An alternative nisin A resistance mechanism affects virulence in *Staphylococcus aureus*

running title: High nisin A resistance in *Staphylococcus aureus*

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Abstract

Nisin A is a bacteriocin produced by *Lactococcus lactis* and is widely used as a food preservative. *Staphylococcus aureus* has the BraRS-VraDE system that provides resistance against low concentrations of nisin A. BraRS is a two-component system that induces the expression of the ABC transporter VraDE. Previously, we isolated a highly nisin A-resistant strain with increased VraDE expression due to a mutation in *braRS*. In this study, we isolated *S. aureus* MW2 mutants with BraRS-VraDE-independent nisin A resistance. These mutants, designated SAN2 (*S. aureus* nisin resistant) and SAN469, had a mutation in *pmtR*, which encodes a transcriptional regulator responsible for the expression of the *pmtA-D* operon. As a result, these mutants exhibited increased expression of PmtA-D, a transporter responsible for the export of phenol soluble modulin (PSM). Characterization of the mutants revealed that they have decreased susceptibility to human β defensin-3 (hBD3) and LL37, which are innate immune factors. Additionally, these mutants showed higher hemolytic activity than the original MW2 strain. Furthermore, in a mouse bacteremia model, the SAN2 strain exhibited a lower survival rate than the original MW2 strain.

These results indicate that the increased expression of *pmtA-D* due to *pmtR* mutation is an alternative nisin A resistance mechanism that also affects virulence in *S. aureus*.

Importance

Recently, the emergence of antibiotic-resistant bacteria has resulted in serious problems for chemotherapy. In addition, many antibacterial agents, such as disinfectants and food additives, are widely used. Therefore, there is a possibility that bacteria are exposing many antibacterial agents and becoming resistant to some antibacterial agents. In this study, we investigated whether *S. aureus* can become resistant to nisin A, one of the bacteriocins applied as a food additive. We isolated a highly nisin A-resistant strain designated SAN2 that displayed increased expression of Pmt proteins, which are involved in the secretion of virulence factors called PSMs. This strain also showed decreased susceptibility to human antimicrobial peptides and increased hemolytic activity. In addition, SAN2 showed increased lethal activity in a mouse bacteremia model. Our study provides new insights that the acquisition of resistance against food preservatives may modulate virulence in *S. aureus*, suggesting that we need to pay more attention to the use of food preservatives together with antibiotics.

Introduction

Staphylococcus aureus is a commensal bacterium in humans; it generally localizes in the nasal cavity, skin and intestine and sometimes causes opportunistic infections, such as suppurative diseases, pneumonia and sepsis (1-3). Additionally, *S. aureus* causes food-borne poisoning because this organism produces several heat-stable enterotoxins (2). To cure *S. aureus* infections, antibacterial agents are generally administered. However, the emergence of methicillin-resistant *S. aureus* (MRSA) sometimes causes difficulties in chemotherapeutic treatment. Glycopeptides such as vancomycin and teicoplanin are now used for MRSA infection, although vancomycin-intermediate and/or vancomycin-resistant bacteria have been reported (4-6). In addition to antibiotics, other antibacterial agents, such as disinfectants and food preservatives, are widely used. Qac genes in *S. aureus* were identified as resistance factors for quaternary ammonium compounds, such as chlorhexidine and mupirocin (7, 8). Therefore, *S. aureus* shows resistance not only to several antibiotics but also to other antibacterial agents.

Nisin A is a bacteriocin produced by *Lactococcus lactis* (9). Nisin A belongs to the lantibiotics, which are antimicrobial peptides containing unusual amino acids, lanthionines (10, 11). The target of nisin A is lipid II, which is involved in cell wall biosynthesis (12, 13). The binding of nisin A to lipid II inhibits cell wall biosynthesis

and eventually causes the formation of pores or disturbances in bacterial membranes. Nisin A has a broad range of antibacterial activities against mainly Gram-positive bacteria, including species of *Streptococcus*, *Staphylococcus* and *Clostridium* (14-19). We and other groups have demonstrated that a two-component system (TCS) named BraRS is responsible for resistance to nisin A (20, 21). When *S. aureus* cells are exposed to nisin A, BraS senses nisin A, and phosphorylation of BraR then occurs. Phosphorylated BraR binds upstream of *vraDE*, which encodes an ABC transporter responsible for nisin A resistance. Inactivation of *braRS* resulted in increased susceptibility to nisin A. However, a high concentration of nisin A is still effective against *S. aureus* cells (18). Due to its broad-spectrum activity, nisin A is widely used as a food additive worldwide for the prevention of food-borne poisoning (14, 22, 23). Additionally, the use of bacteriocins, including nisin A, as clinical antibacterial agents has been investigated (14, 17, 24).

Previously, to determine whether nisin A treatment induces increased nisin A resistance, we tried to isolate mutants that showed increased levels of nisin A resistance by incubation with a subminimum inhibitory concentration (sub-MIC) of nisin A and ultimately obtained three mutants that constitutively expressed high levels of VraDE

(25). Then, we found point mutations in the promoter region of *braRS* in the *braR* or *braS* coding region in each mutant.

In this study, to explore potential nisin A resistance mechanisms independent of the BraRS-VraDE system, we tried to isolate *S. aureus* strains that are highly resistant to nisin A by exposing *S. aureus* to nisin A and eventually obtained mutants with BraRS-VraDE-independent nisin A resistance. Our analysis of these mutants demonstrated that they utilize an alternative nisin A resistance mechanism and that the mutations also affect the virulence of *S. aureus*.

Results

Isolation of *S. aureus* strains highly resistant to nisin A

Previously, we obtained three types of *S. aureus* strains highly resistant to nisin A, which showed increased levels of *vraDE* expression (25). We tried to isolate additional strains highly resistant to nisin A. From two independent experiments, we obtained two mutants that showed no increased expression of *vraDE*. We designated these mutants SAN2 and SAN469. The nisin A MICs for SAN2 and SAN469 were 2,048 μg/ml, while the MIC for the original MW2 strain was 512 μg/ml (Table 1).

We also evaluated the MICs of these mutants against bacitracin, gallidermin and nukacin ISK-1, which also inhibit the lipid cycle for cell wall biosynthesis (Table 1). There was no difference in MICs for these three agents between the mutants and the original MW2 strain.

Analysis of gene expression in MW2 and SAN2 by microarray analysis

To identify the factors responsible for high resistance to nisin A, we investigated the expression of all genes located on the chromosome of MW2. As shown in Table 2, the expression of MW1875 to MW1871 was significantly increased in the SAN2 strain, with more than 30-fold higher expression levels than that of the original MW2 strain in the absence of nisin A. MW1875-MW1871 were previously associated with phenol-soluble modulin (PSM) transport (PmtA-D; MW1874-MW1871) and a transcriptional regulator (PmtR; MW1875) (26). In the strain SAN2, the expression levels of several other genes were also increased but lower extents (2- to 3-fold greater than the MW2 levels).

Effects of MW1875 or MW1874 inactivation on nisin A susceptibility

From microarray analysis, we thought MW1875-MW1871 was associated with high

130 resistance to nisin A. Therefore, we constructed corresponding inactivation mutants to
131 examine whether these genes truly contributed to high nisin A resistance. Since *pmtR*
132 and *pmtA-D* (MW1875-MW1871) formed an operon, we constructed two insertional
133 mutants (MM2278 and MM2153) in which pYT1 was integrated in MW1875 or
134 MW1874 of strain SAN2 and one insertional mutant (MM2202) with an insertion in
135 MW1875 of strain MW2 (Table 3). The two SAN2 mutants showed the same nisin A
136 MIC as MW2. Similarly, the MW2 mutant (MM2202) retained the same MIC as MW2
137 (Table 3). A complemented strain, MM2279, in which the *pmtRABCD* operon
138 (MW1875-MW1871) was expressed by pCL15 in MM2278, also showed a similar MIC
139 as SAN2, indicating that the increased expression of *pmtRABCD* was involved in high
140 nisin A resistance in SAN2. We also constructed one mutant (MM2259) in which only
141 MW1875 was inactivated but MW1874-1871 was constitutively expressed and found
142 that MM2259 showed the same MIC as the SAN2 strain.
143
144 **DNA sequences of the MW1875 to 1871 (*pmtR* and *pmtA* to *D*) regions**
145 The DNA sequences of the MW1875-1871 regions in MW2, SAN2 and SAN469
146 were determined. In SAN2, only one mutation was detected in MW1875 (Fig. 1). This
147 mutation induced an alanine (Ala) at the 43rd amino acid of MW1875 to aspartic acid

148 (Asp) in the SAN2 strain. In SAN469, the fifth amino acid position in MW1875 was
149 mutated to a stop codon.
150
151 **Isolation of strains highly resistant to nisin A derived from other *S. aureus* strains**
152 To determine whether similar *pmtR* mutants were able to be obtained from other
153 strains after exposure to nisin A, we isolated nisin A-resistant mutants from *S. aureus*
154 COL and TY34. From two independent experiments in each strain, we obtained two
155 mutants with increased expression of *pmtR*A*BCD*, one from each strain (Table 3). We
156 designated the mutants SAN233 (from COL) and SAN455 (from TY34). DNA
157 sequencing analysis identified point mutations at the 16^{th} and 93^{rd} amino acids of
158 MW1875 (PmtR) in SAN233 and SAN455, respectively (Fig. 1), while there were no
159 mutations in the *pmtA-D* genes. These two mutations introduced stop codons within the
160 *pmtR* gene. Therefore, SAN233 and SAN455 did not express full-length PmtR.
161
162 **Expression of *pmtR* (MW1875), *pmtA* (MW1874) and *vraD* in MW2 and its**
163 **mutants**
164 We investigated the expression of *pmtR*, *pmtA* and *vraD* by quantitative PCR. In
165 SAN2, the expression of *pmtR* and *pmtA* (more than a 70-fold increase) was

166 significantly increased compared to that in MW2 in the absence of nisin A (Fig. 2A). We
167 observed the same result by immunoblotting analysis (Fig. 2B). We also confirmed the
168 increased expression of *pmtR* and *pmtA* in SAN469 by quantitative PCR (Supplemental
169 Fig. 1). In addition, the PmtR and PmtA expression patterns of MW2 and SAN2 in the
170 absence of nisin A were quite similar to those in the presence of nisin A because the
171 expression of *pmtR* and *pmtA* was not induced by nisin A (Supplemental Fig. 2).
172 Additionally, we investigated the expression of *vraD* and found that SAN2 showed no
173 increase in *vraD* expression at a low concentration of nisin A (32 μg/ml), while MW2
174 showed increased expression of *vraD* (Fig. 2C). However, the inactivation of
175 *pmtRABCD* in SAN2 resulted in increased *vraD* expression in the presence of nisin A.
176 Then, we investigated *vraD* expression in the presence of various concentrations of
177 nisin A (Fig. 3A). In MW2, the expression of *vraD* was induced at concentrations above
178 1/32 MIC nisin A (16 μg/ml), while in SAN2, it was induced by concentrations above
179 1/2 MIC nisin A (1024 μg/ml). We further investigated the induction of *vraD* expression
180 by bacitracin (1 MIC=64 μg/ml). Since the MIC of bacitracin was the same in the MW2
181 and SAN2 strains (Table 1), we analyzed the effect of bacitracin at a range from 1/64
182 MIC to 1 MIC. The *vraD* expression in both strains was similarly induced by bacitracin
183 at each concentration (Fig. 3B).

