CLONING AND EXPRESSION OF AN APH(3′)-III PHOSPHOTRANSFERASE FROM *STAPHYLOCOCCUS AUREUS* IN *STREPTOMYCES LIVIDANS*

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An aminoglycoside 3′ type III phosphotransferase derived from *Staphylococcus aureus* plasmid pRN1956 was cloned on the high copy number Streptomycetes vectors pIJ702 and pIJ704. *Streptomyces lividans* transformants carrying the hybrid plasmids show a resistance pattern towards aminoglycoside antibiotics comparable to the resistance pattern of *S. aureus*. The APH(3′)-III with expanded spectrum of resistance, is a useful additional marker for gene cloning in Streptomycetes.

Aminoglycoside modifying enzymes can be detected in antibiotic-producing organisms such as Streptomycetes[1], *Bacillus*[2] as well as in Gram-positive and Gram-negative nonproducing organisms (for a review see DAVIES and SMITH[3]); they confer resistance to the organisms by inactivation of the antibiotic. It has been demonstrated that aminoglycoside modifying enzymes from *Bacillus*[2] and *Staphylococcus aureus*[4] can be efficiently cloned and expressed in *Escherichia coli*. The development of a cloning system for the genus *Streptomyces* in recent years[5,6] provides the possibility of cloning and expressing genes from different sources in this important group of organisms. Several aminoglycoside modifying enzymes have been cloned in *Streptomyces lividans*[7,8] and used to construct cloning vehicles[9,10]. Using these vectors the tyrosinase gene from *S. antibioticus*[11], aminoglycoside modifying enzymes from *S. fradiae*[2], *S. glaucescens*[12] and genes involved in the biosynthesis of antibiotics[13,14,24] have been cloned and expressed. We report here the cloning and expression of another APH(3′)-III phosphotransferase, that of *S. aureus* in *S. lividans*, which extends the number of vector genes that can be applied.

Materials and Methods

Bacterial Strains and Plasmids

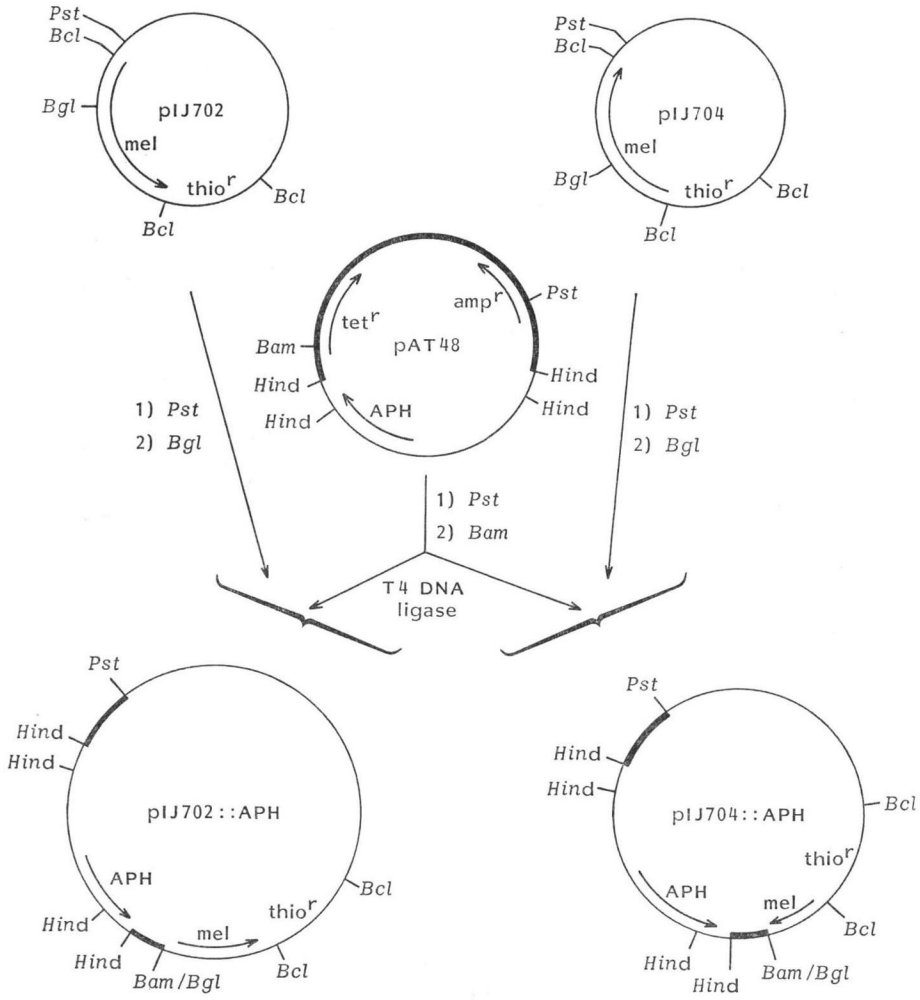
S. lividans strain 1326[15] was used as host for all the transformation experiments described.

