A structural basis for the antibiotic resistance conferred by an N1-methylation of A1408 in 16S rRNA

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ABSTRACT

The aminoglycoside resistance conferred by an N1-methylation of A1408 in 16S rRNA by a novel plasmid-mediated methyltransferase NpmA can be a future health threat. In the present study, we have determined crystal structures of the bacterial ribosomal decoding A site with an A1408m1A antibiotic-resistance mutation both in the presence and absence of aminoglycosides. G418 and paromomycin both possessing a 6′-OH group specifically bind to the mutant A site and disturb its function as a molecular switch in the decoding process. On the other hand, binding of gentamicin with a 6′-NH₃⁺ group to the mutant A site could not be observed in the present crystal structure. These observations agree with the minimum inhibitory concentration of aminoglycosides against Escherichia coli. In addition, one of our crystal structures suggests a possible conformational change of A1408 during the N1-methylation reaction by NpmA. The structural information obtained explains how bacteria acquire resistance against aminoglycosides along with a minimum of fitness cost by the N1-methylation of A1408 and provides novel information for designing the next-generation aminoglycoside.

INTRODUCTION

Aminoglycosides such as neomycin and gentamicin are a class of antibiotics that cause mRNA miscoding (1,2), inhibit translocation (3,4) and inhibit ribosome recycling (5), and have been widely used in treatment of serious bacterial infectious diseases. They are produced by Streptomyces (-mycin) and Micromonospora (-micin) species (6). These aminoglycoside producers have several mechanisms to prevent self-toxicity by nature; (i) modifying the target site, (ii) modifying aminoglycoside itself, (iii) decreasing intracellular drug concentration (7,8). As the WHO (World Health Organization) recently warned about global threat of antibiotic resistance (9), pathogenic bacteria acquired these antibiotic-resistance mechanisms from the antibiotic producers due to the excessive, inappropriate and often unnecessary use of antibiotics.

Aminoglycosides except streptomycin commonly target the ribosomal A-site molecular switch in 16S rRNA composed of highly conserved 15 nt residues (Figure 1) (10). During the decoding process, the molecular switch changes its conformation between ‘off’ (with tucked-in A1492 and/or A1493) and ‘on’ (with bulged-out A1492 and A1493) states, and discriminates a single cognate-tRNA from several near-cognate tRNAs by recognitions between the bulged-out A1492/A1493 residues and the first two base pairs in the tRNA–mRNA complex through A-minor interactions (11–13). The binding specificity of aminoglycosides to the bacterial A-site switch is mainly conferred by its ring I, which makes a pseudo pair with the universally conserved A1408 in the bacterial A site through two hydrogen bonds (Supplementary Figure S1) (12–26). The interaction forces the A site to adopt the ‘on’ state even when near-cognate tRNAs are delivered to the A site, thereby reducing the accuracy of the decoding process resulting to cell death. Therefore, it is reasonable that the aminoglycoside producers possess chromosomally encoded 16S rRNA methyltransferase (e.g. KamA, KamB, CmnU and Kmr) as a self-defense system that methylates the N1 position of A1408 to disturb the pseudo-pair formation (27). However, in 2007, Wachino et al. first isolated an aminoglycoside-resistant Escherichia coli strain possessing a plasmid-encoded A1408-N1-methyltransferase NpmA from Japanese clinical settings (28). Since the npmA gene is encoded in a plasmid, rapid spread of antibiotic-resistant bacteria by its horizontal transfer between pathogens can be a future threat. Recently, structural insights into the methylation of A1408 by A1408-N1-methyltransferases have been revealed by crystal structures of NpmA and KamB themselves and NpmA in complex with the substrate 30S ribosome (29–31). These structural information are useful for designing A1408-N1-methyltransferase inhibitors. In the present study, in order to obtain a structural basis for the intrin-
sic and acquired antibiotic resistance conferred by the N1-methylation of A1408, we have determined crystal structures of the m1A1408-modified A site both in the presence and absence of aminoglycosides.

### MATERIALS AND METHODS

#### Sample preparations and crystallizations

An RNA duplex designed to fold as a double helix containing two bacterial A sites with the A1408m1A modification and chemical structure of 1-methyladenosine (m1A). The rRNA residues are numbered according to the numbering used in Escherichia coli 16S rRNA. The A/m1A1408, A1492 and A1493 residues are colored in orange, blue and red, respectively.

