ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2009, p. 193–201 Vol. 53, No. 1
0066-4804/09/$08.00+0 doi:10.1128/AAC.00873-08

Inactivation of KsgA, a 16S rRNA Methyltransferase, Causes Vigorous Emergence of Mutants with High-Level Kasugamycin Resistance▽

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Received 2 July 2008/Returned for modification 2 September 2008/Accepted 31 October 2008

The methyltransferases RsmG and KsgA methylate the nucleotides G535 (RsmG) and A1518 and A1519 (KsgA) in 16S rRNA, and inactivation of the proteins by introducing mutations results in acquisition of low-level resistance to streptomycin and kasugamycin, respectively. In a *Bacillus subtilis* strain harboring a single *rrn* operon (*rrnO*), we found that spontaneous *ksgA* mutations conferring a modest level of resistance to kasugamycin occur at a high frequency of 10^{-6}. More importantly, we also found that once cells acquire the *ksgA* mutations, they produce high-level kasugamycin resistance at an extraordinarily high frequency (100-fold greater frequency than that observed in the *ksgA*$^+$ strain), a phenomenon previously reported for *rsmG* mutants. This was not the case for other antibiotic resistance mutations (Tsp^r and Rif^r), indicating that the high frequency of emergence of a mutation for high-level kasugamycin resistance in the genetic background of *ksgA* is not due simply to increased persistence of the *ksgA* strain. Comparative genome sequencing showed that a mutation in the *speD* gene encoding *S*-adenosylmethionine decarboxylase is responsible for the observed high-level kasugamycin resistance. *ksgA speD* double mutants showed a markedly reduced level of intracellular spermidine, underlying the mechanism of high-level resistance. A growth competition assay indicated that, unlike *rsmG* mutation, the *ksgA* mutation is disadvantageous for overall growth fitness. This study clarified the similarities and differences between *ksgA* mutation and *rsmG* mutation, both of which share a common characteristic—failure to methylate the bases of 16S rRNA. Coexistence of the *ksgA* mutation and the *rsmG* mutation allowed cell viability. We propose that the *ksgA* mutation, together with the *rsmG* mutation, may provide a novel clue to uncover a still-unknown mechanism of mutation and ribosomal function.

The bacterial ribosome is a major target for antibiotics (6, 25). The aminoglycoside antibiotic kasugamycin, first reported in 1965 (31), has been important in agriculture because of its potent activity against rice blast. In the early 1970s, it was reported that bacterial resistance to kasugamycin involves silencing of the *ksgA* gene (also known as *rsmA*) through natural mutation, leading to inactivation of KsgA and resulting in the loss of dimethylation of two adjacent adenosine bases in 16S rRNA (12, 13). KsgA and the resulting modified adenosine bases (A1518 and A1519) appear to be conserved in all microorganisms examined to date. More recently, using *Escherichia coli* strains in which all of the rRNA is transcribed from a plasmid-encoded *rrn* operon, Vila-Sanjurjo and coworkers (32) reported that three 16S rRNA mutations (A794G, G926A, and A1519C) confer resistance to kasugamycin. The KsgA protein is homologous to another family of RNA methyltransferases, Erm, the members of which methylate a single adenosine base in 23S rRNA and confer resistance to the macrolide group of antibiotics. Recent work on the crystal structure of KsgA demonstrated a strong resemblance between KsgA and the crystal structure of ErmC′ (21).

Previously, we demonstrated that loss of the m^7G modification in 16S rRNA due to *rsmG* (renamed from *gidB*) mutations results in a low-level resistance to streptomycin as shown in *E. coli*, *Bacillus subtilis*, and *Streptomyces coelicolor* (19, 20, 23). The methyltransferase RsmG methylates the N7 position of nucleotide G527 (numbered according to *E. coli*). The nucleotide G527 is situated within a hairpin loop (the so-called 530 loop) that is one of the most highly conserved features of 16S rRNA, and mutations in this loop have been shown to be associated with resistance to streptomycin (17, 26, 30). This region of 16S rRNA is situated close to the ribosomal protein S12, and both of these ribosomal components play major roles in translational fidelity (22, 26). The phylogenetic conservation of RsmG and of the 16S rRNA sequence in the 530 loop suggests that methylation at this rRNA site should confer some selective advantage. However, a growth competition assay revealed that there are no differences in growth fitness between the *rsmG* mutant and the parent strain. Thus, the apparent lack of a disadvantage in cells that can no longer methylate the G527 position raised questions regarding the biological importance of this modification. The *rsmG* mutations arise spontaneously at a high frequency, ranging from 10^{-4} to 10^{-6} (19, 20, 23). Most importantly, in the *rmsG* mutant background, *rpsL* (encoding ribosomal protein S12) mutants with high-level streptomycin resistance emerged at a frequency 200-fold greater than that in the wild-type strain. This elevated fre-

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▽ Published ahead of print on 10 November 2008.

TABLE 1. Strains used in this study

Strain	Relevant genotype or description	Construction, reference, or source
B. subtilis		
168	*trpC2* (Marburg strain)	Laboratory stock
RIK543	Strain harboring a single *rrn* (*rrnO*)	All *rrn* other than *rrnO* were deleted
KO-847	*ksgA* (Pro 92→Phe [Thr 97→stop codon])	Kasugamycin-resistant mutant from 168
KO-827	*ksgA* (Ala 67→Cys [Glu 77→stop codon])	Kasugamycin-resistant mutant from RIK543
KO-875	*ksgA speD*	Mutant with high-level kasugamycin resistance from KO-847
KO-894	*speD* (Gly 7→Trp)	Transformation of 168 by DNA of KO-875
KO-750	*rsmG* (frameshift [Val 70→stop codon])	20
KO-826	*rsmG* (Met 31→Asn [Glu 34→stop codon])	Mutant with low-level streptomycin resistance from RIK543
KO-819	*rplK* (Pro 22−Ala 27→Δ)	Mutant with low-level thiostrepton resistance from RIK543
KO-809	*rpoB* (Ala 478→Val)	Mutant with low-level rifampin resistance from RIK543
KO-671	*rpsL* (Lys 56→Thr)	20
KO-924	*ksgA* (Pro 92→Phe [Thr 97→stop codon]) *rsmG* (Glu 46→stop codon)	Mutant with low-level streptomycin resistance from KO-847
KO-925	*ksgA* (Pro 92→Phe [Thr 97→stop codon]) *rsmG* (Gly-Ala insertion at position 81)	Mutant with low-level streptomycin resistance from KO-847
KO-926	*ksgA* (Pro 92→Phe [Thr 97→stop codon]) *rsmG* (Gly 79, Ala 80→Δ)	Mutant with low-level streptomycin resistance from KO-847
KO-927	*ksgA* (Pro 92→Phe [Thr 97→stop codon]) *rsmG* (frameshift [Val 70→stop codon])	Transformation of KO-847 by DNA of KO-750
E. coli		
BW25113	Wild-type strain	*E. coli* Genetic Stock Center
KO-845	*ksgA* (Gln 39→stop codon)	Kasugamycin-resistant mutant from BW25113
KO-895	*rpsL* (Lys 42→Asn)	Streptomycin-resistant mutant from BW25113
JWK3718	*rsmG* (Δ*rsmG*::*kan* strain isogenic to BW25113)	3
S. coelicolor		
KO-178	*rpsL* (Lys 88→Glu)	19
KO-179	*rsmG* (frameshift [Asp 16→stop codon])	19
M. smegmatis		
JCM5866	Wild-type strain	Japan Collection of Microorganisms
KO-935	*tlyA* (frameshift [656A→Δ])	Mutant with low-level capreomycin resistance from JCM5866
KO-936	*rsmG* (frameshift [205C→Δ])	23

