyces sp.*	DSM 40592< ISP 5592 ('*Streptomyces peruviensis*')< NRRL 2775< Rhone-Poulenc, 6227

[T,] Type strain.

Abbreviations: ATCC, American Type Culture Collection, 1081 University Boulevard, Manassas, U.S.A.; **DSM,** Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124, Braunschweig, Germany; **ETH**, Eidgenössische Technische Hochschule, Institut für spezuekke Botanik, Zurich, Switzerland; **IFO**, Institute for Fermentation, 17-85 Juso-Honmachi, 2-chome, Yodogawa- ku, Osaka, Japan; **INA,** Culture Collection of the Institute of New Antibiotics, Academy of Medical Sciences; Moscow, Russia; **ISP**, International *Streptomyces* Project, Ohio Wesleyan University, Delaware, Ohio, 43015, U.S.A.; **KCC,** Culture Collection of Actinomycetes, Kaken Pharmaceutical Co. Ltd., Tokyo, Japan; **NCIMB**, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, U.K.; **NRRL**, Culture Collection of the Northern Research Laboratories, US Department of Agriculture, Peoria, Illinois, U.S.A.

Table 2. Colour grouping, spore chain morphology and spore ornamentation of strains assigned to the *Streptomyces cyaneus* species-group *sensu* Williams *et al.* (1983)

ISP or laboratory number	Name	Oatmeal agar		Peptone yeast iron agar	Oatmeal agar	
		Substrate mycelium colour	Diffusible pigment	Melanin production[a]	Spore chain morphology	Spore ornamentation[a]
A. Aerial spore mass colour-Blue						
5228^T	*S. afghaniensis*	Red	Red	+	Spiral[a]	Spiny[a]
5106^T	*S. azureus*	No distinctive colour	None	+	Spiral	Smooth[a]
5185^T	*S. bellus*	Yellowish white	None	+	Open loops	Spiny[a]
5140	*'S. bicolor'*	No distinctive colour	Yellow	+	Spiral[a]	Spiny[a]
5084^T	*S. caelestis*	No distinctive colour	None	+	Open loops	Smooth
5085^T	*S. chartreusis*	No distinctive colour	None	+	Spiral[a]	Spiny[a]
5144^T	*S. coeruleofuscus*	Light grayish brown	None	+	Spiral[a]	Spiny[a]
5145^T	*S. coeruleorubidus*	Cream/dark grayish blue	None	+	Spiral	Spiny[a]
5146^T	*S. coerulescens*	No distinctive colour	None	+	Spiral	Spiny[a]
5107^T	*S. curacoi*	No distinctive colour	None	+	Spiral	Spiny[a]
5090^T	*S. lanatus*	Orange yellow	Yellow	+	Spiral[a]	Spiny[a]
B. Aerial spore mass colour-Gray						
5293^T	*S. arenae*	No distinctive colour	None	+	Spiral[a]	Spiny[a]
5129	*S. collinus*	No distinctive colour	None	+	Spiral	Smooth[a]
5108^T	*S. cyaneus*	Dark grayish blue	Pale violet	+	Spiral/open loops	Spiny[a]
5013^T	*S. echinatus*	Brown	Yellow brown	+	Spiral	Spiny[a]
5499^T	*S. griseochromogenes*	Grayish greenish yellow	None	+	Spiral/ open loops	Spiny[a]
5469^T	*S. griseorubiginosus*	Light olive brown	Light grayish yellow brown	+	Straight	Smooth[a]
5482^T	*S. iakyrus*	Olive grey	Yellow	+	Spiral	Spiny[a]

Table 2. (continued)

