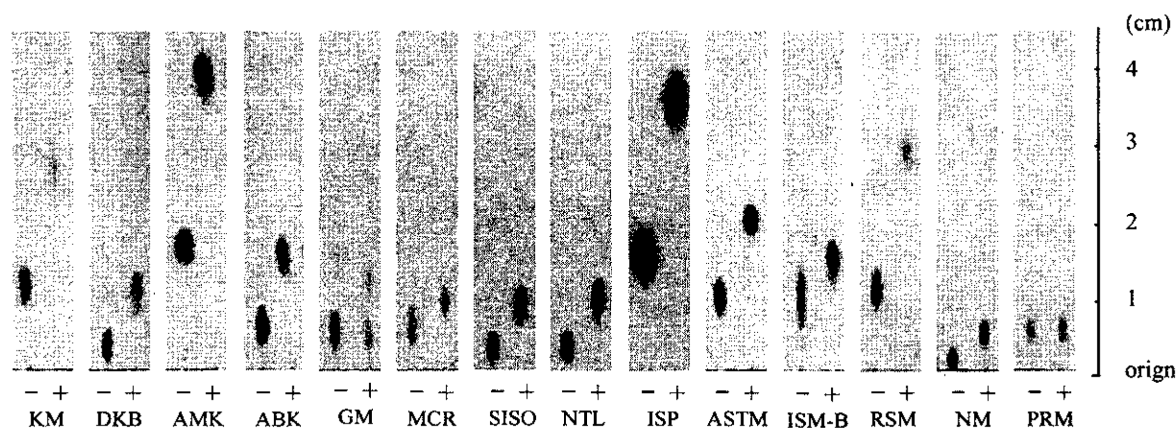
Table 1. Resistance to and acetylation of aminoglycoside antibiotics due to the cloned gene.

Group	Antibiotics	Resistance [a] (μg/ml)			TK21/pANT-S2	
		#8	TK21	TK21/ pANT-S2	Acetylation [b]	Activity [c] (%)
Kanamycin	KM	100	<2.5	50	++	<1
	DKB [d]	100	<2.5	50	++	<1
	AMK [d]	25	<2.5	10	++	<1
	ABK [d]	25	<2.5	5	++	30
Gentamicin	GM	≦2.5	<2.5	<2.5	+	nt
	SISO	25	<2.5	<50	++	<1
	MCR	25	<2.5	10	++	<1
	ISP [d]	100	<2.5	25	++	<1
	NTL [d]	10	<2.5	50	++	<1
Astromicin	ASTM	50	<2.5	50	++	<1
	ISM- B	100	<2.5	100	++	<1
Neomycin-Paromomycin	RSM	200	<2.5	50	++	<1
	NM	25	<2.5	≦2.5	++	50
	PRM	<2.5	<2.5	<2.5	−	100

a: Strains were streaked on ISP No. 2 agar plates containing AGs (2.5-200 μg/ml).
b: Complete (++), incomplete(+) and no(-) acetylations were observed upon TLC.
c: Antibiotic activity of the incubated reaction mixtures with acetylCoA relative to that of the mixtures without acetylCoA. Paper disk assay using ISP No. 2 agar plate seeded with *B. subtilis* ATCC6633 was carryed out.
d: Semisynthetic AGs.
Abbreviation: SISO: sisomicin, MCR: micromomicin, ISM-B: istamycin-B.

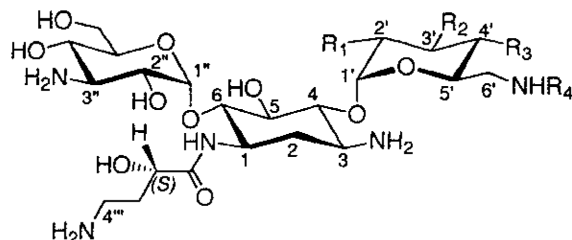
Fig. 2. TLC of the reaction mixtures of aminoglycosides incubated with the cell free extract from *S. lividans* TK21/pANT-S2 in the presence (+) or absence (−) of acetylCoA.



Determination of Acetylation

Structures of the acetylated products of ABK, AMK and ASTM were determined by spectral analyses of FAB-MS (Jeol JMX-SX102) and ^{1}H and ^{13}C NMR in D_2O at pD 1.7~1.9 (Jeol JNX-EX400). These products turned out to be identical with the known compounds; 6′-*N*-acetylABK (Fig. 3)[12], 6′-*N*-acetylAMK (Fig. 3)[22] and 6′-*N*-

Fig. 3. Structures of the enzymatically acetylated derivatives of ABK and AMK.



Arbekacin (ABK)	$R_1 = NH_2$	$R_2 = H$	$R_3 = H$	$R_4 = H$
6'-*N*-AcetylABK	$R_1 = NH_2$	$R_2 = H$	$R_3 = H$	$R_4 = Ac$
Amikacin (AMK)	$R_1 = OH$	$R_2 = OH$	$R_3 = OH$	$R_4 = H$
6'-*N*-AcetylAMK	$R_1 = OH$	$R_2 = OH$	$R_3 = OH$	$R_4 = Ac$

acetylASTM[23], respectively.

6'-*N*-acetylABK: FAB-MS (positive) *m/z* 595 $(M+H)^+$; The 6'-H signals (δ 3.32 and 3.43) shifted to lower field than those of ABK, and β-carbon shift of C-5' (δ 69.6) was also observed.

6'-*N*-acetylAMK: FAB-MS (positive) *m/z* 628 $(M+H)^+$; The 6'-H signals (δ 3.51 and 3.59) and C-5' (δ 72.0) signal shifted due to the 6'-*N*-acetylation.

6'-*N*-acetylASTM: FAB-MS (positive) *m/z* 448 $(M+H)^+$; the 6'-H (δ 3.99) and the β-carbon shifts (C-5' at δ 71.9 and 6'-CCH_3 at δ 16.1) were observed.

6'-*N*-acetylABK showed 8% activity of ABK, whereas neither 6'-*N*-acetylAMK nor 6'-*N*-acetylASTM showed significant antibiotic activities (0.3% and 0.2% activities of AMK and ASTM, respectively).

Discussion

The acetyltransferase encoded by the cloned gene turned out to be AAC(6') responsible for the 6'-*N*-acetylation of AGs as well as the multiple resistance to AGs with 6'-NH_2 except for ABK and NM. Therefore, it was concluded that the AAC(6') substantially contributed to the multiple AG resistance of the strain #8. However, since the strain #8 is resistant to both ABK and NM, the strain #8 should possess some additional resistance determinant(s) distinct from AAC(6'). Actually, we demonstrated that the strain #8 contained AAC(1) and perhaps other AACs in addition to AAC(6')[21].

It was noted that the AAC(6') of strain #8 was capable of acetylating all of the examined AGs with 6'-NH_2 including semisynthetic ones such as ABK and ISP that have been known to be rather refractory to the action of AAC(6')s of clinical origin. Since ASTM was also acetylated at 6'-NH_2, the AAC(6') of strain #8 was regarded to have similarity in substrate specificity to AAC(6')-Ie[5]. In fact, the deduced amino acid sequence of the former showed similarity to that of the latter (unpublished). Furthermore, it was remarked that acetylation by AAC(6') of ABK and NM did not result in inactivation, whereas that of the other AGs resulted in inactivation. The antibiotic activity of 6'-*N*-acetylNM[24] as well as the NM sensitivity of *E. faecalis* with AAC(6')-Ii[25] have been known, but the weak antibiotic activity of 6'-*N*-acetylABK has never been reported. In this context, we reported that the acetylation derivatives by AAC(3) and AAC(2') of ABK retain substantial antibiotic activity[13,14]. By contrast, NM was inactivated by AAC(3) although data was not shown. Thus, ABK is distinct from the other AGs in terms of the antibiotic activity of acetylation products and therefore we may call ABK the double stage active antibiotic.

ABK is an anti-MRSA agent with a broad antimicrobial spectrum. Although it has been widely used since its approval in 1990, the emergence of ABK-resistant MRSA has remained low and all of the emerged ABK-resistant MRSA strains have so far been dependent on AAC(6')/APH(2")[1,26,27]. Based on our findings, AAC-dependent ABK-resistant MRSA may hardly emerge although AAC(6') as well as AAC(6')/APH(2") have been increasing their importance in clinical AG resistance[8~11], provided that ABK-resistant MRSA with two different AACs may emerge.

Acknowledgements

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REVIEW

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Semisynthetic aminoglycoside antibiotics: Development and enzymatic modifications

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Abstract
The critical resistance mechanisms of aminoglycoside antibiotics in bacteria of clinical importance are the enzymatic *N*-acetylation, *O*-phosphorylation, and *O*-nucleotidylation that generally result in the inactivation of aminoglycosides. To overcome such resistance mechanisms, dibekacin (3′,4′-dideoxykanamycin B) was developed as the first rationally designed semisynthetic aminoglycoside, based on the enzymatic 3′-*O*-phosphorylation of kanamycin. Subsequently, amikacin, netilmicin, and isepamicin were developed by introducing (*S*)-4-amino-2-hydroxybutyryl (AHB), ethyl, and (*S*)-3-amino-2-hydroxypropionyl side chains into the 1-amino group of kanamycin, sisomicin, and gentamicin B, respectively. These side chains are believed to block the access of a variety of aminoglycoside-modifying enzymes to their target sites. The latest semisynthetic aminoglycoside of clinical use in Japan is arbekacin (1-*N*-AHB-dibekacin), which has been extensively used since its approval as an anti-methicillin-resistant *Staphylococcus aureus* (MRSA) agent in 1990. Although it has several possible modification sites for aminoglycoside acetyltransferases (AACs), arbekacin-resistant MRSA strains that have emerged in the past 8 years have been those with a low or moderate level of resistance, due to a bifunctional enzyme, AAC(6′)/APH(2″), at low incidence. To overcome AAC(6′)/APH(2″)-dependent arbekacin-resistant MRSA strains, 2″-amino-2″-deoxyarbekacin and its 5-epiamino derivative have been already synthesized. However, simulative modification studies using AACs from aminoglycoside-producing *Streptomyces* strains have revealed that AAC(3) and AAC(2′) converted arbekacin to 3″-*N*-acetyl and 2′-*N*-acetyl derivatives, respectively, which retain high antibiotic activity. By contrast, the same acetylations of amikacin (3″-*N*-) and dibekacin (3-*N*-) resulted in their inactivation. Thus, these new findings confirmed the steric hindrance effect of the 1-*N*-acyl side chain and illuminated the novel aspect of arbekacin distinct from the other semisynthetic aminoglycosides, indicating that MRSA strains cannot be arbekacin-resistant even if they have acquired the *aac(3)* or *aac(2′)* gene.

