Two types of genetic carrier, the IncP genomic island and the novel IncP-1 β plasmid, for the *aac(2)-IIa* gene that confers kasugamycin resistance in *Acidovorax avenae* ssp. *avenae*

ATSUSHI YOSHII^{1,2,*}, TSUTOMU OMATSU³, YUKIE KATAYAMA³, SATOSHI KOYAMA⁴, TETSUYA MIZUTANI³, HIROMITSU MORIYAMA² AND TOSHIYUKI FUKUHARA²

¹Central Research Laboratories, Hokko Chemical Industry Co., Ltd., Toda 2165, Atsugi, Kanagawa 243-0023, Japan

²Department of Applied Biological Sciences, Tokyo University of Agriculture and Technology, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan

³Research and Education Center for Prevention of Global Infectious Diseases of Animals, Tokyo University of Agriculture and Technology, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan

⁴Cooperative Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan

SUMMARY

A unique aminoglycoside antibiotic, kasugamycin (KSM), has been used to control many plant bacterial and fungal diseases in several countries. The emergence of KSM-resistant *Acidovorax avenae* ssp. *avenae* and *Burkholderia glumae*, which cause rice bacterial brown stripe and rice bacterial grain and seedling rot, respectively, is a serious threat for the effective control of these diseases. Previously, we have identified the *aac(2)-IIa* gene, encoding a KSM 2'-N-acetyltransferase, from both KSM-resistant pathogens. Although all KSM-resistant isolates from both species possess the *aac(2)-IIa* gene, only *A. avenae* strain 83 showed higher resistance than other strains. In this research, kinetic analysis indicates that an amino acid substitution from serine to threonine at position 146 of AAC(2)-IIa in strain 83 is not involved in this increased resistance. Whole draft genome analysis of *A. avenae* 83 shows that the *aac(2)-IIa* gene is carried by the novel IncP-1 β plasmid pAAA83, whereas the genetic carrier of other strains, the IncP genomic island, is inserted into their chromosomes. The difference in the nucleotides of the promoter region of *aac(2)-IIa* between strain 83 and other strains indicates an additional transcription start site and results in the increased transcription of *aac(2)-IIa* in strain 83. Moreover, biological characterization of pAAA83 demonstrates that it can be transferred by conjugation and maintained in the host cells. These results demonstrate that acquisition of the *aac(2)-IIa* gene takes place in at least two ways and that the gene module, which includes *aac(2)-IIa* and the downstream gene, may be an important unit for the dissemination of antibiotic resistance.

Keywords: *Acidovorax*, antibiotic resistance, *Burkholderia*, genomic island, IncP-1 β plasmid, kasugamycin, rice.

INTRODUCTION

Kasugamycin (KSM), an aminoglycoside antibiotic, is used as an agrochemical to control fungal and bacterial diseases that affect rice, sugar beet, kiwi fruit, Japanese apricot and other crops in over 30 countries (Spadafora *et al.*, 2010). Rice blast, caused by the fungus *Magnaporthe grisea*, is the most important target for KSM in Japan and other Asian countries (Ishiyama *et al.*, 1965). Many bacterial diseases caused by members of the genera *Pseudomonas* and *Erwinia* are also controlled by KSM in various countries (Hiramatsu *et al.*, 1989). Recently, KSM has been demonstrated to be an effective bactericide against fire blight caused by *Erwinia amylovora* in the USA, one of the most destructive diseases of pome fruit trees, especially apple and pear (Adaskaveg *et al.*, 2011; Jurgens and Babadoost, 2013; McGhee and Sundin, 2011).

KSM consists of two sugars, a D-chiro-inositol and kasugamine (2,4-diamino-2,3,4,6-tetradeoxy-D-arabino-hexose), with a side-chain (carboxy-imino-methyl group) (Flatt and Mahmud, 2007). As KSM lacks the (deoxy)streptamine moiety common among aminoglycosides, translational miscoding activity is not detected (Ikekawa *et al.*, 1966). KSM binds to the mRNA-binding tunnel in the E- and P-sites of the 30S bacterial ribosomal subunit (Schluenzen *et al.*, 2006; Schuwirth *et al.*, 2006). As a result, KSM inhibits translational initiation by perturbing the mRNA-tRNA codon-anticodon interaction. This mode of action is different from that of streptomycin, also used in agriculture, and of other antibiotics (Tanaka *et al.*, 1965).

Resistance to antibiotics is a serious problem in the struggle to control bacterial infectious diseases. As with many other antibiotics used clinically, the emergence of KSM-resistant *Burkholderia glumae* and *Acidovorax avenae* ssp. *avenae*, the causative Gram-negative bacteria of seed-transmitted rice diseases, has been reported in Japan (Hori *et al.*, 2007; Takeuchi and Tamura, 1991). *Burkholderia glumae* infects not only rice seedlings, but also grains at the heading stage, and causes serious damage to rice production in nursery boxes and paddy fields (Goto and Ohata, 1956; Uematsu *et al.*, 1976). Bacterial seedling and grain rot

*Correspondence: Email: yoshii-a@hokkochem.co.jp

caused by *B. glumae* has been reported in East Asia, Latin America and the USA (Ham *et al.*, 2011). *Acidovorax avenae* ssp. *avenae* causes brown stripe disease of rice seedlings and also infects many other economically important plants, including corn, oats, sugarcane and millet (Schaad *et al.*, 2008; Tominaga *et al.*, 1983). Recently, an *A. avenae* ssp. *avenae* rice strain has been proposed for reclassification as *Acidovorax oryzae* as it can be distinguished from corn and oat strains. It is also distinct from *A. avenae* ssp. *citrulli* infecting plants in the Cucurbitaceae and *A. avenae* ssp. *cattleyae* infecting orchids (Schaad *et al.*, 2008). Throughout this article, we refer to this subspecies as *A. avenae*. Bacterial brown stripe caused by *A. avenae* has also become the centre of attention in Asia (Xie *et al.*, 2011). Therefore, it is important to control these seed-borne pathogens in nursery boxes and paddy fields for the production of disease-free rice.

The most prevalent bacterial resistance mechanism to antibiotics is inactivation of the drugs by pathogen-produced modifying enzymes (Vakulenko and Mobashery, 2003). The aminoglycoside *N*-acetyltransferases, AACs, are divided into four groups on the basis of the site specificity of the modification: AAC(1), AAC(2'), AAC(3) and AAC(6') (Vakulenko and Mobashery, 2003). Recently, we have identified a novel KSM-specific 2'-*N*-acetyltransferase gene, *aac(2')-IIa*, from both KSM-resistant rice pathogens, *B. glumae* and *A. avenae* (Yoshii *et al.*, 2012). AAC(2')-IIa transfers the acetyl group of acetyl-CoA to the 2'-amino residue of the kasugamine ring, and acetylated KSM loses its antibacterial activity. The *aac(2')-IIa* gene is carried by the genomic island, the IncP island, which is inserted into the 3' end of the *guaA* gene on the chromosome of *B. glumae* KSM-resistant strain 5091 (Yoshii *et al.*, 2012). The region named IncP, which encodes certain homologous proteins with IncP incompatibility group plasmids, was originally found in the chromosome of *Brucella suis* 1330 (Lavigne *et al.*, 2005). In contrast, KSM-sensitive strains do not possess the IncP island with the *aac(2')-IIa* gene. Almost all KSM-resistant strains of *B. glumae* (18 strains) and *A. avenae* (eight strains) exhibit similar KSM resistance levels. *Acidovorax avenae* strain 83, however, exhibits a four-fold higher resistance than the other KSM-resistant *A. avenae* strains (Yoshii *et al.*, 2012). Strain 83 contains an amino acid substitution from serine to threonine at position 146 (S146T) in AAC(2')-IIa. The role of this amino acid substitution in higher resistance remains to be determined. The elucidation of this resistance mechanism in *A. avenae* 83 may provide information on new aspects of KSM resistance.

