Impact of acquisition of 16S rRNA methylase RmtB on the fitness of *Escherichia coli*

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ABSTRACT

The aim of this study was to elucidate the biological phenotypes of 16S rRNA methylase RmtB in *Escherichia coli* and the impact of RmtB acquisition on the fitness of the target bacterium. An *rmtB* in-frame deletion mutant in *E. coli* was constructed using a suicide vector (pDMS197)-based double crossover allelic exchange, and its corresponding complemented strain was established. Combined studies of microdilution susceptibility testing, conjugation experiments, growth kinetics assays, competitive experiments, biofilm formation tests and motility assays were performed to study the *rmtB*-mediated fitness among the prototype *E. coli* strain, its isogenic mutant and the corresponding complemented strain. The minimum inhibitory concentrations (MICs) of 4,6-disubstituted 2-deoxystreptamines for the *rmtB* wild-type strain, its isogenic mutant and the complemented strain were ≥ 1024 , ≤ 2 and ≥ 1024 mg/L, respectively. Both the growth rates and the competitive abilities of the wild-type and complemented strains were relatively inferior to the $\Delta rmtB$ mutant. There was no significant difference in biofilm formation and motility among the three strains. In conclusion, the data presented here suggest that acquisition of the 16S rRNA methylase gene *rmtB* in *E. coli* can exact a fitness cost on the bacteria, subsequently reducing the growth rate slightly and decreasing the competitive capacity of the bacterium, whereas it does not affect biofilm formation or motility.

1. Introduction

Aminoglycosides are potent antibiotics active against aerobic Gram-negative bacteria and some Gram-positive bacteria. The mechanism of antibacterial activity involves binding to the highly conserved A site of 16S rRNA in the 30S small ribosomal subunit, leading to interference with the decoding of mRNA [1]. The 16S rRNA methyltransferase exists in antibiotic-producing bacteria and prevents the bacterium from being eliminated by its own secreted antibiotics; in the past this self-protective mechanism was once thought to be irrelevant in clinical settings [2]. However, a study by Yokoyama et al. [3] showed the existence of a drug resistance determinant, designated 16S rRNA methylase RmtA, in clinical settings. The unique resistance mechanism of these enzymes could involve blocking of aminoglycosides from binding to their target site by methylating one or several bases in the A site of the 16S ribosomal subunit. This might involve using S-adenosylmethionine as the methyl donor, leading to a high level of resistance to aminoglycosides, thus resulting in the failure of antibiotic treatment. So far, ten kinds of 16S rRNA methylases have been reported in Gram-negative bacteria from different countries and regions, including ArmA, RmtA, RmtB, RmtC [4], RmtD or RmtD₁ [5,6], RmtD₂ [5,6], RmtE [7], RmtF [8], RmtG [9], RmtH [10] and NpmA [11], which are encoded by at least 12 types of gene alleles.

It is known that antimicrobial resistance carries a fitness cost expressed in terms of reduced growth rate, competitive ability or virulence [12]. This cost of antimicrobial resistance is predicted to play a key role in the evolutionary dynamics of resistance, because the presence of resistance generates selection against resistance. However, some recent studies have found that the costs of aminoglycoside methylases are highly variable owing to a wide variety of factors, and thus estimating their potential distribution and evolutionary dynamics is difficult [13–16]. Recent epidemiological investigations have frequently reported the presence of 16S rRNA methylase RmtB in clinical and veterinary strains from various geographic areas, including East Asia, Europe and the Americas [4,17–20], notably increasingly involving bacterial strains isolated and identified in animal breeding industries. Studying the effects of 16S rRNA methylase genes on bacterial fitness is meaningful for evaluating the evolution and transmission of this class of genes. In this study, *rmtB*, one of the most common 16S rRNA methylase genes found in clinical settings, was chosen to probe the fitness impact and phenotypes of 16S rRNA methylase RmtB in order to better understand its persistence of transmitted resistance.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmids used in the study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) broth (Oxoid Ltd., Basingstoke, UK; Sinopharm, Shanghai, China) or on LB agar (Oxoid Ltd.; Sinopharm) plates and, when appropriate, media were supplemented with the appropriate antibiotics.

