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Characterization of a Bifunctional Aminoglycoside-Modifying Enzyme with Novel Substrate Specificity and Its Gene from a Clinical Isolate of Methicillin-Resistant *Staphylococcus aureus* with High Arbekacin Resistance

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A clinical isolate (designated PRC104) of methicillin-resistant *Staphylococcus aureus* was discovered with a novel aminoglycoside resistance profile, including unusually high resistance (MIC 128 μg/ml) to arbekacin (an effective anti-MRSA drug in Japan). We characterized the activity and gene of its bifunctional aminoglycoside-modifying enzyme, AAC(6′)/APH(2″), in comparison with those of a regular one that has been known as the critical resistance basis to both gentamicin and arbekacin in methicillin-resistant *Staphylococcus aureus*. The *aac(6′)/aph(2″)* gene of strain PRC104 contained a single base alteration at a novel site (G1126A) resulting in one amino acid substitution (S376N) in the phosphorylation catalytic motif. The phosphorylation activity of the PRC104 enzyme was enhanced for arbekacin and reduced for gentamicin. Both strain PRC104 and *S. aureus* RN4220 containing the cloned gene were identical in terms of the substrate specificity of the enzyme as well as the aminoglycoside resistance profile, although both mRNA and aminoglycoside resistance levels were markedly high in strain PRC104. Therefore, the cloned *aac(6′)/aph(2″)* gene may represent the molecular basis for the novel aminoglycoside modification capability as well as novel aminoglycoside resistance profile of *S. aureus* PRC104.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been and still is one of the most serious microorganisms in the nosocomial infection problem in Japan, because of relatively high incidence of MRSA strains with multiple resistance to wide varieties of antibiotics[1,2]. Therefore, a limited number of antibiotics are available as anti-MRSA drugs such as vancomycin (VCM) and teicoplanin. In addition, arbekacin (ABK), a semisynthetic aminoglycoside (AG) antibiotic, has also been used as the anti-MRSA orphan drug in Japan since 1990[3].

These antibiotics still keep good anti-MRSA activity clinically[1,2]. In other words, incidence of MRSA isolates with resistance to these antibiotics still remains very low. Actually, neither distinct VCM-resistant MRSA (VRSA) such as the one emerged in US[4] nor incidence increase of ABK-resistant MRSA has been reported[1,2]. Concerning ABK, MRSA strains with low level ABK resistance emerged soon after ABK was introduced into clinics[5]. Subsequent analysis of the ABK resistance revealed a bifunctional AG-modifying enzyme, AAC(6′)/APH(2″) as the critical factor to ABK resistance. This enzyme has been known to play important roles in the multiple AG resistance of MRSA since it confers its host organisms with the capability of 6′-*N*-acetylation and/or 2″-*O*-phosphorylation of AGs that contain 6′-NH_2 and/or 2″-OH as shown in Fig. 1[6~8].

In our epidemiological survey on clinical isolates of MRSA, we learned that over 90% of MRSA strains with AAC(6′)/APH(2″) isolated during 1980 to 2000 at hospitals in Japan were sensitive to ABK (MIC: <8 μg/ml) but highly resistant to GM (MIC: >128 μg/ml). The effectiveness of ABK might be due to the hindrance effect

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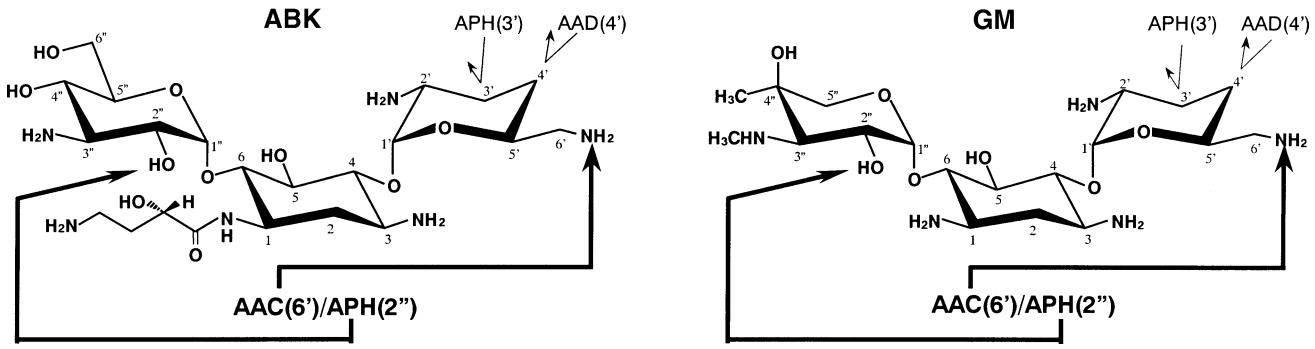
Fig. 1. Structures and enzymatic modification sites of arbekacin and gentamicin.



