MUTACIN IV PRODUCTION IN *STREPTOCOCCUS MUTANS* UA159: CHARACTERIZATION, REGULATION, AND MECHANISM OF SELF-IMMUNITY

BY

Mohammad Shahnoor Hossain

Submitted to the graduate degree program in Microbiology, Molecular Genetics and Immunology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dissertation Committee:

_____
Indranil Biswas, Ph.D., Chairperson

_____
Joe Lutkenhaus, Ph.D.

_____
Wolfram R. Zückert, Ph.D.

_____
Philip R. Hardwidge, Ph.D.

_____
Philippe Prochasson, Ph.D.

Date defended: June 22, 2012

The Dissertation Committee for Mohammad Shahnoor Hossain certifies that

this is the approval version of the following dissertation:

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Indranil Biswas, Ph.D., Chairperson

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Abstract

Streptococcus mutans, an oral pathogen, is considered as a principle etiological agent of dental caries. To colonize successfully in the oral cavity, this organism encounters fierce competition with other oral microbiota and this process is facilitated by ribosomally synthesized small cationic peptides called mutacins (bacteriocins) secreted by *S. mutans*. Although for long time mutacins are regarded as important virulence factors, it is not clear how these peptides work, what is the mechanism of self-immunity, and how they are regulated. In this study, we have characterized the activity, mechanism of self-immunity, and regulation of expression of a well-known bacteriocin, mutacin IV.

To clarify the activity of mutacin IV, we studied the contribution of NlmA and NlmB peptides to mutacin IV activity. Using genetic and biochemical approaches, we showed that the presence of both genes is required for optimum mutacin IV activity. We also showed that mutacin IV is active against multiple *Streptococcus* species. Our studies revealed that mutacin IV has comparatively broader inhibitory spectrum than mutacin V; and both of them can work synergistically to inhibit various microorganisms.

Next, we investigated the immunity activity of a putative immunity protein, SMU.152, which lies just downstream of the *nlmAB* operon. We observed that heterologous expression of SMU.152 in two sensitive strains converted the strains to become resistant to mutacin IV. We identified that C-terminal charged residues of SMU.152 is indispensible for conferring immunity to susceptible cells. In addition, our studies demonstrated that in absence of cognate immunity protein, *S. mutans* can employ other paralogous proteins to prevent the self-toxicity, which implies the presence of cross-immunity or immune redundancy in *S. mutans* UA159.

To get a clear understanding on regulation of mutacin IV production, we carried out pGh9:IS*S1*-mediated random transposon mutagenesis in a reporter strain containing the *nlmAB* promoter with a promoter less *gusA* (glucuronidase) gene. Mapping the position of transposon insertion sites revealed 22 unique genes of diverse cellular activities, which are involved in regulation of mutacins production. Highlight of our finding is that an extracellular ATP-independent Lon-like protease functions specifically on maturation of peptide pheromone, CSP. Here, we show that functional form of CSP (18-amino acids) is generated by a specific post export cleavage with this Lon-like protease. Our study suggests that ComDE two-component system is primarily involved in regulation of mutacins production in *S. mutans* UA159. We also show that both the peptide pheromone (CSP) and mutacins use the same ABC transporter, NlmTE, for secretion. During the course of this study, we discovered two more novel regulators for gene expression in this bacterium. We show that a small hypothetical protein, SMU.2137, is required for normal cellular processes in *S. mutans* UA159 and a protein, SMU.832, required for glucose side chain formation, can also regulate mutacin production in this organism.

Collectively, our data encourage us to propose a new model for regulation of mutacins production in *S. mutans* UA159. According to our model, peptide pheromone (BipC) is secreted as 21-amino acids and a specific Lon-like protease cleaves the C-terminal three amino acids to generate functional peptide pheromone (18-residue). BipC activates a two-component signal transduction system (BipDE), which in turn stimulates the expression of mutacins encoding genes (such as *nlmAB* and *nlmC*). A common ABC transporter, BipTE (NlmTE), is required for both mutacins and BipC secretion and proteolytic cleavage of leader peptide after GG-motif. In sum, our study has unraveled new pathways of gene regulation in *S. mutans* and strengthened our understanding on overall biology of mutacins production in *S. mutans*.

Acknowledgements

First and foremost, I am thankful to my parents and my wife for their love and best wishes for me throughout my life. I want to take this opportunity to thank my mentor Indranil Biswas for his guidance and support of my research all these years. Thanks also go to all previous and current members of Dr. Biswas lab for their great company and support. I have learned very much in the time I spent in this lab.

I would like to thank my dissertation committee members Joe Lutkenhaus, Wolfram Züeckert, Philip R. Hardwidge and Philippe Prochasson for their comments and valuable suggestions during my research. I would also like to thank IGPBS program for getting me into the graduate school at the University of Kansas Medical Center.

Lastly, I would like to extend my appreciations to all of my friends for their support and encouragement throughout my life.

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Abbreviations

ATP: Adenosine triphosphate

DNA: Deoxyribonucleic acid

LB: Luria-Bertani medium

OD: Optical density

SDS: Sodium dodecyl sulfate

PAGE: Poly acrylamide gel electrophoresis

Man-PTS: Mannose phosphotransferase system

CSP: Competence stimulating peptide

Com: Competence

Em: Erythromycin

Km: Kanamycin

GAS: Group A streptococcus

CHAPTER ONE

LITERATURE REVIEW

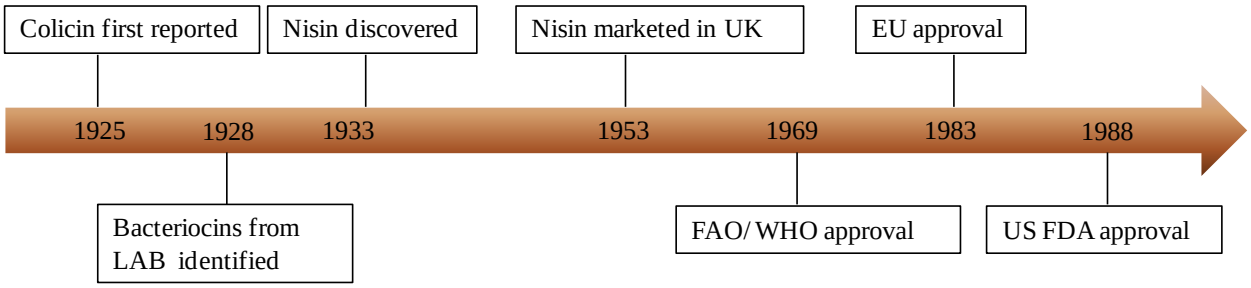
All forms of life developed innate immune system to resist the challenge of other competing organisms. Ribosomally synthesized small antimicrobial peptides (AMPs) are one of the oldest and widely present antimicrobial strategies in living system, from bacteria to humans, plants to insects (54, 219). These peptides play vital roles in conferring immunity against microbial infection in plants and animals, such as α- defensins produced by neutrophils in the small intestine maintain the integrity of the barriers of mucosal surfaces (54). AMPs produced by bacteria are commonly referred as bacteriocins and are used for out-competing other bacteria present in the same micro-ecological niche. Both AMPs and bacteriocins are similar; they are cationic, hydrophobic or amphiphillic and membrane permeabilizing in nature (217). Bacteriocins might have either narrow spectrum (acting toward the same species) or broad spectrum of activity (acting across the genera) and are more potent than the eukaryotic AMPs; while AMPs show activity at micro molar concentration, bacteriocins are active at nano molar concentration (219). Therefore, they pose the potential for developing species-specific therapeutics to fight against the ever increasing antibiotic resistant bacterial pathogens.

Bacteriocin was first discovered in 1925 when inhibition between *Escherichia coli* strains first noticed (98). Originally it was named ‘colicin’ after the producer bacteria *E. coli*. Despite the commercial use of bacteriocins as food preservative was first proposed in 1951, it is likely that we human being are using bacteriocins coincidentally since the first fermentation process was developed 8000 years ago (54). In fact, many studies have found that lactic acid bacteria (LAB) involved in cheese manufacturing also produce bacteriocins to maintain the composition

of complex microflora by inhibiting the unwanted bacteria (193, 260, 313). Nisin (group N inhibitory substances) was first reported in 1933 (313) and marketed in England in 1953 and to date, 48 countries have approved its use in dairy industries (54) (Fig.1). Importantly, nisin was tested as safe for food use by the Joint Food and Agriculture Organization/ WHO Expert Committee on food additives in 1969. US Food and Drug Agency (FDA) approved its use in pasteurized, processed cheese industry in 1988 (54).

The story of the successful commercialization of nisin from initial microbiological observation is a model that rebounded bacteriocins research in recent days in all over the world. However similar stories of other bacteriocins yet to be developed in the same scale (54). Despite this reality, we predict that bacteriocins can be exploited to use in various food industries to prevent the spoilage of foods, can be used in pharmaceutical industries to develop species-specific therapeutic to target the specific pathogen without harming beneficial microorganisms or producer bacteria can be used as probiotic to inhibit the potential pathogens. To realize this potential, it is essential to study the biology of various bacteriocins; specifically their spectrum of activity, mode of action, immunity and regulation of biosynthesis. With this goal in mind, we have studied the bacteriocins from *S. mutans* UA159, an oral bacterium believed to be involved in tooth decay (176, 177). Human oral cavity harbors one of the most heterogeneous microbial population (>700 species) and this micro-ecological niche is an ideal model system to study the inter-species interaction (1, 275). Diversity of bacterial population and shortage of food supply trigger deadly competition among microbial species in this microenvironment. Interspecies competition between *S. mutans* and other bacteria in the oral cavity has been reported by various groups (22, 38). To inhibit the growth of competing bacteria and to establish a successful colonization in the plaque biofilm, *S. mutans* secretes a wide variety of bacteriocins, called

Fig. 1. Landmark of the bacteriocin development over the period of time [adapted from Cotter et al. (54)]. Bacteriocin was first identified in 1925 and marketed in 1953. US Food and Drug Agency (FDA) approved it in 1988.


Colicin first reported
Nisin discovered
Nisin marketed in UK
EU approval
1925
1928
1933
1953
1969
1983
1988
Bacteriocins from LAB identified
FAO/ WHO approval
US FDA approval

'mutacins' that exhibit various inhibitory spectrum (8, 15). In this study, we have clarified the genetic organization and inhibitory spectrum of one of such bacteriocins called 'Mutacin IV' along with the mechanism of self-immunity and regulation of biosynthesis in *S. mutans* UA159.

Antimicrobial peptides (AMPs) of eukaryotic origin

AMPs maintain the first line of defense and also provide innate immunity in higher eukaryotes. Therefore, they help in the existence of producer organisms in a given ecological niche against the infections or competitor organisms (50, 217). Table-1 shows some examples of AMPs from eukaryotic origin with their activity spectrum. They might act against a particular group of organisms or have broad spectrum of activity (50). AMPs protect the producer organisms mostly by disrupting the target cell membrane causing irreversible leakage of cellular materials, although they have various other mode of actions (50).

Antimicrobial peptides of bacteria (bacteriocins)

Bacteriocins are defined as ribosomally produced, small peptides that are active against closely related bacteria. However, the producer bacteria is immune to its own bacteriocin due to the presence of specific immune mechanism (54). The first identified bacteriocins, colicins, comprise of a group of antibacterial proteins that kill other bacteria by diverse mechanisms such as blocking the cell wall synthesis, permeabilizing the cell membrane or by inhibition of RNase or DNase activity (50). Lactic acid bacteria have been extensively studied as an ample source of antimicrobial peptides for application in food industries (Table-2). Nisin is now effectively used as a preservative in cheese, skim milk, sausage, lean beef and Kimchi preparations to prevent spoilage caused by different bacteria (45, 57, 60, 83, 307).

Table-1: Antimicrobial peptides (AMPs) from eukaryotes [adapted from Cleveland et al.(50)]

AMPs	**Source**	**Mode of action**	**Spectrum of activity**	**References**
Pardexin	*Pardachiros maroratus*	Release neurotransmitter by forming pores	Gram +; Gram -	(228)
Melittin	Bee venom	Membrane insertion	Gram +; Gram -	(228)
Ceratotoxin	*Ceratitis capita*	Not known	Gram +; Gram -	(186)
Histatins	Human saliva	Pore formation in membrane	Bacteria; Fungi	(117)
Trichorzins	*Trichoderma spp.*	Voltage gated ion channel formation	*Staphylococcus aureus*	(97)
Magainins	Frogs & amphibian	Create anion permeable channel in membrane	Bacteria; Fungi	(110)
Defensins	Mammalian neutrophils	Voltage gated ion channel formation	Bacteria; Fungi; Enveloped virus	(121)

Table-2: Potential food applications of various bacteriocins

Bacteriocin	Producer	Application and target bacteria	Ref.
Nisin	*L. lactis*	To inhibit *L. monocytogenes* in cheese & *B. cereus* in skim milk	(83, 307)
Pediocin PA-1/AcH	*Pediococcus acidilactici*	To inhibit *L.monocytogenes* & *S. aureus* in cheese; *C. botulinum* in chilled soup	(73, 258)
Divercin V41	*Carnobacterium divergens* V41	To inhibit *L.monocytogenes* in smoked salmon	(74)
Enterocin A	*Enterococcus faecium* CTC492	To inhibit *L.monocytogenes* in dry fermented sausage and soy milk	(164-166)
Enterocin CCM4231	*Enterococcus faecium* CCM4231	To inhibit *S. aureus* in soy milk	(165)
Leucocin A	*Leuconostoc carnosum* UAL187	To inhibit *L.monocytogenes* in ground beef	(73)
Plantaricin	*L. plantarum* 423	To inhibit *L.monocytogenes* in ostrich meat salami	(68)
Sakacin A	*L. sakei* 790	To inhibit *L.monocytogenes* in chicken cold cut	(303)
Sakacin P	*L. sakei* 790	To inhibit *L.monocytogenes* in cold smoked salmon & LAB in brined shrimp	(80, 143)

Bacteriocins and antibiotics are sometimes confusing but there are clear distinctions between them (73). Antibiotics are secondary metabolites and have broad target spectrum. Antibiotics are clinically used to kill pathogens; however, they have various side effects. They can target the cell wall, cell membrane or intracellularly but don't provide any immunity to host cell. On the other hand, bacteriocins are ribosomally produced and mostly form pore in the membrane. They have narrow spectrum of activity without any side effects. Bacteriocins also provide the first line of defense to the host cells (73).

Classification of bacteriocins

Bacteriocins include heterogeneous group of small, extracellular antibacterial peptides produced by bacteria (54). They have been classified into different groups based on their molecular structures and mode of actions (54, 118, 151, 209, 212). Cotter et al. (54) suggested two main classes of bacteriocins based on their structure such as lanthionine-containing bacteriocins in class I and non-lanthionine containing bacteriocins in class II. However, they expelled heat-labile peptides from the definition of bacteriocins. This attempt unjustifiably excluded the first described bacteriocin 'colicin'. To redress this problem, Heng and Tegg (118) proposed a more inclusive approach of classification where they divided bacteriocins into four distinct classes. Due to the extensive diversity of bacteriocins, we propose this recent classification should be modified slightly; specifically for the classification of class II. Here, we offer a new classification scheme (Table-3) based on the classification systems proposed by Heng and Tegg (118) and Drider D. et al.(73).

Class I bacteriocins (lantibiotics): The lantibiotics are lanthionine-containing small peptides (19-38 amino acids long) that are formed when an enzymatically dehydrated serine

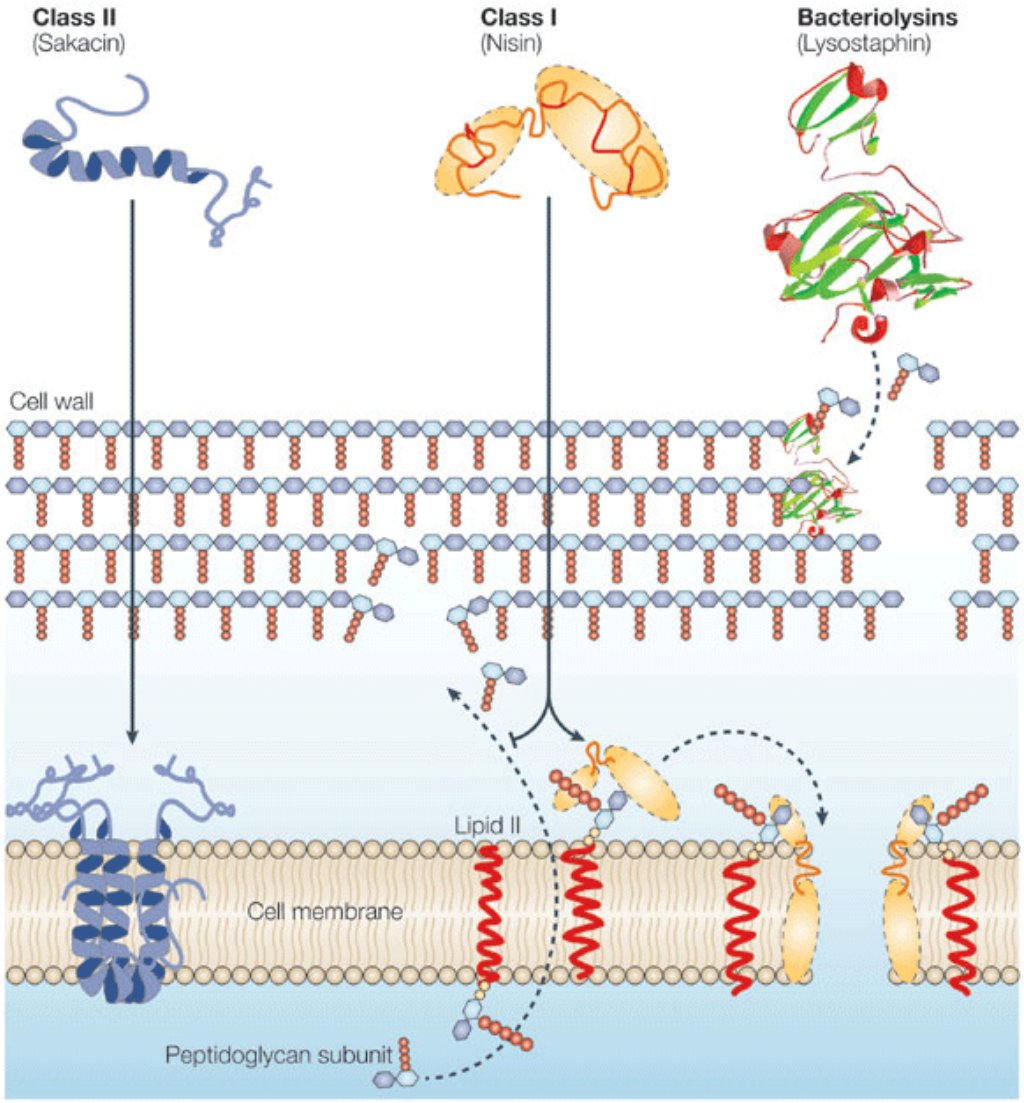
Table-3: Classification of bacteriocins

	Class I	Class II	Class III	Class IV
Type	Lantibiotics (Post translationaly modified)	Non-lanthionine, heat-stable peptides	Large heat-labile proteins	Cyclic peptides (N- and C- termini covalently linked)
Molecular Weight	<5kDa	<10kDa	>10kDa	<10 kDa
Subgroups	**Type Ia:** Elongated peptides with a net positive charge **Type Ib:** Globular peptides with a net negative charge or no charge **Type Ic:** Multi-component	**Type IIa:** monopeptide and a YGNGVXC N-terminal motif **Type IIb:** Two-peptide and GXXXG motif **Type IIc:** Heterogeneous	**Type IIIa:** Bacteriolytic **Type IIIb:** Non-lytic	None
Examples	Nisin, lacticin 481, lacticin 3147, mutacin I,II, III, Smb, BHT etc.	Pediocin PA1, aureocin A53, plantaricin, mutacin IV& V	Lysostaphin, helveticin J	Enterocin AS-48

(dehydroalanine) forms a covalent bond (thioether) with the sulphydryl group of a neighboring cysteine. This forms an internal ring which is the distinctive feature of lantibiotics. This ring can also be formed between threonine and cysteine and is termed as β-methyllanthionine (54, 170). Moreover, lantibiotics might have some unusual residues such as substitution of D-alanine for L-serine (132, 232, 319). Due to these unusual modifications, lantibiotics are highly resistant to extreme environmental conditions. For example, they are resistant to 121^0C for 15 min and pH from 2-12 (64, 167). Lantibiotics are also divided into three subtypes based on their structure and function. Class Ia includes the elongated amphiphilic molecules of less than 4-kDa in mass and cationic in nature such as nisin (54, 73). They act by forming pores in the membrane (Fig. 2), which leads to disruption of membrane potential and the efflux of metabolites from the target cells (54). In contrast, class Ib (globular molecules with low molecular mass of 1.8 to 2.1 kDa such as mersacidin) inhibits the enzyme activity (141). However, it is now clear that nisin have both mode of actions (54). Two-peptide lantibiotic, such as lacticin 3147, shows the co-operative activity of individual peptides. This complex nature make them difficult to sub classify and recently it has been proposed that lantibiotics can be sub classified into 11 subgroups based on their primary amino acid sequences (53). Inhibitory concentration of lantibiotics resides in nano molar range toward the target species and their activity is several order higher than the antimicrobial peptides of innate immune system (54).

Despite the lack of accurate molecular mechanism of action for all lantibiotics, several studies have established that a docking molecule, such as lipid II, is necessary to exhibit their activities (35-37, 128, 314). The interaction of nisin with the lipid II blocks peptidoglycan synthesis by preventing the transport of peptidoglycan precursors from cytoplasm to cell wall.

Fig. 2. Mode of actions of bacteriocins. Based on the mode of action, bacteriocins from lactic acid bacteria can be classified into three categories (54). Class I (lantibiotics) has dual mode of actions. They can bind to the main transporter (lipid II) of peptidoglycan subunits which are involved in transport of peptidoglycan from cytoplasm to cell wall. Moreover, they can use lipid II as docking molecule to initiate membrane insertion and pore formation. Class II peptides, in general, have an amphiphilic helical structure which enables them to insert into the membrane of the target cells, leading to depolarization and death. Large bacteriocins (bacteriolysins) lyse the cell wall directly which leads to cell death.


Class II
(Sakacin)
Class I
(Nisin)
Bacteriolysins
(Lysostaphin)
Cell wall
Lipid II
Cell membrane
Peptidoglycan subunit

Moreover, lipid II acts as docking molecules for membrane insertion and pore formation (35, 314). Lacticin 3147, a two peptide lantibiotic, has these bi-functional activities imparted by individual peptides (188), whereas globular peptide, mersacidin, can bind to the lipid II only (54). It has been identified that membrane phospholipids, phosphatidylethanolamine, acts as the docking molecule for cinnamycin like lantibiotics (126, 181). Besides this, enterococcal two-peptide lantibiotic (enterocin 1071) has broad spectrum of activity. They can target gram-positive bacteria as well as eukaryotic cells such as human, bovine and horse erythrocytes; retinal cells; polymorphonuclear leukocytes; and intestinal epithelial cell (55).

Class II bacteriocins (non-lanthionine containing bacteriocins): Class II bacteriocins are small (<10kDa), heat-stable peptides without any post-translational modification (54). Most of the class II bacteriocins cause disruption of proton-motive force by permeabilizing the cell membrane and act in nano molar range (Fig. 2). Class II can be further divided into several subclasses (54, 219). Class IIa consists of listeria-active pediocin like peptides, class IIb consists of two-peptide bacteriocins, and other multi-component peptides are included into class IIc (Table-3).

Class IIa (mono-peptide bacteriocins): Class IIa bacteriocins consist of pediocin-like mono-peptides produced by various lactic acid bacteria and show narrow spectrum of activity but highly specific for *Listeria monocytogenes* (116, 202). They are also active against various *Lactobacillus, Lactococcus, Leuconostoc, Clostridium perfringens, Bacillus cereus* and *Staphylococcus aureus* (73, 79, 131). They are cationic, amphiphilic or hydrophobic and contain 37- to 48-amino acid residues (54, 219). They have conserved N-terminal ‘pediocin box’ motif which harbors the consensus sequence YGNGVXCXXXXVXV (X denotes any amino acid) (54, 73) and is believed to facilitate non-specific interaction with the target cell surface (44, 144). The

C-terminal domain, which is present after the hinge region, is less conserved and involved in membrane insertion due to their hydrophobic nature (86, 198). Generation of hybrid peptides suggested that C-terminal domain is involved in target cell specificity determination (84, 140) and interacts with the lipids or proteins in the hydrophobic parts of the cell membrane. It has been reported that membrane-associated mannose phosphotransferase system permease is involved in the sensitization of the target cells to pediocin-like bacteriocins (58, 115, 252, 253, 321). Recently, a study suggested that MptC and/or MptD subunit of mannose phosphotransferase permease binds with the pediocin-like bacteriocin (70). In addition, this study revealed that specific immunity protein binds strongly with the permease complex and subsequently protects from bacteriocin-mediated cell lysis.

Class IIb (two-peptide bacteriocins): Two-peptide bacteriocins contain two different peptides and both peptides are required in equal amount for optimum bacteriocin activity (219). They exhibit very low activity when they were tested individually. More than 15 class two-peptide bacteriocins have been isolated and characterized so far (219), such as thermophilin 13, lactococcin G, mutacin IV, plantaricin E/F, lacticin F etc. All of them alter the permeability of the membrane to small molecules that results in dissipation of proton-motive force and decreasing of intracellular ATP concentration which eventually leads to cell lysis (95). Based on the types of small molecules, these bacteriocins show some specificity and different bacteriocins differ in their specificities. For examples, lactococcin G permeabilizes the cell for monovalent cations but not for divalent cations, anions or H^+ (199, 200). Plantaricin E/F or J/K permeabilizes membrane for monovalent cations and H^+ but not for divalent cations or anions (201).

Each of the peptides of two-peptides bacteriocins has distinct features and they are cationic, 30-50 amino acids long, hydrophobic and/or amphiphilic and all of them translated as

prepeptide with 15-30 amino acids N-terminal leader sequence (Table-4). This leader sequence has double glycine motif, which is cleaved off at the C-terminal side of the double glycine motif by the peptidase activity of a dedicated ABC-transporter during export of the peptides from the cell (95, 217, 219, 225). Individual peptide of some two-peptide bacteriocins displays some antimicrobial activity, such as lacticin F (10) and plantaricin E/F and plantaricin J/K (11), although few others didn't show any activity when tested individually. For example, lactococcin G, a two-peptide bacteriocins, didn't show any activity when tested individually at concentration as high as 50 µM, but showed activity at 50 pM when both of the peptides were present (200, 216). It has also been reported that a peptide from two-peptide bacteriocin only exhibit potent antimicrobial activity only if combined with the complementary peptide of the same two-peptide bacteriocin or in some cases combined with the peptide from a homologous two-peptide bacteriocin. For example, lactococcin G does not show any activity when combined with any of the peptide from plantaricin E/F or plantaricin J/K (11). However, they show activity when combined with the complementary peptide from lactococcin Q or enterocin 1071 (224, 225, 327). This reality indicates that the two peptides of class IIb bacteriocins act together to display antimicrobial activity (219). This is in agreement with the facts that the genes encoding the two-peptide bacteriocins are next to each other in a single operon along with a single immunity gene and both of them are expressed equally (18, 21, 89, 219, 247, 327). In addition, structural studies of the three two-peptide bacteriocins (lactococcin G, plantaricin E/F and plantaricin J/K) by circular dichrosim (CD) suggest that the complementary peptides interact in a structure-inducing manner upon arrival at the membrane (111, 112, 219). So, the synergistic activity of the two-peptide bacteriocins is due to inter-peptide interactions, but not the activity of individual peptides at different sites of target cell (219).

Table-4: Amino acid sequences of two-peptide bacteriocins. The GXXXG-motifs are shaded yellow. Substitution of G with S has also been considered and shaded yellow along with GXXXG- motif.

Name	Chain	Sequences	Ref.
Lactococcin G	LcnGα	GTWDDIGQGIGRVAYWVGKAMGNMSDVNQASRINRKKKH	(216)
	LcnGβ	KKWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI	
Enterocin 1071	Entα	ESVFSKIGNAVGPAAYWILKGLGNMSDVNQADRINRKKH	(89)
	Entβ	GPGKWLPWLQPAYDFVTGLAKGIGKEGNKNKWKNV	
Plantaricin S	Plsα	RNKLAYNMGHYAGKATIFGLAAWALLA	(280)
	Plsβ	KKKKQSWYAAAGDAIVSFGEGFLNAW	
Lactocin 705	705α	GMSGYIQGIPDFLKGYLHGISAANKHKKGRLGY	(297)
	705β	GFWGGLGYIAGRVGAAYGHAQASANNHHSPING	
Plantaricin E/F	PlnE	FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR	(69)
	PlnF	VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG	
Plantaricin J/K	PlnJ	GAWKNFWSSLRKGFYDGEAGRAIRR	(69)
	PlnK	RRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK	
Salivaricin P	Sln1	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	(21)
	Sln2	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH	
Mutacin IV	NlmA	KVSGGEAVAAIGICATASAAIGGLAGATLVTPYCVGTWGLIRSH	(247)
	NlmB	DKQAADTFLSAVGGAASGFTYCASNGVWHPYILAGCAGVGAVGSV VFPH	

Although the three dimensional structure of two-peptide bacteriocins is not well known as like class IIa peptides, CD and NMR studies of three peptides suggest that these peptides are unstructured in aqueous solution but gain helical structure when exposed to hydrophobic or membrane-like environment, such as trifluroethanol, micelles or liposome (87, 111, 112, 261). Almost all of the two-peptide bacteriocins carry GXXXG-motif (where X denotes any amino acid) which is believed to be involved in helix-helix interaction in membrane proteins (270). However, in some cases substitution of well-conserved G with S/A residue have been reported (81, 226). It has been proposed that α and β peptides of these two-peptide bacteriocins interact through GXXXG-motif (219). The proposed structural model suggests that α and β peptides in the helix-helix structure reside in a parallel orientation and staggered in a mode relative to each other (261), which in turn generate and/or stabilize helical structure in the GXXXG-motif and more unstructured region between the N- and C- terminal helical region in the α and β peptides. Proposed structural model also indicates that the immunity proteins of class IIb bacteriocin might interact with the N-terminal region of the α-peptide and C-terminal region of the β-peptide (219). Replacement of glycine residues in the GXXXG-motif is detrimental for antimicrobial activity of lactococcin G, suggesting that they are indeed involved in helix-helix interaction (226).

Class III (bacteriolysins): Bacteriolysins are large heat-labile proteins that cause hydrolysis of cell wall peptidoglycan structure (54). They have different domains to exert their activities, in which every domain has its specific function, such as translocation, receptor binding and lethal activity. So far, genetic characterization has been done for four bacteriolysins from LAB strains (24, 135, 214, 273). These modular proteins have N-terminal catalytic domain which has homology to endopeptidase and a C-terminus probable target recognition site (140,

162). In contrary to other classes of bacteriocins, they do not always have specific immunity proteins, rather depends on producers' cell wall modification to prevent the self-toxicity (54).

Class IV (cyclic bacteriocins): This class of bacteriocins has covalently linked N- and C- terminal structure (219). They are cationic, hydrophobic and their size varies from 3.4 kDa to 7.2 kDa. All of them permeabilize the target cell membrane to small molecules that cause the disruption of membrane potential, which ultimately results in cell lysis (219). Enterocin AS-48 is the best characterized cyclic bacteriocin which contains 70 residues and produced by several species of enterococcus (96, 189, 190). Cyclic structure might protect the bacteriocins from proteolysis (219) and are active against both gram-positive and gram-negative bacteria (184).

Mutacins (bacteriocins of *S. mutans*): Mutacins, bacteriocins of *S. mutans* origin, were first studied by Kelstrup and Gibbons (145) and the word 'mutacin' was coined by Hamada and Ooshima in 1975 (106). Interest was raised for their possible use as anti-caries agents, role in the colonization of the oral cavity and as epidemiological fingerprinting tools (100, 107). New interests are being developed for their potential use as food preservatives and as new antimicrobial therapeutics (205, 212). Several mutacins from both lantibiotics and non-lantibiotics groups have been characterized to date (Table-5) (196). Mota-Meira et al. (205) and Morency et al. (203) showed that mutacin producing bacteria can inhibit food-borne pathogenic bacteria, such as *L. monocytogenes*, *B. cereus*, *C. perfringens*, *S. aureus* and *Campylobacter jejuni*. Mutacins can also inhibit various streptococci and enterococci, including some multi-drug resistant strains (156, 206, 247, 248). Two prominent pathogens, *Helicobacter pylori* and *Neisseria gonorrhoeae* have also been shown to be sensitive to mutacins (206).

Table-5: Characteristics of known mutacins from *Streptococcus mutans*

Class	Name of Mutacins	Producer strains	Mature peptide	Activity spectra
I	I	UA140; CH43	FSSLSLCSLGCTGVKNPSFNSYCC	*L. monocytogenes*, *C. perfringens*, *Bacillus, C. sporogenes*, LAB, *Campylobacter jejuni*,& *Staphylococcus aureus* (17, 75, 196, 205, 247)
	II	T8;UA96	NRWWQGVVPTVSYECRMNSWQHVFTCC	
	III (1140)	JH1140; UA787	FKSWSLCTPGCARTGSFNSYCC	
	B-NY266	NY266	FKSWSFCTPGCAKTGSFNSYCC	
	SmbA	GS5	STPACAIGVVGITVAVTGISTACTSRCINK	*L. monocytogenes*, *S. aureus* and LAB (196, 212, 239, 325)
	SmbB	GS5	GTTVVNSTFSIVLGNKGYICTVTVECMRN CSK	
	K8	K8	MGKGAVGTISHECRYNSWAFLATCCS	Similar to mutacin I & *C. botulinum* (257, 298)
II	IV (NlmA)	UA140; UA159	KVSGGEAVAAIGICATASAAIGGLAGATL VTPYCVGTWGLIRSH	*Listeria, Enterococcus, Lactobacillus, Bacillus , Streptococcus, Clostridium* & *Leuconostoc* (103, 212, 247, 325)
	IV (NlmB)	UA140; UA159	DKQAADTFLSACGGAASGFTYCASNGVW HPYIL AGCAGVGAVGSVVFPH	
	V	UA140; UA159	GRGWNCAAGIALGAGQGYMATAGGTAF LGPYAIGTGAFGAIAGGIGGALNSCG	*L. lactis* & Non-streptococcal spp (104, 299)
	VI	UA159	GMIRCALGTAGSAGLGFVGGMGAGTVT LPVVGTVSGAALGGWSGAAVGAATFC	*Enterococcus faecium* (196, 320)
	N	N	SRQAADTFLSGAYGAAKGVTARASTGVY VVPATL VALGVYGAGLNIAFP	Similar to class I & oral streptococci (16, 102)

Mutacin B-Ny266 has inhibitory activity against many nisin-A resistant strains (*L. monocytogenes* Scott A, *Pediococcus acidilactici*), oxacillin-resistant strains (*Enterococcus faecalis, S. aureus* and *S. epidermidis*) and vancomycin-resistant strains (*N. gonorrhoeae, E. faecalis*) (205). Mutacins I and III have shown to have more potency than nisin to inhibit methicilin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) and *S. epidermidis*, displaying minimum inhibitory concentrations lower than 10 µg/ml (246, 249). Mutacins I, II, III and IV can inhibit the group A streptococci (GAS) and penicillin-resistant *S. pneumoniae* with MICs below 1µg/ml (249).

Lantibiotic mutacins show activity against the wide variety of gram-positive bacteria, whereas non-lantibiotic mutacins (Nlm) are mainly active against the closely related bacteria (196). So far, six lantibiotic mutacins have been characterized which include mutacin I (248), mutacin II (220), mutacin III/ mutacin 1140 (122, 246), mutacin B-NY266 (206), mutacin K8 (257) and mutacin Smb (325). All of the necessary genes (such as precursor, modification, transporter and immunity genes) required for lantibiotic mutacins production are organized in the same gene cluster (Fig.3) (196). On the other hand, the non-lantibiotic mutacins are widely present in every *S. mutans* strain analyzed so far (142, 196). In general, each of the strains encodes more than one non-lantibiotic mutacin genes. However, they have comparatively narrow spectrum of activity than the lantibiotic mutacins. For instance, mutacin IV is active against various non-mutans streptococcal species, staphylococci or enterococci (103, 247). So far, four different non-lantibiotic mutacins have been characterized, such as mutacins IV, V, VI and mutacin N (16, 103, 247, 320). In contrast to lantibiotic mutacins, genes necessary for non-lantibiotic mutacins are not organized in the same gene cluster, despite the same ABC transporter is required for processing and transport of multiple non-lantibiotic mutacins (103).

Although the genome sequence of *S. mutans* UA159 (7) suggests the presence of several other non-lantibiotic mutacins encoding genes based on the presence of well-conserved GXXXG and GG motifs (Table-6) (212, 299), two of them are well recognized such as non-lantibiotic mutacin IV (NlmAB) and mutacin V (NlmC) (104). Mutacin IV is a two-peptide bacteriocin produced by *S. mutans* UA140 and UA159 which is active against the mitis group of streptococci (247). Both of the peptides are encoded by *nlm* operon, which contains two genes, *nlmA* and *nlmB*, organized in tandem (7). Both peptides contain signal sequences with well conserved GG motifs, the site where peptidase cleavage occurs during export. NlmTE, a transporter complex, cleaves the signal sequence and mediates the export of these peptides (103). Each peptide also contains a single GXXXG motif, which is essential for bacteriocin activity due to its involvement in the formation of the helix-helix interaction (219). Mutacin V (also called CipB), a single peptide bacteriocin, is encoded by *nlmC*. The NlmC peptide is 52 residues long, with a signal peptide and three GXXXG motifs (104). This mutacin is also secreted by the NlmTE export system (103) and appears to have broad antimicrobial activity, ranging from mitis streptococci to lactococci and micrococcus (104). However, unlike mutacin IV, which was purified from the culture supernatant (247), mutacin V activity has never been purified.

Biosynthesis and export: At least four genetic components are required for production of class II bacteriocin (82, 219), such as (i) structural bacteriocin gene, encoding a prebacteriocin; (ii) a gene encoding an ABC transporter (ATP-binding cassette) for secretion; (iii) a gene encoding the immunity gene which provides protection to self-toxicity; and (iv) a peptide pheromone mediated two-component system to activate the bacteriocin gene expression. Bacteriocins are usually expressed as pre-peptide with an N-terminal leader sequence. This leader sequence is cleaved by site-specific protease while exporting through the ABC transporter

Table-6: Putative bacteriocins of *S. mutans* UA159 based on the presence of GG- and GXXXG-motifs in the respective peptides. Substitution of G with S has also been considered and shaded yellow along with GXXXG- motif.

Gene ID	Size	Mature peptide
SMU.150	67	KVSGGEAVAAIGICATASAAIGGLAGATLVTPYCVGTWGLIRSH
SMU.151	78	DKQAADTFLSAVGGAASGFTYCASNGVWHPYILAGCAGVGAVGSVVFPH
SMU.281	92	VLGAGTGFTAGFKPAQFLWRVPVAGPYLALGTLDLTTVAGVYIGAREGWGDGGFW
SMU.283	72	FDVKGVAASYLAMGTAALGGLACTTPVGAVLYLGAEVCAGAAVIYYGAN
SMU.299	72	GLYDGANGYAYRDSQGHWAYKVTKTPAQALTDVVVNSWASGAASFAAYA
SMU.423	76	GMIRCALGTAGSAGLGFVGGMGAGTVTLPVVGTVSGAALGGWSGAAVGAATFC
SMU.1719	82	TFIARKQMEKYLEENPPLNEDVIRNMMSQMGQKPSEAKVQQVVRQMNKQQKAAKA KAKKKK
SMU.1882	117	YNSSIGYSAQGNRGFSNYPYDVSMDSDNSSSSSNTTGGYVNYNQSFNSGW
SMU.1889	88	LAGAGTGAAVSAPAAEGGGLGPIAGAAIGWDLGAISGAGLGWANFCQ
SMU.1892	61	SENIARFAAAFENEQVVSYARWFRRSWRGSGSSSRF
SMU.1895	53	MTWAEIGAIVGATIGSFYIPNPVIVPFRVR
SMU.1896	100	FGWDSIWRGFKCVAGTAGTIGTGALGGSATGGLTLPIIGHVSGGIIGGISGAGVGIASF C
SMU.1905	62	GRAPRCAALVGASIYDGLAVVGDPVGVAMAAGTIAAGSFC
SMU.1906	70	GCSWKGADKAGFSGGVGGLIGAGGNPVGGVLGIAGGLDAYGELVGGN
SMU.1914	76	GRGWNCAAGIALGAGQGYMATAGGTAFLGPYAIGTGAFGAIAGGIGGALNSCG

and the matured peptide is secreted (114, 302) or, sometimes, by general secretion pathway (Sec) of the cell (54). The C-terminal cytosolic domain of the transporter contains the ABC-binding cassette which propels the secretion of the peptides out of the cell with the hydrolysis of ATP and the proteolytic N-terminal domain of cysteine protease family is involved in cleavage and maturation of the pre-peptide (13, 219). The pre-bacteriocins are cleaved off at the C-terminal site of the GG- motif (88) and transmembrane domains of the transporter as well as some accessory proteins are required for translocation of the mature bacteriocin through the membrane (82). For lantibiotics, the structural bacteriocin genes are normally organized with the genes needed for regulation, export, immunity and modifications (54). The leader sequence is believed to maintain the bacteriocins in inactive state in the cytosol to prevent self-toxicity. It might also act as specific signal sequence which is recognized by transporter system for export and required for post-translational modification by the modification apparatus in case of lantibiotics (43, 208, 318). Supporting this hypothesis, it has recently been shown that the secretion rate and the proteolytic efficiency are dependent on the amino acid sequences of the leader peptide (13).

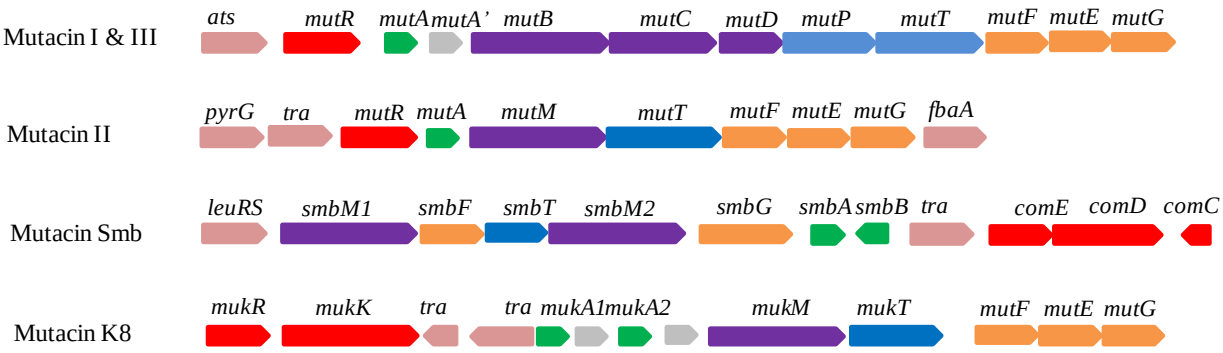
Regulation of biosynthesis: Bacteriocins production is regulated by various environmental and genetic factors. Temperature, pH, aeration, concentration of nitrogen and carbon sources, presence of essential elements, vitamins and cell density reportedly influence the bacteriocins production by altering the transcription of structural genes or operons (196, 212). However, it is still unknown how these environmental factors regulate the mutacins genes expression (196). Many studies have suggested that mutacins production in liquid medium is relatively low in compared to agar plate and yeast extract shown to be activator of mutacin production (211, 212, 233, 259). It has been shown that cell density has direct correlation with bacteriocin expression in *S. mutans*. Studies on the expression of lantibiotic mutacin I and non-

lantibiotic mutacin IV suggested relatively low level of expression in liquid media, which can be boosted to higher level if incubated at cell pellet (156, 158, 195) or allowed to form colonies onto agar plate (157).

The genetic components required for mutacin biosynthesis are located in the chromosome and display little variation in their organization (Fig. 3). Many lantibiotics show auto-inducing ability and involve the activation of two-component regulatory pathway which are organized in the same operon (152). In case of class II bacteriocins, inducing peptide is called peptide pheromone and is not present in the same operon (212). However, non-lantibiotic mutacins do not exhibit auto-inducing ability and they don't have membrane-histidine kinase and cognate response regulator in their biosynthetic operon except for mutacin K8 (257). The presence of the homologous of Rgg-family (regulator gene of glycosyltransferase) transcriptional regulator has been reported in the biosynthetic operon of mutacin I, II and III and named MutR (42, 246). MutR is indispensable for mutacin genes expression in case of mutacin I and II and mutation of *mutR* gene cause drastic reduction of transcription of the downstream mutacin operon and diminished mutacin activity in deferred antagonism assay (154, 246) (Fig. 4). Several other factors have been reported to be involved in mutacin production recently. CiaH, a two-component sensor kinase, and an interspecies signaling system, LuxS, have been shown to regulate mutacin I expression (195, 250). However, role of CiaH in mutacin production among different strains vary considerably. While inactivation of *ciaH* in *S. mutans* T8 and UA140 does not have any role in expression of mutacin II and IV respectively (250), mutacin IV production in UA159 was reduced more than 100-fold (28). Merrit et al. (195) suggested that LuxS regulates mutacin I production and ComDE controls mutacin IV production in *S. mutans*. Deletion of another two-component sensor kinase, LiaS, and an AAA^+ protease, ClpP, also reduced the

Fig. 3. Genetic organization of mutacins-encoding genes with their accessory factors in *S. mutans* [adapted from Merrit et al. (196)]. Green indicates the mutacin structural genes; red indicates the transcriptional regulator for mutacin production; purple color indicates the genes involved in post translational modification of lantibiotics; blue represents the genes necessary for transport and processing of pre-peptides; and immunity proteins are marked with orange color.

Lantibiotics



Non-lantibiotic mutacins

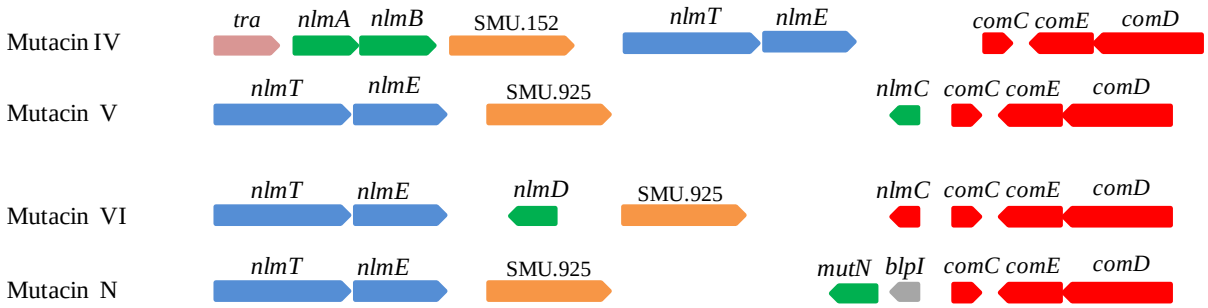
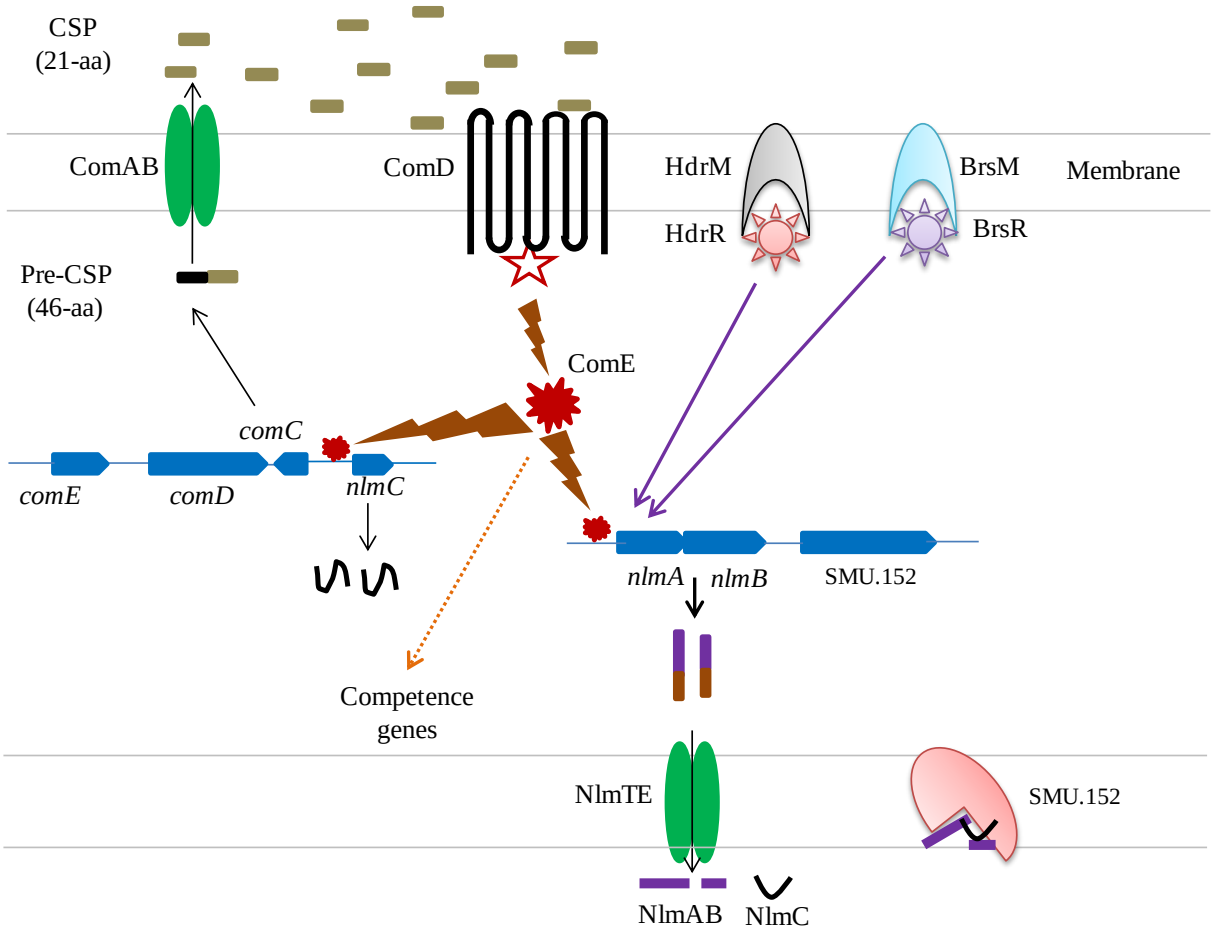


Fig. 4. Summary of the regulation of mutacins production. Various regulatory pathways involved in mutacin production are colored differently. Lightning bolt indicates direct transcriptional regulation and purple colored lines indicate conditional regulation. Dashed line indicates the indirect activation by unknown mechanism. Cell density is the most prominent extrinsic factor for mutacin genes expression which directly or indirectly activates ComCDE, HdrRM and BsrRM pathways. ComDE directly senses the external CSP and activates the expression of mutacin genes. HdrR and BrsR can activate the expression of mutacins only in the absence of their cognate repressors, HdrM and BrsM. All bacteriocins are exported outside the cell through a dedicated ABC transporter, NlmTE.