2.2. Construction of an *rmtB* in-frame gene deletion mutant, its complemented strain and relevant recombinant strains

The in-frame *rmtB* gene of wild-type *Escherichia coli* 3A11-16 was deleted according to a previously described method with modifications [21,22]. A 1059-bp DNA fragment containing the DNA upstream of *rmtB* and a 719-bp DNA fragment carrying the DNA downstream of *rmtB* of *E. coli* strain 3A11-16 were PCR-amplified using an ExpandTM High Fidelity PCR System (TaKaRa, Dalian, China) with two primer pairs: P1/P2 and P3/P4 (Table 2; Fig. 1). Both products were then cloned into the intermediate vector pBluescriptII KS(+) to generate the recombinant pBluescriptII KS(+)Δ*rmtB* containing the *rmtB* gene with an in-frame *rmtB* deletion. The resulting *rmtB* in-frame deletion fragment in pBluescriptII KS(+)Δ*rmtB* was excised with the restriction enzymes *Xba*I and *Kpn*I and was inserted into the same restriction enzyme sites in the suicide vector pDMS197, which carries both the *sacB* gene (conferring sucrose sensitivity) and tetracycline resistance genes. The recombinant plasmid pDMS197Δ*rmtB* was transformed into *E. coli* CC118 λ*pir* for replication. Subsequently, the purified recombinant plasmid DNA with the in-frame deletion of *rmtB* was electroporated into the wild-type strain *E. coli* 3A11-16. Following electroporation, positive recombinant colonies containing single recombination events were streaked on fresh LB plates containing tetracycline for selection of recombination of plasmid DNA in the bacterial genome. The selected isolates were then grown in nutrient broth containing 10% sucrose lacking both NaCl and tetracycline to screen for loss of the integrated *sacB1* gene. Colonies sensitive to tetracycline were selected and confirmed by PCR using the primer pair Δ*rmtB*YZ1/Δ*rmtB*YZ2 (Table 2; Fig. 1) and their genotypes were examined by PCR using the primer pair P1/P4 (Table 2; Fig. 1) and were sequenced. The deletion mutant was named *E. coli* 3A11-16Δ*rmtB*. Meanwhile, the encoding gene of *rmtB* was cloned into the pBR322 plasmid for engineering the complemented strain, named *E. coli* 3A11-16Δ*rmtB*/pr*rmtB*, and complementation studies were conducted.

In addition, in order to study the antibacterial resistance of engineering strains *E. coli* DH5 α and *E. coli* BL21(DE3) carrying RmtB, the open-reading frame of *rmtB* was cloned into multicopy expression vector pET28a and was transformed into *E. coli* BL21(DE3) resulting in *E. coli* BL21(DE3)/pET28a-*rmtB*, and the recombinant plasmid pBR322-*rmtB* was transformed into *E. coli* DH5 α to generate *E. coli* DH5 α /pBR322-*rmtB*.

2.3. Conjugation experiments

To determine whether *rmtB* is located on a conjugative plasmid, conjugation experiments were conducted for *E. coli* 3A11-16, *E. coli* 3A11-16 Δ *rmtB* and *E. coli* 3A11-16 Δ *rmtB*/prmtB used as donor bacteria and *E. coli* C600 as a recipient strain according to a previously described method [17,23]. As the plasmids from three types of strains have been confirmed by PCR with primers *bla*_{TEM-1}F/*bla*_{TEM-1}R (Table 2) to carry the same *bla*_{TEM-1} gene, transconjugants were selected on MacConkey agar (Shanghai Zhongke Insect Biology Ltd., Shanghai, China) plates containing both 256 mg/L ampicillin (Sangon Biotech, Shanghai, China) and 2048 mg/L streptomycin (Sangon Biotech). For the conjugant conjugated from the wild-type strain, transfer of the *rmtB* and *bla*_{TEM-1} genes was confirmed by PCR with primers *rmtB*-U/*rmtB*-D and *bla*_{TEM-1}F/*bla*_{TEM-1}R (Table 2). For the conjugant conjugated from the mutant and complemented strains, transfer of Δ *rmtB* and *bla*_{TEM-1} were confirmed by PCR with primers Δ *rmtB*YZ1/ Δ *rmtB*YZ2 and *bla*_{TEM-1}F/*bla*_{TEM-1}R (Table 2).