RESULTS

KSM acetyltransferase activity in *A. avenae* strains

To elucidate the mechanism by which resistance is increased, KSM-acetylating activity in *A. avenae* strains was determined. Crude extracts were prepared from 1- or 2-day-old liquid shake

cultures of KSM-resistant *B. glumae* strain 5091 and *A. avenae* strains 1-1 and 83. The rates of KSM acetylation were similar in *B. glumae* 5091 and *A. avenae* 1-1 from both culture periods (Fig. 1a). In contrast, *A. avenae* 83 showed about a four-fold higher activity than *A. avenae* 1-1 (Fig. 1a). The nucleotide sequences of the *aac(2')-IIa* gene were the same in *B. glumae* 5091 and *A. avenae* 1-1, but *A. avenae* 83 harboured an S146T amino acid substitution.

Biochemical characterization of two types of AAC(2')-IIa

To verify the role of the S146T substitution, both types of C-terminal 6 × histidine (6 × His)-tagged AAC(2')-IIa proteins were overexpressed in *Escherichia coli* BL21(DE3). His-tagged proteins were affinity purified and analysed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1b). The molecular mass of the AAC(2')-IIa proteins was 30 kDa, which corresponded to the estimated mass calculated from its deduced amino acid sequence. Initially, the optimum conditions for the enzyme reaction were analysed. An enzyme assay with strain 5091 of AAC(2')-IIa at various pH values showed that enzymatic activity was highest at pH 7.6 (Fig. 1c). Enzymatic activity greater than 50% of maximum activity ranged from pH 6.8 to pH 8.8. We further determined that the optimum temperature for the activity of AAC(2')-IIa (strain 5091) ranged from 20 to 25 °C in 50 mM sodium phosphate buffer (pH 7.6) (Fig. 1d). At higher temperatures, the activity of the enzyme declined sharply.

To assess the contribution of S146T in AAC(2')-IIa, kinetic studies were carried out with a fixed concentration (80 µM) of acetyl-CoA for KSM, and with a fixed concentration (800 µM) of KSM for acetyl-CoA. Both proteins showed no significant difference in K_m , K_{cat} and K_{cat}/K_m values for KSM (Table 1). In addition, kinetic parameters for acetyl-CoA were not significantly different between AAC(2')-IIa of strains 5091 and 83 (S146T), indicating that the S146T substitution was not involved in the higher resistance of *A. avenae* 83 (Table 1). These results suggest that the amount of AAC(2')-IIa protein in *A. avenae* 83 might have increased.

Determination of the genetic carrier for the *aac(2')-IIa* gene in *A. avenae* strain 83

Although the IncP island region was conserved among other KSM-resistant *B. glumae* strains 4-1-9, 9-1-1 and 11-1-2 and *A. avenae* strains 1-1, 3-2 and 213, no regions of the IncP island were amplified from *A. avenae* 83 by polymerase chain reaction (PCR) analysis, except for the *aac(2')-IIa* to *bglu_5091g13* gene region (Fig. S1, see Supporting Information).

The flanking regions of the *aac(2')-IIa* gene in *A. avenae* 83 were sequenced by whole draft genome sequence analysis using a next-generation sequencer. After annotating the genome

Fig. 1 Purification and activity of AAC(2')-IIa. (a) Kasugamycin (KSM) acetyltransferase activity of crude lysates from *Burkholderia glumae* and *Acidovorax avenae*. All values are the means $\pm$ standard deviations from triplicate experiments. (b) Purification of AAC(2')-IIa-His protein. Left lanes of each protein: total proteins extracted from isopropyl- β -D-thiogalactopyranoside (IPTG)-induced cells. Right lanes: purified proteins. Arrowhead indicates purified AAC(2')-IIa protein. (c) pH dependence of acetyltransferase activity of AAC(2')-IIa (5091 type). (d) Temperature dependence of acetyltransferase activity of AAC(2')-IIa (5091 type).

Table 1 Kinetic parameters of AAC(2')-IIa.

AAC(2')-IIa type	Substrate	K_m (μ M)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
5091, 1-1	Kasugamycin	475.3 ± 141.2	0.91 ± 0.18	1.9×10^3
	Acetyl-CoA	25.8 ± 4.5	0.56 ± 0.05	2.2×10^4
83	Kasugamycin	473.2 ± 67.0	0.80 ± 0.07	1.7×10^3
	Acetyl-CoA	32.5 ± 6.2	0.55 ± 0.05	1.7×10^4

sequence of the maize strain of *A. avenae* ssp. *avenae* (strain ATCC 19860), a total of 461 contigs was obtained. One (contig 110) contained the region from *aac(2')-IIa* to *bglu_5091g13*, which could not be annotated to *A. avenae* ATCC 19860, and was denoted as pAAA83 (Fig. 2a). pAAA83, a circular plasmid (59 534 bp) with an average G + C content of 64.5%, harbours a typical backbone of the IncP-1 β group plasmid and is most similar to pB8 isolated from activated sludge bacteria of a wastewater

treatment plant (Fig. 2b,c). pB8 confers multidrug resistance to amoxicillin, spectinomycin, streptomycin and sulfonamides on host bacteria (Schlüter *et al.*, 2005).

Backbone structure of plasmid pAAA83

The backbone of pAAA83 consists of Tra 1 (*tra* genes for mating pair formation, blue in Fig. 2a), Tra 2 (*trb* genes for conjugative DNA transfer, blue in Fig. 2a), Ctl (central control for plasmid maintenance, partitioning, purple in Fig. 2a) and Rep (replication initiation, yellow in Fig. 2a), with the origin of vegetative replication (*oriV*) and transfer (*oriT*). The backbone nucleotide sequence of pAAA83 is more than 97% identical to pB8 (Fig. 2c). In addition, the conserved nucleotide motifs, such as the palindromic repeats and iterons in *oriV*, which control plasmid replication and copy number, are 100% identical to those of pB8 and the prototype

Fig. 2 The IncP-1 β plasmid, pAAA83. (a) Map of the pAAA83 plasmid. Outer circle, gene map; middle circle, G + C content (window, 100 bp; step, 50 bp); inner circle, GC skew (window, 500 bp; step, 100 bp). On the genetic map, the replication initiation module (Rep), *tra* region (Tra 1) and *trb* region (Tra 2), plasmid maintenance, partitioning and controlling region (Ctl), and accessory regions are drawn in yellow, blue, purple and red, respectively. Green or brown in the middle or inner circle represents higher than average G + C content or zero GC skew. Orange or blue represents opposite values. (b) Phylogenetic tree of the IncP-1 plasmid backbone. Maximum likelihood tree is inferred from concatenated backbone regions from *traL* to *traC2* and from *trbL* to *trbC* of the 18 IncP-1 plasmids. The enlarged subtree of IncP-1 β plasmids is shown in the box. The scale bar represents the nucleotide substitution rate. (c) Alignment of IncP-1 β plasmid pAAA83 and pB8. The region of sequence covering the coloured block reveals homology between both plasmids.

IncP-1 β resistance plasmid R751 (Table S1, see Supporting Information) (Schlüter *et al.*, 2005; Thorsted *et al.*, 1998). Interestingly, the IncP island of *B. glumae* 5091 possesses some *tra* and *trb* genes, and there are weak similarities between pAAA83 and the IncP island (identities of the TrbL, TrbJ, TraJ and TraI proteins are 29.8%, 25.5%, 24.7% and 25.0%, respectively, and their similarities are 43.7%, 39.3%, 33.7% and 36.4%, respectively).

Accessory regions of pAAA83

In pAAA83, three accessory regions were detected: (i) the *kluA*–*kluB* gene region; (ii) the region downstream of the replication initiation gene *trfA*; and (iii) the region between Tra 1 and Tra 2 (red in Fig. 2a). The second and third accessory regions have a relatively lower G + C content than the backbone of the plasmid (66.3%); the G + C content of the first accessory region is 66.3%, that of the second is 61.0% and that of the third is 60.1% (middle circle of Fig. 2a). In the first region, *kluA*–*kluB* genes, which were also detected in pB8 and R751, may function as a post-segregational killing system to promote plasmid stability

(Heuer *et al.*, 2004; Schlüter *et al.*, 2007). In the downstream region of the *trfA* gene, the *strA*–*strB* and *mer* genes, which encode streptomycin phosphotransferases and mercury resistance proteins, respectively, were identified as the second accessory region (Figs 2a and 3a). The downstream region of *trfA* in pAAA83 showed high homology to the accessory regions of the IncP-1 β plasmid, pB4 and pB10 (Schlüter *et al.*, 2003; Tauch *et al.*, 2003), indicating that the streptomycin-resistant transposon Tn5393c had inserted into the *merE* and *orf-2* genes of the mercury-resistant transposon Tn501 (Fig. 3a). It was confirmed that other KSM-sensitive and KSM-resistant *A. avenae* strains were sensitive to streptomycin, although strain 83 exhibited resistance to streptomycin [minimal inhibitory concentration (MIC) > 200 μ g/mL] (Fig. 3b).

