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Philosophiae Doctor (PhD) Thesis 2011:55

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Characterization Of Fecal Lactic Acid Bacteria Isolated From Healthy Ethiopian Infants: Bacteriocin Production And Antibiotic Susceptibility

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KARAKTERISERING AV FEKALE MELKESYREBAKTERIER ISOLERT FRA ETIOPISKE SPEDBARN: BAKTERIOCIN PRODUKSJON OG ANTIBIOTIKA SENSITIVITET

Philosophiae Doctor (PhD) Thesis

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Summary

In this thesis lactic acid bacteria (LAB) were isolated from fecal samples of healthy Ethiopian infants, identified to species level, screened for bacteriocin production and tested for antibiotic susceptibility. Enterococci were also screened for the production of cytolysin and gelatinase.

Among 150 LAB isolates, 81 lactobacilli, 54 enterococci and 15 streptococci were identified by 16S rRNA gene sequence analysis. Most of the lactobacilli were obtained from breast-fed infants, while most of the enterococci and the streptococci were from mixed-fed infants. *Lactobacillus fermentum*, *Enterococcus avium* and *Enterococcus faecalis* were the most frequently isolated species.

About 10% of the LAB produced bacteriocins. The majority of the producers were enterococci, indicating a higher prevalence of bacteriocin production in enterococci compared to lactobacilli. For some of the bacteriocins, the producers were found to occur in high numbers in fecal samples, suggesting that bacteriocin renders the producers the capacity to outcompete other bacteria. Six new bacteriocins were detected, two of which showed strong activity against pathogenic bacteria and thus were purified and characterized at biochemical and genetic levels. These are a 4288.2 Da pediocin-like bacteriocin (avicin A) and a 3466.55 Da trypsin-resistant, nisin-like lantibiotic bacteriocin (salivaricin D). Avicin A, produced by *E. avium* strains, is active against the food-borne pathogen *Listeria monocytogenes*. Sequence analysis showed that avicin A gene was found on a 7 Kb locus that contains genes encoding bacteriocin synthesis, export, immunity and regulation. Avicin A is similar to mundticin KS and enterocin CRL 35, but its locus resembles that of sakacin X. We showed that the production of avicin A is inducible and regulated by a quorum sensing regulatory system. Salivaricin D is produced by *S. salivarius* isolates which appeared to account for over 60% of the fecal LAB flora of an infant. It is active against the important pathogens *S. pyogenes* and *S. pneumoniae*, which cause a wide variety of diseases. Sequence analysis showed that salivaricin D is located on a 16.5 kb locus that consists of genes responsible for its synthesis, modification, export, processing, immunity and regulation. The primary structure and locus of salivaricin D is similar to that of nisin Q.

Avicin A and salivaricin D may be potential chemotherapeutic agents that might control infections due to the respective pathogens they inhibit. Moreover, the producing strains may

be used as potential probiotic strains in appropriate settings. Avicin A and salivaricin D share some common features that may increase the chance of the two bacteriocins or their producers to be used as chemical or biological control agents (probiotics), respectively.

Different patterns of antibiotic susceptibility were observed among the LAB. Nearly all lactobacilli were sensitive to chloramphenicol, erythromycin and tetracycline. Most lactobacilli were resistant to aminoglycosides and vancomycin which is intrinsic. Many enterococci showed resistance mainly to tetracycline which might have been acquired, but ampicillin and vancomycin resistance was almost absent. Multidrug resistance as well as resistance to high level of aminoglycosides was common among the *E. faecalis* and *E. faecium* strains. The streptococci were generally susceptible to the antibiotics. These results suggest that transferrable antibiotic resistance is common among the enterococci, but not among the lactobacilli and streptococci. A very low prevalence of cytolysin and gelatinase production was observed among the enterococci, suggesting that infant enterococci may not cause diseases.

Sammendrag

I denne avhandlingen ble melkesyrebakterier (LAB) isolert fra fekale prøver av friske etiopiske spedbarn, identifisert til artsnivå, screenet for bacteriocin produksjon og testet for sensitivitet av antibiotika. Enterokokker ble også screenet for produksjon av cytolysin og gelatinase.

Blant 150 LAB isolater, ble 81 laktobasiller, 54 enterokokker og 15 streptokokker identifisert ved 16S rRNA gensekvensanalyse. De fleste av laktobasiller ble isolert fra diende spedbarn, mens det meste av enterokokker og streptokokker var fra blandings-matede spedbarn. Rundt 10% av LAB produserte bakteriociner. Flertallet av produsentene var enterokokker, noe som indikerer en høyere prevalens av bacteriocinproduksjon i enterokokker sammenlignet med laktobasiller.

For noen av bakteriocinene, ble produsentene funnet oftere i høye tall i fecal prøver, som kan tyde på at bacteriociner øker produsentenes kapasitet til å utkonkurrere andre bakterier. Seks nye bakteriociner ble oppdaget, to av dem viste sterk aktivitet mot sykdomsfremkallende/patogene bakterier og bel derfor renset og karakterisert biokjemisk og genetisk. Disse er et 4288,2 Da pediocin-lignende bacteriocin (avicin A) og en 3466,7 Da trypsinresistent, Nisin-lignende lantibiotisk bacteriocin (salivaricin D). Avicin A, produsert av *E. avium* stammer, er aktivt mot mat-borne patogen *Listeria monocytogenes.* Sekvensanalyse viste at avicin A lokuset er 7,5 Kb og inneholder gener som koder for produksjon, eksport, immunitet og regulering av avicin A. Avicin A er lik mundticin KS og enterocin CRL 35, men dens locus minner om Sakacin X. Vi viste at produksjonen av avicin A er induserbar og regulert av et quorum sensing system.

Salivaricin D er produsert av *S. salivarius* isolater som utgjorde over 60% av fekal LAB flora hos et spedbarn. Den er aktiv mot viktige patogener inkludert *S. pyogenes* og *S. pneumoniae* som forårsaker en rekke sykdommer. Sekvensanalysen viste at salivaricin D ligger på et 16,5 kb locus som består av gener ansvarlig for produksjon, modifikasjon, eksport, prosessering, immunitet og regulering. Primærstrukturen og av salivaricin D og genlocuset ligner Nisin Q.

Avicin A og salivaricin D er potensielle kjemoterapeutika som kan kontrollere infeksjoner av respektive patogener de ble vist å hemme. Videre kan de produserende stammene potensielt brukes som probiotiske stammer i visse betingelser. Avicin A og salivaricin D har noen felles funksjoner som kan fremme muligheten for at de to bakteriocinene eller deres produsenter

kan brukes som henholdsvis kjemiske eller biologiske kontrollagenser (probiotika).

Ulike mønstre av mottakelighet for antibiotika ble observert blant LAB. Nesten alle lactobacilli var følsomme for kloramfenikol, erytromycin og tetracyklin. De fleste lactobaciller var iboenderesistente mot aminoglykosider og vankomycin. Mange enterokokker viste ervervet tetracyklinresistens, mens resistens mot ampicillin og vankomycin var nesten fraværende. Multiresistens samt resistens mot høye nivåer av aminoglykosider var vanlig blant *E. faecalis* og *E. faecium* stammer. Streptokokker var generelt sett antibiotikasensitive. Disse resultatene tyder på at overførbar antibiotikaresistens er utbredt blant enterokokker, men ikke blant laktobasiller og streptokokker. En svært lav prevalens av cytolysin og gelatinase produksjon ble observert blant enterokokker, som kan tyde på at enterokokker fra spedbarn ikke forårsaker sykdommer.

List of papers

This thesis consists of the following three papers:

Paper I

Birri DJ, Brede DA, Tessema GT and Nes IF.2011. Bacteriocin production, antibiotic susceptibility, and prevalence of cytolysin and gelatinase production in fecal lactic acid bacteria isolated from healthy Ethiopian infants. (Manuscript)

Paper II

Birri DJ, Brede DA, Forberg T, Holo H, Nes IF.2010. Molecular and genetic characterization of a novel bacteriocin locus in *Enterococcus avium* isolates from infants. Appl Environ Microbiol 76(2):483-492

Paper II

Birri DJ, Brede DA, Nes IF .2011. Biochemical and genetic characterization of salivaricin D, an intrinsically trypsin resistant lantibiotic from *Streptococcus salivarius* 5M6c isolated from a healthy infant. (Submitted)

1. Introduction

1.1. The human gut microflora

1.1.1. Adults

The adult gut microflora is a complex community characterized by high density and diversity which increases from the stomach to the colon (143, 171). An estimated number of at least 1000 bacterial species are found in the human gut, with an individual containing at least 160 species (199). The gut microflora consists of archaea, bacteria, eukarya and viruses (22, 69, 183, 199, 202). The bacteria are the most diverse and dominant (> 99%), represented by members of 9 phyla, of which Firmicutes and Bacteroidetes predominate (> 90%), followed by members of Actinobacteria and Proteobacteria (9, 199). The proportion of Bacteroidetes is lower than Actinobacteria in obese individuals (243). The archaea and eukarya are represented by members of one phyla each (202). The viruses represent 1200 genotypes (22).

Community composition of the gut microflora differs from individual to individual (57). In spite of this, it has been shown that there is no significant difference in community structure of the gut microflora between monozygotic and dizygotic twin pairs, and family members contain more similar community structure compared to unrelated individuals (243). Moreover, a metagenomic study that involved 124 individuals has shown that there is a common group of bacterial species (common bacterial core) that are shared among individuals (199). For example, 18 species were common to all study subjects, 75 to ≥ 50% and 57 to ≥ 90% of the individuals (199).

1.1.2. Infants

The gut of a new born infant is sterile. Soon after and/or at birth microbes start to colonize it. The gut of infants are colonized early by higher proportion of aerobic or facultative anaerobic bacteria (enterobacteria, enterococci and streptococci) which after about one week reduce the gut environment, making it conducive for subsequent colonization by anaerobic bacteria, such as bifidobacteria, *Bacteroides* and clostridia (2, 247). The diversity, complexity and stability of the intestinal microflora of infants increases with age until it becomes adult-like microflora after about one year of age (190, 247).

Mother, food and environment are the primary sources of microbes that colonize the gut of infants (143). Many factors affect the colonization pattern, development and composition of the intestinal microflora of infants. These include feeding type, mode of delivery,

environment, and hygiene and living style (2, 3, 62, 189, 191). In addition, host genetics (270) and ingestion of antimicrobial substance (2) can influence gut microbial diversity.

1.1.3. Function of gut flora

The intestinal flora have been shown to serve various functions, including host nutrition, regulation of epithelial development (203), regulation of host fat storage (8), stimulation of intestinal angiogenesis (231), inflammatory immune responses (182, 212) and pathogen resistance (148).

1.2. Lactic acid bacteria

Lactic acid bacteria (LAB) are low GC content (< 55%) Gram-positive cocci, coccobacilli, or rods, nonsporulating, catalase negative, acid tolerant and facultative anaerobic bacteria that produce lactic acid as a major end-product of hexose fermentation (6, 249). LAB include species belonging to the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Oenococcus*, *Leuconostoc*, *Carnobacterium*, *Vagococcus*, *Weissella* and *Tetragenococcus* (6, 249). The LAB are divided into two physiological groups based on their fermentation pathway. The homolactics (e.g., *Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus* and some species of *Lactobacillus*) produce mainly lactic acid as an end-product of fermentation of glucose, whereas heterolactics (e.g., *Oenococcus*, *Leuconostoc* and some species of *Lactobacillus*) produce other end-product besides lactic acid (115). The LAB are found in nutrient rich habitats such as milk and dairy products, vegetables and plants, cereals, meat and meat products and GIT of humans and animals.

The LAB have various beneficial properties. They play important roles in production and preservation of fermented food products (90). Their metabolic products allow them to impart foods with characteristic flavor and aroma. The LAB also produce a variety of antimicrobial substances, such as organic acids, diacetyl, ethanol, hydrogen peroxide and bacteriocins that can inhibit the growth of food spoilage or pathogenic bacteria in foods. Furthermore, the LAB can be used as probiotics (140), which may promote the health of the host (86). Some strains of *Lactobacillus, Enterococcus* and *Streptococcus* are currently in use as probiotics (132).

1.2.1. Lactobacilli

The lactobacilli are the largest group of LAB that contain at least 145 species. They are facultative heterofermenters, obligate heterofermenters, or obligate homofermenters, and

have complex nutritional requirements (233, 249). The lactobacilli can grow in habitats containing high levels of soluble carbohydrates, protein digests, vitamins and low level of oxygen (89). They occur in the oral cavity, gastrointestinal tract, and vagina of humans and animals, on plants and materials of plant origin, and in manure, sewages and food (89, 233).

Lactobacilli are generally regarded as safe or nonpathogenic but some species are occasionally associated with certain infections such as endocarditis, bactremia, peritonitis, abscesses, and meningitis (26, 221). Lactobacilli can show resistance to several antibiotics (42). For example, many species of lactobacilli display intrinsic resistance to vancomycin and acquired resistance to tetracycline and erythromycin. Antibiotic resistance in lactobacilli is a concern since they are associated with food, and can thus serve as potential reservoirs for transmissible antibiotic resistance genes (158).

1.2.2. Enterococci

Enterococci are coccoid LAB that occur singly, in pairs, or in chains. They ferment glucose mainly to lactic acid. Enterococci grow best at 35 °C, but most species can grow between 10 and 45 °C (71). They can also grow in the presence of 6.5% NaCl, 40% bile salts at pH 9.6, and are relatively heat resistant, capable of surviving at 60 °C for 30 min (187).

Enterococci are typically found in the intestinal tract of mammals and birds (47). The number of enterococci in human digestive tract ranges from 10^2 to 10^8 per gram of digestive content (187). Some are found in the soil, food, water, sewage and plants. They are also less commonly found in vagina, oral cavity and on skin.

Enterococci were under group D streptococci before they were placed in the genus *Enterococcus* (222). Currently, there are 28 species in the genus *Enterococcus,* most of which have been divided into 8 groups (*E. faecium* group, *E. avium* group, *E. gallinarum* group, *E. dispar* group, *E. saccharolyticus* group, *E. cecorum* group, *E. faecalis* group and *Tetragenococcus*) on the basis of 16S rRNA sequence homology analysis (73, 131). Among the enterococci, *E. faecalis* and *E. faecium* are the most common and probably the most important species in health, food and probiotics (72).

Enterococci play important roles in food and health (71, 73). They are involved in the production and preservation fermented food products, such as cheeses (71). They are also used as probiotics (51). Enterococci are opportunistic pathogens that are capable of causing

both community-acquired and hospital acquired (nosocomial) infections, such as urinary tract infections, bacteremia, bacterial endocarditis, diverticulitis, wound infection and meningitis (126), *E. faecalis* alone accounting for about 80% of the infections (104). Many enterococci have virulence factors that can promote their ability to cause infections. These include gelatinase, cytolysin, aggregation substance and other (71, 72, 126, 237). Gelatinase and cytolysin are tissue-damaging virulence factors that are more common in clinical than non-clinical isolates (56, 224).

Enterococci can be resistant to many antibiotics. Both intrinsic and acquired resistance are observed among the enterococci (168). They show intrinsic resistance to cephalosporins, penicillinase resistant semi-synthetic penicillins (e.g., oxacillin, methicillin), monobactams, polymyxins, quinupristin–dalfopristin, nalidixic acid, lincosamides, low levels of aminoglycosides and low levels of clinidamycin, and vancomycin resistance in *E. gallinarum* and *E. casseliflavus.* Resistance to high levels of aminoglycosides, high levels of trimethoprim, and high levels of clindamycin, chloramphenicol, tetracyclines, penicillins (due to β-lactamase), fluoroquinolones, macrolides (e.g., erythromycin), glycopeptides and oxazolidinones (linezolid) are acquired. Not only does acquired resistance pose a challenge in treatment, but also it can be transferred through pheromone-mediated, conjugative plasmids or transposons to other enterococci (165). Vancomycin resistance also attracts a special attention since this antibiotic has been used as a last choice in the treatment of infections caused by multidrug resistant enterococci (73, 164).

1.2.3. Streptococci

The streptococci are cocci or short rods that most commonly occur in chains or pairs. They are oxidase-negative, homofermentative and have complex nutrition requirements. There are about 55 species of *Streptococcus* which are widely distributed in nature (61). They occur mainly on the mucosal surfaces (mouth, upper respiratory tract, alimentary canal, genitourinary tract) of man and animals. Some are found in the soil, water, dairy products and other foods, and on plants.

Some species of *Streptococcus* are human or animal pathogens (30). *S. pyogenes*, being the most pathogenic species of streptococci, causes a wide variety of diseases, such as pharyngitis and impetigo, scarlet fever, erysipelas, cellulitis, necrotizing fasciitis, wound infections, toxic shock syndrome, puerperal fever, rheumatic fever and glomerulonephritis

(40, 61). *S. pneumoniae* causes pneumonia and meningitis (61). *S. agalactiae* is the major cause of neonatal sepsis and also causes mastitis in cattle (223). *S. thermophilus* is used as a starter culture in dairy products.

Antibiotic resistance is rare amongst the streptococci and is not a major problem in treatment (91). The streptococci are generally sensitive to penicillin, the antibiotic of choice for treatment of streptococcal infections. However, resistance of some viridians streptococci (including *S. pneumoniae*) to penicillin and macrolides, especially erythromycin and tetracycline, is currently increasing (24, 141, 142, 211, 213, 230, 238).

There are 11 species of streptococci that are β-haemolytic, the most important ones being *S. pyogenes* and *S. agalactiae* (61). The non-β-haemolytic include α-haemolytic (the viridan streptococci) and γ-haemolytic species.

1.2.4. Intestinal lactic acid bacteria

The LAB account for a significant proportion of the complex intestinal microflora of humans and animals. They are among the early colonizers of the intestinal tract of infants (103, 163). LAB species belonging to the genera *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Streptococcus* are commonly detected in intestinal samples (19, 70, 251).

1.2.5. Lactic acid bacteria as probiotics

According to World Health Organization, probiotics are defined as "live microorganisms which when ingested in sufficient quantities can confer health benefits on the host" (63). The possible health benefits include protection against/treatment of infections, inflammatory diseases, bowel syndromes, allergy and cancer (140, 192, 207, 239). It has been suggested that probiotics control pathogens through, for example, production of antimicrobial substances, such as bacteriocins and organic acids, competition with pathogens for binding site and nutrients, enhancement of gut epithelial function, and modulation of the host immune response (159, 175). Today, the most important group of bacteria that are being used or promising for future use as probiotics are the bifidobacteria and the LAB, mainly lactobacilli, some enterococci and streptococci. Selection of bacterial strain for probiotic use is based on the following criteria: the strain has to be preferably of healthy human origin, acid and bile tolerant, adhere to gut cells, persist in GIT, produce antimicrobial substances, antagonistic against carcinogenic and pathogenic bacteria, be safe in clinical and food use (non-pathogenic), and show clinically validated and documented health effects (214).

1.3. Bacteriocins

1.3.1. Definition and classification

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins that are produced by bacteria and may kill members of the same or a wide range of species (130). Bacteriocins are produced inside the cells but are excreted to the exterior environment where they kill target cells. The producing cells have immunity mechanisms to protect themselves from their own bacteriocins (236). Both Gram-negative and Gram-positive bacteria are known to produce bacteriocins (84, 114, 236).

Gram-negative bacteria, mainly members of the *enterobacteriaceae* produce bacteriocins called colicins and microcins (84). Colicins are large (30-75 kDa), plasmid-borne bacteriocins produced by *Escherichia coli* under stress conditions, and act by diverse mode of action, including depolarization of the cytoplasmic membrane, a non-specific DNase activity, a highly specific RNase activity or inhibition of murein synthesis (28). Microcins (<10 kDa) may be linear and unmodified (class I) or post-translationally modified (class II) and act by depolarizing cell membranes or interfering with intracellular functions (55).

Gram-positive bacteria produce a great diversity of bacteriocins and bacteriocin-like peptides which are currently divided into two major classes, excluding lytic proteins: class I (post-translationally modified bacteriocins) and class II (non-modified bacteriocins) (39, 205). Bacteriocins of Gram-positive bacteria will be discussed in detail because of their relevance to this thesis.

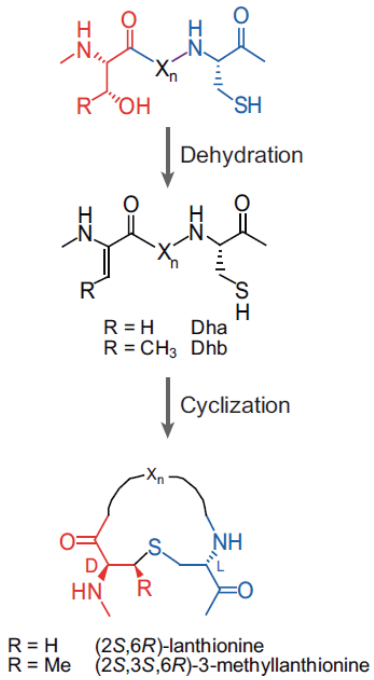

Figure 1. Formation of thioether amino acids (red- derived from Ser or Thr and blue- derived from cysteine (259).

1.3.1.1. Class I (Modified bacteriocins)

1.3.1.1.1. Structure, function and classification

Class I is subdivided into three: lantibiotics (class Ia), labyrinthopeptins (class Ib) and sactibiotics (class Ic). Lantibiotics are small (< 5 kDa), heat stable, post-translationally modified bacteriocins that contain unusual amino acid residues such as the thioether amino acids lanthionine (Lan) and methyllanthionine (MeLan), the dehydrated (unsaturated) amino acids 2,3-didehydroalanine (Dha) and 2,3-didehydrobutrine (Dhb). Lantibiotics may also contain other less commonly modified amino acids (259).

During modification process (Fig. 1), the hydroxy amino acids serine and threonine are selectively dehydrated to Dha and Dhb, respectively. Lan and MeLan are formed as a result of intramolecular addition of the nearby sulfhydryl amino acid cysteine to the double bonds of Dha and Dhb, respectively. Lan is made up of two alanine residues (L-alanine from serine and D-alanine from cysteine) and MeLan is composed of α-aminobutyric acid and D-alanine linked to each other by ether bonds through their β-carbons (31, 180). The thioether linkage

results in the formation of a polycyclic structure which is important for the function, heat stability and resistance to proteases of mature peptide lantibiotic.

Class Ia (lantibiotics) consist of the great majority of modified bacteriocins and are further subdivided into 4 subclasses (I- IV) based on how they are modified and presence or absence of antimicrobial activity (194). Subclass I lantibiotics are linear peptides that are modified by two different enzymes: LanB enzyme (dehydratase) and LanC enzyme (cyclase) (135), exported by LanT (a dedicated ABC transporter), and their leader peptides, which contain the motif FNLD, are removed by LanP enzyme, a subtilisin-like serine protease (216). This subclass includes 5 groups: nisin, epidermin, streptin, pep5 and planosporicin. Subclass II are modified by a single enzyme (LanM) which has both dehydratase and cyclase activity and contains zinc ligand, and exported by LanT which also cleaves the leader peptides at GG or GA or GS (a double glycine motif). This LanT contains a conserved N-terminal cysteine protease domain and is different from LanT of subclass I. Subclass II include 6 groups: lacticin 481, mersacidin, LtnA2, cytolysin, lactosin S and cinnamycin. Subclass III are lantibiotics-like peptides that include morphogenetic peptides, such as SapT, Amfs and SapB which do not have antimicrobial activity but are important in hyphae formation in filamentous bacteria and fungi (133, 134, 240, 245). They appear to be modified by a C-terminal LanM-like enzyme which is devoid of a zinc ligand. Subclass IV comprise lantibiotic-like peptides lacking antimicrobial activity (lantipeptides) which are modified by new lanthionine synthetases (LanL), which generate dehydroamino acids by a mechanism other than direct dehydration that involves phosphorylation and dephosphorylation of hydroxyl amino acids (85).

Class Ib (labyrinthopeptins) include carbacyclic bacteriocins that are characterized by containing labionin, a post-translationally modified amino acid (161). They are active against Herpes simplex virus.

Class Ic (Sactibiotics) are characterized by formation of cross-linkages between the sulfurs of the cysteine residues and α-carbon of other residues. Bacteriocins with this feature include the cyclic bacteriocin subtlosin A, and the two-peptide bacteriocin thuricin CD (125, 157, 206).

Not all modified peptides bacteriocins can be placed in the above classification scheme. Nonlantibiotic bacteriocins that contain other types of modifications, such as glycosylation in sublancin (188) and glycocin F (232), and formylation of N-terminal methionine in the leaderless, two peptide bacteriocins enterocin 7A and enterocin 7B (139) have been reported very recently. Therefore, future bacteriocin classification schemes must take these into account and also the lantibiotic-like peptides that lack antimicrobial activities should not be grouped with bacteriocins or else the definition of bacteriocins must be updated.

1.3.1.1.2. Genetics

A lantibiotic is produced as an inactive prepeptide comprising an N-terminal leader peptide (23-59 amino acids) and C-terminal propeptide (19-39 amino acids) and must undergo maturation which involves modification, export and removal of the leader to be active. The genes required for the biosynthesis, immunity and regulation of lantibiotics are generally clustered in a locus on chromosomes, transposons or plasmids. The prepeptide is encoded by *lanA* gene. Modification of the prepeptide to mature biologically active peptide occurs in two steps: dehydration of Ser and Thr which is catalyzed by gene product of *lanB* (123) or *lanM* (dehydratase) and cyclization/formation of thioethers which is mediated by gene product of *lanC* or *lanM* (cyclase) (135). The cyclic prepeptide is exported by gene product of *lanT* (a dedicated ABC transporter). Removal of the leader is performed by the gene product of *lanP* (248) or *lanT* itself. The producer cell is protected from its own lantibiotic by *lanI*(*H*) that encodes immunity protein and/or *lanEF*(*G*) which encode for 3 peptides that are located in the cell membrane and that form ABC transporters that are able to protect the producer cells (53, 227). The *lanF* gene encodes the ATPase domain while *lanE* and *lanG* encode the integral membrane domain of the transporter (53). The production of lantibiotics and their immunity proteins is regulated by one or more regulatory proteins (LanR, LanK, LanQ or LanX) in cell-density dependent manner, and many lantibiotics, including nisin, subtilin, bovicin HJ50, mersacidin and salivaricin A are inducers of their own production (31, 160, 176).

1.3.1.1.3. Mode of action of lantibiotics

Many lantibiotics bind to lipid II, a precursor in the bacterial cell formation, and inhibit cell wall biosynthesis and/or form pore in the cell membrane which leads to cell death (17). Nisin and epidermin groups, plantaricin C and lactcin 3147 have dual mode of action, whereas mersacidin and actagradine do not form pores (17). Nisin and probably other lantibiotics with

rings A and B remove lipid II from the septum, blocking synthesis of the cell wall (92). Mersacidin and actagradine act specifically by blocking the transglycosylation step in the peptidoglycan synthesis (23). The duramycin group binds to membrane phospholipids and inhibit phospholipase A2 (150).

1.3.1.2. Class II (Non-modified bacteriocins)

Class II bacteriocins include small, heat stable, non-lanthionine containing antimicrobial peptides (39). They do not undergo extensive post-translational modifications and act most commonly by causing permeabilization of the bacterial cell membrane (37). Non-modified bacteriocins are subdivided into four subclasses: class IIa (pediocin-like), class IIb (two-peptide), class IIc (cyclic), class IId (non-pediocin single peptide linear) bacteriocins (39, 205).

