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ARTICLE

Instability of the 16S rRNA methyltransferase-encoding *npmA* gene: why have bacterial cells possessing *npmA* not spread despite their high and broad resistance to aminoglycosides?

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Abstract

The NpmA bacterial 16S rRNA methyltransferase, which is identified from *Escherichia coli* strains, confers high resistance to many types of aminoglycoside upon its host cells. But despite its resistance-conferring ability, only two cases of its isolation from *E. coli* (14 years apart) have been reported to date. Here, we investigated the effect of the *npmA* gene on aminoglycoside resistance in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and its stability in *E. coli* cells by comparing it with *armA*, another 16S rRNA methyltransferase gene currently spreading globally. As a result, we found that *npmA* conferred resistance to all types of aminoglycoside antibiotics we tested (except streptomycin) in both *P. aeruginosa* and *K. pneumoniae*, as well in *E. coli*. In addition, co-expression of *armA* and *npmA* resulted in an additive effect for the resistance. However, in return for the resistance, we also observed that the growth rates and the cell survivability of the strains transformed with the *npmA*-harboring plasmids were inferior than those of the control strains and that these plasmids were easily disrupted by IS*10*, IS*1*, and IS*5* insertion sequences. We discuss these data in the context of the threat posed by pathogenic strains possessing *npmA*.

Introduction

The history of antibiotics is also the history of the fight against drug-resistant pathogens. As the World Health Organization has reported, the global spread of drug-resistant pathogenic bacterial strains is becoming an increasingly urgent issue to be addressed [1]. In particular, infectious diseases caused by drug-resistant Gram-negative bacteria are particularly concerning. Gram-negative bacteria originally have natural resistance to various classes of antibiotics, but worse still, new drug-resistant genes, such as the colistin resistance *mcr-1* gene [2], the carbapenem resistance bla_{NDM-1} gene, bla_{KPC} gene, and other β-lactamase-encoding genes [3], have been isolated from various pathogenic species worldwide. In this study, we focused on another two genes of concern, *armA* and *npmA*, which each encode 16S rRNA methyltransferases that confer high-level resistance to aminoglycoside drugs.

armA, which was originally identified on a plasmid from a *Citrobacter freundii* strain [4], was found to be widely spread in Asia, Europe, and the United States [5]. It should also be noted that *armA* is often found with other antibiotic-resistant genes on highly transmissible plasmids [6–8]. The *armA* gene product, ArmA, catalyzes the methylation of guanine at position 1405 in the 16S rRNA located in the decoding site of 30S ribosomes [9]. The strains expressing enzymes related to ArmA, such as RmtB and RmtC, are also found in various parts of the world [5].

In contrast, *npmA* encodes a 16S rRNA methyltransferase that catalyzes the methylation of adenine at position

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1408 located in the decoding site of 30S ribosomes [9]. *npmA*, which was originally found in an *Escherichia coli* strain isolated in 2003 in Japan, confers high resistance to many aminoglycoside types upon the host *E. coli* cells [10]. But surprisingly, despite it contributing to strong resistance against a broad range of aminoglycoside antibiotics, the second report of the isolation of the bacteria possessing *npmA* did not appear in the scientific literature until August 2017 from China [11].

Hence, we performed a comparative study on *armA* and *npmA*, in which we evaluated their activities against aminoglycosides, their stabilities, and their effects on *E. coli*, *P. aeruginosa*, and *K. pneumoniae* as host bacterial cells.

Results

Drug resistance in an *E. coli* DH5α derivative expressing rRNA methylase genes

For the purpose of understanding the properties of *armA* and *npmA*, *E. coli* DH5α strains possessing *armA* and/or *npmA* were constructed (Table 1) and they were used to evaluate their drug resistance patterns against various aminoglycoside types. As shown in Table 2, the NCGM58 strain was highly resistant to dibekacin, arbekacin, gentamicin, sisomicin, and fortimicin A, as compared with the NCGM57 vector control strain, indicating that *armA* confers resistance to these compounds. In contrast, the NCGM59 strain showed intermediate or high resistance against all the aminoglycosides we tested except streptomycin, indicating that *npmA* confers resistance to various kinds of aminoglycoside compounds. Moreover, the NCGM60 strain transformed by a plasmid containing both *armA* and *npmA* displayed the resistance properties of both NCGM58 and NCGM59. In particular, the differences we observed in the MIC values for paromomycin and lividomycin A among the test strains were clearly related to the co-expression of *armA* and *npmA*.

Effect of *armA* and *npmA* on *E. coli* proliferation

Although both *armA* and *npmA* were determined to be responsible for aminoglycoside resistance, these gene products might adversely affect *E. coli* cells because the enzymes they encode alter the partial structure of a decoding site in the ribosome by methylating the 16S rRNA. Therefore, we examined the effect of *armA* and *npmA* on proliferation of the DH5α derivative strains in LB medium supplemented with 30 µg/ml of chloramphenicol. As a result, the growth rate of the NCGM58 strain possessing *armA* was similar to the vector control NCGM57 strain, as shown in Fig. 1. In contrast, the NCGM59 strain, which

