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

Philosophiae Doctor (PhD)
Thesis 2023:74

Bacteriocins: from discovery to characterization and applications

Bakteriociner: fra identifisering til karakterisering og anvendelse

Thomas Førland Oftedal

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To Tine,
Who have been a constant source of love and encouragement.

To the late Prof. Dzung B. Diep
For his dedication to his students and to science, for his belief in others, and for his endless curiosity.

Thomas Førland Oftedal
Ås, July 2023

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Abbreviations and definitions

BCG – Bacteriocin gene cluster
RiPP – Ribosomally synthesized and post-translationally modified peptide
NRP – Non-ribosomal peptide
PTM – Post-translational modification
GRAS – Generally Recognized as Safe
MoA – Mode of action
TMS – Transmembrane segment
VRE – Vancomycin-resistant enterococci
aa – amino acids
LAB – Lactic acid bacteria
ECF – extracytoplasmic function
TCS – two-component regulatory system
EntK1 – Enterocin K1
EntEJ97 – Enterocin EJ97
EntEJ97s – Enterocin EJ97 short
EntQ – Enterocin Q
H1 – Hybrid 1
Man-PTS – Mannose phosphotransferase system
RseP – Regulator of sigma-E protease
S1P – Site-1 protease
S2P – Site-2 protease
I-CLiP – Intramembrane cleaving proteases
Ecs - effect on exoproteins, defect in competence and sporulation
RseP – Regulator of sigma-E protease
AMR – Antibiotic resistance

List of papers

Paper I

Oftedal, T. F., Ovchinnikov, K. V., Hestad, K. A., Goldbeck, O., Porcellato, D., Narvhus, J., Riedel, C. U., Kjos, M., & Diep, D. B. (2021). Ubericin K, a new pore-forming bacteriocin targeting mannose-PTS. Microbiology Spectrum, 9(2), e00299-21. doi: https://doi.org/10.1128/Spectrum.00299-21

Paper II

Oftedal, T. F.*, Rosenbergová, Z.*, Ovchinnikov, K. V., Thiyagarajah, T., Rebroš, M., & Diep, D. B. (2022). Identification of a novel two-peptide lantibiotic from *Vagococcus fluvialis*. Microbiology Spectrum, 10(4), e00954-22. doi: https://doi.org/10.1128/spectrum.00954-22

Paper III

Oftedal, T. F.*, Ovchinnikov, K. V.*, Reich, S. J., Bar, N. S., Holo, H., Skaugen, M., Riedel, C. U. and Diep, D. B. (2022). Genome-assisted identification, purification, and characterization of bacteriocins. Bio-protocol 12(14): e4477. doi: https://doi.org/10.21769/BioProtoc.4477.

Paper IV

Oftedal, T. F., Kjos, M. & Diep, D. B. (2023). Design of novel saposin-like bacteriocins with antimicrobial activity using a hybrid approach. Manuscript.

Paper V

Oftedal, T. F.*, Kristensen, S. S.*, Røhr, Å. K., Eijsink, V. G. H., Mathiesen, G., & Diep, D. B. (2022). The extracellular domain of site-2-metalloprotease RseP is important for sensitivity to bacteriocin EntK1. Journal of Biological Chemistry, 102593. doi: https://doi.org/10.1016/j.jbc.2022.102593

Paper VI

Oftedal, T. F. & Diep, D. B. (2023). Flow cytometric detection of vancomycin-resistant *Enterococcus faecium* in urine using fluorescently labelled enterocin K1. Scientific Reports 13, 10930. doi:https://doi.org/10.1038/s41598-023-38114-9

* These authors contributed equally to their respective works.

Abstract

Multidrug resistant bacteria have become a major concern in both human and veterinary medicine. Due to the waning efficacy of many antibiotics, new antimicrobial agents are needed. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria to kill other bacteria. Bacteriocins are typically membrane-active antimicrobials with a mechanism of action that differs from antibiotics and are therefore equally potent against antibiotic-resistant strains as their susceptible counterparts. Bacteriocins generally exhibit low toxicity, low bioaccumulation, and a narrow spectrum of inhibition, making them a promising alternative or supplement to antibiotics.

Papers I-IV focus on the discovery of novel bacteriocins. **Paper I** describes the discovery, purification, and initial characterization of a novel bacteriocin, ubericin K. In this study, samples of raw bovine milk were screened for the presence of bacteria producing bacteriocins inhibiting *Enterococcus faecalis*, *Streptococcus dysgalactiae*, or *Staphylococcus aureus*. These species are implicated as the causative agents in bovine mastitis, and bacteriocins have previously been shown to be able to reduce the incidence of mastitis in dairy cows. Additionally, *S. aureus* and *E. faecalis* are opportunistic pathogens also in humans. In **Paper II**, a bacteriocin producer that inhibited a multidrug resistant strain of *E. faecium*, an emerging opportunistic human pathogen, was found. The bacteriocin named vagococcin T was identified as a two-peptide lantibiotic and shown to be active also against *Listeria monocytogenes* and *E. faecalis*. **Paper III** describes the techniques and methods used in our laboratory for purifying and identifying new bacteriocins and investigating their mode of action. In **Paper IV**, we show that leaderless bacteriocins can be engineered to change their target organism and potency. This was achieved by constructing a library of hybrid peptides consisting of the N- and C-terminal halves of saposin-like leaderless bacteriocins. We hypothesized that these peptides have N-terminal and C-terminal halves with distinct properties related to membrane insertion and the recognition of a molecular target. Using *in vitro* protein synthesis and spot-on-lawn assays, we identified novel peptides with a spectrum and potency that differed from the parental peptides. We believe that these hybrid bacteriocins are good candidates for future therapeutics and/or probes.

There are some major challenges to the clinical application of bacteriocins, such as their susceptibility against proteases, low solubility under physiological conditions, and rapid clearance from blood/plasma. An alternative to using bacteriocins as therapeutic agents is to use them as "molecular probes" for the detection of their target organism(s). For example, the bacteriocin enterocin K1 (EntK1) exhibits high potency only towards *E. faecium*, a specificity determined by a receptor protein, RseP, on target cells. The high potency is believed to be due to a high binding affinity to the receptor.

In **Paper V,** the interaction between EntK1 and its receptor, RseP, was studied in detail using sensitivity and binding experiments. In contrast to UbeK and VcnT, EntK1 is a small, unmodified, and leaderless bacteriocin ideally suited for synthetic production and chemical modification. In this study, we show that the binding of EntK1 to cells solely depends on RseP and that the spectrum of activity of EntK1 is due to the subtle sequence differences in RseP between species.

The potential of EntK1 as a diagnostic "probe" was also explored. Despite recent advances in clinical and diagnostic technologies, diagnosing a given infection is often time-consuming and complex. The diagnosis of an infection and identification of the causative microorganism can often only be obtained by molecular detection or traditional culture-based techniques. Because of this, infections are frequently treated blindly with broad-spectrum antibiotics. The unnecessary use of antibiotics is undesirable due to the spread of resistance and off-target killing of non-pathogenic (good) bacteria. Rapid and cost-effective diagnostic methods could reduce health costs and antibiotic use.

In **Paper VI,** we employed EntK1, which was chemically synthesized with an N-terminal fluorescent tag, to develop a flow cytometry-based detection method for *E. faecium*. The method was shown to be highly specific for *E. faecium*, which was detected with higher fluorescent signals than *S. aureus* and *E. coli*. Further, the detection method was evaluated on urine samples containing less than 10^5 CFU/ml of bacteria, the laboratory diagnostic criterium for a urinary tract infection. Samples containing *E. faecium* were positively identified and could be distinguished from samples containing *S. aureus* or *E. coli*. The work presents a proof of concept for using bacteriocins as specific probes for rapid detection and diagnosis.

Norsk sammendrag

Multiresistente bakterier er blitt et betydelig problem i både human- og veterinærmedisin. På grunn av redusert effekt av mange antibiotika, er det behov for nye antimikrobielle midler. Bakteriociner er ribosomalt-syntetiserte antimikrobielle peptider produsert av bakterier, for å drepe andre bakterier. Bakteriociner er typisk membran-aktive forbindelser med virkningsmekanismer som er forskjellig fra antibiotika. De er derfor like effektive mot multiresistente som antibiotika-sensitive bakterier. Bakteriociner generelt viser lav toksisitet, liten grad av opphoping i miljøet, og de har et smalt hemmingsspekter, Tilsammen gjør dette bakteriociner til et lovende alternativ eller supplement til antiobika.

Artikkel I-VI er fokusert på identifisering av nye bakteriociner. **Artikkel I** beskriver identifiseringen, isolering, og initiell karakterisering av et nytt bakteriocin som vi kaller ubericin K (UbeK). I denne studien ble prøver av upasteurisert melk undersøkt for tilstedeværelsen av bakteriocinproduserende bakterier som kunne hemme *Enterococcus faecalis*, *Streptococcus dysgalactiae*, og *Staphylococcus aureus.* Dette er arter som forårsaker mastitt hos kyr, og bakteriociner har tidligere vært vist å kunne redusere forekomsten av slike infeksjoner. *S. aureus* og *E. faecalis* er også opportunistiske patogene bakterier hos mennesker. I **Artikkel II** ble ble det oppdaget en bakteriocinprodusent som hemmet en multiresistent stamme av *E. faecium*, en patogen som er et økende problem hos mennesker. Bakteriocinet, navngitt vagococcin T (VcnT), ble identifisert som et to-peptide lantibiotikum og vist å være aktiv mot *Listeria monocytogenes* og *E. faecalis*. **Artikkel III** beskriver de teknikkene og metodene som anvendes av vår forskningsgruppe for identifisering og karakterisering av nye bakteriociner. I **Artikkel IV** viser vi at lederløse bakteriociner kan konstrueres syntetisk til å endre målorganisme og antimikrobiell aktivitet. Dette ble muliggjort ved å lage et bibliotek av hybrid-bakteriociner som bestod av N- og C-terminale halvdelene til saposin-lignende lederløse bakteriociner. Vi antok at disse peptidene har N- og C-terminale halvdeler med distinkte funksjoner, relatert til membraninnsettelse og reseptor-gjenkjenning. Ved bruk av *in vitro* proteinsyntese og spot-on-lawn analyse, identifiserte vi nye peptider med hemmingsspektere og antimikrobiell aktivitet som var forskjellig fra bakteriocinene de er satt sammen av. Disse hybrid-bakteriocinene er gode kandidater for videre bruk til behandling eller diagnostikk.

Det er store utfordringer ved bruk av bakteriociner til behandling av infeksjoner, for eksempel høy sensitivitet mot proteaser, lav løselighet, og hyppig utskillelse fra blodet. Et alternativ til å bruke bakteriociner for behandling er å bruke dem som gjenkjennelsesmolekyler ("markører") for deteksjon av deres målorganisme. For eksempel viser bakteriocinet enterocin K1 (EntK1) god aktivitet bare mot *E. faecium*, en spesifisitet som kommer av et reseptor protein, RseP, på målorganismen. Den gode aktiviteten er antatt å være på grunn av en sterk binding mellom bakteriocinet og reseptoren.

I **Artikkel V** blir interaksjonen mellom bakteriocinet EntK1 og dens reseptorprotein RseP studert i detalj, ved bruk av sensitivitet og bindingsanalyser. I motsetning til UbeK og VcnT, er EntK1 et lite, umodifisert, og lederløst bakteriocin som enkelt kan syntetiseres og modifiseres. I tillegg så har EntK1 et veldig smalt hemmingsspektrum hovedsakelig mot *E. faecium*. I denne studien viser vi at bindingen mellom EntK1 og målcellen bare er avhengig av RseP, og at hemmingsspekteret kommer av sekvensforskjeller i RseP mellom arter.

Potensialet til EntK1 som et gjenkjennelsesmolekyl for deteksjon ble også utforsket. På tross av nylige utviklinger innen medisin og diagnose, er diagnostiseringen av infeksjoner ofte tidkrevende og vanskelig. Diagnose av en infeksjon og identifiseringen av den forårsakende mikroorganismen kan ofte bare gjøres ved bruk av molekylære metoder eller ved kultivering av mikroorganismen. På grunn av dette er infeksjoner ofte behandlet med bredspektret antibiotika uten å identifiere organismen. Unødvendig bruk av antibiotika er ikke ønskelig på grunn av spredningen av antibiotika-resistens og påvirkningen slike antibiotika-kurer har på den sunne tarmfloraen. Raske og kostnadseffektive diagnostiske metoder kan redusere bruken av antibiotika og byrden på helsesystemet.

I **Artikkel VI** utnyttet vi EntK1, som ble kjemisk syntetisert og modifisert med et fluorescent molekyl festet til N-terminus, til å utvikle en flowcytometri-basert analysemetode for deteksjon av *E. faecium*. Metoden ble vist å være veldig spesifikk for *E. faecium*, som kunne bli detektert med høyere fluorescens-signaler enn *S. aureus* og *E. coli*. Videre ble metoden testet på urinprøver som inneholdt mindre enn 10^5 CFU/ml bakterier, et mye brukt diagnostisk kriterium for urinveisinfeksjon. Urinprøver som inneholdt *E. faecium* ble identifisert, og det kunne skilles mellom prøver som inneholdt *E. faecium* og prøver med *S. aureus* eller *E. coli*. Dette arbeidet beviser konseptuelt hvordan bakteriociner kan brukes for rask deteksjon og diagnose.

1 Introduction

Virtually all forms of life produce antimicrobial proteins and peptides as part of their defense against competing or invading organisms. The first report of an antimicrobial protein has been credited to André Gratia, who was investigating agents capable of killing bacteria [1]. In 1925, he observed growth inhibition in *E. coli* from one strain caused by another virulent strain [1]. Later characterization of the antimicrobial, which was named colicin V, revealed that it was heat-labile, proteinaceous, and gene-encoded [2]. Subsequently, the term "bacteriocin" was introduced for toxic proteins produced by bacteria that kill related species but not the producer [2]. Although research on bacteriocins continued, it was overshadowed by the discovery of the antibiotic penicillin in 1928. A primary reason why penicillin became such a revolutionary drug was its ability to inhibit a wide range of bacterial species, making it possible to treat bacterial infections even when the causative agent was unknown.

The discovery and development of new antibiotics continued during the twentieth century, and antibiotics quickly became an essential part of medicine. Antibiotics have not only contributed to increasing the average lifespan of humans by over 20 years, but these compounds have also enabled medical advancements such as organ transplants, cancer treatments, and various surgeries [3]. Unfortunately, an increasing number of bacteria are becoming resistant to many of the antibiotics currently in use. Antimicrobial resistance among bacteria is not a new phenomenon, *Streptococcus pyogenes* resistant to sulfonamide emerged in hospitals in the 1930s, and *Staphylococcus aureus* resistant to penicillin was found in London hospitals shortly after penicillin was introduced in the 1940s [4,5]. Similarly, *Mycobacterium tuberculosis* resistant to streptomycin emerged only a few years after streptomycin was first isolated, while multidrug resistance was detected in *Escherichia coli*, *Shigella*, and *Salmonella* in the 1950s and early 1960s [6,7]. However, antimicrobial resistance garnered little attention at the time, especially in the industrialized world. Possibly because resistant strains were primarily a problem in developing nations, but also because new antibiotics and antibiotic classes were frequently being discovered and characterized during 1945–1965, a period often referred to as the "golden age of antibiotic discovery" [3]. This allowed alternative antibiotics to be used to treat infections caused by resistant strains.

Since then, antibiotic resistance (AMR) has become widespread and turned some of the most ubiquitous bacteria into high-priority pathogens. In 2017, the World Health Organization (WHO) published a press release announcing 12 families of bacteria that posed the greatest threat to human health [8]. Among the high-risk pathogens were vancomycin-resistant *Enterococcus faecium* (see section 1.4) and methicillin-resistant *S. aureus*. The threat posed by these bacteria is primarily due to their resistance to multiple existing antimicrobial agents. Because of this, these bacteria are capable of causing untreatable infections that are often deadly. A report published in 2022 estimated that 1.27 million (0.911–1.71; 95% CI) deaths in 2019 were directly attributable to AMR [9]. The rise and dissemination of AMR globally is believed to be caused by the overuse and improper use of antibiotics in both human medicine and agriculture. To deal with the global health crisis posed by AMR, the WHO and numerous other organizations have stressed the importance of developing new antimicrobial agents.

The immense costs and regulatory hurdles of bringing new drugs to market practically restrict such developments to large pharmaceutical companies [10]. However, these companies are governed by financial incentives, and new antimicrobials must compete with all other products such companies may want to develop. In fact, new antibiotics are deemed unprofitable by most pharmaceutical companies [10]. At least two factors contribute to this unprofitability: I) antibiotics are typically only used by patients in small quantities over a short period of time, and II) the use of new antimicrobials will likely be restricted to emergency use only as a last resort [11]. In contrast, drugs for chronic illnesses can be prescribed to patients over decades. Since the golden age of antibiotic discovery in the early 1960s, only four new classes of antimicrobials have been developed, and most new antibiotics are chemical modifications of existing scaffolds [11,12]. Additionally, none of the new antibiotic classes have made much difference to the pharmaceutical market or the AMR crisis [11].

As more antibiotics lose efficacy and few new useful antibiotics are being developed, alternative strategies for treating microbial infections must be explored. Promising alternatives include bacteriophages and antimicrobial peptides (AMP), which are agents that kill bacteria with a mechanism different from antibiotics [13]. Especially promising are bacterial AMPs, known as bacteriocins, because of their high potency (also towards antibiotic-resistant strains), broad- or narrow-spectrum activity, and stability [14].

1.1 Bacteriocins

In the natural environment of the producer cells, a diversity of microbial species exists together in communities that compete for the same nutrients and niche space. The ecological role of bacteriocins is still unclear, but they are believed to provide the producer with a competitive advantage. Bacteriocins may function as offensive agents, enabling the invasion of one producing strain into another microbial community. Alternatively, bacteriocins may assist the producing strain in defending its niche from invasion by other bacteria. Many bacteriocins are narrow-spectrum, inhibiting only species closely related to the producer. The narrow targeting is often achieved by the requirement of specific molecular targets, such as "docking molecules" or "receptors", on target cells. In Gram-positive bacteria, proteins known to be targeted by bacteriocins are typically membrane-embedded proteins with an extracellularly exposed domain. The producer is immune to its own bacteriocin, typically by dedicated immunity proteins that are co-expressed with its cognate bacteriocin.

Almost all bacteriocins are initially synthesized as a longer precursor peptide encoded by a structural gene (see **Figure 1**). An N-terminal extension called a leader sequence or leader peptide is attached to the core peptide, and the core peptide becomes the active or "mature" bacteriocin. The leader sequence is important for immunity and for directing the peptide to enzymes for modification and export. Removal of the leader sequence can occur as part of the secretion process by dedicated ABC-transporters with a peptidase domain, or after secretion by extracellular proteases. The cleavage of leader sequences typically occurs at a double-glycine motif or a glycine-alanine motif.

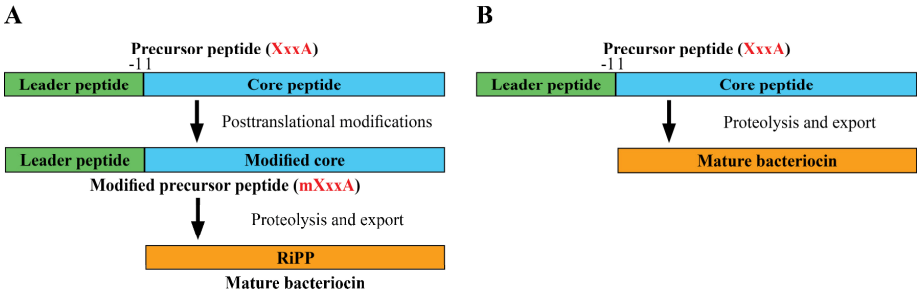

Figure 1. General biosynthetic pathway and terminology of bacteriocins. (A) bacteriocins that are posttranslationally modified, also known as ribosomally synthesized and post-translationally modified peptides (RiPPs). The precursor peptide is designated "A" and encoded by the gene *xxxA*. The modified precursor is given the prefix m and abbreviated mXxxA. (B) Unmodified bacteriocins.

To effectively inhibit the invasion of a competing strain, bacteriocin must be produced in sufficient quantity, which imposes a significant metabolic burden on the producer. Indeed, the production of bacteriocins is often regulated by a quorum-sensing mechanism. The quorum sensing regulation ensures a concerted effort by the entire population only when the population has reached a certain density. Depending on the number of different competitors in each niche, the population may benefit from producing broad-spectrum bacteriocins versus narrow-spectrum bacteriocins. The prevalence of narrow-spectrum bacteriocins is likely indicative of the high degree of specialization within bacteria. Evolutionary modeling suggests that narrow-spectrum bacteriocins provide the most benefit to the producer in a majority of niches [15]. Narrow-spectrum bacteriocins focus their attack on the most direct competitors, whose ecological niche has the greatest overlap with the bacteriocin producer [15]. In addition, it greatly minimizes the loss of bacteriocin peptides to non-relevant targets with little niche overlap.

Generally, broad-spectrum antimicrobials have been preferred for infection treatment because they allow for the treatment of infections when the causative agent is not known. However, increasing attention to the importance of "healthy" and commensal bacteria favors narrow-spectrum antimicrobials like bacteriocins. In addition, the overuse of broad-spectrum antibiotics has been suggested as a primary selection pressure for the rise and dissemination of antibiotic resistance genes [16]. Thus, antibiotic use should be reduced or avoided as much as possible to counteract the rise of antibiotic resistance. Although bacteriocin resistance is also widespread in nature, some bacteriocins exploit important cellular targets, where resistance

may come at a considerable fitness cost [17]. One such example is the membrane protein RseP, a bacterial protease involved in mediating environmental signaling important for stress tolerance and virulence in many pathogens [18].

1.2 Classification of Bacteriocins

In contrast to antibiotics, which are secondary metabolites made by multi-function/domain enzymes known as non-ribosomal peptide synthetases (NRPSs), bacteriocins are directly derived from gene-encoded peptides that are translated by the ribosome. Bacteriocins are also often extensively modified after translation, and these peptides are known as ribosomally synthesized and post-translationally modified peptides (RiPPs).

Numerous efforts have been made to systematically classify and organize bacteriocins. Various proposed classification schemes have been suggested based on their chemical structure, mechanism of action, spectrum of activity, biosynthetic similarities, phylogenetic relationships, and so on [19–25]. A simple and/or universal classification system has been difficult because of overlaps in both structural and genetic characteristics between peptides that differ in other important aspects [26]. In addition, very little is still known about the molecular targets (receptors) and mechanism of action of bacteriocins (see section 1.2.5). In recent years, the low molecular weight bacteriocins primarily produced by lactic acid bacteria (LAB) have gained the most attention, and bacteriocins from Gram-positive species, primarily LAB, have been the basis for current classification schemes.

LAB constitute a widespread group of Gram-positive bacteria in nature, including genera such as *Streptococcus*, *Lactococcus*, *Enterococcus,* and *Vagococcus*, that is associated with food preservation and fermentation, but also colonizes the cavities of humans and animals [27,28]. LAB are characterized by their ability to metabolize carbohydrates to produce lactic acid and numerous other metabolites with desirable nutritional and sensory properties. For this reason, LAB has been extensively used in food production and preservation for centuries. The acid produced by LAB lowers the pH of the environment, thus making it unfavorable for foodborne pathogenic bacteria. In addition, LAB has been shown to produce a plethora of bacteriocins, further inhibiting pathogenic and food spoilage bacteria. Due to the presence of LAB in human food for centuries, many LAB strains have been given GRAS (generally recognized as safe) status [29].

A comprehensive classification scheme is presented in **Figure 2**, which is intended to include bacteriocins from both Gram-positive and Gram-negative bacteria [26].

As stated previously, bacteriocins with a low molecular weight (less than 10 kDa) are classified as class I if they are post-translationally modified (RiPPs), or class II if they are unmodified [26]. The class I bacteriocins comprise the largest and most diverse collection of bacteriocins. Currently, class I bacteriocins are subdivided into at least the following subclasses, most of which are named after their characteristic modifications: lantibiotics, head-to-tail cyclized bacteriocins, sactibiotics, linaridins, thiopeptides, glycocins, bottromycins, cyanobactins, lasso-peptides, linear azol(in)-containing peptides, siderophore-containing bacteriocins, and nucleotide-peptide bacteriocins [26]. The class II unmodified bacteriocins are subdivided into: IIa) pediocin-like, IIb) two-peptide, IIc) leaderless bacteriocins, and IId) non-pediocin-like single peptides.

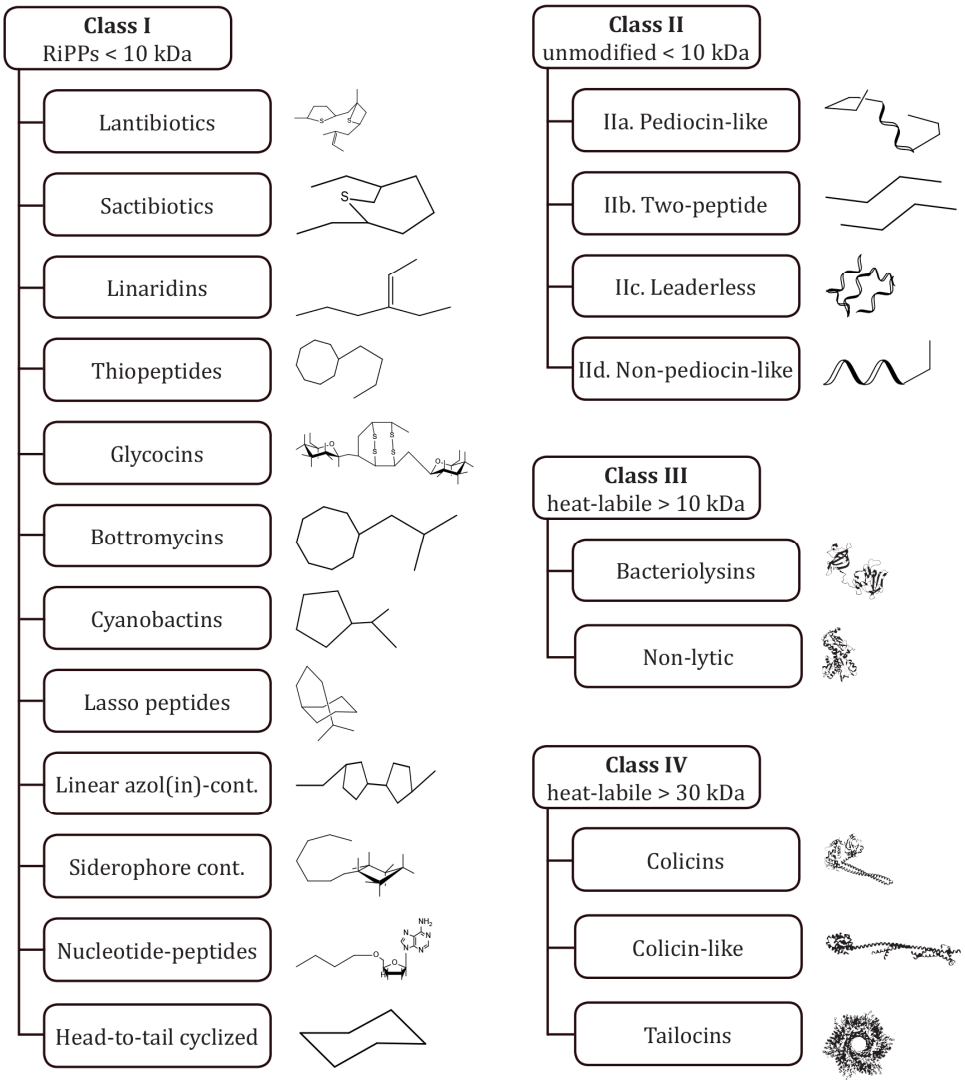

Figure 2. Classification scheme for bacteriocins (cont.: containing).

In the following subsections, the class I (lantibiotics), IId (non-pediocin-like single peptides), and IIc (leaderless bacteriocins) will be covered in depth since these are particularly relevant to the work presented in this thesis. The remaining subclasses will only be described briefly. The large and heat-labile bacteriocins are placed in class III (> 10 kDa) or class IV (>30 kDa) will not be covered here.

1.2.1 Class I bacteriocins

1.2.1.1 Lantibiotics

Lantibiotics constitute a subgroup of class I bacteriocins, defined by the presence of β-thioether crosslinks, which are added posttranslationally from a dehydrated Ser/Thr residue to a Cys residue. The biosynthesis of lantibiotics follows the general scheme for RiPPs (see **Figure 1A**). The enzyme performing the dehydration reaction is designated LanB, the cyclase LanC, and the exporter and peptidase are designated LanT and LanP respectively. Immunity to lantibiotics is conferred by a dedicated immunity protein, LanI, often in conjunction with an ABC-transporter, LanFE(G). Lantibiotic production may be regulated by quorum sensing using a dedicated two-component system, LanRK, consisting of a response regular (R) and histidine kinase (K) that are often autoregulated (activated by the lantibiotic) as presented in **Figure 3** (see section 1.3.1).

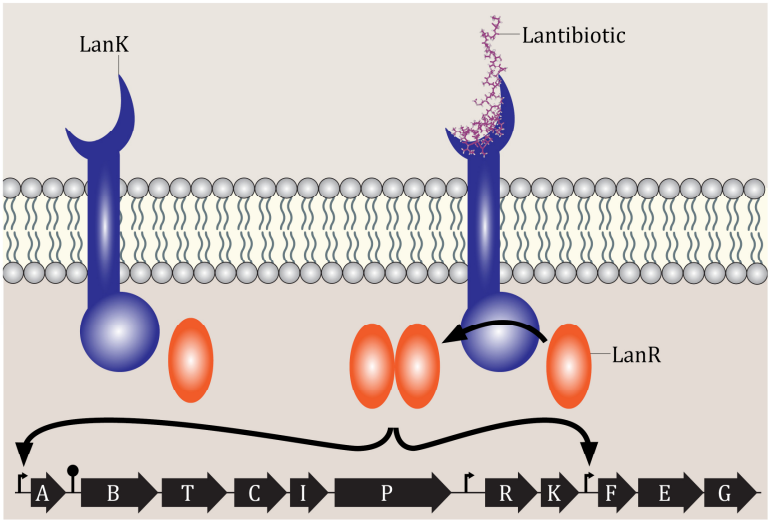

Figure 3. General scheme for the quorum sensing and autoregulatory mechanism of lantibiotic production. A transmembrane histidine kinase (LanK) senses the mature lantibiotic, resulting in the activation of a response regulator (LanR), typically by dimerization. Activated LanR binds to the promoter regions of biosynthetic genes to activate transcription.

The unifying structural features of the lantibiotics are the thioether crosslinks and the mechanism by which they are installed. The first step involves the dehydration of either a serine or a threonine residue, which results in didehydroalanine (Dha) or

didehydrobutyrine (Dhb), respectively. The subsequent addition of a cysteine to the dehydrated serine produces a lanthionine (Lan) or a methyllanthionine (MeLan) if the dehydrated amino acid is a threonine. The presence of a (methyl)lanthionine derived from a dehydrated of Ser/Thr attacked by a cysteine is the defining characteristic of lantibiotics; however, the dehydration process is carried out differently within lantibiotics. For this reason, lantibiotics have been grouped into four subclasses: I, II, III and IV (see **Figure 4**).

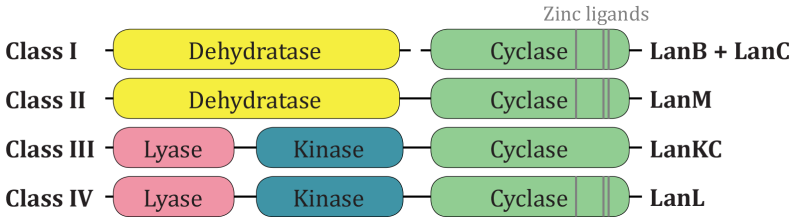

Figure 4. Overview of lanthipeptide synthetases that define the four subclasses of lanthipeptides.

Subclass I lantibiotics are produced by dedicated dehydratase (LanB) and cyclization (LanC) proteins, subclass II lantibiotics are made by a single dual-function enzyme designated LanM with both a dehydratase- and cyclase domains. Subclass III and IV lantibiotics are made by a trifunctional enzyme containing a lyase-, kinase- and cyclase domain (LanKC and LanL). Subclass III and IV are distinguished by the presence of a zinc-binding motif in LanL that is absent in LanKC. Many subclass III lantibiotics display no or little antimicrobial activity and therefore are not strictly lantibiotics; for this reason, many are named with the suffix "-peptin" (e.g., labyrinthopeptins) as opposed to "-cin" or "-cidin" which implies killing (e.g., cinnamycin) [30,31].

In 1928, it was observed that one strain of *L. lactis* inhibited *Lactobacillus bulgaricus* in milk fermentations, leading to the discovery of nisin, the founding member of the lantibiotics (Nisin; Group N [*Streptococcus*] inhibitory substance) [32,33]. The potential of nisin was realized soon after its discovery, as it was shown to be small, heat-stable, and displayed a wide inhibition spectrum encompassing many Gram-positive species [34]. It was not until the late 80s and early 90s that the genetic determinants of nisin and its production became known. In this strain, the nisin biosynthetic genes were located on a conjugative transposon (Tn*5276*) and organized as four operons, *nisABTCIPRK*, *nisI*, *nisRK,* and *nisFEG* [34]. The nisin structural gene (*nisA*) and biosynthetic machinery are regulated by a NisRK two-

component system together with *nisFEG*. The remaining operons are constitutive [34]. Nisin A is initially synthesized as a precursor peptide of 57 aa (NisA), NisBC then performs dehydration and cyclization to the precursor peptide. The modified precursor (mNisA/prenisin) is exported by NisT before the leader sequence is removed by the cell-wall anchored NisP. Biologically active nisin A features five thioether rings formed by 1 Lan and 4 MeLan, and contains 1 Dhb, 2 Dha, and 21 unmodified residues. The potent activity of nisin depends on lipid II, an essential precursor molecule in the biosynthesis of peptidoglycan in the bacterial cell wall. The structure of nisin forms a pocket, or "cage", with high binding affinity for the pyrophosphate groups on lipid II. Nisin has a dual mode of action, at low concentrations, nisin is thought to primarily inhibit the peptidoglycan synthesis pathway by binding to and sequestering lipid II. As the concentration of nisin increases, lipid II-nisin complexes assemble into pores in membrane, thus resulting in membrane depolarization [35].

The nisin producer is immune to the action of nisin due to the concerted effort of the immunity proteins NisI and NisFEG. NisFEG is an ABC-transporter that maintains a low concentration of nisin in the membrane by active expulsion. NisI is a 245 aa lipoprotein anchored to the extracellular side of the membrane that directly sequesters secreted nisin before it reaches the cell membrane [36].

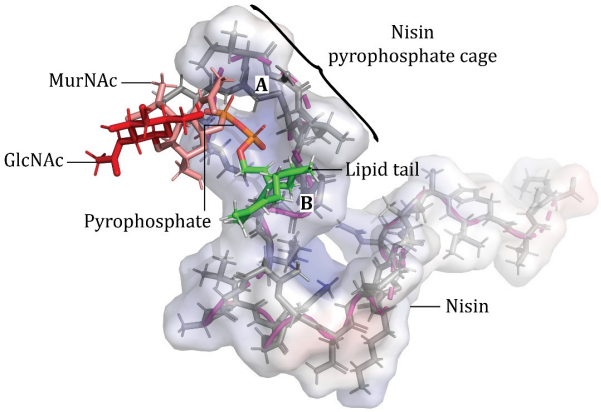

Figure 5. Pyrophosphate cage formed by rings A and B of nisin formed around the pyrophosphate moiety of a modified variant of lipid II (containing 3 isoprene units instead of 11). The pentapeptide attached to MurNAc is not shown. Based on PDB 1WCO [37].

The lipid II binding motif, the "pyrophosphate cage", of nisin is formed by the first two rings of nisin (see rings A and B in **Figure 5**); ring structures of lantibiotics are designated alphabetically in order of appearance (from N- to C terminus) [37,38].

The same motif is present in numerous lantibiotics with the same mode of action as nisin, referred to as the nisin-group, such as subtilin, gallidermin, mutacin 1140, NAI-107, and bovicin HC5 [39]. A different structural motif binds lipid II by members of the mersacidin-group of lantibiotics. Members of this group appear to bind the head group of lipid II as opposed to the pyrophosphate moiety [39]. The mersacidin-group contains a conserved acidic residue (E/D) in the lipid II-binding ring, and they do not induce membrane damage like members of the nisin-group [39]. Instead, members of the mersacidin-group are potent inhibitors of cell wall biosynthesis and include lacticin 481, nukacin ISK-1, mutacin II, and plantaricin C.

An interesting addition to the previously mentioned single-peptide lantibiotics are the two-peptide lantibiotics, where a second peptide acts synergistically to exacerbate the effect of the other peptide [40]. Two-peptide lantibiotics consist of an α- and β-peptide (Lanα and Lanβ), where the α-peptide is lipid II-binding but unable to permeabilize the membrane. Only in the presence of the β-peptide is pore formation believed to occur. The α-peptides of the two-peptide lantibiotics lacticin 3147 and haloduracin contain mersacidin-like lipid II-binding motifs but, in the presence of the β-peptide will permeabilize the membrane, presumably by pore formation. A complex formed between the α-peptides and lipid II is believed to recruit the β-peptide which assembles into a pore-forming complex like that observed for the nisin-group lantibiotics.

1.2.1.2 Other class I bacteriocins

Head-to-tail cyclized bacteriocins are peptides translated with 2-48 residues leader sequences. Following enzymatic removal of the leader sequence, the new N-terminus is covalently linked by a peptide bond to the C-terminus end [41]. It is still unknown which enzymes catalyze the leader peptide removal and cyclization of circular bacteriocins [41,42]. The resulting circular bacteriocins have many desirable properties for practical applications, such as especially good stability against peptidases. Currently, at least 14 circular bacteriocins are described, including enterocin AS-48, garvicin ML and enterocin NKR5-3B. Although all circular bacteriocins vary considerably in sequence, most appear to share a similar saposin-like fold structure (containing 4-5 α-helices in a compact arrangement to form a

small hydrophobic core) [43]. Circular bacteriocins are believed to exert their antimicrobial action through direct interaction with the bacterial membrane [42,44]. However, sensitivity to garvicin ML is to some degree dependent on a maltose ABC-transporter [45].

Sactibiotics contain unusual thioether cross-links like the lantibiotics (see section 1.2.1.1) but are unique in that the cross-links are between a cysteine and an α-carbon as opposed to a β-carbon [46]. Examples in this group include subtilosin A, thuricin CD, thurincin H, and propionicin F. Unlike most lantibiotics, the spectrum of activity of sactibiotics is relatively narrow, suggesting a mode of action unrelated to lipid II and lantibiotics [46].

Linaridins (linear + arid) are bacteriocins that contain dehydrated residues like dehydrobutyrine, similar to the lantibiotics, but are linear and have a very different biosynthesis [47]. In addition, the type A linaridin cypemycin is N-terminally methylated, and has a heterocyclic S-2-aminovinyl-d-cysteine (AviCys) group at the C-terminus [47]. Only six members of this class of bacteriocins have been described in some detail, cypemycin, grisemycin, legonaridin, mononaridin, salinipeptins, and corynaridin [48]. Corynaridin has a bacteriocidal mode of action without pore-formation [48].

Thiopeptides are extensively modified sulfur-containing cyclic peptides, most of which inhibit bacterial protein synthesis [49]. Modifications common to thiopeptides include a central pyridine ring, as well as dehydropiperidine and hydroxypyridine [50]. Most thiopeptides also contain dehydrated amino acids, and oxazole and thiazole ring structures. For this reason, the thiopeptides were believed for over six decades to be antibiotics and are often referred to as antibiotics to this day [51]. Aided by bioinformatics tools, a structural gene for thiocillin was discovered in 2009 [50,52]. The structural gene was shown to encode a 52 aa precursor with a 38 aa leader sequence, resulting in a core peptide of only 14 aa. Characterization of the biosynthetic gene cluster found 24 different genes involved in the production and maturation of the thiopeptide, which was further shown to carry out 13 different posttranslational modifications to the core peptide.

One of the best-characterized members of the thiopeptide family is micrococcin (P1), which has been shown to inhibit protein synthesis by directly binding to ribosomal protein L11 [53,54]. The binding to L11 prevents the proper interaction

between ribosomal proteins L11 and L7, which consequently inhibits the binding and hydrolysis of elongation factor EF-G [53]. The protein EF-G is essential for translation elongation by catalyzing GTP-dependent ribosomal translocation. Target cells are thus inhibited by the cessation of protein synthesis. Immunity to micrococcin P1 is conferred by alternative variants of the ribosomal protein L11 that replace the native protein [55].

How thiopeptides enter target cells to reach the ribosome is not well understood, no surface receptor molecule has been identified, and they exhibit a wide inhibition spectrum, including many diverse Gram-positive species [49,56]. Therefore, thiopeptides are believed to cross the membrane of Gram-positive bacteria spontaneously due to their small size and high hydrophobicity. Gram-negative bacteria are protected by an outer membrane containing lipopolysaccharide that effectively sequester hydrophobic molecules. Some thiopeptides can target the Gram-negative bacteria *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in these bacteria, the bacteriocins exploit the outer membrane siderophore receptors FpvA, FpvB, and FoxA to gain entry into the cells [57–61].

Glycocins are glycosylated bacteriocins, meaning that the core peptide contains at least one sugar moiety [62]. The sugar group is covalently attached to a cysteine, threonine, or serine. Currently characterized glycocins include glycocin F (GccF), sublancin (SunA), thurandacin A (ThuA) and enterocin F4-9. GccF is a 43 aa core peptide covalently linked to N-acetylglucosamine at S^{18} and N-acetylhexosamine at C^{43} [63]. GccF and enterocin F4-9 are said to be 'glycoactive' because at least one sugar is essential for antimicrobial activity [62]. This differs from SunA and ThuA which are both active without the glucosyl moiety. The mechanism by which glycocins inhibit growth is not known, but a glucose PTS transporter is implicated as a receptor for GccF, and a glucose transporter PtsG is involved in sensitivity to SunA [64,65].

Bottromycins are a group of highly modified small peptides with sequence and structure similarities to bottromycin, the first member to be purified from a culture of *Streptomyces bottropensis*, isolated from the region of Bottrop, Germany [66]. Bottromycins bind to the A site of the 50S bacterial ribosome, thus blocking translation [67]. Bottromycin precursor peptides (BotA) lack the N-terminal leader sequence that is common for most bacteriocins, but instead have a follower peptide, a C-terminal extension that does not become part of the mature peptide. The

follower peptide is important for recognition by some of the biosynthetic enzymes, such as BotP, which removes the N-terminal methionine in the first step of bottromycin biosynthesis. Later removal of the follower peptide results in a core peptide derived from only 8 residues (GP-V/A/L-VVFDC). All bottromycins characterized so far are produced by *Streptomyces* spp. [67].

Cyanobactins is the name given to a class of cyclic peptides isolated from various marine animals, primarily ascidians (tunicates/sea squirts; sac-like invertebrate filter feeders) [68]. These cyclic peptides are characterized by azole/azoline rings and prenyl groups [69]. The source of these peptides was unknown for 25 years, until the cyanobacterium *Prochloron* was shown to produce some of them. Although the producing organisms for most of these peptides remain to be identified, they are all believed to be produced by symbiotic cyanobacteria living in the ascidians, hence the name "cyanobactins" [70]. However, of the more than 100 cyanobactins identified to date, only a few have been demonstrated to be produced by cyanobacteria.

Lasso-peptides are characterized by an unusual structure where the C-terminus of the peptide is sterically locked in a macrocyclic ring formed by seven to nine N-terminal residues [71]. The compact, "locked" topology confers high thermal and peptidase stability to the lasso peptides [72]. Well known members include microcin J25 (MccJ25) and lariatin. Different molecular targets ("receptors") have been implicated in the mode of action of some lasso-peptides, such as RNA polymerase, lipid II and ClpC protease [73–75].

Nucleotide peptide bacteriocins contain a nucleotide as part of their structure, the only member characterized to date is microcin C (previously also called microcin C7, C51, or C7/C51). MccC is the smallest known bacteriocin and encoded by the smallest known gene in *E. coli*, encoding only 7 amino acids (heptapeptide) [76]. Microcin C is translated without any leader sequence, and post-translationally modified with a covalent linkage of a nucleotide (adenosine monophosphate) at a C-terminal aspartate residue [30]. The mechanism of action of microcin C is highly unique among bacteriocins. The peptide gains entry into Gram-negative cells using the outer membrane porin OmpF and the inner membrane transporter YejABEF [77]. To become active, microcin C has to undergo a two-step process inside the target cell: first, the formyl group is removed from the N-terminal methionine (N-formylmethionine) by a deformylase [77]. Second, an aminopeptidase cleaves the

peptide bond Ala^{6}-Asp^{7} to release a non-hydrolyzable molecular mimic of aspartyl adenylate. The resulting molecule inhibits aspartyl-tRNA synthase, which leads to inhibition of translation [77]. Self-immunity to microcin C is conferred by an acetyltransferase (MccE) and carboxypeptidase (MccF) which inactivate the bacteriocin by acetylation and/or hydrolysis, respectively [78].

Siderophore bacteriocins are antimicrobial peptides decorated with a siderophore molecule, thereby exploiting siderophore receptors/importers to gain access to target organisms [79]. The mechanism exploited by siderophore bacteriocins to enter cells has been called a "trojan horse" strategy, as the bacteriocin mimics the natural iron-siderophore complexes that surface receptors (FepA, Fiu, Cir, and IroN) recognize [79,80]. These bacteriocins are primarily produced by the enterobacteria *E. coli* and *Klebsiella pneumoniae*, therefore often referred to as siderophore-microcins [79]. Characteristic members are microcin E492, MccH47, and MccM. Upon interaction with the target cell, these peptides cause membrane depolarization, either by targeting the mannose phosphotransferase system (Man-PTS) resulting in pore formation, or by targeting the ATP synthase, causing unregulated passage of protons [79,80].

Linear azol(in)-containing (LAPs) bacteriocins contain thiazol(in)e and/or (methyl)oxazol(in)e heterocyclic structures, arising from the cyclization of Cys and Ser/The residues. Representative members of the LAPs subclass are microcin B17, klebsazolicin, and plantazolicin. Although these bacteriocins share similar chemical features, they differ considerably in mode of action. Microcin B17 is known to target and inhibit DNA gyrase, thus inhibiting DNA replication [81]. Plantazolicin targets the membrane, and klebsazolicin inhibits the ribosome [82,83].

1.2.2 Class II bacteriocins

Most class II bacteriocins possess a conserved double-glycine cleavage motif in their leader sequences (positions -1 and -2). Although the defining characteristic of class II bacteriocins is their lack of PTMs (see **Figure 1B**), some have disulfide bridges that are essential for activity and dedicated "chaperone"-like proteins that ensure that the correct disulfide bonds are formed [84]. Members of this class are generally amphipathic, hydrophobic, and cationic.

Class IIa pediocin-like bacteriocins are peptides of approximately 35 to 50 residues, that are characterized by a conserved YGNG(V/L) motif at the N-terminal end ("pediocin-box") [85]. These peptides exploit the mannose-phosphotransferase system (Man-PTS) to kill target cells. The interactions of the bacteriocins with Man-PTS result in pore formation, dissipation of membrane potential, depletion of ATP, and loss of essential ions and amino acids [86]. Over 90 members of class IIa are currently known, and they range in size from 17 aa (bacteriocin PE-ZYB1) to 58 aa (acidocin A) [87,88]. Interestingly, most pediocin-like bacteriocins exhibit potent anti-listerial activity [89].

Class IIb two-peptide bacteriocins consists of two peptides (α and β) that exhibit the highest antimicrobial activity when assayed in equimolar concentrations but have little- to- no activity individually. An interesting feature of these peptides is the presence of GxxxG-like motifs (or variations where G is substituted with A/S, which is the case for plantaricin Sβ and plantaricin NC8β). These motifs are involved in helix-helix interactions and are important for activity. For example, plantaricin EF consists of the two peptides PlnE and PlnF (33 and 34 residues, respectively). PlnE has two GxxxG motifs while PlnF has a GxxxG-like motif, SxxxG. In artificial membrane-mimicking environments, the PlnEF peptides interact in an anti-parallel orientation. PlnEF causes cation efflux in exposed cells via a receptor protein CorC, a predicted magnesium/cobalt exporter. The mechanism of action of PlnEF is to some degree distinct from the related plantaricin JK (PlnJK), which has been reported to cause efflux of anions via a receptor protein of the APC transporter family [90]. Furthermore, a membrane-spanning protein UppP (undecaprenyl pyrophosphate phosphatase, or BacA) is involved in the mode of action of other class IIb bacteriocins, including lactococcin G and enterocin 1071 [91].

1.2.2.1 Linear non-pediocin bacteriocins (IId)

All class II bacteriocins not belonging to any of the other subclasses (see section 1.2.2.2 for class IIc) are collectively referred to as the linear non-pediocin-like bacteriocins (class IId). For this reason, this class of bacteriocins contains peptides with a wide diversity in both structure, biosynthesis, and mode of action. Members vary in size from 7 to over 100 amino acids, some are exported by the Sec-pathway (for example, lactococcin 972 and enterocin P) and some are produced by Gram-negative bacteria, such as microcin N [92–94]. A subgroup within this class, known as the lactococcin A subgroup, uses Man-PTS as a receptor [26]. The mode of action

of lactococcin A (LcnA), the founding member of this subgroup, has been studied in some detail (see section 1.2.5).

1.2.2.2 Leaderless bacteriocins (IIc)

Leaderless bacteriocins are directly synthesized on the ribosome into their bioactive forms [41]. Thus, the amino acid sequence of the bacteriocin structural gene (the open reading frame) corresponds exactly to the purified and isolated bacteriocin. Interestingly, this means that most leaderless bacteriocins start with a N-formylmethionine residue [26]. As of 2022, at least 39 leaderless bacteriocins have been described, and they include single-, two-, three- and four-peptide bacteriocins (e.g., enterocin K1, enterocin DD14, garvicin KS, and aureocin A70, respectively) [26]. How bacteria can synthesize bacteriocins without a leader sequence is not well-understood. It is known that leader sequences serve as a recognition signal for biosynthetic enzymes and keep the bacteriocin inactive prior to export and full deployment of immunity. It also remains unknown how producer cells protect themselves intracellularly. This is especially puzzling because most leaderless bacteriocins are not believed to require a receptor protein or docking molecule [41]. The only exception are members of the LsbB family of leaderless bacteriocins (see section 1.2.3).

Aureocin A53 (Aur53) family are a family of highly cationic and tryptophan-rich peptides. Some members of this family are presented in **Figure 6** and include epidermicin NI01 (EpiNI01), lactolisterin BU (LliBU), BHT-B, and lacticin Q (LnqQ).

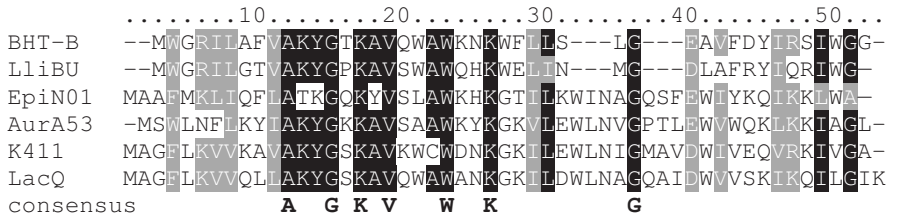

Figure 6. Multiple sequence alignment of some members of the AurA53-group of leaderless bacteriocins. Conserved residues are shown as a consensus. Alignment was performed using Clustal Omega and colored using BoxShade [95,96].

Aur53 is a 51 aa bacteriocin produced by *S. aureus* A53. The biosynthetic genes are located on a plasmid pRJ9 and organized as three operons, consisting of the AurA53 structural gene *aucA*, an ABC transporter *aurEFG*, and a pair of immunity proteins *aucIB* and *aucIA*. Many leaderless bacteriocin gene clusters are small, as only a transport and immunity protein are necessary in addition to the structural gene. AurA53 is most potent against *Micrococcus luteus*, but is also active against *E. faecium*, *L. innocua* and *S. aureus* [97].

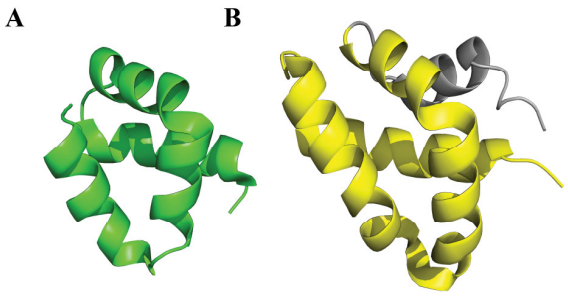

Figure 7. Structural comparison of (A) AurA53 (PDB: 2N8O), and (B) saposin D (PDB: 2RB3).

AurA53 and several other members of this family have been shown to share a saposin-like fold structural motif (see **Figure 7**). The saposin-fold is comprised of a compact bundle of 4-5 α-helices that form two V-shaped "wedges". Saposins are small (10-20 kDa) membrane-interacting proteins that play an important role in sphingolipid metabolism in humans.

Enterocin L50 (EntL50) family contains the two-peptide bacteriocins enterocin L50 (EntL50A and EntL50B) and enterocin MR10 (EntMR10A and EntMR10B), and the single-peptide salivaricin C, and weissellicin Y (see **Figure 8**). EntL50 was the first leaderless bacteriocin to be reported (1998) [98–101]. Several other leaderless two-peptide bacteriocins with various names have been described in the literature but are identical or nearly identical in sequence to EntMR10 (such as enterocin DD14; EntDD14A and EntDD14B, and enterocin 7; Ent7A and Ent7B) [41]. Both the two-peptide and multi-peptide leaderless bacteriocins have very simple gene clusters. Two operons are responsible for EntL50 biosynthesis, one operon contains the two structural genes (*entL50AB*) and one contains the immunity protein and transporter (*orfCD*).

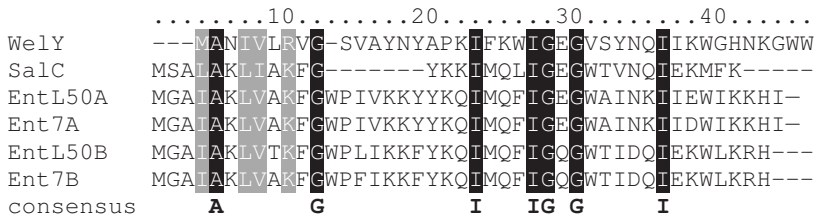

Figure 8. Multiple sequence alignment of the EntL50 family of bacteriocins. Conserved residues are shown as a consensus in bold. Alignment was performed using Clustal Omega and colored using BoxShade [95,96].

Multipeptide leaderless bacteriocins comprise a family of bacteriocins whose activity depends on three to four different peptides for optimal activity [102,103]. Current members include garvicin KS (GarKS), aureocin A70 (AurA70), and cereucins V, X and H (CerV, CerX, and CerH). The four-peptide leaderless bacteriocin aureocin A70 was described already in 2001, however, several new members were described recently [103]. All multipeptide leaderless bacteriocins described thus far are transcribed together in tandem, and only require an ABC transporter and immunity protein to be produced, although the gene cluster for garvicin KS (GarKS) also encodes a putative transcription regulator with a DNA-binding motif [103]. In contrast to GarKS, which is chromosomally encoded, the genes for AurA70 are encoded on an 8 kb mobilizable plasmid, pRJ6, in *S. aureus* A70. GarKS is produced by *L. garvieae* KS1546, isolated from bovine raw milk [103,104].

GarKS is composed of the three peptides GakA, GakB and GakC (32, 34 and 34 aa), that exhibit considerable synergy. Against *L. lactis* IL1403 the individual peptides have a MIC of 0.36, >12, and 6 μM respectively, while the combination gives a MIC of 10 nM (corresponding to a 36-fold increase compared to the most active peptide GakA) [103]. Importantly, no synergy is observed by combining any pair of peptides, which confirms the three-peptide nature of GarKS. Similarly, the individual peptides of CerX all have a MIC > 6 μM, while it is 45 nM for the combination (>130-fold). Surprisingly, the three peptides of GarKS show considerable sequence identity with each other, especially at the N-terminal half. GarKS exhibit an especially broad inhibition spectrum, inhibiting most Gram-positive species tested (e.g., *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium*, *E. durans*, *P. pentosaceus*, *L. garvieae*, *B. cereus*, *Listeria*) [103]. No molecular target (receptor) necessary for sensitivity to multipeptide leaderless bacteriocins has so far been found.

1.2.3 LsbB family of leaderless bacteriocins

The LsbB family of bacteriocins is a small group of sequence-related leaderless peptides that, in contrast to what is known for other leaderless bacteriocins, exploit a molecular target in order to kill sensitive cells [105–107]. The molecular target (receptor) for all members of the LsbB family, including synthetic derivatives, is the membrane-bound protease RseP (see section 1.3.3). The four native members of this bacteriocin family are LsbB (lactococcal small bacteriocin B), enterocin K1 (EntK1), enterocin Q (EntQ) and enterocin EJ97 (EntEJ97) (see **Figure 9**).

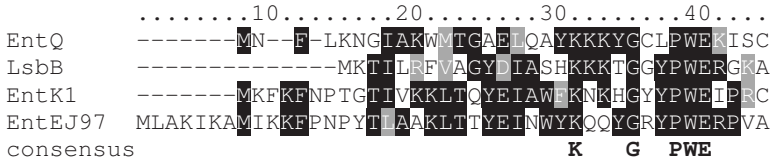

Figure 9. Multiple sequence alignment of all natural members of the LsbB family of bacteriocins. Conserved residues are shown in bold.

The family also includes two synthetically engineered variants called H1 (hybrid 1) and EntEJ97s [108]. H1 consists of the N-terminal half of EntK1 fused to the C-terminal half of EntEJ97s. EntEJ97s is a short (s) variant of EntEJ97 where the first 7 residues have been removed [109]. All members are small (30-44 aa), cationic, and share the same KxxxGxxPWE motif at the C-terminal end (see **Figure 9**) [26]. However, the antimicrobial spectrum of each member differs greatly. LsbB is very narrow-spectrum, with activity only towards *L. lactis* IL1403 [107]. Both EntK1 and EntQ exhibit a somewhat broader spectrum, EntQ kills some strains of *Latilactobacillus sakei* and *E. faecium*, while EntK1 targets *L. lactis*, *E. faecium*, and *Enterococcus hirae* [107,110]. EntEJ97 and H1 have the broadest spectra, targeting most Gram-positive species with varying potency [111]. The concentration needed to inhibit the growth of a bacterial culture by 50% or more, referred to as the MIC_{50} (minimum inhibitory concentration), is 0.5 nM for LsbB towards *L. lactis* IL1403, 10-85 nM for EntK1 towards *E. faecium,* and 145-295 nM for EntEJ97 towards *E. faecalis* [107].