184

185 **Binding of the wild-type and mutated PmtR protein to the upstream region of**

186 **MW1875.**

187 Fig. 4A shows the previously reported promoter region of *pmtR*, the transcriptional

188 start site and the binding region of PmtR (27). The results of our EMSA revealed that

189 MW2-rPmtR bound the upstream region of *pmtR* (*pmtR*-F), while SAN2-rPmtR did not

190 bind its region (Fig. 4B). This binding of MW2-rPmtR was inhibited by the addition of

191 an excess amount of unlabeled DNA fragments.

192

193 **Hemolytic activity of MW2, SAN2, MM2278 and MM2279**

194 Since PSM transported by PmtA-D has been demonstrated to be involved in hemolytic

195 activity (28), we hypothesized that the increased expression of PmtA-D would affect

196 hemolytic activity, and we next analyzed the hemolytic activities of the strains MW2

197 and SAN2 on sheep blood agar. Compared to wild-type MW2, SAN2 produced a larger

198 hemolytic zone (Fig. 5A). When *pmtR* and *pmtA-D* (*pmtR-D*: MW1875 to MW1871)

199 were inactivated in SAN2 (MM2278), the hemolytic zone became smaller than that of

200 SAN2. The hemolytic zone produced by the complemented strain MM2279 was similar

201 in size to that of SAN2.

It was reported that PSM or δ-hemolysin enhanced the activity of β-hemolysin (28). Since MW2 (and SAN2) do not produce β-hemolysin, we investigated the synergistic hemolytic activity of MW2 and its derivatives with RN4220 (which produces β-hemolysin). An enhanced hemolytic zone with RN4220 was produced (white arrows) by SAN2, MM2279 and SAN469, while MW2 and MM2278 did not have an enhanced hemolytic effect with RN4220 (Fig. 5B). We also investigated the hemolytic activity of COL, SAN233, TY34 and SAN455 and found similar results (Supplemental Fig. 3).

Susceptibility to hBD3 and LL37 in MW2, SAN2, MM2278 and MM2279

Since it was reported in a previous study that *pmtR-D* inactivation increased the susceptibility to hBD3 and LL37 (29), we investigated the effect of the increased expression of *pmtA-D* in SAN2 on susceptibility to hBD3 (Fig. 6) and LL37 (supplemental Fig. 4). As shown in Fig. 6, compared to the original MW2 strain, SAN2 showed a decrease in susceptibility to hBD3, while the inactivation of *pmtRABCD* increased susceptibility to the peptides, resulting in the same susceptibility as that of MW2. The complemented strain (MM2279) showed susceptibility similar to that of SAN2.

Mouse survival rate after injection of MW2, SAN2, MM2278 and MM2279

We performed a mouse survival experiment using a bacteremia model. Injection of the MW2 strain killed only one out of 8 mice 3 days after injection, while 5 of 8 mice were killed between 1 to 5 days after the injection of SAN2 (Fig. 7A, P=0.010, log-rank test). No mouse was killed by injection of the SAN2 *pmtR-D* inactivation strain (MM2278), while 4 of 5 mice were killed between 1 to 3 days after the injection of the complemented strain (MM2279) (Fig. 7B; P=0.002, Fig. 7C; P=0.604, log-rank test).

Discussion

In this study, we demonstrated a novel high nisin A resistance mechanism that functions independently of the BraRS-VraDE system. We obtained 4 mutant strains with the increased expression of PmtA-D from MW2, COL and TY34 that acquired high nisin A resistance. All these mutants had a point mutation in the *pmtR* gene, yielding a mutant PmtR with an Ala43Asp substitution (SAN2 from MW2) or truncated PmtR (SAN469 from MW2, SAN233 from COL and SAN455 from TY34). EMSAs showed that the SAN2-derived PmtR protein (SAN2-rPmtR) did not bind the DNA region upstream of *pmtR-D*. Since PmtR is a negative transcriptional regulator of the

238 *pmtRABCD* operon (27), SAN2-PmtR and the three truncated PmtRs could not suppress
239 the expression of the *pmtR-D* operon, resulting in the increased expression of *pmtR-D*.
240 The mutation site of PmtR in SAN2 is within the helix-turn-helix DNA-binding region.
241 Based on our EMSA, a mutated-PmtR in SAN2 lost the ability to bind to the target
242 DNA region. This result implies two possibilities. One possibility is that the mutation
243 site is critical for DNA binding. Another is that the mutation causes a structural change
244 in PmtR leading to loss of DNA binding.
245 PmtA-D form an ABC transporter from two membrane proteins (PmtA and C) and
246 two ATPases (PmtB and D) (26). PmtA-D is involved in the transport of PSMs and
247 δ-hemolysin (Hld) from the cytoplasm to the extracellular space (26, 28). PSMs have
248 broad virulence activities, such as surface spreading activity responsible for epithelial
249 colonization, biofilm formation, proinflammatory activity, cytolytic activity and
250 antimicrobial activity (30, 31, 32). In addition, Cheung GYC et al. recently reported that
251 the Pmt transporter is also associated with human-derived antimicrobial peptides, such
252 as hBD3 and LL37 (29). We also found that high expression of Pmt transporters in
253 SAN2 resulted in high resistance against hBD3 and LL37. These results suggest the
254 association of Pmt transporters with the susceptibility of human antimicrobial peptides.
255 Although there are no clear structural similarities among PSMs, δ-hemolysin, hBD3 and

LL37, we speculate that PmtA-D may be associated with the export of these peptides with membrane insertional activity (Fig. 8). However, we also evaluated the susceptibility to bacitracin, gallidermin and nukacin ISK-1 and found no difference between the MW2 and SAN2 strains. Nukacin ISK-1 is functionally similar to nisin A because these are both lantibiotics with the same target, lipid II (12, 33). Furthermore, we found a different response of *vraD* expression: bacitracin induced *vraD* expression in both the MW2 and SAN2 strains with the same low concentration of nisin A, while only a high concentration of nisin A induced the expression of *vraD* compared to that for MW2 (Fig. 3A and 3B). These results indicate that the Pmt system recognizes the limited structure of antimicrobial peptides, such as nisin A and LL37, with membrane insertional activity.

Joo HS et al. demonstrated that PSMα1-3 binds PmtR to release it from its target DNA region, which is followed by induction of the expression of the *pmt* operon (27). PSM expression is upregulated by the *agr* system (28, 31). Since *agr* expression increases in the late exponential phase (34, 35), the expression levels of PSM and PmtA-D are increased at the late exponential phase. In this study, we collected bacterial cells for the evaluation of gene expression at mid exponential phase (OD=0.5) and found strong expression of *pmtA* in SAN2, but not MW2 (Fig. 2A and S1), indicating

that in the SAN2 mutant, PmtA-D expression is independent of the growth phase due to the lack of functional PmtR. Therefore, the expression of PmtA-D is constitutively increased; thus, PSMs are constantly exported from the cell during SAN2 growth. As mentioned above, PSMs have broad virulence activities, so the increased PSM transport may modulate the virulence of *S. aureus*. We found that the hemolytic activity of SAN2 was greater than that of the wild-type MW2, likely because PSMα has an enhanced effect on hemolytic activity (28). Additionally, susceptibility to human antimicrobial peptides was decreased in the mutants. Finally, in a mouse experiment, we found that compared to the wild-type MW2, SAN2 showed an increase in fatality rate due to its increased virulence. The Pmt-dependent nisin A resistance identified in this study is different from the previously identified BraRS-VraDE system in terms of the modulation of virulence in *S. aureus*.

Previously, we isolated strains that are highly resistant to nisin A that showed high constitutive expression of VraDE (25). VraDE, which is regulated by BraRS, is an intrinsic resistance factor against nisin A, bacitracin, gallidermin and nukacin ISK-1. In MW2, sub-MIC (1/16 MIC) nisin A induced the expression of VraDE, while sub-MIC nisin A did not induce its expression in SAN2. However, *pmtA-D* inactivation in SAN2 caused inducible expression of VraDE by sub-MIC nisin A. Additionally, induction of

292 *vraD* expression occurred at high concentrations of nisin A in SAN2, suggesting that the
293 Pmt system is a dominant system contributing to nisin A resistance in SAN2 (Fig. 8).
294 In this study, we used nisin A obtained from Sigma-Aldrich with 2.5% formulation.
295 Since MIC of nisin A in MW2 showed 512 μg/ml in this study, MIC of purified nisin A
296 is estimated to 12.8 μg/ml. There are several reports regarding to the concentration of
297 nisin A in several foods such as cheese, sausage and milk (36, 37). Although nisin A
298 concentration is varied among foods, its concentration is mostly above 10 μg/g.
299 Therefore, the concentration of nisin A for the isolation of the resistant mutant in this
300 study is close to that for food preservatives, speculating the possibility that nisin A
301 resistant *S. aureus* might be generated by food preservatives.
302 In conclusion, we found a new mechanism for high nisin A resistance that is mediated
303 by the increased expression of Pmt proteins in *S. aureus*. It is important to note that the
304 increased expression of Pmt proteins causes high nisin A resistance and enhances
305 virulence. Since nisin A is widely used as a food additive, it is important to use it
306 cautiously.
307
308 **Materials and Methods**
309

Bacterial strains and growth conditions

The bacterial strains used in this study are shown in Table 3. *S. aureus* and *Escherichia coli* XL-II were grown in trypticase soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and Luria-Bertani (LB) broth, respectively. Tetracycline (WAKO Chemicals, Osaka, Japan) (TET, 5 μg/ml) and chloramphenicol (Sigma Aldrich, St. Louis, MO, USA) (CHL: 5 μg/ml) were used for *S. aureus*, and ampicillin (WAKO Chemicals, Osaka, Japan) (AMP, 100 μg/ml) was used for *E. coli* when necessary.

