

Antagonism between Aminoglycosides and β -Lactams in a Methicillin-Resistant *Staphylococcus aureus* Isolate Involves Induction of an Aminoglycoside-Modifying Enzyme

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We encountered three clinical isolates of methicillin-resistant *Staphylococcus aureus* which were susceptible to netilmicin and arbekacin in the absence of β -lactam antibiotics but which were resistant to them in the presence of β -lactam antibiotics. One of these strains, KU5801, was used to further investigate the antagonism between aminoglycosides and β -lactam antibiotics. β -Lactam antibiotics induced bacterial synthesis of aminoglycoside-6'-*N*-acetyltransferase and 2'-*O*-phosphotransferase [AAC(6')-APH(2'')] in association with decreased antimicrobial activities of aminoglycosides. A 14.4-kb *Eco*RI fragment that included the genes that control for β -lactam-inducible aminoglycoside resistance was cloned from a 31-kb conjugative plasmid present in KU5801. Restriction fragment mapping and PCR analysis suggested that a Tn4001-like element containing a gene encoding AAC(6')-APH(2'') was located downstream from a truncated *blaZ* gene. The DNA sequence between *blaR1* and a Tn4001-like element was determined. The Tn4001-IS257 hybrid structure was cointegrated into the *blaZ* gene, and the typical sequences for the termination of transcription were not found between these regions. We deduced that antagonism of aminoglycosides by β -lactam antibiotics in isolate KU5801 involved transcription of the *aac(6')-Ie-aph(2'')-Ia* gene under the influence of the system regulating penicillinase production.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections, and these bacteria have acquired resistance to multiple antibiotics among the wide range of antibiotics used to treat infections caused by these organisms, including aminoglycosides (9, 10, 11). The frequencies of isolation of gentamicin- and tobramycin-resistant (MICs > 8 μ g/ml) MRSA strains in Japan are 61.7 and 95.3%, respectively (9). The activity of netilmicin against gentamicin- and tobramycin-resistant MRSA isolates was found to be more potent than those of gentamicin and tobramycin (20, 24). Arbekacin, a new aminoglycoside antibiotic, showed strong activity against these MRSA isolates because it was less modified by aminoglycoside-6'-*N*-acetyltransferase and 2'-*O*-phosphotransferase [AAC(6')-APH(2'')] or aminoglycoside-4'-*O*-phosphoryltransferase I [ANT(4')-I] than gentamicin or tobramycin (1, 9, 10, 29). This antibiotic was approved for clinical use against MRSA infections by the Japanese Ministry of Health and Welfare in 1990. Since the effects of aminoglycosides used in combination with β -lactam antibiotics are frequently shown to be synergistic (2, 12), arbekacin is often used in combination with a β -lactam antibiotic against MRSA infections. The effects of combinations of arbekacin with β -lactam antibiotics such as ampicillin and sulbactam have been shown to be synergistic and have successfully been used to treat serious infections caused by MRSA (8).

