Opposing genetic polymorphisms of two ABC transporters contribute to the variation of nukacin resistance in *Streptococcus mutans*

Naoki Sadaoka,[a,b] Mi Nguyen-Tra Le,[a,c] Miki Kawada-Matsuo,[a,c]# Sopongselamuny Eng,[a] Takeshi Zendo,[d] Jun Nakanishi,[b] Katsuhiro Takeda,[b] Hideki Shiba,[b] Hitoshi Komatsuzawa[a,c]#

[a]Department of Bacteriology, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, Japan.

[b]Department of Biological Endodontics, Graduate School of Biomedical and Health Sciences,Hiroshima University.

[c]Project Research Centre for Oral Infectious Diseases, Hiroshima University, Hiroshima, Japan

[d]Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University.

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#Address correspondence to Miki Kawada-Matsuo, mmatsuo@hiroshima-u.ac.jp or Hitoshi Komatsuzawa, komatsuz@hiroshima-u.ac.jp

ABSTRACT

We investigated the susceptibility of 127 *Streptococcus mutans* strains to nukacins produced by *Staphylococcus* spp. and detected diverse susceptibilities. Nineteen strains had a disrupted LctF (type I), which is responsible for nukacin susceptibility, whereas the remaining 108 strains had an intact LctF (type II) and displayed resistance to nukacins. However, the type I strains still showed low resistance to nukacins to some extent, indicating that other factors are involved in this resistance. Interestingly, 18/19 (94.7%) type I strains carried a *mukA-T* locus, which is related to the synthesis of mutacin K8, and *mukFEG*, an ABC transporter. In contrast, among type II strains, only 6/108 strains (5.6%) had both the *mukA-T* locus and *mukFEG*, 19/108 strains carried only *mukFEG*, and 83/108 strains (76.9%) harbored neither *mukA-T* nor *mukFEG*. We also found that MukF had two variants: 305 amino acids (type α) and 302 amino acids (type β). All type I strains showed a type α MukF (MukFα), whereas most type II strains with *mukFEG* (22/25 strains) had a type β MukF (MukFβ). To determine the contribution of both types of MukF to nukacin resistance, we constructed a *mukFEG*-deletion mutant complemented with MukFαEG or MukFβEG and found that only MukFαEG was involved in nukacin resistance. The nukacin resistance capability of type II-LctFEG (LctF$_{II}$EG) was stronger than that of MukFαEG. In conclusion, we identified a novel nukacin resistance factor, MukFEG, and either LctFEG or MukFEG was active in most strains via genetic polymorphisms depending on *mukA-T* genes.

IMPORTANCE

Streptococcus mutans is an important pathogenic bacterium for not only dental caries but also systemic diseases. *S. mutans* is known to produce a variety of bacteriocins and retain

resistance to these bacteriocins. In this study, we found that two ABC transporters, LctFEG and MukFEG, were associated with nukacin resistance. Each ABC transporter has two subtypes: active and inactive. Of the two ABC transporters, only one ABC transporter was activated, whereas the other ABC transporter was inactivated by genetic mutation. Interestingly, this phenomenon was defined by the presence or absence of the mutacin K8 synthesis gene region, one of the bacteriocins of *S. mutans*. This finding suggests that the acquisition of resistance factors is tightly controlled in each strain. This study provides important evidence showing that the insertion of bacteriocin synthesis genes is involved in the induction of genetic polymorphisms and suggests that bacteriocin synthesis genes may play an important role in bacterial evolution.

INTRODUCTION

S. mutans is a known cariogenic bacterium, and one of its pathogenic properties is the ability to form dental plaques (1), which facilitate the occurrence of dental caries and periodontitis (1, 2). The major virulence factors for this bacterium include three glucosyltransferases, which synthesize sticky water-insoluble glucans for dental plaque formation, and organic acids, mainly lactic acid, for the demineralization of teeth. In addition, *S. mutans* produces various bacteriocins to compete with other bacteria in the oral cavity (3–5).

Bacteriocins are antibacterial peptides that are ribosomally produced by bacteria, and studies have identified various bacteriocins, especially those of the genera *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Enterococcus* (6–11). Bacteriocins are generally effective against the same bacterial species or closely related species. Therefore, bacteriocins are considered the weapons used by bacteria to maintain and expand their

own survival area by excluding other bacteria within a bacterial community (12, 13). Most bacteriocins have a length of 20-70 amino acid residues and show stability to heat and acids (10, 11). There are two types of bacteriocins: posttranslationally modified peptides, which contain unusual amino acids such as lantibiotics, and unmodified peptides. Bacteriocins kill bacteria by physically disrupting the cell membrane or forming channels. Nisin, a type-AI lantibiotic, binds to lipid II on the cell membrane and ultimately disrupts the cell membrane, causing cell death (14, 15).

Nukacins are lantibiotics produced by *Staphylococci* (16–19) and are categorized as type-AII lantibiotics, which contain one dehydrobutyrin, one 3-methyl lanthionine, and two lanthionine residues (20). Analyses of their mode of action have revealed that their initial target are lipid II, similar to that of nisin, but unlike nisin, nukacins do not exert a pore-forming effect on the cell membrane, resulting in bacteriostatic activity (21). Nukacins are found within the lacticin 481 group, which includes lacticin 481 of *L. lactis*(8), mutacin II(9) and mutacin K8 (22) of *S. mutans*, salivaricin A2 of *S. salivarius*(23), and streptococcin A-F22 of *S. pyogenes* (24) (Fig. S1). Additionally, we previously identified nukacin KSE650 produced by *S. epidermidis* isolated from the oral cavity (19). Therefore, nukacins and closely related bacteriocins are thought to be produced by oral bacteria. We previously demonstrated that the transporter LctFEG is associated with nukacin ISK-1 resistance in the *S. mutans* UA159 strain (25). The expression of *lctFEG* is significantly induced by the addition of nukacin ISK-1. In this study, we investigated the susceptibility of 127 *S. mutans* strains to nukacins produced by staphylococcal species and found variations in susceptibility. A genomic analysis of the 127 strains identified MukFEG, a novel factor involved in nukacin resistance. We then investigated the relationship between MukFEG and LctFEG, both of which are involved

in nukacin resistance.

Results

Susceptibility of 127 *S. mutans* strains to nukacins

The susceptibility of 126 clinical isolates of *S. mutans* and UA159 to nukacin ISK-1 and nukacin KSE650 was tested (Fig. 1A). In the nukacin ISK-1 susceptibility test, 99/127 strains (78%) showed no inhibition zone, whereas 28/127 strains (22%) exhibited a range of inhibition zones (Fig. 1A, left). In contrast, in the nukacin KSE650 susceptibility test, 121 strains (95.3%) showed no inhibition zone, whereas 6 strains (4.7%) presented a range of inhibition zones (Fig. 1A, right).

Gene structure of the *lctFEG* and *mukFEG* regions

We previously performed whole-genome sequencing of 126 clinically isolated *S. mutans* strains (5). Using these 126 *S. mutans* genomes and the UA159 genome (obtained from the NCBI database), we focused on the *lctEFG* genes, which are responsible for nukacin resistance. The results showed that 19 strains carried a disrupted LctF due to introduction of a premature termination codon in *lctF* (Fig. 1B and 2A), whereas the amino acid sequence of LctEG was intact in all 127 strains. The strains with a disrupted LctF were designated type I (LctF$_{I}$), whereas the strains with an intact LctF were designated type II (LctF$_{II}$). The susceptibility of types I and II to nukacin ISK-1 and nukacin KSE650 are shown in Fig. 1C. Against nukacin ISK-1, the type I strains showed higher susceptibility than the type II strains, but the susceptibility varied among the type I strains. No