Plasmid pAT48 is pBR322 harboring a cloned neomycin-kanamycin resistance gene derived from *S. aureus* plasmid pRN1956[16] and was used as a source for the aminoglycoside (3′)-III phosphotransferase gene[4]. Plasmids pIJ702 and pIJ704, derivatives of the pIJ101 multicopy plasmid[17] containing the tyrosinase gene from *S. antibioticus* in different orientations[11] were used as cloning vectors.

Culture Conditions and Transformation Procedure

For protoplast preparation spores and mycelial fragments of *S. lividans* were grown in yeast-malt extract medium containing sucrose 34%, $MgCl_2$ 5 mM and glycine 0.5%[5]. Protoplast formation and transformations were carried out as described by THOMPSON *et al.*[9], except that the transformed protoplasts were spread directly on regeneration medium without further washing according to KIESER *et al.*[17]. Mycelia for cell-free extracts as well as for plasmid preparations were obtained from growth in Tryptic soya broth CMI129 (Oxoid).

Fig. 1. Derivation of hybrid plasmids pIJ702:: APH(3')-III and pIJ704:: APH(3')-III from pAT48, pIJ702 and pIJ704 respectively.



MIC (minimal inhibitory concentration) value determinations were done on yeast extract - malt extract agar[18] according to ONO *et al.*[19].

Detection of Transformants

Transformants were detected by the overlay method[17] using 2.5 ml soft agar containing 200 μg/ml thiostrepton. Colonies containing inserts were isolated as clones unable to produce black melanin halos[11]. Direct selections were done using kanamycin at a concentration of 200 μg/ml.

Plasmid Preparations and DNA Manipulations

Plasmids were isolated after alkaline lysis and phenol - chloroform extraction according to KIESER[20]. Restriction analysis and ligations were performed according to THOMPSON *et al.*[8]. Restriction enzymes and T4 DNA ligase were of commercial source (BRL) and used according to the suppliers recommendations. Agarose gel electrophoresis was carried out according to MANIATIS *et al.*[6] using Tris-acetate or Tris-borate buffer.

Preparation of Cell-free Extracts

Cell-free extracts were prepared according to BENVENISTE and DAVIES[1].

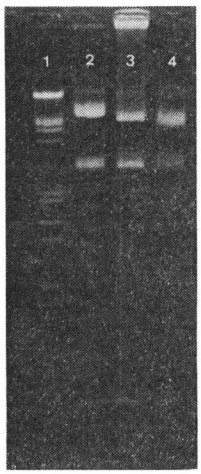
Assay for Aminoglycoside-phosphotransferase

Cell-free extracts were first tested for the presence of modifying enzymes using a biological test. A typical inactivation reaction was prepared as follows: cell-free extract 250 μl, buffer containing per liter Tris-HCl pH 7.4 (2 mM), $MgCl_2$ (0.8 mM), NH_4Cl (0.1 mM), dithiothreitol (0.1 mM), ATP (0.3 mM) and antibiotic (0.15 mM); 250 μl. The mixtures were incubated at 30°C for different times and the residual antibiotic activity was determined using *S. aureus* strain 209P as tester organism[19]. Radiochemical assays were carried out using the phosphocellulose paper binding method[1] with [γ-^{32}P]ATP as substrate.

