J Ind Microbiol Biotechnol (2003) 30: 472–479
DOI 10.1007/s10295-003-0038-0

ORIGINAL PAPER

F. V. Ritacco · B. Haltli · J. E. Janso · M. Greenstein
V. S. Bernan

Dereplication of *Streptomyces* soil isolates and detection of specific biosynthetic genes using an automated ribotyping instrument

Received: 3 January 2003 / Accepted: 7 February 2003 / Published online: 5 April 2003

Abstract The discrimination of distinct cultures among morphologically similar *Streptomyces* soil isolates (dereplication) and the detection of specific biosynthetic pathways in these strains are important steps in the selection of microorganisms to include in a natural products library. We have developed methods for analysis of actinomycetes using the RiboPrinter microbial characterization system, an automated instrument that performs ribotyping on bacterial samples. To evaluate our dereplication method, 26 *Streptomyces* isolates, obtained from soil samples collected in Maui, Hawaii, were ribotyped and compared with each other, using the RiboPrinter. The strains were also compared by 16S rDNA sequence analysis, MIDI fatty acid analysis, and LC-MS profiling of fermentation extracts. The RiboPrinter was able to identify closely related isolates and to discriminate between morphologically similar isolates with unique genetic, fatty acid and fermentation profiles. For the detection of biosynthetic genes, a 1,006-bp probe containing a portion of an adenylation domain of a non-ribosomal peptide synthetase (NRPS) was employed. Using this alternate probe in place of the standard ribosomal probe, the RiboPrinter was able to detect NRPS genes in several strains of *Streptomyces*. These results demonstrate that the RiboPrinter has multiple applications in a natural products research program.

Keywords Ribotyping · DNA fingerprinting · RiboPrinter

F. V. Ritacco · B. Haltli · J. E. Janso · M. Greenstein
V. S. Bernan
Natural Products Microbiology, Wyeth Research,
Pearl River, New York, USA

F. V. Ritacco (✉)
Bldg. 205 rm. 465, Wyeth Research,
401 North Middletown Rd., Pearl River,
NY 10965, USA
E-mail: ritaccf@wyeth.com
Tel.: +1-845-6023482
Fax: +1-845-6025687

Introduction

The search for novel natural products with useful pharmacological activities often includes the isolation of actinomycetes, such as *Streptomyces* species, from soil samples. These soil isolates become part of a collection of cultures that are fermented and analyzed for the production of bioactive secondary metabolites. Members of the genus *Streptomyces* are abundant in many soil samples, and multiple species and strains with very similar morphology often exist within a single sample. In order to avoid duplication and maximize biodiversity in the development of a culture collection, a rapid means of dereplicating morphologically similar isolates is essential.

Many methods exist for the identification of actinomycetes, each of which is useful for characterization to a certain taxonomic level. Because different subspecies or strains within a given species may produce different secondary metabolites, a method sensitive enough to discriminate at the strain level is valuable for dereplication of soil isolates [5]. Genetic fingerprinting methods, such as pulsed-field gel electrophoresis, restriction fragment length polymorphisms, randomly amplified polymorphic DNA and ribotyping, provide a level of sensitivity suitable for discrimination of strains below the species level [2, 5, 9, 14]. These methods, however, can be fairly labor-intensive and do not provide the rapid results needed when screening many soil isolates at a time.

The RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, Del.) is an automated ribotyping instrument. Ribotyping is a genetic fingerprinting method in which genomic DNA is digested using a restriction endonuclease and the resulting DNA fragments are separated by gel electrophoresis. Following electrophoresis, the DNA is transferred to a membrane and is then hybridized with a ribosomal DNA (rDNA) probe by Southern blotting [4, 9]. The RiboPrinter employs an rDNA probe derived from the entire ribosomal operon from *Escherichia coli*. The pattern of DNA fragments hybridizing with this

probe serves as a genetic fingerprint, which can be used to distinguish between distinct strains in a population of bacterial isolates. The RiboPrinter automates this process, and the ribotype pattern generated is captured by a digital camera. The pattern of bands detected is then compared with a standard marker set, and each band is normalized for position and intensity. In this way, the ribotype pattern is converted into a standardized RiboPrint, which can be compared against a database [9].

We have developed methods for using the RiboPrinter for the dereplication of actinomycete isolates in our natural products discovery program. To demonstrate these methods, we characterized a set of *Streptomyces* isolates from Maui, Hawaii, using the RiboPrinter, and compared these results to those obtained by MIDI (Newark, Del.) fatty acid profiling, 16S rDNA sequence comparison, and LC-MS profiling of fermentation extracts. We have also developed methods for the detection of specific biosynthetic genes using this system with a non-ribosomal peptide synthetase (NRPS)-derived probe in place of the rDNA probe. A 1,006-bp DNA probe containing a portion of an adenylation domain from an NRPS was labeled by sulfonation and used with the RiboPrinter in place of the rDNA probe. Several strains of *Streptomyces*, including known species and unidentified soil isolates, were probed for the presence of NRPS genes using this method.

Materials and methods

Isolation of actinomycetes from soil

Soil was collected in Maui, Hawaii. Soil (1 g) was air-dried overnight and then suspended in 5 ml of a solution containing 6% yeast extract, 0.05% SDS and 0.01 M $CaCl_2$. The soil suspension was heated at 50 °C for 10 min and then diluted to 20 ml in 0.1% agar/H_2O. The diluted suspension was then inoculated into a variety of selective agar-based isolation media containing various antibiotics. The plates were incubated at 28 °C and checked every day for the appearance of actinomycete colonies. Actinomycete isolates were cultured and purified on an agar medium containing (per liter): 5 g dextrose, 2.5 g yeast extract, 15 g agar, 10 g soluble starch, 2.5 g NZ-amine A and 0.5 g calcium carbonate. Before inoculation, agar plates were overlaid with a sterile 0.2-μm polycarbonate membrane for easier colony removal for ribotyping.

