Sequence and interspecies transfer of an aminoglycoside phosphotransferase gene (*APH*) of *Bacillus circulans*

Self-defence mechanism in antibiotic-producing organisms

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The *APH* gene of a butirosin-producing *Bacillus circulans* was cloned and shown to be expressed in *Escherichia coli* and *Streptomyces lividans*. The gene was sequenced and a possible developmentally regulated promoter identified. When the deduced protein sequence was compared with those from transposon Tn5, transposon Tn903, *Streptomyces fradiae*, *Staphylococcus aureus* and *Streptococcus faecalis*, significant homology was found, indicating that the genes may have a common origin.

INTRODUCTION

Micro-organisms, both prokaryotic and eukaryotic, produce several thousand antibiotics of diverse chemical structure (Berdy, 1974). One unique problem that all these organisms face is to avoid being killed by their own antibiotics. In recent years it has become apparent that the mechanisms used by antibiotic-producing micro-organisms to protect themselves from their own antibiotics are similar to those found in clinical isolates of antibiotic-resistant bacteria (reviewed in Davies & Smith, 1978; Foster, 1983; Cundliffe, 1984). This has led to the hypothesis that antibiotic-producing organisms could be the original source of clinically significant antibiotic-resistance (Walker & Walker, 1970; Benveniste & Davies, 1973).

When the spectrum of antibiotic producers is surveyed, one of the most striking features is that the vast majority are differentiating micro-organisms. The observations that antibiotic production and antibiotic-resistance only appear at certain stages in the life of a culture (Demain, 1974) and that some mutations that affect antibiotic metabolism also have pleiotropic effects on differentiation (Martin & Demain, 1980; Hopwood, 1981) raise the possibility that the control mechanism for the two processes may be similar or linked. In the genus *Bacillus* one level of developmental control is the production at different stages of the life cycle of distinct RNA polymerase σ factors, which modify the RNA polymerase apoenzyme and cause the recognition of different sets of promoters (Losick & Pero, 1981; Johnson *et al.*, 1983).

In an attempt to answer some of these questions concerning the inter-relationship of antibiotic-resistance genes and the control of these genes in producing organisms, we have elucidated the full nucleotide sequence of the aminoglycoside phosphotransferase (*APH*) gene from *Bacillus circulans*, which produces the aminoglycoside antibiotic butirosin. The deduced protein sequence is compared with the sequences of aminoglycoside phosphotransferases from other sources. In addition, possible regulatory sequences in the *APH* gene of *B. circulans* are identified. This work is taken in part from

a Ph.D. thesis (Herbert, 1983) and a preliminary account of part of this work has already been published (Herbert *et al.*, 1983).

MATERIALS AND METHODS

Materials

[α - ^{32} P]dATP (400 Ci/mmol; catalogue no. PB164) and [γ - ^{32} P]ATP were obtained from Amersham International, Amersham, Bucks., U.K. Dideoxynucleoside triphosphates and M13 15-base universal primer were obtained from P-L Biochemicals, Northampton, U.K.

DNA polymerase Klenow fragment was obtained from BCL-Boehringer Corp., Lewes, East Sussex, U.K.

Reagents for oligonucleotide synthesis were obtained from Cruachan (now Cruachem) Chemicals, Livingstone, Scotland, U.K.

Restriction endonucleases and phage- T_4 DNA ligase were obtained from Bethesda Research Laboratories (U.K.), Cambridge, U.K.

Butirosin and ribostamycin were a gift from Dr. K. Richardson, Pfizer Central Research, Sandwich, Kent, U.K.; however, the latter antibiotic is also available from Sigma Chemical Co., Poole, Dorset, U.K.

Bacterial strains

Bacillus circulans was a gift from Dr. J. Davies, Biogen, Geneva, Switzerland. *Escherichia coli* HB101 was a gift from Dr. M. Pocklington, Department of Biology, University of Southampton. *Escherichia coli* JM103 and M13 phages mp8 and mp9 were obtained from Bethesda Research Laboratories (U.K.). *Streptomyces lividans* 66 and M180 (*Strep. lividans* 66 harbouring the plasmid SLP1.2) were gifts from Professor D. A. Hopwood, John Innes Institute, Norwich, U.K.

DNA sequencing

DNA was sequenced by using the chain-termination method of Sanger *et al.* (1977) in conjunction with the M13 phage system of Messing (1982), with the 'universal primer' or specific oligodeoxynucleotides.