2.4. Antimicrobial susceptibility testing for aminoglycosides

Minimum inhibitory concentrations (MICs) were determined for the wild-type (*E. coli* 3A11-16) strain, the Δ *rmtB* mutant (*E. coli* 3A11-16 Δ *rmtB*), the complemented strain (*E. coli* 3A11-16 Δ *rmtB*/prmtB) and some relevant recombinant strains using the microdilution susceptibility testing method [24]. *Escherichia coli* ATCC 25922 was used as a control strain and eight kinds of aminoglycosides were tested, including gentamicin, sisomicin, amikacin, kanamycin, neomycin, apramycin, streptomycin and spectinomycin, of which gentamicin, sisomicin, amikacin and kanamycin are 4,6-disubstituted 2-deoxystreptamines (Table 3).

2.5. Growth kinetics and growth competition experiments

Growth kinetics was determined for *E. coli* 3A11-16, *E. coli* 3A11-16 Δ *rmtB* and *E. coli* 3A11-16 Δ *rmtB*/prmtB. An inoculum of 10⁷ CFU per strain was grown in 50 mL of LB broth in the absence of antibiotics. Cultures were incubated for 14 h at 37 °C with shaking at 200 rpm. The

optical density at 600 nm (OD_{600}) was measured every 30 min during the first 4 h and then sequentially every hour after the first 4 h.

Escherichia coli 3A11-16, *E. coli* 3A11-16 $\Delta rmtB$ and *E. coli* 3A11-16 $\Delta rmtB/prmtB$ were used in competition experiments as described previously [25]. Bacteria were incubated for 16 h at 37 °C in LB medium and then 10^6 CFU were mixed in 50 mL of antibiotic-free LB medium and were grown at 37 °C with agitation at 100 rpm. The cultures were diluted 1:250 into 50 mL of fresh LB medium every 24 h. Samples were collected every 24 h for 10 days. Competition experiments were designed such that one competitor carried the aminoglycoside resistance gene *rmtB* while the other strain did not (i.e. *E. coli* 3A11-16 vs. *E. coli* 3A11-16 $\Delta rmtB$ and *E. coli* 3A11-16 $\Delta rmtB/prmtB$ vs. *E. coli* 3A11-16 $\Delta rmtB$). Aliquots of bacterial cultures were plated on non-resistance selective LB agar. The proportion of resistant colonies was deduced by replica-plating 100 colonies onto LB agar supplemented with amikacin (256 mg/L) and gentamicin (256 mg/L).

The competition index (CI) represents the relative fitness of the bacteria and was calculated as the ratio of the mean CFU in independent competition experiments for the resistant and susceptible strains at a given time point (t_n) divided by the same ratio at initial time (t_0). The selection coefficient (s) was calculated as the slope of the linear regression model: $s = \ln(CI)/\ln(d)$, where d is the dilution factor [26]. The selection coefficient (s) estimated the difference between the relative fitness of the two competitors over the entire competition experiment.

2.6. Biofilm formation and motility assays

Biofilm assays were performed on microtitre plates as previously described [27] with some modifications. For a quantitative analysis of biofilm production, an overnight culture (OD_{600} of ca. 2.0) was diluted 1:20 and was grown in biofilm-inducing medium [27]. Culture medium (100 μ L) was added to each well of a round-bottomed 96-well microtitre plate (Corning Inc., Corning, NY) and was incubated for 48 h at 30 °C without shaking. To remove loosely associated bacteria, the culture medium was removed from the wells, which were rinsed three times with sterile distilled water. Crystal violet (Sinopharm) 1% solution was added to each well to stain the bacteria. Following incubation at room temperature for 15 min, the wells were rinsed with Milli-Q water and then 95% ethanol was added to each crystal violet-stained well. The OD_{600} was measured using a spectrophotometer (BioTek, Winooski, VT). Six replicates were used in each experiment and each experiment was performed three times.

