LANTIBIOTIC SMB: CHARACTERIZATION OF THE IMMUNITY PROTEIN, IDENTIFICATION OF A NOVEL RECEPTOR-LIKE PROTEIN, AND A NEW PERSPECTIVE ON REGULATION

By

SASWATI BISWAS

March 13, 2014

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.

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This is to attest that the work represented in this dissertation was performed by Saswati Biswas in my laboratory, except where indicated.

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Abstract

Dental caries, commonly known as tooth decay, is a chronic disease that develops slowly and requires formation of biofilm on tooth surfaces, commonly known as dental plaque. Dental plaque is a highly complex multispecies biofilm containing over ~700 different microorganisms. In this microbiota, *Streptococcus mutans* is considered to be the primary etiological agent for cariogenesis. To colonize and maintain its dominance over competing non-cariogenic species, *S. mutans* secretes various antimicrobial peptides called bacteriocins. *S. mutans* produces two types of bacteriocins: linear unmodified peptides known as non-lantibiotics and extensively modified nonlinear peptides called lantibiotics. *S. mutans* GS-5 strain is a highly virulent isolate that has been extensively used for genetic and biochemical studies. This strain produces a broad-spectrum lantibiotic called Smb. This lantibiotic is one of the arsenals that *S. mutans* GS-5 uses to shift the established bacterial flora associated with dental health towards the flora associated with dental caries.

A lantibiotic producer strain must contain a self-protection mechanism to protect itself from the lantibiotic-mediated damage. Immunity mechanisms against Smb have not been identified. A previous report by Kuramitsu's group described SmbG, a putative ABC transporter with a peptidase domain, as the immunity protein for Smb. This proposed function of SmbG in providing immunity is not supported by their experimental data. In this study we show that an ABC-transporter encoded by SmbFT functions as an immunity complex. We show that GS-5 becomes sensitized to Smb upon deletion of *smbT,* which makes the ABC transporter non-functional. We demonstrate that SmbFT can confer protection against Smb when expressed heterologously in four different sensitive streptococci. We also demonstrate that SmbFT can confer protection against structurally similar two-petide lantibiotics such as haloduracin. We conclude that SmbFT truly displays immunity function and confers protection against Smb and structurally similar lantibiotics.

Lantibiotics are potent bactericidal agents and usually functional at nanomolar range, whereas other antimicrobial peptides are effective at micromolar concentrations. This fact indicates that the interplay between the lantibiotics and the target organisms must be specific and perhaps it occurs through receptor-

mediated interaction. However, to date, no such receptor molecules have been identified for any lantibiotics. In this study we identify in *S. pyogenes* (a human pathogen) a membrane-bound protein that exhibits a receptor-like function for Smb. This protein, which we named LsrS, belongs to CAAX-protease family. LsrS is widely present in streptococci including *S. mutans* and is highly conserved. Deletion of the LsrS homolog in sensitive *S. mutans* strains makes them refractory to Smb inhibition. However, neither LsrS nor its homolog can recognize other structurally similar two-peptide lantibitics. Nevertheless, this is the first protein that displays a receptor-like function for any lantibiotics.

It is of great importance to understand how the producer strain regulates the expression of its immunity protein to counteract the cognate lantibiotic produced by the cell as well as by the neighbours. An auto-sensing mechanism may exist to maintain a constant ratio of the immunity protein and the lantibiotic. Little is known about the transcriptional regulation of the *smb* operon. Unlike most of the other lantibiotic loci that encode their own regulatory factors, *smb* locus does not encode any factor that can function as an auto-regulator. We provide experimental evidences that Smb peptides function as signaling molecules and auto regulate the *smb* operon through some yet to be discovered regulators. We attempted to identify the unknown regulators by transposon mutagenesis and identified an operon that seems to be involved in activation of *smb* operon. Further analysis indicated that a transcriptional regulator encoded within the operon indeed regulates *smb* production. Our results show that a new regulator and perhaps a new regulatory pathway might control *smb* expression.

Lantibiotics, such as Smb, are highly potent, stable, and active at nanomolar concentrations. Because of the stability and potency lantibiotics are widely used in food industry as preservative. Few other lantibiotics are in clinical trials with the prospective to be used as antimicrobial agents in the healthcare industry. Since Smb can inhibit many pathogenic streptococci, it has the potential to be used as an antimicrobial agent in food and/or healthcare industry.

Acknowledgements

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Abbreviation

ABC	ATP binding cassette
Ap	Ampicillin
BP	Base pairs
CI	Clinical isolate
CSP	Competence stimulating peptide
Em	Erythromycin
GAS	Group A streptococcus
GBS	Group B streptococcus
IE	Infective endocarditis
IS	Insertion sequence
Km	Kanamycin
LB	Luria-Bertini
MIC	Minimum inhibitory concentration
OD	Optical density
ORF	Open reading frame
RGP	Rhamnose-glucose polysaccharide
RT-PCR	Reverse transcriptase polymerase chain reaction
SMU.	Designation for ORFs of *S. mutans*
TCS	Two-component signal transduction system
THY	Todd-Hewitt with yeast extracts broth
Ts	Thermosensitive
ZOI	Zone of inhibition

Chapter 1: Introduction

1.1. *Streptococcus mutans*: the pathogen and its community

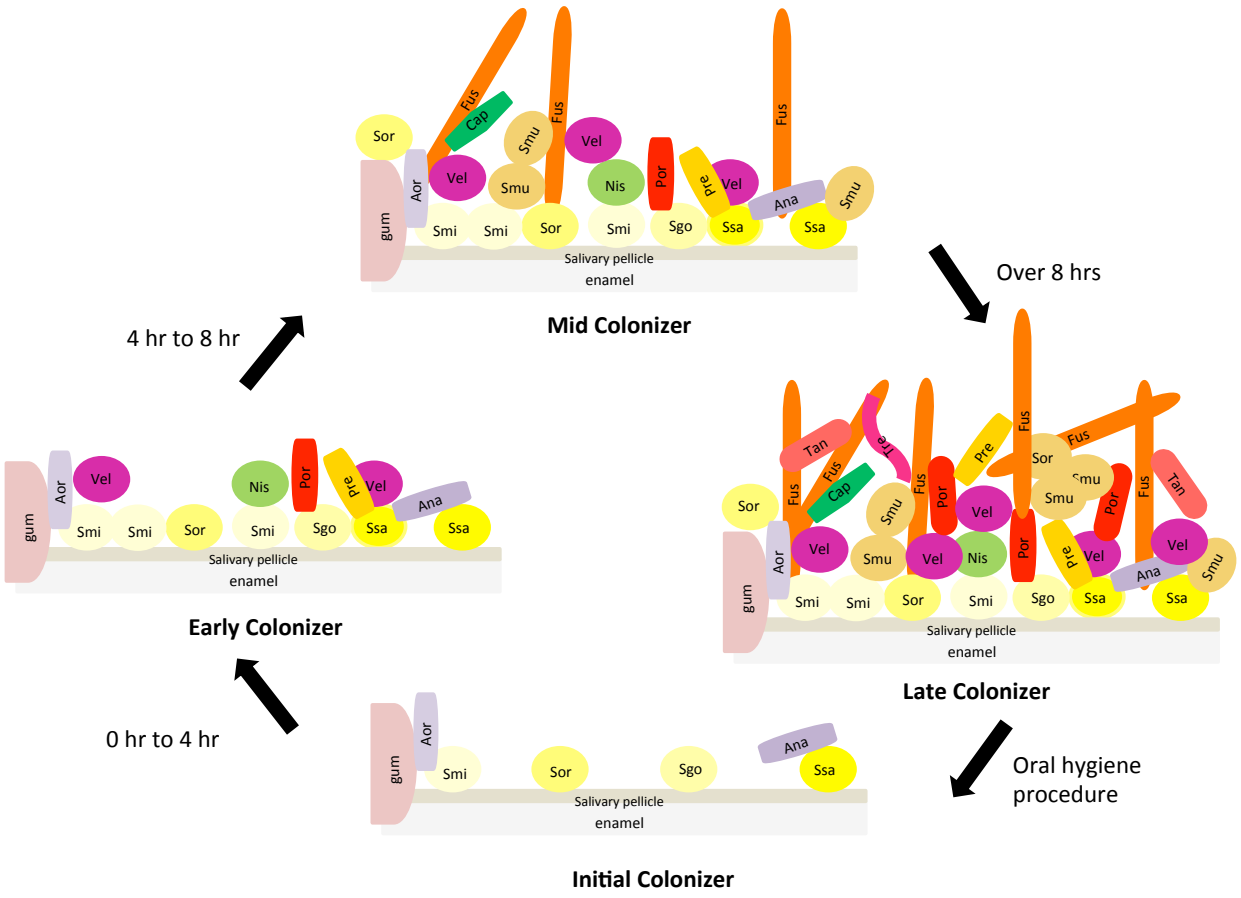
The oral cavity, which is sterile at birth, is immediately colonized by bacteria acquired primarily from the mother or other family members of the infant. Right after the eruption of the first tooth, the oral cavity microbial community diversifies and eventually becomes a complex consortium of bacteria with more than 700 species [1]. In healthy individuals, this community provides protection against pathogens by inhibiting their survival as well as interfering with their colonization in the oral cavity. However, some potential opportunistic pathogens are members of this community and can cause diseases when the conditions become favorable. Although bacteria can adhere to different mucosal surfaces in the oral cavity, they are periodically shed during epithelial cell turnover. One exception is the tooth, where bacteria can colonize firmly and form biofilms, unless proper hygiene is maintained. Biofilms formed on the tooth surfaces are known as dental plaques. Dental plaque formation is a prerequisite to dental caries, which is one of the most prevalent and somewhat benign human diseases. In 1924, Clarke and colleagues have isolated *Streptococcus mutans*, a Gram-positive and chain forming cocci, from cariogenic tooth of humans [2]. The prevalence of this organism in human dental caries entitled the bacterium the name of the causative agent for the tooth decay. *S. mutans* has exceptional ability to adhere to tooth surfaces and to produce high amount of acid to de-mineralize the tooth enamel causing dental caries. Although, *S. mutans* and few other streptococci are the predominant species during initial stages of dental plaque formation, other Gram-positive rods such as *Actinomyces oris* and filamentous bacteria such as *Fusobacterium nucleatum* start accumulating with the progression of the dental caries. Other species of bacteria such as *Actinomyces* spp. and *Veillonella* spp. are also associated with caries formation; however their exact role in the disease pathogenesis is not well understood.

1.1.1. The oral environment

The diverse community of oral bacteria often shows distinct tissue-specific tropisms. Various bacteria colonize different surfaces of the oral cavity, such as the tooth enamel, tongue, soft and hard palate, gingiva and buccal mucosa [3]. Several studies have been conducted to characterize which biological surfaces in oral cavity are colonized by which group of bacteria. For example, *Actinomyces* spp. seem to colonize on tooth surfaces, while *Prevotella melaninogenica* and *Veillonella parvula* colonize soft tissue surfaces in higher proportions [3]. Other Gram-negative organisms such as *Capnocytophaga gingivalis* and *V. parvula* primarily colonize the tongue, which is also a soft tissue [3]. Gram-positive organisms, specifically streptococci such as *S.mitis, S. oralis,* and *S. salivarius* predominantly colonize the soft tissues and are found in higher proportion in the saliva as compared to other organisms [4]. Other pathogenic streptococci, such as *S. agalactiae*, *S. dysgalactiae*, and *S. pyogenes* are all able to colonize the human nasopharynx [5]. The rate of colonization of *S. pyogenes* in the oral cavity specifically in the oropharynx of children can reach as high as 10% [6]. The dominance of certain bacterial species in the specific regions of the oral cavity is due to the fact that various organisms express different cell-surface associated adhesins that determine the tissue tropism [3].

Dental plaque formation requires extensive bacterial colonization on the tooth surfaces. Colonization by oral bacteria in biofilms requires attachment to a surface such as to teeth, epithelial cells, or to the bacterial surface. This initial adherance is the most crucial step in building dental plaque. The best-studied oral biofilms are the two types of dental plaques. The first one is the dental plaque that develops on the tooth enamel and known as supragingival plaque. The second one is the plaque below the gum line on the root surface known as subgingival plaque. Development of a mature dental plaque occurs in four successive stages involving different colonizing bacteria (Fig. 1). The bacteria involved in these four stages are called *initial, early, mid*, and *late* colonizers. These bacteria either directly interact with the epithelial cell or to tooth surfaces through adhesin-receptor interaction, or to another bacteria by specific protein-protein interaction known as coaggregation.

Figure 1: Pictorial representation of oral bacterial colonization and biofilm formation on pellicle coated tooth surface. This colonization is detected within few hours of oral hygiene procedure. *Streptococcus oralis* (Sor), *Streptococcus gordonii* (Sgo), *Streptococcus snaguinis* (Ssa), *Streptococcus mitis* (Smi) *Actinomyces oris* (Aor), and *Actinomyces naeslundii* (Ana) bind to salivary receptors or coadhere to the other initial colonizers. Early colonizers *Veillonella* spp. (Vei), *Prevotella* spp. (Pre), and *Porphyromonas* spp. (Por) appear within four hours of dental hygiene. Growth of initial colonizers is shown by increased numbers of streptococci and actinomyces. Mid-colonizers such as *Fusobacterium nucleatm* (Fus), *Capnocytophaga* spp. (Cap), and *Streptococcus mutans* (Smu) coadhere with the already present initial and early colonizers. Late colonizers such as *Tanerella forsythia* (Tan), *Treponema denticola* (Tre) coaggregate with the bacteria in the already established biofilm. The color scheme follows the Socransky classification of dental bacteria. [Adapted from Kolenbrander and Periasamy, Oral Microbial Communities]


Sor
Fus
Cap
Smu
Fus
Vel
Fus
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Por
Pre
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Ana
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Sor
Smi
Sgo
Ssa
Ssa
Salivary pellicle
enamel
Mid Colonizer
Over 8 hrs
4 hr to 8 hr
Vel
Nis
Por
Pre
Vel
Ana
Aor
gum
Smi
Smi
Sor
Smi
Sgo
Ssa
Ssa
Salivary pellicle
enamel
Early Colonizer
Late Colonizer
0 hr to 4 hr
Oral hygiene procedure
Aor
Ana
gum
Smi
Sor
Sgo
Ssa
Salivary pellicle
enamel
Initial Colonizer

The initial colonization step is perhaps the most crucial stage in biofilm development since it dictates the community structure of the matured dental plaque. The initial colonizers of dental plaque are capable of directly binding to the salivary glycoproteins in pellicle, which immediately coats the tooth surface after oral hygiene measures [7,8]. Results from culturing bacteria in dental plaque indicated that streptococcal species belonging to the viridians group, primarily *S. mitis* and *S. oralis* dominated the initial stage. Two other streptococci, *S. sanguinis* and *S. gordonii*, were also found but at a lesser frequency and *Actinomyces* spp. were also present in the initial development [8-10]. After the establishment of the initial community, coaggregation and coadherence between different bacterial species continues as a result biofilm microflora becomes more complex [9]. Surface component mediated cell-cell interactions of two species have been extensively studied and various adhesins that promote co-aggregation have been identified [10]. For example, cell wall anchored adhesins SspA and SspB on different streptococci have been shown to mediate co-aggregation with *Actinomyces naeslundii* and *S. gordonii* [10]. This interaction introduces genus diversity in early plaque formation. Although *S. gordonii* is an initial colonizer, streptococcal adhesin SspA/B expressed by *S. gordonii* shown to interact with fimbriae of *Porphyromonas gingivalis,* involved in periodontal disease. Initial colonization by streptococci is also influenced by the intrageneric coaggregation among all the initial colonizing bacteria [11]. Intrageneric coaggregation is widely observed among various oral streptococci and not so much with other species. However, other oral bacteria such as *A. naeslundi* and *Actinomyces oris* display intergenic coaggregation with the initial streptococcal colonizer and other genera, and diversify the initial developing community ([12,13]; Fig 1).

After coadherence and coaggregation, the initial colonizers start multiplying and provide a bacterial surface for the attachment of the early colonizing bacteria. The early colonizing bacteria include genera such as *Eikenella, Neiseeria, Porphyromonas, Prevotella*, and *Rothia* all of them have the ability to coaggregate with the already attached cells. However, an important group, *Veillonella* spp., has the ability to readily coaggregate with the attached streptococci and actinomyces. *Veillonella* spp. can reach high proportion in the biofilm and can constitute between 2 to 9% of the microbiota in the early

colonizing stage [14,15]. This observation suggests that Veillonellae can efficiently exhibit both intergeneric and intrageneric interactions on tooth enamel. Another importatnt trait is that Veillonellae cannot utilize sugars for their growth but are able to ferment organic acids such as lactic acid. Both streptococci and actinomyces present in the early biofilm produce lactic acid as metabolic by product. This lactic acid provides a nutritional source for Veillonellae growth.

Another notable coaggregation in the early biofilm community is between *S. gordonii* with *S. oralis* and *P. gingivalis*. The coaggreation between *S. gordonii* and *P. gingivalis* is very well characterized. The interaction involves Mfa, a short fimbriae present on the *P. gingivalis* cell and SspA/B on the *S. gordonii* cell [16]. This interaction is regulated by several factors including an auto inducer molecule, a LuxR family transcription factor, and involvement of arginine deiminase pathway [17-19]. Likewise, *S. oralis* is also an important contributor in the early biofilm community. A two-species biofilm study suggests that *S. oralis* can grow mutualistically with *Veilonella* sp. [20]. However, another two-species biofilm study involving *S. oralis* and *P. gingivalis* show no growth of either species [21]. By comparison, a three-species biofilm study involving all three organisms (*S. oralis*, *P.gingivalis*, and *Veillonella*) show growth of all, indicating that the interfering effects observed in the two-species study can be overcome by the addition of a third species [20]. These in vitro studies indicate that intricate multispecies partnerships are the key for succession of species during dental plaque development.

The mid-colonizing bacteria need to interact with both the initial and early colonizers as well as with the late colonizers. These bacteria also need to compete with the numerous streptococci as well as other initial and early colonizers to establish themselves in the biofilm. The mid colonizers include mainly two species*:* *Fusobacterium nucleatum* and *Capnocytophaga gingivalis*. These bacteria exhibit different community interactions and strategies than those employed by the initial and early colonizers. For example, *F. nucleatum* has the ability to coaggregate with all the initial and early colonizers [22]. Fusobacteria are also able to co-aggregate with the mid colonizer *C. gingivalis* [7,9]. This trait provides *F. nucleatum* an added advantage over already adherent all other species including *C. gingivalis.* Both of these species are ubiquitously present in non-carious sites of healthy oral tissues. However, both *F.*

nucleatum and *C. gingivalis* bacteria appear predominantly in the oral sites that are subsequently affected by periodontitis, these middle colonizers are called "the crossroads between health and disease."

F. nucleatum expresses an arginine-sensitive adhesin, RadD, on its cell-surface that is used for coaggregation between *F. nucleatum* and other streptococci [23,24]. *F. nucleatum* can also form coaggregates with numerous Gram-negative species that are lactose-inhibitable. However, one initial colonizer, *S. oralis*, appears to negatively influence the growth of *F. nucleatum*. On the other hand, another initial colonizer, *A. oris*, appears to positively influence *F. nucleatum* growth. Therefore, the relationship among the species in the mid colonization phase seems to be highly dynamic with some interactions being synergistic while others being antagonistic.

It is important to mention here that while *S. mutans* is considered as an early colonizer and can directly interact with the tooth enamel via surface expressed adhesins (see below), as well as can form coaggregates with early colonizers such as *Neisseria* spp. and *Actinomyces* spp. [25], some researcher believe that *S. mutans* may actually be a mid colonizer. This is because some studies have found that *S. mutans* is noticeably absent from the initial and early biofilms, while many other streptococci, including *S. mitis, S. oralis, S. gordonii, S. infantis,* and *S. sangunis* are dominant in the early stages [26,27]. Interestingly, these dominant streptococci produce hydrogen peroxide, which inhibits the growth of *S. mutans*. Therefore, in healthy and non-cariogenic lesions, *S. mutans* may be outcompeted by other streptococci. But situation is differnt in the cariogenic tooth where using multiple antagonistic approaches *S. mutans* establishes itself in the biofilm at the early stages and suppresses the growth of other competing species.

As the microbial community develops and diversifies, late colonizers start to accumulate in the biofilm. These late colonizers are frequently associated with the periodontal disease. Three organisms, *Tannerella forsythia*, *Treponema denticolla*, and *P. gingivalis,* are commonly classified as red complex and cause periodontal disease. Another organism, *Aggregatibacter actinomycetemcomitans,* which is often involved in an aggressive form of periodontitis, is also a late colonizer [28]. As expected, all four species can efficiently coaggregate with fusobacter, the mid colonizer. However, as mentioned before, *P. gingivalis*

is the exception among the red complex bacteria since it can coaggregate with the initial colonizer *S. gordonii*. Red complex bacteria can also interact with each other in the oral biofilm. *P. gingivalis* can coaggregate with *T. denticola* through the involvement of fimbriae and treponema PrtP protease [29].

Established biofilm communities are found not only on the tooth surfaces but also in the mucosal surfaces of the tongue, buccal mucosa, and gingival crevices. The population structure of the bacterial flora in these established biofilms is highly complex and dynamic. Competitive and mutualistic interactions among the species determine the ultimate composition of the biofilm. However, the population structure is subjected to changes by parameters such as diet and underlying health conditions [30]. Environmental factors such as nutrient availability and red-ox potential as well as host factors such as presence of receptors and immune chemicals (cytokines and antibodies) determine the composition of the biofilm at a given niche. Most bacteria in the biofilm display commensalism, which benefits both host and the bacteria. However, as mentioned before, among the innocuous bacterial flora, some opportunistic pathogens with virulence capability exist and can cause disease within and beyond the confines of oral cavity. When the condition is shifted from healthy state to a disease state, the entire consortia of bacterial population changes. There are no bacterial species that are exclusively associated with the healthy oral environments. The proper balance between pro-healthy and pro-disease causing bacterial community is critical and the balance can be disturbed due to nutritional variations, exposure to antibacterial agents, underlying health conditions, and other factors. Oral diseases such as dental caries occur when the pro-disease causing bacterial community increases in abundance relative to the pro-healthy microbiota. The exact reasons responsible for the emergence of certain disease causing pathogens and the shift from heathy state to disease state are not very well understood.

1.1.2. Diseases caused by *S. mutans*

Changing diet to a more sugar-based (carbohydrates), more frequent feeding, and consumption of acidic drinks shift the bacterial flora to more acidogenic population. In this acidogenic population, *S. mutans* is

the dominant species and is considered to be the principal etiological agent in human dental caries. *S. mutans* encodes for different virulence factors that help them to cause diseases (Fig 2). Apart from *S. mutans,* various lactobacilli, *S. sobrinus* and *F. nucleatum* also show some association with dental caries; however, *S. mutans* is the predominant one. It is believed that *S. mutans*, which is an early or mid colonizer, could out compete other bacteria in the dental plaque. Often, this organism is involved in causing infective endocarditis (IE) and *S. mutans* accounts for more than 20% of cases of viridians streptococcus-induced endocarditis. The induction of dental caries and the development of endocarditis are discussed below.

1.1.2. a. Dental caries

Dental caries is initiated by a breach in the tooth enamel caused by bacteria present in the dental plaque. These acidogenic bacteria produce lactic acid from dietary sugars during carbohydrate metabolism, which demineralizes the tooth enamel [31,32]. The disease develops from this initial area of deminaralization that exposes the underlying dentine to the condition where bacteria gain access to the tubular network of dentine reaching to dental pulp. There are at least three major hypotheses behind the development of dental caries that includes the specific plaque hypothesis, the non-specific plaque hypothesis, and the ecological plaque hypothesis [2,33-35]. In 1924, Clarke [2] first proposed the specific plaque hypothesis suggesting that only a few species of bacteria, such as *S. mutans*, are involved in the caries development. Towards this end, several studies have indicated the presence of higher levels of *S. mutans* at human carious lesions compared to other organisms and for this reason, *S. mutans* is attributed as the principal etiological agent of caries formation [31,32,36,37].

Miller in the late 1800s [33] proposed the non-specific plaque hypothesis. He suggested that all bacteria in the mouth have the potential to be cariogenic and few reports indicate several non-mutans bacteria can successfully produce caries lesions [35,38,39]. The non-specific plaque hypothesis is an attractive and highly accepted alternative explanation for dental caries formation. Finally, the ecological plaque hypothesis suggests that dental caries and other plaque-related diseases are formed by imbalances in the

resident community microflora. For example, imbalances due to high acidic conditions may favor emergence of a plaque community enriched in cariogenic streptococci [40]. This hypothesis proposes that any acidogenic organisms in the oral cavity, such as *S. mutans* and others, can initiate caries formation provided that the local environmental conditions support the overall process. Several recent microbiome studies as well as other targeted studies [41] involving children with healthy and cariogenic tooth suggest that complex cariogenic community might be responsible for dental caries. Nevertheless, most of the studies, whether microbiome based or targeted, have always identified *S. mutans* in the cariogenic population. Therefore, *S. mutans* truly involved in development of dental caries in human.

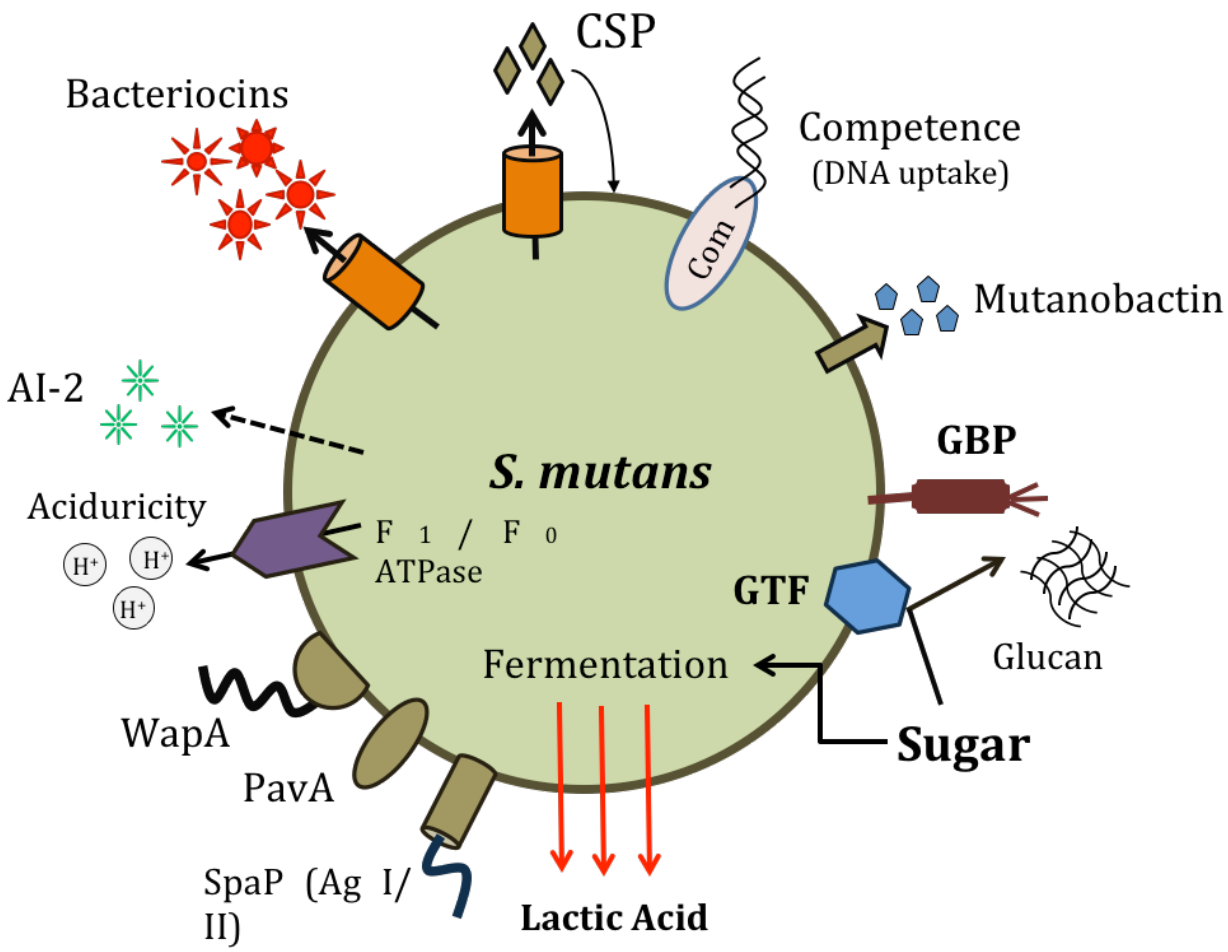
Cariogenic lesions on the tooth surfaces progresses rather slowly. The pH inside oral plaque is always in constant flux. The pH can rapidly decrease due to acid production by acidogenic bacteria following intake of dietary carbohydrate and can rise due to alkali production by other plaque bacteria under nutrient limited condition [35,39,42]. Since *S. mutans* can metabolize a diverse range of carbohydrates, this organism has the innate ability for initiation and progression of dental caries [43-45]. Carbohydrates such as fructose, glucose, galactose, and sucrose can be easily fermented by *S. mutans* via the glycolytic pathway to produce lactic acid from pyruvate [31]. Lactic acids generated by *S. mutans* and other acidogenic plaque bacteria such as lactobacilli and *S. sobrinus*, acidify the local environment below the pH 5.5; the critical point required for remineralisation of tooth enamel [32,43,46]. Extended period of exposure to low pH leads to continuous demineralisation of the tooth enamel causing caries formation [47].

1.1.2.b. Infective endocarditis (IE)

S. mutans is also responsible for the induction of infective endocarditis. IE is a bacterial infection of the endocardium (a lining that covers the inside of the heart) and the heart valve [48,49]. Predominantly, IE is caused by Gram-positive cocci, which account for more than 80% of all endocarditis in humans [49]. The viridans group of streptococci are the most common players of endocarditis involving native heart valves in patients with congenital heart disease [48]. IE results from a combination of systemic infections

including bacteremia following oral surgery or various dental procedures and a predisposing condition of damaged heart valve [50,51]. *S. mutans* accounts for about 20% of IE cases attributed to viridans streptococci [47,52]. Viridans streptococci are a group of streptococci that produce greeninsh halo on blood agar plate (see later). Success in causing the diseases depends on two strategies: adherence to cardiac tissue and evasion of host immune response. To adhere to cardiac tissue, *S. mutans* expresses various cell surface associated adhesins. The serotype specific rhamnose-glucose polysaccharide (RGP) acts as a putative adhesins for binding of *S. mutans* cells to human monocytes, fibroblasts, and platelets [53]. Recently, two surface associated collagen-binding proteins Cnm and Cbm are also shown to be involved in adhesion and invasion human endothelial cells [54]. Once the organism reaches to heart valve, the next step is to facilitate growth of a coagulum at the site of adherence. Within this protective niche covered by platelets, bacteria multiply and damage host tissues that lead to formation of thrombotic vegetation. The dynamic or sequential events of bacteria-platelet interactions during colonization and vegetation formation on the heart valves are currently unclear.

Figure 2: Schematic representation of different communications and virulence strategies employed in dental plaque by *S. mutans*. *S. mutans* ferments different sugars to produce acid and uses ATPase pump for acidifying its environment while maintaining neutrality inside the cell. *S. mutans* also extracellularly synthesizes different polysaccharides such as glucan and fructan to adhere to tooth surface. These bacteria express different surface antigens, WapA, PavA, and SpaP. These proteins are help in coaggregation with other bacteria. *S. mutans* employs both AI-2 and CSP mediated quorum sensing mechanisms to communicate in a multispecies community. *S. mutans* produces inhibitory molecules mutanobactin and bacteriocins to destroy competing species.


CSP
Bacteriocins
Competence
(DNA uptake)
Com
Mutanobactin
AI-2
GBP
S. mutans
Aciduricity
F 1 / F 0
ATPase
H+
H+
H+
GTF
Glucan
Fermentation
Sugar
WapA
PavA
SpaP (Ag I/
II)
Lactic Acid

1.1.3. Classification of streptococci and diseases caused by major streptococcal pathogens

The genus streptococcus is a heterogeneous group of bacteria containing about 103 different species; many of them are involved various human and animal associated diseases. Based on 16S ribosomal RNA sequences, streptococci are phylogenetically divided into six groups: anginosus, bovis, mitis, mutans, pyogenic, and salivarius (Fig 3). This phylogenetic classification is further supported by numerous genome sequences and is the current method of choice for classifying streptococci.

There are two other traditional methods of classifications, one that uses Lancefield group antigens that are present on the cell surface and the other one is based on bacterial ability to lyse red blood cells. Lancefield classification contains twenty described serotypes named Lancefield groups A to H and K to V. There are some streptococci that have no described Lancefield group antigens and some with new antigens. Among the various Lancefield groups, group-A and group-B are by far the most common human pathogens. Streptococci are also classified into three hemolytic groups: α, β, and γ. The α-hemolytic group includes the viridans group of streptococci (oral streptococci) and produces partial hemolysis or green color around the colony by oxidizing the hemoglobin of the red blood cells. The β-hemolytic group includes mostly group-A and group-B streptococci that produce complete lysis of the red blood cells. Finally the γ-hemolytic group does not lyse the red blood cells at all; bacteria such as enterococci, lactococci (both of which are not considered as streptococci anymore), *S. salivarius*, and *S. thermophilus* are included in this group. It is important to mention that Lancefield group antigen does not always correlate with the species and the classification based on hemolysis is also not accurate.

Among the various streptococci, *S. pyogenes* (classified as group-A), *S. agalactiae* (classified as group-B), and *S. pneumonia* belonging to non Lancefield group antigen are perhaps the major human pathogens. *S. pyogenes* causes variety of diseases in humans ranging from mild to severe in nature. Infections such as pharyngitis (strep-throat) and impetigo (skin infections) are self-limiting and mild. In contrast, invasive diseases such as faciitis, bacteremia, and toxic-shock are more severe and life threatening. Although, *S. pyogenes* is generally found on the skin as commensal organism, it is often isolated from the oral cavity and saliva along with the other oral bacteria. The rate of colonization of *S. pyogenes* in the oral

cavity specifically in the oropharynx of children can reach as high as 10%. Perhaps less frequently, *S. pyogenes* can cause infective endocarditis along with the primary causative agents such as *S. mutans* and other viridans streptococci.

