Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Philosophiae Doctor (PhD)
Thesis 2018: 4

Garvicin KS, a bacteriocin with wide inhibitory spectrum and potential application

Garvicin KS, et bakteriocin med bredt hemmespektrum og potensiell anvendelse

Hai Chi

Garvicin KS, a bacteriocin with wide inhibitory spectrum and potential application

Garvicin KS, et bakteriocin med bredt hemmespektrum og potensiell anvendelse

Philosophiae Doctor (PhD) Thesis

Hai Chi

Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Ås, 2018

Thesis number 2018: 4
ISSN 1894-6402
ISBN 978-82-575-1494-5

Contents

Acknowledgements

The work presented in this thesis was carried out at the Department of Chemistry, Biotechnology and Food Science at the Norwegian University of Life Sciences from 2012 to 2018 with financial support from the China Scholarship Council and East China Sea Fisheries Research Institute, Chinese Academy of Fishery Science (2016M03).

First, I would like to thank to my main supervisor, Prof. Helge Holo, for all his outstanding supervisions, endless encouragements, patience and inspirations. Your door is always open for me. Your valuable advice and 'tricks' make all my work interesting and effective, which leads new idea to my project, as well as my scientific views for the future. Thank you for providing me with more spaces and freedom to let me understand and recognize who I am and what I could do. I have to admit, I could not complete my Ph.D. without you.

I also would like to thank my co-supervisor Dr. Cathrine Arnason Bøe for her great supervision, smiles during Western-blot. Your amiability and attitudes to people are unforgettable. I also would like to thank Prof. Dzung B. Diep for offering me the opportunity to work with nice people around in the group. I have learnt what I should do and what I should not do from you. The memories working with you would be valuable for rest of my life.

Many thanks to Dr. Ibrahim Mehmeti, Cyril Frantzen and Dr. Juan José Jiménez Martinez. You are always supportive and nice to me. The communications with you not only help my work, but also make my life colorful in Norway. I am also grateful to Dr. Kirill Ovchinnikov for contribution to the project, and Linda Godager, May-Britt Hovet for the technical supports.

Special thanks to Mr. Svein Thalberg, Dr. Karen Eg Taraldrud and Mrs. Kari Elise Moxnes for your supports. Your supports and helps draw me out from my tough time, without that I might end up with nothing. Your helps will be membered forever.

I also would like to express my gratitude to all my past and present colleagues and officemates for making me enjoyable time in the lab.

At last, I am deeply grateful to my parents and my wife for your endless love and supports. I think I am the luckiest person in the world to have you. No words could describe how much I love you. You mean everything to me.

我知道，

如果您在这，一定会为我骄傲的！

致慈父迟殿臣（1955 年 3 月 16 日-2015 年 2 月 15 日）

Ås, Jan. 2018

Hai Chi

Summary

In this study, we screened for bacteriocin producer(s) from a large collection of bacterial isolates (over 1800) obtained from raw milk using a panel of indicator strains of different bacterial species and genera. Fourteen isolates with a broad inhibition spectrum were found and identified as *Lactococcus garvieae* (10 isolates) and *Enterococcus faecalis* (4 isolates). Due to the identical characteristics (such as fermentation capacity, inhibitory spectrum and genetic profile) of ten *L. garvieae* isolates, further analysis on one out of ten isolates of *L. garvieae* was carried out. The bacteriocin producer showed a broad inhibition spectrum, against many important problematic bacteria of genera *Listeria*, *Bacillus*, *Staphylococcus* and *Enterococcus*, as well as Gram-negative bacteria of *Acinetobacter* genus.

The purification of bacteriocin from *L. garvieae* was carried out using cation exchange followed with reverse phase chromatography. The N-terminal amino acid sequencing was done by Edman degradation, and was confirmed by the whole genome sequencing. The whole genome sequence results showed that the bacteriocin (termed as garvicin KS) consists of three similar leaderless peptides of 32 to 34 amino acids. Moreover, the whole genome sequencing demonstrated that it contains structural genes followed with an immunity gene and an ABC-transporter gene. Based on a protein BLAST searching, a four-peptide bacteriocin produced by *S. aureus* A70 and an unannotated a three-peptide putative bacteriocin produced by *B. cereus* showed similar homology to garvicin KS. The synthesized peptide confirmed that the bacteriocin requires an equal amount of all three peptides for its activity. Both purified and synthesized garvicin KS showed the same broad inhibition spectrum, indicating that garvicin KS is a promising bacteriocin with potential applications in medicine and food industry.

In order to have a comprehensive understanding of the mechanism of action of garvicin KS, the whole genome sequencing method was used for the identification of putative receptor of garvicin KS. Garvicin KS resistant mutants of *Lactococcus lactis* IL1403 were isolated, and six out of them with the highest resistance levels were subjected to the whole genome sequencing. The results of whole genome sequencing showed that all six mutants contained the mutation within the same gene, encoding for phage shock protein C (PspC), an integral transmembrane protein. A *pspC* knockout mutant exhibited increased resistance levels as compared to the wild type strain, and complementation with *pspC* restored the sensitivity to garvicin KS in garvicin KS resistant mutants. Interestingly, the mutants also lost sensitivity to LcnG, a bacteriocin that uses undecaprenyl pyrophosphate phosphatase (UppP) for its activity. Cloning of *pspC* in *Lactobacillus sakei* also conferred sensitivity to LcnG. Binding assays were performed to study the binding of different bacteriocins to cells. The mutants showed less binding to garvicin KS and LcnG than the wild type. No difference was observed between wild type and mutants binding to nisin. The mutants and wild type checked by immunoprecipitation showed no binding to nisin. However, binding to both the bacteriocins (LcnG and garvicin KS), as complete bacteriocin or as individual subunits, was demonstrated by immunoprecipitation, indicating that PspC might act as a receptor for these bacteriocins.

We also evaluated the activity of garvicin KS, alone and in combination with other antimicrobial agents, against important pathogens from Gram-positive and Gram-negative pathogenic bacteria. The synergistic potential of garvicin KS with other antimicrobial agents was assessed using checkerboard assay and time-kill analysis. Garvicin KS showed potent antimicrobial activity against many Gram-positive bacteria and notably Gram-negative bacteria of Acinetobacter genus. However, high concentrations of garvicin KS alone were required to

inhibit *A. baumannii* and *S. aureus*. When used in combination, garvicin KS and other antimicrobial agents could inhibit *A. baumannii* and *S. aureus* at lower concentrations, as well as a garvicin KS-resistant strain of *E. coli*, revealing a strong synergistic effect. Such combinations can dramatically reduce the required concentrations of the antimicrobial agents, enhance their efficacy, and can lower the probability of development of resistant strains.

Sammendrag

En samling med mer enn 1800 bakterie-isolater fra rå melk ble undersøkt for bakteriocinproduksjon med et panel av indikatorbakterier fra flere ulike arter og slekter. I alt fjorten isolater hadde bredt hemmespektrum. Ti av disse ble identifisert som *Lactococcus garvieae* og fire var *Enterococcus faecalis*. Da alle *L. garvieae* isolatene hadde samme inhibitoriske spekter, fermenteringsegenskaper og genetiske profil ble bare en av disse isolatene studert videre. Bakterioicinprodusenten hadde et hemmespekter som inkluderte mange viktige problemorganismer i slektene *Listeria*, *Bacillus*, *Staphylococcus* og *Enterococcus*, og Gram-negative bakterier i slekten *Acinetobacter*.

Bakteriocinet fra *L. garvieae* ble renset med kationbytter og revers fase kromatografi. N-terminal sekvens ble bestemt med Edman degradering, og hel-genom sekvensering viste at bakteriocinet, kalt garvicin KS, består av tre lederløse peptider med liknende sekvens med 32-34 aminosyrer. Videre viste sekvenseringen at de strukturelle genene etterfølges av et immunitetsgen og genet for en ABC transporter. Databasesøk med protein BLAST viste at garvicin KS har sekvens homologi med et fire-peptid bakteriocin produsert av by *S. aureus* A70 og et ikke-annotert putativt tre-peptid bakteriocin i *Bacillus cereus*. Ved hjelp av kjemisk syntetisert garvicin KS ble det vist at full bakteriocinaktivitet krever like mengder av hver av de tre peptidene. Kjemisk syntetisert garvicin KS hadde det samme brede hemmespekteret som bakteriocin opprenset fra bakteriekultur. Dette gjør garvicin KS til et lovende bakteriocin med potensielle anvendelser innenfor medisin og næringsmiddelindustri.

For å få innblikk i virkningsmekanismen til garvicin KS ble hel-genom sekvensering benyttet til å identifisere reseptoren til garvicin KS. Garvicin KS resistente mutanter av *Lactococcus lactis* IL1403 ble isolert, og genomene til seks av de med høyest resistens ble

sekvensert. Resultatene viste at alle hadde en mutasjon i samme gen, genet som koder for «phage shock protein C» (PspC), et integralt transmembranprotein. En *pspC* delesjonsmutant var mer resistent enn villtypen, og komplementering med *pspC* brakte garvicin KS følsomhet i resistente mutanter tilbake til normalnivå. De resistente mutantene var ikke bare resistente mot garvicin KS, de var også mindre følsomme for lactococcin G (LcnG), et bakteriocin som trenger enzymet undecaprenyl pyrofosfat fosfatase (UppP) for å være aktivt. Ved å klone *pspC* i *Lactobacillus sakei* ble også denne bakterien følsom for LcnG. Bakterienes evne til å binde bakteriocin ble studert. Mutantene viste mindre binding av garcicin KS og LcnG enn villtypen, men mutantene bandt normale mengder nisin. Ved hjelp av immunoprecipitering ble det vist at pspC kan binde garvicin KS og LcnG, enten som hele bakteriociner, eller deres individuelle peptider. Dette indikerer at PspC kan være en reseptor for disse bakteriocinene.

Effekter av å kombinere garvicin KS med andre antimikrobielle stoffer ble undersøkt med viktige patogene Gram-positive og Gram-negative bakterier. Synergier ble målt ved å studere drapskinetikk og veksthemming med ulike blandingsforhold av disse stoffene. Garvicin KS viste potent antimikrobiell aktivitet mot mange Gram-positive bakterier og Gram-negative bakterier i slekten *Acinetobacter*. Men høye konsentrasjoner av garvicin KS måtte til for å hemme *A. baumannii* og *S. aureus*. Når de ble brukt i kombinasjon kunne garvicin KS og de andre antimikrobielle forbindelsene hemme *A. baumannii* og *S. aureus* ved lavere konsentrasjoner, og i tillegg *E. coli* som viste resistens mot garvicin KS alene. Effekten av disse kombinasjonene forsøkene demonstrerte sterk synergi mellom gravicin KS og de andre antimikrobielle stoffene som ble testet. I slike kombinasjoner kan man redusere dosering av de antimikrobielle stoffene, øke effekten og redusere faren for resistensutvikling.

List of papers included in this thesis

Paper I

A novel antimicrobial peptide produced by *Lactococcus garvieae* with a broad inhibition spectrum

Hai Chi, Ibrahim Mehmeti, Kirill Ovchinnikov, Helge Holo, Ingolf F. Nes, Dzung B. Diep. Oral Presentation in 19th International Conference on Agricultural, Biological and Ecosystems Sciences, March 9-10, 2017, Miami, USA

Paper II

Novel Group of Leaderless Multi peptide Bacteriocins fromGram-Positive Bacteria

Kirill V. Ovchinnikov, Hai Chi, Ibrahim Mehmeti, Helge Holo, Ingolf F. Nes, Dzung B. Diep. (2016). Applied and Environmental Microbiology, 82(17): 5216-5224.

Paper III

Phage shock protein C of *Lactococcus lactis* is receptor for multiple peptide bacteriocins

Hai Chi, Cathrine A. Bøe, Camilla Oppegård, Tom Kristensen, Beatriz Martínez, Helge Holo (Manuscript).

Paper IV

Synergistic antimicrobial activity between the broad spectrum bacteriocin Garvicin KS and nisin, farnesol and polymyxin B against Gram-positive and Gram-negative bacteria

Hai Chi, Helge Holo. (2017). Current Microbiology, (4):1-6. doi:10.1007/s00284-017-1375-y

1. INTRODUCTION

Serious bacterial infection is a very challenging global concern and threatening to human health. During the last decade, scientists have put many efforts to combat bacterial infections, and to develop new antibiotics. Unfortunately, the slow pace of development of new antibiotics could not match the rapidly increasing problem of antibiotic resistance in both Gram-positive and Gram-negative bacteria [6,52,93]. The number of multidrug-resistant (MDR) pathogens, in particular ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) is increasing, which causes the majority of the infections [14,15,35]. The National Nosocomial Infection surveillance System recently reported that *Acinetobacter* spp. are responsible for 6.9% of pneumonias cases, 2.4% of bloodstream infections, 2.1% of surgical infections and 1.6% of urinary tract infections [35]. The data from Control of Pathogens of Epidemiologic Importance in the USA reported relatively high infection rates of *Pseudomonas* spp. and coagulase-negative *Staphylococci* [31,51,112]. These facts suggest that the emergence of the MDR pathogens and slow development of new antimicrobial agents should be compensated by searching for alternatives [36,56]. Thus, searching for alternatives to control/inhibit resistant pathogens is essential. Screening and identification of novel bacteriocins could be one of the alternative strategies.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by both Gram-negative and Gram-positive bacteria [23]. In general, bacteriocins have antimicrobial activity against closely related species. Unlike traditional antibiotics, most bacteriocins often have narrow antimicrobial inhibition spectrum, but some possess broad inhibition spectrum against food-borne pathogens and spoilage microorganisms, as well as antibiotic resistant strains [24]. Furthermore, most of the bacteriocins are more potent against closely related species, while

higher concentrations of traditional antibiotics are needed to kill/inhibit target cells. Most importantly, many bacteriocins are of proteinaceous nature and can be inactivated by proteinases. Taken together, bacteriocins are considered as one of the most novel alternatives to prevent the development of the antibiotic resistance, with many potential applications in the medicine and food industries.

Bacteriocins from Gram-negative bacteria

The first bacteriocin produced by Gram-negative bacteria was isolated from *Escherichia coli* in 1925 [19]. The bacteriocin was referred to as colicin as it could kill *E. coli*. This promising discovery resulted in the impetus to study bacteriocin genetics, ecology, and biochemistry. These studies have provided insights into colicin and other close relatives (colicin-like bacteriocins produced by members of the *Enterobacteriaceae*). Later, many bacteriocins produced by Gram-negative bacteria, such as microcin, klebicin, marcecin and cloacin, have been identified and characterized [17,26,54,99]. These discoveries illustrated that bacteriocins produced by Gram-negative bacteria had huge diversity. They could further be divided into three groups based on their molecular weight: 1) colicins and large colicin-like bacteriocins whose molecular weight ranges from 25 to 80 kDa, 2) microcins whose molecular weight is less than 10 kDa, and 3) phage tail-like bacteriocins, which are multimeric peptide assemblies [19]. Colicins and microcins are the most well-studied and well-characterized bacteriocins produced from Gram-negative bacteria.

Colicins and microcins have been intensively studied in the past years. Their potential applications in livestock [8] and human health [53] have been gradually recognized. To date, over 30 colicins have been discovered and characterized. Most of the colicins are relatively large (usually more than 20 kDa) and heat-labile proteins, which possess a bactericidal mode of action

by attacking the specific receptors. The mode of action of different colicins ranges from membrane penetration to protein synthesis inhibition via DNA or RNA degradation. The microcins are low molecular weight (ranging from 1 to 10 kDa) bacteriocins mostly produced by *E. coli* under stress and poor nutrient conditions [7,30]. Microcins also kill target cells via the receptor identification. Microcins, in the nanomolar rage, can interact directly with phospholipid membrane bilayers to kill sensitive bacteria. Other microcins can inhibit bacterial enzymes, for example, DNA gyrase and RNA polymerase can be inhibited by microcin B17 [58] and microcin J25 [2], respectively.

Bacteriocins from Gram-positive bacteria

Baceriocins produced by Gram-positive bacteria, are generally small, heat-stable peptides, whereas some are large complex molecules. So far, the majority of Gram-positive bacteriocins discovered are from lactic acid bacteria (LAB) that are generally considered as safe microorganisms for human use, because they are present in high numbers in diverse fermented meat and vegetable products as well as are common inhabitants in the gastrointestinal tracts of humans and animals [3]. Bacteriocins from LAB also have huge diversity, varing from simple unmodified peptides to post-translationally modified peptides [33,64,80]. The latest classification of bacteriocins from Gram-positive bacteria was summarized by Bastos (2015) and Bali [5] (Table 1).

Table 1. Classification scheme for bacteriocins produced by Gram-positive bacteria (modified from Bastos [9] and Bali (2016)).

Classification	Relevant Features	Types/Subclasses	Examples	References
Class I (lantibiotics)	Small, heat-stable peptides (<5 kDa), containing modified amino acids (lanthionine, 3-methyl-lanthionine, dehydrated amino acids, S-aminovinyl-cystein, among others)	Type A (linear) Type B (globular) Type C (two components) Type D (reduced antimicrobial activity)	Nisin Mersacidin Lacticin 3147 SapT	[80] [4] [96] [63]
Class II	Small, heat-stable peptides (<10 kDa), containing no-modified amino acids	IIa (linear; pediocin-like) IIb (linear; two components) IIc (cyclic peptides) IId (leaderless single linear peptide) IIe (linear; more than two components)	Pediocin PA-1 Lactococcin G Garvicin ML Lactococcin A Aureocin A70	[59] [88] [60] [48] [85]
Class III	Large, heat-labile proteins	Type IIIa (bacteriolysins) Type IIIb (non-lytic)	Lysostaphin Helveticin J	[97] [65]

Class I bacteriocins, also called lantibiotics, are small, heat-stable peptides with modified amino acids that are formed by post-translational modifications [11]. Nisin is one of the best-studied lantibiotics with 34-amino acids, including unusual amino acids like lanthionine (Lan), methyllanthionine (MeLan), didehydroalanine (Dha), and didehydroaminobutyric acid (Dhb). These uncommon amino acids are generated during post-translational modification of the precursor peptide. The original 57-amino acid peptide is enzymatically cleaved and subsequently converted to the mature peptide during the posttranslational modification.

Class II bacteriocins are also small, heat-stable peptides. The Class II bacteriocins can be further divided into five subclasses (Table 1). Class IIa bacteriocins are 'pediocin-like' bacteriocins that exhibit antimicrobial activity against *Listeria* spp. as well as some foodborne pathogens. The Class IIa bacteriocins are synthesized as precursors containing an N-terminal leader sequence. The leader sequence is cleaved off by site-specific proteolytic activity during the maturation [57]. The mature peptide of Class IIa bacteriocins ranges in length from 25 to 58 amino acids for mutacin F-59.1 [86] and acidocin A [71]. The mature peptides of Class IIa

bacteriocins are linear peptides with a consensus sequence YGNGVXaaC at the N-terminal. However, the C-terminal region of Class IIa bacteriocins is less conserved, which subdivide this group of bacteriocins into 4 subclasses according to the sequence alignments at the C-terminal region [10,41].

Class IIb bacteriocins are linear, two-component bacteriocins that consist of two different peptides that are translated by two separate genes in the same operon [89]. These bacteriocins require both the peptides in an equal amount for their antimicrobial activity. Normally, Class IIb bacteriocins are synthesized as pre-peptides that contain 15 to 30 amino acids residues with a double-glycine leader sequence at N-terminal region. The leader sequence interacts with dedicated ABC-transporter to transfer the mature bacteriocin peptides across the cell membrane to make them active.

Class IIc contains circular bacteriocins whose N-terminal region covalently links with the C-terminal part to form circular peptides. Class IIc bacteriocins are synthesized as a linear pre-peptide with varying length of leader sequence at N-terminal region. The leader sequence is removed by cleavage by an unidentified peptidase [77]. The cleaved linear peptide is then circularized by linking of N-terminal region to C-terminal region to form mature circular peptide. The peptide is exported from the cell by a dedicated ABC-transporter. The mature peptides contain 58 to 70 amino acid residues and exhibit broad antimicrobial activity against Gram-positive bacteria, including common foodborne pathogens, such as *Clostridium* and *Listeria* spp. [46,78,98].

Class IId bacteriocins are categorized as non-pediocin-like, one-peptide linear bacteriocins. In this class, there are a considerable number of bacteriocins that are uncommon and fail in classifying. The characteristics of this class of bacteriocins are rather broad and

variable as compared to that of the bacteriocins in the other Class II subclass [62]. Thus, Class IId bacteriocins are further divided into 3 subgroups according to Iwatani et al. [62], 1) *sec*-dependent bacteriocins, 2) leaderless bacteriocins, and 3) nonsubgrouped bacteriocins.

Class IIe bacteriocins are linear, multi-component bacteriocins that consist of more than two different peptides that are translated by genes present in the same operon [85]. To date, only one bacteriocin produced by *S. aureus* A70 has been isolated from commercial milk [85]. This bacteriocin has four peptides that are small (approximately 30-31 amino acid residues for each peptides), strongly cationic, and highly hydrophobic. The bacteriocin has broad inhibition spectrum against Gram-positive bacteria including *L. monocytogenes* and *S. aureus*. Garvicin KS, a new bacteriocin produced by *L. garvieae* KS1546, consists of three peptides, which are required in equal amounts for the activity of the bacteriocin [92]. Garvicin KS has broad inhibition spectrum, against many distantly related genera of Firmicutes, problematic species of *Listeria*, *Staphylococcus*, and *Acinetobacter*, as well as their antibiotic resistant derivatives.

Class III bacteriocins are large (>30kDa) and heat-labile proteins. This class of bacteriocins can also be divided into 2 groups. Type IIIa bacteriocins are bacteriolysins. They are the bacteriolytic enzymes, such as lysostaphin [97] and Enterolisin A [87], that kill the sensitive strains by lysing their cell walls, Type IIIb bacteriocins are lytic proteins such as Caseicin 80 [82] and Helveticin J [65]

Mode of action

In general, several genetic elements are required to make bacteriocins functionally active. These genes include a structural gene encoding a precursor, an immunity gene encoding an immunity protein, a gene encoding a transporter (such as ATP-binding cassette transporter, referred to as ABC-transporter) and a gene encoding an accessory protein for extracellular

translocation of bacteriocin [28,84]. It was previously predicted that genes present in one or two operons are required to produce a functional bacteriocin [84]. Nowadays, the whole genome sequencing method and other considerable gene expression and structural studies are used to elucidate the regulatory mechanisms involved in the bacteriocin production [46,47,108].

The structural gene of LAB-derived bacteriocins

Bacteriocins are synthesized either as inactive pre-peptides (precursors) that contain an N-terminal leader sequence that is cleaved off during the maturation or as leaderless peptides that do not require post-translational processing for activity. Most of the bacteriocins from Class II have the structural genes that encode for the pre-peptides with leader sequence at the N-terminal region. The leader sequence presumably maintains the bacteriocins in an inactive form within the cell, and facilitates their interaction with the transporter. The pre-peptide is eventually cleaved at different spots during the maturation.

Immunity protein protects bacteriocin producer

Bacteriocin-producing strains are protected from their own products through immunity systems. The immunity systems are expressed concomitantly with the bacteriocins in the same operons. Normally, the immunity genes are identified next to or downstream of the bacteriocin structural genes [84]. The immunity proteins are relatively small, ranging from 51 to approximately 150 amino acid residues. The homology between the bacteriocins and their immunity proteins is quite low, indicating that no direct interaction occurs between them [40,66]. The mechanisms of action of immunity proteins for most of the bacteriocins are still poorly understood. Few cases demonstrated that the immunity proteins either locate on the membrane surface [29] or embed in the membrane [21]. These proteins are trapped in the membrane because of their size and block the insertion of bacteriocin into the membrane to protect the

producer cells from their bacteriocins. Diep *et al*. [27] demonstrated that the immunity protein (LciA) for lactococcin A and Class IIa bacteriocins binds the bacteriocin to intervene the penetration. Kjos *et al*. [74] proposed another immunity mechanism of Class II bacteriocins stating that membrane-bound proteins might act as mediators, either by depredating the bacteriocin receptor or by modifying them. In cases of lantibiotics and aureocin A53, immunity proteins are responsible for binding the bacteriocin on the membranes and recruiting ABC-transporters to remove the bacteriocin from the cell [1].

Transport of bacteriocin from the cell

Bacteriocin is exported from the cell by the transporter systems or the secretory pathway [57]. The transporter system typically consists of an ABC transporter protein and an accessory protein [57]. The N-terminal of ABC-transporter is hydrophobic and integrated into the cell membrane, while the C-terminal has ATP-binding region. Normally, the ABC-transporter recognizes the immature bacteriocin (with a leader peptide), and once the leader peptide is removed by a proteinase, it translocates the mature peptide across the membrane in an ATP-dependent manner.

An accessory protein is also required for the production of extracellular bacteriocin. The first accessory protein LcnD was identified by Stoddard *et al*. [103]. LcnD shares homology with other proteins involved in the ATP-dependent translocation processes. Therefore, the protein was considered as an accessory protein needed in the ABC-transporter-dependent translocation process. The topological studies on LcnD have predicted that the N-terminus of LcnD is intracellularly located; one transmembrane helix spans the cytoplasmic membrane and the C-terminus projects outside the cell [44,84]. Another similar study has reported that the accessory

protein for pediocin PA-1 is required for the externalization of the bacteriocin [110]. However, the special roles of the accessory proteins in the translocation process have yet not been resolved.

Identification of receptors of LAB-derived bacteriocins

It is generally recognized that bacteriocins from LAB kill the target cells by forming pores in their membrane [16,32]. The formation of pores in the membrane causes the leakage of low weight molecules, leading to the dissipation of the proton motive force [16]. There has been a long-term controversial discussion that the pore formation by bacteriocins might occur without recognizing the specific receptor. However, later studies showed that bacteriocins were active against the protein-containing cellular membranes but not against the protein-free ones, leading to the hypothesis that bacteriocins might employ the proteins to confer the sensitivity [20,107]. So far, few receptors have been identified and characterized for (A) class IIa (Pediocin PA-1) and some class IId bacteriocins (Lcn A) (involving Man-PTS system subunits) [27]; (B) class I lantibiotics, (like nisin and lactococcin 972) involving lipid II and related peptidoglycan precursors [55]; (C) the class IIc bacteriocin, garvicin ML (involving the maltose ABC-transporter) [45]; (D) the class IId bacteriocin, LsbB (involving a Zn-dependent metallopeptidase) [106] and (E) Class IIb bacteriocins LcnG (involving UppP) [73]. Lipid II is an essential precursor for bacterial cell wall biosynthesis. It links to the peptidoglycan through the pyrophosphate bridge. Lantibiotics, like nisin, bind to lipid II using the ring-structured region in the N-terminal part of the peptide, and form the pore in the cell membrane [55,61]. The receptor identification was not established until 2007 when Diep et al. [27] found that mannose PTS system acts as a receptor for pediocin-like bacteriocin (Class IIa bacteriocin) and lactococcin A and lactococcin B (Class IId bacteriocin). The identification of mannose PTS system as a receptor shed light on the mode of action of bacteriocins produced by LAB. The related studies

by Gabrielsen et al. [45] observed that garvicin ML-resistant *L. lactis* IL1403 were unable to grow on maltose or starch. The mutants generated by deleting the gene were resistant to garvicin ML. However, the complementation with maltose ABC-transporter conferred the sensitivity to garvicin ML. Both data indicated that maltose ABC-transporter is responsible for the sensitivity of *L. lactis* IL1403 to garvicin ML. In case of a Zn-dependent metallopeptidase was found to be responsible for the sensitivity to LsbB (Class IId bacteriocin) [106]. Additionally, Kjos *et al*. identified the receptor for LcnG (Class IIb bacteriocin) using genome sequencing of bacteriocin resistant mutants and wild type [73], which for the first time, extended our knowledge of bacteriocin receptors identification. The genome sequencing approach provided us with a new mean to identify the receptor, such as for the two-peptide bacteriocin plantaricin JK [91].

