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ORIGINAL PAPER

Molecular and phenotypic comparison of phaeochromycin-producing strains of *Streptomyces phaeochromogenes* and *Streptomyces ederensis*

Frank V. Ritacco · Douglas E. Eveleigh

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Abstract *Streptomyces* strain LL-P018 produces the phaeochromycins, novel anti-inflammatory polyketides. This organism was identified as a strain of *Streptomyces phaeochromogenes* by physiological and genetic taxonomic analysis. In order to gain greater taxonomic perspective, LL-P018 was compared to related strains from major culture collections by 16S rRNA gene sequence, ribotype, HPLC–MS metabolite profile, and *rpoB* sequence. Using BioNumerics software, genetic and chemical fingerprint data were integrated via multivariate cluster analysis into a single, robust comparison. Based upon this analysis, strain LL-P018 is very closely related to the type strains of both *S. phaeochromogenes* and *Streptomyces ederensis*, indicating that these two types may in fact represent a single species. This novel comparative multi-cluster analysis is most useful for clarifying relationships between closely related species.

Keywords *Streptomyces* · Phaeochromycins · Ribotyping · Metabolite profiling

Introduction

Streptomycetes are abundant soil bacteria capable of producing a vast array of complex and biologically active secondary metabolites. They have been studied extensively over the past 50 years and are the source of a large and diverse range of medically important and highly valuable pharmaceutical products including antibacterial, antifungal, antiparasitic, anticancer and immunosuppressant drugs [4, 5, 8–10, 13, 14, 19, 20, 23, 27, 28, 30, 37–40].

Streptomyces strain LL-P018 was isolated from a soil sample taken from the bank of the Aa river in Westerenger, Germany. This strain produces the phaeochromycins (Fig. 1), novel polyketide inhibitors of MAPKAP-2 (MK-2) [12]. MK-2 is a kinase involved in the regulation of tumor necrosis factor (TNF-α) biosynthesis, and is a promising target in the search for new anti-inflammatory drugs [17]. The phaeochromycins are analogous to intermediates and shunt products of other type II polyketide biosynthetic pathways, differing primarily in the presence of a characteristic propyl side chain, possibly derived from a butyrate starter unit [12].

Strain LL-P018 was taxonomically identified using a combination of traditional and molecular biological techniques. While 16S rRNA gene sequencing and classical taxonomic methods (such as morphological observation, carbohydrate utilization, sporulation and pigment production) are often sufficient for species identification, more sensitive methods are needed to detect strain variation within a species [2, 18, 25]. In order to determine the uniqueness of this strain with regard to its production of novel secondary metabolites, genetic and chemical fingerprinting methods were used to compare LL-P018 to 15 closely related strains from major culture collections. Genetic fingerprinting of all strains was performed using the RiboPrinter®, an automated ribotyping instrument capable of discriminating between strains of *Streptomyces* [22, 25]. For comparison of chemical fingerprints, all strains were grown in three liquid media under identical conditions. Culture extracts were analyzed by HPLC–MS. BioNumerics® software (Applied Maths, Austin, TX, USA) was used to integrate RiboPrint® patterns and multiple

F. V. Ritacco (✉)
Wyeth Research, Pearl River, NY, USA
e-mail: ritaccf@wyeth.com

D. E. Eveleigh
School of Environmental and Biological Sciences,
Rutgers University, New Brunswick, NJ, USA

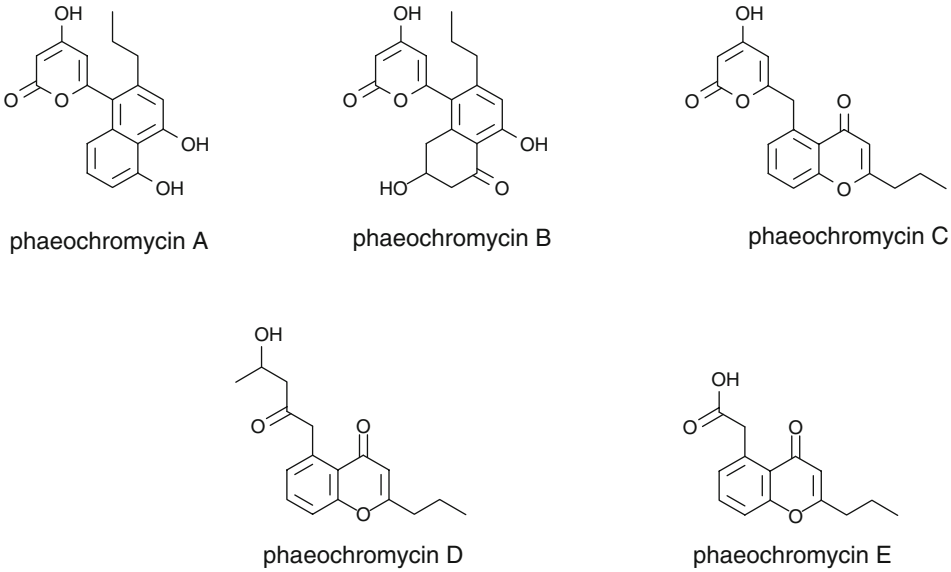

Fig. 1 Polyketide structures of the phaeochromycins [12]

HPLC chromatograms in a single cluster analysis, allowing the simultaneous comparison of molecular typing data and fermentation metabolite profiles. This novel approach to classification is most useful in clarifying relationships between closely related strains and species.

Materials and methods

Strains

Streptomyces phaeochromogenes strain LL-P018 has been previously documented [12] and was obtained from the Wyeth Research Natural Products Culture Collection, Pearl River, NY, USA. Comparative strains were obtained from the Agricultural Research Service (ARS) Culture Collection, Peoria, IL and the American Type Culture Collection (ATCC), Manassas, VA (Table 1).

Three *S. phaeochromogenes* type strains, NRRL B-1248^T, ATCC 3338^T and ATCC 23945^T, were used for comparison in this study. *S. phaeochromogenes* was first described as *Actinomyces phaeochromogenus* by H. J. Conn in 1917, but the oldest existing representative of this type is ATCC 3338^T, which was deposited by Selman Waksman in 1963 (ATCC technical services, personal communication). NRRL B-1248^T and ATCC 23945^T are derived from ATCC 3338^T (Dr. David Labeda, Curator, NRRL-ARS actinobacterial culture collection, personal communication; ATCC technical services, personal communication).

16S rRNA gene sequencing

Complete (>1,400 bp) 16S rRNA genes were amplified by polymerase chain reaction (PCR) using standard primers [24]. PCR amplification was carried out in 100 µL reaction volumes containing approximately 5–10 ng of genomic DNA, 1 mM each of the eight FPL forward and 1,492 RPL reverse primers and 50 µL of Jumpstart PCR mix (Sigma, St Louis, MO, USA). The PCR was performed using a BioMetra T Gradient thermocycler (Whatman-BioMetra, Goettingen, Germany) as follows: one cycle of denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 55°C for 45 s and 72°C for 90 s, with one extension cycle at 72°C for 5 min and a pause at 4°C. The amplified PCR product was purified using the DNA Clean and Concentrator-5 kit (Zymo Research, CA, USA) and directly sequenced using an ABI 3700 sequencer with the ABI Prism DNA sequencing kit and Big Dye terminators version 3.0 (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on the PCR reaction using the 16S forward and reverse PCR primers described above, and the sequence was compared to the GenBank database by nucleotide BLAST search [1]. Sequences were aligned using ClustalX [31]. The phylogenetic tree was based on neighbor-joining analysis of 16S rRNA gene sequences with 1,000 bootstrap replicates using TREECON software [15, 33]. The 16S rRNA gene sequence of the evolutionarily distant species *Actinoplanes utahensis* IFO-13244^T was chosen as an outgroup and used to root the phylogenetic tree.