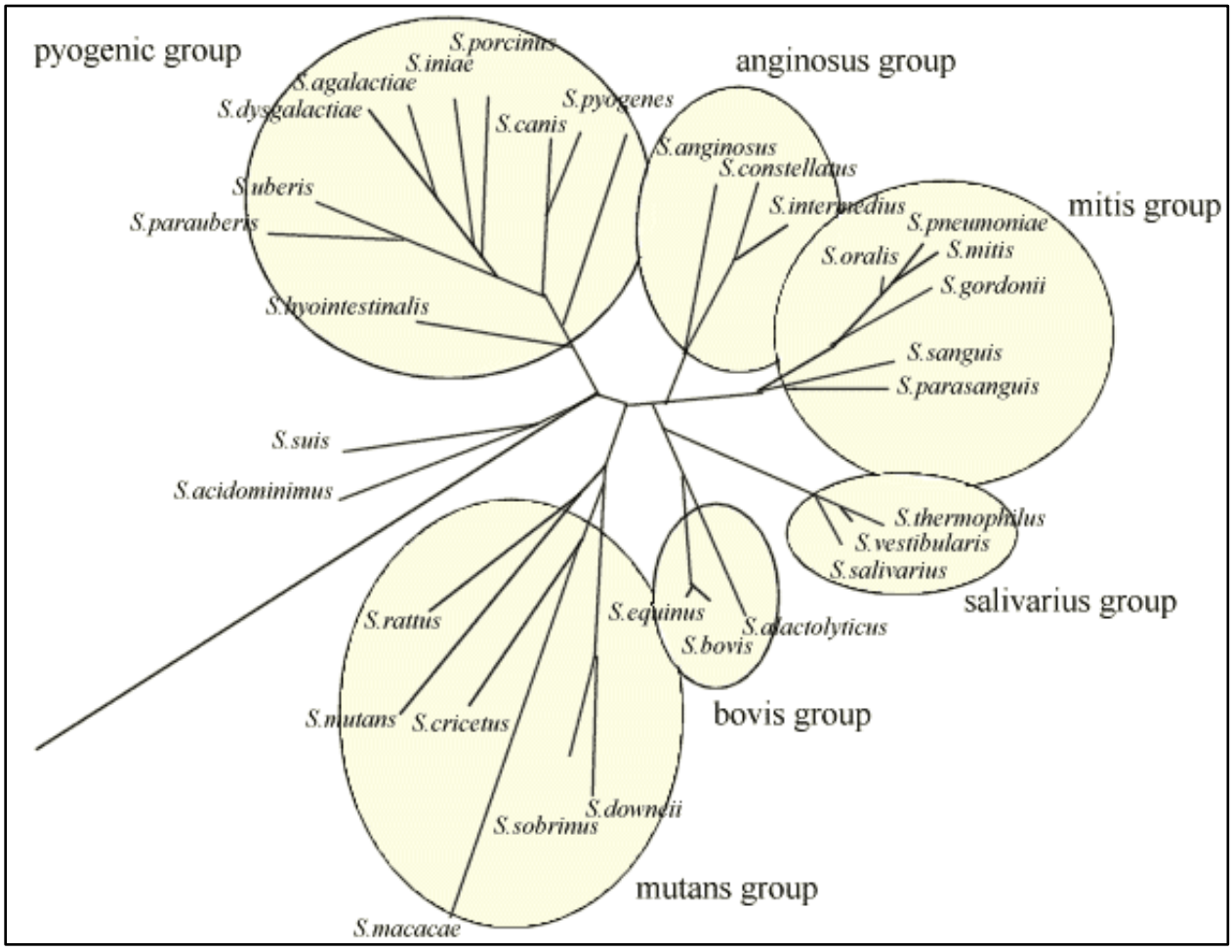
On the other hand, *S. agalactiae* is generally colonized in the female genital tract. Occasionally, *S. agalactiae* can be found in the nasopharynx. This organism commonly causes pneumoniae, meningitis, and sepsis in the newborns. *S. agalactiae* is also an important animal pathogen and can cause bovine mastitis. And finally, *S. pneumoniae* is an upper respiratory tract pathogen and the carrier rate of *S pneumoniae* in the normal human nasopharynx is as much as 20-40%. This capsular coccus has over 90 different serotypes. Although usually commensal, it is capable of causing pneumoniae, sinusitis, conjunctivitis, meningitis, bacteremia, sepsis, osteomyelitis, and endocarditis in immunocompromised individuals.

Apart from the streptococci that are discussed above, a few others streptococci can cause various human diseases. For example, viridans group of strptococci are the causative agents in 40-60% of endocarditis. Viridans streptococci are a heterogeneous group containing mainly α-hemolytic streptococci. The most important clinical representatives, other than *S. mutans,* are: *S. oralis*, *S. mitis*, *S. sanguinis*, and *S. gordonii*. Because these streptococci are part of the normal oral flora and are relatively avirulent, the course of endocarditis caused by these streptococci is slow as opposed to endocarditis caused by other bacteria. Streptococci belonging to bovis group also cause endocarditis. In particular, *S. gallolyticus* is often cuases IE in patients with colon cancer [55]. *S. gallolyticus* is an emergent pathogen and recently it has been shown to be associated with colon cancer as well as bacteremia, meningitis, and other illnessess [56].

Interestingly, there are many streptococci that are beneficial to humans as they play a protective role when present in the microbiota. For example, *S. salivarius*, an oral bacterium, can antagonize caries formation and pharyngitis because it produces antimicrobial peptide called bacteriocins. Oral

streptococci in general are a prolific producer of various bacteriocins (see below). Bacteriocins produced by commensal streptococci are of major interests in the drug industry because they have the potential to inhibit many important pathogens including the pathogenic streptococci.

Figure 3: Classification of streptococci based on 16S rRNA analysis. Phylogenetic relationship among various streptococci species is depicted here. *S. suis* and *S. acidominimus* could not be placed into any of these six groups. Not all streptococci are listed. [Adapted from Kawamura et. al., 1995 [26]]


pyogenic group
S.porcinus
S.iniae
S.agalactiae
S.dysgalactiae
S.pyogenes
S.canis
S.uberis
S.parauberis
S.hyointestinalis
anginosus group
S.anginosus
S.constellatus
S.intermedius
mitis group
S.pneumoniae
S.oralis
S.mitis
S.gordonii
S.sanguis
S.parasanguis
S.suis
S.acidominimus
S.thermophilus
S.vestibularis
S.salivarius
salivarius group
S.rattus
S.equinus
S.bovis
S.dactolyticus
bovis group
S.mutans
S.cricetus
S.downei
S.sobrinus
mutans group
S.macacae

1.1.4. Cell-cell signaling in dental plaque

Multispecies colonization initiates from direct molecular interaction between two organisms, which eventually establishes a community. In this community of dental plaque recycling of metabolic byproducts among various bacterial species takes place. Community supplied metabolic byproducts can be potentially used as a source of nutrition by another organism [57]. For example, Veillonellae are unable to utilize sugar but can ferment lactic acid which is a common metabolic end product produced by different streptococci as well as actinomyces [57,58]. A similar symbiotic interaction between *S. oralis* and *A. naeslundii* was observed. When nutrient-limited saliva was provided as the sole energy source both species could grow together, suggesting that these species are fitted for each other. However, when cultivated independently neither of these two could survive [59]. This observation is reinforced in the case of *P. gingivalis,* which stimulates biofilm formation by *F. nucleatum*. Surprisingly, *S. sanguinis*, an early colonizer produces para-aminobenzoic acid that is utilized as a source of vitamin by *S. mutans* during anaerobic growth [58]. Various physiological parameters such as pH, oxygen, metabolites, and toxins of the dental plaque can govern the composition of microbial diversity in the biofilm. Different incompatible microorganisms can coexist within the heterogeneous environment of the oral biofilm and facilitates the creation of complex symbiotic networks [60].

Bacterial cells also communicate within and across the species using various signaling molecules (Fig 2). Although, the majority of the studies were done to understand the intrasepcies communication using monospecies culture, only a few studies have been reported about the interspecies communication using multispecies systems. In this section we will focus on how the interspecies communication occur in oral biofilm between *S. mutans* and other organisms, as well as how *S. mutans* senses and responds to different environmental cues using two-component signal transduction systems.

Bacterial communication that depends on cell density is generally termed as quorum sensing. Quorum sensing usually occur when an organism senses and responds to a critical concentration of small extracellular molecules, generally termed as autoinducer (AI). Autoinducers are either small chemical moieties or small peptides that are produced constitutively as the cell population grows. When a

threshold concentration is achieved, autoinducer molecules trigger a change in expression of various genes in the population. In this way, a bacterial population can behave in a highly coordinated fashion, similar to multicellular organisms. Quorum sensing can control antibiotic production, expression of virulence genes, formation of biofilm, competence development, sporulation, and several other biological processes [61]. Three different signaling molecules mediate quorum sensing: AI-1, AI-2, and small peptides. AI-1 signaling is restricted to only Gram-negative bacteria and is involved in intraspecies communication. Hence, we will only discuss the function of AI-2 in the following section.

1.1.4. a. Auto inducer (AI)-2 signaling

Both Gram-positive and Gram-negative organisms produce AI-2. AI-2 is generally referred as LuxS/autoinducer-2 system. They are generated from a common metabolite SAM by the action of LuxS enzyme. AI-2 is a collection of interconvertable forms of 4, 5-dihydroxy-2, 3-pentadione (DPD), which undergoes structural changes depending on the environmental cues. In dental biofilm, actinomyces, fusobacterium, porphyromonas, and streptococcus are producers of AI-2. In *S. mutans,* AI-2 signal production occurs at mid-exponential growth phase. A total of 585 genes (30% of total genes) were differentially expressed among the wild type, a *luxS* mutant, and chemically complemented mutant strains [62]. These genes are involved in cell division, cell growth and stress response. Moreover, the *luxS* mutant was also biofilm defective [63]. Interestingly, this defect in biofilm formation by *S. mutans luxS*-deficient strain can be restored by the addition of diffusible molecules produced by *S. anginosous*, *S. gordonii*, and *S. sobrinus*; but not by *S. salivarius*, *S. sanguinis*, or *S. oralis* [63]. The *luxS* gene appears to be well conserved among many oral bacteria and can be involved across the species signaling. Another phenotype assigned to an *S. mutans luxS* mutant is the production of a particular type of bacteriocin, called mutacin I [64]. Bacteriocins are small antimicrobial peptides used for intra- and interspecies competition by inhibiting the growth of competing bacteria (See section 1.2). Deletion of *luxS* completely abolished the mutacin I expression; however the molecular mechanisms by which LuxS represses mutacin expression is yet to be uncovered. The control of bacteriocin expression in the dental

plaque by AI-2 signaling is highly significant in the context of competition because it can determine which species will be successfully establised in the oral biofilm.

1.1.4.b. Two-component signal transduction systems

Oral bacteria need to constantly adapt to various fluctuations in their surrounding environments. They also need to compete with other organisms within the complex multispecies biofilm for nutrient acquisition by responding to available dietary products. To survive in the oral cavity, bacteria employ various sensory systems commonly known as two-component signal transduction systems (TCS) to monitor and respond to the environmental cues. This system is composed of two protein molecules, one of which is a membrane-bound sensor kinase and the other one is a cytoplasmic response regulator. The function of sensor kinases is to perceive the environmental signals where as the response regulators integrate that information by changing the gene expression pattern as an adaptive response. The number of TCS varies greatly depending on the organism. One recent comprehensive study of TCSs from eight *S. mutans* strains revealed that total 18 orthologs of TCSs, two orphan sensor kinases, and two orphan response regulators are present in *S. mutans* [65]. Not all the TCSs are present in every *S. mutans* isolates. Some key TCSs, necessary for surviving in the oral cavity, are present in all the isolates. Most of the *S. mutans* strains contain about 14 TCSs of which eight are common to all the isolates. These eight TCSs are involved in various stress responses, competence development, various virulence gene expression, biofilm formation, and bacteriocin production [66,67]

1.1.4.c. Competence-stimulating peptide (CSP) mediated signaling

S. mutans and streptococci encode a peptide mediated quorum sensing pathway that also requires a TCS. This system is required for development of competence in many streptococci. Central to this pathway is a small peptide called competence-stimulating peptide (CSP). In *S. mutans* the CSP, which is encoded by the *comC* gene, is synthesized as a 46-mer prepeptide. NlmTE, an ABC transporter, processes this prepeptide to a mature 21-mer peptide during secretion. In *S. mutans*, but not in other streptococci, this

21-mer peptide is further processed to a 18-mer active peptide by an extracellular protease named SepM [68]. When this active 18-mer peptide reaches a critical dentisty, it acts as a signal to induce ComD, a histidine kinase. ComD then activates the cognate response regulator ComE, which then activates expression of various genes including those required for competence development. The genes encoding *comC*, *comD*, and *comE* are organized in an operon and this operon is under the positive feedback regulation. In *S. mutans* and some other streptococci, CSP also activates expression of various bactertiocin and bacteriocin-like genes. CSP mediated signaling is also ubiquitous among early colonizers in the dental plaque. This includes *S. mitis*, *S. oralis*, *S. gordonii*, *S. sanguinis* from the mitis group, and *S. constellatus*, *S. intermedius*, *S. anginosus* from the anginosus group. Notably, CSP signals are highly variable among different streptococcal strains including *S. mitis*, but in the anginosus group, CSP signals are highly similar. Thus, it indicates that CSP might be involved in interspecies communication at least in the anginosus group of streptococci. Since CSP is involved in the expression of bacteriocin genes to inhibit the growth of other competing species, this signaling pathway is also crucial for the establishment of a species in the dental plaque.

1. 2. Bacteriocins produced by Gram-positive organisms

In order to survive in a competitive multispecies environment such as in the dental plaque, many bacteria produce different antimicrobial peptides that are active against other bacteria. These ribosomally synthesized small antimicrobial peptides produced by bacteria are called "bacteriocins". These bacteriocins can be either broad or narrow spectrum. When the bacteriocins are active against a diverse species, they are called broad spectrum and the bacteriocins that are only active against related species are called narrow spectrum. The first bacteriocin, colicin, was identified from the Gram-negative organism *E. coli* in 1925. Bacteriocins produced by Gram-negative bacteria are generally narrow spectrum and only active against its own species or very closely related species. On the other hand, bacteriocins produced by Gram-positive organisms including lactic acid bacteria (LAB) are of prime interest in the pharmaceutical industry because these bacteriocins are usually broad spectrum.

In order to get FDA approval of any bacteriocin to be used in food industry the producer strain is required to be nonpathogenic in nature. The GRAS (generally recognized as safe) status assigned by FDA to lactic acid bacteria made them an attractive choice as bacteriocin producer for industrial application. LAB used in cheese production is believed to secrete bacteriocins that determine the complex nature of the microflora needed for cheese manufacturing and prevent the growth of spoilage and pathogenic organisms.

A large, diverse array of Gram-positive bacteriocins has been discovered and their structures have been reported [69], along with their mode of action, synthesis, and self-immunity mechanisms. Furthermore, a growing interest to modify the existing bacteriocin by using different modification enzymes is highy apparent. This approach might improve the stability and efficacy of the exsiting bacteriocins already tested for commercial application. Although, naturally occurring modified bacteriocins are more resistant to heat and protease degradation than the unmodified bacteriocins, nevertherless they are unstable under alkaline pH and are degraded by proteases present in stomach. Therefore, unless the stability is improved by bioengineering, even the modified bacteriocins are only good to be used for topical applications in the pharmaceutical industry. In the food industry a modified bacteriocin, nisin, has been used over 50 years to inhibit spoilage bacteria mainly *Clostridium difficile* and *Listeria monocytogenes*. The encouraging news is that there is no resistance against nisin is observed till today and nisin is the most used commercial food preservetive. This phenomenon suggests that bacteria do not develop resistance easily against bacteriocins and remain susceptible to the bacteriocin inhibitions. Therefore, overall bacteriocins have a potential to be used as an alternative to antibiotics in the era when multidrug resistant bacteria such as methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *E. faecalis* (VRE) are highly prevalent. Recently, few modified bacteriocins showed promising activity against MRSA and VRE. Bacteriocins have two properties that prevent the development of resistant organism. First, the mode of inhibition by bacteriocins towards susceptible organism is highly rapid therefore the time required to generate resistant organims is not enough. Second, since the bacteriocins are protineaceous in nature, they are easily degraded inside the human body leaving little chance for resistance development in the

environment. In the following section, we will describe how various bacteriocins are classified.

1. 2.1. Classification of bacteriocins

Bacteriocins produced by Gram-positive organisms are broadly classified into two distinct categories. The class I bacteriocins are post-translationally modified bacteriocins and are called lantibiotics because they contain polycyclic thioether amino acids lanthionine or methyl lanthoinine. The class II bacteriocins are unmodified linear or cyclic and are called nonlantibiotics. Table-1 summarizes the classification of various bacteriocins.

1.2.1. a. Lantibiotics

Lanthionine or methyl lanthionine containing bacteriocins are called lantibiotics. The characteristic properties of lantibiotics are the presence of post-translationally modified unusual amino acids. The most common modifications are serine residue modified to dehydroalanine and threonine modified to dehydrobutyrine. These two modified residues are present in almost all lantibiotics. The chemical bonds between dehydroalanine and dehydrobutyrine to cystine are called lanthionine and methyl lanthionine bonds, respectively. These modifications and ring structures make those lantibiotics more tolerant to heat, proteolysis, and oxidation. These lantibiotics are further classified into two groups depending on their modified structure type-A (I) and type-B (II) [70]. Type-A lantipeptides are elongated in nature whereas type-B lantipeptides are globular ([71]). Nisin produced by *L. lactis* and pep5 produced by *S. epidermidis* are two examples of type-A lantibiotics. Globular mersasidin from *B. subtillis* and actagerdin from *Actinoplanes sp.* are examples of type-B lantibiotics. Additionally, lantibiotics can consist of a single peptide (one-peptide) or two separate peptides (two-peptide). One-peptide lantibiotic can be either type-A or type-B. As mentioned above, nisin, which is a type-A and mersasidin, which is a type B, are both one-component lantibiotics. On the other hand the two-component lantibiotics usually contain one of each type-A and type-B components. Lacticin 3147 is a well-known example of two-peptide lantibiotics, which comprises of globular Ltnα (type-B) and elongated Ltnβ (type-B) components. Another classification scheme is devised on the basis of the pathway of modification of the peptide

lantibiotics [71]. This scheme is simple and flexible enough to include all lantithionine-containing peptides. Moreover, this inclusive method may accommodate the lantibiotics that are yet to be discovered.

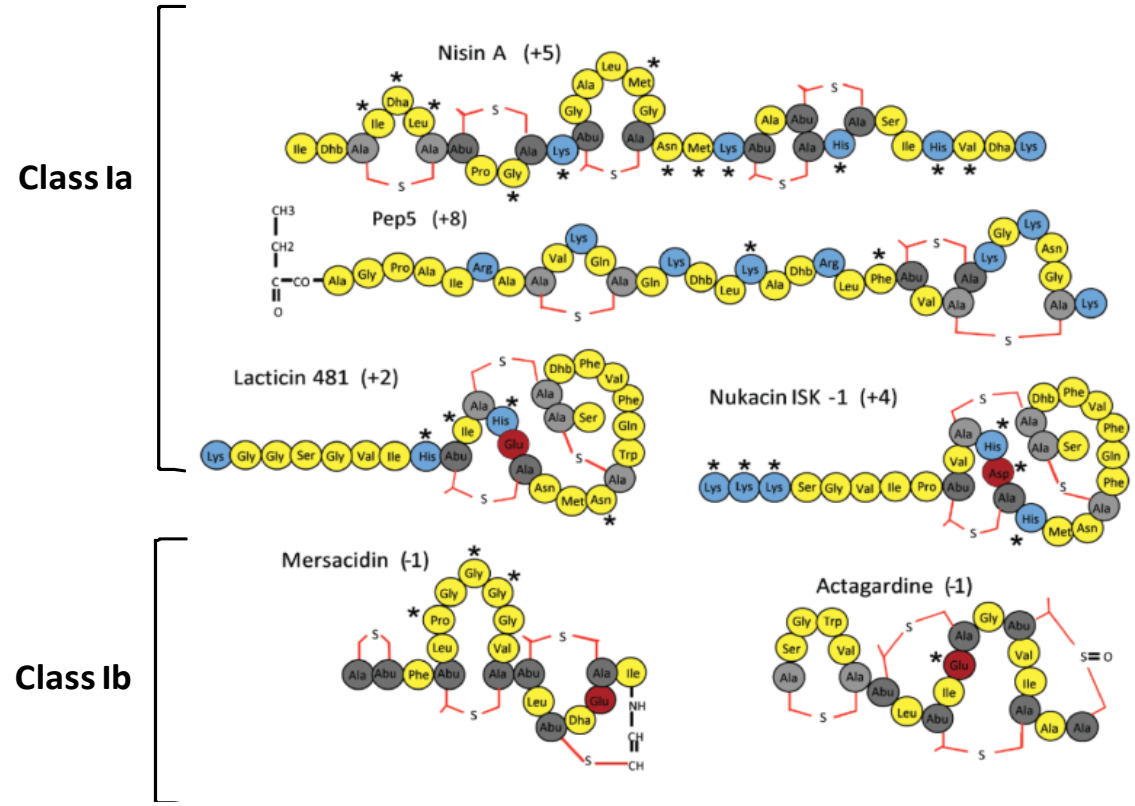
Class-I lantibiotics are modified by two modification enzymes, one is specific for serine/threonine dehydration and the other one is specific for thioether cyclization. In general the dehydratase and cyclase enzymes are termed as LanB and LanC enzymes, respectively. The modified peptides generated by these two enzymes are more linear in nature than the class-II peptides. Class-II lantibiotics are modified by a single bifunctional enzyme termed as LanM that posses both dehydratase and cyclase activity in their N- and C-terminal domains, respectively. However, the homology between LanM with LanB or LanC is not significant. Class-II group is structurally more diverse and represents a larger body of members than class-I lantibiotic. The unique members of class-II lantibiotics are the two-component lantibiotics. Lantibiotics belonging to class-III are modified by a trifunctional enzyme containing a lyase domain, a kinase domain, and a cyclase domain; however, this enzyme lacks many conserved active site residues found in LanC/LanM. And finally, class-IV lantibiotics are synthesized by LanL enzyme, which is similar to class-III enzyme, except that the cyclase domain is analogous to LanC. The classifications just described above are based on the modification on the lantithionine peptides (Fig 4). However, there is one other classification exists where the latibiotics are subdivided into 11 groupes based on the sequence homology with the unmodified lantibiotics [72]. The groups are named after the 11 representatives from each group and they are: cinnamycin, cytolysin, epidermin, lacticin 481, lactocin S, LtnA, mersacidin, nisin, pep5, streptin, and sublancin. To date, seven two-peptide lantibiotics have been identified; among them six are mersacidin-like systems (lacticin 3147 produced by *L. lactis* DPC3147, staphylococcin C55 produced by *S. aureus* C55, plantaricin W produced by *Lactobacillus plantarum*, Smb prodcced by *S. mutans* GS-5, BhtA produced by *S. rattus* BHT, and haloduracin produced by *Bacillus halodurans*). The seventh two-component lantibiotic is cytolysin, produced by *E. faecalis.* Both the components of cytolysin are similar to each other and it is the only example of this type.

Table 1: Classification of bacteriocins produced by Gram-positive bacteria

Classification	Description	Examples
Class I	Post-translationally modified peptide lantibiotic	
Type Ia	Elongated peptide with net positive charge	Nisin, Pep5
Type Ib	Globular peptide net negative or no charge	Mersacidin, actagardin
Type Ic	Multi component	Lacticin3147, SmbAB
Class II	Unmodified, non-lanthionine, heat stable	
Type IIa	Monopeptide with YGNGVGVXC motif	PediocinPA1, Leucocin A
Type IIb	Two peptide GxxxG motif	NlmAB, Lactococcin G
Type IIc	Heterogeneous	Lactococcin A, Lacticin Q
Class III	Large heat labile	
Type IIIa	Bacteriolytic	Lysostaphin
Type IIIb	Nonbacteriolytic	Helveticin J
Class IV	Cyclic peptide; no sub-class	Enterocin AS-48, Gassericin A

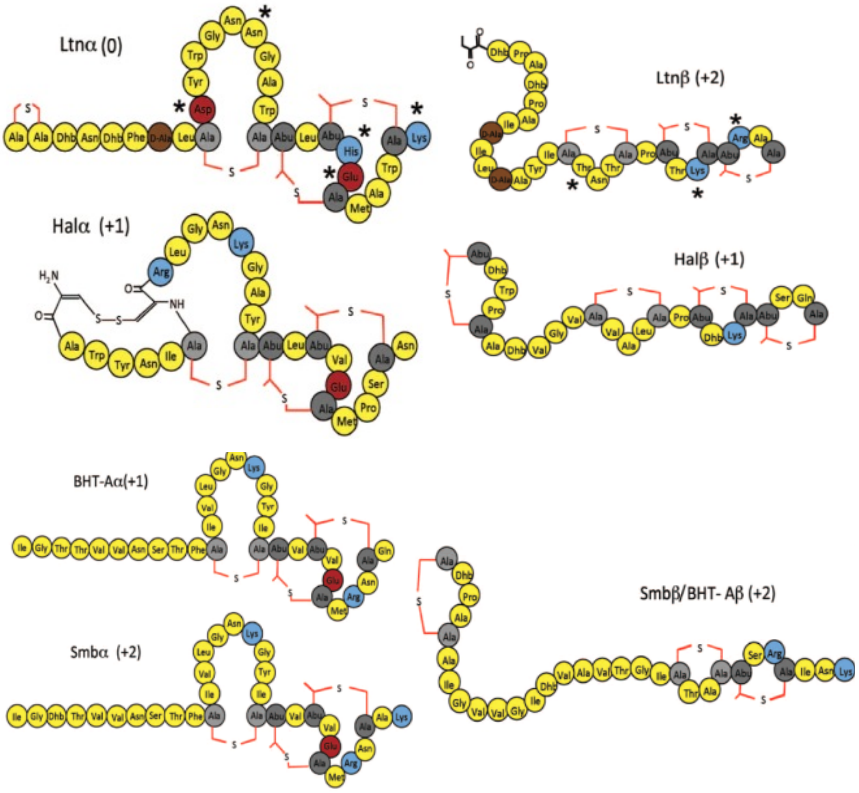
Figure 4: Representative examples of lantibiotics, a class I bacteriocin. (A) Examples of one-peptide lanticbiotics belonging to class Ia (net positive charge containing elongated lantibiotiics) and class Ib (globular lantibiotics). (B) Examples of some two-peptide lantibiotics belonging to class Ic are shown here. Positively charged residues are colored blue, negatively charged residues are colored maroon, where as lanthionine and methyl lanthionine are colored as light and dark grey [Adapted from Suda et. al. 2010, [73]].

(A)


Class Ia
Nisin A (+5)
Pep5 (+8)
Lacticin 481 (+2)
Nukacin ISK -1 (+4)
Class Ib
Mersacidin (-1)
Actagardine (-1)

(B)

Class Ic



1.2.1. b. Non-lantibiotics

Bacteriocins belonging to class-II are not post-translationally modified; in other words, they are non-lanthionine containing bacteriocins. It is important to point out that class-II lantibiotics, which are just described, and class-II bacteriocins are entirely different. Class-II bacteriocins are relatively simple in structure and mostly linear peptides, although some of them can be circular (Table 1). Class II bacteriocins are subdivided into four groups: class IIa, class IIb, class IIc, and class IId. Class IIa bacteriocins are called pediocin-like bacteriocins becuase pediocin, the first bacteriocin in this class, is prouced by *Pediococcus sp*. All pediocin-like bacteriocins, of which more than 20 have been identified, contain an N-terminal consensus sequence YGNGVXC, called "pediocin-box" motif. Pediocins-like bacteriocins are highly active against listeria and are used as bioperservative [72]. Class IIb bacteriocins are two-peptide bacteriocins. Lactococcin G produced by *L. lactis* and mutacin IV produced by *S. mutans* are the member of class IIb bacteriocin family. Class IIc bacteriocins are circular bacteriocin where the N- and the C- terminals are covalently linked by a peptide bond. Enterococcin AS-48 produced by a variety of enterococci was the first class IIc bacteriocin identified. The last group of unmodified bacteriocins (class IId) constitutes the remaining linear, non pediocin-like, one-component bacteriocins. This group of bacteriocins is highly diverse in nature both in terms structure wise and activity wise, and therefore it requires further sub-classification. Some members of this group have a leader peptide that is processed during their transport by the Sec dependent mechanism. For example, the plasmid-encoded lactococcin 972 that is produced by certain strains of *L. lactis* utilizes Sec dependent pathway for secretion. In contrast, there are a group of class IId peptides that do not contain a leader sequence and are called leaderless bacteriocins. Two prominant examples are lacticin Q and lacticin Z, both secreted by different *L. lactis* strains. Despite the differences in structure (linear, cyclic, or circular), biochemical properties (dehydrated residues, thioether bonded residue, or presence of motifs), as well as the composition (one-component or two-component), most of the bacteriocins function in a quite convergent manner and they all interfere with the bacterial cell-wall or membrane integrity.

1.2.2. Mode of action of lantibiotics

As mentioned before, bacteria employ bacteriocins as a weapon to inhibit other competing species. These antimicrobial peptides are very potent and active at a nanomolar concentration. Modes of action of a given bacteriocin can be predicted from the primary structure of the peptides. In this section, we will mainly focus on the mechanisms of inhibition by lantibiotics (Fig 5).

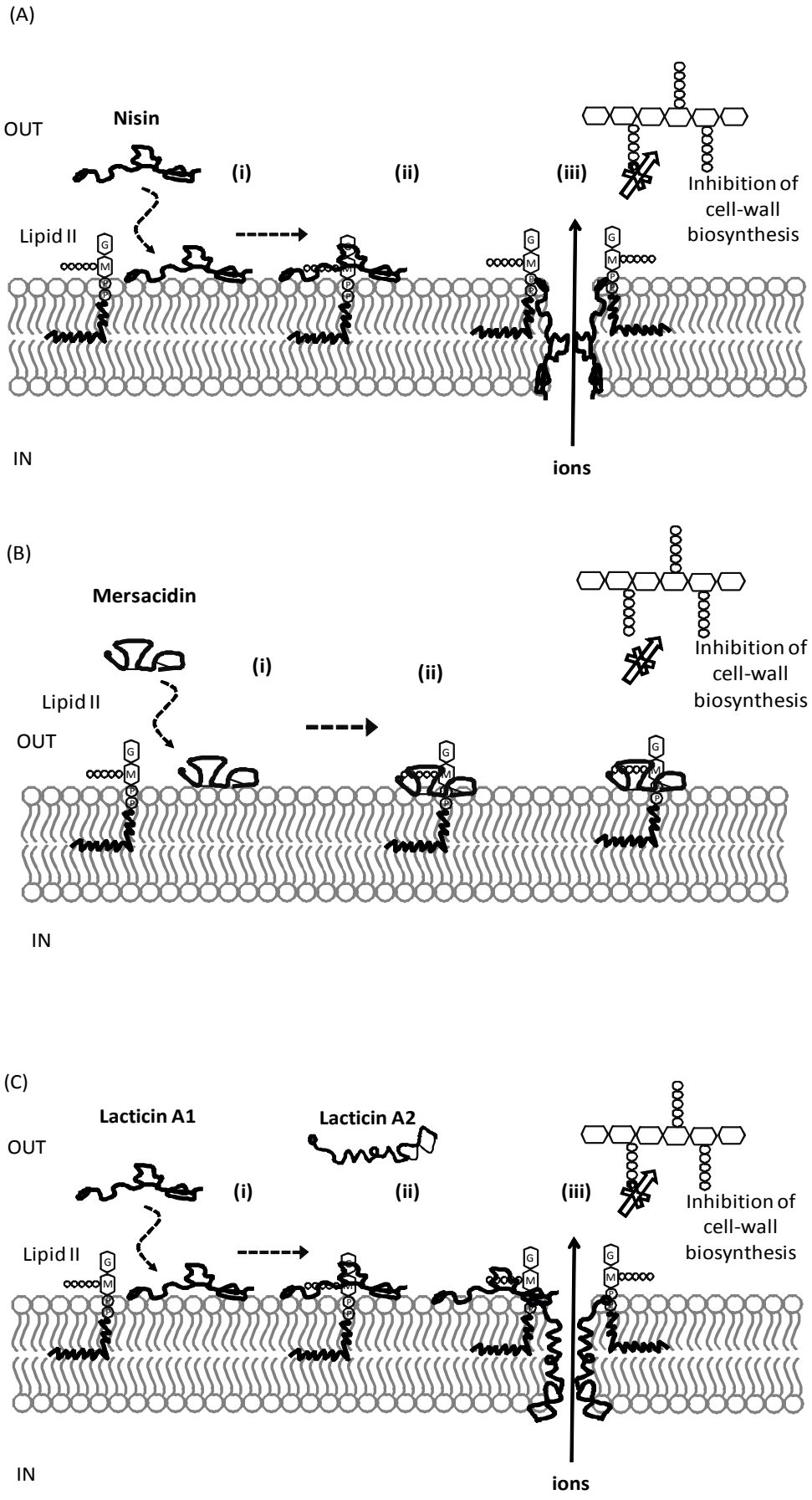
Type-A lantibiotic is elongated, amphillic, or hydrophobic peptide and often contains a net positive charge. Nisin is one such kind whose mode of action was experimentally characterized. Nisin and related lantibiotics have activity against a wide range of Gram-positive bacteria. Studies using artificial and physiological membrane revealed that nisin adheres to the membrane by electrostaic interaction with the membrane lipids (Fig 5A). Mutagenesis studies suggested that the initial interaction of nisin with the membrane is through the C-terminal of the peptide that contains most of the cationic residues. Nisin inserts into the membrane mediated by hydrophobic residues on the N-terminal side of the peptide. The first ring or ring A on the N-terminal side is involved in insertion in the membrane; any change in that ring region interferes with the insertion and eventual decrease in antimicrobial activity. However, C-terminal side of nisin has no effect in the membrane insertion process. Not only the modified part but also the linear or the hinge region of nisin (ie. region between the ring C and ring D) is required for membrane insertion. Finally, nisin molecules oligomerize to form a pore complex at the membrane.

Lipid II is a conserved precursor of bacterial cell wall biosynthesis. Nisin can form pores at a much lower concentration and more efficiently if lipid II is present at the artificial membrane. Afterwards, NMR analysis revealed that nisin interacts with the lipid II moiety at the pyrophosphate bridge between the lipid carrier and the disaccharide. Moreover, for this interaction, only first ten amino acids (ring A and ring B) are necessary for binding (Fig 6). A conserved binding sequence, CTxTxEC, was identified among a number of lantibiotics that target lipid II. Some lipid II binding lantibiotics such as mersacidin, plantaricin C, or actagerdin do not have ability to cross the membrane bilayer to form pore, rather they inhibit cell wall biosynthesis by interfering with lipid II avalability (Fig 5B). In contrast, epidermin like lantibiotics such as gallidermin, epidermin, and mutacin 1140 bind to lipid II, but can only cross thin

layer of membrane as shown in some *in vitro* studies. Interestingly, gallidermin is active against only micrococcus and staphylococcus but not against lactococcus. Evidently, it was reported that short chain lipids are present in micrococcus and staphylococcus membranes but not in lactococcus. A new member of lipid II binding lantibiotic, michiganin, contains the conserved CTxTxEC motif, but it is not inside the ring structure as in the case of mersacidin or actagardin. However, the mode of action for this new type has not been evaluated.