Type cultures

S. coelicolor A3(2) was obtained from the John Innes Institute, Norwich, UK. *S. akiyoshiensis* (ATCC 13480) and *S. griseus* (ATCC 11746) were obtained from the American Type Culture Collection, Rockville, Md. *S. hygroscopicus* (NRRL 30439) was obtained from Wyeth Research, Pearl River, N.Y.

Ribotyping

Approximately two 10-μl loopfuls containing 2–5 colonies were scraped from agar plates overlaid with polycarbonate membranes and resuspended in 200 μl of RiboPrinter sample buffer (Qualicon). The sample was then ground with a sterile, motorized pestle, and 40 μl of the ground cell suspension was placed in the RiboPrinter sample carrier, which was placed in the RiboPrinter heat-treatment station and heat-treated for approximately 20 min. Two lysis buffers (Qualicon) 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 et al. [4]. Phenograms were constructed using BioNumerics software (Applied Maths, Austin, Tex.).

16S rDNA amplification and sequencing

Genomic DNA was isolated according to a modification [1] of a method described by Hopwood et al. [10]. PCR amplification of 16S ribosomal subunit genes was carried out in 100-μl reaction volumes containing approximately 5–10 ng of genomic DNA, 1 mM each of the 8 FPL forward and 1492 RPL reverse primers described by Reysenbach et al. [13] and 50 μl of Jumpstart PCR mix (Sigma). The PCR was performed on a BioMetra T Gradient thermocycler 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. Direct sequencing was performed using an ABI 3700 sequencing machine with the ABI Prism DNA sequencing kit and Big Dye terminators ver 3.0 (Applied Biosystems, Foster City, Calif.). Sequencing was performed on the PCR reaction using the 16S forward and reverse PCR primers described above.

16S rDNA sequence comparison

Multiple sequence alignments were performed using CLUSTAL X 1.81 [15]. Phylogenetic trees were constructed using TREECON software (neighbor-joining, 500 bootstrap replicates [11, 16]).

Fatty acid profiling

Cultures were grown on trypticase soy agar (BBL) and cells were harvested before the appearance of aerial mycelia or spores. Fatty acid profiles were obtained using the MIDI microbial identification system according to the manufacturer's instructions. Phylogenetic trees were constructed using Sherlock software ver 4.5 (MIDI)

Fermentation profiling

All cultures were fermented in a liquid medium containing (per liter): 2.5 g NZ-amine A, 12.5 g dextrose, 12.5 g soy flour, 1.5 g ammonium chloride, 0.4 g agar and 1 g calcium carbonate. Fermentation was at 28 °C and 200 rpm for 6 days. Whole broth was extracted in methanol (1:1) and centrifuged to remove insolubles. The supernatant was dried to completion and resolubilized in methanol to a 2× final concentration. Reverse phase HPLC of extracts was performed using a Hewlett Packard model HP1100 liquid chromatograph with photodiode array detection, coupled to a ThermoFinnigan LCQ classic ion trap mass spectrometer fitted with an electrospray ionization source. Extracts were resolved by reverse phase chromatography using a YMC ODS-A HPLC column (4.6×150 mm, 5 μm, 120 Å), 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 25 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.

Use of NRPS probe with the RiboPrinter

The 807-bp adenylation domain core (A3–A8 region) of a putative serine-activating NRPS module was amplified from the chromosome of *S. hygroscopicus* NRRL 30439 and cloned into pCR2.1TOPO (Invitrogen). For the purpose of generating a probe to be used in conjunction with the RiboPrinter, the adenylation domain fragment was amplified using the M13 forward and reverse primers located in pCR2.1TOPO. Several PCR reactions were pooled and then electrophoresed on a 1% Tris-acetate EDTA agarose gel. The resulting 1,006-bp fragment was cut from the gel and eluted from the agarose using the QIAquick gel extraction kit (Qiagen). The eluted samples were pooled and ethanol-precipitated. The DNA was reconstituted in water at a concentration of 325 ng/μl, and 50 μl of probe DNA was labeled using the SulfoProbe labeling kit (Sigma), according to the manufacturer's specifications. An entire labeling reaction was used for each hybridization.

Results

Comparison of soil isolates by ribotyping

Ribotype analysis of the *Streptomyces* isolates with the RiboPrinter is illustrated in Figs. 1 and 2. Comparison of the isolates by ribotype pattern is depicted as a phenogram in Fig. 3. Isolates were clustered based on the percent similarity of their ribotypes. While this tree does not show true evolutionary distance, it summarizes the comparison of all of the isolates using the RiboPrinter. Isolates that did not match any other by greater than 92% were considered to represent unique strains. Those that were at least 92% similar by ribotype were considered ribotype clusters. Six clusters containing multiple strains at least 92% similar to each other by ribotype were identified. A seventh cluster was assigned to contain isolates 122 and 124, for which useful ribotype patterns were not obtained. These clusters are highlighted in the phenogram with brackets and are identified with numbers 1–7. Additionally, nine isolates were identified as unique strains, which did not match any other isolate by ribotype. Isolates that did not cluster with any other were not assigned a cluster number.

