The *aacA-aphD* Gentamicin and Kanamycin Resistance Determinant of Tn4001 from *Staphylococcus aureus*: Expression and Nucleotide Sequence Analysis

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The *aacA-aphD* aminoglycoside resistance determinant of the *Staphylococcus aureus* transposon Tn4001, which specifies resistance to gentamicin, tobramycin and kanamycin, has been cloned and shown to express these resistances in *Escherichia coli*. The determinant encoded a single protein with an apparent size of 59 kDa which specified both aminoglycoside acetyltransferase [AAC(6′)] and aminoglycoside phosphotransferase [APH(2″)] activities. Nucleotide sequence analysis of the determinant showed it to be capable of encoding a 479-amino-acid protein of 56·9 kDa. Analysis of Tn1725 insertion mutants of the determinant indicated that resistance to tobramycin and kanamycin is due to the AAC activity specified by, approximately, the first 170 amino acids of the predicted protein sequence and is consistent with the gentamicin resistance, specified by the APH activity, being encoded within the C-terminal region of the protein. Comparison of the C-terminal end of the predicted amino acid sequence with the reported sequences of 13 APHs and a viomycin phosphotransferase revealed a region which is highly conserved among these phosphotransferases.

INTRODUCTION

Enzymic modification of aminoglycosides is a ubiquitous mechanism of resistance to these antibiotics among clinical isolates of both Gram-negative and Gram-positive bacteria. These modifying enzymes can be plasmid- or chromosome-mediated and are often encoded on transposable elements. Three types of enzyme activity are recognized: aminoglycoside-*O*-phosphotransferase (APH), aminoglycoside-*N*-acetyltransferase (AAC), and aminoglycoside-*O*-adenyltransferase (AAD). Typically, a particular enzyme catalyses a single type of modification reaction which is capable of inactivating a characteristic subset of the aminoglycosides (for reviews see Bryan, 1984; Phillips & Shannon, 1984).

An exception to this property is the bifunctional modifying enzyme found in strains of *Staphylococcus aureus*, *Staphylococcus epidermidis* (Le Goffic *et al.*, 1977; Martel *et al.*, 1983; Ubukata *et al.*, 1984) and *Streptococcus faecalis* (Courvalin *et al.*, 1980), which catalyses both 6′-acetyltransferase [AAC(6′)] and 2′-phosphotransferase [APH(2″)] reactions. These activities, which in *S. aureus* are encoded by a determinant designated *aacA-aphD* (Lyon & Skurray, 1987), collectively mediate resistance to the aminoglycosides gentamicin, kanamycin and tobramycin.

The composite transposon Tn4001, which has been responsible for the gentamicin resistance among epidemic strains of *S. aureus* from Australian hospitals (Lyon *et al.*, 1984; Gillespie *et al.*, 1987) and shares homology with a determinant which occurs in isolates from North America (Lyon *et al.*, 1987a), produces a similar resistance pattern to that mediated by AAC(6′)-APH(2″)

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Abbreviations: AAC, aminoglycoside-*N*-acetyltransferase; AAD, aminoglycoside-*O*-adenyltransferase; APH, aminoglycoside-*O*-phosphotransferase; SPH, streptomycin phosphotransferase; VPH, viomycin phosphotransferase; URF, unidentified reading frame.

activity; Tn4001 may, therefore, encode an AAC(6')-APH(2'') bifunctional enzyme. Support for this hypothesis, and further information on the genetic basis of the two enzyme activities, is provided by the structural and expression analysis of the Tn4001 aminoglycoside resistance determinant presented here.

METHODS

Bacterial strains and plasmids. The *S. aureus* strains used were: SK982 (rifampicin^r, novobiocin^r), Lyon *et al.* (1984); SK2201 = SK982 carrying plasmid pSK1 [gentamicin^r (Gm^r), tobramycin^r (Tm^r), kanamycin^r (Km^r), trimethoprim^r, acriflavine^r, ethidium bromide^r, quaternary ammonium compound^r], Lyon *et al.* (1987b); SH6 carrying plasmid pSH6 (Gm^rTm^rKm^r), McDonnell *et al.* (1983); RN2397 = RN450 carrying plasmid pUB110 [neomycin^r (Nm^r) Km^r], Novick *et al.* (1981). The *Escherichia coli* host strains employed were: JM101, *supE thi Δ(lac-proAB) [F' traD36 proAB lacI^a Δ(lacZ)]M15*, Yanisch-Perron *et al.* (1985); MC1022, *araD-139 Δ(ara-leu)7697 galU galK strA Δ(lacZ)M15*, Casadaban & Cohen (1980); CSR603, *F⁻ thr-1 leuB6 proA2 hisG4 argE3 thi-1 ara-14 lacY1 galK xyl-5 mtl-1 rpsL31 tsx-33 supE44 sup-37 phr-1 recA1 uvr-6*, Sancar *et al.* (1979). *E. coli* plasmids used or constructed in this study are listed in Table 1.

Recombinant DNA techniques. Construction of recombinant DNAs was according to Ray & Skurray (1983). DNA manipulations were performed with C1 physical containment as laid down by the Recombinant DNA Monitoring Committee, Australia.

Tn1725 mutagenesis. Transposon mutagenesis, using an F₁*lac*⁺ plasmid which carried Tn1725, was performed as described previously (Cram *et al.*, 1984) except that the *recA*⁺ strain MC1022 was used for mutagenesis and ampicillin (Ap) was used to select for the target plasmid, pSK330.

