Antibiotic-producing ability by representatives of a newly discovered lineage of actinomycetes

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The discovery of new antibiotics and other bioactive microbial metabolites continues to be an important objective in new drug research. Since extensive screening has led to the discovery of thousands of bioactive microbial molecules, new approaches must be taken in order to reduce the probability of rediscovering known compounds. The authors have recently isolated slow-growing acidophiles belonging to the novel genera *Catenulispora* and *Actinospica* within the order *Actinomycetales*. These strains, which likely belong to a new suborder, grow as filamentous mycelia, have a genome size around 8 Mb, and produce antimicrobial activities. In addition, a single strain harbours simultaneously genes encoding type I and type II polyketide synthases, as well as non-ribosomal peptide synthetases. The metabolite produced by one strain was identified as a previously reported dimeric isochromanone. In addition, at least the *Catenulispora* strains appear globally distributed, since a PCR-specific signal could be detected in a significant fraction of acidic soils from different continents, and similar strains have been independently isolated from an Australian soil (Jospeh *et al.*, *Appl Environ Microbiol* **69**, 7210–7215, 2003). Thus, these previously uncultured actinomycetes share several features with *Streptomyces* and related antibiotic-producing genera, and represent a promising source of novel antibiotics.

INTRODUCTION

Programmes aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture. However, it soon became apparent during screening programmes that some microbial metabolites were discovered more frequently than others. As the number of described microbial metabolites increased, so did the probability of rediscovering known compounds. At the present time, with several thousands of described microbial metabolites, strategies must be introduced into screening programmes to increase the chances of discovering novel compounds (Bull *et al.*, 2000; Lancini *et al.*, 1995). The filamentous actinomycetes account for a significant fraction of microbial metabolites and, among them, *Streptomyces* is by far the most prolific genus. The pharmaceutical industry, over several decades, has probably isolated and screened millions of *Streptomyces* strains. Consequently, the chances of isolating a novel *Streptomyces* strain have substantially diminished, and so has the probability of discovering a novel

compound. Therefore, while it has been estimated that only a fraction of the antibiotics produced by *Streptomyces* strains have been discovered (Watve *et al.*, 2001), identifying the undiscovered portion will require a substantial effort.

Genomic studies indicate that the genetic potential for producing secondary metabolites is not uniformly distributed within the bacterial world. In fact, most bacterial genomes lack any detectable gene cluster for secondary metabolism. On the other hand, *Streptomyces coelicolor* (Bentley *et al.*, 2002) and *Streptomyces avermitilis* (Ikeda *et al.*, 2003; Omura *et al.*, 2001) each possess more than 20 gene clusters devoted to the synthesis of secondary metabolites. Multiple gene clusters for secondary metabolism appear also to be a hallmark of several non-*Streptomyces* filamentous actinomycetes (Sosio *et al.*, 2000) and of the myxobacteria (Gerth *et al.*, 2003). It is interesting to point out that genomic data parallel the observed discovery events and indicate that streptomycetes, pseudomonads and bacilli have yielded large numbers of metabolites since they can be easily isolated and have a relatively large genetic potential for producing secondary metabolites. It is thus likely that most microbial strains that could be isolated in large numbers have actually been screened for bioactivities.

Historically, significant advances in the discovery of new antibiotics occurred when previously little-known actinomycete genera were isolated in high numbers and rapidly

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Abbreviations: ITS, internal transcribed spacer; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are given in Tables 1–3.

screened (Parenti & Coronelli, 1979; Wagman & Weinstein, 1980). In recent years, the family *Micromonosporaceae* has been enlarged by the isolation of previously unreported genera from marine sources (Magarvey *et al.*, 2004; Mincer *et al.*, 2002). We also reasoned that previously uncultured actinomycete strains might represent a potential source of novel bioactive metabolites (Donadio *et al.*, 2005), and have recently proposed the new genera *Catenulispora* (Busti *et al.*, 2006) and *Actinospica* (Cavaletti *et al.*, 2006), which belong to the novel suborder *Catenulisporinae* (Cavaletti *et al.*, 2006). However, frequent production of secondary metabolites is confined to selected families within the order *Actinomycetales*. Here, we show that representatives of the genera *Catenulispora* and *Actinospica* share several features typical of antibiotic-producing *Streptomyces* and related actinomycetes.

