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A novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways

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Abstract The actinomycetes traditionally represent one of the most important sources for the discovery of new metabolites with biological activity; and many of these are described as being produced by polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS). We present a strain characterization system based on the metabolic potential of microbial strains by targeting these biosynthetic genes. After an initial evaluation of the existing bias derived from the PCR detection in a well defined biosynthetic systems, we developed a new fingerprinting approach based on the restriction analysis of these PKS and NRPS amplified sequences. This method was applied to study the distribution of PKS and NRPS biosynthetic systems in a collection of wild-type actinomycetes isolated from tropical soil samples that were evaluated for the production of antimicrobial activities. We discuss the application of this tool as an alternative characterization approach for actinomycetes and we comment on the relationship observed between the presence of PKS-I, PKS-II and NRPS sequences and the antimicrobial activities observed in some of the microbial groups tested.

Introduction

In recent decades, natural-product screening programs devoted an enormous effort to discovering new microbial

secondary metabolites with interesting biological activities. One of the most important steps in the drug discovery process from microbial natural products is the selection of new microorganisms, such as members of the actinobacteria group. The selection criteria applied to wild-type actinomycete strains evolved in response to these needs and include a vast array of morphological, chemo-taxonomic and molecular fingerprinting methods (Peláez and Genilloud 2003). In the past decade, multiple molecular fingerprinting methods were developed for *Streptomyces* and other members of the actinomycete group, based on random amplifications of repetitive sequences from microbial genomes (Anderson and Wellington 2001). Alternatively, the amplification of specific ribosomal gene sequences (Jensen et al. 1993; Welsh and McClelland 1991) or the analysis or restriction patterns of rDNA PCR products (Cook and Meyers 2003) were frequently used to evaluate diversity and to distinguish among large actinomycete populations.

There are several examples where PCR screens for genes associated with secondary metabolism are used to evaluate the biosynthetic potential of actinomycetes. These include non-ribosomal peptide synthetases (NRPS), modular and aromatic polyketide synthases (PKS-I, PKS-II), hydroxymethylglutaryl coenzyme A reductases and aminoglycoside resistance genes (Metsa-Ketela et al. 1999; Anderson et al. 2002; Sigmund et al. 2003; Ritacco et al. 2003; Ayuso and Genilloud 2004). The drawback to these methods is that they are not effective at “dereplicating” or identifying unique isolates without extensive sequencing of the PCR products obtained. It is not currently known whether the detection of high amplification frequencies in a given actinomycete population reflects or not a potential to produce secondary metabolites with biological activity. If so, combining the data derived from traditional molecular fingerprinting methods and new fingerprinting approaches based on the metabolic potential to produce secondary metabolites would enable screening efforts to focus on the most talented groups, increasing the chances of finding secondary metabolites with interesting biological activities.

For this purpose, we initially evaluated the potential bias of the PCR amplification approach when targeting con-

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served sequences within biosynthetic domains, before developing a fingerprinting approach based on the restriction analysis of amplified metabolic gene sequences. This method was later applied to study the occurrence of these biosynthetic systems in a collection of wild-type actinomycetes isolated from tropical soil samples where the presence of PKS-I, PKS-II and NRPS sequences could be related to the antimicrobial activity observed in some of the microbial groups tested. Here, we discuss the application of this approach as an alternative dereplication method for actinomycetes on the basis of their potential to produce bioactive compounds.

Materials and methods

Bacterial strains and cosmids

The wild-type actinomycetes used in this study were isolated from three tropical soils collected on Martinique (Windward Islands, Central America). Soils were serially diluted and plated onto soil extract- and humic acid-based agar media supplemented with 20 µg/ml nalidixic acid and cycloheximide; and colonies were isolated under a stereoscope after observation of their distinct morphology.

The type strains used were *Saccharopolyspora erythraea* MA6655 (ATCC 11635), *S. hygroscopicus* NRRL5491 (ATCC 29253) and *S. avermitilis* MA6847 (ATCC 31267). Cosmids that contained the avermectin biosynthetic pathway (pVE855, pVE859, pVE 923, pVE 924) were obtained from D.J. MacNeil (MacNeil et al. 1992).

All strains were grown at 28°C on YME agar medium (0.4% yeast extract, 1% malt extract, 0.4% glucose, 0.2% Bacto-agar) and ATCC-2 liquid medium (0.5% yeast extract, 0.3% beef extract, 0.5% peptone, 0.1% dextrose, 0.2% potato starch, 0.1% CaCO₃, 0.5% NZamine E).

DNA manipulation

DNA extraction

Total genomic DNA from the different actinomycetes used in this study was recovered and purified as described by Innis et al. (1990).

PCR primers

Consensus ketosynthase (KS) PCR primers KS-BEF (5'-CCGCGCGAGGCGCTGGCCGTCGAC-3') and KS-BER (5'-CCGCGCCGGGCGGGGTCTCGTCGTTCCGCATCAGCGGCACCAACGCG-3') were designed to universally detect KS domains from PKS-I systems. Degenerate PCR primers target specifically NRPS adenylation domains (A3, A7R), PKS-I KS domains and methyl malonyl transferase domains (K1F, M6R), as described by Ayuso and Genilloud (2004). A second degenerate KS primer, K2R

(5'-CVTTCGGVVTCAGCGGSACBAA-3') derived from KS_BER, was used in combination with K1F to amplify specifically a shorter fragment of KS domains. PKS-II sequences were amplified specifically using the primers KS α and KS β (5'-TSGRCTACRTCAACGGSCACGG-3', 5'-TACSAGTCSWTCGCTGGTTC-3'), designed to target conserved sequences in KS α and KS β domains (A. Ayuso, unpublished data). Primers G1 and L1 were used to amplify the variable ribosomal spacer regions between the 16S and 23S rDNAs (Jensen et al. 1993). All primers were supplied by ECOGEN.

PCR amplifications

PCR amplifications were performed in a Peltier PTC-200 thermal cycler in a final volume of 50 µl containing 10% of extracted DNA, 0.4 µmol of each primer, 0.2 mmol of each of the four dNTPs (Roche), 5 µl of extracted DNA, 1 unit of Appligene Taq polymerase (with its recommended reaction buffer) and 10% of DMSO. PKS-I amplifications with primers KS-BE F/R were performed according to the following profiles: 5 min denaturation at 95°C and 35 cycles of 1 min at 94°C, 1 min at 62°C and 1 min extension at 72°C, followed by 10 min at 72°C, 5 min denaturation at 95°C and 15 cycles of 1 min at 94°C, 1 min at 62°C and 1 min extension at 72°C, followed by 10 min at 72°C. NRPS, PKS-I and PKS-II amplifications with degenerate primers were performed according to the following profile: 5 min at 95°C and 35 cycles of 30 s at 95°C, 2 min at either 55°C (for K1F/M6R, K1F/K2R), 58°C (for KS α /KS β) or 59°C (for A3F/A7R) and 4 min at 72°C, followed by 10 min at 72°C. Amplification products were analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide.

