AJPMHS GLOBAL RESEARCH NETWORK

AMERICAN Journal of Pediatric Medicine and Health Sciences
Volume 02, Issue 2, 2024 ISSN (E): 2993-2149

ONE FACTOR CONTRIBUTING TO STAPHYLOCOCCUS AUREUS PATHOGENICITY IS RNA RIBOSOMAL METHYLTRANSFERASES

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Abstract

The balance between translation precision and speed is influenced by rRNA changes, although the exact processes involved are still unknown. By studying alterations in ribosomal RNA, it may be possible to understand the functions of different bacteria species, even if they are taxonomically distant. The methyltransferases responsible for these alterations play a role in adjusting ribosomal function, especially during stressful situations. In the case of Escherichia coli, two methyltransferases (RsmI and RsmH) have been found to be responsible for cytidine dimethylation in the ribosome's decoding site. However, the physiological implications of these rRNA alterations are not yet clear.Using a silkworm infection model. rsmI & rsmH have been discovered by research to be novel s. aureus virulence genes. These genes cause specific methylations of the S. aureus 16S rRNA, resulting in changes in the bacterium's susceptibility to oxidative stress and its virulence in silkwormsThe loss of virulence results from the elimination of both rsmI or rsmH. While under stress from oxidation, a double-knockout strain exhibits decreased translated accuracy. Additionally, the inactivation of rlmQ, which affects methylation at a different location (G2601) in S. aureus rRNA, has a significant impact on the

bacterium's proliferation, cytotoxicity, and biofilm formation.To further understand the relationship between translation, virulence, and rRNA changes in dangerous gram-positive bacteria, researchers injected N-acetyl-l-cysteine, a scavenger of free radicals, which restored the ability of the double-knockout strain to kill silkworms. Researchers have discovered that the pathogenicity of Staphylococcus aureus is influenced by RNA ribosomal methyltransferases. They conducted a study in which they administered N-acetyl-l-cysteine, a free radical scavenger, into a double knockout strain of the bacteria Staphylococcus aureus. The injection had restored the strain's capacity to kill silkworms. Based on these data, it can be concluded that the methyl modifications of cytidine at site [1412] in the 16S r RNA contribute to the pathogenesis of Staphylococcus auras, especially through the organism's response to oxidative stress.

Key words: *pathogenesis of bacteria immunological reaction Translational fidelity and oxidative stress rRNA modification*

INTROUCTION

All living things have rRNA that has undergone chemical changes. Because rRNA chemical modifications such as methylation occur across the ribosome's functional zones, it is assumed that they have a role in regulating ribosome structure as well as function (1-3). Escherichia coli's 16S rRNA and 23S rRNA have 11 and 14 methyl modifications, respectively. Each methylation site has its own set of catalysts enzymes genes [3]. Accurate translational start [6, 7] and ribosome biogenesis [4, 5] are facilitated by the rRNA methylations. Certain rRNA methyltransferases have more significant roles when the environment is stressed [1]. Antibiotic resistance is impacted by some rRNA methyltransferases (8–12). Knocking down the 23 S rRNA methyltransferase RlmG in E. coli increases osmotic & oxidative- stress sensitivity [13]. Deletion of the 16 S rRNA methyltransferase RsmF either the 23S rRNA methyltransferase RlmKL causes starvation-induced growth defects (14, 15) A phenotype that is susceptible to cold is caused by the 16S rRNA methyltransferase RsmJ knockout [16]. Furthermore, oxidative stress, low temperatures, and heat all affect the expression of many rRNA methyl transfers [1]. These results suggest the significance of these rRNA methyltransferases in stressful environments. The ribosome's P-site, which binds peptidyl-tRNA and lengthens polypeptide chains in concert with the A-site's binding of aminoacyl-transfer RNA, is essential to the synthesis of proteins. Positions 1496–1502 and 1400–1405 of the 16S rRNA residues that are conserved make up the P-site. Plants and bacteria have a high degree of conservation for cytotidine 1402 as of (C1402) at the P-site [17]. E. coli exhibits a slow-growth phenotype when U1402 is substituted for C1402 [18], demonstrating the critical function of C1402 to the ribosomal. Two methyltransferases, RsmI and RsmH, change the C1402 of 16S rRNA in E. coli to N4, 2′-O-dimethylcytidine (m4Cm) [19]. Dimethylation of C1402 is thought to help fine-tune ribosome structure as well as function since deletion of rsmI or rsmH results in lower translation fidelity. The physiological relevance of (C1402) dimethylation in RsmI & RsmH, however, is unclear, due to the deletion strains are alive and only minor growth abnormalities are seen under the examined circumstances. [19]. The multipurpose pathogenic bacterium Staphylococcus aureus is responsible for a number of illnesses in humans, including food poisoning, meningitis, and skin infections. During the 1960s, methicillin-resistant S. aureus, in particular, has affected a large number of individuals globally due to its resistance to a wide variety of medications. It's critical to comprehend the molecular basis of S. aureus pathogenicity in order to develop efficient treatment approaches against methicillin-resistant strains of the bacteriaOur previously discovered novel Staphylococcus aureus genes associated with virulence using the silkworm disease model (20, 21). S. aureus pathogenicity against

