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The detection of diverse aminoglycoside phosphotransferases within natural populations of actinomycetes

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The conserved nature of the genes that code for actinomycete secondary metabolite biosynthetic pathways suggests a common evolutionary ancestor and incidences of lateral gene transfer. Resistance genes associated with these biosynthetic pathways also display a high degree of similarity. Actinomycete aminoglycoside phosphotransferase antibiotic resistance enzymes (APH) are coded for by such genes and are therefore good targets for evaluating the bioactive potential of actinomycetes. A set of universal PCR primers for APH encoding genes was used to probe genomic DNA from three collections of actinomycetes to determine the utility of molecular screening. An additional monitoring of populations for the predominance of specific classes of enzymes to predict the potential of environmental sites for providing isolates with interesting metabolic profiles. Approximately one-fifth of all isolates screened gave a positive result by PCR. The PCR products obtained were sequenced and compared to existing APH family members. Sequence analysis resolved the family into nine groups of which six had recognizable phenotypes: 6′-phosphotransferase (APH(6)), 3′-phosphotransferase (APH(3)), hydroxyurea phosphotransferase (HUR), peptide phosphotransferase, hygromycin B phosphotransferase (APH(7″)) and oxidoreductase. The actinomycetes screened fell into seven groups, including three novel groups with unknown phenotypes. The strains clustered according to the environmental site from where they were obtained, providing evidence for the movement of these genes within populations. The value of this as a method for obtaining novel compounds and the significance to the ecology of antibiotic biosynthesis are discussed.
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Introduction

Aminoglycosides are an important class of clinically relevant chemotherapeutic compounds that are used for their antimicrobial and antiprotozoal activities (e.g., neomycin, streptomycin and tobramycin). Actinomycetes, particularly from the *Streptomyces* genus, have proved to be an effective source of aminoglycoside compounds [33]. Resistance mechanisms have developed in bacteria that are associated with exposure to the relevant aminoglycoside antibiotic, notably in clinical [27] and agricultural [42] environments. This highlights the need for natural product discovery programs to identify novel compounds from this class to replace drugs that have lost their efficacy. There are three types of resistance mechanisms to aminoglycosides: (1) mutations to the bacterial ribosome, the target site for the antibiotic [29], (2) prevention of uptake [36] and (3) enzymatic alteration of the compound or the ribosome. The most prevalent method of resistance in clinical isolates is the enzymatic modification of the compound either by acetylation, adenylation or phosphorylation, all of which render the compound inactive [37]. The aminoglycoside phosphotransferases (APH) are a large group within the protein kinase family [9]. They are composed of seven classes with many isozymes within each [49]. APH resistance enzymes are also present in aminoglycoside-producing bacteria, actinomycetes in particular. They can render the producing bacteria resistant to its own antibiotic. It has been suggested that the genes that code for these enzymes originated in the antibiotic-producing organisms and transferred into the populations where selective pressure has rendered the presence of resistance genes a necessity [10]. Enzymes from the producing bacteria and clinical isolates share common functions involved with antibiotic modification and have a high degree of similarity at the amino acid level. They share similar amino acid sequence motifs that correspond to the ATP binding site and a characteristic structural recognition site that is specific for the substrate antibiotic. Enzymes include hygromycin B phosphotransferase from the hygromycin B producer *Streptomyces hygroscopicus* [50]; 3′ phosphotransferases from the neomycin producer *Streptomyces fradiae* [43], the clinical species *Streptococcus faecalis* [14] and *Bacillus circulans* [16]; viomycin phosphotransferase from the viomycin producer *Streptomyces vinaceus* [4] and two 6′ phosphotransferases from the streptomycin producer *Streptomyces griseus* (APHD) [26] and the hydroxystreptomycin producer *Streptomyces glaucescens* (SPH) [17].

It is usual for the genes that code for antibiotic resistance enzymes to be clustered with the antibiotic biosynthetic cluster in the producing organism [8]. Exceptions include the 3′ aminoglycoside phosphotransferase (APHE), which confers resistance to streptomycin in the producing strain *S. griseus* [15], and hydroxyurea phosphotransferase (HUR), which confers resistance to the synthetic antibiotic hydroxyurea in *Streptomyces aurofaciens* [25]. Hoshiko *et al* [20] demonstrated that there was cross-substrate specificity between neomycin and ribostamycin phosphotransferase as both have substrates that are 4,5-distributed deoxystreptamine derivatives. It is plausible that HUR and APHE confer resistance to as-yet-undetected antibiotics with structural similarities to hydroxyurea and streptomycin, respectively.