quency in the emergence of high-level streptomycin resistance was facilitated by a mutation pattern in *rpsL* more varied than that obtained by selection of the wild-type strain. As *rsmG* mutation (conferring a low level of streptomycin resistance) and *ksgA* mutation (conferring a modest level of kasugamycin resistance) share a common characteristic—failure to methylate the 16S rRNA bases—we examined whether *ksgA* mutants also display such a peculiar phenotype as that observed in *rsmG* mutants. To characterize *ksgA* mutation, we chose *B. subtilis* strain 168, as genomic information and numerous tools for genetic, biochemical, and physiological analyses are available for this well-characterized system (7, 24). Here, we report that acquisition of high-level resistance at an extraordinarily high frequency is common for *rsmG* mutants and *ksgA* mutants, while *ksgA* but not *rsmG* mutants display a disadvantage in overall fitness compared to the parent strain.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *B. subtilis* and *E. coli* were grown in LB medium at 37°C, except for *ksgA* mutants that were grown in LB medium without NaCl because the absence of NaCl rendered cells more sensitive to kasugamycin. LB medium consisted of 1% tryptone (Difco, Detroit, MI), 0.5% yeast extract (Difco), and 0.5% NaCl. *S. coelicolor* was grown in glucose-yeast extract-malt extract medium (29) at 30°C. *Mycobacterium smegmatis* was grown on rich (R) agar at 37°C. R agar contained 1% peptone (Difco), 0.5% yeast extract (Difco), 0.2% beef extract (Difco), 0.2% glycerol, 0.1% $MgSO_4 \cdot 7H_2O$, 2% agar, and 50 mg/liter of Tween 80. The strains used in this study are listed in Table 1. Spontaneous *B. subtilis* mutants with low-level kasugamycin resistance were generated from the RIK543 strain (MIC, 500 μg/ml) and wild-type strain 168 (MIC, 1,500 μg/ml) on agar plates of LB medium minus NaCl containing 1,000 μg/ml and 3,000 μg/ml kasugamycin, respectively. Drug-resistant colonies developed after 2 to 3 days of incubation at 37°C. On the other hand, spontaneous *E. coli* mutants with low-level kasugamycin resistance were generated from the BW25113 strain (MIC, 200 μg/ml) on agar plates of LB medium minus NaCl containing 500 μg/ml kasugamycin. Spontaneous mutants with low-level resistance to streptomycin, thiostrepton, or rifampin (i.e., KO-826, KO-819, and KO-809) were generated from the RIK543 strain on LB medium containing 5 μg/ml streptomycin, 0.02 μg/ml thiostrepton, or 0.01 μg/ml rifampin, respectively. Spontaneous mutants with low-level resistance to capreomycin were generated from the *Mycobacterium smegmatis* wild-type strain JCM5866 (MIC, 5 μg/ml) on R medium containing 10 μg/ml of capreomycin. Serial dilutions of the cell suspension were also plated on media without any antibiotic to determine the number of viable cells in the original suspension. To measure the frequency of resistant mutants, single colonies were isolated, and cells originating from each of about 10 clones were examined separately. MICs to fully inhibit the growth were determined by spotting cell suspensions ($\sim 10^6$) onto drug-containing plates, followed by incubation for 24 h to 48 h at 37°C.

Construction of the *B. subtilis* strain harboring a single *rrn* operon. *B. subtilis* strain RIK543 contains only a single *rrn* operon (*rrnO*) in the genome, deleting the other nine *rrn* operons. The detailed procedures for the construction of RIK543 will be reported elsewhere. In brief, two methods were utilized for the construction of the deletion mutations. One method is to delete the target *rrn* operon by gene conversion, as follows. First, the target operon was disrupted by a chloramphenicol resistance gene (*cat*), and then the *cat* gene was deleted by the recombinant plasmid carrying the upstream and downstream sequence of the target *rrn* operon. A shuttle vector, pCHE11 (18), derived from pBR322 and pUB110ts-2 carrying a temperature-sensitive replication mutation in the *repU* gene of pUB110, was used as a vector for deleting the *cat* gene in the target *rrn* operon via gene conversion. After the selection for a kanamycin-resistant and chloramphenicol-sensitive phenotype at 30°C on LB plates, the resulting transformants were grown at 37°C on LB plates without any added antibiotics to eliminate the recombinant plasmid. Then, the transformant exhibiting a kanamycin- and chloramphenicol-sensitive phenotype at 30°C on LB plates was selected. The deletion mutation of *rrnHG* (Δ*rrnHG1*) was obtained in this way.