G1/L1 PCR amplifications were performed as described by Hirsch and Sigmund (1995). PCR products were analyzed by electrophoresis in 4–20% acrylamide gradient mini-gels (Criterion TBE gels, BioRad).

RFLP analysis

PCR products were cloned using the TOPO TA cloning kit (Invitrogen). Restriction analysis of the clones was performed using *Hinf*I or *Sau*3A1 according to Sambrook and Russel (2001) and separation on 4–20% gradient polyacrylamide gels (Criterion precast gel 4–20% TBE; Bio-Rad).

Cosmid library construction

A total of 3.0 g (wet weight) of *Streptomyces* sp. strain DC328 mycelia was used for genomic DNA isolation. Genomic DNA was prepared for cloning by random shearing and then end-repair was used to generate blunt ends; and sized DNA was then ligated into pWEB::TNC

(Epicentre Technologies) as per the manufacturer's instructions. The DNA was then packaged using MaxPlack packaging extracts and the cosmid was used to infect *Escherichia coli* EPI100-T1, according to the instructions of the manufacturer (Epicentre Technologies). A total of 1,800 cosmid-containing clones were isolated and individually frozen at -80°C in microtiter dish wells.

DNA sequencing

Cloned products were sequenced using universal primers M13R-28 and M13F-20 in an ABI Prism dye terminator cycle sequencing kit (Perkin Elmer).

Evaluation of antimicrobial activity

In vitro antimicrobial susceptibility tests were performed using the following panel of strains: *Bacillus subtilis* MB964 (ATCC 6633) and clinical isolates *Staphylococcus aureus* MB 5393, *E. coli* MB4926 and *Candida albicans* MY1055, all from the Merck Culture Collection. The inoculum and assay plates for bacteria and yeast were prepared as described by Suay et al. (2000). Agar plugs from wild-type actinomycete strains cultivated in YME agar for at least 7 days were applied to the surface of the assay plates, which were incubated at either 28°C (yeast) or 37°C (bacteria). Growth inhibition zones were measured after 24 h of incubation.

Data analysis of G1/L1 PCR amplifications and *HinF1* restriction fingerprinting

G1/L1 PCR amplification patterns and restriction fingerprinting patterns were analyzed with the BioNumerics ver. 2.5 program (Applied Maths). A similar matrix was generated using the Pearson correlation moment; and cluster analysis was performed with the UPGMA algorithm. Sequence database searching was done using BLAST (Altschul et al. 1990). Sequence alignments were done using CLUSTAL W (Thompson et al. 1994) and phylogenetic analysis was done in PHYLIP (Felsenstein 1981), using the neighbor algorithm. The analysis was bootstrapped using 100 replicates.

Results

Development of the type I PKS-I KS consensus PCR screen

The previously described degenerate primers K1F and M6R targeted specifically to PKS-I KS domains and methylmalonyl transferase domains in actinomycetes and had shown the limitations of their use to detect the frequently occurring malonyl-transferase domains in PKS-I biosynthetic pathways (Ayuso and Genilloud 2004). To broaden the detection range, we focused exclusively on KS domains and designed two new pairs of KS PCR primers. One pair was non-degenerate (KS-BE F/R) and encompassed a region of 1,014 nt. The second was degenerate (K2R/K1R) and am-

Table 1 Detection of KS domains in the four model systems studied

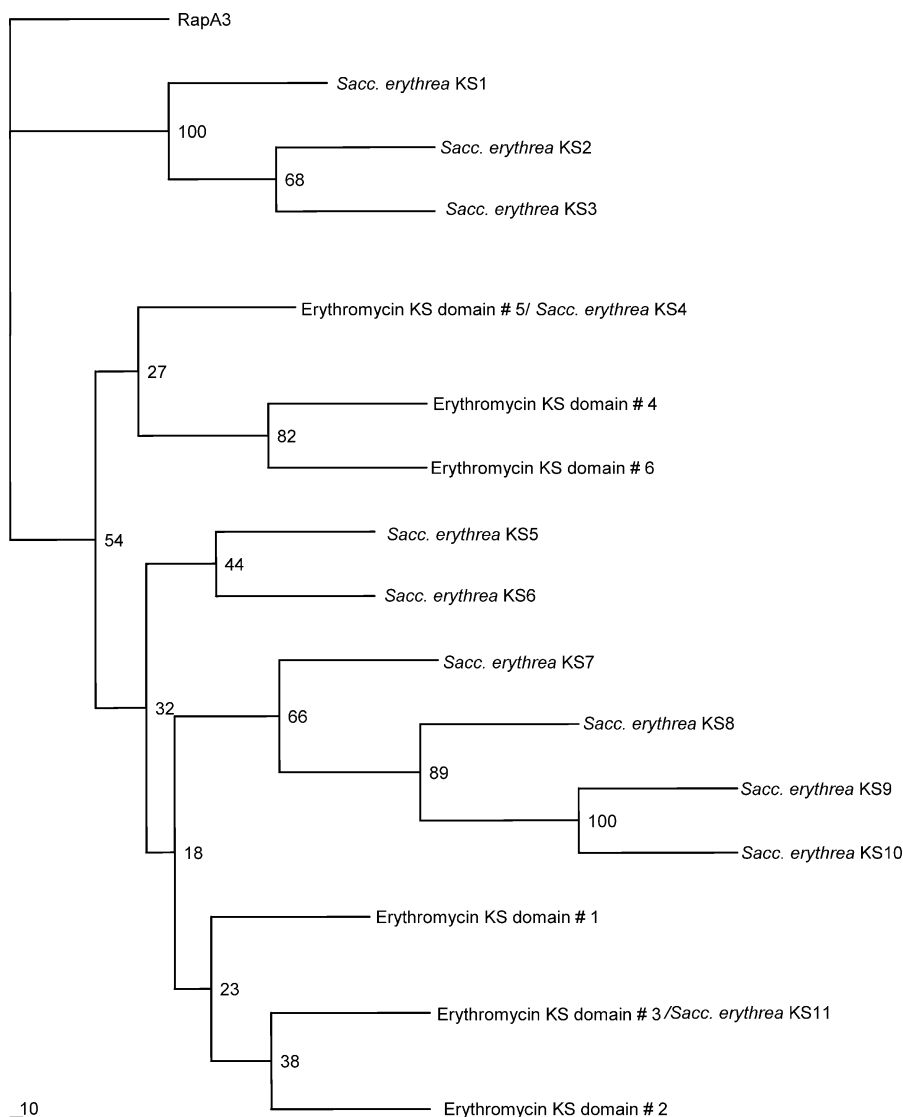
Strain	PCR primers	PKS type I pathways	Target pathway	KS domains in target pathway	KS domain clones screened	KS domains detected from target pathway	KS domains detected from other pathways	Comment
<i>Saccharopolyspora erythraea</i>	KS-BE	Not known	Erythromycin 6	6	10	1	1	Standard PCR: 1 $\mu\text{g}/\text{ml}$ DNA template concentration, $\times 35$ cycles
					60	2	5	100 ng/ml DNA template concentration, $\times 35$ cycles
					60	2	9	100 ng/ml DNA template concentration, $\times 15$ cycles
<i>Streptomyces avermitilis</i>	KS-BE	8	Avermectin	12	60	1	10	Chromosomal DNA
<i>Streptomyces</i> sp DC328	KS-BE	Not known	Not known	Not known	30	-	5	Avermectin cosmids
				known	250	-	25	Chromosomal DNA
<i>Streptomyces hygroscopicus</i>	K1F-K2R	Not known	Rapamycin	12	25	2	14	<i>Streptomyces</i> sp DC328 cosmids
								Chromosomal DNA