MIC determination

The MICs of several antibacterial agents were determined by microdilution with liquid culture (TSB), as described previously (38). Nukacin ISK-1 was purified as described previously (20). Nisin A with 2.5% formulation (Sigma Aldrich, St. Louis, MO, USA) was used in this study. Bacitracin and gallidermin were obtained from WAKO Chemicals, Osaka, Japan and Santa Cruz Blotechnology, TX, USA, respectively. *S. aureus* strains (10^5 cells) were inoculated into 100 μl of TSB containing various concentrations of nisin A (from 4096 to 4 μg/ml), nukacin ISK-1 (from 128 to 0.125 μg/ml), gallidermin (from 128 to 0.125 μg/ml) and bacitracin (from 256 to 0.25 μg/ml).

MICs were determined after incubation for 24 h at 37°C. MIC determination was repeated independently three times.

Isolation of mutants highly resistant to nisin A

S. aureus mutants highly resistant to nisin A were obtained by a method described elsewhere (25). Briefly, a microdilution method that is generally used to evaluate the minimum inhibitory concentration (MIC) of antibacterial agents was used for isolation of the mutants. An overnight culture of *S. aureus* MW2 was diluted to 10^7 cells/ml, and 10 µl of the diluted culture was applied to each well (100 µl), which contained a 2-fold dilution series of nisin A (16 to 16384 µg/ml). After incubation at 37°C overnight, bacterial cells that grew in 1/2 MIC nisin A were collected and diluted 100-fold. Ten microliters of this diluted sample were then applied to each well (100 µl), which contained serial 2-fold dilutions of nisin A (16 to 16384 µg/ml). This procedure was repeated three times. Ultimately, bacterial cells grown in the presence of 1/2 MIC nisin A were appropriately diluted and plated on tryptic soy agar (TSA). After overnight incubation, 14 colonies were picked and incubated in 5 ml of TSB. Overnight culture was used for the determination of the MIC of nisin A. This experiment was independently performed 3 times. Then, we investigated the *vraD* expression of the

346 strains with high nisin A resistance by quantitative PCR and found no increase in *vraD*
347 expression.
348 We also tried to isolate strains highly resistant to nisin A from *S. aureus* COL and
349 TY34 by the same method described above.
350
351 **Microarray analysis.**
352 Overnight cultures of *S. aureus* (10^8 cells) were inoculated into 30 ml of fresh TSB
353 and cultured at 37°C with shaking. When the OD_{660} reached 0.4, the bacterial cells were
354 collected by centrifugation at 5,000 x *g* for 5 min at 4°C. Total RNA was extracted by
355 using a FastRNA Pro Blue Kit (MP Biomedicals, Cleveland, OH, USA) according to the
356 manufacturer's protocol. Then, cDNA from each sample was synthesized from 10 μg of
357 total RNA using a FairPlay III Microarray Labeling Kit (Agilent Technologies, Santa
358 Clara, CA, USA) according to the manufacturer's instructions. The Agilent eArray
359 platform (Agilent Technologies) was used to design the microarray: 13,939 probes
360 (60-mers) were designed to target the 2,628 protein-coding genes of *S. aureus* MW2 (up
361 to five probes per gene). Microarray analysis was performed by a method described
362 elsewhere (39). The experiments were performed using three biological replicates (three
363 technical replicates for each set of conditions), and the expression data were deposited

364 into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession
365 GSE131352. Statistical analysis was determined by student's *t*-test. The *p* values were
366 indicated in Table 2.
367
368 **DNA sequences of MW1875-1871 regions**
369 Since we found the increased expression of genes corresponding to MW1875-1871 in
370 the SAN2 strain, we determined the DNA sequence of the MW1875-1871 regions.
371 Primers were constructed to amplify MW1875-1871 with their corresponding flanking
372 regions, including the promoter regions of MW1875 (Table 4). To prepare chromosomal
373 DNA from the original MW2 strain and the mutant strains, the cells from 1 ml of
374 overnight cultures were collected. The cells were suspended in 100 µl of 10 mM
375 Tris-HCl (pH 6.8) containing 10 µg of lysostaphin (Sigma Aldrich), incubated at 37°C
376 for 20 min, and then incubated at 95°C for 15 min. After centrifugation, cell lysates
377 were used as template DNA for PCR. PCR was performed using the Takara Ex Taq
378 system, and the amplicons were purified using a QIAquick kit (QIAGEN, Hilden,
379 Germany). The nucleotide sequences of each DNA fragment were determined using
380 specific primers. The primers used to amplify DNA sequences are listed in Table 4.
381

Inactivation of MW1875 and MW1874 in *S. aureus* and their complementation

The strains used in this study are listed in Table 3. Specific gene inactivation by the insertion of the thermosensitive plasmid pYT1 was performed by a method described elsewhere (40). An internal DNA fragment of MW1875 or MW1874 was amplified by PCR using specific primers and then cloned into a pYT1 vector. The plasmid was electroporated into *S. aureus* RN4220, and then the plasmid in RN4220 was transferred into respective strains by transduction using phage 80α. The obtained strains were grown overnight at 30°C. Appropriate dilutions of the culture were spread on TSA plates containing TET (10 μg/ml) and then incubated at 42°C overnight. Colonies were picked and replated on TSA containing TET. Disruption of the target gene was checked by PCR. For gene complementation, the vector pCL8, which is a shuttle vector for *E. coli* and *S. aureus* (41). A DNA fragment for complementation was PCR-amplified using chromosomal DNA from the MW2 or mutant strains. The DNA fragment was cloned into pCL8 using *E. coli* XL-II competent cells. The obtained plasmid was electroporated into *S. aureus* RN4220 and was subsequently transduced into the appropriate strain using phage 80α.

To obtain a mutant that constitutively expresses MW1874-1871 by its own promoter upstream of MW1875 without MW1875 expression, we constructed a plasmid by PCR

400 cloning of a DNA fragment containing the internal region of MW1875 (295 bp) and the
401 promoter region of MW1875-1871 (149 bp). Two PCR fragments were obtained and
402 then cloned into the pYT1 vector by using restriction enzymes. The plasmid was finally
403 transduced into the MW2 strain. The plasmid integration was performed using the
404 method described above. Two types of mutants were obtained (Supplemental Fig. 5).
405 Finally, the strain that contained inactivated MW1875 and expressed MW1874-1871
406 was verified by PCR and DNA sequencing.
407
408 **Quantitative PCR and immunoblotting analysis**
409 Quantitative PCR was performed to investigate the expression of MW1875 (*pmtR*),
410 MW1874 (*pmtA*) and *vraD*. A small portion of overnight culture (10^8 cells) was
411 inoculated into 5 ml of fresh TSB and then grown at 37°C with shaking. When the
412 optical density at 660 nm reached 0.5, nisin A (16 to 2048 μg/ml) or bacitracin (1 to 64
413 μg/ml) was added to the bacterial culture. After incubating for 15 min (for quantitative
414 PCR) and 2 h (for immunoblotting), the bacterial cells were collected. For quantitative
415 PCR, RNA extraction, cDNA synthesis and PCR were performed as described
416 previously (25). Statistical analysis was determined by $*p < 0.01$, $**p < 0.005$, as
417 determined by Dunnett's post hoc tests compared to the control in each experiment.

418 For immunoblotting, antiserum against MW1875 and MW1874 was obtained by
419 immunizing rabbit with the recombinant protein, as described previously (42). Briefly,
420 the coding region of MW1875 or MW1874 amplified by PCR using specific primers
421 was cloned into pQE30 (QIAGEN, Tokyo, Japan), which is used for the construction of
422 histidine-tagged recombinant proteins. The obtained plasmid was transformed into *E.*
423 *coli* M15 (pREP4). The resulting recombinant protein was purified according to the
424 manufacturer's instructions. Antiserum was obtained using the recombinant protein. We
425 also immunoblotted VraD using anti-rVraD antibodies obtained previously (25).
426 Collected bacterial cells were resuspended in 200 µl of Tris-HCl (pH 6.8) containing
427 10 µg of lysostaphin and incubated for 20 min at 37°C. The cells were then heated at
428 95°C for 15 min. After centrifugation, the supernatant was obtained as a whole-cell
429 lysate. Lysate proteins mixed with an equal volume of sample loading buffer were
430 resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE). Then, the proteins
431 were transferred to a nitrocellulose membrane. After blocking with 2% skim milk in
432 Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl [pH 8.0]) containing 0.05%
433 Tween 20 (TBS-T), the membrane was incubated with specific antiserum (diluted
434 1:1,000 in 1% skim milk in TBS-T) for 1 h at 37°C. The membrane was then washed
435 with TBS-T and incubated with horseradish peroxidase-conjugated anti-mouse IgG

436 (diluted 1:1,000 in TBS-T) (Promega, Madison, WI, USA) for 1 h at 37°C. The
437 membrane was then washed 5 times with TBS-T, and the protein band reacting with the
438 antiserum was detected using a chemiluminescence detection system (PerkinElmer,
439 Waltham, MA, USA).
440
441 **Hemolysis assay**
442 Hemolysis assays were performed by using TSB agar plates containing 5% sheep
443 blood (Becton, Dickinson and Company). To evaluate the hemolysis activity of
444 individual strains, three microliters of a 10-fold diluted overnight culture of MW2,
445 SAN2, MM2278 (*pmtR*, *pmtA-D* inactivated strain) and MM2279 (complemented strain
446 of MM2278) was spotted individually on a sheep blood agar plate and incubated at
447 37°C for 2 days. The hemolytic area of each strain was calculated by using Image J
448 analysis. This experiment was performed independently 3 times. To evaluate the
449 synergistic effect on β-hemolysin of individual strains, three microliters of a 10-fold
450 diluted overnight culture of the RN4220 strain (a β-hemolysin-producing strain) was
451 spotted. Additionally, three microliters of a 10-fold diluted overnight culture of MW2,
452 SAN2, MM2278 or MM2279 was spotted around RN4220 (distance from the center of
453 the RN4220 spot was 17 mm). The agar plate was incubated at 37°C for 20 h and then

kept at 4°C for 2 days. This experiment was performed independently 3 times to confirm the results. We quantified hemolytic activity by measuring the hemolysis zone of each strain. Using Image J analysis. Briefly, we excised same area covering hemolysis zone (1.5 cm ×1.5 cm). Then, percentage of hemolysis area (%) in MW2, SAN2, MM2278, MM2279 and SAN469 was evaluated by Image J. Statistical analysis was determined by $*p < 0.01$, $**p < 0.005$, as determined by Dunnett's post hoc tests compared to MW2.