Another method used for the construction of the deletion mutation was based on the "ampicillin screening" technique. To utilize this technique, kanamycin and erythromycin resistance genes were first integrated in tandem into the *trpB trpA* region in the genome, and then point mutation was introduced into either one of the resistance genes, resulting in the kanamycin-resistant, erythromycin-sensitive phenotype (Km^r Em^s) or kanamycin-sensitive, erythromycin-resistant phenotype (Km^s Em^r). Using these markers, the deletion mutation of the target *rrn* operon was constructed as follows. First, the *cat* gene was inserted downstream of the promoter region of the target *rrn* operon in the strain carrying Km^s Em^r markers. Next, a PCR fragment with the deletion mutation of the target *rrn* operon and a DNA fragment containing Km^r Em^s markers were simultaneously added to the competent-cell culture of the strain constructed as described above. After incubation at 37°C for 90 min, the culture was diluted with LB medium containing kanamycin and incubated at 37°C overnight. The overnight culture was again diluted with LB medium containing kanamycin and incubated at 37°C with shaking. Two types of transformants existed in this culture; one carries both kanamycin resistance and chloramphenicol resistance genes, and the other carries the kanamycin resistance gene but lacks the chloramphenicol resistance gene as a result of cotransformation with the kanamycin resistance gene and the deletion of the target *rrn* operon. To obtain the transformants carrying the deletion of the target *rrn* operon more efficiently, chloramphenicol was added to the culture at a final concentration of 50 μg/ml when cells were growing exponentially in the medium. Then, the culture was further incubated for 30 min to cause the growth arrest of the transformants carrying the deletion of the target *rrn* operon. In contrast, transformants carrying the *cat* gene grew normally in the presence of chloramphenicol. Next, ampicillin was added to the culture at a final concentration of 1 mg/ml. Since this antibiotic exhibits a bactericidal activity toward only the growing cells, most of the transformants carrying the *cat* gene were expected to be killed by this treatment. After further incubation for 2 or 3 h, cells were plated on LB plates containing kanamycin. The transformants which exhibited a kanamycin-resistant, chloramphenicol-sensitive phenotype were selected, and then proper introduction of the deletion mutation of the target *rrn* operon was confirmed by PCR amplification and DNA sequencing. The deletion mutations *rrnD* (Δ*rrnD1*), *rrnE* (Δ*rrnE1*), *rrnB* (Δ*rrnB2*), *rrnA* (Δ*rrnA1*), *rrnI* (Δ*rrnI2*), and *rrnW* (Δ*rrnW2*) were obtained in this way.

To introduce the deletion mutations into one strain successively, cotransformation experiments were carried out using both *trpC2* and *hisC101* genetic markers. In brief, the strain carrying the *cat* gene inserted downstream of the promoter region of the target *rrn* operon was used as the recipient, and the strain carrying the deletion of the target *rrn* operon was used as the donor strain, respectively. If the recipient strain contained the *trpC2* mutation, the *hisC101* mutation was introduced into the donor strain. The recipient strain was transformed with the DNA extracted from the donor strain, and Trp^+ transformants were first selected. Among the Trp^+ transformants, the chloramphenicol-sensitive transformant, which is generated as a result of the cotransformation event, was selected. In most cases, the resulting transformants also showed a His^- phenotype because of the close genetic linkage between *trpC* and *hisC* genes. Thus, the obtained transformant containing the *hisC101* mutation was subjected to the next transformation experiment as described above, using the donor DNA carrying the *trpC2* mutation. As a result of these successive transformation experiments, RIK543 (Δ*rrnHG1* Δ*rrnD1* Δ*rrnE1* Δ*rrnB2* Δ*rrnA1* Δ*rrnI2* Δ*rrnW2* Δ*rrnJ1*::*cat trpC2*), which contained only a single *rrnO* operon, was obtained. The existence of only one *rrnO* operon in the genome of RIK543 was confirmed by Southern blot analysis (data not shown).

Mutation analysis. The primers used to amplify the candidate DNA fragments are listed in Table 2. PCR amplification was carried out with ExTaq (Takara, Otsu, Japan). Purified PCR products were directly sequenced with BigDye Terminator cycle sequencing kits (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequence data were aligned using the GENETIX program (Software Development Co., Tokyo, Japan). Comparative genome sequencing, a new method of identifying unknown mutations, was performed as described previously (19).

Analysis of intracellular polyamine profiles. Polyamines were identified as described previously (8, 27). Aliquots of bacterial cultures were pelleted, washed, and extracted with 0.2 M perchloric acid. Polyamines were subsequently dansylated and extracted with toluene for analyses by thin-layer chromatography (TLC). TLC was performed on silica gel G plates (Merck) and developed in ethyl acetate/cyclohexane (2:3, vol/vol). Spots were visualized under UV light.

Competition assay between mutant and parent strains. A competition assay was performed to compare the overall fitness of each strain. Briefly, equal numbers of cells from drug-resistant mutant and parent strains were inoculated into LB medium or into sterilized soil. After incubation (with shaking in LB medium but with standing in sterilized soil) for appropriate times as indicated above, the mixed cultures were inoculated (inoculation size, 1%) into new medium or new sterilized soil. This procedure was repeated 5 to 10 times. Finally, the ratios of mutant and parent strains were determined by spreading the cultured broth on LB medium with or without the corresponding antibiotic.

RESULTS

Development of *ksgA* mutations in *B. subtilis*. We initially assumed that high-level kasugamycin resistance may involve a mutation within the *rrn* gene encoding the 16S rRNA, as Vila-Sanjurjo and coworkers successfully identified kasugamycin resistance mutations (A794G, G926A, and A1519C) in the 16S rRNA gene of engineered *E. coli* (32). However, it is generally difficult to identify rRNA mutations because wild-type *E. coli* and *B. subtilis* strains have 7 and 10 chromosomal copies of the rRNA genes, respectively. One way to circumvent the difficulties due to the presence of multiple *rrn* operons is to use an engineered strain harboring only a single set of *rrn*. Thus, Kawamura and coworkers constructed the *B. subtilis* strain RIK543, in which all nine *rrn* genes other than *rrnO* have been deleted. The construction of the strain is described in Materials and Methods. As expected, RIK543 grew more slowly than the wild-type strain 168 and showed increased sensitivity to the antibiotic tested (Table 3). The details of the procedure for strain construction will be reported elsewhere. Using the *B. subtilis* wild-type (168) and engineered (RIK543) strains, we first attempted to isolate *ksgA* mutants. Mutants with low-level kasugamycin resistance developed at a high frequency (10^{-6}) in each strain on plates containing a twofold MIC amount of kasugamycin. These kasugamycin-resistant mutants (19 mutants derived from RIK543 were tested) were all found to have a mutation within the *ksgA* gene and were characterized by the frequent appearance of deletion or insertion mutations that resulted in stop codons just downstream of the mutations (Table 4). Likewise, the wild-type strain 168 also produced a wide variety of *ksgA* mutants at a high frequency (data not shown), indicating independence of this phenomenon of the number of rRNA gene copies. Unexpectedly, none of the RIK543-derived kasugamycin-resistant mutants had a mutation in the rRNA gene (i.e., *rrnO*) as determined by DNA sequencing. These results suggest that the frequency of emergence of rRNA mutation conferring kasugamycin resistance (if any in *B. subtilis*) is much lower than that of the *ksgA* mutation. In fact, mutants with a 16S rRNA mutation (A802G or G935A, corresponding to A794G and G926A in *E. coli*, respectively) were readily detected, though at a low frequency of 10^{-8} to 10^{-9}, when