mice requires the identified genes [21]. The bulk of the most well-known Staphylococcus aureus virulence genes discovered in mammalian species are also required for the pathogen's lethal effects in the silkworms [22], making the silkworm an excellent animal model for finding the genes required for S. aureus pathogenicity. In this study, we employed a silkworm system for searching for genes required for S. aureus pathogenicity. We observed that rsmI & rsmH, that encode a 16S rRNA methyltransferase, are essential for both Staphylococcus aureus pathogenicity and resistance to oxidative stress... These adjustments are made during ribosome assembly and contribute to the harmony between precision and speed of translation (Siibak and Remme 2010) . Staphylococcus aureus has been shown to have a paralog of E. coli RlmI in the current study. Remarkably, we have discovered that this enzyme catalyzes the methylation process at position 7 of G2601 in vivo Nucleotide G2601 is found in 23S rRNA helix H90 Ribosomal messenger RNA the enzymes methyl contributes to the virulence of the bacteria Staphylococcus aureus., this methylation process is important. A. Notably, this location remains unchanged in E. Coli (G2574) This suggests a specific function in S. aureus. Because of the lack of methyl at location (C1989) of Staphylococcus aureus 23S's rRNA, which is analogous to E. coli (C1962) modified at location 5, RlmQ was the new name given to this methylase.,. The rlmQ-disruption mutant strain's phenotypic investigation showed a decreased cytotoxicity, a somewhat shorter doubling time in nutrient-poor illnesses and additionally Bahena-Ceron enhanced generation of biofilms. We will also talk about evolutionary aspects of in the following The biological effects of two very similar enzymes, RlmQ and RlmI, with different specificities for 23S methylation in different bacterial species.

RESULTS

Recent research has uncovered two novel virulence genes, SA0447 and SA1022, within Staphylococcus aureus. To investigate their role in pathogenicity, a study focused on conserved putative genes and created 73 deletion strains of S. aureus. The pathogenicity of these strains was then evaluated using a silkworm infection model.

The findings revealed that knockout strains lacking SA0447 or SA1022 exhibited reduced lethality towards silkworms, as indicated by decreased mortality rates and a significantly higher LD50 compared to the original strain. Interestingly, reintroducing the SA0447 and SA1022 genes into their respective knockout strains restored their killing capacity, emphasizing the pivotal role of these genes in S. aureus pathogenicity.Moreover, 20 hours post-injection, the hemolymph of silkworms infected with the SA0447 and SA1022 knockout strains contained fewer viable bacterial cells compared to those infected with the parent strain, further highlighting the impact of these genes on the bacterium's pathogenic potential. In conclusion, the study's outcomes suggest a strong correlation between the presence of SA0447 and SA1022 genes and the pathogenicity of Staphylococcus aureus, shedding light on potential targets for future therapeutic interventions. The provided Table 1 details the silkworm-killing potential of various S. aureus strains, including ΔSA0447, ΔSA1022, and ΔSA0447/ΔSA1022, offering valuable insights into the differential impact of these genes on pathogenicity.