Carbohydrate utilization, pigmentation, and sporulation

Carbohydrate utilization, pigment production, and sporulation were observed according to the methods defined by the International *Streptomyces* Project [29]. For analysis of carbohydrate utilization, agar media containing appropriate carbohydrate sources (4 mL) was added to the wells of a

Table 1 *Streptomyces phaeochromogenes* and closely related strains

Culture number	Presumptive identification	Source
LL-P018	*Streptomyces* strain LL-P018	Wyeth Research, Natural Products Culture Collection, Pearl River, NY
ATCC 3338^T	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	American Type Culture Collection, Manassas, VA
ATCC 23945^T	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	American Type Culture Collection, Manassas, VA
NRRL B–1248^T	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-1131	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-1517	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-2031	*Streptomyces phaeochromogenes* subspecies *chloromyceticus*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-2123	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-3559	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-5333	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-5478	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL.
NRRL B-16392	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
ISP-5170	*Streptomyces phaeochromogenes* subspecies *phaeochromogenes*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
WC-3776	*Streptomyces phaeochromogenus* subspecies *chloromyceticus*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-8146^T	*Streptomyces ederensis*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL
NRRL B-12497^T	*Streptomyces tauricus*	Agricultural Research Service (ARS) Culture Collection, USDA, Peoria, IL

(^{T}) = type strain

12-well Falcon culture plate (part number 35-3043, Becton-Dickinson, Franklin Lakes, NJ, USA) and inoculated with 25 µL of cells prepared and washed as described [29]. For observation of sporulation and pigment production, 50 µL washed cells were inoculated to ISP sporulation agars and grown according to defined methods [29]. Growth, sporulation, and pigment production were observed after 7, 14, and 21 days.

Ribotyping

Ribotype analysis was performed using the RiboPrinter Microbial Characterization System (DuPont Qualicon, Wilmington, DE) as previously described [25]. Two 10 µL loopfuls containing 2–5 colonies were scraped from agar plates overlaid with polycarbonate membranes. Colonies were ground with a sterile, motorized pestle in 200 µL of RiboPrinter sample buffer (DuPont Qualicon, Wilmington, DE). Forty microliters of each ground cell suspension was heat-treated in the RiboPrinter heat-treatment station for approximately 20 min. Lysis buffers (DuPont Qualicon, Wilmington, DE) were then added to each sample and the samples were loaded into the RiboPrinter. Disposable reagents, including *Pvu*II, DNA preparative enzymes and reagents, probe, conjugate and substrate solutions, agarose gel and membrane were loaded into the RiboPrinter, according to the manufacturer's instructions (Qualicon) and the methods of Bruce [7]. Genomic DNA obtained from cell lysates was digested with *Pvu*II, and chromosomal DNA fragments were hybridized with the ribosomal probe provided by the manufacturer (DuPont Qualicon, Wilmington, DE).

Culture conditions

All cultures were grown in three liquid media. Liquid medium 1 contained (grams per liter): $MgSO_4 \cdot 7H_2O$ (0.5), KCl (0.5), K_2HPO_4 (2.5), NaCl (5), agar (0.4), glycerol (10), soy peptone (5). Liquid medium 2 contained (grams per liter): D-mannitol (10), Bacto-peptone (Difco/Becton-Dickinson, Franklin Lakes, NJ, USA) (1), soluble starch (Difco/Becton-Dickinson, Franklin Lakes, NJ) (15), $MgSO_4 \cdot 7H_2O$ (0.1), KH_2PO_4 (0.5), and $CaCO_3$ (1). Liquid medium 3 contained (grams per liter): D-glucose (1), D-mannitol (10), Bacto-peptone (Difco/Becton-Dickinson, Franklin Lakes, NJ) (1), soluble starch (Difco/Becton-Dickinson, Franklin Lakes, NJ) (15), $MgSO_4 \cdot 7H_2O$ (0.1), KH_2PO_4 (0.5), and $CaCO_3$ (1). Cultures were grown at 28°C and 200 rpm for 6 days. One milliliter (1 mL) of whole broth was extracted in 1 mL ethyl acetate (1:1) and centrifuged to separate phases. The organic phase was dried to completion and the product was resolubilized in 100 µL methanol, resulting in a tenfold final concentration. Reverse phase HPLC of extracts was performed using an Agilent model 1100 liquid chromatograph with photodiode array detection, coupled to an Agilent LC–MSD ion trap mass spectrometer fitted with an electrospray ionization source. Extracts were resolved by reverse phase chromatography using a Zorbax Eclipse XDB-C18 column (3.0 × 75 mm, 3.5 µm particle size) with a mobile phase of 0.025% formic acid in water (solvent A) and 0.025% formic acid in acetonitrile (solvent B). For elution, a linear gradient from 5% A to 95% B in 15 min was used, holding at 95% acetonitrile for 10 min, with a flow rate of 0.8 mL/min. A split was used to divert 25% of the flow into the mass spectrometer. UV detection was performed using a photodiode array with a scan range of 200–600 nm. The MS electrospray was performed in positive mode with a scan range of 200–2,000 m/z.

Cluster analysis of ribotype data and HPLC chromatograms

Cluster analysis of RiboPrints and HPLC–MS chromatograms was performed using BioNumerics software (Applied Maths, Austin, TX, USA). RiboPrint patterns were exported from the RiboPrinter database as text files and uploaded into a BioNumerics database using a custom import script (BNScripts60) designed by software engineers at Applied Maths (Austin, TX). HPLC chromatograms were exported from Agilent ChemStation software as comma-delimited Excel files (Microsoft) and uploaded into the BioNumerics database using another custom import script (import_curves_background). Using this script, each exported chromatogram file was uploaded into BioNumerics along with a "blank" chromatogram to be used for background subtraction. During import into BioNumerics, an HPLC chromatogram derived from an uninoculated media blank was subtracted from each sample chromatogram to eliminate unwanted and non-specific medium components from the resulting comparisons. RiboPrint patterns and HPLC chromatograms were analyzed as densitometric curves and compared using the Pearson product–moment correlation [21, 34]. Hierarchical cluster analysis of Riboprints and HPLC chromatograms was performed using the unweighted pair group method using arithmetic averages (UPGMA). For composite data sets comprised of data from multiple experiments, RiboPrint patterns and metabolite profiles were equally weighted, and the resemblance matrices of the individual data sets were averaged [34].

rpoB sequencing

Ribosomal polymerase β-subunit (*rpoB*) genes (354 bp) were amplified by polymerase chain reaction (PCR) using defined primers [16]. PCR amplification was carried out in

100 µL reaction volumes containing approximately 5–10 ng of genomic DNA, 1 mM each of the SRPOF1 forward and SRPOR1 reverse primers [16] and 50 µL of Jumpstart RedTaq PCR mix (Sigma, St Louis, MO, USA). The PCR was performed under conditions recommended by Dr. David Labeda (USDA, Peoria, IL, personal communication) using a BioMetra T Gradient thermocycler (Whatman-BioMetra, Goettingen, Germany) as follows: one cycle of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 45 s, with one extension cycle at 72°C for 5 min and a pause at 4°C. The amplified PCR product was purified using the DNA Clean and Concentrator-5 kit (Zymo Research, CA, USA) and sequenced using an ABI 3700 sequencer with the ABI Prism DNA sequencing kit and Big Dye terminators version 3.0 (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on the PCR reaction using the forward and reverse PCR primers described above, and the sequence was compared to the GenBank database by nucleotide BLAST search [1]. Sequences were aligned using ClustalX [31]. The phylogenetic tree was based on neighbor-joining analysis of *rpoB* sequences with 1,000 bootstrap replicates using TREECON software [15, 33].