121 significant difference in the susceptibility to nukacin KSE650 was found between the type
122 I and type II strains, and no inhibition zone was detected with all type I (19 strains) and
123 most type II strains (102/108 strains). The varied susceptibility of most type I strains
124 (17/19 strains) to nukacin ISK-1 suggests that other factors may also be involved.
125 Therefore, we focused on another ABC transporter, namely, MukFEG (gene ID,
126 SMU_RS08310- RS08300 in *S. mutans* UA159 from the NCBI database), which is highly
127 homologous to the ABC transporter ScnFEG (*S. pyogenes*), a known immunity factor for
128 streptococcin A-FF22 (26, 27) (Fig.S2???). An analysis of the genomic data of the 126 *S.*
129 *mutans* strains and the UA159 strain detected a *mukFEG* locus in all (19/19 strains) type
130 I strains and some (25/108 strains) type II strains (Fig. 2A). Furthermore, MukF was
131 divided into two types, which were designated type α and type β (Fig. 1B and 2A): the
132 sequence of type α is three amino acids longer than that of type β (Fig. 1D), whereas the
133 other sequences are similar.
134 Comparison of the type I and type II strains showed that all type I strains (19 strains)
135 were MukFα, whereas among the type II strains (108 strains), 3 strains were MukFα, 22
136 strains were MukFβ, and 83 strains (77%) did not harbor *mutFEG* and were designated
137 type IIγ (Fig. 1B and Fig. 2A). In addition, the genetic structure upstream of *mukFEG* of
138 each type (type Iα, IIα, and IIβ) showed variation depending on the structure of *mukA1-*
139 *T* (Fig. 2A). Most type Iα strains (18/19 srains) carried the *mukA1-T* locus, while most
140 type IIβ strains (19/22 strains) did not harbor this locus. Among the three type IIα strains,
141 two strains (KSM125 and 153) had a complete *mukA1-T* locus, and one isolate (KSM55)
142 carried an incomplete locus with deletion of *mukA1* and *mukA2*.
143
144 **Variation in the susceptibility of 127 *S. mutans* strains to nukacins**

We compared susceptibilities of the four above-mentioned types to nukacin ISK-1 and nukacin KSE650. No type IIγ strains showed an inhibition zone against nukacin ISK-1 or KSE650 (Fig. 2B), and no type Iα strains presented an inhibition zone against nukacin KSE650, whereas the type Iα strains exhibited diverse susceptibilities ranging from low (no inhibition zone) to high (15-mm inhibition zone) susceptibility (Fig. 2B). Among the three type IIα strains, one strain showed no inhibition zone against nukacin ISK-1, whereas two strains presented no inhibition zone against nukacin KSE650. Among the 22 type IIβ strains, 9 and 5 strains exhibited an inhibition zone against nukacin ISK-1 and KSE650, respectively, whereas the remaining strains did not (Fig. 2B). Three type IIβ strains with the *mukA1-T* region (Fig. 2A) showed varied susceptibility against nukacin KSE650 (2 strains with an inhibition zone) and nukacin ISK-1 (3 strains with an inhibition zone).

Relationship between nukacin susceptibility and *mukF* and *lctF* gene expression

We previously reported that *lctFEG* expression is induced by nukacin ISK-1 (25). Here, we investigated the gene expression of *mukF* (type Iα, IIα and IIβ) and *lctF* (all types) with or without nukacin ISK-1 in each group by quantitative PCR (Fig. 3 and 4). Based on the results of susceptibility testing, all type Iα (19 strains), 17/22 type IIβ strains, and all type IIγ (83 strains) strains did not show inhibition zones against nukacin KSE650 (Fig. 2B). The expression of *mukF* and *lctF* was examined in three representative strains of these three groups (Fig. 3). The type Iα strains (KSM8, KSM97, KSM182) significantly induced *mukF* expression, but not *lctF* expresson, upon addition of nukacin ISK-1. On the other hand, the type IIβ strains (KSM95, KSM110, UA159) significantly induced *lctF* expression, but not *mukF* expression, upon addition of nukacin ISK-1. The

amount of *lctF* expression by nukacin ISK-1 observed in the type IIβ strain was comparable to that seen in the type IIγ strain(KSM16, KSM20, KSM25), which does not have the *mukFEG* gene.

Among the 108 type II strains with $LctF_{II}$, 11 strains (10.2%) (including two type IIα strains and nine type IIβ strains) showed an inhibition zone against nukacin ISK-1, and 6 strains (5.6%) (including one type IIα strain and five type IIβ strains) exhibited an inhibition zone against nukacin KSE650. To determine the relationship between *lctF/mukF* expression and susceptibility to nukacin, we investigated *lctF* and *mukF* expression in one type IIα strain and 5 type IIβ strains, which showed an inhibition zone against nukacin KSE650, and in two type IIα strains and one type IIβ strain, which exhibited no inhibitory zone (Fig. 4). The type IIα strain KSM153, which presented no inhibition zone against either nukacin (indicated as (R) in Fig. 4), showed increased expression of *lctF* but not of *mukF*. KSM125 and KSM55, which had an inhibition zone against nukacin ISK-1, showed *mukF*α expression but not *lctF* expression (Fig. 4, left). Among five type IIβ strains (KSM2, KSM60, KSM117, KSM154, and KSM183) with an inhibition zone against nukacin KSE650, increased expression of *lctF* was observed in only two strains (KSM2 and KSM154), but their expression level was significantly lower than that found in KSM110, which showed no inhibition zone. In contrast, three strains (KSM60, KSM117, and KSM183) did not show increased *lctF* expression. In addition, increased *mukF* expression was observed only in one strain (KSM2), whereas the other strains, including KSM110, did not show increased expression of *mukF*.

Association of MukF and LctF types with susceptibility to nukacins

Because two types of MukF (type α and type β) were found among the MukFEG-

193 harboring strains, we investigated the contribution of MukF variation to nukacin
194 susceptibility (Fig. 5). We constructed *mukFαEG*-deletion mutants (Δ*mukFEG*) of
195 KSM182 (type Iα) and two types of *mukFEG* (*mukF*α from one type α isolate
196 [KSM182] and *mukF*β from one type β isolate [UA159])-complemented strains
197 (Δ*mukFEG::mukF*α*EG* or Δ*mukFEG::mukF*β*EG*). The Δ*mukFEG* strain showed
198 increased susceptibility to nukacin ISK-1, and Δ*mukFEG::mukF*α*EG* exhibited reduced
199 susceptibility to nukacin ISK-1, whereas Δ*mukFEG::mukF*β*EG* showed no change in
200 susceptibility. Introduction of *lctF*II*EG* into the Δ*mukFEG* of KSM182 reduced
201 susceptibility.
202
203 **Discussion**
204 In this study, we observed a phenomenon in which conflicting genetic polymorphisms
205 contribute to the mechanism underlying the nukacin resistance phenotype of *S. mutans*.
206 Diversity in nukacin susceptibility was observed among 127 *S. mutans* strains. This
207 diversity was apparently caused by the presence of conflicting combinations of amino
208 acid sequence patterns in the previously reported nukacin resistance factor LctF and the
209 newly identified resistance factor MukF in the genome. Nukacins are lantibiotics
210 categorized as type-AII lantibiotics. We previously identified nukacin KSE650 of *S.*
211 *epidermidis* isolated from the oral cavity (19). Because *S. epidermidis* and *S. warneri* are
212 commensal bacteria in the skin and nasal cavity, these bacteria have also been isolated
213 from the oral cavity (28, 29). Additionally, nukacins belong to the lacticin 481 group,
214 which includes lacticin 481 produced by *L. lactis*, mutacin II and mutacin K8 (22)
215 produced by *S. mutans*, salivaricin A2 produced by *S. salivarius* (23) and streptococcin
216 A-F22 produced by *S. pyogenes* (24) (Fig. S1). Therefore, *S. mutans* may come into

contact with nukacins and nukacin-like bacteriocins in the oral cavity.

