The Novel Kasugamycin 2′-N-Acetyltransferase Gene *aac(2′)-IIa*, Carried by the IncP Island, Confers Kasugamycin Resistance to Rice-Pathogenic Bacteria

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Kasugamycin (KSM), a unique aminoglycoside antibiotic, has been used in agriculture for many years to control not only rice blast caused by the fungus *Magnaporthe grisea* but also rice bacterial grain and seedling rot or rice bacterial brown stripe caused by *Burkholderia glumae* or *Acidovorax avenae* subsp. *avenae,* respectively. Since both bacterial pathogens are seed-borne and cause serious injury to rice seedlings, the emergence of KSM-resistant *B. glumae* and *A. avenae* isolates highlights the urgent need to understand the mechanism of resistance to KSM. Here, we identified a novel gene, *aac(2′)-IIa,* encoding a KSM 2′-N-acetyltransferase from both KSM-resistant pathogens but not from KSM-sensitive bacteria. AAC(2′)-IIa inactivates KSM, although it reveals no cross-resistance to other aminoglycosides. The *aac(2′)-IIa* gene from *B. glumae* strain 5091 was identified within the IncP genomic island inserted into the bacterial chromosome, indicating the acquisition of this gene by horizontal gene transfer. Although excision activity of the IncP island and conjugational gene transfer was not detected under the conditions tested, circular intermediates containing the *aac(2′)-IIa* gene were detected. These results indicate that the *aac(2′)-IIa* gene had been integrated into the IncP island of a donor bacterial species. Molecular detection of the *aac(2′)-IIa* gene could distinguish whether isolates are resistant or susceptible to KSM. This may contribute to the production of uninfected rice seeds and lead to the effective control of these pathogens by KSM.

T he aminoglycoside kasugamycin (KSM) is produced by *Streptomyces kasugaensis* isolated from soil at Kasuga Shrine in Nara, Japan (49). KSM is absolutely effective on rice blast caused by the fungus *Magnaporthe grisea* and has been widely used in agriculture since 1965 (22). KSM also has good activities against plant bacterial diseases and has been used to control rice bacterial grain and seedling rot caused by the proteobacterium *Burkholderia glumae* (17) and fire blight of apple and pear caused by *Erwinia amylovora* (29). Unlike other aminoglycosides, KSM has a unique structure: it is composed of two sugars, a D-*chiro*-inositol moiety and a kasugamine moiety (2,4-diamino-2,3,4,6-tetradeoxy-D-arabino-hexose), with a carboxy-imino-methyl group (11). KSM inhibits protein synthesis in prokaryotes and *M. grisea* (27, 46), but it fails to induce translational misreading due to lack of the deoxystreptamine moiety common among aminoglycosides (20). KSM is known to bind the bacterial ribosome 30S subunit in the region of the mRNA-binding tunnel in the E-site and P-site and indirectly inhibits tRNA binding at the P-site by perturbing the mRNA-tRNA codon-anticodon interaction during translational initiation (35, 36).

Bacterial resistances to aminoglycoside antibiotics are conferred by several mechanisms such as decreased drug uptake, modification of the ribosomal target, efflux of drugs, and enzymatic modification of drugs (50). In the clinical scene, modifications of the amino or hydroxyl groups of antibiotics are primary mechanisms of bacterial resistance and are mediated by three major groups of modifying enzymes: *N*-acetyltransferases (AACs), *O*-adenyltransferases, and *O*-phosphotransferases (50).

As with other aminoglycosides, resistance mutations for KSM have been found in bacteria. First, a dimethyltransferase, the *ksgA* gene was mapped in *Escherichia coli* (38). Loss of methylation of the two adjacent nucleotides A1518 and A1519 in the helix 45 of the 16S rRNA due to *ksgA* mutation indirectly influenced the con-

formation of helix 24, which is involved in KSM binding (10, 15, 16). The mutations of *ksgA* then confer modest resistance to KSM (15). Higher resistance to KSM is known to result from the mutations of A794, G926, or A1519 in 16S rRNA (52). Recent studies by a chemical probing assay and structural analysis of the KSM-30S subunit complex show that universally conserved residues A794 and G926 are involved in binding KSM (35, 36, 53). Furthermore, mutations in three other genes (*ksgB, ksgC,* and *ksgD*) in *E. coli* cause resistance to KSM independently (13, 39, 54). Mutation of *ksgC* alters the amount of ribosomal protein S2 (54). Cell-free protein synthesis with ribosomes from *ksgA* and *ksgC* mutants show resistance to KSM; in contrast, ribosomes from *ksgB* mutant are sensitive to KSM *in vitro,* indicating that a mutation of *ksgB* results in altered cell membrane permeability to KSM (39). However, detailed molecular mechanisms of KSM resistance in *ksgB, ksgC,* and *ksgD* mutants have not been elucidated yet.

The emergence of two phytopathogenic bacteria resistant to KSM in rice plants is known in Japan. KSM-resistant field isolates of *Proteobacteria, Acidovorax avenae* subsp. *avenae,* which is the causal agent of rice bacterial brown stripe, appeared in 1990 (43). Fourteen years later, KSM-resistant *B. glumae* was isolated (19). Because these are seed-borne diseases, bacterial pathogens infest rice seedlings in nursery boxes and result in serious losses in yield.

TABLE 1 Bacterial strains used in this study

Strain	MIC (μg/ml)[a]					Susceptibility[b]		Presence of $aac(2')$-IIa[c]	Source
	KSM	OX	CuCl$_2$·3Cu(OH)$_2$	Cu(OH)$_2$	TMTD	KSM	OX		
Burkholderia glumae									
210	12.5	0.8	25	25	100	S	S	−	This study
216	25	0.4	25	25	50	S	S	−	This study
3	25	100	25	50	50	S	R	−	This study
5091	3,200	0.4	25	50	50	R	S	+	This study
5096	3,200					R	S	+	This study
5321	3,200					R	S	+	This study
2-1	3,200					R	S	+	This study
4-1-1	3,200	0.4	50	50	50	R	S	+	This study
4-1-2	3,200					R	S	+	This study
4-1-3	3,200					R	S	+	This study
4-1-4	3,200					R	S	+	This study
4-1-5	3,200					R	S	+	This study
4-1-6	1,600					R	S	+	This study
4-1-8	1,600					R	S	+	This study
4-1-9	1,600	0.4	50	50	50	R	S	+	This study
4-2-1	3,200					R	S	+	This study
4-2-2	3,200					R	S	+	This study
9-1-1	3,200	0.4	50	50	50	R	S	+	This study
9-1-2	3,200					R	S	+	This study
11-1-1	1,600	0.4	25	50	50	R	S	+	This study
11-1-2	1,600					R	S	+	This study
Acidovorax avenae subsp. *avenae*									
161	6.3	0.8				S	S	−	This study
199	6.3	0.4				S	S	−	This study
5	6.3	>100				S	R	−	This study
83	3,200	0.4				R	S	+	This study
1-1	800	0.4				R	S	+	This study
2-1	800	0.4				R	S	+	This study
3-2	800	0.4				R	S	+	This study
4-2	800	0.4				R	S	+	This study
5-1	800	0.4				R	S	+	This study
213	800	100				R	R	+	This study
214	800	100				R	R	+	This study
215	800	100				R	R	+	This study

[a] The MIC was determined by using the agar streak method with peptone-glucose medium. KSM, kasugamycin; OX, oxolinic acid.
[b] S, susceptible; R, resistant.
[c] The presence (+) or absence (−) of the $aac(2')$-IIa gene is indicated.