Results

16S rRNA gene sequencing

A phylogenetic tree depicting comparison of all strains by 16S rRNA gene sequence showed four clusters that were at least 99% similar by 16S rRNA sequence (Fig. 2). The largest of these, Cluster 1, contained seven strains including *Streptomyces* strain LL-P018. The 16S rRNA gene of strain LL-P018 was 99.5% similar to that of the type strains of *S. phaeochromogenes* included in this study (Fig. 2). Two other strains of *S. phaeochromogenes* (NRRL B-5478 and ISP-5170) and *S. ederensis* NRRL B-8146^T were also clustered with this highly similar group. Of the strains in Cluster 1, five (*S. phaeochromogenes* ATCC 23945^T, *S. phaeochromogenes* NRRL B-5478, strain LL-P018, *S. phaeochromogenes* ISP-5170, and *S. ederensis* NRRL B-8146^T) exhibited heterogeneity in the hypervariable α-region of the 16S rRNA gene (Fig. 3), indicating slight sequence differences between multiple copies of the gene within each organism [32].

Streptomyces tauricus NRRL B-12497^T was a divergent offshoot from Cluster 1, sharing 98.3% sequence similarity with the *S. phaeochromogenes* type strains (Fig. 2). The remaining eight strains were highly divergent from the *S. phaeochromogenes* cluster. Comparison of these sequences to the GenBank database suggested that these strains are more closely related to other species of *Streptomyces* than *S. phaeochromogenes* (Fig. 2).

Cluster 2 contained strains NRRL B-5333 and NRRL B-3559, whose 16S rRNA gene sequences were 99.8% similar and most closely matched that of a *S. viridobrunneus* type strain in GenBank.

Strains NRRL WC-3776 and NRRL B-2031 comprised Cluster 3. These strains are 99.9% similar by 16S rRNA gene sequence and most closely related to *S. viridobrunneus*.

The fourth cluster contained strains NRRL B-2123 and NRRL B-1517, which shared 99.1% sequence identity and most closely resembled the *S. melanogenes* type strain.

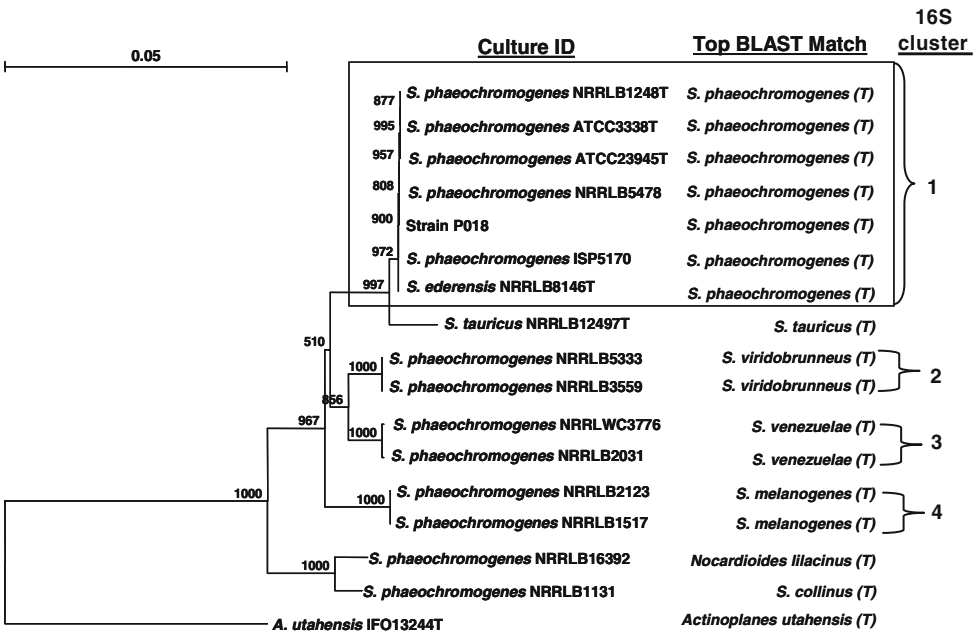

Fig. 2 16S rRNA gene sequence comparison of *S. phaeochromogenes* and closely related strains. Complete (>1,400 bp) 16S ribosomal RNA (16S rRNA) gene sequences were aligned using ClustalX [31]. The tree was constructed with TREECON software using the neighbor-joining method with 1,000 bootstrap replicates [15, 33]. Strains sharing greater than 99% sequence similarity were assigned a 16S rRNA gene cluster number for reference purposes. (^{T}) = type strain

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                                        165                 185
NRRL B-5478      CGGGGTCTAA TACCGGATAA CACTYSYMSA GGCATCTSKG KGGGTTGAA
Strain LL-P018   CGGGGTCTAA TACCGGATAA YACTYKYMSA GGCATCTSKG KGGGTTGAA
ISP-5170         CGGGGTCTAA TACCGGATAA CACTCSYSSM SGCATSKSKG KGGGTTGAA
NRRLB-8146T      CGGGGTCTAA TACCGGATAA CACTCSYSSM SGCATSKSKG KGGGTTGAA
ATCC3338T        CGGGGTCTAA TACCGGATAA CACTCGCCGA GGCATCTCGG TGGGTTGAA
NRRLB1248T       CGGGGTCTAA TACCGGATAA CACTCGCCGA GGCATCTCGG TGGGTTGAA
ATCC23945T       CGGGGTCTAA TACCGGATAA CACTCGCCSA GGCATCTCGG KGGGTTGAA
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Fig. 3 Sequence heterogeneity within the 16S rRNA genes of *S. phaeochromogenes* and closely related strains. Sequences were aligned using ClustalX [31]. Numbers shown refer to the corresponding nucleotide position in the LL-P018 16S rRNA gene sequence. Sequence heterogeneity codes: Y= C or T; K= G or T; S= C or G; M= A or C

Three strains, *S. tauricus* NRRL B-12497^T, *S. phaeochromogenes* NRRL B-16392, and *S. phaeochromogenes* NRRL B-1131 did not share greater than 99% sequence identity with any other strain and were not assigned to a cluster.

GenBank accession numbers for all sequences generated in this study are listed in Table 2.

Carbohydrate utilization

Carbohydrate utilization patterns confirmed identification of strain LL-P018 as a strain of *S. phaeochromogenes* (Table 3). All carbon sources tested (except cellulose) were utilized readily by strain LL-P018, *S. phaeochromogenes* ATCC 3338^T, *S. ederensis* NRRL B-8146^T, and *S. tauricus* NRRL B-12497^T. Slight growth on cellulose (microcrystalline, Sigma, St Louis, MO, USA) was observed only for strain LL-P018 and *S. ederensis* NRRL B-8146 ^{T}.

Sporulation and pigment production

Strain LL-P018 was highly similar to ATCC 3338^T, supporting placement of strain LL-P018 within the species *S. phaeochromogenes* (Table 4). A strong similarity between the *S. ederensis* type strain and the *S. phaeochromogenes* type strain was also observed.