Among 127 *S. mutans* strains, 44 (35%) had a *mukFEG* region with two types of amino acid patterns of MukF, type α and type β: three amino acids found in type α are absent in the 243-245 region (EYG) of type β. Interestingly, all 22 strains with MukFβ showed LctF type II. In contrast, 19 (86%) of the 22 strains with MukFα showed LctF type I. In addition, 21 (87.5%) of the 24 strains with the *mukA-T* region were of the MukFα type, whereas 19 (95.0%) of the 20 strains without the *mukA-T* region were classified into the MukFβ type. These results suggest that the *mukA-T* region may be a key factor defining the diversity of MukF and LctF. The *mukA-T* region is a gene cluster associated with the synthesis of mutacin K8 (22), a lantibiotic of the same type as nukacin (30), although we found that the 23 strains with the *mukA-T* region did not exhibit mutacin K8 expression under our conditions (Fig. S3). Therefore, whether mutacin K8 itself is involved in the diversity of MukF and LctF remains unknown. The results presented in Fig. 5 show that the MukFα type, which has three additional amino acids, is responsible for resistance to nukacins, whereas the MukFβ type has no effect on resistance. Therefore, the factors responsible for resistance to nukacins are LctFIIEG and MukFαEG. The comparison of nukacin ISK-1 susceptibility between the type Iα (MukFαEG and LctFIEG) and type IIγ (LctFIIEG, no MukFEG) strains (Fig. 2B) revealed that all type IIγ strains showed resistance without forming an inhibition zone, whereas many type Iα strains presented an inhibition zone. However, the results of the nukacin KSE650 susceptibility test showed that all type Iα strains did not form an inhibition zone, although the amino acids of nukacin ISK-1 and nukacin KSE650 were quite similar (Fig. S1). In addition, when purified nukacins were used for the evaluation of MIC values against *S. mutans* UA159, we found no difference in the MIC values between the two nukacins (Table S1). The

direct assay results for *S. warneri* ISK-1 (nukacin ISK-1) and *S. epidermidis* KSE650 (nukacin KSE650) using *M. luteus* as an indicator showed that the inhibition zone of *S. warneri* was larger than that of *S. epidermidis* KSE650 (Table S1). These results suggest that *S. warneri* ISK-1 produces greater amounts of nukacin than *S. epidermidis* KSE650. Based on these findings, it can be hypothesized that MukFαEG contributes to sufficient resistance to low concentrations of nukacin but it may not fully contribute to sufficient resistance to high nukacin concentrations. Furthermore, LctFIIEG acts more strongly as a resistance factor against nukacins than MukFαEG, which could be inferred from the results of complementation experiments (Fig. 5).

Among the strains harboring *lctF*II*EG*, some were less resistant to nukacins. Because the induction of *lctF* and *mukF* expression by nukacin ISK-1 in these strains was low (Fig. 4), we inferred an association between nukacin susceptibility and gene expression level in these strains. For example, among the two type IIα strains harboring *lctF*II and *mukF*α, one strain (KSM153) was resistant to nukacins, whereas the KSM125 and the KSM55 exhibited a reduced degree of resistance compared with strain KSM153. Gene expression analysis showed that the susceptible strains (KSM125 and KSM55) showed no induction of *lctF* but did show induction of *mukF*, whereas the resistant strain KSM153 exhibited induction of *lctF* but not of *mukF* (Fig. 4, left). We speculated that the observed differences in expression could be due to differences in the promoter activity of each gene and the involvement of transcriptional regulatory factors. However, because no differences in the sequences of the *lctF* promoter regions of KSM125 and KSM153 were found, the differences in expression could have been caused by other unknown factors, such as regulatory factors. A similar tendency was observed for type IIβ (Fig. 4, right).

The results obtained in the present study speculate that the insertion of a bacteriocin production-related gene leads to a high frequency of modification in existing immunity factors. In a previous study, we showed that the presence of gene variants of the ABC transporter MutFEG and its neighboring regions in *S. mutans* defined its individual responsiveness to mutacin I (4), III (31), and IIIb (known as mutacin B-Ny226) (32), which are *S. mutans*-derived bacteriocins belonging to the group of type-AI lantibiotics (33). In this study, we found variation between two ABC transporters, LctF and MukF, depending on the presence or absence of the *mukA-T* region involved in mutacin K8 synthesis. Mutacin K8 is a bacteriocin belonging to the same class (type-AII lantibiotics) as nukacins and has a similar structure, as shown in Fig. S1. However, our genetic analysis showed that only 23 out of the 127 strains had *mukA-T* regions associated with mutacin K8 synthesis and that strains with *mutA-T* regions tended to function via MukFEG, suggesting the existence of an immune response against mutacin K8, and those without *mutA-T* regions tended to function via LctFEG. Based on these findings, we proposed the occurrence of a polymorphism between the two ABC transporters, LctFEG and MukFEG, with or without the *muk* region. In the presence of two factors responsible for the resistance function, 'switching' is thought to occur, and in this process, gene insertion results in genetic modification of the unwanted ABC transporter, which would allow only the more effective factor to function in resistance. The studies suggest that bacteriocin synthesis genes play a central role in this switching function. However, the mechanism of this gene switching is unknown, and this study suggests the existence of a novel mechanism of gene mutation that allows bacteria to acquire bacteriocin resistance mechanisms. The results of this study are important for understanding the evolution of bacteria through gene acquisition.

289

290 **Materials and Methods**

291

292 **Bacterial strains**

293 The bacterial strains used in this study are listed in Table 1. *S. mutans* UA159 ((34) and
294 126 clinical strains (5) of nukacin KSE650/ISK-1 producing *Staphylococcus epidermidis*
295 (19, 25) were used. *S. mutans* strains were grown in trypticase soy broth (TSB) (Becton,
296 Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C under 5% CO_2.
297 *Staphylococci* were grown in TSB at 37°C under aerobic conditions. When necessary,
298 erythromycin (10 mg/ml) or spectinomycin (500 mg/ml) was added to the medium.

299

300 **Genome analysis**

301 The whole-genome data of 126 clinically isolated *S. mutans* strains were obtained
302 previously (5). The *S. mutans* UA159 genome sequence was obtained from the NCBI
303 database (NC_004350.2). The amino acid sequences of each ORF from the *S. mutans*
304 genomes were extracted and compared using BLAST
305 (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
306 The genomic data from 127 *S. mutans* strains were analyzed using SnapGene v5.3.2 (GSL
307 Biotech LLC), and the amino acid sequence identity was verified using NCBI BLAST.

308

309 **Construction of gene knockout and complement strains**

310 The methods used for gene deletion and complementation in *S. mutans* are described
311 elsewhere (35). Briefly, the erythromycin resistance gene (Em^r) or the spectinomycin
312 gene without a terminator (Spc^r) was amplified by PCR from pAMβ1 (36) or pDL55 (37),

313 respectively. Approximately 500 bp of the 5' and 3' flanking regions of the target gene
314 was amplified by PCR from the chromosomal DNA of the respective strain. The PCR
315 amplicons were manipulated with complementary sequences for cloning at both ends of
316 the Em^r or Spc^r gene, thus creating a gene cassette consisting of antibiotic resistance genes
317 flanked by upstream and downstream sequences of the target gene. The PCR amplicons
318 were transformed into *S. mutans*, and the corresponding deletion mutants were
319 constructed and selected based on erythromycin or spectinomycin resistance.
320 For genetic complementation, we constructed a DNA fragment to insert Em^r and the
321 target gene into the *ftf* gene, which encodes fructosyltransferase. A gene cassette
322 containing four regions, the upstream region of the *ftf* gene (ftf-UP), the Em^r gene, the
323 coding region of the target gene without its putative promoter region and the upstream
324 region of the *ftf* gene (ftf-DW), was constructed. The target genes ftf-UP, ftf-DW, and Em^r
325 were amplified using specific primers containing complementary sequences. Then,
326 overlap extension PCR was performed, and the complementary sequences facilitated
327 amplicon assembly and ligation. The entire cassette [fttUP-Em^r-target gene-ftfDW] was
328 then transformed into a deletion mutant to generate a complementary strain via
329 erythromycin and spectinomycin resistance selection. The primers used are shown in
330 Table S2.
331
332 **Susceptibility tests**
333 To assess the antimicrobial activity of bacteriocins, two methods, MIC determination and
334 direct assay, were used as previously reported (5). In the direct assay, overnight cultures
335 of each bacteriocin-producing strain were spotted on TSA plates and incubated for 24 h
336 at 37°C with (*S. mutans*) or without (*Staphylococci*) 5% CO_2. After confirming that the

diameter of the growth zone of the bacteriocin-producing strain was uniformly 5 mm, 5 ml of prewarmed TSA soft agar (1%) containing indicator bacteria (10^7 cells/ml) was poured onto the TSA plate and incubated at 37°C for 16 h under appropriate conditions. The diameter of the growth inhibition zone surrounding the bacteriocin-producing strains was measured in two directions. Because the colony size was 5 mm, the value obtained by subtracting 5 mm from the actual value was evaluated as the antimicrobial activity (range of inhibition zone). Three independent experiments were performed, and the average value (mm) was calculated.

