Molecular mechanism for the enhancement of arbekacin resistance in a methicillin-resistant *Staphylococcus aureus*

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Abstract **We have clinically isolated a methicillin-resistant *Staphylococcus aureus* (MRSA) K-1 which exhibits enhanced arbekacin (Abk) resistance. In this study, we investigated a molecular mechanism for the overproduction of a bifunctional enzyme catalyzing both 2″-*O*-phosphorylation and 6′-*N*-acetylation of aminoglycoside antibiotics that is encoded by *aacA-aphD* and designated [AAC(6′)/APH(2″)] and is expressed in MRSA K-1. The sequence analysis of the 5′-adjacent region of the *aacA-aphD* structural gene in MRSA K-1 showed that 12 bp are deleted from the *aacA-aphD* promoter region when compared with that in MRSA B-26, which exhibits lower resistance to Abk than K-1. By artificially deleting the 12 bp from the corresponding region in MRSA B-26, we confirmed that the strain increases Abk resistance to the same level as seen in MRSA K-1, which suggests that the 12 bp deletion from the 5′-adjacent region of the *aacA-aphD* structural gene created a strong promoter to overexpress the bifunctional enzyme.**
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Key words: *aacA*; Aminoglycoside antibiotic-inactivating enzyme; *aphD*; Arbekacin resistance; Methicillin-resistant *Staphylococcus aureus*

1. Introduction

Because of the clinical use of methicillin and related antibiotics such as cephems of the third generation, methicillin-resistant *Staphylococcus aureus* (MRSA) has appeared and diffused throughout the world. MRSA expresses resistance to the antibiotic by producing a specific penicillin-binding protein, designated PBP2′ [1]. The protein, encoded by *mecA* [2], has a decreased binding affinity for methicillin [1]. The appearance of MRSA has given rise to a serious problem, namely, that MRSA exhibits drug resistance to many kinds of antibiotics in addition to methicillin. We have in fact found that almost all strains of MRSA isolated at Hiroshima University Hospital, Japan, were resistant even to an anti-tumor agent, bleomycin (Bm), which had not been used as an antibacterial agent [3–5].

Vancomycin, a glycopeptide antibiotic, has been used to treat MRSA infections worldwide. In Japan, arbekacin (Abk), a semi-synthetic aminoglycoside antibiotic that exhibits lower side effects than vancomycin, has also been used. Abk has both amino and hydroxyl groups, which can be modified by several aminoglycoside antibiotic-inactivating enzymes. However, Abk expresses anti-bacterial activity to a wide variety of aminoglycoside antibiotic-resistant bacteria [6]. This is because a 4-amino-2-hydroxybutyric acid group contained in the Abk molecule plays a key role to protect the antibiotic from inactivation by a variety of aminoglycoside antibiotic-modifying enzymes [6].

Methicillin-sensitive *S. aureus* (MSSA) FDA209P, a standard strain for the measurement of drug resistance, is very sensitive to Abk, which for this strain exhibits a minimum inhibitory concentration (MIC) below 0.2 μg/ml. MRSA B-26 is a Bm-resistant strain isolated at Hiroshima University Hospital [3–5]. MRSA B-26 was less susceptible to Abk (MIC = 1.56 μg/ml) than FDA209P, an effect mediated by a bifunctional enzyme [AAC(6′)/APH(2″)] that catalyzes both 2″-*O*-phosphorylation and 6′-*N*-acetylation [7]. We have recently found that a strain isolated from a patient infected with MRSA exhibited a higher resistance to Abk than B-26. The MIC to Abk in the strain, designated MRSA K-1, is 12.5 μg/ml. The objective of this study was to elucidate the reason that MRSA strain K-1 exhibits enhanced Abk resistance.

2. Materials and methods

2.1. Bacterial strains and plasmids used

MSSA FDA209P was used as a *S. aureus* strain that is very susceptible to Abk. Ten MRSAs, which were clinically isolated at hospitals in Hiroshima during 1991–1996, are coagulase type VII strains. MRSA B-26 [3–5], a coagulase type II strain isolated as a Bm-resistant strain at the Hiroshima University Hospital, is more resistant (MIC = 1.56 μg/ml) to Abk than FDA209P. MRSA K-1, which was clinically isolated recently at the Hiroshima Prefectural Hospital, is more resistant to Abk (MIC = 12.5 μg/ml) than the B-26 strain. The bacterium, which is a coagulase type II strain, is also resistant to Bm. *Escherichia coli* JM109 and pUC18, pUC19, and pUC119 were used as a host and vectors for cloning experiments, respectively. *E. coli* XL1-Blue was used as a host for the assay of chloramphenicol acetyl-

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Abbreviations: AAC, aminoglycoside antibiotic acetyltransferase; Abk, arbekacin; APH, aminoglycoside antibiotic phosphotransferase; Bm, bleomycin; CAT, chloramphenicol acetyltransferase; Dbk, dibekacin; DTT, dithiothreitol; Gm, gentamicin; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*

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transferase (CAT) gene expression. *S. aureus* RN4220 [8] was used as a host strain to introduce *aacA-aphD*.