ISP or laboratorynumber	Name	Oatmeal agar		Peptone yeast iron agar	Oatmeal agar	
		Substrate mycelium colour	Diffusible pigment	Melanin production[a]	Spore chain morphology	Spore ornamentation[a]
5432	*'S. indigocolor'*	Grayish red	Pale violet	+	Spiral[a]	Spiny[a]
5483^T	*S. luteogriseus*	Olive brown	Light brown	+	Spiral	Smooth[a]
5588^T	*S. neyagawaensis*	Dark olive brown	Light gray	+	Spiral[a]	Smooth[a]
MAV^T	*S. paradoxus*	Light brown	None	-	Spiral[a]	Spiny[a]
5592	*'S. peruviensis'*	No distinctive colour	None	+	Spiral	Spiny or Smooth[a]
5212^T	*S. pseudovenezuelae*	Light brown	None	+	Straight[a]	Smooth[a]
5133^T	*S. resistomycificus*	Dark reddish brown	None	+	Spiral/ open loops	Smooth[a]
5227	*'S. thermotolerans'*	Brown	Trace of yellow	+	Spiral	Spiny
C. Aerial spore mass colour-Red						
5467^T	*S. cinnabarinus*	Pink	Pink	+	Straight	Smooth
5154^T	*S. fumanus*	Grayish yellow/mild yellow	None	-	Spiral	Smooth[a]
5206^T	*S. janthinus*	Cream	None	+	Spiral	Spiny[a]
5531	*'S. pallidus'*	Light yellow	Trace of yellow	+	Open loops	Smooth[a]
5310^T	*S. purpurascens*	Dark yellowish pink	None	+	Spiral	Spiny[a]
5277^T	*S. roseviolaceus*	Mild red	Light pink	+	Spiral	Spiny
5205^T	*S. violarus*	Mild red	Light pink	+	Spiral	Spiny
5209^T	*S. violatus*	Mild red	Light pink	+	Spiral	Spiny[a]
5207	*'S. violochromogenes'*	Deep red	Grayish pink	+	Spiral	Smooth
D. Aerial spore mass colour -Yellow						
5042	*S. hawaiiensis*	Reddish brown	Light orange yellow	+	Open loops	Spiny[a]
5166	*S. longisporus*	Yellow	None	+	Spiral[a]	Spiny[a]

+, positive; -, negative.
[a] Spore chain morphology and spore ornamentation data taken from the ISP descriptions (Shirling & Gottlieb, 1968a, 1968b, 1969, 1972).

the tree. It is evident that *S. cyaneus*, the type strain of the cluster 18, had little 16S sequence homology in common with the other strains and was separated from the remaining strains in the cluster. Our study supports the suggestion that members of the species-group demonstrated a high degree of heterogeneity.

DISCUSSION

The 16S rDNA sequence data generated in the present investigation indicate that the *Streptomyces cyaneus* species-group *sensu* Williams *et al*. (1983) is markedly heterogeneous, a finding which underpins the results of earlier DNA: DNA pairing (Labeda and Lyons, 1991), fatty acid (Saddler *et al*., 1987) and total protein pattern (Manchester *et al*., 1990) studies. However, an important advantage of the 16S rDNA sequence data over the results of these earlier studies is that they provide a measure of the taxonomic diversity encompassed within the *Streptomyces cyaneus* species-group. In the present study, it was particularly interesting that the type strains of validly described species assigned to the taxon by Williams *et al*. (1983) were recovered in two out of the four 16S rDNA clades recognized in the streptomycete tree. It was also interesting that 19 out of the 31 type strains were assigned to 16S rDNA clade A together with 14 representatives of validly described *Streptomyces* species. It is evident from the 16S rDNA sequence data that streptomycetes which produce a blue aerial spore mass on oatmeal agar form a markedly heterogeneous group. These results although interesting are not surprising as it is now realised that streptomycete species need to be delineated not on the basis of a few subjectively chosen morphological and pigmentation properties but by using a combination of reliable genotypic and phenotypic data (Manfio *et al*., 1995; Atalan *et al*., 2000; Sembiring *et al*., 2000). The results of the 16S rDNA sequencing studies carried out in the present investigation have gone someway towards clarifying relationships between members of the *Streptomyces cyaneus* species-group and between them and representatives of validly described *Streptomyces* species included in the RDP database. The next step is to determine the taxonomic status of the 16S rDNA subclades by evaluating their composition in light of available genotypic and phenotypic data.