Recently, we encountered three clinical isolates of MRSA which were susceptible to netilmicin and arbekacin in routine testing but which were resistant to them in the presence of β -lactam antibiotics. These MRSA isolates were simultaneously isolated from different patients in a neurosurgery ward. Their genomic DNA fingerprinting patterns obtained by pulsed-field gel electrophoresis were identical (data not shown). We presumed that these MRSA isolates originated from one clone and spread to patients in the same ward. The present report is a description and characterization of the antagonism between aminoglycosides such as netilmicin and β -lactam antibiotics in this strain. To better understand the mechanism of antagonism, we studied the influences of β -lactam antibiotics on bacterial aminoglycoside-modifying enzyme (AME) production and analyzed the genetic sequence located between penicillinase regulatory genes and the gene encoding an AME.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* KU5801 was isolated from a patient who developed a respiratory tract infection following subarachnoid hemorrhage. Strain KU5801 was serendipitously discovered by routine susceptibility testing in a hospital's clinical laboratory, in which truncation of the aminoglycoside zone of inhibition on the side of the adjacent disk containing β -lactam antibiotics was observed by disk diffusion susceptibility testing. For reference, KU5801 was resistant to methicillin, erythromycin, tobramycin, gentamicin, tetracycline, minocycline, and ofloxacin, while it was susceptible to streptomycin, netilmicin, arbekacin, chloramphenicol, and rifampin. No production of β -lactamase could be detected in this strain by an iodometric assay (15). We used *S. aureus* RN4220 and RN4220RIF, a rifampin-resistant mutant arising from RN4220, as recipients in transformation and conjugation studies.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
<i>S. aureus</i>		
KU5801	Clinical isolate	This study
RN4220	Recipient strain for transformation	13
RN4220RIF	Rifampin-resistant mutant of RN4220 used as a recipient strain for conjugation	This study
<i>E. coli</i> JM109	Cloning host strain	Takara Shuzo Co., Ltd.
Plasmids		
pND50	Shuttle cloning vector	30
pT7blueT	PCR-amplified fragment cloning vector	Novagen Inc.
pI258	Reference plasmid	25
pKU111	A 31-kb plasmid isolated from KU5801	This study
pKU112	Recombinant plasmid containing a 14.4-kb <i>EcoRI</i> fragment from pKU111 cloned into pND50	This study
pKU113	Derivative plasmid of pKU112 with reversed 2.5-kb <i>ClaI</i> fragment (see Fig. 4)	This study
pKU114	Recombinant plasmid containing a 2.2-kb PCR-amplified fragment by P1 and A3 primers from pKU111 in pT7blueT	This study

A 31-kb plasmid isolated from KU5801 was designated pKU111. pKU112 is a recombinant plasmid containing a 14.4-kb *EcoRI* fragment encoding a β -lactam-inducible aminoglycoside resistance gene from pKU111 that was inserted into pND50, a shuttle cloning vector for *Escherichia coli* and *S. aureus* (30). In pKU113, a plasmid derived from pKU112, a 2.5-kb *ClaI* fragment was recloned in the opposite orientation within the 14.4-kb *EcoRI* fragment (see below).

Conjugation and transformation. Mating (16) and transformation (19) were carried out as described previously. Transconjugants were selected by using agar plates containing 25 μ g of kanamycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan) per ml and 50 μ g of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml. Transformants were selected by using agar plates containing 25 μ g of kanamycin per ml.

Antibiotics and susceptibility testing. Powders of the different antibiotics of known potency were obtained from the indicated sources: arbekacin and streptomycin, Meiji Seika Kaisha, Tokyo, Japan; netilmicin and gentamicin, Schering-Plough Co., Ltd., Osaka, Japan; imipenem, Banyu Pharmaceutical, Tokyo, Japan; aztreonam, Bristol-Myers Co., Tokyo, Japan; and methicillin, cefazolin, cefotaxime, and lidovomycin, Sigma Chemical Co. All antibiotics were dissolved in distilled water. Susceptibility disks containing 50 μ g of antibiotic per disk were prepared from 8-mm paper circles (Advantec, Tokyo, Japan).

MIC determinations and disk diffusion tests were performed in Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.) by the protocol of the National Committee for Clinical Laboratory Standards (17, 18). Antagonism between aminoglycosides and β -lactam antibiotics was confirmed by the double-disk diffusion test. The MICs of the aminoglycosides were determined with or without the addition of aztreonam (final concentration, 25 μ g/ml) to the agar plates.

AME activity. Cultures of RN4220RIF(pKU111) were grown to the early logarithmic phase with shaking at 37°C in Luria-Bertani (LB) broth (containing tryptone, 10 g; yeast extract, 5 g; and NaCl, 5 g per liter). Induction was then carried out by adding methicillin (Sigma Chemical Co.) at various concentrations and allowing growth to continue. After 2 h of induction, the bacterial cells were centrifuged, washed in 200 ml of 50 mM Tris-HCl buffer (pH 7.5), and resuspended in 5 ml of lysing solution (50 mM Tris-HCl [pH 7.5], 25 μ g of lysostaphin per ml). After 30 min of incubation at 37°C, the cell extracts were separated from the precipitate by centrifugation. AME activity was measured in cell extracts by a bioassay with netilmicin as the substrate, as described previously (7). The assays for determination of AME activity were conducted in triplicate for each sample. The AME activity calculated from the remaining activity of netilmicin in the bioassay is indicated as the number of micrograms of netilmicin inactivated per hour per milligram of protein.