TABLE 2. The primers used in this study

Purpose	Primer	Oligonucleotide sequence (5′→3′)
PCR for *ksgA* of *B. subtilis*	BS-ksgA-F	CTGCGGCCAAATGCCGCCGGGAACGACTG
	BS-ksgA-R	CAGCAATCAGCCTGACTTCATCTCCATGC
Sequence for *ksgA* of *B. subtilis*	BS-ksgA-seqF	GATTTTATGAGTGCGCTCGATACCGTTATG
	BS-ksgA-seqR	GGTCCTTCTCTAGCCTATGTAGCGGCAGTC
PCR for *rsmG* of *B. subtilis*	BS-gidB-F	CGCAATTAAGGGGATTGCGACCGAGGCGC
	BS-gidB-R	CACTCAAGCGATTGCACCGCTCTCCAGCG
Sequence for *rsmG* of *B. subtilis*	BS-gidB-seq	GAGCAGGGACGCATTGCGAAGATAGC CGAG
PCR for *rrnO* of *B. subtilis*	Bs-16S-F	TGGAGAGTTTGATCCTGGCT
	Bs-16S-R2	GCGCACGCCTGATAAGCGTGA
Sequence for *rrnO* of *B. subtilis*	Bs-16S-mF	GGGGAACTTGAGTGCAGAAG
	Bs-16S-mR	GGGTATCTAATCCTGTTC
	Bs-16S-1kF	TCGTGTCGTGAGATGTTGGG
PCR for *rplK* of *B. subtilis*	BS-rplK-F	GTTTGGCCGTGAAACGCCGGTTGAGCTGG
	BS-rplK-R	CGAGAGCTACTGCTTCAGAGACGTCG
Sequence for *rplK* of *B. subtilis*	BS-rplK-seq	GAATGAAACCTTGAGTGGGAGGG
PCR for *rpoB* of *B. subtilis*	BS-rpoB-F	GCTTCAGGTGAATTGTGGATCGTCATACAG
	BS-rpoB-R	GCCCGTGTTACTTCGACTCCGCAGCGGTC
Sequence for *rpoB* of *B. subtilis*	BS-rpoB-seq500R	CGCATCAGTTTCGTATTCTAACCATGCGC
	BS-rpoB-seq410F	CATTAACGGTGCGGAACGTGTTATCGTTTC
	BS-rpoB-seq870F	GAATCGCCTCTTCAATCAGAGACTTG
	BS-rpoB-seq1320F	GTTCGTGAGAGAATGTCAATTCAAGATACG
	BS-rpoB-seq1760F	GCTCGTCTTGATGACGAAGGCGCC
	BS-rpoB-seq2210F	GTCCGATCGTAAGTGTCGGCGATGAAG
	BS-rpoB-seq2650F	GATACTTCTCTTCGTGTGCCTCATGGCGG
	BS-rpoB-seq3100F	GATAACCGTGTATCTGTCGGTATCATG
PCR and sequence for *speD* of *B. subtilis*	speD-F	AAAGGGTTAGGACCTCTC
	speD-R	GCTTCAGCTCCCTTTGTG
PCR for *ksgA* of *E. coli*	Eco-ksgA-F	CCAGGGCTTCGGGCGCGGTGTGAACATTAC
	Eco-ksgA-R	GCCATTGGTGATCAGCCAGTAACGCCCC
Sequence for *ksgA* of *E. coli*	Eco-ksgA-seq1	GAACTGGCGGGACGTGGCAAAGCCGATG
	Eco-ksgA-seq2	CGTAACGTTCATTATCAGGTGAAGATTGAG
PCR for *tlyA* of *M. smegmatis*	Ms-tlyA-F	CGCAGGCCAATACCGTTG
	Ms-tlyA-F	GCAGCCAAGCTGGAACAG
Sequence for *tlyA* of *M. smegmatis*	Ms-tlyA-1F	CGCAGGCCAATACCGTTG
	Ms-tlyA-2F	GGTGTCAGATCGCGAA

TABLE 3. Antibiotic susceptibility of *B. subtilis* strains 168 and RIK543

Antibiotic examined	MIC (μg/ml) for strain[a]:	
	168	RIK543
Kasugamycin	1,500	500
Streptomycin	15	5
Thiostrepton	0.04	0.02
Rifampin	0.1	0.01

[a] Determined after a 15-h incubation at 37°C on LB agar (for streptomycin, thiostrepton, and rifampin) or LB agar without NaCl (for kasugamycin).

selection was done for high-level (3,000 μg/ml or more) kasugamycin resistance, under the conditions which the *ksgA* mutants could no longer develop (data not shown). We noted that the *ksgA* mutation conferred no resistance to any of the other antibiotics tested, including streptomycin, kanamycin, spectinomycin, gentamicin, thiostrepton, lincomycin, erythromycin, and fusidic acid. Strains KO-827 (derived from RIK543) and KO-847 (derived from 168) were used for further study of the *ksgA* mutation. We also isolated mutants with low-level streptomycin resistance (KO-826), thiostrepton resistance (KO-819), and rifampin resistance (KO-809) from RIK543 for use as the control mutants in the following experiments (Table 1).