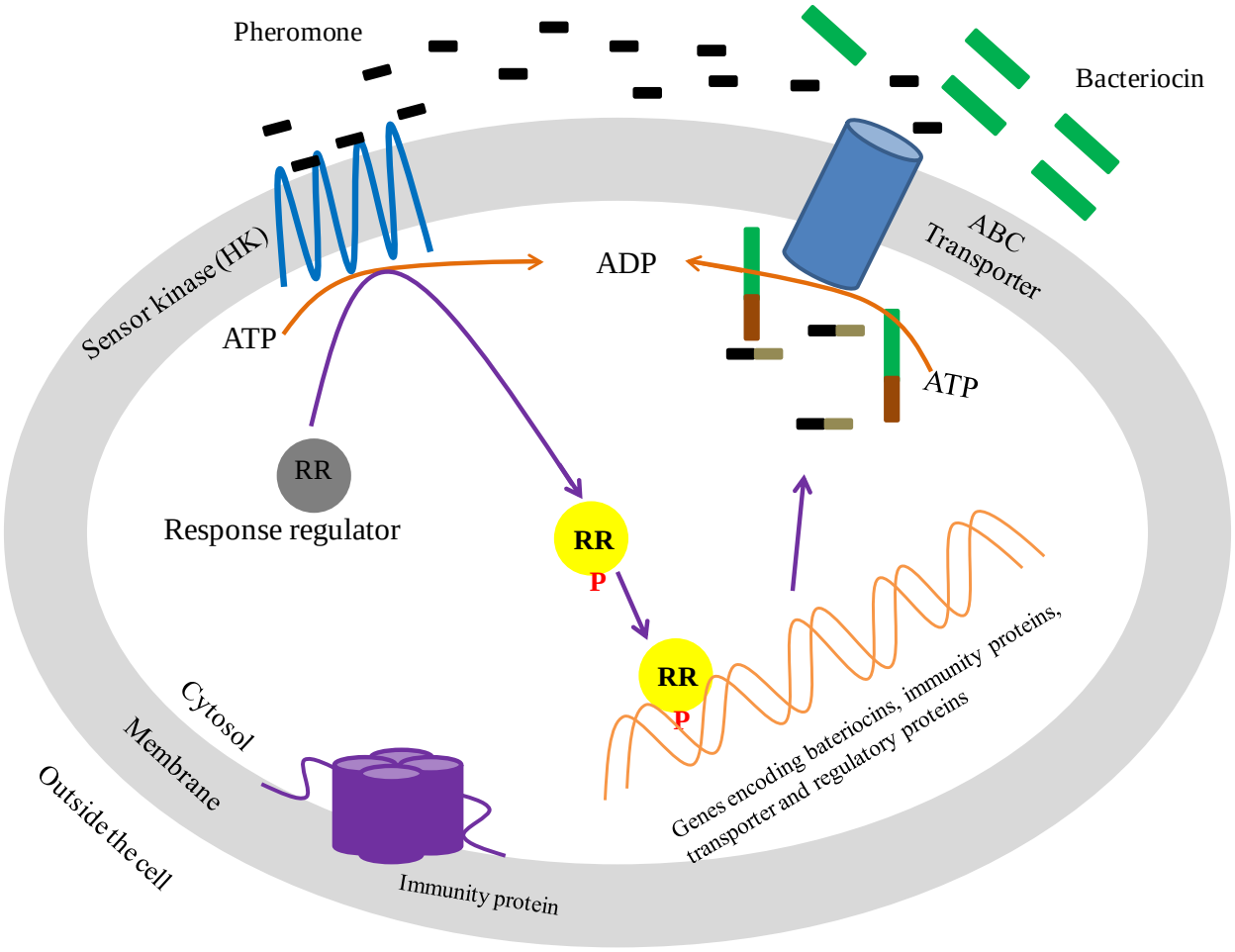

CSP
(21-aa)
ComAB
ComD
HdrM
HdrR
BrsM
BrsR
Membrane
Pre-CSP
(46-aa)
ComE
comC
comE
comD
nlmC
nlmA
nlmB
SMU.152
Competence
genes
NlmTE
SMU.152
NlmAB
NlmC

mutacin IV production in UA159 (41, 46). Random insertional mutagenesis revealed 25 additional genes for the expression of mutacin I in the virulent strain UA140 (291). They include two-component signal transduction system (*vicRK* and *hk03/rr03*), a stress response regulator (*hrcA*), metabolic enzymes (*pttB, adhE*) and some genes involved in central cellular processes. In the same manner, Merrit et al.(197) identified a novel high-cell-density-responsive membrane bound protein, HdrM, which regulates mutacin production and competence development in *S. mutans*. Recently, another novel regulator for mutacin gene expression has been reported (BrsRM) and both of the new regulators bypass the classic ComCDE system for mutacin production (221, 222, 320). Both of these genes show similarity with LytTR family two-component regulator of various gram-positive bacteria (212). These newly identified regulatory systems (HdrRM and BrsRM) contain a membrane-bound inhibitor protein (M) and an associated LytTR family transcription factor, where inhibitor proteins (HdrM and BrsM) antagonize the activity of the transcriptional regulators (HdrR and BrsR)(196). For this reason, mutation of *hdrM* and *brsM* caused constitutive expression of *hdrR* and *bsrR*. As a result, mutacin genes were expressed greatly and increased mutacin production was observed during deferred antagonism bacteriocin assay (221, 320). Further studies suggested that BrsR can bind ComE-response-element (another LytTR family transcriptional activator) present in the promoter region of bacteriocin genes (320). However, it is still unknown which cellular or environmental factors activate these genes.

Expression of mutacins production in *S. mutans* UA159 is mostly driven by a two-component signal transduction system through the quorum-sensing mechanism (Fig.5) (54, 196). In this organism, a two-component system (ComDE) is induced by a peptide pheromone called, CSP (competence stimulating peptide) in response to high cell density and some other

environmental stresses (172, 237, 299). CSP-mediated response is also implicated in biofilm formation, competence development and acid-tolerance (6, 173). CSP is encoded by *comC* gene and translated as prepeptide with a leader sequence containing double-glycine (GG) motif which is cleaved off while exporting through the ABC transporter, CslAB (competence-related secretory locus) (103, 173, 212, 241). When a certain level of CSP reaches in the medium, it is recognized by membrane sensor kinase (ComD) and become autophosphorylated which in turn activates the response regulator (ComE) by transferring the phosphate group. The activated ComE then induces the expression of mutacins and related genes by recognizing an conserved imperfect 11-bp direct repeats present in the promoter region of the bacteriocins encoded genes (1, 129, 153, 156, 158, 168, 237, 299). Due to this ability of binding to direct repeat motif, ComE is included to the group of AlgR/AgrA/LytR family transcription activators (213). The CSP-stimulated signal transduction system also activates a competence-controlling alternative transcription factor, ComX (also known as SigX), by an unknown indirect mechanism (156), since ComE binding site is absent in the promoter region of *comX* gene. Instead, they contain a com-box (also termed cin-box) consensus sequence (TACGAATA) in the -10 promoter region of the responsive genes (173). In streptococci, ComX regulates the expression of late competence genes *comYA-G* which encode proteins involved in DNA uptake and recombination process essential for competence development (187). Based on these outcomes, it was proposed that mutacin production and competence development in *S. mutans* is co-coordinately regulated by ComE and/or ComX (173). Recently, Mashburn-Warren et al. (191) showed that ComR/ComS is essentially required for competence development through the activation of SigX and ComE can't activate the *sigX* expression in *comR* mutant, suggesting that ComE acts upstream of ComR/ComS regulon by an still unknown mechanism and can't bypass it. Furthermore, *comE*

Fig. 5. Schematic diagram of the biosynthesis of class II bacteriocins by two-component signal transduction pathway. Mature inducer peptides (pheromone) pass through the ABC transporter and activate sensor histidine kinase (HK) which is autophosphorylated at the cytosolic side. Activated HK can now transfer the phosphate group to cytosolic response regulator (RR), which in turn activates the expression of bacteriocins related genes.


Pheromone
Bacteriocin
Sensor kinase (HK)
ABC Transporter
ADP
ATP
ATP
RR
Response regulator
RR
P
RR
P
Cytosol
Membrane
Outside the cell
Immunity protein
Genes encoding bateriocins, immunity proteins, transporter and regulatory proteins

deletion neither blocked *sigX* expression, nor CSP could activate the transformation in *comR* mutation background. In addition, deletion of *comC* didn't exhibit any significant effect on *comX* expression and transformation in *S. mutans* UA159 (6), which raised the question of the role of CSP in competence development.

Bacteriocin immunity: Bacteria that produce bacteriocins always have immunity mechanism which protects them against the cognate bacteriocin. This system is highly specific and usually don't provide protection against other bacteriocins (278, 297), even though, some exceptions have been reported (79, 90). For instance, lactococcin G immunity protein provides protection against lactococcin G, but not against enterocin 1071. However, lactococcin G immunity protein can protect enterococci against enterocin 1071 (212). In case of lantibiotics, the immunity system is conferred by three genes (*lanFEG*) which encodes the LanFEG ABC transporter which pumps out lantibiotics from the membrane to the external medium (212). These proteins might be associated with another gene (*lanI*) which encodes a lipoprotein that has membrane-bound specific lantibiotic-binding activity, thereby protecting the producer bacteria (286). These two immunity systems function either independently or cooperatively to provide the immunity activity against the cognate lantibiotic (12, 40, 279).

For class II bacteriocins, immunity is generally conferred by one gene located just downstream of the bacteriocin structural gene(s) in a same operon (147, 212). For this conserved genetic organization, it is easy to identify the putative immunity genes for cognate bacteriocins and their functions have been tested by heterologous expression in sensitive cells. For instance, LagC immunity protein rendered the sensitive bacteria immune to cognate class II bacteriocin, lactococcin G (223) and EntqC provides immunity activity to enterocin Q (56). Although immunity proteins for class II bacteriocins differ to a great extent in their size and sequences,

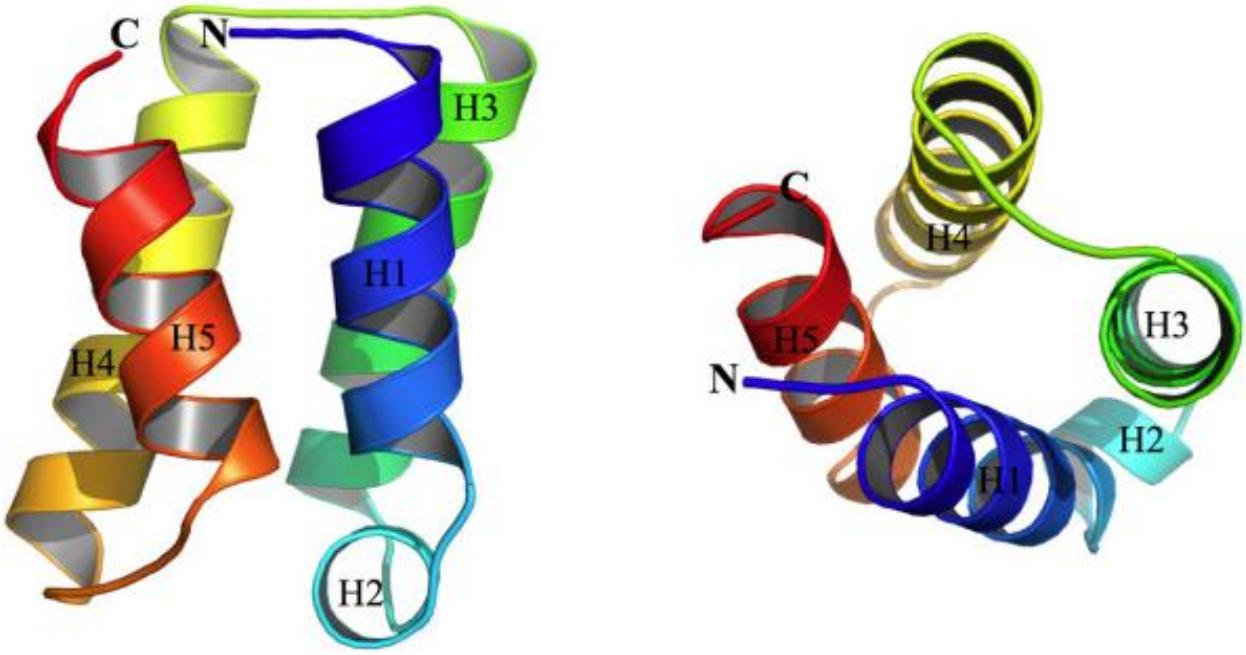
exact molecular mechanisms of immunity is still unclear for most of the bacteriocins. Recently, few studies have tried to understand the immunity mechanisms for some class II bacteriocins (70, 93, 149). To date, at least 20 tentative immunity proteins for class IIa bacteriocins have been identified based on their DNA sequences (73). They are highly charged (containing 25-35% charged residues) and mostly localized in the cytosol or loosely associated with the inside of the cell membrane (61, 251). Expression of these proteins can protect the sensitive cells to externally added cognate bacteriocins (85, 251). However, external addition of both the bacteriocins and immunity proteins can't provide protection to sensitive cells, which indicates that immunity proteins to class IIa act from inside the cells. Similar results were also found for the immunity protein of non-class IIa bacteriocin, lactococcin A (215, 277, 301).

Three-dimensional structure of at least two class IIa immunity proteins have been illustrated recently. Immunity proteins for carnobactericin B2 (ImB2) was studied by NMR (277) and immunity protein for enterocin A (EntA-im) was studied by X-ray crystallography (59, 138). Both of the studies revealed that they have the same 3D structure despite the presence of different amino acids and immunity activity toward two different class IIa bacteriocins. Both of them are globular in nature and cytosolic with four antiparallel left-turning alpha helices linked with short loops and the helices are oriented relative to each other forming a hydrophobic core in the center of the protein, whereas the hydrophilic and charged residues are present at the surface of the proteins (Fig. 6). ImB2 and EntA-im shows structural differences at the C-terminal parts the helix-4. Whereas ImB2 has distinct short alpha helix with a C-terminal extension, C-terminal ends of EntA-im is relatively flexible (138). Homology modeling revealed that the four-helix bundle is a conserved structural motif for class IIa immunity proteins and the length of the alpha helices are relatively constant with minor variation in the loops (73, 138). Presence of positively

charged residues on the surface of immunity proteins has been predicted to be involved in interaction with the negatively-charged phospholipids of the membrane (138, 277). However, structural analysis with circular dichroism of EntA-im while exposing to membrane-mimicking entities did not provide any further structuring, indicating that class IIa bacteriocins don't interact with the cell membrane extensively (139). But the immunity protein to non-class II bacteriocin (lactococcin A) interacts with the membrane to display immunity activity (215, 301). Recently, Chang et al. (39) solved the crystal structure of the gene-product of locus Spy_2152 from *S. pyogenes* and found that it also contains an anti-parallel four-helix bundle that is structurally similar to other class IIa bacteriocin immunity proteins. This protein can be a possible immunity protein to class IIb bacteriocin based on the fact that *S. pyogenes* does not contain any class IIa pediocin-like proteins but has class IIb bacteriocins encoding genes. It has been shown that the first methionine and C-terminal 23 residues are disordered and the four helices are organized tightly together around a well-defined hydrophobic core. However, its cognate bacteriocin has not been identified yet.

The immunity proteins exhibit specificity to their cognate bacteriocins, even though their 3D structures show high level of similarity (139, 251). Fimland et al. (85) demonstrated that immunity proteins to pediocin-like bacteriocins can protect against their cognate bacteriocins as well as to other class IIa bacteriocins, although the extent of immunity was lesser in case of non-cognate bacteriocin. Cross-immunity was mainly directed toward closely related bacteriocins. For example, pediocin PA-1/AcH and sakacin P are very similar and immunity protein of pediocin or sakacin displayed immunity activity against both of them, even though they were only 28% similar (85). Cross-immunity is not limited to class IIa bacteriocins and it has been found that immunity protein for carnobacteriocin A can protect structurally related bacteriocin

Fig. 6. Crystal structure of a typical bacteriocin immunity protein (pyogenecin) isolated from *Streptococcus pyogenes* (39). The structure shows a four helix bundle in two-orthogonal view and the N- and C-termini are labeled blue and red color respectively.


C
N
H3
H1
H5
H4
H2
C
H4
H5
H3
N
H2
H1

enterocin B (90). Studies with hybrid immunity proteins, Johnsen et al. (139, 140) demonstrated that the C-terminal, disordered region of immunity proteins specifically interacts with the C-terminal part of the cognate class IIa bacteriocin (140, 277). According to this model, there is no direct contact between bacteriocins and immunity proteins and both of them reside in opposite sides of the cell membrane (Fig. 7a). The mechanisms by which the C-terminal part of the immunity protein recognize the cognate bacteriocins and protect against it is still unknown (73).

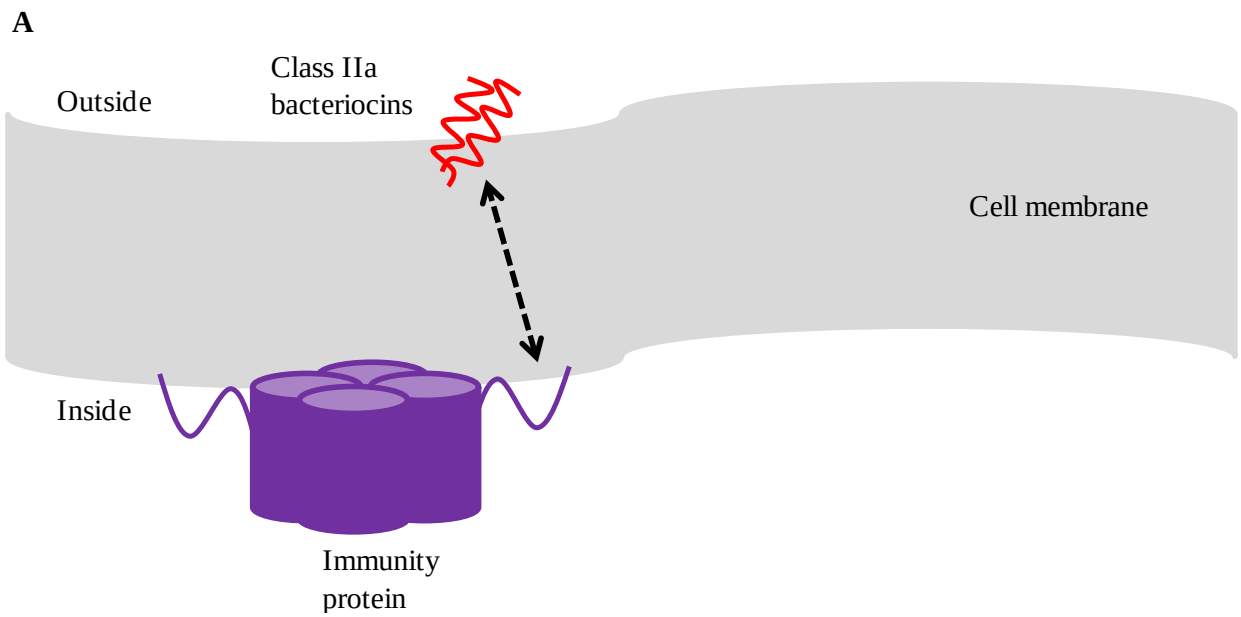
The length of the immunity proteins to Class IIa bacteriocins and lactococcin G varies in size from 88 to 118 amino acids and display very poor similarities in their sequences in compared to high sequence similarities among their target bacteriocins (79, 85). Immunity system to lactococcin G, a class II bacteriocin, is well studied and revealed the details mechanism of immunity functions in *L. lactis*. Cloning of *lciA* (lactococcin A immunity gene) to a sensitive strain has rendered it to be immune to lactococcin G (125). It has been proposed that mannose phosphotransferase system (man-PTS) is involved in target recognition for these bacteriocins to induce pore formation in the membrane (147), since resistant strains were found to lack in expression of man-PTS (99, 253). In addition, introduction of Man-PTS from a sensitive cell converted the resistant lactococcal cells to become sensitive to various class IIa bacteriocins (70, 252). It has been reported that immunity proteins are always associated with the membrane fraction in producer bacteria although they are normally localized in the cytosol (301), suggesting two different conformations in producer cells. Recently, Diep et al. (70) showed that immunity protein, LciA, behaves differently whether bacteriocins are present or not. In the presence of bacteriocin, the immunity protein is strongly associated with the bacteriocin-man-MPT complex, thereby providing immunity to the producer cells (Fig.7b). On the other hand, immunity proteins were found unassociated in the absence of bacteriocin. This study

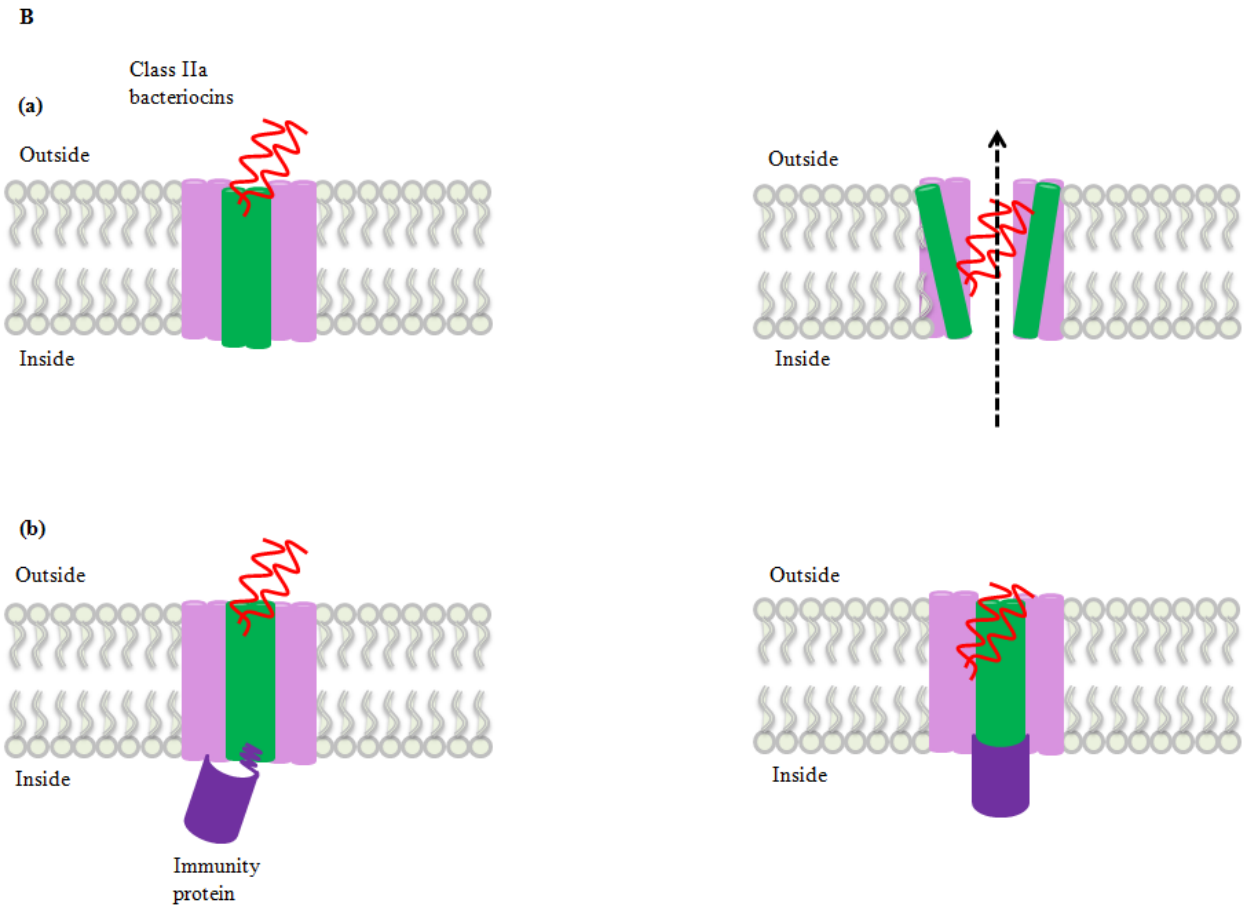
indicated that similar bind-and-lock immunity mechanism is present to several other class IIa bacteriocins (70), suggesting the convergence of receptor targeting (man-PTS) in class II bacteriocins despite the lack of similarities in amino acid sequence of bacteriocins and their cognate immunity proteins (147).

Transporter systems have also been shown to be involved in immunity activity against two bacteriocins (67, 93). Multidrug resistance (MDR) transporter system provides immunity to bacteriocins LsbA and LsbB in a natural isolate of *Lactococcus lactis* where the transporter LmrB is involved in secretion and immunity to this class II bacteriocin (93). In case of enterocin AS-48, immunity activity is conferred by specialized ABC transporters which are presumed to pump out the bacteriocins from the membrane (67).

Immunity mechanisms to two-peptide bacteriocins are poorly understood. Recent studies suggest that a proteolytic mechanism might be involved in conferring immunity to class IIb bacteriocin (150). It has been reported that Abi family proteases determine the immunity activity in several class IIb bacteriocins encoding loci, such as *pnc* locus of *S. pneumoniae* (179), *pln* locus of *Lactobacillus plantarum* and the *skk* locus of *Lactobacillus sakei* (150). While immunity activity to class II bacteriocins show high level of specificity, Abi family immunity proteins display cross-immunity to different bacteriocins (150), which indicates that they provide immunity via a common proteolytic mechanism (147). Despite the fact that substrates of this proteases family are yet to be identified in bacteria; it has been shown that bacteriocin itself is not the proteolytic substrate of this proteases (150).

Fig. 7. Proposed model for immunity mechanism. (A) A model showing the presence of bacteriocins on the extracellular side of the cell membrane and cognate immunity protein on the cytosolic side. C-terminal part of the immunity protein recognizes the bacteriocins and protects against it; (B) this cartoon shows the mode of action (a) and immunity mechanism (b) for class IIa bacteriocin system. The N-terminal β-sheet of bacteriocin (red color) interacts with the extracellular loop of Man-PTS IIc protein (green bar) and thereafter C-terminal helical part inserts into the membrane by interacting with the transmembrane helices of the IIC and/or IID proteins (right panel) which leads to pore formation and cell death (a). During immunity, the specific immunity protein blocks the pore formation by binding tightly to the Man-PTS in response to bacteriocin-mediated conformational change (b).


A
Outside
Class IIa
bacteriocins
Cell membrane
Inside
Immunity
protein


B
(a)
Class IIa
bacteriocins
Outside
Inside
Outside
Inside
(b)
Outside
Inside
Immunity
protein
Outside
Inside

In this study, I explored the role of NlmA and NlmB peptide in mutacin IV activity, mechanism of self-immunity and CSP-mediated regulation of *nlmAB* expression. By using genetic approaches and with semi-purified peptides, we showed that optimum activity is dependent on the presence of both peptides. We also showed that mutacin IV is active against multiple streptococci and lactococcal species. Our study suggests that a gene, SMU.152, located just downstream of *nlmAB* operon confers immunity activity to mutacin IV in *S. mutans* UA159. However, this immunity is not specific; several other putative immunity proteins could also provide cross-immunity to mutacin IV in producer strain in absence of cognate immunity protein, SMU.152. By using pGhost9::IS*S1* mutagenesis, we have identified several new factors required for expression of mutacin IV. Further studies revealed that a well-conserved Lon protease-mediated cleavage of CSP is required for the activation of *nlmAB* genes expression. By site directed mutagenesis, we identified that S-235 and K-280 are essential for Lon protease activity of SMU.518. Our studies also demonstrated that a gene involved in glucose side chain formation, SMU.832, and a conserved hypothetical protein, SMU.2137, are also required for optimum mutacins production by *S. mutans* UA159.

CHAPTER TWO

METHODS AND MATERIALS

Bacterial strains and growth conditions: *Escherichia coli* strain DH5α and T-10 were grown in Luria-Bertani medium supplemented, when necessary, with ampicillin (100 μg/ml), erythromycin (300 μg/ml), and kanamycin (100 μg/ml). Various streptococci strains were routinely grown in Todd-Hewitt medium (BBL; Becton Dickson) supplemented with 0.2% yeast extract (THY) at 37°C. *Lactococcus lactis* MG1363 was grown in M17 medium supplemented with 0.5% glucose or THY medium at 30°C. When necessary, erythromycin (5-10 μg/ml), kanamycin (300-500 μg/ml) or spectinomycin (300 μg/ml) was added to the sterile growth media. Bacterial growth was monitored with a Klett-Summerson colorimeter as described previously (30).

Transformation assay: To carry out transformation assay, S. *mutans* strains were grown up to desired growth stage (A_{595} of 0.2 until or unless mentioned) and CSP (200nM) was added if needed followed by 10 min incubation at 37^0C. 1.0 μg of linear or circular DNA was added to 1.0 ml culture; followed by incubation at 37^0C for 2-hr and plating onto THY-agar plate with or without antibiotics. Colonies were appeared after 2-3 days of incubation and a single colony was inoculated into THY-broth with respective antibiotics for further analysis. Transformation efficiency was measured as transformants/10^9 cfu/μg of DNA if needed. Total counts were measured by plating onto THY agar plate without any antibiotic.

Deletion of *nlmAB* operon: *nlmAB* operon was deleted using a Cre-*loxP* based method (20). First, *nlmAB* operon with approximately 700-bp flanking regions was amplified with primer

set SMU. NlmA Nested-F1and Xho-SMU-Nlm-R1 (for all the primers, see Table-9) using *S. mutans* UA159 chromosomal DNA as template and cloned into the pGEM-T Easy cloning vector (Promega) to create the plasmid, pIB-D10. A Km resistant cassette with flanking modified *loxP* sites, was amplified from pUC4 ΩKm (235) using the primers lox71-Km-F and lox66-Km-R and cloned into BbsI and BstEII digested, T4 DNA polymerase blunted pIB-D10, to generate pIB-D16. Plasmid pIB-D16 was linearized with EcoRI and transformed into *S. mutans* UA159 as previously described (31). Transformants were selected on THY plates containing Km; one such transformant was named IBS-D1. To eliminate the *loxP*-Km^r cassette from the chromosome, IBS-D1 was transformed with pCrePA(20), which expresses the *cre* recombinase gene from a temperature-sensitive replicon (pWV01). Em^r and Km^s colonies were then selected which contain pCrePA and lost the Km^r resistance gene from the chromosome. To cure the plasmid, one selected colony was inoculated into THY broth without antibiotics and grown overnight at 37^0 C and plated onto THY-agar plate. Em^s colonies were isolated by replica patching; one such clone was selected for further analysis and named IBS-D3. Deletion of *nlmAB* operon was verified by PCR analysis.

Deletion of *nlmA* gene: *nlmA* gene was deleted using a Cre-*loxP*- based method as previously described (20). First, approximately 1000-bp upstream and downstream regions were amplified with primer set SMU-NlmA-Nested-F1/EcoR-V SMU. NlmA-5'Fusion and EcoR-V SMU. NlmA-3'Fusion/SMU. Nlm Nested –R1 (for all the primers, see Table-9) using *S. mutans* UA159 chromosomal DNA as template. Two fragments were then digested with EcoRV followed by ligation and cloning into the pGEMT-Easy cloning vector (Promega) to create pIB-D15. A Km resistant cassette with flanking modified *loxP* sites, was amplified from pUC4ΩKm using the primers lox71-Km-F and lox66-Km-R and cloned into the EcoRV digested site to

generate pIB-D17. Plasmid pIB-D17 was linearized with *Eco*RI and transformed into *S. mutans* UA159 as described above. Transformants were selected on THY plates containing Km; one such transformant was named IBS-D2. To eliminate the *lox*P-Km^r cassette from the chromosome, IBS-D2 was transformed with pCrePA, which expresses the *cre* recombinase gene from a temperature-sensitive replicon (pWV01). Subsequently, Em^r and Km^s colonies were selected; those colonies contain pCrePA and lost the Km resistance gene from the chromosome. To cure the plasmid, one selected colony was inoculated into THY broth without antibiotics and grown overnight at 37^0 C and plated onto THY-agar. Em^s colonies were isolated by replica patching; one such clone was selected for further analysis and named IBS-D4. Deletion of *nlmA* gene was verified by PCR analysis (Fig. 8B) and sequencing.

Deletion of *nlmB* gene: *nlmB* gene was deleted by overlapping fusion PCR. First, approximately 1000-bp upstream and downstream regions were amplified with primer set SMU-NlmA-Nested-F1/ *nlmB* 5' fusion-R and *nlmB* 3' fusion-F / XhoI SMU. Nlm-R using *S. mutans* UA159 chromosomal DNA as template. A Km resistant cassette was amplified from pIB-D7 (created by cloning Km cassette into pGEMT-EZ vector) using primers Kan D7 F and Kan D7 R which have the 20-bp overlapping region with the two fusion primers used for amplifying upstream and downstream sites of *nlmB* gene. Equal amount of PCR products from each of the three products were further used as template for overlapping PCR using the primer set SMU-NlmA-Nested-F2/ SMU- Nlm-Nested-R1. Amplified product was PCR purified and transformed into UA159 as described above. Transformants were selected on THY plates containing Km and one such transformant was named IBS-D10. Deletion of *nlmB* gene was verified by PCR analysis (Fig. 8B) and sequencing.

Deletion of *nlmAB* and *nlmC* gene: To delete the *nlmC* gene, a fragment containing *nlmC* locus was amplified with ~1000-bp flanking regions from the genomic DNA of an *nlmC* deletion strain (containing Em^r cassette; kindly provided by C. Levesque, University of Toronto) with primers SMU NlmC F1 and SMU. 1913-R (237). Amplified product was PCR purified and transformed into IBS-D3 as described above. Transformants were selected on THY plates containing Em; one such transformant was named IBS-D13. Deletion of *nlmC gene* was verified by PCR analysis.

Deletion of SMU.152: SMU.152 was deleted by fusion PCR as previously described (127). Briefly, 1000-bp upstream and downstream regions were amplified with the primers sets SMU. NlmA Nested F/SMU.152 5' Fusion-R and SMU.152 3' Fusion-F/SMU.152 3' R using *S. mutans* UA159 chromosomal DNA as template. A Km resistant cassette was amplified from pIB-D38 with NcoI-Kan-F and PstI-Kan-R primers and overlapping fusion PCR was carried out using equal amount of each PCR products with the primers SMU. NlmA Nested F and SMU.152 3' R. The amplified products were purified and transformed into *S. mutans* UA159 as described above. Deletion of the SMU.152 locus was verified by PCR analysis and named, IBS-16. To eliminate the *lox*P-Km^r cassette from the chromosome, IBS-D16 was transformed with pCrePA. This plasmid has *cre* recombinase gene, an Em^r gene for selection, and a temperature- sensitive replicon (pWV01). Transformants were grown at 30^0C on THY-Em plates, and selected colonies were then grown into THY-Em broth and plated onto THY-Em plates. Colonies were patched onto THY plates containing Km. Selected Em^r and Km^s colonies were inoculated into THY broth and incubated overnight at 37^0C to cure the pCrePA. After overnight growth, they were spread onto THY-agar without antibiotic. Colonies from THY-agar plate were then patched onto THY-

agar and THY-Em plate. After two days of incubation, Em^s colony was selected for further analysis. One of such clone was verified by PCR analysis and named IBS-D56.

Deletion of SMU.1909 to SMU.1913: The region spanning from SMU.1909 to SMU.1913 was deleted by fusion PCR as described above with the primer sets SMU.1908 F/ EcoRV SMU.1909 5' Fusion and EcoRV-SMU.1913-3' Fusion/SMU.1915-R using *S. mutans* UA159 chromosomal DNA as template for generating upstream and downstream fragments. Two fragments were then digested with EcoRV, followed by ligation and cloning into the pGEM-T Easy cloning vector (Promega) to create pIB-D22. A Km-resistant cassette with flanking modified *loxP* sites was amplified from pUC4ΩKm using the primers lox71-Km-F and lox66-Km-R and cloned into the EcoRV-digested site to generate pIB-D23. Plasmid pIB-D23 was linearized with EcoRI and transformed into UA159 and IBS-D3 to generate IBS-D6 and IBS-D8 respectively. Positive clones were verified by PCR analysis.

Construction of multiple gene knockout mutant: Genomic DNA was isolated from ΔSMU.925-Em (237) and digested with EcoRV. Digested DNA products were then transformed into IBS-D8 and selected on THY medium plates containing Em; one such transformant was named IBS-D58.

Deletion of PTS locus: PTS locus containing the genes SGO.1679 to SGO.1682 was deleted by fusion PCR as previously described (127). At first, approximately 1000-bp upstream and downstream regions were amplified with primer set SGO-PTS-F/SGO-PTS-5'Fusion-R and SGO-PTS-3'-Fusion-F/ SGO-PTS-3'-R using *S. gordonii* DL-1 chromosomal DNA as template. A Km resistant cassette was amplified from pIB-D38 (Constructed by cloning the Km cassette into pGEMT-Easy) using the primers Nco-Kan-F and Pst- Kan-R, each of which has 20-bp

overlapping region with the upstream and downstream fragments respectively. Overlapping fusion PCR was carried out using the equal amount of each PCR products with the primers SGO-PTS-F/ SGO-PTS-3'-R. The amplified products were purified and transformed into *S. gordonii* DL-1 as previously described (20). Deletion of the PTS locus was verified by PCR analysis.

Insertional inactivation of SMU.518: The gene encoding the SMU.518 was insertionally inactivated using a Cre-*loxP*- based method as previously described (20). SMU.518 operon was amplified from *S. mutans* UA159 chromosomal DNA using the primers, SMU.518 5' F and SMU.518 3' R and cloned into the pGEM-T Easy cloning vector (Promega) to create pIB-D48. A Km resistant cassette with flanking modified *loxP* sites, was amplified from pUC4ΩKm (235) using the primers lox71-Km-F and lox66-Km-R and cloned into EcoRV-digested pIB-D48 to generate the plasmid, pIB-D50. Plasmid pIB-D50 was linearized with EcoRI and transformed into *S. mutans* UA159 as previously described (31).The transformants were selected on THY-Km plates containing Km and named IBS-D20. To eliminate the *lox*P-Km^r cassette from the chromosome, IBS-D20 was transformed with pCrePA. This plasmid has *cre* recombinase gene, an Em^r gene for selection, and a temperature- sensitive replicon (pWV01). Transformants were grown at 30^{0}C on THY-Em plates, and selected colonies were then grown into THY-Em broth and plated onto THY-Em plates. Colonies were patched onto THY plates containing Km. Selected Em^r and Km^s colonies were inoculated into THY broth and incubated overnight at 37^{0}C to cure the pCrePA. After overnight growth, they were spread onto THY-agar without antibiotic. Colonies from THY-agar plate were then patched onto THY-agar and THY-Em plate. After two days of incubation, Em^s colony was selected for further analysis. Deletion of SMU.518 was verified by PCR analysis and named IBS-D29.

Deletion of SMU.518: SMU.518 was deleted by advanced Cre-loxP based marker-less gene deletion system (20). Briefly, SMU.518 with the upstream and downstream regions was amplified with the primers set SMU.518 5' F and SMU.518 3'R using *S. mutans* UA159 chromosomal DNA as template and cloned into the pGEMT-Easy cloning vector (Promega) to create pIB-D48. A Km resistant cassette with flanking modified *loxP* sites, was amplified from pUC4 ΩKm (235) using the primers lox71-Km-F and lox66-Km-R and cloned into BbsI and EcoRV digested, T4 DNA polymerase blunted pIB-D48, to generate pIB-D54. Plasmid pIB-D54 was then linearized with EcoRI and transformed into *S. mutans* UA159 as previously described (31). Transformants were selected on THY plates containing Km; one such transformant was named IBS-D30. To eliminate the *loxP*-Km^r cassette from the chromosome, IBS-D30 was transformed with pCrePA (20), which expresses the *cre* recombinase gene from a temperature-sensitive replicon (pWV01). Em^r and Km^s colonies were then selected which contain pCrePA and lost the Km^r resistance gene from the chromosome. To cure the plasmid, one selected colony was inoculated into THY broth without antibiotics and grown overnight at 37^0 C and plated onto THY-agar. Em^s colonies were isolated by replica patching; one such clone was selected for further analysis and named IBS-D31. Deletion of SMU.518 was verified by PCR analysis.

Deletion of *comC*: SMU.1915 encoding the competence stimulating peptide (CSP) was deleted by overlapping fusion PCR. Briefly, 1000-bp upstream and downstream regions were amplified with the primers set XhoI SMU. 1913 R / SMU.1915 5' fusion-R and SMU.1915 3' fusion-F/ SMU.1915 3' R using *S. mutans* UA159 chromosomal DNA as template. A Km resistant cassette was amplified from pIB-D38 with the same primers as mentioned above and overlapping fusion PCR was carried out using the equal amount of each PCR products with the primers XhoI SMU.1913 R / SMU.1915 3' R The amplified products were purified and

transformed into *S. mutans* UA159 as described above to get the clone, IBS-D36. To eliminate the *loxP*-Km^r cassette from the chromosome, IBS-D36 was transformed with pCrePA (20), which expresses the *cre* recombinase gene from a temperature-sensitive replicon (pWV01). Em^r and Km^s colonies were then selected which contain pCrePA and lost the Km^r resistance gene from the chromosome. To cure the plasmid, one selected colony was inoculated into THY broth without antibiotics and grown overnight at 37^0 C and plated onto THY-agar. Em^s colonies were isolated by replica patching; one such clone was selected for further analysis and named IBS-D39. Deletion of the *comC* locus was verified by PCR analysis.

Deletion of *nlmTE*: *nlmTE*, encoding the transporter of non-lantibiotic mutacins, was deleted by overlapping fusion PCR as described above. Briefly, 1000-bp upstream and downstream regions of *nlmTE* locus (SMU. 286 & SMU.287) were amplified with the primers set SMU. NlmT Nested F1/SMU. NlmT 5' Fusion R and SMU. NlmTE 3' Fusion F/ SacII NlmTE R1 using *S. mutans* UA159 chromosomal DNA as template. A Km resistant cassette was amplified from pIB-D38 with the same primers as mentioned above and overlapping fusion PCR was carried out using the equal amount of each PCR products with the primers SMU. NlmT Nested F and SacII NlmTE R1. The amplified products were purified and transformed into *S. mutans* UA159 as described above to get the clone, Δ*nlmTE*::*loxP-km*^{r}. To eliminate the *loxP-km*^{r} cassette from the chromosome, pCrePA (20) was introduced into the mutant bacteria, which expresses the *cre* recombinase gene from a temperature-sensitive replicon (pWV01). Em^r and Km^s colonies were then selected which contain pCrePA and lost the Km^r resistance gene from the chromosome. To cure the plasmid, one selected colony was inoculated into THY broth without antibiotics and grown overnight at 37^0 C and plated onto THY-agar. Em^s colonies were

isolated by replica patching; one such clone was selected for further analysis and named IBS-D32. Deletion of the *nlmTE* locus was verified by PCR analysis.

Deletion of *cslAB*: The genetic locus spanning the *cslAB* gene was also deleted by overlapping fusion PCR as described above. Briefly, 1000-bp upstream and downstream regions of *cslAB* region (SMU. 1897 to SMU.1900) were amplified with the primers, SMU.1898 F/ SMU.1898 5' Fusion and SMU.1898 3' Fusion F/ SMU.1898 R using *S. mutans* UA159 chromosomal DNA as template. A Km resistant cassette was amplified from pIB-D38 with the same primers as mentioned earlier and overlapping fusion PCR was carried out using equal amount of each PCR products with the primers SMU.1898 F and SMU.1898 R. The amplified products were purified and transformed into *S. mutans* UA159 as described above to get the clone IBS-H4. Deletion of the *cslAB* locus was verified by PCR analysis.

Deletion of SMU.832: SMU.832 was deleted by Cre-*loxP*- based method as previously described (20). Briefly, SMU.832 operon was amplified from *S. mutans* UA159 chromosomal DNA using the primers set, SMU.832 5' F and SMU.832 3' R and cloned into the pGEM-T Easy cloning vector (Promega) to create pIB-D64. A Km resistant cassette with flanking modified *loxP* sites, was amplified from pUC4ΩKm (235) using the primers lox71-Km-F and lox66-Km-R and cloned into BbsI-digested, T4 DNA polymerase blunted pIB-D64 to generate the plasmid, pIB-D67. Plasmid pIB-D67 was linearized with EcoRI and transformed into *S. mutans* UA159 as previously described (31). The transformants were selected on THY-Km plates containing Km and named IBS-D48. To eliminate the *lox*P-Km^r cassette from the chromosome, IBS-D48 was transformed with pCrePA. This plasmid has *cre* recombinase gene, an Em^r gene for selection, and a temperature- sensitive replicon (pWV01). Transformants were grown at 30^0C on THY-Em

plates, and selected colonies were then grown into THY-Em broth and plated onto THY-Em plates. Colonies were patched onto THY plates containing Km. Selected Em^r and Km^s colonies were inoculated into THY broth and incubated overnight at 37^0C to cure the pCrePA. After overnight growth, they were spread onto THY-agar without antibiotic. Colonies from THY-agar plate were then patched onto THY-agar and THY-Em plate. After two days of incubation, Em^s colony was selected for further analysis. One of such clone was named IBS-D51. Deletion of SMU.832 was verified by PCR and RT PCR analysis and named, IBS-D51.

Deletion of SMU.2137: SMU.2137 was deleted by overlapping fusion PCR. Briefly, 1000-bp upstream and downstream regions were amplified with the primers sets SMU.2137 5'F/ SMU.2137 5'Fusion R and SMU.2137 3' Fusion F/ SMU.2137 3' R using *S. mutans* UA159 chromosomal DNA as template. A Km resistant cassette was amplified from pIB-D38 with the same primers as mentioned earlier and overlapping fusion PCR was carried out using equal amount of each PCR products with the primers SMU.2137 5' F and SMU.2137 3' R. Amplified products were purified and transformed into *S. mutans* UA159 as described above to get the clone IBS-D53. The fragment containing the ΔSMU.2137:: *loxP-km* was again amplified with the primers SMU.2137 5' F and SMU.2137 3' R and retransformed to get the clone, IBS-D55. Deletion of the SMU.2137 locus was verified by PCR and RT PCR analysis.

