MODIFICATION OF AMINOGLYCOSIDE ANTIBIOTICS BY CLINICAL ISOLATES OF *STREPTOCOCCUS FAECALIS*

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Clinical isolates of *Streptococcus faecalis* were found to contain streptomycin (SM)-phosphotransferase, kanamycin (KM)-acetyltransferase and KM-adenylyltransferase. The existence of a new type of KM-phosphotransferase was suggested from aminoglycoside substrate profiles.

It is known that antibiotic resistance in *Streptococcus faecalis* can be governed by resistance plasmids[1,2]. There are many strains which show high levels of resistance to SM and KM. Recently, the occurrence of strains resistant to gentamicin was reported[3]. The biochemical mechanisms of resistance to aminoglycoside antibiotics in *Streptococcus faecalis* have been reported by COURVALIN and his coworkers[2,4]. We recently reported the sensitivity of 51 clinical isolates of *Streptococcus faecalis* to various antibiotics and the transferability and stability of the resistance determinants[5]. The present paper deals with aminoglycoside modification mechanisms on the basis of aminoglycoside substrate profiles in crude extracts from multiantibiotic resistant strains of *Streptococcus faecalis*. The role of aminoglycoside-modifying enzymes in the resistant strains is also described.

Materials and Methods

Bacterial strains

The bacteria used in this study and minimum inhibitory concentrations (MICs) of antibiotics are listed in Table 1; strains were isolated from clinical specimens. Strain KSf24m was used as a comparative sensitive strain. *Bacillus subtilis* ATCC 6633 was used as a test organism for the bioassay of aminoglycoside antibiotics.

Media

Brain heart infusion (BHI, Difco) broth and BHI agar were used for bacterial growth.

MIC determinations

An overnight broth culture was spotted on BHI agar plates containing serial two fold dilutions of each antibiotic. After culturing overnight at 37°C, the MIC was determined.

Preparation of crude extract

The crude extract was prepared as follows: 500 ml of an exponential culture was harvested by centrifugation, and washed with TMK solution (0.1 M Tris-hydrochloride buffer, pH 7.8, 0.06 M KCl, 0.01 M $MgCl_2$, and 6 mM 2-mercaptoethanol). The washed cells were resuspended in 3 ml of TMK solution and the suspended cells were treated with 500 μg/ml of lysozyme (Sigma) for 60 minutes at 37°C and disintegrated by sonic oscillation (Ohtake Sonicator, Tokyo, 100 w) for 5 minutes. The supernatant fluid obtained was centrifugated at 105,000$\times g$ for 2 hours and the supernatant thus obtained was used as a crude extract[6,7].

To study the inactivation of aminoglycoside antibiotics, the crude extract was prepared by using modified TMK solution which consisted of 0.1 M Tris-hydrochloride buffer, pH 7.8, 0.06 M KCl, 0.01 M magnesium acetate and 6 mM 2-mercaptoethanol.

Inactivation of aminoglycoside antibiotic by crude extract

Crude extract (0.3 ml), 0.2 ml of 40 mM ATP, 0.1 ml of 2 mM CoA, 0.1 ml of 1 mM antibiotic, were mixed and incubated at 37°C for 24 hours. After heating at 100°C for 3 minutes, the residual antibiotic activity in the reaction mixture was determined by using *B. subtilis* ATCC 6633 as a test organism[5].

Incorporation of isotope from labelled adenosine 5′-triphosphate (ATP) into aminoglycoside antibiotic

The reaction mixture consisted of 40 μl of the crude extract, 5 μl of labelled ATP (ATP-γ-^{32}P or ATP-8-^{14}C, 1 μCi), and 5 μl of 1 mM antibiotic. After reaction at 37°C for 24 hours, 10 μl of the mixture was absorbed onto phosphocellulose paper (Whatman P81) (0.75 cm^2), rinsed with 20 ml of hot water 5 times, and dried, followed by counting radioactivity in a Packard Tri-carb Liquid Scintillation Spectrometer (Model 3330). Toluene based fluid was prepared by dissolving 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene in 1 liter of toluene. The non-specific binding of labelled ATP was determined by the reaction in the absence of enzyme or antibiotic[7].