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Description	
Escherichia coli strains		
DH5α		TaKaRa Bio Inc.,
NCGM57	DH5α/GS1	This work
NCGM58	DH5α/GS73	This work
NCGM59	DH5α/GS79	This work
NCGM60	DH5α/GS81	This work
BKKZ1054	DH5α/pSTV-aac	This work
AG1		Agilent Technologies Japan
BKKZ1594	AG1/GS1	This work
BKKZ1595	AG1/GS73	This work
BKKZ1596	AG1/GS79	This work
BKKZ1597	AG1/GS81	This work
MDS42 Δ*recA*	MG1655 derivative, 663,316 bp deleted (IS-less)	Scarab Genomics, LCC
BKKZ1751	MDS42/GS1	This work
BKKZ1752	MDS42/GS73	This work
BKKZ1753	MDS42/GS79	This work
BKKZ1754	MDS42/GS81	This work
Klebsiella pneumoniae strains		
PCI 602		Laboratory collection
BKKZ1436	PCI 602/pBHGS1	This work
BKKZ1437	PCI 602/pBHGS73	This work
BKKZ1438	PCI 602/pBHGS79	This work
BKKZ1443	PCI 602/pBHGS81	This work
Pseudomonas aeruginosa strains		
PAO1		Laboratory collection
BKKZ1461	PAO1/pBHGS1	This work
BKKZ1463	PAO1/pBHGS73	This work
BKKZ1465	PAO1/pBHGS79	This work
BKKZ1467	PAO1/pBHGS81	This work
Plasmids		
GS1	pACYC184 replicon, *lacZα*, *cat*, Equivalent to a low-copy cloning vector pSTV28	TaKaRa Bio Inc.,
GS73	GS1 derivative, *lacZα*::*armA*	This work
GS79	GS1 derivative, *lacZα*::*npmA*	This work
GS81	GS1 derivative, *lacZα*::[*armA*, *npmA*]	This work
pSTV-aac	GS1 derivative, *lacZα*::*acc(3)-IV*	This work
pBHR1	broad-host-range plasmid, *cat*, *aph(3′)-Ia*	MoBiTec GmbH
pBHGS1	replicon of pSTV28 and pBHR1, *lacZα*, *cat*, *aph(3′)-Ia*	This work
pBHGS73	pBHGS1 derivative, *lacZα*::*armA*	This work

Table 1 (continued)

Strains/plasmids	Description	
pBHGS79	pBHGS1 derivative, *lacZα*:: *npmA*	This work
pBHGS81	pBHGS1 derivative, *lacZα*:: [*armA npmA*]	This work

The genes shown encode the following enzymes: *lacZα*; α-fragment of β-galactosidase from *E. coli*, *cat*; chloramphenicol acetyltransferase, *aph(3′)-Ia*; aminoglycoside phosphotransferase (kanamycin resistance)

Table 2 Antibacterial activities (μg/ml) of the aminoglycoside compounds tested against the *E. coli* DH5α derivatives NCGM57, NCGM58, NCGM59, and NCGM60

Aminoglycoside compounds tested	MIC (μg/ml) for *Escherichia coli* strains			
	NCGM57 (control)	NCGM58 (*armA*)	NCGM59 (*npmA*)	NCGM60 (*armA*, *npmA*)
Dibekacin	0.25	128	64	128
Arbekacin	0.25	>128	8	>128
Gentamicin	0.13	>128	16	>128
Sisomycin	0.13	>128	128	>128
Butirosin A	0.25	0.25	>128	>128
Paromomycin	0.5	1	4	64
Lividomycin A	2	8	16	>128
Fortimicin A	0.5	>128	>128	>128
Apramycin	4	4	>128	>128
Streptomycin	1	1	1	1

possesses *npmA*, grew slower, while the NCGM60 strain, which possesses both *armA* and *npmA*, grew much slower than the other strains.

The effect of the *npmA* gene on aminoglycoside resistance and its proliferation patterns in *K. pneumoniae* and *P. aeruginosa*

As there have been no reports of a strain possessing *npmA* except for two cases in *E. coli*, it was unclear whether *npmA* could function in other Gram-negative species as well as in *E. coli*. Therefore, we introduced either or both *armA* and *npmA* into *K. pneumoniae* PCI 602 and *P. aeruginosa* PAO1 using a broad-host-range plasmid (Table 1). We then evaluated the resistance of these bacterial species to aminoglycoside drugs and their proliferation rates.

The results (Table 3) of these experiments showed that *armA* possessing *K. pneumoniae* BKKZ1437 and *P. aeruginosa* BKKZ1463 displayed resistance to arbekacin and gentamicin, whereas the *npmA*-possessing BKKZ1438 and BKKZ1465 strains displayed resistance to apramycin and