In the case of two-peptide lantibiotics, the α-peptides are the type-B globular peptides and the β-peptides are the type-A elongated peptides. The α-peptides contain the lipid II binding motif. A concerted effort involving both the component peptides is required for the antimicrobial activity. A three-step model has been proposed to explain the mode-of-action of the two-component lantibiotics (Fig 5C). In this model, the inhibition process is initiated by the α-peptide, which first binds to lipid II molecule. Next the β-peptide recognizes the complex of α-peptide bound lipid II and forms a tripartite complex. This tripartite complex formation helps the β -peptide to get inserted inside the membrane and subsequently to form pores. It has been shown that the two ring structures present at the C-terminal end of the α-peptide of lacticin 3147 are involved in lipid II binding as well as binding to the β-peptide. This C-terminal domain is necessary for the activity of the peptide complex. Type-B lantibiotic cinnamycin and most likely duramycin also bind to phospholipid phosphatidylethanolamine and inhibit phospholipase activity. At present, it is not clear why the efficient inhibitory activity against the peptidoglycan synthesis as well as pore formation capacity of the lantibitics function only against certain strains or species instead of all the strains or species.

Figure 5: Proposed models for the mode of action of different classes of lantibiotics. (A) Class Ia lantibiotic such as nisin, adsorbs to the membrane of the target cell and eventually interacts with the lipid II molecule. While bound to lipid II nisin not only sequesters this cell wall precursor it also forms pore complex at the target site. (B) Class Ib lantibiotic like mersacidin also binds to the lipid II and inhibits wall synthesis of the target cell, but do not for pore. (C) Class Ic two-peptide lantibiotic such as lacticin 3147 contains a globular component, lacticinA1, which interacts with the lipid II molecule and inhibit cell wall synthesis. The second peptide, lacticinA2, interacts with lipid II bound lacticinA1 and forms pore in the target cell membrane.


(A)
OUT
Nisin
(i)
(ii)
(iii)
Inhibition of cell-wall biosynthesis
Lipid II
IN
ions
(B)
Mersacidin
(i)
(ii)
Inhibition of cell-wall biosynthesis
Lipid II
OUT
IN
(C)
Lacticin A1
Lacticin A2
OUT
(i)
(ii)
(iii)
Inhibition of cell-wall biosynthesis
Lipid II
IN
ions

Most of the nonlantibiotic bacteriocins form pores but the size of the pore varies depending on the type of bacteriocins. The mode of action for class IIa bacteriocins, such as pediocin, involves mannose phosphotransferase system (Man-PTS), which acts as a recruiter for the bacteriocins to membrane to form pore on the susceptible organisms. These pores are relatively small but allow loss of proton gradient to occur that ultimately leads to death of the sensitive strains. Class IIb two-peptide bacteriocin consisting two different unmodified peptides functions at equimolar ratio. Class IIb bacteriocin such as lactococcin G and lactococcin Q permeabilize membrane of the sensitive strain by pore formation [74]. The α- and β-components of the above mentioned bacteriocins are interchangeable without significant loss of activity. The pore formed by these two-peptide bactericins is relatively large that allows leakage of monovalent cations, phosphate ions, and ATP through the pore leading to death of the susceptible bacteria. A similar mechanism has been proposed for class IIc bacteriocins. As mentioned before, class IId bacteriocins are structurally diverse. Lactococcin A and lactococcin B both are suggested to form pores in the membrane and loss of solutes kills the susceptible bacteria. Lacticin Q, on the other hand is a leaderless bacteriocin, and it interacts with the lipid II molecule to form a relatively large peptide-lipid pore [75]. It is the first example of a nonlatibiotic that interacts with the lipid II molecule. Another class-IId bacteriocin, lactococcin 972 (lcn972), interacts with lipid II and inhibits cell wall biosynthesis. Although it interacts with lipid II, Lcn972 does not contain N-terminal ring structure like nisin. The binding site of Lcn972 might divulge a novel lipid II binding mechanism. For class-IV bacteriocin, enterocin AS-48 forms nonselective pores that allow loss of low molecular weight substances leading to cell death [76]. In contrary, carnocyclin A, another class-IV bacteriocin, forms anion specific pores and causes depolarization of the membrane. Thus, while most of the nonlantibiotic peptides form pores on the target cell membrane, the size of the pore and solutes that escaped through them varies significantly.

1.2.3. Immunity proteins

Bacteriocin producer strains are usually protected from their own antimicrobial activity by concomitant expression of one or more immunity protein complexes. Three different immunity mechanisms that are

present in the producer strain: an ABC transporter complex that exports out bacteriocins, a membrane protein that sequester the bacteriocins before they reach to the membrane, and antagonizing the receptor of the bacteriocins. Immunity proteins for nisin and lacticin 3147 have been well characterized and they function in somewhat similar manner. In case of lacticin 3147, an ABC transporter complex, LanFEG, and a membrane bround protein LanI or LanH that sequesters bacteriocin, provide self-immunity [77]. LanF of the ABC-transporter complex has nucleotide-binding domain and functions as ATPase component of the complex. This complex is known to scavenge out the self-produced lantibiotic from its own membrane. This type of immunity provided by ABC-transporter is very common. For instance, SpaFEG of subtilin producer *B. subtilis* and NisFEG of nisin producer *L. lactis* both function in similar manners.

The LanI proteins are subdivided into three groups: lipoproteins such as NisI and SpaI, immunity peptides residing on the membrane or at the extracellular space such as PepI and EciL, and transmembrane proteins such as LtnI, CylI, and SunI. The degree of immunity provided by these proteins can vary greatly and the exact mechanisms of their function are not well studied. For example, LanI or LanH cannot provide complete protection against lacticin 3147 in the absence of corresponding ABC transporter complex. For one LanI member, NisI, the C-terminal domain is involved in the binding of lantibiotics (nisin) and the N-terminal domain is involved in interacting with the NisFEG complex. In contrast, PepI has been shown to attach to the outer side of the membrane and shields the target from the lantibiotic action.

In case of nonlantibiotic bacteriocins, sequences of the immunity protein and their sizes vary significantly and the detail mechanisms of protection are also not well understood [78]. However, studies on lactococcin A revealed the detail mechanism of how the immunity protein LciA confers immunity. During purification, LciA was found to be associated with the membrane fraction of the producer strain. It was demonstrated that in the presence of the cognate bacteriocin lactococcin A, the immunity protein associates tightly with the membrane components IIC and IID of man-PTS system; but in the absence of the bacteriocin, LciA remained mostly unassociated [79]. Although there are variations in the sequence

and in the size of the immunity proteins, for class IIa bacteriocins most of the immunity proteins function in similar manners [80]. For class IIb bacteriocins the lactococcin G immunity protein, LagC, was predicted to bind with a yet to be indentified membrane-associated receptor to interact the lactococcin G [81]. Recently, it has been shown that immunity protein might use protease activity to confer immutnity [80]. In particular, a CAAX family protease, called as Abi-family protein, is a putative transmembrane associated protein involved in the immunity function against sakacin [82]. The immunity protein, SkkI, was isolated from *Lactobacillus sakei,* which also produces sakacin (a class IIb bacteriocin). It was shown that the immunity function is lost if the putative protease active site residues are mutated in SkkI [82]. Though class II bacteriocin family immunity proteins are somewhat specific, Abi-family immunity proteins are observed to confer cross-immunity [82]. It is hypothesized that a common proteolytic mechanism may confer the immunity; however the bacteriocin itself is not the substrate. Therefore, the exact mechanism by which the Abi-family proteins confer immunity in bacteria is currently unknown. For class IIc and class IId family bacteriocins the immunity proteins are suggested to be either ABC transporters or other hydrophobic peptides. Further elucidation is needed to understand these diverse mechanisms of bacteriocin immunity in bacteria.

1.2.4. Protein receptors for bacteriocins

An organism is susceptible to a given bacteriocin because they carry a receptor protein. Initial studies with bacteriocin resistant mutants of *E. faeccalis* and *L. lactis* indicated that man-PTS might act as a receptor for class IIa bacteriocins [83]. The bacterial man-PTS system contains three proteins: IIAB, IIC, and IID, encoded by a single operon. When the entire locus encoding man-PTS was deleted from a sensitive lactococci strain, the strain became resistant to the lactococcin A bacteriocin [83]. Moreover, introduction of IIC and IID together in a resistant *L. sakei* strain made the cells sensitive to lactococcin A. Other pediocin-like bacteriocins, enterocin P, sakacin A, pediocin PA1, and penocin A have inhibitory activity against *L. sakai* but not against *L. lactis* IL1403 while both contains man-PTS. However, when the man-PTS from *L.sakai* was expressed in *L. lactis*, it became sensitive to the bacteriocin [83]. This

observation confirms that in general for class II bacteriocin, components of man-PTS function as potential receptor. The consensus sequence at the N-terminal of pediocin-like bacteriocins probably interacts with the receptor. Subsequently, the C-terminal α-helix gets inserted in the membrane and interacts with the man-PTS. This interaction causes structural changes in the man-PTS and the permease opens as a pore. This eventually leads to the loss of solutes that ultimately kills the bacteria. Understanding the motif present on bacteriocin molecule involved in man-PTS interaction will aid in designing more potent bacteriocin by bioengneering method. Since man-PTS is absent in eukaryotes, use of this target by class II bacteriocin will not have any adverse effect on the host.

So far no lantibiotics receptors have been identified for lantibiotics. However, for some lantibiotics, lipid II has been proposed as a docking molecule (i.e. receptor), but how the initial interaction between the lipid II and the lantibiotics at the membrane happens is currently not known.

Figure 6: Schematic diagram of nisin processing and maturation. The leader peptide of the precursor nisin directs this peptide to the modification machinery and to the transporter for processing and secretion. Specific serine and threonine residues are dehydrated by NisB and converted to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Next, NisC cyclase forms lanthionine bond between Dha and cyscteine, and methyllanthionine bond between Dhb and cysteine. NisT transports out this fully modified nisin and NisP protease removes the leader peptide to generate active nisin [Adapted from Cotter et. al. 2005, [72]].

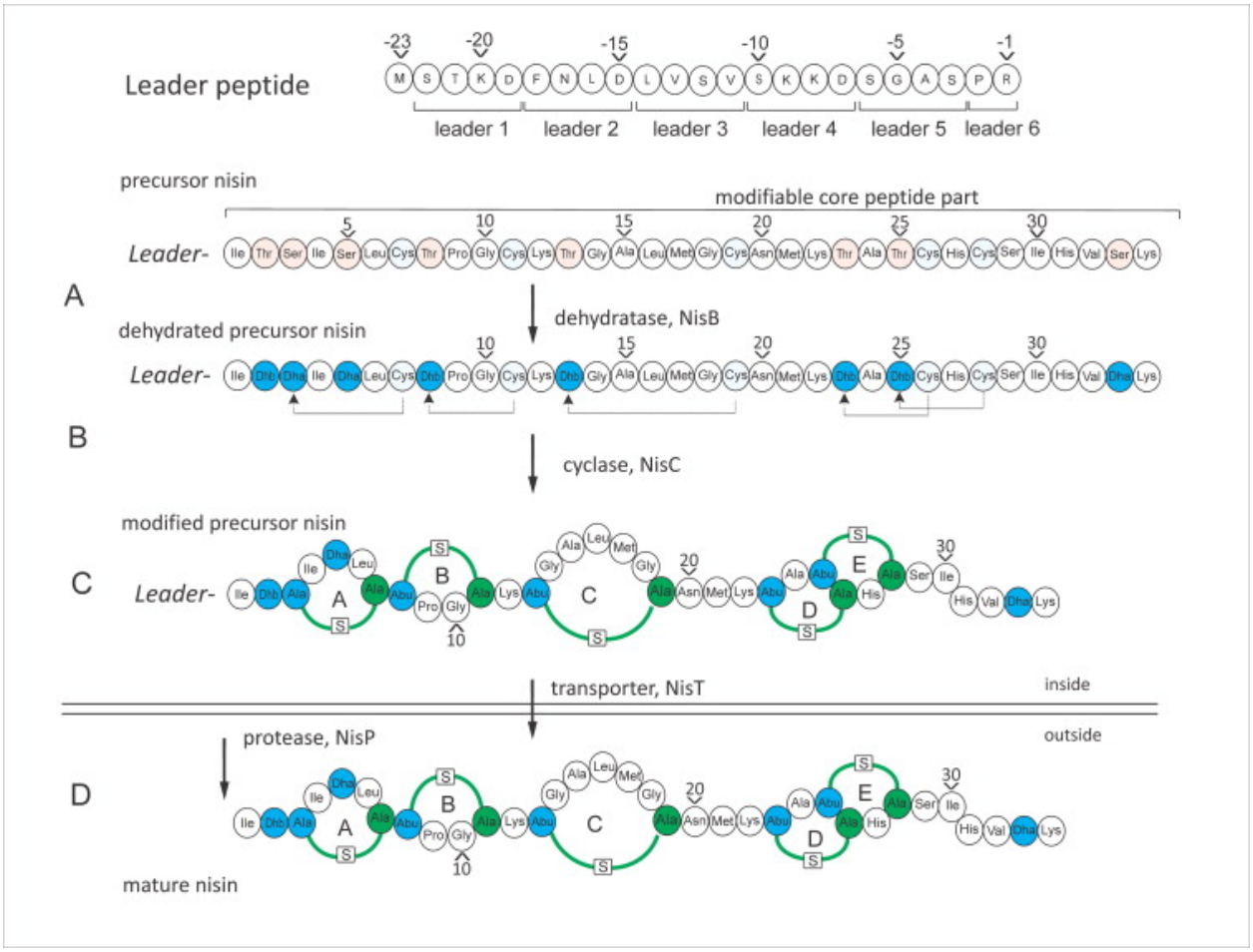
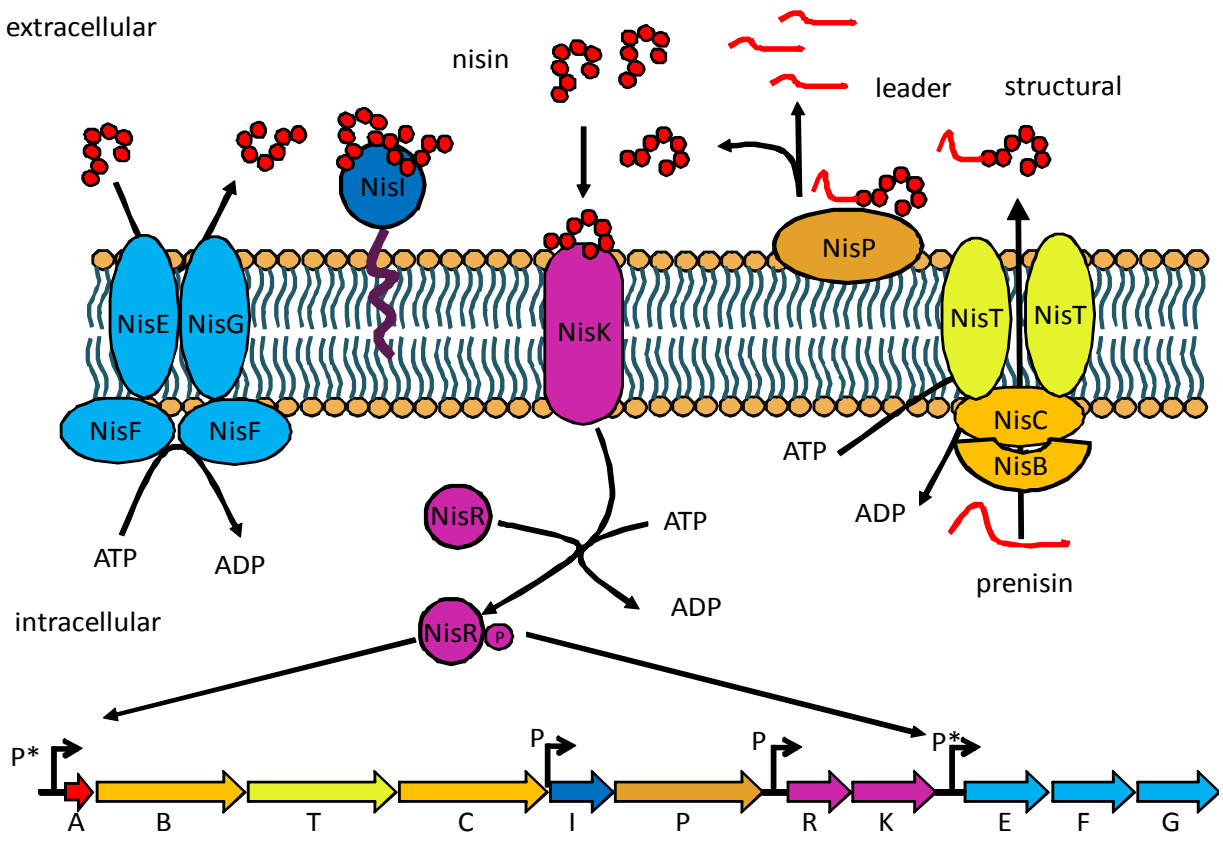

Leader peptide
leader 1
leader 2
leader 3
leader 4
leader 5
leader 6
precursor nisin
modifiable core peptide part
Leader-
A
dehydrated precursor nisin
dehydratase, NisB
B
cyclase, NisC
modified precursor nisin
C
transporter, NisT
inside
outside
protease, NisP
D
mature nisin

Figure 7. Diagrammatic presentation of all the mechinary needed for lantibiotic production. Nisin is encoded by a locus containing 11 genes needed for nisin production, maturation, export, immunity, and regulation of biosynthesis. Yellow color represents modification and transport machinery, blue color represents immunity machinery, purple color represents the regulatory molecules and the red color represents nisin molecule.


extracellular
nisin
leader
structural
NisI
NisP
NisE
NisG
NisK
NisT
NisT
NisC
NisB
NisF
NisF
ATP
ADP
ATP
ADP
NisR
ATP
ADP
prenisin
intracellular
NisR
P
P*
P
P
P*
A
B
T
C
I
P
R
K
E
F
G

1.2.5. Regulation of bacteriocin production

Bacteriocin production is regulated by genetic as well as environmental factors. Two-component systems are known to regulate the expression of both lantibiotics and nolantibiotics. The lantibiotic synthetic genes are encoded in a gene cluster that sometime encodes regulatory proteins. The gene clusters for nisin, salivaricin A, and subtilin all harbor a two-component regulatory system that regulates the gene expression of the respective lantibiotic and the associated immunity proteins (Fig. 7). Many lantibiotics encode orphan regulators in their gene clusters. For example, lacticin 3147, mersacidin, and epidermin all contain regulators in the biosynthetic operon. Growth and culture conditions also influence the lantibiotic production for some lantibiotics. Subtilin (produced by *B. subtilis*) is regulated by SpaRK two-component system, which is expressed by σ^H a stationary phase sigma factor, but σ^H did not influence the production of mersacidin. Epidermin is active in stationary phase like subtilin but controlled by the agr two-component system. The drop in pH can also induce bacteriocin production such as Lacticin 481 production. The presence of some host factors can also regulate bacteriocin production such as cytolysin expression by *E. faecalis*. Finally, some bacteriaocins can act as signaling molecules to induce their own expression, as in the case of nisin (Fig 8). A number of class II bacteriocins such as curvacin A, sakacin P, and plantaricin E/F are also regulated by two-component regulatory systems that are activated by peptide pheromones. Regulation of some bacteriocins produced by *S. mutans* is discussed in the following section.

1.3. Mutacins: Bacteriocins produced by *S. mutans*

S. mutans is not only a prolific producer of bacteriocin they produce multiple types of bacteriocin as well. Bacteriocins produced by *S. mutans* were first studied by Kelstrup and Gibson [84] and named mutacins by Hamada and Ooshima [85]. Mutacin production frequency in S. mutans can vary from 70 to 100% depending on the conditions of the tests and the indicator strains used [86]. A population based study by Kamiya and colleagues [87] showed that over 70% clinical isolates from caries-active and caries-free individuals produced one or more bacteriocin like activities *in vitro*. Most *S. mutans* bacteriocins

characterized to date are lantibiotics. Mutacin I, II, III, BNy266, K8, H29B, and 1140 are lantibiotic monopeptide, where as Smb (produced by *S. mutans* strain GS-5) and BHT (produced *S. ratti*) are examples of dipeptide lantibiotic [88]. Mutacins I and III are the most thoroughly characterized members of all mutacins. Mutacin I is encoded by *S. mutans* UA140 and CH4 strains and the biosysnthesis locus encodes 14 ORFs (See Fig. 8). On the other hand, mutacin III is produced by *S. mutans* UA787 and the biosynthetic operon encompasses eight genes. Mutacin I and III harbor significant sequence similarities and can confer similar antibacterial activities. For both, the alanine t-RNA synthetase (SMU.650) gene is found upstream of the biosynthetic operon, suggensting that some strains might have acquired the genes by horizontal gene transfer. Furthermore, the sequence of the genes encoding mutacin 1140 produced by *S. mutans* JH1140 is identical to mutacin III. Similarly, the primary sequences for mutacin II produced by *S. mutans* T8 and mutacin H29B peptides are completely identical. However, while the biosynthetic locus is known for mutacin II (Fig. 8), the locus is not identified for H29B; therefore a direct comparison between these two loci is not possible. Apart from mutacin I, II, and III, mutacin B-Ny266 produced by *S. mutans* Ny266 is somewhat well studied. It is the first lantibiotic mutacin to be completely sequenced. Subsequent studies were carried to to detremine the MIC and in vivo acticities of B-Ny266. *S. mutans* K8 strain produces another lantibiotic called mutacin- K8. The biosyntheis locus for mutacin K8 consists of 13 genes and shows high homology with the genes encoding lantibiotic SA-FF22 produced by *S. pyogenes* (Fig. 8).

The only two-component lantibiotic produced by *S. mutans* is Smb. Smb was originally identified by Paul and Slade [89] as mutacin GS-5. Later Yonezawa and Kuramitsu [90] identified a biosynthesis locus for two-component lantibiotic and named as "Smb" (*S. mutans* bacteriocin). The Smb peptides were subsequently purified from culture supernatant as suggested by Petersen and colleagues [91]. Hyink and colleagues [92] also purified a variant of Smb that is produced by *S. ratti* strain BHT, and named as BHT-A. Like most lantibiotic mutacins, very little is known about the biochemical properties or the mode of action for Smb.

S. mutans also produces several monopeptide non lantibiotics such as mutacin V, mutacin N, IT-9, and a dipeptide non-lantibiotic mutacin IV. These mutacins are not well characterized and for many the inhibitory spectra are unknown. Apart from these above-mentioned non-lantibiotics, the first *S. mutans* sequenced strain UA159 encodes at least eight additional genes with putative non-lantibiotics like sequences designated as *bsm*. A recent study suggests that these uncharacterized *bsm* genes are more frequent than the characterized mutacin genes in the clinical isolates. Furthermore, some of these *bsm* genes are highly expressed in many clinical isolates. Interestingly, the study by Kmaiya and colleagues (2008) have indicated that over 12% clinical isolates do not encode any known mutacin encoding genes, yet they produce antimicrobial substances that kill other bacteria. This observation suggests that there could be many more mutacin encoding genes that are yet to be discovered.

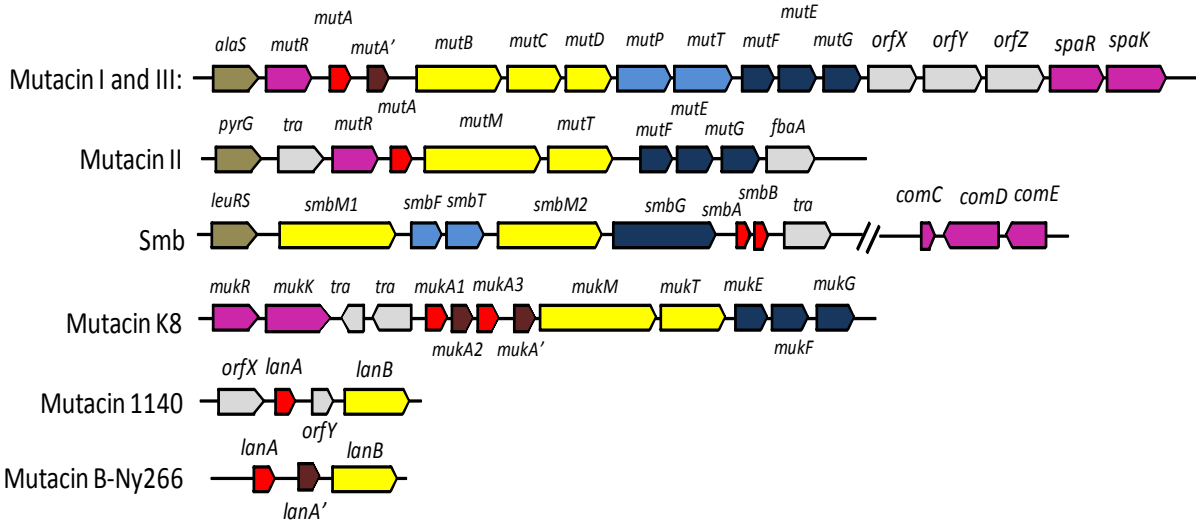
Expression of mutacins is highly coordinated and is regulated by several genetic and environmental factors [93]. At least three types of regulatory systems have been described for mutacin expression. The first category is Rgg-like stand-alone regulators referred to as MutR. The genetic loci for mutacin I, II, and III, each encodes a MutR like transcriptional activator. In fact MutR is an essential activator for mutacin operon expression and mutacin activity is undetected when MutR is inactivated. Rgg family proteins require a small peptide that acts as a signal. However, no such small peptides have been identified in the mutacin operons. The second category regulators belong to LytTR family. So far only two such systems, HdrRM and BrsRM have been described for *S. mutans*. A LytTR regulatory system consists of a membrane bound inhibitory portein that antagonizes the activity of a cognate trasncription factor that activates the gene expression. At present the signals that induce HdrRM and BrsRM pathways are unknown. However, inactivation of the membrane-bound inhibitory proteins lead to increase mutacin gene expression and production. The third regulatory systems that control mutcin expression are the two-component signal transduction systems. Among the 14 two component systems, perhaps the ComDE is the most important and best-studied system. ComDE plays a direct role in the expression of mostly non-lantibiotics such as mutacin IV. A direct interaction of the ComE response regulator with the mutacin

encoding gene promoters has been demonstrated. The two peptide lantibiotic Smb is also regulated by ComDE system as shown by Kuramitsu's group. Some of the other two-component systems involved in mutacin gene regulation are CiaRH, VicRK, and LiaRS systems. However, a direct regulation of mutacin genes by these systems has not been shown. Interestingly, the locus that encodes mutacin K8 contains both an Rgg-like activator MukR and a two-component system MukRK. Perhaps both these regulatory systems involved in mutacin K8 regulation. An analogous to MukRK system known as ScnRK (SMU.1814-SMU.1815) is also present in UA159, a strain that does not produce lantibiotics. The role of ScnRK and other two-component systems in mutacin production has not been evaluated.

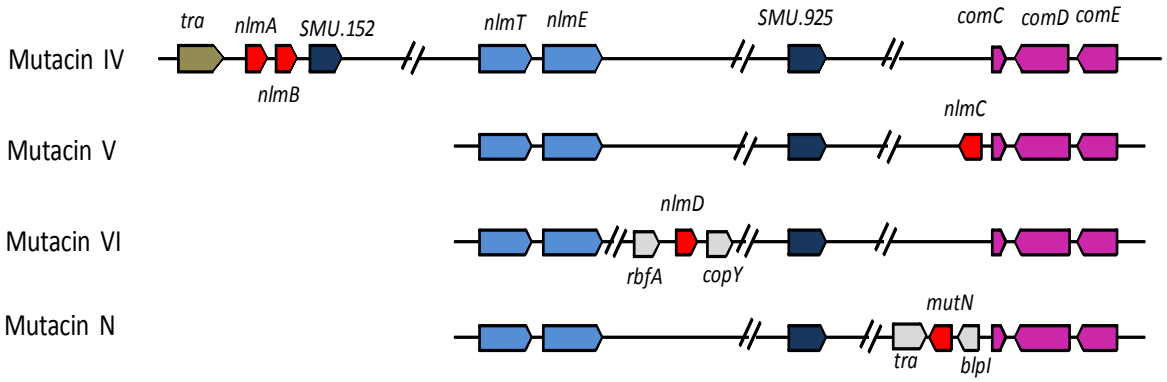
Mutacin production is also modulated by environmental conditions. How environmental conditions regulate mutacin expression has not been demonstrated, but it is assumed that the regulatory pathways mentioned above might be involved. The two most important environmental factors are cell density and nutritional conditions. Studies with mutacin I (lantibiotic) and mutacin IV (non-lantibiotic) have shown that transcription of both of these mutacins is relatively weak in liquid culture but is increased drastically if the cells are grown on solid media or cells are incubated as cell pellet. As for the nutrient conditions are concerned, phosphate seems to have a negative effect on mutacin gene expression. On the other hand, easily fermentable carbohydrates and yeast extract seem to stimulate various mutacin production. The exact molecular mechanisms by which nutritional conditions exert their effect on mutacin production are currently unknown. However, mutacin production is induced when the cells experience stresses. In the dental plaque, *S. mutans* encounters multiple types of stress conditions that might induce mutacin production. This leads to the inhibition of competing species within the biofilm and proliferation of *S. mutans*.

Figure 8: Genetic organization of the biosynthetic gene cluster for various mutacins produced by *S. mutans* isolates. Various colored block arrows represent the following: red, structural genes; dark blue, immunity genes; yellow, modification genes; light blue, transporter genes; and purple, regulatory genes. Other genes not involved with mutacin biosynthesis or regulation are shown with either brown or gray arrows [Adapted from Merritt et. al. 2012, [93]]

Lantibiotic mutacin



Non-lantibiotic mutacin



1.4. Scope of the study

S. mutans strain GS-5 was originally isolated from a cariogenic tooth and was first reported by Gibbons RJ in 1966. Initially this strain was widely used for the characterization of virulence and physiologic properties of *S. mutans*. GS-5 was also the strain of choice to study bacteriocin production. However, only recently Kuramitsu and colleagues (2005) reported the presence of a two-component lantibiotic Smb in GS-5 strain. Currently, Smb is the only known two-component lantibiotic encoded by *S. mutans*. A lantibiotic producer strain must contain a self-protection mechanism to protect itself from the lantibiotic-mediated damage. The self-protection is achieved by three mechanisms: sequestration by a membrane protein, efflux by an ABC transporter, or proteolytic activity. Immunity mechanisms against Smb have not been identified. A previous report by Kuramitsu's group described SmbG, a putative ABC transporter with a peptidase domain, as the immunity protein for Smb. This proposed function of SmbG in providing immunity is not supported by their experimental data. For example, the authors found that SmbG deficiency alone has no effect on immunity or protection against Smb. However, the authors observed that deletion of the entire *smb* locus makes the strain susceptible to the lantibiotic. This observation implies that SmbG is not the authentic immunity protein; rather another gene present in the operon may encode the immunity function. Interestingly, *smb* locus encodes another ABC transporter complex, SmbFT, whose function is not yet characterized. Therefore, it is possible that SmbFT might function as an immunity complex. The multispecies biofilm community in the dental plaque provides a favorable environment for horizontal gene exchange because of spatial proximity and constant selection pressure. Therefore, an Smb non-producer strain can easily acquire the immunity function by horizontal gene transfer and become resistant to Smb. It will be interesting to examine if the immunity protein can function in a heterologous hosts and can provide protection against Smb.

Lantibiotics have more potent bactericidal activity compared to the mammalian antimicrobial peptides such as defensin. Lantibiotics usually function at nanomolar range, whereas other antimicrobial peptides are effective at micromolar concentrations. This fact indicates that the interplay between the lantibiotics and the target organisms must be specific and likely it occurs through receptor-mediated interaction. So

far, only two receptor proteins have been identified for non-lantibiotics. However, to date, no receptor molecules have been identified for any lantibiotics. Since Smb is a broad-spectrum lantibiotic, we speculate that the receptor protein (or its homologs) is widely expressed in bacteria and present in all the susceptible organisms that were tested.

It is of great importance to understand how the producer strain regulates the expression of its immunity protein to counteract the cognate lantibiotic produced by the cell as well as by the population in the biofilm. An auto-sensing mechanism must exist to maintain a constant ratio of the immunity protein and the lantibiotic. Little is known about the transcriptional regulation of the *smb* operon. Recent studies have shown that a two-component system, ComDE, encoded elsewhere in the genome, is required for the *smb* expression. However, ComDE might not be the regulatory system for the auto-sensing. Unlike most of the other lantibiotic loci that encode their own regulatory factors, *smb* does not encode any factor that can function as an auto-regulator. We speculate that Smb peptides function as signaling molecules and auto regulate the *smb* operon through some yet to be discovered regulators.

1.5. Hypothesis

Based on the current knowledge of immunity protein and receptor functions we hypothesize that inactivation of the putative immunity protein (ABC transporter) will make the producer strain sensitive to Smb. And, Smb itself coordinates the immunity protein expression to facilitate the protection of the organism against its own antimicrobial activity. Moreover we predict that Smb recognizes specific receptors that are present on the target strains. Inactivation of the receptor encoding genes will generate resistance against Smb.

Above hypotheses will be tested by means of the following three specific aims:

Aim 1: Identify the self-immunity proteins in GS-5 that are specific to Smb and evaluate their activity in heterologous organisms, since there is a possibility that immunity functions can be transferred by horizontal gene transfer to other streptococci in the densely packed dental biofilm.

Aim 2: Identify the putative receptor molecule(s) from a medically important streptococcus and evaluate its function.

Aim 3: Identify new regulators for *smb* locus and test whether Smb could induce the operon.

Chapter 2: Materials and Methods

2.1. Bacterial strains and growth conditions

Escherichia coli strains DH5α[(F- φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) *deo*R *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-)] (NEB) and Top10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara- leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen) were used for cloning. *E. coli* TG1Rep+ [(*supE hsd*-5 *thi* Δ(*lac-proAB*) F'(*traD6 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) (*repA* from pWVO1)] was use for propagation of pGhost9::*IS*S1 at 37°C. These strains were grown in Luria-Bertani (LB) medium, and when necessary, ampicillin (100 μg ml^{-1}, Ap), erythromycin (100 μg ml^{-1}, Em), and kanamycin (100 μg ml^{-1}, Km) were included.

Streptococci and other Gram-positive bacteria (Table 4) were routinely grown at 37°C in Todd-Hewitt medium (BBL, Becton Dickinson) supplemented with 0.2% yeast extract (THY) under microaerophilic condition. Some organisms were also grown in BHI under microaerophilic condition. For *Bacillus subtilis* LB medium was used and cultured aerobically at 37°C. When needed, erythromycin (10 μg ml^{-1}) and kanamycin (300 μg ml^{-1}) were included in the media. Bacterial growth was measured using a Klett-Summerson colorimeter with a red filter and the turbidity was expressed in Klett unit (KU).

S. gordonii, *S. mutans*, and *S. sanguinis*, strains were transformed by means of natural transformation according to previously described protocol. In brief, streptococcal strains were grown in presence of horse serum to an OD of 0.2 at A_{595} wavelength. Transforming DNA and CSP (when necessary, at final concentration of 200nM) were added, incubated at 30°C for three hours, and plated on THY agar plates with appropriate antibiotics. The plates were then incubated under microaerophilic condition at 37°C. Colonies appeared after two or three days of incubation.

For *S. pyogenes*, electrotransformation was carried out as previously described. Briefly, strains were grown to an OD of 0.25 at A_{560}. Cells were harvested, washed twice with 0.5% sucrose solution, and resuspended to one hundredth of the original volume in the same buffer. Electroporetion was carried out using ~500ng DNA in chilled cuvettes (gap width 1mm) at 1.8KV. Transformed cultures were incubated for two hours at 37°C and plated on THY agar plates with antibiotics.