plified a 250 bp region. The specificity of both pairs of primers was evaluated using the erythromycin biosynthetic cluster of *Saccharopolyspora erythraea* MA6655 and the rapamycin biosynthetic system of *Streptomyces hygrosopicus* NRRL 5491 respectively as model systems. After amplification of *Saccharopolyspora erythraea* genomic DNA (1 µg) with primers KS-BE F/R, PCR fragments were cloned and analyzed by restriction analysis with *Sau3A1*. The erythromycin biosynthetic pathway from *S. erythraea* contains six KS domains, from which only one was detected among the two distinct clones selected according to their restriction profile. Further PCR optimization was done, which included reducing the number of PCR cycles and reducing the DNA template concentration (Table 1). Ultimately, 11 KS domains were obtained, which included the previously detected KS domains and two additional new ones (Fig. 1). Similarly, genomic DNA from *Streptomyces hygrosopicus* was amplified with the degenerate primers KS1F/KS2R and cloned. Twenty-five clones were selected

for analysis and 16 different KS sequences were identified, of which two were from a rapamycin cluster and the rest were uncharacterized KS domains.

To investigate the distribution of PKS-I KS domains within actinomycete isolates further, we compared the PCR detection of KS domains from isolates having cosmid libraries with the KS domains obtained by direct PCR of the source DNA. To do this, we used *Streptomyces avermitilis*, which has eight documented PKS-I pathways which include avermectin and oligomycin (Omura et al. 2001), and a soil isolate, *Streptomyces* sp DC328, which had an unknown polyketide production capacity but was positive by PCR using the KS-BE PCR primers. Eleven KS domains were detected by screening *Streptomyces avermitilis* chromosomal DNA, of which one was from the avermectin biosynthetic pathway, seven from the oligomycin biosynthetic pathway and an additional three from uncharacterized PKS-I pathways in this strain. Analysis of the four overlapping cosmids that contained the avermectin

Fig. 1 Sequence diversity of KS domains isolated from *S. erythraea* by PCR and comparison of the KS domains obtained from *S. erythraea* with KS domains from the erythromycin biosynthetic pathway. A KS domain from the rapamycin biosynthetic cluster of *Streptomyces hygrosopicus* was included as a root (*RapA3*; accession number X86780). The bootstrap values for 100 replicates are given. Asterisks represent novel KS domains. The accession numbers for the *Saccharopolyspora erythraea* KS domains are: *S. erythraea* KS1 AY678081, *S. erythraea* KS2 AY678082, *S. erythraea* KS3 AY678080, *S. erythraea* KS4 AY678079, *S. erythraea* KS5 AY678073, *S. erythraea* KS6 AY678078, *S. erythraea* KS7 AY678077, *S. erythraea* KS8 AY678076, *S. erythraea* KS9 AY678075, *S. erythraea* KS10 AY678074, *S. erythraea* KS11 AY678083



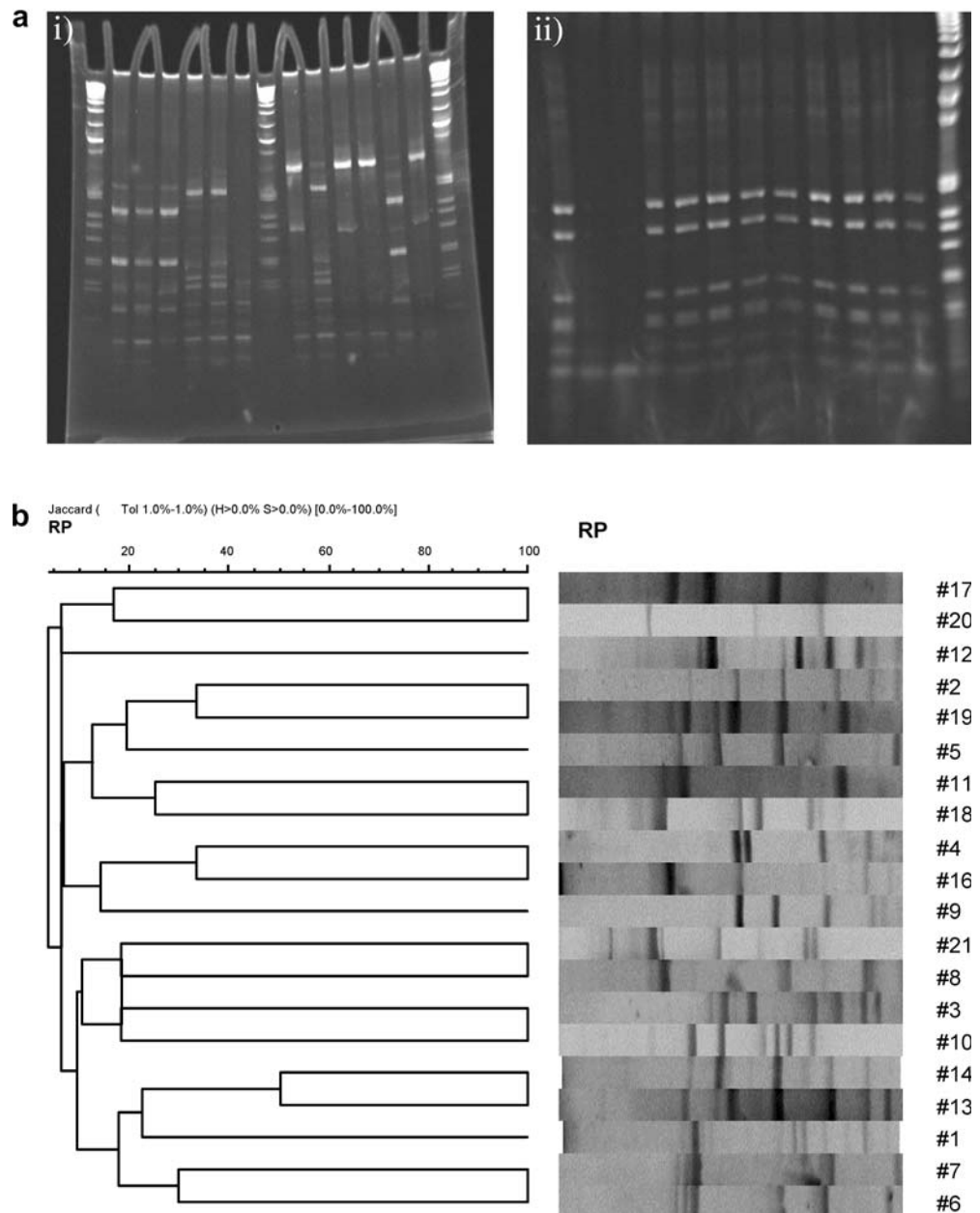
biosynthetic pathway enabled the detection of six of the 12 KS domains (Table 1). Finally, we also tested this approach with *Streptomyces* sp. DC328. Chromosomal DNA was screened by PCR using the KS-BE PCR primers, the PCR products were cloned and 30 clones were characterized by restriction digestion with *Sau3A1*. After sequencing, five unique KS domains were identified. A systematic approach was taken to further evaluate this strain for KS domains by screening a cosmid library that contained 1,800 cosmids; and 49 were positive for KS domains by PCR (Table 1). This represented 2.6% of the library. PCR products were cloned and 250 clones were analyzed by restriction digestion. Eleven cosmids were identified that contained multiple KS domains. Single KS domains were detected from the remaining 38 cosmids that were positive for KS

