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Identification of four genes responsible for antimicrobial resistance of MEL-B against *S. aureus*

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ABSTRACT

There is increasing interest in the antimicrobial activity of mannosylerythritol lipids-B (MEL-B) against Gram-positive bacteria such as *Staphylococcus aureus* (*S. aureus*). However, the specific molecules involved in MEL-B's antimicrobial action against *S. aureus* have not been identified. This study utilized the Nebraska transposon mutant library (NTML), which contains 1920 mutants, each lacking three-quarters of the genes found in *S. aureus*. The NTML was screened to identify mutants resistant to MEL-B. Four mutants (Accession Number: SAUSA300_0904, SAUSA300_0752, SAUSA300_0387, and SAUSA300_2311) largely unaffected by incubation with MEL-B, indicating MEL-B resistance. Despite the strong binding of MEL-B to these mutants, the four molecules encoded by the deleted genes (*yjbI, clpP, pbuX, or brpS*) in each mutant were not directly recognized by MEL-B. Given that these molecules are not localized on the outer surface of *S. aureus* and that the antibacterial activity of MEL-B against *S. aureus* is facilitated by the effective transfer of two antibacterial fatty acids (caprylic acid and myristoleic acid) to *S. aureus* via ME, the deletion of each of the four molecules may alter the peptidoglycan structure, potentially inhibiting the effective transfer of these antimicrobial fatty acids into *S. aureus*.

1. Introduction

Mannosylerythritol lipids (MELs), produced by culturing various fungi, are classified as glycolipid bio-surfactants [1,2]. MELs exhibit amphiphilic properties attributed to their structure, which comprises a hydrophilic sugar and two hydrophobic fatty acids [3,4]. Their low toxicity and exceptional moisture retention qualities have garnered significant interest across various fields, including the food industry and skincare [5,6]. Moreover, MELs have emerged as a potential antibacterial agent against Gram-positive bacteria, presenting a promising alternative to antibiotics and mitigating the risk of antibiotic-resistant bacterial strains [7,8].

Staphylococcus aureus (*S. aureus*), a harmful Gram-positive bacterium, poses a threat to both human and animal health by producing various toxins that enhance pathogenicity, such as panton-valentine leukocidin, toxic shock syndrome toxin-1, and staphylococcal enterotoxin [9,10]. In addition to their direct impact on health, these toxins contribute to environmental disturbances [11]. The rise of antimicrobial

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resistance (AMR) in *S. aureus* increases the risk of human mortality [12]. Therefore, adhering to the "one-health" principle of infection prevention, there is a pressing need to devise alternative strategies to antibiotics that can impede the growth of pathogenic bacteria without fostering AMR [13].

Several studies have explored the potential of MEL as an alternative to antimicrobial agents, with a particular focus on MEL-B. Our laboratory and others have extensively investigated the antimicrobial activity of MEL-B against Gram-positive bacteria, including *S. aureus* [14–16]. The effectiveness of MEL-B against *S. aureus* has been linked to the length of fatty acids that are efficiently transported to *S. aureus* via ME, a component of MEL-B [15,16]. However, no studies have been conducted to identify molecules expressed by *S. aureus* that are not only targeted by MEL-B but also play a role in inhibiting bacterial growth.

In this study, we utilized the Nebraska transposon mutant library (NTML) to identify mutants resistant to MEL-B. The goal was to identify genes (or the molecules they encode) that play a role in the antimicrobial activity of MEL-B against *S. aureus*.

2. Methods

2.1. Bacterial strains and culture conditions

The NTML, comprising 1920 strains [17], was utilized to screen *S. aureus* strains resistant to MEL-B. The NTML was created by inserting a transposon carrying the erythromycin resistance gene into a wild-type *S. aureus* JE2 [17]. Both JE2 and NTML were cultured in Tryptone soya (Nissui) with or without 5 μg/ml of erythromycin. The transposon's locus was confirmed via inverse PCR, as per a previous study [17], with modifications for selecting MEL-B-resistant mutants. Specifically, strains were disrupted using 100 μg/ml of lysostaphin to elute genomic DNA, which was then extracted using an automated DNA extractor, PI-480α (Kurabo). Next, 4 μg of bacterial DNA was digested using 20 U of AciI (New England Biolabs) at 37 °C for 1 h. The fragments were ligated using a DNA Ligation Kit (Takara Bio). The cyclic DNA was subjected to PCR using MartnF primer (5′-TTTATGGTACCATTTCATTTTCCTGCTTTTTC-3′) and Martn-ermR primer (5′-AAACTGATTTTTAGTAAACAGTTGA CGATATTC-3′) [18]. The amplicons were directly sequenced to identify the locus of transposon insertion on the genomic DNA using a 3730xl DNA analyzer (Applied Biosystems).