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Huddleston and coworkers [21,22] developed three sets of PCR primers for detection of 6′ aminoglycoside phosphotransferase (APH(6)) genes from actinomycetes that produce compounds from the streptomycin group of antibiotics including streptomycin (*S. griseus*), hydroxystreptomycin (*S. glaucescens*) and dihydrostreptomycin (*Streptomyces humidus*). Primers were designed that were (1) specific for APHD, (2) specific for SPH and (3) consensus primers to detect both APHD and SPH genes. These were designated STR. The STR primers were designed around the central conserved ATP motif and the structural recognition site of the 6′ phosphotransferase. They gave a positive result with all the strains that produced streptomycin and associated compounds and with strains that had not been documented as producing streptomycin-type compounds [21]. The APHD PCR primers were subsequently used to monitor a lateral gene transfer (LGT) event in a population of actinomycetes isolated from Brazilian soils [22]. Six taxonomically distinct isolates were detected, which contained a gene that was 99% similar to APHD at the nucleotide level. Subsequent studies were unable to determine whether these genes were linked to the production of streptomycin or an additional compound [12].

In this investigation the specificity of STR PCR primer sets was investigated to determine whether they could universally detect genes that code for APH(6) enzymes from actinomycetes and therefore be used to assess the bioactive potential of isolates. In addition, two distinct environmental populations were screened with the STR PCR primers to evaluate whether molecular approaches are useful tools for monitoring the ecology of antibiotic biosynthesis by detecting populations that have undergone selective pressure and are enhanced for populations of potentially bioactive bacteria.

Materials and methods

Bacterial strains and growth conditions

Two populations of actinomycetes were investigated. The first set of 69 actinomycetes (designated DC) was isolated from an agricultural field site in Costa Rica. The second population (48isolates) was obtained from a coastal salt marsh in the USA and was designated SMA. Isolations were performed using the method described by Huddleston [21]. In addition to these natural isolates, a collection of standard type strains was also included (Table 1). All actinomycetes were grown in tryptone soy broth (Sigma, St. Louis, MO) supplemented with sucrose (10%) at 28°C for 24 h at 150 rpm. Strains were stored as spores in a 10% glycerol solution at −20°C using the method described by Hopwood *et al* [19].

DNA extraction, PCR and sequencing

Chromosomal DNA was extracted using the method of Fisher [19]. The STR PCR primers used were as described by Huddleston [21]: STR-F (5′-CGG CTG CTC GAC CAC GAC-3′) and STR-R (5′-GTC CTC GAT GTC CCA CAG-3′). The expected product size for these primers is 600 bp using *aph*D as the template. PCR mixtures contained high-fidelity PCR Master-mix (Invitrogen, Carlsbad, CA) supplemented with dimethyl sulfoxide (10%) and 30 nmol of each primer. Approximately 100 ng of genomic DNA was used in each reaction mixture. Two reaction protocols were used. The first, designated as low stringency, consisted of a 5-min denaturation step, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and a 1-min extension at 72°C. A final incubation for 10 min

Table 1 Actinomycetes used in this investigation and results for the STR PCR assay

No. in study	Microorganisms tested[a]	Compound produced	No. amplifiable with str primers (55°C annealing)	No. amplifiable with str primers (62°C annealing)
48	SMA actinomycetes	N/A	13 (27%)	6 (12.5%)
69	DC actinomycetes	N/A	22 (32%)	12 (17%)
1	*S. griseus* MA 565	Streptomycin	1	1
1	*S. antibioticus* MA 6096 (ATCC 11891)	Oleandomycin	0	0
1	*Actinomyces* sp. MA 2857	Macrolide	0	0
1	*Streptomyces* sp. MA 6287 (ATCC 53527)	Virginiamycin analogs	0	0
1	*S. azureus* MA 5816 (ATCC 14921)	Thiostrepton	0	0
1	*S. clavuligerus* MA 4251 (ATCC 27064)	Clavulanic acid and cephalosporin analogs	1	1
1	*S. hygroscopicus* MA 6434 (ATCC 29253)	Hygromycin	0	0
1	*Actinomyces* sp. MA 1416		0	0
1	*Streptoverticillium* sp. MA 5881 (ATCC 33160)	Leucomycin	0	0
1	*Kibdelosporangium aridum* MA 7133 (ATCC 39922)	Glycopeptide	0	0
1	*S. aureofaciens* MA 6538 (ATCC 13304)	Chlortetracycline and tetracycline	0	0
1	*S. ambiofaciens* MA 6502 (ATCC 15154)	Spiramycin	0	0
1	*S. lincolnensis* MA 2824 (ATCC 25466)	Lincomycin	1	0
1	*S. thermotolerans* MA 5961 (ATCC 11416)	Carbomycin	0	0
1	*Streptomyces* sp. MA 5875	Macrolide	1	0
1	*Stv. cinnamoneum* MA 5991 (ATCC 23897)	Leucomycin derivatives	0	0
1	*S. ambiofaciens* MA 5043 (ATCC 23877)	Spiramycin	1	1
1	*S. antibioticus* MA 6223 (ATCC 31771)	Erythronolide complex	1	1
1	*S. avermetilis* MA 4680 (ATCC 31267)	Avermectin	1	0
1	*Sacc. erythrea* MA 6655 (ATCC 11635)	Erythromycin	1	1
1	*S. hygroscopicus* MA 6531	FK520/523	0	0
1	*Streptomyces* sp. MA 6949	FK506	0	0
1	*Actinomyces* sp. MA 6927	Macrolide	1	0
1	*Streptomyces* sp. MA 6548 (ATCC 53770)	FK506	0	0
1	*S. halstedii* MA 6545 (ATCC 33140)	Deltamycins and carbomycins	0	0
1	*S. coelicolor* A3(2)	Actinorhodin	1	1
Total	143		45 (31%)	24 (17%)

[a]ATCC accession number given for deposited cultures.