Susceptibility to hBD3 and LL37

An antibacterial assay was performed as described elsewhere (43). Briefly, overnight cultures of *S. aureus* strains were collected and washed with 10 mM sodium phosphate buffer (PB). The bacterial suspension was diluted to 10^7 cells/ml with PB, and 10 µl of the bacterial suspension (10^5 cells) was inoculated into 500 µl of PB with or without human antimicrobial peptides (β-defensin-3: Peptide Institute Inc., Osaka, Japan; or LL37; 0.5, 0.4, 0.2, 0.1, 0.05 µM) and incubated aerobically for 10 min and 2 hours at 37°C. Dilutions of the reaction mixture (100 µl) were plated on agar medium and incubated at 37°C overnight. The colony-forming units (CFU) were determined as the total number of colonies on each plate. The antibacterial effect was calculated as the

ratio of the number of surviving cells (survival rate [%]) to the total number of bacteria incubated in a control PB solution after exposure to antimicrobial peptides. Statistical analysis was determined by $*p < 0.05$, as determined by Dunnett's post hoc test, compared to untreated MW2.

Electrophoretic mobility shift assay (EMSA)

For an EMSA, two 6× histidine-tagged recombinant PmtR (rPmtR) proteins from the original MW2 strain and SAN2 were used. Construction of the rPmtRs was performed as described above. Briefly, a DNA fragment encoding *pmtR* (MW1875) was amplified with specific primers by using chromosomal DNA of MW2 and SAN2. Then, DNA fragments were subsequently cloned into pQE30. The plasmid was then transformed into *E. coli* M15 (pREP4). The recombinant protein was purified according to the manufacturer's instructions. To assess the binding of rPmtR to a region upstream of *pmtR*, an EMSA was performed as described previously (25). A DNA fragment encompassing the region upstream of *pmtR, pmtA-D* was amplified with the specific primers listed in Table 1. The DNA fragments were labeled at the 3′ end with digoxigenin (DIG) using a DIG Gel Shift Kit, 2nd Generation (Roche, Mannheim, Germany). The DIG-labeled fragment (5 ng) was reacted with rPmtR protein (50 mM)

490 in the labeling buffer provided with the kit. When necessary, a nonlabeled DNA
491 fragment (10 ng) was added to the reaction mixture. After native PAGE on a 6%
492 polyacrylamide gel, the DNA fragments were transferred to a positively charged nylon
493 membrane (Roche, Mannheim, Germany) and visualized according to the
494 manufacturer's protocol.
495
496 **Mouse bacteremia experiment**
497 Since we found the alteration of virulence in SAN2 strain, we then performed the
498 animal experiment to see whether SAN2 strain show strong virulence in vivo. A mouse
499 bacteremia experiment was performed as described previously (44). Six-week-old
500 female Slc:ddY mice were purchased from SLC (Shizuoka, Japan). Small portions of
501 overnight cultures of *S. aureus* MW2, SAN2, SAN2 with MW1875-1871 inactivation
502 (MM2278) and MM2278 complemented with MW1875-1871 (MM2279) were
503 inoculated into 5 ml of fresh TSB and incubated at 37°C with shaking. When the OD_{660}
504 reached 0.8, bacterial cells were collected and washed with PBS. Then, the cells were
505 resuspended in PBS at a concentration of 1.0×10^9 CFU/ml. An aliquot of 100 μl ($1.0 \times$
506 10^8 CFU) was injected into the tail vein of the mice. We divided 3 groups for this
507 experiment: (A) survival comparison between MW2 and SAN2, (B) the comparison

between SAN2 and MM2278, (C) the comparison between SAN2 and MM2279. Number of trials: (A) MW2=8, SAN2=8; (B) SAN2=8, MM2278=6; (C) SAN2=6, MM2279=5. Mouse survival was monitored for 6 days. Survival statistics were calculated by the log-rank test (Mantel-Cox). The animal experimentation performed in this study was conducted according to a protocol approved by the President of Kagoshima University after review by the Institutional Animal Care and Use Committee (Ethical number: D18015).

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Conflict of interest

The authors declare no competing financial interests in relation to the work described.

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661
662

663 **Figure Legends**

664

665 **Fig. 1. ORF map of the *pmt* region (MW1875-1871) and mutation sites in *pmtR* of**

666 **isolated mutants.**

667 The mutation sites in the *pmtR* region are indicated by white arrows.

668

669 **Fig. 2. Expression of *pmtR, pmtA* and *vraD* in the mutants.**

670 (A) Expression of *pmtR* and *pmtA* in MW2, SAN2, MM2278 (SAN2 with inactivation

671 of *pmtR-pmtD*) and MM2279 (the complemented strain in MM2278) was evaluated by

672 quantitative PCR using specific primers. *p <0.001, as determined by Dunnett's post

673 hoc test, compared to untreated MW2. (B) Expression levels of PmtR and PmtA in *S.*

674 *aureus* strains were evaluated by immunoblotting analysis using respective specific

675 antibodies. (C) Expression of *vraD* in *S. aureus* strains in the presence or absence of

676 1/16 MIC nisin A (32, 128, 32 and 512 μg/ml in MW2, SAN2, MM2278 and MM2279,

677 respectively) was evaluated by quantitative PCR using specific primers. *p <0.005, as

678 determined by Dunnett's post hoc test, compared to untreated MW2.

679

680 **Fig. 3. Expression of *vraD* in MW2 and SAN2 with various concentrations of nisin**

681 **A and bacitracin.**

682 The induction of *vraD* expression in *S. aureus* MW2 and SAN2 by the addition of
683 various concentrations of nisin A (A) or bacitracin (B) was evaluated by quantitative
684 PCR using specific primers. *p <0.05 and **p <0.01, as determined by Dunnett's post
685 hoc test, compared to untreated MW2 or SAN2.
686
687 **Fig. 4. EMSAs of PmtR**
688 (A) The nucleotide sequence of the *pmtR* promoter region. Squares, -35, -10 box; *,
689 *pmtR* transcriptional start site; bold, PmtR-binding region**.** (B) EMSAs of PmtR with a
690 DNA fragment labeled with DIG were performed with the method described in the
691 Materials and Methods.
692
693 **Fig. 5. Hemolytic assay of *S. aureus* mutants**
694 (A) Three microliters of a 10-fold diluted overnight culture of MW2, SAN2, MM2278
695 (SAN2 with inactivation of *pmtR-pmtD*) and MM2279 (the complemented strain in
696 MM2278) was spotted individually on a sheep blood agar plate and incubated at 37°C
697 for 2 days. Percentage of hemolysis area was evaluated by ImageJ. *p <0.01, **p
698 <0.005, as determined by Dunnett's post hoc tests compared to MW2. (B) Three
699 microliters of a 10-fold diluted overnight culture of RN4220 (a β-hemolysin-producing

700 strain) was spotted, and the same volume of MW2, SAN2, MM2278, MM2279 and
701 SAN469 was spotted around RN4220 (distance from the center of the RN4220 spot was
702 17 mm). The agar plate was incubated at 37°C for 20 h and then kept at 4°C for 2 days.
703 White arrows indicate a synergistic effect. Percentage of hemolysis area was evaluated
704 by ImageJ. *p <0.01, **p <0.005, as determined by Dunnett's post hoc tests compared
705 to MW2.
706
707 **Fig. 6. Susceptibility of *S. aureus* mutants to hBD3**
708 An antibacterial assay for hBD3 was performed as described in the Materials and
709 Methods section. *S. aureus* was reacted with various concentrations of hBD3 for 10 min
710 (A) and 2 hours (B). *p <0.05, as determined by Dunnett's post hoc test, compared to
711 untreated MW2.
712
713 **Fig. 7. Mouse survival experiment**
714 Survival percentage of Slc:ddY mice after being challenged with intravenous injection
715 of 1.5×10^8 CFU of *S. aureus*. (A) Survival comparison between MW2- and
716 SAN2-inoculated mice. (B) Survival comparison between wild-type SAN2 and
717 MM2278 (SAN2 with inactivation of *pmtR-pmtD*). (C) Survival comparison between

718 wild-type SAN2 and MM2279 (the complemented strain in MM2278). Significant
719 differences between the two strains are shown. Survival statistics were calculated by the
720 log-rank test (Mantel-Cox).
721
722 **Fig. 8. Proposed high nisin A resistance mechanism mediated by the Pmt system.**
723 The MW2 strain shows resistance to low concentrations of nisin A by the BraRS system.
724 In the SAN2 strain, the Pmt system contributes to high nisin A resistance due to the
725 mutation in PmtR.
726
727

Table 1. susceptibility of nisin A, bacitracin, gallidermin and nukacin ISK-1 against *S. aureus* strains.

	MIC (μg/ml)			
strain	nisin A	bacitracin	gallidermin	nukacin ISK-1
MW2	512	64	8	16
SAN2	2048	64	8	16
SAN469	2048	64	8	16
COL	1024	64	8	16
SAN233	2048	64	8	16
TY34	512	64	8	16
SAN455	2048	64	8	16

Table 2. Genes up-regulated in *S. aureus* SAN2

gene ID [1]	gene name	function	Fold-difference[2]	SD	*p* value[3]
MW1873	*pmtB*	ABC transporter, membrane domain	62.2	13.7	0.01
MW1874	*pmtA*	ABC transporter, ATPase domain	54.7	2.8	<0.001
MW1875	*pmtR*	transcription regulator, GntR family	49.0	3.3	0.001
MW1872	*pmtC*	ABC transporter, ATPase domain	47.7	3.2	0.002
MW1871	*pmtD*	ABC transporter, membrane domain	38.1	6.2	0.007
MW1869		hypothetical protein	2.6	0.3	0.008
MW1868		hypothetical protein	2.4	0.9	0.004
MW1867		hypothetical protein	2.1	0.3	0.02

[1] Gene IDs are from the GEO of the NCBI Database (http://www.ncbi.nlm.nhi.gov/geo/)

[2] expression level of SAN2 related to that of MW2

[3] The *p* values were analyzed for statistically significant differences by Student's *t*-test

Table 3. Strains used in this study

Strains	character	MIC of nisin A (μg/ ml)	reference
S. aureus			
MW2	clinical strain, methicillin-resistant (*mecA*+)	512	45
COL	clinical strain, methicillin-resistant (*mecA*+)	1024	46
TY34	clinical strain, methicillin-resistant (*mecA*+)	512	47
MM2202	pYT1 insertion to MW1875 (MW1875-1871 inactivation) in MW2, TET^r	512	This study
MM2280	Overexpression of MW1875-71 (pCL8) in MW2, CHL^r	2048	This study
MM2259	pYT1 insertion to MW1875 (MW1874-1871 overexpression) in MW2, TET^r	2048	This study
SAN2	nisin A resistant mutant from MW2	2048	This study
MM2278	pYT1 insertion to MW1875 (MW1875-1871 inactivation) in SAN2, TET^r	512	This study
MM2153	pYT1 insertion to MW1874 (MW1874-1871 inactivation) in SAN2, TET^r	512	This study
MM2279	MW1875-1871 (SAN2) complementation in MM2278, TET^r, CHL^r	2048	This study
SAN469	nisin A resistant mutant from MW2	2048	This study
SAN233	nisin A resistant mutant from COL	2048	This study
SAN455	nisin A resistant mutant from TY34	2048	This study
E. coli			
XL-II	*endA1 supE44 thi-1 hsdR17 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*q*ZΔM15* Tn*10* (TET^r) Amy CHL^r]	-	Stratagene
MM1127	His-tag fused MW1875 (MW2) gene in XL-II, AMP^r	-	This study
MM1128	His-tag fused MW1875 (SAN2) gene in XL-II, AMP^r	-	This study