Genotype	LD_{50} (× 10^6 CFU)	LD_{50} ratio	*P*-value
Experiment 1			
Parent	3.0	1	
ΔSA0447 [Δ*rsmI*]	9.2	3.1	0.0045 (versus parent)
SA0447$^+$ [*rsmI*$^+$]	2.9	0.97	0.0001 (versus Δ*rsmI*)
Experiment 2			
Parent	2.3	1	
ΔSA1022 [Δ*rsmH*]	8.3	3.6	< 0.0001 (versus parent)
SA1022$^+$ [*rsmH*$^+$]	4.2	1.8	0.0050 (versus Δ*rsmH*)
Experiment 3			
Parent	3.9	1	
ΔSA0447/ΔSA1022 [Δ*rsmI*/Δ*rsmH*]	12	3.1	< 0.0001 (versus parent)
SA0447$^+$/SA1022$^+$ [*rsmI*$^+$/*rsmH*$^+$]	5.9	1.5	< 0.0001 (versus Δ*rsmI*/Δ*rsmH*)

In our investigation, we conducted an analysis of the growth curves in tryptic soy broth (TSB) for the strains ΔSA0447, ΔSA1022, and ΔSA0447/ΔSA1022 (refer to Fig. 1E). Notably, at three distinct temperatures, the ΔSA0447/ΔSA1022 and ΔSA1022 strains exhibited slightly prolonged TSB doubling times, as outlined in Table 2. Specifically, at 27°C, 37°C, and 43°C, the ΔSA1022 strain demonstrated 1.08-fold, 1.07-fold, and 1.04-fold longer doubling durations compared to the parent strain. Similarly, the ΔSA0447/ΔSA1022 strain displayed 1.14-fold, 1.11-fold, and 1.08-fold longer doubling durations at the same respective temperatures

Furthermore, we evaluated the growth of the bacteria ΔSA0447, ΔSA1022, and ΔSA0447/ΔSA1022 on tryptic soy agar (TSA) plates. It was observed that the colonies of ΔSA0447/ΔSA1022 and ΔSA1022 strains appeared relatively smaller in comparison to the parent strain (refer to Fig. 1F) .

These findings provide valuable insights into the growth characteristics of Staphylococcus aureus strains, shedding light on the potential impact of SA0447 and SA1022 genes on bacterial growth and development. The detailed data presented in Table 2 offers a comprehensive overview of the differences in doubling times at varying temperatures, providing a deeper understanding of the growth dynamics of these S. aureus strains.

The smaller size of the colonies observed in the ΔSA0447/ΔSA1022 and ΔSA1022 strains could be attributed to several factors related to the genetic modifications and the impact on bacterial growth and development. Some potential explanations for the smaller colonies include:

Reduced Growth Rate: The deletion of the SA0447 and SA1022 genes may have led to a reduced growth rate of the bacterial cells, resulting in smaller colonies on the agar plates.

Alteration in Metabolic Pathways: The absence of SA0447 and SA1022 genes could have disrupted essential metabolic pathways or regulatory mechanisms, affecting the overall growth and colony formation of the bacterial strains.

Cellular Stress Response: The genetic modifications may have induced stress responses within the bacterial cells, leading to alterations in cell division and colony formation.

Changes in Cell Wall Composition: The absence of specific genes could have influenced the composition and structure of the bacterial cell wall, impacting the formation and size of colonies on the agar plates .

Nutrient Utilization: The genetic modifications may have altered the ability of the bacteria to effectively utilize nutrients present in the agar medium, affecting their growth and colony size.

These factors collectively demonstrate the intricate relationship between genetic modifications, bacterial growth dynamics, and the resultant colony morphology, providing valuable insights into the impact of SA0447 and SA1022 genes on the growth characteristics of Staphylococcus aureus strains.

Table 2: Staphylococcus aureus strains ΔSA0447, ΔSA1022, and ΔSA0447/ΔSA1022 doubling times

Genotype	Doubling time (min)		
	27 °C	37 °C	42 °C
Parent	65.8 ± 3.8	30.1 ± 3.8	31.0 ± 2.0
ΔSA0447 [Δ*rsmI*]	65.9 ± 4.8	30.8 ± 2.8	30.9 ± 2.5
ΔSA1022 [Δ*rsmH*]	71.3 ± 3.6	32.3 ± 2.3	32.4 ± 2.1
ΔSA0447/ΔSA1022 [Δ*rsmI*/Δ*rsmH*]	75.1 ± 5.3	33.5 ± 2.9	33.5 ± 2.0

In the study, the ΔSA1022 strain of Staphylococcus aureus was subjected to RNase T1 or RNase A digestion, followed by examination using LC/MS. The results revealed intriguing findings related to the cleavage patterns and methylated nucleosides within the bacterial RNA .