Applications and future perspectives

Food preservation

The bacteriocins from LAB are widely used as bio-preservatives, because of their low/zero toxicity and physical stability for eliminating or controlling pathogens and spoilage microorganisms in food [81]. Nisin, the first commercially used bacteriocin approved by FDA in 1988, has many potential applications in the food industry. In some homemade cheeses, nisin produced by *L. lactics* resulted in inhibitory activity against *Listeria* and *staphylococcus* spp. [25,76]. The isolation and identification of nisin and other bacteriocin producers in dairy products indicate that they might participate in certain fermentation processes as the starter culture. Some purified (or partially purified) bacteriocins are added to the ready-to-eat food as their antimicrobial capacity is of great interest for controlling the unwanted pathogens and for extending the shelf life. Neetoo *et al*. [83] used different concentrations of nisin on smoked salmon to inhibit the *Listeria monocytogenes*, highlighting the potential of nisin for enhancing

the safety by controlling the unwanted microorganisms. The Pediocin PA-1, isolated from fermented pork sausage, also has the potential to control *Listeria monocytogenes* without affecting the quality of the fermented food [72]. Some other studies also showed that the incorporation of different bacteriocins resulted in significant reduction in the number of pathogenic bacteria, like *Listeria monocytogenes* [113].

Clinical application

Because of the activities of bacteriocins against pathogenic bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) strains, Shiga toxin-producing *E. coli* [101] and Enter-toxigenic *E. coli* (ETEC), the clinical potential of bacteriocins has been increasingly investigated [23,49,90]. Jordi et al. [68] found around 20 different *E. coli* that could produce colicin, and used the bacteriocin produced from five different *E. coli* to inhibit ETEC. Similar results were found with purified colicin that could strongly inhibit ETEC *in vitro* [100]. The reduction in pathogens indicated that purified bacteriocins could be used with other dietary intakes for animal feeding. Some studies used nisin for feeding the broiler chickens and found a significant reduction in number of *Enterobacteriaceae* in nisin-supplemented chickens [69]. Stern et al. [102] used purified bacteriocin OR-7 treated in different ways (treated with lysozyme or lipase, heated at 90 ℃, and exposed to pH ranging from 3.0 to 9.1) against human gastroenteritis pathogens.

Bacteriocins produced by *Lactococcus garvieae*

Lactococcus garvieae is a gram-positive, facultative anaerobic, non-spore forming coccus [109]. Similar to other LAB, *L. garvieae* produces lactic acid as the final product of fermentation. It is therefore tolerant to acidic and high pH (9.6). It can grow at different ranges of temperature (from 4 to 45 °C) and in medium with 6.5% NaCl [109]. Initially, *L. garvieae* was considered as a pathogenic agent for fish but it has also been isolated from infected cows and buffalos [34,104]. This bacterium has been found in faecal samples from healthy humans, indicating that this bacterium might dwell or transfer in the gastrointestinal tract of humans [18]. However, the virulence of *L. garvieae* to humans is relatively low and the role of *L. garvieae* as a causative agent of human infection has not been reported. Interestingly, various *L. garvieae* strains have been isolated from dairy products, suggesting that *L. garvieae* might participate into the certain fermentation processes [43]. Taken together, *L. garvieae* might yet prove to be more of an opportunistic bacterium rather than a pathogenic bacterium for humans [39,95].

Bacteriocins produced by *L. garvieae* have not been completely identified. To our knowledge, only five bacteriocins are known from *L. garvieae*. These are garvicin A, Q, L1-5, ML and LG34, produced by strains isolated from different ecological sources: garvicin A from a clinical source [75], garvicin Q from fermented pork sausage [105], garvicin L1-5 from bovine milk [111], garvicin ML from the intestine of Mallard duck [13] and garvicin LG34 from the Chinese traditional fermented cucumber [50]. The first bacteriocin reported from *L. garvieae* was garvicin L1-5. Garvicin L1-5, isolated form cow milk, is a heat stable bacteiroicn with a low molecular weight (2.5 kDa). Its nature is still unknown; nevertheless, its inhibition spectrum consists of mostly closely related species and some strains of Listeria and Clostridium but not Pediococcus. Garvicin Q is a Class IId bacteriocin. It is synthesized as a 70 amino acid pre-

peptide without modified residues. The pre-peptide is cleaved off at a double glycine residue in the leader sequence to form a mature peptide with 50 amino acid residues. Garvicin Q has a broad antimicrobial spectrum mostly against strains of *L. garvieae* and closely related species. Garvicin A is also a Class IId bacteriocin that contains a leader sequence to that of garvicin Q. This bacteriocin is cleaved at double glycine residue in the leader to form the mature peptide. However, garvicin A has relatively narrow antimicrobial spectrum [75]. Garvicin ML is a circular bacteriocin and belongs to Class IIc. Garvicin ML is synthesized as a 63-amino acid pre-peptide. During the processing, the amino acids between Asp^3 and Leu^4 from the N-terminal region are circularized with Ala^{63} at the C-terminal region to form the mature circular 60 amino acid peptide [13]. Garvicin ML has apparently wider inhibition spectrum than the aforementioned bacteriocins. Like other circular bacterioins, it shows strong antimicrobial activity against those most closely related to *L. garvieae* and other lactococci. It also shows high activity against different strains, including *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Listeria*, and *Clostridium*. Most recently identified bacteriocin produced by *L. garvieae* is garvicin LG34 [50]. This bacteriocin belongs to Class IIa group and possesses a broad inhibition spectrum not only against Gram-positive bacteria, but also shows promisingly against Gram-negative bacteria, like *Salmonella typhimurium*, *E. coli* and *Shigella flexneri* [50].

2. AIMS OF THE STUDY

The antibiotic resistance has become a serious issue throughout the world. Therefore, alternatives of antibiotics are highly demanded. Bacteriocins are considered as promising antimicrobial agents for replacing the antibiotics, owing to their broad antimicrobial inhibition spectra and safe nature. However, only a few bacteriocins (nisin and pediocin PA-1) are commercially used as the knowledge of the mode of action of bacteriocins is limited. In addition,

the Gram-positive bacteria found in various foods show resistance to nisin, hence compromising its activity [12,22,79]. Therefore, searching for new bacteriocin(s) with a broad antimicrobial inhibition spectrum is essential. Meanwhile, to understand the mode of action of the new bacteriocin(s) and to apply the bacteriocin(s) for clinical/food-related applications is important.

In this current study, our aims are to:

1) Screening for the new bacteriocin(s) with broad antimicrobial inhibition spectrum (Paper I);

2) Purification and characterization of the new bacteriocin(s) (Paper II);

3) Understanding the mode of action of the new bacteriocin(s) against target cells (Paper III);

4) Study of potential applications of the new bacteriocin(s) that act synergistically with other antimicrobial agents (Paper IV).

3. MAIN RESULTS

Paper I:

A novel antimicrobial peptide produced by *Lactococcus garvieae* with a broad inhibition spectrum

Hai Chi, Ibrahim Mehmeti, Kirill Ovchinnikov, Hegle Holo, Ingolf F. Nes, Dzung B. Diep.

In this paper, we performed a systematic analysis of the microbial quality of raw bovine milk from many different farms in Kosovo and isolated a large collection (over 1800 isolates) of LAB. We used this collection to screen for strains that produce bacteriocins with broad inhibition spectra for potential antimicrobial applications. From this collection, fourteen isolates showed bacteriocin-like activity. Using 16 rDNA sequencing, these fourteen isolates were found to belong to *Lactococcus garvieae* (10 isolates) and *Enterococcus faecalis* (4 isolates) with 100% and 99% similarity, respectively. Further analysis revealed that all ten *L. garvieae* isolates were very similar, if not identical, to each other in metabolic and genetic features as they had the same fermentation profiles on different types of sugars, similar repetitive sequence-based PCR (rep-PCR) DNA pattern and the same inhibition profile towards over fifty isolates of different species. One of the ten isolates of *L. garvieae* therefore was used for further analysis.

The bacteriocin which was termed garvicin KS, was found to be heat-stable and proteinase-labile and its inhibition spectrum included many distantly related genera of Firmicutes, comprising mostly LAB as well as problematic species of *Bacillus*, *Listeria*, and *Staphylococcus* and their antibiotic resistant derivatives (e.g. VRE, MRSA). Taken together, the results indicate that this is a potent bacteriocin from *L. garvieae* and its broad inhibition spectrum can be a very useful property for use in food preservation as well as in the treatment of infections caused by Gram-positive pathogens and their antibiotic-resistant derivatives.

Paper II:

Novel Group of Leaderless Multi peptide Bacteriocins from Gram-Positive Bacteria

Kirill V. Ovchinnikov, Hai Chi, Ibrahim Mehmeti, Helge Holo, Ingolf F. Nes, Dzung B. Diep.

In this paper, we purified a new bacteriocin produced by *Lactococcus garvieae* KS1546 by a classic procedure involving ion exchange and reverse phase chromatography. Based on the whole genome sequencing, the bacteriocin was found to be composed of three similar leaderless peptides of 32 to 34 amino acids. The structural genes followed with genes involved in ABC-transporter (bacteriocin transporter) and immunity are located in an operon-like structure. Moreover, the bacteriocin demonstrated sequence homology to a four-peptide bacteriocin produced by *Staphylococcus aureus* A70, and a few unannotated putative multi-peptide bacteriocins, found in public database, produced by *Bacillus cereus*. All these multi-peptide bacteriocin loci showed conserved genetic organization, including being located adjacent to conserved genetic determinants (Cro/cI and integrase) that are normally associated with mobile genetic elements or genomic rearrangements. The antimicrobial activity of all multi-peptide bacteriocins was confirmed using synthetic peptides, and all the multi-peptide bacteriocins require an equal amount of each peptide for their activity.

The newly-identified bacteriocin produced by *L. garvieae* has broad antibacterial spectrum. Thus, it shows great potential for antimicrobial application in the food industry and medical purpose.

Paper III:

Phage shock protein C of Lactococcus lactis is receptor for multiple peptide bacteriocins

Hai Chi,Cathrine A. Bøe, Camilla Oppegård, Tom Kristensen, Beatriz Martínez, Dzung B. Diep, Helge Holo.

Bacteriocins from LAB have great potentials in diverse antimicrobial applications, such as natural food preservatives, and antimicrobials in infection therapy. However, poor understanding of the mode of action of various bacteriocins impedes their developments and applications. To gain a better understanding of the broad spectrum bacteriocins, we generated garvicin KS resistant mutants of *Lactococcus lactis* IL1403. Thirteen spontaneous resistant mutants were isolated and sequenced. Notably, all the mutants showed either a point of a truncated mutation in the same gene, encoding phage shock protein C (PspC), a stress response protein. The *pspC* completely knock-out strain of *L. lactis* showed increased resistance. Additionally, complementation with *pspC* conferred the sensitivity of mutants to garvicin KS. Interestingly, the mutants also lost sensitivity to LcnG a two peptides bacteriocin that uses UppP for its activity, and cloning *pspC* in *Lactobacillus sakei* resulted in sensitivity to LcnG as well as an increase in garvicin KS sensitivity.

In order to study whether garvicin KS, LcnG and other bacteriocins use the same target, we conducted the binding assay of bacteriocins to cells. The results showed that the garvicin KS resistant mutants bound less garvicin KS and Lcn G than the wild type, and binding to nisin showed no difference between wild type and mutants, but no binding was observed when PspC was pulled down by immunoprecipitation. In addition, binding to both bacteriocins, as complete bacteriocin or in the form of individual subunits, was checked by immunoprecipitation, demonstrating that PspC could act as a receptor for them. The role of PspC appears to capture the

multi-peptides bacteriocins at low concentrations, and possibly to facilitate the interactions between the individual peptides, and likely to transfer the bacteriocin to its true target.

Paper IV:

Synergistic antimicrobial activity between the broad spectrum bacteriocin Garvicin KS and nisin, farnesol and polymyxin B against Gram-positive and Gram-negative bacteria

Hai Chi and *Helge Holo*.

The increasing spread of multidrug-resistant Gram-positive and Gram-negative bacteria has become a global concern, and the occurrence of the multidrug resistant bugs threatens human health because of the lack of progress of development of new drugs. Alternatives are urgently required to combat the multidrug resistant bacteria. The bacteriocins are considered as one of the alternative strategies to fight the bacteria. In addition, use of different antimicrobial agents in combination offers a potential for increasing the efficacy of antimicrobial treatment and for reducing the evolution of multi-drug resistance. In addition, combination antimicrobial therapy is widely used in the treatment of serious infections.

In this paper, we described the potentials of a new bacteriocin (garvicin KS) to increase the efficacy of other antimicrobial agents (such as nisin, polymyxin B and farnesol), by particularly focusing on the application of preventing important clinical pathogens (such as *S. aureus*, *E. coli* and *A. baumannii*). The results showed that garvicin KS was successful in combination with other antimicrobial compounds and it improved the killing kinetics and eradicated all the sensitive bacteria tested. The minimum inhibition concentration (MIC) determinations and time-kill analysis demonstrated that the antimicrobial activity of garvicin KS substantially increased by combining it with selected antimicrobial agents. The combination of garvicin KS with nisin and polymyxin B could effectively be against *A. baumannii* and *E. coli* with rapid killing of the organisms. In addition, rapid killing of *S. aureus* was also observed by combining garvicin KS, nisin and farnesol. The combination of garvicin KS with nisin and

farnesol proved to be highly efficient by improving killing kinetics and eradication, as well as by lowering the rate of resistance development. Importantly, the data proposed the idea that garvicin KS in combination with other antimicrobial agents could be one of the alternative strategies to combat the clinical pathogens. Such combinational therapy may yield added benefits by reducing toxicity through the administration of significantly lower levels of some antimicrobial agents.

4. DISCUSSION

Screening and characterization of garvicin KS

Traditionally, most of the known bacteriocin producers are identified by searching for antimicrobial activity against chosen indicators. The time-consuming screening is carried out normally against a panel of bacteria from different genera and species. However, such screening assays often lead to the identification of narrow-spectrum bacteriocins because these are relatively more common in nature. In our study, we employed a different approach to search for broad-inhibition bacteriocin producers, by employing five indicators from distantly related genera for the first round of screening. Subsequently, four well-known bacteriocin producers that are frequently known in dairy products were used for our second round of screening owing to their self-immunity protection system. Eventually, the number of potential bacteriocin producer(s) was drastically reduced from 107 to 14. Amongst the fourteen potential candidates, ten were identified as *L. garvieae* with 100% similarity while the remaining four as *E. faecalis* with 99% similarity. It appeared that the ten *L. garvieae* isolates were genetically and phenotypically very similar, if not identical, as they shared the same profiles when analyzed based on their rep-PCR, bacteriocin inhibition spectrum and sugar fermentation. It is reasonable to believe that these ten isolates are of the same clone because of their genetic- and phenotypic-likeness.

One out of the ten isolates (*L. garvieae* KS 1546, termed as garvicin KS) appears to produce a novel bacteriocin producer with a wide inhibition spectrum, against many important problematic bacteria of genera *Listeria*, *Staphylococcus*, *Streptococcus* and *Enterococcus*. The broadness of inhibition spectrum was comparable to that of nisin that has been approved by FAO/WHO for use as a food preservative in many countries. Like nisin, garvicin KS was also capable of killing antibiotic-resistant bacteria, such as *L. monocytogenes*, MRSA and VRE which

are common problematic bacteria in dairy and/or hospital environments. Unlike nisin and other bacteriocin with broad inhibition spectra, garvicin KS could kill *Acinetobacter* spp., although relative high amount of garvicin KS was required. The broad inhibition spectrum of garvicin KS is of great interests to control many pathogens, which encouraged us to purify this bacteriocin.

The purification of garvicin KS was carried out using a classic procedure involving cation exchange and reverse phase chromatography. Based on the whole genome sequencing, the bacteriocin was confirmed to be composed of three similar leaderless peptides of 32 to 34 amino acids. To our knowledge, only five bacteriocins are known from *L. garvieae*. These are garvicin A, Q, L1-5, ML and LG34, produced by strains isolated from different ecological sources: garvicin A from a clinical source [75], garvicin Q from fermented pork sausage [105], garvicin L1-5 from bovine milk [111], garvicin ML from the intestine of Mallard duck [13], and garvicin LG34 from Chinese traditional fermented cucumber [50]. The difference of amino acid sequence of garvicin KS from other bacteriocins produced by *L. garvieae* showed that garvicin KS is a new bacteriocin. According to the information available in NCBI, garvicin KS showed similar homology to A70, a four-peptide bacteriocin produced by *S. aureus*, and *B. cereus*. Both bacteriocins have similar features, for example, they consist of 3 or 4 leaderless peptides, and require an equal amount of each peptide for their antimicrobial activities.

Mutiple roles of PspC

The Psp proteins in both Gram-positive and Gram-negative bacteria have similar functions, and are involved in the response to extra-cytoplasmic stress and protection of the cells probably by maintaining the integrity of the cytoplasmic membrane [42]. Nevertheless, the compositions of Psp proteins differ between Gram-positive bacteria and Gram-negative bacteria. In *L. lactis strains*, PspC is not only required to sense the stress signals, it also provides

resistance to cell-wall targeting antimicrobial agents, and eventually protects the cells. Some studies reported that PspC protein is able to sense the stress and can increase the resistance to lysozyme and nisin directly or indirectly via interactions with SpxB [38,67,94]. Thus, PspC in *L. lactis* IL1403 is likely to play a key role in genetic reprogramming directed to restore a physiological proton motive force and to adjust energy utilization to favor the maintenance of steady states [38].

Our results clearly demonstrate the importance of PspC in conferring garvicin KS sensitivity to *L. lactis*. Surprisingly, PspC is a target not only for garvicin KS but also for LcnG that shows no apparent sequence similarity to garvicin KS. However, the UppP mutants showed similar sensitivity to garvicin KS and similar bacteriocin binding characteristics as *L. lactis* IL1403, while the PspC mutants showed reduced sensitivity and binding to both the bacteriocins. In addition, both bacteriocins, individually and in combination, showed different binding to bacteriocins, indicating that PspC could act as a magnet and facilitate the interaction between bacteriocin subunits.

Garvicin KS acts synergistically with other antimicrobials against pathogens

Combining of antimicrobials offers a potential for increasing treatment efficacy and for reducing resistance evolution [70], and combinatorial antibiotic therapy is widely used in the treatment of serious infections. In our study, garvicin KS was successful in combination with other antimicrobial compounds and showed improved killing kinetics, resulting in eradication of all the sensitive bacteria tested. The results from MIC determinations and time-kill analysis demonstrated that the antimicrobial activities were substantially increased by combining garvicin KS with selected antimicrobial agents, such as polymyxin B and farnesol. The potential benefits associated with combining the antimicrobial agents with new bacteriocins, like garvicin KS that

act synergistically, are obvious. Importantly, the amount of polymyxin B needed to eradicate the bacteria was significantly reduced beyond the recommended dosage for intravenous treatment when it was used in combination with garvicin KS and nisin [37].

Strong synergy between nisin and garvicin KS was also observed in *S. aureus*. This synergy indicates that garvicin KS and nisin have different mode of actions as compared to the classical antibiotics. Moreover, the mixture of farnesol, nisin and garvicin KS completely killed all the *S. aureus* tested. In the presence of polymyxin B, nisin and garvicin KS also showed synergistic effects. In *E. coli* we did not see the synergy between polymyxin B and garvicin KS except in the presence of nisin, and only the combination of polymyxin B, nisin and garvicin KS could fully eradicate the *E. coli*. However, synergy unfortunately was not found with garvicin KS in combination with other antimicrobial compounds against *P. aeruginosa*. This finding may because of less penetration of garvicin KS and nisin inside the cells, or difference in the structures of outer membrane of *P. aeruginosa* from other Gram-negative strains tested.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The present work describes a novel three-peptides-leaderless bacteriocin, garvicin KS that has a broad inhibition spectrum. Its receptor was identified, and its synergistic potential in combination with other antimicrobial agents against pathogens including Gram-positive and Gram-negative bacteria was studied. The findings in this work have brought a new insight into the field of antimicrobial therapy. Moreover, the analysis of the mode of action of garvicin KS suggested a new strategy for antimicrobial therapy. Importantly, the synergistic potential of garvicin KS with other antimicrobial agents is also of great interests for applications in the medicine and food industries.

Several aspects, however, still need to be addressed. One of the important tasks is to identify the true target of garvicin KS. It could be critical to dig deep into the whole genome sequence of both mutants and wild type. One approach to find out the true target might be by isolating the garvicin KS once it binds to its receptor, and then analyzing the structure and function of the garvicin KS receptor complex. In addition, garvicin KS has antimicrobial activity against Gram-negative, *Acinetobacter* strains. This interesting finding might lead to the understanding mechanism of cell killing employed by garvicin KS. At last, garvicin KS, in combination with farnesol, shows great potential against *S. aureus* and *A. baumannii in vitro*. Adding garvicin KS into perfume (containing a high amount of farnesol) and spraying it on the skin can provide additional protection against *S. aureus* and *A. baumannii*. The incorporation of garvicin KS into perfume might not only provide a cheap treatment method against skin infection caused by *S. aureus* and *A. baumannii*, but also provide insight into the future potential of all the bacteriocins.

6. REFERENCES

1. Acedo JZ, van Belkum MJ, Lohans CT, Towle KM, Miskolzie M, Vederas JC (2016) Nuclear Magnetic Resonance Solution Structures of Lacticin Q and Aureocin A53 Reveal a Structural Motif Conserved among Leaderless Bacteriocins with Broad-Spectrum Activity. Biochemistry 55 (4):733-742. doi:10.1021/acs.biochem.5b01306

2. Adelman K, Yuzenkova J, La Porta A, Zenkin N, Lee J, Lis JT, Borukhov S, Wang MD, Severinov K (2004) Molecular mechanism of transcription inhibition by peptide antibiotic Microcin J25. Mol Cell 14 (6):753-762. doi:10.1016/j.molcel.2004.05.017

3. Allen HK, Trachsel J, Looft T, Casey TA (2014) Finding alternatives to antibiotics. Ann N Y Acad Sci 1323:91-100. doi:10.1111/nyas.12468

4. Altena K, Guder A, Cramer C, Bierbaum G (2000) Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. Appl Environ Microbiol 66 (6):2565-2571

5. Bali V, Panesar PS, Bera MB, Kennedy JF (2016) Bacteriocins: Recent Trends and Potential Applications. Critical Reviews in Food Science and Nutrition 56 (5):817-834. doi:10.1080/10408398.2012.729231

6. Baquero F (1997) Gram-positive resistance: challenge for the development of new antibiotics. J Antimicrob Chemother 39 Suppl A:1-6

7. Baquero F, Moreno F (1984) The microcins. FEMS Microbiology Letters 23 (2-3):117-124. doi:10.1111/j.1574-6968.1984.tb01046.x

8. Barton MD, Hart WS (2001) Public Health Risks: Antibiotic Resistance - Review. Asian Australas J Anim Sci 14 (3):414-422. doi:10.5713/ajas.2001.414

9. Bastos Mdo C, Coelho ML, Santos OC (2015) Resistance to bacteriocins produced by Gram-positive bacteria. Microbiology 161 (Pt 4):683-700. doi:10.1099/mic.0.082289-0

10. Belguesmia Y, Naghmouchi K, Chihib N-E, Drider D (2011) Class IIa Bacteriocins: Current Knowledge and Perspectives. In: Drider D, Rebuffat S (eds) Prokaryotic Antimicrobial Peptides: From Genes to Applications. Springer New York, New York, NY, pp 171-195. doi:10.1007/978-1-4419-7692-5_10

11. Bierbaum G, Sahl HG (2009) Lantibiotics: mode of action, biosynthesis and bioengineering. Curr Pharm Biotechnol 10 (1):2-18

12. Blake KL, Randall CP, O'Neill AJ (2011) In vitro studies indicate a high resistance potential for the lantibiotic nisin in Staphylococcus aureus and define a genetic basis for nisin resistance. Antimicrob Agents Chemother 55 (5):2362-2368. doi:10.1128/aac.01077-10

13. Borrero J, Brede DA, Skaugen M, Diep DB, Herranz C, Nes IF, Cintas LM, Hernández PE (2011) Characterization of Garvicin ML, a Novel Circular Bacteriocin Produced by Lactococcus garvieae DCC43, Isolated from Mallard Ducks (Anas platyrhynchos). Applied and Environmental Microbiology 77 (1):369-373. doi:10.1128/aem.01173-10

14. Boucher HW, Corey GR (2008) Epidemiology of Methicillin-Resistant Staphylococcus aureus. Clinical Infectious Diseases 46 (Supplement 5):S344-S349. doi:10.1086/533590

15. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J (2009) Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. Clinical Infectious Diseases 48 (1):1-12. doi:10.1086/595011

16. Bruno ME, Montville TJ (1993) Common mechanistic action of bacteriocins from lactic Acid bacteria. Appl Environ Microbiol 59 (9):3003-3010

17. Buffenmyer CL, Rycheck RR, Yee RB (1976) Bacteriocin (klebocin) sensitivity typing of klebsiella. J Clin Microbiol 4 (3):239-244

18. Chan JF, Woo PC, Teng JL, Lau SK, Leung SS, Tam FC, Yuen KY (2011) Primary infective spondylodiscitis caused by Lactococcus garvieae and a review of human L. garvieae infections. Infection 39 (3):259-264. doi:10.1007/s15010-011-0094-8

19. Chavan MA, Riley MA (2007) Molecular Evolution of Bacteriocins in Gram-Negative Bacteria. In: Riley MA, Chavan MA (eds) Bacteriocins: Ecology and Evolution. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 19-43. doi:10.1007/978-3-540-36604-1_3
20. Chikindas ML, Garcia-Garcera MJ, Driessen AJ, Ledeboer AM, Nissen-Meyer J, Nes IF, Abee T, Konings WN, Venema G (1993) Pediocin PA-1, a bacteriocin from Pediococcus acidilactici PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Appl Environ Microbiol 59 (11):3577-3584
21. Coelho ML, Coutinho BG, Cabral da Silva Santos O, Nes IF, Bastos Mdo C (2014) Immunity to the Staphylococcus aureus leaderless four-peptide bacteriocin aureocin A70 is conferred by AurI, an integral membrane protein. Res Microbiol 165 (1):50-59. doi:10.1016/j.resmic.2013.11.001
22. Collins B, Curtis N, Cotter PD, Hill C, Ross RP (2010) The ABC transporter AnrAB contributes to the innate resistance of Listeria monocytogenes to nisin, bacitracin, and various beta-lactam antibiotics. Antimicrob Agents Chemother 54 (10):4416-4423. doi:10.1128/aac.00503-10
23. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3 (10):777-788. doi:10.1038/nrmicro1273
24. Cotter PD, Ross RP, Hill C (2013) Bacteriocins - a viable alternative to antibiotics? Nat Rev Microbiol 11 (2):95-105. doi:10.1038/nrmicro2937
25. Cui HY, Wu J, Li CZ, Lin L (2016) Anti-listeria effects of chitosan-coated nisin-silica liposome on Cheddar cheese. J Dairy Sci 99 (11):8598-8606. doi:10.3168/jds.2016-11658
26. de Graaf FK (1973) Effects of cloacin DF13 on the functioning of the cytoplasmic membrane. Antonie Van Leeuwenhoek 39 (1):109-119
27. Diep DB, Skaugen M, Salehian Z, Holo H, Nes IF (2007) Common mechanisms of target cell recognition and immunity for class II bacteriocins. Proc Natl Acad Sci U S A 104 (7):2384-2389. doi:10.1073/pnas.0608775104
28. Drider D, Fimland G, Hechard Y, McMullen LM, Prevost H (2006) The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 70 (2):564-582. doi:10.1128/mmbr.00016-05
29. Dubois JY, Kouwen TR, Schurich AK, Reis CR, Ensing HT, Trip EN, Zweers JC, van Dijl JM (2009) Immunity to the bacteriocin sublancin 168 Is determined by the SunI (YolF) protein of Bacillus subtilis. Antimicrob Agents Chemother 53 (2):651-661. doi:10.1128/aac.01189-08
30. Duquesne S, Destoumieux-Garzon D, Peduzzi J, Rebuffat S (2007) Microcins, gene-encoded antibacterial peptides from enterobacteria. Nat Prod Rep 24 (4):708-734. doi:10.1039/b516237h
31. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP (1999) Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis 29 (2):239-244. doi:10.1086/520192
32. Eijsink VGH, Axelsson L, Diep DB, Håvarstein LS, Holo H, Nes IF (2002) Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie van Leeuwenhoek 81 (1):639-654. doi:10.1023/a:1020582211262
33. Ennahar S, Sashihara T, Sonomoto K, Ishizaki A (2000) Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol Rev 24 (1):85-106
34. Eyngor M, Zlotkin A, Ghittino C, Prearo M, Douet DG, Chilmonczyk S, Eldar A (2004) Clonality and diversity of the fish pathogen Lactococcus garvieae in Mediterranean countries. Appl Environ Microbiol 70 (9):5132-5137. doi:10.1128/aem.70.9.5132-5137.2004
35. Falagas ME, Bliziotis IA (2007) Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? International Journal of Antimicrobial Agents 29 (6):630-636. doi:http://dx.doi.org/10.1016/j.ijantimicag.2006.12.012
36. Falagas ME, Bliziotis IA, Kasiakou SK, Samonis G, Athanassopoulou P, Michalopoulos A (2005) Outcome of infections due to pandrug-resistant (PDR) Gram-negative bacteria. BMC Infect Dis 5:24. doi:10.1186/1471-2334-5-24
37. Falagas ME, Kasiakou SK, Tsiodras S, Michalopoulos A (2006) The use of intravenous and aerosolized polymyxins for the treatment of infections in critically ill patients: a review of the recent literature. Clin Med Res 4 (2):138-146