2.2. Inactivation of genes by gene replacement

A previously described fusion PCR method was used for construction of various mutant strains. In short, ~500-bp upstream (up) and downstream (dn) flanking regions of the targeted genes were separately PCR amplified using appropriate primer pairs as listed in Table 2. GS-5, UA159, or V403 genomic DNA was used as template when necessary. A third fragment (middle) containing either erythromycin resistant cassette (Em) or kanamycin resistant cassette (Km) was amplified from pIBM01 or pIBD38, respectively, using primer pair NcoI-kanD7F and PstI-KanD7R (Table 2). The 5' end of this middle PCR fragment has a 20-bp complementary sequence to the 3' end of the up fragments, whereas the 3' end of this middle fragment has a 20-bp complementary region to the dn fragments to generate fusion PCR products, equal molar ratios of up, dn, and the middle fragments were mixed and used as a template for the next round of PCR amplification with the flanking primers of the targeted genes. The final PCR products were verified by gel electrophoresis for correct sizes and transformed in *S. mutans* strains. Transformants were selected on THY agar plates containing either Em or Km. Gene replacement events were confirmed in the transformants by isolating genomic DNA and confirmed by PCR.

2.3. Construction of P*smb-gusA* reporter strains

Plasmid pIB107 was used to construct this reporter fusion strain. Plasmid pIB107 contains a promoter less *gusA* gene flanked by SMU.1405 locus to integrate into GS-5 chromosome by homologous recombination. A fragment of ~200-bp containing the promoter of *smb* locus was amplified using the genomic DNA of GS-5 with the primers upPsmbBamF and upPsmbXhoR (Table 2). The amplified fragment was digested with *Bam*HI and *Xho*I, and cloned into *Bam*HI-*Xho*I digested pIB107 to create pIBA32 (Table 3). The promoter fusion construct was verified by DNA sequencing. Plasmid pIBA32 was linearized with *Not*I and transformed into GS-5 strain to create strain IBSA71. The reporter construct was verified by PCR for the presence of the promoter fragment.

2.4. Construction of plasmids for complementation

Plasmid pIB184-Km was used as vector for cloning [68]. This plasmid contains P23 promoter from lactococcal phage (pOri23). To clone the *smbFT* genes, a fragment containing the coding regions was amplified from GS-5 genomic DNA. The upstream primer was designed in such a way that it also includes the ribosome-binding site (Fig. 10). The amplified fragment was digested with *Bgl*II and *Xho*I and cloned *into Bam*HI-*Xho*I-digested pIB184-Km to generate pIBM09. This plasmid and the vector were introduced into various streptococci.

A PCR fragment containing the entire SPy1384 coding region plus 36-bp upstream sequence (containing ribosome binding site) was amplified from GAS JRS4 genomic DNA using the primers NewCBam-1384F and NewCXho-1384R which introduced a unique *Bam*HI site at the 5′-end and a unique *Xho*I site at the 3′-end. The resulting ~0.7-kb fragment was digested with *Bam*HI plus *Xho*I and ligated into *Bam*HI-*Xho*I-digested pIB184-Km to create pIBA35. This plasmid was also introduced into selected streptococci.

2.5. Bacteriocin assay (zone of inhibition)

GS-5 and its mutant derivatives were stabbed on THY-agar plates and incubated overnight under microaerophilic condition at 37^0C [94]. The following day, the plates were overlaid with freshly grown indicator strain cultures by mixing with soft agar. When the indicator strains contain plasmids, kanamycin was also included in the soft agar. The overlaid plates were incubated again overnight under the same condition as above. The diameter of the clearing zone was measured afterwards. Assays were repeated at least twice with four replicates.

2.6. Antibiotic sensitivity assay

Disk diffusion assays were performed to evaluate the antibiotic susceptibility of different *S. pyogenes* and *S. mutans* strains as described previously [95]. Briefly, antibiotic disks (6 mm diameter; Becton Dickinson) were placed on THY agar plates that were overlaid with 10 ml of THY soft agar containing

400µl of freshly grown cultures. The plates were incubated overnight at 37°C under microaerophilic condition, and the zones of inhibition were measured. For some chemicals such as nisin and tunicamycin, stock solutions were prepared at indicated strengths and 20µl was spotted directly or on an empty disk (6mm diameter).

2.7. Transposon mutagenesis, screening and identification of IS*S1* integration site

The procedure described by Maguin et al. [96] was used to generate insertion mutants of GAS. Briefly, JRS4 strain was electroporated with pGhost9::IS*S1* and transformants were selected on THY agar containing erythromycin at 30°C. An overnight culture was made from a single transformed colony at 30°C with erythromycin. Cultures were diluted 100-fold in the same medium without antibiotics, grown for 2 h at 30°C, and then shifted to 37°C for 2.5 h to select for transposition events. This culture was then stored at -80°C with 20% glycerol as transposon library. GS-5 was stabbed on THY-agar plates (about four to six stabs per plate) and incubated overnight under microaerophilic condition at 37^0C [94]. The stabbed plates were overlaid with the 100µl of library that was freshly revived in 500µl THY. Colonies that appeared inside the zone of inhibition were inoculated in THY broth containing erythromycin at 37°C. The location of the inserted IS*S1* element was identified by one of two methods. A template generated by self-ligation of *Hin*dIII-digested chromosomal DNA was subjected to inverse PCR by using IS*S1*Rout2 and IS*S1*For4 primers. The PCR product was sequenced with primer IS*S1*-Rout2 to identify the flanking sequences. The insertion sequences were identified by comparison to the serotype M1 (SF370) and serotype M6 (MGAS10394) genome sequences.

2.8. Identification of positive regulators for P*smb* promoter

Insertional mutagenesis was also performed with the plasmid pGhost9:IS*S1*. S. *mutans* strain IBSA71 was transformed with pGhost9:IS*S1* and transformants were selected on THY agar containing Em, and incubated at 30°C. An overnight-grown liquid culture was made from a single transformed colony. Cultures were diluted 100-fold in the same medium, grown for 2 hrs at 30°C, and then shifted to growth

at 37°C for 2.5 hrs to select for transposition events. Cultures were then plated on THY-Em plates containing X-gluc (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 300µg/ml) at 37°C. Approximately, 10000 colonies were evaluated and about 43 colonies were found to be white. These colonies were further verified for color generation on THY-Em plate containing X-gluc (200µg/ml) and evaluated for Smb production using GS-5 and *ΔsmbAB* as controls. Low or no Smb producing clones were selected and the location of IS*S1* insertion sites were analyzed using inverse PCR as described above. The flanking sequences obtained from sequencing of the inverse PCR product were mapped on the genomes of *S. mutans U*A159 and GS-5 by BLAST search.

2.9. Curing of pGhost9::IS*S1* from the selected mutants

S. mutans or *S. pyogenes* cells carrying chromosomally inserted pGhost9:IS*S1* were subjected to multiple growth cycles in liquid THY medium at permissive and nonpermissive temperatures, in the absence of antibiotic, to induce plasmid DNA excision. For each growth cycle, a saturated culture grown at 37°C was diluted 1,000-fold in fresh THY medium, followed by incubation at 30°C for 16 h. After 16 h the cells were diluted and plated on THY agar. Colonies were then replica-patched on THY agar with or without erythromycin to determine the efficiency of plasmid excision and to isolate pGhost9:IS*S1*-cured strain. Erythromycin sensitive colonies were confirmed for the loss of the plasmid sequence by PCR with primers homologous to the flanking regions.

2.10. Isolation of RNA from bacterial cultures

Total RNA was isolated from bacterial cultures according to the protocol described below. *S. mutans* UA159 and its derivatives were grown in THY medium with appropriate antibiotics to mid-exponential phase (70 Klett units), and the cultures were harvested by centrifugation. The cell pellets were then suspended in 10 ml of RNAprotect bacterial reagent (Qiagen) and incubated at room temperature for 10 min. Total RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's instructions, with a modified bacterial-lysis step. Briefly, cells were broken by the addition of an equal

volume of 0·10 mm glass beads (MP Biomedicals) and vortexing the suspensions for 45 sec. at speed 6 in a bead beater (Thermo Electron). The supernatants were loaded onto RNeasy mini columns and purified using the manufacturer's protocol. The purified RNA samples were further treated with Turbo DNase (Ambion) according to the manufacturer's instructions to remove residual DNA contamination. The quality and integrity of the purified RNA samples were ascertained on a 1.2% agarose gel electrophoresis. Total RNA was quantitated in a UV spectrophotometer (Shimadzu) according to the optical density at 260 nm (OD_{260}) (1 unit = 40 µg/ml).

2.11. Semi-quantitative RT-PCR

Total RNA (DNA free) was used to prepare cDNA, using Superscript II reverse transcriptase (Invitrogen). Briefly, RNA samples (500 ng) were mixed with random decamer primer (100 ng) and deoxynucleotide triphosphates (dNTP) (0.5 mM), and the cocktail was heated at 65°C for 5 min, followed by quick chilling on ice. First-strand buffer (SuperScript-II reverse transcriptase; Invitrogen), 10 mM dithiothreitol (DTT), and RNase inhibitor (40 U; Roche) were added to the cocktail and incubated at room temperature for 2 min, and the reverse transcriptase was added to the reaction mixture. The reaction mixture was further incubated at room temperature for 10 min, followed by incubation at 42°C for 50 min to synthesize cDNA. The reaction mixture was heat inactivated at 70°C for 15 min. To degrade the DNA-RNA hybrid, RNase H (2 U; Invitrogen) was added to the reaction mixture and incubated at 37°C for 45 min. Finally, the cDNA was purified using a PCR purification kit (Qiagen), and the cDNA concentration was determined using a UV spectrophotometer (Shimadzu). Five to 20 ng of cDNA was used to carry out the second-step PCR, using ReadyMix *Taq* PCR mix with $MgCl_2$ (Sigma). Twenty to 22 PCR cycles were carried out to amplify the cDNA products of interest. The amplified PCR products were then electrophoresed on a 2% agarose gel. As an internal control, the *gyrA* gene was used to ensure that equal amounts of cDNA were used in each reverse transcription (RT)-PCR.

2.12. Partial purification of Smb from culture supernatant of *S. mutans*

Overnight cultures of GS-5 were prepared in 0.5 X APT broths supplemented with 4% yeast extract. The supernatant was collected and the residual bacteria were removed from the supernatant by passing through 0.45µm filter. After cooling at 4°C, ammonium sulphate was added to a final concentration of 80% (w/v). The precipitate was collected by centrifugation at 10,000g for 30 min. The pellet was dissolved in PBS and dialyzed against PBS using a dialysis membrane with a 3000Da molecular weight cut-off. The partially purified Smb was verified against *S. salivarius*.

2.13. Site-directed mutagenesis of *lsrS*

For site-directed mutagenesis of the putative CAAX protease domain, the coding region of *Spy1384* was amplified by using the primers NewCBam-1384F and NewCXho-1384R using JRS4 genomic DNA as template and inserted into pGEMT-EZ by TA cloning, to generate pIBA40. Site-directed mutagenesis was performed using high fidelity *Pfu* polymerase (Quick Change, Agilent Tech.) with the mutagenic primers that encode the mutations either EE145/146AA or H178M with an additional recognition site for *Bbv*I (to facilitate screening by restriction digestion) to create intermediate plasmids either pIBA43 or pIBA42, respectively. The mutations were confirmed by sequencing. Spy1384 was then amplified from pIBA42 and pIBA43 with primers NewCBam-1384F and NewCXho-1384R, digested with *Bam*HI plus *Xho*I and ligated into *Bam*HI-*Xho*I-digested pIB184Km, to create pIBA45 and pIBA44, respectively. Sequencing the entire coding region reconfirmed the mutations in these constructs. Plasmids pIBA44 and pIBA45 were transformed into *S. pyogenes* by electroporation and into *S. mutans* by natural transformation as described above.

2.14. Membrane topology prediction for LsrS

Various bioinformatic software searches such as TMHMM, HMMTOP, TMpred, ToPhred were used to determine the hydrophobic region of LsrR receptor protein. We used β-galactosidase (*lacZ*) fusion to experimentally verify the LsrS membrane topology. For this pNM480 plasmid was used as a vector to

clone various N-terminal regions of LsrS and constructed in-frame fusion with the LacZ gene encoded by pNM480. β-galactosidase activity was measured as described by Miller. Briefly, *E. coli* cells were grown in 10 ml LB until A_{600} was reached ~0.5 and cells were collected by centrifugation followed by resuspension in 1ml Z-buffer. Cells were permeabilized with 0.1% SDS and chloroform. Subsequently, 4mg / ml 2-nitrophenyl- β-galactopyranoside (ONPG) was added to the permeabilized cells. The mixture was incubated at 25°C until the development of yellow color and the reaction was stopped with 1M sodium carbonate. Enzymatic activities were expressed in Miller units using the formula [(522xOD420 of reaction mixture)/(OD600 of culture X volume per ml of culture used x time of reaction)].

Table 2: List of oligonucleotides used in this study

Name	Sequence (5'→3')	Purpose
FsnsmbAupF	GTTGGCGAAAGTGGTTCTGGTAAG	Inactivation of *smbA, smbB, smbAB* (up)
FsnsmbAupR	GCCGCCATGGCGGCCGGGAGTAATAAATTACTTTTCATTTTA	Inactivation of *smbA, smbAB* (up)
FsnsmbBdnF	CGCGGCCGCCTGCAGGTCGACCGAATGCATGAGAAATTGTAG	Inactivation of *smbAB* (dn)
FsnsmbAdnR	GACCGCTTTCATATTGTTCAGCAC	Inactivation of *smbA, smbB smbAB* (dn)
FsnsmbAdnF	CGCGGCCGCCTGCAGGTCGACCGCACATCTCGCTGTATTAAC	Inactivation of *smbA ?(up)*
FsnsmbBupR	GCCGCCATGGCGGCCGGGAGCCTGCTTTTTGAATCTCTTTC	Inactivation of *smbB (up)*
LocussmbAR	GTTATTTGTTAATACAGCGAGATG	*smbA* amplification
LocussmbBF	GGAGTAAATTATAATGAAAGAGATTC	*smbB* amplification
LocussmbAF	GGAAGGAATATAGGGTGAAAAG	*smbA, smbAB* amplification
LocussmbdnR	CGTATTACTTACTACAATTCTCATGC	*smbB, smbAB* amplification
LocussmbupF	CTTGGTAAGGGGGAAGTTGTGGAAAC	*smb locus* amplification
NcoI-Kan D7 F	CTCCCGGCCGCCATGGCGGCCGC	Antibiotic cassette (middle)
PstI-Kan D7 R	GGTCGACCTGCAGGCGGCCGCG	Antibiotic cassette (middle)
FsnSmbTupF	CCAGCCACATCTTACAAAATTTGGAGC	Deletion of *smbT* (up)
FsncrtSmbTupR	GCGGCCGCCATGGCGGCCGGGAGGGTCCTTTTAAATTCTCAATAG	Deletion of *smbT* (up)
FsnSmbTdnF	CGCGGCCGCCTGCAGGTCGACGCTGATTTGACTAAATATATTCC	Deletion of *smbT* (dn)
FsnSmbTdnR	CCTTTGAATATAATTACAAATAACAAC	Deletion of *smbT* (dn)
SMBFT-XHO-R	GCCCTCGAGCCACTATGCATAACCCCATTTACGATAAATC	Complementation of *smbFT*
SMBFT-BGLII-RBSGTF-	CGCAGATCTTGGAGGTTCCTAATGAGAATTTTAGACATTCAAAATTTAAA	Complementation of *smbFT*
NewCBam1384F	AGTGGATCCAGACAATTTTACCGTTAGCCTAAAAGG	LsrS complementation
NewCXho1384R	GTTCTCGAGCCGAAGCTTTTTATTATATGACTCC	LsrS complementation
FSN662upF	CAATTTTACTTTGTTTTGTTTTTCTGCCAAGAAG	SMU.662 deletion
FSN662upR	CGGCCGCCATGGCGGCCGGGAGCAAGTGATAATAAAATCAGTCCAATAAC	SMU.662 deletion
FSN662dnF	CGCGGCCGCCTGCAGGTCGACCTACGGCGCTTTATTTCTTATTTATAGC	SMU.662 deletion
Fsn662dnR	GGACATTGACAAAATGACTGGACTCTGACAAGACCTTGCC	SMU.662 deletion
EE145AABbv1F	GCTTTTATCGCTCCTATTATGGCAGCACTAGTCTTTAGAGGATTTCCTATG	EE146/146AA mutation LsrS
EE145AABbv1R	CATAGGAAATCCTCTAAAGACTAGTGCTGCCATAATAGGAGCGATAAAAGC	EE146/146AA mutation of LsrS
H178ABbv1F	CTTGTTTTTGCTTTACCAGCAGCCACCAATAGTGTTGAA	H178A mutation of LsrS
H178ABbv1R	TTCAACACTATTGGTGGCTGCTGGTAAAGCAAAAACAAG	H178A mutation of LsrS
PJRS-F	TAAGGCTATTGGTGTTTATGGC	LsrS-LacZ fusion (upstream)
M74LZHind-R	CCTAAGCTTCCATTTTTTGCTGTTTAATAAAAGTGTCTTGCTTAGC	LsrS-LacZ fusion (downstream)
V148LzHindR	CCTAAGCTTCGACTAGTTCTTCCATAATAGGAGCGATAAAAGCTAT	LsrS-LacZ fusion (downstream)

Table 2: Contd…

Name	Sequence (5'→3')	Purpose
PBam-smbAB	CACGGATTCGTGACAAAATAATTGTTTTAAAAGACG	SmbAB complementation
PXho-smbAB	CAGCTCGAGCTTTTCATTTTATATTCCTTCCTATTC	SmbAB complementation
SmbGsqF	GCCAAACAGAATGTGGATTATGTG	sQ-RT-PCR (*smbG*)
SmbGsqR	GTCCTCCGATTCTATAAGCGATGCC	sQ-RT-PCR (*smbG*)
SmbM1sqF	GCATCGGTGCTTACAACGGAATAGCT	sQ-RT-PCR (*smbM1*)
SmbM1sqR	CACACGATTATTTAAACAGTCACTC	sQ-RT-PCR (*smbM1*)
SmbTsqF	GCAGCTATCAACGCCTTTGGTAGAGAG	sQ-RT-PCR (*smbT*)
SmbTsqR	GCATAAACTAAGTAAGCCGGTACCAG	sQ-RT-PCR (*smbT*)
SmbwholelcsF	CGTTGTCAATCCTGATGATGTTGTCG	*smb* locus amplification
SmbwholelcsR	GCACATGAATAGAGCACTCAAGCAAACC	*smb* locus amplification
UpPsmbBam-F	CAGGGATCCTGAAGGCTGAAATAGTC	Psmb amplification
UpPsmbXho-R	CAGCTCGAGCAGTCCTTGTTTCCACA	Psmb amplification
ISS1 Rout2	AATAGTTCATTGATATATCCTCGCTGTCA	Inverse PCR for ISS1
ISS1 For 4	GGTCTTAATGGGAATATTAGC	Inverse PCR for ISS1

Table 3: List of plasmids used in this study

Plasmid	Relevant Characteristic(s)	Source or Reference
pGEM T-EZ	Commercial TA cloning vector, Ap^r	Promega
pGhost9::ISS1	Thermosensitive delivery plasmid for IS*S1*, Km^r	Maguin
pIB184-Km	*E. coli – Streptococcus* shuttle plasmid, Km^r	Biswas
pIBD38	pGEM T-EZ containing *loxP*-Km cassette, Ap^r, Km^r	Biswas
pNM480	*E. coli* vector for *lacZ* fusion, Ap^r	Milton
pIB107	Vector for *gusA* reporter fusion	Biswas
pIBM01	pGEMT-EZ containing *ermB* gene, Ap^r, Em^r	This study
pIBM08	pIB184Km containing *smbFT* with native RBS, Km^r	This study
pIBM09	pIB184Km containing *smbFT* with GtfB RBS, Km^r	This study
pIBA32	pIB107 containing P*smb* promoter fused with *gusA*	This study
pIBA35	pIB184Km containing SPy1384, Km^r	This study
pIBA39	pNM480with *lacZ*-fused to SpPy1384 at codon 75 (M75), Ap^r	This study
pIBA40	pGEMT-T-EZ containing SPy1384, Ap^r	This study
pIBA41	pNM480 with *lacZ* fused to SpPy1384 at codon 149 (V149), Ap^r	This study
pIBA42	pGEMT-T-EZ with H178A mutation in SPy1384, Ap^r	This study
pIBA43	pGEMT-T-EZ with EE145/146AA mutations in SPy1384, Ap^r	This study
pIBA44	pIB184Km with EE145/146AA mutations in SPy1384, Km^r	This study
pIBA45	pIB184Km with H178A mutation in SPy1384, Km^r	This study

Table 4: List of Gram-positive strains used in this study

Name	Genotypes and descriptions	Reference or Source
IBSA63	*S. mutans* GS5 with *ΔsmbAB*, Km^r	This study
IBSA66	*S. pyogenes* JRS4::ISS1, clone #1, insertion not mapped, Em^R	This study
IBSA67	*S. pyogenes* JRS4::ISS1, clone #4, insertion in Spy1384, Em^R	This study
IBSA68	*S. pyogenes* JRS4::ISS1, clone #13, insertion in Spy1384, Em^R	This study
IBSA69	IBSA67, pGhost 9 cured, Em^S	This study
IBSA70	IBSA68, pGhost 9 cured, Em^S	This study
IBSA71	*S. mutans* GS5 with P*smb-gusA* reporter fusion, Km^R	This study
IBSA72	*S. mutans* GS5 with ΔSMU.1704, Em^r	This study
IBSA73	IBSA71 with *ΔsmbAB*, Em^r, Km^R	This study
IBSA74	IBSA71 with *ΔsmbA*, Em^r, Km^R	This study
IBSA75	IBSA71 with *ΔsmbB*, Em^r, Km^R	This study
IBSA76	IBSA71 with *ΔsmbAB*, Em^r, Km^R	This study
IBSA77	*S. mutans* GS5 with *ΔsmbT*, Em^r	This study
IBSA81	*S. mutans* GS5 with Δ[SMU.1704 – SMU1706], Em^r	This study
IBSA82	IBSA71 with ΔSMU.1704, Em^r, Km^R	This study
IBSA83	IBSA71 with Δ[SMU.1704 – SMU1706], Em^r, Km^R	This study
IBSA89	*S. mutans* GS5 with *ΔsmbG*, Em^r	This study
IBSA91	IBSA71 with Δ*smbT*, Em^r, Km^R	This study
IBSA93	IBSA71 with Δ*smbG*, Em^r, Km^R	This study
IBSA98	*S. mutans* with V403 with ΔSMU.662, Em^r	This study
IBSA99	*S. mutans* with UA159 with ΔSMU.662, Em^r	This study
OG1X	*Enterococcus faecalis*	ATCC
MG1363	*Lactococcus lactis*	INRA
RB2006	*Lactobcillus rhamnosos*	Clinical isolate
1457	*Staphylococcus epidermidis*	UNMC
A909, NEM306	*S. agalactiae*	ATCC
CI-SA	*S. anginosus*	Clinical isolate
CI-SC	*S. constellatus*	Clinical isolate
AHT	*S. criceti*	Krasse
ATCC33798	*S. downeii*	ATCC
CI-SD	*S. dysgalactiae*	Clinical isolate
ATCC700410	*S.equines*	ATCC
43143,TX20005 BAA2069	*S. gallolytics*	ATCC and others
ATCC10588, DL-1, M-5	*S. gordonii*	ATCC and others
K388	*S. iniae*	Clinical isolate
CI-MT	*S. mitis*	Clinical isolate

Table 4: Contd…

Names	Genotypes and descriptions	Reference or source
109c, 262RF, 8VS3, DP1, GS-5, NG-8, OMZ175, SJ32, SM3209, SP-2, T8, UA130, UA159, V1, V100, V403	*S. mutans*	ATCC and others
NCTC7864	*S. oralis*	NCTC
ATCC6303, ATCC43079, ATCC49619	*S. pneumoniae*	ATCC
AM3, JRS4, K32	*S. pyogenes*	Clinical isolates
BHT, FA-1	*S. ratii*	ATCC
BAA491, ATCC25975	*S. salivarius*	ATCC
SK36	*S. sanguinis*	VCU
6715, ATCC27352, ATCC33478	*S. sobrinus*	ATCC
UT387	*S. uberis*	UT

Chapter 3:

SmbFT, a putative ABC transporter complex, confers protection against the lantibiotic Smb in streptococci

3.1. Abstract

Streptococcus mutans, a dental pathogen, secretes different kinds of lantibiotic and non-lantibiotic bacteriocins. For self-protection, a bacteriocin producer strain must possess one or more cognate immunity mechanisms. Here we report the identification of one such immunity complex in *S. mutans* strain GS-5 that confers protection against Smb, a two-component lantibiotic. The immunity complex that we identified is an ABC transporter composed of two proteins: SmbF (the ATPase component) and SmbT (the permease component); both the protein-encoding genes are located within the *smb* locus. We show that GS-5 becomes sensitized to Smb upon deletion of *smbT,* which makes the ABC transporter non-functional. To establish the role SmbFT in providing immunity, we heterologously expressed this ABC transporter complex in four different sensitive streptococci species and demonstrated that it can confer resistance against Smb. To explore the specificity of SmbFT in conferring resistance we tested mutacin IV (a non-lantibiotic), nisin (a single peptide lantibiotics), and three peptide antibiotics (bacitracin, polymyxin B, and vancomycin). We found that SmbFT does not recognize these structurally different peptides. We then tested whether SmbFT can confer protection against haloduracin, another two-component lantibiotic that is structurally similar to Smb; SmbFT indeed conferred protection against haloduracin. SmbFT can also confer protection against an uncharacterized but structurally similar lantibiotic produced by *Streptococcus gallolytics.* Our data suggest that SmbFT truly displays immunity function and confer protection against Smb and structurally similar lantibiotics.

3.2. Introduction

Oral biofilm is complex and diverse in nature. As many as 700 different bacterial species colonize in our mouth; unfortunately the identity of many of them is still unknown [1,97]. The complexity of biofilm greatly depends on the interspecies interaction during early as well as maturing phase of biofilm formation. The participation of these organisms is not restricted to dental disease only, as they can cause bacterimia and in some cases infective endocarditis [98-100]. Endocarditis is generally caused by viridans group of streptococci. Some of these viridians streptococci are commensal in oral cavity and the others are involved in disease formation [100,101]. Some of the commensal streptococci have a protective role against oral disease development [102]. The exact in vivo mechanisms for this interspecies interference are currently not well understood, however several studies strongly suggest an important role for hydrogen peroxide and bacteriocin in this process [103-105].

In general, bacteriocins are ribosomally synthesized small peptides with bactericidal or bacteriostatic activity on other species. Bacteriocins are broadly categorized into two groups: non-lantibiotic and lantibiotic. The non-lantibitic group contains peptides that do not require any modifications for their biological activity [106]. In contrast, the lantibiotic groups contain peptides that require posttranslational modification for their antimicrobial activity [70,107]. In lantibiotics, most of the serine and threonine residues are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, by modifying enzymes encoded within the lantibiotic synthesis operon. With neighboring cysteine residues, Dha and Dhb can form thioetherlinked lanthionine and 3-methyl lanthinoine bridges, respectively. Sometimes Dha, Dhb and other modified residues can be present as unlinked residues (for reviews see [70,71,108-110]). Based on the nature of the enzymes responsible for the modifications, lantibiotics are divided into three classes [70]. Class I lantibiotics (such as nisin and subtilin) are modified by two enzymes generally referred to as LanBC system. Class II lantibiotics (such as lacticin 481 and mersacidin) are modified by a single enzyme often referred to as LanM-type enzyme. Class II lantibiotics also include two-component lantibiotics (such as lacticin 3147 and haloduracin) and the antimicrobial activity requires synergistic interaction of both peptides [111,112]. Lantibiotics that belong to class III are lanthionine peptides that

have no antimicrobial activity [113,114]. The advantage of bearing modified residues as well as cyclization is to make lantibiotics more stable against heat, pH, and protease degradation compared to non-lantibiotics [115]. This stable nature of lantibiotics makes them a major candidate for commercial application; for example, nisin is extensively used as a preservative in the food industry [72].

Lantibiotics are generally produced by Gram-positive bacteria and usually have a variable spectrum of inhibition [72,109,115-117]. The producer organisms usually encode specific immunity proteins that protect themselves from the deleterious effect of their own lantibiotics and the immunity protein encoding genes are often present within the same lantibiotic biosynthesis operon. Currently, two types of immunity proteins have been identified. The first type includes dedicated ABC transporters that presumably pumps lantibiotics out of the membrane and thus prevent accumulation to inhibitory levels (for reviews see, [108,118]). The second type includes small proteins that are weakly associated with the membrane and often sequester specific lantibiotics at the cell surface before they could cause cell damage [119-121].

Streptococcus mutans, an oral streptococci and a major etiological agent of human dental caries, often produces several kinds of bacteriocins, collectively known as mutacins. A majority of the mutacins characterized to date belong to lantibiotics, such as mutacins I, II, III, K8, B-Ny266, Smb, and1140 [64,122-128]. Based on their primary amino acid sequences, mutacins belonging to lantibiotics are further subdivided into two classes: AI and AII [88,129,130]. Subclass AI contains the most well-characterized peptides such as mutacin I and III (1140), which are similar to nisin and subtilin. Detailed structural information of these mutacins is currently lacking. Although the lanctibiotic mutacins are widely present in *S. mutans* [86,128], surprisingly the first sequenced reference strain UA159 does not encode any lantibiotic, it only encodes non-lantibiotics [44]. In contrast, another recently sequenced *S. mutans* strain GS-5 encodes both Smb and non-lantibiotics [126,131]. Majority of the mutacins encoding genes are acquired by the *S.mutans* by horizontal gene transfer mechanism. For example, the strains that produce mutacin K8 contains the K8-encoding *muk* locus that is inserted in between SMU.1811 and SMU.1812 genes of the corresponding UA159 genome [128]. Similarly, the *smb* locus, which contains all the genes necessary for Smb biosynthesis, is integrated in between SMU.1942 and *syl* locus. Smb is the only two-

component lantibiotic identified so far in *S. mutans* and appears to be encoded only by a handful of strains [128]

The *smb* locus contains seven open reading frames in the following order: *smbM1, -F, -T, -M2*, *-G, -A*, and *–B*. Putative transposase encoding genes are also present near the *smb* locus [126]. Two genes, *smbA* and *smbB*, are the structural genes for the Smb pre-peptides and two putative modification enzymes encoded by *smbM1* and *smbM2* are also present in the *smb* locus. A recent report indicated that SmbG appears to play a role in Smb immunity and sensitivity to antimicrobial agents [132]. Two other genes, *smbF* and *smbT*, show homology with the ATP binding cassette transporter and were previously predicted to be involved in processing and secretion of Smb pre-peptides [126]. We noticed that this SmbFT ABC transporter complex does not have a signal peptidase domain, and hence might not be involved in the processing and secretion of the Smb pre-peptide. We predicted that SmbFT might provide immunity to Smb producing *S. mutans* strains. In this study we provide evidences that SmbFT indeed confer resistance towards Smb in the producer strain and show that deletion of *smbT* gene in GS-5 leads to sensitivity towards Smb. We also demonstrate that SmbFT can confer resistance to sensitive streptococci belonging to non-mutans group. Furthermore, we show that SmbFT has a narrow specificity since it only recognizes structurally similar two-peptide lantibiotics.

3.3. Results

3.3.1. Smb sensitive strains do not encode the *smb* locus. Smb is the only lantibiotic that is secreted by *S. mutans* GS-5 [131] and a previous study suggested that Smb is active against many oral streptococci [91]. Since most of the lantibiotics including nisin have wider spectrum than the non-lantibiotics [106,115,130], we wanted to determine whether Smb could inhibit various streptococci belonging to different phylogenetic groups [133]. We first constructed a *smbAB*-deleted mutant strain (Δ*smbAB*) to include in our inhibition assays to make sure that the activity that we will observe is due to lantibiotic Smb and not due to other bacteriocins. We then used GS-5 and Δ*smbAB* strains as tester against 18 streptococci and 16 *S. mutants* isolates (Fig 9A). All the streptococci strains, except some *S. mutans* isolates (see below), were inhibited by Smb. We divided the activity spectra in to two classes, a zone-of-inhibition (ZOI) smaller than 10mm and larger than 10 mm (Fig 9A). Two groups of streptococci, the pyogenic and the bovis groups, consistently produced larger ZOI than the other groups suggesting that the streptococci belonging to these groups are more susceptible to Smb than the other groups. *S. mutans* strains generally do not inhibit the growth of other *S. mutans* strains. However, we found that nine out of 16 *S. mutans* also produced large ZOI (Table 5). Furthermore, two *S. ratti* strains were also produced some ZOI but FA strain produced small (~8mm) but clear ZOI while BHT strain produced diffused ZOI (~10mm). Among the mitis group, most of the isolates produced smaller ZOI except *S. sanguinis* SK36, which produced a large ZOI (~20mm).

It was surprising to observe that many of the *S. mutans* isolates were insensitive to Smb. Since the lantibiotics have the potential to attack the producer strains, the producer strains must contain some self-protection mechanisms to protect against their own lantibiotics. Therefore, it is possible that the *S. mutans* strains that were insensitive may actually encode the *smb* locus. To examine the presence of *smb* locus, we PCR amplified the *smbAB* structural genes from all the 16 *S. mutans* isolates. Eleven of these isolate belongs to serotype c, including common laboratory strains (UA159, NG8, and GS5); one each from serotype e (V100) and serotype f (OMZ175); and the rest were taken from unknown serotypes.

Figure 9. Smb activity spectrum and distribution of *smb* locus in various *S. mutans* strains (A) Member species of different phylogenetic group of streptococci were tested against mutacin Smb produced by GS-5. GS-5 and a *ΔsmbAB* mutant strain were stabbed on THY-agar plate and incubated overnight in microaerophilic condition at 37^0C. Next day, overnight grown culture of indicator strain was mixed with soft agar and overlaid on the plate. The overlaid plates were incubated again overnight under the same condition. The following day the diameter of the zone of inhibition (ZOI) was measured. The observation is based on two separate experiments with four replicates. Diamonds and squares indicate ZOI greater than or less than 10 mm in diameter, respectively. (B) The presence of *smb* locus in 16 different *S. mutans* strains. Analysis of PCR products of *smbAB* genes by agarose gel electrophoresis. Solid circles indicate the strains that are susceptible to Smb inhibition (Table 6). The observation is based on at least three independent experiments.