Motility assays were performed on semisolid agar plates as described previously [28] with some modifications. Briefly, 50 mL of bacterial stock from overnight cultures was re-inoculated into 5 mL of sterile LB broth and was incubated at 37 °C with aeration in a bacterial shaker at 178 rpm until an OD₆₀₀ of ca. 1.0 was achieved, indicating that the culture had entered logarithmic phase. Then, 1 µL of the culture samples from the *E. coli* 3A11-16 parent strain, the mutant *E. coli* 3A11-16 Δ *rmtB* strain and the complemented *E. coli* 3A11-16 Δ *rmtB*/*prmtB* strain were seeded onto 0.3% semisolid tryptone agar plates (Oxoid Ltd., Sinopharm). Motility was observed and valued following incubation for 18 h at 37 °C by measuring the diameter of radial diffusion around the primary inoculum.

3. Results and discussion

3.1. Construction of the Δ *rmtB* mutant and the complemented strain

Upstream and downstream homologous fragments of *rmtB* covering lengths of 1059 bp and 719 bp, respectively, were successfully amplified by PCR, yielding products with the expected size. After confirming the DNA sequence of the PCR product, 30 bp (residues 25–54) of the *rmtB* gene in *E. coli* 3A11-16 was successfully deleted using the double selection strategy of allelic exchange mutagenesis with suicide vector pDMS197-based recombination. Generally the *rmtB* resistance gene is located on conjugative or non-conjugative plasmids [17,29,30] and, in this study, we confirmed that the *rmtB* gene of the *E. coli* strain used was located on a conjugative plasmid using conjugation experiments. Edwards et al. [22] demonstrated that genes on the chromosome and on plasmids could be modified through suicide vector-mediated recombination. In the current study, we demonstrated that the gene on the conjugative plasmid could be modified using suicide vector-based recombination, and homologous recombination could occur on both the chromosome and the plasmid in the prokaryotic cells [22].

For the complemented strain, after tetracycline-resistant transformants were selected and confirmed with proof primers Δ *rmtB*YZ1/ Δ *rmtB*YZ2, the recombinant plasmid pBR322 carrying the *rmtB* gene was confirmed by both PCR analysis using primers pBR322-F/pBR322-R and *rmtB*-U/*rmtB*-D and DNA sequencing, the complemented strain *E. coli* 3A11-16 Δ *rmtB*/*prmtB* was constructed successfully.

3.2. *RmtB*-harbouring strains show high-level resistance to 4,6-disubstituted 2-deoxystreptamines

As shown in Table 3, the MICs of the 4,6-disubstituted 2-deoxystreptamines (i.e. gentamicin, sisomicin, amikacin and kanamycin) for the $\Delta rmtB$ mutant were significantly reduced to the same level as the control strain *E. coli* ATCC 25922. However, there was no significant difference between the *rmtB* wild-type and the $\Delta rmtB$ mutant in the MICs of non-4,6-disubstituted 2-deoxystreptamines. As expected, there was a marked difference observed in the MICs of the 4,6-disubstituted 2-deoxystreptamines between the $\Delta rmtB$ mutant and the complemented strain. The MICs of the 4,6-disubstituted 2-deoxystreptamines of the complemented strain were restored to wild-type levels. However, differences in the MICs of non-4,6-disubstituted 2-deoxystreptamines between the wild-type and the $\Delta rmtB$ mutant were not significant, and similar results were observed both in *E. coli* DH5 α carrying pBR322 and *E. coli* BL21(DE3) carrying pET28a whether or not they express the methylase gene *rmtB*.

