Full circle: Genetics, biosynthesis, mode of action and resistance mechanisms of the bacteriocin garvicin ML

Genetikk, biosyntese, virknings- og resistensmekanismer for det sirkulære bakteriosinet garvicin ML

Philosophiae Doctor (PhD) thesis

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“Science - If you don’t make mistakes, you’re doing it wrong. If you don’t correct those mistakes, you’re doing it really wrong. If you can’t accept that you’re mistaken, you’re not doing it at all”
-Unknown

Table of contents

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Ås, September 2013

Christina Gabrielsen

Summary

Bacteriocins are ribosomally synthesized antimicrobial peptides, produced by many lactic acid bacteria, which show high promise as antimicrobial agents for use in both food industry and for medical applications. In this work, we have studied the bacteriocin garvicin ML (GarML), which is a head-to-tail ligated circular bacteriocin that has a broad spectrum of activity and is active against a range of pathogenic bacteria. This class of bacteriocins is furthermore attracting interest due to their favourable characteristics for potential industrial use, i.e. high pH and thermal stability in addition to resistance to many proteases. However, there are many aspects of circular bacteriocin biology that are still not known, and in this work, we have attempted to shed light on the processes which govern the biosynthesis, mode of action and resistance to this bacteriocin.

Circular bacteriocins are synthesized with a leader sequence, and maturation of these peptides is thought to occur through three steps: cleavage of the leader sequence, head-to-tail circularization and export out of the cell. However, the mechanisms involved or indeed the enzymes responsible have not yet been characterized. Furthermore, the sequence of events and potential coupling of these processes is unknown. In paper I and II we have sequenced the producer strain of GarML, which allowed identification and characterization of the gene cluster involved in biosynthesis and immunity to GarML. The gene cluster was shown to share several traits, both in genetic organization and in the putative functions of the encoded proteins, with other circular bacteriocin gene clusters. Functional analysis combined with mass spectrometry of deletion mutants of the GarML operons revealed new insights into biosynthesis of GarML, which may thus apply to circular bacteriocins in general. Firstly, we have provided evidence for leader sequence cleavage occurring without subsequent circularization in two knock-out mutants (Δ*garBCDE* and *garX*::pCG47), which demonstrates not only that these processes are independent, but that leader sequence cleavage precedes circularization in time (paper II). Furthermore, the evidence suggests that leader sequence cleavage is not performed by any of the proteins encoded by the GarML gene cluster, i.e. *garX*, *garBCDE* or *garFGH*, because we still observe cleavage in their absence (paper II). Two of the operons, namely *garX*, *garBCDE*, were implicated in biosynthesis of GarML, specifically in the circularization reaction, as well as providing immunity towards GarML, while the third operon (*garFGH*) was demonstrated to be non-essential.

For circular bacteriocins it has been and remains a controversial issue whether these peptides require a target receptor or docking molecule like the class Ia lantibiotics and IIa pediocin-like bacteriocins for antimicrobial activity, or whether the peptides interact unspecifically with the target cell membrane to create pores. A few circular bacteriocins have been demonstrated to act on liposomes and/or lipid bilayers, which may indicate that a target receptor is not required, at least at high bacteriocin concentrations. In paper III we however provide evidence for a maltose ABC transporter being implicated in sensitivity to GarML in *L. lactis*. The deletion of this complex led to 6-11-fold lowered sensitivity to GarML, whereas complementation restored high-level sensitivity to the

bacteriocin. However, consistent with other circular bacteriocins, we observe receptor-independent killing at higher concentrations of GarML. These results therefore suggest that this class of bacteriocins may indeed require a specific interaction with a target receptor/mediator for antimicrobial activity at low concentrations.