The class IIa bacteriocins are listeria-active cationic peptides that are characterized by having a YGNGV/L motif which is followed by two conserved cysteine residues that form a disulfide bond separated by four amino acids in their N-terminal regions (Fig. 2) (15, 59, 60). Like class I bacteriocins, class IIa bacteriocins are produced as inactive prepeptides consisting of the N-terminal leader peptide (15-30 amino acids) and the C-terminal propeptide (37-58 amino acids), and in order for the bacteriocin to be active, the leader must be cleaved off. The leaders of most class IIa bacteriocins have two conserved glycine residues (GG) at their C-terminal end (double glycine leader) where cleavage occurs (172). Cleavage and export is carried out by a dedicated ABC transporter, resulting in a mature biologically active bacteriocin (94). The cytosolic N-terminal domain contains proteolytic activity, while the cytosolic C-terminal domain has ATP-binding cassette that provides energy for transport (94). But those with sec-dependent leaders, such as enterocin P, bacteriocin 31 and listerocin 743A are secreted by a sec-dependent transport system (33, 121, 242).

Based on their primary structures class IIa bacteriocins are divided into a cationic, hydrophilic and highly conserved N-terminal region and a more hydrophobic and less conserved C-terminal region (65, 68). Sequence similarities and differences in the C-terminal region were used to classify class IIa bacteriocins into four subgroups (Fig. 2) (180).

The three dimensional structure of some class IIa bacteriocins has been elucidated by circular dichroism and nuclear magnetic resonance, showing that the conserved N-terminal region

assumes a three-stranded antiparallel β-sheet-like structure stabilized by a disulfide bridge between the two conserved cysteine residues, while the more hydrophobic C-terminal region generally forms a hairpin-like structure consisting of an α-helix followed by an extension of C-terminal tail that folds back on to the central α-helix (Fig. 3A) (93, 246). The hairpin-like structure is stabilized, in some class IIa bacteriocins, by a disulfide bridge between a cysteine residue in the middle of the α-helix and a cysteine residue at the C-terminus and by two conserved tryptophan residues (one at the center and the other at C-terminal end) in those that lack these cysteine residues (Fig. 3B) (67). The two regions are separated by a flexible hinge that allows their movement relative to each other (76). The hydrophilic N-terminal regions orient themselves on the outside target cells membranes, while the hydrophobic C-terminal region penetrates the membranes (32, 127, 162). The class IIa bacteriocins are structured when they come in contact with membrane-mimicking entities, but not structured in solution (180).

Although class IIa bacteriocins share high sequence similarity, they differ in their target cells specificity (Fig. 2). It has been shown that the C-terminal part of class IIa bacteriocins determines the specificity between these bacteriocins and the target cells (119). The sensitive cells recognize the bacteriocin through the C-terminal part of subunit IIC of their mannose phosphotransferase system (49, 128).

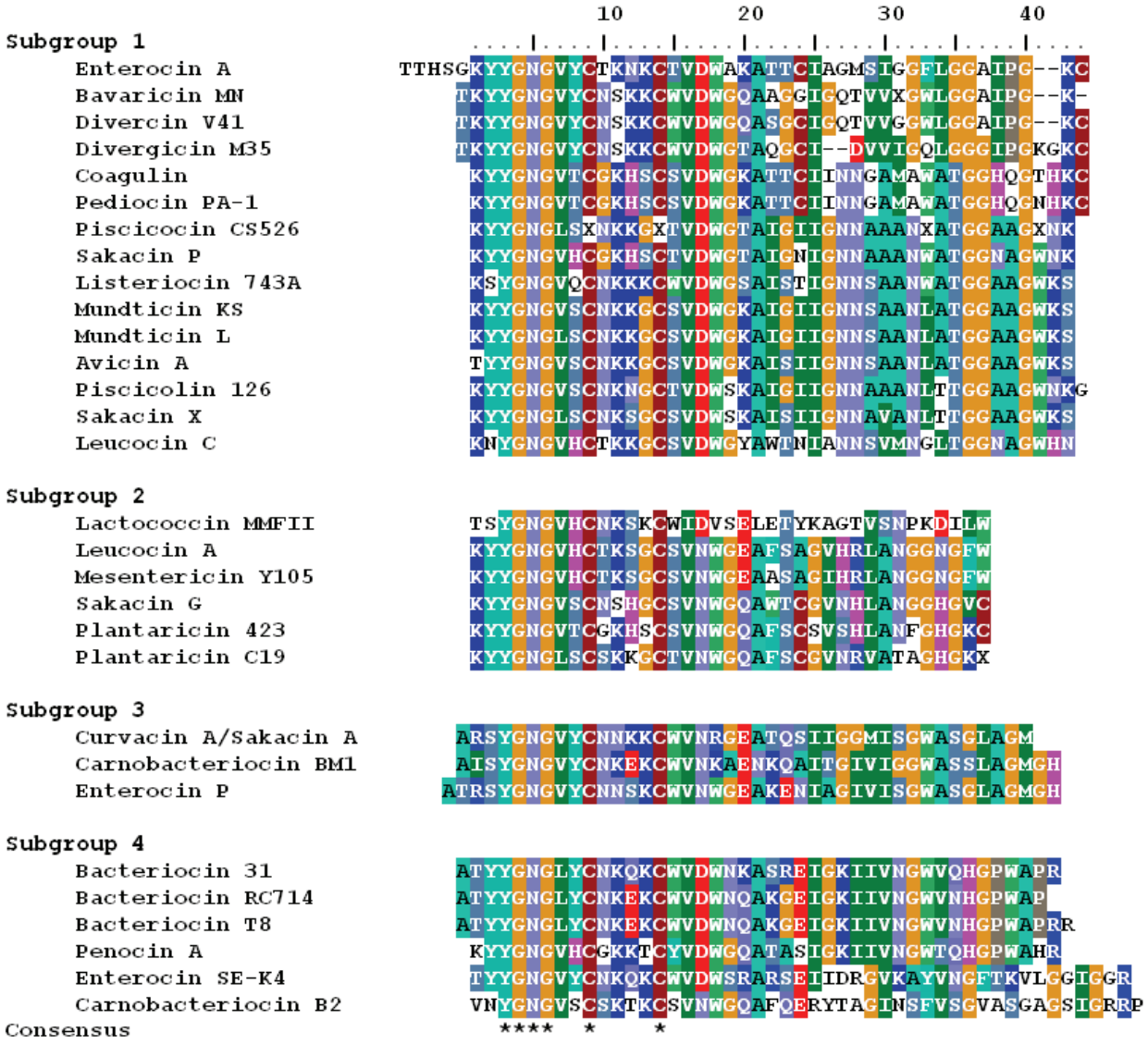

Figure 2. Multiple alignment of subgroups of class IIa bacteriocins (180). The highly conserved residues in the N-terminal part of class IIa bacteriocins are indicated by asterisks.

Immunity proteins for class IIa bacteriocins are highly charged cytosolic proteins which are thought to have association with the cell membrane (118). They are divided into three groups (A, B, C) based on the similarities of their primary structures (68). The immunity proteins for class IIa bacteriocins are very specific to their own bacteriocins, and the C-terminal part of the immunity proteins specifically recognize the C-terminal part of their cognate bacteriocins (118). Although they are very specific to their cognate bacteriocins (118), the immunity proteins may provide cross-protection when either the bacteriocins or the immunity proteins are very closely related or belong to the same subgroup (66). They can also provide protection when they are heterologously expressed in bacteriocin-sensitive cells (66, 201).

Very recently, it has been shown that the Abi proteins also confer immunity function against cognate bacteriocins (129).

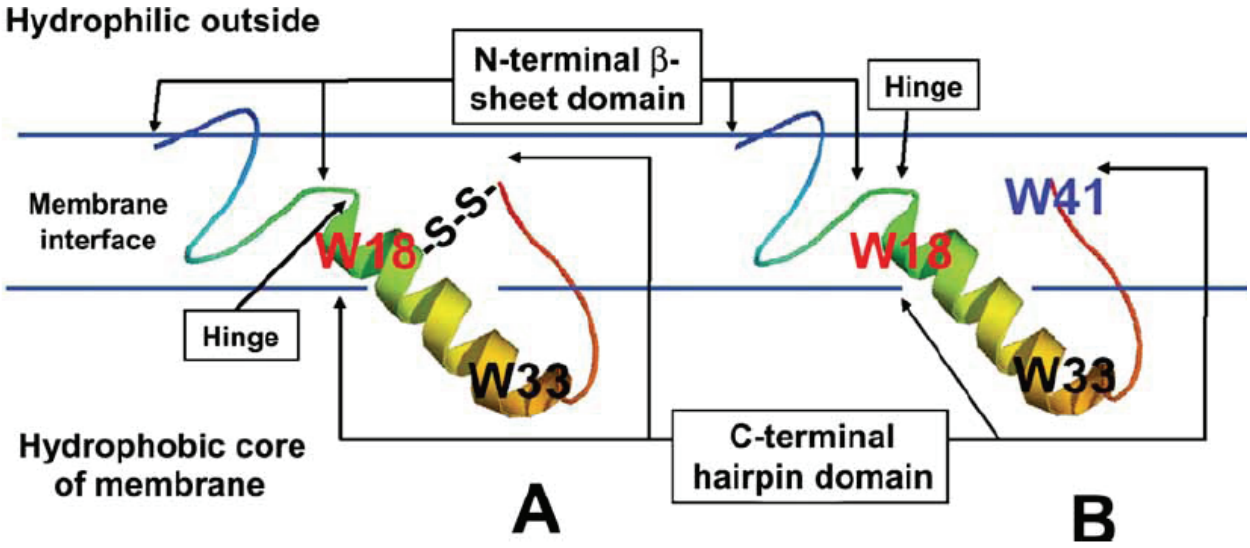

Figure 3. The structure of class IIa bacteriocins belonging to subgroup 1 and 2 (180). The β-sheet-like and the hairpin-like structures are stabilized by (A) a disulfide bride and (B) tryptophan residues, respectively.

The production of class IIa bacteriocins usually requires four genes: a bacteriocin gene (encodes bacteriocin precursor), an immunity gene (protects the producer form its bacteriocin), and the ABC transporter and transport accessory genes (172, 229). The biosynthesis of class IIa bacteriocins is frequently regulated by a quorum sensing regulatory mechanism that consists of a peptide pheromone (inducing peptide) which acts as a signal in a phosphorylation reaction with the receptor histidine protein kinase followed by the phosphorylation of the response regulator to activate the gene expression of the various operons required for bacteriocin production (174). The genes for most class IIa bacteriocins production and regulation are located on plasmids (some on chromosomes) in two or more operons. Details about class IIa bacteriocins can be found in recent reviews (15, 54, 180).

Class IIb bacteriocins are two-peptide bacteriocins that act synergistically when the two peptides are combined in about equal amounts. One or both of the two peptides have no or little activity separately (178). Like class IIa bacteriocins, i) they have a double glycine leader (15-30 amino acids) that is removed by a dedicated ABC transporter, ii) the production of some of them is regulated by a three-component quorum sensing system, iii) they are not structured in solution but when they come in contact with membrane mimicking entities and

iv) they kill cells by forming pores in the target cell membrane. Class IIb two-peptides and their immunity proteins are encoded by a single operon (178), suggesting that the peptides are produced proportionally. All class IIb bacteriocins have the GxxxG motifs (Fig. 4). For detail information about class IIb bacteriocins very recent reviews can be read (178, 179).

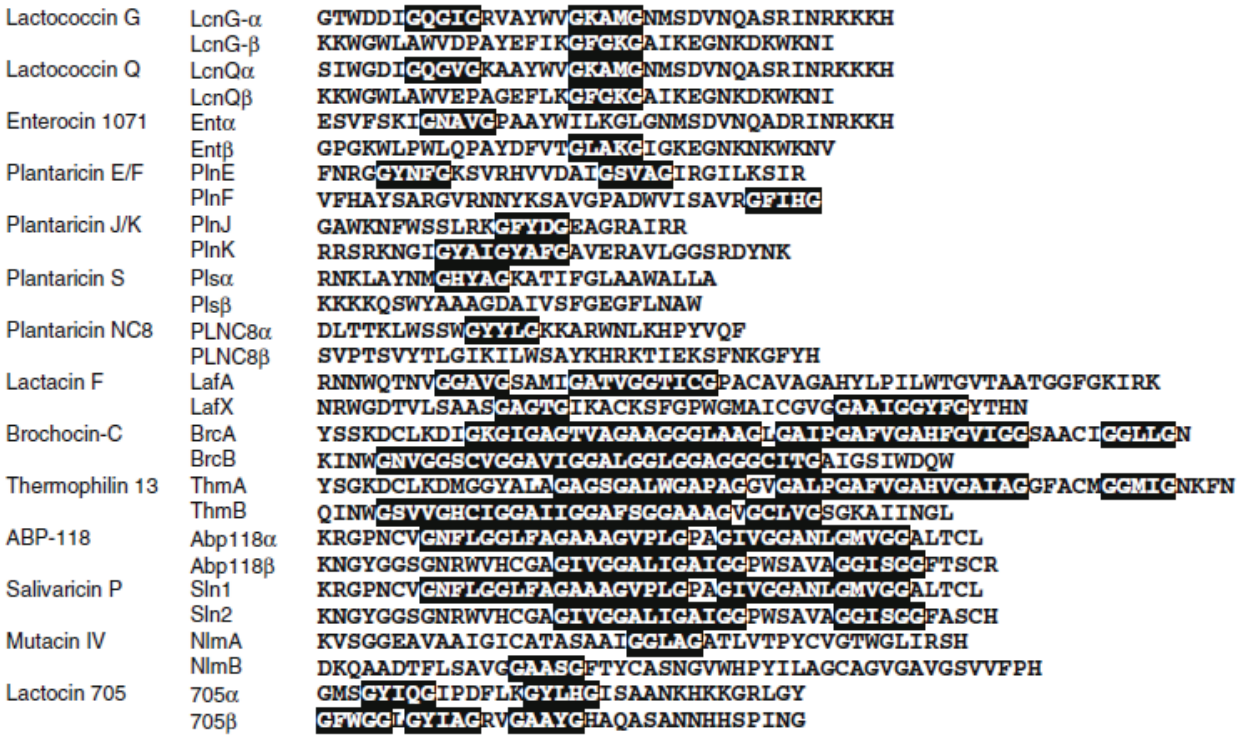

Figure 4. Class IIb bacteriocins (178).

Class IIc bacteriocins have a cyclic primary structure in which the N- and C-termini are covalently linked by amide/peptide bonds (153). The circular structure renders these bacteriocins conformational, pH and heat stability, and resistance to digestion by proteases (147, 154, 220). At least 9 cyclic bacteriocins have been characterized so far (gassericin A, enterocin AS-48, enterocin 4, carnocyclin A, butyrivibriocin AR10, lactocyclicin Q, uberolysin, cirularin, garvicin ML) (152).

Nearly all class IIc bacteriocins are cationic, relatively hydrophobic and their mass is between 3400 to 7200 Da. The number of amino acids in their leaders and mature peptides ranges from 2-35 and 35 to 70, respectively (147, 220). Like many other bacteriocins, cyclic bacteriocins act by permeabilization of the target cell membrane (180).

The three dimensional structure has been elucidated for enterocin AS-48 and the recently characterized carnocyclin A (83, 153, 154), revealing that these two bacteriocins are structured in solution, unlike other class II bacteriocins, and fold into a compact globular shape in which four (in carnocyclin A) to five (in enterocin AS-48) α-helicies surround a hydrophobic core.

The class IId bacteriocins are linear non-pediocin-like bacteriocins that do not share sequence similarity with the pediocin-like bacteriocins. They are very diverse in their amino acid sequences and mechanism of action (112). Many of them have no leader sequences (e.g., enterocin EJ97, Enterocin L50A/L50B, enterocin Q, Aureocin A70, Aureocin A53). Some have a double glycine leader (e.g., lactococcin A, enterocin B). Others have sec-dependent leaders (e.g., divergicin A, propionicin T, lactococcin 972). The class IId also contain ungrouped bacteriocins such as lactococcins A, B and M.

1.3.2. Diversity of bacteriocins of lactic acid bacteria

Most characterized bacteriocins are produced by lactic acid bacteria and they are very diverse. Seven genera of LAB are known to produce bacteriocins. These are *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Lactococcus*, *Carnobacterium*, *Leuconostoc* and *Pediococcus*. However, most of the LAB bacteriocins have been characterized from *Lactobacillus* (> 40), *Enterococcus* (> 30), *Streptococcus* (> 30), *Lactococcus* (> 15) and *Carnobacterium* (> 10). Except *Streptococcus*, which mainly produces lantibiotics, these genera commonly produce class II bacteriocins. Bacteriocins of enterococci and streptococci will be discussed in the following sections because of their relevance to this thesis.

1.3.2.1. Bacteriocins of enterococci

Enterococci produce many bacteriocins (called enterocins) which have been recently reviewed (74, 173). Almost all enterocins are class II bacteriocins, cytolysin and columbicin A being the only class I (lantibiotics) produced by enterococci (Table 1). Class II enterocins are diverse and

Table 1. Bacteriocins of enterococci[a]

Bacteriocin	Class*	Producer strain	Length (aa)	Mass* (Da)	Reference/ Accession
Avicin A	IIa	*E. avium* XA83, *E. avium* 208	43	4288	(18)
Bac 32	IId	*E. faecium* VRE200	70	7998	(111)
Bacteriocin 31	IIa, sec dep.	*E. faecalis* YI717	43	ND	(242)
Bacteriocin 51	ND	*E. faecium* VRE38	105	~ 12000	(265)

Bacteriocin E 50-52	IIa	*E. faecium* NRRL B-30746	39	3340	(234)
Bacteriocin GM-1	IIa	*E. faecium* GM-1	44	4630	(122)
Bacteriocin RC714	IIa, sec dep.	*E. faecium* RC714	42	~ 3000	(46)
Bacteriocin T8, Hiracin JM79, Bacteriocin 43	IIa, sec dep.	*E. faecium* T8, *E. hirae* DCH5, *E. faecium* VRE82	44	5090	(43, 218, 241)
Columbicin A	I	*E. columbae* PLCH2	33	ND	ABJ98063
Cytolysin Cyl_L, Cyl_S	I, two peptide	*E. faecalis* strains	38, 21	3458, 2032	(87)
Durancin L28-1A	IId	*E. durans* L28-1	ND	~ 3400	(266)
Durancin TW-49M	IId, GG leader	*E. durans* QU 49	54	5228	(102)
Enterocin 012	ND	*E. gallinarum* 012	ND	~ 3400	(116)
Enterocin 1071A & Enterocin 1071B	IIb	*E. faecalis* BFE 1071	39, 35	4285, 3897	(11)
Enterocin 3Da	ND	*E. faecium* 3D		3893	(13)
Enterocin 3Db	ND	*E. faecium* 3D		4203	(13)
Enterocin 62-6	IId, leaderless	*E. faecium* 62-6		5206, 5219	(48)
Enterocin 7A (Ent7A), enterocin 7B (Ent7B)	IId, leaderless, N-terminally formylated	*E. faecalis* 710C	44, 43	5200.8, 5206.65	(139)
Enterocin 96	II, GG leader	*E. faecalis* WHE 96	48	5494	(113)
Enterocin A	IIa	*E. faecium* T136, *E. faecium* DPC1146	47	4829	(7, 184)
Enterocin AS-48	IIc	*E. faecalis* S-48	70	7166	(146)
Enterocin B	IId	*E. faecium* T136 *E. faecium* BFE 900	53	5479	(27, 75)
Enterocin E-760	II	*Enterococcus* species	62	5362	(137)
Enterocin EJ97	IId, leaderless	*E. faecalis* EJ97	44	5328	(79, 217)
Enterocin L50A & Enterocin L50 B	IId, leaderless	*E. faecium* L50	44, 43	5190, 5178	(34, 35)
Enterocin LR/6	ND	*E. faecium* LR/6	ND	~ 6100	(136)
Enterocin P	IIa, sec dep.	*E. faecium* P13, *E. faecium* L50	44	4493	(33, 34)
Enterocin Q	IId, leaderless	*E. faecium* L50	34	3980	(34)
Enterocin RJ-11	IId, leaderless	*E. faecalis* RJ-11	44	5049	(264)
Enterocin S37	ND	*E. faecalis* S37	ND	4000-5000	(14)
Enterocin SE-K4	IIa	*E. faecalis* K-4	43	5356	(50, 58)
Enterocin x	II	*E. faecium* KU-B5	40, 37	4420, 4069	(101)
Enterocin-HF	IIa	*E. faecium* HS, *E. faecium* TA29	43	ND	P86183
MR10A, MR10B	IId, leaderless	*E. faecalis* MRR 10-3	44, 43	5202, 5208	(151)
Mundticin L	IIa	*E. mundtii* CUGF08	43	ND	(64)
Mundticin, Mundticin KS, Enterocin CRL35	IIa	*E. mundtii* ATO6, *E. mundtii* NFRI 7393, *E. mundtii* CRL35	43	4287	(16, 124, 215)

* ND, not determined; *sec dep., sec dependent; [a] Not all bacteriocins of enterococci are included in this table

include many pediocin-like, sec-dependent, leaderless, circular and non-pediocin-like linear peptide bacteriocins (Table 1). Most of the bacteriocins are produced by strains of *E. faecalis* and *E. faecium* (Table 1). A study has shown that 47% of 218 enterococcal isolates (*E. faecalis and E. faecium*) obtained from different sources produced bacteriocin (46). Bacteriocin production by other enterococcal species is rare. Only four bacteriocins (all of which are nearly identical) from *E. mundtii*, two from *E. avium* and *E. durans* each, one from *E. hirae, E. columbae* and *E. gallinarum* each, have been reported (Table 1).

Of the two bacteriocins reported from *E. avium,* one is avicin A which is produced by *E. avium* XA83 and 208 and which were isolated from two different babies (18). Avicin A is a 4288.2 Da class IIa bacteriocin that is very active against listeria. It has been characterized biochemically and genetically. It is found in a 7 kb locus that consists of genes responsible for its production, immunity, export and expression. It shares very high similarity with mundticins (18). The other bacteriocin (~ 6k Da) is produced by *E. avium* PA1 isolated from honey bee (5). It is also active against listeria but it has not been characterized genetically.

Bacteriocins of enterococci are considered important because they are generally active against food-borne pathogens such as *Listera moncytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* (33, 34).

1.3.2.2. Bacteriocins of streptococci

Streptococci, like the enterococci, are also known to produce many bacteriocins, most of which are lantibiotics (Table 2). Many of the bacteriocins are produced by oral streptococci, such as *S. salivarius* and *S. mutans* (Table 2).

S. salivarius produces bacteriocins called salivaricins (salivaricin A, A2-A5, A9, B, D, G32) and streptin (Table 1), all of which are lantibiotics (20, 107, 185, 210, 253, 256, 257). The bacteriocins are frequently found on megaplasmids (160-220 kb) which usually carry multiple bacteriocins; consequently, some *S. salivarius* strains produce two or more bacteriocins (252). For example, a 220 kb plasmid from *S. salivarius* JH bears salivaricin A3, salivaricin G32 and streptin, *S. salivarius* 9 contains a 170 kb plasmid that encodes salivaricin 4 and 9, and the oral probiotic *S. salivarius* K12 has a 190 kb plasmid that carries salivaricin A2 and B (252, 257).

Table 2. Bacteriocin of streptococci[a]

Bacteriocin	**Class***	**Producer strain**	**Length (aa)***	**Mass (Da)***	**Reference**
BHT-A	I, two peptide	*S. rattus* strain BHT, *S. mutans*	30, 32	2802, 3375	(106)
Bovicin 255	IId	*S. gallolyticus LRC0255*	56	5968	(258)
Bovicin HC5	I	*S. bovis* HC5	ND	2440	(145)
Bovicin HJ50	I	*S. bovis* HJ50	33	3428	(138, 263)
BTH-B	IId	*S. rattus* strain BHT, *S. mutans*	44	5195	(106)
Dysgalacticin	Heat-labile, nonlytic	*S. dysgalactiae* subsp. *equisimilis*	192	21493	(96, 98)
Macedocin	I	*S. macedonicus* ACA-DC 198	26	2795	(80)
Mutacin 1140	I	*S. mutans* JH1005	22	2263	(100)
Mutacin B-Ny266	I	*S. mutans* Ny266	22	2270	(166)

Mutacin F-59.1	IIa	*S. mutans* 59.1	25	ND	(177)
Mutacin I	I	*S. mutans* CH43, *S. mutans* UA140	24	2364	(196, 197)
Mutacin II	I	*S. mutans* T8	27	3245	(181, 262)
Mutacin III	I	*S. mutans* UA787	22	2266	(198)
Mutacin IV	I, two peptide	*S. mutans* UA140	44, 49	4169, 4826	(196)
Mutacin K8	I	*S. mutans* K8	26	2734	(209)
Mutacin N	II	*S. mutans* N	49	4806	(10)
Nisin U	I	*S. uberis* 42	32	3029	(260)
Phocaecin PI80	ND	*S. phocae PI80*	ND	9244	(219)
Salivaricin 9	I	*S. salivarius 9*	24	2560	(253, 257)
Salivaricin A	I	*S. salivarius* 20P3	22	2315	(210, 256)
Salivaricin A1	I	*S. pyogenes* 148, *S. dysgalactiae* 4003, *S. agalactiae* 120	22	2327	(256)
Salivaricin A2	I	*S. salivarius* K12, *S. salivarius* DPC6481	22	2368, 2366	(107, 185)
Salivaricin A3	I	*S. salivarius* JH	22	2319	(256)
Salivaricin A4	I	*S. salivarius* 9	22	2342	(256)
Salivaricin A5	I	*S. salivarius* H21f, *S. salivarius* DPC6490	22	2329	(185, 256)
Salivaricin B	I	*S. salivarius* K12*, S. mitis*	25	2740	(107)
Salivaricin G32	I	*S. salivarius* JH	25	2667	(253)
Salivaricin D	I	*S. salivarius* 5M6c	34	3466.55	(20)
Smb	I, two peptide	*S. mutans* GS5	ND	ND	(268)
Streptin 1	I	*S. salivarius*	23	2424	(253, 255)
Streptin 2	I	*S. pyogenes*	26	2821	(255)
Streptocin STH	IIb	*S. gordonii* DL1	ND		(97)
Streptococcin A-FF22	I	*S. pyogenes* FF22	26	2795	(108)
Streptococcin A-M49	I	*S. pyogenes* serotype M49	26		(109)
Streptococcin A-M57	Non-lytic	*S. pyogenes* FF22 M-type 57	ND	~17000	(98)
Thermophilin 1277	I	*S. thermophilus* SBT1277	33	3700	(120)
Thermophilin 13	IIb	*S. thermophilus* SFi13	62,43	5776, 3910	(149)
Ubericin A	IIa	*S. uberis* E	49	5271	(95)
Uberolysin	IIc	*S. uberis* 42	70	7048	(261)

* ND, not determined; [a] This table does not contain all bacteriocins from streptococci

Salivaricin A and its variants (A1-A5) are autoinducible bacteriocins that are produced not only by *S. salivarius,* but also by other streptococcal species such as *S. pyogenes*, *S. dysgalactiae* and S. *agalactiae* (254, 256) and inhibit *S. pyogenes*. Salivaricin A production is prevalent in at least 10% of *S. salivarius* isolates (254). Salivaricin B is another autoinducible salivaricin which kills *S. pyogenes* and inhibits several other streptococci (253). Salivaricin G32 differs from SA-FF22 only by one amino acid and it has double copies of its structural gene (254). Salivaricin 9 is kills *S. pyogenes* and some enterococci (254). Streptin, also produced by *S. pyogenes*, inhibits many species of streptococci including *S. pneumoniae*, *S. salivarius* and *S. mutans* (254). Salivaricin D, which is characterized in this thesis, is active against *S. pyogenes, S. pneumoniae* and other bacteria (20).