Treating rice seeds in nursery boxes with KSM granules (0.3 to 0.4 g as the active ingredient per box [30 cm by 60 cm by 3 cm]) or with KSM liquid (0.125 to 0.25 g as the active ingredient per box) is highly efficacious in the control of KSM-sensitive rice bacterial stripe and bacterial seedling rot. Unfortunately, KSM fails to control KSM-resistant isolates of *A. avenae* and *B. glumae* (19, 43). Moreover, the mechanism of resistance to KSM in *A. avenae* and *B. glumae* remains obscure.

The aim in the present study was to clarify the genetic determinants of KSM-resistance in these rice-pathogenic bacteria. Our results demonstrate that acetylation of the 2′-amino residue of the KSM by the novel acetyltransferase, AAC(2′)-IIa, encoded by the IncP genomic island confers specific resistance to KSM.

MATERIALS AND METHODS

Bacterial strains. Eighteen KSM-resistant *B. glumae* isolates (isolated from grain or diseased seedlings) and three KSM-susceptible *B. glumae* isolates (one of them showing resistance to oxolinic acid) were used in the present study, as were nine KSM-resistant *A. avenae* subsp. *avenae* isolates

(isolated from grain or diseased seedlings) and three KSM-susceptible *A. avenae* isolates (one of them showing resistance to oxolinic acid). All isolates were supplied by Hokko Chemical Industry Co., Ltd., collections in Japan (Table 1). Liquid cultures of *B. glumae* and *A. avenae* strains were grown aerobically in Luria-Bertani (LB) broth at 28°C with shaking at 120 rpm. *E. coli* DH5α was grown either on LB agar or in LB broth at 37°C except for the antibiotic susceptibility assay.

Chemicals. Kasugamycin hydrochloride, semisynthetic 2′-acetyl-kasugamycin, oxolinic acid, and *bis*(dimethylthiocarbamyl) disulfide (TMTD) were supplied by Hokko Chemical Industry Co., Ltd. Oxolinic acid was dissolved in 100 mM sodium hydroxide, which, at a concentration of 1 mM in the medium, did not affect bacterial growth. The copper compounds copper chloride oxide hydrate [CuCl$_2$·3Cu(OH)$_2$] and copper (II) hydroxide [Cu(OH)$_2$] were used from the agrichemical products Germany-Bord'eaux A and KOCIDE, respectively. Streptomycin sulfate, kanamycin sulfate, gentamicin sulfate, chloramphenicol, and tetracycline hydrochloride were obtained from Wako Pure Chemical Industries, Ltd. Neomycin trisulfate, paromomycin sulfate, ribostamycin sulfate, tobramycin sulfate, sisomicin sulfate, and spectinomycin dihydrochloride were obtained from Sigma-Aldrich Corp.

Antibiotic susceptibility test. The MICs of antibiotics and antibacterial compounds were determined by the agar dilution method. Briefly, 2-fold serial dilutions of drugs were plated in peptone-glucose medium (peptone, 5 g; glucose, 5 g; agar, 18 g; deionized water, 1 liter [pH 6.8]) or Muller-Hinton medium (Eiken Chemical Co., Ltd.). Bacterial suspensions were then streaked on each medium, and the results were observed after 2 days of incubation at 28°C for *B. glumae* and *A. avenae* or after overnight incubation at 37°C for *E. coli*. The lowest concentration of each drug, which completely inhibited bacterial growth, was defined as the MIC.

DNA techniques. Procedures for general DNA techniques, such as *E. coli* transformation, plasmid extraction, restriction enzyme digestion, ligation, and agarose gel electrophoresis were performed essentially as described previously (5). Genomic DNA of *B. glumae* and *A. avenae* was prepared using a Qiagen RNA/DNA kit (Qiagen) or a Bacteria GenomicPrep Mini Spin kit (GE Healthcare).

The sequences of *ksgA* and 16S rRNA genes from *B. glumae* were determined directly by using Ksg-F (5′-ACCGCGGGCATGACGG-3′) and Ksg-831R (5′-TTCGTTTGATTCTTTCGATGTCGA-3′) primers for the *ksgA* gene and S705-F (5′-ATGTGGAGGAATACCGATGG-3′) and S1536-R (5′-AAAGGAGGTGATCCAGCC-3′) primers for the 16S rRNA gene with a BigDye Terminator cycle sequencing kit (Applied Biosystems, Inc.). DNA sequences were performed on a 3100 DNA sequencer (Applied Biosystems, Inc.). Sequence alignment was conducted using the CLUSTAL W program (47).

Cloning of the KSM acetyltransferase *aac(2′)-IIa* gene. The KSM acetyltransferase gene from *B. glumae* 5091 was cloned from libraries of EcoRI-digested genomic DNA constructed in vector pBluescript II SK(+). The ligation products were transformed into *E. coli* DH5α by heat shock. Then, KSM- and ampicillin-resistant transformants were selected on LB agar containing 200 μg of KSM/ml and 50 μg of ampicillin/ml. The recombinant plasmid contains a 5.6-kb insert. DNA fragment analysis confirmed that the responsible gene for KSM-resistance was present in the 5.6-kb insert at the HindIII site.

Nucleotide sequences of 5.6-kb DNA fragments from three independent KSM-resistant clones were determined by the primer-walking method. Sequence analyses were conducted by using CLC Sequence Viewer version 6.5.1 (CLC Bio) and BLASTN and BLASTP algorithms (4). The phylogenetic positions of AAC(2′)-IIa and other *N*-acetyltransferases were determined using the software MEGA 5.05 with the neighbor-joining method (45).

The *aac(2′)-IIa* gene was amplified by PCR using 5′-phosphorylated primers aac(2′)IIa-2F (5′-CAATGAAAGACAGATCCCATGA-3′) and aac(2′)IIa-R (5′-AACAATTTCAGCCTATCAGGC-3′) or the primers aac(2′)IIa-92F (5′-AGATTTGGATTGTGCAATCC-3′) and aac(2′)IIa-R, and the amplicons were cloned into the HincII site of pUC118 (TaKaRa Bio, Inc.) to produce KSM acetyltransferase without the 91-bp promoter region of the *aac(2′)-IIa* gene [pUC118-aac(2′)-IIa P−] or with the promoter region (P+). To verify whether the *aac(2′)-IIa* gene was present among KSM-resistant *B. glumae* and *A. avenae* isolates, the following primers were used for PCR analysis: aac(2′)IIa-57F (5′-GAACCACTGGCTGAAGACCGATTAT-3′) and aac(2′)IIa-710R (5′-ATGTTGTCTCGGTCCGTGCTGTAG-3′). PCR amplification was performed with 1 cycle of 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 1 min.

Detection of the acetyltransferase activity. *E. coli* BL21(DE3) harboring pUC118-aac(2′)-IIa P+ was grown to the mid-log phase. The bacterial pellet was resuspended in 100 mM sodium phosphate buffer (pH 7.6). Bacterial cells were disrupted by ultrasonication, and insoluble materials were removed by centrifugation at 30,000 × *g* for 1 h. Then, the soluble fraction was prepared by using PD-10 columns (GE Healthcare).