Key words Aminoglycoside antibiotics · Resistance mechanism · Aminoglycoside-modifying enzymes · Semisynthetic · Arbekacin · *Streptomyces* origin

Introduction

The first aminoglycoside antibiotic, streptomycin,[1] was discovered by Waksman in 1944, and has been widely used for the treatment of bacterial infections, in particular, tuberculosis. A few years later, another aminoglycoside antibiotic was found independently by Umezawa et al.[2,3] who termed it streptothricin B (fradiomycin) and by Waksman and Lechevalier who termed it neomycin.[4] The clinical use of penicillin, streptomycin, chloramphenicol, and tetracycline resulted in the emergence of drug-resistant bacteria, and staphylococci and gram-negative bacteria resistant to all these antibiotic agents caused serious infections. Kanamycin (produced by *Streptomyces kanamyceticus*), discovered by Umezawa et al.[5] in 1957, has been used clinically as an effective agent for the treatment of infections with these drug-resistant bacteria, including streptomycin-resistant tuberculosis. However, as a result of its widespread use, in 1965 kanamycin-resistant strains appeared in patients, at a low incidence. Umezawa et al.[6–11] then began studies of the biochemical mechanisms of resistance to aminoglycoside antibiotics, and in 1967 the enzymatic mechanisms were first elucidated, demonstrating three types of aminoglycoside-modifying enzymes in clinically isolated resistant bacteria carrying R plasmids. These are aminoglycoside acetyltransferases (AAC), phosphotransferases (APH), and adenylyltransferases (AAD), which

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modify the amino and hydroxyl groups at specific sites in the antibiotics. On the basis of these findings, Umezawa et al. predicted structures refractory to these enzymes, and synthesized many aminoglycoside derivatives in order to develop agents that would be effective against resistant bacteria. In 1975, dibekacin was selected for chemotherapeutic use as the first rationally designed semisynthetic aminoglycoside.

In this article, we discuss the enzymatic mechanisms of resistance to aminoglycoside antibiotics, and the semisynthetic modifications of antibiotics including the development of arbekacin, which is effective against methicillin-resistant *Staphylococcus aureus* (MRSA). The enzymatic modifications of arbekacin by new AACs of *Streptomyces* origin are also described.

Useful aminoglycoside antibiotics

In general, the term "aminoglycoside antibiotics (or aminoglycosides)" refers to a group of antibiotics possessing a glycosidic linkage(s) with aminosugar(s) or pseudo-aminosugar (aminocyclitol). Basic glycosides containing aglycones, such as the macrolide, nucleoside, and anthracycline antibiotics, are not included. The stereostructures of all aminoglycoside antibiotics described in this review are based on absolute structures confirmed by X-ray crystallographic studies on kanamycin, streptomycin, and other antibiotics.

Most aminoglycoside antibiotics exhibit a broad antimicrobial spectrum and strongly bactericidal activity against mycobacteria, staphylococci, and gram-negative bacteria, including pseudomonads, but have little effect in inhibiting the growth of streptococci, pneumococci, and anaerobic bacteria. More than 150 naturally occurring aminoglycosides have been isolated from culture broths of actinomycete and bacterial strains.[11-13] Most aminoglycoside antibiotics important for chemotherapy contain a 1,3- or 1,4-diaminocyclitol named streptidine, actinamine, 2-deoxystreptamine, or fortamine (Table 1 and Fig. 1). Among these, streptomycin, spectinomycin, neomycins, paromomycins, ribostamycin, kanamycin, bekanamycin (kanamycin B), tobramycin, gentamicins, sisomicin, micronomicin, and astromicin (fortimicin A) are available as chemotherapeutic agents. Five semisynthetic aminoglycosides; dibekacin and arbekacin (derived from bekanamycin), amikacin (derived from kanamycin), netilmicin (derived from sisomicin), and isepamicin (derived from gentamicin B) are marketed as chemotherapeutic agents active against resistant bacteria. Hygromycin B and destomycin A are used as animal anthelmintics. Kasugamycin, validamycin A, and some aminoglycosides are used for the prevention of plant diseases. Geneticin (G-418) and a few other aminoglycosides are available as biochemical reagents. The structures of clinically used kanamycin and gentamicin antibiotics are shown in Fig. 2.

Enzymatic mechanisms of resistance in clinical isolates

The most important mechanism of resistance to aminoglycoside antibiotics among resistant bacteria of clinical origin arises from enzymatic *N*-acetylation, *O*-phosphorylation, and *O*-nucleotidylation of specific sites in the antibiotics. The genes for these aminoglycoside-modifying enzymes are located mainly on plasmids. Organisms with resistance due to permeability barriers to agents, have

Table 1. Useful aminoglycoside antibiotics containing 1,3- or 1,4-diaminocyclitols

Diaminocyclitol	Glycosidic substitution	Antibiotic group	Naturally-occurring	Semisynthetic
Streptidine	4-	Streptomycin	Streptomycin[a]	
Actinamine	4,5-	Spectinomycin	Spectinomycin[a]	
2-Deoxystreptamine	4-	Apramycin	Apramycin	
	5-	Destomycin	Destomycin A[b]	
			Hygromycin B[b]	
	4,5-	Neomycin	Neomycins[a]	
			Paromomycins[a]	
			Lividomycins	
			Ribostamycin[a]	
			Butirosins	
	4,6-	Kanamycin	Kanamycin[a]	Dibekacin[a]
			Bekanamycin[a]	Amikacin[a]
			Tobramycin[a]	Arbekacin[a]
	4,6-	Gentamicin	Gentamicin C[a]	Netilmicin[a]
			Gentamicin B	Isepamicin[a]
			Micronomicin[a]	
			Sisomicin[a]	
			Geneticin[c]	
Fortamine	6-	Fortimicin	Astromicin[a]	

[a] Clinical chemotherapeutic.
[b] Veterinary anthelmintic agent.
[c] Biochemical reagent.

also been isolated. But ribosomal resistance to aminoglycosides is very rare in clinically isolated bacteria. These enzymatic mechanisms have been reviewed by Umezawa,[8,9] Davies and Smith,[14] and Umezawa and Kondo.[10,11] Many genes encoding aminoglycoside-modifying enzymes were appropriately reviewed by Shaw et al.[15] The nomenclature and abbreviations for these aminoglycoside-modifying enzymes and resistant genes were proposed by Mitsuhashi.[16]

In 1965 Okamoto and Suzuki[17] reported that an intracellular enzyme in *Escherichia coli* K12 R5 transferred the acetyl group of acetyl coenzyme A (CoA) to chloramphenicol. The strain was obtained by transmission of an R plasmid from a natural isolate of multiple drug-resistant dysentery bacteria. In 1967, the Umezawa group[6,18] isolated the reaction product of kanamycin with the homogenate of this strain, and determined the structure to be 6′-*N*-acetylkanamycin. Following this, Mitsuhashi obtained *Eschericia coli* K12 ML1629, which was highly resistant to all kanamycins and neomycins, by transmission of an R plasmid from a clinically isolated resistant strain of *E. coli* to nalidixic acid-resistant *E. coli* K12 ML1410. The Umezawa group[7,19,20] demonstrated that the homogenate of the strain catalyzed the transfer of the terminal phosphate group of ATP to the 3′-hydroxyl of kanamycin. In 1968 they also first found an enzymatic modification of streptomycin by this strain, and the structure of the reaction product was determined to be streptomycin 3″-adenylate.[21,22] Independently, Yamada et al.[23] reported the inactivation of streptomycin by the adenylyltansferase in *E. coli* JE254.

Thus, the method of clarifying the enzymatic mechanisms of resistance to aminoglycoside antibiotics by structural elucidation of the enzymatic reaction products (which were purified by ion-exchange chromatography)[24] was established by the Umezawa group.[10,25] Besides these three

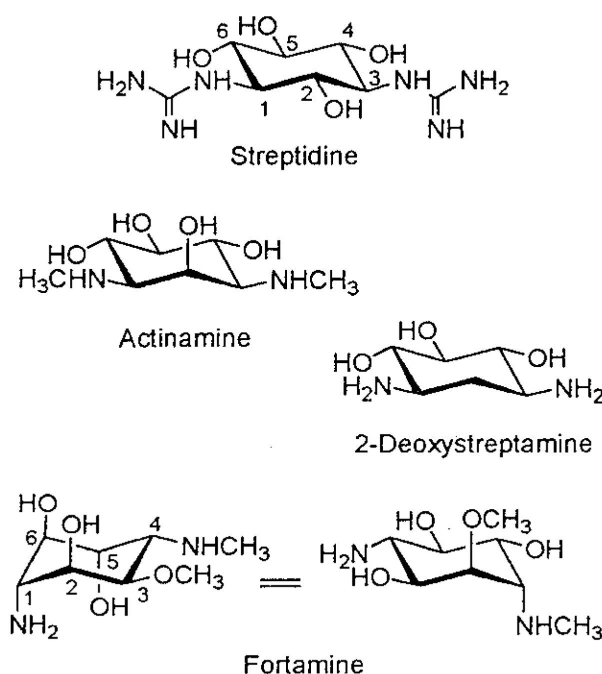

Fig. 1. Diamnocyclitols

aminoglycoside-modifying enzymes – AAC(6′), APH(3′), and AAD(3″) – a large number of other AACs, APHs, and AADs have been found in resistant strains by many researchers, as shown in Table 2. It is interesting that neither an acetyltransferase that modifies streptomycin nor a single enzyme that inactivates both streptomycin and kanamycin have been found. Recently, structures of enzymatic reaction products have been elucidated solely by spectrometric methods, including ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and mass spectrometric analyses.[10] In particular, the progress of two-dimensional NMR experiments has contributed to the accurate and rapid determination of the structure. Enzymatic modifications of kanamycin and bekanamycin by resistant bacteria are summarized in Fig. 3.