domains by PCR (Fig. 2a). The different KS domains identified by restriction analysis were all sequenced and found to represent 25 KS domains, including the five domains that were also detected by PCR of chromosomal DNA (Fig. 2b). The diversity of novel KS domains from *Streptomyces* sp. DC328 was compared with KS domains from characterized PKS pathways (Fig. 2c).

Detection of PKS-I, PKS-II and NRPS sequences in wild-type actinomycetes

Taking into account the inherent limitations of the direct PCR method, we focused our study on a wild-type actinomycete population of 329 strains isolated from three

Fig. 2 Evaluation of PKS-I screen using *Streptomyces* sp. DC328. **a** example of multiple-restriction profiles obtained from amplification products from a single cosmid (*i*), compared with single-restriction profiles that were obtained for 38 of the 49 positive cosmids (*ii*). Each gel represents the results for a single cosmid. **b** Dendrogram of 20 of the unique restriction profiles obtained from *Streptomyces* sp. DC328, using *Sau3A1* digests. **c** Cluster analysis of the KS sequences obtained for *Streptomyces* sp. DC328 compared with known PKS-I KS domains. *Streptomyces* sp. DC328 sequences are in numbered red boxes; and all the PKS-I KS domains from characterized pathways are color-coded with the key given in the figure



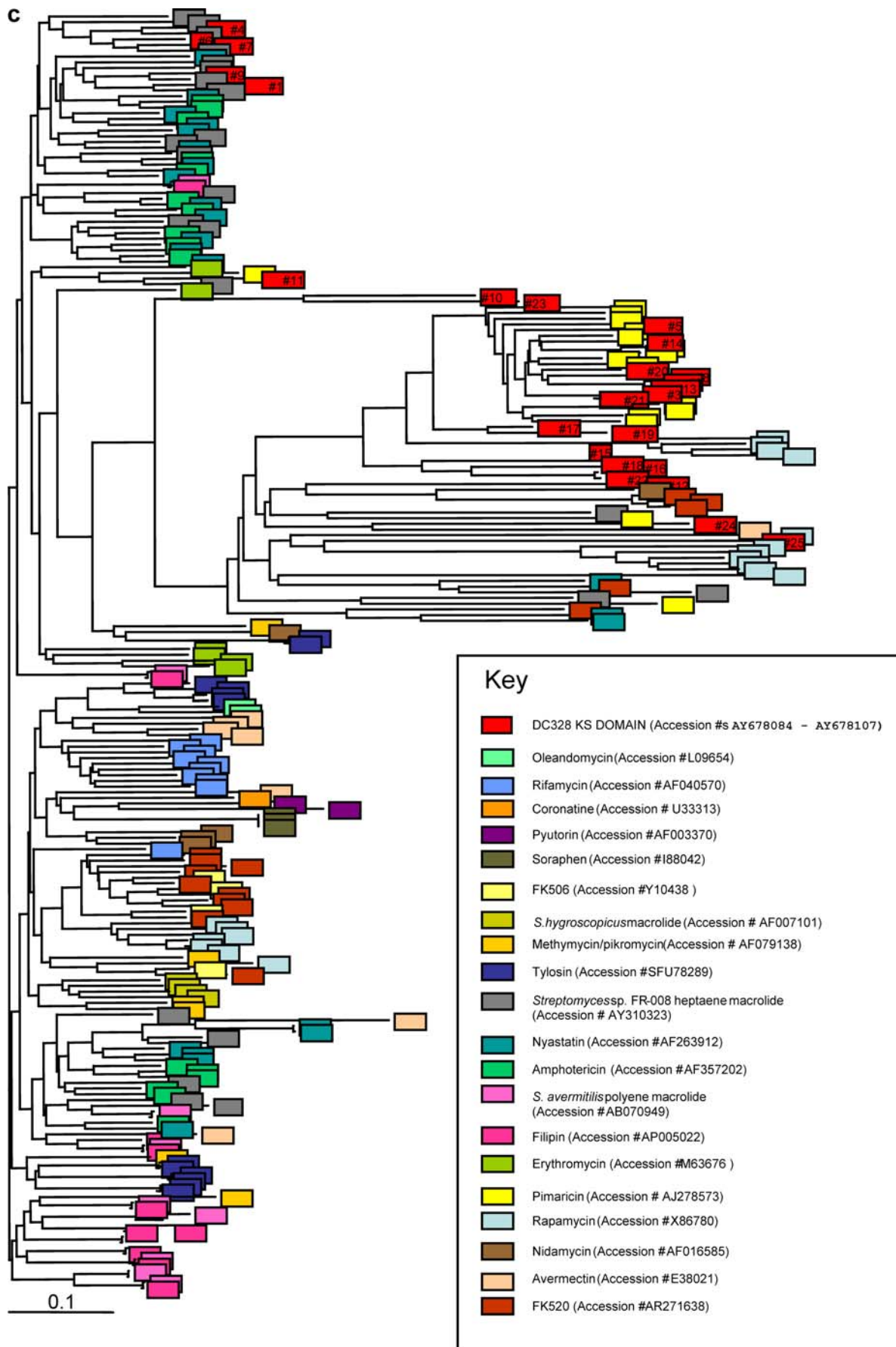


Fig. 2 (continued)

tropical soil samples collected in Martinique, to evaluate the diversity of biosynthetic sequences that could be detected by standard amplification in a microbial population from a tropical environment (Table 2).