Because these bacteriocins rely on RseP for killing, sensitive cells with altered RseP (e.g., by spontaneous mutations) can become completely insensitive to these bacteriocins, presumably by interfering with the bacteriocin-receptor interaction. Indeed, subtle sequence differences in the RseP sequence between species and/or

strains are sufficient to explain differences in inhibition spectrum between the members of the LsbB family [106]. However, in nature, variable levels of expression of the receptor RseP are also likely to play a role. The LsbB family of bacteriocins is especially attractive because of their small unmodified structure and receptor-mediated mechanism. In addition, the peptides exhibit high potency, making it possible to reach therapeutic concentrations (e.g., *in vivo*).

The first member of this family to be discovered EntEJ97 is produced by *Enterococcus faecalis* EJ97, a strain isolated from municipal wastewater [111]. The biosynthetic genes are located on a conjugative plasmid, pEJ97. EntEJ97 is encoded by the gene *ej97A* (132-nucleotides) producing a 44 aa polypeptide. The bacteriocin peptide contains 48% hydrophobic residues and has a predicted pI of 10.8 (neutrally charged a pH 10.8; cationic). Downstream of *ej97A* is a gene encoding an ABC-transporter (584 aa), followed by two hypothetical proteins of 66 and 64 codons, *ej97C* and *ej97D*. The latter two hypothetical proteins are thought to have a role in immunity and/or as accessory proteins in the export of bacteriocin.

Enterocin K1 (EntK1) was discovered by database search (BLAST) of NCBI with the DNA sequence of the EntEJ97 transporter. By manual analysis of significant hits, a small ORF (37 codons) was identified that contained the same conserved motif present in LsbB and EntEJ97 (K. V. Ovchinnikov, personal communication, June 2, 2023). Peptide synthesis and antimicrobial testing confirmed its bioactivity. No experimental data has been published on its biosynthesis; however, the genome sequence is very similar to that of the other members of the LsbB family. EntK1 was discovered on a whole-genome shotgun sequence assembly of *E. faecium* E2039 (Accession number: AHXS01000032). The EntK1 locus is shown in **Figure 10**.

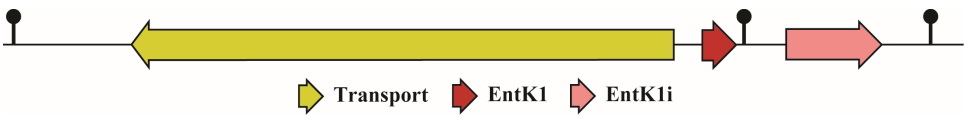

Figure 10. Genetic organization of the EntK1 bacteriocin cluster. Putative rho-independent transcription terminators were predicted using ARNold (lollipops) [112].

Downstream of the EntK1 structural gene is a 67 aa hypothetical protein with physicochemical properties like the immunity proteins to EntQ and *ej97C*, and therefore named EntK1i. An ABC-transporter (567 aa) is located on the opposite

strand upstream of the EntK1 gene. EntK1 is particularly active against *E. faecium* irrespective of antibiotic resistance profiles.

LsbB was first purified from the culture supernatants of *L. lactis* BGMN1-5 [113]. The bacteriocin is encoded on a 5.65 kb plasmid pMN5 together with another gene, *lsbA* (encoding LsbA, reported to be a bacteriocin with a leader sequence), and a gene encoding a multidrug ABC transporter LmrB. The LmrB transporter, is reported to be responsible for both export of- and immunity to both LsbB and LsbA.

Enterocin Q is produced by the multibacteriocinogenic strain *E. faecium* L50, a strain isolated from dry fermented Spanish sausage [110]. The strain also produces EntL50 (two-peptide leaderless) and EntP (pediocin-like). EntQ is encoded on a plasmid pCIZ2 (7.4 kb) by the gene designated *entqA* and regulated separately from the two other genes, *entqB* and *entqC* of the EntQ gene cluster. The gene *entqB* encodes an ABC-transporter with sequence similarity to both Ej97B and LmrB, the transport proteins of EntEJ97 and LsbB, respectively. *entqC* encodes a dedicated immunity protein of 67 aa with no significant sequence similarity to other proteins of known function [114]. Expression of *entqC* in a plasmid-free derivative of the wild-type producer *E. faecium* L50 provides resistance towards EntQ equal to that of the plasmid-harboring strain [114]. Interestingly, EntQ is the only member containing two cysteine residues; these are likely to oxidize spontaneously to form a disulfide bridge.

Peptides of the LsbB family are intrinsically unstructured in aqueous solutions but become structured in membrane-mimicking environments such as in DPC-micelles or in TFE (trifluoroethanol) [107]. The NMR structures of EntK1 and LsbB in 50% TFE have been solved and revealed a similar structure for both peptides. The N-terminal half is primarily α-helical, while the latter 10-15 residues at the C-terminus end is unstructured [107]. Structure prediction tools suggest a similar structure for EntEJ97 and EntQ, although this has not been determined experimentally (unpublished). The C-terminal tail containing the conserved PWE motif has been suggested to be directly involved in receptor binding, as the antimicrobial activity of LsbB is completely inhibited by the presence of a 100-fold molar excess of only the last 10 residues of LsbB (HKKKTGGYPWERGKA) [115]. The blocking activity of this peptide is presumably due to competitive binding to the receptor. This blocking is lost if the tryptophan (W^{25}) in the PWE motif is replaced by alanine. The latter 20

residues of both EntEJ97 and EntK1 also block the activity of LsbB, suggesting a common mechanism among the LsbB family [115].

1.2.4 Regulation of bacteriocin biosynthesis

One of the simplest signaling cascades (or communication modules) in bacteria consists of a pair of proteins with a sensor and response function. The protein serving as the sensor is typically located in the cytoplasmic membrane and monitors an environmental signal, the response regulator is in the cytoplasm and mediates the response, usually by changing gene expression. This general scheme or signaling cascade is carried out by so-called two-component regulatory systems (TCRSs) in bacteria (see section 1.3.1). A TCS is comprised of a histidine protein kinase (HPK; "sensor") and response regulator (RR; "response"). These signal transduction systems are crucial in bacteria and permit their adaptation to a changing environment. HPKs typically contain an N-terminal input domain and a transmitter domain at the C-terminus. When a signal is detected, autophosphorylation occurs at the C-terminal transmitter domain. This phosphoryl group is subsequently received by the response regulator, causing its activation. Response regulators are often activated by dimerization promoted by the phosphorylation, once activated most RRs are DNA-binding proteins that function as repressors or activators of transcription.

As mentioned in the introduction to this section, bacteriocin production is likely costly for the cell. For this reason, bacteriocin synthesis is often controlled by such regulatory systems. Indeed, many bacteriocin gene clusters encode two-component regulatory systems, and sometimes an inducing factor (IF) [116]. The IF may be the mature bacteriocin itself, as is the case for nisin, or a dedicated peptide pheromone encoded by the cluster (e.g., plantaricin A). In some cases, proteins with DNA-binding motifs are encoded in or near bacteriocin loci, that likely have a role as transcription activators or repressors [103]. Additionally, promoters in bacteriocin loci are often quite dissimilar to the strong consensus promoter, having a poor -35 box, -10 box, or distance between them [116–118]. Which suggests that other factors are likely necessary to promote transcription from these promoters. The presence of direct sequence repeats near promoters strongly suggests that they are DNA-binding motifs for bacterial response regulators. Repeats separated by multiples of 10-12 bp are strongly indicative of regulatory elements for response

regulators. Bacterial response regulators often bind as homodimers, with one molecule binding to one repeat. Repeats separated by multiples of 10-12 bp will face the same side on DNA. For most bacteriocins, the regulation of their biosynthesis is poorly understood, and likely involves exogenous inducing factors, and/or DNA-binding proteins other than typical RRs. Two examples where the regulation of biosynthesis has been studied in detail are nisin and the plantaricins EF and JK.

NisK is a histidine sensor kinase that acts as a receptor for mature nisin. Binding of nisin to NisK results in autophosphorylation, and the phosphoryl group is subsequently transferred to the response regulator NisR. The response regulator NisR act as a transcriptional activator of *nisABTCIPRK* and *nisFEG*, binding of NisR to the promoter regions of these operons recruits RNA polymerase to initiate transcription. A pair of direct repeats of the sequence "TCT" separated by an 8-bp AT-rich spacer are present in the promoter region. These repeats (TCT-N_8-TCT) are located at positions −107 to −94 and −39 to −26 relative to the transcription start site of *nisA,* and the latter repeat is essential for NisR binding [119,120].

L. plantarum C11 contains a multibacteriocin gene cluster encoding two bacteriocins, PlnEF and PlnJK, and a peptide pheromone, plantaricin A (PlnA). The ORFs of the *pln* locus are organized as five operons preceded by a pair of repeats of the consensus sequence ('5-TACGTTAAT-3') separated by 12 nucleotides (approximately 1 helical turn of β-DNA; 10.5 bp/turn). The genes *plnBCD* encode a TCS-like quorum-sensing system that is activated by PlnA, encoded by the first gene of the operon. Regulatory systems where the inducer is part of the same transcriptional unit as its TCS are often called a three-component regulatory systems.

1.2.5 Mode of action and immunity of bacteriocins

As evident from the overview of the different bacteriocins mentioned above (see section 1.2), there is a great deal of variation between bacteriocins when it comes to structure and antimicrobial mechanism. Bacteriocins inhibit cell wall synthesis, protein synthesis or form pores in bacterial membranes of target bacteria. For the majority of bacteriocins produced by lactic acid bacteria, it is generally recognized that the mode of action is pore formation of the cytoplasmic membrane, resulting in leakage of intracellular molecules and/or ions such as K^+, H_3O^+, PO_4^{3-}, and ATP, with subsequent loss of membrane potential, which is lethal to cells. In general, very little

is understood about the mechanism of action of bacteriocins, but a general scheme has emerged [36]. Bacteriocins, especially class II, are typically cationic, hydrophobic, amphiphilic, and sometimes contain critical hydrophobic residues such as tryptophan [22]. The positively charged residues are believed to be important for the initial interaction with the cell surface. Bacterial membranes are negatively charged due to teichoic acids and carboxylate groups. At the membrane interface, the hydrophobic side of the bacteriocin is thought to interact with the lipids or a receptor protein, which leads to the insertion of the bacteriocin peptides into the membrane. Once inside the membrane, the peptides associate to form a pore complex. Some bacteriocins form specific pores that only conduct some ions, while others form dynamic or unspecific pores. It is not known if the receptor and/or bacteriocin becomes structural parts of the pore or not.

An example is lactococcin G (LcnG), a two-peptide bacteriocin depending on the complementary action of two peptides α and β. The two peptides form helix to helix interactions in membrane-mimicking environments, facilitated by residues in a GxxxG conserved motif present in most two-peptide bacteriocins [91,121,122]. LcnG is believed to insert into the bacterial membrane of sensitive cells to form defined pores, likely in a manner that depends on UppP/BacA [91,123,124]. The pores permit the efflux/influx of monovalent cations only, including choline, Na^+, K^+, Cs^+, Li^+, and Tris, but not protons (H^+) or the cations Mg^{2+} or HPO_3^{2-}/PO_4^{3-} [123,124]. Consequently, the efflux of ions results in a loss of transmembrane electrical potential, but this is not accompanied by a collapse in the pH gradient across the membrane. The killing by LcnG is believed to be a result of several effects, the disturbance in cation homeostasis causes an osmotic imbalance and imbalance in turgor pressure. The collapse of the sodium gradient across the membrane and electrical potential leads to ATP depletion. Depletion of ATP further leads to the cessation of active transport systems needed to sustain metabolic processes [123,124]. Immunity to LcnG is conferred by the immunity protein LagC (110 aa), which appears to recognize and bind both α- and β-peptides [125]. Additionally, immunity by LagC has been shown to depend on another cellular component, suggesting a similar mechanism of immunity as described for lactoocccin A (see below) [125].

Lactococcin A (LcnA) was one of the first class IId bacteriocins to be isolated and is one of the best characterized. LcnA increases the permeability of the membrane of sensitive cells, leading to the free diffusion of ions and amino acids out of the cell

[126]. Consequently, the leakage in the membrane dissipates the membrane potential [126]. The bioactivity of LcnA depends on Man-PTS, a primary PEP (phosphoenylpyruvate)-dependent sugar uptake system for various sugars in many bacteria [127]. Strains of *L. lactis* where the genes encoding Man-PTS have been removed become insensitive to LcnA [128]. Man-PTS is a complex consisting of several enzymes, the permease component is comprised of subunits IIAB, IIC, and IID (encoded by the *ptn* operon in *L. lactis*; *ptnABCD*). Subunits IIC and IID form the membrane-embedded channel, while IIAB is associated with IICD from the cytosolic side. The immunity protein LciA has been shown to co-purify with components of Man-PTS in the presence of LcnA (from *L. lactis* lysates) [128]. Additionally, *L. lactis* exhibits a reduced growth rate with mannose/glucose as the sole carbon source when expressing LciA with added LcnA. This growth deficit was not present when galactose was used as the sole carbon source, this sugar is imported by an uptake system other than Man-PTS. Further, heterologous expression of *ptnABCD* in *Lactobacillus sakei* Lb790, a naturally LcnA resistant strain, showed that expression of the *ptnCD* pair alone was sufficient to confer sensitivity [128].

Taken together, these data suggest a model where LcnA permeabilizes the membrane in a IICD-dependent manner. The immunity protein is only very loosely or transiently associated with Man-PTS in the absence of LcnA but becomes tightly associated in the presence of LcnA to form a bacteriocin-immunity-receptor complex. Very recently, the ternary complex LcnA-LciA-man-PTS was solved by cryo-electron microscopy [129]. Indeed, LcnA appears to act like a “wedge” by binding and pushing apart two domains of the Man-PTS complex, thereby forming a pore/channel. LciA recognizes the newly opened cleft from the intracellular side and partially penetrates it with its flexible C-terminal tail. The remaining structure of LciA forms a four-helix bundle that binds at the pore opening, thus acting like a plug and preventing leakage through the pore [129,130]. Structural data suggests a similar mechanism also for class IIa bacteriocins targeting Man-PTS, including pediocin PA-1 and sakacin A [129,131].

For the lantibiotic nisin, the initial interaction with lipid II blocks cell wall biosynthesis. Higher concentrations of nisin lead to the formation of nisin-lipid II complexes that initially form stable 2 nm diameter pores in the membrane [132]. The pore complex is believed to consist of 8 nisin and 4 lipid II molecules. However, these complexes are likely to continue to associate into larger-scale aggregates consisting of thousands of nisin-lipid II complexes, causing drastic lipid

rearrangements and membrane damage [133,134]. A breach of membrane integrity in target cells causes a loss of membrane potential, leakage of intracellular components, and cell death [135].

The leaderless class IIc aureocin A53 (AurA53) primarily kills by membrane permeabilization, efflux of cell nutrients, and membrane depolarization. Membrane permeabilization by AurA53 probably occurs through a general membrane disruption rather than the formation of structured pores. The model proposed to be utilized by AurA53 is SMH (Shai-Matsuzaki-Huang), which suggests that antimicrobial peptides cause lipid displacements, leading to changes in the membrane structure, and potentially internalization of the bacteriocins to the inside of cells. In contrast to most class II bacteriocins, AurA53 is reported to have a defined rigid structure in aqueous solution where all five tryptophan residues are externally exposed. Interestingly, AurA53 interacts with equal affinity with both neutrally charged and negatively charged phospholipids. This observation undermines the conventional "wisdom" that the negatively charged lipids serve an important role in the peptide-membrane interaction.

Another leaderless bacteriocin, lacticin Q (LnqQ), shows strong antimicrobial activity towards *Bacillus*, *Enterococcus*, and *Staphylococcus*, and has been shown to form large pores in their membrane [136,137]. The model proposed for the mode of action of LnqQ is the formation of so-called huge toroidal pores (HTPs); in this model, LnqQ initially binds to the outer leaflet of the membrane due to electrostatic interactions, followed by folding of the peptides into an α-helical structure that transverses the membrane and oligomerizes into a pore of 4.6-6.6 nm in diameter [137]. However, pore formation is not thought to be the final antimicrobial mechanism of the bacteriocin. Instead, exposed cells experience oxidative stress from an accumulation of hydroxyl radicals that eventually kills the cell [138]. A mode of action like HTP proposed for LnqQ highlights the puzzle of intracellular self-immunity, as the Gram-positive cell membrane is often reported to be symmetric (same lipid composition in both inner and outer leaflet) [139]. In addition, LnqQ displays a rather specific and variable antimicrobial activity both within and between species [41,140]. The variability in sensitivity is thought to be caused by subtle membrane lipid composition differences (and thus differences in cell surface affinities), and the ability of a cell to respond to and/or tolerate oxidative stress [41,140].

Very little is known about the mode of action of the LsbB family of bacteriocins. EntEJ97 is reported to be bactericidal and lytic against *E. faecalis* [111]. *L. lactis* protoplasts exposed to LsbB showed leakage of intracellular β-galactosidase [141]. It is known that these peptides are dependent on the site-2 protease RseP as a receptor on target cells, however, it is not known what role the receptor protein RseP has in their mechanism of action [107,141–143].

1.3 Bacterial stress response

Bacteria have a remarkable ability to tolerate changes in their local environment, including changes in temperature, salinity, drought, and acidity. In addition, microorganisms and animals have competed in an arms race for billions of years. As part of the innate immune system, animals secrete a wide array of antimicrobial peptides (AMPs) with inhibitory effects against bacteria, fungi, parasites, and even viruses [144]. Simultaneously, many bacteria have acquired elaborate systems (virulence factors) to aid in colonizing and invading the tissues of animal hosts. The success of a pathogen is completely dependent on its ability to counteract the onslaught of the immune system of a foreign host. To tolerate changes in the environment and counteract a foreign immune system, a cell must be able to sense and respond to its environment. The bacterial cell surface is laden with proteins, many of which act as sensors by initiating a signaling cascade in response to specific stimuli [145]. Due to the impermeable nature of the cytoplasmic membrane, transmembrane signaling cascades are used to relay information from the environment to the interior of the cell. Examples of regulatory cascades mediating such signals include TCSs and the use of ECF σ-factors.

1.3.1 Two-component regulatory systems

In bacteria, environmental sensing is primarily carried out by two-component signal transduction systems (TCRSs) (see section 1.2.4). These signal transduction systems are crucial in bacteria and permit their adaptation to a changing environment. Although TCSs can activate or repress a wide range of processes, such as nisin production via NisRK (see section 1.2.4), one of their most important roles is arguably as envelope stress response systems. Upon exposure to sublethal concentrations of antimicrobial compounds like antibiotics, an adaptive response can be induced in bacteria that contributes to tolerance and resistance. Envelope stress-responsive TCSs are exemplified by the LiaRS-system of *B. subtilis*, VraSR of *S. aureus* and CesSR of *L. lactis*. These TCSs have all been shown to be important for sensitivity to various antimicrobials, including bacteriocins [146–149].

LiaRS (lipid II interacting antimicrobials) responds to cell wall-active antibiotics such as vancomycin, bacitracin, and ramoplanin, as well as cationic antimicrobial peptides (CAMPs) [146,150]. In addition, the degree or extent of activation of LiaRS depends on the net charge of the cell envelope [151]. This TCS is often referred to as

a three-component system because an additional protein, LiaF, is genetically and functionally connected to LiaRS. LiaF is a strong inhibitor of LiaRS, and is encoded on the same operon (*liaGFSR*). During activation of LiaRS, the inhibitory effect of LiaF ceases, leading to phosphorylation of LiaR by the HPK LiaS. The main operon regulated by LiaR is *liaIH*. LiaI is small hydrophobic protein of unknown function with predicted transmembrane localization. LiaH is a PspA orthologue that belongs to the family of phage shock proteins (Psp) [152]. In *E. coli* PspA is important for the maintenance of the proton motive force during cell envelope stress [153]. Because of similarities between the Psp response and LiaRS, LiaH is thought to serve a similar role as PspA while LiaI is thought to function as a membrane anchor that recruits LiaH to the membrane during stress [154].

CesSR (cell envelope stress) is one of six TCSs identified in *L. lactis* MG1363. This TCS is induced in the presence of bacitracin, vancomycin, plantaricin C, and lactococcin 972 [148]. The CesR regulon includes 23 genes, all encoding predicted membrane- and/or stress-related proteins. Disruption of CesR results in increased susceptibility to the aforementioned antimicrobials [148]. One of the strongly upregulated genes is *spxB* (*yneH*), a transcriptional regulator positively regulating the expression of *oatA*. The encoded protein OatA is an O-acetyltransferase acting on peptidoglycan, and increased acetylation of PG confers reduced sensitivity to, e.g., nisin and lysozyme [155].

VraSR (vancomycin resistance associated) TCS of *S. aureus* is a LiaRS-like system implicated in antibiotic-resistant phenotypes of *S. aureus* [156]. Thus, VraSR is important because of the clinical relevance of *S. aureus*. Analogous to the LiaRS system in *B. subtilis*, *vraSR* is located on an operon together with a *liaF* homolog (*yvqF*/*vraT*). Disruption of either VraS or VraR increases sensitivity to various antibiotics such as vancomycin, daptomycin, and methicillin [157]. However, *liaF* (*yvqF*/*vraT*) is necessary for maximal resistance towards the antibiotics, suggesting a more elaborate involvement of *liaF/vraT* in the VraTSR three-component system [157,158]. The VraSR TCS is induced primarily by disruption of cell wall biosynthesis and not by general stress (osmotic shock, pH, heat) [159]. The VraR regulon encompasses approximately 46 genes and includes genes encoding proteins involved in cell envelope biosynthesis and maintenance such as *pbp2* (penicillin binding protein 2; peptidoglycan transpeptidase and transglycosylase), *sgtB* (glycosyltransferase), *tagA* (teichoic acid biosynthesis), and *murZ* (UDP-N-

acetylglucosamine enolpyruvyl transferase, involved in the first committed step in PG biosynthesis) [159].

1.3.2 ECF σ-factors

Initiation of transcription in bacteria is carried out by the RNA polymerase (RNAP) holoenzyme, consisting of the RNA polymerase core enzyme and a sigma (σ) factor that guides RNAP to specific promoters [160]. A primary ("housekeeping") σ-factor recognizes the promoters of most genes required for normal growth under typical conditions, such as the σ^{70} in *E. coli* or σ^{A} in many other bacterial species [161].

As environmental conditions change and a different set of genes are required by the cell, alternative σ-factors with smaller and more specific regulons are activated [161]. Some alternative σ-factors such as the *E. coli* σ^{38} starvation/stationary phase σ-factor, share the highest homology to the primary σ-factors, but unlike the primary sigma factors, alternative σ-factors are not essential for growth [160]. The most divergent group of σ-factors is known as the extracytoplasmic function (ECF) σ-factors (extra, from latin; outside, beyond). The ECF σ-factors are small, diverse, and widespread in both Gram-negative and Gram-positive bacteria.

The ability of many bacteria to respond to certain environmental signals, such as stress, depends on the activation of ECF σ-factors. ECF σ-factors direct RNA polymerase (RNAP) to a narrow set of promoters to express genes that produce an adaptive response. Under non-inducing conditions, some ECF σ-factors are held inactive by an association with a cognate anti-σ factor which is typically co-transcribed with its σ-factor. Most anti-σ factors are transmembrane proteins with an intracellular inhibitory domain and an extracytoplasmic sensing domain, the latter of which permits the cell to sense environmental stimuli [162]. For the cell to respond to the environment, signals must be relayed from outside the cell to the inside. One mechanism used to achieve this is RIP (regulated intramembrane proteolysis).

1.3.3 Regulated intramembrane proteolysis

RIP is a mechanism of signal transduction involving the sequential proteolysis of a transmembrane protein where cleavage by a 'site-1' protease (S1P) must precede the cleavage by a 'site-2' protease (S2P) (6). The site-2 cleavage occurs inside the

lipid bilayer of the membrane, as such, proteolysis is mediated by an intramembrane-cleaving protease (i-CliP). The RIP signaling cascade is activated by external stimuli, and results in the liberation of a cytosolic signaling molecule that initiates a defined biological response [163]. Bacterial i-CliPs are divided into three families based on their catalytic mechanism, the aspartyl-, serine-, and zinc metallo-proteases [164]. S2Ps belong to the zinc metalloproteases, a group of proteins found in both bacteria and humans. The important role of S2P-mediated RIP signaling in bacterial fitness and virulence has been widely recognized [165,166].

A key site-2 protease and i-CliP is the protease RseP (regulator of sigma-E protease), also known as RasP in *B. subtilis*, and Eep in *E. faecalis*. The active site of RseP is located within the cytoplasmic membrane, and orthologues of RseP are involved as the site-2 protease in the liberation of stress responsive ECF σ-factors in numerous species, such as σ^E in *E. coli*, σ^W in *B. subtilis*, and σ^V in *E. faecalis* [167–171]. Importantly, RseP is also the receptor for the LsbB family of bacteriocins (see section 1.2.3). This opens the possibility of exploiting RseP as a drug target in these bacteria. However, these bacteriocins only target the RseP of some Gram-positive bacteria. A better understanding of RseP and its structure, and the mechanism of the LsbB bacteriocins may allow us to engineer and modify these bacteriocins to target RseP in other important pathogens.

1.3.3.1 RseP of *E. coli*

The best characterized RIP-activated ECF σ-factor in *E. coli* is σ^{24} (heat shock, also called σ^E or RpoE; E for envelope), which is activated and required for the survival of *E. coli* at temperatures above 42°C [172]. Under normal conditions, σ^E is held inactive by being anchored to the inner membrane by the anti-σ factor RseA (see **Figure 11**), a single-pass membrane protein with a cytoplasmic inhibitory N-terminal domain and a periplasmic C-terminal sensing domain [173]. A stress signal such as misfolded periplasmic proteins triggers the removal of the periplasmic domain of RseA ($RseA^{149-216}$; residues 149-216) by the site-1 protease DegS [173,174]. RseA lacking the periplasmic domain is permitted to enter the active site of the site-2 protease RseP, which liberates σ^E into the cytoplasm with the N-terminal domain of $RseA^{1-108}$ still attached [175]. An adapter protein SspB guides the complex to the cytoplasmic protease ClpXP (caseinolytic protease), which removes the bound N-terminal fragment of RseA allowing the interaction of σ^E with RNAP and subsequent transcription of stress-responsive genes [175,175,176].

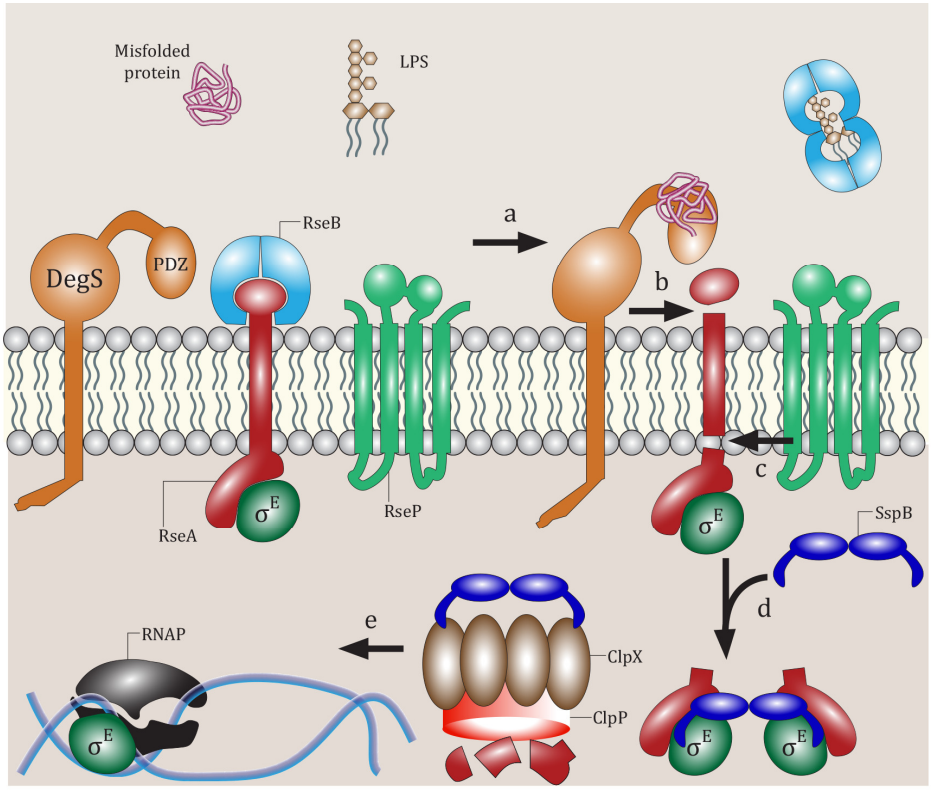

Figure 11. Activation of σ^E (RpoE) in *E. coli*. Two separate events regulate the site-1 cleavage of RseA by DegS. The PDZ domain of DegS interacts with misfolded proteins to expose the active site, and the regulatory protein RseB is released from RseA upon binding LPS. (a) Site-1 cleavage by DegS removes the periplasmic domain of RseA. (b) RseA, devoid of a periplasmic domain is permitted to enter the active site of RseP, which performs site-2 cleavage. (d) Degradation of leftover residues of RseA in complex with σ^E is facilitated by SspB via ClpX-ClpP. (e) Liberated σ^E associates with RNAP to direct transcription of stress-associated genes.

RseP is similarly involved in the activation of the ECF σ-factor FecI (iron starvation sigma factor; σ^{19}), a sigma factor responsible for expression of the *fecABCDE* operon encoding proteins for ferric iron uptake (citrate-dependent iron(III) transport system) [177–180]. Iron is an essential micronutrient for bacteria, which is often limiting, especially for pathogenic bacteria that must reproduce inside a host [181,182]. In *E. coli*, the iron uptake system encoded by *fecABCDE* is specifically for ferric dicitrate import, an operon regulated by the gene products of *fecIR* [178].

The protein FecR serves a similar role as an anti-σ factor for FecI because FecR keeps FecI inactive in the absence of citrate, however, FecR is strictly not an anti-σ factor because FecI is also inactive in the absence of FecR (the term pro-σ-factor have been proposed for FecR) [178]. An N-terminal "tail" of FecA extends to the inner membrane, where it interacts with the C-terminal periplasmic domain of FecR. This interaction triggers a cleavage by the protease Prc (processing involving the C-terminal cleavage), which releases the C-terminal domain of FecR into the periplasm [177]. Analogously to the RIP cascade, FecR without the periplasmic domain undergoes intramembrane cleavage by RseP. The N-terminal fragment of FecR liberated in the cytoplasm associates with FecI and promotes the association of the complex with RNA polymerase. Although *E. coli* has numerous iron-uptake systems, the ferric citrate (*fec*) system is important for fitness and virulence in uropathogenic strains during a urinary tract infection (UTI).

In addition to recognizing and cleaving RseA and FecR in *E. coli*, RseP can cleave a diverse group of unrelated proteins with intramembrane helices. However, the promiscuous activity of RseP *in vivo* appears to be controlled by the necessity of prior cleavage by a signal peptidase. In *E. coli* most leader peptides are removed by Lep (leader peptidase, also called Signal Peptidase I), cleavage by Lep is a key step in both the Sec-dependent- and Tat-translocation pathways; the two major pathways in bacteria for translocating proteins across the cytoplasmic membrane. Proteins that are to be translocated across the membrane contain 18-30 residues long N-terminal signal sequences; in both translocation pathways, the N-terminal signal becomes embedded in the membrane as the protein is translocated. Cleavage by Lep releases the protein in the periplasmic space, thus leaving the signal peptide behind in the membrane. The accumulation of remnant signal peptides in the membrane can be toxic to cells and interfere with further protein export, however, not much is known about their degradation and removal in bacteria [183,184]. The only proteins implicated in signal peptide degradation in *E. coli* is SppA (signal peptide peptidase A) and OpdA (oligopeptidase A), but SppA has only been demonstrated to cleave signal peptides *in vitro*, and OpdA has only been shown to cleave peptides in the cytosol [185]. Interestingly, RseP has been demonstrated to cleave the remnant signal peptides from the translocated proteins OmpF, LivK, SecM, PhoA, LivJ, OmpC, and Lpp [186]. Further, RseP has been shown to cleave the transmembrane region of several proteins unrelated to RseA both *in vitro* and *in vivo* [187]. Cleavage of substrates unrelated to RseA depends on the presence of helix-destabilizing residues such as proline (residues found infrequently in alpha helices) [187].

RseP in *E. coli* belongs to the M50 family of metallopeptidases in the clan MM (membrane-embedded metalloendopeptidases) according to the MEROPS classification of peptidases [188]. For each family in this classification, a "type enzyme" is assigned that is characteristic for that family. The type enzyme of the M50 family is the human MBTPS2 (membrane-bound transcription factor site-2 protease; Uniprot O43462). However, another member of the M50 family has been characterized in detail and differs substantially in amino acid sequence from MBTPS2, namely SpolVFB from *Bacillus subtilis* (sporulation factor IV B protease). For this reason, the M50 family has been further subdivided into M50A and M50B, with *E. coli* RseP placed in M50B together with SpolVFB (the type enzyme of the M50B subfamily). The S2P proteins have been further grouped into four subfamilies based on their phylogenetic relationships, and are thus assumed to stem from a common ancestor protein. Human MBTPS2 and RseP homologs belong to the Group I S2Ps together with RasP from *B. subtilis*.

E. coli RseP contains 450 amino acids and four transmembrane segments (TMS) with both the N- and C-termini located in the periplasm (see **Figure 12**). The first TMS (TMS1) spans residues 1 to 33 and encompasses two histidine residues that chelate a catalytic divalent zinc ion (**H**^{22}EFG**H**).

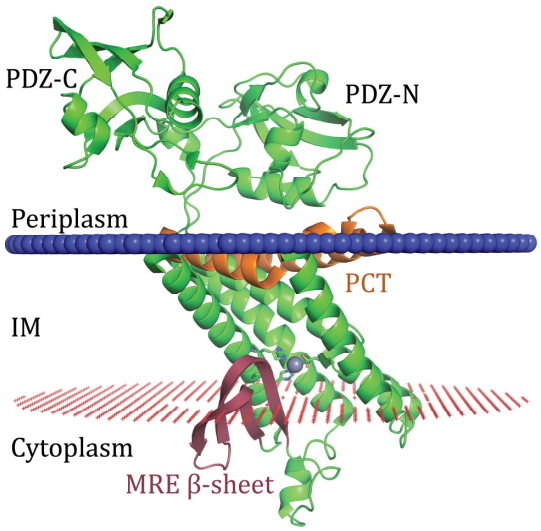

Figure 12. Structure of *Ec*RseP with some of the important features indicated. The activate site is shown as sticks, with a central zinc atom shown as a sphere (in gray). The transmembrane topology was predicted using the PPM 3.0 web server [189]. Structure is based on PDB 7W6X [190]. Figure was generated using PyMOL.

Following the first TMS is a region forming a four-stranded β sheet (residues 37-72) named the MRE β-sheet (membrane-reentrant β-sheet). Between TMS2 (94-122) and TMS3 (376-415) are two PDZ domains arranged in tandem (PDZ; post-synaptic density protein, disc large and zo-1 proteins). PDZ domains are small protein-protein interaction domains of approximately 90 aa. Generally, the fold of the PDZ domain consists of two α-helices and six β-strands. These domains are believed to interact with and recognize 5-10 residues at the C-terminus of incoming proteins. Although these domains have also been reported to bind internal sequences. In humans, PDZ-domain containing proteins are important for cellular trafficking, the regulation of ion channels, and neuron signaling. However, the function of PDZ domains in prokaryotes is poorly characterized. The PDZ domain(s) of RseP in many bacteria are believed to function as a "size exclusion filter" instead of recognizing specific C-terminally located peptide sequences [191]. As such, membrane proteins with large and/or intact extracytoplasmic domains are prevented from accessing the active site by steric hindrance.

The region after the PDZ domains is referred to as PCT (323-374; PDZ C-terminal) and contains two α-helices positioned almost parallel to the membrane situated at the membrane-periplasm interface (see **Figure 12**). Two important regions of PCT are PCT-SH (355-358) and PCT-H2 (362-374) which reside near the active center and participate in the regulation of substrate access to the active site. Another zinc ligand, D^{402} is in TMS3 (376-415) which is followed by the last transmembrane segment, TMS4 (423-450). The highly conserved motifs H^{22}ExxH and N^{394}xxP-xxxLDG^{403} indicate amino acids that are crucial for the function of RseP.

Very little is understood about substrate specificity and recognition by bacterial RseP homologs. The current model for RseP proposes that substrates with periplasmic domains are only permitted to pass through the size exclusion filter following site-1 cleavage. The transmembrane segment of the substrate then enters the active site when PCT-H2 and TM4 shift apart. This shift is caused by an electrostatic attraction between PCT-H2 and D^{446}. A reorientation of the PDZ domain accommodates the substrate, after which the PDZ domain recloses on the substrate, leading to unwinding by strand addition and "kinking" of the substrate helix. The kink exposes the amide bond for hydrolysis. Upon hydrolysis, the PDZ "gate" re-opens to release the substrate.

The active site of *Ec*RseP is shown in **Figure 13**. Residues H^{22}, H^{26}, and D^{402} function as metal ligands that coordinate the divalent zinc ion. A glutamic acid (E^{23}) is the only active site residue directly involved in the catalytic reaction. Although the reaction mechanism of the M50 family of proteases has not been determined, all monometallic metallopeptidases are believed to follow the same generally accepted catalytic mechanism [192].

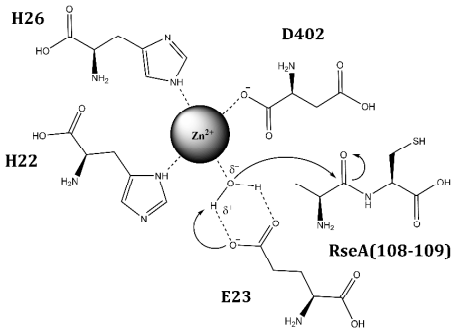

Figure 13. The active site of *Ec*RseP and the general steps of electron pair transfers resulting in peptide bond cleavage. RseA is cleaved between alanine[108] and cysteine[109].

Central to the catalytic mechanism is a water molecule that becomes polarized in proximity to zinc and aspartic acid (E^{23}). The activated water/hydroxide molecule attacks the carbonyl atom in the peptide bond of the substrate (nucleophilic addition), forming a tetrahedral intermediate. The intermediate is resolved by bond breakage and electron pair transfer from the newly formed α-amino group. The importance of water in the proposed mechanism of RseP with an active site located within the membrane appears counterintuitive, as the hydrophobic membrane environment is impermeable to water. However, an RseP homologue from *Methanocaldococcus jannaschii*, *mj*S2P (locus tag MJ_RS02060), forms a potential water access channel from the cytosolic side to the catalytic center [193]. Similarly, the MRE β-sheet of *Ec*RseP contains several charged amino acids that likely facilitate water diffusion to the active site.

1.3.3.2 RasP of *B. subtilis*

An RseP orthologue in *B. subtilis* named RasP (regulating anti-sigma factor protease) is the best characterized Gram-positive member of this group of proteins, and is responsible for the activation of σ^W , σ^V, and σ^I via a RIP mechanism analogous to

that in *E. coli* [194]. The *B. subtilis* σ^W factor is encoded by the *sigW* gene located just upstream of its cognate anti-sigma factor *rsiW* (regulation of σ^W) in the same operon [194]. The extracellular region of RsiW is removed by the site-1 protease PrsW (protease responsible for activating σ^W) prior to processing and release by RasP [195]. The σ^W regulon consists of over 60 genes that are upregulated upon acid/base shock, high salinity, and some cell wall targeting antimicrobial compounds [162,196–199]. Most genes of this regulon have been referred to as "antibiosis" genes, as they function in the defense of some antibiotics like fosfomycin and antimicrobial peptides/bacteriocins [200]. In addition, many genes are involved in cell envelope synthesis/maintenance, and detoxification by inactivation, sequestration, or elimination of toxic compounds [199].

The anti σ-factor of σ^V RsiV (regulator of σ^V) is processed by the site-1 protease SipS (signal peptidase S) in a manner that depends on the presence of lysozyme [201]. Subsequent site-2 cleavage is performed by RasP. The σ^V ECF is primarily responsible for resistance to lytic enzymes such as its induced lysozyme. The sigV operon is positively autoregulated, and the operon consists of *sigV*, *rsiV*, *oatA*, and *yrhK*. OatA performs O-acetylation of peptidoglycan, which is associated with resistance to cell-wall targeting endoglycosidase enzymes. Also upregulated by σ^V is the *dlt* operon, the Dlt system fortifies the cell wall by modifying teichoic acids by D-alanylation [202].

The sigma factor SigI (σ^I) is more closely related to σ^{70} than ECF σ-factors, and therefore not classified as an ECF σ-factor [203]. However, its regulon is small, and its activation appears to be via RIP analogous to that of the ECF σ-factors [204]. Expression of σ^I is induced by heat and encoded on an operon together with a cognate anti σ-factor RsgI (regulation of *sigI*), a single-pass membrane protein. *B. subtilis*, lacking σ^I, cannot grow at high temperatures [205,206]. Similarly to the other anti-σ factors, the N-terminal domain of RsgI sequesters σ^I to the inner cytoplasmic membrane [203]. Upon heat-shock, a signaling cascade involving several proteases leads to the degradation of RsgI and the release of σ^I. The signaling cascade resulting in the degradation of RsgI involves RasP, as deletion of *rseP* significantly reduces *sigI* expression [204]. However, this is not observed in mutants lacking RsgI, suggesting that RasP affects SigI via RsgI, likely by proteolytic cleavage as part of its degradation [204]. In addition to the role of RasP in the activation of σ-factors in *B. subtilis*. RasP is also reported to be involved in the

control of cell division and sporulation by proteolysis of FtsL, an essential cell division protein [207,208].

Curiously, an ABC transporter, EcsAB, is essential for the function of RasP in *B. subtilis*, as cleavage of the anti σ-factor RsiW is fully inhibited in strains lacking EcsAB [209]. Moreover, cells lacking either RasP or EcsAB exhibit a similar phenotype, including reduced competence and biofilm formation. The function of this transporter in *B. subtilis* is not known, but an orthologue of EcsAB in *E. faecalis* has been shown to be involved in sex pheromone expulsion from the membrane (see section 1.3.3.3) [210]. Interestingly, strains of *S. haemolyticus* and *E. faecalis* with mutations in *ecsA* or *ecsB* show substantially reduced sensitivity to bacteriocins targeting RseP (H1 and EntEJ97) [107,108].

1.3.3.3 Eep of *E. faecalis*

The RseP homolog in *E. faecalis* is known as Eep [211]. *E. faecalis* is a regular commensal in the mammalian gastrointestinal tract, but also a major cause of nosocomial infections [212]. This species is known to be highly resistant to lysozyme, an important antibacterial enzyme in the human innate immune system [213]. Lysozyme cleaves β-1,4-bonds between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan, leading to cell death. This resistance to lysozyme is attributed to the activation of the ECF σ-factor SigV [169,170]. In the same manner as RseP is involved in the activation of σ^E in *E. coli*, Eep is required for the activation of SigV in *E. faecalis* via RIP. Normally, SigV is sequestered to the cytoplasmic membrane by the anti-σ factor RsiV. As a trigger for this cascade, RsiV is believed to act as a receptor for lysozyme by changing conformation upon binding to lysozyme, which triggers site-1 cleavage by a signal peptidase. SigV is then released into the cytosol by subsequent site-2 cleavage performed by RseP [214].

Activated SigV induces the expression of genes that modify the cell envelope, such as *oatA*, *dltA*, and *pgdA*, which then provide the cell with protection against lysozyme and other cationic antimicrobial peptides [169]. OatA is an O-acetyltransferase that acetylates peptidoglycan (C^6 hydroxyl group of N-acetylmuramic acid; O-acetylation), and O-acetylation is linked to lysozyme resistance and virulence in other species, including *S. aureus* [215]. DltA, an enzyme involved in D-alanylation of lipoteichoic acids, has been shown to increase the packing density of peptidoglycan and reduce the net negative charge of the cell surface. The consequence is a

reduction in susceptibility to cationic antimicrobial peptides [216–218]. The SigV protein further protects *E. faecalis* against many general stressors, such as heat, acid, ethanol, and salt [168]. However, *E. faecalis* with disrupted RseP not only exhibits reduced stress tolerance, but also reduced virulence. In *E. faecalis* OG1RF *rseP* was shown to be upregulated in the early stages of infection [219]. In the same study, an *rseP* deletion showed 10 000-fold lower bacterial counts in the heart valve of rabbits, indicating reduced virulence in an endocarditis model [219].

E. faecalis is capable of exchanging plasmids through an induced conjugation system. To receive plasmids from neighboring cells, *E. faecalis* produces an array of sex pheromones [220–222]. Potential nearby donors respond by producing adhesion proteins, conventionally called "aggregation substance" which promotes plasmid transfer between cells [223]. Because of its medical importance, the tetracycline resistance plasmid pCF10 has been studied in detail. The pCF10 and related plasmids have likely contributed to the dissemination of antibiotic resistance genes among nosocomial enterococci [224,225]. The transfer of pCF10 is initiated by a sex pheromone producing non-harboring strain, which will trigger conjugative transfer from a harboring donor strain [220]. In *E. faecalis* the peptide sex pheromone for pCF10, cCF10, is derived from a prolipoprotein encoding gene *ccfA* located on the chromosome [226]. The pheromone peptide is only 8 aa (LVTLVFV) and located internally in the N-terminal signal sequence of CcfA [226]. To yield the functional pheromone, the N-terminal signal sequence is first removed by signal peptidase II (SPase II) [227]. The signal sequence is then further processed by Eep which reduces the peptide to 22 aa. A final cleavage at the C-terminal by an exopeptidase results in functional sex pheromone. Eep has similarly been implicated in the production of the sex pheromones cCF10, cAD1, cOB1, cAM373 and cPD1 [210,211]. A general overview of the sex pheromone maturation pathway proposed for *E. faecalis* is shown in **Figure 14**.

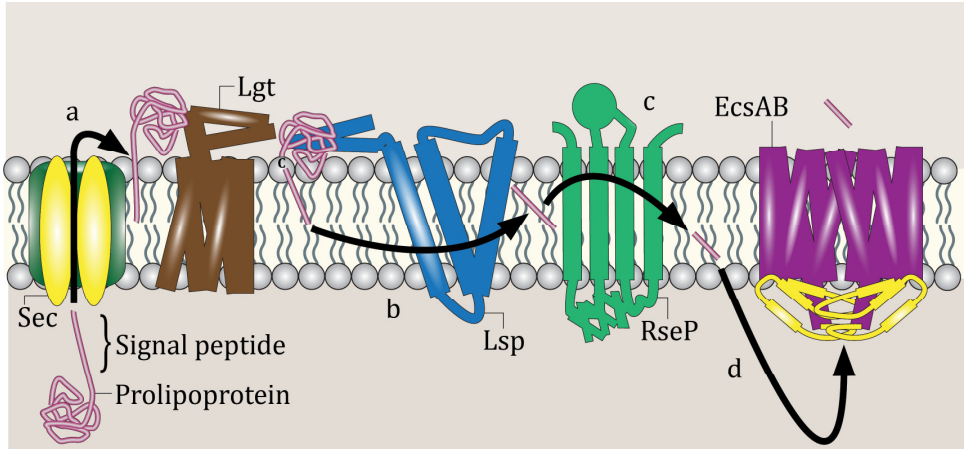

Figure 14. Sex pheromones in *E. faecalis* are derived from the signal peptides of prolipoproteins. (a) The prolipoprotein is translocated across the membrane by the general Sec pathway. The lipoprotein is anchored to the membrane by a diacylglyceryl group, which is added to a sulfhydryl group on the lipoprotein. (b) Removal of the signal peptide is catalyzed by Lsp. (c) The signal peptide is processed by RseP (Eep), and then (d) exported to the extracellular milieu by EcsAB (PptAB).

The RseP homolog in *E. faecalis* was therefore named Eep (enhanced expression of pheromone). Intriguingly, all these sex pheromones are derived from the signal sequences of different putative lipoproteins [228]. For the pheromones cCF10, cOB1, and cAM373 it has been shown that EcsAB is involved in their export out of the cell [210,211,227,228].

RseP is the receptor for the LsbB family of bacteriocins (see section 1.2.3) and is necessary for killing in all target species of these bacteriocins. Intriguingly, in some species, spontaneous mutants resistant to the LsbB bacteriocins harbor mutations in *ecsAB* and not *rseP*. Mutations in *ecsAB* that confer resistance are seen particularly in *S. haemolyticus*, but also *E. faecalis* and *E. faecium*, although less frequently [108,142,143]. For *E. faecalis*, mutations in *ecsAB* only result in an intermediate resistance to EntEJ97, while *ecsAB* mutants of *E. faecium* can be fully resistant [142,143]. Although a direct connection between RasP and EcsAB has only been shown in *B. subtilis*, these observations suggest an intriguing interplay between RseP and EcsAB in other species as well.

1.4 *Enterococcus faecium* – an emerging pathogen

Enterococci were originally part of the genus *Streptococcus* and were discovered in human feces in 1899 [229]. The species currently known as *E. faecalis* was first reported in 1906 as the causative agent in a patient diagnosed with endocarditis. The species now known as *E. faecium* was discovered shortly thereafter, in 1919 [230]. Increased knowledge about the metabolism and genetic makeup of these species led to their transfer to the genus *Enterococcus* in the early to mid-1980s [231]. Although *E. faecalis* and *E. faecium* appear similar in name, they are located on opposite sides of the phylogenetic tree of the genus *Enterococcus* [232].

Enterococci are among the most relevant nosocomial multidrug-resistant organisms. Enterococcal infections are particularly prevalent in immunocompromised patients or patients with concurrent illness. *E. faecalis* has historically been the major causative agent of enterococcal infections, however, *E. faecium* has recently emerged as the more challenging enterococcal pathogen. Enterococci are facultative anaerobe Gram-positive cocci (oval-shaped), naturally found in the gastrointestinal (GI) tract of humans and animals. In addition, enterococci have been used as probiotically in both humans and animals to alleviate gastrointestinal problems (e.g., diarrhea) [233]. Despite enterococci being part of the normal flora of human, these bacteria are inherently resistant to many antimicrobials (e.g., clindamycin, aminoglycosides, and cephalosporins) and has an excellent ability to acquire and share antibiotic resistance gene clusters, such as vancomycin-resistance (a glycopeptide antibiotic) [234,235]. Further, enterococci exhibit high genome plasticity which likely contributes to adaptation [236]. *E. faecium* can survive on dry surfaces for months, which allows these cells to persist in hospital environments and sometimes contaminate medical implants and catheters [236].

An outbreak of vancomycin-resistant enterococci (VRE) was first reported at the Dulwich Public Health Laboratory in 1986 [237]. During the outbreak, 55 strains of VRE were collected from 22 patients suffering from renal failure or multiple organ failure. Of the 55 strains, 48 isolates were identified as *E. faecium* and 7 as *E. faecalis*. The outbreak had been preceded by a regimen of vancomycin and ceftazidime in patients with sepsis and/or renal failure, specifically in patients where the causative bacterium had not yet been determined. This regimen may have led to subinhibitory levels of vancomycin in the tissues of these patients for prolonged periods. It has

been speculated that this promoted or exacerbated the development of VRE [237]. In addition, the diversity of strains that arose with a VRE phenotype suggested a dissemination of VRE determinants among the enterococci in the clinic [232].

In the past 20-25 years, ampicillin-resistant phenotypes of *E. faecium* has overtaken *E. faecalis* in causing nosocomial infections in Europe [238]. The European Antimicrobial Resistance Surveillance System (EARSS) reported the highest increase in *E. faecium* infections during 2002-2008 compared to almost all other pathogens, including *E. coli*, *S. aureus*, and *S. pneumoniae* [239]. Although *E. faecium* can cause serious infections such as endocarditis, bloodstream infections, and sepsis, the most frequent infections by *E. faecium* are urinary tract infections (UTI). These UTIs are often catheter-associated (CAUTI). UTIs is the most common hospital-associated infection, and it poses a considerable burden on the health system. In addition to patients with indwelling catheters, UTIs primarily affect pregnant and sexually active females. Bacteria most commonly implicated in uncomplicated UTIs are the Gram-negative *E. coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*, and the Gram-positive *Enterococcus* spp. and *S. saprophyticus* [240]. However, enterococci are more than twice as frequently implicated as the causative agent in complicated UTIs [240].

While the *E. faecalis* genome encodes four ECF σ-factors, including SigV, *E. faecium* is predicted to encode just one (locus tag: EfaeDraft_1628, *E. faecium* DO) [241]. Despite the importance of *E. faecium* as a human pathogen, the role of this ECF σ-factor or RseP has not yet been studied. However, the involvement of RseP in the stress response, virulence, and conjugation in many related pathogens such as *E. faecalis* suggests an important role for RseP also in *E. faecium*. The importance of RseP in *E. faecium* is supported by phenotypic experiments showing reduced fitness of *rseP* disruption mutants. In these experiments, mutants showed 6-to 8-fold higher sensitivity towards lysozyme, and severely reduced tolerance to desiccation, from at least 55 days in the wild type to only 4 days for the *rseP* mutants [242]. Further, *rseP* mutants lack the ability to form chains (chaining) [242]. Disruption of *rsiV* (encoding the anti-SigV factor) in *E. faecalis*, which leads to constitutive activation of SigV, induces a chaining phenotype [170]. Although little is known about the importance of chaining in enterococci, in other species, chaining is an important contributor to virulence, complement evasion, and adhesion to host cells [243,244].

1.5 Applications of bacteriocins

The rise of antibiotic resistance, regarded by many as the new global health crisis, has reinvigorated an interest in bacteriocins as an addition or alternative to antibiotics. There is a growing need for new antimicrobials to combat pathogens that affect both humans and animals. Bacteriocins comprise a large and diverse group of compounds with high potency against many of the most important pathogens. In addition, bacteria normally present in our environment are arguably an inexhaustible source of new bacteriocins [245]. However, commercial applications of bacteriocins have thus far been limited to agriculture and the food industry to improve the shelf-life of food [246].

Nisin is approved for use as a food preservative in over 50 countries and was granted the generally recognized as safe (GRAS) status by the FDA in the late 1980s [247,248]. Nisin is the only commercially produced bacteriocin, and the only bacteriocin approved for use as a food additive (approved in the EU as food additive E324) [249]. The commercially available nisin products are sold under the trademarks Nisaplin™ and Novasin™ by Danisco A/S, a subsidiary of DuPont. Nisaplin™ is a partially purified (crude) milk fermentate containing 2.5% active nisin. Nisaplin™ has been used to control foodborne pathogens in a variety of food products for decades [250].

Many investigators have shown that Nisaplin™ and other nisin preparations also has the potential to control infections caused by (antibiotic resistant) Gram-positive bacteria [251]. Goldstein et al. (1998) showed that Nisaplin™ had excellent activity *in vitro* towards clinical isolates of *S. pneumoniae* and intravenous administration protected mice from *S. pneumoniae* infection [252]. In a study by Dosler & Gerceker (2011) Nisaplin™ acted synergistically with ciprofloxacin and vancomycin against MRSA and MSSA (susceptible), thus lowering the amount of antibiotic needed [253]. Despite the promising results of bacteriocins in animal and in vitro studies, the estimated cost of bringing a new drug to market is in excess of $161 million USD [254].

As an alternative to therapeutic applications, some studies have investigated the use of bacteriocins for diagnostic purposes. This can help overcome the financial and regulatory hurdles of drug development, while providing rapid, low-cost detection of pathogens. Faster diagnosis of patients with infections reduces the burden on the

health care system and can reduce the use of broad-spectrum antibiotics. However, the literature on using bacteriocins for detection and diagnostics is scarce. Lupetti et al. (2003) showed that a peptide corresponding to residues 29-41 of a murine microbicidal protein ubiquicidin, radiolabeled with ^{99m}Tc, would locate to sites of infection in mice [255]. The authors showed that the peptide was able to discriminate between inflamed tissues infected with *Pseudomonas aeruginosa* and tissues inflamed by injection of LPS (lipopolysaccharide). A similar discrimination was also shown towards some fungi and Gram-positive bacteria [255].

The same peptide (UBI29-41) labeled with a near-infrared (NIR) dye ICG-Der-02 was shown to localize to the site of *S. aureus* infection in mice by Liu & Gu (2013) [256]. Wavelengths of light in the near-infrared (IRDye 800CW emits at 780-792 nm) can pass through thin sections of tissue (< 20 mm), which allows for the *in vivo* imaging of an ongoing infection in living hosts. A similar approach was employed by van Oosten et al. (2013) using vancomycin labeled with an IRdye 800CW [257]. Vancomycin is an antibiotic with strong affinity for the Gram-positive cell wall, as it binds to the d-Ala-d-Ala terminus of peptidoglycan [257]. Indeed, the authors show that labeled vancomycin (vanco-800CW) accumulated *in vivo* at the infection site caused by *S. aureus*. This was tested in a mouse myositis model inoculated with both *E. coli* (or sterile inflammation) and *S. aureus* prior to intravenous injection of vanco-800CW [257].

Antimicrobial peptides (AMPs) produced by animals, particularly insects and amphibians, exhibit good activity against different Gram-negative bacteria [258,259]. Although the activity is highly variable and not specific (not receptor-mediated), most show a strong affinity for the Gram-negative cell envelope [258]. Arcidiacono et al. (2008) labeled three AMPs (cecropin P1, SMAP29 and PGQ) with the fluorescent label Cy5 to demonstrate binding to pathogenic *E. coli* O157:H7 [260]. Using Cy5 labeled cecropin P1, the authors measured binding directly in whole-cell suspensions with a sensitivity 10-fold higher than for Cy5 labeled anti-*E. coli* O157:H7 antibodies [260].