TABLE 4. Location of mutation in the *ksgA* gene and resulting amino acid exchange in KsgA[a]

Mutant	Position of mutation in *ksgA* gene	Amino acid exchange	MIC to kasugamycin (μg/ml)
RIK543 (parent)	WT[b]		500
1	253–261→Δ	85–87 Ile, Leu, Lys→Δ	1,500
2	708–717→Δ	Lys 237→Ser (Leu 240→stop codon)	1,500
3	301C→T	Gln 101→stop codon	1,500
4	709–850→Δ	Lys 237→Leu (frameshift)	1,500
5	478–492→Δ	Glu 160, Ala 164→Δ	1,500
6	272C→G	Ser 91→stop codon	1,500
7	198T→TT	Ala 67→Cys (Glu 77→stop codon)	1,500
8	515G→GGG	Ser 173→Ala (Met 188→stop codon)	1,500
9	515G→GG	Ser 173→Leu (Asn 200→stop codon)	1,500
10	706C→T	Arg 236→Cys	1,500
11	274–295→Δ	Pro 92→Phe (Thr 97→stop codon)	1,500
12	597–615→Δ	Asn 200→Phe (Asp 202→stop codon)	1,500
13	393C→G	Tyr 131→stop codon	1,500
14	608C→T	Ser 203→Phe	1,500
15	324C→CTC	Lys 109→Ser (Val 125→stop codon)	1,500
16	181G→GG	Ala 61→Gly (Glu 77→stop codon)	1,500
17	709–850→Δ	Lys 237→Leu (frameshift)	1,500
18	495–525→Δ	Asp 165→Glu (Ala 177→stop codon)	1,500
19	230A→G	Glu 77→Lys	1,500

[a] Numbering originated from the start codon (ATG) of the open reading frame.
[b] WT, wild-type *ksgA* gene.

High-frequency emergence of high-level kasugamycin resistance in *ksgA* mutants. Spontaneous mutations that lead to high-level antibiotic resistance (often a 100-fold increase in the MIC) generally emerge at a low frequency in bacteria ($\leq 10^{-8}$). Consistently, in *B. subtilis* RIK543 (MIC, 500 μg/ml), spontaneous mutants conferring high-level kasugamycin resistance (MIC, at least 5,000 μg/ml) arose at a low frequency, between 8×10^{-9} and 5×10^{-8}. Strikingly, but consistent with previous observations for streptomycin resistance (20, 23; this study), the *B. subtilis ksgA* mutant produced spontaneous mutants

TABLE 5. Effect of *ksgA* mutation on the emergence of mutants with high-level kasugamycin resistance

Strain	Antibiotic used for selection (μg/ml)	Frequency of mutants with high-level resistance[a]	Relative frequency
B. subtilis			
RIK543	Kasugamycin (5,000)	8×10^{-9} to 5×10^{-8}[b]	1
KO-827 (*ksgA*)	Kasugamycin (5,000)	6×10^{-7} to 5.5×10^{-6}	100
RIK543	Kasugamycin (10,000)	$<3 \times 10^{-9}$	1
KO-827 (*ksgA*)	Kasugamycin (10,000)	2×10^{-7} to 8×10^{-7}	>100
RIK543	Streptomycin (1,000)	3×10^{-9} to 1.5×10^{-8}	1
KO-826 (*rsmG*)	Streptomycin (1,000)	5×10^{-7} to 1.5×10^{-6}	100
RIK543	Thiostrepton (1)	1×10^{-8} to 5×10^{-8}	1
KO-819 (*tsp*)	Thiostrepton (1)	1.5×10^{-8} to 9×10^{-8}	2
RIK543	Rifampin (30)	1×10^{-9} to 6×10^{-9}	1
KO-809 (*rif*)	Rifampin (30)	4×10^{-9} to 1.5×10^{-8}	2.5
168	Kasugamycin (5,000)	1.5×10^{-8} to 9×10^{-8}[c]	1
KO-847 (*ksgA*)	Kasugamycin (10,000)	4×10^{-7} to 1.2×10^{-5}	110
KO-894 (*speD*)	Kasugamycin (10,000)	3×10^{-8} to 1.3×10^{-7}	1.5
E. coli			
BW25113	Kasugamycin (1,000)	$<1 \times 10^{-8}$	1
KO-845 (*ksgA*)	Kasugamycin (1,000)	1.5×10^{-6} to 5×10^{-6}	>200
BW25113	Kasugamycin (2,000)	$<4 \times 10^{-10}$	1
KO-845 (*ksgA*)	Kasugamycin (2,000)	1.5×10^{-7} to 7×10^{-7}	>500
B. subtilis			
168	Kasugamycin (5,000)	2.5×10^{-8} to 1.5×10^{-7}	1
KO-750 (*rsmG*)	Kasugamycin (5,000)	2×10^{-8} to 1×10^{-7}	0.8
168	Streptomycin (2,000)	1×10^{-8} to 2.5×10^{-8}	1
KO-847 (*ksgA*)	Streptomycin (2,000)	5×10^{-9} to 8×10^{-9}	0.4

[a] Cells (10^7 to 10^{10}) were plated on LB agar (without NaCl for kasugamycin) containing each antibiotic and incubated at 37°C for 2 to 3 days.
[b] Similar frequency was detected when 3,000 μg/ml of kasugamycin was used instead of 5,000 μg/ml.
[c] Frequency was $<2 \times 10^{-9}$ when 10,000 μg/ml of kasugamycin was used instead of 5,000 μg/ml.

showing resistance to a high level of kasugamycin (5,000 μg/ml) at a frequency on the order of 10^{-6} to 10^{-7}. The data for *ksgA* mutant KO-827 are shown in Table 5 and indicate that there was a 100-fold greater frequency of mutations to high-level kasugamycin resistance than in the wild-type strain. Likewise, high-frequency emergence was detected when the *ksgA* mutant KO-847, derived from the wild-type strain 168, was used (a 110-fold greater frequency) or when the *E. coil ksgA* mutant KO-845 was compared with its parent strain, BW25113 (a 200- to 500-fold-greater frequency) (Table 5). Thus, this peculiar event occurs irrespective of the number of rRNA gene copies. Despite such marked increases in the frequency of high-level kasugamycin resistance in the genetic background of *ksgA*, only slight increases in the frequency of high-level resistance to the corresponding antibiotics were detected in the genetic background of Tsp^r or Rif^r, mutations of which confer a low level of resistance to thiostrepton and rifampin, respectively (Table 5). In agreement with previous work (20), the *rsmG* mutant KO-826 with low-level resistance to streptomycin produced spontaneous mutants showing resistance to a high level of streptomycin (1,000 μg/ml) at a 100-fold greater frequency. It is notable that the *ksgA* mutation did not lead to a greater frequency of the appearance of high-level streptomycin-resistant mutants and vice versa for the *rsmG* mutation (Table 5). In addition, the *ksgA* mutation did not affect the frequency at which mutants resistant to antibiotics other than kasugamycin (spectinomycin, lincomycin, erythromycin, chloramphenicol, and rifampin were tested) emerged, indicating that the observed effect of the *ksgA* mutation is limited to kasugamycin.