Enzyme assay

Aminoglycoside phosphotransferase activity was assayed in crude cell extracts by using the semi-quantitative phosphocellulose-paper binding assay method of Haas & Dowding (1975).

Media

B. circulans was grown in Difco nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 1% (w/v) glucose. *E. coli* strains were grown in L-broth (Miller, 1972). Minimal agar for *Streptomyces* contained asparagine (0.5 g/l), K_2HPO_4 (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), $FeSO_4 \cdot 7H_2O$ (0.01 g/l) and Bacto-agar (15 g/l); after autoclaving sterile glucose solution was added to 1% (w/v). For minimal ribostamycin agar, sterile ribostamycin solution was added to minimal agar to give a final concentration of 25 µg/ml. The other media used for *Streptomyces* were as described in Chater *et al.* (1982).

Oligodeoxynucleotide synthesis

Oligodeoxynucleotides were synthesized as described in detail by Ner *et al.* (1986). The salient features of the method are as follows: 5'-dimethoxytritylthymidine-3'-succinyl-polystyrene resin (t-resin) with a loading of 230 µmol/g of resin was prepared by the method of Ito *et al.* (1982), and oligodeoxynucleotide chain was assembled on this resin essentially by using the method of Gait *et al.* (1982), which involves selective flow of solvents (1 ml/min) through a column containing the resin. After the removal of 5'-dimethoxytrityl group from the resin (25 mg) with 10% (w/v) trichloroacetic acid in chloroform (2 min), the resin was successively washed with chloroform (3 min), dimethylformamide (3 min) and pyridine (4 min). Appropriately *N*-protected 5'-*O*-dimethoxytrityl-2-deoxynucleoside-3'-(2-chlorophenyl) phosphate (50 mg) and the condensing agent 1-(mesitylenesulphonyl)-3-nitro-1,2,4-triazole (60 mg) in 300 µl of doubly distilled dry pyridine were then injected over a 5 min period and the reaction was allowed to proceed for 40 min. The excess reagents were then removed by washing with pyridine (4 min) and chloroform (3 min).

The cycle of deprotection and coupling was repeated until a 16-base-long oligomer was obtained. The overall yield was about 32%.

The removal of the oligodeoxynucleotide chain from the resin and various deprotections were performed as described by Efimov *et al.* (1982). The completely deprotected oligodeoxynucleotide was purified to greater than 95% purity by gel electrophoresis on a 20% polyacrylamide denaturing gel. After placement of the gel on a fluorescent t.l.c. plate, the slowest-moving band, which was most intense when viewed under 254 nm light, was excised, and oligodeoxynucleotide was eluted with 25 mM-triethylammonium bicarbonate, pH 7.6, at 37 °C for 24 h and supernatant passed through Sep-pak C_{18} cartridge (Waters Associates, Milford, MA, U.S.A.). The oligodeoxynucleotide was then eluted with 30% (v/v) acetonitrile in 25 mM-triethylammonium bicarbonate, pH 7.6, and evaporated to dryness.

Protein assay

Protein was assayed by the method of Bradford (1976), with bovine γ -globulin as the standard.

Restriction-endonuclease digestion, ligations and agarose-gel electrophoresis

These were performed by using the standard methods and buffers described in Davis *et al.* (1980).

Transformation

E. coli strains were transformed by using the methods of Davis *et al.* (1980), and *Strep. lividans* 66 was transformed by using the method of Chater *et al.* (1982).

RESULTS

Cloning of the *B. circulans* APH gene in *E. coli*

The previous demonstration that the APH gene of *B. circulans* could be expressed in *E. coli* (Courvalin *et al.*, 1977) simplified the cloning of the gene, as clones could be detected by the antibiotic-resistance phenotype. Total DNA from *B. circulans* (2 µg) was digested with restriction endonuclease *EcoRI* and mixed with *EcoRI*-

Table 1. Phosphotransferase activity in the APH-gene clones

Phosphotransferase activity was assayed in crude cell extracts by using the semi-quantitative phosphocellulose-paper binding assay method (Haas & Dowding, 1975) with ribostamycin as the phosphate acceptor (A) or butirosin as the phosphate acceptor (B). The assays were carried out for 20 min in a reaction mixture that contained 7.5 nmol of ATP and 3 nmol of antibiotic. The transfer of 10000 d.p.m. to the antibiotic is equivalent to the production of approx. 0.8 nmol of phosphorylated antibiotic.