Acetyltransferase assays were carried out by measuring the increase in absorbance at 412 nm due to the formation of 5-thio-2-nitrobenzoate (TNB; $\varepsilon_{412} = 14,150\ \text{M}^{-1}\ \text{cm}^{-1}$), generated by the reaction between sulfhydryl group of CoA-SH, the product of the acetyltransferase reaction,

and 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB). The reaction mixture (1 ml) contained 0.2 mM acetyl coenzyme A (acetyl-CoA), 1 mM DTNB, 100 mM sodium phosphate (pH 7.6), and cell extract (600 μg of total protein), and the reaction was initiated by the addition of 1 mM antibiotics at room temperature. The degree of enzymatic reaction was calculated by subtracting from that of a control reaction that lacked antibiotic substrate. For high-performance liquid chromatography (HPLC) analysis, the reactions were performed in 100 μl of a reaction mixture of 2 mM acetyl-CoA, 1 mM KSM, 50 mM sodium phosphate (pH 7.6), and cell extract (300 μg of total protein). After 2 h of incubation at 30°C, heating at 80°C for 10 min terminated the reactions. HPLC analyses were performed by utilizing a YMC-pack ODS-AQ column (4.6 by 250 mm) with UV detection (230 nm) and a mobile phase of acetonitrile-water (20:80 [vol/vol]) at pH 3.5 containing 0.1% sodium decane sulfonate as the ion-pairing agent.

Cloning of the IncP island. To clone the full length of the IncP island, long PCR DNA amplification was performed using KOD plus DNA polymerase (Toyobo Co., Ltd.) with 1 cycle of 94°C for 2 min, followed by 35 cycles of 10 s at 98°C (DNA denaturation), then 10 min at 74, 72, and 70°C for 5 cycles each, and finally 10 min at 68°C for 20 cycles (primer annealing and DNA synthesis). guaA-77F (5′-ACGTCTATTGCGAGATTCATCCGAACGAT-3′) and E1-404R (5′-GGTACCGCTGGTATGGATTTATGAGGCATT-3′) primers were used to clone the IncP island from *B. glumae* 5091. These 5′-phosphorylated primers were designed based on the complete nucleotide sequence of *B. glumae* BGR1 (26) and on the partial sequence of the IncP island from 5091 strain. A 9.6-kb fragment of the IncP island was cloned into pUC118 and sequenced by the primer-walking method. Comparative nucleotide sequence analyses were conducted using Mauve 2.3.1 (7).

Filter-mating assay. KSM-resistant *B. glumae* 5091 was used as the donor strain in filter matings with the recipient *B. glumae* strain 3 or *A. avenae* strain 5, which were KSM-sensitive and oxolinic acid-resistant strains, respectively. Conjugational transfers were conducted by mixing a donor and recipient strain in a 1:1 ratio, and mixed cultures were filtered using a 0.45-μm-pore-size filter (Millipore). After 8 h of incubation on an antibiotic-free peptone-glucose plate, the cells were resuspended from the filter in 3 ml of 50 mM potassium chloride. The transconjugants that could grow on peptone-glucose plates with 100 μg of KSM/ml and 10 μg of oxolinic acid/ml were counted. Conjugation frequencies were expressed as the number of transconjugants per number of donor cells recovered after 2 days of incubation at 28°C.

Detection of the circular forms containing *aac(2′)-IIa*. Inverse nested PCR was used to analyze the circular intermediates containing the *aac(2′)-IIa* and *bglu_5091g13* genes from genomic DNA of *B. glumae* or *A. avenae*. First, inverse PCR was conducted using the aac(2′)IIa-308R (5′-AGTGAAAGATAACGTTCAAGATGCT-3′) and aac(2′)IIa-451F (5′-AGAGTACCGGAAGCTTTGTTTGAG-3′) primers, but no detectable PCR product was obtained. Second, inverse nested PCR was carried out using the aac(2′)IIa-240R (5′-TTCTTCGAGCTTTAGTGCTATGTCA-3′) and hypo-F (5′-TTCATCTGCATTCTGACGACTT-3′) primers from the diluted DNA amplified by the first PCR. Then, the *att*P sequences in the obtained PCR products were analyzed.

Reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated as described previously (2). First-strand cDNA was synthesized from 500 ng of DNase-treated RNA using a gene-specific primer, hypo-R (5′-GCCTGTCCATCGGTAACTAAAA-3′), and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. After first-strand cDNA synthesis, RNA template was removed by treatment with RNase H. PCR amplification was conducted using aac(2′)IIa-57F and aac(2′)IIa-710R primers for the *aac(2′)-IIa* gene and hypo-F and hypo-R primers for the *bglu_5091g13* gene.

Nucleotide sequence accession number. The nucleotide sequence of the IncP island and surrounding region from *B. glumae* 5091 was deposited in the GenBank nucleotide sequence database under the accession number AB669090.

TABLE 2 MICs of various antibiotics for KSM-sensitive or -resistant *Burkholderia glumae*

| *B. glumae* strain | MIC (μg/ml)[a] | | | | | | | | | | | |
| | Aminoglycoside | | | | | | | | | | | |
	KSM	SM	NM	PM	RSM	KM	TOB	GM	SISO	SPC	CP	TC
210	12.5	1.6	25	25	3.1	1.6	3.1	3.1	6.3	3.1	100	3.1
216	25	1.6	25	12.5	3.1	0.8	3.1	3.1	6.3	3.1	100	3.1
3	25	1.6	25	25	3.1	1.6	3.1	6.3	6.3	3.1	50	3.1
5091	3,200	1.6	25	25	3.1	0.8	3.1	6.3	6.3	3.1	50	3.1
4-1-1	3,200	1.6	25	25	3.1	0.8	3.1	6.3	6.3	3.1	50	3.1
4-1-9	1,600	0.8	12.5	12.5	0.8	0.2	0.8	0.8	3.1	0.8	50	3.1
9-1-1	3,200	3.1	25	25	3.1	1.6	3.1	3.1	6.3	3.1	50	3.1
11-1-1	1,600	3.1	25	25	3.1	1.6	3.1	6.3	6.3	3.1	50	3.1

[a] The MIC was determined using the agar streak method with peptone-glucose medium. SM, streptomycin; NM, neomycin; PM, paromomycin; RSM, ribostamycin; KM, kanamycin; TOB, tobramycin; GM, gentamicin; SISO, sisomicin; SPC, spectinomycin; CP, chloramphenicol; TC, tetracycline.

RESULTS

Antibacterial susceptibility of KSM-resistant isolates. To clarify the mechanism of bacterial KSM-resistance in field isolates, we collected KSM-resistant bacterial pathogens from Japan. First, we examined the antibiotic susceptibility of KSM-sensitive and -resistant isolates of *B. glumae*. Although KSM-sensitive strains (strains 210, 216, and 3) exhibited MICs from 12.5 to 25 μg/ml to KSM, 18 KSM-resistant strains exhibited MICs from 1,600 to 3,200 μg/ml to KSM (Table 1). These KSM-resistant isolates caused rice seedling rot in nursery boxes, even after treatment with KSM granules or KSM liquid, which is consistent with a previous report (19). We also examined the susceptibility to other ribosome-targeting antibiotics such as other aminoglycosides, chloramphenicol (inhibition of peptidyl transferase activity), and tetracycline (inhibition of binding aminoacyl-tRNA). Although strain 4-1-9 was more susceptible to other aminoglycoside antibiotics than other strains, there was no significant difference between the susceptibility of KSM-sensitive and -resistant *B. glumae* to streptomycin, neomycin, paromomycin, ribostamycin, kanamycin, tobramycin, gentamicin, sisomicin, and spectinomycin, as well as chloramphenicol and tetracycline (Table 2). KSM resistance was also not correlated with susceptibility to other agrichemicals such as copper, oxolinic acid, and TMTD, which are used to control bacterial plant diseases in Japan (Table 1). These results indicated that KSM-resistant *B. glumae* isolates were resistant to KSM specifically, revealing no cross-resistance to other antibiotics or agrichemicals.

