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Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids

(antibiotic resistance/streptomycete cloning/evolution of resistance determinants)

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ABSTRACT The DNA sequence of an aminoglycoside phosphotransferase gene (*aph*) from *Streptomyces fradiae* ATCC 10745 (a neomycin producer) was determined. The gene was localized by *in vitro* subcloning and insertional inactivation. Molecular weight, amino acid composition, and amino-terminal analysis of the purified *aph* gene product confirmed the accuracy and position of the *aph* gene sequence. Pairwise comparisons of *S. fradiae aph* with the *aph* genes encoded by bacterial transposons Tn5 and Tn903 showed significant nucleotide and amino acid homologies which indicated a common evolutionary origin for these antibiotic-resistance genes.

The morphological complexity of the streptomycetes, as well as their medical and industrial importance as the producers of the majority of the known antibiotics, makes the study of the genetics of this genera of considerable interest. The unusually high G+C content (72–74%) (1) of *Streptomyces* DNA could have interesting consequences; for example, most regulatory sequences examined in bacteria are typically A+T rich (2, 3). The structure of streptomycete genes and their associated regulatory sequences have not been examined. Advances in streptomycete genetics and cloning technology (reviewed in refs. 4 and 5) provide an opportunity to study genes involved in nutritional pathways (6) as well as those encoding antibiotic-resistance mechanisms (6–9).

Aminoglycoside-modifying enzymes, such as the aminoglycoside 3′-phosphotransferase [APH(3′)] present in *Streptomyces fradiae*, are also found in many antibiotic-resistant bacteria of clinical origin (10). Benveniste and Davies (11) suggested that antibiotic-resistance mechanisms found in clinical isolates may have evolved by the recruitment of resistance genes from antibiotic-producing actinomycetes. Recently, the sequences of the APH(3′) genes (*aph*) of bacterial transposons Tn903 (12) and Tn5 (13) have been determined, and the *S. fradiae aph* sequence described here provided an opportunity to evaluate the Benveniste and Davies hypothesis.

Because the resistance genes of streptomycetes function by enzymatic detoxification of the antibodies or by target site modification (9), they are dominant characters that are useful for the development of streptomycete cloning vectors (14, 15). The *aph* gene of *S. fradiae* promises to be a particularly useful marker of selection and expression because high levels of APH activity were found when this gene was cloned into a low-copy-number plasmid (SLP1.2) with *S. lividans* as host (9).

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MATERIALS AND METHODS

DNA Sequence Analysis. DNA sequence analysis was performed according to the method of Maxam and Gilbert (16). DNA fragments were labeled at the 5′ end by using [γ-^{32}P]ATP and polynucleotide kinase or at the 3′ end by using [α-^{32}P]cordycepin 5′-triphosphate and terminal transferase or [α-^{32}P]dNTP and DNA polymerase I (Klenow fragment). Labeled DNA fragments for sequence analysis were prepared by secondary restriction enzyme cleavage or strand separation and analyzed by electrophoresis on 8% or 20% polyacrylamide gels (0.4 mm thick).

Analysis of *S. fradiae aph* Product. Extracts of mycelial soluble protein from early stationary phase *S. lividans* growing in yeast extract/malt extract medium (YEME) were prepared by sonication and centrifugation (9). The proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (17) and APH(3′) was assayed as described (9). APH(3′) activity was purified from extracts by ammonium sulfate precipitation (60–90% saturation) and HPLC. A Waters HPLC system was used with a Brownlee column (0.046 cm) packed with a RP300 C_{18} matrix for reverse-phase chromatography. APH(3′) activity eluted as a single peak (90% recovery) at 91% in a 40–100% acetonitrile gradient containing 0.1% trifluoroacetic acid. The fractions containing APH(3′) were pooled, lyophilized, dialyzed against water, and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis or complete amino acid analysis. Protein hydrolysis was carried out under standard conditions (6 M HCl, 0.01% mercaptoacetic acid, 108°C, 2 hr) and the hydrolysate was analyzed on an LKB 4400 amino acid analyzer. After further purification by $NaDodSO_4$ gel electrophoresis, APH(3′) was analyzed for amino-terminal amino acids on a gas phase protein sequence analyzer (Applied Biosystems model 470A).

