Three Distinct Two-Component Systems Are Involved in Resistance to the Class I Bacteriocins, Nukacin ISK-1 and Nisin A, in *Staphylococcus aureus*

Miki Kawada-Matsuo1,*, Yuuma Yoshida1,2,*, Takeshi Zendo3, Junichi Nagao4, Yuichi Oogai1, Yasunori Nakamura2, Kenji Sonomoto3, Norifumi Nakamura2, Hitoshi Komatsuzawa1*

1 Department of Oral Microbiology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan, 2 Department of Oral and Maxillofacial Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan, 3 Laboratory of Microbial Technology, Division of Applied Molecular Microbiology and Biomass Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka, Japan, 4 Department of Functional Bioscience, Section of Infection Biology, Fukuoka Dental College, Fukuoka, Japan

**Abstract**

*Staphylococcus aureus* uses two-component systems (TCSs) to adapt to stressful environmental conditions. To colonize a host, *S. aureus* must resist bacteriocins produced by commensal bacteria. In a comprehensive analysis using individual TCS inactivation mutants, the inactivation of two TCSs, graRS and braRS, significantly increased the susceptibility to the class I bacteriocins, nukacin ISK-1 and nisin A, and inactivation of vraSR slightly increased the susceptibility to nukacin ISK-1. In addition, two ABC transporters (BraAB and VraDE) regulated by BraRS and one transporter (VraFg) regulated by GraRS were associated with resistance to nisin ISK-1 and nisin A. We investigated the role of these three TCSs of *S. aureus* in co-culture with *S. warneri*, which produces nukacin ISK-1, and *Lactococcus lactis*, which produces nisin A. When co-cultured with *S. warneri* or *L. lactis*, the braRS mutant showed a significant decrease in its population compared with the wild-type, whereas the graRS and vraRS mutants showed slight decreases. Expression of vraDE was elevated significantly in *S. aureus* co-cultured with nisin A/nukacin ISK-1-producing strains. These results suggest that three distinct TCSs are involved in the resistance to nisin A and nukacin ISK-1. Additionally, *braRS* and its related transporters played a central role in *S. aureus* survival in co-culture with the strains producing nisin A and nukacin ISK-1.

**Introduction**

Many bacteria produce antibacterial agents, called bacteriocins, which interfere with other bacteria in the bacterial community [1]. Bacteriocins are peptides or proteins that are ribosomally synthesized and show antimicrobial activity, mostly against bacterial species that are closely related to the producers [2]. In gram-positive bacteria, bacteriocins are classified into two major types, classes I and II bacteriocins [3,4,5]. Class I bacteriocins (≤5-kDa peptides) are called lantibiotics because they contain the unusual amino acids, lanthionine and methyllanthionine, which are posttranslationally modified, whereas class II bacteriocins contain unmodified amino acids. Lantibiotics are further divided into two types (types A and B) [6]. Type A lantibiotics include two subtypes, type A(I) such as nisin A and type A(II) such as nukacin ISK-1. The mode of action of lantibiotics, especially nisin A, has been well characterized [7,8]. Nisin A exhibits pore-forming activity and the inhibition of cell wall biosynthesis. The docking molecule of nisin A is lipid II, which is a membrane component consisting of one GlcNAc–MurNAc pentapeptide subunit linked to a polyisoprenoid and is associated with peptidoglycan biosynthesis in the membrane [9]. Nukacin ISK-1 also binds to lipid II [10]. However, the mode of action of nukacin ISK-1 is not pore-forming, but the inhibition of cell wall synthesis causes a bacteriostatic effect [10,11]. These bacteriocins are considered to affect other bacterial populations. In addition, some bacteriocins, such as nisin A, are used as preservatives for foods and other surfaces [1].

*Staphylococcus aureus* is a major pathogen in humans that can cause a variety of suppurative diseases, food poisoning, and toxic shock syndrome [12,13,14]. Furthermore, clinically isolated strains, particularly methicillin-resistant *S. aureus* (MRSA), exhibit multiple antibiotic resistances [15,16], resulting in serious problems with regard to therapy against *S. aureus* infectious diseases. Several two-component systems (TCSs) such as vraSR, agrCA, and graRS/aopRS, were recently reported as being associated with susceptibility to antibacterial agents [17,18,19,20]. A TCS, which is thought to function as a monitor and adapt to specific environmental conditions [21], is a prokaryote-specific signal transduction system that contains a sensor that encodes a sensory histidine-kinase and a regulator that encodes a cognate response regulator (RR) [22]. Recently, we and others identified one TCS,
called BceRS [23], BraRS [24] or NsaRS [25], which affects susceptibility to bacitracin. In addition, this TCS is associated with resistance to nisin A [24,25,26]. Because this TCS was designated separately by three different groups, we used the name BraRS in this study because Hiron et al. well characterized this TCS and the name (bacitracin resistance associated) is representative of its characteristics [24]. These findings suggest that S. aureus has several systems for resisting bacteriocins. Given that S. aureus is a commensal bacterium in the nasal cavity, skin, and intestine, this organism is faced with many other bacterial species, including other staphylococci such as S. epidermidis and S. warneri [27,28]; thus it is considered that S. aureus must resist bacteriocins to survive when it co-exists with bacteriocin-producing bacteria. Herein, we investigated the association of TCSs with susceptibility to the class I bacteriocin, nukacin ISK-1.

