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Involvement of the Novel Two-Component NsrRS and LcrRS Systems in Distinct Resistance Pathways against Nisin A and Nukacin ISK-1 in *Streptococcus mutans*

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The novel two-component systems NsrRS and LcrRS are individually associated with resistance against the distinct lantibiotics nisin A and nukacin ISK-1 in *Streptococcus mutans*. NsrRS regulates the expression of NsrX, which is associated with nisin A binding, and LcrRS regulates the expression of the ABC transporter LctFEG.

Streptococcus mutans, a commensal bacterium in the oral cavity, is a cariogenic pathogen in humans (1, 2, 3). *S. mutans* forms dental plaques with other bacterial species. Therefore, *S. mutans* has evolved mechanisms that facilitate competition or cooperation with other oral bacteria in dental plaques. Many bacteria produce antibacterial agents, known as bacteriocins, to ensure survival within this community (4, 5). Bacteriocins are primarily classified into classes I and II (6). Class I bacteriocins (peptides of <5 kDa), called lantibiotics, contain a ring bridged by lanthionine and 3-methyllanthionine residues (7), whereas class II bacteriocins comprise unmodified amino acids (8). *S. mutans* produces several types of bacteriocins known as mutacins (9–12). However, the mechanism underlying the resistance of *S. mutans* to the bacteriocins of other bacteria has not been elucidated. Recently, two-component system (TCS)-based resistance mechanisms against antibacterial agents, including bacteriocins, have been identified in several bacterial species (13–17). In this study, we evaluated the roles of the *S. mutans* TCSs in resistance to several types of bacteriocins.

We first examined 14 *S. mutans* TCS mutants for susceptibility to class I and II bacteriocins by using previously described direct and MIC methods (18, 19) (Table 1 and Fig. 1). The strains, plasmids, and primers are listed in Tables S1 and S2 in the supplemental material. The methods for the construction of the mutants are described in the supplemental materials. Nisin A and nukacin ISK-1 were purified as previously described (20). One TCS encoded by the SMU.659-660 mutant (designated *nsrRS* [nisin A-resistant TCS] in this study) displayed increased susceptibility to nisin A compared with the wild type. Because we identified the function of this TCS, previously named SpaKR (21), we designated this TCS NsrRS. Another TCS encoded by the SMU.1146-1145 mutant (designated *lcrRS* [lacticin 481-resistant TCS]) displayed increased susceptibility to nukacin ISK-1 and lacticin 481. Each complemented strain was able to restore the respective mutation (Fig. 1). The susceptibilities of all TCS mutants against individual class II bacteriocins were not significantly different than those of wild-type cells.

DNA microarray experiments were performed to characterize the transcriptional control mediated by NsrRS and LcrRS. The Agilent eArray platform was used to design a microarray; 14,028 probes (60-mers) were designed for the 2,012 protein-coding genes of *S. mutans* UA159 (up to seven probes per gene) (detailed methods are provided in the supplemental materials). A comparison of the transcription profiles of UA159 and *nsrRS* mutant cells

TABLE 1 Susceptibilities of TCS deletion mutants to various bacteriocins

Strain	Susceptibility[a] to:						
	Class I						
	Nisin A	Nukacin ISK-I	Lacticin 481	Class IIa, munditicin	Class IIb, mutacin IV	Class IIc, lactocyclicin Q	Class IId, lacticin Q
UA159	1 (64)	1 (64)	1	5	0	3	8
TCS45	1 (64)	1 (64)	1	5	0	3	8
TCS1	1 (64)	1 (64)	1	5	0	3	8
TCS2	1 (64)	1 (64)	1	5	0	3	8
TCS3	8 (4)	1 (64)	1	5	0	3	8
TCS4	1 (64)	1 (64)	1	5	0	3	8
TCS5	1 (64)	1 (64)	1	5	0	3	8
TCS6	1 (64)	1 (64)	1	5	0	3	8
TCS7	1 (64)	1 (64)	1	5	0	3	8
TCS8	1 (64)	8 (8)	7	5	0	3	8
TCS9	1 (64)	1 (64)	1	5	0	3	8
TCS10	1 (64)	1 (64)	1	5	0	3	8
TCS11	1 (64)	1 (64)	1	5	0	3	8
TCS12	1 (64)	1 (64)	1	5	0	3	8
TCS13	1 (64)	1 (64)	1	5	0	3	8
TCS14	1 (64)	1 (64)	1	5	0	3	8

