ABSTRACT

Title of dissertation: TRANSFER OF NISIN GENE CLUSTER FROM *Lactococcus lactis* INTO THE CHROMOSOME OF *Bacillus subtilis* 168

Sahru Yuksel, Doctor of Philosophy, 2003

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The posttranslationally modified antimicrobial peptide nisin belongs to the family of lantibiotics, which constitute a group of small peptides that contain a high proportion of unusual amino acids. These unusual residues confer unique properties that are essential for its biological activity. Nisin is produced by *Lactococcus lactis*, and has a broad spectrum of activity against gram-positive bacteria including food-spoilage bacteria and pathogens.

The aim of this work was to express nisin from *Bacillus subtilis* 168 by transferring the nisin gene cluster from its natural host into the chromosome of *B. subtilis* 168.This would allow the exploration of the use of genetic engineering techniques to create structural analogs of nisin and also enable a more facile study of the post-

translational modification machinery. A variety of strategies to achieve this transfer were explored. In one strategy, a λ genomic library of *L. lactis* 11454 was constructed. The clones containing the 16.5 kb nisin gene cluster and immediate neighboring genes was then subcloned into a cassette vector, pLPVcat, and transformed into *B. subtilis* ermΔsunA, which was then integrated into the *B. subtilis* chromosome by a double recombination. This recombinant was tested for its ability to express mature nisin into the culture supernatant by using mass spectrometry. However, no nisin or nisin like peptide could be detected when the culture supernatant was analyzed. Integration of a constitutive promoter in front of the nisin structural gene in an attempt to insure that the nisin gene was expressed did not solve the problem. There could be several explanations for this result. One possibility is that transcription of the nisin gene cluster was inadequate despite the presence of the integrated promoter. Another possibility is that transcription was adequate, but there was a lesion in the posttranslational processing pathway. RT-PCR was employed to establish that all of the *nis* ABTCIPRK genes were transcriptionally active, but the possibility that the level of transcription was too low to give detectable levels of nisin could not be ruled out. It is noted that nisin biosynthesis is a complex process, and that one or more of the posttranslational events could be inefficient in the *B. subtilis* 168 host.

An important achievement in this work was the construction of a *B. subtilis* 168 strain that contains the entire nisin gene cluster integrated into the chromosome. This will greatly facilitate future work on expression of nisin in a heterologous strain that is more suitable for laboratory studies than is the natural producer strain.

TRANSFER OF NISIN GENE CLUSTER FROM *Lactococcus lactis* INTO THE CHROMOSOME OF *Bacillus subtilis* 168

by

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Dissertation submitted to the Faculty of the Graduate School of the
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DEDICATION

This work is dedicated to the following individuals:

To my husband, Kerrar, for his support and patience,
To my parents, Nihal and Fahrettin Aral, for their support and encouragement,
And to Samiha Ayverdi, for her spiritual guidance and enlightenment

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LIST OF ABBREVIATIONS

amp	ampicillin
amu	atomic mass units
APS	ammonium persulfate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BME	β-mercapto ethanol
bp	base pairs
BSA	bovine serum albumin
cat	chloramphenicol transacetylase gene
CIAP	calf intestinal alkaline phosphatase
Cm	chloramphenicol
cm	centimeters
Da	daltons
ddH2O	distilled deionized water
Dha	dehydroalanine
Dhb	dehydrobutyrine
DMF	dimethylformamide
DNA	deoxyribnucleic acid
dNTP (dATP, dTTP, dCTP, dGTP)	deoxyribonucleoside triphosphate
ddNTP (ddATP, ddTTP, ddCTP, ddGTP)	dideoxyribonucleoside triphosphate
ds	double-stranded
EDTA	ethylenediaminetetraacetic acid
erm	erythromycin
erm	erythromycin resistance gene
EtBr	ethidium bromide
HPLC	high performance liquid chromatography
hr	hour
IPTG	isopropyl-β-thiogalactopyranoside

kb	kilobase
kDa	kilodalton
kV	kilovolts
LB	Luria-Bertani medium
l	liter
MALDI-TOF-MS	matrix assisted laser desorption/ionization-time of flight mass spectrometry
μg	microgram
μm	micrometer
μl	microliter
ml	milliliter
mm	millimeter
μM	micromolar
mM	millimolar
msec	millisecond
M	molar
min	min
mcs	multi-purpose cloning site
MS/MS	mass spec/mass spec
m/z	mass/charge
ESI-MS	electrospray ionization mass spectrometry
NBT	Nitro blue tetrazolium chloride
ng	nanogram
nt	nucleotide
PAB	Penassay broth (antibiotic medium 3)
PAGE	polyacrylamide gel electrphoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming units
P_i	Phosphate (inorganic)
rpm	revolutions per min

RT	room temperature
sec	seconds
ss	single-stranded
SDS	sodium dodecyl sulfate
SSC	standard saline-sodium citrate
TAE	tris-acetate EDTA
TBST	tris-buffered saline-Tween 20
TEMED	N, N, N’,N’, tetramethylenediamine
TFA	trifluoroacetic acid
Tris	tris(hydroxylmethyl)aminomethane
wt	wild type
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

Introduction

Bacteria are a very large group of single-cell microorganisms. Bacteria were first discovered with the advent of the microscope in the 17th century. However, it was not until Louis Pasteur studied them in the mid-19th century that it became clear that bacteria were responsible for many human diseases.

The new era in modern medicine began with the discovery of the first antibiotic drug penicillin by Alexander Fleming in 1928 and it was first used to cure soldiers in World War II. Since then, many antibiotics have been developed and discovered. The advent of antibiotics has revolutionized the treatment of serious bacterial illness that has not been controlled by the immune system. These medications work either by killing bacteria directly, or else by stopping their growth in order that the immune system can more effectively eliminate them. Unfortunately, bacteria frequently develop resistance to the antibiotics, which once killed them. In effect, a war has begun between the scientists who keep developing new antibiotics, and the bacteria, which seem to develop resistance to those antibiotics at an ever-increasing rate. In the search for new antibiotics, scientist have discovered that bacteria as well as non-bacterial cells fight for niche and nutrients against other bacteria and as one of the many ways to combat this environmental take over, some have evolved the ability to secrete antimicrobial peptides. Today, we can broadly classify peptide antibiotics into two categories. One category consists of peptides, which are synthesized by multienzyme complexes, and another category consists of peptides, which are encoded by structural genes and synthesized ribosomally.

Peptide antibiotics that contain unusual amino acids are common, and most of them, such as valinomycin and Gramicidin S, are synthesized by a non-ribosomal mechanism. In contrast nisin, another antimicrobial peptide, is derived from posttranslational modifications of a ribosomally synthesized polypeptide. Nisin's being gene encoded has the great advantage over the antibiotics that are biosynthesized by multistep enzymatic pathways in that their structure can be modified by mutagenesis of the gene. This allows us to construct antibiotics that may have much improved antimicrobial activities or to design new antibiotics that can be used to treat diseases to which no treatments are known.

Part I. The lantibiotics

Gene-encoded antimicrobial peptides are widely distributed in nature, being produced by mammals, birds, fish, amphibia, insects, plants, fungi, and microorganisms (68;3;68). Lantibiotics are a subgroup of antimicrobial peptides that are produced by gram-positive bacteria of different genera: *Staphylococcus, Lactococcus, Streptococcus, Bacillus and Streptomyces* (29). The activity of nisin, the prototype of the lantibiotics, was first observed in 1928 (67). Subtilin, which is produced by *Bacillus subtilis* ATCC 6633, is a natural analogue of nisin and was discovered in 1948 (29). They exhibit 60% identity in their amino acid residues.

Lantibiotics have high proportion of modified residues that are introduced by posttranslational modification machinery. For example, in both nisin and subtilin one third of the amino acids are modified, including three dehydro residues, two dehydroalanine (Dha) and one dehydrobutyrine (Dhb), and five lanthionine rings each

contain two modified residues. Initially, it was thought that nisin was synthesized by a non-ribosomal mechanism due to the presence of high proportion of unusual amino acids. However, Hurst's observation that nisin biosynthesis was inhibited by protein synthesis inhibitors suggested that protein synthesis was required, which argued for a ribosomal mechanism (33). This was confirmed when the nisin gene was first cloned in the Hansen laboratory in1988 (8).

Lantibiotics originate *via* enzymatic modification of ribosomally synthesized precursor molecules, the so-called prelantibiotics (Figure 2). These polypeptides consist of a leader peptide and the unmodified prolantibiotic. The name lantibiotic is derived from their content of the thioether amino acids lanthionine and 3-methyllanthionine (73) (Figure 1). These amino acids arise through a multistep process; first, β-hydroxy-α-amino acids in the pro-peptide portion of the precursor are enzymatically dehydrated to their respective α,β-unsaturated equivalents and then the thiol group of a neighboring Cys residue is added to the double bond in an enzyme mediated reaction to generate the corresponding thioether.

Part I-1. Classification of lantibiotics

Lantibiotics can be categorized according to their structural features (Figure 3). Type A lantibiotics, of which nisin is the most thoroughly characterized, are elongated, screw shaped, and amphiphilic and cationic; and they have a molecular weight in the range of 2,000-5,000. Examples of this class are nisin, subtilin, Pep5, epidermin, and gallidermin. Type B lantibiotics are more globular in shape with a low net charge and

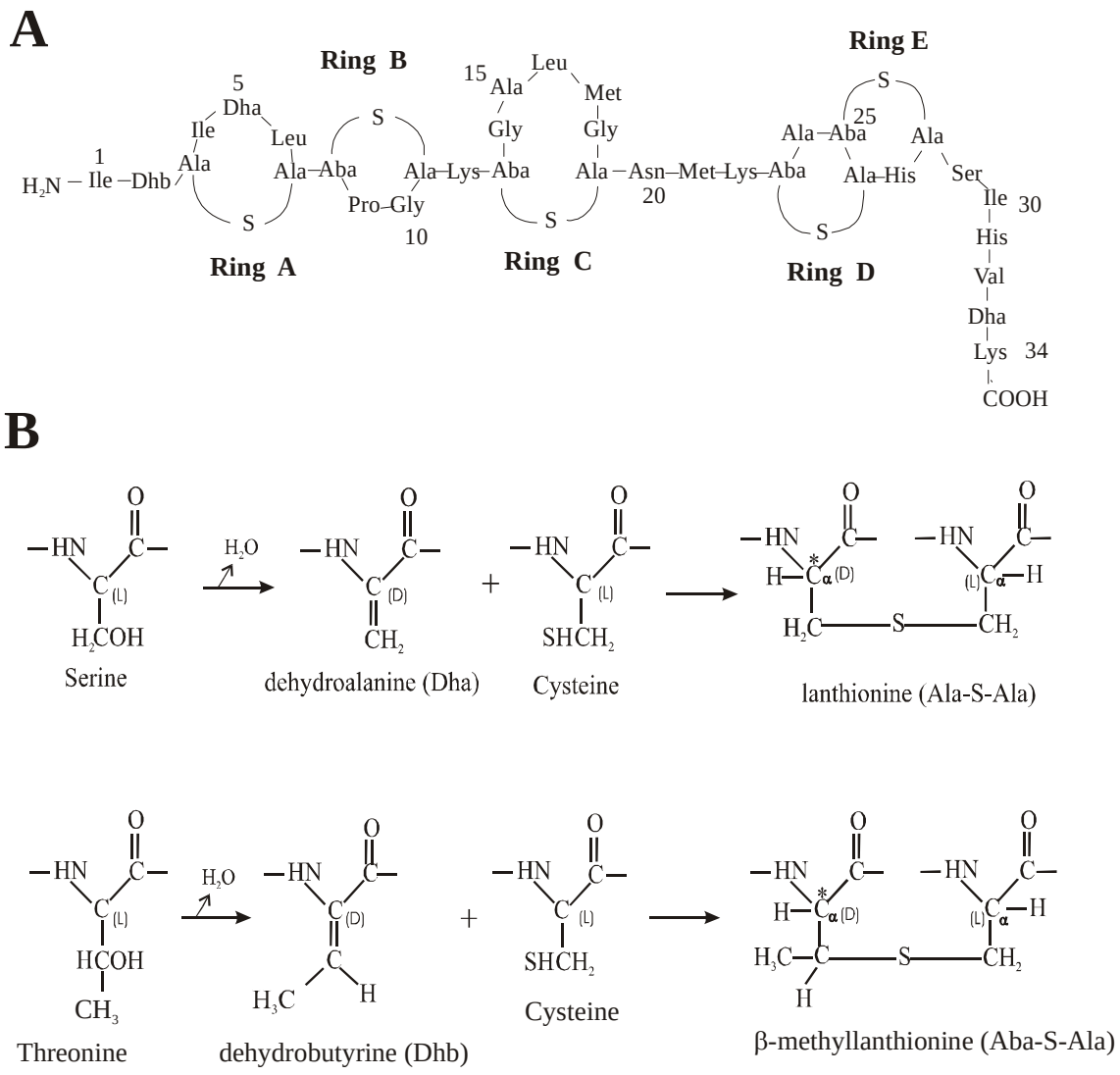


Figure 1. Nisin and unusual amino acids present in nisin.

A) Structure of modified nisin. B) Formation of dehydro and lanthionine residues Isomeric configurations of the amino acids are indicated in paranthesis.

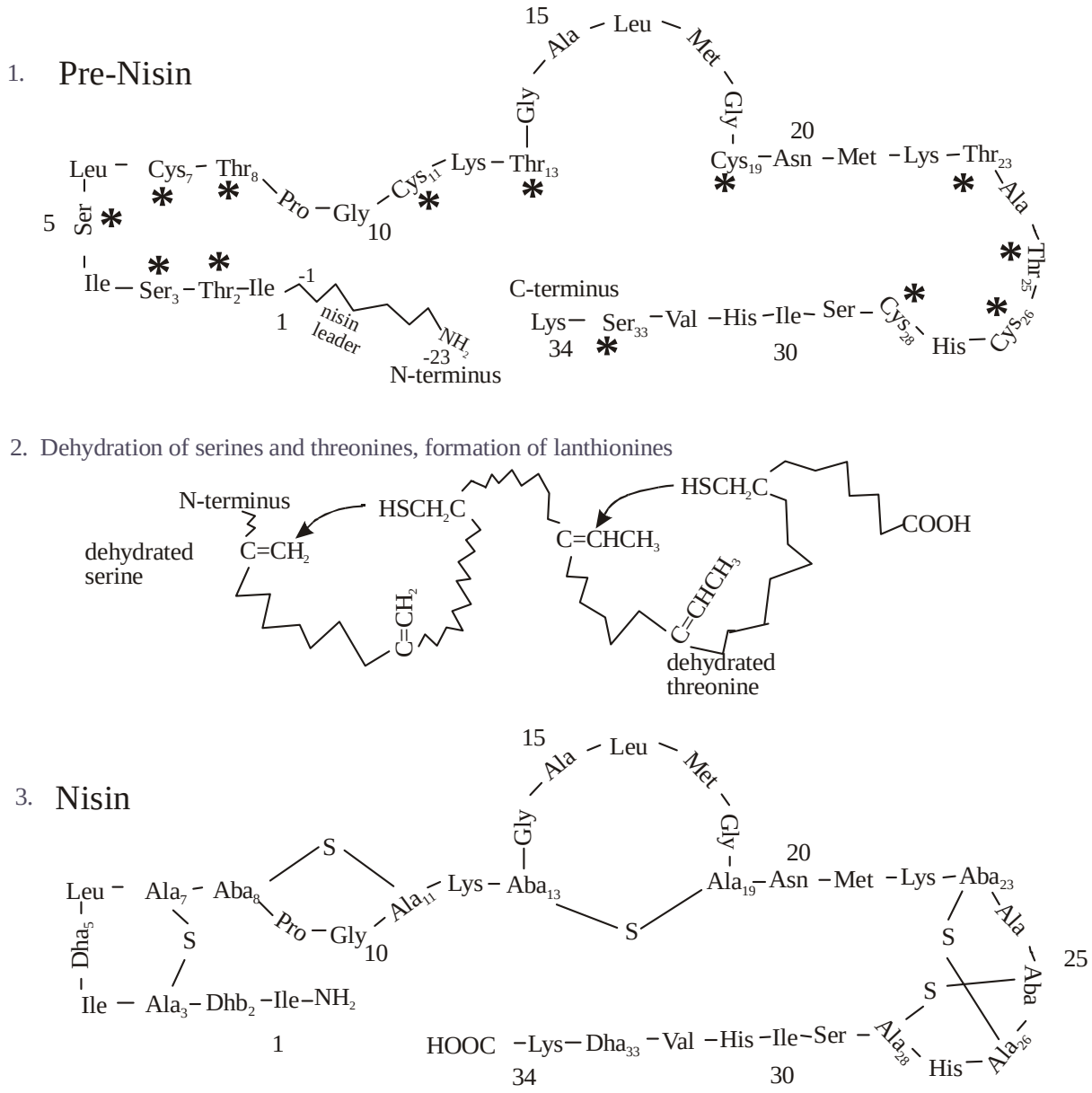
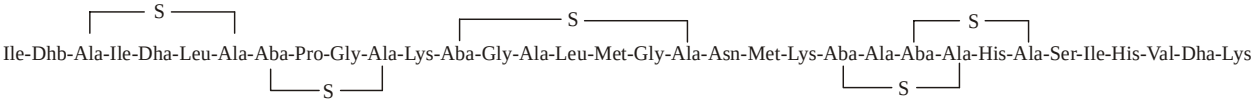


Figure 2. Scheme for posttranslational modification of prenisin into nisin.

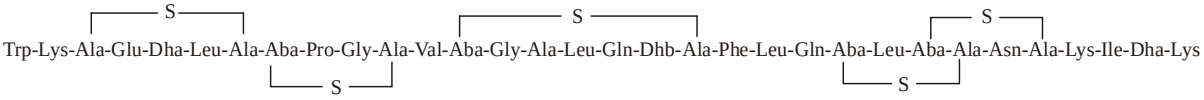
The serines and threonines (marked with asterisks) undergo sequence-specific dehydration reaction to create modified amino acids with electrophilic centers. Stereospecific addition of the sulfhydryl group of the cysteine to the dehydro residues result in the formation of thioether cross-linkages called lanthionine and β-methyllanthionine. This scheme was originally proposed by Ingram (34).
Ala-S-Ala: Lanthionine, Ala-S-Aba: β-methyllanthionine, Dha: Dehydroalanine, Dhb: Dehydrobutyrine.

Type A

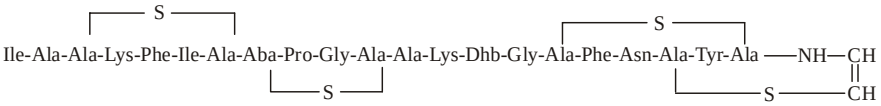
Nisin



Subtilin

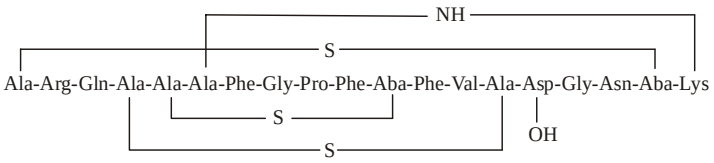


Epidermin



Type B

Cinnamycin



Type C

Mersacidine

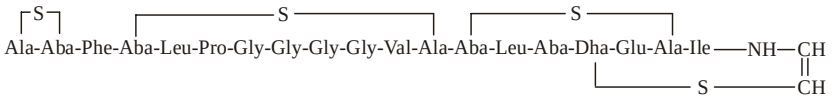


Figure 3. Structures of the representative Type A, B, and C lantibiotics

Ala-S-Ala: Lanthionine, Ala-S-Aba: β-methyllanthionine, Dha: Dehydroalanine, Dhb: Dehydrobutyrine, Ala-NH-Lys: Lysinoalanine, Asp-OH: Hydroaspartic acid.

have molecular weights of about 2,000. Examples of this class are cinnamycin, duramycins, and ancovenin. The type A lantibiotics interact with the membrane of the susceptible cells and form transient voltage-dependent pores (55), whereas at least some type-B lantibiotics act by inhibiting the functioning of certain enzymes. Mersacidin and actagardine are categorized as type-C lantibiotics since they do not fit into either type-A and type-B category (27;29;61). Type-C lantibiotics interfere with the cell wall synthesis in gram-positive bacteria (70).

Part I-2. Genes involved in the biosynthesis of lantibiotics.

Lantibiotics begin as ribosomally synthesized prepeptides (Lan A peptide) and undergo a complex series of events including posttranslational modification of the mature region, cleavage of the leader peptide, and secretion outside the cell. However, our current knowledge of these events, such as the order in which they occur or the location is incomplete. Besides the structural genes for the individual lantibiotic prepeptides (*lanA* genes), there are additional genes which code for a number of other functions that are clustered near the structural gene (Figure 4) in the host chromosome, or alternatively, on plasmids. Genes that are found as homologs in more than one type of lantibiotic gene cluster are denoted with prefix *lan*. These include: i) proteins with sequence similarity to the family of two-component regulatory proteins (proteins designated LanR and LanK); ii) proteins with sequence similarity to serine proteases (LanP proteins); iii) proteins with sequence similarity to translocators of the ATP-binding cassette transporter protein family (LanT proteins); iv) proteins without similarity to known proteins which were found to be involved in producer –self-protection (‘immunity’, LanI proteins); and v) two

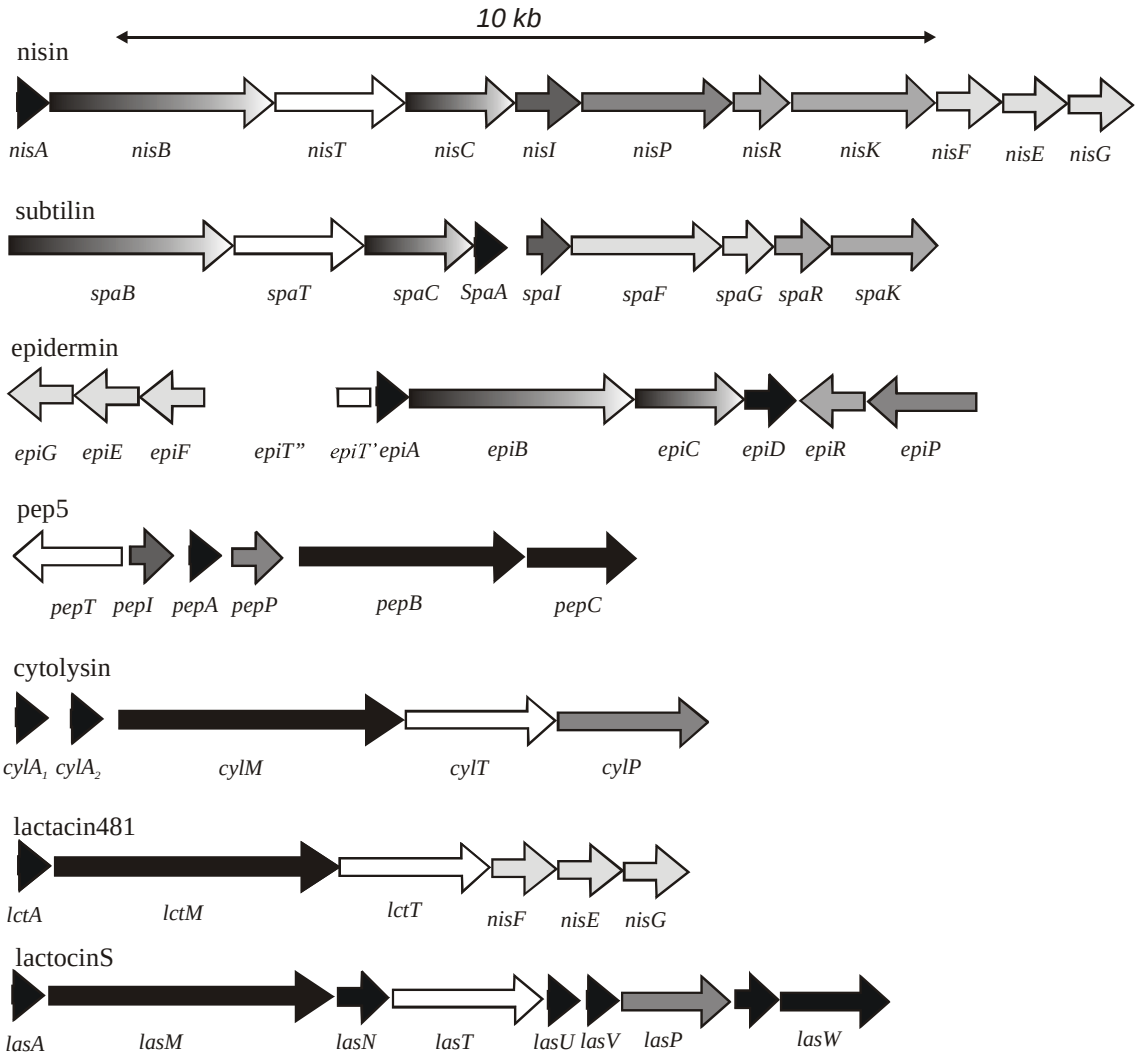


Figure 4. Organization of the selected lantibiotic gene clusters.

Genes with similar proposed function or substantial sequence similarity are given similar suffixes and are highlighted in the same manner. The arrowheads indicate the transcription of each *lan* gene. The functions of the genes are described in the text. LactocinS gene cluster contains several open reading frames with no counterparts in other gene cluster with known functions. The gene names used are standardized nomenclature proposed by deVos *et al* (13).

proteins with no obvious sequence similarity to proteins in databases (LanB and LanC proteins).

The *lan* genes are all these genes involved in the lantibiotic synthesis, and those of a particular lantibiotic have their specific names, such as the *nis* genes involved in nisin biosynthesis and the *spa* genes involved in subtilin biosynthesis. LanB and LanC proteins seem to be novel, as they have no homology to other known proteins in the databases. Thus it has been speculated that these genes may be responsible for the formation of the thioether rings which are characteristics of the lantibiotics (23);(37). The first experimental evidence showing that *nisB* is required for dehydration and *nisC* for correct lanthionine formation in nisin maturation was published recently (43). The *lanB* and *lanC* genes appear to be missing in the gene clusters responsible for the production of lacticin S, cytolysin and in some other lantibiotics. These clusters do however contain a gene encoding a LanM protein, which is much longer. The C-terminal domain shows 25% sequence identity to the LanC proteins, arguing that they may perform both LanB and LanC functions (35).

Many lantibiotic-producing strains have three genes in common considered as *lanB*, *lanC*, and *lanT*. With respect to the nisin biosynthesis, the *nisT* gene product shares strong homology with several ATP-dependent tranport proteins having two ATP-binding sites and a very hydrophobic region at the N terminus with six potential membrane-spanning domains.

It is also interesting to note that these modifications are only found in the lantibiotics and not any other proteins inside the bacteria. This suggests that the leader sequence, or region in the mature sequence, may contain some signals that may direct

them to the NisB and NisC proteins in producing the modified peptide. Identifying these specific signals might allow us to design novel biomolecules that would contain these modified amino acids, which would confer novel activities or chemical properties that cannot be attained by the common 20 amino acids.

Part I-3. Leader peptide.

The last step in the biosynthesis of a lantibiotic is the removal of the leader peptide from the lantibiotic precursor. In the gene clusters for the lantibiotics nisin, epidermin, Pep5, epilancin K7, lacticin S, cytolysin and epicidin 280 a *lanP* gene was found encoding a subtilisin-like protease. The location, at which processing of the leader peptide occurs, varies with the lantibiotic. NisP, responsible for cleavage of the nisin precursor is anchored to the cellular membrane at the outside of the host cell (80). In contrast to nisin, the *spa* gene cluster does not contain a gene encoding a peptidase, and it is assumed that processing of subtilin occurs by a general serine protease of the host *Bacillus subtilis* (76).

Leader peptides of type A lantibiotics are hydrophilic, highly charged, devoid of cysteine residues and do not show the characteristics of *sec*-dependent signal sequences (14;11;70). The leader peptides are not post-translationally modified as shown for nisin and Pep5 (10;81). The leader peptide part of precursor nisin, the synthetic leader peptide, and probably also the leader peptide in the primary translation product are predominantly random coil in aqueous solution (79;84). Thus, the conserved sequence Phe-Asn-Leu-Asp of the nisin leader peptide does not adopt a well-defined structure that could act as a rigid

recognition site. However, conformational adjustment is possible to binding sites of modifying enzyme(s), of proteins involved in the transport, or at a membrane.

Four possible functions have been proposed for type A lantibiotic leader peptides (16). First, the leader peptide may keep precursor molecules biologically inactive. Indeed, the final intermediate in biosynthesis of nisin is almost inactive. Second, assistance of the precursor peptide in transport across cytoplasmic membrane is possible. In this respect, it is noted that the leader peptide part of the precursor of nisin was observed to be in contact with, but not traversing, the membrane like surface of micelles. The biological relevance of this observation is not clear. Third, it may act as recognition or binding site for biosynthetic enzymes. Fourth, an interaction between the leader peptide part and the rest of the molecule may favor a conformation essential for correct modification, preventing nonspecific reactions. In precursor nisin, which is already modified, the leader peptide part and the nisin part do not interact, so that at this point in the maturation there are no indications for such a function.

Part I-4. Immunity of producer organisms

The *nis*I gene was identified in the middle of the nisin gene cluster and shown to be involved in the development of immunity. The deduced sequence of the 245-residue NisI protein suggests that it is a lipoprotein, probably anchored in the membrane with its C-terminus toward the outer side of the cell (45). The nisin and subtilin (165 residue) immunity proteins are similar in amino acid sequence and general properties, most likely as a reflection of the high degree of similarity of these lantibiotics; however there is no cross immunity between producing strains, demonstrating the specificity of the immunity

mechanisms (70). Cross immunity was only observed between strains producing natural variants such as nisin A and nisin Z (15). Nisin Z differs from nisin A by a single mutation (58). Overexpression of LanI proteins in cells that do not possess the lantibiotic biosynthetic machinery only yields very low protection levels (1-4%) against the corresponding lantibiotic (45). An in-frame disruption of *nisI* in *Lactococcus lactis* yielded a strain that could still produce nisin, albeit to levels five times lower than the wild-type (64), which suggests that the immunity level could be the first limiting factor in reaching high production levels. These results already indicate that additional factors are involved in the full self-protection.

In several lantibiotic gene clusters, three other proteins are encoded, named LanF, LanE, and LanG. The LanFEG proteins belong to the group of ABC transporters, where LanF contains the intracellular ATP-binding domain and LanEG the membrane spanning subunits. The genes *lanFEG* have been found in the gene clusters for nisin (74), subtilin (40). It is tempting to speculate that these proteins have a function in the removal of the corresponding lantibiotic at a certain stage of its membrane interaction. Expression of the LanFEG proteins, without LanI, yields a significant level of immunity although it remains below the wild type level. The synergistic function of all the immunity proteins could thus reside in the first recognition and binding or ‘immobilization’ of the lantibiotic in the membrane by NisI, followed by active removal, in which the LanFEG proteins are involved. It can not be excluded, however that also other proteins encoded in the lantibiotic gene clusters display a synergistic effect in the immunity mechanism, for instance by being involved in a multi-protein complex that spans the membrane.

Candidates to be involved in such a complex are LanBTC proteins, which have already been shown to form a membrane associated complex (75;38;59).

Interestingly, in some cases, e.g. nisin and subtilin production, the expression of the immunity genes is also regulated by the concentration of the lantibiotic in the medium, which means that by sensing low (subinhibitory) amounts of the antimicrobial peptide in the medium, cells can rapidly increase the immunity level, concominant with or even faster than the biosynthesis rate (12).

Part I-5. Regulation of biosynthesis

Biosynthesis of lantibiotics appears to be under the control of two-component response regulatory proteins, a large family of proteins involved in regulation of a variety of physiologically important processes. Nisin also plays an important role in the regulation of its own biosynthesis. In fact, nisin can be regarded as a peptide pheromone, which is sensed by the histidine kinase NisK, which resides at the outer side of the membrane, probably by direct protein-peptide interaction. By analogy with other known two-component regulatory systems (39), NisK will autophosphorylate at a specific histidine residue when it senses a certain concentration of nisin in the medium and subsequently transfer the phosphate moiety to the response regulator NisR. The response regulator is assumed to get phosphorylated at a specific Asp residue, which is supposed to trigger its binding to two regulated promoters in the nisin gene cluster, *i.e.* the *nisA* and *nisF* promoter, thereby activating transcription of the structural gene *nisA* and the downstream genes *nisBTCIP* by limited read through from *nisA*, and the genes *nisFEG* located at the end of gene cluster (44;12;56). The regulatory genes *nisRK* themselves are

transcribed from their own promoter which is assumed to be not dependent on nisin induction (12).

The transcription from the *nisA* and *nisF* promoter in the nisin gene cluster is directly related to the concentration of nisin in the medium. This property is extremely useful for the development of a controlled gene expression system, since a linear dose-response for the expression of target genes is highly desirable for industrially-relevant production organisms. In the case of subtilin production, the autoregulatory process has been shown to be very similar to the nisin case, because the *spaB*, *spaI* and *spaS* promoters could be activated by subtilin in the medium. Knockouts of *spaRK* and *nisRK* have been shown to be detrimental for lantibiotic production (30;54).

Both inducer and sensor engineering have been used to study the molecular interaction between these molecules. First, it was shown that variants and fragments of nisin were able to act as inducer with variable efficiencies and that their induction capacity was unrelated to their antimicrobial activity, demonstrating that the mechanisms for the induction and pore formation are different (20;42).

Part I-6. Bacteriocidal activity.

Type A lantibiotics exert bacteriocidal activity toward a broad range of Gram-positive bacterial strains, including Streptococci, Bacilli, Listeriae, Clostridia, and Staphylococci (32). The antibacterial effect is strong, with nM concentrations of a lantibiotic being sufficient to kill the bacterial cells. Fungal cells, yeast cells or human cells are very insensitive to lantibiotics, even when treated with mM concentrations of the peptides (82). In general, gram-negative bacteria are also insensitive to lantibiotics. It has

been shown that their outer membrane functions as a barrier for the lantibiotics. When the outer membrane of the gram-negative strains was weakened by treatment with EDTA or osmotic shock, the susceptibility of cells towards nisin (78) or Pep5 strongly increased.

Positive charge and amphipathicity are general features of antimicrobial peptides, which kill bacteria by permeabilization of their membranes. It has been shown that nisin most efficiently interacts with anionic membrane lipids (3;18), and that positive charges in the C-terminal domain of nisin are important for this interaction (4). The peptides appear to interfere with the membrane function of sensitive cells by increasing the permeability of the bilayer for small molecules and disrupting the membrane potential, resulting in cell death. Within several minutes, the lantibiotics induce the release of ions, small molecules and ATP from sensitive bacterial cells. The efflux of high-molecular-weight compounds (>500 Da) was not observed (21), indicating that lantibiotics do not completely disrupt the barrier function of the membrane, as detergents do. Recent studies have also indicated that nisin binds specifically to Lipid II (6). The presence of Lipid II, a specific docking molecule, has increased the pore formation efficiency of nisin in the model membranes (5). Lipid II molecules provide the peptidoglycan precursors necessary for biosynthesis of extracellular cell wall. In contrast to recent reports on resistance or tolerance to vancomycin, no resistance to nisin has been reported, despite its use as a preservative for the last 50 years. Complete elucidation of events in nisin's mechanism of action could be invaluable in terms of development of a new class of highly efficient antibiotics.

A second mode of action that several type-A lantibiotics possess is inhibition of spore outgrowth. There is some evidence supporting the fact that nisin and subtilin may

become covalently attached to nucleophilic membrane sulfhydryl groups on the surface of germinating spores by reacting with one or more electrophilic dehydro residues. It has been shown that Dha5Ala mutant of subtilin (51) and nisin (9) is unable to inhibit the outgrowth of spores but is fully active against vegetative cells.

Part II. Nisin.

Nisin is the most extensively studied lantibiotic due to its being stable at high temperatures and at low pH, its non-toxic nature (LD_{50} 5g/kg), and commercial availability. It is produced by food-grade strains of *Lactococcus lactis*. Nisin has been given the generally recognized as safe (GRAS) status for certain food applications in 1988 and its currently being used as a food preservative worldwide. It has many other potential industrial applications. The genes involved in biosynthesis of lantibiotic nisin are located on a 70 kb conjugative transposon (65), which also contains the genetic information for sucrose metabolism. The first gene of the cluster is the structural gene *nis*A, which encodes for the precursor consisting of a 23-residue leader region and a 34-residue structural region. The mature peptide has a molecular weight of 3353.4 Da and overall positive charge. The structural gene is followed by ten other genes i.e. *nisB, nisT, nisC, nisI, nisP, nisR, nisK, nisF, nisE, nisG,* encoding regulatory proteins, proteases, transport proteins and immunity proteins (14).

In aqueous solution, nisin does not adopt a preferred conformation. In a lipophilic environment, it adopts an amphiphilic α-helix shape with two domains. The N-terminal domain that contains ring A-C is connected to the C-terminal domain (rings D and E) through a flexible hinge region. The exact role of thioether bridges is not clear but they

are likely to constrain the peptide into a conformation that is required for activity and/or confer protease resistance, possiblity to prevent degradation by proteases of competing bacteria.

Part II-1. Applications of nisin and other lantibiotics

Nisin has a very broad spectrum of activity against many species that are important in dairy and food industry and has been approved for use in about 50 countries (17). Applications of nisin as a food preservative includes the use in processed cheese, canned foods, meat, milk, high moisture baked products, and fermented beverages such as beer and wine (28). It can also be used in cosmetics to increase the shelf life of certain products. The proteases of the human stomach and intestinal tract hydrolyze it completely and it does not damage the gram-negative bacteria of the intestinal flora. Nisin is a naturally occurring product in various fermented foods due to the fact that nisin producing *L. lactis* is commonly used as a starter culture in dairy industry. Thus, humans have consumed nisin safely for hundreds, or perhaps thousands of years. It is widely used in prevention of bovine mastitis and has potential for treatment of peptic ulcer caused by *Helicobacter pylori* (28). Another area of interest for nisin is dental applications, since it has been shown to be effective in killing *S. mutans*, the prime cause of dental cavities, and in prevention of oral plaque and gingivitis (28).

Epidermin, another type-A lantibiotic, is used for treatment of skin infections (28). Ancovenin can inhibit the angiostatin-converting enzyme and thus has potential for use in treatment of high blood pressure (28). Cinnamycin, a type B lantibiotic has been shown to inhibit replication of Herpes simplex virus, HSV1 (28).

In spite of nisin's frequent use as a biopreservative for nearly 50 years, no bacterial strains, which have developed resistance to nisin, have been detected. This is in great contrast with the case of regular antibiotics. Perhaps the unusual residues present in the lantibiotics play a crucial role in their distinct mechanism of action. Elucidation of the ways that lantibiotics orchestrate the introduction of these unusual residues, determination of their mode of action against vegetative cells and spores, and the set of events that take place during the biosynthesis would be of great importance to the scientific and medical communities. It is likely that this knowledge could be applied to the development of novel antimicrobial agents to fight bacterial resistance and may be even targeted against infectious or other diseases that are currently incurable.

Part II-2. Heterologous expression of nisin and subtilin.

The ability to produce an antibiotic is a trait that can be easily transmitted between bacteria. Usually, the genes necessary for production of the antibiotic are clustered together within the chromosome or within some kind of genetic element such as plasmid or transposon. This facilitates the transfer of antibiotic production between bacterial species. Being gene encoded makes the lantibiotics particularly amenable to expression in other bacteria since they could adapt to different environments by the mechanism of mutation and selection that is available to all proteins.

We are particularly interested in expressing lantibiotics in hosts that are suitable for industrial production. This is because if these antbiotics can be produced inexpensively on a large scale, their use in a variety of new applications is more likely to be explored and thus widely used. Another goal is to identify all the genes involved in the

biosynthetic pathway of lantibiotics. To date, the genes were assigned to functions by comparison of their sequences with the protein database, however genetic and biochemical analysis should be done ultimately to confirm these functions. The details of the biosynthesis machinery also need to be explored. However not every lantibiotic producer can be easily manipulated by genetic engineering.

The genes responsible for subtilin production have been transferred from *B. subtilis* 6633 to *B. subtilis* 168 (50) in this laboratory. Derivatives of *B. subtilis* 168 have been widely exploited for industrial production of biological materials. There are extensive genetic information and methodologies available for *B. subtilis* 168. Being a gram-positive bacteria and not producing an endotoxin are other great advantages of this organism for lantibiotic gene transfer and expression.

The presence of nisin genes on a conjugative transposon, a mobile genetic element, argues the fact that nisin could be expressed in other bacteria. In fact, transfer of nisin production ability between species and strains of *L. lactis* has been achieved by several labarotories (27). Since nisin and subtilin are so closely related, it is presumed that information about one will provide useful insight about the other such as the specificity of leader peptides and/or the role of leader peptide in the introduction of unusual residues etc.

A primary goal of this project has been to transfer nisin producing ability into *B. subtilis* 168 so that all the genes involved in nisin biosynthesis could be characterized in this new host. This would create opportunities to engineer the nisin structure in order to study structure/function relationships and to design analogs with modified and novel biophysical, biochemical, and biological properties.