Table4. Primers used in this study

target gene ID	primer-forward	primer-reverse
Construction of gene-inactivatied mutants		
pmtR+*pmtA*-*D*	5’- ttggatccacaatagtgattttccgatt -3’	5’- caaagctttggcttgcgcttcattaa -3’
pmtA-*D*	5’- agggatccatatggctctcaatccg -3’	5’- aaaagcttcactcacaacttgatatc -3’
Construction of the plasmid for gene complementation		
pmtR+*pmtA*-*D*-pCL15	5’- ataagcttatacagaaagtgataggg -3’	5’- ttggatccaactgatcacttgaataatt - 3’
pmtA-*D*-pCL15	5’- ataagctttttaagcttcatttatgag -3’	5’- ttggatccaactgatcacttgaataatt - 3’
pmtR+*pmtA*-*D*-pCL8	5’- ctaagcttttgaagtagacaatgcaag -3’	5’- atcccgggttcccaacctcaaaattat -3’
Pmt promoter+*pmtA*-*D*-pYT1	5’- caagatctaatggtagtgtcatttcatt -3’	5’- caggatcccaacgtccccctatcac -3’
	5’- ttggatccacaatagtgattttccgatt -3’	5’- caaagctttggcttgcgcttcattaa -3’
Amplification of DNA fragments used in gel shift assay		
pmtR-F1	5’- aatggtagtgtcatttcattt -3’	5’- caacgtccccctatcac -3’
Construction of the plasmid for recombinant protein		
rPmtR	5’- ccggatccatgaaaataattttaaaaaacaat -3’	5’- ttaacgtttcatgatgattcctcctca -3’
rPmtA	5’- ccggatccatgaatgccatagaattaag -3’	5’- ttaagcttttaaaaaccttcttccatca -3’
Primers for quantitative PCR		
pmtR	5’- aattggttaatgaagcgcaag-3’	5’- gattcctcctcataaatgaacg-3’
pmtA	5’- taaagcttcgttcatttatgaggagg -3’	5’- acgataaaaaggggcaatca -3’
vraD	5’- cacttgccaaattccgta -3’	5’- aatacctaatgctgtcgtga -3’
gyrB	5’- aggtcttggagaaatgaatg -3’	5’- caaatgtttggtccggtt -3’

Primers used for DNA sequence		
pmtR, A-D-seq-F-500	5’- tgaaattcaataacttattaaa -3’	
pmtR, A-D –F-26	5’- atacagaaagtgataggg-3’	
pmtR, A-D –F351	5’- aacgttcatttatgaggagg -3’	
pmtR, A-D –F967	5’- attatatcattcacttaagtg -3’	
pmtR, A-D –F1429	5’- tttggattttagatgctggtca -3’	
pmtR, A-D –F1967	5’- attacaaaaaaatacggctc -3’	
pmtR, A-D-F2409	5’- atggctcaattgatgtgctg -3’	
pmtR, A-D-F3047	5’- cgcgtgatttttcacaaggt -3’	
pmtR, A-D-seq-R		5’- tttaaaattcccaacctca -3’

Underlines indicate the recognition site of the respective restriction enzyme

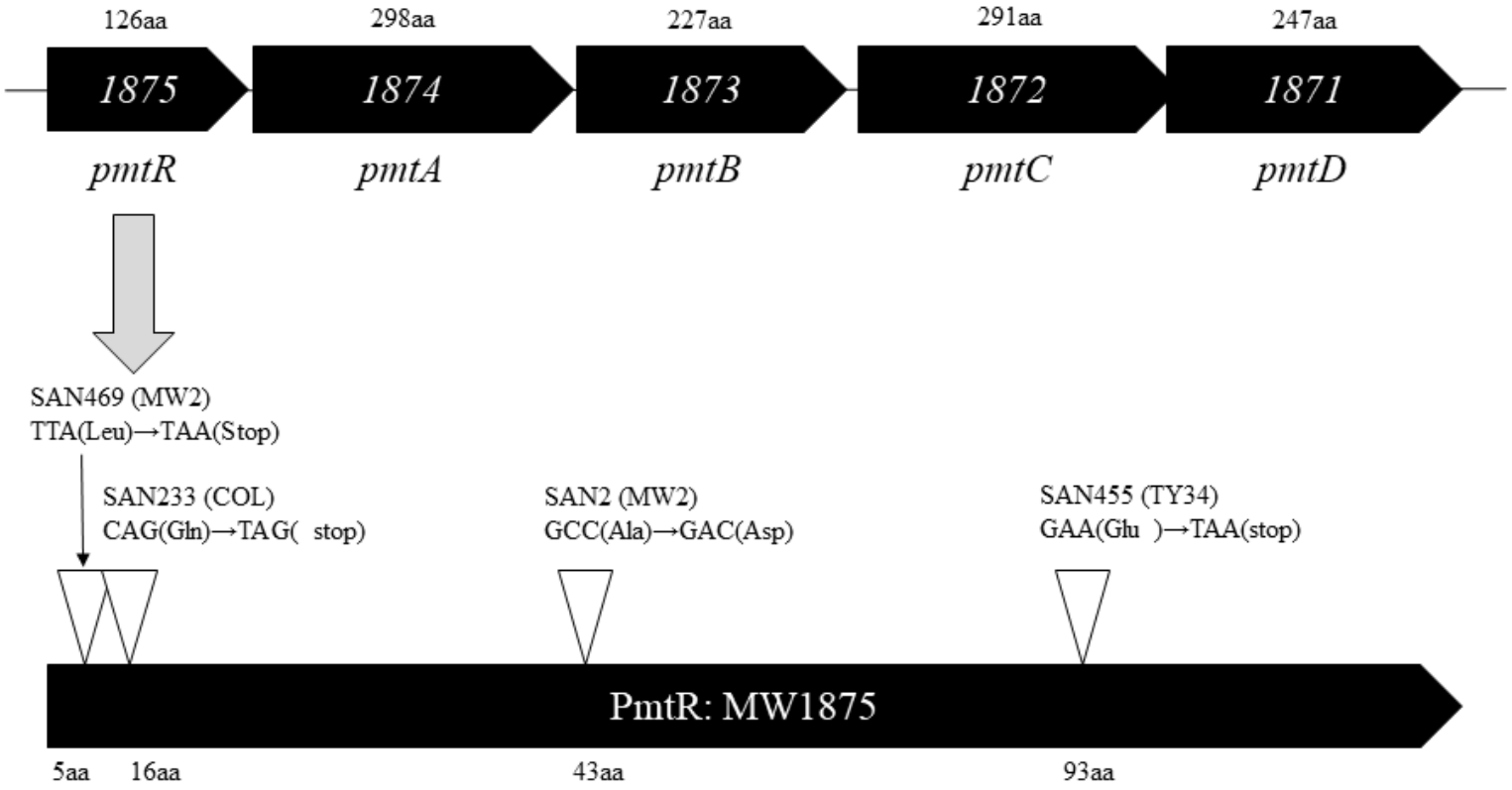

126aa
298aa
227aa
291aa
247aa
1875
1874
1873
1872
1871
pmtR
pmtA
pmtB
pmtC
pmtD
SAN469 (MW2)
TTA(Leu)→TAA(Stop)
SAN233 (COL)
CAG(Gln)→TAG(stop)
SAN2 (MW2)
GCC(Ala)→GAC(Asp)
SAN455 (TY34)
GAA(Glu)→TAA(stop)
PmtR: MW1875
5aa
16aa
43aa
93aa

Fig. 1.

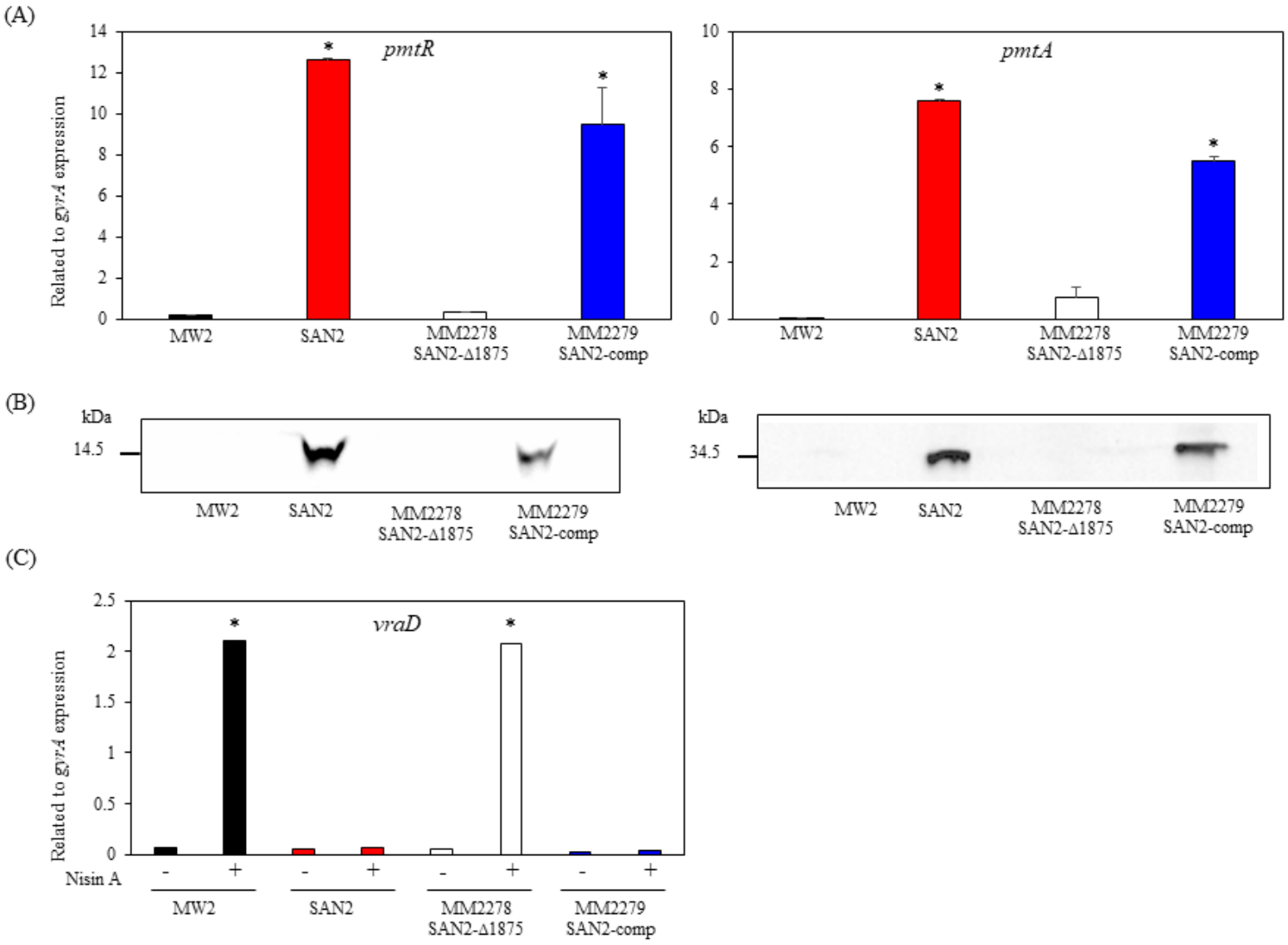

(A)
pmtR
Related to gyrA expression
MW2
SAN2
MM2278 SAN2-Δ1875
MM2279 SAN2-comp
pmtA
(B)
kDa
14.5
34.5
(C)
vraD
Nisin A

Fig. 2.