Mutation of the KSM interacting site. Bacterial resistance to KSM by inactivation of the dimethyltransferase gene (*ksgA*) is the most frequent mechanism of spontaneous mutation *in vitro* (29, 33). We detected nucleotide variations, but not changes in amino acids, in *ksgA* gene sequences between KSM-susceptible *B. glumae* strains 210 and 3 (see Fig. S1 in the supplemental material). The sequence of *ksgA* in strain 210 was identical to that of strain BGR1, whose genome has already been sequenced (26). In contrast, the nucleotide sequence of the *ksgA* gene in KSM-resistant *B. glumae* (strains 5091, 4-1-9, and 9-1-1) differed from that of KSM-sensitive strains (strains 210 and BGR1) only by a silent C-to-T substitution at position 282 (see Fig. S1 in the supplemental material).

Previously, it had been reported that KSM-resistance is conferred by three independent 16S rRNA mutations (A794G,

G926A, and A1519C; *E. coli* numbering) (52), whose residues are involved in binding the drug (35, 36). The 3′ sides of 16S rRNA gene sequences, including these KSM binding sites of KSM-resistant *B. glumae* (strains 5091, 4-1-9, and 9-1-1), were identical to those of KSM-sensitive strains (strains 210 and 3) and strain BGR1 (data not shown), indicating that KSM's interacting sites on 16S rRNA are not involved in KSM resistance in *B. glumae*.

Isolation of the KSM acetyltransferase gene. To identify the gene(s) that confer KSM resistance to *B. glumae*, a plasmid library constructed from EcoRI-digested genomic DNA of KSM-resistant 5091 strain was introduced into KSM-susceptible *E. coli* DH5α, and three KSM-resistant transformants were isolated. These three transformants harbored an identical 5.6-kb DNA fragment in the plasmid vector. DNA fragment analysis with HindIII revealed that the gene responsible for KSM resistance was present at the HindIII site. Sequence analysis of transformants revealed that there was an open reading frame (ORF) encoding a general control nonrepressed 5-related *N*-acetyltransferase (GNAT) gene (51) at the HindIII site (Fig. 1). As described below, we denoted this *N*-acetyltransferase gene as *aac(2′)-IIa* because of the regiospecificity of the modification and the specificity of the substrate.

To determine whether the *aac(2′)-IIa* gene was responsible for resistance to KSM, we transformed *E. coli* DH5α with the *aac(2′)-IIa*-expressed plasmid lacking the 91-bp promoter region of the *aac(2′)-IIa* gene [pUC118-aac(2′)-IIa P−] or including the promoter region of the *aac(2′)-IIa* gene [pUC118-aac(2′)-IIa P+]. Compared to *E. coli* DH5α, which harbored the control plasmid (pUC118), *aac(2′)-IIa*-expressed DH5α showed a notable reduction in susceptibility to KSM, except for the plasmid, which harbored an opposite direction of the *aac(2′)-IIa* gene with the *lac* promoter (Fig. 2). It was indicated that the 91-bp promoter region of *aac(2′)-IIa* functioned in *E. coli* constitutively. Susceptibility to neomycin, kanamycin, tobramycin, and gentamicin was unaffected (Fig. 2). Thus, the *aac(2′)-IIa* gene could contribute to KSM resistance by inactivating its target.

Acetylation of KSM by AAC(2′)-IIa. The *aac(2′)-IIa* gene consists of 783 nucleotides and encodes a 260-amino-acid protein. AAC(2′)-IIa shares sequence similarity with the GNAT superfamily of acetyltransferases, which includes the aminoglycoside *N*-acetyltransferases. We analyzed the acetyltransferase activity of AAC(2′)-IIa by a spectrophotometric assay that quantifies the conversion of acetyl-CoA to CoA-SH. The cell extract from AAC(2′)-IIa-expressed *E. coli* converted KSM into an acetylated form (Fig. 3A). On the other hand, neomycin, kanamycin, tobramycin and gentamicin were not modified by AAC(2′)-IIa (Fig. 3A). The reaction mixture that lacked acetyl-CoA showed clear antibiotic activity against KSM-sensitive *B. glumae*. Conversely, when a complete reaction with KSM was carried out, no antibacterial activity was observed (data not shown), indicating the inactivation of KSM. HPLC elution peaks of products from the AAC(2′)-IIa reaction were identical to those of standard 2′-acetyl-KSM (Fig. 3B).