Isolates were screened for the presence of NRPS, PKS-I and PKS-II sequences by specific amplification of total DNA. All three pathways were detected at a high frequency in the *Streptomyces*, *Micromonosporaceae* and *Nocardiaceae* populations (Table 2). The detection levels of PKS-I sequences varied according to the specificity of the pair of primers used, K1F/K2R or K1F/M6R. However, the ratios between both pairs remained quite similar in the different actinomycetes groups. Some PKS and NRPS sequences were also detected among the few members of the families *Thermomonosporaceae* and *Streptosporangiaceae* included in the study and in many of the sterile actinomycete strains that could not be assigned to a defined taxonomic group (Table 2).

Antimicrobial activity and presence of biosynthetic gene sequences

The production of antimicrobial activity by the soil isolates was evaluated against a test panel of three bacteria (*B. subtilis*, *Staphylococcus aureus*, *E. coli*) and a yeast (*C. albicans*). Over half (62%) of the *Streptomyces* showed some antimicrobial activity against at least one of the test strains, mostly against the Gram positive test strains *B. subtilis* and *Staphylococcus aureus* (55%). The activity levels against *E. coli* and *C. albicans* were 25% and 14%, respectively. In contrast, very little activity was observed among representatives of the family *Micromonosporaceae* (1.3%) and no activity was obtained within the family

Nocardiaceae. We also detected the production of activity in both members of the *Thermomonosporaceae* and in one of the two strains of the family *Streptosporangiaceae* (Table 2).

To determine any existing relationship between the presence of NRPS and PKS sequences and the potential to produce antimicrobial compounds, isolates were divided into two groups of active and inactive isolates and were analyzed for the presence of these biosynthetic gene sequences. We observed that higher detection rates were obtained for the target pathways in the group of active strains than in the group of inactive cultures (Table 3). In the case of *Streptomyces*, the percentages of positive NRPS and PKS-I amplifications were almost two-fold higher in the active compared with the inactive group; and this difference was not extended to PKS-II sequences (Table 3). This correlation was not seen for the *Micromonosporaceae* and *Nocardiaceae* isolates, due to their low performance in the bioactivity screen (Table 3).

Metabolic PCR fingerprinting and diversity of wild-type actinomycetes

To evaluate the diversity of the wild-type actinomycete population used in the study, we used the amplification of the variable spacer region between ribosomal genes 16S and 23S with primers L1/G1 to generate reproducible fingerprints that allowed strain discrimination below the species level (Hirsch and Sigmund 1995). We compared the amplification patterns of wild-type *Streptomyces* and members of the families *Micromonosporaceae* and *Nocardiaceae* containing NRPS, PKS-I and PKS-II sequences; and we built three dendrograms. The strains were grouped

Table 2 PCR detection of PKS-I, PKS-II and NRPS biosynthetic sequences and antimicrobial activities in wild-type soil actinomycetes. For taxonomy, isolates were characterized to the genus level,

using micromorphological characters. Antimicrobial test panel: *BS Bacillus subtilis*, *SA Staphylococcus aureus*, *EC Escherichia coli*, *CA Candida albicans*

Taxonomic group	Total strains	Biosynthetic sequences				Active strains	Antimicrobial test panel activity			
		NRPS A3F/A7R	PKS-I K1F/K2R K1F/M6R		PKS-II KS α /KS β		BS	SA	EC	CA
Streptomycetaceae										
<i>Streptomyces</i>	221	134	160	114	153	138	110	64	54	33
Micromonosporaceae										
<i>Actinoplanes</i>	17	12	8	5	8	1	1	0	0	0
<i>Micromonospora</i>	26	20	16	8	11	5	4	1	4	1
<i>Dactylosporangium</i>	1	0	0	0	0	0	0	0	0	0
Sterile cultures ^a	23	20	14	13	13	0	0	0	0	0
Nocardiaceae										
<i>Rhodococcus</i>	1	1	1	1	1	0	0	0	0	0
<i>Nocardia</i>	17	17	16	16	15	0	0	0	0	0
Thermomonosporaceae										
<i>Actinomadura</i>	2	1	1	1	1	2	2	2	0	0
Streptosporangiaceae										
<i>Streptosporangium</i>	2	2	2	1	2	1	1	0	0	0
Non-identified	19	14	14	8	4	6	5	1	4	0

^aThese isolates were assigned to this family after specific PCR amplification with *Micromonosporaceae* primers

Table 3 PCR detection of NRPS, PKS-I and PKS-II biosynthetic systems in wild-type soil actinomycetes. Wild-type strains were divided into two groups: active strains, which included strains

showing antimicrobial activity against at least one of the four control strains tested, and inactive strains, which included strains for which no activity was observed in the antimicrobial assays

Taxonomic group	Active strains	NRPS	PKS-I		PKS-II	Inactive strains	NRPS	PKS-I		PKS-II
		A3F/A7R positive	K1F/K2R positive	K1F/M6R positive	KS α /KS β positive		A3F/A7R positive	K1F/K2R positive	K1F/M6R positive	KS α /KS β positive
Streptomycetaceae	138	101	110	90	106	83	32	50	24	47
Micromonosporaceae	6	4	4	2	3	61	48	34	24	29
Nocardiaceae	0	0	0	0	0	18	18	17	17	16
Thermomonosporaceae	2	1	1	1	0	0	0	0	0	0
Streptosporangiaceae	1	1	1	1	1	1	1	1	0	1
Non-identified	6	4	6	3	3	13	10	8	5	1
Total	153	109	122	98	114	176	109	106	72	98

into 14 clusters of closely related strains with above 75% similarity in both *Streptomyces* and the *Micromonosporaceae*, whereas only two clusters were outlined in the *Nocardiaceae* (data not shown).

An alternative fingerprinting approach based on restriction analysis of the amplified biosynthetic sequences was explored to determine whether isolates could be simply characterized for their biosynthetic potential. *Hin*I restriction patterns were studied for the NRPS, PKS-I and PKS-II amplification products of the strains included in the three major groups in study. We observed in almost all cases a high pattern heterogeneity among the strains included in the clusters defined on the amplification of ribosomal sequences, suggesting a quite diverse biosynthetic potential even among closely related strains. To define any potential relatedness among the restriction patterns observed within each of the microbial groups studied, we built three dendrograms for each of the major taxonomic groups studied, on the basis of a similarity matrix generated by the addition of the three restriction fingerprints (Figs. 3, 4, 5).

Most of the wild-type strains analyzed present distinct NRPS and PKS restriction patterns and correspond to different isolates, in spite of their closely related ribosomal fingerprint patterns. Nevertheless we observed in the three major groups studied some cases of similar restriction patterns in ribosomally related isolates (Figs. 3, 4, 5). The similarity of restriction patterns was also paralleled by a similar antimicrobial profile in *Streptomyces*, suggesting the presence of duplicate isolates within the population studied. In addition, in *Streptomyces* and the *Micromonosporaceae* we observed cases of high similarity of restriction patterns between unrelated strains assigned to different ribosomal clusters (Fig. 3, 4).