1.3.3. Applications of bacteriocins

The ability of bacteriocins to kill or inhibit the growth of other bacteria, especially pathogens and food-spoilage bacteria can be exploited to control microbes in several settings. Bacteriocins act against diverse group of pathogens. In this respect, bacteriocins can have potential applications in food and in medicine (39, 44, 77, 78, 208). In food they can be used to control food spoilage bacteria and food-borne pathogens (such as listeria, bacilli and clostridia, staphylococci), increasing the shelf-life of food products and reducing the risk of getting food-borne infections and food intoxication. Bacteriocins can be used in food in several ways. Purified or semi-purified bacteriocin can be added to the food, or bacteriocin-producing strain can be added or bacteriocin can be used in combination with other hurdles (45). Currently, two bacteriocins (nisin and pediocin PA-1) are in use as food additives to preserve foods and feeds (45). Bacteriocin-producing starter cultures are commercially used both in dairy and meat fermentation industry.

Bacterial resistance to traditional antibiotics has been increasing, and under such condition bacteriocins may be potential candidates that can replace some antibiotics. Either purified bacteriocins or bacteriocin-producing bacteria can be used in this respect. Some bacteriocins have proven their potential in the treatment of human and animal infections. For example, the use of nisin in 1) the treatment of peptic ulcer disease by inhibiting growth and colonization of *Helicobacter pylori* 2) the prevention of *Clostridium difficile* from colonizing the colon and 3) killing multidrug resistant *Streptococcus pneumoniae* (226). Salivaricin A and B producing *S. salivarius* strains were capable of protecting against sore throats caused by *S. pyogenes* and salivaricin B has been implicated in the treatment of halitosis (25, 235). *Lb. salivarius* UCC118, a probiotic strain that produces bacteriocin Abp118, has been shown to prevent mice from infection by *Listeria monocytogenes* (38). A very recent study has shown that thuricin CD, a narrow-spectrum bacteriocin, effectively killed *Clostridium difficile* without significant effect on the other colon bacteria, indicating that thuricin CD may be used for selective control of *C. difficile* infection (204, 206).

Although so many studies have been conducted to identify new bacteriocins and exploit them for human and animal benefit, a few were approved for use in food and health. Therefore, the search for new bacteriocins must be continued until the best ones are discovered. The present study has been conducted in light of this issue.

2. Objectives of the study

a. General objectives

i. To characterize LAB (lactobacilli, enterococci and streptococci) isolates obtained from fecal samples of healthy Ethiopian infants with emphasis on bacteriocin production and antibiotic susceptibility

ii. To identify and characterize new bacteriocins that may have potential applications in food and health

iii. To study the occurrence of tissue-damaging virulence factors in the enterococci isolated from the infants

b. Specific objectives

i. To isolate LAB from fecal samples of healthy infants and identify them to species-level by 16S rRNA gene sequence analysis

ii. To screen LAB strains for production of bacteriocins

iii. To purify selected bacteriocins that may have potential applications in food and health (that kill pathogens) by chromatography

iv. To sequence purified bacteriocins by Edman degradation and/or mass spectrometry

v. To identify the genetic basis for production of new bacteriocins

vi. To determine if the production of the bacteriocins is inducible

vii. To test the susceptibility of LAB strains to nine selected antibiotics

viii. To identify potentially transferrable and multidrug resistance phenotypes

ix. To determine the prevalence of cytolysin and gelatinase production among the enterococci isolated from the infants

3. Main results

Paper I

Bacteriocin production, antibiotic susceptibility, and prevalence of cytolysin and gelatinase production in fecal lactic acid bacteria isolated from healthy Ethiopian infants

Birri DJ, Brede DA, Tessema GT and Nes IF. (2011). Manuscript

In this paper, LAB were isolated from fecal samples of healthy Ethiopian infants and screened for bacteriocin production and antibiotic susceptibility. In addition, the prevalence of cytolysin and gelatinase production was determined in the enterococci. Out of 150 LAB isolates obtained from 28 infants (17 breast-fed and 11 mixed-fed), 81, 54 and 15 were identified to be lactobacilli, enterococci and streptococci, respectively. Most lactobacilli were isolated from breast-fed infants, whereas most enterococci and streptococci were from mixed fed-infants. About 10% of the LAB were found to produce bacteriocins. The majority of bacteriocin producers were enterococci. The bacteriocins included a new class IIa bacteriocin called avicin A from *Enterococcus avium*, a new nisin-like lantibiotic called salivaricin D form *Streptococcus salivarius*, a two-peptide bacteriocin gassericin T from *Lb. gasseri*, a class IIa bacteriocin and an unknown bacteriocin from *E. faecalis* strains and two unknown bacteriocins from *Lb. fermentum* strains. The producers of some of these bacteriocins appear to occur in a relatively higher numbers in the gut of the infants. Avicin A and salivaricin D were genetically characterized in papers II and III, respectively, while the rest were microbiologically characterized in paper I. Antibiotic susceptibility test showed that lactobacilli were generally more susceptible to chloramphenicol, erythromycin and tetracycline than to aminoglycosides and glycopeptides. Kanamycin resistance was highly prevalent (97.5%) among the lactobacilli. Most *Lactobacillus* species (except *Lb. gasseri* and *Lb. johnsonii*) were intrinsically resistant to vancomycin. The streptococci were generally susceptible to the nine antibiotics used. The enterococci showed high prevalence of resistance to tetracycline, erythromycin and kanamycin which was possibly acquired. Vancomycin resistance was very rare. Multidrug resistance as well as resistance to high level of

aminoglycosides was common among the *E. faecalis* and *E. faecium* strains. It seems that the prevalence of acquired transferrable antibiotic resistance is low among the lactobacilli, but high among the enterococci. A very low prevalence of cytolysin and gelatinase production was observed among the enterococci.

Paper II

Molecular and genetic characterization of a novel bacteriocin locus in *Enterococcus avium* isolates from infants

Birri DJ, Brede DA, Forberg T, Holo H, Nes IF. (2010) Appl Environ Microbiol 76(2):483-492

In this paper, a new class IIa bacteriocin (avicin A) was purified and characterized biochemically and genetically from *E. avium* isolates obtained from two infants. Avicin A is a 43- amino acid, trypsin and proteinase K sensitive bacteriocin with a molecular mass of 4288.2 Da. The N-terminal peptide sequence of mature avicin A was obtained by Edman degradation, and PCR with degenerate primers was used to identify the structural avicin gene and the whole bacteriocin locus (~7 Kb) was sequenced by primer-walking strategy. In addition to the structural gene that encodes avicin A, the bacteriocin locus contained genes for putative: divergicin A-like bacteriocin, immunity protein, export (ABC transporter and transport accessory protein), and regulation (sensor histidine protein kinase (HPK), response regulator (RR), peptide-pheromone (IP)). The genetic organization is similar to that of sakacin X from *Lb. sakei* 5. Avicin A shares a very high similarity with mundticins and enterocin CRL35 from *E. mundtii.* The putative proteins associated with bacteriocin production are similar to their corresponding partners in the sakacin X locus. This paper showed that avicin A production is regulated by quorum sensing regulatory system that consists of IP, HPK and RR. Avicin A has inhibitory activity against many genera of Gram-positive bacteria, including the food-borne pathogen *Listeria monocytogenes*. This suggests that avicin A may be a potential antimicrobial agent that can control this pathogen in foods and the gut.

Paper III

Biochemical and genetic characterization of salivaricin D, an intrinsically trypsin resistant lantibiotic from *Streptococcus salivarius* 5M6c isolated from a healthy infant

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This paper describes purification, and biochemical and genetic characterization of a new lantibiotic bacteriocin from *Streptococcus salivarius* 5M6c which was isolate from fecal sample of a healthy human infant. The bacteriocin, called salivaricin D, is completely different from other bacteriocins known to be produced by *S. salivarius* strains but similar to nisins. Salivaricin D has a mass of 3466.55 Da, consists of 34 amino acids and is trypsin resistant. Genome sequencing revealed salivaricin D locus which comprises 12 bacteriocin related genes that include genes for a presalivaricin D, a presalivaricin N, modification enzymes (dehydratase and cyclase), an ABC transporter for export, a protease for cleave of leader peptide, four immunity proteins, and regulatory proteins (RR and HPK). The genetic content of the locus is similar to that of nisin Q but with different arrangements. One of the immunity genes (*SalI*) was heterologously expressed in a sensitive strain and provided significant protection against salivaricin D, verifying the functionality of the immunity gene and the identity of the locus to salivaricin D. Salivaricin D appears to be an autoinducible bacteriocin as the locus contains RR, HPK and binding site (*nis*-box) for RR. Salivaricin D is inhibitory to members of seven genera of Gram-positive bacteria, including the important human pathogens *S. pyogenes* and *S. pneumoniae*, suggesting its potential application in the control of infections cause by these streptococcal pathogens.

4. General discussions

Antibiotics have been used as major weapons to combat infectious diseases nearly for a century. However, they have some drawbacks. One is that the wide spread use of antibiotics has resulted in the emergence and increasing spread of drug resistant pathogens to the extent that treatment of infections cause by such microbes has become difficult. Antibiotic resistance can develop and spread relatively easily since antibiotics have usually a single target of action and resistance genes are usually carried on transferable genetic elements, respectively. The other limitation is that

antibiotics have a broad spectrum of activity and are less specific in targeting a specific pathogen; consequently, their use destroys not only the pathogens but also the normal flora of the host.

In order to overcome the above-mentioned shortcomings inherent in antibiotics, alternative antimicrobial agents for use in human and animal health must be sought. One such agent is bacteriocins. Unlike antibiotics, many bacteriocins (lantibiotics) such as nisins have dual targets which would make it difficult for bacteria to develop resistance to bacteriocins. Resistance to bacteriocins has not yet been reported to be transmissible. Moreover, compared to traditional antibiotics, many bacteriocins have narrow spectrum of inhibitory activity and may have no or little negative effect on the normal flora. Furthermore, unlike antibiotics, bacteriocins are gene-encoded and can be genetically manipulated to improve their activity and stability. For these reasons, bacteriocins may be used as better/alternative antimicrobial agents to control infections. Alternatively, bacteriocin-producing strains may be used as biological control agents or as probiotics to control pathogens in the body and in food (38). A probiotic strain should not be pathogenic/virulent or resistant to antibiotics. The three papers included in this thesis describe isolation and identification of LAB from fecal samples of healthy Ethiopian infants and their characterization with emphasis on production of bacteriocins and antibiotic susceptibility, and production of the enterococcal tissue-damaging virulence factors, such as cytolysin and gelatinase.

Almost all infants involved in the current study were vaginally delivered, and comprised two feeding groups: breast-fed and mixed-fed. The lactobacilli were more prevalent among the breast-fed infants, whereas the enterococci and the streptococci were more prevalent in mixed-fed infants. The lactobacilli prevailed in the former possibly because they were acquired by the infants from two major sources: vagina during delivery and mother breast milk (52, 155, 156) and the ingested milk can favor the proliferation of the lactobacilli. A previous study has shown that lactobacilli were predominant LAB in vaginally delivered infants, as compared to those delivered by Caesarean section, suggesting that vagina is an important source of the lactobacilli for the infant gut (163). The predominance of enterococci as a group of LAB in mixed-fed Ethiopian infants is in agreement with the findings in the infants from the developed counties (Greece and Norway) in which the abundance of enterococci

surpassed that of the lactobacilli (70, 99, 163). Foods other than milk might have contributed to the difference since enterococci can be commonly found in foods (73, 82).

Many bacteriocins have been characterized from food or environmental bacterial isolates. Only a few were identified from the normal flora of GIT. O'shea et al. (2009) have intensively screened a very large number of mammalian gut isolates, including those of healthy humans, for bacteriocin production using a large number of indicators and found 84 bacteriocin producers in at least 40 000 isolates (about 0.2%). Paper I shows that the prevalence of bacteriocin production in the intestinal LAB was about 10% (14 bacteriocin producers in 150 LAB isolates), regardless of the limitation that we used only 5 indicators for screening. This suggests that bacteriocin-producing strains are abundant in the infants gut, and that more bacteriocin producers would have been identified if many indicators had been used. Bacteriocins can have a narrow spectrum of activity and thus the use of a few indicators for screening would result in underestimation of the number of bacteriocin producers per total number of isolates screened. Even though the majority of infants did not harbor bacteriocin-producing isolates (Paper I), it is interesting that many isolates that produced identical bacteriocins were identified in the same infant and this was true for four infants (Papers I-III). Similar results were also found by other studies (70, 185). The finding suggests that bacteriocin production might help the producers to outnumber other bacteria in a complex ecosystem such as the GIT or food, supporting the competitive exclusion principle (185). This ecological property may have a profound effect on enhancing the potential of such bacteria as candidate probiotics. However, more studies have to be done not only on the GIT microflora of infants, but also on the more complex gut flora of adults to confirm this finding. Moreover, we do not know if the relative dominance of bacteriocin producers would persist in the gut for a long period of time; thus, longitudinal studies have to be conducted to answer this question.

It is evident from Bactibase (bacteriocins database) or PubMed search that most LAB bacteriocins are produced by enterococci (88). Several studies have also shown that bacteriocin production is widely distributed among the enterococci (21, 46, 241). In consistent with these observations or studies, paper I shows that bacteriocins were

produced mainly by the enterococci from the infants. This, in turn, suggests that bacteriocin production in LAB is more prevalent in the enterococci than in the other LAB groups (lactobacilli and streptococci) and the former may be better sources from which bacteriocin production can be screened efficiently and effectively.

Among several bacteriocins identified from the LAB in this thesis (Paper I), only two novel bacteriocins were purified and characterized genetically from two different LAB species: one from *E. avium* (Paper II) and the other from *S. salivarius* (Paper III). These two bacteriocins were selected, in accordance with our objective, for purification and further characterization because they displayed strong antimicrobial activity against pathogenic Gram-positive bacteria.

Even though *E. faecium* and *E. faecalis* are known to produce many bacteriocins, other *Enterococcus* species produce a few bacteriocins (Table 1). Only one bacteriocin, which was about 6 KDa, heat-stable and listeria-active, has been reported from *E. avium* (honey isolate) before, but it has not been purified or genetically characterized (5). In paper II, a new class IIa bacteriocin (avicin A) has been characterized from *E. avium* strains that were isolated from fecal samples of two healthy infants (Ethiopian and Norwegian). Like the previously identified bacteriocin from *E. avium*, avicin A is heat-stable and listeria active, but smaller in size, suggesting that it is a different and a new bacteriocin produced by *E. avium*. Avicin A is highly similar to mundticin KS or enterocin CRL 35 which were identified from *Enterococcus mundtii* strains (124, 215), suggesting that they might have arisen from common ancestral gene as a result of mutation. Most of the genes in the locus of avicin A and their organization is similar to that of sakacin X which is produced by *Lb. sakei 5* (250). We showed, in paper II, that *Lb. sakei 5* was immune to avicin A and this could be due to the similarity between the immunity proteins of sakacin X and avicin A, but the vice versa was not true, probably due to production of multiple bacteriocins by *L. sak*ei 5 (250) to one or more of which *E. avium* XA83 can be sensitive.

We showed that avicin A is an inducible bacteriocin whose production is regulated by a three-component regulatory system. This was evidenced by identification of IP, RR and HPK in avicin A locus. The functionality these regulatory genes was confirmed

by inducing avicin A production by addition of CFS and synthetic IP to non-producing cell cultures.

An interesting feature about the antimicrobial activity of avicin A is that it kills the important food-borne pathogen *Listeria monocytogenes* in a very small concentration (< 0.3 nM). For this reason, avicin A can have a potential application in food industry where it can be added to foods to control listeria. It is also interesting that avicin A producers were isolated from three different infants from two different countries (paper I and II), and that the producing strains occur in large numbers in all the three babies, which suggests that bacteriocin production gives the producing strains a competitive advantage over other gut flora. This may increase the likelihood of avicin A producer being used as a potential probiotic bacterium. Also the strain is susceptibility to all antibiotics tested and does not produce virulence factors such as cytolysin and gelatinase, suggesting that it could be safe to use it as a probiotic.

The second bacteriocin (salivaricin D) was purified and characterized genetically from *S. salivarius* 5M6c (paper III) and is a new lantibiotic. A number of bacteriocins, most of which are lantibiotics, are produced by *S. salivarius* strains (Table 2). But salivaricin D is not similar to any of these bacteriocins. However, it is similar (59-62%) to the nisins, sharing highest similarity with nisin Q and Z (167, 267, 269), with major variations occurring in the N-terminal region. The other bacteriocin-related genes in salivaricin D locus are also similar to those of nisin Q and Z, although the arrangement of the genes is different. The presence of two transposase genes in the locus, suggests that mobile genetic elements have been involved in the acquisition of the salivaricin D locus by *S. salivarius* 5m6c. It seems that salivaricin D production is controlled by quorum sensing regulatory system as RR and HPK, and possible RR binding site (*nis* box) are identified in the locus. The locus was identified by whole genome sequencing of *S. salivarius* 5M6c and confirmed by cloning and expression of the immunity gene (*SalI*) in a sensitive indicator, proving evidence for the self-protection functionality of the immunity protein. However, full protection was not obtained since the ABC system was not cloned.

Like avicin A, salivaricin D has interesting biochemical features. Salivaricin D exerts its antimicrobial activity against many species of bacteria, including the major

pathogenic streptococci such as *S. pneumoniae* and *S. pyogenes*. In these regard, it is similar to other salivaricins. Therefore, salivaricin D and other salivaricins may be used as antimicrobial substances to control these pathogens. Moreover, the producing strains, which are human commensal flora, might be used as oral probiotics or biological control agents to control the growth of pathogenic streptococci. Nevertheless, salivaricin D does not inhibit staphylococci, enterococci and listeria, even though it shares similarity with the nisins, which are inhibitory to these bacteria, and this could be explained by multiple substitutions that occur mainly in the C-terminal half of salivaricin D.

Another important property of salivaricin D is its ability to resist trypsin digestion which was due to absence of internal cleavage sites for trypsin in its primary structure. This implies that salivaricin D can remain stable in the intestine, where trypsin is secreted, maintaining its antimicrobial activity. Bacteriocins with such properties are rare in nature, and for this reason, some investigators tried to engineer bacteriocins to generate trypsin resistant variants, but activity and stability of such variants might be reduced (186). So, it is interesting to identify salivaricin D which is engineered by nature to be trypsin resistant. This is a useful characteristic if *S. salivarius* 5M6c is intended to be used as probiotic bacterium because the activity of the bacteriocin will not be affected by intestinal trypsin. Still another important feature is the stability of salivaricin D in fridge and at room temperature which might make it suitable its applications at these temperatures.

Finally, salivaricin D, like avicin A, is produced by strains that appeared to occur in large numbers in the fecal microbial population. This can be explained by the antimicrobial activity of salivaricin D that might have made producing strains outcompete other bacteria.

In short, avicin A and salivaricin D share some common features: 1) both are produced by LAB strains of healthy human origin, 2) both have good storage and heat stability 3) both kill important human pathogens, 4) the producers of both appear to dominate the fecal/intestinal flora of the infants, 5) the producers of both are sensitive to the antibiotics used in this study and lack virulence traits. These important features

may promote the ability of the two bacteriocins or their producers to be used as chemical or biological control agents (probiotics), respectively.

The LAB (except enterococci and streptococci) are very rarely involved in causing diseases. However, it is important to test their antibiotic susceptibility because the LAB can serve as potential reservoirs for transmission of antibiotic resistance genes to non-pathogens or opportunistic pathogens in the GIT or foods (158). In the present study, lactobacilli, enterococci and streptococci showed different patterns of susceptibility or resistance to antibiotics. The lactobacilli were generally more susceptible to chloramphenicol, erythromycin and tetracycline than aminoglycosides (especially to kanamycin) and glycopeptides in consistent with a previous study (42). However, low prevalence of resistance to erythromycin and tetracycline has been detected and we suggest that this might be transferrable resistance since such resistances have been shown to be transferred to other bacteria (81, 170). Resistance of lactobacilli to aminoglycosides (kanamycin and streptomycin) and glycopeptides (vancomycin) may be intrinsic (42, 244). Kanamycin and streptomycin resistance may be natural (42) or could be due to high level of spontaneous mutation in chromosomal DNA and may not be transferrable (41).

Not many studies have been conducted on the susceptibility of enterococci isolated from healthy infants, even though it has been done on isolates obtained from healthy children (12). Our result that high prevalence of resistant to tetracycline, erythromycin and kanamycin and absence of ampicillin resistance among the enterococci is in agreement with the study on healthy children (12). In many studies done on enterococci, tetracycline resistance has been shown to be the most prevalent regardless of the source of the enterococci (12, 29, 105, 195), indicating widespread transmission of resistance determinants.

Multidrug resistance among the enterococci is increasing and posing a challenge in the treatment infections (4). The prevalence of multidrug resistant enterococci in the present study is lower than that of enterococci isolated from neonates (103). The multidrug resistance and high level of aminoglycoside resistance are both common in *E. faecalis* and *E. faecium*, and this has to be a concern since these two species are important nosocomial pathogens.

Cytolysin is considered an important virulence factor in enterococci (36, 110, 117) and it is widely distributed among the enterococci (at least 10 species) (224). Gelatinase is an extracellular zinc-endopeptidase that hydrolyses gelatin, collagen, casein, hemoglobin and other biologically active peptides (144) and studies have suggest its virulence role in animal models and humans (169, 193, 200, 228). The occurrence of these two traits in enterococci has been demonstrated to prevail in clinical isolates as compared to non-clinical ones (1, 224, 225). It is not surprising that we found a low incidence of cytolysin and gelatinase production in this study since the isolates are non-clinical. This suggests that most of the isolates might not have virulence potentials and it is thus less likely that the infants harbor virulent enterococci.

5. Conclusions and recommendations

In this thesis, LAB were isolated from healthy Ethiopian infants and species belonging to the genera *Lactobacillus*, *Enterococcus* and *Streptococcus* were identified. The lactobacilli appeared to be dominant LAB in breast-fed infants, while enterococci and streptococci predominated the LAB flora of mixed-fed infants.

Bacteriocin production appears to be common among the infant isolates, especially among the enterococci. The two new bacteriocins identified and characterized in this thesis have good killing efficacy against pathogens and may be potential therapeutic agents that may be used to control the pathogens. Together with the good antimicrobial activity of the bacteriocins, the occurrence of the producer strain in significantly higher numbers in the gut, and absence of virulent factors and antibiotic resistance in producers may promote their potential to be used as probiotics. But in vivo studies in animal models has to be done to confirm the protective/treatment efficacy and safety of the strains before they are intended to be used as probiotics.

It seems that bacteriocins may render their producers a competitive advantage over other bacteria in their ecological niche. Nevertheless, since there are no strong

evidences available in support of this view, many investigations must be done to prove and confirm it.

The two new bacteriocins identified and characterized in this thesis suggest that the effort to search new antimicrobial substances, especially bacteriocins must be continued since there is a possibility of discovering new ones with desirable and interesting features that can make a big difference in the fight against pathogens.

Antibiotics resistance was common among the infants isolates and the three LAB groups showed different patterns of susceptibility to antibiotics. The lactobacilli were generally more susceptible to chloramphenicol, erythromycin and tetracycline than aminoglycosides and glycopeptides. Kanamycin and vancomycin resistance were the most prevalent among the lactobacilli. In general, the enterococci were susceptible to ampicillin, vancomycin, chloramphenicol and gentamycin, although tetracycline and kanamycin were the most prevalent resistant phenotypes. Multidrug resistance and resistance to high level of aminoglycosides were mainly due to *E. faecalis* and *E. faecium,* which are the leading causes of nosocomial infections. The streptococci were generally sensitive to all antibiotics. It seems that transferrable antibiotic resistance is rare among the lactobacilli while it could be common among the enterococci (mainly resistance to tetracycline and high level of aminoglycosides). Production of cytolysin and gelatinase was rare among the enterococci.

6. References

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PAPER I

Bacteriocin production, antibiotic susceptibility, and prevalence of cytolysin and gelatinase production in faecal lactic acid bacteria isolated from healthy Ethiopian infants

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Abstract

The objective of this study was to characterize lactic acid bacteria (LAB) isolated from faecal samples of healthy Ethiopian infants, with emphasis on bacteriocin production and antibiotic susceptibility. One hundred and fifty LAB were obtained from 28 healthy Ethiopian infants. The isolates belonged to *Lactobacillus* (81/150), *Enterococcus* (54/150) and *Streptococcus* (15/150) genera. *Lactobacillus* species were enriched in the breast-fed infants while *Enterococcus* dominated the mixed-fed population. Bacteriocin-producing LAB species were isolated from 8 of the infants. Seven different bacteriocins were identified, including one new bacteriocin from *S. salivarius*, avicin A (class IIa) from *E. avium*, one class IIa bacteriocin from *E. faecalis* strains, one unknown bacteriocins from *E. faecalis* and two unknown bacteriocins from *Lb. fermentum* strains and the two-peptide gassericin T from the *Lb. gasseri* isolates. Susceptibility tests performed for nine antibiotics suggest that some lactobacilli might have acquired resistance to erythromycin (2.5%) and tetracycline (3.8%) only. The streptococci were generally antibiotic sensitive except for penicillin, to which they showed intermediate resistance. All enterococci were susceptible to ampicillin, while 13.2% showed penicillin resistance. Only one *E. faecalis* isolate was vancomycin resistant. Tetracycline (50.9%) and erythromycin (26.4%) resistance was prevalent among the enterococci, but multidrug resistance was confined to *E. faecalis* (46.7%) and *E. faecium* (33.3%).

Screening of enterococcal virulence traits revealed that 1.9 % were β-haemolytic. The structural genes of cytolysin were detected in 28.3% of the isolates in 5 enterococcal species, the majority being *E. faecalis* and *E. raffinosus*. This study shows that antibiotic resistance is a common trait of intestinal LAB of Ethiopian infants.

Key words: lactic acid bacteria, infants, enterococci, lactobacilli, antibiotic resistance, haemolysis, gelatinase, bacteriocin

Introduction

The gut of a human-being is sterile at birth, but it is instantly colonized by bacteria that increase fast in number and complexity and being established as a durable microflora of the growing infant (60). The composition of the gut bacteria differs from one to another and is distinct for an individual (60). This is because the development and composition of intestinal microflora of infants is determined by various factors (3, 59, 61), including feeding type (e.g., breast milk, formula or other foods), delivery mode (vaginal or caesarean), environment and hygiene conditions. It has been shown that the colonization of the gut with lactobacilli and bifidobacteria was delayed in infants delivered by caesarean section compared to vaginally delivered infants (34, 51). There is also a difference in colonization pattern between infants in developing and developed countries. The former are colonized early by enterobacteria (including *E. coli)*, bifidobacteria, enterococci and lactobacilli and contain a more diverse microflora than the latter ones (3).

The LAB play an important role as probiotics (46, 66, 78). For safety reasons, LAB of healthy human origin, which are non-pathogenic and not resistant to antibiotics, are often preferred for selection as potential probiotics. The probiotic potential of LAB may promoted by their ability to produce antimicrobial compounds, the most prominent being bacteriocins. Bacteriocins are antimicrobial peptides produced by bacteria and kill other bacteria. Bacteriocin production has also been implicated in the protection of mice against the food-borne pathogen *Listeria monocytogenes* (18). Thus, bacteriocin-producing LAB can be potential candidates for use as probiotics or as biological control agents.

Even though the lactobacilli are generally considered non-pathogenic and clinically less important than the enterococci and streptococci, it is important to screen them for antibiotic susceptibility. This is because there is a concern that lactobacilli (and other normal flora) may act as potential reservoirs of several antibiotic resistant genes which might be transferred to pathogenic or opportunistic bacteria during passage in the GIT, contributing to the spread of antibiotic resistance (67, 77). Several studies have shown that the transfer of such genes or plasmids bearing the genes from the lactobacilli to pathogens is possible (32, 42, 80). In particular, *erm(B)* and *tet(M)* genes that confer resistance to erythromycin and tetracycline , respectively, have been identified in intestinal lactobacilli (13).