Aminoglycoside-modifying enzyme assays. APH, AAC and AAD activities were assayed by the method of Shannon & Phillips (1983). Enzymic activities were measured as the amount of radio-labelling of an aminoglycoside mediated by the cell-extract of a plasmid-bearing strain after subtraction of the value for the parental strain alone. RN2397(pUB110), which encodes an AAD(4')(4'') activity (Sadaie *et al.*, 1980), served as a positive control for AAD assays. Gentamicin components A, B, C1, C1a and C2, and sisomicin, were kindly donated by the Schering Corporation. Amikacin, kanamycin A and kanamycin B were a gift from Bristol-Myers Pty Ltd. Neomycin B and neomycin C were a gift from Upjohn Pty Ltd. Warner-Lambert Co. donated paromomycin.

Maxicell analysis of plasmid-encoded proteins. Plasmid-encoded proteins were analysed using the maxicell expression system of Sancar *et al.* (1979), with CSR603 as the host strain. The [³⁵S]methionine labelling of proteins, SDS-PAGE and subsequent autoradiography were as previously described (Ray & Skurray, 1983). Protein standards were: bovine serum albumin, 67.0 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; soybean trypsin inhibitor, 20.0 kDa; β-lactalbumin, 14.4 kDa.

Nucleotide sequencing. DNA nucleotide sequence was determined with [³⁵S]dCTP label (Amersham), by the dideoxy chain termination method of Sanger *et al.* (1977) using restriction fragments cloned into the M13 derivatives mp8 and mp9 (Messing, 1983) or pUC8. Plasmid DNA templates were prepared according to Azad *et*

Table 1. *E. coli* plasmids

Plasmid	Description*	Reference/source
pACYC184	Cm ^r Tc ^r vector	Chang & Cohen (1978)
pUC8	Ap ^r vector	Messing & Vieira (1982)
pPV33-H	Promotor-probe vector	West & Rodriguez (1982)
<i>F</i> ₁ <i>lac</i> ⁺ :: Tn1725	Tn1725 carrier	P. Reeves
pSK310	pSK1 <i>Hind</i> III D fragment in pACYC184	Lyon <i>et al.</i> (1987)
pSK330	pSK1 <i>Hind</i> III D fragment in pUC8	This study
pSK343-pSK365	Independent Tn1725 insertion derivatives of pSK330	This study
pSK483	Deletion derivative of pSK344	This study
pSK484	Deletion derivative of pSK345	This study
pSK489	Deletion derivative of pSK353	This study
pSK491	Deletion derivative of pSK355	This study
pSK492	Deletion derivative of pSK358	This study
pSK493	Deletion derivative of pSK359	This study

* Cm^r, Tc^r and Ap^r indicate that resistance is specified to chloramphenicol, tetracycline and ampicillin, respectively.

al. (1985) and subjected to alkali denaturation prior to sequencing (Chen & Seeburg, 1985). Sequence analysis was performed using the programs of Garnier *et al.* (1978) and Staden (1986) as modified by R. Maxwell (Department of Biochemistry, Monash University) and A. Kyne (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), respectively; protein secondary structures were predicted by the algorithm of Robson (Garnier *et al.*, 1978).

RESULTS

Expression of Tn4001-encoded aminoglycoside resistance in E. coli

The aminoglycoside-resistance determinant of Tn4001 present on the multiresistant *S. aureus* plasmid pSK1 has been cloned on a 2.5 kb *Hind*III fragment into pACYC184, forming the plasmid pSK310 (Lyon *et al.*, 1987*a*). This *Hind*III fragment, which consists of the 1.9 kb unique region flanked by 0.3 kb of each of the 1.35 kb IS256 copies contained within Tn4001 (Lyon & Skurray, 1987; Lyon *et al.*, 1987*b*), was subcloned from pSK310 into pUC8, to form plasmid pSK330 (Table 1). The *E. coli* strain MC1022 harbouring pSK330 expressed Gm^r, Tm^r and Km^r at similar levels to those of the *S. aureus* strain SK2201, which carries Tn4001 located on pSK1; minimum inhibitory concentration (MIC) values for Gm, Tm and Km for MC1022(pSK330) were 16, 32 and 256 µg ml⁻¹, respectively. Furthermore, the 2.5 kb *Hind*III fragment, when recloned into the promoter-probe vector pPV33-H (West & Rodriguez, 1982), conferred a similar level of resistance to that of pSK330, irrespective of its orientation (data not shown). Since transcriptional activity does not cross the cloning site in pPV33-H, the elements required for Gm^r, Tm^r and Km^r expression in *E. coli* are contained within the 2.5 kb *Hind*III fragment.

Tn1725 insertion mutagenesis and deletion analysis

In order to define the functional areas within the 2.5 kb *Hind*III fragment necessary for expression of Gm^r, Tm^r and Km^r, pSK330 (Table 1) was subjected to Tn1725 mutagenesis. Of the 22 independent insertion derivatives obtained (designated pSK343 to pSK365), 18 possessed insertions within the cloned *Hind*III fragment and produced four phenotypic mutant classes (Fig. 1, top): (i) insertions at either end of the clone within IS256 sequences had no apparent effect on resistance, (ii) insertions between the arbitrary *Hind*III fragment coordinates 0.85–1.20 resulted in a Gm^sTm^sKm^s phenotype, (iii) insertions between coordinates 1.25–2.10 gave a Gm^sTm^rKm^r phenotype, and (iv) insertions mapping between coordinates 0.45–0.70 produced a low level of resistance to all three aminoglycosides (MICs being 4, 8 and 32 µg ml⁻¹ for Gm, Tm and Km, respectively). From these results the minimum span of the coding region for Tm^r and Km^r is 0.35 kb (coordinates 0.85–1.20; delineated by the insertions in pSK352 and pSK356) and the maximum span lies between coordinates 0.70–1.25 (defined by pSK351 and pSK358).