Discussion

In this investigation, we evaluated the specific detection of KS domains from four model systems and demonstrated that, despite the abundance of these target domains within the strains studied, one can only obtain a proportion of the

domains that are present, leaving the metabolic potential of the isolates underestimated as a result of the bias introduced by the PCR method itself (Ishii and Fukui 2001; Suzuki and Giovannoni 1996). Even using the most optimized procedures, as in the case of *Streptomyces avermitilis*, only half of the 12 avermectin domains were detected despite using cosmids that contained the pathway as templates. Many novel KS domains were detected using these model systems, highlighting the abundance of putative novel PKS pathways. To further evaluate this, novel KS domains from *Streptomyces* sp. DC328 were compared with published KS domains from characterized macrolide biosynthetic pathways, using a phylogenetic approach (Fig. 2c). The trend for characterized PKS pathways is for KS domains within a module to cluster together. The hypothetical KS domains detected in this study appeared to form distinct clusters, but one cannot use this as a predictive tool for KS pathways without a more extensive analysis of the cosmids from which they were detected. Direct PCR analysis has the obvious limitations that it may underestimate diversity and may detect irrelevant pathways. Despite this, we obtained data that demonstrate that the metabolic potential of these isolates was clearly under-represented.

We went on to use our optimized procedures to evaluate our wild-type population. Previous studies focused on how similar methods can be used to assess the metabolic potential of members of the *Streptomyces* genus (Sosio et al. 2000; Metsa-Ketela et al. 2002). This study confirmed these findings and expanded our knowledge about the distribution of NRPS, PKS-I and PKS-II pathways in members of the families *Micromonosporaceae* and *Nocardiaceae* and in other minor population components, where few producers have been described.

The results we derived from a comparison of the microbial populations with and without antimicrobial activity suggest that, in strains of the genus *Streptomyces*, a clear relationship can be established between the occurrence of biosynthetic gene sequences and the production of antimicrobial activities. This correlation was not seen for the other major families studied. It should be considered that not all

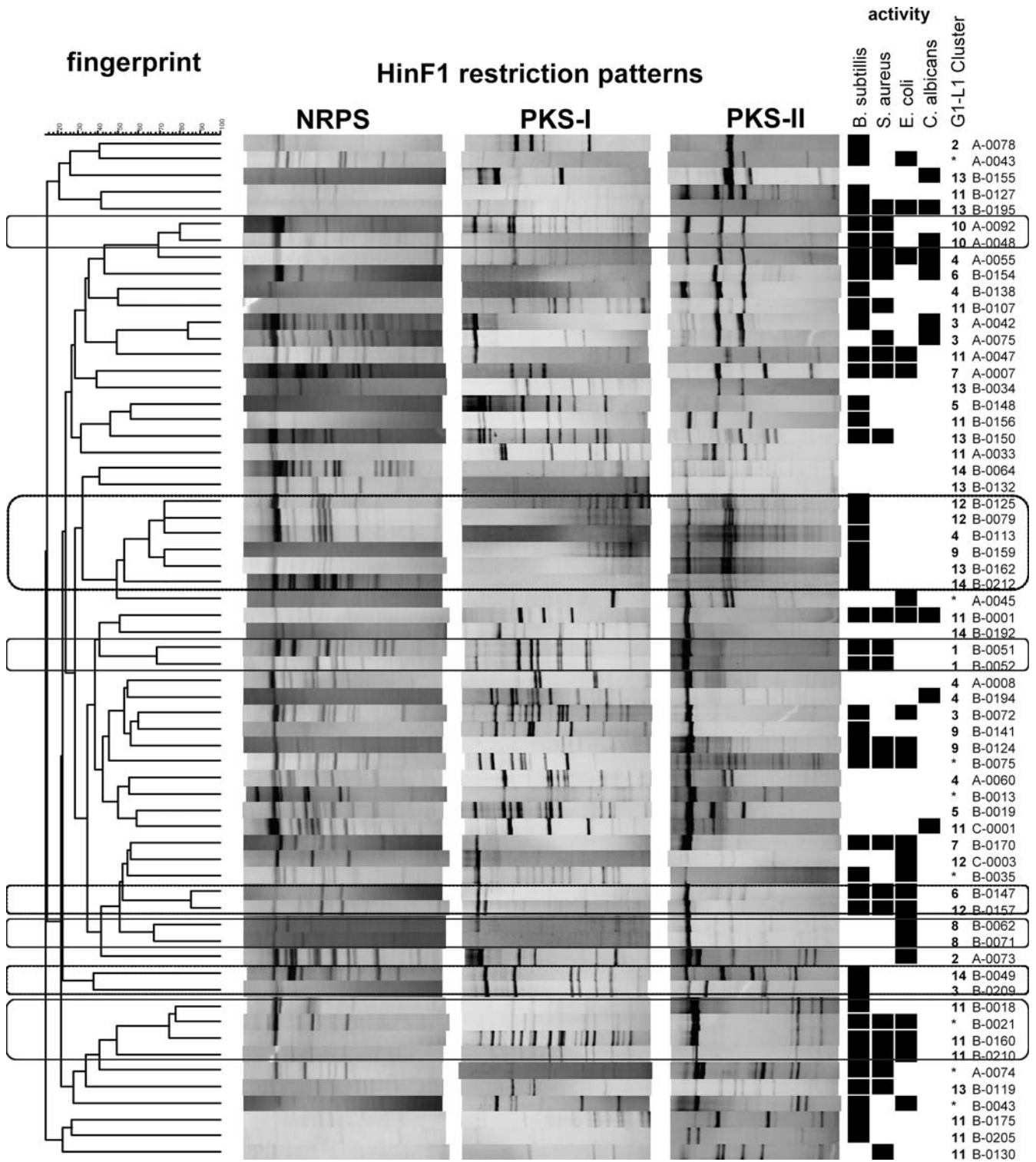


Fig. 3 Diversity of wild-type *Streptomyces*. Dendrogram showing similarity, based on the *Hin*I restriction patterns of NRPS, PKS-I and PKS-II amplification products. The L1/G1 ribosomal fingerprint cluster assignment is indicated for each isolate. *Solid lines* Duplicate

strains with similar restriction profiles, *dashed lines* unrelated isolates with similar restriction profiles. *Right* The antimicrobial activity pattern is shown by *squares* for each isolate

the sequences detected with our primers are necessarily involved in the synthesis of industrially relevant bioactive metabolites and that the amplification products can reflect the presence of other genes involved in the biosynthesis of

other types of metabolites, such as pigments or structural components of the microbial cell (Portevin et al. 2004). It is also important to note that biological activities of interest are governed by the screen to which the isolate is