Table 1. Bacterial strains.

| Strain | Inactivated Gene ID | Gene Name | Characteristics | Reference |
|--------|---------------------|-----------|-----------------|-----------|
| Staphylococcus aureus |  |  |  |  |
| MW2 | – | – | Clinical strain, methicillin-resistant (mecA+) S. aureus | 18 |
| MM30 | – | – | MW2 harboring pCL8, CP | This study |
| FK61 | MW0198-99 | unassigned | MW0199: pCLS2.1 in MW2, TC | 18 |
| FK62 | MW0236-37 | lySRS | lyS: pCLS2.1 in MW2, TC | 18 |
| FK64 | MW0621-22 | graRS/opsRS | graR: pCLS2.1 in MW2, TC | 18 |
| FK65 | MW0667-68 | saeRS | saeR: pCLS2.1 in MW2, TC | 18 |
| FK66 | MW1208-09 | unassigned | MW1208: pCLS2.1 in MW2, TC | 18 |
| FK67 | MW1304-05 | arRS | arR: pCLS2.1 in MW2, TC | 18 |
| FK68 | MW1445-46 | srAB | srA: pCLS2.1 in MW2, TC | 18 |
| FK69 | MW1536-37 | phoPQ | phoP: pCLS2.1 in MW2, TC | 18 |
| FK71 | MW1789-90 | unassigned | MW1790: pCLS2.1 in MW2, TC | 18 |
| FK72 | MW1824-25 | vraSR | vraS: pCLS2.1 in MW2, TC | 18 |
| FK73 | MW1962-63 | agrCA | agrC: pCLS2.1 in MW2, TC | 18 |
| FK74 | MW2002-03 | kdpDE | kdpD: pCLS2.1 in MW2, TC | 18 |
| FK75 | MW2282-83 | hssRS | hssR: pCLS2.1 in MW2, TC | 18 |
| FK76 | MW2313-14 | nreBC | nreB: pCLS2.1 in MW2, TC | 18 |
| FK77 | MW2544-45 | braRS | braR: pCLS2.1 in MW2, TC | 18 |
| MM08 | MW2544 | braS | braS: pCLS2.1 in MW2, TC | 23 |
| MM09 | MW2544 | braS | pMM09(braS) in MM08, TC, CP | 23 |
| MM01 | MW0623-24 | vraFG | vraF: pCLS2.1 in MW2, TC | This study |
| MM02 | MW0623-24 | vraFG | pMM17(vraFG) in MM01, TC, CP | This study |
| MM07 | MW2543-42 | braAB | braA: pCLS2.1 in MW2, TC | 23 |
| MM07 | MW2542 | braB | braB: pCLS2.1 in MW2, TC | 23 |
| MM10 | MW2542 | braB | pMM10(braB) in MM07, TC, CP | 23 |
| MM03 | MW2620-21 | vraDF | vraD: pCLS2.1 in MW2, TC | 23 |
| MM11 | MW2620-21 | vraDE | pMM11(vraDE) in MM03, TC, CP | 23 |
| MM12 | MW0621-21 | graRS/opsRS | pMM12(graR) in FK64, TC, CP | This study |
| MM31 | MW0621-22 | graRS/opsRS | pMM31(vraDE) in FK64, TC, CP | This study |
| MM23 | MW1824-25 | vraSR | pMM231(vraSR) in FK72, TC, CP | This study |
| Escherichia coli |  |  |  |  |
| XL-2 | – | – | endA1 supF44 thi-1 hsdR17 recA1 gyrA96 relA1 lac [F’ proAB lacIq ZM15 Tn10 (Tet)] Amy Cam ] | Stratagene |
| mm01 | MW0623-24 | vraFG | pMM01/E. coli XL-2 for vraF inactivation, Amp | 4 |
| mm17 | MW0623-24 | vraFG | pMM17/E. coli XL-2 for vraFG complementation, Amp | This study |
| mm12 | MW0621-21 | graRS/opsRS | pMM12/E. coli XL-2 for graR complementation, Amp | 4 |
| mm31 | MW0621-22 | vraDE | pMM31/E. coli XL-2 for vraDE complementation, Amp | This study |
| mm23 | MW1824-25 | vraSR | pMM231/E. coli XL-2 for vraSR complementation, Amp | This study |

1Gene ID in S. aureus MW2.
2Chloramphenicol resistance.
3Tetracycline resistance.
4Ampicillin resistance.

doi:10.1371/journal.pone.0069455.t001
Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Tables 1 and 2. *S. aureus* inactivation mutants were constructed previously [23]. *S. aureus* and *S. warneri* were grown in trypticase soy broth (TSB; Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) at 37°C. *Escherichia coli* XL-II was grown in Luria-Bertani (LB) broth at 37°C. *Lactococcus lactis* was grown in De Man, Rogosa, Sharpe (MRS) broth (Becton Dickinson Microbiology Systems) at 37°C. Tetracycline (TC; 5 µg/ml) or chloramphenicol (CP; 10 µg/ml) was added to *S. aureus* when necessary.