There is some evidence that only streptomycetes from certain phyletic lines can produce specific metabolites, as exemplified by the production of clavulanic acid from members of the *Streptomyces clavuligerus* subclade (Payne *et al.*, 2001). In the present study, anthracycline-like antibiotics were detected in eleven out of the thirty-seven members of the *Streptomyces cyaneus* species-group (Dr. E. Lacey,

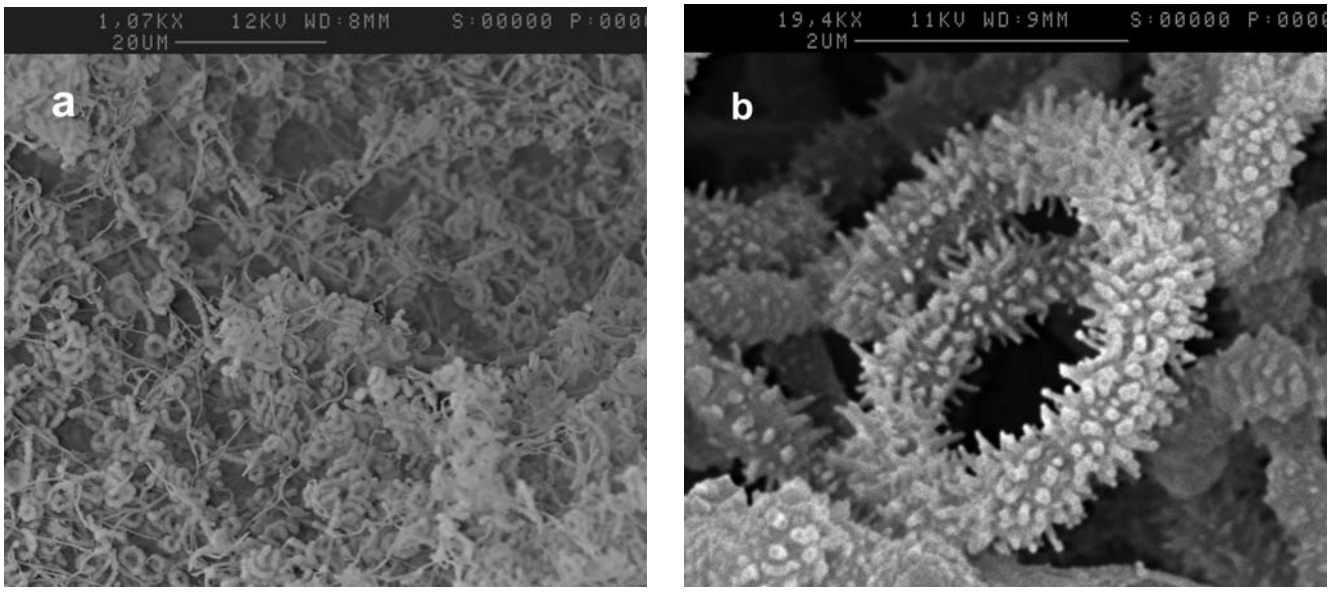

Figure 1. Scanning electron micrographs of "*Streptomyces thermotolerans*" ISP 5227 grown on oatmeal agar plates for 21 days at 28°C: (**a**) Spiral spore chains; (**b**) Spiny spores.

