INCREASED PRODUCTION OF AMINOGLYCOSIDES ASSOCIATED WITH AMPLIFIED ANTIBIOTIC RESISTANCE GENES

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The 6′-*N*-acetyltransferase derived from *Streptomyces kanamyceticus* strain M1164 was cloned on to the high copy plasmid vector pIJ702 and introduced into *S. kanamyceticus* (ATCC 12853, a kanamycin producer) and *S. fradiae* (ATCC 10745, a neomycin producer). In both cases transformants containing the recombinant plasmid showed increased resistance to a number of aminoglycoside antibiotics and substantially increased production of kanamycin and neomycin. This demonstrates that specific amplification of gene products associated with antibiotic biosynthesis provides a means for improving antibiotic production.

Aminoglycoside/aminocyclitol antibiotics constitute a large group of chemically related antibiotics containing aminosugar residues[1]. They are produced by a variety of different bacterial genera such are *Streptomyces*, *Micromonospora*, *Bacillus* and *Pseudomonas*. In spite of their toxicity[2] they remain one of the most potent group of chemotherapeutic agents against serious Gram-positive and Gram-negative bacterial infections. Natural fermentation products (kanamycins, neomycins, gentamicins and tobramycin), as well as semisynthetic derivatives (amikacin, dibekacin and netilmicin) are used[3]. The antimicrobial activity of these drugs results from an interaction with the ribosomes of sensitive microorganisms leading to inhibition of protein synthesis[4]. The extensive clinical use of aminoglycoside antibiotics has led to the selection of organisms resistant to their inhibitory action. The primary mechanism of resistance in clinical situations is the inheritance of plasmids encoding aminoglycoside detoxification mechanisms (*O*-phosphorylation, *N*-acetylation and *O*-adenylylation). The newer, semisynthetic aminoglycosides have been designed to be less susceptible to enzymatic modification; to some extent this approach has been successful.

Similar mechanisms of resistance have been characterised in aminoglycoside-producing microorganisms and it has been suggested that antibiotic-producing organisms may have provided the source from which resistance mechanisms in clinical isolates could have evolved[5]. The precise role of the aminoglycoside modifying enzymes in aminoglycoside production remains obscure. It is quite possible that they provide a mechanisms by which the producing organisms avoid autotoxicity during antibiotic biosynthesis. On the other hand some studies have suggested that the aminoglycoside modifying enzymes are actually involved in the pathways of biosynthesis. Biologically inactive *N*-acetyl derivatives of neomycin[6], kanamycin B[7], and ribostamycin[8] have been isolated from fermentation broths of producing strains. In addition, it has been known for some time that aminoglycoside producing organisms possess both inactivating enzymes and enyzmes able to restore biologically active antibiotics from the inactivated forms. For example the presence of streptomycin 6-phosphotransferases[9] and streptomycin-phosphate phosphohydrolase[10,11] in streptomycin-producing strains and the presence of both kanamycin 6′-*N*-acetyltransferase and acetylkanamycin iminohydrolase in

the kanamycin producer *S. kanamyceticus*[12]. High level aminoglycoside resistant strains are often overproducers[13] and in some cases increased levels of aminoglycoside production correlate with increased levels of aminoglycoside modifying enzymes[14]; moreover loss of production frequently correlates with the loss of the modifying activities[14~17].

In order to establish the nature of the relationship between aminoglycoside production and resistance we have used *Streptomyces* cloning systems[18,19] to test directly the effects of changed aminoglycoside modifying enzyme content on antibiotic resistance and productivity in producing strains.

In this report we describe the effects of a cloned aminoglycoside 6'-*N*-acetyltransferase on antibiotic yields, antibiotic resistance and modification activities in two aminoglycoside producing strains.