Quantitative analysis of mRNA. The expression of mRNA coding the *aac(6')-Ie-aph(2'')-Ia* gene was analyzed by a real-time (TaqMan) PCR assay. Cultures of RN4220RIF(pKU111) were grown to the early logarithmic phase with shaking at 37°C in 200 ml of LB broth. Then, induction was carried out by adding aztreonam (Bristol-Myers Co.) at 25 μ g/ml and allowing growth to continue. After 2 h of induction, the bacterial cells were centrifuged and washed in distilled water. Total cellular RNA was extracted from the bacterial pellet described above by using the TRIzol reagent by the protocol provided by the manufacturer (Life Technologies, Inc., Rockville, Md.). The RNA solution was treated with DNase

I (Roche Molecular Biochemicals, Mannheim, Germany) and was purified by phenol-chloroform extraction and ethanol precipitation. Total RNA was reverse transcribed for single-strand cDNA synthesis with random hexadeoxynucleotide primers (Promega Co., Madison, Wis.) and Ready-to-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.). The primers and internal probes for amplification of cDNA reverse transcribed from AAC(6')-APH(2'') mRNA were determined with the Primer Express computer program (Applied Biosystems Japan Ltd., Tokyo, Japan) and were prepared by Takara Shuzo Co., Ltd. (Kyoto, Japan), and Applied Biosystems Japan Ltd. The oligonucleotide sequences of the forward and reverse primers were 5'-CCAAG AGCAATAAGGGCATACC-3' and 5'-CATTGCCTAACATTTGTGGCA-3', respectively. The sequence of the internal probe with a 6-carboxyfluorescein fluorescent label was 5'-CCAGAACATGAATTACACGAGGGCAAAAAA-3'. The PCR mixture was prepared with the TaqMan Universal PCR Master Mixture (Applied Biosystems) according to the instructions of the manufacturer, except that the final PCR mixture volume was 25 μ l instead of 50 μ l. PCR amplification was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). To quantify the gene transcripts precisely, 16S rRNA was monitored as an internal control, and each sample was normalized on the basis of its 16S rRNA transcript content. The primers and internal probe for 16S rRNA were determined and prepared as described above, and their oligonucleotide sequences were as follows: forward primer, 5'-GTGAAATGCGCAGAG ATATGGA-3'; reverse primer, 5'-TCGCACATCAGCGTCAGTTAC-3'; and probe, 5'-ACACCAGTGGCGAAGGCGACTTCT-3'. Standard curves for AAC(6')-APH(2'') mRNA and 16S rRNA were generated by using a serially diluted solution of total DNA extracted from RN4220RIF(pKU111) as the template. The amount of the *aac(6')-Ie-aph(2'')-Ia* gene expression was calculated from these standard curves, and quantitative normalization of the cDNA in each sample was performed by use of expression of the genes encoding 16S rRNA as an internal control. Real-time PCR assays were conducted in triplicate for each sample, and a mean value was indicated as the number of micrograms converted into the value of 16S rRNA.

PCR. PCR primers chosen on the basis of previous reports were synthesized by using published DNA sequences (5, 19, 21, 22). These primers, obtained from Takara Shuzo Co., Ltd., are shown in Table 2. Total DNA from KU5801 was purified as described previously (19). PCR amplification was performed with a

TABLE 2. Primers used for PCR

Primer	Sense or antisense	Sequence	Positions ^a
P1	Sense	5'-AATCCTGCAAGAAGAGTTAG-3'	5153-5172
P2	Antisense	5'-TTCCTTCATTACACTCTTGG-3'	6238-6219
A1	Sense	5'-GGACTTGACTGAGTTTATGG-3'	290-309
A2	Antisense	5'-ATGGCAAGCTCTAGGATTAC-3'	618-599

^a Sequences and positions were derived from the nucleotide sequences of Tn 552 reported by Rowland and Dyke (22) and that of *aac(6')-Ie-aph(2'')-Ia* reported by Rouch et al. (21).