Construction of plasmids for complementation: Plasmid pIB184-Km was used as the vector for cloning (127). To clone *nlmA* gene, a fragment containing *nlmA* gene with the native promoter was amplified from the genomic DNA of UA159 using the primers BamHI-NlmA-F and Xho-NlmA-R. The amplified fragment was digested with BamHI and XhoI, and cloned into BamHI - XhoI digested pIB184-Km to generate pIB-D28. Similarly, *nlmB* gene was amplified

from IBS-D4 where *nlmA* was absent using the primer set Bam-NlmA-F and Xho-SMU. Nlm-R. The amplified fragment was digested with BamHI and XhoI, and cloned into BamHI - XhoI digested pIB184-Km to generate pIB-D20. *nlmC* gene was also cloned using the similar approach with the primer set BamHI NlmC-F and XhoI NlmC-R. Complementing plasmids were verified with restriction digestion, RT PCR and sequencing.

Cloning of SMU.152, SMU.925 and SMU.1909: To clone SMU.152, a fragment containing the SMU.152 gene and the promoter region was amplified from the genomic DNA of UA159 using the primers BamHI- SMU.152-F and XhoI-Nlm-R. The amplified fragment was digested with BamHI and XhoI, and cloned into BamHI - XhoI digested vector, pIB184Km to generate pIB-D8. SMU.925 and SMU.1909 were also cloned using the similar approach with the primer set BamHI SMU.925-F/XhoI SMU.925-R and BamHI-SMU.1909/ PstI SMU.1909 respectively (for all the plasmids, see Table-7). Complementing plasmids were verified with restriction digestion. SKNK-residues were added to the 3' end of SMU.1909 and SMU.925 by adding the coding sequences of SKNK from SMU.152 to the XhoI SMU.925+SKNK-R and XhoI SMU.1909 +SKNK R and cloned similarly as described above.

Different versions of truncated SMU.152 were generated using the common forward primer BamHI SMU.152-F and respective reverse primers carrying desired truncation (for all the primers, see Table-9). The amplified fragments were digested with BamHI and XhoI, and cloned into BamHI - XhoI digested vector, pIB184Km to generate the respective plasmids (for all the plasmids, see Table-7). Insertion of the cloned fragments was verified by restriction digestion.

Cloning of SMU.518: SMU.518 with ribosome binding site was amplified from the genomic DNA of UA159 with high fidelity Accutaq DNA polymerase (Sigma) using the

primers, BamHI SMU.518 F and XhoI SMU.518 R. After digestion with BamHI and XhoI, fragment was cloned into the BamHI - XhoI digested vector, pIB184-Km. Positive clones were verified with restriction digestion and named pIB-D52.

Cloning of SMU.1915: SMU.1915 with promoter region was amplified from the genomic DNA of UA159 with high fidelity Accutaq DNA polymerase (Sigma) using the primers, BamHI-SMU.1915 F and XhoI SMU.1915 R. After digestion with BamHI and XhoI, fragment was cloned into the BamHI - XhoI digested vector, pIB184-Km. Positive clones were verified with restriction digestion and named pIB-D58. Similarly, 18-amino acids version of *comC* was cloned by same approach except the reverse primers XhoI 18-aa *comC* R and named pIB-D57.

Cloning of SMU.832: SMU.832 with promoter region was amplified from the genomic DNA of UA159 with high fidelity Accutaq DNA polymerase (Sigma) using the primers, BamHI SMU.832 F and XhoI SMU.832 R. After digestion with BamHI and XhoI, fragment was cloned into the BamHI - XhoI digested vector, pIB184-Km. Positive clones were verified with restriction digestion and named pIB-D71.

Cloning of SMU.2137: SMU.2137 with promoter region was amplified from the genomic DNA of UA159 with high fidelity Accutaq DNA polymerase (Sigma) using the primers, BamHI SMU.2137 F and XhoI SMU. 2137 R. after digestion with BamHI and XhoI, SMU.2137 was cloned into BamHI - XhoI digested pIB-D13. Positive clones were verified with restriction digestion and named pIB-D73.

Construction of pIB-D13: Plasmid pMM223 (305) which is 8.0 kb in size was used for construction of pIB-D13 to make more convenient cloning vector. pMM223 was digested with EcoRI, HindIII and AlwN1 to release 4.2kb fragments containing the MCS region, *aad* (encoding the spectinomycin gene) and *trp-lacZ* genes, followed by blunting and ligation to generate the vector pIB-D11. A MCS region containing the promoter region of P_{23} was amplified from pIB184Km and cloned into the PvuII digested pIB-D11 to generate the plasmid pIB-D13. Newly generated vector pIB-D13 now contains the broad host range lactococcal replicon pWV01 with erythromycin resistant cassette Em^r and is 4.1kb in size.

Site-directed mutagenesis of SMU.518: pIB-D48, generated from cloning of SMU.518 into pGEM-T Easy vector, was used as a template to mutate the Lon active residues, S-235 and K-280. Site-directed mutagenesis was performed using the high fidelity *Pfu* polymerase (Quick change II XL site-directed kit; Agilent technologies, CA) with the mutagenic primers that encode the mutation S235A and K280A having additional recognition site of PvuII and PstI respectively. SMU.518 was then amplified from the intermediate mutated plasmids with BamHI SMU.518 F and XhoI SMU. 518 R and cloned into the BamHI - XhoI digested vector, pIB184km (127) to generate pIB-D86 and pIB-D87 respectively. The mutation was checked by restriction digestion and the expression was confirmed by western blotting.

Construction of P*nlmA-gus* reporter strains: Plasmid pIB107 was used to construct different reporter strains (29). To construct the P*nlmA-gus* fusion, a fragment carrying the promoter region of *nlmA* gene was amplified from the genomic DNA of UA159 using the primers EcoRI SMU. NlmA F and XhoI NlmA IGS R. The amplified fragment was digested with EcoRI and XhoI, and cloned into EcoRI - XhoI digested pIB107 (a suicidal vector which

has sequences of SMU.1405) to generate the plasmid, pIB-D21. Plasmid pIB-D21 and pIB107 were then digested with BglI and transformed into UA159 to get the strains, IBS-D5 and IBS-D44 respectively. Construction was verified with PCR and sequencing. Similarly, BglI digested pIB-D21 was also transformed into ΔSMU.518, Δ*comC*, Δ*nlmTE*, and ΔSMU.518 Δ*comC* to generate respective reporter strains IBS-D37, IBS-D40, IBS-D41 and IBS-D46 (Table-8).

Transposition assay, screening and identification of IS*S1* integration sites: To identify the factors that regulate *nlmA* expression, we performed insertional mutagenesis with the plasmid pGh9:IS*S1* according to the methods described by Maguin et al. (183) and later modified by Zhang et al.(328). IBS-D5 was transformed with pGh9:IS*S1* and transformants were selected on THY agar containing Em at 30°C. A single positive colony was grown in liquid culture for overnight and diluted 100-fold in the same medium, grown for 2-hr at 30°C, and then transferred to water bath at 37°C for 4-h to select the transposition events. This culture represents the starting transposon library. This culture was then plated on THY-Em plates containing the X-gluc and incubated at microaerophilic condition at 37°C. Transformants were selected based on the blue/white colony appearance. Selected white colonies were than tested for NlmAB production using *Streptococcus gordonii* DL-1 as indicator bacteria. After screening of ~12000 CFU (colony forming units), we got 25 positive clones. Chromosomal DNA from the selected colonies were then isolated and digested by HindIII, followed by heat inactivation at 65°C. 2 µg of digested DNA was taken for self-ligation by T4 DNA ligase, and the ligated samples were subjected to inverse PCR by using the primers IS*S1*R-out 2 and IS*S1* For-4. The PCR products were purified from agarose gel and sequenced with the primer IS*S1*-Rout-2. The flanking sequences obtained from sequencing analysis were mapped on the genome of *S. mutans* UA159 by a BLAST search.

Curing of integrated pGhost9:IS*S1*: To induce the excision of chromosomally integrated pGh9:IS*S1*, cells were subjected to several round of growth at permissive and non-permissive temperature in THY-broth in the absence of antibiotic. During each cycle, overnight culture of IBS-D5 (grown at 37^0C) carrying chromosomally inserted pGh9:IS*S1* were diluted (1:1000) and inoculated into fresh THY-broth in the absence of antibiotic, followed by incubation at 30°C for 16 h. After 16-hr, the cells were diluted and plated on THY agar. Colonies were then replicated on THY agar with or without Em to isolate the pGh9:IS*S1*-cured strain.

DNA extraction and PCR screening of various genes: Total DNA was extracted using a Master Pure DNA Purification kit (Epicentre Biotechnologies), according to the manufacturer's instructions. The mutacin-producing strains were grown into THY-broth for overnight at 37^0C under microaerophilic condition. 5-ml culture was centrifuged (5000 RPM for 5 min) and DNA was extracted from the pellet using the manufacturer protocol. The DNA purity was determined by calculating the A260/A280 ratio. The detection of various genes was performed by PCR using primers specific to each type of gene. Primers for the intended genes were designed based on sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) (7)**.** PCR amplification was performed using a PCR Master Cycler (Eppendorf). The PCR reaction was carried out using 2X Taq mix or high fidelity Accutaq DNA polymerase (Sigma, USA) when necessary using 50 ng templates DNA. In addition to the strains being tested, purified genomic DNA from *S. mutans* UA159 was used as a positive control and distilled water was used as a negative control in each PCR. The PCR products were analyzed by electrophoresis in 1.0% agarose gel.

Isolation of RNA from bacterial cultures: Total RNA was isolated from bacterial cultures according to the protocol described earlier (27). Briefly, *S. mutans* cultures were grown

until the desired optical density was reached, and the bacterial cell pellet was resuspended into 5 ml of RNA protect bacterial reagent (Qiagen). Total RNA was isolated using an RNeasy mini-kit (Qiagen) with a modified bacterial lysis step. The supernatant obtained after bacterial cell lysis was loaded onto an RNeasy mini column, and DNA contaminants were removed by on-column DNase I treatment following the manufacturer's instructions. RNA was purified according to the supplier's suggested protocol. The RNA concentrations were determined by UV spectrophotometer.

Semi quantitative RT-PCR: 500 ng of RNA samples isolated from various *S. mutans* cultures were used for reverse transcription using the Titan one-tube reverse transcription-PCR (RT-PCR) system (Roche) as described previously (27). cDNA was purified using QIAGEN PCR purification kit and 10 ng of cDNA was used for PCR amplification of each gene. *gyrA* gene was used as an internal control to ensure that equal amount of RNA was being used in all RT-PCR mixtures. The PCR products were separated on 2.0 % agarose gel electrophoresis and quantified by Doc-It-LS (UVP) software.

β-Glucuronidase assay (GusA assay): β-glucuronidase assays were performed as described previously by Biswas et al (29). Reporter strains were grown overnight at 37^0C and dilute 1:20 in THY-broth followed by incubation at 37^0C until the growth reached OD_{595} of 0.2. CSP (intact CSP or CSP's processed with various strains) or the culture supernatants (40% ammonium sulfate precipitations from overnight cultures) of various strains were added and incubated until the OD_{595} reached 1.00. 10-ml of culture was precipitated at this point, washed in saline water and resuspended in Z-buffer. 500-ml of resuspended culture was then disrupted by bead beating (Fast Prep-24; M.P. Biomedical, California) to get the cell lysate. The protein

concentration of the lysate was measured by Bradford protein assay (Bio-Red) method standardized with BSA (34). The Gus activities of the culture lysates were standardized by comparison with known concentration of glucuronidase (Sigma-Aldrich). Equal amount of each lysates was mixed with p-nitro phenyl-β-D- glucoside (Sigma) to a final concentration of 0.8 mg/ml and the reaction was initiated at 37^0C. As soon as color developed in the reaction mixture, the reaction was stopped by the addition of Na_2CO_3 to a final concentration of 0.25M. The OD value at 420 nm and the time of sodium carbonate addition were noted. Gus activity was calculated as $[1000 \times A_{420}]$/ [time (min) × cell A_{595}] in Miller units (MU).

For the isolation of culture supernatants, bacterial cultures were grown overnight and diluted 1:20 into THY broth and incubated at 37^0C in shaker incubator for overnight. Culture supernatants were separated from the bacterial cells by passing through 0.45-µM membrane filter and precipitated with 40% ammonium sulfate. After resuspending into 50 mM Tris-Cl (pH-7.4), equal amount of each sample was added to the actively growing reporter strains (OD_{595} of 0.2), followed by incubation for another 4-hour.

Purification of mutacin IV activity from the culture supernatant: For purification of active mutacin IV molecules, wild-type UA159 or complemented strains (IBS-D3 with P*nlmA*, P*nlmB*, and P*nlmAB* respectively) were inoculated into 10 ml Todd-Hewitt medium and incubated for overnight. 4-ml each overnight culture was then inoculated into 200 ml of fresh Todd-Hewitt medium and incubated for 24 h at 37°C in a shaker incubator at 150 rpm. An equal volume of chemically defined media (27) was then added and incubated for another 24-hr. Bacterial culture was then passed through a 0.45-µm membrane filter, followed by ammonium sulfate fractionation (40% saturation) as described by Yamamoto et al. (322).

Cleavage of synthetic CSP (21-amino acid) by *S. mutans*: Competence stimulating peptide of *S. mutans* UA159 was synthesized commercially by Genscript (Genescript Corporation, USA) with a purity grade of >95%. The lyophilized peptides were resuspended into dimethyl formamide at 2.0 mg/ml concentration. Bacterial cultures were grown overnight or late logarithmic phase at 37^0C. 1-ml of culture was precipitated and washed with PBS. Culture pellet was resuspended into 100 µl of PBS and CSP was added to a concentration of 120 µM and incubated at 37^0C for an hour. Cells were pelleted again at 12000 RPM for 5-min and the supernatant containing the CSP was taken out. Processing of CSP was checked by spotting at different concentration onto X-gluc plate seeded with the reporter strain, ΔSMU.518::P*nlmA-gus*.

Mass spectrometry of the processed CSP's: ESI spectra were acquired on a SYNAPT G2 hybrid quadruple / ion mobility / Tof mass spectrometer (Waters Corp., Milford, MA) from the University of Kansas Mass Spectrometry/Analytical Proteomics Laboratory. The instrument was operated in a sensitivity mode with all lenses optimized on the MH+ ion from the Leucine Enkephalin. The sample cone voltage was 30eV. Argon was admitted to the trap cell that was operated at 4eV for maximum transmission. Spectra were acquired at 9091 Hz pusher frequency covering the mass range from 100 to 3000 u and accumulating data for 1.5 seconds per cycle. Time to mass calibration was made with NaI cluster ions acquired under the same conditions. Mass spectra of [Glu^1]-Fibrinopeptide B were acquired in parallel scans and doubly charged ions at m/z 785.8426 were used as a lock mass reference.

Samples were desalted on a reverse phase C_4 column, 1cm, 1mm I.D. (Vydac, 300 Å pore size, 5 µm particles packed by Micro-Tech Scientific) using a NanoAcquity chromatographic system (Waters Corp., Milford, MA). The solvents were A (99.9% H2O, 0.1% formic acid) and B (99.9% acetonitrile, 0.1% formic acid). A short gradient was developed from

1 to 70% B in 4 minutes with a flow rate of 20 µL/min. Mass Lynx 4.1 software was used to collect the data. MaxEnt 3 routine was used for processing data to convert peaks of multiple charged ions in continuum spectra into centroid singly charged spectra.

Western blotting: Bacterial strains with the HA-tagged SMU.518 or indicated mutants (C-terminal HA tag) were grown up to 70 KU and harvested at 5000K for 5min. Cell pellets were resuspended in tissue and cell lysis solution (Epicenter) and homogenized with a Bead Beater. Protein lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (161) and blotted onto membranes. Western blotting was carried out using standard techniques with anti-HA monoclonal antibody (Sigma) as the primary antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma). The blots were developed with Pierce ECL plus reagent (Thermo Scientific) and the signals were detected by Typhoon FLA 9000 (GE Healthcare).

Deferred antagonism bacteriocin assay: After overnight growth on THY agar plate, isolated colonies were stabbed into THY agar plate with a toothpick and grown overnight (~18-hr) at 37°C under microaerophilic conditions. Indicator strains were grown to mid exponential growth phase in THY broth and 0.4-ml of the indicator culture was mixed with 10 ml of soft agar and overlaid on agar plates that were stabbed with the tester strains. Overlaid plates were then incubated overnight under same conditions and the diameter of the zones of inhibition around the mutacin-producing strains was measured. *Streptococcus gordonii* (DL-1, ATCC 10558 and M5) and *S. cristatus* (BHT) were used as indicator strain for mutacin IV or NlmAB activity; while *Lactococcus lactis* MG1363 was used as indicator for mutacin V or NlmC activity (104).

Measurement of growth kinetics: Bacterial cultures were grown in THY-broth for overnight at 37^0C. Equal amount of each inoculum (based on OD at 595) was added into the respective Klett Flasks as 1:20 dilution and incubated at 37^0C in the water bath. Bacterial growth was monitored with a Klett-Summerson colorimeter (MP Biomedical) at different time intervals and recorded as Klett unit.

Table-7: List of plasmids constructed in this study

Plasmid	Marker	Origin	Construction	Purpose
pIB-D1	Ap	pGEM-T Easy	pGEM-T Easy::STP	Clean deletion of STP
pIB-D2	Ap	pGEM-T Easy	pGEM-T Easy::STK	Clean deletion of STK
pIB-D3	Ap	pGEM-T Easy	pGEM-T Easy::STP-STK	Clean deletion of STP-STK
pIB-D4	Km	pGEM-T Easy	pIB-D1::*loxP-km*	Clean deletion of STP
pIB-D5	Km	pGEM-T Easy	pIB-D2::*loxP-km*	Clean deletion of STK
pIB-D6	Km	pGEM-T Easy	pIB-D3::*loxP-km*	Clean deletion of STP-STK
pIB-D7	Km	pGEM-T Easy	pGEM-T Easy::*loxP-Km*	For overlapping PCR
pIB-D8	Km	pIB184Km	pIB184Km::SMU.152	Cloning of SMU.152
pIB-D9	Em	pORI23	pORI23::SMU.1909	Cloning of SMU.1909
pIB-D10	Ap	pGEM-T Easy	pGEM-T Easy::*nlmAB*	Clean deletion of *nlmAB*
pIB-D11	Em	pMM223	ΔpMM223	Cloning vector
pIB-D12	Em	pMM223	pIB-D11::MCS of pIB184Km	Cloning vector
pIB-D13	Em	pMM223	pIB-D11::MCS of pIB184Km	Cloning vector
pIB-D14	Em	pIB-D11	pIB-D11:: *nlmAB* operon	Complementation of *nlmAB*
pIB-D15	Ap	pGEM-T Easy	pGEMT-Easy::5'-3' fusion fragments of *nlmAB* operon	For clean deletion of *nlmA*
pIB-D16	Km	pIB-D10	ΔpIB-D10::*loxP-km*	For clean deletion of *nlmAB*
pIB-D17	Km	pIB-D15	pIB-D15::*loxP-km*	Clean deletion of *nlmA*
pIB-D18	Km	pIB104	ΔSMU.198::k*m*^{r}	For transformation assay
pIB-D19	Km	pIB184Km	pIB184Km::*nlmAB*	Complementation of *nlmAB*
pIB-D20	Km	pIB184Km	pIB184Km::*nlmB*	Complementation of *nlmB*
pIB-D21	Km	pIB107	pIB107::P*nlmA-gus*	Cloning of *nlmA* IGS region
pIB-D22	Ap	pGEM-T Easy	pEz:: 5' & 3' SMU.1909-1913	Clean deletion of full region
pIB-D23	Km	pGEM-T Easy	pIB-D22::*loxP-Km*	Clean deletion
pIB-D24	Km	pIB184Km	pIB184Km::HA *nlmA*	To study the mode of action
pIB-D25	Km	pIB184Km	pIB184Km::*irvR*	Complementation of *irvAR*
pIB-D26	Km	pIB-D19	GXXXG to AXXXA	Structure-function relationship
pIB-D27	Em	pIB190	pIB190::N-His SMU.152	To study the immunity activity
pIB-D28	Km	pIB184Km	pIB184Km::*nlmA*	To study the NlmA only
pIB-D29	Km	pIB184Km	pIB184Km::*nlmC*	Complementation of *nlmC*
pIB-D30	Km	pIB-D19	GXXXS to AXXXA of *nlmB*	Structure-function relationship
pIB-D31	Km	pIB-D19	GXXXG to AXXXA	Structure-function relationship
pIB-D32	Km	pIB184Km	pIB184Km::*nlmC*	Complementation of *nlmC*
pIB-D33	Km	pIB184Km	pIB184Km::SMU.1909	To study the immunity activity
pIB-D34	Km	pIB184Km	pIB184Km::SMU.925	To study the immunity activity
pIB-D35	Km	pIB184Km	pIB184Km::SMU.152ΔC	To study the immunity activity
pIB-D36	Km	pIB184Km	SMU152ΔKRRSKNK	To study the immunity activity
pIB-D37	Km	pIB184Km	pIB184Km::SMU.152ΔSKNK	To study the immunity activity
pIB-D38	Km	pGEM-T Easy	pGEMT-Easy::*loxP-km*	Cloning of *loxP-Km*
pIB-D39	Km	pIB184Km	GXXXG to AXXXA of *nlmB*	Not good
pIB-D40	Km	pIB184Km	pIB184Km::SMU.152ΔKNK	To study the immunity activity
pIB-D41	Ap	pGEM-T Easy	pGEMT-Easy::SMU.152	To study the immunity activity
pIB-D42	Ap	pIB-D31	GGXXSG to AAXXAA	Structure-function relationship
pIB-D43	Km	pIB184Km	pIB184Km::*nlmAB*	Structure-function relationship
pIB-D44	Ap	pIB-D41	SMU.152::KRR to KAA	To study the immunity activity

Plasmid	Marker	Origin	Construction	Purpose
pIB-D45	Ap	pIB-D44	SMU.152::KRR to AAA	To study the immunity activity
pIB-D46	Km	pIB184Km	SMU.152(KRR to KAA)	To study the immunity activity
pIB-D47	Km	pIB184Km	SMU.152(KAA to AAA)	To study the immunity activity
pIB-D48	Ap	pGEM-T Easy	pGEMT-Easy::SMU.518	Clean deletion of SMU.518
pIB-D49	Km	pIB-D48	pIB-D48::*km*^{r}	Inactivation of SMU.518
pIB-D50	Km	pIB-D48	pIB-D48::*loxP-km*	Inactivation of SMU.518
pIB-D51	Sp	pGEM-T Easy	pGEMT-Easy::Sp	Cloning of spectinomycin
pIB-D52	Km	pIB184Km	pIB184Km::SMU.518	Complementation of SMU.518
pIB-D53	Km	pIB184Km	pIB184Km::HA-SMU.518	Expression of SMU.518
pIB-D54	Km	pIB-D48	Δ518: *loxP-km*	Clean deletion of SMU.518
pIB-D55	AP	pGEM-T Easy	pGEMT-Easy::SMU.1915	Deletion of SMU.1915
pIB-D56	Ap	pIB-D48	pIB-D48ΔSMU.518	Deletion of SMU.518
pIB-D57	Km	pIB184Km	pIB184Km::*comC (*18residue)	Study the activity of 18-residue
pIB-D58	Km	pIB184Km	pIB184Km::*comC*	Complementation of *comC*
pIB-D59	Km	pIB184Km	pIB184Km::HA-*comC*	To study the secretion of CSP
pIB-D60	Km	pIB184Km	pIB-D54ΔSMU.518	Inverse PCR to delete SMU.518
pIB-D61	Ap	pGEM-T Easy	pGEMT-EZ::SMU.611	Deletion of SMU.611
pIB-D62	Km	pIB184Km	pIB184Km::His-*comC*	To study the transporter of CSP
pIB-D63	Km	pGEM-T Easy	pIB-D61::*loxP-km*	Deletion of SMU.611
pIB-D64	Ap	pGEM-T Ez	pGEMT-EZ:: SMU.832	Deletion of SMU.832
pIB-D65	Ap	pGEM-T Ez	pGEMT-EZ:: SMU.1509	Deletion of SMU.1509
pIB-D66	Ap	pGEM-T Ez	pGEMT-EZ:: SMU.2137	Deletion of SMU.2137
pIB-D67	Km	pIB-D64	pIB-D64::*loxP-km*	Deletion of SMU.832
pIB-D68	Km	pIB-D65	pIB-D65::*loxP-km*	Deletion of SMU.1509
pIB-D69	Km	pIB-D66	pIB-D66::*loxP-km*	Deletion of SMU.2137
pIB-D70	Km	pIB184Km	pIB184Km::SMU.2137	Complementation of SMU.2137
pIB-D71	Km	pIB184Km	pIB184Km::SMU.832	Complementation of SMU.832
pIB-D72	Km	pIB184Km	pIB184Km::SMU.1909 SKNK	Cloning of SMU.1909+SKNK
pIB-D73	Em	pIB-D13	pIB-D13:: SMU.2137	Complementation of SMU.2137
pIB-D74	Em	pIB-104	pIB-104::Em^r	Transformation assay
pIB-D75	Km	pIB184Km	pIB184Km::SMU.925+SKNK	Cloning of SMU.925+SKNK
pIB-D76	Ap	pIB-D48	SMU.518::N296 to A	Mutagenesis
pIB-D77	Ap	pIB-D48	SMU.518::R254 to A	Mutagenesis
pIB-D78	Km	pIB184Km	SMU.518:R254 to A	Mutagenesis
pIB-D79	Km	pIB184Km	SMU.518:N296 to A	Mutagenesis
pIB-D80	Ap	pGEM-T Ez	pEz::SMU.518 (GPS to AAA)	Mutagenesis
pIB-D81	Km	pIB184Km	SMU.518 (GPS to AAA)	Mutagenesis
pIB-D82	Ap	pGEM-T Ez	SMU.518 (S235A)	Mutagenesis
pIB-D83	Ap	pGEM-T Ez	SMU.518 (K280A)	Mutagenesis
pIB-D84	Ap	pGEM-T Ez	SMU.518 (S235A)	Mutagenesis
pIB-D85	Ap	pGEM-T Ez	SMU.518 (K280A)	Mutagenesis
pIB-D86	Km	pIB184Km	SMU.518 (S235A)	Mutagenesis
pIB-D87	Km	pIB184Km	SMU.518 (K280A)	Mutagenesis
pIB-D88	Km	pIB184Km	SMU.518 -HA(S235A)	Expression of SMU.518
pIB-D89	Km	pIB184Km	SMU.518-HA (K280A)	Expression of SMU.518
pIB-D90	Km	pIB184Km	SMU.518 (N296 A)	Expression of SMU.518

Table-8: List of strains used in this study

Name	Construction/Description/Strain	Comment
Streptococcus mutans UA159		Wild type
IBS-D1	Δ*nlmAB*::*loxP-km*	This study
IBS-D2	Δ*nlmA*::*loxP-km*	This study
IBS-D3	Δ*nlmAB* (clean deletion)	This study
IBS-D4	Δ*nlmA* (clean deletion)	This study
IBS-D5	ΔSMU.1405::P*nlmA-gus*	This study
IBS-D6	ΔSMU.1909-SMU.1913:: *loxP-km*	This study
IBS-D7	ΔSMU.1909-SMU.1913:: *loxP-km* in OMZ175	This study
IBS-D8	Δ*nlmAB*::ΔSMU.1909-SMU.1913	This study
IBS-D9	P*nlmAB::lacZ*	This study
IBS-D10	Δ*nlmB*::*loxP-km*	This study
IBS-D11	ΔSMU.61::Sp^r	This study
IBS-D12	ΔSMU.1882::Sp^r	This study
IBS-D13	*ΔnlmAB;ΔnlmC*	This study
IBS-D14	ΔSMU.381::Sp^r	This study
IBS-D15	ΔPTS::*loxP-km* in *S. gordonii* DL-1	This study
IBS-D16	ΔSMU.152::*loxP-km*	This study
IBS-D17	IBS-D13::P*nlmA-gus*	This study
IBS-D18	IBS-D3::*PnlmA-gus*	This study
IBS-D19	ΔSMU.518:: *loxP-km*	This study
IBS-D20	ΔSMU.518:: *loxP-km*	This study
IBS-D21	ΔSMU.518::IS*S1*	This study
IBS-D22	ΔSMU.518::IS*S1*	This study
IBS-D23	IBS-D5::pGhost9-IS*S1*	This study
IBS-D24	ΔSMU.287::IS*S1*	This study
IBS-D25	ΔSMU.287::IS*S1*	This study
IBS-D26	ΔSMU.121::IS*S1*	This study
IBS-D27	ΔSMU.121::IS*S1*	This study
IBS-D28	ΔSMU.286::IS*S1*	This study
IBS-D29	ΔSMU.518 (clean deletion)	This study
IBS-D30	ΔSMU.518::*loxP-km*	This study
IBS-D31	ΔSMU.518 (clean deletion)	This study
IBS-D32	Δ*nlmTE* (clean deletion)	This study
IBS-D33	ΔSMU.1915::ISS1	This study
IBS-D34	ΔSMU.121::*loxP-km*	This study
IBS-D35	ΔSMU.518::*loxP-km*	This study
IBS-D36	ΔSMU.1915::*loxP-km*	This study
IBS-D37	IBS-D29::P*nlmA-gus*	This study
IBS-D38	ΔSMU.518::*loxP-km*	This study
IBS-D39	ΔSMU.1915 (clean deletion)	This study
IBS-D40	ΔSMU.1915::P*nlmA-gus*	This study
IBS-D41	Δ*nlmTE::PnlmA-gus*	This study
IBS-D42	ΔSMU.518; Δ*comC::loxP-km*	This study
IBS-D43	ΔSMU.518::*loxP-km*	This study
IBS-D44	ΔSMU.1405::*gus* (control)	This study

Name	Construction/Description/Strain	Comment
IBS-D45	ΔSMU.518; Δ*comC* (clean deletion)	This study
IBS-D46	IBS-D45::P*nlmA-gus*	This study
IBS-D47	Δ SMU.611::*loxP-km*	This study
IBS-D48	ΔSMU.832::*loxP-km*	This study
IBS-D49	ΔSMU.1509::*loxP-km*	This study
IBS-D50	ΔSMU.2137::*loxP-km*	This study
IBS-D51	ΔSMU.832 (clean deletion)	This study
IBS-D52	ΔSMU.1509::*loxP-km*	This study
IBS-D53	ΔSMU.2137 *loxP-km*	This study
IBS-D54	ΔSMU.832::P*nlmA-gus*	This study
IBS-D55	ΔSMU.2137 (retransformation)	This study
IBS-D56	ΔSMU.152 (clean deletion)	This study
IBS-D57	ΔSMU.832;ΔClpX:: *loxP-km*	This study
IBS-D58	Δ*nlmAB;*ΔSMU.1909-SMU.1913; ΔSMU.925	This study
IBS-H4	Δ*cslAB*:: *loxP-km*	This study
S. mutans UA159	ΔSMU.1914::Em^r (Δ*nlmC*)	(237)
S. mutans UA159	Δ SMU.925::Em^r	(237)
Streptococcus gordonii	DL-1, ATCC 10558, M5	Wild type
Lactococcus lactis	MG1363	Wild type
S. agalactiae	NEM306	Wild type
S. uberis	UT387	Wild type
S. iniae	K388	Wild type
S. pyogenes	K-56, AM3, JRS4	Wild type
S. anginosus	CI	Wild type
S. constallatus	CI	Wild type
S.pneumoniae	ATCC 6303	Wild type
S. cristatus	ATCC 51100	Wild type
S. sanguis	SK36	Wild type
S salivarious	ATCC 25975	Wild type
S. bovis	TX20005	Wild type
S. downeii	ATCC 33798	Wild type
S. sobrinus	6715	Wild type
S. criceti	AHT	Wild type
S. ratti	BHT	Wild type
S. mutans	UA130, GS-5, NG8,SM3208, OMZ175	Wild type

Table -9: List of oligonucleotides used in this study

Name of the Primer	Sequence	Purpose
SMU. Nlm Nested F1	GGCGTGACCATTCATGAAGCGAATGCC	Amplification of *nlmAB* operon
SMU. Nlm Nested F2	GTGTTTCGTCTTTCATGGGATATTTAAAGGG	Amplification of *nlmAB* operon
SMU. Nlm Nested R1	GCCTACAGCTAAGGCGATTAATACACC	Amplification of *nlmAB* operon
SMU. Nlm Nested R2	GGCAGCAAAAAGTATCCAATAGACC	Amplification of *nlmAB* operon
Bam HI SMU. NlmA F	GCGGGATCCGGCAATCGAAGTTTTGGTGTAGA GGC	Cloning of *nlmAB* operon
Xho1-SMU.Nlm R	GCGCTCGAGACAACTATTAGGCTTCGGCCTTA GC	Cloning of *nlmAB* operon
EcoR-V SMU. NlmA-5' Fusion	GCGGATATCGTTTGGCTGTCCATTACATC	Deletion of *nlmA*
EcoR-V SMU. NlmA-3' Fusion	GCGGATATCGGGGACTCATTCGATCTCAT	Deletion of *nlmA*
XhoI-SMU. NlmA-R	GCGCTCGAGCCATGGTATTAATTCTCCATTCC	Cloning of *nlmA*
XhoI NlmA R	GCGCTCGAGTTTAATGAGATCGAATGAGTCCC CAAGTGC	Cloning of *nlmA*
Kan D7 F	CTCCCGGCCGCCATGGCGGCCGC	Amplification of Km^r cassette
Kan D7 R	GGTCGACCTGCAGGCGGCCGCG	Amplification of Km^r cassette
NlmB 5' fusion-R	GCCGCCATGGCGGCCGGGAGTTCTCCATTCCA TTTAATTC	Deletion of *nlmB* by overlapping PCR
NlmB 3' fusion-F	CGCGGCCGCCTGCAGGTCGACCCCACACTAAA TTGATATAATGG	Deletion of *nlmB* by overlapping PCR
loxP-KanF	CGATAACTTCGTATAATGTATGCTATACGAAGT TATGAGGATGAAGAGGATGAGGAGGCAG	For *cre* mediated clean deletion
loxP-KanR	CGATAACTTCGTATAGCATACATTATACGAAG TTATGCTTTTTAGACATCTAAATCTAGG	For *cre* mediated clean deletion
BamHI NlmC-F	GCGGGATCCCATTTTATATCTCCTTTTTTTG	Cloning of *nlmC*
XhoI NlmC-R	GCGCTCGAGTTAAATTACTAACCACAGGAAT	Cloning of *nlmC*
NlmT-F	TTAATCGTCATAGCCGTTAACATTC	RT PCR
NlmT-R	CCTTACTCATCCTAGTCACCTTAAC	RT PCR
NlmB RT- F	GGAATGGAGAATTAATACCATG	RT PCR
NlmB RT- R	GTGTGGAAAAACTACAGATCCA	RT PCR
SMU. GyrA-F	CTATGCTATGAGTGTTATTGTTGCTCGGGC	RT PCR
SMU. GyrA-R	GGCCATTCCAACAGCAATACCTGTCGCTCC	RT PCR
SMU.1914c.F	CGATGAATACACAAGCATTTGA	RT PCR
SMU.1914c.R	CTAACCACAGGAATTAAGAGCT	RT PCR
SMU.1915-F	CCGGATGAAAAAAACACTATCA	RT PCR
SMU.1915-R	CCGGTTATTTTCCCAAAGCTTGT	RT PCR
NlmA-F	ATGGATACACAGGCATTTG	RT PCR
NlmA-R	ATGAGATCGAATGAGTCC	RT PCR

Name of the Primer	Sequence	Purpose
ComE-F	GACGTCTTGAAACCACCATTG	RT PCR
ComE-R	CTTCATTCATTTTGCTCTCCTT	RT PCR
KanR-For	GAGGATGAAGAGGATGAGGAGGCAGATTGCC	To amplify Km^r cassette
KanR-Rev	GCTTGTAGTTAAAGCTTTTTAGACATCTAAATC TAGG	To amplify Km^r cassette
lox77-KanF	CGTACCGTTCGTATAGCATACATTATACGAAGT TATGAGGATGAAGAGGATGAGGAGGCAG	Clean deletion
lox66-KanR	CGTACCGTTCGTATAATGTATGCTATACGAAGT TATGCTTTTTAGACATCTAAATCTAGG	Clean deletion
SMU. NlmT-F	GTCTTGGCTTTTGTTAGCCACGGGCCTTGC	To amplify *nlmTE*
SMU. NlmT-R	CATTGAGAATGGTATTACGGGAAACTGC	To amplify *nlmTE*
SMU.NlmC.F-1	GCAACTCCTAATAATAAAATAGAGGAGGCC	Sequencing of *nlmC* and *comC*
SMU.NlmC-R-1	GTCCATCAATGTAAAATAAACAAAGG	Sequencing of *nlmC* and *comC*
SMU.1916-F	GCAATCATATTCTTTATCTTGGATGGAAC	To amplify *comD*
SMU.1916-R	GATATTATAACGGGTATCCTGCAATTG	To amplify *comD*
BamHI SMU.152 F	GCGGGATCCGGATCTGTAGTTTTTCCACAC	Cloning of SMU.152
XhoI-SMU. Nlm R	GCGCTCGAGGACAACTATTAGGCTTCGGCCTT AGC	Cloning of SMU.152
XhoI Δ3'SMU.152 R	GCGCTCGAGTTTAAAAGATGAGTAAGATGGCA GC	Generation of truncated SMU.152
XhoI ΔRRSKNK SMU.152 R	GCGCTCGAGTTTTATTTTAGCTCTTTATAGAGT TCAACAGC	Generation of truncated SMU.152
XhoI ΔKNK SMU.152 R	GCGCTCGAGTTTAGCTTCTTCTTTTTAGCTCTTT ATAG	Generation of truncated SMU.152
XhoI ΔKRRSKNK SMU.152 R	GCGCTCGAGTTTTATAGCTCTTTATAGAGTTCA ACAGC	Generation of truncated SMU.152
NcoI Kan F	CTCCCGGCCGCCATGGCGGCCGC	To amplify Km^r gene
PstI Kan R	GGTCGACCTGCAGGCGGCCGCG	To amplifyKm^r gene
BamHI-SMU.925-F	GCGGGATCCGACGCTGCCCTTGCAGCAGTG	Cloning of SMU.925
Xho1-SMU.925-R	GCGCTCGAGGCTGCCATAACCACAAAAAGCC	Cloning of SMU.925
BamH1-SMU.1909	GCGGGATCCGTCCTTGGTTCGTTAATAGATTGG G	Cloning of SMU.1909
Pst1-SMU.1909 –R	GCGCTGCAGGCCTGAAAGTAAAAACTGTTTAG CGTC	Cloning of SMU.1909
XhoI SMU.1909+ SKNK R	GCGCTCGAGTTTACTTATTCTTGCTGCGTCTCT TATGCAGTTTAATGAG	Cloning of SMU.1909+SKNK
SGO PTS F	CCGCCAGATAGATTGATAAAGTCC	Deletion of *mpt* locus
SGO PTS 5' fusion R	GCCGCCATGGCGGCCGGGAGGTTTGACCAAAC TTTAAGAAAAG	Deletion of *mpt* locus
SGO PTS 3' fusion F	CGCGGCCGCCTGCAGGTCGACCGGCAAATTTT TTAAAAACCTATTTGC	Deletion of *mpt* locus
SGO PTS 3' R	GCTCATAGCTGTGAAGTAGATTGG	Deletion of *mpt* locus
SMU.518 F	GAAAACAAACAAAAAATTTAAATG	To amplify SMU.518
SMU.518 R	GTGTTTTCTTAGGTAATCAATAGC	To amplify SMU.518
SMU.152 3' R	CTGCGTTACATATGAACATAGACC	Deletion of SMU.152

Name of the Primer	Sequence	Purpose
SMU.152 5' fusion-R	GCCGCCATGGCGGCCGGGAGCCTCTCTTGAAA TTCGACATTA	Deletion of SMU.152
SMU.152 3' fusion-F	CGCGGCCGCCTGCAGGTCGACCGCTAAGGCCG AAGCCTAATAGTTG	Deletion of SMU.152
XhoI SMU.925 +SKNK R	GCGCTCGAGTTTACTTATTCTTGCTGCGTTTTCT AAAAAAGCTGG	Cloning of SMU.925+SKNK
SMU. 1908-F	GTACTTATCCTTATTTTGTTTTTATCTTGCTG	Deleting SMU. 1909 to SMU.1913
EcoR-V SMU.1909 5' Fusion	GCGGATATCGAACTCATTAAACTGCATAAGAG ACGC	Deleting SMU.1909 to SMU.1913
EcoR-V SMU. 1913-3' Fusion	GCGGATATCGCTGCGCTGTTAAAAGTCTTAAT AACC	Deleting SMU.1909 to SMU.1913
SMU.1915-R	CGGAAAAATGTTGATAGGCTTCCG	Deleting SMU.1909 to SMU.1913
SMU. 518 5' F	GCTGTTATTGAAGCTAATATCAGGATG	Deletion of SMU.518
SMU.518 3' R	CTGCTGCCGCTACCTCTGGATTTTCC	Deletion of SMU.518
SMU.518 5' Fusion-R	GCCGCCATGGCGGCCGGGAGGTTTGTTTTCAC ATTTTTTCCCCA	Deletion of SMU.518
SMU.518 3' Fusion-F	CGCGGCCGCCTGCAGGTCGACCTTACCTAAGA AAACACTAATAAAGG	Deletion of SMU.518
BamHI SMU.518 F	GCGGGATCCTTGGGGCAGACATCAGTCCTT	Cloning of SMU.518
XhoI SMU.518 R	GCGCTCGAGTTAGTGTTTTCTTAGGTAATC	Cloning of SMU.518
XhoI-HA-SMU.518-R	GCGCTCGAGTTATTCTAATTTAGCAGCATAATC AGGAACATCATAAGGATAGTGTTTTCTTAGGT AATCAATA	To study the expression of SMU.518
SMU.518 inverse F	GTGCAGGAGGCTATTGATTACCTAAG	Deleting SMU.518
SMU.518 inverse R	CTGATTAGCCACCATTTAAATTTTTTG	Deleting SMU.518
SMU.518 F2	GACTCTTAATACTGTTACTTAGAATG	To amplify SMU.518
SMU.518 R2	GGATTTGTGAAGGTCTGCCTTAGTAAG	To amplify SMU.518
SMU.1906F	GAATACACACGTATTGGAAC	RT PCR
SMU.1906R	CCTCCAACAAGTTCACCGTA	RT PCR
SMU.423 F	GAATACACAAGCATTTGAAC	RT PCR
SMU.423R	CAACAAAAAGTAGCAGCACC	RT PCR
IS*S1*-R-OUT 2	AATAGTTCATTGATATATCCTCGCTGTCA	Mutagenesis
IS*S1*-FOR4	GGTCTTAATGGGAATATTAGC	Mutagenesis
EcoRI SMU. NlmA F	GCGGAATTCGGCAATCGAAGTTTTGGTGTAGA GGC	Cloning of *nlmA* promoter
XhoI SMU. NlmA IGS R	GCGCTCGAGTGTATCCATATGATAAACACCCC TTTTTC	Cloning of *nlmA* promoter
SMU. NlmC F	CCAGCCTTGTTTTATTCCAATG	To amplify *nlmC*
SMU. NlmC R	GAACGCTACTGTGTCTTTTTTAC	To amplify *nlmC*
XhoI SMU.1913 R	GATTTTTTTGAACGCTACTGTGTCTTTTTTAC	Deletion of *comC*
SMU.1915 5' fusion	GCCGCCATGGCGGCCGGGAGGTGTTTTTTTCAT TTTATATCTCC	Deletion of SMU.1915
SMU.1915 3'fusion	CGCGGCCGCCTGCAGGTCGACCGATAGGCTAA CATTGGAATAAAAC	Deletion of SMU.1915
SMU.1915 R	CTTTACAAGGATATTCGAAGTTTCC	Deletion of *comC*
SMU.2137 5' F	CCAGATCAACTTATTATTTTAGCTGC	Deleting SMU.2137

Name of the Primer	Sequence	Purpose
SMU.2137 3’ R	GTGACCGTGATTTACAAATTGACGAG	Deleting SMU.2137
SMU.2137 5’ fusion R	GCCGCCATGGCGGCCGGGAGCTGCAAATGCGT CACTCATTGTTTC	Deleting SMU.2137
SMU.2137 3’fusion F	CGCGGCCGCCTGCAGGTCGACCCTGAGAAAAC ATTAATACGTTATTTTG	Deleting SMU.2137
SMU.832 5’ fusion R	GCCGCCATGGCGGCCGGGAGCTCTCCTTCTAT ATATTAGCATTG	Deleting SMU.832
SMU.832 3’fusion F	CGCGGCCGCCTGCAGGTCGACCGATATCCCTT ACTTAAAGAAAAGGTG	Deleting SMU.832
SMU.832 5’ F	GAGTTGTTATTCACCCATCTTCGG	Deleting SMU.832
SMU.832 3’ R	CACGTTCTCGATTCTCATAAGCTAC	Deleting SMU.832
SMU.832 RT F	GATTGCTTATTGAATCAGTGACG	RT PCR
SMU.832 RT R	GCAAAGCCGTATAAATAATCAAC	RT PCR
Xho1 ComC 6X His R	GCGCTCGAGTTTAGTGATGGTGATGGTGATGTT TTCCCAAAGCTTGTGTAAAAC	To study the ComC transport
XhoI HA-ComC-R	GCGCTCGAGTTTAAGCGTAATCTGGAACATCG TATGGGTATTTTCCCAAAGCTTGTGTAAAAC	To study the ComC transport
BamHI SMU.832 F	GCGGGATCCGTTAATTCTAGTGATTTTACAGC	Complementation
XhoI SMU.832 R	GCGCTCGAGCCTTGAAAATTAATAATTGGATT	Complementation
BamHI SMU.2137F	GCGGGATCCGTAAAATTAATGTTCCAAAAAG	Complementation
XhoI SMU.2137 R	GCGCTCGAGACTAATCAAAATAACGTATTAAT G	Complementation
BamH1 ComC F	GCGGGATCCGCTAATTTTACATTATGTGTCC	Complementation
XhoI 18aa ComC R	GCGCTCGAG TTAAGCTTGTGTAAAACTTCTGTTA	Complementation
XhoI ComC 21-aa R	GCGCTCGAGTTATTTTCCCAAAGCTTGTGTAAA A	Complementation
SMU.1915 inverse F-out	GCTAACATTGGAATAAAACAAGG	Deletion of *comC*
SMU.1915 R	CTTTACAAGGATATTCGAAGTTTCC	Deletion of *comC*
SMU.925-F	GAAAAGTCGGAAACTGAAGTTATTGAG	To amplify SMU.925
SMU.925-R	CCATCAAAAGCAAAAACACGACAG	To amplify SMU.925
SMU.1898 F	GGTATATCAGGAGCTGGTGTAGG	Deleting *cslAB*
SMU.1898 R	CATATAAAAAAAAGCGGAGCGTTC	Deleting *cslAB*
SMU.1898 5' Fusion	GCCGCCATGGCGGCCGGGAGCAATCAGTGTCA CCATTAAACTGG	Deleting *cslAB*
SMU.1898 3' Fusion F	CGCGGCCGCCTGCAGGTCGACCCCTCTAAAGA TGTCACCCGCCTC	Deleting *cslAB*
SMU.121 RT F	GGAAATCTTGTGGGTACAAAAG	RT PCR
SMU.121 RT R	GCCATTTTAAATGGAACAAGTCAG	RT PCR
SMU.121 F	CTATAGCGATTAAAAGGAGCAG	To amplifySMU.121
SMU.121 R	ATTTCTGTTATGCTTAACCTTG	To amplifySMU.121
SMU.121 5' fusion-R	GCCGCCATGGCGGCCGGGAGCACTTCCCTTGG TTAAGTCCGTC	Deletion of SMU.121
SMU.121 3' fusion-F	CGCGGCCGCCTGCAGGTCGACCCTGATGCGCT TAATCTATTTCAG	Deletion of SMU.121
SMU.121 5'F	CTTTGAAATTGGATACGGGTTTAG	Deletion of SMU.121
SMU.121 3'R	CGAACGGCTGAGCATAGGTTGGTC	Deletion of SMU.121
SMU.611 5’F	GGTAACTTACAGTTCAAATACAGTG	Deletion of SMU.611

Name of the Primer	Sequence	Purpose
SMU.611 3’ R	TGTATAAGCTCCCACAAATCCTTC	Deletion of SMU.611
SMU.611 5’ fusion R	GCCGCCATGGCGGCCGGGAGGTGCGAAGCTAC CTTATCAAACGTTAG	Deletion of SMU.611
SMU.611 3’ fusion F	CGCGGCCGCCTGCAGGTCGACCGGTGACAGAT AACCATTCAAATTC	Deletion of SMU.611
SMU.611 F	GACATTCTTTCTGCTGTTGCAAAAG	To amplify SMU.611
SMU.611 R	CTTTGCGATTTTTGTTAGAAGTCC	To amplify SMU.611
SMU.121 RT F	GGAAATCTTGTGGGTACAAAAG	RT PCR
SMU.121 RT R	GCCATTTTAAATGGAACAAGTCAG	RT PCR
NlmE F	ATGGATCCTAAATTTTTACAAAG	To amplify *nlmE*
NlmE R	GATATATAGAATATCTAATTATCC	To amplify *nlmE*
N296A SMU.518 F	ATCTTTTTTGTTCCCGCTAACCCGGTAAGTAAG GCA	Mutagenesis
N296A SMU.518 R	TGCCTTACTTACCGGGTTAGCGGGAACAAAAA AGAT	Mutagenesis
SMU.518 S235A F	CAAATGGTATTGGAGGGCCAGCTGCCGGTCTC ATGTTTACC	Mutagenesis
SMU.518 S235A R	GGTAAACATGAGACCGGCAGCTGGCCCTCCAA TACCATTTG	Mutagenesis
SMU.518 K280A F	GGCGGTGCCGATATGGCAGTGGCTGCAGCAGA TAGGATTAATG	Mutagenesis
SMU.518 K281A R	CATTAATCCTATCTGCTGCAGCCACTGCCATAT CGGCACCGCC	Mutagenesis
SMU. NlmTE F	GTCTTGGCTTTTGTTAGCCACGGGCCTTGC	Deletion of *nlmTE*
SMU. NlmTE R	CATTGAGAATGGTATTACGGGAAACTGC	Deletion of *nlmTE*
SMU. NlmTE 5’ Fusion	GCCGCCATGGCGGCCGGGAGCTCTAAGAGAAT GTTAACGGCTATGAC	Deletion of *nlmTE*
SMU. NlmTE 3’Fusion	CGCGGCCGCCTGCAGGTCGACCCTATATATCA GCAGCAATCTTTGC	Deletion of *nlmTE*

CHAPTER THREE

CHARACTERIZATION OF MUTACIN ACTIVITY

Abstract

Streptococcus mutans UA159, whose genome is completely sequenced, produces two nonlantibiotic mutacins, mutacin IV (encoded by *nlmAB*) and mutacin V (encoded by *nlmC*). In this study, we investigated the contribution of *nlmA* and *nlmB* to mutacin IV activity and demonstrated by performing genetic studies as well as by using semipurified molecules that, in contrast to a previous report, both of these genes are required for optimum mutacin IV activity. We also showed that mutacin IV is active against multiple *Streptococcus* species. In contrast, mutacin V displayed a narrower inhibitory range than mutacin IV. Our results suggest that mutacin IV and mutacin V may act synergistically to inhibit various organisms.