Comparison of the third accessory region of pAAA83 between *parA* (Tra 1 region) and the *upf31.0* gene (Tra 2 region) with IncP-1 β pAKD1 indicated that the Tn21-like transposon was inserted into this region of pAAA83 (Fig. 3c) (Sen *et al.*, 2011). The partitioning gene *parA*, encoding resolvase, is found in some IncP-1 β plasmids (e.g. pB3, pBP136, pTP6, pRSB111, pA1 and

Fig. 3 Accessory region of pAAA83. (a) Alignment of the streptomycin-resistant transposon Tn5393c (grey) and the mercury-resistant transposon Tn501 (white) from pAAA83, pB4 and pB10. Rectangles under the gene map indicate the terminal inverted repeats (IR) of transposons: grey; IR of Tn5393c; black, IR of Tn501 (*IR_{mer}*). (b) Minimal inhibitory concentration (MIC) of streptomycin (Sm) for *Acidovorax avenae* strains. (c) Alignment of the Tn21-like transposon (grey) of pAAA83 and pAKD1. The *aac(2)-Ila* and *bglu_5091g13-like* genes are shown in black. Arrowheads indicate the 19-bp direct repeats (black) and 36–37-bp direct repeats (white). Rectangles under the gene map indicate the IR (black) to be almost the same as the IR of Tn501 (Tn21 subgroup).

pJP4), but not in pB8 and R751 (Harada *et al.*, 2006; Heuer *et al.*, 2004; Kamachi *et al.*, 2006; Schlüter *et al.*, 2007; Smalla *et al.*, 2006; Szczepanowski *et al.*, 2007; Trefault *et al.*, 2004). The ParA protein of pAAA83 has much lower homology (identities are 60.9–63.1% and similarities are 70.4–72.7%) to the proteins of other

IncP-1 β plasmids. The ParA protein of pAAA83 also has much lower homology to the proteins of IncP-1 α plasmids, RP4, pTB11 and pBS228 (identity is 60.9% and similarity is 69.8%). Furthermore, the insertion of mobile elements in other plasmids (e.g. pB3, pTP6, pRSB111 and pA1) occurred between *traC* and *parA*. These observations indicate that the origin of *parA* in pAAA83 differs from that of other IncP-1 β plasmids.

The *aac(2)-Ila* gene and a hypothetical gene, which is identical to the *bglu_5091g13* gene of *B. glumae* 5091 (termed *bglu_5091g13-like*), are found in the region between the *parA* and *upf31.0* genes, but are not inserted into the Tn21-like transposon (Fig. 3c). The 36–37-bp direct repeats (*attL₂* and *attR₂*), which form closed circular DNA by joining at both sites (formation of the *attP* site) in *A. avenae* 83 and 1-1, and in *B. glumae* 5091 (Yoshii *et al.*, 2012), were confirmed on either side of the *aac(2)-Ila* and *bglu_5091g13-like* genes in pAAA83. These results demonstrate that the genetic carrier of the *aac(2)-Ila* gene in *A. avenae* 83 is different from the carriers of other KSM-resistant strains of *A. avenae* and *B. glumae*.

Comparative analysis of the region downstream of the *guaA* gene

The maize strains of *A. avenae* ssp. *avenae* ATCC 19860 and *A. avenae* ssp. *citruilli* AAC00-1 harbour mobility-related genes (e.g. integrase) in the 3' end of the *guaA* gene on the chromosome, indicating that genomic islands are integrated into the region downstream of *guaA* (Fig. 4). In contrast, whole draft genome sequence analysis of *A. avenae* 83 indicates that a DNA helicase gene exists in the region downstream of the *guaA* gene, but no other genomic islands were detected (Fig. 4). Moreover, the 19-bp left border sequence (*attL₁*) of the IncP island from *A. avenae* 1-1 and *B. glumae* 5091 was slightly different from the similar sequences of *A. avenae* 83, ATCC 19860 and *A. avenae* ssp. *citruilli* AAC00-1 (Table S2, see Supporting Information). In other words, the acquisition of pAAA83 in *A. avenae* 83 has no relation with the occupation of the downstream region of *guaA* by insertion of other mobile genetic elements.

Comparative analysis of the promoter region of the *aac(2)-Ila* gene

To verify the up-regulated transcription of the *aac(2)-Ila* gene in *A. avenae* 83, the nucleotide sequences of the promoter region of the *aac(2)-Ila* gene were analysed by comparison with strain 83 (plasmid pAAA83 type) and strain 1-1 (IncP genomic island type). Both promoter regions are highly similar from the start codon of *aac(2)-Ila* to the –35 box, whereas regions upstream of the –35 box are clearly different from each other (Fig. 5). Interestingly, an *attL₁*-like sequence was found between the –35 and –10 boxes in pAAA83 (Fig. 5 and Table S2). Although we detected circular

Fig. 4 Alignment of the region downstream of the *guaA* gene (encoding GMP synthase) in *Acidovorax avenae* strain 83, *A. avenae* ATCC 19860 (NC_015138) and *A. avenae* ssp. *citrulli* AAC00-1 (NC_008752; reverse complement sequence). Annotated genes are shown as white or grey boxes, with genes transcribed from the reverse strand shifted downward. Grey boxes indicate mobility-related genes. The similarity plot in grey indicates a homologous region among the three genomes. The genomic islands of *A. avenae* ATCC 19860 and *A. avenae* ssp. *citrulli* AAC00-1 (AAC00-1G^{guaA}) are enclosed in large boxes. The small white arrowheads indicate the 19-bp direct repeat at the boundaries of the genomic island (see Table S2).

Fig. 5 Comparison of the promoter region of the *aac(2)-Ila* gene from kasugamycin (KSM)-resistant *Acidovorax avenae* strain 83 with that of strain 1-1. Promoter sequences are shown from the gene upstream of *aac(2)-Ila* to the start codon of the *aac(2)-Ila* gene. Identical nucleotides are represented by a dot and substitutions are indicated by the relevant letter. The transcriptional start sites of the *aac(2)-Ila* gene are denoted by bent arrows. The predicted -35, -10 regions and SD sequence are boxed. The 19-bp direct repeat-like sequence of strain 83 is shown as a dotted line.

intermediate DNA of the *aac(2)-Ila* gene by joining *attL₂* and *attR₂* in *A. avenae* 83 (Yoshii *et al.*, 2012), the role of the *attL₁*-like sequence in forming circular DNA is unknown.

The 5' rapid amplification of cDNA ends (RACE) analyses indicated that the transcriptional start site of the *aac(2)-Ila* gene was present between the -10 box and the Shine–Dalgarno (SD) sequence in both strains (Fig. 5). In addition, one more transcriptional start site of *aac(2)-Ila* was detected between the -35 and -10 boxes in *A. avenae* 83, indicating a different transcriptional

regulation of this gene, thus providing a good explanation for the four-fold higher KSM-resistance and KSM-acetylating activities.