Although AAC(2′)-IIa catalyzed the same substrate as Kac[273], namely, KSM acetyltransferase from *Streptomyces kasugaensis* (18, 21), the deduced amino acid sequences were quite distinct from each other (11.9% identity) (Fig. 1). AAC(2′)-IIa shares sequence similarity with several GNAT acetyltransferases in *Sinorhizobium meliloti* 1021, *Burkholderia cenocepacia* J2315, and *Burkholderia* sp. strain 383 [deduced amino acid sequence identity to AAC(2′)-IIa is 65.8, 56.3, and 55.9%, respectively], and yet their substrates are unknown (Fig. 1B). Moreover, AAC(2′)-IIa has low homology
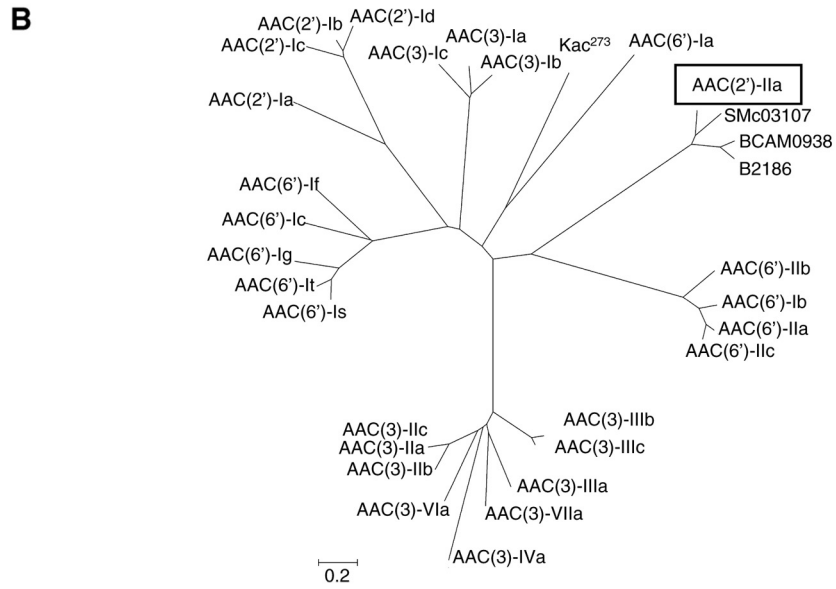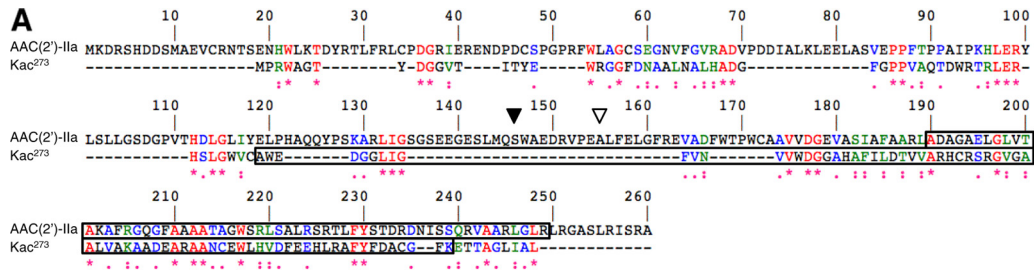
FIG 1 Comparison of the AAC(2′)-IIa protein from *B. glumae* 5091 with other *N*-acetyltransferases. (A) Comparison of the AAC(2′)-IIa with Kac273 from *Streptomyces kasugaensis* 273. The closed arrowhead reveals the amino acid substitution site from serine to threonine of AAC(2′)-IIa from *Acidovorax avenae* subsp. *avenae* 83. The HindIII restriction site in *aac(2′)-IIa* is shown by an open arrowhead. GNAT domains are boxed. Symbols: asterisk (*), identical amino acid; colon (:), strongly similar; period (.), weakly similar. (B) Phylogenetic tree of the AAC enzymes, including SMc03107 from *Sinorhizobium meliloti* 1021 (NC_003047), BCAM0938 from *Burkholderia cenocepacia* J2315 (NC_011001), and Bcep18194_B2186 from *Burkholderia* sp. strain 383 (NC_007411). AAC accession numbers: AAC(2′)-Ia from *Providencia stuartii*, L06156; AAC(2′)-Ib from *Mycobacterium fortuitum*, U41471; AAC(2′)-Ic from *M. tuberculosis*, U72714; AAC(2′)-Id from *M. smegmatis*, U72743; AAC(3)-Ia from *Pseudomonas aeruginosa*, X15852; AAC(3)-Ib from *P. aeruginosa*, L06157; AAC(3)-Ic from *P. aeruginosa*, AJ511268; AAC(3)-IIa from *Enterobacter cloacae*, X51534; AAC(3)-IIb from *Serratia marcescens*, M97172; AAC(3)-IIc from *E. coli*, X54723; AAC(3)-IIIa from *P. aeruginosa*, X55652; AAC(3)-IIIb from *P. aeruginosa*, L06160; AAC(3)-IIIc from *P. aeruginosa*, L06161; AAC(3)-IVa from *E. coli*, X01385; AAC(3)-VIa from *E. cloacae*, M88012; AAC(3)-VIIa from *Streptomyces rimosus*, M22999; AAC(6′)-Ia from *Citrobacter diversus*, M18967; AAC(6′)-Ib from *Klebsiella pneumoniae*, M21682; AAC(6′)-Ic from *S. marcescens*, M94066; AAC(6′)-If from *E. cloacae*, X55353; AAC(6′)-Ig from *Acinetobacter haemolyticus*, L09246; AAC(6′)-Is from *Acinetobacter* sp., AF031327; AAC(6′)-It from *Acinetobacter* sp., AF031328; AAC(6′)-IIa from *P. aeruginosa*, M29695; AAC(6′)-IIb from *P. fluorescens*, L06163; and AAC(6′)-IIc from *P. aeruginosa*, AF162771.

FIG 2 Susceptibility of *E. coli* DH5α expressing the *aac(2′)-IIa* gene to aminoglycosides. The 91-bp promoter region (P) of *aac(2′)-IIa* gene is not contained (P−) or contained (P+) in pUC118 vector. NM, neomycin; KM, kanamycin; TOB, tobramycin; GM, gentamicin.

with other classes of the AAC enzymes. On the basis of sequence similarity and enzymatic properties, the *aac(2′)-IIa* gene is a novel KSM-specific 2′-*N*-acetyltransferase (Fig. 3C).

Distribution of the *aac(2′)-IIa* gene among KSM-resistant rice-pathogenic bacteria. To clarify the distribution of the *aac(2′)-IIa* gene among KSM-resistant *B. glumae*, we examined whether this gene is present or absent by PCR analysis. Of the 18 resistant isolates analyzed, all of them harbored the *aac(2′)-IIa* gene. In contrast to the resistant isolates, the gene was absent in KSM-sensitive strains (Table 1). Because the acetylation of KSM seemed to be major cause for KSM resistance in field isolates of *B. glumae*, we examined whether the *aac(2′)-IIa* gene is present in KSM-resistant *A. avenae* subsp. *avenae*, which causes rice bacterial brown stripe. In addition to KSM-resistant *B. glumae*, of the nine isolates of KSM-resistant *A. avenae* analyzed, all of them harbored the *aac(2′)-IIa* gene. In contrast to the resistant isolates, the gene was absent in KSM-sensitive strains (Table 1).
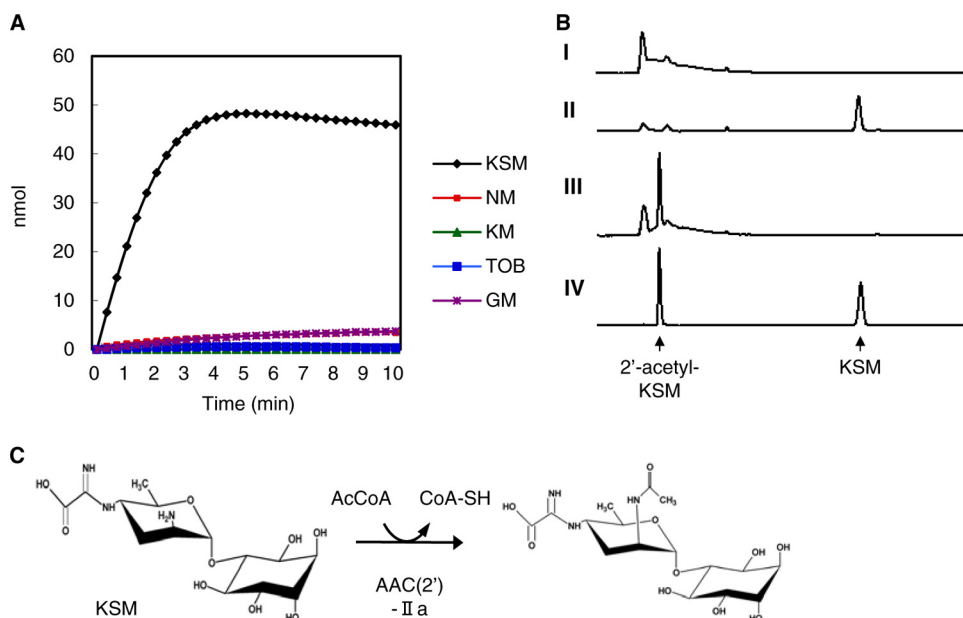
FIG 3 Enzymatic properties of AAC(2′)-IIa. (A) Time course of the production of acetylated antibiotics by the cell extract from *E. coli* expressing the AAC(2′)-IIa protein. NM, neomycin; KM, kanamycin; TOB, tobramycin; GM, gentamicin. (B) HPLC elution profiles of the product of the acetyltransferase reaction of KSM. I, no KSM control; II, no acetyl-CoA control; III, acetyltransferase reaction; IV, standard 2′-acetyl-KSM and KSM. (C) AAC(2′)-IIa catalyzes the transfer reaction of an acetyl group to the 2′-*N*-kasugamine moiety of KSM.