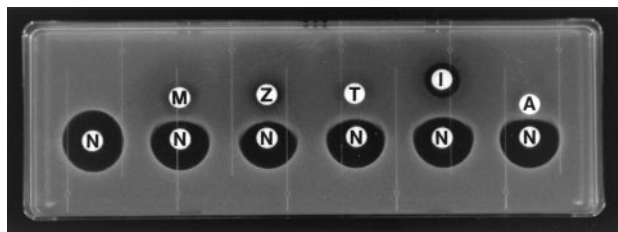


FIG. 1. Antagonism between netilmicin and β -lactam antibiotics for *S. aureus* KU5801 in the double-disk diffusion test. M, methicillin; Z, cefazolin; T, cefotaxime; I, imipenem; A, aztreonam; N, netilmicin. Each of the susceptibility test disks contains 50 μ g for the β -lactam antibiotics and 125 μ g for netilmicin.

DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.). The cycling program was repeated for 25 cycles and included a denaturing step at 94°C for 1 min, an annealing step at 55°C for 2 min, and an extension step at 72°C for 3 min. Samples of the reaction products were analyzed by electrophoresis on 1 or 2% agarose gels in TBE buffer (89 mM Tris-HCl, 89 mM borate, 2 mM EDTA [pH 8.0]). PCR products were detected by ethidium bromide staining followed by UV illumination.

DNA sequence. The DNA fragment amplified by PCR with primers P1 and A2 (Table 2) was cloned into the pT7blueT vector (Novagen, Inc., Madison, Wis.), and a recombinant plasmid designated pKU114 was constructed. The nucleotide sequence of this fragment was determined with universal M-13 RV and M4 primers and with synthetic primers (Takara Shuzo Co.) by the dideoxynucleotide termination method of Sanger et al. (23).

Aminoglycoside and β -lactam antagonism in *aac(6')*-*Ie-aph(2'')*-*Ia* reverse recombinant. To prove that AAC(6')-APH(2'') expression is influenced by *blaZ* transcriptional regulation, pKU113 containing *aac(6')*-*Ie-aph(2'')*-*Ia* in the reverse orientation was constructed from pKU112 by self-ligation after *Cl*A1 digestion (see Fig. 4). pKU113 was transformed into *S. aureus* strain RN4220, and its recombinant strain was used in the study of antagonism between aminoglycosides and β -lactam antibiotics.

Nucleotide sequence accession number. The DNA sequence of the 2,158-bp DNA fragment amplified from RN4220RIF(pKU111) by PCR with primers P1 and A2 cloned into vector pT7blueT was determined and appears in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB074882.

RESULTS

Aminoglycoside and β -lactam antagonism. In the double-disk diffusion test, antagonism between arbekacin and some β -lactam antibiotics was evident as truncation of the arbekacin zone of inhibition on the side of the adjacent disk. By the double-disk diffusion test with KU5801, all β -lactam antibiotics studied produced such a distortion of the zone of inhibition (Fig. 1). When the MICs of aminoglycosides such as arbekacin, gentamicin, netilmicin, streptomycin, and lividomycin with or without aztreonam were determined for KU5801 (Table 3), aztreonam decreased the susceptibilities to arbekacin, gentamicin, and netilmicin; the MICs obtained when aztreonam was