of the novel semi-synthetic side chain, 1-*N*-(*S*)-4-amino-3-hydroxyl-butyryl group stemmed out of 1-NH_2 of the deoxystreptamine moiety of ABK on the access to the modification sites of AAC(6′)/APH(2″) as shown in Fig. 1[1,9,10]. An additional novel characteristic that might assist ABK to resist inactivation by AAC(6′)/APH(2″) is that the acetylation products of ABK by AAC(3), AAC(2′) and AAC(6′) retained antibiotic activities, while those of GM were inactive except for the acetylated GM by AAC(6′) as HOTTA *et al.* demonstrated[11].

Recently, an exceptional MRSA strain with novel AG resistance profile was isolated[12]. The strain showed high ABK resistance (MIC 128 μg/ml) comparable with GM resistance (MIC: 256μg/ml). This is very unusual, compared with the resistance profile of the dominant type MRSA isolates in Japanese hospitals which are so called NY/Japan type ones[13]. They usually show coagulase serotype II and at least 16～32 fold higher resistance to GM than to ABK when they contain the *aac(6′)/aph(2″)* gene. In this study, we characterize the activity and gene of AAC(6′)/APH(2″) of strain PRC104 and demonstrate a single base alteration in the APH(2″) region of *aac(6′)/aph(2″)* gene that resulted in a novel substrate specificity of the enzyme.

Materials and Methods

Bacterial Strains and Culture Conditions

The bacterial strains and plasmids are described in Table 1. *S. aureus* strains were grown in Brain Heart Infusion (BHI; Difco Lab. ML) with shaking or on BHI agar plates at 37°C. *Esherichia coli* strains were grown in Luria Bertani broth with shaking at 37°C. Oligonucleotide primers were synthesized by Sigma genosis (Japan). Plasmid pAW8 containing *aac(6′)aph(2″)* was constructed as follows; a 2.0 kb fragment spanning *aac(6′)aph(2″)* gene was amplified by PCR using KOD-plus-DNA polymerase (TOYOBO, Japan) and the following two primers, each containing *Bam*HI sites (5′-TATTGGATCCGTTCTTATGGACCTACAT-3′ and 5′-TTCTGGATCCATATATTATATAATCAATC-3′). Subsequently the fragment was ligated to the *Bam*HI site of pAW8 and 1 μg of the ligated plasmid DNA was mixed with *S. aureus* RN4220[14] cells which were suspended in 100 μl of a buffer (1.1 M sucrose and 2 mM $MgCl_2$). Electroporation was carried out with an electric pulse of 1.9 kV and 186 Ω using the electro cell manupulator ECM 600 (BTX Inc. CA). The cells were then transferred into 0.9 μl of 1.1 M sucrose in BHI broth and incubated for 1 hour at 37°C. Transformants were selected by using agar plates containing 10 μg/ml tetracycline and 10 μg/ml kanamycin. The transformants RN4220/w19 and RN4220/w25 contained regular *aac(2′)/aph(2″)* genes from strain BG0021. The transformants RN4220/m33 and RN4220/m47 contained a novel *aac(2′)/aph(2″)* genes from strain PRC104.