Resistance mechanisms to bacteriocins, both developed and innate, are poorly understood for many classes of bacteriocins. Gaining insight into these processes is essential in order to be able to minimize resistance, which is an important prerequisite for the potential use of bacteriocins in many applications. In this work, we have demonstrated examples of both adaptive and inherent resistance to GarML. In paper III, we have shown that *L. lactis* can develop resistance to GarML by loss of the maltose ABC transporter, which occurs at relatively low frequencies (from 10^{-7} to10^{-8}) compared to adaptive response of class Ia lantibiotics and class IIa pediocin-like bacteriocins. However, no resistance development occurs at high bacteriocin concentrations (>250 BU mL^{-1}), which indicates that killing is receptor-independent above this level (paper III). In paper IV, we have however provided evidence for an inherent resistance mechanism against GarML, which is conserved in a lineage of *L. lactis* ssp. *cremoris* strains. This mechanism appears to be specific for GarML, as it does not affect sensitivity towards other bacteriocins targeting lactococci, even including another circular bacteriocin (paper IV). Thus, we have evidence for a new, specific and inherent mechanism of resistance to GarML in this lineage of *L. lactis* ssp. *cremoris* strains, which contributes to the understanding of how dissemination of resistance factors leads to intraspecies variations in sensitivity to bacteriocins.

Sammendrag

Bakteriosiner er ribosomalt syntetiserte antimikrobielle peptider som blant annet produseres av mange melkesyrebakterier, og som har stort potensial som antimikrobielle forbindelser til bruk i matindustri og i medisinske applikasjoner. I dette arbeidet har vi studert bakteriosinet garvicin ML (GarML), som er et peptid med sirkulær peptidkjede med bredt aktivitetsspektrum og som er aktivt mot mange patogene bakterier. Denne klassen av bakteriosiner anses som interessante fordi de har egenskaper som gjør dem godt egnet til eventuelle industrielle formål, dette er blant annet høy pH- og temperaturstabilitet i tillegg til resistens mot en rekke proteaser. Det er allikevel flere aspekter ved sirkulære bakteriosiner som ikke er tilstrekkelig forstått, og i dette arbeidet har vi ønsket å undersøke nettopp de prosessene som bestemmer biosyntese, virkningsmekanisme og resistensmekanismer for dette bakteriosinet.

Sirkulære bakteriosiner syntetiseres med en ledersekvens, og modning av peptidene er antatt å omfatte tre steg: kløyving av ledersekvensen, sirkulering ved ligering av N- og C-terminus, og eksport ut av cellen. Imidlertid er mekanismene involvert og de ansvarlige enzymene ikke kjent. I tillegg er rekkefølgen av disse stegene, og de mulige koblingene mellom dem, ennå ukjent.

I artikkel I og II har vi sekvensert produsentstammen av GarML, som igjen tillot identifisering og karakterisering av gruppen av gener, bestående av fire operoner, som er involvert i biosyntese av og immunitet mot GarML. Denne gruppen av gener ble vist å ha mye til felles, både når det gjelder organisering og antatte funksjoner av de proteinene disse genene koder for, med tilsvarende gener for andre sirkulære bakteriosiner. Funksjonell analyse kombinert med massespektrometri ga ny innsikt i biosyntesen av GarML, som dermed kan gjelde også for sirkulære bakteriosiner generelt. Først og fremst har vi påvist at kløyving av ledersekvensen skjer uten sirkularisering i to knock-out mutanter (Δ*garBCDE* and *garX*::pCG47), noe som demonstrerer at disse to prosessene er uavhengige, men også at kløyving skjer forut for sirkularisering i tid (artikkel II). Videre viser resultatene at kløyving av ledersekvensen ikke utføres av noen av proteinene som er kodet for i GarML operonene, det vil si *garX*, *garBCDE* eller *garFGH*, fordi man observerer kløyving også uten deres tilstedeværelse (paper II). To av operonene i gruppen, *garX* og *garBCDE*, ble vist å være involvert i biosyntesen av GarML, spesifikt i sirkulariseringsreaksjonen, og samtidig gi immunitet mot GarML, mens et tredje operon (*garFGH*) ble vist å være ikke-essensielt.