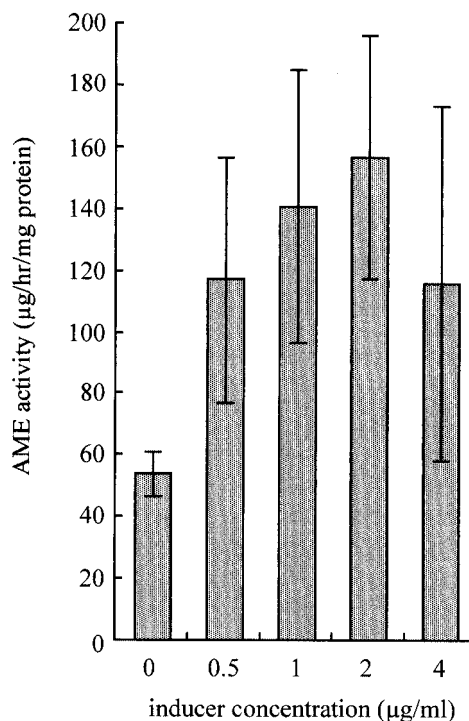


FIG. 2. AME activity of *S. aureus* RN4220RIF(pKU111) with methicillin present as an inducer. Data are expressed as means \pm standard deviations for triplicate tests.

present were four to eight times higher than those when aztreonam was absent. The MICs of streptomycin and lividomycin were not changed by aztreonam.

Conjugal transfer and cloning. Transconjugants with β -lactam-inducible aminoglycoside resistance were obtained from KU5801 at frequency of 6.4×10^{-9} . A large 31-kb plasmid identified in all transconjugants (data not shown) was designated pKU111. The 14.4-kb *Eco*RI fragment was cloned from pKU111 into pND50, and the resulting recombinant plasmid was designated pKU112. Both the transconjugant [RN4220RIF(pKU111)] and the transformant [RN4220(pKU112)] showed β -lactam-inducible aminoglycoside resistance identical to that observed for KU5801 (Table 3).

AME activity. AME activity in RN4220RIF(pKU111) was measured with different concentrations of methicillin (Fig. 2). When RN4220RIF(pKU111) was grown in the absence of methicillin, AME activity was 53.9 μ g/h/mg of protein. AME activity was induced by the addition of 2 μ g of methicillin per ml, resulting in AME activity of 157 μ g/h/mg of protein. De-

TABLE 3. Susceptibilities of *S. aureus* KU5801 and *S. aureus* RN4220RIF(pKU111) to aminoglycosides with or without aztreonam

Strain	MIC (μ g/ml) ^a									
	Netilmicin		Gentamicin		Arbekacin		Streptomycin		Lividomycin	
	Azt(-)	Azt(+)	Azt(-)	Azt(+)	Azt(-)	Azt(+)	Azt(-)	Azt(+)	Azt(-)	Azt(+)
KU5801	4	32	128	1,024	1	4	8	8	32	32
RN4220RIF	0.25	0.25	0.5	0.5	0.5	0.5	8	8	4	4
RN4220RIF(pKU111)	4	64	64	>1,024	1	16	8	8	4	4

^a Azt(-), without aztreonam; Azt(+), with aztreonam at 25 μ g/ml.

spite induction by methicillin, the cell extract could not inactivate streptomycin or lividomycin (data not shown).

Gene expression. Expression of the *aac(6′)-Ie-aph(2′′)-Ia* gene in RN4220RIF(pKU111) was analyzed under conditions with or without induction with 25 μg of aztreonam per ml. When RN4220RIF(pKU111) was grown in the absence of aztreonam, the amount of AAC(6′)-APH(2′′) mRNA was 0.0032 ± 0.00035 μg/μg of 16S rRNA (mean ± standard deviation). On the other hand, the amount of AAC(6′)-APH(2′′) mRNA detected when aztreonam was present was 0.10 ± 0.0074 μg/μg of 16S rRNA. This amount was 32 times higher than the amount of AAC(6′)-APH(2′′) mRNA detected when aztreonam was absent.

Cloning and mapping. The restriction map of the 14.4-kb *EcoRI* fragment of pKU112 was compared with those of Tn552 (22) and Tn4001 (5, 21) (Fig. 3). In the central region of the 14.4-kb *EcoRI* fragment, some restriction sites corresponded to a portion of Tn552 or Tn4001. The Tn4001-like element appeared to be just downstream of the region corresponding to the *bla* operon carried on Tn552.