DNA Sequencing

The DNA fragments for sequencing were amplified by PCR using genomic DNA as template and purified with a PCR purification kit (Roche, Germany). The PCR products were directly sequenced with a DYEnamic ET Terminator sequencing kit (Amersham Bioscience, Corp. NJ) using a synthetic primer. The sequence was read with ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan Ltd.,

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Remarks	Sources
Strains		
S. aureus		
BG0021	Clinical MRSA isolate with regular *aac(6')/aph(2")*	This study
PRC104	Clinical MRSA isolate with novel *aac(6')/aph(2")*	This study
RN4220	Restriction negative MSSA strain	14)
RN4220/pAW8	RN4220 transformed with shuttle vector, pAW8	This study
RN4220/w19, RN4220/w25	RN4220 transformed with regular *aac(6')/aph(2")*	This study
RN4220/m33, RN4220/m47	RN4220 transformed with novel (S376N) *aac(6')/aph(2")*	This study
E. coli		
XL1 Blue MR	Cloning host strain	Novagen Inc.
Plasmids		
pAW8	*E.coli-S.aureus* shuttle cloning vector, Tet^r	Dr.Wada
pwac/ap8	pAW8 containing a 2.0-kb PCR-amplified fragment of regular *aac(6')/aph(2")*	This study
pmac/ap8	pAW8 containing a 2.0-kb PCR-amplified fragment of novel *aac(6')/aph(2")* with S376N alteration	This study

Japan).

Antibiotic Resistance

MRSA strains were examined for their resistance against 8 AGs: Kanamycin (KM), dibekacin (DKB), amikacin (AMK), ABK, GM, sisomicin (SISO), isepamicin (ISP), netilmicin (NTL). They were used at concentrations ranging from 1 to 1,028 μg/ml by agar dilution method using Mueller Hinton II (Becton Dickinson and com. NJ) medium as previously described[11].

Aminoglycoside-modifying Enzyme (AME) Activity

Cultures (10 ml) were grown to the logarithmic phase with shaking at 37°C in BHI. The bacterial cells were centrifuged, washed once with 3 ml of TE, and resuspended in a lysis solution consisting of 50 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$ and 20 μg/ml lysostaphin. After 15 minutes of incubation at 37°C, the reaction mixture was centrifuged and the resulting supernatant was used as the cell free extract or crude enzyme solution. Acetylation or phosphorylation reactions were carried out at 37°C in a 50 μl reaction mixture with the following composition; 200 μg/ml of AG, 0.1 M phosphate buffer (pH 7.0), 10% (v/v) cell free extract and 4 mM acetyl coenzyme A sodium salt (nacalai tesque., Japan) or ATP disodium salt (Sigma Chemical Co., St. Louis, Mo). The AME activity was monitored by ninhydrin reaction after TLC using a silica gel plate (Merck 5712, Germany) and 5% KH_2PO_4 as the developing agent.

Isolation and Structure Determination of the Phosphorylation Product of Arbekacin

The phosphorylation product of ABK was isolated and its structure was determined by NMR analysis as previously described[5].

Northern Blotting

Bacterial cells were suspended into 100 mM Tris-HCl (pH 8.0) buffer and lysed using lysostaphin (50 μg/ml). Total RNA was isolated from the cell lysate by treating with acid-phenol TRIzol (Invitrogen, CA) followed by incubation for 15 minutes with 1 unit/μg of DNaseI (Roche, Germany). Total RNA (20 μg/ml) was electrophoresed in a formaldehyde containing agarose gel and blotted onto a nylon membrane. Labeling of probe and hybridization were carried out using AlkPhos direct labeling system (Amersham Bioscience corp., NJ). A part of the *aac(6')aph(2")* gene amplified as a fragment of

Table 2. Aminoglycoside resistance of MRSA isolates and *S. aureus* RN4220 with the cloned novel *aac(6′)/aph(2″)*.

