The sequence of an antibiotic resistance gene from an antibiotic-producing bacterium

Homologies with transposon genes

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The *APH* gene of a butirosin-producing *Bacillus circulans* has been cloned and sequenced; a comparison of the translated protein sequence with those from TN5 and TN903 indicates that they may have a common origin.

Aminoglycoside phosphotransferase *Antibiotic-producing bacteria* *Antibiotic resistance*
Bacillus circulans *Butirosin* *DNA sequence*

1. INTRODUCTION

The production of antibiotics by differentiating micro-organisms is of considerable commercial importance, and the genes involved in their metabolism appear to be developmentally regulated [1]. The observation that diverse strains produce identical, or similar, antibiotics argues for considerable homology between the genes involved in their metabolism. Aminoglycoside phosphotransferases (APH) confer resistance to a variety of aminoglycoside antibiotics and are found in diverse groups of bacteria [2]. *Bacillus circulans* produces the antibiotic, biturosin, which is a member of the neomycin family of aminoglycoside antibiotics [3]. Strains that produce these antibiotics all possess a phosphotransferase activity, and an aminoglycoside acetyltransferase (AAC) activity, but it is the phosphotransferase activity which seems to be responsible for the resistance of the strain to its antibiotic [2]. It has been suggested that antibiotic-producing organisms might be the primary source of clinically significant antibiotic resistance [4]. The sequences of the *APH* genes from the antibiotic resistance transposons, Tn5 [5] and Tn903 [6], are known; also, the *APH* genes from *B. circulans* [7] and *Streptomyces fradiae* [8] have been cloned and shown to confer antibiotic resistance in their recipient strains. We now report the cloning and sequencing of the *APH* gene from *B. circulans* and the comparison of its translated protein sequence with those from the transposons.

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2. MATERIALS AND METHODS

Phosphotransferase activity was assayed in crude extracts as in [9]. Restriction endonuclease digestion and electrophoresis were carried out as in [10]. Sequencing was done using the chain-termination method in [11] in conjunction with the M13 phage cloning system in [12]. *B. circulans* was a gift from Dr J. Davies of BIOGEN SA.

3. RESULTS AND DISCUSSION

Total genomic DNA from *B. circulans* was completely digested with *Eco*RI, and ligated into

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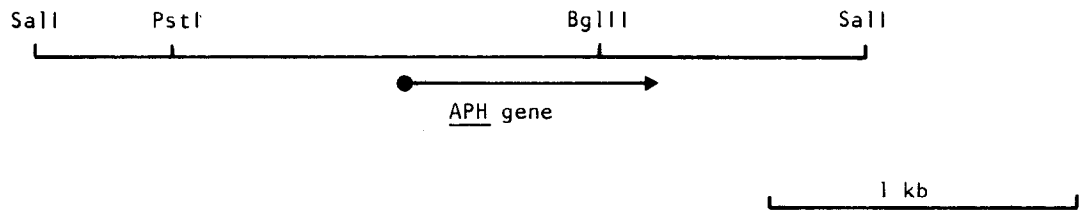

Fig.1. Partial restriction map of the 2.7 kb insert from pCH5 showing the position of the *APH* gene.

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                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
AGGAGGAACTGACAAATGAACGAAAGTACGCGTAATTGGCCGGAGGAACTTCTTGAGCTTCTCGGGCAGACGGAACTAACCGTCAACAAA
     -6         5         15         25         35         45         55         65         75

 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
ATCGGATATTCCGGAGATCACGTCTATCACGTGAAAGAGTACAGGGGCACCCCCGCATTTCTGAAAATCGCCCCCAGTGTATGGTGGAGA
     85         95         105        115        125        135        145        155        165

 T  L  R  P  E  I  E  A  L  A  W  L  D  G  K  L  P  V  P  K  I  L  Y  T  A  E  H  G  G  M
ACGCTCCGGCCCGAAATTGAAGCGCTCGCTTGGCTGGACGGGAAGCTCCCGGTTCCCAAAATTTTGTACACGGCCGAACACGGCGGGATG
     175        185        195        205        215        225        235        245        255