Strain	Concn. of protein (mg/ml)	Activity (d.p.m. transferred/10 µl of extract)	Relative activity (d.p.m. transferred/µg of protein)
(A) <i>B. circulans</i>	2.0	2740	137
<i>E. coli</i> HB101 [pCH1]*	6.7	19670	296
<i>E. coli</i> HB101 [pCH4]†	6.1	17850	293
<i>E. coli</i> HB101 [pCH5]‡	5.8	15560	268
<i>E. coli</i> [pBR322]	4.5	230	5
(B) <i>B. circulans</i>	3.2	11440	358
<i>Strep. lividans</i> 66 [pCL1]	3.4	10840	319
<i>Strep. lividans</i> 66 SLP1.2	2.8	890	32

* 7.5 kb *EcoRI*-digest fragment of *B. circulans* in pBR322.

† 5.7 kb *HindIII*-digest fragment of *B. circulans* in pBR322.

‡ 2.7 kb *SalI*-digest fragment of *B. circulans* in pBR322.

Fig. 1. Southern-blot experiment

The DNA was isolated from various clones and aminoglycoside-producing organisms, digested with *Eco*R1, and subjected to electrophoresis in an agarose gel. The DNA was transferred to a nitrocellulose filter, and the filter was hybridized by using the 2.7 kb *Sal*I-digest fragment of pCH5 that had been labelled with ^{32} P by nick-translation in the presence of [α - ^{32} P]dCTP. After this, the nitrocellulose filter was exposed to X-ray film. A comparison of the original gel and autoradiograph showed that the 2.7 kb

cleaved plasmid pBR322. After ligation, the mixture was used to transform *E. coli* HB101, and transformants were selected for ampicillin-resistance and ribostamycin-resistance [ribostamycin (25 μ g/ml) was used in most experiments because it was more readily available than butirosin]. Plasmids from 11 transformants were isolated and restriction analysis showed that all contained the same 7.5 kb *Eco*R1 insert. The phosphotransferase from *B. circulans* is reported to have a monomer of M_r 28 500 (Davies & Smith, 1978), which corresponds to a coding region of some 800 base-pairs and a total gene length of approx. 1200 base-pairs. Two deletion derivatives were isolated from one of the recombinant plasmids (pCH1) that contains direct repeat of pBR322 as well as the 7.5 kb *Eco*R1-digest insert, after digestion with *Hind*III or *Sal*I restriction endonuclease. These contained a 5.5 kb *Hind*III-digest insert (pCH4) or a 2.7 kb *Sal*I-digest insert (pCH5) at the corresponding site in pBR322. Both these derivatives expressed ampicillin- and ribostamycin-resistance in *E. coli*.

Expression of the *B. circulans* APH gene in *Strep. lividans*

In attempts to express the *B. circulans* APH gene in *Strep. lividans* 66, the 2.7 kb *Sal*I-digest insert of pCH5 was transferred to the *Streptomyces* vector SLP1.2 (Bibb *et al.*, 1980) by ligating a mixture of a *Sal*I digest of pCH5 (1 μ g) and a partial digest of SLP1.2 (0.5 μ g) cut at one or two sites of its three *Sal*I sites. Protoplasts of *Strep. lividans* 66 were transformed, spread on regeneration agar, and incubated for 10 days at 28 °C. This resulted in well-sporulated lawns that contained approx. 1500 'pocks' (zones of growth inhibition) due to the L_T^+ reaction associated with the transfer of SLP1.2-derived plasmids into a plasmid-free host (Bibb *et al.*, 1978). The regeneration plates were replicated on to minimal agar containing ribostamycin. After incubation, 51 patches of drug-resistant growth were seen. One of these, *Strep. lividans* 66 [pCL1], was purified and found to contain a plasmid (pCL1) in which the 2.7 kb *Sal*I-digest fragment derived from pCH5 had been cloned into intact SLP1.2.

For further proof that ribostamycin-resistance is encoded by pCL1, the plasmid was purified from one of the colonies above and used to transform protoplast of *Strep. lividans* 66 and the regeneration performed as above for 10 days, when well-sporulated lawns with several pocks were seen. The replica plating of the latter on to minimal agar with ribostamycin showed that each pock on the master plate had given rise to a ribostamycin-resistance colony on the replica. In control experiments, purified pCL1 was spread on to protoplast-regeneration agar plates, but no colonies grew, indicating that the DNA was not contaminated with cells. This demonstrated that the ribostamycin-resistance is linked to the plasmid.

