ISOLATION AND CHARACTERISATION OF AN AMINOGLYCOSIDE PHOSPHOTRANSFERASE FROM NEOMYCIN-PRODUCING *Micromonospora chalcea*; COMPARISON WITH THAT OF *Streptomyces fradiae* AND OTHER PRODUCERS OF 4,6-DISUBSTITUTED 2-DEOXYSTREPTAMINE ANTIBIOTICS

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The gene (*aphA-5c*) encoding for aminoglycoside-*O*-phosphoryltransferase (APH(3')-Vc) from *Micromonospora chalcea* 69-683 was cloned by expression in *Streptomyces lividans*; it showed strong nucleic and amino acid similarities with previously described streptomycetes *aph* genes. S1 mapping of the 5' region indicated that, as with several streptomycete genes, transcription apparently initiates at the translation start codon, with no extragenic ribosome binding site. Comparison of the flanking sequences of the actinomycete *aph* genes indicates considerable divergence, which is not consistent with the notion that clustered biosynthetic genes for structurally related (or identical) antibiotics are disseminated in their entirety between microbial species.

The processes conferring antibiotic resistance in aminoglycoside antibiotic-producing streptomycetes appear various[1]. Mechanisms of self protection can involve aminoglycoside modifying enzymes, target site modifications, or alterations in antibiotic transport; these different mechanisms may also play roles in antibiotic biosynthesis and its regulation. The close coordination of antibiotic modification and antibiotic biosynthesis implies that resistance mechanisms are an integral part of antibiotic synthesis and that resistance genes would be linked to the biosynthetic genes on the chromosome. This notion has now been established by genetic analysis for a number of antibiotics; for example streptomycin biosynthesis in *Streptomyces griseus*[2]. In the case of *Streptomyces fradiae* ATCC 10745, a neomycin producer, an *O*-phosphotransferase (APH) encoded by the *aphA-5a* gene and an *N*-acetyltransferase (AAC(3)) have been characterised[3] (Table 1). The APH is assumed to be the more important function and the *aph* gene is not closely linked to *aac*. The biosynthetic gene cluster(s) for this class of antibiotics has yet to be identified. Neomycin is also

Table 1. 4,6-Disubstituted 2-deoxystreptamine antibiotic producing-strains cited in this study; produced antibiotics, genes and aminoglycoside modifying encoded enzymes.

Strain	Antibiotic produced	Gene	Encoded enzyme
Streptomyces ribosidificus SF 733	Ribostamycin	*aphA-5b*	APH(3')-Vb[19]
S. fradiae ATCC 10745	Neomycin	*aphA-5a*	APH(3')-Va[18]
		aacC8	AAC(3)-VIII[5]
Micromonospora chalcea 69-683	Neomycin	*aphA-5c*	APH(3')-Vc
		aacC9	AAC(3)-IX[5]
Streptomyces rimosus forma *paromomycinus*	Paromomycin	*aph*[a]	APH(3')[27]
		aacC7	AAC(3)-VII[41]
Bacillus circulans NRRL B3312	Butirosin	*aphA-4*	APH(3')-IV[21]

[a] Note that this *aph* sequence is not yet published, and the encoded enzyme is not classified.

produced by *Micromonospora chalcea* 69-683[4] (Table 1) which, according to accepted classification procedures is not closely related to *Streptomyces*. *M. chalcea* 69-683 was shown to possess an APH, and with a view to comparing the pathways of neomycin biosynthesis in the two organisms, we have cloned, expressed and characterised the *aph* from *M. chalcea*. In separate studies[5] we have characterised the *M. chalcea aac* gene.

Determination of the nucleotide sequence of genes controlling antibiotic resistance in producing organisms, especially those of *Streptomyces*, has provided insights into the structural regions that regulate transcription and translation in this genus. A variety of promoters have been found, sharing little or no sequence resemblance with other bacterial promoters, suggesting that unique sigma factors may be involved in transcription[6]. One approach to identifying regulatory functions in antibiotic production is to compare gene sequences and organisation in producers of the same antibiotic belonging to different genera. Such analyses may also suggest evolutionary relationships, for example if biosynthetic gene clusters or their component genes diverged from a common ancestor, or if gene clusters are transferred intact between species.

APH enzymes are widely distributed in Gram-positive and Gram-negative organisms and have been studied as a model of gene evolution[7]. While the seven reported APH (3′) do not show extensive DNA similarities, several conserved amino acid sequence motifs have been identified. Similar motifs have been described in a variety of kinase-type enzymes and nucleotide binding factors, including adenylate kinase, viral oncogene kinases and bacterial elongation factors[8], which suggests that the APH may have evolved from other kinases. In comparing *aph* gene sequences of closely related antibiotic producers such as *S. fradiae* ATCC 10745, *M. chalcea* 69-683, *Streptomyces ribosidificus* SF 733 (ribostamycin) and *Bacillus circulans* NRRL B3312 (butirosin) (Table 1) we provide additional information on this question, and on the relationship between organisms producing the same class of antibiotic.

Materials and Methods

Bacteria and Growth Conditions

Neomycin producing *M. chalcea* 69-683 was kindly provided by A. HORAN (Schering, Bloomfield N. J., U.S.A.). *Streptomyces lividans* 66 and plasmid pIJ 702[9] were from D. A. HOPWOOD (John Innes Institute, Norwich, England).

M. chalcea was grown in liquid medium as recommended by WAGMAN for neomycin production. *S. lividans* was grown on liquid YEME medium[10] supplemented with 34% sucrose, 5 mM $MgCl_2$ and thiostrepton 20 μg/ml (Squibb) plus neomycin 10 μg/ml (Upjohn) when required. Resistance to neomycin after protoplast regeneration was examined in R5 medium with an overlay of thiostrepton plus neomycin[10].