 D  Y  L  L  M  E  A  L  G  G  K  D  G  S  H  E  T  I  Q  A  N  R  K  L  F  V  K  L  Y  A
GACTACTTGCTGATGGAGGCGCTAGGCGGAAAAGACGGCTCCCACGAAACGATCCAGGCGAACCGGAAACTGTTTGTGAAGCTGTACGCG
     265        275        285        295        305        315        325        335        345

 E  G  L  R  S  V  H  G  L  D  I  R  E  C  P  L  S  N  G  L  E  K  K  L  R  D  A  K  R  I
GAAGGGCTCCGAAGCGTGCATGGCCTCGATATCCGCGAATGTCCGCTGTCGAACGGGCTGGAGAAGAAGCTCCGGGATGCGAAAAGAATA
     355        365        375        385        395        405        415        425        435

 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  K
GTCGATGAGAGCCTGGTGGACCCGGCCGATATAAAAGAGGAGTACGATTGCACGCCGGAGGAATTGTACGGGCTATTGCTTGAGAGTAAG
     445        455        465        475        485        495        505        515        525

 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
CCGGTAACCGAAGATCTGGTTTTTGCGCACGGAGATTACTGTGCTCCGAATCTGATTATCGACGGTGAGAAGCTGTCGGGATTTATCGAT
     535        545        555        565        575        585        595        605        615

 L  G  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
CTCGGACGTGCGGGCGTGGCGGACCGTTATCAGGACATCAGCCTGGCGATCCGCAGCCTCCGGCACGATTACGGCGACGACCGCTACAAA
     625        635        645        655        665        675        685        695        705

 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  *
GCGCTCTTCCTGGAACTTTACGGGCTGGACGGGCTTGACGAGGACAAGGTCCGGTATTACATCCGGCTGGATGAATTTTTTTGACGAAAC
     715        725        735        745        755        765        775        785        795
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Fig.2. Sequence of *B. circulans APH* gene with the translated protein sequence.

*Eco*RI-cleaved pBR322. The ligation mixture was transformed into *E. coli* HB101 and plated onto a nutrient agar containing 25 μg ribostamycin/ml, an aminoglycoside antibiotic. In this way 11 clones were isolated which all carried a 7.5 kb insertion conferring aminoglycoside resistance. This insert was too large to be used for sequence analysis, so smaller fragments were subcloned; one clone was isolated (CC1105) that carried a 2.7 kb *Sal*I insert in pBR322 (pCH5) which conferred resistance to aminoglycosides. When tested on solid media, CC1105 and *B. circulans* have a similar spectrum of resistance to aminoglycoside antibiotics, and in crude extracts the specific activity of the phosphotransferase in CC1105 was about twice that of *B. circulans*.

The 2.7 kb insert was mapped (fig.1) and found to be divided into conveniently sized fragments by unique *Bgl*II and *Pst*I sites. These smaller fragments were cloned and tested for their ability to confer aminoglycoside resistance. Only the large *Sal*I–*Pst*I fragment was able to do this, indicating that the *Bgl*II site was within the *APH* gene. This was confirmed by inserting a fragment of phage λ DNA into the *Bgl*II site of pCH5 causing the inactivation of the gene. Phosphotransferase activity was regained when the phage λ DNA was excised and the plasmid religated.

Libraries for sequencing were generated by polymerising the purified insert, partially digesting this material with frequent cutting enzymes such as *Sau*3A, *Hpa*II and *Taq*I, and ligating the fragments into appropriately cut M13mp9 replicative form DNA. This produced a library of overlapping fragments that enabled the sequence of the whole insert to be determined. Examination of the sequence in the region of the *Bgl*II site showed one long open reading frame of 786 bases. This would code for a protein of M_r 29800 (fig.2) which is in broad agreement with the observed M_r of 28500 [2]. The +1 and −1 reading frames contain a total of 20 stop codons in this region.