METHODS

Soils, strains and culture conditions. All soil samples were from the historical collection in our laboratories. Samples were air-dried at 30 °C for 2–7 days and stored in a sealed plastic container before use. Source 11943 is an acidic (pH 4.2) soil sample collected from a temperate forest in Gerezano, Italy. Source 8505 is an acidic soil sample (pH 5.0) from Nicaragua. Source 12228 (pH 6.8) was collected in a chestnut wood near Salerno, Italy.

The strains used in this study are reported in Table 1. They were isolated in three distinct campaigns from independent aliquots of soils 11943 and 8505. Strains were maintained on HSA (Suzuki *et al.*, 2001), BTT-agar or ISP3 plates (Atlas & Park, 1993), all adjusted to pH 4.5–5.8 with HCl after autoclaving. As liquid media, BTT and ATSB (Busti *et al.*, 2006) were used for the Neo and Gamma strains, respectively. Both media were adjusted to pH 6 with HCl before autoclaving. Growth temperature was 28 °C and, depending on inoculum, strains required 4–21 days for vigorous growth.

Phylogenetic and automated ribosomal intergenic spacer analyses. Genomic DNA was extracted with the GenElute Bacterial Genomic DNA kit (Sigma). PCR amplification and sequence of the 16S rRNA gene, and phylogenetic analyses, were performed as described by Monciardini *et al.* (2002). The 16S–23S internal transcribed spacer (ITS) was amplified as described by Mazza *et al.* (2003), except that the forward primer was 5'-end labelled with the fluorochromes NED (yellow), FAM (blue) and HEX (green). The sizes of amplified PCR products were determined by capillary electrophoresis using an ABI 310 genetic analyser (Applied Biosystems). Sample preparation and run conditions were as described by Fisher & Triplett (1999). The internal size standard was GeneScan-1000 and data were analysed by using the GENESCAN 3.1 software (Applied Biosystems). Only the number and apparent sizes of peaks were considered; relative peak area was not used for strain comparison. Sizes obtained in different runs for the three independent PCR products corresponding to each strain (each labelled with a different dye) were compared, and peak size was calculated as the mean of the three measures.

Genome size determination. Strains were grown under shaking conditions until sufficient growth was observed. At this point, 3 mm glass beads were added, and flasks were shaken for a further 3–7 h. Then the dispersed mycelium was reinoculated into fresh medium and grown for a further 1–3 days. Agarose plugs were prepared by a modification of the procedure described by Beyazova *et al.* (1995). Briefly, mycelia were harvested by centrifugation and, if necessary,

Table 1. Strains analysed

Strain	Source*	16S rRNA accession no.	ITS size(s)†
Neo1	11943	AJ865855	565
Neo2	11943	AJ865856‡	565, 579 and 582
Neo3§	11943	AJ865857	566 and 582
Neo6	11943	AJ865858	561 and 567
Neo15	11943	AJ865860‡	565 and 575
Neo9	11943	AJ865859	565 and 579
Gamma3¶	11943	AJ865861	637
Gamma4	11943	AJ865862	637 and 641
Gamma6¶	11943	AJ865863#	574 and 619
Gamma25	8505	AJ865864	590, 592 and 623
Gamma22	11943	AM157335#	574 and 619
Gamma26	8505	AJ865865	587, 593, 621 and 626
Gamma28	8505	AJ865866#	589 and 623

*Numbers correspond to the internal source collection codes.

†Mean size (nt) of the ITS peak(s), rounded to the nearest unit.

‡Strains Neo2 and Neo15 have identical 16S rRNA gene sequences.

§Strain Neo3 corresponds to *C. acidiphila* ID139908^T (Busti *et al.*, 2006).

||Strains Neo3 and Neo9 have identical 16S rRNA gene sequences.

¶Strains Gamma3 and Gamma6 correspond to *A. acidiphila* GE134766^T and *A. robiniae* GE134769^T, respectively (Cavaletti *et al.*, 2006).