Enterococci, especially *E. faecalis* and *E. faecium*, are the major cause of nosocomial infections (30, 33). Enterococci display both intrinsic and acquired resistance to antibiotics that entails a challenge to treatment of enterococcal infections. Tolerance to chloramphenicol, erythromycin, tetracycline, high levels of clindamycin and high levels of aminoglycosides, high levels of β-lactams and glycopeptides is acquired resistance (54, 55). Acquired resistance is a major concern in infectious diseases not only for posing challenges in the treatment of infections but also for its transmissibility among bacteria. Vancomycin resistance also deserves a particular attention since this antibiotic was considered as 'a last line of defense' in the treatment of infections caused by multidrug resistant enterococci (30, 52).

The ability of enterococci to cause diseases can be promoted by production tissue-damaging virulence factors such as cytolysin and gelatinase(43). Cytolysin, a two-peptide lantibiotic haemolysin, production in enterococci is dictated by a cytolysin operon that consists of 8 genes: *cylR1, cylR2, cylL*$_L$, *cylL*$_S$, *cylM*, *cylB*, *cylA* and *cylI* (72). The operon is mostly located on pheromone-responsive plasmids and sometimes on the chromosome within a pathogenicity island (72). Its occurrence is more prevalent in clinical isolates than in food isolates (1, 70). Gelatinase production is more frequent in *E. faecalis* and is mainly associated with clinical or veterinary isolates (22, 62).

The objectives of this study were to characterize LAB from faecal samples of infants with emphasis on i) bacteriocin production ii) susceptibility to selected antibiotics, and iii) detection of cytolysin and gelatinase production, among the enterococci.

Materials and methods

Subjects and sample collection

Faecal samples were collected from 28 healthy Ethiopian infants (Dilla town, Southern Ethiopia), aged between 3 and 26 weeks, of whom 16 were females and 12 males. Seventeen were breast-fed and eleven were mixed-fed. All were vaginally delivered except one. Eight were born at home and 20 in hospital (Table 1). Informed consent of parents was obtained before samples were collected. One faecal sample was collected in sterile screw-capped plastic tubes from each of the infants.

Isolation and identification of bacteria

The LAB were isolated on de Man-Rogosa-sharpe (MRS) with cysteine-HCl (0.5 g/L), reinforced clostridial (RC) agar and bile aesculin (BA) agar (all from Oxoid, England). Faecal samples were homogenised in broths (1 g faeces/mL) and10-fold serial dilutions were done. Samples (0.1 mL) were plated from each of the three highest dilutions (10^{-5} to 10^{-7} or 10^{-6} to 10^{-8}) on MRS with cysteine-HCl (0.5 gL^{-1}), RC and BA agar plates. The plates were incubated under anaerobic condition at 37 ^{o}C for 24-48 hrs. Three to four colonies from each plate were picked and inoculated in appropriate stab agar, and transported to Norway. Then, the bacteria in the transport media were streaked on appropriate agar plates to recover the isolates. Single colonies were picked and streaked on plates to confirm their purity. The resulting isolated colonies were subcultured in appropriate broths and subsequently maintained at -80 ^{o}C with 13% glycerol containing appropriate medium. Species identification was done by partial 16S rRNA gene sequence analysis.

DNA isolation, PCR and Sequencing

DNA isolation was done using the Bacterial Genomic DNA Purification Kit (Edge Biosystems, Gaithersburg, USA).The purified DNA was resuspended in 1 x TE buffer pH 8.

PCR was done on genomic DNA to amplify genes for the16S rRNA, bacteriocins and cytolysin with appropriate primers (Table 2). The PCR mixture (25 µl) contained 100-200 ng template, 0.5 unit DyNAzyme DNA polymerase (Finnzymes), 1x DyNAzyme buffer, 0.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM of each primer. PCR conditions: initial denaturation at 95 ^{o}C for 1 min and 35 cycles of 95^{o}C for 15 sec, 50-54 ^{o}C for 30 sec and 72 ^{o}C for 90 sec. The PCR products were purified using QIAquick PCR purification Kit (QIAGEN, Germany). Cycle sequencing was done by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied

Biosystems, USA) and the PCR products were sequenced by ABI Prism 377 DNA sequencing system (Applied Biosystems, USA).

Tests of bacteriocin production and activity

Antimicrobial activity was detected by soft agar overlay assay as described previously (53). Briefly, the isolated bacteria were spotted onto MRS or GM17 agar plates and incubated for 16 hrs at 37^{o}C. The colonies were overlaid with appropriate soft agar seeded with an overnight culture (diluted 400-fold) of the indicator strain. Five indicators (*Lactobacillus sakei* NCDO 2714, *Lb. plantarum* LMGT 2003*, E. faecalis* LMGT2708, *Listeria innocua* BL86/26 B, *Staphylococcus aureus* LMGT 3242) were used for detecting bacteriocin production (Table 4). After overnight incubation, the formation of growth inhibition zones around the colonies were examined as indication of antimicrobial activity. To investigate whether the antimicrobial activity observed was caused by a non-protein compound or a bacteriocin, the antimicrobial activity was tested of being sensitive to proteinase K.

In order to further characterize the bacteriocins detected in two *E. faecalis* strains (14M5c and 21M8a), the sensitivity of these strains to other enterococci that produce known bacteriocins was tested using soft agar assay as described above. *E. faecalis* LMGT2708, which is sensitive to many bacteriocins including pediocin-like bacteriocins, is used as a reference indicator. The producer enterococci used and their bacteriocins (in bracket) are: *E. faecalis* 100 (enterolysin A), *E. faecalis* 189 (enterolysin A and cytolysin), *E. faecalis* 3207 (Enterocin L50 and AS-48), *E. faecalis* 3208 (Enterocin P, L50 and AS-48), *E. faecalis* 26c and 116c (Enterocin 1071), *E. faecium* L50 (enterocin P, Q, L50) and *E. faecium* LMGT 2384 (enterocin AB) (29, 38).

Haemolytic activity

The haemolytic activity of enterococci isolates was tested on Brain heart infusion (BHI) agar supplemented with 1% (W/V) glucose, 0.03% (W/V) L-arginine and 5% (V/V) defibrinated horse blood (12). The isolates were streaked on the agar, followed by anaerobic incubation at 37 ^{o}C for 24 hrs. *E. faecalis* HJ2SS (PAD1) (44) and *E. faecalis* 111A (73) were used as positive controls while *E. faecalis* V583 and *E. faecalis* Symbioflor1 were included as negative controls. Haemolytic activity was detected by the appearance of a clear zone (resulting from complete lysis of red blood cells) around the colony on the blood agar. The

presence of the cytolysin structural genes (*cylL*$_L$ and *cylL*$_S$) was verified by PCR and DNA sequencing as described above.

Gelatinase production

The ability of the enterococci to produce gelatinase was tested on Todd Hewitt agar (Oxoid, England) supplemented with 3% bovine gelatine (65). The bacteria were spotted on the gelatine containing agar plates and incubated at 37^{o}C for 40 to 48 hrs, after which they were stored at 4 ^{o}C for 5 hrs. Gelatinase production was evidenced by the formation a turbid zone around the colonies.

Antibiotic sensitivity analysis

The sensitivity of the LAB to the following antibiotics (concentration range in μg mL^{-1}) was tested using microtiter plate 2-fold dilution assay: ampicillin (0.25-512), chloramphenicol (0.5-64), erythromycin (0.125-1024), gentamycin (16-2048), kanamycin (16-8192), penicillin G (2-8192), streptomycin (16-8192), tetracycline (0.25-32) and vancomycin (0.25-32). Enterococci and streptococci were grown and tested in GM17 and the lactobacilli in MRS. A two-fold serial dilution (in broth) of 50 μl the antibiotic solution was prepared in a microtiter plate well containing 50 μl broth to which 150 μl diluted overnight culture (400-fold diluted in broth) of the test strain was added. The plate was incubated at least for 16 hrs after which growth inhibition was measured turbidometrically at 620 nm by a microtiter plate reader (Labsystems Acsent Reader MF, Labsystems, Helsinki, Finland). Resistance was determined by using NCCLS (National Committee for Clinical Laboratory Standards) breakpoints for enterococci and streptococci and EFSA (European Food Safety Authority) breakpoints for the lactobacilli (25, 57).

Statistical analysis

Fisher's Exact test was used to compare proportions. P value < 0.05 was considered statistically significant.

Results

Diversity of the LAB among infants

A total of 150 LAB were isolated from 28 Ethiopian infants and identified to species level by 16S rRNA sequencing analysis. The isolates belonged to *Lactobacillus* (81/150), *Enterococcus* (54/150) and *Streptococcus* (15/150) genera (Table 3). Significant differences were observed between breast-fed and mixed-fed infants with respect to distribution of the three groups of LAB (Table 3). Sixty of the *Lactobacillus* isolates (64%) were found in breast-fed infants which is significantly higher than number of lactobacilli found among the mixed-fed (21 isolates, 36%) infants (Odds Ratio (OR), 5.357; P-value, < 0.00001). Conversely, a significant enrichment of enterococci among isolates from mixed-fed infants (59%) was observed (OR, 2.652; P-value, 0.0061). The streptococci are more abundant in mixed-fed infants (13 isolates) than in breast-fed infants (2 isolates) (OR, 10.057; P-value, 0.0006) (Table 3).

Bacteriocin production

The 150 LAB isolates were screened for bacteriocin production using a universal indicator *Lb. sakei* NCDO2714 that allows detection of most known class I and II bacteriocins (9, 11, 27). Fourteen strains (Table 4) isolated from 8 different infants were found to produce antimicrobial compounds that were susceptible to proteolytic treatment. The isolates comprise six *E. avium*, four *E. faecalis*, two *Lb. fermentum,* one *Lb. gasseri* and one *S. salivarius* isolates. From one of the bacteriocinogenic *E. avium* isolates (XA83) a class IIa bacteriocin (avicin A) has already been purified and characterized (9). PCR and DNA sequence analysis showed that the other 5 *E. avium* strains (Table 4) from two different infants also contain the structural gene of avicin A. The four *E. faecalis* strains (Table 4) produced increased antimicrobial activity up to 6-7 h of growth in liquid GM17 medium at 37^{o}C, but the activity was gradually lost when incubation time was prolonged. The instability of bacteriocin produced by *E. faecalis* 21A8a could be due to degradation by gelatinase (68) since this strain produces gelatinase. By testing two different indicators; the class IIa bacteriocins sensitive indicator *E. faecalis* LMGT2708, and the class IIa resistant mutant *E. faecalis* LMGT 2708RA that one can conclude if pediocin-like bacteriocins are produced or not (9). The faecal isolates *E. faecalis* 14M5a, 14A6b and 14M5c inhibited growth of *E. faecalis* LMGT2708 while *E. faecalis* LMGT 2708RA was insensitive to the same antibacterial producers. The results strongly suggest that the bacteriocins produced by 14M5a, 14A6b and

14M5c are pediocin-like bacteriocins. PCR and its partial DNA sequence showed that the bacteriocin from *Lb. gasseri* 10M7c is most likely identical to the two-peptide bacteriocin gassericin T which inhibits *Lb. delbrueckii* subsp. *bulgaricus* (45). *S. salivarius* 5M6c produced a new lantibiotic that is similar to the nisins (10). We have no sequence information of the bacteriocins produced by remaining bacteriocin producing LAB isolates (Table 4).

β-Haemolytic activity and gelatinase production are rare in gut enterococci

Fifty-three enterococci were tested for their ability to haemolyse red blood cells**.** One *E. faecalis* train (21A8a) was found to be β-haemolytic. The remaining isolates, including the enterococcal negative controls, showed varying degrees of haemolytic activities which were weaker than β-haemolysis. PCR showed that 15/53 enterococci harbored the structural genes for biosynthesis of cytolysin and the genes were found most frequent in *E. faecalis* and *E. raffinosus* (Table 5).

Gelatinase activity has been observed earlier in faecal enterococci from infants (73). In this study, 2 of the 53 tested enterococci (*E. faecalis* and *E. maldoratus*) were found to produce gelatinase. The gelatinase producing *E. faecalis* (strain 21A8a) showed also bacteriocin and cytolysin activity.

Antibiotic resistance is common among intestinal LAB from Ethiopian infants.

Antibiotic resistance pattern is different for the three LAB groups (Table 6). The lactobacilli were generally susceptible to chloramphenicol, erythromycin and tetracycline, but resistant to aminoglycosides and glycopeptides. Even though many lactobacilli showed sensitivity to ampicillin, most *L. fermentum* strains and the majority of *Lb. gasseri* did not. Kanamycin resistance was highly prevalent (97.5%). One *Lb. oris* and one *Lb. salivarius* strain were resistant to kanamycin. Among the *Lactobacillus* species, only *Lb. gasseri* and *Lb. johnsonii* were sensitive to vancomycin, the rest (83%) were intrinsically resistant.

Most of the streptococcus isolates appeared to be more susceptible to the nine antibiotics than the enterococci (Table 6). All streptococci were susceptible to ampicillin except two isolates which showed intermediate resistance. All streptococci showed intermediate resistance to penicillin (MIC: 0.25-2 μg mL^{-1}), while all were sensitive to chloramphenicol, erythromycin and vancomycin. One *streptococcus* isolate was resistant to tetracycline (MIC > 8 μg mL^{-1}).

***E. faecalis* and *E. faecium* constitute a reservoir for antibiotic resistance among the gut enterococci**

Out of the 53 enterococci isolates tested for antibiotic sensitivity, 34 isolates were resistant to one or more antibiotics. Resistance includes both full resistance and intermediate resistance, unless otherwise stated. Tetracycline resistance was the most prevalent (50.9%), followed by erythromycin (26.4%) and kanamycin (20.8%) and was present in five species. Resistance to ampicillin was not observed. Only one *Enterococcus* isolate showed high vancomycin resistance (MIC > 32 μg mL^{-1}) and this *E. faecalis* strain was also resistant to penicillin and erythromycin (MIC = 32 μg mL^{-1} for both). In addition, two *E. gallinarum* isolates showed intermediate resistance to vancomycin (MIC = 8-16 μg mL^{-1}).

High level of aminoglycosides resistance was observed in *E. faecalis* and *E. faecium* isolates. Over all, the prevalence of high level of aminoglycosides resistance was observed in 5 of 14 *E. faecalis* and 5 of 9 *E. faecium* isolates. Among these, two *E. faecalis* isolates were resistant to the three aminoglycosides (streptomycin, gentamycin and kanamycin).

Ten isolates (18.9%) of the enterococci showed multidrug (more than two antibiotics) resistance and they included only *E. faecalis* and *E. faecium* isolates. So, multidrug resistance is significantly more prevalent in *E. faecalis* and *E. faecium* than in other enterococci (P value, 0.0001). The multidrug resistance observed combined for the most part tetracycline, aminoglycosides and erythromycin resistance.

Discussion

In the present study, 150 faecal LAB from 28 healthy Ethiopian infants were isolated and identified to species level. Their ability to produce bacteriocins and their susceptibility to antibiotics was investigated. Moreover, haemolytic and gelatinase activities were determined. Our study indicates that lactobacilli and enterococci dominate the faecal LAB microflora of Ethiopian infants, consistent with studies of infant gut microflora in other part of the world (23). Our result implies that the lactobacilli are predominant in the breast-fed infants but they also constitute a significant part of the faecal LAB flora among the mixed-fed infants. The gut microbiota of the infants is partly acquired during delivery from the vaginal microflora (21) but the mother's milk has also been shown to be an important source and growth medium for the lactobacilli (49). A previous study showed that Ethiopian infants contain more enterococci and lactobacilli during the first 2 weeks of life as compared to infants from Sweden (8). This observation is also in agreement with our result, indicating that this trend extends beyond 3

weeks after birth. Furthermore, two previous studies have shown that enterococci and lactobacilli are the prevailing LAB in breast-fed Norwegian infants (29, 38), providing more support to our finding. The high level of occurrence of enterococci in the faeces of mixed-fed than breast-fed infants has previously been attributed to foods other than breast milk (75, 83).

A previous study showed that in 45% of Swedish breast-fed infants lactobacilli reached their highest number at the age of 6 months, the most common being *L. rhamnosus* and *L. gasseri* (4). *L. rhamnosus*, *L. johnsonii* and *L. paracasei* prevail in the intestinal flora of Greek neonates but no infants contained more than one species at a time (51). In our study, these lactobacilli species were also common in the faecal samples of Ethiopian infants; however, the most prevailing species was *L. fermentum*. The most common enterococci isolated from infants are *E. faecalis* and *E. faecium* (2, 3, 39). Also *E. avium*, *E. raffinosus* and *S. salivarius* have been identified from neonates (26, 29). Consistent with these studies, we isolated *E. avium* and *E. faecalis* from many infants, and *E. faecium, E. raffinosus* and *E. gallinarum* from several samples (Table 3**).** In this study the colonization rate of enterococci (68%) is higher than what has been reported in a study from Germany in which only 23% of the neonates were colonized with enterococci (39).

Recently, a large number of bacteriocin-producing LAB strains has been isolated from the gut of mammals, including man (58). Bacteriocin-producing LAB were also found frequently in faecal samples of Norwegian infants (29, 38). This study also shows that potential bacteriocin producers are commonly found in faecal LAB of Ethiopian infants (10%) (Table 4). Identification of 14 bacteriocin-like compounds from 150 LAB producers obtained from 28 infants is a relatively high number when compared to isolation of bacteriocin producing LAB from different environments (31, 69). Most bacteriocin production was found in enterococci and this is in line with a similar study of faecal samples from Norwegian infants (29, 38). It is interesting to note that enterococci which occasionally occur as pathogens also are among the most frequent bacteriocin producers among the gut bacteria of infants (29, 38). This suggests that enterococci may be better targets (sources) for bacteriocin screening compared other LAB. In some infants at least 3 to 6 bacteriocin producing strains belonging to same species were found, suggesting that bacteriocin producers may predominate in their resident gut microflora.

Haemolytic phenotype and genotype have been studied in enterococci obtained from several sources. One study that investigated enterococci from poultry showed that 1% was β-haemolytic but 39% contained the *cylL$_S$* gene which was most prevalence among the *E. faecalis* isolates (62) and this is similar to the result we obtained. In another study, both cytolysin production and cytolysin operon were detected in 13% of clinical enterococci (35). On the contrary, enterococci isolated from faecal samples of wild boars did not show cytolysin activity or presence of the complete cytolysin operon (64). Still in another study, β-haemolysis was observed in 33% and 6% of clinical and food enterococci, respectively, with high prevalence of *cylL* gene in *E. faecalis* (88%) and *E. faecium* (70%) (70). In *E. faecalis* isolated from healthy Norwegian infants, the prevalence of cytolysin production and cytolysin structural genes was 9/31 and 15/31, respectively (73). In contrary to the above mentioned studies, we found very low frequency (1.9%) of β-haemolysis activity after 24 h growth on blood plates.

Cytolysin production is common in the *E. faecalis*, but rare among other enterococcal species. In our study PCR dectected cytolysin structural genes (*cylL$_L$* and *cylL$_S$*) also in *E. faecium*, *E. avium*, *E. maldoratus* and *E. raffinosus* (Table 5). Interestingly, one previous study (70), which examined 164 enterococci representing 20 species, detected β-haemolysis under anaerobic incubation in 16% of the isolates. However, the structural genes of cytolysin were detected only in 9% of the haemolytic strains, compared to our study which detected the structural genes in 28.3% of the enterococci. Haemolysin structural genes were not detected in some haemolytic enterococci. This can be explained by variation in the sequence the structural genes of cytolysin or it might be conferred by different haemolysins other than cytolysin, as seen in another study (70). The detection of structural genes in some non-haemolytic strains (e.g., *E. avium*) might be due to mutations and/or non-functional genes or gene products.

Gelatinase production is more common in *E. faecalis* (22, 62) although it has been reported in other species such as *E. faecium*, *E. durans* and *E. hirae* (47). In one study almost all *E. faecalis* isolated from poultry produced gelatinase (62). About half of the 31 *E. faecalis* strains isolated from the Norwegian infants produced gelatinase (73). In the current study only 1 out of 15 *E. faecalis* produced gelatinase. These results show that gelatinase production is highly variable among intestinal enterococci, which could indicate that gelatinase may not

promote colonization or persistence in the infant gut. In contrast, there are several reports that show that gelatinase production *E. faecalis* is most prevalent among clinical isolates (17, 22).

Many studies have reported high prevalence of tetracycline resistance among the enterococci (14, 63). This was conferred by acquired resistance mainly due to tetracycline resistant genes *tetL*, *tetM*, *tetS* or *tetO* (14, 40) that is more pronounced in poultry isolates. In one study it has been shown that tetracycline resistance occurred in 24% of food isolates (40). In enterococci isolates from healthy Portuguese children, 28.7% of the isolates were resistant to tetracycline, 21.8% were resistant to erythromycin and 8.9% were resistant to kanamycin (6). In another study, very high level of resistance to tetracycline and erythromycin was present in poultry and pet animals compared to isolates from faeces of healthy humans (63). In the same study, no ampicillin resistance was found in human isolates and no vancomycin resistance in any of the isolates (63). In consistent with these studies we found high prevalence of tetracycline resistance in the enterococci, followed by erythromycin resistance. Also tetracycline resistance was the most prevalent (55%) among *E. faecalis* isolated from healthy Norwegian infants (73), comparable to the present study (53%). It has been shown in several studies that *E. faecium* is more resistant to penicillin than *E. faecalis* (28, 37). Our result is in agreement with these studies since 44% of the *E. faecium* were resistant to penicillin compared to 13% of *E. faecalis*.

Higher prevalence of multidrug resistance (57%) was reported in enterococci isolated from German neonates (39), as compared to our study (19%) (P-value, 0.0001%). The former study also reported absence of vancomycin resistant. Recently, it has been shown that multidrug resistance was frequent in *E. faecalis* clinical isolates (1). *E. faecalis* was also the most frequent and showed the highest levels of multidrug resistance in the present study. High level aminoglycosides resistance was also observed among the *E. faecalis* and *E. faecium* isolates and this might be due to aminoglycoside-modifying enzymes (16). In contrast to our study, high level of aminoglycoside resistance occurred in all species of enterococci isolated from poultry but with high prevalence among *E. faecium* and *E. faecalis*.

Many studies have documented the resistance of most lactobacilli, including *Lb. casei* (19, 76), *Lb. salivarius*, *Lb. acidophilus* and *L. plantarum*, *L. delbreuckii*, *Lb. paracasei*, and *Lb. brevis* (36, 48), to vancomycin which is due to intrinsic resistance. However, some lactobacilli belonging to the *Lb. acidophilus* group (in our case *Lb. gasseri* and *Lb. johnsonii*)

were sensitive to vancomycin. This phenotypic characteristic can be used to differentiate the latter group from the rest of lactobacilli (36). Similarly, the high prevalence of aminoglycoside resistance in the lactobacilli in this study might be due to intrinsic resistance. Also the resistance of most *L. fermentum* to ampicillin could be due to intrinsic resistance. Eleven *Lb. casei* strains isolated from Italian hard cheeses were all susceptible to ampicillin, penicillin G, tetracycline and vancomycin, but 1 and 8 were resistant to erythromycin and gentamycin, respectively (7). In our study, all the 12 *Lb. casei* were susceptible to tetracycline, chloramphenicol, erythromycin and ampicillin (except one isolate) but all were resistant to vancomycin, gentamycin, kanamycin and most to streptomycin.
A study done on antibiotic susceptibility of intestinal lactobacilli of healthy children showed that all were susceptible to ampicillin, erythromycin, gentamycin and the majority were susceptible to tetracycline while 73% were resistant to vancomycin (48). Except for gentamycin this study is generally in agreement with our study.

Lb. casei and *Lb. johnsonii* in this study were kanamycin resistant which is in agreement with a previous work of probiotic *Lb. casei* and *Lb. johnsonii* isolates that were also shown to be resistant to kanamycin (76). The same work also showed that the *Lb. johnsonii* strains were susceptible to chloramphenicol, penicillin and tetracycline which is also consistent with our study. Also the high prevalence of kanamycin resistance among the lactobacilli was observed in both studies (81% in the published probiotic study (76) and 97.5% in our study). Lactobacilli are generally more susceptible to chloramphenicol, erythromycin and tetracycline but more resistant to the aminoglycosides (15). Our result is in agreement with this since nearly all trains were susceptible to chloramphenicol and tetracycline, and erythromycin (see Table 4). The only exceptions were the three tetracycline resistant *Lb. ruminis* isolates, and the only one *Lb. vaginalis* isolate and one of the *Lb. gasseri* isolates that were erythromycin resistant.

It has been shown that nearly 70% of lactobacilli, including, *Lb. fermentum*, *Lb. gasseri, Lb. johnsonii* and *Lb. casei*, isolated from human faeces were found to harbour *tet*(M) and or *erm*(B) genes (13). Also *Lb. fermentum*, *Lb. salivarius*, *Lb. casei* and *Lb. vaginalis* from food were found to contain these genes and they were resistant to erythromycin and tetracycline (56) . Therefore erythromycin resistance observed in our *Lb. gasseri* and *Lb. vaginalis* isolates could be due to presence of *erm*(B) genes in these bacteria. In contrast to a study by Delgado et al. (20), we found high prevalence of multidrug resistance in human intestinal lactobacilli.

However, the multidrug resistance is a combination of glycopeptides and vancomycin, and should not impose a big concern since most of these resistances are most likely intrinsic and not transferrable.

This work demonstrates that probiotic properties such as bacteriocin production is a major feature among faecal LAB. Also putative virulence properties like gelatinase and haemolytic activities are rarely found among faecal LAB. The present study also shows that antibiotic resistance is frequently found among most of the faecal LAB.