Materials and Methods

Bacterial Strains and Plasmids

S. lividans strain 66[20], *S. kanamyceticus* strain ATCC 12853 and *S. fradiae* strain ATCC 10745 were used as hosts in transformation. *Staphylococcus aureus* strain 209P (ATCC 6538P) was used for detection of both antibiotic production and presence of aminoglycoside modifying enzymes[17]. Plasmid pMS18[21] was used as source of a 6'-*N*-acetyltransferase (AAC6') gene derived from the chromosome of *S. kanamyceticus* strain M1164. As cloning vectors pIJ702[22] and the low copy number plasmid pR2, a derivative of pIJ61[23] carrying a deletion extending into the aminoglycoside 3'-*O*-phosphotransferase (APH 3') gene (unpublished result), were used.

Media, Strain Storage and Culture Conditions

Stock cultures of *Streptomyces* strains were grown and maintained on solid agar media[24]. Stock suspensions of spores were stored on 50% sterile glycerol at −20°C. For protoplast preparation, strains were grown on YEMEG medium[25] containing sucrose (34%), $MgCl_2$ (5 mM) and glycine (0.6%). For regeneration transformed protoplasts were plated on a osmotically stabilized medium as described by HINTERMANN *et al.*[26]. For antibiotic production and aminoglycoside modifying enzyme determinations strains were grown on 1,000-ml Erlenmeyer flasks with 4 baffles containing 100 ml medium at 30°C on a rotary shaker (220 rpm); *S. fradiae* on Tryptic soya broth CM-129 (Oxoid), *S. kanamyceticus* on a minimal medium according to BASAK and MAJUMDAR[27]. Minimal inhibitory concentrations (MIC) were determined according to ONO *et al.*[17] on yeast extract - malt extract agar containing antibiotics at various concentrations. *Staphylococcus aureus* was grown overnight at 37°C on a rotary shaker in Tryptic soya broth CM-129. After centrifugation (5 minutes, 10,000 rpm) and washing with sterile deionized water, cells were stored in a 50% glycerol suspension at −70°C.

Protoplast Preparations, Transformations and Selection for Transformants

Streptomyces protoplasts were prepared and transformed according to THOMPSON *et al.*[23]. Transformants were selected by the overlay method[28] using thiostrepton (500 μg/ml, 2.5 ml soft agar/plate) and inserts were detected by insertional inactivation of the melanin gene[22] or directly selecting for aminoglycoside resistance using amikacin (500 μg/ml, 2.5 ml soft agar/plate).

Plasmid Preparations and DNA Manipulations

For mini-lysates as well as for large scale preparations plasmids were isolated and purified according to KIESER[29]; restriction analyses and ligations were performed according to THOMPSON *et al.*[23]. 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.*[30] using Tris-acetate or Tris-borate buffer.

Preparation for Cell-free Extracts

Cell-free extracts were prepared according to BENVENISTE and DAVIES[5] and stored at −70°C until used.

Determination of Neomycin and Kanamycin Production

During the course of fermentation 2 ml samples were removed, centrifuged (10 minutes, 10,000 rpm at 4°C) and the supernatants stored at −20°C. The estimation of kanamycin and neomycin production (μg/ml) was according to ONO *et al.*[17] by measuring the inhibition zones against *S. aureus* strain 209P. Commercial kanamycin and neomycin sulfate (Sigma) were used as standards to quantify the amounts of antibiotic produced.

Determination of Aminoglycoside Modifying Activities

Extracts of wild type strains and of transformants were tested for their ability to transfer [^{14}C]-acetyl groups from [^{14}C]acetyl-coenzyme A to kanamycin as previously described[5]. Acetylated antibiotic was then separated from unreacted cofactor by absorption onto phosphocellulose paper as described by HAAS and DOWDING[31]. Reaction mixtures contained 25 μM kanamycin, cell free-extract to 20 μg total protein, 3 μl of [1-^{14}C]acetyl-coenzyme A (20 μCi/ml, 59.3 mCi/mmol) and buffer (10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM NH_4Cl, 3 mM 2-mercaptoethanol) to a total volume of 40 μl. Incubation was continued for 60 minutes at 30°C. Enzymatic activities were expressed as arbitary units *i.e.* cpm incorporated into kanamycin per 1 μg total protein per hour $\times 10^{-3}$. Controls contained all components except cell-free extracts.