Ribotyping

Three type strains of *S. phaeochromogenes* (NRRL B-1248^T, ATCC 3338^T and ATCC 23945^T) were greater than 95% similar to one another by ribotype, confirming that these are identical strains (Fig. 4). Strain LL-P018 grouped closely with these strains, forming a distinct branch 92% similar to the type strain cluster. This variation is clearly due to the presence of an approximately 11 kb band in the ribotype fingerprint of strain LL-P018 which is not seen in those of the type strains, indicating genomic divergence and

Table 2 GenBank accession numbers for all sequences used in this study

Strain		GenBank accession numbers	
		16S rRNA Gene sequence (complete)	*rpoB* Gene sequence (partial)
Streptomyces phaeochromogenes	ATCC 23945T	EU594468	EU621822
Streptomyces phaeochromogenes	ATCC 3338T	EU594469	EU621823
Streptomyces phaeochromogenes	ISP 5170	EU594470	EU621825
Streptomyces phaeochromogenes	NRRL B-1131	EU594471	Not sequenced
Streptomyces phaeochromogenes	NRRL B-1248T	EU594472	EU621820
Streptomyces tauricus	NRRL B-12497T	EU594473	EU621821
Streptomyces phaeochromogenes	NRRL B-1517	EU594474	Not sequenced
Streptomyces phaeochromogenes	NRRL B-16392	EU594475	Not sequenced
Streptomyces phaeochromogenes	NRRL B-2031	EU594476	Not sequenced
Streptomyces phaeochromogenes	NRRL B-2123	EU594477	Not sequenced
Streptomyces phaeochromogenes	NRRL B-3559	EU594478	Not sequenced
Streptomyces phaeochromogenes	NRRL B-5333	EU594479	Not sequenced
Streptomyces phaeochromogenes	NRRL B-5478	EU594480	EU621819
Streptomyces ederensis	NRRL B-8146T	EU594481	EU621824
Streptomyces phaeochromogenes	WC-3776	EU594482	Not sequenced
Streptomyces phaeochromogenes	LL-P018	EU594483	EU621826

Table 3 Carbohydrate utilization by *S. phaeochromogenes* and closely related species

Carbohydrate utilization				
Carbon source	*Streptomyces* strain LL-P018	*Streptomyces phaeochromogenes* ATCC 3338^T	*Streptomyces ederensis* NRRL B-8146^T	*Streptomyces tauricus* NRRL B-12497^T
Glucose	+	+	+	+
Arabinose	+	+	+	+
Sucrose	+	+	+	+
Xylose	+	+	+	+
Inositol	+	+	+	+
Mannitol	+	+	+	+
Fructose	+	+	+	+
Rhamnose	+	+	+	+
Raffinose	+	+	+	+
Cellulose	±	−	±	−
No carbon	−	−	−	−

Utilization of various carbohydrates was compared according to methods defined by the International *Streptomyces* Project [29]. "+"Good growth, "±" weak growth, "−" no growth. (^{T}) type strain

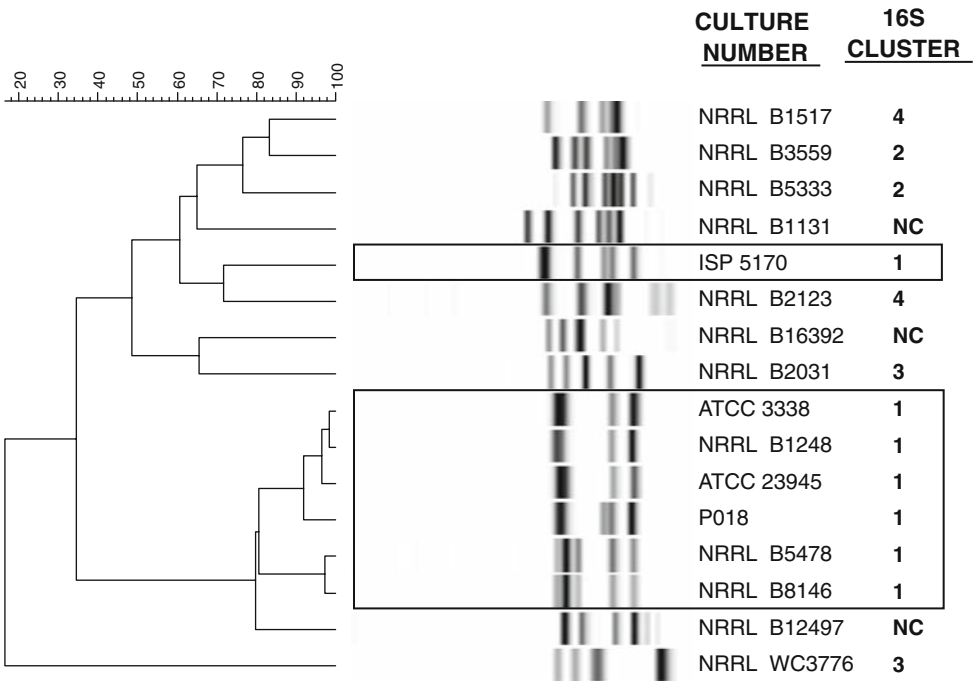

Fig. 4 Ribotype comparison of *S. phaeochromogenes* and closely related strains. Ribotyping was performed using the RiboPrinter Microbial Characterization System (Dupont Qualicon, Wilmington, DE) and cluster analysis of Riboprints was performed using BioNumerics software (Applied Maths, Austin, TX). Boxes highlight the strains corresponding to 16S rRNA gene cluster 1 (Fig. 2). *NC* not clustered

therefore strain variation. The *S. ederensis* type strain, NRRL B-8146^T, was 97.4% similar to *S. phaeochromogenes* strain NRRL B-5478 by ribotype. These strains both clustered with strain LL-P018 and the *S. phaeochromogenes* type strains by 16S rRNA gene sequence comparison (Fig. 2, "Cluster 1"), but were only 80% similar to these strains by ribotype and therefore formed a distinct ribotype cluster (Fig. 4). *S. phaeochromogenes* ISP-5170, which also grouped in 16S rRNA gene cluster 1 (Fig. 2), was very divergent from the group by ribotype, sharing only 21–34% similarity to other strains in this group (Fig. 4).

The remaining eight strains, which were not closely related to *S. phaeochromogenes* or *S. ederensis* by 16S rRNA gene sequence or ribotype (Figs. 2, 4), exhibited ribotype strain variation within their respective 16S rRNA gene clusters. Strains NRRL B-5333 and NRRL B-3559 (Fig. 2, "Cluster 2"), which were 99.8% identical in 16S rRNA gene sequence, shared only 75.9% similarity by ribotype (Fig. 4). Strains NRRL WC-3776 and NRRL B-2031 (Fig. 2, "Cluster 3") were 99.9% similar in 16S rRNA gene sequence but only 13.9% similar by ribotype (Fig. 4). Strains NRRL B-2123 and NRRL B-1517 (Fig. 2, "Cluster 4") were only 79.4% similar by ribotype (Fig. 4), despite 99.1% similarity in 16S rRNA gene sequence (Fig. 2). Based on 16S rRNA similarity values, these strain pairs clearly represent species-level clusters. However, the

divergence in ribotype patterns just described suggests subspecies or strain-level variation within these clusters. These apparent strain variations were investigated further by comparison of metabolite profiles, and will be discussed in detail in this paper.

Metabolite profiling

Strain LL-P018 and the *S. phaeochromogenes* type strains were all highly similar by metabolite profile (Fig. 5). In liquid media 1 and 2, these strains produced only the phaeochromycins (Fig. 6). In liquid medium 3, however, these strains produced only the related polyketide alnumycin. Metabolite profiles for these strains correlated well with ribotype comparisons and confirmed a high degree of similarity between Strain LL-P018 and *S. phaeochromogenes.* For example, in a composite analysis combining metabolite profiles from all three media, these four strains formed a cluster sharing 83–97% similarity to one another (Fig. 5).

The *S. ederensis* type strain, NRRL B-8146^T was very similar to *S. phaeochromogenes* NRRL B-5478 (88.3%) and *S. phaeochromogenes* ISP-5170 (92.2%) by composite metabolite profile (Fig. 5). These three strains grouped closely with strain LL-P018 and the *S. phaeochromogenes* type strains in 16S rRNA gene sequence (Fig. 2, "Cluster 1") but showed distinct differences in metabolite profile (Fig. 5). All of the strains in 16S rRNA gene cluster 1 (Fig. 2) produced the phaeochromycins in liquid medium 1 (Fig. 6) and alnumycin in liquid medium 3. However, differences in metabolite production among members of this group were observed in liquid medium 2. In this medium, NRRL B-8146^T, NRRL B-5478 and ISP-5170 produced alnumycin. In contrast, strain LL-P018 and the *S. phaeochromogenes* type strains produced the phaeochromycins (Fig. 5). As a result, strains NRRL B-8146^T, NRRL B-5478 and ISP-5170 formed a separate cluster in the composite HPLC comparison, only 64.6% similar in metabolite profile to the cluster containing strain LL-P018 and the *S. phaeochromogenes* type strains (Fig. 5).