Materials and Methods

Part I. Material sources

Part I-1. Reagents and Chemicals

Acetic Acid, Glacial	Baker Chemical, Phillipsburg, NJ
Acetonitrile, HPLC grade	Fisher Scientific Co., Fairlawn, NJ
Acrylamide	Bio-Rad Laboratories, Hercules, CA
Agarose	BRL, Gaithersburg, MD
Amino acids	Sigma Chemical Company, St. Louis, MO
Ammonium acetate	Fisher Scientific Co., Fairlawn, NJ
Ammonium bicarbonate	Sigma Chemical Company, St. Louis, MO
Ammonium hydroxide (30%)	Baker Chemical, Phillipsburg, NJ
Ammonium persulfate	U. S. Biochemicals, Cleveland, OH
Ammonium phosphate, dibasic	Sigma Chemical Company, St. Louis, MO
Ammonium sulfate	Baker Chemical, Phillipsburg, NJ
[γ-^{32}P]ATP	New England Nuclides, Boston, MA
BCIP	Bio-Rad Laboratories, Hercules, CA
BioGel Agarose	Bio 101, Vista, CA
Bisacrylamide	Bio-Rad Laboratories, Hercules, CA
Bovine serum albumin	Sigma Chemical Company, St. Louis, MO
Bromophenol Blue	Sigma Chemical Company, St. Louis, MO
Calcium Chloride	Fisher Scientific Co., Fairlawn, NJ
Cesium chloride	Sigma Chemical Company, St. Louis, MO
Chloroform	Baker Chemical, Phillipsburg, NJ

Cellulose, non ionic powder	Bio-Rad Laboratories, Hercules, CA
Citric acid (anhydrous)	Fisher Scientific Co., Fairlawn, NJ
dGTP, dATP, dTTP, dCTP	U. S. Biochemicals, Cleveland, OH
DEPC	Sigma Chemical Company, St. Louis, MO
Dimethylformamide	Fisher Scientific Co., Fairlawn, NJ
Disodium EDTA dihydrate	Fisher Scientific Co., Fairlawn, NJ
Ethanol	Fisher Scientific Co., Fairlawn, NJ
Ethidium Bromide	Sigma Chemical Company, St. Louis, MO
Ferric Chloride	Acros Organics, New Jersey, USA
Formaldehyde	Sigma Chemical Company, St. Louis, MO
Formamide	Baker Chemical, Phillipsburg, NJ
Glycine	Baker Chemical, Phillipsburg, NJ
Goat Anti-Rabbit IgG-AP Conjugate	BRL, Gaithersburg, MD
Glycerol	Fisher Scientific Co., Fairlawn, NJ
Herring sperm DNA	BRL, Gaithersburg, MD
Hydrochloric acid (30%)	Baker Chemical, Phillipsburg, NJ
IPTG	Sigma Chemical Company, St. Louis, MO
Isopropanol	Baker Chemical, Phillipsburg, NJ
Manganous chloride tetrahydrate	Baker Chemical, Phillipsburg, NJ
Magnesium chloride hexahydrate	Fisher Scientific Co., Fairlawn, NJ
Magnesium sulfate	Sigma Chemical Company, St. Louis, MO
β-mercaptoethanol	Sigma Chemical Company, St. Louis, MO
MOPS	Sigma Chemical Company, St. Louis, MO

NBT/BCIP 1 Step™ Solution	Pierce Biotechnology Inc., Rockford, IL
Paraffin oil	Baker Chemical, Phillipsburg, NJ
Phenol	Fisher Scientific Co., Fairlawn, NJ
Phosphoric acid	Fisher Scientific Co., Fairlawn, NJ
Polyethylene glycol-8000	Fisher Scientific Co., Fairlawn, NJ
Potassium chloride	Baker Chemical, Phillipsburg, NJ
Potassium hydroxide	Fisher Scientific Co., Fairlawn, NJ
Potassium phosphate, monobasic	Fisher Scientific Co., Fairlawn, NJ
Potassium phosphate, dibasic	Fisher Scientific Co., Fairlawn, NJ
Sinapinic acid	Sigma Chemical Company, St. Louis, MO
Sodium acetate trihydrate	Baker Chemical, Phillipsburg, NJ
Sodium chloride	Fisher Scientific Co., Fairlawn, NJ
Sodium citrate, anhydrous	Fisher Scientific Co., Fairlawn, NJ
Sodium dodecyl sulfate	Fisher Scientific Co., Fairlawn, NJ
Sodium hydroxide	Fisher Scientific Co., Fairlawn, NJ
Sodium phosphate, monobasic,monohydrate	EM Science, Gibbstown, NJ
Sodium phosphate, dibasic,12-hydrate	Baker Chemical, Phillipsburg, NJ
Sodium sulfate	Fisher Scientific Co., Fairlawn, NJ
Sucrose	Fisher Scientific Co., Fairlawn, NJ
TEMED	Sigma Chemical Company, St. Louis, MO
Tricine	Sigma Chemical Company, St. Louis, MO
Tris base	Fisher Scientific Co., Fairlawn, NJ
tRNA	Sigma Chemical Company, St. Louis, MO

Tween-20	Sigma Chemical Company, St. Louis, MO
X-Gal	Sigma Chemical Company, St. Louis, MO
Xylene cyanol FF	Baker Chemical, Phillipsburg, NJ
Zinc Chloride	Sigma Chemical Company, St. Louis, MO

Part I-2. Strains, Enzymes and kits

Alkaline Phosphatase, calf intestinal	New England Biolabs, Boston, MA
DyNAzyme™ EXT	MJ Research, Waltham, MA
Eppendorf Perfectprep® Plasmid Mini kit	Eppendorf, Westbury, NY
Geneclean™ DNA purification	Bio 101, Vista, CA
Klenow DNA polymerase	Fermentas Inc., Hanover, MD
Lysozyme	Sigma Chemical Company, St. Louis, MO
Mutanolysin	Sigma Chemical Company, St. Louis, MO
One step RT-PCR kit	Qiagen Inc., Valencia, CA
Proteinase K	Sigma Chemical Company, St. Louis, MO
Restriction enzymes	New England Biolabs, Boston, MA
Restriction enzymes	Promega Corporation, Madison, WI
Restriction enzymes	Fermentas Inc., Hanover, MD
Ribonuclease A	Sigma Chemical Company, St. Louis, MO
RNase free DNase set	Qiagen Inc., Valencia, CA
RNeasy® Mini Kit	Qiagen Inc., Valencia, CA
T_4 DNA ligase	Fermentas Inc., Hanover, MD
T_4 polynucleotide kinase	Fermentas Inc., Hanover, MD

TA Cloning® kit	Invitrogen, Carlsbad, CA
Taq DNA polymerase	Fermentas Inc., Hanover, MD
100 bp DNA ladder	Fermentas Inc., Hanover, MD

Part I-3. Media

Bacto Agar	Difco, Detroit, MI
Bacto casamino acids	Difco, Detroit, MI
Bacto lactose	Difco, Detroit, MI
Bacto tryptone	Difco, Detroit, MI
Yeast extract	Becton Dickinson, Sparks, MD
M17 media	Difco, Detroit, MI
NZY Broth	Gibco Laboratories, Madison, WI
SOC media	Invitrogen, Carlsbad, CA
Penassay broth (PAB) antibiotic medium 3	Difco, Detroit, MI

Part I-4. Antibiotics, oligonucleotides

Ampicillin	U. S. Biochemicals, Cleveland, OH
Chloramphenicol	Sigma Chemical Company, St. Louis, MO
Erythromycin	Sigma Chemical Company, St. Louis, MO
Oligonucleotides	Sigma/Genosys, St. Louis, MO

Part I-5. Apparatus

Aerosol spray unit	Sigma Chemical Company, St. Louis, MO

Air and water incubating shakers	New Brunswick Scientific, New Brunswick
Chromatography columns	BIO-RAD Laboratories, Hercules, CA
Centrifuge, Avanti J25-I	Beckman, Irvine, CA
Centrifuge Bottles	VWR Scientific, Baltimore MD
Corex glass centrifuge tubes, 30 ml	DuPont
Dialysis tubing	VWR Scientific, Baltimore MD
Digital photo documentation system	Eastman Kodak, Rochester, NY
Electrophoretic transfer cell	BIO-RAD Laboratories, Hercules, CA
Electroporation cuvette Plus™, Model 610	BTX Inc, San Diego, CA
Electroporation system, Model ECM 600	BTX Inc, San Diego, CA
ESI-Mass Spectrometer (LCQ)	Finnigan, San Jose, CA
HPLC column, Zorbax	Agilent Technologies, Palo Alto, CA
HPLC column, Microsrob	Rainin Instrument Co., Inc, Woburn, MA
HPLC	Hewlett-Packard, Avondale, PA
Immobilon™-NC nitrocellulose membrane	Millipore Inc, Bedford, MA
Eppendorf Microcentrifuge	VWR Scientific, Baltimore MD
Gel electrophoresis horizontal units	BRL, Gaithersburg, MD
Gel electrophoresis horizontal units	Owl scientific
Gel electrophoresis vertical units	BioRad Laboratories, Hercules, CA
Gel electrophoresis vertical unit glass plates	VWR Scientific, Baltimore, MD
Hybridization bags	Kapak, St. Louis, MI
Hybridization bag sealer (Kapak)	VWR Scientific, Baltimore, MD
Klett-Somerson photoelectric colorimeter	Klett Manufacturing, Inc., New York, NJ

MALDI-TOF MS	Bruker Instruments, Manning Park, MA
MALDI-TOF MS sample stages	Bruker Instruments, Manning Park, MA
Microscope	Nikon, Japan
Molecular biology disposable supplies	VWR Scientific, Baltimore, MD
Nitrocellulose membranes	Millipore, Bedford, MA
Nitrocellulose membranes	Schleicher and Schuell, Keene, NH
NanoESI Capillaries, PicoTip™ Emitter, EconoTip™	New Objective, www.newobjective .com
Orbital platform shaker	Hoefer Scientific, San Francisco, CA
pH meter, Accumet AB15	VWR Scientific, Baltimore MD
Phosphor Imager (Storm™)	Molecular Dynamics, Sunnyvale, CA
Phosphor Imager intensifying screen	Molecular Dynamics, Sunnyvale, CA
Pipetteman, P20, P200, P1000	Rainin Instruments company Inc.
Power Supply, Model 494	ISCO, Lincoln, NE
Power Supply, Model EC105	E-C Apparatus, St. Petersburg, FL
SpeedVac	Savant Instruments, Farmingdale, NY
Syringes, 10cc, 20cc, 60cc	VWR Scientific, Baltimore, MD
Syringe Filters	VWR Scientific, Baltimore, MD
Syringe needles 18G11/2, 20G1, 27G11/2	VWR Scientific, Baltimore, MD
Toyopearl® Butyl 650M HIC resin	Tosohaas, Montgomeryville, PA
Ultracentrifuge, Preparative, Model L3-50	Beckman, Irvine, CA
UV transluminator	Fotodyne, Inc., New Berlin, WI
UV-VIS Spectrophotometer	Hewlett-Packard, Avondale, PA

XAR-5 X-ray films	VWR Scientific, Baltimore, MD
Water bath, constant temperature	Precision Scientific, Chicago IL
Whatmann Filter Paper (1MM and 3MM)	VWR Scientific, Baltimore, MD
X-ray film developer	Konica, Newark, NJ

Part II. Methods

Part II-1. Bacterial strains, cloning vectors, and oligonucletide sequences.

Strain name/ plasmid name	**Description**	**Source of reference**
B. subtilis BE1010	*B. subtilis* 168 that carries a deletion in chromosomal genes encoding subtilisin and neutral protease.	(63)
B. subtilis 168 BR151	Wild-type *B. subtilis* 168 (*trp*C2, *met*B10, *lys*-3)	Bacillus Genetics Stock Center
B. subtilis ermΔ sunA	*B. subtilis* BR151 in which, *sunA*, sublancin structural has been replaced with *erm* gene. Non-sublancin producer.	(62)
B. subtilis SYA2NE	*B.subtilis* SYNcat in which the *cat* gene has been replaced with the erm gene and A2 promoter (see Figure 26).	This work
B. subtilis SYcat	*B.subtilis* BR151 that was transformed with pSYcat. Integrated the *sac*R, *transposase* homology regions and the *cat* gene (see Figure 6).	This work
B. subtilis SY2cat	*B. subtilis* BR151 that was tansformed with pSY2cat. Integrated the nisin gene cluster (see Figure 10).	This work
B. subtilis SY20N	*B.subtilis* BE1010 that was transformed with *B. subtilis* SYNEC chromosomal DNA. Integrated the nisin gene cluster with double crossover.	This work
B. subtilis SYNcat	*B. subtilis* ermΔsunA in which the *erm* gene has been replaced wit the *cat* gene and nisin gene cluster (see Figure 15).	This work
B.subtilis SYNEC	*B. subtilis* SYcat that was transformed with pSYNE. Integrated the *erm* gene and nisin gene cluster by single cross over (see Figure 18).	This work

Lactococcus lactis 11454	Natural nisin-producing *L. lactis* strain.	(7)
E. coli JM101	Standard *E. coli* cloning strain.	BRL
E. coli INVαF′	An *E. coli* cloning strain included with the TA Cloning Kit.	Invitrogen
pTZ18R	Standard cloning vector.	USB
pCR2.1	Cloning vector included with the TA cloning kit.	Invitrogen
pLPv*cat*	*sunA* gene mutagenesis cassette vector.	(62)
pSYcat	pTZ18R derivative, contains *amy*E, *transposase*, *sac*R homology regions (see Figure 5).	This work
pGh9:ISS1	Plasmid that can replicate in *E. coli*, *B. subtilis*, *L. lactis*. Contains a temperature sensitive origin of replication.	(52)
pGH9USR	pGh9:ISS1 that contains the upstream *sac*R homology region (see Figure 7).	This work
pCR2.1nisin	pCR2.1 vector that contains nisin gene cluster (see Figure 10).	This work
pSY2cat	pCR2.1nisin that was fused with pEZamycat (see Figure 10).	This work
pEZamycat	pEZcat that contains *amy*L homology region (see Figure 10).	This work
pEZcat	Plasmid which contains mpc restriction sites at either end of *cat* gene.	(36)
pEZerm	Plasmid which contains mpc restriction sites at either end of *cat* gene.	(19)
pSYNcat	pLPvcat which contains nisin gene cluster (see Figure 15).	This work
pSYNE	pCR2.1 that contains the *erm* gene and nisin gene cluster (see Figure 18).	This work
pSYA2NE	pSYNcat in which the *yol*F gene has been replaced with A2 promoter and upstream *nis*A homology region (see Figure 24).	This work
pLPerm	pLPvcat in which the *cat* gene has been replaced with the *erm* gene (see Figure 24).	This work
pA2trpA	Plasmid containing the constitutive phage promoter which functions in *B. subtilis* 168 (see Figure 24).	Personal gift from Dr. George Spiegelman, at the University of British Columbia, in Vancouver.

Table 1. Bacterial Strains and plasmids.

Part II-2. Frequently used stock solutions, buffers, enzymes and growth media.

2-1. Solutions

Ammonium acetate: A 7.5 M stock of ammonium acetate was prepared by dissolving 57.8 g of NH_4OAc in 70 ml ddH_2O. The solution was adjusted to 100 ml with ddH_2O. The solution was autoclaved 20 min at 120 °C and stored at room temperature.

Antibiotic stock solutions: A stock solution of 100 mg/ml ampicillin was prepared by dissolving 100 mg of sodium ampicillin in 1 ml of sterile ddH_2O and stored at –20 °C. A working concentration of 100 μg/ml was used. The solution was prepared fresh every month. A chloramphenicol stock solution (10 mg/ml) was prepared by dissolving 100 mg of chloramphenicol in 10 ml of 95% ethanol and stored at –20 °C. A working concentration of 10 μg/ml was used for *B. subtilis* strains and 5 μg/ml used for *E. coli* strains. A 10 mg/ml stock of erythromycin was prepared by dissolving 100 mg of erythromycin in 10 ml of 95% ethanol. The solution was stored in a lightproof bottle at –20 °C. A working concentration of 10 μg/ml was used for *B. subtilis* strains and 500 μg/ml used for *E. coli* strains.

Calcium chloride: A 1 M stock solution of calcium chloride was prepared by dissolving 18.4 g of $CaCl_2.2H_2O$ in 125 ml ddH_2O. 50 mM $CaCl_2$ was prepared by dissolving 7.35 g of $CaCl_2.2H_2O$ in 1 liter of ddH_2O and used for *E. coli* transformations. The solutions were autoclaved for 20 min at 120 °C and stored at 4 °C.

Denhardt's solution: A 100x stock of Denhardt's solution was prepared by dissolving 20 g of Ficoll type 400, 20 g of polyvinylpyrolidone, 20 g of bovine serum albumin fraction V in 800 ml ddH_2O. The final volume was adjusted to 1 L. The stock

was aliquoted into 50 ml polypropylene tubes, and stored at –20 °C. A single aliquot was kept at 4 °C for use in hybridizations.

EDTA: 0.5 M EDTA was prepared by adding 93.05 g of $Na_2EDTA2H_2O$ to 300 ml ddH_2O. The pH was adjusted to 8.0 with 1 N NaOH (EDTA will not go into the solution until pH is around 7.0). The volume was then adjusted to 500 ml with ddH_2O. The solution was autoclaved for 20 min at 121 °C and stored at room temperature.

Ethidium Bromide: A 10 mg/ml solution was prepared by dissolving 0.1 g of ethidium bromide in 10 ml ddH_2O. The solution was stored in a lightproof bottle at room temperature.

Herring sperm DNA: A 2 mg/ml stock was prepared by dissolving Herring sperm DNA in 50 mM Tris-HCl, pH 7.6, and 0.4 M NaCl. The DNA was sheared by using a French press at 20,000 psi. The DNA was then extracted twice with 1 volume of 1:1 phenol-chloroform and once with 1 volume of chloroform. The DNA was precipitated by using 2 volumes of 95% ethanol and then resuspended in TE to a final concentration of 2 mg/ml. The stock was stored at –20 °C.

IPTG: A 0.1 M stock solution was made by dissolving 250 mg of isopropyl β-thiogalactoside (IPTG) in 10.5 ml ddH_2O. The solution was sterilized by filtering through 0.2 μm filter and stored in 0.5 ml aliquots at –20 °C. A volume of 20 μl was used per plate.

Nisin: A 10 mg/ml stock solution was prepared by dissolving 10 mg of nisin in 1 ml of 0.05 % TFA.

Phenol, buffer saturated: A buffer saturated phenol was used in all experiments and was prepared by adding the following reagents to a bottle containing 500 g of Fisher-

loose crystals phenol (A92-500): 110 ml of 2 M Tris-HCL, pH 7.6, 143 ml of deionized, distilled water, 27.5 ml m-cresol, 1.1 ml 2-mercaptoethanol, and 550 mg 8-hydroxyquinoline. The reagents were combined in the phenol bottle and placed in a 37 °C water bath. The bottle was swirled occasionally until the two phases were liquid. The phases were mixed thoroughly overnight. The phases were then allowed to separate over several hours. The saturated phenol was stored in brown phenol bottle and was stable for up to one year at room temperature.

Sodium acetate (NaOAc): A 1 M NaOAc pH 4.0 solution, used for HIC column purification, was prepared by adding 57.2 ml glacial acetic acid to 400 ml ddH_2O. The pH was adjusted to 4.0 with 10 N NaOH. The volume was then adjusted to 500 ml with ddH_2O.

3M NaOAc was prepared by dissolving 204 g sodium acetate trihydrate in 300 ml ddH_2O. The pH was adjusted to 6.0 with glacial acetic acid, and the volume brought up to 0.5 L with ddH_2O. The solution was autoclaved for 20 min at 120 °C and stored at room temperature.

Sodium chloride: A 5 M stock of sodium chloride, used for HIC column purification, was prepared by dissolving 146.1 g into ~480 ml of ddH_2O. The volume was then adjusted to 500 ml with ddH_2O.

Sodium dodecyl sulfate (SDS): 20% (w/v) SDS was prepared by adding 400 ml ddH_2O into the SDS bottle containing 100 g of SDS while slowly stirring. Moderate heat was applied to dissolve SDS if it was necessary. The volume was then adjusted to 500 ml and the solution was stored at room temperature.

Sodium Hydroxide: 10 N NaOH was prepared by dissolving 40.0 g NaOH in a final volume of 100 ml ddH_2O.

Standard saline citrate (SSC): A 20x SSC stock (3 M NaCl, 0.3 M sodium citrate) was prepared by dissolving 175.3 g of NaCl and 88.2 g sodium citrate in 800 ml ddH_2O. The volume was adjusted to 1 L with ddH_2O and stored at room temperature.

Tracking dye (6X): 0.25% Xylene cyanol FF, 0.25% bromophenol blue, and 30% glycerol. 0.25g of xylene cyanol FF and bromophenol blue were dissolved in 50 ml ddH_2O. 30 ml of glycerol was added to the mixture and the volume was adjusted to 100 ml. The solution was kept at 4 °C.

Tris-HCl: 2 M stocks of Tris-HCl were prepared by dissolving 121.11 g of Tris Base in 300 ml ddH_2O and adjusting the desired pH with concentrated HCl. The volume was then adjusted to 0.5 liter with ddH_2O. Solutions were then autoclaved 20 min at 120 °C and stored at room temperature.

Yeast t-RNA: A 10 mg/ml stock soltion was prepared by dissolving 10 mg in 1 ml sterile distilled H_2O.

X-Gal: A 40 mg/ml stock was prepared by dissolving 200 mg of 5-bromo-4-chloro-3-indolyl galactoside (X-Gal) in 5 ml dimethylformamide. The 0.5 ml aliquots of solution were stored in the dark at –20 °C. Any solutions that turned yellow or orange-red over time were discarded.

2-2. Buffers

TE Buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA was prepared from stocks of 2 M Tris-HCl, pH 8.0 and 500 mM EDTA, pH 8.0.

Phage Buffer: Phage buffer was prepared from the following components in ddH_2O to the indicated final concentration: 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$. The solution was autoclaved for 20 min at 120 °C and stored at room temperature.

SM buffer: SM buffer was prepared from the following components in ddH_2O to the indicated final concentration: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin. The solution was autoclaved for 20 min at 120 °C and stored at room temperature.

Tris Acetate-EDTA (TAE): A 50x TAE electrophoresis buffer was prepared from the following components: 2 M Tris-OAc, 0.01 M EDTA. The stock was prepared by adding 242 g Tris base, 57.1 ml glacial acetic acid, 18.6 g $Na_2EDTA.2H_2O$ to 800 ml ddH_2O and pH was adjusted to 7.2. The volume was adjusted to 1 liter.

2-3. Solutions for peptide purification

Hydrophobic interaction column (HIC) equilibration/wash buffer: 50 mM NaOAc pH 4.0, 1 M NaCl. The solution was prepared by adding 50 ml of 1M NaOAc pH 4.0 and 200 ml of 5 M NaCl to 750 ml of ddH_2O.

Hydrophobic interaction column (HIC) elution buffer: 60% Acetonitrile was prepared by adding 60 ml acetonitrile to 40 ml ddH_2O.

Non-ionic cellulose (NIC) column equilibration/wash buffer: 100 mM NaP*i* at pH 7.8 was prepared by dissolving 13.8 g of monobasic sodium phosphate in 900 ml of ddH2O. The pH was adjusted to 7.8 by 10 M NaOH and the final volume was brought up to 1 liter.

Non-ionic cellulose (NIC) column elution buffer: 5.4 M HOAc was prepared by adding 155 ml of glacial acetic acid into 345 ml of ddH_2O.

2-4. Solutions for RNA work.

Formaldehyde loading dye: 10x formaldehyde loading dye was prepared from the following components in DEPC-ddH_2O to the indicated final concentration: 50% glycerol, 1mM EDTA, 0.25 % bromophenol blue, 0.25% xylene cyanol.

MOPS buffer: 10X stock was prepared by mixing 41.8 g MOPS, 16.6 ml of 3M NaAc (pH 7.0) and 20 ml of 0.5M EDTA (pH 8.0) in 800 ml of DEPC-ddH2O. The volume was adjusted to 1 liter. The solution was then filter sterilized and stored in dark (or wrapped with aluminum foil).

Pre-mix: Pre-mix was prepared by mixing 50 μl of 10X MOPS and 87 μl of 37% formaldehyde (buffered) and 250 μl of deionized formamide. Pre-mix was added to RNA in such a way that the amount if premix added is 2x volume of RNA.

2-5. Solutions for SDS-PAGE electrophoresis.

Acrylamide/bisacrylamide: A 49.5 % acrylamide, 3% bisacrylamide stock solution was prepared by dissolving 48 g of acrylamide and 1.5 g of bisacrylamide in 75 ml ddH_2O. The volume was then adjusted to 100 ml with ddH_2O. The solution was filter sterilized with 0.45-μm filter and stored at room temperature in a light proof bottle.

Ammonium persulfate: A 10% stock solution was prepared by dissolving 0.5 g in 5 ml ddH_2O. The solution was stored at 4 °C.

Anode buffer: A 5X stock of anode buffer (1 M TrisHCl, pH 8.9) was prepared by dissolving 121.11 g of tris base in in 750 ml of ddH_2O. The pH was adjusted to 8.9

with HCl. The volume of the solution was brought up to 1 liter with ddH_2O. The solution was stored at room temperature.

Cathode buffer: A 5X stock of cathode buffer (0.5 M Tris HCl, pH 8.25, 0.5M tricine, 0.5% SDS) was prepared by dissolving 60.55 g tris base, 89.6 g of tricine and 5 g of sodium dodecyl sulfate in 750 ml of ddH_2O. The volume was then adjusted to 1 liter. The solution was stored at room temperature. The pH does not need to be adjusted.

Gel buffer: A 5X gel buffer (3 M Tris, pH 8.45, 0.3% SDS) was prepared by dissolving 36.33 g of tris base and 0.3 g of sodium dodecyl sulfate in 70 ml of ddH_2O. The pH was adjusted to 8.45 with HCl and then volume was brought up to 100 ml with ddH_2O.

SDS-PAGE gel loading buffer: A 2X loading buffer was prepared by adding 4 g of SDS, 12 g of anhydrous glycerol, 5 ml of 1 M TrisHCl pH 6.8, 10 g of bromophenol blue, and 4 ml of β-mercaptoethanol to 25 ml of ddH_2O. The volume was then adjusted to 50 ml. The solution was stored at room temperature.

2-6. Solutions for western blotting

Transfer buffer: 25 mM Tris, 192 mM glycine, and 20 % methanol. The solution was prepared by dissolving 12.11 g of tris base and 57.65 g of glycine in 2 liter of ddH_2O. 800 ml of methanol was added and then the volume was adjusted to 4 liters with ddH_2O. The buffer was stored at room temperature.

TBST: 50 mM TrisHCl pH 7.4, 150 mM NaCl, 0.05 % Tween-20. The solution was prepared by dissolving 6.05 g of tris base and 8.77 g of NaCl in 800 ml of ddH_2O. 500 μl of Tween-20 was added and stirred thoroughly. The pH was adjusted to 7.4 with HCl and then the volume was brought up to 1 liter.

Blocking buffer: 1 % BSA in TBST was prepared by dissolving 0.5 g of BSA in 50 ml of TBST.

2-7. Enzymes

DNAase I: A 1 mg/ml stock solution was prepared by dissolving the DNAase I in 50 mM Tris-HCl pH 7.6, and 50% glycerol and stored at –20 °C.

Lysozyme: A 10 mg/ml stock solution was prepared by dissolving 10 mg of lysozyme in 1 ml of sterile ddH_2O and stored at –20 °C.

Proteinase K: A 10 mg/ml stock solution was prepared by dissolving 10 mg of proteinase K in 1 ml of sterile ddH_2O and stored at –20 °C.

Ribonuclease A (RNAase): A 10 mg/ml was prepared by dissolving 10 mg of RNAase in 1 ml of 20 mM sodium acetate, pH 6.0. The solution was boiled for 15 min in a water bath and slowly cooled to room temperature. 0.5 ml aliquots were stored at –20 °C. A working concentration of 10 μg/ml was used unless otherwise was indicated.

Mutanolysin: A 10 units/μl stock of mutanolysin was prepared by dissolving 1,000 units of mutanolysin in 100 μl of ddH_2O and stored at –20 °C.

2-8. Growth Media

Culture media were sterilized by autoclaving for 20 min at 120 °C and stored at room temperature unless otherwise indicated. To make plates, 15 g of Bacto-agar was added per liter of media before autoclaving. The appropriate antibiotic was added to the media only after it had cooled to 50 °C.

Luria-Bertani (LB) Medium: 10 g of Bacto tryptone, 5 g of yeast extract, and 5 g of NaCl were dissolved in the final volume of 1 liter ddH_2O.

Penassay Broth (PAB): The media was prepared by dissolving 17.5 g of antibiotic medium No. 3 in final volume of 1 liter ddH_2O.

M17 media: M17 media was prepared by dissolving 37.25 g of dehydrated M17 broth into 800 ml ddH_2O. The volume was then adjusted to 950 ml with ddH_2O, and autoclaved. This media was stored at 4 °C. 50 ml of the sterile 10% glucose was added to the 950 ml of M17 just before inoculation with the bacteria. 10% (w/v) glucose solution was made by dissolving 10 g of glucose in the final volume of 100 ml ddH_2O. The solution was then sterilized by filtering through 0.2 μm filter, and stored at 4 °C.

Medium A: This medium was originally designed for the production of subtilin in *B. subtilis* 6633 (25). It was later modified for production of subtilin and sublancin in 168 derivatives (60). 0.5 liter of this medium was prepared just before inoculation with the *B. subtilis* strain by mixing 390 ml of solution I, 100 ml of solution II and 5 ml of solution III and IV at room temperature. Solutions I and II were autoclaved and stored at 4 °C. Solutions III and IV were both sterilized by filtering through 0.2 μm filter and stored at 4 °C.

Solution I consists of 2% sucrose and 0.2% PAB and was prepared by dissolving 10 g of sucrose and 1 g of PAB in a final volume of 390 ml of ddH_2O in a 2 L baffled flask. Solution I was always prepared fresh the day before inoculation.

Solution II was prepared as a 5X stock by dissolving 35.1 g of citric acid (anhydrous), 12.6 g $(NH_4)_2HPO_4$, 12 g of Na_2SO_4, and 15 g of yeast extract into 500 ml ddH_2O. The pH was adjusted to 6.9 with concentrated ammonium hydroxide followed by adjustment of the volume to 600 ml with ddH_2O.

Solution III was prepared as a 100x stock by dissolving 7.62 g KCl, 4.18 g $MgCl_2 \cdot 6H_2O$, 0.543 g $MnCl_2.4H_20$, and 0.11 g $ZnCl_2$ in 100 ml ddH_20.

Solution IV was prepared as a 100x stock by dissolving 0.25 g of $FeCl_3$ (anhydrous) in 100 ml ddH_2O.

NZCYM Medium: 21 mg of NZY broth (composed of 10 g of enzymatic casein hydrolysate, 5 g of NaCl, 5 g of bacto-yeast extract, 0.94 g of $MgSO_4$) and 1 g of casamino acids were dissolved in 950 ml of ddH_2O. The pH was adjusted to 7.0 with NaOH. Then the volume was brought up to 1 L by adding ddH_2O. The solution was autoclaved 20 min at 120 °C.

SOC Medium: 20 g of bacto-tryptone, 5 g of bacto-yeast extract and 0.5 g of NaCl was dissolved in 950 ml of ddH_2O. Then 10 ml of a 250 mM KCl solution was added. The volume was adjusted to 1 l and autoclaved 20 min at 120 °C. Just before use, 5 ml sterile solution of 2 M Mg_2Cl and 20 ml of a sterile1 M solution of glucose were added.

Top agarose: 1.0 g of Bacto-tryptone, 0.8 g NaCl and 0.6 g agarose were mixed in 100 ml ddH_2O. The solution was autoclaved 20 min at 120 °C and stored at room temperature.

Part II-3. Maintenance and growth conditions.

Strains of *E. coli* were stored on LB plates at 4 °C for up to 2 months. Appropriate antibiotic was added to the plate if the strain had been transformed with a plasmid carrying an antibiotic selection gene. Cultures were grown in LB media at 37 °C with shaking at 250 rpm.

Strains of *Bacillus subtilis* were kept on PAB plates with no or appropriate antibiotic added at room temperature and a parafilm wrapped around the plates. Cultures were grown in PAB media at 37 °C with shaking at 250-300 rpm.

Strains of *Lactococcus lactis* were maintained on M17 plates with no or appropriate antibiotic added at 4 °C for up to one month. Cells were cultured anaerobically at 32 °C in M17 media.

For long-term storage, each bacterial strain was stored at –80 °C in 15-20% glycerol mixed with the medium. To prepare a glycerol stock, a single colony was streaked heavily onto a fresh plate, with the appropriate antibiotic added if necessary, and incubated overnight. 1 ml of sterile 50% glycerol solution in ddH_2O was added to 2 ml of the appropriate liquid media. No antibiotic was added to this mixture. This solution was poured onto the plate with the freshly grown cells, which were then gently resuspended by pipeting. A 1 ml aliquot was then transferred to a labeled 1.5 ml cryotube, and frozen in a dry ice/ethanol bath. The tubes were immediately placed at –80 °C in a labelled archive box. All information concerning the particular strain including location of the archived sample was entered into the archive books.

Part II-4. Plasmid DNA isolation.

Depending on the subsequent analysis steps, plasmids were isolated from strains of *E. coli* by using either one of two protocols. Eppendorf Perfectprep® kit was used for rapid isolation of plasmid DNA for restriction endonuclease screening enzyme analysis of the clones or transformation into *B. subtilis* host strain. The DNA isolated this way was obtained quickly and in a highly pure form. Plasmid DNA was isolated by the rapid

alkaline extraction method when large amounts of DNA were necessary for the subsequent analysis steps. These methods are described below.

4-1. Purification of plasmid DNA using the rapid alkaline extraction method.

The procedure followed was modified from the method initially described by Brinboim and Doly (1979). A single colony of the *E. coli* strain from a LB plate, containing the appropriate antibiotic, was inoculated into 3-5 ml of LB media containing the appropriate antibiotic and incubated at 37 °C overnight in an orbital shaker. 1.5 ml aliquot of the culture was then transferred to a 1.7 ml microfuge tube, and centrifuged at 14,000 rpm for 1 min in a microfuge. The media was aspirated and the cell pellet was resuspended in 200 μl of solution I (50 mM glucose, 25 mM TrisHCl pH 8.0, 10 mM EDTA), with vigorous vortexing. The suspension was incubated for 5 min at room temperature. Then 400 μl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and mixed by gently inverting several times. The solution had become transparent which was an indication of cell lysis. The lysate was incubated on ice for 5 min. Then 300 ml of solution III (7.5 M ammonium acetate, pH 7.2) was added and mixed by inverting several times. The sample was incubated on ice for 10 min and then centrifuged at 14,000 rpm, for 5 min to pellet the cell debris. The supernatant was then transferred to a new microfuge tube. At this point the sample could be treated with RNAase if desired. RNA might sometimes interefere with the restriction digests by decreasing the visibility of low moleculer weight DNA bands. In this case, 5 μL of 10 mg/ml Rnase I was added to the sample and incubated at 37 °C for 30 min.

A series of phenol/chloroform and chloroform extractions were then performed. First an equal volume of (1:1) phenol:chloroform was added (For 900 μl of sample, 450

μl phenol and 450 μl of chloroform was added) followed by vortexing, and centrifuging at 14,000 rpm for 2 min. The top aqueous layer was transferred to a new tube and extracted twice with equal volume of chloroform. The plasmid DNA was then precipitated by adding 0.6 volumes of isopropanol followed by mixing and incubating for 10 min on ice. Precipitated DNA was then recovered by centrifugation at 14,000 rpm, 10 min. The liquid was decanted and the DNA pellet was washed by the addition of 1 ml ice-cold 70% ethanol. Then the sample was centrifuged at 14,000 rpm, 2 min and the wash was aspirated. The DNA was dried in the speed vacuum for 5-10 min. The pellet was then resuspended in 20-50 μl of TE pH 8.0, with vortexing. The purified plasmid DNA was stored at –20 °C.

This procedure could be adapted to isolate a stock supply of plasmid DNA by scaling up the volumes appropriately. For the medium scale plasmid isolation, 50 ml culture was grown overnight. The cells were harvested by centrifugation at 4,000 rpm for 5 min in a SS-33 rotor at room temperature. Solution volumes were increased to 3 ml for solution I, 6 ml for solution II, and 4.5 ml for solution III. The same temperature and incubation times were used. The centrifugation to pellet the cell debris was then carried away in 30 ml corex tubes for 10 min at 10,000 rpm in a SS-34 rotor. The volumes of extraction agents were also scaled up accordingly. After the final wash, the tube was air-dried for 1-2 hr. Then the DNA was resuspended 100-300 μl of TE.

4-2. Purification of plasmid DNA using the Eppendorf Perfectprep® Plasmid Mini kit.

The manufacturer's method was exactly followed for purification of plasmid DNA. A single colony of the *E. coli* strain from an LB plate was used to inoculate 3-5 ml

of LB medium with the appropriate antibiotic and incubated in an orbital shaker at 37 °C for overnight or 8-10 hr. 1.5 ml of the culture was then transferred to a 1.7 ml microfuge tube, and centrifuged at 14,000 rpm for 1 min. The supernatant was aspirated and the pellet was resuspended in 100 μl of solution I by vortexing, followed with the addition of 100 μl solution II and sample was mixed with gentle inversions. Finally, 100 μl of solution III was added and mixed thoroughly by vigourous inversions of the tube. The tube was centrifuged at 14,000 rpm for 1 min. The supernatant was transferred to a PERFECTprep spin column in a collection tube followed by the addition of 450 μl of DNA binding matrix. The solution was mixed well and briefly centrifuged. The DNA bound matrix was washed by 400 μl of diluted purification solution. The tube was briefly centrifuged and spin column was transferred to a new tube and centrifuged again. Finally the DNA was eluted from the matrix by addition of 50 μl of elution buffer. The purified DNA was stored at a –20 °C.

Part II-5. Isolation of chrosomal DNA from bacteria.

5-1. Isolation of chrosomal DNA from *B. subtilis* strains.

A single colony of *B. subtilis* was used to inoculate 50 ml PAB with no or appropriate antibiotic added. This was grown for overnight at 37 °C with shaking. The cells were precipitated by centrifuging at 4,000 rpm for 5 min. The supernatant was decanted. The cell pellet was resuspended in 10 ml of Solution I (10 mg lysozyme, 25 mM Tris-HCl, pH 8.0, 50 mM glucose and 10 mM EDTA) by vortexing. The cell suspension was incubated at 37 °C for 20 min without shaking followed by addition of

0.25 ml 20% SDS and 0.25 ml of 10 mg/ml proteinase K. The solution was mixed by gentle inversions and incubated at 37 °C for 15 min or overnight without shaking. Overnight incubation slightly increases the yield of DNA. Proteins were extracted by adding 1:1 ratio of phenol/chloroform (5 ml phenol and 5 ml chloroform), which was mixed by gently rocking for 10 min followed by centrifugation for 10 min at 10,000 rpm at 4 °C in corex tubes. Aqueous (upper) layer was transferred to a new 50 ml polypropylene tube and the phenol/chloroform extraction was repeated once more. The aqueous upper layer was again transferred to a new tube and extracted twice with equal volume of chloroform. A pasteur pipette with a broken tip was used to collect the aqueous phase in order not to disturb white protein precipitate at the interface of the organic and aqueous layers. The mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. To precipitate the DNA 0.1 volumes of 3 M NaOAc, pH 6.0 and 2 volumes of ice-cold 95% ethanol were added to the aqueous phase, gently mixed and the tube was put at –80 °C for 10 min. The sample was centrifuged at 10,000 rpm for 10 min at 4 °C to pellet the DNA. The ethanol was decanted and wash step was repeated with 2 ml of 80% ethanol. The DNA pellet was air-dried. The DNA was then resuspended in 250-500 μL of TE and stored at –20 °C. At this point the sample could be treated with RNAase if a cleaner sample was required and the RNAase could then be removed by a series of phenol/chloroform and chloroform extractions. Amount of DNA was estimated by running a 2-3 μl aliquot on a 0.8 % gel or by UV spectrophotometry.

5-2. Isolation of chromosomal DNA from *L. lactis* 11454.