#Strains Gamma6 and Gamma22 have identical 16S rRNA gene sequences.

frozen at –80 °C in 10% (v/v) glycerol. After thawing, cells were washed twice with 0.5 M EDTA pH 8 and resuspended in 10 ml of the same buffer. After addition of 2 ml phenol saturated with TE (10 mM Tris/HCl, 1 mM EDTA, pH 8) and a 15 min incubation at 65 °C, cells were washed three times with chloroform/isoamyl alcohol (24:1, v/v) and then incubated in 2% SDS, 2 mM EDTA for 15 min at 65 °C. After washing once with 1 mg BSA ml^{–1} in 5 mM EDTA and three times with TE, the harvested cells were suspended in TESuc (0.3 M sucrose in TE) to obtain a final OD₆₀₀ between 2 and 3. The cell suspension was mixed with an equal volume of melted 2% low-melting-point agarose and kept at 50 °C before pouring into plug moulds, where it was allowed to solidify at 4 °C. Agarose plugs were treated with 1 mg lysozyme ml^{–1} in TESuc for 2–3 h at 37 °C, then overnight with 1 mg subtilisin ml^{–1} in TE at 50 °C. Finally, plugs were washed three times, 1 h each, with TE at 50 °C. Restriction digestions were performed by pre-incubating plugs in 500 µl of suitable buffer for 15 min at 37 °C, followed by 5–6 h at 37 °C with 40 U of enzyme with occasional shaking (for *AseI*, after 2–3 h an additional 10 U were added).

Pulsed-field gel electrophoresis was performed on 1% agarose gels (PFGE certified, Bio-Rad) in 0.5 × TBE (Tris/borate/EDTA; Sambrook & Russell, 2001), containing 50 mg thiourea l^{–1}, in a CHEF-DRIII apparatus (Bio-Rad). Running conditions were 6 V cm^{–1} at 14 °C. The run consisted of 15 h at a switch time of 70 s and a field angle of 120°, followed by 11 h at a switch time of 120 s and a field angle of 120°. Fragment sizes were estimated with the help of size standards such as *Saccharomyces cerevisiae* chromosomes (BioLabs) or *AseI*-digested *Streptomyces coelicolor* genomic DNA, prepared as described by Kieser *et al.* (1992).

Amplification of secondary metabolism genes. For the amplification of the ~700 bp segment encoding the KS domain from modular polyketide synthases (PKS-I), we used the primers described by Courtois *et al.* (2003), with a slight modification of their PCR protocol. Briefly, the PCR mixture consisted of 500 nM each primer, 200 µM dNTPs, 10 mM Tris/HCl (pH 8.3), 2.5 mM MgCl₂, 50 mM KCl, 7% dimethyl sulfoxide, 2 U RedTaq DNA polymerase and 10–20 ng genomic DNA, in a final volume of 50 µl. After a 5 min incubation at 95 °C, three cycles (each consisting of 45 s at 95 °C, 45 s at 65 °C and 1 min at 72 °C) were followed by 30 cycles under identical conditions, except that the annealing temperature was lowered to 62 °C. To amplify a portion of the ~600 bp segment encoding KSz from PKS-II genes, we used the conditions described by Metsä-Ketelä *et al.* (1999). To amplify the 450 bp segment encoding the adenylation (A) domain of non-ribosomal peptide synthetases (NRPSs), we used the primers ADEdom5 (5'-ACSGGCNNNCCSAAGGGCGT-3') and ADEdom3 (5'-CTCSGTS-GGSCCGTA-3'). The PCR mixture was as above, except for 1 µM each primer, 1.5 mM MgCl₂ and 3% DMSO. After a 5 min incubation at 96 °C, 10 cycles (each of 1 min at 95 °C, 45 s at 50 °C and 1 min at 72 °C) were followed by 20 cycles under identical conditions, except that the annealing temperature was raised to 55 °C. All PCR reactions were terminated with a 10 min elongation at 73 °C.

PCR products were cloned in the pGEMT-Easy vector (Promega), and the ligation mixture was used to transform *E. coli* by standard procedures (Sambrook & Russell, 2001). Plasmids were recovered with the Genelute Plasmid Miniprep Kit (Sigma) and inserts sequenced using vector primers. Sequences were compared to GenBank through FASTA (Pearson, 1990). All other sequence analyses were performed with programs of the Wisconsin Package, Version 9.1 (Genetics Computer Group). Phylogenetic analyses were performed with programs of the PHYLIP package (Felsenstein, 1993). Putative substrate specificity of A domains was determined by comparison with the NRPS8AA database maintained at the NP Biogene web site (<http://www.npbiogene.com>).

Antimicrobial activities. The production of bioactive metabolites was assayed against *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (L819), *Micrococcus luteus* (ATCC 9341) and *Escherichia coli* (L47) on AM1 plates containing 5×10^5 c.f.u. ml⁻¹ of the test micro-organism. All strains were from the Vicuron strain collection. Bioactivity was determined employing either agar plugs cut from 7–10-day-old plates, or 10 µl from a 4-day broth culture.