Chemicals

All chemicals were of highest available purity grade from Sigma or Merck.

Fig. 2. Restriction analysis of pIJ702:: APH(3′)-III, pIJ704:: APH(3′)-III and pAT48 with *Hin*d III, (1) λDNA, *Eco*R I+*Hin*d III digest. (2) pIJ702:: APH(3′)-III. (3) pIJ704:: APH(3′)-III. (4) pAT48.



Results

Construction of Recombinant Plasmids and Detection of Transformants

The strategy used to subclone the APH(3′)-III phosphotransferase gene from plasmid pAT48 into the *Streptomyces* vectors pIJ702 and pIJ704 is summarized in Fig. 1. Ligation mixtures were used to transform *S. lividans* protoplasts. White, thiostrepton resistant colonies due to insertional inactivation of the melanin gene were isolated and plasmid mini-lysates of such strains screened for inserts. Two transformants containing plasmids of the expected size were analysed by agarose gel electrophoresis after restriction with *Hin*d III (Fig. 2). The results confirm, that an insert containing the four expected *Hin*d III restriction sites was present.

Properties of the Transformants

Phenotypic Properties

The MIC values of the wild type strain *S. lividans*, transformants containing pIJ702, and the two *in vitro* constructs were determined against a variety of aminoglycoside antibiotics (Table 1). The results are consistent with the broad substrate range of the *S. aureus* phosphotransferase[4].

The gene dosage is apparently not sufficient to confer resistance to amikacin *in vivo*, but cell-free extracts of strains containing the hybrid plasmids phosphorylate this antibiotic (see later). This is probably due to the lower affinity (high *Km* value) of the APH(3′)-III for amikacin[2,3]. Both recombinant constructions are very stable in *S. lividans* even after long term fermentation without selective pressure (not shown).

Enzymatic Properties

The aminoglycoside substrate profile of aminoglycoside phosphotransferase activity extracted from *S. lividans* transformants was determined (Table 2). The profile is consistent with that found in *S. aureus* containing pRN1956 plasmid[4]. Tobramycin is, as expected, not a substrate for the phosphotransferase and the resistance levels *in vivo* against this antibiotic are unchanged (Table 1).

Table 1. Minimal inhibitory concentrations (μg/ml) of different strains against aminoglycoside antibiotics.

Strain	KAN	AMI	BUT	RMS	PAR	NEO	LVDM	TOB
Streptomyces lividans 1326	<4	<2	<5	10	10	<1	20	<5
S. lividans 1326 pIJ702	<4	<2	<5	10	10	<1	20	<5
S. lividans 1326 pIJ702:: APH(3′)-III	>150	>2<5	35	>300	>50	>40	>60	<5
S. lividans 1326 pIJ704:: APH(3′)-III	>150	>2<5	35	>300	>50	>40	>60	<5

Abbreviations: KAN kanamycin, AMI amikacin, BUT butirosin, RMS ribostamycin, PAR paromomycin, NEO neomycin, LVDM lividomycin, TOB tobramycin.

Table 2. Incorporation of [γ-^{32}P]ATP into different aminoglycoside antibiotics by cell-free extracts of different strains (cpm/10 μl cell-free extract) for abbreviations see Table 1.