Når det gjelder sirkulære bakteriosiner, så er det kontroversielt hvorvidt disse peptidene trenger en målreseptor eller et dokking-molekyl for antimikrobiell aktivitet som klasse Ia lantibiotika og IIa pediocin-liknende bakteriosiner eller om de interagerer uspesifikt med cellemembranen for å danne porer. I noen tilfeller har det blitt vist at sirkulære bakteriosiner virker på lipid bilag og/eller liposomer, noe som kan indikere at et målmolekyl ikke er nødvendig, i hvert fall ved høye konsentrasjoner av bakteriosin. I artikkel III viser vi derimot at en maltose ABC transporter medvirker

til sensitivitet mot GarML i *L. lactis*. Delesjon av dette komplekset gav 6-11-ganger lavere sensitivitet til GarML, mens komplementering gjenopprettet høy sensitivitet til bakteriosinet. Allikevel ble det ved svært høye konsentrasjoner av bakteriosin observert reseptor-uavhengig dreping. Disse resultatene indikerer dermed at det ved lave konsentrasjoner av bakteriosin kan være nødvendig med en spesifikk interaksjon med et målmolekyl for antimikrobiell aktivitet også for denne klassen bakteriosiner.

Resistensmekanismer mot bakteriosiner, bade utviklede og iboende, er ikke godt forstått for mange klasser av bakteriosiner. Det å få innsikt i disse prosessene er essensielt for å kunne minimere nettopp resistensutvikling, noe som er en forutsetning for den potensielle utnyttelsen av bakteriosiner til ulike formål. I dette arbeidet har vi vist eksempler på både utviklet og iboende resistens til GarML. I artikkel III har vi vist at *L. lactis* kan utvikle resistens mot GarML ved tap av maltose ABC transporter komplekset, som skjer ved en relativt lav frekvens (fra 10^{-7} til10^{-8}) sammenliknet med utviklet resistens for klasse Ia lantibiotika og klasse IIa pediocin-liknende bakteriosiner. I tillegg ble det ikke observert noen resistensutvikling ved høy konsentrasjon av bakteriosin (>250 BU mL^{-1}), noe som indikerer at over dette nivået så er drepingen ikke reseptor-mediert. I artikkel IV har vi derimot påvist en iboende resistensmekanisme mot GarML som er konservert i en avstamming av *L. lactis* ssp. *cremoris*. Denne mekanismen ser ut til å være spesifikk for GarML, da den ikke påvirker sensitivitet mot andre bakteriosiner som virker mot laktokokker, bl.a. et annet sirkulært bakteriosin. Derav tyder resultatene på at vi har en ny, spesifikk og iboende resistensmekanisme mot GarML i denne avstammingen av *L. lactis* ssp. *cremoris* stammer, noe som bidrar til forståelsen av hvordan spredning av resistensfaktorer fører til variasjon i sensitivitet mot bakteriosiner innad i arter.

List of papers

List of papers included in this thesis:

Paper I

Gabrielsen, C., Brede, D.A., Hernandez, P.E., Nes, I.F., and Diep, D.B. (2012) Genome sequence of the bacteriocin-producing strain *Lactococcus garvieae* DCC43. *Journal of bacteriology* **194**: 6976-6977.

Paper II

Gabrielsen, C., Brede, D.A., Nes, I.F., Salehian, Z. and Diep, D.B.(2013) Functional genetic analysis of the garvicin ML gene cluster in *Lactococcus garvieae* DCC43 gives new insights into circular bacteriocin biosynthesis. (manuscript)

Paper III

Gabrielsen, C., Brede, D.A., Hernandez, P.E., Nes, I.F., and Diep, D.B. (2012) The maltose ABC transporter in *Lactococcus lactis* facilitates high-level sensitivity to the circular bacteriocin garvicin ML. *Antimicrobial agents and chemotherapy* **56**: 2908-2915.