2.2. Determination of the MIC

The MIC of Abk to MRSA was determined according to the methods established by the Japanese Society of Chemotherapy [9].

2.3. Assay of aminoglycoside antibiotic acetyltransferase (AAC) and aminoglycoside antibiotic phosphotransferase (APH) activities

Bacterial cells, grown in LB medium at 37°C for 12 h, were collected by low-speed centrifugation, washed using an AAC assay buffer (10 mM Tris–HCl pH 7.4, 10 mM magnesium acetate, 10 mM ammonium chloride, and 1 mM 2-mercaptoethanol) or an APH assay buffer (20 mM Tris–HCl pH 7.4, 10 mM magnesium acetate, 2.5 mM ammonium chloride, 1 mM KCl, and 0.2 mM dithiothreitol (DTT)) and suspended in the same buffer. To obtain the cell-free extract, the *S. aureus* cells were incubated for 1 h at 37°C together with 20 μg lysostaphin before sonication, but the *E. coli* cells were sonicated without prior treatment. The cell-free extract was centrifuged at 16 000×*g* for 10 min at 4°C, and the resulting supernatant was used as an enzyme solution. AAC or APH activity was analyzed as described previously by the phosphocellulose paper-binding assay method [10]. One unit of AAC or APH activity was defined as the amount of enzyme that catalyzes conversion of 1 μmol of aminoglycoside antibiotic in 1 min.

2.4. Southern blotting

S. aureus cells grown in LB medium for 12 h at 37°C were washed with solution I (25 mM Tris–HCl pH 8.0, 50 mM glucose, and 25 mM EDTA) and suspended in the same solution. After the cells had been lysed by lysostaphin supplemented with 10% sodium dodecyl sulfate (SDS), the chromosomal DNA was isolated. Southern blot hybridization was principally performed according to the general method [11]. The chromosomal DNA was digested with the given restriction enzymes (*Xba*I, *Pst*I, and *Hin*dIII), electrophoresed in agarose gel, and followed by blotting onto a nylon membrane (Hybond N+, Amersham, Sweden). DNA hybridization was done at 55°C overnight in a 5×SSPE, 0.5% SDS, 5×Denhard't solution, and 20 μg/ml sheared calf thymus DNA supplemented with the DNA probe labeled with [α-^{32}P]dATP using the Nick Translation Kit (Takara Shuzo, Japan). The probe was part of the *aacA-aphD* gene (+212–+900; A in the ATG start codon of *aacA-aphD* = +1) [12], obtained by polymerase chain reaction (PCR), with primers 5′-GTTCCTATTGGATATGGACAAATAT-3′ and 5′-TCGTTTTAACAAATTTTGTTCTTCT-3′ using chromosomal DNA from MRSA B-26 as a template. After hybridization, the nylon membrane was washed twice with 2×SSPE containing 0.1% SDS at 55°C for 10 min and then rinsed with 1×SSPE containing 0.1% SDS for 15 min at the same temperature.

2.5. Northern blotting

S. aureus cells grown until 1 OD_{600nm} in LB medium were suspended in STET buffer (10 mM Tris–HCl pH 8.0, 8% sucrose, 0.5% Triton X-100, and 50 mM EDTA) and lysostaphin added to 100 μg/ml for cell lysis. Total RNA from the cell lysate was isolated using Isogen (Nippon Gene, Japan) based on the method of Chomczynski and Sacchi [13]. The RNA (40 μg) was electrophoresed in formaldehyde containing agarose gel and blotted onto a nylon membrane (Hybond N+) [11]. After pre-hybridization of RNA had been carried out at 45°C for 1–2 h, the ^{32}P-labeled DNA was hybridized to RNA on the membrane at 45°C for 12 h. The probe DNA was the same as that used for Southern blotting analysis.