16S rDNA sequence comparison

A phylogenetic tree depicting comparison of the isolates by 16S rDNA sequence is shown in Fig. 4. Clustering of isolates based on the ~1,400-bp 16S rDNA sequence was very similar to the clustering by ribotype similarity. All isolate pairs or groups that were at least 92% similar by ribotype also clustered together by 16S rDNA comparison. In some cases, strain variations that were detected by the RiboPrinter were not identified by 16S sequence comparison. For example, ribotype clusters 1 (isolates 82, 87, 128, 132, 133) and 2 (isolates 113, 136), which are distinct from each other by ribotype, formed a single group by 16S rDNA comparison. Similarly, ribotype cluster 4 (isolates 83, 84) became part of a larger cluster in the 16S phylogenetic tree, including isolates 107 and 130, which are not closely related by ribotype. Isolates 122 and 124 (ribotype cluster 7), for which useful ribotype patterns were not obtained, formed a cluster by 16S sequence comparison.

Fatty acid analysis

Comparison of the isolates by fatty acid profiling is shown as a phylogenetic tree in Fig. 5. Many of the strain cluster groups defined by ribotyping were also observed by fatty acid analysis. For example, ribotype cluster 3 (isolates 108, 134) clustered as expected based on ribotype. Also, isolates 122 and 124 (ribotype cluster 7) clustered together as expected based on 16S sequence comparison and their similar lack of a useful

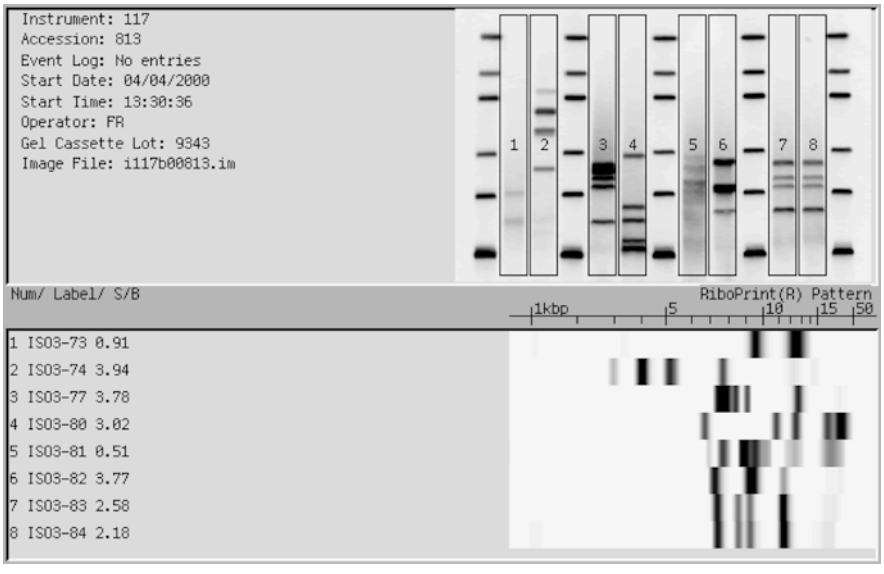
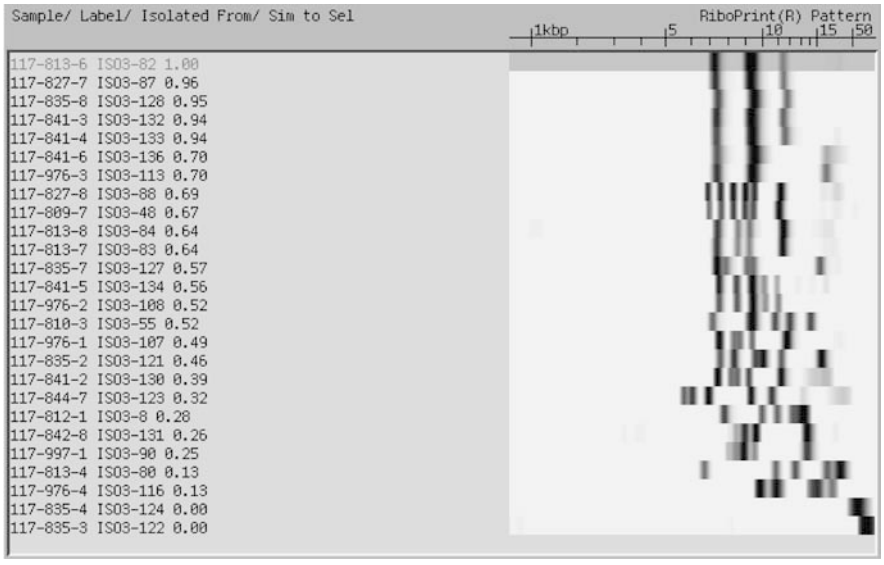


Fig. 1 Ribotype analysis of *Streptomyces* isolates with the RiboPrinter. Ribosomal probe hybridization patterns are captured by the RiboPrinter's digital camera (*top right*). Eight samples can be run at one time. Each pattern is normalized to standards in an adjacent lane and converted to a standardized RiboPrint (*bottom*). *ISO* Isolate number