Plasmid and Phage Constructions

The procedures for plasmid constructions, the preparation of chromosomal DNA and transformation of *S. lividans* was as described[10]. Other DNA manipulations were according to MANIATIS *et al.*[11]. Restriction enzymes were purchased from Amersham International or Appligene (Illkirch, France). DNA fragments for sequencing were subcloned into the polylinker of the replicative form of bacteriophage M13 derivatives mp 18 and mp 19[12]. *E. coli* JM101[13] was transfected with the recombinant phage and single-stranded DNA was prepared.

DNA Sequence Analysis

Nucleotide sequences were determined by the dideoxynucleotide chain termination method[14] using Sequenase (United States Biochemical Corporation).

RNA Preparation and S1 Nuclease Mapping

RNA was isolated from *M. chalcea* 69-683 and from *S. lividans* harbouring the cloned *aph* gene by a modification of the method of KIRBY[10]. Contaminating DNA was removed by centrifugation through a cushion of CsCl[15].

RNA was hybridized with a ^{35}S-labelled single strand DNA probe prepared from bacteriophage T7 polymerase using single strand M13 matrix constructed for sequencing. After annealing the M13 matrix containing the antisense strand (2 μg) and a primer (10 pmol) (18 mer synthetic oligonucleotide), the polymerisation was performed in the presence of a limiting concentration (the same conditions as for sequencing) of ^{35}S-dATP and a large excess (1.5 mM) of the remaining dNTPs. The reaction was terminated by adding 1.5 mM dATP. The second strand of the M13 phage was complete but not ligated with the primer at its extremity. For isolation of the probe, restriction endonuclease digestion was performed on the primer-opposed point of the polylinker of M13. The labelled probe was then eluted overnight at 42°C from a denaturing 5% polyacrylamide gel containing 7 M urea with 0.3 M sodium acetate buffer pH 5.2 after localization by autoradiography and excision with a scalpel.

This probe was hybridized overnight to 20 μg of RNA at 42°C in 20 μl of hybridization buffer containing 50% formamide and digested with S1 enzyme (Bethesda Research Laboratories[16]).

Aminoglycoside Phosphorylation

Cell free extracts of soluble protein from early stationary phase *S. lividans* mycelium containing the cloned *aph* gene were prepared by sonication and centrifugation. Aminoglycoside modifying enzyme assays were performed by the phosphocellulose paper technique[17]. Labelled chemicals were purchased from Amersham International.

Results

Cloning of the *aphA-5c* Gene

A genomic library was prepared by shotgun cloning of *Sau*3AI partial fragments of *M. chalcea* 69-663 DNA into the *Bgl*II site of pIJ 702. From this library, five *S. lividans* transformants resistant to neomycin were isolated after protoplast regeneration, grown in YEME and assayed for APH activity. All five clones expressed APH activity. After plasmid DNA extraction, pDSA2 containing a 3.2 kb DNA insert was chosen for analysis (Fig. 1); the *aph* gene was subcloned on a *Pst*I - *Sph*IDNA fragment of 1.28 kb (plasmid pDSA212).

Fig. 1. Cloning of *aphA-5c* in pDSA2 and subcloning in pDSA212.

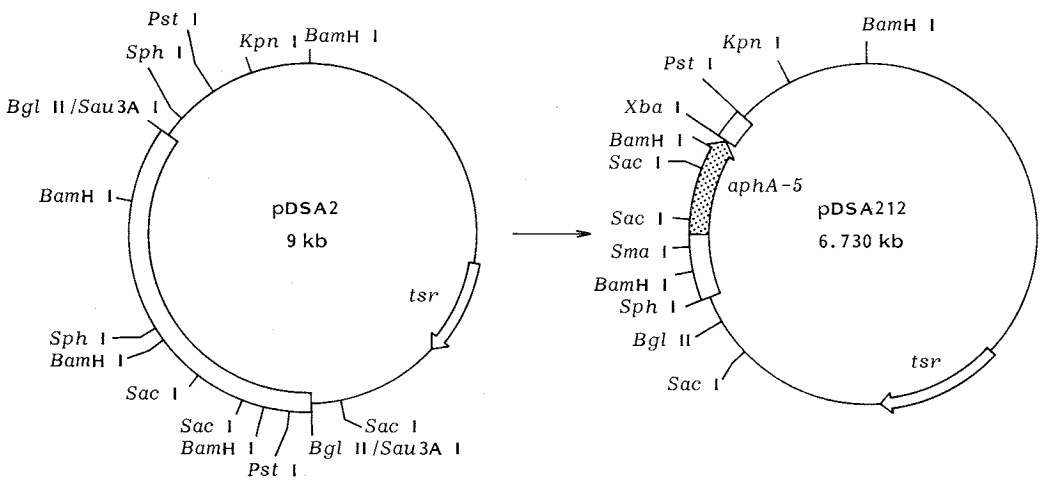

Fig. 2. Nucleotide sequence of the DNA fragment of *Micromonospora chalcea* containing the *aphA-5c* gene.

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1                                       31
GCC GGA CCG GTA GCG GGT CCG CTC GTG GTC GGC GAC GCG GAG TTC GCG CCC GAG ACC GAC

61                                      91
ACC GCC GAG GCC GAC GTC ACC CAG GAC GCG GAG GCC GCC GTG AGC ACC GGC GCG CCG GGC

121                                     151
GTC ATC GGG CCG ACC GCC GAC GGG AGC ACC GCG GCC GAG GCG GAC GAG CAG TCG GGC ACC

181                                     211
CGG GCG AGA TCC TCA GTG AGC CGA ACC GCT CGT GTC CGA AAT TGT TCA AGC AGT ATC AAC