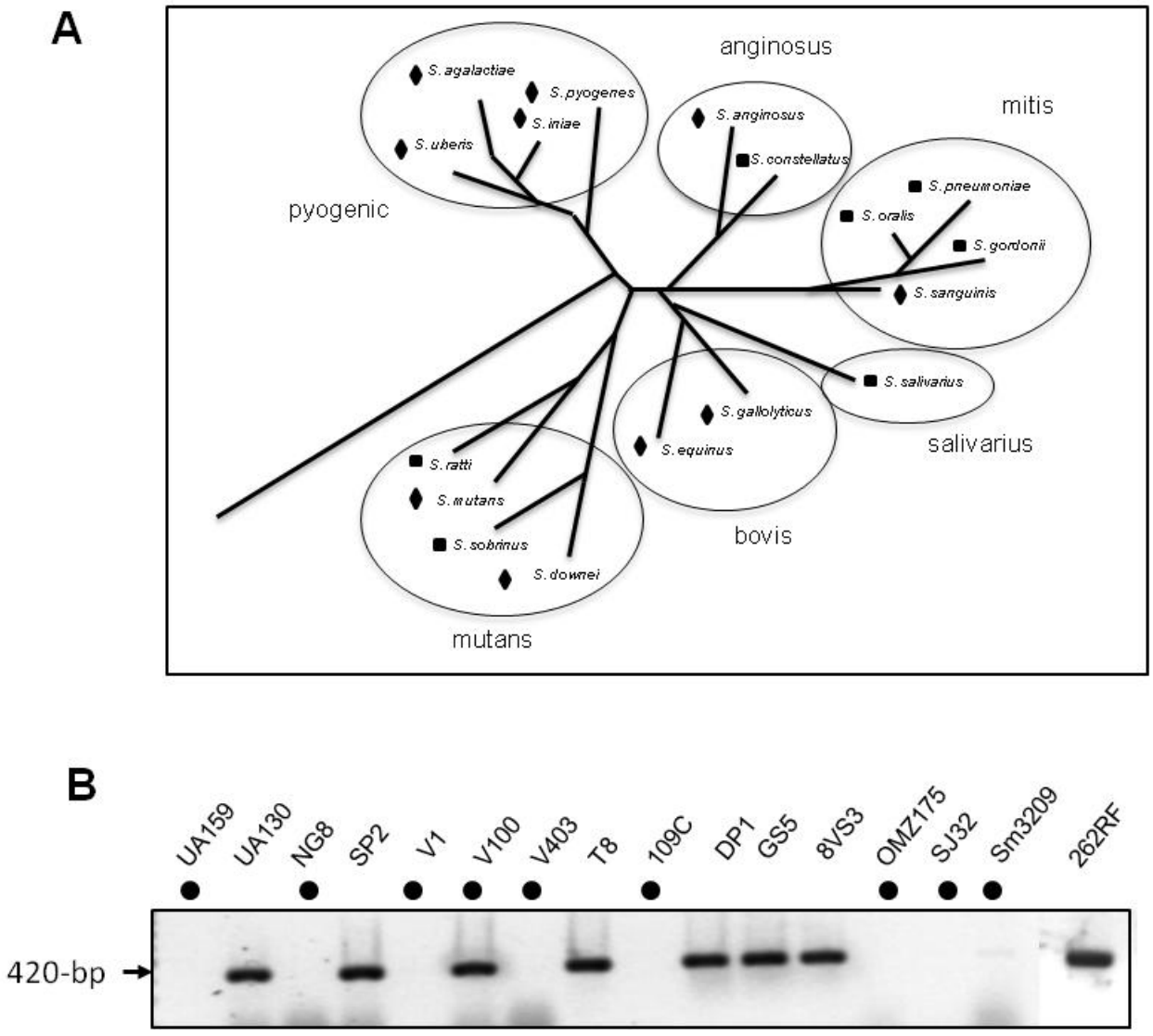

A
anginosus
S. agalactiae
S. pyogenes
S. iniae
S. uberis
pyogenic
S. anginosus
S. constellatus
mitis
S. pneumoniae
S. oralis
S. gordonii
S. sanguinis
S. salivarius
salivarius
S. gallolyticus
S. equinus
bovis
S. ratti
S. mutans
S. sobrinus
S. downei
mutans
B
UA159
UA130
NG8
SP2
V1
V100
V403
T8
109C
DP1
GS5
8VS3
OMZ175
SJ32
Sm3209
262RF
420-bp

Table 5: Sensitivity of various Gram-positive bacteria to Smb

Species (indicator strain)	Group	Zone of inhibition diameter (mm)	
		GS-5	*ΔsmbAB*
S. uberis (UT387)	Pyogenic	26.0 ± 2.0	12.0 ± 1.0
S. agalactiae (NEM306)	Pyogenic	18.0 ± 0.5	4.0 ± 1.0
S. agalactiae (A909)	Pyogenic	19.0 ± 1.0	4.0 ± 1.0
S. dysgalactiae (CI)	Pyogenic	26.0 ± 1.0	0.0 ± 0.0
S. iniae (K388)	Pyogenic	31.0 ± 1.0	7.0 ± 1.0
S. pyogenes (JRS4)*	Pyogenic	26.0 ± 1.0	9.5 ± 1.5
S. pyogenes (AM3)	Pyogenic	24.0 ± 1.0	10.5 ± 1.5
S. pyogenes (K32)	Pyogenic	28.0 ± 1.0	10.5 ± 1.5
S. anginosus (CI)	Anginosus	16.0 ± 1.0	4.5 ± 0.5
S. constellatus (CI)	Anginosus	10.0 ± 1.0	4.5 ± 0.5
S. gordonii (DL-1)*	Mitis	9.5 ± 0.5	4.0 ± 0.5
S. gordonii (M5)	Mitis	11.5 ± 1.5	6.5 ± 0.5
S. gordonii (ATCC10558)	Mitis	12.5 ± 3.5	6.0 ± 1.0
S. pneumonia (ATCC 6303)	Mitis	10.0 ± 1.0	1.0 ± 0.5
*S. pneumonia (*ATCC49619)	Mitis	11.5 ± 1.5	0.0 ± 0.0
S. pneumonia (ATCC43079)	Mitis	13.5 ± 0.5	1.5 ± 1.0
S. sanguinis (SK36)*	Mitis	23.0 ± 1.0	0.0 ± 0.0
S. oralis (NCTC7864)	Mitis	7.0 ± 1.0	0.0 ± 0.0
S. mitis (CI)	Mitis	4.5 ± 0.5	3.5 ± 0.5
S. salivarius (ATCC25975)	Salivarius	15.0 ± 1.0	6.0 ± 1.0
S. salivarius (BAA491)	Salivarius	24.0 ± 1.0	0.0 ± 0.0
S. gallolyticus (TX20005)	Bovis	12.0 ± 1.0	1.5 ± 0.5
S. gallolyticus (BAA2069)	Bovis	22.0 ± 2.0	3.0 ± 0.5
S. gallolyticus (43143)	Bovis	25.0 ± 1.0	1.0 ± 0.5
S. equines (ATCC700410)	Bovis	20.0 ± 1.0	0.0 ±0.0
S. downeii (ATCC 33798)	Mutans	14.0 ± 1.0	0.0 ± 0.0
S. sobrinus (6715)	Mutans	7.0 ± 1.0	0.0 ± 0.0
S. sobrinus ATCC 27352	Mutans	7.0 ± 0.5	0.0 ± 0.0
S. sobrinus ATCC33478	Mutans	5.5 ± 0.5	0.0 ± 0.0
S. criceti AHT	Mutans	7.0 ± 0.5	0.0 ± 0.0
S. ratti FA-1	Mutans	6.0 ± 0.5	0.0 ± 0.0
S. ratti BHT	Mutans	9.0 ± 0.5	0.0 ± 0.0
L. lactis MG1363	Other	20.0 ± 6.0	2.0 ± 2.0
E. faecalis OG1X	Other	7.5 ± 0.5	3.5 ± 0.5
L. rhamnosus (CI)	Other	22.0 ± 1.0	7.0 ± 1.0
Staph. epidermidis 1457	Other	0.0 ± 0.0	0.0 ± 0.0

CI, Clinical isolate. *, Strains used for heterologous expression of SmbFT

Table 6: Susceptibility of various *S. mutans* strains to Smb

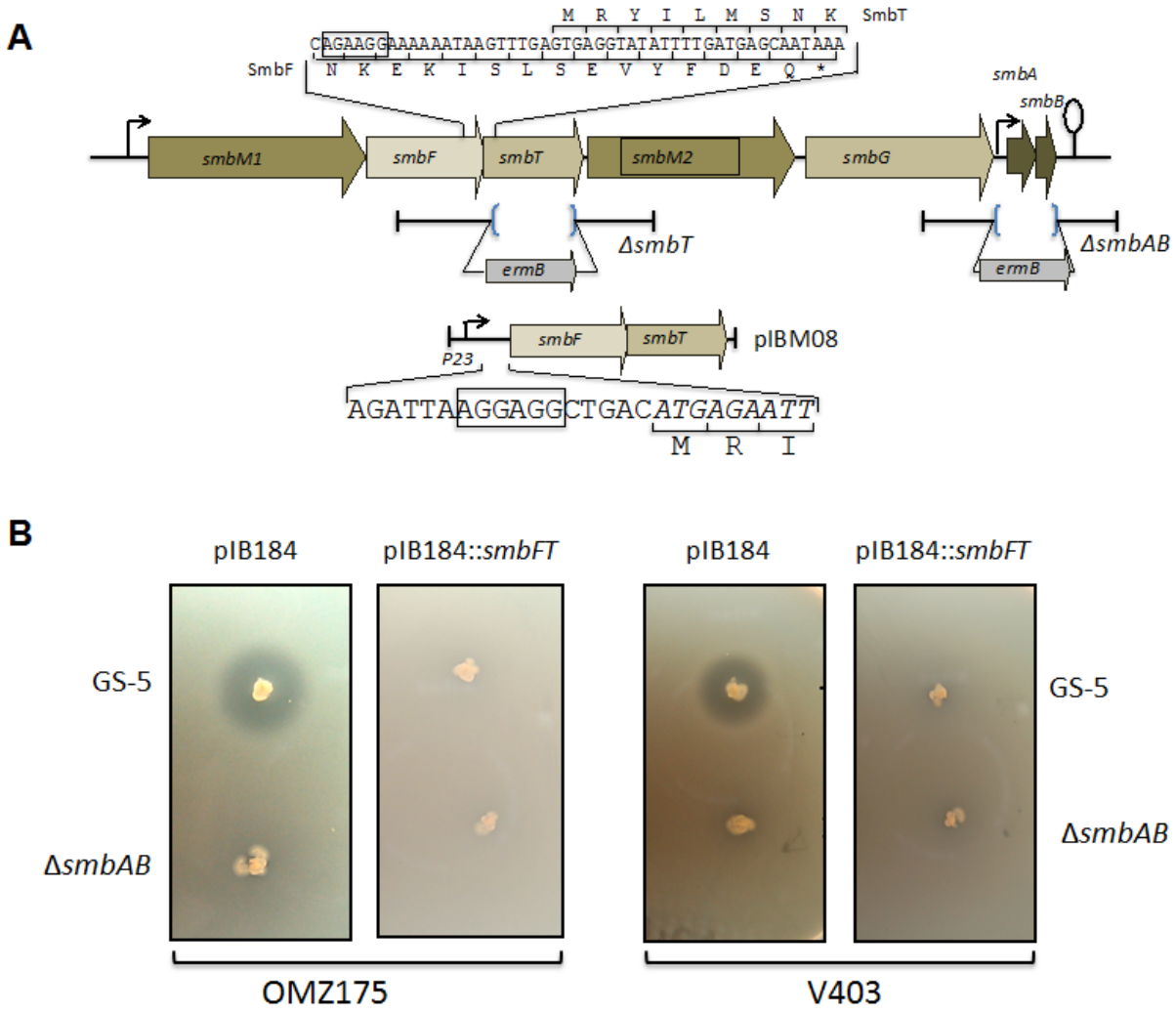
Species (strain)	Zone of Inhibition [diameter, mm]	
	GS-5	*ΔsmbAB*
S. mutans UA159*	11.0 ± 1.0	2.0 ±2.0
S. mutans NG-8*	11.0 ± 1.0	0.0 ± 0.0
S. mutans GS-5	0.0 ± 0.0	0.0 ± 0.0
S. mutans SP-2	0.0 ± 0.0	0.0 ± 0.0
S. mutans Sm3209	9.5 ± 1.5	2.5 ± 1.5
S. mutans T8	0.0 ± 0.0	0.0 ± 0.0
S. mutans SJ32	13.5 ± 1.5	0.0 ± 0.0
S. mutans 8VS3	4.1 ± 1.0	0.0 ± 0.0
S. mutans 109C*	16.5 ± 0.5	0.0 ± 0.0
S. mutans OMZ175*	15.5 ± 0.5	0.0 ± 0.0
S. mutans V100	11.5 ± 1.5	0.0 ± 0.0
S. mutans 262RF	0.0 ± 0.0	0.0 ± 0.0
S. mutans V403*	18.0 ± 2.0	0.0 ± 0.0
S. mutans UA130	2.0 ± 0.0	0.0 ± 0.0
S. mutans DP1	1.0 ± 0.0	0.0 ± 0.0
S. mutans V1	10.0 ± 1.0	0.0 ± 0.0

CI, Clinical isolate. *, Strains used for heterologous expression of SmbFT

We found the presence of expected 420-bp size band in eight of the isolates. As expected, these strains were also found immune to Smb, indicating presence of the same immune mechanism(s) in these strains as in the producer strain. The only exception was V100 strain that encodes the *smb* locus but was sensitive to Smb mediated inhibition. All other eight strains that did not produce a PCR product were all produced clear ZOI against Smb. This data support the notion that the strains that encode the *smb* locus are all immune to Smb mediated killing, except strain V100. V100 is unique among the *S. mutans* strains since does not develop natural competence (data not shown). We speculate that it does not express the *smb* locus.

3.3.2. SmbFT, a putative ABC transporter complex, provides immunity against Smb. The Smb encoding operon also encodes *smbFT* that shares high homology to a putative ABC transporter complex. Since ABC transporters often function as immunity proteins, we wanted to verify whether SmbFT can protect *S. mutans* from Smb mediated killing. SmbFT transporter is composed of two polypeptides. SmbT encodes permease component and is 238 residues long with six transmembrane domains. SmbF, on the other hand, encodes a 274- residues long protein with ATPase signature sequences [134]. The promoter for *smb* locus lies about 3.0-kb upstream of the *smbFT* genes. Sequence analysis suggests the presence of a weak ribosome-binding site at 5-nt upstream of *smbF* start codon and an overlap of nine codons between the *smbF* and *smbT* coding sequences (Fig 10A). Because the promoter lies far from the *smbF* and since *smbFT* are linked together, we decided to express both *smbFT* from a low-copy plasmid under a heterologous promoter (P23) as a single transcript. We selected a stable theta-replicating plasmid, pIB184Km [135], to clone and express *smbFT*. We also replaced the native weak ribosome-binding sequence with a strong sequence from the *gftB* gene. To examine the role in immunity, we heterologously expressed SmbFT in five *S. mutans* strains that are susceptible to Smb. These strains are UA159, NG-8, V403, 109c, and OMZ175.

Figure 10. Smb and its immunity protein (A) Genomic organization of *smb* locus in GS 5. Regions used for fusion PCR for deletion constructions are shown at the bottom. Brackets indicate the regions that were deleted from the genome and an erythromycin resistance gene (*ermB*) was inserted. Plasmid used for heterologous expression of *smbFT* (pIBM09) is shown. This plasmid contains a RBS from *gftB* gene and a P23 promoter used for *smbFT* expression. Relevant sequences near the *smbF* start codon and the overlapping region between *smbF* and *smbT* is shown. Bent arrows and lollipop indicate promoters and transcription termination site. Block arrows indicate gene orientation. (B). Immunity activity of SmbFT in *S. mutans* strains. GS-5 and a *ΔsmbAB* strain 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 ZOI of the indicator strains was evaluated after overnight incubation. The indicator strains are OMZ175 (left panel) and V403 (right panel) containing the vector (pIB184) or the vector with SmbFT. These plates are representative of three independent assays.


A
M R Y I L M S N K SmbT
CAGAAGGAAAAAATAAGTTTGAGTGAGGTATATTTTGATGAGCAATAAA
SmbF N K E K I S L S E V Y F D E Q *
smbA
smbB
smbM1
smbF
smbT
smbM2
smbG
ΔsmbT
ermB
ΔsmbAB
ermB
P23
smbF
smbT
pIBM08
AGATTAAGGAGGCTGACATGAGAATT
M R I
B
pIB184
pIB184::smbFT
pIB184
pIB184::smbFT
GS-5
ΔsmbAB
GS-5
ΔsmbAB
OMZ175
V403

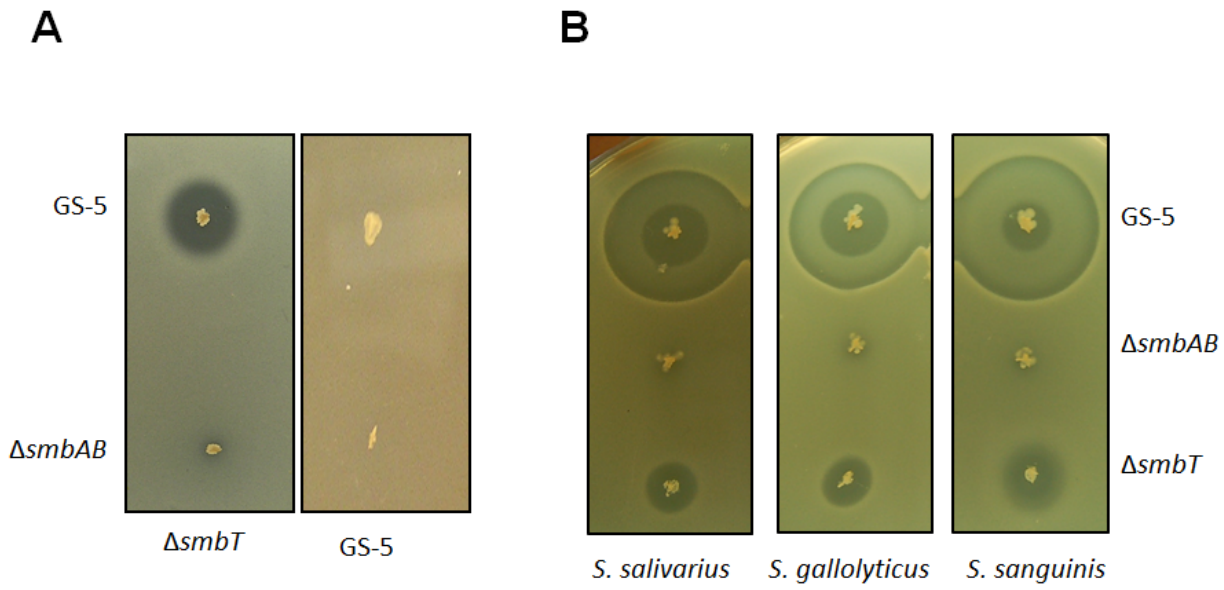
.

Heterologous expression of SmbFT in all the five *S. mutans* strains provided complete protection against Smb compared to the empty-vector carrying strains (Fig 10B, data not shown). Thus our findings support the prediction that SmbFT indeed functions as an immunity protein against Smb.

3.3.3. Inactivation of *smbT* makes the strain susceptible to Smb. Since SmbFT can confer protection against Smb lantibiotic, we wanted to determine whether SmbFT has a redundant function in providing immunity against Smb in GS-5. This is because a previous study suggests that two other factors, Bip (SMU.1914) and SmbG (encoded by *smb* operon), also provide protection against Smb [132]. We first constructed a *smbT*-deleted mutant (Δ*smbT*) in GS-5 by replacing the *smbT* coding sequence with a non-polar Em resistance cassette. We used Δ*smbT* as an indicator strain in previously described plate assays against Smb producer. As shown in Fig. 11A, we observed that Δ*smbT* is more susceptible to Smb compared to GS-5 strain. The ZOI inhibition produced by Δ*smbT* strain was very clear and as large as the ZOI produced by *S. salivarius* strain. This result supports that SmbFT functions primarily as an immunity protein in GS-5 and its absence makes the strain more susceptible to the lantibiotic.

To investigate the influence of SmbFT in Smb production, we performed a plate assay to measure ZOI formation by Δ*smbT* strain against *S. salivarius* indicator strain with GS-5 and Δ*smbAB* as controls. While GS-5 produced a larger and clear ZOI including a secondary halo, the ZOI produced by Δ*smbT* was clear and small. On the other hand Δ*smbAB* did not produce a visible ZOI. The larger secondary ZOI that was seen with GS-5 is not always visible and it depends on the age of the indicator culture (Fig 11B, data not shown). To confirm that Δ*smbT* could produce a clear ZOI, we used two other indicator strains, *S. galolyticus* and *S. sanguinis*. As shown in Fig 11B (right panels), Δ*smbT* produced a clear ZOI in both the strains as compared to Δ*smbAB* control, which did not produce a halo. As expected, GS-5 strain produced a larger ZOI with a secondary halo against both the indicator strains. Taken together, our data suggest that Smb is secreted at low level even in the absence of *smbT*.

Figure 11. Sensitivity of *ΔsmbT* to Smb and secretion of Smb by *ΔsmbT* (A) Agar diffusion assays were carried out with GS-5 and *ΔsmbAB* as tester strains with GS5 and *ΔsmbT* as indicator strains. (B). Production of Smb by *ΔsmbT* was verified by agar diffusion assay using *S. salivarius* BAA491, *S. galllolyticus* BAA2069 and *S. sanguinis* SK36 as indicator strains. GS-5 and *ΔsmbAB* was used as positive and negative controls, respectively. The plates are representative of three independent replicates.

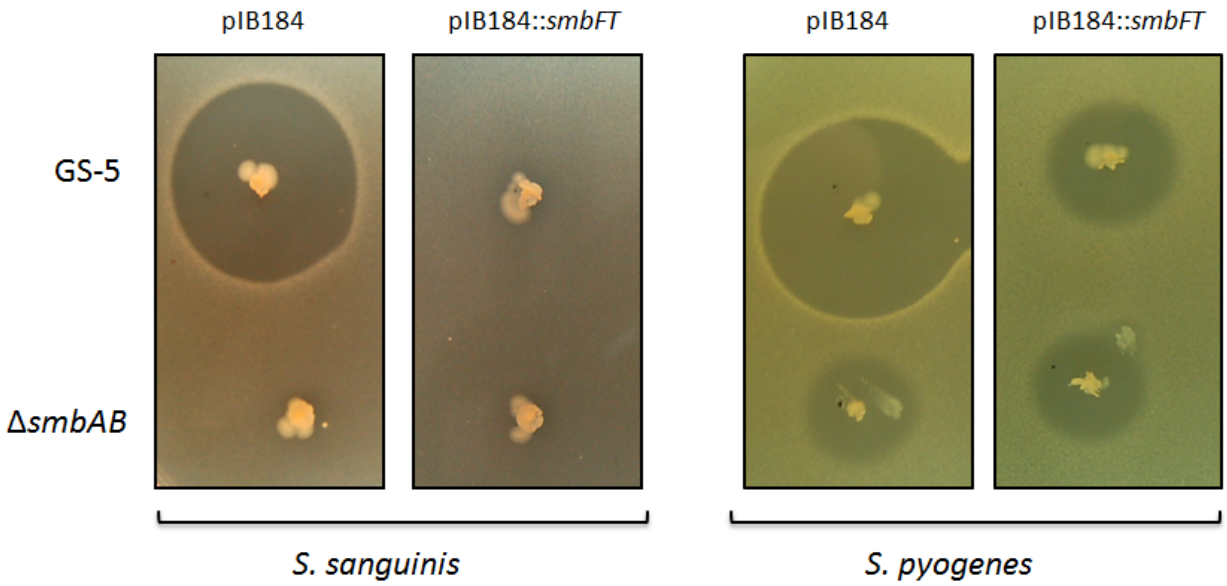

A
B
GS-5
ΔsmbAB
ΔsmbT
GS-5
GS-5
ΔsmbAB
ΔsmbT
S. salivarius
S. gallolyticus
S. sanguinis

3.3.4. SmbFT can confer immunity in heterologous streptococcal hosts. The oral cavity harbors over 700 different bacterial species, and streptococci constitute the majority [41]. Many streptococci contain ABC transporters that are similar to SmbFT yet they are sensitive to Smb. To verify whether SmbFT can confer resistance in other oral streptococci we selected a mitis group of streptococci, *S. sanguinis,* which plays a beneficial role in oral cavity and known to compete with *S. mutans* for subsequent colonization [103,105]. Another reason for selecting *S. sanguinis* was that the organism is naturally competent and therefore easy to manipulate [136]. When we overexpressed SmbFT from the plasmid in *S. sanguinis* SK36, we observed a complete protection against Smb mediated killing (Fig 12). In fact, there was very little or no ZOI in strain overexpressing SmbFT whereas ZOI of about 20mm diameter was observed in strain containing the vector only (Fig 12).

Since SmbFT conferred protection against Smb in streptococcus belonging to mitis group, we wanted to test whether it can confer protection in other groups. For this, we selected the pyogenic group since all the four tested species produced larger and clear ZOI (Fig 9A). We introduced the plasmid overexpressing SmbFT and the vector control in *S. pyogens* JRS4 strain, an M6 serotype strain. As shown in Fig. 12, overexpression of SmbFT in *S. pyogenes* also provided protection against Smb in *S. pyogenes* strain. However, the level of protection was less drastic and the Δ*smbAB* strain, which was included as control, also produced a clear ZOI in both vector and SmbFT overexpressing strain. Nevertheless, SmbFT conferred protection against this lantibiotic in *S. pyogenes*. Taken together, our results suggest that SmbFT can function as a universal immunity protein for Smb in streptococci.

3.3.5. The immunity provided by SmbFT is specific toward Smb and related lantibiotics. Cross-immunity is a rare occurrence among lantibiotic producer strains and has only been found in strains that produces very closely related lantibiotics, such as nukacin ISK-1 and lacticin 481 [137]. Since SmbFT was able to confer protection in various streptococci, we wondered whether SmbFT could provide cross-protection against other bacteriocins. Towards this end, we first tested against haloduracin, a two-component lantibitic produced by *Bacillus halodurans* and shares structural similarity with Smb [111].

Figure 12. SmbFT confers protection in heterologous hosts. Agar diffusion assays were carried out with GS-5 and *ΔsmbAB* as tester strains. The indicator strains are *S. sanguinis* SK36 (left panel) and *S. pyogenes* JRS4 (right panel) containing the vector (pIB184) or the vector with SmbFT. These plates are representative of three independent assays.


pIB184
pIB184::smbFT
pIB184
pIB184::smbFT
GS-5
ΔsmbAB
S. sanguinis
S. pyogenes

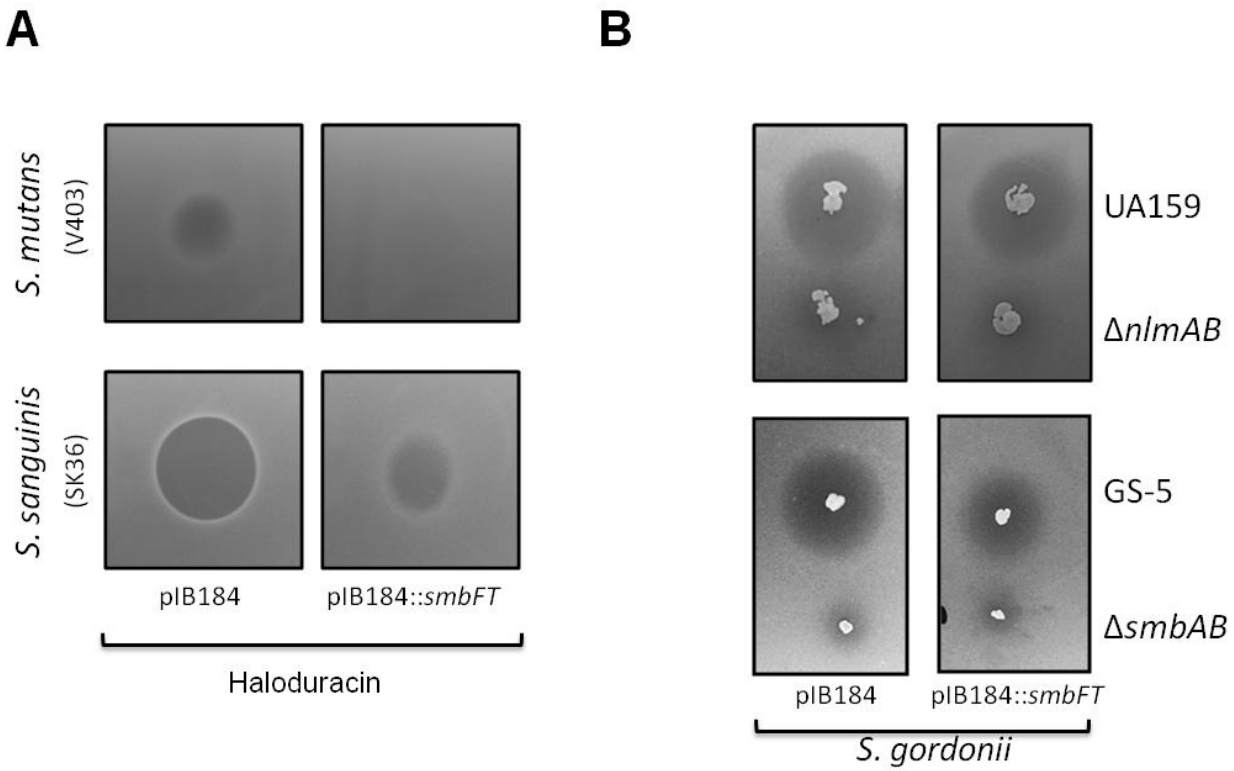
S. sanguinis and *S. mutans* V403 strains containing either smbFT expression plasmid or the vector alone were used as indicator strains in agar diffusion assay with 10µl of 100µM purified haloduracin (kindly provided by Dr. van der Donk) spotted on plate and the results are shown in Fig. 13. Haloduracin produced a clear and larger ZOI in *S. sanguinis* containing the vector while *S. sanguinis* expressing SmbFT produced a much smaller and cloudy ZOI. The ZOI in *S. mutans* V403 with the vector was much smaller and cloudy, suggesting that haloduracin is not very effective against *S. mutans*. However, no ZOI was observed in V403 expressing SmbFT indicating that as in *S. sanguinis*, SmbFT provided protection against haloduracin in *S. mutans*.

S. gallolyticus BAA 2069 possess a unique 23 kb genetic island in the genome, coding for bacteriocin-associated genes (SGGBAA2069_c00810-c00960). These genes encode for a lantibiotic that share high degree of similarity with Smb and haloduracin. Hinse et al [138] have shown that BAA 2069 strain indeed produces a lantibiotic that is active. Therefore, we tested *S. mutans* V403, *S. sanguinis* SK36, and *S. pyogenes* JRS4 strains overexpressing SmbFT or containing the vector. All the streptococci produced much smaller ZOI when SmbFT was present as compared to vector only strains (data not shown). This indicates that SmbFT is also active against the lantibiotic produced by BAA 2069.

We then tested whether SmbFT could provide protection against other bacteriocins. For this we chose *S. mutans* UA159 that primarily produces mutacin IV (NlmAB, a two-component non-lantibiotic. *S. gordonii* is generally used as an indicator strain for mutacin IV. As shown in Fig. 13, *S. gordonii* with or without SmbFT produced similar size ZOI indicating that SmbFT could not confer protection (control Δ*nlmAB* did not produce any ZOI in either cases*)*. Next we checked the effect SmbFT on protection against purified nisin, a well-studied monopeptide lantibiotic that is structurally different from Smb. As expected, SmbFT did not provide protection against nisin (data not shown). ABC transporters are often associated with tolerance to various peptide antibiotics such as vancomycin and bacitracin [139].

Therefore, we also checked sensitivity to different cell-wall specific peptide antibiotics, such as bacitracin, polymyxin B, and vancomycin by disc diffusion assay. We did not observe any noticeable difference between the strain containing SmbFT or the vector control (data not shown). Taken together our results imply that SmbFT is specific to confer protection against Smb or closely related lantibiotics.

Figure 13. Substrate specificity of SmbFT. (A) Purified haloduracin (1:1 mixture of Halα and Halβ) was spotted on THY plate and then overlaid with indicator strains containing the vector or the vector with SmbFT. (B). UA159 and *ΔnlmAB* strains were stabbed into THY agar and deferred antagonism assay was carried out with *S. gordonii* DL-1 strain containing the vector or the vector with SmbFT. GS-5 and *ΔsmbAB* strains were used as control. Assays were repeated at least three times, and a representative plate is shown.


A
S. mutans (V403)
S. sanguinis (SK36)
pIB184
pIB184::smbFT
Haloduracin
B
UA159
ΔnlmAB
GS-5
ΔsmbAB
pIB184
pIB184::smbFT
S. gordonii

3.4. Discussion

Lantibiotic producer strains possess multiple mechanisms to protect themselves against their own inhibitory activity. One of the most effective mechanisms that provide the maximum protection is through various dedicated ABC transporters. ABC transporters that are involved in self-immunity or protection against other lantibitics are generally classified into multiplecategories [140]. ABC transporters such as NisT and SunT family members are encoded by a single polypeptide where the ATPase domain is fused with the permease domain. Some members of SunT also encode an N-terminal peptidase C39 domain. SmbG, which was shown to confer protection against Smb [132], belongs to the SunT family. The other ABC transporters families all contain two separate polypeptides one with permease activity and the other with ATPase function. SmbFT, the ABC transporter that we studied here, belong to BcrAB family that contains two separate polypeptides. ABC transporters belonging to BcrAB family are not extensively characterized except for two transporters from Bacilli [141,142]. A recent evolutionary relationship study indicates that BcrAB family proteins are closely related to LanFEG family transporters. The latter family proteins, which contain two separate permeases (LanE and LanG) and one ATPase (LanF), are all involved in self-immunity of lantibiotic producing bacteria. On the other hand BcrAB family transporters are involved in resistance against bacitracin [141,143]. Since the genes are encoded within the biosynthesis locus for bacitracin, BcrAB family proteins appear to confer self-immunity [143]. The SmbFT transporter that we studied here is the first example of a BcrAB family protein that is involved in lantibiotic resistance.

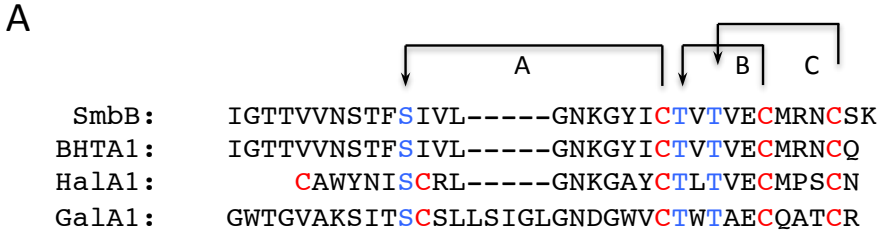
As mentioned before the SmbFT transporter is composed of two polypeptides, SmbT, which encodes the permease component and SmbF, on the other hand, encodes the ATPase where two third of the N-terminal region comprises of P-loop containing nucleoside triphosphate hydrolase signature sequences [144]. Although *S. mutans* encodes numerous permease proteins [44], a sequence similarity search with SmbT against the genome returned no results suggesting that SmbT encodes a unique sequence. In contrary, search using SmbF as query against the genome returned several ABC transporter related ATPAses. Four ATPases, SMU.238, SMU.654, SMU.1035, and SMU.1811 showed maximum similarity

(E-value >-30) with SmbF (data not shown). At present we do not know whether any of these ATPases can form a productive complex with SmbT permease to counteract Smb.