For the parent strain of S. aureus, RNase T1 treatment produced a tetramer (m4) with the sequence CmCCGp, while RNase A treatment detected a trimer (Gm4 CmCp). These outcomes indicated that RNase T1 cleaved at the 3' terminus of guanosine, while RNase A cleaved at the 3' terminus of cytidine or uridine, with 2'-O-methylation of cytidine preventing cleavage by RNase A.

Furthermore, the nucleoside analysis uncovered that the dimethylated cytidine of S. aureus 16S rRNA shared the same mass and retention time as N4-, 2'-O-dimethylcytidine from E. coli 16S rRNA, suggesting that N4-, 2'-O-dimethylcytidine is the 1412 nucleoside of S. aureus 16S rRNA .

In the case of the SA0447 strain, RNase T1 treatment revealed a tetramer (m4 CCGp), while RNase A treatment identified a dimer (Gm4 Cp). Similarly, the ΔSA0447/ΔSA1022 strain exhibited distinct cleavage patterns, indicating the potential involvement of the SA0447 and SA1022 genes in the methylation process.

The study also delved into the contribution of S. aureus RsmI and RsmH methyltransferases to translation fidelity, as well as the comparison of S. aureus RlmQ and E. coli RlmI methyltransferases. Additionally, the crystal structure of SAV1081, a S. aureus methyltransferase, was determined, providing valuable insights into the structural characteristics of this enzyme.

In summary, the research shed light on the intricate mechanisms of RNA methylation in Staphylococcus aureus, uncovering the roles of specific genes and methyltransferases in the bacterial translation process. These findings have significant implications for understanding bacterial pathogenicity and the development of targeted interventions.

The presence of dimethylated cytidine in S. aureus 16S rRNA holds significant biological and functional implications for the bacterium. This modification plays a crucial role in various aspects of bacterial physiology and pathogenicity. Some of the key significance of dimethylated cytidine in S. aureus 16S rRNA includes:

Translation Fidelity: Dimethylation of specific cytidine residues within the 16S rRNA is known to contribute to the accuracy and fidelity of the bacterial translation process. This modification ensures the precise decoding of mRNA and the faithful synthesis of proteins, thereby influencing the overall efficiency and accuracy of protein production in S. aureus.