Paper IV

Gabrielsen, C., Brede, D.A., Nes, I.F., Salehian, Z. and Diep, D.B.(2013) Functional genomic analysis of *Lactococcus lactis* ssp. *cremoris* reveals a novel resistance mechanism to the circular bacteriocin garvicin ML. (manuscript)

1. Introduction

1.1. Bacteriocins

1.1.1. Characteristics and classification

Production of antimicrobial peptides is an evolutionary conserved antagonistic mechanism which is found in all classes of life, from the host defence peptides of the innate immune system in plants and animals (Zasloff, 2002) to the archaeocins produced by Archaea (O'Connor and Shand, 2002) and the bacteriocins produced by Bacteria. Bacteriocins are defined as ribosomally synthesized antimicrobial peptides produced by Gram-negative and Gram-positive bacteria (Tagg *et al.*, 1976), which are presumed to give the producer strain a competitive advantage over closely related bacteria in the contest for resources in an ecological niche (Eijsink *et al.*, 2002). Bacteriocin production generally commences at high cell densities when resources become scarce, in response to quorum sensing mechanisms, and as such also serve as a means of intraspecies bacterial communication (Eijsink *et al.*, 2002). The overwhelming majority of bacteriocins discovered so far come from the Gram-positive lactic acid bacteria (LAB). Because lactic acid bacteria are ubiquitous in plants and mammalian microflora, in addition to their use as starter cultures for a wide range of dairy and fermented foods, humans have likely benefited from their effects as inhibitors of common food-borne pathogens since long before their discovery in 1925 (Gratia, 2000).

LAB bacteriocins constitute a large and heterogeneous group, from which bacteriocins are mainly classified based on features such as size, homology, structure and posttranslational modifications. The classification of LAB bacteriocins is frequently revised as more bacteriocins are characterized (Cotter *et al.*, 2005a; Klaenhammer, 1993; Nes *et al.*, 1996; Nissen-Meyer *et al.*, 2009; Rea *et al.*, 2011), but the main distinction is made between the class I lantibiotics, which undergo posttranslational modifications, and the class II non-modified peptides. Previously, a third class consisting of large heat-labile lytic proteins (bacteriolysins) was included in this classification scheme; however according to Cotter *et al.* (2005a) and some subsequent classifications, these are no longer regarded as bacteriocins.

The class I bacteriocins are mainly comprised of a large group known as the lantibiotics. The lantibiotics, which include the prototype bacteriocin nisin, are small peptides (approx. 19-38 residues) defined by the thioether-containing amino acids lanthionine (Lan) and β-methyllanthionine (MeLan). These residues are introduced by a two-step posttranslational process catalyzed by dedicated dehydratase, cyclase or synthetase enzymes. The Lan and MeLan residues form crosslinks between amino acid residues, resulting in characteristic intramolecular cyclic structures as shown in figure 1 (Bierbaum *et al.*, 1996; Havarstein *et al.*, 1995; Karp *et al.*, 2005). Class I lantibiotics may also contain other posttranslationally modified amino acids, e.g. didehydroalanine (Dha) and

didehydrobutyrine (Dhb), and can consist of a single or two peptide moieties. The lantibiotics have previously been subgrouped based on mode of action or structural features, but a more recent classification based on sequence similarity of the unmodified peptides subdivides the lantibiotics into 11 groups (Cotter *et al.*, 2005b). Recently, two new subclasses have been proposed included into the post-translationally modified bacteriocins (Rea *et al.*, 2011): The first group (designated as class Ib) are called labyrinthopeptins and contain the modified amino acid residue labionin (Lab). Labionins are carbacyclic amino acid residues that form thioether and methylene bridges, resulting in the complex cyclic structures of these peptides (figure 1) (Meindl *et al.*, 2010). The second group (class Ic) are known as the sactibiotics, and are characterized by several α-carbon to sulphur crosslinks (Murphy *et al.*, 2011). The first characterized bacteriocin containing these unusual bridges was the head-to-tail circularized bacteriocin subtilosin A produced by *Bacillus subtilis* (Kawulka *et al.*, 2004) while another well-known example is thuricin CD, a linear two-peptide bacteriocin produced by *B. thuringiensis* (Rea *et al.*, 2010).