(A)

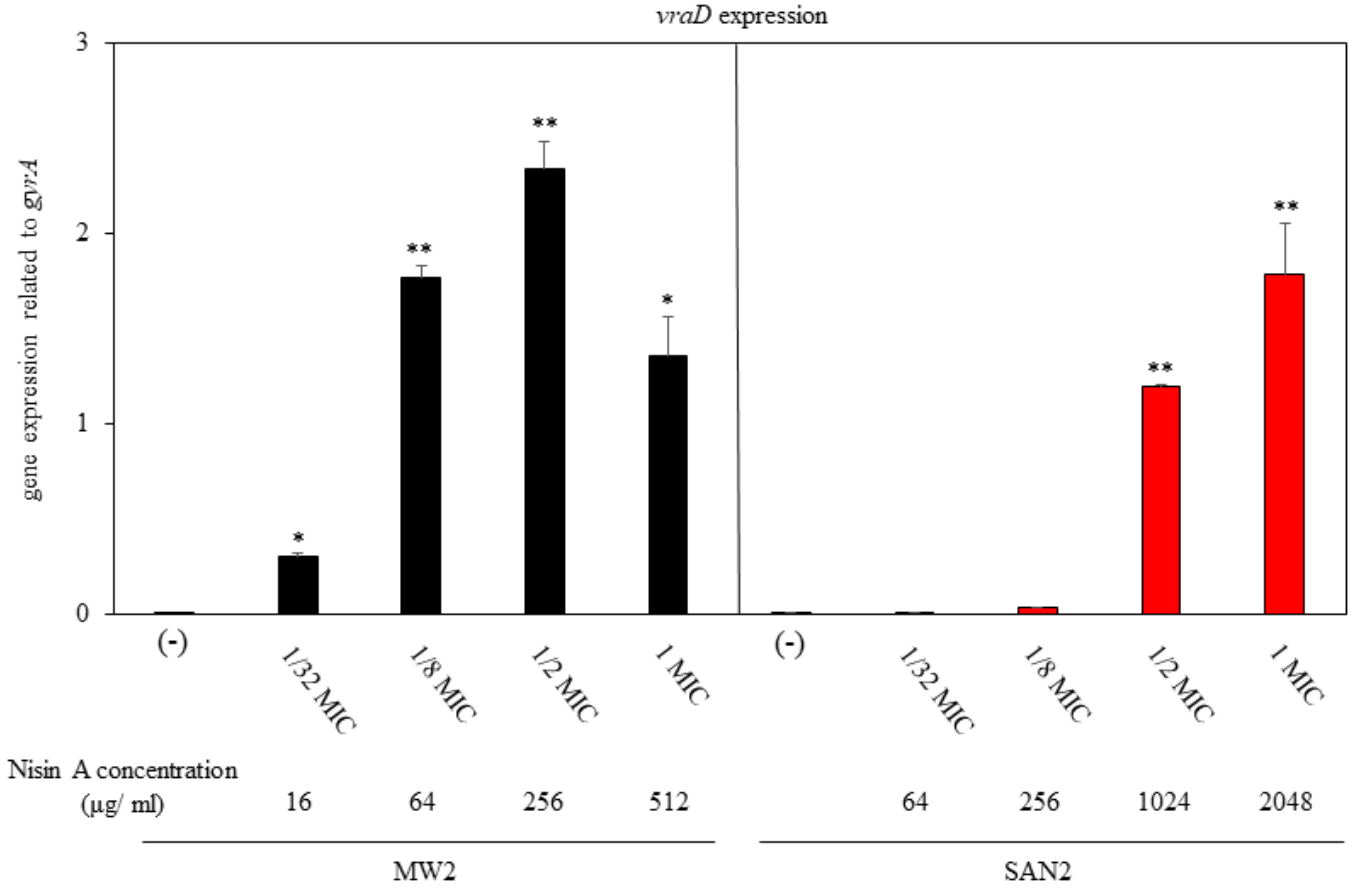

vraD expression
gene expression related to gyrA
3
2
1
0
**
*
(-)
1/32 MIC
1/8 MIC
1/2 MIC
1 MIC
Nisin A concentration
(μg/ ml)
16
64
256
512
64
256
1024
2048
MW2
SAN2

Fig. 3.

(B)

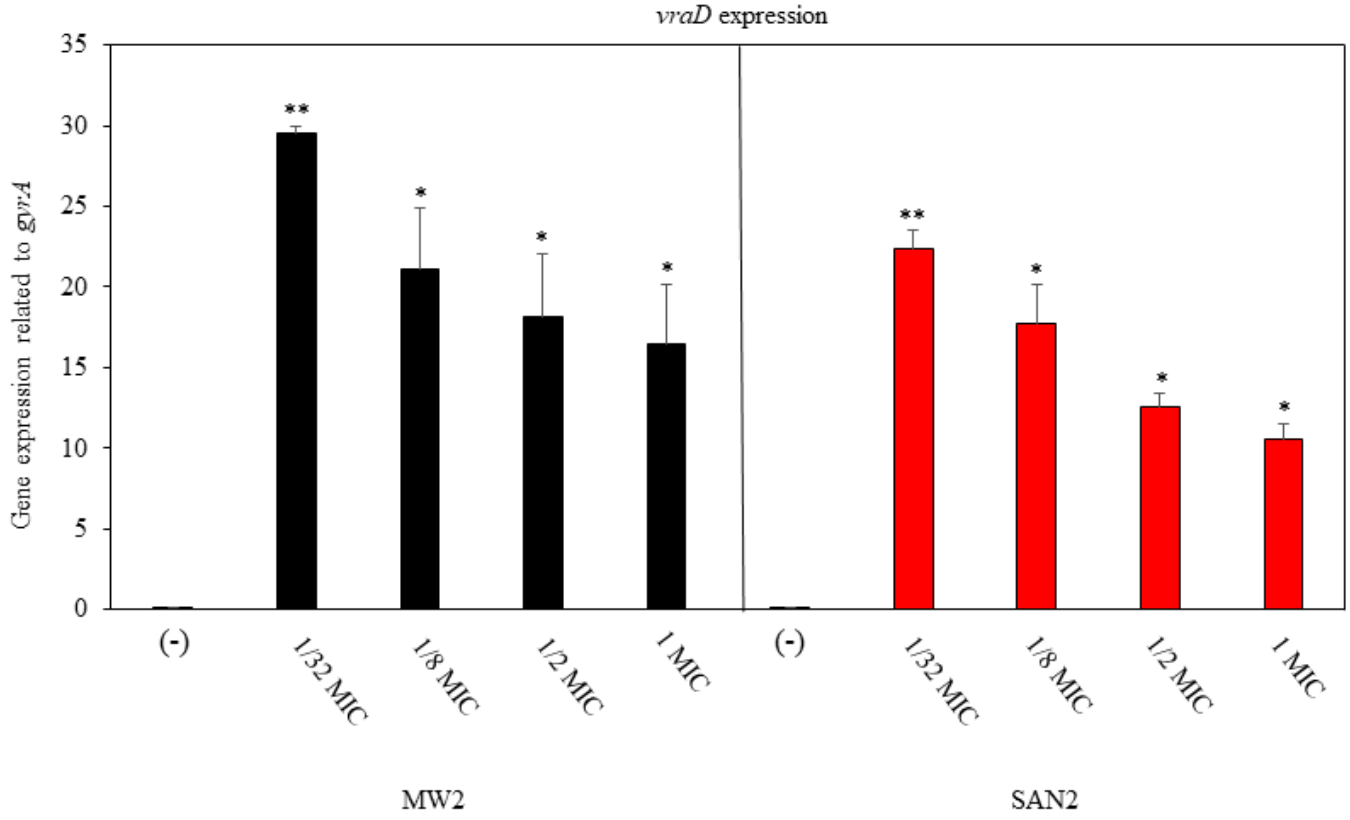

vraD expression
Gene expression related to *gyrA*
35
30
25
20
15
10
5
0
**
*
*
*
**
*
*
*
(-)
1/32 MIC
1/8 MIC
1/2 MIC
1 MIC
(-)
1/32 MIC
1/8 MIC
1/2 MIC
1 MIC
MW2
SAN2

Fig. 3.

(A)

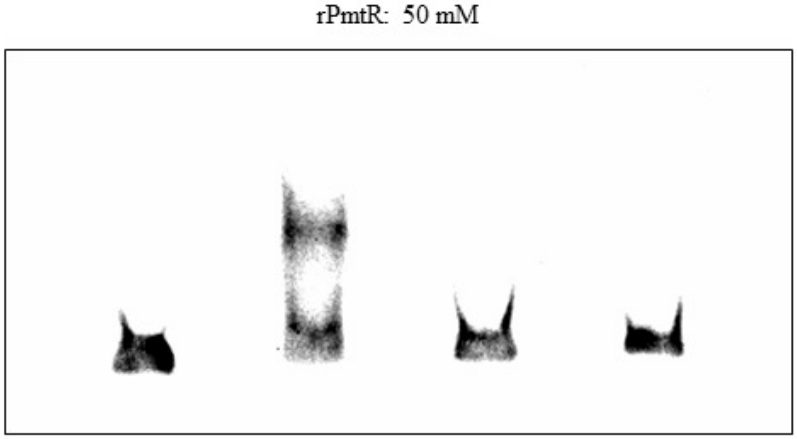
AATGGTAGTGTCATTTCATTTTATTCAACTGCTAGTAATACAATACTCAGTGTTATTTCTGAAGACTGG

TTGATAATGAGACATGCATATTTACAAAA**CTGTGTATATTGTG**TATATT**GTATATA***TACAGAAAGTG

→*pmtR*

ATAGGGGGACGTTGATG

(B)



MW2-rPmtR	-	+	-	+
SAN2-rPmtR	-	-	+	-
DIG-*pmtR*-F	+	+	+	+
Unlabeled *pmtR*-F	-	-	-	+

Fig. 4.