Importantly, although the collective importance of rRNA modifications for protein synthesis has been shown (11), *rsmG* mutants developed even in the genetic background of *ksgA* when selected for low-level streptomycin resistance (e.g., KO-924 to KO-926) (Table 1), suggesting that *ksgA* and *rsmG* mutations can coexist without a loss of cell viability. Allowance of coexistence was confirmed by transformation (using KO-847 as the recipient and KO-750 as the donor DNA), in which *ksgA rsmG* transformants readily developed (e.g., KO-927) (Table 1). The *ksgA rsmG* double mutants not only grew as well as the wild-type strain 168 but also sporulated well (see Table 8).

***speD* mutation is responsible for high-level kasugamycin resistance.** We next determined mutations leading to high-level kasugamycin resistance from low-level resistance. As expected, the mutants with high-level resistance derived from KO-809 (Rif^r) and KO-826 (Sm^r) were all found to have a mutation within the *rpoB* and *rpsL* genes, respectively, although the majority of mutants with high-level thiostrepton resistance showed no mutations within the *rplK* gene (data not shown). Unexpectedly, no mutations were found in the 16S rRNA gene when the mutants with high-level kasugamycin resistance (more than 20 strains were tested) derived from KO-827 (*ksgA*) were subjected to DNA sequencing. Therefore, we utilized comparative genome sequencing (19), a new method that uses microarray-based DNA sequencing to identify single-nucleotide polymorphisms and insertion-deletion sites within the genome. The mutant KO-875, derived from KO-847 (*ksgA*), with high-level kasugamycin resistance, was utilized as the source of mutant genomic DNA, and the stain KO-847 was utilized as the source of reference genomic DNA.

TABLE 6. Location of mutation in the *speD* gene and resistance level to kasugamycin in *B. subtilis* mutants

Strain	Position of mutation in *speD*[a]	Amino acid exchange	MIC to kasugamycin (μg/ml)[b]
168 (wild-type)			1,500
KO-847 (*ksgA*)			5,000
KO-894 (*speD*)[c]	19G→T	Gly 7→Trp	4,000
KO-875 (*ksgA speD*)	19G→T	Gly 7→Trp	15,000
KO-876 (*ksgA speD*)	22C→GT	Frameshift	15,000
KO-877 (*ksgA speD*)	191A→T	Glu 64→Val	15,000
KO-878 (*ksgA speD*)	193T→A	Ser 65→Thr	15,000
KO-879 (*ksgA speD*)	236G→T	Ser 79→Ile	15,000

[a] Numbering originated from the start codon (ATG) of the open reading frame.
[b] Determined 2 days after incubation on LB agar (without NaCl).
[c] Transformant of 168 by DNA of KO-875.

As a result, we successfully identified a putative single-nucleotide polymorphism within the *speD* gene (formerly called *ytcF* but renamed *speD* by Sekowska et al. [28], which was confirmed by direct sequencing to be a point mutation [19G→T representing Gly 7→Trp]). The *speD* gene encodes *S*-adenosylmethionine decarboxylase (28). Strikingly, four other isolates with high-level kasugamycin resistance (KO-876 to KO-879) (Table 6) were all found to carry a mutation within the *speD* gene, including a frameshift mutation, thus strongly suggesting that the *speD* mutations are responsible for the acquisition of high-level resistance to kasugamycin (Table 6). The causal relationship was confirmed by *speD* transformation; the *speD* transformants (e.g., KO-894) showed kasugamycin resistance as did the *ksgA* mutant KO-847 (Table 6). This observation also indicated that the *speD* mutation alone is able to confer resistance to kasugamycin. Importantly, in contrast to the *ksgA* mutants, the *speD* mutant KO-894 did not produce mutants with high-level kasugamycin resistance at a high frequency (Table 5). Unlike the case for the *B. subtilis ksgA* mutant, none of the mutants with high-level kasugamycin resistance derived from *E. coli ksgA* strain KO-845 carried a mutation in *speD* and *speE* (coding for spermidine synthase), although the *ksgA* strain KO-845 also produced mutants with high-level kasugamycin resistance at a high frequency, which was more than 200-fold greater than that in the parental strain BW25113 (Table 5). The mutation(s) that arose in these *E. coli* mutants was not studied further.

***speD* mutation causes a marked reduction of intracellular spermidine.** Sekowska et al. (28) recently reported that the *B. subtilis* SpeD protein (encoded by the *speD* gene), together with SpeE, participates in spermidine biosynthesis in this organism; *speD*-disrupted mutants exhibit a total lack of spermidine. Therefore, we performed TLC analyses of polyamine contents in wild-type and *speD* mutant cells. As shown in Fig. 1, spermidine concentrations in both the *speD* and *ksgA speD* mutant cells were significantly lower than those in wild-type cells, although they were not entirely diminished, perhaps due to the uptake of spermidine present in the medium. As expected, the kasugamycin resistance acquired in the *speD* transformant KO-894 was negated entirely or to some extent when 5 mM or 1 mM spermidine, respectively, was included in the medium (data not shown). In contrast, the addition of pu-

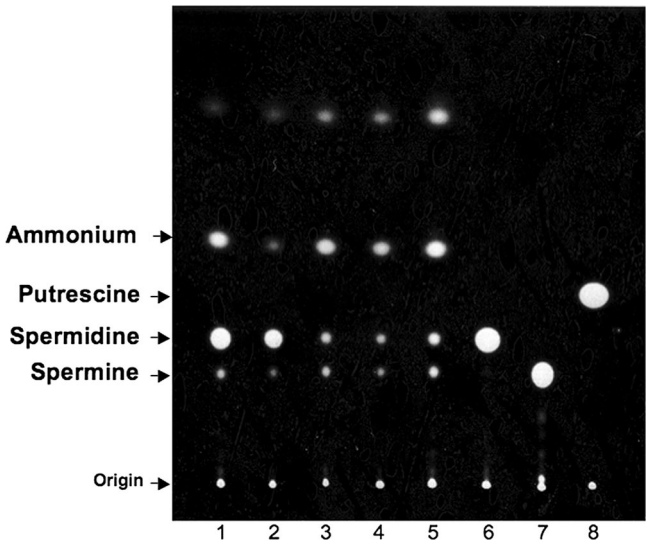

FIG. 1. Identification of polyamines by TLC. Dansyl polyamine derivatives of cell extracts were analyzed by TLC in ethyl acetate-cyclohexane (2:3, vol/vol). Lane 1, wild-type (168); lane 2, *ksgA* (KO-847); lane 3, *speD* (KO-894); lane 4, *ksgA speD* (KO-875); lane 5, *ksgA speD* (KO-876); lane 6, spermidine standard; lane 7, spermine standard; and lane 8, putrescine standard.

trescine (5 to 10 mM) did not negate the *speD* mutation-induced kasugamycin resistance in KO-894. Thus, we concluded that the reduced spermidine concentrations caused by *speD* mutations are responsible for the phenotype with high-level kasugamycin resistance observed in the *ksgA speD* double mutants.