The nucleotide sequences of the *aac(2′)-IIa* gene from *B. glumae* 4-1-9, 9-1-1, and 11-1-1 are identical to that of *B. glumae* 5091. Furthermore, the nucleotide sequences of *aac(2′)-IIa* from KSM-resistant *A. avenae* strain 1-1, 3-2, 4-2, 5-1, and 213 were identical to that of *B. glumae* 5091. On the other hand, KSM-resistant *A. avenae* strain 83 contains three substitutions of a nucleotide sequence at position 234 from C to G, at position 258 from T to G, and at position 436 from T to A within the coding region. T436A leads to the substitution of an amino acid from serine to threonine at position 146 in AAC(2′)-IIa (Fig. 1A). MIC of KSM was relatively higher in *A. avenae* strain 83 (3,200 μg/ml) than in other strains 1-1, 3-2, 4-2, 5-1, and 213 (800 μg/ml) (Table 1). We are currently investigating whether the substitution from serine to threonine at position 146 in AAC(2′)-IIa contributes to an increased MIC to KSM.

Identification of the IncP Island. The genetic organization of regions flanking the *aac(2′)-IIa* gene on the 5.6-kb EcoRI fragment is shown in Fig. 4. At the right end of the sequence is a part of the hypothetical gene (*bglu_1g13010*), which was identified in a genome sequence of *B. glumae* BGR1 chromosome 1 (26). Although the upstream region of *bglu_1g13010* contains the *guaA* gene encoding GMP synthase in BGR1, the 5′ upstream of *aac(2′)-IIa* in strain 5091 encodes two ORFs, oriented in the same direction as *aac(2′)-IIa*: the putative *traJ* gene and the *traI* gene (Fig. 4). In addition to these genes, a part of the putative *traC* gene was located on the left end of the 5.6-kb EcoRI fragment. These *tra* genes are known to be involved in horizontal gene transfer and originated from phages or plasmids (1, 9). A comparison of the *B. glumae* BGR1 and 5091 genomes indicated that a genetic element carrying the *aac(2′)-IIa* gene was inserted between the *guaA* and *bglu_1g13010* genes.

To identify the genetic element carrying *aac(2′)-IIa*, the region between *guaA* and *traC* was amplified by PCR based on the BGR1

and 5091 genome sequences. A 9.6-kb fragment was amplified from KSM-resistant *B. glumae* 5091; however, no PCR product was observed when genomic DNA from the KSM-sensitive strain 210 was used as a template. The insertion in the *B. glumae* 5091 genome is 12,724 bp and contains 13 ORFs (*bglu_5091g01* to *bglu_5091g13*) between *guaA* and the hypothetical (*bglu_1g13010*) gene (Fig. 4). The protein encoded by *bglu_5091g01* is a member of the Int family of site-specific recombinase. CP4-like integrases are found in temperate bacteriophages, integrative plasmids, and other mobile genomic islands (24). The proteins encoded by *bglu_5091g02* to *11* are putative AlpA (transcription regulator), putative RepA (replication protein), hypothetical protein, putative TrbL (conjugative transfer protein), putative TrbJ (conjugal transfer/entry exclusion protein), hypothetical protein, putative TraC (primase), putative TraK (auxiliary protein for relaxase), putative TraJ (auxiliary protein for relaxase), and putative TraI (relaxase). TrbL and TrbJ are components of the mating pair formation apparatus, which forms a channel between the donor and recipient cells (1). TraI recognizes the DNA strand at an origin of transfer (*oriT*) with TraJ and TraK, yielding a single-stranded DNA break for DNA transfer to the recipient cell via the conjugation machinery (1, 9). The genetic organization from the *integrase* gene to the *traI* gene in *B. glumae* 5091 reveals high similarity to the IncP island in the genome of *Brucella suis* 1330 and *Klebsiella pneumoniae* 342 (12, 25) (see Fig. S2 in the supplemental material). These IncP islands are integrated into the 3′ end of the *guaA* gene, which has been recognized as a hot spot for the insertion of genomic islands (37). While the G+C content of chromosome 1 of *B. glumae* BGR1 is 68.11% (26), that of the IncP island of *B. glumae* 5091 is 57.24%. The difference in G+C content from the surrounding chromosome suggests an acquisition of the IncP island of *B. glumae* 5091 by horizontal gene transfer.
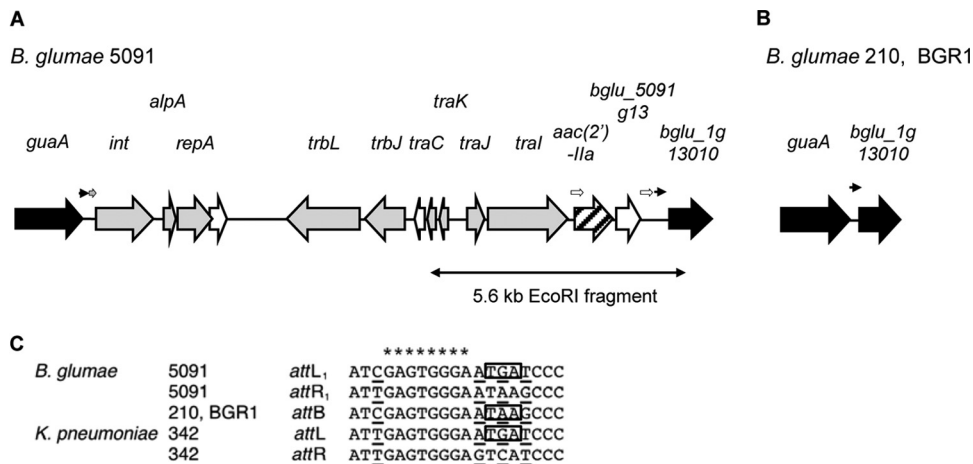
FIG 4 Structure of the IncP island from KSM-resistant *B. glumae* 5091 (A) and the *guaA*-hypothetical (*bglu_1g13010*) gene region of the KSM-sensitive strain 210 and the genome-sequenced strain BGR1 (B). Genes related to the phage or plasmid origin, hypothetical genes in the IncP island, and *aac(2′)-IIa* are indicated by gray arrows, open arrows, and a slashed arrow, respectively. The small black arrows above the gene map indicate the 19-bp direct repeats at the boundaries of the IncP island, whereas small open arrows indicate the 36- to 37-bp direct repeats flanking the *aac(2′)-IIa* and *bglu_5091g13* genes. The small gray arrow indicates the 52-bp inverted repeat sequence which functions as an *att* site for *Brucella suis* 1330 (25). (C) Comparison of the site-specific integration sites (19-bp direct repeats) of the IncP islands of *B. glumae* 5091 with *Klebsiella pneumoniae* 342. Asterisks indicate a consensus sequence, which is the putative excision site of the *guaA*-associated genomic islands (37). Open boxes indicate the translational termination codon of the *guaA* gene.