Figure 1 Phylogenetic analysis of actinomycete APH enzymes (A) and nucleotide sequences obtained from the STR screen of actinomycetes (B). Analysis was performed using 100 bootstraps. Strain accession numbers are given in Table 2.

at 72°C ensured completion of strand synthesis. A high-stringency protocol was also used that differed from the low-stringency protocol by having a 62°C annealing temperature instead of one at 55°C. Three reactions were run for each DNA template. PCR products were gel purified using standard techniques and sequenced. One microgram of DNA was subjected to cycle sequencing with dye terminators (ABI model 373A automatic sequencer) with 3.2 pmol of the forward or reverse primer in each sequence reaction. Sequence database searching was done using BLAST [1]. Sequence alignments were done using CLUSTAL W [44] and phylogenetic analysis was done in PHYLIP [13] using the neighbor algorithm. The analysis was bootstrapped using 100 replicates. All the DC and SMA isolates included in the phylogenetic study were identified by 16S rDNA sequencing using methods described by Wiener *et al* [47].

Resistance profiles

Resistances to streptomycin, neomycin and hydroxyurea were investigated by bioassay. Susceptibility discs (Difco Laboratories, BD and Co., Sparks, MD) containing a defined concentration of antibiotic (neomycin (30 μg) and streptomycin (10 μg)) were overlaid with 0.75% nutrient broth seeded with actinomycete spores (10^6 spores/ml inoculum). Plates were incubated at 28°C for 5 days, after which zones of inhibition were measured. Isolates were then divided into three groups: resistant (no zone of inhibition), sensitive (zone measuring up to 25 mm) and hypersensitive (zone measuring 30 mm or over). No zones were observed between 25 and 30 mm. Hydroxyurea resistance was assessed by supplementing nutrient agar plates with 15 mM hydroxyurea and inoculating them with the actinomyces spores. Duplicate plates were also inoculated that did not contain hydroxyurea. Plates were scored for growth after

Table 2 Characterization of STR sequences

Phylogenetic group	Characteristic group phenotype	Genus/species/strain[a]	Accession number	Function demonstrated	APH catalytic domain present[b]	Ref
1-1	APH(6)	*S. griseus*	X05647	Yes	Yes	[11]
1-2		*S. glaucescens*	X05648	Yes	Yes	[45]
1-3		*Streptomyces* sp. ASB33	AF012547	No	Yes	[47]
1-4		*Streptomyces* sp. ASB37	AF012548	No	Yes	[47]
1-5		*Streptomyces* sp. ASB27	AF012546	No	Yes	[47]
1-6		*Streptomyces* sp. ASSF22	AF012545	No	Yes	[47]
1-7		*Streptomyces* sp. ASSF15	AF012544	No	Yes	[47]
1-8		*Streptomyces* sp. ASSF13	AF012543	No	Yes	[47]
1-9		*Nocardioides* sp. DC274	AF372851	str[s]	Yes	This study
1-10		*Micrococcus* sp. DC275	AF372852	str[s]	Yes	This study
2-1	Hydroxyurea-like	*S. aurofaciens*	M81739	Yes[c]	Yes	[25]
2-2		*S. ambiofaciens* MA 5043	AF372860	Hur[r]	Yes	This study
2-3		*S. coelicolor* A3(2)	SCD49	Hur[s]	Yes	This study
2-4		*Kitasatosporia kifunense* DC 271	AF372859	Hur[r]	Yes	This study
2-5		*S. ornatus* SMA185	AF372855	Hur[r]	Yes	This study
2-6		*S. glaucescens* DC 193	AF372852	Hur[s]	Yes	This study
2-7		*S. aureofaciens* DC 262	AF372856	Hur[r]	Yes	This study
2-8		*Kitasatospora* sp. DC 320	AF372857	Hur[r]	Yes	This study
2-9		*S. antibioticus* MA 6223	AF372858	Hur[r]	Yes	This study
2-10		*Streptomyces* sp. SMA 69b2	AF372862	Hur[s]	Yes	This study
2-11		*S. globisporus* SMA 67	AF372861	Hur[r]	Yes	This study
3-1	APH(3)	*S. fradiae*	K00432	Yes	Yes	[34]
3-2		*S. ribosidificus*	M22126	Yes	Yes	[20]
3-3		*S. griseus*	M37378	Yes[c]	Yes	[15]
3-4		*S. cyaneus* SMA161	AF372850	Neo[s]	No	This study
3-5		*K. kifunense* DC271	AF372871	No	No	This study
3-6		*S. diastatochromogenes* SMA216	AF372849	Neo[s]	No	This study
4-1	Peptide (APH(2) like)	*S. vinaceus*	X02393	Yes	Yes	[4]
4-2		*S. capreolus*	SCU13078	Yes	Yes	[39]
5-1	Hygromycin (APH(7))	*S. hygroscopicus*	X03615	Yes	Yes	[50]
6-1	Unknown Gp 1	*Streptomyces* sp. DC 259	AF372867	No	Yes	This study
6-2		*Saccharothrix* sp. DC 244	AF372866	No	Yes	This study
6-3		*S. galbus* DC 261	AF372868	No	Yes	This study
6-4		*Saccharothrix espanaensis* DC 238	AF372869	No	Yes	This study
6-5		*Kitasatosporia azaticus* DC 237	AF372870	No	Yes	This study
7-1	Unknown Gp 2	*Sacc. erythrea* MA 6655	AF372865	No	No	This study
7-2		*Streptomyces* sp. SMA 96	AF372864	No	Yes	This study
7-3		*S. clavuligerus* MA 4251	AF372863	No	Yes	This study
8-1	Unknown Gp 3	*S. coeruleofuscus* DC 265	AF372846	No	No	This study
9-1	Oxidoreductase	*S. cyanogenus*	AF080235		No	[46]
9-2		*K. azaticus* DC237	AF372847	No	No	This study
9-3		*S. violaceoruber*	SVI011500		No	[23]
9-4		*S. purpurascens*	SPU10405		No	[31]
9-5		*Zymonomas mobilis*	M97379	Yes	No	[24]
9-6		*Amycolatopsis mediterranei*	AF040570		No	[3]