Strains	AG resistance (MIC)							
	KM	DKB	AMK	ABK	GM	SISO	ISP	NTL
BG0021	>128	>128	128	8	128	>128	>128	16
PRC104	>1024	>1024	512	128	256	256	>1024	16
RN4220 / pAW8	2	<1	<1	<1	<1	<1	1	<1
RN4220 / w19	>1024	128	8	4	128	256	8	16
RN4220 / w25	>1024	128	8	4	128	256	8	16
RN4220 / m33	>1024	512	8	8	8	4	8	<1
RN4220 / m47	>1024	512	8	8	8	4	8	<1

Strains RN4220/w33 and RN4220/w47 contain the cloned novel *aac(6')aph(2")*

approximately 700 bp by PCR was used as a probe. A fragment of 16S ribosomal RNA gene was used as the control probe. The target sequence was visualized with CDPstar (Amersham Bioscience corp., NJ).

Real Time RT-PCR

DNA-free total RNA prepared from *S. aureus* cells by treating with TRIzol followed by DNaseI was subjected to the reverse transcription (RT) reaction using Rever Tra Ace α (TOYOBO, Japan) according to the manufacture's instructions. Real time PCR reactions were carried out in a total volume of 10 μl containing 200 nM each of gene specific primers, 1 μl of a 10 fold dilution of cDNA and SYBR Premix Ex Taq (TAKARA, Japan). To confirm the absence of DNA in each RNA sample, the control reaction without reverse transcriptase was carried out. The ABI Prism 7000 sequence detection system (Applied Biosystems, CA) was run with the following cycle profiles: 1 cycle at 95°C for 10 seconds, 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. A standard curve was plotted for each primer set with critical threshold cycle values obtained from the amplification of known quantity of DNA isolated from PRC104. The quantity of *aac(6′)/aph(2″)* cDNA was normalized to that of *16SrRNA* cDNA in each strain. Each RNA sample was tested in duplicate. The forward and reverse primers for *aac(6′)/aph(2″)* were 5′-TACAGAGCCTTGGGAAGATG-3′ and 5′-CATTTGTGGCATTATCATCATATC-3′, and those for *16SrRNA* were 5′-GGATTAGATACCCTG-GTAGTC-3′ and 5′-AGGGTTGCGCTCGTTG-3′, respectively.

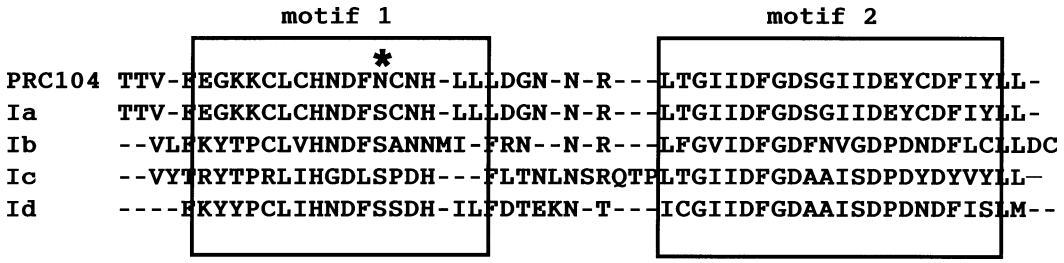
Results

Aminoglycoside (AG) Resistance of Strain PRC104

AG resistance profiles of strain PRC104 with unusually high ABK resistance and strain BG0021 with regular ABK resistance were shown in Table 2. The resistance (MIC) to ABK and GM were 128 and 256 μg/ml, respectively, in strain PRC104, whereas 8 and 128 μg/ml in strain BG0021. PCR typing demonstrated that these strains carried bifunctional AG-modifying enzyme (AME) gene *aac(6′)/aph(2″)*. In addition, strain PRC104 carried *aad(9)* and *aph(3′)* genes and strain BG0021 carried *aad(9)* and *aad(4′,4″)* though data were not shown. As represented by strain BG0021, MRSA isolates with AAC(6′)/APH(2″) gene usually show weak or low resistance to ABK, but distinctive resistance to GM and the other AGs. Usually, GM resistance level is 16~32 fold higher than ABK-resistance level. In this regard, strain PRC104 is exceptional because of its similar levels of resistance to both ABK and GM.