Therefore, the relative mRNA expression level of the *aac(2)-Ila* gene was examined in *A. avenae* 83 and 1-1 by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. The level of *aac(2)-Ila* transcript in *A. avenae* 83 was 3.5-fold higher than that in *A. avenae* 1-1 (Fig. 6a). In contrast, the relative amount of DNA of the *aac(2)-Ila* gene in *A. avenae* 83 was almost equal to that of *A. avenae* 1-1 (Fig. 6b). These results strongly

Fig. 6 Relative mRNA expression levels of the *aac(2)-Ila* gene (a) and relative amount of DNA of the *aac(2)-Ila* gene (b) between *Acidovorax avenae* strain 1-1 and strain 83. All values are the means $\pm$ standard deviations from triplicate experiments. Significant differences ($P < 0.01$) are indicated by an asterisk (*t*-test).

suggest that the increased resistance to KSM in *A. avenae* 83 is caused by the high expression level of *aac(2)-Ila*, but not by the difference in DNA copy numbers of the plasmid and chromosome.

Biological characterization of pAAA83

Most IncP plasmids have a broad host range and are transferred to other bacterial cells by conjugation. Therefore, we characterized the transferability and stability of pAAA83. Although conjugative transfer of the IncP island from *B. glumae* 5091 to oxolinic acid-resistant *B. glumae* 3 was not detected (Yoshii *et al.*, 2012), the transfer of pAAA83 from *A. avenae* 83 to *A. avenae* 3 occurred at a frequency of 3.3×10^{-5} transconjugants per donor, and from *A. avenae* 83 to *B. glumae* 3 at a frequency of 3.8×10^{-4} transconjugants per donor, after mating on filter paper for 5 h.

The stability of pAAA83 in *A. avenae* 83 and transconjugants *A. avenae* 5+ and *B. glumae* 3+ was examined by serial propagation in liquid medium without KSM. The strains *A. avenae* 1-1 and

B. glumae 5091, in which the *aac(2)-Ila* gene was integrated into the chromosome, were used as controls for stable KSM resistance in the assay. In transconjugants *A. avenae* 5+ and *B. glumae* 3+, 'sporadic loss' or 'no detectable loss' of pAAA83 was observed over about 250 generations, respectively (Fig. 7). In contrast, the fraction of KSM-resistant cells in *A. avenae* 83 gradually decreased to 56.7% at the endpoint of the assay, indicating 'low instability'. These results indicate that pAAA83 was maintained and inherited in *A. avenae* and *B. glumae*, but that the stability varied, even within the same species.

DISCUSSION

Isolation of the novel IncP-1 β plasmid, pAAA83

Whole draft genome analysis of *A. avenae* 83 demonstrated that the *aac(2)-Ila* gene was carried by the novel IncP-1 β plasmid (Fig. 2). In contrast, other KSM-resistant *B. glumae* and *A. avenae* strains possess the IncP genomic island, which is inserted into the 3' terminal region of the chromosomal *guaA* gene (Fig. S1). IncP-1 plasmids are widely distributed in Gram-negative bacteria, and phylogenetic analysis of TrfA classified them into six subgroups: α , β , γ , ϵ , δ and ζ (Popowska and Krawczyk-Balska, 2013). The structure and organization of the backbone genes, which regulate plasmid replication, maintenance, partition and transfer, are highly conserved among IncP-1 β subgroup plasmids, including pAAA83 (Fig. 2). IncP-1 β plasmids have been identified from environmentally or clinically important bacteria, such as *Bordetella pertussis* (the agent of human whooping cough) and *Mycobacterium abscessus* ssp. *bolletii* (the agent of human pulmonary disease) (Kamachi *et al.*, 2006; Leão *et al.*, 2013). Thus far, occurrence of the IncP-1 β plasmid in phytopathogenic bacteria has not been reported. This report is the first description of the isolation of the IncP plasmid from rice pathogenic bacteria.

The regions between Tra 1 and Tra 2, and/or between *oriV* and the *trfA* gene, are known as hotspots for the integration of various mobile genetic elements (Popowska and Krawczyk-Balska, 2013; Schlüter *et al.*, 2007). These accessory elements confer advantages to their host bacteria to survive unfavourable environmental conditions. For example, pB8 contains *oxa-2* (conferring β -lactam resistance), *aadA4* (conferring aminoglycoside resistance), *qacE Δ 1* (conferring small multidrug resistance), *sul1* (conferring sulfonamide resistance) and *qacF* (conferring quaternary ammonium compound resistance) in its transposons of the accessory region (Schlüter *et al.*, 2005). In pAAA83, the *kluA-kluB* region is present downstream of *oriV* (Fig. 2). The genes *kluA* (transcriptional regulator) and *kluB* (ParE-like plasmid stabilization protein) are found in R751, pB8, pADP1 and pTP6, but not in any of the other IncP-1 β plasmids, indicating that this region is acquired (Heuer *et al.*, 2004; Schlüter *et al.*, 2007). The second accessory region, containing streptomycin- and mercury-resistant transposons, is located

Fig. 7 Stability of plasmid pAAA83 in *Acidovorax avenae* 83 (▲), *A. avenae* 5+ (●) (a) and *Burkholderia glumae* 3+ (●) (b). The strains *A. avenae* 1-1 and *B. glumae* 5091, in which the *aac(2)-Ila* gene is integrated into the chromosome, were used as controls (□). Data points and error bars represent means $\pm$ standard deviations from three independent cultures.

between *trfA* and *oriV* (Fig. 3). The streptomycin-resistant transposon Tn5393 has been identified on many plasmids, such as pB4 and pB10 from bacteria isolated from wastewater treatment plants (Schlüter *et al.*, 2003; Tauch *et al.*, 2003), pPSR1 from the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* (Sundin *et al.*, 2004), pEa34 from *E. amylovora* (McGhee *et al.*, 2011) and pRAS2 from the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida* (L'Abée-Lund and Sørum, 2000). Streptomycin-resistant *Xanthomonas oryzae* pv. *oryzae*, the causal pathogen of bacterial leaf blight of rice, was isolated from various locations in Japan; at most of them streptomycin has never been applied (Wakimoto and Mukoo, 1963). There seems to be no correlation between insertion of the streptomycin-resistant transposon into pAAA83 and streptomycin usage. However, KSM has been rated as a highly effective drug against streptomycin-resistant *E. amylovora* in the USA (Adaskaveg *et al.*, 2011; Jurgens and Babadoost, 2013; McGhee and Sundin, 2011). Monitoring the susceptibility of KSM to other phytopathogenic bacteria is important for the sustainable use of antibiotics.

Biological properties of pAAA83

Plasmids are extrachromosomal elements which may contribute significantly to bacterial evolution by adaptation to unfavourable environments with regard to their hosts and the presence of toxic compounds (Vivian *et al.*, 2001). Antibiotic resistance genes located on plasmids may spread to diverse phylogenetic groups of bacteria by conjugation. The IncP plasmids are important mobile genetic elements for antibiotic resistance. The transfer of plasmids consists of three steps: replication in donor cells; transfer to a new host; and persistence and inheritance in the recipient cells

(Popowska and Krawczyk-Balska, 2013). In this study, we confirmed that pAAA83 was a functional conjugative plasmid and maintained in the absence of antibiotic selection (Fig. 7). *Acidovorax avenae* 83 showed slow plasmid loss over 250 generations and sporadic loss was found in strain 5+, whereas no detectable loss was demonstrated for *B. glumae* 3+. Gelder *et al.* (2007) also indicated that plasmid stability is influenced by strain-specific traits. Despite this, even in the absence of selection pressure, the majority of bacterial cells possessed pAAA83 over the experimental period of our study.

The KSM acetyltransferase gene module is a minimum unit for dissemination of KSM resistance

The third accessory region, which carries the *aac(2)-Ila* gene, resides between the Tra 1 and Tra 2 regions. In *B. glumae* 5091, the *aac(2)-Ila* and *bglu_5091g13* genes are inserted into the IncP island, and this gene region, named KAM (KSM acetyltransferase and *bglu_5091g13* genes module), can be released from its chromosome by forming a closed circular intermediate of 36–37-bp direct repeats (Yoshii *et al.*, 2012). KAM from *A. avenae* 83 (pAAA83) and 1-1 (IncP island) also forms circular intermediate DNA (Yoshii *et al.*, 2012). KAM is conserved in the two different genetic carriers, and only four nucleotides are substituted in this region (three substitutions are in *aac(2)-Ila* and one substitution is in *bglu_5091g13*), a common feature between *B. glumae* 5091 and *A. avenae* 83. These results demonstrate that acquisition of the *aac(2)-Ila* gene is possible in two ways (Fig. 8). KAM may have an important role in the dissemination of KSM resistance by both the genomic island and episomal plasmid.