The class IIa bacteriocin leucocin A (LeuA) has been utilized in various detection platforms to selectively detect *Listeria monocytogenes* from other Gram-positive bacteria [261–265]. LeuA is a 37 aa bacteriocin with potent activity towards *Listeria* (minimum inhibitory concentration ≈ 0.1 nM) [263]. Azmi et al. (2014) immobilized LeuA to the surface of a microcantilever (MCL)-based biosensor. A microcantilever

is a device that detects changes in deflection or vibrational frequency upon changes in the weight/load of the cantilever. In a suspension of 10^5 CFU/ml of *L. monocytogenes*, the authors measured a significant deflection of the MCL, indicating detection of the bacterium in the sample. A similar detection sensor was demonstrated by Etayash et al. (2014), in this study, LeuA was immobilized on an interdigitated impedimetric array sensor [264]. This sensor changes its impedance (electrical resistance) when biomolecules associate with the surface. The impedance signal was higher for *L. monocytogenes* than other Gram-positive bacteria, including *E. faecalis*, *L. innocua* and *S. aureus* [264].

2 Purpose of study and outline of thesis

Bacteriocins are a class of compounds with high specificity and potency against many important pathogens. Because most bacteriocins are membrane-active, pore-forming antimicrobials, their mode of action differs from that of antibiotics. This makes bacteriocins equally potent also against antibiotic-resistant strains. Despite the success of bacteriocins in controlling pathogens *in vitro* and in animal studies, bacteriocins have so far seen very little or no use in human medicine. This is arguably due to the success of antibiotics, and consequently, the very limited investment spent on research, discovery, and characterization of bacteriocins with potential for therapeutic use. This is further exacerbated by the high costs involved in drug development, clinical trials, and approvals for human use. The goal of this thesis is; **(I)** discover, isolate, and characterize novel bacteriocins active against important pathogens. **(II)** obtain a better understanding of the interaction of the bateriocin EntK1 with its receptor RseP, and **(III)** investigate the potential of using EntK1 as a diagnostic tool for the detection of *E. faecium*.

The thesis is divided into two parts, the first part (**Papers I-IV**) is on the discovery, isolation, and characterization of novel bacteriocins active against important Gram-positive pathogens, with a particular emphasis on *E. faecium*. The second part (**Papers V-VI**) is an in-depth investigation of the bacteriocin-receptor interaction for EntK1 and RseP, and the development of a bacteriocin-based assay employing EntK1 for detecting *E. faecium*.

3 Main Results

I Ubericin K, a new pore-forming bacteriocin targeting mannose-PTS

Bacteriocins are known to generally target closely related organisms. In this study, we sought to discover new bacteriocins active against the bovine mastitis-associated pathogens *S. aureus*, *S. dysgalactiae* and *E. faecalis*. To do this, we screened 53 samples of raw milk for bacteriocin-producing bacteria that inhibited the growth of these organisms. The samples had been obtained from individual Norwegian Red cows from two dairy herds in south-eastern Norway as part of a study by Porcellato et al. (2020) [266]. From the screening, an isolate of *Streptococcus uberis* was found that inhibited both *S. dysgalactiae* and *E. faecalis,* but not *S. aureus.* The antimicrobial activity was present in the supernatant, and was heat-stable and protease-sensitive, properties typical of bacteriocins.

Whole-genome sequencing of the isolate revealed a gene cluster predicted to encode five uncharacterized bacteriocin-like peptides, one class IIb two-peptide bacteriocin (ORF6/7) and three class IId bacteriocins (ORF3, ORF10, and ORF13). The bacteriocin gene cluster appeared to be intricately regulated by a quorum sensing-like three-component system, consisting of a response regulator (ORF1), histidine protein kinase (ORF2) and peptide pheromone (ORF3). Additionally, a pair of direct repeats was found near the predicted promoters for *orf5* and *orf12* both upstream and downstream of the promoter, suggesting the presence of transcription factors with both activator and repressor functions acting on this cluster.

The antimicrobial activity was purified using ammonium sulfate precipitation, cation-exchange- and reversed-phase chromatography. To identify the bacteriocin, active fractions were analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). A mass consistent with the expected mature and unmodified product of ORF10, which we named ubericin K (UbeK), was identified. Sequence alignments showed that it belonged to the lactococcin A subgroup of linear, non-pediocin like bacteriocins. The highest sequence identity with a known bacteriocin was to bovicin 255. In addition to inhibiting *S.*

dysgalactiae and *E. faecalis*, UbeK showed potent activity against *E. faecium* and *Listeria*. However, we could not exclude the possibility that other bacteriocins were also responsible for some or all of the activity from this strain. To show that UbeK was the bacteriocin responsible for the activity, the gene was cloned and expressed *in vitro*. Assays of *in vitro* synthesized UbeK showed nearly identical activity and inhibition spectrum as purified UbeK. The activity of UbeK was shown to be receptor-mediated and rely on Man-PTS. A man-PTS deletion mutant of *L. lactis* IL1403 (strain B464) was shown to be insensitive to UbeK, while its wild type counterpart was highly sensitive. We further show, by using a ratiometric variant of green fluorescent protein (pHluorin), that the MoA of UbeK involves membrane disruption (i.e., pore formation).

II Identification of a novel two-peptide lantibiotic from *Vagococcus fluvialis*

In this study, we screened a varied selection of fermented fruit and vegetables (40 samples) for bacteriocin producers specifically able to inhibit a vancomycin-resistant strain of *E. faecium* (LMGT 20705; VRE). The strain was confirmed to be multi-drug resistant by the disc diffusion test, according to EUCAST. Additionally, WGS of the strain revealed resistance genes for several antibiotic classes. A total of 17 isolates were found to produce an inhibitory substance against *E. faecium* LMG 20705. However, using repetitive element PCR (Rep-PCR) only 9 unique profiles were found, suggesting that some isolates were clonal. Using whole-genome sequencing and bacteriocin-mining tools (AntiSMASH and Bagel4), one isolate belonging to the species *Vagococcus fluvialis* (later designated LMGT 4216) was found to encode a putative novel two-peptide lantibiotic that we named vagococcin T (VcnT).

In silico analysis of the genes believed to belong to the VcnT cluster predicted 11 genes organized as two transcriptional units. One operon appeared to encode all biosynthetic genes, at the start of the cluster were two genes encoding a LanFE-like immunity system (VcnFE), which was followed by a tandemly arranged pair of genes (*vcnA2-vcnM2-vcnA1-vcnM1*) encoding a lantibiotic precursor peptide and its modification enzyme. Downstream was a gene predicted to encode a bacteriocin ABC-transporter and peptidase (VcnT/VcnT$_P$), followed by a small ORF that may serve a role in immunity (VcnI). The second predicted operon encode proteins resembling a three-component quorum sensing system. In addition to a RR (VcnR) and HPK (VcnK), two ORFs (VcnQ1 and VcnQ2) encode proteins with sequence similarity to FsrD and FsrB. Fsr is a quorum sensing system in *E. faecalis*, where FsrD is a prepeptide that is processed by FsrB into a small cyclic peptide pheromone.

Purification of the antimicrobial activity produced by *V. fluvialis* LMGT 4216 and subsequent MALDI-TOF mass spectrometry found molecular masses corresponding to the theoretical masses predicted for mature VcnA1 (Vcn Tα) and VcnA2 (VcnTβ). The purified Vcn T was shown to be active against all Gram-positive species tested except for *S. aureus*. Spontaneous mutants of *E. faecium* resistant to VcnT (up to 256-fold reduced sensitivity) could be isolated from cultures exposed to the

bacteriocin. Sequencing of these mutants revealed mutations in a gene encoding the cell wall-active antibiotics response protein LiaF (stress regulator protein). LiaF serves an important regulatory role in the activation of stress-responsive two/three-component systems in *B. subtilis* (LiaFSR) and *S. aureus* (VraTSR; VraT is a LiaF homologue). Similar TCSs to Lia exist in most Firmicutes, and all regulate the expression of genes that protect the cell against damage to the cell envelope. The connection between high-level resistance to membrane-targeting antimicrobials and the regulator LiaF suggests this protein as a good target for future drug development.

III Genome-assisted identification, purification, and characterization of bacteriocins

Bacteriocins exhibit many properties of a good drug candidate, such as high potency, small molecular weight, low toxicity, and a narrow inhibition spectrum. However, bacteriocins have so far not been used therapeutically beyond preliminary experiments on animals.

In this paper, we provide a detailed step-by-step protocol for the set of methods used in our laboratory for bacteriocin purification, identification, and preliminary characterization. These methods have been used successfully on many new bacteriocins belonging to both class I (e.g., vagococcin T) and class II (e.g., lactococcin A, ubericin K, and garvicin KS). The protocol involves ammonium sulfate precipitation followed by cation-exchange- and reversed-phase chromatography. Fractions obtained from each purification step is assayed for antimicrobial activity using a serial-dilution technique in microtiter plates. By proceeding only with the most active fraction(s), the method provides a highly pure bacteriocin sample. MALDI TOF MS is used to estimate the purity of the sample and to verify or identify the bacteriocin. For new bacteriocin producers, whole-genome sequencing and bacteriocin mining tools are used to identify the structural gene and other biosynthetic genes. Structural prediction of the bacteriocin combined with the molecular mass(es) obtained by MALDI TOF MS is often sufficient for verification.

To obtain some insight into the mode of action of a bacteriocin, we present two pore formation assays. The first method is based on the fluorescent dye propidium iodide (PI), which has a higher quantum yield (and consequently fluorescence intensity) when intercalated with DNA than in solution. Intact membranes are impermeable to PI, however, when the membrane is disrupted by a bacteriocin, it will diffuse into the cell, resulting in an increase in fluorescence. This can be easily measured by a microplate reader with fluorescence capability. The second assay uses a pHluorin biosensor strain (*L. monocytogenes* pNZ-pHin2^{Lm}), the fluorescence characteristic of the strain is dependent on the intracellular pH (pH_{in}). Membrane disruption results in a decline in pH_{in} if the surrounding medium is kept at a lower pH. This is measured by a change in emission intensity at 520 nm from excitation at 400 nm relative to 480 nm.

IV Design of novel saposin-like bacteriocins with antimicrobial activity using a hybrid approach

In this work, we used a synthetic biology approach to identify new-to-nature bacteriocins. We constructed a library of synthetic genes encoding hybrid peptides derived from seven saposin-like leaderless bacteriocins; lactolisterin BU (LliBU), mutacin BHT-B (BHT-B), aureocin A53 (AurA53), K411, lacticin Q (LacQ), epidermicin NI01 (EpiNI01), and salivaricin C (SalC). To construct the library, we hypothesized that these bacteriocins are bifunctional, where the C-terminal part binds and recognizes certain lipids, while the N-terminal part inserts into the membrane. Consequently, each peptide was split in two based on sequence features thought to be important for these roles (hydrophobic/hydrophilic character, presence of acidic residues). The resulting two peptide sequences of all seven bacteriocins were exchanged in all combinations to create a library of 49 sequences. The final library consists of 42 new hybrid peptides in addition to the seven bacteriocin sequences from which they were derived.

The library was then expressed *in vitro* to assess their bioactivity. Antimicrobial activity from each synthesis was assessed against a panel of indicators; *L. lactis*, *E. faecium*, *E. faecalis*, *L. monocytogenes*, *S. dysgalactiae*, *S. aureus*, *S. haemolyticus*, and *E. coli*. Testing revealed 11 new hybrid bacteriocins that were active against at least one of the indicators. Furthermore, six of the new hybrid bacteriocins appeared highly potent and active against several of the indicators, including *S. aureus*, *L. monocytogenes*, and *E. faecium*. Interestingly, swapping the C-terminal part of the bacteriocins changed both the inhibition spectrum and apparent potency.

A better understanding of the mechanism of binding and killing by bacteriocins will make it possible to rationally engineer these peptides for improved potency and to potentially target any bacterial species of interest.

V The extracellular domain of site-2-metalloprotease RseP is important for sensitivity to bacteriocin EntK1

Enterocin K1 (EntK1) is a small (37 aa), unmodified, and leaderless bacteriocin that exploits the membrane-bound protease RseP as a receptor to kill target cells. RseP is a well-conserved protein with orthologs present in most bacteria. A better understanding of how EntK1 interacts with RseP to cause cell death will allow us to potentially engineer new and improved variants of these peptides. Such peptides could potentially be targeted against RseP homologs present in important pathogens or to improve potency.

In this work, we combine sensitivity and binding assays to better understand the binding of EntK1 to RseP. To do this, we developed a heterologous expression system for *rseP* in *L. plantarum*, a naturally EntK1-insensitive species. Using this system, we expressed RseP fusions of various regions of RseP from both sensitive and non-sensitive species. Additionally, alanine substitutions of specific amino acids in a sensitive variant of RseP were performed to examine the effect of each substitution on binding and sensitivity. As expected, heterologous expression of *rseP* from the sensitive *E. faecium* (*Efm*RseP) rendered *L. plantarum* highly sensitive to EntK1. To measure binding, we developed a binding assay for *L. plantarum*. To do this, EntK1 was chemically synthesized with an N-terminal FITC fluorescent tag (FITC-EntK1). Following incubation with FITC-EntK1, the fluorescence signal of the cells was measured by flow cytometry and expressed as the median fluorescence intensity (MFI) of the entire population. *L. plantarum* expressing *Efm*RseP showed ~250-fold higher MFI values compared to a non-expressing control, indicating a strong RseP-dependent association of FITC-EntK1 to these cells.

Previous studies have shown that residues of the active site of RseP are important for killing by the LsbB family of bacteriocins. Additionally, these bacteriocins have similar characteristics to the natural substrates of RseP. Based on this, we hypothesized that EntK1 interacts with RseP in a similar manner as the natural substrate. Several regions of RseP have been proposed to be important for substrate recognition and cleavage, these include the membrane-reentrant β-hairpin–like loop (MRE β-loop), the GxG motif, the PDZ domain, and the extended LDG region. By constructing hybrids of RseP containing these regions from either *Lp*RseP (non-sensitive) or *Efm*RseP (sensitive), we showed that residues of the PDZ domain and

extended LDG region are crucial for binding and killing by EntK1. Alanine substitutions in the active site residues (H18A, H19A, and H22A) did not drastically alter the sensitivity towards EntK1, indicating that catalysis is not essential. Although a triple mutant with all three substitutions was significantly less sensitive, this suggests that the structure of the active site region is likely important. Substitutions in the extended LDG region revealed one mutant, N359A which completely lost sensitivity and binding to EntK1.Taken together, we were able to show that binding and sensitivity towards EntK1 depend on key residues in the PDZ domain in conjunction with N359.

VI Flow cytometric detection of *E. faecium* in urine using fluorescently labelled enterocin K1

The rapid diagnosis of bacterial infections is a major challenge in medicine. Identification of microorganisms by conventional methods typically involves cultivation and plating techniques, which are laborious and time-consuming. Many bacteriocins target bacteria in a specific receptor-mediated manner and can be active in the femto- to nanomolar range, suggesting a strong interaction with the receptor. In this work, we sought to explore the possibility of using the bacteriocin EntK1 as a specific probe for the detection of its target organism, *E. faecium*. We further demonstrate the potential of EntK1 to detect *E. faecium* in urine in a simulated case of a urinary tract infection (UTI). UTIs are one of the most common infections in hospitals and account for considerable health-care costs. The typical laboratory diagnostic criterion for a UTI is the presence of 10^5 CFU/ml of at least one bacterium in urine. Identification of the causative agent is most commonly done by urine culture, which takes 24-48 h. An estimated 3% of UTIs are caused by *E. faecium*.

In this work, we developed and optimized a method for detecting *E. faecium* in a sample using fluorescently labeled EntK1 (FITC-EntK1) and flow cytometry. The method was fast (<40 min) and reproducible, which made it possible to distinguish samples containing *E. faecium* (e.g., from infected individuals) from control samples. We further show that the method could distinguish between urine samples containing *E. faecium* and samples containing *E. coli* or *S. aureus* at 10^5 CFU/ml. *E. coli* and *S. aureus* were included as controls as Gram-negative and Gram-positive bacteria implicated in UTIs, and both were insensitive to EntK1. Furthermore, EntK1 was shown to preferentially bind *E. faecium* cells also in mixed cultures containing an equal number (CFU) of *E. coli* or *S. aureus*.

The work demonstrates the diagnostic potential of bacteriocins and provides a proof of concept for using bacteriocins as specific “probes” for detecting bacteria in a sample. The method is fast and showed good sensitivity and specificity, positively identifying samples containing *E. faecium*. Methods could most likely be developed for other bacteriocins with specificty towards selected pathogens, targeting other organisms, and for other bodily fluids where infections occur.

4 Discussion

4.1 Identification of novel antimicrobials

Paper I and **II** describe the discovery, purification, and characterization of two novel bacteriocins that we named ubericin K (UbeK) and vagococcin T (VcnT). Both bacteriocins were found to inhibit the growth of important pathogens, such as *E. faecium* and *L. monocytogenes*.

It has been well-documented that bacteriocin production is ubiquitous among bacteria, and many new bacteriocins are yet to be identified and exploited. Samples from nature that are rich in bacteria, such as raw bovine milk (**Paper I**) or fermented fruits and vegetables (**Paper II**) will almost certainly contain bacteriocin producers. By using simple screening techniques, bacteria producing inhibitory substances against a given "indicator" can be isolated from such samples. Given the recent advances in genomics, isolates can be whole-genome sequenced at low cost to identify potential bacteriocin biosynthetic genes. The continued effort to identify new bacteriocins may help discover antimicrobials suited for therapeutic or diagnostic applications.

For both bacteriocins, purification and characterization were carried out using methods described in **Paper III**. This work describes a general purification scheme that works well with most bacteriocins. As most bacteriocins are positively charged, an initial purification step using cation-exchange chromatography is well-suited for removing the majority of unwanted molecules. However, this step will not work well for peptides that are neutral or only have a small positive charge. Indeed, initial purification of VcnT revealed substantial loss of the bacteriocin following cation-exchange chromatography. We found that simply diluting the sample 10-fold prevented loss and allowed efficient binding of the peptides to the column. This indicates that the presented purification scheme can easily be adapted also to more diverse molecules. Subsequent purification using reversed-phase chromatography should be sufficient to isolate the bacteriocin peptide(s) for identification by mass spectrometry. **Paper III** describes the use of MALDI-TOF MS, a technique that can be used by most researchers without special training to facilitate identification and/or confirmation of the bacteriocin.

In **Paper IV** we describe the construction and discovery of novel hybrid saposin-like bacteriocins using synthetic DNA and cell-free protein expression. Many circular and leaderless bacteriocins have been shown to have a saposin-like fold, saposins are a group of proteins involved in lipid binding and metabolism (see section 1.2.2.2 and **Figure 7**). A mechanism of action has been proposed for the saposin-like circular bacteriocin AS-48, which has been shown to exist in two dimeric forms depending on pH [267]. Upon interacting with the membrane, the acidic environment is thought to protonate four acidic residues of AS-48, which then interact with the phosphate moiety of a phospholipid [267]. The interaction results in the rearrangement of AS-48 into a membrane-bound dimer. Many linear saposin-like bacteriocins contain two or more acidic residues, primarily at their C-terminal half (see **Paper IV**, Figure S1). Because of this, we hypothesized a similar mechanism also for the saposin-like linear bacteriocins. Where the C-terminal half is involved in lipid recognition and the N-terminal half inserts into the membrane.

Additionally, many saposin-like bacteriocin gene clusters encode proteins with bacterial pleckstrin homology (bPH2) domains, with a presumed role in transport and/or immunity. These proteins are found in the gene clusters encoding LacQ, SalC, EpiNI01, K411, and AurA53, among others. The pleckstrin homology (PH) domain is very common in eukaryotic proteins with diverse roles, but one of the best known is binding phosphatidylinositol lipids and targeting proteins to the membrane [268]. It is tempting to speculate that these bPH2/PH domain containing proteins are also involved in lipid binding in bacteria, possibly in a manner that confers immunity to producer cells.

Normally, the biosynthesis of bacteriocins requires at least two other proteins, a dedicated immunity protein and a transporter for secretion. Both proteins are relatively specific for their cognate bacteriocins, which hinders the production of new and modified peptides in bacteria. One method to overcome this is using cell-free protein expression systems.

A cell-free expression approach for bacteriocin production is not new, a library of synthetic bacteriocin genes for in vitro synthesis of over 100 bacteriocins, known as the PARAGEN 1.0 library, was reported in 2019 [269]. Inspired by this and the possibilities of synthetic DNA, we constructed a library of hybrid (novel) peptides derived from leaderless bacteriocins. Using this hybrid approach, we identified new

peptides with antimicrobial activity against *E. faecium*, *L. monocytogenes*, *S. aureus*, and *S. haemolytics* isolated from human and animal infections.

Due to limitations in cell-free expression of bacteriocins, we sought to establish a bacterial expression and purification scheme for these hybrid bacteriocins. This would allow us to obtain larger quantities of the peptides for further characterization. Because no cognate immunity or transporter exists for these peptides, production was attempted in the cytosol of *E. coli*, a species insensitive to all bacteriocins in the library. To establish the expression and purification scheme, one hybrid bacteriocin showing good broad-spectrum activity, designated ISP26 (in vitro synthesized peptide), was used. However, expression of this peptide in *E. coli* was unsuccessful, even when the bacteriocin peptide was fused to the C-terminus of MBP (maltose-binding protein). We could not determine why expression in *E. coli* failed. One possibility is that *E. coli* harbors effective mechanisms to recognize and degrade unstructured peptides/proteins in the cytosol.

Due to the failure of expressing the fusion in *E. coli*, the MBP-ISP26 fusion was cloned into a lactoccocal expression vector based on the nisin promoter (P_{nisA}). When the strain *L. lactis* NZ9000 (which harbors a chromosomal insertion of *nisRK*) was transformed with the plasmid, expression of the fusion was successfully induced. Purification of the fusion using amylose resin followed by SDS-PAGE showed a protein of the expected size (49.6 kDa) which was bigger than the protein purified from the control expressing only MBP (45.5 kDa).

To liberate the hybrid bacteriocin ISP26 from MBP, a TEV cleavage tag was present at the C-terminal end of MBP. The bacteriocin ISP26 had been fused to MBP such that the start codon (Met) was in the P1' position of the TEV protease recognition site, which is reported to be tolerant to several residues at that position (ENLYFQ|S/G/A/M/C/H) [270]. However, TEV cleavage of the purified fusion protein was not successful. Treatment with TEV protease did not yield active bacteriocin, nor was any cleavage detectable by SDS-PAGE. The addition of denaturing agents (urea, SDS, and guanidine HCl) during cleavage was also attempted without success. More work is needed to find good production and purification strategies for these peptides.

In summary, **Paper V** describes the construction of new-to-nature antimicrobials with apparently improved potency and inhibition spectrum. The antimicrobial activity data presented in this work can provide important clues to the antimicrobial determinants of leaderless bacteriocins. An improved understanding of the features necessary for killing will allow us to engineer new and improved peptides in the future. Importantly, leaderless bacteriocins are ideally suited for chemical synthesis, modification, and engineering.

4.1.1 The ubericin K cluster encode other active bacteriocins

The strain *S. uberis* LMGT 4214 was shown to harbor a bacteriocin gene cluster encoding multiple bacteriocin-like peptides (Blp) designated ORF3, ORF6, ORF7 and ORF13. However, purification of the antimicrobial, which was active towards the indicators, only revealed ubericin K (UbeK). One possibility is that these Blps may be remnants of genes that once encoded functional bacteriocins but have been lost by selection. Alternatively, these genes may be regulated differentially and require an unknown stimulus for expression. To investigate this, we have since cloned all Blps from the cluster and expressed them *in vitro* as described for UbeK in **Paper I**. Activity assays of the *in vitro* synthesized peptides revealed that all were, in fact, active bacteriocins except for ORF3 (unpublished). Furthermore, ORF6 and ORF7 were only active in combination and not individually, thus confirming these peptides as a new class IIb two-peptide bacteriocin. ORF6/7 inhibited a strain of *E. avium* and *Lactobacillus sakei* that were both otherwise insensitive to UbeK and ORF13. A strain of *Pediococcus acidilactici* and *L. garvieae* were sensitive to all three bacteriocins.

Interestingly, ORF13 produced a larger zone of inhibition than UbeK towards the *S. uberis* LMGT 3912 strain used as an indicator for purification of UbeK (unpublished). Although we did not estimate the yield of ORF13 from the *in vitro* expression, however, it indicates considerable activity of the bacteriocin ORF13 that should permit its discovery during purification. Despite assaying all fractions obtained during purification towards multiple strains sensitive towards *in vitro* synthesized ORF13 and ORF6/7, no activity other than UbeK was found.

Mature ORF3 is a short (27 aa), hydrophobic, and cationic peptide, properties similar to those of the pheromone plantaricin A. Furthermore, ORF3 is located just downstream of the putative TCS (ORF1; RR and ORF2; HK). ORF3 is likely part of a

quorum-sensing system and necessary for induction of the TCS and activation of the RR. Putative promoters with two direct repeats predicted to be regulatory elements are located just upstream of *orf5* and *orf12*, but not *ubeK*. Instead, a putative promoter, TTGACA-20nt-TATAAT is located upstream of *ubeK* with a -35 and -10 sequence exactly matching the *E. coli* consensus promoter. Overexpression from this promoter is likely only limited by the 20 bp spacing between the -35 and -10 boxes, which is suboptimal, but still present in many highly expressed genes [271]. In fact, the strength of promoters also depends on an AT-rich 17-20 nt region just upstream of the -35 box known as an UP-element, which often compensates for suboptimal spacing [271]. The 20-nt region just upstream of the putative *ubeK* promoter has a GC-content of only 10% (while the genome is 37%). Upstream of *orf12* is a predicted weak terminator (ΔG_s -3.7 kcal/mol) that is likely partially responsible for the lack of transcription of *orf13/14* from the *ubeK* promoter. Additionally, DNA-binding proteins predicted to act on the direct repeats at P_{orf12} probably also act as a roadblock for RNA polymerase.

The mature peptide encoded by ORF3 was chemically synthesized and added to the culture of the producer strain to see if it could stimulate the production of ORF6/7 and ORF13. However, only UbeK could be purified from the culture. It could be that RR encoded by the cluster acts as a transcription activator and is activated by ORF3, but that other repressors are present that require other signals. Analogously to the *lac* operon in *E. coli*, the Lac repressor (LacI) remains bound and blocks transcription even if the activator CAP is present. Further work is needed to identify the conditions necessary for expression of the remaining bacteriocins, and the possible significance of their role. The differential regulation and varied inhibition spectrum of all three bacteriocins suggest a complex ecological role for this bacteriocin locus.

4.2 The LsbB-like leaderless bacteriocin EntK1 binds specifically to the site-2 protease RseP on target cells

In **Paper V**, we show that the binding of EntK1 to target cells, a peptide belonging to the LsbB family of leaderless bacteriocins (see section 1.2.3), depends on RseP (see section 1.3.3). We further explored the role of the different regions of RseP in binding EntK1. For this purpose, heterologous expression of RseP was established in *Lactiplantibacillus plantarum* using the pSIP system. *L. plantarum* is naturally insensitive to EntK1, and the expression of RseP variants using this system would ensure the same background for all experiments. If factors other than RseP are important for binding EntK1, they would likely vary between species, making it difficult to differentiate effects due to RseP and species-specific effects. As expected, expression of RseP from sensitive species (e.g., *E. faecium*) in *L. plantarum* rendered the cells highly sensitive to the bacteriocin. However, it could be that EntK1 associated equally with both sensitive and insensitive cells. It is believed that the initial interaction of bacteriocins with the bacterial cell surface is dominated by electrostatic interactions. The cell surface is negatively charged due to phosphoryl and carboxylate groups on phospholipids and lipopolysaccharides.

To measure binding, EntK1 was chemically synthesized with a FITC (fluorescein isothiocyanate) fluorescent tag attached to the N-terminus. Importantly, the modified EntK1 was still able to bind RseP as it retained high potency (only a 4-fold reduction in MIC) towards *E. faecium*. The FITC tag was chosen because of its small size, as larger fluorescent tags could interfere with the receptor interaction of EntK1 and thus be unsuitable. However, FITC has the disadvantages of being prone to photobleaching, being pH sensitive, and having a low fluorescence intensity compared to other dyes.

Interestingly, *L. plantarum* cells expressing RseP from insensitive species showed almost no binding, as assessed by flow cytometry. Further, the degree of binding measured by flow cytometry correlated well with differences in sensitivity of the various clones (based on MIC). These observations strongly suggest that EntK1 depends on a specific RseP present on the cell surface for binding and that other factors, such as surface charge, have little measurable contribution.

To better understand the specific regions of RseP involved in the interaction, various RseP fusions were constructed that contained parts of RseP from *L.*

plantarum (*Lp*RseP) and parts from *E. faecium* (*Efm*RseP). Using binding and sensitivity assays, the PDZ domain of RseP was shown to be crucial for sensitivity and binding. Furthermore, by constructing single alanine substitutions in RseP, we identified an amino acid, N359, which was also crucial for binding and sensitivity. This residue is predicted to be located just above the active site.

Taken together, this suggests that binding of EntK1 to RseP is a two-step process, where an initial binding to the PDZ domain is essential. *In silico* modeling and structural prediction indicate that the C-terminal tail of EntK1 binds to a groove or pocket formed by the PDZ domain (**Figure 15**).

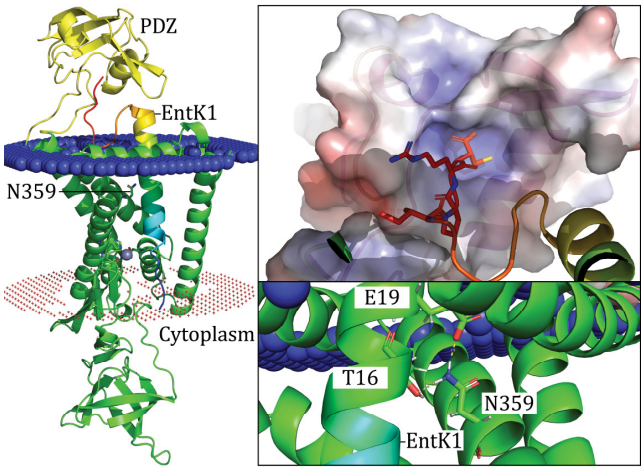

Figure 15. Structure prediction of the complex between *E. faecium* RseP and EntK1. The C-terminal tail of EntK1 is colored red and seen to interact in the peptide binding groove ("pocket") of the PDZ domain (upper right panel). The residue N359 of RseP interacts by hydrogen-bonding with T16 and E19 of EntK1 (lower right panel). The structure was predicted using AlphaFold-Multimer (v2.3.2), the zinc atom (gray sphere) was placed using AlphaFill, and the transmembrane topology was predicted using the PPM 3.0 web server [189,272,273].

Only then can the N-terminal half penetrate through the transmembrane core of RseP down near the active site. However, the subsequent events leading to cell death are unknown. Further research to understand the mode of action of these bacteriocins is ongoing and will be discussed in the subsections below.

4.2.1 Mode of action of the LsbB family

Understanding how bacteriocins of the LsbB family bind to RseP and cause cell death will be crucial for fully utilizing RseP as an antimicrobial target. It can be envisaged that a good understanding of the mechanism of these bacteriocins will allow us to rationally engineer these peptides to target RseP in other species and increase their potency. Currently, nothing is known about the mechanism of action of the LsbB family of bacteriocins. A plausible mechanism for pore forming bacteriocins targeting transporters such as Man-PTS, CorC, or APC can be proposed based on what is known for lactococcin A (LcnA). Similar to the mechanism described for LcnA, simply locking, or wedging these transporters into an open or unregulated conformation would likely result in leakage and cell death. However, RseP has only four transmembrane helices and is unlikely to have any transport function. However, SDS-PAGE and size-exclusion chromatography of purified *Efm*RseP indicate that the protein exists as a dimer *in vivo* or possibly in larger oligomeric states [274]. A protein complex of RseP would likely contain larger cavities that could be exploited by the bacteriocin for pore formation. Another attractive hypothesis is that a pore can form from the water access channel going to the active site. Normally, this channel only spans approximately halfway through the lipid bilayer, however, binding of the bacteriocin could act like a wedge to force the channel further open to span through the membrane. Structures of RseP from *E. coli* and *Methanocaldococcus jannaschii* (*mj*S2P) suggest that this channel is narrow, possibly only permitting the diffusion of water, protons (hydronium) or the smallest cations such as potassium (including the radius of the hydration shell) [190,193,275].

An essential function of bacterial membranes is the maintenance of a proton motive force (PMF) which ensures an electrochemical proton gradient across the membrane necessary for ATP synthesis. The PMF is a force resulting from two phenomena, the electrical potential gradient ($\Delta\Psi$) and the proton gradient (ΔpH) across the membrane. The electric potential difference across the bacterial membranes is due to a net imbalance of cations to anions in the cytoplasm, while the proton gradient is an excess of protons outside the cell relative to the inside, or *vice versa*. As the PMF is crucial for normal growth and provides the energy necessary for many intracellular processes, the disruption of either its $\Delta\Psi$ or ΔpH component can be lethal [276].

The propidium iodide-based pore formation assay described in **Papers I** and **III** has also been performed on the LsbB family of bacteriocins on both *E. faecium* (EntK1, EntEJ97, H1) and *L. lactis* (LsbB) with negative results (unpublished). Suggesting that these members of the LsbB family do not form large pores that permit the passage of PI and/or DNA. To investigate if these bacteriocins disrupt the ΔpH, the pHluorin-based pore assays using biosensor strains of both *L. monocytogenes* and *L. lactis* were performed, also with negative results (unpublished). *L. monocytogenes* was tested against H1 which exhibits weak activity towards the strain, while *L. lactis* was tested with LsbB, EntK1, and EntEJ97, all of which exhibit good potency against *L. lactis*. The other likely possibility is the formation of ion-specific pores that are not proton-conductive, such as those described for LcnG. To test this, we have performed preliminary adsorption and release experiments using the voltage-sensitive dye DiSC(3)5. This dye binds to polarized membranes (charged), resulting in the quenching of its fluorescence, as the membrane potential is lost, e.g., by the formation of ion-conducting pores, the dye releases from the membrane increasing its fluorescence. Preliminary data did not show any clear loss of membrane potential in *E. faecium* or *L. lactis* upon exposure to EntK1 or LsbB, respectively. Additionally, potassium-leakage experiments on *L. lactis* cultures exposed to LsbB were performed. The results did indicate K^+-release from *L. lactis* following exposure to LsbB (unpublished). However, the biological significance of this K^+-release is uncertain because valinomycin, a K^+-specific ionophore, resulted in higher K^+-leakage than LsbB but had no antimicrobial activity.

The low activity of valinomycin may be due to the acidic (pH 6.5) used in the K^+-leakage buffer. This can be explained by considering the relative contributions of $\Delta\Psi$ and ΔpH to the PMF at different pH. At acidic, pH the ΔpH is the main component contributing to the PMF, only at alkaline pH is the main contributor $\Delta\Psi$ [277]. Additionally, $\Delta\Psi$ disrupting agents may show reduced activity towards *L. lactis* and other LAB in many assays because LAB can rapidly acidify their surroundings (to as low as pH 4-4.5) by excretion of lactic acid [278,279]. An interesting observation with many bacteriocins is a reduction of activity/potency at high salt concentrations. The potency of EntK1 towards *E. faecium* has also been shown to be reduced approximately 64-fold (MIC_{50}) in the presence of 250 mM KCl (unpublished). As discussed in **Paper VI**, this phenomenon has been attributed to a reduction in the electrostatic interaction of the peptides with the cell surface. However, as many bacteriocins kill bacteria by dissipating the PMF, the influence of a high salt concentration on $\Delta\Psi$ must also be considered.

It remains to be investigated whether a potential pore formed by these bacteriocins is selective for essential ions other than H^+ and K^+. One interesting example is the two-peptide bacteriocin plantaricin JK, which has been shown to cause efflux of specific anions such as glutamate [280]. Most essential ions are actively transported in bacteria by energy-demanding ATP-driven importers. It could be imagined that leakage of such ions through pores could lead to continuous re-import, leading to ATP-depletion and cell death.

4.2.2 Immunity to the LsbB family

A characterization of the immunity proteins to the LsbB bacteriocins may improve our understanding of the mechanism of action of these bacteriocins. It remains a mystery how the producer of leaderless bacteriocins protects itself from the action of its own bacteriocin. Especially puzzling is that no receptor has been identified (or suggested) for most of the leaderless bacteriocins, which suggests that these peptides act directly on the membrane. This would suggest that immunity proteins act by directly associating with the bacteriocin immediately after synthesis to detoxify the peptide. Alternatively, specific molecular structures (e.g., lipids, cell wall precursors) are present only in the upper leaflet of the membrane.

If the immunity proteins must associate with RseP to confer immunity, it would be expected that immunity also depends on the structure of RseP. Thus, the immunity protein would only provide immunity if the structure of RseP is very similar to that of the producer strain. A preliminary characterization of the immunity proteins to the LsbB family has been performed by Tymoszewska (2015). In this work, it was shown that LsbA is the immunity protein for LsbB and not another bacteriocin, as previously claimed. This is not surprising given the similar genetic organization of the LsbB gene cluster to the other bacteriocins in the family, and the similar physicochemical properties of LsbA with the other immunity proteins.

Expression of LsbA in *L. lactis* IL1403 provides complete immunity to the strain against LsbB, EntK1, and EntEJ97 [281]. Interestingly, the sequence of RseP (YvjB) in *L. lactis* IL1403 differs from the original producer, *L. lactis* BGMN1-5, in only one amino acid position (A57T). Conversely, the EntK1 immunity protein (EntK1i) in *L. lactis* IL1403 provides no immunity towards LsbB and only poor or incomplete immunity towards EntK1 and EntEJ97 [281]. This could be due to a low affinity of

EntK1i towards the lactococcal RseP, which differs considerably in sequence from the enterococcal RseP in the original EntK1 producer (54.1% identity). As such, the highly potent LsbB is most likely able to outcompete the immunity protein for binding to RseP. Furthermore, the immunity protein EntEJ97i cannot provide immunity to EntEJ97 in *L. lactis* IL1403, but does provide some immunity towards EntK1, a bacteriocin with lower potency towards this strain than EntEJ97 [281]. The species dependency of immunity function together with cross-immunity seems to support a model where the immunity protein binds to RseP, thereby blocking binding of the bacteriocin, however, this needs to be further tested.

4.2.3 Bacteriocin 'receptors'

RseP is a 'true' bacteriocin receptor, as spontaneous mutants with disrupted *rseP* become completely insensitive to the bacteriocins. However, for some bacteriocins, resistant mutants cannot easily be isolated. In such cases, cells with reduced sensitivity can sometimes be obtained by subculturing sensitive cells in successively higher concentrations of the bacteriocin. The question then arises whether these bacteriocins depend on a receptor protein or molecule for their activity, or if they utilize a "non-specific" mode of killing. One possibility is that the receptor is an essential protein, and that amino acids important for its function are simultaneously involved in bacteriocin interaction. Another possibility is that these bacteriocins exploit a non-proteinaceous moiety as a docking molecule or "receptor", such as lipids or lipid precursors (see **Paper IV**).

The term "receptor" in the context of bacteriocins is not well defined and is arguably misused. A receptor can be broadly defined as "a molecule inside or on the surface of a cell that binds to a specific substance and causes a specific effect in the cell." (National Cancer Instititute Dictionary). For a bacteriocin, the receptor is thus a molecule responsible for the molecular events leading to growth inhibition or cell death. In the absence of the receptor, a bacteriocin can no longer target a cell via the specific mechanism attained through adaptation/evolution. As a consequence, the antimicrobial activity of the bacteriocin towards a cell lacking the receptor should be nonexistent. Although some bacteriocins exert a secondary non-specific antimicrobial activity just due to their amphiphilic and cationic properties. However, this secondary mechanism is usually only observed at concentrations considerably higher than those biologically relevant. In principle, a non-specific activity should be similar to any random peptide sequence with similar structure

and chemical properties (e.g., charge, hydrophobicity, and size). A distinction ought to be made between receptors necessary for killing (e.g., RseP and Man-PTS), where the absence of the receptors results in complete insensitivity to the bacteriocin, and those that only have some effect on sensitivity (e.g., maltose ABC transporter).

As mentioned previously (see sections 1.3.3.2 and 1.3.3.3) an ABC transporter, EcsAB (PptAB), is involved in sensitivity to the LsbB-like bacteriocins. Spontaneous mutants fully resistant to the bacteriocins sometimes harbor mutations in *ecsAB* but not *rseP* [108,142,143]. The importance of EcsAB for the function of RasP (RseP) in *B. subtilis* suggests that resistance is likely mediated via RseP. The involvement of EcsAB in the export of sex pheromones following cleavage by RseP raises the possibility that EcsAB is required for substrate release by RseP [108]. Alternatively, a non-functional EcsAB results in the accumulation of RseP cleavage products in the membrane, which retains binding affinity for RseP. RseP is suggested to be a very dynamic protein *in vivo*, switching between "open" and "closed" conformations to allow substrate entry and cleavage, respectively [190]. Consequently, the LsbB family of bacteriocins can no longer target RseP because of competitive binding, or because RseP is structurally "locked" (see **Figure 16**).

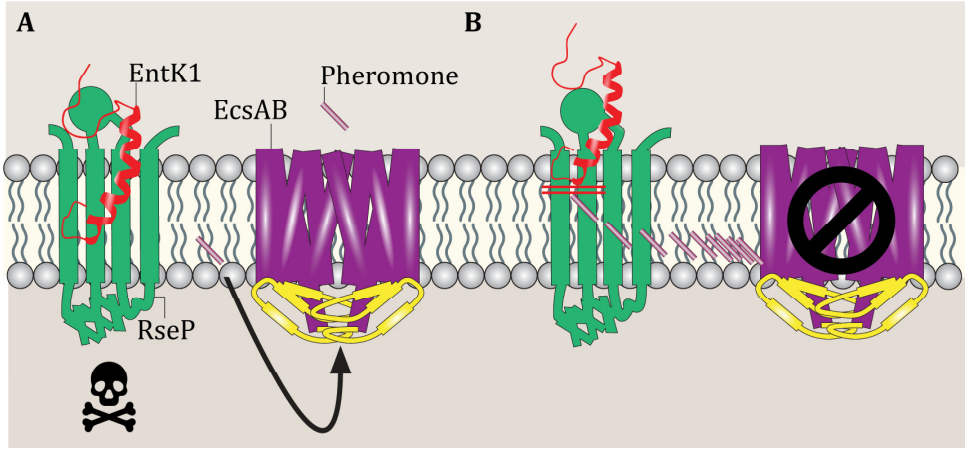

Figure 16. The proposed model for bacteriocin resistance in *ecsAB* mutants. (A) When EcsAB is functional, sex pheromones are removed from the membrane, and EntK1 can interact with RseP leading to cell death. (B) A non-functional EcsAB leads to the accumulation of pheromones that outcompete EntK1 for binding to RseP.

4.3 EntK1 as a diagnostic tool

Paper VI describes the development of a proof-of-concept binding assay for EntK1, which could identify samples containing *E. faecium*. The binding was shown to be sufficiently specific to allow for the differentiation of samples containing *E. faecium* from samples containing *E. coli* or *S. aureus*. The binding assay developed in **Paper VI** is fast (<40 min) and cost-effective, requiring only 6 nmol of fluorescent EntK1. Furthermore, the method was able to detect *E. faecium* at very low cell density, showing a limit of detection of only 3×10^3 CFU/ml, a clinically relevant limit for urinary tract infections. The method can potentially be used for the detection of *E. faecium* from a wide range of samples, from water sources as indicators of water quality to other bodily fluids where infections occur.

Many bacteriocins in nature are highly potent and exhibit very narrow inhibition spectra, killing target cells via a specific interaction with a receptor molecule [2,282,283]. As such, it seems that bacteriocin peptides can function as specific recognition elements for the detection of target bacteria. EntK1 is one such bacteriocin, exhibiting high potency and narrow activity primarily towards *E. faecium* [107]. This peptide is small, leaderless, and unmodified, enabling low-cost chemical synthesis and modification (**Papers V-VI**).

Indeed, in **Paper V,** the bacteriocin EntK1 with a FITC fluorescent tag at the N-terminus was shown to only bind bacterial cells expressing RseP from sensitive cells, with very little binding due to other factors. Based on these findings, it appeared likely that EntK1 could be used as a "molecular probe" to detect the presence of *E. faecium* (or other sensitive cells) in a sample. However, the binding experiments had only been performed on *L. plantarum* expressing RseP using the pSIP system. The binding characteristics of the *L. plantarum* clones differed substantially from those of *E. faecium* cells. Even though the *E. faecium* strain used in the binding assay was more sensitive to EntK1, the binding was much lower (20-fold). As such, a binding assay for *E. faecium* had to be developed and optimized.

Most studies using bacteriocins for the purpose of detection employ an immobilization strategy where the bacteriocin is attached to a surface (see section 1.5). A suspension of bacteria is then allowed to interact with the surface, which is acting as the immobilized phase, before the bound bacteria on the surface is measured in various ways. Presumably, the bacteriocin is thought to interact with

cells in a similar manner when bound to a surface as in solution (e.g., by binding to its molecular target on the cell surface). A benefit of this idea is that bacteria become concentrated at the surface, which will aid in detection in fluids with low cell density. Because the aim of this work was to develop EntK1 as a diagnostic tool, samples were to be obtained from urine containing 10^3-10^5 CFU/ml of bacteria that are typical for UTIs [284,285].

An immobilization strategy was initially attempted when developing a detection assay for *E. faecium* using EntK1. To do this, EntK1 was immobilized on magnetic beads functionalized with NHS ester reactive groups. The bacteriocin was coupled to the beads via primary amines at the N-terminus and the side chains of lysine (EntK1 has 6 lysines; $K^{2,4,13,14,24,26}$). However, no binding of *E. faecium* to the magnetic beads could be demonstrated with this approach.

This is, however, not very surprising considering that bacteriocins are quite small (<70 aa) compared to the thickness of the Gram-positive cell wall. The cell wall of Gram-positive bacteria is typically measured to have a thickness of 15-50 nm, and *Enterococcus* is reported to be at the higher end of that range (30-50 nm) [286–288]. Considering that many bacteriocins have an α-helical structure, which contains 3.6 aa per turn at 0.54 nm per turn (pitch), the peptide would need to be 100 aa to span 15 nm (or as much as 333 aa for 50 nm). It therefore appears highly unlikely that immobilized bacteriocins can reach the bacterial membrane to interact with membrane proteins (e.g., RseP, Man-PTS, CorC, UppP). A solution that could warrant further investigation is the use of long linker molecules (e.g., polyglycine). However, for the purpose of this work, flow cytometry was also pursued for detection.

Flow cytometry is a technique used to measure cells and particles in solution using their light scattering characteristics and fluorescence. Due to the small size of bacteria, they are indistinguishable from dust, debris, and other particles using light scatter with most conventional flow cytometers. This is especially problematic when measuring impure samples and/or bacteria at low density. Furthermore, for good resolution (less interference from impurities), a flow cytometer must be operated at a low flow rate of 10-50 µl/min, which means that large sample volumes (>1 ml) will take a long time to be analyzed [289]. On the other hand, bacteria can be distinguished via labeling with fluorescence, such as binding of FITC-EntK1 (see **Paper VI**). For these reasons, a method was developed to concentrate bacteria and

remove impurities from the sample prior to analysis. A simple sample preparation scheme of filtration (20 μm) and centrifugation was sufficient to selectively detect *E. faecium* at 10^5 CFU/ml, a commonly used clinical threshold for diagnosing UTIs. To determine the limit of detection, a gating strategy had to be used because of the high proportion of signals due to noise and impurities relative to cells.

Paper IV presents a proof of concept for using bacteriocins with a narrow spectrum as 'probes' for the detection of their target bacteria. We believe that other bacteriocins could also be used for detection purposes, providing rapid and specific detection of their target species in various samples. However, more work is needed to assess the potential of bacteriocins for detection and potential applications.

5 Concluding remarks

The results presented in this thesis describe the various aspects of bacteriocin discovery, characterization, and potential application. The rise of antimicrobial resistance is arguably one of the most urgent global health concerns. Addressing this issue will require a multitude of approaches, including new drug targets and alternative antimicrobials such as bacteriocins. Continued discovery and characterization of new antimicrobials will undoubtedly lead to the discovery of compounds that will serve an important role in the future. Additionally, more research is needed to fully explore the potential of existing bacteriocins, such as EntK1 and its target, RseP.

It is clear that RseP serves a very important role in the stress response, virulence, biofilm formation, and plasmid exchange in several important pathogens. The crucial importance of RseP highlights this site-2 protease as an attractive antimicrobial target. EntK1, which belongs to a small family of leaderless and unmodified bacteriocins, already targets RseP to exert its antimicrobial activity. These bacteriocins are ideally suited for chemical synthesis, modification, and rational design of improved derivatives. Receptor-targeting peptides have been extensively pursued in drug design to improve binding affinity and specificity. RseP is a highly conserved protein present in multiple species, including high priority pathogens.

A good understanding of how these bacteriocins are able to exploit RseP to kill target cells will allow us to rationally design new and improved peptides able to bind the RseP of important pathogens. Rational design of improved peptides can be aided by *in vitro* protein expression, which enables large-scale synthesis and screening of modified peptides. However, more work is needed to understand the mechanism of these bacteriocins and to allow us to fully exploit their potential.

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Publications

Paper I

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

RESEARCH ARTICLE

Ubericin K, a New Pore-Forming Bacteriocin Targeting mannose-PTS

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ABSTRACT Bovine mastitis infection in dairy cattle is a significant economic burden for the dairy industry globally. To reduce the use of antibiotics in treatment of clinical mastitis, new alternative treatment options are needed. Antimicrobial peptides from bacteria, also known as bacteriocins, are potential alternatives for combating mastitis pathogens. In search of novel bacteriocins against mastitis pathogens, we screened samples of Norwegian bovine raw milk and found a *Streptococcus uberis* strain with potent antimicrobial activity toward *Enterococcus*, *Streptococcus*, *Listeria*, and *Lactococcus*. Whole-genome sequencing of the strain revealed a multibacteriocin gene cluster encoding one class IIb bacteriocin, two class IId bacteriocins, in addition to a three-component regulatory system and a dedicated ABC transporter. Isolation and purification of the antimicrobial activity from culture supernatants resulted in the detection of a 6.3-kDa mass peak by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, a mass corresponding to the predicted size of one of the class IId bacteriocins. The identification of this bacteriocin, called ubericin K, was further confirmed by *in vitro* protein synthesis, which showed the same inhibitory spectrum as the purified antimicrobial compound. Ubericin K shows highest sequence similarity to the class IId bacteriocins bovicin 255, lactococcin A, and garvieacin Q. We found that ubericin K uses the sugar transporter mannose phosphotransferase (PTS) as a target receptor. Further, by using the pHlourin sensor system to detect intracellular pH changes due to leakage across the membrane, ubericin K was shown to be a pore former, killing target cells by membrane disruption.

IMPORTANCE Bacterial infections in dairy cows are a major burden to farmers worldwide because infected cows require expensive treatments and produce less milk. Today, infected cows are treated with antibiotics, a practice that is becoming less effective due to antibiotic resistance. Compounds other than antibiotics also exist that kill bacteria causing infections in cows; these compounds, known as bacteriocins, are natural products produced by other bacteria in the environment. In this work, we discover a new bacteriocin that we call ubericin K, which kills several species of bacteria known to cause infections in dairy cows. We also use *in vitro* synthesis as a novel method for rapidly characterizing bacteriocins directly from genomic data, which could be useful for other researchers. We believe that ubericin K and the methods described in this work will aid in the transition away from antibiotics in the dairy industry.

KEYWORDS bacteriocin, ubericin, mastitis, *in vitro* translation, pore formation, quorum sensing, pHluorin

Bovine mastitis is the most common infection in dairy cattle worldwide and is a major cause of economic losses for the dairy industry due to reduced milk production and quality as well as increased drug and veterinary costs (1). Organisms implicated in bovine mastitis include several species within the genera *Streptococcus*, *Enterococcus*,

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and *Staphylococcus* (2, 3). One of the main treatment strategies for bovine clinical mastitis is the use of antibiotics, which is increasingly undesirable due to rising antibiotic resistance and waning efficacy (4). Alternative strategies and agents against mastitis pathogens are therefore needed. A potential alternative to antibiotics for treating bovine mastitis is the use of antimicrobial peptides such as bacteriocins.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a broad range of organisms for defense or niche competition, typically targeting closely related species (5). For Gram-positive bacteria, at least two main classes of bacteriocins have recently been established, the posttranslationally modified bacteriocins (class I) and the unmodified bacteriocins (class II) (6–8). Class II bacteriocins are further subdivided into several subclasses. Class IIa consists of the pediocin-like bacteriocins, which contain a conserved YGNG(VL) motif (pediocin box) located near the N-terminal end. These bacteriocins show strong antilisterial activity and kill sensitive cells by membrane disruption and loss of the proton gradient across the membrane (9). Class IIb consists of the two-peptide bacteriocins whose activity requires the presence of two different peptides, normally in equimolar concentrations (10). Class IIc bacteriocins are leaderless and are produced as active peptides without a leader sequence (6). Finally, bacteriocins that are linear and missing the sequence motif characteristic of pediocins are designated class IId (11). A subfamily within class IId is bacteriocins sharing sequence similarity, such as lactococcin A, bacteriocin SJ, garvieacin Q, and bovicin 255 (12–15). These bacteriocins are all translated with a double glycine-type leader sequence and are located near a gene encoding an ABC transporter and peptidase. Maturation of the bacteriocin prepeptide occurs by cleavage at the GG motif (positions −1 and −2), which is coupled to the export of the peptide out of the cell (16).

Unlike lantibiotics, such as nisin, which use the cell wall precursor lipid II as a docking molecule on target cells, the class II bacteriocins appear to use different membrane-located proteins as target receptors. One of them is the sugar transporter mannose phosphotransferase system (man-PTS), which is used as a receptor for most, if not all, class IIa bacteriocins and for some class IId bacteriocins, such as lactococcin A, bacteriocin SJ, and garvieacin Q (17–19). Bacteriocin producers are immune to the action of their own bacteriocins due to immunity proteins that are generally cotranscribed with the bacteriocin gene (17, 20). Immunity proteins are small (50 to 150 amino acids) and are believed to protect the producer by forming a strong complex with the receptor protein and bacteriocin (17).

Lactococcin A kills sensitive cells by forming pores in the cytoplasmic membrane in a manner that depends on the presence of the man-PTS receptor protein (17). Pore formation results in depolarization of the membrane potential of sensitive cells, inhibition of amino acid uptake, and efflux of amino acids already imported (21). This efflux of amino acids is independent of the membrane potential and occurs with membrane vesicles of sensitive cells but not liposomes prepared from phospholipids of sensitive cells (21). This mode of action results in a very potent antimicrobial, as lactococcin A is active at picomolar concentrations (12). Similarly, class IIa bacteriocins as well as other class IId bacteriocins have also been shown to target man-PTS on sensitive cells, and this sugar transport system appears to be an attractive target for different antimicrobial agents (17–19, 22, 23). Man-PTS-targeting bacteriocins are generally highly potent and are, as such, an attractive option for combating pathogens. However, it should also be noted that mutants with resistance to such bacteriocins are observed (24).

To search for novel antimicrobials with the potential to fight bovine mastitis pathogens, we screened a selection of bovine raw milk samples shown to have diverse microbial content (25). Here, we report the identification, purification, and characterization of a new class IId bacteriocin from one isolate of *Streptococcus uberis*. The bacteriocin, called ubericin K, shows antimicrobial activity toward many relevant mastitis pathogens in addition to a potent antilisteria activity. Further, we also show that man-PTS is required for the action of this bacteriocin and that it causes depletion of the proton gradient in target cells.

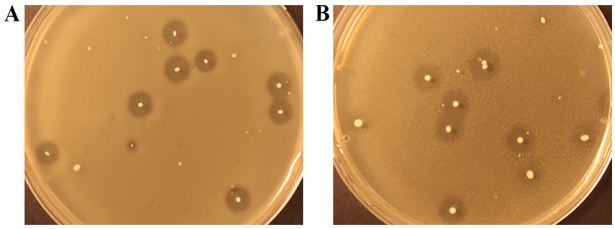

FIG 1 Screening for bacteriocin producers from sample 385 (10-fold dilution of sample) using *En. faecalis* (A) and *Str. dysgalactiae* (B) as the indicators. Colonies from this sample show clear and defined inhibition zones indicative of bacteriocin production.

RESULTS

Bacteriocin screening. A collection of 53 raw milk samples was screened for antimicrobial producers inhibiting bacteria known to cause various etiological conditions in cows. Only one sample (sample 385) contained several colonies displaying clear inhibition zones against both *En. faecalis* and *Str. dysgalactiae* (Fig. 1). None of the samples had obvious antimicrobial activity against *Sa. aureus*. Sample 385 was obtained from a cow with a high abundance of *Streptococcus* in the udder microbiota, as show in data published previously (25). To determine the identity of the producing colonies and to avoid isolates from the same clone, we performed 16S rRNA gene sequencing and repetitive element PCR of 10 randomly selected colonies with inhibition zones from sample 385. Two unique producers were found, one of *En. faecalis* and one of *Str. uberis* (data not shown). Whole-genome sequencing of both producers was performed to assist identification of the antimicrobials. Using the online bacteriocin prediction tool BAGEL4 (26), the genome of the *En. faecalis* strain was revealed to contain the known enterolysin A gene (27) that encodes enterolysin A, a well-characterized cell wall-degrading protein with a broad inhibitory spectrum that includes *Enterococcus faecalis* (27). The *Str. uberis* strain, however, encoded an uncharacterized bacteriocin-like gene cluster. Due to the novelty of the encoded bacteriocin-like peptides, the *Str. uberis* strain (hereafter called Laboratory of Microbial Gene Technology [LMGT] 4214) was chosen for further study.

Bacteriocin purification. Initial physicochemical analyses demonstrated that the antimicrobial activity in the supernatant of LMGT 4214 resisted heating for 10 min at 95°C but was labile to proteinase K treatment (data not shown), properties typical for bacteriocins (28). The antimicrobial activity in the supernatant was purified with a standard bacteriocin purification protocol consisting of three steps: ammonium sulfate precipitation, cation-exchange, and reverse-phase chromatography (29). Purification resulted in a 2,000-fold increase in activity from 40 bacteriocin units (BU)/ml to 81,920 BU/ml with a calculated total yield of 204% (Table 1). A possible explanation for this apparent increase in the amount of bacteriocin is provided in the discussion below. All fractions from reversed-phase fast protein liquid chromatography (RP-FPLC) were assayed for antimicrobial activity against the mastitis pathogen *Str. uberis* LMGT 3912, and only three fractions (10 to 12) were found to have bactericidal activity (Fig. 2). The active fractions eluted in a distinct peak at 31% isopropanol, and the highest activity was found in fraction 11.