Integration site of the IncP island and excision activity. Genomic islands are often flanked by short (16- to 20-bp) almost-perfect direct repeats, called attachment sites (*att*s) (24). Direct repeats are usually produced by the site-specific recombination of genomic islands into the insertion sites. Integrases control both functions of the integration into and the excision from the genome. Site-specific integration of DNA into an *att*B site in a bacterial chromosome generates two junctions (*att*L and *att*R). Upon comparison of the *att* sequence of the IncP islands from *B. glumae* 5091 to those from *B. suis* 1330 and *K. pneumoniae* 342, short direct repeats (19 bp) are observed on either side of the IncP islands from *B. glumae* 5091 and *K. pneumoniae* 342 (12) (Fig. 4). These 19-bp direct repeats contain a consensus sequence (5′-GAGTGGGA-3′), which is the putative excision site of the *guaA*-associated genomic islands (37). In contrast, the 52-bp almost-perfect direct repeats are used for *att* sites in the IncP island of *B. suis* 1330 (25). The IncP island of *B. glumae* 5091 contains the same 52-bp nucleotide sequence on the left side only, which exists inside of the 19-bp short border sequence (Fig. 4).

To investigate whether integrase could mediate the recombination in *B. glumae* 5091 between *att*L₁ and *att*R₁ sites, we tried to detect the excision of the IncP island from its genome, the first step of the gene transfer. Whereas excision of the IncP island was previously reported in *B. suis* 1330 (25), its excision was not detected in *B. glumae* 5091. The excision and transfer of some genomic islands is facilitated by environmental stress or growth conditions (3, 14, 48). However, in our experimental conditions, and even in other bacterial strains (4-1-1, 4-1-9, 9-1-1, and 11-1-1), excision of IncP islands from KSM-resistant *B. glumae* was not observed (see Fig. S3 in the supplemental material). Furthermore, in a mating assay, transfer of the IncP island from the donor *B. glumae* 5091 to the recipient, *B. glumae* 3 or *A. avenae* 5 (oxolinic acid-resistant strains) was not detected (the transfer frequency from *B. glumae* 5091 to *B. glumae* 3 or to *A. avenae* 5 was $<2.2 \times 10^{-9}$ or $<1.3 \times 10^{-10}$ per donor, respectively).

Generation of the circular intermediates containing the *aac(2′)-IIa* and *bglu_5091g13* genes. Compared to the IncP islands from *B. glumae* 5091 with those from *B. suis* 1330 and *K. pneumoniae* 342, the 5′ sequences (from *integrase* to the *traC* gene) in the IncP island are conserved among the three species; however, the remaining 3′ sequences are quite different to each other (see Fig. S2 in the supplemental material). The *aac(2′)-IIa* and hypothetical genes (*bglu_5091g13*) exist in the IncP island of *B. glumae* 5091 only. We found that the second 36- to 37-bp direct repeats (*att*L₂ and *att*R₂) were observed on either side of the *aac(2′)-IIa* and *bglu_5091g13* genes (Fig. 4 and Fig. 5C). Interestingly, the closed circular forms containing the *aac(2′)-IIa* and *bglu_5091g13* genes were detected in *B. glumae* 5091, *A. avenae* 1-1, and 83 by inverse PCR, and the *att*P sequences were generated by joining *att*L₂ to *att*R₂ (Fig. 5). In addition, we determined whether *aac(2′)-IIa* and *bglu_5091g13* were polycistronically transcribed by using RT-PCR. As shown in Fig. 6, the *aac(2′)-IIa* and *bglu_5091g13* genes were amplified from a single transcript. These results indicate that the cassette of *aac(2′)-IIa* and *bglu_5091g13* genes are inserted into the IncP island by another recombination and that the recombination activity may still be active.

DISCUSSION

To elucidate the mechanism of KSM resistance, we first investigated spontaneous mutations in the *ksgA* gene of KSM-resistant *B. glumae* isolates. Previous *in vitro* studies showed that spontaneous KSM-resistant mutants of *E. amylovora*, *E. coli*, and *Bacillus subtilis* harbored mutations in the *ksgA* methyltransferase gene (29, 33). However, our results clarified that a nucleotide substitution of the *ksgA* gene is not involved in KSM resistance in field isolates of *B. glumae*. Moreover, the possibility that *ksgA*-related KSM-resistant bacteria emerged in the field is low because of decreased fitness in the growth rate *in vitro* and virulence to plants (29, 33). Vila-Sanjurjo et al. identified KSM-resistant mutations (A794G, G926A, and A1519C) in the single 16S rRNA gene of genetically
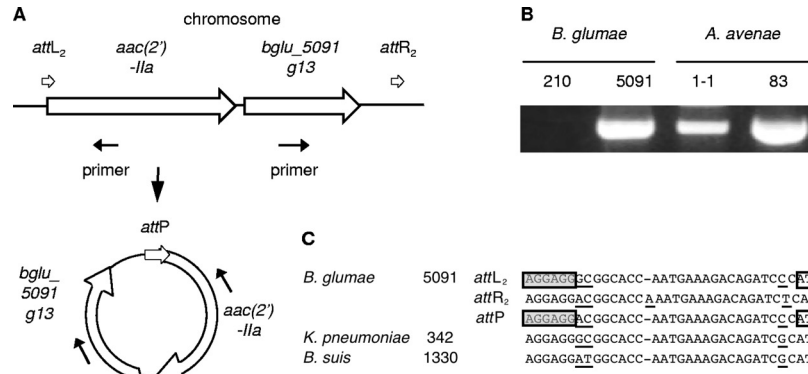
FIG 5 Circularization of the *aac(2′)-IIa* and hypothetical genes (*bglu_5091g13*). (A) Generation of the closed circular form of the *aac(2′)-IIa* and *bglu_5091g13* genes at 36- to 37-bp direct repeats (*att*). PCR primer positions are shown under the gene map. (B) Detection of the circularization by PCR. (C) Comparison of the 36- to 37-bp direct repeats in *B. glumae* 5091 with other similar sequences in the IncP islands from *K. pneumoniae* 342 and *Brucella suis* 1330. The open boxes indicate the translational initiation codon of the *aac(2′)-IIa* gene. The gray boxes indicate the putative Shine-Dalgarno (SD) sequence of the *aac(2′)-IIa* gene. Underlined letters show differences in nucleotides in the direct repeats.

engineered *E. coli* (52). However, most bacteria harbor multiple copies of rRNA genes in their chromosomes; *B. glumae* BGR1 also harbors five copies of the rRNA operons (26). These multiple copies of rRNA genes would mask a mutation in the single rRNA gene. Indeed, the nucleotide sequences of 16S rRNA genes in KSM-resistant *B. glumae* isolates (strains 5091, 4-1-9, and 9-1-1) are identical to those of KSM-susceptible strains (strains 210 and 3).