In the MIC evaluation, the concentration of purified nukacin ISK-1/KSE650 was adjusted to 0.5 mg/ml in TSB. The bacteriocin solution was subjected to 2-fold serial dilutions (2-fold to 128-fold dilution), and bacterial cells (10^5 cells/100 µl) were then inoculated into each well. After incubation at 37°C for 24 h, the MIC value was determined.

Quantitative PCR analysis

Quantitative PCR was performed to assess the expression of *lctF* and *mukF*. cDNA generation from extracted RNA was performed according to previously published methods (38). A small portion of the overnight culture (10^8 cells) was inoculated into 5 ml of fresh TSB and grown at 37°C with 5% CO_2. When the optical density at 660 nm reached 0.5, nukacin ISK-1 at 1/8 MIC was added to the culture. After incubation for 15 min, bacterial cells were collected. The collected bacterial cells were subjected to RNA extraction followed by cDNA synthesis and quantitative PCR according to the manufacturer's protocol as described elsewhere (38). RNA extraction was performed using the FastRNA Pro Blue Kit (MP Biomedicals, Solon, OH, USA), and cDNA

361 synthesis was performed using the First Strand cDNA Synthesis Kit (Roche, Tokyo,
362 Japan). Quantitative PCR was performed using FastStart Essential DNA Green Master
363 Mix and a LightCycler 96 instrument (Roche, Tokyo, Japan). The primer sequences are
364 shown in Table S1.
365
366 **Purification of nukacin ISK-1 and KSE650**
367 The purification of nukacin ISK-1 and nukacin KSE650 from supernatants of *S. warneri*
368 ISK-1 and *S. epidermidis* KSE650 cultures, respectively, was performed according to
369 previously published methods (19). Briefly, overnight cultures (500 ml) of *S. warneri*
370 ISK-1 were centrifuged at 4,000 x g for 15 min. Macro-Prep cationic resin (1.5 ml) (Bio-
371 Rad, USA) was added to the supernatant and stirred for 12 h at 4°C. The resin was then
372 collected into an open column and washed three times with 10 ml of 25 mM ammonium
373 acetate (pH 7.5). To elute the bacteriocin, the resin was treated with 500 µl of 5% acetic
374 acid. This elution was repeated 10 times. After each fraction was evaporated completely,
375 the samples were dissolved in 50 µl of distilled water. Each solution was tested for
376 antibacterial activity against *Micrococcus luteus*. Overnight cultures of *M. luteus* (100 µl)
377 were inoculated on TSA plates. Then, 5 µl of each solution was spotted on TSA. Samples
378 with antibacterial activity were subjected to HPLC using a C18 column, and a linear
379 gradient from 0 to 60% acetonitrile was then used for 30 min. Each peak was fractionated,
380 and the samples were evaporated and then dissolved in 50 µl of distilled water.
381 Subsequently, the antibacterial activity of each fraction was tested using the above-
382 described method.
383
384 **Statistical analysis**

Student's t test (for comparisons of susceptibility, Fig. 1) and one-way ANOVA (for comparison of the susceptibility test results (Figs. 2B and 5) and for comparisons of gene expression (Figs. 3 and 4)) were performed using GraphPad Prism version 10.1.0 (GraphPad Software, San Diego, CA, USA).

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Acknowledgements

This study was supported in part by Grant in-Aid for Scientific Research (C) (Grant No. 21K09858) from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan.

Author contributions

N. S., M. N. L., M. KM., T. Z., N. J., and H. K. performed the majority of the experiments and participated in interpreting the data and writing the manuscript.

N. S., M. KM., T. Z., and S. E performed the purification nukacin.

N. S., and S. E performed the direct assay and MIC.

N. S. and M. KM. performed a construction of deletion mutants and complement mutants. M.N.L. and M. KM. analyzed genome data.

All authors read and approved the manuscript.

Competing interests.

The authors declare no competing interests.

Additional informat

Correspondence and requests for materials should be addressed to M. KM.

529

530 **Figure legends.**

531 **Fig. 1. Variation in nukacin susceptibility among 126 *S. mutans* clinical strains and**
532 **UA159.**

533 (A) Susceptibility against *S. warneri* ISK-1 (Sw ISK-1) and *S. epidermidis* KSE650 (Se
534 KSE650). The number in brackets represents the number of resistant strains (no inhibition
535 zone).

536 (B) Classification of 127 *S. mutans* strains by variation in LctF and MukF. Type I,
537 truncated LctF (LctFI); type II, intact LctF (LctFII); type α, 301AA (MukFα); type β,
538 298AA (MukFβ); type γ, no MukF.

539 (C) Comparison of susceptibility against *S. warneri* ISK-1 and *S. epidermidis* KSE650
540 between type I (19) and type II (108) strains.

541 The number in brackets represents the number of resistant strains (no inhibition zone). *,
542 $p < 0.0001$ (Student's t test)

543 (D) Alignment of MukF between UA159 (type β) and KSM182 (type α). *, site with
544 amino acid deficiency in the UA159 strain.

545

546 **Fig. 2. ORF map of *mukFEG* and *lctFEG* loci and susceptibility to nukacin ISK-1**
547 **(*Sw* ISK-1) and nukacin KSE650 (*Se* KSE650) in each group.**

548 (A) ORF map of *mukFEG* and *lctFEG* in representative strains of each type (left and
549 middle) and number of resistant strains (no inhibition zone) of each type obtained by the
550 direct method (right). (B) Distribution of zones of inhibition obtained with all strains of
551 each type by the direct method.

552 *Sw* ISK-1, susceptibility against *Staphylococcus warneri* ISK-1; *Se* KSE650,

susceptibility against *Staphylococcus epidermidis* KSE650.

Fig. 3. *lctF* and *mukF* expression among types Iα, IIβ, and IIγ under nukacin ISK-I induction.

Gene expression of the ABC transporters *mukF* (upper) and *lctF* (lower) with and without nukacin ISK-1 induction in representative type Iα, IIβ, and IIγ strains which showed no inhibition zone against nukacin KSE650.

*, $p < 0.05$; **, $p < 0.01$ (Student's *t* test)

Fig. 4. Comparison of *lctF* and *mukF* expression among strains of the same type.

mukF and *lctF* gene expression with and without nukacin ISK-1 was investigated in all type IIα strains (3 strains) and type IIβ (5 strains with the inhibiton zone, 1 strain with no inhibition zone against nukacin KSE650) strains. S and R represent susceptible (inhibition zone observed) and resistant (no inhibition zone), respectively.

*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$ (one-way ANOVA followed by Tukey's post hoc multiple comparison test.)

Fig. 5. Susceptibility of *S. mutans* wild types and their mutants against *S. warneri* ISK-1.

Direct assay was performed using host strain, *S. warneri* ISK-1.

*, $p < 0.01$; **, $p < 0.001$ (One-way ANOVA for comparison between group by Dunnett's post hoc multiple comparison test.)

577 **Supplemental figure legends.**

578

579 **Fig. S1. Similarities of type-AII lantibiotics.**

580 Alignment of lacticin 481 group lantibiotics. Red arrow, consensus AA. (B) Structures of
581 nukacin ISK-1, nukacin KSE650, mutacin K8, salivaricinA2, mutacin II, and lacticin 481.
582 Red, amino acids different from those of nukacin ISK-1; gray, no amino acids from
583 Nukacin ISK-1; blue and green, unusual amino acid.

584

585 **Fig. S2. Amino acid sequence alignment among MukF and LctF.**

586 1, *Streptococcus mutans*; 2, *Streptococcus pyogenes*; 3, *Lactococcus lactis*.