TABLE 1 Purification scheme for ubericin K

Fraction no.	Fraction	Vol (ml)	Activity (BU/ml)	Total activity (BU)	Yield (%)
I	Culture supernatant	1,000	40	40,000	100
II	Ammonium sulphate precipitation	100	320	32,000	80
III	Cation-exchange chromatography	100	160	16,000	40
IV	Reversed-phase chromatography	1	81,920	81,920	204

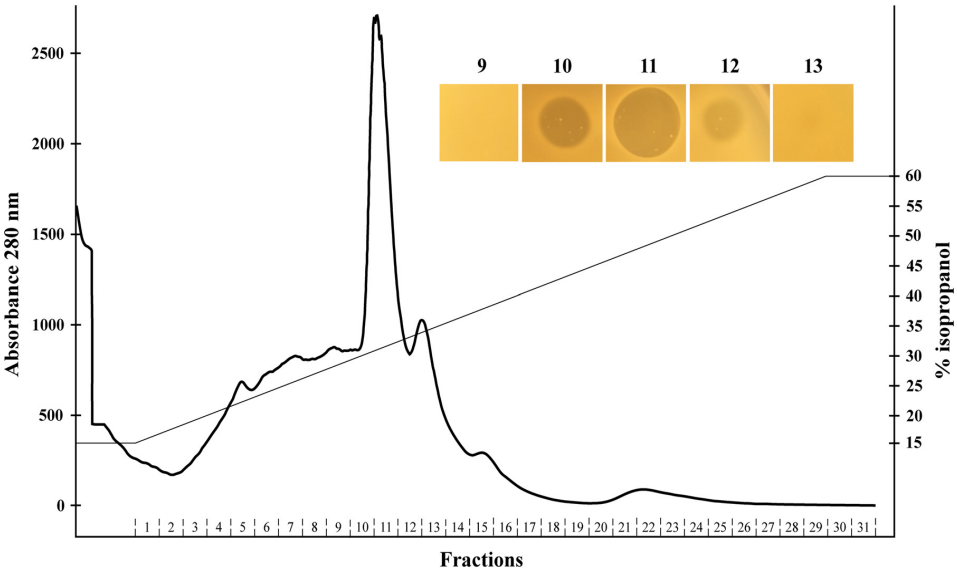

FIG 2 Reversed-phase chromatography elution profile of crude bacteriocin concentrate obtained from cation-exchange chromatography. Bacteriocin activity against the indicator *Str. uberis* LMGT 3912 was detected in fractions 10 to 12 with the highest activity in fraction 11, which eluted at 32% isopropanol (0.1% [vol/vol] TFA). The inhibition zone produced by a 3-μl drop of the active fractions is pictured in the upper right.

Genome sequencing and gene analysis. Given the proteinaceous nature of the antimicrobial, data from the whole-genome sequencing of LMGT 4214 were used to identify potential bacteriocin genes. Annotation combined with an *in silico* search for bacteriocin genes by the online program BAGEL4 revealed a gene cluster containing bacteriocin-like biosynthetic genes (Fig. 3). A search for homologous DNA in public databases identified several published genomes of *Str. uberis* containing the same locus; however, to our knowledge, genes from this locus have not been studied experimentally. The locus included one open reading frame (ORF) encoding a C39 family bacteriocin-type ABC transporter and peptidase (*orf4*) and several ORFs (*orf3*, *orf6*, *orf7*, *orf10*, and *orf13*) encoding bacteriocin-like peptides, each with a double glycine-type leader sequence (Fig. 3; Table 2). The length of the predicted mature bacteriocin-like peptides varies from 27 amino acids (derived from ORF3) to 58 amino acids (derived

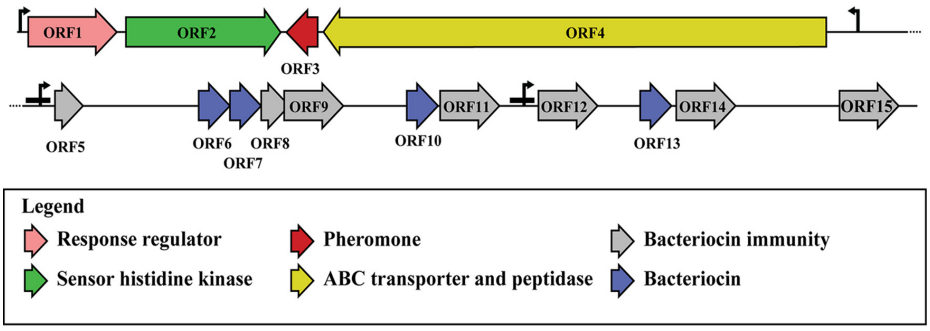

FIG 3 Genetic organization of the ubericin K bacteriocin cluster. Putative bacteriocins (bacteriocin-like peptides) (blue) and immunity proteins (gray) are located downstream of an ABC transporter and C39 family peptidase (yellow), a putative bacteriocin/pheromone (red), GHKL domain-containing sensor histidine kinase (green), and a LytTR DNA-binding transcriptional regulator (pink). Arrows indicate putative promoters, while the bars upstream of *orf5* and *orf12* indicate potential regulatory repeats. The figure depicts the continuous genomic region from ORF1 to ORF15.

TABLE 2 List of the encoded proteins from the bacteriocin locus of *Str. uberis* LMGT 4214

ORF	Predicted function[a,b]	Size (amino acids)	Mass (kDa)	pI	Homologs and/or possible role
orf1	Response regulator	246	29.09	9.4	Response regulator transcription factor (WP_012658029.1)
orf2	Histidine protein kinase	439	52.47	6.7	Two-component system (TCS) sensor kinase (KKF42577.1)
orf3	Peptide pheromone	27	3.11	12.03	ComC/BlpC family leader-containing pheromone/bacteriocin (WP_080502297.1)
orf4	ABC transporter	717	81.51	9.38	Peptide cleavage/export ABC transporter (WP_046392064.1)
orf5	Unknown	55	6.61	9.14	Hypothetical protein AF69_00955 (KKF59227.1)
orf6	Bacteriocin-like prepeptide	53	4.99	9.31	Blp family class II bacteriocin (WP_046388584.1)
orf7	Bacteriocin-like prepeptide	48	4.80	9.45	Blp family class II bacteriocin (WP_046389168.1)
orf8	Immunity	70	8.46	9.52	Hypothetical protein SAMN05216423_1865 (SEI90296.1)
orf9	Immunity	103	12.03	10.01	Membrane protein (KKF59107.1)
orf10	Bacteriocin-like prepeptide	58	6.3	9.04	Garvicin Q family class II bacteriocin (WP_154629194.1)
orf11	Immunity	99	11.51	9.60	Bacteriocin immunity protein (WP_012658037.1)
orf12	Unknown	91	10.61	9.06	Hypothetical protein AF68_02745 (KKF60419.1)
orf13	Bacteriocin-like prepeptide	54	5.87	8.68	Bacteriocin (WP_154590650.1)
orf14	Immunity	119	14.01	9.71	Bacteriocin immunity protein (MTB58145.1)
orf15	Unknown	99	11.34	9.52	Bacteriocin immunity protein (WP_154617908.1)

[a]Predicted function is based on sequence homology, genetic location, or/and physicochemical properties; for immunity proteins, the hydrophobic characteristic, and their genetic location (right after the bacteriocin structural gene) are used for the prediction.
[b]For bacteriocin-like peptides and the pheromone, only their mature sequence was used to calculate size (amino acids), mass (kilodaltons), and pI (isoelectric point).

from ORF10) and all have an alkaline pI (above 8.6). A double glycine leader motif and an alkaline pI are features typical for most unmodified bacteriocins (28, 30). The amino acid sequence of these bacteriocin-like precursors and the expected cleavage site are shown in Fig. 4A.

The bacteriocin-like *orf6* and *orf7* are located next to each other, resembling the genetic organization of a typical two-peptide bacteriocin system (31). These two genes are followed by two small ORFs (*orf8* and *orf9*) encoding small hydrophobic proteins with a probable role in immunity function. The mature sequence of ORF6 and ORF7 share sequence similarities with the class IIb two-peptide bacteriocin lactacin F, 44% and 39% sequence identity with LafA and LafX, respectively (32).

Downstream of *orf9* are *orf10* and *orf11*, which encode a bacteriocin-like peptide and a predicted hydrophobic immunity-like protein, respectively. The predicted mature sequence