Chemical modifications based on resistance mechanism

On the basis of enzymatic mechanisms of resistance to aminoglycoside antibiotics, the Umezawa group[8,10,13,26] initiated synthetic studies of derivatives that do not undergo the enzymatic reactions and inhibit the growth of resistant strains. The first agent synthesized based on the finding of the resistant mechanism, 6′,3″-di-*N*-methylkanamycin, inhibited the growth of *E. coli* producing AAC(6′), but showed weaker antibacterial activity against kanamycin-sensitive strains than the parent antibiotic.[27] Although 3′-*O*-methylkanamycin had only weak activity,[28] 3′-deoxykanamycin, which was synthesized by a complicated glycosidation method, showed excellent activity against both gram-positive and gram-negative bacteria, including resistant strains due to APH(3′).[29] This provided complete proof of the enzymatic mechanisms of resistance. Subsequently, 3′,4′-dideoxykanamycin B (dibekacin) was prepared, starting from kanamycin B (bekanamycin), and showed strong activity not only against resistant staphylococci and gram-negative bacteria, but also against *Pseudomonas*.[30] Dibekacin has been used in Japan since 1975 and is a useful chemotherapeutic agent. The successful result boosted the syntheses of numerous 3′-deoxy and 3′,4′-dideoxy derivatives active against resistant bacteria having APH(3′).

Another approach to semisynthetic aminoglycosides active against resistant bacteria is the acylation or alkylation of the 1-amino group in 2-deoxystreptamine-containing aminoglycosides. Kawaguchi et al.[31] first synthesized amikacin by the 1-*N*-acylation of kanamycin with (*S*)-4-amino-2-hydroxybutyric acid (AHB). The amino acid is contained in butirosins, aminoglycoside antibiotics produced by *Bacillus circulans*, which have shown activity against a variety of resistant bacteria.[32] Amikacin inhibits the growth of resistant bacteria having APH(3′)-I and AAD(2″) and has been used since 1977 for treating infections caused by resistant bacteria. Netilmicin (1-*N*-ethylsisomicin),[33] which has good activity against sensitive and resistant bacteria, has been marketed as a chemotherapeutic agent since 1985. Isepamicin[34] which was synthesized

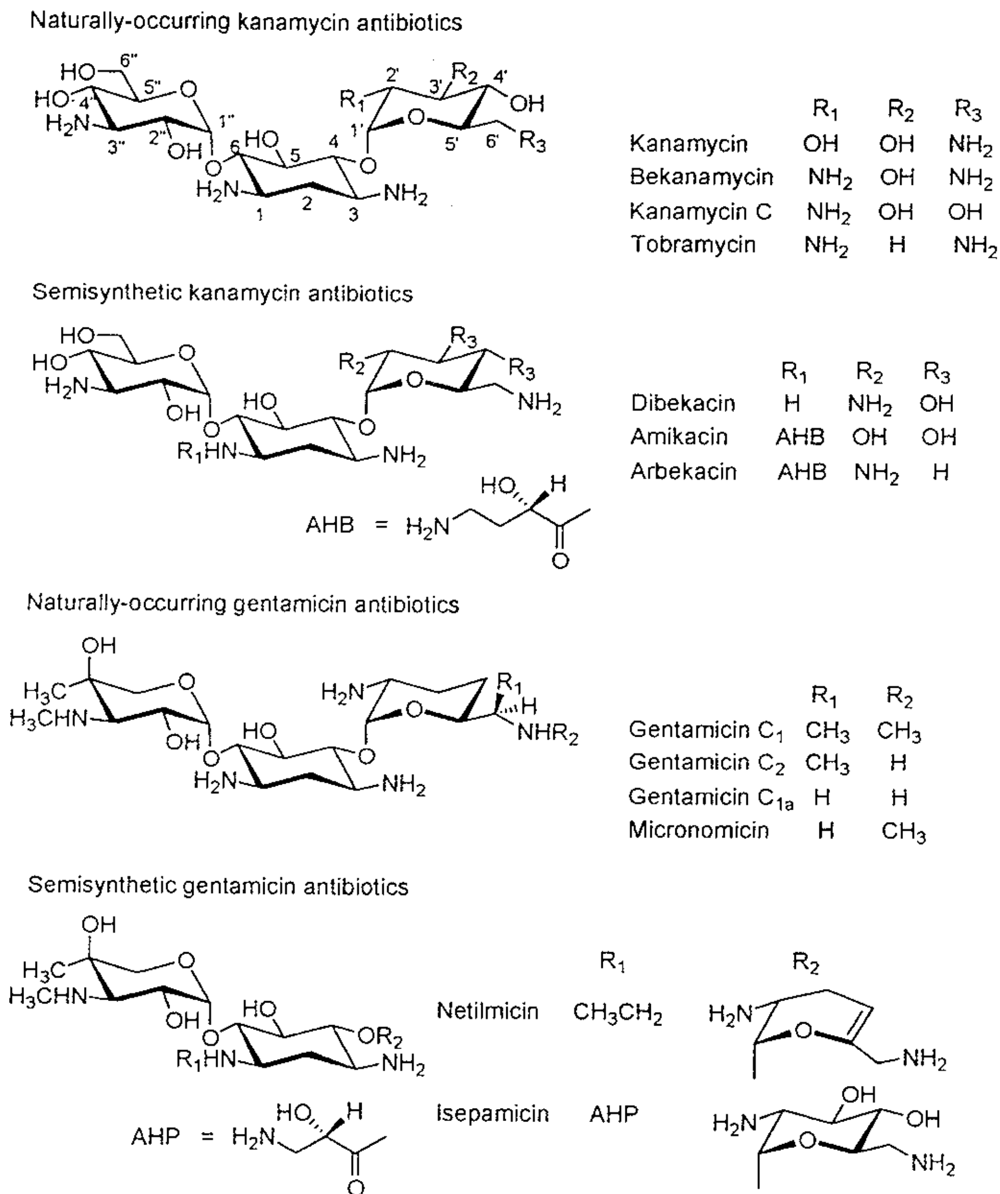

Fig. 2. 4,6-Disubstituted 2-deoxystreptamine aminoglycosides

by the 1-*N*-acylation of gentamicin B with (*S*)-3-amino-2-hydroxypropionic acid and launched in 1988.

The combination of deoxygenation and 1-*N*-acylation has provided further effective derivatives. Arbekacin, synthesized by the 1-*N*-acylation of dibekacin with AHB, showed strong activity against resistant bacteria, including *Pseudomonas*.[35] Accordingly, many deoxygenated derivatives of aminoglycoside antibiotics were synthesized and screened in efforts to develop new chemotherapeutic agents.[13] Polydeoxy derivatives, 5,2′,3′,4′,4″,6″-hexadeoxykanamycin and 5,3′,4′,4″,6″-pentadeoxykanamycin B, which have only one hydroxyl group at the 2″-position, were also active against gram-positive and gram-negative bacteria, except for *Pseudomonas* and some resistant bacteria producing AAC(6′) and AAD(2″). But 5,2′,3′,4′,2″,4″,6″-heptadeoxykanamycin, which had no hydroxyl group, had very weak activity. The AHB derivatives of hexadeoxykanamycin and pentadeoxykanamycin B showed strong activities.[36,37] It was concluded that the amino groups of kanamycin antibiotics play a critical role in antibacterial activity, and the 2″-hydroxyl and the AHB moiety on the 1-amino group markedly augments this activity. It has now become possible to prepare semisynthetic aminoglycosides which will be effective against resistant strains producing aminoglycoside-modifying enzymes which may appear in the future.

Development of arbekacin an agent effective against MRSA

In 1973, Kondo et al.[35,38] synthesized arbekacin, starting from dibekacin, by the acylation of the 1-amino group with AHB. Arbekacin[39–41] was refractory to most aminoglycoside-modifying enzymes in resistant bacteria

Table 2. Typical aminoglycoside-modifying enzymes in resistant bacteria of clinical isolates

Enzyme	Phenotype (modifying position)	Bacterial strain
AAC(3)-I	GMr, ASTMr(1-NH_2)	*Pseudomonas aeruginosa, Escherichia coli, Enterobacter*
AAC(3)-II	GMr, KMr	*Klebsiella*
AAC(3)-III	GMr, KMr, PRMr	*P. aeruginosa*
AAC(3)-IV	GMr, KMr, PRMr, APr	*E. coli*
AAC(2′)	GMr, TOBr, KMs	*Providencia*
AAC(6′)-I	KMr	*E. coli, Shigella*
AAC(6′)-II	KMr, GMr	*Moraxella*
AAC(6′)-III	KMr, GMr, DKBr	*P. aeruginosa*
AAC(6′)-IV	KMr, GMr, DKBr, AMKr	*P. aeruginosa*
AAC(6′)/APH(2″)	KMr, GMr, TOBr, ABKs	*Staphylococcus aureus* (MRSA)
APH(3′)-I	KMr, RSMr, LVr(5″-OH), GMs	*E. coli, P. aeruginosa, S. aureus*
APH(3′)-II	KMr, RSMr, BTr, GMs	*E. coli, P. aeruginosa*
APH(3′)-III	KMr, RSMr, BTr, LVr(5″-OH)	*P. aeruginosa*
APH(5″)	RSMr	*P. aeruginosa*
APH(6)	SMr	*P. aeruginosa*
APH(3″)	SMr	*E. coli, P. aeruginosa, S. aureus*
AAD(4′,4″)	KMr, TOBr, AMKr, DKBr(4″-OH)	*S. epidermidis, S. aureus*
AAD(2″)	KMr, GMr, AMKs	*E. coli*
AAD(6)	SMr	*S. aureus*
AAD(3″)	SMr, SPCMr(9-OH)	*E. coli*

AAC, aminoglycoside acetyltransferase; APH, aminoglycoside phosphotransferase; AAD, aminoglycoside adenylyltransferase; GM, gentamicin; ASTM, astromicin; KM, kanamycin; PRM, paromomycin; AP, apramycin; TOB, tobramycin; DKB, dibekacin; AMK, amikacin; ABK, arbekacin; RSM, ribostamycin; LV, lividomycin; BT, butirosin; SM, streptomycin; SPCM, spectinomycin; r, resitant; s, sensitive.