Evaluation of Bacteriocin Susceptibility

Two methods (the minimum inhibitory concentration [MIC] and direct methods) were used to evaluate susceptibility to bacteriocins. MICs of nisin A, nukacin ISK-1, and bacitracin were determined by micro-dilution method as described previously [29]. Nisin A [30] and nukacin ISK-1 [31] were purified as described elsewhere. MICs were determined after 10 h of incubation. Three independent experiments were performed.

In the direct method, modified from a previous method [32], 2 µl of an overnight culture of *S. warneri* and *L. lactis* were spotted on an MRS agar plate. After overnight incubation at 37°C, 5 ml of pre-warmed TSB soft agar (0.75%) containing wild-type *S. aureus* or the mutants at 10^6 cells/ml was poured over the TSB agar plate. Plates were incubated for 20 h at 37°C. We confirmed that the diameter of the producing colony was uniformly 7 mm among all strains. The diameter of the inhibition zones surrounding bacteriocin-producing strains was measured in three directions. Three independent experiments were performed for the direct method, and the average result of the three experiments was calculated. Statistical analysis was performed with Dunnett’s method.

Effect of Nukacin ISK-1 and Nisin A on the Expression of TCSs and Transporters

A small portion (10^8 cells) of *S. aureus* cultured overnight was inoculated into 10 ml fresh TSB, and then grown at 37°C with shaking. When the optical density reached 0.5 at 660 nm, various concentrations of nukacin ISK-1 or nisin A were added to the medium. After the appropriate incubation, bacterial cells were collected. Total RNA was extracted from the bacterial cells with a FastRNA Pro Blue kit (MP Biomedicals, Solon, OH, USA) in accordance with the manufacturer’s protocol. A 1-µg aliquot of total RNA was reverse-transcribed to cDNA using a first-strand cDNA synthesis kit (Roche, Tokyo, Japan). Using cDNA as template, quantitative PCR was performed using a LightCycler system (Roche). Primers for *braR*, *vraR*, and *graR* (TCS), as well as for *braB*, *vraD*, and *vraF* (ABC transporter) were constructed and used to determine the optimal conditions for analysis of their expression, and *graA* was used as an internal control. Three independent experiments were performed, and the mean was calculated. Statistical analysis was performed with Dunnett’s method. The primers are listed in Table S1.

Co-culture of *S. aureus* with *S. warneri* or *L. lactis*

The method for the co-culture experiment is summarized schematically in Figure S1. Overnight cultures of *S. aureus* MM30 (MW2 harboring pCL8 [33]), *S. warneri* ISK-1, *S. warneri* ISK-1’, *L. lactis* ATCC 11454, and *L. lactis* NZ9000 were adjusted to OD_{660} = 1.0 and diluted ten-fold. Next, 100 µl of bacterial culture (*S. aureus* [10^7, 10^6, 10^5, 10^4 cells] and *S. warneri* [10^6 cells], *S. aureus* [10^7, 10^6, 10^5, 10^4 cells], and *L. lactis* [10^5 cells]) was mixed well. After overnight incubation at 37°C, the bacterial colonies growing on the agar plate were scraped and suspended in 1 ml of TSB. The appropriate dilutions were plated on TSA and TSA containing chloramphenicol (for selection of *S. aureus*). After 1 day, the colony-forming units (CFUs) grown on TSA and TSA containing antibiotics were determined, and we calculated the percent population of the *S. aureus* strain. We also extracted total RNA from scraped cells and performed cDNA synthesis using the method described above; gene expression analysis was conducted by quantitative PCR. The statistical

Table 2. Bacteriocin-producing and non-producing strains used in this study.

| Strain            | Bacteriocin          | Reference |
|-------------------|----------------------|-----------|
| *Lactococcus lactis* ATCC 11454 | Nisin A (class I) | 50        |
| *Lactococcus lactis* NZ9000 | Nisin A non-producing | 10       |
| *Staphylococcus warneri* ISK-1 | Nukacin ISK-1 (class I) | 31       |
| *Staphylococcus warneri* ISK-1’ | pPl-1 cured strain, nukacin ISK-1 non-producing | 51       |

doi:10.1371/journal.pone.0069455.t002

Table 3. Susceptibility to nisin A, nukacin ISK-1 and bacitracin of *S. aureus* mutants.