Plasmid Constructions. The plasmid constructions are shown in Fig. 1. *S. lividans* 66 was used as host for all streptomycete plasmids (4) and *Escherichia coli* ED8767 was used as host for pBR322 (18). The procedures for plasmid isolation, construction of recombinant plasmids *in vitro*, and transformation have been described (4, 19, 20).

RESULTS

Identification of the Neomycin Resistance Gene and Gene Product. A summary of the plasmids constructed to identify

Abbreviations: kb, kilobase pair(s); *aph*, aminoglycoside phosphotransferase gene; APH, aminoglycoside phosphotransferase enzyme; YEME, yeast extract/malt extract medium.

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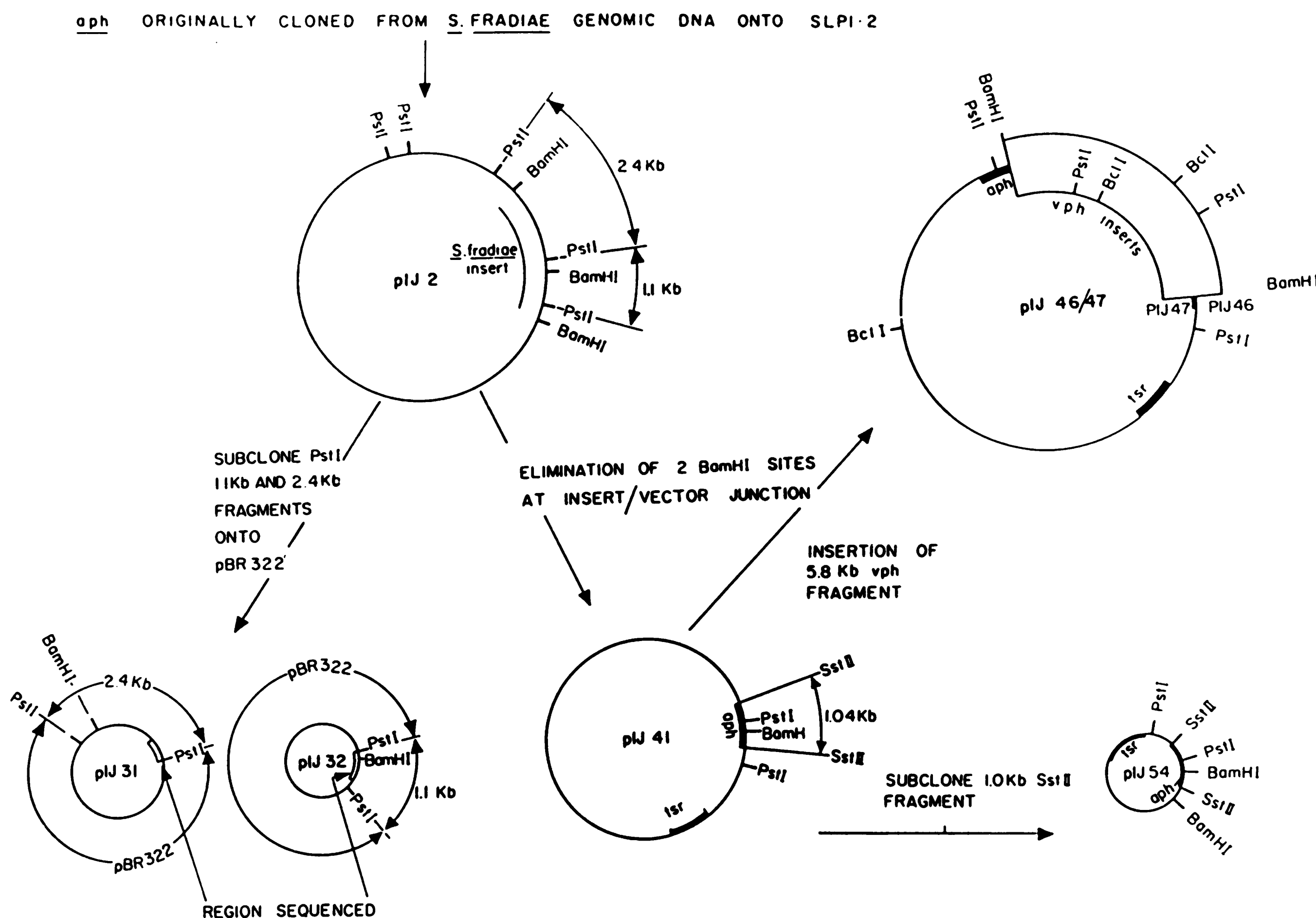