For metabolite extraction, strains Neo3 and Gamma22 were grown on a BTT or ISP3 agar plate, respectively, for 7–10 days at 28 °C. Then 7 ml methanol per g solid medium was added and thoroughly mixed; after agar removal, the resulting liquid phase was extracted twice with 1 vol. ethyl acetate. The organic phase was dried under vacuum and dissolved in 0.5 ml 10% DMSO. An aliquot was fractionated by HPLC using a 5 µ-symmetry C₁₈ column (Waters Chromatography), and the effluent was subjected to mass spectrometry and diode-array UV-VIS analysis. Fractions from the HPLC column were collected and tested for biological activity against *Staph. aureus*. The UV and MS parameters of the biologically active fraction(s) were searched against Vicuron's proprietary database of microbial products (Lazzarini *et al.*, 2000).

Neo-specific PCR protocol. Following described procedures (Monciardini *et al.*, 2002), the 16S rRNA gene sequences from Neo strains (Table 1 and additional isolates) were used to identify the group-specific primers NeoF178 (5'-GGGTGGAAAGTTTTTCGG-TGGA-3') and NeoR940 (5'-CCCCACATCTCTGCAGGTAG-3'), which are expected to yield a 762 bp PCR product. The PCR mixture consisted of 500 nM each primer, 200 µM each dNTP, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 3 µl 10× Denhardt's solution, 2 U RedTaq DNA polymerase and 5 ng DNA,

in a final volume of 50 µl. Cycling parameters were as follows: 5 min denaturation at 95 °C, followed by 30 cycles of 45 s at 94 °C, 30 s at 65 °C and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. For amplification directly from soil DNA, conditions were as above, except that 16 cycles, each consisting of 45 s at 94 °C, 30 s at 65 °C and 1 min at 72 °C (but decreasing the annealing temperature by 0.25 °C after each cycle), were followed by 20 cycles, each consisting of 30 s at 94 °C, 30 s at 60.5 °C and 1 min at 72 °C. The PCR protocol, validated as described by Monciardini *et al.* (2002) using genomic DNA from several actinomycete strains, showed a sensitivity of 1–5 pg, estimated with purified DNA from Neo strains. The selectivity of the PCR protocol was assessed on soil DNA, by end-sequencing 17 independent clones obtained after cloning the PCR product from sources 11943 and 12228. All sequenced clones showed between 99.1 and 100% identity to those of the Neo strains from Table 1 (data not shown).

Nucleotide accession numbers. The sequences described here have been deposited in GenBank, and the corresponding accession numbers are reported in Tables 1–3.

RESULTS

Strain diversity

Descriptions of *Catenulispora acidiphila* and of *Actinospica robiniae* and *A. acidiphila* are reported elsewhere (Busti *et al.*, 2006; Cavaletti *et al.*, 2006). The strains analysed in this study were derived from three independent isolation campaigns from two different sources and are referred to with the Neo- and Gamma- prefixes to designate *Catenulispora*- and *Actinospica*-related strains, respectively (Table 1). All strains show the typical characteristics of filamentous actinomycetes, with branching vegetative hyphae and production of aerial hyphae that differentiate into spore chains upon ageing.

We analysed the isolates in order to assess the level of phylogenetic diversity. The strains were initially grouped according to their ITSs, applying to single strains the technique originally developed for community analysis (Fisher & Triplett, 1999). For each strain, the ITS was PCR-amplified and the apparent sizes of the amplified products were recorded. We analysed 29 Gamma strains (isolated from sources 8505 and 11943) and 35 Neo strains (isolated from source 11943 only). Each strain was characterized by a reproducible pattern, comprising one to four peaks of different sizes (data not shown). For each lineage, six distinct ITS profiles were obtained. Next, we randomly chose one representative strain from each profile type, leading to the 12 strains with unique profiles reported in Table 1, which were further characterized through 16S rRNA sequence analysis (Fig 1).