The translated protein sequences of the *B. circulans*, Tn5 and Tn903 *APH* genes were compared using the DIAGON program in [13]. This uses a score matrix to search for homology, and regions which exceed a minimum value are displayed (fig.3). This revealed regions of homology between all the proteins; the transposon encoded proteins showed homology throughout their length. There is also significant homology between the *B. circulans* protein and the transposon proteins; this is concentrated in the C-terminal portion of the sequences. The output of the DIAGON program can be used to line up the homologous regions of the proteins and highlight the conserved sequences (fig.4). This clearly shows that the most strongly conserved sequences are in the last 100 amino acids, and identifies regions that could be involved in substrate binding and catalytic activity. One striking feature is the high level of conservation of the proline residues, which implies that they are needed to maintain the tertiary structure of the protein.

Phosphotransferase enzymes have been divided into a total of 5 classes on the basis of in vitro

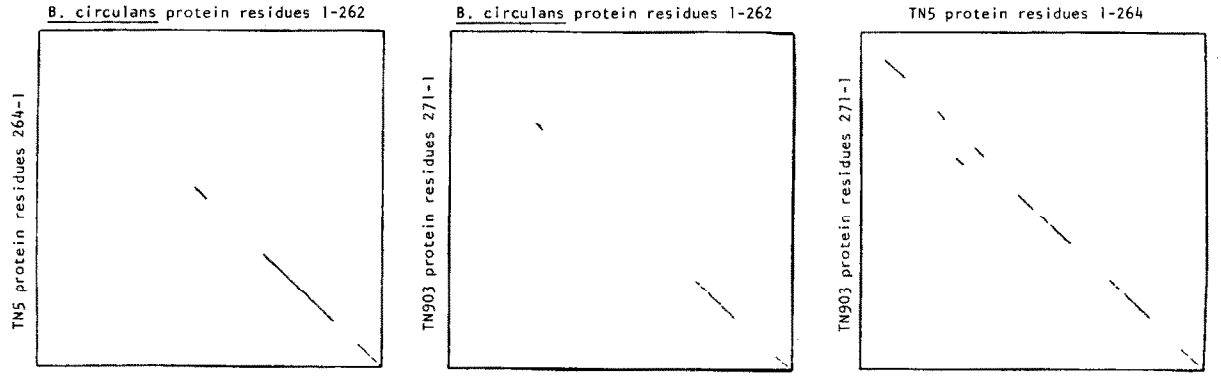

Fig.3. DIAGON plots of homology between pairs of phosphotransferase sequences obtained using the proportional matching algorithm of the DIAGON program. The figures were obtained using a double-matching expectation score equivalent to a 0.001% probability level, and a window length of 21 residues.