587

588 **Fig. S3. Gene expression of *mukA*1 in type Iα strains.**

589 The gene expression of *mukA1*, which encodes mutacin K8, was investigated in type Iα
590 strains.

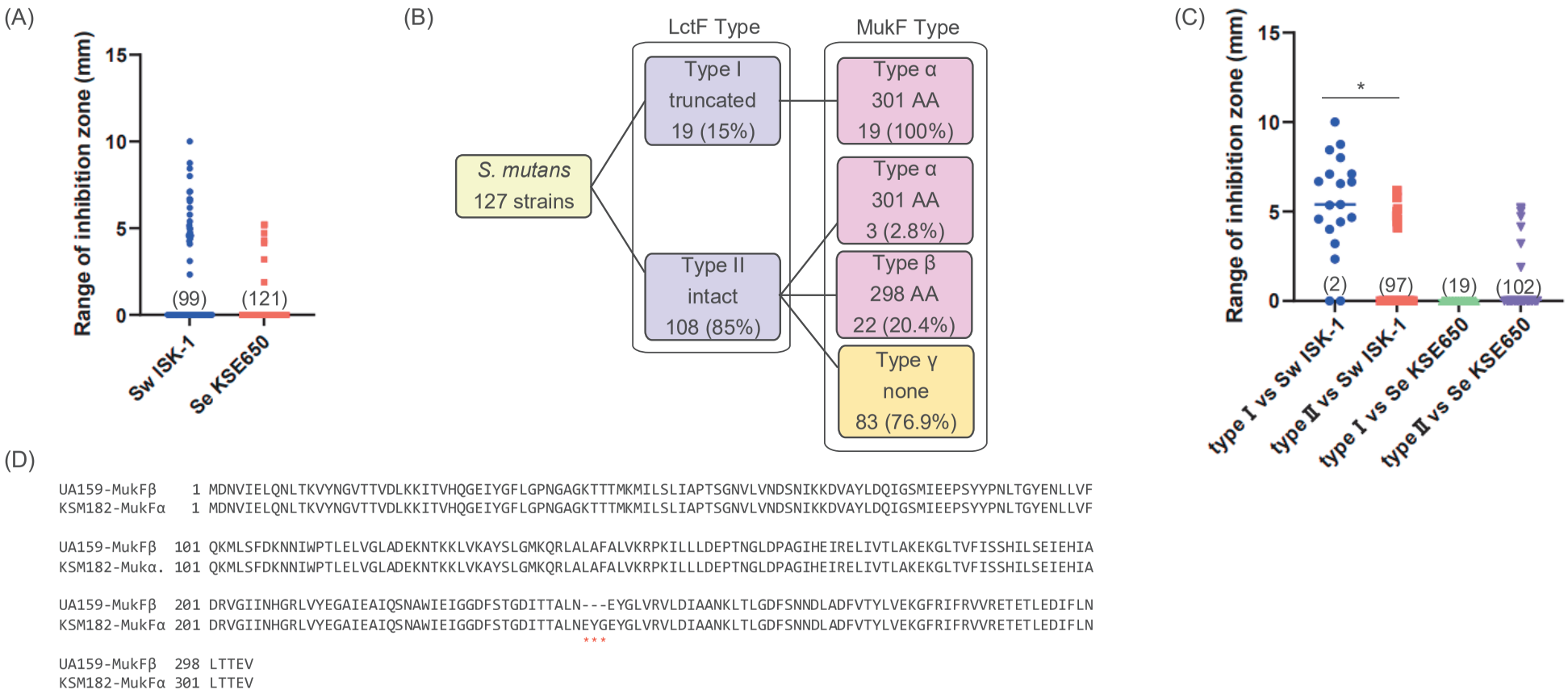

Fig. 1. Susceptibility variation of 126 *S. mutans* clinical isolated strains and UA159 against nukacins.

(A) Susceptibility against *S. warneri* ISK-1 (*Sw* ISK-1) and *S. epidermidis* KSE650 (*Se* KSE650). Score represents the number of strains without halo. (B) Classification of *S. mutans* 127 strains by variation of LctF and MukF. Type I, truncated LctF (LctFI) ; type II, intact LctF (LctFII) : Type α, 298AA (MukFα); Type β, 301AA (MukFβ); type γ, no MukF. (C) Comparison of susceptibility against *S. warneri* ISK-1 and *S. epidermidis* KSE650 between Type I (19 isolates) and Type II (108 isolates). Score represents the number of strains without halo. *, $p < 0.0001$ (student's t-test)

(D) Alignment of MukF between UA159 (Type β) and KSM182 (Type α). *, deficient site of amino acid in UA159 strain.

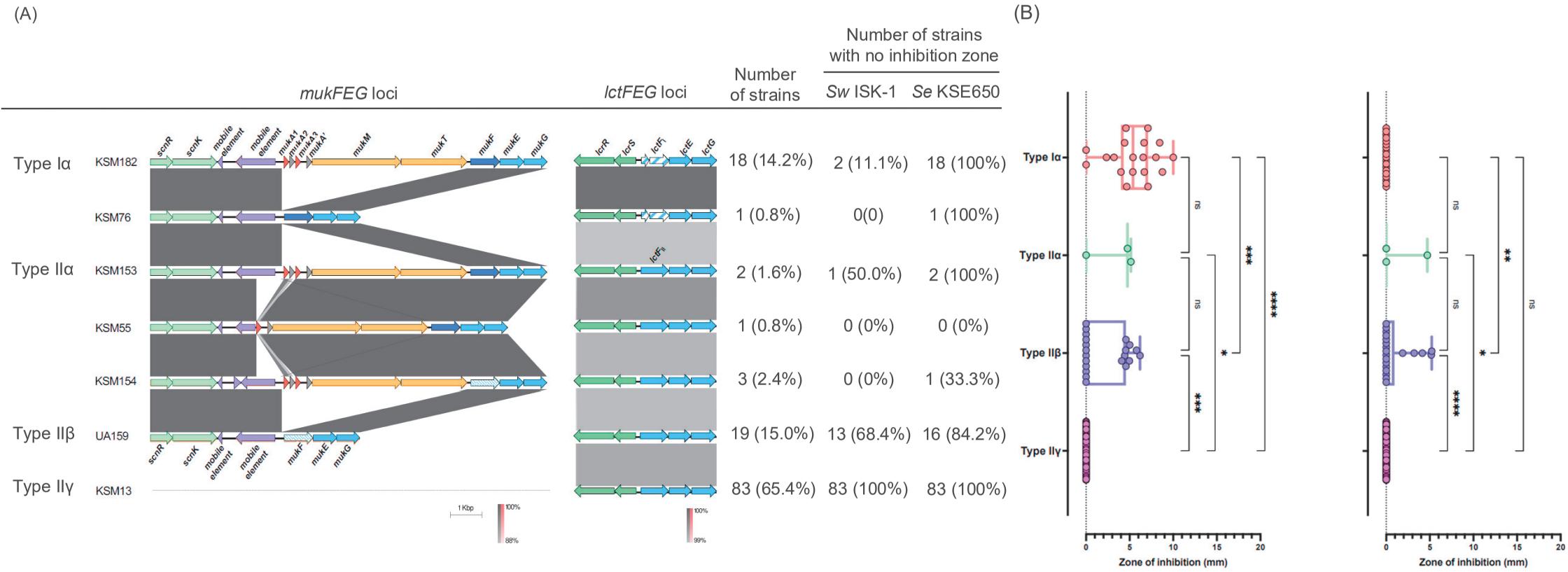

Fig. 2. ORF map of *mukFEG* loci and *lctFEG* loci and the susceptibility to nukacin ISK-1(*Sw* ISK-1) and nukacin KSE650(*Se* KSE650) in each group.
(A) ORF map of *mukFEG* and *lctFEG* in representative strains of each type (left and middle) and the averages of nukacins susceptibility for individual type by direct method (right).
(B) Type-specific susceptibility in all strains by direct method for each type.
Sw ISK-1, indicator as *Staphylococcus warneri* ISK-1; *Se* KSE650, indicator as *Staphylococcus epidermidis* KSE650.