Results

Subcloning of the AAC6′-gene from pMS18 onto pIJ702 and pR2

pMS18, a plasmid containing a 1.9 kb fragment encoding an aminoglycoside 6′-acetyltransferase derived from *Streptomycetes kanamyceticus* strain M1164[20] was digested with restriction endonuclease *Bcl* I and ligated with pIJ702 previously cut with *Bgl* II or pR2 cut with *Bcl*-I; ligation mixtures were transformed into *S. lividans* protoplasts. Transformants were selected as colonies able to grow in the presence of thiostrepton (50 μg/ml) and kanamycin (50 μg/ml). The presence of the 1.9 kb fragment coding for the AAC6′ was detected by agarose gel electrophoresis after restriction with *Bcl* I (not shown) and *in vivo* expression was demonstrated by growth of the transformants on media containing a variety of aminoglycoside antibiotics (Table 1). As shown in this table, transformants containing the high copy number construction (pIJ702:: AAC6′) are more resistant against aminoglycosides than transformants containing the low copy number construction (pR2:: AAC6′).

Transformation of the pIJ702 Hybrid Construction into Production Strains and Effects on the Resistance Patterns

The chimeric plasmid pIJ702:: AAC6′ isolated from *S. lividans* was used to transform *S. kanamyceticus* and *S. fradiae* protoplats. Transformants were isolated as colonies able to grow in the

Table 1. Effects of a cloned AAC6′ on the resistance levels of *S. lividans* 66 against different aminoglycoside antibiotics (μg/ml).

Plasmid	KMA*	AMI	RMS	BUT	NEO	PAR+	LVDM+
None	2	<1	10	<5	<1	10	20
pIJ702	2	<1	10	<5	<1	10	20
pR2	2	<1	10	<5	<1	10	20
pIJ702:: AAC6′	360	>200	>400	>200	10	10	20
pR2:: AAC6′	230	80	>400	>200	5	10	20

+ 6′ substitution R-OH: No modification site for AAC6′.

* Abbreviations: KMA; Kanamycin A, AMI; amikacin, RMS; ribostamycin, BUT; butirosin, NEO; neomycin, PAR; paromomycin, LVDM; lividomycin, AAC6′; aminoglycoside 6′-*N*-acetyltransferase. pIJ 702:: AAC6′ and pR2:: AAC6′; Chimeric plasmids containing the cloned AAC6′ gene.

Table 2. Effects of a cloned AAC6′ acetyltransferase on the resistance levels of *S. kanamyceticus* ATCC 12853 against different aminoglycoside antibiotics (μg/ml).

Plasmid	KMA[a]	AMI	RMS	BUT	PAR[+]	LVDM[+]
None	140	25	>200	100	10	25
pIJ702:: AAC6′	750	260	>200	100	10	25
*	550	180	>200	100	10	25

* This single transformant shows an intermediate resistance.

[+] 6′ substitution R-OH: No modification site for AAC6′.

[a] See the footnotes in Table 1.

Table 3. Effects of a cloned AAC6′ acetyltransferase on the resistance levels of *S. fradiae* ATCC 10745 against different aminoglycoside antibiotics (μg/ml).

Plasmid	KMA[a]	AMI	RMS	BUT	NEO	PAR	LVDM
None	90	30	>500	30	180	>100	>40
pIJ702	90	30	>500	30	180	>100	>40
pIJ702:: AAC6′	>200	>100	>500	>160	750	>100	>40

[a] See the footnotes in Table 1.

Fig. 1. Kanamycin production by *S. kanamyceticus* ATCC 12853.

● Wild type strain, △ single transformant containing pIJ702:: AAC6′ and showing an intermediate kanamycin resistance, ▲ kanamycin high resistant transformants containing pIJ702:: AAC6′.