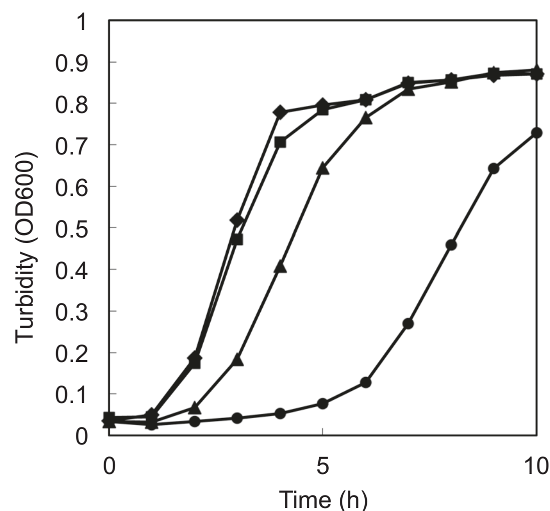

Fig. 1 Turbidity time course of the *E. coli* DH5α derivatives possessing *armA* and/or *npmA* methyltransferase genes. Overnight cultures of each strain were inoculated into LB medium containing chloramphenicol and then incubated at 37 °C at 140 spm. Symbols: diamonds, NCGM57 vector control strain; squares, NGCM58 possessing *armA*; triangles, NCGM59 possessing *npmA*; circles, NCGM60 possessing *armA* and *npmA*

butirosin A. It was interesting that *npmA* conferred higher resistance to arbekacin and gentamicin in *P. aeruginosa* compared with *E. coli* and *K. pneumoniae*. All the aminoglycoside drugs we tested, except streptomycin, were ineffective against BKKZ1443 and BKKZ1467, which both possess *armA* and *npmA*, as does *E. coli* NCGM60.

Slower growth was also observed in *K. pneumoniae* BKKZ1438 and BKKZ1443 and in *P. aeruginosa* BKKZ1465 and BKKZ1467, both of which harbored the *npmA*-containing plasmid (Fig. 2 and Table 4). *K. pneumoniae* BKKZ1437 and *P. aeruginosa* BKKZ1463, which both possess *amrA*, also had slightly slower growth rates than their respective BKKZ1436 and BKKZ1461 vector control strains.

Evaluation of methylase gene stability in the plasmids

Incidentally, we observed that the NCGM59 strain had lost its resistance to apramycin after several subculturing procedures. We hypothesized that this may have occurred via the disruption of *npmA*. Therefore, we performed continuous subculturing experiments with the strains and analyzed the plasmids obtained from each successive culture. Another host strain, *E. coli* AG1, was also used for this experiment. Strains BKKZ1594, BKKZ1595, BKKZ1596, and BKKZ1597, which are AG1 derivatives transformed via the uptake of GS1, GS73, GS79, and GS81 plasmids, respectively, showed similar growth properties to those of the NCGM strains (data not shown). As expected, the size

Table 3 Antibacterial activities (μg/ml) of the aminoglycoside compounds tested against *E. coli*, *K. pneumoniae*, and *P. aeruginosa* strains possessing *armA* and/or *npmA* methyltransferase genes

Aminoglycoside compounds tested	MIC (μg/ml) for							
	Klebsiella pneumoniae strains				*Pseudomonas aeruginosa* strains			
	BKKZ1436 (control)	BKKZ1437 (*armA*)	BKKZ1438 (*npmA*)	BKKZ1443 (*armA*, *npmA*)	BKKZ1461 (control)	BKKZ1463 (*armA*)	BKKZ1465 (*npmA*)	BKKZ1467 (*armA*, *npmA*)
Streptomycin	2	1	1	2	16	16	16	16
Apramycin	2	2	>128	>128	4	4	>128	>128
Butirosin A	1	1	>128	>128	16	16	>128	>128
Arbekacin	0.25	>128	8	>128	0.5	>128	64	>128
Gentamicin	0.25	>128	16	>128	1	>128	>128	>128

of the plasmid had altered over the 16 rounds of subculturing that had been performed in LB medium for all of the quintuple cultures in all the cultures of strains possessing *npmA* (Fig. 3A). On the one hand, eight subcultures (nearly equivalent of an eightieth generation) were needed to detect the plasmid alteration in NCGM59 and BKKZ1596 (Fig. S2A) and the cells harboring the original plasmid were not completely replaced by the new derivatives harboring the altered plasmid within the 16 subcultures. On the other hand, it is notable that only two subcultures were needed to detect the plasmid alteration and five subcultures were enough for the derivative strains to replace the original NCGM60 and BKKZ1597 strains completely (Fig. S2B).

The sequencing analysis revealed that the GS79 and GS81 plasmids had their *npmA* or *armA* genes (including the untranslated regions) disrupted via addition of the insertion sequence (IS) in the all the cultures (Fig. 3B). In the DH5α derivative NCGM59 and NCGM60 strains, IS*10* played a major role in plasmid disruption. In contrast, in the AG1 derivative strains BKKZ1596 and BKKZ1597, IS*10* was not present in their genomic DNAs and, therefore, no IS*10*-inserted plasmids were identified in them. Alternatively, it is possible that these plasmids were disrupted by insertion of IS*1* or IS*5*.

It is not unreasonable that the IS fragments were preferentially inserted into the *npmA* gene of the GS79 plasmid. But interestingly, all the IS fragments were preferentially inserted into the *armA* gene in the case of GS81, with the exception of one culture. Additionally, it should be noted that base substitutions, deletions, or insertions (other than transposons) were not observed in both *armA* and *npmA* throughout this experiment.

Effect of *npmA* on transposon movement

It was surprising to us that the *npmA* and *armA* genes in the *npmA*-containing plasmids were easily disrupted by the transposons. Therefore, we examined whether *npmA* could activate movement of the transposon. In this experiment, NCGM59, NCGM60, and BKKZ1054, which is the control strain possessing the *aac(3)-IV* apramycin acetyltransferase encoding gene, were cultivated continuously and the collected cells were analyzed by Southern blotting to detect the movement of IS*10*. Apramycin was used to prevent disruption of the *npmA* gene.