[a] Susceptibility was based on direct analysis (the diameter of inhibition [in mm]), with the exception of the two class I drugs on which this study was focused, nisin and nukacin ISK-I, for which MICs were also determined (MIC values [in μg/ml] are shown in parentheses).

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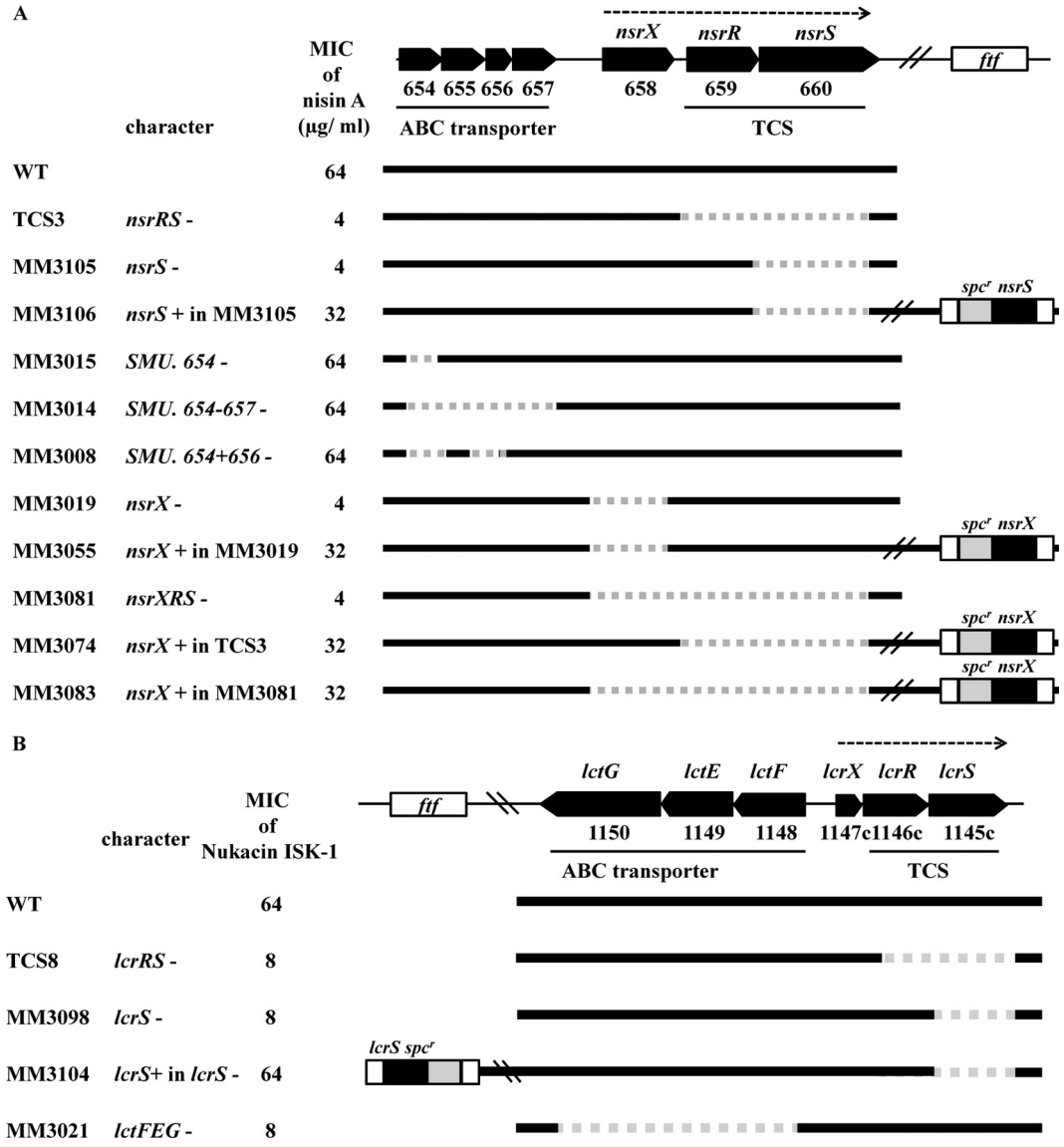