FIG. 1. Subcloning and localization of the *S. fradiae aph* structural gene from pIJ2 (7).

the *aph* gene and its product is shown in Fig. 1. The neomycin-resistance determinant of *S. fradiae* was cloned in plasmid pIJ2 from genomic DNA by using SLP1.2 as vector (6) in a *S. lividans* host. *S. lividans* strains that contained either pIJ2 or pIJ41 [a vector derived from pIJ2 (15)] were found to express a high level of APH(3′) activity (2.6 units/mg for pIJ2 and 2.1 units/mg for pIJ41) and an abundant, insert-specific, 32,000-dalton protein that was detected by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2).

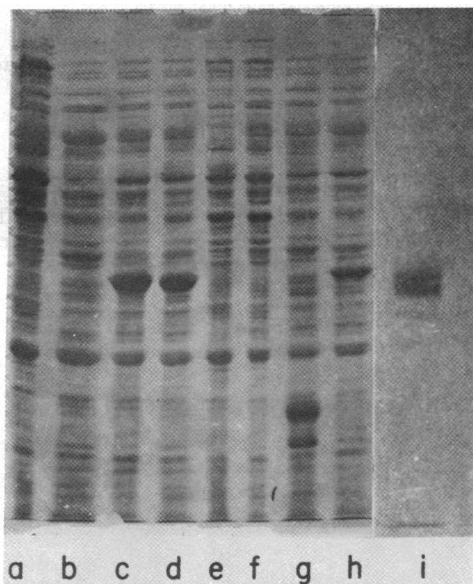

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of *S. fradiae aph* gene product. Lanes show sonic extracts of *S. lividans* 1326 containing: a, no plasmid; b, SLP1.2; c, pIJ2; d, pIJ41; e, pIJ46; f, pIJ47; g, pIJ350; h, pIJ54; i, pIJ54 (purified *aph*).

A *Bam*HI restriction fragment encoding viomycin resistance (15) was inserted, in either orientation, into the *Bam*HI site of the pIJ41 *aph* gene; strains containing these plasmids—pIJ46 and pIJ47—were sensitive to neomycin and lacked both the APH activity (less than 0.002 unit/mg) and the 32,000-dalton protein. The neomycin APH gene could be localized to a 1.1-kilobase-pair (kb) pIJ41 *Sst* II fragment (which contained the *Bam*HI site) by subcloning neomycin resistance onto the multicopy plasmid pIJ350 [thus generating pIJ54 (Fig. 1)].

The APH(3′) activity was purified by HPLC; the protein eluted as a unique peak that contained two components of apparent size 31,000 and 33,000 daltons by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2). The amino-terminal sequence of this APH(3′) preparation was found to be NH_2-?-Asp-Asp-Ser-Thr-Leu-?-?-Lys-Tyr. Under the conditions used for sequence analysis, proteins with blocked (formylated) amino termini would not have been detected. The composite amino acid content of the *S. fradiae* APH(3′) is compared to that predicted from the DNA sequence (see below) in Table 1.