| Strain       | Relevant Genotype | MIC (mg/L) |
|--------------|-------------------|------------|
|              | Nisin A | Nukacin ISK-1 | Bacitracin |
| MW2          | Wild-type | 6.4 | 64 | 64 |
| FK77         | ΔbraRS | 3.2 | 16 | 32 |
| MM08         | ΔbraS  | 3.2 | 16 | 32 |
| MM09         | braS complement in ΔbraS | 6.4 | 64 | 64 |
| FK64         | ΔgraRS | 3.2 | 16 | 64 |
| MM12         | graR complement in ΔgraRS | 6.4 | 64 | 64 |
| FK72         | ΔvraSR | 6.4 | 32 | 32 |
| MM231        | vraSR complement in ΔvraSR | 6.4 | 64 | 64 |
| MM02         | ΔbraAB | 3.2 | 16 | 32 |
| MM07         | ΔbraB  | 3.2 | 16 | 32 |
| MM10         | braB complement in ΔbraB | 6.4 | 64 | 64 |
| MM03         | ΔvraDE | 3.2 | 8 | 16 |
| MM11         | vraDE complement in ΔvraDE | 6.4 | 64 | 64 |
| MM01         | ΔvraFG | 3.2 | 32 | 32 |
| MM17         | vraFG complement in ΔvraFG | 6.4 | 64 | 64 |

doi:10.1371/journal.pone.0069455.t003

Resistance to Class I Bacteriocins in *S. aureus*
analysis was conducted by Dunnnett’s method for the percentage ratio of the S. aureus population and the expression of braA, vraD, and vraF were performed as described in the Materials and Methods. (A) braA, vraF, vraD, braR, vraR, and graR expression in S. aureus MW2 exposed to various concentrations of nukacin ISK-1 (5-min exposure). *, statistically significant difference from the wild-type as tested using Dunnett’s method (p<0.05). (B) Time course experiment of braA, vraF, and vraD expression in S. aureus MW2 exposed to nukacin ISK-1 (4 μg/ml). (C) braA, vraF, and vraD expression in S. aureus MW2 and three mutants (braRS, graRS, and vraSR mutant) exposed to nukacin ISK-1 (4 μg/ml). *, statistically significant difference from the wild-type as tested using Dunnett’s method (p<0.05).

doi:10.1371/journal.pone.0069455.g001

Figure 1. Expression of TCSs and ABC transporters in S. aureus exposed to nukacin ISK-1. Analysis of expression levels of braR, graR, vraR, braA, vraD, and vraF were performed as described in the Materials and Methods. (A) braA, vraF, vraD, braR, vraR, and graR expression in S. aureus MW2 exposed to various concentrations of nukacin ISK-1 (5-min exposure). *, statistically significant difference from the wild-type as tested using Dunnett’s method (p<0.05). (B) Time course experiment of braA, vraF, and vraD expression in S. aureus MW2 exposed to nukacin ISK-1 (4 μg/ml). (C) braA, vraF, and vraD expression in S. aureus MW2 and three mutants (braRS, graRS, and vraSR mutant) exposed to nukacin ISK-1 (4 μg/ml). *, statistically significant difference from the wild-type as tested using Dunnett’s method (p<0.05).

Next, the co-culture of S. aureus TCS or ABC transporter mutants with S. warneri or L. lactis was investigated using the method described above. The concentrations of bacterial cells used in this assay were 10^7 cells/ml S. aureus mutant and 10^6 cells/ml S. warneri or 10^6 cells/ml S. aureus mutant and 10^8 cells/ml L. lactis. For the co-culture of S. aureus with S. warneri, TSA containing chloramphenicol (wild-type S. aureus, 10 μg/ml) and tetracycline (S. aureus mutants, 10 μg/ml) were used for S. aureus selection.

Susceptibility of Bacitracin-treated S. aureus to Nukacin ISK-1 and Nisin A

To investigate whether the VraDE expression level affects susceptibility to nisin A and nukacin ISK-1, we evaluated the susceptibility of bacitracin-pretreated S. aureus to nisin A and nukacin ISK-1 using the MIC method, as described above, and the spot-on-lawn method described elsewhere [11]. Previously, we reported that bacitracin at a sub-MIC induced VraDE expression significantly [23]. S. aureus pretreated with or without bacitracin was used for both methods. S. aureus cells (10^7/ml) were exposed to a sub-MIC (1/8 MIC: 0 μg/ml) of bacitracin (Sigma-Aldrich,
Resistance to Class I Bacteriocins in *S. aureus*

**Results**

Susceptibility of TCS-inactivated Mutants to nisin A and Nukacin ISK-1

We determined the susceptibility of TCS mutants to nukacin ISK-1 using the MIC and the direct method (Table 3, Figure S2). The *graRS* and *braRS* mutants exhibited higher susceptibility to nukacin ISK-1, and the *vraSR* mutant exhibited higher susceptibility to nukacin ISK-1. The susceptibilities of other TCS mutants other than *braRS*, *graRS* and *vraSR* to nukacin ISK-1 did not change (data not shown). We also evaluated the susceptibility of TCS mutants to nisin A and nukacin ISK-1 using the direct method (Figure S2), and obtained similar results to those obtained using the MIC method. Furthermore, we determined the susceptibility of the mutants and their complemented strains. We found that each complemented strain could restore the respective mutation (Table 3). Also, we investigated the susceptibility of TCS mutants against nisin A, and found similar results to nukacin ISK-1 except that the susceptibility of the *vraRS* mutant to nisin A did not increase (Table 3, Figure S2).