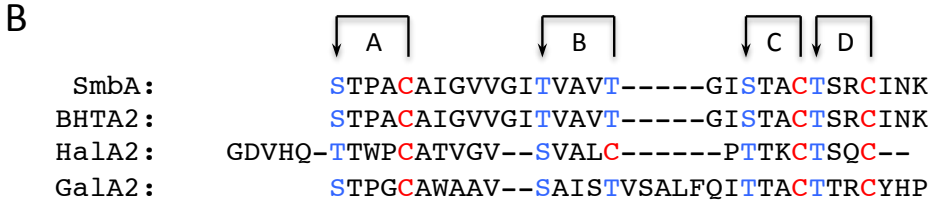
We expressed SmbFT in at least four different streptococci other than *S. mutans.* In each of these streptococci, heterologous expression of SmbFT conferred full protection against Smb lantibiotic. This means SmbFT does not need any other accessory proteins for their activity. Alternatively, the accessory protein could be a highly conserved protein present all the streptococcal strains tested here. As previously mentioned, lantibiotic producer strains often encode two types of transporters for full protection. For example, *Lactococcus lactis* strain that produces lacticin 3147, a two-component lantibiotic similar to Smb, possesses both LtnI, a lipid anchored membrane protein, and LtnFE systems [77]. Draper et al [77] have demonstrated that in a heterologous system, LtnI and LntFE act synergistically to confer complete protection. The *smb* locus does not encode any LntI homolog but it encodes SmbG, a SunT homolog, containing ABC transporter motif and a C39 peptidase motif. It has been proposed that SmbG functions as an immunity protein against Smb [132]; however, an *smbG* mutant strain is not susceptible to Smb. An immunity-like protein encoded by SMU.1913 (mentioned as Bip in ref.[132]) has also been proposed to function as Smb immunity protein. Surprisingly, a *bip* single mutant strain is also not susceptible against Smb indicating other proteins can confer resistance. In contrast, our data suggest that SmbFT alone can confer complete protection against Smb in a variety of streptococcal strains; all these strains neither contain SmbG nor Bip. Therefore, unlike other lantibiotic immunity systems, we speculate that SmbG and SmbFT provide different functions in *S. mutans*. We believe that the primary role of SmbG is to secrete Smb peptides from cytoplasm to the milieu and when SmbG is absent, the strain does not produce active Smb. Indeed we found that a *smbG* mutant is unable to produce Smb and functionally behave like the Δ*smbAB* mutant.

Figure 14: Sequence alignment of two-peptide lantibiotics closely related to Smb. The α-peptide (A1) is encoded by SmbB while the β-peptide (A2) by SmbA. Other sequences were retrieved from GenBank and they are: *S. ratti* BHT strain BHTA1 (AAZ76603) and BHTA2 (AAZ76602); *Bacillus halodurans* C-125 HalA1 (NP_241320) and HalA2 (NP_241319); and *S. gallolyticus* BAA 2069 SGALA1 (YP_004287012). The GALA2 peptide was not annotated in the BAA 2069 genome and was identified by analyzing the untranslated sequence near the GALA1 coding region. Curved arrows indicate residues involved in putative and/or established ring structure formation and the letters inside the arrows indicate the ring designation in the structure.

A


A
B
C
SmbB: IGTTVVNSTFSIVL-----GNKGYICTVTVECMRNCSK
BHTA1: IGTTVVNSTFSIVL-----GNKGYICTVTVECMRNCQ
HalA1: CAWYNISCRL-----GNKGAYCTLTVECMPSCN
GalA1: GWTGVAKSITSCSLLSIGLGNDGWVCTWTAECQATCR

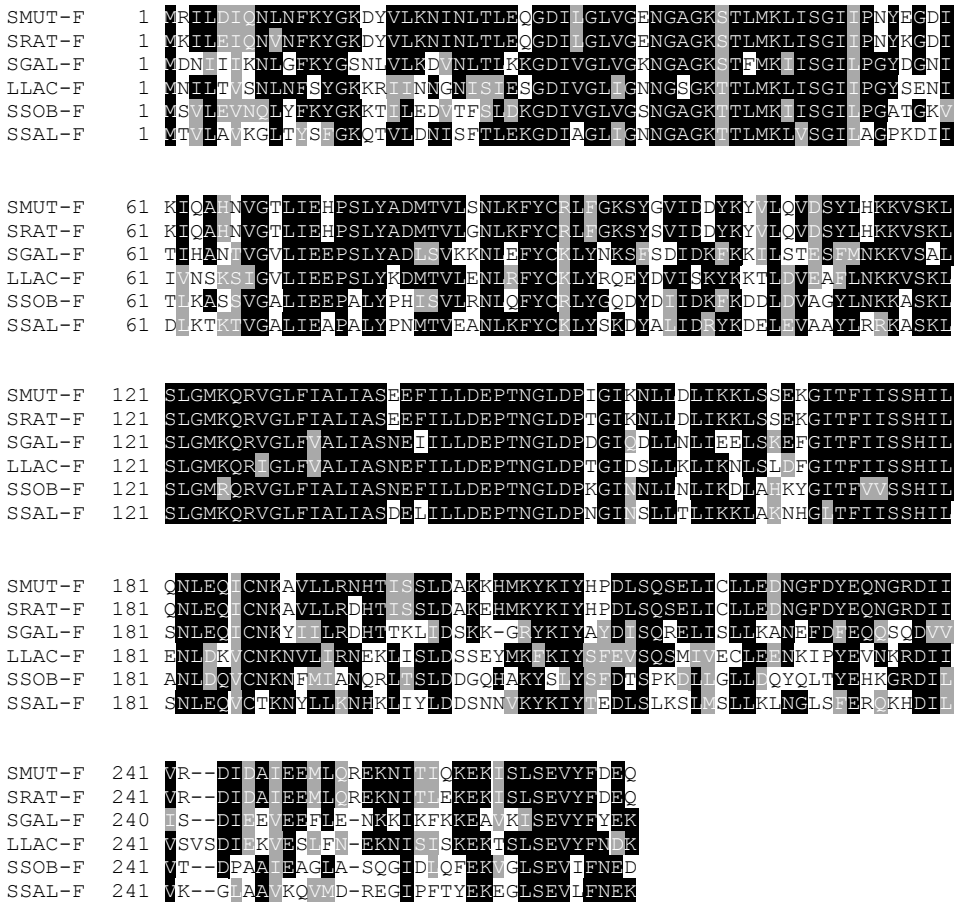
B


A
B
C
D
SmbA: STPACAIGVVGITVAVT-----GISTACTSRCINK
BHTA2: STPACAIGVVGITVAVT-----GISTACTSRCINK
HalA2: GDVHQ-TTWPCATVGV--SVALC------PTTKCTSQC--
GalA2: STPGCAWAAV--SAISTVSALFQITTACTTRCYHP

We also believe that the primary function of SmbFT is to confer self-protection and not secretion. This is because we found that the Δ*smbT* mutant produced a clear ZOI against multiple strains (Fig.11) indicating Smb production; however, the ZOI was much smaller than the GS-5 strain. There are several reasons that can explain the smaller ZOI produced by the Δ*smbT* strain. Since the strain was constructed by insertion of an *ermB* gene (erythromycin resistance), it might have interfered with the transcription of the downstream genes in the *smb* operon as a result less *smbAB* was produced. Often lantibiotic production is under feedback inhibition and positively influenced by the immunity proteins [145,146]. Therefore, it is also possible that SmbFT positively influences *smb* production. Whether SmbFT has a positive role in Smb production remains to be tested.

Our results suggest that SmbFT could confer protection not only against Smb but also against haloduracin and an uncharacterized lantibiotic produced by BAA 2069 (we named it as gallolacticin). All the three lantibiotics share high degree of sequence similarity (Fig. 14) and probably structurally similar as well. All three lantibiotics are composed of two modified peptides, α and β. While no information is available for Smb or gallolacticin, a detailed structure has been proposed for haloduracin that is based on mass-spectrometry data and modeled on NRM structure of lacticin 3147 [147]. The structural information, together with the sequence similarity, suggests that all α-peptides have the same topology with three C-terminal rings ([70], Fig 14). The β-peptides appear to contain three or four rings, however the B ring is present only in haloduracin and absent in all others. Since SmbFT recognized all the three lantibiotics we speculate that the transporter complex predominantly recognizes the conserved topology and structurally similar lantibiotics. Based on this observation, we also speculate that SmbFT will confer protection against two-component lantibiotics that share this overall topology. Interestingly, the β-peptides of two lantibiotics, lacticin 3147 and staphylococci C55 lack the first ring (A ring) but contain three other rings (B-D). It would be of interest to test whether SmbFT could recognize lacticin 3147 and staphylococcin C55.

Figure 15: Multiple sequence alignment of SmbF (A) and SmbT (B) with their closest homologs. The alignment was performed using Clustal-W. Degree of shading was done using BoxShade where black and gray blocks indicate identical and similar amino acids, respectively. Sequences were obtained from GenBank (accession numbers are in parenthesis). Strains are: *S. ratti* (SRAT-F, WP_003086779.1; SRAT-T: WP_003086777.1); *S. gallolyticus* (SGAL-F, YP_004287007.1; SGAL-T, YP_004287008.1); *L. lactis* (LLAC-F, YP_004761484.1; LLAC-T, YP_004761485.1); *S. sobrinus* (SSOB-F, WP_019770819.1;SSOB-T, WP_019776383.1); and *S. salivarius* (SSAL-F, YP_004728647.1 SSAL-T, YP_004728646.1)



So far all the ABC transporters that belong to BcrAB family have been shown to act specifically against cyclic peptide bacitracin ([141,143]). Although sequence similarity indicates that SmbFT belongs BcrAB family, we found that SmbFT did not recognize bacitracin, nor it recognized vacomycin and polymyxin B, two other cyclic peptide antibiotics. SmbFT also did not recognize nisin, a heavily modified single peptide lantibiotics. A BLASP search using SmbF or SmbT peptides as query sequences against the GenBank database returned sequences mostly from streptococci. The top hit sequences include *S. ratti* (strains BHT and FA-1), *S. gallolyticus* BAA 2069, *S. sobrinus* DSM20742, *S. salivarius* CCHSS3, and *Lactococcus lactis* (Fig 15). Thus we propose that SmbFT and its homolog constitute an ABC transporter family distinct from BcrAB and specifically recognize two-component lantibiotics. It is noteworthy to mention that although *S. gallolyticus* BAA 2069 produces gallolactacin and encodes a SmbFT homolog, which we named GalFT, the strain was sensitive to Smb (data not shown). Therefore, we speculate that while SmbFT confer protection against gallolacticin, GalFT from BAA 2069 cannot confer protection against Smb. Sequence alignment between SmbF and SmbT with the corresponding GalF and GalT sequences identified presence of several unique amino acid residues in both GalF and GalT. A hybrid approach is currently underway where we are combining SmbF with GalT and vice versa to identify the subunit necessary for substrate recognition and/or discrimination.

Although mechanisms related to protection against lantibiotics have been extensively studied, the molecular mechanism of lantibiotic transport is still poorly understood. Several recent studies suggest that unlike general ABC transporters that transport substrates across the membranes, the ABC transporters related to immunity function transport specific lantibiotics from the membrane to the extracellular space [120,121,137]. A recent study identifies the presence of a conserved motif, termed as E-loop, in the ABC transporters belonging to LanFEG and BcrAB families [134]. This E-loop structure plays an important role in the function of these transporters and perhaps induces structural changes in the transmembrane domains. Whether E-loop is involved in substrate recognition and/or binding remains to be evaluated. Additional structure/function studies are necessary to understand the molecular mechanism of lantibiotic transport by the immunity related ABC transporter systems.

Chapter 4: A conserved streptococcal membrane protein, LsrS, exhibits a receptor-like function for lantibiotics

4.1. Abstract

Streptococcus mutans strain GS-5 produces a two-component lantibiotic, Smb, which displays a broad-spectrum inhibitory activity that includes other streptococci. Lantibiotics must recognize specific receptor molecules present on the sensitive bacterial cells for inhibition. However, so far no such receptor proteins have been identified for any lantibiotic. In this study, using a powerful transposon mutagenesis approach, we have identified a gene in *Streptococcus pyogenes* that exhibits a receptor-like function for Smb. The gene-encoded protein, which we named LsrS, is a membrane protein belonging to CAAX protease family. We also found that nisin, a mono-peptide lantibiotic, requires LsrS for its optimum inhibitory activity. However, we found that LsrS is not required for inhibition by haloduracin and galolacticin, both these are two-peptide lantibiotics closely related to Smb. LsrS appears to be a well-conserved protein that is present in many streptococci including *S. mutans*. Inactivation of SMU.662, an LsrS homolog, in *S. mutans* strains UA159 and V403 rendered the cells refractory to Smb-mediated killing. Furthermore, overexpression of LsrS in *S. mutans* causes the cells to be more susceptible to Smb. Although LsrS and its homologs encode CAAX protease domain, we demonstrate that inactivation of the putative active sites on the LsrS protein has no effect on its receptor-like function. This is the first report describing a highly conserved membrane protein that displays a receptor-like function for lantibiotics.

4.2. Introduction

Lantibiotics are a group of ribosomally synthesized small peptides containing bactericidal or bacteriostatic activity. These peptides are post-translationally modified involving multiple residues [70,107,115,148]. In general, lantibiotic synthesis operon encodes various enzymes that dehydrate most of the serine and threonine residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. When cysteine residues are present in the vicinity, Dha and Dhb can form thioetherlinked lanthionine and 3-methyl lanthinoine bridges, respectively. Occasionally, Dha, Dhb and other modified residues can be present as unlinked residues [for reviews see [70,71,108-110]]. On the basis of the biochemical activities of the modifying enzymes lantibiotics are grouped into three classes [70]. Lantibiotics that belong to class I include nisin, streptin, and Pep5, and are modified by two enzymes, LanB and LanC [71,149]. Class II lantibiotics are generally globular peptides with the prototype lantibiotics mersacidin and cinnamycin that modified by a single enzyme often referred to as LanM-type enzyme. Class II lantibiotics also include two-component lantibiotics (such as lacticin 3147, plantaracin W, and haloduracin) and the antimicrobial activity requires synergistic interaction of both peptides [111,112,150]. Class III lantibiotics, such as SapT and SapB, constitute an emerging group of lantibiotics that has mainly morphogenetic functions and displays very limited antibacterial activities [2,71,113,114].

Based upon their mode of action, lantibiotics can also be classified into several categories. Lantibiotics such as Pep5 directly targets the bacterial membrane to form pores that leads to release of ions and molecules from the sensitive bacteria, eventually leading to cell death [151]. Other lantibiotics such as mersacidin and nukacin ISK-1 bind to lipid II and thereby inhibit peptidoglycan biosynthesis in the target bacteria, a mechanism similar to that of vancomycin that also binds to lipid II [107,152]. Lantibiotics belonging to the next category function by a complex double mode-of-action mechanism where they inhibit cell wall biosynthesis by binding to lipid II molecules as well as create pore formation in bacterial membranes. Often both functions can be combined into a single polypeptide as in nisin and epidermin [153,154]. However, a combination of two functionally specialized peptides, known as two-peptide lantibiotics, is required for the activity. Two-peptide lantibiotics contain a globular α-peptide with

homology to mersacidin that binds to lipid II and an elongated β-peptide that forms a complex with the α-peptide bound lipid II complex. Subsequently the β-peptide forms a pore by inserting inside the bacterial membrane [155,156]

The lactic acid bacteria such as enterococci, lactococci, and streptococci secrete a wide range of lantibiotics with variable spectra of inhibition [72,109,115-117,157]. Among the lantibiotics, nisin, which is secreted by lactococci, is one of the most well-studied and widely used lantibiotics [72,116]. Nisin has a broad range of inhibitory spectrum and can inhibit several gram-positive bacteria including *Staphylococcus aureus*, *Listeria monocytogenes*, and as well as a variety of streptococci and enterococci [158,159]. Furthermore, nisin can inhibit Bacillus spore outgrowth and germination [160]. Among the two-peptide lantibiotics, lacticin 3147, which is also secreted by some strains of lactococci, inhibits many gram-positive bacteria including *L. monocytogenes*, *S. aureus* and *Clostridium difficile* in addition to streptococci, enterococci and mycobacteria [161,162].

Streptococcus mutans, an oral lactic acid bacteria and a major causative agent of dental caries in humans, secretes several types of lantibiotics, commonly known as mutacins [64,122-128]. Most of these lantibiotics such as mutacin I, II, and III (1140) are monopeptide and presumably function like nisin or mersacidin. The only two-peptide lantibiotic so far identified in *S. mutans* is Smb produced by GS-5 and some other strains [126,131]. Although the lantibiotic mutacins are widely present in *S. mutans* [86,128], surprisingly the first sequenced reference strain UA159 does not encode any lantibiotic, it only encodes non-lantibiotics [44]. It appears that *S. mutans* has acquired many mutacins encoding genes by horizontal gene transfer mechanism. For example, the strains that produce mutacin II contains the *mut* operon that is inserted after the alanyl t-RNA synthetase (*ats*, SMU.650) in the corresponding UA159 genome [128]. Likewise, the *smb* locus that encodes genes necessary for Smb biosynthesis appears to be integrated in between SMU.1942 and *syl* locus.

The frequency of the presence of *smb* locus among various *S. mutans* clinical isolates has not been systematically studied. However, we recently showed that as many as 50% *S. mutans* isolates in our laboratory collection encode the *smb* locus [163]. Although very little is known about the structure or the

mode-of-action of Smb, primary sequence analysis suggests that Smb is similar to lacticin 3147 and haloduracin [163]. Smb also has a broad inhibitory spectrum. It can inhibit growth of streptococci belonging to all six phylogentic groups as well as lactococci and enterococci [91,163]. However, it appears that Smb may not inhibit *Staphylococcus epidermidis* and *Bacillus subtilis* [91,163].

One of the streptococci that Smb very efficiently inhibits is the human pathogen *S. pyogenes,* also known as group A streptococcus (GAS). GAS causes a wide variety of diseases, including relatively mild and self-limiting infections of the throat and skin as well as life-threatening invasive diseases like septicemia, myositis, necrotizing fasciitis, and streptococcal toxic shock syndrome (for recent reviews, see reference [164,165]. Earlier observations suggest that *S. pyogens* and other sensitive bacteria express cell surface molecules that can act as receptors [166,167]. Theses molecules are different from the surface polymers such as group and type antigens [89]. The study by Perry and Slade [167] suggests that a partially purified fraction of sonicated extracts of *S. pyogenes* can inhibit the lantibiotic activity produced by GS-5 strain presumably because a receptor-like molecule sequesters one or both the peptides. In this study, we attempted to identify a receptor molecule in *S. pyogenes* for Smb by using a transposon mutagenesis approach. We identified a previously uncharacterized membrane protein that exhibits a receptor-like function for Smb.

4.3. Results

4.3.1 Identification of a receptor gene in *S. pyogenes*

A previous study indicated that *S. pyogenes* might encode some cell surface proteins that function as receptor for the lantibiotic Smb [167]. We wanted to identify the genes that encode those putative receptor molecules. Towards this end, we used the insertion sequence IS*S1* because it randomly inserts into the genome of gram-positive bacteria, including various streptococci and because it rarely inserts itself more than once into the same cell [96,168-171]. We introduced this transposon into JRS4, an M6 serotype strain, on pGhost9::IS*S1*, a plasmid whose replication is temperature sensitive [172]. An erythromycin-resistant (Em^r) transformant containing pGhost9::IS*S1* was grown overnight at 30°C, and Em^r colonies containing the transposon were isolated at 37°C. We reasoned that inactivation of a receptor molecule on GAS would produce a strain that would be recalcitrant to Smb-mediated inhibition. We plated a transposon library on THY agar plates that were previously stabbed with *S. mutans* GS-5 strain that produces lantibiotic Smb. While most of the stabbed GS-5 produced clear zones of inhibition (ZOI) with diameters of 24±1 mm, three stabbed cultures produced ZOI each with a single colony that grew inside the halo. Using an inverse PCR method, as described in Material and Methods, we attempted to identify the IS*S1* insertion sites in these survivor mutants that grew inside the halo. Two of the insertion sites were located within the SPy1384 genes (M1 GAS SF370 is the reference strain) while the insertion site could not be determined for the third mutant.

SPy1384 encodes a polypeptide of 231 residues; the ISS*1* insertions occurred in this gene at codon positions 8 and 148 (Fig 16). We renamed this gene as *lsrS* for lantibiotic Smb receptor like function in streptococci. It appears that *lsrS* is the last gene of a three-gene operon. SPy1386, which encodes a putative transcriptional regulator protein (71aa) with a Helix-Turn-Helix XRE-family like motif and SPy1385, which encodes a hypothetical protein with DUF3169 domain, are the two other genes in the operon. Just upstream of the operon is *alaS* gene that encodes alanyl-tRNA synthetase. An intergenic

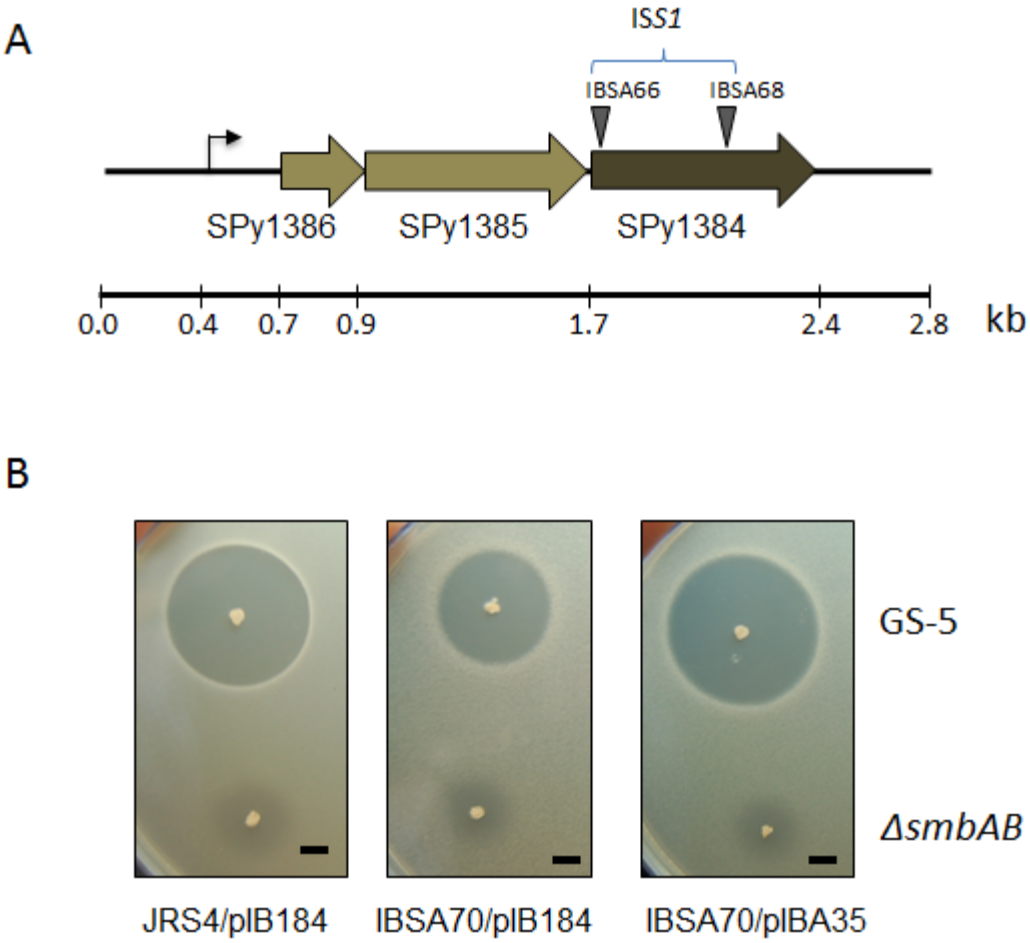
region of 259-bp lies between *alaS* and SPy1386 loci. Analysis by BPROM (Softberry) software indicate the presence of a -35 box (TTGTCA) and a -10 box (TACAAT) within at a position 250-bp upstream of the ATG start codon of SPy1386 (Fig 16).

To confirm that *lsrS* indeed plays a role in Smb mediated inhibition, we selected the mutant strain (IBSA68) in which IS*S1* was inserted at the 148th codon. We generated a clean mutant derivative strain (IBSA70) by curing the integrated pGhost9:IS*S1* plasmid from strain IBSA68 to create IBSA70. We also cloned the *lsrS* gene in plasmid pIB184-Km under a heterologous promoter (P23) for complementation purposes. Both the vector plasmid (pIB184-Km) and the complementing plasmid (pIBA35) were introduced into IBSA70. The vector plasmid was also introduced into JRS4 for uniformity. These strains were then tested against GS-5 for sensitivity. As shown in Fig 16, IBSA70 carrying only the vector plasmid produced a ZOI with a diameter of 18±1 mm where as JRS4 with the vector plasmid produced a ZOI with a diameter of 24±1 mm, about 40% reduction in the total area of inhibition in the mutant strain(Fig 16). When we complemented IBSA70 with the plasmid pIBA35 carrying the *lsrS,* the ZOI became 25±1 mm in diameter suggesting that the observed reduction in ZOI is indeed due to inactivation of *lsrS.*

4.3.2. LsrS plays a role in nisin and tunicamycin sensitivity

Smb is a two-component lantibiotic and like other lantibiotics, it is expected to interact with the lipid II molecules. Because LsrS is involved in the optimum function of Smb to inhibit *S. pyogenes*, we wanted to test whether LsrS is also involved in inhibition by other two-component lantibiotics. For this purpose, we selected haloduracin, a well-characterized lantibiotic that targets lipid II, and galolacticin that is produced by *S. gallolyticus* BAA2069 with sequence similarity to Smb [163]. As shown in Fig. 17, sensitivities of both JRS4 and IBSA70 strains were similar against the purified haloduracin and galolacticin. This result suggests that LsrS is specific to Smb and does not recognize other two-component lantibiotics.

Figure 16: Isolation of a receptor protein for lantibiotic Smb in *S. pyogenes*. (A) Genetic organization of the receptor locus (SPy1384). SPy1384 is the last gene of a three-gene operon. The first gene, SPy1386 encodes a putative transcription factor of HTH_XRE superfamily. The second gene, SPy1385, encodes a hypothetical membrane protein with DUF3169 motif. The site of IS*S1* insertions and their relative positions are shown. The bent arrow indicates the putative promoter sequence. (B). Deferred antagonism assay for receptor activity. Bacterial cultures were stabbed on THY-agar plate and incubated overnight at 37^0C under microaerophilic condition. The plates were then overlaid with soft agar containing indicator strains. The zones of inhibition (ZOI) of the indicator strains were measured after overnight incubation. The observation is based on four separate experiments and a representative area of interest is shown. The ZOI values for Smb mediated inhibition are: JRS4/pIB184, 24±1; IBSA70/pIB184, 18±1; and IBSA70/pIBA35, 25±1. The length of the scale bar equals to 5mm.


A
ISS1
IBSA66
IBSA68
SPy1386
SPy1385
SPy1384
0.0
0.4
0.7
0.9
1.7
2.4
2.8
kb
B
GS-5
ΔsmbAB
JRS4/pIB184
IBSA70/pIB184
IBSA70/pIBA35

Nisin is a one-component lantibiotic that functions similar to two-component lantibiotics, i.e. it binds to lipid II and inhibits cell-wall biosynthesis as well as form pores in the membrane. Therefore, we tested whether LsrS could display a receptor like function for nisin. We observed that IBAS70 produced a ZOI with 14±1 mm diameter while the wild type JRS4 produced a ZOI with 18±1 mm diameter, about a net reduction of 40% in total area. This indicates that in addition to Smb, LsrS plays an important role, either directly or indirectly, for nisin recognition.

Since Smb and nisin both bind to lipid II, and since LsrS is a putative membrane protein, we wanted to know whether inactivation of LsrS renders the cell sensitive to antibiotics that target cell-wall biosynthesis. We tested antibiotics specific for lipid II synthesis such as fosfomycin (inhibits MurA), D-cycloserine (inhibits D-ala ligase), tunicamycin (inhibits MraY), bacitracyin (blocks lipid carrier recycling), vancomycin (blocks transglycosylation), and penicillin (blocks transpeptidation). We also included polymyxin B and colistin, which target cell membranes. We found that among these antibiotics, only tunicamycin produced a 40% smaller halo in IBSA70 as compared with JRS4 (Fig 17). All other antibiotics produced similar halo in both the strains (Table7, data not shown). This indicates that LsrS plays a role in tunicamycin sensitivity in *S. mutans*.

4.3.3. LsrS homologs are present in other streptococci and function as Smb receptor

Bioinformatics analysis suggests that LsrS belongs to COG1266, a highly conserved family predicted to encode a zinc dependent CAAX prenyl metalloprotease domain. Furthermore, the C-terminal region of LsrS contains a domain called Abi (abortive infection, Pfam02517), which is a subfamily of CAAX protease. A BLAST-P search showed that LsrS is present in all the sequenced *S. pyogenes* genomes. Furthermore, the search also fetched numerous streptococci including many oral streptococci with E-values lower than -50. Surprisingly, it appears that all the sequenced *S. mutans* strains also encode an LsrS homolog protein with an E-value of -61. The homolog in *S. mutans* UA159 is SMU.662, which showed 41% identity and 66% similarity with the LsrS sequence (Fig 18).

Figure 17: Sensitivity of the *lsrS* mutant to various antimicrobial agents. THY agar plates containing the indicator strains were either pre-seeded with galolacticin producing strain (BAA2069), or spotted directly on the overlaid plates and incubated overnight at 37^0C under microaerophilic condition. Experiments were repeated at least three times and representative areas of interest are shown. Both the strains also contain vector pIB184-Km. Bars, 5mm.

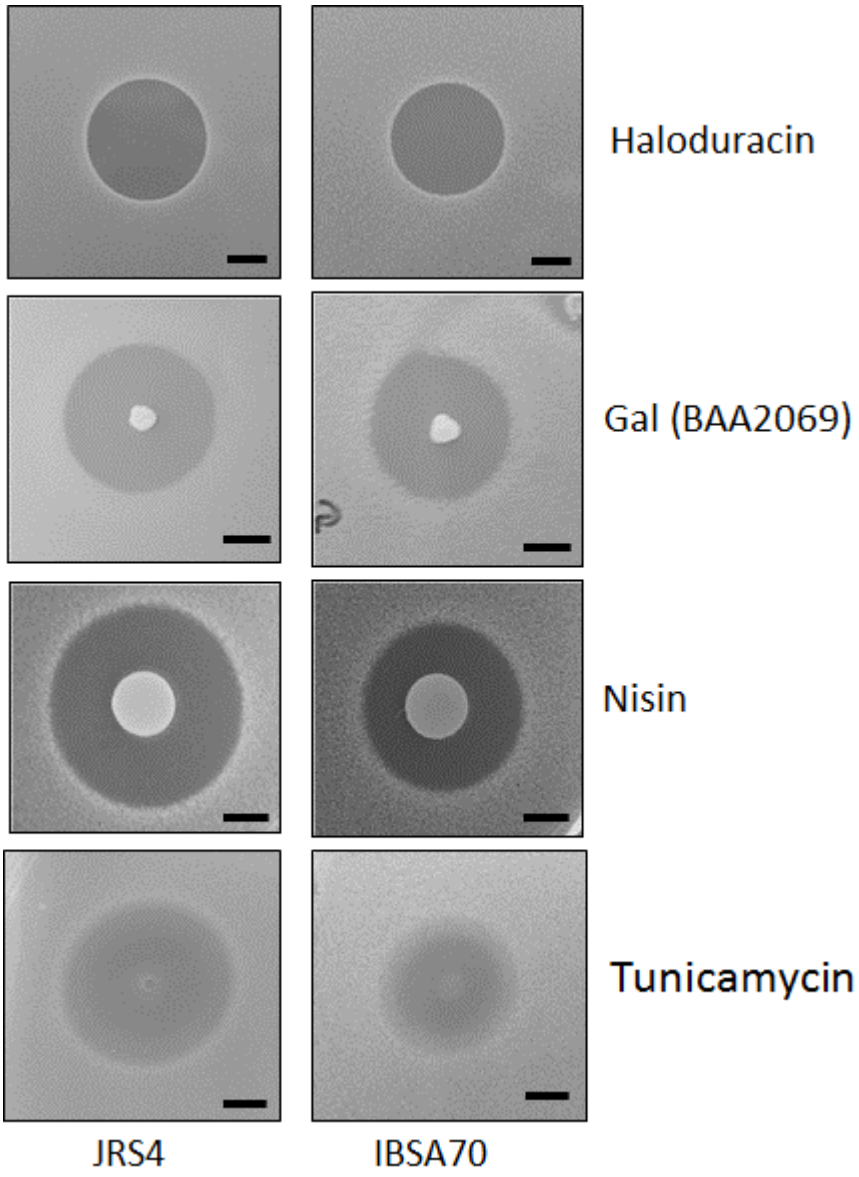

Haloduracin
Gal (BAA2069)
Nisin
Tunicamycin
JRS4
IBSA70

Table 7: Effect of various bacteriocins and antibiotics on *lsrS* mutant

Compounds/Strains	Indicator Strains	
	JRS4/pIB184	IBSA70/pIB184
Lantibiotics:		
Smb*	24.0 ± 1.0	18.0 ± 1.0
Haloduracin	15.0 ± 1.0	15.0 ± 1.0
Gal	15.0 ± 1.0	16.0 ± 1.0
Nisin*	18.0 ± 1.0	14.0 ± 1.0
Cell-wall antibiotics:		
Amidinocillin (AMD10)	18.0 ± 1.0	20.0 ± 1.0
Bacitracin (B10)	27.0 ± 2.0	27.0 ± 2.0
Colistin (CL10)	9.0 ± 1.0	9.0 ± 1.0
Cycloserine (100mg/ml)	35.0 ± 2.0	37.0 ± 2.0
Fosfomycin (F300)	24.0 ± 2.0	24.0 ± 2.0
Penicillin (P2)	29.0 ± 1.0	30.0 ± 1.0
Polymyxin B (PB300)	12.0 ± 1.0	13.0 ± 1.0
Tunicamycin (5mg/ml)*	19.0 ± 1.0	13.0 ± 1.0
Vancomycin (V5)	17.0 ± 1.0	17.0 ± 1.0
Strains producing bacteriocins:		
UA159	18.0 ± 1.0	17.0 ± 1.0
UA159::Δ*nlmAB*	15.0 ± 1.0	15.0 ± 1.0
UA159::Δ*nlmC*	16.0 ± 1.0	16.0 ± 1.0
UA159::Δ*nlmABC*	14.0 ± 1.0	13.0 ± 1.0

To study whether SMU.662 could function as a receptor protein, we selected two *S. mutans* strains (UA159 and V403) that are sensitive to Smb [163]. The entire SMU.662 coding region was replaced in these strains with an erythromycin resistant gene by fusion PCR and tested these strains against Smb mediated inhibition. As shown in Fig 18B, inactivation of SMU.662 nearly abolished the sensitivity towards Smb lantibiotic in both the strains. This finding suggests that SMU.662 indeed encodes a receptor like protein for Smb.