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Tables

Table1. Distribution of faecal lactic acid bacteria from 28 Ethiopian infants

Infant code	Sex	Age (weeks)	Delivery mode	Delivery place	Nutrition	Number of isolates	Number of species	Genera LAB[a]
A	M	5	vaginal	hospital	breast-fed	**10**	3	EL
C	M	4	vaginal	home	breast-fed	**9**	3	L
E	F	11	vaginal	hospital	breast-fed	**8**	3	EL
F	F	14	vaginal	hospital	breast-fed	**4**	3	EL
G	F	9	vaginal	hospital	breast-fed	**3**	2	E
I	M	18	vaginal	hospital	mixed-fed	**2**	2	ES
M	F	16	vaginal	hospital	breast-fed	**9**	3	L
N	M	26	vaginal	hospital	mixed-fed	**10**	4	EL
P	F	7	vaginal	hospital	mixed-fed	**5**	2	L
R	M	8	vaginal	home	breast-fed	**12**	3	L
S	F	18	vaginal	home	mixed-fed	**10**	4	ELS
T	F	26	vaginal	hospital	mixed-fed	**5**	1	S
U	F	15	vaginal	home	mixed-fed	**13**	6	ELS
W	F	7	vaginal	hospital	breast-fed	**5**	2	L
X	F	26	vaginal	hospital	mixed-fed	**7**	3	E
Y	F	10	vaginal	home	breast-fed	**2**	2	EL
Z	M	25	vaginal	hospital	mixed-fed	**4**	1	E
3	F	17	vaginal	hospital	breast-fed	**3**	2	EL
5	F	13	vaginal	hospital	breast-fed	**3**	2	LS
7	M	11	vaginal	hospital	breast-fed	**2**	2	L
10	M	10	CS	hospital	breast-fed	**4**	3	L
11	M	7	vaginal	hospital	breast-fed	**2**	2	EL
12	M	15	vaginal	home	mixed-fed	**4**	2	EL
13	M	12	vaginal	hospital	mixed-fed	**3**	2	EL
14	F	14	vaginal	hospital	breast-fed	**3**	1	E
18	F	22	vaginal	home	mixed-fed	**3**	2	EL
20	M	13	vaginal	home	breast-fed	**1**	1	E
21	F	10	vaginal	hospital	breast-fed	**4**	4	ELS

[a]E, Enterococci; L, Lactobacilli; S, Streptococci

Table 2. DNA primers used in this study

Name	Sequence (5'-3')	Position	Target gene	Reference
1F	GAGTTTGATCCTGGCTCAG	9-27	16S rRNA	(81)
11F	TAACACATGCAAGTCGAACG	50-70		(24)
5R	GGTTACCTTGTTACGACTT	1510-1492		(74)
*gasT*F1	GGCTAACATAGTTGGTGGGAGA	2012-2033	Gassericin T	(45)
*gasT*R1	TTTCCGAATCCACCAGTAGC	2191-2172		
*salA*F1	TGATTGCCATGAAAAACTCAA	433-453	Salivaricin A	(41)
*salA*R1	CAACAAACGAATACTGAGTTTGGA	583-560		
*sboA*F1	GGCTAAACAACAAATGAATCTCG	19094-19116	Salivaricin B	(41)
*sboA*R1	CAAGTAAACAAGAACTGCCA	19252-19233		
BF1	ATGATGAATGCTACTGAAAACCAA	156-179	Bovicin HJ50	(82)
BF2	AATTGTACCTGCACAAATTGAAGA	305-282		
*cylL*F	CAACAATTTTATGATGGAGGGTAA	42303-42326	Cytolysin	(71)
*cylS*R	TCTTCCATGTAAGCACTCCTTTT	42831-42809		
AS-48F	TTTTTGGGGTTAGCCTTGTT	182-201	AS-48	(50)
AS-48R	GCTGCAGCGAGTAAAGAA	372-355		
Bac31 F	TTTGTGGCATTATTGGGATT	4276-4295	Bacteriocin 31	(79)
Bac31 R	CCATGTTGTACCCAACCATT	4441-4422		
Ent1071F	ATGCTGTAGGTCCAGCTGC	761-743	Enterocin 1071	(5)
Ent1071R	TTTCCAGGTCCTCCACCAGT	552-571		
AVCf2	ACAAGAAAGGCTGTTCAG	2010-2027	Avicin A	(9)
AVCr2	GACTTCCAACCAGCAGCAC	2109-2091		

Table 3. Distributions LAB species among infants

LAB Species	Number of isolates			The infants[b]
	Total	Breast-fed	Mixed-fed	
E. avium	16	5	11	G,U,X,3,18,21
E. canintestini	1	1	0	A
E. faecalis	15	4	11	I,N,S,X,Z,14,21
E. faecium	9	4	5	E,S,12, 20
E. maldoratus	2	1	1	X,Y
E. raffinosus	6	4	2	A,E,U,
E. gallinarum	5	3	2	F,G,11,13
Subtotal	**54**	**22**	**32**	
L. fermentum	27	19	8	C,F,M,P,U,W,10,12,18
L. mucosae	14	12	2	E,R,S,U
L. casei	12	8	4	A,F,N,P
L. gasseri	9	7	2	C,M,R,U,Y,10
L. johnsonii	5	1	4	N,R
L. ruminis	4	4	0	W
L. salivarius	8	7	1	3,5,10,11,13,21
L. oris	1	1	0	7
L. vaginalis	1	1	0	7
Subtotal	**81**	**60**	**21**	
S. salivarius	1	1	0	5
S. infantarius	13	1	12	S,T,U,21
S. oralis	1	0	1	I
Subtotal	**15**	**2**	**13**	
Total	**150**	**84**	**66**	

[b]Breast-fed infants are underlined

Table 4. Bacteriocin production in LAB obtained from faecal samples of Ethiopian infants

Bacteriocin producer [a]	**Infant**	**Indicators (Inhibition zone diameter in mm)** [b]						
		***Lb. plantarum* LMGT 2003**	***L. lactis* subsp. *cremoris* 393**	***Lb. sakei* NCDO 2714**	***Lb. delbrücki* NCDO 213**	***E. faecalis* LMGT 2708RA**	***E. faecalis* LMGT 2708**	***Listeria innocua* BL86/26 B**
E .avium XM63	X	0		25		0	15	7
E. avium XA64	X	0		20		0	12	16
E. avium XA83	X	0		25		0	13	8
E. avium XM84	X	0		20		0	11	16
E. avium 3B7b	3	0		25		0	13	15
E. avium 3M7a	3			26		0	12	15
E. faecalis 14M5a	14	0		9		0	6	7
E. faecalis 14M6b	14	0		9		0	7	7
E. faecalis 14M5c	14	0		8		0	7	8
E. faecalis 21A8a	21	0	0	12		0	0	0
Lb. fermentum 12M7b	12	10		11		0	0	0
Lb. fermentum 18M7b	18	0		12		0	0	0
Lb. gasseri 10M7c	10	0	8	15	17	0	0	0
S. salivarius 5M6c	5	0	12	13	15	0	0	0

[a] All strains were grown in MRS medium except the *E. faecalis* strain that were grown in GM17

[b] E, *Enterococcus*; L, *Lactococcus*; Lb. *lactobacillus*

Table 5. Haemolytic activity and *cylLS* genes in enteroccoci

Species	Number of isolates tested	Number of β-haemolysis	Number with *cylLS* gene
E. avium	16	0	1
E. canintestini	1	0	0
E. faecalis	14	1	6
E. faecium	9	0	2
E. gallinarum	5	0	0
E. maldoratus	2	0	1
E. raffinosus	6	0	5
Total	**53**	**1**	**15**

Table 6. MIC range (μg mL^{-1}) and susceptibility of LAB to nine antibiotics

Species (Number)	Antibiotic[a]																	
	Ampicillin				Chloramphenicol				Erythromycin				Gentamycin				Kanamycin			
	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R
E. avium (16)	0.25-2	16			1-4	16			0.5	16			32	16			32-64	16		
E. canintestini (1)	0.5	1			2	1			16			1	32	1			256	1		
E. faecalis (14)	0.25-2	14			1-16	12	2		0.5-32	8	5	1	32-512	12		2	32->2048	8		6
E. faecium (9)	1-4	9			1-2	9			0.5-8	3	4	2	32-256	9			64-1024	4		5
E. gallinarum (5)	0.5 -1	5			1- 4	5			0.5	5			32-128	5			128-256	5		
E. maldoratus (2)	0.5	2			1- 4	2			0.5-1	1	1		32	2			32-64	2		
E. raffinosus (6)	0.25-2	6			4	6			0.5	6			32-64	6			32-256	6		
Total (53)		**53**				**51**	**2**			**39**	**10**	**4**		**51**		**2**		**42**		**11**
Lb. fermentum (27)	1-32	5		22	1-2	27			0.25	27			16-128	19		8	128-1024			27
Lb. casei (12)	2-8	11		1	0.5-2	12			≤ 0.25	12			32-64			12	128-512			12
Lb. gasseri (9)	1-4	4		5	0.5-4	9			≤ 0.25-128	8		1	16-128	5		4	128-512			9
Lb. johnsonii (5)	1-2	4		1	1-2	5			≤ 0.25	5			64-128			5	256-512			5
Lb. mucosae (14)	0.25-4	13		1	1-4	14			≤ 0.25-0.5	14			16-64	5		9	32-512			14
Lb. oris (1)	8			1	0.25	1			0.125	1			8	1			32			1
Lb. ruminis (3)	0.25-0.5	3			1	3			0.25	3			16	3			64-512			3
Lb. salivarius (8)	4-32	6		2	0.5-1	8			0.5-1	8			16-128	4		4	16-2048	1		7
Lb. vaginalis (1)	512			1	0.5	1			32			1	16	1			16	1		
Total (80)		**46**		**34**		**80**				**78**		**2**		**38**		**42**		**2**		**78**
S. infantarius (11)	0.06-0.5	11			0.25-2	11			0.06-0.5	11			4-256				4-2048			
S. oralis (1)	0.0325	1			0.25	1			0.0625	1			32				64			
S. salivarius (1)	0.0325	1			1	1			0.5	1			32				64			
Streptococcus spp (1)	0.125	1			0.5	1			0.0625	1			64				256			
Total (14)		**14**				**14**				**14**										

$ One vancomycin resistant isolate MIC 32μg mL^{-1} [a] S, susceptible; I, intermediate resistant; R, resistant *Intrinsic resistance

Table 6 continued.

Species (Number)	Antibiotic														
	Penicillin G				Streptomycin				Tetracycline				Vancomycin			
	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R
E. avium (16)	2	16			32-128	16			0.5 -16	10	5	1	0.5-1	16		
E. canintestini (1)	2	1			32	1			0.5	1			1	1		
E. faecalis (14)	2-64	12		2	32->2048	9		5	0.5->32	7	1	6	1->32	13		1[$]
E. faecium (9)	2-32	5		4	64-256	9			0.5->32	3	3	3	1-2	9		
E. gallinarum (5)	2-4	5			32-128	5			0.5->32	3		2	0.5-8	3	2	
E. maldoratus (2)	2-128	1		1	32-64	2			0.5	2			1	2		
E. raffinosus (6)	2-8	6			128-256	6			>32			6	1-2	6		
Total (53)		**46**		**7**		**48**		**5**		**26**	**9**	**18**		**50**	**2**	**1**
Lb. fermentum (27)	1-32				32-256	13		14	0.5-2	27			>16			27*
Lb. casei (12)	2-4				64-256	3		9	0.25-0.5	12			>16			12*
Lb. gasseri (9)	1-4				16-128	5		4	0.5-2	9			1-2	9		
Lb. johnsonii (5)	1-2				16-32	2		3	0.5-1	5			1-2	5		
Lb. mucosae (14)	2-16				32-1024	5		9	1-2	14			>16			14*
Lb. oris (1)	4				8	1			0.5	1			>16			1*
Lb. ruminis (3)	1				16	3			8-16			3	≥ 16			3
Lb. salivarius (8)	1-2				16-512	2		6	0.5-2	8			>16			8*
Lb. vaginalis (1)	8192				256	1			1	1			>16			1*
Total (80)						**35**		**45**		**77**		**3**		**14**		**66**
S. infantarius (11)	0.25-2		11		8->1024				0.125-0.5	11			0.5-1	11		
S. oralis (1)	0.25		1		32				0.125	1			0.5	1		
S. salivarius (1)	2		1		32				0.5	1			0.5	1		
Streptococcus spp (1)	0.5		1		128				128			1	0.5	1		
Total (14)			**14**							**13**		**1**		**14**		

[a]Resistance breakpoints are not available for penicillin for lactobacilli, and for glycopeptides for streptococci.

PAPER II

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Molecular and Genetic Characterization of a Novel Bacteriocin Locus in *Enterococcus avium* Isolates from Infants[▽]

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Enterococci are among the most common human intestinal lactic acid bacteria, and they are known to produce bacteriocins. In this study, fecal enterococci were isolated from infants and screened for bacteriocin production. Bacteriocin-producing *Enterococcus avium* isolates were obtained, and a new pediocin-like bacteriocin was purified and characterized. This bacteriocin, termed avicin A, was found to be produced by isolates from two healthy infants. It was purified to homogeneity from culture supernatant by ion-exchange and reversed-phase chromatography, and part of its amino acid sequence was obtained. The sequence of a 7-kb DNA fragment of a bacteriocin locus was determined by PCR and DNA sequencing. The bacteriocin locus was organized into four operon-like structures consisting of (i) the structural genes encoding avicin A and its immunity protein, (ii) a divergicin-like bacteriocin (avicin B) gene, (iii) an ABC bacteriocin transporter gene and two regulatory genes (histamine protein kinase- and response regulator-encoding genes), and (iv) induction peptide pheromone- and transport accessory protein-encoding genes. It was shown that the production of avicin A was regulated by the peptide pheromone-inducible regulatory system. Avicin A shows very high levels of similarity to mundticin KS and enterocin CRL35. This bacteriocin showed strong antimicrobial activity against many species of Gram-positive bacteria, including the food-borne pathogen *Listeria monocytogenes*. The avicin A locus is the first bacteriocin locus identified in *E. avium* to be characterized at the molecular level.

Bacteriocins are ribosomally synthesized antimicrobial peptides and proteins. Production of these compounds is widespread in Gram-negative and Gram-positive bacteria (23). Bacteriocins produced by lactic acid bacteria (LAB) have recently been classified into two major categories: the lantibiotics or lanthionine-containing bacteriocins (class I) and the non-lanthionine-containing bacteriocins (class II) (5). According to this classification, the former class III bacteriocins (large heat-labile bacteriocins) were considered nonbacteriocins and hence designated bacteriolysins. The class II bacteriocins are further subdivided into four subclasses: subclass IIa (pediocin-like bacteriocins), subclass IIb (two-peptide bacteriocins), subclass IIc (cyclic bacteriocins), and subclass IId (nonpediocin linear peptide bacteriocins). Class II bacteriocins are most commonly found in enterococci. The subclass IIa bacteriocins are known for their strong antilisterial activity, and they are distinguished by their N-terminal conserved YYGNG motif and two covalently S-S-linked cysteines separated by four amino acid residues (11).

The production of subclass IIa bacteriocins usually requires four genes: a bacteriocin gene (which encodes the bacteriocin precursor), an immunity gene (which protects the producer from its bacteriocin), and the ABC transporter and transport accessory genes (31, 44). Bacteriocins are synthesized as biologically inactive prepeptides (precursors) containing an N-terminal leader peptide that is cleaved off during maturation and exported (23, 31) by a dedicated ABC transporter. The biosynthesis of subclass IIa bacteriocins is frequently regulated by a quorum-sensing regulatory mechanism that consists of a peptide pheromone (inducing peptide) that acts as a signal in a phosphorylation reaction with the receptor histidine protein kinase; this is followed by phosphorylation of the response regulator to activate gene expression in the various operons required for bacteriocin production (32).

Bacteriocins have a narrow spectrum of antimicrobial activity, killing only strains of the same species or closely related species (26, 27), but they can be very potent and are able to kill other bacteria at nanomolar concentrations. Most bacteriocin-producing LAB have been isolated from foods, and very few bacteriocin producers have been isolated from humans so far (14). It has been proposed that bacteriocins can be used not only in food preservation but also in medicine as selective antimicrobials to inhibit pathogens without affecting the normal flora (17). Bacteriocin production is considered a probiotic feature, and it has convincingly been shown that bacteriocin-producing LAB can effectively inhibit the growth of and kill listeriae in mice (4). Thus, identification and characterization of bacteriocin-producing LAB of human origin are needed in order to develop probiotic bacteria with diverse antimicrobial potential. In line with this goal, we aimed at isolating bacteriocin-producing LAB from healthy human infants. In two independent screenings for bacteriocin-producing LAB in fecal samples from healthy infants, a new bacteriocin from *Enterococcus avium* was purified and characterized at the molecular and genetic levels.

MATERIALS AND METHODS

Isolation and identification of bacteriocin-producing strains. In order to screen for bacteriocin-producing LAB, we isolated LAB from feces of 13 healthy Norwegian infants in 2005 and 20 Ethiopian infants in 2007. Bacteriocin-pro-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference[b]
Strains		
Carnobacterium divergens NCDO 2763^T	Sensitive to avicin A	NCDO
Carnobacterium divergens NCDO 2306	Sensitive to avicin A	NCDO
Carnobacterium piscicola NCDO 2762^T	Sensitive to avicin A	NCDO
Carnobacterium piscicola NCDO 2764	Sensitive to avicin A	NCDO
Carnobacterium piscicola UI 49	Bacteriocin producer	45
Escherichia coli DH5α	ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Promega
Enterococcus avium 208	Avicin A producer, Norwegian infant isolate	This study
Enterococcus avium GM62	Sensitive to avicin A	This study
Enterococcus avium UA62	Sensitive to avicin A	This study
Enterococcus avium UM83	Sensitive to avicin A	This study
Enterococcus avium XA83	Avicin A producer, Ethiopian infant isolate	This study
Enterococcus faecalis 185	Infant isolate, sensitive to avicin A	This study
Enterococcus faecalis LMGT 2708RA	Pediocin-resistant mutant of *E. faecalis* EF BRIDGE (B)	LMGT
Enterococcus faecium P21		LMGT
Enterococcus maldoratus XM83	Infant isolate, sensitive to avicin A	This study
Enterococcus faecalis EF BRIDGE (B)	Indicator sensitive to avicin A	LMGT
Lactobacillus fermentum KLD		LMGT
Lactobacillus plantarum 965		LMGT
Lactobacillus rhamnosus 205	Infant isolate, sensitive to avicin A	This study
Lactobacillus sakei NCDO 2714	Indicator sensitive to avicin A	NCDO
Lactobacillus sakei 5	Bacteriocin producer	V. Eijsink
Lactococcus lactis IL 1403	Host for transformation, contains *lcnC* and *lcnD* analogues	49
Lactococcus lactis CM4		
Leuconostoc gelidum Ta 11a		LMGT
Listeria innocua BL86/26 B	Sensitive to avicin A	LMGT
Pediococcus acidilactici	Pediocin producer	LMGT
Pediococcus pentosaceus NCDO 559		NCDO
Plasmids		
pBluescript KS	3.0-kb cloning vector, α*lacZ*, Ap^r	Stratagene
pMG36e	3.7-kb lactococcal expression vector, Em^r	47
pMG36eDV	pMG36e containing divergicin-like ORF, *avcB*	This study
pCR2.1-TOPO	3.9-kb cloning vector, Ap^r	Invitrogen

[a] Ap^r, ampicillin resistant; Em^r, erythromycin resistant.
[b] NCDO, National Collection of Dairy Organisms (Reading, United Kingdom); LMGT, Laboratory of Microbial Gene Technology, Norwegian University of Life Sciences.

ducing strains were obtained from fecal samples from one Norwegian infant and one Ethiopian infant. The strains were isolated on reinforced clostridial medium or de Man-Rogosa-Sharpe medium (MRS) after fecal samples were diluted. The species was identified by using 16S rRNA gene sequencing and BLAST analysis.

Bacterial strains, plasmids, and culture conditions. Strains and plasmids used in this study are shown in Table 1. Solid media and soft agar were prepared with 1.5% and 0.7% agar, respectively. All strains were maintained as frozen stocks at −80°C in 13% glycerol. *Enterococcus* species were grown in GM17 or MRS at 37°C. *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Carnobacterium* species were grown in MRS at 25°C or 30°C. *Lactococcus* species were grown in GM17 or MRS at 30°C. *Escherichia coli* was grown in Luria-Bertani (LB) medium at 37°C. *Listeria* species were grown in GM17 at 37°C.

Bacteriocin antimicrobial activity assay. Antimicrobial activity was detected by using a deferred antagonism assay (41). The isolated bacteria were spotted onto MRS agar plates and incubated for 16 h at 37°C. The colonies were overlaid with an overnight culture (diluted 400-fold) of the indicator strain (Table 1). After overnight incubation, the formation of growth inhibition zones around the colonies was used as an indication of antimicrobial activity. To investigate whether the antimicrobial activity observed was caused by a proteinaceous compound, the sensitivity to proteinase K was tested. Only proteinase K-sensitive activities were used in further studies.

Quantitative determination of the antimicrobial activity of the bacteriocin in cell-free culture supernatant (CFS) was performed by using a microtiter assay system (21). A twofold serial dilution (in MRS) of 100 μl CFS (with the pH adjusted to about 7) was prepared in a microtiter plate well containing 50 μl MRS to which 150 μl of a diluted (400-fold in MRS) overnight culture of the indicator strain *Lactobacillus sakei* NCDO 2714 was added. The plate was incubated for 16 h, after which growth inhibition was measured turbidometrically at 620 nm with a microtiter plate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). One bacteriocin unit was defined as the amount of bacteriocin that inhibited the growth of the NCDO 2714 indicator strain by 50%. Likewise, the MICs of the purified bacteriocin for sensitive strains were determined by using a microtiter assay. Purified bacteriocin was used to determine the molar MICs for the individual indicator strains tested.

Bacteriocin induction assay. Quorum-sensing-dependent regulation of bacteriocin production was tested by using a slight modification of a previously described method (7). In order to obtain non-bacteriocin-producing *E. avium* XA83 (bac$^-$ phenotype) from the bacteriocin-producing culture (bac$^+$ phenotype), this culture was serially diluted in MRS and grown overnight, and a bac$^-$ culture was obtained with the highest dilutions. In order to induce bacteriocin production, 50 μl of CFS from a bacteriocin-producing overnight culture was added to 5 ml of a bac$^-$ strain-inoculated culture in MRS broth and incubated overnight. Also, the inducing peptide AvcP, which was synthesized and purified (>95% pure) by EZBiolab (United States), was used in induction experiments.

Purification of bacteriocin. The supernatant from a 500-ml overnight culture (21 h at 37°C in MRS) of *E. avium* XA83 was collected. Ammonium sulfate (40 g per 100 ml) was added to the supernatant, which was then agitated for 30 min at 4°C. The bacteriocin was precipitated from the supernatant by centrifugation (14,000 rpm for 30 min at 4°C) and dissolved in 50 ml sterile distilled water, and the pH was adjusted to 3.5 with 1 M HCl.

The preparation was then passed through a 5-ml SP Sepharose Fast Flow column (GE Healthcare Biosciences, Uppsala, Sweden) equilibrated with 10 mM acetic acid. The column was washed twice with 20 ml of 20 mM sodium phosphate (pH 6.8). The column was eluted with a stepwise gradient consisting of 10 ml of 0.1 NaCl (two runs), 10 ml of 0.3 NaCl (two runs), and 10 ml of 1 M NaCl (two runs) at a flow rate of 1 ml per min. All fractions except the second 10-ml

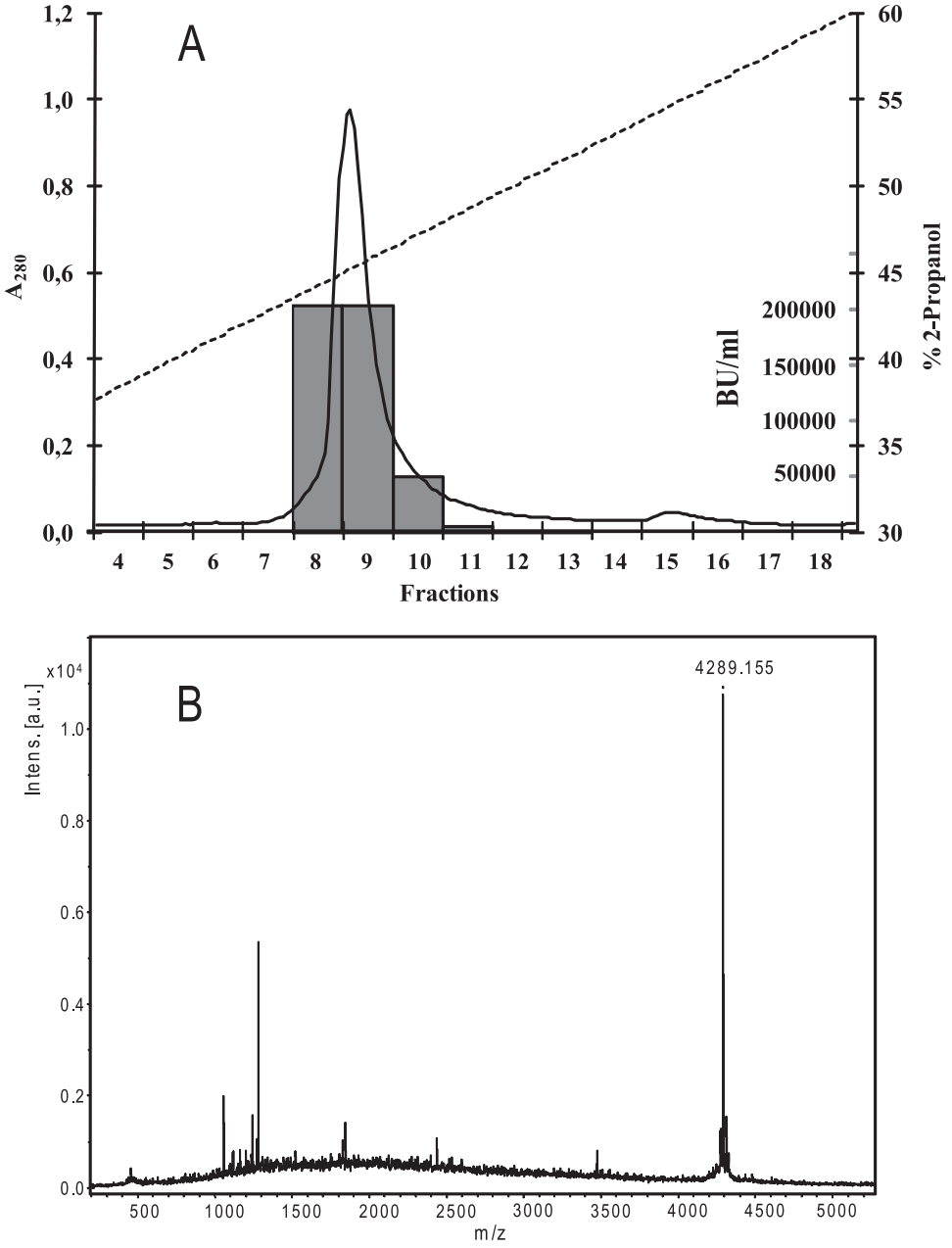

FIG. 1. (A) Results of second reversed-phase chromatography of avicin A. Elution was performed by using a 30-CV linear gradient of 0 to 100% 2-propanol containing 0.1% TFA. Solid line, absorbance at 280 nm; dashed line, isopropanol gradient; bars, bacteriocin units (BU) in eluted active fractions. A sample for mass spectroscopy analysis was obtained from fraction 9. (B) Mass spectrometry analysis of avicin A obtained from the second reversed-phase chromatography. Intens., intensity; a.u., arbitrary units.

0.1 M NaCl fraction contained bacteriocin activity, and the most activity was found in the first 10-ml 0.3 M NaCl eluate. Subsequent purification was performed by using reversed-phase chromatography with an Äkta Purifier fast protein liquid chromatography system. The most active fraction (0.3 M eluate) from the ion-exchange chromatography step was applied to a reversed-phase column (Resource I; Pharmacia Biotechnology) equilibrated with 0.1% trifluoroacetic acid (TFA) in water. Elution was performed by using a 30-column volume (CV) linear gradient from 0 to 100% 2-propanol containing 0.1% TFA, and 2-ml fractions were collected. The two fractions with the most activity (fractions 9 and 10) were combined and diluted in sterile water (final volume, 20 ml), and then they were applied to a Source 5RPC ST 4.6/415 column (Pharmacia Biotechnology) and eluted with a 5-CV linear gradient as described above in 1-ml fractions (Fig. 1A). The fractions were assayed for antimicrobial activity, and the two most active fractions (fractions 8 and 9) coincided with the single peak of absorbance at 280 nm (Fig. 1A) and were stored at −20°C until further analysis.