*Sal*I-digest fragment hybridized to DNA in tracks 1, 2, 4 and 5: track 1, 7.5 kb *Eco*R1-digest fragment of pCH3; track 2, 2.7 kb *Sal*I-digest fragment of pCL1; track 3, *Hind*III-digested λ -phage DNA; tracks 4 and 5, 7.5 kb *Eco*R1-digest fragment of *B. circulans* DNA. The other tracks, 6, 7, 8 and 9, represent unsuccessful attempts at hybridization with DNA from other aminoglycoside-synthesizing organisms, namely *Eco*R1-digested *Strep. fradiae* DNA, *Eco*R1-digested *Strep. ribosidificus* DNA, *Eco*R1-digested *Strep. rimosus* forma *paromomycinus* DNA and *Eco*R1-digested *Strep. lividus* DNA respectively.

Table 2. Sequences of synthetic oligodeoxynucleotides used for sequencing the *APH* gene

The four primers were synthesized and purified as described in the Materials and methods section. These were then used to sequence various stretches of the 2.7 kb *Sal*I-digest DNA containing the *APH* gene as indicated below. The strand of the gene used in the sequencing for each oligodeoxynucleotide is shown in parenthesis. The full sequence is outlined in Fig. 3.

Sequence of oligodeoxynucleotide	Stretch of 2.7 kb <i>Sal</i> I-digest DNA sequenced
(A) 3'TTTCGAGGCCGTCTAA5'	192→538 (coding strand)
(B) TTCGCTCGCGAAGTTA	492→866 (coding strand)
(C) TGAGAGTTCGTTATCG	815→1126 (coding strand)
(D) TGTTTACTTGCTTTCA	17←255 (non-coding strand)

Fig. 2. Diagrammatic representation of the gel readings used to construct the sequence shown in Fig. 3

DNA pieces in the range 400–1000 base-pairs, obtained from the 2.7 kb *Sal*I-digest fragment, were cloned in phages M13mp8 and M13mp9 and sequenced as described in the text. The consensus sequence of the essential portion of the *APH* gene (Fig. 3) was further confirmed by 'gene-walking' with four synthetic primers (Table 2). Arrows below the line show the direction and extent of sequencing data obtained with universal primer, whereas arrows above the line were with synthetic primers.

Biochemical characterization of the *APH*-gene clones

To confirm that the antibiotic-resistance phenotype observed in the clones was due to an aminoglycoside phosphotransferase, the amount of the enzyme was measured in crude cell extracts by using the phosphocellulose-paper binding assay method of Haas & Dowding (1975). The results of these assays (Table 1) show that the activity cloned is indeed a phosphotransferase. In *E. coli* harbouring the *APH*-gene-carrying derivatives of pBR322 (a relatively high-copy-number vector) the amount of the enzyme is about twice that found in *B. circulans*. However, in *Strep. lividans* 66, transformed with the appropriate derivative of SLP1.2 (a low-copy-number vector; Bibb *et al.*, 1980), the activity is about the same as in *B. circulans*. When the activity of the enzyme was measured in the three different hosts (*B. circulans*, *E. coli* and *Strep. lividans*) with a variety of aminoglycosides as the phosphate acceptor, the spectrum of activity was found to be essentially the same in each case (Herbert, 1983).

To confirm that the *APH* gene cloned is derived from *B. circulans*, the 2.7 kb *Sal*I-digest insert from pCH5 was purified, labelled by nick-translation and used to probe restriction-endonuclease digests of *B. circulans* DNA in a Southern-blot experiment (Southern, 1975). As expected,

when the genomic DNA was digested with *Eco*RI the fragment hybridized to a 7.5 kb band (Fig. 1).