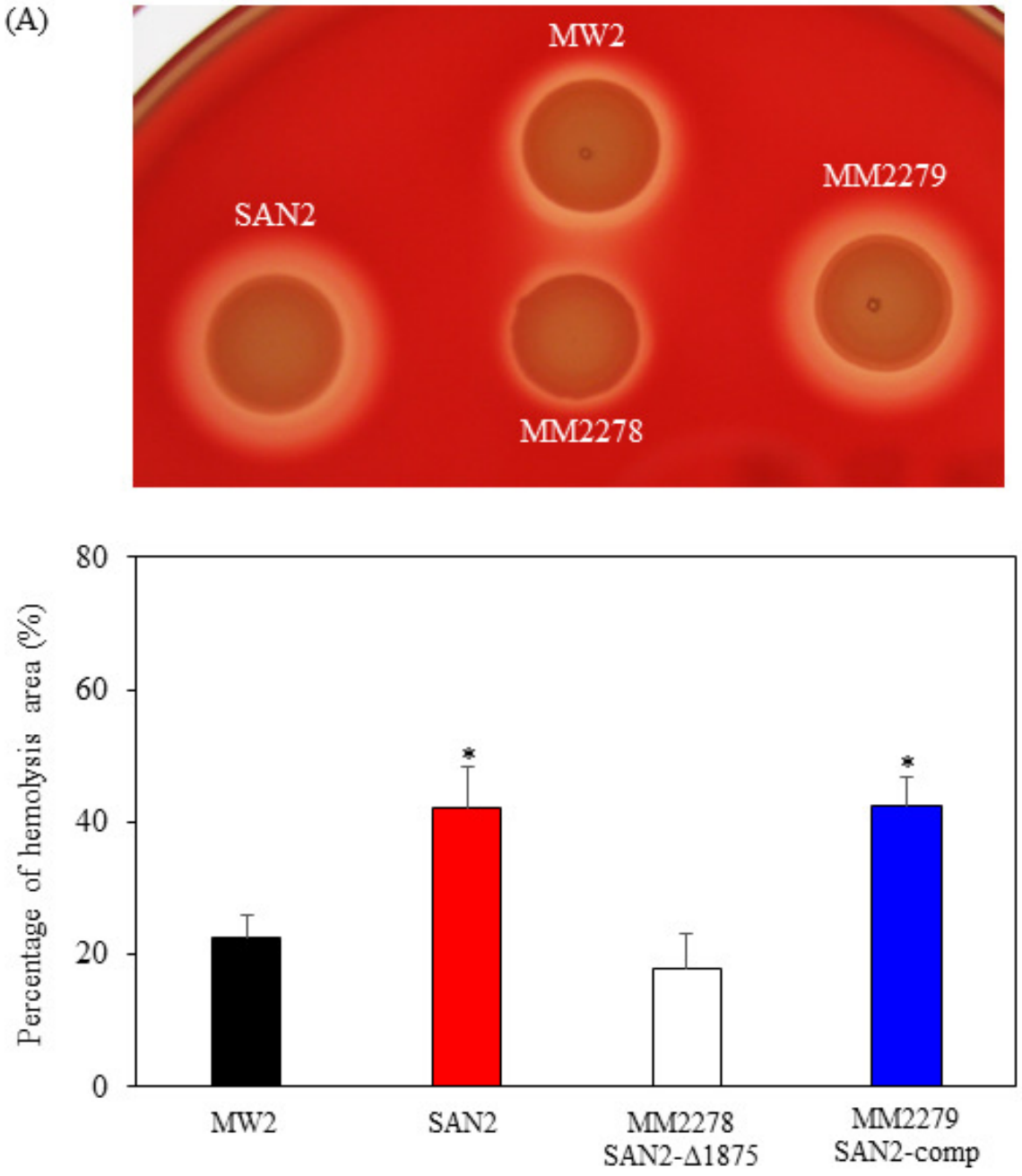
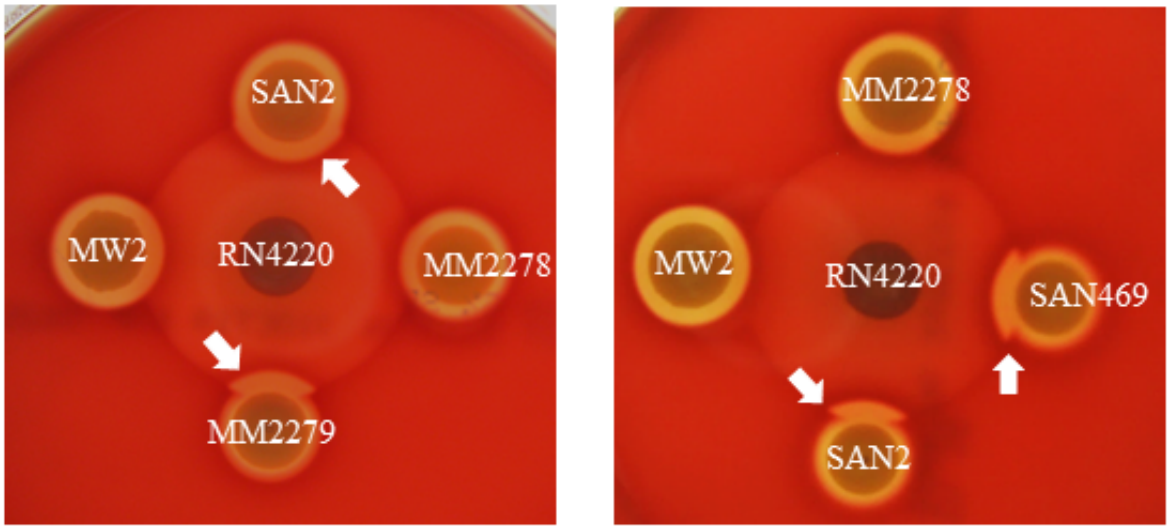

(A)
MW2
SAN2
MM2279
MM2278
Percentage of hemolysis area (%)
80
60
40
20
0
*
*
MW2
SAN2
MM2278
SAN2-Δ1875
MM2279
SAN2-comp

Fig. 5.

(B)


SAN2
MW2
RN4220
MM2278
MM2279
MM2278
MW2
RN4220
SAN469
SAN2

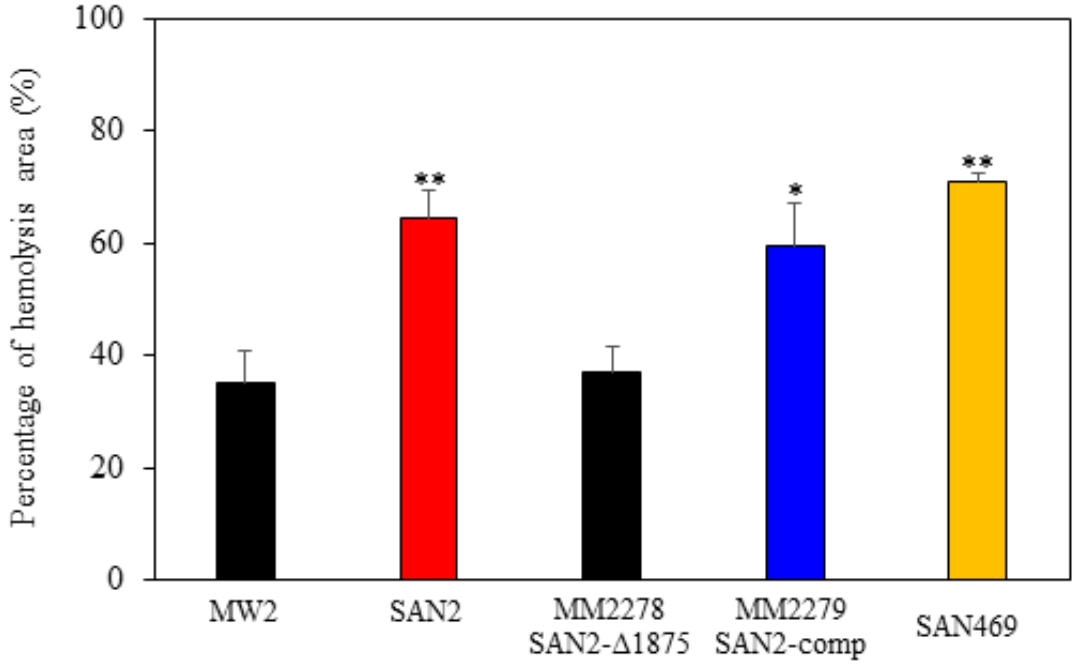

Percentage of hemolysis area (%)
100
80
60
40
20
0
**
*
**
MW2
SAN2
MM2278
SAN2-Δ1875
MM2279
SAN2-comp
SAN469

Fig. 5.