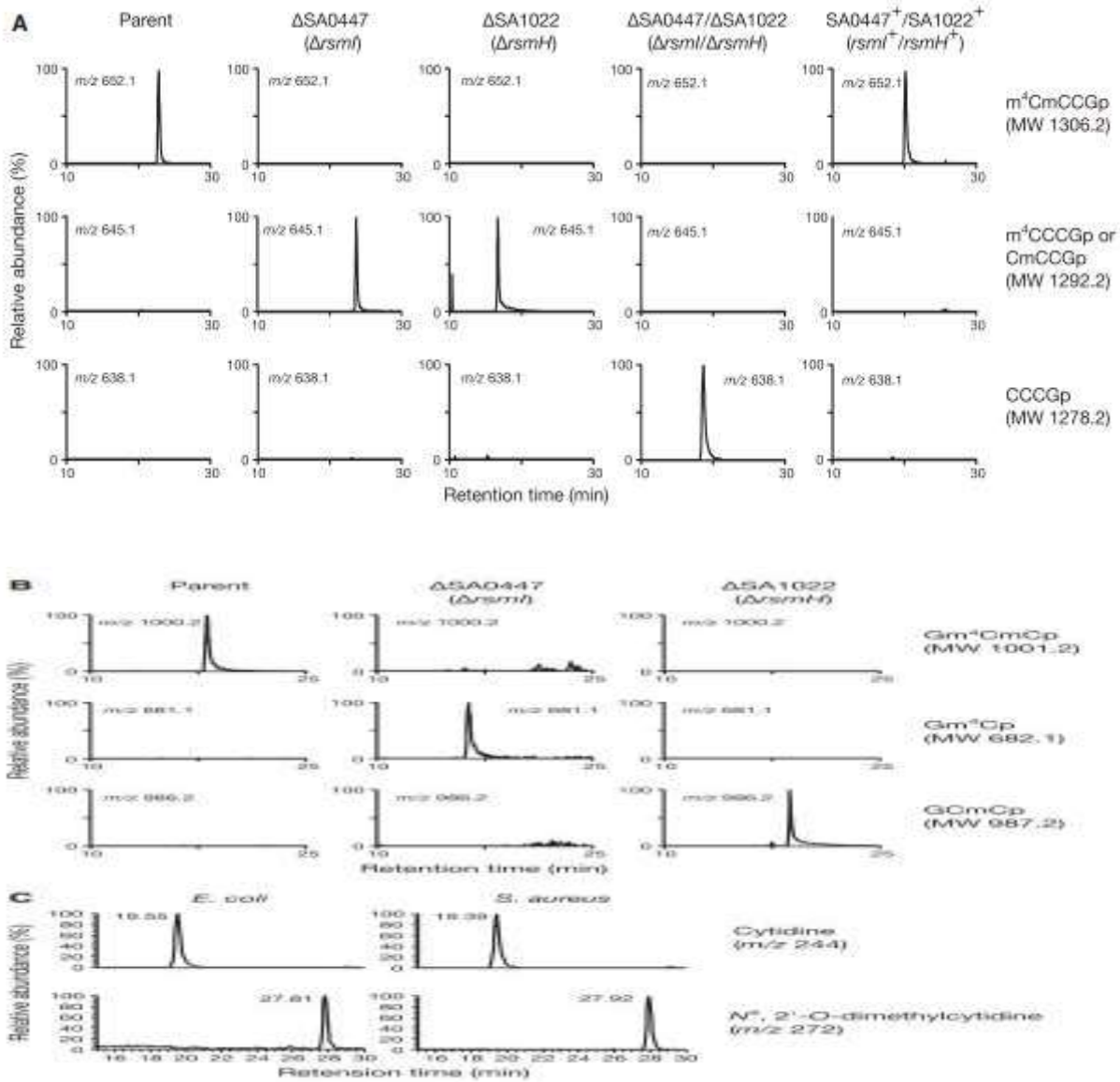
Ribosomal Function: The presence of dimethylated cytidine in the 16S rRNA is linked to the optimal functioning of the bacterial ribosome, which is essential for protein synthesis. This modification likely affects ribosomal stability, dynamics, and interaction with other components involved in translation .

Regulation of Gene Expression: Dimethylated cytidine may also play a role in regulating the expression of specific genes in S. aureus, influencing the bacterium's adaptation to different environmental conditions, stress responses, and virulence factor production.

Pathogenicity: The presence of dimethylated cytidine in S. aureus 16S rRNA may contribute to the bacterium's pathogenicity by influencing its ability to adapt to host environments, evade host immune responses, and promote infection.

Antibiotic Resistance: RNA modifications, including dimethylation, can impact the susceptibility of bacteria to certain antibiotics, potentially influencing the effectiveness of antibiotic treatments against S. aureus infections.

Overall, the significance of dimethylated cytidine in S. aureus 16S rRNA underscores its roles in fundamental biological processes, bacterial adaptation, and pathogenicity, making it a key target for further research into the development of novel therapeutic strategies and antimicrobial interventions.



Exploring Cytosine Methylation in S. aureus 16S rRNA and Its Influence on Translation Fidelity and Virulence

RNA Extraction and Analysis:

Total RNA was extracted from S. aureus parent strains, ΔSA0447 (ΔrsmI), ΔSA1022 (ΔrsmH), ΔSA0447/ΔSA1022 (ΔrsmI/ΔrsmH), and SA0447+ / SA1022+ (rsmI+ / rsmH+). A partial segment of the 16S rRNA (1388–1436) was isolated using RNase T1 and subsequently purified for LC/MS analysis. Additionally, the same partial segment was separated by RNase A, purified, and subjected to LC/MS analysis.