Incorporation of ^{14}C-labelled acetate into aminoglycoside antibiotic

The reaction mixture contained 30 μl of crude extract, 5 μl ^{14}C-labelled acetate (1 μCi), 5 μl of 2 mM CoA, 5 μl of 1 mM antibiotic and 5 μl of 40 mM ATP. The reaction mixture was incubated at 37°C for 24 hours, and 10 μl was pipetted onto a 0.75 cm^2 square of phosphocellulose paper. Radioactivity bound to the phosphocellulose paper was counted according to the method described above[6].

Antibiotics and chemicals

The following antibiotics were commercial products: Tetracycline (TC), erythromycin (EM), chloramphenicol (CM), and neomycin B (NMB) were purchased from Sigma Chemicals Co., streptomycin (SM), kanamycin (KM), and kanamycin B (KMB) from Meiji Seika Kaisha, Ltd., gentamicin complex (GM) and tobramycin (TOB) from Shionogi & Co., Ltd., respectively. Amikacin (AMK) and spectinomycin (SPC) were kindly supplied by Sankyo Co., Ltd., and Daiichi Seiyaku Co., Ltd., respectively. GMC_1 and GMC_{1a} were gifts from Yamanouchi Pharmaceutical Co., Ltd.

ATP-γ-^{32}P (specific activity, 7.1 Ci/mmole), ATP-8-^{14}C (specific activity, 56.0 mCi/mmole) and ^{14}C-sodium acetate (specific activity, 2.4 mCi/mmole) were all commercial products of New England Nuclear.

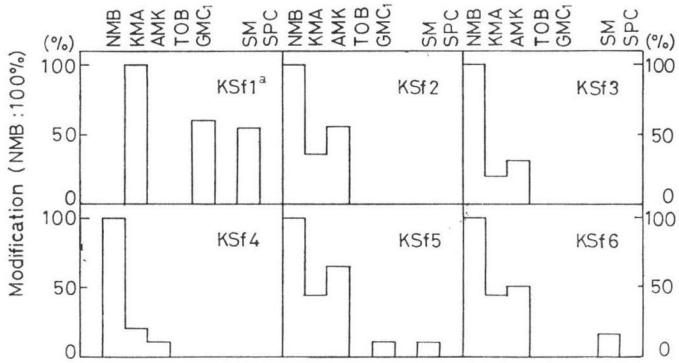
Results

Phosphorylation of Aminoglycosides

As shown in Fig. 1, crude extracts from strains KSf2, KSf3, KSf4, KSf5 and KSf6 were found to catalyze the phosphorylation of NMB, KMA, and AMK, whereas TOB which is a 3′-deoxy-aminoglycoside was not modified. This finding indicated that the enzyme was aminoglycoside 3′-phosphotransferase APH(3′). The aminoglycoside substrate profiles of crude extracts from strains KSf2, KSf3, KSf5 and KSf6 were very similar to those reported for APH(3′)-III[8,9]. On the other hand, AMK was a poor substrate for the phosphorylating enzyme extracted from strain KSf4: this has been reported in *Streptococcus faecalis* strains[2].

The crude extract from strain KSf1 gave a different aminoglycoside substrate profile compared to other strains described above. The fact that phosphorylation of both KMA and GMC_1 took place but not that of NMB suggested that the crude extract from strain KSf1 phosphorylates the 2″-hydroxyl group. However, TOB, a 4,6-diglycosylated 2-deoxystreptamine aminoglycoside, was not a substrate for this enzyme. On the assumption that there are two different enzymes catalyzing the phosphorylation of KMA and GMC_1 the aminoglycoside modification mechanism was suggested as follows. Phosphorylation of GMC_1 is by a typical APH(2″) with aminoglycoside 6′-acetyltransferase AAC(6′)

Fig. 1. Substrate profiles of phosphotransferase extracted from strains KSf1 through KSf6.
Phosphorylation is expressed relative to NMB as 100%.
a: Phosphorylation of KSf1 is expressed relative to KMA as 100%, since NMB is not phosphorylated.



activity as described later. The second enzyme did not phosphorylate NMB, and showed a more restricted substrate profile as shown in Fig. 1. Accordingly, this enzyme might be a new type of APH(3′) which is different from APH(3′)-I, APH(3′)-II and APH(3′)-III.