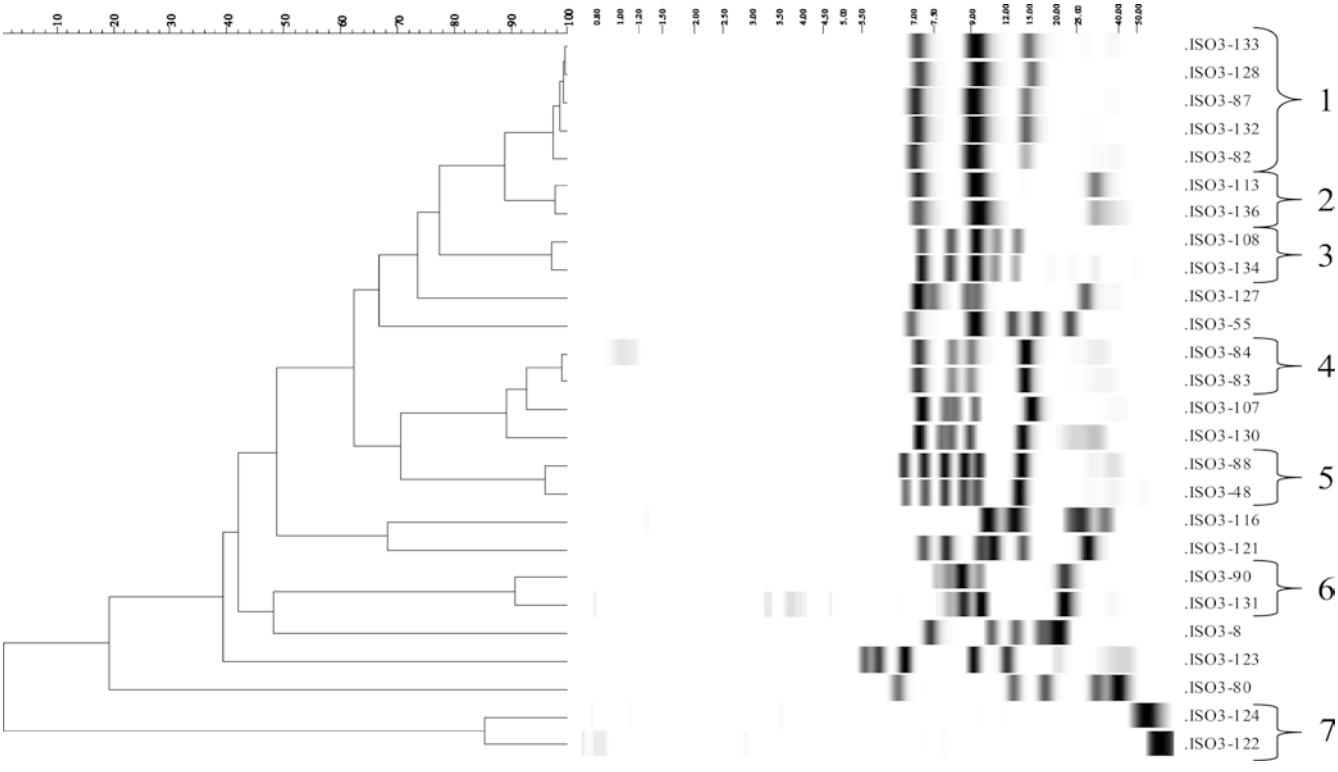


Fig. 2 Database analysis using the RiboPrinter. All RiboPrints are automatically compiled into a database. Each pattern can be quickly compared against all others by similarity percentage. Numbers to the right of sample identifiers represent percent similarity to the selected culture (*highlighted at the top*)

Fig. 3 Phenogram of RiboPrints of 26 *Streptomyces* isolates. Phylogenetic trees were constructed using BioNumerics software (Applied Maths, Austin, Tex.). The isolates were clustered by the percent similarity of their ribotypes. Isolates that do not match any other by greater than 92% are here considered to represent unique strains. Those that are least 92% similar by ribotype are considered strain clusters. Strain clusters are marked with brackets and numbered 1–7. Cluster 7 contains two isolates for which useful RiboPrints could not be obtained

ribotype pattern. In some cases, however, this method was not able to identify certain unique strain groups defined by ribotyping. For example, isolates 82, 87, 128, 132 and 133 (ribotype cluster 1), isolate 113 (from ribotype cluster 2) and isolates 83 and 84 (ribotype cluster 4) all clustered together at the subspecies level (Euclidean distance = 6). By ribotype, these eight isolates formed three distinct strain groups (see Fig. 3), which were not clearly identified by comparison of fatty acid profiles. Similarly, isolates 90 and 131 (ribotype cluster 6) grouped together with isolate 116, which is unrelated by ribotype. Furthermore, isolates 113 and 136 (ribotype cluster 2) clustered somewhat distantly by fatty acid profile, together with several strains unrelated by ribotype. These two isolates were