2.2. MEL-B, NBD-labeled MEL-B, and fatty acids

Pseudozyma tsukubaensis (NBRC1940) was cultured at 25 °C with 10 % (v/v) olive oil, 0.1 % (w/v) yeast extract, 4 % (w/v) glucose, 0.3 % (w/v) $NaNO_3$, 0.03 % (w/v) $MgSO_4{\cdot}7H_2O$, and 0.03 % (w/v) KH_2PO_4. MEL-B was then purified from the culture supernatant using high-performance liquid chromatography. To synthesize fluorescent-labeled MEL-B, MEL-B was reacted with 4-N-chloroformylmethyl-N-methyl (amino)-7-nitro-2,1,3-benzoxadiazole (NBD) for 60 min in dry acetone. This was used to detect the binding of MEL-B to *S. aureus* mutants selected from the NTML. Caprylic acid (C8:0) (Sigma) and myristoleic acid (C14:1) (Sigma) were used at concentrations of 200 and 2000 μg/ml, and 10 and 100 μg/ml, respectively, to investigate their antimicrobial activity against *S. aureus* mutants.

2.3. High-throughput screening

Wild-type JE2 and *S. aureus* mutants selected from the NTML were cultured in 500 μl of fresh TS broth in 96 deep-well plates. This was done in the presence or absence of 100 μg/ml of MEL-B at 1000 rpm and 37 °C for 3.5 h. After incubation, the culture medium was diluted 1:50 with 0.85 % NaCl. The number of bacteria was estimated by measuring the amount of adenosine triphosphate (ATP) using a CheckLite HS set (Kikkoman). The survival rates were calculated using the values obtained from 1920 NTML strains and wild-type JE2 as follows: Survival rate = (the amount of ATP from the bacterial cultured with MEL-B)/(the amount of ATP from the bacterial cultured without MEL-B). Nongrowth strains were excluded from the analysis by monitoring ATP levels that were less than 10,000 RLU under normal culture conditions. MEL-B-resistant strains (top 5 in survival) selected by screening, along with wild-type JE2, were cultured in 5 ml of fresh TS broth. This was done in the presence or absence of 100 μg/ml of MEL-B at 37 °C in a rotary shaker (120 rpm). The effect of survival inhibition by MEL-B was evaluated over time by measuring the optical density at 600 nm (OD_{600}).

2.4. Colony-forming units (CFU) count

To verify the reduced susceptibility of selected MEL-B resistant strains to MEL-B, the number of bacteria in the logarithmic growth phase was counted. This was done during culture with or without 100 μg/ml of MEL-B by obtaining CFU. Additionally, to examine the antimicrobial activity of caprylic acid and myristoleic acid against selected strains from the NTML, the numbers of bacteria were cultured. This was done during culture with either caprylic acid (at concentrations of 200 and 2000 μg/ml) or myristoleic acid (at concentrations of 10 and 100 μg/ml) by obtaining CFU.

2.5. Binding analysis

For the binding analysis, wild-type JE2 and NTML strains were cultured for 8 h with or without MEL-B. The bacteria were then treated with various concentrations of NBD-MEL-B for 30 min. Following this,