A

```
ORF3        MNNFKQFSTLSESELNQIIGG  KSLWRTINSIFNKTTKNAAIKIGNFSR
ORF6        MNSNIEFDSIDTELLEKVIGG  KNNWQANVSGILAAGAAGAAIGAPVCGLACGYIGAKTAITLWAGVTGATGGFK
ORF7           MDKHLVLSNQQLLDVVGG  NAPGNAVLGGLGGLQTGIKYCKVPHPVLKGVCIVGFTTAGAYLAYKAN
ORF10    MKTKSSKQFTELTVKDLSAVIGG  AKGVCKYVYPGSNGYACRYPNGEWGYIVTKSNFEATKDVIVNGWVSSLGGGYFHGNRG
ORF13       MSKMKQFKVLNEELLKKTLGG  TNVAPGIYCVDKNGKAKCSVDYKELWGYTGQVIGNGWINYGPWAPRPGFGVIIP
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B

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Ubericin K          AKGVCKYVYP-GSNGYA-CRYPNGEWGYIVTKSNFEATKDVIVNGWVSSLGGGYFHGNRG
Lactococcin A       KLT----FIQSTAACDLYYNTNTHKYVYQQTQNAFCAAANTIVNGWMGGAAGGFGLH--H
Bovicin 255         GKGYCKPVVY-AANGYS-CRYSNGEWGYVVTKGAFQATTDVIANGWVSSLGGGM------
Bacteriocin SJ      -YS----YFG-GSNGYS-WRDKRGHWHYTVTKGGFETVIGIIGDGWGSAGAPGPG-Q--H
Garvieacin Q        EYH----LMN-GANGYL-TRF-VNGKYVYRVTKDPVSAVFGVISNGWGSAGA-GFGPQ--H
consensus                 ..  ...*.   .  .... * .*. .. .   .* .**......*..    .
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FIG 4 List of bacteriocin-like peptides in the ubericin K cluster (A). Predicted leader sequence and mature bacteriocin are separated by a space. The predicted cleavage site is indicated by a triangle (▼). Multiple sequence alignment of known bacteriocins sharing significant sequence identity with ubericin K (ORF10), lactococcin A (M90969.1), bovicin 255 (AF298196), bacteriocin SJ (FM246455), and garvieacin Q (JN605800) (B). The alignment was generated using T-Coffee (http://tcoffee.crg.cat/apps/tcoffee) and colored using BoxShade. Similar amino acids are shaded gray, and identical amino acids are shaded black.

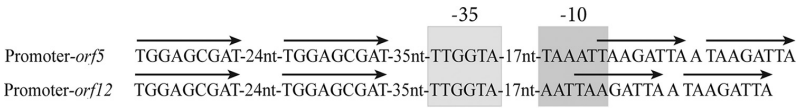

FIG 5 Putative promoter sequences and potential regulatory elements found upstream of *orf5* and *orf12*. Two direct repeats indicated by the arrows are conserved in the promoter region of both genes.

of ORF10 shows highest similarities to class IId bacteriocins of the garvicin Q family, as shown with lactococcin A, garvieacin Q, and bovicin 255 in Fig. 4B.

Like ORF10, the bacteriocin-like ORF13 is also followed by a small ORF (*orf14*) encoding a small hydrophobic immunity-like protein. The predicted mature part of ORF13 shows sequence homology to hiracin-JM79 (40% identity) (33).

The last bacteriocin-like gene, *orf3*, is located at the other end of the gene cluster between the genes encoding the ABC transporter (*orf4*) and the histidine protein kinase (HPK; *orf2*). Initially, *orf2* was the gene located at the very end of the DNA contig obtained from the Illumina sequencing. HPK genes are often involved in regulation of bacteriocin biosynthesis, where each HPK gene is closely associated with a gene encoding a response regulator (RR) (34). As the HPK gene was located at the very end of this contig, we suspected an RR gene may be in the upstream region of the genome. To prove this, we used the HPK gene as a query to identify nearby genes in sequenced genomes with a similar gene cluster to LMGT 4214. Several homologs to the HPK gene were found. The best hit was the genome of *Str. uberis* NCTC3858 (accession number LS483397.1) in which an annotated RR gene (locus tag NCTC3858_00615) is indeed located next to the searched HPK gene (locus tag NCTC3858_00616). The same RR gene (100% identity) was found in our Illumina sequencing results at the very end of another contig. To confirm that the two contigs were indeed located next to each other in the genome, we performed PCR and Sanger sequencing of the missing region in LMGT 4214. Indeed, using this sequence information, the assembly resulted in a contig with the complete bacteriocin gene cluster (Fig. 3). The RR gene (*orf1*) was located directly upstream of the HPK gene (*orf2*) as expected. The organization of *orf1*, *orf2*, and *orf3* shows a typical genetic organization of a three-component regulatory system containing an HPK (ORF2), an RR (ORF1), and a pheromone peptide (mature product of ORF3), which together are known to be involved in the regulation of biosynthesis in many bacteriocin regulons through a mechanism normally referred to as quorum sensing (35, 36).

An *in silico* search for promoters and regulatory elements was performed. Putative promoters were found just upstream of *orf1*, *orf4*, *orf5*, and *orf12*, suggesting the presence of four operons in the gene cluster (Fig. 3). Interestingly, the two putative promoters situated upstream of *orf5* and *orf12* show only limited homology to consensus promoter sequences, but both are preceded by a pair of direct repeats (TGGAGCGAT) 35 nucleotides (nt) upstream of the −35 box (Fig. 5). The two direct repeats are separated by a spacer sequence of 24 nt. Thus, the distance between the middle of each repeat is 32 to 33 nt, a length corresponding to about three complete helical turns (10.5 bp/turn in B-DNA double helix). This arrangement would allow the two repeats to face toward the same side of DNA, hence resembling regulatory DNA elements for activator binding. Furthermore, downstream of and partially overlapping the −10 box of the two predicted promoters are another pair of direct repeats (TAAGATTA), a location resembling an operator-like element (Fig. 5). The promoters predicted upstream of *orf1* and *orf4* had no obvious regulatory sequences, and the *orf1* promoter was similar to the strong canonical *Escherichia coli* promoter (37). A summary of all relevant properties of the encoded proteins in the bacteriocin gene cluster is presented in Table 2.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry reveals a bacteriocin peptide in active fractions. Given that there were several bacteriocin-like peptides that could potentially contribute to antimicrobial activity, we subjected all active fractions from the purification (fractions 11 to 13) to matrix-assisted

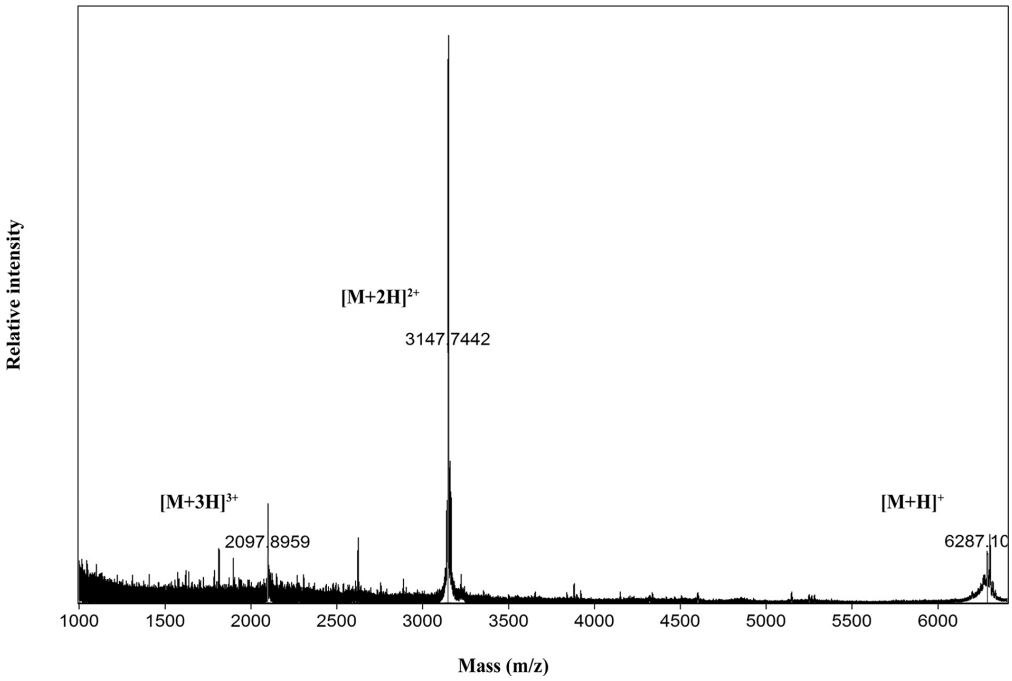

FIG 6 MALDI-TOF mass analysis of the most active fraction from RPC. The peak labeled at 6,287.10 Da indicates the presence of ubericin K. The peaks at 2,097.90 *m/z* and 3,147.74 *m/z* likely represent the triply and doubly charged ions, respectively.

laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for mass determination. A distinct peak closely corresponding to the mass of the predicted mature peptide of ORF10 could be seen at 6,287.10 *m/z* (Fig. 6); the highest intensity peak at 3,147.74 *m/z* likely represents the doubly charged ion of the same molecule. Similarly, the peak at 2,097.8959 *m/z* is the triply charged ion. The theoretical monoisotopic mass of the mature sequence of ORF10 with oxidized cysteines is 6,290 Da (2,097.89 $\times$ 3 $-$ 3 = 6,290.67). Although other masses were also present in purified samples, they did not correspond to the mass of any of the other bacteriocin-like peptides found in the cluster. This result suggests that the predicted mature part of ORF10, hereafter called ubericin K, was primarily responsible for the antimicrobial activity observed.

In vitro expression of ubericin K, bioactivity, and antimicrobial spectrum. Previous research has shown that bacteriocins are sometimes incorrectly identified due to the copurification of small and undetectable amounts of other bacteriocin peptides (38). As the producer in our case was predicted to encode numerous bacteriocin-like peptides, we could not exclude the possibility that other antimicrobial peptides in the sample also contributed to the observed antimicrobial activity. To avoid this potential problem, we sought to synthesize the mature peptide of ubericin K by *in vitro* synthesis (IVS) to confirm its bioactivity in the absence of any copurified molecules. As shown in Fig. 7, IVS-ubericin K inhibited the growth of the same indicators used in the initial screening, thus confirming the bioactivity of ubericin K.

As mentioned above, ubericin K shows sequence similarities to lactococcin A, garvieacin Q, and bovicin 255. The antimicrobial activity of lactococcin A appears to be confined to members of the genus *Lactococcus* (12), while garvieacin Q inhibits a broader range of organisms that include *En. faecium*, *La. garvieae*, and *Listeria monocytogenes* (13). Bovicin 255 was also active against a species of *Enterococcus*; however, this bacteriocin was only tested against ruminal bacteria (15). To examine the

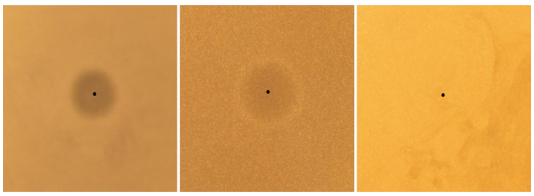

FIG 7 Reaction mixture (3 μl) from *in vitro*-synthesized ubericin K spotted on a lawn of each of the three indicators used in the screening. *En. faecalis* LMGT 2333 (left), *Str. dysgalactiae* LMGT 3890 (middle), and *Sa. aureus* LMGT 3023 (right).

inhibitory range of ubericin K, a selection of microorganisms was tested for growth inhibition by both *in vitro*-synthesized and reverse-phase column (RPC)-purified ubericin K. As shown in Table 3, the IVS-ubericin K displayed an almost identical inhibition spectrum as the purified ubericin K. Most notable was a very potent antilisterial activity, with strong inhibition also of *En. faecium*, *Str. uberis*, and *La. lactis*. We also noted that resistant colonies were readily visible in the inhibition zones from both IVS-ubericin K and purified ubericin K with *Li. monocytogenes*, *Enterococcus*, and *La. lactis*, a phenomenon also seen with other man-PTS-targeting bacteriocins (data not shown) (24).

Ubericin K is a pore former targeting the mannose PTS system on sensitive cells. Lactococcin A and garvieacin Q are both known to use the sugar transporter man-PTS as the receptor to target sensitive cells (17, 39), and we wanted to know whether this was also true for ubericin K. To examine this, we exposed *La. lactis* IL1403 (40) and a mutant where the operon encoding the man-PTS system has been deleted (strain B464) (17) to the *in vitro*-synthesized ubericin K. In this experiment, we also included the RPC-purified ubericin K and nisin A, a lantibiotic with a mechanism of action independent of man-PTS, for comparison. As seen in Fig. 8, both IVS-ubericin K and the purified ubericin K were active against the wild-type strain but not the man-mutant. Nisin A, which uses lipid II as a docking molecule, was active against both strains. These results together provide strong evidence that ubericin K was produced by *Str. uberis* LMGT 4214 and that an intact man-PTS is required for the sensitivity toward this bacteriocin.

TABLE 3 Inhibition spectrum of RPC-purified ubericin K and *in vitro*-synthesized (IVS) ubericin K in a spot-on-lawn assay

Indicator strain[a]	Purified ubericin K[b]	IVS-ubericin K[b]
Streptococcus dysgalactiae LMGT 3890	++	+
Streptococcus dysgalactiae LMGT 3899	++	++
Lactococcus lactis IL1403	+++	+++
Bacillus cereus LMGT 2805	−	−
Bacillus cereus ATCC 9136B	−	−
Enterococcus faecalis LMGT 2333	+	+
Enterococcus faecalis LMGT 3088	+	+
Enterococcus faecium LMGT 2763	+++	+++
Enterococcus faecium LMGT 2772	+++	+++
Lactococcus lactis IL1403	+++	+++
Lactobacillus curvatus LMGT 2353	++	++
Lactobacillus garvieae LMGT 3390	++	++
Listeria monocytogenes LMGT 2651	+++	+++
Listeria monocytogenes LMGT 2604	+++	++
Listeria innocua LMGT 2785	+++	+++
Staphylococcus aureus LMGT 3023	−	−
Staphylococcus aureus LMGT 3242	−	−
Streptococcus uberis LMGT 3912	+++	+++
Escherichia coli TG1	−	−

[a]LMGT, Laboratory of Microbial Gene Technology, Norwegian University of Life Sciences, Ås, Norway.
[b]+++, clear zone of inhibition; ++, smaller clear zone of inhibition; +, visible/diffuse inhibition; −, no inhibition.

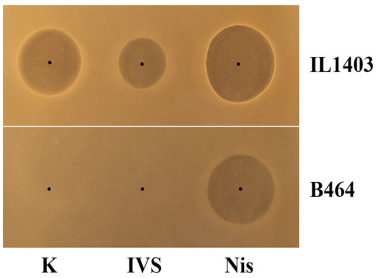

FIG 8 Inhibition of wild-type *Lactococcus lactis* IL1403 and a *ptn* (man-PTS) deletion mutant of IL1403 (B464) by *in vitro*-synthesized ubericin K (IVS), RPC-purified active fraction of ubericin K (K), and nisin A (Nis). No inhibition is observed by ubericin K with the mutant. Nisin A was used as a positive control.

We have previously constructed a sensor strain of *Li. monocytogenes* expressing pHlourin (41). pHluorin is a pH-sensitive green fluorescent protein that has different fluorescence emission spectra dependent on the local pH (42). Thus, by keeping the intra- and extracellular pH at different values, one can observe a shift in emission pattern if there is a leakage of protons across the membrane (41). The activity of ubericin K toward *Li. monocytogenes* that we observed previously allowed us to use this sensor strain to investigate whether ubericin K kills cells by forming pores on target cells. When the sensor strain was exposed to nisin A, which is a known pore former, a significant reduction in the fluorescence emission at 510 nm was seen (excitation at 400 nm over 470 nm) as expected (Fig. 9). The same was seen with the detergent cetyltrimethylammonium bromide (CTAB), which is commonly used as a positive control for a membrane disruption agent. Interestingly, ubericin K also caused a similar shift in emission, thus indicating that this bacteriocin has a mode of action that involves pore formation. Thus, ubericin K kills sensitive cells by man-PTS-dependent pore formation. Pore formation was also evident for pediocin PA-1, a class IIa bacteriocin targeting man-PTS (43).

DISCUSSION

In this study, we screened milk samples from dairy herds in Norway and succeeded in isolating a strain of *Str. uberis* that produces a new bacteriocin that kills closely related species as well as *En. faecium* and *Listeria* spp. *Str. uberis* is an organism frequently detected

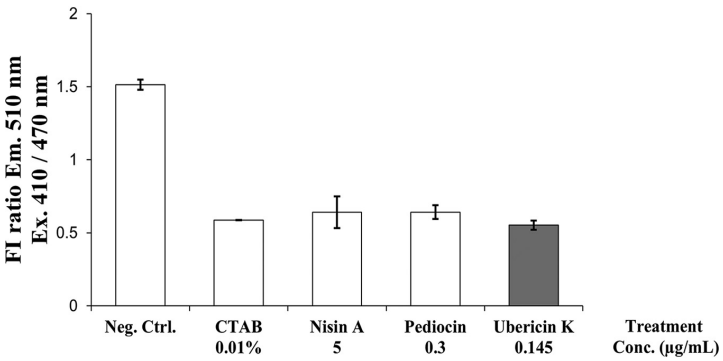

FIG 9 Assay for measuring a drop in intracellular pH during exposure to antimicrobials. A ratiometric pH-sensitive variant of green fluorescent protein (GFP) is expressed in *Li. monocytogenes* EGDe/pNZ-P_{help}-pHluorin, and a lower ratio of fluorescence from excitation at 410 nm over 470 nm indicates reduced pH. Ubericin K was equally as effective as the positive control CTAB (0.01%) in causing a drop in intracellular pH.

from the milk and udders of dairy cows and is also recognized as one of the primary causative agents of mastitis in Norway and worldwide (44–47). Virulence determinants required for host invasion and colonization of *Str. uberis* have not been clearly defined and likely involve more complex population dynamics and interaction (48). A key component in population dynamics of streptococci is the intercommunication of strains with peptide pheromones and the intra- and interspecies competition by the production of bacteriocins (49). *Str. uberis* LMGT 4214 shows strong antagonism toward other mastitis-associated strains and could therefore potentially outcompete virulent strains in the udder. However, the pathogenic potential of *Str. uberis* LMGT 4214 itself has not yet been established. Optionally, purified bacteriocin from the producer could have potential in treatment and prevention of mastitis, as has been demonstrated previously for lacticin 3147 and micrococcin P1 (50, 51).

Antimicrobial activity was purified from the culture supernatant by methods commonly used for bacteriocins based on their general physicochemical characteristics, including small size and cationic and hydrophobic nature. Genomic analysis of *Str. uberis* LMGT 4214 revealed a multibacteriocin gene cluster potentially expressing three novel bacteriocins in addition to a three-component regulatory system (composed of an HPK, an RR, and a peptide pheromone) and a dedicated ABC transporter. However, when analyzing the active fractions from RP-FPLC, only one peak showed antimicrobial activity.

The three-component system suggested that bacteriocin production is regulated by a quorum-sensing mechanism, as has previously been described in other Gram-positive bacteria, such as for the plantaricins EF and JK, sakacin A, and the Blp bacteriocins in *Lactobacillus plantarum*, *Lactobacillus sakei*, and *Streptococcus pneumoniae*, respectively (38, 52, 53). In this system, an activated response regulator binds to conserved direct repeats at regulated promoters as a homodimer (54, 55). In the bacteriocin cluster of *Str. uberis* LMGT 4214, two putative promoters were found with proximal direct repeats, suggesting the presence of at least two regulated promoters. The arrangement of the direct repeats is such that they face toward the same side of the DNA, thereby facilitating a dimeric binding of a regulator, a function that is likely performed by the response regulator encoded by *orf1*. It is feasible that a dimeric regulator bound to the direct repeats can assist RNA polymerase in binding to its promoter located only 35 nt downstream. Another interesting feature identified is the second pair of direct repeats, which are partially overlapping the predicted −10 box of the two predicted regulated promoters. In view of its location, this feature resembles an operator sequence for which a repressor could bind and sterically block access to the promoter. Regulated promoters involving both activating regulatory elements and operator are known in nature. An example of this is the classical *lac* operon, which involves the catabolite gene activator protein (CAP) site and the *lac* operator in *Es. coli* (56). However, such a regulation has, to our knowledge, not yet been found for any bacteriocins, and whether the repeats identified in the bacteriocin cluster of *Str. uberis* LMGT 4214 serve such functions requires further investigation in future work, as this aspect is beyond the scope of the present study.

Of the bacteriocins found in the cluster, two of the bacteriocins belong to the class IId family and one to the class IIb family, all being followed by a gene or genes encoding hydrophobic proteins, which likely play a role in immunity. The predicted bacteriocin-like peptides is of a relatively small size (<7 kDa) with a high pI and an N-terminal 15- to 30-amino acid leader sequence with a GG-type cleavage motif. Maturation and export of bacteriocins with this type of leader sequence normally use a dedicated ABC transporter and peptidase where maturation occurs concomitant with export, a function likely executed by the encoded ABC transporter in the locus. Nevertheless, calculating the expected mass of the four bacteriocin-like peptides found in the bacteriocin gene cluster only provided one match to the mass spectrometry peak at approximately 6,290 Da (*m*/*z*), which is the mass of mature ubericin K. To ensure that the antimicrobial activity measured from the purified sample was due to ubericin K and not to unknown antimicrobial contaminants, the peptide was synthesized *in vitro*. This showed that IVS-ubericin K has an identical inhibition spectrum as purified ubericin K and that both required an intact man-PTS for antimicrobial activity, thus confirming that ubericin K

alone could be responsible for the antimicrobial activity. Resistant colonies in the inhibition zones from both IVS-ubericin K and purified ubericin K indicates that resistance to the bacteriocin is likely prevalent in nature; this is a challenge that must be addressed for the clinical application of this class of antimicrobials.

Ubericin K was *in vitro* synthesized as the mature peptide but with an added initiator methionine (*N*-formylmethionine) that is not present in the native mature bacteriocin. It is worth mentioning that IVS-ubericin K activity was the highest immediately after synthesis. Activity of the reaction mixture was significantly reduced following storage, showing a reduction in the diameter of inhibition zones by about 4-fold after 24 h at room temperature and only a faint zone of inhibition following overnight storage at 4°C or a freeze-thaw cycle (data not shown). A possible explanation for this loss could be the formation of multimeric complexes or aggregates due to the cationic and hydrophobic properties and low solubility under the basic aqueous conditions required for *in vitro* synthesis. The formation of inactive precipitates following storage at 4°C has been documented for lactococcin A (12). Such aggregates would also be expected to form in the supernatant and aqueous buffers during purification. The apparent increase in the amount of bacteriocin (approximately 200% yield) following reverse-phase purification is then probably from the dissociation and resolubilization of aggregates by the isopropyl alcohol/trifluoroacetic acid (TFA). The loss of activity could also be a result of, or exacerbated by, oxidation of the added initiator methionine, a phenomenon that also has been observed with pediocin PA-1 and lactococcin B (57, 58). In addition, attempts at purifying *in vitro*-synthesized ubericin K from the reaction mixture using 100,000-molecular weight cutoff filters to remove the macromolecules necessary for the reaction, as recommended by the manufacturer, were not successful, as activity was lost in the filtrate. We did not ascertain if this was due to aggregates or from the adsorption of ubericin K to the filter material. Thus, to fully take advantage of *in vitro* synthesis to characterize bacteriocins mined from genomic databases, this instability needs to be solved in future research.

Ubericin K was shown to disturb the pH homeostasis of sensitive cells in the same manner as the pore-forming bacteriocin nisin A and the potent antilisterial bacteriocin pediocin PA-1. Pore formation in target cells by the pediocin-like class IIa has been well established (59). In addition, the one-peptide nonpediocin-like class IId bacteriocin lactococcin A also causes pore formation and loss of the proton motive force (21). The pore-forming ability of garvieacin Q and bovicin 255 has not been established, but the results presented here strongly suggest a similar mechanism based on their sequence homology.

Despite having a multibacteriocin gene cluster, the presence of other bacteriocins than ubericin K was not apparent. It is possible that the other putative bacteriocin-like peptides (ORF6, ORF7, and ORF13) have no or low activity against the chosen indicator or they are differently regulated. For ORF6 and ORF7 that likely constitute a two-peptide bacteriocin, no activity would be expected if the peptides were separated into different fractions during purification. This notion is in fact relatively common, as we have previously encountered similar problems during the purification of the multipeptide plantaricins (38) and garvicin KS (29). The regulation, inhibitory spectrum, and activity of these bacteriocin-like peptides thus remain to be determined, and further characterization of this bacteriocin cluster by heterologous expression and *in vitro* synthesis together will help answer those questions.

Genomes uploaded to public databases often contain bacteriocin clusters, and programs, such as BAGEL4 and other annotation software, are continuously getting better at correctly identifying bacteriocin-like peptides and bacteriocin biosynthetic proteins. Indeed, BLAST searches of all bacteriocin-like peptides in the cluster had significant matches to protein sequences annotated as bacteriocins. As such, using data mining to find new bacteriocin-like genes is one possible approach (60–62). However, the major bottleneck in discovering novel bacteriocins with desired properties is their characterization experimentally. *In vitro* protein synthesis is a promising rapid method for

TABLE 4 Microorganisms used in this study with relevant characteristics

Bacterial strain[a]	Relevant characteristics	Reference[a]
Streptococcus uberis LMGT 4214	Bacteriocin producer	This study
Lactococcus lactis IL1403	Indicator strain	40
B464	*ptn* deletion mutant of IL1403	17
Escherichia coli DH5α	Cloning and plasmid propagation host	Invitrogen (Cat. No. 18265-017)
Listeria monocytogenes EGDe/pNZ-P_{help}-pHluorin	A clone expressing a pHluorin protein used to measure pore formation ability of bacteriocins	41
Enterococcus faecalis LMGT 2333	Indicator strain used in the screening	Lab collection (LMGT), Norway
Staphylococcus aureus LMGT 3023	Indicator strain used in the screening	Lab collection (LMGT), Norway
Streptococcus dysgalactiae LMGT 3890	Indicator strain used in the screening	Lab collection (LMGT), Norway
Streptococcus uberis LMGT 3912	Indicator strain used to monitor activity for bacteriocin purification. Mastitis pathogen.	Lab collection (LMGT), Norway

[a]LMGT, Laboratory of Microbial Gene Technology, Norwegian University of Life Sciences, Ås, Norway.

screening and testing new bacteriocins, as the small unmodified and leaderless bacteriocins are largely unstructured in aqueous solution. This method allows for a streamlined pipeline for characterizing bacteriocins from sequence data (63). Further characterization of the many bacteriocins from streptococci, among others, will be valuable for devising alternative strategies for the treatment and prevention of mastitis and other infections as well as to understand the interstrain competition in these environments.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains used in this study are listed in Tables 3 and 4. All bacterial strains in Table 4 were grown in brain heart infusion (BHI) (Oxoid) at 37°C, except for strains of *Lactococcus lactis*, which were propagated in M17 broth (Oxoid) supplemented with 0.5% (wt/vol) glucose (GM17) at 30°C, and *Escherichia coli* NEB 5-alpha (New England BioLabs), which was grown in LB (Oxoid) containing 100 μg/ml ampicillin at 37°C with shaking. All strains used for the determination of the antimicrobial spectrum (Table 3) were grown in BHI at 30°C.

Bacteriocin screening. Bovine raw milk samples were collected from individual cows selected from two dairy herds. Sample collection as well as the microbiota content of the samples have been described previously by Porcellato et al. (25). Screening of bacteriocin producers was performed using a multisoft agar overlay method as follows. Samples of raw bovine milk were first diluted in saline (0.9% NaCl) to ensure a good distribution of colonies (50 to 500 colonies/plate) before being mixed with soft agar and poured onto BHI agar. A second layer of BHI soft agar was poured on top before the plates were incubated at 30°C overnight. On the following day, an overnight culture of the indicator strain (*Enterococcus faecalis* LMGT 2333, *Staphylococcus aureus* LMGT 3023, or *Streptococcus dysgalactiae* LMGT 3890) was diluted 50-fold in BHI soft agar and poured evenly as a top layer. Following incubation at 37°C overnight, the plates were inspected for zones of growth inhibition of the indicator. The colonies producing inhibition zones were restreaked on new BHI plates to obtain pure cultures, and bacteriocin production was confirmed by a second inhibition test before frozen cultures in 15% glycerol were made and stored at −80°C until use.

DNA extraction, repetitive element PCR fingerprinting and 16S rRNA gene sequencing. Genomic DNA isolation and purification was performed using a GenElute bacterial genomic DNA kit (Sigma-Aldrich). Isolated DNA was used as a template for both repetitive element PCR (rep-PCR) and 16S rRNA gene amplification. rep-PCR was performed using the primer pair ERIC1R and ERIC2 (Table 5), as described by Versalovic et al. (64). The 16S rRNA gene was amplified using the primers 16S-11F and 16S-12R (Table 5), and the resulting PCR product was sequenced using the same primers by Sanger sequencing (Eurofins Genomics). A contig of the resulting reads was constructed using CAP3 (65) and searched against the NCBI rRNA/ITS database.

Bacteriocin purification. The bacteriocin was purified from the supernatant of 1 liter of overnight culture. Cells were removed by centrifugation at 10,000 $\times$ *g* for 30 min, and the bacteriocin in the supernatant was precipitated by the addition of ammonium sulfate (60% saturation, 4°C). After centrifugation for 40 min at 12,000 $\times$ *g*, the bacteriocin precipitate was dissolved in Milli-Q water (Merck Millipore) and adjusted to a pH of 4 by the addition of 1 M hydrochloric acid and subjected to cation-exchange chromatography using a HIPrep 16/10 SP-XL column (GE Healthcare Biosciences). The column was washed with 5 column volumes (CV) of 20 mM sodium phosphate buffer at a pH of 6.8 before the bacteriocin was eluted from the column with 5 CV of 1 M sodium chloride (unbuffered). The eluate containing the bacteriocin was applied on a Resource reverse-phase chromatography (RPC) column (1 ml) (GE Healthcare Biosciences) connected to an ÄKTA purifier system (Amersham Pharmacia Biotech). The column was equilibrated with 20 CV of 0.1% TFA before loading the sample and eluted with a linear gradient of 15% to 60% isopropyl alcohol (Merck) containing 0.1% (vol/vol) TFA at a rate of 1 ml/min. The concentration of ubericin K in the final RPC-purified fraction was estimated using the Qubit protein assay kit (Invitrogen). Activity from each step of the purification procedure was assessed using the indicator strain *Str. uberis* LMGT 3912, which was isolated from a case of clinical mastitis.

TABLE 5 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)
16S-11F	TAACACATGCAAGTCGAACG
16S-12R	AGGGTTGCGCTCGTT
ERIC1R	ATGTAAGCTCCTGGGGATTCAC
ERIC2	AAGTAAGTGACTGGGGTGAGCG
KC1F	CGTAATTCATATGGCTAAAGGTGTCTGTAAGTATG
KC1R	ATGGATCCGTTTACCCTCTATTTCCGTGG
KIF	TGCTAGCCCCGCGAAATTAATACG
KGAP1F	ACATCGACTTATCTTGCACG
KGAP2R	CATACAACTCTTCAACATGTCG

Bacteriocin assays. Antimicrobial activity in solutions obtained from each step in the purification procedure was determined using a microtiter plate assay (12). Twofold dilutions of sample in BHI were prepared in microtiter plates to a volume of 100 μl per well. Each well was then inoculated with 100 μl of a 25-fold diluted overnight culture of the indicator *Str. uberis* LMGT 3912 (50-fold final dilution). After incubation at 37°C for approximately 8 h, the turbidity was measured spectrophotometrically at 600 nm using a SPECTROstar Nano reader (BMG Labtech). One bacteriocin unit (BU) was defined as the minimum amount of the antimicrobial that inhibited growth of the indicator strain by at least 50% in 200 μl of culture.

The inhibition spectrum of RPC-purified ubericin K (most active fraction) and *in vitro*-synthesized ubericin K was performed as a spot-on-lawn assay. Indicator strains were grown overnight in BHI at 30°C and then diluted 50-fold in 5 ml of BHI soft agar (0.8% agarose) and poured over a base layer of BHI agar (1.5% agarose). After solidification, 3 μl of purified antimicrobial or *in vitro*-synthesized ubericin K was spotted on the plates. Plates were inspected visually for inhibition zones after overnight incubation at 30°C. Nisin A (N5764, Sigma-Aldrich) was included as a comparison and prepared with a potency of ≥40,000 IU/ml in 0.05% (vol/vol) acetic acid, insolubles were removed by centrifugation, and remaining nisin A solution was sterile filtered (0.22-μm pore size; Millipore).

Pore formation in target cell membranes was tested using the recently published fluorescent reporter strain *Li. monocytogenes* EGDe/pNZ-P_{help}-pHluorin (41). This strain expresses the fluorescent protein pHluorin that has a bimodal excitation spectrum showing ratiometric pH-dependent changes in fluorescence intensity (42). In *Listeria* minimal buffer (LMB) at pH 6.5, untreated *Li. monocytogenes* EGDe/pNZ-P_{help}-pHluorin cells are able to maintain intracellular pH. However, in the presence of membrane-damaging compounds, the intracellular pH rapidly drops, resulting in a characteristic change in fluorescence at the two excitation peaks. For assays, an overnight culture of *Li. monocytogenes* EGDe/pNZ-P_{help}-pHluorin was harvested by centrifugation (3,000 × *g* for 10 min at 4°C), washed once in phosphate-buffered saline, and resuspended in LMB at pH 6.5 (41) to an optical density at 600 nm (OD_{600}) of 3. One hundred microliters of this suspension was added to individual wells of the 96-well screening plates. Then, 100 μl of a sample was added and plates were vortexed for 10 s, wrapped in aluminum foil, and incubated for 1 h at room temperature in the dark. Fluorescence was measured using a Tecan Infinite M200 microplate reader with excitation at 400/9 and 470/9 nm and emission at 510/20 nm. The ratios of emission intensities after excitation at 400 and 470 nm were calculated.

MALDI-TOF mass spectrometry. Acquisition of mass spectrometry data was performed on an UltrafleXtreme III TOF/TOF (Bruker Daltonics) MALDI-TOF mass spectrometer operated in reflectron mode. The instrument was set to analyze positively charged ions in the range of 1,400 to 6,600 *m/z* and had been externally calibrated in the *m/z* range of 700 to 3,100 using the peptide calibration standard II (Bruker Daltonics). The RPC-purified active fraction was mixed 1:1 with matrix solution (α-cyano-4-hydroxycinnamic acid [HCCA]) as recommended by the supplier (Bruker Daltonics) and applied to a stainless steel MALDI target plate (Bruker Daltonics).

Whole-genome sequencing. Genomic DNA isolation and purification was performed using a GenElute bacterial genomic DNA kit (Sigma-Aldrich). The sequencing libraries were prepared using a Nextera XT DNA sample prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Sequencing was performed using the Illumina MiSeq platform (Illumina) and V3 chemistry. Reads were error corrected and assembled *de novo* using SPAdes v3.14.1 (66). The obtained contigs were annotated by InterProScan (67) as well as submitted to the BAGEL4 web server to search for potential bacteriocin gene clusters (26). Initial assembly did not result in a complete cluster due to a lack of coverage upstream of ORF2. Therefore, primers KGAP1F and KGAP2R (Table 5) were designed based on the initial assembly and used to fill the gap region. The two primers were used in PCR, and the product was sequenced by Sanger sequencing (Eurofins Genomics).

***In vitro* protein synthesis.** The mature bacteriocin peptide sequence was synthesized *in vitro* using the PURExpress *in vitro* protein synthesis kit (New England BioLabs). First, the DNA sequence encoding the mature peptide was amplified from the producer using the primer pair KC1F and KC1R containing a start codon (ATG) and restriction sites NdeI and BamHI. All primers used in his study are listed in Table 5. The amplified product was purified with Macherey-Nagel PCR cleanup and gel extraction kit (Macherey-Nagel). The resulting amplicon and the DHFR control plasmid supplied with the PURExpress kit were digested with NdeI and BamHI (Thermo Fisher Scientific) according to manufacturer's recommendations. Digests were mixed in a molar ratio of 3:1 (insert to vector) and ligated with T4 DNA ligase (New England BioLabs) at room temperature for 10 min, and the ligation mixture was cloned into competent *Es. coli* NEB 5-alpha (C2987) cells following the high efficiency transformation protocol supplied by the manufacturer. The construct was isolated with an EZNA plasmid minikit I (Omega Bio-Tek) and verified by sequencing using the K1F primer before *in vitro* synthesis. Approximately 280 ng of plasmid, as estimated by a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific), was used

as the template for *in vitro* protein synthesis. The reaction mixture was incubated at 37°C for 4 h, diluted 2-fold with 20 mM magnesium acetate (Sigma-Aldrich), and used immediately without further purification.

Data availability. The entire bacteriocin gene cluster has been deposited in GenBank under accession number MZ189362.

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Paper II

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RESEARCH ARTICLE

Identification of a Novel Two-Peptide Lantibiotic from *Vagococcus fluvialis*

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ABSTRACT Infections caused by multiresistant pathogens have become a major problem in both human and veterinary medicine. Due to the declining efficacy of many antibiotics, new antimicrobials are needed. Promising alternatives or additions to antibiotics are bacteriocins, antimicrobial peptides of bacterial origin with activity against many pathogens, including antibiotic-resistant strains. From a sample of fermented maize, we isolated a *Vagococcus fluvialis* strain producing a bacteriocin with antimicrobial activity against multiresistant *Enterococcus faecium*. Whole-genome sequencing revealed the genes for a novel two-peptide lantibiotic. The production of the lantibiotic by the isolate was confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, which revealed distinct peaks at 4,009.4 *m/z* and 3,181.7 *m/z* in separate fractions from reversed-phase chromatography. The combination of the two peptides resulted in a 1,200-fold increase in potency, confirming the two-peptide nature of the bacteriocin, named vagococcin T. The bacteriocin was demonstrated to kill sensitive cells by the formation of pores in the cell membrane, and its inhibition spectrum covers most Gram-positive bacteria, including multiresistant pathogens. To our knowledge, this is the first bacteriocin characterized from *Vagococcus*.

IMPORTANCE Enterococci are common commensals in the intestines of humans and animals, but in recent years, they have been identified as one of the major causes of hospital-acquired infections due to their ability to quickly acquire virulence and antibiotic resistance determinants. Many hospital isolates are multiresistant, thereby making current therapeutic options critically limited. Novel antimicrobials or alternative therapeutic approaches are needed to overcome this global problem. Bacteriocins, natural ribosomally synthesized peptides produced by bacteria to eliminate other bacterial species living in a competitive environment, provide such an alternative. In this work, we purified and characterized a novel two-peptide lantibiotic produced by *Vagococcus fluvialis* LMGT 4216 isolated from fermented maize. The novel lantibiotic showed a broad spectrum of inhibition of Gram-positive strains, including vancomycin-resistant *Enterococcus faecium*, demonstrating its therapeutic potential.

KEYWORDS bacteriocin, *Vagococcus*, lantibiotic, antimicrobial, vagococcin T, pore formation

Enterococci such as *Enterococcus faecium* and *E. faecalis* are regular commensals of human and animal intestines (1, 2). However, in recent years, enterococci have become a concern in both human and veterinary medicine as they have emerged as some of the most prevalent nosocomial pathogens (3, 4). In addition to their ability to effectively acquire, harbor, and distribute antimicrobial resistance (AMR) determinants, enterococci are robust and able to survive on nonbiotic surfaces for prolonged periods

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(5, 6). There is increasing evidence that the overuse of antibiotics is a primary selection pressure for the acquisition and dissemination of antibiotic resistance in bacteria (7). To reduce the dissemination of AMR and to combat resistant bacteria, alternatives to antibiotics are needed. One such promising alternative is bacteriocins, natural proteinaceous compounds produced by bacteria with antimicrobial activity mostly against closely related species, including pathogenic and antibiotic-resistant strains.

Small bacteriocins (<10 kDa) are classified based on their biosynthesis: posttranslationally modified bacteriocins belong to class I, while unmodified bacteriocins are members of class II (8, 9). Lanthipeptides, which belong to class I, are characterized by thioether linkages formed between cysteines and dehydrated serine and threonine residues to yield lanthionine and methyllanthionine, respectively (10). The organization of the ring structures then recognizes a specific target on sensitive cells, such as lipid II, which is the docking molecule for most lantibiotics (11). The bacteriocin producer must protect itself from the lethal action of its own bacteriocin. For lantibiotics, self-immunity is achieved by the production of immunity proteins commonly named LanI and/or LanFE(G) (12, 13). The LanFE(G) proteins compose a specialized ABC transporter that mediates the efflux of mature lanthipeptides from the cell, while LanI is thought to protect the producer extracellularly against the secreted lanthipeptide (12).

Lantibiotics are further subdivided into at least two types based on differences in the modification enzymes (14). Type I lanthipeptides, of which nisin is the founding member, use two separate enzymes for the dehydration (LanB) and cyclization (LanC) steps that produce the (methyl)lanthionine rings. Type II employs a single bifunctional enzyme (LanM) that catalyzes both steps (10, 14). LanM modification enzymes usually carry out the modification of two-peptide lantibiotics, each of which consists of two different peptides exhibiting considerable synergy when combined but having little or no activity when assessed individually (15). The most well-studied two-peptide lantibiotic, lacticin 3147, has potent activity against numerous pathogenic Gram-positive species, including vancomycin-resistant enterococci (VRE) (16). Lacticin 3147 also attenuates the growth of *Staphylococcus aureus* in a murine infection model and disrupts *Streptococcus mutans* biofilms, demonstrating the clinical potential of lantibiotics (17, 18).

Vagococcus fluvialis belongs to a genus of motile lactic acid bacteria most closely related to *Enterococcus* and *Carnobacterium* and was first described as a phylogenetically distinct genus in 1989 (19, 20). Not much is known about *V. fluvialis*; most characterized isolates originated from wounds of animals (pigs, horses, and cattle) and from human clinical cases (20, 21). However, the species has also been isolated from the urine of healthy cattle and was described as a potential probiotic in fish (22, 23). In this work, we describe the discovery and characterization of a novel two-peptide lantibiotic produced by *Vagococcus fluvialis* LMGT 4216. The bacteriocin was active against most Gram-positive strains tested, including animal and human pathogens, such as multidrug-resistant *E. faecium* and mastitis-associated *Streptococcus uberis* (24). The bacteriocin gene cluster had an atypical organization and included what resembles a quorum-sensing system. To our knowledge, this is the first bacteriocin characterized from *Vagococcus*. We believe that this bacteriocin could serve an important role as a therapeutic in the future.

RESULTS

Screening for bacteriocin producers against *Enterococcus faecium*. *E. faecium* LMG 20705 is a multidrug-resistant opportunistic pathogen. The resistance pattern was determined by AMRFinderPlus (see Table S1 in the supplemental material) and a disc diffusion test according to EUCAST methods (data not shown) (25, 26). The strain was shown to be resistant to vancomycin, ampicillin, and streptomycin, all of which are first-line therapeutics for enterococcal infections (27). In addition, the strain exhibited resistance to quinupristin-dalfopristin, a mixture of streptogramins B and A used for the treatment of serious VRE-related infections (28).

A total of 40 different samples of fermented fruits and vegetables were screened for the presence of bacteriocin producers that could inhibit the growth of *E. faecium*

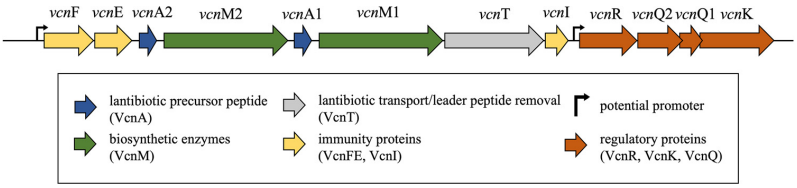

FIG 1 Gene organization of the vagococcin T cluster in *V. fluvialis* LMGT 4216. Bifunctional modification enzyme genes (green) are located downstream of lantibiotic precursor genes (blue). A lantibiotic transporter gene with a leader removal function (gray) is located downstream of *vcn*M1 and upstream of *vcn*I, encoding a potential immunity protein (yellow). Other genes involved in bacteriocin immunity are located at the beginning of the cluster. A group of genes resembling a quorum-sensing system (red) is located at the end of the cluster.

LMG 20705. From all samples, 17 colonies exhibited a distinct inhibition zone indicative of antimicrobial production. Repetitive element PCR (rep-PCR) was performed to examine the genetic similarity of these isolates. Nine unique DNA band profiles were observed after gel electrophoresis (data not shown). One representative from each group was selected for whole-genome sequencing to identify novel bacteriocin genes. The genomes were analyzed for bacteriocins by BAGEL4 and antiSMASH (29, 30). The analysis revealed that all but two isolates had genes for previously characterized bacteriocins known to be active against enterococci: subtilosin A (31), ericin S (32), enterolysin A (33), and NKR-5-3B (34). One of the two isolates with a potentially novel bacteriocin was a strain isolated from fermented maize; the genome of this isolate contained a gene cluster with an organization similar to those of the two-peptide lantibiotic gene clusters. The best database hit for the predicted bacteriocin was the lantibiotic flavecin from *Ruminococcus flavefaciens* (35), with only 45% identity, suggesting that the isolate, identified as *Vagococcus fluvialis*, likely produced a novel two-peptide lantibiotic.

Genome analysis and identification of the vagococcin T gene cluster. The search for putative bacteriocin genes by antiSMASH resulted in the identification of a type II lantibiotic gene cluster (Fig. 1). Two bacteriocin genes, *vcn*A1 and *vcn*A2, were identified and predicted to represent the α (*vcn*A1) and β (*vcn*A2) peptides of a two-peptide lantibiotic hereafter named vagococcin T (Vcn Tα and Vcn Tβ). Located downstream of each of the *vcn*A1 and *vcn*A2 genes are genes encoding lantibiotic biosynthesis proteins, *vcn*M1 and *vcn*M2, respectively. Both gene products, VcnM1 and VcnM2, showed sequence similarity with MrsM, the modification enzyme for the lantibiotic mersacidin (36). The predicted functions of all proteins encoded by the *vcn* gene cluster are listed in Table 1.

TABLE 1 Encoded proteins from the vagococcin T cluster of *V. fluvialis* LMGT 4216 with their homologs and predicted functions

Gene product	Putative function(s)	Homolog, % sequence identity (GenBank accession no.)
VcnF	Bacteriocin immunity	NisF, 47 (AAC43327.1)
VcnE	Bacteriocin immunity	MrsE, 22 (CAB60257.1)
VcnA2	Vagococcin T β-peptide	FlvA2b, 46 (P0DQL4.1)
VcnM2	VcnA2 dehydratase and cyclase	MrsM, 44 (CAB60261.1)
VcnA1	Vagococcin T α-peptide	FlvA1a, 42 (P0DQM1.1)
VcnM1	VcnA1 dehydratase and cyclase	MrsM, 48 (CAB60261.1)
VcnT	Bacteriocin maturation and export	MrsT, 45 (KAF1340276.1)
VcnI	Bacteriocin immunity	
VcnR	Response regulator	FsrA, 39 (EIA6660097.1)
VcnQ2	Pheromone maturation/export	FsrB, 35 (EGO8521395.1)
VcnQ1	Pheromone/signaling molecule prepeptide	FsrD, 37 (CDK37795.1)
VcnK	Protein histidine kinase	FsrC, 35 (EIP8082021.1)

A

VcnA1

MERNPILREKKQQQFLSTSGLEEVNQNIEFIENLSGG NNVWVTILQGVVGCVASWAVGNKGKVCTWTVECQKNCS

VcnA2

MRTSNDIKNKTGYVEESKLKEMIEEPDYSGG AWTTLPCIGGIIAATLNFDACPTSACTKSCNK

B

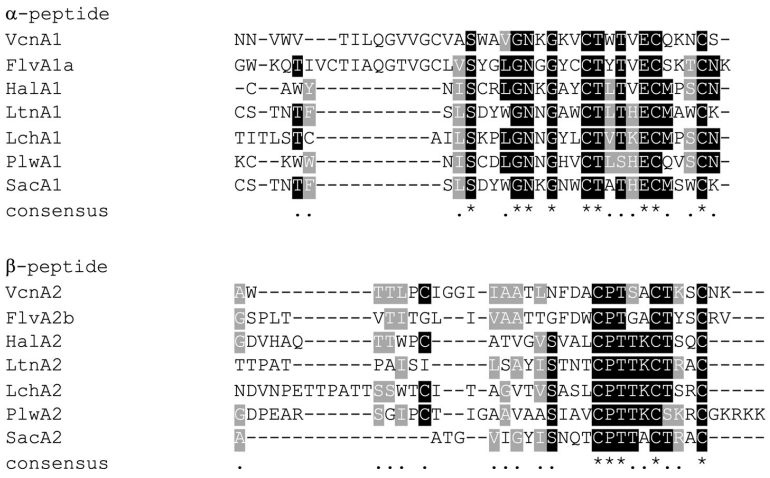

FIG 2 (A) Predicted amino acid sequences of vagococcin T prepeptides. Leader sequences are underlined and separated from the mature peptides by a space. (B) Multiple-sequence alignment of α- and β-peptides of the known two-peptide lantibiotics flavecin (Flv) (UniProt accession numbers P0DQM1 and P0DGL4), haloduracin (Hal), lacticin 3147 (Ltn) (accession numbers O87236 and O87237), lichenicidin (Lch) (accession numbers P86475 and P86476), plantaricin W (Plw) (accession numbers D2KR94 and Q9AF68), and staphylococcin C55 (Sac) (accession numbers Q9S4D3 and Q9S4D2). The sequence alignment was performed using T-Coffee and colored with BoxShade; black and gray shading correspond to identical and similar amino acids, respectively.

The *vcn*T gene is located downstream of *vcn*M1 and encodes a C39 peptidase that shows 45% identity with MrsT, the mersacidin transport enzyme that cleaves the leader after the GG/GA motif, a typical cleavage site for many bacteriocin leaders (37). A GG motif is indeed present in both the VcnA1 and VcnA2 prepeptides (Fig. 2A). The mature peptides showed the highest homology to the flavecin FlvA1a and FlvA2b peptides (42% and 46%, respectively) (35). Sequence alignment of Vcn Tα with other lantibiotic α-peptides (Fig. 2B) showed that Vcn Tα contains the same conserved CTxTxEC motif believed to be essential for lipid II docking (38). Similarly, the conserved sequence (CPTxxCt/sxxC; variable residues are shown in lowercase, threonine/serine) typical for all β-peptides was found in Vcn Tβ (Fig. 2B).

The types of immunity genes present in lantibiotic gene clusters vary, and the encoded immunity proteins often show little sequence identity with each other (39). Two genes of the LanFE(G) immunity system are present in the *vcn* cluster, *vcn*F and *vcn*E, located at the start of the operon. VcnF showed 47% identity to the ATP-binding domain NisF of the NisFEG transporter and contained the conserved sequences for both Walker A and B motifs (40).

The last four genes in the cluster resembled an analog of the Fsr quorum-sensing system of *E. faecalis*; this type of quorum-sensing system has not previously been identified in other lantibiotic clusters (41). The product of the first open reading frame (ORF), designated *vcn*R, showed 39% identity to the response regulator (RR) FsrA (Table 1). An FsrB homolog is encoded by the gene designated *vcn*Q2, with 36% identity (Q for quorum). The third component, a sensor histidine protein kinase (HPK)

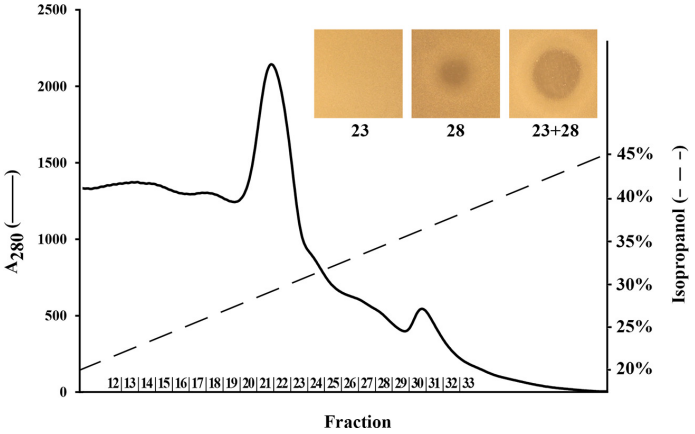

FIG 3 Reversed-phase chromatography elution profile of the sample obtained by cation-exchange chromatography. All collected fractions exhibited relatively low bacteriocin activity against *E. faecium* LMG 20705, but two fractions showed a significant increase in potency when assayed together (1:1 [vol/vol]), indicating the presence of a two-peptide bacteriocin. The inhibition of *E. faecium* LMG 20705 by individual and combined fractions is pictured in the top right corner.

encoded by *vcn*K, showed 35% identity to FsrC. A search for small reading frames that could encode the pheromone component of the quorum-sensing system revealed a small ORF between *vcn*Q2 and *vcn*K. The product of this ORF gave no hits to any known peptides by a BLAST search; however, sequence alignment showed 37% identity to FsrD, the gelatinase biosynthesis-activating pheromone (GBAP) prepeptide (42). It is therefore possible that the processed product of *vcn*Q1 is a pheromone.

Another small ORF located between *vcn*T and *vcn*R also showed no sequence homology to known proteins by a BLAST search; however, the gene product had a size, charge, and hydrophobicity similar to those of known lantibiotic immunity proteins. LanI proteins with comparable physicochemical properties include EciI, PepI, and LasJ, the LanI immunity proteins for epicidin 280, Pep5, and lactocin S (39). The ORF located between *vcn*T and *vcn*R was therefore named *vcn*I and is further discussed in Discussion below.

Because of the novelty of vagococcin T, the antimicrobial produced by the isolate of *V. fluvialis*, named *V. fluvialis* LMGT 4216 here, was chosen for further characterization. Cell-free supernatants from the isolate contained an antimicrobial substance that was heat stable and sensitive to proteinase K (data not shown), properties expected for bacteriocins like vagococcin T (8).

Purification of bacteriocin. The purification of the predicted two-peptide lantibiotic produced by *V. fluvialis* LMGT 4216 was achieved by a three-step purification scheme consisting of ammonium sulfate precipitation, cation-exchange chromatography, and reverse-phase chromatography (RPC) (43). During the RPC elution, two peaks corresponding to 29% and 36% isopropanol were observed in the elution profile (Fig. 3). The collected fractions were assayed against *E. faecium* LMG 20705; a low antimicrobial activity of 400 bacteriocin units (BU)/mL was found only in the second peak (fractions 26 to 30), which would be expected due to the separation of the two peptides into separate fractions (44). To test this notion, fractions 21 to 24 were individually combined with fractions 26 to 30 in a 1:1 (vol/vol) ratio to find any combination of fractions exhibiting synergy (Fig. S1). Indeed, the highest synergy was observed between fractions 23 and 28, which in combination had an antimicrobial activity of 51,200 BU/mL, representing a 1,200-fold increase in activity with a yield of 128% (Table 2).

With purified bacteriocin, the biological activity of vagococcin T against a number of bacteria was determined using a spot-on-lawn assay (Table 3). Lantibiotics are known to

TABLE 2 Bacteriocin purification

Sample	Vol (mL)	Activity (BU/mL)	Total activity (BU)	Yield (%)
Supernatant	1,000	80	80,000	100
Ammonium sulfate precipitate	150	320	48,000	60
Cation-exchange chromatography	100	160	16,000	20
Reversed-phase chromatography	2	51,200	102,400	128

be very potent against Gram-positive bacteria but have limited activity against Gram-negative bacteria, as observed for nisin (45), lichenicidin (46), and thusin (47). In addition to showing potent antimicrobial activity against the indicator strain *E. faecium* LMG 20705, vagococcin T displayed a broad inhibition spectrum, including all Gram-positive bacteria tested except for *Staphylococcus aureus*. The Gram-negative bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium were not inhibited, which is expected for the lipid II-targeting type A lantibiotics.

Molecular mass and bacteriocin identification. Given the synergism of fraction 23 with fraction 28, these fractions were analyzed further using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The acquired spectra revealed the presence of only one distinct peak in each fraction. A peak at 4,009.5 *m/z* can be seen in fraction 23 (Fig. 4A), which correlated well with the mass predicted for one of the two peptides by antiSMASH (30) (assuming 1 unmodified Ser/Thr residue). The peak in fraction 28 (Fig. 4B) at 3,181.7 *m/z*, however, differed from the prediction by 70.1 Da (3,111.6 Da, assuming 2 unmodified Ser/Thr residues). The reasoning behind this difference is given in Discussion below. A schematic representation of all posttranslational modifications of Vcn Tα and Vcn Tβ consistent with the measured masses is shown in Fig. 5. These results confirm that the antimicrobial activity produced by *V. fluvialis* LMGT 4216 was indeed caused by the predicted two-peptide lantibiotic vagococcin T.

Pore-forming nature of vagococcin T. To assess whether vagococcin T is a pore former, a propidium iodide (PI) assay was conducted. PI is a membrane-impermeant dye that increases its fluorescence efficiency/quantum yield when bound to double-stranded

TABLE 3 Inhibition spectrum of reversed-phase chromatography-purified vagococcin T (2 μL)

Indicator strain[a]	Sensitivity[b]
Bacillus cereus LMGT 2805	++
Bacillus cereus LMGT 2731	+
Enterococcus faecalis LMGT 2333	++
Enterococcus faecalis LMGT 3331	++
Enterococcus faecium LMGT 2772	++
Enterococcus faecium LMGT 3104	++
Lactobacillus curvatus LMGT 2353	+++
Lactobacillus plantarum LMGT 2352	++
Lactococcus garvieae LMGT 3390	++
Lactococcus lactis LMGT 2081	++
Listeria innocua LMGT 2710	+++
Listeria monocytogenes LMGT 2604	++
Listeria monocytogenes LMGT 2650	+
Pediococcus acidilactici LMGT 2002	+++
Streptococcus dysgalactiae LMGT 3890	+
Streptococcus thermophilus LMGT 3555	+++
Streptococcus uberis LMGT 3912	++
Staphylococcus haemolyticus LMGT 4133	+
Staphylococcus aureus LMGT 3242	−
Salmonella Typhimurium B1377	−
Escherichia coli TG1	−

[a]Laboratory of Microbial Gene Technology (LMGT), Norwegian University of Life Sciences, Ås, Norway.
[b]Inhibition zone diameters of 5 to 9 mm (+), 10 to 14 mm (++), or >15 mm (+++) or no inhibition (−).

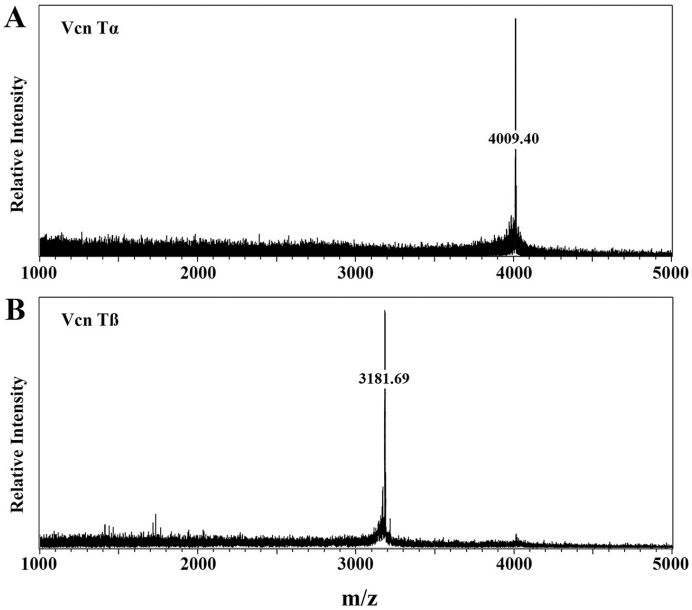

FIG 4 MALDI-TOF mass spectrometry analysis of fractions 23 (A) and 28 (B) from reversed-phase chromatography. The peaks at 4,009.40 *m/z* and 3,181.69 *m/z* represent Vcn Tα and Vcn Tβ peptides, respectively.

DNA (48). After exposing the indicator strain to the known pore-forming lantibiotics nisin A and nisin Z in the presence of extracellular PI, an increase in the emission was detected (Fig. 6). Similar results were also obtained for vagococcin T, implying that vagococcin T has a similar mode of action involving pore formation. The negative control, micrococcin P1, a bacteriocin that kills cells by inhibiting protein synthesis (49), caused little or no increase in fluorescence as it does not form pores.

To further corroborate our results showing that vagococcin T is membrane active, the indicator cells exposed to vagococcin T were examined by scanning electron microscopy (SEM). Clear differences were observed for bacteriocin-treated compared to untreated cells (Fig. 7). Treated cells appeared collapsed/shriveled, suggesting a loss of turgor pressure. Irregular dark spots were visible on some cells, possibly indicating pores or damage to the cell envelope. In addition, an extracellular matrix-like material was visible only in the treated cells. In comparison, the cell surface of untreated cells was smooth, without ruptures or signs of cell damage.

Stress response involved in resistance to vagococcin T. Resistant colonies of *E. faecium* LMG 20705 were occasionally visible within the inhibition zones of vagococcin T. The increased tolerance to vagococcin T of four randomly selected spontaneous mutants was tested and showed a 64- to 256-fold increase in the MIC compared to that for the wild type (Table 4). The frequency of resistant mutants was estimated to be 8.7×10^{-7} based on plating techniques. Whole-genome sequencing was performed on the four mutants to identify the possible mechanism for the increased tolerance to vagococcin T. Three of the four mutants had mutations in *liaF* (M1 to M3), two with nonconservative missense mutations (Ile108Asn and Trp141Ser) and one with a frameshift from amino acid position 9 (Val9fs) (M2). Several mutations were found in various genes of mutant M4, none of which could be directly linked to the increased tolerance to vagococcin T (Table 4). *liaF* encodes a negative regulator of LiaRS, a two-component regulatory system involved in the cell envelope stress response induced by lipid II-interacting antimicrobials

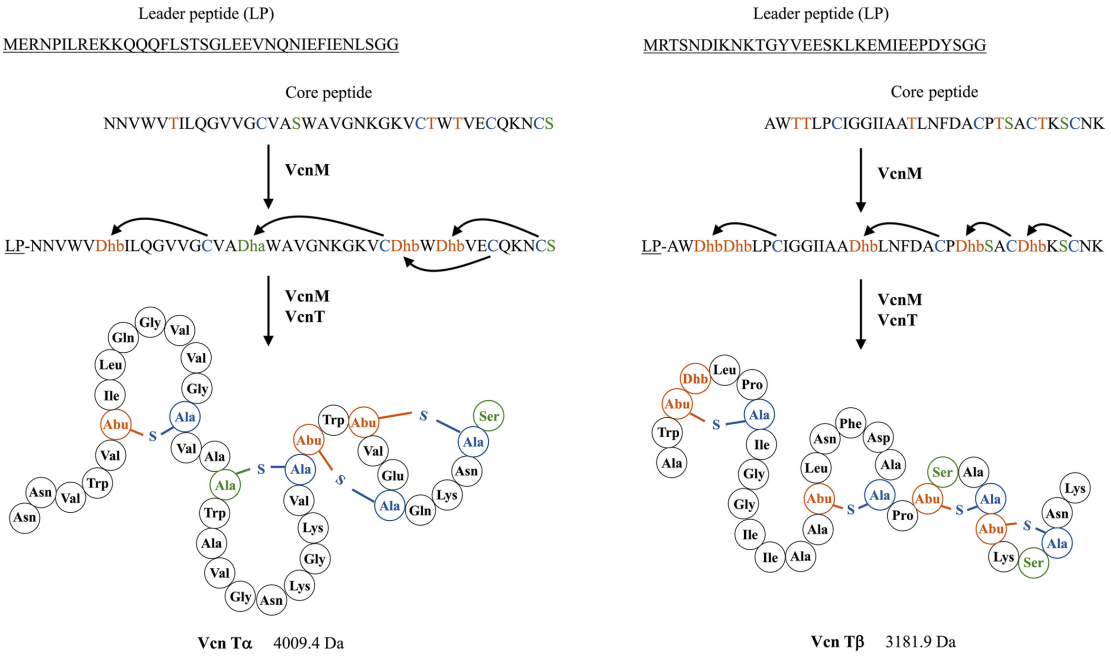