AG resistance profiles of *S. aureus* RN4220 clones transformed with *aac(6′)/aph(2″)* genes from strains PRC104 and BG0021 were also shown in Table 2. The transformants *S. aureus* RN4220/m33 and RN4220/m47 (hereafter m33 and m47, respectively) showed the same AG resistance profile as that of strain PRC104, although their resistance levels were distinctively lower (16~64 fold) except for KM resistance. Their resistance profile was, on the other hand, distinct from that of the other transformants *S. aureus* RN4220/w19 and RN4220/w25 (hereafter w19 and w25, respectively) containing regular type *aac(6′)/aph(2″)*. The transformants w19 and w25 showed similar AG resistance to that of strain BG0021, except for

Fig. 2. Alignment of PRC104 APH(2″) motif domain in comparison with those of staphylococci and enterococci.



Asterisk indicates the position of the S376N substitution. Motif 1 and 2 may represent the catalytic site and the ATP-Mg^{2+}-binding domain, respectively. Ia: *S. aureus*, Ib: *E. faecium* (AF207840), Ic: *E. gallinarum* (U51479), Id: *E. casseliflavus* (AF016483).

resistance to AMK and ISP. This should be due to that w19 and w25 lack *aad(4′,4″)* that may inactivate both AMK and ISP by modifying their 4′-OH.

Alteration Detected in *aac(6′)/aph(2″)* Cloned from Strain PRC104

Nucleotide sequencing revealed that the coding region of *aac(6′)/aph(2″)* from PRC104 contained a single nucleotide alteration (G1126A) resulting in S376N substitution at a unique position in the highly conserved catalytic domain for 2″-*O*-phosphorylation (Fig. 2). Additionally, *aph(3′)* was also sequenced but no alteration was detected.

AG Modification Activity of Strain PRC104 and Transformants

Activities of acetylation and phosphorylation of ABK and GM were examined with cell free extracts (CFEs) from strain PRC104 and w19 as shown in Fig. 3A. It turned out that strain PRC104 showed a markedly higher ABK phosphorylating activity and a distinctly lower GM phosphorylating activity, compared with those of the strain w19. Structure determination of the phosphorylated ABK by NMR analysis indicated that all of the NMR signals were identical with those of authentic 2″-*O*-phosphorylated ABK.

Fig. 3B shows phosphorylation of ABK and GM using CFEs from strains w19 and w25 containing the regular type AAC(6′)/APH(2″) as well as strains m33 and m47 containing the novel S376N variant enzyme. ABK phosphorylation was markedly faster with CFEs from strains m33 and m47 than with those from strains w19 and w25. In contrast, the phosphorylation of GM was faster with CFEs from strains w19 and w25. These results were closely similar to those obtained with CFE from strain PRC104. The acetylation of ABK and GM was faster with CFEs from strains w19 and w25 (data not shown). It was notable that the ABK reaction mixtures retained weak but distinct antibiotic activity even after complete acetylation.