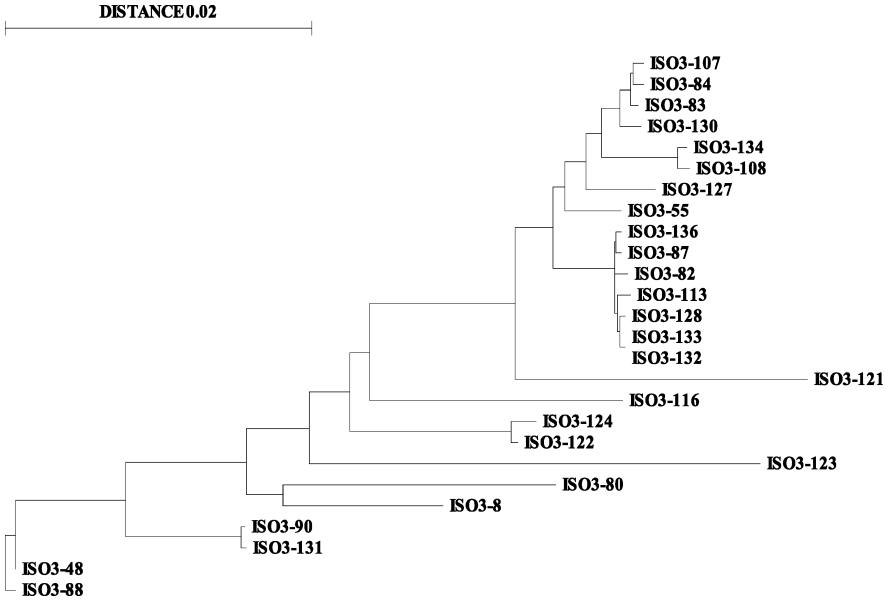


Fig. 4 Phylogenetic relationship of isolates based on 16S rDNA sequence comparison. The phylogenetic tree was based on neighbor-joining analysis of ~1,400-bp 16S rDNA sequences for all isolates, with 500 bootstrap replicates [11, 16]

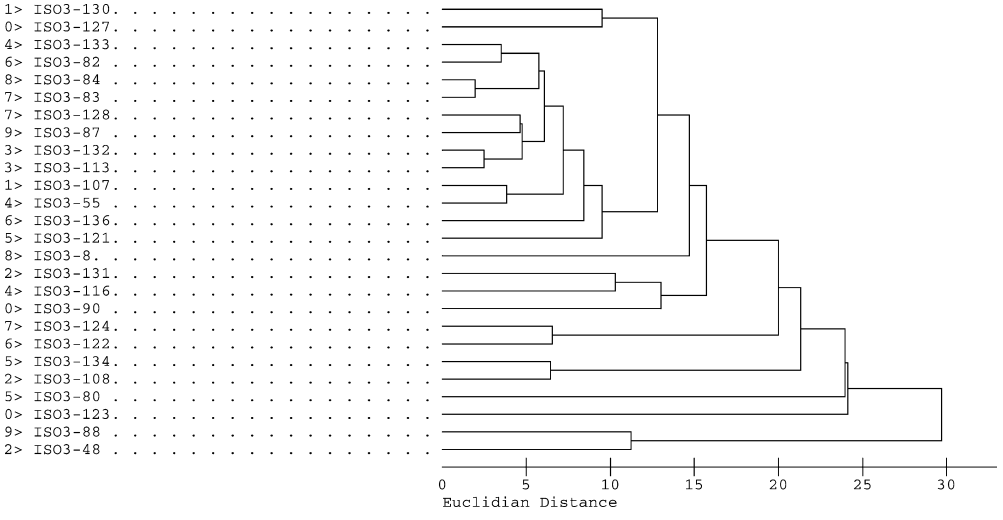


Fig. 5 Phylogenetic tree based on fatty acid profiles. Fatty acid profiles were obtained using the MIDI microbial identification system according to the manufacturer's instructions (MIDI, Newark, Del.). Phylogenetic trees were constructed using Sherlock software ver 4.5 (MIDI)

not identified as a unique strain cluster by fatty acid profiling.

LC-MS profiling of fermentation extracts

Fermentation profiles of cultures related by ribotype (>92%) showed a high degree of similarity, while cultures with very distinct ribotypes showed differences in their fermentation components. For example, isolates 108 and 134 (ribotype cluster 3) had nearly identical fermentation profiles, which were clearly different from the profiles of unrelated isolates, such as isolate 90 (Fig. 6). In some cases, minor variations in ribotype did not correlate with detectable differences in fermentation profile. For example, ribotype clusters 1 (isolates 82, 87, 128, 132, 133) and 2 (isolates 113, 136), which are distinct from each other by ribotype, all had very similar fermentation profiles.

Use of an NRPS probe with the RiboPrinter

The results of a RiboPrinter run with a sulfonated NRPS probe are shown in Fig. 7. Several hybridization signals were observed in all lanes containing *Streptomyces* genomic DNA. Two intense hybridization signals present in lane 4 (*S. hygroscopicus*) corresponded to *Pvu*II fragments predicted to contain portions of the 807-bp serine adenylation domain amplified from the *S. hygroscopicus* genome. The observed size of these signals (2.0 kb, 2.5 kb) correlates quite well with the signal sizes of 2.171 kb and 2.646 kb predicted from analysis of the gene cluster DNA sequence. Two signals (1.3 kb,