Fig. 8 A model of the acquisition of the *aac(2)-Ila* gene. (a) Circularized DNA could be integrated into the IncP island of donor bacteria (*Burkholderia glumae* 5091 and *Acidovorax avenae* 1-1 type). (b) Circularized DNA could be integrated into the IncP-1 β plasmid of donor bacteria (*A. avenae* 83 type). The nucleotide sequences of direct repeats (*attP*, *attL2* and *attR2*) have been reported previously (Yoshii *et al.*, 2012), whereas the *attB* sequence has not been determined experimentally.

Another genetic element, class I integron, can incorporate one or several gene cassettes, which are small mobile elements consisting of a promoterless single gene and a recombination site (*attC*, previously called 59-be) (Domingues *et al.*, 2012). Circular forms of the gene cassette are inserted into the integron by recombination between the *attC* and *attI* sites of the integron by the action of integrase. Over 100 gene cassettes are known, the majority of which encode various antibiotic resistance genes, including aminoglycosides. As the transcription of these genes is dependent on the promoter of the integron (*Pc*), expression levels of gene cassettes vary with different variants of *Pc* (Domingues *et al.*, 2012). Similarly, differences in the promoter region of the *aac(2)-Ila* gene between strain 83 (pAAA83 type) and strain 1-1 (IncP island type) result in different transcriptional levels of the *aac(2)-Ila* genes, and in a different level of resistance to KSM. Furthermore, the *attC* sites of the gene cassettes can form secondary structures by self-combination of the DNA strand (Recchia and Hall, 1995). These single-stranded *attC* sites are specific binding targets for integron integrase (Francia *et al.*, 1999). Although the integrase gene is also encoded in the IncP island, it is not encoded in pAAA83. In addition, the folding secondary structure of the 36–37-bp direct repeats cannot be predicted by *in silico* analysis. These observations indicate that integration of KAM occurs through a mechanism that is distinct from the gene cassettes.

Finally, the geographical origin of *A. avenae* 83 was different from that of the other strains. Strain 83 was isolated from Hokkaido, the northern island of Japan, whereas other all

KSM-resistant strains of *A. avenae* and *B. glumae* were isolated from the main island of Japan, Honshu. There is no trade of rice seeds between Honshu and Hokkaido because of differences in climate and cultivation requirements of the distinct cultivars (Abe, 2005). A future investigation may clarify the geographical diversity and significance of the genetic carriers of the *aac(2)-Ila* genes which will help to manage KSM resistance.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

The bacterial strains used in this study are shown in Table 2. *Acidovorax avenae*, *B. glumae* strains and *E. coli* were grown in Luria–Bertani (LB) medium. For the antibiotic susceptibility assay, *A. avenae* strains were cultured in peptone–glucose medium (Yoshii *et al.*, 2012). All isolates, except for *E. coli* (Table 2), were supplied by Hokko Chemical Industry Co., Ltd, Atsugi, Kanagawa, Japan.

DNA manipulation

Standard DNA manipulations, including DNA extraction, enzyme assays, cloning and PCR, were performed as described by Ausubel *et al.* (1994). DNA sequences were determined with an ABI 3100 DNA sequencer (Applied Biosystems Inc., Life Technologies Japan, Tokyo, Japan). Sequences were aligned with CLC Sequence Viewer version 6.6.2 (CLC bio Japan, Tokyo, Japan).

Table 2 Bacterial strains used in this study.

Strain	Relevant characteristics	Source or reference
<i>Acidovorax avenae</i> ssp. <i>avenae</i>		
83	KSM resistant (pAAA83), oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
1-1	KSM resistant (IncP island), oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
3-2	KSM resistant (IncP island), oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
213	KSM resistant (IncP island), oxolinic acid resistant	Yoshii <i>et al.</i> (2012)
161	KSM resistant, oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
5	KSM resistant, oxolinic acid resistant	Yoshii <i>et al.</i> (2012)
5+	Transconjugant, KSM resistant (pAAA83), oxolinic acid resistant	This study
<i>Burkholderia glumae</i>		
5091	KSM resistant (IncP island), oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
4-1-9	KSM resistant (IncP island), oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
9-1-1	KSM resistant (IncP island), oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
11-1-2	KSM resistant (IncP island), oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
210	KSM resistant, oxolinic acid susceptible	Yoshii <i>et al.</i> (2012)
3	KSM resistant, oxolinic acid resistant	Yoshii <i>et al.</i> (2012)
3+	Transconjugant, KSM resistant (pAAA83), oxolinic acid resistant	This study
<i>Escherichia coli</i>		
DH5 α	F ⁻ , ϕ 80d <i>lacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169, <i>endA1</i> , <i>recA1</i> <i>hsdR17</i> (rk ⁻ mk ⁺), <i>deoR</i> , <i>thi-1</i> , <i>supE44</i> , λ^- , <i>gyrA96</i> , <i>relA1</i> , <i>phoA</i>	TAKARA BIO INC.
BL21(DE3)	F ⁻ , <i>ompT</i> , <i>hsdS</i> (rB ⁻ mB ⁻), <i>gal</i> , <i>dcm</i> , λ (DE3)	Life Technologies

KSM acetyltransferase activities of crude bacterial lysates

Acidovorax avenae and *B. glumae* cells were collected from liquid shake cultures at 120 rpm and 28 °C after 1 or 2 days. Each bacterial pellet was resuspended in extraction buffer [10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 25 mM NH₄Cl, 0.6 mM β -mercaptoethanol], cells were disrupted on ice 15 times each for 10-s pulses with a 10-s interval using an Astrason XL-2020 (Misonix Inc., Farmingdale, NY, USA) and insoluble materials were removed by centrifugation at 20 000 *g* for 20 min. Acetyltransferase assays were carried out as described previously (Yoshii *et al.*, 2012) with 100 mM sodium phosphate buffer (pH 7.6), 80 μ M acetyl-CoA, 1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), 0.4 mM KSM and cell lysate. Acetyltransferase activity (nmol/mg of protein/min) was calculated from the initial rate of generation of acetylated KSM.

Overexpression and purification of AAC(2')-IIa of strains 5091 and 83

The KSM acetyltransferase gene from *B. glumae* 5091 was amplified by PCR using the primers *aac*(2')IIa-*NdeI* (5'-TCGTCATATGAAAGACAGATCCCATGACG-3') and *aac*(2')IIa-*XhoI* (5'-TACACTCGAGGGCGCGTGATATTCGCA-3'), containing the restriction sites for *NdeI* and *XhoI* (italic in the primer sequences), respectively. An amplified DNA fragment was cloned into the *NdeI* and *XhoI* sites of pET-22b(+) (Merck Millipore, Billerica, MA, USA) to produce an AAC(2')-IIa (*B. glumae* strain 5091 and *A. avenae* strain 1-1, subsequently called 5091) fused C-terminal 6 $\times$ His tag. To overexpress strain 83 of AAC(2')-IIa, an S146T substitution was introduced into pET22b-AAC(2')IIa-His (strain 5091) by Quickchange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA, USA) as follows. The whole pET22b-AAC(2')IIa-His (strain 5091) plasmid was amplified by PCR with one cycle of 95 °C for 30 s, followed by 12 cycles of 95 °C for 30 s, 55 °C for 30 s and 68 °C for 12 min, using primers *aac*(2')IIa-421mF (5'-

GAGAGTCTGATGCAGACTTGGGCGGAGGAC-3') and *aac*(2')IIa-450mR (5'-GTCCTCCGCCCAAG7CTGCATCAGACTCTC-3') containing the substitution sites in italic. This methylated template plasmid was digested with *DpnI*, and the resulting pET22b-AAC(2')IIa-His (strain 83) was sequenced to assess whether the substitution had been introduced.

pET22b-AAC(2')IIa-His (strain 5091 or 83) was overexpressed in *E. coli* BL21(DE3) following the addition of 1 mM isopropyl- β -D-thiogalactopyranoside at an optical density at 600 nm (OD_{600 nm}) of 0.5. Cells were allowed to grow at 37 °C for an additional 3.5 h prior to harvest. The soluble AAC(2')-IIa-His proteins were affinity purified by Ni²⁺-NTA agarose according to the manufacturer's protocol (Qiagen K.K., Tokyo, Japan).