We also tested whether SMU.662 can be effective against haloduracin, galolacticin, and nisin. However, we found no difference between the wild type and the SMU.662 inactivated strains (data not shown). Thus, at least in *S, mutans*, SMU.662 is very specific and only recognizes Smb. Furthermore, we also observed that SMU.662 deleted strains were as susceptible to tunicamycin as their isogenic wild type strains. Therefore, it seems that although SMU.662 recognizes Smb for lantibiotic activity, the LsrS protein in *S. pyogenes* might have additional functions that are absent in SMU.662.

4.3.4. Overexpression of LsrS in a heterologous host increases sensitivity

It appears that Smb produced a smaller ZOI in *S. mutans* than in *S. pyogenes* strains. We speculated that SMU.662 might not function as efficiently as LsrS, therefore we decided to overexpress LsrS in UA159. When we used UA159 containing pIBA35, we observed that the ZOI was increased about 2.5 times as compared to UA159 containing the vector plasmid (Fig. 19). To rule out the possibility that observed effect is not a strain specific phenomenon, we also used V403 strain and observed the increased ZOI when LsrS was overexpressed. Taken together, these results suggest that LsrS can efficiently function in heterologous host and overexpression can lead to increased sensitivity.

Since LsrS deficient *S. pyogenes* strains showed decreased sensitivity towards nisin and tunicamycin, we wanted to test whether overexpression of LsrS in *S. mutans* makes the strain more sensitive to these compounds. We found that LsrS, when expressed in *S. mutans*, does not affect the sensitivity toward these reagents. This result indicates that in *S. pyogenes*, additional proteins, which are absent in *S. mutans*, are necessary for the observed LsrS functions.

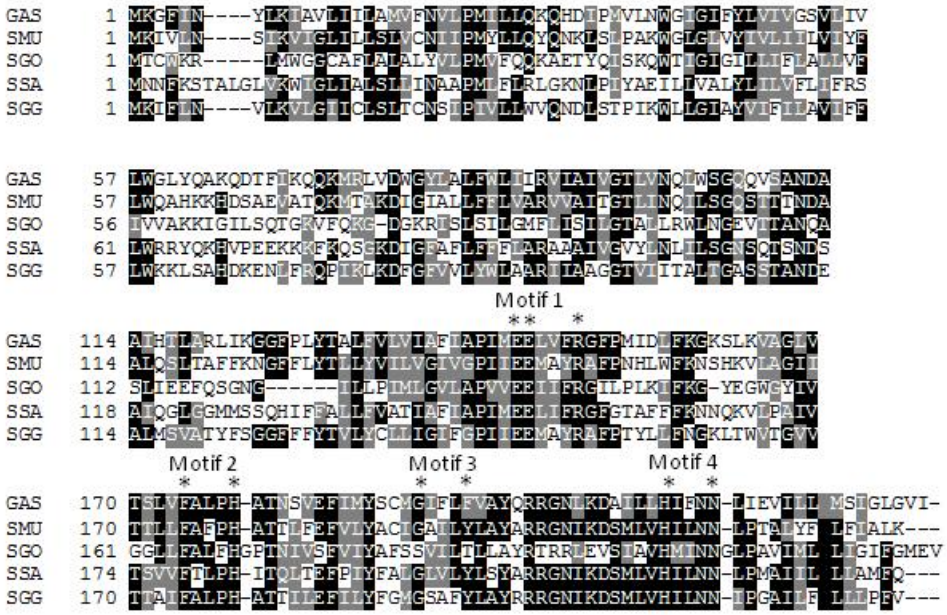
4.3.5. Protease activity of LsrS is not required for the receptor-like function

In eukaryotes, Abi-domain containing proteins are known to be involved in protein prenylation [173]. These membrane proteases belong to a zinc metalloprotease family and cleave within CAAX of the substrate (A denotes an aliphatic residue where as X denotes one of several allowed residues that dictates the specificity of prenyltransferases). Abi-domain itself encodes four transmembrane helices (TH) with the conserved active site residues. The catalytic glutamic acids in motif 1 and histidines in motif 2 and 4 are predicted to coordinate zinc ions (Fig 18A). We used TMpred and TopPred2 to determine the membrane topology of the LsrS and as shown in Fig 5A, LsrS appears to contain six THs. We verified the orientation of TH3 and TH4 with the help of LacZ translational fusions at positions M74 and V148 (Fig 20). *E. coli* XL-1 strain containing these translational fusions generated blue color colonies on agar plate containing X-gal, suggesting that the predicted TH orientations correlate with the experimentally verified ones.

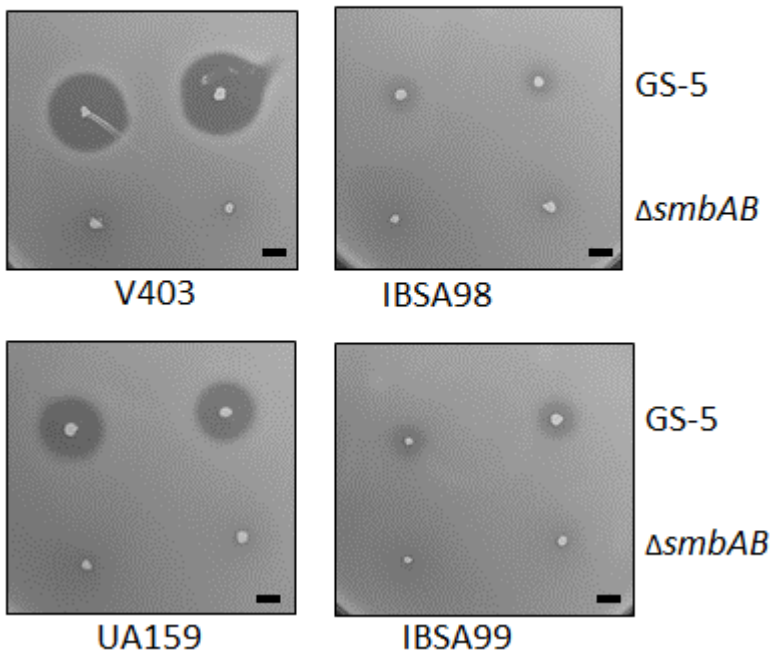
We then tested whether the protease-like mechanism of LsrS is necessary for the receptor function. For this, we made alanine substitutions at the conserved glutamic acids at positions 145 and 146, and histidine residue at position 178 described as critical for the metalloprotease activity with alanine residues (EE145/146AA, pIBA44; and H178A, pIBA45). As shown in Fig 20B, both the mutations in the conserved active site residues of LsrS have no effect on the receptor activity in *S. pyogenes* or in *S. mutans*. Thus, the putative protease activity of LsrS is not necessary to exhibit the receptor-like functions.

Figure 18: Deletion of *lsrS* homolog in *S. mutans* makes the strains resistant to Smb-mediated inhibition. (A). Multiple sequence alignment of LsrS and its homolog from various streptococci. Sequences were aligned with Clustal-W and the degree of relatedness was displayed with BoxShade where black and gray indicate identical or similar residues, respectively. Sequences were obtained from GenBank (accession numbers are in parenthesis). The strains are: *S. pyogenes* (GAS, NP_269484), *S. mutans* (SMU, NP_721090), *S. gordonii* (SGO, YP_001449790), *S. sanguinis* (SSA, YP_001034746), and *S. gallolytics* (SGG, YP_004287423). The four conserved putative metalloprotease motifs along with the active side residues (asterisks) are also indicated. (B). Deferred antagonism assay using two *S. mutans* isolates and their mutant derivatives. Assays were carried out with GS-5 and *ΔsmbAB* as tester strains as described in figure 1 and repeated at least four times. The length of the scale bar equals to 5mm.

A


GAS
SMU
SGO
SSA
SGG
Motif 1
Motif 2
Motif 3
Motif 4

B


GS-5
ΔsmbAB
V403
IBSA98
GS-5
ΔsmbAB
UA159
IBSA99

4.4. Discussion

One of the noteworthy features of bacteriocins, specifically lantibiotics, is that the peptides are highly potent and active in the nanomolar range. On the other hand antimicrobial peptides produced by human and animal (such as defensins and LL-37) are active in the micromolar range, a difference of 1,000-fold in the concentration [155,174]. It is assumed that the primary reason for this extreme potency is due to the fact that bacteriocins recognize specific receptors on the target cells while antimicrobial peptides of eukaryotic origin interacts non-specifically with their targets. This assumption was first validated by the identification of mannose-phosphotransferase system (Man-PTS) as a receptor for some non-lantibiotics (class II) belonging to pediocin-like bacteriocins of sub-class IIa [79], and also for some non-pediocin-like linear bacteriocins of subclass IId such as lactococcin A and B [175]. Subsequently, another sugar transporter, a maltose-ABC transporter, was also found to be required in target cells for sensitivity to garvicin ML, a circular bacteriocin belonging to subclass IIc [176]. Furthermore very recently, Uzelac et. al. [177] have recently identified a membrane bound Zn-dependent metaloprotease in *L. lactis* that seems to act as a receptor for yet another non-lantibiotics, LsbB, produced by some strains of *L. lactis*. So far no receptor molecules have been identified for lantibiotic peptides including nisin, one of the most extensively studied lantibiotics. In the present study we report the discovery of a new protein, LsrS, employed by Smb to target sensitive strains.

The locus that encodes *lsrS* is organized in a three-gene operon and is present in all the sequenced *S. pyogenes* strains. A BLAST-P search with LsrS as query against the *S. pyogenes* genomes did not return any other proteins suggesting that LsrS does not have any paralogs. The inactivation of *lsrS* generated about 40% reductions in ZOI, but did not completely abolish the sensitivity to Smb. This indicates that in *S. pyogenes,* LsrS may not be the only protein with receptor like function. Since no other LsrS paralogs are present in *S. pyogenes*, we speculate that Smb utilizes other molecules unrelated to LsrS as receptors to inhibit this organism. To this line, it is noteworthy to mention that Perry and Slade [167] first isolated an inhibitory factor with a molecular weight of 93-kd from *S. pyogenes* strain E14 (a sensitive strain) that

neutralizes bacteriocins produced by GS-5. Soon after, Franker [166] isolated another factor, which is 74-kd, from *S. pyogenes* strain MJP-2 (also sensitive to GS-5) that also demonstrates inhibitory activity against GS-5. The exact identities for both the factors are not known and we speculate that these factors might act as additional receptors for Smb.

We observed that LsrS has a receptor like activity for Smb and not for other closely related two-peptide lantibiotics, such as haloduracin and galolacticin (Fig 17). This was surprising to us since the immunity protein, SmbFT, can recognize all the three lantibiotics. While haloduracin and galolacticin are structurally similar to Smb, several differences in the sequence also exist. We speculate that some critical residues that are present in either one or both the components of Smb might be important for peptide-receptor interactions, and those critical residues are absent in haloduracin and galolacticin. Since these two lantibiotics inhibit *S. pyogenes* very efficiently, they might utilize other cell-surface molecules as receptors.

In contrast, we found that LsrS facilitates nisin activity. This was also surprising since nisin, which is a mono-peptide lantibiotic, has very little sequence or structural similarity with Smb. Furthermore, the mechanism of inhibition by nisin and two-peptide lantibiotics is different. In the case of two-peptide lantibiotics, the α-peptide component interacts with the lipid II that most likely involves the mersacidin-like binding motif and form a complex. The β-peptide then binds to the α-peptide/lipid II complex and adopt a transmembrane conformation to form a defined pore. Although LsrS displays a receptor like activity for both nisin and Smb, the molecular mechanism might be very different. It is possible that an accessory protein acts as the primary receptor for nisin and the function of LsrS is to enhance or stabilize the interaction. We speculate this because, when we overexpressed LsrS in *S. mutans*, it did not enhance the nisin-mediated inhibition, only the Smb-mediated inhibition was enhanced (Fig 19, data not shown). Since in *S. pyogenes*, the LsrS encoding gene is genetically linked to SPy1385, it is possible that SPy1385 might be involved in nisin recognition. SPy1385 is a hypothetical protein that is present in all the

sequenced *S. pyogenes* strains that encodes LsrS. In fact the entire operon is very highly conserved in *S. pygenes* and other pyogenic streptococci. When we performed a BLAST-P search, we did not find any Spy1385 homolog in *S. mutans*, strengthening our hypothesis. Furthermore, SPy1385 contains six transmembrane helices (data not shown) and thus it is also appears to be a membrane protein. We also found that pyogenic group of streptococci are more sensitive to nisin as compared to mutans group (data not shown). Thus, we believe that for pyogenic group of streptococci, both LsrS and SPy1385 play an important role for nisin-mediated inhibition.

In addition to forming pores in the membrane, both nisin and Smb interfere with the cell-wall biosynthesis. Therefore we tested the susceptibility of LsrS-deficient strains to various antibiotics that target enzymatic steps leading lipid II biosynthesis and post lipid II pathways leading to cell-wall formation. To our surprise, except for tunicamycin, we did not find any significant differences in the sensitivity to any other antibiotics including vancomycin that also binds to lipid II (Table 7). Thus, we speculate that LsrS has no negative effect in overall cell-wall synthesis. However, we found that LsrS-deficient *S. pyogenes* strains were significantly resistant to tunicamycin action. The chemical composition of tunicamycin is complex and it contains uracil, N-acetyl glycosamine, an 11-carbon aminodialdose called tunicamine, and a fatty acid linked to the amino group. Tunicamycin inhibits the enzymatic activity of MraY, the phospho-MurNAc-pentapeptide translocase that catalyzes the synthesis of lipid I in the conserved pathway for peptidoglycan biosynthesis. Since MraY is also a transmembrane protein, it is possible that LsrS, either alone or in combination with other proteins, interferes with the MraY activity in *S. pyogenes*. Hence, in the absence of LsrS, the enzymatic activity of MraY is enhanced. Alternatively, LsrS itself acts as a receptor for tunicamycin. We believe that this latter possibility is unlikely because when we overexpressed LsrS in *S. mutans*, we did not observe any change in tunicamycin sensitivity (data not shown).

Figure 19: Overexpression of LsrS in *S. mutans* causes increased sensitivity. Deferred antagonism assays were carried out with GS-5 and *ΔsmbAB* as tester strains and were performed as described in figure 16. These plates are representative of three independent assays. The length of the scale bar equals to 5mm.

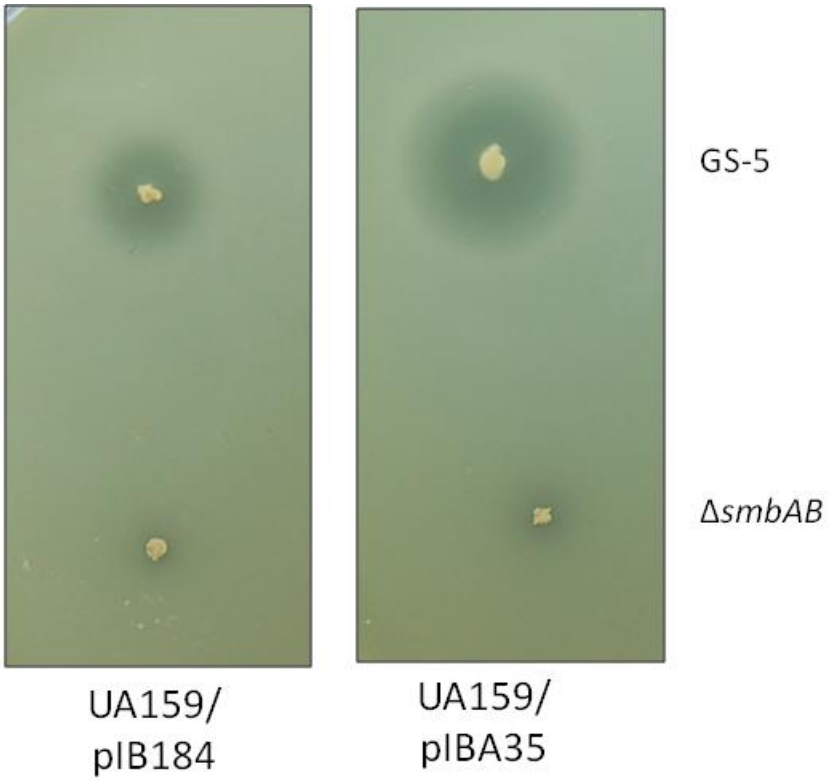
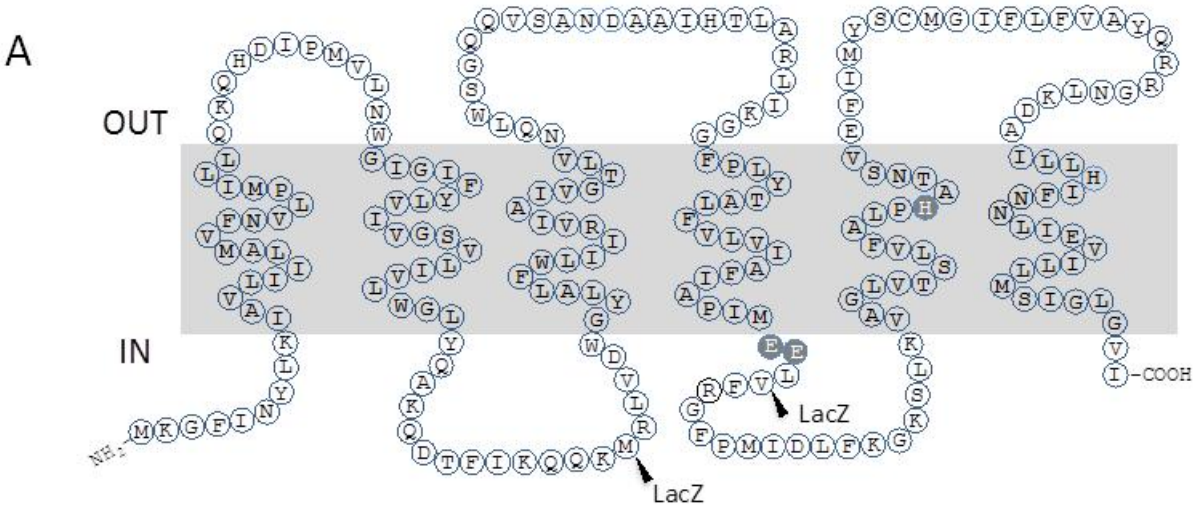
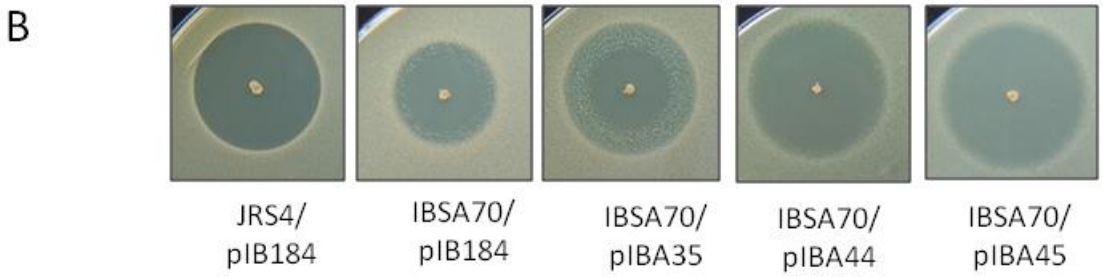
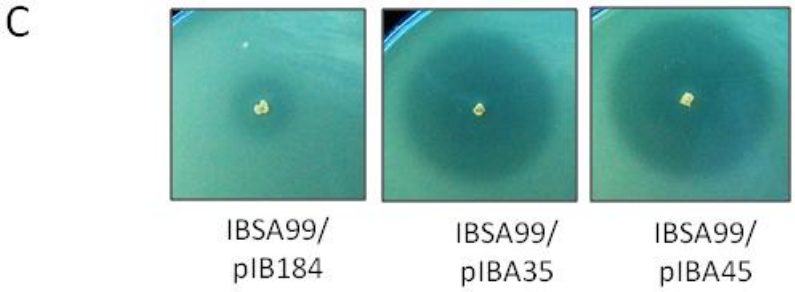

GS-5
ΔsmbAB
UA159/
pIB184
UA159/
pIBA35

Figure 20: Putative protease activity is not required for LsrS activity. (A) Proposed transmembrane topology of LsrS. The hydrophobicity plots predicted from TopPred 2, TM-Pred and TMHMM, are similar. The predicted six putative transmembrane α-helices are indicated. The positions (residues) for LacZ fusions are shown. The residues with dark background are putative active sites for the CAAX protease activity. (B-C). Site-directed mutations in the conserved active site motifs do not affect LsrS receptor function in *S. pyogenes* (B) and *S. mutans* (C). Deferred antagonism assays were repeated at least three times, and representative plate areas are shown.


A
OUT
IN
NH2
LacZ
LacZ
-COOH


B
JRS4/
pIB184
IBSA70/
pIB184
IBSA70/
pIBA35
IBSA70/
pIBA44
IBSA70/
pIBA45


C
IBSA99/
pIB184
IBSA99/
pIBA35
IBSA99/
pIBA45

In *S. pyogenes*, the *lsrS* is encoded by a three-gene operon (Fig. 16). Our bioinformatics searches found that the entire operon is present in all the sequenced strains of *S. pyogenes*. We also found that this operon is present in some of isolates of *S. anginosus, S. constellatus, S. dysgalactiae*, *S. pneumoniae*, and *S. suis*. Since these streptococci are pathogenic, we speculate that in addition to receptor like function for Smb, the genes encoded within this operon might play a role in virulence. Additional experiments are required to determine the true role of the genes encoded by this operon.

While the operon that encodes LsrS is present in a handful of streptococci, a BLAST-P search with LsrS as query yielded several additional streptococci with an E value of -35 or less. The streptococci that we found are *S. gallolyticus*, *S. intermedius*, *S. mutans*, and *S. sangunis*. Apart from streptococci, the only other organism that we found is Carnobacterium sp 17-4, a lactic acid bacterium often associated with seafood and dairy products. However, LsrS showed the highest degree of homology (E^{-60}) to SMU.662 and its counterpart encoded by various *S. mutans* strains. This was surprising to us since Smb is also secreted by *S. mutans.* We showed that SMU.662 alone could function as a receptor for Smb and deletion of SMU.662 makes the strains insensitive to Smb. Two recent large-scale genome-sequencing studies indicate that SMU.662 is a part of the core genome [65,178]. Furthermore, the upstream region [SMU.651-SMU.658] and the downstream region [SMU.681-SMU.687] appear to be genomic islands and are present in some but not all *S. mutans* strains [179]. Whether SMU.662 is only a receptor for lantibiotics or it plays a role in other biological processes remains to be evaluated.

LsrS is a member of a highly conversed protein family with a putative CAAX prenyl protease domain. This family, which is recently renamed as CPBP (*C*AAX *p*rotease and *b*acteriocin-*p*rocessing enzymes), encompasses more than 5000 proteins [180]. Members of the CPBP family are involved in diverse biological functions. For example, Kjos et. al. have shown that SkkI functions as a bacteriocin immunity protein for sakacin secreted by *Lactobacillus plantarum* [82]. These authors have also shown that protease activity is necessary for the immunity function. In *S. pneumonia*, PcnO is both necessary for the

production of bacteriocin Pnc as well as involved in the immunity against Pnc [181]. The exact mechanism by which PncO regulates bacteriocin production or mediates immunity is currently unknown. CPBP proteins are also shown to be involved in expression of surface proteins containing YSIRK signal peptide as in the case of *Staphylococcus aureus* Spd proteins [182]. Recently, Frion and colleagues have shown that in *S. agalactiae*, a CPBP protein, Abx, forms a signaling complex with the histidine kinase CovS and regulates expression of virulence factors [183]. The number of CPBP family proteins varies greatly depending on the organism. For example, while *S. pyogenes* encodes only two or three CPBP family proteins (depending on the isolates), some streptococci, such as *S. sanguinis,* contain as many as 21 CAAX-family proteins and the roles of most of these proteins remain largely unknown. In this study we added another role for a CPBP family protein to the growing list of functions.

Chapter 5: Regulation of *smb*: auto-regulation and identification of a new regulator SMU.1704

5.1. Abstract

A post translationally modified two-peptide lantibiotic, Smb, is produced by *Streptococcus mutans* strain GS-5. The structural genes, *smbA* and *smbB,* are part of a seven-gene cluster: *smbM1-F-T-M2-G-A-B*. Unlike other lantibiotic biosynthesis operons, the *smb* locus does not encode any regulator that might act as autoregulator. In the present study we showed that the promoter of this locus is auto-regulated by the mature Smb peptides. We observed that the transcription levels of the *smbM1* (encoding a modification enzyme), *smbF* (encoding an immunity protein), and *smbG* (encoding transporter) are all reduced more than two-fold in a *smbAB* deleted strain. As expected, the *smbAB* mutant strain was more sensitive to Smb compared to the wild type strain. This increased sensitivity to Smb was also apparent in a *smbG* mutant strain, presumably because the strain is unable to secrete the mature Smb and thus unable to autoinduce the operon. To identify other potential regulators for this operon, we performed a transposon mutagenesis assay using a reporter fusion with the promoter of the operon. We found several insertions on various components of the Com pathway, which is known to regulate *smb*. We also obtained insertions in five hypothetical protein encoding genes. Two of them, SMU.1704 and SMU.1706 appear to be organized in an operon. The first gene, SMU.1704 encodes a PadR family regulator, while SMU.1705 and SMU.1706 both encode putative membrane proteins. Deletion of SMU.1704 generates a strain that produces a lower amount of Smb compared to GS-5. We also found that the SMU.1704 deleted strain is more susceptible to Smb. Taken together our results provide preliminary evidences for a novel example of bacterial signalling mediated by a two-peptide lantibiotic Smb. Our results also show that a new regulator and perhaps a new regulatory pathway may control *smb* expression.

5.2. Introduction

Lantibiotics are small, ribosomally synthesized peptides with antimicrobial properties. These peptides are postranslationally modified and contain polycyclic thioether amino acids lanthionine or methyl lanthoinine as well as unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid. Gram-positive organisms including lactic acid bacteria such as streptococci, lactococci, and enterococci predominantly secrete lantibiotics. Lantibiotics produced by Gram-positive bacteria are relatively broad spectrum and are relatively resistant to heat and protease degradation. These properties of lantibiotics have generated significant attention in the pharmaceutical industry to explore the possibility to use as an alternative to antibiotics to treat infections. To increase the yield of this natural bacteriocin one needs to increase the production by manipulating the regulation of the biosynthetic genes.

Regulation of lantibiotic has been well studied for nisin operon. Nisin has been widely used in the food industry over 50 years and it is perhaps the most extensively studied lantibiotics. Nisin, a mono-component lantibiotic, is encoded by *nisA*, which is a part of an 11-gene cluster containing all the genes needed for the production of modified active nisin (Fig 7). Besides the structural gene, this locus contains genes encoding modification enzymes, transporters, immunity proteins, and regulators. A two-component signal transduction system, NisRK, encoded within the locus is shown to regulate the expression of *nisA*. The matured nisin peptide itself acts as a signal to activate the sensor kinase, NisK, to induce the expression in a quorum dependent manner. Interestingly, over expression of the response regulator, NisR, alone was sufficient to induce the operon. A similar observation was made in case of lantibiotic epidermin where overexpression of EpiQ (response regulator) increases epidermin transcription [184]. However for subtilin production the presence of both the sensor kinase SpaK and the response regulator SpaR are necessary [185]. Infact in most cases, a two-component system is associated and often essential for lantibiotic expression. The production of lantibiotic is also growth phase dependent as in the case of plantaracin A and subtilin [186]. But the signal necessary for the growth phase dependent regulation has not been identified.

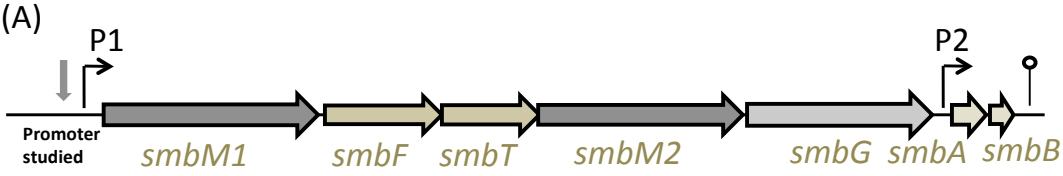
S. mutans GS-5 is a highly virulent strain that is known to produce a two-peptide lantibiotic named Smb. The Smb biosynthetic operon contains seven genes (*smbM1, -F, -T,-M2, -G, -A*, and *-B*) and is regulated by ComDE two-component system that responds to competence stimulating peptide (CSP), a quorum sensing molecule. The locus that encodes ComDE and CSP (*comC*) is located elsewhere in the genome and genetically unlinked. In addition to the *smb* operon, ComDE system regulates several other operons. Previous studies have shown that *smb* operon contains two promoters, one upstream promoter (P1) that drives the expression of all the seven genes and one down stream promoter (P2) that drives the last two structural genes *smbAB*. ComDE appears to regulate (positively) only the P1 promoter and the effect on P2 promoter is unknown.

A previous study showed that *smbG*, the fifth gene in the operon, can provide resistances in the organism against some antimicrobial substances [126]. However, the authors did not observe any increase in sensitivity in the *smbG* deficient strain [132]. They observed sensitivity only when the indicator strain contained two mutations, one in the promoter of *smb* and another in an unrelated immunity protein (Bip) expressed from a different region in the chromosome. However, we showed that SmbFT ABC transporter complex alone can provide immunity against Smb and a strain devoid of *smbT* is sensitive to Smb (Chapter 3). Since SmbG contains a protease motif, it might be involved in the processing and transport of mature peptide Smb.

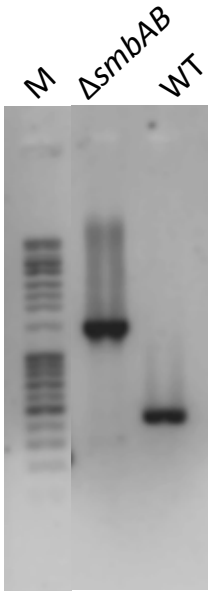
The present study shows that the mature peptide Smb functions as a signaling molecule to induce its own expression. Since the *smb* locus does not encode any two-component regulatory system, a search for the regulator involved in mediating the signal to the promoter identified a new protein. This regulator is predicted to be a PadR family protein. We show that this protein positively influences the production of Smb.

Figure 21: Genetic organization of *smb* locus and construction of the *smbAB* mutant (A). The locus encodes seven genes that are transcribed from a major promoter, P1. Another promoter (P2) that is located just downstream of the fifth gene, *smbG*, and putatively transcribes *smbAB* genes. A rho-independent terminator maps downstream of the *smbAB* genes. (B). PCR verification of Δ*smbAB* mutant. The mutant was created by gene replacement with an kanamycin resistant cassette.

(A)


P1
P2
Promoter studied
smbM1
smbF
smbT
smbM2
smbG
smbA
smbB

(B)


M
ΔsmbAB
WT

5.3. Results

5.3.1. Inactivation of the structural genes and evaluation of the mutants for susceptibility to Smb. Since nisin works as a signaling molecule to activate its own locus we wanted to examine whether the structural genes *smbA* and *smbB* are also involved in enhancing transcription of its own locus. Because the immunity protein (SmbFT) is also encoded in this locus, we wanted to test the sensitivity of the strains lacking *smbAB* against GS-5. We inactivated both the structural genes by gene replacement with a Km resistance cassette. The mutant was evaluated as a tester strain against Smb produced by GS-5. Indeed the *smbAB* mutant (IBSA63) showed increased susceptibility to Smb. This result suggests that the *smbAB* genes are necessary for complete protection of the producer strain.

5.3.2. Induction of P1 (*smb*) by SmbAB functioning as a signal. To further verify whether Smb functions as a signaling molecule to activate the promoter P1, we performed semiquantitative RT-PCR of the three following genes - *smbM1*, *smbT*, and *smbG* form GS-5 and the mutant strain IBSA63. We found that expressions of all three genes were at least two-fold reduced in the mutant strain compared to the wild-type GS-5. Growth of both the strains was measured for any apparent growth defect. We observed that both strains grew equally well at 37°C. This finding indicates that the matured form of Smb functions as a signal to activate its own locus.

5.3.3. Effect of putative transporter SmbG on promoter activation. The *smb* locus contains only two ABC transporters, the SmbFT complex and SmbG. We showed that SmbFT functions as an immunity protein for Smb. Here we propose that the other ABC transporter, SmbG, which contains a cysteine protease domain, is the exporter of the processed Smb. We wanted to verify if the *smbG* mutant (IBSA89) is also sensitive to Smb like IBSA63. We observed that this mutant is more sensitive to Smb than GS-5. Moreover, when we compared the susceptibility with the *smbFT* mutant (IBSA77), we found that IBSA89 is not as susceptible as IBSA77. This observation suggests that SmbFT is expressed at a basal level even in the absence of Smb signaling.

5.3.4. Identification of positive regulators for P1 (*smb*). To understand the *smb* promoter regulation further, we constructed a P*smb-gusA* reporter strain (IBSA71) and subjected this strain to IS*S1* mutagenesis. Since we wanted to identify positive regulators for this promoter, we selected mutants that produce white or pale blue color colonies on X-Gluc plate. We screened approximately 6000 colonies of which 45 colonies were either pale blue or white. Among the 45 colonies, only 20 were defective in mutacin production. These colonies were analyzed further to identify the IS*S1* insertion site. We classified the mutants into two groups. The first group contains mutants that were mapped in the genes involved in the ComDE signaling pathway. These genes are: *comD* (the histidine kinase), *comE* (the response regulator), *nlmTE* (the transporter of CSP) and *sepM* (the protease that processes CSP to its active form). For each of these genes, at least one mutant was identified and in some cases two independent insertions were obtained (*comD*). The second group includes genes that are annotated as hypothetical proteins. These genes are SMU.831, SMU.833, SMU.1077, SMU.1704, and SMU.1706. Interestingly, two independent isertions had ocurred into a SMU.1706. The operon that encodes SMU.1706 is a three-gene operon. The first gene of this operon, SMU.1704, is a putative PadR family regulator and we obtained an insertion into this gene. The following two genes are SMU.1705 and SMU.1706; both encode putative membrane bound proteins. Since, competence related genes including ComE are known to activate the P1 promoter we did not study them further, rather we decided to focus on the uncharacterized operon SMU.1704-6 that we identified.

We inactivated the regulator, SMU.1704, by gene replacement (IBSA72). We also constructed a strain where we replaced the entire SMU.1704-6 locus with an Em cassette (IBSA81). We tested whether IBSA72 produces less Smb compared to GS-5 and is more sensitive to Smb. We observed that IBSA72 indeed produces less Smb compared to GS-5 (Fig 25) and is more susceptible to Smb (data not shown). However, IBSA81 seemed to produce a slightly lower amount of Smb compared to GS-5 on agar plate. This observation suggests that this newly identified operon SMU.1704-6 might act as a positive regulator of the P1 promoter. Further experiments are required to confirm whether P1 expression is directly

regulated by SMU.1704 protein, or indirectly regulated by one or more gene products encoded by this operon.