Following deletion of the target gene in the 16S rRNA methylase-producing strain and phenotypic analysis for the clinical prototype strain, its isogenic mutant and the complemented strain, the resistance mechanism could be further elucidated. As already known, methylation of the N1 position of A1408 in 16S rRNA of bacteria confers resistance to 4,6-disubstituted 2-deoxystreptamines (kanamycin and gentamicin groups), 4,5-disubstituted 2-deoxystreptamines (neomycin group) and apramycin, a monosubstituted 2-deoxystreptamines, whereas methylation of N7 G1405 confers resistance to only 4,6-disubstituted 2-deoxystreptamines [4,11,30]. 16S rRNA methylase *RmtB* may act by directly blocking aminoglycoside binding to the target site, i.e. N7 G1405, in the A site of 16S rRNA, and this would confer bacterial resistance to various aminoglycosides belonging to the 4,6-disubstituted 2-deoxystreptamines. The susceptibility assay results show a significant difference between the wild-type and mutant strains against 4,6-disubstituted 2-deoxystreptamines. The susceptibility of the $\Delta rmtB$ mutant to 4,6-disubstituted 2-deoxystreptamines showed that deleting the amino acid residues 9–18 (corresponding to base pairs 25–54) of *RmtB* likely disrupts the whole protein structure. Schmitt et al. [31] demonstrated that the N-terminal domain specific to the aminoglycoside resistance family (Agr) of methyltransferases is required for enzymatic activity and amino acid residues 10–19 of *RmtB* are strictly conserved, as observed by analysis of the three-dimensional structure of *RmtB*. Based on a mutation of 10 amino acid residues from positions 9–18 of *RmtB*, leading to the $\Delta rmtB$ mutant's sensitivity to 4,6-disubstituted 2-deoxystreptamines, we reason that amino acid residues 9–18 of *RmtB* may play a critical role in methylating the A site of 16S rRNA by S-adenosylmethionine, which needs to be confirmed.

3.3. The growth rate is relatively slower in *rmtB*-harbouring strains

From the bacterial growth curves (Fig. 2) it can be observed that the growth rates both of *E. coli* 3A11-16 and *E. coli* 3A11-16 Δ *rmtB*/*prmtB* were slightly lower than that of the Δ *rmtB* mutant after the exponential phase. The fitness cost associated with most resistance mechanisms is an important biological parameter that influences the emergence and dissemination of antimicrobial resistance and its persistence in the absence of selective pressure [32]. Considering that the growth rate of *E. coli* 3A11-16 Δ *rmtB*/*prmtB* was significantly lower compared with *E. coli* 3A11-16 Δ *rmtB*, whereas the growth rate of *E. coli* 3A11-16 was only slightly lower (Fig. 2), we suggest that the 16S rRNA methylase RmtB, which confers methylation at N7 G1405 of the A site of 16S rRNA, relates to a small disadvantage that causes a slightly decreased growth after the exponential phase, especially in the condition when *rmtB* is multicopy-expressed. Benítez-Páez et al. reported that aminoglycoside methylase RsmG, methylating m⁷G527 in 16S rRNA, stimulates ppGpp accumulation and downregulates rRNA synthesis when cells were grown in nutrient-limitation conditions that cause growth arrest or were entered into the stationary phase [33]. The current research results perhaps suggest that a similar mechanism exists in 16S rRNA methylase RmtB in *E. coli*.

3.4. The competitive capacity of the *rmtB*-harbouring strain is reduced

In vitro competition experiments were performed between three strains that differed by the presence or absence of the 16S rRNA methylase RmtB gene in its active or inactive form. An obvious competitive disadvantage was observed in *rmtB* wild-type compared with the Δ *rmtB* mutant, with a loss of 3.25% per generation (Fig. 3A). Moreover, a major competitive disadvantage was observed in cells transformed with the recombinant plasmid pBR322-*rmtB* expressing RmtB compared with the mutant, with a loss of 20.89% per generation (Fig. 3B). A marked competitive disadvantage was observed for the wild-type and cells with multicopy-expressing RmtB, thus confirming that 16S rRNA methylase RmtB, leading to N7 G1405 methylation, is responsible for exacting a fitness cost to the cells.