38. Fallico V, Ross RP, Fitzgerald GF, McAuliffe O (2011) Genetic response to bacteriophage infection in Lactococcus lactis reveals a four-strand approach involving induction of membrane stress proteins, D-alanylation of the cell wall, maintenance of proton motive force, and energy conservation. J Virol 85 (22):12032-12042. doi:10.1128/jvi.00275-11
39. Fihman V, Raskine L, Barrou Z, Kiffel C, Riahi J, Bercot B, Sanson-Le Pors MJ (2006) Lactococcus garvieae endocarditis: identification by 16S rRNA and sodA sequence analysis. J Infect 52 (1):e3-6. doi:10.1016/j.jinf.2005.04.021
40. Fimland G, Eijsink VG, Nissen-Meyer J (2002) Comparative studies of immunity proteins of pediocin-like bacteriocins. Microbiology 148 (Pt 11):3661-3670. doi:10.1099/00221287-148-11-3661
41. Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J (2005) Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. J Pept Sci 11 (11):688-696. doi:10.1002/psc.699
42. Flores-Kim J, Darwin AJ (2016) The Phage Shock Protein Response. Annu Rev Microbiol 70:83-101. doi:10.1146/annurev-micro-102215-095359
43. Florez AB, Reimundo P, Delgado S, Fernandez E, Alegria A, Guijarro JA, Mayo B (2012) Genome sequence of Lactococcus garvieae IPLA 31405, a bacteriocin-producing, tetracycline-resistant strain isolated from a raw-milk cheese. J Bacteriol 194 (18):5118-5119. doi:10.1128/jb.00975-12
44. Franke CM, Leenhouts KJ, Haandrikman AJ, Kok J, Venema G, Venema K (1996) Topology of LcnD, a protein implicated in the transport of bacteriocins from Lactococcus lactis. J Bacteriol 178 (6):1766-1769
45. Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB (2012) The maltose ABC transporter in Lactococcus lactis facilitates high-level sensitivity to the circular bacteriocin garvicin ML. Antimicrob Agents Chemother 56 (6):2908-2915. doi:10.1128/aac.00314-12
46. Gabrielsen C, Brede DA, Nes IF, Diep DB (2014) Circular bacteriocins: biosynthesis and mode of action. Appl Environ Microbiol 80 (22):6854-6862. doi:10.1128/aem.02284-14
47. Gabrielsen C, Brede DA, Salehian Z, Nes IF, Diep DB (2014) Functional genetic analysis of the GarML gene cluster in Lactococcus garvieae DCC43 gives new insights into circular bacteriocin biosynthesis. J Bacteriol 196 (5):911-919. doi:10.1128/jb.01115-13
48. Gajic O, Buist G, Kojic M, Topisirovic L, Kuipers OP, Kok J (2003) Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal multidrug resistance proteins. J Biol Chem 278 (36):34291-34298. doi:10.1074/jbc.M211100200
49. Galvin M, Hill C, Ross RP (1999) Lacticin 3147 displays activity in buffer against gram-positive bacterial pathogens which appear insensitive in standard plate assays. Lett Appl Microbiol 28 (5):355-358
50. Gao Y, Li D, Liu S, Zhang L (2015) Garviecin LG34, a novel bacteriocin produced by Lactococcus garvieae isolated from traditional Chinese fermented cucumber. Food Control 50:896-900. doi:http://dx.doi.org/10.1016/j.foodcont.2014.10.040
51. Gaynes R, Edwards JR (2005) Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis 41 (6):848-854. doi:10.1086/432803
52. Giamarellou H (2010) Multidrug-resistant Gram-negative bacteria: how to treat and for how long. Int J Antimicrob Agents 36 Suppl 2:S50-54. doi:10.1016/j.ijantimicag.2010.11.014
53. Gillor O, Kirkup BC, Riley MA (2004) Colicins and Microcins: The Next Generation Antimicrobials. In: Advances in Applied Microbiology, vol Volume 54. Academic Press, pp 129-146. doi:http://dx.doi.org/10.1016/S0065-2164(04)54005-4
54. Gordon DM, Oliver E, Littlefield-Wyer J (2007) The Diversity of Bacteriocins in Gram-Negative Bacteria. In: Riley MA, Chavan MA (eds) Bacteriocins: Ecology and Evolution. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 5-18. doi:10.1007/978-3-540-36604-1_2
55. Hasper HE, Kramer NE, Smith JL, Hillman JD, Zachariah C, Kuipers OP, de Kruijff B, Breukink E (2006) An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science 313 (5793):1636-1637. doi:10.1126/science.1129818
56. Hauser AR, Mecsas J, Moir DT (2016) Beyond Antibiotics: New Therapeutic Approaches for Bacterial Infections. Clin Infect Dis 63 (1):89-95. doi:10.1093/cid/ciw200

57. Havarstein LS, Diep DB, Nes IF (1995) A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol Microbiol 16 (2):229-240
58. Heddle JG, Blance SJ, Zamble DB, Hollfelder F, Miller DA, Wentzell LM, Walsh CT, Maxwell A (2001) The antibiotic microcin B17 is a DNA gyrase poison: characterisation of the mode of inhibition. J Mol Biol 307 (5):1223-1234. doi:10.1006/jmbi.2001.4562
59. Henderson JT, Chopko AL, van Wassenaar PD (1992) Purification and primary structure of pediocin PA-1 produced by Pediococcus acidilactici PAC-1.0. Arch Biochem Biophys 295 (1):5-12
60. Holo H, Nilssen O, Nes IF (1991) Lactococcin A, a new bacteriocin from lactococcus lactis subsp. cremoris: isolation and characterization of the protein and its gene. J Bacteriol 173 (12):3879-3887
61. Hsu ST, Breukink E, Tischenko E, Lutters MA, de Kruijff B, Kaptein R, Bonvin AM, van Nuland NA (2004) The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nat Struct Mol Biol 11 (10):963-967. doi:10.1038/nsmb830
62. Iwatani S, Zendo T, Sonomoto K (2011) Class IId or Linear and Non-Pediocin-Like Bacteriocins. In: Drider D, Rebuffat S (eds) Prokaryotic Antimicrobial Peptides: From Genes to Applications. Springer New York, New York, NY, pp 237-252. doi:10.1007/978-1-4419-7692-5_13
63. Izaguirre G, Hansen JN (1997) Use of alkaline phosphatase as a reporter polypeptide to study the role of the subtilin leader segment and the SpaT transporter in the posttranslational modifications and secretion of subtilin in Bacillus subtilis 168. Appl Environ Microbiol 63 (10):3965-3971
64. Jack RW, Tagg JR, Ray B (1995) Bacteriocins of gram-positive bacteria. Microbiological Reviews 59 (2):171-200
65. Joerger MC, Klaenhammer TR (1986) Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by Lactobacillus helveticus 481. J Bacteriol 167 (2):439-446
66. Johnsen L, Fimland G, Mantzilas D, Nissen-Meyer J (2004) Structure-function analysis of immunity proteins of pediocin-like bacteriocins: C-terminal parts of immunity proteins are involved in specific recognition of cognate bacteriocins. Appl Environ Microbiol 70 (5):2647-2652
67. Jordan S, Hutchings MI, Mascher T (2008) Cell envelope stress response in Gram-positive bacteria. FEMS Microbiology Reviews 32 (1):107-146. doi:10.1111/j.1574-6976.2007.00091.x
68. Jordi BJ, Boutaga K, van Heeswijk CM, van Knapen F, Lipman LJ (2001) Sensitivity of Shiga toxin-producing Escherichia coli (STEC) strains for colicins under different experimental conditions. FEMS Microbiol Lett 204 (2):329-334
69. Jozefiak D, Kieronczyk B, Juskiewicz J, Zdunczyk Z, Rawski M, Dlugosz J, Sip A, Hojberg O (2013) Dietary nisin modulates the gastrointestinal microbial ecology and enhances growth performance of the broiler chickens. PLoS One 8 (12):e85347. doi:10.1371/journal.pone.0085347
70. Jumbe N, Louie A, Leary R, Liu W, Deziel MR, Tam VH, Bachhawat R, Freeman C, Kahn JB, Bush K, Dudley MN, Miller MH, Drusano GL (2003) Application of a mathematical model to prevent in vivo amplification of antibiotic-resistant bacterial populations during therapy. J Clin Invest 112 (2):275-285. doi:10.1172/jci16814
71. Kanatani K, Oshimura M, Sano K (1995) Isolation and characterization of acidocin A and cloning of the bacteriocin gene from Lactobacillus acidophilus. Appl Environ Microbiol 61 (3):1061-1067
72. Kingcha Y, Tosukhowong A, Zendo T, Roytrakul S, Luxananil P, Chareonpornsook K, Valyasevi R, Sonomoto K, Visessanguan W (2012) Anti-listeria activity of Pediococcus pentosaceus BCC 3772 and application as starter culture for Nham, a traditional fermented pork sausage. Food Control 25 (1):190-196. doi:http://dx.doi.org/10.1016/j.foodcont.2011.10.005
73. Kjos M, Oppegard C, Diep DB, Nes IF, Veening JW, Nissen-Meyer J, Kristensen T (2014) Sensitivity to the two-peptide bacteriocin lactococcin G is dependent on UppP, an enzyme involved in cell-wall synthesis. Mol Microbiol 92 (6):1177-1187. doi:10.1111/mmi.12632
74. Kjos M, Snipen L, Salehian Z, Nes IF, Diep DB (2010) The Abi Proteins and Their Involvement in Bacteriocin Self-Immunity. Journal of Bacteriology 192 (8):2068-2076. doi:10.1128/jb.01553-09
75. Maldonado-Barragan A, Cardenas N, Martinez B, Ruiz-Barba JL, Fernandez-Garayzabal JF, Rodriguez JM, Gibello A (2013) Garvicin A, a novel class IId bacteriocin from Lactococcus garvieae that

inhibits septum formation in L. garvieae strains. Appl Environ Microbiol 79 (14):4336-4346. doi:10.1128/aem.00830-13
76. Malheiros Pda S, Sant'Anna V, Barbosa Mde S, Brandelli A, Franco BD (2012) Effect of liposome-encapsulated nisin and bacteriocin-like substance P34 on Listeria monocytogenes growth in Minas frescal cheese. Int J Food Microbiol 156 (3):272-277. doi:10.1016/j.ijfoodmicro.2012.04.004
77. Martinez-Bueno M, Valdivia E, Galvez A, Coyette J, Maqueda M (1998) Analysis of the gene cluster involved in production and immunity of the peptide antibiotic AS-48 in Enterococcus faecalis. Mol Microbiol 27 (2):347-358
78. Masuda Y, Ono H, Kitagawa H, Ito H, Mu F, Sawa N, Zendo T, Sonomoto K (2011) Identification and characterization of leucocyclicin Q, a novel cyclic bacteriocin produced by Leuconostoc mesenteroides TK41401. Appl Environ Microbiol 77 (22):8164-8170. doi:10.1128/aem.06348-11
79. Mazzotta AS, Crandall AD, Montville TJ (1997) Nisin Resistance in Clostridium botulinum Spores and Vegetative Cells. Appl Environ Microbiol 63 (7):2654-2659
80. McAuliffe O, Ross RP, Hill C (2001) Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev 25 (3):285-308
81. Morgan SM, O'Connor P M, Cotter PD, Ross RP, Hill C (2005) Sequential actions of the two component peptides of the lantibiotic lacticin 3147 explain its antimicrobial activity at nanomolar concentrations. Antimicrob Agents Chemother 49 (7):2606-2611. doi:10.1128/aac.49.7.2606-2611.2005
82. Muller E, Radler F (1993) Caseicin, a bacteriocin from Lactobacillus casei. Folia Microbiol (Praha) 38 (6):441-446
83. Neetoo H, Ye M, Chen H, Joerger RD, Hicks DT, Hoover DG (2008) Use of nisin-coated plastic films to control Listeria monocytogenes on vacuum-packaged cold-smoked salmon. Int J Food Microbiol 122 (1-2):8-15. doi:10.1016/j.ijfoodmicro.2007.11.043
84. Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H (1996) Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek 70 (2-4):113-128
85. Netz DJ, Sahl HG, Marcelino R, dos Santos Nascimento J, de Oliveira SS, Soares MB, do Carmo de Freire Bastos M (2001) Molecular characterisation of aureocin A70, a multi-peptide bacteriocin isolated from Staphylococcus aureus. J Mol Biol 311 (5):939-949. doi:10.1006/jmbi.2001.4885
86. Nicolas GG, LaPointe G, Lavoie MC (2011) Production, purification, sequencing and activity spectra of mutacins D-123.1 and F-59.1. BMC Microbiol 11:69. doi:10.1186/1471-2180-11-69
87. Nilsen T, Nes IF, Holo H (2003) Enterolysin A, a cell wall-degrading bacteriocin from Enterococcus faecalis LMG 2333. Appl Environ Microbiol 69 (5):2975-2984
88. Nissen-Meyer J, Holo H, Havarstein LS, Sletten K, Nes IF (1992) A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J Bacteriol 174 (17):5686-5692
89. Nissen-Meyer J, Oppegård C, Rogne P, Haugen HS, Kristiansen PE (2010) Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. Probiotics and Antimicrobial Proteins 2 (1):52-60. doi:10.1007/s12602-009-9021-z
90. Okuda K, Zendo T, Sugimoto S, Iwase T, Tajima A, Yamada S, Sonomoto K, Mizunoe Y (2013) Effects of bacteriocins on methicillin-resistant Staphylococcus aureus biofilm. Antimicrob Agents Chemother 57 (11):5572-5579. doi:10.1128/aac.00888-13
91. Oppegard C, Kjos M, Veening JW, Nissen-Meyer J, Kristensen T (2016) A putative amino acid transporter determines sensitivity to the two-peptide bacteriocin plantaricin JK. Microbiologyopen 5 (4):700-708. doi:10.1002/mbo3.363
92. Ovchinnikov KV, Chi H, Mehmeti I, Holo H, Nes IF, Diep DB (2016) Novel Group of Leaderless Multipeptide Bacteriocins from Gram-Positive Bacteria. Appl Environ Microbiol 82 (17):5216-5224. doi:10.1128/aem.01094-16
93. Rice LB (2006) Antimicrobial resistance in gram-positive bacteria. Am J Med 119 (6 Suppl 1):S11-19; discussion S62-70. doi:10.1016/j.amjmed.2006.03.012
94. Roces C, Campelo AB, Veiga P, Pinto JP, Rodriguez A, Martinez B (2009) Contribution of the CesR-regulated genes llmg0169 and llmg2164-2163 to Lactococcus lactis fitness. Int J Food Microbiol 133 (3):279-285. doi:10.1016/j.ijfoodmicro.2009.06.002

95. Russo G, Iannetta M, D'Abramo A, Mascellino MT, Pantosti A, Erario L, Tebano G, Oliva A, D'Agostino C, Trinchieri V, Vullo V (2012) Lactococcus garvieae endocarditis in a patient with colonic diverticulosis: first case report in Italy and review of the literature. New Microbiol 35 (4):495-501
96. Ryan MP, Jack RW, Josten M, Sahl HG, Jung G, Ross RP, Hill C (1999) Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. J Biol Chem 274 (53):37544-37550
97. Schindler CA, Schuhardt VT (1964) LYSOSTAPHIN: A NEW BACTERIOLYTIC AGENT FOR THE STAPHYLOCOCCUS. Proc Natl Acad Sci U S A 51:414-421
98. Scholz R, Vater J, Budiharjo A, Wang Z, He Y, Dietel K, Schwecke T, Herfort S, Lasch P, Borriss R (2014) Amylocyclicin, a novel circular bacteriocin produced by Bacillus amyloliquefaciens FZB42. J Bacteriol 196 (10):1842-1852. doi:10.1128/jb.01474-14
99. Sproat D, Brown A (1979) Bacteriocin typing of Serratia marcescens. A simplified system. Am J Clin Pathol 71 (2):172-176
100. Stahl CH, Callaway TR, Lincoln LM, Lonergan SM, Genovese KJ (2004) Inhibitory activities of colicins against Escherichia coli strains responsible for postweaning diarrhea and edema disease in swine. Antimicrob Agents Chemother 48 (8):3119-3121. doi:10.1128/aac.48.8.3119-3121.2004
101. Steckbeck JD, Deslouches B, Montelaro RC (2014) Antimicrobial peptides: new drugs for bad bugs? Expert Opin Biol Ther 14 (1):11-14. doi:10.1517/14712598.2013.844227
102. Stern NJ, Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP, Pokhilenko VD, Levchuk VP, Svetoch OE, Seal BS (2006) Isolation of a Lactobacillus salivarius strain and purification of its bacteriocin, which is inhibitory to Campylobacter jejuni in the chicken gastrointestinal system. Antimicrob Agents Chemother 50 (9):3111-3116. doi:10.1128/aac.00259-06
103. Stoddard GW, Petzel JP, van Belkum MJ, Kok J, McKay LL (1992) Molecular analyses of the lactococcin A gene cluster from Lactococcus lactis subsp. lactis biovar diacetylactis WM4. Appl Environ Microbiol 58 (6):1952-1961
104. Teixeira LM, Merquior VL, Vianni MC, Carvalho MG, Fracalanzza SE, Steigerwalt AG, Brenner DJ, Facklam RR (1996) Phenotypic and genotypic characterization of atypical Lactococcus garvieae strains isolated from water buffalos with subclinical mastitis and confirmation of L. garvieae as a senior subjective synonym of Enterococcus seriolicida. Int J Syst Bacteriol 46 (3):664-668. doi:10.1099/00207713-46-3-664
105. Tosukhowong A, Zendo T, Visessanguan W, Roytrakul S, Pumpuang L, Jaresitthikunchai J, Sonomoto K (2012) Garvieacin Q, a novel class II bacteriocin from Lactococcus garvieae BCC 43578. Appl Environ Microbiol 78 (5):1619-1623. doi:10.1128/aem.06891-11
106. Uzelac G, Kojic M, Lozo J, Aleksandrzak-Piekarczyk T, Gabrielsen C, Kristensen T, Nes IF, Diep DB, Topisirovic L (2013) A Zn-dependent metallopeptidase is responsible for sensitivity to LsbB, a class II leaderless bacteriocin of Lactococcus lactis subsp. lactis BGMN1-5. J Bacteriol 195 (24):5614-5621. doi:10.1128/jb.00859-13
107. van Belkum MJ, Kok J, Venema G, Holo H, Nes IF, Konings WN, Abee T (1991) The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. J Bacteriol 173 (24):7934-7941
108. van der Ploeg JR (2005) Regulation of bacteriocin production in Streptococcus mutans by the quorum-sensing system required for development of genetic competence. J Bacteriol 187 (12):3980-3989. doi:10.1128/jb.187.12.3980-3989.2005
109. Vendrell D, Balcazar JL, Ruiz-Zarzuela I, de Blas I, Girones O, Muzquiz JL (2006) Lactococcus garvieae in fish: a review. Comp Immunol Microbiol Infect Dis 29 (4):177-198. doi:10.1016/j.cimid.2006.06.003
110. Venema K, Kok J, Marugg JD, Toonen MY, Ledeboer AM, Venema G, Chikindas ML (1995) Functional analysis of the pediocin operon of Pediococcus acidilactici PAC1.0: PedB is the immunity protein and PedD is the precursor processing enzyme. Mol Microbiol 17 (3):515-522

111. Villani F, Aponte M, Blaiotta G, Mauriello G, Pepe O, Moschetti G (2001) Detection and characterization of a bacteriocin, garviecin L1-5, produced by Lactococcus garvieae isolated from raw cow's milk. J Appl Microbiol 90 (3):430-439
112. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39 (3):309-317. doi:10.1086/421946
113. Woraprayote W, Kingcha Y, Amonphanpokin P, Kruenate J, Zendo T, Sonomoto K, Benjakul S, Visessanguan W (2013) Anti-listeria activity of poly(lactic acid)/sawdust particle biocomposite film impregnated with pediocin PA-1/AcH and its use in raw sliced pork. Int J Food Microbiol 167 (2):229-235. doi:10.1016/j.ijfoodmicro.2013.09.009

Paper I

Antimicrobial Peptide Produced by Lactococcus garvieae with a Broad Inhibition Spectrum

Authors : Hai Chi, Ibrahim Mehmeti, Kirill Ovchinnikov, Hegle Holo, Ingolf F. Nes, Dzung B. Diep

Abstract : By using a panel of multiple indicator strains of different bacterial species and genera, we screened a large collection of bacterial isolates (over 1800 isolates) derived from raw milk, for bacteriocin producers with broad inhibition spectra (BIS). Fourteen isolates with BIS were identified, and by 16S rDNA sequencing they were found to belong to Lactococcus garvieae (10 isolates) and Enterococcus feacalis (4 isolates). Further analysis of the ten L. garvieae isolates revealed that they were very similar, if not identical, to each other in metabolic and genetic terms: they had the same fermentation profile on different types of sugars, repetitive sequence-based PCR (rep-PCR) DNA pattern as well as they all had the same inhibition profile towards over 50 isolates of different species. The bacteriocin activity from one of the L. garvieae isolates was assessed further. The bacteriocin which was termed garvicin KS, was found to be heatstable and proteinase-labile and its inhibition spectrum contained many distantly related genera of Firmicutes, comprising most lactic acid bacteria (LAB) as well as problematic species of Bacillus, Listeria, Streptococcus and Staphylococcus and their antibiotic resistant derivatives (e.g. VRE, MRSA). Taken together, the results indicate that this is a potent bacteriocin from L. garvieae and that its very broad inhibition spectrum can be a very useful property for use in food preservation as well as in infection treatments caused by gram-positive pathogens and their antibiotic-derivatives.

Keywords : bacteriocin, lactic acid bacteria, Lactococcus garvieae, antibiotics resistance
Conference Title : ICABES 2017 : 19th International Conference on Agricultural, Biological and Ecosystems Sciences
Conference Location : Miami, USA
Conference Dates : March 09-10, 2017

Antimicrobial peptide produced by *Lactococcus garvieae* with a broad inhibition spectrum

Hai Chi, Ibrahim Mehmeti, Kirill Ovchinnikov, Helge Holo, Ingolf F. Nes, Dzung B. Diep

***Abstract*—** By using a panel of multiple indicator strains of different bacterial species and genera, we screened a large collection of bacterial isolates (over 1800 isolates) derived from raw milk, for bacteriocin producers with broad inhibition spectra (BIS). Fourteen isolates with BIS were identified, and by 16S rDNA sequencing they were found to belong to *Lactococcus garvieae* (10 isolates) and *Enterococcus feacalis* (4 isolates). Further analysis of the ten *L. garvieae* isolates revealed that they were very similar, if not identical, to each other in metabolic and genetic terms: they had the same fermentation profile on different types of sugars, repetitive sequence-based PCR (rep-PCR) DNA pattern as well as they all had the same inhibition profile towards over 50 isolates of different species. The bacteriocin activity from one of the *L. garvieae* isolates was assessed further. The bacteriocin which was termed garvicin KS, was found to be heatstable and proteinase-labile and its inhibition spectrum contained many distantly related genera of Firmicutes, comprising most lactic acid bacteria (LAB) as well as problematic species of *Bacillus*, *Listeria*, *Streptococcus* and *Staphylococcus* and their antibiotic resistant derivatives (e.g. VRE, MRSA). Taken together, the results indicate that this is a potent bacteriocin from *L. garvieae* and that its very broad inhibition spectrum can be a very useful property for use in food preservation as well as in infection treatments caused by gram-positive pathogens and their antibiotic-derivatives.

***Keywords*—**Bacteriocin, Lactic acid bacteria, *Lactococcus garvieae*, antibiotics resistance

I. Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by many different bacteria [1]-[2]. Their antimicrobial activity is often towards closely related species, but some can have broad inhibition spectra including food spoilage and pathogenic microorganisms [3]. A large number of bacteriocins are produced by LAB which are generally considered as safe (GRAS) microorganisms for human use because they are numerous in diverse fermented meat and vegetable products as well as being common inhabitants in the gastrointestinal tract (GIT) of humans and animals. Both bacteriocins and their LAB producers have therefore been considered to hold a great potential in diverse antimicrobial applications, such as natural food preservatives, and antimicrobials in infection therapy [4]-[5]. The latter is of special interest because there is an urgent need for a new source of antimicrobials to fight antibiotic resistant pathogens causing serious problems in infection treatments worldwide, and indeed many bacteriocins have the ability to kill antibiotic resistant pathogens [6].

Dairy products are common sources for bacteriocins where species of *Lactococcus* are often prevalent as active producers. However, lactococcal bacteriocins from non-dairy sources have also been reported [7]-[8]. Some known lactococcal bacteriocins nisin, lacticin 481, lactococcins A, B, M, G, LsbB, 972 and Q, are all produced by different *Lactococcus lactis* strains[7]-[13]. Garvicin ML and garvicin A are also produced by *Lactococcus garvieae* strains isolated from the GIT of Mallard duck [14] and from a human clinical case [15], respectively. Among the aforementioned bacteriocins nisin and lacticin 481 belong to the class consisting of heavily modified peptides known as lantibiotics [11] while the remaining ones belong to the non-lantibiotic class which consists of non-modified peptides or peptides with limited modifications [1]. Like most bacteriocins, these lactococcal bacteriocins have relatively narrow inhibition spectra, which includes mostly lactococcal cells, except nisin and garvicin ML that have relatively broad inhibition spectra [14].

We have recently performed a large and systematic survey on the microbial quality of raw bovine milk from many different farms in Kosovo and a large collection (over 1800 isolates) of LAB has been isolated [16]. In the present work, we have used this collection to screen for bacteriocin producers with BIS for potential antimicrobial applications. From this collection, fourteen isolates showed BIS activity. Ten isolates were identified as *L. garvieae* and the rest were *Enterococcus faecalis*. Among those isolates, one *L. garvieae* isolate was found to produce a very potent bacteriocin with a broad inhibition spectrum. We provide strong evidences that this is a novel bacteriocin from *L. garvieae*, and that it has a great potential value as it can kill many gram-positive pathogens including the problematic species of *Enterococcus*, *Listeria* and *Staphylococcus* and their antibiotic-resistant derivatives.

II. Experiments

A. Bacterial strains and growth conditions

The bacterial collection of LAB used in the screening assay was from raw milk samples collected from 221 farms in Kosovo from the period of November 2011 to June 2012 [16]. The large collection of LAB later was re-streaked out on de man, Rogosa, Sharpe (MRS) (Oxoid, UK) agar plates, single colonies were picked up and transferred into MRS broth tubes, subsequently

Hai Chi, Ibrahim Mehmeti, Kirill Ovchinnikov, Hegle Holo, Ingolf F. Nes, Dzung B. Diep are with the Norwegian University of Life Sciences, 1432 Ås, Norway (corresponding author to provide phone: +4767232482; e-mail: chi.hai@nmbu.no).

incubated at 30 ℃ for 24 h. The indicator strains were routinely grown in brain heart infusion (BHI) (Oxoid, UK) broth at 30 ℃ in aerobic condition. When appropriate, some indicator strains, like Clostridium species, were grown anaerobically in BHI broth at 37 ℃.