                                                    ↓
241                                     271
ATT CCC CGA GAA AGC CGC GTG ACC TCT GCC ATG ATT CCG ACC ATG TAC GCC ATG TTG CGC
      -35                               -10           met tyr ala met leu arg
301/7                                   331/17
CGG AAA TAC CAG CAC TAC GAA TGG ACC TCC GTG AAC GAA GGA GAT TCG GGC GCC TCC GTT
arg lys tyr gln his tyr glu trp thr ser val asn glu gly asp ser gly ala ser val
361/27                                  391/37
TAC CGC CTC GCC GGA CAG CAG CCC GAG CTC TAT GTG AAA TTC GCT CCG CGC GAA CCG GAA
tyr arg leu ala gly gln gln pro glu leu tyr val lys phe ala pro arg glu pro glu
421/47                                  451/57
AAT TCC GCG TTC GAC CTG GCG GGC GAG GCC GAC CGG CTC ACC TGG CTC ACC CGC CAC GGC
asn ser ala phe asp leu ala gly glu ala asp arg leu thr trp leu thr arg his gly
481/67                                  511/77
ATC CCG GTT CCG TGC ATT GTC GAG TGC GGC GGC GAC GAC ACC TCG GTT TTC CTC GTC ACC
ile pro val pro cys ile val glu cys gly gly asp asp thr ser val phe leu val thr
541/87                                  571/97
GAG GCC GTC ACC GGC GTA TCG GCC GCC GAG GAG TGG CCG GAG CAC CAG CGC TTC GCC GTC
glu ala val thr gly val ser ala ala glu glu trp pro glu his gln arg phe ala val
601/107                                 631/117
GTC GAG GCG ATG GCC GAC CTC GCC CGC ACC CTG CAC GAA CTG CCC GTT GGT GGC TGC CCC
val glu ala met ala asp leu ala arg thr leu his glu leu pro val gly gly cys pro
661/127                                 691/137
TTC GAT CGC AGC CTG GCG GTG ACG GTT GCC GAA GCC CGC CAC AAC CTA CGC GAG GGC CTC
phe asp arg ser leu ala val thr val ala glu ala arg his asn leu arg glu gly leu
721/147                                 751/157
GTG GAC CTG GAC GAC CTC CAA GAG GAG CAC GCC AAC TGG TCC GGT GAC CAG CTT CTC GCC
val asp leu asp asp leu gln glu glu his ala asn trp ser gly asp gln leu leu ala
781/167                                 811/177
GAG CTC GAC CGA ACG CGG CCC GAG AAA GAG GAT CTG GTC CTC TGC CAC GGG GAC CTG TGC
glu leu asp arg thr arg pro glu lys glu asp leu val leu cys his gly asp leu cys
841/187                                 871/197
CCC AAC AAC GTG CTG CTC GAT CCC GAG ACA TGC CGA GTC ACC GGA ATG ATC GAT GTG GGC
pro asn asn val leu leu asp pro glu thr cys arg val thr gly met ile asp val gly
901/207                                 931/217
CGC CTC GGC CGC GCC GAT CGC CAC GCC GAC CTG GCC CTC GCC GCC CGC GAG CTG GAG ATC
arg leu gly arg ala asp arg his ala asp leu ala leu ala ala arg glu leu glu ile
961/227                                 991/237
GAC GAG GAT CCC TGG TTT GGC CCC GAG TAC GCC CAG CGG TTC CTC GAA CGC TAC GGC GCG
asp glu asp pro trp phe gly pro glu tyr ala gln arg phe leu glu arg tyr gly ala
1,021/247                               1,051/257
CAC CAC GTC GAC GAG AAC AAG ATG GCC TTT TAC CAG CTG CTC GAC GAG TTT TTC TAG AAA
his his val asp glu asn lys met ala phe tyr gln leu leu asp glu phe phe AMB
1,081                                   1,111
GGC TCC GTT AGG TCT TCG GGT GCG GTC TGT TCC TGG ACA TGG TGG ATG GAT GGA CGC ACA
                                        ←
1,141                                   1,171
TGA AGT GAA CCG TGT CCG GGC GAA GTT GAC GTT GTA CGT GGC TGA CGT GTT CGC ATC GGC
      →
1,201
GCC GCG TAA GGA CCA GCG GGC
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The deduced amino acid sequence of this gene is reported under the corresponding nucleotide codons. The vertical arrow indicates the 5′ end of the *aph* mRNA as determined by S1 mapping. The horizontal arrows indicate the two inverted sequences which could be involved in a putative transcription terminator. The −35 and −10 regions are underlined.

Expression of APH Activity

APH activity was assayed in crude extracts of *M. chalcea* 69-683, and *S. lividans* or *E. coli* carrying recombinant plasmids. *M. chalcea* 69-683 and pDSA212/*S. lividans* extracts had characteristic aminocyclitol 3′-*O*-phosphotransferase type V activity; the substrate range includes neomycin, ribostamycin and paromomycin but weak activity with butirosin (Table 2). Therefore, *M. chalcea* 69-683 APH is in the same group as the *S. fradiae* APH. No other aminocyclitol modifying enzyme activity (AAC or ANT) was detected with the *M. chalcea* 69-683 recombinant plasmids used in this work. APH activity was not detected in *E. coli* after transformation with plasmids harbouring the *aphA-5c* containing *Pst*I-*Sph*I fragment.

Sequence of *aphA-5c*

The nucleotide sequence of the *Sph*I-*Pst*I *aph*-containing fragment is shown in Fig. 2. Comparison with the APH(3′)-Va of *S. fradiae*[18] and APH(3′)-Vb of *S. ribosidificus*[19] permitted the localization of the phosphotransferase open reading frame according to protein sequence similarities.

The 5′ extremity of *aphA-5c* showed three ATG codons in the same phase. We were unsuccessful in obtaining amino terminal sequence of the purified APH, since the amino terminal appeared to be blocked. Cleavage of the APH protein with CNBr was also unsuccessful, presumably because of the presence of sulfoxydized methionine.

S1 mapping, performed with the 0.4 kb *Sac*I-*Bam*HI fragment as probe, indicated the transcription start in the region of the ATG (nucleotide 283) (Fig. 3). Comparison with the sequences of the *aph* genes of *S. fradiae*[18] and *S. ribosidificus*[19] (Fig. 4) strongly suggests that this ATG is the initiation codon, as judged by similarities in nucleotide sequence and *N*-terminal amino acid sequence of the three APH proteins. At present only the amino acid sequence for the *S. fradiae* protein has been determined.