Inactivation of TlyA does not cause a high-frequency emergence of mutants with high-level capreomycin resistance. Like RsmG and KsgA, which methylate the base moiety of 16S rRNA, TlyA, encoded by the *tlyA* gene, methylates the ribose moiety of nucleotide C1409 of 16S rRNA and C1920 of 23S rRNA in *Mycobacterium tuberculosis* (14, 16). Inactivation of the *tlyA* gene confers low-level resistance to the ribosome-targeting drug capreomycin, which has been used as a second-line antibiotic for tuberculosis chemotherapy. Although many bacterial species lack the *tlyA* gene, including *E. coli*, *B. subtilis*, and *S. coelicolor*, we studied using *M. smegmatis* whether or not inactivation of *tlyA* (i.e., failure to methylate the ribose moiety of rRNA) results in a high-frequency emergence of mutants with high-level capreomycin resistance. The *tlyA* mutant KO-935, derived from the wild-type strain JCM5866 (MIC, 7 μg/ml) by spontaneous mutation (Table 1), revealed a low-level resistance (MIC, 20 μg/m) to capreomycin. The *M. smegmatis tlyA* mutant, however, did not give rise to mutants with high-level capreomycin resistance at a high frequency; the emergence of mutants with high-level resistance (MIC, ≧100 μg/ml) was as low as 2×10^{-10} or less in either wild-type or *tlyA* mutant strains, thus discriminating the *tlyA* mutation from the *rsmG* and *ksgA* mutations in resistance emergence (data not shown).

***ksgA*, *speD*, and *tlyA* mutations result in a fitness disadvantage.** Although mutations that confer drug resistance often have a biological cost, causing mutant bacteria to grow more slowly (2), *rsmG* mutants grow as well as the parental strain, as demonstrated by *E. coli*, *B. subtilis*, and *S. coelicolor* (19, 20, 23). Moreover, the growth competition assay demonstrated that the *B. subtilis rsmG* mutants are as fit as the wild-type strain. These findings were in contrast with previous studies of several other 16S rRNA methylases (1, 4, 15), which showed that knockout mutants were less fit than the wild-type strain. Although the *ksgA* mutants (and also the *speD* mutants) of *B. subtilis* and *E. coli* grew as well as the parental strain in LB medium, we examined whether the *B. subtilis ksgA* and *speD* mutants were as fit as the parental strain. The results of growth competition assays using strains of *B. subtilis*, *E. coli*, *S. coelicolor*, and *M. smegmatis* are summarized in Table 7. It is evident that the *ksgA* mutations resulted in a substantial disadvantage in growth fitness and that this was especially pronounced in *speD* mutations and *rpsL* mutations. A modest level of disadvantage in growth fitness was also detected in the *M. smegmatis tlyA* mutant. In contrast, no substantial disadvantages resulting from the *rsmG* mutation were detected in any bacteria tested, irrespective of culture conditions using LB medium (representing a rich medium) or sterilized soil (representing a poor medium) (Table 7). The *B. subtilis* mutant strains used here all displayed abundant sporulation, the ability of which might affect the overall growth fitness of each strain. In particular, *rsmG* mutant KO-750 displayed an ability for sporulation that was superior to that of the wild-type strain 168

TABLE 7. Growth fitness of *ksgA* and *speD* mutants compared to that of *rpsL* and *rsmG* mutants

Culture conditions and microorganism examined	Fitness test[a]	Ratio after multiple-cycle cultivation
Liquid culture		
B. subtilis	168/KO-847 (*ksgA*)	1:0.004
	168/KO-894 (*speD*)	1:0.00001
	168/KO-750 (*rsmG*)	1:1.2
	168/KO-671 (*rpsL*)	1:0.0002
	168/KO-924 (*ksgA rsmG*)	1:0.007
	168/KO-875 (*ksgA speD*)	1:0.000005
E. coli	BW25113/KO-845 (*ksgA*)	1:0.0008
	BW25113/KO-895 (*rpsL*)	1:0.005
	BW25113/JWK3718 (*rsmG*)	1:1
S. coelicolor	1147/KO-178 (*rpsL*)	1:0.00001
	1147/KO-179 (*rsmG*)	1:0.4
M. smegmatis	JCM5866/KO-935 (*tlyA*)	1:0.06
	JCM5866/KO-936 (*rsmG*)	1:0.8
Soil culture		
B. subtilis	168/KO-750 (*rsmG*)	1:1.3
E. coli	BW25113/JWK3718 (*rsmG*)	1:0.9
S. coelicolor	1147/KO-179 (*rsmG*)	1:0.3

[a] In the liquid culture, strains were mixcultured in LB medium at 37°C (for *B. subtilis* and *E. coli*) or glucose-yeast extract-malt extract medium at 30°C (for *S. coelicolor*) with five-cycle cultivation. One cycle was 2 days. *M. smegmatis* strains were mix cultured in R medium at 37°C with five-cycle cultivation. One cycle was 3 days. In the soil culture, strains were mix cultured in the sterilized soil with 10-cycle (for *B. subtilis* and *E. coli*) or 4-cycle (for *S. coelicolor*) cultivation. One cycle was 2 weeks. The initial mix ratio was always 1:1.

TABLE 8. Ability of *B. subtilis* mutant strains to sporulate in certain media

Strain	No. of heat-resistant spores/ml in[a]:		
	Sporulation medium	2× sporulation medium	LB medium
168 (wild-type)	5.4×10^7	4.2×10^8	6.1×10^7
KO-847 (*ksgA*)	5.1×10^7	3.5×10^8	7.3×10^6
KO-750 (*rsmG*)	1.0×10^8	1.2×10^9	6.5×10^7
KO-894 (*speD*)	9.1×10^6	8.1×10^8	5.5×10^6
KO-875 (*ksgA speD*)	1.3×10^7	8.3×10^8	4.8×10^6
KO-924 (*ksgA rsmG*)	5.9×10^7	3.8×10^8	9.0×10^6

[a] Strains were incubated with sporulation medium (for 36 h), 2× sporulation medium (for 24 h), or LB medium (for 48 h). Heat-resistant spores were determined by treating the cultured broth at 80°C for 15 min. Sporulation medium consisted of 0.8% nutrient broth (Difco), 0.2% KCl, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 μM $FeSO_4$, and 10 μM $MnCl_2$. 2× sporulation medium contained 1.6% nutrient broth and 0.1% glucose in the components described above.