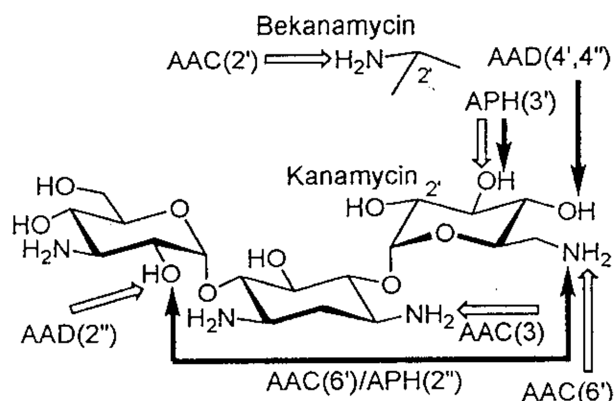

Fig. 3. Enzymatic modifications of kanamycin (2′-OH) and bekanamycin (2′-NH_2) by resistant bacteria. *Hollow arrows*, By gram-negative bacteria; *black arrows*, by methicillin-resistant *Staphylococcus aureus* (MRSA). *AAC*, Aminoglycoside acetyltransferase; *AAD*, Aminoglycoside adenylyltransferase; *APH*, Aminoglycoside phosphotransferase

(Fig. 4) and inhibited not only gram-negative bacteria, including *Pseudomonas*, but also staphylococci. An increase in the industrial yield to more than 70% led to clinical studies in the 1980s. Excellent effects and a low incidence of adverse reactions were confirmed in various clinical fields.[39] In 1984, Ubukata et al.[42] found that arbekacin was stable to aminoglycoside-modifying enzymes such as APH(3′), AAD(4′,4″),[43,44] and AAC(6′)/APH(2″)[45,46] in multiple drug-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. The clinical efficacy of arbekacin against MRSA infections was confirmed, as expected.[47] Therefore, Professor Konno suggested developing arbekacin as an anti-MRSA agent for emergency use. Arbekacin has been approved for the specific treatment of MRSA infections since 1990 in Japan, and is still extensively used for this indication.

Arbekacin derivatives stable to enzymatic modification by MRSA

A small number of MRSA strains with a moderate level (minimum inhibitory concentration [MIC]; 6.25-25 μg/ml) of arbekacin resistance have been clinically isolated, but no highly resistant strains have been isolated. When arbekacin was modified by an in-vitro reaction using an excess amount of a crude enzyme preparation extracted from an arbekacin-resistant MRSA strain (12.5 μg/ml), three inactivated products, consisting mainly of arbekacin 2″-phosphate, along with small amounts of 6′-*N*-acetylarbekacin and doubly modified arbekacin, were isolated by Kondo et al. in 1993.[48,49] It was confirmed that the arbekacin resistance of clinically isolated MRSA strains was due mainly to a bifunctional enzyme, AAC(6′)/APH(2″), which has the capacity for both 2″-*O*-phosphorylation and 6′-*N*-acetylation in arbekacin. Very recently, Fujimura et al.[50] reported the acetylation of the 4‴-amino group of arbekacin in an in-vitro reaction using a crude enzyme preparation from an MRSA strain with low arbekacin resistance. According to their discussion, this acetylation was due to AAC(4‴) derived from AAC(6′)/APH(2″).[51]

Replacement of the 2″-hydroxyl group by an amino group in dibekacin or in arbekacin was designed by Kondo[40] to obtain potent active derivatives against the MRSA with

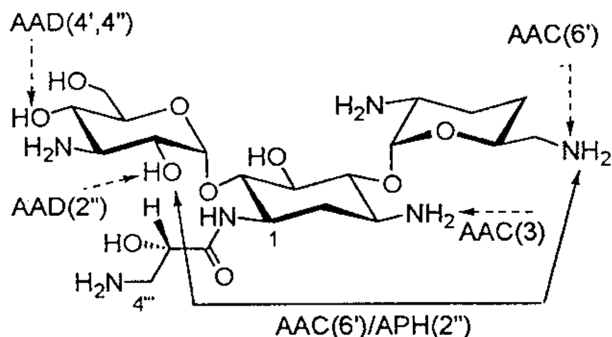

Fig. 4. Enzymatic modification of arbekacin by resistant bacteria. *Solid arrows*, by MRSA; *dotted arrows*, by gram-negative bacteria, to which arbekacin is refractive

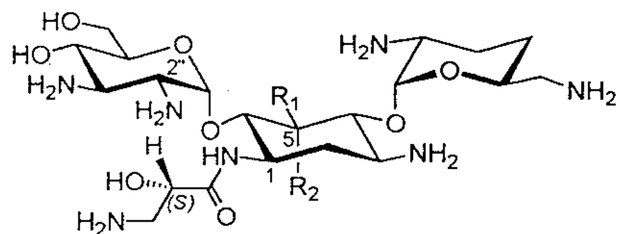

Fig. 5. Derivatives of arbekacin

the bifunctional enzyme. Conversion of the 2″-hydroxyl group by selective oxidation followed by reductive amination gave 2″-amino-2″-deoxydibekacin and 2″-amino-2″-deoxyarbekacin.[52] Their 5-deoxy, 5-epifluoro and 5-epiamino derivatives were also synthesized.[53] As expected, all 2″-amino-2″-deoxyarbekacin derivatives showed excellent activities against MRSA as well as against gram-negative bacteria. Among the derivatives 2″-amino-2″-deoxyarbekacin (AmABK) and its 5-epiamino derivative (Am_2ABK) (Fig. 5) were selected for further evaluation. Two derivatives showed in-vivo activity which paralleled the in-vitro MICs (Table 3), and were less toxic than arbekacin in terms of acute toxicity in mice (Table 4) and nephrotoxicity in rats.[54]

Modifications of arbekacin by new acetyltransferases of *Streptomyces* origin

According to a recent survey of clinical aminoglycoside resistance,[55] AACs were shown to be the most frequently occurring resistance factors. In this regard, arbekacin has modification sites for AAC(3), AAC(2′), and AAC(6′). Although MRSA strains with these AACs have not been reported thus far, one cannot rule out the possibility that such MRSA strains will emerge in the future. To check the possibility, Hotta et al.[56] attempted to use AACs available from *Streptomyces*, such as aminoglycoside producers. First, arbekacin and dibekacin were exposed to AAC(2′) derived from a kasugamycin-producing strain, *Streptomyces kasugaensis* MB273. Subsequently arbekacin was readily converted to 2′-*N*-acetylarbekacin, which retained antibiotic activity (42% of the activity of arbekacin against *Bacillus subtilis* PCI219 by an ordinary cup assay method), indicating that AAC(2′)-dependent aminoglycoside-resistant bacteria were not resistant to arbekacin.[56] By contrast, 2′-*N*-acetyldibekacin showed almost no activity. A small amount of the 2′,6′-di-*N*-acetyl derivatives of arbekacin and dibekacin were also formed by this enzymatic reaction. If these derivatives are produced by a single enzyme, then the AAC(2′) of *Streptomyces* origin should be a novel one (Fig. 6).

A new enzyme, AAC(3)-X, which acetylates the 3-amino group of kanamycin, was prepared from *S. griseus* SS-1198PR, (a kanamycin-resistant mutant derived from a wild type streptomycin-producing strain [*S. griseus* SS-1198]) by Hotta et al.[57] Subsequently, arbekacin, amikacin, and dibekacin were exposed to AAC(3)-X. Interestingly, arbekacin and amikacin, which have the 1-*N*-acyl side chain, were modified by acetylation at the 3″-amino group, which has never been reported in any aminoglycosides (Fig. 6), whereas dibekacin, which lacks the AHB side chain, was converted to the 3-*N*-acetyl derivative, as in the case of kanamycin.[58] On the other hand, two known enzymes, AAC(3)-III and AAC(3)-IV, produced by *Pseudomonas aeruginosa* PST1[59] and *Escherichia coli* JR225,[60] respectively, did not acetylate arbekacin and amikacin, but readily converted kanamycin and dibekacin to the 3-*N*-acetyl derivatives. A new product, 3″-*N*-acetylarbekacin, showed substantial antibiotic activity (55% of the activity of arbekacin against *Bacillus subtilis* PCI219), as in the case of 2′-*N*-acetylarbekacin.[56] By contrast, 3″-*N*-acetylamikacin and 3-*N*-acetyldibekacin showed 3% and 0.2% of the activities, respectively, of their parent antibiotics. Thus, the high antibiotic activities of these monoacetylated arbekacin derivatives represent a striking aspect of arbekacin distinct from the other aminoglycoside antibiotics. Arbekacin may be regarded as representing a new generation of aminoglycoside antibiotics, as shown in Fig. 7.

The 3″-*N*-acetylation should reflect a steric hindrance effect of the acyl side chain common to both arbekacin and amikacin. The 3″-*N*-acetylation will take place on the opposite side of the 3-amino group, possibly due to the effect of the side chain. The long arm of the panthoteine residue in the acetyl CoA molecule may also be critical for the 3″-*N*-acetylation. It should also be noted that AACs derived from *P. aeruginosa* and *E. coli* failed to produce the 3″-*N*-acetylation, although these enzymes produced 3-*N*-acetyl derivatives from kanamycin and dibekacin. This means that AAC(3)-X of *Streptomyces* origin has a unique catalytic property.