str[s], sensitive to streptomycin (10 mg); hur[r], resistant to hydroxyurea (15 mM); hur[s], sensitive to hydroxyurea (15 mM); Neo[s], sensitive to neomycin (15 mg).
[a]Identification of DC and SMA isolates determined by 16S rDNA sequencing.
[b]The APH catalytic domain is defined as presence of motifs 1 and 2 as defined in Ref. [36].
[c]*hur* and *aphE* have both been assigned phenotypes (hydroxyurea resistance and streptomycin resistance, respectively). Their *in vivo* function is not known.

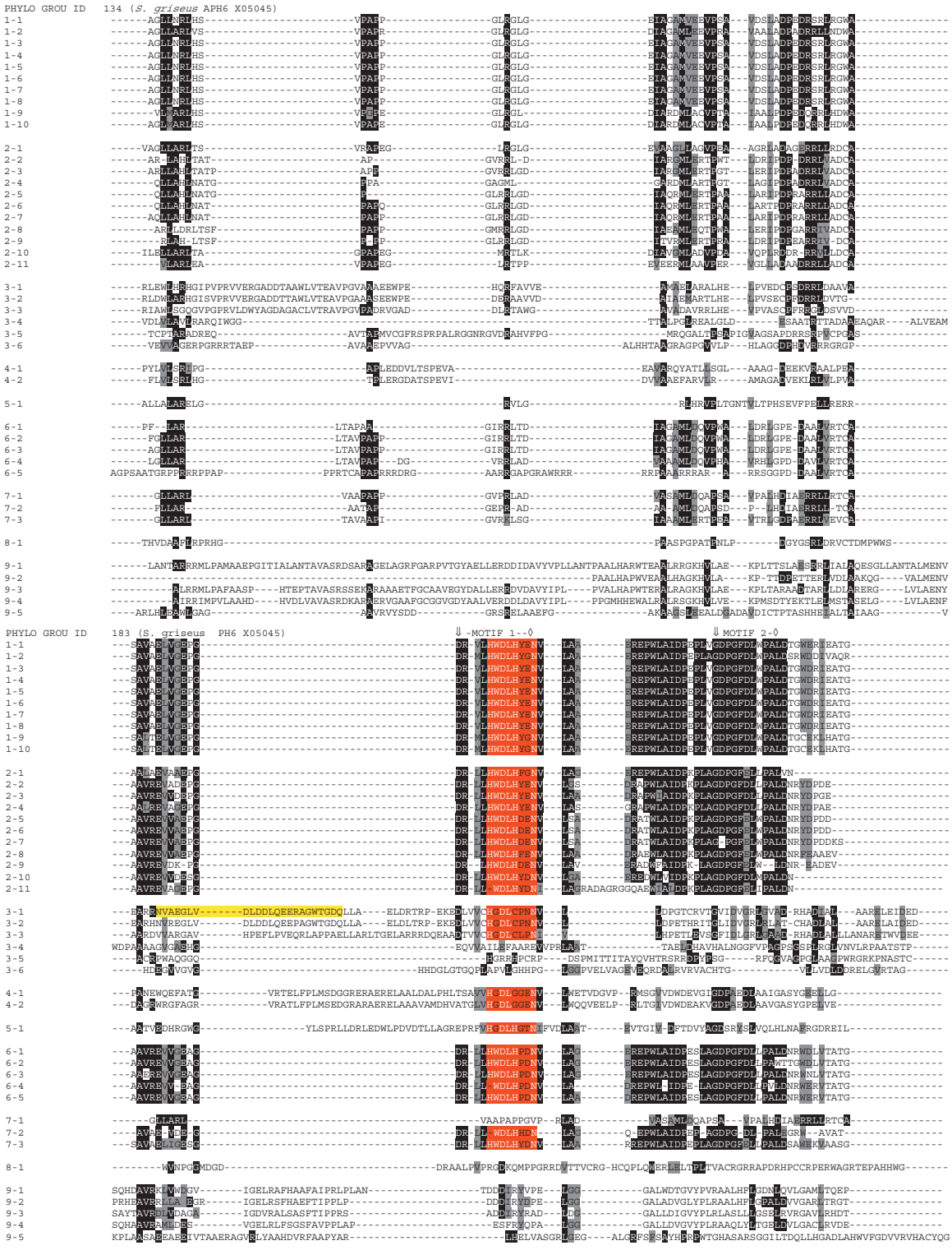

Figure 2 Alignments of amino acid sequences obtained from the STR PCR screen. Sequences are labeled using the phylogenetic group code given in Table 2. Conserved amino acids are boxed in black and functionally conserved residues are boxed in gray. The Universal APH catalytic site (HGDXXXXN) is highlighted in red [18]. Characteristic motifs as defined in Ref. [37] are marked and the substrate binding pocket defined by Burk *et al* [6] is highlighted in yellow. The amino acid numbering is according to APHD, X05045.

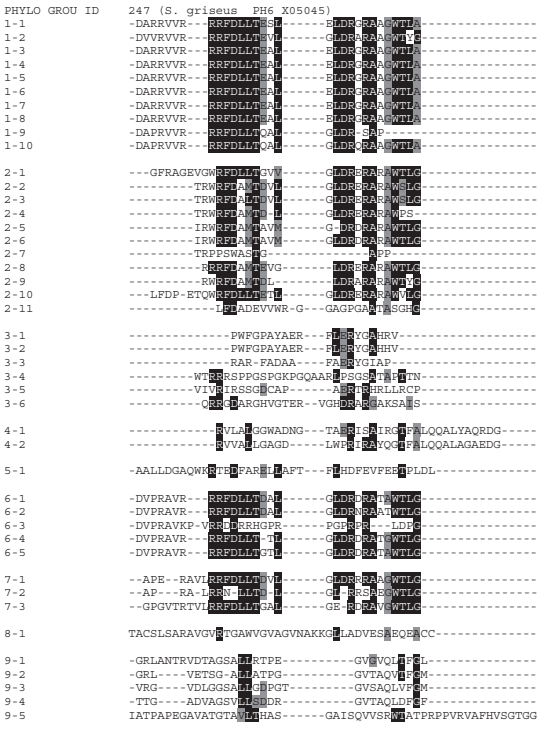

Figure 2 (*Continued*)

5 days incubation at 28°C. Test error was determined using the method described by Sneath and Johnson [40].

Results

STR PCR assay

A strain documented to contain an APH(6) enzyme was used for the positive control: *S. griseus* MA 565. *Streptomyces coelicolor* A3(2) was also included as a positive control; The sequence of the entire genome of *S. coelicolor* A3(2) has been made available by the *Streptomyces coelicolor* A3(2) Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/Projects/S_coelicolor/) and it was surveyed for the presence of genes that may code for enzymes from this family. One such gene was detected (SCD49). A negative control was harder to define due to the limited amount of total genomic sequence data available for actinomycetes. *S. hygroscopicus* was included. This strain confers resistance to its product, hygromycin, with an APH(7″) enzyme. *Streptomyces