Since transcription of the area encoding the three aminoglycoside resistances most probably occurs from left to right in Fig. 1 (see below), Tn1725 insertions that are located between coordinates 0.85–2.10 and resulted in loss of Gm^r (with or without corresponding loss of Tm^r and Km^r), may have done so for two possible reasons: they may have occurred within the structural coding region for Gm^r, or, alternatively, blocked transcription into this region. The low level of resistance to all three aminoglycosides resulting from the Tn1725 insertions between coordinates 0.45–0.70 suggests that these insertions fall not within the structural coding regions, but within a region necessary for their normal expression.

To further clarify the extent of DNA required for the normal expression of aminoglycoside resistance, a series of deletions, starting at the left-hand end of the *Hind*III fragment (coordinate 0.0), were constructed (Fig. 1, bottom). These were produced from plasmids containing Tn1725 insertions, by deleting the region between the *Eco*RI site in the pUC8 vector moiety (adjacent to the *Hind*III site at coordinate 0.0) and the rightmost *Eco*RI sites of the Tn1725; only 18 bp of the Tn1725 sequence remained after deletion (Ubben & Schmitt, 1986). pSK483, with a deletion spanning coordinates 0.0–0.20, conferred wild-type resistance. In contrast, pSK484, with a deletion between coordinates 0.0–0.45, demonstrated approximately 20-fold lower MICs for Gm, Tm and Km. Plasmids with larger deletions were sensitive to all three aminoglycosides.

Fig. 1. *Tn1725* insertion map and deletion analysis of the *Tn4001* aminoglycoside determinant. The arrow indicates the long open reading frame determined from the sequence of the 2.5 kb *Hind*III fragment from *Tn4001*. The IS256 sequences present on the fragment are shown as the hatched areas. *Tn1725* insertion points are marked by the squares, with associated plasmid (pSK) numbers. The phenotypes of the *Tn1725* mutants, shown within the squares, are: ■, wild-type resistance; □, $Gm^sTm^rKm^r$; ▤, low-level resistance to Gm, Tm and Km; ▥, $Gm^sTm^rKm^r$. Plasmids with 5' deletions of the 2.5 kb *Hind*III fragment contained in pSK330 are designated at the lower left; the remainder of the insert fragment left after deletion is indicated. Marked at the top are the main promoter region for the determinant and the AAC(6')IV and APH(2'') coding regions inferred from this and other experiments described in this study.

The region spanning coordinates 0.20–0.45, then, is important for the expression of aminoglycoside resistance, suggesting that it may contain promoter elements. The level of resistance conferred by the deletion plasmid pSK484 was fivefold lower than that of its parental plasmid, pSK345; the higher resistance encoded by the latter may be due to weak promoter activity from *Tn1725* directing transcription of the aminoglycoside-resistance-coding regions.

Maxicell analysis of plasmid products

The maxicell expression system was employed to examine the products encoded by the cloned *Hind*III fragment and selected *Tn1725* mutants (Fig. 2). Maxicells which contained plasmids conferring wild-type resistance, viz. pSK330, pSK365 (pSK330 with *Tn1725* inserted in the vector moiety), pSK343, and pSK362, produced a single insert-specific protein, estimated to be 59 kDa in size (lanes B, C, D and L, respectively), suggesting that the Gm^r , Tm^r and Km^r are specified by a single protein. In contrast, maxicells containing pSK348, which mediated low-level resistance to Gm, Tm and Km, produced the 59 kDa protein at a markedly reduced level (lane E), as did pSK351 (data not shown), indicating that the low-level resistance phenotype may result from reduced synthesis of the 59 kDa protein.

A number of the plasmids in which a *Tn1725* insertion caused complete loss of one or more aminoglycoside resistances, e.g. pSK355, pSK356, pSK359 and pSK360 (lanes G, H, I, J), did not direct synthesis of the 59 kDa protein, but specified unique proteins of 18, 20.5, 31 and 38 kDa, respectively. These products appear to be truncated forms of the 59 kDa protein. Given this, the concordant increase in size of the protein produced with increased distance of the site of *Tn1725* insertion from the left, in Fig. 1, indicates that the direction of transcription and translation of the 59 kDa protein is from left to right. pSK361, which gave a $Gm^sTm^rKm^r$ phenotype, still encoded a polypeptide of approximately 59 kDa (lane K), probably due to the insertion of *Tn1725* near the end of the coding region. This is consistent with the position of the open reading frame defined by the nucleotide sequence analysis (Figs 1 and 4). Two plasmids

Fig. 2. Autoradiograph of [^{35}S]methionine-labelled polypeptides in lysates of maxicells harbouring: pUC8 (lane A), pSK 330 (lane B), pSK 365 (lane C), pSK 343 (lane D), pSK 348 (lane E), pSK 353 (lane F), pSK 355 (lane G), pSK 356 (lane H), pSK 359 (lane I), pSK 360 (lane J), pSK 361 (lane K), pSK 362 (lane L). The migration positions of protein standards of the indicated sizes (kDa) are shown at the left. The sizes (kDa) of the plasmid-encoded polypeptides, including the β -lactamase (BLA) of pUC8 and the chloramphenicol acetyltransferase (CAT) of Tn1725, are indicated on the right. The aminoglycoside resistance phenotypes are summarized at the bottom; symbols are as defined in Fig. 1.

specifying $\text{Gm}^s\text{Tm}^s\text{Km}^s$ phenotypes, pSK353 (lane F) and pSK354 (data not shown), failed to direct the synthesis of any detectable unique proteins. This may be due to synthesis of a product too small to be detected, since the insertions in these plasmids are close to the proposed translation initiation site for the 59 kDa protein.