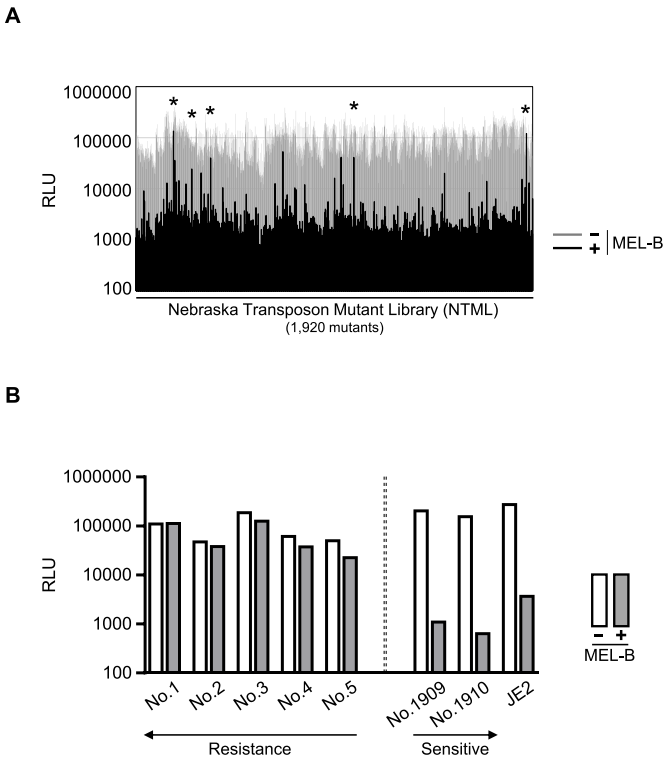


Fig. 1. Identification of resistant mutants in MEL-B using the Nebraska transposon mutant library (NTML). (**A**) A total of 1920 mutants from the NTML and the wild-type JE2 were cultured in the presence or absence of MEL-B. The growth of these cultures was monitored by measuring intracellular ATP levels, facilitating the selection of mutants exhibiting resistance to MEL-B. The top five resistant mutants are highlighted. (**B**) The intracellular ATP levels of the top five and least resistant mutants, along with the wild-type JE2, cultured with or without MEL-B, are presented. The mutants are identified by their respective numbers: No.1: SAUSA300_0904, No. 2: SAUSA300_0752, No. 3: SAUSA300_0387, No. 4: SAUSA300_2311, and No. 5: SAUSA300_2357, No.1909: SAUSA300_2024, No.1910: SAUSA300_0585.