Mass spectrometry and N-terminal amino acid sequencing. The molecular weight of the purified bacteriocin was determined by mass spectrometry as previously described (6). Briefly, a bacteriocin sample (fraction 9 [Fig. 1A]) was mixed 1:1 with a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA-acetonitrile (2:1) and deposited on a ground steel matrix-assisted laser desorption ionization target. Mass spectra were recorded in reflection mode with an Ultraflex TOF/TOF (Daltonics), using a pulsed ion extraction setting of 40 ns and an acceleration voltage of 25 kV. The spectra displayed the accumulated signals of 200 laser shots with the laser power adjusted to just above the threshold level. A peptide mass similarity search was done at ExPASy with the TagIdent

TABLE 2. PCR primers

Primer	Sequence (5′–3′)
Ur4	AACTGCTTTAATTGTGGCTAA
F1	GGAGTGTGTTGATATGACAAG
AvcF4	GCTTTGCGAAATGATGGAGT
Df5	TGCGACATTTAATATGAAGAG
R1	TCATTCCATACCTGACGGTG
Dr8	TCATGAATCCCACCACAATC
Df8	ACAGGAGTTTTCGCACTTGG
Df10	AGCATGACGAAAAGGATTGC
Df11	GTATTAACAGCAGTCCCAGACG
Dr15	CCCAATAATCCCAAATCCTG
Df12	AGCAAACGAGGAACAGATGG
Dr11	TTCCCAGAACCACTCATACC
Df13	GAACTAGCGCAAATAAGGGAG
Df14	CATGATAGGAAGTGAGTCTGTG
Df20	GTTGCTTGGGTTAAAAGCTG
Dr12	TTACAGTTAATCCCGTTTGCTC
Df16	GATGTGATTAATATGGATGAGG
Df17	CAGCTTTGGCTAATGTCGAG
Dr13	CTCGACATTAGCCAAAGCTG
Df18	AATTGTTTCGTTTTGCGACA
Dr14	TAGAGAAATGCAACCCTTCAG
T7	GTAATACGACTCACTATAGGG
M13rev	CAGGAAACAGCTATGAC
AvFwr	ACNTAYTAYGGNAAYGGNGT
AvRev	GCNCCNCCNGTNGCNARRTTNGC
TFF2	TGACTGGGGAAAGCCATCAG
TFR2	GGCTTTGCCCCAGTCACCTGA
aviB-F1[a]	TGGTGGTCTAGATAG**TGCGACATTTAATATGAAGAGC**
aviB-R1[a]	TGGTGGCTGCAG**CTAACCAAAACACCCACCTG**

[a] Primers used for cloning, not for primer walking. Underlining indicates XbaI and PstI restriction sites; bold type indicates sequences complementary to the template DNA.

tool (http://au.expasy.org/tools/tagident). The N-terminal amino acid sequence of the peptide (bacteriocin) was determined by Edman degradation as previously described (21).

General molecular techniques. Genomic DNA was isolated from LAB strains by the cetyltrimethylammonium bromide method (1), with some modifications, and was purified using a QIAquick PCR purification kit (Qiagen, Germany). Plasmid DNA was isolated from *E. coli* and *Lactococcus lactis* using a Qiagen plasmid miniprep kit (Qiagen, Germany). All enzymes used for DNA manipulation were purchased from New England Biolabs and were used according to the manufacturer's instructions. Primers were synthesized by Thermo Scientific (Germany). PCR products and DNA digests were purified with a QIAquick PCR product purification kit. PCR products were purified from an agarose gel using a Wizard SV gel and the PCR Clean-Up system (Promega, United States).

PCR and sequencing. Genomic DNA of the bacteriocin producers XA83 and 208 were digested with DraI, RsaI, ClaI, EcoRV, HindIII, and SpeI, and purified fragments were ligated into plasmid pCR2.1-TOPO digested with DraI and RsaI and plasmid pBluescript KS digested with ClaI, EcoRV, HindIII and SpeI, respectively. Each ligation product in pCR2.1-TOPO was used as a PCR template to amplify part of the bacteriocin gene (113 bp) by using the degenerate primers AvFwr and AvRev (Table 2) deduced from the N-terminal peptide sequence. The PCR product was cloned into the vector pCR2.1-TOPO and sequenced with the T7 universal primer. T7 and new primers that were designed based on the sequence were used to amplify and sequence flanking regions of the bacteriocin gene using ligation products in pBluescript KS as PCR templates. This procedure was repeated until the bacteriocin locus was completely sequenced. Primers used in this study are listed in Table 2. Cycle sequencing was done by using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, United States), and the PCR products were sequenced by using the ABI Prism 377 DNA sequencing system (Applied Biosystems, United States). Contigs were assembled by using the BioEdit software, version 7.0.0. The integrity of the contigs was confirmed by performing PCR with genomic DNA and sequencing the products. Both strands were sequenced to make sure that the whole sequence was error-free. Open reading frames (ORFs) were identified by ORF finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A similarity search was done by using blastn and/or blastx at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Cloning of the divergicin-like ORF. Primers aviB-F1 and aviB-R1 (Table 2) containing XbaI and PstI restriction sites were designed for the regions flanking the two ends of the divergicin-like ORF (*avcB*) to amplify a 309-bp product from genomic DNA. The PCR product was cloned in plasmid pMG36e using standard protocols (43). The recombinant plasmid was used to transform competent *E. coli* DH5α prepared by the $CaCl_2$ method (43) and grown on LB plates containing 200 μg/ml erythromycin. The plasmid extracted from positive transformants was digested with XbaI and PstI to check for the presence of the insert. The presence and integrity of the insert were confirmed by PCR and sequencing. pMG36e with the correct *avcB* sequence was designated pMG36eDV. pMG36eDV was electroporated into competent *L. lactis* IL 1403 (19, 20), and the transformation mixture was grown on SR medium containing 1 μg/ml erythromycin. pMG36eDV was extracted from positive transformants after they were lysed as described below. Cells were pelleted by centrifugation, washed with 0.5 ml TES buffer (10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA; pH 8), resuspended in 250 μl GTE buffer (50 mM glucose, 25 mM Tris-HCl [pH 8], 10 mM EDTA; pH 8) containing 5 mg/ml lysozyme and 100 μg/ml RNase A, and incubated at 37°C for 30 min. The presence of the insert in pMG36eDV was confirmed by PCR. In order to determine if the *L. lactis* IL 1403 *avcB* clone expressed antimicrobial activity, both a CFS and an ammonium sulfate precipitate of a CFS from an overnight culture were tested with strains sensitive to the *E. avium* bacteriocin (avicin A) and avicin A-resistant mutants of *Enterococcus faecalis* and *E. avium*.

Nucleotide sequence accession number. The nucleotide sequence obtained in this study has been deposited in the NCBI database under accession number FJ851402.

RESULTS

Identification of bacteriocin-producing strains. A total of 103 fecal LAB isolates were obtained from 20 Ethiopian infants, and *E. avium* isolates were obtained from 3 infants. The *E. avium* isolate from one infant produced bacteriocin-like activity. The 16S rRNA gene sequence of this strain showed 99% sequence identity to the 16S rRNA gene sequence of *E. avium* ATCC 14025 (type strain) (36). This isolate of *E. avium* (XA83) was selected for further study. Only 1 of 13 Norwegian infants was found to carry *E. avium* that produced a bacteriocin identical to the bacteriocin produced by the *E. avium* isolate from Ethiopia (15). Inactivation by protease K treatment suggested that the antimicrobial activity was a due to a proteinaceous substance.

Purification of avicin A. A supernatant from a 0.5-liter overnight culture of *E. avium* XA83 grown in MRS was used for bacteriocin purification. This supernatant was precipitated with ammonium sulfate (40%) and subsequently purified by ion-exchange chromatography, followed by two-step reversed-phase chromatography (Table 3). The culture supernatant contained 1,280 bacteriocin units/ml as determined with the indicator strain *L. sakei* NCDO 2714. Ammonium sulfate precipitation resulted in 80% recovery and an approximately 26-fold increase in the specific activity. The level of recovery by ion-exchange and reversed-phase chromatography was low (due to loss of activity to other fractions). The results of the purification procedure are summarized in Table 3. The specific activity of the final purified elute from the second reversed-phase chromatography was about 29,000-fold higher than that of the culture supernatant (Fig. 1A). The monoisotopic molecular mass of the purified bacteriocin (termed avicin A) was determined by mass spectrometry to be 4,288.2 Da (M+1, 4,289.2) (Fig. 1B), which is close to the molecular mass of the subclass IIa bacteriocin mundticin, whose monoisotopic molecular mass is 4,285 Da (3). The following sequence was ob-

TABLE 3. Purification of avicin A

Purification step	Vol (ml)	Recovery (%)	Protein concn (mg/ml)[a]	Antimicrobial activity (bacteriocin units/ml)	Sp act (bacteriocin units/mg)	Increase in sp act (fold)
Culture supernatant	500	100	19.9	1.3×10^3	65	1.0
Ammonium sulfate precipitate	50	80	6.06	1.0×10^4	1,650	26
Ion-exchange chromatography	10	16	0.28	1.0×10^4	35,700	570
First reversed-phase chromatography	2	26	0.8	8.2×10^4	1.02×10^5	1,600
Second reversed-phase chromatography	1	32	0.11	2.05×10^5	1.86×10^6	29,000

[a] The protein concentration was determined either by determining the optical density at 280 nm or by using the calculated absorbance value for purified avicin A (from the second reversed-phase chromatography fraction) (1 mg/ml = 3.257).

tained by N-terminal peptide sequencing: TYYGNGVSCNK KGCSVDWGKAI.

Inhibition spectrum of avicin A. Members of several species of Gram-positive bacteria (*Listeria*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Carnobacterium* species) were susceptible to avicin A (Table 4), while the *Lactococcus* strains tested were not affected. Based on the MICs, *Lactobacillus rhamnosus* 205 and *Listeria* species seem to be the organisms most sensitive to avicin A, whereas pediococci appear to be less sensitive (Table 4). The subclass IIa bacteriocin-resistant strain *E. faecalis* LMGT 2708RA was also found to be resistant to avicin A (Table 4), as expected for a subclass IIa bacteriocin. The antimicrobial spectrum of the avicin A producer was also tested with the same indicators using the deferred overlay assay. The results are consistent with the results obtained using purified avicin A, which indicates that no other bacteriocins are produced by *E. avium* XA83.

Identification of the bacteriocin gene locus. Based on information for the N-terminal amino acid sequence, the gene sequence of avicin A was obtained by performing PCR with degenerate primers (AvFwr and AvRev) and sequencing the 113-bp PCR product. New primers were designed based on the nucleotide sequence for further sequencing by primer walking, and the avicin locus sequence was obtained. Specific fragments generated by restriction digestion and PCR were sequenced and assembled into an 8,660-bp contig. Southern blot analysis showed that the avicin A locus is located on chromosomal DNA (data not shown).

TABLE 4. Inhibition spectrum of avicin A

Strain	Inhibition[a]	MIC (nM)[b]
Carnobacterium divergens NCDO 2763^T	+	1.25
Carnobacterium divergens NCDO 2306	+	2.51
Carnobacterium piscicola NCDO 2762^T	+	0.31
Carnobacterium piscicola NCDO 2764	+	0.31
Enterococcus avium GM62	+	0.63
Enterococcus avium UA62	+	5.02
Enterococcus avium UM83	+	0.31
Enterococcus faecalis EF BRIDGE (B)	+	10.04
Enterococcus faecalis LMGT 2708RA	−	
Enterococcus maldoratus XM83	+	5.02
Lactobacillus plantarum LMG 2003	−	
Lactobacillus rhamnosus 205	+	<0.31
Lactobacillus sakei NCDO 2714	+	1.25
Lactobacillus sakei 5	−	
Lactococcus lactis IL 1403	−	
Lactococcus lactis CM4	−	
Leuconostoc lactis NCDO 533	+	5.02
Leuconostoc mesentereroides NCDO 529^T	+	2.51
Leuconostoc gelidum Ta 11a	−	
Listeria innocua BL86/26 B	+	<0.31
Listeria monocytogenes 223 serotype 1	+	<0.31
Listeria monocytogenes 400 serotype 4	+	<0.31
Pediococcus acidilactici NCDO 1851	+	40.16
Pediococcus acidilactici PacI	−	
Pediococcus pentosaceus NCDO 814	+	20.08

[a] +, inhibition; −, no inhibition. Antimicrobial tests with *E. avium* XA83 were performed by using the deferred assay. The MICs were not determined for insensitive indicators. The MICs varied within ±30%.
[b] MICs of purified avicin A.

DNA sequence analysis. DNA sequence analysis of the 8,660-bp contig identified eight putative ORFs, seven of which are apparently bacteriocin related and localized in a 7-kb locus on the sequenced fragment. The unrelated ORF is probably involved in carbohydrate metabolism, as the deduced amino acid sequence encoded by it showed significant similarity (95% identity) to an annotated maltose-6′-phosphate glucosidase of *Clostridium* sp. 7_2_43FAA (accession number ZP_05129714). A similarity search in public databases revealed that the seven ORFs include the genes that encode a structural bacteriocin (*avcA*) and its immunity protein (*avcI*), a divergicin A-like bacteriocin (*avcB*), a dedicated ABC-type transporter (*avcT*), a peptide induced two-component regulatory system (histidine kinase [*avcK*], a response regulator [*avcR*], a peptide pheromone [*avcP*]), and a transport accessory protein (*avcD*) (Table 5). All ORFs were unidirectionally oriented (Fig. 2A) and, with the exception of *avcR*, preceded by a putative ribosome binding site. The bacteriocin gene cluster is organized into four predicted operon structures, all of which contain putative −10 and −35 sites in the upstream noncoding region of the operon (Fig. 2B).

The first operon (*avcAI*) includes the structural gene for avicin A (*avcA*) and the immunity gene (*avcI*), which is located 40 nucleotides downstream of *avcA*. The promoter region upstream of *avcA* contains a pair of 9-nucleotide identical direct repeats that are separated by 12 nucleotides (Fig. 2B). Right after the stop codon of *avcI*, there is a pair of 9-nucleotide inverted repeats separated by 12 nucleotides.

The second operon was predicted to be a monosistronic transcript unit consisting of a single ORF (*avcB*), which is located 368 nucleotides downstream of *avcI*. The product shows significant similarity to divergicin A (51). No repeats were found in the promoter region of *avcB*. A pair of 10-nucleotide inverted repeats separated by 24 nucleotides is located 15 nucleotides downstream of the termination codon of *avcB*.

The third operon (*avcTKR*) contains genes encoding an ABC transporter (*avcT*), a histidine protein kinase (*avcK*), and a response regulator (*avcR*). As in the promoter of *avcAI*, a

TABLE 5. Proteins similar to ORF products of the avicin A locus

ORF	Length of product (amino acids)	Similar protein							
		Designation	Length (amino acids)	Producer(s)	% Identity	% Similarity	E value(s)	Function	Reference(s)
avcA	61	MunA	58	*Enterococcus mundtii* strains	87	89	9×10^{-20}, 10^{-19}	Precursor of mundticin KS, enterocin CRL35	25, 42
avcI	97	SakXIM	97	*Lactobacillus sakei* 5	65	80	2×10^{-27}	Sakacin X immunity protein	48
avcB	67	Divergicin A	75	*Carnobacterium divergens* LV13	45	60	7×10^{-10}	Divergicin A	51
avcT	725	StxT	723	*Lactobacillus sakei* 5	70	86	0	ABC transporter	48
avcK	442	StxK	437	*Lactobacillus sakei* 5	58	80	4×10^{-144}	Histidine protein kinase	48
avcR	244	StxR	252	*Lactobacillus sakei* 5	77	90	6×10^{-113}	Response regulator	48
avcP	45	IP-TX	45	*Lactobacillus sakei* 5	62	77	10^{-8}	Inducer peptide	48
avcD	171	BrcD	158	*Brochothrix campestris* ATCC 43754	45	69	8×10^{-36}	Transport accessory protein	28

pair of 9-nucleotide direct repeats with one mismatch that were separated by 12 nucleotides were identified upstream of the −35 site of the *avcTKR* promoter (Fig. 3B). In addition, a pair of 17-nucleotide inverted repeats having four mismatches and separated by 12 nucleotides is located 42 nucleotides downstream of the termination codon of the ORF *avcR*.

The fourth operon comprises genes encoding a putative peptide pheromone (*avcP*) and a transport accessory protein (*avcD*). A pair of direct repeats similar to those identified in the promoter regions of the *avcAI* and *avcTKR* operons was also found in the promoter region of this operon (Fig. 2B). These repeats contain 9 nucleotides with four mismatches (three mismatches are consecutive), and one of the pairs is identical to the pairs that occur in *avcAI*. One nucleotide downstream of the termination codon of *avcD*, a perfect 16-nucleotide inverted repeats pair separated by 19 nucleotides was identified.

The inverted repeats at the 3′OH end of the mRNAs may function as rho-independent transcription terminators, although no obvious U tract follows the stem structure. The direct repeats identified in the promoter region may serve as binding sites for a response regulator (8, 39, 40).

Protein sequence analysis. *avcA* encodes a 61-amino-acid prepeptide that includes an N-terminal double-glycine leader consisting of 18 amino acid residues and a mature peptide consisting of 43 residues. The pI is 9.18, and the theoretical monoisotopic molecular mass of avicin A (the two cysteines are assumed to form a disulfide bridge) was estimated to be 4,288.2 Da. The extinction coefficient at 280 nm (path length, 1 cm) was calculated to be 3.26 (1 U of optical density at 280 nm = 3.26 mg/ml).

The mature peptide (43 amino acids) contains the N-terminal conserved motif YYGNG (Fig. 3A), as well as the two conserved cysteine residues (at positions 9 and 14) typical of subclass IIa bacteriocins.

The deduced amino acid sequence of the prepeptide showed high levels of similarity to other IIa bacteriocins (Table 5). Mature avicin A is 97% identical to mundticin KS (25) and enterocin CRL35 (42) and differs only at amino acid residues 1 and 23. Also, mature avicin A is 95%, 85%, and 82% identical to mundticin L (12), sakacin X (48), and piscicolin 126 (24), respectively. The double-glycine leader peptide of avicin A exhibits high levels of similarity (64% identity) to the leader peptides of sakacin X (48), mundticin KS (25), mundticin L (12), and enterocin CRL35 (45).

avcI encodes a 97-amino-acid peptide that is significantly similar to the immunity proteins of sakacin X (48) and piscicolin 126 (Table 5) (18, 24). The immunity proteins of pediocin-like bacteriocins have recently been divided into subgroups A, B, and C (13). Avicin A immunity protein is a

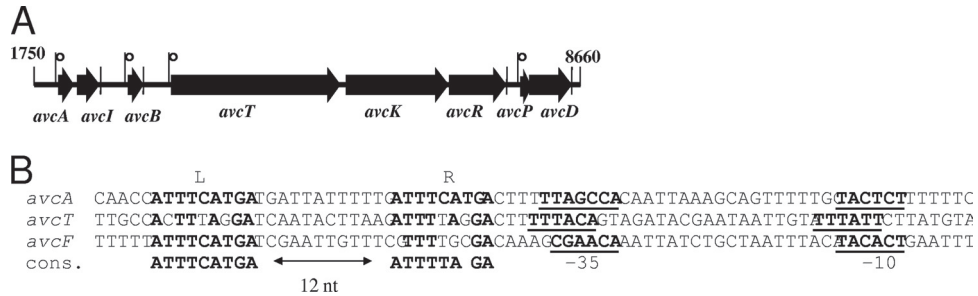

FIG. 2. (A) Genetic organization of avicin A locus (GenBank accession number FJ851402). *avcA* is the avicin A precursor gene (183 bp), *avcI* is the immunity gene (291 bp), *avcB* is the divergicin-like bacteriocin gene (201 bp), *avcT* is the ABC transporter gene (2,175 bp), *avcK* is the histidine kinase gene (1,326 bp), *avcR* is the response regulator gene (732 bp), *avcP* is the peptide-pheromone gene (135 bp), and *avcD* is the transport accessory protein gene (513 bp). p indicates promoter positions. The vertical bars immediately after arrows indicate inverted repeats. (B) Alignment of putative promoter sequences. Putative −10 and −35 sequences are underlined. Direct repeats (L and R) are enclosed in boxes, and the consensus sequence (cons.) is indicated below the boxes. The direct repeats were not identified in the promoter of *avcB*.

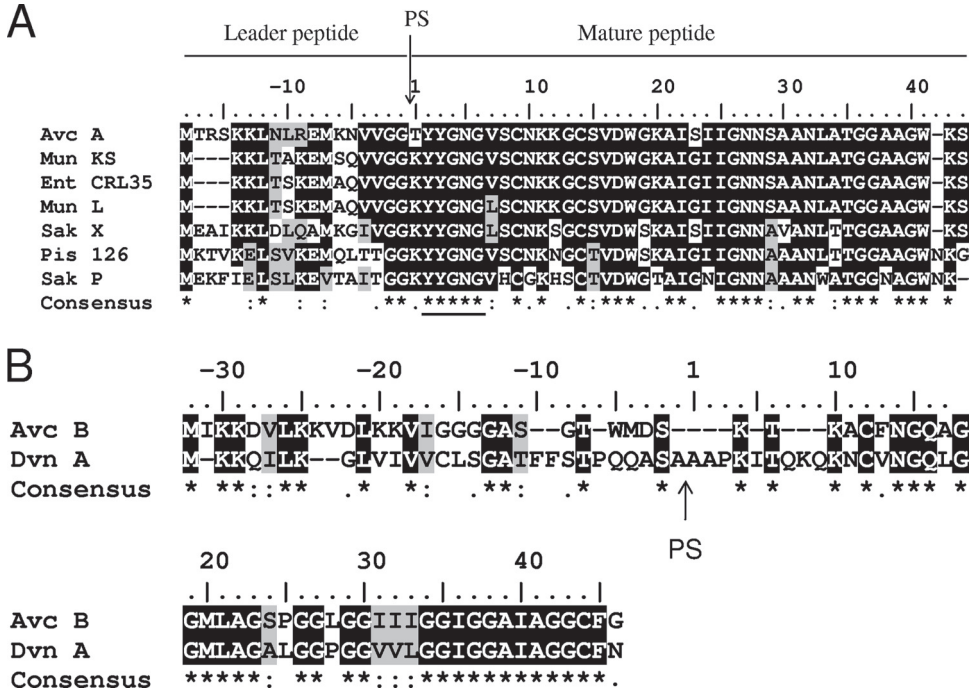

FIG. 3. (A) Multiple alignment of avicin A precursor with precursors of closely related subclass IIa bacteriocins. Mun KS, precursor of mundticin KS; Mun L, precursor of mundticin L; Ent CRL35, precursor of enterocin CRL35; Sak X, precursor of sakacin X; Pis 126, precursor of piscolin 126; Sak P, precursor of sakacin P. The vertical arrow indicates a cleavage site where the leader peptide is removed from the mature peptide. The YYGNG motif is underlined. (B) Pairwise alignment of avicin B (Avc B) and divergicin A precursor (Dvn A). PS indicates the processing site for divergicin A.

member of subgroup B, which includes the immunity proteins of sakacin X, piscicolin 126, sakacin P, and mundticin KS. Because of the similarity between AvcI and sakacin X immunity protein produced by *L. sakei* 5, we examined whether there is cross-protection between *E. avium* XA83 and *L. sakei* 5. The results showed that neither *E. avium* nor purified avicin A inhibited *L. sakei* 5 but *L. sakei* 5 inhibited *E. avium* XA83, demonstrating that the immunity protein of avicin A is not sufficient to provide protection against the plethora of bacteriocins produced by *L. sakei* 5.

avcB may code for a 67-amino-acid glycine-rich (30%) peptide that shares 45% identity (60% similarity) with divergicin A, a bacteriocin that inhibits *Carnobacterium divergens* (Table 5 and Fig. 3B) (51). Both avicin-producing strains contain the *avcB* gene, but neither strain had any apparent immunity-encoding ORF. In order to investigate whether *avcB* encodes a functionally expressed bacteriocin, we tested the inhibition of bac^+ strain XA83 using pediocin-resistant mutants of *E. avium* and *E. faecalis*, but no activity was observed. Notably, no *sec* leader sequence was found in the N-terminal part of translated *avcB*; however, a potential double-glycine leader was identified (Fig. 3B.).

In order to investigate whether the *avcB* gene encodes a functional bacteriocin, it was cloned in pMG36e and expressed in *L. lactis* IL 1403, which contains the lactococcin A transport gene apparatus (49). The resulting clone did not show antibacterial activity against the bacteria tested. Neither CFS nor the ammonium sulfate precipitate fraction inhibited the growth of strains that are sensitive or resistant (*E. avium* and *E. faecalis*) to avicin A. However, we cannot exclude the possibility that the lack of antimicrobial activity could have been due to improper processing of the AvcB leader peptide in IL 1403 or due to lack of expression, although the host carried the proper processing and transport mechanisms for such bacteriocins. When a very small inoculum of *E. avium* XA83 is added to new broth (dilution factor, 10^{-6}), avicin A is not produced because its quorum-sensing regulatory system is not turned on (see below). The *avaB* gene is apparently not regulated by quorum sensing since no binding site for the response regulator was found, but no antimicrobial activity was observed in this culture, which suggests that *avcB* was not expressed. Based on the cumulative evidence it is very unlikely that avicin B is a functional bacteriocin.

avcT encoded a 725-amino-acid protein that showed a high level of similarity to a putative ABC transporter of sakacin X and significant levels of similarity to other bacteriocin ABC transporters, suggesting that it is involved in the export of avicin A (Table 5) (48).

The *avcK*, *avcR*, and *avcP* genes, which constitute a three-component regulatory system, are located adjacent to one another but in two different operons (Fig. 2A), and they encode proteins composed of 442, 244, and 45 amino acids, respectively. These ORFs share significant similarity with genes encoding histidine protein kinase, a response regulator, and an inducing peptide of sakacin X, respectively (Table 5) (48).

avcD encodes a 171-amino-acid peptide that shares similarity with a transport accessory protein of brochocin C, a two-peptide bacteriocin (Table 5) (28). The N-terminal half of AvcD is 56% identical to the deduced amino acid sequence of ORF 5 in the TX locus of *L. sakei* 5 (48). This region of AvcD

is only 38% identical to the corresponding region in the transport accessory protein of brochocin C.

Induction of avicin A production. A bac$^-$ phenotype could be obtained by extensive dilution of a bacteriocin-producing culture; 10^{-6} dilution of an overnight bacteriocin-producing culture resulted in a non-bacteriocin-producing culture (bac$^-$). Only addition of cell-free culture supernatant (CFS) from a bacteriocin-producing culture or a synthetic inducing peptide (AvcP) could then induce bacteriocin production in the bac$^-$ culture.

The induction activity was tested with a synthetic AvcP peptide pheromone. The minimum concentration of the synthetic peptide that induced bacteriocin production in a bac$^-$ culture was 1 ng/ml; 0.1 ng/ml could not induce bacteriocin production. This result confirms that *avcP* encodes the peptide pheromone that regulates avicin A production. Although the inducer peptides of avicin A and sakacin X share significant similarity, CFS from *L. sakei* 5 did not induce bacteriocin production in a strain with the avicin A-negative phenotype.

DISCUSSION

In this study, we report biochemical and genetic characterization of a novel subclass IIa bacteriocin produced by two different *E. avium* strains (XA83 and 208) isolated from two healthy human infants from two different countries, Ethiopia and Norway. The structural and immunity genes of the bacteriocins produced by these two strains are identical. PCR confirmed that the gene structures of the two strains are identical. For these reasons, their bacteriocins were given the same designation, avicin A. An interesting observation was that the levels of bacteriocin-producing isolates XA83 and 208 were higher ($>10^{-6}$ CFU g^{-1} feces) than the levels of nonproducing isolates of *E. avium* (10^{-5} CFU g^{-1} feces). The bacteriocin producers also accounted for 25 to 45% of the total bacterial plate counts on the LAB selective growth media. Moreover, the frequent occurrence of *E. avium* in healthy infants (both Norwegian and Ethiopian) suggests that bacteriocin-producing *E. avium* may play an important role in the succession of a healthy gut microbiota in some neonates. It is not uncommon that different bacterial strains or species produce identical bacteriocins. For example, mundticin ATO6 and mundticin KS are two identical subclass IIa bacteriocins isolated from two different strains (ATO6 and NFRI 7393) of *E. mundtii* obtained from different sources (25, 42). To our knowledge, this the first report of a thorough elucidation of the activity spectrum, gene structure, and regulation of a bacteriocin system of *E. avium*.