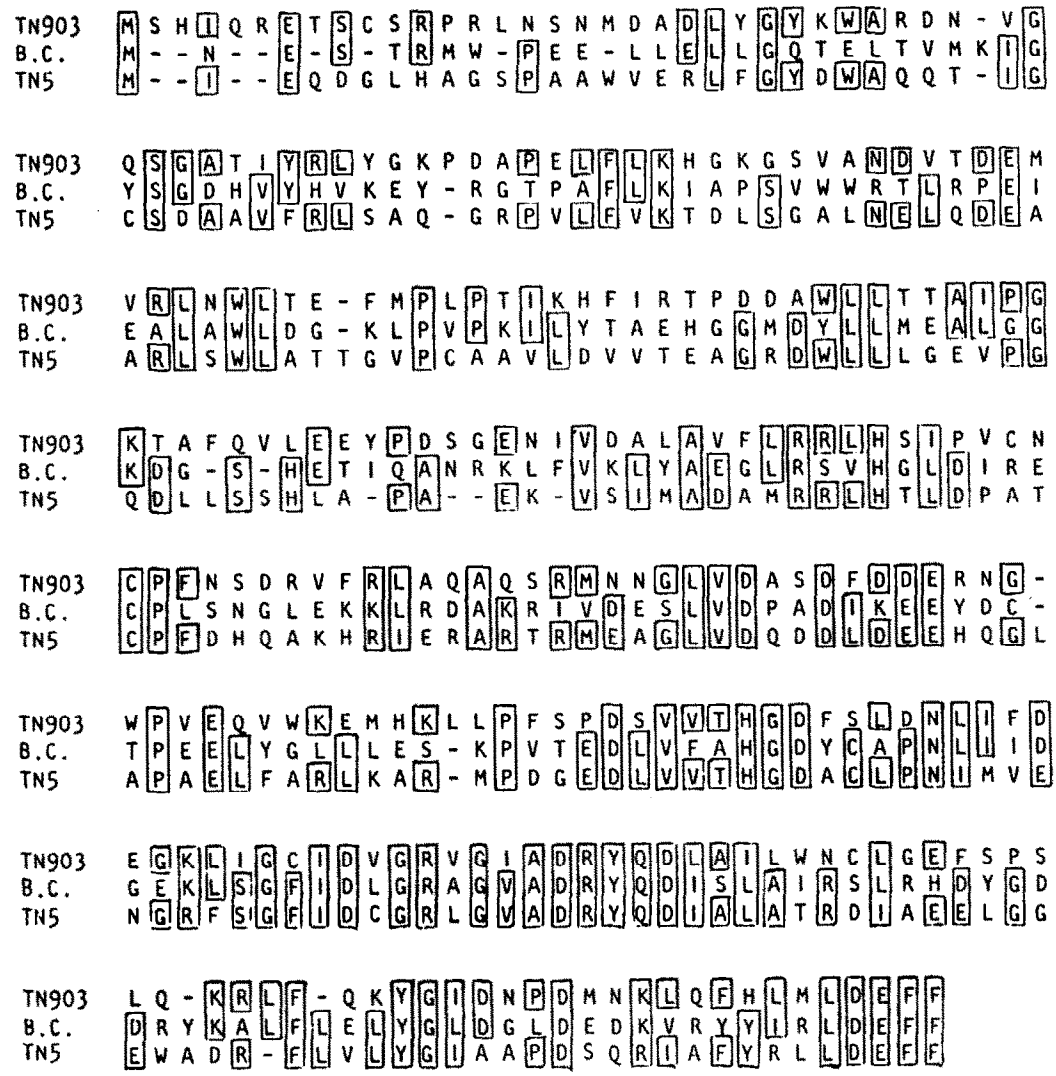

Fig.4. Comparison of the *APH* protein sequences showing the regions of homology. Initially the sequences were lined up using the regions of homology revealed by the DIAGON maps; the intervening regions were then fitted using as few padding characters as possible. Pairs of amino acids (I/L, D/E and K/R) are considered to be equivalent.

substrate specificity [2,14]. The ones considered in this study all belong to different types: Tn903 codes for a type I enzyme, Tn5 a type II, and *B. circulans* a type IV. The homology between these classes was not detected using immunological [2] or DNA hybridisation techniques [15] and the sequence data offers a possible explanation of this. At the nucleic acid level the homology is not strong enough for stable hybridisation. In the protein, if the conserved sequences are involved in substrate-binding and buried in the interior, then the variable sequences will be present at the surface functioning as the antigenic determinants. The conservation of sequences between the phosphotransferase from *B. circulans* and those from the transposons indicates that they may have a common origin, but this cannot be taken as evidence that the antibiotic producing organisms are the original source. The sequence of the *S. fradiae APH* gene has now been completed; a comparison of this and the two transposon sequences also indicates significant homology [16]. In future

all these conclusions will require confirmation by investigations on the proteins involved. As more information becomes available about the control of development in the genus *Bacillus* an examination of the regulatory sequences of the *APH* gene of *B. circulans* may cast new light on the link between differentiation and antibiotic metabolism.

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