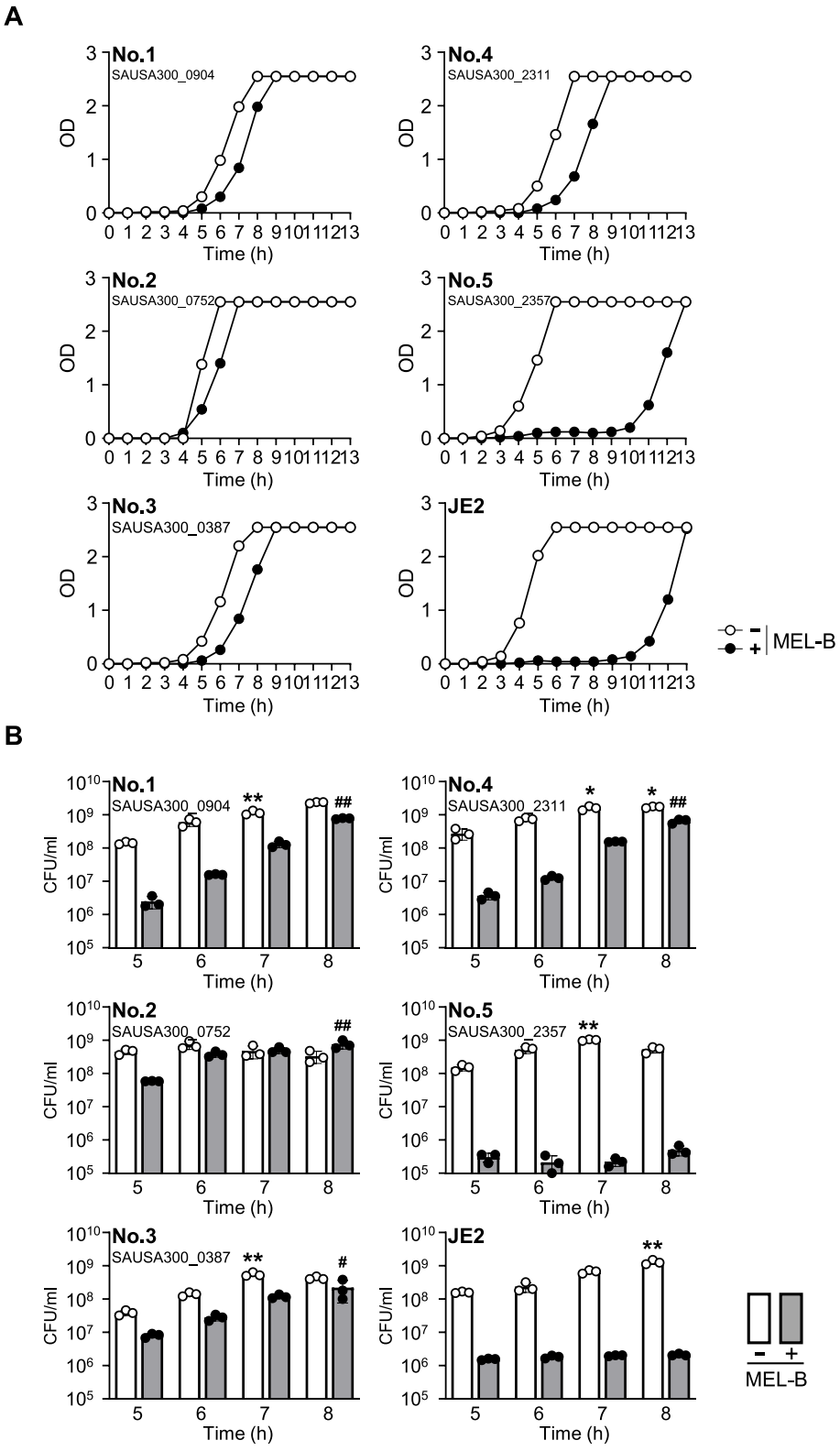
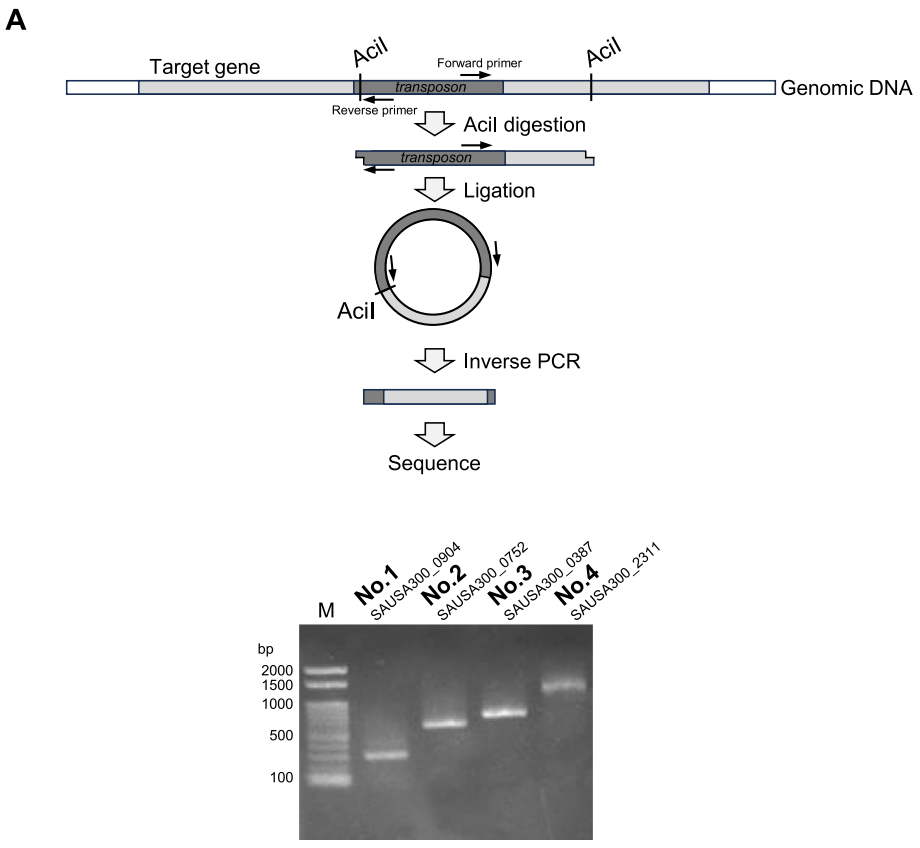


Fig. 2. Verification of initial screening results through turbidity measurement and colony-forming unit estimation. (**A**) The top five mutants selected and the wild-type JE2 were cultured with MEL-B. The turbidity of the medium, represented by optical density values, was measured. (**B**) The bacterial count of the selected mutants and JE2 during the logarithmic phase was estimated using colony-forming units (CFU). Statistical significance is indicated as follows: $^{*\ \text{or}\ \#}p < 0.05$, $^{**\ \text{or}\ \#\#}p < 0.01$ (compared to the 5 h mark). The data shown are representative of three independent experiments.



B

No	Accession Number	Predicted size	Gene
1	SAUSA300_0904	295	*yjbI*
2	SAUSA300_0752	634	*clpP*
3	SAUSA300_0387	768	*pbuX*
4	SAUSA300_2311	1308	*brpS*

Fig. 3. Determination of genes contributing to *Staphylococcus aureus* resistance to MEL-B. (**A**) The location of transposon insertion within the genome of the selected mutants was verified using inverse PCR. Each inverse PCR resulted in the detection of a single band. (**B**) Gene deleted in each mutant was identified through sequencing of the PCR amplicon.

the cells were washed twice with 0.85 % NaCl and fixed in 4 % (w/v) paraformaldehyde (Nacalai Tesque) for 1 h at 4 °C. A flow cytometric analysis was performed using an Accuri C6 (DB) to analyze the binding ability of MEL-B to wild-type JE2 and NTML strains.