avermitilis* was also included as a negative control. The genome of this strain is the only other publicly available actinomycete genome sequence [32]. No APH enzymes were detected in the reported hypothetical biosynthetic pathways of this strain. Low- and high-stringency PCR reactions were done. A PCR product of the expected size (600 bp) was detected for the positive controls using both the low- and high-stringency reactions. A PCR product of the expected size was detected for only one of the negative controls (*S. avermitilis*) with the low-stringency PCR reaction. No PCR products were detected for the negative controls using the high-stringency PCR. The two environmental populations and the cohort of type strains were screened using both the low- and high-stringency protocols. PCR products were obtained for approximately one-third of the strains using the low-stringency PCR. These products ranged in size from 500 to 2000 bp and several PCR products of different sizes were obtained for some isolates. The high-stringency PCR reduced this figure to 17%, of which all were single PCR products of approximately 600 bp. A PCR product was obtained for a slightly higher proportion of the agricultural isolates (17%) than for the coastal marsh isolates (12.5%). PCR products were also obtained from four of the type strains that had not previously been associated with phosphotransferase resistance mechanisms (Table 1). Triplicate STR PCR products from the 25 positive reactions were sequenced. Two different sequences were obtained for two of the agricultural isolates (DC271 and DC237). Sixteen of the sequences obtained gave a high identity to the APH(6) from *S. glaucescens*, one (DC 237a STR PCR product) was highly similar to a putative oxidoreductase from *Streptomyces cyanogenus* (*lanT*) and seven of the products sequenced did not match to anything in the database. The PCR products from the *S. griseus* (MA565) and *S. coelicolor* A3(2) positive controls matched to the expected published sequences.