Agarose gel electrophoresis of the plasmids (Fig. 4A) and genomic Southern blotting analysis (Fig. 4B) revealed that movement of two IS*10* elements, which DH5α possesses, was not observed in any of the triplicate cultures of BKKZ1054. By contrast, one extra band was detected in the Southern blot of the NCGM59 strain. This band probably comes from the plasmid disrupted by IS*10*, although the plasmid amount was too small to be detected by electrophoresis. In the case of the NCGM60 strain, electrophoresis and sequencing analyses revealed that the *armA* gene in the plasmid from all the triplicate cultures was disrupted by ISs and the original plasmids were completely excluded. Therefore, the additional band we detected probably comes from a plasmid disrupted by IS*10*, as detected by Southern blotting. However, no other extra bands were observed.

Plasmid stability in the IS-less *E. coli* strain

To estimate the effect of the IS elements on the mutation frequency in the *npmA*-expressing *E. coli* cells, continuous subculture was performed using the transformants of the IS-less strain, MDS42 Δ*recA*. After 12 rounds of subculturing, the plasmids were extracted and analyzed. Although no drastic change in the plasmid size was observed (Fig. S3), deletions or base substitutions were observed in the GS79 plasmid in three of the quintuple cultures of BKKZ1753 and in the GS81 plasmid in four of the quintuple cultures of the BKKZ1754 strain (Table 5). Unlike the cases of NCGM60 and BKKZ1597, all the mutations in the bacteria from the BKKZ1754 cultures were observed in the *npmA* gene.

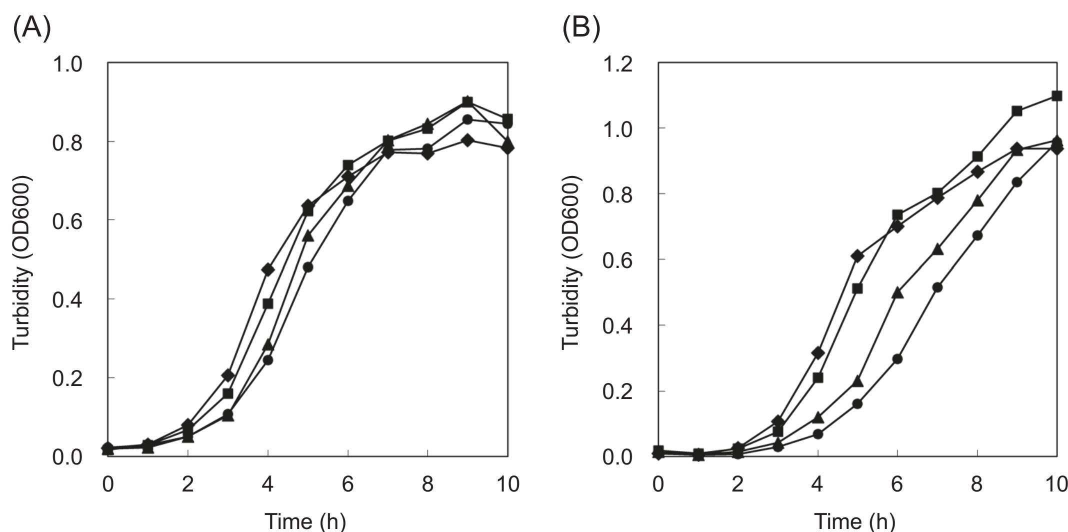

Fig. 2 Turbidity time course for *K. pneumoniae* PCI 602 (**A**) and *P. aeruginosa* PAO1 (**B**) derivatives harboring *armA* and/or *npmA* methyltransferase genes. Overnight cultures of each strain were inoculated into LB medium containing kanamycin and then incubated at 37 °C at 140 spm. Symbols: diamonds, BKKZ1436 (**A**) and BKKZ1461 (**B**) vector control strains; squares, BKKZ1437 (**A**) and BKKZ1463 (**B**) possessing *armA*; triangles, BKKZ1438 (**A**) and BKKZ1465 (**B**) possessing *npmA*; circles, BKKZ1443 (**A**) and BKKZ1467 (**B**) possessing *armA* and *npmA*

Table 4 The mean and the standard deviation of doubling times (in three independent trials) for *K. pneumoniae* PCI 602 (A) and *P. aeruginosa* PAO1 (B) derivatives harboring *armA* and/or *npmA* methyltransferase genes

Strain (resistant gene)	Doubling time (min)
(A)	
BKKZ1436 (control)	46.4 ± 1.1
BKKZ1437 (*armA*)	46.2 ± 1.6
BKKZ1438 (*npmA*)	53.5 ± 1.9*
BKKZ1443 (*armA, npmA*)	56.1 ± 1.0**
(B)	
BKKZ1461 (control)	34.8 ± 5.8
BKKZ1463 (*armA*)	39.7 ± 1.7
BKKZ1465 (*npmA*)	51.6 ± 6.0**
BKKZ1467 (*armA, npmA*)	57.1 ± 6.5**

Asterisks represent significant differences from the control strains (*p value <0.01, **p value <0.001, Student's t test)

E. coli cell viability in the competition cultures

Lastly, we observed the time courses of cell viability of NCGM59 strain cocultured with NCGM57 or NCGM58. As shown in Fig. 5, CFU of NCGM59 strain was decreased to 1/100 of the end of growth phase even in the mono-culture. In the competition cultures, CFU of NCGM59 strain was drastically decreased after stationary phase and was superseded by competition strain, regardless of the initial cell numbers.