mRNA Level of *aac(6′)/aph(2″)* in PRC104

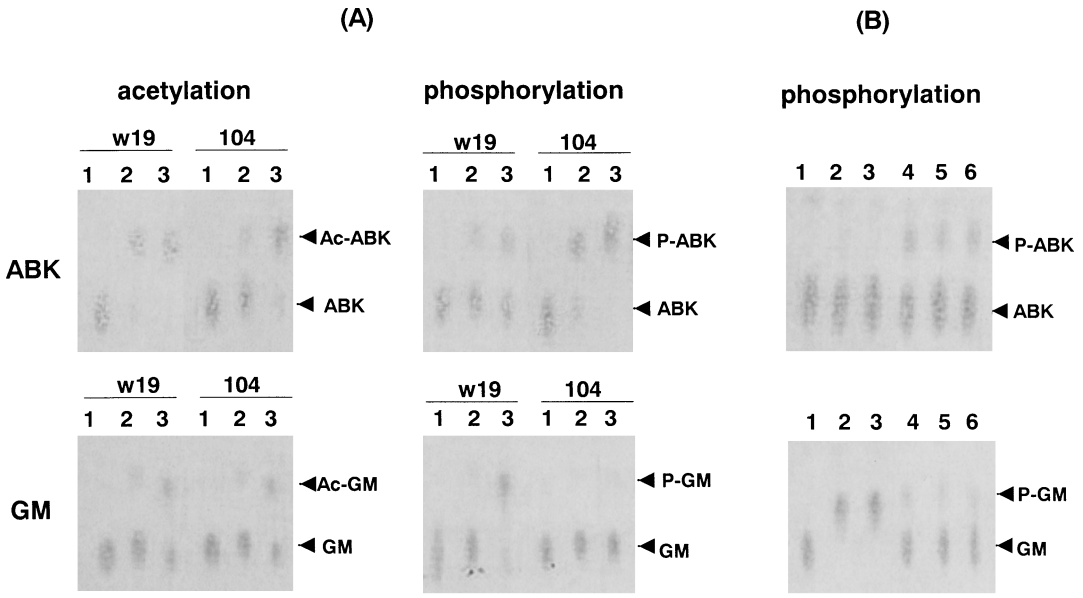
Fig. 4A shows Northern blot analysis results. The mRNA level of *aac(6′)/aph(2″)* was markedly higher in strain PRC104 than in the other strains (w19, w25, m33 and m47) that contained the cloned *aac(6′)/aph(2″)* genes. In the real time RT-PCR (Fig. 4B), it was confirmed that *aac(6′)/aph(2″)* mRNA level from PRC104 was about 5 folds higher than those of the other strains. No substantial difference in the mRNA level of *aac(6′)/aph(2″)* was observed among the strains other than strain PRC104. It was thus obvious that high level expression of the novel type *aac(6′)/aph(2″)* gene in strain PRC104 markedly high AG resistance.

Discussion

Compared to known clinical MRSA strains with AG resistance, strain PRC104 is unusual because of its unusually high level of ABK resistance comparable to that of GM resistance.

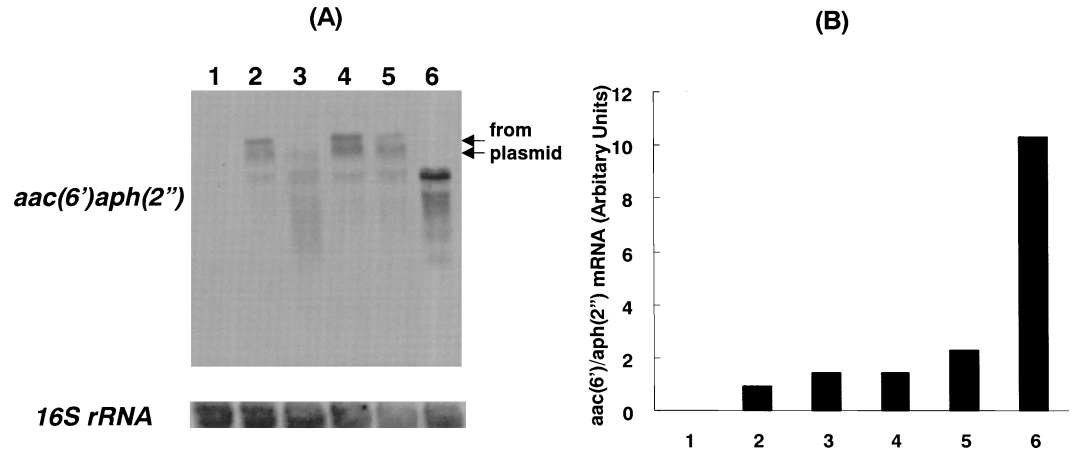
Since *aac(6′)/aph(2″)* has been known as the sole

Fig. 3. AG modifying activity of strain PRC104.



(A) TLC after acetylation or phosphorylation reaction for 30 minutes with CFEs (Lanes 1, 2 and 3 refer to 0, 250, 500 μg/ml protein used, respectively) prepared from strains RN4220/w19 and PRC104. (B) TLC after phosphorylation reaction (1 hour) of ABK or GM with CFEs prepared from strains containing regular or novel types of AAC(6′)/APH(2″). Lanes 1, 2, 3, 4 and 5 refer to cell free extracts prepared from RN4220 clones containing pAW8, w19, w25, m33, and m47, respectively. Lane 6 refers to CFE from strain PRC104.