Strain KSf1 was found to contain an enzyme that catalyzed the phosphorylation of SM but not SPC. This is the first report of the presence of SM-phosphotransferase in *Streptococcus faecalis*. The site of phosphorylation is probably either the 3-hydroxyl group of the glucosamine moiety or the 6-hydroxyl group of the streptidine moiety.

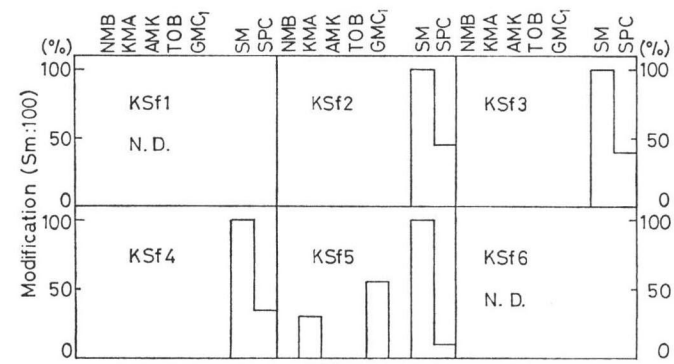
Phosphorylation of GMC_1 was also detected in crude extracts of strain KSf5, and SM-phosphotransferase was found in strains KSf5 and KSf6.

Adenylylation of Aminoglycosides

As shown in Fig. 2, both SM and SPC were adenylylated by the crude extracts from four strains (KSf2, KSf3, KSf4 and KSf5), though the SM-adenylylating activity was less effective against SPC as a substrate. The same type of activity in which SM is an excellent substrate for the enzyme but SPC is not has been reported for *Staphylococcus aureus*[8] and in *Streptococcus faecalis*[2]. It is probable that modification may occur on the 3″-hydroxyl group of SM; the enzyme is AAD(3″)[9].

In addition, strain KSf5 was found to contain an aminoglycoside adenylyltransferase that was

Fig. 2. Substrate profiles of adenylyltransferase extracted from strains KSf1 through KSf6.
Adenylylation is expressed relative to SM as 100%. N.D.: not detected.



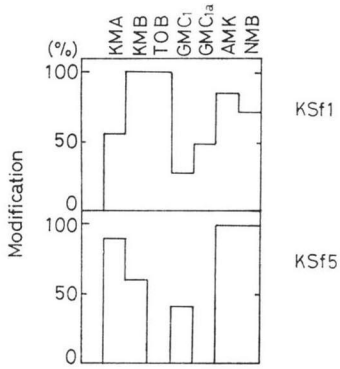
effective against GMC_1 and KMA, which probably modified the 2″-hydroxyl group since NMB was not adenylylated. Previously, no adenylylation of deoxystreptamine antibiotics has been reported in *Streptococcus faecalis*.

Acetylation of Aminoglycosides

As shown in Fig. 3, the presence of acetylating enzyme was indicated in strains KSf1 and KSf5. Presently, three types of enzyme [AAC(6′), AAC(2′) and AAC(3)] are known to be capable of acetylating 2-deoxystreptamine aminoglycosides. The fact that KMA was acetylated indicates that the enzyme present is not AAC(2′). LE GOFFIC[10] and COURVALIN *et al.*[4] reported that the deoxystreptamine aminoglycoside 6′-acetyltransferase, AAC(6′), was also capable of phosphorylating the 2″-hydroxyl group of the antibiotics. The speculation was supported by UMEZAWA[11]. As described earier, strain KSf1 possessed two modifying activities: an acetylating activity that modified GMC_{1a}, TOB, KMA, AMK and NMB, and a phosphorylating activity that modified KMA and GMC_1. Accordingly, the acetylating enzyme produced by this strain was considered to be the *bifunctional* AAC (6′)-APH(2″).

Fig. 3. Substrate profiles of acetyltransferase extracted from strains KSf1 and KSf5.

Acetylation is expressed relative to KMB as 100% in the case of KSf1 and to NMB as 100% in the case of KSf5.



The fact that KMA, AMK and NMB, but not TOB and GMC_{1a}, were acetylated indicates the possibility that strain KSf5 contained a different form of aminoglycoside 6′-acetyltransferase AAC(6′).