2.6. Scanning electron microscopy analysis

The impact of gene disruption on cellular structure was investigated using scanning electron microscopy (SEM). Both wild-type JE2 and selected strains from the NTML were cultured for 24 h, with or without the presence of 100 μg/ml of MEL-B. Following this, the cells were washed twice with distilled water and then fixed in a 2.5 % (v/v) glutaraldehyde solution (Polysciences) for 1 h at 4 °C. After another round of washing, the cells were attached to clean glass slides that had been coated with a layer of platinum and palladium. Images of these cells were then captured using a Hitachi SEM SU8000 at an operating voltage of 3.0 kV.

2.7. Statistics

The statistical analyses were performed employing a one-way analysis of variance (ANOVA) in conjunction with the Kruskal–Wallis test. All these computations were executed using Prism 7 (GraphPad).

3. Results

3.1. Identification of four mutants resistant to MEL-B

To pinpoint the genes that contribute to the antibacterial activity of MEL-B against *S. aureus*, a comprehensive screening was carried out using the NTML. This library has transposons inserted at the locus of each of the 1920 genes. Each mutant was cultured either in the presence or absence of 100 μg/ml MEL-B, and their growth was assessed by measuring the intracellular ATP in viable cells (Fig. 1A and Table S1). Out of the 1920 mutants, 10 were not considered for this study due to their limited proliferative potential (Fig. 1A and Table S1). The growth of most mutants was inhibited when treated with MEL-B, similar to the wild-type JE2. However, five mutants (namely, SAUSA300_0904,