For the six Neo strains, 16S rRNA variability was low, with pairwise sequence identity between 99.6 and 100%. The Australian isolates Ellin5034, -5119 and -5116 (Joseph *et al.*, 2003) have 16S rRNAs highly similar (99.3–99.8% pairwise identity) to those of the six Neo strains. The Gamma strains were divided into two groups: one consisting of Gamma3 and -4 (sharing 99.6% sequence identity), and the second

Fig. 1. Phylogenetic tree of 16S rRNA genes from *Catenulispora* and *Actinospica* strains, rooted using the 16S rRNA sequence of *Conexibacter woesei* DSM 14684^T (GenBank AJ440237). The tree was constructed substantially as described by Cavaletti *et al.* (2006). Accession numbers for the sequences are in parentheses. Numbers at nodes are bootstrap values based on 100 resamplings; values lower than 70 are not shown. The scale bar indicates 1 inferred nucleotide substitution per 100 nt.

consisting of the other four strains (99.8–100% identity). It should be noted that Gamma6 and -28, characterized by 99.9% identical 16S sequences, were isolated from soils collected from locations far apart (Table 1).

Genome size

Agarose-embedded DNA from Neo1 and Neo2 was prepared as described in Methods, and restricted with *AseI*, *DraI* and *SspI*. Neo1 and Neo2 DNAs yielded distinct fragments when cut with *AseI* and *DraI*, while the *SspI* fragments

were poorly resolved (see Fig. 2). Summing the *AseI* or *DraI* fragments observed with Neo1 DNA yielded genome sizes of 8.3 or 8.6 Mb, respectively. For Neo2, size estimates with the two enzymes were 8.3 and 9.9 Mb, respectively. Similar experiments performed with Gamma3 DNA yielded resolved fragments with *AseI* and *DraI* (data not shown), from which we could calculate genome sizes of 8.3 and 8.6 Mb, respectively. These results indicate that the genome sizes of the Neo and Gamma strains are very similar to those of other filamentous actinomycetes, which range from 8 to 10 Mb (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Redenbach *et al.*, 2000).

Fig. 2. Pulsed-field gel electrophoresis of Neo1 and Neo2 DNA. Lane 1, *Saccharomyces cerevisiae* chromosomes; lane 2, *Asel*-digested *Streptomyces coelicolor* DNA; lanes 3–5, Neo1 DNA digested with *Asel* (lane 3), *Dral* (lane 4) or *Sspl* (lane 5); lanes 6–8, Neo2 DNA, digested with *Asel* (lane 6), *Dral* (lane 7) or *Sspl* (lane 8). Sizes of selected DNA fragments (in kb) are indicated on the left.

Genes for secondary metabolism

A substantial fraction of the metabolites produced by *Streptomyces* and related actinomycetes consist of a polyketide or a polypeptide scaffold. Most peptide-derived metabolites are synthesized by NRPSs (Marahiel, 1997), while two major routes exist for polyketide synthesis in

actinomycetes (Hopwood, 1997): one employs type I enzymes (PKS-I), also known as modular PKSs; and the other uses type II systems (PKS-II), mostly responsible for the synthesis of aromatic polyketides. Typical of actinomycetes is the presence, in a single strain, of genes encoding PKS-I, PKS-II and NRPSs.

We screened the strains listed in Table 1 for the presence of PKS-I, PKS-II and NRPS genes. All strains analysed yielded a distinct band of the expected size with all primer sets (data not shown). In order to confirm that the amplicons did correspond to the target genes, the products from selected strains were subcloned and sequenced. The results are summarized in Tables 2 and 3. In all cases analysed, the retrieved sequences corresponded to the expected gene segments. The translated DNAs were highly related (69.6–82.1 % identity) to known PKS-I or PKS-II sequences, while for NRPSs the identity scores were lower (35.6–68.1 %). The lower level of identity of the NRPS segments was expected, since the amplified segment encodes a relatively variable portion of the A domain (Challis *et al.*, 2000; Stachelhaus *et al.*, 1999).

PKS-I and NRPS genes present a modular organization, with a repetition of similar gene segments within a single gene cluster, so each amplified band may consist of different sequences originating from a single cluster. In addition, a single actinomycete strain normally contains more than one PKS-I or NRPS cluster. However, since KS and A domains present considerable sequence variation, primer pairs are seldom universal and can amplify only a fraction of the genes present. Consequently, only a fraction of the entire PKS-I or NRPS modules can be accessed through PCR. Among seven PKS-I clones sequenced (all deriving from strain Neo6), four different sequences were detected (Table 2). For the NRPSs, we analysed 13 independent clones obtained from