Introduction

Bacteriocins are ribosomally synthesized small antimicrobial peptides secreted by a wide variety of bacteria to inhibit the growth of other bacteria. The antimicrobial activity of the bacteriocins may or may not be against species that are closely related to the producer organism (133). In most cases, producer organisms secrete multiple bacteriocins, each displaying a distinct inhibitory spectrum, either narrow or broad, to provide a competitive advantage by eliminating organisms that share the same ecological niche with the producer organism. Bacteriocins produced by lactic acid bacteria (LAB) are active against clostridia, listeria, and other pathogens; therefore, these bacteriocins have the potential to be used as antimicrobial therapy.

S. mutans, a lactic acid bacterium, is considered to be the primary causative agent of dental caries, commonly known as tooth decay (177). *S. mutans* resides in the dental plaque, a multispecies biofilm community that harbors over 600 different types of microorganisms (1). To inhibit the growth of competing bacteria and to establish a successful colonization in the plaque biofilm, *S. mutans* secretes a wide variety of mutacins that display a diverse inhibitory spectrum (8, 15). Based on the chemical structure, mutacins are generally classified into the following two groups: class I, the lantibiotics, and class II, the heat-stable nonlantibiotics (54, 151). Both lantibiotics and nonlantibiotics are synthesized ribosomally; however, lantibiotic peptides are post-translationally modified and contain either a lanthionine or methyllanthionine ring structure as well as dehydrated amino acids (315). On the other hand, nonlantibiotics are unmodified peptides and consist of either one or two separate molecules (219).The genome sequence of the reference strain *S.mutans* UA159 does not encode any genetic determinant for lantibiotic mutacins (7). However, this strain carries and produces at least two nonlantibiotics, mutacin IV

and mutacin V (104). The NlmA peptide consists of 44 residues with a molecular mass of 4169 Da, while the NlmB peptide consists of 49 amino acid residues with the molecular mass of 4826 Da. This strain also carries several other nonlantibiotics- like small peptides with unknown functions (299).

Qi et al. (247) first purified mutacin IV activity from the culture supernatant of the UA140 strain and found that the active fractions contained both the NlmA and NlmB peptides. However, attempts to separate the complex of these two peptides into individual peptides by various high-pressure liquid chromatography (HPLC) protocols were unsuccessful (247). In contrast, when Hale et al. (104) deleted the *nlmB* gene, the mutant strain apparently retained its mutacin activity and thus raised doubt about the two-peptide action of the mutacin IV activity and suggested that NlmA was solely responsible for mutacin IV mediated inhibition of various bacteria.

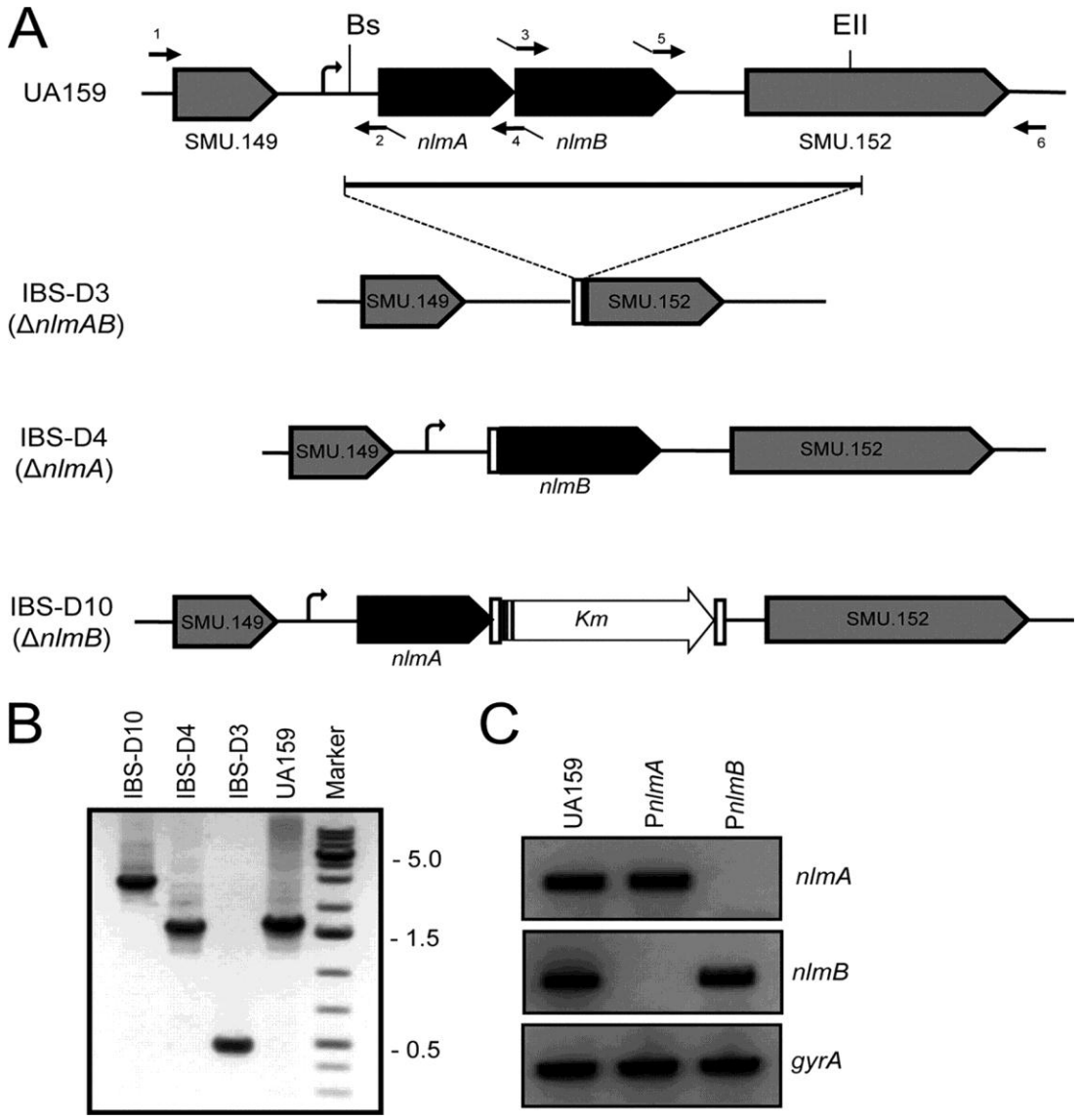
In this chapter, we focused strictly on the characterization of mutacins IV and V produced by the UA159 strain. We found that, contrary to the previous report, both the NlmA and NlmB peptides are required for optimum mutacin IV activity. Furthermore, we showed that mutacin IV activity has a broader inhibitory spectrum than previously described.

Results

Mutacin IV activity requires both the NlmA and NlmB peptides: To clarify the role of individual peptides in mutacin IV activity, we constructed individual single-deletion mutants and a double-deletion mutant. Since *nlmA* and *nlmB* are organized in an operon, insertion of any antibiotic resistance gene in *nlmA* would disrupt the normal expression of *nlmB*. To avoid this polar effect, we constructed a marker less *nlmA* mutant strain, IBS-D4, using the Cre-*loxP*

system (Fig. 8). We also deleted the *nlmB* gene and replaced it with a kanamycin resistance cassette that lacks a transcription terminator at its 3' end and was inserted in the same orientation as the *nlmB* gene, therefore eliminating the concern of a polar effect on the *nlmA* gene (Fig. 8). Expression of *nlmA* in *nlmB* and *nlmB* in *nlmA* mutation background was verified by sqRT-PCR. The downstream SMU.152 gene contains its own promoter, and its expression was not affected by the replacement, as measured by semi quantitative reverse transcription- PCR (RT-PCR) assay (data not shown). We also constructed a marker less *nlmAB* double-deletion mutant, IBS-D3, by using the Cre-*loxP* method (Fig. 8). These strains were evaluated for mutacin IV production against two indicator strains, *S. gordonii* and *S. cristatus*. These two indicator strains are sensitive to mutacin IV only when used against *S. mutans* strain UA159 (41). As shown in Fig. 9A, deletion of either *nlmA* or *nlmB* drastically reduced the zone of inhibition against both of the indicator strains, although there was some residual mutacin IV activity present in both of the mutants, with the Δ*nlmA* strain displaying slightly more activity than the Δ*nlmB* strain. As expected, deletion of the *nlmAB* genes completely abolished the mutacin IV production. To confirm that both the NlmA and NlmB peptides are necessary for the observed bacteriocin activity, we used a complementation assay. The IBS-D3 strain was complemented with plasmids carrying *nlmA*, *nlmB*, or *nlmAB* and tested against the same two indicator strains. We also used IBS-D3 carrying only the vector plasmid as a control. Expression of *nlmA*, *nlmB*, and *nlmAB* from the plasmids was confirmed by semi quantitative RT-PCR analysis (Fig. 8C and data not shown). To facilitate the selection of the complementing plasmids with antibiotics, the indicator strains were transformed with the same vector plasmid. As shown in Fig. 9B, expression of the *nlmAB* genes successfully restored mutacin activity, whereas expression of the *nlmA* gene alone

Fig. 8. Construction of *nlmAB* deletion mutants. (A) Schematic diagram of various deletions constructs. A Cre-loxP-based marker less gene deletion method was used for the construction of *nlmA* (IBS-D4) - and *nlmAB* (IBS-D3)-deletion strains. IBS-D3 was created by deleting the *nlmAB* operon by BbsI (Bs) and BstEII (EII) digestion and replacing it with a kanamycin resistance cassette (Km) that is flanked by *loxP* sites. The Km cassette was subsequently cured with Cre recombinase. An overlapping PCR method was used for the generation of IBS-D4. Two PCR fragments were generated by primers 1 and 2 and primers 3 and 6 and were fused with a loxP-flanked Km resistance cassette, which was later removed with Cre recombinase. Similarly, two PCR fragments were generated with primers 1 and 4 and primers 5 and 6 and fused with the *loxP*-flanked Km resistance cassette to create IBS-D10. The Km cassette was not cured in IBS-D10. Symbols: block arrows, open reading frames; bent arrows, promoters; arrowheads, primers; arrowheads with tails, primers with regions overlapping; white box, loxP site. (B) PCR verification of the constructs using primers 1 and 6. (C) Verification of plasmid-driven expression of *nlmA* and *nlmB* by semi quantitative RT-PCR. Total RNA was harvested from the indicated strains and subjected to cDNA synthesis. Five nanograms of cDNA from each strain were used for semi quantitative RT-PCR. The *gyrA* gene was included to ensure that equal amounts of RNA were used for all reactions. The data are representative of RT-PCR analyses resulting from at least two different RNA isolations.


A
UA159
Bs
EII
1
2
3
4
5
6
SMU.149
nlmA
nlmB
SMU.152
IBS-D3
(ΔnlmAB)
IBS-D4
(ΔnlmA)
IBS-D10
(ΔnlmB)
Km
B
IBS-D10
IBS-D4
IBS-D3
UA159
Marker
- 5.0
- 1.5
- 0.5
C
UA159
PnlmA
PnlmB
nlmA
nlmB
gyrA

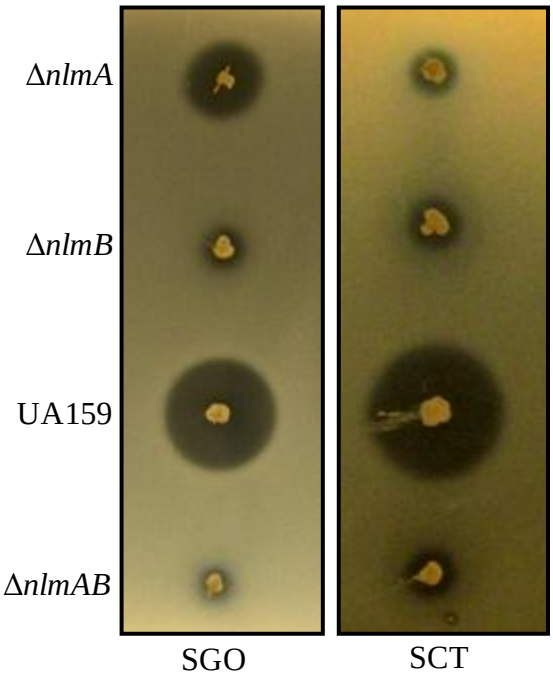
did not restore the mutacin activity compared with the wild type. Surprisingly, we found that expression of *nlmB* could restore the mutacin production to some extent; however, the activity was much less than that restored by the strain with *nlmAB*. Thus, taken together, our results suggest that optimum mutacin IV activity requires both the NlmA and NlmB peptides.

To further confirm our observation, we purified the active molecules by ammonium sulfate precipitation from the culture supernatant. The purified molecules were tested for mutacin IV activity against the *S. gordonii* indicator strain. As a positive control, we also included the wild-type UA159 strain. As shown in Fig. 9C, deletion of the *nlmAB* genes completely abolished the mutacin activity. Although the overproduction of *nlmB* restored the mutacin activity, overproduction of both of the peptides significantly augmented the activity, confirming that optimum mutacin IV activity requires both the NlmA and NlmB peptides.

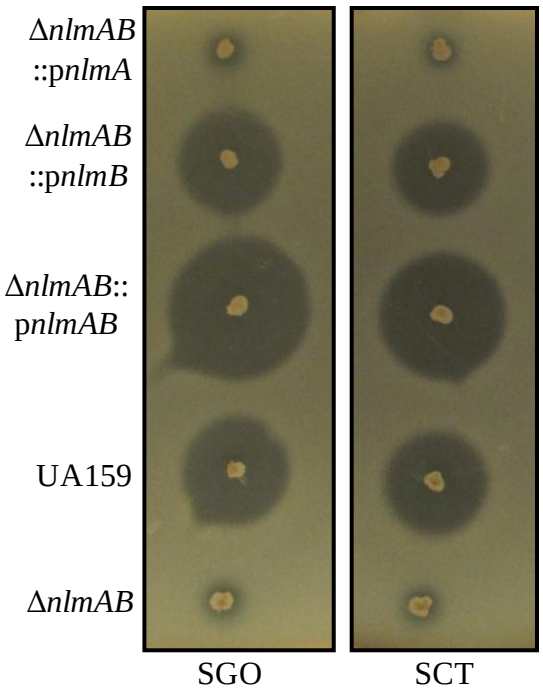
Bacteriocins produced by *S. mutans* UA159 are active against *Streptococcus pyogenes*: *S. pyogenes*, a pathogenic streptococcus that also resides in the oral cavity, was shown by Hale et al. to be inhibited by mutacin IV (104). To refine and expand the observation, we tested our mutant strains against three *S. pyogenes* strains, JRS4, K56, and AM3, belonging to three different serotypes, M6, M12, and M3, respectively. As shown in Fig. 10A, deletion of *nlmA*, *nlmB*, or *nlmAB* caused a significant reduction in the zone of inhibition formation against the *S. pyogenes* strains. Although the reduction was not as drastic as that observed against the *S. gordonii* strain, nevertheless, the reduction was easily measurable. We then tested the effect of mutacin V against *S. pyogenes* strains. For this, we used either the *nlmC* mutant or the *nlmAB nlmC* triple mutant strains. As shown in Fig. 10B, deletion of *nlmC* also leads to a reduction of zone of inhibition compared with that of the wild type. However, the reduction was higher in

Fig. 9. Deferred antagonism assay for mutacin IV production by *S. mutans* strains. (A) Ability to produce mutacin IV by *nlmA* (IBS-D4)-, *nlmB* (IBS-D10)-, and *nlmAB* (IBS-D3)-deleted strains. (B) Complementation analysis of the Δ*nlmAB* strain (IBS-D3) with various levels of plasmid-derived expression of *nlmA*, *nlmB*, or *nlmAB*. For the mutacin IV assay, *S. mutans* cultures were stabbed into THY agar and incubated overnight at 37°C under microaerophilic conditions. The plates were overlaid with soft agar containing *S. gordonii* DL-1 (SGO) or *S. cristatus* ATCC 51100 (SCT) as indicator strains. The zones of inhibition of the indicator strains were evaluated after overnight incubation (*, strain also contains plasmid pIB184-Km). (C) Activity profile of semi purified mutacin IV from culture supernatant. Ammonium sulphate extractions from the clarified culture supernatant from the representative strains were tested for mutacin IV activity against the *S. gordonii* DL-1 indicator strain. A representative antagonism assay from at least three independent extractions is shown. WT, wild type.

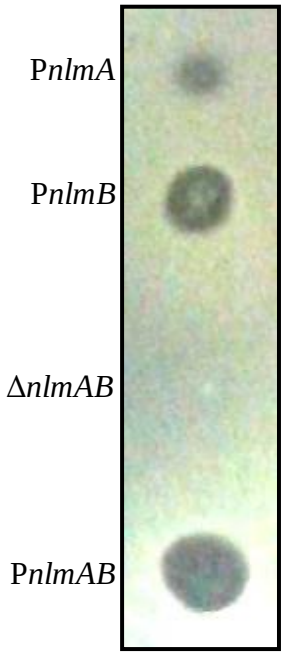
A



B


Δ*nlmAB* ::p*nlmA*
Δ*nlmAB* ::p*nlmB*
Δ*nlmAB*:: p*nlmAB*
UA159
Δ*nlmAB*
SGO
SCT

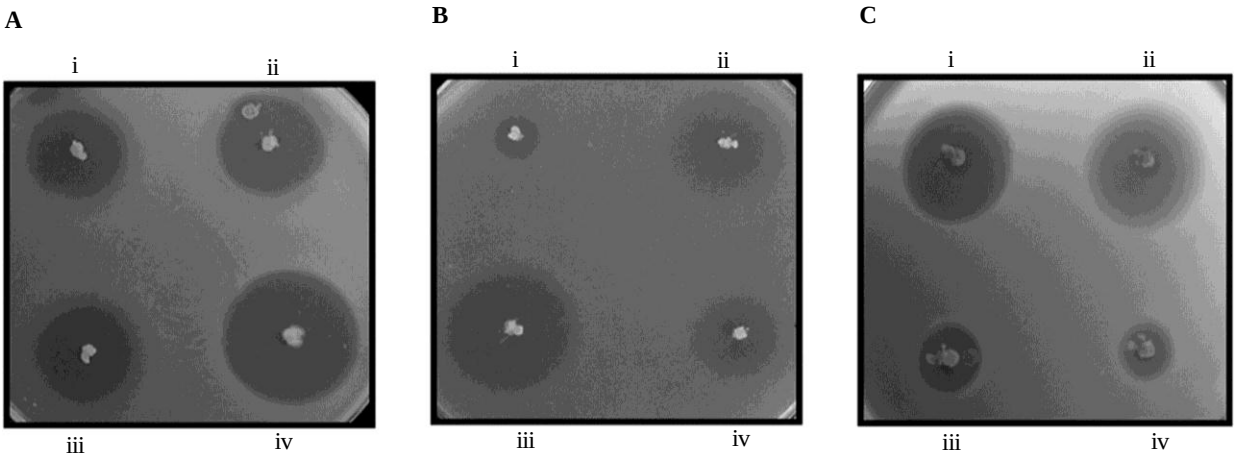
C


P*nlmA*
P*nlmB*
Δ*nlmAB*
P*nlmAB*

case of the *nlmC* mutant than that in the case of *nlmAB* mutant, suggesting that mutacin V is more active against *S. pyogenes* than mutacin IV (Fig. 10B, comparison of ii and iv). As expected, the zone of inhibition produced by the triple mutant was further reduced, although the mutant produced a very small but noticeable zone of inhibition. To confirm our results, we complemented the triple mutant strain (IBS-D13) with plasmid-based *nlmAB* or *nlmC*. As shown in Fig. 10C, expression of the *nlmAB* genes in IBS-D13 from a complementing plasmid completely restored the mutacin IV activity (comparison of i and ii). Similarly, expression of *nlmC* in IBS-D13 also restored mutacin V production; however, the efficiency of complementation was below the expected level (Fig. 10B, comparison ii and iii). Taken together, our results suggest that both mutacin IV and mutacin V inhibit *S. pyogenes* and that their effect is synergistic.

Growth of the indicator strain dictates sensitivity to mutacin: It has been previously shown that *L. lactis* is not inhibited by mutacin IV (104). When we tested our mutant strains against *L. lactis* MG1363, we observed that depending on the growth stage of the culture, mutacin IV activity could inhibit *L. lactis* growth. When mid-exponential-growth-phase *L. lactis* culture was used for the overlay, we observed that the Δ*nlmC* strain produced a noticeable zone of inhibition that was absent when the indicator culture used was from the stationary growth phase (Fig.11A & B). As expected, the triple mutant strain IBS-D13 did not produce any zone of inhibition irrespective of the growth stage of the indicator strain. Thus, mutacin IV appears to inhibit *L. lactis* growth. It is known that mutacin IV inhibits *S. gordonii* growth, but very little is known about the inhibitory spectrum for mutacin V. Therefore, we tested the Δ*nlmC* and Δ*nlmABC* (IBS-D13) strains against *S. gordonii* culture that was grown to early stationary phase. As shown in Fig. 11C, the zone of inhibition produced by the Δ*nlmC* strain was drastically

Fig. 10. Bacteriocin activity of *S. mutans* UA159 against group A streptococcus. (A) Inhibitory effect of mutacin IV on *S. pyogenes* strain JRS4. i, Δ*nlmA* strain; ii, Δ*nlmB* strain; iii, Δ*nlmAB* strain; iv, strain UA159. (B) Activity of mutacin IV- or V-deficient mutant strains against *S. pyogenes* JRS4. i, Δ*nlmABC* strain; ii, Δ*nlmAB* strain; iii, UA159; iv, Δ*nlmC* strain. (C) Complementation analysis of the Δ*nlmABC* strain. i, UA159/pIB184-Km; ii, IBS-D13/pIB-D19 (*nlmAB*); iii IBS-D13/pIB-D29 (*nlmC*); iv, IBS-D13/pIB184-Km. These plates are representative of at least three independent assays.


A
i
ii
iii
iv
B
i
ii
iii
iv
C
i
ii
iii
iv

reduced compared to that produced by the wild-type strain. On the other hand, the Δ*nlmAB* Δ*nlmC* mutant strain produced virtually no zone of inhibition against *S. gordonii*. These results indicate that mutacin V also can inhibit the growth of *S. gordonii* in addition to that of *L. lactis*. Interestingly, when we used the Δ*nlmAB* strain, we observed very little mutacin activity against the *S. gordonii* indicator strain, suggesting that mutacin V alone cannot work efficiently; the presence of mutacin IV augments the inhibitory activity of mutacin V. In this regard, these two mutacins seem to work synergistically.

Inhibition of various streptococci by mutacin produced by *S.mutans* UA159:

Because we found that both mutacin IV and mutacin V have wider ranges of inhibitory spectra than expected, we tested several other *Streptococcus* spp. belonging to all phylogenetic subgroups for sensitivity to both mutacins (Fig.12). For this, we used the same two mutant strains, IBS-D3 and IBS-D13, as well as the Δ*nlmC* strain, and tested them against 18 different *Streptococcus* spp. As shown in Table-10, mutacin IV inhibited growth of all the streptococcus subgroups, except the mutans group and *S. pneumoniae*, a mitis subgroup species. On the other hand, mutacin V inhibited growth of only five streptococci belonging to four subgroups (pyogenic, anginosus, mitis, and bovis). However, we found that not all species belonging to a particular subgroup were susceptible to mutacin V. For example, in the case of the pyogenic subgroup, only *Streptococcus iniae* and *S. pyogenes* were inhibited by mutacin V. Thus, we conclude that mutacin V has a narrower inhibitory spectrum than mutacin IV, which has a much broader inhibitory spectrum. Because we observed different inhibitory spectra for mutacin IV and mutacin V, it argues that the mechanisms of immunity against these two mutacins are different.

Fig. 11. Mutacin activity against other species. Derivatives of *S. mutans* UA159 were tested for inhibitory activity against *L. lactis* (LLC) (A and B) or *S. gordonii* (SGO) (C). For *L. lactis*, either freshly grown (A) or 1-day old (B) cultures were tested for growth inhibition. These plates are representative of three independent experiments.

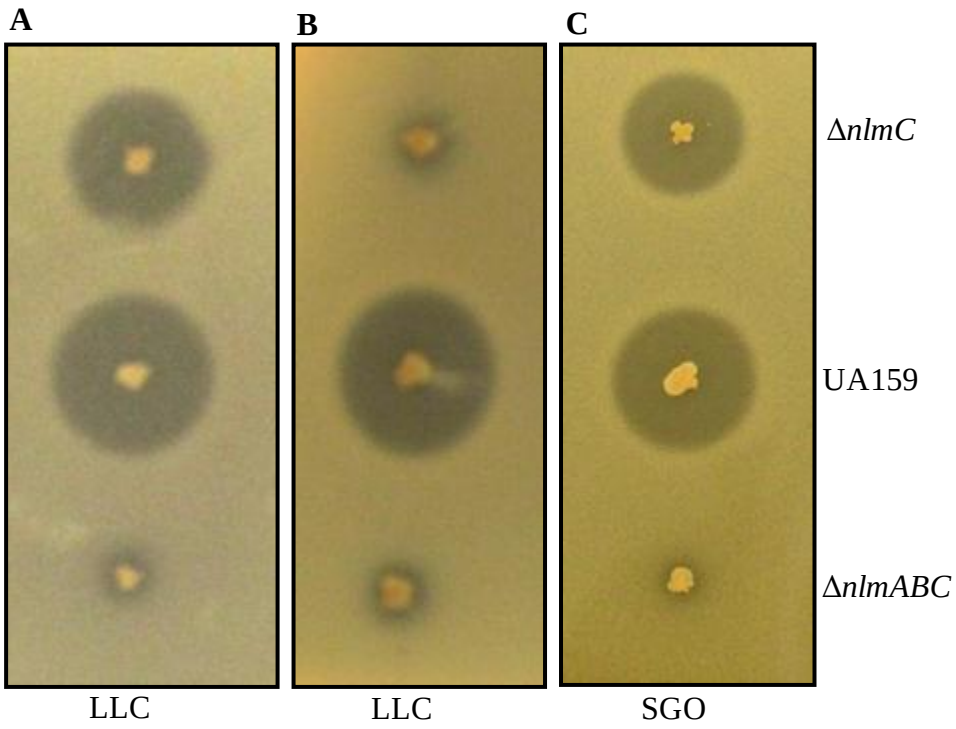

A
B
C
ΔnlmC
UA159
ΔnlmABC
LLC
LLC
SGO

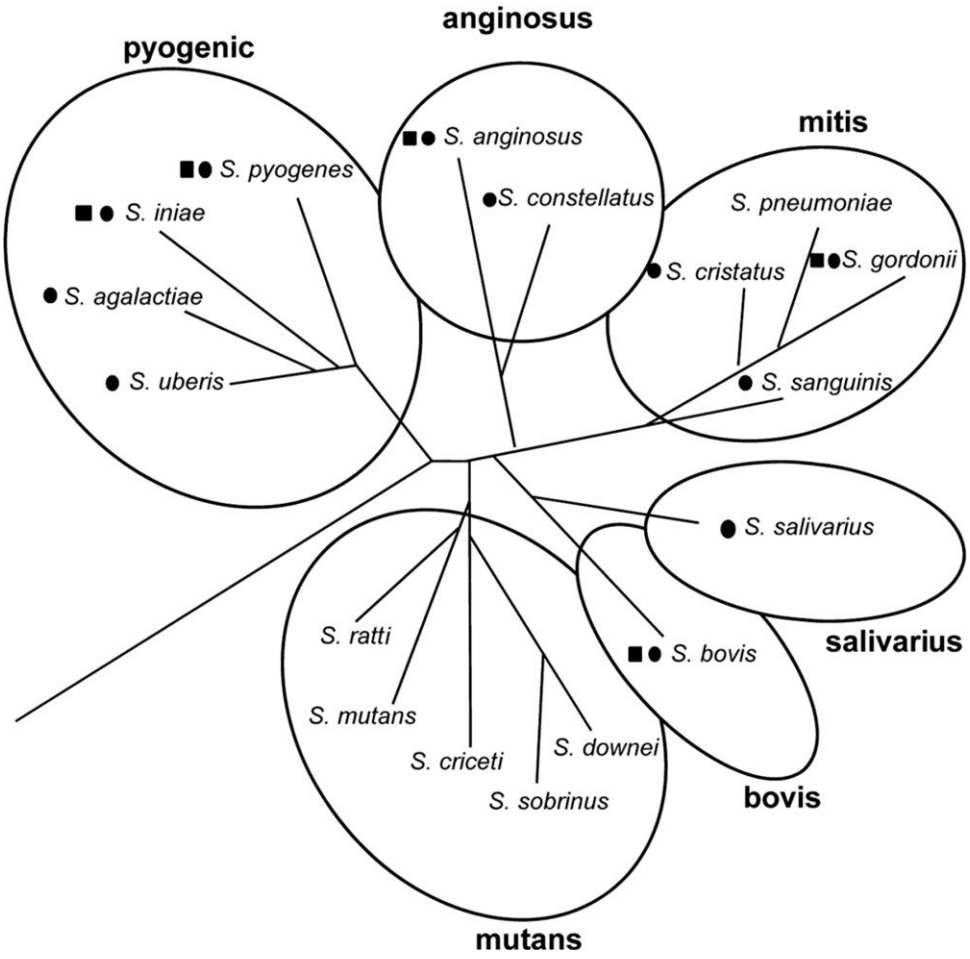
Table-10: Inhibitory spectrum of *S. mutans* UA159

Species (indicator strain[s][b]	Group	Zone of inhibition in diameter (mm)[a]			
		Wild type	Strain carrying:		
			Δ*nlmAB*	Δ*nlmC*	Δ*nlmABC*
S. uberis (UT387)	pyogenic	22±2	-	22±2	-
S. agalactiae (NEM306)	pyogenic	16±1	-	13±1	-
S. iniae (K388)	pyogenic	14±1	13±1	9±1	5±0.5
S. pyogenes (JRS4, K56, AM3)	pyogenic	24±1	14±1	17±1	10±1
S. anginosus (CI)	anginosus	23±1	16±1	17±1	5±0.5
S. constallatus (CI)	anginosus	25±2	-	17±1	-
S.pneumoniae (ATCC6303)	mitis	10±1	10±1	10±1	10±1
S. cristatus (ATCC51100)	mitis	14±1	4±0.5	11±1	4±0.5
S. gordonii (DL-1, M5, ATCC 10558)	mitis	17±1	-	17±1	-
S. sanguinis (SK36)	mitis	10±1	-	9±1	-
S salivarious (ATCC 25975)	salivarious	8±1	-	8±1	-
S. bovis (TX20005)	bovis	9±1	4±0.5	-	-
S. downeii (ATCC 337908)	mutans	-	-	-	-
S. sobrinus (6715)	mutans	-	-	-	-
S. criceti (AHT)	mutans	-	-	-	-
S. mutans (UA159, UA130, GS-5)	mutans	-	-	-	-
S. ratti BHT	mutans	-	-	-	-

↲a: −, none or a < 4-mm zone of inhibition. Values are means ± standard deviations resulting from at least three independent experiments performed in duplicates.

↲b: three different strains for three streptococci were tested, as indicated. CI, clinical isolates.

Fig.12. Mutacin activity against various streptococci. Phylogenetic classification of various streptococci based on 16S rRNA sequence analysis. The species tested for sensitivity against mutacin IV (circle) or mutacin V (square) produced by *S. mutans* UA159 are shown.


anginosus
pyogenic
mitis
S. anginosus
S. pyogenes
S. constellatus
S. pneumoniae
S. iniae
S. gordonii
S. cristatus
S. agalactiae
S. uberis
S. sanguinis
S. salivarius
salivarius
S. ratti
S. bovis
S. mutans
S. downei
S. criceti
S. sobrinus
bovis
mutans

Discussion

Streptococcus mutans, a lactic acid bacterium, is one of the primary causative agents of dental caries (108, 177). Occasionally, *S. mutans* can enter the blood stream and cause transient bacteremia and infective endocarditis (177). The organism possesses several properties which allow it to successfully colonize and survive in the human host. In the oral cavity, the bacterium can form dental plaque, a type of biofilm, on the tooth surfaces (19). Dental plaque is home to one of the most complex bacterial floras associated with human body. So far, more than 700 different bacterial species have been identified from human oral cavity, and the majority of them are associated with dental plaque (2, 65). However, *S. mutans* has ability to outcompete other bacteria by producing various bacteriocins known as mutacins (8, 15). In this study, we have characterized the role of a well recognized bacteriocin called Mutacin IV. Mutacin IV is a two-peptide bacteriocin produced by *S. mutans* UA140 and UA159 and is active against the mitis group of streptococci (247). By definition, both of the peptides should be present at eqiomolar concentration to exert their activities (219). Qi et al. (247) first purified mutacin IV activity from the culture supernatant of the UA140 strain and found that the active fractions contained both the NlmA and NlmB peptides. However, Hale et al. (104) showed that deletion of the *nlmB* gene produced the same phenotype as compared to wild type, suggesting the sole role of NlmA in mutacin IV activity.

To clarify the role of individual peptide in mutacin IV activity, we constructed a panel of clean mutants of each peptide and tested their activity against the susceptible bacteria. Our deletion and complementation studies clearly established that both of the peptides are required for optimum mutacin IV activity, despite the fact that NlmB displayed killing activity against *S.*

gordonii to a certain extent in *nlmA* background but not against *S. cristatus*, where both of the mutants lost activity completely. Further study with semi-purified molecules clearly showed that both of the peptides should be present in equal amount to display optimum inhibition of the susceptible cells (Fig. 9). Although it is not entirely clear why Hale et al. (104) failed to observe any function for the NlmB peptide, it is possible that the inhibition assay and the growth media used by those authors were different from the ones used here. In our case, a direct overlay of the indicator strain was used to avoid the chloroform vapor. Our data also suggest that overproduction of the NlmB peptide is able to inhibit the growth of the indicator strains. We speculate that NlmB itself can form a homodimeric transmembrane helical structure needed for the activity since it also contains the GXXXG motif. In this regard, mutacin IV is similar to lactacin F, which is also a dipeptide bacteriocin, composed of LafA and LafX. The individual peptides display inhibiting activity, although the efficiency is lower than when both the peptides are used together (10). Alternatively, in the absence of NlmA, NlmB can form the heterodimeric helix-helix structure with other bacteriocin-like peptides such as SMU.283, which shows high homology to NlmA. Further *in vitro* studies with purified or synthetic peptides may tell us whether NlmB can form such structures *in vivo*. Our study showed that mutacins produced by *S. mutans* UA159 have much broader inhibitory spectra than expected. We also found that maximum mutacin IV activity requires both the NlmA and NlmB peptides. In this regard, mutacin IV truly belongs to class IIb bacteriocins, which require complementary interaction of two separate peptides.

In this study, we also analyzed and characterized the NlmC activity and found that NlmC can work with NlmAB to produce optimum mutacin IV phenotype. Interestingly, we found that NlmAB also imparts in mutacin V activity depending on the status of indicator bacteria. An early

or mid exponential culture of *L. lactis* is prominently susceptible to mutacin IV activity; whereas stationary phase culture was exclusively inhibited by stationary phase culture. These findings suggest the expression of different membrane receptors at different growth stages. Our studies revealed that mutacin IV activity can inhibit more bacterial species than reported before. We found that streptococci from almost all groups, except mutans group, can be inhibited by mutacin IV activity and this spectrum of activity is often associated with mutacin V activity.

Interestingly, while some streptococci are susceptible to both of the mutacins, most of the strains are not, suggesting that immunity against these two mutacins is different. Mechanisms of immunity against a given bacteriocin can vary widely. The immunity factors can interact directly with the bacteriocin itself, thereby blocking interaction with the membrane receptors, or facilitate expulsion of intracellularly accumulated bacteriocins (40, 192). Another novel mechanism by which immunity protein blocks the interaction of bacteriocin with its cognate membrane receptor is mannose phosphotransferase (man-PTS) (70). Since very little is known about the mode of action for these two mutacins, further studies are required to understand the molecular mechanism of target specificity as well as immunity against the activity of these mutacins.

CHAPTER FOUR

MECHANISM OF SELF-IMMUNITY

Abstract

Streptococcus mutans, principal causative agent of dental caries, secretes antimicrobial peptides known as mutacins to suppress the growth of competing species to establish a successful colonization. *S. mutans* UA159, a sequenced strain, produces at least two major mutacins, mutacin IV and V. Mutacin IV is a two-peptide bacteriocin encoded by *nlmAB* genes, which are mapped just upstream of a putative immunity-encoding gene, SMU.152. Here we explored the function of SMU.152 as an immunity protein. We observed that over expression of SMU.152 in two sensitive host strains converted the strains to become immune to mutacin IV. To identify the residues that are important for immunity function; we sequentially deleted residues from the C-terminal region of SMU.152. We observed that deletion of as few as seven amino acids, all of which are highly charged (KRRSKNK), drastically reduced the immunity function of the protein. Furthermore, we identified two other putative immunity proteins, SMU.1909 and SMU.925, which lack the last four charged residues (SKNK) that are present in SMU.152 but contains the KRR residues. Synthetic addition of SKNK residues to either SMU.1909 or SMU.925 to reconstitute the KRRSKNK motif and expressing these constructs in sensitive cells rendered the cells resistant to mutacin IV. We also demonstrated that deletion of Man-PTS system from a sensitive strain made the cells partially resistant to mutacin IV, indicating the Man-PTS system plays a role in mutacin IV recognition.

Introduction

Bacteriocinogenic bacteria also contain the specific immunity mechanism to its own bacteriocin (54). This system determines the distinction between 'self' vs. 'non-self' and provides protection to the producer strains. Two different classes of immunity mechanisms are prevalent among the producer bacteria (93) and these immunity mechanisms are highly specific and don't confer protection against other bacteriocin (54, 278). Immunity can be achieved by specific immunity protein or a specialized ABC-transporter which pumps the bacteriocin from the producers membrane (101, 229, 238, 256). In case of lantibiotics, one or both of these systems are involved for providing immunity (278), whereas specific immunity protein is present for class II bacteriocins and cyclic peptides are dependent on ABC-transporters (67). In most cases, immunity proteins either sequester the bacteriocin peptides or compete for the receptor binding to confer protection (124, 302). Immunity to bacteriolysins are mediated by alteration of the peptidoglycan structure of the producer strain (25). For some pediocin-like (class IIa) and some class IIc bacteriocins, the proteins that confer immunity have been shown to directly bind to the bacteriocin receptor and therefore block the pore formation by the bacteriocin (70). Recently, it has been shown that Abi family proteins consist of membrane bound putative metalloproteases could confer protection against two-peptide bacteriocins such as plantaricins (PlnEF and PlnJK) produced by *Lactobacillus plantarum* (150). Furthermore, Abi family proteins display extensive cross-immunity against non-cognate two-peptide bacteriocins. Because Abi encodes putative metalloproteases, it has been proposed that Abi-mediated immunity could involve direct proteolytic degradation of the bacteriocins (150).

Immunity activity to mutacins has been studied on lantibiotics (mutacin I, II, III) where immunity function is conferred by *mutFEG* genes that are closely related to the LanFEG immunity system (194, 246, 248). For dipeptide lantibiotic (Smb), immunity system is dependent on two proteins, SmbF and SmbG respectively (212, 325). However, no cognate immunity proteins have been identified so far for non-lantibiotic dipeptide mutacin IV. After the first isolation of a two-peptide bacteriocin, lactococcin G (216), several two-peptide bacteriocins have been isolated and characterized (218). The genes encoding the two-peptide bacteriocins are always found next to each other, along with a gene that encodes a membrane associated immunity protein that seems to protect the bacteriocin producer strain from being killed by its own bacteriocin.

Immediately downstream of the *nlmAB* operon lies another gene, SMU.152, which is annotated in the GenBank database as an immunity protein. In this study, we have characterized this gene and showed that SMU.152 can confer protection against mutacin IV in *S. gordonii*, an indicator strain traditionally used for mutacin IV activity. Furthermore, we show that the last seven charged amino acid residues at the C-terminal domain of the protein are essential for optimum immunity.

Results

SMU.152 can confer immunity against mutacin IV: SMU.152 encodes a hypothetical protein that is topologically linked to *nlmAB* operon, which encodes the structural genes for mutacin IV. An intergenic sequence (IGS) of 193-bp is present in between the stop codon of *nlmB* gene and the initiation codon of SMU.152. A schematic diagram of the SMU.152 locus and the upstream *nlmAB* genes are shown in Fig.13A. Sequence analysis, confirmed using BPROM

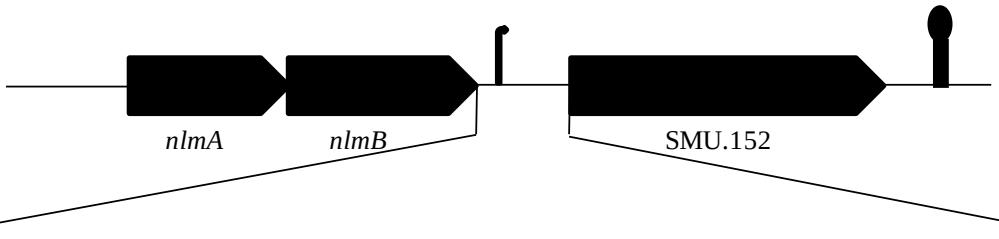
online software (prediction of bacterial promoters, Softberry, http://linux1.softberry.com), indicates the presence of putative -35 (TTAGAA) and -10 (TATACT) box motifs 78- and 55-bp, respectively, upstream of the putative start codon with a putative ribosomal binding site (AGAGG) 11-bp upstream of the start codon. A putative rho-independent terminator sequence (AATTATGGAGGAGGA) was also identified at position 66-bp downstream of SMU.152 by TransTermHP (http:// transterm. cbcb.umd.edu) program (146). Thus, it appears that the SMU.152 is a single gene stand-alone operon.

We analyzed the primary amino acid sequences of SMU.152 by several topology predicting algorithms {(TopPred (304), HMMTOP (293)} and found that SMU.152 contains at least four transmembrane helices (Fig 13B). SMU.152 primary sequence was also subjected to a protein threading algorithm {MUlti-Sources ThreadER (317)} to predict the 3-dimensional structure of SMU.152, the predicted structure is shown in Fig. 13C. From the structure, it appears that the last few residues are not part of any transmembrane helix and are disorganized.

To evaluate the function of SMU.152 as an immunity protein, we cloned the entire coding region and the promoter region of SMU.152 into a shuttle plasmid and introduced the plasmid into *S. gordonii*, which was used as the indicator strain. As shown in Fig.14A, heterologous expression of SMU.152 in *S. godonii* confers protection against mutacins secreted by *S. mutans* UA159. This result suggests that SMU.152 alone can give protection against mutacin IV and therefore acts as an immunity protein. We also over expressed SMU.152 in S. *constellatus*, a strain that is susceptible to mutacin IV (127). We found that as in *S. gordonii*, the

Fig. 13. Mutacin IV and its immunity protein. (A) Genetic organization of *nlmAB* and SMU.152. The intergenic sequence along with the putative promoter region is shown. Co-expression of SMU.152 with *nlmAB* genes is verified by linkage PCR. (B) Predicted secondary structure of SMU.152 determined by HMMTOP transmembrane topology prediction software (http://www.enzim.hu/hmmtop). Symbols are: i, inside the cell; H, helical (within membrane); o, outside of the membrane. (C). Predicted three dimensional structure of SMU.152 as determined by MUlti-Sources ThreadER (317).