#### RESULTS

**Aminoglycoside binding to the m1A1408-modified A site**

In the A1408m1A-G418 and A1408m1A-Paromomycin crystals, aminoglycosides G418 and paromomycin both possessing a 6′-OH group specifically bind to the m1A1408-modified A site and force it to adopt the ‘on’ state conformation with fully bulged-out A1492 and A1493 (Figure 3, top). It is very important to note that these bulged-out adenines recognize two consecutive Watson–Crick G = C base pairs in a symmetry-related molecule through the A-minor interactions (Supplementary Figure S3). This crystal packing interaction perfectly mimics the recognition between the A site and the tRNA–mRNA complex occurring in the ribosome (12,13,39). Ring I of G418 and paromomycin stack on top of G1491. A short distance between the CH3 group attached to N1 of m1A1408 and the 6′-OH group of ring I suggests that a C-H...O interaction may exist between them and supports formation of a pseudo pair between m1A1408 and ring I (Figure 3, middle). Another possibility is that 6′-OH of ring I simply accommodates in the binding pocket without any steric/electrostatic hindrance with m1A1408. On the opposite side of ring I, the O3′ and O4′ atoms make hydrogen bonds to the phosphate oxygen atoms of A1492 and A1493 (Figure 3, bottom). In addition, ring II, which is also referred to as 2-DOS (2-deoxystreptamine) ring and important for the specific recognition of the A site, makes hydrogen bonds from its N3 atom to the phosphate oxygen atoms of A1493 and A1494 (Figure 3, bottom), so that A1492 and A1493 are fixed in the bulged-out conformation. G418 that belongs to the 4,6-disubstituted subclass makes 17 direct contacts (13 hydrogen bonds and 4 C-H...O interactions) to the mutant A site (Figure 3A, bottom). On the other hand, paromomycin that belongs to the 4,5-disubstituted subclass makes 11 direct

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**Figure 1.** Secondary structure of the bacterial ribosomal A site with the antibiotic-resistant A1408m1A modification and chemical structure of 1-methyladenosine (m1A). The rRNA residues are numbered according to the numbering used in Escherichia coli 16S rRNA. The A/m1A1408, A1492 and A1493 residues are colored in orange, blue and red, respectively.

**Figure 2.** Crystal packing interactions mimic a molecular crowding environment around the A site in the ribosome (see examples below) (39). A chemically synthesized RNA oligomer (Gene Design Inc., Japan) was purified by 20% polyacrylamide gel electrophoresis under a denaturing condition containing 3.2 M urea and then desalted by C18 reversed-phase chromatography.

**Figure 3.** Aminoglycoside binding to the m1A1408-modified A site. The rRNA residues are numbered according to the numbering used in Escherichia coli 16S rRNA. The A/m1A1408, A1492 and A1493 residues are colored in orange, blue and red, respectively.

**Table S1.** Crystallization conditions are summarized in Supplementary Table S1.

**Table 1.** Molecular drawings were made using the program PyMOL (46). The statistics of structure refinements are summarized in Table 1. Molecular drawings were made using the program PyMOL (46).
Figure 2. Chemical structures of G418 (geneticin), gentamicin and paromomycin.

Table 1. Crystal data, statistics of data collections and structure refinements

| Crystal code | A1408m<sup>1</sup>A | A1408m<sup>1</sup>A-G418 | A1408m<sup>1</sup>A-Paromomycin | A1408m<sup>1</sup>A-Gentamicin |
|--------------|---------------------|--------------------------|-------------------------------|------------------------------|
| PDB-ID       | 4WCP                | 4WCQ                     | 4WCR                          | 4WCS                         |

**Crystal data**
- **Space group**: C2, P<sub>2</sub>1<sub>2</sub>1<sub>2</sub>, P<sub>2</sub>1<sub>2</sub>1<sub>2</sub>, P<sub>2</sub>1<sub>2</sub>1<sub>2</sub>
- **Unit cell (Å)**: 
  - a = 159.7, a = 33.4, a = 31.6, a = 32.6
  - b = 46.5, b = 89.8, b = 90.8, b = 92.0
  - c = 38.8, c = 46.8, c = 46.8, c = 47.7
  - β = 96.4
- **Z**: 1.5, 1, 1, 1