Table 2. PKS sequences

Seq. ID*	Strain	Accession no.	Best match		
			Accession no.	Organism†	Identity (%)
PKSI-2‡	Neo6	AJ865872	Q07017	<i>S. antibioticus</i>	70.5
PKSI-3§	Neo6	AJ865873	Q9S0R4	<i>S. avermitilis</i>	76.4
PKSI-8	Neo6	AJ865874	Q846X6	<i>S. cinnamomensis</i>	69.6
PKSI-12	Neo6	AJ865875	O52789	<i>A. mediterranei</i>	74.9
KSx-3N1	Neo1	AJ865867	Q93HM2	<i>S. avermitilis</i>	80.8
KSx-5N2	Neo2	AJ865868	Q54495	<i>S. nogalater</i>	82.1
KSx-3N6	Neo6	AJ865869	Q02578	<i>S. curacoi</i>	79.6
KSx-G6	Gamma6	AJ865870	Q02578	<i>S. curacoi</i>	78.7
KSx-G26	Gamma26	AJ865871	Q9LCH6	<i>S. venezuelae</i>	78.9

*PKSI and KSx prefixes indicate clones deriving from PKS-I and PKS-II PCRs, respectively.

†Genus names abbreviated as: A., *Amycolatopsis*; S., *Streptomyces*.

‡Two other clones have identical sequences.

§Another clone has an identical sequence.

||Clone 5 from strain Neo3 has the same sequence.

Table 3. NRPS sequences from strain Neo6

Seq. ID	Accession no.	Best match			aa specificity*
		Organism†	Accession no.	Identity (%)	
PS1	AJ865876	<i>S. coelicolor</i>	Q9Z4X5	54·7	Asn
PS2	AJ865877	<i>S. avermitilis</i>	Q93H39	68·1	Ser
PS3	AJ865878	<i>A. lactamdurans</i>	Q6WS98	35·6	?
PS4	AJ865879	<i>P. putida</i>	Q88F77	42·3	Asp
PS6	AJ865880	<i>P. putida</i>	Q88F77	43·1	Asp
PS7	AJ865881	<i>P. putida</i>	Q88F77	44·0	Asp
PS11	AJ865882	<i>S. coelicolor</i>	Q9Z4X6	51·9	?
PS13	AJ865883	<i>P. putida</i>	Q88F77	43·0	Asp
PS16	AJ865884	<i>P. putida</i>	Q88F78	40·6	Asx

*Predicted amino acid activated by the corresponding A domain.

†Genus names abbreviated as: *P.*, *Pseudomonas*; other abbreviations as in Table 2.

Neo6 DNA, obtaining nine different nucleotide sequences (Table 3). The NRPS primers were designed to amplify most of the DNA segment encoding the amino acid specificity pocket of A domains, including 7 out of 8 diagnostic residues (Challis *et al.*, 2000; Stachelhaus *et al.*, 1999). Thus, sequencing of the amplified products obtained from one strain could provide information on the minimum number of NRPS modules, as well as on the amino acids recognized by the corresponding A domains. The residues lining the binding pocket of the putative Neo6 A domains were determined by alignment with GrsA (CAA33603). Comparison with a database of 442 A domain sequences allowed the identification of the amino acid likely to be recognized by the putative Neo6 A domains (Table 3). Four different domains are likely to activate Asp, and one each is likely to recognize either Asp/Asn, Asn or Ser. Interestingly, for the two remaining domains, the signature sequence had no correspondence in the database, even if both sequences were highly similar to known A domains. Thus, we can conclude that strain Neo6 contains at least nine different A domains, and presumably an equivalent number of NRPS modules, likely to recognize at least three distinct amino acids.

Since PKS-II systems do not have a modular organization, a gene cluster usually encodes a single KS α . However, some strains possess more than one PKS-II cluster, and this route is also used for the synthesis of spore pigments (Hopwood, 1997). In a survey of 85 *Streptomyces* isolates, 60 % could be sequenced directly while the remaining showed multiple sequences that needed cloning (Metsä-Ketelä *et al.*, 2002). To investigate whether a single strain contained more than one PKS-II cluster, some PCR products were directly sequenced. In all instances we observed mixed sequences, suggesting that more than one DNA segment had been amplified. We thus cloned six PCR products and analysed four clones each. All clones deriving from a single strain always yielded the same sequence, which corresponded to the major signal read from direct analysis of the corresponding PCR product (data not shown). We suspect that

the analysed strains may encode more than one KS α , but that amplification is biased towards one of them, and several clones should be analysed in order to identify other KS α sequences.