FIG 1 Evaluation of nisin A and nukacin ISK-1 susceptibilities. The characteristics of the *nsrRS* (A) and *lcrRS* (B) loci in the mutant strains used in this study are indicated. The dotted arrows indicate the same operon. The MICs of nisin A and nukacin ISK-1 are also shown.

revealed that the nisin A-mediated induction of seven of these genes (including SMU.654-657, which encodes an ABC transporter, and *nsrXRS*) was reduced in the *nsrRS* mutant (Table 2). A comparison of the transcription profiles of UA159 and *lcrRS* mutants demonstrated that the nukacin ISK-1-mediated induction of six of these genes (including *lctEFG*, which encodes an ABC transporter, and *lcrXRS*) was reduced in the *lcrRS* mutant (Table 2). We further confirmed the expression of these genes by using quantitative PCR (data not shown).

To identify the genes directly involved in nisin A resistance, we constructed SMU.654-657 and *nsrXRS* deletion mutants. The *nsrXRS* deletion mutant displayed increased susceptibility to nisin A, while the susceptibilities of the three SMU.654-657-related mutants were not altered (Fig. 1A). We also constructed an *nsrX* single mutant through substitution with the terminatorless Em^r gene. We confirmed that the mutation did not affect *nsrRS* expression downstream of *nsrX* (data not shown). Similar to the *nsrXRS* deletion mutant, the *nsrX* single mutant also displayed increased susceptibility to nisin A. The complementation of *nsrX* in the *nsrX* single mutant and *nsrXRS* mutant restored *nsrX* expression and susceptibility to nisin A (Fig. 1A). These results indicated that *nsrX* is associated with the NsrRS-regulated resistance to nisin A. NsrX comprises 280 amino acids and nine predicted transmembrane helices. NsrX does not show homology with any other known immunity proteins, such as NukH, NisI, or LtnI (22, 23, 24). Instead, NsrX shares homology with several acetyltransferases, including those of the TraX family, which is associated with F pilin acetylation in *Escherichia coli* (25). Thus, we performed a nisin A-binding assay to characterize the binding of nisin A to *S. mutans* cells, using a previously described method with some modifications (22, 26). The results of the nisin A-binding assay revealed that more nisin A bound to MM3055 cells (an *nsrX* deletion mutant chromosomally complemented with the cloned *nsrX* gene) than to MM3019 cells (an *nsrX* deletion mutant) (Table 3). This

TABLE 2 Genes up- or downregulated in *S. mutans* UA159 following nisin A or nukacin ISK-1 treatment