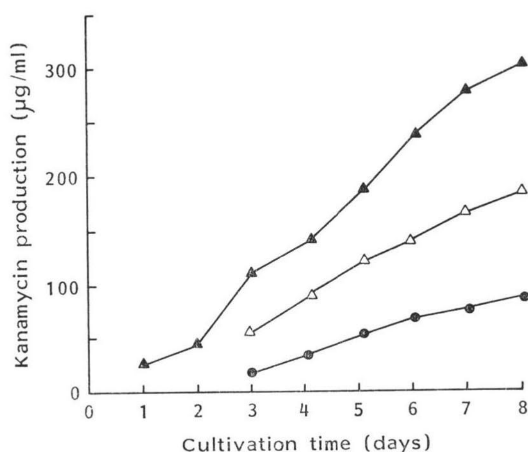
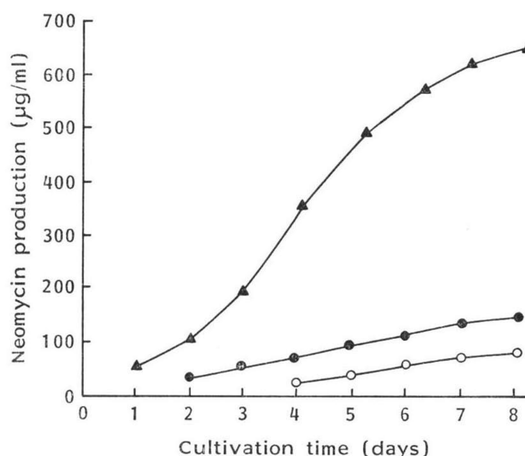


Fig. 2. Neomycin production by *S. fradiae* ATCC 10745.

● Wild type strain, ○ wild type strain containing pIJ702, ▲ transformants containing pIJ702:: AAC6′.



presence of thiostrepton (50 μg/ml) and amikacin (50 μg/ml); the presence of the intact plasmid was confirmed by agarose gel electrophoresis and restriction analysis. The resistance pattern of transformants against aminoglycoside antibiotics was determined. As shown in Tables 2 and 3 the cloned AAC6′ markedly increased the levels of resistance against aminoglycoside antibiotics in both *S. kanamyceticus* and *S. fradiae*.

Influence of Increased Copies of AAC6′ on Antibiotic Production

The wild type strains of *S. kanamyceticus* and *S. fradiae* as well as transformants containing the cloned AAC6′ were grown in liquid media and the antibiotic productivity determined. Results are

summarized in Figs. 1 and 2. To exclude the possibility that high producers were induced by protoplast formation and regeneration, plasmids isolated from *S. kanamyceticus* and *S. fradiae* were retransformed into protoplasts of the respective wild type strain. Five retransformants per strain were tested for production level and resistance pattern. All exhibit production and resistance levels comparable to that of the original transformants (not shown). Note that one transformant of *S. kanamyceticus* showing an intermediate resistance level between wild type and "high" producing trans-

Fig. 3. Acetyltransferase activity in *S. fradiae* ATCC 10745.
■ Protein content, ● acetyltransferase activity.
(a) Wild type strain.
(b) Transformants containing pIJ702:: AAC6′.