Discussion

In this study, we investigated the properties of *E. coli*, *P. aeruginosa*, and *K. pneumoniae* strains that possess two RNA methyltransferase genes, *armA* and *npmA*. The different aminoglycoside-resistant spectrums among the strains possessing either or both of the methyltransferase genes seems to result from the difference of the mode of binding to the ribosome among aminoglycoside drugs. Broader drug-resistant spectrum in the strain possessing both *armA* and *npmA* is a reasonable outcome considering that more methylation of the decoding site makes it more difficult for aminoglycoside drugs to approach the site. Unlike the other aminoglycoside compounds we tested, streptomycin does not interact directly with positions 1405 and 1408 of 16S rRNA [12], and this may be the reason why streptomycin is active against the control strains. Additionally, acquisition of resistance against aminoglycosides by *npmA* was observed in three species of Gram-negative bacteria, implying that *npmA*-expressing strains of various Gram-negative genera could potentially be spread and isolated at any moment in time.

However, this does not actually happen, implying that methylation by NpmA is probably a double-edged sword; it would also disturb ribosome assembly and/or smooth and accurate translation, which would lead to decreased cell proliferation and poor survivability after the growth phase. This means that in the absence of aminoglycoside antibiotics, bacterial strains expressing NpmA will be easily excluded from the environment. In fact, *npmA* is inactivated