2.6. DNA sequencing

The staphylococcal DNA fragment, subcloned into pUC119, was used for the construction of deletion mutants with a Kilo-Sequence Deletion Kit (Takara Shuzo, Japan). DNA was sequenced by the dideoxy chain termination method [14] using an AutoRead Sequencing Kit and the A.L.F. DNA sequencer (Pharmacia LKB Biotechnology, Sweden). The sequence data were analyzed for an open reading frame and for a promoter region with GENETYX (Software Development, Japan).

2.7. Determination of the transcriptional starting point by the primer extension method

An annealing reaction mixture consisting of RNA (10 or 30 μg) and a biotin-labeled primer (1.5 pmol) were incubated at 65°C for 10 min and then cooled to 37°C. A 20 μl portion of the annealing sample was mixed with 10 μl of a 5×first-strand buffer, 5 μl of 0.1 M DTT, 2.5 μl of 10 mM dNTP, 5 μl of actinomycin D (1 mg/ml), and 7.5 μl of water and incubated at 42°C for 2 min before the addition of reverse transcriptase (Superscript II, Gibco, USA). After the primer extension experiment had been done at 42°C for 20 min, the reaction was stopped by incubation at 37°C for 20 min after the addition of 10 μl RNase A (2 mg/ml). The resulting DNA was treated with phenol/chloroform and purified by ethanol precipitation. The purified DNA was denatured and applied to a 6% acrylamide gel containing 7 M urea. After electrophoresis at 35 W for 2–6 h, the DNA was transblotted onto a nylon membrane (Biodyne B, Japan Genetics, Japan). Detection of DNA was carried out using a Phototope-Star Detection Kit (New England Biolabs, UK) according to the supplier's instruction manual. Primer A (5′-TATACATATTTCATTTTCAACTATATTCAT-3′), which anneals to the 5′-adjacent region of the *aacA-aphD* structural gene, and primer B (5′-AAAAGTGTATAGCAATATGCAAATCCTATA-3′), which hybridizes to the nucleotide sequence located 270 bases upstream of the *aacA-aphD* structural gene, were used in the primer extension.

2.8. Assay of the MRSA's promoter activity in E. coli

To amplify the *aacA-aphD* structural gene together with P*alr* or P*ahr* in K-1 and B-26 by PCR, three kinds of sense primers, P1B (5′-TCAAGGATCCACATCAATTTTGATAAGTAG-3′), P2B (5′-TATAGGATCCTACACAGGAGTCTGGACTTG-3′), and P3B (5′-ATATGGATCCTGATATTGATAATTTAAAAG-3′), and an antisense primer, PEE (5′-CACTTGAATTCTTATTTTTCATTTTTTTAA-3′), were synthesized. Each *aacA-aphD*, including P*alr* or P*ahr*, was amplified using the chromosomal DNA from K-1 and B-26 with the antisense primer and each sense primer in turn. The resulting amplified DNA was cloned into pUC18 to generate p18D1B-26, p18D2B-26, p18D1K-1, p18D2K-1, and p18D3K-1 (see Fig. 4A). *E. coli* JM109, transformed with one of these plasmids, was analyzed for the [AAC(6′)/APH(2″)] activity.

Promoter activities were also evaluated using a CAT assay. Using the sense primers P1B and P2B, and the antisense primers P1′H (5′-ATTAAAGCTTCTTCCATAAACTCAGTCAAG-3′) and P2′H (5′-TATCATTAAGCTTTCCTTTTAAATTATCAA-3′), the P*alr* or P*ahr* promoter from the K-1 and B-26 strains was amplified by PCR. Each PCR-amplified promoter was inserted into the CAT assay vector, pKK232-8 [15], to generate p1CATB-26, p2CATB-26, p1CATK-1, and p2CATK-1 (see Fig. 4B). The CAT activity in *E. coli* XL1-Blue harboring one of these plasmids was analyzed as follows: a 4 μl portion of seed culture grown at 37°C overnight was inoculated into 4 ml of an LB medium and cultivated until 0.5 OD_{600nm}. The culture was induced with 0.3 mM IPTG and grown for 4 h. The cells collected by centrifugation at 1800×*g* for 5 min were washed with 0.25 M Tris–HCl (pH 7.6), suspended into the same buffer, and sonicated for 10 min. The supernatant obtained by centrifugation was used as an enzyme solution for the CAT assay, which was performed as described elsewhere [11]. One unit of CAT activity was defined as the amount of enzyme that acetylated 1 nmol of the chloramphenicol in 1 min.