A



ATTGATATAATGGTGGGCAATGTCAATTCAGGGCGAGATTTCTGAATGGGTACAGTGGCATTGTCC

ATTTTTCGTCCAATAGAATTTTTTATTTAAGCTGGGCTATAACTA**TTAGAA**TATTAAGTATTGACTT
-35

A**TATACT**TGTTAAAAAGATTTATATTATATAATGTCGAATTTCAAG**AGAGG**GAAAAAATAAG**ATG**
-10 **RBS**

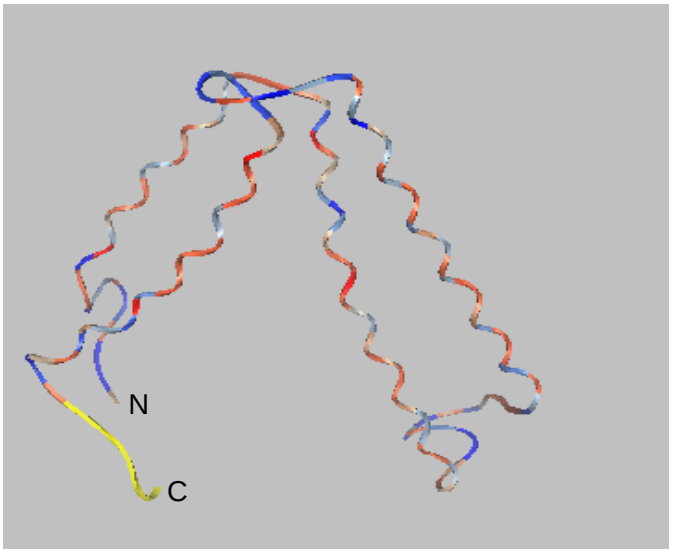
B

```
seq  MKKLERISTK ILRWAYLILT TIVFVAALII YNWLYYGKSM LFWLPAVLVL    50
pred IIiiiiiiii iiiHHHHHHH HHHHHHHHHH HooooooooH HHHHHHHHHH

seq  LVTALLAYLV KKQLEARLEP LLIFNTLQQI IGVLIALAVG LFALAYKALN   100
pred HHHHHHHHHi iiiiiiiiii iiiiiiiiiH HHHHHHHHHH HHHHHHHHHo

seq  TKINDGNEVY WILFAAILLI FSWNAVELYK ELKRRSKNK  139
pred ooooooooooH HHHHHHHHHH HHHHHHHHHi iiiiiiiii
```

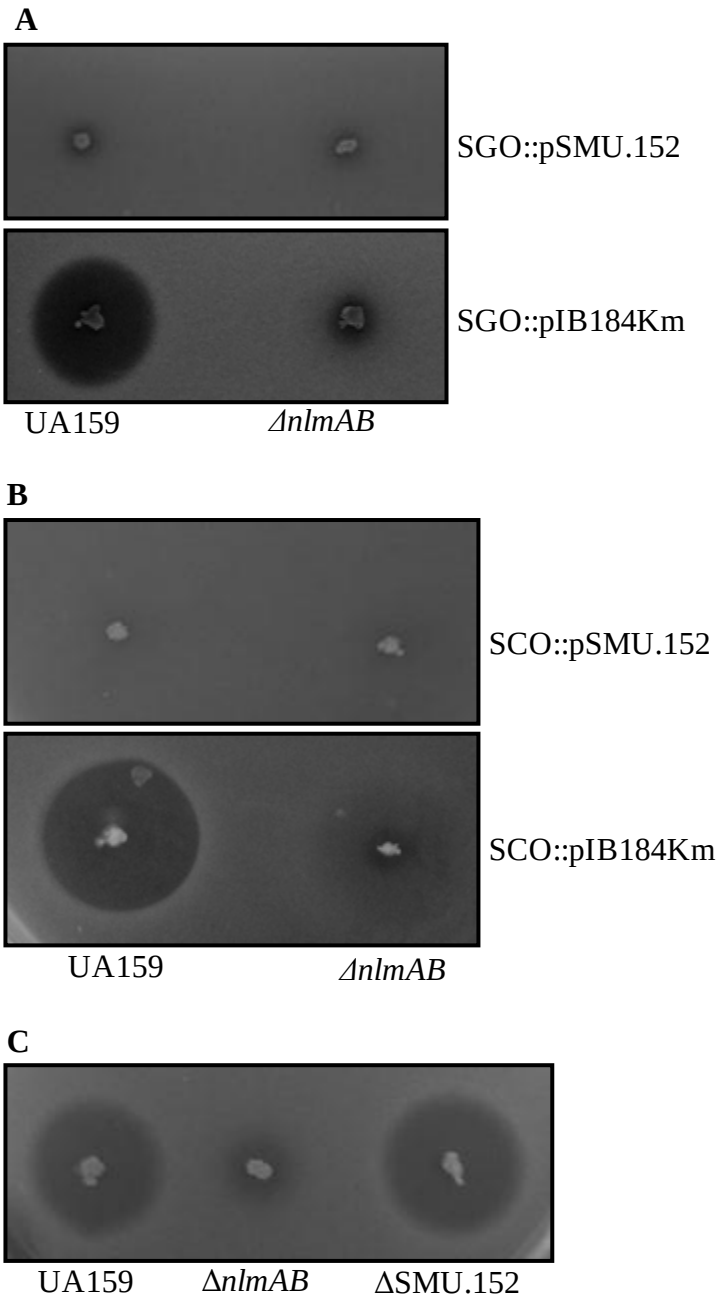
C



S. constellatus strain containing SMU.152 is also refractory to lysis by mutacin (Fig. 14A-B). To explore further, we attempted to delete the SMU.152 locus from UA159 leaving the *nlmAB* loci intact. To our surprise, we were able to construct SMU.152 deleted strain (IBS-D16) and found no significant difference in growth in liquid medium between IBS-D16 and UA159 (data not shown). We then tested whether IBS-D16 strain produces mutacins and as shown in Fig. 14C, IBS-D16 produces mutacin and there was no difference in the level of production between UA159 and IBS-D16. We also used IBS-D16 as an indicator strain in a deferred antagonism assay against UA159 and found that IBS-D16 is not susceptible to mutacins produced by UA159 (data not shown). Therefore, our data suggest that UA159 probably encodes other immunity proteins that confer protection.

***S. mutans* UA159 immunity proteins show cross-reactivity:** According to KEGG database, three other SMU.152 paralogous genes are encoded by *S. mutans* UA159. One of them, SMU.925, which is also known as CipI, is previously shown to function as an immunity protein against mutacin V (encoded by *nlmC*) (237). Two other putative immunity proteins, SMU.1909 and SMU.1913, are located near putative bacteriocin-like proteins (Blp) such as SMU.1906 and SMU.1914. Since SMU.152 shows sequence homology with SMU.925 as well as with SMU.1909 and SMU.1913, we wanted to test the possible role of these proteins for immunity to mutacin IV. We constructed various deletion derivatives of these putative immunity genes and used the strains as indicator against UA159. When we used as indicator, a strain in which SMU.925 was deleted (ΔSMU.925) or a strain in which both SMU.1909 and SMU.1913 were deleted (ΔSMU.1909 – SMU.1913), we did not observe any significant changes in the sensitivity to mutacin IV or V (data not shown). We then constructed a multiple deletion strain in

Fig. 14. Immunity activity of SMU.152 in *S. gordonii*. *S. mutans* UA159 and an *nlmAB* strain (IBS-D3) were stabbed into THY agar and incubated overnight at 37°C under microaerophilic conditions. The plates were overlaid with soft agar containing the indicator strains. The zone of inhibition of the indicator strains was evaluated after overnight incubation. The indicators strains are *S. gordonii* DL-1 (A) and *S. constellatus* (SCO) (B) containing the vector (pIB184Km) or the vector with SMU.152 (pSMU.152). (C). Mutacin activity of ΔSMU.152 (IBS-D16) strain. These plates are representative of three independent assays.


A
SGO::pSMU.152
SGO::pIB184Km
UA159
ΔnlmAB
B
SCO::pSMU.152
SCO::pIB184Km
UA159
ΔnlmAB
C
UA159
ΔnlmAB
ΔSMU.152

the *ΔnlmAB* background, where we knocked out SMU.152, SMU.925, as well as SMU.1909 to SMU.1913. When we tested this IBS-D58 strain as indicator, to our surprise we found that the strain became sensitive to its own mutacins (Fig. 15). However, the zone of lysis was not as large as the zones produced against *S. gordonii* or other indicator bacteria. Taken together, our results suggest that SMU.152 may interact with other immunity proteins to confer complete protection against its own mutacins.

C-terminal charged residues of SMU.152 are required for optimum activity: Since SMU.152 confers immunity against mutacins, and because the predicted three dimensional structures indicate a disordered coil at the C-terminus, we wanted to evaluate the role of C-terminus in mutacin immunity. We made sequential deletions of 19, 7, 6, and 3 residues from the C-terminal region and tested for immunity activity. As shown in Fig.16, deletion of as few as six residues from the C-terminal region caused drastic reduction in the immunity activity. Therefore, it appears that charged residues KRRSKNK at the C-terminal regions are required for optimum immunity activity against mutacins.

SMU.1909 with SKNK residues can confer immunity against mutacin IV: As mentioned above, SMU1909 shows significant homology to SMU.152 and is annotated as a paralogous gene for SMU.152. When we performed a sequence alignment (Fig.17A), we found that SMU.1909 protein shares 49% similarity (including 30% identity) with SMU.152. Most importantly, we found that SMU.1909 lacks the last four residues of the KRRSKNK sequence. To verify whether SMU.1909 can confer immunity against mutacin IV, we over expressed SMU.1909 and SMU.1909 carrying SKNK residues in *S. gordonii*. As shown in Fig. 17B, expression of SMU.1909 does not provide protection (compare the zone of lysis for the top two

Fig: 15. Immunity activity of different mutants. UA159, Δ*nlmAB*, Δ*nlmC*, and Δ*nlmABC* were stabbed into THY agar and mutacin activity was measured against IBS-D16 (ΔSMU.152) and IBS-D58 (Δ *nlmAB,* ΔSMU.152, Δ SMU.1909 -SMU.1913, Δ SMU.925). These plates are representative of three independent assays.

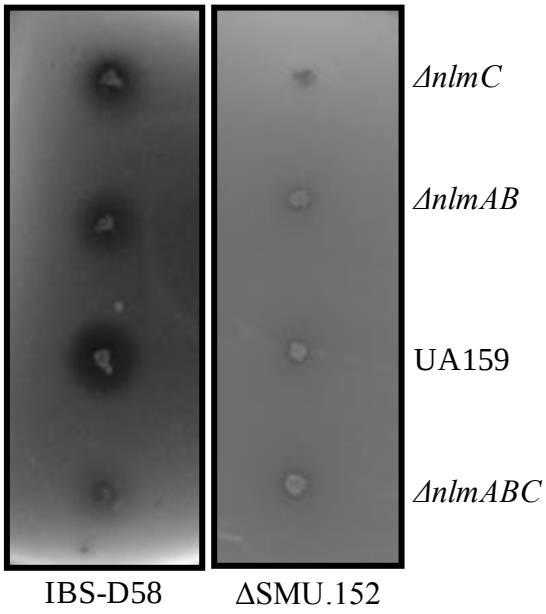

ΔnlmC
ΔnlmAB
UA159
ΔnlmABC
IBS-D58
ΔSMU.152

Fig.16. Immunity activity of truncated SMU.152. Various C-terminal deletions of SMU.152 were created and tested for immunity activity in *S. gordonii* as described in Figure 2. The data shown (in millimeter) are means ± standard deviations (error bars) of at least three independent experiments. Student's t-test was used to calculate the significance of the difference between the mean diameters of the zone of lysis. Asterisks indicate the P values that are <0.001.

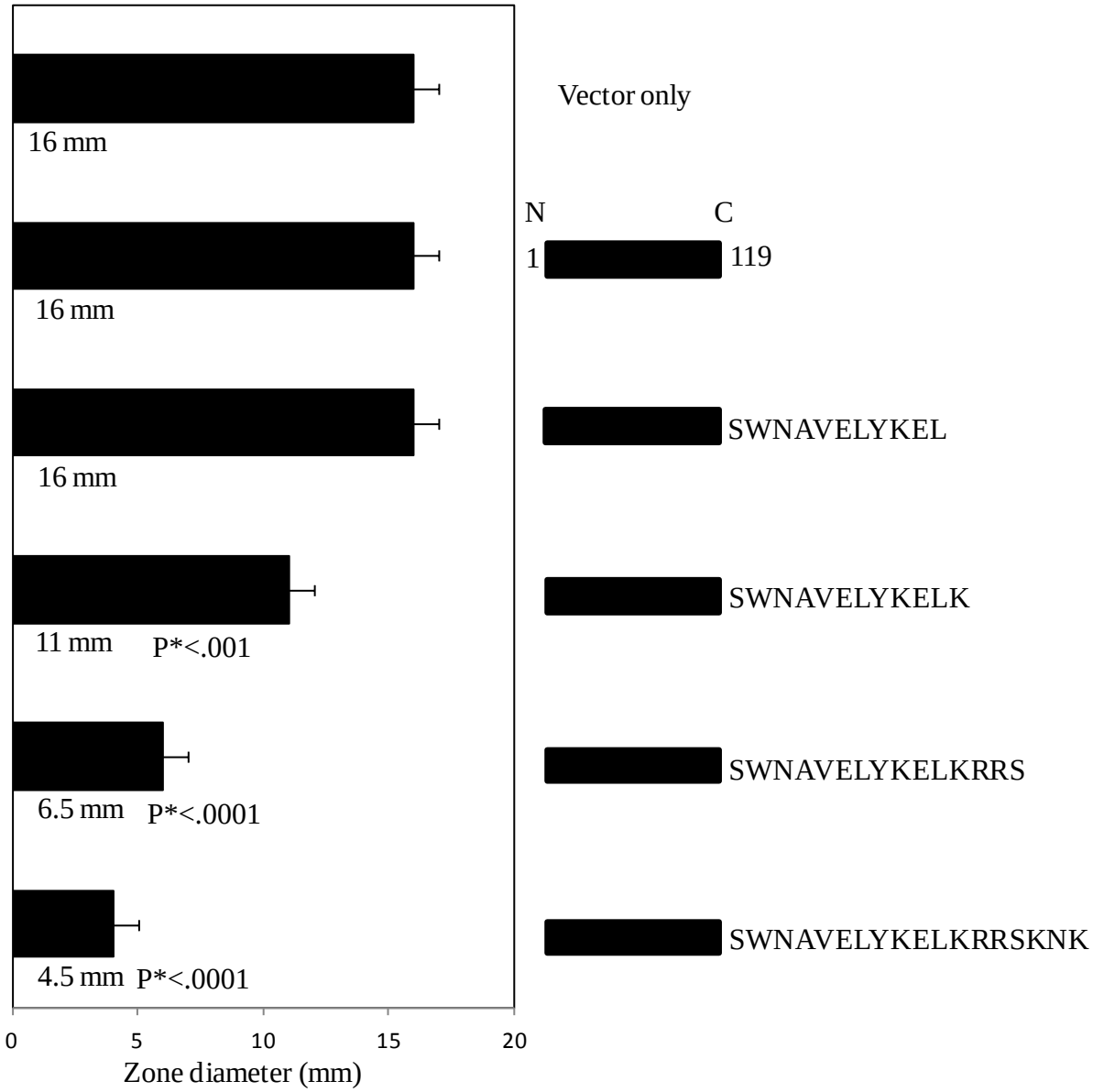

Vector only
16 mm
N
C
1
119
16 mm
SWNAVELYKEL
16 mm
SWNAVELYKELK
11 mm
P*<.001
SWNAVELYKELKRRS
6.5 mm
P*<.0001
SWNAVELYKELKRRSKNK
4.5 mm
P*<.0001
0
5
10
15
20
Zone diameter (mm)

Fig. 17. Immunity activity of SMU.1909 (A) Sequence alignment between SMU.152 and SMU.1909. An asterisk (*) indicates positions which have a single, fully conserved residue; a colon (:) indicates conservation between groups of strongly similar properties; and a period (.) indicates conservation between groups of weakly similar properties (B) *S. gordonii* strains containing SMU.1909 alone or SMU.1909 with four additional residues (SKNK) were tested for sensitivity against mutacin IV produced by UA159. Assays were repeated at least three times and a representative plate is shown. Student's t-test was used to calculate the significance of the difference between the mean diameters of the zone of lysis ($P<0.001$).

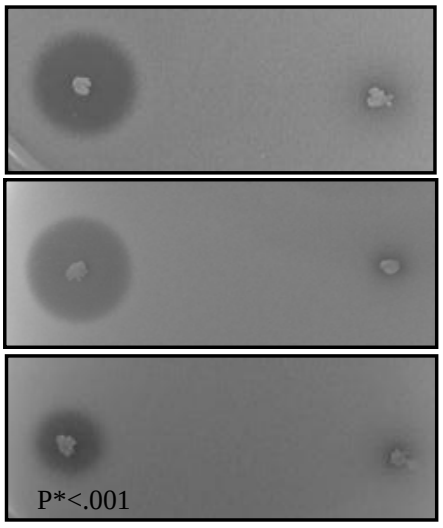
A

```
SMU.152         MKKLERISTKILRWAYLILTTIVFVAALIIYNWLYYGKSMLFWLPAVLVLLVTALLAYLV
SMU.1909        -MRLSKCSSRVLRFLRLGAVLSGFLALDLCFHMIDSKSSIWLWLPLGILSLLVFLVAYLA
                  :*.: *:::**:  *  .   *:*  : :: :   .*: :***  :: *:. *:***.

SMU.152         KKQLEARLEPLLIFNTLQQIIGVLIALAVGLFALAYKALNTKINDGNEVYWILFAAILLI
SMU.1909        YKELNQRPIAEQQFTQYQTQTAVTIFSFLVLLVCSYRAITTNDTTQRTALIIVNILLVIV
                 *:*: *  .   *.  *   .* *   : *:. :*:*:.*: .  . .  *:   ::::

SMU.152         FSWNAVELYKELKRRSKNK
SMU.1909        IIWNLIELIKLHKRR----
                : ** :** *  ***
```

B



panels). However, when we added SKNK sequence to SMU.1909, the zone of lysis was reduced to approximately half as compared to the vector control. Thus, it appears that while SMU.1909 alone does not confer immunity against mutacin IV, when we restored the complete KRRSKNK sequence that is present in SMU.152, we observed significant amount of protection against mutacin IV activity conferred by the modified SMU.1909.

SMU.925 with SKNK also can confer immunity to mutacin IV: As mentioned above, SMU.925 also shows significant similarity with SMU.152. Since, SMU.925 also shares sequence homology with SMU.152 (Fig. 18A), we wanted to test whether over expression of SMU.925 in *S. gordonii* can confer protection against mutacin IV. As shown in Fig. 18B, over expression of SMU.925 did not produce noticeable difference in the zone of lysis (compare the top panel with the middle panel). Like SMU.1909, SMU.925 also lacks the last four charged amino acids that are present in SMU.152. We added these residues (SKNK) to SMU.925 and tested sensitivity against mutacin IV. We observed a slight but reproducible decrease in the zone of lysis produced by wild-type UA159 (Fig. 18B, bottom panel). To further confirm that SMU.925+SKNK can confer immunity against mutacin IV, we tested against an *nlmC* deletion strain that predominantly produces mutacin IV (127). As observed before, the zone of lysis produced by the Δ*nlmC* strain in *S. gordonii* is smaller than the zone produced by the wild type strain (127). However, when we over expressed SMU.925+SKNK in *S. gordonii*, the zone of lysis were significantly smaller (~25% reduction) compared to the vector control strain. Thus, it appears that addition of SKNK to SMU.925 can confer some level of immunity against mutacin IV.

Man-PTS system is required for optimum mutacin IV activity: The Man-PTS, which is a major sugar uptake system in *Firmicutes*, was recently shown to act as a receptor for class-

Fig: 18. Immunity activity of SMU.925. (A) Sequence alignment between SMU.152 and SMU.925. Symbols are described in Fig. 17A (B). Deferred antagonism assay was carried out with *S. gordonii* DL-1 strain as described in Fig. 17B, except that the indicator strain also contains the vector plasmid to facilitate selection during antagonism assay. Assays were repeated at least three times and a representative plate is shown. Student's t-test was used to calculate the significance of the difference between the mean diameters of the zone of lysis and the P values are shown.

A

```
SMU.152         ---------------MKKLERISTKILRWAYLILTTIVFVAALIIYN-WLYYGKSMLFWL 44
SMU.925         MLYCTANYMNIILGEEKDMSRKKSRKLKLLRLLTAQLIAICLLIMVLTQAIFKSPLLVQV 60
                              *.:.* .:: *:   *: : :: :. **:      : ..:*. :

SMU.152         PAVLVLLVTA-LLAYLVKKQLEARLEP----LLIFNTLQQIIGVLIALAVGLFALAYKAL 99
SMU.925         CFLIITLCTAGLNYYFIKEAREANAQESSAATVTSQVIFTVVFLLLMVLSGYKAIQVSEF 120
                  ::: * ** *  *::*:  **. :      :  :.:  :: :*: :  *  *:  . :

SMU.152         NTKINDGNEVYWILFAAILLIFSWNAVELYKELKRRSKNK 139
SMU.925         ASKIIFSAAAVISFIVSLLLLWGIRYVKKTSFFRKR---- 156
                 :**  .  .   ::.::**::. . *:  . :::*
```

B

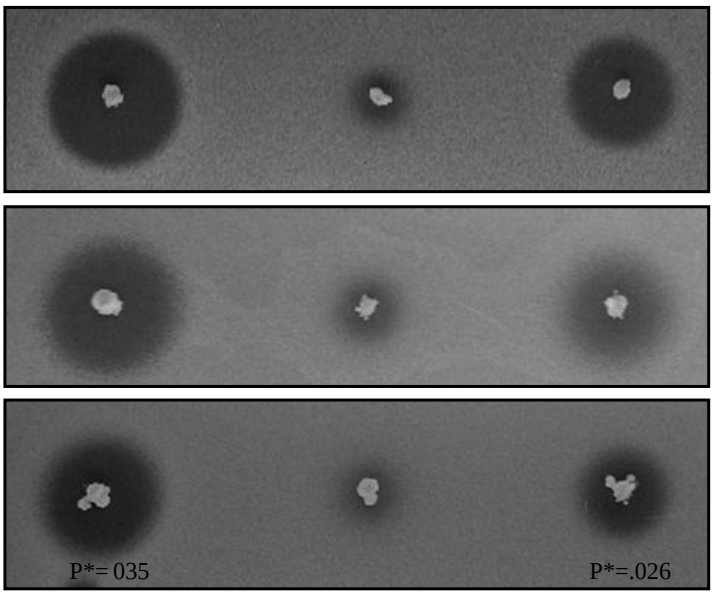
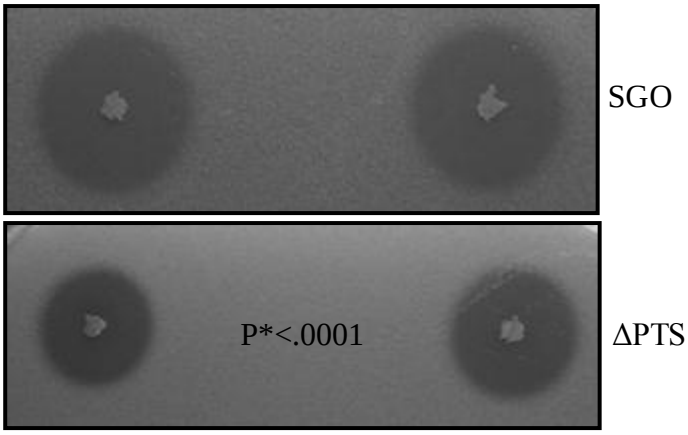


Fig. 19. Mannose-PTS is required for optimum sensitivity. Assays were done as described in Fig. 14. UA159 was stabbed in THY agar plate. The indicator strains were wild type *S. gordonii* DL-1 (SGO) and a mutant DL-1 without a functional Man-PTS system (ΔPTS, IBS-D15). Assays were repeated at least three times and a representative plate is shown. Student's t-test was used to calculate the significance of the difference between the mean diameters of the zone of lysis ($P<0.001$).


SGO
P*<.0001
ΔPTS

IIa bacteriocins and for lactococcocci A, a class-IIc bacteriocin (70, 148, 149). However, these two classes differ from class-IIb bacteriocins since they are single peptide bacteriocins. To verify whether Man-PTS system plays a role as a receptor for mutacin IV, we deleted the entire Man-PTS system from *S. godonii* and subjected the deletion strain in a deferred antagonism assay for mutacin IV sensitivity. As shown in Fig. 19, the zone of lysis was approximately 30% smaller in the Man-PTS deleted strain as compared to the wild type of *S. gordonii* strain. Thus, Man-PTS system appears to play a role in mutacin IV sensitivity. Since we observed some degree of lysis in the Man-PTS deleted strain, it suggests that the Man-PTS is not the only complex that acts as receptor for mutacin IV in *S. godonii*.

Discussion

Bacteriocins that belong to class II generally kill target cells either by pore formation or by interfering with the integrity of the cell membrane; however, the exact molecular pathways by which the bacteriocins exert their effect on membrane are unknown (73). Since each bacteriocin display a specific inhibitory spectrum, it indicates that individual bacteriocin might recognize a particular receptor on the target cells. Some immunity proteins, specifically those that are related to class-IIa and -IIc bacteriocin have been shown to bind directly to these cell surface receptors and block pore formation (70). However, no immunity mechanism is known for any class-IIb two peptide bacteriocins, except for the Abi-family proteins that presumably degrade the bacteriocins. In this study, we have characterized a self-immunity protein for mutacin IV, a two-peptide bacteriocin.

The genes that encode self-immunity proteins for class-IIb are often located just downstream of the structural genes for bacteriocin. Because of this genetic organization, putative

immunity proteins for most of the bacteriocins can be readily identified. However, their function as an immunity protein has been demonstrated for only few of them (223). SMU.152 is also located downstream of *nlmAB* operon and therefore, it was an appropriate candidate to test for immunity activity. Indeed our results indicate that SMU.152 when expressed in heterologous sensitive hosts can confer complete protection against mutacin IV, suggesting that SMU.152 alone is sufficient to counteract mutacin IV activity. However, surprisingly we found that when SMU.152 was deleted from *S. mutans*, the deletion strain was still refractory to mutacin IV activity. Several possibilities can explain why SMU.152 deficient strain is resistant to mutacin killing. For example, there may be other immunity proteins in *S. mutans* to offer cross protection when the cognate immunity protein for a specific mutacin is absent. However, when we tested two putative immunity proteins (SMU.1909 and SMU.925) in *S. gordonii*, including the one (SMU.925) that confers protection against mutacin V (237), we did not observe any protection. Although this does not rule out that these two proteins cannot function as an immunity protein in the native host and may require a cellular component as suggested for LcnG, the lactococcal G immunity protein (223). Moreover, genome analysis indicates that *S. mutans* encodes several other proteins with similar structural features and sizes, and some of these proteins could function as immunity protein. Furthermore, this possibility has been corroborated by the findings that *S. mutans* OMZ175, which does not carry the *nlmAB* operon, is insensitive to mutacin IV (data not shown). Moreover, deletion of SMU.1909 in a *nlmAB* deficient strain (127), and OMZ175 (which lacks *nlmAB* locus) do not show sensitivity against mutacin IV (data not shown). Our data also suggest that SMU.152 may interact with other putative immunity proteins to confer full protection in the native host since deletion of multiple putative immunity proteins leads to sensitivity.

Our data strongly indicate that SMU.152 is sufficient to provide immunity to sensitive strains. We also showed that the C-terminal seven charged residues are important for the immunity activity. Theoretical structure prediction suggested that SMU.152 contains four helical domains and the last ten residues are highly disorganized. Deletion of last seven amino acids completely abolished the immunity function of this protein. Thus, these charged amino acids are important for the activity. We speculate that this region of the protein directly interacts with the mutacin peptides and therefore provides specificity. A recent study on lactococcal G suggests that the N-terminal 13 residues of one peptide (Lcnα) and 11 residues at the C terminal half of the other peptide (Lcnβ) are involved in the recognition by the immunity protein (223). Since mutacin IV is highly homologous to lactococcin G, we speculate a similar situation where the N-terminal portion of NlmA and the C-terminal portion of NlmB will interact with SMU.152. It is possible that this two-peptide mutacin IV might interact with a membrane integrated protein that functions as the receptor and induce membrane leakage. Binding of SMU.152 to the mutacin IV-receptor complex blocks the membrane leakage. This mechanism is similar to what has been demonstrated for class IIa pediocin-like bacteriocin involving Man-PTS system as the receptor (70). It is important to point out that we also identified Man-PTS as one of the putative receptors for mutacin IV activity since deletion of the Man-PTS rendered the *S. gordonii* strain less sensitive to mutacin IV. Since we did not observe complete protection against mutacin IV when Man-PTS was deleted, it indicates that there may be other cell surface proteins in *S. gordonii* that function as mutacin IV receptor.

Spontaneous resistance can occur for some class-II bacteriocins when the sensitive cells are continuously exposed to bacteriocins. The frequency of such occurrence depends on the type of bacteriocins as well as the type of the sensitive cell. *Listeria monocytogenes* can achieve

resistance frequency as high as 10^{-4} when exposed to class-IIa type bacteriocins including leucocin A and pediocins (99). The mechanisms of resistant generation can vary depending on the nature of the bacteriocin and it has been extensively studied for class-IIa type bacteriocins. In most cases expression of Man-PTS is down regulated in the resistant mutants (99, 148, 227, 287). However, Man-PTS independent generation of resistance cells has also been reported in *L. monocytogenes* where changes in the cell envelop have been found to be associated with the resistant mutants (295, 296). So far no spontaneous resistance mutant has been isolated for mutacin IV and we speculate that frequency of resistant occurrence would be very low.

Mutacin IV produced by *S. mutans* can inhibit growth of all groups of streptococci except the mutans group of streptococci that include *S. criceti, S. downei, S. ratti,* and *S. sobrinus* (127). The exact mechanisms by which these mutans streptococci are immune to mutacin IV are not known. It is possible that the entire mutans streptococci group lacks a functional receptor for mutacin IV and possibly for other mutacins as well. Alternatively these mutans streptococci encode the immunity genes without the corresponding mutacin genes, a phenomenon known as immune mimicking mechanism and demonstrated to be present for sakacin and pediocin immunity (85, 204). Mutans streptococci may also encode transporters to pump peptides out from the cell envelope; thereby, conferring resistance against mutacins, as has been shown for some lantibiotics (9, 25). Further investigation such as comparative genome analysis of the sensitive and resistant streptococci may reveal additional mechanisms of immunity against mutacins secreted by *S. mutans.*

CHAPTER FIVE

REGULATION OF MUTACIN PRODUCTION

PART-A

Abstract

S. mutans, a gram-positive pathogen, is primarily responsible for dental caries. Its outstanding ability to produce a variety of bacteriocins confers it selective advantage for successful colonization and thus considered as an important virulence factor. Although several studies have shed light on regulation of mutacin production in *S. mutans*, a comprehensive picture is necessary to unravel the entire molecular mechanisms of mutacin production. To search for new regulatory factors associated with mutacin IV production, we employed a random insertional mutagenesis by pGh9::IS*S1* on the reporter strain, P*nlmA-gus*. We screened ~12,000 clones for defective mutacin phenotypes (pale blue or white colonies) on media containing chromogenic substrate (X-gluc) and identified 22 unique genes including six previously reported regulators by mapping the insertion of transposon. One of these genes is SMU.518 that encodes a PDZ domain containing hypothetical protein with a Lon-like proteolytic motif. Gene inactivation coupled with complementation studies confirmed SMU.518's role as one of the key regulators of mutacin production. Further studies revealed that SMU.518 works in the ComDE pathway. Our genetic, biochemical and mass spectrometric analyses suggested that SMU.518 is required for post-transport cleavage of CSP and the processed CSP is the active component to induce the mutacin genes expression. Moreover, by site-directed mutagenesis, we found that S-235 and K-280 residues are essential for proteolytic function of SMU.518. We have also characterized the role of two more novel regulators, SMU.832 and SMU.2137, in mutacins production. We found

that SMU.832 (encodes a protein required for glucose side chain formation on ramnose-glucose polysaccharide) controls mutacins production by regulating the expression of *comC,* a gene encoding the competence stimulating peptide. We demonstrated that a conserved short hypothetical protein, SMU.2137, is also involved in regulation of mutacins production by severely down regulating the regulatory genes necessary for expression of mutacins in this organism. Taken together, our results suggest that the mutacin IV production is controlled at multiple levels by several regulatory factors of diverse cellular activities and this process is intricate with normal cellular homeostasis.

Introduction

Streptococcus mutans, an oral pathogen, leads a biofilm lifestyle in the oral cavity and considered as the primary etiological agent of dental carries (177, 320). In the oral habitat which harbors more than 700 different species, this organism undergoes fierce competition with other bacteria for successful colonization to initiate biofilm formation (210, 320). This process is aided by the ribosomally produced small cationic peptides called mutacins (107). Ability to produce mutacins, biofilm formation, competence developments, to produce acid from the carbohydrate and to survive at low pH is considered as important virulence factors to cause tooth decay (19, 160, 233). Most of the mutacins identified so far from *S. mutans* are lantibiotics and the production of two well characterized mutacins (mutacin I and mutacin 1140) is dependent on the presence of agar or agarose in culture medium (122, 247, 299). Nonlantibiotic class II bacteriocin, mutacin IV, was first purified from *S. mutans* UA140 (247), which also produces lantibiotic mutacin I. The genome sequence of the reference strain *S.mutans* UA159 does not encode any genetic determinant for lantibiotic mutacins (7); however, this strain carries and produces nonlantibiotics, mutacin IV and mutacin V(104).

Various studies suggest that mutacins production and competence development in *S. mutans* UA159 are coordinately regulated (153, 156, 173, 237, 299, 325). An inducer peptide together with a two-component signal transduction system is required for mutacins expression along with the stimulation of competence development in *S. mutans* UA159 (103, 156, 158, 168, 237, 299). During growth, this bacterium produces the peptide pheromone, CSP (competence stimulating peptide), encoded by *comC* and secretes through the dedicated ABC transporter CslAB (also called ComAB) as matured peptide (21-amino acids) while N-terminal leader

peptide is cleaved off by the proteolytic activity of the transporter (103, 173, 241). When the CSP concentration reaches a certain threshold, it is sensed by membrane sensor kinase ComD and gets activated by autophosphorylation and transfer the phosphate group to response regulator, ComE (158, 320). The activated ComE then stimulates the expression of mutacins and mutacins-associated genes by recognizing a set of conserved direct repeat present in the promoter region along with indirect activation of ~20 early competence genes (153, 168, 237, 299). In addition to this classic view of regulation, CiaH, LiaS, ClpP, HdrRM, BrsRM and VicKR also influence the bacteriocin expression at different levels (28, 41, 46, 221, 269, 320).

However, this classical notion bears some scientific limitations which need to be clarified. Specifically, the origin of this notion is *S. pneumoniae* which does not show any coordinated regulation of bacteriocin production and competence development. Instead they contain two distinct regulon of bacteriocin production and competence development (168, 187). BlpABCRH regulon activates the bacteriocin production and comprises of several bacteriocin-like peptides (Blp) with double glycine motif, a peptide pheromone named BIP (Blp inducing peptide and encoded by *blpC*), a ComAB paralogue transporter system BlpAB, a sensor histidine kinase BlpH and response regulator BlpR (63, 254). On the other hand, ComABCDE regulon in *S. pneumoniae* exclusively controls competence development which contain a peptide pheromone, CSP (an unmodified 17-residue mature peptide) (113). CSP is secreted and processed by an ABC transporter (ComAB), which processes the 45-residue pre-CSP right after GG-motif and generate mature peptide (114). ComDE is the two-component system where ComD senses the extracellular CSP and activates response regulator ComE by phosphotranfer. ComE is included to AgrA/AlgR/LytR family response regulators (213) and activated ComE stimulates the *com* genes expression for competence development (310).

Based on the genetic organization and sequence similarities, it has been shown that ComABCDE regulon of *S. mutans* is more similar to BlpABCRH system of *S. pneumoniae* (179, 187). Interestingly, *S. mutans* UA159 contains two ortholog loci for BlpAB or ComAB of *S. pneumoniae* and Martin et al.(187) confirmed that *S. mutans' comAB-cslAB* genes arrangement is highly similar to BlpAB of S. *pneumoniae.* Several experimental evidences also indicate that ComABCDE system of S. *pneumoniae* and *S. mutans* differ in several aspects. It has been shown that *S. mutans* CSP needs post-export cleavage at the C-terminal end by an unknown factor for efficient activation of ComDE regulon (239) and the synthetic CSP of *S. mutans* works at comparatively much higher concentration than any other characterized CSPs to exhibit the maximum response (242). For instance, maximum response in *S. pneumoniae* and *S. intermedius* is achieved at 20 nM concentration (113, 240), whereas *S. mutans* requires 0.5 µM to 1.0 µM concentration of CSP to get optimum response (156, 242, 299, 326). In addition, it takes ~2-hr to get maximum transformation efficiencies in *S. mutans* (156), indicating that expression of *comX* is not directly regulated by ComE. In consistent with this idea, there are no ComE-binding sites in front of *comX* gene. In contrast, the expression of *nlmAB* (mutacin IV) is induced rapidly upon addition of CSP (156) and *comCDE* mutants cannot express mutacin genes in *S. mutans* UA159 (153, 299) suggesting an exclusive requirement for ComCDE in *nlmAB* expression which relies on the presence of conserved direct repeat in the upstream of *nlmAB* and other mutacin related genes (299). In *S. pneumoniae, comE* mutation caused abolition of transformation phenotypes, which hinted that ComE is essential for ComX activation (168). Surprisingly, *comC* mutation didn't change the transformation phenotype in *S. mutans* UA159 (6) and *comDE* mutations only affect the CSP mediated transformation keeping the basal level of transformation intact which suggest that ComCDE system up regulates transformation phenotype by indirect activation of

ComX (6, 172, 191). Recently, Mashburn-Warren et al. (191) showed that a novel regulatory system, ComR/ComS (encodes XIP/Shp61peptide pheromone), is exclusively required for *comX* gene expression and competence development. A mutation in *comR/comS* abolished the transformation phenotype and ComCDE system can't activate *comX* gene in *comR/comS* background suggesting the auxiliary role of ComCDE system upstream of ComRS regulon.

Another marked point of difference exists in regard to nature of CSPs from *S. mutans* and *S. pneumoniae* (9). To date, six distinct pherotypes of CSP have been identified from *S. pneumoniae* and most of the strains demonstrate competence only in the presence of their cognate CSP (245, 312). Variability among competence pherotypes in *S. pneumoniae* is based on the variation of hydrophobic patch in the CSP and on the sensor domain of ComD (130, 137). *S. mutans* CSP does not exhibit any variation in its hydrophobic patch and it has been shown that two distinct functional domains control binding to and activation of ComDE system to trigger the responsive genes expression (9, 284). Although *comC* gene locus shows little allelic variability, there was no correlation between the CSP genotype and transformability of these strains (9, 239). Moreover, various strains of *S. mutans* can sense and trigger the transformability upon addition of synthetic CSP from other strains (9). In addition, response to CSP in *S. pneumoniae* is a global phenomenon where ~100% of the cells develop competence in the laboratory condition (48). Whereas only a subpopulation of *S. mutans* develop competence temporarily, expression of *nlmAB* genes is global (~100%) in response to CSP addition (168). Based on all of these facts, it was assumed that ComC is essentially involved in induction of bacteriocins production and CSP has the dual function as bacteriocin inducing peptide (BIP) and competence stimulating peptide (CSP) and clarification of the entire regulatory process was warranted (187). To clarify these contrasts and confusions and to get a comprehensive view of regulation of mutacins production

in *S. mutans* UA159, we carried out a transposon mediated insertional mutagenesis. In this study, we have found that mutacin IV production is regulated by numerous regulatory factors at different levels and characterized the role of a membrane-associated extracellular Lon protease in regulation of mutacin production in *S. mutans* UA159.

Results

Identification of the genes involved in activation of mutacin IV: To identify the genes that are involved in regulation of mutacins production, we carried out IS*S1*-mediated transposition mutagenesis which integrates into the chromosome of streptococci randomly (30, 289, 328) and rarely inserts more than one place into the genome of the same cell (30, 32). Plasmid pGhost9:IS*S1* (182, 183) was used to deliver the insertion element into the strain IBS-D5 (P*nlmA-gus*), which has the blue phenotype on THY-X-gluc plate. The insertion frequency was less than 0.5% which is in consistent with several previous reports (30, 32, 289). We performed three independent screening of the master transposon library and tested ~12,000 CFU for white/pale blue colonies onto THY-X-gluc plate. We excluded most of the whitish colonies which displayed severe growth defect or small colony phenotype. We obtained ~200 tentative positive clones after first round of blue/white screening. These colonies were rescreened for mutacin production against *S. gordonii* DL-1, which is the indicator for mutacin IV (104, 247). We obtained 32 mutants after second round of screening which displayed either no or significantly reduced (<10 mm in diameter) level of mutacin production in compared to IBS-D5 (16-mm in diameter).

Mapping the position of IS*S1* in the genome of IBS-D5: To map the position of transposon insertions, we did inverse PCR as described in methods and materials. Chromosomal

DNA from the selected colonies was isolated and digestion by HindIII, followed by heat inactivation and self-ligation by T4 DNA ligase. The ligated DNA was subjected to inverse PCR by using the primers IS*S1*R-out 2 and IS*S1* For-4. The PCR products were sequenced with the primer IS*S1*-Rout-2. The flanking sequences (after the GAACC) obtained from sequencing analysis were mapped on the genome of *S. mutans* UA159 by a BLAST search. We were able to determine the location of insertion explicitly for all 32 mutants, which contain 22 unique genes (Table-11). Among them, we found double insertion for only three mutants. The position of insertion was further confirmed by PCR using the gene-specific primers. We observed consistent results upon deferred antagonism bacteriocin assay for most of the cured mutants, except SMU.121, which displayed variable phenotypes. Some of the mutants were already known for their role in regulation of mutacin IV (*comC, comD, comE, nlmT, nlmE, ciaH*) along with other unknown factors. We have classified all of them based on the mutacin production against the indicator bacteria. Class I includes the mutants that displayed white colony morphology and no mutacin production. Among them five were already documented for their proven role in mutacin production. A two-component signal transduction system (ComC, ComD and ComE) and a dedicated ABC transporter (NlmTE) were in class I along with a Lon-like domain containing conserved hypothetical protein, SMU. 518. Class II produced very little mutacin (< 6-mm zone of lysis) and includes one sensor kinase (*ciaH*), one gene involved in glucose side chain formation on ramnose-glucose backbone (SMU.832), two conserved hypothetical proteins (SMU.2137 and SMU.1634), and five other genes involved in energy metabolism and central cellular processes. Class III produced intermediate level of mutacin and includes six genes of diverse functions. Only one gene (SMU.121) has been categorized as class IV, which displayed variable properties in initial mutacin assay. This gene got seven insertions at the same place in all

three independent screening and designated as MATE efflux family/damage inducible protein/ cation efflux pump (DinF). To further understand the role of some unknown factors, we made deletion construct of SMU.121, SMU.611, SMU.518, SMU.832 and SMU.2137. Two of the deletion constructs (ΔSMU.611 and ΔSMU.121) did not produce any phenotype, whereas other three mutations affected mutacin production significantly and were used for further investigations. Among them, SMU.518 encodes a conserved hypothetical protein with lon-like proteolytic domain, SMU.832 encodes a membrane protein involved in glucose side-chain formation on *S. mutans* rhamnose-glucose polysaccharide (RGP) (231) and SMU.2137 encodes small cytoplasmic protein of unknown function.

Characterization of SMU.518 locus: SMU.518 encodes a 346-residue long polypeptide which is highly conserved among gram-positive bacteria and mycobacterium (Fig. 20B). It contains an N-terminal uncleavable signal sequence for secretion with a transmembrane domain (from residue 10-26) (http://www.oralgen.lanl.gov/oralgen/bacteria/smut), a eukaryotic type PDZ domain (from residue 131-195) and a C-terminal Lon-like protease (S16) domain (from residue 233-314) (http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=smu:SMU_518). C-termini also contain a domain of Birnavirus VP4 protein (residue 221-287), which overlaps with the Lon-like domain. However, this protein does not contain any canonical ATP binding motifs (walker A or B). SMU.518 appears to be organized in a single operon with SMU.516 and a gene encoding phosphopantetheine adenylyltransferase (*kdtB*) and involved in biosynthesis of lipopolysaccharide) with a common promoter (Fig. 20A). 3' region of *kdtB* gene has 23-base pair overlapping with the 5'region of SMU.518. The IS*S1*insertions occurred in SMU.518 gene at position 116 and 229-amino acid. There is no promoter region just before SMU.518. In silico

Table-11: Genes involved in regulation of mutacin IV (NlmAB) production

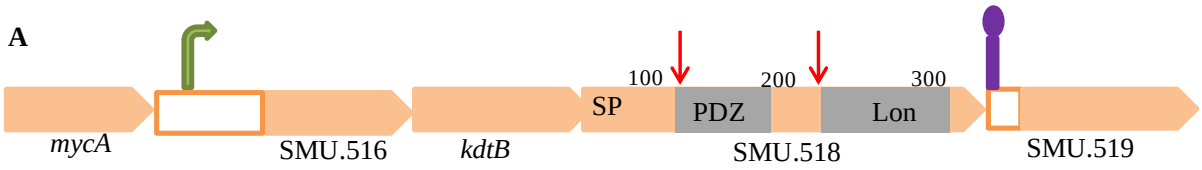
Class	Gene ID	Function/Definition in oralgen database
Class I (No mutacin production)		
1	SMU.1915 (3)	Competence stimulating peptide (CSP) precursor
2	SMU.1916 (2)	Histidine kinase (ComD)
3	SMU.1917 (2)	Response regulator (ComE)
4	SMU.286 (2)	Competence factor transporting ATP-binding/ protein (NlmT)
5	SMU.287 (1)	ABC transport protein ComB, accessory factor for ComA (NlmE)
6	SMU.518 (2)	Conserved hypothetical protein
Class II (6-mm or less mutacin production)		
7	SMU.1128 (1)	Two-component sensor histidine kinase (CiaH)
8	SMU.832 (1)	Hypothetical protein
9	SMU.2137 (1)	Conserved hypothetical protein
10	SMU.611 (1)	ATP-dependent RNA helicase/DEAD family*
11	SMU.1634 (1)	Conserved hypothetical protein, MutT/nudix family*
12	SMU.421 (1)	Translation initiation factor IF-2
13.	SMU.1086 (1)	Thymidine kinase
14	SMU.26 (1)	Fatty acid/phospholipids synthesis protein; fatty acid metabolism (PlsX)*
15	SMU.29 (1)	Phosphoribosylaminoimidazole-succinocarboxamide synthase; Purine metabolism (PurC)*
Class III (8-mm to 10-mm mutacin production)		
16	SMU.241 (1)	Transport and binding proteins; amino acid ABC transporter, ATP-binding protein
17	SMU.1235 (1)	Thiophene and furan oxidation protein; tRNA modification GTPase; Protein synthesis; tRNA and rRNA base modification(ThdF)
18	SMU.502 (1)	Conserved hypothetical protein*
19	SMU.1174 (1)	ATP-dependent DNA helicase* (PcrA)
20	SMU.475 (1)	Conserved hypothetical protein
21	SMU.1914 (1)	Bacteriocin (NlmC)
Class IV (various level of mutacin production)		
22	SMU.121 (7)	MATE efflux family, damage inducible protein, cation efflux pump (DinF)

*indicate the double insertions in the genome of a single mutant

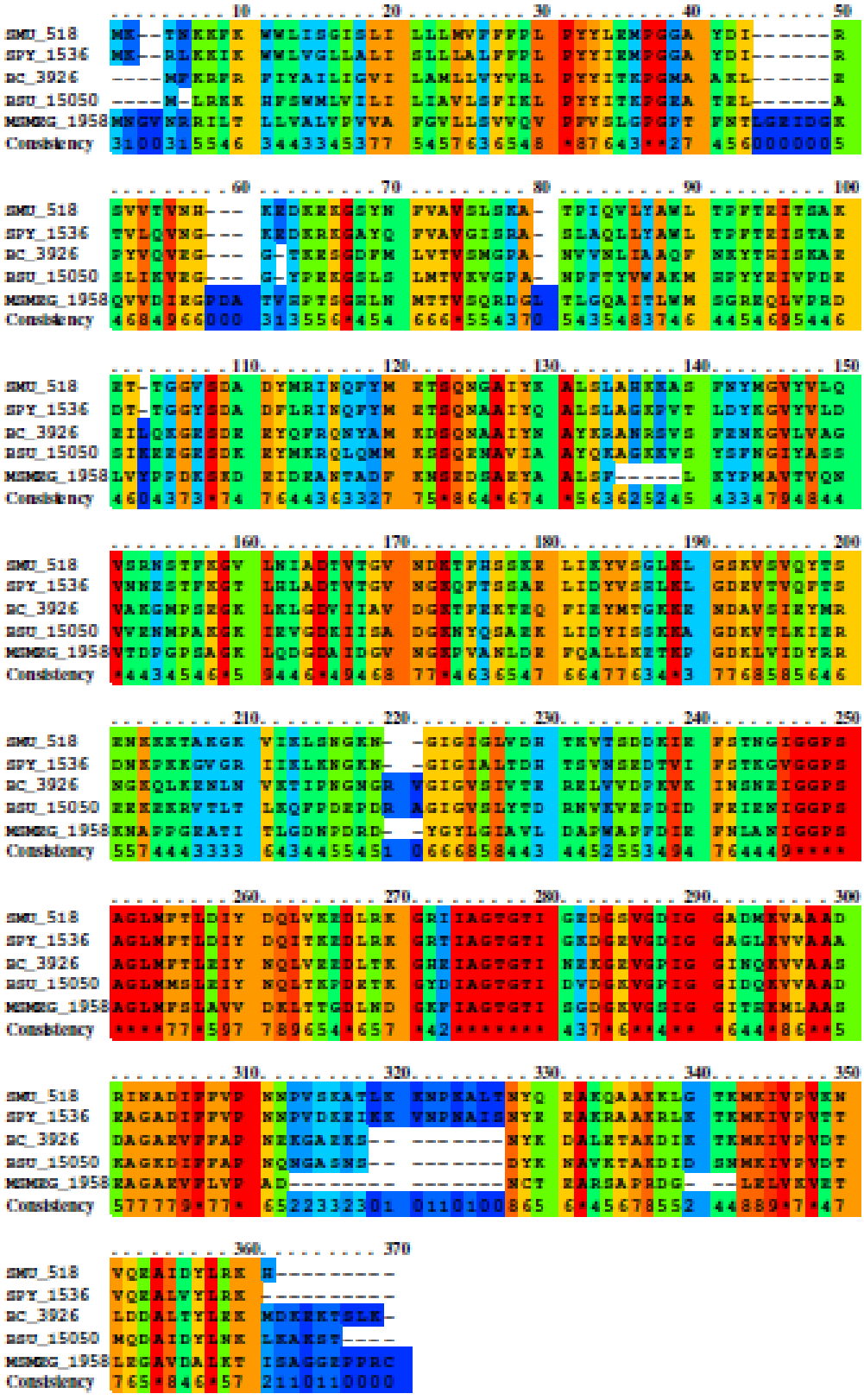
analysis of the intergenic region present upstream of SMU.516 by BPROM software (Softberry, http:// Linux1.softberry.com) indicates that this region contains a strong promoter sequence (-10 box [AGTTATAAT] at position 90 and -35 box [TTGTTA] at position 72). This suggests that SMU.518 is co-transcribed with the SMU.516 and *kdtB*. Analysis of the downstream IGS region of SMU.518 by TransTermHP (http:// transterm. cbcb.umd.edu) program (146) suggests a rho-independent terminator sequence (GTTCTAGA**ATT**TCTAGGAC) just after the stop codon of SMU.518.