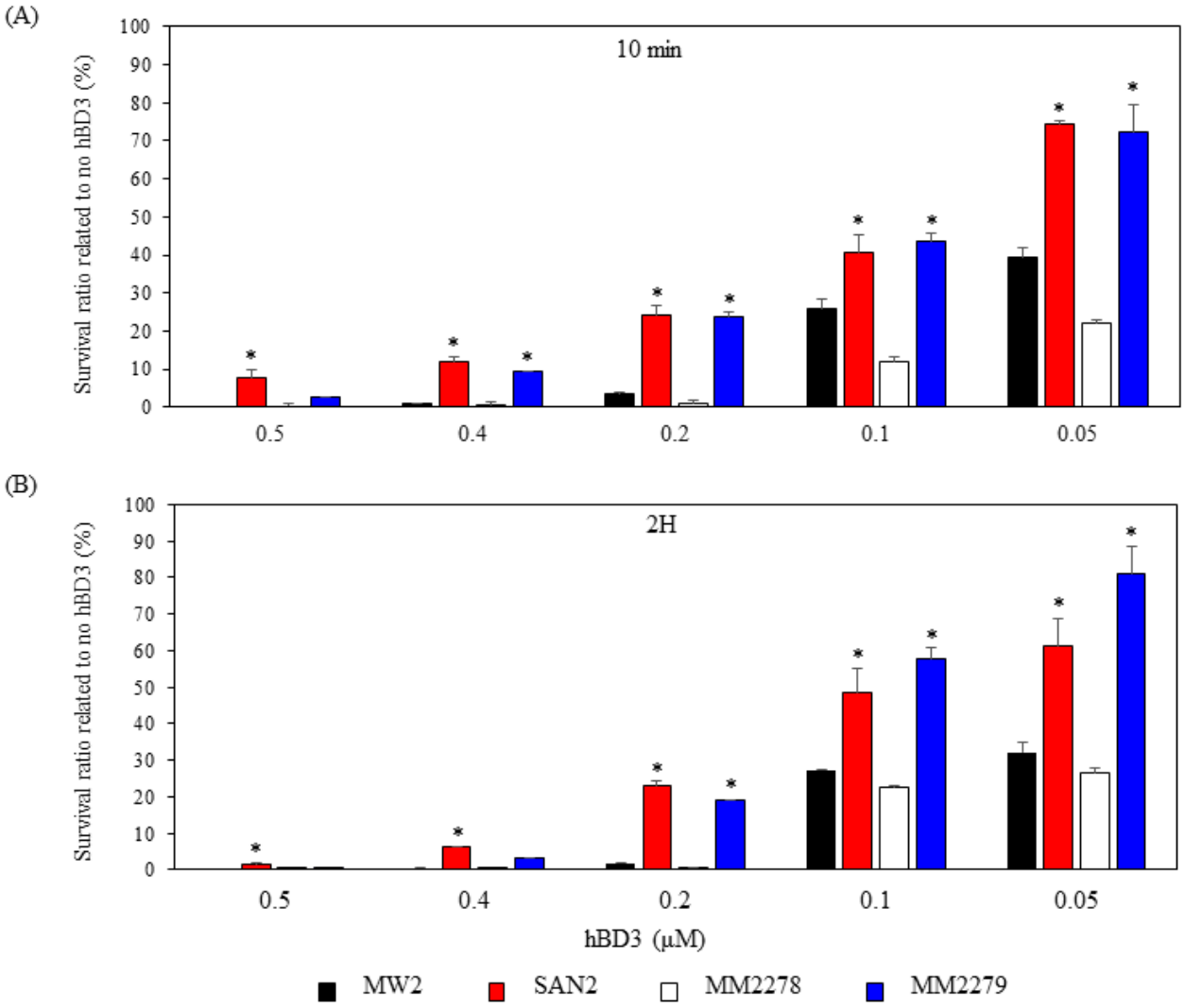

(A)
10 min
Survival ratio related to no hBD3 (%)
100
90
80
70
60
50
40
30
20
10
0
0.5
0.4
0.2
0.1
0.05
(B)
2H
Survival ratio related to no hBD3 (%)
hBD3 (μM)
MW2
SAN2
MM2278
MM2279

Fig. 6.

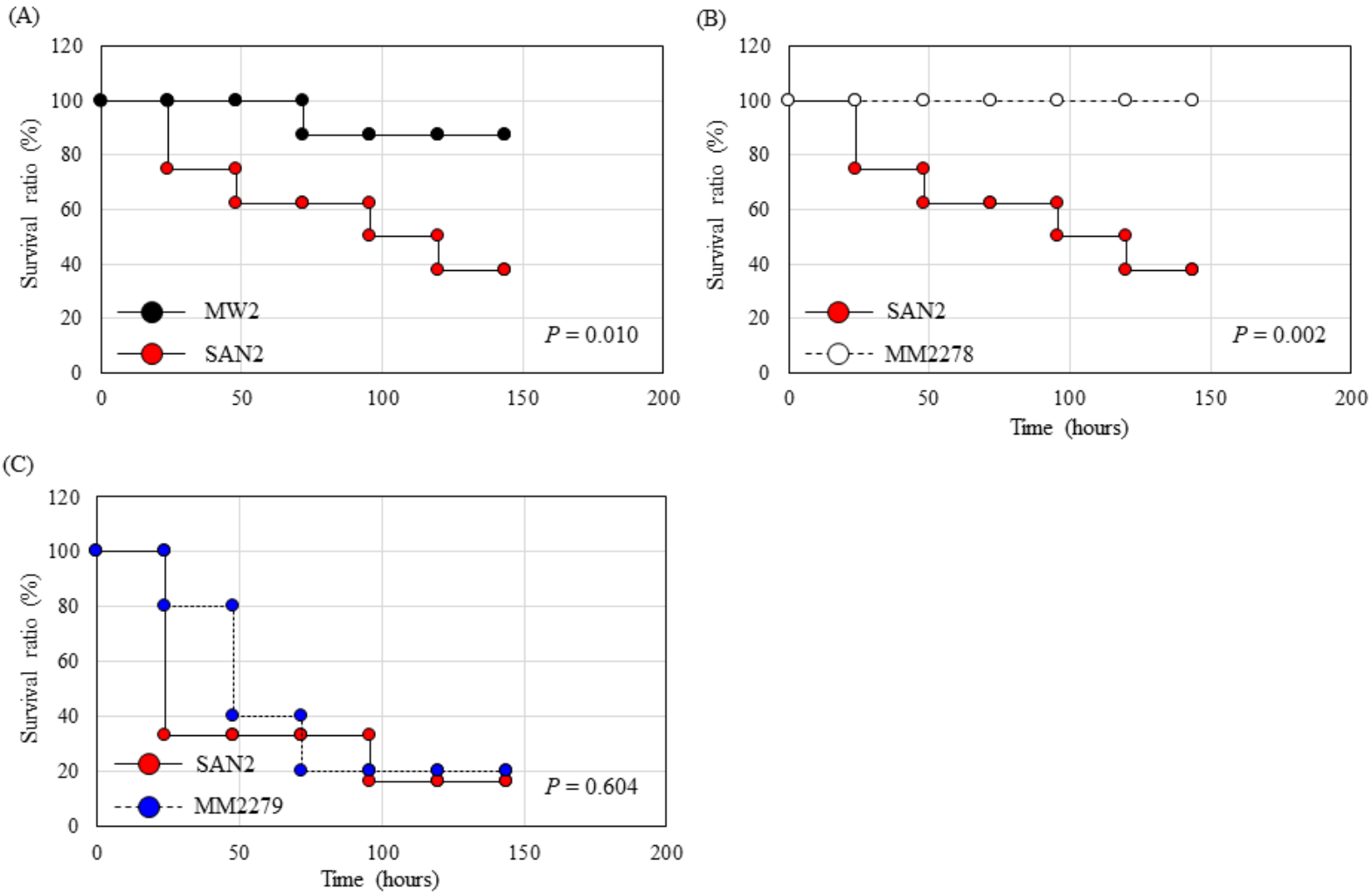

(A)
Survival ratio (%)
MW2
SAN2
P = 0.010
(B)
Survival ratio (%)
SAN2
MM2278
P = 0.002
Time (hours)
(C)
Survival ratio (%)
SAN2
MM2279
P = 0.604
Time (hours)

Fig. 7.

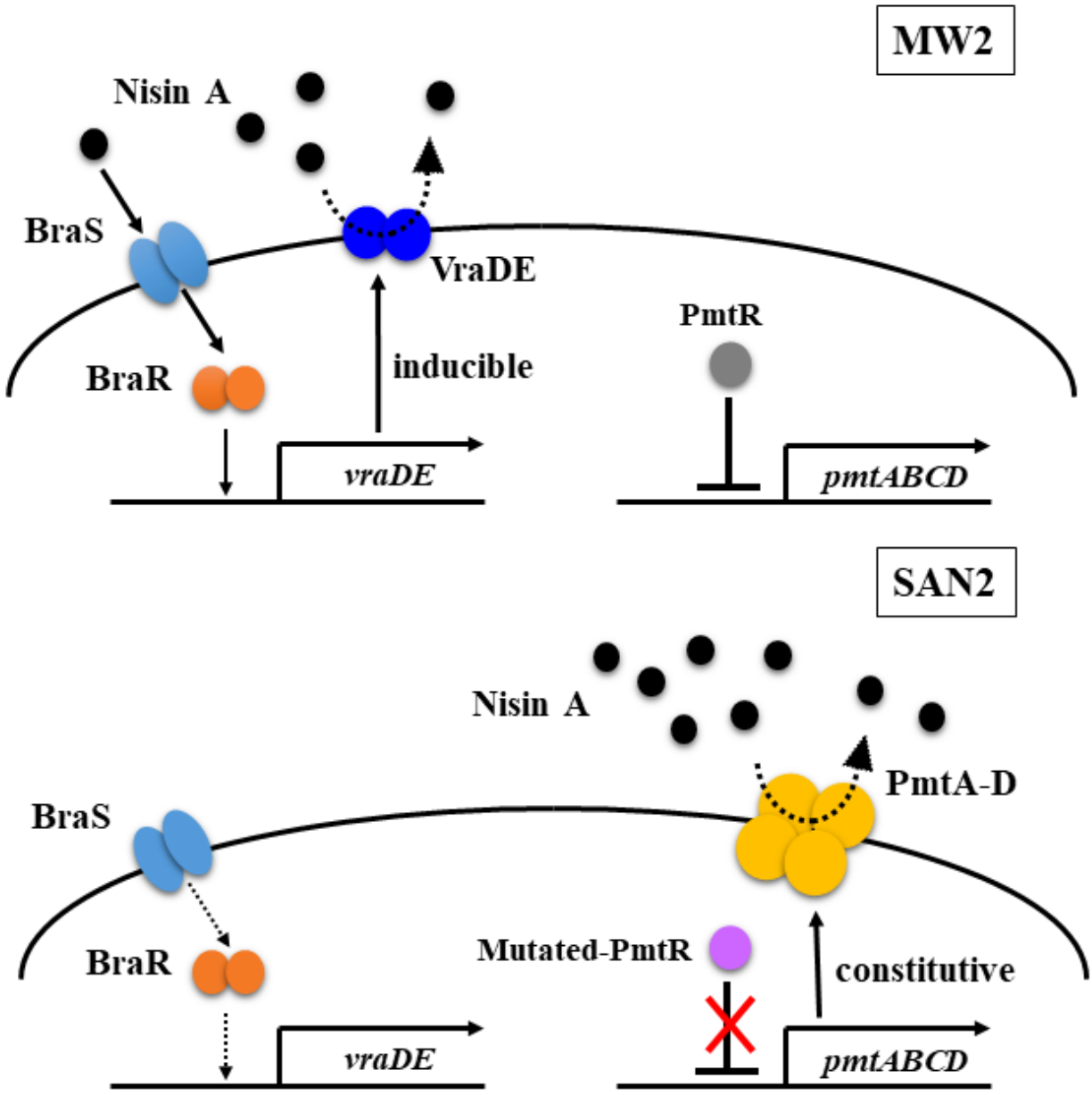

MW2
Nisin A
BraS
VraDE
BraR
inducible
PmtR
vraDE
pmtABCD
SAN2
Nisin A
PmtA-D
BraS
BraR
Mutated-PmtR
constitutive
vraDE
pmtABCD

Fig. 8.