Table 3. Antimicrobial spectra of arbekacin and its 2″-amino derivatives

Test organism	Aminoglycoside-modifying enzyme	MIC (μg/ml)		
		ABK	AmABK	Am_2ABK
Staphylococcus aureus 209P		0.20	0.39	0.20
S. aureus Smith		≤0.10	≤0.10	≤0.10
S. aureus Ap01	AAD(4′,4″)	0.78	1.56	1.56
S. aureus MS16502 (MRSA)	AAC(6′)/APH(2″)	6.25	1.56	1.56
S. aureus MS16526 (MRSA)	AAC(6′)/APH(2″)	12.5	1.56	0.78
S. epidermidis 109	AAD(4′,4″)	0.78	1.56	0.78
Bacillus subtilis PCI219		≤0.10	0.20	0.20
Corynebacterium bovis 1810		0.39	0.78	3.13
Escherichia coli NIHJ		0.39	0.39	0.78
E. coli K-12		0.20	0.78	0.78
E. coli K-12 R5	AAC(6′)-1	12.5	12.5	50
E. coli K-12 J5 R11–2	APH(3′)-I	0.20	0.39	0.78
E. coli K-12 ML 1629	APH(3′)-I	0.78	1.56	3.13
E. coli K-12 ML 1410		0.78	3.13	1.56
E. coli K-12 ML 1410 R81	APH(3′)-I	0.78	1.56	1.56
E. coli K-12 LA290 R55	AAD(2″)	1.56	1.56	1.56
E. coli K-12 C600 R135	AAC(3)-I	0.39	1.56	3.13
E. coli W677		0.20	0.78	0.78
E. coli JR66/W677	APH(3′)-II, AAD(2″)	1.56	3.13	3.13
E. coli JR225	AAC(3)-IV	0.39	0.78	0.78
Klebsiella pneumoniae PCI602		0.78	1.56	0.78
K. pneumoniae 22#3038	APH(3′)-II, AAD(2″)	1.56	3.13	1.56
Shigella dysenteriae JS11910		1.56	3.13	3.13
Salmonella typhi T-63		0.78	0.78	0.78
S. enteritidis 1891		1.56	6.25	3.13
Proteus vulgaris OX19		0.78	1.56	1.56
Providencia sp. Pv16	AAC(2′)	1.56	1.56	0.78
Providencia sp. 2991	AAC(2′)	6.25	6.25	0.78
Serratia marcescens		6.25	6.25	3.13
Pseudomonas aeruginosa A3		≤0.10	0.78	0.78
P. aeruginosa No. 12		3.13	6.25	3.13
P. aeruginosa H9	APH(3′)-II	3.13	6.25	6.25
P. aeruginosa TI-13	APH(3′)-I	3.13	3.13	1.56
P. aeruginosa GN315	AAC(6′)-4	6.25	12.5	25
P. aeruginosa 99	AAC(3)-I	6.25	12.5	6.25
P. aeruginosa B-13	APH(3′)-I, -II	6.25	12.5	6.25
P. aeruginosa 21-75	APH(3′)-III	25	50	12.5
P. aeruginosa PST1	AAC(3)-III	6.25	12.5	3.13

ABK, Arbekacin; AmABK, 2″-amino-2″-deoxyarbekacin; Am_2ABK, 2″-amino-5,2″-dideoxy-5-epiaminoarbekacin; MIC, minimum inhibitory concentration (in vitro).

Table 4. In-vivo antibacterial activity and intravenous acute toxicity of arbekacin and its 2″-amino derivatives

Antibiotic	*S. aureus* MS16526 (MRSA)			*P. aeruginosa* GN10362		Acute toxicity
	MIC (μg/ml)	ED_{50}[a] (mg/mouse)	ED_{50}[b] (mg/mouse)	MIC (μg/ml)	ED_{50}[c] (mg/mouse)	LD_{50}[d] (mg/kg)
ABK	12.5	0.25	0.75	3.13	0.42	118
AmABK	1.56	0.33				>150
Am_2ABK	0.78		0.17	6.25	0.44	168

ED_{50}, Effective dose for 50% of group; LD_{50}, lethal dose for 50% of group.
[a] Eight ICR-Jcl male mice were used in each group, and antibiotics were administered intravenously. Challenge dose, 1.7×10^5 CFU/mouse (ip).
[b] Challenge dose, 7.1×10^5 CFU/mouse (ip).
[c] Challenge dose, 4.9×10^4 CFU/mouse (ip).
[d] Antibiotics were injected intravenously into ICR-Jcl male mice (five in each group).

The other point to note is that 3″-*N*-acetylarbekacin showed substantial antibiotic activity, whereas no significant activity was observed with 3″-*N*-acetylamikacin, despite their structural similarity. The differences can be seen at the 2′-, 3′- and 4′-positions. It seems possible that the presence of an extra amino group in arbekacin in comparison with amikacin plays a critical role in the antibiotic activity. However, this explanation cannot be acceptable for the substantial activity of 2′-*N*-acetylarbekacin compared with the activity of 2′-*N*-acetyldibekacin, as there is no difference in the numbers of free amino groups between them (Fig. 6).[56] Therefore, the reason for the antibiotic activity of the monoacetyl derivatives of arbekacin remains to be elucidated.

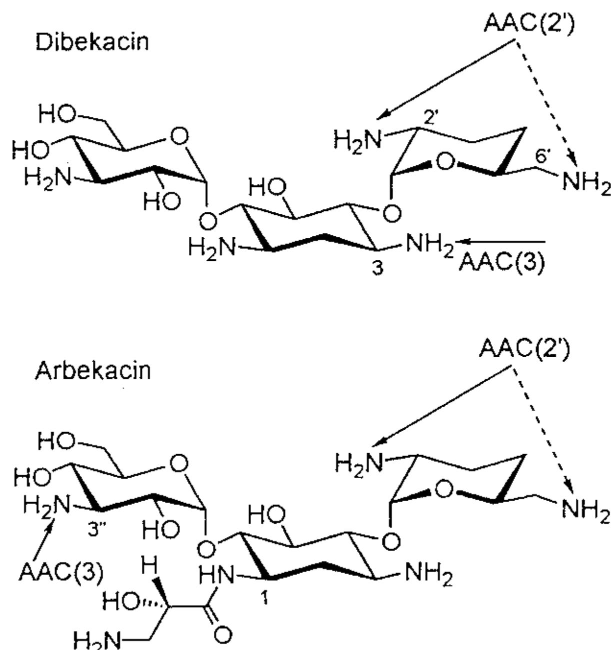

Fig. 6. Modification of dibekacin and arbekacin by aminoglycoside-modifying enzymes of *Streptomyces* origin

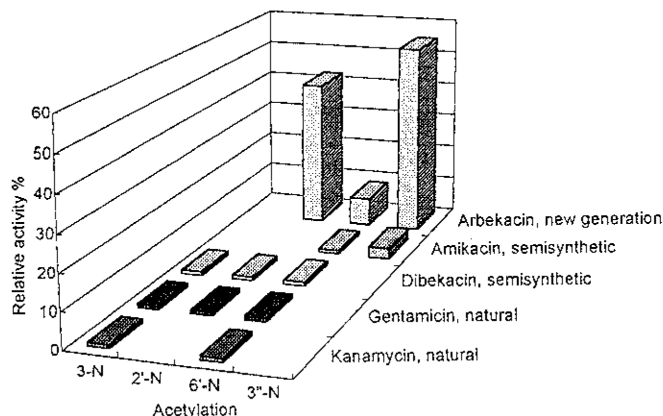

Fig. 7. Relative activities of monoacetylated aminoglycoside antibiotics

Concluding remarks

Refractoriness to aminoglycoside-modifying enzymes of clinical origin has been the key stimulus for the development of new semisynthetic aminoglycoside antibiotics. Dibekacin (1975), amikacin (1977), netilmicin (1985), isepamicin (1988), and arbekacin (1990), which are marketed as chemotherapeutic agents, were developed by deoxygenation of the 3′-hydroxyl group as the modification site for APH(3′), and by 1-*N*-acylation, in order to synthesize dibekacin and the others, respectively. However, novel resistant bacteria to these antibiotics emerged sooner or later, and again were shown to be dependent on new types of aminoglycoside-modifying enzymes. In this regard, arbekacin, which is approved as an anti-MRSA agent, has been characterized by a low-to-moderate level of resistance, low incidence, and AAC(6′)/APH(2″)-dependence, in terms of the emergence of arbekacin-resistance in MRSA strains. Further, unexpected novel properties of arbekacin have been revealed by simulative modification studies using AACs of amnoglycoside-producing strains of *Streptomyces* (i.e., that the monoacetylated derivatives of arbekacin have substantial antibiotic activities). Therefore, we believe that arbekacin can be regarded as representing a new-generation aminoglycoside antibiotic, and that it provides a new direction, "double-stage activity" for developing new semisynthetic aminoglycoside antibiotics.

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ORIGINAL ARTICLE

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Inhibitory action of clarithromycin on glycocalyx produced by MRSA

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Abstract
We determined whether clarithromycin (CAM) had the ability to eliminate glycocalyx and biofilm produced by methicillin-resistant *Staphylococcus* aureus (MRSA) using an in-vitro experimental system (consisting of a bladder model and a kidney model) simulating complicated urinary tract infection (UTI). We also examined whether a combination of CAM and vancomycin (VCM) was effective for eliminating the MRSA biofilm. VCM (urinary concentration simulating drip infusion of 500mg twice a day for 5 days; minimum inhibitory concentration (MIC) 0.5µg/ml) eliminated MRSA from the bladder model medium at 48h, but reproliferation occurred immediately after withdrawal of the agent. No disappearance of MRSA biofilm was noted, and this appeared to be the cause of the bacterial regrowth. CAM (urinary concentration simulating oral administration of 200mg twice a day for 5 days; MIC, 128µg/ml) allowed microbial recovery to the initial level within 48h, but led to the disappearance of the glycocalyx-forming biofilm. A combination of VCM and CAM caused microbial elimination from the bladder model medium at 46h with no regrowth after withdrawal of the antimicrobial agents. Scanning electron microscopy confirmed that the MRSA biofilm disappeared completely and no microbial adhesion was noted. These results suggest that CAM has an inhibitory action on glycocalyx and biofilm of MRSA, and that the combined use of VCM and CAM may be efficacious for the treatment of MRSA UTI.

Key words MRSA · Biofilm · Clarithromycin · Vancomycin

Introduction

Complicated urinary tract infection (UTI) is often refractory to antibacterial therapy. One of the reasons for this is that the infection involves biofilm formation on the surface of the bladder mucosa or a foreign body.[1–3] Researchers have studied biofilm infections caused by *Pseudomonas aeruginosa*, which is frequently isolated from complicated UTI.[1–6] In biofilm infections caused by *P. aeruginosa*, clarithromycin (CAM), a macrolide antimicrobial agent, has been reported not to have any antiproliferative effect, but to eliminate the glycocalyx component of the biofilm, which results in reduced biofilm formation, suggesting the possibility of CAM employment for the treatment of respiratory infections.[7] We have already confirmed this action of the drug against *P. aeruginosa* in an in-vitro experimental model simulating complicated cystitis.[4–6] However, biofilm infections caused by Gram-positive cocci such as methicillin-resistant *Staphylococcus aureus* (MRSA) have not been sufficiently investigated.