PCR analysis. Two sets of primers (Table 2), one for detection of the promoter region of the *aac(6′)-Ie-aph(2′′)-Ia* gene and another for detection of the *blaZ* gene, were used. Amplification of DNA fragments from total DNA isolated from RN4220RIF(pKU111) and also RN4220(pI258) (a control strain carrying the complete Tn552 sequence) was confirmed by agarose gel electrophoresis (data not shown). PCR with primers A1 and A2, specific for the promoter region of *aac(6′)-Ie-aph(2′′)-Ia*, yielded a fragment of 329 bp. This DNA fragment was amplified only from total DNA isolated from RN4220RIF(pKU111). PCR with primers P1 and P2, specific for the *blaZ* gene, yielded a fragment of 1,086 bp. This DNA fragment was amplified only from total DNA isolated from RN4220(pI258) and not from total DNA isolated from RN4220(pKU111). PCR analysis was performed with primers P1 and A2 (Fig. 3), and a 2.2-kb DNA fragment was amplified from total DNA isolated from RN4220RIF(pKU111).

DNA sequence. The 2,158-bp DNA fragment amplified from RN4220RIF(pKU111) by PCR with primers P1 and A2 was cloned into vector pT7blueT (Fig. 3). The sequence of the upstream region (nucleotide [nt] positions 1 to 246) corresponded to portions of *blaR1* and *blaZ* carried on Tn552; the level of identity was 97.4%. The region downstream of the *blaZ* gene carried on pKU111 was deleted beginning at nt 625. A nonsense mutation causing defective *blaZ* translation was found at nt 650, 17 bp downstream from the *blaZ* deletion position. The DNA sequence of the downstream region (nt positions 245 to 2157) corresponded to the sequences of IS257, truncated IS256, and *orf132*; these genes are located upstream from *aac(6′)-Ie-aph(2′′)-Ia* carried on pSK41 (3), a staphylococcal conjugative multidrug resistance plasmid. The level of identity between the downstream region of our sequence and the pSK41 sequence was 99.7%. These results show that *aac(6′)-Ie-aph(2′′)-Ia* is located approximately 2 kb downstream from the *blaZ* promoter region and that both the *aac(6′)-Ie-aph(2′′)-Ia* gene and the *blaZ* gene are on the sense DNA strand.

Aminoglycoside and β-lactam antagonism in an *aac(6′)-Ie-aph(2′′)-Ia* reverse recombinant. The MICs of netilmicin with or without aztreonam for RN4220(pKU113) were compared