Assay of aminoglycoside-modifying enzymes

The similarity of the resistance pattern specified by Tn4001 to that conferred by the AAC(6') and APH(2'') activities encoded by *S. aureus* plasmids such as pSH6 (McDonnell *et al.*, 1983) suggested that Tn4001 might encode for similar aminoglycoside-modifying activities. To verify this notion, assays of aminoglycoside-modifying enzymes were performed on both *S. aureus* and *E. coli* strains harbouring plasmids carrying the determinant (Table 2). Plasmids pSK1 (which carries Tn4001) and pSH6 both conferred substrate profiles specifically indicative of AAC(6')IV and APH(2'') activities (Foster, 1983); neither specified any significant AAD activity (data not shown). The *E. coli* recombinant plasmid pSK330, containing the resistance determinant of Tn4001, produced AAC and APH substrate profiles essentially similar to those of pSK1, but

Table 2. Assay of aminoglycoside-modifying enzymes

Assay for:	Plasmid/host	Enzyme modification activity against*:													
		GmA	GmB	GmC1	GmC1a	GmC2	Si	Ak	Tm	KmA	KmB	NmB	NmC	Pm	Sm
APH	pSK1/SK982	M	L	M	M	L	M	M	–	–	L	–	–	–	–
	pSH6/SH6	M	L	M	M	L	M	L	–	–	L	–	–	–	–
	pSK330/MC1022	M	M	M	M	M	M	M	–	–	L	–	–	–	–
	pSK344/JM101	M	M												
	pSK353/JM101	–	–												
	pSK360/JM101	–	–												
AAC	pSK1/SK982			L	M	–	L	L	H	H	H	L			
	pSH6/SH6			L	M	–	L	L	H	H	H	L			
	pSK330/MC1022			M	M	L	L	L	H	H	H	L			
	pSK344/JM101				M				H	H					
	pSK353/JM101				–				–	–					
	pSK360/JM101				M				H	H					

* Assays for APH and AAC modification of the aminoglycoside indicated were performed twice; duplicates were within 20% of each other. Average enzyme activities are shown: —, negative, 0–99 c.p.m.; L, low, 100–299 c.p.m.; M, medium, 300–999 c.p.m.; H, high, 1000–3000 c.p.m. Blank spaces denote reactions not tested. Abbreviations: GmA, GmB, GmC1, GmC1a, GmC2, gentamicin components; Si, sisomicin; Ak, amikacin; Tm, tobramycin; KmA, KmB, kanamycin components; NmB, NmC, neomycin components; Pm, paromomycin; Sm, streptomycin.

activities were in general slightly elevated. The resistance determinant of *Tn4001* appears, therefore, to encode both AAC(6') and APH(2'') activities only and has, accordingly, been designated *aacA-aphD*.

Examination of the enzyme substrate profiles (Table 2) suggested that the AAC activity conferred Tm^r and Km^r, and that the APH activity conferred Gm^r. These associations were investigated further by determination of the enzyme activities specified by different classes of the *Tn1725* mutants. pSK360 (Gm^sTm^sKm^s) specified AAC but not APH activity, whereas pSK353 (Gm^sTm^rKm^r) did not confer AAC or APH activity. The activities of pSK344 (Gm^rTm^rKm^r) were similar to those specified by pSK330, indicating that *Tn1725* insertion *per se* had no effect. Taken together, these results are consistent with Tm^r and Km^r being due to AAC activity and with the APH activity being responsible for the Gm^r.

Nucleotide sequence of the aminoglycoside-resistance determinant

To further delineate the functional regions within the *aacA-aphD* determinant, the nucleotide sequence of the 2.5 kb *Hind*III fragment encompassing the determinant was obtained from subclones of pSK330 derivatives containing *Tn1725* insertions. The sequencing strategy is shown in Fig. 3. This strategy made use of the inserted *Tn1725* copies as each effectively constituted a new *Eco*RI site, thereby facilitating subcloning (Ubben & Schmitt, 1986).

The sequence (Fig. 4) contains an open reading frame, bp 700–2136, of sufficient size to encode a 479-amino-acid protein of 56850 Da. This open reading frame appears to be the structural coding region for aminoglycoside resistance since *Tn1725* insertions which resulted in complete loss of resistance to one or all aminoglycosides mapped within it (Fig. 1). Furthermore, the sizes of the normal and apparent truncated forms of this protein (specified by pSK330 and *Tn1725* insertion derivatives of it) predicted from the sequence were comparable to those produced in maxicells (Table 3). For the prediction of protein sizes the contribution of *Tn1725* sequences was taken into account since the translation stop codons for the reading frames entering *Tn1725* occur at significant distances from the ends of the transposon (the same as for *Tn1721*; Schoffl *et al.*, 1981). The hydrophilicity along the primary structure of the deduced *aacA-aphD* product was evenly distributed, with no large hydrophobic regions (data not shown), which is typical of soluble proteins, and is consistent with the proposed cytoplasmic location for aminoglycoside-modifying enzymes (Foster, 1983).

The coding region which specifies the AAC activity (Tm^rKm^r) within the *aacA-aphD* determinant, as deduced from the *Tn1725* insertion mutagenesis, occurs at a minimum between bp 846–1200 and at a maximum between bp 700–1245 (Figs 1 and 4). These occur in the 5'-proximal region of the *aacA-aphD* coding frame and thus the AAC activity is specified by between 167 and 180 amino acids at the N-terminal end of the deduced *aacA-aphD* product.