Extract source	Antibiotic (cpm)								
	—	KAN	AMI	BUT	RMS	PAR	NEO	LVDM	TOB
None	167								
Streptomyces lividans 1326	227	126	52	367	132	112	20	122	122
S. lividans 1326 pIJ702	141	92	50	287	161	89	34	134	134
S. lividans 1326 pIJ702:: APH(3′)-III	147	11,192	15,031	21,237	10,567	18,435	20,097	22,536	136
S. lividans 1326 pIJ704:: APH(3′)-III	162	18,623	12,521	28,529	11,610	29,529	20,545	24,384	164

Discussion

The results reported here confirm that a gene for aminoglycoside antibiotic modification originally derived from the Gram-positive bacterium *S. aureus* can act as antibiotic determinant in *S. lividans*. The new resistance plasmids obtained by the cloning of the neomycin-kanamycin phosphotransferase from *S. aureus* into *S. lividans* confer an antibiotic resistance phenotype comparable to those due to the naturally occurring plasmid in *S. aureus* (Table 1)[4].

The phosphorylation profile using a variety of aminoglycoside antibiotics confirms that the cloned gene corresponds to an APH(3′) type III aminoglycoside phosphotransferase (lack of phosphorylation with tobramycin as substrate, but ability to phosphorylate both butirosin and lividomycin, Table 2)[3].

The fragment containing the APH(3′)-III gene was expressed in both orientations into the *Bgl* II site with respect to the *mel* gene orientation into pIJ702 and pIJ704 (Fig. 1), therefore we can exclude expression controlled by the promotor of the melanin gene. On the other hand we cannot exclude expression coming from a vector plasmid promotor reading counter clockwise through the *Pst* I site (Fig. 1). The gene is in opposite orientation with respect to the tet^R and amp^R genes from the *E. coli* plasmid pBR322 and the start of the phosphotransferase coding sequence is preceded from 779 *E. coli* base pairs followed by 1,288 base pairs of *S. aureus* DNA[22]. This could support the idea, that translational regulatory sequences from *S. aureus* are recognised by *S. lividans*. On the other hand no obvious homologies exist between the pre-gene sequence of a APH 3′ phosphotransferase from *S. fradiae* and the ribosome binding site or promoter sequences found in other bacterial systems[22,23]. In contrast the *S. aureus* APH 3′ pre- and post-gene sequences contain signal sequences closely resembling to those observed for other bacterial and bacteriophage genes[16,22]. Since the *S. aureus* gene has been sequenced[16] the function of the regulatory regions can now be studied in *Streptomyces*.

The cloning and expression of the typical *S. aureus* APH(3′)-III in Streptomycetes, provides a useful, broad substrate selective marker for cloning in this species. The staphylococcal gene expresses high level resistance to a number of aminoglycoside antibiotics and since the gene has only limited nucleic acid homology with analogous Streptomycete genes[16], the APH(3′)-III can be used in studies of antibiotic production in organisms containing an amplified phosphotransferase gene that is less susceptible to loss as a result of recombination between plasmid and chromosome.