2.9. Induction of plasmid DNA into the S. aureus cells

Competent *S. aureus* were prepared according to the method of Schenk and Laddaga [16]. A 70 μl portion of the cell suspension was mixed with 5 μl of DNA solution (< 1 μg) and transferred into a 1 cm gap cuvette. Electroporation was done with an Electroporator II (Invitrogen, USA) with 70 Ω, 50 μF, and 1.7 kV. After the addition of 390 μl of a B2 medium [16], the cell suspension was kept for 1–4 h at 28–37°C and spread on a plate containing LB medium supplemented with the given antibiotic.

2.10. Homologous recombination experiment of the S. aureus chromosome

A 2.5 kb *Hin*dIII-*Hin*dIII fragment containing *aacA-aphD*, which was excised from p19AMEK, was subcloned into pTS-1 [17] to generate pTSAGK. The chimeric plasmid was introduced into *S. aureus* RN4220 by electroporation described above. The plasmid, pTSAGK, purified from the transformant, was used for homologous recombination into MRSA B-26 according to the methods described previously [18,19]. This yielded MRSA B-26, in which the promoter of the *aacA-*

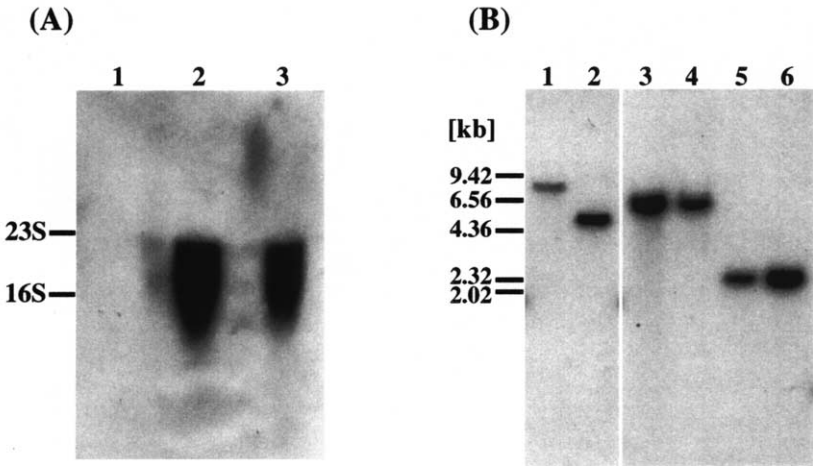

Fig. 1. A: Northern blot analysis of the transcription of the *aacA-aphD* gene. Total RNA (40 μg) isolated from FDA209P, K-1, and B-26 strains was analyzed using an incomplete *aacA-aphD* fragment as a probe. The rRNA positions (16S and 23S) are indicated on the left side of the panel. Lanes: 1, FDA209P; 2, K-1; 3, B-26. B: Analysis of the copy number of the *aacA-aphD* gene in MRSAs B-26 and K-1. The chromosomal DNAs from the B-26 and K-1 strains, digested with the given restriction enzyme, were analyzed by the Southern blotting technique using the same *aacA-aphD* probe as used in A. Molecular sizes are indicated on the left side of the panel. Lanes: 1, *Xba*I-digested B-26; 2, *Xba*I-digested K-1; 3, *Pst*I-digested B-26; 4, *Pst*I-digested K-1; 5, *Hin*dIII-digested B-26; 6, *Hin*dIII-digested K-1.

aphD gene was replaced by that of the K-1 strain. The accuracy of the recombination was confirmed by sequencing of the *aacA-aphD* gene and Southern blot analysis.

3. Results and discussion

3.1. *Abk resistance of MRSA isolated clinically at a hospital in Japan*

Eleven MRSA strains including B-26, which were clinically isolated at hospitals in Hiroshima, Japan, from 1991 to 1996, were randomly selected. They exhibited MICs from 0.78 to 3.13 μg/ml of Abk, compared to 0.2 μg/ml for the MSSA FDA209P. Interestingly, the MIC of Abk for MRSA K-1, which was recently isolated at the Hiroshima Prefectural Hospital, increased to 12.5 μg/ml (Table 1). All of these MRSAs were highly resistant to dibekacin (Dbk) and gentamicin (Gm) over 100 μg/ml.

3.2. *Relationship between Abk resistance and [AAC(6′)/APH(2″)]*

We determined the [AAC(6′)/APH(2″)] activity in cell-free extracts from the above 11 MRSAs and K-1 strains. Dbk, an antibiotic lacking a 4-amino-2-hydroxybutyryl group contained in the Abk molecule, was also used as a substrate to assay the [AAC(6′)/APH(2″)] activity. As listed in Table 1, enzyme activity was observed in MRSA K-1 and in 11 MRSAs from hospitals in Hiroshima; however, none was observed in *S. aureus* FDA209P. The [AAC(6′)/APH(2″)] activity in MRSA K-1 was much higher than that in other MRSA strains, suggesting that the enhanced Abk resistance in MRSA K-1 may depend on the overexpression of [AAC(6′)/APH(2″)].