The results from selected PKS-II gene segments are summarized in Table 2. The deduced KS α sequences from strains Neo1 and Neo6 were 94 % identical, and those from Gamma6 and Gamma26 were 98 % identical. In addition, two KS α clones, obtained from strains Neo2 and Neo3, had identical sequences. In phylogenetic trees of KS α sequences from various *Streptomyces* strains, the enzymes for antibiotic biosynthesis were found to clearly separate from those involved in spore pigment formation, although two KS α 's from non-*Streptomyces* actinomycetes did not belong to any of the two clades (Metsä-Ketelä *et al.*, 2002). When the five different sequences were compared with those of selected KS α 's, only KS α 5-N2 clearly grouped with enzymes for antibiotic biosynthesis (Fig. 3). The other four sequences formed two separate branches in the tree.

Antimicrobial activities

The production of bioactive metabolites by the strains was assayed by the agar plug method. Strains Neo2, -3 and -6 showed significant activity against *B. subtilis*, *Staph. aureus* and *M. luteus*, while no activity was detected against *E. coli*. Similarly, Gamma22 produced significant anti-Gram-positive activity (data now shown). Occasionally, Neo1 produced a weak activity against *E. coli* (data not shown).

Chromatographic analysis indicated that Neo2 and Neo3 produced different bioactive metabolites (not shown). The active metabolites produced by Neo3 were extracted with ethyl acetate and analysed, as described in Methods. This led to the identification of an HPLC peak eluting at 24·2 min that contained the major anti-staphylococcal activity. This peak exhibited UV-VIS maxima at 234, 269 and 437 nm, and showed a [M-H]⁺ signal at 629·1 upon MS analysis. Database searches indicated that these values

Fig. 3. Phylogenetic tree of KSα sequences. The translated sequences of the KSα clones (Table 2) were aligned over a 145 aa span with selected actinomycete KSα sequences. Bootstrap values greater than 50% are shown at nodes. The tree was rooted using the sequence of a type I PKS (Q07017, KS from module 5 of the *Streptomyces antibioticus* oleandomycin PKS) as outgroup. The scale bar indicates 10 inferred substitutions per 100 aa. CurA, WhiE and SppA are responsible for spore pigment formation, while Act, PraA and DpsA are involved in the synthesis of actinorhodin, pradimicin and daunorubicin, respectively. Accession numbers of reference sequences are indicated.

closely matched those of the bioactive dimeric isochroman-quinone GTRI-BB, which has a molecular mass of 630, a UV-VIS spectrum compatible with that observed for the 24·2 min peak, and is reported to have anti-Gram-positive but not anti-Gram-negative activity (Yeo *et al.*, 2002). Thus, it is quite likely that Neo3 produces this same compound. Using the same methodology, the active peak produced by Gamma22 did not find a match with any database entry, indicating that it represents a potentially new antibiotic.

Distribution of Neo strains in different soils

The Neo-specific PCR protocol described in Methods was employed to screen soil DNAs for the presence of 16S rRNA sequences ascribable to Neo strains. We selected several soil samples from different environments, and of different pH. In total, 100 soil DNAs were surveyed by PCR analysis. We could detect a distinct signal from 33% of the soils with a pH of 5·5 or lower. No signal was observed from 19 soils with pH > 7. From this analysis, the Neo strains that can be targeted by our PCR conditions appear to be present above the detection limit only in acidic soils. This result is consistent with their growth behaviour as acidophiles (Busti *et al.*, 2006). On the other hand, we were able to obtain a PCR product from soil samples collected in distinct locations from different continents (i.e. Europe, Africa and America), indicating that Neo-like sequences are widespread on the planet. This result is consistent with the isolation of the Neo-related Ellin strains from an Australian soil (Joseph *et al.*, 2003).

The 16S rRNA sequences of several strains, identified as 'Acidophilic Actinobacterium', have recently been deposited in databases (e.g. GenBank accession numbers AB180758, AB180762 and AB180777). These sequences are highly related to those of the Gamma strains, suggesting a global distribution for these strains also.