Comparison Between *aphA* from Strains Producing Neomycin-like Antibiotics

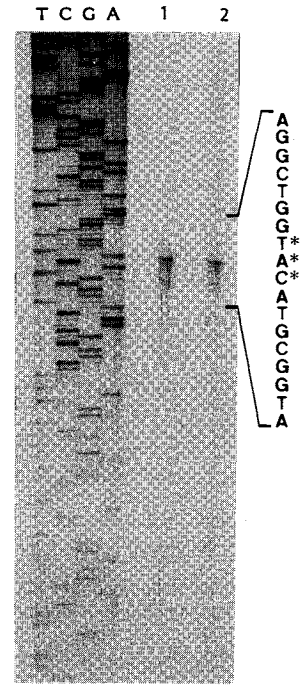
1) Intragenic: Strong nucleotide sequence

Table 2. Substrate specificity of *Micromonospora chalcea* and *Streptomyces fradiae* APH.

Substrate	*M. chalcea*	*S. fradiae*
Neomycin	100	100
Lividomycin	0	5
Butirosin	11	8
Ribostamycin	54	81
Paromomycin	107	98

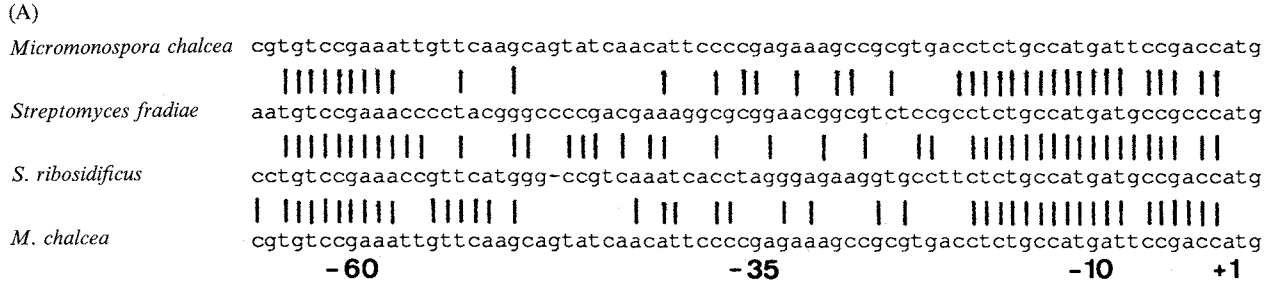
The results are expressed in percentage of activity against neomycin. Amikacin, gentamicin C1a, kanamycin, tobramycin, streptomycin and hygromycin B were not substrates (typical results).

Fig. 3. S1 mapping of *aphA-5c*.



Lanes T, C, G and A correspond to Sanger reactions. Lane 1 is *aph* DNA protected by mRNA from *Micromonospora chalcea* and lane 2 is *aph* DNA protected by mRNA from *Streptomyces lividans* harbouring pDSA 212 plasmid. The 18 mer synthetic oligonucleotide used as primer was 5′-GGGCTGCT-GTCCGGCGAG-3′ (anneals to nucleotides 367 to 384 of sequence shown in Fig. 2). Asterisks indicate possible transcription starting points.

Fig. 4. Comparisons of the promoter regions (A) and of the region just after the begining of transcription (B) of the three *aphA-5* genes.



The high degree of homology suggests that the initiation codon of the *M. chalcea aph* is the codon where transcription starts, as for *S. fradiae* and *S. ribosidificus*. The one letter amino acid code is used.

Table 3. Number of matched bases (A) or amino acids (B) between the three *aphA-5* (A) and their products (B).

(A)

	Micromonospora chalcea	*Streptomyces fradiae*	*Streptomyces ribosidificus*
Number of nucleotides	795	807	792
M. chalcea		641 (80%)	635 (80%)
S. fradiae			681 (86%)

(B)

	M. chalcea	*S. fradiae*	*S. ribosidificus*
Number of amino acids	264	268	263
MW of APH	29,794	29,953	29,542
M. chalcea		223 (84%)	216 (82%)
S. fradiae			230 (87%)

The percentages are indicated in parentheses. The MW's of the APH are calculated upon number of each amino acid. We have considered here identical or conserved (ile=leu=val; ser=thr; asp=glu; lys=arg; phe=tyr) amino acid residues.

similarities (at least 80%) were revealed by WILBUR and LIPMAN algorithm analysis[20] between the three *aphA-5* genes (Table 3); only weak similarities were observed between the *aphA-4* of *B. circulans* and the *aphA-5* group[21]. The same high degree of similarity was also seen in APH(3')-V protein sequences; APH(3')-IV has only 40% similarity to APH(3')-V[22]. However, the hydrophobicity patterns of the three APH(3')-V and other APH(3') proteins are very similar (Fig. 6), and the conserved amino acid sequence motifs[23] identified in the known APH(3') enzymes and protein kinases[24], are present. The G+C% and codon usage of the *aph* of *Micromonospora* and *Streptomyces* are different, consistent with the probability of finding G or C in third position in the codons (84% for *M. chalcea* against 97% for *S. fradiae*, and 92% for *S. ribosidificus*)[25].

2) Flanking Sequences: Upstream of the three *aphA-5* genes (Fig. 4), very strong nucleotide sequence homologies were found in the -1 to -20 region, but not in the -35 region. Another conserved region was identified near -60. Downstream of the *aph* genes, after the translation stop codon (TAG in all three cases), the similarity becomes very poor, even between *S. fradiae* and *S. ribosidificus* (38% for the 50 first nucleotides, against 46% between *M. chalcea* and *S. fradiae* and 36% between *M. chalcea* and *S. ribosidificus*, using the best alignment). A 12-base-pair imperfect repeated sequence determining a 25-base hairpin-loop was found at positions $1112 \sim 1160$, 34 bp after the amber codon ($\Delta G = -28.8$ kcal/mol)[26]; this may be implicated in transcription termination.