Fig. 3. Sequencing strategy and partial restriction map of the 2.5 kb *Hind*III fragment of Tn4001. The positions of the Tn1725 insertions used to facilitate subcloning for sequencing (see text) are marked by triangles; hatched areas denote the inverted IS256 sequences. Solid and dashed arrowed lines below the map depict the extent and direction of sequences obtained from M13 and pUC8 recombinants, respectively.

Table 3. Observed and expected sizes of truncated *AacA*–*AphD* polypeptides

Plasmid	Polypeptide size (kDa)	
	Inferred from DNA sequence*	Observed†
pSK355‡	18.9	18
pSK356‡	22.9	20.5
pSK359‡	32.9	31
pSK360‡	40.7	38
pSK361‡	57.9	59
pSK330	56.8	59

* In calculation of the expected polypeptide sizes the translation start point was taken as bp 700 in the nucleotide sequence of the 2.5 kb *Hind*III fragment (Fig. 4); the translation end point for the Tn1725 insertion derivatives was determined from the sequenced site of insertion, as shown in Fig. 4, accounting for translation read-through into Tn1725 (see text), with the exception of pSK356, where the site of Tn1725 insertion was determined by restriction mapping and the contribution of Tn1725 sequences to the expected polypeptide size was taken as the average of that predicted for the different reading frames.

† Sizes of polypeptides produced in maxicells, as described in Fig. 2.

‡ Derivative of pSK330 with a Tn1725 insertion causing an altered aminoglycoside resistance phenotype, as described in Figs 1 and 4.

Within the *Hind*III fragment is an unidentified reading frame (URF), 5' to *aacA*–*aphD* between bp 370–696, capable of encoding a protein of 12.6 kDa. Both the *aacA*–*aphD* reading frame and the URF, which read in the same direction, are preceded by strong ribosome-binding sites that are typical of genes from Gram-positive bacteria (McLaughlin *et al.*, 1981).

Upstream from the *aacA*–*aphD* coding frame are three strong potential promoter sequences. In order of decreasing fit to the *E. coli* promoter consensus sequence, which is the same as that of Gram-positive bacteria (McLaughlin *et al.*, 1981; Moran *et al.*, 1982), these are P1, P2 and P3 (Fig. 4). For example, P1 shows a five-out-of-six fit to the consensus for both the –35 and –10 boxes [the consensus being TTGACA and TATAAT, respectively (Rosenberg & Court, 1979)] and has the optimal spacing of 17 bp between them. Both P1 and P2 occur upstream from the URF, which precedes the *aacA*–*aphD* reading frame. So, a polycistronic mRNA, encompassing the URF and *aacA*–*aphD* coding region, could possibly be initiated from P1 or P2. Interestingly, the P1 sequence lies across the boundary between the left IS256 inverted repeat and the unique central region of Tn4001 (Fig. 4).

1300 1350
 GCAATGAAATATTTAATTGAGCATFACTTTTGATAATTTCAAAGTAGATAGTATTGAAATAATCGGTAGTGGTTATGATAGTGTGGCATAT
 AlaMetLysTyrLeuIleGluHisTyrPheAsnPheLysValAspSerIleGluIleIleGlySerGlyTyrAspSerValAlaTyr
 359 1400
 TTAGTTAATAATGAATACATTTTTAAACAAAATTTAGTACTAATAAGAAAAAGGTTATGCAAAAGAAAAAGCAATATATAATTTTTTA
 LeuValAsnAsnGluTyrIlePheLysThrLysPheSerThrAsnLysLysLysGlyTyrAlaLysGluLysAlaIleTyrAsnPheLeu
 1450 1500
 AATACAAATTAGAACTAATGTAAAAATTCCTAATATGAATATTCGTATATTAGTGATGAATTATCTATACTAGGTTATAAGAAATTT
 AsnThrAsnLeuGluThrAsnValLysIleProAsnIleGluTyrSerTyrIleSerAspGluLeuSerIleLeuGlyTyrLysGluIle
 1550 1600
 AAAGGAACCTTTTTAACACCAGAAATTTATCTACTATGTGAGAAGAAGACAAAATTTGTTAAAAACGAGATATTGCCAGTTTTTTAAGA
 LysGlyThrPheLeuThrProGluIleTyrSerThrMetSerGluGlnAsnLeuLeuLysArgAspIleAlaSerPheLeuArg
 360
 CAAATGCACGGTTTAGATTATACAGATATTAGTGAATGTACTATTGATAATAACAAAATGTATTAGAAGAGTATATATTGTTCCGTGAA
 GlnMetHisGlyLeuAspTyrThrAspIleSerGluCysThrIleAspAsnLysGlnAsnValLeuGluGluTyrIleLeuLeuArgGlu
 1650 1700
 ACTATTTATAATGATTTAACTGATATAGAAAAAGATTATAGAAAGTTTATGGAAAGACTAAATGCAACAACAGTTTTTGGAGGGTAAA
 ThrIleTyrAsnAspLeuThrAspIleGluLysAspTyrIleGluSerPheMetGluArgLeuAsnAlaThrThrValPheGluGlyLys
 1750 1800
 AAGTGTTTATGCCATAATGATTTTAGTTGTAATCATCTATTGTTAGATGGCAATAATAGATTAACTGGAATAATGATTTTGGAGATTCT
 LysCysLeuCysHisAsnAsnPheSerCysAsnHisLeuLeuLeuAspGlyAsnAsnArgLeuThrGlyIleIleAspPheGlyAspSer
 1850
 GGAATTATAGATGAATATTTGTGATTTTATATACTTACTTGAAGATAGTGAAGAAGAAATAGGAACAAATTTTGGAGAAGATATATTAAGA
 GlyIleIleAspGluTyrCysAspPheIleTyrLeuLeuGluAspSerGluGluGluIleGlyThrAsnPheGlyGluAspIleLeuArg
 1900 1950
 ATGTATGGAATATAGATATTGAGAAAAGCAAAAGAAATATCAAGATATAGTTGAAGAATATTATCCTATTGAAACTATTGTTTATGGAATT
 MetTyrGlyAsnIleAspIleGluLysAlaLysGluTyrGlnAspIleValGluGluTyrTyrProIleGluThrIleValTyrGlyIle
 2000 2050
 AAAAATATTAACAGGAATTTATCGAAAATGGTAGAAAAGAAATTTATAAAAGGACTTTATAAGATTGATTATATAATATATGAAACGCT
 LysAsnIleLysGlnGluPheIleGluAsnGlyArgLysGluIleTyrLysArgThrTyrLysAsp***
 2100 2150
 ATTATAAAGACATTAGTATTAATAGTTTAAAAAATGAAAAATAATAAGGAAGTGAAGTCAAGTCCAGACTCCTGTGTAATGCTAT
 ACAATGTTTTTACCATTCTACTTATCAAAATGATGTATTTTCTTGAAGAATAAATCCATTTCATCATGTAGGTCCATAAGAACGGCTCC
 2200 2250
 AATTAAGCGATTGGCTGATGTTTGATTGGGAAGATGCGAATAATCTTTTCTCTTCTGCGTACTTCTTGATTCACTGTTCAATTAGATT
 2300 2350
 GGTACTCTTTAGTCGATTGTGGAAATTTCTTGACGGTATATTGAAAGCGCTCTTCAATCCATCATCCAATGATGCGCAAGCTT
 2400 2450 2500 HindIII