References

1) Benveniste, R. & J. Davies: Aminoglycoside antibiotic-inactivating enzymes in Actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. U.S.A. 70: 2276~2280, 1973
2) Courvalin, P.; B. Weisblum & J. Davies: Aminoglycoside-modifying enzyme of an antibiotic-producing bacterium acts as a determinant of antibiotic resistance in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 74: 999~1003, 1977
3) Davies, J. & D. I. Smith: Plasmid-determined resistance to antimicrobial agents. Ann. Rev. Microbiol. 32: 469~518, 1978
4) Courvalin, P. & M. Fiandt: Aminoglycoside-modifying enzymes of *Staphylococcus aureus*: Expression in *Escherichia coli*. Gene 9: 247~269, 1980
5) Chater, K. F.; D. A. Hopwood, T. Kieser & C. J. Thompson: Gene cloning in *Streptomyces*. Curr. Top. in Microbiol. & Immun. 96: 69~95, 1982
6) Maniatis, T.; E. F. Fritsch & J. Sambrook: Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982
7) Murakami, T.; C. Nojiri, H. Toyama, E. Hayashi, K. Katumata, H. Anzai, Y. Matsuhashi, Y. Yamada & K. Nagaoka: Cloning of antibiotic-resistance genes in *Streptomyces*. J. Antibiotics 36: 1305~1311, 1983
8) Thompson, C. J.; J. M. Ward & D. A. Hopwood: DNA cloning in *Streptomyces*: Resistance genes from antibiotic producing species. Nature 286: 525~527, 1980
9) Thompson, C. J.; Ward J. M. & D. A. Hopwood: Cloning of antibiotic resistance and nutritional genes in *Streptomyces*. J. Bacteriol. 151: 668~677, 1982
10) Thompson, C. J.; T. Kieser, J. M. Ward & D. A. Hopwood: Physical analysis of antibiotic resistance genes from *Streptomyces* and their use in vector construction. Gene 20: 51~62, 1984
11) Katz, E.; C. J. Thompson & D. A. Hopwood: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129: 2703~2714, 1983
12) Hintermann, G.; R. Crameri, M. Vögtli & Hütter: Streptomycin-sensitivity in *Streptomyces glaucescens* is due to deletions comprising the structural gene coding for a specific phosphotransferase. Mol. Gen. Genet. 196: 513~520, 1984
13) Feitelson, J. S. & D. A. Hopwood: Cloning of a *Streptomyces* gene for an *O*-methyltransferase involved in antibiotic biosynthesis. Mol. Gen. Genet. 190: 394~398, 1983
14) Gil, J. A. & D. A. Hopwood: Cloning and expression of a *p*-aminobenzoic acid synthetase gene of the candicidin producing *Streptomyces griseus*. Gene 25: 119~132, 1983
15) Lomovskaya, N. D.; N. M. Mkrtumian & V. N. Danilenko: Characterization of temperate actinophage C31 isolated from *Streptomyces coelicolor* A3(2). J. Virol. 9: 258~262, 1972
16) Gray, S. G. & W. M. Fitch: Evolution of antibiotic resistance genes: The DNA sequence of a kanamycin resistance gene from *Staphylococcus aureus*. J. Mol. Biol. & Evol. 1: 57~66, 1983
17) Kieser, T.; D. A. Hopwood, H. M. Wright & C. J. Thompson: pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: Functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. 185: 223~238, 1982
18) Pridham, T. G.; P. Anderson, C. Foley, L. A. Lindenfelser, C. W. Hesseltine & R. G. Benedict: A selection of media for maintenance and taxonomic studies of *Streptomyces*. Antibiot. Ann. 1956-1957: 947~953, 1957
19) Ono, H.; R. Crameri, G. Hintermann & R. Hütter: Hydroxystreptomycin resistance and production in *Streptomyces glaucescens*. J. Gen. Microbiol. 129: 529~537, 1983
20) Kieser, T.: Factors affecting the isolation of cccDNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12: 12~19, 19~36, 1984
21) Courvalin, P. & J. Davies: Plasmid mediated aminoglycoside phosphotransferase of broad substrate range that phosphorylates amikacin. Antimicrob. Agents Chemother. 11: 619~624, 1977
22) Gray, S. G.: Aminocyclitol resistance in *Staphylococcus aureus*: Analysis of the resistance enzymes and plasmids and comparison of aminocyclitol 3′-phosphotransferases. Ph. D. Thesis, Univ. Wisconsin-Madison, 1982
23) Thompson, C. J. & G. S. Gray: Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase and its relationship to phosphotransferases encoded by resistance plasmids. Proc. Natl. Acad. Sci. U.S.A. 80: 5190~5194, 1983
24) Martin, J. F. & J. A. Gil: Cloning and expression of antibiotic production genes. Biotechnology 1984: 63~72, 1984