Discussion

M. chalcea aphA-5c Encodes an APH(3')-V

Antibiotic-producing strains avoid suicide by self protection mechanisms[1]. The producers of aminoglycoside antibiotics generally possess aminoglycoside modifying enzymes although other mechanisms of resistance such as ribosome modification are known. *S. fradiae* ATCC 10745, the neomycin producer, possesses both APH and AAC activities; APH is assumed to be the primary mechanism of resistance, and this function is also believed to be involved in biosynthesis although direct genetic or biochemical evidence

is not available. The *S. fradiae* enzyme is classified APH(3′)-V and the corresponding gene has been cloned and sequenced. As *M. chalcea* 69-683 a member of a different genus also produces neomycin, it was of interest to compare these two organisms; does *M. chalcea* 69-683 have similar APH and AAC activities to *S. fradiae* and what is the organisation of the resistance and biosynthetic genes in the two organisms? Is it possible that the neomycin biosynthetic genes were transferred *in toto* between these different genera? The APH of *M. chalcea* is also classified as (3′)-V. Among strains producing antibiotics of the neomycin type (4,5-substituted 2-deoxystreptamines), two classes of APH(3′) have been characterized: APH(3′)-V in

Fig. 5. Comparisons of amino acid sequences of APH(3′) from aminoglycoside-producing strains.

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IV  M N E S T R N W P E E L L E L L G Q T E L T V N K I G Y S G D H V Y H V
Va            M D D S T L R R K Y P H H E W H A V N E G D S G A F V Y Q L
Vb            M E - S T L R R T Y P H H T W H L V N E G D S G A F V Y R L
Vc            M Y A - M L R R K Y Q H Y E W T S V N E G D S G A S V Y R L 29
                                              *   *       * *

IV  K E Y R - - G T P A F L K I A P S V W W R - - - T L R P E I E A L A W L
Va  T G G P E P Q P E L Y A K I A P R A P E N S A F D L S G E A D R L E W L
Vb  T G - H G - - P E L Y A K I A P R T P E N S A F H L D G E A D R L D W L
Vc  A G - - Q - Q P E L Y V K F A P R E P E N S A F D L A G E A D R L T W L 62
                  *   *                   *       *       * *

IV  D G K - L P V P K I L Y T A E H G G M D Y L L M E A L G G K D G S H E T
Va  H R H G I P V P R V V E R G A D D T A A W L V T E A V P G V A A A E E W
Vb  A R H G I S V P R V V E R G A D D T T A W L V T E A V P G A A A S E E W
Vc  T R H G I P V P C I V E C G G D D T S V F L V T E A V T G V S A A E E W 98
                    *                   *               *

IV  I Q A N R K L F V K L Y A E G L R S V H G L D I R E C P L S N G L E K K
Va  P E H Q R F A V V E A M A E L A R A L H E L P V E D C P S D R R L D A A
Vb  P E D E R A A V V D A I A E M A R T L H E L P V S E C P F D R R L D V T
Vc  P E H Q R F A V V E A M A D L A R T L H E L P V G G C P F D R S L A V T 134
                    *                   *               * *

IV  R L D A K R I V D E S L V D P A D I K E E Y D C T P E E L Y G L L L E S
Va  V A E A R R N V A E G L V D L D D L Q E E R A G W T G D Q L L A E L D R
Vb  - G E A R H N V R E G L V D L D D L Q E E P A G W T G D Q L L A E L D L
Vc  V A E A R H N L R E G L V D L D D L Q E E H A N W S G D Q L L A E L D R 170
                      *   *

IV  - K P V T E D L V F A H G D Y C A P N L I I D G E - - K L S G F I D L G
Va  T R P E K E D L V V C H G D L C P N N V L L D P G T C R V T G V I D V G
Vb  T R P E K E D L V V C H G D L C P N N V L L D P E T H R I T G L I D V G
Vc  T R P E K E D L V V C H G D L C P N N V L L D P E T C R V T G M I D V G 206
                      * * *       *                       *

IV  R A G V A D R Y Q D I S L A I R S L R H D - - - Y G D D R Y K A L F L E
Va  R L G V A D R H A D I A L A A R E L E I D E D P W F G P A Y A E R F L E
Vb  R L R L A T C H A D L A L A A R E L A I D E D P W F G P A Y A E R F L E
Vc  R L G R A D R H A D L A L A A R E L E I D E D P W F G P E Y A Q R F L E 242
  *     *         * *             *

IV  L Y G L D G L D E D K V R Y Y I R L D E F F
Va  R Y G A H R V D K E K L A F Y Q L L D E F F
Vb  R Y G A H H V D Q E K M A F Y Q L L D E F F
Vc  R Y G A H H V D E N K M A F Y Q L L D E F F 264
              *   *   *       * * *
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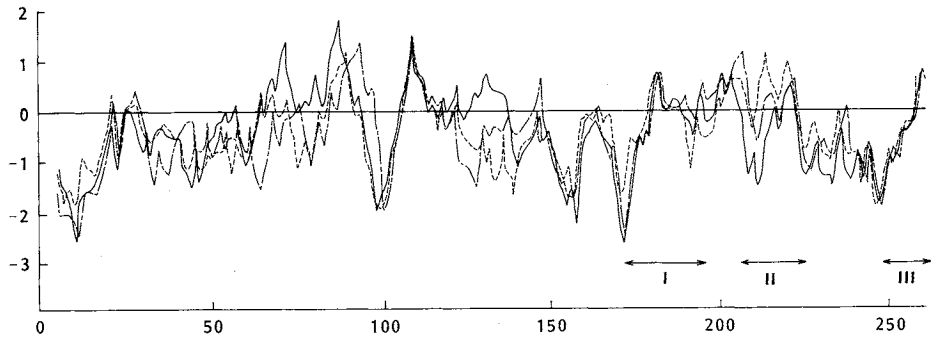
Conserved or identical amino acids are boxed (L=V=I, F=Y, K=R, S=T, D=E). The asterisks indicate amino acids conserved among the seven reported types of APH(3′). The sequence of *Streptomyces rimosus* forma *paromomycinus* APH is not yet available.