Enzyme kinetics

The optimum pH and temperature for AAC(2')-IIa-His (strain 5091) were determined by a microtitre plate assay. The buffers used for the pH assay were 50 mM sodium citrate (pH 4.2–5.8), 50 mM sodium phosphate (pH 5.8–8.0) and 50 mM Tris-HCl (pH 7.2–8.8). Temperature assays were performed by incubation of the reaction mixture at the selected temperatures. For kinetic assays of purified AAC(2')-IIa-His protein, various concentrations of KSM were added to 1 mL of the assay mixture, as described in the enzyme assay with crude lysate. The concentration of acetyl-CoA was 80 μ M for determination of the kinetic parameters of KSM, and that of KSM was 800 μ M for acetyl-CoA. Triplicate samples were assayed for each substrate concentration, and initial velocities were fitted to the Michaelis–Menten equation using Hyper 32 1.0 (<http://hyper32.software.informer.com/>).

Sequence and bioinformatics analyses of pAAA83 and the *A. avenae* 83 genome

pAAA83 and the *A. avenae* 83 genome were sequenced with a next-generation sequencer: MiSeq (Illumina K.K., Tokyo, Japan). A DNA library

was synthesized with a Nextera DNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol. The DNA sequences obtained were annotated by CLC genomics workbench ver. 6.0.1 (CLC bio). The open reading frames (ORFs) were predicted using GeneMark.hmm 2.0 (Lukashin and Borodovsky, 1998) and manually. The G + C content and GC skew of pAAA83 were calculated by arcWithColor 1.35 (<http://www.ige.tohoku.ac.jp/joho/gmProject/gmhomeJP.html>). To infer the genetic distance of pAAA83 with other IncP plasmids, full nucleotide sequences of the 18 plasmids were first aligned using Mauve 2.2.0 (Darling *et al.*, 2010). Then, two large conserved regions (between *traI* and *traC2* and between *trbL* and *trbC*) were extracted and connected to a single alignment. The insertion element between *trbD* and *trbF* of the IncP-1 α plasmid pBS228 was eliminated for subsequent analysis. A phylogenetic tree was constructed based on the alignment of the concatenated sequences using maximum likelihood with MEGA 5.05 (Tamura *et al.*, 2011). The region of the genomic island in *A. avenae* ATCC 19860 was estimated using IslandViewer (Langille and Brinkman, 2009).

The accession numbers of the other IncP-1 plasmids used in bioinformatics analyses were as follows: pA1 (NC_007353), pA81 (CP002288), pADP1 (DQ448807), pAKD1 (JN106164), pAKD4 (GQ983559), pAKD16 (JN106167), pB3 (NC_006388), pB4 (NC_003430), pB8 (AJ863570), pB10 (NC_004840), pBP136 (NC_008459), pBS228 (NC_008357), pJP4 (AY365053), pMCBF1 (AY950444), pQKH54 (NC_008055), R751 (NC_001735), pTP6 (NC_007680) and pUO1 (NC_005088).

Antibiotic susceptibility test

The MIC of streptomycin was determined by the agar dilution method as described previously (Yoshii *et al.*, 2012).

Determination of transcriptional start sites of the *aac(2)-IIa* gene

Total RNA was isolated as described by Aiba *et al.* (1981). To determine the transcriptional start sites of the *aac(2)-IIa* gene from *A. avenae* strains 1-1 and 83, 5' RACE by anchored PCR, using a 5' RACE System for Rapid Amplification of cDNA ends ver. 2.0 (Life Technologies), was carried out. After synthesis of the first-strand cDNA using SuperScript III reverse transcriptase (Life Technologies) with a gene-specific *aac(2)-II-R* primer (Yoshii *et al.*, 2012), a poly(C) tail was added to the 3' end of the first-strand cDNA by terminal deoxynucleotidyl transferase. The first PCR was performed with a primer specific for the poly(C) tail, AP-1 (5'-CCGGATCCGGGIIIGGGIIGGIG-3'), and *aac(2)-II-710R* (Yoshii *et al.*, 2012). Nested PCR was performed with primer AP-1 and *aac(2)-II-308R* (5'-AGTGAAAGATAACGTTCAGATGCT-3'). The PCR fragment was subcloned into pGEM-Teasy (Promega KK, Tokyo, Japan) and the nucleotide sequences of plural clones were analysed.

To determine the precise position of the additional start site of the *aac(2)-IIa* gene in *A. avenae* 83, 5' RACE by inverse PCR, using the 5'-Full RACE Core Set (TAKARA BIO Inc., Otsu, Shiga, Japan), was carried out. After synthesis of the first-strand cDNA with a gene-specific 5' phosphorylated *aac(2)-II-R* primer, the single-strand cDNA was self-ligated. First, inverse PCR was performed with primers *aac(2)-II-308R* and *aac(2)-II-336F* (5'-CGATCTTGGTCTGATTACGAGT-3'). Nested PCR was

performed with primers *aac(2)-II-240R* (5'-TTCTTCGAGCTTTAGTGTATG TCA-3') and *aac(2)-II-451F* (5'-AGAGTACCGGAAGCTTTGTTGAG-3'). The PCR fragment was subcloned into pGEM-Teasy (Promega) and the nucleotide sequences of the plural clones were analysed.

qPCR analysis

To analyse the levels of the *aac(2)-IIa* gene transcript in *A. avenae*, qRT-PCR was performed with TAKARA SYBR Premix Ex Taq II (Tli RNaseH Plus). RNA was purified by the method mentioned above and cDNA was synthesized from 1 μ g of RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TAKARA BIO Inc.), following the manufacturer's instructions. Then, qPCR was performed with one cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, using a Thermal Cycler Dice Real Time System TP800 (TAKARA BIO Inc.). For the qPCR assay, the following primers were used for the *aac(2)-IIa* gene, *aac(2)-IIa-316F* (5'-AGTGATGGCCCTGTACCC-3') and *aac(2)-IIa-486R* (5'-ACGGAAGCCAAGCTCAAAC-3'), and for the *gyrA* gene, *gyrA1829F* (5'-GGCTGTACTGGCTCAAGTC-3') and *gyrA1931R* (5'-TTGATCTTCTCGCTTCCTG-3'). Amplification data were analysed by standard curves to determine the gene expression relative to the *gyrA* gene.

To analyse the relative DNA amount of the *aac(2)-IIa* gene in *A. avenae*, qPCR was performed as indicated above. Total DNA was isolated as described previously (Yoshii *et al.*, 2012).

Filter mating assay

KSM-resistant *A. avenae* 83 was used as the donor strain for filter mating with the recipient *A. avenae* strain 5 or *B. glumae* strain 3, which are KSM-sensitive and oxolinic acid-resistant strains, respectively (Table 2). Conjugative transfer was conducted on the membrane filter for 5 h as described previously (Yoshii *et al.*, 2012). Some transconjugants were verified by detection of the *aac(2)-IIa* gene.

Plasmid stability assay

The stability of pAAA83 in *A. avenae* or *B. glumae* was examined according to Gelder *et al.* (2007). Briefly, three independent colonies were inoculated into 5 mL of LB broth amended with 100 μ g/mL KSM, and each culture was incubated for 24 h at 28 °C with shaking at 140 rpm. Subsequently, 4.88 μ L of culture were transferred to 5 mL of fresh LB without KSM (approximately 10 generations per day). Successive cultures for 25 days were conducted under the above conditions every 24 h using 1 : 2¹⁰ dilution rates for each transfer (approximately 250 generations). Every 2 or 3 days, the culture was diluted and spread onto peptone-glucose medium. Fifty randomly chosen colonies from each culture were analysed with regard to whether or not cells contain plasmid by growth on peptone-glucose agar medium amended with 100 μ g/mL of KSM. Plasmid stability was defined as follows: high instability, rapid plasmid loss within 100 generations; low instability, much slower plasmid loss; stable, no detectable loss after 200 generations (Gelder *et al.*, 2007). To confirm whether plasmid-free segregants were derived from *A. avenae* 83, three KSM-sensitive cells were used for inoculation of three-leaf-stage rice

seedlings (*Oryza sativa* L. cv. Asahi) by injecting the bacterial cell suspension into the base of the stem. Symptoms of brown stripe were evaluated 10 days after inoculation.