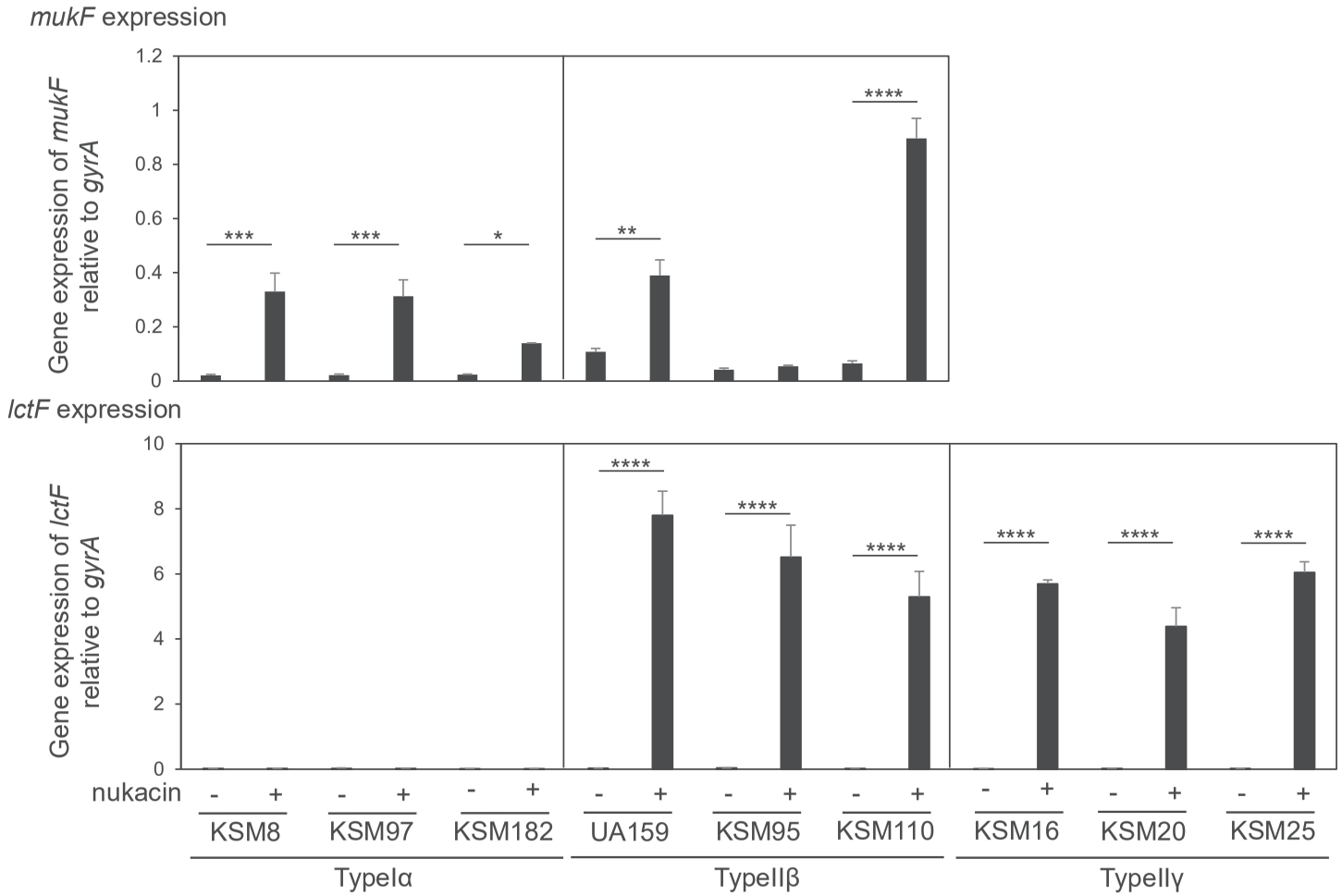

Fig. 3. Gene expression among representative type Iα, IIβ and IIγ strains with no inhibition zone against nukacin KSE650.
Gene expression of the ABC transporters *mukF* (upper) and *lctF* (lower) with and without nukacin ISK-1 in representative strains for each type.
*, p <0.05; **, p < 0.005; ***, p < 0.0005; ****, p < 0.0001 (One-way ANOVA for comparison between group by Tukey's post hoc multiple comparison test)

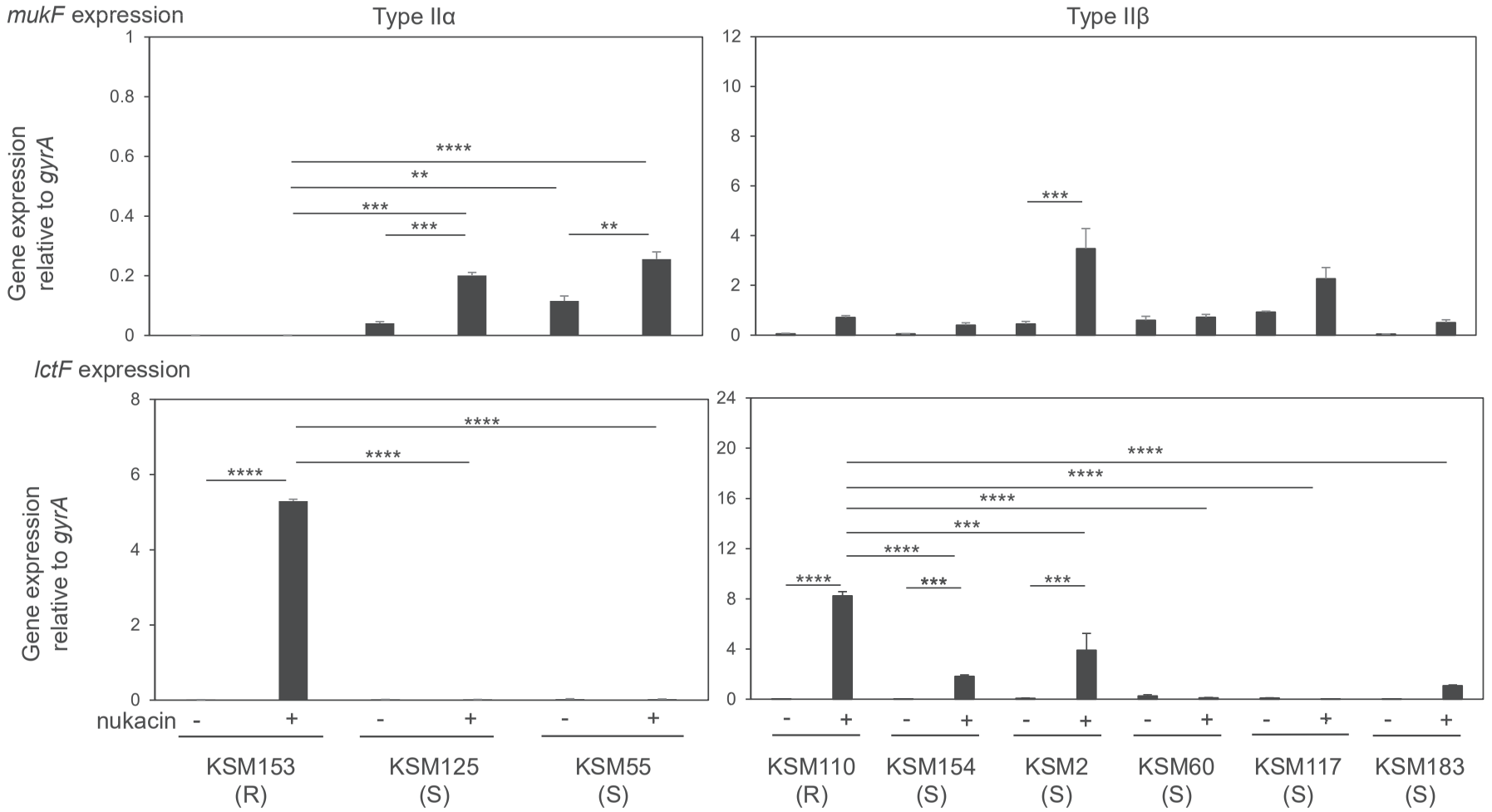

Fig. 4. Comparison of *lctF* and *mukF* expression among strains of same type.
mukF (upper) and *lctF* (lower) gene expression with and without nukacin ISK-1 were investigated in Type IIα (left) and Type IIβ (right). S and R represent susceptible and resistant, respectively.
*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$ (One-way ANOVA for comparison between group by Tukey's post hoc multiple comparison test.)