Figure 22: Growth characteristics and gene expression profile of the wild type and the *smbAB* mutant. (A). RNA was isolated from the wt and the mutant strains and subjected to semiquantitative RT-PCR analysis to measure the relative amounts of the targeted genes as shown. The experiment was performed twice. (B). Analysis of growth of the wt and the mutant in THY broth at 37◦C under microaerophilic condition. The experiment was repeated twice. (C). Schematic diagram showing the *smb* operon. Genes used for RT-PCR analysis are shown with vertical arrows.

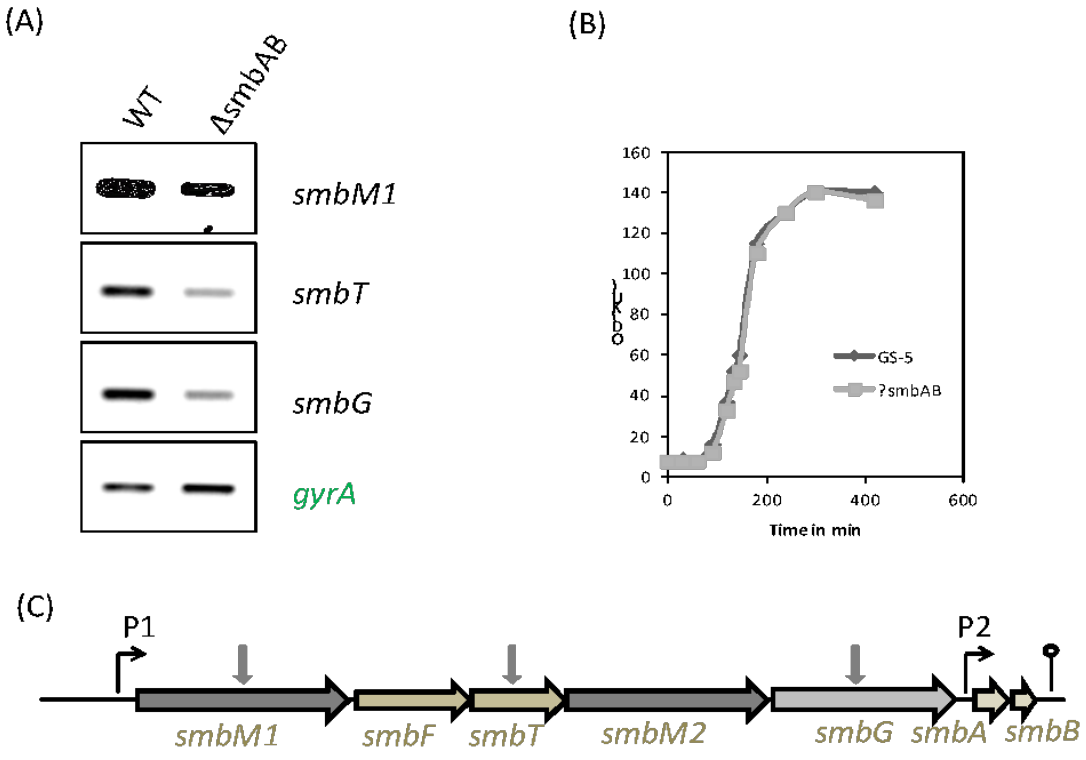

(A)
WT
ΔsmbAB
smbM1
smbT
smbG
gyrA
(B)
160
140
120
100
80
60
40
20
0
0
200
400
600
Time in min
GS-5
?smbAB
(C)
P1
P2
smbM1
smbF
smbT
smbM2
smbG
smbA
smbB

Figure 23: Susceptibility of *smbAB* deletion mutant to Smb. Deferred antagonism assays were performed as described in Chapter 2. The plates were overlaid with either GS5 or the mutant starin. A diffused ZOI is clearly visible in case of the Δ*smbAB* strain. The experiments were performed twice.

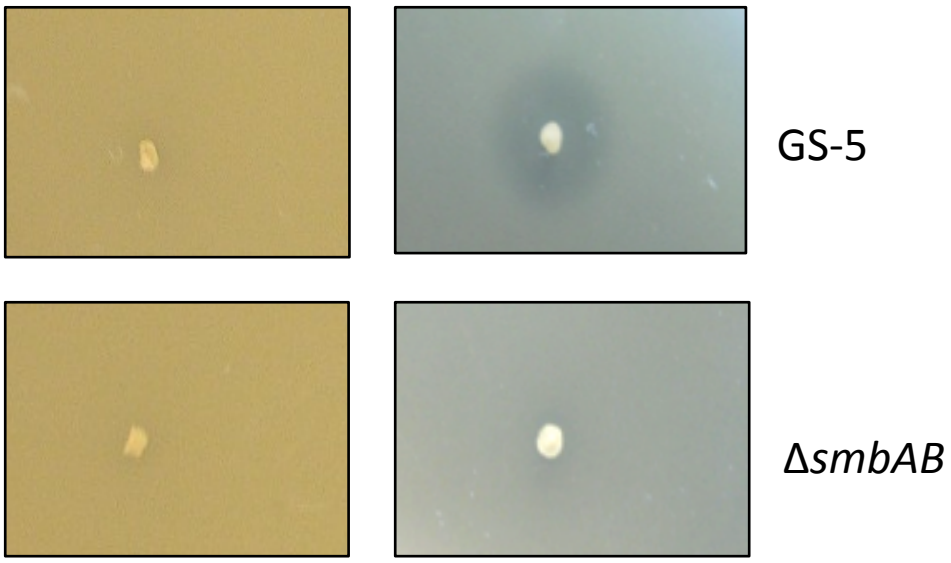

GS-5
ΔsmbAB
GS-5
ΔsmbAB

Figure 24. Susceptibility of *smbG* mutant against Smb. Deferred antagonism assay using the wild type and two mutant strains. Indicator strains were overlaid on THY agar plates that were pre stabbed with GS5 and Δ*smbAB*. Note, Δ*smbG* strain clearly produces a diffused ZOI, whereas Δ*smbT* strain produces a clear ZOI.

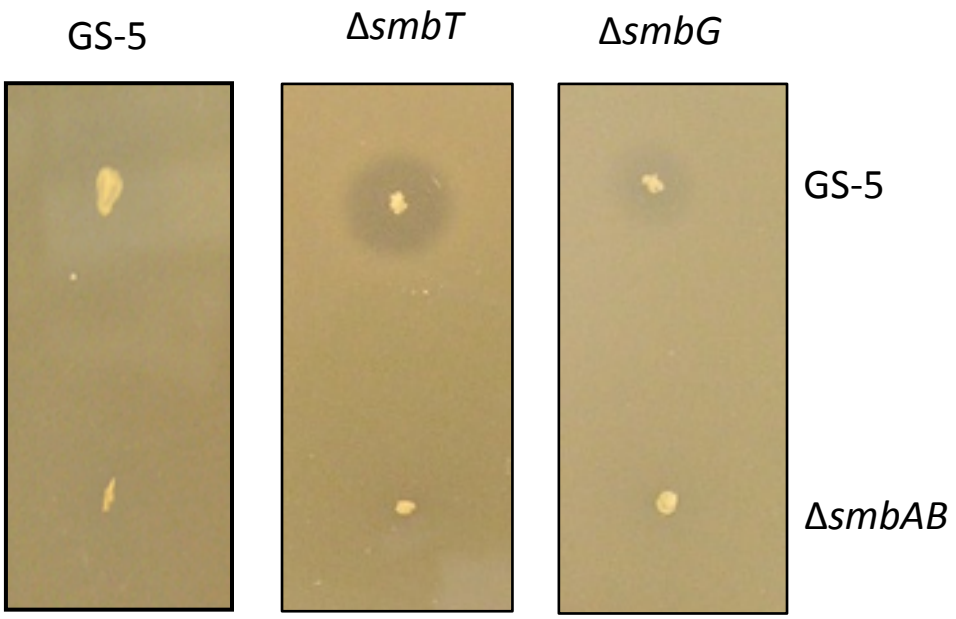
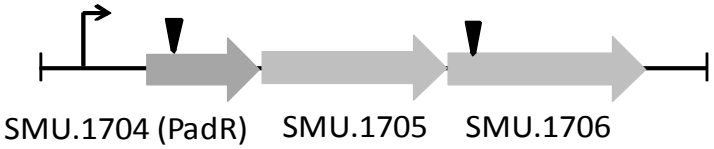

GS-5
ΔsmbT
ΔsmbG
GS-5
ΔsmbAB

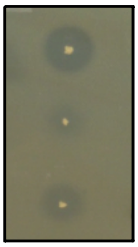
Figure 25: Assessment of the role of SMU.1704 -1706 operon in *smb* promoter (P1) expression. (A). Schematic diagram of the SMU.1704 -1706 operon. A putative promoter like sequence is shown with the bent arrow. Site for two IS*S1* insertions are also shown with inverted triangles. (B). Deferred antagonism assay with ΔSMU.1704 (IBSA72) and ΔSMU.1704 -1706 (IBSA81) strains as indicator. (C). Growth kinetics of the wt and two mutants were measured in THY broth at 37◦C under microaerophilic condition. Experiments were performed twice.

(A)



(B)

S. salivarius



(C)

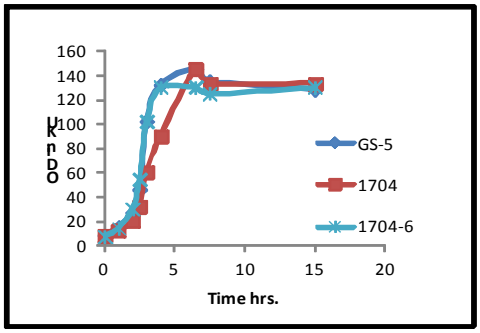
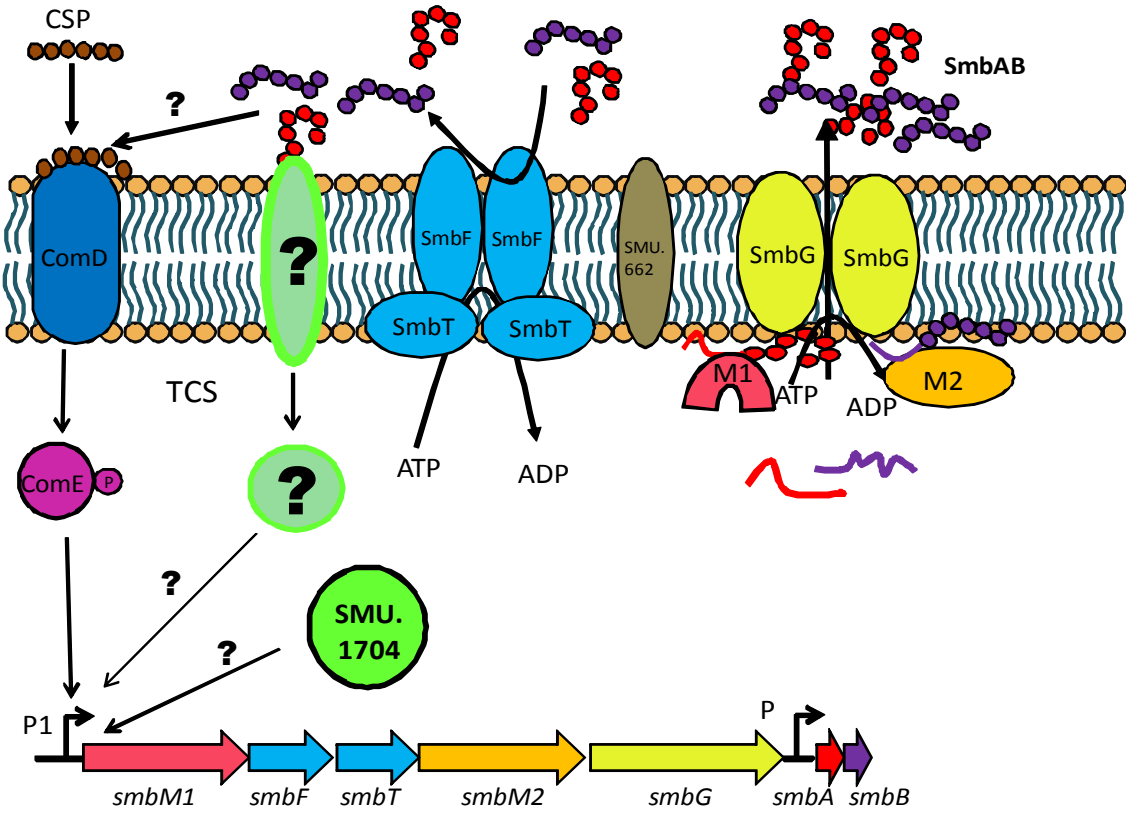


Figure 26. A working model for *smb* promoter regulation. In this model we predict SmbG (yellow oval) is the transporter that secretes the matured Smb peptides. Whether SmbA and SmbB peptides are secreted individually or as a complex is not known. The mature Smb peptide then binds to an unknown sensor protein, most probably a sensor kinase (green oval) to activate the cognate response regulator, which in turn induces P1 promoter. Since SMU.1704 is a transcriptional regulator, we speculate that SMU.1704 also directly regulates this operon. The P1 promoter is also directly regulated by ComDE pathway. However, we believe Smb does not interact with ComDE pathway. The exact function of SMU_GS5_02915 (the receptor, SMU.662) in the *smb* regulation is unknown.


CSP
?
ComD
?
SmbF
SmbF
SmbT
SmbT
SMU.
662
SmbG
SmbG
SmbAB
M1
ATP
ADP
M2
TCS
ComE
P
?
ATP
ADP
?
?
SMU.
1704
P1
P
smbM1
smbF
smbT
smbM2
smbG
smbA
smbB

5.4. Discussion

Nisin and subtilin are two well-studied lantibiotics that are involved in self-regulation. It was known that both nisin and subtilin regulate their promoters by working as a signaling molecule through a two-component system. However, to our knowledge, the role of a two-peptide lantibiotic working as a signaling molecule to activate its own transcription has never been reported. In the present study, we wanted to test whether Smb is functioning as a signaling molecule for its own promoter. We observed that *smbAB* inactivated strain was susceptible to exogenous Smb produced by GS-5. This indicates that the P1 promoter of *smb* is autoregulated by Smb. Either the intracellular Smb prepeptides or the secreted form of matured and processed Smb promotes the transcription induction of the *smb* operon.

Because we observed increased sensitivity of the *smb* mutant against GS-5, we wanted to examine whether the immunity protein SmbFT expression is reduced and therefore becomes sensitive to Smb. We also wanted to verify whether the transcription of the whole locus is reduced. Our semiquantitative RT-PCR data show that the expression of *smbM1* (the modification enzyme), *smbF* (the immunity complex protein), and *smbG* (the putative transporter) are reduced two-fold in the *smb* mutant strain. This indicates that Smb signaling enhances the expression of the genes encoded in the operon. In other words, without Smb signaling, genes in this operon are expressed at a basal level. We also observed that *smbG* (a putative transporter) mutant strain, which is deficient in secreting mature Smb, is sensitive as the *smb*-deficient strain. This suggests that the secreted mature form of Smb and not the intracellular form of Smb is involved in the signaling. Moreover, we also observed that the *smbT*-deficient strain is significantly more sensitive to Smb compared to the *smb*- or *smbG*-deficient strains (Fig 25). This indicates that Smb signaling only enhances the expression of the operon encoding the immunity protein SmbFT.

S. rattus is very closely related to *S. mutans* and belongs to mutans group of streptococcus. *S. rattus* encodes a two-peptide lantibiotic operon, *bhtA*, which is nearly identical to *smb* except that the *bhtA* locus also encodes a regulator protein encoding gene upstream to the operon locus. This regulator protein most

probably regulates the expression of *bhtA* operon. In the case of *smb* operon, since the regulator is absent, we speculate that ComDE maintains a certain level of expresssion of Smb. When the cells reach a certain density or when the extracellular concentration of Smb reaches a certain threshold, Smb positively autoregulates its own expression.

Finally, our transposon mutagenesis study identified several activators of the P1 promoter. Since we identified several genes that belong to the ComDE pathway and since ComDE pathway genes are known to regulate P1 promoter, it indicates that our screening method was successful for the identification of the activators. Interestingly, we identified a new operon SMU.1704-6 that is involved in the activation of this operon. We observed that the SMU.1704 mutant strain is deficient in Smb production. Furthermore, the mutant strain is also susceptible to Smb like the *smb*-deficient strains (data not shown). Altogether, this observation suggests that the putative PadR family repressor SMU. 1704 regulates the P1 promoter. The exact mechanism by which SMU.1704 regulates the P1 promoter is currently unknown. Since both SMU.1705 and SMU.1706 encode membrane bound proteins, we speculate that these two proteins might form a complex and function as a sensor. When this complex recognizes appropriate signals, they activate SMU.1704. This activated SMU.1704 then induces the *smb* operon. Alternatively, SMU.1704 might function with a yet to be indentified partner sensor protein. In this regard, SMU.1704 might function the same way as the unusual two-component system Stp/Stk where the partner protein is unknown. Further experiments on SMU.1704 might unravel a new regulatory pathway in bacteria (Fig 26).

Chapter 6: Conclusions and Discussions

Both one- and two-peptide lantibiotics are potential alternatives to the already existing antibiotics in the market. However, lantibiotics have not been properly explored for therapeutic purposes in the healthcare industry. The emergence of multidrug-resistant bacteria generated a renewed interest in these naturally existing antimicrobials because the lantibiotics use different targets on bacteria than the commercially available antibiotics. Usually, most of the lantibiotics use the lipid II as their docking molecule to inhibit the target cells. But the initial binding sites on lipid II for lantibiotics and antibiotics such as vancomycin are different. Thus, lantibiotics has even the potential for treatment against vancomycin-resistant enterococcus (VRE) or vancomycin-resistant *S. aureus* (VRSA).

Among the lantibiotics, it is not known whether two-peptide lantibiotics are more potent than the one-peptide ones. One-peptide lantibiotics such as mersacidin or actagardin can bind to lipid II and inhibit cell wall biosynthesis. In contrast, two-peptide lantibiotics such as lacticin 3147 (Ltnα and Ltnβ) contains a lipid II binding component, Ltnα, which inhibits cell wall biosynthesis. The second component, Ltnβ, can act synergistically to form pores in the target cell membrane and cause ion loss leading to rapid cell death. In general the two-peptide lantibiotics are more potent and have wider inhibitory spectra. For example lacticin 3147 has much wider inhibitory spectra than mersacidin. However, nisin, which is a one-peptide lantibiotic, is an exception and can inhibit a wide range of organisms. This is because nisin can inhibit both the cell-wall biosynthesis and can form pores in the target cell membrane. Two-peptide lantibiotics could provide additional options to generate mutations aiming to improve the potency. For one-peptide lantibiotics the options to improve the potency by mutagenesis is theoretically limited to half. Furthermore, the modification enzymes may not be able to recognize or modify if too many residues are changed in a single peptide. Furthermore two separate peptides provide lantibiotics a chance to pair with a homologous counterpart from another two-peptide lantibiotic to create a hybrid lantibiotic with much wider inhibitory spectra, increased potency and/or stability. Smb, the two-peptide lantibiotic that we studied here, has not been explored for bioengineering purposes. Moreover, to our knowledge Smb has

never been extensively studied for its activity or never been paired with another two-peptide lantibiotic for enhanced potency.

Smb is naturally very potent and has a broad inhibitory spectrum that includes most pathogenic streptococci such as *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae*. Few other less pathogenic streptococci, such as *S. dysgalactiae* (causes osteomyletis) and *S. gallolyticus* (causes encodcarditis and perhaps colon cancer), are also inhibited by Smb (Chapter 3). Furthermore, Smb is active against many commensal but opportunistic viridians group of streptococci including many *S. mutans* isolates, and these viridans streptococci can cause systemic infections leading to infective endocarditis. Based on the inhibitory spectra it appears that the natural form of Smb carries a strong therapeutic potential.

To evaluate the therapeutic applicability of Smb, it is fundamental to understand how Smb recognizes and inhibits the target organisms. An organism becomes a target when it carries a receptor for the lantibiotic and at the same time lacks the immunity protein necessary for protection against the lantibiotic (Fig 27). Identities of neither of these molecules were known for Smb. In addition, how *S. mutans* regulates the level of Smb production during initial colonization and how the organism sustains bacterial warfare in the dental plaque are also unknown. Our study is the first to shed some lights on these various unexplored questions.

Lantibiotics are generally purified from the culture supernatant of the producer strain, but the yield of the purified product is often not high enough for economic benefit. In many cases, lantibiotics are purified from agar media for better yield, presumably because the lantibiotics are expressed in higher amounts in solid media than in liquid broth. However, an alternative production strategy is to over express the immunity protein leading to higher secretion of the cognate lantibiotics [146]. In this dissertation work, we identified SmbFT as the immunity protein for Smb (Chapter 3). We also verified that over expression of SmbFT in GS-5 leads to increased ZOI indicating increased Smb production (preliminary observation).

Identification of the cognate immunity protein for Smb also opens up new avenues of research. For example, it is now possible to explore various mutated forms of SmbFT for better protection against Smb. However, if a more potent bioengineered form of Smb is developed in the future, it has to be kept in mind that the immunity protein also needs to be modified accordingly to provide proper protection to the producer strain. Identifying the critical residues on SmbFT required for lantibiotic recognition as well as for protection will provide the necessary information for such modifications. These studies will further explore the therapeutic potential of Smb lantibiotic beyond the natural limits.

To our surprise we found that SmbFT can confer cross-protection against related lantibiotics such as haloduracin and gallolacticin. We speculate that in a given multispecies community there are bacteria that are protected from each other's bacteriocins to coexist together and to employ a concerted inhibitory effect against their competitors. This group of bacteria are perhaps metabolically diverse and do not compete within themselves for nutritional resources. We also found that SmbFT alone is sufficient to provide protection against Smb in *S. sanguinis*, a cocolonizer of *S. mutans*. If *S. sanguinis,* which is naturally competent, acquires just the *smb*FT or the entire *smb* locus by horizontal gene transfer, then *S. mutans* will not be able to inhibit *S. sanguinis*. We speculate that *S. mutans* might use other strategies, such as interfering with the competence of *S. sanguinis,* so that the Smb is still active against this organism.

Cross-immunity conferred by an ABC transporter complex has been reported previously. A staphylococcin C55 producer *S. aureus* strain is also cross immune to lacticin 3147 produced by *L. lactis*. Therefore, a repository of Smb or lacticin 3147 resistant strains, other than the expected producer organisms, exist in the nature and perhaps are present in the same environmental niches [187]. If a given lantibiotic is considered for therapeutic purposes, it is necessary to consider the resistance problems before hand so that a proper strategy can be developed to counter act the emergence of resistance due to cross-immunity.

Although the exact mode of action for SmbFT was not characterized in this study, an equivalent system encoded by NisFEG has been shown to export the membrane-inserted nisin to the outer milieu. An additional protein, NisI, sequestrates the extracellular expelled nisin and prevents their reinsertion into the membrane. For SmbFT it is currently unknown what prevents Smb from coming back to the membrane once it is exported outside, since the *smb* locus does not encode a sequestering protein like NisI. We speculate that the exporter protein SmbG that is involved in the secretion and processing of Smb might provide a sequestration function. This is because SmbG bears a large extracellular domain that might be suitable for binding the expelled Smb and prevent its re-association with the membrane. Alternatively, Bip, a membrane protein that was proposed by Kuramitsu's group to function as an immunity protein, might function as a sequestration protein and prevent Smb to re-associate with the membrane. However, this is just a speculation that needs to be verified experimentally.

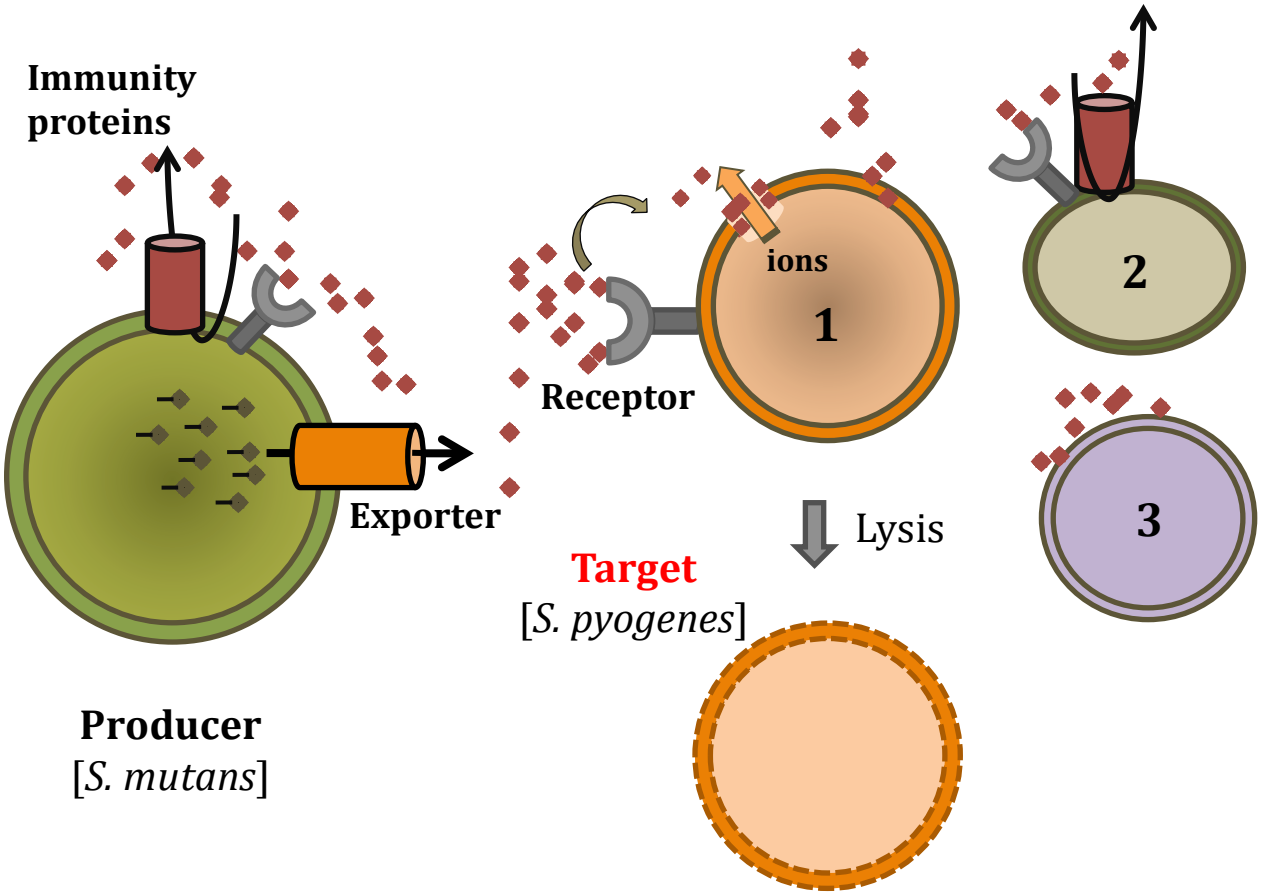
Although it is known that immunity proteins can enhance the production of cognate lantibiotics, the exact mechanism of enhanced production has not been evaluated at the molecular level. Some immunity proteins such as BceAB type ABC transporter interact with a signal transduction pathway and regulate the production of bacteriocin [139]. In case of BceAB, the transmembrane domain of the transporter complex interacts with the histidine kinase present at the membrane and this interaction is necessary for the signal transduction that ultimately regulate bacteriocin production. Interestingly, the ATPase activity of the immunity protein is also necessary for the signaling. It seems that when the immunity protein exports out the bacteriocins from the membrane, the ATPase activity influences the sensor kinase to activate transcription therefore more immunity protein is produced. We also observed that when SmbFT is over expressed Smb production is also increased; however, the detailed molecular mechanisms by which SmbFT influences Smb production remains to be explored.

In general, lantibiotics have a very well defined inhibitory spectra and this led us to believe that the target selectivity is due to specific interaction between the lantibiotic and a receptor-like component on the target organism. Lipid II is still essential for inhibition but the absence of a particular receptor or receptor-like protein makes an organism resistant (Figure 27). Identification of a receptor for a non-lantibiotic bacteriocin such as pediocin is a very recent event; although, the pediocin-like bacteriocins were discovered decades ago. No receptors or receptor-like proteins have been identified for any lantibiotics despite the intense research in this area. In this work, we have identified a protein with receptor-like function towards Smb (Chapter 4). This protein, which we named LsrS, is a zinc metalloprotease and shows a receptor-like function in *S. pyogenes* for Smb. The molecular mechanisms by which LsrS causes Smb mediated toxicity are currently unknown. However, we speculate that the LsrS-Smb interaction is direct since we isolated an *lsrS*-deficient mutant colony as a survivor inside the ZOI of Smb. Although, the ZOI in *S. pyogenes* was not reduced to zero, the ZOI was reduced to zero in a SMU.662 mutant of *S. mutans* V403. Since the *lsrS*-mutant of *S. pyogenes* produced a moderate ZOI it is possible that this organism encodes multiple receptors or receptor-like proteins for Smb, while *S. mutans* encodes only one. Since all the members of pyogenic group of streptococci that we tested produced consistently larger ZOI compared to the other organisms, we speculate that pyogenic streptococci perhaps encode multiple receptors for Smb. Furthermore, the streptococci tested in this study were sensitive to Smb because of the presence of the receptor or receptor-like proteins although some encode homologs of the immunity protein as evident from our *in silico* searches. These immunity-like proteins either are pseudogenes or do not recognize Smb. Surprisingly, we found that the Smb producer strain GS-5 also encodes the receptor protein (SMU_GS5_02915). Our preliminary studies suggest that this protein shows receptor-like function. At present we do not know the reason why a producer strain also encodes the receptor. Since LsrS is the only receptor so far identified, we do not know how frequent is the presence of both the receptor and the cognate lantibiotic in bacteria. One possibility is that when the producer strain encodes a receptor, the receptor may handover the lantibiotic immunity proteins. We speculate that the immunity proteins and receptors reside on the producer membrane juxtaposed to each

other to efficiently handover the receptor bound Smb to SmbFT for export. Furthermore, it is possible that the presence of receptor bound to Smb might negatively influence the secretion of Smb. Future studies will unravel the significance of the presence of receptors in producer organisms.

LsrS is a CAAX family protease that belongs to CPBP subfamily. CBPB subfamily proteins are widely present in bacteria but they are very poorly characterized. Only a handful of CPBP proteins have been experimentally characterized and they show a diverse range of functions. These CBPB proteins contain various functional domains at the N-terminal side, which are not characterized. So far the identified receptor proteins for unmodified bacteriocins are sugar transporters and the most recent one is a zinc metallo-protease. These proteins are all well conserved in bacteria. LsrS is also a conserved protein and its presence in the producer strain indicates that the lantibiotics, in addition to killing activity, may possess a signaling activity to regulate various cellular functions that are needed for the growth in the multispecies community. Deciphering this signaling pathway mediated by lantibiotics within and beyond the producer population might open up new avenues in bacteriocin research.

Figure 27: A model for Smb mediated inhibition. The SmbG transporter protein secretes Smb to the outside. Accumulated Smb is then recognized by a cell-surface receptor protein, SMU_GS5_02915 (SMU.662), which probably interacts with the immunity complex, SmbFT (an ABC transporter) that pumps out the Smb before it can exert its inhibition. The target cells that contain only the receptor protein, and not the immunity protein, will be killed. For example, *S. pyogenes* which conatins LsrS but not the immunity protein is susceptible. Whereas Smb will not inhibit cells containing both the receptor and the immunity proteins. Similarly, cells that are devoid of both the receptor and the immunity proteins are also refractory to Smb mediated inhibition.


Immunity proteins
Exporter
Producer
[S. mutans]
Receptor
ions
1
2
3
Lysis
Target
[S. pyogenes]

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Appendix: Complete Genome Sequence of *Streptococcus mutans* GS-5, a Serotype *c* Strain

Abstract

Streptococcus mutans, a principal causative agent of dental caries, is considered to be the most cariogenic among all oral streptococci. Of the four *S. mutans* serotypes *(c, e, f,* and *k*), serotype *c* strains predominate in the oral cavity. Here we determine the complete genome sequence of *S. mutans* GS-5, a serotype *c* strain originally isolated from human carious lesions, which is extensively used as a laboratory strain worldwide.

Text

Streptococcus mutans, a colonizer of the supragingival tooth-surface, is a part of a complex microflora comprising ~700 species. To maintain its dominant presence and to cause dental caries, this acidogenic and aciduric organism can drastically and quickly reduce the pH of its surrounding leading to demineralization of tooth enamel (10). *S. mutans* also secretes antimicrobial peptides (mutacins) to suppress the growth of other competing species. Of the four serotypes of *S. mutans*, the predominant oral isolates are of serotype *c* (14). GS-5 is a representative serotype c strain originally isolated from carious lesions forty-five years ago (7), and has been extensively used in genetic and biochemical studies of virulence (10). This strain also produces a unique two-peptide lantibiotic mutacin, known as SmbAB (17), which is encoded by only ~8% of the isolates (16). Recent completion of three *S. mutans* genome sequences (UA159, NN2025, and LJ23; (1, 2, 13), indicates a large degree of diversity and genome rearrangement within the species. Here we determine the complete genome sequence of GS-5, which will allow us to gain further insight into overall genetic variation in *S. mutans*.

Genome sequencing was performed using a combination of strategies that include Illumina GA-IIx and Roche GS-Jr. technology at Cofactor Genomics (St. Louis, USA). Illumina sequencing used two genomic libraries SIPE (~300bp) and LIMP (~3-kb), which generated ~21 and ~15 million paired reads, respectively. Roche 454 GS-Jr. generated 142,832 reads (average length ~450nt) that covered ~26X of the entire genome. Several assembly procedures were applied and manual editing was performed with the 454 GS-Jr. data. Remaining gaps and unassertive assembled regions were verified by PCR/ABI

sequencing to obtain a single contig onto which Illumina data were subsequently mapped for further refinement. The genome was annotated using IGS Annotation Engine (5) and The NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) was employed for submission (http://www.ncbi.nlm.nih.gov/genomes /static/Pipeline.html).

GS-5 genome encodes a 2,027,088-bp circular molecule with a G+C content of 36.8%. The sequence information is consistent with a previously generated physical map of the GS-5 genome (3). Putative origin of replication was mapped near position 1553-nt by OriFinder (6). GS-5 harbors 65 tRNA genes, 15 rRNA genes, and 1985 CDS. Among the CDS, 91 exported-proteins, 23 lipo-proteins, 11 wall-proteins, and 462 membrane-proteins were identified by SLEP (8). No putative phages or prophages were predicted by Prophinder (12). However, two complete and one incomplete CRISPRs (clustered regularly interspaced short palindromic repeats), and numerous insertion sequences were identified with CRISPRFinder (9) and ISfinder (15), respectively. Furthermore, in addition to SmbAB, at least ten other putative mutacins were also identified by BAGEL2 (4). Several large (>1.0-kb) duplicated regions were identified in the genome by REPuter (11). Sequence alignment indicated that genome rearrangement occurred between GS-5 and NN2025 across the replication axis, but not between GS-5 and UA159. The GS-5 complete genome will allow for in depth comparative genomics to unravel the extent of genome rearrangements and dynamics in *S. mutans* and to better understand the adaptive life style of this pathogen.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER: The complete genome sequence of S. mutans GS-5 was deposited in GenBank under the accession number CP003686.

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