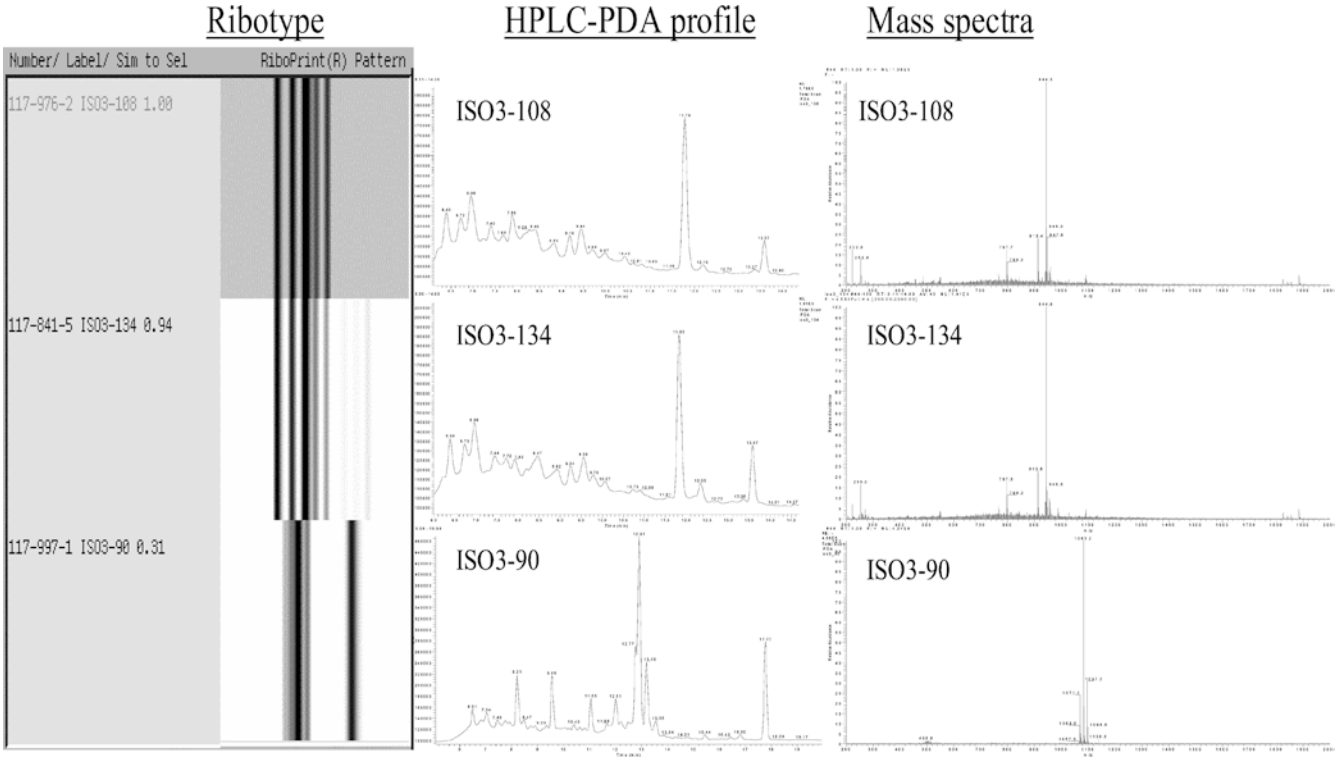


Fig. 6 Fermentation profiles of isolates based on LC-MS analysis. All cultures were fermented under identical conditions. Fermentation broths were extracted and analyzed by HPLC-MS in order to compare fermentation profiles. Fermentation profiles of cultures matching by ribotype (> 92%) showed a high degree of similarity, while cultures with very distinct ribotypes showed differences in the fermentation components

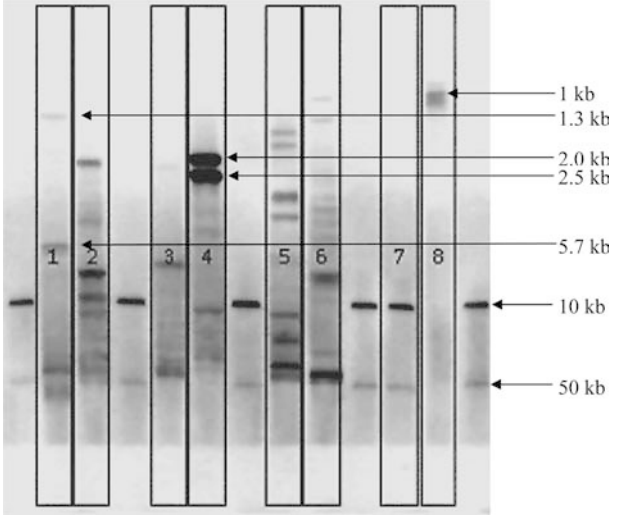


Fig. 7 Detection of non-ribosomal peptide synthetase (NRPS) biosynthetic genes in *Streptomyces* strains using the RiboPrinter. The rDNA probe used for ribotyping was replaced by a 1,006-bp adenylation domain probe amplified from an NRPS gene cluster isolated from *S. hygroscopicus* NRRL 30439. Lane 1, *S. coelicolor* A3(2), lane 2, *S. griseus* ATCC 11746, lane 3, *S. akiyoshiensis* ATCC 13480, lane 4, *S. hygroscopicus* NRRL 30439, lane 5, Maui soil isolate ISO3-48, lane 6, Maui soil isolate ISO3-90, lane 8, positive control (~0.3 ng of unlabeled probe DNA). Lane 7 and unmarked lanes contain RiboPrinter DNA standards (Qualicon)

5.7 kb) were observed in lane 1, containing *S. coelicolor* DNA. A nucleotide BLAST search of the GenBank database indicated that the probe was most similar to bases 200, 634–201, 439 of the *S. coelicolor* genome [3]. These bases correspond to the threonine-activating adenylation domain of the calcium-dependent antibiotic (CDA) peptide synthetase gene cluster [8]. The deduced sizes of these signals also correlate well with the predicted sizes of 1.113 kb and 5.721 kb. Hybridization signals were present in lanes containing DNA from *S. griseus* (lane 2) and *S. akiyoshiensis* (lane 3), indicating the presence of NRPS-like genes in the genomes of these organisms. The NRPS probe also hybridized to several fragments in the lanes containing DNA from Maui soil isolates 48 (lane 5) and 90 (lane 6), indicating the presence of NRPS genes in these isolates. A weak hybridization signal was observed in lane 8, which contained the positive control (0.3 ng of the unlabeled probe DNA). The weak nature of the signal generated by the positive control was due to the low amount of DNA loaded in this lane. The unlabeled lanes and lane 7 contained the standard RiboPrinter molecular mass marker. Signals corresponding to 10 kb and 50 kb were observed in these lanes. The ability of the NRPS probe to hybridize to the molecular weight marker was likely due to homology between the vector sequences flanking the 807-bp adenylation domain sequence and the observed bands of the marker, which are known to contain some common vector sequences (Qualicon, personal communication).