A single colony of *L. lactis* 11454 was used to inoculate a 50 ml of M-17 medium in a 50 ml polypropylene tube. The culture was incubated overnight without shaking at 28 °C to get the OD_{600} between 0.6-1.0. The following morning, the OD was checked and DL-threonine was added to a final concentration of 20 mM to weaken the cell walls and incubated at 32 °C for an additional 1-2 hr. The cells were then pelleted by spinning down at 4,000 rpm for 10 min at room temperature. The supernatant was decanted and the pellet was washed with 50 ml TE-wash three times by resuspending and centrifuging. TE-wash buffer consisted of 20 mM Tris-Hcl, pH 8.0 and 50 mM Na_2EDTA, pH 8.0. After the last wash, the cells were resuspended in 4 ml of the TE-wash and lysozyme (10 mg/ml) to a final concentration of 2 mg/ml followed by the addition of equal volume of 24% PEG. Then mutanolysin (10 units/μl) was added to a final concentration of 0.5 units/μl and sample was incubated at 37 °C for 2 hr. The protoplasts were collected by centrifuging at 4,000 rpm for 5min and the pellet was resuspended in 1 ml of 12% PEG buffered with TE (0.5 ml TE and 0.5 ml 24% PEG). The protoplasts were then lysed with the addition of 10 ml TE buffered 1% SDS. The mixture was rocked gently for 5 min (or until the solution had cleared). Proteinase K (20 mg/ml) was added to a final concentration of 20 μg/ml and incubated at 50 °C for 2 hr. The lysate was extracted with equal volume of chloroform, then equal volume of phenol:chloroform (1:1) and finally again with an equal volume of chloroform. Then the DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 95% ethanol to the aqueous phase. The DNA was incubated at –80 °C for 7 min and the DNA was spun down at 10,000 rpm, 4 °C, for 10 min the ethanol was decanted and the DNA pellet was air-dried. Finally, the DNA was resuspended in 500 μl of TE.

Part II-6. Restriction enzyme digestion of nucleic acids.

Restriction enzyme digestions are often performed to prepare compatible ends of the plasmid DNA and the fragment of interest for subcloning. After the fragment was cloned, 6-10 colonies were screened by diagnostic restriction enzyme digest(s) for the presence of the fragment or a unique restriction site introduced by the insert. The number of colonies to be analyzed was decided by looking at the efficiency of transformation. If the fragment had contained new restriction sites that were going to be used in the subsequent cloning steps, functioning of these sites were tested at each subcloning step.

For a typical restriction digest, 0.5-1 μg of plasmid DNA (usually 3-5 μl of purified plasmid DNA by PERFECTprep kit) or chromosomal DNA, 4 μl of the suggested 10x digestion buffer (supplied with the enzyme), 0.5 μl of enzyme (5-10 units), were used in a reaction volume of 40 μl in microfuge tube. The digestions were performed at 37 °C for an hour unless otherwise indicated by the supplier. To visualize the digests, 8 μl of 6X tracking dye was added and the photograph of the gel was taken on an UV illuminator. If further manipulations were required, the band of interest was excised from the gel and cleaned by Geneclean^{TM} kit (Part II-8). More DNA could also be digested in a single tube by increasing the reaction volume and the amount of enzyme.

Part II-7. Agarose gel electrophoresis.

Agarose gels of various concentrations were often used to resolve different sizes of DNA fragments and then specific fragments could be further purified if necessary. A 100 bp DNA ladder (Fermentas) or λ DNA digested with Eco RI and Hind III was often run along with the sample as a size standard. 1% (w/v) agarose gels in 1X TAE were used

to resolve restriction digests of DNA between 0.4-10 kb. For fragments bigger than 10 kb or chromosomal DNA, 0.8% (w/v) agarose gels were used and finally to resolve fragments smaller than 0.4 kb 3% (w/v) BioGel agarose was used.

Agarose gels were prepared according to procedures described by Maniatis *et al* (71). To make the 1% gel, 1 g of agarose was mixed with 2 ml of 50X TAE and 98 ml of ddH_2O in a 250 ml Erlenmeyer flask (Proper amounts of agarose were used for different concentrations). Agarose was dissolved by microvawing the flask until the solution had boiled. The solution was swirled or stirred to dissolve any undissolved agarose and allowed to cool to 50 °C. 3 μL of Ethidium Bromide (10 mg/ml) was then added to the flask and mixed by swirling. The agarose solution was then poured onto the mini (7 cm) or midi (13 cm) gel casts that had been taped at the open ends. A comb was inserted to create wells and the gel was allowed to solidify at room temperature. 1x TAE was used to run the gels at a constant voltage of 100 mV. Samples were run until the appropriate resolution of DNA bands was obtained and then visualized by an UV transilluminator. Gels were photographed by Kodak Digital Science DC120 Zoom Digital camera.

Part II-8. Geneclean™ purification of DNA.

This method was used for either isolation of DNA from agarose gels or from solutions containing unwanted materials such as enzymes and other chemical reagents. The protocol supplied by the manufacturer was followed and used for DNA fragments ranging from 0.2-10 kb. For purification of DNA fragments from agarose, the bands were excised from the gel and placed in a microfuge tube. Then 3 volumes of the 6 M sodium iodide buffer was added and incubated at 55 °C until the gel had melted (~5 min). For

DNA in solution, the incubation step was omitted and directly went on to the next step, which was the addition of 5 μl of Glass Milk for up to 5 μg of DNA. The suspension was mixed by vortexing and incubated on ice for 5 min with vortexing every 1-2 min. The sample was centrifuged at 14, 000 rpm for 5 sec. The supernatant was decanted and the pellet was washed twice with 500 μl of cold NEW ^{TM} solution. The pellet was dried in the speed vacuum for 3-4 min and then resuspended in 15 μl of 1X TE or ddH_2O followed by incubation at 55 °C for 5 min. The mixture was centrifuged for 1 min at 14,000 rpm. The 10 μl of supernatant containing the DNA was transferred to a new tube. To increase the yield of DNA the pellet was again resuspended in 10 μl of 1X TAE or ddH_2O and incubated and centrifuged the same way described above. The amount of DNA was estimated by running 3-4 μl on an agarose gel.

Part II-9. Subcloning.

Subcloning fragments of DNA from one type of vector to another is one of the most frequently used procedure in molecular cloning. Subcloning is a simple matter when the restriction sites at the termini of the target fragment are identical to, or compatible with, those of the new vector. However, this was not the case in many of the cloning experiments. The procedures used during the course of this work to create DNA with compatible ends were listed in this section.

9-1. Preparation of linkers by annealing of two synthetic single-stranded oligonucleotides.

A problem often faced during designing cloning strategies is the lack of appropriate restriction sites. This problem can be overcomed by cloning short (usually 20-30 bp) DNA pieces that were designed to contain appropriate restriction site(s). The oligonucleotides were designed to have complementary central region (16-20 bases) and protruding bases at the 5' ends, representing the digested version of the restriction site(s) to which the linker is going to be cloned. The oligonucleotides were chemically synthesized by Sigma-Genosys. Each oligonucleotide was resuspended in ddH_2O to a final concentration of 1 μg/ml. To anneal the two DNA strands to each other; 1 μg of each strand was added to a microfuge tube along with 2 μl of 10X NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) or 4 μl of 5X annealing buffer (50mM Tris pH 7.8, 20 mM $MgCl_2$), and ddH_2O to a final volume of 20 μl. The tube was vortexed and incubated in a boiling water bath for 10 min on a hot plate to denature the oligonucleotides. The heat was turned off and the tube was allowed to cool down to room temperature in the water bath. After this, DNA was purified by precipitating with 0.4 volume of 7.5 M ammonium acetate and ethanol and then the DNA pellet was air-dried. The DNA was then resuspended in 20 μl of ddH_2O and was ready to be used in the ligation reaction. If the oligonucleotides have tendency to make secondary interactions or dimers, then the volume of annealing mixture could be increased to 250 μl since the bigger volumes will enhance the proper complementary annealing of two strands by decreasing the chance of self-interaction.

9-2. Klenow filling-in reaction

Another way to overcome the lack of appropriate restriction site in subcloning is to blunt a restriction site with Klenow Polymerase to make the vector and insert ends

compatible. After digesting the DNA with a restriction endonuclease that generated a 5′ overhang, the DNA sample was treated with a Klenow Polymerase that fills in the flanking fragments with the complementary nucleotides. If both sites were going to be blunted, the insert could be ligated to the vector in two ways. In this case, diagnostic restriction enzyme digestions might need to be done to determine the orientation of the insert after cloning. The reaction is carried out according to protocol provided by the manufacturer of the Klenow Fragment (DNA Polymerase I large fragment). To prepare the reaction mixture the following were added in a microfuge tube; 2 μl of 10x reaction buffer (500 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$, 10 mM DTT), 0.1-4 μg of DNA, 0.5 μl of 2 mM 4dNTP mix (to the final concentration of 0.05 mM), 1-5 units of Klenow fragment, and ddH_2O to the final volume of 20 μl. The mixture was incubated at 37 °C for 10 min. The DNA was purified by using Geneclean™ DNA purification kit (Part II-8).

Part II-10. PCR of DNA fragments.

The PCR technique was often used during the course of this work to amplify fragments ranging from 500 bp to 14 kb from the appropriate templates. Software called "Primer3" (69) was used to pick PCR primers and hybridization probes that were used in this work (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). This product includes software developed by the Whitehead Institute for Biomedical Research. The oligonucleotides chemically synthesized by Sigma-Genosys. The manufacturer provided the parameters such as molecular weight, Tm, OD and nmoles of nucleotides for each sample. 250 μM stock of each primer was prepared by resuspending in ddH_2O

unless otherwise indicated and stored at –20 °C. To prepare the working stock of 2.5 μM, oligonucleotides were diluted 100 times with ddH_2O. During the sample preparation, sterilized solutions and equipments were used to prevent any contamination and gloves were worn at all times. Prior to the preparation of samples, the dNTP mix was allowed to thaw on ice. The tube was briefly vortexed, then several 20 μl aliquots were prepared in microfuge tubes and stored at –20 °C for future use. Preparing aliquots saved time as the thawing time shortened and prevented the degradation of dNTPs in the stock due to repeated freezing and thawing cycles. dNTPs were always kept on ice and placed at –20 °C immediately after usage. The thermocycler was turned on for about half an hour to allow the machine warm up.

10-1. PCR of fragments shorter than 10 kb.

The samples were prepared on ice in 0.5 ml thermowell^{TM} tubes and the reaction components were added in the following order. First, sterile ddH_2O was pipetted into the tubes to brought up the final volume to 100 μl then 10 μl of 10X *Taq* PCR buffer, 10 μl of the 25 mM $MgCl_2$ (to a final concentration of 2.5 mM), 10 μl of the forward (2.5 μM) and the reverse primer (2.5 μM) to a final concentration of 0.25 μM, 2 μl of template DNA (15 ng/μl), 1 μl of 100 mM dNTP mix (25 mM of each dNTP) to a final concentration of 1 mM and finally 0.25 μl of *Taq* polymerase (5 unit/μl) were added. Tube was vortexed briefly and spun down for 3-5 sec. 50 μl of paraffin oil was then carefully pippetted onto the samples to prevent evaporation and recondensation during the reactions. The cycles were started and when the temperature in the wells reached to 94 °C, tubes were placed into the PCR thermocycler.

Three cycles/profiles were generally used to amplify the target sequence from the template. The first step involved the initial denaturation of template DNA and primers by keeping the mixtures at 94 °C for 2 min. Then the cycler advanced to the second profile. In this step samples were denatured at 94 °C for 30 seconds and then the temperature ramped down to 50 °C and was kept there for 1.5 min to allow annealing of primers to the template DNA followed by ramping up to 72 °C for 3 min for the extension of the sequence from the primers. The second step repeated 30 times and then the cycler switched to the last profile in which the tubes were kept at 72 °C for 10 min for the final extension and then ramped down to 4 °C indefinitely. The ramp rate was set up to the machine's maximum during all the cycles. The samples were pipetted into a clean tube to get rid of the paraffin oil and 5 μl aliquots were run on a 1 % agarose gel along with a size standard. Then a ligation reaction was step up for immediate ligation with the pCR 2.1 vector (Part II-11, 11-2). *Taq* polymerase was used for the amplification because of its ability to add 3'-adenine overhangs at the ends of the nascent polynucleotide strands.

10-2. PCR of fragments longer than 10 kb.

Long range PCR was used to amplify 14 kb nisin gene cluster from *L. lactis* 11454. DyNAzyme^{TM} EXT (MJ Research) was used as an enzyme since it was optimized for long range PCR, has proofreading activity, more thermostable than *Taq* and has non-template dependent 3' adenine addition activity for TA cloning.

Two reaction master mixes were prepared on ice. To prepare the first master mix, 14.2 μl of ddH_2O, 4 μl of 10 μM forward and the reverse primer (to a final concentration of 0.8 μM each), 1 μl (100 ng) of the *L. lactis* 11454 genomic DNA and 1.44 μl of the 12.5 mM dNTP mix (to a final concentration of 360 μM) were added into one tube. To prepare the

second master mix, 17.3 μl of ddH_2O, 5 μl of the 10X Mg^{2+}-free DyNAzyme EXT buffer, 1.7 μl of 50 mM $MgCl_2$ and 1 μl of the DyNAzyme™ EXT (1 unit/μl) were mixed in another tube. The tubes were vortexed, briefly centrifuged and then combined in a 0.5 ml Thermowell™ tube. 50 μl of paraffin oil was pipetted and then placed into the thermocycle when the temperature reached to 94 °C.

In the first cycle, the tubes were denatured at 94 °C for 2 min. The second cycle composed of 3 stages. First stage was 30 seconds denaturation at 94 °C, then 30 seconds annealing at 62 °C, followed by 11.5 min extension at 70 °C. The second cycle was set to repeat 10 times. Then the machine advanced to third cycle. The first stage of the third cycle was 30 seconds denaturation at 94 °C, then 15 seconds annealing at 62 °C, followed by 12 min extension at 70 °C. The third cycle was set to repeat 16 times. In the fourth and the final stage, the extension continued for 10 more minutes at 70 °C and the temperature ramped down to 4 °C indefinitely.

The sample was pipetted into a clean tube to get rid of the paraffin oil. 5 μl aliquot of the sample was run on a 0.8 % agarose gel along with a size standard and then immediately ligated to pCR 2.1 vector (Part II-12, 12-2).

Part II-11. 5-prime dephosphorylation of vector DNA.

5-prime dephosphorylation of linearized vector significantly decreases the number of background colonies during the subcloning steps. Backgroung colonies formed when the vector circularizes by ligating to itself instead of ligating to insert. Since the ligases cannot ligate the DNA pieces without 5′ phosporyl termini, calf instestinal alkaline phosphatase (CIAP) was used to prevent the re-circularization of the vector. The protocol

suggested by the vendor was followed. For the reaction, DNA was suspended in 1x NEBuffer 3 (10 mM NaCl, 5 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM DTT, pH 7.9) to a concentration of 0.5 μg/10 μl. 0.5 units of enzyme was added per μg of DNA and incubated at 37 °C for exactly 60 min. Excess amounts of enzyme on the pipette tip was wiped with a Kimwipe since too much enzyme might result in the removal of nucleotides from the ends. After the reaction was over, the DNA was purified immediately by Geneclean™ DNA purification kit.

Part II-12. Ligation of insert and vector DNA.

12-1. Sticky and blunt end ligations

After the vector and the plasmid containing the insert were digested with the appropriate restriction enzyme(s) they were run on an agarose gel. The fragments that were going to be ligated to each other were excised from the gel and purified by Geneclean™ kit (Part II-8). To estimate the amount of purified DNA, 3-4 μl aliquot of each sample were run on a gel and ethidium bromide-DNA complexes were visualized. Best ligation efficiencies were obtained with freshly cut samples since the DNA overhangs might be degraded over time, especially in unbuffered environments. Ligation efficiencies were also increased when the insert was used in excess of the vector (usually vector:insert ratios of 1:3, 1:4 gave optimal results). Several reactions in varying amounts of vector:insert ratio were set up. For a typical reaction, vector to insert ratios of 1:3, 1:1 and 3:1 were used. The amount of vector used was usually about 100 ng and the amount of insert was adjusted acordingly. Linear vector by itself and linear vector in the presence of ligase were also set up as control reactions. If the vector had been

dephosphorylated, ligase treated vector was expected to yield no transformants since the linear vector was not suppose to recircularize.

The reaction was set up according to the vendor's protocol. For a typical ligation reaction, following components were added to a microfuge tube placed on ice: appropriate volumes of insert and vector DNA, 2 μL of 10x ligase buffer (containing 10 mM ATP), 0.5 μl (2 units for sticky ends) or 1μl (4 units for blunt ends) of T_4 DNA ligase (Fermentas), and ddH_2O to the final reaction volume of 20 μL. The solutions were thoroughly mixed and incubated at room temperature for 1 hr or overnight. Better results were achieved when the incubation was carried out overnight at room temperature especially with blunt end ligations. 2 μl of 50% (w/v) PEG 4000 solution was also added to the reaction mixture for only blunt end ligations.

12-2. Ligation of PCR amplified fragments into pCR2.1 vector.

For cloning of PCR products amplified by *Taq* polymerase, the TA Cloning® kit (Invitrogen) was used. In this kit, a linearized vector (pCR2.1) with single 3′ deoxythymidine (T) overhangs was provided. Since *Taq* polymerase has a non-template dependent activity that adds a single deoxyadenosine (A) residue to the 3′ ends of PCR product, any fragment amplified by *Taq* polymerase can be ligated to this vector. The advantage of this vector is that it does not require designing long PCR primers with restriction sites, which can sometimes complicate PCR reactions. For the ligation reactions, the protocol provided by the manufacturer was followed. To set up 10 μl of the reaction, 1 μl of 10X ligation buffer, 2 μl of linearized pCR2.1 vector (25 ng/μl), 1μl of T4 DNA ligase (4.0 units/μl), 1.5 μl of the fresh PCR product and 4.5 μl of sterile ddH_2O

was mixed in a microfuge tube. The reaction mixture was incubated at 14 °C overnight. The next morning 2 μl of this mixture was transformed into *E. coli* INVαF′ or JM101 cells.

Part II-13. Preparation of competent cells and transformation.

13-1. Transformation into competent *E. coli* JM101, JM109 strains.

E. coli JM101 and JM109 competent cells are prepared and transformed as described by Maniatis *et al* (71). *E. coli* cells were streaked out onto a LB plate from the archieve and incubated overnight at 37 °C. The plates older than several days were not used for competent cell preparation since the efficiency of the transformantions dropped significantly when the cells stored at 4 °C for prolonged times. However, once the competent cells were prepared, glycerol stocks of them can be stored at –80 °C and could be used up to 6 month without any significant drop in the competency.

A single colony of *E. coli* was used to inoculate 3-5 ml of LB in a 15 ml sterile polypropylene culture tube. The tube was shaken at 250 rpm, 37 °C overnight in an orbital shaker. The following day, 1 ml of the overnight culture was used to inoculate 50 ml of LB in a sterile 250 ml Erlenmeyer flask. The culture was grown at 37 °C with shaking for 1.5 hr or until the OD_{600} was 0.5-0.8. After the inoculation 30 ml of 50 mM $CaCl_2$ was transferred into a sterile 50 ml tube with printed graduations and chilled on ice while the culture was growing. Cells were then harvested by transferring the culture to a sterile 50 ml polypropylene tube and centrifuged at 4000 rpm for 5 min at room temperature. The supernatant were discarded and cells were resuspended in 25 ml of

sterile, chilled 50 mM $CaCl_2$ by gentle up-and-down pipetting using a motorized Pipetman. During the pipetting the tube was kept on ice as much as possible. The tube was incubated on ice for 1 hr after which the cells were pelleted by centrifugation at 4000 rpm for 5 min. The supernatant was discarded and the tube was placed on ice. The cells were resuspended in 5 ml of sterile, chilled 50 mM $CaCl_2$ very gently the same way described above. At this point the cells are competent and were usually used within 30 min. For long-term storage, sterile 50% glycerol added to a final concentration of 15% and cells were quickly frozen in dry ice ethanol bath in 1 ml aliquots at -80°C. Once thawed, the cells were used immediately

Transformation of the ligation mixtures and controls were done as follows. 200 μL of the competent cell preparation was added into the chilled ligation mixtures, which was mixed by very gentle pipetting. For transformation controls, one microfuge tube was set up with only competent cells to test the ampicillin plates, another tube was set up with a known concentration of an uncut plasmid and competent cells to check the efficiency of cells. The tubes were incubated on ice for 30 min. The samples were then heat shocked in a 42 °C water bath for exactly 2 min and placed back on ice. 0.8 ml LB was added to the each microfuge tube and incubated at 37 °C water bath for 2 hr. A 100 μL aliquot of the transformed cells were spreaded on an LB plate usually containing 100 μg of ampicillin (or appropriate antibiotic). The tubes were centrifuged at 14,000 rpm for approximately 1 min to pellet the remaining cells. 750 μl of media was discarded and the cells were resuspended in the remaining media by vortexing. The cells were spreaded on another plate. Plates incubated at 37 °C overnight. If the colonies were going to be screened for *Lac* Z α-complementation, 20 μl of 40mg/ml X-Gal and 40 μl of 0.1 M IPTG were mixed

with the cells and spreaded onto plates. Transformant colonies were screened for the presence of recombinant DNA constructs by restriction enzyme analysis and Southern blots or colony lifts/hybridization techniques.

13-2. Transformation into competent *E. coli* INVαF′ cells.

E. coli INVαF′ cells were provided as frozen 50 μl aliquots with the TA Cloning® kit. They were made competent chemically and had high transformation efficiency. The protocol for the transformation was provided by the vendor. The required number of tubes was thawed on ice. 2 μl of the ligation mixture as described in Part II-12, 12-2 was gently pipetted into the thawed competent cells and mixed gently by moving around the pipette tip in the suspension of cells. The tubes were incubated on ice for 30 min and then heat shocked for exactly 30 seconds in 42 °C water bath without shaking and the placed on ice for 2 min. SOC medium provided with the kit was prewarmed to room temperature. 250 μl of prewarmed SOC medium was added onto each sample after the heat shock. The samples were incubated at 37 °C shaking horizontally at 225 rpm for 1 hr. 50 μl of each sample was spreaded onto a prewarmed LB/amp plate with 40 μl of X-Gal from the 40mg/ml stock. No IPTG was added, since this strain does not express the *Lac* repressor. The remaining culture was centrifuged at 14,000 rpm in a microfuge for 1 min and supernatant was decanted. Pelleted cells were resuspended in 100 μl of the SOC and plated onto LB/amp plates the same way. After 15 minutes, the plates were inverted and incubated at 37°C for at least 16 hours. The following day, the plates were placed in the refigerator for 2 hours for color development.

13-3. Transformation into competent *Bacillus subtilis* strains.

In contrast to *E. coli* strains, *Bacillus subtilis* 168 becomes competent naturally during the late stationary phase when grown in appropriate media. To prepare competent *B. subtilis* 168 strains the following solutions were prepared in advance. Bott and Wilson (BW) salts solution, Bott and Wilson (BW) amino acids solution, a stock of 0.1M $MgSO_4$, a stock of 20% (w/v) glucose, a stock of 2% (w/v) yeast extract, a stock of 1 M $MgCl_2$, a stock of 1M $CaCl_2$ and 4X PAB. These solutions were sterilized by autoclaving at 120 °C for 20 min except the glucose and BW amino acids solution, which were sterilized by filtering through 0.2 μm nitrocellulose filters. 4X Bott and Wilson (BW) salts solution were prepared by adding 4.96% (w/v) K_2HPO_4, 3.04% (w/v) KH_2PO_4, 0.4%(w/v) sodium citrate, and 2.4% (w/v) $(NH_4)_2SO_4$, pH 7.0. The pH of this solution was then adjusted to 7.0 with 1N KOH if necessary. A 100 ml of Bott and Wilson (BW) amino acids solution was prepared by dissolving 500 mg of each of the following amino acids in ddH_2O: valine, lysine, threonine, glycine, aspartic acid, methionine, histidine, tryptophan, and arginine. Calcium chloride, yeast extract, BW amino acids (wrapped with aluminium foil), glucose were stored at 4 °C. All the solutions were prepared in ddH_2O.

On the day of inoculation GM1 media was prepared fresh in a sterile 100 ml graduated cylinder by adding 12.5 ml BW salts, 1.25 ml 20% glucose, 2.5 ml BW amino acids, 0.5 ml of 0.1M $MgSO_4$, and 2.5 ml 2% yeast extract and sterile ddH_2O to a final volume of 50 ml. 20 ml of GM1 was transferred to an autoclaved Klett flask and inoculated by a single colony of *B. subtilis* 168. 10 ml of GM1 was transferred to a sterile 250 ml Erlenmeyer flask and stored at 4 °C to prepare GM2 media the next day. Remaining 20 ml was used as a blank to calibrate the Klett-Somerson photoelectric

colorimeter. The inoculated GM1 was kept at room temperature overnight without shaking. The following day a slight turbidity was observed in the media. The culture was then placed in a gyrotory water bath shaker and shaken at level 7. The turbidity was measured every 15-min using a Klett-Summerson colorimeter to monitor the growth. A growth curve was made by plotting turbidity (Klett units) vs. time (min) on semi-log graph paper. Usually after 2 hours, cells had reached the stationary phase, which was marked by constant turbidity readings. After the beginning of stationary phase, cells were incubated 90 more minutes. In order to determine the beginning of stationary phase experimentally, a best line was drawn both on the log phase and the stationary phase readings on the growth curve. The intersection of these lines was taken as the beginning of stationary phase. At the end of 90 minutes, 1 ml of the culture was transferred into 10 ml of prewarmed (to 37 °C) GM2, which was prepared by adding 5 μl of 1 M $CaCl_2$ and 25 μl of 1 M $MgCl_2$ to the 10 ml of GM1 prepared the day before. This culture was shaken at 37 °C for 1 hr at the end of which the cells became competent.

To transform the DNA, 0.5 ml of GM2 culture was transferred to a 15 ml sterile polypropylene tube and 0.5-5 μg of uncut or linear plasmid DNA or chromosomal DNA was added. There was an increase in the recombination as the amount of DNA was increased up to a certain limit. If extraordinarily complex recombination events were expected to happen such as big pieces of DNA was targeted to be transferred into the chromosome of the host by using small homology regions, the chances of recombination were increased by adding DNA up to 50 μg. The tubes were shaken in an orbital shaker at 37 °C for 30 min and then 1 ml of 4X PAB was added to each tube. Incubation continued for an additional 90 min. The selection was usually done on choloramphenicol

or erythromycin both of which inhibits protein synthesis. In order to induce the expression of the antibiotic, the cells were incubated with small amount (to a final concentration of 0.5μg/ml) of the appropriate antibiotic for an additional 30 min. A 100 μL aliquot of the transformed cells was spread on a PAB/Cm plate. The remaining culture was centrifuged briefly and the pellet was resuspended in 150 μl of PAB and spreaded on a PAB/Cm plate. Plates were inverted and incubated at 37 °C until colonies appeared which took 24-72 hr.

13-4. Transformation into electrocompetent *E. coli* JM101 strain.

A single colony of the *E. coli* strain from a freshly streaked LB plate was inoculated into 3 ml of LB media and incubated at 37 °C overnight in an orbital shaker. Next morning, a 1 ml aliquot of this culture was transferred to a 500 ml flask containing 200 ml of LB medium and incubated at 37 °C in an orbital shaker until the OD_{600} had reached 0.6 (about 2.5-3 hr). These mid-log phase cells were chilled on ice for 15 min and then aseptically transfered to a sterile, ice-cold centrifuge tube. Always well-chilled cells, rotors and containers were used in the subsequent steps of the process. The cells were precipitated by centrifuging at 5000 rpm for 20 min at 4 °C. The supernatant was decanted and the cells were resuspended in 200 ml of ice cold, sterile ddH_2O by vortexing. The cells were recovered by centrifuging at 5000 rpm for 20 min at 4 °C and this wash step was repeated one more time. After centrifugation the pellet was resuspended in 40 ml of ice cold, sterile 10 % glycerol and pelleted at the same speed and temperature for 10 min. Finally, the volume of the pellet was estimated and the cells were resuspended in an equal volume of 10 % ice cold, sterile glycerol. 50 μl aliquots in

microfuge tubes were snap frozen in dry ice ethanol bath and stored at –80 °C for up to a year with a little loss in electrocompetence or immediately used.

For electroporation, 1 μl of the ligation mixture or uncut DNA was mixed with 50 μl of electrocompetent cells and transferred to a prechilled sterile BTX disposable electroporation cuvette (1 mm gap). The cuvette was chilled on ice 10 more minutes and placed in the chamber in BTX ECM 600 electroporation system. High voltage mode (field strengths >2.5 kV/cm) was chosen, the capacitance was fixed at 50 μF and the resistor number was set to 5 (129 ohms) to generate a 5 msec pulse when 1. 70 kV charging voltage was applied. 1 ml of the ice-cold SOC medium was added immediately after the pulse was over and placed on ice. The mixture was then transferred to a sterile microfuge tube and incubated at 37 °C water bath for 2 hr. The cells were spreaded on LB-amp plates and incubated overnight at 37 °C. The transformation was more efficient when the cells were pulsed only once instead of twice.

13-5. Transformation into electrocompetent *L. lactis* 11454 strain.

In contrast to *E. coli*, the best transformation efficiency was obtained when the stationary phase *L. lactis* cells were used for electroporation (53). A single colony of the *L. lactis* 11454 from a freshly streaked M-17 plate was inoculated into 50 ml of M-17 media in a sterile 50 ml polypropylene tube and incubated at 32 °C without agitation until the OD_{600} reached 1.2. These stationary phase cells were chilled on ice for 15-30 min and then aseptically transfered to a sterile, ice-cold centrifuge tubes. Cells were pelleted by centrifuging at 5,500 g at 4 °C for 10 min. Supernatant was discarded and pellet was resuspended in 10 ml ice cold, sterile ddH_2O. The cell suspension was centrifuged at 12,000 g at 4 °C for 10 min. This wash step was repeated one more time. This time cells

were spun at 12,000 g at 4 °C for 15 min. The wash was repeated the third time by resuspending the sample in 1 ml ice cold, sterile ddH_2O instead of 10 ml. The cells were pelleted 12,000 g at 4 °C for 3 min. These washes were necessary since contaminating ions from the media could decrease the transformation efficiency by altering the ionic strength of the cell suspension. Finally, the cell pellet was resuspended in 1.25 ml of ice cold, sterile ddH_2O and was now ready for electroporation.

Electrocompetent *L. lactis* cells were always prepared fresh for electroporation. 70 μl of the elecrocompetent cells were mixed with 3 μl of the plasmid DNA, chilled on ice for 10 min and exposed to either one or two high-voltage (1.70 kV) electric pulses, which lasted about 5 msec. The same settings described for the electroporation system in the above section (13-4) were also used for *L. lactis* cells. Immediately after the pulse(s) 1 ml of M-17 media was added and the cuvette was placed on ice. The sample was then transferred to a microfuge tube and incubated in a 37 °C water bath for 2 hr. Then the cells were spreaded on M-17 plates containing the appropriate antibiotic and incubated at 37 °C until the colonies were observed (24-72 hr).

Part II-14. Construction of λ library of *Lactoccocus lactis* 11454.

A genomic DNA library of *L. lactis* 11454 was constructed in order to be able to isolate the piece of DNA that contains the whole nisin gene cluster. The protocols for the Lambda GEM®-11 BamH I arms plus packagene system kit were provided by the manufacturer.

14-1. Digestion of *L. lactis* 11454 DNA with *Bgl* II.

Analysis of restriction enzyme map of the ~18 Kb region including nisin gene cluster of *L. lactis* 11454 genomic DNA revealed the presence of *Bgl* II sites that will release a 16561 bp fragment upon digestion. This fragment would contain the whole nisin gene cluster and part of the upstream and downstream sequences.

The digestion mixture was set up as follows. 3 μg of genomic DNA (0.3 μg/μl), 25 μl of NEBuffer 3, 3 μl of Bgl II (10 units/μl) were mixed in a microfuge tube and the volume was brought up to 250 μl with ddH_2O. The mixture was incubated at 37 °C, overnight. The following morning the DNA was extracted by adding equal volume of 1:1 ratio of phenol:chloroform and extraction repeated once more with an equal volume of chloroform. 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 95 % ethanol were added to the aqueous phase to precipitate the DNA. The sample was incubated at –80 °C until the solution had thickened and then centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was washed with 70% ethanol once and DNA was lyophilized in speedVac. The dried DNA pellet was then resuspended in 50 μl of TE. 10 μl aliquot was run on a 0.8 % agarose gel along with the uncut DNA to be able to visualize the efficiency of digestion. λ DNA that had been digested with EcoR I was also included as a size standard.

14-2. Ligation of *L. lactis* 11454 *Bgl* II digest with Lambda GEM®-11 *Bam*H I arms.

The flanking ends produced with *Bgl* II genomic DNA digest are compatible with *Bam*H I ends of the λ DNA provided with the Lambda GEM®-11 *Bam*H I arms plus packagene system. And the λ *Bam*H I arms were already dephosphorylated to prevent the

self-ligation. The ligation reactions were set up as follows. Reaction mixtures were incubated at room temperature for 3 hr.

Component	Reaction I (-) control	Reaction II (+) control	Reaction III *L. lactis* digest
Lambda vector *Bam*H I arms (0.5 μg/μL)	2 μl	2 μl	2 μl
Positive control insert (0.5 μg/μL)*	—	2 μl	—
Bgl II *L. lactis* digest (~0.5 μg/μl)	—	—	2 μl
10x ligase buffer	1 μl	1 μl	1 μl
ddH_2O	6.5 μl	4.5 μl	4.5 μl
T4 DNA Ligase (5 units/μL)	0.5 μl	0.5μL	0.5 μl
Total reaction volume	10 μL	10 μl	10 μl

* Test insert was provided with the kit.

14-3. Packaging of ligated DNA and titration of the recombinant phage

The ligated λ DNA was packaged into bacteriophage particles using the packaging extract supplied by Promega in the Lambda GEM®-11 *Bam*H I arms plus Packagene system. Three tubes containing the Packagene® Extract (50 μl/ tube) was thawed on ice. Then 5 μl of the ligation reaction was pipeted into tube containing the extract and mixed by gently tapping the bottom of the tube. The DNA/extract mixes were incubated at 22 °C (room temperature) for 3 hr. Then 445 μl of phage buffer and 25 μl of chloroform was added to each tube and mixed by gentle inversions. At this point, the packaged phage could be stored at 4 °C for a week without a significant drop in the titer. The packaging reactions were diluted 1:1,000 and 1:10,000 fold with phage buffer for tittering. 100 μl of the diluted phage was added to the 100 μl of log phase (OD_{600} 0.6-0.8) *E. coli* LE392 cells in a 5 ml sterile tubes and incubated at 37 °C for 30 min to allow adsorbtion of phage particles onto bacteria. Then 3 ml of molten (45 °C) LB top agar was

poured onto the mixture, mixed by quick inversions and immediately poured onto pre-warmed LB plates. The plates were inverted and incubated overnight at 37 °C after the top agar had hardened. To prepare log phase cells, a single colony from a freshly streaked plate was inoculated into a 3 ml of LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. The culture was shaken at 37 °C until the OD_{600} was between 0.6-0.8 (~5hr).

14-4. Screening of the λ library for the presence of nisin gene cluster.

Lambda clones containing the nisin gene cluster were identified by hybridizing a number of plaques with a probe against the nisin structural gene, *nis*A, (see table 3). 10 plates were prepared to yield ~200 plaques/plate for screening with hybridization as follows. 100 μl of the log phase *E. coli* LE392 cells (see Part II-14-3) were mixed with 2 μl of the undiluted phage in 5 ml sterile tubes and incubated at 37 °C for 30 min. Then 3 ml molten LB top agar was poured onto the mixture, mixed by quick inversions and immediately poured onto pre-warmed LB plates. The plates were inverted and incubated overnight at 37 °C and then stored at 4 °C for at least 1 hr prior to the hybridization.

The nitrocellulose filters were labeled with a pen. Then the filter was gently placed at one edge of the plate and more of the filter was progressively laid down as it wetted. Formation of any air bubble between the filter and the top agar and repositioning of the filter was avoided. The filters were left on the plates for an hour at room temperature. To record the orientation of the filter to the plate, a 20-gauge needle was stabbed through the filter at 12, 3, 6, 9 o'clock postions. The filters were slowly removed with the help of a forceps, placed on a paper towel plaque side up and dried for 30 min at room temperature. The dry filters (plaque side up) were placed on a piece of Whatman 3

MM paper saturated with 0.2 M NaOH, 1.5 M NaCl for 2 min and then transferred onto another Whatman paper saturated with 0.4 M Tris-HCL (pH 7.6), 2X SSC for 2 min. Finally, the filters were placed on a piece of Whatman paper saturated with 2X SSC for 2 min and dried on a clean paper towel (plaque side up). The filters were then baked at 80 °C for 2 hr to immobilize the DNA. The filters were hybridized with end labeled *nis A* probe (see part II-16, 16-1) as described in part II-17.

Two of the plaques hybridizing with the *nis A* probe were chosen for large-scale preparation of recombinant bacteriophage λ. Plaques were scraped of from the top agarose with a pipette tip and put in a microfuge tube containing 100 μl of the phage buffer and placed at 4 °C overnight. The phage plug eluate was assumed to contain 10^{10} pfu/ml. The recombinant λ DNA from the chosen clones were amplified at low multiplicity and isolated according the protocol of Sambrook *et. al*. (71). 100 ml of NZCYM media prepared in a 500 ml flask was inoculated with a single colony of *E. coli* LE392 and incubated at 37 °C, shaking at 300 rpm, overnight (~12 hr). The following morning, OD_{600} of the culture was measured and recorded (OD_{600}= 1.02). 1 OD_{600} assumed to have 8x 10^8 cells. 2 aliquots containing 10^{10} (12.5 ml) cells were withdrawn and centrifuged at 4000 rpm for 10 min at room temperature seperately. Pellets were resuspended in 3 ml of SM buffer. ~5 x 10^7 pfu was pipetted onto bacterial cell suspensions, mixed and incubated at 37 °C for 20 min with intermittent shaking. Each infected aliquot was added onto the 500 ml of prewarmed (37 °C) NZCYM medium in a 2 L flask. The culture was observed for cell lysis after 4 hr. The white thread like cell debris was observable at this point. The cultures were incubated for a total of 11 hr, after which, 10 ml of chloroform was added to each culture. The flasks were shaken at 300

rpm for 10 more minutes at 37 °C. Recombinat bacteriophage λ was purified from the culture as described in part II-14, 14-5.

14-5. Purification of the recombinant bacteriophage λ.

The lysed cultures were cooled down to room temperature and pancreatic DNAse I and RNAse were added to the final concentration of 1 μg/ml and incubated at room temperature for 30 min. Then solid NaCl was added to the final concentration of 1 M to promote dissociation of bacteriophage particles from bacterial debris and precipitate the particles efficiently from polyethyleneglycol (PEG). After the NaCl had dissolved, the culture was left at 4 °C overnight (or at least for an hour on ice). The following morning, debris was removed by centrifuging at 11,000 g for 10 min at 4 °C. The supernatants were pooled into a clean flask and PEG 8000 was added to a final concentration of 10% w/v and dissolved with slow stirring at room temperature. The solution was incubated on ice for at least an hour to allow phage particles to form a precipitate. The precipitated phage particles were recovered by centrifuging at 11,000 g for 10 min at 4°C. The supernatant was discarded and any remaining fluid was drained by keeping the bottles in a tilted position. Phage particles were then resuspended in 8 ml of SM buffer and the walls of the bottles were carefully washed to remove any stuck phages. The PEG and the cell debris were extracted from the bacteriophage suspension by adding an equal amount of chloroform and vortexing for 15 sec. The organic and the aqueous phase were separated by centrifugation at 3,000g for 15 min at 4 °C. Then 0.5 g of solid CsCl was added per milliliter of aqueous phase containing the phage suspension and mixed gently to dissolve. When the CsCl had dissolved, bacteriophage suspension was carefully layered onto CsCl step gradients that were preformed in a centrifuge tubes and the top of

the tubes were sealed. Solutions of CsCl with different densities (1.45 g/ml, 1.50 g/ml, 1.70 g/ml) were prepared in SM buffer. The step gradients were prepared by carefully layering the solutions of decreasing density on top of one another. A mark was made on the outside of the tube opposite to the position of the interface between the densities of 1.50 g/ml and 1.45 g/ml. The tubes were centrifuged for 4 hr 30 min at 70,000 rpm in a VTi80 rotor in a Beckman preparative ultracentrifuge. A band of phage particles was observable as a blue band when a light was shined from the side of the tube at the interface between the 1.50 g/ml layer and 1.45 g/ml layer. The top of the tube was cut off and a 20 gauge needle was stabbed underneath the blue band and collected into a tube. The phage suspension was dialyzed against 1000-fold volume of 10 mM NaCl, 50 mM Tris pH 8.0, 10 mM $MgCl_2$ solution for an hour twice. The buffer was changed and dialyzed the third time overnight at 4 °C.

The next morning, the dialyzed solution was transferred into a 50 ml polypropylene tube and EDTA was added to the final concentration of 20 mM followed by the addition of proteinase K and SDS to a final concentration of 50 μg/ml and 0.5% w/v, respectively. The solution was incubated at 56 °C for an hour. The sample was then extracted with an equal amount of phenol: chloroform mixture at 1:1 ratio and centrifuged at 3000 g for 5 min at room temperature and then extracted again with an equal volume of chloroform. The DNA was precipitated by adding 3 M sodium acetate to a final concentration of 0.3 M and 2 volumes of 95% ethanol. The mixture was incubated at – 20 °C for 30 min and centrifuged at 11,000g for 10 min at 4 °C to pellet the DNA. The DNA was washed with 1 ml of 70% ethanol and centrifuged again at 11,000g for 10 min at 4 °C. The ethanol was discarded and the DNA was air-dried. The dry DNA was

resuspended in 200 μl of TE. The recombinant phage DNA was analyzed with restriction enzyme analysis (Part II-6) and Southern blot analysis and hybridization (Part II-15, 15-2 & 17) for the presence of nisin gene cluster.