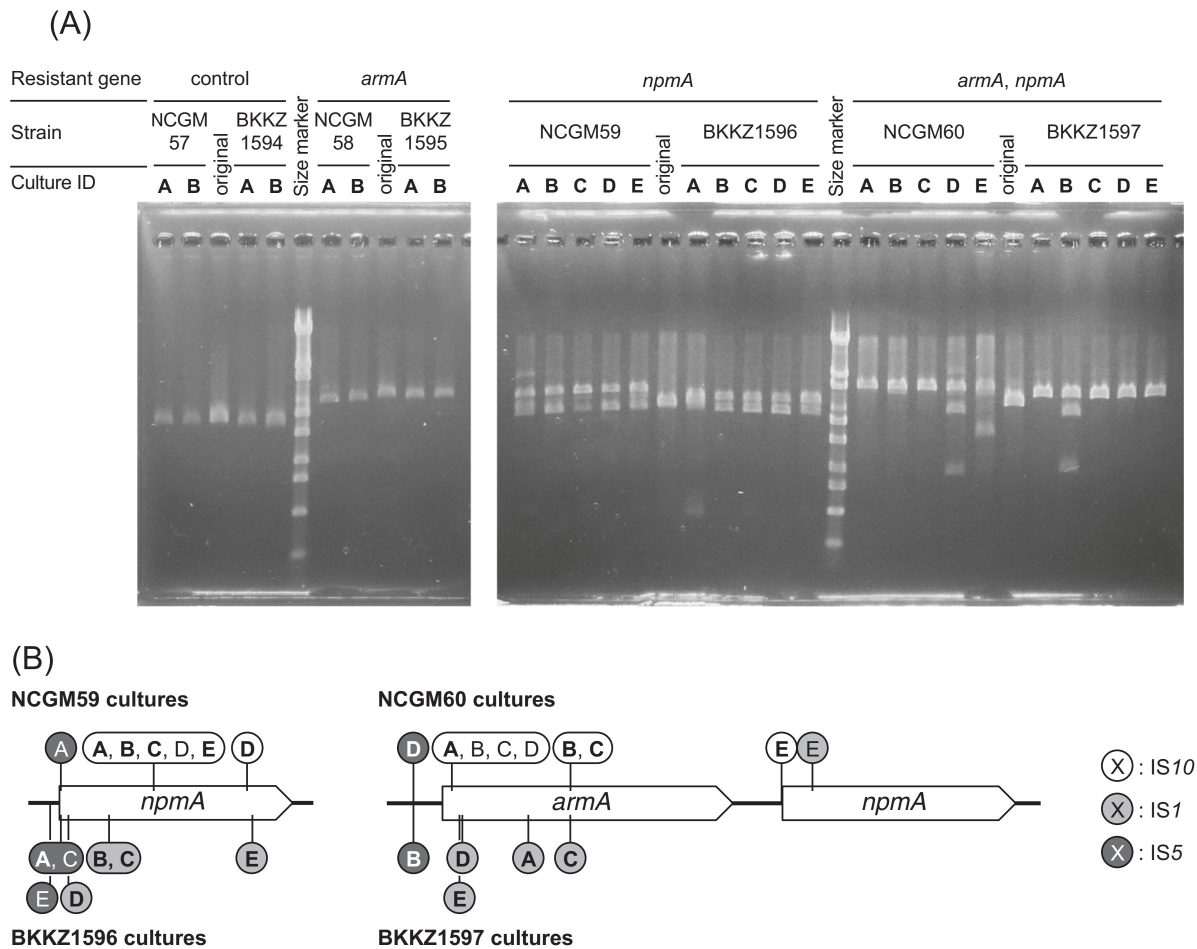

Fig. 3 A Agarose gel electrophoresis of the EcoRI digestion patterns of the plasmids extracted from the double or quintuple cultures of *E. coli* strains subjected to 16 rounds of subculturing. The strains were transformed with the original plasmids shown in the figure. The EcoT14I digest of λ DNA was used as the size marker. The size marker contains the following 11 discrete fragments (in base pairs): 19,329, 7743, 6223, 4254, 3472, 2690, 1882, 1489, 925, 421, and 74 (invisible). **B** Outline of the transposon insertion positions detected preferentially in *npmA* or *armA* from the plasmids shown in Fig. 3 (**A**). IS*10*, IS*1*, and IS*5* are shown by white, pale gray, and dark gray circles, respectively. Alphabetical letters represent the culture IDs for each strain and bold font mean that the disruptant was found to be the most preferential

by IS elements with great ease. Although the *npmA* gene was also inactivated by deletion or base substitution in the IS-less MDS42 strain, the frequency of mutation induction was much less than in the case of the DH5α derivatives and AG1, indicating that IS insertion is the principal cause of inactivation of *npmA* and *armA* co-expressed with *npmA*. Recently, a causal relationship has been established between IS elements and adaptation to some unfavorable environmental condition [13] such as high osmolarity [14], metal limitation [15], or nutrient-limitation [16]. Although we did not obtain any evidence that *npmA* stimulates the movement of IS elements, it seems likely that expression of this gene is unfavorable to cells and cells can easily adapt to this condition via IS elements.

If so, are we free from the threat of the rapid spread of *npmA*-possessing pathogens? This is possibly true, but we need to consider why the second report of an *npmA*-possessing strain took such a long time (14 years), because although this strain did not expand to become dominant, it did survive long enough to spread between Japan and China, at least. The two following possibilities may explain these observations.

The first possibility is that the stable maintenance of *npmA* in bacterial cells requires additional genetic variation in the bacterial chromosomal genes, such as those of the 16S rRNA or those encoding ribosomal proteins. In this scenario, the *npmA* transmission frequency would be lower than that of strains carrying other drug-resistant genes, even when *npmA* is located on highly transmittable plasmids. If additional genetic variation is needed in all of the multicopy 16S rRNA genes for *npmA* maintenance, then transmission would rarely occur. Genome analysis and biochemical analyses of the two isolates possessing *npmA* should provide us with clues to support or refute this possibility.