B. Screening for broad-spectrum bacteriocin producers

To screen for bacteriocin producers with BIS, five indicative strains from distantly related genera (*L. lactis*, *L. sakei*, *L. plantarum*, *L. innocua* and *S. aureus*) were used for the first round screening. The agar diffusion bioassay was used to check the antimicrobial activity as previously described [17]. Briefly, indicator cells from overnight cultures were diluted 100 fold in 5 mL of BHI soft agar and plated out as a lawn on BHI agar plates. 3 μL of potential bacteriocin producers were spotted on the indicator lawn. The plates subsequently incubated at appropriate temperatures. The inhibition zone shown on the plates was detected after at least 16 h. Proteinase K at concentration of 20 μg/mL was spotted close to the indicative cells. Proteinase-sensitivity was seen as cell inhibition was prevented close to where proteinase K was applied. Heat-treatment assay was detected by applying the sample at 100 ˚C for 15 min for the bacteriocin activity.

C. DNA isolation, PCR , 16S rDNA gene sequencing and rep-PCR

Total genomic DNA was isolated by using Fastprep (Bio101/Savant) and DNA mini kit (Omega Bio-tek Inc., GA). Amplification of 16S rRNA gene by PCR was carried out using the primers 5F (5'-GGTTACCTTGTTACGACTT-3') and 11R (5'-TAACACATGCAAGTCGAACG-3') as previously described [18]. PCR products were purified as described from the company's introduction, and the samples were sent to for sequencing in Germany. For genetic comparison, rep-PCR was performed as previously described [19]. Amplicons were visualized under UV light after electrophoretic migration through a 1.0 % agarose gel.

D. API test-fermentation profiling

Carbohydrate fermentation was determined by using API test according to the manufacturer's instructions (bioMerieux®sa, France). Color changes were detected after 24 h at 30 ˚C. Fermentation of a carbohydrate was confirmed when the color in the medium was changed from purple to yellow after 48 h.

III. RESULTS

A. Screening for broad inhibition spectrum bacteriocins producers

In the study of microbial quality of cow's raw milk in Kosovo, a large collection (1854 isolates) of LAB has been isolated [16]. We used this bacterial collection to screen for BIS antimicrobials with potential application in food preservation or/and medical treatment. In the first screening, we applied a panel of 5 genetically different indicators, with some being frequently found in milk (*L. lactis*), being normally associated with contaminated milk (*L. innocua, S. aureus*) and some being less common in dairy environment (*L. sakei* and *L. plantarum*). Dependent on the chosen indicators (Table I), between 15–25 % of the isolates were found to have antimicrobial activity, with lowest score against the *L. plantarum* strain (273 out of 1854; 14.7 %) while with highest scores against the problematic bacteria *L. inocua* (467 out of 1854; 25. 2 %) and *S. aureus* (402 out of 1854; 21.7 %). Among the antimicrobial producers, 107 isolates could be considered as BIS producers because they were active against all these 5 different indicators.

TABLE I
THE PORTION OF ISOLATES PRODUCING ANTIMICROBIAL ACTIVITY AGAINST THE FIVE INDICATORS

Indicators	Isolates with antimicrobial activity[a]
L. lactis IL 1403	380 (20.5 %)
L. sakei LMGT 2313	291 (15.7 %)
L. innocua LMGT 2710	467 (25.2 %)
S. aureus LMGT 3242	402 (21.7 %)
L. plantarum LMGT 2003	273 (14.7 %)
All five[b]	107 (12.5 %)

a Total isolates screened for antimicrobial activity were 1854;
b Number of isolates with antimicrobial activity against all the five indicators concurrently

Lactococcus species are frequently found in raw milk and dairy products, and many of them are known as bacteriocin producers. To avoid identification of known or related bacteriocins such as nisin, lactococcins G, A, B and M produced by dairy-associated lactococci, we therefore used characterized lactococcocal producers of these bacteriocins as indicators for the next round of screening. Our rational is that these bacteriocin-producing indicators will be immune to other producers of the same or related bacteriocins due to dedicated immunity and cross immunity mechanisms [20]. Among the 107 BIS isolates, only fourteen of these were found to be capable to kill all these bacteriocin producing lactococcal indicators (data not shown). Subsequent physicochemical analysis conformed that the antimicrobial activity from these fourteen isolates had typical bacteriocin characteristics, i.e., being sensitive to proteinase K and heat-stable.

The fourteen isolates were subsequently genotyped by 16S rDNA gene sequencing. The sequencing results revealed that ten of these showed 100 % sequence identity to *L. garvieae* while the remaining four showed highest sequence identity to *E. faecalis* (over 98 %).

B. Characterization of the ten bacteriocin producing L. garvieae isolates

The ten *L. garvieae* isolates selected were from 10 different farms of 4 geographically different regions in Kosovo: Gjakova (2 isolates), Rahoveci (4 isolates), Skenderaji (2 isolates) and Sharri (2 isolates) [16]. Interestingly, during the inhibition assay described above, we noticed that the ten *L. garveae* isolates had identical inhibition spectra, indicating that they might produce the same bacteriocin(s) and hence possibly have the same genetic background. To assess their genetic similarity, rep-PCR was performed. As control, we used *L. garveae* DCC 43 which is the producer of the known bacteriocin garvicin ML and was isolated from the intestine of Mallard duck [14]. All ten *L. garvieae* isolates from Kosovo appeared to have the same pattern of amplified DNA bands in rep-PCR but this pattern was different from that of DCC 43 (data not shown). In addition, the ten *L. garvieae* isolates also shared the same fermentation

profile of different sugars tested (Table II). In the fermentation profile, all ten Kosovo-derived *L. garvieae* isolates gave positive signals on the same 18 of the sugars tested while the DCC 43 strain gave positive signals only on 13 of them. Further, all these 13 sugars fermented by DCC 43 were within the list of the 18 fermentable sugars for the Kosovo isolates, indicating that the dairy-derived isolates have a much larger fermentation capacity than the Mallard-duck gut-derived DCC 43. It is also noteworthy that the sugars lactose, galactose and sucrose which are common in milk were fermented by the Kosovo-derived isolates but not by DCC 43, thus providing a strong evidence that the growth of the Kosovo-derived isolates are adapted to dairy environments while DCC 43 is not. As the ten Kosovo-derived isolates appear identical in terms of inhibition spectra, fermentation profiles, and genetic profiles by rep-PCR, we reckoned that they all were probably very similar, if not identical, genetically and present the same bacteriocin activity. Therefore, only one of them, termed *L. garvieae* KS 1546, was assessed further.

TABLE II
FERMENTATION PROFILE OF THE TEN DAIRY-DERIVED BACTERIOCIN PRODUCERS OF *L. GARVIEAE* FROM KOSOVO COMPARED WITH THAT OF THE GUT-DERIVED *L. GARVIEAE* DCC 43

Active ingredients	KS 1564[a]	DCC 43
Mannitol	+	- [b]
Lactose	+	-
Sucrose	+	-
Mannitol	+	-

a All ten *L. garveae* isolates from Kosovo had the same fermentation profile. Therefore only, KS 1546, was shown here
b " - " means no fermentation while " + " means fermentation detected

A more extended comparison between the bacteriocin activity of *L. garvieae* KS 1546 (termed garvicin KS) and *L. garvieae* DCC 43 (garvicin ML producer) can also be seen in Table III. We noticed that garvicin KS was more potent (i.e., relatively lower MIC values) than garvicin ML toward several genera of known pathogens, including *Enterococcus*, *Listeria* and *Staphylococcus*.

TABLE III
COMPARISON OF THE INHIBITORY SPECTRA OF *L. GARVIEAE* KS 1546 WITH *L. GARVIEAE* DCC 43

Indicators	Relative MIC value[a]	
	KS 1546	DCC 43
Bacillus cereus	128-256	64
Clostridium bifermentans	64-128	16-32
Enterococcus faecium	≤0.25	≤0.25
Listeria monocytogenes	16	32
Staphylococcus aureus	256-512	≥512

a Minimum inhibition concentration (MIC) was defined as the minimum amount of bacteriocin that inhibited at least 50 % of the growth of the indicator in 200 μL of culture. The relative MIC value is relative to the MIC value for the indicator *L. lactis* IL 1403. Hence, the MIC value of *L.lactis* IL 1403 was referred to 1 while MIC values of other indicators were relative to that of *L.lactis* IL 1403
b " NI " means no inhibition activity in the conditions tested

C. Antimicrobial activity against problematic or potentially problematic bacteria

As the antimicrobial activity of garvicin KS is relatively broad, we explored further its potential to kill a larger panel of important pathogens. The list contained 147 problematic or potentially problematic bacteria of species belonging to *Listeria*, *Staphylococcus*, *Streptococcus* and *Enterococcus*, isolated from food and clinical sources. In this assay, we compared the antimicrobial activity of garvicin KS with that of garvicin ML and nisin, the last one being known as a broad inhibition-spectrum bacteriocin [21]. In general, garvicin ML was, as we expected, much less active compared to nisin and garvicin KS (Table IV): among the 147 strains tested, only 51 strains (34.6 %) were killed by garvicin ML while 112 strains (76.1 %) by nisin and remarkably, 139 strains (94.6 %) by garvicin KS. At genus and species level, only against *E. faecium* appeared garvicin ML more active than nisin (7/7 for garvicin ML and 3/7 for nisin) but it was equally active as garvicin KS (7/7). Otherwise, garvicin ML was much less active toward any of the other species. In all cases, garvicin KS was either equal or better than nisin, regardless whether the isolates were from clinical or food environments, except for the activity toward clinical isolates of *S. aureus* where nisin appeared marginally better (24/25 for nisin and 23/25 for KS).

TABLE IV
COMPARISON OF THE ANTIMICROBIAL ACITIVITY OF GARVICIN KS WITH THAT OF NISIN AND GARVICIN ML, AGAINST PROBLEMATIC BACTERIA

Indicators[a]	Original sources[b]					
	Clinical			Food		
	Nisin	DCC 43	KS 1546	Nisin	DCC 43	KS 1546
L. monocytogenes (n = 24)	4/4	0/4	4/4	20/20	0/20	20/20
L. innocua (n = 6)	-	-	-	6/6	1/6	6/6
L. grayi (n = 2)	-	-	-	2/2	0/2	2/2
L. seelingeri (n = 1)	-	-	-	1/1	0/1	1/1
Staph. aureus (n = 53)	24/25	9/25	23/25	18/28	4/28	27/28
Strep. thermophylus (n = 8)	-	-	-	8/8	8/8	8/8
S. agalactia (n = 1)	1/1	1/1	1/1	-	-	-
S. pneumonia (n = 2)	1/1	1/1	1/1	1/1	1/1	1/1
S. salivarius (n = 1)	1/1	1/1	1/1	-	-	-
E. faecalis (n = 42)	0/2	0/2	2/2	22/40	18/40	35/40
E. faecium (n = 7)	-	-	-	3/7	7/7	7/7
Total	31/34	12/34	32/34	81/113	39/113	107/113

a The numbers in parentheses are referred to the number of strains tested, e.g, for *L. monocytogenes*, 24 strains were tested, of which 4 and 20 strains are from clinical and food sources, respectively

b The numerators are referred to the number of strains being sensitive while the denominator to the number of strains tested, e.g., 4 strains of *L. monocytogenes* from clinical sources were tested; all 4 were sensitive to nisin and gavicin KS (KS) but none to garvicin ML (DCC 43)

D. Activity against antibiotic resistant bacteria

Use of antibiotics is a common practice in many farms in Kosovo that has resulted in a relatively high prevalence of antibiotic resistant bacteria [16]. The most commonly used antibiotics in Kosovo are penicillin, streptomycin, oxitetracyclin and ampicillin, the first two normally in combination as a mixture known as PenStrep. To examine whether garvicin KS can kill strains of methicillin-resistant *S. aureus* (MRSA; a causative of mastitis) and antibiotic-resistant strains of *L. monocytogenes* (an important food-borne pathogen) and vancomycin-resistant *E. faecium* (VRE), a test on agar plates was performed. As depicted in Figure 1, these antibiotic resistant strains were sensitive to garvicin KS and to some extent to the control nisin.

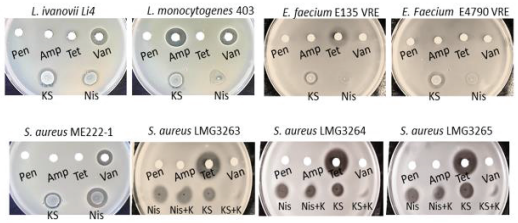

Fig. 1 Antimicrobial activity of nisin (Nis) and garvicin KS (KS) against important antibiotic-resistant strains of *Listeria* spp, vancomycin-resistant strains of *E. faecium* (VRE) and multi-resistant strains of *S. aureus* (MRSA). Dics containing antibiotics (penicillin, ampicillin, tetracyclin and vancomycin) at concentration of 1 mg/mL (2 μL) were placed on lawns of tested strains. For bacteriocin activity, 3 μL of bacteriocin producers were spotted in the first 5 plates while boiled culture of supernatants (3 μL) were spotted in the last three plates. Proteinase K (2 μL) (+K) was added the near the spotted bacteriocins to demonstrate proteinaceous nature. Proteinase sensitive is seen when the inhibition zones are reduced.

IV. DISCUSSION

Traditionally, most known bacteriocin producers are identified by the observation that they have antimicrobial activity against a chosen indicator. Their inhibition broadness are assessed in a later stage toward a panel of bacteria from different genera and species. Such screening assays often lead to the identification of narrow-spectrum bacteriocins because these are relatively more common in nature. Hence, such an approach can easily overlook broad-spectra bacteriocins which may be otherwise very useful in certain applications, e.g., in food preservation or infection treatments where the bacterial activities are unwanted. In the present study, we employed a different approach to search for broad-inhibition bacteriocin producers, by employing five indicators from distantly related genera for our first round screening. Furthermore, four well-known bacteriocin producers who are frequently found in dairy products were used for the second round of screening, eventually, the number of potential bacteriocin producer(s) were reduced drastically due to the self-immunity system. In our case, from 107 to 14 which is a more doable number in subsequent detailed analysis.

Amongst the fourteen potential candidates, ten were identified as *L. garvieae* while the remaining four as *E. faecalis*. It appeared that the ten *L. garvieae* isolates were very similar, if not identical, genetically and phenotypically, as they shared the same profiles when analyzed by rep-PCR, bacteriocin inhibition spectrum and sugar fermentation. Interestingly, these ten lactococcal isolates were isolated from geographically different regions in Kosovo: Gjakova (2 isolates), Rahoveci (4 isolates), Skenderaji (2 isolates) and Sharri (2 isolates). It is reasonable to believe that these ten isolates are of the same clone because of their genetic and phenotypic likeness. Nonetheless, the probability to find the same clone/strain in several geographically different regions of Kosovo is quite low even the size of Kosovo is relatively small (for comparison, the

size of Kosovo is 10,908 km^2 which is about 35 times smaller than Norway, 385,178 km^2). However, to define the mechanism of spreading is beyond the scope of the present study.

Although the identity of antimicrobial activity of garvicin KS has yet to be revealed, it has properties typical for most bacteriocins, i.e., heat stablity and proteinase sensitivity. Nevertheless, the bacteriocin activity of garvicin KS is much broader compared to most known bacteriocins. To our knowledge, only four bacteriocins are known from *L. garvieae*. These are garvicins A, Q, L1-5 and ML, produced by strains isolated from different ecological sources: garvicin A from a clinical source [15], garvicin Q from fermented pork sausage [22], garvicin L1-5 from bovine milk [23] and garvicin ML from the intestine of Mallard duck [14]. Garvicins A and Q belong to the subclass IId which consists of linear and non-pediocin-like bacteriocins, and both bacteriocins display relatively narrow inhibition spectra containing mostly strains of *L. garvieae* and closely related species [15]-[22]. The nature of L1-5 is still unknown, nevertheless its inhibition spectrum consists of mostly closely related species and some strains of *Listeria* and *Clostridium* but not *Pediococcus* [23]. Garvicin ML is a circular bacteriocin with an apparently wider inhibition spectrum than the aforementioned ones [14], but definitely narrower compared to that of garvicin KS. The recent study showed that purified garvicin KS was consist of three similar leaderless peptides of 32 to 34 amino acids [24].

Garvicin KS appears to be a BIS bacteriocin with an inhibition spectrum containing many important problematic bacteria of genera *Listeria*, *Staphylococcus*, *Streptococcus* and *Enterococcus*. The broadness of inhibition is comparable to that of nisin that has been approved by FAO/WHO for use as a food preservative in many countries [25]. Like nisin, garvicin KS was also capable to kill antibiotic-resistant bacteria of *L. monocytogene*s, MRSA and VRE which are common problematic bacteria in dairy environments and/or hospital environments. As such, garvicin KS has a great potential as a preservative or an antimicrobial in applications dealing with pathogens and food spoilage bacteria.

The fermentation profiling showed that the ten bacteriocin-producing *L. garvieae* from Kosovo had fermentation profiles different from that of *L. garvieae* DCC 43, the latter being a strain isolated from the intestine of Mallard ducks [14]. The five sugars: galactose, mannitol, lactose, sucrose and gluconate were found to be fermented by the dairy strains but not by the one from the duck's intestine. Among these sugars lactose and galactose are common constituents in raw milk of animals and humans but relatively rare in other environments. These results imply that the Kosovo strains have specialized to grow in milk. Similar findings have also been reported in some previous studies [26]-[27]. Fortina et al. [26] compared the lactose metabolism of *L. garvieae* strains isolated from dairy products and fish, and found that the strains from fish could grow in milk but were not able to assimilate lactose. A study by Fernández et al. [28] provided further evidence that *L. garvieae* strains isolated from milk possessed the galactose permease or Leloir pathway, which allowed these strains to import and grow on this sugar.

The current increasing demands by consumers for natural preservatives in foods and drinks and the urgent need for novel drugs to fight antibiotic resistant pathogens highlight the importance of searching for novel sources of potent antimicrobials. Bacteriocins appear to be great candidates for these purposes as they have the appreciated properties: (1) they are natural antimicrobials often produced by LAB that are GRAS organisms and (2) many of them can kill problematic pathogens including antibiotic resistant ones. The present study identified one such candidate, namely the bacteriocin garvicin KS of a LAB strain isolated from raw bovine milk in Kosovo. This bacteriocin has a wide inhibition spectrum including problematic species *Listeria*, *Enterococcus*, *Streptococcus* and *Staphylococcus*. These problematic bacteria are of great concern in many developing countries, especially in dairy farms such as in Kosovo where large amounts of antibiotics are used that not only have caused a high prevalence of antibiotic resistant bacteria but also contaminated the surrounding environments (e.g., ground water). Development of potent bacteriocins such as garvicin KS into useful applications should therefore be of high priority in order to find sustainable and environmental friendly solutions in our path to deal with problematic bacteria.

V.ACKNOWLEDGEMENTS

Hai Chi was financial granted by Chinese Scholarship Council, and his project No.2016M02 supported by special scientific research funds for central non-profit institute (East China Sea Fisheries Research Institute).

REFERENCES

[1] Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3:777-788.
[2] Diep DB, Nes IF. 2002. Ribosomally synthesized antibacterial peptides in Gram positive bacteria. Curr Drug Targets 3:107-122.
[3] Gillor O, Ghazaryan L. 2007. Recent advances in bacteriocin application as antimicrobials. Recent Pat Antiinfect Drug Discov 2:115-122.
[4] Cleveland J, Montville TJ, Nes IF, Chikindas ML. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 71:1-20.
[5] O'Sullivan L, Ross RP, Hill C. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84:593-604.
[6] Allen HK, Trachsel J, Looft T, Casey TA. 2014. Finding alternatives to antibiotics. Ann N Y Acad Sci 1323:91-100.
[7] Iwatani S, Zendo T, Yoneyama F, Nakayama J, Sonomoto K. 2007. Characterization and structure analysis of a novel bacteriocin, lacticin Z, produced by Lactococcus lactis QU 14. Biosci Biotechnol Biochem 71:1984-1992.
[8] Fujita K, Ichimasa S, Zendo T, Koga S, Yoneyama F, Nakayama J, Sonomoto K. 2007. Structural analysis and characterization of lacticin Q, a novel bacteriocin belonging to a new family of unmodified bacteriocins of gram-positive bacteria. Appl Environ Microbiol 73:2871-2877.
[9] Kojic M, Strahinic I, Fira D, Jovcic B, Topisirovic L. 2006. Plasmid content and bacteriocin production by five strains of Lactococcus lactis isolated from semi-hard homemade cheese. Can J Microbiol 52:1110-1120.
[10] Morgan S, Ross RP, Hill C. 1995. Bacteriolytic activity caused by the presence of a novel lactococcal plasmid encoding lactococcins A, B, and M. Appl Environ Microbiol 61:2995-3001.
[11] Piard JC, Muriana PM, Desmazeaud MJ, Klaenhammer TR. 1992. Purification and Partial Characterization of Lacticin 481, a Lanthionine-

Containing Bacteriocin Produced by Lactococcus lactis subsp. lactis CNRZ 481. Appl Environ Microbiol 58:279-284.
[12] Nissen-Meyer J, Holo H, Havarstein LS, Sletten K, Nes IF. 1992. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J Bacteriol 174:5686-5692.
[13] Martinez B, Suarez JE, Rodriguez A. 1996. Lactococcin 972 : a homodimeric lactococcal bacteriocin whose primary target is not the plasma membrane. Microbiology 142 (Pt 9):2393-2398.
[14] Borrero J, Brede DA, Skaugen M, Diep DB, Herranz C, Nes IF, Cintas LM, Hernandez PE. 2011. Characterization of garvicin ML, a novel circular bacteriocin produced by Lactococcus garvieae DCC43, isolated from mallard ducks (Anas platyrhynchos). Appl Environ Microbiol 77:369-373.
[15] Maldonado-Barragan A, Cardenas N, Martinez B, Ruiz-Barba JL, Fernandez-Garayzabal JF, Rodriguez JM, Gibello A. 2013. Garvicin A, a novel class IId bacteriocin from Lactococcus garvieae that inhibits septum formation in L. garvieae strains. Appl Environ Microbiol 79:4336-4346.
[16] Mehmeti I, Muji S, Diep DB, Nes IF. 2015. High frequency of the potential pathogen Lactococcus garvieae in raw milk from Kosovo. Food Control 53:189-194.
[17] Holo H, Nilssen O, Nes IF. 1991. Lactococcin A, a new bacteriocin from Lactococcus lactis subsp. cremoris: isolation and characterization of the protein and its gene. J Bacteriol 173:3879-3887.
[18] Birri DJ, Brede DA, Tessema GT, Nes IF. 2013. Bacteriocin production, antibiotic susceptibility and prevalence of haemolytic and gelatinase activity in faecal lactic acid bacteria isolated from healthy Ethiopian infants. Microb Ecol 65:504-516.
[19] Mohammed M, Abd El-Aziz H, Omran N, Anwar S, Awad S, El-Soda M. 2009. Rep-PCR characterization and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt. Int J Food Microbiol 128:417-423.
[20] Jack RW, Tagg JR, Ray B. 1995. Bacteriocins of gram-positive bacteria. Microbiol Rev 59:171-200.
[21] AlKhatib Z, Lagedroste M, Fey I, Kleinschrodt D, Abts A, Smits SH. 2014. Lantibiotic immunity: inhibition of nisin mediated pore formation by NisI. PLoS One 9:e102246.
[22] Tosukhowong A, Zendo T, Visessanguan W, Roytrakul S, Pumpuang L, Jaresitthikunchai J, Sonomoto K. 2012. Garvieacin Q, a novel class II bacteriocin from Lactococcus garvieae BCC 43578. Appl Environ Microbiol 78:1619-1623.
[23] Villani F, Aponte M, Blaiotta G, Mauriello G, Pepe O, Moschetti G. 2001. Detection and characterization of a bacteriocin, garviecin L1-5, produced by Lactococcus garvieae isolated from raw cow's milk. Journal of Applied Microbiology 90:430-439.
[24] Ovchinnikov K, Chi H, Mehmeti I, Holo H, Nes IF, Diep BD. 2016. Novel Group of Leaderless Multipeptide Bacteriocins from Gram-Positive Bacteria. Applied Environmental and Microbiology, 82(17):5216-24.
[25] Ross P, Morgan S, Hill C. 2002. Preservation and fermentation: past, present and future. International Journal of Food Microbiology 79:3-16.
[26] Fortina MG, Ricci G, Borgo F. 2009. A study of lactose metabolism in Lactococcus garvieae reveals a genetic marker for distinguishing between dairy and fish biotypes. J Food Prot 72:1248-1254.
[27] Montel Mendoza G, Pasteris SE, Otero MC, Fatima Nader-Macias ME. 2014. Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. J Appl Microbiol 116:157-166.
[28] Fernández E, Alegría Á, Delgado S, Mayo B. 2010. Phenotypic, genetic and technological characterization of Lactococcus garvieae strains isolated from a raw milk cheese. International Dairy Journal 20:142-148.

Paper II

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Novel Group of Leaderless Multipeptide Bacteriocins from Gram-Positive Bacteria

Kirill V. Ovchinnikov,[a] Hai Chi,[a] Ibrahim Mehmeti,[a,b] Helge Holo,[a] Ingolf F. Nes,[a] Dzung B. Diep[a]

Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway[a]; Faculty of Agriculture and Veterinary University of Prishtina, Prishtina, Kosovo[b]

ABSTRACT

From raw milk we found 10 *Lactococcus garvieae* isolates that produce a new broad-spectrum bacteriocin. Though the isolates were obtained from different farms, they turned out to possess identical inhibitory spectra, fermentation profiles of sugars, and repetitive sequence-based PCR (rep-PCR) DNA patterns, indicating that they produce the same bacteriocin. One of the isolates (*L. garvieae* KS1546) was chosen for further assessment. Purification and peptide sequencing combined with genome sequencing revealed that the antimicrobial activity was due to a bacteriocin unit composed of three similar peptides of 32 to 34 amino acids. The three peptides are produced without leader sequences, and their genes are located next to each other in an operon-like structure, adjacent to the genes normally involved in bacteriocin transport (ABC transporter) and self-immunity. The bacteriocin, termed garvicin KS (GarKS), showed sequence homology to four multipeptide bacteriocins in databases: the known staphylococcal aureocin A70, consisting of four peptides, and three unannotated putative multipeptide bacteriocins produced by *Bacillus cereus*. All these multipeptide bacteriocin loci show conserved genetic organization, including being located adjacent to conserved genetic determinants (Cro/cI and integrase) which are normally associated with mobile genetic elements or genome rearrangements. The antimicrobial activity of all multipeptide bacteriocins was confirmed with synthetic peptides, and all were shown to have broad antimicrobial spectra, with GarKS being the most active of them. The inhibitory spectrum of GarKS includes important pathogens belonging to the genera *Staphylococcus*, *Bacillus*, *Listeria*, and *Enterococcus*.

IMPORTANCE

Bacterial resistance to antibiotics is a very serious global problem. There are no new antibiotics with novel antimicrobial mechanisms in clinical trials. Bacteriocins use antimicrobial mechanisms different from those of antibiotics and can kill antibiotic-resistant bacteria, but the number of bacteriocins with very broad antimicrobial spectra is very small. In this study, we have found and purified a novel three-peptide bacteriocin, garvicin KS. By homology search, we were able to find one known and three novel sequence-related bacteriocins consisting of 3 or 4 peptides. None of the peptides has modified amino acids in its sequence. Thus, the activity of all bacteriocins was confirmed with chemically synthesized peptides. All of them, especially garvicin KS, have very broad antibacterial spectra, thus representing a great potential in antimicrobial applications in the food industry and medicine.