The slight biological cost associated with resistance could lead to the selection of compensatory mutations that restore the fitness of the host [34]. However, in contrast to RmtC, the results of growth rate and growth competition experiments showed that the 16S rRNA methylase RmtB leads to a fitness burden to the host bacteria, as observed for most other antimicrobial resistance enzymes.

3.5. Acquisition of 16S rRNA methylase RmtB has no impact on biofilm formation, motility or conjugation of the host bacterium

Although the amount of biofilm formation for the mutant was found to be consistently higher than that of the wild-type strain, no significant difference was found in biofilm formation among the three types of strains (Fig. 4A). Biofilm formation and its contribution to resistance to antimicrobial agents may be a major cause of many persistent and chronic infections. The environment of the bacterial biofilm shows increased tolerance to antibiotics and disinfectant chemicals [35], since the dense extracellular matrix and the outer layer of cells protect the interior of the bacterial community. In some cases, antibiotic resistance could be increased 1000-fold owing to the existence of biofilms [36]. Høiby et al. reported that conventional resistance mechanisms, such as chromosomal β -lactamase, upregulated efflux pumps and mutations in antibiotic target molecules in bacteria, also enhance biofilm survival [35]. To understand how bacteria adapt to acquisition of the *rmtB* gene, the biofilm formation experiment and motility tests were performed. Contrary to the report of Høiby et al. mentioned above [35], we found that expression of 16S rRNA methylase RmtB did not enhance biofilm formation and instead led to a slight decline in biofilm formation. Moreover, no significant discrepancy was found in the motility of the three strains (Fig. 4B).

In addition, transconjugants were easily obtained for each of the three types of strains in conjugation experiments. The resistance plasmid of the $\Delta rmtB$ mutant can still be transferred into the recipient strain, confirming that the 16S rRNA methylase *rmtB* gene does not influence the function of the conjugation apparatus (or *tra* genes) of the conjugative resistance plasmid.

The effects of aminoglycoside methylases on bacterial fitness have been reported as being highly variable. Puneekar et al. stated that knockout of *rlmJ* (*yhiR*), encoding methylase RlmJ (YhiR) methylating m⁶A2030 in 23S rRNA, does not affect the growth rate [14,15] but lowers the competitive fitness at long-term growth in stationary phase [15,16] and provides a small growth advantage under anaerobic conditions [14,15]. However, no fitness cost coupled with the methylation pattern at N7 G1405 of the 16S rRNA of acquired 16S rRNA methylase (or Arm/Rmt methylase) RmtC was observed, which suggests that evolution of the ribosome ultimately led to the selection and maintenance of resistance even in the absence of selective pressure [25]. Different from that of the 16S rRNA methylase RmtC, acquisition of 16S rRNA methylase RmtB located on a conjugation plasmid decreases the host bacterial fitness by reducing the growth rate slightly and decreasing the competitive power of *E. coli*. Therefore, the fitness costs of the acquired 16S rRNA methylase RmtB do not explain the phenomenon of the increasing prevalence of the acquisition of

the 16S rRNA methylase gene *rmtB* in clinical settings, and more research work should be carried out to illustrate the mechanism in the future.

Moreover, exploration of the difference between the wild-type *rmtB* strain and its isogenic $\Delta rmtB$ mutant may facilitate the potential exploitation of effective inhibitors of 16S rRNA methylase, which will be of vital importance in the near future.

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Fig. 1. Schematic diagrams for construction of deletion mutations. (A) Schematic diagram for the position of primers (grey boxes represent the gene open reading frame). (B) Schematic diagram for *rmtB* deletion; 30 base pairs (residues 25–54) of the *rmtB* gene were deleted and a 57-bp exogenous gene was inserted into the original place [hatched part represents exogenous fragment from the pBluescriptII KS(+)].