M. chalcea (neomycin), *S. fradiae* (neomycin), *S. ribosidificus* (ribostamycin) and *Streptomyces rimosus* forma *paromomycinus* (paromomycin)[27]; whereas *B. circulans* (butirosin) contains APH(3')-IV (Table 1). The two types of APH differ substantially in substrate range[28]. The 4,5-substituted 2-deoxystreptamine antibiotics possess a common biosynthetic intermediate in ribostamycin. The butirosin molecule harbours an unusual substituent on the 1-position (hydroxy amino butyric acid), and it is not surprising that butirosin modifying activity is specific to APH(3')-IV. Butirosin can be modified by other APH(3') enzymes found in Gram-positive and Gram-negative bacteria (types II and III); APH(3')-IV possesses no more similarity with types II and III than with type V[8,22].

We have cloned and determined the nucleotide sequence of the *M. chalcea* neomycin-resistance gene *aphA-5* and compared with other *aph* genes and their derived proteins. Strong nucleotide similarities are noted in the intragenic regions of the three *aphA-5*, but none with *aphA-4*, even though the latter may be

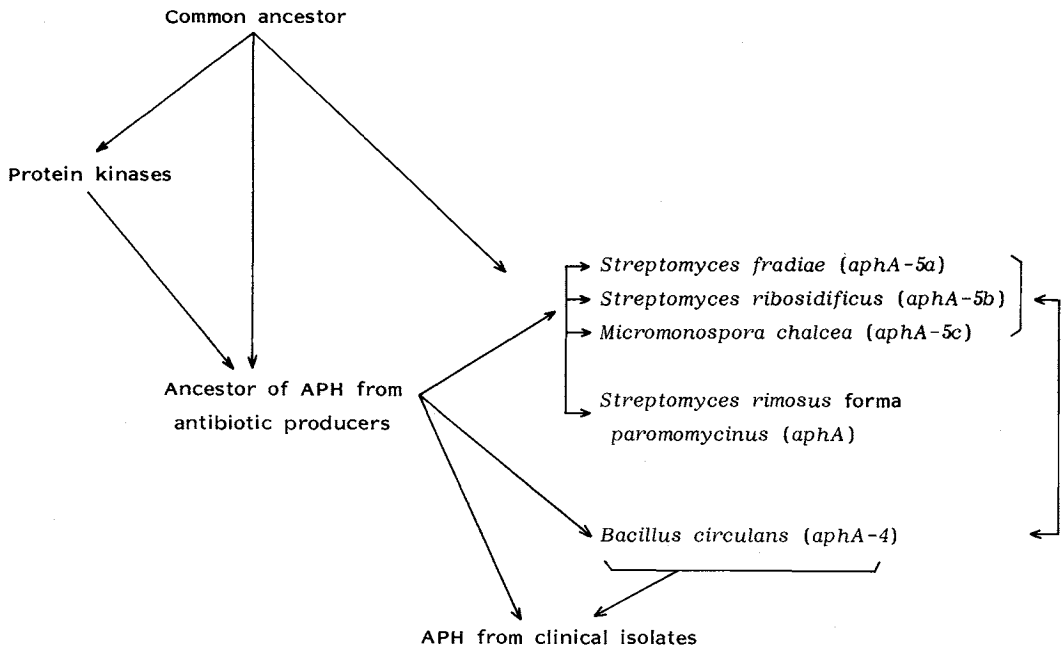
Fig. 6. Comparisons of hydrophobicity patterns of a number of APH(3').

—— APH(3')-Vc, —·— APH(3')-Va, ------ APH(3')-Vb.



Amino acid position is noted in abscissa while polarity is indicated in ordonate and expressed in units defined by KYTE and DOOLITTLE[42]. Arrows indicate conserved motifs throughout the majority of APH(3').

Fig. 7. The possible evolutionary path from a common ancestor to protein kinases and APH of antibiotic-producing and non producing bacteria.



considered essentially identical with respect to its roles in resistance and biosynthesis. Although the APH(3')-V protein from *M. chalcea* has no more than 30% sequence similarity to most of the clinical isolates, it is similar to the seven types of APH(3') previously reported[18,21~23,29~31] possessing the conserved amino acid motifs[8,22] (Fig. 5). Despite protein sequence differences among the seven APH(3') proteins, comparison of hydropathy profiles reveals that the physicochemical properties of certain sequence stretches throughout the majority of the sequence are conserved[32] (Fig. 6). DISTLER *et al.*[33] proposed that the *N*-terminal conserved region corresponded to the substrate recognition site of the enzyme, while the *C*-terminal region was involved in substrate binding. Sequences similar to those of proteins interacting with ATP[34] have also been deduced[8,22,33]. There is a plausible evolutionary route to APH of antibiotic-producing organisms or clinical isolates from the protein kinase genes of intermediary metabolism, or alternatively they may have evolved divergently from a common ancestor (Fig. 7).

The fact that amino acid sequence similarities are more numerous among *aphA-5* than with *aphA-4* translation products is probably due to a divergent evolution of the latter (Fig. 7). The same feature has been observed between the *aphA-1* genes from transposon Tn 903 and plasmid RP4[32], where the amino acid similarities are higher than with the other genes. It was proposed these latter two genes were the result of a more recent divergent evolution. In the case of the *aph* associated with production of neomycin-like antibiotics, the low G+C content of *B. circulans* obviously accentuates the differences. The divergence between the *Streptomyces* and *Micromonospora aph* may be due to the fact that the frequency of G or C in the third position in codon triplets is significantly lower in *Micromonospora*. Until more *Micromonospora* sequences are available, it will be difficult to identify cases of adaptation of a *Streptomyces* gene to *Micromonospora*, as might have followed gene transfer from one genus to the other. The strong similarities between *aph* genes of *S. fradiae*, *S. ribosidificus* and *M. chalcea* could be explained by interspecies transfer within related organisms.