Accession numbers

The nucleotide sequences of pAAA83 (contig 110) and downstream of the *guaA* gene (contig 28) from *A. avenae* strain 83 were deposited in the GenBank nucleotide sequence database under accession numbers AB852526 and AB852527, respectively.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

- Abe, H. (2005) Domestic distribution of rice seed. In: *Abstracts of the 15th Symposium of the Research Committee on Fungicide Resistance*, pp. 29–34. Shizuoka, Japan: The Phytopathological Society of Japan, Tokyo (in Japanese).
- Adaskaveg, J.E., Forster, H. and Wade, M.L. (2011) Effectiveness of kasugamycin against *Erwinia amylovora* and its potential use for managing fire blight of pear. *Plant Dis.* **95**, 448–454.
- Aiba, H., Adhya, S. and de Crombrughe, B. (1981) Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J. Biol. Chem.* **256**, 11 905–11 910.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) *Current Protocols in Molecular Biology*, Vol. 1. New York: Greene Publishing Associates and John Wiley and Sons, Inc.
- Darling, A.E., Mau, B. and Perna, N.T. (2010) Progressive mauve: multiple genome alignment with gene gain, loss, and rearrangement. *PLoS ONE*, **5**, e11147.
- Domingues, S., da Silva, G.J. and Nielsen, K.M. (2012) Integrins: vehicles and pathways for horizontal dissemination in bacteria. *Mob. Genet. Elements*, **2**, 211–223.
- Flatt, P.M. and Mahmud, T. (2007) Biosynthesis of aminocyclitol-aminoglycoside antibiotics and related compounds. *Nat. Prod. Rep.* **24**, 358–392.
- Francia, M.V., Zabala, J.C., de la Cruz, Z. and Garcia-Lobo, J.M. (1999) The *Int1* integrin integrase preferentially binds single-stranded DNA of the *attC* site. *J. Bacteriol.* **181**, 6844–6849.
- Gelder, L.D., Ponciano, J.M., Joyce, P. and Top, E.M. (2007) Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. *Microbiology*, **153**, 452–463.
- Goto, K. and Ohata, K. (1956) New bacterial diseases of rice (brown stripe and grain rot). *Ann. Phytopathol. Soc. Jpn.* **21**, 46–47 (in Japanese).
- Ham, J.H., Melanson, R.A. and Rush, M.C. (2011) *Burkholderia glumae*: next major pathogen of rice? *Mol. Plant Pathol.* **12**, 329–339.
- Harada, K.M., Aso, Y., Hashimoto, W., Mikami, B. and Murata, K. (2006) Sequence and analysis of the 46.6-kb plasmid pA1 from *Sphingomonas* sp. A1 that corresponds to the typical IncP-1 β plasmid backbone without any accessory gene. *Plasmid*, **56**, 11–23.
- Heuer, H., Szczepanowski, R., Schneiker, S., Pühler, A., Top, E.M. and Schlüter, A. (2004) The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1 β group without any accessory genes. *Microbiology*, **150**, 3591–3599.
- Hiramatsu, M., Wada, T., Kanda, M. and Yamamura, H. (1989) Effect of kasugamycin on the control of bacterial diseases. In: *Abstracts of Plant pathogenic bacteria: Proceedings of the 7th International Conference on Plant Pathogenic Bacteria* (Klement, Z., ed.), pp. 219–224. Hungary: Akadémiai Kiadó, Budapest.
- Hori, T., Kuroda, T. and Ishikawa, K. (2007) Occurrence of kasugamycin-resistant *Burkholderia glumae*. *Ann. Phytopathol. Soc. Jpn.* **73**, 278 (in Japanese).
- Ikekawa, T., Umezawa, H. and Iitaka, Y. (1966) The structure of kasugamycin hydrobromide by X-ray crystallographic analysis. *J. Antibiot. (Tokyo)*, **19**, 49–50.
- Ishiyama, T., Hara, I., Matsuoka, M., Sato, K., Shimada, S., Izawa, R., Hashimoto, T., Hamada, M., Okami, Y., Takeuchi, T. and Umezawa, H. (1965) Studies on the preventive effect of kasugamycin on rice blast. *J. Antibiot. (Tokyo)*, **18**, 115–119.
- Jurgens, A.G. and Babadoost, M. (2013) Sensitivity of *Erwinia amylovora* in Illinois apple orchards to streptomycin, oxytetracycline, kasugamycin, and copper. *Plant Dis.* **97**, 1484–1491.
- Kamachi, K., Sota, M., Tamai, Y., Nagata, N., Konda, T., Inoue, T., Top, E.M. and Arakawa, Y. (2006) Plasmid pBP136 from *Bordetella pertussis* represents an ancestral form of IncP-1 β plasmids without accessory mobile elements. *Microbiology*, **152**, 3477–3484.
- L'Abée-Lund, T.M. and Sørum, H. (2000) Functional Tn5393-like transposon in the R plasmid pRAS2 from the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida* isolated in Norway. *Appl. Environ. Microbiol.* **66**, 5533–5535.
- Langille, M.G. and Brinkman, F.S. (2009) IslandViewer: an integrated interface for computational identification and visualization of genomic islands. *Bioinformatics*, **25**, 664–665.
- Lavigne, J.P., Vergunst, A.C., Bourg, G. and O'Callaghan, D. (2005) The IncP island in the genome of *Brucella suis* 1330 was acquired by site-specific integration. *Infect. Immun.* **73**, 7779–7783.
- Leão, S.C., Matsumoto, C.K., Carneiro, A., Ramos, R.T., Nogueira, C.L., Junior, J.D., Lima, K.V., Lopes, M.L., Schneider, H., Azevedo, V.A. and da Costa da Silva, A. (2013) The detection and sequencing of a broad-host-range conjugative IncP-1 β plasmid in an epidemic strain of *Mycobacterium abscessus* subsp. *bolletii*. *PLoS ONE*, **8**, e60746.
- Lukashin, A.V. and Borodovsky, M. (1998) GeneMark.hmm: new solutions for gene finding. *Nucleic Acids Res.* **26**, 1107–1115.
- McGhee, G.C. and Sundin, G.W. (2011) Evaluation of kasugamycin for fire blight management, effect on nontarget bacteria, and assessment of kasugamycin resistance potential in *Erwinia amylovora*. *Phytopathology*, **101**, 192–204.
- McGhee, G.C., Guasco, J., Bellomo, L.M., Blumer-Schuette, S.E., Shane, W.W., Irish-Brown, A. and Sundin, G.W. (2011) Genetic analysis of streptomycin-resistant (Sm^R) strains of *Erwinia amylovora* suggests that dissemination of two genotypes is responsible for the current distribution of Sm^R *E. amylovora* in Michigan. *Phytopathology*, **101**, 182–191.
- Popowska, M. and Krawczyk-Balska, A. (2013) Broad-host-range IncP-1 plasmids and their resistance potential. *Front. Microbiol.* **4**, 44. doi: 10.3389/fmicb.2013.00044.
- Recchia, G.D. and Hall, R.M. (1995) Gene cassettes: a new class of mobile element. *Microbiology*, **141**, 3015–3027.
- Schaad, N.W., Postnikova, E., Sechler, A., Claflin, L.E., Vidaver, A.K., Jones, J.B., Agarkova, I., Ignatov, A., Dickstein, E. and Ramundo, B.A. (2008) Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citrulli* Schaad et al., 1978) comb. nov., and proposal of *A. oryzae* sp. nov. *Syst. Appl. Microbiol.* **31**, 434–446.
- Schluenzen, F., Takemoto, C., Wilson, D.N., Kaminishi, T., Harms, J.M., Hanawa-Suetsugu, K., Szaflarski, W., Kawazoe, M., Shirouzu, M., Nierhaus, K.H., Yokoyama, S. and Fucini, P. (2006) The antibiotic kasugamycin mimics mRNA nucleotides to destabilize tRNA binding and inhibit canonical translation initiation. *Nat. Struct. Mol. Biol.* **13**, 871–878.
- Schlüter, A., Heuer, H., Szczepanowski, R., Forney, L.J., Thomas, C.M., Pühler, A. and Top, E.M. (2003) The 64508 bp IncP-1 β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1 β group. *Microbiology*, **149**, 3139–3153.
- Schlüter, A., Heuer, H., Szczepanowski, R., Poler, S.M., Schneiker, S., Pühler, A. and Top, E.M. (2005) Plasmid pB8 is closely related to the prototype IncP-1 β plasmid R751 but transfers poorly to *Escherichia coli* and carries a new transposon encoding a small multidrug resistance efflux protein. *Plasmid*, **54**, 135–148.
- Schlüter, A., Szczepanowski, R., Pühler, A. and Top, E.M. (2007) Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol. Rev.* **31**, 449–477.
- Schuwirth, B.S., Day, J.M., Hau, C.W., Janssen, G.R., Dahlberg, A.E., Cate, J.H. and Vila-Sanjurjo, A. (2006) Structural analysis of kasugamycin inhibition of translation. *Nat. Struct. Mol. Biol.* **13**, 879–886.
- Sen, D., Van der Auwera, G.A., Rogers, L.M., Thomas, C.M., Brown, C.J. and Top, E.M. (2011) Broad-host-range plasmids from agricultural soils have IncP-1 backbones with diverse accessory genes. *Appl. Environ. Microbiol.* **77**, 7975–7983.
- Smalla, K., Haines, A.S., Jones, K., Krögerrecklenfort, E., Heuer, H., Schlöter, M. and Thomas, C.M. (2006) Increased abundance of IncP-1 β plasmids and mercury resistance genes in mercury-polluted river sediments: first discovery of IncP-1 β plasmids with a complex mer transposon as the sole accessory element. *Appl. Environ. Microbiol.* **72**, 7253–7259.