Sequencing of the *B. circulans* *APH* gene

The source of the *B. circulans* *APH* gene used for the sequencing experiments was the 2.7 kb *Sal*I-digest insert cloned in pCH5. The method used was the chain-termination method of Sanger *et al.* (1977) in conjunction with the M13 phage cloning system of Messing (1982) to provide single-stranded DNA templates. The sequences of the ends of the 2.7 kb fragment were determined after the fragment had been cloned in both orientations in the *Sal*I site of the phage M13mp8. Libraries for sequencing were generated by polymerizing the purified 2.7 kb *Sal*I-digest fragment with phage-*T*₄ DNA ligase (to avoid end effects) and then partially digesting this polymerized material with *Sau*3AI, *Taq*I or *Hpa*II restriction endonuclease. Fragments in the range of 400–1000 bp were purified and cloned in M13mp9 cut with *Bam*HI (for *Sau*3AI-digest fragments) or *Acc*I (for *Taq*I-digest and *Hpa*II-digest fragments). From the data obtained from these libraries it was possible to produce a continuous sequence for the 2.7 kb *Sal*I-digest fragment. The consensus sequence of the 2.7 kb fragment was further analysed by 'gene-walking' with four specially synthesized

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AGATCGGCCCTAAAAAGGAATTTTGTTCCTAACGCGGAATAACAGTAATTTCCGTTTAAAGCGGGCCCTTTTAAAAAGCGGAGAGGATCTACTGTATAGTTCTTTACTTTGGTAATGTTCT
10 20 30 40 50 60 70 80 90 100 110 120

GGTGGATCGGCTATGAATGTACAAGATTTTTTTGCAAACTGCGGAGCTTTACACGGAATGAACGTTACTAAGAAAAATTAGGCCGAGGACGAATCGGGCCTTGCAGGAGGATCAAGA
130 140 150 160 170 180 190 200 210 220 230 240

TCCGTATTAAAGCCGAATTTTAAGGAGGAACCTGACAAATGAACGAAAGTACGCGTAATTTGGCCGAGGAACTTCTTGAGCTTCTCGGGCAGACGGAACCTAACCGTCAACAAAAATCGGATAT
250 260 270 280 290 300 310 320 330 340 350 360

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 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 D
TCCGAGATCAGCTATCAGTGAAAGAGTACAGGGGCACCCCGCATTTCTGAAAAATCGCCCCAGIGTATGTTGGAGAACGCTCCGGCCCGAAATTTGAAGCGCTCGCTTGGCTGGAC
370 380 390 400 410 420 430 440 450 460 470 480

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 I Q A K R K
GGGAAGCTCCCGGTTCCCAAAATTTTGTACACGCGCAACACGCGGGGATGGACTACTTGTGATGGAGCGCTAGGCGGAAAGACGGCTCCACGAAACGATCCAGGGGAAGCGGAAA
490 500 510 520 530 540 550 560 570 580 590 600

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 L R D A K R I V D E
CTGTTGTGAAGCTGTACGCGGAAGGCTCCGAAGCGTGATGCGCTCGATATCGCGAATGTCGCTGTGCGAAGGCTGGAGAAGAGCTCCGGGATCGGAAAGAAATAGTCGATGAG
610 620 630 640 650 660 670 680 690 700 710 720

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 K P V T E D L V F A H G D Y
AGCCTGGTGGACCGCGCGATATAAAGAGGAGTACGATTGCACGCGGAGGAATGTACGGGCTATTGCTTGAGAGTAAGCCGGTAACCGAAGATCTGGTTTTTTCGCACGCGGATTAAC
730 740 750 760 770 780 790 800 810 820 830 840

C A P N L I I D G E K L S G F I D L G R A G V A D R Y Q D I S L A I R S L R H D
TGTGCTCGGAATCTGATTATCGACGGTGAGAAAGCTGTGCGGATTTATCGATCTCGGACGTGCGGGCGTGGCGGACCGTTATCAGGACATCAGCCTGGCGATCCGACGCTCCGSCACGAT
850 860 870 880 890 900 910 920 930 940 950 960

Y G D D R Y K A L F L E L Y G L D G L D E D K V R Y Y I R L D E F F *
TAGGGCAGCAGCAGCTACAAAGCGCTCTTCCTGGAACCTTACGGGCTGGACGGGCTTGACGAGGACAAGGTCCGGTATTACATCCGGCTGGATGAATTTTTTTGACGAAACGGAAGGAGA
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080

CAGCCGGAATGTTAAAGAGCTGCAAAACCATGGGAGTAAACCGCAGCGGATCGAAATCGGATTCCGTTAAGAAAGGGGGACAAACCGTGCTCTATCATTTTCAGCGAAACCCCGGCATC
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200

AAGACGTTGTCCTCCCGGAAAAACCGCCCGGCTCGTGGCGGCTTGCCCGCTGTGCTGGCGGTCGATGAAGCGCATGCGCATCTGTATTATTTCGGGGGATTGCCCGGCATCGTCTACG
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320