**Data collection**
- **Beamline**: NW12A of PF, BL-17A of PF, BL-17A of PF, BL-5A of PF
- **Wavelength (Å)**: 1.0, 0.98, 0.98, 1.0
- **Resolution (Å)**: 38.5-2.4, 44.9-2.1, 32.6-3.5, 42.3-3.1
- **Resolution range (Å)**: 38.5-2.4, 44.9-2.1, 30.0-3.5, 40.0-3.1
- **Used reflections**: 11197, 8418, 1847, 2847
- **R<sub>merge</sub>**<sup>b</sup> (%): 4.2, 5.9, 14.3, 6.8
  - in the outer shell (%): 9.4, 14.3, 14.3, 6.8
- **Completeness (%)**: 99.4, 96.6, 97.2, 99.3
- **Redundancy**: 7.0, 6.4, 5.8, 6.3
- **I/σ**: 22.4, 15.4, 8.2, 13.0
- **Structure refinement**
  - **Resolution range (Å)**: 38.5-2.4, 44.9-2.1, 30.0-3.5, 40.0-3.1
  - **Used reflections**: 11197, 8418, 1847, 2847
  - **R<sub>factor</sub>** (%): 17.7, 20.7, 20.5, 25.5
  - **R<sub>free</sub>** (%): 21.6, 25.1, 22.8, 28.4
  - **Average B-factor**
    - of RNA: 48.1, 39.1, 137.8, 107.5
    - of ligand: 32.6, 32.6, 132.2, -
    - of water: 51.6, 42.9, 78.7, 126.0
  - **No. of atoms**
    - of RNA: 1461, 920, 937, 920
    - of ligand: 68, 42, -
    - of water: 137, 142, 8, 19
  - **R.m.s.d. bond length (Å)**
    - 0.005, 0.021, 0.058, 0.007
  - **R.m.s.d. bond angles (°)**
    - 1.0, 1.6, 1.0, 1.1

<sup>a</sup>Number of RNA duplex in the asymmetric unit.
<sup>b</sup>R<sub>merge</sub> = 100 × Σ<sub>hkij</sub> |I<sub>hkij</sub> - <I<sub>hkij</sub>| / Σ<sub>hkij</sub> |<I<sub>hkij</sub>|.
<sup>c</sup>R-factor = 100 × Σ|F<sub>o</sub> - |F<sub>c</sub>| / Σ|F<sub>o</sub>|, where |F<sub>o</sub>| and |F<sub>c</sub>| are optimally scaled observed and calculated structure factor amplitudes, respectively.
<sup>d</sup>Calculated using a random set containing 10% of observations.
contacts (9 hydrogen bonds and 2 C-H...O interactions) (Figure 3B, bottom).

In the A1408m1A-Gentamicin crystal obtained in the presence of gentamicin with a 6′-NH3+ group, binding of the aminoglycoside to the mutant A site could not be observed (Supplementary Figure S2). We cannot exclude a possibility that gentamicin did not bind only in the present crystal form due to, for example, crystal packing effect or degrade of gentamicin. However, it can be assumed by a molecular modelling that ring I with the 6′-NH3+ group is less likely to form a pseudo pair with m1A1408, because the NH3+ group may repel the CH3 group attached to the positively-charged N1 atom (Figure 4).

**DISCUSSION**

**Minimum inhibitory concentration**

The present crystal structures shown above agree with the minimum inhibitory concentrations (MICs) of aminoglycosides against *E. coli* (Supplementary Table S2) (28). *Escherichia coli* CSH-2 strain without the *npmA* gene is susceptible to both paromomycin (MIC_{CSH-2} = 0.5 µg/ml) and gentamicin (MIC_{CSH-2} ≤ 0.06 µg/ml). On the other hand, the clinically isolated antibiotic-resistant *E. coli* ARS3 strain and *E. coli* CSH-2 strain possessing a conjugative plasmid carrying the *npmA* gene (CSH-2 (pARS3)) exhibit high-level resistance to gentamicin with the 6′-NH3+ group (MIC_{ARS3} > 256 µg/ml; MIC_{CSH-2 (pARS3)} = 125 µg/ml). These antibiotic-resistant strains exhibit low to moderate level resistance to paromomycin with the 6′-OH group
Figure 5. Local structures of the ‘off’ states of the wild-type (A; PDB-ID = 3BNL (33)) and m1A1408-modified (B) A sites. A dashed line indicates a hydrogen bond. The CH₃ group attached to N1 of m1A1408 is shown as an orange sphere.