SMU.518 is involved in mutacin production: To verify that the observed phenotype of SMU.518 IS*S1*insertion mutation was not due to additional spontaneous mutations elsewhere in the genome, we constructed a marker-less clean deletion of SMU.518 gene in strain UA159 using a Cre-*loxP* based system as described in materials and methods. SMU.518 deletion mutant, IBS-D29, did not show any significant growth difference (data not shown) in compared to wild type UA159. We then tested its ability for mutacin production by deferred antagonism bacteriocin assay as described in methods and materials. As shown in Fig. 21A, deletion of SMU.518 abolished the mutacin activity of both mutacin IV and V. To further confirm that this effect was due to deletion of SMU.518 only, we performed complementation analysis. We cloned DNA fragment containing SMU.518 with ribosome binding site into the *E. coli*/streptococci shuttle vector pIB184Km (127) to generate pIB-D52 and introduced it into IBS-D29. As shown in Fig. 21B, plasmid mediated SMU.518 completely rescued the mutant phenotypes for both NlmAB and NlmC. Taken together, our results suggest that SMU.518 is involved in mutacins production in *S. mutans* UA159.

Fig. 20. (A) Genetic organization of SMU.518. Gene map along with the putative promoter (bent arrow) and terminator (purple bar with a circle on top) is shown. Open reading frames and orientation of the transcriptions are indicated with block arrows. Red vertical arrows indicate the position of IS*S1* insertion. SP indicates the signal peptide. Shaded box represents the respective domain in SMU.518 as mentioned. (B) Multiple sequence alignment of SMU.518 and its orthologous genes from different bacteria by PRALINE multiple sequence alignments tool. Orthologous genes were SPY.1536 (*S. pyogenes*), BC.3926 (*Bacillus cereus*), BSU.15050 (*Bacillus subtilis*) and MSMEG.1958 (*Mycobacterium smegmatis*). Red color indicates fully conserved residue (score 10) and blue color indicates least conserved residue (score 0).

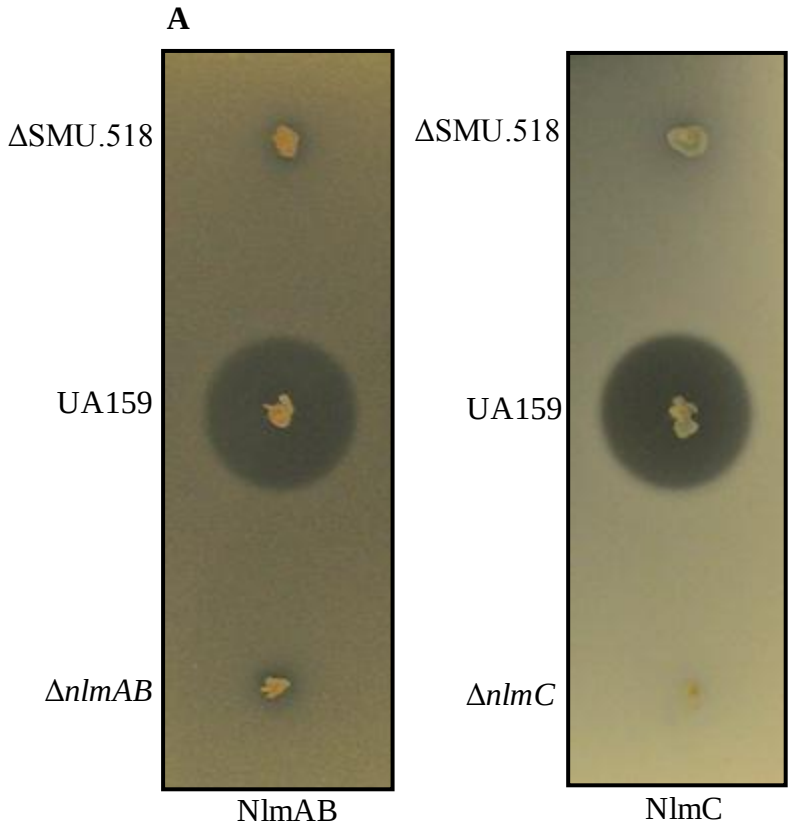

A
100
200
300
SP
PDZ
Lon
mycA
SMU.516
kdtB
SMU.518
SMU.519

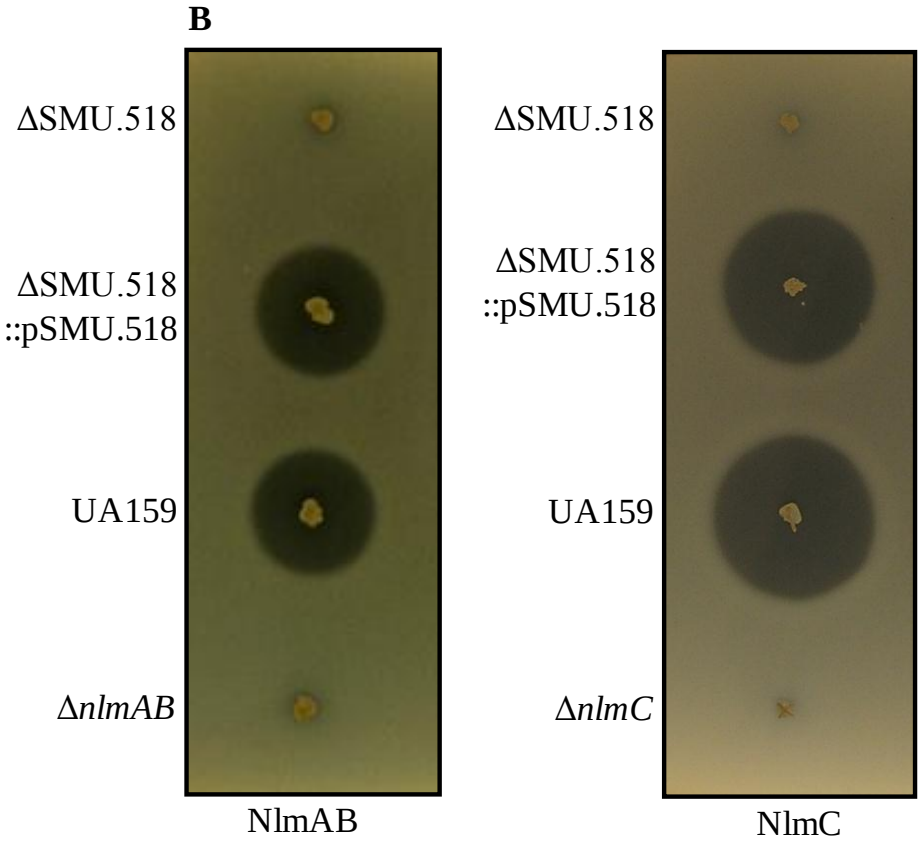
B



(unconserved 0 1 2 3 4 5 6 7 8 9 10 conserved)

Fig. 21. Mutacin IV and V production by SMU.518 mutant. (A) Ability to produce mutacin IV and V by SMU.518 deletion strain. (B) Complementation analysis of the ΔSMU.518 strain (IBS-D29) with plasmid-derived expression of SMU.518. *S. mutans* cultures were stabbed into THY agar and incubated overnight at 37°C under microaerophilic conditions. The plates were overlaid with soft agar containing *S. gordonii* DL-1 (NlmAB) or *Lactococcus lactis* MG1363 (NlmC) as indicator strain. The zones of inhibition of the indicator strains were evaluated after overnight incubation. UA159, deletion strain and indicator bacteria also contain the plasmid, pIB184-Km. A representative antagonism assay from at least three independent experiments is shown.


A
ΔSMU.518
UA159
ΔnlmAB
NlmAB
ΔSMU.518
UA159
ΔnlmC
NlmC


B
ΔSMU.518
ΔSMU.518
::pSMU.518
UA159
Δ*nlmAB*
NlmAB
ΔSMU.518
ΔSMU.518
::pSMU.518
UA159
Δ*nlmC*
NlmC

SMU.518 regulates several putative bacteriocin genes at transcriptional level: To understand the exact molecular basis of defective mutacin phenotype in ΔSMU.518, we tested the expression of two well-known mutacins, mutacin IV (encoded by *nlmA* and *nlmB* genes) and V (*nlmC*) by sqRT-PCR. As shown in Fig. 22A, deletion of SMU.518 caused the reduction of *nlmA* expression below the detection level and the *nlmC* expression was reduced around 15-fold in compare to wild type UA159. To get better understanding of the regulation of mutacin production by SMU.518, we also tested two other mutacins encoding genes (SMU.423 and SMU.1906) expression. Surprisingly, we found that both of them were remarkably down regulated in mutant strain (Fig. 22A). However, plasmid mediated complementation of SMU.518 could fully restore the expression pattern as like wild type strain. Taken together, our semi-quantitative RT-PCR results suggest that SMU.518 works at the transcriptional level and the presence of SMU.518 can activate the transcription of mutacin-encoding genes in *S. mutans* UA159.

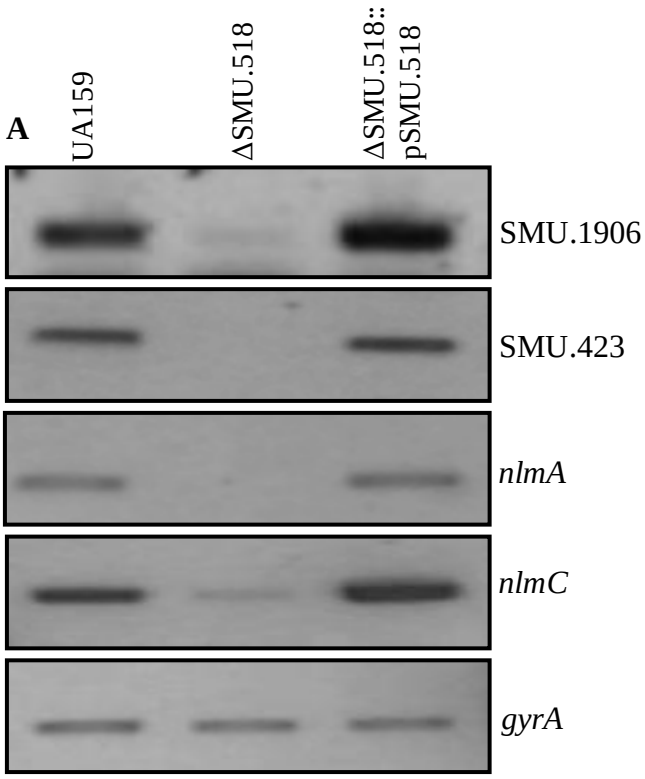
Transcription of *comC* was unaffected in SMU.518 mutant: It is interesting to note that all of these four mutacins encoding genes carry the highly conserved consensus binding sequences for AlgR/AgrA/LytR family transcriptional regulator in their promoter region (Fig. 22C)(196, 299). This conserved region contains 9-bp direct repeats separated by a 12-bp spacer (153, 299). Several previous studies have demonstrated that all of these four genes were CSP-inducible and controlled by the ComDE two-component signal transduction system (158, 168, 237, 299, 320). The ComCDE system is an intraspecies quorum sensing system which is also involved in competence development. Moreover, *comCDE* mutation displayed defective mutacin production in both genotypic and phenotypic level (156, 299). In addition, our initial insertion mutagenesis studies also revealed that SMU.518 mutant produces the same phenotype as like

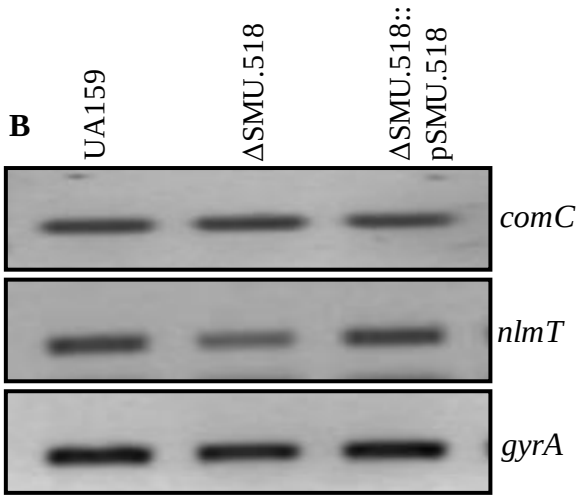
comCDE mutants. All of these facts impelled us to hypothesize that the observed phenotypes were due to defect in CSP expression. To test whether the CSP expression was affected by the mutation of SMU.518, we did semi-quantitative RT PCR of *comC* gene and its putative transporter, *nlmT*. As shown in Fig. 22B, there was no significant change in *comC* transcription as compared to UA159. In contrast, we observed modest reduction (2-fold) in *nlmT* transcription. We also tested the expression of *comDE* and didn't find any significant difference between wild type and mutant strains (data not shown). Altogether, above findings suggest that the expression of CSP, its cognate two-component pathway and CSP-secretion apparatus were not perturbed in the mutant. Therefore, the observed phenotype might be due to a potential post-export role of SMU.518 on CSP. This notion was further supported by the finding that addition of synthetic CSP was not able to restore the Gus activity in reporter strain, IBS-D37 (ΔSMU.518::P*nlmA-gus*) as compared to wild type (IBS-D5), which strikingly stimulated Gus activity upon addition of external CSP (Fig. 23A).

Synthetic CSP cannot stimulate the competence development in SMU.518 mutant:

Development of competence in *S. mutans* is known for some strains, although the frequency was less than other streptococci (172, 178). In *S. mutans*, alternative sigma factor, SigX, is absolutely required for the development of competence for natural transformation (191). Nonetheless, presence of ComCDE quorum sensing system is required for optimum transformation phenotype (172, 191, 284). External addition of synthetic CSP has been shown to remarkably augment the transformation efficiency of *S. mutans* (6, 9, 156, 172, 191, 221, 237, 239, 284). Synthetic CSP can up regulate the *sigX* (*shp61*) expression up to 15-fold which might be the reason for enhanced transformation phenotype (168). To prove the potential post-export role of SMU.518 on CSP, we carried out the transformation assay in presence or absence of synthetic CSP. To our

Fig. 22. Semi-quantitative RT PCR analysis of mutacins and related genes. (A) sqRT-PCR of *nlmA, nlmC,* SMU.423 and SMU.1906. (B) sqRT-PCR of *comC* and *nlmT*. Total RNA was harvested from the indicated strains and subjected to cDNA synthesis. Five nanograms of cDNA from each strain were used for semi-quantitative RT PCR. The *gyrA* gene was included to ensure that equal amounts of RNA were used for all reactions. The data are representative of RT-PCR analyses resulting from at least two different RNA isolations. (C) Sequence conservation of upstream regions from four bacteriocins encoding genes. General consensus binding sites for AlgR/AgrA/LytR family are shown as capital letters and highlighted with underlines (299).


A
UA159
ΔSMU.518
ΔSMU.518::
pSMU.518
SMU.1906
SMU.423
nlmA
nlmC
gyrA


B
UA159
ΔSMU.518
ΔSMU.518::
pSMU.518
comC
nlmT
gyrA

C

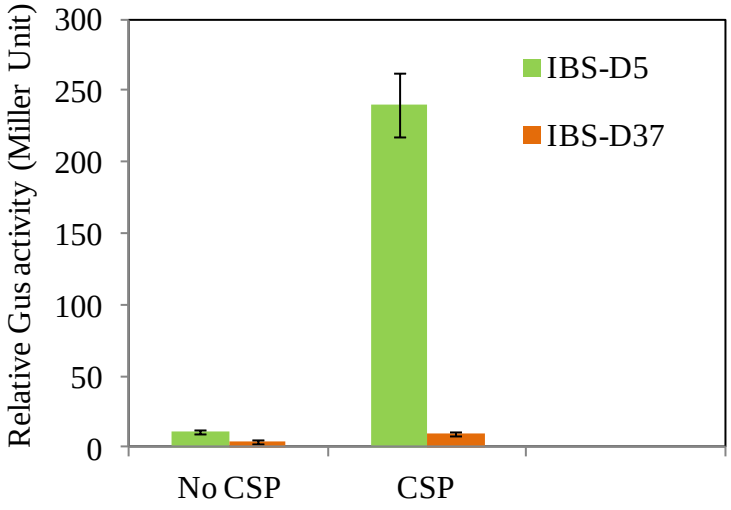
nlmA	ACCGTTTAAgacaaaatagctACCATTTAG
nlmC	ACCGTTTAGgacaaaatagctACCATTTAG
SMU.423	ACCGTTTAGgacaaaatagctACCGTTTAG
SMU.1906	ACCGTTTAGgacaaaatagctACCGTTTAG

surprise, we found significantly reduced level of transformation phenotypes in repeated experiments in mutant background as compared to UA159 and complemented strain (Fig. 23B). Nevertheless, mutant bacteria were transformable without external CSP, although the efficiency was lower than the wild type. As shown in Fig. 23B, exposure to exogenous CSP was inept to up regulate the transformability in ΔSMU.518. This basal level of transformation phenotype is the hall mark of ComCDE mutation (6, 172, 191, 221, 237). As expected, we observed ~100-fold increased transformability in wild type and SMU.518 complemented strain upon addition of synthetic CSP. This study further substantiated the idea that post-export association of CSP and SMU.518 occurs outside the cell and SMU.518 works on ComCDE pathway.

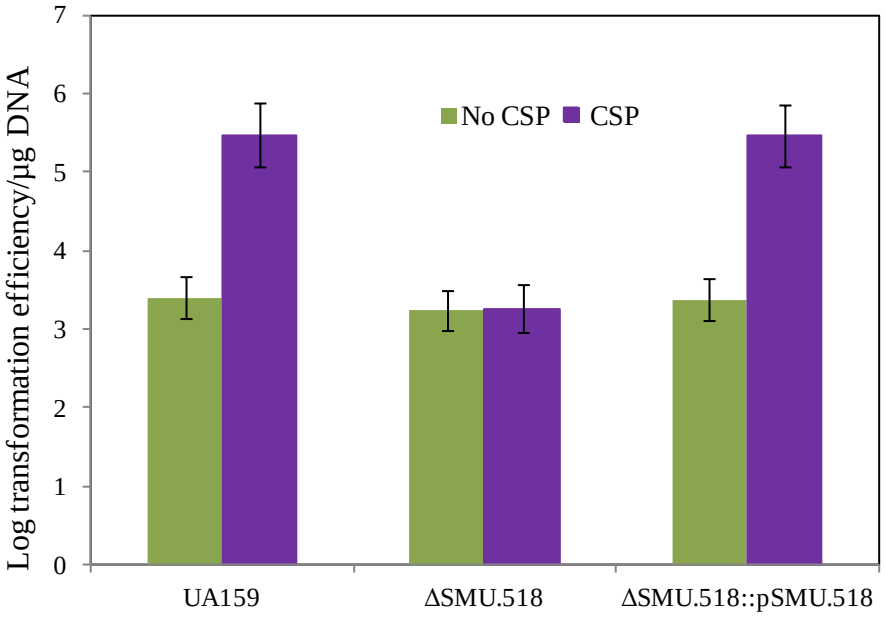
Synthetic CSP undergoes proteolytic maturation by SMU.518: Because of the lack of CSP-responsive *nlmAB* induction and development of competence, due to its predicted Lon protease domain with extracellular localization (membrane-associated), it is reasonable to hypothesize that SMU.518 is involved in post-export maturation of CSP and synthetic CSP (21-residue) cannot function in absence of SMU.518. This idea is further supported by the fact that CSP is present as 18-residue form in culture supernatant and 18-residue was shown to be more potential than 21-residue form (239). Moreover, its orthologous gene in group A streptococcus (GAS) is involved in binding to human extracellular matrix and antibody to it was protective in mice (91), which further confirm its predicted surface localization. To investigate whether SMU.518 can process synthetic CSP outside the cell and make it more potential, we first separated the cells from culture supernatants and incubated 21-residue CSP with the cell pellet of UA159, ΔSMU.518 and SMU.518 complemented strain as mentioned in materials and methods and tested the P*nlmA-gus* gene expression in the reporter strain, IBS-D37(ΔSMU.518:: P*nlmA-gus*). Surprisingly, we found that CSP processed with UA159 and complemented strain could

Fig. 23. Effect of synthetic CSP on expression of P*nlmA-gus* and transformation efficiency. (A) P*nlmA-gus* expression from the reporter strains, IBS-D5 (P*nlmA-gus* insertion in SMU.1405 locus) and IBS-D37 (ΔSMU.518:: P*nlmA-gus*). Strains were grown in THY broth at 37^0C and harvested at 0.9 OD of 595 nm. When needed, CSPs (200 nM) was added to the actively growing culture (0.2 OD at 595 nm). The values were normalized with Gus activity obtained from IBS-D44 (*gusA* gene without any promoter inserted in SMU.1405). Experiments were repeated at least three times and the mean values are shown. (B) Linear DNA (SMU.198::Em^r) was transformed into indicated strains after grown up to 0.15 OD at 595 nm and in THY medium with 10% horse serum. CSP was added 10-minutes prior to addition of DNA when needed. 2-hr after incubation at 37^0 C, bacterial strains were plated onto THY agar plates with respective antibiotics. Antibiotic resistant colonies were counted after 3-days of incubation. Total counts were measured by plating onto THY agar plate without antibiotic. Transformation efficiency was measured as log transformants/10^9 cfu/µg of DNA. The data shown are means ± standard deviations (error bars) of at least three independent experiments.

A


Relative Gus activity (Miller Unit)
300
250
200
150
100
50
0
IBS-D5
IBS-D37
No CSP
CSP

B


Log transformation efficiency/µg DNA
7
6
5
4
3
2
1
0
No CSP
CSP
UA159
ΔSMU.518
ΔSMU.518::pSMU.518

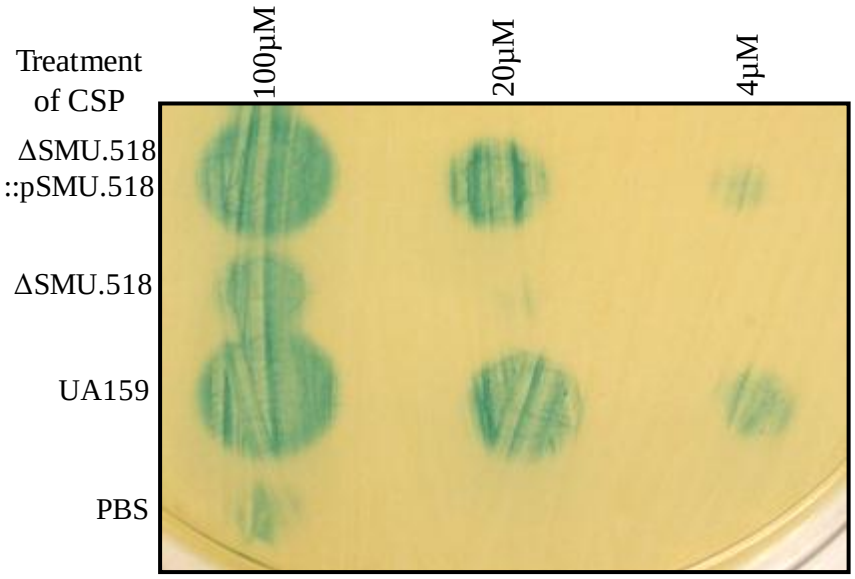
turn on Gus phenotype prominently on THY-X-gluc plate at much lower concentration than the CSP treated with ΔSMU.518 cells, where Gus phenotype was turned on to a limited extent only with undiluted CSP (Fig. 24A). On the other hand, control CSP (incubation with PBS) could produce very little phenotype on THY-X-gluc plate. In addition, we performed quantitative Gus assay with the CSPs treated with different strains. In consistent with our qualitative Gus assay, we also found that CSP processed with UA159 or SMU.518 complemented strain could turn on *gus* gene expression efficiently at lower concentration (50 nM) in compared to CSP treated with the mutant or PBS (Fig. 24C). This study confirmed that Lon protease of SMU.518 is responsible for processing of synthetic CSP and the processed CSP is more potential in *gus* gene expression in compare to intact CSP. The increased activity of ΔSMU.518-treated CSP in compared to untreated CSP or the very little activity of untreated CSP can be attributed to the non-specific proteolytic activities of other proteases present on the surface of *S. mutans* cells as like processing of CSF (competence and sporulating factors) in *Bacillus subtilis* where different surface proteases impart in generation of pentapeptide competence and sporulating factor, CSF (163). Indeed, another surface localized serine protease (HtrA) of *S. mutans* UA159 has been reported to be involved in regulation of competence development (5).

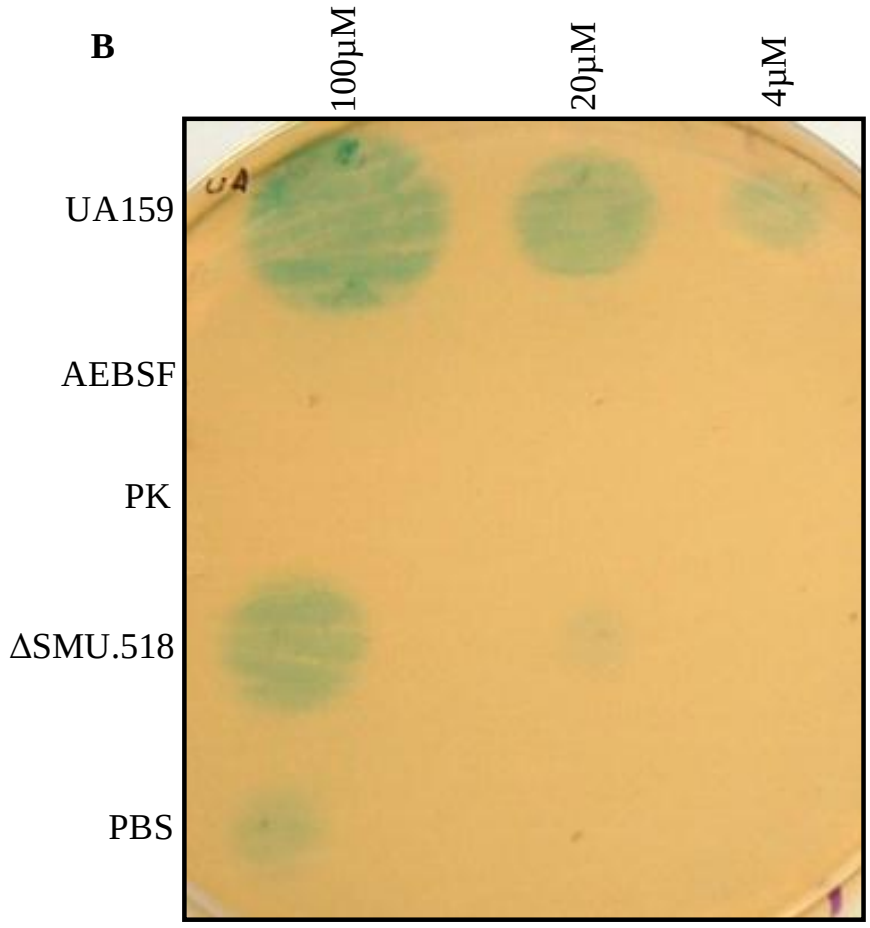
SMU.518 is localized outside the membrane and contains a Lon-like serine protease: Lon is a member of AAA+ (ATPases associated with a variety of cellular activities) super family enzymes which is involved in protein quality control system in all domains of life (bacteria, archaea and eukaryotes), thereby maintaining cellular homeostasis (77, 92, 264). They can degrade specific short lived regulatory proteins and defective or abnormal proteins inside the cells (33). All Lon proteases contain a serine-lysine dyad in their active site (33, 264, 300). Lon proteases from *E. coli* and *Bacillus subtilis* have been characterized (47, 255, 283). Whereas

most of the Lon proteases function in the cytosol, Lon protease of *Bacillus subtilis* found to be expressed in the forespore compartment (274). Archaeal Lon protease shown to be membrane bound and has ATP-independent activity toward unfolded proteins and ATP-dependent activity toward folded proteins (92). Predicted structure of SMU.518 suggests that it has signal peptide at the N-terminal region along with a transmembrane region and a C-terminal Lon protease domain without any canonical ATP binding motif. As the Lon proteases contain a serine residue in its active site and because of its predicted surface localization, treatment with serine protease inhibitor or proteinase K should inhibit the SMU.518 mediated CSP cleavage and we should not detect the Gus phenotypes on X-gluc plate with the reporter strain, IBS-D37. To test this hypothesis, we first treated the cells (UA159) with serine protease inhibitor, AEBSF[4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride] (230) or proteinase K and separated the culture pellet from the supernatant and washed twice to remove residual AEBSF and proteinase K, followed by incubation with synthetic CSP without any external ATP. In consistent with our hypothesis, we found that AEBSF or proteinase K treatment were able to completely abolish the SMU.518-mediated CSP cleavage (Fig. 24B) and as a result, we did not see any Gus phenotype on X-gluc plate with reporter strain, IBS-D37. In contrast, control CSP (treated with only UA159 cell pellet) exhibited expected Gus phenotype on X-gluc plate with reporter strain, IBS-D37. Interestingly, very little Gus activity associated with only CSP as shown in earlier experiment (Fig. 24A) was also absent with the treatment of AEBSF or proteinase K, which indicates that residual Gus activity associated with ΔSMU.518 was indeed the result of very limited CSP processing by a non-specific serine protease other than SMU.518. This study also confirmed the surface localization of SMU.518 as none of the AEBSF or proteinase K is permissible to cell membrane. Altogether, these studies suggest that SMU.518 is an ATP-

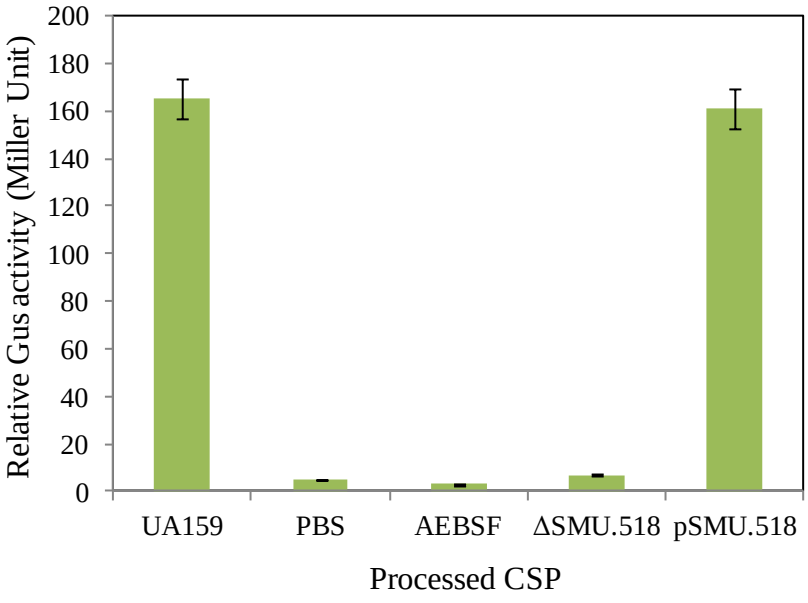
Fig. 24. Expression of P*nlmAB-gusA* from the reporter strain, IBS-D37 (ΔSMU.518::P*nlmA-gus*) with processed CSPs. (A) Indicated bacteria were grown overnight and pelleted. After washing with PBS, CSP was added at a concentration of 120 µM and incubated at 37^0C for an hour. Cells were separated from the supernatant containing the CSP and the processing of CSP was checked by spotting at different concentration onto X-gluc plate seeded with the reporter strain IBS-D37. (B) Effect of AEBSF on CSP processing. (C) Gus assay was carried out as described in Fig. 23A, except the addition of processed CSPs at a concentration of 50 nM. The data shown are means ± standard deviations (error bars) of at least three independent experiments. AEBSF is a specific serine protease inhibitor. PK, proteinase K.

A


Treatment of CSP
100μM
20μM
4μM
ΔSMU.518 ::pSMU.518
ΔSMU.518
UA159
PBS


B
100µM
20µM
4µM
UA159
AEBSF
PK
ΔSMU.518
PBS

C


Relative Gus activity (Miller Unit)
200
180
160
140
120
100
80
60
40
20
0
UA159
PBS
AEBSF
ΔSMU.518
pSMU.518
Processed CSP

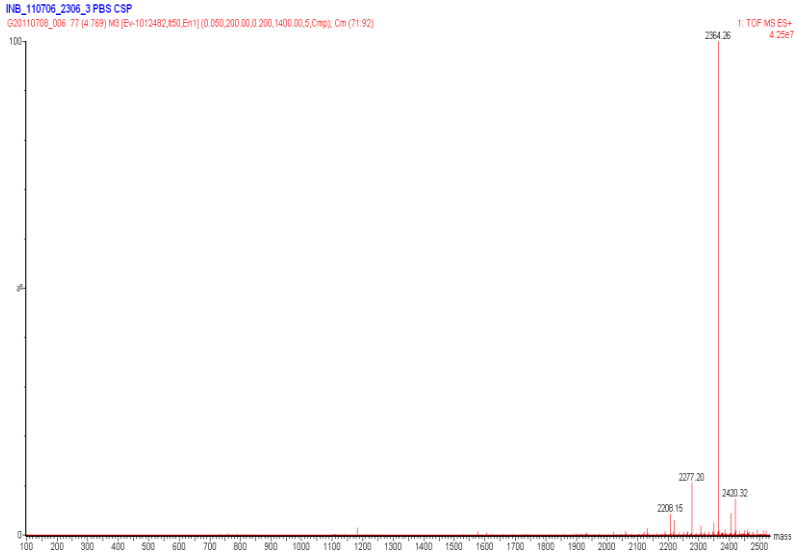
independent Lon protease which contains a serine residue in the active site and localized on the surface of *S. mutans*.

SMU.518-mediated cleavage removes three C-terminal residues of 21-residue CSP: It was previously proposed that three C-terminal residue must be cleaved to generate the functional CSP and induce the competence development in *S. mutans* (239). Our reporter assay also suggested that CSP is processed by SMU.518. To further confirm that SMU.518 cleaves the CSP and produces functional or more potential CSP, we did mass spectrometry to identify the functional form of CSP. Three samples were prepared to verify the post-export cleavage of CSP by SMU.518, such as (1) 21-residue CSP plus PBS as control; (2) 21-residue CSP plus UA159 cells; (3) 21-residue CSP plus ΔSMU.518 cells. After incubation for 1-hr and separating the cell pellets by repeating centrifugation, the samples were subjected to MALDI-TOF ESI mass spectrometry as described in methods and materials.

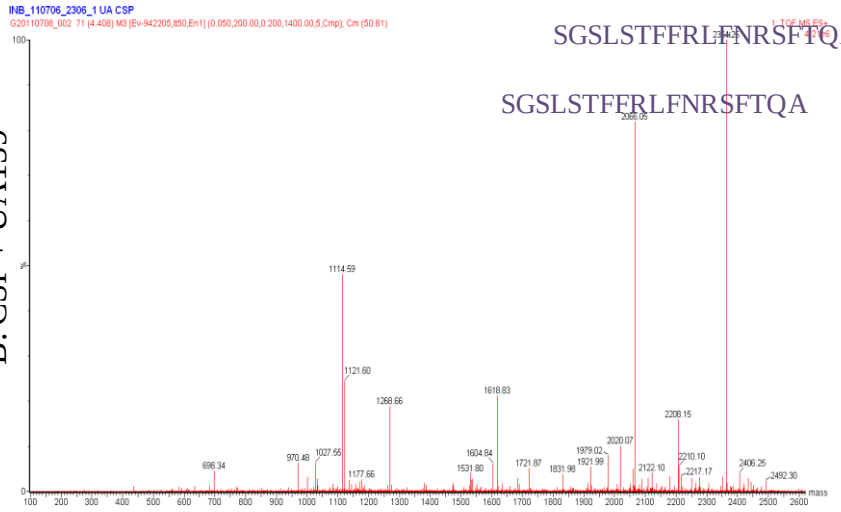
As shown in Fig. 25, only 21-residue CSP was identified with a single major peak at 2364-Da in control CSP treatment. Sample treated with UA159 generated a single major peak at 2066-Da which corresponds to 18-residue CSP without the three C-terminal residues along with several truncated versions and one major peak of 21-residue CSP. Interestingly, sample treated with ΔSMU.518 also produced all of the truncated versions of CSP except 18-residue CSP. Thus 18-residue CSP is the unique cleavage product of SMU.518. Taken together, our mass spectrometry data suggested that 18-amino acids version of CSP is the functional form to activate ComDE pathway and SMU.518 is essential for maturation of CSP. Other small fragments might be due to non-specific cleavage of CSP by other surface proteases. In fact, *S. mutans* contains five other membrane associated or extracellular protease such as, *mutP* (serine protease), SMU.648, SMU.1438, SMU.1929 (*htpX*) and SMU.2163 (*htrA*, serine protease). We

Fig. 25. Mass spectrometry of processed CSP. Three samples were prepared to verify the post-export cleavage of CSP by SMU.518, such as (A) 21-residue CSP plus PBS as control; (B) 21-residue CSP plus UA159 cells; (C) 21-residue CSP plus ΔSMU.518 cells. After incubation for 1-hr and separating the cell pellets by repeating centrifugation, the samples were subjected to MALDI-TOF ESI mass spectrometry as described in methods and materials.

A. CSP + PBS



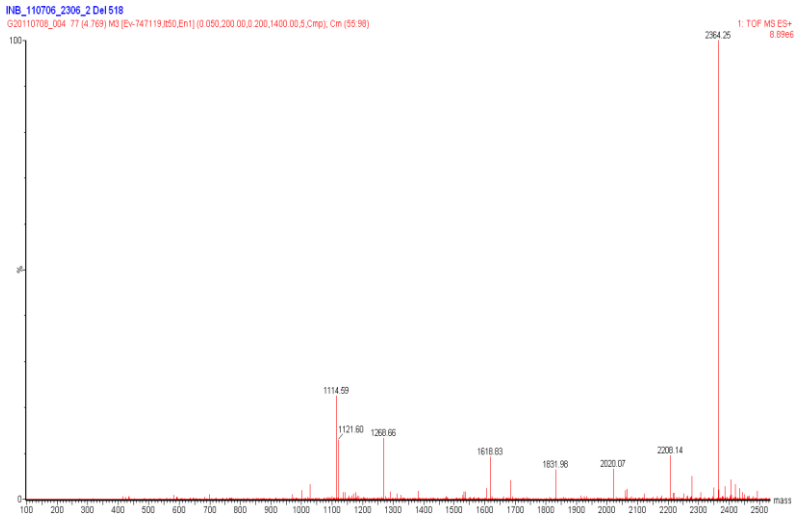
B. CSP + UA159



SGSLSTFFRLFNRSFTQALGK

SGSLSTFFRLFNRSFTQA

C. CSP + Δ SMU.518



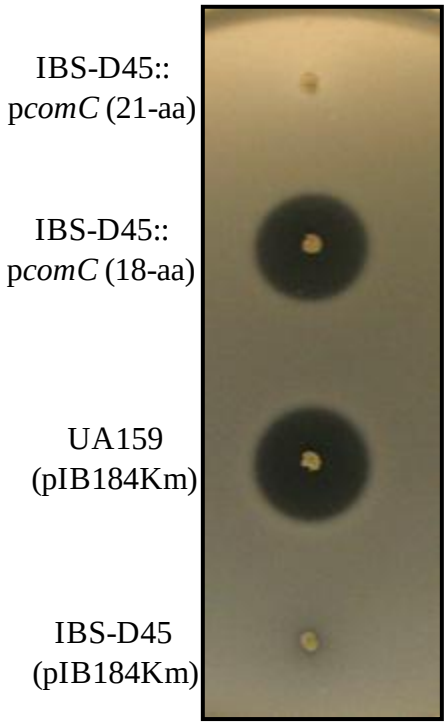
presume that at least one of these two serine proteases can non-specifically cleaves the CSP to various fragments and speculate that anyone of the fragments can activate the ComDE pathway for P*nlmAB* gene expression slightly when reaches a certain concentration as we observed in our previous experiments. HtrA has been shown to be associated with competence development and involved in processing of extracellular proteins in *S. mutans* (5, 66).

18-amino acids but not the 21-amino acids is the functional form of CSP: If 18-residue CSP is the functional form of CSP, then plasmid mediated expression of the N-terminal 18-residue without the three C-terminal residues should restore the mutacin phenotype in ΔSMU.518 Δ *comC* double mutations background. To demonstrate that 18-residue CSP is the functional form, we cloned 18- residue CSP (excluding the nucleotide sequences for last three amino acids) and 21-residue CSP into shuttle vector pIB184Km and introduced it into the double mutant, IBS-D45 (ΔSMU.518 Δ *comC*), which was constructed by Cre-*loxP* based clean mutation system. As expected, plasmid carrying the nucleotide sequences for first 18-amino acids could restore the mutacin phenotype in double mutation background (Fig. 26A). However, the mutant carrying the full length *comC* gene could not alter the mutacin phenotype of double mutant, IBS-D45.

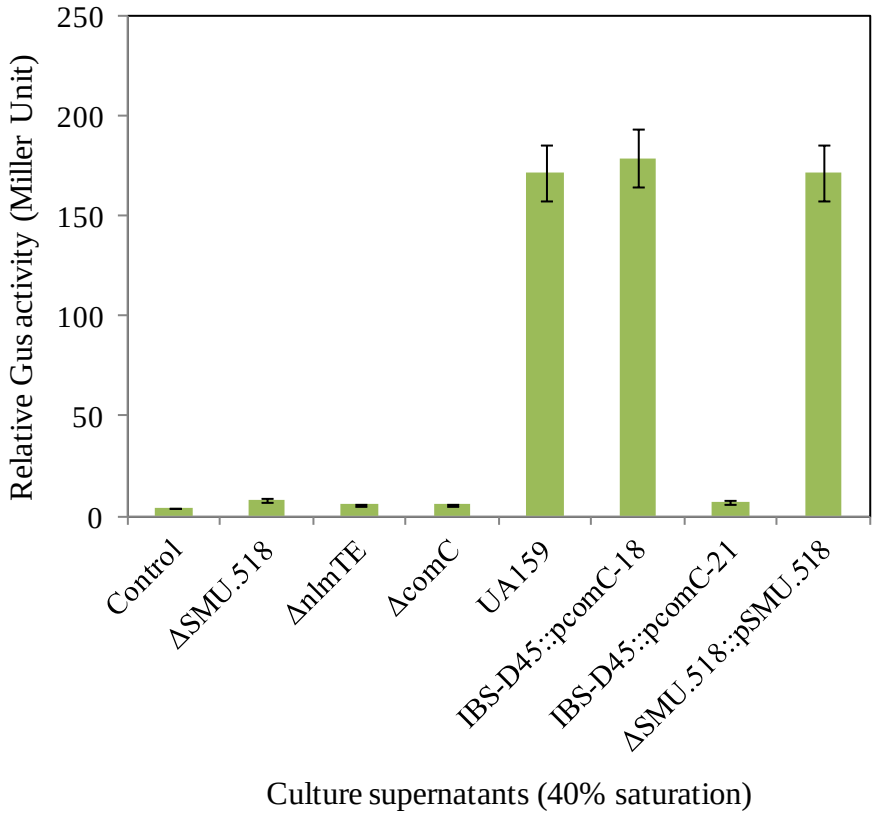
To further confirm that 18-amino acids is the functional form of CSP, we added the culture supernatants (40% saturation with ammonium sulfate) of IBS-D45 carrying either 18- or 21- amino acids version of *comC* to the actively growing culture of reporter strain, IBS-D46 (ΔSMU.518 Δ*comC*::P*nlmA-gus*) and measured the Gus activity. As shown in Fig. 26B, culture supernatants of wild type UA159, IBS-D45 with 18-residue form of CSP and ΔSMU.518:: SMU.518 showed the similar Gus phenotype. However, IBS-D45 with 21-residue form of CSP could not restore the mutant Gus phenotype. We used three different controls such as culture

Fig. 26. Mutacin production by 18-amino acids CSP. (A) NlmAB activity of 18-amino acids *comC* was tested as like Fig. 21. 18-CSP and 21-CSP versions of *comC* were cloned into the vector, pIB184Km and transformed into the double mutation background (IBS-D45). Each experiment was performed at least three times and a representative plate is shown. (B) Gus assay was performed as mentioned in Fig. 23A, except the addition of CSP, culture supernatants (40% ammonium sulfate precipitation) was added to the actively growing culture of IBS-D46 (ΔSMU.518 Δ*comC::*P*nlmA-gus*). The data shown are means ± standard deviations (error bars) of at least three independent experiments.

A


IBS-D45::
p*comC* (21-aa)
IBS-D45::
p*comC* (18-aa)
UA159
(pIB184Km)
IBS-D45
(pIB184Km)

B


250
200
150
100
50
0
Relative Gus activity (Miller Unit)
Control
ΔSMU.518
ΔnlmTE
ΔcomC
UA159
IBS-D45::pcomC-18
IBS-D45::pcomC-21
ΔSMU.518::pSMU.518
Culture supernatants (40% saturation)

supernatants from Δ*nlmTE*, Δ*comC* and ΔSMU.518 along with the mock (PBS) control for better representation of the reporter assay. Taken together, our data suggest that 21-amino acids CSP is non-functional and cleavage of three C-terminal residues is required to generate functional CSP.

S-235 and K-280 form the catalytic dyad of *S. mutans* Lon protease: SMU.518 and its orthologous genes are widely present among bacteria. Multiple sequence alignment suggests that they are highly conserved among the species from streptococci to mycobacterium (Fig. 20). A typical Lon protease contains an N-terminal substrate recognition domain, an ATPase domain for substrate unfolding and translocation to the protease chamber and a protease domain at the C-termini (77, 264). Lon proteases function as an oligomeric complex as like other AAA+ proteases in the cytosol of the cell (77). Archaeal Lon proteases contain a transmembrane domain present in between walker A- and walker B- motifs of ATPase domain which attaches the protein to the cytoplasmic side of the membrane (263). A non-canonical Lon protease has been characterized from Birnavirus which does not have any ATPase domain (26). Lon proteases contain Ser-Lys dyad in their catalytic center, which are separated by 40-45 amino acids and does not show any similarity with the classical catalytic Ser-His-Asp triad of serine proteases (33, 264). Lon protease of SMU.518 is unique as it does not have any ATPase domain and it is localized outside the membrane with an N-terminal transmembrane sequence. Moreover, its size (345-residue) is comparatively smaller than LonA (581-1133 residue) and LonB (621-1127 residue) (264). Nevertheless, it contains a PDZ domain which has the ability to bind the peptides and lipids (72, 290) .

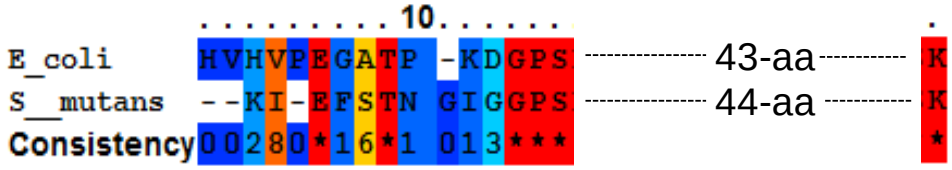
To characterize the Lon catalytic sites, we performed multiple sequence alignment of Lon signature sequences from *E. coli* to SMU.518 and its orthologous genes from various gram-positive bacteria (Fig. 27A & B). We found that Lon signature is well conserved across the

species. Sequence comparison revealed that S-235 and K-280 are the homologue of S-679 and K-722 of *E.coli* Lon protease. Three dimensional structure prediction by multiple threading alignment algorithm [I-TASSER (265)] suggests that these two residues are closely localized and have the potential to form hydrogen bond (Fig. 27C). To verify the function of these two residues as possible catalytic dyad in SMU.518, we did site-directed mutagenesis by substituting S235A and K280A and investigated the effect of these mutations in CSP processing and mutacin production. Substitution of these residues did not change the stability of protein as shown in Fig. 27D.To elucidate the effect of these mutations, we introduced them into ΔSMU.518 mutant and investigated for mutacin production and CSP processing. As shown in Fig. 28A, both of the mutations caused the abrogation of mutacin production as compared to wild type strain. To understand further, we tested the processability of CSP with these mutants SMU.518. In accordance with our mutacin assay, we observed that S235A or K280A mutations were unable to process the synthetic CSP into functional form (Fig. 28B). This result underscores that S-235 and K-280 have key role in Lon protease domain of SMU.518. Taken all of these facts under consideration, we infer that S-235 and K-280 form the catalytic dyad in SMU.518.