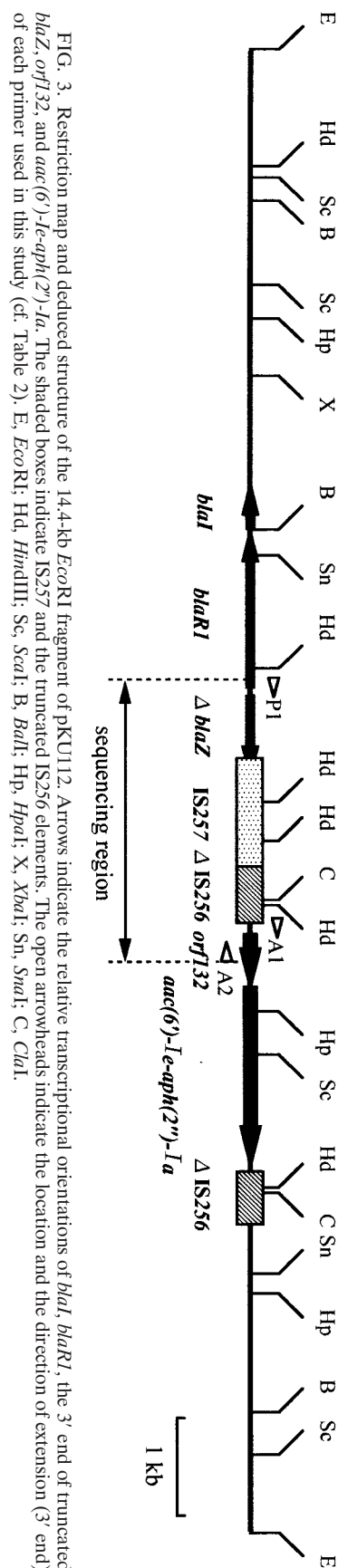


FIG. 3. Restriction map and deduced structure of the 14.4-kb *EcoRI* fragment of pKU112. Arrows indicate the relative transcriptional orientations of *blaI*, *blaR1*, the 3′ end of truncated *blaZ*, *orf132*, and *aac(6′)-Ie-aph(2′′)-Ia*. The shaded boxes indicate IS257 and the truncated IS256 elements. The open arrowheads indicate the location and the direction of extension (3′ end) of each primer used in this study (cf. Table 2). E, *EcoRI*; Hd, *HindIII*; Sc, *ScaI*; B, *BalI*; Hp, *HpaI*; X, *XbaI*; Sn, *SnaI*; C, *ClaI*.

Plasmid	MIC of netilmicin ($\mu\text{g/ml}$)	
	Azt(-)	Azt(+)
pKU112	16	256
pKU113	8	4
pND50 (vector)	0.25	0.25

FIG. 4. Influence of transcriptional orientation of *aac(6')-Ie-aph(2'')-Ia* on netilmicin resistance induced by aztreonam. Arrows indicate the relative transcriptional orientations of *blaI*, *blaR1*, *blaZ*, and *aac(6')-Ie-aph(2'')-Ia*. The 3' end of the *blaZ* gene was deleted. The MICs of netilmicin were determined with or without aztreonam for *S. aureus* RN4220 transformed by each of the plasmids. Azt(-), without aztreonam; Azt(+), with 25 μg of aztreonam per ml.

with those for RN4220(pKU112) (Fig. 4). The MICs of netilmicin without aztreonam for RN4220(pKU113) and RN4220(pKU112) were 8 and 16 $\mu\text{g/ml}$, respectively. The addition of aztreonam increased the MIC of netilmicin for RN4220(pKU112) 16 times and decreased the MIC for RN4220(pKU113) by half. As mentioned above, antagonism between aminoglycosides and β -lactam antibiotics was not observed in RN4220(pKU113).

DISCUSSION

Generally, transcription of genes encoding AME activities is believed to be constitutive; although it is costly in terms of cellular energy, such expression provides constant protection against aminoglycosides (26). Two known exceptions to this generalization are expression of the *aac(6')-Ic* gene of *Serratia marcescens* (27) and the *aac(2')-Ia* gene of *Providencia stuartii* (P. N. Rather, E. Orosz, K. J. Shaw, R. Hare, and G. H. Miller, Program Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 436, 1992). Transcriptional regulation of these genes depends on unusual promoter regions. However, in KU5801, a clinical MRSA isolate, we found a novel aminoglycoside resistance phenotype that was induced by β -lactam antibiotics. In the present study of antagonism between aminoglycosides and β -lactam antibiotics, all β -lactam agents tested induced resistance to gentamicin, netilmicin, and arbekacin but not resistance to streptomycin or lividomycin (Fig. 1; Table 3). Gentamicin, netilmicin, and arbekacin all are modified by AAC(6')-APH(2''), while streptomycin and lividomycin are not (26). Moreover, the AME activity in RN4220RIF(pKU111) under our experimental conditions with methicillin as the inducer was increased 2.9-fold by 2 μg of methicillin per ml. These results demonstrate that resistance to aminoglycosides in KU5801 involves enzymatic modification of these classes by AAC(6')-APH(2'') induced by β -lactam antibiotics.