Resistance to aminoglycoside antibiotics is often conferred by enzymatic modifications. The aminoglycoside *N*-acetyltransferase (AAC) can be divided into four groups, based on the regiospecificity of the modification: AAC(1), AAC(2′), AAC(3), and AAC(6′) (50). These enzymes utilize acetyl-CoA as the donor of the acetyl group to modify drugs at positions 1 and 3 of the 2-deoxystreptamine ring and positions 2′ and 6′ of the amino-sugar ring. The majority of AACs are encoded by plasmids, which permits the transfer of resistance genes among bacteria. On the other hand, representatives of the AAC(2′)-I subclass of aminoglycoside

acetyltransferase are unique because they are chromosomally encoded (34). In phytopathogenic bacteria, the aminoglycoside streptomycin and KSM are usually used to control them. Streptomycin-resistant isolates of *E. amylovora*, *Pseudomonas syringae*, and *Xanthomonas campestris* harbor the *strA-strB* gene pair encoding phosphotransferase, APH(3′)-Ib-APH(6)-Ic, on the transposon (variants of Tn*5393*) in the large conjugative plasmid, or on the small nonconjugative plasmid RSF1010 (28, 32, 40, 50). As well as clinical pathogens, the primary mechanism of streptomycin resistance in phytopathogenic bacteria involves the acquisition of streptomycin modifying enzyme genes via horizontal gene transfer.

In the present study, we isolated the *aac(2′)-IIa* gene encoding the KSM 2′-*N*-acetyltransferase from *B. glumae* and *A. avenae* subsp. *avenae*. AAC(2′)-IIa could not react on other aminoglycoside antibiotics tested, indicating the KSM-specific enzyme. The substrate specificity of AAC(2′)-IIa is consistent with the unique structure of KSM. The amino acid sequence of AAC(2′)-IIa exhibits low homology with AAC(2′)-Ia in *Providencia stuartii* (deduced amino acid sequence identity to AAC(2′)-IIa is 9.4%) and AAC(2′)-Ib, -Ic, and -Id in mycobacterial species (13.0, 11.7, and 4.9%, respectively). Also, AAC(2′)-IIa exhibits low homology with Kac[273], a self-resistant KSM acetylating enzyme of a KSM-producer, *Streptomyces kasugaensis* (18, 21). Based on the regiospecificity of the modification and the specificity of the substrate, the *aac(2′)-IIa* gene is classified as a novel subclass of *aac(2′)* (Fig. 1).

In agriculture, the available antibiotics are limited to inhibit the occurrence and spread of antibiotic resistance in clinically important pathogens (8). KSM possesses the least potential to affect any mechanism of aminoglycoside resistance in their emergence and spread in human and animal pathogenic bacteria due to its unique structure and low antibacterial activity against clinical pathogens (44). Our results also indicate that if *aac(2′)-IIa* genes are transferred from phytopathogenic bacteria to clinical pathogens, they would not influence the efficacy of clinically used antibiotics.

Generally, the intensive use of drugs increases the risk of the development of their resistance. The accumulation of resistant pathogens will be enhanced by frequent use of drugs, by the pres-
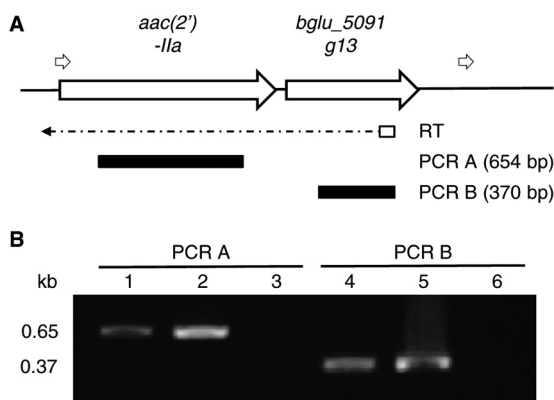

FIG 6 Polycistronic transcription of the *aac(2′)-IIa* and hypothetical genes (*bglu_5091g13*) from *B. glumae* 5091. (A) Organization of the *aac(2′)-IIa* and hypothetical genes. The small open arrows above the gene map indicate the 36- to 37-bp direct repeats. The dotted arrow below the gene map represents the direction of the reverse transcription (RT) reaction. The black thick bars below the RT arrow indicate the PCR products from the corresponding RT reactions. (B) Analysis of the RT-PCR products of *aac(2′)-IIa* (PCR A) and *bglu_5091g13* (PCR B). Lanes 1 and 4, RT-PCR products; lanes 2 and 5, PCR products from the DNA template; lanes 3 and 6, PCR products from the RNA template.

ence of larger pathogen populations before the application of drugs, and by greater descendant production (e.g., large number of spore production in fungi) and shorter generation times in the pathogen (6). Resistance of the fungus *M. grisea* to KSM in the field was found in 1971 in Yamagata prefecture, where KSM had been used intensively for several years (30). Although the mycelial growth, sporulation, and virulence of these resistant isolates were almost the same as for the sensitive ones (31), the competitive ability of the resistant isolates was inferior to sensitive ones (23). When the application of the antibiotic was stopped, the population of KSM-resistant *M. grisea* decreased rapidly (31). Currently, the combinations of KSM plus melanin biosynthesis inhibitors (MBIs) are used effectively to control rice blast. Ascospore analyses revealed that the occurrence of resistance to KSM in *M. grisea* is controlled by at least three independent chromosome loci (41, 42).

In plant bacterial pathogens, KSM-resistant *A. avenae* subsp. *avenae* emerged in some areas of Japan in 1990 (43). In these regions, to hasten germination and control rice bacterial diseases, rice seeds had been incubated at a high temperature in a solution of KSM with air circulation for 1 day before sowing seeds. These conditions during seed disinfection were also suitable for bacterial growth, leading to the occurrence of KSM-resistant *A. avenae*. In our research, all KSM-resistant rice-pathogenic bacteria (*B. glumae*; 18 isolates, *A. avenae*; 9 isolates) harbored the *aac(2′)-IIa* gene whose molecular detection can distinguish whether bacterial strains are resistant or susceptible to KSM. Furthermore, our studies reveal that the *aac(2′)-IIa* gene of *B. glumae* 5091 is located in the IncP genomic island of the bacterial chromosome. Acquiring the *aac(2′)-IIa* gene may result from the horizontal gene transfer by activity of *integrase* and *tra* genes in the IncP island. Lavigne et al. reported that the IncP island in the genome of *Brucella suis* 1330 is excised from its chromosome and forms circular intermediates (25). Although no excision activity of the IncP island in KSM-resistant *B. glumae* was observed, the circular forms containing the *aac(2′)-IIa* and *bglu_5091g13* genes were generated at the second *att* recombination site, suggesting that the *aac(2′)-IIa* gene had been integrated into the IncP island of a donor bacterial species. Because the ability of IncP island transfer in KSM-resistant *B. glumae* may be tightly regulated, the occurrence of KSM-resistant isolates in various parts of the country occurs at a relatively low frequency. It is important to inhibit the spread of KSM-resistant pathogens through the distribution of rice seeds.

In conclusion, we demonstrated that the mechanism of KSM resistance in rice-pathogenic bacteria in the fields is mainly due to acetylation of the drug and that KSM-resistant isolates can be detected whether the KSM acetyltransferase, *aac(2′)-IIa* gene, is present or absent. The rapid distinction between KSM-sensitive and -resistant isolates by PCR analysis will serve to inhibit the spread of KSM-resistant rice seed-borne diseases and spur the effective use of the drug. To characterize the detailed function of the *aac(2′)-IIa* gene will provide hopeful information to combat KSM-resistant pathogens in the future.

ACKNOWLEDGMENTS

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