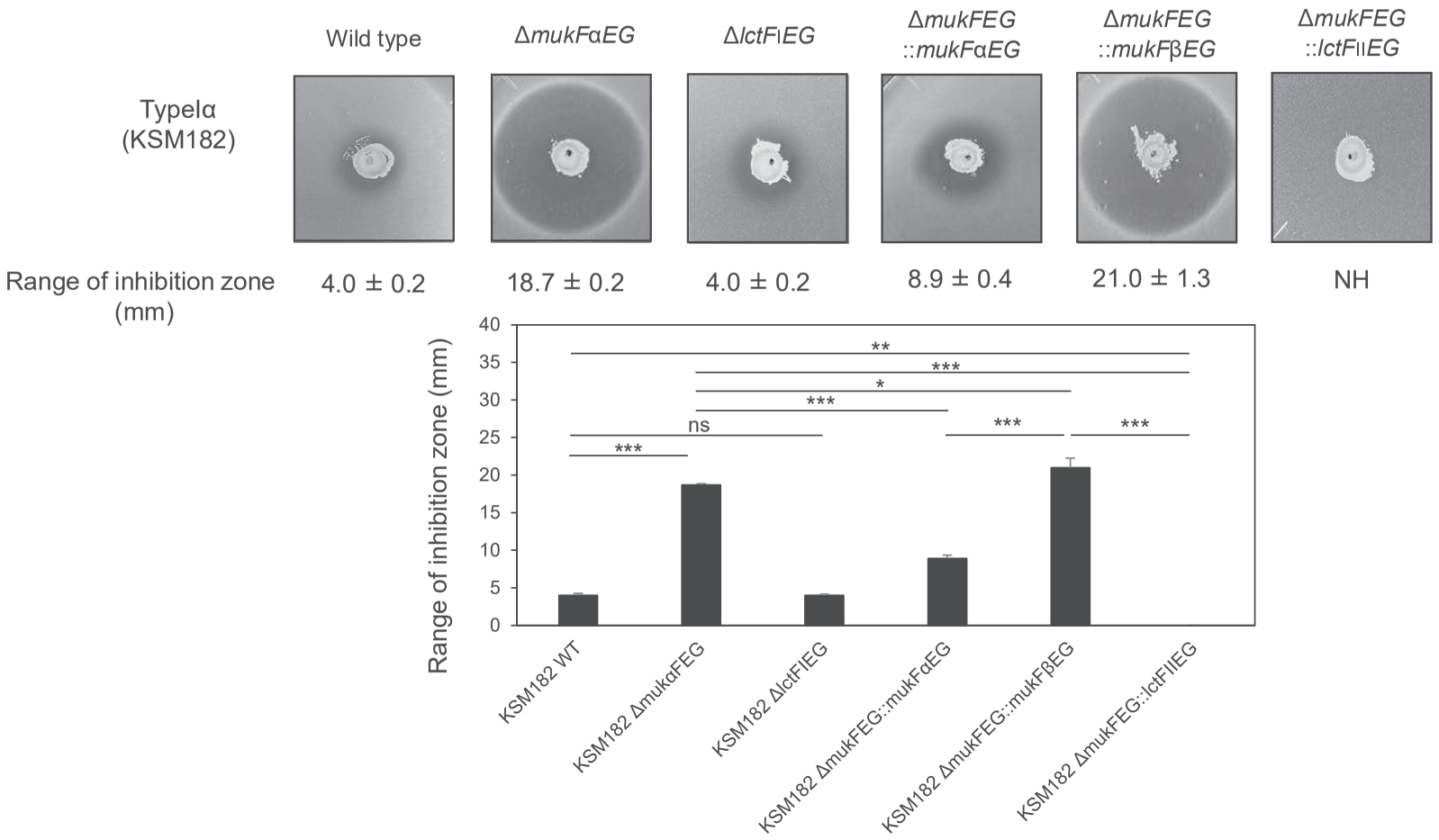

Fig. 5. Susceptibility of *S. mutans* wild types and their mutants against *S. warneri* ISK-1.
Direct assay was performed using host strain, *S. warneri* ISK-1.
*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$ (One-way ANOVA for comparison between group by Dunnett's post hoc multiple comparison test.)

Table1. Strains used in this study.

Strains	Character	origin
Staphylococcus epidermidis KSE650	Wild type, nukacin KSE650 production	19
Staphylococcus warneri ISK-I	Wild type, nukacin ISK-1 production	21
Streptococcus mutans	Clinical isolate (KSM strains)	5
Streptococcus mutans UA159	Wild type	33
Streptococcus mutans KSM182	Wild type	5
Δ*lctFEG*	Δ*lctFEG* inactivation, Em^r	This study
Δ*mukFEG*	Δ*mukFEG* inactivation, Em^r	This study
Δ*lctFEG*	Δ*lctFEG* inactivation, Spc^r	This study
Δ*mukFEG*	Δ*mukFEG* inactivation, Spc^r	This study
Δ*mukFEG::lctFIIEG*	Complementation, Em^r and Spc^r	This study
Δ*mukFEG::mukF*α*EG*	Complementation, Em^r and Spc^r	This study
Δ*mukFEG::mukF*β*EG*	Complementation, Em^r and Spc^r	This study

Em^r, Erythromycin resistance

Spc^r, Spectinomycin resistance

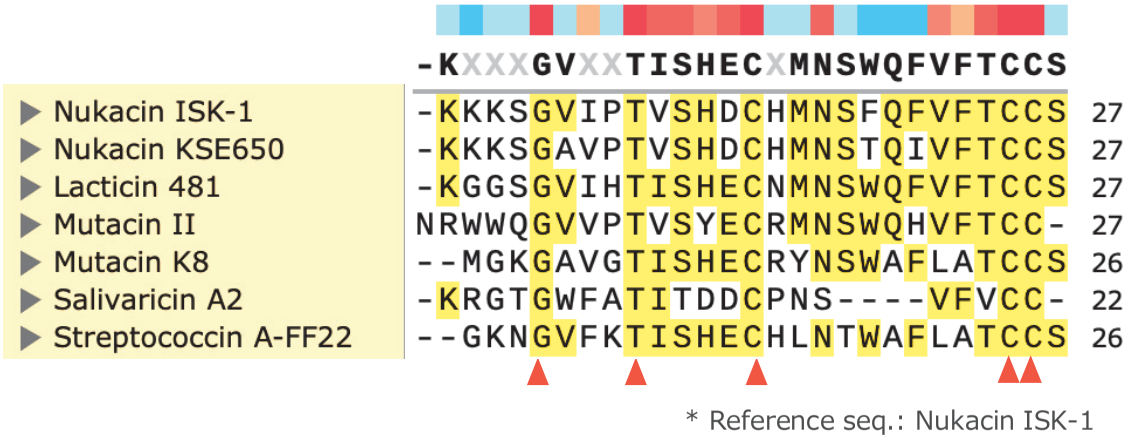

Fig. S1. Similarities of Type-AII lantibiotics.
Alignment of Lacticin481 group lantibiotics. Red arrow, consensus AA