Nucleoside Analysis via LC/MS:

The partial 16S rRNA fragment from E. coli BW25113 or S. aureus RN4220 was enzymatically digested to nucleosides and analyzed using LC/MS. Ion-trap MS was utilized to identify N4, 20-O-dimethylcytidine (m/z 272) and cytidine (m/z 244).

Investigation of Translation Fidelity:

By employing reporter plasmids, the read-through frequency of UGA and frameshift frequencies were evaluated. The ΔrsmI strain exhibited a slightly higher read-through frequency of UGA compared to the parent strain. Conversely, the ΔrsmH and ΔrsmI/ΔrsmH strains displayed marginally lower read-through and frameshift frequencies compared to the parent strain, indicating a potential impact on translation fidelity.

Contribution of Ribosomal RNA Methyltransferases to S. aureus Virulence:

It was observed that ribosomal RNA methyltransferases (RsmI and RsmH) play a role in the virulence of Staphylococcus aureus, underscoring their significance in the pathogenicity of the bacterium.

This comprehensive investigation delved into the intricate mechanisms of cytosine methylation in S. aureus 16S rRNA and its implications for translation fidelity and virulence, offering valuable insights into the molecular processes underlying the pathogenicity and biological functions of S. aureus.

DISCUSSION

This study revealed the impact of the rsmI and rsmH genes, responsible for encoding 16S rRNA methyltransferase, on the pathogenicity of S. aureus. Interestingly, it was found that these genes play a crucial role in maintaining translation fidelity and conferring resistance to oxidative stress conditions. The presence of RsmI and RsmH was shown to support the preservation of translation accuracy and enhance the bacterium's ability to resist oxidative stress, ultimately serving as a vital defense mechanism against radical damage.