Bacterial resistance to antibiotics has become a serious worldwide problem (1). In spite of that, only two new classes of antibiotics—oxazolidinones (linezolid) and cyclic lipopeptides (daptomycin)—have reached the market during the last 3 decades (2), and development of resistance against these antibiotics has been reported (3). The situation is getting worse, as there are no new antibiotic classes in phase II or III clinical trials and none in the preregistration stage (4). Consequently, there is an urgent need for new antimicrobial agents with different killing mechanisms and new strategies to overcome multidrug-resistant bacteria (5).

One alternative to antibiotics is a diverse group of antimicrobial peptides called bacteriocins (6). In Gram-positive (G^+) bacteria, most bacteriocins are small, ribosomally synthesized peptides able to inhibit growth or kill other G^+ bacteria in competition for nutrients or habitats. Most bacteriocins have narrow inhibitory spectra, targeting species or genera closely related to the producer (7), but some have wide inhibitory spectra (8). Unlike most antibiotics, which are enzyme inhibitors, bacteriocins are membrane-active antimicrobials; i.e., they act by disrupting the membrane integrity of sensitive cells, causing leakage of intracellular solutes and eventually cell death (9). Thus, due to different modes of action, bacteriocins are normally active against both antibiotic-sensitive pathogens and their antibiotic-resistant counterparts. To date, the best-studied bacteriocins are from lactic acid bacteria (LAB) because of their status as generally recognized as safe (GRAS) for human consumption. These bacteriocins can be safely used as natural preservatives in foods and drinks (10). However, so far only two bacteriocins, both with broad antimicrobial spectra, nisin and pediocin PA-1, have been autho-

Received 8 April 2016 **Accepted** 10 June 2016

Accepted manuscript posted online 17 June 2016

Citation Ovchinnikov KV, Chi H, Mehmeti I, Holo H, Nes IF, Diep DB. 2016. Novel group of leaderless multipeptide bacteriocins from Gram-positive bacteria. Appl Environ Microbiol 82:5216–5224. doi:10.1128/AEM.01094-16.

Editor: C. M. Dozois, INRS–Institut Armand-Frappier

Address correspondence to Dzung B. Diep, dzung.diep@nmbu.no.

K.V.O. and H.C. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01094-16.

rized as preservatives in the food industry (11). The need for new bacteriocins with broad antimicrobial spectra is still great (12).

Generally, the heat-stable bacteriocins from Gram-positive bacteria are divided into two major classes: class I (lantibiotics) and class II (nonlantibiotics). Lantibiotics are small peptides, of 19 to 38 amino acids, containing posttranslationally modified residues such as lanthionine or β-methyllanthionine (6). Class II bacteriocins contain peptides without modified residues or with minor modifications (e.g., disulfide bridge or circularization) and are divided further into four subclasses. Subclass IIa contains medium-length bacteriocins (37 to 48 residues) with strong antilisterial activity. They are often called pediocin-like bacteriocins after pediocin PA-1, the first member of the class (13). All members of the group contain a conserved N-terminal sequence—the YGNGV "pediocin box"—and one or two intrachain disulfide bonds (14). Subclass IIb consists of two-peptide bacteriocins whose antimicrobial activity relies on the complementary action of the two different peptides (15). Subclass IIc consists of circular bacteriocins with N- to C-terminal covalent linkage. Their circular nature makes the peptides extremely resistant to environmental abuses as well as to many proteolytic enzymes (16). Subclass IId is a miscellaneous group encompassing bacteriocins that do not fit into the three other groups. This group is relatively diverse in terms of amino acid sequence, structure, mechanisms of secretion, and action. It also includes a leaderless bacteriocin subgroup whose members are different from most bacteriocins in that they do not involve an N-terminal leader sequence for export (6).

Dairy products are common sources for bacteriocins; species of *Lactococcus* are often prevalent as active producers. We have recently performed a large and systematic survey on the microbial quality of raw bovine milk from many different farms in Kosovo, and a large collection (over 1,800 isolates) of LAB has been isolated (17). In the present work, we used this collection to screen for bacteriocin producers. We describe here the screening assay, purification, and identification of a novel and broad-inhibitory spectrum bacteriocin with potent activity against many important pathogens. It is a multipeptide leaderless bacteriocin, produced by an isolate of *Lactococcus garvieae*. We also performed genome sequencing to identify the genes involved in bacteriocin synthesis. By homology search we were able to identify three unannotated sequence-related multipeptide bacteriocins produced by *Bacillus cereus* and to prove their activity. Based on this, we propose a separate subgroup for these multipeptide bacteriocins due to their related biochemical composition and genetic organization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial collection of LAB which was used in the screening assay was from raw bovine milk samples collected from 221 farms in Kosovo from November 2011 to June 2012 (17). Cells from the collection and the indicator strains (see below) were routinely grown in brain heart infusion (BHI) (Oxoid, United Kingdom) broth at 30°C under aerobic conditions without shaking.

Screening for broad-spectrum bacteriocin producers. To screen for wide-inhibition-spectrum bacteriocin producers, strains of *Lactococcus lactis*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Listeria innocua*, and *Staphylococcus aureus* were used as indicators in the first round of screening. The antimicrobial screening was performed using the agar diffusion bioassay as previously described (18). Briefly, indicator cells from overnight cultures were diluted 100-fold in 5 ml of BHI soft agar and plated out as a lawn on BHI agar plates. Potential bacteriocin producers at volumes of 3 μl were spotted on the indicator lawn and then incubated at 30°C for 24 h for cell growth and cell inhibition. Inhibition was detected as clear zones around the spotted cells.

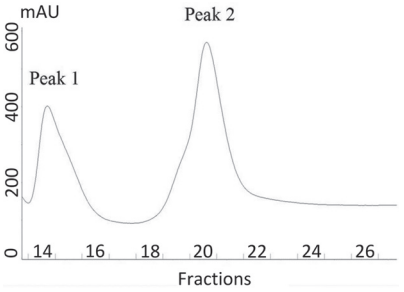

FIG 1 Elution profile of garvicin KS with 2-propanol in the first RPC. Peaks 1 and 2 represent fractions with antimicrobial activity.

For protease sensitivity, 2 μl of proteinase K (Sigma-Aldrich) at 20 μg/ml was applied near the spotted cells. Sensitivity was seen when indicator cell growth was not affected in the region close to where proteinase K had been applied. Heat sensitivity was assessed at 100°C for 5 min before samples were tested for bacteriocin activity.

DNA technologies. Total genomic DNA was isolated by using FastPrep (Bio101/Savant) and DNA minikit (Omega Bio-tek Inc., GA). Amplification of the 16S rRNA gene by PCR was carried out using the primers 5F (5′-GGTTACCTTGTTACGACTT-3′) and 11R (5′-TAACACATGCA AGTCGAACG-3′) as previously described (19). PCR products were purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and sent to GATC Biotech, Germany, for sequencing. For genetic fingerprinting, repetitive sequence-based PCR (rep-PCR) was performed using oligonucleotide primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) and a protocol previously described (20). Amplicons were visualized under UV light after electrophoretic migration through a 1.0% agarose gel. The whole-genome sequencing service was provided by Norwegian Sequencing Center (University of Oslo, Oslo, Norway). Quality-filtered reads were assembled into contigs using CLC Genomics workbench 5.5 (CLC Inc., Aarhus, Denmark) as previously described (21). Genome annotation was performed using the RAST (Rapid Annotation using Subsystem Technology) server (22).

API test-fermentation profiling. Carbohydrate fermentation was determined by using the API 50CH test according to the manufacturer's instructions (bioMérieux SA, France).

Bacteriocin purification and assay. The bacteriocin-producing strain *L. garvieae* KS1546 was grown in M17 medium (Oxoid) supplemented with 0.5% (wt/vol) glucose at 30°C without shaking. Purification was done as described by Holo et al. (18). The bacteriocin was purified from a 1-liter culture. The cells were grown to the early stationary phase and removed by centrifugation at 10,000 × *g* for 15 min at 4°C. The bacteriocin was precipitated from the culture supernatant with ammonium sulfate (45% saturation at 4°C) and harvested by centrifugation (15,000 × *g* and 4°C for 30 min). The protein pellet containing the crude bacteriocin was dissolved in 100 ml of water containing 0.1% (vol/vol) trifluoroacetic acid (TFA; Sigma-Aldrich) (buffer A). The sample was applied on a HiPrep 16/10 SP-XL column (GE Healthcare Biosciences) equilibrated with buffer A. The column was washed with 100 ml of 20 mM sodium phosphate buffer at pH 6.8 before elution of the bacteriocin with 50 ml of 0.2 M NaCl. The eluate was applied to a Resource reverse-phase chromatography (RPC) column (1 ml) (GE Healthcare Biosciences) connected to an ÄKTA purifier system (Amersham Pharmacia Biotech). A linear gradient of isopropanol (Merck) with 0.1% (vol/vol) TFA (buffer B) at a flow rate of 1.0 ml min^{-1} was used for elution. The crude bacteriocin was eluted in two peaks with 31 and 34% buffer B, respectively (Fig. 1). Since the second (34% of isopropanol) peak fractions were about 16 times more

active, this peak was chosen for further purification. Active fractions of the second peak were diluted in buffer A five times and applied on an RPC C_8 column (Amersham Biosciences). Bacteriocin peptides were eluted with 36% buffer B. Fractions showing antibacterial activity were chosen for mass spectrometry (MS) analysis.

Bacteriocin activity was determined using a microtiter plate assay (18). The plates were incubated at 30°C for 8 h, and growth was measured spectrophotometrically at 600 nm (A_{600}) at 15-min intervals using SPECTROstarNano (BMG LABTECH, Germany). The MIC was defined as the bacteriocin concentration (bacteriocin units [BU] per ml) that inhibited the growth of the indicator strain by at least 50% in 200 μl of culture (i.e., 50% of the turbidity of the control culture without bacteriocin).

MS analysis and N-terminal amino acid sequencing. Acquisition of MS data was performed on an Ultraflex MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) instrument operated in reflection mode with delayed extraction. Positively charged ions in the *m/z* range of 200 to 6,000 were analyzed using an acceleration voltage of 25 kV. The sample spectra were calibrated externally with a calibration standard covering the *m/z* range from 700 to 3,100 (Bruker Daltonics, Bremen, Germany). Two of the most active fractions after the second RPC step (C_8 column) were chosen for N-terminal amino acid sequencing by Edman degradation using ABI Procise 494 sequencer (Alphalyse, Denmark).

Synthetic peptides. All the peptides were synthesized by Pepmic Co., Ltd., China, with 90 to 99% purity, except for CehA, CehB, CexA, and CevA (85% purity) due to technical difficulties (poor purification). All the synthesized peptides were not formylated. The peptides were solubilized to concentrations of 0.1 to 2 mg/ml in 0.1% (vol/vol) trifluoroacetic acid and stored at −20°C until use.

Accession number(s). The sequence for the garvicin KS DNA locus has been submitted to GenBank under accession number KU821057.

RESULTS

Screening for broad-inhibitory-spectrum bacteriocin producers. In a previous study of microbial quality of raw bovine milk in Kosovo, a large collection (1,854 isolates) of LAB was created (17). We used this collection to screen for broad-inhibitory-spectrum bacteriocin producers using a panel of five indicators: *Lactococcus lactis*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Listeria innocua*, and *Staphylococcus aureus*. Of the 1,854 isolates, 107 were active against all five indicators.

Lactococcus species are frequently found in raw milk and dairy products, and many of them are known as bacteriocin producers. To avoid identification of known lactococcal bacteriocins such as nisin and lactococcins G, A, B, and M, we used producers of these bacteriocins as indicators for the next round of screening. Our assumption is that these known bacteriocin-producing indicators will be immune to their own bacteriocins (23). Among the 107 isolates, only 10 were found to be capable of killing all these producers (data not shown). The 10 isolates were genotyped by 16S rRNA gene sequencing and showed 100% sequence identity to *L. garvieae*.

Characterization of the 10 bacteriocin-producing *L. garvieae* isolates. The 10 *L. garvieae* isolates were from 10 different farms in 4 geographically different Kosovo regions (17). Their antimicrobial activity was heat stable and proteinase sensitive, properties typical of bacteriocins. They had identical inhibition spectra against 46 Gram-positive bacteria from 8 different genera (data not shown), indicating that they might produce the same bacteriocin(s). rep-PCR showed that all 10 *L. garvieae* isolates had the same pattern of amplified DNA bands (data not shown), and API 20E tests showed that the 10 isolates shared the same profile of fermentation of different sugars (see Table S1 in the supplemental material). Based on that, we reckoned that they all were probably very similar genetically and produced the same antimicrobial activity. Therefore, only one of the bacteriocin producers, termed *L. garvieae* KS1546, was chosen for bacteriocin purification.

Purification and characterization of the bacteriocin activity. Purification was accomplished by established methods for bacteriocins, including cation-exchange chromatography followed by two reverse-phase chromatography (RPC) steps. In the first RPC step, two peaks of antimicrobial activity were identified that corresponded with peaks absorbance at 215 nm (Fig. 1). The first peak of activity was eluted with 31% 2-propanol and the second with 34%, indicating the presence of molecules with different levels of hydrophobicity. MS analysis of the peaks' fractions revealed several predominant masses from about 3,000 to 3,500 Da (Fig. 2) in both of them. Fractions from both peaks were active against *L. lactis*, *L. innocua*, *S. aureus*, *L. sakei*, and *L. plantarum* (data not shown). Peak 2 had antimicrobial activity about 16 times higher than that of peak 1 and was rechromatographed.

The second RPC active fractions were obtained with 36% 2-propanol; however, significant loss of bacteriocin activity was observed, as the final yield was only 0.3% of the starting activity (Table 1). MS analysis of two most active fractions of second RPC showed that both contained a predominant 3,478.6-Da peptide (data not shown). Subsequent N-terminal amino acid sequencing by Edman degradation revealed identical amino acid sequences of 20 residues in both fractions: MGAIIKAGAKIVGKGVLGGG.

Identification of the multiple peptides and the bacteriocin-encoding operon. In a separate work, the genome of *L. garvieae* KS1546 was sequenced (unpublished data). Based on the amino acid sequence obtained, we searched for the corresponding DNA sequence in the bacterial genome of the producer. An open reading frame (ORF) was found to encode a peptide of 34 amino acid residues, of which the first 20 N-terminal amino acids perfectly matched the peptide sequence obtained by the Edman degradation (Table 2). However, the theoretical monoisotopic mass of the gene-derived peptide was 3,450.9 Da, which is 28 Da less than the mass determined by the MS analysis of the purified peptide (3,478.6 Da). Further analysis of the flanking regions revealed two additional small ORFs that encoded putative peptides with high sequence similarity to the aforementioned ORF. The additional two peptides were of 34 and 32 amino acids, with theoretical monoisotopic masses of 3,158.8 and 3,097.7 Da, respectively (Table 2). Interestingly, these two masses were also about 28 Da smaller than the two for the predominant peaks (3,186.6 and 3,125.5 Da) identified in the MS analysis of peak 2 (Fig. 2B). The three putative bacteriocin-like ORFs were named *gakA*, *gakB*, and *gakC* in the order in which they are aligned in the DNA.

Confirmation of the bacteriocin activity with chemically synthesized peptides. The three identified peptides termed GakA, GakB, and GakC (Tables 2 and 3) were chemically synthesized and tested for antimicrobial activity against *L. lactis* IL1403. The individual peptides had poor activity, with MICs of 360 nM for GakA and 6 μM for GakC and GakB having no measurable activity at the highest concentration tested (12 μM). However, when the three peptides were combined at equal molar concentrations, the MIC of the mixture decreased drastically, to 10 nM. Combinations of any two peptides did not show any increased antimicrobial activity over that of the individual peptides (data not shown). These results strongly indicate that the bacteriocin unit, here called garvicin KS (GarKS), is a multipeptide bacteriocin composed of the three peptides GakA, GakB, and GakC.

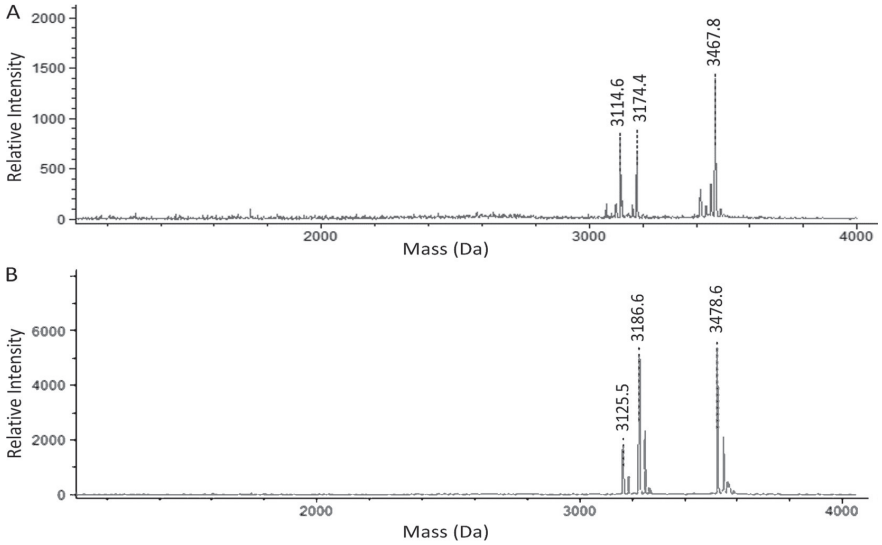

FIG 2 Mass spectrometry analysis of active fractions of garvicin KS after the first RPC. (A) Peak 1; (B) peak 2.

Bioinformatics search for GarKS-related ORFs in the sequence databases. By using the peptide sequences of GarKS, we performed a protein BLAST search in the bacterial data banks. Three homologues—putative leaderless bacteriocins—and one known bacteriocin were identified (Table 2). The known bacteriocin is a four-peptide bacteriocin, aureocin A70 (AurA70), produced by *S. aureus* (24). The other three putative bacteriocins were identified in the different genomes of *B. cereus* and comprised of three or four peptides. All their peptides were annotated as hypothetical proteins. The three putative bacteriocins from *Bacillus* isolates were named cereucin X (CerX; three peptides), cereucin H (CerH; four peptides), and cereucin V (CerV; three peptides) (Table 2). As can be seen from Table 2 and Fig. 3, GakA is very similar to the CexA, CehA, CevA, and CehB peptides. GakB is even more similar to the CexC, CehC, AurB, and CevB peptides.

The peptides comprising aureocin A70 and the three putative *B. cereus* bacteriocins were chemically synthesized (Table 2). As demonstrated with GarKS, the new *B. cereus* bacteriocins were highly active only when all peptides were combined. When the peptides were assessed individually, little, if any, activity was seen (Table 3). To examine and compare their inhibitory spectra, these bacteriocins were tested against a panel of 43 bacteria of different species and genera; they showed high antimicrobial activity (Table 4). Among the bacteriocins, garvicin KS was the most potent.

CerH: three- or four-peptide bacteriocin? The DNA sequence suggests that CerH consists of four peptides (Table 2; Fig. 4). Surprisingly, two of them, CehA and CehB, are very similar at their C termini (Table 2). We were therefore interested to know whether one of these is dispensable in terms of constituting the bacteriocin activity. We compared the activities of these combinations: CehACD, CehBCD and CehABCD. It turned out that CehACD and CehBCD were equally active only against *B. subtilis* LMGT 3131 and some *S. aureus* strains but, when tested against the rest of the strains, the CehBCD mixture was up to 30 times more active than the CehACD mixture (Table 4). CehBCD was generally even more active than CehABCD. These observations suggest that CehA might be redundant for optimal bacteriocin activity, as it performs more poorly than CehB when combined with the other two peptides (CehCD) and also because it can be replaced by CehB without a loss of activity.

Comparison of bacteriocin loci. DNA sequences of CerH, CerV, CerX, and AurA70 loci were taken from the NCBI database (GenBank accession numbers AHDX01000055.1, AHFF01000058.1, AHCW01000073.1, and AF241888.2, respectively) to compare to that of GarKS. In addition to bacteriocin structural genes, one putative bacteriocin ABC transporter gene was found in each bacteriocin locus (Fig. 4). Moreover, GarKS, CerH, and CerX loci contain an ORF encoding a protein of 150 to 156 residues which was found to share high similarity with the AurA70 immunity protein (25) (Fig. 4 and 5). Interestingly, some other genes in these bacteriocin loci were also very similar: at the protein level, the genes encoding mercury resistance proteins in the loci of CerX and CerV were 93% identical to each other. The same level of identity was found between the integrases in the CerH and CerX loci. The genes encoding the prophage Cro/cI

TABLE 1 Purification of garvicin KS

Fraction	Vol (ml)	Total activity (10^4 BU)	Yield (%)
Culture supernatant	1,000	63	100
Ammonium sulfate precipitate	100	51	81
Cation-exchange chromatography	50	26	41
Reverse-phase chromatography			
HiPrep[a]	5	10	16
C_8	5	0.2	0.3

[a] The activity shown is from peak 2 in Fig. 1.

TABLE 2 Sequences of GarKS and related bacteriocin peptides

Bacteriocin	Peptide	Sequence	M_w (monoisotopic)	pI
GarKS	GakA	MGAIIKAGAKIVGKGVLGGGASWLGWNVGEKIWK	3,450.9	10.18
	GakB	MGAIIKAGAKIIGKGLLGGAAGGATYGGLKKIFG	3,158.8	10.30
	GakC	MGAIIKAGAKIVGKGALTGGGVWLAEKLFGGK	3,097.7	10.18
AurA70	AurA	MGKLAIKAGKIIGGGIASALGWAAGEKAVGK	2,922.6	10.18
	AurB	MGAVAKFLGKAALGGAAGGATYAGLKKIFG	2,795.5	10.18
	AurC	MGALIKTGAKIIGSGAAGGLGTYIGHKILGK	2,952.7	10.18
	AurD	MGAVIKVGAKVIGWGAASGAGLYGLEKIFKK	3,084.8	10.00
CerV	CevA	MGAVVKGGLKIIGGTAASWLGWEAGTRIWK	2,974.6	10.28
	CevB	MGAAVKMLGKAFAGGVAGGATYGGLKKIFG	2,827.5	10.18
	CevC	MGAVVKGALKIIGGGAASGGAVYGLERIFGR	3,112.7	10.29
CerX	CexA	MGKKIGKWIITGAAGWAGWEIGEGIWK	2,942.5	9.52
	CexB	MKYLGTLIKGAAGGAGAYVGEKIYNWYKN	3,135.6	9.52
	CexC	MGALFKAALKAAGGGAAGGATYGGLKHFFG	2,796.4	10.00
CerH	CehA	MAKIGKWVVKGAAGYLGWEIGEGIWK	2,846.5	9.40
	CehB	MGALVKGGLKLIGGTAASWLGWEAGERVWK	3,140.7	9.70
	CehC	MGAIIKGGLKLVGGGAAGGFTYGGLKKIFG	2,837.6	10.18
	CehD	MGAIIKGAAKVLGKGAATGGVIYGLEKLFR	2,988.7	10.17

family proteins of CerX and CerH were even 100% identical and shared 62% and 49% identity with AurA70 and GarKS Cro/cI genes, respectively.

W26 is crucial for antimicrobial activity of GakA peptide as well as for GarKS bacteriocin. GakA is a very hydrophobic peptide and difficult to synthesize and purify, probably due to the presence of three tryptophan residues in the C-terminal half. The purity of the synthetic peptide in our experiment was at best only 90%. As an attempt to improve purification, we reduced the hydrophobicity of GakA by replacing its tryptophan residues at the C-terminal end one by one with alanine (W23A, W26A, and W33A). As expected, the modified peptides all attained higher purity (95%). However, when assessed for antimicrobial activity against *L. lactis* IL1403, W26A could not restore activity either as a single peptide or when mixed with GakB and GakC. W23A and W33A peptides were found to be about two times less active (MIC = 0.72 mM) than the wild-type peptide when assessed alone or as a mixture with GakB and GakC (MIC = 20 nM).

TABLE 3 MICs of single peptides and their combinations against *L. lactis* IL1403

Bacteriocin	Peptide	MIC (μM)	MIC of peptide mixture (nM)
Gak	A	0.36	
	B	>12	10
	C	6	
Aur	A	2	
	B	>6	95
	C	>6	
	D	>6	
Cev	A	1.3	
	B	>5	80
	C	5	
Cex	A	>6	
	B	>6	45
	C	>6	
Ceh	A	>2.5	
	B	2.5	46
	C	>2.5	
	D	5	

DISCUSSION

In this study, we aimed to isolate novel bacteriocins with broad inhibition spectra. To achieve this we employed a set of several indicators of distantly related genera (*Listeria*, *Lactobacillus*, *Lactococcus*, and *Staphylococcus*) in our initial screening. Further, by using a second set of indicators of known bacteriocin producers that are frequently found in the same or similar environments (dairy environment in our study), we were able to reduce the number of potential new broad-spectrum bacteriocins drastically,

```
CexA   -------MGKKIGKWIITGAAGWAGWEIGEGIWK
CehA   --------MAKIGKWVVKGAAGYLGWEIGEGIWK
GakA   MGAIIKAGAKIVGKGVLGGGASWLGWNVGEKIWK
CevA   MGAVVKGGLKIIG----GTAASWLGWEAGTRIWK
CehB   MGALVKGGLKLIG----GTAASWLGWEAGERVWK
                  :*      .*.: **: *  :**

CexC   MGAL----FKAALKAAGGGAAGGATYGGLKHFFG
CehC   MGAIIKGGLKLV----GGGAAGGFTYGGLKKIFG
GakB   MGAIIKAGAKIIGKGLLGGAAGGATYGGLKKIFG
AurB   ----MGAVAKFLGKAALGGAAGGATYAGLKKIFG
CevB   ----MGAAVKMLGKAFAGGVAGGATYGGLKKIFG
                 *       **.*** **.***::**
```

FIG 3 Clustal Omega alignment of GakA and GakB homologues peptides. Tryptophan residues at C termini are shown in bold. Stars indicate identical amino acids, colons very similar amino acids, and dots periods similar amino acids.

TABLE 4 MICs of GarKS, AurA70, CerV, CerX, and CerH against different bacterial species

Indicator strain	MIC (nM)						
					CerH[a]		
	GarKS	AurA70	CerV	CerX	A–D	A, C, D	B, C, D
S. aureus							
LMGT 3310	160	1,500	5,000	6,000	1,500	3,000	1,500
LMGT 3264	320	6,000	5,000	6,000	3,000	6,000	1,500
LMGT 3260	2,500	>6,000	>5,000	6,000	3,000	3,000	3,000
LMGT 3266	630	750	5,000	1,500	1,500	1,500	1,500
LMGT 3305	1,250	1,500	5,000	3,000	1,500	1,500	1,500
LMGT 3258	2,500	3,000	5,000	3,000	3,000	3,000	3,000
LMGT 3289	2,500	3,000	5,000	3,000	3,000	3,000	3,000
LMGT 3272	2,500	1,500	5,000	1,500	3,000	3,000	3,000
S. epidermidis LMGT 3026	320	3,000	2,500	1,500	750	1,500	1,500
E. faecalis							
LMGT 3199	160	6,000	2,500	3,000	1,500	6,000	730
LMGT 3330	160	6,000	2,500	3,000	740	3,000	730
LMGT 3359	320	6,000	5,000	6,000	3,000	6,000	1,500
LMGT 3333	160	6,000	5,000	6,000	1,500	3,000	1,500
LMGT 3143	320	6,000	5,000	6,000	3,000	6,000	1,500
LMGT 3351	160	3,000	2,500	1,500	740	1,500	730
LMGT 3200	320	6,000	2,500	1,500	1,500	3,000	730
E. faecium							
LMGT 3108	160	6,000	2,500	3,000	1,500	3,000	730
LMGT 3104	80	3,000	2,599	1,500	740	1,500	370
LMGT 2722	160	3,000	2,500	1,500	740	6,000	730
LMGT 2787	160	>6,000	3,000	1,500	370	6,000	730
LMGT 2783	320	>6,000	5,000	3,000	1,500	3,000	1,500
E. durans LMGT 3191	160	3,000	1,269	1,500	740	3,000	730
P. pentosaceus LMGT 2001	40	185	315	45	92	190	45
L. garvieae							
LMGT 1546[b]	6,000	370	5,000	1,500	740	1,500	730
LMGT 3390	40	745	1,300	740	370	1,500	370
LMGT 2217	80	3,000	5,000	3,000	370	3,000	370
L. lactis							
IL1403	10	95	80	45	46	380	22
LMGT 2084	40	370	315	90	185	760	90
LMGT 2095	5	185	160	25	23	50	10
LMGT 2057	2	45	80	3	10	90	3
LMGT 2233	10	95	160	45	25	380	10
L. sakei							
LMGT 2334	40	740	630	670	370	1,500	180
LMGT 3353	160	1,500	1,260	740	370	1,500	370
L. plantarum LMGT 2329	320	6,000	2,500	6,000	3,000	3,000	1,500
B. subtilis LMGT 3131	320	745	315	370	370	380	370
B. cereus							
LMGT 2805	80	370	315	45	90	190	45
LMGT 2731	160	370	630	45	370	380	180
LMGT 2711	80	370	315	23	92	380	45
LMGT 2735	320	1,500	630	185	185	1,500	180
L. monocytogenes							
LMGT 319	160	745	1,260	370	370	750	370
LMGT 2605	160	370	1,260	1,500	370	3,000	370
L. innocua							
LMGT 2785	160	745	2,500	1,500	1,500	3,000	730
LMGT 2710	160	745	1,260	370	370	750	370

[a] CerH was assessed in three different combinations: all peptides together (A to D) and three-peptide combinations: (i) A, C, and D and (ii) B, C, and D.
[b] GarKS producer.

from 107 to 10. These 10 producers were subsequently shown to be very similar isolates of *Lactococcus garvieae*.

