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Research paper

16S rRNA methyltransferase KsgA contributes to oxidative stress resistance and virulence in *Staphylococcus aureus*

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ABSTRACT

We previously reported that the rRNA methyltransferases RsmI and RsmH, which are responsible for cytidine dimethylation at position 1402 of 16S rRNA in the decoding center of the ribosome, contribute to *Staphylococcus aureus* virulence. Here we evaluated other 16S rRNA methyltransferases, including KsgA (RsmA), RsmB/F, RsmC, RsmD, RsmE, and RsmG. Knockout of KsgA, which methylates two adjacent adenosines at positions 1518 and 1519 of 16S rRNA in the intersubunit bridge of the ribosome, attenuated the *S. aureus* killing ability against silkworms. The *ksgA* knockout strain was sensitive to oxidative stress and had a lower survival rate in murine macrophages than the parent strain. The *ksgA* knockout strain exhibited decreased translational fidelity in oxidative stress conditions. Administration of N-acetyl-L-cysteine, a free-radical scavenger, restored the killing ability of the *ksgA* knockout strain against silkworms. These findings suggest that the methyl-modifications of 16S rRNA by KsgA contribute to maintain ribosome function under oxidative conditions and thus to *S. aureus* virulence.

1. Introduction

rRNA is post-transcriptionally modified by methylation. Because methyl modifications of rRNA are observed at the decoding center, peptidyl transferase center, and the interface of the ribosome subunit, they are considered important for fine-tuning ribosome function [1]. In *Escherichia coli*, 11 and 14 positions in 16S rRNA and 23S rRNA, respectively are methylated [1]. Some of the methylations are involved in ribosome biogenesis [2,3] and accurate translation initiation [4,5]. Expression of *E. coli* rRNA methyltransferases is altered by environmental stressors, including antibiotic treatment, temperature shift, and an oxidative treatment, supporting the importance of methyl modifications of rRNA under stress conditions [6]. Most rRNA methyltransferase studies are conducted in *E. coli*, which are Gram-negative bacteria, and thus the functions of rRNA methyltransferases in Gram-positive bacteria are unclear.

KsgA, which methylates two adenosines at positions 1518 and 1519 of 16S rRNA in *E. coli*, is conserved among bacteria, archaea, and eukaryotes [7,8]. The methylations are important for the packing of helices 45, 44, and 24a of 16S rRNA [9], and locate at the surface of the 30S subunit as an intersubunit bridge between the 30S and 50S subunits. KsgA is important for small subunit biogenesis [2,3] and ribosome recycling [10]. In *E. coli* and other Gram-negative bacteria, KsgA is not essential for cell growth, but contributes to growth at low temperatures [2,11,12]. A functional analysis of KsgA in Gram-positive bacteria was recently reported in *Bacillus subtilis* and *Staphylococcus aureus* [13,14]. In *B. subtilis*, KsgA is not essential for growth, but is required for competition with the wild-type strain in liquid culture [13]. In *S. aureus*, KsgA is also not essential for growth, but contributes to growth at low temperatures [14]. In Gram-positive bacteria, however, the physiologic role of KsgA is obscure.

We previously identified *S. aureus* virulence factors using a silkworm infection model [15–17]. In the course of our studies, we found that the 16S rRNA methyltransferases RsmI and RsmH are required for oxidative stress resistance and *S. aureus* virulence in animals [18]. Our findings suggested that cytidine dimethylation by RsmI and RsmH at the decoding center of the ribosome maintains ribosome function under oxidative conditions. Functional investigation of rRNA methyltransferases in the bacterial infectious process will advance our understanding of the physiologic roles of the methyl modifications. In the present study, we hypothesized that other rRNA methyltransferases have roles in bacterial virulence and evaluated the virulence of several knockout mutants.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

S. aureus strains were aerobically cultured at 37 °C in tryptic soy broth (TSB; Becton Dickinson). When culturing bacterial strains transformed with plasmids, 12.5 μg/ml chloramphenicol, 50 μg/ml kanamycin, 10 μg/ml erythromycin, or 5 μg/ml tetracycline was added to the medium. The bacterial strains and plasmids used in this study are listed in Table 1.

2.2. Knockouts of S. aureus rRNA methyltransferases

Knockout of *S. aureus* genes was performed by integrating a suicide vector by single recombination [19]. DNA fragments homologous to the internal region of 16S rRNA methyltransferase genes were amplified by polymerase chain reaction using oligonucleotide primers (Table 2) and *S. aureus* RN4220 genomic DNA as a template. The DNA fragments were inserted into pCK20 or pSF151, resulting in targeting vectors. The *S. aureus* RN4220 strain was transformed with the targeting vectors and spread onto TSB agar plates containing 12.5 μg/ml chloramphenicol or 50 μg/ml kanamycin. Genomic DNA was extracted from the developing colony and gene knockout was confirmed by Southern blot analysis.