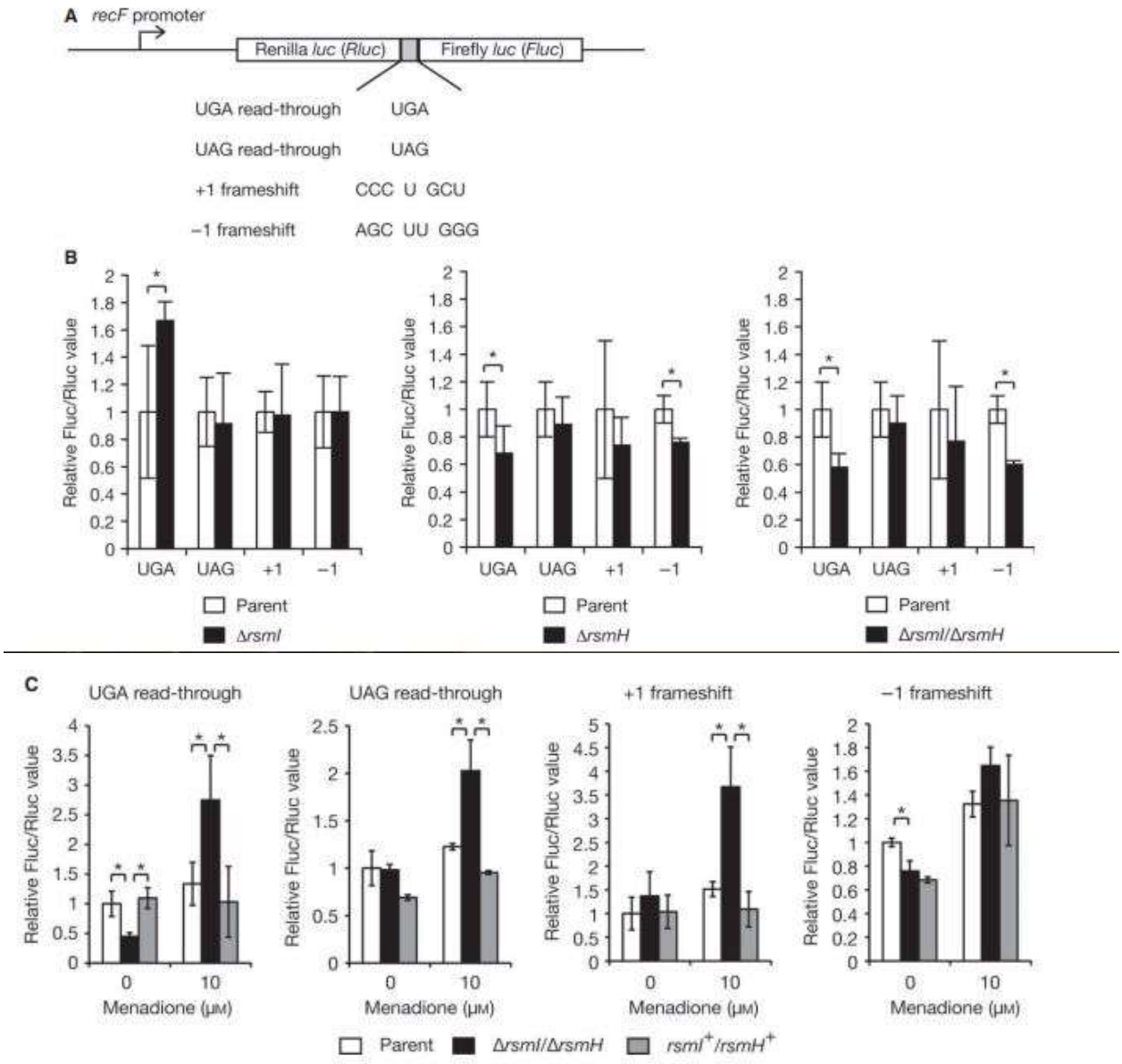


Figure 3: Influence of rsmI and rsmH Knockouts on Translation Fidelity and Oxidative Stress Resistance

Experimental Set-up and Analysis:

Representative plasmids containing Renilla luciferase (Rluc), Firefly luciferase (Fluc), stop codons (UGA or UAG), or frameshifts were utilized to assess translation fidelity in S. aureus parent strain, ΔrsmI strain, ΔrsmH strain, and ΔrsmI/ΔrsmH strain under normal culturing conditions and oxidative stress. The activities of Rluc and Fluc in S. aureus cell lysates were measured, and the Fluc/Rluc values were compared to those of the parent strain.

Impact of rsmI and rsmH Knockouts:

The results demonstrated the effects of rsmI and rsmH knockouts on translation fidelity and oxidative stress resistance. Specifically, the ΔrsmI/ΔrsmH strain exhibited a similar trend in affecting translation fidelity as the ΔrsmH strain, indicating the significant role of RsmH's N4-methylation of cytidine in maintaining translation accuracy. Additionally, under standard culture conditions, a radical scavenger mitigated the enhanced doubling time of the ΔrsmI/ΔrsmH strain, suggesting a link between oxidative stress response and the absence of methylations.

Correlation with Previous Findings:

These observations align with similar trends observed in E. coli, emphasizing the conserved impact of these methylations on translation fidelity. The absence of one of the unique methylations of C1412 was speculated to induce distinct alterations in ribosome structure, potentially explaining the divergent effects observed.

Virulence Implications:

Furthermore, the study revealed that while a radical scavenger counteracted the enhanced doubling time of the ΔrsmI/ΔrsmH strain, it did not mitigate the weakened virulence observed in silkworms infected by this strain, highlighting complex implications for bacterial pathogenicity.

This comprehensive analysis provides valuable insights into the interplay between rsmI and rsmH knockouts, translation fidelity, oxidative stress response, and virulence in S. aureus.

Hence, the presence of RsmI and RsmH in S. aureus contributes to the bacterium's virulence by bolstering the host animals' resilience to oxidative stress. Interestingly, the ΔrsmI/ΔrsmH strain exhibited reduced translation fidelity (UGA read-through, UAG-read-through, and +1 frameshift) in the presence of oxidative stress, while displaying a marginal enhancement in UGA read-through fidelity under normal culturing conditions. These findings suggest that ribosomes containing non-methylated C1412 of 16S rRNA adopt unique configurations under both normal and oxidative stress conditions, leading to varying impacts on translation fidelity.

Furthermore, it is inferred that the dimethylated C1412 of 16S rRNA, mediated by S. aureus, serves to safeguard the ribosome structure against oxidative damage. Notably, a recent transcriptome study coupled with RNA sequencing in E. coli revealed that heat stress and oxidative stress influence the expression of RsmH and RsmI, respectively, highlighting the role of these methyltransferases in stress response.