Discussion

Genetic fingerprinting methods and other techniques capable of discriminating between bacterial isolates at the subspecies or strain level are typically very labor-intensive and time-consuming. We have developed methods to use the RiboPrinter microbial characterization system for automated dereplication of *Streptomyces* soil isolates.

The RiboPrinter is marketed primarily as a tool for epidemiology, clinical microbiology and quality control [4, 9]. As such, the methods and reagents recommended by the manufacturer are designed for the identification of bacterial pathogens and food contaminants and are not optimal for the analysis of *Streptomyces* or other actinomycetes. One difficulty encountered in the analysis of *Streptomyces* isolates with the RiboPrinter was the preparation of colonies from agar plates. Because contamination of a colony preparation with agar can potentially inhibit enzymatic activity, a method was designed to allow agar-free colony removal from plates. When streptomycete cultures are grown on agar plates overlaid with polycarbonate membranes, the colonies can be easily lifted from the membrane using an inoculating loop, avoiding agar contamination. Because of the typically leathery nature of streptomycete colonies, a motorized pestle is used to grind the colonies in the provided sample buffer. Also, because the ribosomal probe is based on ribosomal DNA sequence from *E. coli*, the amount of *Streptomyces* colony material required to achieve sufficient hybridization is greater than that recommended by the manufacturer. These methods allow, in most cases, effective cell lysis, DNA digestion and generation of useful RiboPrints from streptomycetes.

The restriction endonuclease suggested for use with the RiboPrinter is *Eco*RI, and the RiboPrinter database primarily includes RiboPrints generated with this enzyme. Initial experiments (data not shown) indicated that the use of *Eco*RI with *Streptomyces* DNA did not yield reproducible ribotype data. These inconsistencies were likely due to the infrequent occurrence of the *Eco*RI recognition site in *Streptomyces* DNA. *Pvu*II was found to be much more effective for the discrimination of *Streptomyces* strains.

Comparison of *Streptomyces* soil isolates by ribotype has proven to be a very effective means of dereplication. Strain groupings determined by ribotyping correlate very closely with those determined by fatty acid analysis and 16S rDNA sequence comparison. Ribotyping of isolates with the RiboPrinter has several advantages over these other methods, including greater sensitivity to strain variation, automated sample analysis and database utility.

Strain delineation by ribotyping appears to correlate well with chemical diversity, as observed by comparing the fermentation profiles of the isolates analyzed. In all cases, isolates that were closely related by ribotype had very similar fermentation profiles. In most cases, isolates that were less than 92% similar by ribotype had substantial differences in their fermentation profiles in terms of major UV peaks or mass spectra observed. Because ribotyping is highly sensitive, strain variations detected by this method may not always reflect differences in secondary metabolite production. However, variations in fermentation profiles among these strains might be observed if a wide variety of media and fermentation conditions are used.

Occasionally, analysis with the RiboPrinter does not yield a useful ribotype. For example, in the analysis of Maui soil isolates 122 and 124 (Fig. 3), the DNA isolated from the organisms remained uncut and the RiboPrint obtained consisted of only a single band of high-molecular-mass DNA. Analysis of fatty acids and 16S rDNA sequence (Figs. 4, 5) indicated that these are closely related strains. It is possible that these strains either modify their DNA, making it resistant to digestion with *Pvu*II, or produce enzyme inhibitors. There are also occasional instances where certain isolates are not sufficiently lysed by the methods and reagents supplied by the manufacturer, possibly due to differences in the bacterial cell wall. For these strains, no ribotype band pattern can be seen at all. In cases such as these, where a useful ribotype cannot be easily obtained, other methods may be necessary for true strain discrimination.

The use of polyketide synthase or NRPS probes derived from one pathway to identify similar pathways in a different organism is a common technique. This process usually involves several tedious steps, including DNA isolation, DNA digestion, agarose gel electrophoresis, transfer of DNA to a membrane and finally the hybridization procedure itself. One goal of these experiments was to determine the compatibility of a heterologous probe with the RiboPrinter. The results shown in Fig. 7 indicate that a sulfonated NRPS probe is compatible with the RiboPrinter technology, as hybridization signals were observed in each lane containing *Streptomyces* genomic DNA. Hybridization of the probe to the expected *Pvu*II fragments in the lanes containing DNA from *S. hygroscopicus* (100% homologous) and *S. coelicolor* (75.6% homologous) attests to the ability of this probe to identify true NRPS-encoding genes. The probe was also successful in identifying putative NRPS genes in *S. griseus*, *S. akiyoshiensis* and in two Maui soil isolates. *S. griseus* ATCC 11746 is a known candicidin-producer, although the production of this polyene antibiotic does not require an NRPS [6]. The hybridization signals evident in the lane containing *S. griseus* DNA are likely due to hybridization of the probe to genes possibly involved in the biosynthesis of actinomycin or some other peptide secondary metabolites which are not normally detectable in this strain. Hybridization of the probe to *S. akiyoshiensis* ATCC 13480 DNA indicates that this strain possesses one or more NRPS-like gene sequences. No NRPS-derived antibiotics have been reported from this strain, but it is known to produce the anti-tuberculosis [12] and anti-fungal [18] agent 5-hydroxy-4-oxonorvaline (HON). HON is a non-protein