Part II-15. Immobilization of DNA on nitrocellulose membranes.

15-1. Screening of recombinant plasmid clones by colony lift.

The colony lift/hybridization technique was used when it was necessary to screen large numbers of ampicillin resistant *E. coli* transformants. Usually 100 or more colonies could be screened with relative ease with this technique. Also the modified form of the protocol was used to screen the plaques obtained from the λ library of *L. lactis* 11454 chromosome (Part 14, 14-4).

The ampicillin resistant *E. coli* transformants were grided onto duplicate LB/ampicillin plates with a sterile toothpick. The orientantion of the plate was marked at 12 and 3 o'clock positions with a marker. The plates were incubated overnight at 37 °C. Next morning, one of the plates was stored at 4 °C to be used as a source of viable positive clones and the replica plate was used for colony lift/hybridization analysis. The replica plate was also cooled to 4 °C for at least 30 min. A nitrocellulose filter with a diameter of 82 mm and a pore size of 0.45 μm was labeled with a water insoluble marker and placed on the surface of the each replica plate. After 5 minutes, filters were marked at the same positions by making holes on the filter and gently removed from the surface of the plate by a tweezer. Filters were dried at room temperature for 30 min colony side facing up on a dry paper towel. Nitrocellulose filters were always handled with gloves or with a tweezer in order to prevent contamination from the skin. A piece of Whatman 3

MM filter paper was placed on a glass plate and wetted with 0.5 N NaOH. The filter was placed on the saturated Whatman paper colony side up for 10 min to lyse the cells and denature the DNA. The filters were then transferred to Whatman paper soaked in 1.0 M TrisCl pH 7.6 neutralizing solution and allowed to stand for 10 min. Finally, filters were placed on a Whatman paper saturated with 1.0 M TrisHCl pH 7.6, 1.5 M NaCl for 20 min. The filters were then removed and air-dried on a piece of dry paper towel for 30 min. The DNA was immobilized on the membrane by baking at 80 °C in a vacuum oven for 2 hr. The membranes were then ready for prehybridization. The filters could be stored between the paper towels until the preparations of the hybridization experiment were done.

15-2. Screening of recombinant DNA's with a Southern blot.

Recombinant chromosomal DNA from *B. subtilis* cells or plasmid DNA from *E. coli* cells were digested with appropriate restriction enzymes including positive and negative controls such as DNA from the wild type strain that did not contain the cloned sequence and a source of DNA that did contain the insert. These were run on an agarose gel with an appropriate DNA ladder. The upper right corner of the gel was cut of with a razor blade to keep track of the orientation. The gel was placed on a UV transilluminator and a ruler was placed next to the gel and its picture was taken. This way bands on the X-ray film could be compared to the actual bands on the gel later on. The DNA was soaked in 500 ml of denaturization solution (0.5 M NaOH, 1.5 M NaCl) for 1 hr in a plastic dish with gentle agitation. The gel was rinsed several times with ddH_2O and then placed in 500 ml of neutralization solution (1 M Tris-HCl, pH 7.6, 0.6 M NaCl) for 1 hr with gentle agitation.

To prepare the transfer set up, a pyrex dish was filled with 500 ml of 20X SSC and a glass plate was placed across the top of the dish. 3 MM Whatman paper was cut in a size so that two ends could be dipped in 20X SSC when placed across the top of glass plate and would serve as a wick. This Whatman paper with a width slightly bigger than the gel was briefly wetted in 20X SSC and placed on the glass plate. A 10 ml glass pipette was used to roll over the layers to eliminate any air bubbles. This step was repeated every time until the last whatman paper was layered on top of the nitrocellulose filter. It is important to smooth out the layers because trapped air bubles could interfere with the transfer of DNA. After neutralization the inverted gel was placed on the wick. A nitrocellulose paper was cut to the size of the gel and dropped flat to a dish filled with ddH_2O. The filter was left in water for about 5 min. It is important not to submerge the filter into the water since it will not get wet if water touches the both surface at the same time. The nitrocellulose filter was then placed on top of the inverted gel. 3 MM Whatman paper cut in slightly bigger size than the gel was also wetted briefly in ddH_2O and placed on top of the filter. Air bubbles were eliminated one last time. Saran wrap was placed on all sides of the gel to prevent any contact between the wick and the stack of paper towels placed on top of the gel making the transfer more efficient. A stack of dry paper towels that were about 8 cm was placed onto the last layer of Whatman paper, followed by a small glass plate and weight such as a bottle filled with 0.5 liter of water. The transfer of DNA onto the filter was achieved by the capillary action that took place between the SSC solution that was sucked by the paper towels. Next morning the assembly was dismantled. The places of the wells were marked on the nitrocellulose filter with a pen and then the filter was air-dried for 30 min on a dry paper towel. The DNA was

immobilized by baking at 80 °C in a vacuum oven for 2 hr. The membrane was then ready for prehybridization and could be stored at room temperature.

Part II-16. Radioactive labeling of DNA

16-1. 5' end labeling of oligonucletide probes.

Synthetic oligonucleotides that were end labeled with a radioactive ^{32}P-phosphate were used to screen recombinant plasmid DNA's in the hybridization of immobilized DNA from either colony lift or Southern blot experiments for the presence of fragment of interest. Unlike restriction fragments, synthetic oligonucleotides have 5'-hydroxyl ends that are required by the enzyme polynucleotide kinase. The oligonucleotides were usually designed to have 20 complementary base pairs.

The following components were mixed in a microcentrifuge tube for the kinase reaction. 1μl of the 0.5 μg/μl oligonucleotide, 2.5 μl of 10x kinase buffer, 10 μl of ddH_2O, 10 μl of [γ-^{32}P]ATP (100μCi) (behind the plexiglass shield) and 1.5 μl of 10 unit/μl T_4 polynucleotide kinase. The sample was incubated at 37 °C for 30 to 45 min. The reaction was stopped by adding 5 μl of 0.5 M EDTA. Labeled oligonucleotide was precipitated by adding 2 μl of 10 μg/μl tRNA, 10.7 μl of 7.5 M NH_4OAc, 120 μl of 95% ethanol. The mixture was vortexed and incubated on dry ice for 10 min followed by centrifugation at 14,000 rpm for 10 min. The radioactive supernatant was properly discarded into the radioactive waste container. The DNA pellet was allowed to air-dry and then resuspended in 600 μl of TE pH 8.0. The end labeled probe was stored in a lead container at –20 °C and were usable within 2 months.

16-2. Labeling of DNA by HexaLabel Plus^{TM} DNA labeling kit.

Uniform radioactive label was incorporated into longer pieces of DNA by priming the polymerase reaction on the template DNA with random hexanucleotide primers in the presence of labeled deoxyribonucleoside triphosphates. These probes were used in hybridization with the immobilized chromosomal DNA fragments.

The protocol provided by the vendor was followed. The reaction was set up in a microfuge tube by adding the following components. 10 μl of DNA template (100 ng), 10 μl of 5X hexanucleotide reaction buffer and ddH_2O up to 40 μl. The mixture was briefly vortexed and spun down in a microfuge for 3-5 seconds. The tube was incubated in a boiling water bath for 5-10 min and then cooled on ice. The tube was spun down briefly. The following components were also added to the same tube. 3 μl of Mix C, 6 μl of [α-^{32}P]CTP (50 μCi) and 1 μl of Klenow fragment exo- (5 units). The tube was vortexed and centrifuged for 3-5 seconds followed by incubation at 37 °C for 10 min. 4μl of dNTP mix added and incubated for 5 more min at 37 °C. The reaction was stopped with the addition of 1 μl 0.5 M EDTA, pH 8.0. The labeled DNA was used directly in hybridization or stored in a lead container at –20 °C. The reaction could also be carried out by using two α-^{32}P labeled deoxynucleoside triphosphates.

Part II-17. Hybridization

The nitrocellulose filters from either the colony lift or Southern blot experiments were placed in a covered plastic container composed of 100 ml of prehybridization solution (6X SSC and 4X Denhardt's, 1% SDS) and incubated in a 65 °C water bath for 2

hr. After which the membranes were placed on a dry paper towel to absorb excess solution and then transferred to a heat sealable plastic bag.

The hybridization solution was prepared in the mean time by adding the components in the following order into a 15 ml polypropylene tube behind a plexiglass shield. 250 μl of 2 μg/μl Herring sperm DNA, 200 μl of the end labeled probe (or 20 μl of probe labeled with HexaLabel Plus^{TM} DNA labeling kit). The DNA was denatured by addition of 200 μl of 1 N NaOH and vortexed. This was followed by neutralization with 50 μl of 2 M Tris pH 7.6, 200 μl of 1 N HCl, and 5.65 ml (or 5.83 ml if added 20μl of the probe) of ddH_2O. The solution was vortexed and then pH was checked with a pH stick to make sure it was neutral. The pH was adjusted to pH 7-8, if necessary and then 3.0 ml of 20x SSC, 400 μl of 100x Denhardt's solution, and 50 μl of 20% SDS were added. This mixture was poured into a plastic bag containing the membranes and sealed with a bag sealer with a heating element, which was prewarmed by plugging in to an electrical outlet. The bag was then immersed in a 50 °C water bath upright, and taped to keep the position. Incubation continued overnight. Different bags were used for hybridizing with different probes.

The next day, the hybridization bag was cut open and the liquid was properly discarded into the radioactive liquid waste container. Filters were gently removed with a tweezers and placed into a plastic container. 100 ml of post hybridization wash solution (2x SSC and 0.5 % SDS) was prepared by adding 10 ml of 20X SSC, 2.5 ml of 20% SDS and ddH_2O up to 100 ml was poured onto the filters. SDS was added last while preparing the solutions in order to prevent the formation of white precipittate. The container was then placed in a gently rotating orbital shaker for 5 min. Then the solution was discarded

into liquid radioactive waste container. The filters were then incubated in 250 ml of the wash solution (2x SSC and 0.5 % SDS) at 55 °C water bath for 45 min followed by the disposal of the solution same way described above. The last wash step was repeated once more and then the filters were placed on a paper towel to remove excess liquid and analyzed by autoradiography (Part II-18).

Part II-18. Autoradiography

The wet filters were placed colony-side-up on an old piece of Kodak X-ray film. If there were more than one filter, they were placed assymetricaly on the support for easier identification of the filter later on the developed X-ray film. The filter were covered with Saran wrap and placed in a cassette with intensifying screen. A piece of unexposed Kodak X-Omat XAR-5 film was placed into the cassette containing the filters in the dark room. The cassette was closed tightly and the film was exposed to the filters at –80 °C for at least 2 hours. Exposure times were adjusted depending on the freshness of the labeled probe and/or the intensity of the signal on the filters after the washes. The films were developed using an automatic developer machine.

Part II-19. Integration of DNA into *B. subtilis* by single (Campbell integration) and double crossover.

Recombination is the exchange of homologous segments between the two DNA molecules. Foreign DNA that is introduced into a host cell could integrate itself into the host chromosome with a single or a double crossover depending on the form of DNA (linear or circular) and/or the number of homology regions. Single crossover (Campbell

integration) takes place if the recombination happens between a single homology region between the two DNA molecules and this results in the integration of the entire foreign genetic material into the recipient chromosome. The DNA should be in the circular form in order for this to happen since the integration of a linear DNA with a single crossover would result in the breakage of the host chromosome and the recombinant clone will not be viable. On the other hand, a double crossover occurs if the recombination takes place between two homology regions between the host and the foreign DNA. Unlike the single crossover, a double crossover results in the exchange of genetic material between the homology regions. It has been noted that for a double crossover event to occur most efficiently, 400 or more nucleotides of homology on either side of the gene of interest are necessary (24).

It was possible to differentiate the type of recombination with antibiotic selection when using vectors derived from pLPcat cassette vector (62). These vectors contain the *cat* gene in between homology regions and the target site in *B. subtilis* chromosome contains the *erm* gene in between the homology regions. Therefore, a *B. subtilis* cell that integrates the transformed plasmid by a single crossover results in the integration of the entire plasmid with no displacement of the *erm* gene from the chromosome and the resulting strain becomes both chloramphenicol and erythromycin resistant. However, if the double recombination occurs, the resulting strain becomes chloramphenicol resistant and erythromycin sensitive since the *erm* gene was displaced with the *cat* gene and the piece of DNA that was intended to be placed into the host chromosome.

The *B. subtilis* cells transformed with either of these plasmids were spread on chloramphenicol plates. Then the chloramphenicol resistant transformants were grided

first onto chloramphenicol (10 μg/ml) containing plate with the help of a sterile toothpick and then onto erythromycin (10 μg/ml) containing plate at the identical positions. The plates were inverted and incubated up to 48 hr at 37 °C to observe the colony formation. Both single and double crossover colonies were analyzed with halo assay to see if the biological activity was different between any of them. The clones that produced bigger halos and were chloramphenicol resistant and erythromycin sensitive were selected for further manipulations since they had integrated the desired piece of DNA through double crossover. The presence of a plasmid sequence in the chromosome is not desirable because it might interfere with further genetic manipulations if one has such plans.

Part II-20. Expression of peptides in *B. subtilis* strains.

B. subtilis strains containing the nisin gene cluster were selected as described in part II-15 and grown in Medium A which is used for the production of sublancin and subtilin from the *B. subtilis* 168 strains. A single colony of the transformant from an overnight plate was inoculated into 5 ml of PAB with appropriate antibiotic in a sterile 15 ml polypropylene tube. The culture was grown overnight with shaking at 250 rpm at 37 °C. Next morning, this 5 ml culture was used to inoculate a flask of prewarmed Medium A (2% sucrose, 0.2% PAB) medium to determine if the transformant strain was producing nisin. The solutions for Medium A (Part II-2, 2-5) were prepared in advance and 500 ml of the media was prepared fresh by mixing the four solutions just before the inoculation as described previously. The 2 L baffled flask containing the culture was plugged with a foam stopper and shaken at 300 rpm in an orbital shaker at 37 °C for 26-28 hr. The color change of the culture to a dark maroon and a drop in the pH to 5.8-6.2

was usually the indications of a good lantibiotic production in strains of *B. subtilis* 168. The production of nisin was also expected to produce the same effects. At the end of the incubation, the cultures were acidified to pH ~2.5 with phosphoric acid (85%). Then the cells were pelleted in a 250 ml centrifuge bottles by spinning at 10,000 rpm in a Beckman JLA-16.250 rotor for 10 min at room temperature. The supernatants were then transferred to another flask. At this point the supernatant was ready for the isolation of the peptide of interest or could be stored at –20 °C for later manipulation. For the initial purification of nisin, two different column chromatography approaches were used. The culture supernatants were either applied to a hydrophobic interaction column (HIC) as described in part II-21 or to a non-ionic cellulose (NIC) column as described in part II-23.

Part II-21. Purification of peptides with a hydrophobic interaction column (HIC).

The culture supernatant described in Part II-17 was prepared for the HIC purification by adding 1 M NaOAc, pH 4.0 and 5 M NaCl (Part-II-2, 2-2) to a final concentration of 50 mM and 1 M, respectively. A Bio-Rad econo column (11 cm in height, 1.5 cm in diameter, and 20 ml in volume) was prepared by packing with Toyopearl® Butyl 650M hydrophobic interaction resin. The resin was then equilibrated by passing 100 ml of wash buffer (50 mM NaOAc pH 4.0 and 1 M NaCl, see Part II-2, 2-3) through the column using a peristaltic pump. The equilibrated culture supernatant was placed on ice and applied to the equilibrated hydrophobic interaction column by peristaltic pump at a flow rate of 1.5-2 ml/min. After all of the supernatant had flowed through, the column was washed by 100 ml of equilibration/wash buffer. Bound proteins were then eluted from the column with passing the elution buffer, 60 % acetonitrile, (see Part II-2, 2-3) through the column. About 30 ml of eluate was collected into a 300 ml

lyophilizer jar. The collection started after the front of eluting materials had reached to the bottom of the column (in other words after approximately 1 bed volume of buffer had passed through the column), this can be usually observed as a dark brown band. The jar was then frozen in a dry ice-ethanol bath and the contents were lyophilized overnight. The next morning, the dried eluate was dissolved in 2 ml of 100 mM HOAc. The solution was filtered through a 0.22 μm syringe filter and purified further by reversed phase HPLC as described in Part II-22.

Part II-22. Reversed phase high performance liquid chromatography (RP-HPLC).

Reversed phase high performance liquid chromatography (RP-HPLC) was used for further purification of peptides. RP-HPLC was performed on a Hewlett-Packard Series 1050 equipped with a photodiode array detector. A 250 mm x 4.6 mm MICROSORB 300 Å C-18 reversed phase column with 5 μm pore size was used to resolve the peptides. For the mobile phase, buffer A was composed of 0.5 % acetonitrile, 0.1 % acetic acid and 0.01 % trifluoroacetic acid, in ddH_2O and buffer B was composed of 0.5 % ddH_2O, 0.1 % acetic acid 0.01% trifluoroacetic acid in acetonitrile. Linear gradient at a flow rate of 1.2 ml/min was applied. The elution profiles were monitored using absorbencies at 254 nm for dehydro residues and 214 nm for peptide bonds and 280 nm for aromatic residues. Before sample injection, the column was washed twice or until completely cleaned with an acetonitrile gradient (0-100%) over 2 min, holding at 100% for 3 min, reversing the gradient over an additional 2 min and holding at 0% several 2 min to equilibrate. Samples were then loaded into the loop and injected onto the column. After injection the mobile phase was maintained at 100% buffer A for 4 min to allow for

binding of peptides to the column and flow-through of any nonbinding material. The gradient was initiated by ramping the mobile phase from 0% to 100% buffer B over the next 21 min to gradually eluate the bound materials with respect to their hydrophobicity. The gradient was held at 100% buffer B for 3 min and and then reversed back to 0% over the next 2 min. Fractions were collected at one-min increments into clean 1.5 ml microfuge tubes and tested for biological activity using halo assay (see Part II-20). Active fractions were further analyzed with mass spectroscopy for the presence of lantibiotics. Collected fractions were frozen at -80 °C and then lyophilized in a vacuum centrifuge. The dried fractions were then resuspended in 100 μL of 100 mM HOAc and stored at –20 °C. If the peptide yield was low, the halo assay was performed after concentrating the fractions with lyophilization.

Part II-23. Cellulose column purification of peptides produced by *Lactococcus lactis* 11454 and recombinant *B. subtilis* strains.

The ability of nisin to bind to the non-ionic cellulose was characterized by a former group member Amer Villaruz (83). In order to isolate pure recombinant nisin species from the cultures of appropriate *B. subtilis strains* a non-ionic cellulose column was used. 50 g of non-ionic cellulose powder was resuspended in ddH_2O and allowed to settle. The fine particles that remained in the liquid phase were gently decanted. This was repeated couple of times to get ride of the cellulose fine particles as much as possible. The cellulose was then resuspended in 100 ml ddH_2O and was now ready for use. The suspension was poured into the column until about 20 ml resin was packed in while the water was running through the column. This allowed homogeneous packing. The column

was equilibrated with about 100 ml of 0.1 M NaP_i pH 7.8. Culture supernatants were also equilibrated to pH 7.8 by titrating with NaOH. When working with cellulose column extra care was taken not to let the column to go dry. If the column had dried out, the resin was taken out and resuspended in a beaker and packed again.

Lactococcus lactis 11454 (natural host of nisin) culture was prepared by inoculating a single colony from an M17 agar plate to a 50 ml of sterile polypropylene tube filled to top with M17 media and incubated at 32 °C overnight. The next day, if the high turbidity of *L. lactis* cells was observed, the entire 50 ml of this culture was then transferred to 950 ml of sterile M17 media in a 1 L flask, which was sealed with parafilm and incubated at 32 °C overnight. The following morning pH of this culture was adjusted to pH 2.5 with 85% phosphoric acid, and centrifuged at 10,000 rpm for 10 min at room temperature to pellet the cells. The supernatant was then collected, and the pH was adjusted to 7.8 with 10 N NaOH.

The recombinant *B. subtilis* strains were cultured in Medium A as described in Part II-20. After the cells had pelleted the pH was adjusted to 7.8 as described for *L. lactis* cells. After the equilibration of the supernatant the same protocol was followed for both cell types.

The equilibrated supernatants were pumped into the cellulose column at a flow rate of 1.5-2 ml/min at room temperature. Then the column was washed with ~100 ml of 100 mM NaP_i pH 7.8. The bound materials were then eluted by applying 5.4 M HOAc to the column. The eluate was collected into a lyophilizer jar and frozen using a dry ice-ethanol bath, and lyophilized. The dried residue was resuspended in 2 ml of 100 mM HOAc.

The cellulose column eluate was then further purified on RP-HPLC using a linear water-TFA-acetonitrile gradient described in Materials and Methods, Part II-22. The fractions were collected at one-min intervals, frozen at –80 °C, lyophilized in a Speedvac, and resuspended in 100 μL of 100 mM HOAc. A 10 μL aliquot of each fraction was tested for biological activity as described in Materials and Methods, Part II-24. The fractions were tested for the presence of nisin with MALDI- MS and analyzed by nanoESI MS as described in Materials and Methods, Part II-25, 25-2.

Part II-24. Testing the biological activity with spore outgrowth inhibition assay (Halo Assay).

After the HPLC purification, native or recombinant proteins were tested for biological activity by a halo assay (inhibition zone assay). The active HPLC fractions were determined by their ability to inhibit spore outgrowth. 10 μl of the HPLC fractions were pipetted onto a prewarmed PAB plate and allowed to air-dry for 10-15 min at room temperature. A thin layer of spores was sprayed onto the plate in a ventilated hood and allowed to sit at room temperature for 10-15 min. Plates were then inverted and incubated at 37 °C until the halos develop (~ 5 hr) or overnight. Plates were placed in a sealable plastic bag during incubation to avoid contamination. The plates were then photographed.

After the transformation of a construct into the *B. subtilis* cells, recombinant clones were also screened with halo assay to quickly detect if there was any change in the in the size of halos around the recombinant and wild type strains. The recombinant clones with bigger and clearer zones of inhibition were chosen for further investigation. Usually 8 of the clones were grided per plate with a sterile toothpick and incubated overnight at

37 °C. The following day the plates were sprayed with a thin layer of *B. cereus T* spores and allowed to sit at room temperature for 10 min. Plates were placed in a sealable plastic bag, inverted and incubated at 37 °C. The halos were usually visible in 5 hr. The pictures of the plates were taken.

B. cereus T spores was prepared by suspending 250 mg of dried spores in 20 ml of ddH_2O. The spores were dispersed using a ground-glass homogenizer and then placed in a 50 ml polypropylene tube. The spore suspension was heat shocked at 65 °C for two hr. The spores were centrifuged at 6,000 rpm for 5 min. The spore pellet was resuspended in 40 ml of 10% ethanol. The spore suspension was then transferred to an aerosol spray bottle and stored at room temperature.

Part II-25. MALDI-TOF and nanoESI Mass Spectrometry.

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and nanoelectrospray ionization mass spectrometry (nanoESI MS) techniques were used for quick identification and/or analysis of lantibiotic species from the cultures of bacteria.

25-1. MALDI-TOF MS.

Samples to be analyzed were crystallized on sinapinic acid (SA) matrix by the 'sandwich method' as described by Kussman *et al* (49). A small metal disc, which was about 2 cm in diameter, was used as a sample stage.10 etched circles located on the disk allowed to analyze 10 separate samples at a time. To prepare the samples, 0.5 μl of the SA matrix was deposited onto desired number of circles and allowed to dry in a fume

hood. Then about 0.5 μl of the sample were pipetted onto the matrix and dried in a fume hood. Finally, 0.5 μl of the SA matrix was deposited onto each sample and dried the same way as described above. The samples were always dissolved in low pH solutions such as 100 mM HOAc or 0.05% TFA to facilitate ionization. External standards (such as nisin) were used to calibrate the instrument. Samples were analyzed on a Bruker Proflex MALDI-TOF mass spectrometer which was interfaced to a Sun Solaris workstation. The measurements were averaged over the multiple laser shots and displayed on the screen as mass/charge ratio. The SA matrix solution (20 mg/ml) was prepared by dissolving 20 mg of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 1 ml of a 70% 0.1% TFA, 30% CH_3CN. Undissolved matrix was spun down. The solution was stored at 4°C.

25-2. NanoESI MS.

The peptides purified from the culture supernatant with the described methods (Part II.20-23) were further analyzed by nanoESI mass spectrometry to be able to identify the mass peaks that might belong to nisin or nisin variants. The lyophilized RP-HPLC fractions were resuspended in 100 μl of 0.1 M HOAc. 2 μl aliquot of the sample was loaded on a nanospray capillary (New Objective) by a Hamilton syringe (10 μl). Voltage (1.00-1.5 keV) was applied to the nanospray capillary to charge the spraying droplets. The capillary inlet inside the machine was heated to 200 °C to facilitate the evaporation. The measurements were done on a Finnigan LCQ quadrapole ion trap mass spectrometer connected to a Windows 2000 workstation. The LCQ TunePlus software package was used to analyze the ions in the range of 150-2000 amu. The positive ion mode was used to analyze all the samples. Since the peaks were displayed as *m/z* values on the spectrum,

the charged state of a particular molecular ion had to be determined in order to calculate the molecular weight of that ion. Zoom scan mode was used to determine the charged state of a particular ion. In this mode, one peak within a 1 amu unit indicates a singly charged peptide ion whereas two peaks indicate a doubly charged peptide ion. Scans were collected for 3-10 min and saved into a file. Later on, a mass spectrum was obtained by averaging this collected data.

Part II-26. Isolation of total RNA and RT-PCR of nisin genes.

Proper microbiological, aseptic technique was always used when working with RNA. Gloves were worn at all times to prevent RNase contamination from the skin. In order to create and maintain an RNase free environment, the following precautions were taken while working with RNA. All tips and microfuge tubes were sterilized by autoclaving for 20 min at 120 °C. All glassware was filled up with 0.1% DEPC (in ddH_2O) and stirred overnight at room temperature. DEPC treated glassware was then autoclaved for 20 min at 120 °C to remove any residual DEPC. The electrophoresis tank, cuvettes and non-disposable plasticware were treated with 0.3 N NaOH and then rinsed with DEPC-H_2O. Solutions that were used in RNA work were either prepared with DEPC treated H_2O and glassware or treated with 0.1% DEPC afterwards. When preparing Tris buffers, the water was treated first with DEPC and then used to make the appropriate Tris buffer because DEPC is highly unstable in the presence of Tris buffers.

26-1. Isolation of total RNA.

Total RNA was isolated from strains of *B. subtilis* 168 and *L. lactis* 11454 by using RNeasy® Mini Kit in accordance with the manufacturer protocol (Qiagen). The on

column Dnase digestion was performed by using the RNase free DNase set which was manufactured by Qiagen to be used with RNeasy kits.

The bacterial cultures were grown overnight at 37 °C with shaking except the *L. lactis* culture, which was grown overnight 28 °C without shaking. 500 µl of the bacterial cell culture was added onto 1 ml of RNA Protect™ Bacteria Reagent in order to stabilize the RNA in the cells. For the lysis of the *L. lactis* culture, DL threonine was added to a final concentration of 20 mM when the OD_{600} was between 0.6-1.0 and the incubated at 30 °C for 1-2 hr to weaken the cell wall. In addition to the lysozyme (to a final concentration of 3 mg/ml) that was used to lyze *B. subtilis* cells, mutanolysin (10 units/µl) was also added to a final concentration of 0.5 units/µl to the *L. lactis* 11454 cells.

RNA was visualized on 1.2% formaldehyde agarose gels by staining with EtBr (Materials and Methods Part II-26, 26-2). The concentration and purity of the samples were determined taking readings at OD_{260} and OD_{280} with the UV-visible spectrophotometer.

26-2. Analysis of RNA on formaldehyde containing gels.

The following RNase free solutions were prepared in advance; 0.1 M Tris pH 7.9, 0.5 M EDTA pH 8.0, 3 M NaOAc pH7.0, 10X MOPS buffer.

1.2 % agarose gels were prepared by melting 1.2 g agarose in 57 ml DEPC-ddH2O in a microwave oven (10-15 seconds were counted after the solution had boiled). After the solution had cooled down to 50-55 °C, 10 ml 10X MOPS buffer (200 mM MOPS, 50 mM NaOAc, pH 7.0 and 10 mM EDTA, pH 8.0) and 30 ml 37% Formaldehyde (buffered) was added and mixed by swirling. The solution was then

poured onto midi (13 cm) gel casting stand that had been taped at the open ends. A comb was inserted to create wells and the gel was allowed to solidify at room temperature

To run the samples, appropriate volumes of RNA that contained 0.5-2.0 μg of RNA were prepared. If it was necessary, the SpeedVac was used to concentrate the samples. To each sample, a 2X volume of the pre-mix was added and mixed by vortexing. The tubes was briefly centrifuged to bring down all the solution and then incubated at 65 °C for 5 min. 2-5 μl of the 10X formaldehyde loading dye (50% Glycerol, 1mM EDTA, 0.25 % bromophenol blue, 0.25% xylene cyanol) was added prior to loading the samples on the gel. A 100 bp DNA ladder was used as a size marker. The gel was run at 45V at constant current (50 mA) in 1X MOPS buffer (10X MOPS buffer was diluted with DEPC-ddH_2O) until bromophenol blue migrated to the halfway of the gel. This took about 3-6 hrs.

The gel was rinsed with DEPC-ddH_2O several times and then stained in DEPC-ddH_20 which was containing 0.5 μg/ml EtBr. Staining was continued for at least 30 min (or overnight) on a shaker. Then it was washed with DEPC-ddH_2O overnight on a shaker to remove the excess EtBr. The next day, UV transilluminator was used to visualize the RNA bands.

26-3. RT-PCR of nisin genes.

The RT-PCR experiments were performed by using the Qiagen one step RT-PCR kit. The presence of a special enzyme combination in this kit allows the efficient and sensitive reverse transcription of any RNA quantity from 1 pg to 2μg. The enzyme mix also contains the HotStarTaq DNA polymerase which was activated by heating to 95 °C for 15 min after the reverse transcription is complete. This step also inactivates the

reverse transcriptases and eliminates extension from nonspecifically annealed primers and primer dimers in the first cycle.

A 240 μM stock of each primer was prepared by resuspending in RNAse free ddH_2O and stored at –20 °C. To prepare the working stock of 2.4 μM, oligonucleotides were diluted 100 times with ddH_2O. During the sample preparation, sterilized solutions and equipments were used to prevent any contamination and gloves were worn at all times. Prior to the preparation of samples, template RNA, primer solutions, dNTP mix, 5x Qiagen OneStep RT-PCR buffer, and RNase free water was thawed and placed on ice.

The samples were also prepared on ice in 0.5 ml RNase free thermowell^{TM} tubes and the reaction components were added in the following order. First, RNase-free ddH_2O was pipetted into the tubes to brought up the final volume to 50 μl then 10 μl of 5X RT-PCR buffer, 12.5 μl of the forward (2.4 μM) and the reverse primer (2.4 μM) to a final concentration of 0.6 μM, 2 μl of dNTP mix (10 mM of each dNTP) to a final concentration of 400μM, 2 μl of Qiagen OneStep RT-PCR enzyme mix and finally 2 μl of template RNA (≤2 μg/reaction) were added. Tubes were vortexed briefly and spun down for 3-5 sec. 50 μl of paraffin oil was then carefully pipetted onto the samples to prevent evaporation and recondensation during the reactions. The cycles were started and when the temperature in the wells reached 50 °C, tubes were placed into the PCR thermocycler directly from ice.

Four cycles/profiles were used to amplify cDNA of the target sequence from the RNA template. The first profile involved the reverse transcription from the template RNA by incubating the tubes at 50 °C for 30 min. Then the cycler advanced to second profile, which was initial PCR activation step. In this step, tubes were kept at 95 °C for 15

min in order to activate HotStarTaq DNA polymerase and inactivate reverse transcriptases. The cDNA template was also denatured. In the next profile, the cDNA was amplified through 30 repetitive denaturation, annealing and extension cycles. First, the denaturation was done at 94 °C for 30 seconds then the temperature ramped down to 51 °C and kept there for 30 seconds to allow annealing of primers to the template cDNA followed by ramping up to 72 °C for 1 min for the extension of the sequence from the primers. Finally, the cycler switched to the last profile in which the tubes were kept at 72 °C for 10 min for the final extension and then ramped down to 4 °C indefinitely. The ramp rate was set up to the machine's maximum during all the cycles. A negative control was included in each experiment that did not contain any template RNA to detect possible contamination of the reaction components. Also a control reaction was set up to detect any DNA contamination by setting up the reaction and placing it on the thermocycler only after it had reached 95 °C for the HotStarTaq DNA polymerase activation step. This high-temperature step inactivates the reverse transcriptases and in the absence of the reverse transcription, the only DNA template for PCR is contaminating DNA. Therefore, formation of PCR products in these reactions indicates the presence of DNA contamination.

After the cycles had completed, the samples were pipetted into clean tubes to get rid of the paraffin oil and 5 µl aliquots were run on a 1 % agarose gel along with a size standard to visually determine if the amplification was successful.

Part II-27. SDS Polyacrylamide gel electrophoresis

All analyses were done by using the tricine SDS-PAGE that was initially described by Schagger and Jagow (72). 16.5 % separating gel was prepared by mixing 10

ml of 49.5 % acrylamide, 3 % bisacrylamide stock solution, 10 ml of gel buffer (3.0 M TrisHCl, pH 8.45, 0.3 % SDS), 6.4 ml of 50 % glycerol. The volume was brought up to 30 ml. 100 μl of APS and 10 μl TEMED was added just before pouring the gel.

14 cm x 16 cm gel plates were assembled with spacers of 1 mm thickness using clamps. The edges and the bottom were sealed with 1 % molten agarose. The assembly was filled with the separating gel by using a 60 ml syringe and then a comb was placed immediately at the top. The gel was allowed to polymerize for 45-60 min. After the gel had solidified, the comb and the clamps were removed and the gel was placed into the electrophoretic apparatus. 400 ml of the 1X cathode buffer was poured into the upper reservoir and 400 ml of the 1X anode buffer was poured into the lower reservoir. The samples were prepared by adding equal amount of the 2X gel-loading buffer and incubated in a boiling water bath for 2-5 min. The samples were then loaded onto the wells, which have been flushed with the 1X cathode buffer. The gel was electrophoresed at 100 mV for about 60 min and then at 15 mV overnight. The next morning, the gel was electroblotted onto a nitrocellulose membrane for western blot analysis.

Part II-28. Western blot analysis.

The peptides from the purified culture supernatants were analyzed for presence of nisin or nisin related peptides by doing a western blot analysis. The peptides separated on a SDS-PAGE gel were soaked into the transfer buffer for 30 min. The nitrocellulose membrane, two sheets of 3MM Whatman paper, and the two filter pads were also equilibrated in the transfer buffer for about 10 min. The transfer block was opened up and the layers were assembled on the clear side (that will be closest to the positive electrode).

The layering was done in the following order: a transfer filter pad, a 3MM Whatman paper, the nitrocellulose membrane, the polyacyrlamide gel, another 3MM Whatman paper, and finally the other transfer filter pad. The black side of the plate was placed on top and the assembly was clamped shut. The transfer cell was filled with 3 liters of the transfer buffer and the assembly was placed in it with clear side facing the positive electrode. The cell was electrophoresed at a constant current 250 mA for 3-4 hr. The nitrocellulose membrane was then immediately probed with polyclonal nisin antibodies or wrapped in plastic wrap and stored at 4 °C.

Part II-29. Immunodetection.

The membrane-immobilized peptides were probed by using rabbit anti-nisin IgG. The nitrocellulose filter was incubated with 50 ml of the blocking buffer (1 % BSA in TBST) for 1 hr, while rocking gently. The filter was then immersed in 25 ml of TBST containing the 50 μl of the primary antibody (1:500 dilution) and gently agitated at room temperature for 45 min. The membrane was then washed twice in 50 ml of TBST for 5 min with agitation. The membrane was immersed in 25 ml of TBST containing 5 μl (1:5,000 dilution) from the 1 mg/ml secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase) solution. The membrane was incubated for 45 min in this solution with gentle agitation. The membrane was then washed twice in 50 ml of TBST for 5 min with agitation to get rid of the unbound secondary antibody. 30 ml of the 1 Step™ NBT/BCIP (Pierce) was poured on the membrane and incubated until the bands reached the desired color intensity. The reaction was stopped by rinsing the membrane

with water twice for 5 min and air dried on a clean paper towel. The developed membrane was photographed and stored in dark.

Results

Part I. Transfer of nisin genes into *Bacillus subtilis* BR151.

Rationale:

Nisin is an antimicrobial peptide that is very effective in killing food-spoilage bacteria and its non-toxic nature has led to its application as a food preservative in various food products in over 50 countries. Nisin is notable in containing a high proportion of unusual amino acids that are introduced by post-translational processing. These unusual residues confer highly unusual and useful properties to nisin. Novel dehydro residues (dehydroalanine and dehydrobutyrine) convert it into an electrophilic agent, and lanthionine residues confer remarkable chemical stability and protease resistance. In order to explore the potential of utilizing genetic engineering techniques to make structural analogs of nisin, and to explore the mechanism of posttranslational modification, it is useful to transfer the nisin biosynthetic machinery from its natural host (*Lactococcus lactis*) to a well characterized bacterial strain that is amenable to genetic manipulation. *Bacillus subtilis* 168 is an ideal host strain for the following reasons: The entire genome has been sequenced; it is already in use for industrial production of bioengineered materials; it is naturally competent and readily amenable to introduction and expression of foreign genes; it is a natural producer of a lantibiotic (sublancin) and is therefore compatible with lantibiotic processing proteins.

In the long run, understanding the mechanism of formation of these unusual residues might also allow us to introduce them into different peptides to create novel antimicrobial agents directed towards many diseases. Genetic engineering techniques

could also be used to increase the efficiency of production. This would greatly facilitate the use for for its industrial and pharmaceutical applications.

The goal of this work was to express nisin out of *B. subtilis* 168 through transfer of the nisin gene cluster from natural host into *B. subtilis* 168 chromosome. In pursuing this project, a major problem was to devise a suitable selection strategy. Different approaches were undertaken to transfer the 11 genes that are involved with the nisin biosynthetic machinery. In the following sections these experimental strategies for transfer and selection of nisin gene cluster are presented and evaluated.

Part I-1. Construction of pSYcat to provide recombination target sites from *L. lactis*.

The plasmid pSYcat was constructed to introduce the flanking regions of the nisin gene cluster from *Lactococcus lactis* into the *amy*E locus of *Bacillus subtilis* 168. The *amyE* gene was chosen as the target site to transfer nisin genes because it is a non-essential gene and is widely used as a target for integration of cloned genes into the *Bacillus subtilis* 168 chromosome.