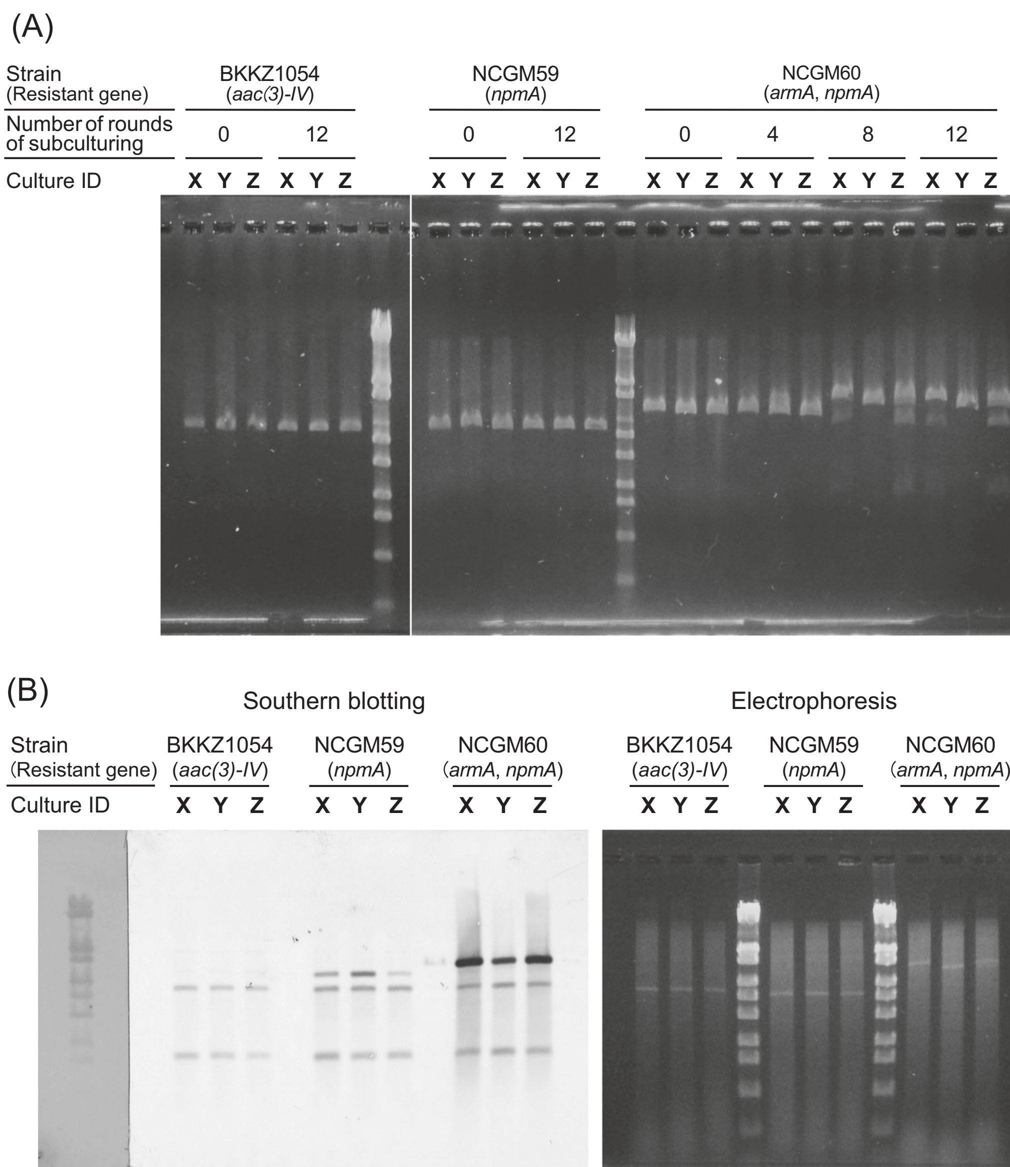

Fig. 4 **A** Agarose gel electrophoresis showing the EcoRI digestion patterns of the plasmids extracted from triplet cultures of *E. coli* strains subjected to 0, 4, 8, and 12 rounds of subculturing. **B** Southern blot analysis of the genomic DNA from triplet cultures of *E. coli* strains subjected to 12 rounds of subculturing. Samples of the genomic DNA were digested with ClaI and the IS*10* sequences were detected by Southern blotting. The size marker on the membrane (EcoT14I digest of λ DNA digest) was stained with azure C

The second possibility is that the *npmA* gene spreads as a disruptant by ISs (or by other transposable elements). Given that *npmA* is easily inactivated by the insertion of IS elements, it is conceivable that these elements could also be easily eliminated from *npmA* and gain aminoglycoside antibiotic resistance again. If so, this could represent a novel, unique mechanism that could be classified as "ready to use IS-dependent resistance", because *npmA* is usually inactivated by a transposon and only when the host cell is exposed to aminoglycoside does *npmA* become active by eliminating the IS elements. Although a bacterium harboring an IS-inactivated *npmA* gene has not yet been identified, the accumulating genome sequence data from clinical and community-acquired isolates, including data for aminoglycoside-sensitive strains, will disclose whether this IS-dependent mechanism actually exists.

It appears likely that the presence of the *npmA* gene is unfavorable for the growth and survival of general pathogenic bacteria, making it possible that the explosive spreading of *npmA* may not occur. However, further investigation to understand the mechanism of *npmA* maintenance in bacterial cells will also be needed to make a final judgment on the potential threat to public health posed by *npmA*-possessing pathogens.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α (TaKaRa Bio Inc., Shiga, Japan), AG1 (Agilent Technologies Japan, Ltd., Tokyo, Japan), the IS-less MDS42 strain [17] (Scarab Genomics, LCC, Madison, WI), *P. aeruginosa* PAO1, and *K. pneumoniae* PCI 602 were used to host the plasmids containing *armA* and/or *npmA*.

Plasmid construction and transformation

The plasmids used in this study are listed in Table 1. Plasmids GS73, GS79, and GS81 are derivatives of pSTV28 (TaKaRa Bio Inc., designated as GS1 in this study), which contains *armA* or *npmA* or both genes, respectively, in the multi-cloning site in *lacZ*α. Plasmid pSTV-aac is a pSTV28 derivative containing the *aac(3)-IV* gene, which encodes an aminoglycoside 3-*N*-acetyltransferase. These plasmids were used to transform the *E. coli* strains. Plasmids pBHR-GS1/73/79/81 were made from GS1/73/79/81 by inserting the kanamycin-resistant *aph(3′)-Ia* gene and the genes for replication from the broad-host-range plasmid, pBHR1 [18] (MoBiTec GmbH, Göttingen, Germany). These plasmids were used to transform *K. pneumoniae* and *P. aeruginosa* by chemical transformation [19] and electroporation [20], respectively.

Table 5 Sequence analysis of the *npmA* gene from plasmids isolated from IS-less BKKZ1573 and BKKZ1574 strains after 12 round of subculturing, the results of which are shown in Fig. S3

Culture ID	Variant(s) found	
	BKKZ1573	BKKZ1574
A	c.196delA[a], c.3delG[a]	c.196delA[a]
B	ND	c.196delA[a]
C	c.114_115delTA[a]	c.67C>T[b]
D	ND	c.182_644del[a]
E	c.196delA[a]	ND

The description of the genetic variants is in accordance with the Human Genome Variation Society [21]

ND no variants were detected

[a]Frameshift mutation

[b]Amber mutation

Evaluation of the antibacterial activities of the aminoglycoside compounds

Antibacterial activities of the aminoglycosides were evaluated by the agar dilution method. Overnight cultures of the test strains were diluted and spotted onto BD™ Difco™ Muller-Hinton agar (Thermo Fisher Scientific Kabushiki Kaisha, Kanagawa, Japan) containing the test compounds at concentrations of 0.031–128 µg/ml in a twofold dilution series. After incubation for 16 h at 37 °C, the antibacterial activities of the bacterial species were evaluated using the ten aminoglycoside compounds shown in Fig. S1. All of these compounds were of analytical grade or were ethical pharmaceuticals.