FIG 5 Proposed biosynthetic scheme for vagococcin T α- and β-peptides. The structures of Vcn Tα and Vcn Tβ were deduced from the known structures of other two-peptide lantibiotics. Lanthionine rings (Ala-S-Ala) are formed between didehydroalanine (Dha), derived from serine (green) and cysteine (blue) residues; methyllanthionine (Abu-S-Ala) rings are formed between didehydrobutyrine (Dhb), derived from threonine (orange) and cysteine residues.

(50). We examined the cross-resistance of *liaF* mutants to other membrane-active bacteriocins, nisin A and garvicin KS (43). As expected, both nisin A and garvicin KS showed reduced bioactivity (4- to 32-fold) toward the mutants compared to the wild-type strain.

DISCUSSION

Bacteriocins are a promising alternative to traditional antibiotics, as they display activity against antibiotic-resistant pathogens and have many desirable properties for the control of microorganisms. They are often produced by probiotic species with GRAS

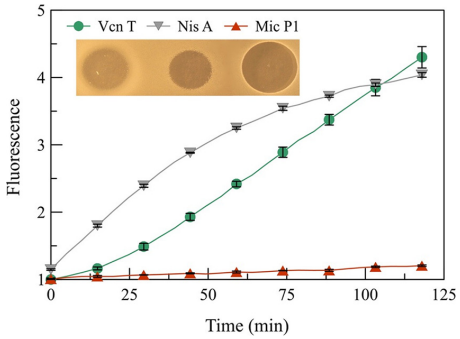

FIG 6 Bacteriocin-induced pore formation assay. Shown is the propidium iodide fluorescence intensity over time in the presence of *E. faecium* and the antimicrobials vagococcin T (Vcn T), nisin A (Nis A), and micrococcin P1 (Mic P1). An increase in emission is observed for the pore-forming nisin A. Micrococcin P1, a non-pore-forming bacteriocin, was used as a negative control. The inhibition zone produced by each bacteriocin (2 μL) is shown at the top left.

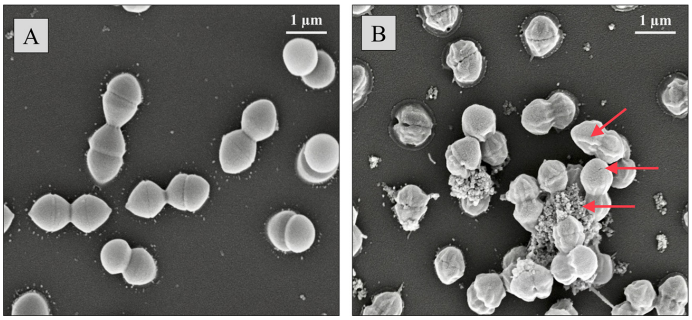

FIG 7 Scanning electron microscopy (SEM) showing the effect of vagococcin T on *E. faecium* cells (magnification, ×30,000). Cells incubated without vagococcin T showed no visible cell damage (A), while the vagococcin T-treated cells had a shriveled appearance following a 2-h incubation with 10× MIC of vagococcin T (B). Signs of cell damage and lysis are indicated by red arrows.

(generally regarded as safe) status and have high potency and low toxicity (51). In addition, bacteriocins are arguably more amenable to biotechnological manipulation as they are defined by structural genes. Given the high potency and potential clinical applications of bacteriocins, we sought to find new bacteriocins with possible therapeutic use. To this end, we screened for bacteriocin producers in fermented fruits and vegetables that inhibited the growth of the indicator strain, a multidrug-resistant *E. faecium* isolate. From a sample of fermented maize, we successfully isolated a strain of *V. fluvialis* producing a two-peptide lantibiotic named vagococcin T (*V. fluvialis* LMGT 4216).

To our knowledge, vagococcin T is the first bacteriocin characterized from the genus *Vagococcus*. The two bacteriocin genes *vcn*A1 and *vcn*A2 are separated by a *vcn*M gene, which is an unusual arrangement: two-peptide bacteriocin genes are most often located adjacent to each other in tandem. Because of the low sequence similarity of the two vagococcin T prepeptides (20% sequence identity), each of the two *vcn*M gene products is likely dedicated to modifying its cognate bacteriocin peptide. Upstream of the bacteriocin genes in the same operon is the gene pair *vcn*FE encoding an ABC transporter that likely has a dual role in the export of the bacteriocin peptides and immunity, a property that is common for other lantibiotics, including nisin, mersacidin, and lacticin 3147 (39). At the end of the *vcn* cluster is an operon encoding proteins with homology to the Fsr quorum-sensing system from *E. faecalis*. In the Fsr system, the FsrD propeptide is exported and processed by FsrB into a small 11-amino-acid cyclic peptide pheromone. A membrane-bound sensor HPK, FsrC (VcnK), then responds to the pheromone and activates the intracellular RR FsrA (VcnR) (30). VcnQ2 and VcnQ1 show 35% and 37% sequence identities to FsrB and FsrD, respectively (Table 1). The majority of circular peptide pheromones have been reported to form a thiolactone linkage between the C-terminal amino acid (methionine, phenylalanine, or leucine) and a cysteine located 3 or 4 residues from the N-terminal cleavage site (52).

TABLE 4 Mutations identified in *E. faecium* LMG 20705 spontaneous mutants with increased tolerance to vagococcin T

E. faecium mutant	Fold increase of MIC[a]			Mutations[b]	Protein	RefSeq accession no.
	Vcn T	Nis A	Gar KS			
M1	256	16	4	c. 323T>A; p. Ile108Asn	Stress regulator protein LiaF	WP_002328613.1
M2	256	32	4	c. 24dupT; p. Val9fs	Stress regulator protein LiaF	WP_002328613.1
M3	256	32	8	c. 422G>C; p. Trp141Ser	Stress regulator protein LiaF	WP_002328613.1
M4	64	8	4	c. 605T>A; p. Val202Glu	Aldose 1-epimerase	WP_002328285.1
				c. 514G>A; p. Gly172Arg	Metal-dependent hydrolase	WP_002287133.1
				c. 187A>G; p. Ile63Val	Hypothetical protein	WP_100970561.1
				c. 277A>T; p. Thr93Ser	Mg^{2+} cation transporter (CorA family protein)	WP_002318987.1

[a]Vcn T, vagococcin T; Nis A, nisin A; Gar KS, garvicin KS.
[b]c., coding DNA; p., protein; >, substitution; dup, duplication; fs, frameshift.

However, the peptide processed from FsrD contains a lactone linkage between the C-terminal methionine and the hydroxyl group of a serine residue (42). In addition, an autoinducing peptide containing a lactone ring between the C-terminal phenylalanine and a serine residue has been identified in *Staphylococcus intermedius* (53). VcnQ1 may be processed similarly, forming a lactone linkage between serine and the C-terminal phenylalanine. Interestingly, the closest homolog to VcnQ1 was found to be an unannotated ORF (159 nucleotides [nt]) in the locus of the circular bacteriocin enterocin NKR-5-3B (Ent53B) produced by *E. faecium* strain NKR-5-3 (GenBank accession number LC068607) (54). The ORF is arranged similarly to *vcn*Q1 between genes encoding an HPK and an FsrB-like protein (*orf5* and *orf6*). The predicted mature product of this ORF contains an 11-amino-acid sequence showing 73% identity (100% similarity) to the putative VcnQ1-derived pheromone. *E. faecium* NKR-5-3 produces multiple bacteriocins: enterocins NKR-5-3A, -B, -C, -D, and -Z (Ent53A, Ent53B, Ent53C, Ent53D, and Ent53Z) (55). An inducing peptide, Ent53D, has been shown to regulate the transcription of the above-mentioned bacteriocins except for NKR-5-3B (55). A derivative of the unannotated ORF in the *E. faecium* NKR-5-3 genome may be involved in the regulation of NKR-5-3B. However, it is presently not known if VcnQRK constitutes a functional quorum-sensing system in *V. fluvialis* LMGT 4216; characterization of the *vcn* regulatory system is beyond the scope of the present study.

The production of vagococcin T by *V. fluvialis* LMGT 4216 was confirmed by bacteriocin purification and MALDI-TOF MS. Vagococcin T was purified from the cell-free supernatant using a typical scheme for bacteriocin purification in our laboratory, starting with ammonium sulfate precipitation at 60% saturation (4°C), a concentration determined to be a good compromise between yield and purity for many bacteriocins. By reversed-phase chromatography, a significant increase in potency (51,200 BU/mL) was observed for the combination of fractions 23 and 28. Despite not corresponding to the two peaks in the elution profile, the noticeably higher activity observed for the combination was strong evidence of a two-peptide bacteriocin.

Mass determination of each fraction revealed single distinct peaks at 4,009.4 *m/z* and 3,181.69 *m/z* for fractions 23 and 28, respectively. Analysis of the *V. fluvialis* LMGT 4216 genome by the ribosomally synthesized and post-translationally modified peptides (RiPP) mining tool antiSMASH (30) identified a lanthipeptide gene cluster encoding two putative lanthipeptide precursors. In addition to predicting lanthipeptide genes, antiSMASH predicts the leader cleavage site, dehydrations, cross-links, and expected masses. The mass predicted for Vcn Tα (4,010.6 Da), assuming one unmodified serine or threonine residue, corresponded well with the measured value of 4,009.4 *m/z*. However, the mass predicted for Vcn Tβ (3,111.6 Da) was approximately 71 Da lower than the mass obtained by MALDI-TOF MS. The reason for this discrepancy is likely inaccurate leader peptide prediction. The predicted Vcn Tα leader peptide is a typical double-glycine-type leader with a GG| cleavage site, while the Vcn Tβ leader cleavage site was predicted to be (G)GA|. The predicted mass of Vcn Tβ with the addition of alanine is 3,181.5 Da, which is consistent with the measured mass of *m/z* 3,181.67. The close correspondence between the measured and the theoretical masses provides strong evidence that the purified bacteriocin vagococcin T is the gene product of *vcn*A1 and *vcn*A2. The predicted structures of Vcn Tα and Vcn Tβ peptides are consistent with the structures of other two-peptide lantibiotics (Fig. 5).

The α-peptide of most two-component lantibiotics employs lipid II as a docking molecule to exert its antimicrobial activity (56, 57). A lipid II-binding motif was found in Vcn Tα (Fig. 2B), suggesting a lipid II-dependent mode of action of vagococcin T. It is believed that the β-peptide of lipid II-targeting two-component lantibiotics binds to the complex formed between lipid II and the α-peptide, which then leads to pore formation. The predicted mode of action involving pore formation was consistent with SEM showing *E. faecium* with a shriveled appearance, lysed cells, and cell debris following exposure to vagococcin T (Fig. 7). The extracellular matrix-like material likely consists of cell debris cross-linked by the fixing agent. The pore formation property is further supported by the fact that Vcn T

showed a pore-forming ability comparable to that of nisin A, a known pore-forming lantibiotic (58, 59).

For many lantibiotics, the type of immunity system appears to correlate with the mode of action of the lantibiotic (12, 13). It is believed that producers of pore-forming lantibiotics require both the LanI and LanFE(G) components for immunity (13, 60). However, no LanI component was immediately apparent in the *vcn* cluster despite the evident pore-forming mode of action of vagococcin T (Fig. 6). Upon further analysis, a small ORF was found downstream of *vcn*T, encoding a predicted transmembrane, cationic, 50-amino-acid protein (charge 5 at pH 7). The protein sequence shows no homology to known proteins but shares similar properties with PepI, EciI, and LasJ (LanI components of Pep5, epicidin, and lactocin S, respectively), all predicted transmembrane proteins 57 to 69 amino acids long with a charge of 4 to 6 (at pH 7). Due to this similarity, we believe that this ORF is involved in lantibiotic immunity, and it is thus named *vcn*I.

Upon challenging the *E. faecium* indicator strain with the bacteriocin, we observed resistant cells at a frequency of 8.7×10^{-7}. Three randomly selected isolates with the highest tolerance to vagococcin T all had mutations in *liaF*, a negative regulator (repressor) of the LiaRS (lipid II-interacting antibiotic response regulator and sensor) cell envelope stress response system. Previous studies have shown that membrane-active antimicrobials decouple the repression by LiaF, allowing the HPK LiaS and its cognate RR LiaR to trigger genes involved in resistance (61). The effect of the genetic disruption of *liaF* is likely similar to that of the decoupling of LiaF-mediated repression. Orthologs of the Lia system exist in most *Firmicutes*, and all systems investigated so far regulate the expression of genes that protect the cell against perturbations in the cell envelope (50). In *Bacillus subtilis*, the LiaFSR system is one of the primary response systems against lipid II-interacting antibiotics such as vancomycin and bacitracin (62) but is also induced by cationic antimicrobial peptides, organic solvents, and detergents (63–65). The genes regulated by the Lia system vary between species; in *Staphylococcus aureus*, the LiaRS homolog (VraSR) upregulates genes encoding penicillin-binding proteins and proteins involved in teichoic acid synthesis, chaperones, and membrane lipid biosynthesis that together confer resistance to β-lactam antibiotics (66–69). Even though the LiaFSR regulon in enterococci remains unknown, the LiaFSR system has been implicated in resistance to daptomycin and antimicrobial peptides due to the redistribution of cardiolipin microdomains away from the division septum (70, 71). All *liaF* mutants displayed low-level cross-resistance to nisin A, another lipid II-interacting lantibiotic (Table 4). These results confirm the role of LiaFSR in mediating resistance to vagococcin T, which further supports the lipid II-mediated mode of action of the bacteriocin.

The appearance of vagococcin T-resistant colonies of *E. faecium* exemplifies the hardiness of enterococcal populations. Combination therapies will likely be needed to effectively control enterococcal populations in the future. Formulations combining bacteriocins with different modes of action have been developed and showed increased potency and a broader inhibition spectrum with a very low frequency of resistance (72, 73).

In summary, in this work, we describe the isolation and characterization of a new two-component lantibiotic, vagococcin T, showing a broad antimicrobial spectrum against Gram-positive species, including multidrug-resistant strains. Furthermore, we show that mutations in the *liaF* gene confer resistance to vagococcin T and other antimicrobials. This connection highlights LiaF and the stress response system as appealing targets for future drug development and combination therapies. Further work is required to establish the potential of vagococcin T as a therapeutic in human or veterinary medicine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The indicator strain *E. faecium* LMG 20705 (FAIR-E 102) was obtained from the LMG collection (BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium). *E. faecium* LMG 20705 was grown in M17 broth supplemented with 0.5%

(wt/vol) glucose (GM17) and incubated at 37°C without shaking. All other bacterial strains were grown in brain heart infusion (BHI) broth at 30°C without shaking.

Screening for bacteriocin producers. A selection of 40 different fruits and vegetables was purchased from a local market (Oslo, Norway) and prepared as described previously (74). Samples were screened for bacteriocin producers using a multilayer soft-agar technique. Briefly, 10-fold serial dilutions of samples were prepared in sterile saline. An aliquot (10 mL) of each dilution was mixed with 5 mL of BHI soft agar (0.7% [wt/vol] agar), plated onto a BHI agar plate (1.5% [wt/vol] agar), and allowed to solidify. A second layer of BHI soft agar was poured on top, and the plates were incubated overnight at 30°C. Next, a culture of the indicator strain grown overnight was diluted 1:100 in 5 mL BHI soft agar and poured over the plate. After an additional incubation at 30°C overnight, colonies showing a clear zone of inhibition were restreaked to obtain pure cultures. The pure culture was retested against the indicator strain before being stored in 20% glycerol at −80°C for later use.

DNA sequencing and repetitive element PCR fingerprinting. Genomic DNA was isolated and purified using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the provided protocol. The 16S rRNA gene was amplified using the universal primers 11F (5′-TAACACATGCAAGTCGAACG-3′) and 4R (5′-ACGGGCGGTGTGTRC-3′). The PCR product was purified using a NucleoSpin gel and PCR cleanup kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and sent to Eurofins Genomics for Sanger sequencing. Repetitive element PCR (rep-PCR) fingerprinting was performed using primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′), and LL-rep1 (5′-TACAAACAAAACAAAAAC-3′) as previously described (74, 75).

Whole-genome sequencing was performed by BGI (Beijing Genomics Institute) (Beijing, China) using the DNBSeq sequencing platform (150-bp paired-end reads). Reads were error corrected and assembled using SPAdes v3.14.1 (76). The resulting contigs were submitted to antiSMASH and BAGEL4 for the identification of potential bacteriocin genes (29, 30). For submission, the whole-genome assembly was assembled using Unicycler v0.5.0 and annotated using the NCBI prokaryotic genome annotation pipeline (PGAP) (77, 78).

Bacteriocin purification. *V. fluvialis* LMGT 4216 was cultivated in 1 L of BHI broth at 30°C for 24 h. Cells were removed by centrifugation (10,000 × *g* for 30 min at 4°C), and the bacteriocin was precipitated from the culture supernatant with ammonium sulfate (60% saturation at 4°C overnight). The precipitate was harvested by centrifugation (15,000 × *g* for 40 min at 4°C), redissolved in 700 mL of distilled water, and adjusted to pH 3.5 with 1 M hydrochloric acid. The sample was applied to a Hi-Prep 16/10 SP-XL column (GE Healthcare, Chicago, IL, USA). Unbound material was washed from the column with 150 mL of 25 mM sodium citrate-phosphate buffer (pH 3.5). The bacteriocin was eluted with 100 mL of 0.5 M sodium chloride, and the eluate was then applied to a 1-mL Resource RPC column (GE Healthcare) connected to an Äkta purifier system (Amersham Pharmacia Biotech, Amersham, UK). The column was previously equilibrated with 0.1% (vol/vol) trifluoroacetic acid (TFA), and the bacteriocin was eluted from the column using a linear gradient (40 column volumes [CV]) of isopropanol containing 0.1% (vol/vol) TFA at 1 mL/min.

Bacteriocin activity assays. Bacteriocin activity was assayed in microtiter plates as previously described (79). A culture of the indicator strain *E. faecium* LMG 20705 or mutants grown overnight was diluted 50-fold in GM17 broth containing 2-fold dilutions of the sample to a total volume of 200 μL. The plate was incubated at 37°C for approximately 4 h, after which the absorbance at 600 nm was measured using a SPECTROstar Nano plate reader (BMG Labtech, Ortenberg, Germany). Bacteriocin activity was expressed in bacteriocin units (BU) per milliliter: 1 BU is the amount of bacteriocin that inhibits the growth of the indicator strain by at least 50% in 200 mL of culture (79). Nisin A was prepared by thoroughly resuspending milk solids containing 2.5% nisin A in 0.05% acetic acid (catalog number N5764; Sigma-Aldrich, St. Louis, MO, USA) and discarding the remaining solids by centrifugation. Micrococcin P1 was purified as previously described (73).

A spot-on-lawn assay was used to obtain the inhibition spectrum of purified vagococcin T. A vagococcin T solution was prepared by mixing fractions with the highest synergy in a 1:1 ratio. Fresh cultures grown overnight were diluted 1:100 in 5 mL of BHI soft agar and poured onto a BHI agar plate. Once the layer solidified, 2 μL of the vagococcin T solution was spotted onto the lawn. The plates were incubated overnight at 30°C, and the inhibition zones were measured.

Propidium iodide assay. The pore-forming mode of action of vagococcin T was investigated using a propidium iodide (PI) method (80–82). A culture of the indicator strain grown overnight was washed twice in phosphate-buffered saline (PBS) and adjusted to an optical density at 600 nm (OD_{600}) of 0.7 with PBS in the wells of a black microtiter plate containing 20 μM PI (final concentration) and vagococcin T. Fluorescence was measured at 5-min intervals for 2 h using a Hidex (Turku, Finland) Sense microplate reader with excitation at 535/20 nm (515 to 555 nm) and emission at 610/20 nm (590 to 630 nm). Each data point is the mean from three biological replicates, and error bars indicate ±1 SD (sample standard deviation).

MALDI-TOF mass spectrometry. MALDI-TOF MS was performed on an ultrafleXtreme mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in reflectron mode. The instrument was externally calibrated with peptide calibration standard II (Bruker Daltonics), and positively charged ions in the range of 1,000 to 6,000 *m/z* were analyzed. The RPC-purified fractions and matrix (α-cyano-4-hydroxycinnamic acid [HCCA]) were mixed in a 1:1 ratio and applied on a Bruker MTP 384 steel target plate (Bruker Daltonics) for analysis.

Scanning electron microscopy. The indicator strain was grown to mid-log phase (OD_{600} of ~0.6) and incubated with vagococcin T (10× MIC) for 2 h at 37°C with gentle shaking. A culture with no bacteriocin added was used as a control. After incubation, cells were harvested by centrifugation (10,000 × *g* for 5 min),

washed twice in PBS, and resuspended in fixing solution (1.25% [wt/vol] glutaraldehyde, 2% [wt/vol] formaldehyde, PBS) for incubation overnight at 4°C. Fixed cells were then washed three times in PBS and allowed to sediment/attach on poly-L-lysine-coated glass coverslips at 4°C for 1 h. Subsequently, attached cells were dehydrated with an increasing ethanol series (30, 50, 70, 90, and 96% [vol/vol]) for 10 min each and finally washed four times in 100% ethanol. Cells were dried by critical-point drying using a CPD 030 critical-point dryer (Bal-Tec, Los Angeles, CA, USA). Coverslips were sputter coated with palladium-gold using a Polaron Range sputter coater (Quorum Technologies, Lewes, UK). Microscopy was performed on an EVO50 EP scanning electron microscope (Zeiss, Oberkochen, Germany) at 20 kV with a probe current of 15 pA.

Mutant analysis. To characterize mutants of *E. faecium* LMG 20705 resistant to vagococcin T, a total of 20 plates were made as described above for the spot-on-lawn assay. However, to avoid sequencing clones of the same mutant, the lawn on each plate was prepared from genetically independent cultures (inoculated with different single colonies). Colonies that were observed at or near the center of the inhibition zone from vagococcin T following incubation overnight were picked.

Colonies from several agar plates were restreaked to obtain pure cultures. Resistance to vagococcin T was confirmed and quantified by determining the bacteriocin activity toward the mutants compared to the wild-type strain. Genomic DNA of mutant strains was isolated with a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions and sent to Novogene Bioinformatics Technology Co., Ltd. (Beijing, China), for sequencing (NovaSeq 150-bp paired end). Reads from the wild type were assembled using SPAdes v3.15.3 to obtain reference contigs. Snippy was used to identify variants by mapping the reads from mutant isolates to the reference contigs using default settings (83).

Accession number(s). The DNA sequence of the vagococcin T gene cluster was submitted to GenBank under accession number OM959625. The whole-genome shotgun project has been deposited in the DDBJ/ENA/GenBank database under accession numbers PRJNA836177 (BioProject) and SAMN28154986 (BioSample).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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Supplementary tables and figures.

Table S1 Antibiotic resistance of *Enterococcus faecium* LMG 20705.

Antibiotic	Gene[a]	Gene product	Accession number)
ampicillin[b]	-	-	-
aminoglycoside	*aac*(6')-Ii	aminoglycoside 6'-N-acetyltransferase	WP_002293989.1
clindamycin[b]	*lnu*B	lincosamide nucleotidyltransferase	WP_002294514.1
erythromycin[b]	*erm*B	rRNA adenine N-6-methyltransferase	WP_001038795.1
kanamycin[b]	*aph*(3')-IIIa	aminoglycoside O-phosphotransferase	WP_001096887.1
pleuromutilin	*eat*A	ABC-F type ribosomal protection protein	WP_002296175.1
spectinomycin	*ant*(9)-Ia	aminoglycoside nucleotidyltransferase	WP_002294509.1
streptogramin A[b,*]	*lsa*E	ABC-F type ribosomal protection protein	WP_002294513.1
streptogramin B[b,*]	*msr*C	ABC-F type ribosomal protection protein	WP_063854349.1
streptomycin[b]	*ant*(6)-Ia	aminoglycoside nucleotidyltransferase	WP_001255866.1
streptothricin	*sat*4	streptothricin N-acetyltransferase	WP_000627290.1
tetracycline[b]	*tet*L	tetracycline efflux MFS transporter	WP_002294500.1
	*tet*M	tetracycline resistance ribosomal protection protein	WP_063856394.1
vancomycin[b]/teicoplanin[b]	*van*A	D-alanine-(R)-lactate ligase	WP_001079845.1
	*van*HA	D-lactate dehydrogenase	WP_001059542.1
	*van*RA	DNA-binding response regulator	WP_001280781.1
	*van*SA	histidine kinase	WP_002305818.1
	*van*XA	D-Ala-D-Ala dipeptidase	WP_000402348.1
	vanYA	D-Ala-D-Ala carboxypeptidase	WP_001812592.1
	vanZA	glycopeptide resistance protein	WP_000516404.1

[a] Found in *E. faecium* LMG 20705 genome with AMRFinderPlus
[b] Tested and confirmed by disc diffusion method according to EUCAST
[*] Quinopristin/dalfopristin resistance

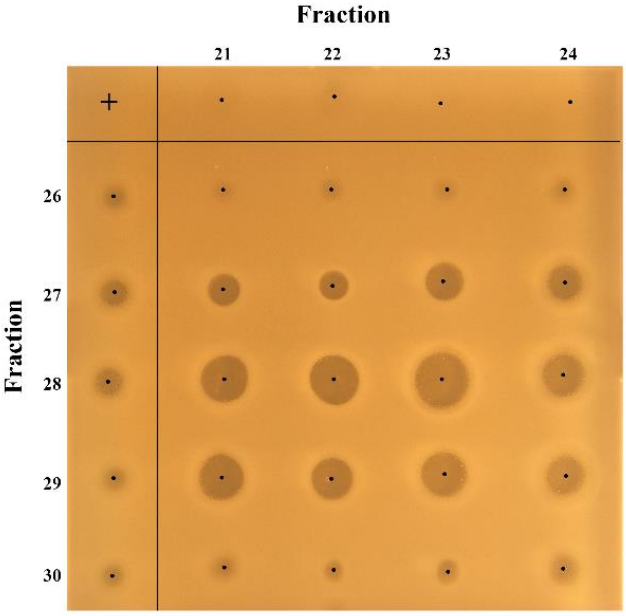

Figure S1 Fractions (1 µl) from reversed-phase chromatography corresponding to the first (21 to 24) and second peak (26 to 30) were spotted individually (to the left and above black bars) and in combination (1:1 v/v ratio) on a lawn of *E. faecium* LMG 20705. Fractions spotted individually produced no or only small/diffuse inhibition zones, some fractions produced large inhibition zones when spotted in combination with the largest zone produced by a combination of fractions 23 and 28.

Paper III

bio-protocol

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Genome-assisted Identification, Purification, and Characterization of Bacteriocins

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Abstract

Bacteriocins are antimicrobial peptides with activity against antibiotic resistant bacterial pathogens. Here, we describe a set of methods aimed at purifying, identifying, and characterizing new bacteriocins. The purification consists of ammonium sulphate precipitation, cation-exchange chromatography, and reversed-phase chromatography. The yield of the bacteriocin is quantified by bacteriocin antimicrobial activity in a microtiter plate assay after each purification step. The mass of the purified bacteriocin is assessed by MALDI TOF MS analysis of the active fractions after reversed-phase chromatography. The mass is compared with the theoretical mass based on genetic information from the whole genome sequencing of the bacteriocin producer strain. Physicochemical characterization is performed by assessing antimicrobial activity following heat and protease treatments. Fluorescent techniques are used to examine the capacity of the bacteriocin to disrupt membrane integrity. Herein a set of protocols for purification and characterization of the bacteriocin nisin Z is used as a typical example in this paper.

Keywords: Bacteriocin, Purification, Peptides, Antimicrobial peptide, Antibiotic resistance, Chromatography, MALDI, MALDI TOF, Biosensor, pHluorin, Propidium iodide, Pore formation

This protocol was validated in: Microbiol Spectr (2021), DOI: 10.1128/Spectrum.00299-21

Background

Antibiotics used to treat bacterial infections are becoming increasingly less efficacious due to the emergence of antibiotic-resistant pathogens (Högberg *et al.*, 2010). In addition, such pathogens are often resistant to two or more antibiotics. As a consequence, first line therapies often involve the administration of either multiple or broad-spectrum antibiotics (Hagihara *et al.*, 2012; Khameneh *et al.*, 2016; Frieri *et al.*, 2017). The overuse of antibiotics is thought to be the primary selection pressure driving the dissemination of resistance (World Health Organization, 2018). In addition, broad-spectrum treatments are known to cause long lasting alterations to the healthy gut microbiota, which is likely to have unforeseen health consequences (Willing *et al.*, 2011). For these reasons, there is a need for alternative antimicrobials, such as bacteriocins, that could be used therapeutically.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria to kill or inhibit other closely related bacteria for nutrients and/or niche competition (Eijsink *et al.*, 2002). Bacteriocins comprise a very diverse group of peptides, from extensively post-translationally modified molecules (class I) to small unmodified peptides (class II) (Alvarez-Sieiro *et al.*, 2016; Acedo *et al.*, 2018). Most bacteriocins produced by Gram-positive bacteria are cationic (pI > 7) and hydrophobic/amphiphilic peptides (Diep and Nes, 2002). They are often of small size ranging from 40 to 70 amino acids, heat-stable, and do not lose activity after storage in organic solvents (2-propanol, acetonitrile, etc.). Most bacteriocins are protease-sensitive (especially unmodified peptides) and have narrow inhibitory spectra, targeting species or genera closely related to the producer (Nissen-Meyer and Nes, 1997), but some have wide inhibitory spectra (Field *et al.*, 2015; Ovchinnikov *et al.*, 2016). Unlike most antibiotics, bacteriocins normally exploit membrane proteins as receptors and disrupt the membrane integrity of sensitive cells upon binding, causing leakage of intracellular solutes and eventually cell death (Nes *et al.*, 2007). Thus, due to different modes of action, bacteriocins are most often equally active against both antibiotic-sensitive pathogens and their antibiotic-resistant counterparts. Bacteriocins have many desirable properties for clinical use, such as high potency, low toxicity, specific inhibition spectrum, and the potential probiotic use of producer organisms (Cotter *et al.*, 2013). However, bacteriocins have not been used in human clinical settings so far. There are a few challenges to the clinical use of bacteriocins, such as their sensitivity towards proteases and low solubility under physiological conditions. Another major factor is likely the insufficient investment spent on the discovery and characterization of new bacteriocins to find candidates more suitable for medical applications.

Here, we describe a set of methods for bacteriocin purification, identification, and characterization. The methods have been used to characterize several bacteriocins in our laboratory (Holo *et al.*, 1991; Ovchinnikov *et al.*, 2016; Desiderato *et al.*, 2021; Goldbeck *et al.*, 2021; Oftedal *et al.*, 2021; Weixler *et al.*, 2022). We believe that this scheme is a good starting point for most bacteriocins and could easily be optimized for special cases, such as multi-peptide bacteriocins, circular bacteriocins, or bacteriocins with relatively low isoelectric points.

Materials and Reagents

1. 0.2 µm syringe filter, Filtropur S, PES (Sarstedt, catalog number: 83.1826.001)
2. Eppendorf Safe-Lock microcentrifuge tubes (Sigma-Aldrich, catalog number: EP0030123611)
3. 1.5 mL microcentrifuge tubes (Eppendorf Safe-Lock, catalog number: EP0030123611)
4. Micro test plate, 96-well, transparent (Sarstedt, catalog number: 82.1581.001)
5. Nalgene™ PPCO Centrifuge Bottles (Thermo Scientific, catalog number: 3141-0250PK)
6. Glass laboratory bottles (VWR, catalog numbers: 215-1514, 215-1515 , 215-1517, 215-1518)
7. 15 mL reaction tubes (Sarstedt, catalog number 62.554.002)
8. Black microtiter plates (Sarstedt, catalog number: 82.1581.120)
9. D-(+)-Glucose monohydrate (Sigma-Aldrich, catalog number: 49159)
10. M17 broth (Oxoid, catalog number: CM0817)
11. Bacteriological agar (Oxoid, catalog number: LP0011T)
12. Ammonium sulphate (Sigma-Aldrich, catalog number: 7783-20-2)
13. $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich, catalog number: 71643)
14. NaH_2PO_4 (Sigma-Aldrich, catalog number: S0751)

15. NaCl (Sigma-Aldrich, catalog number: S7653).
16. Proteinase K (Sigma-Aldrich, catalog number: P2308)
17. 2-Propanol ≥99.0%, GPR RECTAPUR® (VWR Chemicals, catalog number: 20839.366)
18. Hydrochloric acid (Sigma-Aldrich, catalog number: 320331)
19. Trifluoroacetic acid, suitable for HPLC, ≥99.0% (Sigma-Aldrich, catalog number: 302031)
20. Acetonitrile, LiChrosolv® Reag. Ph Eur. (Merck Millipore, catalog number: 1000302500)
21. α-cyano-4-hydroxycinnamic acid (Bruker, catalog number: 8201344)
22. Bruker MTP 384 Target Plate Ground Steel BC (Bruker, catalog number: 8280784)
23. Peptide Calibration Standard II (Bruker LabScape Daltonics, catalog number: 8222570)
24. Propidium Iodide (Fisher Scientific, Invitrogen, catalog number: P1304MP)
25. Brain Heart Infusion Broth (Dehydrated) (Thermo Scientific, Oxoid, catalog number: CM1135B)
26. GenElute™ Bacterial Genomic DNA Kits (Sigma-Aldrich, catalog number: NA2120-1KT)
27. Chloramphenicol (Sigma-Aldrich, catalog number: C0378)
28. KH_2PO_4 (Sigma-Aldrich, catalog number: 795488)
29. $MgSO_4$ (Sigma-Aldrich, catalog number: M7506)
30. $(NH_4)_2SO_4$ (Sigma-Aldrich, catalog number: A4418)
31. Nisin (Sigma-Aldrich, catalog number: N5764-5G)
32. Micrococcin P1 (Cayman Chemical, catalog number: 17093)
33. MOPS (3-Morpholino-propanesulfonic acid) (Sigma-Aldrich, catalog number: 69947)
34. Solution A (see Recipes)
35. Solution B (see Recipes)
36. Sodium phosphate wash buffer (see Recipes)
37. PBS (Phosphate-buffered saline) (see Recipes)
38. HCCA matrix solution (see Recipes)
39. Listeria minimal buffer (LMB) (see Recipes)

Equipment

1. ÄKTA purifier w/ Box-900, pH/C-900, UV-900, P-900, Frac-900 (Pharmacia Biotech)
2. HiPrep SP XL 16/10 (GE Healthcare, catalog number: 28936540)
3. RESOURCE RPC 1 mL (Cytiva, catalog number: 17118101)
4. TS-100 Thermo-Shaker (Biosan, catalog number: BS-010120-AAI)
5. -86°C ULT Chest Freezer (Thermo Scientific, model: 8708)
6. Incubator (Termaks, model: KBP6395LL)
7. Microfuge 16 (Beckman Coulter)
8. Finnpipette™ F2 GLP Kits (Thermo Scientific, catalog number: 4700880)
9. Finnpipette™ F2 Multichannel Pipette (Thermo Scientific, catalog number: 4662030)
10. pH meter (Mettler Toledo® F20)
11. SPECTROstarNano (BMG LABTECH, Germany)
12. High-speed centrifuge Avanti J-26 XP w/ JA-14 rotor (Beckman Coulter)
13. Merck Milli-Q Integral 10 (Merck Millipore)
14. Ultrasonic bath (VWR, model: USC100T)
15. NanoDrop 2000/2000c (Thermo Scientific, catalog number: ND-2000C)
16. Hidex Sense Multi-Mode Microplate Reader
17. Rotary shaking incubator (I26, New Brunswick Scientific)
18. Infinite M200 fluorescence microplate reader (Tecan)
19. IKA RCT magnetic stirrer (IKA, catalog number: 0003810000)

Software

1. Unicorn 5.11 to support ÄKTA (Cytiva, https://www.cytivalifesciences.com/en/us/shop/unicorn-5-11-p-03388)

Procedure

A. Bacteriocin purification

1. Take the vial of bacteriocin producing culture *Lactococcus lactis* LMGT 4215 (nisin Z producer) from deep freezer (-80°C) and place it on ice or in a cold block.
2. Aseptically transfer culture to M17 agar plates supplemented with 0.5% wt/vol glucose (GM17) with sterile loop and streak out to obtain single colonies.
3. Incubate at 30°C for 24 h.
4. Take a single colony from the plate with a sterile loop and transfer it to a sterile culture tube containing 10 mL of the GM17 broth. Leave the tube O/N (overnight) at 30°C without shaking.
5. The following day, inoculate 1 L of GM17 with the culture prepared earlier (1% inoculum, v/v). Leave the bottle for 20–24 h without shaking at 30°C.
   Note: Bacteriocin production usually peaks at the early stationary growth phase, but this can vary from strain to strain. Therefore, this has to be monitored for each new bacteriocin producer if yield is important. If the culture is incubated for a prolonged period of time, bacterial proteases can digest the bacteriocin of interest and reduce or even abolish antimicrobial activity. It is important to optimize the incubation time for each individual bacteriocin producer. Also, bacteriocin production can depend on the growth medium, temperature, and aeration—all those parameters should also be optimized for a particular bacteriocin producer (Telke et al., 2019).
6. Transfer the culture (1 L) of the bacteriocin producer to centrifuge bottles (floor centrifuge) at room temperature. Spin down the cells (10,000 × *g*, 20 min, 4°C). Distribute the supernatant (SN) carefully into a new 1.5–2 L bottle. Discard the cell pellet.
   Note: Continue immediately to the next step to avoid bacterial growth in the supernatant.
7. Take an aliquot of the SN (1–2 mL) to analyze the initial bacteriocin concentration in the SN. Immediately heat the SN aliquot for 5 min at 100°C to sterilize and inactivate proteases. Store at -20°C until use.
8. Add ammonium sulphate dry salt to the cold cell-free SN (at 4°C) to reach 50% (w/v) saturation; mix well using a magnetic stirrer until all salt is dissolved. Leave the SN with ammonium sulphate O/N at 4°C for protein precipitation.
   Notes:
   a. *Use an ammonium sulphate saturation calculator (such as: http://www.encorbio.com/protocols/AM-SO4.htm). Increasing the ammonium sulphate concentration up to 70% saturation can increase yield as more of the bacteriocin will precipitate.*
   b. *Bacteriocins are unstructured in water, meaning that there is no need for careful and gradual addition of ammonium sulphate. In our laboratory, the procedure takes only a few min. After adding the ammonium sulphate, the solution can be left at 4°C for a few days without loss of bacteriocin activity, as proteases are unlikely to be active at high concentrations of ammonium sulphate. However, some bacteriocins lose activity due to oxidation, such as pediocin PA-1 (Fimland et al., 2000); in this case, prolonged storage in ammonium sulphate is not recommended.*
9. Centrifuge the ammonium sulphate solution (12,000 × *g*, 45 min, and 4°C), and carefully discard the SN from the centrifuge bottles to avoid resuspension of the protein pellet because loss of the protein pellet will reduce yield.
10. Gently resuspend all protein pellets in Milli-Q water to a total volume of 150 mL (100–150 mL/L of

starting volume of supernatant). Use a 5 mL pipet to dislodge and resuspend the pellets. Transfer the total volume to a new bottle or beaker. Adjust the pH of the protein solution to 4 by the addition of 1 M HCl.
Note: Reducing the pH ensures that bacteriocin peptides are positively charged, which improves binding to the cation-exchange column.

11. Connect the cation exchange column HiPrep 16/10 SP-XL column to the ÄKTA purifier system equipped with the fraction collector. Set the maximum pressure limit to 0.5 MPa (highest pressure limit for that column) to avoid potentially damaging the column.
12. Wash pump A with Milli-Q water with a pH of 4 and pump B with 1 M NaCl (unbuffered).
13. Equilibrate the column with 5 CV (column volumes; 100 mL) of Milli-Q water adjusted to pH 4.
14. Place pump A inlet into the protein solution and apply it to the column at a flow rate of 1–7 mL/min. Collect the flow-through in a new bottle.
15. Wash the column again with 5 CV of Milli-Q pure water at pH 4 (this step can be omitted).
16. Place pump A inlet in 20 mM phosphate buffer (pH 7) and wash the column with 5 CV (100 mL). Collect the flow-through in a new bottle.
17. Elute the bacteriocin with a linear gradient from 0 M to 1 M NaCl (unbuffered) at a flow rate of 5 mL/min over 20 min. Set the fraction collector to collect 20 fractions of 5 mL each. Each fraction, as well as the initial heat-treated SN, the cation-exchange flow-through, and the "wash" fractions, are checked for antimicrobial activity using the microtiter plate assay (see below). The active fractions eluted with NaCl are pooled for the reversed-phase chromatography (RPC) purification.
Note: To prevent bacteriocin aggregation, it is recommended to reduce the pH of the pooled active fractions to 2 with 1 M HCl. This is especially important if the cation-exchange eluate will be stored for a long time and/or the purified bacteriocin molecules are large (>40 residues) and hydrophobic. The recommended temperature to store bacteriocins is -20°C.
18. RPC purification is performed with a resource RPC column (1 mL) connected to ÄKTA purifier system. First, prepare 200 mL Milli-Q water with 0.1% (v/v) trifluoroacetic acid (TFA; solution A) and 200 mL 2-propanol with 0.1% (v/v) TFA (solution B). Wash pump A and pump B with solution A and B, respectively. Set the maximum pressure limit to 4 MPa (highest pressure limit for that RPC column) to avoid potentially damaging the column. Equilibrate the RPC column with at least 10 mL of solution A.
19. Change pump A inlet from solution A to the pooled active fractions from cation-exchange chromatography (eluate). Apply the eluate to the column at 3–5 mL/min flow rate.
20. Use a linear gradient (0–100%) of solution B at the flow rate of 1.0 mL/min for elution of the bacteriocin.
Note: Normally, bacteriocins are eluted at 25–50% solution B.
21. Set the fraction collector to collect 1 mL per fraction from the RPC column. Collect a total of 30–40 fractions.
22. Take a 10 µL aliquot from each fraction for antimicrobial activity test in a microtiter plate assay (see microtiter plate protocol below).

B. Microtiter plate assay

1. Add 100 µL of GM17 broth medium to the wells A1 to A11 of a microtiter plate. Add 200 µL to well A12 as a control; this well should have no growth.
2. Add 100 µL of the heat-treated supernatant or cation-exchange fractions (flow-through, wash flow-through, elution fraction) to A1 to a total volume of 200 µL.
Notes:
   a. *For reversed-phase fractions, use 10 µL of each reversed-phase fraction plus 90 µL of growth medium, so that the total added volume is 100 µL to each well.*
   b. *If the purified bacteriocin is predicted (BAGEL4, AntiSMASH, see below) to consist of two or more different peptides, the individual peptides can be eluted into different fractions, thereby resulting in low or no antimicrobial activity. In this case, we recommend pooling the fractions corresponding to the peaks and seeing if the antimicrobial activity is restored. A checkboard assay can then be performed to determine which two fractions the peptides are in.*

c. *The growth of mutants resistant to the bacteriocin can supersede the growth of the wild-type strain during incubations longer than 5–6 h; hence, avoid overnight incubations.*

3. Mix the liquid in A1 by pipetting up and down 4–5 times.
4. Take 100 µL from A1 and transfer to A2, and pipet up and down 4–5 times to mix. Move 100 µL from A2 to A3 and so on until A10.
5. After mixing in A10, discard the tip containing 100 µL of liquid.
6. Make 25 times diluted O/N culture of indicator (known to be sensitive towards your bacteriocin, such as *Lactococcus lactis* IL1403 or *Listeria innocua* LMGT 2785 for nisin), *e.g.*, 1 mL O/N culture into 24 mL of BHI broth.
7. Add 100 µL of the diluted culture to well A11. Now there is 200 µL in A11 with no bacteriocin; this is a positive control for normal cell growth.
8. Continue adding 100 µL of diluted indicator culture to A10, then A9, and so on, up to A1 without changing the tip(s). Now there is 200 µL of liquid in all A1–A12. A12 will always be transparent (pure broth), and A11 will become turbid (only bacteria).
   Note: Other fractions can be tested using the rest of the microtiter plate (B1-H1). After application of the test samples into the wells, use a multichannel pipette.
9. Incubate the plate at 30°C for 5–6 h.
10. Measure growth at A_{600} using a spectrophotometer such as the SPECTROstar Nano. One bacteriocin unit (BU) is defined as the amount of bacteriocin that inhibits the growth of the indicator strain by at least 50% in 200 µL culture (*i.e.*, ≤ 50% of the turbidity of the control culture without bacteriocin). The amount of antimicrobial in column 5 of Figure 1 is then 1 BU or 5 BU/mL. The RPC fraction then has an activity of 3,200 BU/mL if 10 µL of the RPC fraction was added to well No. 1 at the beginning.
    Note: Using the bacteriocin purification protocol presented here, we typically achieve 60–70% yield starting from 320 BU/mL in the cell-free supernatant.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,082	0,078	0,077	0,092	0,216	0,587	0,886	0,924	0,883	0,949	0,95	0,059
B	0,082	0,076	0,078	0,088	0,218	0,566	0,937	0,896	0,947	0,943	0,922	0,058
C	0,076	0,075	0,083	0,085	0,218	0,7	0,965	0,957	0,954	0,903	0,914	0,065
D	0,083	0,082	0,086	0,092	0,214	0,599	0,956	0,997	0,936	0,944	1,006	0,057
E	0,085	0,082	0,086	0,091	0,204	0,6	0,829	0,93	0,953	0,93	0,942	0,069
F	0,085	0,086	0,084	0,092	0,182	0,518	0,939	0,923	0,935	0,939	0,946	0,067
G	0,086	0,085	0,085	0,092	0,155	0,497	0,836	0,95	0,903	0,979	0,911	0,058
H	0,088	0,089	0,092	0,109	0,181	0,549	0,763	0,924	0,94	0,908	0,89	0,06
											No bacteriocin	No cells

Figure 1. An example of the microtiter plate assay.
Eight aliquots of nisin Z (10 µL each) from the same RPC fraction were tested for antimicrobial activity against *L. lactis* IL1403. The plate was incubated for 5 h at 30°C before optical density was read at 600 nm. Wells with clear inhibition (more than 50% compared to the OD in wells of column 11) are shown in yellow (columns 1–5), cultures without bacteriocin in brown (positive control; column 11), cultures with no or less than 50% inhibition in white, and GM17 broth control in blue (negative control; column 12).

C. Protease-sensitivity

1. Reconstitute proteinase K in Milli-Q water to 20 mg/mL.
2. Dilute indicator strain *L. lactis* IL1403 (for nisin Z) in GM17 soft agar (0.8% agarose) cooled to 45°C; pour evenly over a GM17 agar plate. Leave the soft-agar to solidify for 3–5 min with the lid partly off.
3. Drop 2–3 µL of the bacteriocin producing O/N culture (or a bacteriocin containing solution). Drop 2 µL of proteinase K solution 4–5 mm near the bacteriocin-producing culture (make sure the drops do not mix). Let the plate dry for 5–10 min before incubation O/N at appropriate temperature. Next day a "crescent moon" shape will appear on the plate if the antimicrobial is susceptible to proteinase K, see

Figure 2.
Note: Circular and highly modified bacteriocins can be resilient to proteinase K.

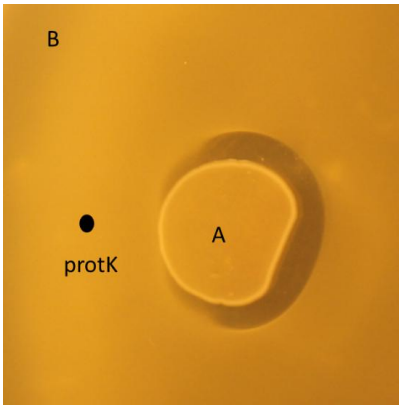

Figure 2. Proteinase K drop (indicated with the black dot) degrades nisin Z produced by *L. lactis* LMGT 4215 (A), making it inactive against indicator culture *L. lactis* IL1403 (B).

D. Heat-stability

1. Spin down the O/N culture of the bacteriocin producer using a centrifuge (10,000 × *g*, 3 min). It is also possible to further sterilize the SN by filtration using a 0.2 µm filter.
2. Take 1 mL of the cell-free SN and distribute it equally into two centrifuge tubes. Leave one tube at room temperature, and place the other in a heating block (or water bath) at 100°C for 5 min. Compare antimicrobial activity in the two tubes (heated and non-heated) using a microtiter plate assay as described above.
   Note: Bacteriocins are heat-stable molecules and do not lose their activity after heating. If the antimicrobial activity is lost after heating, it is most likely to be due to antimicrobial enzymes/proteins (of high molecular weight).

E. MALDI TOF MS

1. Prepare the MALDI matrix solution as described in the recipes section. Thoroughly dissolve the α-cyano-4-hydroxycinnamic acid (HCCA) by vortexing, followed by sonication (5 min).
   Note: Although a number of alternative matrices, such as 2,5-didroxybenzoic acid (DHB), could work well, we find that HCCA is particularly useful for bacteriocin analysis.
2. Prepare the calibration standard by dissolving the Peptide Calibration Standard II in 0.1% TFA according to the manufacturer's instructions. Store the peptide calibration standard as 4–5 µL aliquots at -20°C until use.
   Note: For optimal results, it is recommended to calibrate the mass axis frequently. This is achieved by acquiring spectra from calibration standards covering the useful mass range, which may be obtained from several vendors.
3. To 1–2 µL of RPC purified bacteriocin sample, add an equal volume of matrix solution in, *e.g.*, a 0.2 mL PCR tube, and mix thoroughly by pipetting up and down several times. Apply a small drop (0.5–1 µL) to a spot on the MALDI target plate and let the droplet air dry.
   Note: For best mass accuracy results, always apply your sample next to a calibration spot, which is prepared the same way as the sample spot.
4. Mount the target plate in the target frame, insert the frame into the instrument, and wait for the complete

evacuation of the ion source. Load an appropriate (positive reflectron mode) acquisition method. Set the instrument to an acceleration voltage (ion source 1) of 20 kV, ion source 2 of approximately 18 kV, reflectron voltage 1 and 2 of approximately 21 kV and 11 kV, respectively, and a PIE (delayed extraction) setting of approximately 140 ns. To suppress low mass (mainly matrix) signals, use a deflection setting of 400–600.

Note: The procedure described applies to analysis performed using the Bruker Daltonics Ultraflex and Ultraflextreme MALDI-TOF/TOF instruments. For other systems, adjustments to sample preparation as well as to instrument settings may be required.

5. Position the cursor on the appropriate spot and start firing the laser. Adjust the laser intensity to achieve maximum resolution. Achieve the required peak intensity and signal/noise ratio by accumulating several shots; increasing the laser intensity instead may lead to poor resolution and mass accuracy. Once an acceptable spectrum has been accumulated, calibrate the instrument by assigning the peaks to a list of theoretical monoisotopic m/z values, using a cubic enhanced function. A calibration with <5 ppm error is acceptable; normally, <2 ppm is achieved. Once the calibration spectrum has been accepted, the instrument's mass axis is calibrated, proceed to acquire data from your sample spot(s). A representative example for nisin is shown in Figure 3.

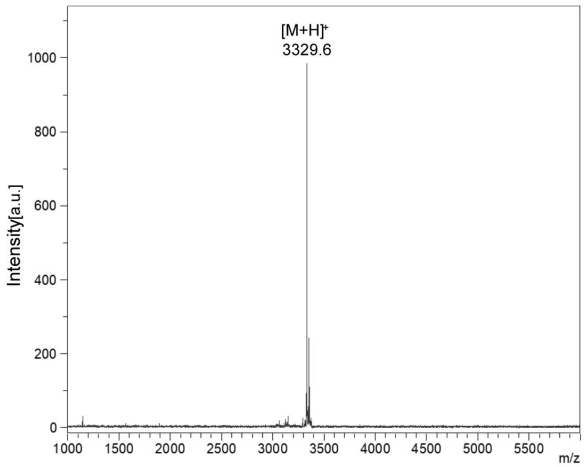

Figure 3. MALDI TOF MS spectrum obtained from the most active RPC fraction.
The mass of 3329.6 m/z corresponds well with the predicted mass for nisin Z (containing one unmodified Ser/Thr).

F. Whole genome sequencing and analysis

1. Prepare genomic DNA from the bacteriocin producer from 1.5 mL of overnight culture using the GenElute Bacterial Genomic DNA kit according to the manufacturer's instructions.
2. Ensure that the sample meets the minimum requirements set by the sequencing laboratory. For microbial genome sequencing by Novogene, the sample concentration should be ≥ 10 ng/μL by Qubit with a minimum volume of 20 μL. The total amount of DNA should be ≥ 200 ng. The DNA should migrate on an agarose gel as a single band at approximately 20–25 kb, and $OD_{260/280}$ should be 1.8–2.0 by NanoDrop.
3. Ship the sample to your sequencing provider (*e.g.*, Novogene) for bacterial whole genome sequencing (100× coverage, paired-end 150 bp).

4. Download the sequencing results using the web interface from your sequencing provider. Two files should be associated with the sample and have a suffix _R1/_1 for forward reads and _R2/_2 for reverse reads.
5. Create an account with https://www.patricbrc.org, which provides free bioinformatic analyses such as assembly.
   Note: Most assembly software is freely distributed and can be executed on your personal computer (such as SPAdes, MEGAHIT, ALLPATHS-LG, IDBA-UD, MIRA, and Velvet). However, these tools are inaccessible to most researchers because they require familiarity with the command-line interface and GNU/Linux (or Windows Subsystem for Linux).
6. In the web interface, go to WORKSPACES and "Genome Groups". Go to "Upload", then "Select Files". Select both sequencing files. Then "Start Upload".
7. Select the first file, then "Edit type" and select "reads" in the drop-down menu. Do this for both files.
8. Go to "Services" then "Assembly". In the "Paired read library" box, select read file 1 as the file named _R1 or _1. For read file 2, select the file named _R2 or _2.
9. In the box "Parameters" select SPAdes under "Assembly strategy", set the output folder to "/home/Assemblies". Give the assembly an output name.
10. Click the right arrow in the "Paired read library" box, then click "Assemble" at the bottom.
11. When the assembly is finished, go to "Workspaces" and select "home". Double-click on the "Assemblies" directory, then on the directory with the assembly name chosen previously.
12. Select the file named *contigs.fasta, then download the file by clicking "DWNLD" in the green bar to the right.
13. Submit the file to http://bagel4.molgenrug.nl and https://antismash.secondarymetabolites.org to identify bacteriocin genes, see Figure 4A.
14. Analyze the identified genes and compare the theoretical monoisotopic mass with that obtained by MALDI TOF MS. The mass can be calculated using a tool such as PeptideMass (https://web.expasy.org/peptide_mass/), see Figure 4B.

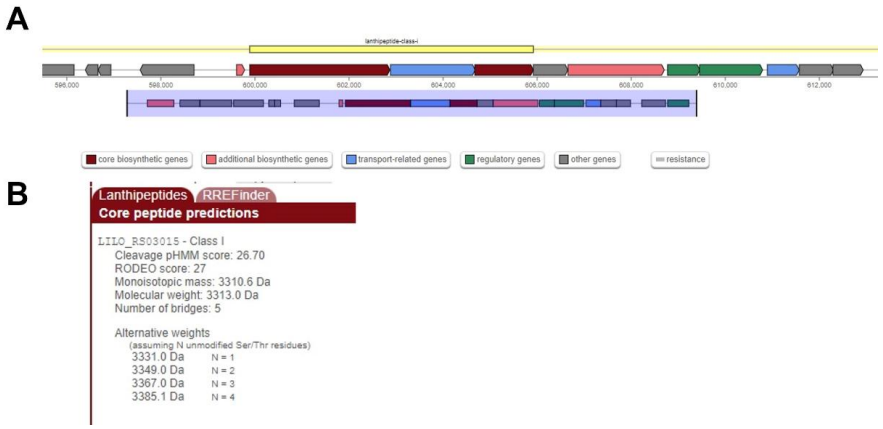

Figure 4. AntiSMASH search result.
AntiSMASH correctly identifies a lanthipeptide cluster in the assembled contigs from the nisin Z producer (A). Theoretical monoisotopic mass of the predicted core peptides is provided in a panel located at the lower right of the AntiSMASH window (B). The mass measured by MALDI TOF MS is correctly predicted in the alternative weights assuming 1 unmodified Ser/Thr (3331.0 Da).

G. Propidium iodide pore formation assay

1. Dilute RPC purified antimicrobial in PBS containing 40 µM propidium iodide to total volume of 100 µL in the well of a black 96-well plate. Include three controls, one containing no antimicrobial, one containing an antimicrobial that does not form pores such as micrococcin P1, and one containing a known pore-former such as commercially obtained nisin A.
2. Prepare 20–50 mL overnight culture of your indicator, such as *L. lactis* IL1403 in GM17. Wash the cells once in PBS and resuspend to an OD_{600} of 1.
3. Add 100 µL of cell suspension to the wells containing diluted antimicrobial and the negative controls.
4. Immediately place the 96-well plate in the Hidex Sense plate reader and measure the fluorescence every 10 min (kinetic) for 3 h with excitation at 535/20 nm (515–555 nm) and emission at 630/40 nm (590–670 nm); see Figure 5 for a representative result and Figure 6 for the principle of the Propidium iodide assay.
   Note: Many fluorometers can produce very large values depending on the settings and dynamic range of the instrument. Here, we are only interested in the difference between samples; relative fluorescence values are obtained by dividing readings from all samples by the same constant (e.g., the first reading from the well containing micrococcin P1, making this value equal to 1).

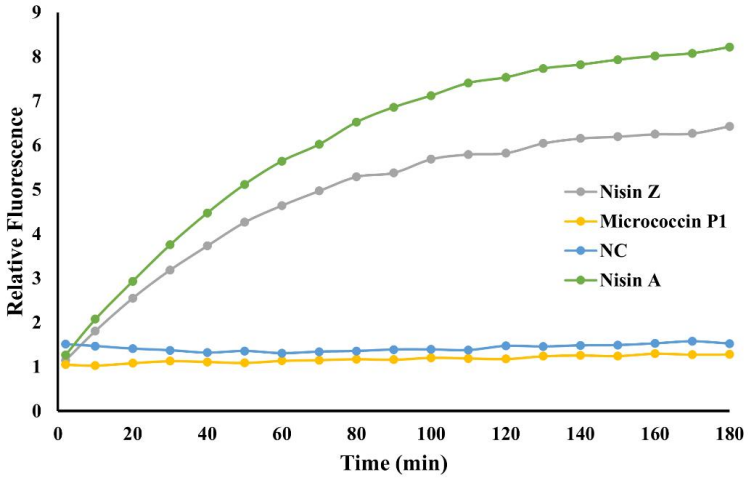

Figure 5. Propidium iodide pore formation assay.
An increase in fluorescence indicative of pore formation is observed for commercial nisin A (green) as well as for the purified fraction of nisin Z (grey). Wells with micrococcin P1 (yellow), which does not form pores but kills cells by inhibiting protein synthesis, or the control with no added antimicrobial (NC; blue), show no increase in fluorescence.

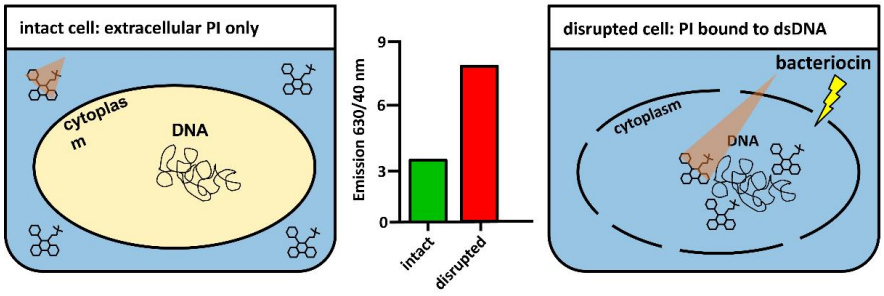

Figure 6. Principle of the propidium iodide-based pore formation assay.
Intact bacterial membranes are impermeable to propidium iodide molecules (left). Membrane disruption allows PI to diffuse into bacteria and interact with DNA, causing an increase in the fluorescence intensity (right). The triangle represents the fluorescence emission from PI in solution (small triangle, left) and when interacting with dsDNA (larger triangle; right).

H. Assessment of pore formation using pHlourin biosensors

1. Inoculate 5–10 mL of BHI containing 10 µg/mL chloramphenicol from a single bacterial colony of the biosensor bacteria, *e.g.*, *Listeria innocua*/pNZ-pHin2^{Lm} (biosafety level 1) or *Listeria monocytogenes*/pNZ-pHin2^{Lm} (BSL 2) (Reich *et al.*, 2022), and incubate overnight at 37°C under shaking conditions (130 rpm).
2. Next morning, harvest the bacteria by centrifugation at 4,500 × *g* for 10 min, wash with an equal volume of PBS, and measure the OD_{600} (typically between 3–4). Centrifuge again and resuspend bacteria at an OD_{600} of 3 in Listeria minimal buffer (LMB) (Crauwels *et al.*, 2018).
3. Prepare your samples in a black 96-well microtiter plate as follows:
   a. For general analysis of activity in multiple samples, distribute 100 µL per sample to the wells of the microtiter plate. Include controls of 100 µL LMB (no pore formation) and 100 µL LMB containing 10 µg/mL of a commercial nisin A preparation (maximum pore formation).
   b. For closer analysis of activity in up to eight samples, fill as many rows of the plate as samples to be analyzed with 100 µL of LMB per well. Add 100 µL of sample to the first well of a row and mix by pipetting. Prepare horizontal dilution rows by transferring 100 µL to the next well using a multichannel pipette, ensuring always to mix well. Repeat until column 11, then discard 100 µL from the wells in column 11. Use wells in column 12 for positive and negative controls as described in step 3a.
   Note: Column 12 is used for both negative and positive controls, e.g., by using wells A12/B12/C12 for negative and D12/E12/F12 for positive controls.
4. Using a multichannel pipette, add 100 µL of LMB-suspended sensor bacteria to all wells. Mix carefully by shuffling the plate on the lab bench.
5. Incubate at RT in the dark for 30 min.
6. Measure fluorescence emission at 520 nm of each well using an infinite M200 multiplate reader (Tecan) with excitation at 400 and 480 nm.
7. In the results file, divide the emission value for excitation at 400 nm by the emission value for excitation at 480 nm. Compare ratio values by plotting, *e.g.*, as bar chart (Figure 7). Pore formation leads to collapse of intracellular pH, leading to a decrease in fluorescence ratio.

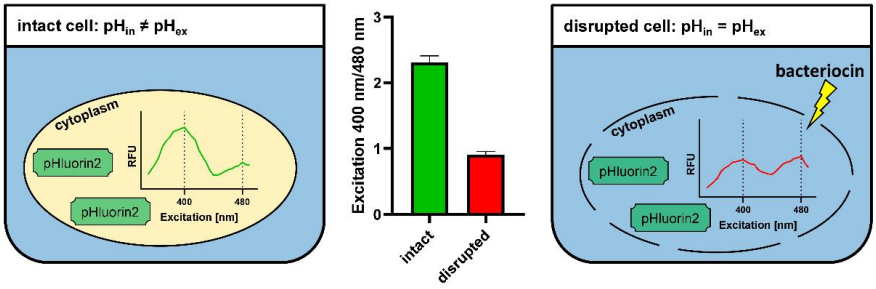

Figure 7. Principle of assessing membrane integrity via pHluorin2 fluorescence.
Biosensor bacteria constitutively express the pH-sensitive fluorescent protein pHluorin2, which shows a bimodal excitation spectrum with maxima at 400 and 480 nm. In intact cells, the ratio of fluorescence intensities at the two excitation peaks is defined by the intracellular pH (left). If pH homoeostasis is disrupted by membrane-damaging compounds, intracellular pH drops to the pH of the assay buffer (pH 6.2), and this leads to a ratiometric change in the fluorescence intensity of pHLuorin2 at the two excitation peaks right). Calculation of fluorescence intensity ratios at the two excitation maxima (400 and 480 nm) allows discrimination between intact and disrupted cells (middle).

Recipes

1. Solution A

Reagent	Final concentration	Amount
Trifluoroacetic acid (99%)	0.1%	0.1 mL
H_2O	n/a	99.1 mL
Total	n/a	100 mL

2. Solution B

Reagent	Final concentration	Amount
2-Propanol (≥99.0%)	n/a	99.9 mL