Treatment, regulation change, and gene ID[a]	Gene name	Regulation by TCS	Fold difference[b]	Gene function
Nisin A				
Upregulated genes				
SMU.655	*mutE*	+[c]	17.3	ABC transporter, permease
SMU.1961	*levD*	−	17.1	Fructose-specific enzyme IIA component
SMU.654	*mutF*	+	16.8	ABC transporter, ATP binding
SMU.656	*mutE2*	+	14.8	ABC transporter, ATP binding
SMU.657	*mutG*	+	14.6	ABC transporter, permease
SMU.658	*nsrX*	+	12.9	Conserved hypothetical protein
SMU.1958	*levF*	−	10.8	Fructose-specific enzyme IIC component
SMU.659	*nsrR*	+	10.2	Response regulator
SMU.1957	*levG*	−	9.0	Fructose-specific enzyme IID component
SMU.1960	*levE*	−	7.6	Fructose-specific enzyme IIB component
SMU.1956		−	7.2	Conserved hypothetical protein
SMU.660	*nsrS*	+	6.3	Histidine kinase
SMU.1195		−	4.0	ABC transporter permease protein
SMU.1193	*yhcF*	−	3.9	Transcriptional regulator, GntR family
SMU.1194	*yurY*	−	3.9	ABC transporter, ATP binding
SMU.862		−	3.5	Conserved hypothetical protein
Nukacin ISK-1				
Upregulated genes				
SMU.1148	*lctF*	+[d]	74.4	ABC transporter, ATP binding
SMU.1149	*lctE*	+	67.3	ABC transporter, permease
SMU.1150	*lctG*	+	53.3	ABC transporter, permease
SMU.1705		−	6.5	Conserved hypothetical protein
SMU.1706		−	5.7	Conserved hypothetical protein
SMU.1704		−	5.2	Conserved hypothetical protein
SMU.1147	*lcrX*	+	4.7	Hypothetical protein
SMU.1146	*lcrR*	+	4.4	Response regulator
SMU.1145	*lcrS*	+	4.2	Histidine kinase
SMU.753		−	4.1	Conserved hypothetical protein
Downregulated genes				
SMU.1960	*levE*	−	0.5	Fructose-specific enzyme IIB component
SMU.1961	*levD*	−	0.3	Fructose-specific enzyme IIA component
SMU.1877	*manL*	−	0.3	Mannose PTS component IIAB
SMU.1878	*manM*	−	0.3	Mannose PTS component IIC
SMU.1879	*manN*	−	0.3	Mannose PTS component IID

[a] Gene identification (ID) numbers are from the GEO database of NCBI (http://www.ncbi.nlm.nih.gov/geo/).
[b] The fold change between bacteriocin-treated cells versus nontreated cells.
[c] Regulated by NsrRS.
[d] Regulated by LcrRS.

finding suggested that NsrX or an as-yet-unidentified factor modified through NsrX binds to nisin A and inhibits the binding of nisin A to lipid II. To identify the genes that are directly involved in nukacin ISK-1 resistance, we constructed an *lctFEG* deletion mutant, which exhibited a reduced MIC against nukacin ISK-1 (Fig. 1B). We determined that the LcrRS-LctFEG system is involved in lacticin 481 resistance in *Lactococcus lactis* CNRZ481 (Table 2). The structure of lacticin 481 is similar to nukacin ISK-1 (6), indicating that LcrRS responds to the lacticin 481 group of type AII lantibiotics.

We performed a coculture assay using the method described in the supplemental material to determine whether TCS-mediated resistance was directly involved in colocalization with other bacteriocin-producing bacteria. *S. mutans* UA159 wild-type, *nsrRS*

TABLE 3 Quantitative nisin A-binding assay results with the *nsrX* deletion mutant and *nsrX*-expressing cells

Strain	Genotype	Fraction	Nisin A binding[a] (μg/ml)	*P* value[b]
MM3019	*nsrX* mutant	Bound	10.7 ± 0.11	
MM3055	$nsrX^+$ in *nsrX* mutant	Bound	17.8 ± 0.23	0.008
MM3019	*nsrX* mutant	Unbound	21.1 ± 0.70	
MM3055	$nsrX^+$ in *nsrX* mutant	Unbound	16.5 ± 0.94	0.015

[a] Mean ± standard deviation binding activity, as measured by liquid chromatography/mass spectrometry.
[b] Statistical significance for the difference between the *nsrX* deletion mutant versus the *nsrX*-expressing strain, based on Student's *t* test.