Strains NRRL B-5333 and NRRL B-3559 (Fig. 2, "Cluster 2") were only 52% similar by composite metabolite profile (Fig. 5). This result supports the strain variation suggested by ribotype comparison (76% similarity) (Fig. 4). Very low metabolite similarity (6.9%) between strains NRRL WC-3776 and NRRL B-2031 (Fig. 2, "Cluster 3", and Fig. 5) was in agreement with the low ribotype similarity between these strains (13.9%), but stood in stark comparison to the 99% similarity in their 16S rRNA gene sequences (Fig. 2). Strains NRRL B-2123 and NRRL B-1517 (Fig. 2, "Cluster 4") were 69.6% similar by metabolite profile (Fig. 5), sharing several major metabolite peaks but showing differences in the presence and intensity of many analytes as well.

Combined ribotype and metabolite profile comparison

Integration of genetic and chemical fingerprint data into a single comparison yielded a robust analysis combining elements of each method (Fig. 7). For this analysis, one ribotype pattern and three HPLC metabolite profiles were combined with equal weighting. The seven strains comprising 16S rRNA gene cluster 1 (Fig. 2) formed two distinct clusters in this composite analysis (Fig. 7). One contained strain LL-P018 and the three *S. phaeochromogenes* type strains, which were all highly similar by ribotype and metabolite profile (Fig. 2, 5, 7). The other contained *S. ederensis* NRRL B-8146^T, *S. phaeochromogenes* NRRL B-5478 and *S. phaeochromogenes* ISP-5170 (Figs. 2, 7). This group differed from strain LL-P018 and the *S. phaeochromogenes* type strains in both ribotype (Fig. 4) and in production of the phaeochromycins in liquid medium 2 (Fig. 5). Strain ISP-5170, which was highly divergent from the other Cluster 1 strains by ribotype (Fig. 4), rejoined the group in this composite analysis due to overall similarities in metabolite profile (Fig. 7). Due to its unique ribotype pattern, however, ISP-5170 still formed a distinct branch within the cluster.

Strains NRRL B-5333 and NRRL B-3559 (Fig. 2, "Cluster 2") were only 58% similar in this combined analysis due to significant variations in both ribotype and metabolite profile (Fig. 7). Strains NRRL WC-3776 and NRRL B-2031 (Fig. 2, "Cluster 3") were only 8.7% similar and did not cluster at all in this analysis (Fig. 7), despite a high degree of 16S rRNA gene similarity (Fig. 2). Strains NRRL B-2123 and NRRL B-1517 (Fig. 2, "Cluster 4") were 72.6% similar in the combined analysis (Fig. 7), highlighting a degree of strain variation between these closely related organisms (see "Discussion").

rpoB sequencing

Strain LL-P018 was compared to all strains from 16S rRNA gene cluster 1 (Fig. 2) as well as *S. tauricus* NRRL B-12497^T by *rpoB* sequence (354 bp). A phylogenetic tree depicting this comparison is shown in Fig. 8. All of the strains from 16S rRNA gene cluster 1 (Fig. 2) were 99.7–100% similar in *rpoB* sequence (Fig. 8), consistent with intraspecies similarity values reported for *Streptomyces* species [16]. The *S. ederensis* type strain, NRRL B-8146^T, was 100% identical to *S. phaeochromogenes* ATCC 3338^T,

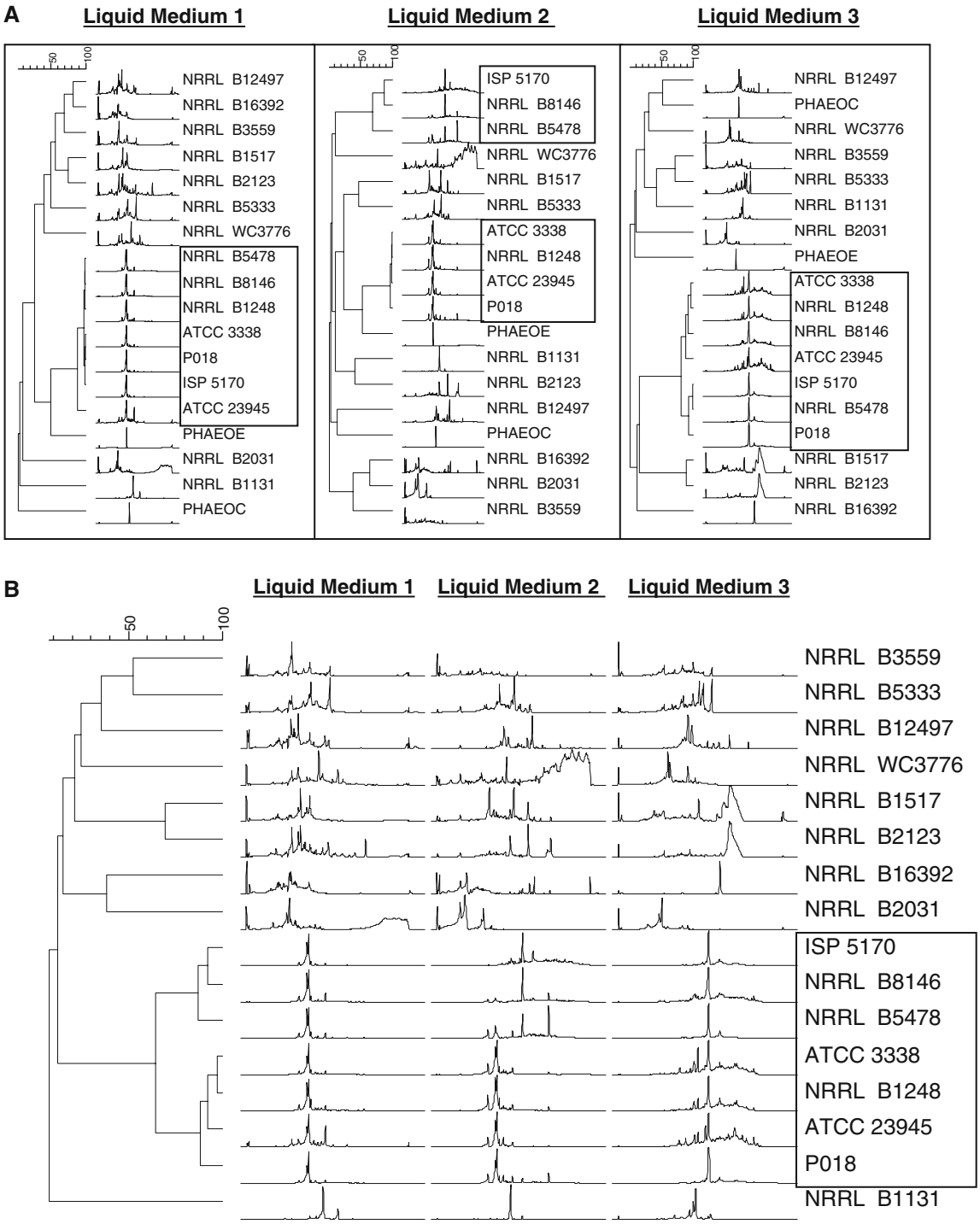

Fig. 5 Metabolite profiles of *S. phaeochromogenes* and closely related strains in three liquid media. Cluster analysis of HPLC chromatograms was performed using BioNumerics software (Applied Maths, Austin, TX) using the unweighted pair group method using arithmetic averages (UPGMA). Boxes highlight the strains corresponding to 16S rRNA gene cluster 1 (Fig. 2). 5A: Separate comparisons of metabolite profiles in individual media. 5B: Composite comparison of all three metabolite profiles. For composite comparison, each data set was weighted equally

S. phaeochromogenes ATCC 23945^T, and *S. phaeochromogenes* NRRL B1248^T (Fig. 8), further suggesting that *S. ederensis* and *S. phaeochromogenes* represent a single species. *Streptomyces tauricus* NRRL B-12497^T shared 95.2% *rpoB* sequence similarity with the strains from 16S rRNA gene cluster 1 (Figs. 2, 8), consistent with previously reported interspecies similarity values for species within the genus *Streptomyces* [16].