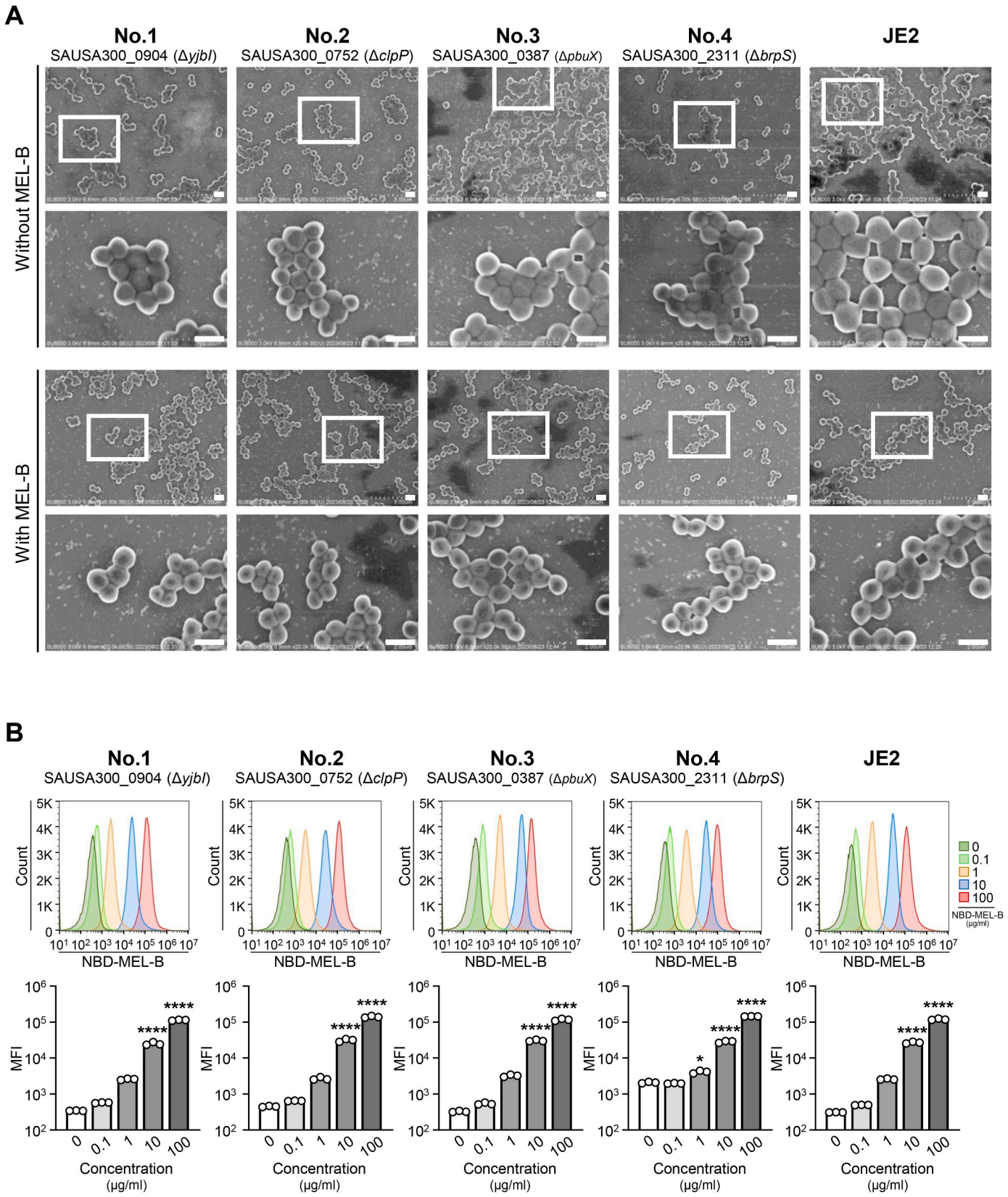


Fig. 4. Analysis of mutant surface structure and MEL-B Binding. (**A**) The surface structures of the selected mutants and JE2, cultured with or without MEL-B, were examined using scanning microscopy. The scale bar represents 1 μm. (**B**) The binding of MEL-B to the selected mutants and JE2 was validated using flow cytometry, following incubation with varying concentrations of NBD-MEL-B. The data presented are representative of three independent experiments. $^{*}p < 0.05$, $^{****}p < 0.0001$ (compared to 0 μg/ml).