Sequence of *aph*. The *Sst* II DNA fragment containing the gene *aph* was analyzed by the Maxam and Gilbert technique. The restriction endonuclease cleavage map and sequence analysis strategy are shown in Fig. 3. The sequence shown in Fig.

Table 1. Amino acid composition of APH

	Residues	
	Predicted from nucleotide sequence	Found by amino acid analysis
Ala	35	34
Arg	23	23
Asp/Asn*	30	30
Cys	4	3
Glu/Gln*	36	38
Gly	18	20
His	10	10
Ile	5	6
Leu	28	29
Lys	5	7
Met	2	2
Phe	8	9
Pro	17	21
Ser	5	7
Thr	8	10
Tyr	6	6
Val	22	22

*Because acid hydrolysis of proteins results in deamination of asparagine and glutamine, these amino acids are not distinguished from aspartate and glutamate, respectively. The quantitation of methionine represents a minimal value because it may be lost by oxidation during the analysis.

4 was confirmed by multiple independent sequence determinations. The structural gene was identified as the sequence with an open reading frame coding for a protein with an amino acid composition and amino-terminal sequence similar to those determined for purified APH(3′).

The DNA sequence of the *Sst* II fragment was analyzed by computer (Intelligenetics, Palo Alto, CA) for G+C content, inverted repeat sequences, and codon usage. In the region 5′ to the structural gene, a relatively A+T-rich segment occurs at positions 230–251 (G+C% = 43). The 3′ terminus of the structural gene (positions 1,067–1,110) is also relatively A+T rich (G+C% = 52). An inverted repeat sequence was found at positions 1,133–1,173. This 16-base-pair repeated sequence occurred 20 base pairs after the amber stop codon [$\Delta G = -43$ kcal/mol (21); 1 kcal = 4.184 kJ].

Table 2 indicates the distribution of nucleotides in the codon triplets within and adjacent to the *aph* gene sequence. Within the structural gene, adenine and thymine are rarely found in the first position (G+C content = 80%), are abundant in the second position (G+C content = 43%), and virtually excluded from the third position (G+C content = 97%). This reflects the nonrandom choice of codons as shown in Table 3. Twenty-five of the 64 coding triplets are not used. In contrast, the sequences that precede and follow the structural gene do not show

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  Nar 1
CGCGGCGCCCGCGCCGCCAGCTCGGCGGGGCGGACCCGGACCCGGCCGCCGAGGTCCTCG   60

CCGCCGACCGGGAGGCGTCGGCCTCGCCGCCGAAGACCGCCGTCCTGCTGCGGCTCACGG  120

  Rsa1
AGGCGTACCTCTCGCCCTGCGCGCGGGCCTTCGACCCCGCCGGGACCTCCGGCACCGGGC  180

 Sst2                            Nar1
CCGCGGCGACGCCGGGCGCACCGGGTCCACCGGCGCCCCCCCACCCCGCACAGAATGTC  240

CGAAACCCCTACGGGCCCCGACGAAAGGCGCGGAACGGCGTCTCCGCCTCTGCCATGATG  300

                                  Rsa1
CCGCCCATGGACGACAGCACGTTGCGCCGGAAGTACCCGCACCACGAGTGGCACGCAGTG  360
      MetAspAspSerThrLeuArgArgLysTyrProHisHisGluTrpHisAlaVal

         Hinf1  Nar1
AACGAAGGAGACTCGGGCGCCTTCGTCTACCAGCTCACCGGCGGCCCCGAGCCCCAGCCC  420
AsnGluGlyAspSerGlyAlaPheValTyrGlnLeuThrGlyGlyProGluProGlnPro

 Sst1
GAGCTCTACGCGAAGATCGCCCCCCGCGCCCCCGAGAACTCCGCCTTCGACCTGTCCGGC  480
GluLeuTyrAlaLysIluAlaProArgAlaProGluAsnSerAlaPheAspLeuSerGly