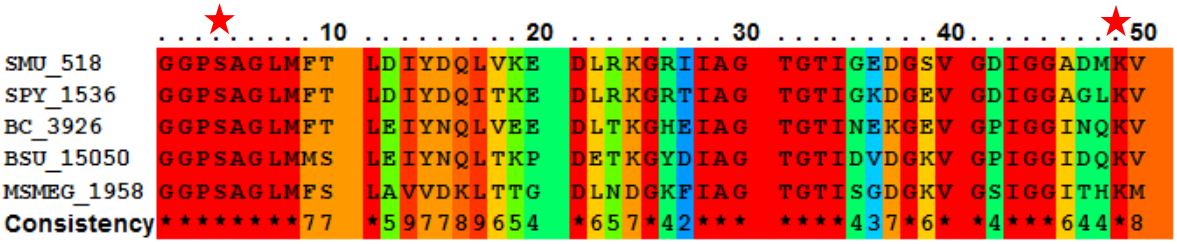
CSP is essential for mutacin production: Results presented above suggested that competence stimulating peptide (CSP) is the sole regulator for mutacins production in *S. mutans* UA159. Our initial insertional mutagenesis screening also revealed that ComCDE system is essential for bacteriocin production (Table-11).This finding is in agreement with a previous report (299), which showed that mutation of any of the components of ComCDE abolished bacteriocin production. To assess the involvement of *comC* in mutacin production, we constructed a marker less clean *comC* deletion mutant and tested it against the indicator bacteria

Fig. 27. Characterization of the Lon active residues in SMU.518. (A) Alignment of Lon signature sequences from *Escherichia coli* (264) and SMU.518. (B) Multiple sequence alignment of Lon domains of SMU.518 and its orthologous genes from different bacteria by PRALINE multiple sequence alignments tool. Orthologous genes were SPY.1536 (*S. pyogenes*), BC.3926 (*Bacillus cereus*), and BSU.15050 (*Bacillus subtilis*) and MSMEG.1958 (*Mycobacterium smegmatis*). Red color indicates fully conserved residue (score 10) and blue color indicates least conserved residue (score 0). Red star indicates the possible catalytic serine and lysine required for Lon protease activity. (C) Predicted three-dimensional structure of SMU.518 as determined by I-TASSER. (D) Western blotting of whole cell lysates to detect the stability of SMU.518 mutants (S235A and K280A). Protein lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting was carried out using anti-HA monoclonal antibody as the primary antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody. For each sample, loading volume was equal to 150 µg of whole cell lysate for HA-tagged SMU.518 detection and 30 µg of whole cell lysate for enolase detection.

A

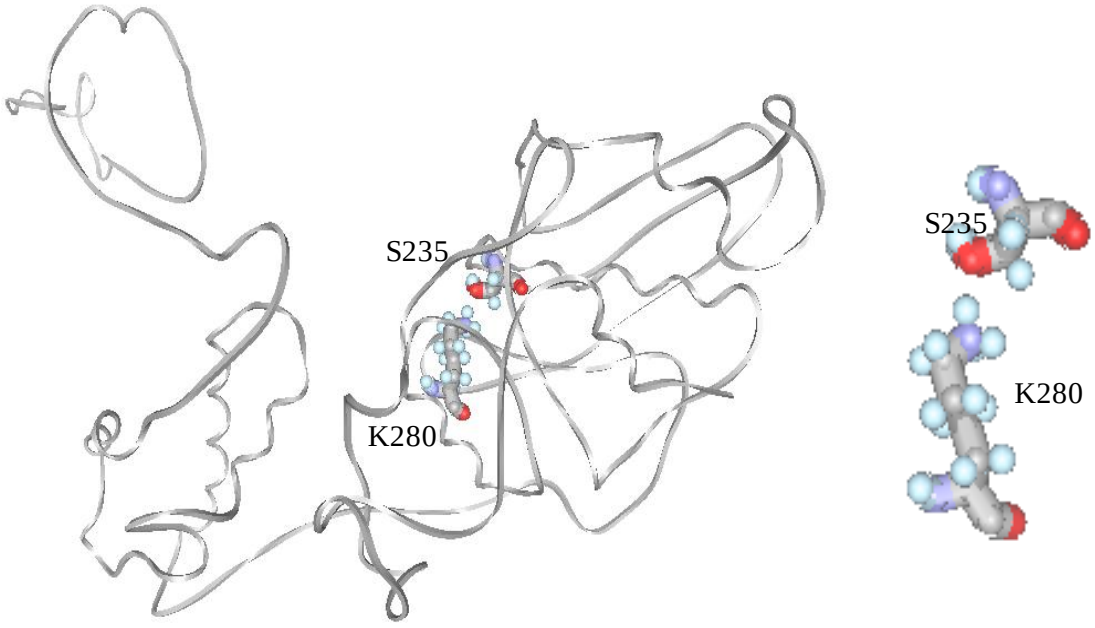


B



(unconserved 0 1 2 3 4 5 6 7 8 9 10 conserved)

C



D

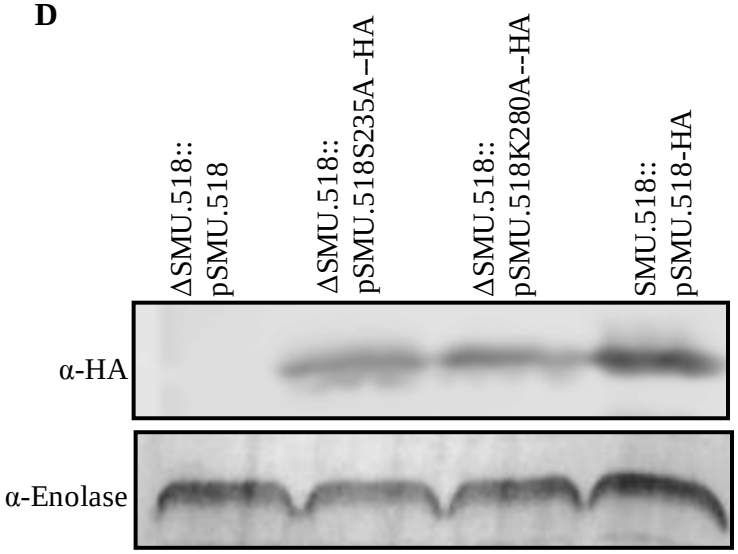
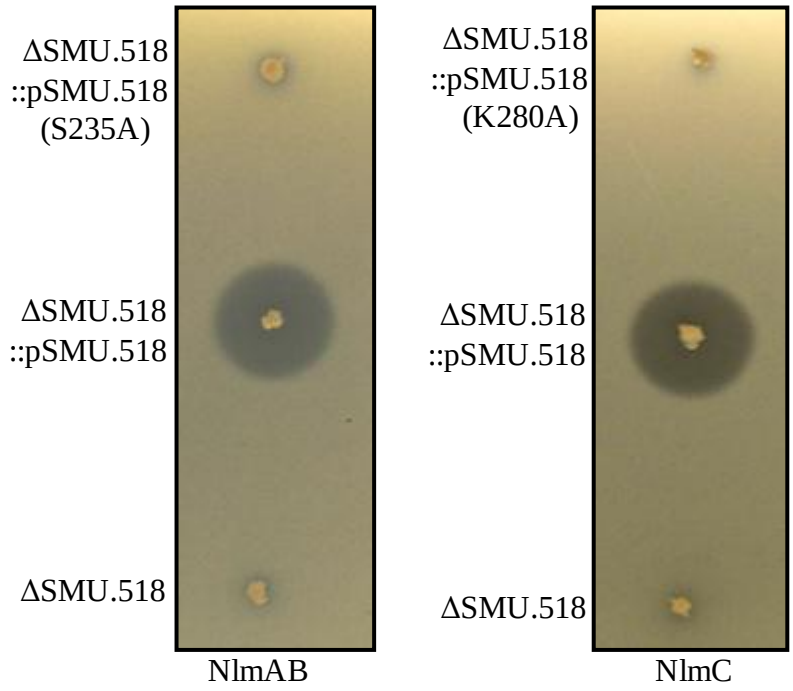
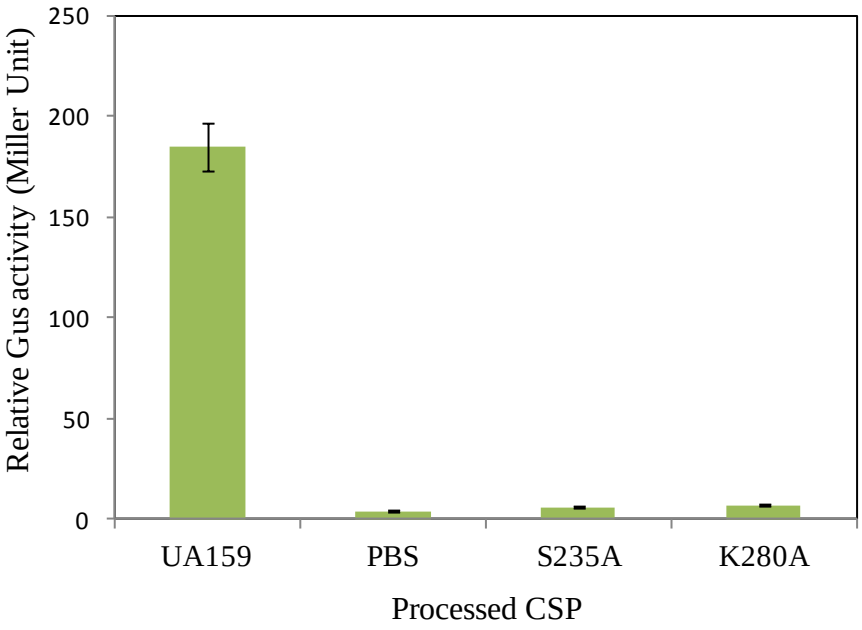

ΔSMU.518::
pSMU.518
ΔSMU.518::
pSMU.518S235A–HA
ΔSMU.518::
pSMU.518K280A--HA
SMU.518::
pSMU.518-HA
α-HA
α-Enolase

Fig. 28. Mutacins production by S235A and K280A mutants. (A) NlmAB and NlmC activity of the indicated mutants. Deferred antagonism bacteriocins assay was done as like Fig. 21. Each experiment was performed at least three times and a representative plate is shown. (B) Gus assay was performed as mentioned in Fig. 23A. CSP, treated with different mutants, was added to the actively growing culture of IBS-D37. The data shown are means ± standard deviations (error bars) of at least three independent experiments.

A


ΔSMU.518
::pSMU.518
(S235A)
ΔSMU.518
::pSMU.518
ΔSMU.518
NlmAB
ΔSMU.518
::pSMU.518
(K280A)
ΔSMU.518
::pSMU.518
ΔSMU.518
NlmC

B

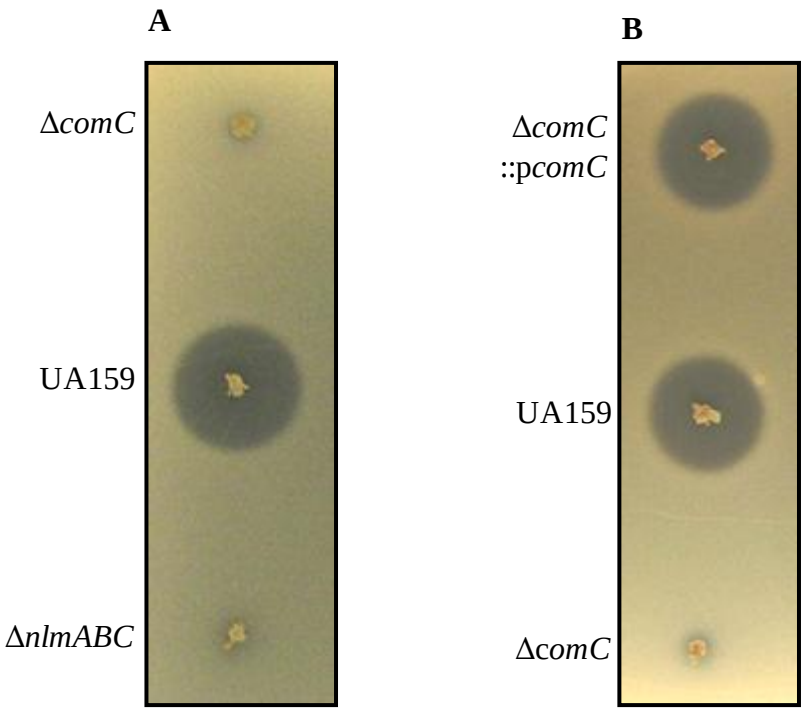

Relative Gus activity (Miller Unit)
250
200
150
100
50
0
UA159
PBS
S235A
K280A
Processed CSP

for mutacin IV and V. In consistent with the previous findings, we did not see any mutacins activity in mutant background (Fig. 29A). In addition, plasmid mediated expression of *comC* could restore the mutacins phenotype for both mutacin IV and V (Fig. 29B). Furthermore, external addition of synthetic CSP could restore the Gus phenotype of the reporter strain Δ*comC*:: P*nlmA-gus* (Fig. 29C). Together with the previous reports, our present results suggest that CSP is essential for induction of mutacin genes expression in *S. mutans* UA159.

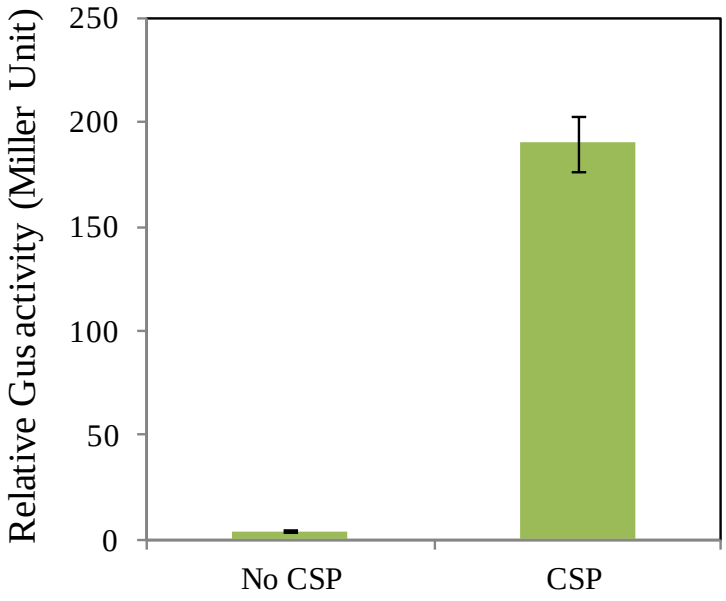
NlmTE (ComAB) is the transporter of CSP: Based on the sequence homology and transformation phenotype, it was previously proposed that CslAB (encoded by SMU.1897 to SMU.1900) functions as competence related secretion (241) and transporter of CSP in *S. mutans* (103). However, 'com' system of *S. mutans* is strikingly similar to 'blp' system of *S. pneumoniae* (187). Co-ordinated expression of mutacin production and competence development has been demonstrated in *S. mutans* UA159 (156) and *comC* mutation had little or no influence on transformation phenotype (5, 172, 221), but diminished the mutacin production (299).Thus, regulation of bacteriocin was proposed to be the primary function of CSP (187).

Since CSP (encoded by *comC*) is essential for mutacins production in *S. mutans* UA159, then mutation of any of the related genes such as, transporter of CSP and the cognate two-component system (ComDE) should also abrogate mutacin production in this organism. To clarify this, we constructed *nlmTE* and *cslAB* deletion mutants and carried out mutacin assay against the indicator bacteria. In accordance with our hypothesis, we found that deletion of *nlmTE* caused the complete abrogation of NlmAB production; however, there was no discernible change in mutacin production in case of *cslAB* mutation (Fig. 30A). Our result is in agreement with two previous results where deletion of *nlmTE* blocked mutacin production in *S. mutans* UA159 (103, 237, 299), but *cslAB* mutation did not produce any mutacin phenotype (103).

Fig. 29. Mutacin production by *comC* mutant. (A) NlmAB activity of *comC* deletion strain. Experiment was done as like Fig. 21. (B) NlmAB activity of the complemented strain. Each experiment was performed at least three times and a representative plate is shown. (C) Gus assay was performed as mentioned in Fig. 23A, except the reporter strain, IBS-D40 (ΔSMU.1915:: P*nlmA-gus)*. The data shown are means ± standard deviations (error bars) of at least three independent experiments.


A
ΔcomC
UA159
ΔnlmABC
B
ΔcomC
::pcomC
UA159
ΔcomC

C

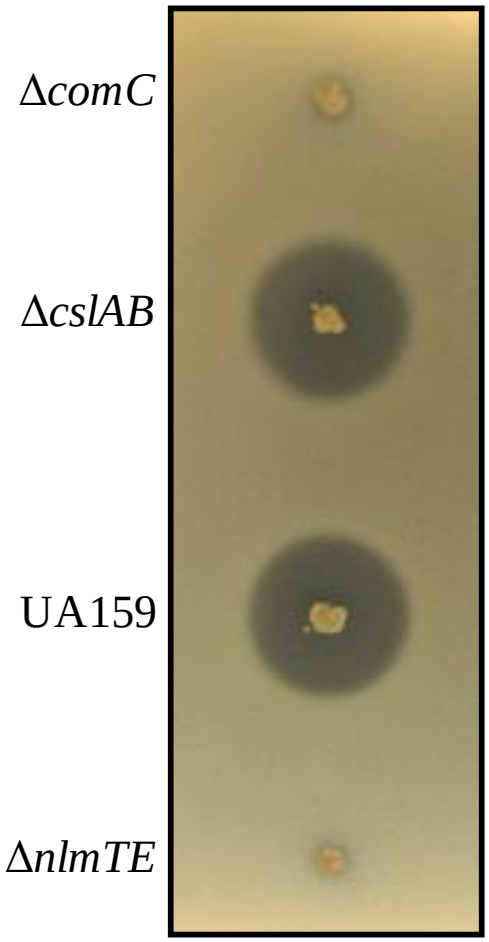

Relative Gus activity (Miller Unit)
250
200
150
100
50
0
No CSP
CSP

Since addition of external CSP can stimulate the mutacin genes expression (Fig. 23A) (156, 168, 237, 299); because a mutation in CSP-transporter should not permit the endogenous CSP to secrete into the culture supernatant, then addition of culture supernatant from CSP-transporter mutation should not induce the *gus* gene expression in the reporter strains, IBS-D37(ΔSMU.518::P*nlmA-gus*), and Δ*comC*:: P*nlmA-gus* (IBS-D40). To further confirm that NlmTE, not the CslAB, is the transporter of CSP in *S. mutans* UA159, we precipitated the culture supernatants of both *nlmTE* and *cslAB* mutants by 40% ammonium sulfate fractionation and added them into the actively growing culture of reporter strains, IBS-D37, and IBS-D40. After incubation for several hours, we have harvested the cells and performed Gus assay. We also included culture supernatants of UA159 as positive control and Δ*comC,* ΔSMU.518 strains as negative control in this reporter assay. As shown in Fig. 30B, addition of culture supernatant of Δ*nlmTE* mutant could not stimulate the Gus gene expression in reporter strain, whereas culture supernatant from *cslAB* mutation could completely restore the Gus phenotype as like UA159. To further validate this result that NlmTE is the transporter of CSP, we constructed another reporter strain named, IBS-D41 where P*nlmA-gus* gene was transformed into Δ*nlmTE* mutant background which lacks the Gus phenotype. In consistent with above reporter assays, Gus phenotype was restored upon addition of culture supernatant of *cslAB* mutant but not from *nlmTE* mutant (Fig. 30B). This reality is further corroborated by our initial insertional mutagenesis where we found that insertion at either *nlmT* or *nlmE* locus produced white colonies on the THY-X-gluc plate and was mutacin defective. Taken together, our studies highlight that CSP uses the same transporter, NlmTE, as like mutacins in *S. mutans* UA159.

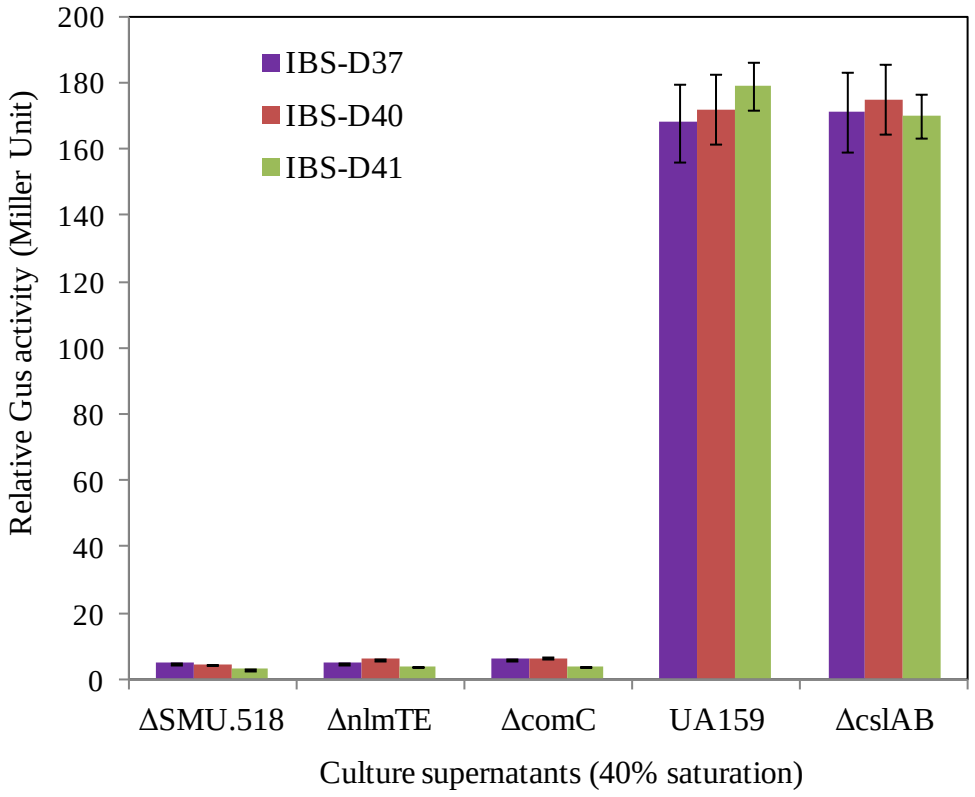
CSP can up regulate the competency development at early logarithmic phase:

Fig. 30. Mutacin production by different mutants. (A) NlmAB (mutacin IV) activity of *comC*, *nlmTE* and *cslAB* deletion strains. Experiment was done as like Fig. 21 and a representative plate from at least three independent experiments is shown. (B) Gus assay was performed as mentioned in Fig. 23A, except the addition of CSP, culture supernatants from the indicated strains (40% ammonium sulfate precipitation) was added to the actively growing culture of three reporter strains, IBS-D37 (SMU.518*::PnlmA-gus*), IBS-D40 (Δ*comC::PnlmA-gus*) and IBS-D41(Δ*nlmTE::PnlmA-gus*). The data shown are means ± standard deviations (error bars) of at least three independent experiments.

A



B


200
180
160
140
120
100
80
60
40
20
0
Relative Gus activity (Miller Unit)
IBS-D37
IBS-D40
IBS-D41
ΔSMU.518
ΔnlmTE
ΔcomC
UA159
ΔcslAB
Culture supernatants (40% saturation)

Competence development is a transient physiological state which triggers the natural genetic transformation by activation of a set of regulatory genes which allow the competent cells to uptake DNA from the environment (49, 136). Natural transformation was first identified in *S. pneumoniae* (14) and later found in *S. mutans* (236). Development of competence in streptococci occurs at particular growth stage (normally at the early to mid-logarithmic phase of growth) for a limited period of time, although there are marked differences prevailed among the species and strains (6). In *S. mutans*, alternative sigma factor, SigX, is absolutely required for the development of competence for natural transformation (191). Nonetheless, presence of ComCDE quorum sensing system is required for optimum transformation phenotype (172, 191, 284). However, external addition of CSP could not influence the transformation phenotype of *comR/comS* mutants (191). Moreover, only a subpopulation of bacterial cells develop competence temporarily (shuts down the competence machinery after ~3 to 4 hr of induction) in a given population of *S. mutans* cells in response to external CSP, whereas all of the bacterial cells activate the expression of bacteriocin-related genes (168).

To further elucidate the role of CSP in competency development, we studied the transformation efficiency at different growth stages of *S. mutans* UA159 with or without any external CSP addition (Fig. 31). We found that competence development is limited to early logarithmic phase (OD_{595} from 0.1 to 0.6), which reached maximum level when we added DNA at OD_{595} of 0.2 to 0.25. *S. mutans* UA159 lost the competence below detection level at late logarithmic phase (after OD_{595} of 0.7) even though we add CSP or not. In sum, our results suggest that competence development is a transient phenomenon in early logarithmic phase of *S. mutans* and CSP can stimulate the transformation efficiency at the early logarithmic phase.

CSP is not essential for transformation but can augment its activity: Competence stimulating peptide (CSP) has been shown to remarkably enhance the transformation efficiency of *S. mutans* when added externally (6, 9, 156, 172, 191, 221, 237, 239, 284). However, *comC* mutation had little or no influence on transformation phenotype (5, 172, 221). Since our studies suggested that NlmTE is the transporter of CSP, then deletion of *nlmTE* should provide similar transformation phenotype as like *comC* mutation. To prove this hypothesis, we did transformation assay with *comC* and *nlmTE* mutants. In addition, we used a *comR* deletion strain as negative control. In accordance with our expectation, we found that *comC* and *nlmTE* mutants exhibited similar transformation efficiency and the level of transformation was ~2-fold less than the wild type strain in absence of exogenous CSP (Fig. 32). However, addition of external CSP rescued the transformation phenotype in both of the mutants as to the extent of wild type bacteria. Interestingly, transformation efficiency was further less in mutant strain of SMU.518, which exhibited ~6-fold reduced transformation phenotype in our assay conditions. This unexpected finding can be ascribed to the reduced level of NlmC in SMU.518 mutant background, since optimum transformability is also dependent on the availability of NlmC (76).

Fig. 31. Effect of CSP on transformation efficiency. Transformation efficiency was measured at different growth stages as mentioned in Fig. 23B. A representative of three independent experiments is shown. Linear DNA (SMU.198:: Em^r) was added at different growth stages. CSP was added (200 nM) 10 minutes prior to addition of DNA. Black solid and dotted lines indicate the competence development in the presence or absence of CSP respectively.

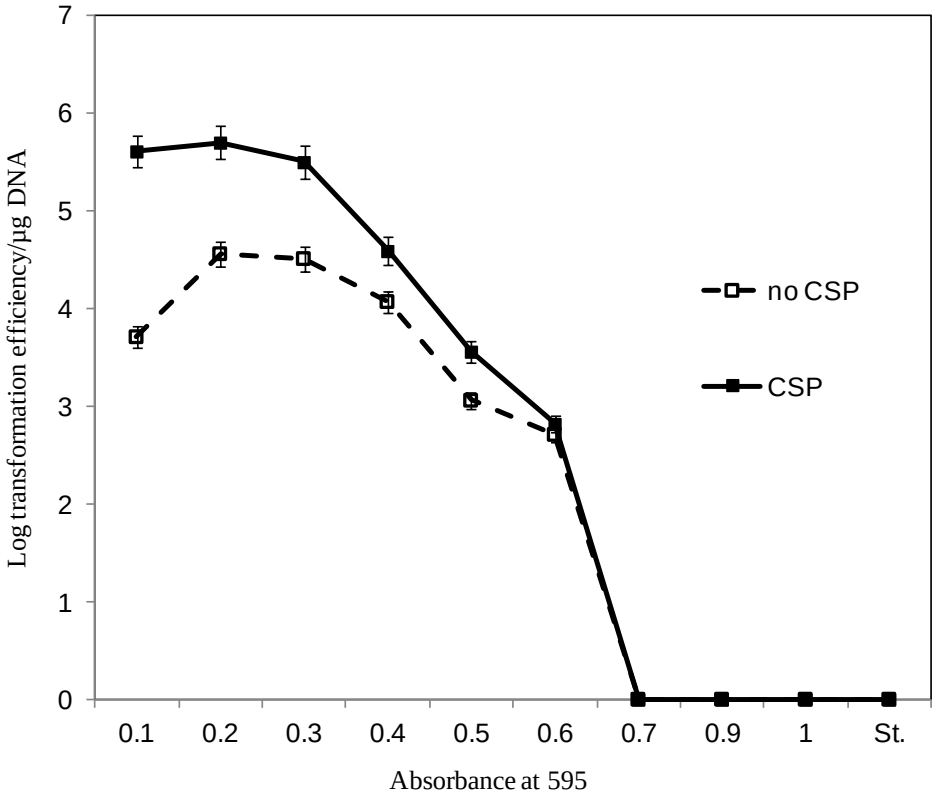
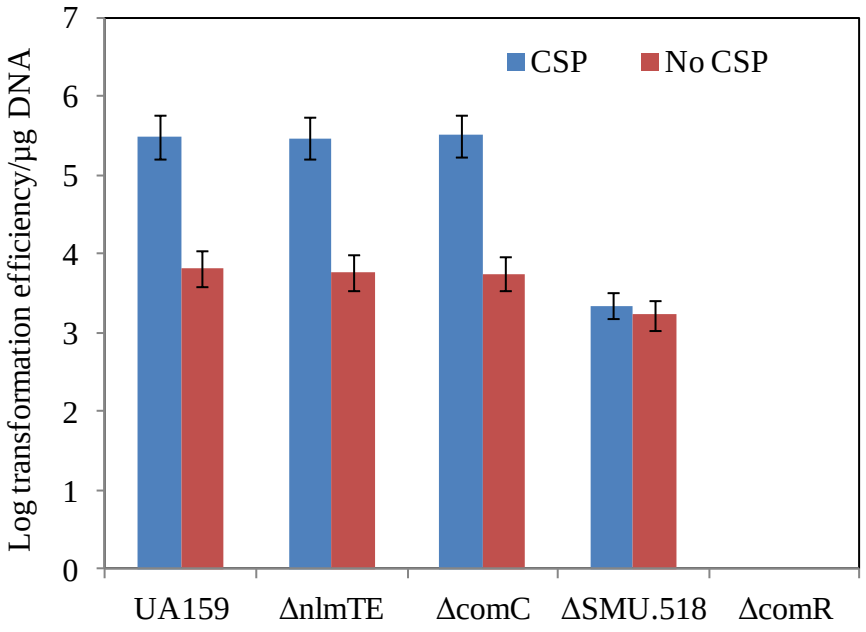

Log transformation efficiency/µg DNA
7
6
5
4
3
2
1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.9
1
St.
Absorbance at 595
no CSP
CSP

Fig. 32. Transformation efficiencies of various mutants. Transformation efficiency was measured as described in methods and materials. Linear DNA (SMU.198::Em^r) was added at OD_{595} of 0.15. CSP was added (200 nM) 10 minutes prior to addition of DNA. The results presented here are the average of three independent transformation assays.


Log transformation efficiency/µg DNA
7
6
5
4
3
2
1
0
CSP
No CSP
UA159
ΔnlmTE
ΔcomC
ΔSMU.518
ΔcomR

REGULATION OF MUTACINS PRODUCTION

PART-B

(Regulation of mutacins production by SMU.832 and SMU.2137)

Introduction

Cellular responses to environmental cues depend on diverse dynamic posttranslational modifications of their integral proteins which play vital roles in signal transduction pathway (311). Glycosylation of proteins, a posttranslational modification by carbohydrate moieties, has important function in modulating protein structure and function (123). Glycoproteins are widely present on cell surface and extracellular matrices of eukaryotes and are involved in cell-cell communication and cell-matrix recognition which are essential for numerous biological processes; from immune recognition to cancer development (285). However, glycoproteins are not limited to eukaryotes; there have been increasing evidences on protein glycosylation in prokaryotes, specifically among the mucosal-associated pathogens (123, 285). Two major types of proteins glycosylation are present among all domains of life: N-glycosylation, where the oligosaccharide is added to the asparagines residue of Asn-X-Ser/Thr consensus sequence (where X represents any amino acid except proline) and O-glycosylation, where oligosaccharide is attached to a serine, threonine or tyrosine residue (123). For several proteins, O-linked β-N-acetylglucosamine (O-GlcNAc) and O-phosphate can alternatively occupy the same or adjacent sites which suggests the potential regulatory function of O-GlcNAc in signal transduction cascades (4, 311). Reciprocity between O-GlcNAc and O-phosphorylation has been documented by several groups (3, 51, 105) and a single serine/threonine residue can exist as three forms: unmodified, phosphorylated and glycosylated which indicates the possibility of controlling

signal transduction system other than classical phosphorylation-mediated on/off system (311). This dynamic process has been reported on various nuclear and cytoplasmic proteins, including transcription factors, translations initiation factors, cytoskeleton proteins, oncogene products and tumor suppressors (51, 94, 109, 311, 324).

Presence of lipopolysaccharides (LPS) and lipooligosaccharides are hallmark of several bacteria. Both N-and O-linked glycosylations are present in bacteria (285, 288, 306). Polysaccharides are the major determinant of serological classification and identification of streptococci which are present in the cell wall (272). *S. mutans* contains rhamnose-glucose polysaccharides (RGP) which have backbone structure of α 1,2 and α 1,3-linked rhamnan along with glucose side chains and linked to alternate rhamnoses of the cell wall (323). This structural backbone is also present in the group-specific antigens of Lancefield group A, C and E streptococci (272) Studies on surface-layers also revealed the protein glycosylation in prokaryotes (285) and glycosylation of surface appendages such as, flagellum and pilli have been reported (23, 244, 267). Several studies have demonstrated that protein glycosylation in bacteria have diverse roles such as, adhesion (159, 174), antigenic variation (71), protein assembly (281), solubility (185), protection from proteolytic cleavage (120) and protective immunity (262). However, regulation of gene expression by glycosylation is yet to be identified in prokaryotes. In this chapter, we show that SMU.832, encoding a glucosyl transferase gene, is involved in mutacin genes expression in *S. mutans* UA159.

Gene expression in bacteria is also influenced by various small molecules beside the well known quorum sensing peptide, such as small non coding RNA and proteins (76). These small proteins or peptide mediated regulation of gene expression is relatively novel mechanism in bacteria and assumed to be involved in diverse physiological functions, such as cell killing,

modification of the DNA-binding capacity of a transcription factor, protein degradation/ stabilization, activation of sensor kinase and alteration of the specificity of a membrane transporter (76). A small protein named MgrB (47-residue) in *E. coli* interacts directly with the sensor kinase PhoQ of the PhoQ/PhoP two-component system which in turn represses multiple genes in the PhoQ/PhoP regulon (175). In *B. subtilis*, the competence-specific transcription factor ComK forms a ternary complex with ClpC and MecA which remains sequestered until the small protein ComS interacts with ClpC and free it to activate the transcription of genes essential for genetic competence (292). Recently, the role of a small peptide, XIP, has been reported in *S. mutans* (191) which forms a complex with ComR and activates the transcription of alternative sigma factor, ComX. Dufour et al. (76) also demonstrated the role of a small peptide, CipB, in global gene expression and competence development in *S. mutans*. In this chapter, we show that another small peptide, encoded by SMU.2137, regulates diverse physiological functions in *S. mutans* UA159. A mutation in this gene severely impaired the normal growth phenotype, mutacin production and competence development in this organism.

Results

SMU.832 is involved in mutacins production: Our initial insertional mutagenesis demonstrated that an insertion in SMU.832 drastically reduced the mutacin production in *S. mutans* UA159. SMU.832 (also called *rgpH*) encodes a protein which is involved in glucose side chain formation on RGP backbone (231). This gene is organized in *rgp* locus that regulates the biosynthesis of rhamnose-glucose polysaccharide (RGP) backbone in *S. mutans* (231, 323). To confirm that the observed phenotype was due to SMU.832, we made clean mutation in SMU. 832 locus by advanced Cre-*loxP* based method (20).We then carried out mutacin assay against two different indicator strains and found that mutacin production was notably reduced as

compared to wild type strain (Fig. 33A). To further illuminate that SMU.832 is involved in reduced mutacin phenotype, we constructed a complemented plasmid which contains SMU.832 and introduced this plasmid into ΔSMU.832. As expected, complementation could rescue the mutacins phenotype in deferred antagonism bacteriocin assay (Fig. 33A). Collectively, our results suggest that SMU.832 is required for optimum mutacin production in *S. mutans* UA159.

SMU.832 controls mutacin production via transcriptional regulation of *comC*:

According to oralgen database, SMU.832 is an integral membrane protein which contains a signal sequence followed by 12-transmembrane regions. To better understand how SMU.832 imparts in regulation of mutacin production, we investigated the expression of *nlmA*, *nlmC* and *comC* genes by sqRT-PCR. As shown in Fig. 34 (A, B), we observed significant reduction (~2-fold) of *nlmA* and *nlmC* genes expression in mutant background; whereas we noticed marked reduction in *comC* (~4-fold) gene expression. Plasmid mediated complementation of SMU.832 restored the mutant phenotypes in all three genes expression. We predict that impaired *comC* expression is responsible for deficient mutacins phenotypes and *comC* expression is down regulated due to perturbation of autogenic regulation of this peptide pheromone. We hypothesize that SMU.832 is required for maintaining proper orientation of NlmTE, a transporter of CSP and mutacins. Therefore, a mutation in SMU.832 caused reduced level of CSP secretion which in turn down regulated mutacin genes expression by less activation of ComDE pathway. This idea is supported by the finding that addition of external CSP could fully restore the Gus phenotype in the reporter strain, IBS-D54 (ΔSMU.832:: P*nlmA-gus*) (data not shown). In sum, our data suggest that SMU.832 regulates the transcription of mutacin encoding genes by down regulating the expression of bacteriocin inducing peptide, CSP.

Fig. 33. Mutacin IV and V production by SMU.832 mutant. Mutacin assay was done as described in Fig. 21. A representative of at least three independent experiments is shown.

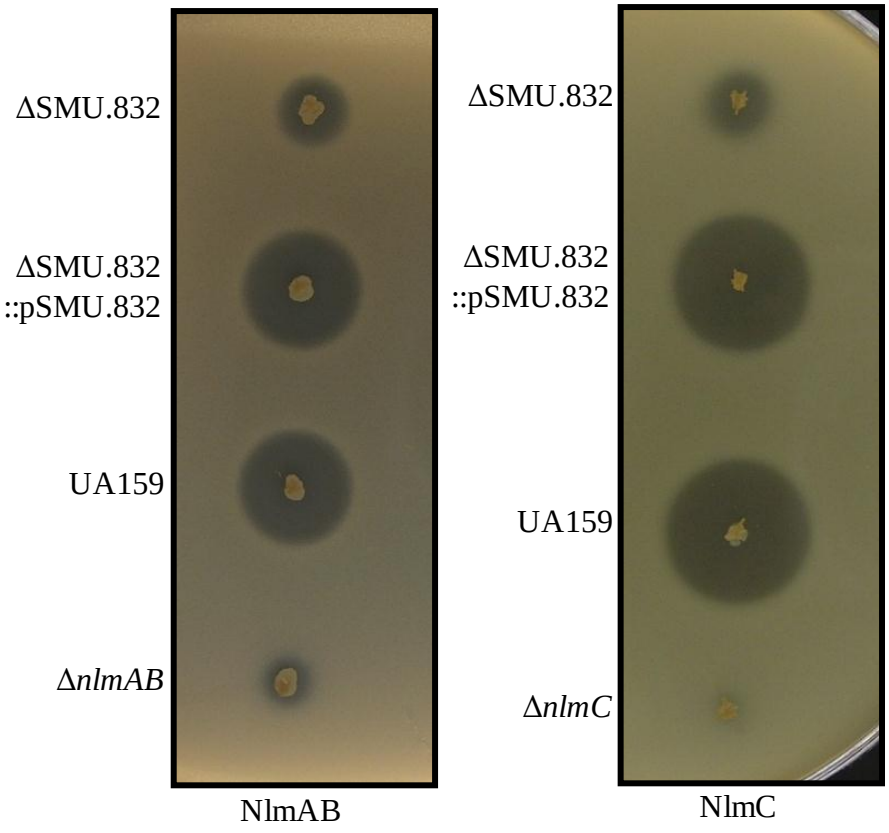
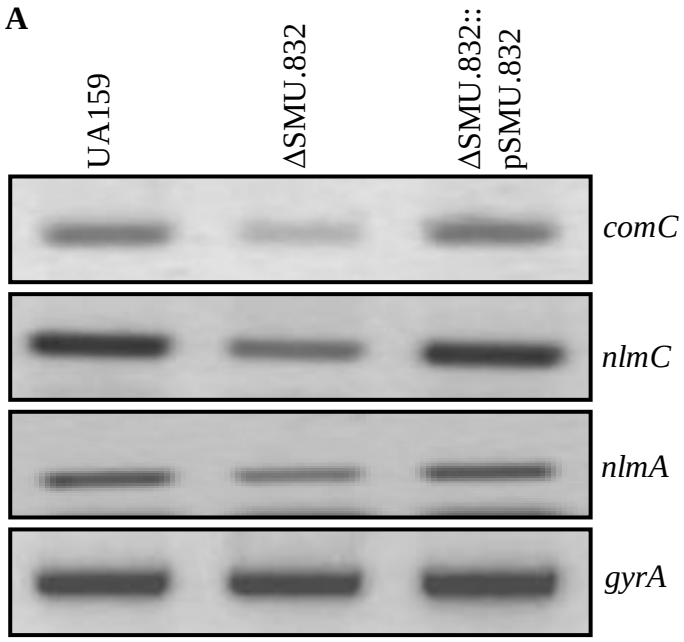
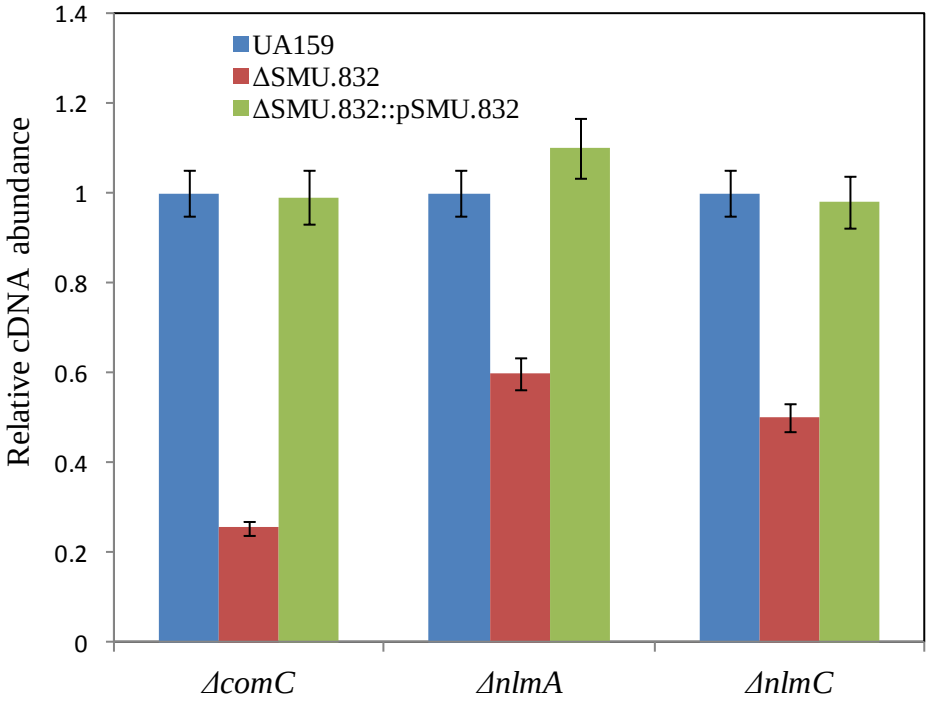

ΔSMU.832
ΔSMU.832
::pSMU.832
UA159
ΔnlmAB
NlmAB
ΔSMU.832
ΔSMU.832
::pSMU.832
UA159
ΔnlmC
NlmC

Fig. 34. Semi-quantitative RT PCR analysis of *nlmA*, *nlmC* and *comC*. (A) sqRT-PCR experiments were done as like Fig. 22. The data are representative of two different RT-PCR analyses resulting from a single RNA isolation. (B) Quantification of cDNA abundance of various genes. The PCR products were separated on 2.0 % agarose gel electrophoresis and quantified by Doc-It-LS (UVP) software. The data shown are means ± standard deviations (error bars) of two experiments from a single RNA isolation.


A
UA159
ΔSMU.832
ΔSMU.832::
pSMU.832
comC
nlmC
nlmA
gyrA

B


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative cDNA abundance
UA159
ΔSMU.832
ΔSMU.832::pSMU.832
ΔcomC
ΔnlmA
ΔnlmC

SMU.832 influences the competence development: As our sqRT-PCR data demonstrated that *comC* and *nlmC* genes were down regulated in ΔSMU.832, since *comC* and *nlmC* expression is required for optimum transformation phenotype (76, 191, 221), we anticipated that a mutation in SMU.832 should exhibit impaired transformation phenotype. In accordance with our proposition, we found that competence development has been impaired greatly in SMU.832 mutant (Fig. 35); however, addition of synthetic CSP was able to restore the mutant phenotype to a certain extent as compared to wild type or complemented strain. This impaired phenotype can be attributed to the reduced level of *nlmC* expression which is mandatory for maximum transformability in *S. mutans* (76). This finding further verified the role of SMU.832 in expression of *comC* and *nlmC*. Taken together, our results suggest that SMU.832 is involved in regulation of *comC* and *nlmC* mediated phenotypes in *S. mutans* UA159.

Characterization of SMU.2137 locus: SMU.2137 encodes a short hypothetical protein (90-residue long) that is located just after the *dnaC* and followed by SMU.2136. A short intergenic sequence (IGS) is present before and after the start and stop codon of SMU.2137. A schematic diagram of the SMU.2137 locus and the neighboring genes are shown in Fig. 36, Sequence analysis, confirmed using BPROM online software (prediction of bacterial promoters, Softberry, http://linux1.softberry.com), indicates the presence of putative -35 (TTCCAA) and -10 (TAGTAT) box motifs 234- and 254-bp, respectively, upstream of the putative start codon with a putative ribosomal binding site (AGGAGA) 10-bp upstream of the start codon. A putative rho-independent terminator sequence (GGCTTTGTAAAGCC) was also identified at position 109-bp downstream of SMU.2137 by TransTermHP (http://transterm.cbcb.umd.edu) program (146).

Fig. 35. Transformation efficiency of SMU.832 mutant. Transformation efficiency was measured as described in methods and materials. Linear DNA (SMU.198:: Em^r) was added at OD_{595} of 0.15. CSP was added (200nM) 10 minutes prior to addition of DNA. The results presented here are the average of three independent transformation assays.