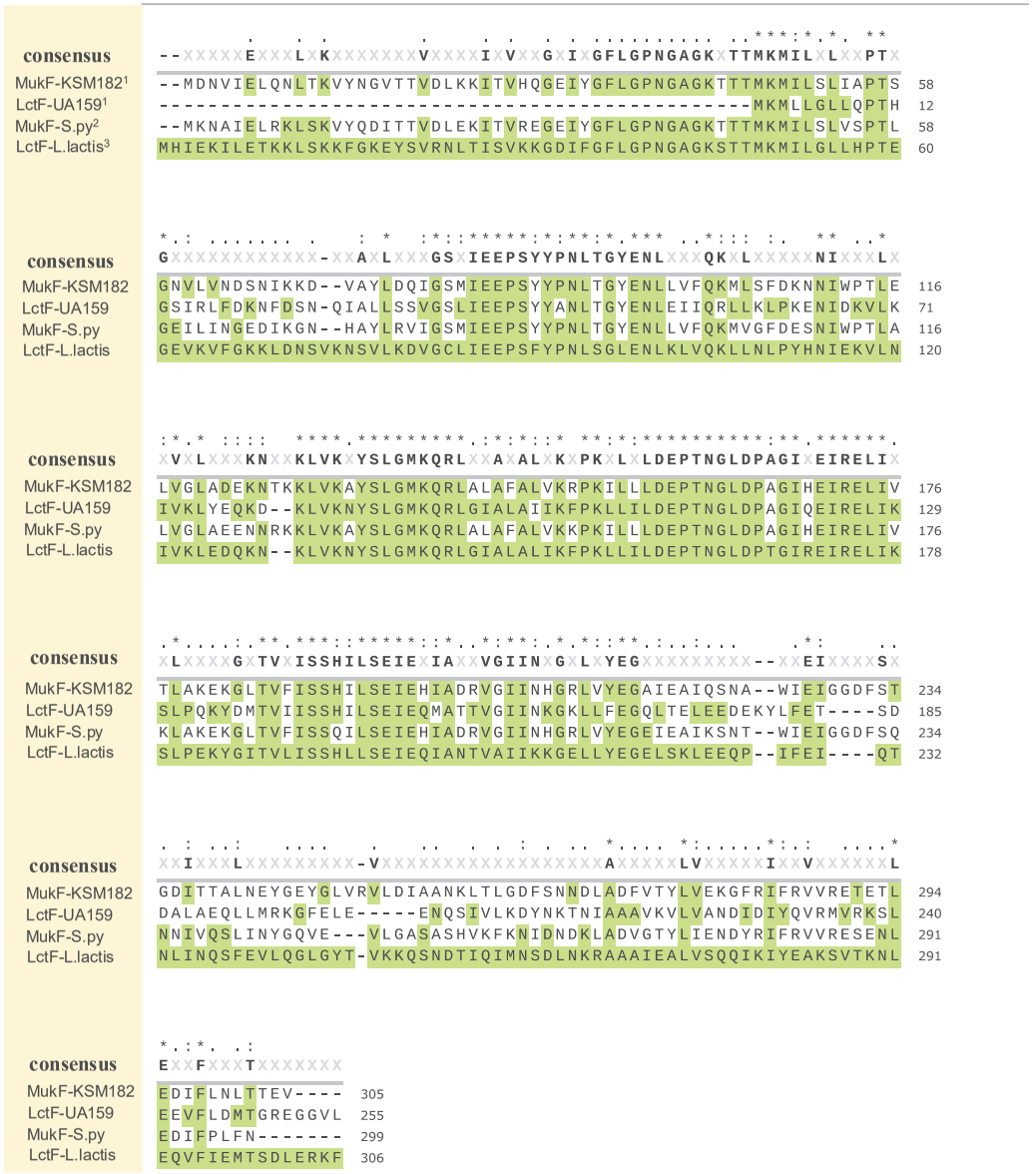

Fig. S2. Amino acid sequence alignment among MukF and LctF.
1, *Streptococcus mutans;* 2, *Streptococcus pyogenes;* 3, *Lactococcus lactis*

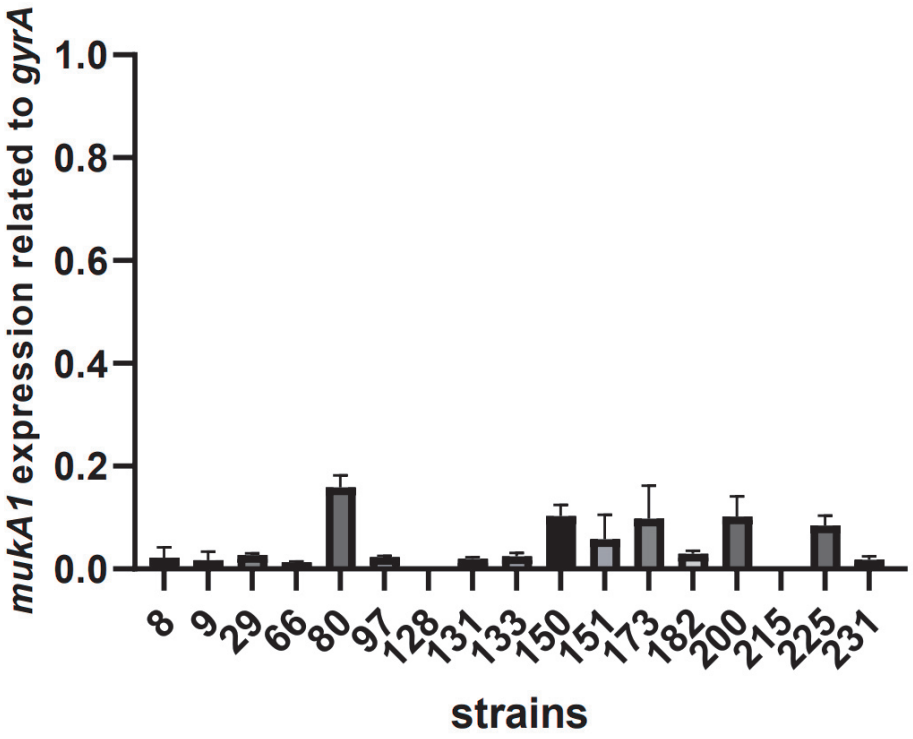

Fig. S5. Gene expression of *mukA1* in Type Iα strains
Gene expression of *mukA1* encoding mutacin K8 was investigated in Type Iα strains.

Table. S1. Activity assessment between nukacin ISK-1 and nukacin KSE650 against *S. mutans* UA159.

	MIC[1] (μg/ml)	Direct assay[2] (mm)
Sw ISK-1	64	16.7 ± 0.5
Se KSE650	64	21.3 ± 0.6

1. Purified nukacin ISK-1 (*Sw* ISK-1) and nukacin KSE650 (*Se* KSE650) were utilized
2. Direct assay of *Sw* ISK-1 and *Se* KSE650 against *Micrococcus luteus*.

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Table S2. primers used in this study

target gene ID	primer-forward	primer-reverse	reference
	Construction of gene-deletion mutants		
lctFEG-UP	5'-aaaagcttcactcacaacttgatatc-3'	5'-cagtcgaggattagccgtgcttttgcca-3'	1
lctFEG-DW	5'-gctgacctagtttagcagtcagtcagctt-3'	5'-aagcagcagcagatcgta-3'	1
mukFEG (for UA159)-UP	5'-tagaaggtctttgaaagtc-3'	5'-cagtcgaggatgttttcactcctttggtta-3'	This study
mukFEG (for UA159)-DW	5'-gctgacctagtagaatcaaagattagaaaattc-3'	5'-tacttactcccaaaatacat-3'	This study
mukFEG (for KSM182)-UP	5'-attatttgagtgtggtgct-3'	5'-cagtcgaggatgttttcactcctttggtta-3'	This study
mukFEG (for KSM182)-DW	5'-gctgacctagtagaatcaaagattagaaaattc-3'	5'-attttagcataattttctatca-3'	This study
lcrRS-UP	5'-aatttaagcaaacgttga-3'	5'-cagtcgaggatcatgctgttacctcgata-3'	1
lcrRS-DW	5'-gctgacctagtttgaaagtgacgattgtc-3'	5'-attgactttgacggctga-3'	1
scnRK (for UA159)-UP	5'-gtcgtccgcatcaagatagtg-3'	5'-cagtcgaggatggatatgctttttcaataatt-3'	1
scnRK (for UA159)-DW	5'-gctgacctagttacttctggttcagacaat-3'	5'-acacgagagaaatcaatga-3'	1
scnRK (for KSM182)-UP	5'-gtcgtccgcatcaagatagtg-3'	5'-cagtcgaggatggatatgctttttcaataatt-3'	This study
scnRK (for KSM182)-DW	5'-gctgacctagttacttctggttcagacaat-3'	5'-acacgagagaaatcaatga-3'	1
	Construction of complement mutants		
ftf-UP	5'-aagaaacaaagaaagctcatcatgtttcaac-3'	5'-gcacaggctattcctatt-3'	This study
ftf-DW	5'-aatgtggttaaattaggaga-3'	5'-actaggtcagcttatttcctcccgttaaa-3'	This study
lctFEG-comp	5'-gctgacctagtatgacaggacactaaacg-3'	5'-tctttgtttctttattgttatgaagtaagacc-3'	This study
*muk*FEG-comp	5'-gctgacctagttaaccaaaggagtgaaaac-3'	5'-tctttgtttcttgaattttctaatctttgattc-3'	This study
	Primers for quantitative PCR		
lctF	5'-tgatgatgcactcgctgaa-3'	5'-aacagccgctgcgatatt-3'	1
mukF	5'-tggtgaaaaggccaaaaatc-3'	5'-cccacacgatctgcaatatg-3'	This study
	Primers used for DNA sequence		
lctF-seq-F6	5'-caattacatcattgaaacca-3'		This study
lctF-seq-R909	5'-ttacaagacacctccttctc-3'		This study
mukF-seq-F3	5'-ggacaatgttattgaactacag-3'		This study
mukF-seq-R901	5'-ccgttgttaaattaaggaaa-3'		This study