Moreover, our investigation unveiled that neutrophils' azurophilic granule proteins, containing oxidative radicals that produce myeloperoxidase, upregulate the expression of rsmH in S. aureus. This insight, obtained from the S. aureus transcriptome database, sheds light on the intricate interplay between host immune responses and bacterial stress adaptation. Additionally, oxidative stress was found to impact the expression of various rRNA methyltransferases, with the rlmG gene, responsible for N2-methylation of G1835 of 23S rRNA, being significantly affected. These findings underscore the ribosome's capacity for conformational resilience against diverse stressors, including oxidative stress, due to the chemical modifications of rRNA.

Considering the location of dimethylated C1412 in the decoding center of the 30S ribosomal subunit and the influence of endoribonuclease MazF on the 30-region of the 16S rRNA, our study provides valuable insights into the intricate mechanisms underlying translation fidelity, stress response, and virulence in S. aureus.

Ribosomes containing non-methylated C1412 of 16S rRNA are implicated in impacting translation fidelity under both normal and oxidative stress circumstances. The absence of methylation at C1412 has been observed to result in unique configurations of ribosomes, leading to distinct effects on translation fidelity. Specifically, under normal culturing conditions, ribosomes lacking this methylation marginally enhance translation fidelity, particularly in the context of UGA read-through. However, under oxidative stress circumstances, the same ribosomes exhibit reduced translation fidelity, evident in the decreased UGA read-through, UAG-read-through, and +1 frameshift. These observations highlight the intricate interplay between ribosomal modifications, stress conditions, and the fidelity of protein synthesis.

The methyl alteration at C1412 of 16S rRNA may also impact the translation of specific mRNA-encoding proteins involved in oxidative stress resistance. This modification is known to play a role in triggering the translation of particular proteins associated with stress resistance. Further research is

essential to unravel the molecular pathways linking rRNA methylation to the resistance to oxidative stress, shedding light on the intricate mechanisms underlying bacterial adaptation to hostile host environments.

Oxidative stress serves as a defense strategy employed by host animals to combat harmful pathogens. Upon engulfing bacteria, neutrophils and macrophages release free radicals generated by NADPH oxidase or superoxide dismutase, posing a challenge to the survival of pathogenic bacteria. Consequently, bacterial enzymes are essential for defending against oxidative stresses to maintain virulence. For instance, studies have shown that S. aureus knockout strains with defective catalase or superoxide dismutase exhibit reduced pathogenicity in mice, underscoring the significance of oxidative stress resistance in bacterial virulence.

The disruption of the translation process of the bacterium in host mammals underscores the critical role of rRNA modifications in bacterial adaptation to the host environment. Understanding the physiological importance of rRNA alteration in pathogenic bacteria will provide valuable insights into the chemical changes of rRNA and their implications for bacterial survival and virulence.

MATERIALS AND METHODS

DNA manipulation

Experimental Techniques and Bacterial Manipulation

The experimental procedures involved in the study encompassed a series of molecular and microbiological techniques. These methodologies included Southern blotting, PCR, plasmid extraction, and E. coli transformation, which were conducted as previously described. Additionally, electroporation was employed to induce genetic changes in S. aureus, while phage 80a was utilized for transduction.

Plasmid Construction and Genetic Modification

DNA fragments comprising specific regions of interest, such as the upstream sequences of SA0445, SA0445, SA0446, SA0447, SA1021, and SA1022, were amplified through PCR and subsequently integrated into plasmids pHY300E and pKE516 to generate targeted constructs. Notably, the creation of plasmids prsmI and prsmH harboring complete rsmI and rsmH genes involved the amplification and self-ligation of specific DNA fragments, as outlined in the experimental procedure.

In vivo Experiments and Drug Sensitivity Evaluation

In vivo experiments involving the administration of N-acetylcysteine (NAC) to silkworms were carried out by injecting saline or NAC solutions, followed by the assessment of LD50 values. Furthermore, the drug sensitivity of S. aureus strains was evaluated by culturing them on plates containing menadione, paraquat, and hydrogen peroxide, allowing for the determination of their susceptibility to these substances.

These diverse experimental approaches provided valuable insights into genetic manipulation, in vivo experimentation, and drug sensitivity assessment in the context of bacterial pathogenicity and stress response.

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