Hospital-acquired MRSA infection, including urinary MRSA infection, has recently become an issue of clinical importance.[8–10] Most urinary infections are associated with complicated UTIs related to an indwelling catheter in the urinary tract, and thus may involve biofilm formation on the catheter surface by MRSA. In this study, we determined whether CAM has an inhibitory action on glycocalyx production by MRSA as well as by *P. aeruginosa* using an in-vitro experimental system simulating complicated UTI. We also investigated whether a combination of CAM and vancomycin (VCM; which has an antimicrobial effect on MRSA), has a more favorable therapeutic effect on MRSA UTI than CAM alone.

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Materials and methods

Bacteria and antimicrobial agents

MRSA isolated from urine (coagulase type II) was used in the experiment. The minimum inhibitory concentrations (MIC) of VCM and CAM against MRSA were 0.5µg/ml and 128µg/ml, respectively.

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Distribution of the *cfiA* Gene among *Bacteroides fragilis* Strains in Japan and Relatedness of *cfiA* to Imipenem Resistance

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The *cfiA* gene, encoding an imipenem-hydrolyzing metallo-β-lactamase produced by *Bacteroides fragilis*, and insertion-like elements were detected by PCR amplification with *B. fragilis* strains isolated in Japan. The *cfiA* gene was found in 1.9 and 4.1% of the imipenem-susceptible *B. fragilis* isolates collected from 1987 to 1988 and from 1992 to 1994, respectively. Insertion-like elements adjacent to the *cfiA* gene were found in all nine metallo-β-lactamase-producing imipenem-resistant strains tested but not in nine *cfiA*-positive strains with no detectable metallo-β-lactamase activity.

Bacteroides fragilis is an anaerobic bacterium most frequently isolated from suppurative anaerobic infections and exhibits a broad spectrum of resistance to antimicrobial agents (17). Nationwide surveys in Japan and the United States showed imipenem to be very active against *B. fragilis* (2, 4). However, the emergence of resistance to imipenem among *B. fragilis* strains has been reported (1, 3, 5). It has been suggested that the production of an imipenem-hydrolyzing metallo-β-lactamase contributes to imipenem resistance among *B. fragilis* strains (1, 10).

The metallo-β-lactamase produced by *B. fragilis* is encoded by the *cfiA* gene (22), which has also been called the *ccrA* gene (15). A recent study demonstrated that an insertion element (IS), IS*1186*, located immediately upstream of the *cfiA* gene promoted the expression of this carbapenemase gene (13) as well as other insertion elements (14). Podglajen et al. suggested that a one-step mutation can allow the silent *cfiA* gene to be expressed (12). If so, *B. fragilis* strains carrying the silent *cfiA* gene would be expected to be eradicated in clinical settings before mutation occurs.

The aim of this study was to investigate the distribution of the *cfiA* gene among *B. fragilis* strains in Japan and to analyze the relationships between susceptibility to imipenem, metallo-β-lactamase production, and the presence of the *cfiA* gene adjacent to IS-like elements. A one-step mutation of *cfiA*-positive, imipenem-susceptible *B. fragilis* strains was also tested.

B. fragilis clinical strains used were placed into one of three groups. (i) The first group consisted of 21 stock strains, including 7 imipenem-resistant strains (MIC, ≥256 μg/ml, 4 strains; 32 μg/ml, 1 strain; and 16 μg/ml, 2 strains) from our laboratory, which were collected between 1986 and 1994 from various hospitals in Japan, and 13 imipenem-susceptible strains (MIC, 4 μg/ml, 1 strain; 1 μg/ml, 5 strains; and 0.5 μg/ml, 7 strains), and 1 imipenem-intermediate strain (MIC, 8 μg/ml), which were collected before 1987. (ii) The second group included 162 isolates, collected between 1987 and 1988, from a central clinical laboratory in Tokyo, Japan. (iii) The third group consisted of 124 isolates collected at Gifu University Hospital, Gifu, Japan, between 1992 and 1994.

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Susceptibility was tested by an agar dilution method (8). Imipenem of known potency was obtained from Banyu Pharmaceutical, Tokyo, Japan.

Metallo-β-lactamase activity was assayed by both a spectrophotometric technique (1) and a biological method. For the biological assay, a 2-day culture of *B. fragilis* on modified Gifu anaerobe medium (GAM) agar (Nissui Pharmaceutical, Tokyo, Japan) was suspended in Anaerobe Broth MIC medium (Difco Laboratories, Detroit, Mich.). The cell suspension of 10^6 CFU/ml was mixed with the same volume of 200 mM 3-(*N*-morpholino)propanesulfonic acid–potassium hydroxide buffer (pH 7.2) containing imipenem at a final concentration of 6.3 μM or with imipenem solution supplemented with 2 mM EDTA. The mixture was incubated anaerobically for 18 h at 37°C. Imipenem alone and a mixture of imipenem and EDTA were incubated in parallel as controls.

To measure the remaining imipenem bioactivity, blank paper disks (Toyo-roshi, Tokyo, Japan) were impregnated with 30 μl of the mixture and placed on Antibiotic Medium 3 (Difco) plus 1.5% agar which was seeded with *Bacillus subtilis* MB-32 as an indicator strain. Plates were read for the presence of inhibition zones after overnight aerobic incubation at 37°C.

Bacterial DNA was obtained by heating cells for 10 min at 95°C. The primers for detection of the *cfiA* gene and IS-like elements and the predicted size of PCR products with primer sets are listed in Table 1. PCR amplification was run for 35 cycles consisting of 20 s at 95°C and 2 min at 64°C as described elsewhere (9). Southern hybridization was performed as described previously (7). Oligonucleotide probe GBI-3 was used for a PCR product with GBI-1 and GBI-2 primers, and oligonucleotide probe GBI-2 was used for an amplicon with GBI-3 and GBI-4 primers (Table 1).

Four *cfiA*-positive and four *cfiA*-negative imipenem-susceptible strains were tested for a one-step mutation resulting in imipenem resistance. A 48-h culture of each of these strains was suspended in Anaerobe Broth MIC medium at a concentration of 10^9 CFU/ml. A 100-μl aliquot of cell suspension was spread on modified GAM agar containing 16 μg of imipenem per ml and incubated anaerobically for 72 h at 37°C. Ten colonies on each agar plate, if available, were subcultured on modified GAM agar and subjected to imipenem susceptibility testing as described above.

To detect the *cfiA* gene, PCR amplification with three

TABLE 1. Sequences of oligonucleotide primers and probes

Genetic element and oligonucleotide	Sequence (5′–3′)	Position[a]	Usage	Amplicon (predicted size)
cfiA gene				
GBI-1	CCCAACTCTCGGACAAAGTG	624–643	Forward primer	GBI-1–GBI-2 (340 bp)
GBI-2	AGTGAATCGGTGAATCCATG	944–963	Reverse primer and probe for a GBI-3 and GBI-4 primer set	
GBI-3	CGAACCAGATGACGATAGAC	891–910	Forward primer and probe for a GBI-1 and GBI-2 primer set	GBI-3–GBI-4 (358 bp)
GBI-4	ACGATCTGCTTGGTATGCTC	1229–1248	Reverse primer	GBI-1–GBI-4 (625 bp)
IS				
G[b]	CGCCAAGCTTTGCCTGCCATTAT	Upstream of *cfiA*	Forward primer	G-E (approx. 2 kbp)
E[b]	CTTCGAATTCGGCGAGGGATACATAA	Inside of *cfiA*	Reverse primer	

[a] Thompson and Malamy (22).
[b] Podglajen et al. (13).

primer sets (GBI-1 and GBI-2, GBI-3 and GBI-4, and GBI-1 and GBI-4) was carried out. A positive PCR test was detected in seven imipenem-resistant laboratory stock strains of *B. fragilis* which produced detectable levels of metallo-β-lactamase by spectrophotometric assay or bioassay; in one imipenem-susceptible strain, which produced no detectable metallo-β-lactamase; and in one imipenem-intermediate strains, which generated no detectable metallo-β-lactamase. Twelve other imipenem-susceptible strains, which had no detectable metallo-β-lactamase, had a negative PCR test. Representative PCR results are shown in Fig. 1A to C. The results of the Southern hybridization agreed with those of the PCR assay (data not shown). All seven imipenem-resistant strains were PCR positive for IS-like elements; a PCR product of approximately 2 kbp in size was generated (Fig. 1D, lanes 2 and 5). One imipenem-susceptible strain, which gave a positive PCR test for *cfiA*, was PCR negative for IS-like elements with an amplicon of approximately 400 bp (Fig. 1D, lane 6), a DNA size which indicates that there is no IS-like element immediately upstream of *cfiA*.

Prevalence of *cfiA*, susceptibility to imipenem, metallo-β-lactamase production, and carriage of IS-like elements were studied in two cohorts of *B. fragilis* strains (Table 2). Based on the results from the stock strains mentioned above, a primer set of GBI-1 and GBI-4 was used to detect *cfiA*. All *cfiA*-positive strains were subjected to a test for metallo-β-lactamase production by both spectrophotometric assay and bioassay.

Imipenem resistance was found in 2 (1.2%) of 162 strains recovered between 1987 and 1988 and 1 (0.8%) of 124 strains isolated between 1992 and 1994. Two resistant isolates collected between 1987 and 1988 had the *cfiA* gene and IS-like elements and produced metallo-β-lactamase, whereas one resistant strain (MIC of imipenem, 32 μg/ml) isolated between 1992 and 1994 was *cfiA*- and IS-negative and showed no detectable metallo-β-lactamase activity. The *cfiA* gene was detected in 1.9% of the 159 imipenem-susceptible strains isolated between 1987 and 1988 and in 4.1% of the 122 imipenem-susceptible strains recovered between 1992 and 1994. Regardless of the susceptibility to imipenem, the *cfiA* gene was found in 6 (3.7%) of the 162 strains isolated between 1987 and 1988 and 5 (4.0%) of the 124 strains isolated between 1992 and 1994.