To complement the *ksgA* knockout mutant with the *ksgA* gene, a DNA fragment containing the open reading frame and the native promoter region of the *ksgA* gene was amplified by polymerase chain reaction using oligonucleotide primers (Table 2) and *S. aureus* RN4220 genomic DNA as a template. The DNA fragment was inserted into pHY300E or pSF151, resulting in pksgA or plksgA. The *ksgA* knockout mutant was transformed with pksgA or plksgA. For plksgA, the desired integration into the *ksgA* gene locus was confirmed by Southern blot analysis.

2.3. Silkworm infection experiment

Infection of silkworms was performed as described previously [16,20]. Fertilized eggs of *Bombyx mori* were purchased from Ehime sansyu (Ehime, Japan) and incubated at 27 °C. The hatched larvae were fed an artificial diet (Silkmate 2S, Nosan Corporation, Yokohama, Japan) and raised to 5th instar larvae. The fifth instar larvae were fed an antibiotic-free diet for 1 d and injected with 50 μl of 2-fold serial dilutions of *S. aureus* overnight cultures using a tuberculin syringe equipped with a 27-gauge needle (Terumo). Survival was monitored by picking up larvae with tweezers. Bacterial numbers injected into the larvae were counted by spreading the bacterial solution onto TSB agar plates. The dose required to kill 50% of the animals (LD_{50}) was calculated by logistic regression analysis [21].

Table 2
Primers used in this study.

Target		Sequence (5′–3′)
ksgA (SA0451)	F	AAGATATTGCAACACCATCAAGAA
	R	CATCACCACGTAGCCATCAA
rsmB/F (SA1060)	F	CGAAAACGTGAGAAGTCTTGC
	R	GACCATAATGTGTTGCCCAAT
rsmC (SA0499)	F	CAAGTGTAATTAGCAATGAACAACGTATTC
	R	GCAGACAAAGCATCACTTTCCTTTACG
rsmD (SA0972)	F	AGGCCGTAATACGAGACCAACTATGGATAAAG
	R	GGTGGATCTAAGAAAATGACATCAAATTGAATATC
rsmE (SA1406)	F	AAGAAGCTTGCTGATGTAAGTCAGCGTTTTT
	R	GGAGGATCCGCTTTGTTCAGCAGCTTCTTT
rsmG (SA2499)	F	GGTTAGCAGAACAATTAAAAGAACA
	R	TCCCTGTAGACACCCTTACCA
ksgA	F	GTCGTCGACGCTTATGAATCAATTGATAAATCTGTGC
	R	GGAGGATCCTAATTTTCTAATTGAGGGAATTTTTTCTTTTC
16S rRNA	F	ACCGTGAGGTCAAGCAAATC
	R	AGAAAGGAGGTGATCCA

2.4. Primer extension analysis

Primer extension analysis was performed as described previously [22]. An oligonucleotide primer (Table 2) complementary to the 3′-end of *S. aureus* 16S rRNA was radio-labeled by using T4 polynucleotide kinase and [α^{32}P]-ATP. A sequencing ladder was obtained using the radio-labeled primer, PCR-amplified 16S DNA fragments, and Primer Cycle Sequencing Kit (GE Healthcare). Total RNA was isolated from *S. aureus* overnight cultures. Reverse transcription reaction was performed using the total RNA, the radio-labeled primer, and a SuperScript III reverse transcriptase (Life

Table 1
List of bacterial strains and plasmids used.

Strain or plasmid	Genotypes or characteristics[a]	Source or reference
Strain		
S. aureus		
RN4220	NCTC8325-4, restriction mutant	[32]
M0451	RN4220 *ΔksgA*::pCK20; Cm^r	Present study
M1060	RN4220 *ΔrsmB/F*::pCK20; Cm^r	Present study
M0499	RN4220 *ΔrsmC*::pSF151; Km^r	Present study
M0972	RN4220 *ΔrsmD*::pSF151; Km^r	Present study
M1406	RN4220 *ΔrsmE*::pSF151; Km^r	Present study
M2499	RN4220 *ΔrsmG*::pCK20; Cm^r	Present study
E. coli		
JM109	General purpose host strain for cloning	Takara Bio
Plasmids		
pCK20	*S. aureus* suicide vector for targeting; Cm^r	[33]
pSF151	*S. aureus* suicide vector for targeting; Km^r	[34]
pHY300E	*E. coli-S. aureus* shuttle vector; Erm^r	[35]
pksgA	pHY300E with intact *ksgA* from RN4220	Present study
plksgA	pSF151 with intact *ksgA* from RN4220	Present study
pHY300PLK	*E. coli-S. aureus* shuttle vector; Tet^r	Takara Bio
pUGA	pHY300PLK with UGA window between Fluc and Rluc	[18]
pUAG	pHY300PLK with UAG window between Fluc and Rluc	[18]
pFS1	pHY300PLK with +1 window between Fluc and Rluc	[18]
pFS2	pHY300PLK with −1 window between Fluc and Rluc	[18]

[a] Cm, chloramphenicol; Km, kanamycin; Erm, erythromycin; Tet, tetracycline.