3.3. *Northern blot analysis of the* aacA-aphD *gene transcript in MRSA*

The above observation led us to investigate the reason for the overproduction of [AAC(6′)/APH(2″)] in MRSA K-1. We analyzed the transcript of *aacA-aphD* using the probe described in Section 2.4. Northern blot analysis revealed a 2.5 kb transcript in both MRSA K-1 and B-26 strains but not in *S. aureus* FDA209P (Fig. 1A). The amount of K-1 transcript was higher than in strain B-26, suggesting that the overproduction of [AAC(6′)/APH(2″)] in the K-1 strain may be due to the increased transcription of *aacA-aphD*.

3.4. *Copy number of* aacA-aphD *in MRSA K-1*

The transposon Tn*4001* consists of an *aacA-aphD* gene, a cryptic gene, *orf132*, and two IS*256*s, between which *aacA-aphD* and *orf132* are located. Tn*4001* is integrated into a plasmid or chromosome of *S. aureus* by mediation of a transposase in IS*256* [20]. There is a possibility that an increment of the copy number of Tn*4001*, integrated into the K-1 chromosome, may give rise to overexpression of the *aacA-aphD* transcript. Therefore, we performed Southern blot analysis of the chromosomal DNAs from K-1 and B-26 strains using the shortened *aacA-aphD* probe. Genomic Southern analyses in MRSA K-1 and B-26 strains showed only single bands hybridizing to the probe (Fig. 1B). These observations suggest that there is one copy of the *aacA-aphD* gene per chromosome

Table 1
MIC (Abk) and [AAC(6′)/APH(2″)] activities in clinically isolated MRSAs

strains	[AAC(6′)/APH(2″)] specific activity (U/mg protein)				MIC (μg/ml)
	AAC		APH		
	Abk	Dbk	Abk	Dbk	
B-26	1.22	6.50	1.61	7.98	1.56
K-1	5.04	24.3	6.58	33.2	12.5
K-2	1.20	5.53	1.58	7.58	3.13
K-3	1.24	6.96	1.64	8.46	1.56
K-4	1.20	6.57	1.48	8.06	1.56
K-5	1.03	5.31	1.40	7.31	0.78
D-1	1.09	5.40	1.42	6.78	1.56
D-2	1.33	7.22	1.69	8.78	3.13
D-3	0.73	3.97	1.26	5.43	0.78
D-4	1.56	8.71	1.90	9.90	3.13
S-1	1.35	6.83	1.85	8.22	1.56
S-2	0.68	3.25	0.90	4.63	0.78

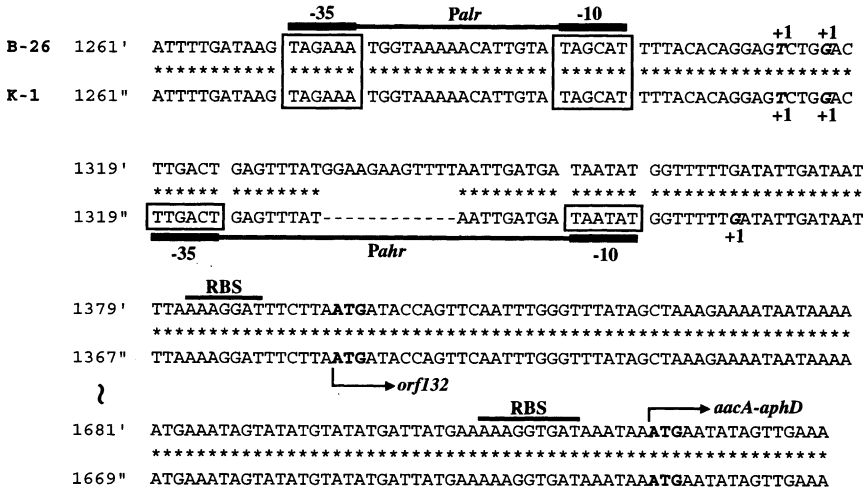

Fig. 2. Nucleotide sequences of the 5′-proximal region of the *aacA-aphD* gene in MRSAs B-26 and K-1. The 12 bp deletion in the K-1 strain is indicated by broken lines. The putative ribosome-binding sites (RBS) for the *orf132* and *aacA-aphD* are indicated by a thick line. The start codons (ATG) for *orf132* and *aacA-aphD* are indicated by bold type. A putative promoter for the *aacA-aphD* gene in MRSA B-26 is designated as P*alr*. P*alr* is also present in the K-1 strain. A putative promoter, P*ahr*, newly generated by the 12 bp deletion, is present in K-1. Deduced −35 and −10 regions for each promoter are indicated by a box. The transcriptional start points are indicated by italicized bold type. The nucleotide number is based on the literature [12].