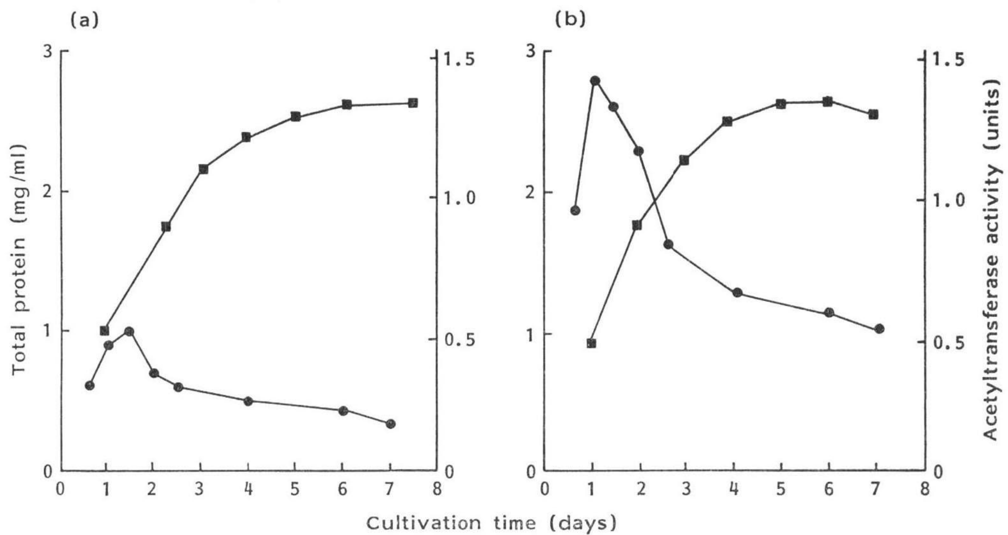
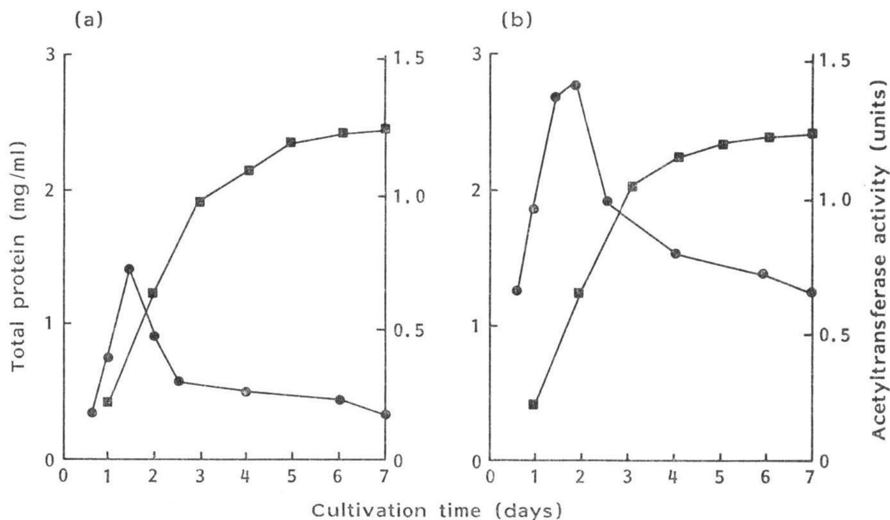


Fig. 4. Acetyltransferase activity in *S. kanamyceticus* ATCC 12853.
■ Protein content, ● acetyltransferase activity.
(a) Wild type strain.
(b) Transformants containing pIJ702:: AAC6′.



formants (Table 2) also shows an intermediate level of kanamycin production (Fig. 1). No differences on the restriction pattern of the plasmids could be detected (not shown).

Acetyltransferase Activities

Acetyltransferase activity during the fermentation was determined for cell-free extracts of wild type strains as well as for transformants. Results reported in Figs. 3a (*S. fradiae*) and 4a (*S. kanamyceticus*) represent the mean of two fermentations runs per strain. Figs. 3b and 4b represents results obtained fermenting three single transformants of *S. fradiae* and *S. kanamyceticus* respectively. It can be seen that acetyltransferase activity, in presence or absence of plasmid, increased rapidly during the early logarithmic phase of growth and then decreased somewhat during later growth phases.

Discussion

Cloned modifying enzymes introduced into a "clean" genetic background confer resistance to the host organism. Many aminoglycoside modifying enzymes have been cloned and expressed in *Streptomyces lividans*. For example both aminoglycoside modifying enzymes present in the neomycin producing streptomycete *S. fradiae*, an APH3′ and a 3-*N*-acetyltransferase (AAC3)[25]. Not surprising was the fact that, in a "shot gun" cloning experiment, some clones contained APH3′ modifying activity, others AAC3 activity. Both genes confer low level of resistance to neomycin to their host[25]; despite of the fact that *S. lividans* strains containing the cloned APH3′ gene produced more than 10% of total protein in form of active aminoglycoside 3′-phosphotransferase[32]. More surprising was the fact that *S. lividans* strains containing both modifying enzymes had aminoglycoside resistance levels comparable to those of the original strain[25]. This indicates that cloning of antibiotic resistance genes into a sensitive genetic background is a convenient way to "purify" discrete genes but not necessarily the way to understand the function of such genes in producing strains.