Susceptibility of ABC Transporter-inactivated Mutants to nisin A and nukacin ISK-1

Previously, we demonstrated that BraRS regulates two transporters (*VraDE* and *BraAB*) for resistance against bacitracin, and that GraRS regulates one transporter (*VraFG*) [19,23]. We evaluated the susceptibility of these three mutants (*vraDE*, *braAB*, and *vraFG* mutants) to nukacin ISK-1 (Table 3, Figure S2). The *vraFG*, *vraDE*, and *braAB* mutants were more susceptible to nukacin ISK-1. Additionally, we found that each complemented strain could restore the respective mutation (Table 3). Also, we investigated the susceptibilities of these mutants to nisin A, and obtained similar results to those for nukacin ISK-1 (Table 3, Figure S2).

Effect of Nukacin ISK-1 and Nisin A on the Expression of TCSs and Transporters

Given that the susceptibility to nisin A and nukacin ISK-1 was changed by inactivation of three TCSs (*braRS*, *graRS*, and *vraSR*) and three transporters (*vraFG*, *vraDE*, and *braAB*), we investigated the expression of those TCSs and transporters upon exposure of bacterial cells to nukacin ISK-1 and nisin A.

Among the three TCSs, the expression levels of the *braR* and *graR* transcripts did not increase upon exposure to nukacin ISK-1, whereas the expression of *vraR* was induced (Figure 1A). Regarding the three ABC transporters, the expression of *braA* and *vraD* in the wild-type MW2 strain was rapidly induced by the addition of nukacin ISK-1 to the medium, whereas *vraF* expression was not (Figures 1A and 1B). This induction occurred after 5 min of exposure, after which the transcript levels of both transporters gradually decreased (Figure 1B). The expressions of both *braA* and *vraD* were dose-dependent (Figure 1A). However, the induction of *vraD* and *braA* expression by nukacin ISK-1 was not observed in the *braRS* mutant, although both genes showed increased expression in the *graRS* and *vraSR* mutants (Figure 1C). In the *graRS* mutant, *vraF* expression decreased irrespective of nukacin ISK-1 addition, but in the other two mutants, the *vraF* expression level was not significantly different from that of the wild-type (Figure 1C).

In addition, we investigated the expression of TCSs and transporters in the wild-type and its mutants by addition of nisin A. We obtained results similar to those of nukacin ISK-1, except that nisin A did not induce *vraSR* expression (Figure S3).

Based on these results, we concluded that the expression of two ABC transporters, *vraD* and *braA*, was induced by nukacin ISK-1 and nisin A, and that this effect was mediated by one TCS, BraRS. Also, the expression of another transporter, *vraF*, was not induced, but *vraF* expression was regulated by GraRS.

Co-culture of *S. aureus* with *S. warneri* or *L. lactis*

Co-cultures of *S. aureus* MM30 with *S. warneri* ISK-1, *S. warneri* ISK-1ApPl-1 (pPl-1 plasmid cured), *L. lactis* ATCC 11454 (nisin A-producing strain), and *L. lactis* NZ9000 (nisin A non-producing strain) were analyzed. When *S. aureus* MM30 was co-cultured with...
S. warneri ISK-1, which produces nukacin ISK-1, the population of the braRS mutant was significantly lower at any S. aureus/S. warneri ratio, compared to that of the wild-type (MM30) (Figure 2A). When S. aureus was co-cultured with S. warneri ISK-1 ΔpPI-1, which does not produce nukacin ISK-1, the population of the braRS mutant was similar to that of the wild-type. We evaluated the expression of vraD and braA under co-culture conditions (Figure 2B). The expression of both increased when S. aureus was co-cultured with S. warneri ISK-1 at various ratios. In particular, both increased gradually as the ratio of S. aureus to S. warneri decreased before spotting on the TSA plate.

S. aureus MM30 was co-cultured with L. lactis ATCC 11454, which produces nisin A, or NZ9000, which does not. Results were similar to those of nukacin ISK-1 (Figure S4).

Figure 3A shows the S. aureus population ratio when 10⁶ cells of S. aureus mutants were mixed with 10⁷ S. warneri ISK-1 cells. The braRS, braAB, and vraDE mutants exhibited drastically decreased population ratios compared with that of the wild-type. In addition, the graRS, vraSR, and vraFG mutants showed slight decreases compared with the wild-type.

Similar results were obtained when 10⁵ cells of S. aureus mutants were mixed with 10⁷ L. lactis cells (Figure 3B). When co-cultured with the ATCC 11454 strain, the population ratios of the braRS, braAB, and vraDE mutants were significantly lowered, compared to that of the wild-type. Additionally, we investigated vraD expression in TCS mutants. The wild-type, graRS and vraSR mutants showed significantly increased vraD expression upon co-culture with bacteriocin-producing strains (Figure 3C). However, vraD expression did not increase in the wild-type, graRS and vraSR mutants when co-cultured with bacteriocin-non-producing strains (data not shown).