The pSYcat construct contained a 527 nucleotide segment from the left end of the *amy*E gene (*amy*L), then a 612 nucleotide segment from the *transposase* gene, followed by chloroamphenicol resistance gene, 640 nucleotides from the *sacR* gene and a 606 nucleotide segment from the right end of the *amy*E gene (*amy*R). The cloning steps that were performed for the construction of the pSYcat are outlined in Figure 5. The construction involved making four PCR products as described in Materials and Methods Part II-10, 10-1. Table 2 shows the sequences of oligos used to make these products. The first step involved the cloning of each PCR fragment into the pCR2.1 vector by making

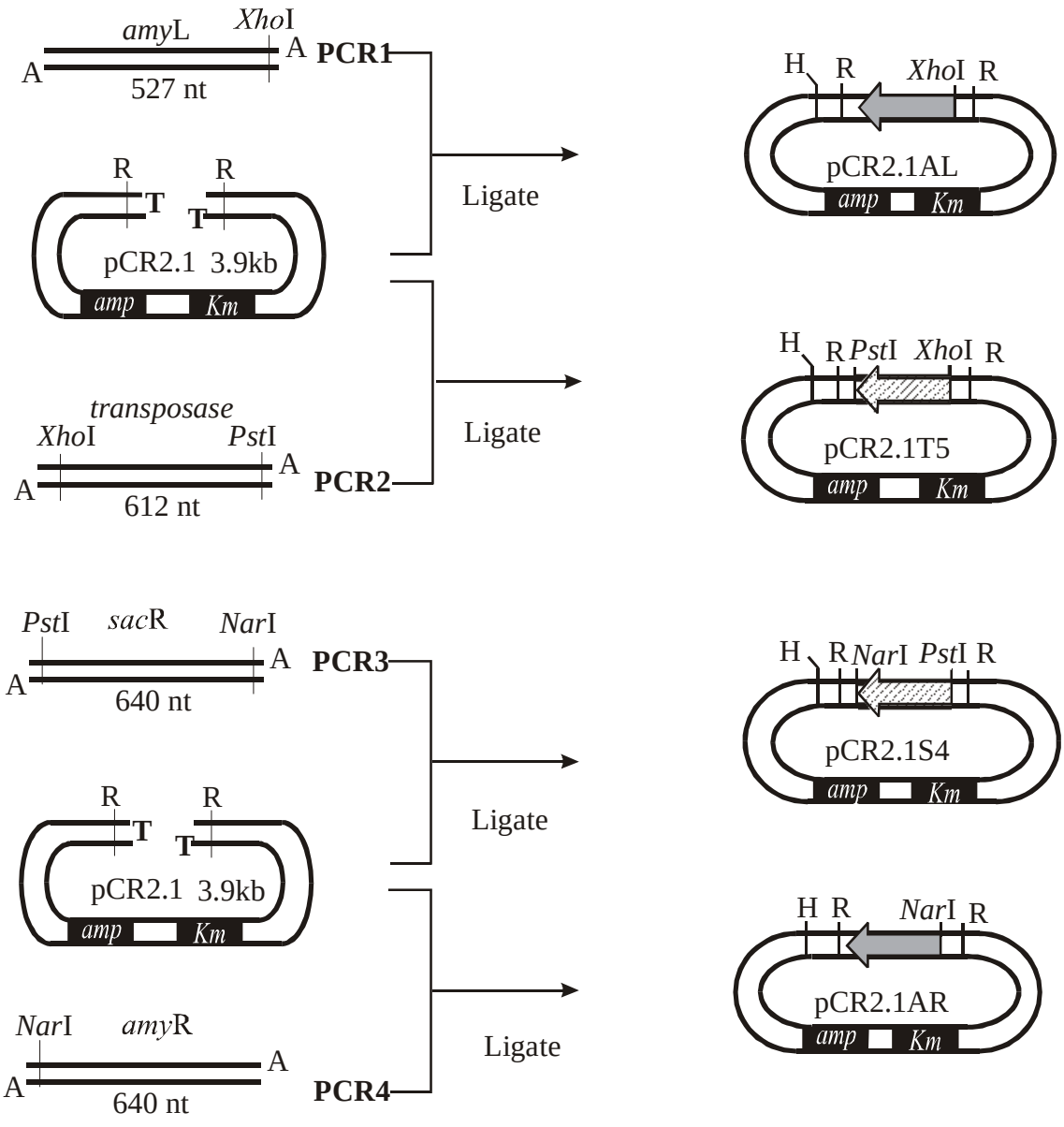
use of AT cloning. Then these fragments were sequentially subcloned into the multipurpose cloning site (mcs) of pTZ18R that contains an origin of replication that works in *E. coli*, which is non-functional in *B. subtilis*. The construct was completed by inserting the *cat* (chloramphenicol acetyl transferase) gene to generate 6.2 kb pSYcat. Each cloning step was subjected to extensive restriction enzyme analysis to confirm the presence of cloned fragments and also colony lifts and/or Southern blot hybridization experiments.

The plasmid pSYcat was then linearized and transformed into competent cells of *B. subtilis* ermΔsunA (Figure 6). The cells were screened for chloramphenicol resistance. Since the vector did not have a *B. subtilis* origin of replication, the only way that the transformed cells could become cm resistant was through the integration of pSYcat into the chromosome. The vector was linearized prior to the transformation in order to integrate the homology regions through a double recombination. A linear plasmid cannot integrate into the host chromosome through a Campbell type of integration because this will break the chromosome. The resulting strain was called *B. subtilis* SYcat.

Part I-2. Transformation of *L. lactis* 11454 genomic DNA and selection on nisin.

In the next step, *L. lactis* 11454 genomic DNA was transformed into *B. subtilis* SYcat (figure 6). The double recombination that would take place through *transposase* and *sacR* homology regions could integrate the nisin gene cluster into the host chromosome by replacing the *cat* gene. A known selection marker was not introduced to the flanking regions of nisin gene cluster because nisin gene cluster contains nisin immunity genes, which confer nisin resistance to the host cell. If these genes work in *B.*

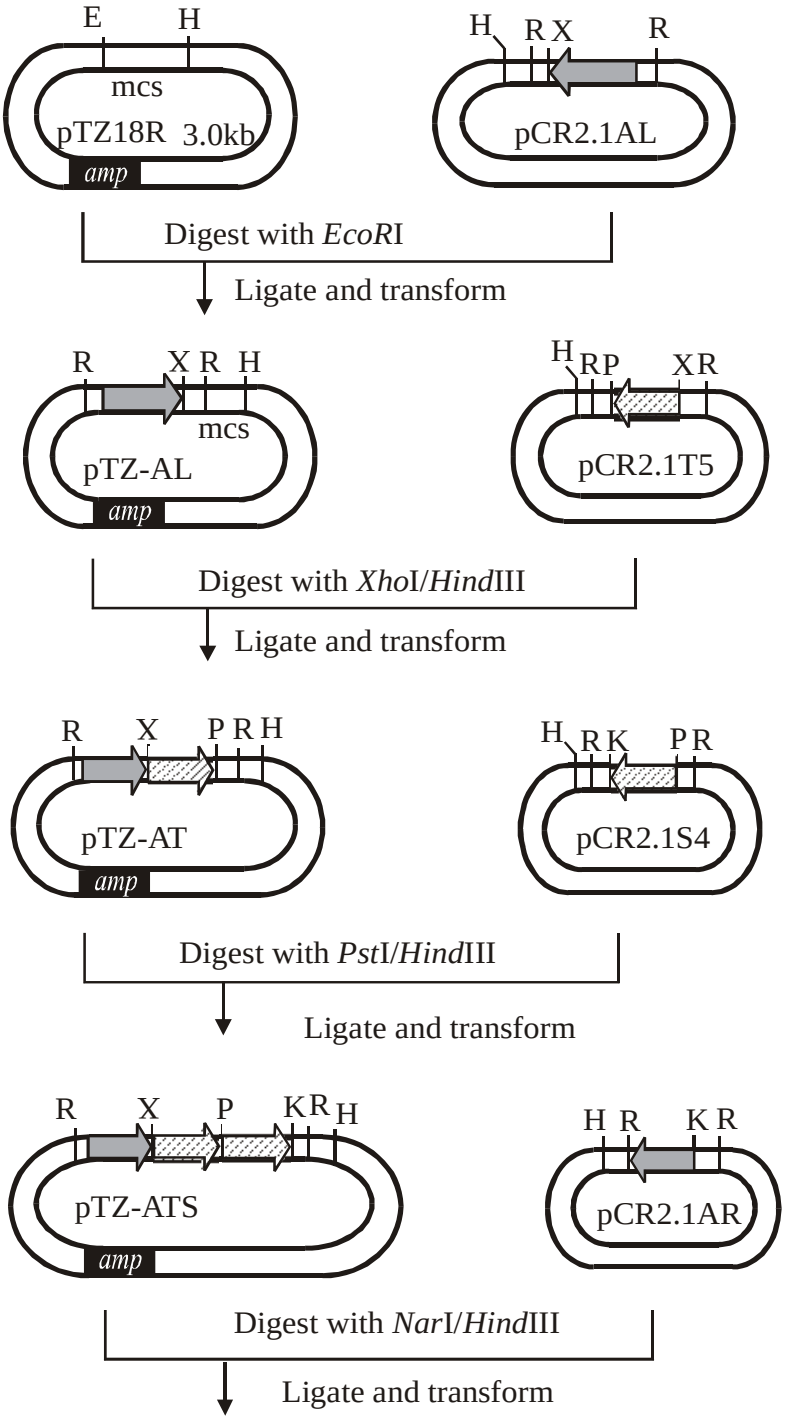
subtilis BR151, transformants could easily be selected on nisin without need to design more elaborate selection strategies. The minimum inhibitory concentration (MIC) of nisin for wt-*B. subtilis* was determined by culturing the wt-*B. subtilis* cells with increasing concentrations of nisin. MIC was found to be ~30 μg/ml. Then the amount of nisin to inhibit the growth of wt-*B. subtilis* BR151 on plate was also determined. When 200 μg of nisin was spread on a plate, it effectively inhibited the growth of wt-*B. subtilis* BR151. Competent *B. subtilis* SYcat cells were prepared and *L. lactis* DNA was transformed as described in materials and methods Part II-13, 13-3. The selection was tried on nisin spread plates several times, but no transformants were observed. Double recombination is a rare event and it happens fewer than 1 per 10^5 cells. One of the possibility of not observing a transformant was that recombination was not efficient enough to transfer ~14 kb nisin gene cluster and its flanking regions into the *B. subtilis* BR151. It was also possible that the nisin immunity genes did not function properly in *B. subtilis* BR151. In order to circumvent these possibilities, a selection marker that is known to work in *B. subtilis* BR151 was employed.

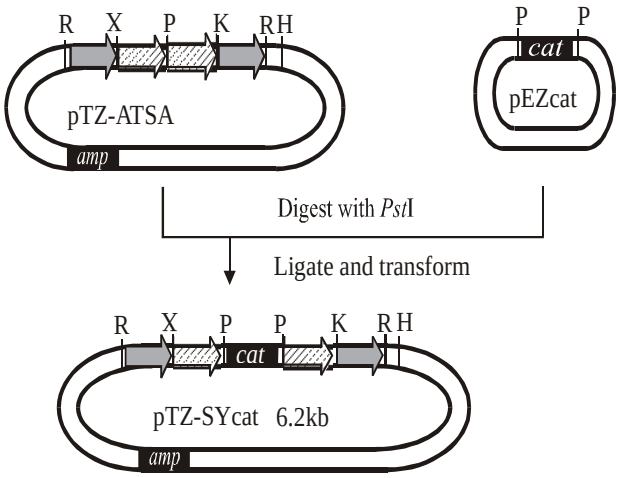


H = *Hind*III, R = *Eco*RI

Figure 5. Construction of pSYcat.

The homology regions were amplified by PCR using Taq polymerase, which has the ability to add single 3' A residues at the end of DNA in a non-template dependant way. *Lactococcus lactis* 11454 genomic DNA was used as a template to amplify regions from *transposase* and *sac*R and *B. subtilis* BR151 genomic DNA was used to amplify *amy*L and *amy*R. PCR products were ligated to pre-linearized pCR2.1 vector with T overhangs Details of the experiments are explained in Materials and Methods partII-6 through 13.


E
H
mcs
pTZ18R 3.0kb
amp
H R X R
pCR2.1AL
Digest with *Eco*RI
Ligate and transform
R X R H
mcs
pTZ-AL
amp
H R P X R
pCR2.1T5
Digest with *Xho*I/*Hind*III
Ligate and transform
R X P R H
pTZ-AT
amp
H R K P R
pCR2.1S4
Digest with *Pst*I/*Hind*III
Ligate and transform
R X P K R H
pTZ-ATS
amp
H R K R
pCR2.1AR
Digest with *Nar*I/*Hind*III
Ligate and transform



E = *Eco*RI, H = *Hind* III, K = *Kas*I, P = *Pst*I, X = *Xho*I

Figure 5. (Continued)

Each of these fragments was subcloned into the multi purpose-cloning site of pTZ18R sequentially. pCR2.1-AL was digested with EcoRI and the *amyL* fragment was ligated into EcoRI site of pTZ18R resulting in pTZ-AL. pCR2.1-T5 and pTZ-AL were digested with XhoI/HindIII and the *transposase* fragment from pCR2.1-T5 ligated with the vector pTZ-AL to create pTZ-AT. PstI/Hind III *sacR* fragment from pCR2.1-S4 was put into the same restriction sites in pTZ-AT, making pTZ-ATS. The plasmid pTZ-ATSA was made by opening up pTZ-ATS with KasI/HindIII and ligating the *amyR* fragment from pCR2.1-AR, which was also digested with KasI/HindIII. Finally *cat* gene from pEZcat, which was contructed by Gonzalo Izaguirre, was digested with PstI to release *cat* gene and it was ligated into pTZ-ATSA to construct pSYcat.

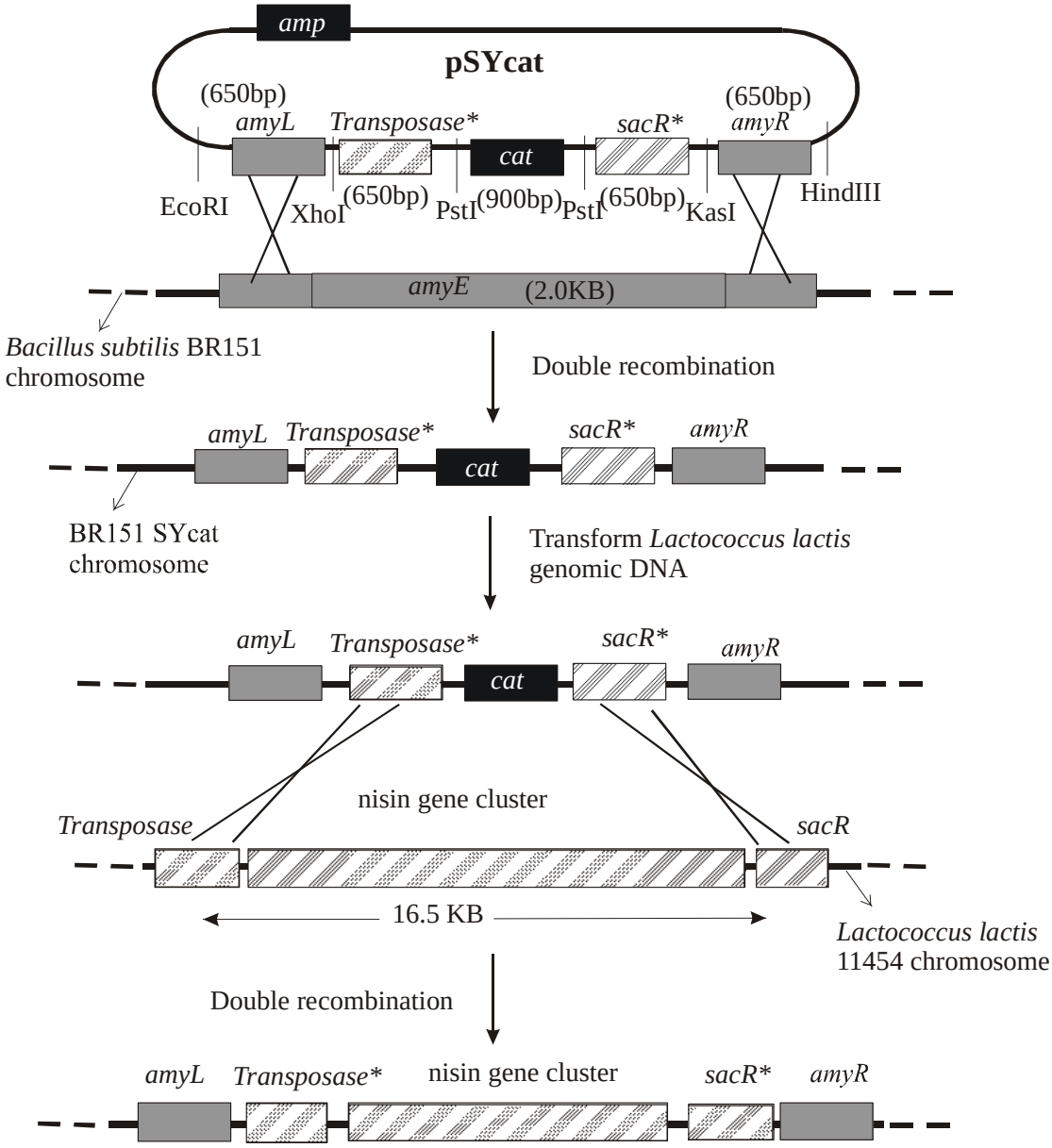


Figure 6. Cloning strategy to transfer nisin genes.

First part of the scheme illustrates the recombination event that took place between pSYcat and *B. subtilis* BR151 chromosome. About 0.5 μg of HindIII linearized pSYcat was transformed into the host, integrating the homologous sequences. The new strain was called *B. subtilis* SYcat. The second recombination event was expected to happen when about 2 μg of *L. lactis* 11454 chromosomal DNA was transformed into *B. subtilis* SYcat replacing the *cat* gene with nisin gene cluster.

Name	Sequence (5’ to 3’)
SYALf	TTGCAAAACGATTCAAAACC
SYALr	CTCGAGGACATCCCATCGATCAGACC
SYALh	GCCATTCAGACATCTCCGAT
SYTf	CCTCGAGCAATGTCTTCACGGATGCAA
SYTr	CCTGCAGATACCTTTGATAAGGTTACT
SYTh	GATTGTCCATACTGATCAAG
SYSf	CCTGCAGGCGTCGATTGATTCTCTCAT
SYSr	TGGCGCCTGGTAGGTTTTGATGGAACTGA
SYSh	TTCTCGGCGAATATGGTC
SYARf	CGGCGCCAGGAACTCTCGAACCCGAAT
SYARr	TCAATTACTCGGCTCCCATC
SYARh	GTGAACGATGGTAAACTGACAG

Table 2. Oligonucleotides used in Results Part I-1.

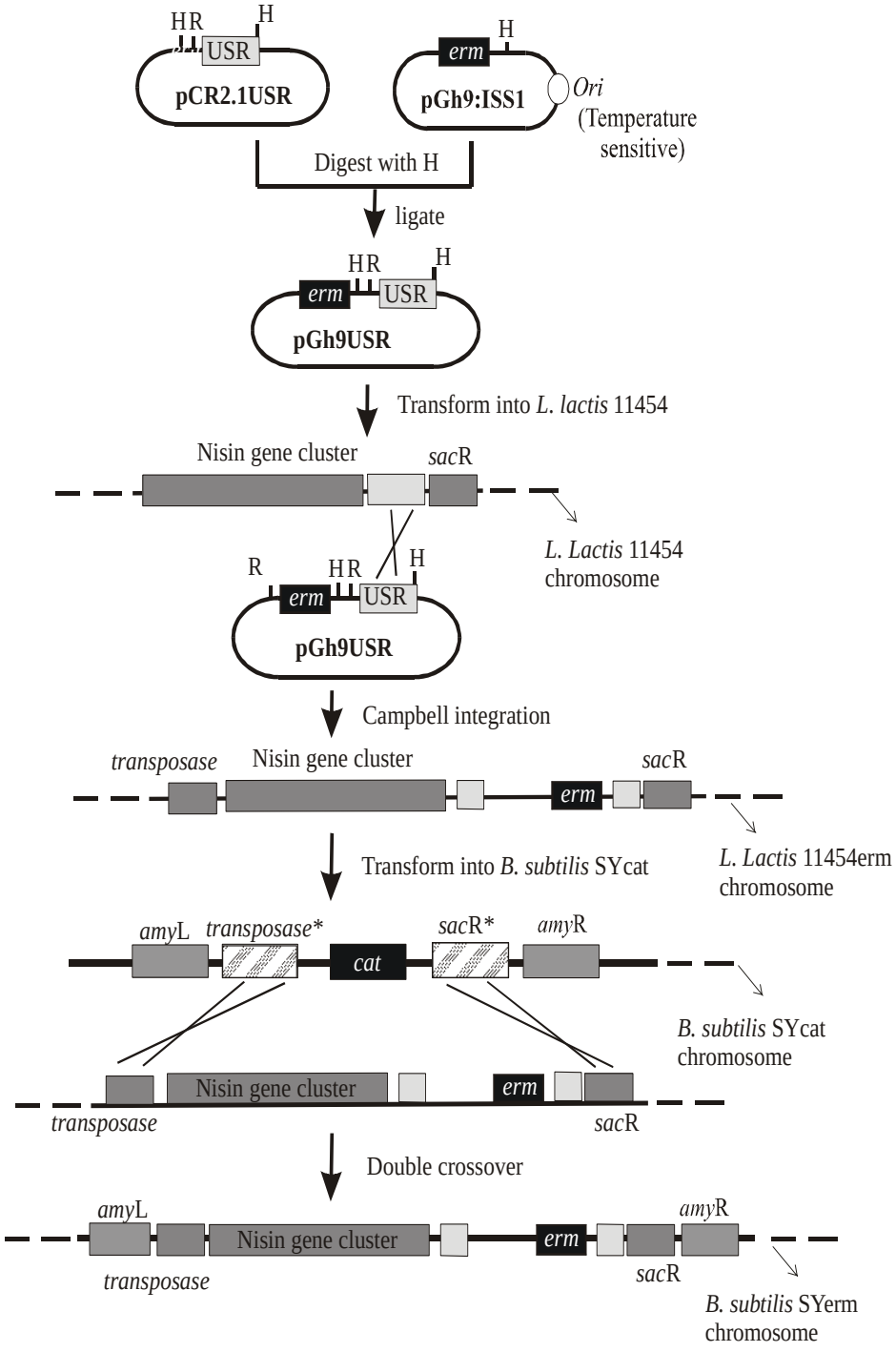
Oligonucleotides SYALf and SYALr were used in the construction of pCR2.1AL, SYTf and SYTr were used in the construction of pCR2.1T5, SYSf and SYSr were used in the construction of pCR2.1S4, SYARf and SYARr were used in the construction of pCR2.1AR and SYALh, SYTh, SYSh, SYARh were used as hybridization probes.

Part II. Selection strategy using the thermosensitive plasmid pGh9:ISS1.

In this strategy, transformants that had integrated the nisin gene cluster were selected by using an *erm* gene that is known to function in *B. subtilis* (Figure 7). The plasmid pGh9:ISS1, which contains the thermosensitive replicon pG+host (52), was used to integrate the erm selection marker into downstream of nisin gene cluster in the *L. lactis* 11454 chromosome. This plasmid can replicate at 28 °C in *L. lactis*, *E. coli* and *B. subtilis* but cannot replicate if the temperature is raised above 37 °C. A detailed description of the strategy was illustrated in Figure 7.

Part II-1. Construction of pGh9USR and electroporation into *L. lactis* 11454.

The 630 bp homology region from the upstream *sac*R gene was amplified by PCR as described in Material and Methods Part II, II-10 and cloned into pCR2.1 vector by AT cloning. The primers used for this reaction were listed in Table 4. The clone containing the upstream *sac*R homology region (pCR2.1USR) was subcloned into pGh9:ISS1. The transformants were analyzed with restriction enzyme digestions. The clone containing the homology region was selected and named pGh9USR. Electrocompetent *L. lactis* 11454 cells were prepared and the pGh9USR was electroporated into the cells as described in Materials and Methods Part II-13, 13-4 in order to integrate the *erm* gene to the downstream region of nisin gene cluster. The cells seemed coagulated after the addition of M-17 medium even though a single pulse at the regular strength was applied as recommended. This did not happen with the *E. coli* control electroporation. The cells were incubated at 28 °C for 1.5 hr then 30 more minutes in the presence of 0.5 μg/ml erm and plated on M-17 erm (5 μg/ml) plates. Even though the plates were incubated at 28 °C



E = *EcoR*I, H = *Hind* III, USR = upstream *sac*R homology region

Figure 7. Construction of the recombinant strain *B. subtilis* SYerm.

A schematic representation of the strategy for the construction of the recombinant strain *B. subtilis* SYerm.The plasmid, pCR2.1USR was digested with *Hind*III to release the upstream *sac*R fragment which was isolated and ligated into *Hind*III linearized, pGH9:ISS1. The construct pGh9USR was then electroporated into *L. lactis* 11454 (Materials and Methods Part II-13, 13-5). The DNA was isolated from the recombinant strain, *L. lactis* 11454erm (Materials and Methods Part II-5, 5-2) and transformed into *B. subtilis* SYcat (Materials and Methods Part II-13, 13-3). The last step shows the double recombination that could take place between the *L. lactis* 11454erm and *B. subtilis* SYcat forming the recombinant strain *B. subtilis* SYerm.

for prolonged times, no colonies were observed on the plates. The electroporation was repeated with several different parameters to optimize the electroporation conditions. For example, an osmotic stabilizer such as sucrose and/or glycerol was included in the cell suspension. Below is a list of conditions tried.

Tube #	Cells (70 μl)	Voltage	Resistance	Pulse length	Amount of DNA
#1	10% glycerol	1.70 kV	129 ohms (R5)	4.15 msec	1.5 μl
#2	10% glycerol	1.30 kV	129 ohms (R5)	4.58 msec	1.5 μl
#3	10% glycerol	1.50 kV	129 ohms (R5)	4.25 msec	3 μl
#4	10% glycerol	1.70 kV	72 ohms (R4)	2.84 msec	3 μl
#5	10% glycerol + 0.5 M sucrose	1.70 kV	129 ohms (R5)	4.33 msec	2 μl
#6	10% glycerol + 0.5 M sucrose	1.20 kV	129 ohms (R5)	4.93 msec	2 μl
#7	10% glycerol	1.70 kV	129 ohms (R5)	4.04 msec	–

The cells in tube #7 was plated on M-17 plate without any antibiotic to make sure that cells were still viable after the applied electric field. A lawn of cells were observed on the plate after an overnight incubation at 37 °C. This confirms that many cells were still viable after the electroporation. Cells from the other tubes were plated on M-17 erm (3 μg/ml) plates and incubated at 28 °C. After 72 hr colonies were observed on some of the plates, and they had mixed phenotypes. Some of the colonies were whiter in color than the wt-*L. lactis* colonies and some of them were not round as the wt-*L. lactis* cells. Nine of the colonies that looked like *L. lactis* 11454 were streaked onto M-17 plates with

a higher concentration of erm (5 μg/ml) and incubated overnight at 28 °C. Also, a master plate was prepared by griding the cells onto M-17 erm (3 μg/ml) to keep track of the colonies. All of the clones grew on these plates but some of the colonies were varied considerably in size, some of them larger than average, and some of them smaller than average.

Part II-2. Testing the integration of pGh9USR into the chromosome of *L. lactis* 11454.

One of the clones was chosen to test whether integration of pGh9USR into the chromosome of *L. lactis* 11454 had occured. With a sterile loop, some of the cells were scooped out from the master plate and resuspended in 300 μl of M-17 medium. The cells were incubated at 30 °C for an hour. Then 50 μl of the undiluted and 1/10 diluted cells were spread onto two M-17 erm (5 μg/ml) plates. One plate with diluted and one plate with undiluted cells was incubated at 37 °C and the other two plates with diluted and the undiluted cells were incubated at 28 °C overnight. The cells that had integrated the plasmid pGh9USR into its chromosome should survive at 37 °C and thus be resistant to erm. However, the ones that have the plasmid in the cytoplasm will not be viable because the plasmid has no active origin of replication. The following morning, the density of the growth on the surface of the plates was compared. It was not possible to compare the plates to which the undiluted cells were spread since the colonies were too dense. However, less growth was observed with the 1/10 diluted plate that was incubated at 37 °C when compared with the one incubated at 28 °C. This was the expected result since the chance of integration of the plasmid into the chromosome is less than the plasmid just transforming into the cytoplasm of the cell. To further confirm the integration of

pGh9USR into the chromosome of *L. lactis* 11454, chromosomal DNA from the two of the recombinant colonies were isolated (Materials and Methods Part II-5, 5-2) and digested with 4 different restriction enzymes (*Xho* I, *Hind* III, *Kas* I, *Sac* I). The digestions were run on a 0.8 % agarose gel and then blotted onto a nitrocellulose paper (Materials and Methods Part II-15, 15-2). This Southern blot was then hybridized with a ^{32}P end labeled probe (Table 3) against upstream *sac*R region to see if there was any shift in the appearance of the band containing the upstream *sac*R region due to the incorporation of the plasmid into the chromosome. A slight shift was observed in the *Sac*I restriction pattern of the 2 of the recombinant DNA tested. This recombinant strain was called *L. lactis* 11454erm.

Part II-3. Transformation of *L. lactis* 11454erm into *B. subtilis* SYcat and testing the expression of peptides.

Chromosomal DNA from *L. lactis* 11454erm was isolated and transformed into competent *B. subtilis* SYcat cells as described in Materials and Methods Part II-13, 13-3. The transformants were selected on PAB erm (5 μg/ml) plates. 15 colonies were observed after incubating the plates at 37 °C for 24-48 hr. The transformants were gridded onto a PAB erm (5 μg/ml) plate with the help of a sterile toothpick (to keep as a master plate) and then onto PAB-Cm (10 μg/ml) plate at an identical position to select for the disappearence of the *cat* gene as a result of the recombination. Six of the transformant strains were sensitive to choloramphenicol (Materials and Methods Part II-19). The Cm sensitive recombinant strain was called *B. subtilis* SYerm. These transformants were grided onto a PAB plate along with *B. subtilis* SYcat to do a halo assay as described in

Materials and Methods Part II-24. There was not any difference between the halos formed around the original and the transformant strains. This was just a preliminary test and the conditions on the plate might not represent how the bacteria behave in the culture medium. Thus, the recombinant strain was tested for the expression of peptides as described in Materials and Methods Part II-20. The *B. subtilis* SYerm was cultured in Medium A for 28 hr. The hydrophobic peptides from the culture supernant were purified on a HIC column (Materials and Methods Part II-21) and then the eluate was resolved on RP-HPLC (Materials and Methods Part II-22). The fractions were analyzed with a spore outgrowth inhibition assay (Figure 8) and then all the fractions were analyzed with MALDI-TOF MS to detect the molecular weight of the peptides present in these fractions. As confirmed from the mass spectra of the fractions, the activity observed in the halo assay was due to the production of sublancin. Sublancin was another lantibiotic naturally produced by *B. subtilis* 168. The characteristics of nisin halo, which leaves all clear zones, were also different from the ones observed in figure 8. No other mass peaks were identified that might be belong to nisin or nisin variants. Since neither nisin nor the nisin variants were detected with the halo assay and MALDI-TOF MS analysis, a Southern blot was performed on five of the Cm sensitive recombinant clones obtained as a result of transformation of *L. lactis* 11454erm into *B. subtilis* SYcat. The chromosomal DNA was isolated from these clones (Materials and Methods Part II-5, 5-1) and three sets of DNA were digested with *Hind* III. The digestions were run on a 0.8% gel and blotted onto three nitrocellulose papers as described previously. Each of the blots was hybridized with three different probes end labeled with ^{32}P (Materials and Methods Part II-16, 16-1). A probe from *nis*A, a probe from *nis*I, and another from *nis*G, which were present at the

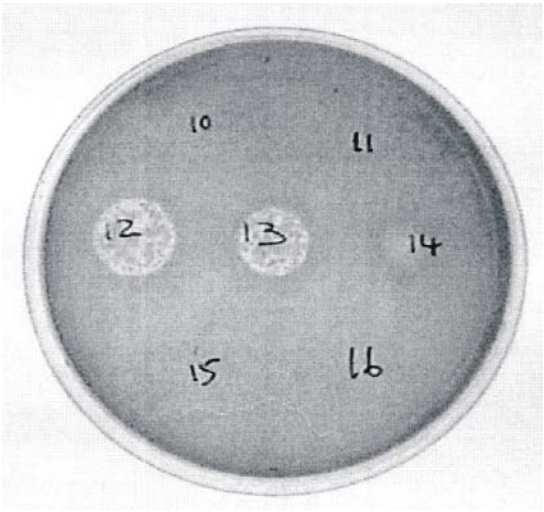


Figure 8. Analysis of the purified culture supernatant from *B. subtilis* SYerm with halo assay.

The extracellular materials from the culture supernatant of a 28 hr Medium A culture of *B. subtilis* SYerm were isolated by HIC and RP-HPLC as previously described. The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores. Activity was observed at fractions 12, 13, and 14 as seen above which belonged to anti microbial peptide sublancin. The sublancin slightly comes early out of the column because a different RP-HPLC column (4.6 mm x 150 mm ZORBAX 300 Extend C-18 column with 5 μm pore size) was used only in this experiment.

beginning, middle and end of the nisin gene cluster respectively was chosen to hybridize with the blots (See Table 3 for the sequence of the oligonucleotide probes). No signal was observed in any of these blots. This was not expected since it was hard to explain the disappearance of the *cat* gene without a double crossover taking place unless the colonies were contaminants. The limited genetic tools for *L. lactis* were one of the main reasons of trying to express nisin genes in *B. subtilis*. The problem in this case was most likely to be related to the inefficient transformation of the plasmid into *L. lactis* 11454. This result showed that the selection approach that did not involve manipulations to *L. lactis* cells would be a better choice.

Name	Sequence (5’ to 3’)
SYUSRf	AAGCTTGAACCAAAGCAAAACTGACG
SYUSRr	AAGCTTGCTCAAGTCCGTCGTCCTTA
SYNISAh	GCATCACCACGCATTACAAG
SYNISGh	GGAGCATGATTATCGCGATT
SYNISIh	AGCTTGCAACGAAGGTAGGA

Table 3. PCR primers for upstream *sac*R homology region and hybridization probes for *nis*A, *nis*G, and *nis*I genes.

Oligonucleotides SYUSRf and SYUSRr were used in the construction of pCR2.1USR. SYNISAh was used to detect the *nisA*gene, SYNISGh was used to detect *nisG* gene, and SYNISIh was used to detect *nisI* gene.

Part III. Selection of transformants integrating the nisin gene cluster by two-step recombination in B. subtilis SYcat.

Summary of the approach:

This strategy was modified so that genetic manipulations would not need to be performed with the *L. lactis* DNA. The strategy employed a plasmid with temperature sensitive origin of replication. This plasmid would be transformed and propogated in the recipient *B. subtilis* strain which would then be transformed with *L. lactis* DNA. Recombination sites would be present in the chromosome so that it would be able to integrate the nisin gene cluster. Then a second recombination in the cytoplasm will be established with the integration of the plasmid into the *B. subtilis* chromosome. The recombination site for the plasmid containing the selection gene will be present in the chromosome only if the first recombination with the *L. lactis* DNA did take place. The cells would then be subjected to the non-permisive temperature. Since the plasmid would stop replicating, the only way a cell could survive on antibiotic selection is to integrate the plasmid. A detailed description of the strategy was illustrated in Figure 9 and explained in the below section.

Part III-1. Transformation of temperature sensitive plasmid pGh9USR into *B. subtilis* SYcat.

Competent *B. subtilis* SYcat was prepared and efficiently transformed with pGH9USR as described in Materials and Methods Part II-13, 13-3 except the cells were incubated at 28 °C instead of 37°C then the cells containing the plasmid were selected at

28 °C by spreading on PAB-erm (5 μg/ml) plates. The permissive temperature was used for selection because the plasmid cannot integrate into the chromosome since there is not any homology between them. The strain containing the plasmid was then made competent and transformed with wt- *L. lactis* chromosomal DNA as described previously except the cells were incubated at 2 hr at 28 °C instead of 37 °C and at the end of this period cells were diluted to 500 ml with PAB. Also a control experiment was set up where no *L. lactis* chromosomal DNA was added to the competent *B. subtilis* SYcat cells. Then the incubation continued at 28°C overnight in the presence of 5 μg/ml erm to allow time for the 2 consecutive recombination events that were described above to take place. The next morning, 50 ml of the culture was again diluted with 500 ml PAB supplemented with 5 μg/ml erm and incubated at non-permisive temperature (37 °C), in order to get rid of non-transformant cells. The OD_{600} of the two cultures were monitored and recorded as seen in below. An aliquot was taken from the

	4hr	7.5 hr	18 hr
OD_{600} Control	0.32	0.60	1.09
OD_{600} Sample	0.32	0.85	1.09

sample after 7.5 hr and serially diluted to 10^{-8} and then 10^{-4}, 10^{-6}, 10^{-7} and10^{-8} dilutions were spreaded on PAB-erm (5 μg/ml) plates. The colonies from the sample plate were then grided on Cm containing PAB plates to see if any of the transformants were Cm sensitive. 1000 cells were screened, however none of the erythromycin resistant clones were sensitive to chloramphenicol. It was possible that after all, the recombination was not efficient enough to produce a double recombinant. Nisin gene cluster was a relatively large segment of DNA that is present in the genomic DNA in a single copy. Perhaps, in

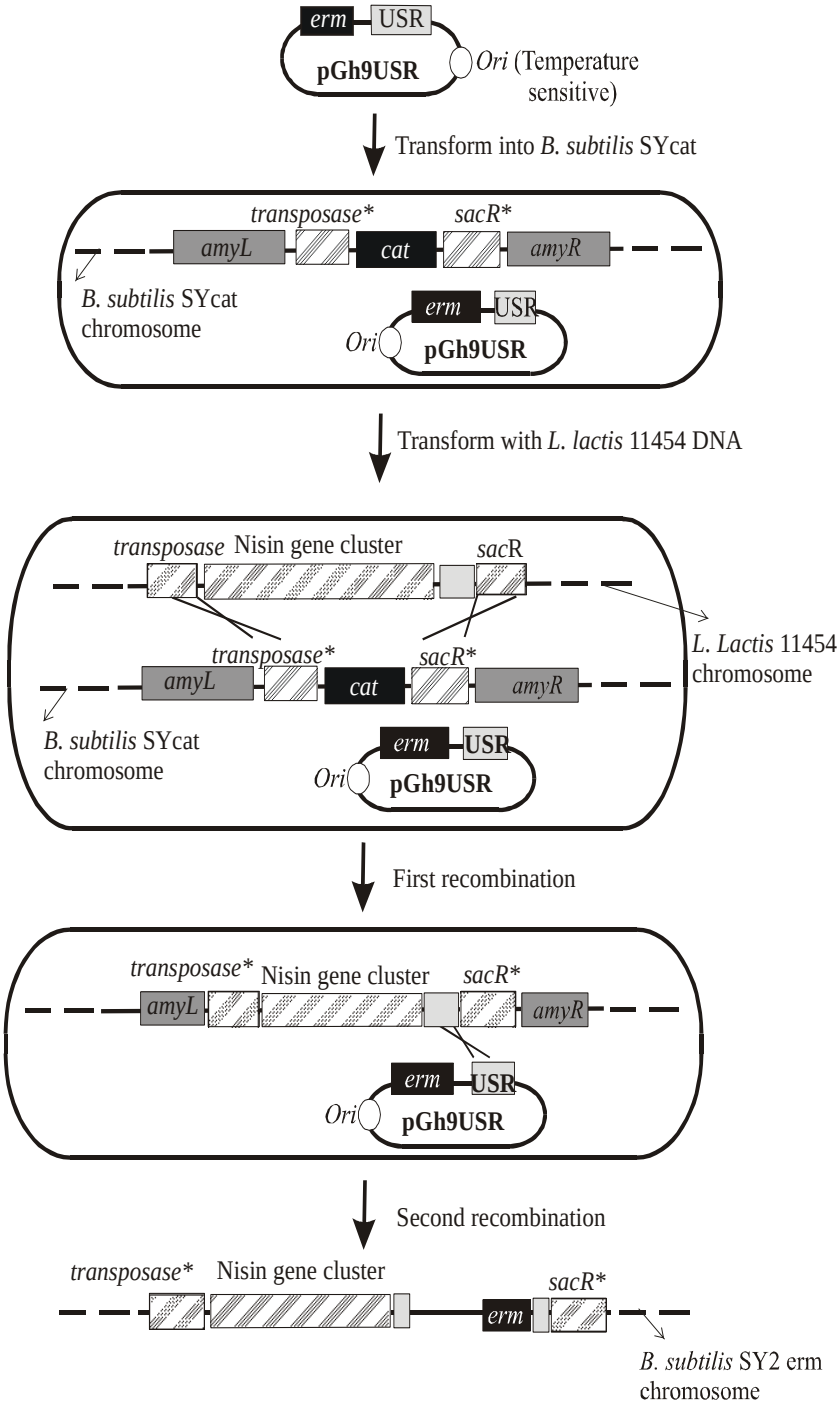

erm
USR
pGh9USR
Ori (Temperature sensitive)
Transform into B. subtilis SYcat
transposase*
sacR*
amyL
cat
amyR
B. subtilis SYcat chromosome
erm
USR
Ori
pGh9USR
Transform with L. lactis 11454 DNA
transposase
Nisin gene cluster
sacR
L. Lactis 11454 chromosome
transposase*
sacR*
amyL
cat
amyR
B. subtilis SYcat chromosome
erm
USR
Ori
pGh9USR
First recombination
transposase*
Nisin gene cluster
sacR*
amyL
amyR
erm
USR
Ori
pGh9USR
Second recombination
transposase*
Nisin gene cluster
sacR*
erm
B. subtilis SY2 erm chromosome

Figure 9. Strategy for construction of the recombinant strain *B. subtilis* SY2erm.

A schematic representation of the strategy to construct the recombinant strain *B. subtilis* SY2erm. Competent *B. subtilis* SYcat cells were prepared and pGH9USR was transformed as described in Materials and Methods Part II-13, 13-3 except the cells were incubated at 28 °C since the plasmid did not have any homology to the chromosomal DNA. Then the chromosomal DNA from *L. lactis* 11454 was isolated (Materials and Methods Part II-5, 5-2 and transformed into *B. subtilis* SYcat (Materials and Methods Part II-13, 13-3). First the cells were incubated at 28 °C to allow the recombination between two chromosomal DNA through homology at *transposase* and *sac*R and then with the pGh9USR through upstream *sac*R homology region. Then the temperature was raised to 37 °C to select for the transformants that had integrated plasmid into the chromosome. The last step shows the single crossover that could take place between the pGh9USR and *B. subtilis* SYcat forming the recombinant strain *B. subtilis* SY2erm.