Fig. 4. *aac(6′)/aph(2″)* mRNA levels in RN4220 transformants and PRC104.



(A) Northern blotting: Lanes 1, 2, 3, 4 and 5 refer to total RNA prepared from RN4220 clones containing pAW8, w19, w25, m33, and m47, respectively. Lane 6 refers to total RNA from strain PRC104. (B) Relative *aac(6′)/aph(2″)* mRNA level to that of *16S rRNA* mRNA. Each bar designates the means of duplicate samples.

resistance factor to both ABK and GM in MRSA, the corresponding gene from strain PRC104 was cloned and sequenced, revealing a single base alteration in comparison with the regular gene sequence. This was deduced to result in a single amino acid substitution (S376N) in the catalytic phosphorylation domain of the encoded product (Fig. 3).

The cloned gene conferred upon *S. aureus* RN4220 a resistance profile similar to that of strain PRC104 and CFEs from both organisms contained enzyme activities with similar substrate specificity in AG phosphorylation. *i.e.* higher phosphorylation of ABK and lower phosphorylation of GM compared with the corresponding activities of strains harbouring the regular *aac(6′)/aph(2″)* gene. These results indicate that the cloned gene may be responsible for the AG resistance of strain PRC104, although the AG resistance levels of *S. aureus* RN4220 containing the cloned gene were markedly lower than those of strain PRC104. This was apparently due to enhanced expression of *aac(6′)/aph(2″)* in PRC104. Thus, when *aac(6′)/aph(2″)* mRNA levels in the two organisms were compared, the level was markedly higher in PRC104 than in *S. aureus* RN4220 containing the cloned gene, although no difference at the nucleotide level was found in the promoter or upstream region of the respective genes. Nor was there any increase in the copy of the gene in PRC104. Therefore, the underlying factor for enhancing the expression of *aac(6′)/aph(2″)* gene in strain PRC104 remains to be resolved.

There are reports on plasmids containing *aac(6′)/aph(2″)* that attribute increased ABK resistance to overproduction of AAC(6′)/APH(2″), although the highest MIC value remained at 32 μg/ml. In this regard, MATSUO *et al.* reported that a 12 bp deletion in the 5′-upstream region of the *aac(6′)/aph(2″)* structural gene produced a strong promoter activity to overexpress the gene[15]. However, the deduced structure of the encoded product remained unchanged. This illuminates the uniqueness of the AAC(6′)/APH(2″) enzyme of strain PRC104 in which a specific change in the phosphorylation catalytic domain was found.

The substrate specificity of AG modifying enzymes has also been studied, based on mutagenesis. LEE *et al.*[16] showed that enhanced levels of AG resistance resulted from single base mutations of H258L and F108L in APH(2″)-Ic. These substitutions correlated with the enhancement of MICs of several AGs. On the other hand, BOEHR *et al.*[17] found that D99 of AAC(6′)-Ie was an active site residue of AAC(6′)-Ie. FUJIMURA *et al.* reported that D80G substitution in AAC(6′)-Ie conferred novel AAC(4‴) activity catalyzing acetylation of the NH_2 group of the 1-N-side chain of ABK[18,19]. The AAC(6′)/APH(2″) with a novel alteration of S376N that we demonstrated in this study may resulted in enhanced ABK phosphorylation and the reduced GM phosphorylation. Concerning the resistance to 8 different AGs, AAC(6′)/APH(2″) with S376N must cause enhanced levels of resistance to KM group including ABK and AMK, and reduced levels of resistance to GM group, especially NTL. Thus, these results indicated that the substitution changed the substrate specificity of the enzyme.

From the point of view of epidemiology, on the other hand, special attention should be paid for the spread of this unusual *aac(6′)/aph(2″)* gene.

Acknowledgements

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