From the results of co-culture assay, we found that the inactivation of one TCS (braRS) and two BraRS-regulated transporters (braAB and vraDE) caused a significant decrease in the S. aureus population when co-cultured with a nukacin ISK-1- or nisin A-producing strain.
Susceptibility of Bacitracin-treated \textit{S. aureus} to nisin A and nukacin ISK-1

Because \textit{vraDE} expression of the \textit{S. aureus} wild-type was induced by nisinA or nukacin ISK-1, we investigated whether the VraDE-overexpressing strain showed higher resistance to nisinA and nukacin ISK-1. We used \textit{S. aureus} pretreated with a sub-MIC of bacitracin, which induced the expression of VraDE [23] but was not bactericidal. The wild-type strain pretreated with bacitracin showed an increased nisin A and nukacin ISK-1 MICs compared to that without bacitracin (Table 4). Also, the MICs of the \textit{graRS} and \textit{vraRS} mutants against nisin A and nukacin ISK-1 were increased by pretreatment with bacitracin, whereas the MIC of the \textit{braRS} mutant did not change. In addition, we constructed a VraDE-overexpression strain using the pCL15 plasmid. VraDE overexpression in the \textit{graRS} mutant caused decreased susceptibility to both nisin A and nukacin ISK-1 (Table 4).

**Discussion**

We performed a comprehensive analysis of the TCSs involved in the susceptibility to the class I bacteriocin, nukacin ISK-1, in \textit{S. aureus} and identified several TCSs to be associated with nukacin ISK-1 susceptibility (Figure S2, Table 3). Previously, BraRS and GraRS were also shown to be associated with bacitracin and nisin A resistance [23,24,25,36]. In addition, Hiron \textit{et al.} demonstrated that BraRS is activated by nisin A, which induces the expression of transporters [24], as confirmed by the results in this study (Figure 1, Figure S3). Therefore, BraRS is involved in the resistance to nukacin ISK-1, nisin A and bacitracin. Bacitracin binds to

---

**Table 4.** Susceptibility of bacitracin-treated and \textit{vraDE}-overexpressing \textit{S. aureus} to nisin A and nukacin ISK-1.

| Strain | Relevant Genotype | Bacitracin (—) | Bacitracin (+) |
|--------|------------------|----------------|----------------|
|        |                  | Nisin A | Nukacin ISK-1 | Nisin A | Nukacin ISK-1 |
| MW2    | Wild-type        | 6.4    | 12.8          | 64     | 128           |
| FK64   | ΔapsRS in MW2    | 3.2    | 12.8          | 32     | 128           |
| FK72   | ΔvraSR in MW2    | 6.4    | 12.8          | 32     | 128           |
| FK77   | ΔbraRS in MW2    | 3.2    | 12.8          | 16     | 128           |

| Strain | Relevant Genotype | Nisin A | Nukacin ISK-1 | Nisin A | Nukacin ISK-1 |
|--------|------------------|---------|---------------|---------|---------------|
|        |                  |         |               |         |               |
| MM31   | vraDE in FK64    | 3.2     | 12.8          | 32      | 128           |

Expression of braR, graR, vraR, vraD, and vraF was determined using the method described in the Materials and Methods. (A) braA, vraF, vraD, braR, graR, and vraF expression in \textit{S. aureus} MW2 exposed to various concentrations of nisin A (5 min exposure). *, statistically significant difference from the wild-type as tested using Dunnett’s method \((p<0.05)\). (B) Time course experiment of braA, vraF, and vraD expression in \textit{S. aureus} MW2 exposed to nisin A (16 \(\mu\)g/ml). (C) braA, vraF, and vraD expression in \textit{S. aureus} MW2 and three mutants (braRS, graRS, and vraSR mutant) exposed to nisin A (16 \(\mu\)g/ml). *, statistically significant difference from the wild-type as tested using Dunnett’s method \((p<0.05)\).

doi:10.1371/journal.pone.0069455.t004

Figure 4. Association of TCSs and ABC transporters with susceptibility to class I bacteriocins, nisin A and nukacin ISK-1.

doi:10.1371/journal.pone.0069455.g004
undecaprenyl pyrophosphate, resulting in inhibition of lipid II formation, whereas nisin A and nukacin ISK-1 bind to lipid II [9,10]. Vancomycin also binds to the D-alanine-D-alanine molecule in lipid II; however, the braRS mutant did not exhibit marked susceptibility to vancomycin [16]. Nisin A binds to the pyrophosphate moiety of lipid II, resulting in pore formation in the membrane [9]. Therefore, we propose that BraRS is associated with susceptibility to antibacterial agents related to the membrane-anchoring region of lipid II. Recently, BraAB was found to act as a cofactor for BraRS but not as a direct resistance factor, suggesting that it is associated with the regulation of vraDE [24]. BraRSs in \textit{S. aureus} shows homology with TCSs of other gram-positive bacteria, including \textit{Enterococcus}, \textit{Bacillus} and \textit{Streptococcus} [37,38,39,40]. Therefore, the BraRS system is widely conserved in gram-positive bacteria.

GraRS is involved in susceptibility to cationic peptides such as defenses, gentamicin, and vancomycin [41,42,43] because it regulates two factors, \textit{dlt} and \textit{mpfF} (\textit{junC}), both of which influence the cell surface charge of bacteria [36,42,43,44]. Inactivation of \textit{gdrRS} causes an increase in the negative charge of the cell surface, resulting in increased attraction of the cationic peptides nisin A and nukacin ISK-1, but not bacitracin, to the cell membrane. In addition, \textit{vraFG} downstream of \textit{gdrRS} is associated with susceptibility to nisin A and nukacin ISK-1, but not bacitracin. Recently, Falord et al. demonstrated that VraFG did not act as a detoxification module but was associated with GraRS activation [45]. Therefore, the difference in susceptibilities between nisin A/nukacin ISK-1 and bacitracin may be due to the charges of these peptides.