                                    Bam H1
GAGGCCGACCGGCTGGAGTGGCTCCACCGCCACGGGATCCCCGTCCCCCGCGTCGTCGAG  540
GluAlaAspArgLeuGluTrpLeuHisArgHisGlyIluProValProArgValValGlu

CGCGGTGCCGACGACACCGCCGCGTGGCTCGTCACGGAGGCCGTCCCCGGCGTCGCGGCG  600
ArgGlyAlaAspAspThrAlaAlaTrpLeuValThrGluAlaValProGlyValAlaAla

GCCGAGGAGTGGCCCGAGCACCAGCGGTTCGCCGTGGTCGAGGCGATGGCGGAGCTGGCC  660
AlaGluGluTrpProGluHisGlnArgPheAlaValValGluAlaMetAlaGluLeuAla

                                              Nar1
CGCGCCCTCCACGAGCTGCCCGTGGAGGACTGCCCCTCCGACCGGCGCCTCGACGCGGCG  720
ArgAlaLeuHisGluLeuProValGluAspCysProSerAspArgArgLeuAspAlaAla

                                                     Pst1
GTCGCCGAGGCCCGGCGGAACGTCGCCGAGGGCTTGGTGGACCTCGACGACCTGCAGGAG  780
ValAlaGluAlaArgArgAsnValAlaGluGlyLeuValAspLeuAspAspLeuGlnGlu

                                         Sst1
GAGCGGGCCGGGTGGACCGGCGACCAGCTCCTGGCGGAGCTCGACCGCACCCGTCCCGAG  840
GluArgAlaGlyTrpThrGlyAspGlnLeuLeuAlaGluLeuAspArgThrArgProGlu

AAGGAGGACCTGGTCGTCTGCCATGGCGACCTGTGCCCCAACAACGTCCTGCTCGACCCC  900
LysGluAspLeuValValCysHisGlyAspLeuCysProAsnAsnValLeuLeuAspPro

            Bst E2
GGGACCTGCCGGGTCACCGGCGTGATCGACGTCGGCCGCCTCGGGGTCGCCGACCGCCAC  960
GlyThrCysArgValThrGlyValIluAspValGlyArgLeuGlyValAlaAspArgHis

GCCGACATCGCCTTGGCCGCCCGCGAGCTGGAGATCGACGAGGACCCCTGGTTCGGCCCC 1020
AlaAspIluAlaLeuAlaAlaArgGluLeuGluIluAspGluAspProTrpPheGlyPro

                              Rsa1 Nar1       Sal1
GCCTACGCCGAGCGGTTCCTGGAGCGGTACGGCGCCCACCGCGTCGACAAGGAGAAGCTG 1080
AlaTyrAlaGluArgPheLeuGluArgTyrGlyAlaHisArgValAspLysGluLysLeu

                                 Xba1
GCCTTCTACCAGCTTCTCGACGAGTTCTTCTAGAGCCGCCCCGCAGGGCGCTCCGCAGGC 1140
AlaPheTyrGlnLeuLeuAspGluPhePhe***

CGCTTCCGGACCACTCCGGAAGCGGCCGTGCGGTCGGAGGUACCCGGCCGCCTTGGAGAC 1200

 Nar1                Sst2
CGGCGCCCGGCCCCCGCTTTCCGCGGCUTGGCCGGAGCCGTCAGAGGCCGTGGTACGGGT 1260

          Kpn1
TGGCGGCGAGGTACCGGGCT   1280
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FIG. 4. Nucleotide sequence of the *S. fradiae aph* gene. The amino acids predicted by the sequence are indicated. The amino terminus of APH was determined by amino acid sequence analysis; the carboxyl terminus was defined by the TAG termination codon.