(Table 8), which may account for the observation of greater fitness of this strain than that of the wild-type 168 (Table 7).

DISCUSSION

The bacterial enzyme KsgA catalyzes the transfer of a total of four methyl groups from *S*-adenosylmethionine to two adjacent adenosine bases (A1518 and A1519 in the loop of helix 45) to produce N^6,N^6-dimethyladenosine in 16S rRNA (12, 13). To date, 10 methylatable nucleosides within *E. coli* 16S rRNA (1), including position G527, which is methylated by the enzyme RsmG (20), are reported. Although the collective importance of these rRNA modifications for protein synthesis has been demonstrated (11), the functions of individual methylations are still unclear, as inactivation of the genes encoding their cognate methyltransferases does not affect cell viability (2, 4, 15). In the present study, we demonstrated the following four characteristic aspects of *ksgA* mutations: (i) *ksgA* mutations emerge at a high frequency of 10^{-6}, conferring a modest level of resistance to kasugamycin that is apparently due to the dispensability of this gene, allowing cells to remain viable; (ii) once cells acquire the *ksgA* mutation, they produce a mutation conferring a high level of resistance to kasugamycin at an extraordinarily high frequency; (iii) the mutation conferring a high level of kasugamycin resistance occurs solely within the *speD* gene (at least in *B. subtilis*), which encodes *S*-adenosylmethionine decarboxylase; and (iv) unlike the *rsmG* mutation, the *ksgA* mutation gives rise to a disadvantage in overall growth fitness. These results clearly indicate similarities (i and ii) and dissimilarities (iii and iv) between *ksgA* and *rsmG* mutations, both of which share common characteristics as represented by the failure to methylate the 16S rRNA bases (not ribose moieties). The mechanism underlying the high-frequency emergence of high-level kasugamycin (and also high-level streptomycin) resistance is not yet clear, but it is unlikely that KsgA (and RsmG) functions as an anti-mutator-like protein, as the *ksgA* (and *rsmG*) mutation did not affect the frequency at which mutants resistant to antibiotics other than kasugamycin or streptomycin emerged (23; this study). In addition, we can exclude the possibility that the observed high frequency of the emergence of mutations conferring high-level kasugamycin resistance is caused by an increase in persistence due to *ksgA* mutation, as mutations conferring a low level of resistance to thiostrepton or rifampin gave rise to only a slight increase (2.5-fold at most) in emergence, possibly due to the increased persistence (Table 5). This conclusion was further supported by the observation that *speD* mutants did not produce mutants with high-level kasugamycin resistance at a high frequency (Table 5), despite the fact that the *speD* mutant and *ksgA* mutant have similar resistance levels to kasugamycin (Table 6). Thus, the mechanism underlying the observed peculiar phenomena, common to *ksgA* and *rsmG* mutants, remains to be studied at the molecular level. Nonetheless, the emergence of mutants with high-level kasugamycin resistance at an extraordinarily high frequency due to *ksgA* mutation is of considerable importance from an agricultural viewpoint, given that kasugamycin has been widely used as a potent anti-rice blast drug. Apart from the kasugamycin resistance, it is also notable that the high-frequency emergence of mutants with high-level streptomycin resistance was detected even in the genetic background of *relA* (coding for ppGpp synthetase) as examined using the *E. coli rsmG relA* double mutant (Y. Tanaka, S. Okamoto, and K. Ochi, unpublished data), indicating that ppGpp is irrelevant to the high-frequency emergence observed.

Polyamines as represented by spermidine, spermine, and putrescine are extremely important for the cell, although they are dispensable under routine laboratory growth conditions. They are involved in macromolecular syntheses and in particular in the modulation of translation accuracy at steps which may be essential for survival of the cell populations (5). Polyamine auxotrophy in *E. coli* due to SpeD inactivation has been implicated in resistance to aminoglycoside antibiotics, including kasugamycin (9, 10). Ribosomes of a polyamine auxotrophic *E. coli* mutant starved of polyamines showed a reduced affinity for streptomycin, and their protein synthesis activity was less sensitive to the drug (10). These previous findings indicate a causal relationship between kasugamycin resistance and intracellular polyamine contents, and in turn account for our observation that *B. subtilis ksgA speD* double mutants showed a higher level of kasugamycin-resistant phenotypes than *ksgA* single mutants (Table 6). Although putrescine did not negate the *speD* mutation-induced kasugamycin resistance in *B. subtilis* (see Results), it is likely that putrescine (present at a high concentration in *E. coli*) (28) participates in the level of kasugamycin resistance in *E. coli*, accounting for the question of why *speD* mutants were not found in *E. coli* with the same screening procedure (see Results). It is also notable that no *speD* mutants were detected in the study when *B. subtilis* $ksgA^+$ strains 168 and RIK543 were used, although *speD* mutation alone could confer resistance to kasugamycin (Table 6). It is conceivable that the emergence of *speD* mutation (in the genetic background of $ksgA^+$) was much lower than that of the *ksgA* mutation, although these genes are both dispensable.

Homologs of *ksgA* (and also *rsmG*) are highly conserved among eubacteria, so it was somewhat surprising that, despite the apparent important contribution made by KsgA and RsmG to ribosomal function, *ksgA* mutations (this study) and disruption of *rsmG* (20, 23) had no effect on growth of *E. coli* and *B. subtilis*. However, it is notable that *ksgA* mutations carried fitness costs, as demonstrated by the growth competition assay (Table 7), implying an important role of 16S rRNA

methylation in survival. In this regard, the complete lack of fitness cost associated with *rsmG* mutation is distinctive, as demonstrated by several bacteria (Table 7). Given the possible involvement of the rRNA methyltransferases in ribosome homeostasis (5a), the *ksgA* mutation, together with the *rsmG* mutation, may provide new insights to uncover unknown mechanisms of mutation and ribosomal function.

ACKNOWLEDGMENTS

This work was supported by grants to K.O. from the Effective Promotion of Joint Research of Special Coordination Funds (the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese government).

We gratefully acknowledge Takayoshi Tamura (Meiji Seika Kaisha) for the generous supply of kasugamycin and Genefrontier Corp., Tokyo, Japan, for supporting the mutation search using the comparative genome sequencing technique.

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