and that the increased transcription of *aacA-aphD* in K-1 is not due to an increase of the Tn*4001* copy number. Interestingly, the *Xba*I-digested hybridizing DNA bands in K-1 and B-26 have sizes of 4.9 and 7.6 kb, respectively (Fig. 1B), suggesting that the integration site of Tn*4001* into the K-1 chromosome may differ from that into the B-26 chromosome. We found that MRSA K-1 possesses a plasmid of 5.5 kb but that *aacA-aphD* is absent in the plasmid (data not shown).

3.5. *Cloning of* aacA-aphD *from K-1 and B-26 strains and determination of their nucleotide sequences*

To clarify the reason for the overexpression of *aacA-aphD* in K-1, we cloned a 2.5 kb *Hin*dIII-*Hin*dIII fragment carrying *orf132*, *aacA-aphD*, and a part of IS*256* from the chromosomal DNA of K-1 and B-26, respectively. The DNA fragments from B-26 and K-1 were inserted into pUC19 to generate p19AMEB and p19AMEK, respectively. Although each 2.5 kb DNA fragment from both strains was inserted into pUC19 in an orientation opposite that of the *lac* promoter, the transformed *E. coli* cells exhibited high resistance to Dbk (MIC > 100 μg/ml), Gm (MIC > 100 μg/ml), and kanamycin (MIC > 100 μg/ml). This suggests that in both cases the expression of *aacA-aphD* is under the control of its endogenous promoter(s). *E. coli* JM109 [p19AMEK] produced [AAC(6′)/APH(2″)] at a six-fold higher rate than *E. coli* JM109 [p19AMEB]. To elucidate the reasons for the increase in enzyme activity, we determined the nucleotide sequence of these two 2.5 kb *Hin*dIII-*Hin*dIII DNA fragments. We found that although *orf132* and *aacA-aphD* are exactly the same in both strains, 12 bp in the 5′-adjacent region of the *aacA-aphD* structural gene in K-1 were deleted when compared with B-26 (Fig. 2). The corresponding regions from all MRSA strains, which exhibit MICs from 0.78 to 3.13 μg/ml to Abk, were exactly the same as in MRSA B-26.

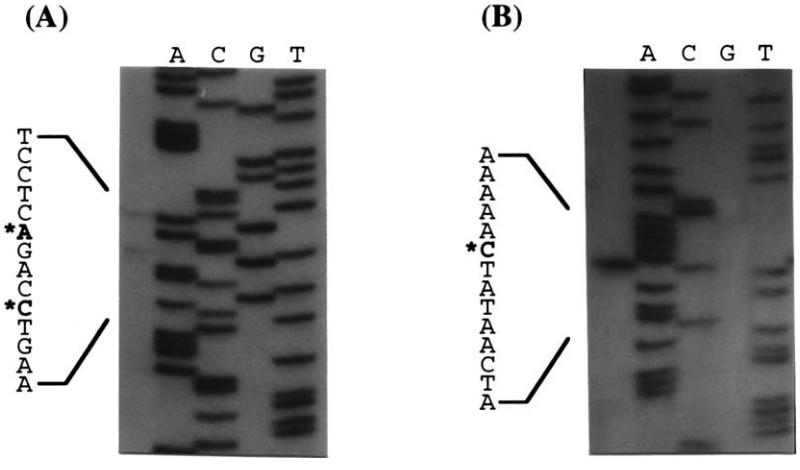

Fig. 3. Transcriptional start points of the *aacA-aphD* gene in MRSAs B-26 (A) and K-1 (B). The transcriptional start point of *aacA-aphD* in B-26 was determined to be guanine or thymine, located approximately 420 bp upstream of the *aacA-aphD* initiation codon by primer extension analysis using primer B. In K-1 the transcriptional start was at a guanine located approximately 360 bp upstream of the *aacA-aphD* initiation codon. The asterisk indicates the transcriptional start point.