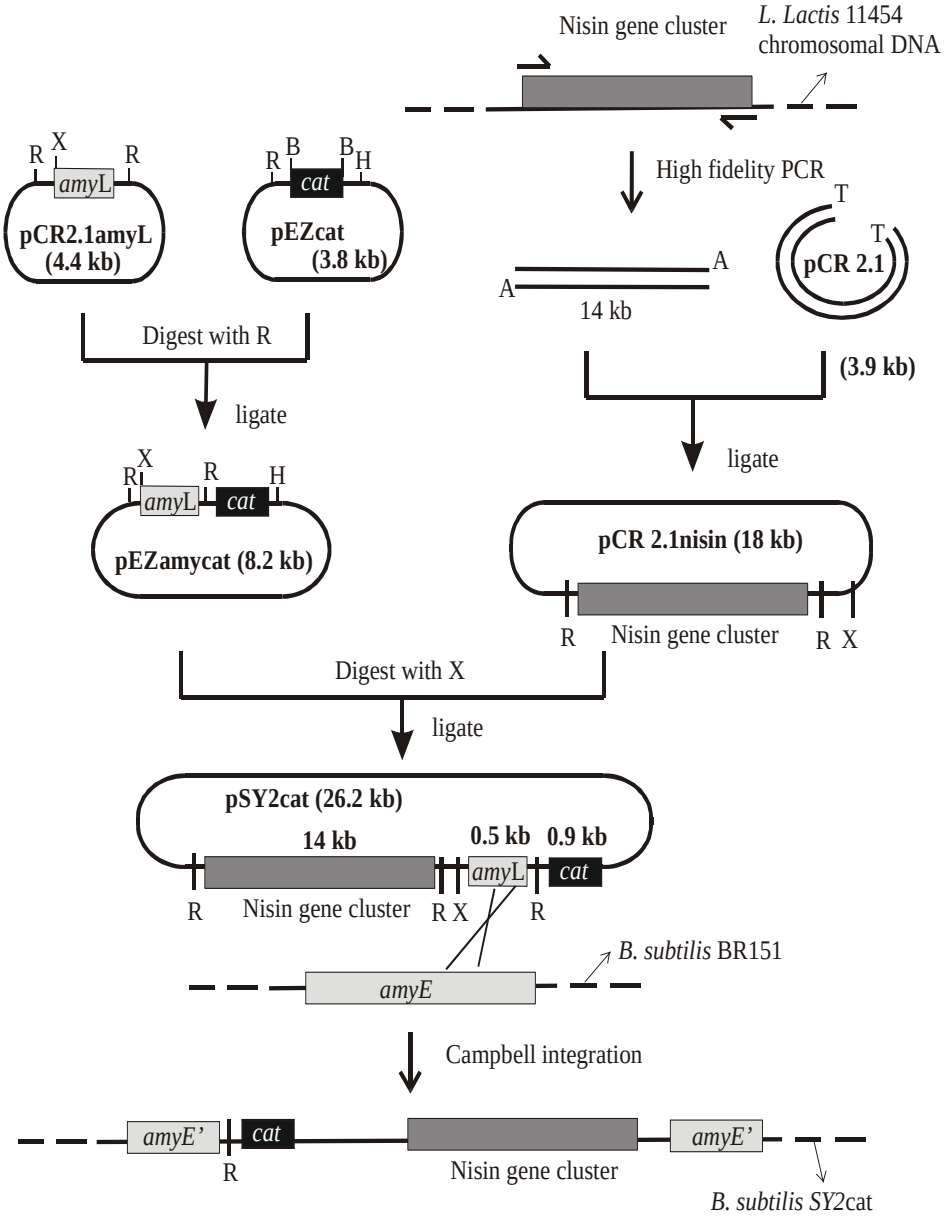
order to get sufficiently efficient recombination, it will be necessary to increase the copy number of the nisin genes. Strategies to achieve this were then developed.

Part IV. Using PCR to amplify the nisin gene cluster and integrate the nisin genes into *B. subtilis* 168.

With today's technology, robust enzymes that are claimed to be able to amplify DNA sequences up to 40 kb in length with high fidelity are available. By using one these commercially available enzymes, it would perhaps be possible to amplify nisin gene cluster from *L. lactis* 11454 genomic DNA and clone this into a plasmid and then transform into *B. subtilis* BR151. Since every transformed cell would contain a copy of the nisin gene cluster, the chance of recombination would be greatly enhanced. This new strategy was developed to increase the chance of recombination. The outline of the strategy was illustrated in Figure 10. The plasmid containing the cloned nisin genes (pCR2.1nisin) was ligated to another plasmid containing the *cat* gene and a single homology region from *amy*E gene of *B. subtilis* 168. A single homology region was placed to integrate the final plasmid into the chromosome of *B. subtilis* 168 with Campbell integration because single recombination tends to be more efficient than the double recombination. This strategy also eliminates the competition by extraneous *L. lactis* genomic DNA.

Part IV-1. PCR of nisin gene cluster and cloning into pCR2.1 vector.

To implement this strategy the 14 kb nisin genes were amplified from the *L. lactis* 11454 chromosome (Figure 11) as described in Materials and Methods Part II-10, 10-2. The primers used were listed in Table 4. An aliquot of the amplified DNA was run on a 0.8 % agarose gel and visualized on a UV-transilluminator. The PCR product appeared at



B = *BamH*I, E = *Eco*RI, H = *Hind* III, X = *Xho*I

Figure 10. Construction of pSY2cat and integration of nisin genes into the chromosome of *B. subtilis* BR151.

A schematic representation for the construction of pSY2cat and the integration of nisin genes into the *B. subtilis* BR151 chromosome. The nisin gene cluster was amplified from *L. lactis* 11454 chromosome as described in Materials and Methods Part-II, II-10 and then cloned into pCR2.1 AT cloning vector forming plasmid pCR2.1nisin. Another vector, pEZamycat, was constructed by ligating the 527 bp *amy*L *Eco*RI fragment from pCR2.1amyL into the *Eco*RI site of pEZcat. Then both pEZamycat and pCR2.1nisin was digested with *Xho*I and ligated to each other forming the final construct, pSY2cat. This construct was then transformed into *B. subtilis* BR151. The chloramphenicol resistant recombinant strain was formed with the integration of the whole plasmid into the *amy*E gene of *B. subtilis* BR151. The new strain was named *B. subtilis* SY2cat.

the correct molecular weight and was free of non-specific products. Amplified DNA was directly ligated into pCR2.1 vector and then transformed into *E. coli* INVαF′ cells (Materials and Methods Part II-). It was possible to do AT cloning since the PCR enzyme being employed has the ability to add non-specific adenosine residue to the 3' end of the amplified sequence just as *Taq* polymerase does. Twenty clones were analyzed with the *Xho*I digestion. Nine of the clones gave the expected band at 18 kb (vector + nisin gene cluster) when linearized. These clones were further analyzed with *EcoR*I and *EcoR*V digestions to see if the fragments matched the expected digestion pattern obtained from the restriction map of the nisin gene cluster (Figure 12). Also, PCR was performed to see if the 1.5 kb region that contains the *nis*A gene could be amplified from these nine transformants. This region was amplified from eight of the plasmid DNA. The clone giving the best match (pCR2.1 nisin) for the restriction enzyme digest patterns was chosen to clone (clone #6) into the pEZamycat. This plasmid contained a single homology region to the *amy*E gene to integrate the construct through single crossover as previously discussed. The plasmid pEZamycat was constructed by subcloning the *amy*L homology region from pCR2.1AL (Results Part I-1) into pEZcat. The resultant plasmid, pEZamycat was ligated to the linearized pCR2.1nisin. The final vector was called pSY2cat and transformed into *B. subtilis* BR151 (Figure 10). Transformants were selected on chloramphenicol containing plates. 8 colonies that were resistant to Cm were obtained. The transformants were tested with halo assay and colonies producing the bigger halos were further tested for sensitivity to nisin with incubating the cells in various concentrations of nisin. The colony producing the best result was chosen and named *B. subtilis* SY2cat and further tested for the expression of nisin.

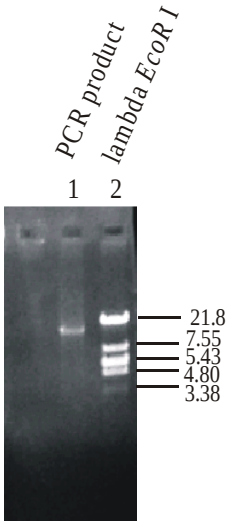
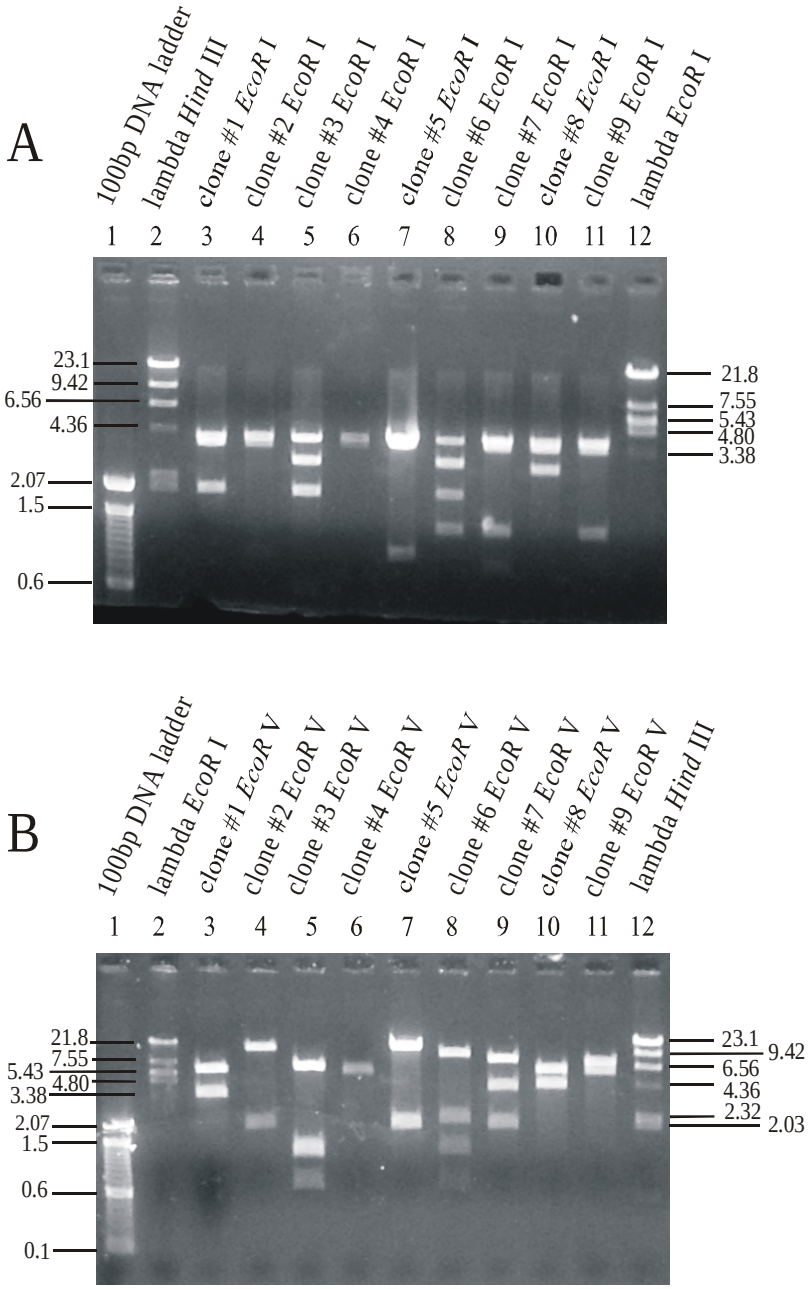


Figure 11. PCR of nisin genes from *L. lactis* 11454.

The picture shows the 5 μl aliquot of the PCR product that was run on a 0.8 % agarose gel along with the size standard, λ DNA *Eco*RI digest. The conditions for amplifying the nisin genes from *L. lactis* with PCR was described in Materials and Methods Part-II, II-10 and the isolation of *L. lactis* genomic DNA was described in Materials and Methods Part-II, II-4.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lambda EcoR I
1 2 3 4 5 6 7 8 9 10 11 12
23.1
9.42
6.56
4.36
2.07
1.5
0.6
21.8
7.55
5.43
4.80
3.38
B
100bp DNA ladder
lambda EcoR I
clone #1 EcoR V
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lambda Hind III
1 2 3 4 5 6 7 8 9 10 11 12
21.8
7.55
5.43
4.80
3.38
2.07
1.5
0.6
0.1
23.1
9.42
6.56
4.36
2.32
2.03

Figure 12. Restriction enzyme analysis of the pCR2.1nisin clones.

PCR amplified nisin genes were cloned into pCR2.1 and transformed into *E. coli* INVαF′ cells. The plasmid DNA was isolated from transformants (Materials and Methods Part II, II-9) and analyzed with *Eco*RI and *Eco*RV restriction enzyme digestions (Materials and Methods Part II, II-9) for the presence of expected band patterns. A lot of variation was observed within the clones. Panel A shows the transformant plasmid DNAs digested with the *Eco*RI. From the restriction map of nisin genes in *L. lactis*11454 and the plasmid pCR2.1, the bands at 1310 bp, 1979 bp, 2917 bp, 3660 bp, 3900bp and 4078 bp were expected. Most of the bands were observed at clone #6 except the band at 2917 bp, and the bands at 3660 bp and 3900 bp probably could not be resolved on the 0.8% gel. Panel B shows the transformant plasmid DNAs digested with *Eco*RV. The bands were expected at 7687 bp, 5900 bp, 2289 bp, 1319 bp and 706 bp. Clone #6 again gave the best match to the expected pattern. However, the band at 5900 bp was missing and the band at 7687 was bigger than expected.

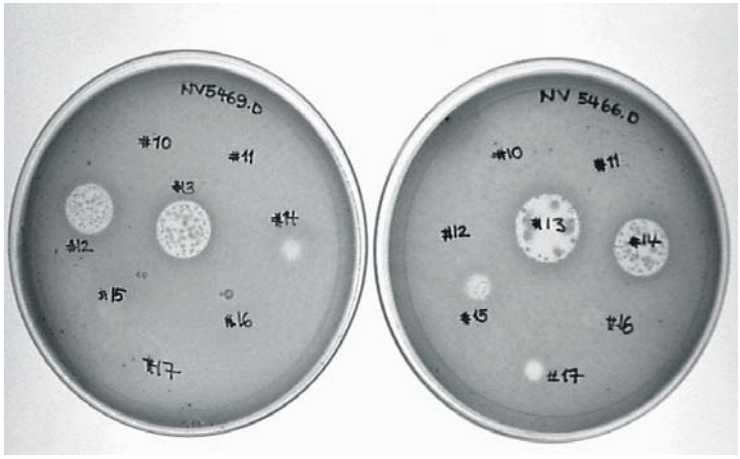
Part IV-2. Testing the expression of nisin from *B. subtilis* SY2cat.

B. subtilis SY2cat was cultured in Medium A for 28 hr as described in Materials and Methods Part II-15 to test the expression of nisin. Nisin expression is under the control of a response regulator that senses the presence of extracellular nisin and switches on the promoter to start the transcription (Introduction Part-V). A similar requirement could possibly be necessary for the expression of nisin genes from *B. subtilis*. The amount needed to turn on the system was relatively small for *L. lactis*, however, it has been reported that nisin sensitivity vary significantly, even between members of the same species (2). Scott *et. al* (22) had found out that there is considerable variation in nisin sensitivity among *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, *B. subtilis*, and *E. faecalis*. When they had tested the *nis*A promoter driven expression in these organisms, they observed the greatest induction from the *nis*A promoter when nisin was added to the culture with a concentration just below the inhibitory level and it was 20 μg/ml for *B. subtilis*168.

Two cultures were set up to test the nisin production from the *B. subtilis* SY2cat. One of the 5 ml overnight cultures were grown in the presence of 10 μg/ml choloramphenicol and the other with 20 μg/ml nisin. The next morning, these cultures were used to inoculate flasks containing 500 ml medium A and incubated for 28 hr. The culture supernatants were applied to a HIC (hydrophobic interaction column) as described in Materials and Methods Part-II, II-21. The eluate was then resolved on RP-HPLC (Materials and Methods Part-II, II-22) and the fractions were tested for biological activity against outgrowing *B. cereus* T spores (Figure 13). All the fractions were also analyzed with MALDI-TOF MS. No differences were observed in the spectra of the

fractions between these two cultures (Figure 13). The activities observed at the halo assays were found to be related to another anti-microbial peptides subtilosin (m= 3398.9 Da) that is naturally produced by wt-*B. subtilis* BR151. There were not any other unidentified peaks. Possible reason of not finding any nisin peak could be the errors introduced into one or more nisin genes, which were essential for nisin biosynthesis, during the PCR. Although the long PCR enzymes were claimed to be high fidelity up to 40 kb, within the 20 clones analyzed there were a lot of variations in the length of the insert and the restriction digest patterns. The clone that had been integrated into the chromosome also lacked at least one observable band at each digestion, and since the resolving power of the agarose gel was not very good, each band was not always clearly identifiable. These size discrepancies suggest that there could be a severe fidelity problem during the PCR amplification of the *L. lactis* DNA. It was concluded that a new strategy should avoid the use of PCR amplification. The next section describes the construction of a λ library of *L. lactis* DNA.

A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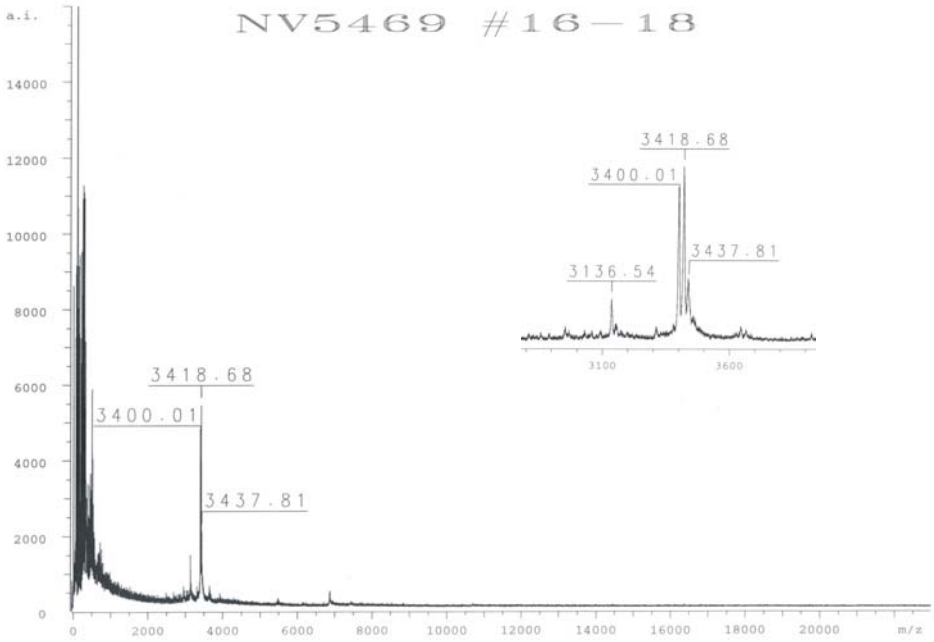
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10000
8000
6000
4000
2000
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3437.81
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6000
8000
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18000
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m/z

C

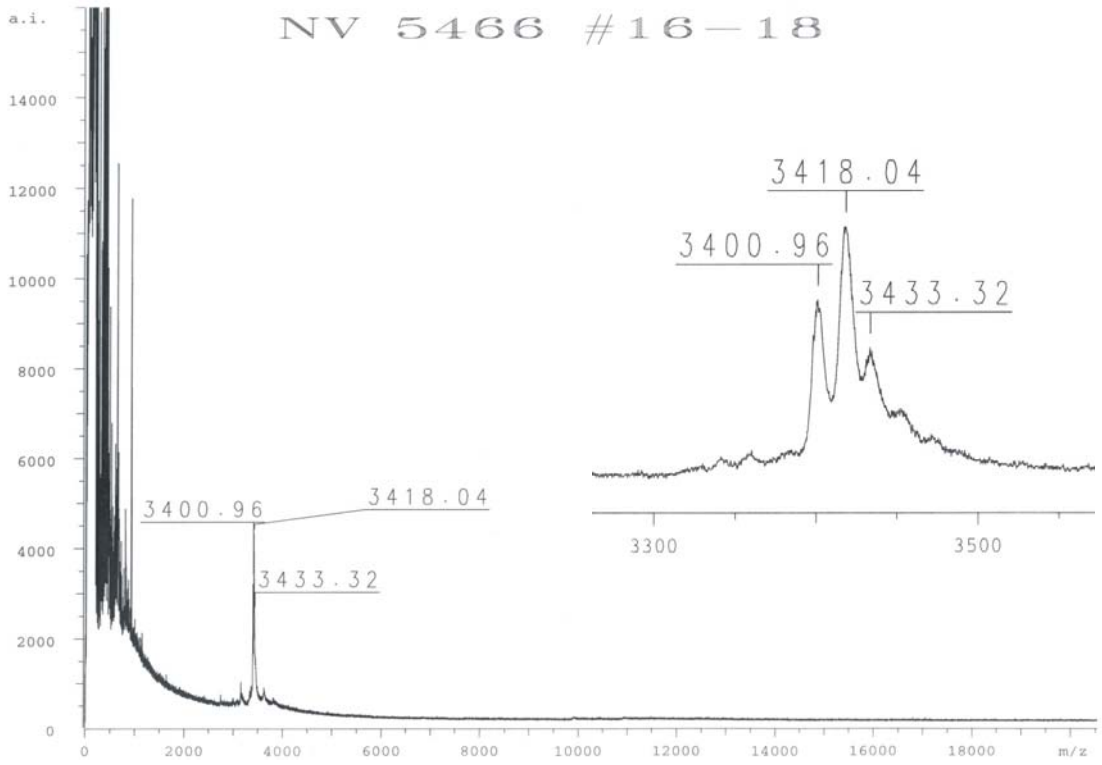


Figure 13. Analysis of the purified culture supernatant from *B. subtilis* SY2cat with halo assay and MALDI-TOF MS.

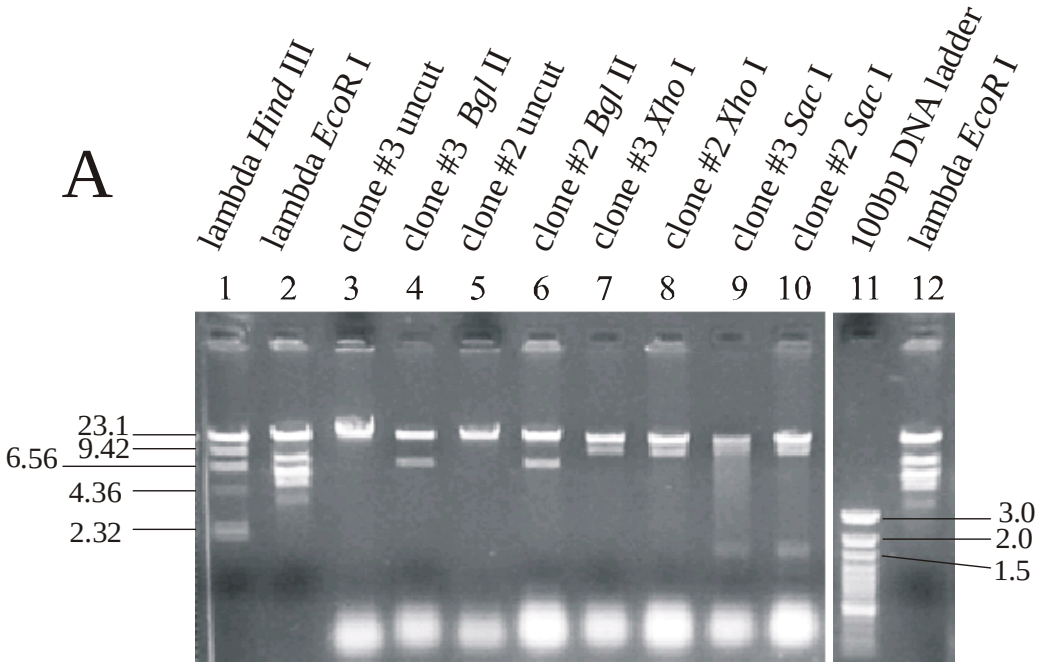
B. subtilis SY2cat was inoculated into 5 ml PAB media in the presence of 20 μg/ml nisin (left plate) or 10 μg/ml Cm (right plate) and were cultured overnight. These 2 cultures were used to inoculate a seperate 500 ml Medium A media and incubated for 28 hr (Materials and Methods Part II-20). The culture supernatant was analyzed by HIC and RP-HPLC as previously described. The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores as previously described. Activity was observed at fractions 12-14 in the nisin added culture and 13-17 in the Cm added culture as seen in Panel A. All the fractions were analyzed with MALDI-TOF. There was only sublancin in fractions 12-14. Panel B shows the MALDI-TOF MS spectra of fractions 16 through18 obtained from the nisin added culture. Panel C shows the MALDI-TOF MS spectra of fraction fractions 16 through18 obtained from the Cm added culture. Both of them gave a peak at *m/z*=3400 which belongs to another antimicrobial peptide subtilosin (m=3398.9 Da).

Part V. Lambda cloning of nisin genes.

A λ library of *L. lactis* 11454 was created by cloning the *Bgl* II digested genomic DNA into Lambda GEM®-11 genomic cloning vector as described in Materials and Methods Part II-8. As described there, the entire nisin gene cluster is contained within a single *Bgl* II fragment and the *Bgl* II sites are located about 600 bp upstream and downstream of the cluster, respectively. The plaques were hybridized with the end labeled probe against *nis*A, nisin structural gene. 11 filters, each containing approximately 100 plaques were hybridized with the *nis*A probe (Table 3), and about 25 of them gave positive signals. The calculated λ library base was 25,000 independent λ clones. λ DNA was isolated from two of the positive clones, clone #2 and clone #3, and digested with four different restriction enzymes. When the Southern blot of the gel was hybridized with a radiolabeled *nisA* probe, the structural gene was observed at the expected molecular weight bands in both of the clones as shown in Figure 14. The clone #2 was named λnisin and digested with *Xho* I to release the nisin gene cluster (16.5 kb fragment), which was then cloned into 2 different shuttle vectors.

Part V-1. Cloning of nisin genes into pLPvcat and integration into the *B. subtilis*ermΔsunA chromosome.

The shuttle vector pLPvcat was constructed and kindly provided by Leena Paul (62). It was used to clone fragments into the sublancin locus of *B. subtilis* 168. It was designed to have homology regions to upstream and downstream regions of the *sun*A gene (sublancin structural gene), which permits cloned genes to be integrated into the *B.*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clone #2 Bgl II
clone #3 Xho I
clone #2 Xho I
clone #3 Sac I
clone #2 Sac I
100bp DNA ladder
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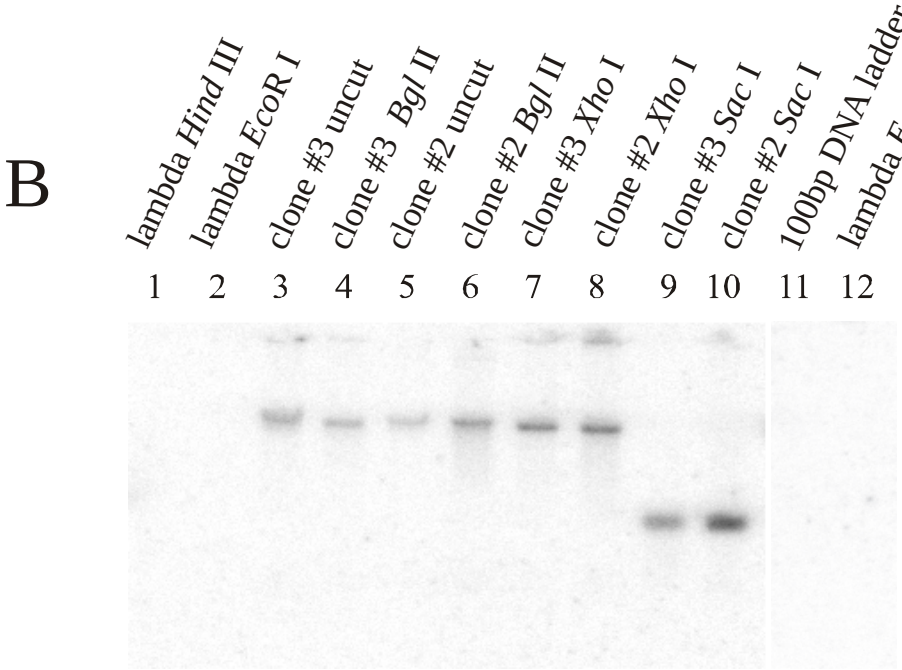
6.56
4.36
2.32
3.0
2.0
1.5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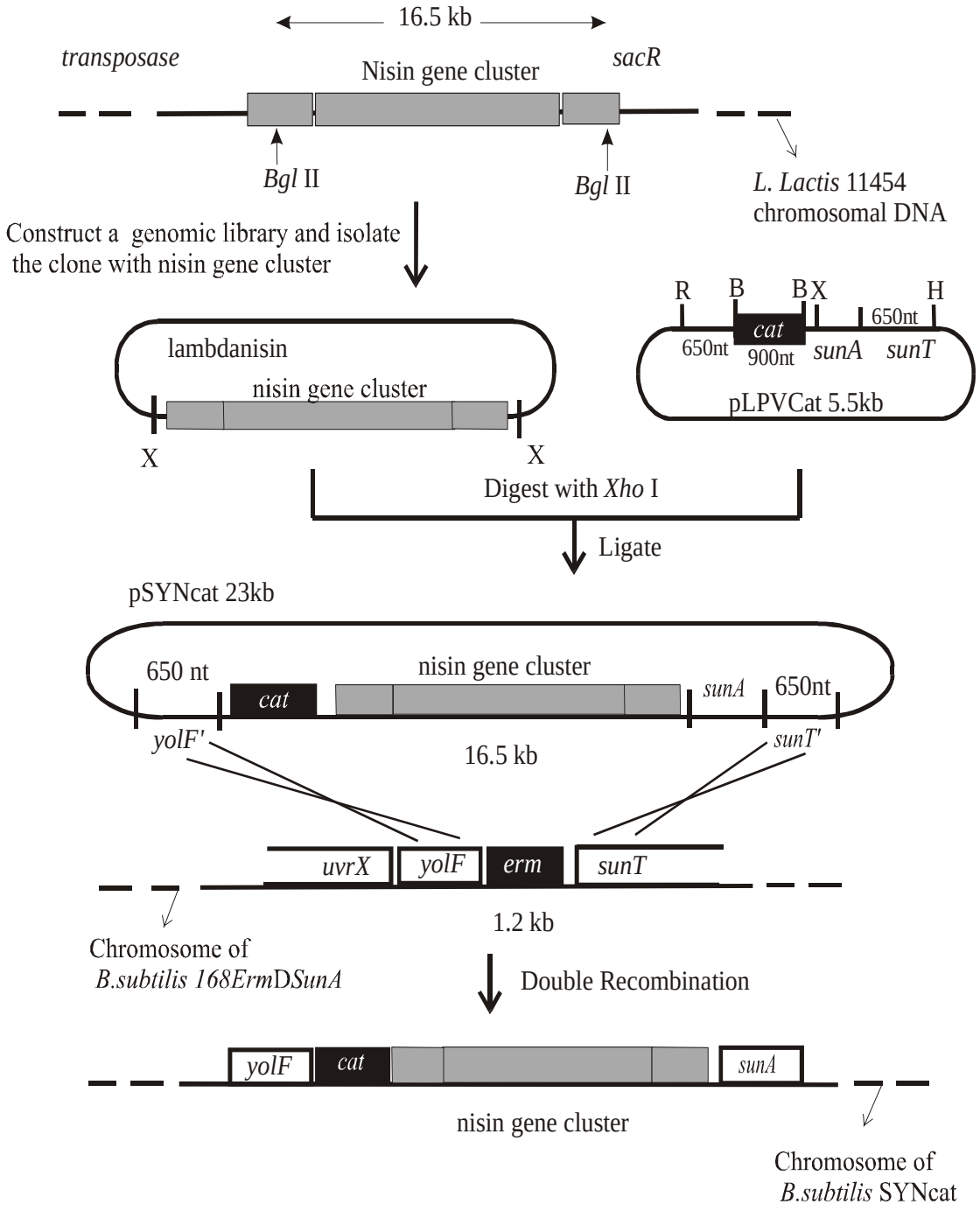
clone #2 Bgl II
clone #3 Xho I
clone #2 Xho I
clone #3 Sac I
clone #2 Sac I
100bp DNA ladder
lambda EcoR I
1 2 3 4 5 6 7 8 9 10 11 12

Figure 14. Analysis of Lambda GEM®-11 clones containing nisin gene cluster with agarose gel electrophoresis and Southern blot.

Unique restriction sites (*EcoR* I, *Sac* I, *Xho* I) were chosen from the multipurpose cloning site of Lambda GEM®-11 vector to digest the clones containing the nisin gene cluster and then the digested DNA were run on a 0.8% agarose gel as seen above (A). The observed bands were compared with the expected pattern that was deduced from the sequence of nisin gene cluster. The blot of the gel (B) was hybridized with a ^{32}P end labeled *nis*A probe to confirm the *nis*A gene appeared at the expected positions. *Xho* I and *Bgl* II sites were not present in the nisin gene cluster so the *nis*A gene appeared in the 16 kb fragment. However, *Sac* I digested the nisin gene cluster into 4 fragments (1444,1265, 916, 12936 bp respectively) and the *nis*A appeared in the 1444bp fragment as anticipated.



B = *BamH*I, E = *Eco*RI, H = *Hind* III, X = *Xho*I

Figure 15. Construction of pSYNcat and integration of nisin gene cluster into the chromosome of *B. subtilis*ermΔsunA.

A *Bgl* II genomic library of *L. lactis* 11454 was constructed in Lambda GEM®-11 cloning vector as described in Materials and Methods Part II-14. Plaques containing the nisin gene cluster was identified by hybridizing with the ^{32}P end labeled *nis*A probe. The DNA was isolated from the positive plaques and digested with *Xho* I to release the nisin gene cluster. Then the fragment was cloned into *Xho* I digested pLPVCat cassette vector. The resulting plasmid, pSYNcat, was transformed into *B. subtilis*ermΔsunA. The chloramphenicol resistant, erythromycin sensitive double recombinants were selected as described in Materials and Methods. The resulting strain was named *B. subtilis* SYNcat.

subtilis 168 chromosome. It contains *cat* gene as a selection marker. The nisin gene cluster was cloned into the *Xho* I site of pLPvcat as illustrated in Figure 15. The transformants were screened with colony hybridization using the *nis*A probe. Positive clones, containing the plasmid pSYNcat were then screened by using several restriction enzyme digestions. Once characterized, the plasmid was transformed into *B. subtilis* ermΔsunA and transformants were selected on chloramphenicol. *B. subtilis* ermΔsunA is a strain derived from *B.* subtilis 168 by replacing the *sun*A gene with the *erm* gene (62). To identify the single-crossover and double-crossover recombinants, transformants were grided on erythromycin and chloramphenicol containing plates (Materials and Methods Part II-19). The ones with a single crossover were resistant to both chloramphenicol and erythromycin. The ones with a double crossover were resistant to chloramphenicol and sensitive to erythromycin since the *erm* gene was deleted by the recombination.

By this criterion, one of the 14 transformants was found to be a double recombinant. Chromosomal DNA from 4 of the transformants was isolated and hybridized with the probe generated from λnisin (bacteriophage λ containing the nisin gene cluster) DNA by random primer extension in the presence of α-^{32}P labeled dCTP. Only the clone containing the double recombinant DNA gave a positive signal (Figure 16). This new strain that appeared to contain the nisin gene cluster integrated into the sublancin locus was named *B. subtilis* SYNcat. The ability of this strain to express nisin was determined as described in Materials and Methods Part II-20. The supernatant of the culture was purified on an HIC column (Materials and Methods Part II-21) and the peptides were resolved on RP-HPLC (Materials and Methods Part II-22). The fractions were tested for biological activity with halo assay (Figure 17) (Materials and Methods

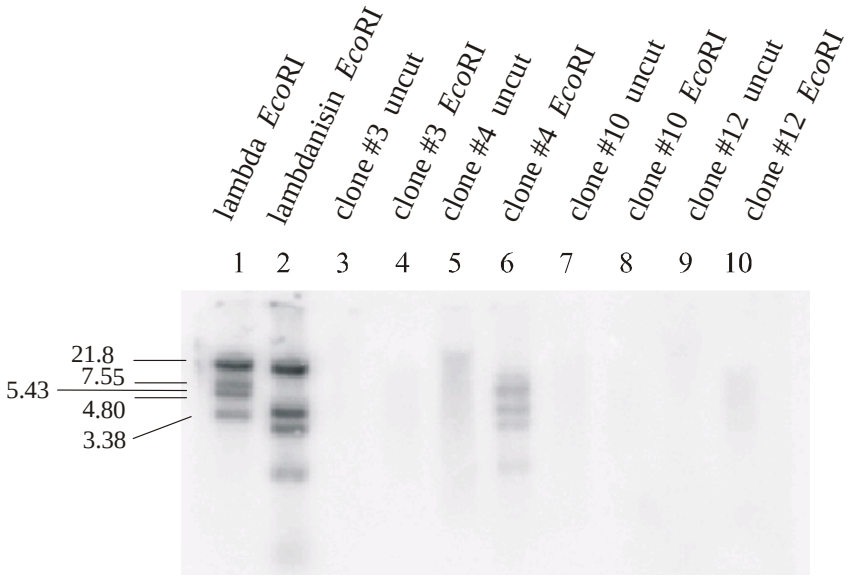


Figure 16. Analysis of pSYNcat transformants with Southern blot.

The shuttle vector pSYNcat was transformed into *B. subtilis* ermΔsunA and the transformants integrating the nisin gene cluster was selected on chloramphenicol plates. The DNA was isolated from the 4 chloramphenicol resistant, erythromycin sensitive recombinants (double crossovers), digested with *EcoR* I and run on a 0.8% agarose gel. The gel was blotted on a nitrocellulose paper and then hybridized with a randomly labeled λnisin (bacteriophageλ DNA containing the nisin gene cluster) DNA with α-^{32}P. Only clone #4 gave a positive signal when hybridized with a nisin probe.