Phylogenetic analysis of APH enzymes

The amino acid sequences of 10 actinomycete APH enzymes and an oxidoreductase enzyme were subject to phylogenetic analysis to determine their relationships to each other (Figure 1A). The analyses included two true APH(6)-modifying enzymes, APHD and SPH from *S. griseus* and *S. glaucescens*, respectively; HUR from *S. ambiofaciens*, which is 50% similar to APHD, but for which the natural substrate is not known; three representatives of the APH(3) group, APH from *S. fradiae*, RPH from *S. ribosidificus* and APHE from *S. griseus*; two peptide modification enzymes that are most similar to the APH(2) modification enzymes [49] VIO and CPH from *S. vinaceus* and *S. capreolus*, respectively; one APH(7) enzyme from *S. hygroscopicus* and SCD47, the hypothetical APH enzyme from *S. coelicolor* A3(2) for which the APH group is not known and the oxidase reductase enzyme which served as a root. The enzymes within the phylogram clustered according to APH type: APH(6), APH(3), APH(2)-like, the enzymes with unknown natural substrates clustering with the APH (6) group (*S. ambiofaciens* and *S. coelicolor*). The APH(7) and the oxidoreductase enzymes both formed single-member clusters. Two of the three APH(3) enzymes showed a higher degree of similarity to each other (APH and RPH) than to APHE. The genes that code for APH and RPH are located within the biosynthetic pathways of the compounds to which they confer resistance. APHE was classed as an APH(3) enzyme as it inactivates streptomycin by 3′ phosphorylation. The gene that codes for this enzyme is present in the streptomycin-producing strain *S. griseus*. It is not, however, clustered within the pathway as APHD the APH(6) enzyme is. It is not known what the intended substrate for APHE is, which may explain its distal relationship to the two other APH(3) enzymes.

Phylogenetic analysis of putative APH enzymes

Actinomycete genes have a high G+C content (0.74). This translates to an extremely high GC codon usage bias for codon usage. Bibb *et al* [5] and Wright and Bibb [48] demonstrated that this bias enables codon usage for actinomyces proteins to be extremely predictable, making it possible to investigate phylogenetic relationships at the nucleotide level when just comparing genes from actinomycete strains. The nucleotide sequences of the PCR products generated in the high-stringency screen were subject to phylogenetic analysis to determine whether the unknown sequences were similar to any of the documented actinomycete

protein kinase resistance genes or whether they were from unrelated genes, such as the oxidase reductase gene *lanT*. The documented APH enzymes were resolved to the same positions by phylogenetic analysis of the nucleotide sequences as they were by analysis of the amino acid sequences, confirming the utility of using nucleotide sequences for this study (Figure 1A and B). The phylogenetic tree resolved the sequences into nine groups of which two represented single species (Figure 1), Table 2. The groups were named according to the known activities within each.

Amino acid analysis of putative APH enzymes

Amino acid alignments were made using ClustalW (Figure 2) to investigate further the validity of the STR screen. APH enzymes possess three characteristic motifs [37] of which two were included in the region analyzed. One of the motifs (1) is the catalytic site that shares catalytic and functional similarities to the eukaryotic serine/threonine protein kinase enzymes [18] and has the consensus sequence **HGD**XXXX**N**. The second is motif 2, a glycine-rich flexible loop (**G**XX**D**X**GR**X**G**), which is involved with Mg^{2+} binding. The functional properties of the APH(3) enzymes have been fully characterized [6], and it has been demonstrated that the substrate-binding pocket is located downstream to the catalytic site. This region is also included in the region amplified by PCR. The substrate-binding pocket has not been confirmed for the other APH enzymes.

The consensus motif 1 sequences detected were: Group 3, **HGD**LC(P/L)N**N**; Group 4, **HGD**LGGE**N**; Group 5, **HGD**-CHGT**N**; Groups 1, 2, 6 and 2/3 from Group 7, **HWD**LHY (E/-G/D)**N**. The motif 2 sequences detected were more diverse: Group 3, **G**(V/L/F)**ID**(V/L)**GRL**(G/R); Group 4, **G**DP**A**E**DL**A**A**; Groups 1, 2, 6 and 2/3 from Group 7, **G**DP**G**F**DL**(L/W/- M/I)**P**. The hygromycin APH did not have a glycine-rich region. The HUR and the unknown 1 and 2 sequences also shared a high degree of similarity around the two motifs and within the region that corresponded to the putative APH(6) substrate-binding pocket. Sequences obtained from the natural isolates that were included in the APH(3) group, one of the three in Group 7, Group 8 and the oxidase reductase group (Group 9) did not match with any of the key APH enzymatic domains (Table 2).