3.6. Transcriptional starting point of aacA-aphD in K-1 and B-26 strains

We determined and compared the transcriptional starting point of *aacA-aphD* between MRSAs K-1 and B-26 by the primer extension method. When using primer A, the resulting reverse transcripts from the K-1 and B-26 RNAs had sizes of approximately 350 and 400 bases, respectively (data not shown). Significantly, the amount of DNA product from K-1 was greater than that from B-26, although the amount of RNA used for B-26 was three-fold higher than that used for K-1. In addition to the 350 base DNA band, four DNA bands were also observed in the K-1 strain. Two of the four bands had the same size as those observed in the B-26 strain. However, these four bands were very faint. These results suggest that, although the *aacA-aphD* of the K-1 strain is weakly transcribed from the same promoter as that of B-26, over-transcription of *aacA-aphD* might be due to the fact that the promoter is different from that of the B-26 strain. Since clarification of the transcriptional starting point using primer A was difficult, it was replaced by primer B. The transcriptional starting point of *aacA-aphD* in K-1, determined by using 10 μg RNA, was guanine, located about 360 bp upstream of the initiation codon (Figs. 2 and 3B), but that in B-26 was guanine or thymine, located about 420 bp upstream of it (Figs. 2 and 3A). When determined with 30 μg RNA, additional transcriptional starting points, which are the same as those of B-26, were also observed in the K-1 strain (data not shown).

3.7. The putative promoter sequence of aacA-aphD in K-1

The consensus promoter sequences in the *E. coli* gene are TTGACA (−35 region) and TATAAT (−10 region). Through sequence analysis with the program GENETYX we presumed that the corresponding sequences are TAGAAA and TAGCAT in the B-26 *aacA-aphD* (Fig. 2). The promoter, designated P*alr*, was also present in K-1. We found an additional promoter sequence (TTGACT and TAATAT) located downstream of the K-1 P*alr*, which was generated by deletion of 12 bp (Fig. 2). The promoter, designated P*ahr*, is highly similar to the *E. coli* consensus sequence.

3.8. Comparison of the promoter activity of Palr and Pahr

The promoter activity of the original promoter P*alr* and the newly generated putative promoter P*ahr* was compared in this study. Each *aacA-aphD*, including P*alr* or P*ahr* from K-1 and B-26, was amplified by PCR. Each amplified DNA was cloned into pUC18 to generate p18D1B-26, p18D2B-26, p18D1K-1, p18D2K-1, and p18D3K-1 (Fig. 4A). For each plasmid transformed into *E. coli* JM109 we determined the MIC of Abk and Dbk and assayed the [AAC(6′)/APH(2″)] activity (Table 2). *E. coli* [p18D1B-26] produced significant amounts of bifunctional enzyme, whereas the same host transformed with p18D2B-26 scarcely produced the enzyme due to deletion of P*alr*. *E. coli* [p18D1K-1] produced the enzyme at a six-fold higher level than the same host carrying p18D1B-26, indicating that the enzyme productivity at the higher level is dependent on P*ahr*. In addition, although *E. coli* transformed with p18D2B-26 did not express the [AAC(6′)/APH(2″)] activity, *E. coli* [p18D2K-1] harboring P*ahr* produced the same amount of enzyme as *E. coli* [p18D1K-1]. These results indicate that P*alr* and P*ahr* function in *E. coli* cells and that the promoter activity of P*ahr* is significantly higher than that of P*alr*.

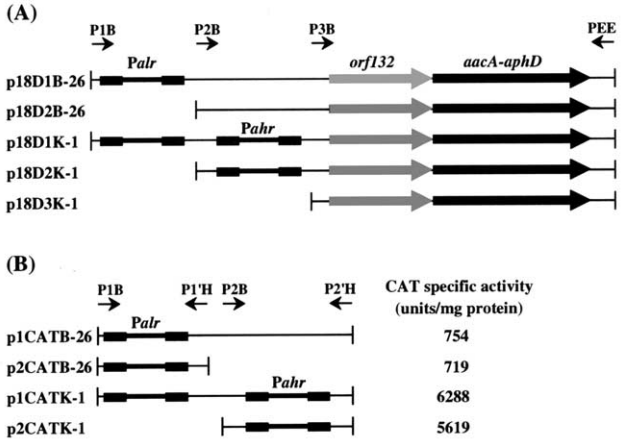

Fig. 4. A: Representation of plasmids used to evaluate the promoter activity of P*alr* and P*ahr*. DNA fragments containing P*alr* and/or P*ahr* together with *orf132* and *aacA-aphD* were amplified by PCR from MRSAs B-26 and K-1, and subcloned into pUC18 to generate the plasmids shown. *E. coli* JM109 harboring each plasmid was analyzed for [AAC(6′)/APH(2″)] activity. The position of primers (P1B, P2B, P3B, and PEE), used to amplify each fragment, is indicated by a small arrow. B: Promoter activity of P*alr* and P*ahr*. DNA fragments containing P*alr* from B-26 and those containing P*ahr*, or P*alr* and P*ahr*, from K-1 were amplified by PCR. Each fragment was inserted into a CAT assay vector, pKK232-8 [15], to generate the plasmids shown. The CAT activity in *E. coli* XL1-Blue harboring each plasmid was analyzed to evaluate the promoter activity. The position of primers (P1B, P2B, P1′H, and P2′H), used to amplify each fragment, is indicated by a small arrow.