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Fig. 3. Nucleotide sequence of the *APH* gene of *B. circulans*

The nucleotide sequence of the non-coding strand of the *APH* gene, in the 5'-3' direction, was deduced by the protocol in Fig. 2 by using the chain-termination method of Sanger *et al.* (1977) in conjunction with the M13 phage system of Messing (1982). The translated protein sequence is shown above the coding region. The DNA sequences upstream of the protein coding region, which contain the putative σ^{37} and σ^{54} — 35 and — 10 (Pribnow) promoter regions and Shine-Dalgarno ribosome-binding site, are further elaborated in Figs. 6 and 7 respectively. A potential termination sequence is indicated by arrows.

Fig. 5. Comparison of *APH*-gene-product protein sequences showing regions of similarity

The alignments were obtained by using the Needleman & Wunch (1970)/Sellers (1980) algorithm, comparing each of the sequences in turn with that for *B. circulans*. The sequences used were for the proteins from (1) Tn903, (2) Tn5, (3) *Strep. fradiae*, (4) *Staph. aureus*, (5) *Strep. faecalis* and (6) *B. circulans*.

oligodeoxynucleotide primers (Table 2). These primers were designed to contain the sequences from the regions of the consensus data that were reinforced from a number of independent clones representing both the DNA strands. These primers were used either to sequence the 2.7 kb insert of pCH5 (pBR322+2.7 kb *SalI*-digest fragment) or one of the M13mp8 derivatives containing the appropriate strand of the 2.7 kb *SalI*-digest fragment. Cumulatively, these experiments gave good overlapping data within the putative coding region of the *APH* gene, and more importantly provided unambiguous information

on the DNA sequence of its 5'-upstream region that could contain the various initiation and binding sites (Figs. 2 and 3). Translation of the sequence from both strands and in all reading frames revealed the possibility of one long open reading frame of 786 bases that could code for a protein of M_r 29917 (262 amino acid residues; Fig. 3). It should be noted that the present analysis confirms the previously reported tentative sequence of the coding region of the gene (Herbert *et al.*, 1983) in all but one respect. The sequence between the nucleotide residues 592 and 597 has now been shown to be AAGCGG instead

Fig. 4. Similarity between *B. circulans* *APH*-gene-product protein and that from five other species

Pairwise comparisons between *B. circulans* *APH*-gene-product protein (running from left to right) with that from the species noted in the text (running from top to bottom) were performed by using the exponentially damped alignment algorithm and the score matrix MDM78. The Figures were obtained by using a damping factor of 0.8 (a notational span length of 9), and the gap start and gap extension penalties were set to 3 and 2 times the mean value of MDM78 respectively. The degree of similarity is indicated by line thickness, a thicker line showing greater similarity. The thresholds chosen correspond to the top 0.3%, 0.1% and 0.03% of the observed scores.

The Figure shows the sequence of the *APH* gene upstream of the protein coding region (lower line) and the sequence of the 3'-end of the 16S rRNA of *B. subtilis* (upper line, *italicized*).

The sequence of the 3'-end of the *B. subtilis* 16S rRNA is known (McLaughlin *et al.*, 1981). When the region immediately before the putative initiation codon of the *B. circulans* APH gene was examined, a seven-base Shine-Dalgarno sequence capable of base-pairing with the 3'-end of the *B. subtilis* 16S rRNA was found ten bases before the initiation codon (Fig. 7). A possible terminator with a six-base-long all-G + C stem was found centred 240 bases after the end of translation.

The hypothesis that antibiotic-producing organisms are the original source of clinically significant antibiotic-resistance (Walker & Walker, 1970; Benveniste & Davies, 1973) has been tested in the case of aminoglycoside phosphotransferases by both immunological and nucleic acid hybridization techniques (Davies & Smith, 1978; Courvalin *et al.*, 1978). However, it was only when deduced protein sequences were compared that homology could be detected between different classes of phosphotransferase (Thompson & Gray, 1983; Herbert *et al.*, 1983).