Effects of the N1-methylation of A1408 on translation efficiency and fitness of the host

From a total of four crystals obtained in this study, nine m1A1408-modified A site structures were observed (Supplementary Figure S2). These structures, as well as accumulated wild-type A site structures obtained from crystal structures of the 30S and 70S ribosomes (12,13) and the A-site RNA model (14–26,38), allow us to speculate and compare the motions of the modified and wild-type A site molecular switches. In the case of the wild-type, the A site molecular switch takes relatively stable ‘off’ states, in which one of the two adenines form a hydrogen bond. The CH₃ group attached to N1 of m1A1408 is shown as an orange sphere.

(MICₐRₛ₃ = 64 µg/ml; MICₐCS₂₉ (pₐRₛ₃) = 4 µg/ml) probably due to attenuation of pseudo-pair stability by the N1-methylation of A1408 (Supplementary Figure S1), but it can also be said that paromomycin is moderately active against the mutant strains. Although MICₜ of G418 against the antibiotic-resistant strains are not reported, it is expected by our crystal structure that the compound with the 6'-OH group should be active against resistant bacteria with N1-methyltransferase.

A possible conformational change of A1408

Based on the crystal structure of NpmA in complex with the substrate 30S ribosome, Dunkle et al. proposed how NpmA binds to the 30S ribosome and changes its conformation for inducing conformational changes of the ribosomal RNA that allows A1408 to enter the active site of the catalytic core of NpmA composed of W107 and W197 (Figure 6). Since the modified A site takes the ‘on’ state with two bulged-out adenines, which is identical to that of the wild-type A site, the m1A1408-modified A site maintains its function as the molecular switch. These observations suggest that the ON/OFF switching might favorably occur in the m1A1408-modified A site compared to the wild-type. Recent study revealed that there is a simple linear trade-off between speed and accuracy of decoding (48). Therefore, it can be expected that the energetically ‘softer’ mutant A-site molecular switch may display lower accuracy of translation compared to the wild-type one.

Recently, effect of m1A1408 modification on translation efficiency and fitness of the host have carefully been analyzed by Grillot-Courvalin and co-workers (49). According to their analyses, several important insights have been elucidated; (i) expression of NpmA interferes with endogenous methylation at the neighboring C1407 residue (m2C1407 modification) by blocking action of a housekeeping methyltransferase RsmF. (ii) Escherichia coli strains with and without the npmA gene had the same growth rate, but in vitro competition experiments between them revealed a small disadvantage of the former cell (loss of 2.7% per generation). On the other hand, (iii) there was no reduction in growth rate of E. coli without the housekeeping rsmF gene relative to the wild-type E. coli, and no fitness decrease in competition experiments performed between E. coli strains with and without the rsmF gene (relative fitness of 1). In addition, dual luciferase assays showed that (iv) a decrease in the +1 reading frame maintenance (16%) and in UGA readthrough (20%) was observed in the presence of NpmA, and a decrease in the UGA readthrough (24%) was also observed in the absence of RsmF. These results are summed up that the exogenous N1-methylation at A1408 is a part of the causes of the slight fitness cost and the decreasing of translation accuracy. The present comparative analysis of the motions of the A-site molecular switches with and without m1A1408 modification suggests at least that the N1-methylation of A1408 affects fitness cost and translation accuracy only at a minimum level but provides a good level of resistance against aminoglycosides.
mutations at A1408 in 16S rRNA are now completely elucidated. Obtained structural information is of great value to overcome a part of the issue of antibiotic resistance.

ACCESSION NUMBERS

PDB IDs: 4WCP, 4WQC, 4WCR and 4WCS.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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