5' and 3' Transcription Signals

The -10 and -35 regions of the three *aph* genes from neomycin and ribostamycin producers show no similarity to the *E. coli* consensus promoter sequence[6]. The -35 regions of three *aph* show limited sequence similarities, whereas the -10 regions situated just before the transcription initiation point as shown by S1 mapping (Fig. 4) are virtually identical. The presence of cytosine residues one, two, and five nucleotides in front of the translational start codon of each of these genes is consistent with the suggestion that an accumulation of C residues is a general feature of translation initiation sites in *Streptomyces*[35]; this conclusion can now be extended to *Micromonospora*. In all three cases, no ribosome binding site consensus sequence was shown. Otherwise, we note the presence of three ATG triplets in phase at the beginning of all three genes, the first at the -10 position in each case (Fig. 4). Is this just a coincidence? Upstream of the proposed promoter region, there is strong similarity in the -65 region of the *aphA-5* genes.

The nature of transcription terminations signals in *Streptomyces* is not well understood[36]. Inverted repeat sequences distal to the 3' ends of coding regions may lead to the formation of hairpin-loop structures which represent termination signals analogous to those observed in *E. coli*. Such a structure is present 3' of the *aph* of *S. fradiae* (16-base-pair repeated sequence 20 base pairs after the amber codon, and $\Delta G = -43$ kcal/mol) and also 34 bases after the stop codon in the *M. chalcea* gene (25 base pairs, $\Delta G = -28.8$ kcal/mol).

S1 mapping with a 5' end fragment of the *aph* gene of *S. fradiae* revealed the presence of two transcription starts[37]. The presence of tandem promoters is common in streptomycetes and has been demonstrated in the genus *Micromonospora*[38]; it has been suggested that such tandem promoters provide a mechanism for selective gene expression under different growth conditions. In the case of the *aph* of *M. chalcea* and *S. ribosidificus*, only one promoter was detected by S1 mapping. S1 mapping of the *aph* of *S. fradiae*[37], indicated five additional promoters near the initiation point, oriented in the opposite direction, but such features were not found for the *aph* of *M. chalcea*.

Genes involved in the biosynthesis of identical antibiotics in different organisms usually have strong similarities in functionality and sequence, for example as described for *S. griseus* and *Streptomyces glaucescens* respectively, streptomycin and hydroxystreptomycin producers[2], and more recently with the various organisms that produce β-lactam antibiotics[39]. In the case of the polyketide family, similar genes have been detected in diverse microbes by hybridisation[40] and this is likely to be the case for other antibiotics,

such as for example, the peptides. Are there common gene sequences associated with the biosynthesis of the neomycin-like antibiotics? This is currently being investigated using sequences flanking the resistance genes. Finally, do these biosynthetic gene clusters diverge from a common ancestor, or are they the result of multiple interspecific gene transfers? An approach to this question may come from studying the organization and the functionality of the sequences flanking the *aph* in a variety of producing organisms. To date the genes of the biosynthetic pathway to neomycin antibiotics remain unidentified and no potential "clusters" have been indicated.

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References

1) CUNDLIFFE, E.: How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. 43: 207~233, 1989
2) MANSOURI, K.; K. PISSOVOTZKI, J. DISTLER, G. MAYER, P. HEINZEL, C. BRAUN, A. EBERT & W. PIEPERSBERG: Genetics of streptomycin production. *In* Genetics and molecular biology of industrial microorganisms. *Ed.*, C. L. HERSHBERGER *et al.*, pp. 61~67, American Society for Microbiology, 1989
3) THOMPSON, C. J.; J. M. WARD & D. A. HOPWOOD: DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. Nature 286: 525~527, 1980
4) WAGMAN, G. H.; J. A. MARQUEZ, P. D. WATKINS, J. V. BAILEY, F. GENTILE & M. J. WEINSTEIN: Neomycin production by *Micromonospora* species 69-683. J. Antibiotics 26: 732~736, 1973
5) SALAUZE, D.; J. A. PEREZ-GONZALEZ, W. PIEPERSBERG & J. DAVIES: Characterization of aminoglycoside acetyltransferase genes of neomycin producing *Micromonospora* and *Streptomyces*. Gene 101: 143~148, 1991
6) WESTPHELING, J.; M. RANES & R. LOSICK: RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature 313: 22~27, 1985
7) TRIEU-CUOT, P. & P. COURVALIN: Evolution and transfer of aminoglycoside resistance genes under natural conditions. J. Antimicrob. Chemother. 18 (Suppl. C): 93~102, 1986
8) PIEPERSBERG, W.; J. DISTLER, P. HEINZEL & J. A. PEREZ-GONZALEZ: Antibiotic resistance by modification: many resistance genes could be derived from cellular control genes in Actinomycetes. A hypothesis. Actinomycetol. 2: 83~98, 1988
9) KATZ, E.; C. J. THOMPSON & D. A. HOPWOOD: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129: 2703~2714, 1983
10) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. L. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic manipulation of *Streptomyces*. A Laboratory Manual. John Innes Foundation, 1985
11) MANIATIS, T.; E. F. FRITSCH & J. SAMBROOK: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1982
12) NORRANDER, J.; T. KEMPE & J. MESSING: Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26: 101~106, 1983
13) YANISCH-PERRON, C.; J. VIERA & J. MESSING: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103~109, 1985
14) SANGER, F.; S. NICKLEN & A. R. COULSON: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74: 5463~5467, 1977
15) GLIŠIN, V.; R. CRKVENJAKOV & C. BYUS: Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13: 2633~2637, 1974
16) MURAKAMI, T.; T. G. HOLT & C. J. THOMPSON: Thiostrepton-induced gene expression in *Streptomyces lividans*. J. Bacteriol. 171: 1459~1466, 1989
17) HAAS, M. J. & J. E. DOWDING: Aminoglycoside-modifying enzymes. Methods in Enzymology 43: 611~628, 1975
18) THOMPSON, C. J. & G. S. GRAY: Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferase encoded by resistance plasmids. Proc. Natl. Acad. Sci. U.S.A. 80: 5190~5194, 1983
19) HOSHIKO, S.; C. NOJIRI, K. MATSUNAGA, E. SATO & K. NAGAOKA: Nucleotide sequence of the ribostamycin phosphotransferase gene and its control region in *Streptomyces ribosidificus*. Gene 68: 285~296, 1988
20) WILBUR, W. J. & D. J. LIPMAN: Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. U.S.A. 80: 726~730, 1983