We have shown that a cloned aminoglycoside modifying enzyme can be used to increase both aminoglycoside resistance and antibiotic yields in the producing strains *S. fradiae* ATCC 10745 and *S. kanamyceticus* ATCC 12853. We cannot completely exclude the possibility that other genes involved in antibiotic biosynthesis could be encoded by the 1.9 kb DNA fragment used and consequently be directly involved in yield improvement. Moreover these results do not answer basic questions about roles of aminoglycoside modifying enzymes in aminoglycoside production. The case of *S. kanamyceticus* is somewhat complex since it produces an aminoglycoside acetyltransferase (AAC6′) during the early growth phase which then decreases rapidly during the logarithmic growth phase of the organism[12] (present paper). This enzyme completely inactivates kanamycin and partially neomycin *in vitro*. During the kanamycin production phase occurring at the end of the growth phase[12] resistance to kanamycin has been shown to be due to an acquired ribosomal resistance as demonstrated by cloning the ribosomal resistance gene into *S. lividans*[33]. The acetyltransferase thus seems not to be responsible for resistance during the kanamycin production phase. Obviously, investigations of cloned genes in different genetic backgrounds do not give conclusive answers about the function of modifying enzymes in producing strains. It seems that, in certain cases, aminoglycoside modifying enzymes are not necessary for resistance or for antibiotic production. There are several examples of aminoglycoside producing strains having resistant ribosomes which do not produce any antibiotic modifying enzymes[34,35]. However from a more practical (industrial) point of view the question concerns primarily the manipulation of genes as a means of increasing the production of antibiotics. As suggested by THOMPSON and DAVIES[36] one of the limiting steps in antibiotic production could be the level of resistance against the antibiotic produced. The isolation of higher producing strains by obtaining mutants with increased resistance levels has been used successfully in the past[13,14]. The development of cloning systems for the genus *Streptomyces* provides rational possibilities to test discrete (cloned) genes for effects on antibiotic production. This paper has investigated the influence of a gene encoding a simple antibiotic modification on antibiotic resistance and antibiotic productivity. An

AAC6′ acetyltransferase cloned on a high copy number plasmid was introduced into two producing organisms: *S. kanamyceticus* ATCC 12853 and *S. fradiae* ATCC 10745. As shown in Tables 2 and 3 transformants containing the chimeric plasmid are markedly more resistant towards aminoglycoside antibiotics than the wild type strains. With respect to antibiotic production, the transformants show an increased level compared to the isogenic parents. We conclude that increased resistance towards an antibiotic can result in a net increase of antibiotic yields. The complete interpretation of this observation must await additional studies since we do not know if the organisms are high producers because they can tolerate more antibiotic or if the result is due to the introduction of additional levels of a limiting enzyme of the biosynthetic pathway. The transformants show higher modifying enzyme levels compared to wild type strains and presumably make more *N*-acetylated aminoglycoside; another limiting step in production could be the reactivation of inactivated antibiotic.

There are several other implications of this study that warrant further investigation. What if the gene for the modifying enzyme is cloned into a producing organism that does not already possess such an enzyme? Would one produce a modified antibiotic? What if the resistance mechanism occurring as a result of a target site modification (ribosome) was introduced into a producing organism, or if this type of resistance was placed under constitutive rather than inducible (developmental) control? The choice of appropriate resistance mechanism is going to be crucial when hybrid antibiotics with broadened spectrum are to be produced by genetic engineering. In this respect, the use of a resistance mechanism found in clinical isolates might be more appropriate than the "natural" resistance mechanisms of producing organisms. We may yet use undesirable resistant organisms to our advantage!

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