amino acid, which is derived from aspartate and a molecule of either acetyl coenzyme A or malonyl coenzyme A [17]. During the biosynthesis of HON, a bidomainal NRPS (adenylation domain and peptidyl-carrier protein) module may activate aspartate prior to condensation of the amino acid with the carboxylic acid component. This scheme would be analogous to that observed in the β-hydroxylation and α,β-desaturations of amino acids incorporated into several peptide antibiotics [7]. Therefore, the hybridization signals present in the lane containing *S. akiyoshiensis* DNA may represent a NRPS possibly involved in the biosynthesis of HON or some other undiscovered peptide natural product. The multiple hybridization signals observed in the lanes containing Maui soil isolates 48 and 90 suggest that these strains contain one or more NRPS-like genes. Additionally, the unique banding pattern observed from each isolate suggests that each strain probably contains different NRPS pathways from the other. These results indicate that the use of an alternative probe in conjunction with the RiboPrinter is a fast and efficient means of identifying and comparing specific secondary metabolic pathways from various *Streptomyces* strains. These results also show compatibility of the SulfoProbe labeling method with the RiboPrinter technology platform. Using this labeling method allows for the future development of other alternative probes that can be used to screen microbial isolates for the presence of other secondary metabolic pathways that often produce potent bioactive molecules (e.g. type I or type II polyketide synthase pathways).

In conclusion, a population of morphologically similar *Streptomyces* soil isolates from a single location may be comprised of multiple species and strains capable of producing unique secondary metabolites. Because of this, dereplication of strains is necessary to achieve maximum diversity and minimum duplication in a collection of microorganisms. Automated ribotyping using the RiboPrinter microbial characterization system is a fast, reliable and rapid means of dereplicating morphologically similar *Streptomyces* isolates from soil. This method is more sensitive than both 16S rDNA sequence comparison and fatty acid analysis and is better suited for the detection of strain variation. In addition, the ribosomal probe normally used for ribotyping with the RiboPrinter can be substituted with alternate probes, allowing rapid, automated detection of specific biosynthetic genes in *Streptomyces* isolates.

Acknowledgements We acknowledge Mia Summers, Jim Bruce, Timothy Dambaugh, Eugene Davis, Dave Fruhling and Charlie Carter for their valuable contributions to this project.

References

1. Aidoo DA (1989) Approaches to the cloning of genes for chloramphenicol biosynthesis in *Streptomyces venezuealae* ISP5230. PhD thesis, Dalhousie University, Halifax, Nova Scotia
2. Anzai Y, Okuda T, Watanabe J (1994) Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening. II. Actinomycetes. J Antibiot 47:183–193
3. Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, et al (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417:141–147
4. Bruce J (1996) Automated system rapidly identifies and characterizes microorganisms in food. Food Technol 50:77–81
5. Bull AT, Ward AC, Goodfellow M (2000) Search and discovery strategies for biotechnology: the paradigm shift. Microbiol Mol Biol Rev 64:573–606
6. Campelo AB, Gil JA (2002) The candicidin gene cluster from *Streptomyces griseus* IMRU 3570. Microbiology 148:51–59
7. Chen H, Thomas MG, O'Connor SE, Hubbard BK, Burkart MD, Walsh CT (2001) Aminoacyl-*S*-enzyme intermediates in β-hydroxylations and α,β-desaturations of amino acids in peptide antibiotics. Biochemistry 40:11651–11659
8. Hojati Z, Milne C, Harvey B, Gordon L, Borg M, Flett F, Wilkinson B, Sidebottom PJ, Rudd BA, Hayes MA, Smith CP, Micklefield J (2002) Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. Chem Biol 9:1175–1187
9. Pfaller MA, Wendt C, Hollis RJ, Wenzel RP, Fritschel SJ, Neubauer JJ, Herwaldt LA (1996) Comparative evaluation of an automated ribotyping system versus pulsed-field gel electrophoresis for epidemiological typing of clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa* from patients with recurrent gram-negative bacteremia. Diagn Microbiol Infect Dis 25:1–8
10. Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrempf H (1985) genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich
11. Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HH (ed) Mammalian protein metabolism. Academic Press, New York, pp 21–132
12. Kanazawa K, Tsuchiya K, Araki T (1960) A new antituberculosis amino acid (5-hydroxy-4-oxonorvaline). Am Rev Respir Dis 81:924
13. Reysenbach AL, Wickham GS, Pace NR (1994) Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl Environ Microbiol 60:2113–2119
14. Sikorski J, Stackebrandt E, Wackernagel W (2001) *Pseudomonas kilonensis* sp. nov., a bacterium isolated from agricultural soil. Int J Syst Evol Microbiol 51:1549–1555
15. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882
16. Van de Peer Y, De Wachter R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 10:569–570
17. White RL, Demarco AC, Smith KC (1988) Biosynthesis of the unusual amino acid 5-hydroxy-4-oxonorvaline. J Am Chem Soc 110:8228–8229
18. Yamaguchi M. Yamaki H, Shinoda T, Yoshitaka T, Suzuki H, Nishimura T, Yamaguchi H (1990) The mode of antifungal action of (*S*)-2-amino-4-oxo-5-hydroxypentanoic acid RI-331. J Antibiot 43:411–416