The subclass IIa bacteriocin inhibition spectrum includes species of the genera *Listeria*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Staphylococcus*, *Micrococcus*, *Streptococcus*, *Clostridium*, *Bacillus*, and *Brochotrix* (10, 30). The inhibition spectrum of avicin A is similar and crosses species borders as this bacteriocin inhibits *Listeria*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Carnobacterium* species in addition to *Enterococcus*.

Amino acid sequence analysis revealed that avicin A contains the N-terminal pediocin consensus motif (YYGNG) and two cysteine residues, indicating that avicin A belongs to bacteriocin subclass IIa. Recently, subclass IIa bacteriocins have been divided into four subgroups on the basis of similarities and differences in their C-terminal regions (33). Avicin A belongs to subgroup 1, which encompasses mundticin KS, piscicolin 126, sakacin X, sakacin P, enterocin A, and pediocin PA-1. Mundticin KS, which is produced by *E. mundtii* NFRI 7393 isolated from grass silage (25), is the bacteriocin most closely related to avicin A both in the leader sequence and in the mature peptide. However, unlike the avicin A gene, which is located on a chromosome, the mundticin KS gene is located on a plasmid. In addition, the structural gene of mundticin A (*munA*) and the immunity gene (*munC*) are located in different operons, whereas in the case of avicin A the structural gene and the immunity gene are in the same operon, like the genes of most subclass IIa bacteriocins characterized to date (23). Moreover, it has been shown by heterologous expression of mundticin KS in *E. faecium* and *Lactobacillus curvatus* that an accessory protein is not needed for transport of mundticin KS, but the data did not confirm the absence of an accessory gene in the cloning host. Other studies have shown by using deletion analysis that an accessory protein is required for export of leucocin A and pediocin PA-1 (46, 50). Since the gene encoding the accessory protein is present in the avicin A locus, this protein might be crucial for bacteriocin externalization.

The biosynthesis of many class II bacteriocins is regulated by a quorum-sensing mechanism through a three-component regulatory system which consists of an inducing peptide, a histidine protein kinase, and a response regulator (31). The presence of genes encoding these three components in the avicin A locus indicates that biosynthesis of avicin A is regulated by a quorum-sensing system. However, some differences have been observed between the genetic organization of the avicin locus and the genetic organization of the loci of other class II bacteriocins. The most surprising difference is the difference in the organization of the three-component regulatory system, where the histidine protein kinase and response regulator genes are in an operon together with the ABC transporter gene, while the peptide pheromone gene is located with the gene encoding the accessory transporter protein AvcD in another operon (Fig. 2A). This organization is different from the organization of most of the regulated class II bacteriocins, where the three genes responsible for regulation are located in the same operon (2, 16, 18, 22, 34, 38). Our results conclusively show that avicin P functions as a pheromone to induce avicin A production in a nonproducing culture, which is consistent with a quorum-sensing regulatory mechanism (32).

Our experiments indicate that *avcB* is not functional and is probably a relic of a previous functional bacteriocin. This hypothesis is also supported by the absence of a dedicated immunity protein, and the fact that *avcB* clones in *L. lactis* IL 1403 did not show antimicrobial activity may support this notion.

Almost all strains that produce subclass IIa bacteriocins characterized thus far were isolated from foods or related products (9). In contrast, avicin A is produced by a bacterium isolated from human feces, suggesting that it is capable of establishing and proliferating in the gut. Human origin and survival in the gut are two of the several selection criteria that must be met by probiotics. In addition, our strains produce a bacteriocin that inhibits pathogenic bacteria, and bacteriocin production is an important probiotic feature (4). Diseases associated with *E. avium* (such as brain abscesses, endocarditis,

and bacteremia) are rarely reported (29, 35, 37). In contrast, our strains might be proven to be beneficial as intestinal LAB and possibly also as probiotics; however, further studies have to be performed to determine this.

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PAPER III

Biochemical and genetic characterization of salivaricin D, an intrinsically trypsin resistant lantibiotic from *Streptococcus salivarius* 5M6c isolated from a healthy infant

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ABSTRACT

In this work, we purified and characterized a newly identified lantibiotic (salivaricin D) from *Streptococcus salivarius* 5M6c. Salivaricin D is a 34-amino acid residue peptide (3467.55 Da), whose gene locus is a 16.5 Kb DNA segment which contains genes encoding the precursor of two lantibiotics, two modification enzymes (dehydratase and cyclase), an ABC transporter, a serine-like protease, immunity proteins (lipoprotein and ABC transporters), a response regulator and a sensor histidine kinase. The immunity gene (*salI*) was heterologously expressed in a sensitive indicator and provided significant protection against salivaricin D, confirming the identity of the bacteriocin locus as well as the immunity function. Salivaricin D is a trypsin-resistant lantibiotic that is similar to nisin-like lantibiotics. It is a relatively broad spectrum bacteriocin that inhibits members of many genera of Gram-positive bacteria, including the important human pathogens *S. pyogenes* and *S. pneumoniae*.

In addition a second bacteriocin encoded gene was identified and a third bacteriocin-like activity was purified but not further characterized. Thus, *Streptococcus salivarius* 5M6c may be a potential biological agent for the control of oro-nasopharynx colonizing streptococcal pathogens or may be used as a probiotic bacterium.

INTRODUCTION

Streptococcus salivarius is a member of the lactic acid bacteria (LAB) that forms part of the normal flora of the oral cavity, throat and upper respiratory tract (21, 26, 38, 41). It has also been observed in nasopharynx and intestinal tract, and can be isolated from human feces (23, 34, 41). *S. salivarius* strains have been shown to produce a number of bacteriocins often referred to as salivaricins, most of which are lantibiotics (20, 37, 47-49).

Lantibiotics are small, heat-stable, ribosomally synthesized, post-translationally modified antimicrobial peptides (bacteriocins) produced by Gram-positive bacteria (2). The lantibiotics, unlike other bacteriocins, are characterized by containing the thioether amino acids lanthionine (Lan) and 3-methyl-lanthionine (MetLan), and the modified amino acids di-dehydroalanine (Dha) and di-dehydrobutyrine (Dhb) (50). Lantibiotics are initially synthesized as inactive linear prepeptides that undergo subsequent extensive modifications to be biologically active. The modifications involve dehydration of the two amino acids serine and threonine residues, forming the di-dehydro amino acids Dha and Dhb, respectively, that react with the nearby residues to form a thioether linkage which results in the formation of Lan and MetLan, respectively. Finally, the modified peptide is exported and cleaved from its leader in order to be active.

A biosynthetic gene cluster that consists of genes encoding the prepeptide (LanA), one (LanM) or two modification enzymes (LanB and LanC) that introduce the thioethers, an ABC transporter (LanT) which exports and/or removes the leader, an extracellular protease (LanP) that removes the leader, and finally a self-protecting system of the producer referred to as immunity proteins (LanI(H) and/or LanFE(G)) are necessary for lantibiotic production

(2). Many lantibiotics are regulated by a quorum sensing system consisting of a response regulator (LanR) and histidine protein kinase (LanK) and a peptide pheromone (induction peptide) that sometime can be the bacteriocin itself (30).

Lantibiotics kill mainly Gram-positive bacteria. In general, lantibiotics kill target cells by inhibiting cell wall biosynthesis by binding to the cell wall precursor lipid II and/or by formation of pores in cell membranes leading to efflux of small molecules and dissipation of membrane potential (1, 2, 4).

Bacteriocins may have potential applications to control food spoilage and food-borne infections (6) and in medicine as selective antimicrobials to inhibit pathogens without affecting the normal flora unlike classical antibiotics that have broad-spectrum activity (36). Bacteriocin production is considered as a probiotic feature, and it has convincingly been shown that bacteriocin producing lactic acid bacteria can effectively inhibit growth of, and kill *Listeria monocytogenes* in mice (8). Thus, identification and characterization of bacteriocin producing LAB of human origin is needed not only to develop probiotic bacteria with a diverse antimicrobial potential, but also to develop bacteriocins into chemotherapeutic agents to control infections. To this end, we aimed at isolation of bacteriocin-producing LAB from healthy human infants. In this study, a new bacteriocin (lantibiotic) was purified from *S. salivarius* 5M6c and characterized at biochemical and molecular level.

MATERIALS AND METHODS

Isolation and identification of bacteriocin-producing strains

Bacteriocin-producing strains were isolated from a fecal sample of a healthy Ethiopian infant. A 10-fold serial dilution of the fecal sample was done in MRS broth, after which 0.1 ml from 10^{-5}, 10^{-6} and 10^{-7} dilutions each was plated on MRS agar and anaerobically incubated at 37^{o}C. Three colonies with different morphologies were randomly picked from each plate, checked for purity and tested for bacteriocin production. Species identification was done by using partial 16S rRNA gene sequencing and BLAST analysis.

Bacterial strains and culture conditions

Strains and plasmids used in this study are listed in Table 1 and 2. Solid media and soft agar were prepared with 1.5% and 0.7% agar, respectively. All strains were maintained as frozen stocks at -80^{o}C in 13% glycerol. *Enterococcus* and *Listeria* species were grown in GM17 at 37^{o}C. *Clostridium, Lactobacillus*, *Pediococcus* and *Leuconostoc* species were grown in MRS at 30^{o}C. *Lactococcus* species were grown in GM17 at 30^{o}C. *Streptococcus* species were grown in MRS, GM17, BHI or TH at 37^{o}C. *Staphylococcus* and *Bacillus* were grown in BHI at 30^{o}C. All media were purchased from Oxoid (England).

Bacteriocin/antimicrobial activity assay

Antimicrobial activity was detected using soft agar overlay assay. The isolated bacteria were spotted onto MRS agar plates and incubated for 16 hours at 37^{o}C. Resulting colonies were over-laid with soft agar containing an overnight culture (diluted 400-fold) of the indicator strain (see Table 1). After overnight incubation, the colonies were examined for the formation of growth inhibition zones, as indication of antimicrobial activity. To investigate whether the antimicrobial activity observed was caused by a non-protein compound or a bacteriocin, the antimicrobial activity was tested in the presence of proteinase K.

Quantitative determination of the antimicrobial activity of the bacteriocin in cell-free culture supernatant (CFS) was conducted using microtiter assay system (18). A two-fold serial dilution (in MRS) of 100 µl CFS was added to microtiter plate wells containing 50 µl MRS (except the first well). To this, 150 µl diluted (400-fold in MRS) overnight culture of the indicator strain *Lactobacillus sakei* NCDO 2714 was added. The plate was incubated for 14 hours, and growth inhibition was measured turbidometrically at 620 nm by a microtiter plate reader (Labsystems iEMS Reader MF, Labsystems, Helsinki, Finland). One bacteriocin unit was defined as the amount of bacteriocin that inhibited the growth of the NCDO 2714 indicator strain by 50%. Similarly, the minimum inhibitory concentration (MIC) of a purified bacteriocin against sensitive strains was done using microtiter assay. MIC was defined as the concentration of pure bacteriocin that inhibited the growth of a sensitive strain by 50%.

Bacteriocin purification

A 2 L overnight culture (22 h) of the bacteriocin producer, *Streptococcus salivarius* 5M6c, was grown in MRS, and supernatant was collected and filtered. Ammonium sulphate was added to the CFS to 40% and the mixture was incubated at 4^{o}C for 30 min with agitation. Bacteriocin was precipitated from the CFS, and collected by centrifugation at 14,000 rpm at 4^{o}C for 30 min. The resulting pellet was resuspended in 200 mL sterile distilled water and the pH was adjusted to 3.5 with 1 M HCl.

The sample was then passed through a 10 mL SP Sepharose^{TM} Fast Flow (GE Healthcare Biosciences, Uppsala) ion-exchanger which had been equilibrated with 10 mM acetic acid. After application, the column was washed twice with 20 mL of 10 mM sodium phosphate buffer pH 6.0. The column was subsequently eluted with a stepwise gradient, consisting of 10

mL each of 0.1, 0.3 and 1.0 M NaCl at a flow rate of 1 mL per min (elution was performed twice with each concentration of NaCl) and elution fractions were stored on ice. The two 0.3 M NaCl fractions contained the highest bacteriocin activity, and were used for further purification by reversed-phase chromatography, using an Äkta Purifier HPLC system. Briefly, the two most active fractions from ion-exchange chromatography were combined and applied to Resource 15 RPC 3 mL reversed-phase column (Pharmacia Biotechnology) equilibrated with 0.1% trifluoroacetic acid (TFA) in water. The sample was eluted with a 10 column volumes (CV) linear gradient of 0 to 100% 2-propanol in 0.1% TFA and collected in 1 mL fractions (RPC I). From RPC I, fractions 9 to 16 were found to be active, and fractions 11, 12 and 13 contained the most bacteriocin activity. Therefore, fractions 11, 12 and 13 were combined (to increase yield), diluted to 30 mL in sterile water, and applied to a Sephasil Peptide C8 5 µm ST 4.6/250 column (Amersham Biosciences). Samples were then eluted with a 7 CV linear gradient from 25 to 45% 2-propanol in 0,1%TFA and collected in 1 mL fractions (RPC II). The two most active elution fractions from RPC II (fractions 17 and 18) were pooled together, diluted to 20 mL, and applied to a Sephasil Peptide C8 5 µm ST 4.6/250 column. The sample was eluted in 0.5 mL fractions with a 7 CV linear gradient of 25 to 35% 2-propanol in 0.1% TFA (RPC III). The antimicrobial activity was separated in two UV absorbance (214 nm) peaks, the low activity peak (fractions 2 and 3) and the high activity peak (fractions 6 to 8) (Fig. 1) and they were stored separately at -20^{o} C for further analysis.

Mass spectrometry, N-terminal sequencing and amino acid composition analysis

The molecular weight of the purified bacteriocin was determined by mass spectrometry as previously described (10). Briefly, the pure bacteriocin samples (fractions 2, 3, 6, 7, and 8) (Fig. 1) were individually mixed 1:1 with a saturated solution of HCCA (α-cyano-4-hydroxycinnamic acid) in 0.1% TFA/acetonitrile (2:1) and deposited on a ground steel MALDI target. Mass spectra were recorded in reflection mode on an Ultraflex TOF/TOF (Bruker, Daltonics, Billerica, MA), using a pulsed ion extraction setting of 40 ns and an acceleration voltage of 25 kV. The spectrum displays the accumulated signals of 200 laser shots, with laser power adjusted to just above threshold level. Peptide mass similarity searches were conducted at ExPASy using TagIdent tool (http://web.expasy.org/tagident/). In order to estimate the number of dehydrated amino acids, salivaricin D was derivatized with ethanethiol and the resulting change in mass was analysed by MALDI-TOF mass spectrometry (28).

Amino acid composition analysis and N-terminal sequencing (Edman degradation) was performed on purified salivaricin D samples by Alphalyse A/S, Denmark.

Genomic DNA isolation for PCR and 16S rDNA sequencing

Genomic DNA isolation was done with modification of the protocol used with Bacterial Genomic DNA Purification Kit (Edge Biosystems). Briefly, 1.5 -3 mL culture was pelleted, washed in 500 µL TES buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl), resuspended in 200 µL spheroplast buffer (10% sucrose, 2 mg/mL lysozyme, 0.4 mg/mL RNase A, 25 U/mL mutanolysin, 25 mM Tris pH 8.0, 25 mM EDTA pH 8.0), and incubated at 37 ^{o}C for 10 to 20 minutes until cell lysis occurred. Then, 50 µl of each of 5% SDS and 5 M NaCl was added, mixed and incubated at 65 ^{o}C for 10 minutes. Buffer N3 (100 µL) (Qiagen) was then added, and samples were mixed and centrifuged in a microcentrifuge at maximum speed at 4^{o}C for 15 minutes. The supernatant was transferred to a new tube, mixed with equal volume of isopropanol and centrifuged in a micro centrifuge at maximum speed at room temperature for 15 minutes to precipitate the DNA. The resulting pellet was washed with 70% ethanol by centrifugation in a micro centrifuge at maximum speed at room temperature for 10 minutes. The final pellet was air-dried and resuspended in 1 x TE buffer pH 8.0.

Genome sequencing and draft assembly

Approximately 100 µg of total DNA from *Streptococcus salivarius* 5M6c was purified using Qiagen genomic tip according to manufacturer's recommendations with minor modifications to optimize lysis as described above.

Genome sequencing was performed (GATC Biotech) using a combination of 454 Life Sciences pyrosequencing and Illumina technologies. The datasets obtained were: GS FLX Titanium, 474256 shotgun reads, average read length 253 nt average coverage 50; GS FLX Titanium, 8 kb mate pair read library, 26013 reads with average read length 128 nt, and Illumina GA pair end library, 6563621 reads, avg read length 31 nt, avg coverage 93. De novo assembly (GATC Biotech) was performed using the GS FLX shot gun data, generating 107 large contigs.

Identification of the salivaricin D gene cluster by genome scanning

The contig 00059 of the GS FLX Titanium genome sequencing of *S. salivarius* 5M6c was found to encode the genes needed for the salivaricin D biosynthesis. The identification was performed by BLAST searches for the presence of LanA, LanB and LanC homologs. Putative salivaricin D gene clusters in contigs of the genome were analyzed by sequence alignment to nisin family lantibiotic biosynthesis clusters like nisin A. Gene prediction was performed on candidate contigs using Prodigal (19) and manual inspection, and gene calling was performed using Artemis (39). Gene functions were assigned based on homology searches using Conserved Domains Blast (27) and BlastP (Table 5). The deduced amino acid sequence of candidate salivaricin D structural genes were compared to the observed mass of purified salivaricin D, and the number of dehydrated residues determined by ethanethiol derivatization of pure salivaricin D (Fig. 2).

Cloning and heterologous expression of the salivaricin D immunity gene

In order to confirm the predicted salivaricin D cluster, the putative immunity gene (*slvI*) was cloned and expressed in the sensitive indicator strain *Lb. sakei* NCDO 2714. A 755 bp fragment that includes *slvI* gene was amplified from genomic DNA of *S. salivarius* 5M6c using the following primers: salxI-f1 5'-GGTGGTGTCGACTAGAAAGGAATCTAAATGGGACGAC -3' and salxI-r1 5'-GGTGGTGCATGCTCATCCTACTCTTCCTTCATTGCAC -3' (SalI and SphI restriction sites are underlined). The resulting PCR product was digested with the restriction enzymes and ligated into pMG36e which had been linearized by the same enzymes using standard protocols. The resulting recombinant plasmid will be hereafter referred to as pLG500. One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen) were transformed with plasmid pLG500. Positive transformants were selected and subcultured in selective LB broth with 200 µg/mL erythromycin. pLG500 was extracted from the *E. coli* and the presence of the insert was checked by restriction digestion with SalI and SphI. The integrity of the insert was confirmed by DNA sequencing. *Lb. sakei* NCDO 2714 was then transformed with the plasmid pLG500, using pMG36e (without insert) as a control. Positive clones were selected on MRS supplemented with 10 µg/ml erythromycin and subsequently grown in selective MRS broth. pLG500 was extracted from *Lb. sakei* NCDO 2714, and the presence of the insert was checked by PCR. The sensitivity of the *Lb. sakei* NCDO 2714 clone containing pLG500

or pMG36e to purified salivaricin D was tested using soft agar overlay and microtiter assays (as described above).

Nucleotide sequence accession number

The GenBank accession number for contig 00059 that contains salivaricin D locus is JN564797.

RESULTS

Bacteriocin production

The novel bacteriocin, called salivaricin D, was produced by *S. salivarius* 5M6c (and other *S. salivarius* strains isolated from the same infant as well; Table 1) at 30^{o}C or 37^{o}C in multiple media, including GM17, MRS, BHI and TH. Maximum production was observed in GM17 (about 10,000 BU/ml) when *Lb. sakei* NCDO 2714 was used as the indicator organism, whereas with the other three media bacteriocin production was approximately 2500 Bu/ml. Based on plate counting a total of 10^7 to 10^9 CFU/g feces were obtained. Colonies were isolated from three MRS agar plates at the highest dilutions, and 5 out of 9 colonies were found to be bacteriocin-producing *S. salivarius* isolates. The result suggests a high prevalence of bacteriocin-producing *S. salivarius* in the fecal sample.

Bacteriocin purification

Purification of bacteriocin was possible from both MRS and GM17. The purification results presented in Table 2 are from 2 L MRS. The third step of reverse phase chromatography (RPC III) revealed the presence of two peaks with antimicrobial activity, suggesting the presence of two bacteriocins (Fig. 1). Salivaricin D was selected for further analysis because it was produced in significantly larger amounts, and had a broader antibacterial activity.

Analysis by mass spectrometry showed that salivaricin D and the second bacteriocin have a mass of 3467.55 Da (Fig. 2) and 3483.02 Da (not shown), respectively. Since the mass of salivaricin D is similar to many known lantibiotics such as nisin, bovicin HJ50 and pep5, we hypothesized that salivaricin D was a lantibiotic. However, it was not possible to detect genes in salivaricin D locus by PCR using designed degenerate primers designed based on conserved regions of modification enzymes. Nor did primers based on structural genes of the above lantibiotics work. We also tried to obtain its amino acid sequence by tandem mass spectrometry, but did not succeed because of poor fragmentation of the bacteriocin as well as

its resistance to trypsination. Only the N-terminal amino acid was determined to be phenylalanine by Edman degradation, but further sequencing failed probably due to presence of a modified residue in the peptide. However, amino acid composition analysis and protein K sensitivity confirmed that it is a protein, and the amino acid composition analysis indicated the absence of arginine, tyrosine, alanine, serine and threonine (Table 4). Absence of Ser and Thr provided additional evidence that salivaricin D is a lantibiotic since these residues undergo dehydration in lantibiotics. An unknown amino acid-like residue was found in the chromatogram at the same retention time of glutamine/glutamate, and this peak may account for some of the modified amino acid residues of lantibiotics (Table 4).

Biochemical properties of salivaricin D

Salivaricin D is sensitive to proteinase K digestion but resistant to trypsin. In addition, it does not absorb UV at 280 nm, indicating the absence of tryptophan and tyrosine in its primary structure. Furthermore, the activity of the bacteriocin was not destroyed by heating at 70^{o}C for 15 minutes, indicating that it is relatively heat stable. Salivaricin D remained stable at 4-6 ^{o}C for at least 10 months and at room temperature for at least 3 weeks, indicating that the bacteriocin is quite stable.

Salivaricin D inhibition spectrum and minimum inhibitory concentrations

Salivaricin D is a relatively broad spectrum bacteriocin that showed inhibitory activity against members of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus, Micrococcus*, *Bacillus* and *Clostridium,* but not against *Staphylococcus* (except some activity against *S. carnosus*), *Enterococcus* and *Listeria* (Table 3). Interestingly, this bacteriocin has a very low MIC value and effectively kills important pathogens like *S. pyogenes* (MIC: 25 ng/mL) and *S. pneumoniae* (MIC: 0.1-41 ng/mL). Thus, the producing strain does not need to synthesize large amounts of salivaricin D to kill target bacteria. The second bacteriocin (fractions 2 and 3 in Figure 1), which has absorbance at 280 nm, inhibited the growth of only two of the tested bacteria (*Lb. sakei* and *B. coagulans*). Furthermore, the two bacteriocins did not act synergistically (data not shown).

The presence of modified amino acid residues in salivaricin D.

Derivatization of salivaricin D with ethanethiol (mass = 62.134 Da) revealed a mass difference between unmodified ($M+H^+$ = 3467.55 Da) and completely modified ($M+H^+$ = 3902.023 Da) (Fig. 2). This indicated the presence of 7 modified amino acid residues

(modification sites) salivaricin D (Fig. 2). Ethanethiol addition to three dehydrated amino acid residues was complete for all molecules of salivaricin D and increased its molecular weight from 3467.5 to 3654.7 Da (Fig. 2). The addition of ethanethiol to the four Lan and MeLan residues is slower and incomplete (from one to four ethanethiol molecules were added), further increased its mass up to 3902.02 (Fig 2B). Consequently, we can identify seven modifications in the derivatized salivaricin D (Fig. 2B*),* which comprise 4 modified entities involved in thioether linked residues, and 3 in dehydrated residues. The peaks with m/z values of 3654.7, 3715.8, 3777.9, 3839.9 and 3902.0 Da resulted from addition of 3, 4, 5, 6 and 7 molecules of ethanethiol to unmodified salivaricin D ($M+H^+$ = 3467.5 Da) (Fig.2), respectively. Remaining peaks were consistent with sodium or potassium adducts of the above set of peaks.

Bioinformatics analysis of the salivaricin D locus

The genome of *S. salivarius* 5M6c was sequenced in order to identify the genes responsible for salivaricin D production. Homology searches identified putative LanA, LanB and LanC family protein encoding genes in the contig 00059 of the draft genome sequence of the strain. Further analysis of the contig 00059 revealed a complete lantibiotic biosynthesis locus, including genes responsible for modification, immunity, export and regulation of salivaricin D (Fig. 3A).

The salivaricin D locus consists of 12 bacteriocin-related putative open reading frames (ORFs) and two transposase-related genes (Fig. 3A). Each ORF is preceded by a strong ribosome binding site (RBS) and all genes (except *orf1* and *orf2*, which are transposase-related genes) encode proteins required for the production of salivaricin D. The 12 ORFs related to bacteriocin production include genes that encode the putative salivaricin D precursor (*slvD*), a nisin family-like precursor bacteriocin (*slvN*), a dehydratase (*slvB*), an ABC transporter (*slvT*), a cyclase (*slvC*), a protease (*slvP*), a response regulator (*slvR*), a sensor histidine kinase (*slvK*) and four immunity proteins (*slvI*, *slvG*, *slvE* and *slvF*). All of the ORFs (with the exception of *slvG*, *slvE* and *slvF* and *orf2*) were encoded on the same DNA strand. The ORFs appear to be organized into 4 operon-like structures (Fig. 3A), all of which contain a putative -10 box and, instead of a -35 box, a pair of pentanucleotide direct repeats (PDR) or *nis*-box, separated by 11 nucleotides, was identified upstream of the -10 box in the promoter regions. A putative -10 box accompanied by one PDR was also identified

upstream of *slvR*, but these were located within the coding region of *slvP*. The PDR are potential binding sites for the response regulator (Fig. 3B) (22).

The first operon consists of the structural gene for salivaricin D (*slvD*). The second operon contains a structural gene for salivaricin N (*slvN*), which is located 148 nucleotides downstream of *slvD*. The third operon (*slvBTCIPRK*) includes genes that encode proteins required for modification of salivaricin D (*slvB* and *slvC*), salivaricin D export (*slvT*), a protease (*slvP*), immunity (*SlvI*) and regulatory proteins (*slvR* and *slvK*). The fourth operon,which is located on the other DNA strand, consists of genes encoding additional immunity proteins (*slvF*, *slvE* and *slvG*).

There are two regions in the locus, each of which is 117 nucleotides long, that share 86% nucleotide identity with each other: The first starts 20 bp upstream of the start codon of *slvD* and ends 94 bp downstream of the start codon; and the second starts 20 bp upstream of the start codon of *slvN* and ends 94 bp downstream of its start codon. Thus, 97 bp of each of the two regions lies in the coding regions of *slvD* and *slvN*, and they share 87% identity at the nucleotide level. Comparison of the deduced peptide (the first 32 amino acids encoded by *slvD* and *slvN* each) from these coding regions revealed 81% identity. Thus, the salivaricin D structural gene (*slvD*) and salivaricin N (*slvN)* appear to be partial duplications.