GenBank accession numbers for all sequences generated in this study are listed in Table 2.

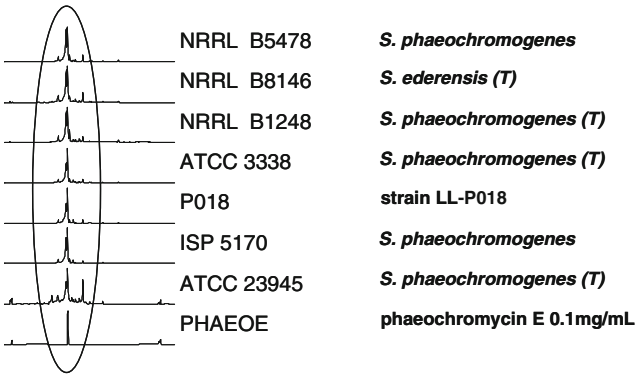

Fig. 6 HPLC analysis of phaeochromycin production by *S. phaeochromogenes* and closely related strains. All of the strains in 16S rRNA gene cluster 1 produce the phaeochromycins (above) and alnumycin (not shown). *PHAEOE* phaeochromycin E, 0.1 mg/mL. (^{T}) = type strain

Discussion

Type strains of *S. phaeochromogenes* and the closely related species *S. ederensis* and *S. tauricus* were obtained for comparison to strain LL-P018. Comparison of these strains by 16S rRNA gene sequence, morphological analysis, sporulation, pigmentation, and carbohydrate utilization supported placement of LL-P018 within the species *S. phaeochromogenes*, and also suggested a high degree of similarity between the type strains for *S. phaeochromogenes* and *S. ederensis* (Fig. 2; Tables 3, 4). In order to assess the uniqueness of strain LL-P018 among members of this species, ten strains identified as *S. phaeochromogenes* were obtained from the USDA-ARS (NRRL) culture collection (Table 1). These strains, along with type strains of *S. phaeochromogenes, S. ederensis* and *S. tauricus* were compared to strain LL-P018 by 16S rRNA gene analysis as well as genetic and chemical fingerprinting methods.

All strains were first compared by 16S rRNA gene sequence analysis (Fig. 2). Strain LL-P018 was 99.5% similar to the type strains of *S. phaeochromogenes* (Fig. 2). Two other strains of *S. phaeochromogenes*, NRRL B-5478 and ISP-5170 were also highly similar to the *S. phaeochromogenes* type strains (99.5 and 100%, respectively, Fig. 2). Interestingly, the type strain for *S. ederensis* also falls within this cluster and shares 99.6% 16S rRNA gene sequence similarity to the *S. phaeochromogenes* type strains (Fig. 2). This result was consistent with comparison of *rpoB* sequences (Fig. 8), as well as morphology, sporulation and carbohydrate utilization profiles of these strains, which were all highly similar (Tables 3 and 4). These results, along with ribotyping and metabolite profiling data indicate that *S. phaeochromogenes* and *S. ederensis* are highly similar, and may in fact represent a single species.

Of the 16 strains compared by 16S rRNA gene sequence, 7 (including strain LL-P018) formed a very tight cluster with 99–100% sequence identity (Fig. 2, "Cluster 1"). Thorough analysis of sequencing chromatograms revealed that slight 16S rRNA gene sequence variation between these highly related strains is due in part to heterogeneity in the hypervariable region of the gene (Fig. 3). Sequence heterogeneity between multiple copies of the 16S rRNA gene within a single strain of *Streptomyces* has been reported, and may occur in up to 6.9% of species within this genus [32]. This heterogeneity occurs within the highly variable α-region of the gene, among the nucleotides corresponding to bases 179–197 of the *S. ambofaciens* 16S rRNA gene, which form a stem-loop in the secondary structure of the subunit [32].

Sequence heterogeneity can be observed if the 16S rRNA gene is directly sequenced from a PCR product, in which case multiple copies of the gene are amplified using the primer set and represented in the sequencing reaction. Analysis of sequencing chromatograms can reveal the presence of multiple nucleotide signals at a single base location. This heterogeneity can be taken into consideration when comparing closely related strains, especially when sequence variation between strains occurs in the stem-loop region. Unfortunately, this heterogeneity is not always represented in sequence data deposited in GenBank. This may be due to the use of a sequencing method where the PCR product is cloned prior to sequencing. By such a method only one copy of the gene is sequenced, leading to a much cleaner sequencing reaction but missing evidence of sequence heterogeneity. Another possibility is that investigators may attempt to "clean up" unclear sequence data, by selecting the stronger of two concurrent nucleotide signals as the correct base identification. This would also ignore the presence of multiple 16S rRNA gene sequences within the organism, and therefore yield an incomplete data set for comparison.

Eight strains deposited in the NRRL culture collection by various investigators as *S. phaeochromogenes* were found to be more closely related to other *Streptomyces* type strains by 16S rRNA gene sequence comparison (Fig. 2). These strains were originally chosen for comparison to strain LL-P018 based on their species identification in the NRRL database. It is likely, however, that these strains were not fully characterized taxonomically prior to deposition. It should be mentioned that only type strains can be assumed thoroughly characterized and identified. Any other strains which may be available from a culture collection, or which may arise as results of a GenBank query, do not necessarily have a valid and reliable taxonomic identification. However, it should also be noted that though eight strains are now realized not to be closely related to *S. phaeochromogenes* or strain LL-P018 (Fig. 2), they still proved

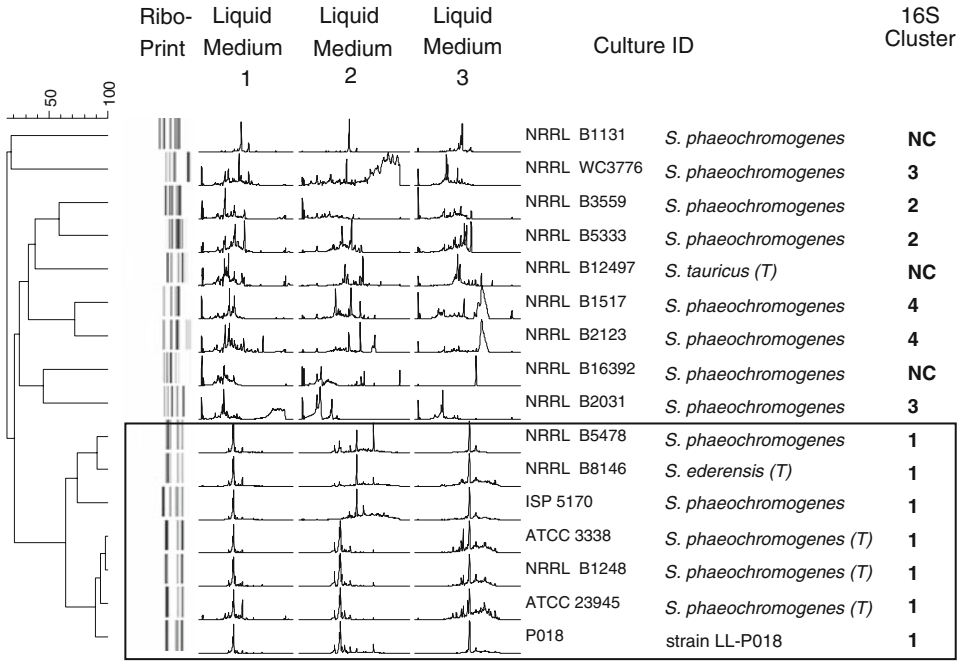

Fig. 7 Combined cluster analysis of *S. phaeochromogenes* and closely related strains by ribotype and metabolite profiles. Cluster analysis of Riboprints and HPLC chromatograms was performed using BioNumerics software (Applied Maths, Austin, TX) using the unweighted pair group method using arithmetic averages (*UPGMA*). For composite comparison, each data set was weighted equally. Boxes highlight the strains corresponding to 16S rRNA gene cluster 1 (Figure 2). *NC* not clustered. (^{T}) = type strain