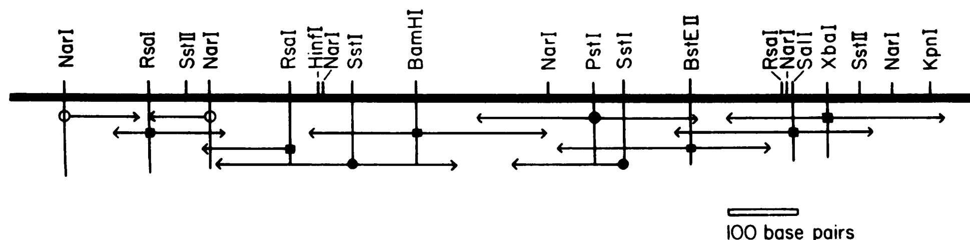

FIG. 3. Restriction endonuclease cleavage map and sequence analysis strategy for the *S. fradiae aph* gene. DNA fragments were labeled at the 5′ end by using polynucleotide kinase or at the 3′ end by using either terminal transferase or DNA polymerase I; analysis was in the direction indicated.

Table 2. Distribution of nucleotides in codon triplets

Region	% G+C content			
	1st	2nd	3rd	Mean
Before *aph*	85	67	84	79
Within *aph*	78	43	97	73
After *aph*	80	82	84	82

this distribution; the G+C contents of the three possible reading frames are similar. The average G+C content of the adjacent sequences is virtually identical to that of the structural gene (73%) and agrees well with the G+C content of *Streptomyces* genomic DNA (72–74%) (1).

Neomycin-Resistance Gene Found in *S. fradiae* Is Similar to That of Tn5 and Tn903. The amino acid sequences of the *S. fradiae* APH(3′) (predicted from the nucleotide sequence) and the APH(3′) encoded by the antibiotic-resistance transposons Tn5 and Tn903 are compared in Fig. 5. The *S. fradiae* sequence is equivalent to Tn903 at 118 of 271 amino acid residues and to Tn5 at 111. Tn5 and Tn903 APH(3′) are equivalent at 100 of 280 positions. The most extensive amino acid homology among these three phosphotransferases is found at the carboxy terminus and within the central regions.

A computerized search for nucleotide sequence homology (a sequence of at least 15 80% matched nucleotides with no sequential three bases mismatched) among the structural genes from the three sources revealed that the *S. fradiae* and Tn5 *aph* genes contained homologous sequences. The most statistically significant region of homology occurs immediately upstream of the TAG stop codon (the most A+T-rich region of the *S. fradiae aph* gene) where the two genes are identical at 26 of 30 nucleotides. This degree of homology would be expected to occur by chance (E) 10^{-4} times in the two sequences. Other regions with homology to the Tn5 *aph* gene occur at *S. fradiae aph* nucleotides 844–869 ($E = 0.02$) and 964–983 ($E = 0.70$). On the other hand, no such sequence nucleotide homologies were noted between the *S. fradiae* and Tn903 *aph* genes or between the *aph* sequences of Tn903 and Tn5.

DISCUSSION

The nucleotide sequence of the *aph* gene of a neomycin-producing *S. fradiae* has been determined. The accuracy of this DNA sequence and location of the structural gene were confirmed by purification of the *aph* gene product and subsequent amino acid analyses. This sequence possesses information related to the regulation of streptomycete genes and to the evolution of clinically important drug-resistance elements.

The possible evolutionary relationship between the structural genes encoding the APH(3′) from *S. fradiae* and the functionally related activities found in the drug-resistance determinants of Tn5 and Tn903 was considered. Pairwise comparisons of the amino acid sequence of three enzymes revealed that there is considerable homology (36–40%). Highly conserved regions at the carboxy terminus of these genes probably represent functionally important domains that may involve binding of one of the two substrates (aminoglycoside antibiotic or ATP). Comparison of the three possible sequence pairs reveals that all three enzymes are equally similar; no one pair exhibits greater homology than the other two. This could indicate the existence of a common ancestor to the *aph* genes found in antibiotic-producing bacteria and R-factor-containing clinical isolates. Acquisition of the *aph* gene by actinomycetes was probably a prerequisite for aminoglycoside antibiotic production (to avoid autotoxicity); acquisition of the gene by other bacteria could have been self-protective in environments containing aminoglycoside antibiotics.