Promoter activities were also evaluated by a CAT assay. The P*alr* or P*ahr* promoter from K-1 and B-26 strains was amplified by PCR. Each PCR-amplified promoter was inserted into the CAT assay vector to generate p1CATB-26, p2CATB-26, p1CATK-1, and p2CATK-1 (Fig. 4B). The CAT activity in *E. coli* XL1-Blue harboring one of these plasmids was analyzed. The activity in *E. coli* [p1CATK-1] was about nine-fold higher than that in *E. coli* [p1CATB-

Table 2
MIC (Abk and Dbk) and [AAC(6′)/APH(2″)] activity in *E. coli* JM109 harboring p18D1B-26, p18D2B-26, p18D1K-1, p18D2K-1 or p18D3K-1

Strain	MIC (μg/ml)		[AAC(6′)/APH(2″)] specific activity (U/mg protein)			
	Abk	Dbk	AAC		APH	
			Abk	Dbk	Abk	Dbk
JM109 [p18D1B-26]	0.78	3.13	0.0076	0.035	0.0081	0.039
JM109 [p18D2B-26]	0.39	1.56	0.0021	0.010	0.0026	0.012
JM109 [p18D1K-1]	1.56	25	0.046	0.221	0.046	0.228
JM109 [p18D2K-1]	1.56	50	0.040	0.210	0.042	0.219
JM109 [p18D3K-1]	0.39	1.56	0.0024	0.012	0.0027	0.013

26], again suggesting that P*ahr* is involved in the high activity. *E. coli* [p2CATK-1] exhibited about eight-fold higher activity than that harboring p2CATB-26, showing that P*ahr* expresses stronger transcriptional activity than P*alr* when introduced into *E. coli*.

3.9. *Effect of 12 bp deletion in the promoter region of* aacA-aphD *in the B-26 strain*

The CAT assay showed that P*ahr* expresses stronger transcriptional activity than P*alr* in *E. coli*. It is likely that P*ahr* shows stronger activity than P*alr* in *S. aureus*, thus explaining why the K-1 strain overexpresses the *aacA-aphD* gene. However, Southern blot analysis indicated that the integration site of Tn*4001* into chromosomal DNA is different between B-26 and K-1 strains (Fig. 1B). The different integration may affect the *aacA-aphD* transcription. To distinguish between these possibilities, we introduced a 12 bp deletion into the promoter region of the *aacA-aphD* gene in B-26 by the homologous recombination method [18,19]. The deletion was confirmed by sequencing the promoter region of the *aacA-aphD* gene from the resulting disruptant, designated MRSA B-26K. Southern blot analysis of the *Xba*I-digested B-26K chromosome confirmed that the position of *aacA-aphD* integrated into the B-26K chromosome was the same as in the original B-26 strain (data not shown). The resistance to Abk (MIC = 12.5 μg/ml) in MRSA B-26K was the same as in MRSA K-1. In addition, the [AAC(6′)/APH(2″)] activity in B-26K was almost the same as that in K-1. These results show that the 12 bp deletion from the *aacA-aphD* promoter region converts the B-26 stain to a K-1-like strain (B-26K), regardless of the difference of the integration position of *aacA-aphD* into the chromosome.

In conclusion, MRSA K-1, which exhibits enhanced Abk resistance, can produce a large amount of [AAC(6′)/APH(2″)] enzyme as a result of the increased transcription of *aacA-aphD*. The present study shows that the transcriptional overexpression is dependent on a strong promoter, newly generated by a 12 bp deletion in the *aacA-aphD* promoter region. Recently, two MRSA strains with high Abk resistance (MIC = 64 μg/ml) have been reported [21]. One strain produces [AAC(6′)/APH(2″)] together with AAD(4′,4″), and the other expresses [AAC(6′)/APH(2″)] together with APH(3′)-III. It will be of interest to investigate the molecular mechanism underlying the high Abk resistance in these MRSAs.

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