The Role of Aminoglycoside-modifying Enzyme in High Resistance to Aminoglycosides

As indicated in Table 1, the multiantibiotic-resistant streptococcal strains possessed high-level resistance to SM (MIC≧3,200 μg/ml). The strains KSf2, KSf3, KSf4 and KSf5 produced a SM-inactivating enzyme, SM-adenylyltransferase AAD(3″). These facts indicate that the mechanism of resistance to SM was due to the production of the SM-adenylyltransferase. Inactivation of SM by SM-phosphotransferase from strains KSf1 and KSf6 was not observed, and we suggest that in these strains the resistance to SM might be due a mechanism other than a drug inactivating enzyme.

KM-3′-phosphotransferase, APH(3′), was detected in all crude extracts from six KM-resistant strains as shown in Fig. 1; the phosphorylated KM had no antibiotic activity. The probable KM resistance mechanism in these strains involves inactivation by APH(3′), although strains KSf1 and KSf5 contain other modifying enzyme.

The crude extracts from strains KSf2, KSf3, KSf4, KSf5 and KSf6 possessed NMB-inactivating activities and also aminoglycoside 3′-phosphotransferase APH(3′) activity. These strains were also resistant to NMB. In contrast, crude extracts from strain KSf1 did not inactivate the drug and no aminoglycoside 3′-phosphotransferase activity was detected. The strain KSf1 produced aminoglycoside 6′-acetyltransferase AAC(6′), but the MIC value of the strain was low (100 μg/ml): almost the same as strain KSf24m. These facts indicate that the mechanism of high-level resistance to NMB

Table 1. Minimum inhibitory concentrations of various antibiotics and inactivation of aminoglycoside antibiotics.

Strain	MIC of antibiotics (μg/ml) (Inactivation)								
	TC	EM	CM	SM	KM	GM[a]	TOB	NMB	AMK
KSf1	200	≧3,200	50	≧3,200 (−)	≧3,200 (+)	≧3,200 (+)	1,600 (−)	100 (−)	200 (−)
KSf2	100	≧3,200	50	≧3,200 (+)	≧3,200 (+)	3.13 (−)	25 (−)	≧3,200 (+)	100 (+)
KSf3	100	≧3,200	50	≧3,200 (+)	≧3,200 (+)	6.25 (−)	100 (−)	≧3,200 (+)	200 (+)
KSf4	100	≧3,200	50	≧3,200 (+)	≧3,200 (+)	6.25 (−)	50 (−)	≧3,200 (+)	200 (+)
KSf5	100	≧3,200	50	≧3,200 (+)	≧3,200 (+)	3.13 (−)	50 (−)	≧3,200 (+)	200 (+)
KSf6	200	≧3,200	100	≧3,200 (−)	≧3,200 (+)	6.25 (−)	50 (−)	≧3,200 (+)	200 (+)
KSf24m	1.56	0.2	6.25	100 (−)	50 (−)	6.25 (−)	25 (−)	25 (−)	100 (−)

a: GMC_1 and GMC_{1a} were used as substrate for the drug inactivation.
(+): inactivated, (−): not inactivated.

was due to the production of the aminoglycoside 3′-phosphotransferase.

Only strain KSf1 showed high-level resistance to GM (MIC≧3,200 μg/ml) (Table 1). Crude extract from the GM resistant strain inactivated GMC_1 and GMC_{1a}. This suggests that the aminoglycoside-modifying enzyme(s) from the strain was associated with the mechanism of resistance to GM. Strain KSf1 was found to possess the activity that catalyzed the phosphorylation and acetylation of GMC_{1a}. However, the inactivated GMC_1 and GMC_{1a} were not reactivated by treatment with alkaline phosphatase (type III, *E. coli*: Sigma) (data not shown). On the other hand, the GMC_1- and KM-adenylylating enzymes found in strain KSf5 do not confer detectable resistance to GM since the strain is susceptible to the drug. Inactivation of GMC_1 and GMC_{1a} was not observed in this strain. Aminoglycoside-phosphotransferase, -adenylyltransferase and -acetyltransferase activity detected in strain KSf5 were not effective against GMC_{1a} as a substrate. These facts indicate that the mechanism of resistance to GMC_{1a} was due to the production of GM-acetyltransferase AAC(6′).