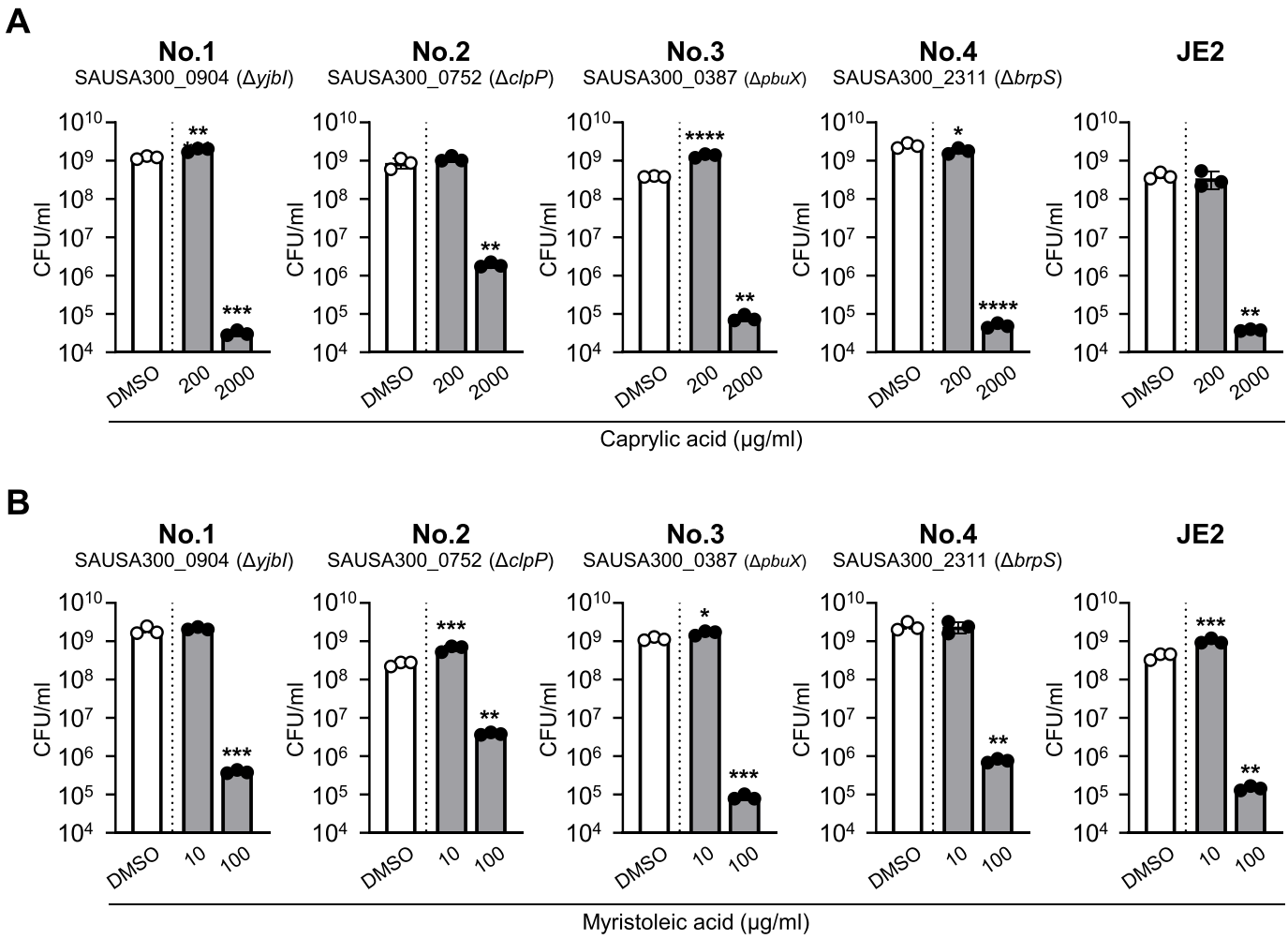


Fig. 5. Evaluation of mutants and JE2 susceptibility to antimicrobial fatty acids. The selected mutants and JE2 were cultured in the presence of either caprylic acid (**A**) or myristoleic acid (**B**) at concentrations of 200 and 2000 μg/ml or 10 and 100 μg/ml, respectively. The data shown are representative of three independent experiments. Statistical significance is denoted as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ (compared to 1 % DMSO).

SAUSA300_0752, SAUSA300_0387, SAUSA300_2311, and SAUSA300_2357) exhibited significant growth in the presence of MEL-B (Fig. 1B and Table S1). The resistance to MEL-B exhibited by these five mutants, identified in the initial screening, was further validated over time by measuring the turbidity of the culture medium. In line with the initial screening, the top four mutants based on the survival rate upon addition of MEL-B demonstrated adequate growth irrespective of the presence of MEL-B in the culture (Fig. 2A). Conversely, the fifth mutant did not exhibit such distinct characteristics when cultured with MEL-B (Fig. 2A). This observation was further corroborated by obtaining CFU during the logarithmic growth phase of the top five mutants and JE2 (Fig. 2B). Consequently, the fifth mutant (SAUSA300_2357) was not included in further analyses to characterize the MEL-B-resistant mutants. Given that the gene deleted in each mutant was not fully available in the NTML database, the loci of the transposon insertion in the four selected mutants were confirmed by inverse PCR. This was done using primers specific for the transposon and AciI-digested genomic DNA extracted from each mutant (Fig. 3A). The single band obtained from each inverse PCR was identified by sequencing as *yjbI*, *clpP*, *pbuX*, and *brpS* (Fig. 3B).

3.2. Characteristics of the four mutants as MEL-B-resistant strains

The surface structure of the four selected mutants was analyzed and compared with that of JE2 to understand their morphological characteristics. This analysis was conducted using SEM. The four mutants exhibited grape-like clusters, and their characteristics showed no discernible differences when compared with JE2 (Fig. 4A). Moreover, no structural alterations were observed when the four mutants and JE2 were cultured with MEL-B (Fig. 4A). To investigate the binding of MEL-B to the mutants, a binding assay was executed using NBD-labeled MEL-B, as per our previous study [16]. The four mutants were incubated with MEL-B at various concentrations (0, 0.1, 1, 10, and 100 μg/ml). The results showed that MEL-B binds to the mutants in a dose-dependent manner, similar to JE2 (Fig. 4B). These findings suggest that the resistance of the four mutants to MEL-B cannot be solely attributed to the structural features of the bacterial surface or the binding properties of MEL-B to the mutants.

3.3. Susceptibility of the four mutants to fatty acids

The antibacterial activity of MEL-B against *S. aureus* is attributed to the effective delivery of antimicrobial fatty acids into the bacteria via hydrophilic ME [16]. To further investigate this, the susceptibility of the four selected mutants to caprylic acid and myristoleic acid, which are components of MEL-B, was investigated. Specifically, the four selected mutants and JE2 were cultured in the presence of either caprylic acid (200 or 2000 μg/ml) or myristoleic acid (10 and 100 μg/ml). Similar to JE2, the growth of the four mutants was inhibited when they were cultured in the presence of high concentrations of caprylic acid (Fig. 5A)

or myristoleic acid (Fig. 5B). These results indicate that the resistance of the four mutants to MEL-B may be due to the inferior ME-mediated delivery of antimicrobial fatty acids into the four mutants, unlike JE2.