valuable in the development of the genetic and chemical fingerprinting tools that were used in this study.

Evaluation of intraspecific relationships and strain variation below the species level requires methods more sensitive than 16S rRNA gene sequence analysis. Various genetic fingerprinting methods exist that have been used to discriminate between closely related *Streptomyces* strains, or to identify strain diversity in a group of environmental isolates [2, 3, 18, 25]. These molecular typing methods provide a "snapshot" of the genome, and can be highly sensitive to slight differences in DNA sequence. As a result, two strains with very similar DNA fingerprints are typically considered to be identical isolates. However, slight variations in DNA fingerprint between two strains do not necessarily reflect differences in secondary metabolite profile or other readily observable phenotypes [25]. In order to take into account both genetic and metabolic strain variation, chemical fingerprinting of culture broth extracts (metabolite profiling) was used along with ribotyping, a well-established genetic fingerprinting method [7, 25], to assess the similarity between strain LL-P018 and the related organisms selected for comparison.

Results of ribotype analysis suggested a close relationship between strain LL-P018 and the type strains of *S. phaeochromogenes* and *S. ederensis* (Fig. 4), which had all grouped together in 16S rRNA gene cluster 1 (Fig. 2). Three type strains of *S. phaeochromogenes* were greater than 96% similar to one another by ribotype (Fig. 4), confirming that these are identical strains. Strain LL-P018 also grouped closely with these strains, forming a distinct branch 92% similar to the *S. phaeochromogenes* type strain cluster (Fig. 4). This variation is clearly due to the presence of an approximately 11 kb band in the ribotype fingerprint of strain LL-P018 which is not seen in those of the type strains. This degree of ribotype variation indicates the presence of some genomic divergence, but does not necessarily suggest obvious phenotypic distinctions. Ribotype patterns generated by the RiboPrinter are typically considered identical if they are at least 93% similar, although a 92% similarity cutoff has been described for use with *Streptomyces* strains, which generate less ribotype bands than typical eubacterial isolates [22, 25]. A high degree of similarity between these four strains was further confirmed by metabolite profiling (Figs. 5, 6), as these strains produce the same suite of compounds under similar culture conditions. In a composite cluster analysis of ribotype and metabolite profiles, these four strains were greater than 89% similar to each other (Fig. 7).

Two other strains from 16S rRNA gene cluster 1 (Fig. 2), *S. ederensis* NRRL B-8146^{T} and *S. phaeochromogenes* strain NRRL B-5478, were 97% similar to one another by ribotype (Fig. 4). These two strains were greater than 99% similar in 16S rRNA gene sequence to strain LL-P018 and the *S. phaeochromogenes* type strains (Fig. 2, "Cluster 1"), but were only 80% similar to these strains by ribotype and therefore formed a distinct cluster in the ribotype analysis (Fig. 4). Metabolite profiling confirmed this strain variation (Fig. 5). Overall, these two strains produce the same family of compounds as strain LL-P018 and the *S. phaeochromogenes* type strains, but the profile of metabolites varies between the two strain clusters under certain culture conditions. This may indicate a difference in regulation of biosynthesis between these strain groups. Strain ISP-5170, also present in 16S rRNA gene cluster 1 (Fig. 2),

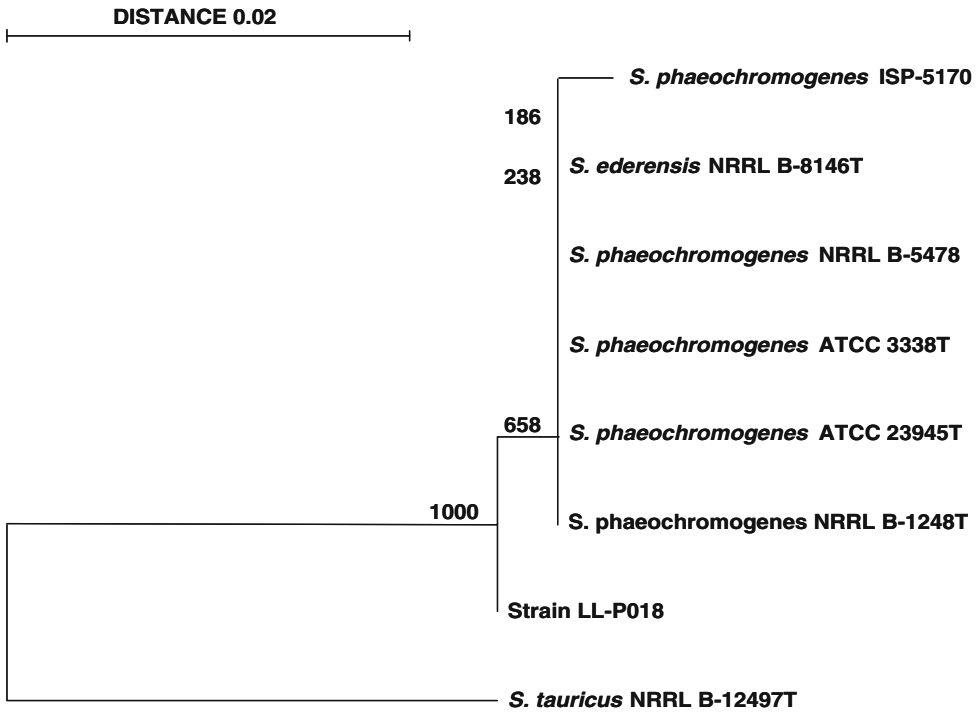

Fig. 8 Comparison of *S. phaeochromogenes* and closely related strains by *rpoB* sequence. Ribosomal polymerase β-subunit (r*poB*) gene sequences (354 bp) were aligned using ClustalX [31]. The tree was constructed with TREECON software using the neighbor-joining method with 1,000 bootstrap replicates [15, 33]. (^{T}) = type strain

was even more divergent from the group by ribotype comparison, sharing less than 35% similarity with the other strains in the cluster (Fig. 4). While this degree of ribotype variation would appear to indicate significant strain variation, metabolite profiling showed that this strain behaves similarly to NRRL B-8146^T and NRRL B-5478 in terms of phaeochromycin and alnumycin production (Fig. 5, 6). In the composite analysis combining ribotype and metabolite profiles, strain ISP-5170 clustered most closely with NRRL B-8146T and NRRL B-5478, but formed a distinct branch within this group due to its unique ribotype pattern (Fig. 7). This confirms the prediction that although ribotyping is a good indicator of strain variation, changes in DNA sequence that result in ribotype variation between two strains may not always affect obvious phenotypic traits such as secondary metabolite profiles [25]. This also confirms the necessity of combining genetic and phenotypic analyses in intraspecific and strain comparisons.