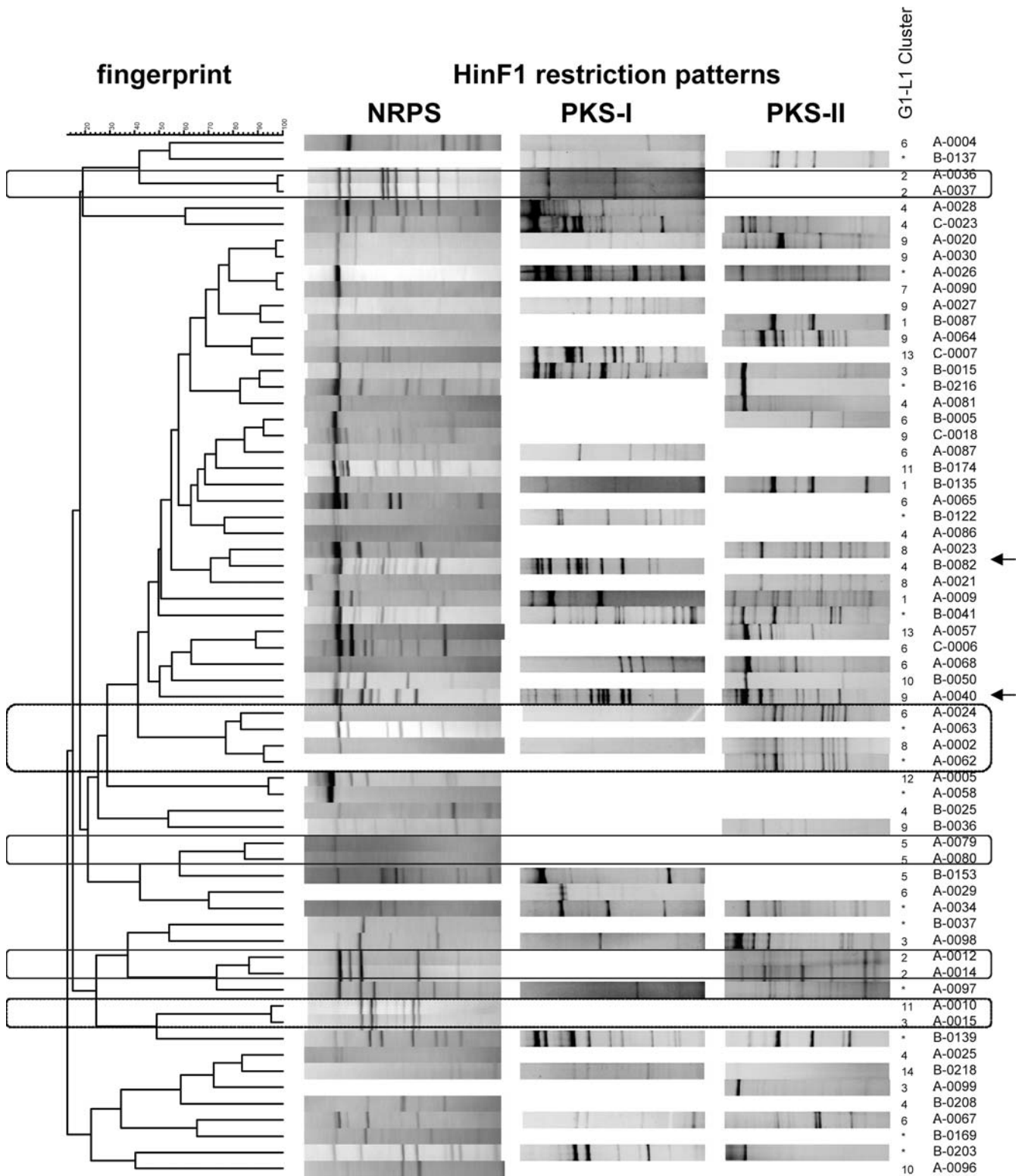


Fig. 4 Diversity of wild-type *Micromonosporaceae*. Dendrogram showing similarity, based on the *HinF1* restriction patterns of NRPS, PKS-I and PKS-II amplification products. The L1/G1 ribosomal fingerprint cluster assignment is indicated for each isolate. Solid

lines Duplicate strains with similar restriction profiles, dashed lines unrelated isolates with similar restriction profiles, arrows strains with antimicrobial activity against *S. aureus* MB5393 and *B. subtilis* MB954

subjected. In this investigation, we looked for antimicrobial activity, because actinomycetes produce compounds that have anti-tumor, anti-helminthic and immunosuppres-

sive properties, to name but a few (Anderson et al. 2000). We can use the detection of a secondary metabolite pathway as an indicator of metabolic potential, but

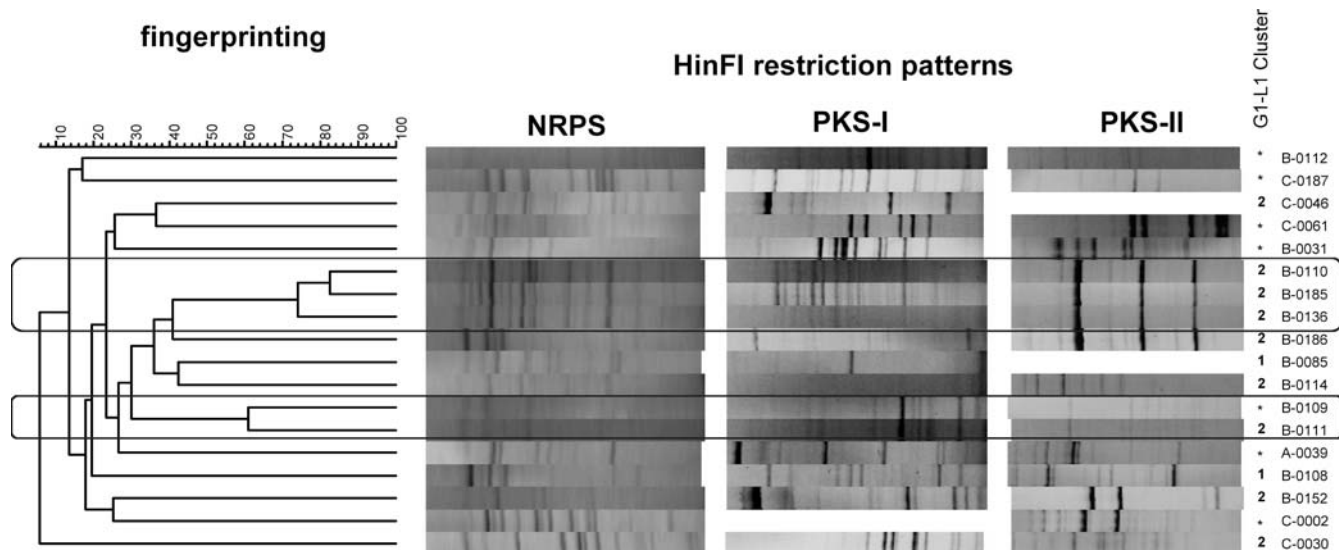


Fig. 5 Diversity of wild-type *Nocardiaceae*. Dendrogram showing similarity, based on the *HinFI* restriction patterns of NRPS, PKS-I and PKS-II amplification products. The L1/G1 ribosomal fingerprint