- Song, L., Pan, Y., Chen, S. and Zhang, X. (2012) Structural characteristics of genomic islands associated with GMP synthases as integration hotspot among sequenced microbial genomes. *Comput. Biol. Chem.* **36**, 62–70.
- Spadafora, V.J., Orr, G., Wade, L. and Wiglesworth, M. (2010) Kasugamycin—a novel antibiotic for North American agriculture. *Phytopathology*, **100**, S166.
- Sundin, G.W., Mayfield, C.T., Zhao, Y., Gunasekera, T.S., Foster, G.L. and Ullrich, M.S. (2004) Complete nucleotide sequence and analysis of pPSR1 (72,601 bp), a pPT23A-family plasmid from *Pseudomonas syringae* pv. *syringae* A2. *Mol. Genet. Genomics*, **270**, 462–476.
- Szczepanowski, R., Krahn, I., Bohn, N., Pühler, A. and Schlüter, A. (2007) Novel macrolide resistance module carried by the IncP-1 β resistance plasmid pRSB111, isolated from a wastewater treatment plant. *Antimicrob. Agents Chemother.* **51**, 673–678.
- Takeuchi, T. and Tamura, O. (1991) Occurrence of kasugamycin-resistant *Acidovorax avenae* subsp. *avenae*. *Ann. Phytopathol. Soc. Jpn.* **57**, 117–118 (in Japanese).
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.
- Tanaka, N., Yoshida, Y., Sashikata, K., Yamaguchi, H. and Umezawa, H. (1965) Inhibition of polypeptide synthesis by kasugamycin, an aminoglycosidic antibiotic. *J. Antibiot. (Tokyo)*, **19**, 65–68.
- Tauch, A., Schlüter, A., Bischoff, N., Goesmann, A., Meyer, F. and Pühler, A. (2003) The 79,370-bp conjugative plasmid pB4 consists of an IncP-1 β backbone loaded with a chromate resistance transposon, the *strA*–*strB* streptomycin resistance gene pair, the oxacillinase gene *bla_{NPS-1}*, and a tripartite antibiotic efflux system of the resistance-nodulation-division family. *Mol. Genet. Genomics*, **268**, 570–584.
- Thorsted, P.B., Macartney, D.P., Akhtar, P., Haines, A.S., Ali, N., Davidson, P., Stafford, T., Pocklington, M.J., Pansegrau, W., Wilkins, B.M., Lanka, E. and Thomas, C.M. (1998) Complete sequence of the IncP β plasmid R751: implications for evolution and organisation of the IncP backbone. *J. Mol. Biol.* **282**, 969–990.
- Tominaga, T., Kimura, K. and Goh, N. (1983) Bacterial brown stripe of rice in nursery box, caused by *Pseudomonas avenae*. *Ann. Phytopathol. Soc. Jpn.* **49**, 463–466 (in Japanese).
- Trefault, N., De la Iglesia, R., Molina, A.M., Manzano, M., Ledger, T., Pérez-Pantoja, D., Sánchez, M.A., Stuardo, M. and González, B. (2004) Genetic organization of the catabolic plasmid pJP4 from *Ralstonia eutropha* JMP134 (pJP4) reveals mechanisms of adaptation to chloroaromatic pollutants and evolution of specialized chloroaromatic degradation pathways. *Environ. Microbiol.* **6**, 655–668.
- Uematsu, T., Yoshimura, D., Nishiyama, K., Ibaraki, T. and Fuji, H. (1976) Occurrence of bacterial seedling rot in nursery flat, caused by grain rot bacterium *Pseudomonas glumae*. *Ann. Phytopathol. Soc. Jpn.* **42**, 310–312 (in Japanese).
- Vakulenko, S.B. and Mobashery, S. (2003) Versatility of aminoglycosides and prospects for their future. *Clin. Microbiol. Rev.* **16**, 430–450.
- Vivian, A., Murillo, J. and Jackson, R.W. (2001) The role of plasmids in phytopathogenic bacteria: mobile arsenals? *Microbiology*, **147**, 763–780.
- Wakimoto, S. and Mukoo, H. (1963) Natural occurrences of streptomycin resistant *Xanthomonas oryzae*, the causal bacteria of leaf blight disease of rice. *Ann. Phytopathol. Soc. Jpn.* **28**, 153–158 (in Japanese).
- Xie, G.L., Zhang, G.Q., Liu, H., Lou, M.M., Tian, W.X., Li, B., Zhou, X.P., Zhu, B. and Jin, G.L. (2011) Genome sequence of the rice-pathogenic bacterium *Acidovorax avenae* subsp. *avenae* RS-1. *J. Bacteriol.* **193**, 5013–5014.
- Yoshii, A., Moriyama, H. and Fukuhara, T. (2012) The novel kasugamycin 2'-N-acetyltransferase gene *aac(2)-IIa*, carried by the IncP island, confers kasugamycin resistance to rice-pathogenic bacteria. *Appl. Environ. Microbiol.* **78**, 5555–5564.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Polymerase chain reaction (PCR) analysis of the IncP island from kasugamycin (KSM)-resistant *Acidovorax avenae* and *Burkholderia glumae*. KSM-sensitive *B. glumae* 210 was used as the negative control. PCR A, *guaA* to *integrase* region; PCR B, *integrase* to *repA* region; PCR C, *trbL* to *trbJ* region; PCR D, *tral* region; PCR E, 92 bp upstream of *aac(2)-IIa* to the end of the *aac(2)-IIa* region; PCR F, *aac(2)-IIa* to the *bglu_5091g13(-like)* region.

Table S1 Conserved motifs between TrfA and KluB proteins in the pAAA83, pB8 and R751 plasmids.

Table S2 Comparison of the *guaA* 3'-terminal sequence of *Acidovorax avenae* 83 with *A. avenae* ATCC 19860 and 1-1, *A. avenae* ssp. *citrulli* AAC00-1, *B. glumae glumae* 5091 and 210, and similar sequence of pAAA83.