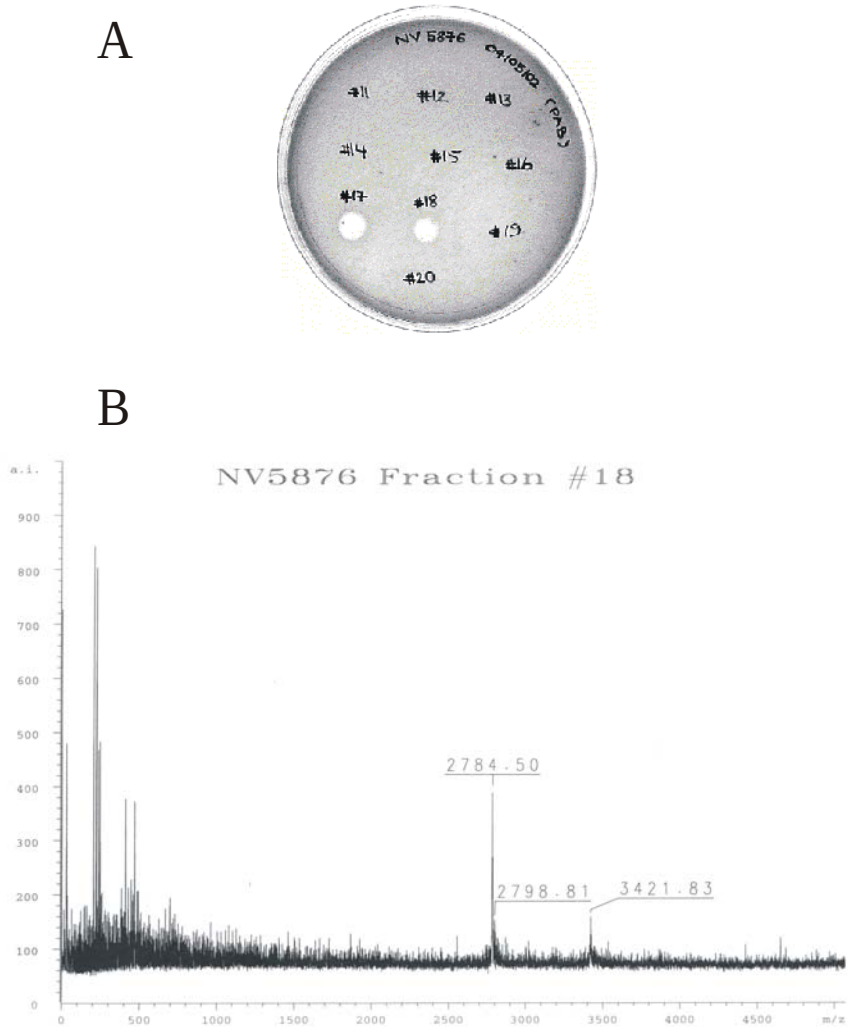


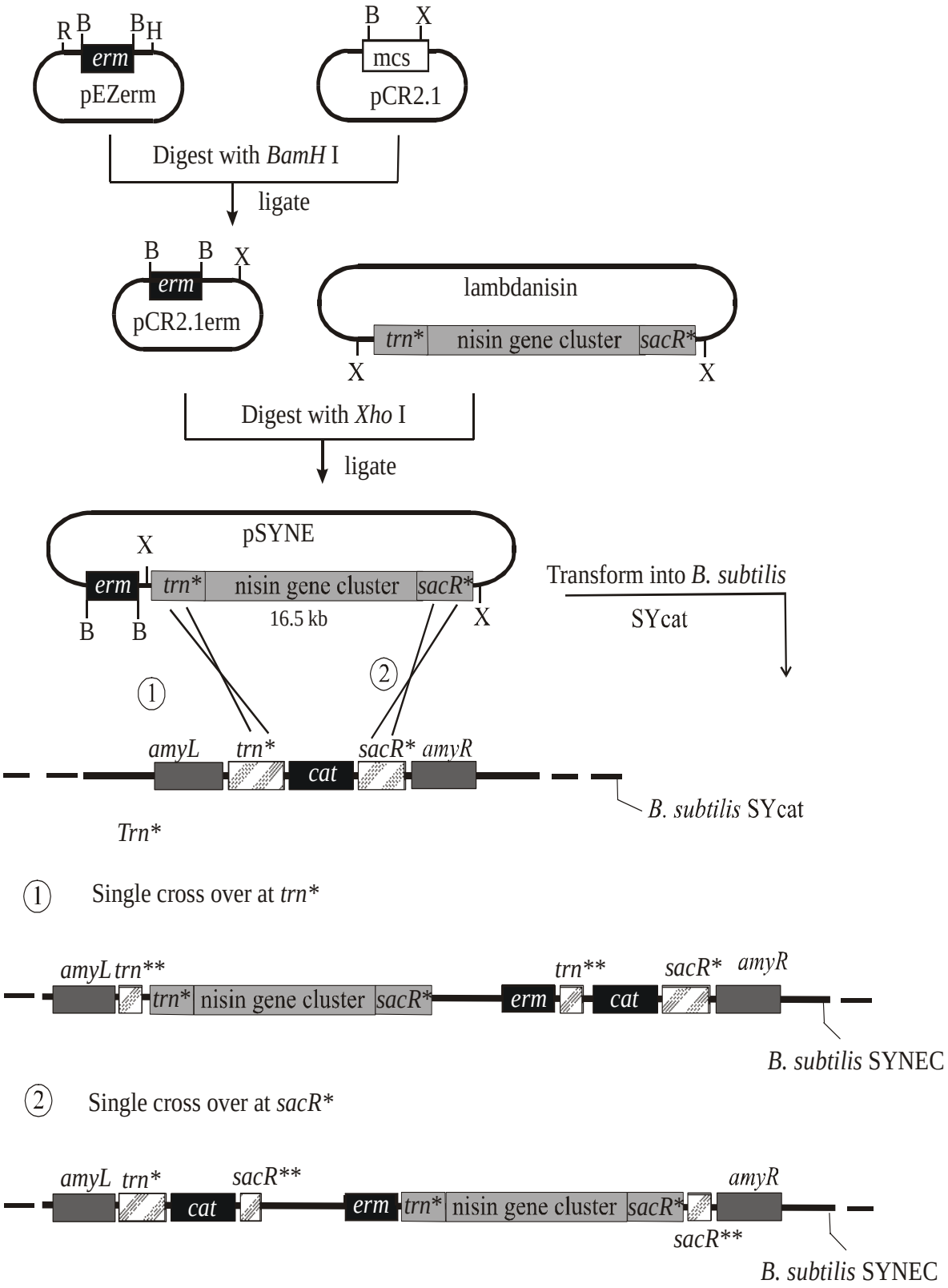
Figure 17. Analysis of the purified culture supernatant from *B. subtilis* SYNcat with halo assay and MALDI-TOF MS.

The extracellular materials from the culture supernatant of a 28 hr Medium A culture of *B. subtilis* SYNcat were isolated by HIC and RP-HPLC as previously described. The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores as previously described. Activity was observed at fractions 17 and 18 as seen in Panel A. All the fractions were analyzed with MALDI-TOF. Fractions #17 and 18 produced the same spectrum. Panel B shows the MALDI-TOF MS spectra of fraction #18.

Part II-24). Clear zones were observed at fraction 17 and 18 where usually nisin comes out of the same column under the same conditions (Figure 17). MALDI-TOF MS was used to further analyze the all the peptide containing fractions. None of the observed molecular weight peaks matched the molecular weight of nisin (*m/z* = 3354). The activity observed at fraction #17 and #18 had the characteristics of nisin halo, which leaves very clear zones of inhibition. Also, the peptide producing the activity had the same chromatographic properties of nisin but had a lower molecular weight. It is therefore possible that this peak is a degradation product of nisin. The differences in the molecular weights might also result from an incompletely processed or aberrantly modified degradation product of nisin.

Part V-2. Cloning of nisin genes into pCR2.1 and integration into the *B. subtilis* SYcat chromosome.

Another shuttle vector, pSYNE was created to integrate the nisin gene cluster into the *amy E* locus of *B. subtilis* BR151 (Figure 18). An erythromycin resistance gene, *erm*, was introduced into the pCR2.1 cloning vector and then the nisin gene cluster was cloned into the *Xho* I site of this plasmid. Resulting plasmid was named pSYNE. Then it was transformed into *B. subtilis* SYcat that contains the *transposase* and *sacR* homology regions. The Campbell integrants containing the nisin gene cluster were selected on erythromycin containing plates. Then the presence of the nisin gene cluster was confirmed with hybridization of chromosomal DNA with the α-^{32}P labeled λnisin (Figure 19). The restriction pattern of the DNA digests did not gave the same fragmentation pattern because the exact place of the recombination was different for every clone tested.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B H
erm
pEZerm
B X
mcs
pCR2.1
Digest with BamH I
ligate
B B X
erm
pCR2.1erm
lambdanisin
trn*
nisin gene cluster
sacR*
X
X
Digest with Xho I
ligate
pSYNE
X
erm
trn*
nisin gene cluster
sacR*
16.5 kb
B B
X
Transform into B. subtilis
SYcat
1
2
amyL
trn*
cat
sacR*
amyR
B. subtilis SYcat
Trn*
1 Single cross over at trn*
amyL trn**
trn*
nisin gene cluster
sacR*
erm
trn**
cat
sacR*
amyR
B. subtilis SYNEC
2 Single cross over at sacR*
amyL
trn*
cat
sacR**
erm
trn*
nisin gene cluster
sacR*
amyR
sacR**
B. subtilis SYNEC

Figure 18. Cloning of nisin genes into pCR2.1 and integration into the *B. subtilis* SYcat chromosome.

A schematic representation for the construction of pSYNE and the integration of nisin gene cluster into the chromosome of *B. subtilis* SYcat was shown above. The *erm* gene was cloned into the *BamH* I site of pCR2.1 forming the plasmid pCR2.1erm and the *Xho* I fragment from λnisin was cloned into pCR2.1erm. The resulting plasmid, pSYNE, was transformed into *B. subtilis* SYcat. The nisin gene cluster was integrated into the chromosome through single crossover at either *sac*R or *transposase* homology regions.

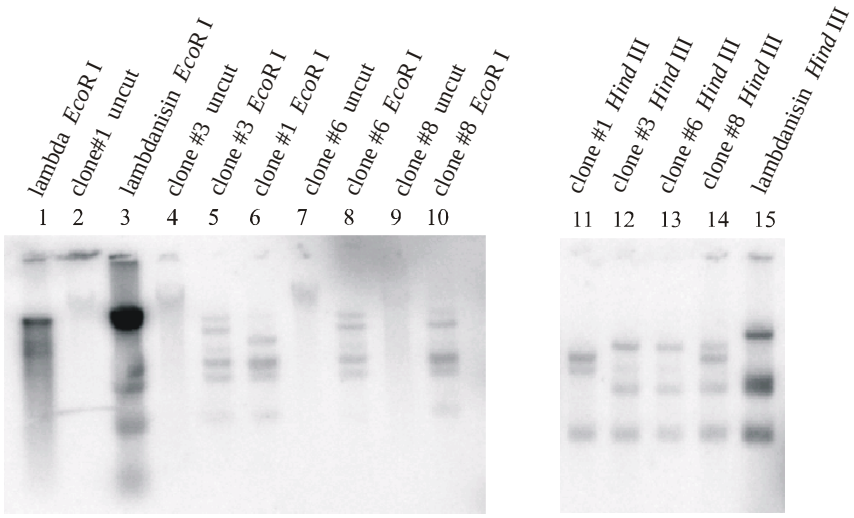


Figure 19. Analysis of pSYNE transformants with Southern blot.

The plasmid pSYNE was transformed into *B. subtilis* SYcat and the transformants containing the nisin gene cluster was selected on erythromycin containing plates. The DNA was isolated from four of the transformants and digested with *EcoR* I and *Hind* III and run on a 0.8% agarose gel. The picture shows the Southern blot of this gel. All four of the transformants gave a positive signal when hybridized with the randomly labeled λnisin with α-^{32}P.

A

B

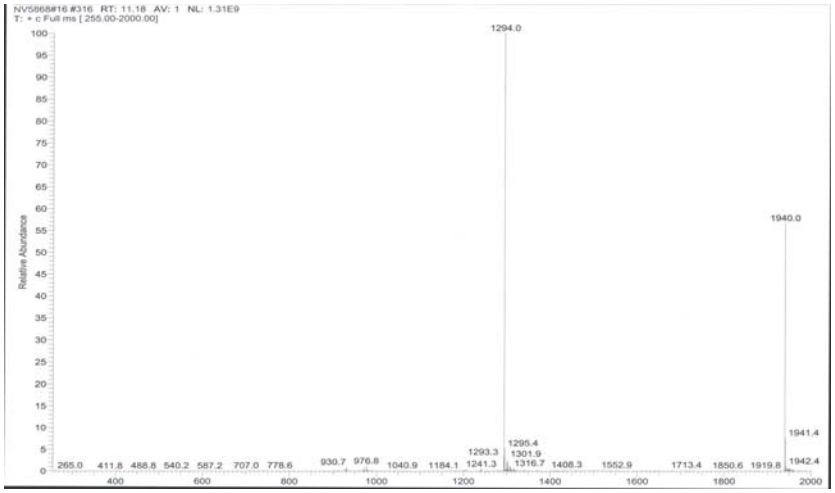


Figure 20. Analysis of the purified culture supernatant from *B. subtilis* SYNEC with halo assay and nanoESI MS.

The extracellular materials from the culture supernatant of a 28 hr Medium A culture of *B. subtilis* SYNcat were isolated by HIC and RP-HPLC as previously described. The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores. Activity was observed at fractions 15, 16 and 17 as seen in Panel A. All the fractions were analyzed with nanoESI MS. When the spectra were screened for nisin or nisin species, nothing was observed. Fractions 15, 16 and 17 produced the same MALDI-TOF MS spectra. Panel B shows the nanoESI MS spectra of fraction #16. A triply charged peak at 929.1 and doubly charged peak at 1394.4 was seen. The calculated molecular weight from both of these peaks corresponds to molecular weight peak of sublancin (3877.8 Da).

The new strain was named *B. subtilis* SYNEC and tested for expression of the nisin gene cluster by culturing in medium A for 28 hr (Materials and Methods Part II-20). The supernatant of the culture was purified on an HIC column (Materials and Methods Part II-21) and the peptides were resolved on RP-HPLC (Materials and Methods Part II-22). The fractions were tested for biological activity with the halo assay (Materials and Methods Part II-24). Activity was observed at fraction #15, #16, and slightly at #17 (Figure 20). However, these halos had ingrowth, which was the characteristic of sublancin halos. Sublancin is an anti-microbial peptide naturally produced by *B. subtilis* 168. Further analyis with MALDI-TOF MS spectra proved the presence of only sublancin at these fractions and any nisin or nisin species could not be detected in the other fractions when analyzed with mass spectroscopy. Sublancin was produced in large quantities in *B. subtilis* 168, which might have suppressed the peaks belonging to the nisin or nisin variants if present any.

Part V-3. Transfer of nisin gene cluster into *B. subtilis* BE1010.

In order to explore the possibility that the activity isolated from the culture supernatant of *B. subtilis* SYNcat consisted of nisin degradation products, the nisin gene cluster was transformed into *B. subtilis* BE1010. This was achieved by transforming the chromosomal DNA isolated from *B. subtilis* SYNEC into *B. subtilis* BE1010. Strain BE1010 is a derivative of *B. subtilis* 168, which lacks all the major proteases. The transformants were selected on chloramphenicol followed by gridding onto plates containing erythromycin. They retained both of the antibiotic selection markers as expected for these transformants. The efficiency of the recombination was very high due

to the extensive homologies between the two chromosomes. Assays were performed on several dozen of the transformants and the several clones that gave relatively large halos were selected for further analysis. The chromosomal DNA was isolated and presence of the nisin gene cluster was confirmed by hybridizing with λnisin DNA that was labeled by random primer extension in the presence of α-^{32}P labeled dCTP. The new strain was called *B. subtilis* SY20N. This strain was cultured to test for nisin expression as described in Materials and Methods Part II-20. Also, wt-*B. subtilis* BE1010 was cultured the same way as a control. The extracellular materials from the culture supernatants were analyzed either by using HIC (Materials and Methods Part II-21) or non-ionic cellulose (NIC) column (Materials and Methods Part II-23) and the eluate was then resolved on RP-HPLC (Materials and Methods Part II-21). The fractions were tested for biological activity using the halo assay (Materials and Methods Part II-23). Non-ionic cellulose columns were also used to analyze the culture supernatants because it was discovered by Amer Villaruz, a former lab member, that nisin has a strong affinity for non-ionic cellulose. He was able to isolate nisin from the culture supernatant of *L. lactis* 11454 in a highly pure form by using a non-ionic cellulose column (83). When the peptides from the culture supernatant of wt- *B. subtilis* BE1010 (protease deficient strain) were purified on NIC column and then resolved on RP-HPLC, the biological activity was observed in fraction #15 and #16 (Figure 21). NanoESI MS analysis of these fractions produced a triply charged molecular ion at *m/z*= 1293.6 (m= 3877.8 Da) which also correspond to the anti-microbial peptide sublancin (m= 3877.8 Da) (Figure 21). When the culture supernatant from *B. subtilis* SY20N was analyzed with NIC column followed by RP-

A

B

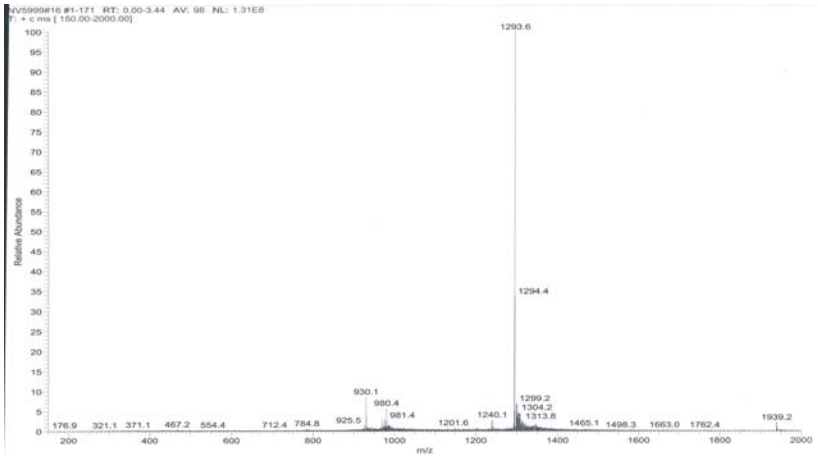
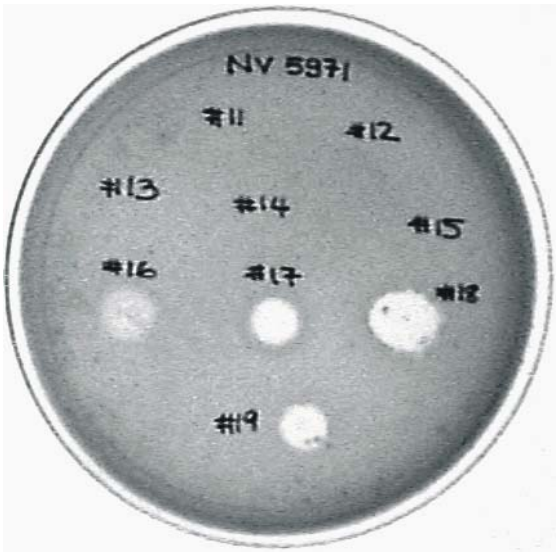


Figure 21. Analysis of the purified culture supernatant from *B. subtilis* BE1010 with halo assay and nanoESI MS.

The extracellular materials from the culture supernatant of a 28 hr Medium A culture of *B. subtilis* BE1010 were isolated by NIC column and RP-HPLC as previously described. The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores as previously described. Activity was observed at fractions 15 and 16 as seen in Panel A. All the fractions were analyzed with nanoESI MS. Fractions 15 and 16 produced the same spectrum. Panel B shows the nanoESI MS spectra of fraction #16. A triply charged peak at *m/*z=1293.6 was seen. The calculated molecular weight from this peak corresponds to molecular weight of sublancin (3877.8 Da).

A


NV 5971
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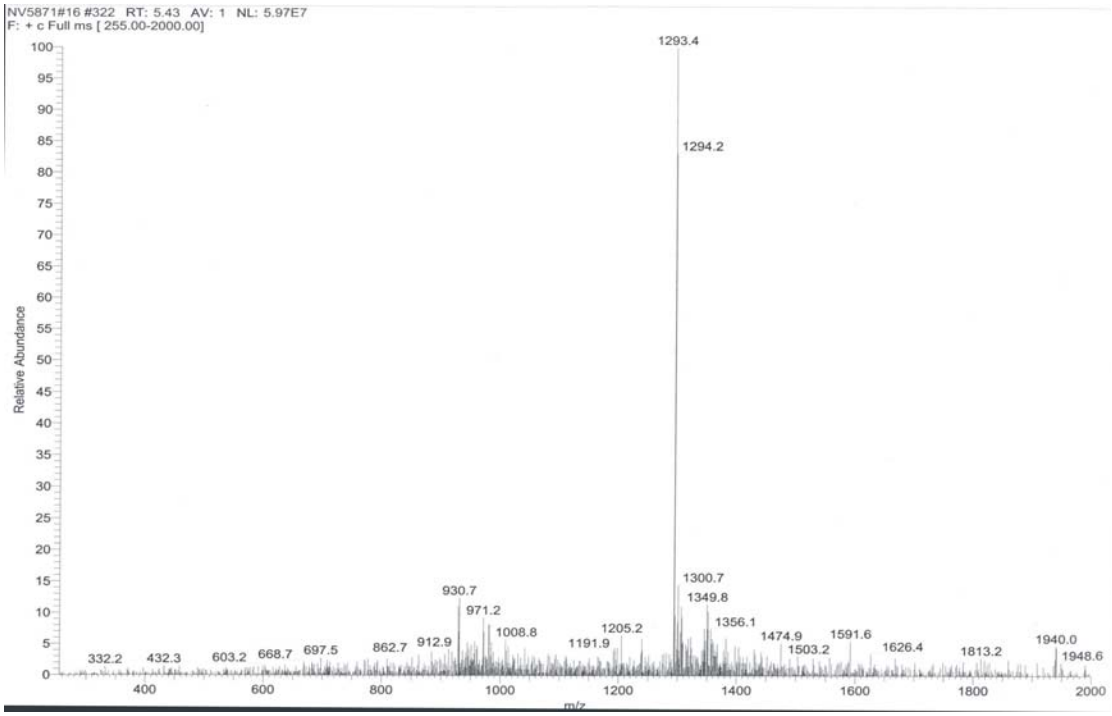
NV5871#16 #322 RT: 5.43 AV: 1 NL: 5.97E7
F: + c Full ms [255.00-2000.00]
Relative Abundance
m/z
1293.4
1294.2
1300.7
1349.8
1356.1
930.7
971.2
1008.8
1205.2
1191.9
912.9
862.7
697.5
668.7
603.2
432.3
332.2
1474.9
1503.2
1591.6
1626.4
1813.2
1940.0
1948.6

C

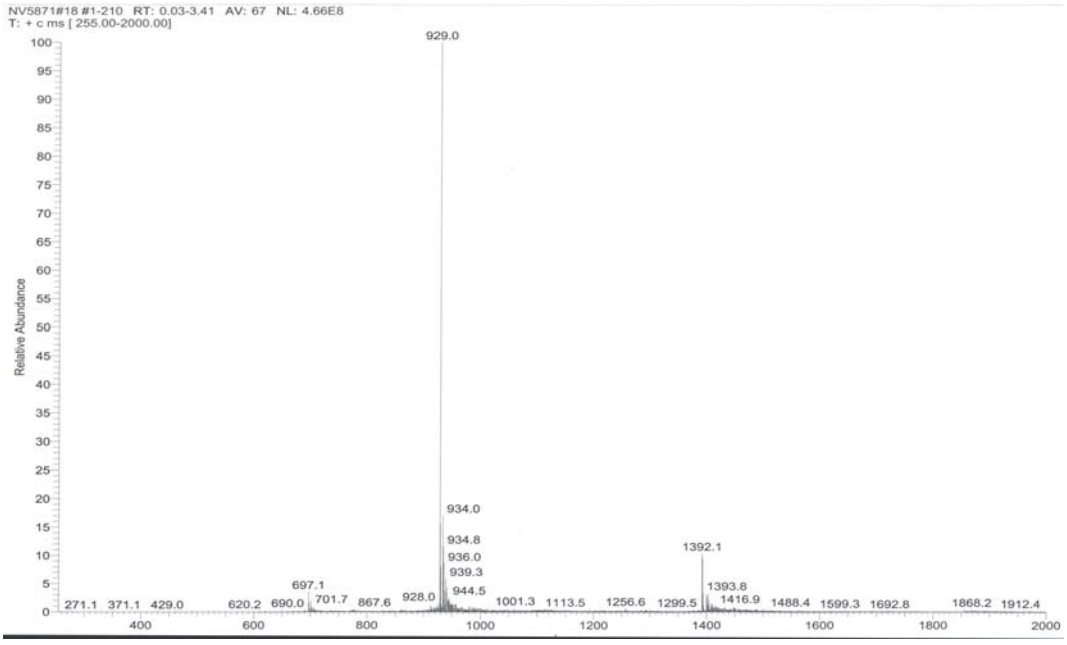

Figure 22. Analysis of the purified culture supernatant from *B. subtilis* SY20N with halo assay and nanoESI MS.

The extracellular materials from the culture supernatant of a 28 hr Medium A culture of *B. subtilis* SY20N were isolated by HIC and RP-HPLC as previously described. The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores. Activity was observed at fractions #16 through 19 as seen in Panel A. All the fractions were analyzed with nanoESI MS. Panel B shows the nanoESI MS spectra of fraction #16. Fraction #16 had a triply charged peak at *m/z*=1293.4. The calculated molecular weight from this peak corresponds to molecular weight of sublancin (3877.8 Da). Panel C shows the nanoESI MS spectra of fraction and #18. A triply charged peak at *m/*z=929.1 (m=2784.3 Da) and doubly charged peak at *m/z*=1392.1 (m=2782.2 Da) as seen. The calculated molecular weight from these two peaks corresponds to molecular weight of an unidentified species.

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NV5955-56
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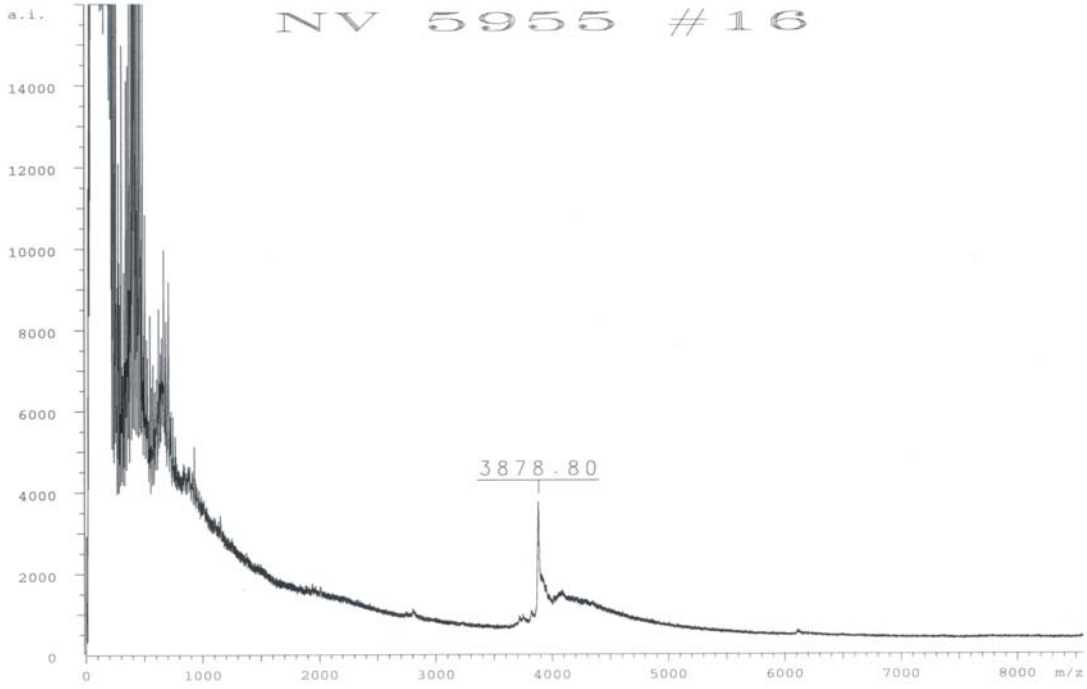
#19

B


NV 5955 #16
a.i.
14000
12000
10000
8000
6000
4000
2000
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3878.80
0
1000
2000
3000
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6000
7000
8000
m/z

C

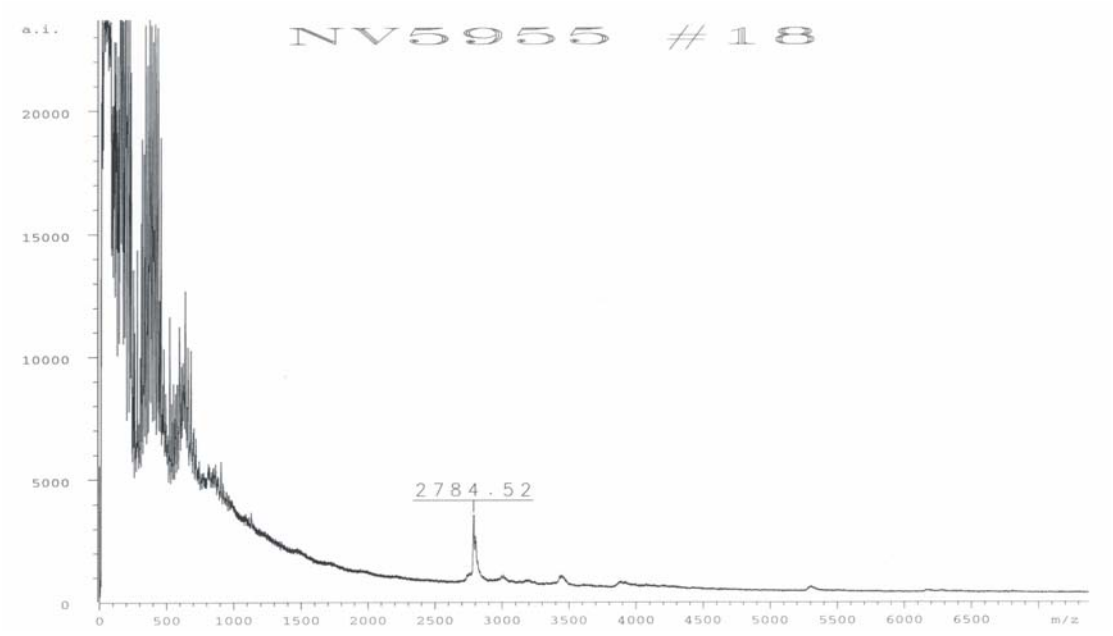


Figure 23. Analysis of the purified culture supernatant from *B. subtilis* BE1010 with halo assay and MALDI-TOF MS.

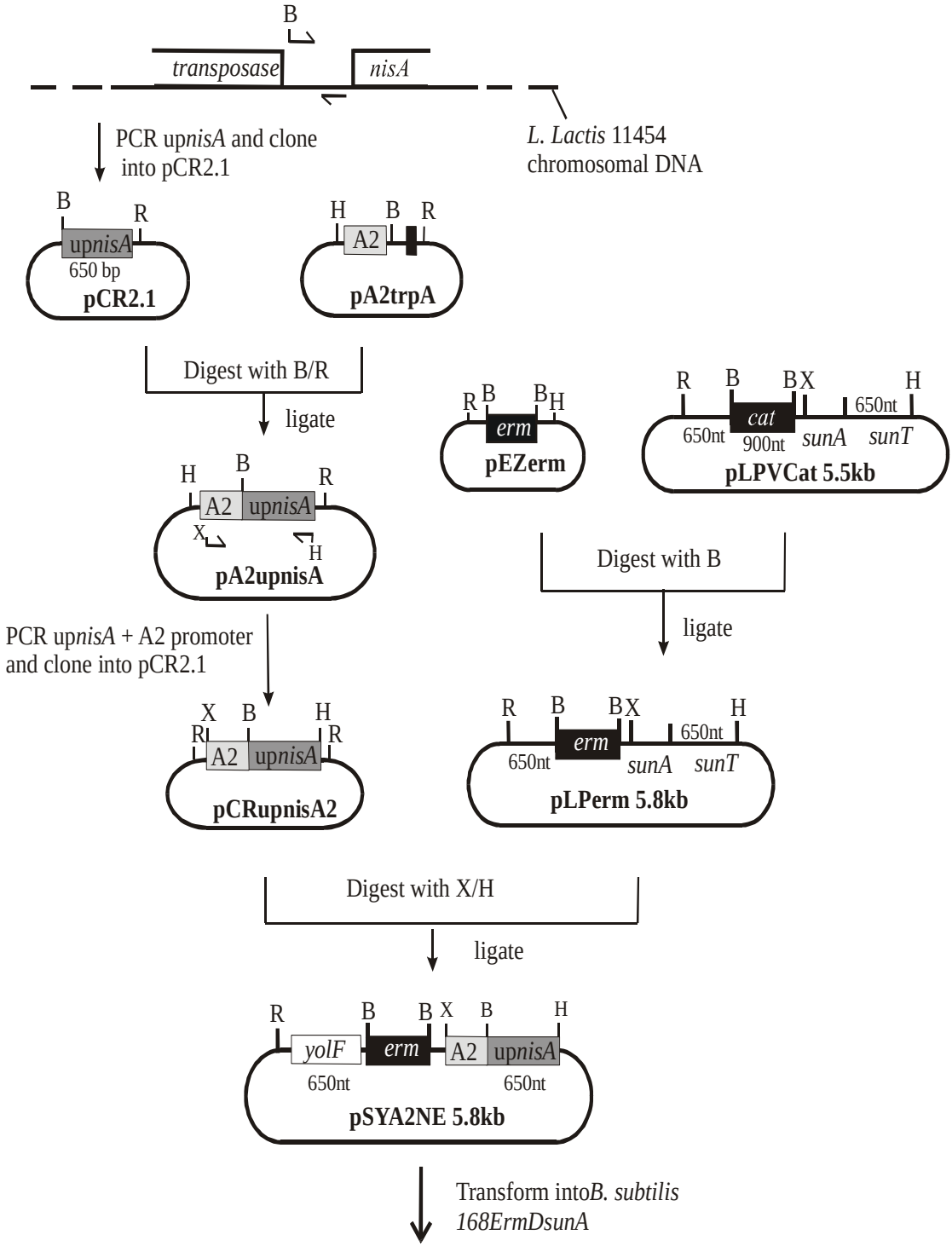
The extracellular materials from the culture supernatant of a 28 hr Medium A culture of *B. subtilis* SY20N were isolated by HIC and RP-HPLC as previously described. The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores. Activity was observed at fractions 14 through 18 as seen in Panel A. All the fractions were analyzed with MALDI-TOF. When the spectra were screened for nisin or nisin species, nothing was observed. Fractions 14, 15 and 16 produced the same MALDI-TOF MS spectra. Panel B shows the MALDI-TOF MS spectra of fraction #16. A singly charged peak at *m/*z=3878.8. Panel C shows the MALDI-TOF MS spectra of fraction #18. A singly charged peak at *m/z*= 2784.52 was observed which belongs to an unknown species.

HPLC, the activities were observed at fractions #16 through #19 (Figure 22). When the fraction #16 was further analyzed with nanoESI MS, a triply charged molecular ion was observed at *m/z*=1293.4 (m=3877.2 Da) (Figure 22) which also corresponded to sublancin. However, when fractions #17-19 were analyzed with nanoESI MS, a triply charged molecular ion at *m/z* = 929.0 was observed which belongs to an unidentified species at 2782 Da. Even though this species produced clear halos that were similar to nisin halos, the molecular weight could not be rationalized as degradation products of nisin. Interestingly, the chromatographic characteristics of this peptide were the same as nisin. The culture supernatant from wt-*B. subtilis* BE1010 was also analyzed by using HIC and then RP-HPLC column. The activities were observed at fractions #14 through #18 (Figure 23) when tested with the halo assay. The MALDI- TOF MS analysis of fractions 14-16 produced a singly charged molecular ion at *m/*z=3878.8 (m=3877.8 Da) (Figure 23). This corresponded exactly to the molecular weight of sublancin. However, the MALDI-TOF MS analysis of fractions #17 and #18 produced a singly charged peak at *m/*z=2784.52 (m=2783.5 Da). This shows that the wild type protease deficient strain was producing the same active peptide as the recombinant strain containing the nisin gene cluster (SY20N). As a result, the activity isolated from *B. subtilis* SY20N was not related to any nisin degradation products.

Part V-4. Cloning of the A2 promoter sequence upstream from the nisin gene cluster in *B. subtilis* SYNcat.

The expression from nisin structural gene, *nis*A, is controlled by its own promoter in *L. lactis*. Expression from this promoter is under the control of a two-component

response regulatory system in which nisin, itself, is the inducer of expression. The system is accordingly turned on in the presence of subinhibitory levels of nisin (Introduction, Part I-5). It is possible that this regulatory system does not function in *B. subtilis* 168. If this is the case, cloning the constitutive A2 phage promoter upstream of *nis*A gene in *B. subtilis* SYNcat might restore the expression of the cluster. This promoter is known to function in *B. subtilis* 168. The construction of the cassette vector for integration of this promoter into the upstream of the nisin structural gene was outlined in Figure 24. The primers used in this part were listed in Table 4. The cassette vector was named pSYA2NE and transformed into *B. subtilis* SYNcat. 6 chloramphenicol (-) double recombinants were obtained out of the 400 transformants tested. These double recombinants are likely to have integrated the promoter into the upstream region of the nisin structural gene. The halo assay was performed on all of the double recombinants and some of the single-crossover recombinants colonies. Two of the double recombinants gave relatively bigger halos when compared with the host strain *B. subtilis* SYNcat suggests possible nisin production. In order to confirm the presence of promoter, PCR reactions were performed to amplify a 501 bp region in which the forward primer was originated within the promoter sequence and a 551 bp region in which the forward primer was originated in the upstream region of the promoter (Figure 25). The primers for this PCR reaction were listed in table 4. Both regions were successfully amplified from clone #10 by PCR, and no bands were observed in the control lanes (lanes 6-9) (Figure 25). Clone #10 was used in the later experiments since both regions were amplified successfully. Clone #10, which contains the A2 constitutive promoter, was named *B. subtilis* SYA2NE. It was cultured for 28 hr to test the expression of nisin (Materials and



B = *BamH*I. E = *Eco*RI. H = *Hind* III. X = *Xho*I

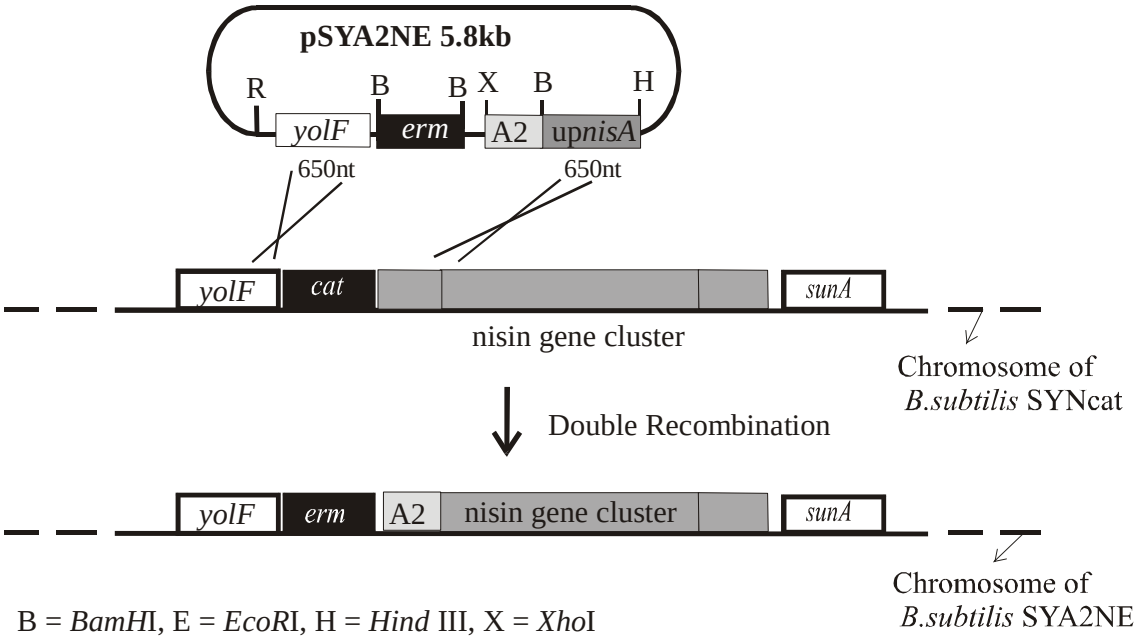


Figure 24. Construction of pSYA2NE and the integration of A2 promoter in front of nisin gene cluster.

A schematic representation for the construction of pSYA2NE and the integration of A2 phage promoter in front of the *nis*A gene in the chromosome of *B. subtilis* SYNcat was shown above. The 646 bp homology region, upnisA, was amplified from the upstream *nis*A region of *L. lactis* 11454 by PCR (see table 4 for primers) and cloned into pCR2.1 by AT cloning. The PCR conditions were described in Materials and Methods, Part II-10, 10-1. Several modifications were made to the indicated conditions. The annealing was performed at 60 °C for 30 sec and the extension was carried out at 70 °C for 2.5 min. The number of cycles was decreased to 25. The reaction volume was decreased to 50 μl while keeping the concentrations of components same except the final primer concentration was doubled to 0.5 μM. Then the upnisA fragment was cloned into the pA2trpA vector by using *BamH* I and *EcoR* I right after the A2 promoter. The A2promoter and the upnisA homology region was then amplified with PCR (see table 4 for primers) from the plasmid pA2upnisA. The amplified fragment was cloned into pCR2.1, making the plasmid pCRA2upnisA The changes described above were also made to this PCR reaction except the annealing temperature was 72 °C and the 0.25 μM final primer concentration was maintained. The *erm* gene was cloned into the *BamH* I site of pLPVCat to form the pLPerm. Then the plasmid pCRA2upnisA and pLPerm was both digested with the *Xho* I and *Hind* III and the A2upnisA fragment was ligated into the pLPerm making the final construct pSYA2NE. This final construct was then transformed into *B. subtilis* SYNcat. The double cross-overed strain, which integrated the *erm* gene and the A2 promoter was named SYA2NE.