DNA homology

The nucleotide sequence of the *aacA-aphD* region is almost identical to that of a *Streptococcus faecalis* plasmid determinant which similarly encodes for AAC(6')-APH(2'') activities (Ferretti *et al.*, 1986). Within the sequenced region of the *Strep. faecalis* determinant, starting at the equivalent of bp 396 of the *S. aureus* sequence and ending at the right-hand *HindIII* site, the only differences are a 1 bp deletion in the *Strep. faecalis* sequence compared to the *S. aureus* sequence at bp 446 and a relative transversion at bp 2157, with A and C occurring in the *Strep. faecalis* and *S. aureus* sequences, respectively. The deletion causes a frameshift in the URF of one sequence compared to the other, and the base pair difference is downstream from the region encoding the AAC(6')-APH(2'') activities. The inferred primary structures of the AAC(6')-APH(2'') proteins from *S. aureus* and *Strep. faecalis* are, therefore, identical.

Enzyme/species of origin	Conserved amino acids
AAC(6')IV-APH(2'') <i>S. aureus</i> / <i>Strep. faecalis</i>	K C L C H N D F S . C N H L L L . . D G N N R L T G I I D P G D S G I I D E Y . C D F I Y 408
APH(3')(5'')I <i>Salmonella typhimurium</i>	S V V T H G D F S L D N L I F D . . E G . . K L T G C I D V G R V G I A D R Y . Q D I A L 232
APH(3')(5'')II <i>K. pneumoniae</i>	L V V T H G D A C P L R I M V E . . N G . . R P S G F I D C G R L G V A D R Y . Q D I A L 223
APH(3')(5'')III <i>S. aureus</i> / <i>Strep. faecalis</i> <i>C. coli</i>	L V F S H G D L G D S N I F V K . . D G . . K V S G F I D L G R G I R A D K W . Y D I A F 222
APH(3')(5'')IV <i>B. circulans</i>	L V F A N G D Y C A P M . 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 221
APH(3')(5'')V <i>Streptomyces fradiae</i>	L V V C H G D L C P N V L L D . . P G T C R V T G V I D V G R L G V A D R H . A D I A L 224
APH(4) <i>E. coli</i>	L V . . H A D F G S M N V L T D . . N G . . R I T A V I D M S E A M F G D S P Q Y E V A N 230
APH(7'') <i>Streptomyces hygroscopicus</i>	F V . . H G D L H G T M I F V D . . L A A T E V T G I V D F T D I Y A G D S R Y S L V Q L 259
VPH <i>Streptomyces vinaceus</i>	A V V . H G D L G G E N V L M E T V D G V P R M S G V V D M D Q V G I G D P A . K D L A A 227
SPH <i>K. pneumoniae</i>	V C P L H G D L H H R N V . L D . . P G D . R G W L A I D P H G L . L G E R T . F D I Y A N 187
APH(6) <i>Streptomyces griseus</i>	D R V L H M D L H Y R V L A A . . E R . . E P W L A I D P E P L . V G D P G . F D L W P 233
APH(6) <i>Streptomyces glaucescens</i>	D R M L H M D L H Y R V L A A . . E R . . E P W L A I D P E P L . A G D P G . F D L W P 233