Trifluoroacetic acid (99%)	0.1%	0.1 mL
Total	n/a	100 mL

3. Sodium phosphate wash buffer

Reagent	Final concentration	Amount
NaH_2PO_4 (1 M)		650 µL
Na_2HPO_4 (0.5 M)		2670 µL
H_2O	n/a	96.68 mL
Total	n/a	100 mL

4. PBS (Phosphate-buffered saline)

Reagent	Final concentration	Amount
NaCl	137 mM	8 g

KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
H_2O	n/a	Up to 1,000 mL
Total	n/a	1,000 mL

5. HCCA matrix solution

Reagent	Final concentration	Amount
HCCA	15 mg/mL	15 mg
TFA (10%)	0.1%	10 µL
Ethanol	50%	500 µL
Acetonitrile	49.9%	490 µL
Total	n/a	1 mL

6. Listeria minimal buffer (LMB)

Reagent	Final concentration	Amount
MOPS	100 mM	2.09 g
KH_2PO_4	4.82 mM	65.6 mg
Na_2HPO_4	11.55 mM	206 mg
$MgSO_4$	1.7 mM	20.5 mg
$(NH4)_2SO_4$	0.6 mg/mL	60 mg
Glucose	55 mM	1.09 g
NaOH (1 M)	NA	to pH 6.5
Total	n/a	100 mL

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Competing interests

The authors declare no competing interests.

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Paper IV

Design of Novel Saposin-like Bacteriocins with Antimicrobial Activity Using a Hybrid Approach

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Abstract

A multitude of approaches will be required to respond to the threat posed by the emergence and spread of antibiotic resistant pathogens. Bacteriocins have recently gained increasing attention as a possible alternative to antibiotics. Bacteriocins utilize a mechanism different from antibiotics, and are therefore equally potent towards antibiotic resistant bacteria as their antibiotic resistant counterparts. A group of saposin-like bacteriocins are believed to act directly on the bacterial membrane. Based on seven saposin-like leaderless bacteriocins, we have constructed a library of hybrid peptides containing all combinations of the N- and C-terminal halves of the peptides. All hybrid peptides were synthesized using *in vitro* protein expression and assayed for antimicrobial activity towards several pathogens. Of the 42 hybrid peptides, antimicrobial activity was confirmed for 11 novel hybrid peptides. Furthermore, several of the hybrid peptides exhibited different antimicrobial spectra and apparent increase in potency compared to the peptides from which they were derived. The activity of the hybrid library provides valuable insights into the design and screening of new active bacteriocin peptides.

Introduction

Antimicrobial resistance (AMR) among bacteria causing infections in humans is increasing. Fewer treatment options are available for infections caused by resistant bacteria, leading to increased morbidity and mortality. An estimated 1.27 million deaths were directly attributable to bacterial antimicrobial resistance (AMR) in 2019 (Murray et al., 2022). A review on AMR published in 2016 estimated that an additional 10 million deaths will be caused by AMR in 2050 if current trends continue (O'Neill, 2014). Additionally, few incentives exist for the development and discovery of new antibiotics (Fischbach & Walsh, 2009). The antibiotic resistance crisis is believed to be exacerbated by excessive and inappropriate use of antibiotics in human medicine and agriculture (Sugden et al., 2016). For this reason, new antimicrobials are sorely needed. One possible addition or supplement to antibiotics are a class of antimicrobials called bacteriocins.

Peptides and proteins with antimicrobial activity are produced by virtually all organisms as part of their innate immune system. Bacteria also produce antimicrobial peptides and proteins, known as bacteriocins, to inhibit each other during competition for common nutrients or niches. Bacteriocins are characterized by a narrow spectrum of activity, often only inhibiting strains closely related to the producer. Furthermore, they often exhibit very potent activity in the pico- to nanomolar range towards their target strains, and are thus in some cases considerably more potent than antibiotics (Hassan et al., 2012). Indeed, bacteriocins have recently received

increasing attention as an alternative or supplement to antibiotics (Cotter et al., 2013; Sang & Blecha, 2008).

Bacteriocins are very diverse, differing in sizes, structures, modes of action, molecular targets, and spectrum of activity. Currently, bacteriocins that are small (< 10 kDa) are typically classified as class I if they are post-translationally modified and class II if they are unmodified (Antoshina et al., 2022). The peptides are further subdivided within both classes based on similarities in biosynthesis, structure, or sequence. However, common to most bacteriocins in both classes is that they are synthesized as precursor peptides with a leader sequence which is removed during or following export to yield the active bacteriocin (Antoshina et al., 2022; Arnison et al., 2012). A notable exception is the class IIc leaderless bacteriocins, which are unmodified peptides synthesized in the cell in their active form (Perez et al., 2018).

To date, class IIc includes over 20 bacteriocins which are either single-, two- or multi-peptide bacteriocins. Among the single-peptide leaderless bacteriocins are the AurA53-like, LsbB-like, and EntL50-like groups of peptides (Perez et al., 2018; Tymoszewska et al., 2021). The LsbB-like bacteriocins depends on the presence of a zinc metalloprotease (RseP/Eep/YvjB) for its antimicrobial activity, and kills target cells via a specific interaction with the metalloprotease (Ovchinnikov et al., 2017; Uzelac et al., 2013). In contrast, all non-LsbB leaderless bacteriocins is generally believed to act directly on the bacterial membrane leading to perturbation and permeation without requiring any specific protein on the cell surface (Perez et al., 2018). Another common feature of the non-LsbB leaderless bacteriocins is that they all seem to have a saposin-like fold (Towle & Vederas, 2017). In fact, it has been suggested to place the leaderless bacteriocins in two classes based on this difference in structure, namely the saposin-like and the LsbB-like (Yi et al., 2022).

As the arsenal of known bacteriocins is growing, discovering new bacteriocin peptides becomes increasingly difficult. The process of sampling, screening, purifying, and identifying bacteriocins is laborious and time-consuming, and often results with the identification of already described bacteriocins. The discovery of new suitable bacteriocins is arguably one of the bottlenecks in developing these peptides for biomedical applications. Recent advances in synthetic DNA combined with *in vitro* protein expression enables the direct synthesis of active bacteriocins (Gabant & Borrero, 2019). In this work we use a hybrid approach to show that existing bacteriocin families are a rich source of new antimicrobial peptides.

Results

Design of hybrid bacteriocins

The saposin-like bacteriocins are remarkably similar in structure, but differ in their activity and spectrum, indicating differences in their mechanism despite the structural similarity (Acedo et al., 2016). A mechanism has been suggested for the circular bacteriocin AS-48 which has a similar saposin-like fold, where protonation of acidic residues are thought to occur at the membrane interface that facilitate membrane insertion (Sánchez-Barrena et al., 2003). Interestingly, many non-LsbB leaderless bacteriocins also contain one or more acidic residues, primarily located at the C-terminal half of the peptides. As such, we hypothesized that these bacteriocins have a bifunctional property where the C-terminal half is involved in binding to certain lipids while the N-terminal half inserts into the membrane. Based on this assumption we constructed a library of genes encoding peptide sequences designated ISP1 through ISP49 containing all combinations of the N- and C-terminal halves of seven leaderless bacteriocins; lactolisterin BU (LliBU; ISP1), mutacin BHT-B (BHT-B; ISP8), aureocin A53 (AurA53; ISP15), K411 (ISP22), lacticin Q (LacQ; ISP29), epidermicin NI01 (EpiNI01; ISP36), and salivaricin C (SalC; ISP43) (Table S1). The resulting library is presented in Table 1 and encode 49 peptide sequences, of which 42 are novel hybrid peptides. The genes encoding the peptide sequences from which the hybrids were derived were included as a comparison and control.

Table 1. Overview of all bacteriocins and hybrid peptides synthesized *in vitro*. The isoelectric point (pI) and hydrophobicity was calculated using the Peptides (v2.4.4) library for R v4.1.1 (Osorio et al., 2015).

Hybrid	ISP	Sequence	pI	Hydrophobicity*
LliBU	ISP1	MWGRILGTVAKYGPKAVSWAWQHKWFLLNMGDLAFRYIQRIWG	11.1	-0.0209
LliBU-BHTB	ISP2	MWGRILGTVAKYGPKAVSWAWQHKWFLLSLGEAVFDYIRSIWGG	10.3	0.1682
LliBU-AurA53	ISP3	MWGRILGTVAKYGPKAVSWAWQHKGKVLEWLNVGPTLEWVWQKLKKIAGL	11.0	-0.1020
LliBU-K411	ISP4	MWGRILGTVAKYGPKAVSWAWQHKGKILEWLNIGMAVDWIVEQVRKIVGA	10.7	0.1580
LliBU-LacQ	ISP5	MWGRILGTVAKYGPKAVSWAWQHKGKILDWLNAGQAIDWVVSKIKQILGIK	11.0	0.0353
LliBU-EpiNI01	ISP6	MWGRILGTVAKYGPKAVSWAWQHKGTILKWINAGQSFEWIYKQIKKLWA	11.0	-0.2265
LliBU-SalC	ISP7	MWGRILGTVAKYGPKAVSWAWQHKIMQLIGEGWTVNQIEKMFK	10.7	-0.1581
BHTB	ISP8	MWGRILAFVAKYGTKAVQWAWKNKWFLLSLGEAVFDYIRSIWGG	10.6	0.2409
BHTB-LliBU	ISP9	MWGRILAFVAKYGTKAVQWAWKNKWFLLNMGDLAFRYIQRIWG	11.3	0.0535
BHTB-AurA53	ISP10	MWGRILAFVAKYGTKAVQWAWKNKGKVLEWLNVGPTLEWVWQKLKKIAGL	11.0	-0.0380
BHTB-K411	ISP11	MWGRILAFVAKYGTKAVQWAWKNKGKILEWLNIGMAVDWIVEQVRKIVGA	10.8	0.2220
BHTB-LacQ	ISP12	MWGRILAFVAKYGTKAVQWAWKNKGKILDWLNAGQAIDWVVSKIKQILGIK	11.0	0.0980
BHTB-EpiNI01	ISP13	MWGRILAFVAKYGTKAVQWAWKNKGTILKWINAGQSFEWIYKQIKKLWA	11.1	-0.1612
BHTB-SalC	ISP14	MWGRILAFVAKYGTKAVQWAWKNKIMQLIGEGWTVNQIEKMFK	10.8	-0.0837
AurA53	ISP15	MSWLNFLKYIAKYGKKAVSAAWKYKGKVLEWLNVGPTLEWVWQKLKKIAGL	10.7	-0.0843
AurA53-LliBU	ISP16	MSWLNFLKYIAKYGKKAVSAAWKYKWFLLNMGDLAFRYIQRIWG	10.7	-0.0023
AurA53-BHTB	ISP17	MSWLNFLKYIAKYGKKAVSAAWKYKWFLLSLGEAVFDYIRSIWGG	10.3	0.1822
AurA53-K411	ISP18	MSWLNFLKYIAKYGKKAVSAAWKYKGKILEWLNIGMAVDWIVEQVRKIVGA	10.5	0.1706
AurA53-LacQ	ISP19	MSWLNFLKYIAKYGKKAVSAAWKYKGKILDWLNAGQAIDWVVSKIKQILGIK	10.7	0.0500
AurA53-EpiNI01	ISP20	MSWLNFLKYIAKYGKKAVSAAWKYKGTILKWINAGQSFEWIYKQIKKLWA	10.8	-0.2060
AurA53-SalC	ISP21	MSWLNFLKYIAKYGKKAVSAAWKYKIMQLIGEGWTVNQIEKMFK	10.5	-0.1364
K411	ISP22	MAGFLKVVKAVAKYGSKAVKWCWDNKGKILEWLNIGMAVDWIVEQVRKIVGA	10.4	0.2885
K411-LliBU	ISP23	MAGFLKVVKAVAKYGSKAVKWCWDNKWFLLNMGDLAFRYIQRIWG	10.6	0.1378
K411-BHTB	ISP24	MAGFLKVVKAVAKYGSKAVKWCWDNKWFLLSLGEAVFDYIRSIWGG	10.1	0.3152
K411-AurA53	ISP25	MAGFLKVVKAVAKYGSKAVKWCWDNKGKVLEWLNVGPTLEWVWQKLKKIAGL	10.7	0.0385
K411-LacQ	ISP26	MAGFLKVVKAVAKYGSKAVKWCWDNKGKILDWLNAGQAIDWVVSKIKQILGIK	10.7	0.1679
K411-EpiNI01	ISP27	MAGFLKVVKAVAKYGSKAVKWCWDNKGTILKWINAGQSFEWIYKQIKKLWA	10.7	-0.0784
K411-SalC	ISP28	MAGFLKVVKAVAKYGSKAVKWCWDNKIMQLIGEGWTVNQIEKMFK	10.4	0.0067
LacQ	ISP29	MAGFLKVVQLLAKYGSKAVQWAWANKGKILDWLNAGQAIDWVVSKIKQILGIK	10.8	0.3000
LacQ-LliBU	ISP30	MAGFLKVVQLLAKYGSKAVQWAWANKWFLLNMGDLAFRYIQRIWG	10.9	0.2933
LacQ-BHTB	ISP31	MAGFLKVVQLLAKYGSKAVQWAWANKWFLLSLGEAVFDYIRSIWGG	10.2	0.4674
LacQ-AurA53	ISP32	MAGFLKVVQLLAKYGSKAVQWAWANKGKVLEWLNVGPTLEWVWQKLKKIAGL	10.8	0.1731
LacQ-K411	ISP33	MAGFLKVVQLLAKYGSKAVQWAWANKGKILEWLNIGMAVDWIVEQVRKIVGA	10.6	0.4231
LacQ-EpiNI01	ISP34	MAGFLKVVQLLAKYGSKAVQWAWANKGTILKWINAGQSFEWIYKQIKKLWA	10.9	0.0588
LacQ-SalC	ISP35	MAGFLKVVQLLAKYGSKAVQWAWANKIMQLIGEGWTVNQIEKMFK	10.6	0.1622
EpiNI01	ISP36	MAAFMKLIQFLATKGQKYVSLAWKHKGTILKWINAGQSFEWIYKQIKKLWA	10.9	-0.0196
EpiNI01-LliBU	ISP37	MAAFMKLIQFLATKGQKYVSLAWKHKWFLLNMGDLAFRYIQRIWG	11.0	0.2044
EpiNI01-BHTB	ISP38	MAAFMKLIQFLATKGQKYVSLAWKHKWFLLSLGEAVFDYIRSIWGG	10.5	0.3804
EpiNI01-AurA53	ISP39	MAAFMKLIQFLATKGQKYVSLAWKHKGKVLEWLNVGPTLEWVWQKLKKIAGL	10.9	0.0962
EpiNI01-K411	ISP40	MAAFMKLIQFLATKGQKYVSLAWKHKGKILEWLNIGMAVDWIVEQVRKIVGA	10.7	0.3462
EpiNI01-LacQ	ISP41	MAAFMKLIQFLATKGQKYVSLAWKHKGKILDWLNAGQAIDWVVSKIKQILGIK	10.9	0.2245
EpiNI01-SalC	ISP42	MAAFMKLIQFLATKGQKYVSLAWKHKIMQLIGEGWTVNQIEKMFK	10.7	0.0733
SalC	ISP43	MSALAKLIAKFGYKKIMQLIGEGWTVNQIEKMFK	10.6	0.1324
SalC-LliBU	ISP44	MSALAKLIAKFGYKKWFLLNMGDLAFRYIQRIWG	10.9	0.3059
SalC-BHTB	ISP45	MSALAKLIAKFGYKKWFLLSLGEAVFDYIRSIWGG	10.2	0.5343
SalC-AurA53	ISP46	MSALAKLIAKFGYKKGKVLEWLNVGPTLEWVWQKLKKIAGL	10.8	0.1512
SalC-K411	ISP47	MSALAKLIAKFGYKKGKILEWLNIGMAVDWIVEQVRKIVGA	10.6	0.4683
SalC-LacQ	ISP48	MSALAKLIAKFGYKKGKILDWLNAGQAIDWVVSKIKQILGIK	10.8	0.3119
SalC-EpiNI01	ISP49	MSALAKLIAKFGYKKGTILKWINAGQSFEWIYKQIKKLWA	10.9	0.0050

* Kyte-Doolittle scale

In vitro synthesis and antmicrobial activity screening of novel hybrids

Genes encoding all the peptides in the library, including the native peptides, were used as a template for *in vitro* protein expression using PURExpress® *In Vitro* Protein Synthesis Kit (New England Biolabs). The resulting products from the *in vitro* synthesis were directly assayed for antimicrobial activity. Using a spot-on-lawn assay, each reaction was tested for antimicrobial activity against a panel of eight indicator bacteria, including strains of *E. faecium*, *L. monocytogenes*, *S. aureus*, *S. dysgalactiae* and *S. haemolytics* isolated from a human or animal infection (Figure 1).

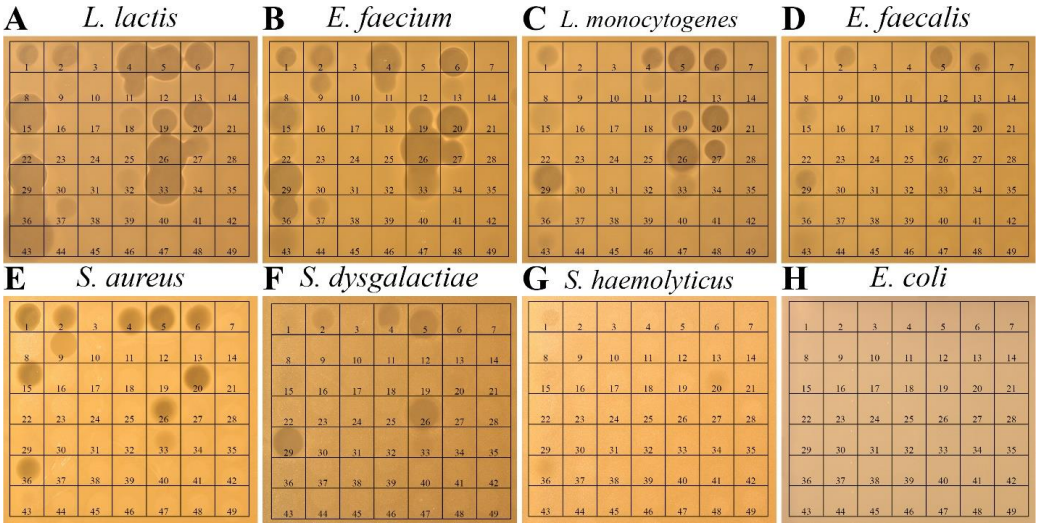

Figure 1. Spot-on-lawn assay assessing antimicrobial activity of the eight leaderless bacteriocins (first column) and hybrid peptides synthesized in vitro. Reaction mixtures of each ISP were spotted inside the corresponding squares (5 µl). Indicators used are (A) *L. lactis* IL1403, (B) *E. faecium* LMGT 3104, (C) *L. monocytogenes* LMGT 2653, (D) *E. faecalis* LMGT 2333, (E) *S. aureus* ATCC 14458, (F) *S. dysgalactiae* LMGT 3890, (G) *S. haemolyticus* LMGT 4071, and (H) *E. coli* DH5α.

Of the seven native bacteriocins tested, six of them displayed activity against at least one indicator. Of all hybrids peptides tested, 11 (11/42; 26%) of them showed activity towards at least one indicator (Table 2). Large zones of inhibition were produced by ISP4-6, ISP20 and ISP26 towards several of the pathogenic strains. Particularly active was ISP26 which inhibited all strains except for *S. haemolyticus* and *E. coli*. ISP4 displayed good activity against *L. lactis*, *E. faecium*, *L. monocytogenes* and *S. aureus*, but was inactive against *E. faecalis*. Similarly, ISP5 was inactive towards *E. faecium* but displayed good activity towards the other strains, except for *S. haemolyticus* and *E. coli*. Four of the hybrids with the N-terminal half from LliBU (4/7;

~60%) showed good activity towards several indicators, while only one hybrid with the C-terminal half from LliBU (ISP37) showed weak activity.

Table 2 Overview of hybrid bacteriocin peptides exhibiting inhibition of at least one of the indicators. Clear zone of inhibition; +, diffuse zone of inbition; (+), no inhibition; -.

Hybrid	ISP	pI	Hyd.*	Activity							
				Lla	Efm	Lmo	Efs	Sau	Sdys	Shae	Ecol
LliBU-BHTB	ISP2	10.3	0.1682	+	+	-	+	+	+	-	-
LliBU-K411	ISP4	10.7	0.1580	+	+	+	-	+	+	-	-
LliBU-LacQ	ISP5	11.0	0.0353	+	-	+	+	+	+	-	-
LliBU-EpiNI01	ISP6	11.0	-0.2265	+	+	+	+	+	-	-	-
BHTB-K411	ISP11	10.8	0.2220	+	+	(+)	-	-	-	-	-
AurA53-LacQ	ISP19	10.7	0.0500	+	+	+	-	-	-	-	-
AurA53-EpiNI01	ISP20	10.8	-0.2060	+	+	+	(+)	+	-	-	-
K411-LacQ	ISP26	10.7	0.1679	+	+	+	(+)	+	+	-	-
K411-EpiNI01	ISP27	10.7	-0.0784	+	+	+	(+)	-	-	-	-
LacQ-K411	ISP33	10.6	0.4231	+	+	(+)	(+)	(+)	(+)	-	-
EpiNI01-LliBU	ISP37	11.0	0.2044	(+)	(+)	-	-	-	-	-	-

* Hydrophobicity (Kyte-Doolittle scale)

Heterologous expression and purification of hybrid peptides

To further characterize and assess the therapeutic potential of these hybrid bacteriocins, we sought to establish a bacterial production scheme that would allow us to obtain larger quantities of the hybrid peptides. To do this, *E. coli* BL21(DE3) were transformed with each pET-3a plasmid containing all genes in the library. Initially, an attempt was made to see if antimicrobial activity could be recovered from culture lysates of transformants encoding all the active hybrids (Table 2). However, growth of clones was severely affected both with and without IPTG, indicating a toxic effect of the peptide on *E. coli*. Despite trying various inducer concentrations of IPTG (0.05 to 3 mM) and harvesting time points post induction (0.5-24 h), no activity could be recovered.

Maltose-binding protein (MBP) is known to solubilize fused proteins and would be expected to detoxify bacteriocin peptides. To test this, we fused ISP26 and ISP29 to the C-terminal end of MBP in pMAL-c6T with an in-frame TEV cleavage tag. The TEV cleavage site is reported to be tolerant to a methionine in the P1' position (Kapust et al., 2002). For this reason, the TEV cleavage tag was fused to ISP26 such that cleavage would leave no additional N-terminal residues that could interfere with antimicrobial activity. Interestingly, clones harboring this plasmid also exhibited severely attenuated growth only reaching an OD_{600} ~0.2-0.3 (compared to > 2 for the control) even without the addition of IPTG. This was observed for all *E. coli* strains tested; DH5α, BL21(DE3) pLysS, and C41(DE3). Cultures would occasionally reach a density comparable to controls, however, these cultures were found to be dominated by disruption

mutants of the construct (revealed by Sanger sequencing of the plasmids), suggesting that the ISP26 and ISP29 peptides are toxic in these fusion constructs in *E. coli*.

The native producers of all bacteriocins in the library are Gram-positive species. Therefore, the NICE expression system established in the Gram-positive *L. lactis* was tried next; the MBP-ISP26 recombinant fusion protein was cloned into pNZ8037 downstream of the nisin-inducible promoter (P_{nis}) and transformed into *L. lactis* NZ9000. The resulting strain exhibited somewhat reduced growth upon nisin induction (10 ng/ml), however, the growth rate was only reduced approximately two-fold. The fusion protein was successfully purified from the lysate of this strain (see Figure S1). However, subsequent cleavage of the fusion protein using TEV protease did not yield active bacteriocin peptide, nor was any cleavage of the fusion protein apparent by SDS-PAGE. We could not demonstrate cleavage of the fusion protein under any of the conditions that we attempted. This could suggest that the cleavage site is buried in the protein core because of the hydrophobic character of the peptide. However, attempts at performing the TEV cleavage in the presence of guanidine HCl (3 M), urea (2 M) and SDS (0.5%) also failed. We also tested the activity of the purified fusion against *L. lactis* IL1403, however, no antimicrobial activity could be observed.

Discussion

The construction of the hybrid bacteriocins presented in this work revealed new active new-to-nature bacteriocins with increased inhibition spectrum compared to their native counterparts. The constructed hybrid bacteriocins were shown to inhibit the growth of WHO priority pathogens *S. aureus* and *E. faecium*, and the relevant pathogens *E. faecalis* and *L. monocytogenes*.

To design the library of genes encoding hybrid bacteriocins in this work, we hypothesized that the saposin-like bacteriocins are “bifunctional”, where the N- and C-terminal parts of the peptides serve different functions. This idea is derived from a proposed mechanism of action for the circular bacteriocin AS-48, and structure and sequence similarities shared between this bacteriocin and other saposin-like bacteriocin (Acedo et al., 2016; Sánchez-Barrena et al., 2003; Towle & Vederas, 2017). In the proposed mechanism for AS-48, protonation of four glutamic acid side chains are believed to occur in the acidic environment of the membrane interface, resulting in the transition to a membrane-bound dimeric form. The protonated glutamic acids of AS-48 recognize and associate with the phosphate moiety of a phospholipid, which was demonstrated by crystallography (Sánchez-Barrena et al., 2003). It is not known if the non-LsbB leaderless bacteriocins form dimers, however, many saposin-like peptides (SAPLIPs) form dimers in their active form. In fact, the transition to the dimeric form for many SAPLIPs is

believed to occur upon interacting with the membrane (Towle & Vederas, 2017). If a similar mechanism is also employed by these bacteriocin, their activity and inhibition spectrum may depend on the presence or abundance of certain phospholipids that will vary between species. Although our hybrid approach successfully resulted in hybrids with improved activites, we could not assign any obvious or distinct role of the N- and C terminal halves of the saposin-like peptides. Understanding the differences in activity between the hybrids will a need more thorough investigation.

Our data shows that *in vitro* protein synthesis is a well-suited tool to screen for active antimicrobials. Nevertheless, there are numerous challenges with *in vitro* synthesis of bacteriocin-like peptides, which practically limits this method to small-scale screening. For example, we have experienced that antimicrobial activity from reactions is lost upon storage/incubation past 24 hours, presumably due to aggregation and/or precipitation (Oftedal et al., 2021). And we have not been successful at purifying the peptides from the reaction mixture. Furthermore, *in vitro* synthesis of bacteriocins sometimes fail, which was also evident in our study. For example, the native bacteriocin BHT-B was included as a control in the screen and shown to be active towards *L. lactis* (Hyink et al., 2005). However, no inhibition zone was produced by the *in vitro* expression in our screen. It is therefore likely that more of the hybrid peptides are active than shown in the screen (Figure 1) due to lack of proper production. The variation in synthesis efficiency also precludes the direct comparison of potency between the peptides. Our results suggest that several of the hybrid peptides have higher potency than the peptides from which they were derived (e.g. ISP4-6 has larger zone than ISP1 towards *L. lactis* in Fig. 1), however, we cannot exclude that this is just a result of different synthesis efficiency in the *in vitro* reactions.

Additionally, due to the length (50-53 aa) and high hydrophobicity of the peptides, commercial peptide synthesis is difficult and costly. For this reason, we sought to produce the hybrid bacteriocins in *E. coli,* the only species insensitive to all peptides tested. However, all our attempts at expressing and purifying these hybrid peptides in *E. coli* were unsuccessful. We could not determine why expression failed in *E. coli*. A failure to express a similar protein fusion in *E. coli* was recently reported by Malesevic et al. (2023), in this work the authors tried to express a fusion of LliBU (ISP1) to MBP (Malesevic et al., 2023). LliBU is a peptide of similar physicochemical properties as ISP29. The authors speculated that the codon usage of this gene has a fitness cost to *E. coli*, as their gene was amplified from *Lactococcus lactis* BGBU1-4. However, our ISP29 gene sequence was codon-optimized for *E. coli*, which suggests that other factors are preventing expression of these fusions in *E. coli*. It could be speculated that these peptides are active when delivered in the cytoplasm of *E. coli* (e.g, inner membrane

permeabilization), and that the lack of activity in our screen is just due to the outer membrane barrier. More work is needed to understand the intracellular toxicity of these peptides in *E. coli*.

In this work we show that previously characterized leaderless bacteriocins can serve as scaffolds for the construction of new-to-nature antimicrobials with improved properties. Additionally, screening new sequences for antimicrobial activity can provide invaluable insight into antimicrobial determinants. Very little is known about the mechanism of most bacteriocins and the factors that determine their potency and spectrum. A better understanding of the features shared between active peptides versus inactive - and those active towards certain species and not others, can allow us to rationally design new peptides targeting high priority pathogens. However, more research is needed to fully characterize these peptides and to assess their therapeutic potential - and to find cost-effective strategies for their production.

Materials and Methods

Strains and growth conditions

All strains used in this study are listed in Table 3. *Lactococcus lactis* was grown at 30 °C in M17 broth (Oxoid) supplemented with 0.4% glucose (GM17), and *E. coli* was grown in LB at 37 °C (180 rpm). All remaining strains were grown in BHI (brain heart infusion; Oxoid) at 37 °C without shaking.

Table 3. Indicator strains used for assessing activity and inhibition spectrum of in vitro synthesized peptides.

Indicator strain	Reference
Lactococcus lactis IL1403	(Chopin et al., 1984)
Enterococcus faecium LMG 20705*	(Rosenbergová et al., 2022)
Listeria monocytogenes LMGT 2653*	Lab collection (LMGT), Norway
Streptococcus dysgalactiae LMGT 3890*	Lab collection (LMGT), Norway
Enterococcus faecalis LMGT 2333	Lab collection (LMGT), Norway
Staphylococcus aureus ATCC 14458*	ATCC
Staphylococcus haemolyticus LMGT 4071*	(Kranjec et al., 2021)
Escherichia coli DH5α	Invitrogen (Cat. No. 18265-017)

Laboratory of Microbial Gene Technology (LMGT), Norwegian University of Life Sciences, Ås, Norway.
* Pathogenic isolates

In Vitro Protein Expression and Antimicrobial Assay

Bacteriocin peptide sequences were reverse translated, and codon optimized for *E. coli* K12 using GENEius (Eurofins Genomics, Germany). All genes were synthesized by Pepmic Co. Ltd

(Suzhou, China) and supplied in pET-3a. Plasmids were solubilized to 250 ng/µl in Milli-Q water and used directly as templates for in vitro protein synthesis using PURExpress® *In Vitro* Protein Synthesis Kit (New England Biolabs). Reactions of 50 µl using 500 ng of template per reaction were assembled according to the protocol provided by the manufacturer in a 96-well plate. The 96-well plate was sealed using heat-sealing film and incubated at 30 °C for 4 hours with vigorous shaking at 1200 rpm using a microplate shaker (PMS-1000i, Grant-Bio, Grant Instruments Ltd., Shepreth, UK). All reactions were immediately assayed for antimicrobial activity using a spot-on-lawn assay. Briefly, an overnight culture of the indicator strain was diluted 50-fold in growth medium (see above) containing 0.8% agar and poured over an agar plate (10x10 cm, square). After solidification, 5 µl of each reaction mixture was spotted onto the plate and allowed to dry. All plates were incubated at 30 °C overnight for the appearance of inhibition zones.

Cloning

The gene encoding ISP26 was amplified from pET-3a using the primers HiFi_ISP26_F and HiFI_ISP26_R (Table S3). The plasmid pMAL-c6T (0.5 µg) was digested for 1 hour at 37°C with SbfI-HF and AlwNI (New England BioLabs). Both were gel-purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel), then combined in a molar ratio of 1:10 (vector:insert) and assembled using a GeneArt ™ Gibson Assembly® HiFi master mix (Thermo Fisher Scientific) according to the manufacturer's instructions. The Gibson assembly mixture was used to transform *E. coli* DH5α. The plasmid was isolated using EZNA Plasmid DNA Mini Kit I (Omega Bio-Tek). The gene fusion was amplified from the plasmid (or from pMAL-c6T) using the primers ISP_BamHI_F and ISP_XhoI_R (Table S3). The PCR product and plasmid pNZ8037 was digested with FastDigest BamHI and XhoI (Thermo Fisher Scientific), then ligated at 16°C overnight in 3:1 ratio (insert:vector) using T4 DNA ligase (New England BioLabs). After deactivation at 65°C for 10 minutes, the ligation mixture (5 µl) was electroporated into *L. lactis* NZ9000. Resulting in two strains harboring pNZ8037-*malE-ISP26* (MBP-ISP26) or pNZ8037-*malE* (MBP).

Table 4. Oligonucleotides.

Primer	**Sequence (3'-5')**
HiFi_ISP26_F	AGAACCTGTACTTCCAGATGGCCGGTTTTCTGAAAGTG
HiFI_ISP26_R	AGCTTATTTAATTACCTGCATTATTTGATCCCTAAAATCTGC
ISP_BamHI_F	AAAGGATCCGTTTAGGTGTTTTCACGAGC
ISP_XhoI_R	AAACTCGAGACGAAAGGCCCAGTCTTTCG

Purification

One liter of GM17 (M17 supplemented with 0.4% glucose; Oxoid) containing 10 µg/ml chloramphenicol was inoculated with 10 ml of culture and incubated at 30°C. When the OD_{600} reached approximately 0.5, nisin (N5764; Sigma) was added to 10 ng/ml. Cultures were incubated for another 2 hours after induction, before being harvested by centrifugation (4000g, 30 min, 4°C). Cells were resuspended in 25 ml of column buffer (CB) (NEBExpress® MBP Fusion and Purification System manual; New England BioLabs) and lysed by three passes through a French pressure cell (Aminco; FA-073) at 15 000 PSI. Intact cells and cell debris were removed by centrifugation (20000g, 40 min, 4°C). Gravity flow columns were prepared with 2 ml of amylose resin (E8021; New England BioLabs) that was washed with 10 ml of CB prior to use. The clarified lysate was applied to the column, and the resin was washed with 10 ml of CB. The sample was eluted with 10 ml of CB (containing 10 mM maltose) in 1 ml fractions. Fractions 2-3 were pooled and concentrated to 100 µl using an Amicon Ultracel-30K centrifugal filter unit (UFC5030; Millipore). Samples were analyzed by SDS-PAGE using Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad).

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Supplementary Materials

Table S1. Bacteriocins used to create the library of hybrid peptides. Each bacteriocin was split at the position indicated by a space resulting in an N- and C- terminal part, both parts were combined in all combinations to produce a library of hybrid peptides shown in Table S2.

	ISP	N-terminal part	C-terminal part	Reference
LliBU*	ISP1	MWGRILGTVAKYGPKAVSWAWQHK	WFLLNMGDLAFRYIQRIWG	Lozo et al. (2017)
BHTB	ISP8	MWGRILAFVAKYGTKAVQWAWKNK	WFLLSLGEAVFDYIRSIWGG	Hyink et al. (2005)
AurA53	ISP15	MSWLNFLKYIAKYGKKAVSAAWKYK	GKVLEWLNVGPTLEWVWQKLKKIAGL	Netz et al. (2002)
K411	ISP22	MAGFLKVVKAVAKYGSKAVKWCWDNK	GKILEWLNIGMAVDWIVEQVRKIVGA	Tymoszewska et al. (2021)
LacQ	ISP29	MAGFLKVVQLLAKYGSKAVQWAWANK	GKILDWLNAGQAIDWVVSKIKQILGIK	Fujita et al. (2007)
EpiNI01	ISP36	MAAFMKLIQFLATKGQKYVSLAWKHK	GTILKWINAGQSFEWIYKQIKKLWA	Sandiford and Upton et al. (2012)
SalC	ISP43	MSALAKLIAKFGYKK	IMQLIGEGWTVNQIEKMFK	Tymoszewska et al. (2021)

* The sequence for LliBU used in this study differs from the published sequence in two positions (E26F and I28L).

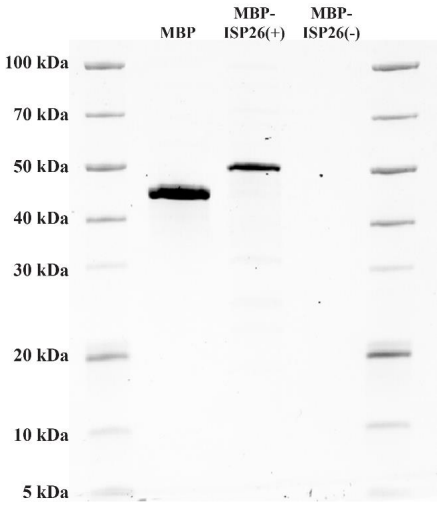

Figure S1. SDS-PAGE of purified MBP and MBP-ISP26 fusions from *L. lactis* NZ9000. Lane MBP: MBP was purified from a control strain expressing the unchanged *malE* gene supplied in the pMAL-c6T vector (calculated mass of 45.5 kDa). Lane MBP-ISP26(+): Purification of the MBP-ISP26 fusion protein (49.6 kDa), purified from a culture induced (+) with 10 ng/ml nisin. Lane MBP-ISP26(-): Purification of MBP-ISP26 from an uninduced (-) culture.

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Paper V

JBC RESEARCH ARTICLE

The extracellular domain of site-2-metalloprotease RseP is important for sensitivity to bacteriocin EntK1

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Enterocin K1 (EntK1), a bacteriocin that is highly potent against vancomycin-resistant enterococci, depends on binding to an intramembrane protease of the site-2 protease family, RseP, for its antimicrobial activity. RseP is highly conserved in both EntK1-sensitive and EntK1-insensitive bacteria, and the molecular mechanisms underlying the interaction between RseP and EntK1 and bacteriocin sensitivity are unknown. Here, we describe a mutational study of RseP from EntK1-sensitive *Enterococcus faecium* to identify regions of RseP involved in bacteriocin binding and activity. Mutational effects were assessed by studying EntK1 sensitivity and binding with strains of naturally EntK1-insensitive *Lactiplantibacillus plantarum*–expressing various RseP variants. We determined that site-directed mutations in conserved sequence motifs related to catalysis and substrate binding, and even deletion of two such motifs known to be involved in substrate binding, did not abolish bacteriocin sensitivity, with one exception. A mutation of a highly conserved asparagine, Asn359, in the extended so-called LDG motif abolished both binding of and killing by EntK1. By constructing various hybrids of the RseP proteins from sensitive *E. faecium* and insensitive *L. plantarum*, we showed that the extracellular PDZ domain is the key determinant of EntK1 sensitivity. Taken together, these data may provide valuable insight for guided construction of novel bacteriocins and may contribute to establishing RseP as an antibacterial target.

Site-2-metalloproteases (S2Ps) are a family of intramembrane-cleaving proteases involved in regulated intramembrane proteolysis (RIP) (1, 2). In the RIP cascade, an S2P cleaves its substrate, for example, a membrane-bound anti-sigma factor, within the cell membrane, thereby mediating transmembrane signaling to trigger an adaptive response. S2Ps are conserved in all kingdoms of life and are crucial in several biological processes, including stress response, sporulation, cell polarity, virulence, and nutrient uptake (3–9). Due to its vital role in both animal and human pathogens, RseP is regarded as an attractive antimicrobial target. In fact, nature itself targets RseP, which is a known target for antimicrobial peptides belonging to the LsbB family of bacteriocins in selected Gram-positive bacteria (10, 11). Little is known about how these bacteriocins recognize and bind RseP and how this interaction eventually leads to killing of target cells. More insight into these issues is crucial for understanding bacteriocin function and for understanding how RseP may be targeted in antimicrobial therapy.

The hallmarks of the S2P family are the conserved catalytic motifs (HExxH and LDG) located on transmembrane segments (TMSs) of the protease (12). The S2P family of proteases is divided into four subgroups based on membrane topology and domain structure (13). Among the four groups, only a few members have been characterized; these include *Escherichia coli* RseP (*Ec*RseP) from group I and the group III members MjS2P and SpolVFB from *Methanocaldococcus jannaschii* and *Bacillus subtilis*, respectively (12, 14, 15). *Ec*RseP is the most extensively studied S2P and was first identified as a key modulator of stress response (16, 17). When *E. coli* cells are exposed to stress, a site-1-protease cleaves the membrane-bound anti-σ^E factor RseA. This primary cleavage triggers a secondary cleavage by the S2P *Ec*RseP, which leads to release of RseA into the cytosol (16–18). RseA is further processed in the cytosol to form the mature σ^E, which activates genes involved in the stress response (19). It is believed that most S2P signaling pathways follow this same general cascade.

Next to the catalytic motifs, several conserved regions are thought to be involved in substrate interaction and catalysis by *Ec*RseP. These include the membrane-reentrant β-hairpin–like loop (MRE β-loop), the GxG motif, and the PDZ domain (Fig. 1) (20–22). The PDZ domain has been suggested to work as a size-exclusion filter, preventing interaction with the substrate prior to site-1-protease cleavage (21, 23).

In addition, conserved residues near the LDG catalytic motif located in the third transmembrane segment (TMS3) have been implicated in substrate binding and recognition, in particular two asparagines and prolines in the sequence motif NxxxxNxxPxPxLDG (24), here referred to as the extended LDG motif. Despite the identification of these potentially important features, the mechanism of substrate recognition and binding by *Ec*RseP remains somewhat enigmatic.

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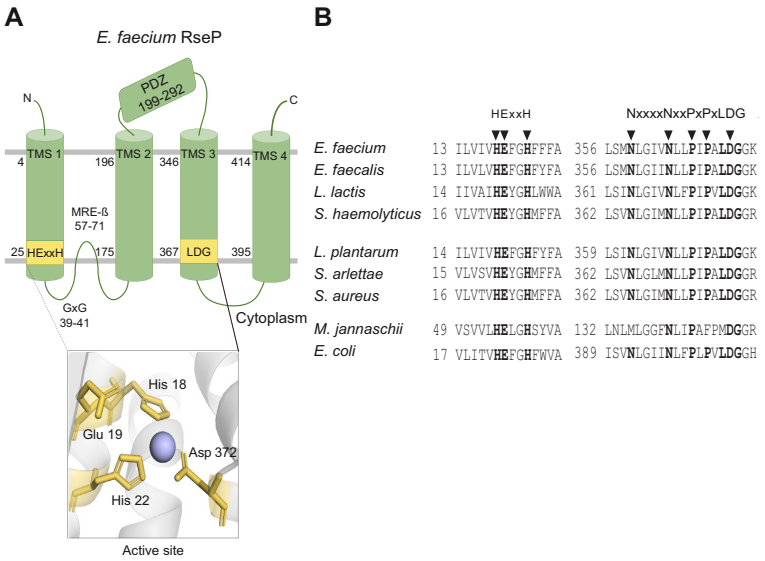

Figure 1. Schematic representation of the topology of *Efm*RseP and alignment of conserved S2P regions. *A*, schematic representation of the predicted topology of *Enterococcus faecium* RseP with conserved S2P motifs indicated. TMS1-4 indicates the four predicted transmembrane segments. The GxG motif, MRE β-loop, and the predicted PDZ domain are indicated. The *box* below shows the predicted active site, consisting of the conserved HEXXH and LDG motifs. *B*, alignment of the amino acid sequences of active site and the extended LDG motif in RseP from four EntK1-sensitive species (*E. faecium*, *Enterococus faecalis*, *Lactococcus lactis*, and *Staphylococcus haemolyticus*) and three EntK1-insensitive species (*Lactiplantibacillus plantarum*, *Staphylococcus arlettae*, and *Staphylococcus aureus*), as well as Gram-negative *Escherichia coli* and *Methanocaldococcus jannaschii*. *Arrow heads* indicate residues subjected to alanine substitutions. EntK1, Enterocin K1; MRE β-loop, membrane-reentrant β-hairpin–like loop; S2P, site-2-metalloprotease; TMS, transmembrane segment.

Bacteriocins are antimicrobial peptides produced by bacteria to inhibit other bacteria in competition for nutrition and ecological niches. They are considered promising alternatives and/or complements to antibiotics, mainly due to their potent activity against multidrug resistant pathogens. We have previously demonstrated that enterocin K1 (EntK1), a leaderless bacteriocin belonging to the LsbB family, is especially potent against *Enterococcus faecium*, including vancomycin-resistant strains (11, 25). Leaderless bacteriocins are synthesized without an N-terminal leader sequence and do not have post-translational modifications, making this group of bacteriocins ideal for synthetic production. Members of the LsbB family are small (30–44 amino acids), cationic, and amphiphilic, with an N-terminal helical structure and a disordered C-terminal end (11, 26). Interestingly, members of the LsbB family of bacteriocins differ in their inhibition spectrum, with LsbB being active only against *Lactococcus lactis*, while the inhibitory spectrum of EntK1 and enterocin EJ97 (EntEJ97) is broader, including high activity toward *E. faecium* and *Enterococcus faecalis*, respectively (11). It has previously been shown that the antimicrobial activity of bacteriocins of the LsbB family depends on RseP being present in target cells (10, 11).

RseP of *E. faecium* (*Efm*RseP) and *Ec*RseP, both from subgroup 1, shares a 28% sequence identity and has the same predicted membrane topology and conserved domains (Fig. 1). Little is known about the function of RseP in *E. faecium*; however, recent phenotypic analysis of *rseP* mutants suggests a role in stress response (25). For *E. faecalis*, it has been shown that RseP (*Efs*RseP) is a key regulator of the stress response through RIP-mediated activation of the sigma factor SigV. Deletion of either *EfsrseP* or *sigV* increases the susceptibility of *E. faecalis* to multiple stressors, such as lysozyme, heat, ethanol, and acid (27). In addition, *Efs*RseP is involved in sex pheromone maturation and is therefore also referred to as Eep (enhanced expression of pheromone) in this organism (28). Lastly, deletion of *EfsrseP* has been shown to result in severely attenuated virulence in a rabbit endocarditis model and a catheter-associated urinary tract infection model, suggesting an important role for *Efs*RseP in pathogenesis (7, 29).

Despite the evident role of RseP in enterococcal virulence, critical features of enterococcal RseP, such as the substrate recognition mechanism, remain unknown. The known substrates of RseP-like S2P share no apparent sequence homology; however, amphiphilic helices in the substrates have been indicated as necessary for recognition (20, 30). Considering the helical structure of EntK1, it is conceivable that EntK1 interacts with enterococcal RseP in a similar manner as the native substrates. Therefore, to gain more insight into bacteriocin action and possibly the interaction between RseP and its natural substrates, we have studied the EntK1–RseP interaction, focusing on the role of conserved regions of RseP. The impact of mutations in these regions was assessed by bacteriocin-binding assays and by analyzing bacteriocin sensitivity of strains carrying mutated RseP. The results shed

light on the interaction between EntK1 and RseP, providing insights into bacteriocin specificity and giving valuable information for the design of novel bacteriocins.

Results

Heterologous expression of RseP renders insensitive Lactiplantibacillus plantarum *sensitive to EntK1*

L. plantarum WCFS1 is a Gram-positive bacterium for which pSIP-based vectors have been developed for heterologous protein expression (31, 32). In addition, the bacterium is insensitive to EntK1 despite having an *rseP* ortholog on the chromosome. Together, these properties make *L. plantarum* a suitable host for expressing *E. faecium* RseP for binding and sensitivity studies. As shown in Table 1, expression of RseP from *E. faecium* renders *L. plantarum* sensitive to EntK1, with a minimum inhibitory concentration (MIC_{50}) of 0.01 μM, while *L. plantarum* carrying the empty vector (pEV) exhibited a MIC_{50} greater than 22 μM, which is considered fully resistant. We also overexpressed *L. plantarum* RseP (*Lp*RseP) in *L. plantarum*, to confirm the inability of *Lp*RseP to be a receptor for EntK1. As expected, the *Lp*RseP-overexpressing strain ($LpRseP^{-His}$; see Table 2 for a description of strain names) remained insensitive (i.e., MIC_{50} greater than 22 μM) (Table 1). These results suggest that the *L. plantarum* strain is a suitable host for heterologous expression of *Efm*RseP. Moreover, a pairwise sequence alignment of *Efm*RseP and *Lp*RseP indicates that subtle sequence differences between *Efm*RseP and *Lp*RseP define the sensitivity toward EntK1 (Fig. S1).

Of note, while the pSIP vectors used for expression (Table 2) have an inducible promoter, regulated by the inducer peptide SppIP (31), all sensitivity and binding experiments were performed using noninducing conditions. Under inducing conditions (3–30 ng/ml SppIP), the transformants showed aberrant growth on agar plates (data not shown), indicating a cytotoxic effect likely due to the high amounts of the membrane-protein RseP. Noninduced cells appeared to grow normally. The inducible promotor *sppA* in the pSIP vector has a low basal activity in *L. plantarum*, which permits low expression of *rseP* genes under noninducing conditions (as demonstrated by the results presented in Table 1).

Antimicrobial activity and binding of EntK1 to sensitive cells depend on RseP

To examine whether the antimicrobial activity observed above is directly linked to the ability of EntK1 to bind target cells, we developed a binding assay for EntK1 to *L. plantarum*. For this assay, EntK1 was chemically synthesized with an N-terminal FITC fluorescent tag. The N-terminal fusion was chosen as the C-terminal half of the LsbB family of bacteriocins and is thought to be necessary for receptor interaction (26). The labeling of EntK1 with FITC reduced the antimicrobial potency, which, however, remained high for *L. plantarum*–expressing *Efm*RseP (Table 1). Fluorescence microscopy of EntK1-sensitive *L. plantarum*–expressing plasmid-encoded *Efm*RseP showed strong fluorescent signals following exposure to FITC-EntK1, consistent with EntK1 binding. In contrast, nonsensitive *L. plantarum* carrying the empty vector (pEV) did not show any visible fluorescent signals under the same conditions, thus confirming lack of EntK1 binding (Fig. 2).

In accordance with the fluorescence microscopy, flow cytometry analysis revealed that FITC-EntK1–exposed *L. plantarum*–expressing RseP derived from *E. faecium* exhibited strong fluorescent signals, while cells containing the empty vector or overexpressing the *Lp*RseP protein showed no signal (Fig. 3). We have previously observed that EntK1 has some antimicrobial activity toward strains of *L. lactis*, *E. faecalis*, and *Staphylococcus haemolyticus* but not strains of *Staphylococcus aureus* and *Staphylococcus arlettae* (33). To confirm that the sensitivity is linked to RseP binding, *rseP* genes derived from these sensitive and insensitive species were heterologously expressed in *L. plantarum*. Table 1 shows that, indeed, *L. plantarum* strains expressing *rseP* genes derived from the sensitive strains of *L. lactis* ($LlRseP^{-His}$), *E. faecalis* ($EfsRseP^{-His}$), and *S. haemolyticus* ($ShRseP^{-His}$) were indeed inhibited by EntK1. In addition, Figure 3 shows that these strains had distinctly higher FITC signals than *L. plantarum* strains expressing RseP from the insensitive strains *S. aureus* ($SasRseP^{-His}$) and *S. arlettae* ($SaeRseP^{-His}$). Taken together, these results provide strong evidence that there is a specific interaction between EntK1 and RseP from bacteria that are naturally sensitive to EntK1 but not between EntK1 and RseP from bacteria that are insensitive to EntK1.

Defining the role of conserved S2P motifs in the EntK1:RseP interaction

To define the regions of RseP involved in EntK1 sensitivity, we initially focused on conserved regions that, based on previous studies of other members of the S2P family, seem to be involved in substrate binding and catalysis. In addition to the

Table 1
MIC for EntK1 and FITC-EntK1 towards *Lactiplantibacillus plantarum* strains expressing heterologous RseP

Strains	Characteristics	MIC_{50} (μM)	
		EntK1	FITC-EntK1
$EfmRseP^{-His}$	Expressing RseP from *Enterococcus faecium*	0.01	0.15
$LpRseP^{-His}$	Expressing RseP from *Lactiplantibacillus plantarum*	>22	>20
$EfsRseP^{-His}$	Expressing RseP from *Enterococcus faecalis*	0.04	0.6
$LlRseP^{-His}$	Expressing RseP from *Lactococcus lactis*	0.09	>20
$ShRseP^{-His}$	Expressing RseP from *Staphylococcus haemolyticus*	0.17	>20
$SaeRseP^{-His}$[a]	Expressing RseP from *Staphylococcus arlettae*	>22	>20
$SasRseP^{-His}$[a]	Expressing RseP from *Staphylococcus aureus*	>22	>20
pEV	Empty vector	>22	>20

[a] Control experiments (Fig. S2) indicated low expression, which may contribute to low sensitivity.

Table 2
Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
Plasmid		
pLp1261_InvS	Spp-based expression vector, pSIP401 backbone, Ery^R	(31, 41)
Strain		
L. plantarum WCFS1 (*Lp*)	Template for *rseP* (*Lp*RseP) and expression host	(53)
E. faecium P21 (*Efm*)	Template for *rseP* (*Efm*RseP)	(54)
E. faecalis V583 (*Efs*)	Template for *rseP* (*Efs*RseP)	NCBI:txid226185
L. lactis IL1403 (*Ll*)	Template for *rseP* (*Ll*RseP)	NCBI:txid272623
S. aureus ATCC 14458 (*Sas*)	Template for *rseP* (*Sas*RseP)	Nofima
S. arlettae LMGT 4134 (*Sae*)	Template for *rseP* (*Sae*RseP)	LMGT
S. haemolyticus LMGT 4106 (*Sh*)	Template for *rseP* (*Sh*RseP)	LMGT
E. coli TOP10	Cloning host	Thermo Fisher Scientific
L. plantarum WCFS1	Harboring pSIP401 encoding various RseP derivates, Ery^R	
pEV	Empty vector	(41)
$EfmRseP^{-His}$	*rseP* from *E. faecium* P21	This study
EfmRseP[a]	*rseP* from *E. faecium* P21, C-terminal 6× His-tag	This study
$EfsRseP^{-His}$	*rseP* from *E. faecalis* V583	This study
$LlRseP^{-His}$	*rseP* from *L. lactis* IL1403	This study
$LpRseP^{-His}$	*rseP* from *L. plantarum* WCFS1	This study
LpRseP[a]	*rseP* from *L. plantarum* WCFS1, C-terminal 6× His-tag	This study
$ShRseP^{-His}$	*rseP from S. haemolyticus* 7067	(33)
$SasRseP^{-His}$	*rseP* from *S. aureus* ATCC 14458	This study
$SaeRseP^{-His}$	*rseP* from *S. arlettae* LMGT 4134	This study
EfmH18A[a]	*Efm*RseP with substitution H18A	This study
EfmE19A[a]	*Efm*RseP with substitution H19A	This study
EfmH22A[a]	*Efm*RseP with substitution H22A	This study
EfmAAxxA[a]	*Efm*RseP with substitutions H18A, H19A, H22A	This study
EfmN359A[a]	*Efm*RseP with substitution N359A	This study
EfmN364A[a]	*Efm*RseP with substitution N364A	This study
EfmP367A[a]	*Efm*RseP with substitution P367A	This study
EfmP369A[a]	*Efm*RseP with substitution P369A	This study
EfmD372A[a]	*Efm*RseP with substitution D372A	This study
Hyb1[a]	Fusion of *Lp*RseP (1–221) and *Efm*RseP (222–422)	This study
Hyb2[a]	Fusion of *Lp*RseP (1–328) and *Efm*RseP (329–422)	This study
Hyb3[a]	Fusion of *Efm*RseP (1–221) and *Lp*RseP (222–425)	This study
Hyb4[a]	Fusion of *Efm*RseP (1–325) and *Lp*RseP (326–425)	This study
Hyb5[a]	Fusion of *Efm*RseP (1–200) and *Lp*RseP (201–425)	This study
Hyb6[a]	Fusion of *Efm*RseP (1–170) and *Lp*RseP (171–425)	This study
Hyb7[a]	Fusion of *Efm*RseP (1–32) and *Lp*RseP (33–425)	This study
Hyb8[a]	Fusion of *Lp*RseP (1–171, 222–425) and *Efm*RseP (172–221)	This study
Hyb9[a]	Fusion of *Lp*RseP (1–201, 222–425) and *Efm*RseP (202–221)	This study
Hyb10[a]	Fusion of *Lp*RseP (1–171, 326–425) and *Efm*RseP (172–325)	This study
Hyb11[a]	Fusion of *Lp*RseP (1–201, 328–425) and *Efm*RseP (202–327)	This study
Trunc[a]	Truncation of *Efm*RseP (1–39, 139–422) Δ40–138	This study

RseP homologs from the respective species are abbreviated with the species initials italicized (*e.g.*, *Sh*RseP is the RseP homolog in *S. haemolyticus*), while the strain names for each *L. plantarum* strain expressing a variant of RseP is not italicized (*e.g.*, ShRseP is *L. plantarum* WCFS1 harboring pSIP401 encoding *Sh*RseP). For cases where the species initials are ambiguous, both the first and last letter of the specific name is used (*e.g.*, *E. faecium* and *E. faecalis*).
Abbreviations: Em^R, erythromycin resistance; LMGT, laboratory of microbial gene technology; Nofima, norwegian institute of food, fisheries and aquaculture research.
[a] Harboring a C-terminal 6× His-tag.

conserved residues of the active site found in all members of the S2P family, *E. faecium* RseP contain multiple other conserved motifs, including the MRE β-loop and the extended LDG motif. These domains are conserved among members of subgroup I and III in the S2P family, as well as the GxG motif and PDZ domain which are only present in subgroup I (Fig. 1). To examine how these conserved motifs of *E. faecium* RseP affect the binding of and sensitivity toward EntK1, mutational analysis of each motif was performed, by site-directed mutagenesis, by creating hybrids of *Efm*RseP and *Lp*RseP, and by a truncation in *Efm*RseP.

The active site

The conserved motifs HExxH and LDG make up the active site of the S2P family (Fig. 1) (14). It has previously been shown that mutations of residues corresponding to *Efm*RseP His18, Glu19, His22, and Asp372 substantially affect the protease activity of RseP homologs from multiple species (12, 14, 34). To examine whether proteolytic activity of RseP is needed for EntK1 sensitivity, alanine substitutions were introduced in all conserved residues in the active site. Single alanine substitutions in the active site (EfmH18A, EfmE19A, EfmH22A, and EfmD372A) resulted in a slight increase of the MIC_{50} from ≤0.002 μM for 6His-tagged WT *Efm*RseP to 0.01 to 0.7 μM for the 6His-tagged mutants (Table 3). In line with these observations, measurements of the populations with the single alanine substitutions in the binding assay described above showed only a slight reduction in binding with 59 to 80% of the median fluorescence intensity of the *L. plantarum* population expressing the native *Efm*RseP. The triple alanine substitution (EfmAAxxA) resulted in a considerable increase in the MIC_{50}, to 2.7 μM (Table 3). However, the triple mutant was still more than 8-fold more sensitive to EntK1 than pEV. The impact of the mutations on the MIC_{50} values could be partly due to variation in RseP expression, which was not assessed in detail. For example, it is conceivable that the triple mutant is rather unstable and was produced in lower amounts, leading to a higher MIC_{50} value and low EntK1 binding. Nevertheless, the fact that all variants remained sensitive and bound the

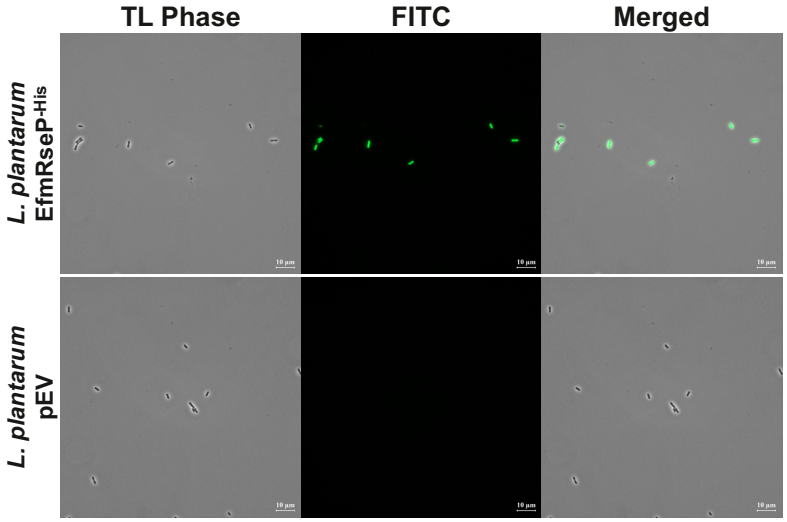

Figure 2. Transmitted light phase contrast and fluorescence microscopy of *Lactiplantibacillus plantarum* expressing *Efm*RseP (EfmRseP^{-His}) or containing the empty vector (pEV) after exposure to FITC-EntK1. EfmRseP cells (*upper panel*) show strong fluorescent signals upon exposure to FITC-EntK1 compared to the negative control containing pEV (*lower panel*). An overlay of the fluorescence and phase-contrast images is shown to the right (Merged). *Efm*RseP, *Enterococcus faecium* RseP; EntK1, Enterocin K1.

bacteriocin clearly shows that the mutant proteins were produced and that the catalytic activity of RseP does not play an essential role in RseP binding and strain sensitivity.

The MRE β-loop and the GxG motif region

Previous studies on *Ec*RseP indicate that the MRE β-loop and the GxG motif region (Fig. 1) interact directly with the substrate (20, 22). To examine the significance of this region for the RseP:EntK1 interaction, residues 39 to 138 encompassing the MRE β-loop and the GxG motif were deleted (Trunc, Fig. S1). The truncation significantly reduced EntK1 sensitivity, as judged by the increase in MIC_{50} of Trunc to 2.7 μM (Table 3). Using the binding assay, we observed that FITC signals were also significantly reduced to 7.4% compared to the full-length protein (Table 3). Nonetheless, the FITC signal reflecting binding (7.4% *versus* 0.4%) and the sensitivity towards EntK1 (MIC_{50} of 2.7 μM *versus* 22 μM) were higher than that of the empty vector control strain (Table 3). It would thus seem that the MRE β-loop and the GxG motif region are not involved in the RseP:EntK1 interaction.

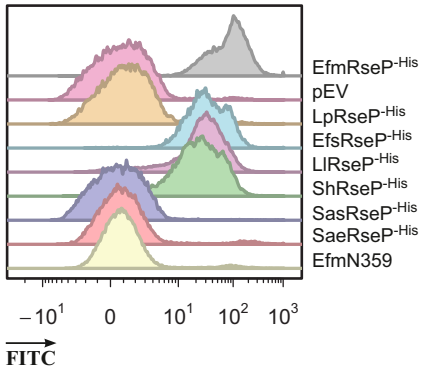

Figure 3. FITC-EntK1 binding assay of *Lactiplantibacillus plantarum* clones expressing RseP from naturally EntK1-sensitive and EntK1-insensitive bacteria. The figure shows representative histograms for *L. plantarum* cells expressing RseP from EntK1-sensitive species *Enterococcus faecium* (EfmRseP^{-His}), *Enterococcus faecalis* (EfsRseP^{-His}), *Lactococcus lactis* (LlRseP^{-His}), and *Staphylococcus haemolyticus* (ShRseP^{-His}), and from EntK1-insensitive species *L. plantarum* (LpRseP^{-His}), *Staphylococcus arlettae* (SaeRseP^{-His}), and *Staphylococcus aureus* (SasRseP^{-His}), in addition to *L. plantarum* carrying the empty vector and the N359A mutant of *Efm*RseP. An increase in fluorescence indicates binding of the bacteriocin to the cells. EntK1, Enterocin K1.

The extended LDG motif

A conserved motif in TMS3 (NxxPxPxLDG), which includes the LDG catalytic site motif (Fig. 1*B*), has been suggested as a prime candidate for S2P substrate binding (13). Moreover, previous substrate-binding studies with *Ec*RseP (24) suggest a longer version of the LDG motif, referred as the extended LDG motif (N359xxxxN364xxP367xP369xLD372G in *Efm*RseP), may be important for substrate binding. The two asparagines and two prolines in the extended motif were individually mutated to alanine. Three of the four mutants remained sensitive to EntK1 and showed strong EntK1 binding (Table 3). However, the alanine substitution of Asn359 in *Efm*RseP (named EfmN359A) resulted in complete resistance to EntK1, and the binding of the bacteriocin was abolished (Fig. 3 and Table 3).

The absence of EntK1 sensitivity and EntK1 binding could be caused by failure to express the *rseP* variant. Therefore,

Table 3
EntK1 sensitivity and EntK1 binding of *Lactiplantibacillus plantarum* expressing variants of RseP

Strain[a]	EntK1 MIC_{50} (μM)	FITC-EntK1 rMFI % (RSD)
EfmRseP	≤0.002	100 (8.3)
pEV	>22	0.4 (4.2)
LpRseP	>22	0.41 (9.7)
Active site		
EfmH18A	0.02	61.6 (3.8)
EfmE19A	0.01	65.4 (3.3)
EfmH22A	0.01	59 (8.1)
EfmAAxxA	2.7	0.5 (5.2)
EfmD372A	0.7	80.6 (3.5)
GxG motif and the MRE β-loop		
Trunc	2.7	7.4 (12.2)
Extended LDG		
EfmN359A	>22	0.41 (22.6)
EfmN364A	0.004	83.4 (3.4)
EfmP367A	0.004	89.1 (4.6)
EfmP369A	≤0.02	84.4 (6.9)
RseP hybrids		
Hyb1	>22	0.42 (25.2)
Hyb2	>22	0.47 (18.3)
Hyb3	0.7	37.6 (24.4)
Hyb4	≤0.002	97.6 (8.5)
Hyb5	>22	0.82 (36.6)
Hyb6	>22	0.51 (6.8)
Hyb7[b]	>22	0.29 (10.4)
Hyb8	>22	0.82 (26.6)
Hyb9	>22	0.53 (18.2)
Hyb10	≤0.002	78 (7.6)
Hyb11	0.09	54.8 (2.4)

The middle column shows MIC for EntK1 towards *L. plantarum* strains expressing various RseP variants (see text, Figs. 1 and S1 for details). The strains are named by the protein variant they express. The right column shows the binding of FITC-labeled EntK1 to indicated strains. The FITC signals, indicating binding of the bacteriocin, are presented as the relative median fluorescence intensity (rMFI) compared to the MFI obtained for EfmRseP6His (100%) with percent relative standard deviations (RSD).
[a] All RseP variants contain a C-terminal 6× His-tag.
[b] Control experiments (Fig. S2) indicated low expression, which may contribute to low sensitivity.

EfmN359A (and all other variants displaying a complete loss of sensitivity, discussed below) were exposed to EntEJ97, another bacteriocin from the LsbB family. EntEJ97 targets RseP but has a different antimicrobial spectrum compared to EntK1 (11), which implies that its interaction with RseP differs from EntK1. Fig. S2 shows that the control pEV clone displayed limited sensitivity towards EntEJ97, while EfmN359A was highly sensitive to the bacteriocin, showing that the alanine substitution did not drastically alter the protein structure nor the expression level and that the removal of the asparagine side chain alone is likely responsible for the alteration in EntK1 sensitivity and binding. Interestingly, Asn359 and the extended LDG motif are highly conserved among both EntK1-sensitive and EntK1-insensitive species (Fig. 1*B*). Thus, the impact of this residue on EntK1 sensitivity must relate to its interaction with other less conserved regions of the protein.

Mapping the regions involved in EntK1 specificity.

To further identify regions determining EntK1 sensitivity, we constructed several hybrid proteins in which parts of the RseP from insensitive *L. plantarum* were replaced with the corresponding parts of RseP from sensitive *E. faecium* (Hyb1-11, Fig. S3). Previous studies had suggested that residues 328 to 428 in the C-terminal region of RseP from *L. lactis* (YvjB) determine the sensitivity of *L. lactis* to LsbB (35). As LsbB and EntK1 target the same receptor, belong to the same bacteriocin family, and have a similar structure (10, 11, 26), we hypothesized that the C-terminal region of RseP from *E. faecium* would confer EntK1 sensitivity. To test the hypothesis, varying parts of the C-terminal region of *Lp*RseP were replaced with the corresponding region of *E. faecium* RseP (Fig. S3). Surprisingly, the resulting hybrid proteins, Hyb1 and Hyb2, did not confer sensitivity to EntK1 (MIC_{50} > 22 μM) nor did they show bacteriocin binding (Table 3). Control experiments with EntEJ97 (Fig. S2) showed that Hyb1 and Hyb2 were produced.