\textit{VraSR} (vancomycin resistance associated sensor/ regulator) was first identified as a factor responsible for vancomycin susceptibility [46]. Further investigations revealed that this TCS regulates many factors involved in cell wall biosynthesis and that it is associated with susceptibility to cell wall synthesis inhibitors including beta-lactams, cycloserine, teicoplanin, and bacitracin [47,48,49]. In this study, the inactivation of \textit{vraSR} led to an increase in susceptibility to nukacin ISK-1, but not nisin A (Table 3 and Figure S2). Moreover, nukacin ISK-1 induced \textit{vraSR} expression (Figure 1A), whereas nisin A did not (Figure S3). Although nisin A also exhibits an inhibitory effect on cell wall biosynthesis, we did not detect elevated \textit{vraSR} expression under our conditions. Nukacin ISK-1 and nisin A are type A lantibiotics, but their subtypes (type A [I] and type A [II], respectively) and structure differ (Figure S5). Also, their modes of action differ; nisin A exhibits a bactericidal effect by causing pore formation and the inhibition of cell wall biosynthesis [7,8], whereas nukacin ISK-1 acts as a bacteriostatic agent by inhibiting cell wall biosynthesis [11]. Therefore, we hypothesize that the different modes of action of these two bacteriocins reflect the different responses in terms of \textit{vraSR} expression.

We evaluated the competition between two bacterial strains using a co-culture method. Notably, the inactivation of three TCSs, but especially \textit{gdrRS} and \textit{braRS}, caused significantly increased susceptibility to nisin A and nukacin ISK-1 by the direct and the MIC methods (Figure S2 and Table 3); however, only one TCS, BraRS, was a major contributor to \textit{S. aureus} survival in co-culture with \textit{S. warneri} or \textit{L. lactis}, which produces bacteriocin (Figures 2, 3 and S4). In particular, the \textit{gdrRS} mutant showed different results between the direct and co-culture methods. We hypothesized that this difference in the \textit{gdrRS} mutant was due to the different level of VraDE expression upon exposure of the mutant to nisin A and nukacin ISK-1. In the direct assay, \textit{S. aureus} cells that expressed VraDE at a very low level were exposed to relatively high concentrations of nisin A and nukacin ISK-1. In the early period after bacteriocin exposure, VraDE expression was not sufficient for BraRS-mediated nisin A/nukacin ISK-1 resistance; thus the \textit{graRS} mutation, which exhibited a more negatively charged cell surface [42,43], showed marked susceptibility to nisin A and nukacin ISK-1. Conversely, \textit{S. aureus} cells in the co-culture are exposed to a low concentrations (non-lethal) of nisin A or nukacin ISK-1 during the early stage of co-culture, and so VraDE expression is induced. Upon exposure to a high concentration of nisin A or nukacin ISK-1 during further incubation, \textit{S. aureus} expressed VraDE at a level sufficient for resistance. Table 4 reflects our hypothesis that pretreatment of the \textit{gdrRS} mutant with bacitracin resulted in marked nisin A and nukacin ISK-1 resistance. Additionally, we obtained the same result when \textit{S. aureus} cells were pretreated with nisin A (data not shown). Furthermore, similar results were obtained using the VraDE overexpression strain (Table 4). Therefore, the different results of the direct and co-culture methods using the \textit{graRS} mutant were due to the different VraDE expression levels upon exposure of \textit{S. aureus} to a high concentration of nisin A or nukacin ISK-1.

Based on our findings, we propose that BraRS and GraRS have distinct functions in terms of resistance to nisin A and nukacin ISK-1. BraRS is an intrinsic factor for such resistance. However, upon exposure of \textit{S. aureus} to a relatively high level of nisin A or nukacin ISK-1, GraRS is important for resistance until significant induction of VraDE expression by BraRS occurs. Therefore, these two TCSs function coordinately in resistance to nisin A and nukacin ISK-1. Furthermore, in addition to these two TCSs, VraSR is independently activated upon inhibition of cell wall biosynthesis. Class I bacteriocins, such as nisin A and nukacin ISK-1, inhibit cell wall biosynthesis, although increased expression of VraSR was identified only in \textit{S. aureus} cells exposed to nukacin ISK-1. Our results strongly indicate that \textit{S. aureus} possesses three distinct class-I-bacteriocin-resistance systems.

In conclusion, we demonstrated that several TCSs and ABC transporters in \textit{S. aureus} are associated with resistance to bacteriocins produced by other bacteria. Notably, \textit{S. aureus} possesses multiple TCSs that resist nisin A and nukacin ISK-1 (Figure 4). In particular, the BraRS system is specific for nisin A and nukacin ISK-1. Conversely, GraRSs and VraSR confer broad-spectrum resistance against cationic peptides and cell-wall synthesis inhibitors, respectively. Our findings suggest that \textit{S. aureus} possesses several TCSs that facilitate its survival in complex bacterial communities.