Phenotypic expression of resistance in soil populations

The soil isolates were screened for resistance phenotypes to the compounds that they grouped with phylogenetically (neomycin, streptomycin and hydroxyurea). The isolates were categorized for resistance to neomycin and streptomycin and grouped according to the levels of resistance exhibited using plate bioassays. A range of antibiotic-producing type strains was also included for comparative purposes. Similar levels of resistant and sensitive strains were detected for all populations. The only significant differences detected were with isolates that exhibited hypersensitivity to streptomycin (Figure 3). Significantly higher levels of the agricultural actinomycetes were hypersensitive to streptomycin than the coastal and type strains. The natural isolates that grouped with the 6′ phosphotransferases did not demonstrate resistance to streptomycin and there was no correlation detected between the isolates that grouped with the 3′ phosphotransferases and neomycin resistance.

The largest genotypic group obtained was Group 2, which consisted of isolates and type strains that clustered with the hydroxyurea resistance gene from *S. aurofaciens*. Hydroxyurea is a potent inhibitor of DNA synthesis in both eukaryotic and

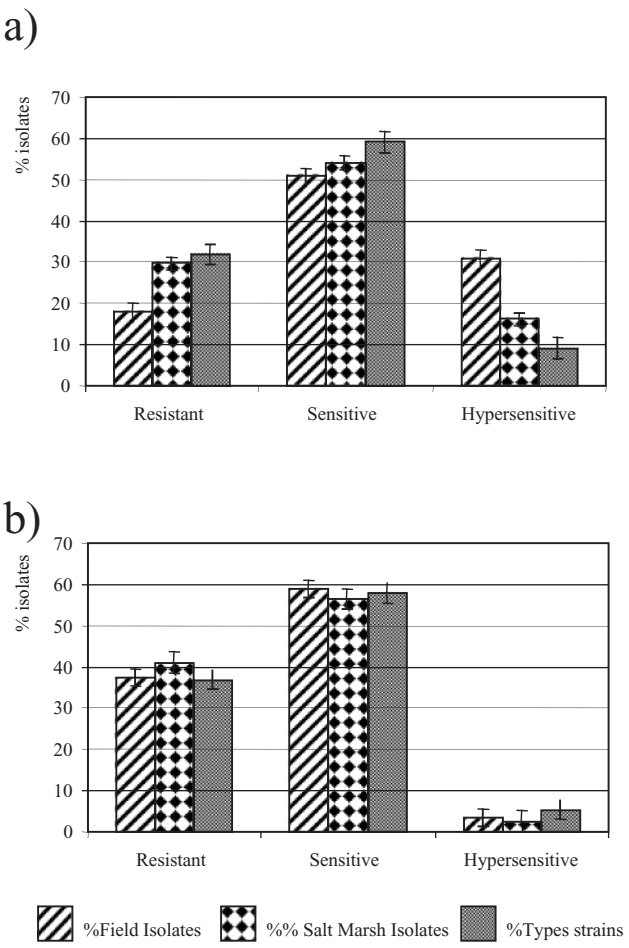

Figure 3 The distribution of antibiotic resistance phenotypes in actinomycete populations. (a) Streptomycin (10 mg/l) and (b) neomycin (30 mg/l).

prokaryotic cells. Resistance mechanisms include phosphorylation of the hydroxy group in the hydroxylamine moiety [25] and overproduction of the target enzyme, ribonucleotide reductase. In this investigation all isolates were screened for resistance to hydroxyurea to determine whether any of the gene sequences that clustered with *hur* conferred resistance to hydroxyurea. Approximately half of the agricultural isolates were resistant to hydroxyurea. The coastal isolates and the type strains contained higher numbers of resistant strains (approximately 70%). Seven of the nine strains that clustered with *hur* were resistant to hydroxyurea (Table 2). It is difficult to conclude that these genes have a similar function due to the high levels of resistance observed in the total population.

Discussion

The distribution of APH genes in environmental populations

The STR PCR primers were designed to be specific for the APH(6) enzymes with the purpose of detecting potential aminoglycoside producers. The screen detected 25 actinomycetes from the 143 screened using the high-stringency PCR. Phylogenetic analysis proved to be a useful method of grouping the sequences obtained, resolving the family into nine groups. Isolates were contained in

seven groups. Analysis of the key APH functional domains was able to further discriminate between putative APH enzymes and false positives. Nineteen of the 25 sequences (76%) had significant structural identity to APH modification enzymes and were closely related to the target APH(6) group. No sequences from the other APH groups were detected, demonstrating the specificity of the STR primers to enzymes from this group.

This investigation highlights that the APH(6) group of enzymes and its close relatives (e.g., HUR) are not ubiquitously distributed in populations of actinomycetes or existing culture collections. From the 19 putative enzymes detected, 11 were from the agricultural strains (16% of the cohort), 4 were from the coastal isolates (8% of the cohort) and 4 were from the type strains. In a similar screen that used PCR primers specific for the polyketide-associated genes that encode ketosynthases, the gene was detected in over 90% of 300 isolates screened [2]. Analysis of the genome sequence of *S. avermitilis* revealed the presence of approximately 11 potential polyketide pathways, out of an expected 25 secondary metabolite pathways, which represent approximately 6% of the genome; no pathways that contained APH resistance enzymes were discovered [32].