TOB was an excellent substrate for the 6′-acetyltransferase produced by strain KSf1 and the strain was highly resistant to the drug. However, crude extracts from the strain could not inactivate the drug. On the other hand, the crude extract from TOB-susceptible strains contained neither aminoglycoside-modifying activity nor -inactivating activity against the drug. It is known that the 6′-acetyltransferase determines resistance to TOB and also inactivates the drug[12]. Accordingly, the biochemical mechanism of TOB resistance in the strain KSf1 remains unclear.

Discussion

COURVALIN *et al.* have studied resistance to aminoglycoside antibiotics in *Streptococcus faecalis* and detected aminoglycoside phosphotransferase and SM-adenylyltransferase in three strains that received plasmids from different clinical origins[2]. The six highly resistant strains studied in our laboratory, showed the MICs of more than 3,200 μg/ml to KM and SM and had transferable plasmids conferring KM and SM-resistance[5]. These six highly resistant strains were divided into 2 classes

based on resistance patterns to aminoglycoside antibiotics other than KM and SM (Table 1). The first class is represented by strain KSf1 which is resistant to GM and TOB but not to NMB. The second class had different resistance patterns from KSfl: they are resistant to NMB but not to GM and TOB. The mechanism of GM resistance in KSf1 was mainly due to AAC (6′)-APH (2″). The five strains in the second class appeared to be divided into three subclasses based on the modifying enzymes carried by each strain (Table 2). Strains KSf2, KSf3 and KSf4 produced aminoglycoside-modifying enzymes APH (3′) and AAD (3″): these enzymes determined resistance to NMB and to SM by inactivation of the respective antibiotics. The three strains presented here were very similar to the strains (JH2-14, JH2-15, JH2-12) which had been reported by COURVALIN *et al.*[2] In strain KSf5, APH (3′) and AAD (3″) contributed to the mechanism of the high NMB- and SM-resistance, respectively, as in the three strains described above. However, KSf5 was found to produce several other types of aminoglycoside-modifying enzymes: they are not responsible for resistance to GM and TOB, although the possibility that these enzymes governed the mechanisms of resistance to antibiotics other than the aminoglycosides tested can not be eliminated. Strain KSf6 was highly resistant to SM and had a SM-phosphorylating enzyme as in strain KSf1. Therefore, it was considered that plasmids in KSf1 and KSf6 did not affect the production of inactivating enzymes. The mechanism of resistance to NMB in KSf6 was considered to be due to APH (3′) as described for strains KSf2, KSf3, KSf4 and KSf5. AMK was a substrate for the APH (3′) enzyme from the strains KSf2 through KSf6 and the drug was also an excellent substrate for the acetyltransferase from strain KSf1 or KSf5. However, the MIC values of AMK to the strains including the plasmid-free strain KSf24m were nearly the same. These phenomena have been reported for *Staphylococcus aureus*[8].

Table 2. Aminoglycoside modifying enzyme detected[a].

Strain	2-Deoxystreptamine aminoglycoside modifying enzyme	Streptomycin/ spectinomycin modifying enzyme
KSf1	APH (3′)[b] APH (2″) AAC (6′)	APH (6 or 3″)
KSf2 KSf3 KSf4	APH (3′)	ADD (3″)
KSf5	APH (3′) APH (2″) AAC (6′)[c] AAD (2″)	ADD (3″) APH (6 or 3″)
KSf6	APH (3′)	APH (6 or 3″)

a: Number in parenthesis in the Table suggests position of the modification. b: This enzyme was different to APH (3′) in its substrate profile. c: This enzyme was different from the APH (2″) and AAC (6′) of strain KSf1 since GMC_1 and TOB were ineffective as substrates.

In *Staphylococcus aureus* and *Streptococcus faecalis,* one single enzyme appears to have both APH (2″) and AAC (6′) activities[4,10]. The same situation exists for the enzymes of strains KSf1 and KSf5 in this paper. Resistance to aminoglycoside antibiotics in *Streptococcus faecalis* appears to be analogous to drug resistance in *Staphylococcus aureus*, primarily governed by the presence of plasmids.

COURVALIN *et al.*[2,4] reported that in *Streptococcus faecalis* SM resistance was due to AAD (3″) and KM resistance was due to APH (3′) and AAC (6′)-APH (2″). We have shown the presence of multiple modifying enzymes in a single strain, some of which have not been reported in *Streptococcus faecalis*.

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