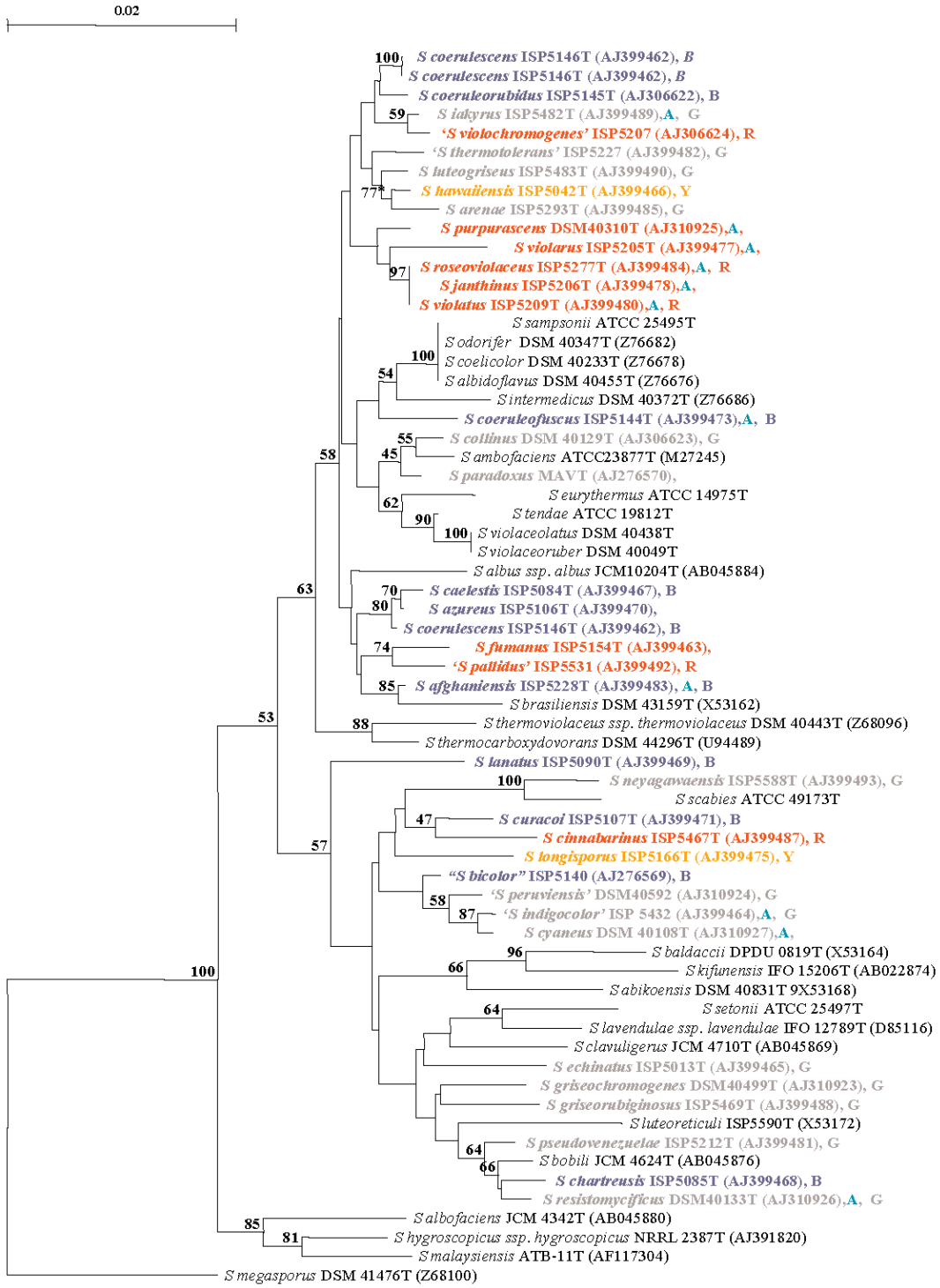

Figure 2. Neighbour-joining tree based on almost complete 16S rDNA sequences showing relationships between members of the *Streptomyces cyaneus* species-group and some representative strains of the genus *Streptomyces*. The numbers at the nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. The scale bar indicates 0.02 substitutions per nucleotide position.

Abbreviation: Letter **A** corresponds to the strains that produce anthracycline compounds (unpublished data provided by Dr. E. Lacey); B=blue, G= gray, R=red and Y=yellow refer to aerial spore mass colours.

unpublished data). However, seven out of the eleven strains were assigned to the *Streptomyces cyaneus* and *Streptomyces purpurascens* subclades, a result which suggest that it may be possible to predict streptomycete metabolite potential from good quality taxonomic data. Additional comparative studies are needed to test this hypothesis.

In general, the results of the present study are very encouraging, especially when taken together with earlier findings, as they indicate that relationships between taxonomically complex groups, such as the *Streptomyces cyaneus* species-group *sensu* (Williams *et al*., 1983) can be resolved using appropriate combinations of genotypic and phenotypic data.

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