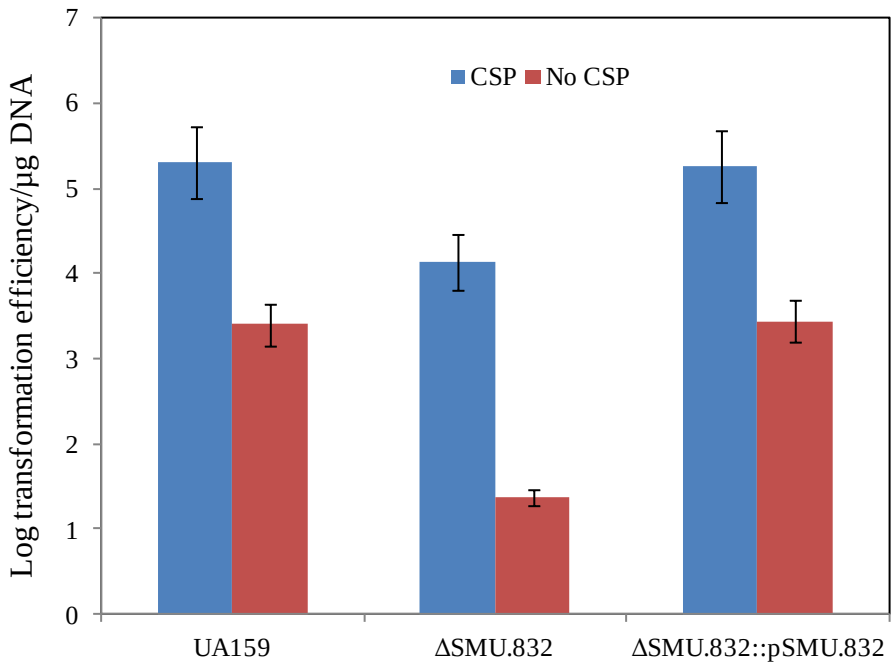
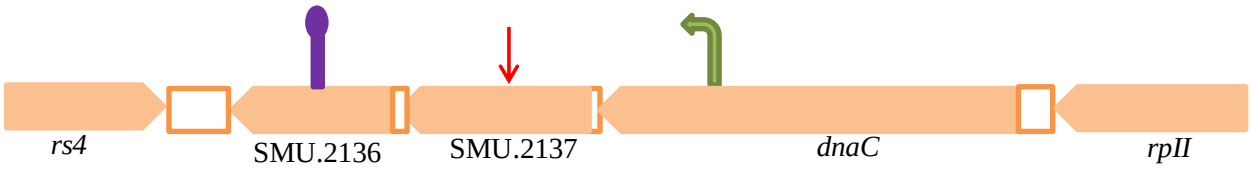

Log transformation efficiency/µg DNA
7
6
5
4
3
2
1
0
CSP
No CSP
UA159
ΔSMU.832
ΔSMU.832::pSMU.832

Fig. 36. A schematic diagram of SMU.2137 locus. Putative promoter (bent arrow) and terminator (purple bar with a circle on top) are shown. Open reading frames and orientation of the transcriptions are indicated with block arrows. Red vertical arrows indicate the position of IS*S1* insertion.


rs4
SMU.2136
SMU.2137
dnaC
rpII

Thus, it appears that the SMU.2137 is a single gene individual operon. This protein is highly conserved among streptococci (Fig. 37) and show high level of similarity with other gram-positive bacteria also.

SMU.2137 is involved in mutacin production: Our initial insertional mutagenesis manifested that an insertion in SMU.2137 caused noticeable reduction in mutacins production and Gus phenotypes on X-gluc plate in compared to wild type bacteria. To illustrate that the observed phenotype is indeed due to SMU.2137, we deleted the SMU.2137 locus by putting a *loxP-km*^{r} cassette and examined the mutacins production against two indicator bacteria. As shown in Fig. 38, a mutation in SMU.2137 drastically reduced the mutacins production as compared to wild type bacteria. To further substantiate the role of SMU.2137 in reduced mutacin phenotype, we constructed a strain with a plasmid-borne copy of SMU.2137. Plasmid mediated expression of SMU.2137 in mutant background restored the mutacin phenotype, showing the loss of mutacin production in the deletion mutant was entirely due to the loss of SMU.2137 function. Taken together, our results implied that SMU.2137 is involved in regulation of mutacins production in *S. mutans* UA159.

SMU.2137 regulates the expression of *nlmA*, *nlmC* and *comC*: To investigate the mechanism by which SMU.2137 regulates mutacins production, we investigated the expression of two mutacins encoding genes, *nlmA* and *nlmC*, and the inducer peptide, *comC* by sqRT-PCR. As shown in Fig. 39, we found that the expressions of all three genes were considerably down regulated in mutant background. Expression of *comC* and *nlmA* were affected prominently (~5- and 6-fold respectively) in compared to *nlmC* which was reduced ~2-fold in mutant strain. However, plasmid mediated expression of SMU.2137 restored the expression pattern of all three

Fig. 37. Multiple sequence alignment of SMU.2137 and its orthologous genes from different streptococci by PRALINE multiple sequence alignments tool. Orthologous genes were selected from *S. agalactiae* (SAG.2138), *S. pyogenes* (SPY.2181) and *S. pneumoniae* (SP.2202). Red color indicates fully conserved residue (score 10) and blue color indicates least conserved residue (score 0).

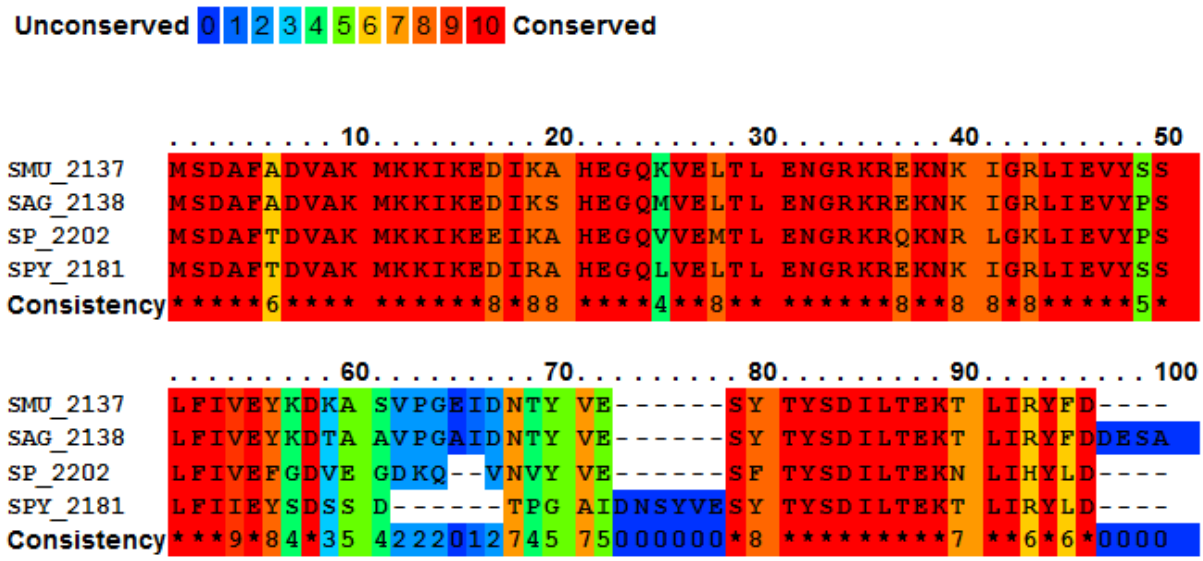
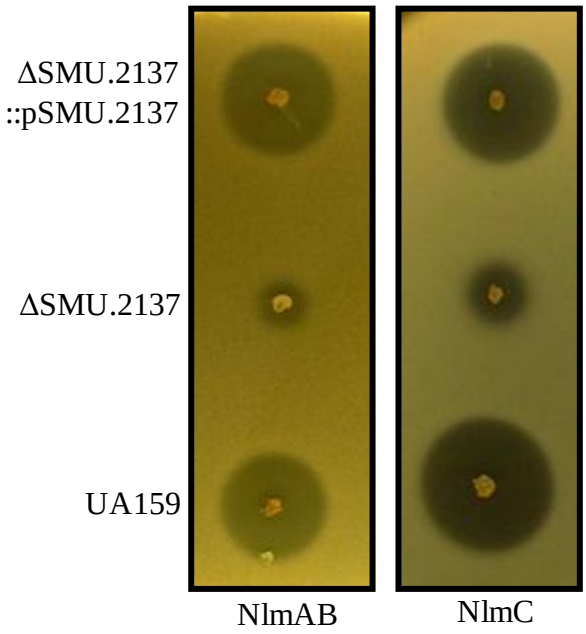

Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved
10 20 30 40 50
SMU_2137 MSDAFADVAK MKKIKEDIKA HEGQKVELTL ENGRKREKNK IGRLIEVYSS
SAG_2138 MSDAFADVAK MKKIKEDIKS HEGQMVELTL ENGRKREKNK IGRLIEVYPS
SP_2202 MSDAFTDVAK MKKIKEEIKA HEGQVVEMTL ENGRKRQKNR LGKLIEVYPS
SPY_2181 MSDAFTDVAK MKKIKEDIRA HEGQLVELTL ENGRKREKNK IGRLIEVYSS
Consistency *****6**** ******8*88 ****4**8** ******8**8 8*8*****5*
60 70 80 90 100
SMU_2137 LFIVEYKDKA SVPGEIDNTY VE------SY TYSDILTEKT LIRYFD----
SAG_2138 LFIVEYKDTA AVPGAIDNTY VE------SY TYSDILTEKT LIRYFDDESA
SP_2202 LFIVEFGDVE GDKQ--VNVY VE------SF TYSDILTEKN LIHYLD----
SPY_2181 LFIIEYSDSS D------TPG AIDNSYVESY TYSDILTEKT LIRYLD----
Consistency ***9*84*35 4222012745 750000008 *********7 **6*6*0000

Fig. 38. Mutacin IV and V production by SMU.2137 mutant. Mutacin assay was done as described in Fig. 21. A representative of at least three independent experiments is shown.

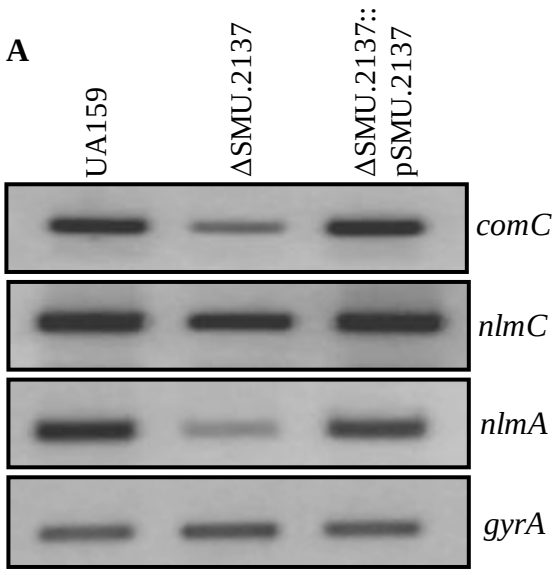

ΔSMU.2137
::pSMU.2137
ΔSMU.2137
UA159
NlmAB
NlmC

genes. Together with the preceding results, this study confirmed that SMU.2137 is required for optimum level of transcriptional activation of mutacins and mutacins-related genes in *S. mutans*.

SMU.2137 mutant is defective in competence development: As we previously mentioned that *comC* and *nlmC* expression is related to competence development in this organism, since our expression studies revealed that SMU.2137 mutant is defective in expression of these genes, it is rationale to assume that ΔSMU.2137 strain is defective in competence development also. To prove this hypothesis, we investigated the mutant strain for transformability with linear DNA (ΔSMU.198:: Em^r). In accordance with our hypothesis, we found severe impairment of competence development in mutant strain (Fig. 40). Further studies with plasmid DNA, pMM223 also confirmed that SMU.2137 mutant is crippled in competence development (data not shown). Surprisingly, reduction of transformation was remarkably less as compared to *comC* or *nlmC* mutant as shown in Fig. 40, indicating a unique role of SMU.2137 to develop competence in *S. mutans* UA159.

Deletion of SMU.2137 caused severe growth defect in *S. mutans* UA159: While working with SMU.2137 mutated strain, we noticed a severe growth defect on agar plate as well as in THY-broth. To further clarify this observation, we examined the growth kinetics of UA159 and ΔSMU.2137 in THY broth at 37^0C. As shown in Fig. 41, the growth of ΔSMU.2137 was much slower than UA159. However, the defective phenotype was restored upon complementation with a plasmid-borne SMU.2137. Apparent defect in growth kinetics suggests that SMU.2137 is involved in expression of key regulatory components needed for normal growth phenotypes also.

Fig. 39. Semi-quantitative RT PCR analysis of *nlmA*, *nlmC* and *comC*. (A) sqRT-PCR experiments were done as like Fig. 22. The data are representative of two different RT-PCR analyses resulting from a single RNA isolation. (B) Quantification of cDNA abundance of various genes. The PCR products were separated on 2.0 % agarose gel electrophoresis and quantified by Doc-It-LS (UVP) software. The data shown are means ± standard deviations (error bars) of two experiments from a single RNA isolation.


A
UA159
ΔSMU.2137
ΔSMU.2137::
pSMU.2137
comC
nlmC
nlmA
gyrA

B

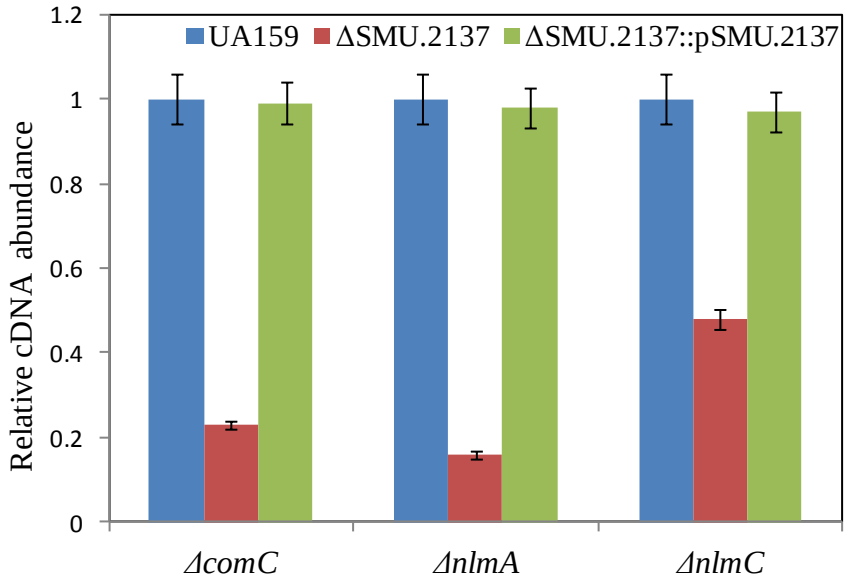

UA159
ΔSMU.2137
ΔSMU.2137::pSMU.2137
Relative cDNA abundance
1.2
1
0.8
0.6
0.4
0.2
0
ΔcomC
ΔnlmA
ΔnlmC

Fig. 40. Transformation efficiency of SMU.2137 mutant. Transformation efficiency was measured as described in methods and material. Linear DNA (SMU.198::Em^r) was added at OD_{595} of 0.15. CSP was added (200 nM) 10 minutes prior to addition of DNA. The results presented here are the average of three independent transformation assays.

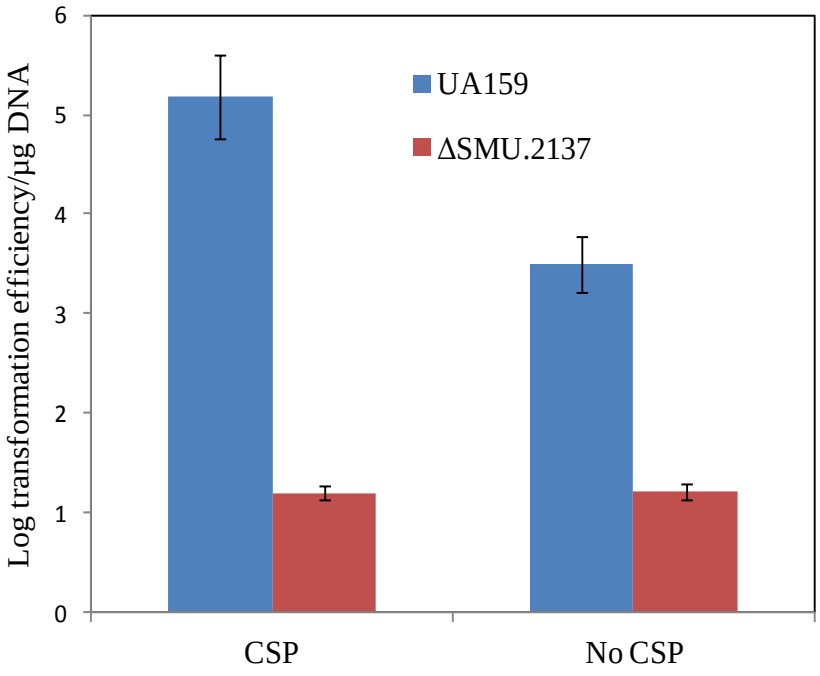
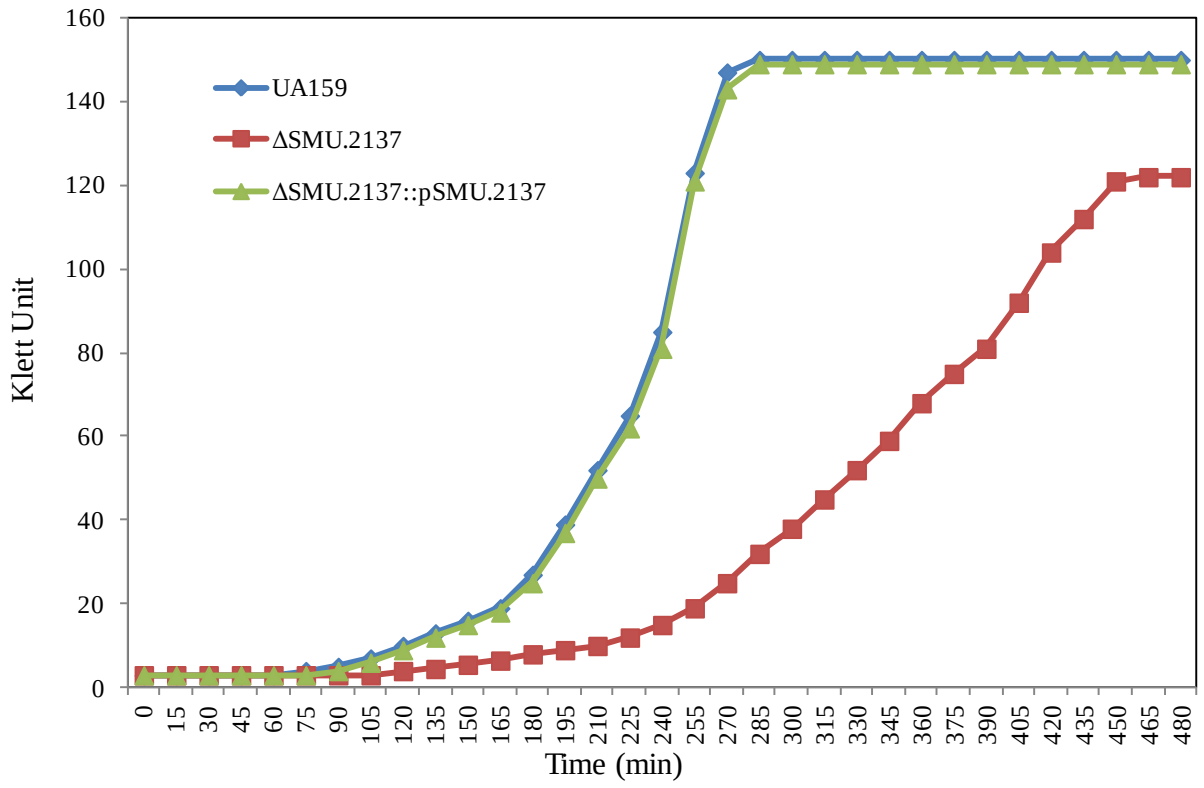

Log transformation efficiency/µg DNA
6
5
4
3
2
1
0
UA159
ΔSMU.2137
CSP
No CSP

Fig. 41. Growth kinetics of *S. mutans* UA159 and SMU.2137 mutant. Overnight cultures were diluted 1:20 into THY broth and incubated at 37^0C. Bacterial growth was monitored with a Klett-Summerson colorimeter (MP Biomedical) at different time intervals. The data shown are from a single experiment representative of three independent growth studies.


160
140
120
100
80
60
40
20
0
Klett Unit
UA159
ΔSMU.2137
ΔSMU.2137::pSMU.2137
0
15
30
45
60
75
90
105
120
135
150
165
180
195
210
225
240
255
270
285
300
315
330
345
360
375
390
405
420
435
450
465
480
Time (min)

Discussion

S. mutans is the primary causative agent of dental caries and leads a biofilm life-style in the oral cavity. Human oral cavity is regarded as the most dynamic microbial habitat in our body due to repeated fluxes of food and water intake, rapid change of temperature, pH, osmotic and oxygen tension. Successful colonization and demineralization of tooth depends on the abilities to adhere and form biofilm on tooth surface, production of acids from carbohydrate metabolism, to withstand the low pH and different environmental stresses in this fluctuating environment. Along with these virulence properties, *S. mutans* can produce antimicrobial peptides (mutacins) to inhibit the growth of other competing species; thereby helping in establishment of flourishing biofilm in the oral cavity (169). Mutacins mediated cell killing is also considered as a source of nutrients during nutrient limited conditions (210). *S. mutans* UA159, genome sequence reference strain, encodes two well known bacteriocins, mutacin IV and V. As almost all of the previous studies on regulation of mutacin IV production were focused on two-component regulatory system (ComCDE), our understanding on global regulatory pathways remains restricted. To get a comprehensive view on regulation of mutacin IV (encoded by *nlmAB*) production in *S. mutans* UA159, we screened a library of random insertion mutations to select clones with no/less mutacin phenotype on THY-X-gluc plate. In this study, we screened ~12,000 colonies in three independent screening and identified 22 unique loci involved in mutacin IV production. However, this approach also suffered some drawbacks as like other insertional mutagenesis. An insertion in essential genes might not have been identified due to growth retardation or weak phenotypes could have been overlooked. Since we only looked for white/pale blue colonies on X-gluc plate, since we were not able to distinguish more blue clones in compared to our wild type reporter strain (IBS-D5); we were not able to identify any repressors associated with

mutacin IV production. In addition, mutacin IV defective phenotypes might have been generated from polar effect on downstream genes. Among the 22 loci we identified in our preliminary analysis, six of them (*comC, comD, comE, nlmT, nlmE and ciaH*) were previously shown to be important for mutacin IV production in *S. mutans* UA159, which further validated the viability of our experimental approach. However, we did not identify *liaS* or *clpP* which were previously reported to influence mutacin production (41, 46). Our inability to identify them might be due to their moderate effect on *nlmA* gene expression and went unnoticed during primary blue/white selection. Based on the level of mutacin production, we have classified all of the mutant clones into four different groups; class I (no mutacin production), class II (<6 mm), class III (intermediate level; 6-10 mm) and class IV (exhibited variable properties). These loci encode proteins for diverse activities, such as two-component signal transduction system, ABC transporter, energy metabolism and central cellular processes. Five of them do not have any known functions and assigned as hypothetical protein in various database (Table-11). To further understand the role of some of these unidentified factors, we made clean deletion in SMU.518 (class I), SMU.611, SMU.832, SMU.2137 of class II and SMU.121 of class IV. We found that a clean mutation in three of them (SMU.518, SMU.832 and SMU.2137) produced prominent phenotypes in deferred antagonism bacteriocin assay and all of them encode the hypothetical proteins of unknown functions. However, a mutation in SMU.121 and SMU.611 did not show any phenotype suggesting the presence of polar effect in initial insertional mutants. In this study, we have further characterized these three loci for better understanding the mechanism of mutacin IV production in *S. mutans* UA159.

Predicted structure of SMU.518 suggests that it contains a Lon-like proteolytic domain at C-terminal end along with a PDZ domain in the middle and an uncleavable signal sequence at

the N-termini (Fig. 20). This protein is highly conserved across the gram-positive bacteria from streptococci to mycobacterium. Its orthologous gene, Spy.1536, in group A streptococci (GAS) was shown to be localized outside the cell and antibody to it was protective in mouse model (91). ATP dependent Lon proteases are primarily responsible for selective intracellular proteolysis, which regulates protein quality control and cellular homeostasis (264). Lon protease was first identified in *E. coli* and later found in every organisms investigated so far (264, 283). These cytosolic proteases are homooligomeric in nature and each of the subunit (784 amino acids long) contains an N-terminal substrate binding domain, a central ATPase domain and C-terminal proteolytic domain with serine-lysine dyad active center (264). In addition to removal of up to 50% aberrant proteins in *E. coli* (47), this protein is also involved in cell division (by degrading cell division inhibitor, SulA), capsule production, plasmid stability and phage replication (47, 77, 78). Gram-positive model bacterium, *B. subtilis,* encodes two *lon* homolog, *lonA* and *lonB* (255, 274). It has been reported that LonA prevents σ^G dependent transcription during vegetative growth (268) and LonB found to be expressed in the forespore compartment under the control of σ^F (271, 274). Archaea contains membrane-bound LonB type protease and compensates the absence of ATP-dependent membrane-bound protease, FtsH (309). Whereas all of the Lon proteases characterized so far contain ATP binding motifs and resides in the cytosol, SMU.518 does not possess any ATP binding motif and suggested to be surface localized in GAS. Thus, this protease is a unique class of Lon protease present in gram-positive bacteria.

In this study, we show evidence that SMU.518 is localized on the surface of *S. mutans* and involved in maturation of peptide pheromone, CSP (competence stimulating peptide). It was previously reported that CSP undergoes post-export cleavage (loss three C-terminal residues) by an unknown factor and activates the two-component signal transduction system in *S. mutans*

(239). Processed CSP (18-amino acids) shown to be functionally more potent than the 21-amino acids peptide. Our study revealed that proteolytic domain of SMU.518 is required for specific ATP-independent cleavage at the C-termini to release the last three amino acids. We demonstrated that this cleavage is essential for generation of functional CSP. In other words, 21-residue CSP is inactive and it must be cleaved at the C-termini to produce functional form of CSP (18-amino acids). We also demonstrated that a serine-lysine dyad (S235 and K280) is responsible for catalytic activity by this protease as like other Lon proteases. Our results suggested that functional form of CSP (18-residue) works at much lower concentration as like other peptide pheromones and consequently, answered the long standing question of the requirement of high concentration of CSP in *S. mutans* (0.5 to 1μM in *S. mutans* as compared to 20 nM in *S. pneumoniae*) (239). Functional form of CSP (18-amino acids) is already present in some of the natural strains along with the C-terminally elongated form (9, 172, 239) and appeared to be not involved in variation in transformation phenotypes among them. One could simply ask about the necessity of having additional residues in the C-termini, while they are not required for functional activity. We are not sure why last three amino acids need to be cleaved off to produce functional form. Although Syvitsky et al. (284) showed that last three amino acids are required for optimum transformation phenotype, our study along with several other studies firmly negate the authenticity of their findings (9, 172, 239). We presume that these three amino acids provide protection against the proteolytic degradation in the dense multispecies biofilm. In support of this assumption, Wang et al. (308) recently showed that the CSP of *S. mutans* is degraded by the proteolytic activity of *S. gordonii*, thereby giving selective advantage to it in the natural habitat. It is also possible that these three amino acids confer protection to its own surface proteases to some extent, such as to HtrA. To support this hypothesis, we found several truncated

versions of CSP in mass spectrometry of ΔSMU.518-treated sample (Fig. 25) and this proteolytic activity was disappeared upon prior treatment with protease inhibitor, AEBSF. It can also be presumed that these three amino acids in full length CSP prevents activation domain of CSP to interact with ComD; therefore, it is required to make an active form of CSP to activate the ComDE system. We can further imagine that intact CSP (21-residue) is an essential defense strategy in natural biofilm condition where *S. mutans* can direct it against other organisms to induce their autolytic activity or inhibit their normal physiological process or 21-residue CSP can be used as bacteriocin to kill other bacteria. Recently it has been demonstrated that CSP can inhibit the formation of hypha in *Candida albicans* (134). Further study is necessary to unravel the exact molecular function of these three amino acids.

Why is it required to have an extracellular protease to activate an intracellular function? While all other Lon proteases are localized inside the cells, why this type of Lon proteases is localized outside the cells? Does it require any association with other proteins? Is CSP the only substrate of SMU.518? It is likely that this protease is also involved in maturation of other peptide pheromones. Indeed, Mashburn-Warren (191) found that competence regulatory peptide (XIP) undergoes proteolytic maturation after secretion. We speculate that this protein is required for degradation of bacteriocins inducing peptide or bacteriocins directed to *S. mutans* by other competitive bacteria in the oral biofilm. It is possible that these proteases have other functions in vivo. In support of this assumption, Fritzer et al. (91) recently showed that this protein is essential for surface proteins expression in both transcriptional and post-transcriptional level as well as involved in binding with extracellular matrix proteins in case of GAS. HtrA, a surface associated protein, has been shown to be involved in surface protein expression in *S. mutans* (31). It is likely that SMU.518 also imparts in same process. As Lon proteases degrade most of

the substrate non-specifically, one can also contemplate that this protease is involved in degradation of extracellular proteins or proteinaceous nutrients to small peptides for energy efficient uptake as nutrients. Further study will tell us about the range of functions executed by SMU.518 among different bacteria.

Two-component signal transduction system controls diverse physiological functions upon induction with peptide pheromone, such as competence development, bacteriocin production, biofilm formation, acid tolerance and virulence expression (282). The results presented in this study suggest that ComCDE, a two-component system, primarily induces the expression of mutacins encoding genes in *S. mutans* UA159. A mutation in *comC, comD* or *comE* locus completely abolished the bacteriocin production in consistent with several previous results (299, 308, 325). Unlike bacteriocin production, our results along with several other previous studies suggest that a mutation in *comCDE* loci still allow competence development but at a lower efficiency than the wild type bacteria (172, 191, 221). Recently, Mashburn-Warren et al. (191) demonstrated that genetic transformation in *S. mutans* is absolutely governed by a peptide pheromone called, XIP, which activates the expression of alternative sigma factor, SigX. Some upstream regulators such as, ComCDE, HtrA, CipB, CiaRH, HdrRM can significantly fluctuate the efficiency of transformation phenotypes under different growth conditions, but none of them are essential for *sigX* induction or competence development (5, 6, 221, 237, 250). Thus, the prevailing idea on CSP-mediated coordination of competence development and bacteriocins production in *S. mutans* is questionable. Our study revealed that *nlmAB* genes expression started at lower level during early logarithmic phase which reached maximum at late exponential phase and then expressed persistently up to stationary phase. In contrast, competence development was a transient phenomenon and limited to early logarithmic phase and diminished its activity below

detection level after mid-logarithmic phase. Our result is in agreement with several previous reports regarding these two phenotypes (156, 207, 236, 299). As our result demonstrated that *comC* expression is growth associated (started at early logarithmic phase and disappeared at stationary phase), one can easily speculate that CSP concentration outside the cell reaches maximum at late logarithmic phase and prevails during stationary phase which in turn can activate the ComDE pathway up to stationary phase. In consistent, we found the maximum level of *nlmAB* expression during stationary phase in sqRT-PCR and the culture supernatant of overnight culture was able to trigger the Gus phenotype during our reporter assay. If CSP is the stimulator of competence development, then we should also detect the transformation phenotypes during late exponential phase or stationary phase. So, it is plausible to conclude that CSP is exclusively required for mutacins production and its effect on competence is indirect. We assume that the decreased level of transformation phenotype in *comCDE* mutations, might be due to the scarcity of intracellular level of NlmC (also called CipB) which has recently been shown to be required for optimum level of competence development (76). We hypothesize that NlmC can interact with ComR for up regulation of *comS* and *comX* transcription. In low level of NlmC (in *comCDE* or SMU.518 mutations), ComR can only transcribe minimum level of XIP and ComX for basal level of competence development. It is also likely that intracellular NlmC can stabilize the ComR-XIP complex, which in turn produces increased transformation phenotypes by stimulating the expression of *comX* and its autogenic circuit. This hypothesis is in agreement with the microarray data of Dufour et al. (76), where they found that a mutation in *nlmC* caused several fold reduction of *comR* and *comX* transcription. In addition, CSP-stimulated ComE can directly bind with the promoter of *shp61* (encodes XIP) and activates its transcription. Therefore, bacteriocin production and competence development is two distinct phenomena in *S.*

mutans as like *S. pneumoniae*, where ComABCDE governs the competence development and BlpABCRH system controls the bacteriocin production (187). Further supports came from the fact that competence induction is also transiently developed in *S. pneumoniae* during early logarithmic phase as like *S. mutans* (243). Moreover, bioinformatics data indicated that Com-system of *S. mutans* is more similar to Blp-system of *S. pneumoniae* (187).

Our results clearly show that *S. mutans* cannot be transformed to a detectable level beyond OD_{595} of 0.7 even though *comC* expression continued until late exponential phase and CSP concentrations outside the cells remained active up to stationary phase. It has also been reported that the expression of competence master regulator, ComX, continued up to stationary phase (168, 191). These facts might instigate someone to speculate that ComX undergoes some sorts of posttranscriptional or posttranslational modification which hinder *S. mutans* to continue competence during late exponential or stationary phase. Interestingly, CSP concentration outside the cell will be the highest at this stage of growth and one can speculate that increased CSP might have some negative feedback role on competence development by blocking ComX. We predict that high level of CSP will also generate high level of truncated versions and one of them might be imported inside to bind with ComX; thereby preventing it from functioning. In the early logarithmic phase, when the CSP concentration is low, low amount of truncated product is not sufficient enough to block ComX completely. Small peptide mediated regulation of competence development has already been reported in *B. subtilis* (292) and *S. mutans* (191). In *B. subtilis*, liberation of ComK (required for transcription of competence related genes) from the ternary complex of ComK-ClpC-MecA depends on the presence of ComS (292). In addition, a pentapeptide named, CSF (competence and sporulating factor), is internalized in *B. subtilis* and interacts with intracellular receptors (RapB and RapC) to inhibit their function (52, 234); which

in turn stimulates the activity of transcription factor ComA (required for genetic competence) and Spo0F (a component of phosphorelay that activates sporulation) (52, 276, 294). In *S. mutans*, XIP (product of ComS) interacts with ComR and activates the expression of competence master regulator, ComX. Further study with purified peptides will elucidate the impaired competence development during late exponential or stationary phase of *S. mutans* (191).

Loss of mutacins production by *nlmTE* mutant and essentiality of the CSP for mutacins expression suggest that the processing and exports of both bacteriocins and CSP are executed by the same transporter, NlmTE. In addition, the culture supernatant of Δ*nlmTE* was not able to trigger the Gus phenotype in reporter strain and external addition of CSP could restore the Gus phenotype in *nlmTE* mutation. Our results firmly nullify the earlier notion that CslAB is the transporter of CSP. A mutation in *cslAB* locus did not produce any phenotype in deferred antagonism bacteriocin assay and culture supernatant from *cslAB* mutant strain was able to trigger Gus gene expression in our reporter assay, which further suggests that NlmTE is the transporter of both mutacins and CSP. This is not surprising because N-terminal leader peptide of both CSP and bacteriocins contain same GG-motif and similar in size. Our results conform the findings of van der Ploeg (299) who previously suggested that NlmTE (also called ComAB) is the transporter of CSP and bacteriocins.

Gene expression is regulated by diverse mechanisms in both prokaryotes and eukaryotes. Role of glycosylation in regulation of gene expression is well established in eukaryotes; however, glycosylation-mediated gene expression is not described in prokaryotes so far. In this study, we found that a gene responsible for glucose side chain formation on RGP backbone regulates the expression of *comC*, which in turn controls the expression of mutacins encoding genes. We speculate that a transcription factor required for *comC* transcription undergoes

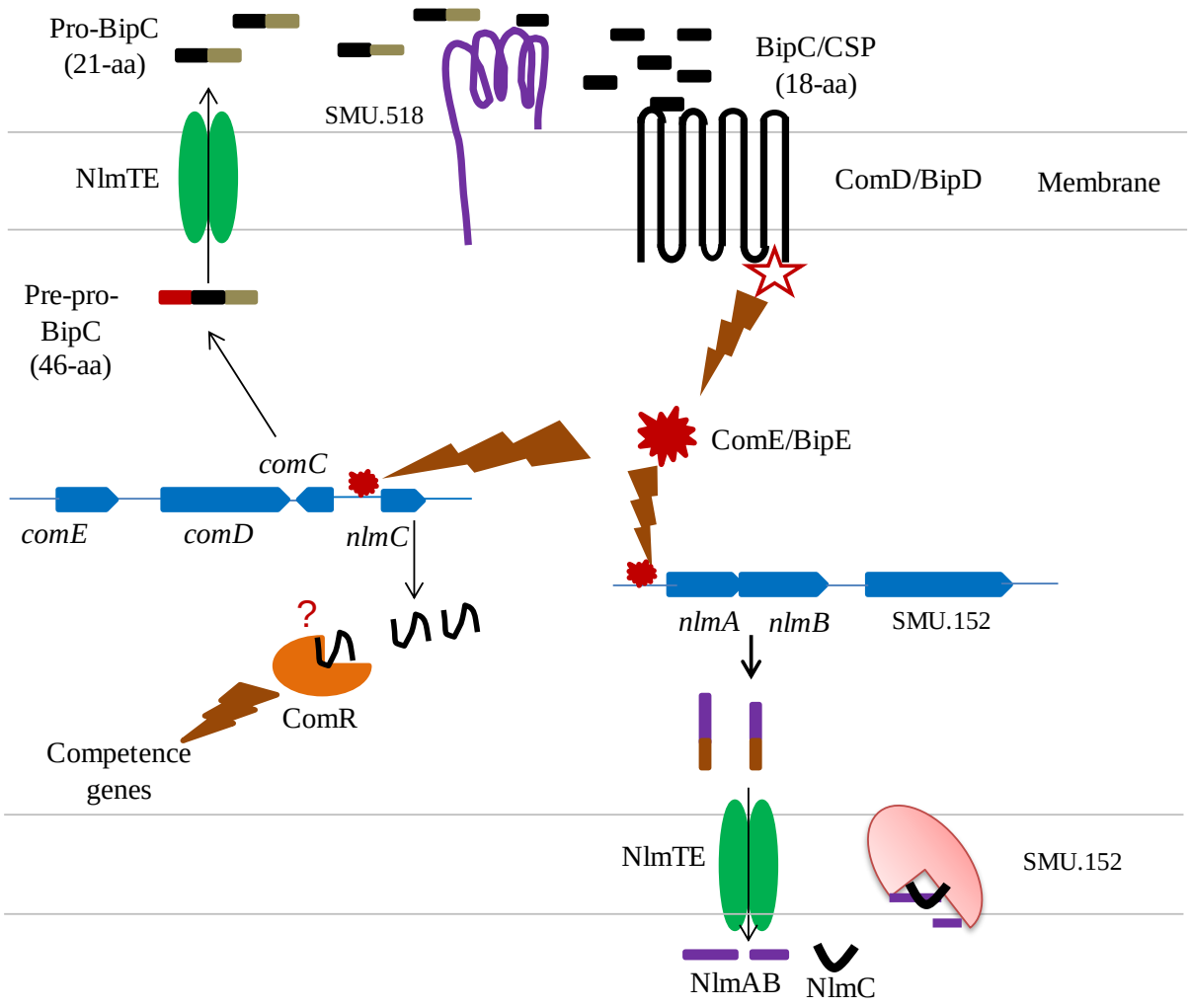
glycosylation for efficient transcription of *comC*. It is likely that ComE undergoes reversible glycosylation and regulates the expression of *comC* and mutacins encoding genes. By glycosylation predicting software, we found four unique sites for glycosylation in ComE (NetNGlyc 1.0 Server) and we predict that anyone of these sites needs glycosylation for efficient transcriptional activation of its responsive genes. It is also possible that SMU.832 down regulates the *comC* gene expression by blocking the autogenic regulation of *comC* gene expression via ComDE pathway. We also speculate that SMU.832 interacts with the NlmTE and helps to maintain functional conformation to secrete the CSP and mutacins. CSP dependent autogenic activation of *comC* has been recently described by Perry et al.(237). Further study will uncover the exact molecular mechanism by which SMU.832 regulates *comC* gene expression in *S. mutans*.

Our study also demonstrated that a small peptide (encoded by SMU.2137) regulates mutacins expression along with diverse physiological functions. This is not completely unexpected, since small proteins or peptides mediated regulation of gene expression has already been reported to be involved in diverse physiological functions, such as cell killing, modification of the DNA-binding capacity of a transcription factor, protein degradation or stabilization, activation of sensor kinase, and alteration of the specificity of a membrane transporter (76). It is likely that this protein forms a functional multiprotein complex with other regulatory proteins and functions in transcriptional or translational level. Intracellular regulation of gene expression is not a discrete entity; rather complex interconnected network is required to exert various cellular activities. Presence or absence of a particular peptide or protein can greatly influence the activity of that protein complex.

Based on our current results and existing evidences on mutacins production, we present a new model for regulation of mutacins production by peptide pheromone in *S. mutans* (Fig. 42). In this model, we propose renaming of 'com' system as 'bip' where bip indicates bacteriocin inducing pathway. We have changed the name of ComC as BipC (bacteriocin inducing peptide C), ComD as BipD and ComE as BipE. According to this model, BipC is translated as pre-pro-peptide and while passing through the dedicated ABC transporter (BipTE/NlmTE), leader sequence is cleaved off after GG-motif by the proteolytic activity of BipTE. Exported peptide again undergoes proteolytic cleavage at the C-termini by a Lon-like protease, BipP. This matured peptide, BipC, is now sensed by the sensor kinase, BipD, which upon phosphorylation activates the response regulator, BipE. BipE induces the transcription of bacteriocins encoding genes expression by binding the direct repeats present in the promoter region of responsive genes. Pre-bacteriocins secrete through the same ABC transporter, BipTE/ NlmTE, and become matured upon cleavage after GG-motif and inhibit the sensitive bacteria. However, producer strain has specific immunity protein for self-protection. BipC can enhance the transformation phenotype indirectly by NlmC, which may interact with ComR, to up regulate the expression of *comR*, *comS* (*xip*) and *comX*, thereby increasing the transformation phenotype.

In summary, our results revealed that mutacins production in *S. mutans* is under multifaceted regulatory mechanisms involving multiple genes of diverse functions, which indicate an intimate link between mutacins production and the normal cellular homeostasis. In addition, regulation of gene expression by reversible glycosylation and multiprotein complex might play an important role in diverse physiological functions in prokaryotic organisms.

Fig. 42. Model of the regulation of mutacins production in *S. mutans* UA159. Bacteriocin inducing peptide, BipC, is translated as pre-pro-Bip and secreted through the ABC transporter, BipTE/NlmTE as pro-Bip. A cell surface Lon-like protease, BipP (SMU.518), cleaves the C-terminal three amino acids of pro-Bip and produces functional peptide pheromone, BipC (18-amino acids). When it reaches a certain threshold concentration, BipC activates the two-component signal transduction pathway, BipDE. Activated response regulator, BipE, stimulates the expression of mutacins encoding genes (such as *nlmAB* or *nlmC*) by binding to the direct repeats present in the upstream promoter region. Mutacins are translated as pre-peptide and secreted through the same ABC transporter BipTE/NlmTE while a proteolytic cleavage removes the leader sequence.


Pro-BipC
(21-aa)
SMU.518
BipC/CSP
(18-aa)
NlmTE
ComD/BipD
Membrane
Pre-pro-
BipC
(46-aa)
ComE/BipE
comC
comE
comD
nlmC
nlmA
nlmB
SMU.152
?
ComR
Competence
genes
NlmTE
SMU.152
NlmAB
NlmC

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

Mutacins play a crucial role in maintaining the favorable ecological niche for *S. mutans* in the oral cavity. Increasing evidences suggest that they provide multiple advantages to the producer bacteria in highly heterogeneous oral habitat. Most apparent advantage is that mutacins help in vying with other early colonizers for successful colonization. Many of its early competitors, such as *S. sanguinis* can colonize the tooth surface earlier and are less susceptible to various stresses (155). Without any countermeasures, it would be very difficult to initiate and sustain a biofilm lifestyle in this extremely competitive micro-ecological milieu. In vitro competition assay clearly demonstrated that mutacin I and IV are required for maintaining dominance over *S. sanguinis* in dual species biofilm condition (157).

The experimental works presented in this study provide a comprehensive understanding on mutacin IV activity, mechanism of self-immunity and regulation of mutacins expression in *S. mutans* UA159. Critical to our understanding is that both NlmA and NlmB peptides are required for maximum mutacin IV activity. By using genetic and biochemical approaches, we resolutely clarified that mutacin IV follows the typical definition of class IIb bacteriocins, where both of the peptides should be present in equimolar concentration to exhibit maximum inhibitory activity. Our studies revealed that the inhibitory spectrum of mutacin IV is much broader than mutacin V, and both of these mutacins work synergistically to inhibit various microorganisms.

We show evidences that *S. mutans* UA159 contains a subtle mechanism to protect itself from its own bacteriocin. The genome of this bacterium encodes a specific immunity gene, SMU.152, which offers immunity to mutacin IV. In addition, in the absence of cognate immunity

protein, *S. mutans* can employ other paralogous proteins to prevent the self-inhibition. We show that C-terminal disordered region of the immunity protein is indispensible for conferring immunity to the susceptible cells. Our studies further demonstrate that Man-PTS is not the only receptor for bacteriocin targeting; other membrane proteins can also function as the target for class II bacteriocin mediated cell inhibition.

Our key finding is that the expression of mutacins is controlled at multiple levels by numerous factors and this process is intricate with cellular homeostasis. Our studies revealed that an extracellular ATP-independent Lon-like protease functions specifically on maturation of peptide pheromone, CSP. Here, we show that functional form of CSP (18-amino acids) is generated by a post-export cleavage with this Lon-like protease. To the best of our knowledge, this is the first report on a Lon-like protease locating outside the cell which does not have any ATP-binding motifs to exhibit its activity. However, this protease contains the same catalytic dyad as other Lon proteases. We demonstrated that the ComCDE two-component system is primarily involved in regulation of mutacins production in *S. mutans* UA159 and proposed the renaming of this entire pathway as BIP (bacteriocin inducing pathway).

Based on the knowledge gained so far on this study, there are several ways to proceed further. As many as 15 putative bacteriocins encoding genes are present in the genome of *S. mutans* (Table-6). We have characterized the activities of only mutacin IV and V. Most of them still remain unexplored. Interestingly, we found that *S. pneumoniae*, a predominant bacterial pathogen, is susceptible to the mutacins other than NlmAB or NlmC (Table-10). Future studies could be directed to unravel the activity spectrum of these uncharacterized mutacins.

Undoubtedly, the mutacins holds a great promise to use as antimicrobial therapeutics against numerous streptococcal species. However, characterization of the activity of a novel

bacteriocin is not enough to find more potent bacteriocins for commercial applications. Structure-function analysis is an important tool for rationale design of a bacteriocin with improved properties, which can be useful for medicinal or biotechnological applications. In addition, specific activity of a bacteriocin can be increased by cloning and expressing in heterologous hosts. Detail structural analysis by NMR spectroscopy, X-ray crystallography in membrane mimicking condition, and site-directed mutagenesis is required to understand how these bacteriocins work on target cells.

Although we have identified a specific immunity protein which rendered protection to mutacin IV, further study is necessary to unravel the mechanism of immunity conferred by this protein. So far, Man-PTS is the only recognized receptor for class II bacteriocin and we found that this is not the only target for mutacin IV activity. Due to the presence of different inhibitory spectrum for each bacteriocin, it is likely that each of them has different specific receptors on target cell membrane. Because of the crucial role of receptors in determining specific inhibitory spectrum of a given bacteriocin, it will be of great interest to identify the receptor and understand the molecular mechanism underlying specific immunity activity of SMU.152.

Bacterial cell-cell communication by quorum sensing has been reported to be associated with virulence genes expression, including acid tolerance, biofilm formation, competence development, sporulation, mating, bioluminescence, and bacteriocin production (62, 119, 171-173, 266, 299). This holds a great promise to rationally design drugs that can block quorum sensing and related pathogenic properties, thereby effectively controlling the pathogens. Development of structural analogs can competitively block the receptor binding and inhibit quorum-sensing regulated virulence expression. Further study on structure-function of CSP will assist in development of new inhibitor that can be used to control the prevalent oral disease,

dental caries, by blocking the CSP-related virulence expression in *S. mutans*. In last decade, several studies have described the role of quorum-sensing antagonist to inhibit the respective regulatory circuits in different bacteria (119, 180, 316).

As our studies revealed that multiple regulatory factors are involved in controlling the mutacins production in *S. mutans*. Here, we have characterized the activity of three hypothetical proteins on regulation of mutacins production. It is interesting that our studies show evidence of the involvement of a non-canonical Lon-like protease in regulation of mutacins production. Further study is necessary to understand how this protease works. Particularly, future investigations on mechanism of surface localization, possible interaction with other proteins or necessity of homooligomerization like other Lon proteases are expected to get insights into this unique protease activity. Regulation of gene expression by glycosylation and multiprotein complex is likely prevalent in prokaryotes as like eukaryotic cells also. Further experiments would be interesting to decipher the exact regulatory mechanisms underlying the genetic regulation by SMU.832 and SMU.2137. At last, but not the least, all other regulators identified in our transposon mutagenesis remain for future studies to uncover the complete regulatory mechanism of mutacin production in S. *mutans* UA159.

Ever increasing trends of antibiotic-resistance among the pathogenic bacteria and the demand of food supply without any chemical additives have accentuated the necessity of novel antimicrobial agents in medicine and food industries. Bacteriocins have the potential to be used as natural preservatives to control food-spoiling bacteria or food-borne pathogens or can be used as species-specific antimicrobial agents to manage the wide-spreading antibiotic resistance problems world-wide. Further studies on mutacin biology will provide valuable insights for its future applications in food or pharmaceutical industries.

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