It was not possible to identify those sequences that control the transcription and translation of the *S. fradiae aph* gene by comparison with the consensus sequences proposed for gene expression in *Escherichia coli* (2, 22) or *Bacillus subtilis* (23). The presence of a streptomycete promoter in the sequence presented is suggested by the observation that a series of plasmids constructed by the insertion of the *Sst* II fragment containing the *aph* gene into vector pIJ350 at different sites, and in both orientations, expressed similar levels of APH(3′) in *S. lividans*: pIJ54, 1.9 units/mg of protein; pIJ57, 1.7; pIJ62, 2.2; pIJ69, 1.6; pIJ70, 1.0; and pIJ71, 1.2 (unpublished data). *E. coli* cells containing a recombinant plasmid (pIJ28) that can replicate in both *E. coli* and *S. lividans* and encoding a functional *S. fradiae aph* gene were sensitive to neomycin and produced less than 0.1% of the APH(3′) activity found in *S. lividans* (15). However, this phosphotransferase gene has been shown to confer neomycin resistance to *E. coli* when transcribed from the pBR325 tetracycline-resistance promoter (24). Although expression of the *aph* gene might suggest the presence of a streptomycete Shine–Dalgarno sequence, DNA sequences immediately preceding the gene showed no homology to either *E. coli* (22) or *S. lividans* (25) 16S rRNA termini.

The high G+C content of the *S. fradiae aph* structural gene is associated with a nonrandom choice of codons, which implies constraints both on the regulation of genes in streptomycetes and on the conservation of their sequence homology to related drug-resistance genes of other bacterial genera. Codon usage relative to an organism's tRNA pools is a genome strategy for gene regulation (26). It is likely that the tRNA populations found in streptomycetes are different from those found in organisms

Table 3. Codon use in the *aph* structural gene

Phe	TTT	0	Ser	TCT	0	Tyr	TAT	0	Cys	TGT	0
	TTC	8		TCC	3		TAC	6		TGC	4
Leu	TTA	0		TCA	0	***	TAA	0	***	TGA	0
	TTG	3		TCG	1	***	TAG	1	Trp	TGG	6
Leu	CTT	1	Pro	CCT	0	His	CAT	1	Arg	CGT	1
	CTC	12		CCC	17		CAC	9		CGC	12
	CTA	1		CCA	0	Gln	CAA	0		CGA	0
	CTG	12		CCG	2		CAG	6		CGG	10
Ile	ATT	0	Thr	ACT	0	Asn	AAT	0	Ser	AGT	0
	ATC	5		ACC	6		AAC	5		AGC	1
	ATA	0		ACA	0	Lys	AAA	0	Arg	AGA	0
Met	ATG	4		ACG	2		AAG	5		AGG	0
Val	GTT	0	Ala	GCT	0	Asp	GAT	0	Gly	GGT	1
	GTC	17		GCC	25		GAC	25		GGC	12
	GTA	0		GCA	1	Glu	GAA	1		GGA	1
	GTG	5		GCG	9		GAG	29		GGG	4