Although tiny colonies were found after eight imipenem-susceptible strains were cultured on imipenem-supplemented agar plates, recovered colonies (irrespective of *cfiA* carriage) developed no resistance to imipenem by susceptibility testing and produced no detectable metallo-β-lactamase.

In this study of two cohorts of *B. fragilis* strains, the prevalence of the *cfiA* gene was 3.7 and 4.0%, respectively. Of imipenem-susceptible *B. fragilis* strains, 1.9% of the first cohort

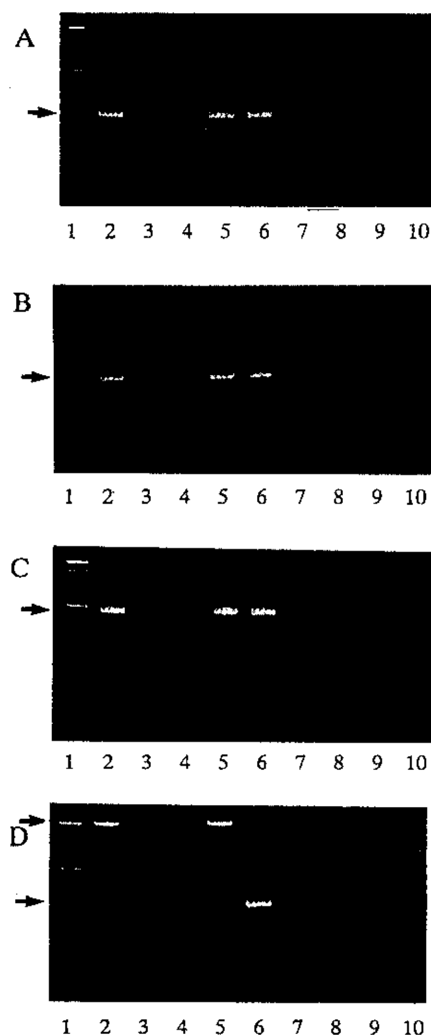

FIG. 1. PCR amplification for detection of the *cfiA* gene with primers GBI-1 and GBI-2 (A), GBI-3 and GBI-4 (B), and GBI-1 and GBI-4 (C) and for detection of IS-like element with primers G and E (D). Lane 1, 100-bp DNA ladder (Gibco BRL); lanes 2 and 5, metallo-β-lactamase-producing, imipenem-resistant *B. fragilis* strains; lanes 3, 4, and 6 to 9, detectable metallo-β-lactamase-negative, imipenem-susceptible strains; lane 10, negative control without DNA sample. Arrows indicate 340-bp (A), 358-bp (B), 625-bp (C), ca. 2-kbp (D), and ca. 400-bp (D) amplicons. Lanes 2, 5, and 6 were PCR positive for the *cfiA* gene. Lanes 2 and 5 were positive for IS-like elements immediately upstream of the *cfiA* gene.

TABLE 2. Distribution of the *cfiA* gene and IS-like element and metallo-β-lactamase production among clinical isolates of *B. fragilis*[a]

No. of strains tested (yr isolated)	Susceptibility to imipenem	*cfiA* gene	IS-like element	Metallo-β-lactamase production	No. of productive strains
162 (1987–1988)	R	+	+	+	2
	I	+	−	−	1
	S	+	−	−[b]	3
	S	−	−[c]	ND	156
124 (1992–1994)	R	−	−	−	1
	I	−	−	ND	1
	S	+	−	−[b]	5
	S	−	−[c]	ND	117

[a] R, resistant with MICs of ≥16 μg/ml; I, intermediate with a MIC of 8 μg/ml; S, susceptible with MICs of ≤4 μg/ml; +, positive; −, negative; ND, not done.
[b] One strain was tested.
[c] Ten strains were tested.

and 4.1% of the second cohort carried the *cfiA* gene. These results are relatively similar to those obtained in previous studies from France showing that approximately 3% of 500 randomly selected strains of *B. fragilis* were *cfiA* positive (14) and that a silent *cfiA* gene was found in 1.6% of the isolates (12, 13). The similarities derived from geographically distinct surveys suggest that the prevalence of *cfiA*-positive strains among *B. fragilis* may be relatively constant in each country.

Our study suggests that metallo-β-lactamase production is clearly related to the presence of the *cfiA* gene and IS-like elements immediately upstream of the metallo-β-lactamase gene. Gene activation by IS elements in *B. fragilis* is being identified: for example, IS*21* (21) activation of the *cepA* gene (20); IS*4351*, IS*942*, and IS*1186* (13, 16) activation of the *ccrA* or *cfiA* gene; IS*4351* activation of the *ermF* gene (18, 19); and IS*1170* and IS*1169* activation of *nimC* and *nimD* (23).

In this study, one strain of *B. fragilis* (MIC of imipenem, 32 μg/ml) lacked production of metallo-β-lactamase. By contrast, *Bacteroides distasonis* (6) and *Enterobacter cloacae* (11) have been shown to have other imipenem resistance mechanisms, including reduced outer membrane permeability and the production of other types of β-lactamase, such as serine β-lactamase. Further studies are needed to determine the other resistance mechanism(s) of *B. fragilis* strains against imipenem.

The intraspecific transfer of imipenem resistance in a *B. fragilis* strain associated with the production of an imipenem-hydrolyzing metallo-β-lactamase has been previously reported (2). However, this earlier study has been the sole report of plasmid-mediated transmission of metallo-β-lactamase. Activation of the silent *cfiA* gene by one-step mutation was not confirmed in this study. Taken together, our data suggest that neither transfer of imipenem resistance by a plasmid nor spontaneous mutation leading to resistance seems to be a common way for *B. fragilis* to acquire resistance to imipenem.

Our study did not prove the conversion of *cfiA* gene-harboring imipenem-susceptible strains to imipenem resistance by a single mutation. This failure may be due to the lack of the IS element necessary for imipenem resistance within the strains tested.

The PCR assay described here, in combining detection of the *cfiA* gene and of IS-like elements immediately upstream of the *cfiA* gene, may be a useful tool to monitor the prevalence of metallo-β-lactamase-mediated imipenem-resistant *B. fragilis* strains.

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FEMS Microbiology Letters 179 (1999) 67–72

FEMS MICROBIOLOGY LETTERS

nalB-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the *mexR* gene of the *Pseudomonas aeruginosa* chromosome

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Abstract

Two mutations, one at the *nalB* and the other at the *mexR* locus, in the *Pseudomonas aeruginosa* chromosome are known to cause overexpression of the MexAB-OprM efflux pump. Based on the following results, we concluded that *nalB* is the mutation that has occurred within the *mexR* gene of the *P. aeruginosa* chromosome. (i) Nucleotide sequencing of the *mex* operon upstream region of 21 independent *nalB*-type mutants including the original *nalB9* revealed that all the mutations were located within the *mexR* gene. The mutations were classified into three different groups and nine types including single base substitutions, single base deletions and base insertions. (ii) Substitution of the mutant *mexR* with the wild-type *mexR* and replacement of the wild-type *mexR* with a defined *mexR* mutation resulted in the expression of wild-type and *nalB*-type MexAB-OprM respectively, which was confirmed by testing the antibiotic susceptibility and β-galactosidase activity of the *mexA-lacZ* translational fusion. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Efflux pump; Multidrug resistance; Antibiotic resistance; *Pseudomonas*; Gene regulation

1. Introduction

Antimicrobial chemotherapy of *Pseudomonas aeruginosa* infections is a major problem in hospitals, since this bacterium exhibits natural and acquired resistance to a broad spectrum of antibiotics. It has been thought that multi-antibiotic resistance in this organism is mainly attributable to a tight outer membrane diffusion barrier [1,2]. However, recent studies have revealed that the wild-type strain of *P. aeruginosa* expresses a low level of the antibiotic efflux pump, MexAB-OprM, that gives the bacterium natural resistance to a broad spectrum of antibiotics [3,4]. Moreover, the bacterium exhibits a higher level of multi-antibiotic resistance than the wild-type strain upon mutation in the *nalB* locus [5–7].

The *nalB* mutation was discovered originally among strains resistant to nalidixic acid and was mapped at 32 min of the *P. aeruginosa* chromosome [5]. More recently, a *mexAB-oprM* operon was cloned and the operon appeared to encode the MexAB-OprM efflux pump [3,4]. The *nalB* mutant over-

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expresses the *mexAB-oprM* operon as evidenced by Northern and Western blotting analyses [4]. In addition, a gene located upstream of the *mexAB-oprM* operon was found to regulate expression of the *mexAB-oprM* operon, designated *mexR* [8]. Mutation in the *mexR* gene overexpressed the *mexAB-oprM* operon and exhibited the *nalB*-type antibiotic resistance profile [8]. The nucleotide sequence of the *mexR* region of the *nalB*-type clinical isolates of *P. aeruginosa* revealed that most carried a mutation in the *mexR* region [9]. However, this result has not clarified the ambiguity about the identity of *nalB* and *mexR*. We addressed this issue by characterizing the *mexR* region by (i) sequencing the *mexR* region of 21 independent *nalB*-type mutants and (ii) replacing the mutant *mexR* with wild-type *mexR* and vice versa.

2. Materials and methods

2.1. Bacterial strains, culture conditions and isolation of the nalB-type mutants

The bacterial strains used are listed in Table 1. Bacteria were grown in L-broth. Vogel-Bonner minimal medium [10] was used for the selection of transconjugants. We isolated spontaneous *nalB*-type mutants by plating PAO4290 on L-agar containing 1 μg ml^{-1} of both ofloxacin and cefsulodin [6,7].

2.2. Recombinant DNA techniques

We manipulated DNA by the standard procedures described previously [10,11]. For PCR amplification of the *mexR* region, a fully grown culture was diluted with a 100-fold volume of distilled water, heated at 100°C for 10 min and this was used as the template. The primers used were P4-forward 5′-TCGGTATTCAGGGTCACC-3′, P4-reverse 5′-TCAAGGCCAAGCTGTACGG-3′, P5-forward 5′-CATGGCCCATATTCAGAACC-3′ and P6-reverse 5′-CCAGTAAGCGGATACCTG-3′.