Of the strains that were not closely related to *S. phaeochromogenes* or *S. ederensis*, several showed strain variation within a species-level cluster. 16S rRNA Clusters 2 and 4 (Fig. 2) both exhibited variations in metabolite profile that correlated well with ribotype variation (Fig. 7). In the case of 16S rRNA gene cluster 3 (Fig. 2), strains NRRL WC-3776 and NRRL B-2031, although highly similar in 16S sequence, did not cluster at all in the combined analysis and appear to represent very different strains (Fig. 7).

These results confirm the ability of genetic and chemical fingerprinting methods to illustrate strain variation among closely related strains, and highlight the value of this polyphasic approach to strain delineation.

There are several issues that must be considered with regards to metabolite profiling as described in this study. A critical factor in comparing HPLC chromatograms from culture extracts is assuring environmental and chromatographic consistency among samples. In this study, strains were inoculated and cultured simultaneously under identical conditions to minimize variation due to environmental factors. Chromatographic equipment was regularly calibrated and standards were run intermittently among samples to assure that no retention time "drift" was occurring. These types of issues can be carefully controlled and monitored in a study such as this one, where a small set of samples is being compared. However, these issues may confound attempts to build a database of chemical fingerprints acquired over time, with new samples being added to the set periodically. In this case, careful normalization with the use of internal or external standards would be necessary to control changes in chromatography over time. Standardization of culture conditions and environmental factors would also be critical for the generation of reliable and reproducible data. It is for precisely these reasons that genetic fingerprinting is a very desirable method for strain delineation, as the genome of an organism is much less sensitive to this type of variability than metabolite production.

Also of importance in metabolite profiling is the processing of data. In this study, BioNumerics software was used for comparison of metabolite profiles. Typical metabolite profiles derived from HPLC analysis of fermentation

Table 4 Morphological comparison of *S. phaeochromogenes* and closely related species

ISP sporulation agar morphology and pigmentation				
ISP Medium	*Streptomyces* strain LL-P018	*Streptomyces phaeochromogenes* ATCC 3338^T	*Streptomyces ederensis* NRRL B-8146^T	*Streptomyces tauricus* NRRL B-12497^T
ISP2 (Yeast extract-malt extract agar)	Sub myc:	Sub myc:	Sub myc:	Sub myc:
	Dark brown	Brown	Dark brown	Pink/tan
	Rev myc:	Rev myc:	Rev myc:	Rev myc:
	Dark brown	Brown	Dark brown	Pink/tan
	Aerial myc:	Aerial myc:	Aerial myc:	Aerial myc:
	White	White	White	None
	Spore mass:	Spore mass:	Spore mass:	Spore mass:
	Grey	Grey	Grey	None
	Pigment:	Pigment:	Pigment:	Pigment:
	Brown	Brown	Brown	None
ISP3 (Oatmeal agar)	Sub myc:	Sub myc:	Sub myc:	Sub myc:
	Brown	Tan	Brown	Pink/brown
	Rev myc:	Rev myc:	Rev myc:	Rev myc:
	Brown	Tan	Brown	Pink/brown
	Aerial myc:	Aerial myc:	Aerial myc:	Aerial myc:
	White/pink	White/grey	White/pink	White/pink
	Spore mass:	Spore mass:	Spore mass:	Spore mass:
	White/pink	White/grey	White/pink	White/pink
	Pigment:	Pigment:	Pigment:	Pigment:
	Brown	None	Trace-brown	None
ISP4 (Inorganic salts-starch agar)	Sub myc:	Sub myc:	Sub myc:	Sub myc:
	Brown	Tan	Brown	Pink
	Rev myc:	Rev myc:	Rev myc:	Rev myc:
	Brown	Tan	Brown	Pink
	Aerial myc:	Aerial myc:	Aerial myc:	Aerial myc:
	White/pink	White	White/pink	White/pink
	Spore mass:	Spore mass:	Spore mass:	Spore mass:
	White/pink	White	White/pink	White/pink
	Pigment:	Pigment:	Pigment:	Pigment:
	None	None	None	None
ISP5 (Glycerol–asparagine agar)	Sub myc:	Sub myc:	Sub myc:	Sub myc:
	Dark brown	Brown	Dark brown	Pink/tan
	Rev myc:	Rev myc:	Rev myc:	Rev myc:
	Dark brown	Brown	Dark brown	Pink/tan
	Aerial myc:	Aerial myc:	Aerial myc:	Aerial myc:
	Grey	Grey	Grey	White/pink
	Spore mass:	Spore mass:	Spore mass:	Spore mass:
	Grey	Grey	Grey	None
	Pigment:	Pigment:	Pigment:	Pigment:
	Tan	Tan	Tan	None

Growth, sporulation, and pigmentation were observed according to methods defined by the International Streptomyces Project [29]. (T) = type strain

extracts contain some artifacts that may not be specific to the strain being analyzed. Strong solvent peaks occur early in the chromatographic profile, while non-polar metabolites and media components may cause high background signal late in the chromatogram. Thus, in this study a 15-min chromatogram was generated, but data generated in the first

2 min and final 3 min of the chromatogram were not included in the comparison. This eliminated nonspecific peaks and areas of high background from the comparison. Also, background subtraction was performed on each chromatogram in the analysis. This was done by subtraction of a chromatogram of an uninoculated media blank from each sample chromatogram. The main effects of background subtraction on a metabolite profile are the elimination of peaks derived from medium components, overall reduction of background signal, and relative intensification of new peaks generated by the fermentation. While this may appear to have obvious benefit in profile analysis, issues such as the generation of "negative peaks" complicate this issue.

Negative peaks may be generated by the subtraction of media components that have already been utilized by a strain. Because the organism has consumed the medium component, the corresponding peak is not present in the sample chromatogram. Therefore, subtraction of the "blank" medium extract containing the peak results in negative values in the culture extract chromatogram. For this study, negative peaks were eliminated using BioNumerics. Although such negative peaks may be considered an important part of an organism's metabolite profile, in this study it was assumed that the consumption of a specific medium component is a characteristic that may be shared by multiple organisms, regardless of their relatedness at any taxonomic level. Negative peaks were therefore considered to be less strain-specific than peaks representing new metabolites produced, especially since the issue of secondary metabolite production is of relevance to this study. All of these considerations regarding the many possible approaches to metabolite profiling are subject to the opinions and the research objectives of the investigator, and certainly a more thorough evaluation of these methods with a larger number of strains may shed more light on the best approaches to this task.

Streptomyces phaeochromogenes was first described by H.J. Conn in 1917 as *Actinomyces phaeochromogenus* [11]. Conn states:

"Description of species of Actinomycetes is at present very difficult. The literature abounds with descriptions that have become invalidated as better methods of study have been developed."

Conn's statement is still relevant and meaningful today, 90 years after it was written. Understanding the genetic and phenotypic relationships between these magnificent soil microorganisms is as complex as it was in Conn's day, even using current technologies. Clearly, no single taxonomic method has yet been described which can define all of the inter- and intra-specific relationships between members of the species *Streptomyces* or the Actinomycetes as a whole. Polyphasic methods incorporating genotypic and phenotypic comparisons have long been necessary for strain and species group comparisons [2], and as new technologies improve our ability to visualize these relationships, we will better understand these organisms and the abundant bioactive compounds they produce. Of particular promise is the fact that the phaeochromycins, a family of novel anti-inflammatory polyketides produced by one of the earliest described members of the species *Streptomyces*, were only just discovered in 2005. This is evidence that these ubiquitous and highly productive soil organisms, which have been studied at great length for more than a century [6, 26, 35, 36], still have some surprises for us. Furthermore, with the implementation of new methods and technologies providing a "fresh" look at "old" strains, more and more of the hidden potential of *Streptomyces* will continue to be discovered.

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