4. Discussion

In our prior research, we demonstrated that the antimicrobial activity of MEL-B, which comprises two types of hydrophobic fatty acids (mostly, caprylic and myristoleic acids) and hydrophilic ME, against *S. aureus* can be attributed to the effective delivery of antimicrobial fatty acids into the bacteria via ME [6,16]. The aim of this study was to verify the existence (or nonexistence) of specific molecules associated with MEL-B (presumably ME) on the outer surface of *S. aureus*. To achieve this, we utilized the NTML to identify MEL-B-resistant bacteria that lack such molecules specifically targeted by MEL-B (or ME). High-throughput screening was successfully conducted by measuring intracellular ATP in NTML cultured with or without MEL-B. Initially, five mutants were selected, and eventually, four mutants were identified as MEL-B-resistant mutants. The genes absent in these four mutants were *yjbI, clpP, pbuX, and brpS,* and the molecules encoded by these genes were localized either at the cell membrane or within the intracellular region (but not on the outer surface) [19–21]. While cell wall-associated proteins, which are linked to peptidoglycan, were suspected to be potential targets of MEL-B, our screening using NTML likely refuted the specific binding of MEL-B to such molecules. Moreover, as observed in JE2, MEL-B is bound to the four mutants irrespective of their resistance to MEL-B. It's worth noting that NTML can be used to determine the involvement of 1920 genes, which constitute three-fourths of the total number of genes in *S. aureus*. Therefore, it might still be plausible to hypothesize that the molecules targeted by MEL-B, particularly at the outer surface, are present due to the absence of mutants lacking the remaining one-quarter of the genes in NTML. However, it should be highlighted that the mutant lacking *srta*, which encodes Sortase A, an enzyme involved in the cell-wall-sorting reaction [22], is susceptible to MEL-B. Since Sortase A is involved in the synthesis of most cell wall-associated proteins, it is probable that the reaction of MEL-B to the outer surface of *S. aureus* is not specific to identified proteins.

Based on the findings of this and previous studies, we propose a hypothesis for the observed resistance of four mutants to MEL-B. We suggest that MEL-B, beyond just caprylic and myristoleic acids, may not be efficiently transported into the bacteria following its binding to the bacterial surface. Gram-positive bacteria, such as *S. aureus*, are characterized by a dense peptidoglycan mesh composed of two types of sugars, N-acetylglucosamine and N-acetylmuramic acid, interconnected by tetrapeptides [23]. We hypothesize that the reduced susceptibility of the four mutants to MEL-B could be attributed to an increased complexity of peptidoglycans resulting from the deletion of specific genes (namely, *yjbI, clpP, pbuX,* and *brpS*). This could lead to MEL-B becoming trapped within the abnormal cell wall structure. Despite SEM analysis not revealing any differences between the four mutants and JE2, several studies have established a link between the identified genes and the cell wall of *S. aureus*. For instance, *yjbI*, which encodes truncated hemoglobin, when disrupted, induces significant alterations in the cell wall composition of *S. aureus* [24]. Similarly, the destruction of *clpP*, which encodes a casein-degrading protease, promotes the formation of highly cross-linked muropeptides on the cell wall of *S. aureus* [25]. However, the roles of *pbux* and *brpS* in peptidoglycan synthesis remain unreported, necessitating further investigation to elucidate the collective involvement of the four molecules encoded by these genes in *S. aureus*'s resistance to MEL-B.

In conclusion, our study has successfully identified four molecules that, while not being direct targets of MEL-B, could potentially influence the permeability of the peptidoglycan layer to MEL-B. This research provides valuable insights into the molecular mechanisms underlying the antimicrobial action of MEL-B against *S. aureus*.

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CRediT authorship contribution statement

Shinya Yamauchi: Conceptualization, Data curation, Formal analysis, Writing – original draft. **So Shimoda:** Data curation. **Akio Kawahara:** Resources. **Tomohiro Sugahara:** Resources. **Shuhei Yamamoto:** Resources. **Masao Kitabayashi:** Resources. **Atsushi Sogabe:** Resources. **Christine A. Jansen:** Writing – review & editing. **Ryuta Tobe:** Writing – review & editing. **Ryota Hirakawa:** Data curation. **Jahidul Islam:** Data curation. **Mutsumi Furukawa:** Data curation. **Hiroshi Yoneyama:** Conceptualization, Writing – original draft. **Tomonori Nochi:** Conceptualization, Writing – original draft.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2024.149566.

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