Fig. 2. Growth kinetics of three *Escherichia coli* strains: wild-type *E. coli* 3A11-16; *rmtB* deletion mutant (*E. coli* 3A11-16 Δ *rmtB*); and the complemented strain (*E. coli* 3A11-16 Δ *rmtB*/*prmtB*). OD₆₀₀, optical density at 600 nm. Data are the mean $\pm$ standard error of the mean.

Fig. 3. Fitness cost of RmtB in different backgrounds. Growth competition curves between three types of strains. (A) *Escherichia coli* 3A11-16 wild-type and *E. coli* 3A11-16 Δ *rmtB* deletion mutant. (B) *E. coli* 3A11-16 Δ *rmtB* and complemented strain *E. coli* 3A11-16 Δ *rmtB*/*prmtB*. Competition curves are depicted as the mean of three independent experiments. Data are the mean $\pm$ standard error of the mean. An obvious competitive disadvantage with a loss of 3.25% per generation was observed for *rmtB* wild-type compared with the Δ *rmtB* mutant; and a major competitive disadvantage with a loss of 20.89% per generation was observed in cells transformed with the recombinant plasmid pBR322-*rmtB* expressing RmtB compared with the mutant. CI, competition index.

Fig. 4. Biofilm formation and motility of the 16S rRNA methylase RmtB-positive strains and their negative counterpart. (A) Quantification of biofilm formation of *Escherichia coli* 3A11-16 wild-type (WT), the *E. coli* 3A11-16 Δ *rmtB* deletion mutant and the *E. coli* 3A11-16 Δ *rmtB*/*prmtB* complemented strain. Surface-adhered biofilm was quantified by measuring the optical density at 600 nm (OD₆₀₀) of ethanol-solubilised crystal violet after biofilm staining from three independent experiments. (B) Motility assay of *E. coli* 3A11-16 WT, the *E. coli* 3A11-16 Δ *rmtB* deletion mutant and the *E. coli* 3A11-16 Δ *rmtB*/*prmtB* complemented strain. Motility was visualised as diameter of radial diffusion around the primary inoculum on semisolid agar plates after 18 h at 37 °C. Data are the mean $\pm$ standard error of the mean.

Table 1

Strains and plasmids used in this work

Strain or plasmid	Genotype	Resource
Strains		
<i>E. coli</i> 3A11-16	<i>rmtB</i> -positive wild-type <i>Escherichia coli</i> from chicken	Clinical collection
<i>E. coli</i> ATCC 25922	Drug-sensitive control strain	ATCC stock
<i>E. coli</i> DH5 α	F ⁻ , ϕ 80d/ <i>lacZ</i> Δ M15, Δ (<i>lacZ</i> YA- <i>argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (<i>rk</i> -, <i>mk</i> +), <i>phoA</i> , <i>supE44</i> , λ -, <i>thi</i> -1, <i>gyrA96</i> , <i>relA1</i>	Takara Ltd.
<i>E. coli</i> CC118 λ <i>pir</i>	<i>araD139</i> Δ (<i>araleu</i>)7697 Δ <i>lacX74</i> Δ <i>phoA20</i> <i>galKthirpsErpoBargE</i> (Am) <i>recA1</i> (λ <i>pir</i>)	[22]
<i>E. coli</i> C600	Streptomycin-resistant (MIC > 2000 mg/L) as recipient strain	[23]
Plasmids		
pDMS197	<i>oriT</i> , <i>oriV</i> , <i>sacB</i> , Tc ^r	[22]
pDMS197- Δ <i>rmtB</i>	pDMS197 carrying in-frame Δ <i>rmtB</i> gene, Tc ^r	This study
pBluescriptII KS(+)	Intermediate vector, Amp ^r	Stratagene, laboratory stock
pBluescriptII KS(+) Δ <i>rmtB</i>	pBluescriptII KS(+) carrying in-frame Δ <i>rmtB</i> gene	This study
pBR322	Expression vector, Tc ^r , Amp ^r	Takara Ltd.
pBR322- <i>rmtB</i>	Intact <i>rmtB</i> ligated into pBR322, Tc ^r	This study
pET28a	Bacterial expression vector, Kan ^r	Takara Ltd.
pET28a- <i>rmtB</i>	Intact <i>rmtB</i> ligated into pET28a, Kan ^r	This study

MIC, minimum inhibitory concentration; Tc^r, tetracycline-resistant; Amp^r, ampicillin-resistant; Kan^r, kanamycin-resistant.