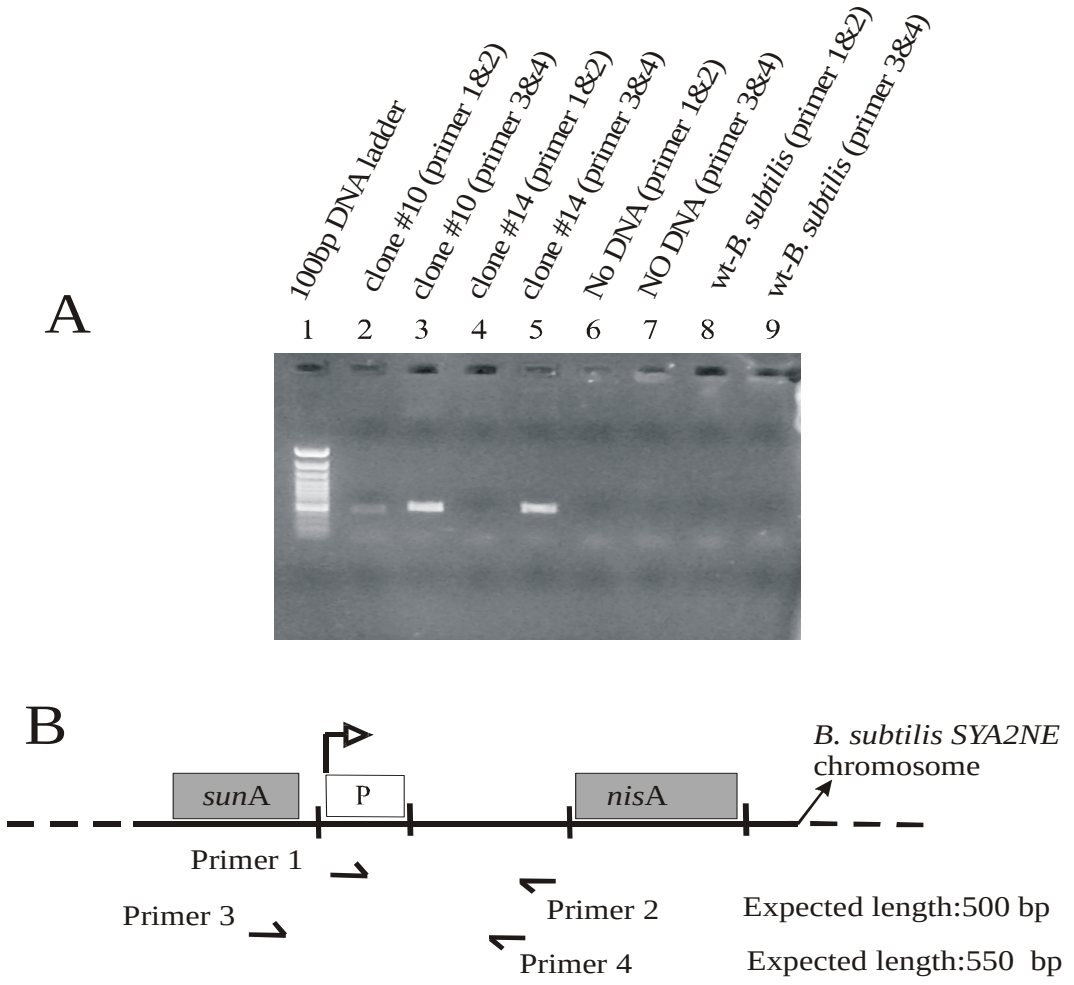


Figure 25. Gel electrophoresis of control PCR reactions that were done to confirm the presence of A2 promoter in the transformants.

The DNA was isolated from two of the double recombinants and PCR reaction was performed to see if the expected fragments could be amplified from the DNA of these transformants. Primer1 and 2, were originated from the promoter sequence and upstream of *nis*A gene, respectively (Panel B). Primer 3 and 4 were originated from the *sun*A gene and upstream of the *nis*A gene, respectively (Panel B). The first set of primers amplified the 501 bp region in clone #10 as expected but not in clone #14. The 551 bp region was amplified from both clone #10 and #14. No bands were observed when the wild type DNA was used. The PCR conditions were described in Materials and Methods Part-II, II-10. Several modifications were made to the indicated conditions. The annealing was performed at 60 °C for 30 sec and the extension was carried out at 70 °C for 2.5 min. The number of cycles was decreased to 25. The reaction volume was decreased to 50 μl while keeping the same molar ratios of the components.

Methods Part II-20). The culture supernatant was then applied to a non-ionic cellulose column as described in Materials and Methods Part II-23 in order to recover any nisin. In parallel, nisin was isolated from supernatant of *L.* lactis11454 by using the non-ionic cellulose column. The eluates were then resolved on RP-HPLC and tested for biological activity with the halo assay (Materials and Methods Part II-24). The inhibition of spore outgrowth was observed at fractions 16, 17 and 18 of the purified peptides from the culture supernatants of both *Lactococcus lactis* 11454 (natural producer of nisin) and the *B. subtilis* SYA2NE (contains the nisin genes) (Figure 26). The halos obtained from the recombinant strain was bigger than the halos obtained from the natural producer even though the amount of starting culture was 0.5 l for *B. subtilis* and 1 l for *L. lactis* 11454. When these fractions were tested with MALDI-TOF MS the molecular weight peak of nisin (*m/*z = 3354) was observed in the active fractions isolated from *L. lactis* culture. However, a triply charged molecular ion peak at *m/*z= 929.1 (m=2784.3 Da) and a doubly charged peak at *m/*z=1392.4 (m=2782.8 Da) and a small triply charged peak at *m/*z=1855.6 (m=5563.8 Da) were observed from the culture of *B. subtilis* SYA2NE (Figure 26). When the mass of the molecular ion at 1855.6 was divided by 2, a mass equal that equals to 2781.9 was obtained. This argues the fact that this unidentified species is a dimer and each of the monomers weighs about 2782 Da. The *B. subtilis* ermΔsunA was also cultured for 28 hr at the same conditions (Materials and Methods Part II-20) to test the expression of the peptides from the wild type strain. The culture supernatant was purified on a NIC column and then eluates were resolved on RP-HPLC as previously described. The fractions were tested for biological activity with the halo assay (Materials and Methods Part II-24). The clear zones were observed at fraction #17,

18 and 19 (Figure 27). This was similar to the activity observed from the fractions obtained from *B. subtilis* SYA2NE culture supernatant. The fractions 17-19 were further analyzed with nanoESI-MS and the three fractions produced the same spectrum. Figure 27 shows the mass spectrum of Fraction 18. The same peaks were observed in the active fractions of *B. subtilis* SYA2NE. The triply charged peak at *m/*z= 929.0 and 1856.2 and a doubly charged peak at *m/*z=1392.3. These peaks were also observed when the culture supernatants of wt-*B. subtilis* BE1010 were analyzed (Results Part V-3). Thus, the peptide producing the activity was present in the wild-type *B. subtilis* 168, and the presence of nisin gene cluster was not responsible for the production of these activities activity. Also, no nisin or nisin like peptide was observed when the purified peptides from the various recombinant strains containing the nisin gene cluster culture were analyzed with the mass spectroscopy.

At this point there is strong evidence that the entire nisin gene cluster has been successfully transferred from the *L. lactis* chromosome, into *B. subtilis* 168 chromosome. Hence a major goal of the project has been achieved. However the presence of the gene cluster did not result in detectable nisin production. This could be attributed to lack of expression of the nisin genes. Alternatively, genes are expressed, but there are either no translation products, or the translation products do not undergo their normal post-translational processing reactions. Introduction of a constitutive promoter, intended to increase mRNA synthesis, did not solve the problem. However, just because a promoter is introduced does not prove it is functional. So a major question is, “Are the nisin genes expressed?” Once this question is resolved, other questions can be adressed.

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NV 5987 L.lactis
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#19
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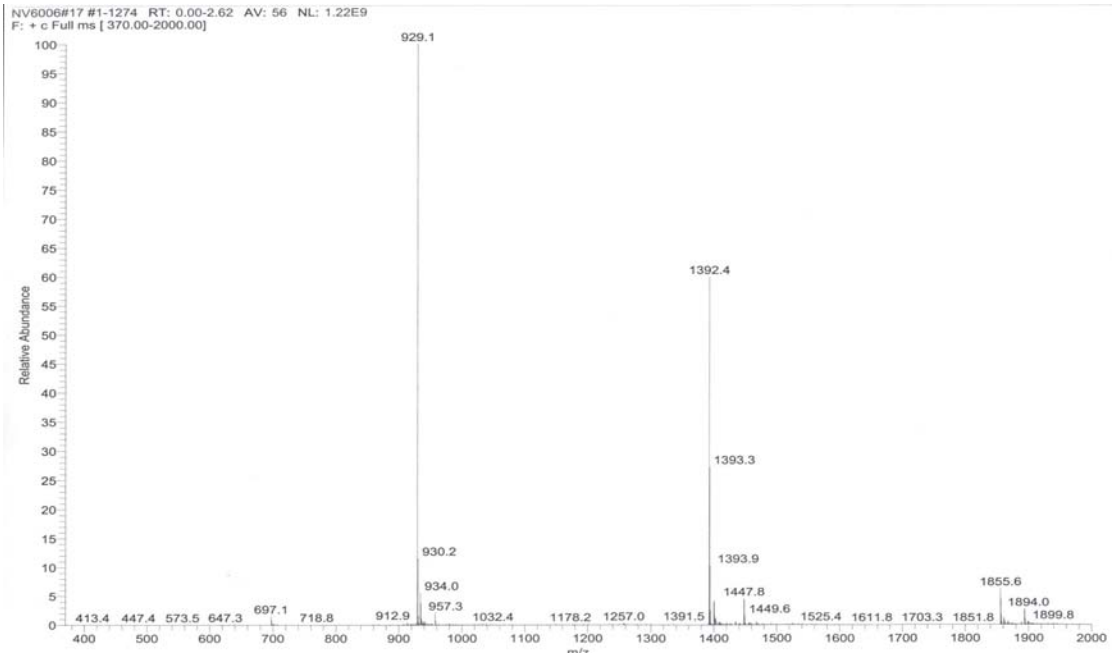
B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F: + c Full ms [370.00-2000.00]
Relative Abundance
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1392.4
1393.3
930.2
1393.9
934.0
957.3
1447.8
1449.6
1855.6
1894.0
1899.8
413.4
447.4
573.5
647.3
697.1
718.8
912.9
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1257.0
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1703.3
1851.8

C

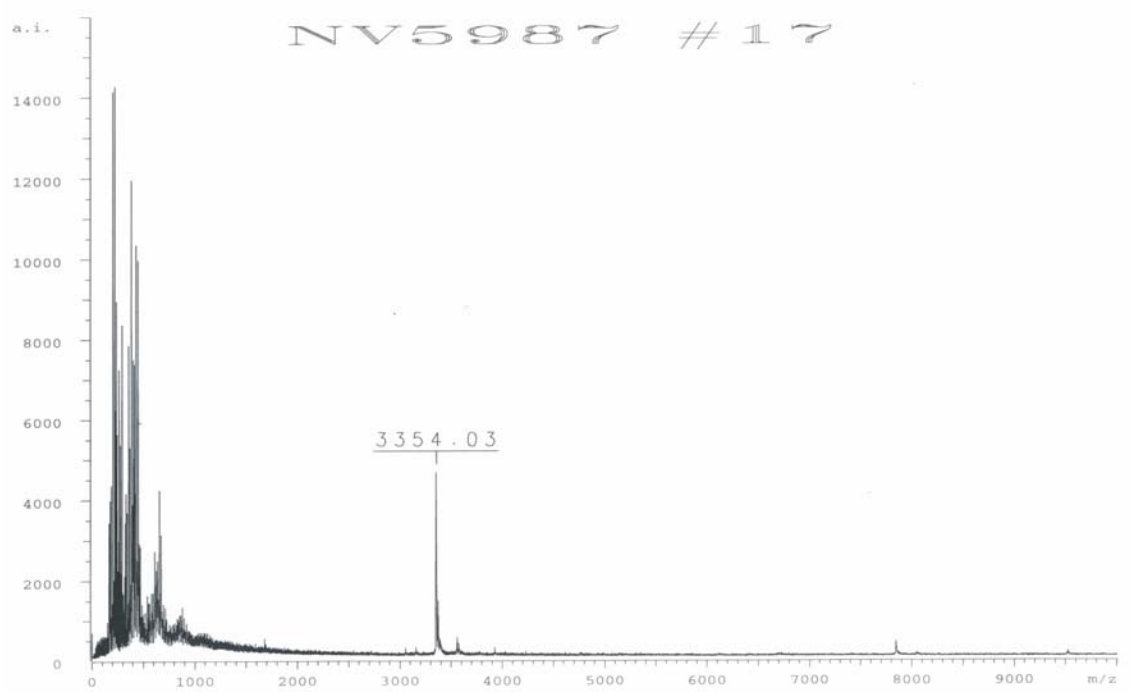
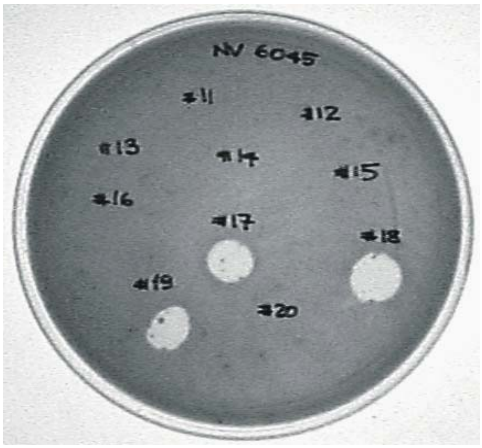


Figure 26. Analysis of the purified culture supernatant from *B. subtilis* SYA2NE and *L. lactis* 11454 with halo assay and mass spectroscopy.

The extracellular materials from the culture supernatant of *B. subtilis* SYA2NE and *L. lactis* 11454 were isolated by NIC column and RP-HPLC (Materials and Methods Part II-23 and 22). The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores as described previously. Activity was observed at fractions #16 through 19 in both of the species as seen in Panel A. All the fractions were analyzed with nanoESI MS. Fractions 16 through 19 produced the same spectrum. Panel B shows the nanoESI MS spectra of fraction #17 of *B. subtilis* SYA2NE. Fraction #17 had a triply charged peak at *m/z*=929.1 (m=2784.3 Da) and 1855.6 (m=5563.8 Da) and a doubly charged peak at *m/z*=1392.4 (m=2782.8 Da). Panel C shows the MALDI-TOF MS spectra of fraction and #17 of *L. lactis* 11454. A singly charged peak at *m/*z=3354.23. The calculated molecular weight from this peak corresponds to molecular weight of nisin (3353.4 Da).

A



B

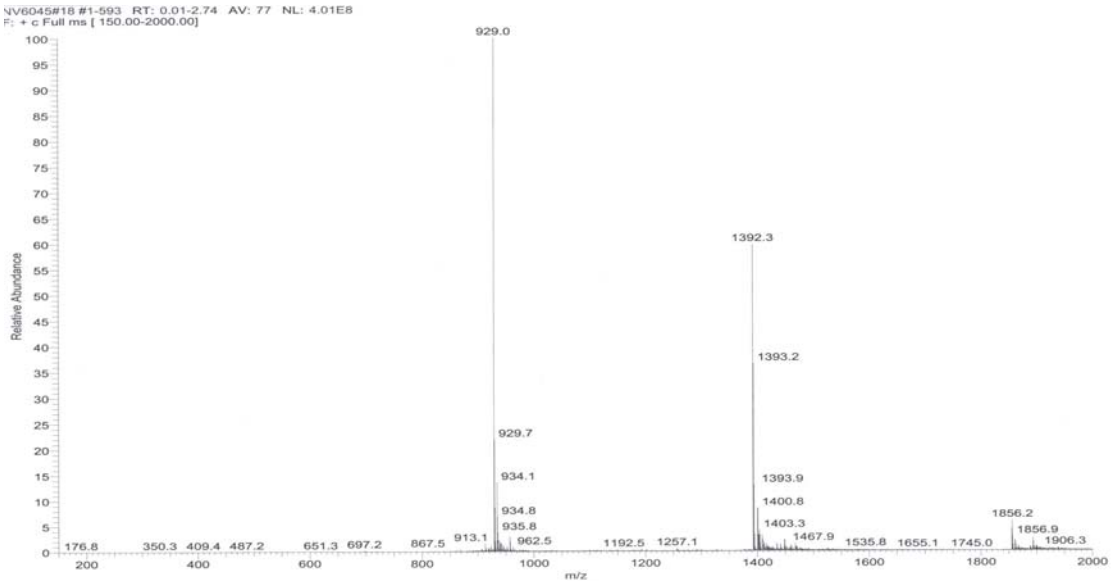


Figure 27. Analysis of the purified culture supernatant from *B. subtilis* ermΔsunA with halo assay and nanoESI MS.

The extracellular materials from the culture supernatant of *B. subtilis* ermΔsunA was isolated by NIC column and RP-HPLC (Materials and Methods Part II-23 and 22). The fractions from the RP-HPLC was tested for biological activity by halo assay against outgrowing *B. cereus* T spores as described previously. Activity was observed at fractions #17 through 19 in both of the species as seen in Panel A. All the fractions were analyzed with nanoESI MS. Fractions 17 through 19 produced the same spectrum. Panel B shows the nanoESI MS spectra of fraction #18. Fraction #18 had a triply charged peak at *m/z*=929.0 (m=2784.0 Da) and 1856.2 (m=5565.6 Da) and a doubly charged peak at *m/z*=1392.3 (m=2782.6 Da).

Name	Sequence (5’ to 3’)
SYNGCf	CCTCGACGATACCATCACTCTTCAT
SYNGCr	CATGAGCTTGACACCAATAACTCCA
SYNISBr	TCAGTTTCTCAATACCTTCACCAA
SYUNISAf	GGATCCTCATGAGTTTGACGCCAATAA
SYUNISAr	TCCTTCGAACGAAATCATTGT
SYA2UNISAf	CTCGAGGCTTGGCTGCAGGTCGAT
SYA2UNISAr	AAGCTTTCCTTCGAACGAAATCATTGT
SYT1F	GTCGATCCGTCGAAGATTTG
SYT1R	ATCGTCGAGGCGTTTAAAGA
SYT2F	ATCCGGATTGCAAACAAATG
SYT2R	TGGTATCGTCGAGGCGTTTA

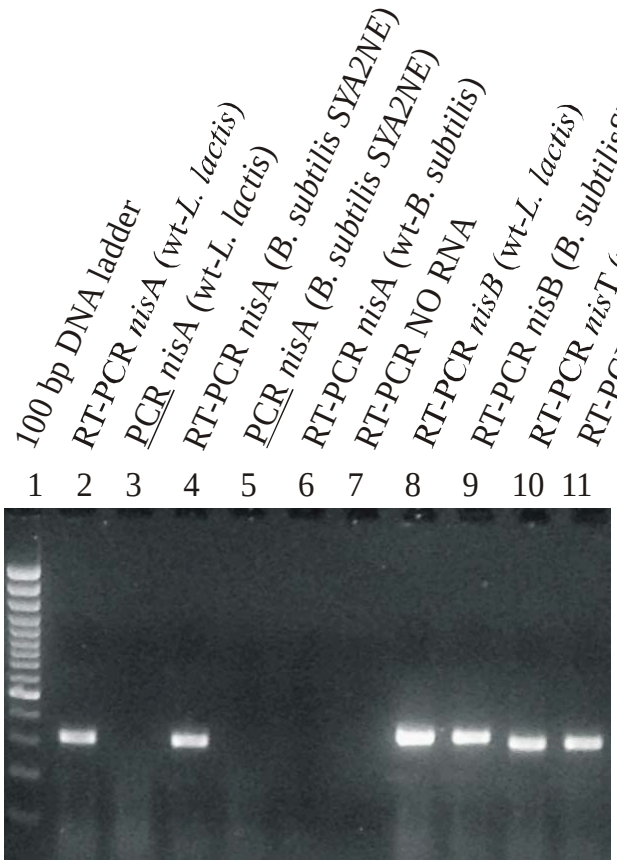
Table 4. PCR primers used in Results Part IV-Part V-4.

Oligonucleotides SYNGCf and SYNGCr were used to amplify nisin gene cluster from *L. lactis* 11454 (Results Part V-1), SYNDCf and SYNISBr were used to amplify 1.5 kb fragment from the beginning of nisin gene cluster (Results Part V-1), SYUNISAf and SYUNISAr were used to in the construction of pCR2.1upnisA, SYA2UNISAf and SYA2UNISAr were used in the construction of pCR2.1A2upnisA, and SYT1f, SYT1r, SYT2f, SYT2r were used in the test PCR in Results Part VI-4 (Figure 25).

Part V-5. RT-PCR of nisin genes.

In order to examine whether the nisin genes were being expressed, the presence of related mRNAs were established using RT-PCR. The Qiagen OneStep RT-PCR kit (Materials and Methods PartII-12) was employed for this purpose. The primers that were used in this section listed in Table 5.

RT-PCR was used to detect the mRNA from each of the *nis*ABTCIPRK genes. To do so, a ~350 bp mRNA segment from each gene was amplified and the amplification mixtures were analyzed on 2.5 % agarose gels (Figure 28). The control lanes consisted of reaction in which the RT part of the reaction was omitted. The control lanes were the only ones that did not show the reaction products and established that the bands were produced by RNA and not DNA. The RT- PCR of *nis*A gene from *B. subtilis* ermΔsunA did not produce any bands, as the nisin gene cluster was not present in this wild type strain. Also, the negative control tube where no RNA was added did not produce any bands showing that the reaction components were not contaminated with RNA. The RT-PCR of the nisin genes was done both with the nisin producer *L. lactis* 11454 and recombinant strain of *B. subtilis* containing the nisin gene cluster (SYA2NE). The mRNA's for the *nis*ABTCIPRK genes in both *L. lactis* 11454 and *B. subtilis* SYA2NE were shown to be present. When the bands obtained from L. lactis11454 were compared with the bands obtained from *B. subtilis* SYA2NE, they had very similar intensities. These results showed that the nisin genes were not only being expressed but also their expression levels were comparable to the natural host. However, this was not a "quantitative RT-PCR" experiment, so the similar intensities of the amplified bands are not conclusive proof that the mRNAs are expressed at the similar levels in both strains.


100 bp DNA ladder
RT-PCR nisA (wt-L. lactis)
PCR nisA (wt-L. lactis)
RT-PCR nisA (B. subtilis SYA2NE)
PCR nisA (B. subtilis SYA2NE)
RT-PCR nisA (wt-B. subtilis)
RT-PCR NO RNA
RT-PCR nisB (wt-L. lactis)
RT-PCR nisB (B. subtilisSYA2NE)
RT-PCR nisT (wt-L. lactis)
RT-PCR nisT (B. subtilisSYA2NE)
1 2 3 4 5 6 7 8 9 10 11

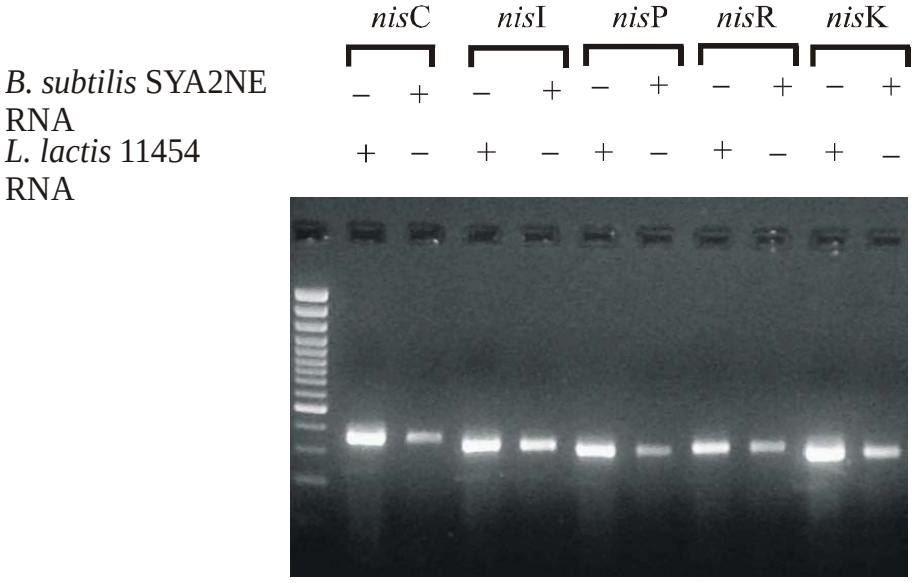

nisC
nisI
nisP
nisR
nisK
B. subtilis SYA2NE RNA
L. lactis 11454 RNA
– + – + – + – + – +
+ – + – + – + – + –

Figure 28. RT-PCR of nisin genes.

The total RNA's were isolated from the bacterial cultures by using the RNeasy mini kit (Qiagen) as described in Material and Methods Part II-26, 26-1. The RT-PCR experiment was done by using the Qiagen OneStep RT-PCR kit (Materials and Methods Part II-26, 26-2). The samples were run on a 2.5 % agarose gel. (Panel A) Lanes 3 and 5 are control lanes where only the PCR part of the reaction for *L. lactis* 11454 and *B. subtilis* SYA2NE were done with total RNA. Lane 6 is the RT- PCR of *nis*A gene from *B. subtilis* ermΔsunA. Lane7 is the negative control tube where no RNA was added. Lanes 2,8,10 are the RT-PCR reactions of *nis*A, *nis*B and *nis*T from *L. lactis* 11454 and Lanes 4, 9, 11 are the reactions for the same genes from *B. subtilis* SYA2NE. (Panel B) The RT-PCR of the rest of the genes was done both with the nisin producer *L. lactis* 11454 and recombinant strain of *B. subtilis* containing the nisin gene cluster (SYA2NE). The + signs indicates the presence of RNA and – signs indicates the absence of RNA in the reaction mixture of the corresponding strain.

Therefore the possibility remains that there was insufficient mRNA being produced to lead to detectable amounts of translation products.

Name	Sequence (5’ to 3’)
SYRTNISAf	CGAGCATAATAAACGGC
SYRTNISAr	GGATAGTATCCATGTCTGAAC
SYRTNISBf	CGATCAACTCCATTTGGATTATTT
SYRTNISBr	CGTCTCCATAGCAAAGCGTTA
SYRTNISTf	AGCTTTTGTTCCGTTGGCTA
SYRTNISTr	TGAGATAAACGATGAAAGCACAA
SYRTNISCf	TCTCTACAGGATTGCCTGGT
SYRTNISCr	TCATATTGCTCGTCGTTGATT
SYRTNISIf	AGGAGATTCCTCAGGAGAACG
SYRTNISIr	CATCAGATACGGTGGTGTCC
SYRTNISPf	TCTTCAACAGAGGGATCAACG
SYRTNISPr	TAAGCGGAGAGGAAGTAACGA
SYRTNISRf	AAAACATTTCACTTCCCTTGGA
SYRTNISRr	TTGACACAGACTCGCCTTTC
SYRTNISKf	GGGCTTGACTTTCTTTCTACGA
SYRTNISKr	ATAAGAAATTGAACGAAGCGTATG

Table 5. RT-PCR primers used in Results Part V

Oligonucleotides SYRTNISAf (57) and SYRTNISAr (57) were used to amplify 321 bp region from the *nis*A gene (Results Part V-1), SYRTNISBf and SYRTNISBr were used to amplify A 355 bp fragment from the *nis*B (Results Part V-1), SYRTNISTf and SYRTNISTr were used to amplify a 340 bp region from the *nis*T gene, SYRTNISCf and SYRTNISCr were used to amplify a 370 bp region from the *nis*C gene, SYRTNISIf and SYRTNISIr were used to amplify a 351 bp region from the *nis*I gene, SYRTNISPf and SYRTNISPr were used to amplify a 340 bp region from the *nis*P gene, SYRTNISRf and SYRTNISr were used to amplify a 358 bp region from the *nis*R gene and finally SYRTNISKf and SYRTNISKr were used to amplify a 340 bp region from the *nis*K gene.

Part V-6. Western blot analysis using antibodies to nisin.

Several fractions that show activity in the halo assay were subjected to SDS-PAGE and Western blot analysis. Figure 29 shows the Western blot obtained using antibodies to nisin. There were two bands in the last lane, which contains nisin. Nisin has been observed to polymerize at high pH (1). The reactive nature of the dehydro residues is likely to be responsible for this polymerization. Nucleophilic side chains such as the ones present in lysine and histidine could possibly react with the electrophilic dehydro residues forming polymers. Up to eight and more nisin molecules has been observed to produce polymers when nisin was incubated at high pH (1). Nisin was also observed to polymerize when stored at –20°C for long periods of time(1). The lower band observed in this lane coresponded to the molecular weight of nisin and the upper band is possibly a dimer of nisin. One of the HPLC fractions from the culture supernatant of *B. subtilis* SYA2NE, the recombinant strain containing the nisin gene cluster, reacted with the nisin antibody. The band had the same molecular weight as nisin dimer. However, no signal was observed where the monomer form appeared. Corresponding fraction from the wt-*B. subtilis* culture did not react with nisin antibodies as expected. This result suggests that a nisin like peptide is being produced and secreted. Since the antibody was able to recognize this peptide, it is highly likely that it contains one or more of the modified residues. It is also possible that nisin was present in the medium as monomers; however, the antibody failed to recognize it due to the lack of appropriate 3-D structure in the monomer form. When this putative nisin molecule forms dimers it might be possible that the appropriate confirmation was achieved and the antibody was able to detect it.

Figure 29. Western blot analysis using anti-rabbit nisin antibodies.

The peptides were isolated from the culture supernatants as described previously except nisin. The HPLC fractions that showed antimicrobial activity (Lanes 2-4), sublancin, and nisin were run on a SDS-poly acrylamide gel and blotted onto a NC-membrane (Materials and Methods Part II, 27-29). Lane 1 is purified sublancin, Lane 2 is Fraction #16 from the culture supernatant of *B. subtilis* SYA2NE (Figure 26), Lane 3 is Fraction #17 (5μl of 0.01 μg/μl) from the culture supernatant of *B. subtilis* SYA2NE (Figure 26). Lane 4 is Fraction #17 (Figure 27) from the culture supernatant of wt-*B. subtilis* 168. Lane 5 is 1 μg of nisin.

The amount of the purified peptide was calculated by determining the sensitivity of nisin antibodies against nisin. Picture 30 shows the western analysis of nisin dilutions. The lowest signal of nisin that could be detected with the nisin antibodies was 0.025 μg. The intensity of the signal obtained with 0.05 μg nisin was about the same as the signal obtained at fraction 17. Acording to this result, concentration of the peptide in fraction #17 was calculated to be 0.01 μg/μl. The amount of the total purified peptide in fraction #17 was 1 μg.

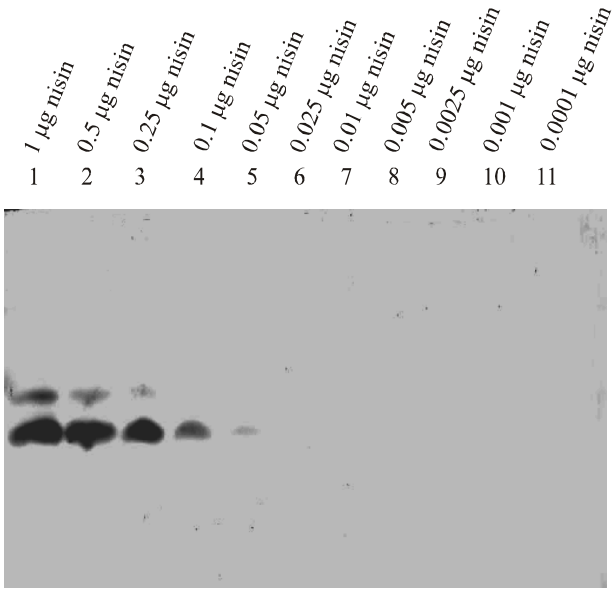


Figure 30 Detection of the purified nisin by western blot analysis.

Various amounts of nisin was run on a SDS-polyacrylamide gel and blotted onto a NC-membrane (Materials and Methods Part II, 27-29).

Discussion

Lantibiotics are a family of gene encoded antimicrobial peptides. They are of particular interest due to the presence of unusual amino acid residues that are introduced by post-translational processing. These residues confer novel biological and chemical properties such as reactive groups that are not present in naturally occurring amino acids (electrophilic dehydro residues) and chemical stability and protease resistance (lanthionine residues).

Nisin is the prototype lantibiotic and the only antibiotic of any kind that has been approved by the FDA to be used as a food preservative. The natural producer of nisin, *L. lactis*, has limited genetic tools available for it. Transfer of the ability to produce nisin into another strain that can be readily manipulated by genetic engineering is important for several reasons. Many structural analogs of nisin could be created which in turn could be used for structure/function studies to elucidate the site and mode of action of this lantibiotic. The current understanding of lantibiotic biosynthetic enzymes is largely based on sequence homology and genetic studies (26;41). The availability of appropriate mutants may make it possible to biochemically characterize the biosynthetic pathway, which includes novel modification enzymes. Finally, the availability of genetic tools might allow us to increase the efficiency of production, which would be of great importance for its industrial and pharmaceutical applications.

Previously, it was thought that nisin could probably be expressed in *B. subtilis* by using the subtilin biosynthetic machinery since the nisin and subtilin are similar and are synthesized by homologous proteins. Various attempts have been made to express nisin

by using just the nisin structural gene in a subtilin producer strain, *B. subtilis* 6633 (31), (66). However, none of them has resulted in the production of nisin. Other attempts to express nisin, such as by attaching the nisin mature segment to the subtilin leader segment in *L. lactis* (46)and in *B. subtilis* 6633 (30;64) has resulted in the secretion of inactive forms of nisin.

The goal of this work was to express nisin in *B. subtilis* 168 by transferring the nisin gene cluster from the natural producer into the chromosome of *B. subtilis* 168. A vector was constructed in order to introduce recombination sites from the flanking regions of the nisin gene cluster so that all the genes that are involved in the nisin biosynthesis could be integrated into *B. subtilis* 168 by a double recombination. The strain 168 has many properties that make it an attractive candidate for heterologous expression of nisin. These include the availability of many genetic tools, already being in use for industrial production of bioengineered proteins, its being a lantibiotic producer, and its being a gram-positive bacterium that is naturaly competent.

The heterologous expression of lantibiotics such as nisin is more complicated than a typical polypeptide, since it requires the presence of not only the structural gene but also modification enzymes, immunity proteins, regulatory proteins, and transport proteins.

The initial strategy was to transfer14 kb nisin gene cluster into *B. subtilis* 168 by a double recombination. A double recombination is a rare event and happens in fewer than 1 in 100,000 cells. Moreover, the two chromosomes do not have any natural homologies and the piece of DNA to be transferred was relatively large. In order to get a recombinant

clone containing the complete nisin gene cluster, we needed a selection strategy that combined efficient recombination with an efficient selection strategy.

Several strategies were used to enhance the chance of recombination. One strategy involved PCR amplification of the entire nisin gene cluster and cloning in a plasmid. Another strategy involved cloning the cluster in λ, and then transfering into a plasmid. These strategies had two advantages. First of all, a double recombination was not absolutely necessary for integration since the plasmid that contained the gene cluster was transformed in the circular form, and thus could integrate by single recombination. Second, these strategies eliminated the competition by extraneous *L. lactis* DNA because a copy of nisin gene cluster would be present in every cell. This enhances the chances of recombination.

Competence in *B. subtilis* 168 is mediated by a set of proteins, which are responsible for binding, transport into the cell, and incorporation of the foreign DNA into its chromosome (77). Upon binding to DNA, one strand is stripped off and left outside whereas the other is drawn inside. During this process, random cuts are introduced to the DNA, so that what was taken inside consists of random fragments. Once the fragments are inside the cell, if there are sufficient regions of homology, recombination can occur. There is probably a limit to the amount of DNA that can be taken inside and this amount is correlated to the number of copies of the binding proteins. It is the saturation of these binding proteins by extraneous DNA that lowers the efficiency of uptake of the desired DNA segment.

If all of the above factors are taken into consideration, integrating all the nisin genes into the *B. subtilis* 168 chromosome is not a simple process. On the other hand,

transfer of large segments of DNA is not uncommon and happens efficiently if the appropriate conditions are established. The transfer of subtilin producing ability from *B. subtilis* 6633 into *B. subtilis* 168 is an example of this. Since the two chromosomes have substantial homologies, the subtilin genes were easily integrated into 168 and then expressed without any need to do further manipulations (50).

Therefore, by devising a selection strategy using a selection marker that can function in *B. subtilis* 168, and by increasing the chances of recombination, we were able to clone the nisin genes into the chromosome of *B. subtilis* 168. Nisin genes are likely to be evolved to function in a variety of hosts, as the genes are located on a 70 kb conjugative transposon which is a mobile genetic element. Thus once all the genes were integrated, nisin production had been expected.

The standard methods for isolation of lantibiotics were used to isolate nisin from culture supernatants of recombinant strains of *B. subtilis* 168 containing the nisin gene cluster. Even though an antimicrobial activity was isolated that has the chromatographic properties that are similar to nisin, no nisin or nisin-like peptide could be detected using MS analysis. The antimicrobial activity was correlated with a molecular weight that was smaller than nisin. To test the possibility that nisin was being degraded by host proteases, nisin genes were transferred into a protease deficient strain of *B. subtilis* 168. The protease deficient strain produced the same active, small peptide that was produced by the normal strain. Moreover, this active peptide was also observed in the control culture of the wild type 168 strain. This proved that this antimicrobial activity was not due to the presence of nisin genes. However, the possibility that the nisin peptides were being degraded by the host enzymes still remains. At least 35 proteases have been identified in

the *B. subtilis* 168 (48) chromosome and any one of these peptides might play a role in the degradation of nisin. The “protease deficient” strain was deficient in just a few of these proteases.

The expression from the nisin gene cluster is controlled by a two-component response regulator system. The presence of subinhibitory levels of nisin is essential to switch on this expression system in the producer strain, since nisin itself is the inducer of transcription of the nisin gene cluster in the natural host. The sensitivity to induction by nisin changes greatly from organism to organism. Studies of the nisin promoter have established that ng/ml levels induced transcription in *L. lactis*, but μg/ml levels are required to induce expression in *B. subtilis* 168. Whether it is sufficient to induce nisin gene expression in the inoculum culture, or it is needed in the actual culture medium is not clear. The latter would require growing the cells in the presence of 20 mg of nisin for each liter of culture just to induce expression.

The possibility that the promoters present in the nisin gene cluster did not function properly in *B. subtilis* 168 was explored by replacing the normal promoter with promoters that are known to function well in *B. subtilis* 168. For example, a constitutive promoter that is known to function in *B. subtilis* 168 was integrated into a location upstream from the nisin structural gene. Despite the presence of the promoter, no nisin or nisin-like peptide could be detected when the purified culture supernatant was analyzed with mass spectrometry. In order to insure that the promoter had been integrated properly, the promoter region was PCR amplified and sequenced. The sequence confirmed the correct placement of the promoter. However, even though the promoter is present, it is not a guarantee that it is functioning properly. Alternatively, the genes are

expressed but there are either no translation products or the translation products do not undergo the normal post-translational processing reactions.

The presence of the mRNA transcripts of genes in the nisin gene cluster was established with an RT-PCR experiment. The amplimers of the mRNAs from each of the nisABTCIPRK genes were detected by running the reaction mixtures on an agarose gel. The intensities of the RT-PCR product bands from the recombinant strain were similar to that of the natural host, which suggests, but does not prove that the genes are transcribed at comparable levels in both strains. This is because this was not a quantitative RT-PCR experiment, so one cannot be certain that mRNAs were expressed at similar levels. Thus there is still a possibility that the mRNA expression was not efficient enough to make a detectable translation product. However, the fact that RT-PCR products were obtained for all the genes in the cluster proves that all of the genes are present and are functional in their ability to produce transcripts.

Even though nisin can be readily isolated from the culture supernatant of its natural producer, no nisin or nisin like peptide could be detected from the recombinant strains of *B. subtilis* 168 that contained nisin gene cluster by mass spectrometry analyses. The correct post-translational modifications of specific residues in lantibiotics have been shown to be prerequisite for processing and secretion of the active peptide (47). The leader peptide plays a critical role in the orchestration of the posttranslational modifications such as dehydrations of serines and threonines, formation of lanthionine rings, proteolytic cleavage of the leader peptide, and transport to the extracellular medium as a mature active peptide. If there is a problem with one or more of these

events, it may prevent the production of the active peptide. Nisin might be produced and secreted but not be in the natural form due to the reasons discussed above.

Some evidence in the production of a nisin like peptide was obtained. Two fractions that showed antimicrobial activity in the halo assay from the culture supernatant of *B. subtilis* 168 containing the nisin gene cluster were analyzed by a western blot. The blot was probed with nisin antibodies and a signal that corresponded to a nisin dimer but not a nisin monomer, was observed in one of these active fractions. The corresponding HPLC fraction from the culture supernatant of the wild type *B. subtilis* 168 did not contain any signals at all. This result argues that a nisin-like peptide was produced and secreted but it was not a normal nisin molecule. The control lane containing the nisin had both monomer and dimer forms. Nisin has been shown to polymerize at high pH or when stored at –20 °C for prolonged periods of time (1). It is possible that the secreted product had various missing or incorrect posttranslational modifications that might favor the polymerization of the molecule or alternatively the monomer form was not recognizable by the antibody molecule. It is not clear at this time what kind of aberrant modification might induce this polymerization. But since the antibody molecule was apparently able to recognize a peptide argues that the peptide is a translation product of the nisin gene, and that it may have undergone some of the posttranslational processing events. It is also possible that the prepeptide was trapped in the cell wall prior to the secretion and only low levels of prepeptide was secreted outside the cell. If the leader peptide had not been cleaved off after the secretion, the molecular weight of the prepeptide would be close to the molecular weight of nisin dimer. Thus, the signal, which was higher than the molecular weight of mature nisin in the western blot could possibly correspond to the

molecular weight of the precursor peptide. Identifying the source of the problem associated with nisin production has been a challenging task. Now that the nisin gene cluster has been incorporated into the *B. subtilis* 168 chromosome, future studies of the expression of these genes, and the biosynthetic pathway that they mediate, will be possible.

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