L. garvieae is a LAB mostly known as a human opportunistic and a major fish pathogen (26, 27), but this species is also commonly found in milk and dairy products (17). So far, four bacteriocins have been found in different *L. garvieae* strains. Garviecin L1-5 is a small bacteriocin, with a molecular mass of about 2.5 kDa, produced by *L. garvieae* L1-5 isolated from a raw cow's milk sample. It inhibits bacteria from the *Lactococcus*, *Listeria*, *Enterococcus*, and *Clostridium* genera (28). Garvicin L1-5 has not been characterized at the protein and genetic level, so its amino acid sequence is not known. The circular bacteriocin garvicin ML, with molecular mass of 6 kDa, consists of 60 residues. It is produced by *L. garvieae* DCC43 isolated from mallard duck intestines (29). It also has a broad antimicrobial spectrum and probably is the best studied among all *L. garvieae* bacteriocins so far (30, 31). Garvieacin Q consists of 50 amino acids (5.3 kDa) and is produced by strain BCC 43578, isolated from fermented pork sausage. It is active primarily against closely related bacteria (32). Garvicin A is a 43-residue class IId bacteriocin produced by *L. garvieae* 21881 (human clinical isolate) with a mass of 4.7 kDa. It has a narrow antimicrobial spectrum (33). Garvicin KS, the bacteriocin identified in the present study, is different from the aforementioned bacteriocins in composition. It is a multipeptide bacteriocin composed of three small similar peptides with a size between 32 and 34 amino acids.

Purification of garvicin KS was carried out by cation-exchange chromatography followed by two steps of RPC. The active fractions after the second RPC showed a 30-fold loss of activity. The reason for that activity decrease became clear after the analysis of the bacteriocin DNA locus, which showed that garvicin KS consists of three structural genes. The residual activity after the purification in the fractions used for amino acid sequence was therefore likely due to GakA alone or to GakA contaminated with trace amounts of GakB and GakC that together restored the observed activity. Such contamination is in fact relatively common during purification of bacteriocin peptides from multiple bacteriocin producers because these peptides often share very similar physi-

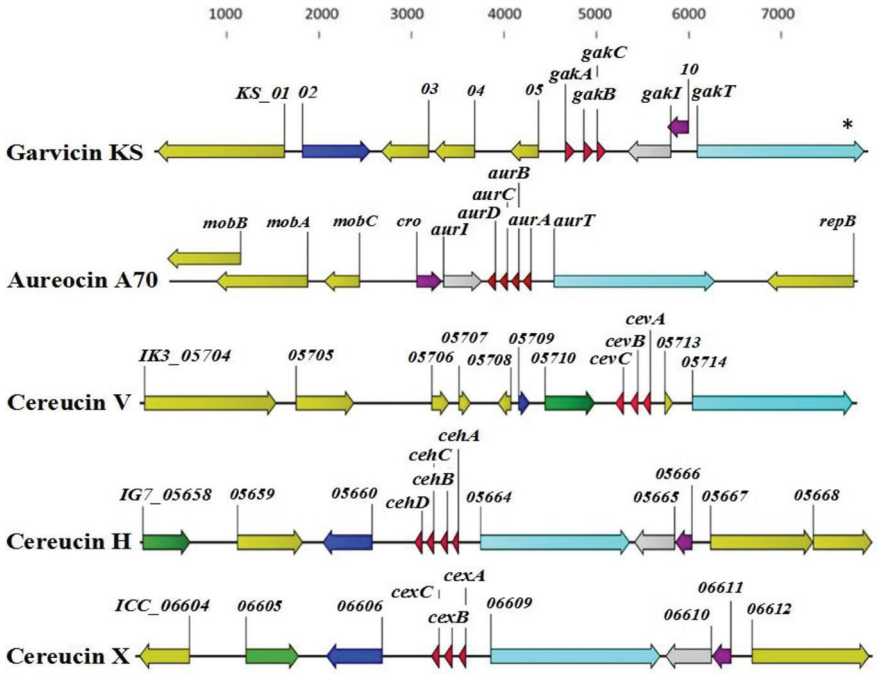

FIG 4 Bacteriocin loci of the multipeptide leaderless bacteriocin family. All loci contain bacteriocin structural genes (red), ABC transporter genes (light blue), and immunity or immunity-like genes (except for the cereucin V locus) (gray). Other conserved genes in the flanking regions are those homologous to the transposase gene (blue), mercury resistance protein gene (green), and Cro/cI protein gene (dark magenta). The remaining genes are homologous to genes for a DNA polymerase (KS_01), an acetyltransferase (KS_03), an AraC family transcriptional regulator (KS_04), and a DNA ligase (KS_05). Also shown are genes for mobilization-associated proteins B, A, and C (*mobB*, *-A*, and *-C*), replication protein Rep (*repB*), mobilization protein IK3_05704, hypothetical protein IK3_05705, replication protein IK3_05706, hypothetical protein IK3_05707, cold shock protein IK3_05708, hypothetical protein IK3_05713, hypothetical protein IG7_05659, RND transporter MFP subunit IG7_05667, macrolide ABC transporter IG7_05668, XRE family transcriptional regulator ICC_06604, and HyD family secretion protein ICC_06612. *, downstream of *gakT* were genes for two hypothetical peptides and a peptide transporter not related to the other proteins.

cochemical properties (most being small, cationic, and amphiphilic or hydrophobic) (34, 35).

The bacteriocin identity was confirmed by the use of synthetic peptides. The peptides individually exhibited no or very poor antimicrobial activity, but when the three peptides were combined in equimolar concentrations, they exhibited a potent activity, with MICs at nanomolar concentrations, confirming that the three peptides form a functional unit (Table 4).

```
ICC_06610   MERLKKWFSLNTHPDERIQQIEMKIWAQSGIIVLLIAFIDFIIRGAYLHRPFLEWAASLA
IG7_05665   MEKLKNWFSLSTHSDERIQQIEMKIWAQSGIVVLLLAFIDFIIRGLYLQRPFLEWAA-TL
aurI        ------------MDERQEIINKNMLIRSFGFLFILLIIYIAFLGVINVTITGHQLILIS
gakI        -MLYFGGKNMKKINDERIIKKDNEIITRTFILMFVLSLFYIVLFNKVVFFREQPQATIFS
                         ***    : ::  ::  .:::: :: : : .

ICC_06610   IIIFYMIFFFIKSILTGIYETDINNKEQLNEKLKEKMSNTLIFCFVAIGTTTYKYNLPED
IG7_05665   IIICYIVFFLIRSVLAGVYETDIHNKEQLNKKLKEKMVNTFIFCFVAISITTYRNQLPEN
aurI        IITLTSIYMWIDSFINKILYYDVQNNKEIKRKLSSCVTTLLVIDVAVLILAFLNKIDINI
gakI        IIIITTVYFIFDSFISKTLFVNIQEKNDVLKKVSHICSLIIAFDTLFILLSLTKKINIDL
            **    ::: : *.:      :::::::: .*:.      : :    :  :  .    :

ICC_06610   FIGWLSVIARFIILFAFLFGIQYLITKYTWYKNNKN
IG7_05665   TIGWLLVILKFIIVFSLIFGIQYLIAKFTWYKNNKN
aurI        SFLFLAILISFNIIVLSVY-YIILKFWLIWYK----
gakI        NLDTIIVLLSLNIFLFISY-YAILRLWVKWIK----
             :  : ::  : *..   :    *     * *
```

FIG 5 Clustal Omega alignment of garvicin KS family immunity proteins. Stars indicate identical amino acids, colons very similar amino acids, and periods similar amino acids.

We observed a 28-Da mass difference between the peptide masses obtained in MS and theoretical masses based on the DNA sequence, indicating formylation of the first methionine residues of the peptides, a feature that distinguishes leaderless bacteriocins from bacteriocins with leader sequences (36). Formylation generally blocks Edman degradation, but *N*-formyl groups are easily removed at low pH (36). Such acidic conditions were encountered during the purification due to the presence of TFA in the fast-performance liquid chromatography buffers. Thus, most of the purified GakA was likely spontaneously deformylated before Edman sequencing.

Interestingly, after the first RPC step of purification, two peaks with antimicrobial activity were detected (Fig. 1). MS of the first peak RPC fractions showed the presence of three peptides with masses of 3,114.6, 3,174.4, and 3,467.8 Da—all about 12 Da smaller than the masses of formylated GarKS peptides (Fig. 2). This mass difference could be due to oxidation (plus 16 Da) and deformylation (minus 28 Da) of the formylated methionine residues during purification or storage, resulting an oxidized methionine [Met(O)] with a mass 12 Da smaller than that of the formylated form. In fact, such modifications have been described for some other leaderless bacteriocins (36). The resulting oxidized peptides would be more hydrophilic. This notion is in line with the observation that these peptides were eluted with a lower concentration of 2-propanol (31%) than the peptides with formylated and nonoxidized methionine residues (34%).

A search for garvicin KS homologues in public sequence databases revealed several hits of significance, all from genome sequences of *Bacillus* and *Staphylococcus* species (Table 2). One of them is the known four-peptide bacteriocin AurA70. This is a plasmid-encoded, four-peptide (30- to 31-residue) bacteriocin produced by *S. aureus* A70 (24). The strain is involved in bovine mastitis and is quite common among *S. aureus* strains in Brazil and Argentina (37, 38).

The remaining three bacteriocins were found in different *B. cereus* genome sequences with some of their peptides annotated just as hypothetical proteins. Some of the peptides were even overlooked by automatic annotation software because of their relatively small sizes (26 to 30 residues).

All these bacteriocins have several features in common: they all are leaderless, each is made up by 3 or 4 small peptides, and their genetic organizations are quite similar, with all structural genes being organized in operon-like structures adjacent to genes homologous to those involved in immunity and transport. Moreover, neighboring the bacteriocin structural genes, there are other genes, which are conserved but apparently not involved in bacteriocin biosynthesis (Fig. 4). These include genes encoding Cro/cI family proteins and integrases/transposases, i.e., genes normally associated with genetic mobile elements. In case of *B. cereus* loci, the identity level between the genes was up to 93 to 100%. Whether this conserved genetic organization is coincident or may have a biological link (e.g., with bacteriocins serving as a toxin-antitoxin system) remains to be investigated.

As shown in Table 4, all bacteriocins have relatively broad inhibitory spectra, with garvicin KS being the most active in the group. Bacteriocins produced by *B. cereus* were slightly more active than GarKS only against *Bacillus* species, which is in line with the general characteristic of bacteriocins, namely, that they are most active against species closely related to the producers.

It has been shown that tryptophan residues in the C termini of bacteriocins are important for their activity (39, 40). As shown in Fig. 3, there are three conserved tryptophan residues in GakA homologous peptides (W23, W26, and W33 in GakA). In the case of GakA, we found W26 to be the most important, since its replacement with A26 led to total loss of activity of the individual peptide GakA as well as the whole bacteriocin GarKS. On the other hand, replacement of W23 and W33 with an alanine reduced the activity of the resulting individual peptides only by half. Whether W26 plays a crucial role in interacting with a hydrophobic environment in the receptor, with the other peptides to form a functional bacteriocin unit, or in other unknown functions remains unknown.

In this study, we have identified a novel group of bacteriocins which share several physicochemical and genetic properties. Most remarkably, they all are leaderless multipeptide bacteriocins whose peptides show significant amino acid sequence similarity to each other, not only within each bacteriocin unit but also across the different bacteriocin units, indicating that these bacteriocins probably share the same ancestor. This group presently contains five members: AurA70, GarKS, CerX, CerH, and CerV, of which only AurA70 has been reported before. AurA70 and CerH consist of four peptides, although the latter can also be viewed as a three-peptide bacteriocin because one of the peptides is dispensable. The remaining are three-peptide bacteriocins. Among these bacteriocins, garvicin KS appears to be the most interesting because it has very potent activity against many pathogenic Gram-positive bacteria (*Listeria*, *Enterococcus*, *Bacillus*, and *Staphylococcus*), representing a great potential for antimicrobial applications.

ACKNOWLEDGMENTS

We thank Cyril A. Frantzen (Norwegian University of Life Sciences) for his help with the whole-genome analysis of *L. garvieae* KS1546.

K.V.O. was supported by a quota scholarship from NMBU, H.C. by a Ph.D. scholarship from the China Scholarship Council, and I.M. by the Norwegian Ministry of Foreign Affairs under the Program for Higher Education, Research and Development (HERD; project no. 332160UW).

REFERENCES

1. **Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, Kishony R, Kreiswirth BN, Kutter E, Lerner SA, Levy S, Lewis K, Lomovskaya O, Miller JH, Mobashery S, Piddock LJ, Projan S, Thomas CM, Tomasz A, Tulkens PM, Walsh TR, Watson JD, Witkowski J, Witte W, Wright G, Yeh P, Zgurskaya HI.** 2011. Tackling antibiotic resistance. Nat Rev Microbiol **9:**894–896. http://dx.doi.org/10.1038/nrmicro2693.
2. **Gupta SK, Nayak RP.** 2014. Dry antibiotic pipeline: regulatory bottlenecks and regulatory reforms. J Pharmacol Pharmacother **5:**4–7. http://dx.doi.org/10.4103/0976-500X.124405.
3. **Kaur DC, Chate SS.** 2015. Study of antibiotic resistance pattern in methicillin resistant Staphylococcus aureus with special reference to newer antibiotic. J Glob Infect Dis **7:**78–84. http://dx.doi.org/10.4103/0974-777X.157245.
4. **Coates AR, Halls G, Hu Y.** 2011. Novel classes of antibiotics or more of the same? Br J Pharmacol **163:**184–194. http://dx.doi.org/10.1111/j.1476-5381.2011.01250.x.
5. **Xu ZQ, Flavin MT, Flavin J.** 2014. Combating multidrug-resistant Gram-negative bacterial infections. Expert Opin Invest Drugs **23:**163–182. http://dx.doi.org/10.1517/13543784.2014.848853.
6. **Cotter PD, Ross RP, Hill C.** 2013. Bacteriocins—a viable alternative to antibiotics? Nat Rev Microbiol **11:**95–105.
7. **Nissen-Meyer J, Nes IF.** 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Arch Microbiol **167:**67–77. http://dx.doi.org/10.1007/s002030050418.
8. **Field D, Cotter PD, Hill C, Ross RP.** 2015. Bioengineering lantibiotics for therapeutic success. Front Microbiol **6:**1363. http://dx.doi.org/10.3389/fmicb.2015.01363.

9. **Kjos M, Borrero J, Opsata M, Birri DJ, Holo H, Cintas LM, Snipen L, Hernandez PE, Nes IF, Diep DB.** 2011. Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. Microbiology **157:**3256–3267. http://dx.doi.org/10.1099/mic.0.052571-0.
10. **Nes IF, Diep DB, Holo H.** 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. J Bacteriol **189:**1189–1198. http://dx.doi.org/10.1128/JB.01254-06.
11. **Benmechernene Z, Fernandez-No I, Kihal M, Bohme K, Calo-Mata P, Barros-Velazquez J.** 2013. Recent patents on bacteriocins: food and biomedical applications. Recent Pat DNA Gene Seq **7:**66–73. http://dx.doi.org/10.2174/1872215611307010010.
12. **Cotter PD, Hill C, Ross RP.** 2005. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol **3:**777–788. http://dx.doi.org/10.1038/nrmicro1273.
13. **Henderson JT, Chopko AL, van Wassenaar PD.** 1992. Purification and primary structure of pediocin PA-1 produced by Pediococcus acidilactici PAC-1.0. Arch Biochem Biophys **295:**5–12. http://dx.doi.org/10.1016/0003-9861(92)90480-K.
14. **Nes IF, Diep DB, Ike Y.** 2014. Enterococcal bacteriocins and antimicrobial proteins that contribute to niche control. *In* Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), Enterococci: from commensals to leading causes of drug resistant infection. Massachusetts Eye and Ear Infirmary, Boston, MA.
15. **Moll GN, van den Akker E, Hauge HH, Nissen-Meyer J, Nes IF, Konings WN, Driessen AJ.** 1999. Complementary and overlapping selectivity of the two-peptide bacteriocins plantaricin EF and JK. J Bacteriol **181:**4848–4852.
16. **Gabrielsen C, Brede DA, Nes IF, Diep DB.** 2014. Circular bacteriocins: biosynthesis and mode of action. Appl Environ Microbiol **80:**6854–6862. http://dx.doi.org/10.1128/AEM.02284-14.
17. **Mehmeti I, Muji S, Diep DB, Nes IF.** 2015. High frequency of the potential pathogen Lactococcus garvieae in raw milk from Kosovo. Food Control **53:**189–194. http://dx.doi.org/10.1016/j.foodcont.2015.01.024.
18. **Holo H, Nilssen O, Nes IF.** 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J Bacteriol **173:**3879–3887.
19. **Birri DJ, Brede DA, Tessema GT, Nes IF.** 2013. Bacteriocin production, antibiotic susceptibility and prevalence of haemolytic and gelatinase activity in faecal lactic acid bacteria isolated from healthy Ethiopian infants. Microb Ecol **65:**504–516. http://dx.doi.org/10.1007/s00248-012-0134-7.
20. **Mohammed M, Abd El-Aziz H, Omran N, Anwar S, Awad S, El-Soda M.** 2009. Rep-PCR characterization and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt. Int J Food Microbiol **128:**417–423. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.09.022.
21. **Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB.** 2012. Genome sequence of the bacteriocin-producing strain *Lactococcus garvieae* DCC43. J Bacteriol **194:**6976–6977. http://dx.doi.org/10.1128/JB.01864-12.
22. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res **42:**D206–D214. http://dx.doi.org/10.1093/nar/gkt1226.
23. **Jack RW, Tagg JR, Ray B.** 1995. Bacteriocins of gram-positive bacteria. Microbiol Rev **59:**171–200.
24. **Netz DJ, Sahl HG, Marcelino R, dos Santos Nascimento J, de Oliveira SS, Soares MB, do Carmo de Freire Bastos M.** 2001. Molecular characterisation of aureocin A70, a multi-peptide bacteriocin isolated from Staphylococcus aureus. J Mol Biol **311:**939–949. http://dx.doi.org/10.1006/jmbi.2001.4885.
25. **Coelho ML, Coutinho BG, Cabral da Silva Santos O, Nes IF, Bastos MDC.** 2014. Immunity to the Staphylococcus aureus leaderless four-peptide bacteriocin aureocin A70 is conferred by AurI, an integral membrane protein. Res Microbiol **165:**50–59. http://dx.doi.org/10.1016/j.resmic.2013.11.001.
26. **Russo G, Iannetta M, D'Abramo A, Mascellino MT, Pantosti A, Erario L, Tebano G, Oliva A, D'Agostino C, Trinchieri V, Vullo V.** 2012. Lactococcus garvieae endocarditis in a patient with colonic diverticulosis: first case report in Italy and review of the literature. New Microbiol **35:**495–501.
27. **Vendrell D, Balcazar JL, Ruiz-Zarzuela I, de Blas I, Girones O, Muzquiz JL.** 2006. Lactococcus garvieae in fish: a review. Comp Immunol Microbiol Infect Dis **29:**177–198. http://dx.doi.org/10.1016/j.cimid.2006.06.003.
28. **Villani F, Aponte M, Blaiotta G, Mauriello G, Pepe O, Moschetti G.** 2001. Detection and characterization of a bacteriocin, garviecin L1-5, produced by Lactococcus garvieae isolated from raw cow's milk. J Appl Microbiol **90:**430–439. http://dx.doi.org/10.1046/j.1365-2672.2001.01261.x.
29. **Sánchez J, Basanta A, Gomez-Sala B, Herranz C, Cintas LM, Hernandez PE.** 2007. Antimicrobial and safety aspects, and biotechnological potential of bacteriocinogenic enterococci isolated from mallard ducks (Anas platyrhynchos). Int J Food Microbiol **117:**295–305. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.04.012.
30. **Gabrielsen C, Brede DA, Salehian Z, Nes IF, Diep DB.** 2014. Functional genetic analysis of the GarML gene cluster in Lactococcus garvieae DCC43 gives new insights into circular bacteriocin biosynthesis. J Bacteriol **196:**911–919. http://dx.doi.org/10.1128/JB.01115-13.
31. **Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB.** 2012. The maltose ABC transporter in Lactococcus lactis facilitates high-level sensitivity to the circular bacteriocin garvicin ML. Antimicrob Agents Chemother **56:**2908–2915. http://dx.doi.org/10.1128/AAC.00314-12.
32. **Tosukhowong A, Zendo T, Visessanguan W, Roytrakul S, Pumpuang L, Jaresitthikunchai J, Sonomoto K.** 2012. Garvieacin Q, a novel class II bacteriocin from Lactococcus garvieae BCC 43578. Appl Environ Microbiol **78:**1619–1623. http://dx.doi.org/10.1128/AEM.06891-11.
33. **Maldonado-Barragán A, Cardenas N, Martinez B, Ruiz-Barba JL, Fernandez-Garayzabal JF, Rodriguez JM, Gibello A.** 2013. Garvicin A, a novel class IId bacteriocin from *Lactococcus garvieae* that inhibits septum formation in *L. garvieae* strains. Appl Environ Microbiol **79:**4336–4346. http://dx.doi.org/10.1128/AEM.00830-13.
34. **Diep DB, Havarstein LS, Nissen-Meyer J, Nes IF.** 1994. The gene encoding plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, is located on the same transcription unit as an *agr*-like regulatory system. Appl Environ Microbiol **60:**160–166.
35. **Nissen-Meyer J, Larsen AG, Sletten K, Daeschel M, Nes IF.** 1993. Purification and characterization of plantaricin A, a Lactobacillus plantarum bacteriocin whose activity depends on the action of two peptides. J Gen Microbiol **139:**1973–1978. http://dx.doi.org/10.1099/00221287-139-9-1973.
36. **Liu X, Vederas JC, Whittal RM, Zheng J, Stiles ME, Carlson D, Franz CM, McMullen LM, van Belkum MJ.** 2011. Identification of an N-terminal formylated, two-peptide bacteriocin from Enterococcus faecalis 710C. J Agric Food Chem **59:**5602–5608. http://dx.doi.org/10.1021/jf104751v.
37. **Ceotto H, Dias RC, Nascimento JDS, Brito MA, Giambiagi-Demarval M, Bastos MDC.** 2012. Aureocin A70 production is disseminated amongst genetically unrelated Staphylococcus aureus involved in bovine mastitis. Lett Appl Microbiol **54:**455–461. http://dx.doi.org/10.1111/j.1472-765X.2012.03226.x.
38. **Ceotto H, Nascimento JDS, Brito MA, Bastos MDC.** 2009. Bacteriocin production by Staphylococcus aureus involved in bovine mastitis in Brazil. Res Microbiol **160:**592–599. http://dx.doi.org/10.1016/j.resmic.2009.07.007.
39. **Ovchinnikov KV, Kristiansen PE, Uzelac G, Topisirovic L, Kojic M, Nissen-Meyer J, Nes IF, Diep DB.** 2014. Defining the structure and receptor binding domain of the leaderless bacteriocin LsbB. J Biol Chem **289:**23838–23845. http://dx.doi.org/10.1074/jbc.M114.579698.
40. **Fimland G, Eijsink VG, Nissen-Meyer J.** 2002. Mutational analysis of the role of tryptophan residues in an antimicrobial peptide. Biochemistry **41:**9508–9515. http://dx.doi.org/10.1021/bi025856q.

Fermentation profile of *L. garvieae* KS1546[a]

Active ingrediens	KS 1546*
Control group	-
Glycerol	-
Erythritol	-
D-arabinose	-
L-arabinose	-
Ribose	+
D-xylose	-
L-xylose	-
Adonitol	-
β-methyl-D-xyloside	-
Galactose	+
D-glucose	+
D-fructose	+
D-mannose	+
L-sorbose	-
Rhamnose	-
Dulcitol	-
Inositol	-
Mannitol	+
Sorbitol	-
α-methyl-D-mannoside	-
α-methyl-D-glucoside	-
N-acetyl-glucoside	+
Amygdalin	+
Arbutin	+
Esculin	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	-
Sucrose	+
Trehalose	+
Inulin	-
Melezitose	-
D-raffinose	-
Starch	-
Glycogen	-
Xylitol	-
β-gentiobiose	+

D-turanose	-
D-lyxose	-
D-fuccose	-
L-fuccose	-
D-arabitol	-
L-arabitol	-
Gluconate	+
Potassium 2-ketogluconate	-
Potassium 5-ketogluconate	-

*The other 9 milk-derived *L. garvieae* isolates had the same fermentation profile as KS1546.
" - " means no fermentation while " + " means fermentation detected

Paper III

Phage shock protein C of *Lactococcus lactis* is receptor for multiple peptide bacteriocins

Hai Chi[1], Cathrine A. Bøe[1], Camilla Oppegård[2], Tom Kristensen[2], Beatriz Martínez[3], Helge Holo[1*]

1. Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway,

2. Section of Biochemistry and Molecular Biology, Department of Biosciences, University of Oslo, Oslo, Norway,

3. DairySafe Group, Department of Technology and Biotechnology of Dairy Products, Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Villaviciosa, Asturias, Spain.

*Corresponding author: Helge Holo

Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, 1432, Norway. E-mail: helge.holo@nmbu.no;

Tel: +47 67232486

Abstract

Bacteriocins from lactic acid bacteria have drawn much attention due to their potency and potential as alternative to traditional antimicrobials. The poor understanding for bacteriocin antimicrobial mechanisms impedes the development of their applications. To gain insight into the mode of action of the broad spectrum bacteriocin garvicin KS we isolated and sequenced mutants of *Lactococcus lactis* IL1403 with reduced sensitivity to the bacteriocin. No fully resistant mutants could be isolated, and the mutants all were mutated in same gene, encoding the phage shock protein C, a stress response protein. The role of *psp*C was confirmed by gene deletion and complementation. Interestingly, the mutants also lost sensitivity to lactococcin G, and cloning pspC in *Lactobacillus sakei* resulted in sensitivity to lactococcin G as well as increased garvicin KS sensitivity.

The mutants bound less garvicin KS and lactococcin G than the wild type, but nisin binding was unaffected. Binding of both bacteriocins, as complete bacteriocins or individual subunits, was demonstrated by immunoprecipitation, showing that PspC can act as a receptor for these bacteriocins. A mutant in UppP, completely resistant to lactococcin G, showed wild type sensitivity to garvicin KS and binding to both bacteriocins. The role of the receptor appears to be capturing the multi peptide bacteriocins at low concentrations and possibly facilitate interactions between the individual peptides.