Next, Hyb3 and Hyb4 (inverts of Hyb1 and Hyb2), containing the N-terminal region of *Efm*RseP and the C-terminal region of *Lp*RseP were constructed (Fig. S3). Unlike Hyb1 and Hyb2, Hyb3 and Hyb4 conferred sensitivity to EntK1 with MIC_{50} values of 0.7 μM and ≤0.002 μM, respectively. Hyb3 and Hyb4 also showed binding of the bacteriocin (Table 3). The results obtained with Hyb1-4 show that the N-terminal region of *E. faecium* RseP (residues 1–324) is involved in EntK1 binding. Although quantitative comparison of MIC_{50} values is risky due to possible differences in expression, it is worth noting that Hyb4, containing the complete *Efm*RseP PDZ domain, was the most sensitive of the four hybrids.

To further narrow down the RseP region needed for EntK1 sensitivity, three additional hybrid proteins containing a decreasing portion of *Efm*RseP were constructed (Hyb5-7, Fig. S3). None of these hybrids, all lacking the PDZ domain from *E. faecium*, could confer sensitivity to or binding of EntK1 (Table 3), indicating that the PDZ domain is required for activity. The control experiments of Fig. S2 showed that Hyb5 and Hyb6 were produced, whereas Hyb7 likely has reduced expression. To confirm the importance of the PDZ region, we constructed four additional hybrid proteins in which different parts of the PDZ domain of *Lp*RseP were replaced with the corresponding sequences of *Efm*RseP (Fig. 3, Hyb8-11). Interestingly, only Hyb10 and Hyb11, which contained the entire PDZ domain from *Efm*RseP were EntK1-sensitive, with MIC_{50} values of 0.002 μM and 0.09 μM, respectively (Table 3). Hyb8 and Hyb9, only containing parts of the *Efm*RseP PDZ domain, were not sensitive to EntK1 with MIC_{50} >22 μM (Table 3). A control experiment showed that both Hyb8 and Hyb9 were highly sensitive to EntEJ97, indicating that these hybrids are produced (Fig. S2).

Importantly, as noted above, all hybrids that did not confer sensitivity or binding to EntK1, except for Hyb7, were sensitive (*i.e.*, inhibition zone >10 mm for EntEJ97; Fig. S2). This indicates that Hyb1-6 and Hyb8-11 were properly expressed and folded. Moreover, all clones of *L. plantarum*–expressing recombinant RseP showed growth comparable to EfmRseP, suggesting that expression of the hybrids had no obvious toxic effect on the host (data not shown).

Discussion

The S2P RseP is highly conserved in multiple species, yet the potency of EntK1 varies considerably between species (11, 33). To further develop EntK1 as a novel treatment option for bacterial infections, a detailed understanding of the determinants of

bacteriocin sensitivity and binding to RseP is essential. Therefore, in this study, we explored the contribution of conserved S2P motifs to the EntK1:RseP interaction and EntK1 sensitivity. To do so, we first needed to establish a sensitivity and binding assay. Although the antimicrobial activity of EntK1 depends on RseP (11), it remains elusive whether the difference in EntK1 sensitivity between species is solely due to variations in the RseP protein or if other factors, such as cell surface composition and gene expression levels, contribute. To avoid potential problems related to these uncertainties, we expressed *rseP* from insensitive and sensitive species in the same expression vector (pSIP) and EntK1-insensitive host (*L. plantarum*). We demonstrated that only *rseP* from sensitive bacterial species confers EntK1 sensitivity to *L. plantarum*. Binding of the bacteriocin to the RseP-producing *L. plantarum* strains was assessed using FITC-labeled EntK1. The levels of FITC-EntK1 signals correlated well with the MIC_{50} values (Table 3; higher binding correlates with lower MIC_{50} values). These observations show that subtle differences in the receptor alone likely determine variation in EntK1 sensitivity.

*Efm*RseP belongs to group I of the S2P family, for which the involvement of several conserved motifs in substrate binding and substrate specificity has been explored to some extent (20, 22, 24, 34). We considered that these motifs could be involved in EntK1 sensitivity and, therefore, we targeted these motifs in the mutagenesis studies to identify their role(s) in RseP as a bacteriocin receptor. We initially focused on the active site of *E. faecium* RseP, as there were indications in the literature that alterations in the active site of RseP in *E. faecalis* affects the sensitivity to a member of the LsbB bacteriocin family (11). However, none of the mutations in the catalytic center, including mutations known to abolish protease activity in *Ec*RseP (16, 36), led to EntK1 resistance, demonstrating that the proteolytic activity of RseP is not essential for interaction and the antimicrobial action of EntK1.

Two motifs of *Ec*RseP known to interact with the substrate are the MRE β-loop and a conserved GxG motif located on a membrane-associated region between TMS1 and TMS2 (Fig. 1) (20, 22). If RseP-targeting bacteriocins mimic the interaction of natural substrates with the receptor, these two regions would likely interact with EntK1. Although deletion of the MRE β-loop and the GxG motif led to a significant reduction in EntK1 sensitivity, the removal of these nearly 100 amino acids did not result in total resistance toward EntK1 (Table 3), indicating that neither the MRE β-loop nor the GxG motif is essential for the EntK1:RseP interaction. The reduced sensitivity and binding upon truncation are likely due to global structural changes in the receptor resulting from the large deletion. Of note, the MRE β-loop and GxG motif of *Efm*RseP are both predicted to be located on the cytoplasmic side of the cell membrane (Fig. 1*A*); such a location would likely not allow direct interaction with the bacteriocin which attacks target cells from the outside. It should be noted that the predicted topology of *Efm*RseP and the RseP hybrids was not confirmed experimentally in this study. However, a similar topology for the group 1 S2P *Ec*RseP and *Sas*RseP has been confirmed by the fusion of alkaline phosphatase to specific regions of RseP (6, 15), suggesting that the predicted topology may be conserved among group 1 S2Ps.

Next, we explored the role of the extended LDG motif in EntK1:RseP interaction. Substituting Asn364, Pro367, and Pro369 with alanine in *Efm*RseP resulted in only minor changes in EntK1 sensitivity and binding (Table 3). This was surprising, as these conserved asparagine and proline residues are known to be important for substrate binding and correct processing in both *Ec*RseP and S2P from *B. subtilis*, known as SpoIVFB (12, 24, 37, 38). On the other hand, Asn359 was shown to be essential for EntK1 sensitivity and binding (Table 3). Under noninduced conditions, we were not able to detect RseP expression from N359A, or any other clone, using a standard Western blot (Fig. S4). However, when induced, expression levels of N359A and *Efm*RseP were comparable, suggesting that the insensitivity of the clone was due to the N359A substitution but not due to a failure in expression. Moreover, EfmN359A remained highly sensitive to EntEJ97, another bacteriocin of the LsbB family targeting RseP, which strongly indicates that the observed changes in sensitivity and binding were not caused by failure to express mutated *rseP* (Fig. S2). Previous studies have exploited the known substrates of RseP homologs to perform cleavage-based activity assays to confirm proper protein folding and expression following the introduction of mutations (24). However, RseP has no known substrates in *E. faecium*, which explains why cleavage-based activity assays could not be used. Interestingly, Asn389 in *Ec*RseP, which corresponds to Asn359 in *Efm*RseP, plays an important role in substrate recognition. When this asparagine was replaced by cysteine, *Ec*RseP showed reduced substrate binding as well as reduced proteolytic activity (24). Despite the evident role of Asn359 in EntK1:RseP binding, it is interesting to note that Asn359 and the surrounding extended LDG domain are highly conserved in the RseP proteins of both EntK1-sensitive and EntK1-insensitive species (Fig. 1*B*). This suggests that other regions of RseP play a role in bacteriocin binding and sensitivity.

Of the 11 constructed *Efm*RseP-*Lp*RseP hybrid proteins, only four (Hyb3, Hyb4, Hyb10, Hyb11) were EntK1 sensitive (Table 3). Importantly, all EntK1-sensitive hybrids contain parts of the *Efm*PDZ domain, with Hyb4, Hyb10, and Hyb11 containing the entire domain. Of the four sensitive hybrids, hybrids containing the entire *Efm*PDZ domain exhibited the lowest MIC_{50} (*i.e.*, most sensitive), underpinning the important contribution of this domain to the EntK1:RseP interaction. Previous studies have shown that the PDZ domain is involved in substrate recognition by RseP-like S2P (21, 23). It has been suggested that the PDZ domain of *Ec*RseP acts as a size-exclusion filter, preventing substrates with large periplasmic domains access to the active site (21). A similar role has been suggested for the PDZ domain of the *B. subtilis* S2P homolog, RasP (23). Several S2Ps process multiple substrates *in vitro* and *in vivo*, yet the substrate specificity of these proteins is poorly understood. We conclude that the PDZ domain of *Efm*RseP is the defining region for EntK1 binding and thus the major determinant of variation in EntK1 sensitivity.

To better understand the positions of *Efm*RseP motifs investigated in this study, we predicted the structure of *Efm*RseP using AlphaFold. AlphaFold is a protein structure prediction

program based on artificial intelligence that predict protein structures with greater accuracy than any other in silico method (39). As illustrated in Figure 4, AlphaFold predicted that the PDZ domain of *Efm*RseP forms a pocket which may prevent direct access to core residues. Among the regions investigated in this study, Asn359 in the extended LDG domain is located the closest to the PDZ domain, while the GxG motif and the MRE-β loop appears to be more distant (Fig. 4). Taken together with our experimental data, it is conceivable that the initial docking of EntK1 to the PDZ domain leads to subsequent interactions with core residues such as Asn359.

During the finalization of this article, AlphaFold-Multimer was published (R. Evans *et al.*, Preprint at bioRxiv). AlphaFold-Multimer is an extension of AlphaFold2 using an artificial intelligence model explicitly trained for multimeric input. This allowed us to predict the EntK1:RseP complex, which strikingly predicted the interaction between EntK1 and RseP to primarily involve the PDZ domain and the region near Asn359 (data not shown). However, while most of the residues of RseP in the complex exhibited a high confidence score (pLDDT > 90), most of the residues of EntK1 were ranked poorly (pLDDT < 50). Confidence scores below 50 is a strong predictor of disorder, suggesting that the peptide chain is unstructured at physiological conditions or only structured as part of a complex. Indeed, EntK1 has been shown to be disordered in an aqueous environment by NMR spectroscopy (11). Due to the low confidence scores produced for EntK1 in the complex, these structure predictions are highly speculative and should be used cautiously.

While it remains unknown how the EntK1:RseP complex eventually leads to cell death, the present study reveals molecular details of the interaction of EntK1 with its receptor. Previous studies have shown that bacteriocins of the LsbB family can be engineered to improve both potency and alter the activity spectrum (33). The interpretation of these previous results, as well as future efforts to develop improved RseP-binding bacteriocins, will benefit from the deeper insight into the bacteriocin–receptor interaction that we provide here. Importantly, LsbB family of bacteriocins are attractive not only because they act on vancomycin-resistant strains but also because the bacteriocins are short, synthesized without an N-terminal leader sequence, and contain no posttranslational modification, which enables low-cost synthetic production. The fact that RseP homologs have important roles in virulence in several animal and human pathogens highlights RseP as an attractive antimicrobial target in multiple species (9, 40). The mutational analysis performed in this study combined with the predicted *Efm*RseP structure may provide a powerful basis for guided construction of novel bacteriocins and may contribute to further development of RseP as a drug target.

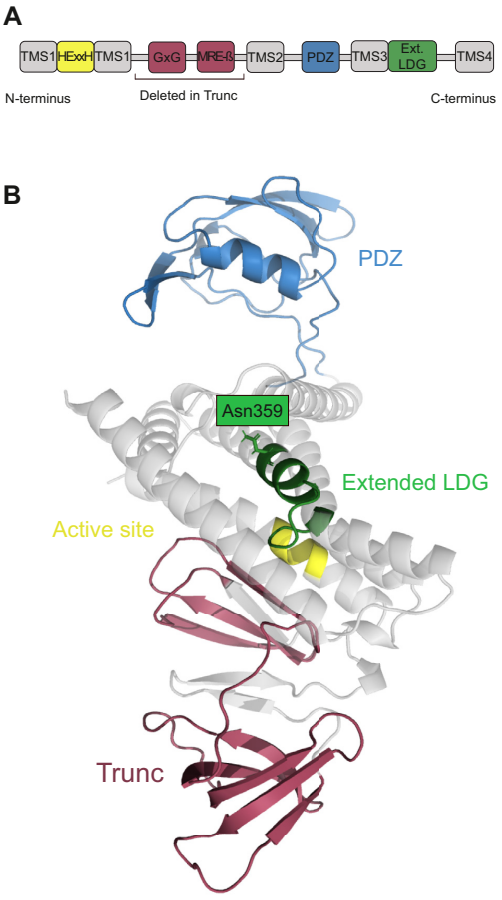

Figure 4. RseP from *Enterococcus faecium* as predicted by AlphaFold. *A*, schematic overview of the conserved RseP-like S2P motifs found in RseP from *E. faecium*. *B*, structure of RseP from *E. faecium* as predicted by AlphaFold with RseP-like S2P motifs highlighted. The HExxH motif of the active site is indicated in *yellow*, the predicted PDZ domain is indicated in *blue*, and the extended LDG domain is indicated in *green*. The region deletion from Trunc, which encompasses the GxG and the MRE β-loop motifs, is indicated in *purple*. MRE β-loop, membrane-reentrant β-hairpin–like loop; S2P, site-2-metalloprotease.

Experimental procedures

Bacterial strains and cultivation conditions

Bacterial strains used in this study are listed in Table 2. The following strains were cultivated in Brain heart infusion broth (Thermo Scientific Oxoid): enterococcal strains (37 °C, without agitation), staphylococcal strains (37 °C, 220 rpm), and *E. coli* (37 °C, 220 rpm). *L. plantarum* and *L. lactis* were cultivated without shaking in DeMan, Rogosa and Sharp (MRS) broth (Thermo Scientific Oxoid) at 37 °C and M17 broth (Thermo Scientific Oxoid) supplemented with 0.5% glucose at 30 °C, respectively. Agar plates were prepared by supplementing the appropriate broth with 1.5% (w/v) agar (VWR chemicals). Erythromycin was added to a final concentration of 200 μg/ml for *E. coli* and 10 μg/ml for *L. plantarum* when appropriate.

Construction of rseP *orthologs,* rseP *hybrids, and site-directed mutagenesis*

Seven orthologs of *rseP* from EntK1-sensitive and EntK1-insensitive species were expressed in *L. plantarum* using the pSIP expression system (31, 32) (Table 2). Briefly,

pLp1261_InvS, a pSIP derivative, was digested with NdeI and Acc65I or XmaI (Thermo Fisher Scientific) (41). Genomic DNA from the seven native *rseP*-containing strains was used as a template for the amplification of *rseP*. PCR amplification of all *rseP* variants was performed using Q5 Hot Start High-fidelity DNA polymerase (New England BioLabs) with In-Fusion primers to yield amplicons with ends complementary to the linearized pSIP vector (Table S1). The amplified PCR fragments were fused with the linearized vector using In-Fusion HD cloning Kit (Takara Bio) and transformed into competent *E. coli* TOP10 (ThermoFisher Scientific).

Site-directed mutants, truncations of RseP and RseP hybrids were constructed using splicing by overlap extension PCR. Briefly, two fragments of the *rseP* sequences were amplified in separate PCR reactions using two primer pairs, each consisting of an inner and outer primer (Table S1). The inner primers generated overlapping complementary ends and acted as mutagenic primers when introducing point-mutations. The overlapping fragments were fused by a second PCR reaction using the outer primers. Fused amplicons containing a mutated *rseP* gene were purified, fused to the linearized vector, and transformed to *E. coli* as described above.

All constructed plasmids were verified by DNA sequencing at Eurofins GATC Biotech (Germany) and subsequently transferred into electrocompetent *L. plantarum* as previously described (42). Fig. S3 shows a schematic representation of all hybrids and the truncated versions of RseP. Protein topology and the PDZ domain were predicted using CCTOP and Pfam, respectively (43, 44).

Antimicrobial assays

The bacteriocins used in this study, EntK1, EntEJ97, and FITC-EntK1, were produced by Pepmic Co, LtD with >95% purity. Bacteriocins were solubilized in 0.1% (vol/vol) TFA (Sigma-Aldrich), except for FITC-EntK1 which was solubilized in dH_2O. For semiquantitative assessment of antimicrobial activity, a spot-on-lawn assay was performed. Briefly, an overnight culture was diluted 1:100 in soft-agar and distributed on agar plates containing appropriate antibiotics. Bacteriocins with various concentrations were applied on designated spots on the solidified soft-agar. The agar plates were incubated at appropriate temperatures overnight and inhibition zones were measured the following day. For more accurate quantification, EntK1 sensitivity was determined using a microtiter plate assay to define MIC_{50} (45). The MIC_{50} was defined as the lowest bacteriocin concentration needed to inhibit bacterial growth by ≥50%. MIC assays were performed with three biological replicates.

Binding assays

Overnight cultures of *L. plantarum* strains were diluted 50-fold and grown until mid-log phase (4 h), after which cells were harvested by centrifugation at 16,000*g* for 3 min and resuspended in sterile 0.9 % (w/v) NaCl to an A_{600} of 1 (assessed using a SPECTROstar Nano reader; BMG Labtech). Cell suspensions were diluted 20-fold in binding buffer [1 μM FITC-labeled EntK1 in 100 μM triammonium citrate pH 6.5 (Sigma-Aldrich)]. The cells were incubated in the binding buffer on a rotator (Multi Bio RS-24, Biosan, Latvia) at 6 rpm for 20 min at room temperature. After incubation, cells were harvested by centrifugation (16,000*g*, 3 min) and the binding buffer was discarded. The cell pellets were resuspended in sterile PBS to an appropriate cell density and analyzed using a MACSQuant Analyzer flow cytometer with excitation at 488 nm and emission at 475 to 575 nm (500 V PMT). The instrument was set to trigger on side-scattered light (SSC-A, 370 V PMT) with the threshold set to 8 to reduce false events.

Data and figures were prepared using the CytoExploreR package (v 1.1.0) for the R programming language (v 4.0.5) (https://github.com/DillonHammill/CytoExploreR [accessed June 25, 2022], https://www.R-project.org/ [accessed June 28, 2022]). All binding assays were performed in triplicate. The median fluorescence intensity (MFI) was calculated as the average of three runs for each strain and expressed as a percent relative to *L. plantarum* expressing RseP from *E. faecium* (rMFI). Percent relative standard deviations were calculated as the ratio of the sample SD to the MFI mean multiplied by 100%.

Phase contrast and fluorescence microscopy

The cells were stained with the FITC-labeled EntK1 as described for the binding assay. After discarding the remaining binding buffer, cells were resuspended in 25 μl of PBS, spotted on a microscopy slide, and overlayed with 2% low melting agarose in PBS to immobilize the cells. Phase-contrast images and FITC fluorescence images were obtained using a Zeiss Axio Observer with ZEN Blue software and an ORCA-Flash 4.0 V2 Digital CMOS camera (Hamamatsu Photonics) using a 100 × phase-contrast objective. The excitation light source was an HXP 120 Illuminator (Zeiss).

AlphaFold and structure analysis

The structure of RseP and complexes between RseP and EntK1 were predicted using the published open source code for AlphaFold according to the instructions by the AlphaFold team (46). All required databases were downloaded on February 10th 2022 and all templates prior to 2022 were included (–max_template_date = 2022-01-01). Interactions present in the predicted complexes were determined by the fully automated protein-ligand interactions profiler (47) and the interactions function implemented in the web-based molecular viewer iCn3D (48, 49). Figures were generated using PyMOL (http://www.pymol.org/pymol). Amino acid sequences used for *Efm*RseP and EntK1 are presented in Table S2.

Data availability

The AlphaFold computations were performed on resources provided by Sigma2 (allocations NN1003K and NS1003K) - the National Infrastructure for High Performance Computing and Data Storage in Norway. For DNA sequence of the

mutants and flow cytometry, the data will be shared upon request.

Supporting information—This article contains supporting information (50–52).

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: *Ec*RseP, *Escherichia coli* RseP; *Efm*RseP, *Enterococcus faecium* RseP; *Efs*RseP, *Enterococcus faecalis* RseP; EntK1, Enterocin K1; *Lp*RseP, *Lactiplantibacillus plantarum* RseP; MFI, median fluorescence intensity; MIC50, minimal inhibitory concentration; MRE β-loop, membrane-reentrant β-hairpin–like loop; RIP, regulated intramembrane proteolysis; S2P, site-2-metalloprotease; TMS, transmembrane segment.

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Supporting information

Table S1| Primers used in this study. Amino acid substitutions are highlighted in bold and underlined.

Name	Sequence (5'-3')
In-Fusion primers for amplification of *rseP*	
EfmRseP_F	GAGTATGATTCATATGAAAACGATTCTGACATTTATC
EfmRseP_R	TCGAACCCGGGGTACCCTAGAAAAAGAATCGTTGAATATCGTTCC
EfmRseP6His_R	CGAACCCGGGGTACCCTAATGATGATGATGATGATGGAAAAAGAATCGTTG
LlsRseP_RseP_F	GGAGTATGATTCATATGATAGAAACACTGATTACTTTTATT
LlsRseP_RseP_R	TCGAACCCGGGGTACCTTAATTTACAAAGGCTCGGAGAATATC
EfsRseP_F	GGAGTATGATTCATATGAAAACAATTATCACATTCATTATT
EfsRseP_R	TCGAACCCGGGGTACCTTAAAAGAAAAAGCGTTGAATATCGTTC
LpRseP_F	GGAGTATGATTCATATGATCGTTACAATTATTACGTTCATTA
LpRseP_R	CTGTAATTTGAAGCTTTTAGAAGAAATATCGCTGAATATCATTC
LpRseP6His_R	CTGTAATTTGAAGCTTTTAATGATGATGATGATGATGGAAGAAATATCGCTGAATATCATTC
SasRseP_F	GGAGTATGATTCATATGGTGAGCTATTTAGTTACAATAATTGCAT
SasRseP_R	TCGAACCCGGGGTACCTTATAAGAAATATCGTCGAATATCATTC
SaeRseP_F	GGAGTATGATTCATATGATAAAAATACGAGGTGTAGTTAATTTGA
SaeRseP_R	TCGAACCCGGGGTACCTTATAAGAAATAACGTTGTATATCATTCCTTCC
Primers for site-directed mutagenesis	
EfmH18A_F	TCTGACATTTATCATCGTTTTTGGTATATTAGTGATTGTT**GCG**GAGTTTGGTCATTTCTTCTTT
EfmH18A_R	AAAGAAGAAATGACCAAACTC**CGC**AACAATCACTAATATACCAAAAACGATGATAAATGTCAGA
EfmE19A_F	GGTATATTAGTGATTGTTCAT**GCG**TTTGGTCATTTCTTCTTTGCG
EfmE19A_R	CGCAAAGAAGAAATGACCAAA**CGC**ATGAACAATCACTAATATACC
EfmH22A_F	ATATTAGTGATTGTTCATGAGTTTGGT**GCG**TTCTTCTTTGCGAAACGATCAGGAATC
EfmH22A_R	GATTCCTGATCGTTTCGCAAAGAAGAA**CGC**ACCAAACTCATGAACAATCACTAATAT
EfmAAxxA_F	CGATTCTGACATTTATCATCGTTTTTGGTATATTAGTGATTGTT**GCGGCG**TTTGGT**GCG**TTCTTCTTTGCGAAACGATCAGG
EfmAAxxA_R	CCTGATCGTTTCGCAAAGAAGAA**CGC**ACCAAA**CGCCGC**AACAATCACTAATATACCAAAAACGATGATAAATGTCAGAATCG
EfmN359A_F	GATGGCGCTTCTTTCAATG**GCT**CTCGGAATCGTCAATCTG
EfmN359A_R	CAGATTGACGATTCCGAG**AGC**CATTGAAAGAAGCGCCATC
EfmN364A_F	AATGAATCTCGGAATCGTC**GCT**CTGCTTCCGATTCCTGCC
EfmN364A_R	GGCAGGAATCGGAAGCAG**AGC**GACGATTCCGAGATTCATT
EfmP367A_F	CGGAATCGTCAATCTGCTT**GCG**ATTCCTGCCTT
EfmP367A_R	AAGGCAGGAAT**CGC**AAGCAGATTGACGATTCCG
EfmP369A_F	CGTCAATCTGCTTCCGATT**GCT**GCCTTAGATGG
EfmP369A_R	CCATCTAAGGC**AGC**AATCGGAAGCAGATTGACG

Efm_D372A_F	TGCTTCCGATTCCTGCCTTA**GCT**GGCGGGAAATTA
Efm_D372A_R	TAATTTCCCGCC**AGC**TAAGGCAGGAATCGGAAGCA

Primers used for construction of *rseP* hybrids

Hyb1_F	GCCCGTACGGCCGGTTTAAAAGAAAATGATGAGGTAGTCAGTGT
Hyb1_R	ATCATTTTCTTTTAAACCGGCCGTACGGG
Hyb2_F	CACGGGTTCAGTTTGGATAAATTAGGCGGACCTGTCA
Hyb2_R	TCCGCCTAATTTATCCAAACTGAACCCGTGAGTGA
Hyb3_F	TGCGGCAGAAGCAGGCATTCAAAAGGGCGATCAAATC
Hyb3_R	TCGCCCTTTTGAATGCCTGCTTCTGCCGCA
LpRseP_XmaI_R	TTGGCGCCTTCGAACCCGGGTTAATGATGATGATGATGATGGAAGAAATATCGCT
Hyb4_F	ACAGGTTTCAGTTTAAACGATTTAGGTGGGCC
Hyb4_R	CCCACCTAAATCGTTTAAACTGAAACCTGTAAATAGTGATC
Hyb5_F	ATGCAAGGTGGTGTTACGAGTACAACGACCCA
Hyb5_R	GGTCGTTGTACTCGTAACACCACCTTGCATAAATGCCA
Hyb6_F	TCGGCTAAATTGTGGCAACGAATGTTGACGAATTTTGC
Hyb6_R	CGTCAACATTCGTTGCCACAATTTAGCCGATTGGA
Hyb7_F	CGATCAGGAATCCTCGTGCGTGAATTTTCTGTCGGGA
Hyb7_F2	CGATCAGGAATCCTCGTGCGTGAATTTTCTGTCGGGA
Hyb8_R	GTTGGTCAGCATACGTTGCCATAACTTGGCCGATT
Hyb8_F	GCCAAGTTATGGCAACGTATGCTGACCAACTTTGC
Hyb9a_R	AGTGTTCGTCACTTGAACACCACCCTGCATAAA
Hyb9b_F	ATGCAGGGTGGTGTTCAAGTGACGAACACTAATCGC

Primers used for construction of truncated *rseP*

Trunc_F	GCGAATTTGCTATCAAAGACGTACAGTTCCAATCGGCT
Trunc_R	GGAACTGTACGTCTTTGATAGCAAATTCGCGGACGA

Table S2| Amino acids sequences employed in AlphaFold-Multimer and structure analysis

Name	Sequence
EntK1	MKFKFNPTGTIVKKLTQYEIAWFKNKHGYYPWEIPRC
RseP from *E. faecium*	MKTILTFIIVFGILVIVHEFGHFFFAKRSGILVREFAIGMGPKIYGHQAKDGTTYTLRLLPIGGY VRMAGNGDDETEMAPGMPLSLLLNSDGIVEKINLSKKIQLTNAIPMELSRYDLEDELTITGY VNGDETEVVTYPVDHDATIIENDGTEIRIAPKDVQFQSAKLWQRMLTNFAGPMNNFILAI VLFIILAFMQGGVQVTNTNRVGEIMPNGAAAEAGLKENDEVVSVDGKEIHSWNDLTTVIT KNPGKTLDFKIEREGQVQSVDVTPKSVESNGEKVGQLGIKAPMNTGFMDKIIGGTRQAFS GSLEIFKALGSLFTGFSLDKLGGPVMMYQLSSEAANQGITTVISLMALLSMNLGIVNLLPIPA LDGGKLVLNIFEGIRGKPLSQEKEGILTLAGFGFLMLLMVLVTWNDIQRFFF	

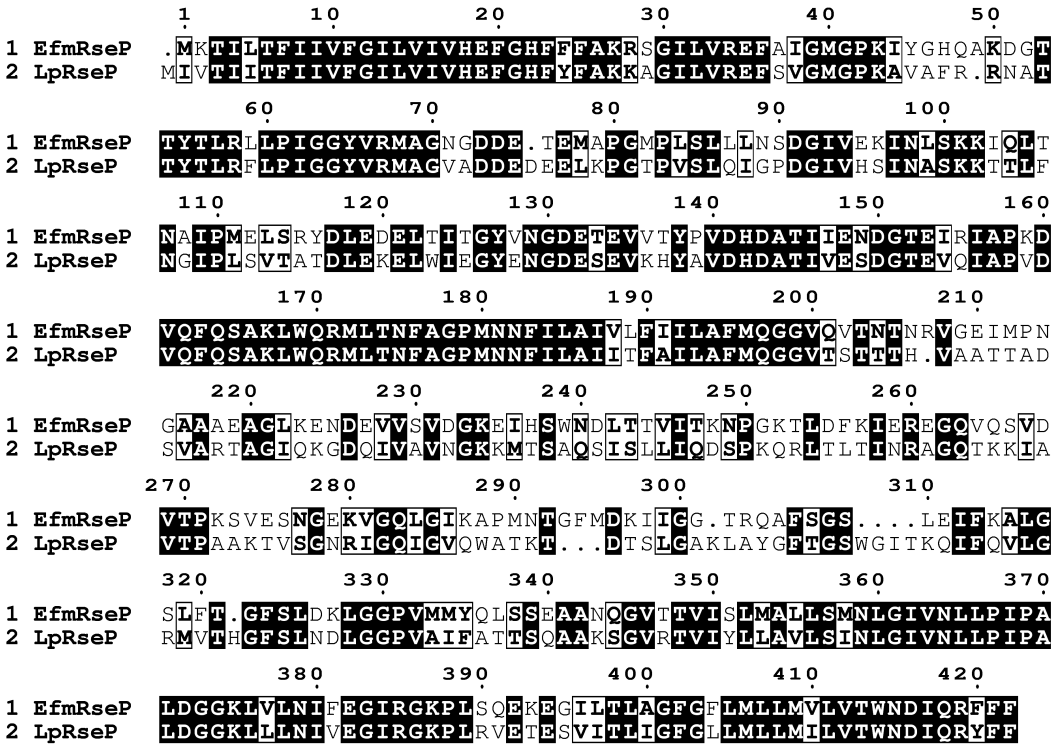

Figure S1| Pairwise sequence alignment of *Efm*RseP and *Lp*RseP. Identical amino acids are shaded in black while amino acids of similar chemical properties are boxed. Alignment was generated using EMBOSS Needle and the figure was exported using ESPript 3 web-server (51, 52).

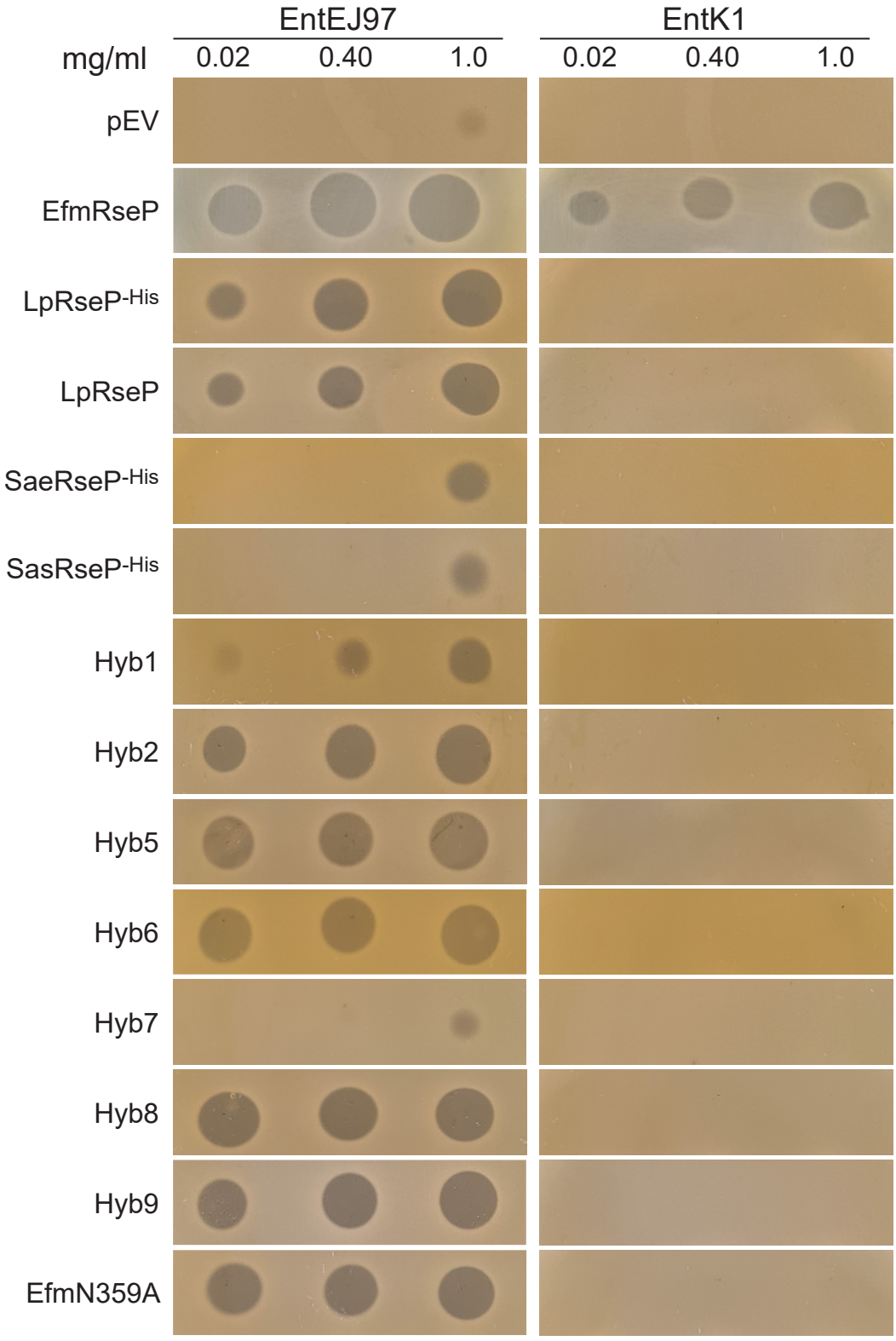
EntEJ97
EntK1
mg/ml
0.02
0.40
1.0
0.02
0.40
1.0
pEV
EfmRseP
LpRseP-His
LpRseP
SaeRseP-His
SasRseP-His
Hyb1
Hyb2
Hyb5
Hyb6
Hyb7
Hyb8
Hyb9
EfmN359A

Figure S2| EntEJ97 inhibition zones in spot-on-lawn assays. 3 µl of EntEJ97 and EntK1 (4–220 µM) was spotted on lawns of *L. plantarum* clones expressing different modified versions of RseP. Since RseP is the receptor for EntEJ97, increased sensitivity towards EntEJ97 compared to the empty vector pEV may indicate proper production and folding of the target protein expression. An inhibition zone of >10 mm was observed for all hybrids and point mutants tested, except for Hyb7, SaeRseP, SasRseP and pEV. A zone comparable to pEV was observed for Hyb7, SaeRseP and SasRseP (<6 mm).

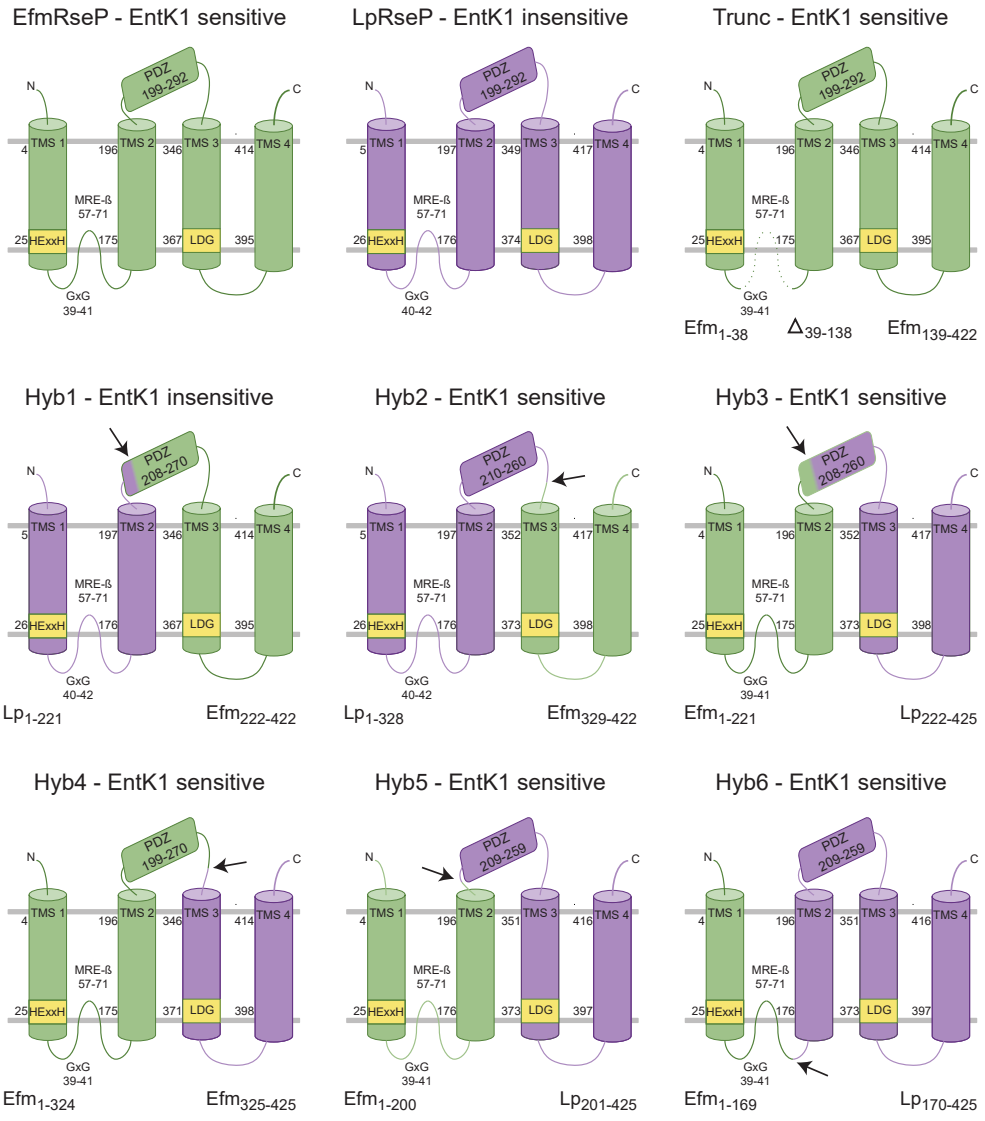

Figure S3A|Schematic representation of all RseP hybrids and truncated RseP. Hybrid proteins in which parts of *Lp*RseP (from the EntK1-insensitive *L. plantarum*, indicated in purple) were replaced with the corresponding sequence from *Efm*RseP (from the EntK1-sensitive *E. faecium*, indicated in green), were constructed. Conserved S2P motifs are indicated (HExxH, GxG, MRE-ß, PDZ, LDG). TMS1-4 indicate the four predicted transmembrane segments (TMS), while the black arrows indicate the point(s) of fusion between *Lp*RseP and *Efm*RseP regions. The gray dashed line indicates part of the full-length *Efm*RseP deleted in Trunc. Protein variants giving a MIC50 ≥22 µM EntK1 are marked as fully EntK1-resistant, while protein variants giving MIC50 values below 2.7 µM are marked as EntK1-sensitive. Note that sensitivity data for Hyb7 are uncertain due to possible expression issues visible in the control experiment of Fig. S2. For MIC50 values, see Table 3.

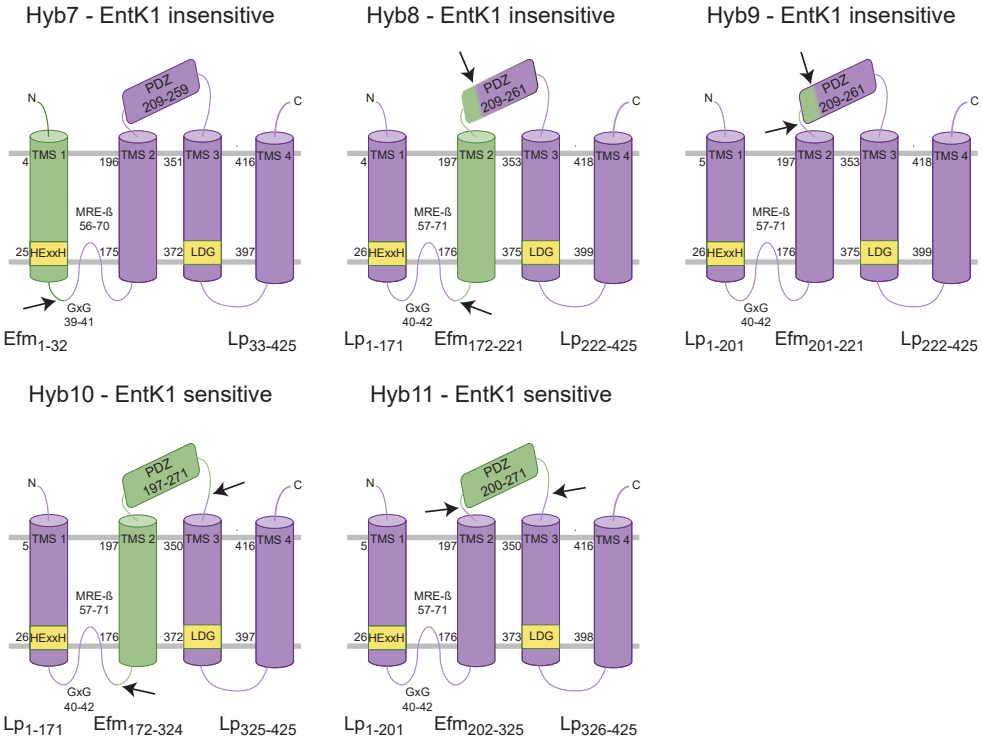

Figure S3B |Schematic representation of all RseP hybrids and truncated RseP.

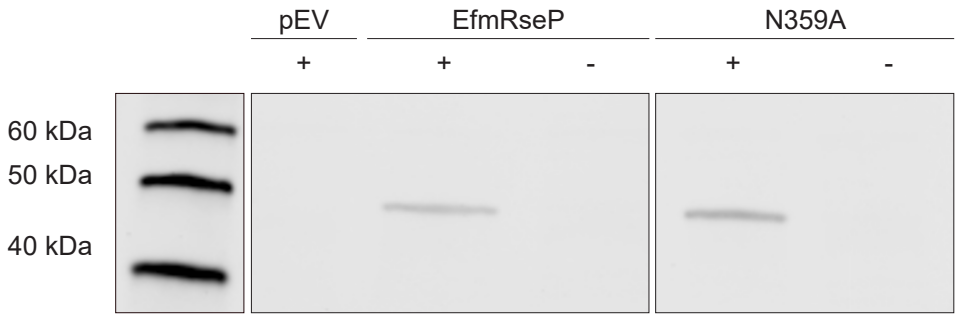

Figure S4|Western blot. pEV, EfmRseP and the N359A mutant under inducing (+) and non-inducing (-) conditions. Cells were harvested, lysed and subjected to SDS-PAGE as previously described (50), with minor modifications. The harvested cells were resuspended in 250 µl NP-40 lysis buffer containing 1 mM PMSF (phenylmethylsulfonyl fluoride). Samples where not boiled prior to SDS-PAGE. Following electrophoresis, proteins were electroblotted onto nitrocellulose mini membranes using iBlot™ Transfer Stack (Invitrogen) and the iBLot Gel transfer device (Invitrogen). The membrane was washed with Tris-Buffered Saline (TBS; 2 × 10 min), then subsequently incubated with blocking buffer (5% Bovine Serum Albumin (BSA) dissolved in TBS) for 1 h and washed again (2 × 10 min Tween-TBS (TTBS; 0.05% Tween-20), 1 × 10 min TBS). The membrane was incubated with Penta-His (Qiagen) (1:1000 in Blocking buffer) for 30 min, following an overnight incubation at 4 °C. The subsequent day the membrane was incubated for 30 min at room temperature, washed (2 × 10 min, TTBS), and incubated with the polyclonal HRP-conjugated anti-mouse IgG (Sigma-Aldrich) secondary antibody (1:5000) in blocking buffer for 1 h. To remove unbound secondary antibodies the membrane was washed 4 × 10 min with TTBS. The blots were subsequently visualized using the SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Fisher Scientific) and image using the Azure c400 system (Azure Biosystem, Dublin, CA).

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Flow cytometric detection of vancomycin-resistant *Enterococcus faecium* in urine using fluorescently labelled enterocin K1

Thomas F. Oftedal[1]✉ & Dzung B. Diep[1,2]

A urinary tract infection (UTI) occurs when bacteria enter and multiply in the urinary system. The infection is most often caused by enteric bacteria that normally live in the gut, which include *Enterococcus faecium*. Without antibiotic treatment, UTIs can progress to life-threatening septic shock. Early diagnosis and identification of the pathogen will reduce antibiotic use and improve patient outcomes. In this work, we develop and optimize a cost-effective and rapid (< 40 min) method for detecting *E. faecium* in urine. The method uses a fluorescently labelled bacteriocin enterocin K1 (FITC-EntK1) that binds specifically to *E. faecium* and is then detected using a conventional flow cytometer. Using this detection assay, urine containing *E. faecium* was identified by an increase in the fluorescent signals by 25–73-fold (median fluorescence intensity) compared to control samples containing *Escherichia coli* or *Staphylococcus aureus*. The method presented in this work is a proof of concept showing the potential of bacteriocins to act as specific probes for the detection of specific bacteria, such as pathogens, in biological samples.

Urinary tract infections (UTIs) are among the most common infections in humans, and account for significant health-care costs and morbidity[1–3]. Women are predominantly affected by UTIs with 13% of women self-reporting having a UTI compared with 3% of men (data from NHANES III, 1988–1994)[4]. UTIs are most commonly caused by bacteria entering the urethra, and usually involve bacteria of the gut microbiota[5]. A UTI is an infection in any part of the urinary tract, such as the bladder, ureters, urethra, or kidneys, but occurs most commonly in the bladder (cystitis), which can progress to pyelonephritis (infection of the kidney)[5]. In pregnant women UTIs are associated with preterm birth and reduced birth weight of the infant[6,7]. If left untreated, UTIs can lead to complications such as kidney stones or systemic bloodstream infections[8,9]. The laboratory diagnostic criterium for UTIs is the presence of at least one bacterial species with a total count $\geq 10^5$ CFU/ml which is determined via urine culture of midstream urine, a diagnostic procedure that typically takes 24–48 h[10]. Because urine culture is slow, determination of the causative microorganism and its antibiotic resistance profile is rarely obtained prior to management of the infection[11]. Consequently, most clinical guidelines currently recommend the diagnosis and management of uncomplicated UTIs based solely on symptoms[11]. In some cases, up to 90% of patients with urinary symptoms receive antibiotics, often without obtaining a urine culture[11]. A faster diagnostic procedure for UTIs would increase positive health outcomes in patients and reduce the unnecessary use of antibiotics[12,13].

In recent years, new methods have been proposed for faster diagnosis of UTIs, such as special-purpose flow cytometers and direct biotyping from urine using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)[14–17]. A major drawback of MALDI-TOF MS is the cost associated with the acquisition of the instrumentation and the proprietary software and databases necessary for its clinical use[18]. Special-purpose flow cytometers such as the Sysmex urinalysis devices are more affordable, easy to use, and claims to rule out potential UTIs within minutes[19]. The Sysmex devices rely on a dedicated mixing chamber where all bacteria are stained with a fluorescent nucleic acid binding dye, which is necessary for detection[15]. Antimicrobial peptides (AMPs) and antibiotics have been explored for the labeling and detection of pathogenic bacteria. Labeled ubiquicidin (29–41) was shown to localize to the sites of infection by *Pseudomonas aeruginosa* or *Staphylococcus aureus* in mice[20,21]. Similarly, fluorescently labelled vancomycin was shown to detect infections by *S. aureus* in a mouse myositis model[22]. Using Cy5-labeled cecropin P1, detection of *Escherichia coli* O157:H7 was enhanced tenfold compared to antibody-based detection[23]. AMPs produced by bacteria are known as bacteriocins, which

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resemble AMPs in many aspects. However, bacteriocins have much higher potency and a narrow spectrum of activity, typically being active only towards species closely related to the producer[24]. The narrow targeting of many bacteriocins is due to specific receptor molecules exploited by these peptides to target cells[25–27]. Bacteriocins show high potency and specificity towards many species of bacteria, including those implicated in UTIs[28–30]. However, the use of bacteriocins for detection remains largely unexplored. Many bacteriocins, especially those that are unmodified, can easily be synthesized commercially with fluorescent labels.

Bacteriocins are a heterogeneous group of ribosomally synthesized antimicrobial peptides produced by virtually all bacterial species[31]. Although bacteriocins are typically only active against species closely related to the producer, there are bacteriocins with broad-spectrum activity[32–34]. Bacteriocins are particularly interesting because of their high specificity and high potency against antibiotic-resistant strains[35]. Members of the LsbB family of bacteriocins include enterocin EJ97 (EntEJ97), enterocin K1 (EntK1), lactococcal small bacteriocin B (LsbB), enterocin Q (EntQ) and the engineered hybrid bacteriocin H1[36–38]. All members are small (30–44 amino acids), leaderless, unmodified, and exploit the same membrane-bound site-2 metalloprotease RseP as a receptor for its antimicrobial activity[38–40]. The C-terminal tail of these bacteriocins is thought to be important for receptor interaction[41]. While enterocin EJ97 displays a broader inhibition spectrum including *E. faecium* and *E. faecalis*, EntK1 and LsbB have a much narrower inhibition spectrum. LsbB is only active against strains of *L. lactis,* while EntK1 mostly toward *E. faecium*, including both nosocomial and vancomycin-resistant (VRE) strains[38]. The target specificity of the bacteriocins is primarily due to subtle sequence differences in RseP between species[38–40]. The small and unmodified nature of the LsbB family of bacteriocins makes them ideal for synthetic production and chemical modifications, that can be used to develop them into useful tools for therapeutic and diagnostic applications.

The aim of this study was to develop the narrow spectrum bacteriocin EntK1 into a molecular probe for cost- and time-effective detection of *E. faecium*. The procedure involves a binding step that allows the fluorescent peptide to bind to target cells, followed by detection using a conventional flow cytometer. We further validated the procedure with urine samples to simulate UTIs. We believe that the potential of a fast and species-specific detection method offered by these peptides would reduce the unnecessary use of antibiotics.

Results

To enable detection of the bacteriocin EntK1, the peptide was chemically synthesized with a FITC fluorescent label conjugated to the N-terminus. FITC is a small (389 Da) and widely used fluorophore with excitation and emission maxima typically measured at 494 nm and 518 nm, respectively[42]. The fluorophore was chosen due to its relatively small size (380 Da) compared to EntK1 (4564 Da) and conjugated to the N-terminus to avoid interfering with the bacteriocin-receptor interaction[25,41]. The minimum inhibitory concentration (MIC) of the labelled EntK1 (FITC-EntK1) was at nanomolar concentrations against *E. faecium* LMGT 3104 (a VRE strain; also designated LMG 20705)[33]. The low MIC of the modified peptide indicates that it is still relatively potent, although the potency was reduced about fourfold compared to the non-modified EntK1 (156 nM for FITC-EntK1 compared to 39 nM for EntK1). A concentration slightly above the MIC_{90} of FITC-EntK1 (0.2 µM) was chosen for further binding experiments. In this work, the term "binding" will be used to describe any measurable association of FITC-EntK1 with cells.

Initial attempts at measuring the binding of FITC-EntK1 to *E. faecium* in physiological buffers (such as PBS) using flow cytometry were not successful. The increase in the fluorescence signal in samples with added FITC-EntK1 was negligible compared to unstained controls, even when the concentration of FITC-EntK1 was increased to 1 µM and the incubation time increased to 2 h (see Fig. 1A). The failure to detect cells with bound FITC-EntK1

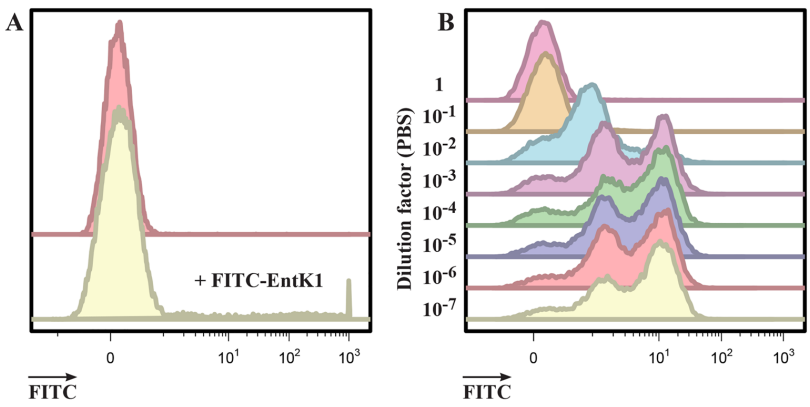

Figure 1. *E. faecium* at approximately 10^5 CFU/ml incubated for 2 h in PBS. (**A**) With and without 1 µM FITC-EntK1, as indicated. (**B**) Binding performed in PBS and a tenfold serial dilution of PBS in pure water from undiluted 1X PBS (top) to 10^{-7} times diluted (bottom).

could be due to cell death and lysis, however, the mode of action appears to be non-lytic, and no morphological changes of the cells were apparent even after 2 h (see Fig. S1). In addition, the number of events measured by the flow cytometer from samples with FITC-EntK1 were comparable to unexposed controls. However, when using diluted buffers during the binding step, the fluorescence intensity of *E. faecium* increased (Fig. 1B).

Although the fluorescence of *E. faecium* increased in dilute PBS, the binding appeared to be inefficient as the distribution had two peaks (bimodal) with a long tail, resulting in reduced median fluorescence intensity values (MFI). To investigate if other buffers could improve binding, a large selection of buffers were tested with 0.2 µM FITC-EntK1 at varying incubation times and ionic strengths. Best results were obtained with the citrate-based buffer triammonium citrate pH 6.8 (TAC) at a concentration of 0.1 mM (Fig. 2). Interestingly, in this buffer, the maximum fluorescence was obtained after only 15 min of incubation, and samples with longer incubation times showed no or only a negligible further increase (see Fig. S2).

The short incubation time of 15 min was confirmed by a killing kinetics assay showing that EntK1 and FITC-EntK1 kill target cells rapidly in 0.1 mM TAC buffer. As seen in Fig. 3, exposure to both EntK1 and FITC-EntK1 at 0.2 µM resulted in a 4-log reduction in viable cells after one minute, with a complete reduction of viable cells after 15 min of exposure to the bacteriocins. The binding buffer alone showed no reduction in cell viability.

Next we attempted to demonstrate the binding of FITC-EntK1 to *E. faecium* originating from urine. To do this, a clinical case of UTI was simulated by adding 10^5 CFU/ml of *E. faecium* LMGT 3104 to urine samples. The measured binding of FITC-EntK1 to *E. faecium* directly in urine was small with a high sample-to-sample variance, likely due to the relatively high and varying salt content. To remove the effect of solutes in urine on

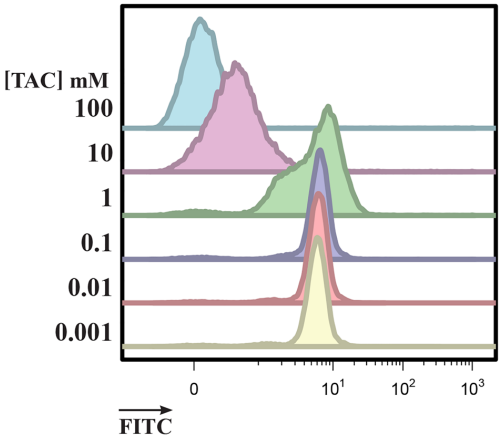

Figure 2. Binding assay of FITC-EntK1 to *E. faecium* (10^5 CFU/ml). The assay was performed in varying concentrations from 100 to 0.001 mM triammonium citrate buffer (TAC) with a 15 min binding step.

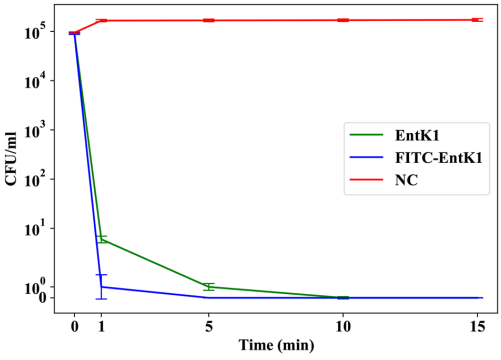

Figure 3. Killing kinetics assay. Number of viable cells in colony-forming units (CFU) in 5 ml of 0.1 mM TAC buffer following the addition of EntK1 (green line) or FITC-EntK1 (blue line) to 0.2 µM. A negative control with no added antimicrobial is shown in red (NC). Error bars are ± SE (standard error). The figure was generated using Python 3.8.8 with a symlog y-axis (linear in the range −5 to 5).

binding, a one-step enrichment of bacteria was first performed by centrifugation. Using flow cytometry, samples containing *E. faecium* showed a 48–59-fold increase in the median fluorescence intensity (MFI) following a 15-min incubation with 0.2 µM FITC-EntK1 in 0.1 mM TAC buffer (see Fig. 4). A similar fold-increase was observed for all biological replicates. Samples containing *E. faecium* with no added FITC-EntK1 had a mean MFI value of 0.1 (n = 12, SD = 0.027, *p*-value = 0.000028). The reproducibility of the method made it possible to distinguish urine samples containing *E. faecium* (i.e., from an infected individual) from a healthy control.

To further investigate if the observed binding was specific to *E. faecium* or if FITC-EntK1 in 0.1 mM TAC buffer would bind unspecifically to any bacteria present in the sample, two species also implicated in UTIs namely *E. coli* and *S. aureus* were included in the assays. Both strains were confirmed to be insensitive to EntK1 and FITC-EntK1 ($MIC_{90} > 200$ µM). As shown in Fig. 5, only urine samples containing *E. faecium* showed a shift in fluorescence intensity.

Samples with *E. coli* or *S. aureus* showed similar fluorescence values to controls with no added FITC-EntK1 (corresponding to background levels of fluorescence). The increase in MFI for *E. faecium* relative to *S. aureus* or *E. coli* was 25–73-fold and reproducible in all assays performed (see Table 1). Density plots of SSC-A and FSC-A for these experiments are presented in Fig. S3.

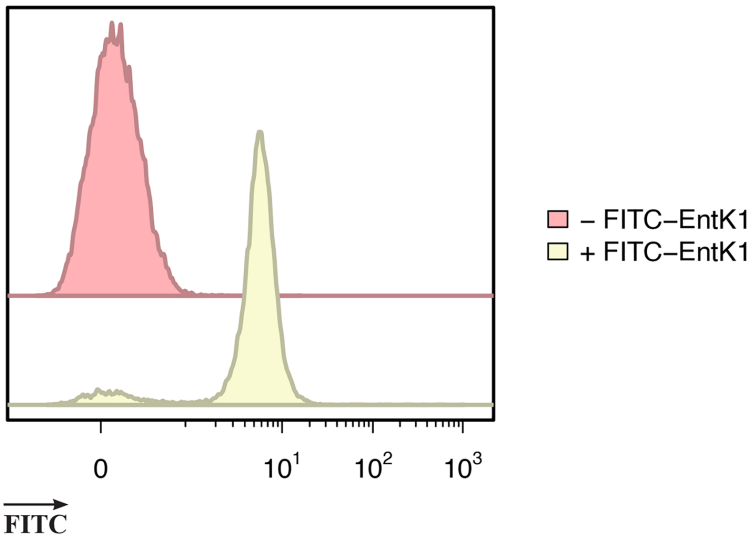

Figure 4. FITC-EntK1 binds to *E. faecium* from urine. Fluorescence of unstained *E. faecium* LMGT 3104 from urine (red), fluorescence following a 15 min incubation with FITC-EntK1 (yellow). Representative figure from twelve independent experiments.

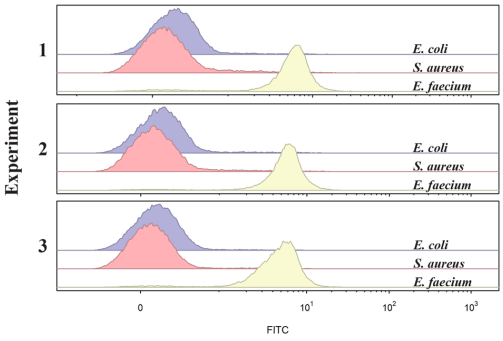

Figure 5. Fluorescence (FITC; 525/50 nm) obtained from urine samples containing *S. aureus*, *E. coli*, and *E. faecium*. Samples containing *E. faecium* show a positive shift in fluorescence (three independent experiments).

Replicate	CFU/ml*	MFI
E. faecium		
1	9.3×10^4	5.88
2	9.6×10^4	5.80
3	8.8×10^4	4.87
S. aureus		
1	1.17×10^5	0.10
2	9×10^4	0.11
3	1.03×10^4	0.08
E. coli		
1	1.07×10^5	0.19
2	1.06×10^5	0.17
3	1.15×10^5	0.13

Table 1. Summary of three independent detection assays. Urine samples containing *E. faecium*, *E. coli* and *S. aureus* (three biological replicates). Total bacteria count determined for each urine sample used in the assay is shown in CFU/ml. *Mean of three technical replicates, rounded to the nearest thousand.

During a UTI, a second microorganism might be present at a high number together with the causative agent, typically with at least one of them present at 10^5 CFU/mL or more. The simultaneous presence of another microorganism could influence and interfere with the binding of FITC-EntK1 to *E. faecium*. To test this, 10^5 CFU/ml of *E. faecium* was pre-mixed with 10^5 CFU/ml of *E. coli* or *S. aureus*. The binding of FITC-EntK1 to *E. faecium* in the presence of another species was examined by confocal laser scanning microscopy (CLSM), which allowed us to distinguish each species based on differences in morphology (see Fig. 6).

Cells with a morphology consistent with *E. faecium* (diplococci) exhibited a visible fluorescence signal. While a lesser signal or no signal was apparent for *S. aureus,* which are predominantly in chains or clusters as single cells (cocci, spherical), while *E. coli* are rod-shaped cells.

Although many laboratories use a bacterial count $\geq 10^5$ CFU/ml as a diagnostic criterion for UTIs, many laboratories have opted to use a lower colony count of 10^3–10^4 CFU/ml[43,44]. The higher threshold has been shown to miss many relevant infections, as healthy urine should otherwise appear sterile by commonly used cultivation techniques. A lower threshold will detect more cases of UTIs and allow for earlier intervention. To determine the ability of the presented method to detect *E. faecium* in urine samples at the lower threshold of 10^3–10^4 CFU/ml, a serial dilution of cells from ~ 10^5 to 3×10^3 CFU/ml was prepared in urine. A sample with no added bacteria was included as a comparison. Because of the high relative proportion of noise at low cell counts, control samples of urine without added *E. faecium* and with *E. faecium* only (without added FITC-EntK1) were used to determine the light scattering characteristics of *E. faecium* bacteria. Based on these controls, a gating strategy was constructed to capture *E. faecium* and reduce noise (Fig. 7A).

As shown in Fig. 7B, urine samples inoculated with *E. faecium* were clearly distinguishable from the control even when present at only 3×10^3 CFU/ml**.** The MFI was 30.1 for the lowest cell count tested, compared to 0.11 for the control with no added cells. Fluorescence and gating data are presented in Table S1 and density plots in Fig. S4.

Materials and methods

Bacteriocin stock preparation. Enterocin K1 (EntK1) and FITC-EntK1 were synthesized by Pepmic Co., Ltd. (Suzhou, China) with >95% purity. The FITC fluorescent label was conjugated to the N-terminal via a 6-aminohexanoic acid linker. Both peptides were solubilized in MilliQ water to a stock concentration of 200 µM for use in all assays.

Minimum inhibitory concentration. Twofold dilutions of EntK1 and FITC-EntK1 in BHI were prepared in 96-well microtiter plates to a volume of 100 µl per well. Each well was then inoculated with 100 µl of a 25-fold diluted overnight culture of *E. faecium* LMGT 3104 (50-fold final dilution). After incubation at 37 °C for 6 h, the turbidity was measured by a spectrophotometer SPECTROstar Nano reader (BMG Labtech) at 600 nm. The MIC_{90} was defined as the concentration of bacteriocin necessary to inhibit growth by 90% or more in 200 µl of culture (having a turbidity equal to 10% or less of a positive control with no antimicrobial).

Killing kinetics assay. A culture of *E. faecium* LMGT 3104 was grown overnight at 37 °C in Brain Heart Infusion broth (BHI). The culture was diluted in BHI to the desired cell count (using a standard curve of turbidity/OD_{600} to CFU per ml) before being added to 5 ml of binding buffer (0.1 mM tri-ammonium citrate, 0.25 M sucrose, pH 6.5) to approximately 10^5 CFU/ml. Actual bacterial counts in each suspension were determined in all assays by plate counting. Briefly, samples were immediately diluted 100-fold in sterile saline (0.9% NaCl) and 0.1 ml of the dilution spread on BHI agar plates. Bacteriocins EntK1 and FITC-EntK1 was added to 0.2 µM final concentration and samples were taken for plate counting as described above at 1, 5, 10 and 15 min. A control with no added antimicrobial was also included to assess any potential antimicrobial effect of the binding buffer.

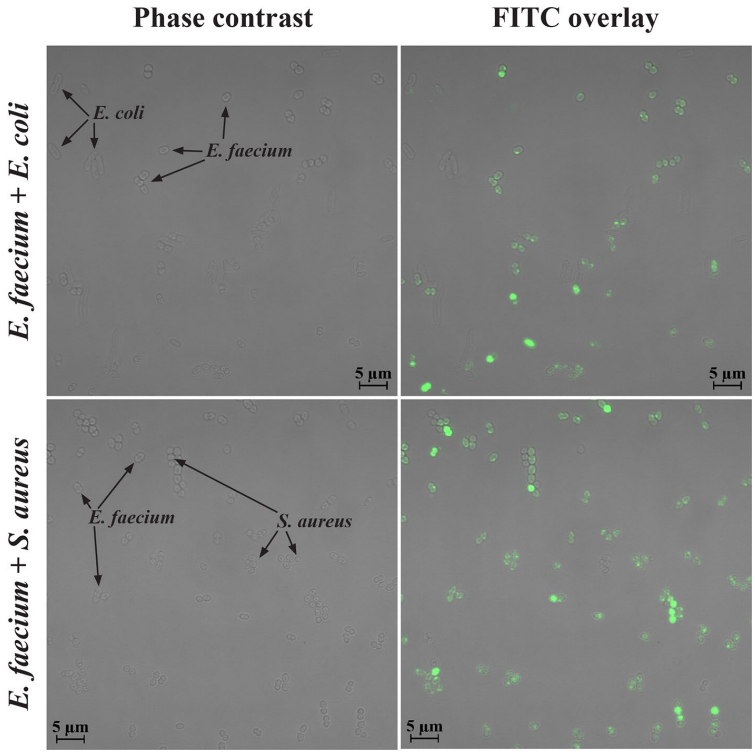

Figure 6. Fluorescence microscopy of mixed cultures. *E. faecium* mixed with *S. aureus* (**A**), and *E. faecium* mixed with *E. coli* (**B**). Images were taken following a 15-min incubation in 0.1 mM TAC buffer with 0.2 µM FITC-EntK1, cells were mixed at equal numbers. Overlay of fluorescence and phase-contrast (transmitted light) channels.

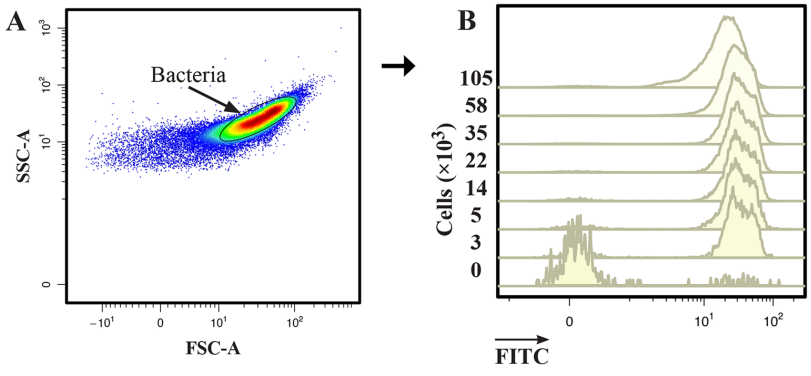

Figure 7. Limit of detection. A gate was constructed for the sample with the highest count of *E. faecium*, and fluorescence was measured on events within the gate (**A**). Fluorescence signal from urine samples containing the indicated number of cells in CFU/ml (**B**). Representative figures from three independent experiments.

The assay was performed in triplicate, and the data presented is the mean of all assays with the corresponding sample standard deviation (SD).

Sample preparation. Urine was sampled from healthy laboratory staff (30 ml per sample) and artificially inoculated with approximately 10^5 CFU/ml of *E. faecium* LMGT 3104, *E. coli* TG1, or *S. aureus* RN4220 as described above. For the limit of detection experiments, a serial dilution of *E. faecium* was prepared in sterile saline (0.9% NaCl) before being added to the urine sample. After adding bacteria to the samples, the actual bacterial counts in all samples were determined by serial dilution in sterile saline and plate counting (three technical replicates per biological replicate). Cells were collected by centrifugation (7500 g, 5 min) and resuspended in binding buffer (0.1 mM tri-ammonium citrate, 0.25 M sucrose, pH 6.5) containing 0.2 µM FITC-EntK1. Samples were incubated for 15 min on a Multi Bio RS-24 rotator (BioSan, Riga, Latvia) at room temperature for binding. Following the binding step, cells were filtered through a 20 µm cell-strainer (EASYstrainer small, Greiner) and washed once in sterile-filtered phosphate-buffered saline (PBS; 137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) by centrifugation as described above, then resuspended thoroughly in 0.5 ml of PBS by vortexing for 5–10 s. The suspension (25 µl) was directly measured by flow cytometry.

Flow cytometry. All samples were analyzed by flow cytometry using a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Events were recorded using a low flow rate (25 µL/min) using the green 488 nm laser for excitation (25 mW laser power) and emission detector B1 (525/50 nm filter) with a detector voltage of 400 V. A trigger threshold was set to 3 using side scattered light (SSC-H; 370 V detector voltage) to reduce excess noise in the measurements. Except for the limit of detection experiments, all flow cytometry data was ungated, and all recorded events were included in the calculations. Statistical comparison was performed using the Mann–Whitney U Test implemented in R. The grating strategy used for the limit of detection experiments is provided in Fig. 7A. Data and figures were prepared using the CytoExploreR package (v 1.1.0) for the R programming language (v 4.1.1).

Microscopy. Urine samples (30 ml) were inoculated with mixed cultures at approximately 5×10^4 CFU/ml of *E. faecium* LMGT 3104, *S. aureus* RN4220 or *E. coli* TG1. The cells were stained with the FITC-labeled EntK1 as described for the sample preparation above. After the washing step, the cells were resuspended in 25 µl of PBS and then spotted on a microscopy slide overlayed with 2% low-melting agarose in PBS to immobilize the cells. Phase-contrast images and FITC fluorescence images were obtained using a confocal laser scanning microscope (LSM700, Axio Observer.Z1, Zeiss, Germany) equipped with an EC Plan-Neofluoar 100x/1.3 objective. Fluorescence was detected with excitation using the 488 nm laser line and measuring emission at wavelengths above 510 nm. Images were processed with ZEN 2012 software.

Discussion

In this study, we show that the bacteriocin FITC-EntK1 can function as a molecular probe that preferentially binds to or associates with *E. faecium*. The detection assay presented in this work allowed us to positively identify urine samples with 10^5 CFU/ml of *E. faecium* present. The assay is both rapid and appears to be species-specific, which could enable early and targeted intervention in a clinical setting. Additionally, a clear shift in fluorescence was observed for urine samples containing as few as 3×10^3 CFU/ml compared to healthy controls, which is below the lowest clinical threshold proposed for the diagnosis of UTIs[45,46]. Although the prevalence of UTIs caused by *E. faecium* is relatively low (~ 2%)[47], the concept of using bacteriocins as probes for detection and diagnosis is largely unexplored. There exists a great diversity of bacteriocins that target various pathogenic species in a specific receptor-mediated manner that could be developed for detection, as demonstrated in this work for EntK1. Bacteriocins active against the most prevalent urinary pathogens have been characterized, such as colicins and microcins against *E. coli*, klebicins against *Klebsiella,* and pyocins against *Pseudomonas*[48–50]. Many bacteriocins targeting Gram-negative bacteria are large proteins (40–70 kDa) and therefore likely impractical as probes. However, it seems plausible that only the smaller receptor-binding domain of such bacteriocins would bind with high affinity to the receptor and could therefore function as probes.

In our study, careful optimization of the binding conditions was necessary to demonstrate the binding of FITC-EntK1 to *E. faecium* populations. Buffers with high ionic strength, such as PBS, showed only a negligible difference in the fluorescent signals produced by *E. faecium* with or without FITC-EntK1. In contrast, all species tested showed binding to FITC-EntK1 in all non-ionic solutions, likely due to unspecific electrostatic interactions with the cell surface. By gradually decreasing the ionic strength of all buffers tested, the binding of FITC-EntK1 to *E. faecium* increased (see Fig. 2). Presumably, the ions in solution shield or neutralize the charges on the cell surface and bacteriocin, thereby reducing electrostatic interactions between the bacteriocin and cells. The effect of solutes on bacteriocin adsorption to cells suggests that defined conditions will be necessary for a reliable detection system.

The low binding measured between FITC-EntK1 and cells in physiological buffers is consistent with previous literature showing reduced sensitivity to bacteriocins in solutions of increasing ionic strength, which is assumed to lower the affinity of the peptides to the cell surface[51–53]. Interactions of bacteriocins such as EntK1 with the cell surface is initially believed to be dominated by electrostatic interactions[54]. Bacteriocins predominantly contain an excess of positively charged amino acids (EntK1 has a net charge of 5 at pH 7 and an isoelectric point at pH 10.17), and the bacterial cell surface possesses a net negative electrostatic charge due to phosphoryl and carboxylate groups[54,55].

Detection of pathogenic bacteria directly from biological fluids without the need for a separation step would reduce the protocol time. However, we believe bacterial separation and enrichment from bodily fluids other than

blood could be performed in minutes by simple filtration and/or centrifugation techniques, as demonstrated for urine. All binding experiments in this work used urine sampled from healthy individuals, urine from infected individuals often contains traces of blood and/or neutrophils (pyuria) that could interfere with the detection assay in a clinical setting. Most eukaryotic cells should not pass the 20 μm filter used in the assay protocol, however, further work is needed to assess the method using urine from infected individuals. In the absence of fluorescent labels, conventional flow cytometers are poor at small-particle detection (< 3 μm) such as bacteria (0.5–2 μm), which appear indistinguishable from noise (e.g., inherent electrical noise, internal reflections, stray light, or dust and debris in the buffers) when analyzed by light scatter. However, by selectively staining bacteria with fluorescent bacteriocins such as FITC-EntK1, bacteria can be detected with sufficient sensitivity.

This work presents a proof of concept of using bacteriocins with specific activity as probes for the detection of target bacteria. The detection assay developed in this work for EntK1 shows good sensitivity and specificity, positively identifying urine samples containing the clinical threshold of 10^5 CFU/ml of *E. faecium*. The detection assay could likely be further developed and optimized for other bacteriocins and for other clinically important bodily fluids such as cerebrospinal, synovial, ascitic, or amniotic fluids. We foresee a role of bacteriocins in the design and development of diagnostic kits and methods, providing rapid and specific identification of their target bacteria. However, further work is needed to establish the potential and broader applicability of the proof of concept presented in this work.

Data availability

The data underlying the results presented in the study are available upon request to T.F.O at thof@nmbu.no.

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Author contributions

T.F.O. designed the experiments, conducted the study, analyzed the data, and wrote the original draft. D.B.D. acquired funding, supervised, and conceived the study. T.F.O and D.B.D contributed to writing, reviewing, and editing the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Materials

Flow cytometric detection of vancomycin-resistant *Enterococcus faecium* in urine using fluorescently labelled enterocin K1

Thomas F. Oftedal[1,*] and Dzung B. Diep[1,†]

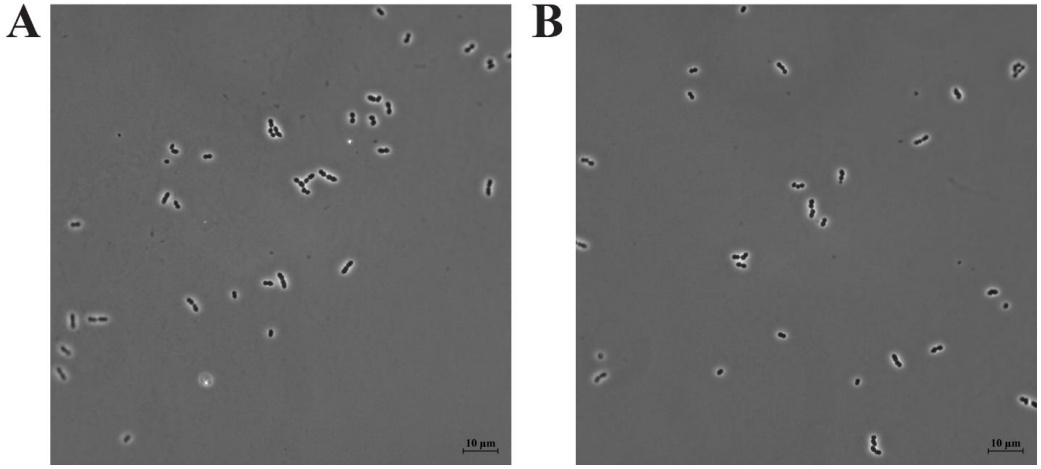

Fig S1. Phase contrast microscopy of *E. faecium* incubated for 2 hours in PBS (A), and with PBS containing 1 µM FITC-EntK1 (B).

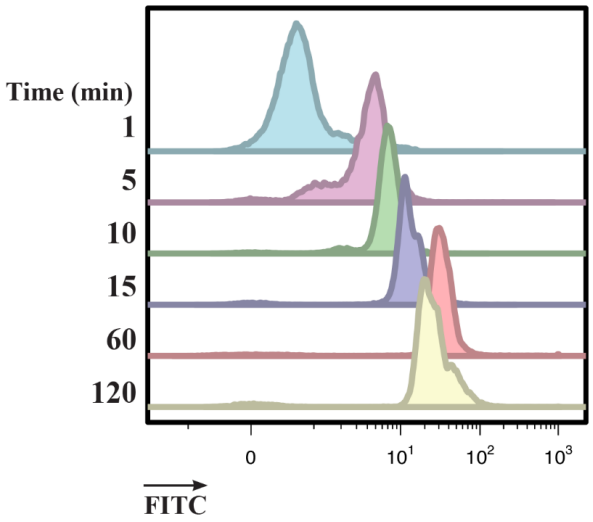

Fig S2. Effect of incubation time on the fluorescence intensity of *E. faecium* (10^5 CFU/ml). Cells were incubated in 0.1 mM triammonium citrate (pH 6.8) containing 0.2 µM FITC-EntK1 at various time points (1-120 minutes).

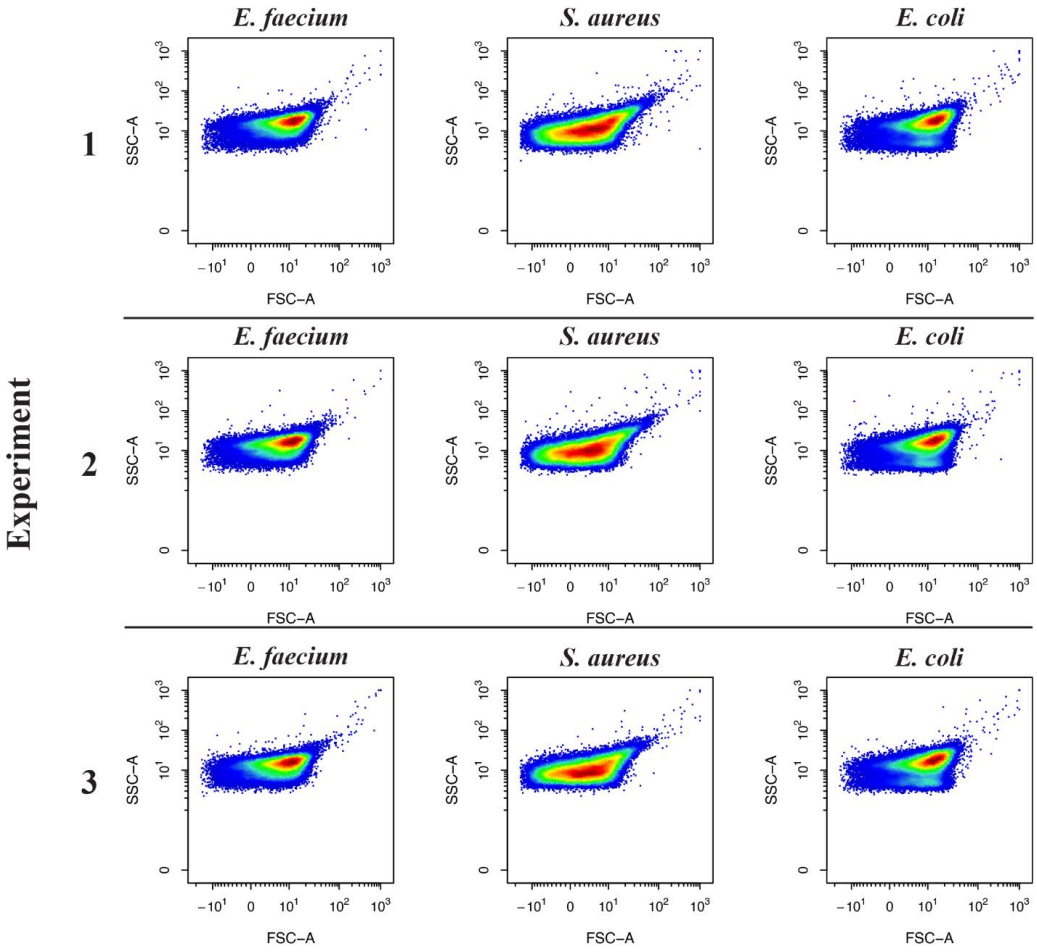

Fig S3. Density plots (dot plots) of flow cytometry measurements presented in Figure 5.

Table S1. Flow cytometry data obtained from the limit of detection experiment; values are representative from three biological replicates.

Cells (×10^3 CFU/ml)	Events	Gated (%)	MFI Gated
105	204431	130836 (64%)	19.4
58	121676	73234 (60%)	29.0
35	69371	37954 (55%)	32.0
22	44024	21157 (48%)	31.8
14	28003	10666 (38%)	31.9
5	21967	6355 (29%)	30.3
3	16809	3518 (21%)	30.1
0	12116	262 (2.2%)	0.11

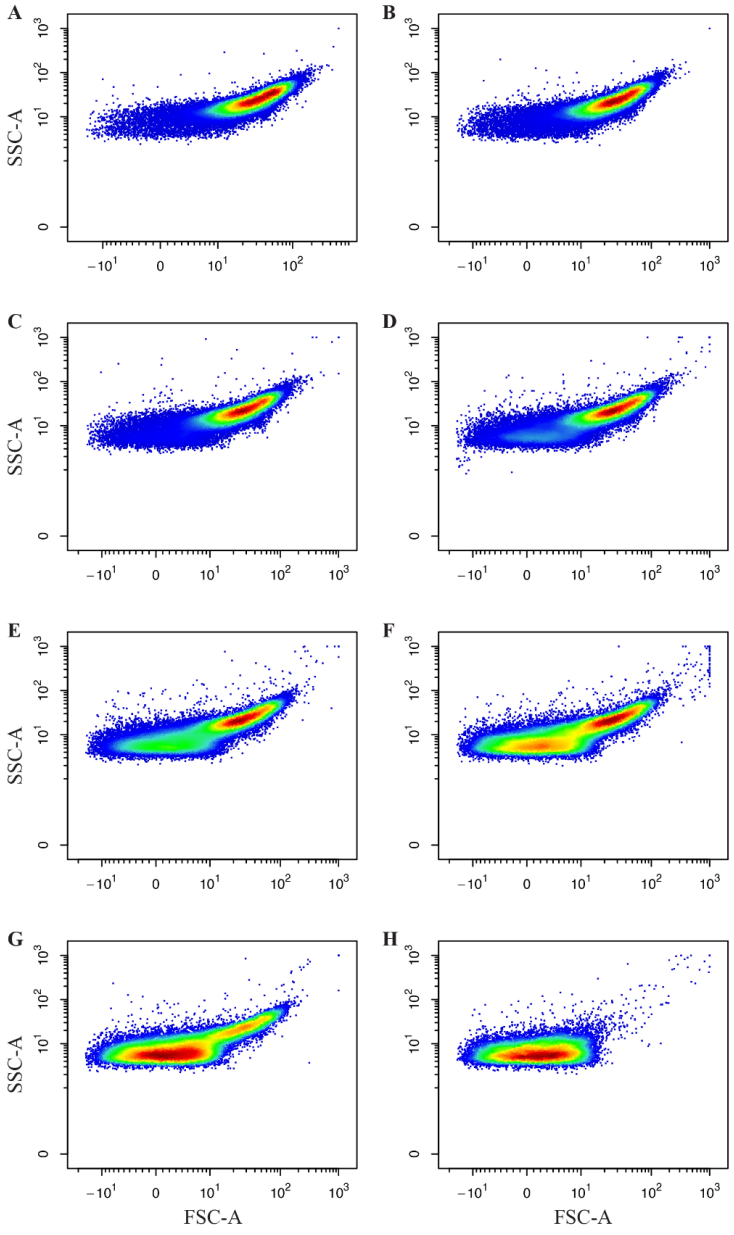

Fig S4. Representative density plots of the limit of detection experiment shown in Figure 7. Measurements were obtained from a urine sample inoculated with 105×10^3 (A), 58×10^3 (B), 35×10^3 (C), 22×10^3 (D), 14×10^3 (E), 5×10^3 (F), 3×10^3 (G), and 0 (H) CFU/ml (see Table S1).

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