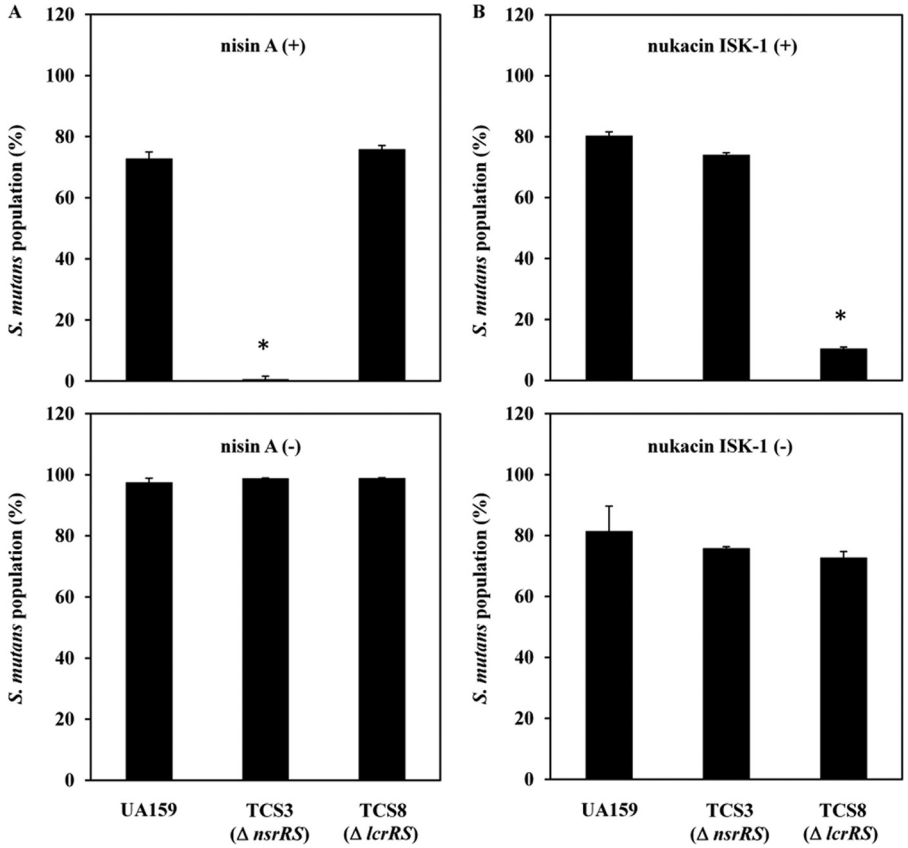

FIG 2 Coculture of *S. mutans* with *L. lactis or Staphylococcus warneri.* The methods for the coculture assay are described in the supplemental material. Populations of *S. mutans* were determined after *L. lactis* (A) or *S. warneri* (B) cells were cocultured with UA159, *nsrRS* mutant, or *lcrRS* mutant cells. *, $P < 0.01$, as determined using Dunnett's method, for the percentage of the *S. mutans* population.

mutant, or *lcrRS* mutant cells were cocultured with nisin A-producing or nonproducing *L. lactis* strains. The *nsrRS* mutant exhibited dramatically decreased population ratios than wild-type or *lcrRS* mutant cells when cocultured with the nisin A-producing strain (*L. lactis* ATCC 11454) but not when cocultured with the non-nisin A-producing *L. lactis* strain (Fig. 2A). Similar results were obtained when *S. mutans* UA159 and mutant cells were cocultured with the nukacin ISK-1-producing or nonproducing strains (Fig. 2B).

In conclusion, we identified two novel TCSs involved in resistance to nisin A and nukacin ISK-1 in *S. mutans* and demonstrated that these two TCSs are important for coexistence with other class I bacteriocin-producing bacteria. These results highlight the roles of bacteriocins in the interactions between different species of oral bacteria and the importance of TCSs in these interactions.

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