Introduction

Bacteriocins from lactic acid bacteria (LAB) are ribosomally synthesized antimicrobial peptides. They normally display narrow inhibition spectra against closely related species, while some possess broad activities towards food borne pathogens and food spoilage microorganisms [6]. They have drawn much attention due to their potency as antimicrobial substances working at nano-molar concentrations as well as being considered as GRAS approved by WHO/FDA [5,21]. Importantly, some studies have shown that bacteriocins exhibit potential activities against multidrug-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci [4,22,23]. The bacteriocins from LAB are classified into two groups. Class I bacteriocins are post-translationally modified peptides, called lantibiotics, and the rest are unmodified peptides, belonging to Class II.

In most of the cases, LAB bacteriocins kill targeting bacteria by disrupting the integrity of the cell membrane via a receptor-mediated approach, which results in dissipation of the proton motive force and depletion of intracellular solutes [8]. The identification of lipid II, a precursor in the cell wall synthesis, as target for nisin as well as for the class II bacteriocin, lactococcin 972 was a milestone breakthrough as receptor identification [3]. Later, components of the mannose phosphotransferase systems were shown to be required for target specificity of class IIa bacteriocins, lactococcin A as well as garvicin Q [7,26]. The maltose ABC-transporter has been found to be responsible for sensitivity to garvicin ML in *Lactococcus lactis* [12]. Zn-dependent metallopeptidase and undecaprenyl pyrophosphate phosphatase (UppP) were recently found to be responsible for the sensitivity to the bacteriocins LsbB and Lactococcin G (LcnG), respectively [19,27]. A latest study reported that APC superfamily transporter is likely to serve

62 as a target for plantaricin JK [26]. However, for the most case of class II bacteriocins, their
63 receptors are still unclear.

64

65 We recently described garvicin KS, a bacteriocin with a broad inhibition spectrum and great
66 potential value, as it is able to kill many gram-positive pathogens including problematic species
67 *Enterococcus*, *Listeria* and *Staphylococcus* and their antibiotic-resistant derivatives [4,22].
68 Garvicin KS consists of three leaderless peptides with highly similarity at the N-terminal part,
69 and all three peptides are required in order to present full antimicrobial activity [22]. While they
70 have been characterized for several one and two peptide bacteriocins, targets for three and four
71 peptides bacteriocins have not been identified. In this work we identified an important role of
72 the phage shock protein C (PspC) of *L. lactis* IL1403 in sensitivity to the three peptide
73 bacteriocin garvicin KS, but also to two peptide bacteriocins.

74

75

Materials and methods

1. Bacterial strains, plasmids, and culturing conditions

The bacterial strains, plasmids used in this study are listed in Table 1. *Lactococcal* strains were grown in M17 medium (Oxoid, Hampshire, UK) supplemented with 0.4 % (w/v) glucose (GM17) at 30 ℃. *Lactobacilli* were grown in deMan, Rogosa and Sharpe (MRS) medium (Oxoid, Hampshire, UK) at 30 ℃. *Escherichia coli* strains were grown in lysogeny broth (LB) medium at 37 ℃ with shaking at 225 rpm. When appropriate erythromycin was added to a final concentration of 300 μg/mL for *E. coli* and 10 μg/mL for *Lactococcus* and *Lactobacillus*.

2. Bacteriocins and antimicrobial assay

Crude concentrated bacteriocin preparations were prepared from overnight culture supernatants by ammonium sulfate precipitation as described by Ovchinnikov et al. [22], Borrero et al. [2], Holo et al. [14] and Holo et al. [16]. Nisin from *Lactococcus lactis* (2.5% purity) was from Sigma-Aldrich, Co. (St. Louis, MO, USA), and synthesized LsbB was from GenScript Company (New Jersey, USA) with 99% purity. The LcnG peptides were isolated according to Rogne et al. [25].

Bacteriocin activity was determined and quantified by using a microtiter plate assay described by Holo et al. [16]. One bacteriocin unit (BU) [18] was defined as the amount of bacteriocin required to cause 50 % growth inhibition of the indicator strain (*L. lactis* IL1403, or

Lactobacillus sakei LMGT2799 for bacteriocins that do not inhibit *L. lactis* IL1403) in 200 µL of culture.

3. Isolation of garvicin KS resistant mutants

Total 100 µL of an overnight culture of *L. lactis* IL1403 was added into GM17 soft agar containing crude garvicin KS with concentrations ranging from 0 to 480 BU/mL. The plates were incubated at 30℃ until colonies appeared. Survivors were streaked on GM17 agar, incubated at 30 ℃, and the isolated colonies were picked and propagated in GM17. The cultures were stored with 15 % glycerol in GM17 at -80 ℃ for further investigation.

4. Genomic DNA isolation and sequence analysis

Genomic DNA was extracted by using a genomic-tip 20/G kit (Qiagen, Valencia, CA, USA) according to the manufacturer's introduction. Genomic libraries were constructed and pair-end sequenced using a Miseq 2000 system (Illumina, USA). Genome sequences were analyzed using CLC Genomics workbench 5.5 (CLC Bio, USA) as previously described [11]. Quality-filtered reads were assembled and the assembled sequences from mutants were mapped to the wild type genome to identify genetic differences.

The *pspC* gene from mutants was amplified by PCR using primers PspF and PspR (Table 2). The PCR products were purified according to manufacturer's instructions and sequenced.

5. Molecular cloning

For pspC expression *pspC* of *L. lactis* IL1403 was amplified by PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs) with primers containing *Sca*I and *Hind*III restriction sites and cloned as ScaI/HindIII fragments behind the strong P32 promoter in pMG36e [28]. PpspcFFLAGN (Table 2) was used as the forward primer for expression of N-terminally flagged pspC, and PpspcR was used as reverse primer. PpspcFFLAGB and PpspcR (Table 2) were used as the forward and reverse primers that amplified flagged tag N-terminal region with three amino acids as control.

Plasmids were introduced into *L. lactis* by electroporation according Holo, Nes [15] and *Lactobacillus sakei* LMGT2799 as described by Jimenez et al. [17].

6. Bacteriocin binding assay

Overnight cultures were 10-fold diluted in 10 mL of GM17 and incubated at 30 ℃ until the OD_{600} reached 0.4 - 0.5, then bacteriocin (500 BU) was added and incubation continued at 30 ℃ for 1 h. The cultures were centrifuged at 5000 g for 5 min and the pelleted cells were washed with 1 mL dH_2O twice. Cells without Flag-tagged PspC, were centrifuged and suspended in 1 mL 100 mM HCl. The mixtures were kept at 90 °C for 10 min, then at room temperature for 30 min followed by centrifugation at 12000 g for 5 min. The supernatants were tested for antimicrobial activity.

After bacteriocin binding and washing in water as described above cells expressing Flag-tagged proteins or peptides were suspended in 400 μL 2x lysis buffer, containing 100 mM Tris-HCl (pH7.4), 300 mM NaCl, 2 mM EDTA, 2% of Triton X-100 and lysozyme (5 mg/mL). After incubation on ice for 30 min, the cells were broken with 0.2 g of glass beads (≤ 106 µm) in a Fastprep under the conditions of 4 m/s for 20 s for three times. The mixtures were centrifuged at 12000 g for 5 min and supernatants were used for immunoprecipitation by using anti-FLAG M2 Agarose Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. From the eluates 50 μL were mixed with 150 μL 100 mM HCl, kept at 90 °C for 10 min, then at room temperature for 30 min, and centrifuged at 12000 g for 5 min. Supernatants were tested for antimicrobial activity.

Results

1. Generation and characteristics of garvicin KS resistant mutants

On exposure the *L. lactis* IL1403 to different concentrations of garvicin KS, colonies appeared at concentrations up to 160 BU/mL after 48 h incubation. The survival rates were about 10^{-7} at 80 BU/mL and 10^{-8} at 120 and 160 BU/mL. No colonies were found at concentration above 160 BU/mL. Survivors were picked and checked for altered garvicin KS sensitivity. Thirteen of them showed at least four-fold increased MIC compared to the wild type (Table 3) and were further characterized.

Whole genome sequencing was performed on five of the isolates (KSR2001, 2002, 2003, 2004 and 2009). The sequencing revealed that all had mutations in the same gene, *psp*C, also denoted *yth*A (Table 3). This gene encodes a protein named phage shock protein C (PspC, a stress response protein) [1].

To investigate if the rest of the isolated colonies also have mutations in the same gene their *pspC* genes were amplified and sequenced. The results showed that all were mutated in *psp*C (Table 3).

2. Expression of *pspC* increases bacteriocin sensitivity

Since all the mutants with reduced sensitivity to garvicin KS were mutated in *psp*C, we also tested strain Δ2164, a variant of *L. lactis* MG1363 from which *psp*C and the downstream gene pspA has been deleted. This strain was 64-fold more resistant to gravicin KS compared to wild

type *L. lactis* MG1363, and had the same MIC level to garvicin KS as the spontaneous mutants of *L. lactis* IL 1403.

To further document the involvement of PspC in garvicin KS sensitivity *pspC* was cloned behind the strong promoter P32 in pMG36e [28] to generate pCH1, and the plasmid was introduced into *psp*C mutants by electroporation. The *psp*C-complemented mutants became equally or even slightly more sensitive to garvicin KS compared to the wild type. The same results were found when *L. lactis* Δ2164 was transformed with pCH1. *Lactobacillus sakei* is sensitive to garvicin KS but not to LcnG. We also introduced pspC into *Lactobacillus sakei* LMGT2799. With pCH1 the strain became more sensitive (8-fold decrease in MIC) to garvicin KS compared to *Lactobacillus sakei* without pCH1 (data not shown). Interestingly, the clone also became sensitive to LcnG, albeit about 30 times less sensitive than *L. lactis* IL 1403.

3. Cross-resistance to other bacteriocins

In a previous study, a mutation in pspC in *L. lactis* IL1403 was found to cause reduced sensitivity to the bacteriocins plantaricin S and LcnG , and a slight reduction in sensitivity to nisin [20]. We therefore checked our mutants for sensitivity to nisin, LsbB, LcnA, garvicin ML, and LcnG. The mutants were as sensitive to nisin, LsbB, LcnA and garvicin ML as the wild type, but they were 8 to 16 fold less sensitive to LcnG. Interestingly, IL11, a mutant of *L. lactis* IL1403 completely resistant to LcnG [19] did not show altered sensitivity to garvicin KS or any of the other bacteriocins tested.

4. Garvicin KS binds to sensitive cells

To further investigate a role for pspC in bacteriocin sensitivity we measured bacteriocin binding of cells with different sensitivity to garvicin KS. After incubation with bacteriocin the bacteriocin was removed by centrifugation and bound bacteriocin was extracted from the cells by acid and heat treatment. The binding of bacteriocins to cells is shown in the Table 4. Garvicin KS, LcnG and nisin could all bind to the cells, but we could not see binding of the two bacteriocins tested that do not inhibit *L. lactis* IL1403, sakacin A or plantaricin W. Different concentrations of garvicin KS (500, 1000 and 2000 BU/mL) were tested in the binding assay, but the results showed no difference in binding to *L. lactis* IL1403. We therefore applied 500 BU/mL for the binding assay.

The binding to nisin showed no difference between mutants and wild type. However, the mutants showing reduced garvicin KS sensitivity also bound less garvicin KS and LcnG than the wild type. However, strain IL11, which is completely resistant to LcnG but shows normal sensitivity to garvicin KS, showed the same binding of LcnG, garvicin KS and nisin as the wild type.

Garvicin KS and LcnG are multiple peptides bacteriocins, and the individual peptides show little or no activity. The binding assay was conducted on cells exposed to the complete bacteriocins as well as the individual peptides. As expected, no bacteriocin activity was found in the extracts from cells exposed to individual LcnG or garvicin KS peptides. However, the individual peptides did bind to the cells, revealed by bacteriocin activity after mixing the extracts from cells treated

with individual peptides, 1:1, for the LcnG peptides, or 1:1:1 for the garvicin KS peptides (Table 4).

The role of PspC in bacteriocin binding was investigated by the used of flag-tagged PspC. The mutant KSR2001 showed wild type bacteriocin sensitivity and bacteriocin binding characteristics when expressing PspC with an N-terminal or C-terminal Flag tag. After bacteriocin binding the cells were broken and extracts subjected to immunoprecipitation using Anti-FLAG M2 Agarose Beads. Garvicin KS and LcnG, presented to the cells as complete bacteriocins or individual peptides, could be recovered by this procedure from cells carrying pCH2. No activity was recovered from the control, cells carrying pCH0. Moreover, the nisin bound to the cells could not be recovered by the immunoprecipitation, showing that nisin does not bind to PspC. The same results were seen using C-terminally flagged pspC (data not shown).

Discussion

Our results clearly demonstrate the importance of PspC in garvicin KS sensitivity in *L. lactis*. The protein is predicted to contain a 60 aa PspC domain at its N terminus and a transmembrane helix spanning aa 39-61. The *psp*C gene is found in both Gram-positive and negative bacteria and is involved in the response to extra-cytoplasmic stress and maintaining the integrity of the cytoplasmic membrane [10]. It has been reported that PspC is able to sense the stress and increase the resistance to lysozyme and nisin directly or indirectly via the interactions to SpxB [9,24].

Garvicin KS is a multiple peptides bacteriocin with broad inhibition spectrum. Its inhibition spectrum, however, shows big variations of sensitivity among species. The species-related sensitivity likely relies on PspC sequence differences, and cloning and expression of *L. lactis psp*C in *Lactobacillus sakei* increased its sensitivity to garvicin KS. Importantly, the cloning also converted the *Lactobacillus sakei* strain into an LcnG sensitive bacterium. It has been shown that LcnG sensitivity depends on UppP, involved in cell-wall synthesis in *L. lactis* [19]. Thus, two targets have been identified for the same bacteriocin. The lethal activity can be ascribed to UppP, but high affinity by binding to PspC. Surprisingly, PspC is a target for multiple bacteriocins, such as LcnG and garvicin KS, with no apparent sequence similarity. However, the UppP mutant showed wild type sensitivity to garvicin KS and the same bacteriocin binding characteristics as *L. lactis* IL1403, while the PspC mutants showed reduced sensitivity and binding to both bacteriocins.

The individual peptides of garvicin KS and LcnG can bind to PspC in the absence of each other. The two peptides of LcnG are able to induce structuring in each other [13] likely to be important for bacteriocin activity. As PspC can capture the individual peptides, it is likely that recruitment by PspC facilitates subunit interactions and promotes downstream bacteriocin killing activities.

The role of pspC appears to be that of a bacteriocin receptor. It can bind the bacteriocin, whereupon the bacteriocin possibly can interact with its target, UppP in the case of LcnG but unknown in the case of garvicin KS. Being able to bind unrelated bacteriocins like garvicin KS and LcnG, PspC appears to be a receptor with broad specificity. However, we could only demonstrate binding to multiple peptides bacteriocins being inhibitory to lactococci.

Like in many similar studies aiming at identifying bacteriocin receptors and mechanisms of killing, we were unable to obtain fully resistant mutants, suggesting the presence of a target with essential function. However, PspC appears to act as a receptor enhancing susceptibility to bacterioicin killing at low concentrations.

References

1. Bernsel A, Viklund H, Hennerdal A, Elofsson A (2009) TOPCONS: consensus prediction of membrane protein topology. Nucleic Acids Res 37 (Web Server issue):W465-468. doi:10.1093/nar/gkp363

2. Borrero J, Brede DA, Skaugen M, Diep DB, Herranz C, Nes IF, Cintas LM, Hernandez PE (2011) Characterization of garvicin ML, a novel circular bacteriocin produced by Lactococcus garvieae DCC43, isolated from mallard ducks (Anas platyrhynchos). Appl Environ Microbiol 77 (1):369-373. doi:10.1128/aem.01173-10

3. Brotz H, Josten M, Wiedemann I, Schneider U, Gotz F, Bierbaum G, Sahl HG (1998) Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol Microbiol 30 (2):317-327

4. Chi H, Holo H (2017) Synergistic Antimicrobial Activity Between the Broad Spectrum Bacteriocin Garvicin KS and Nisin, Farnesol and Polymyxin B Against Gram-Positive and Gram-Negative Bacteria. Curr Microbiol. doi:10.1007/s00284-017-1375-y

5. Cleveland J, Montville TJ, Nes IF, Chikindas ML (2001) Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 71 (1):1-20

6. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3 (10):777-788. doi:10.1038/nrmicro1273

7. Diep DB, Skaugen M, Salehian Z, Holo H, Nes IF (2007) Common mechanisms of target cell recognition and immunity for class II bacteriocins. Proc Natl Acad Sci U S A 104 (7):2384-2389. doi:10.1073/pnas.0608775104

8. Drider D, Fimland G, Hechard Y, McMullen LM, Prevost H (2006) The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 70 (2):564-582. doi:10.1128/mmbr.00016-05

9. Fallico V, Ross RP, Fitzgerald GF, McAuliffe O (2011) Genetic response to bacteriophage infection in Lactococcus lactis reveals a four-strand approach involving induction of membrane stress proteins, D-alanylation of the cell wall, maintenance of proton motive force, and energy conservation. J Virol 85 (22):12032-12042. doi:10.1128/jvi.00275-11

10. Flores-Kim J, Darwin AJ (2016) The Phage Shock Protein Response. Annu Rev Microbiol 70:83-101. doi:10.1146/annurev-micro-102215-095359

11. Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB (2012) Genome sequence of the bacteriocin-producing strain Lactococcus garvieae DCC43. J Bacteriol 194 (24):6976-6977. doi:10.1128/jb.01864-12

12. Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB (2012) The maltose ABC transporter in Lactococcus lactis facilitates high-level sensitivity to the circular bacteriocin garvicin ML. Antimicrob Agents Chemother 56 (6):2908-2915. doi:10.1128/aac.00314-12

13. Hauge HH, Nissen-Meyer J, Nes IF, Eijsink VG (1998) Amphiphilic alpha-helices are important structural motifs in the alpha and beta peptides that constitute the bacteriocin lactococcin G--enhancement of helix formation upon alpha-beta interaction. Eur J Biochem 251 (3):565-572

14. Holo H, Jeknic Z, Daeschel M, Stevanovic S, Nes IF (2001) Plantaricin W from Lactobacillus plantarum belongs to a new family of two-peptide lantibiotics. Microbiology 147 (Pt 3):643-651. doi:10.1099/00221287-147-3-643

15. Holo H, Nes IF (1989) High-Frequency Transformation, by Electroporation, of Lactococcus lactis subsp. cremoris Grown with Glycine in Osmotically Stabilized Media. Appl Environ Microbiol 55 (12):3119-3123

16. Holo H, Nilssen O, Nes IF (1991) Lactococcin A, a new bacteriocin from Lactococcus lactis subsp. cremoris: isolation and characterization of the protein and its gene. J Bacteriol 173 (12):3879-3887

17. Jimenez JJ, Diep DB, Borrero J, Gutiez L, Arbulu S, Nes IF, Herranz C, Cintas LM, Hernandez PE (2015) Cloning strategies for heterologous expression of the bacteriocin enterocin A by Lactobacillus

sakei Lb790, Lb. plantarum NC8 and Lb. casei CECT475. Microb Cell Fact 14:166. doi:10.1186/s12934-015-0346-x
18. Jiménez JJ, Diep DB, Borrero J, Gútiez L, Arbulu S, Nes IF, Herranz C, Cintas LM, Hernández PE (2015) Cloning strategies for heterologous expression of the bacteriocin enterocin A by Lactobacillus sakei Lb790, Lb. plantarum NC8 and Lb. casei CECT475. Microbial Cell Factories 14 (1):166. doi:10.1186/s12934-015-0346-x
19. Kjos M, Oppegard C, Diep DB, Nes IF, Veening JW, Nissen-Meyer J, Kristensen T (2014) Sensitivity to the two-peptide bacteriocin lactococcin G is dependent on UppP, an enzyme involved in cell-wall synthesis. Mol Microbiol 92 (6):1177-1187. doi:10.1111/mmi.12632
20. Kristensen T, Nissen-Meyer J, Oppegård C, Bie E, Kjos M, Diep DB, Nes I Whole genome sequencing as a tool to elucidate the mechanism of action of AMPs. In: Gordon Research Conference on Antimicrobial Peptides, 2015.
21. O'Sullivan L, Ross RP, Hill C (2002) Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84 (5-6):593-604
22. Ovchinnikov KV, Chi H, Mehmeti I, Holo H, Nes IF, Diep DB (2016) Novel Group of Leaderless Multipeptide Bacteriocins from Gram-Positive Bacteria. Appl Environ Microbiol 82 (17):5216-5224. doi:10.1128/aem.01094-16
23. Ovchinnikov KV, Kristiansen PE, Straume D, Jensen MS, Aleksandrzak-Piekarczyk T, Nes IF, Diep DB (2017) The Leaderless Bacteriocin Enterocin K1 Is Highly Potent against Enterococcus faecium: A Study on Structure, Target Spectrum and Receptor. Front Microbiol 8:774. doi:10.3389/fmicb.2017.00774
24. Roces C, Campelo AB, Veiga P, Pinto JP, Rodriguez A, Martinez B (2009) Contribution of the CesR-regulated genes llmg0169 and llmg2164-2163 to Lactococcus lactis fitness. Int J Food Microbiol 133 (3):279-285. doi:10.1016/j.ijfoodmicro.2009.06.002
25. Rogne P, Fimland G, Nissen-Meyer J, Kristiansen PE (2008) Three-dimensional structure of the two peptides that constitute the two-peptide bacteriocin lactococcin G. Biochim Biophys Acta 1784 (3):543-554. doi:10.1016/j.bbapap.2007.12.002
26. Tymoszewska A, Diep DB, Wirtek P, Aleksandrzak-Piekarczyk T (2017) The Non-Lantibiotic Bacteriocin Garvicin Q Targets Man-PTS in a Broad Spectrum of Sensitive Bacterial Genera. Sci Rep 7 (1):8359. doi:10.1038/s41598-017-09102-7
27. Uzelac G, Kojic M, Lozo J, Aleksandrzak-Piekarczyk T, Gabrielsen C, Kristensen T, Nes IF, Diep DB, Topisirovic L (2013) A Zn-dependent metallopeptidase is responsible for sensitivity to LsbB, a class II leaderless bacteriocin of Lactococcus lactis subsp. lactis BGMN1-5. J Bacteriol 195 (24):5614-5621. doi:10.1128/jb.00859-13
28. van de Guchte M, van der Vossen JM, Kok J, Venema G (1989) Construction of a lactococcal expression vector: expression of hen egg white lysozyme in Lactococcus lactis subsp. lactis. Appl Environ Microbiol 55 (1):224-228

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Source or references
Strains		
L. lactis subsp. Lactis IL1403	Indicator strain for garvicin KS	(Bolotin et al. 2001)
L. lactis subsp. lactis MG1363	Indicator strain for garvicin KS	(Gasson 1983)
Lactobacillus sakei LMGT2799	Indicator strain for garvicin KS	(Jimenez et al. 2015)
L. garvieae KS1546	Garvicin KS producer	(Ovchinnikov et al. 2016)
L. garvieae LMGT3390	Garvicin ML producer	(Borrero et al. 2011)
L. sakei LMGT2334	Sakacin A producer	(Katla et al. 2003)
L. planetarium LMGT2379	Plantaricin W producer	(Holo et al. 2001)
L. lactis KSR2001-KSR2013	Spontaneous garKS^r mutants	This study
L. lactis IL11	Spontaneous LcnG^r mutant	(Kjos et al. 2014)
E. coli NEB 10-beta	*E. coli* cloning host	Invitrogen
L. lactis Δ2164	*pspA* and *pspC* complete deletion strain of MG136	(Roces et al. 2009)
Plasmids		
pMG36e	Expression vector, erythromycin resistance	(van de Guchte et al. 1989)
pCH0	pMG36e expressing Flag-tagged control peptide from P32 promoter	This study
pCH1	carrying pspC *gene* behind P32 promoter in pMG36e	This study
pCH2	pMG36e expressing N-terminally Flag tagged PspC from P32 promoter	This study

[a] Em, erythromycin

Table 2. Sequence of specific primers used in this study

Primer name	Sequence (5'-3') *	Purpose
PspF	CGTCGGGGGACTTGGTGATT	Amplification of from 8 garvicin KS resistant mutants
PspR	CCGGAAAGCCACAGATTTAACC	
PpspcF	CATAGAGCTCAGTCTATTGATGACACCGTC	Amplification of pspC from *L. lactis* IL1403
PpspcR	ATGTAAGCTTGGTCATGGTGGACCAGAATT	
PpspcFFLAGN	CATAGAGCTCAGGAGGTTAATTATGGACTACAAAGACGACGACGACAAGTCTCAAAGACAATTAACAAAATC	Amplification of pspC with flag-tag N from *L. lactis* IL1403
PpspcR	ATGTAAGCTTGGTCATGGTGGACCAGAATT	
PpspcFFLAGB	CATAGAGCTCAGGAGGTTAATTATGGACTACAAAGACGACGACGACAAGACCTGGTGATACCTATAATC	Amplification flag terminal from IL1403
PpspcRFLAGB	ATGTAAGCTTGTCTTACCTAGACCAGCTCC	

*Restriction sites (Sca I and Hind III) are underlined in the sequences.

Table 3. Mutations in *pspC* and effects on garvicin KS sensitivity

Mutants	MIC (BU/mL)	Mutation	Consequence
KSR2001, 2005, 2008	80	GGG → GAG	G21E
KSR2002	160	TAT → CAT	Y55H
KSR2010	80	CGT → GTT	R110V
KSR2004, 2006	40	GGG → GTG	G17V
KSR2003, 2012, 2013	80	TGG → TAG	W96*
KSR2007, 2009, 2011	80	AAG → TAG	K138*

The MIC for wild type *L. lactis* IL1403 is 5 BU/mL, corresponding to a mixture of 10 nM of each of the garvicin KS peptides.

Table 4. Binding of bacteriocins to cells and immunoprecipitation of bound bacteriocins.

	Garvicin KS	Garvicin KS separate peptides [a]	LcnG	LcnG separate peptides	nisin	Sakacin A	Plantaricin W
L. lactis IL1403	64	32	32	32	32	<8	<8
L. lactis IL1403 with pCH2	64	32	32	32	32	<8	<8
KSR2001	8	8	8	8	32	<8	<8
KSR2001 with pCH2	64	32	32	32	32	<8	<8
L. lactis IL11	64	32	32	32	32	<8	<8
L. lactis IL1403 with pCH2 immunoprecipitated	32	32	32	16	<8	<8	<8
KSR2001with pCH0 immunoprecipitated	<8	<8	<8	<8	<8	<8	<8
KSR2001with pCH2 immunoprecipitated	32	32	32	16	<8	<8	<8

Amount of bacteriocin bounds to cells in 10 mL culture or immunoprecipited from extracts from the same amount of cells. Bound bacteriocin is given in BU. One BU corresponds to 1.6 ng nisin and 18 ng garvicin KS peptides in 1:1:1 ratio. The detection limit was 8 BU.

[a]Bound to separate peptides. Bacteriocin activity measured after mixing of extracts.

Paper IV

Synergistic Antimicrobial Activity Between the Broad Spectrum Bacteriocin Garvicin KS and Nisin, Farnesol and Polymyxin B Against Gram-Positive and Gram-Negative Bacteria

Hai Chi & Helge Holo

Current Microbiology

ISSN 0343-8651

Curr Microbiol
DOI 10.1007/s00284-017-1375-y

Springer

Curr Microbiol
DOI 10.1007/s00284-017-1375-y

Synergistic Antimicrobial Activity Between the Broad Spectrum Bacteriocin Garvicin KS and Nisin, Farnesol and Polymyxin B Against Gram-Positive and Gram-Negative Bacteria

Hai Chi[1] · **Helge Holo**[1]

Received: 25 April 2017 / Accepted: 12 October 2017

Abstract The increasing emergence of antibiotics resistance is of global concern. Finding novel antimicrobial agents and strategies based on synergistic combinations are essential to combat resistant bacteria. We evaluated the activity of garvicin KS, a new bacteriocin produced by *Lactococcus garvieae*. The bacteriocin has a broad inhibitory spectrum, inhibiting members of all the 19 species of Gram-positive bacteria tested. Unlike other bacteriocins from Gram-positive bacteria, garvicin KS inhibits *Acinetobacter* but not other Gram-negative bacteria. Garvicin KS was tested in combination with other antimicrobial agents. We demonstrated synergy with polymyxin B against *Acinetobacter* spp. and *Escherichia coli*, but not against *Pseudomonas aeruginosa*. Similar effects were seen with mixtures of nisin and polymyxin B. The synergistic mixtures of all three components caused rapid killing and full eradication of *Acinetobacter* spp. and *E. coli*. In addition, garvicin KS and nisin also acted synergistically against *Staphylococcus aureus*, indicating different in modes of action between the two bacteriocins. Both bacteriocins showed synergy with farnesol, and the combination of low concentrations of garvicin KS, nisin and farnesol caused rapid eradication of all the *S. aureus* strains tested. Its broad inhibitory spectrum, rapid killing, and synergy with other antimicrobials makes garvicin KS a promising antimicrobial.