We also showed that the genes encoding aminoglycoside resistance induced by β -lactam antibiotics are located on the 14.4-kb *EcoRI* fragment of pKU111. Sequence analysis showed that IS257 and a truncated IS256 element were integrated into

the *blaZ* gene and that both genes were coded on the same DNA strand. The sequences of IS257 and the truncated IS256 element were identical to that of the upstream region of the *aac(6')-Ie-aph(2'')-Ia* gene carried on the Tn4001-IS257 hybrid structure, which is commonly found in conjugative plasmids that carry gentamicin resistance, e.g., pSK1 (3). Although the central 1.9-kb region of the Tn4001-IS257 hybrid structure including the *orf132* and *aac(6')-Ie-aph(2'')-Ia* genes is identical to this region of Tn4001, the 899-bp region at the 5' end of IS256 is truncated by insertion of IS257 on both sides of the central region (4). A 105-bp inverted repeat, proposed to be involved in prevention of overexpression of *tnp* in response to a foreign upstream promoter (5), is present in the 5'-end region of IS256 carried on Tn4001; however, in the Tn4001-IS257 hybrid structure, this region is truncated by the IS257 insertion and the inverted repeat corresponding to it is not found (3, 4). The observation that the 105-bp inverted repeat at the 5' end of IS256 is deleted is very important for understanding how β -lactam antibiotics induce production of AAC(6')-APH(2'') in KU5801, because transcription of the *blaZ* gene regulated by the BlaI-BlaR1 system can produce an mRNA that includes the *aac(6')-Ie-aph(2'')-Ia* gene. To confirm this hypothesis, we measured the level of *aac(6')-Ie-aph(2'')-Ia* gene expression in RN4220RIF(pKU111) under conditions in which aztreonam was used as the inducer. It was found that induction by aztreonam led to an apparent increase in AAC(6')-APH(2'') mRNA levels. Furthermore, we constructed pKU113 from pKU112; this is a derivative of pKU112 with a reversed 2.5-kb *ClaI* fragment containing *aac(6')-Ie-aph(2'')-Ia* (Fig. 4). When we determined the susceptibility of RN4220(pKU113) to netilmicin with or without aztreonam, aztreonam did not increase the level of resistance to netilmicin. This result shows that antagonism between aminoglycosides and β -lactam antibiotics is caused by transcription of DNA to mRNA encoding the *blaZ* gene operating region through the action of the *aac(6')-Ie-aph(2'')-Ia* gene.

We believe that the sequence of pKU111 reflects one with genetic rearrangement of the *bla* gene and a Tn4001-IS257 hybrid structure from a conjugative staphylococcal plasmid

such as pUW3626, which was among the plasmids isolated from an outbreak of multiple-drug-resistant *S. aureus* infections at a Kentucky hospital (6); this plasmid carried both the *bla* gene and the Tn4001-IS257 hybrid structure (4). Rearrangements caused by IS257-mediated cointegration into the staphylococcal chromosome or plasmid affect expression of resistance to antibiotics. For example, Leelaporn et al. (14) and Simpson et al. (28) reported that near its ends, IS257 carries a potentially outward-directed -35 promoter sequence that influences expression of downstream genes encoding trimethoprim resistance or tetracycline resistance by formation of hybrid promoters. Although the IS257-derived hybrid promoter did not play a direct role in the induction of AAC(6')-APH(2'') production by β -lactam antibiotics in KU5801, IS257 played a role in the increased level of expression of downstream drug resistance genes in both of these instances (14, 28).

We found a difference between increases in AME activity and increases in *aac(6')-Ie-aph(2'')-Ia* gene expression after induction by a β -lactam antibiotic in RN4220RIF(pKU111). The increase in *aac(6')-Ie-aph(2'')-Ia* gene expression after induction by aztreonam was almost consistent with the increase in β -lactamase activity after induction by methicillin in *S. aureus* RN4220(pI258), which harbored complete *blaI*, *blaR1*, and *blaZ* genes (19). However, in enzyme activity assays, RN4220RIF(pKU111) showed a 2.9-fold increase in AME activity after induction by methicillin. The reason for the inconsistency between these two analyses is not clear. Further studies will be required to explore the relationship between gene expression and enzyme activity.

In conclusion, we demonstrated a mechanism for β -lactam-induced expression of AAC(6')-APH(2'') in KU5801, a clinical MRSA isolate. As a result of genetic rearrangement, IS257 had an influence on the expression of adjacent genes, and this influence potentially enhances the ability of the organism to adapt to an environment containing antibiotics.

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