The *slvD* gene consists of 171 nucleotides that encode the 57- amino acid precursor of salivaricin D, which consists of a 23-amino acid leader peptide and a 34-amino acid mature peptide (Fig. 4). The leader peptide contains the conserved motif Phe-Asn-Leu-Asp, which is a characteristic feature of class Ia subclass I lantibiotics (7). It differs from the leader of nisin Q by 2 amino acids, and from those of nisin A, Z or F by 5 amino acid substitutions (Fig. 4). The mature peptide has significant similarity to nisin-type of lantibiotics, with 62% identity to nisin Q (52) and Z (29), 59% to nisin A (5) and F (9) and 55% identity with nisin U (51). The identities lie mainly in the N-terminus, while differences were found close to the C-terminus (Fig. 4). Salivaricin D differs from all nisins at positions 1, 12, 22, 23, 24, 25, 28, and 32, including important differences, such as the absence of internal lysine residues at positions 12 and 22 (Fig. 4), which render salivaricin D resistant to trypsin digestion. In addition, the nisin-like lantibiotics contain 5 cysteines, while salivaricin D has 4 cysteines, whose positions are conserved; salivaricin D lacks the C-terminal cysteine at position 28, which is replaced with a valine residue.

The deduced structure indicates that salivaricin D contains 4 thioether bridges, which are composed of two Lan (Ala-S-Ala) and two MetLan (Abu-S-Ala), as well as two Dha) and one Dhb (Fig. 5). This result is in agreement with the one obtained by derivatization of salivaricin D with ethanethiol, indicating that salivaricin D is encoded by *slvD* identified in the contig.

BlastX analysis identified protein homolog matches for the other ORFs encoded by the salivaricin D gene locus (Table 5), most of which are similar to the gene products of nisin Q locus. The *slvN* translation product appears to be a lantibiotic-like peptide (salivaricin N), and shows significant similarity (65% identity and 80% similarity) to nisin Q and salivaricin D precursors (Fig. 4). The mature peptide is 52% and 41% identical to that of nisin Q and salivaricin D, respectively. However, the leader sequence of salivaricin N shows greater identity to salivaricin D (91%) than to nisin Q (78%).

Heterologous expression of salivaricin D immunity gene

In order to further confirm the identity of the putative salivaricin D locus and that the *slvI* gene encodes the immunity protein of salivaricin D, *slvI* was cloned in the *Lb. sakei* NCDO 2714 indicator strain on the pMG36e plasmid. The resulting clone (pLG500) was 32-fold more resistant to purified salivaricin D than *Lb. sakei* NCDO 2714 carrying only the cloning vector (data not shown), which strongly support our notion that SalI indeed is the immunity protein, providing further confirmation of the identity of salivaricin D locus.

DISCUSSION

A number of bacteriocins have been characterized from *S. salivarius* strains (20, 32, 37, 47-49). Most of these have similar primary structures, with lengths from 22 to 25 amino acids and masses ranging between 2315 and 2767 Da (47). In this study, we report the purification and characterization of a new bacteriocin (salivaricin D), which is different from other bacteriocins produced by *S. salivarius* strains both in size and sequence and showed strong homology to the nisins. It is larger than all known salivaricins. However, salivaricin D is similar to other salivaricins in its inhibition spectrum. For example, salivaricin D inhibits *S. pyogenes*, like most other bacteriocins produced by other *S. salivarius* strains (e.g., salivaricins A to A5, salivaricin B, salivaricin 9 and streptin); and like salivaricin B and streptin, salivaricin D inhibits *S. pneumonae* (47). Thus, salivaricin D and other salivaricins

may have potential applications in the control of infections caused by S. *pyogenes* and *S. pneumonae* (11, 43).

We were unable to obtain the amino acid sequence of salivaricin D by N-terminal sequencing (Edman degradation) except for the very N-terminal residue that is a phenylalanine. Normally, this happens in lantibiotics if the amino acid Thr or Ser are modified (28). The DNA-sequence deduced peptide sequence of salivaricin D confirmed that the first N-terminal amino acid in the mature peptide is phenylalanine, followed by the two possibly dehydrated amino acid residues Thr and Ser, the second of which might be involved in lanthionine formation (Fig. 4 and 5).

During modification of lantibiotics, the amino acids threonine and serine are dehydrated to Dha and Dhb. Derivatization of lantibiotics with ethanethiol is an effective way to determine the number of dehydrated amino acids (28, 31). By using this approach we found an exact match between the results obtained by ethanethiol modification and the number of modified amino acids predicted from the deduced peptide sequence of salivaricin D. As can be seen in Figure 2, three molecules of ethanethiol were associated to all salivaricin D molecules. Moreover, one to four additional ethanethiol molecules were added to the bacteriocin molecules. The results suggest that the addition of 3 ethanethiol molecules corresponds to the number of free didehydro amino acids. The reaction of ethanethiol to Lan and Met-Lan is slower and not complete and results in one to four modifications. Thus, this derivatization method may enable determination of the number of di-dehydro amino acids that are free or involved in the ring formation.

Because of its similarity in its pre-sequence to the pro-nisins (Fig. 4), we deduced the structure of salivaricin D (Fig. 5) using the structure of nisin A as a model. Nisins contain 5 cysteines at positions 7, 11, 19, 26 and 28, while salivaricin D has 4 cysteines, whose positions are conserved but lacks the cysteine at position 28, which is replaced by a valine residue. Nisins A, Z, Q and F have 5 Thr residues at positions 2, 8, 13, 23 and 25, and 3 Ser at positions 3, 29 and 33; in contrast, in salivaricin D residues at positions 25 are replaced by Gly and position28 contains a Val not a Cys (Fig. 4). This means that the four (3-7 (A), 8-11 (B), 13-19 (C), 23-26 (D)) ring structures in nisin A are completely conserved and only the last MetLan structure (ring E composed of amino acid residues 25 and 28) is missing in salivaricin D. Thus, salivaricin D forms apparently 4 rings (A-D), in contrast to the nisins, which form 5 rings (A to E). However, salivaricin D and nisins may have differences in

killing activity since a single amino acid substitution can change/abolish the activity of a protein. Therefore, the inability of salivaricin D to inhibit listeria, staphylococci and enterococci may be associated with the lack of this 5th C-terminal ring (ring E), or differences in amino acid residue of the C-terminal region.

The genetic make-up of the salivaricin D locus is similar to that of class I lantibiotics, especially the nisins. The salivaricin D locus contains all of the genes present in the nisin locus, but in a different arrangement. In nisin A, Z and Q loci, the genes occur in the order *nisABTCIPRKFEG*, while in nisin U the genes occur in the order *nisPRKFEGABTCI*. In contrast, the salivaricin D locus is separated in two gene clusters found on different DNA strands, the *slvABTCIPRK* cluster is located on one strand while the *slvFEG* cluster on the opposite strand. Moreover, these two clusters of genes are separated by a transposase-like coding gene (*orf2*). Another transposase-like encoding gene (*orf1*) also occurs upstream of the structural salivaricin D, suggesting that the locus is inserted in the genome by a transposon. Remnants of transposase genes have also been identified in and at the two ends of nisin U locus (51); and transposons were also found in the loci of nisin A and Z (13, 25) .

Like other members of subclass I lantibiotics, salivaricin D contains genes that encode two modification enzymes (LanB and LanC), a dedicated ABC transporter (LanT) and a serine protease (LanP), suggesting that it belongs to this subclass. Many lantibiotics, including nisin A, nisin U, subtilin, salivaricin A and its variants, have been shown to autoregulate their own production through a two-component regulatory system involving a response regulator and a histidine protein kinase (22, 24, 40, 44, 48, 51). Nisin has been shown not only to induce its own production, but also the production of its associated immunity proteins (14). The presence of genes encoding these two regulatory proteins, as well as a *nis*-box in the salivaricin D locus, suggests that salivaricin D might act as its own signal peptide, autoregulating its own production and immunity. However, this presumption must be proved experimentally. Interestingly, the *nis*-box (**TCTGA**) does show some similarity (indicated in bold face) to direct repeats identified in promoters of the avicin A locus (ATT**TCATGA**), which are also putative binding sites for response regulators (3), suggesting that response regulators for class I and class II bacteriocins could bind to similar DNA sites, and might be involved in cross-functions.

Heterologous expression of the immunity gene (*slvI*) in a sensitive indicator strain resulted in reduced sensitivity to salivaricin D. Full immunity to lantibiotics is usually provided by two systems: the lipoprotein LanI(H) and the ABC transporter LanFE(G) (12).

Another feature that makes salivaricin D different from the nisins is its trypsin resistance; possibly because of the complete absence of arginine residues in salivaricin D and the occurrence of a lysine residue at its C-terminal end. This resistance is advantageous if the producer of salivaricin D is to be used as a probiotic, since it cannot be destroyed by intestinal trypsin, and can still exert its antimicrobial activity against ingested pathogens. Trypsin- resistant bacteriocins can also be produced by bioengineering of natural ones, although this might result in reduced activity (33). Salivaricin D is the first example of a naturally trypsin resistant nisin family lantibiotics and its inhibitory activity (MIC values) is in the sub-nanomolar range, even more potent than many other natural lantibiotics (16, 35, 52). Engineering of nisins has generated variants with improved activity toward selected pathogens, and has identified the hinge region as vital for specificity towards target bacteria (16). Salivaricin D contains His20, Ile21 and Gln22 in the hinge region. The engineered nisin variants with M21I showed enhanced activity against *S. aureus* strains, while N20H and K22Q showed reduced activity compared to natural nisin A (15); and salivaricin D does not inhibit *S. aureus*.

A second putative lantibiotic gene (*slvN*) was found next to *slvD* gene. It shares strong homology to other nisin-like bacteriocins and in particular salivaricin D and nisin Q. We have not identified any antimicrobial activity that could account for *slvN* though we have purified a second bacteriocin (Fractions 2 and 3 in Fig. 1). While salivaricin N does not contain tryptophan, the second purified bacteriocin with low activity seems to contain tryptophan since it shows high absorbance at 280 nm (data not shown). Because of low yield we have not been able to characterize this activity further, but based on our present knowledge (mass analysis) this peptide is not encoded by *slvN*.

Duplication of structural genes among lantibiotics may take place as suggested for lantibiotic salivaricin G32 (47). This may explain the presence of both *slvN* and *slvD*. Another possible explanation is that the *slvN* gene may be reminiscence of a two-peptide bacteriocin which has become a one-peptide bacteriocin. Presently, we do not have any evidence for an active two-peptide bacteriocin. One may also speculate that *slvN* may encode the induction peptide that

activates the *slv* locus, but so far we have not been able to provide any evidence for this hypothesis.

Although *S. salivarius* is predominantly found in the human oral cavity (21, 38), it has also been occasionally isolated from the gut (23, 32, 34, 46). Salivaricin D is produced by a strain which was isolated from the feces of a healthy human infant, indicating that it forms part of the normal flora in the gut as well. In addition, the producer strain appears to occur in high numbers in fecal microflora, suggesting that it might have traits that make it competitive and dominant in the gut flora. Furthermore, the strain was observed to produce extracellular polysaccharides that might help it to adhere to gut mucosal surface, and to evade destruction by the acidic environment of the stomach. For these reasons, *S. salivarius* 5M6c might be a potential probiotic bacterium, and further investigations should be done to evaluate its potential as a probiotic. It should also be pointed out that oral/gut streptococci appear to be a good source for bacteriocins and this may be used to protect the host against invasion of pathogenic streptococci.

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TABLES

TABLE 1. Strains and plasmids used in this study

Strains or plasmids	Description	Source or reference
Lactobacillus sakei NCDO 2714	Sensitive indicator and cloning host	NCDO
Streptococcus salivarius 5M5a	Salivaricin D producer	This study
Streptococcus salivarius 5M5c	Salivaricin D producer	This study
Streptococcus salivarius 5M6a	Salivaricin D producer	This study
Streptococcus salivarius 5M6c	Salivaricin D producer	This study
Streptococcus salivarius 5M7a	Salivaricin D producer	This study
E. coli TOP10	Chemically competent cloning host	Invitrogen
pMG36e	3.7-kb gram positive expression vector, Em^r	(45)
pLG500	pMG36e containing *SlvI* gene	This study

[a] Em^r, erythromycin resistant

[b] NCDO, National Collection of Dairy Organisms (Reading, United Kingdom)

TABLE 2. Purification of salivaricin D

Purification step[a]	**Vol. (ml)**	**Total BU (x 10^3)**	**Recovery (%)**	**A_{214}**	**Protein Conc. (mg/ml)**	**Total Protein (mg)**	**Sp. Act. (x 10^3) (BU/mg)**	**Purification fold**
CFS	2000	5120	100	6.3	0.9	1800	2.8	1.00
AMS	200	2560	50	4.4	0.63	126	20.3	7.1
IEX	30	1536	30	0.356	0.051	1.53	1004	352.9
RPC I	3	3072	60	0.784	0.112	0.336	9143	3214
RPC II	2	2048	40	1.058	0.151	0.302	6781	2384
RPC III	0.5	512	10	0.405	0.058	0.029	17655	6207

[a]CFS, cell free culture supernatant; AMS, ammonium sulphate precipitate; IEX, ion-exchange chromatography; RPC, reversed-phase chromatography; BU, bacteriocin units

TABLE 3. Inhibition spectrum of salivaricin D

Indicators	**Inhibition**	**MIC (ng/ml)[a]**
Bacillus subtilis BD630	+	4.4
Bacillus subtilis OG1	+	18
Clostridium bifermentans NCDO 1711	+	0.35
Clostridium butyricum NCDO 855A	+	22
Enterococcus faecalis v583	-	
Lactobacillus curvatus 89	+	0.8
Lactobacillus curvatus NCDO 2739	+	25
Lactobacillus curvatus ssp. *curvatus* BCS35	+	1.6
Lactobacillus sake NCDO 2714	+	0.5
Lactococcus lactis NCDO 1403	-	
Lactococcus lactis NCDO 497	-	
Lactococcus lactis ssp. *cremoris* 393	+	250
Lactococcus lactis ssp. *cremoris* 730	+	62.5
Leuconostoc lactis NCDO 533	+	3.1
Leuconostoc NCDO 543	+	6.3
Listeria innocua BL86/26 B	-	
Micrococcus luteus ATCC 4698	+	ND
Staphylococcus aureus 2002-60-8452	-	
Streptococcus mitis NCTC 12261	-	
Streptococcus oralis SK 153	-	
Streptococcus pneumoniae D39	+	0.097
Streptococcus pneumoniae TIGR4	+	0.195
Streptococcus pneumoniae R6	+	41
Streptococcus pyogenes 08198	+	25
Streptococcus pyogenes NCTC 700294	+	ND
Streptococcus sanguis SK 36	-	
Streptococcus suis	+	0.391
Streptococcus thermophilus	+	ND
Streptococcus thermophilus	+	ND

[a] ND, not determined

TABLE 4. Amino acid analysis of salivaricin D

Amino acid	**Experimental number of amino acid residues (%)**	**Number of amino acid residues deduced from the gene**
Asp/Asn	1 (3.8)	1
Thr[1]	0 (0)	3
Ser[1]	0 (0.2)	4
Glu/Gln[2]	3-4 (13.9)	1
Gly	4 (15.8)	4
Ala	0 (0.2)	0
Cys	ND	4
Val	2 (7.8)	2
Met	1 (3.6)	1
Ile	4 (17.7)	5
Leu	2 (8.1)	2
Tyr	0 (0)	0
Phe	1 (3.5)	1
His	3-4 (14.7)	4
Lys	1 (3.8)	1
Agr	0 (0)	0
Pro	2 (6.8)	1
Trp	ND	0

[1] All residues were dehydrated/modified and consequently not accessible for detection.

[2] The elution peak of Glu/Gln was probably contaminated with some of the modified amino acid residues (the same retention time). ND - Not determined

TABLE 5. Proteins showing similarity to ORF products of salivaricin D locus

ORF	Length (aa)	Similar protein							
		Designation	Length (aa)	Producer[a]	% Identity	% Similarity	E-value	Function	Reference/Accession
orf1	69	COG2963	172	*S. mitis* NCTC 12261	65	80	$1x10^{-5}$	Transposase	ZP_07639005
slvD	57	NisQ	57	*L. lactis* 61-14	62	79	$3x10^{-15}$	Nisin Q precursor	(52)
savN	53	NisQ	57	*L. lactis* 61-14	66	80	$1x10^{-9}$	Nisin Q precursor	(52)
slvB	984	NiqB	993	*L. lactis* 61-14	52	73	0	Lantibiotic dehydratase	(52)
slvT	612	NiqT	600	*L. lactis* 61-14	62	83	0	ABC transporter	(52)
slvC	406	NiqC	418	*L. lactis* 6F3	53	75	$3x10^{-120}$	Cyclase enzyme	(13)
slvI	244	NiqI	245	*L. lactis* 61-14	37	61	$2x10^{-35}$	Immunity protein	(52)
slvP	538	NiqP	695	*L. lactis* 61-14	66	83	$7x10^{-137}$	Leader peptidase	(52)
slvR	228	NiqR	228	*L. lactis* 61-14	68	83	$9x10^{-86}$	Response regulator	(52)
slvK	445	NiqK	447	*L. lactis* subsp. *lactis* KF-14	53	71	$6x10^{-122}$	Sensor histidine kinase	(42)
orf2	294	DUF772	449	*Lh. bacterium*	50	69	$1x10^{-69}$	Transposase	ZP_07955024
slvG	216	NisG	214	*S. salivarius* CCHSS3	72	86	$4x10^{-107}$	Not given	(52)
slvE	247	NisE	247	*S. salivarius* CCHSS3	90	94	$3x10^{-147}$	Not given	ZP_08069789
slvF	224	NisF	225	*S. vestibularis* ATCC 49124	92	96	$3x10^{-113}$	ABC transporter	ZP_08069789

[a]*S*: *Streptococcus*; *L*: *Lactococcus*; *Lh: Lachnospiraceae*

FIGURES

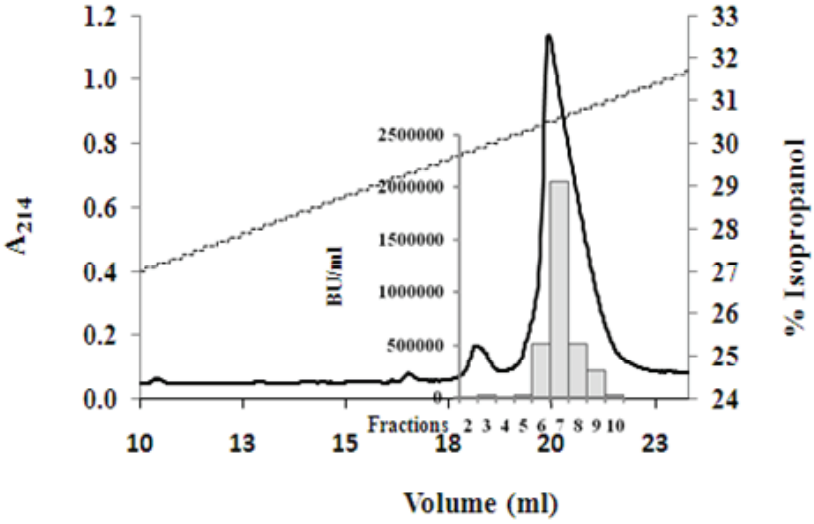


FIG. 1.The chromatographic elution profile from RPC III shows two bacteriocin peaks. The large absorbance peak is salivaricin D and the smaller to the left is a second and unknown bacteriocin-like activity. Solid line and broken line show the absorbance at 214 nm and elution gradient of isopropanol (%), respectively. The antimicrobial activity is shown as grey columns.

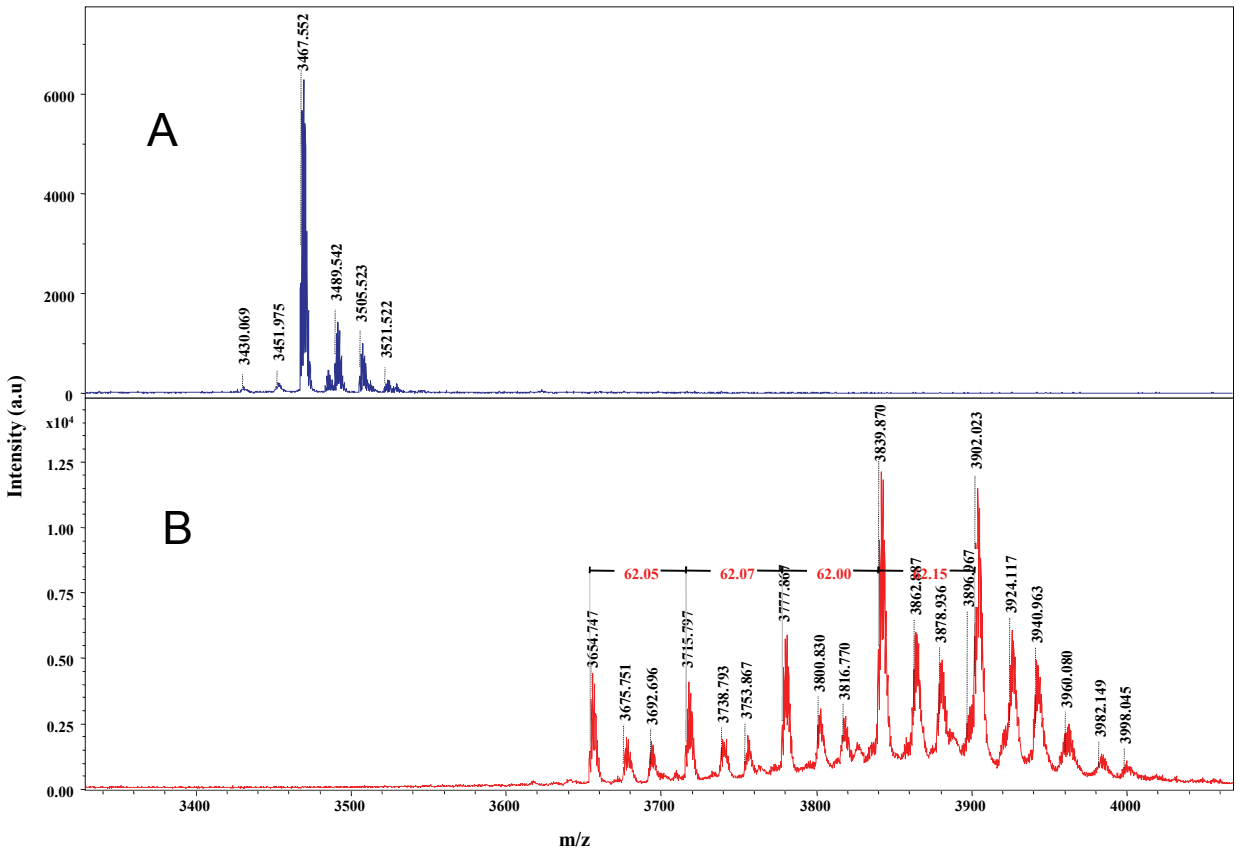
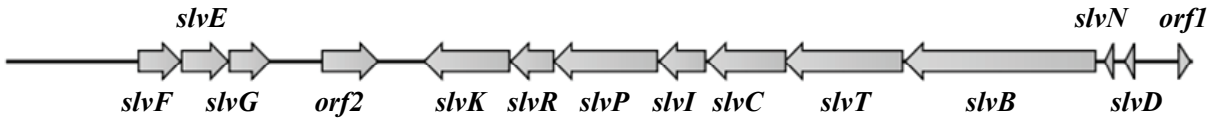


FIG. 2. MALDI-TOF mass spectrometry analysis of salivaricin D and its ethanethiol-derivatized products.

(A) Native salivaricin D (B). Salivaricin D derivatized with ethanethiol

A



B

	PDR	PDR	-10	RBS
slvD	TTCTAAT**TCTGA**ACTAAT**TCTGA**ACTTAA**TATAAT**--35 nt--***AAGGAGGT***ACTCAA**ATG**			
slvN	AAGGAAT**TCTGT**ATATTA**TCTTA**--77 nt--**TATAAT**--13 nt--***AAGGAGGT***GCTTAA**ATG**			
slvB	ATCCTAT**TTTTA**TAAGTT**ACTGT**AAATAGGTA**TTTAAT**--17 nt--***AAGGAGT***CATTATT**TTG**			
slvFEG	AGTTATT**TCTGA**CATAAC**TCTGA**AAATCAGATCT**TATAAT**--19 nt--***AGGAGGT***ATTCAC**ATG**			
Cons.	**TCTGA** --6 nt --**TCTGA**		**TATAAT**	**AAGGAGGT**

FIG. 3. (A) Genetic organization of salivaricin D locus. The ORFs represent genes that encodes salivaricin D precursor (*slvD*), 174 bp; salivaricin N precursor (*slvN*), 162 bp; a dehydratase (*slvB*), 2955 bp; an ABC transporter (*slvT*), 1839 bp; cyclase (*slvC*), 1221 bp; Immunity gene (*slvI),*735 bp; a protease (*slvP*), 1617 bp; a response regulator (*slvR*), 687 bp; a sensor histidine kinase (*slvK*), 1338 bp; and ABC transporter immunity proteins (*slvG*, 651 bp; *slvE*, 744 bp and *slvF* , 675 bp). (B) Alignment of putative promoter sequences. Putative ribosome binding sites (RBS) are in bold italics face. Putative -10 sequences are underlined. The PDR (pentanucleotide direct repeats) upstream of -10 region are in bold face and separated by 6 nucleotides.

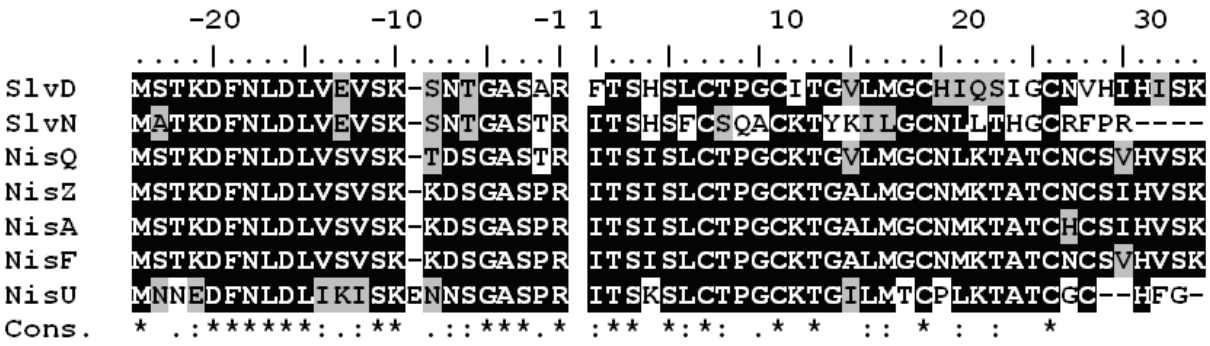


FIG. 4. Alignment of salivaricin D and N with similar lantibiotics. The left and right blocks of the alignment represents the leader and mature peptides, respectively.

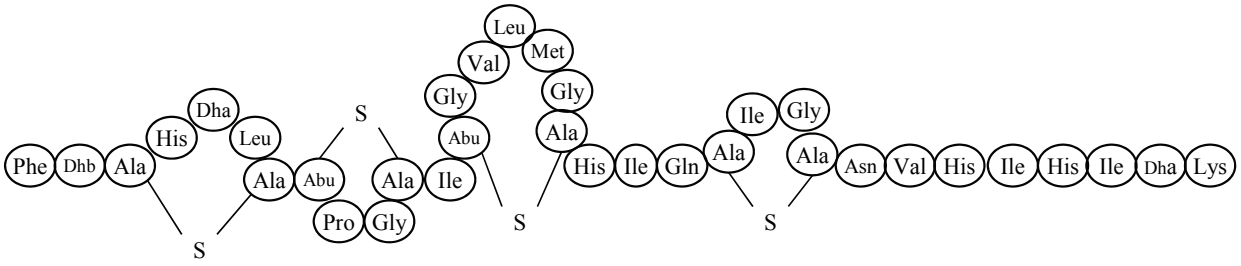


FIG. 5. Proposed structure of salivaricin D. Abu, Aminobutyric acid; Dhb, Didehydrobutyrine; Dha, Didehydroalanine