Keywords Synergy effect · Bacteriocin · Garvicin KS · Antimicrobials · Pathogens

✉ Helge Holo
helge.holo@nmbu.no

[1] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, POB 5003, 1432 Ås, Norway

Introduction

Infections caused by antibiotics resistant pathogens from both Gram-positive and Gram-negative bacteria have become of global concern. In addition, the slow discovery and development of new antibiotics is unable to catch up the rapid increasing of antibiotics resistance [5, 24]. Searching for alternative antimicrobials for inhibition and elimination of the antibiotics resistant pathogens as well as reducing resistance evolution is essential. Antimicrobial peptides represent a source of unexplored compounds with a potential to kill antibiotic resistant bacteria [27]. Their modes of action are not fully understood, but are different from the commonly used antibiotics. The antimicrobial peptides produced by bacteria are known as bacteriocins. Bacteriocins usually have narrow inhibitory spectra, but from Gram-positive bacteria several bacteriocins with wide inhibitory spectra are known. The bacteriocins of lactic acid bacteria are of particular interest, since many are being used in foods and their bacteriocins may contribute to enhance shelf life and food safety. Based on primary structure, these bacteriocins have been grouped into class I, lantibiotics containing modified residues, and class II without modified residues [6].

The best-studied of these bacteriocins is the lantibiotic nisin, which inhibits most Gram-positive bacteria but not Gram-negative [12, 30]. However, in the presence of compounds that can destabilize the outer membrane, even Gram-negatives are inhibited [14, 28].

We recently described garvicin KS, a bacteriocin produced by *Lactococcus garvieae* [24]. We report here on its antimicrobial spectrum, which includes Gram-negative bacteria. We show that it can act synergistically with other antimicrobials, including nisin, against several pathogenic bacteria. Moreover, the synergistic mixtures can improve killing kinetics reducing the risk of resistance development.

Published online: 20 October 2017

Materials and Methods

Bacterial Strains and Growth Conditions

Lactococci were grown in M17 broth (Oxiod, Hampshire, UK) supplemented with 0.4% (w/v) glucose (GM17), *Lactobacilli* and *Pediococci* were grown in deMan, Rogosa and Sharpe (MRS) (Oxoid, Hampshire, UK) medium at 30 °C. *E. coli, Pseudomonas aeruginosa*, and *Acinetobacter* strains were grown in Lysogeny broth (Oxoid, Hampshire, UK) or Mueller Hinton Broth (MHB) (BD Difco, MD, USA) at 37 °C with shaking at 225 rpm. Other strains were grown in Brain Heart Infusion (Oxoid, Hampshire, UK) broth at 30 °C.

Antimicrobial Agents

Nisin from *Lactococcus lactis* (2.5% purity), polymyxin B sulfate and farnesol were from Sigma-Aldrich, Co. (St. Louis, MO, USA). Crude garvicin KS was prepared by following procedure. 1 L of GM17 broth was inoculated with 1% of an overnight culture of *L. garvieae* KS1546 and incubated at 30 °C for 8 h. The cells were removed by centrifugation at 12,000×*g* for 25 min and 258 g of ammonium sulfate was dissolved in the cell-free supernatant, and the mixture was left at 4 °C for 24 h. The crude bacteriocin was precipitated by centrifugation at 15,000×*g* for 30 min at 4 °C, dissolved in 20 mL water and kept in a boiling water bath for 10 min.

Inhibition and Checkerboard Assays

Growth inhibition was determined in microtiter plates with 200 µL of growth medium in each well as described by Holo et al. [16]. The minimum inhibitory concentration (MIC_{50}) refers to the concentration of antimicrobial agent causing 50% growth inhibition, the MIC_{50} was detected once the OD_{600} of testing strains reaches 0.4–0.5. Bacteriocin unit [2] of garvicin KS was defined as the amount of garvicin KS causing 50% growth inhibition of *L. lactis* IL1403 in this assay. The checkerboard assays using mixture of antimicrobials were performed in microtiter plates as described above.

Interactions between antimicrobial agents were determined by using the fractional inhibition concentration (FIC). The FIC was calculated as follows: FIC = FICa + FICb + FICc, where the FICa means MIC of A in combination/MIC of A alone, FICb means MIC of B in combination/MIC of B, and FICc means MIC of C in combination/MIC of C alone. Effects were considered as synergistic if FIC was ≤0.5 for two components mixture [22] and ≤0.75 for three components mixture [3].

Time-Kill Assays

Time-kill assays were performed using cultures grown overnight and then diluted 100× in MHB with different concentrations of antimicrobial agents. The assays were done in triplicate. Total viable count (TVC) was determined by plating on Mueller Hinton agar. The agar plates were incubated at 37 °C, and the TVC was estimated after 24 h incubation. Interactions between antimicrobial agents were interpreted as synergistic when they in mixture caused ≥2-log decrease in TVC compared to the antimicrobial agents alone [25].

Results

Garvicin KS has a Broad Antimicrobial Inhibition Spectrum

Garvicin KS, produced by *L. garvieae* strain, is composed of three similar peptides [24]. Initially, its inhibitory activity was tested using an agar diffusion assay [24] on 250 indicator strains, of which 240 strains (19 species) were Gram-positive. All the Gram-positive bacteria tested, with the exception of three out of 53 *S. aureus* strains, were sensitive (results not shown) tested. The MICs of garvicin KS against a representative selection of the strains are shown in Table 1. Garvicin KS inhibited pathogens like *Listeria monocytogenes, S. aureus*, and vegetative cells of *Bacillus cereus*. The effect of garvicin KS on spores were not tested. The most sensitive bacteria were strains of the lactic acid bacteria *Lactococcus* and *Enterococcus*, with MIC ranging from 5 to 80 BU/mL. 5 BU/mL corresponds to a mixture of 10 nM of each of the pure peptides of garvicin KS [24]. Concentrations to inhibit other Gram-positive strains varied from 320 to 2560 BU/mL.

Notably, garvicin KS was inhibitory to *A. baumannii, A. iwoffii*, and *A. calcoaceticus* (MIC = 2560 BU/mL). However, other Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, did not show any sensitivity to garvicin KS in this assay.

Garvicin KS Acts Synergistically with Other Antimicrobials Against *S. aureus*

Staphylococcus aureus LMGT 3242 was the least sensitive Gram-positive bacterium tested in the microtiter assay, and in time-kill assays we were unable to fully prevent growth of this bacterium even with high concentration of garvicin KS. We therefore used this strain to investigate the effects of combining garvicin KS with other antimicrobials, including nisin, another bacteriocin with a wide inhibitory spectrum [1] and farnesol, shown to act as adjuvant to antimicrobials in *Staphylococci* [17]. Using the checkerboard assay, we

Table 1 MIC_{50} values of garvicin KS against different strains

Bacterial strain	MIC (BU/mL)
Acinetobacter baumannii B1162	2560
Acinetobacter calcoaceticus B1165	2560
Acinetobacter iwoffii B1163	2560
Bacillus cereus LMGT 2805	640–1280
Cellulomonas fimi LMGT3232	10–20
Escherichia coli LMGT 3704	NI
Enterococcus faecalis LMGT 2333	40–80
Enterococcus faecium LMGT 2763	20–40
Lactobacillus curvatus LMGT 2353	80
Lactobacillus plantarum LMGT 2003	80
Lactobacillus sakei LMGT 2361	320
Lactobacillus salivarius LMGT 2787	320–640
Lactococcus garvieae LMGT 3390	80
Lactococcus lactis IL1403	5
Lactococcus lactis LMGT 2122[a]	5
Leuconostoc gelidum LMGT 2386	640–1280
Listeria innocua LMGT 2710	80
Listeria ivanovii LMGT 2813	320
Listeria monocytogenes LMGT 2604	160
Pediococcus acidilactici LMGT 2002	320
Pediococcus pentosaceus LMGT 2001	640
Pseudomonas aeruginosa LMGT 3294	NI
Staphylococcus aureus LMGT 3242	2560
Streptococcus salivarius LMGT 3597	320

NI no inhibition

[a]Nisin producer

found synergy between nisin and garvicin KS (FIC = 0.22) and between bacteriocins and farnesol, (both with FICs of 0.47), and all three compounds in combination (FIC = 0.33) (Table 2). The three components mixture resulted in the reduction of MIC by a factor of 8 for farnesol, and by a factor of 10 for garvicin KS and nisin, respectively.

The effects of garvicin KS, nisin, and farnesol to *S. aureus* were also studied in time-kill assay. In the experiments, concentrations corresponding to the MIC (Table 2) were used. As shown in Fig. 1, none of the three antimicrobials alone could completely kill the bacteria even after 48 h exposure. When tested alone nisin and garvicin KS both caused an initial 3-log reduction in TVC before growth of survivors was detected. The mixtures nisin + garvicin KS caused a complete killing after about 12 h and no regrowth was seen even after 48 h, demonstrating the strong synergy between the two bacteriocins. Killing using the farnesol + garvicin KS mixture was less efficient, causing 4, 5 log reduction of CFU before the growth started. The farnesol + nisin mixture, on the other hand, gave complete killing after 12 h, as did the combination of all three compounds. We tested six other strains of *S. aureus*, including three MRSA, in the time-kill assay using the same concentrations as shown in Fig. 1. All strains were sensitive to nisin and garvicin KS, but none were eradicated by these bacteriocins alone or in combination. Only one of the six strains was completely killed by the mixture of nisin and farnesol. However, the mixture of farnesol, nisin, and garvicin KS caused complete killing of all six strains.

Antimicrobial Synergy Against Gram-Negative Bacteria

A unique feature of garvicin KS is that it has antimicrobial effects on *Acinetobacter* spp., but relatively high amount of garvicin KS was required to inhibit them. Recently, farnesol was found to be a good adjuvant with polymyxin B to *A. baumannii* [18], and like for *S. aureus*; we tested farnesol in mixture with garvicin KS for synergy in inhibiting *Acinetobacter* spp.. No synergy was seen with these combinations (data not shown). We therefore replaced farnesol with polymyxin B in the mixtures.

The *A. baumannii* and *A. calcoaceticus* strains tested were both sensitive to polymyxin B with a MIC of 0.63 µg/mL, while *A. iwoffii* with a MIC of 25 µg/mL is considered resistant [2] (Table 3). Synergy was seen between polymyxin B and garvicin KS against all three *Acinetobacter* strains tested, even the polymyxin B resistant *A. iwoffii*. Similar synergistic effects were seen between nisin and polymyxin B. The combinations of three antimicrobial agents also showed synergistic effects against all the *Acinetobacter* strains. Compared to the individual antimicrobials combining the three compounds caused about 10-fold and 15-fold MIC reductions for garvicin KS and polymyxin B, respectively (Table 3).

The effects on *A. baumannii* of nisin, garvicin KS, polymyxin B alone and in mixtures were assayed in time-kill

Table 2 MIC values of garvicin KS, nisin, farnesol alone and in combinations, and FICs of combinations against *Staphylococcus aureus* LMGT 3242

MIC[a]			MIC (FIC) in mixture			
Garvicin KS	Nisin	Farnesol	Garvicin KS + nisin	Nisin + farnesol	Garvicin KS + farnesol	Garvicin KS + nisin + farnesol
2560	25	0.6	312/2.50 (0.22)	5.00/0.16 (0.47)	512/0.16 (0.47)	256/2.50/0.08 (0.33)

[a]Concentrations are given in BU/mL for garvicin KS, µg/mL for nisin and mM for farnesol

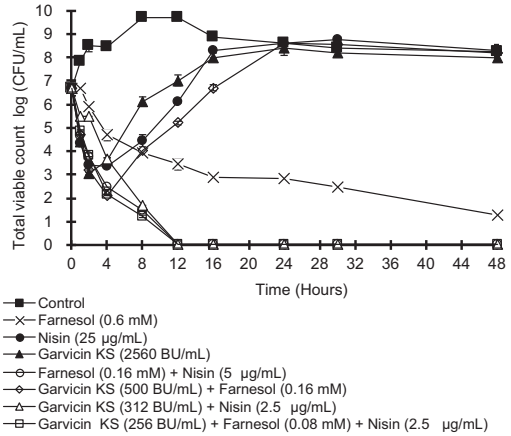

Fig. 1 Time-killing analysis reveals the antimicrobial effects of garvicin KS, nisin and farnesol against *S. aureus* LMGT 3242. Viable counts were determined in triplicate

assays using concentrations corresponding to the MIC values shown in Table 3. As shown in Fig. 2, garvicin KS and polymyxin B could both kill the bacteria, but regrowth was seen after about 8 h and an initial 2–3 log killing in either case. Mixing garvicin KS and polymyxin B prevented regrowth of the bacteria, but the nisin + polymyxin B mixture did not. The bacteria were rapidly killed by the mixture of the three components, reaching eradication after 4 h.

Prompted by the results using *Acinetobacter* strains, we extended the synergy studies to other Gram-negative bacteria. Garvicin KS and nisin were unable to kill *E. coli* alone, but the strain tested was sensitive to polymyxin B (MIC = 0.3 µg/mL). Both bacteriocins showed synergy with polymyxin B. A FIC of 0.4 was found for two- and three components mixture with polymyxin B (Table 3). The synergies were also demonstrated in time-kill assays (Fig. 3). Both bacteriocins improved the killing rates compared to polymyxin B alone, but regrowth was seen. The mixture of all three components, however, caused eradication of the bacteria after 8 h exposure.

We also tested synergy effects by using garvicin KS, nisin, and polymyxin B against *P. aeruginosa*. The MIC for polymyxin B against the strain tested was 0.8 µg/mL. However, there were no synergy effects on their combinations (data not shown).

Discussion

Garvicin KS is a new bacteriocin comprised of three similar peptides of 32–34 amino acids [24]. The bacteriocin shows a broad inhibitory spectrum encompassing all the Gram-positive genera tested. Such a wide spectrum of activity is uncommon among bacteriocins, and has only been reported for certain lantibiotics like nisin and lactacin 3147 [11]. Of note, unlike the lantibiotics, garvicin KS was able to inhibit Gram-negative bacteria of *Acinetobacter* genus. However, the sensitivity towards garvicin KS varied more than 500-fold between the bacteria tested. Relatively high amounts of garvicin KS were required to kill many of the pathogenic species tested, and these bacteria were not completely killed and regrew after initial killing.

Combining antimicrobial agents offers a potential for increasing antimicrobial treatment efficacy and for reducing resistance evolution, and the use of combination antimicrobial therapy is widely used in the treatment of serious infections [4]. Importantly, the individual bacteriocins may act more effectively in combination with other antimicrobials agents [6], and studies demonstrating such effects have recently been reviewed [19]. Here, we have shown that

Table 3 MIC values of garvicin KS, polymyxin B, and nisin alone and in combinations and FICs of combinations against *Acinetobacter* spp. and *Escherichia coli*

Bacterium	MIC[a]			MIC (FIC) in mixture			
	Garvicin KS	Nisin	Polymyxin B	Garvicin KS + nisin	Nisin + polymyxin B	Garvicin KS + polymyxin B	Garvicin KS + nisin + polymyxin B
A. baumannii B1162	2560	NI	0.63	2560/NI (2.0)	1.25/0.05 (0.08)	500/0.15 (0.44)	150/0.80/0.05 (0.20)
A. iwoffii B1163	2560	NI	25.0	2560/NI (2.0)	3.00/5.00 (0.20)	512/6.25 (0.45)	250/2.50/1.50 (0.16)
A. calcoaceticus B1165	2560	NI	0.63	2560/NI (2.0)	5.00/0.31 (0.50)	256/0.20 (0.42)	250/2.50/0.16 (0.35)
E. coli LMGT 3704	NI	NI	0.30	NI (X)	10.0/0.12 (0.40)	3200/0.12 (0.40)	3200/4.0/0.12 (0.40)

NI means no inhibition

X means not valid

[a]Concentrations are given in BU/mL for garvicin KS, µg/mL for nisin and polymyxin B

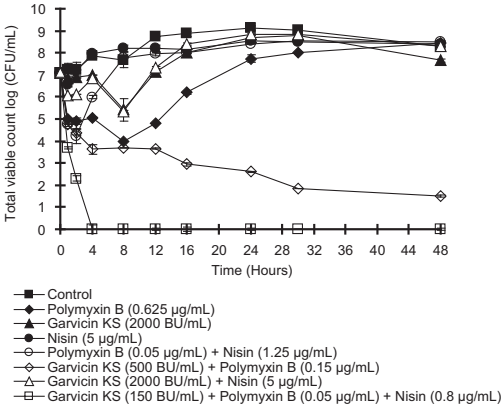

Fig. 2 Time-killing analysis reveals the antimicrobial effects of garvicin KS, nisin and polymyxin B against *A. baumannii* B1162. Viable counts were determined in triplicate

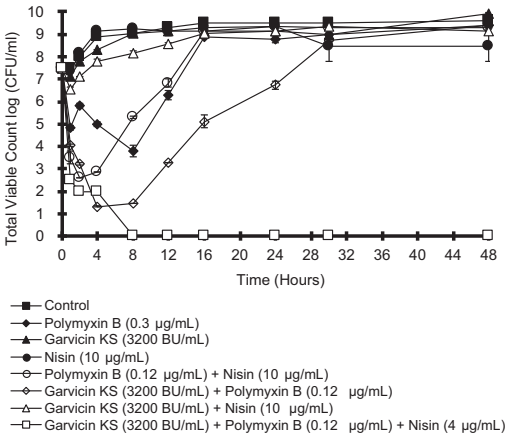

Fig. 3 Time-killing analysis reveals the antimicrobial effects of garvicin KS, nisin, and polymyxin B against *E. coli* LMGT 3704. Viable counts were determined in triplicate

garvicin KS is synergistic with other antimicrobial compounds, improving killing kinetics, and eradication and hence reducing resistance development. It is notable that garvicin KS could act synergistically with all the three compounds tested, indicating differences in mode of killing.

Importantly, garvicin KS showed synergy with polymyxin B, a drug of last resort in the combat infections by multidrug resistant Gram-negative bacteria but which is avoided due to its toxicity at relevant concentrations [9, 26]. Moreover, the emergence of resistance to polymyxins has been reported [8, 10]. Synergy between polymyxins and nisin and lacticin 3147, both bacteriocins from Gram-positive bacteria, against Gram-negative bacteria has been reported [13, 20, 21]. The synergy can be attributed to disruption of the outer membrane by polymyxin B allowing access of the bacteriocins to their target [7]. Polymyxin B is used for treatment of nosocomial infections mostly caused by *A. baumannii*, but because surviving bacteria are frequently found most patients are recommended to receive combination therapy of polymyxin B with other agents active against *A. baumannii* [23, 29]. The potential benefits of combining with new bacteriocins, like garvicin KS that act synergistically are obvious. Importantly, the concentration of polymyxin B needed to eradicate the bacteria was significantly reduced in combination with garvicin KS and nisin and much lower than the dosage recommended for polymyxin B therapy [29].

Strong synergy between nisin and garvicin KS was also observed against *S. aureus*. The synergy indicates that garvicin KS and nisin have different modes of action, which is different from classical antibiotics. Importantly, against *S. aureus* they were both synergistic with farnesol, a cheap and harmless compound which has been considered a promising adjuvant for antibiotics [15]. Moreover, the mixture of farnesol, nisin and garvicin KS completely killed all the *S. aureus* tested.

In conclusion, garvicin KS is a promising antimicrobial agent. We have demonstrated activity against a wide variety of bacteria, including pathogenic species known to account for a large number nosocomial infections, often with multi-resistant strains. The mode of action of garvicin KS is unknown, but different from many other used antimicrobials. Furthermore, garvicin KS mixtures with other antimicrobial compounds can be highly efficient by improving killing kinetics and eradication hence lower resistance development.

Acknowledgements Hai Chi is financially provided by China Scholarship Council, and his project (2016M03) is supported by Special Scientific Research Funds for Central Non-profit Institutes (East China Sea Fisheries Research Institute).

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

References

1. Arques JL, Rodriguez E, Langa S, Landete JM, Medina M (2015) Antimicrobial activity of lactic acid bacteria in dairy products and gut: effect on pathogens. Biomed Res Int 2015:584183. doi:10.1155/2015/584183
2. Bergen PJ, Bulman ZP, Landersdorfer CB, Smith N, Lenhard JR, Bulitta JB, Nation RL, Li J, Tsuji BT (2015) Optimizing polymyxin combinations against resistant Gram-negative bacteria. Infect Dis Ther 4(4):391–415. doi:10.1007/s40121-015-0093-7
3. Bhusal Y, Shiohira CM, Yamane N (2005) Determination of in vitro synergy when three antimicrobial agents are combined against *Mycobacterium tuberculosis*. Int J Antimicrob Agents 26(4):292–297. doi:10.1016/j.ijantimicag.2005.05.005
4. Bollenbach T (2015) Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. Curr Opin Microbiol 27:1–9. doi:10.1016/j.mib.2015.05.008
5. Carlet J, Jarlier V, Harbarth S, Voss A, Goossens H, Pittet D (2012) Ready for a world without antibiotics? The pensieres antibiotic resistance call to action. Antimicrob Resist Infect Control 1(1):11. doi:10.1186/2047-2994-1-11
6. Cotter PD, Ross RP, Hill C (2013) Bacteriocins—a viable alternative to antibiotics? Nat Rev Microbiol 11(2):95–105. doi:10.1038/nrmicro2937
7. Delcour AH (2009) Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta 1794(5):808–816. doi:10.1016/j.bbapap.2008.11.005
8. Di Pilato V, Arena F, Tascini C, Cannatelli A, Henrici De Angelis L, Fortunato S, Giani T, Menichetti F, Rossolini GM (2016) mcr-1.2, a new mcr variant carried on a transferable plasmid from a colistin-resistant KPC carbapenemase-producing *Klebsiella pneumoniae* strain of sequence type 512. Antimicrob Agents Chemother 60(9):5612–5615. doi:10.1128/aac.01075-16
9. Falagas ME, Kasiakou SK (2006) Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. Crit Care 10(1):R27. doi:10.1186/cc3995
10. Falagas ME, Rafailidis PI, Matthaiou DK, Virtzili S, Nikita D, Michalopoulos A (2008) Pandrug-resistant *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections: characteristics and outcome in a series of 28 patients. Int J Antimicrob Agents 32(5):450–454. doi:10.1016/j.ijantimicag.2008.05.016
11. Field D, Cotter PD, Ross RP, Hill C (2015) Bioengineering of the model lantibiotic nisin. Bioengineered 6(4):187–192. doi:10.1080/21655979.2015.1049781
12. Field D, O' Connor R, Cotter PD, Ross RP, Hill C (2016) In vitro activities of nisin and nisin derivatives alone and in combination with antibiotics against *Staphylococcus* biofilms. Front Microbiol 7 (508). doi:10.3389/fmicb.2016.00508
13. Field D, Seisling N, Cotter PD, Ross RP, Hill C (2016) Synergistic nisin-polymyxin combinations for the control of *Pseudomonas* biofilm formation. Front Microbiol 7:1713. doi:10.3389/fmicb.2016.01713
14. Gao Y, van Belkum MJ, Stiles ME (1999) The outer membrane of Gram-negative bacteria inhibits antibacterial activity of brochocin-C. Appl Environ Microbiol 65(10):4329–4333
15. Gomes F, Leite B, Teixeira P, Cerca N, Azeredo J, Oliveira R (2011) Farnesol as antibiotics adjuvant in *Staphylococcus epidermidis* control in vitro. Am J Med Sci 341(3):191–195. doi:10.1097/MAJ.0b013e3181fcf138
16. Holo H, Nilssen O, Nes IF (1991) Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J Bacteriol 173(12):3879–3887
17. Jabra-Rizk MA, Meiller TF, James CE, Shirtliff ME (2006) Effect of farnesol on *Staphylococcus aureus* biofilm formation and antimicrobial susceptibility. Antimicrob Agents Chemother 50(4):1463–1469. doi:10.1128/aac.50.4.1463-1469.2006
18. Kostoulias X, Murray GL, Cerqueira GM, Kong JB, Bantun F, Mylonakis E, Khoo CA, Peleg AY (2016) Impact of a cross-kingdom signaling molecule of *Candida albicans* on *Acinetobacter baumannii* physiology. Antimicrob Agents Chemother 60(1):161–167. doi:10.1128/aac.01540-15
19. Mathur H, Field D, Rea MC, Cotter PD, Hill C, Ross RP (2017) Bacteriocin-antimicrobial synergy: a medical and food perspective. Front Microbiol 8:1205. doi:10.3389/fmicb.2017.01205
20. Naghmouchi K, Baah J, Hober D, Jouy E, Rubrecht C, Sane F, Drider D (2013) Synergistic effect between colistin and bacteriocins in controlling Gram-negative pathogens and their potential to reduce antibiotic toxicity in mammalian epithelial cells. Antimicrob Agents Chemother 57(6):2719–2725. doi:10.1128/aac.02328-12
21. Naghmouchi K, Drider D, Baah J, Teather R (2010) Nisin A and polymyxin B as synergistic inhibitors of Gram-positive and Gram-negative bacteria. Probiotics Antimicrob Proteins 2(2):98–103. doi:10.1007/s12602-009-9033-8
22. Neu HC, Fu KP (1978) Synergy of azlocillin and mezlocillin combined with aminoglycoside antibiotics and cephalosporins. Antimicrob Agents Chemother 13(5):813–819
23. Ouderkirk JP, Nord JA, Turett GS, Kislak JW (2003) Polymyxin B nephrotoxicity and efficacy against nosocomial infections caused by multiresistant Gram-negative bacteria. Antimicrob Agents Chemother 47(8):2659–2662
24. Ovchinnikov KV, Chi H, Mehmeti I, Holo H, Nes IF, Diep DB (2016) Novel group of leaderless multipeptide bacteriocins from Gram-positive bacteria. Appl Environ Microbiol 82(17):5216–5224. doi:10.1128/aem.01094-16
25. Peck KR, Kim MJ, Choi JY, Kim HS, Kang CI, Cho YK, Park DW, Lee HJ, Lee MS, Ko KS (2012) In vitro time-kill studies of antimicrobial agents against blood isolates of imipenem-resistant *Acinetobacter baumannii*, including colistin- or tigecycline-resistant isolates. J Med Microbiol 61(Pt 3):353–360. doi:10.1099/jmm.0.036939-0
26. Siddiqui NU, Qamar FN, Jurair H, Haque A (2014) Multi-drug resistant Gram negative infections and use of intravenous polymyxin B in critically ill children of developing country: retrospective cohort study. BMC Infect Dis 14:626. doi:10.1186/s12879-014-0626-9
27. Steckbeck JD, Deslouches B, Montelaro RC (2014) Antimicrobial peptides: new drugs for bad bugs? Expert Opin Biol Ther 14(1):11–14. doi:10.1517/14712598.2013.844227
28. Stevens KA, Sheldon BW, Klapes NA, Klaenhammer TR (1991) Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl Environ Microbiol 57(12):3613–3615
29. Zavascki AP, Goldani LZ, Li J, Nation RL (2007) Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. J Antimicrob Chemother 60(6):1206–1215. doi:10.1093/jac/dkm357
30. Zendo T (2013) Screening and characterization of novel bacteriocins from lactic acid bacteria. Biosci Biotechnol Biochem 77(5):893–899. doi:10.1271/bbb.130014

ISBN: 978-82-575-1494-5
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

96716 / ANDVORDGRAFISK.NO