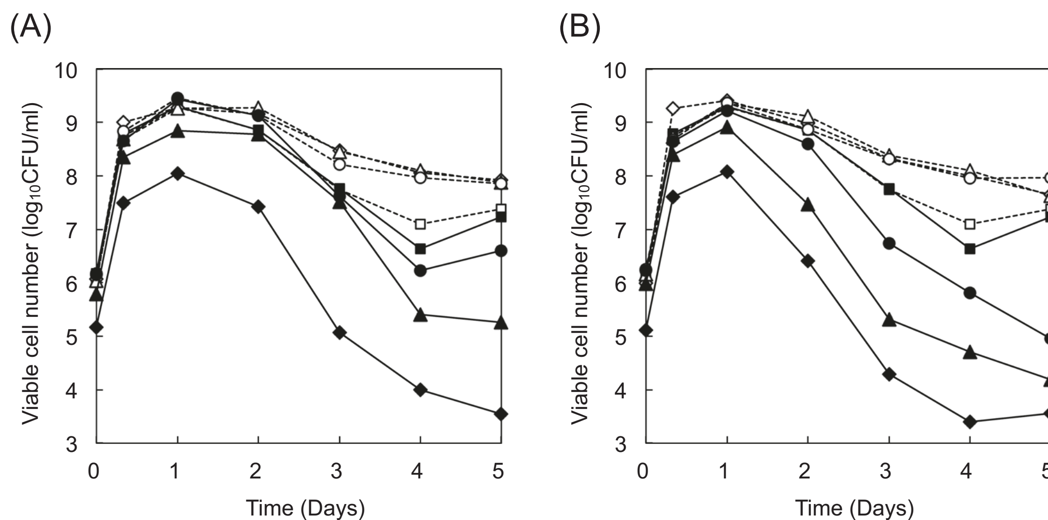

Fig. 5 Time courses of CFU in the competitive culture of NCGM59 with NCGM57 (**A**) or NCGM58 (**B**). Overnight cultures of NCGM59 strain and competitive strains were mixed at the ratio of 1:10 (diamond), 1:1 (triangle), or 10:1 (circle) and these mixtures were used for inoculation source. NCGM59 monoculture (square) was also performed in parallel. LB agar plates supplemented with chloramphenicol or apramycin were used to evaluate the total cell number (opened symbol, dashed line) or viable cell number of NCGM59 (closed symbol, solid line), respectively

Proliferation properties

Overnight cultures of the different bacterial species were inoculated at 1% into fresh LB medium (3 ml) supplemented with chloramphenicol at 30 µg/ml (for *E. coli*) or 50 and 100 µg/ml of kanamycin for *K. pneumoniae* and *P. aeruginosa*, respectively. The cultures were incubated with shaking at 140 spm (strokes per minute) at 37 °C, and their turbidity time courses were monitored.

Continuous subculture

Single colonies of the test strains obtained by transformation were inoculated into 3 ml of LB medium supplemented with 30 µg/ml of chloramphenicol and the cultures were incubated overnight at 37 °C. Three microliters of each culture were added to 3 ml of LB medium supplemented with chloramphenicol, after which one was incubated for 24 h at 37 °C. Aliquots (3 µl) of the cultures were then used for the next subculture. This process was repeated 16 times at most. Where indicated, 30 µg/ml of apramycin was used instead of chloramphenicol.

To detect alterations in the plasmids, they were extracted from each culture using the QuickLyse Miniprep Kit (Qiagen K. K., Toyko, Japan), and then analyzed by EcoRI digestion and subsequent agarose gel electrophoresis. To determine the plasmid sequences, they were digested and tagged using the Nextera XT DNA Library Prep Kit (Illumina Kabushiki Kaisha, Tokyo, Japan), and then analyzed by MiSeq sequencing with the paired-end read 300-cycle option.

To detect movement of the IS*10* insertion sequence, genomic DNA from the strains was extracted using the RBC Genomic DNA Extraction Kit Mini (RBC Bioscience, New Taipei City, Taiwan). Next, the genomic DNA samples were digested with ClaI and subjected to Southern blotting. A DIG DNA Labeling Kit (Roche Diagnostics K.K., Tokyo, Japan) was used to label the DNA probe, which contained the partial fragment (bases 438–765) of the IS*10* transposase gene obtained by PCR amplification.

All reagents and chemicals used in this study were analytical grade and the restriction enzymes were purchased from New England Biolabs Japan (Tokyo, Japan).

Competition cultures

The overnight culture of NCGM59 strain in LB medium with 30 µg/ml of chloramphenicol at 37 °C was combined with the overnight culture of NCGM57 or NCGM58 at the ratio of 1:10, 1:1, and 10:1. Ten microliters of each mixture were inoculated in 10 ml of fresh LB medium supplemented with 30 µg/ml of chloramphenicol and the cultures were incubated at 37 °C. The colony forming units (CFU) of there cultures were periodically counted using LB agar plate supplemented with 30 µg/ml of chloramphenicol or apramycin to evaluate the total cell number or viable cell number of NCGM59, respectively.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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