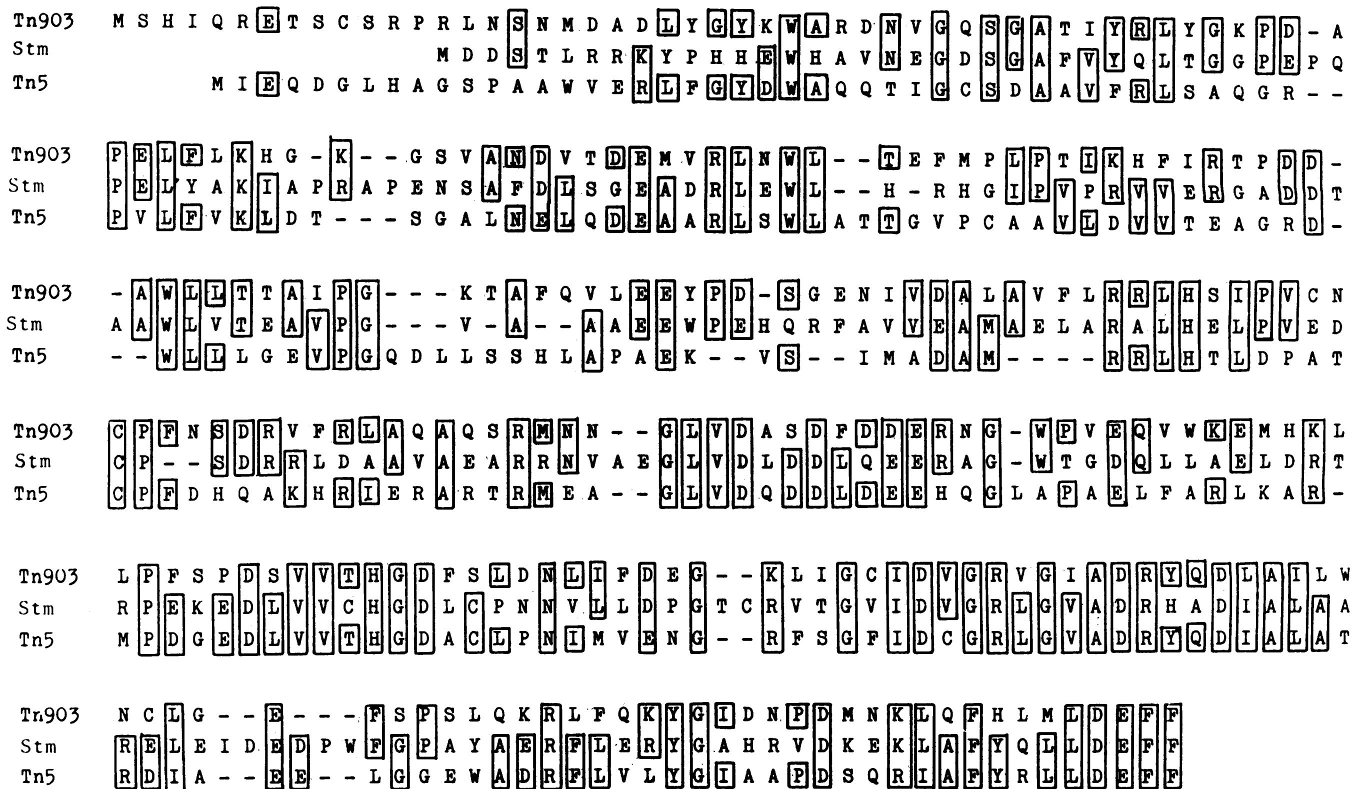

FIG. 5. Homology among the *S. fradiae* (Stm), Tn*5*, and Tn*903* APH proteins. Conserved amino acids (glutamate/aspartate, arginine/lysine, and leucine/isoleucine were considered equivalent) are enclosed in boxes. Dashes indicate amino acids deleted in one sequence relative to another.

with A+T-rich genomes. However, we can rule out the possibility that the codons that are not used in *aph* might result from the complete absence of their cognate tRNAs because several antibiotic-resistance genes of Gram-negative bacteria, including those from Tn*5* (S. G. Foster and J. A. Gil, personal communication; ref. 27) and Tn*903* (28), as well as the chloramphenicol-resistance gene of Tn*9* (28), are expressed in *S. lividans*.

The high level of APH(3′) expression (10% of soluble protein) when the gene is cloned on streptomycete plasmids is probably due to the presence of an active promoter sequence. The *S. fradiae aph* gene has been incorporated into high-copy-number broad-host-range vectors (14, 15), and the uses of vectors carrying this expression system to increase antibiotic yields, to generate hybrid antibiotics, and to produce pharmacologically active proteins are possibilities of considerable economic importance.

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