DISCUSSION

The hypothesis that led us to search for previously uncultured actinomycetes was that these strains were unlikely to have been extensively screened in the past. If they were also able to produce bioactive metabolites, then they should offer a higher probability of discovering novel compounds than previously screened strains (Donadio *et al.*, 2005). While phylogenetic, chemotaxonomic and morphological criteria indicate that *Catenulispora* and *Actinospica* strains belong to the order *Actinomycetales* (Busti *et al.*, 2006; Cavaletti *et al.*, 2006), the ability to produce secondary metabolites is not a uniform trait within this order. In fact, few metabolites have been described as produced by strains belonging to the suborders *Actinomycinae*, *Corynebacterineae*, *Frankineae*, *Glycomycineae*, *Micrococcineae* and *Propionibacterineae*. The data reported here show that at least some strains within the proposed suborder *Catenulisporinae* share with *Streptomyces* and related genera several important features, such as the production of antibacterial activities at high frequency, along with the presence within a single strain of genes encoding NRPSs and type I and II PKSs. To our knowledge, the simultaneous presence of these three biosynthetic routes has been reported so far only within the antibiotic-producing *Actinomycetales*. It is also worth pointing out that, within the *Actinomycetales*, all the prolific producers of secondary metabolites grow as filamentous mycelia, as do the Neo and Gamma strains. In addition, many filamentous actinomycetes possess a chromosome of 8 Mb, or higher (Redenbach *et al.*, 2000), as do the Neo and Gamma strains, while mycobacteria and corynebacteria have smaller genomes (Cole *et al.*, 1998; Correia *et al.*, 1994). Thus, there appears to be a correlation between the ability to produce secondary metabolites on the one hand and genome size and filamentous growth on the other.

Recently, Janssen and coworkers (Joseph *et al.*, 2003; Sait *et al.*, 2002) have isolated a large number of previously

uncultured bacteria. Five of their isolates are highly related to the Neo strains, judging by their 16S rRNA gene sequences. Thus, we share the surprise expressed by Sait *et al.* (2002) that previously uncultured strains could be found also within the intensively isolated actinomycetes. However, the long incubation times required for the appearance of Neo and Gamma strains on isolation plates and their acidophilia might have hindered their retrieval during previous isolation campaigns. Recent isolation efforts (Busti *et al.*, 2006; Cavaletti *et al.*, 2006; Donadio *et al.*, 2005; Joseph *et al.*, 2003; Magarvey *et al.*, 2004; Mincer *et al.*, 2002; Sait *et al.*, 2002) indicate that both marine and terrestrial sources can provide hitherto undescribed actinomycete taxa.

Strain Neo3 produces a bioactive molecule with properties indistinguishable from those of the dimeric isochroman-quinone GTRI-BB, produced by a *Micromonospora* sp. (Yeo *et al.*, 2002). It should be noted that compound GTRI-BB is similar to the well-studied *Streptomyces coelicolor* metabolite actinorhodin, which is synthesized by a PKS-II system (Hopwood, 1997). In addition, Neo3 contains a sequence encoding a KS α related to the actinorhodin enzyme (Fig. 3). Thus, we infer that Neo3 contains at least one complete gene cluster for secondary metabolite production. Apparently, GTRI-BB represents one additional example of the same metabolite produced by phylogenetically unrelated strains (Lancini *et al.*, 1995). On the other hand, Gamma22 produces a compound whose mass and UV properties do not find a match in antibiotic databases. Thus, it may represent a novel antibiotic. It should be noted that the fact that Neo3 produces a known bioactive compound does not imply that all secondary metabolites produced by Neo strains will be known compounds, nor is it reasonable to expect that previously uncultured strains would produce novel metabolites only.

In order to provide a reasonable chance of yielding novel antibiotics, strains belonging to novel taxa must: (1) be able to produce secondary metabolites; (2) possess diversified pathways for secondary metabolism; (3) present significant genetic diversity; (4) be retrievable in large numbers; and (5) be amenable to scale-up for large-volume fermentation. We have provided evidence that the *Catenulispora* and *Actinospica* strains fulfil criteria 1 and 2, and, from the limited number of isolates analysed, possibly also criterion 3. Further work is required to establish the true diversity of these new taxa and the ease with which they can be isolated in large numbers. Preliminary data indicate that these strains can be effectively grown in liquid media.

We assume that phylogenetically distant strains are more likely to possess different genes than phylogenetically related strains; since secondary metabolites are produced by the concerted action of several genes, it is reasonable to expect an increased number of novel genes and gene combinations from strains phylogenetically unrelated to strains highly screened for antibiotics. The Neo and Gamma strains described in this work, and other strains currently under investigation in our laboratory (Donadio *et al.*, 2005),

represent a potential source of novel antibiotics and other biologically active substances. In our opinion, an expansion of the chemical diversity available for pharmaceutical screening is badly needed, especially in the fight against drug-resistant pathogens. Strains belonging to novel actinomycete taxa might fulfil this role.

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