The similarity between the *APH*-gene-product proteins is a strong indication that they have a common origin. However, it cannot be used to argue that antibiotic-producing organisms are the original source of the resistance determinants. A much larger number of sequences from different organisms would be needed

The observation that three different restriction fragments encoding the *B. circulans* APH gene can each be cloned into a different restriction site in pBR322 and the derivatives give similar levels of enzyme activity in *E. coli* HB101 (Table 1), and that the smallest fragment (a 2.7 kb *Sal*I-digest fragment) can be subcloned into the plasmid SLP1.2 and gives rise to phosphotransferase activity in *Strep. lividans* 66 (Table 1) argues that this 2.7 kb *Sal*I-digest fragment carries the required signals for transcription and translation. Precise identification of these regulatory regions by unambiguous experimentation has not as yet been made, but in the meantime a sequence comparison has revealed two potential promoter regions, one being homologous to the *B. subtilis* σ^{55} vegetative promoters and the other being homologous to the *B. subtilis* σ^{37} developmentally regulated promoters (Table 3). Two known genes controlled by σ^{37} promoters are the *spoVC* and the *spoVG* genes (Johnson *et al.*, 1983), and it would appear that in sporulating cells they dictate the most abundant transcripts (reviewed in Losick, 1982). Thus the current thinking that σ^{37} promoters are generally involved in the expression of developmentally regulated genes, when taken in conjunction with a possible link between differentiation and antibiotic metabolism (cf. Introduction section and Demain, 1974), leads to the deduction that in the case of the APH gene from *B. circulans* the expression of antibiotic-resistance may be controlled by a developmentally expressed promoter. The overlapping arrangement for the two promoters proposed here for the *B. circulans* APH gene may appear unusual, but it is not difficult to postulate a reason for this arrangement. Low-level transcription from the σ^{55} promoter during growth could provide continuous low-level resistance. As the cells enter the stationary phase a large increase in the antibiotic production occurs, and high-level transcription from the σ^{37} promoter could provide a high level of resistance. Some precedent for an overlapping σ^{37} promoter does exist, as both the *spoVC* and *spoVG* σ^{37} promoters overlap other promoters. Also, Wang & Doi (1984) have recently used a promoter-cloning plasmid to isolate a 471 bp *Hind*III-digest fragment of *B. subtilis* DNA; this appears to contain overlapping σ^{37} and σ^{55} promoters, and when used to direct the expression of the chloramphenicol acetyltransferase gene gave a pattern of transcription similar to that proposed for the *B. circulans* APH gene. The spacing between the potential σ^{37} -10 and -35 regions of the APH gene is a little large: 21 bases compared with 14 for the *spoVG* σ^{37} promoter and 17 for

Table 4. Similarity between *B. circulans* and *Strep. fradiae* DNA coding for *APH*-gene-product protein

For a given subsequence length (window) all possible subsequences of one DNA were compared with all possible subsequences of the second. For each pair of subsequences the total number of identical matches were counted; the Table reported the maximum score found together with the percentage similarity for each window length. The detail of the region of maximum similarity for a window length of 51 is shown below:

GAATAGTCGA	TGAGAGCCTG	GTGGACCCGG	CCGATATAAA	AGAGGAGTAC	G
* * * * *	*** * * *	***** *	*** * *	*****	*
GGAACGTCGC	CGAGGGCTTG	GTGGACCTCG	ACGACCTGCA	GGAGGAGCGG	G

Window length	Maximum score	Similarity (%)
11	10	90.9
15	13	86.7
21	17	81.0
31	22	71.0
41	29	70.7
51	33	64.7
61	38	62.3
71	43	60.6
81	47	58.0
91	52	57.1
101	56	55.4

the *spoVC* σ^{37} promoter (Johnson *et al.*, 1983). However, as only a few other σ^{37} promoter sequences are known, it is not possible to tell if this is significant.

As σ^{37} promoters are not recognized (Moran *et al.*, 1981) or recognized poorly (Wong & Doi, 1984) by σ^{55} RNA polymerase, the *APH* gene may be transcribed from the σ^{55} promoter when expressed in *E. coli*. At present we have no information to enable us to speculate which, if either, of the two promoters are used when the gene is expressed in *Strep. lividans*. However, as *Streptomyces* and *Bacillus* are both Gram-positive differentiating micro-organisms, it is tempting to speculate that a similar control mechanism may operate in both *B. circulans* and *Strep. lividans*, and that in *Strep. lividans* the gene may also be transcribed (at least in part) from the σ^{37} promoter. In this respect it is interesting to note that a form of *Streptomyces coelicolor* RNA polymerase has been isolated that directs transcription for *B. subtilis* σ^{37} promoters (Westpheling *et al.*, 1985).

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