Fig. 5. The conserved region in the predicted amino acid sequences of 15 phosphotransferases. Sequence locations containing more than six conserved or identical amino acids are enclosed in solid lines. Numbers to the right indicate the sequence position of the last amino acid shown. Conserved means replacements within the groups (I, L, V) (R, K) (D, E) (S, T) (F, Y) (Q, N). Gaps have been introduced to maximize homology. Sequence data were taken for the AAC(6')IV-APH(2'') of *Strep. faecalis* from Ferretti *et al.* (1986); for the APH(3')(5'')I of *Salmonella typhimurium* from Oka *et al.* (1981); for the APH(3')(5'')II of *K. pneumoniae* from Beck *et al.* (1982); for the APH(3')(5'')IIIs of *S. aureus*, *Strep. faecalis* and *Campylobacter coli* from Trieu-Cuot & Courvalin (1983), Gray & Fitch (1983) and Trieu-Cuot *et al.* (1985), respectively; for the *Bacillus circulans* APH(3')(5'')IV from Herbert *et al.* (1983); for the *Streptomyces fradiae* APH(3')(5'')V from Thompson & Gray (1983); for the APH(4) of *E. coli* from Gritz & Davies (1983) and Kaster *et al.* (1983); for the *Streptomyces hygroscopicus* APH(7'') from Zalacain *et al.* (1986); for the *Streptomyces vinaceus* VPH from Bibb *et al.* (1985); for the *K. pneumoniae* streptomycin phosphotransferase (SPH) from Mazodier *et al.* (1985); for the *Streptomyces griseus* APH(6) from Distler *et al.* (1987); and for the *Streptomyces glaucescens* APH(6) from Vögtli & Hütter (1987).

Polypeptide primary structure homology

A comparison of the deduced amino acid sequence of the *aacA-aphD* product with that of other sequenced resistance genes revealed a region of approximately 40 amino acids (368–408) near the C-terminus which is conserved with other APHs and a viomycin phosphotransferase (VPH) (Fig. 5). This suggests that the C-terminal region of the *aacA-aphD* product is responsible for the APH(2'') activity and that the conserved residues represent active-site sequences. The conserved asparagine residues (D), being anionic, may be involved in binding of the cationic aminoglycoside substrates. Although there are further sequence positions at which the phosphotransferases show strong homology, these are scattered.

Significant homology was also found between the deduced primary structure of the *aacA-aphD* product and that of the streptothricin acetyltransferase from *Streptomyces lavendulae* (Horinouchi *et al.*, 1987), between amino acids 110–153 in the AAC(6') region; no significant homology was found with other sequenced acetyltransferases, which include the AAC(3)III and IV of *E. coli* (Bräü *et al.*, 1984; Allmansberger *et al.*, 1985) and the chloramphenicol acetyltransferases (CATs) type I and III from *E. coli* (Shaw *et al.*, 1979; L. Packman, N. Kaye & S. Fitton, unpublished results, cited in Shaw, 1983) and type C and pC194 from *S. aureus* (Shaw *et al.*, 1985; Byeon & Weisblum, 1984). Ferretti *et al.* (1986) noted homology between the amino acid sequences of the AAC(6')-APH(2'') and the CAT from *Bacillus pumilus* (Harwood *et al.*, 1983); our analysis, however, did not register sequence conservation in this case (amino acid identity was less than 30%).

Secondary structure analysis of the *aacA-aphD* product

Ferretti *et al.* (1986) attempted to subclone the APH(2'') domain separately from the *Strep. faecalis* AAC(6')-APH(2''), which has an identical predicted sequence to that of the *aacA-aphD* product, and found that APH activity could only be expressed if most of the N-terminal region, specifying the AAC(6') activity, was present in addition to the C-terminal region proposed to specify the APH(2'') activity; a protein shortened at the N-terminal end by 12% but not 50%

expressed APH activity. Examination of the putative secondary structure of the protein offers an explanation for this; viz., that the C-terminal region takes up a different secondary structure in the whole AAC(6')-APH(2'') protein compared to when it is present in a significantly truncated form (data not shown). Only truncated AAC(6')-APH(2'') polypeptides with less than approximately 18% deleted from the N-terminal end should possess the conformation of the C-terminal domain generated for the wild-type protein, a prediction entirely consistent with the results of Ferretti *et al.* (1986).

DISCUSSION

We report here a genetic and biochemical analysis of the resistance to gentamicin, tobramycin and kanamycin encoded by the transposon Tn4001 from *S. aureus*. Resistance to these aminoglycosides involves AAC and APH modifying enzyme activities, and substrate profiles imply these to be AAC(6')IV and APH(2'') activities, respectively. These activities, which are detected together in staphylococci and streptococci, are specified by a single bifunctional enzyme in the cases of *S. aureus* strains containing plasmids RPAL or pTU053 (Martel *et al.*, 1983; Ubukata *et al.*, 1984), *S. epidermidis* strain TK1265 (Ubukata *et al.*, 1984) and *Strep. faecalis* strains harbouring pIP800 (Courvalin *et al.*, 1980; Ferretti *et al.*, 1986). Likewise, the AAC and APH activities encoded by Tn4001 are also specified by a single enzyme, since expression analysis of the 2.5 kb *Hind*III fragment from Tn4001 [which contains the determinant for AAC(6') and APH(2'') activities, designated *aacA-aphD*], and Tn1725 mutants of it, demonstrated that a single protein with an apparent size of 59 kDa is associated with the activities. This is similar to the sizes reported for purified AAC(6')-APH(2'')s (54–56 kDa; Martel *et al.*, 1983; Ubukata *et al.*, 1984), and is consistent with the nucleotide sequence data, which indicate a polypeptide of 56.9 kDa to be involved.