Supporting Information

Figure S1 Method for the co-culture experiment. (TIF)

Figure S2 Susceptibility of TCS- and ABC transporter-inactivated mutants to nukacin ISK-1 and nisin A. The susceptibilities of \textit{S. aureus} MW2 and its TCS- or ABC transporter-mutants to nukacin ISK-1 and nisin A were evaluated by the direct method. (A) In total, 2 µl of overnight cultures of bacteriocin-producing strains were spotted on an MRS agar plate. After overnight incubation at 37 °C, pre-warmed MRS soft agar (0.75%) containing \textit{S. aureus} was poured over the surface of the MRS agar plate. Plates were incubated for 20 h at 37 °C. (B) The diameters of the inhibition zones surrounding the bacteriocin-producing strain were measured in three directions. Three experiments were performed independently, and the average result of the three experiments was calculated. * statistically significant difference from the wild-type as tested using Dunnett’s method (\(p<0.05\)). The error bar represents the standard deviation. (TIF)
Figure S3  Expression of TCSs and ABC transporters in \textit{S. aureus} exposed to nisin A. (TIF)

Figure S4  Co-culture of \textit{S. aureus} with \textit{L. lactis}. Co-culture experiment was performed as described in the Materials and Methods. (A) Percent ratio of the \textit{S. aureus} population when mixed with various concentrations of \textit{L. lactis} ATCC 11454 and nisin A-non-producing \textit{L. lactis} NZ9000. (B) Expression of ABC transporters (braD and vraD) when mixed with various concentrations of \textit{L. lactis} ATCC 11454. *p<0.05, as determined by Dunnett’s method for expression of the ABC transporters (braD and vraD). (TIF)

Figure S5  Structures of nisin A and nukacin ISK-1. (A) nisin A; (B) nukacin ISK-1. Shaded residues indicate amino acids: A-S-A, lanthionine; Alu-S-A, 3-methylanthionine; Dha, dehydroalnine; Dibh, dehydrobutyryl; Im, N-formylmethionine. (TIF)

Table S1  

Author Contributions

Conceived and designed the experiments: HK MK KS NN. Performed the experiments: MK YO TZ JN YY. Analyzed the data: MK TZ JN YY. Contributed reagents/materials/analysis tools: MK TZ JN. Wrote the paper: HK KS TZ MK NN.

References

1. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3: 777–788.
2. Nissen-Meyer J, Nes IF (1997) Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Arch Microbiol 167: 57–77.
3. Asaduzzaman SM, Sonomoto K (2009) Lantibiotics: diverse activities and unique modes of action. J Biosci Bioeng 107: 475–487.
4. Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12: 59-65.
5. Nissen-Meyer J, Rogne P, Oppegaard C, Haugen HS, Kristiansen PF (2009) Structure-function relationships of non-lanthionine containing peptide (class II) bacteriocins produced by gram-positive bacteria. Curr Pharm Biotechnol 10: 19–37.
6. Jung G (1991) Lantibiotics: a survey, p. 1–34. In G. Jung and H-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
7. Hyde AJ, Parisot J, McNichol A, Bonev BB (2006) Nisin-induced changes in Bacillus morphology suggest a paradigm of antibiotic action. Proc Natl Acad Sci USA 103: 19896–19901.
8. Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G et al. (2001) Analysis and characterization of lacticin Q, a novel bacteriocin belonging to a new family of unmodified bacteriocins of gram-positive bacteria. Appl Environ Microbiol 77: 2671–2077.
9. Foster TJ (2004) The "superbug". J Clin Invest 114: 1693–1704.
44. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G et al. (1999) Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274: 8405–8410.

45. Falord M, Karimova G, Hiron A, Msadek T (2012) GraXSR proteins interact with the VraFG ABC transporter to form a five component system required for cationic antimicrobial peptide sensing and resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 56: 1047–1058.

46. Kuroda M, Kuroda H, Oshina T, Takeuchi F, Mori H et al. (2003) Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol Microbiol 49: 807–821.

47. Belcheva A, Golemi-Kotra D (2008) A close-up view of the VraSR two-component system. A mediator of *Staphylococcus aureus* response to cell wall damage. J Biol Chem 283: 12354–12364.

48. McCallum N, Meier PS, Heusser R, Berger-Bachi B (2011) Mutational analyses of open reading frames within the *vraSR* operon and their roles in the cell wall stress response of *Staphylococcus aureus*. Antimicrob Agents Chemother 55: 1391–1402.

49. Muthaiyan A, Silverman JA, Jayaswal RK, Wilkinson BJ (2008) Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. Antimicrob Agents Chemother 52: 980–990.

50. Chandrapati S, O’Sullivan DJ (2002) Characterization of the promoter regions involved in galactose- and nisin-mediated induction of the nisA gene in *Lactococcus lactis* ATCC 11455. Mol Microbiol 46: 467–477.

51. Aso Y, Koga H, Sashihara T, Nagaor J, Kanemasa Y et al. (2005) Description of complete DNA sequence of two plasmids from the nukacin ISK-1 producer, *Staphylococcus warneri*. Plasmid 53: 164–178.