2.3. Construction of mobilizer plasmids and procedure of gene replacement

To subclone the *mexR* gene, chromosomal *mexR* was amplified from the wild-type strain PAO4290 and its *nalB*-type mutants TNP030#10 and TNP030#11. The PCR products were treated with *Nco*I and *Kpn*I, then subcloned into pMexA [10], yielding pMexR, pMexR (C73T), and pMexR (A388C), respectively. For construction of the revertants TNP077 from *nalB*-type mutants or *mexR* mu-

Table 1
Bacterial strains and plasmids used

	Relevant properties	Parent	Reference
Strains			
P. aeruginosa PAO4290	*lue-10, argF10, aph-9004*, FP−	[10]	
P. aeruginosa TNP030#1 to #20	OFLX- and CFS-resistant #1–#20	PAO4290	This study
P. aeruginosa TNP077#9	wild-type *mexR*	TNP030#9	This study
P. aeruginosa TNP077#10	wild-type *mexR*	TNP030#10	This study
P. aeruginosa TNP077#11	wild-type *mexR*	TNP030#11	This study
P. aeruginosa TNP078#10	C73T mutation in *mexR*	PAO4290	This study
P. aeruginosa TNP078#11	T104G and A388C mutation in *mexR*	PAO4290	This study
P. aeruginosa PAO6006	*proC130, nalB9*		[5]
Plasmids			
pMexA	pNOT19 derivative carrying *mexA*, $ABPC^r$		[10]
pMexR	pMexA derivative carrying downstream region of *mexR*, $ABPC^r$		This study
pMexR(C73T)	pMexA derivative carrying *mexR* (C73T), $ABPC^r$		This study
pMexR(A388C)	pMexA derivative carrying *mexR* (T104G, A388C), $ABPC^r$		This study
pK19*mobsacB*	Broad-host-range mobilizable vector with *sacB*, Km^r		[12]
pHRP309	Broad-host-range promoterless-*lacZ* transcriptional fusion vector, Gm^r		[13]
pMexA-*lacZ*	pHRP309 derivative carrying the *mexR-mexA* intergenic region on the *Sac*I-*Eco*RI fragment of pMexR, Gm^r		This study

tants TNP078 from PAO4290, the *Eco*RI-*Kpn*I fragment containing the *mexR* region of the respective pMexR was subcloned into pK19*mobsacB* [12]. The plasmid harbored in *Escherichia coli* S17-1 was mobilized to *P. aeruginosa* by conjugation. Gene replacement was carried out as reported earlier [10]. All the constructions were confirmed by nucleotide sequencing.

2.4. Other techniques

Western blot analysis has been described previously [10]. The minimum inhibitory concentration (MIC) of antibiotic was determined by the agar dilution method using Mueller-Hinton agar (Becton-Dickinson). The region of the DNA fragment, including a part of *mexR* and *mexA*, was inserted into the plasmid pHRP309 carrying promoterless-*lacZ* [13] in the direction towards *mexA*, yielding the *mexA-lacZ* transcriptional fusion vector. The strain harboring the *mexA-lacZ* fusion vector was cultured overnight in L-broth containing 20 μg ml^{-1} of gentamicin. The β-galactosidase activity was assayed as described previously [11].

3. Results

3.1. Characterization of the nalB-type mutation

We isolated 20 independent spontaneous *nalB*-type mutants from laboratory strain PAO4290 in the presence of both ofloxacin and cefsulodin. The mutation rate was roughly 10^{-8}, suggesting that the mutants most likely had a single mutation. All the mutants isolated of the TNP030 series showed high resistance to the β-lactam antibiotics, chloramphenicol, fluoroquinolone antibiotics and meropenem, which is typical of the *nalB*-type mutant (Table 2). The mutants overexpressed the *mexAB-oprM* operon as judged by Western blot analysis using an anti-OprM antibody (data not shown). All these results suggested that the mutants most likely carried the *nalB* mutation. In the next experiment, we analyzed the nucleotide sequence of the upstream region of the *mexAB-oprM* operon of 20 independent mutants plus one original *nalB* mutant PAO6006 (*nalB9*) [5] and found that all the mutants had a mutation within the coding region of *mexR* (Fig. 1 and Table 2). Ten out of 21 strains including PAO6006 had a C to T substitution at nucleotide 73 resulting in a nonsense mutation. Seven mutants had a single nucleotide substitution at nucleotide 146 (A to C), 209 (G to A), 248 (G to A, two strains) and 388 (A to C, three strains) changing amino acid residues from Gln to Pro, Arg to Gln, Arg to His, and Tyr to Pro, respectively. Three of the four remaining mutants had a single base deletion at nucleotide 218 (ΔG), a 3-bp insertion (TGG) at nucleotide 379 and a 6-bp insertion (ATCCTC) at nucleotide 286 resulting in a frame shift. The *nalB* mutant PAO6006 (*nalB9*) originally isolated in 1982 [5] had one base insertion (A) at nucleotide 251. All these mutants seemed to overexpress the *mexAB-oprM* operon to the same level, since the *mexA* transcriptional reporter expressed a 3.0 times higher β-galactosidase activity than that of the wild-type strain (Table 3). These results suggest that only the *mexR* mutation may be sufficient to overexpress the *mexAB-oprM* operon.

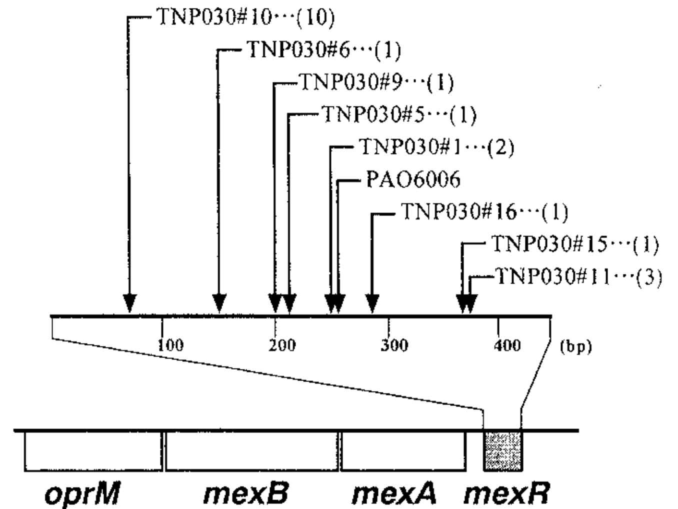

Fig. 1. Physical mapping of the mutations. The figure represents the physical relationship between *mexA,B-oprM* and *mexR*, and the location of the mutations. Physical distance is arbitrary. Number in parentheses represents the number of mutants isolated. (bp) represents nucleotide base pair counting from the initiation codon.

3.2. Replacement of mutant mexR with wild-type mexR and introduction of the defined mutation into the wild-type mexR

It was reported recently that *nalB*-type hyper-ex-

Table 2
The MIC of antibiotics in the representative strains

Strain	Parent strain	Mutation in *mexR*	MIC (μg ml^{-1})[a]					
			AZT	CAZ	CBPC	CP	MPM	OFLX
Wild-type								
PAO4290		wild	3.13	0.78	25	25	0.39	0.39
nalB-type mutant								
TNP030#10	PAO4290	C73T	25	3.13	200	100	1.56	3.13
TNP030#6	PAO4290	A146C	25	3.13	200	200	1.56	3.13
TNP030#9	PAO4290	G209A	25	3.13	200	200	1.56	3.13
TNP030#5	PAO4290	218G-del[b]	12.5	3.13	200	200	1.56	3.13
TNP030#1	PAO4290	G248A	25	3.13	200	100	1.56	3.13
TNP030#16	PAO4290	286 6bp-ins[b]	12.5	3.13	200	200	1.56	3.13
TNP030#15	PAO4290	379 3bp-ins[b]	12.5	3.13	200	200	1.56	3.13
TNP030#11	PAO4290	A388C	12.5	3.13	200	200	1.56	3.13
PAO6006	PAO969	251 A-ins[b]	12.5	3.13	100	200	0.78	3.13
Revertant constructed by gene replacement								
TNP077#9	TNP030#9	wild	3.13	0.78	25	25	0.39	0.78
TNP077#10	TNP030#10	wild	3.13	0.78	25	25	0.39	0.78
TNP077#11	TNP030#11	wild	3.13	0.78	25	25	0.39	0.78
mexR mutant introduced by gene replacement								
TNP078#10	PAO4290	C73T	25	3.13	200	200	1.56	3.13
TNP078#11	PAO4290	T104G, A388C	12.5	3.13	200	200	1.56	3.13

[a]AZT, aztreonam; CAZ, ceftazidime; CBPC, carbenicillin; CP, chloramphenicol; MPM, meropenem; OFLX, ofloxacin.
[b]del, deletion; ins, insertion.

pression of the *mexAB-oprM* operon is not explained simply by a null mutation in *mexR* [8] suggesting that the *nalB* phenotype is dependent upon two genes [14]. This description is confusing at the moment, since it is not clear whether or not *mexR* and *nalB* are identical. To answer this question, we carried out an experiment to replace *mexR* of the *nalB*-type mutant chromosome (TNP030#9, TNP030#10 and TNP030#11) with the wild-type allele by the gene replacement method. The *mexR* revertants (TNP077#9, TNP077#10 and TNP077#11) thus obtained were characterized by nucleotide sequencing analysis, confirming that the wild-type nucleotide sequence is present in *mexR*. All the revertants tested showed antibiotic susceptibility indistinguishable from that in PAO4290 (Table 2). These results suggested that the wild-type *mexR* regulates *mexAB-oprM* expression to a low level in *P. aeruginosa*.

Although it is less likely that the *nalB*-type mutants described above acquired a secondary mutation, this possibility cannot be strictly ruled out because the *nalB*-type mutants of the TNP030 series were selected in the presence of antibiotics. Thus, we designed the experiment to replace the wild-type

Table 3
β-Galactosidase activity of the representative strains

Strain	Mutation in *mexR*	Parent	β-Galactosidase activity	
			×1000 (units)	ratio[a]
PAO4290	wild	–	11.46	–
TNP030(#10)	C73T	PAO4290	32.98	2.9
TNP077(#10)	wild	TNP030(#10)	11.55	1.0
TNP030(#11)	A388C	PAO4290	33.91	3.0
TNP077(#11)	wild	TNP030(#11)	10.54	0.9
TNP078(#11)	T104G, A388C	PAO4290	31.31	2.7

[a]Relative value to PAO4290.