Additionally, an unidentified reading frame (URF), capable of specifying a 12 kDa protein, occurs 5' to the *aacA-aphD* coding area. This region does not, however, appear to be involved in the aminoglycoside resistance since the corresponding region in the nearly identical AAC(6')-APH(2'') determinant from *Strep. faecalis* plasmid pIP800 (Ferretti *et al.*, 1986) contains a frameshift relative to that of Tn4001.

There are a number of potential promoter sequences 5' to the *aacA-aphD* coding frame. Of these, the two strongest, P1 and P2, lie within the area, bp 197–441, defined by the Tn1725 insertion and deletion analysis as being important for expression of aminoglycoside resistance. In contrast, Ferretti *et al.* (1986) suggested that the promoter for the streptococcal AAC(6')-APH(2'') determinant occurs at a position corresponding to bp 623–654 in the *S. aureus* sequence. However, deletions of the *aacA-aphD* region starting at the left-hand *Hind*III site with their end-points occurring 3' to bp 441 resulted in aminoglycoside resistance being expressed at greatly reduced levels, indicating that the P1/P2 region is more likely to be the main promoter region for *aacA-aphD*; conclusive identification of promoter elements awaits transcript analysis. The area between bp 441 and the beginning of the *aacA-aphD* coding frame, at bp 700, contains P3, which has a slightly lesser fit to the promoter consensus sequence than P1 and P2, and a number of much weaker potential promoters. These promoters may be responsible for the low level of aminoglycoside resistance remaining when the region is deleted or when transcription from P1/P2 is blocked with a Tn1725 insertion.

Characterization of Tn1725 insertions in *aacA-aphD* indicates that 167–180 codons from the 5' end of the structural coding region are sufficient to specify the AAC activity, whereas the remaining 3' portion of the gene presumably encodes the APH activity. These results are consistent with the contention that the AAC and APH activities are specified by separate domains in the *aacA-aphD* product, and in full support for this Martel *et al.* (1983) have demonstrated that the AAC and APH active sites of the AAC(6')-APH(2'') enzyme encoded by the *S. aureus* plasmid RPAL occur at two separate locations. As such, it has been suggested that the bifunctional enzyme resulted from the fusion of two genes encoding an AAC(6') and an APH(2'') (Martel *et al.*, 1983; Ferretti *et al.*, 1986). If so, then the failure of the APH domain to specify activity when detached from the AAC domain (Ferretti *et al.*, 1986) implies that the APH domain has undergone structural alteration since the fusion event.

It has been proposed that some resistance mechanisms found in clinical bacterial isolates originated in the antibiotic-producing soil bacteria (Benveniste & Davies, 1973; Koch, 1981; Gray & Fitch, 1983). It is, therefore, of interest that AAC(6') activity with a substrate profile similar to that of the AAC(6')IV of Tn4001 is found in the kanamycin producer *Streptomyces kanamyceticus* (Benveniste & Davies, 1973). However, comparison of the G + C contents of *Streptomyces* and the *aacA-aphD* region of Tn4001, which are 69–73% and 26%, respectively, indicates that *Streptomyces* is unlikely to be the immediate origin of the *aacA-aphD* gene. A possibility, then, is that the direct source of the *aacA-aphD* gene is a soil organism with a low G + C content, such as a *Bacillus*, that cohabits with an aminoglycoside producer. In contrast to the *aacA-aphD* region, with a 26% G + C content, the sequenced portions of the inverted repeat (IS256) sequences which flank this region on Tn4001 have a 47% G + C content. This is suggestive of different origins for the IS256 elements and the *aacA-aphD* determinant of Tn4001.

Enzymes which confer antibiotic resistance *in vivo* may have evolved from pre-existing cellular enzymes (Koch, 1981; Samraoui *et al.*, 1986). Known families of nucleotide-binding proteins and phosphotransferases (kinases) were, therefore, examined for sequence homology with the predicted sequence of the AAC(6')-APH(2'') and other APH enzymes, but no significant homology was detected, suggesting that the APH(2'') of the bifunctional enzyme and related APHs (Fig. 5) represent a new class of kinases. They may have all evolved from an as yet unidentified cellular kinase.

Comparison of the AAC(6') sequence with that of a streptothricin acetyltransferase revealed a significant region of homology, which may thus correspond to the binding site for their common co-factor, acetyl-CoA.

The nucleotide sequence of the *aacA-aphD* determinant and flanking regions is almost identical to that of the AAC(6')-APH(2'') determinant of *Strep. faecalis* plasmid pIP800. Since the *aacA-aphD* determinant is flanked by the IS256 sequences of Tn4001, the result suggests that the *Strep. faecalis* determinant is also flanked, at least on the side that has been sequenced, by a copy of IS256. That is, a Tn4001-like element also occurs in *Streptococcus*. This implies the possibility that all cases of AAC(6')-APH(2'') activities, which have been documented only in staphylococci and streptococci, are due to the presence of Tn4001-like elements. Consistent with this notion is the presence of DNA homologous to Tn4001 in all gentamicin-resistant *S. aureus* strains which we have examined (Lyon *et al.*, 1987a; Gillespie *et al.*, 1987).

Being only 4.7 kb in size, Tn4001 is one of the smallest transposons encoding multiple antibiotic resistance, in part due to the bifunctional nature of the single resistance protein that it encodes. Such a transposon appears to be particularly suited for dissemination on non-conjugative plasmids via transduction, with its intrinsic size limitation, as well as on conjugative plasmids. It is not surprising, therefore that Tn4001-like elements have been detected on both types of plasmids, spreading via non-conjugative plasmids in Australian isolates and via both conjugative and non-conjugative plasmids in North American isolates (Lyon *et al.*, 1987a).

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