cluster assignment is indicated for each isolate. *Solid lines* Duplicate strains with similar restriction profiles

the strain must still be grown in a way that will express the pathway and be subject to the right biological assays. Studies of sequenced bacterial genomes of known strains such as *Streptomyces coelicolor* A(3)2 and *S. avermitilis* have also demonstrated numerous silent pathways for which concerted cloning and fermentation efforts have demonstrated production and activity (Chen et al. 2000; Challis and Hopwood 2003; Hopwood 2003; Knight et al. 2003; Zazopoulos et al. 2003).

The approaches discussed in this study help to identify isolates with a potential talent. We also investigated a rapid method to fingerprint strains using PCR products obtained from metabolic screens as an alternative characterization tool to be applied not only in the study of distinct isolates among ribosomally related groups, but also in the identification of enriched populations with different biosynthetic potentials to be studied for the production of antimicrobial compounds. Our results show the diversity of restriction amplification profiles can suggest the extreme richness in these sequences within each individual strain in the three microbial groups studied. The examples of unrelated wild-type strains sharing similar PCR restriction fingerprints could reflect the occurrence of horizontal gene transfer events within members of a given microbial community, as was characterized by Sigmund et al. (2003) for hydroxy-3-methylglutaryl coenzyme A reductase pathways in natural actinomycete populations.

A concentrated effort is still required to unravel the regulation of how these pathways are expressed, along with fermentation studies, so that this as yet untapped biotechnological resource can be harnessed from these taxa.

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References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Anderson AS, An Z, Strohl WR (2000) Polyketide antibiotics. In: Lederberg J (ed) *Encyclopedia for microbiology*, vol 3. Academic, San Diego, pp 241–254
- Anderson AS, Wellington EMH (2001) The taxonomy of *Streptomyces* and related genera. *Int J Syst Evol Microbiol* 51:797–814
- Anderson AS, Clark D, Gibbons P, Sigmund J (2002) The detection of diverse aminoglycoside phosphotransferases within natural populations of actinomycetes. *J Ind Microbiol Biotechnol* 29:60–69
- Ayuso A, Genilloud O (2004) New molecular tools for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution in major taxonomic groups. *Microb Ecol* (in press)
- Cook AE, Meyers PR (2003) Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. *Int J Syst Evol Microbiol* 53:1907–1915
- Challis GL, Hopwood DA (2003) Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc Natl Acad Sci USA* 100:14555–14561
- Chen G, Wang GY, Li X, Waters B, Davies J (2000) Enhanced production of microbial metabolites in the presence of dimethyl sulfoxide. *J Antibiot* 53:1145–1153
- Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 17:368–376
- Hirsch CF, Sigmund JM (1995) Use of polymerase chain reaction (PCR) fingerprinting to differentiate bacteria for microbial products screening. *J Indust Microbiol* 15:85–93
- Hopwood DA (2003) The *Streptomyces* genome—be prepared! *Nat Biotechnol* 21:505–506
- Innis MA, Gelfand DH, Sninsky JJ, White TJ (1990) *PCR protocols. A guide to methods and applications*. Academic, San Diego
- Ishii K, Fukui M (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. *Appl Environ Microbiol* 67:3753–3755
- Jensen MA, Webster JA, Straus N (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* 59:945–952

- Knight V, Sanglier J-J, DiTullio D, Braccili S, Bonner P, Waters J, Hughes D, Zhang L (2003) Diversifying microbial natural products for drug discovery. *Appl Microbiol Biotechnol* 62:446–458
- MacNeil DJ, Occi JL, Gewain KM, MacNeil T, Gibbons PH, Ruby CL, Danis SJ (1992) Complex organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase. *Gene* 115:119–125
- Metsä-Ketela M, Salo V, Halo L, Hautala A, Hakala J, Mantsala P, Ylihönko K (1999) An efficient approach for screening minimal PKS genes from *Streptomyces*. *FEMS Microbiol Lett* 180:1–6
- Metsä-Ketela M, Halo L, Munukka E, Hakala J, Mantsala P, Ylihönko K (2002) Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal dna genes from various *Streptomyces* species. *Appl Environ Microbiol* 68:4472–4479
- Omura S, Ikeda H, Ishikawa J, Hanamoto A, Takahashi C, Shinose M, Takahashi Y, Horikawa H, Nakazawa H, Osonoe T, Kikuchi H, Shiba T, Sakaki Y, Hattori M (2001) Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proc Natl Acad Sci USA* 98:12215–12220
- Peláez F, Genilloud O (2003) Discovering new drugs from microbial natural products. In: Barredo J-L (ed) *Microorganisms for health care, food and enzyme production*. Research Signpost, Trivandrum, pp 1–23
- Portevin D, Sousa-D'Auria C de, Houssin C, Grimaldi C, Chami M, Daffé M, Guilhot C (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proc Natl Acad Sci USA* 101:314–319
- Ritacco FV, Haltli B, Janson JE, Greenstein M, Bernan VS (2003) Dereplication of *Streptomyces* soil isolates and detection of specific biosynthetic genes using an automated ribotyping instrument. *J Ind Microbiol Biotechnol* 30:472–479
- Sambrook J, Russel DW (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Sigmund JM, Clark DJ, Rainey FA, Anderson AS (2003) Detection of eubacterial 3-hydroxy-3-methylglutaryl coenzyme A reductases from natural populations of actinomycetes. *Microb Ecol* 46:106–112
- Sosio M, Bossi E, Bianchi A, Donadio S (2000) Multiple peptide synthase gene clusters in actinomycetes. *Mol Gen Genet* 264:213–221
- Suay I, Arenal F, Asensio FJ, Basilio A, Cabello MA, Diez MT, Garcia JB, Val AG del, Gorrochategui J, Hernandez P, Pelaez F, Vicente MF (2000) Screening of basidiomycetes for antimicrobial activities. *Antonie Van Leeuwenhoek* 78:129–139
- Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* 62:625–630
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Welsh J, McClelland M (1991) Genomic fingerprints produced by PCR with consensus tRNA gene primers. *Nucleic Acids Res* 19:861–866
- Zazopoulos E, Huang K, Staffa A, Liu W, Bachmann BO, Nonaka K, Ahlert J, Thorson JS, Shen B, Farnet CM (2003) A genomics-guided approach for discovering and expressing cryptic metabolic pathways. *Nat Biotechnol* 21:187–190