Despite the distribution of these enzymes being low, a high degree of diversity was detected within biodiverse cohorts of actinomycetes, indicating the potential for the discovery of novel compounds. *Nocardiodes* sp. DC274 and *Micrococcus* sp. DC275 contained putative APH enzymes with the highest degree of similarity to APHD (78%), The HUR group was the largest group detected and were approximately 55% similar to APHD. This group was composed of isolates from the agricultural site (four isolates), the coastal site (three isolates) and the type strain collection (three isolates). Unique identifications were obtained for all isolates within this group. It is not known whether *hur* is associated with an indigenous antibiotic biosynthetic pathway. Hydroxy urea is a synthetic compound that has antileukemia, antitumour and antiviral activities [35] and is also used for the treatment of sickle cell anemia [30]. Resistance genes that fall into the *hur* category may be indicative of biosynthetic pathways that code for compounds with hydroxyurea-type structural groups. Group 6 contained five potential APH enzymes from two *Streptomyces* sp., two *Saccharothix* sp. and a *Kitasatosporia* sp., all from the agricultural site. The putative function for the enzymes within this group is not known, but they shared 63% homology to APHD at the amino acid level. Two of the three proteins within Group 7 contained key APH functional groups and were 67% identical to APHD.

The agricultural isolates contained the highest number of APH-like enzymes and proteins that shared a high level of identity were detected in taxonomically distinct strains (e.g., Group 6). Such an amplification of a specific gene sequence in an environmental site is implicit of selective pressure, such as antibiosis within a defined environment that leads to LGT and the evolution of novel biosynthetic pathways [47]. Some soils are documented as having disease-suppressive properties [38]. Monitoring populations for the diversity of antibiotic resistance genes either indirectly using isolates or directly using environmental DNA may be one way to target isolation sites for isolates with a high potential for producing novel metabolites.

The contribution of molecular screening to the drug discovery process

Actinomycetes are extremely productive producers of compounds with pharmacological relevance. Traditional screening methodologies relied on the ability to ferment and screen thousands of strains, a laborious and time-consuming process with a relatively low success rate. The large number of strains screened reduced the number of fermentation conditions used, resulting in bioactive metabolites being missed. The number of screens that the strains see is also limited. The isolation process is dynamic. As new cultures are isolated, older ones are either retired to culture collections or destroyed. The identification and analysis of secondary metabolic pathways has made it possible to predict the functions of specific enzymatic domains [28]. Molecular screening strategies can be used to complement drug discovery programs and breathe new life into underutilized culture collections by providing a means for cataloging the metabolic potential of isolates. Once the DNA is obtained it can be screened with multiple probes, to provide a complete metabolic profile of the isolate. Specific microorganisms can be targeted, or removed from screens, thus reducing the numbers of isolates that need to be screened and enabling an increased emphasis on the fermentation conditions of fewer isolates and increasing the probability of obtaining novel compounds. This technique could be applied to various stages of the drug discovery process: (1) With the increasing availability of three-dimensional structural information of therapeutic targets it is possible to predict the structural properties of the desired inhibitor. This has been exploited by synthetic and combinatorial chemistry for the purpose of drug discovery. Utilization of molecular metabolic fingerprinting of microorganisms could be applied in the same way. If an extensive profile of a culture collection is made using probes to detect genes associated with antibiotic biosynthesis, strains could be selected that may synthesize compounds with the desired structure. (2) Once a chemical class is detected as active in a specific screen, a considerable amount of strain improvement and structural modification is required to (a) provide enough material for secondary screens, (b) remove any toxicity associated with the compound and (c) develop a production strain. Molecular fingerprinting of culture collections would enable strains to be identified that contain genes that code for similar structural classes of the active compound and can therefor be "cherry picked" to find additional back up compounds with minimum effort. (3) The increased information would also provide information as to the usefulness of specific environmental sites/niches and specific taxonomic groups of microorganisms.

Potential targets for metabolic probes can be divided into four groups: precursors, core pathways, structural modification genes and resistance genes. The core pathway genes are specific to a class of compounds whereas the other categories are indicative of many different structural classes. Stockmann and Piepersperg [41] demonstrated that genes associated with glycosylation could be used for molecular screening purposes. Sequence analyses of data derived from microbial genome sequencing projects have revealed novel compounds from previously well-characterized strains [7]. Likewise, the APH enzymes are associated with the biosynthetic pathways that they confer resistance to and are also good targets for molecular screens as they are associated with a broad range of chemical classes of compounds. In this investigation, 25 out of 144 isolates were selected for their potential of producing aminoglycoside compounds. Further analysis revealed that at least 25% of them were false positives. These, however, would not have been detected in a drug discovery screen that was looking for functionality. The 19 strains that contain putative APH enzymes all warrant further investigation using aminoglycoside screens and additional probes associated with aminoglycoside biosynthesis to

determine whether these hypothetical enzymes are true APH resistance genes.

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