Table 2

Primers used in this study

Primer name	Sequence	Description
P1	GCTCTAGACATTTTCGTGTCGCCCTTATT (<i>Xba</i> I)	For amplifying homologous sequence; amplified size 1059 bp
P2	CGCGGATCCGGTGAGGGCATCGTTGA (<i>Bam</i> HI)	
P3	CCGCTCGAGCTTTGCCCGGATACC (<i>Xho</i> I)	For amplifying homologous sequence; amplified size 719 bp
P4	GGGGTACCTTATCCATTCTTTTTTATCAAGT (<i>Kpn</i> I)	
<i>rmtB</i> -U	AAAAGTACTATGAACATCAACGATGCCCT (<i>Sca</i> I)	Construction of pBR322- <i>rmtB</i> , amplified size 774 bp
<i>rmtB</i> -D	AAAAGTACTTTATCCATTCTTTTTTATCA (<i>Sca</i> I)	
Δ <i>rmtBYZ1</i>	GGCTGCAGGAATTCGATATCAAG	Δ <i>rmtB</i> mutant confirmation; amplified size 237 bp
Δ <i>rmtBYZ2</i>	CATGCAGCGACAATGCCTT	
<i>bla</i> _{TEM-1} F	AACATTTTCGTGTCGCCCTT	<i>bla</i> _{TEM-1} gene confirmation; amplified size 226 bp
<i>bla</i> _{TEM-1} R	AATACCGCACCATAGCAG	
pBR322-F	ACGCAGTCAGGCACCGTGTAT	pBR322 plasmid confirmation; amplified size 1247 bp
pBR322-R	GTGGTGAATCCGTTAGCGAGG	

Note: Nucleotides underlined represent the restriction sites labelled in parentheses.

Table 3

Antimicrobial susceptibilities of the *rmtB* wild-type strain *E. coli* 3A11-16, the $\Delta rmtB$ deletion mutant *E. coli* 3A11-16 $\Delta rmtB$, the complemented strain *E. coli* 3A11-16 $\Delta rmtB/prmtB$, and some relevant recombinant strains

Strain	MIC (mg/L)							
	GEN	SISO	KAN	AMK	NEO	APR	STR	SPE
<i>E. coli</i> 3A11-16 (wild-type <i>rmtB</i>)	1024	1024	>1024	>1024	4	4	8	64
<i>E. coli</i> 3A11-16 $\Delta rmtB$	1	1	2	1	4	2	4	128
<i>E. coli</i> 3A11-16 $\Delta rmtB/prmtB$	>1024	>1024	>1024	>1024	4	2	4	32
<i>E. coli</i> DH5 α /pBR322	1	1	2	1	2	1	1	32
<i>E. coli</i> DH5 α /pBR322- <i>rmtB</i>	1024	256	1024	1024	1	1	0.5	32
<i>E. coli</i> BL21(DE3)/pET28a	1	1	256	2	32	2	4	32
<i>E. coli</i> BL21(DE3)/pET28a- <i>rmtB</i>	512	512	>1024	>1024	64	4	8	128
<i>E. coli</i> ATCC 25922	1	1	2	1	4	2	4	64

MIC, minimum inhibitory concentration; GEN, gentamicin; SISO, sisomicin; KAN, kanamycin; AMK, amikacin; NEO, neomycin; APR, apramycin; STR, streptomycin; SPE, spectinomycin.

A

B