21) HERBERT, C. J.; I. C. GILES & M. AKHTAR: The sequence of an antibiotic resistance gene from an antibiotic producing bacterium. Homologies with transposon genes. FEBS Lett. 160: 67~70, 1983
22) MARTIN, P.; E. JULIEN & P. COURVALIN: Nucleotide sequence of *Acinetobacter baumanii aphA-6* gene: evolutionary and functional implications of homologies with nucleotide-binding proteins, kinases and other aminoglycoside-modifying enzymes. Mol. Microbiol. 2: 615~625, 1988
23) TENOVER, F. C.; T. GILBERT & P. O'HARA: Nucleotide sequence of a novel kanamycin resistance gene, *aphA-7*, from *Campylobacter jejuni* and comparison to other kanamycin phosphotransferase genes. Plasmid 22: 52~58, 1989
24) HEINZEL, P.; O. WERBITZKY, J. DISTLER & W. PIEPERSBERG: A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin 3″ phosphotransferase, relationships between antibiotic and protein kinases. Arch. Microbiol. 150: 184~192, 1988
25) BIBB, M. J.; P. R. FINLAY & M. W. JOHNSON: The relationship between base composition and codon usage in bacterial genes and its use in the simple and reliable identification of protein coding sequences. Gene 30: 157~166, 1984
26) BORER, P. N.; B. DENGLER, I. TINOCO & O. C. UHLENBECK: Stability of ribonucleic acid double stranded helices. J. Mol. Biol. 86: 843~853, 1974
27) PEREZ-GONZALEZ, J. A. & A. JIMENEZ: Cloning and expression in *Streptomyces lividans* of a paromomycin phosphotransferase from *Streptomyces rimosus forma paromomycinus*. Biochem. Biophys. Res. Commun. 125: 895~901, 1984
28) COURVALIN, P.; B. WEISBLUM & J. DAVIES: Aminoglycoside-modifying enzyme of an antibiotic-producing bacterium acts as a determinant of antibiotic resistance in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 74: 999~1003, 1977
29) OKA, A.; H. SUGISAKI & M. TAKANAMIU: Nucleotide sequence of the kanamycin resistance transposon Tn 903. J. Biol. Mol. 147: 217~226, 1981
30) BECK, E.; G. LUDWIG, E. A. AUERSWALD, B. REISS & H. SCHALLER: Nucleotide sequence and exact location of the neomycin phosphotransferase gene from transposon Tn5. Gene 19: 327~336, 1982
31) TRIEU CUOT, P. & P. COURVALIN: Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3′5″-aminoglycoside phosphotransferase type III. Gene 23: 331~334, 1983
32) PANSEGRAU, W.; L. MIELE, R. LURZ & E. LANKA: Nucleotide sequence of the kanamycin resistance determinant of plasmid RP 4: homology to other aminoglycoside 3′-phosphotransferases. Plasmid 18: 193~204, 1987
33) DISTLER, J.; C. BRAUN, A. EBERT & W. PIEPERSBERG: Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: analysis of a central region including the major resistance gene. Mol. Gen. Genet. 208: 204~210, 1987
34) FRY, D. C.; S. A. KUBY & A. S. MILDVAN: ATP-binding site of adenylate kinase: mechanistic implications of its homology with ras-encoded p21, F1-ATPase, and other nucleotide-binding proteins. Proc. Natl. Acad. Sci. U.S.A. 83: 907~911, 1986
35) BIBB, M. J. & G. R. JANSSEN: Unusual features of transcription and translation of antibiotic resistance genes in antibiotic producing *Streptomyces*. *In* Genetics of Industrial Microorganisms. *Ed.*, M. ALACEVIC *et al.*, pp. 309~318, Pliva Publishers, 1986
36) BIBB, M. J.; M. J. BIBB, J. M. WARD & S. N. COHEN: Nucleotide sequences encoding and promoting expression of three antibiotic resistance genes indigenous to *Streptomyces*. Mol. Gen. Genet. 199: 26~36, 1985
37) JANSSEN, G. R.; M. J. BIBB, C. P. SMITH, J. M. WARD, T. KIESER & M. J. BIBB: Isolation and analysis of *Streptomyces* promoters. *In* Microbiology 1985. *Eds.*, G. HEGEMON & C. L. HERSHBERGER, pp. 392~396, American Society for Microbiology, 1985
38) BAUM, E. Z., M. J. BUTTNER, L.-S. LIN & D. M. ROTHSTEIN: Transcription from the P1 promoters of *Micromonospora echinospora* in the absence of native upstream DNA sequences. J. Bacteriol. 171: 6503~6510, 1989
39) SMITH, D. J.; M. K. R. BURNHAM, J. H. BULL, J. E. HODGSON, J. M. WARD, P. BROWNE, J. BROWN, B. BARTON, A. J. EARL & G. TURNER: β-Lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. EMBO J. 9: 741~747, 1990
40) SENO, E. T. & R. M. BALTZ: Structural organization and regulation of antibiotic biosynthesis and resistance genes in Actinomycetes. *In* Regulation of Secondary Metabolism in Actinomycetes. *Ed.*, S. SHAPIRO, pp. 1~48, CRC, 1989
41) LÓPEZ-CABRERA, M.; J. A. PÉREZ-GONZÁLEZ, P. HEINZEL, W. PIEPERSBERG & A. JIMÉNEZ: Isolation and nucleotide sequencing of an aminocyclitol acetyltransferase gene from *Streptomyces rimosus* forma *paromomycinus*. J. Bacteriol. 171: 321~328, 1989
42) KYTE, J. & R. F. DOOLITTLE: A simple method for displaying the hydropathic character of a protein. J. Biol. Mol. 157: 105~132, 1982