Class II bacteriocins are a heterogeneous group of small (<10 kDa), heat stable peptides defined by their non-modified nature (Cotter *et al.*, 2005a). The common designation of these peptides as "unmodified" is however somewhat misleading: class II bacteriocins may in fact contain modifications like disulphide bonds or formylated N-methionine residues, or have circular backbones. This class of bacteriocins is divided into several subgroups based on structural and functional characteristics (figure 1): Class IIa are pediocin-like one-peptide bacteriocins with a conserved N-terminal sequence motif known as the "pediocin box" and one or two intrachain disulfide bonds. The peptides form a very characteristic structure, consisting of a conserved N-terminal anti-parallel β-sheet connected by a flexible hinge to the more variable C-terminal hairpin-like domain (figure 2) in membrane mimicking environments (Montalban-Lopez *et al.*, 2012; Nissen-Meyer *et al.*, 2009; Rappsilber *et al.*, 2003; Spiro and Dixon, 2010). Class IIa bacteriocins are synthesized as precursors with an N-terminal leader sequence, often of a double-glycine type (Havarstein *et al.*, 1995).This leader is cleaved C-terminally to the double-glycine motif upon export out of the cell; in most cases this process is executed by a dedicated ABC transporter containing an intracellular peptidase domain or accessory protein (Havarstein *et al.*, 1995). The mature peptides generally consist of 37-48 amino acid residues and have a net positive charge and high pI (8-10). Class IIa bacteriocins have narrow activity spectra, and are known for their anti-listerial activity (Cotter *et al.*, 2005a). With approximately 50 characterized members to date (Cui *et al.*, 2012), this constitutes the largest subclass of class II bacteriocins, and they have been extensively studied, both with respect to genetics, structure and mode of action (Drider *et al.*, 2006). Subclass IIb consists of the two-peptide bacteriocins, i.e. bacteriocins where optimal function is dependent on the combined (enhancing or synergistic) activity of two non-identical peptide moieties. Two-peptide bacteriocins are generally cationic, but contain hydrophilic and/or amphiphilic regions, and are synthesized with a double-glycine leader like most class IIa peptides. The structures of two-peptide bacteriocins are in some

cases characterized by a central α-helix with flexible N- and C-terminal regions, and the two peptides are thought to interact via helix-helix interactions (Draper *et al.*, 2008; Nissen-Meyer *et al.*, 2009; Samyn *et al.*, 1994). Class IIc comprises the circular bacteriocins, characterized by a head-to-tail covalent bond forming a perfectly circular structure (figure 1). These bacteriocins are known for a broad activity spectrum and resistance to heat, extreme pH and even many proteases. This group will be discussed in detail in chapter 1.2, as it is the focus of this thesis. The final class, class IId is generally viewed as a collection of the remaining non-modified bacteriocins, which includes all other non-pediocin-like linear one-peptide bacteriocins. This class can be further divided based on leader sequences: The sec-dependent bacteriocins contain a leader sequence which directs the peptides for export by the general secretory pathway (GSP)(Kramer *et al.*, 2006; Verheul *et al.*, 1997), whereas the leaderless bacteriocins, as the name suggests, lack a leader sequence, and are often secreted by dedicated ABC transporters (Kastin, 2006). Some of the leaderless bacteriocins have been shown to retain the formyl group of the N-terminal methionine residue (Liu *et al.*, 2011; Netz *et al.*, 2001).

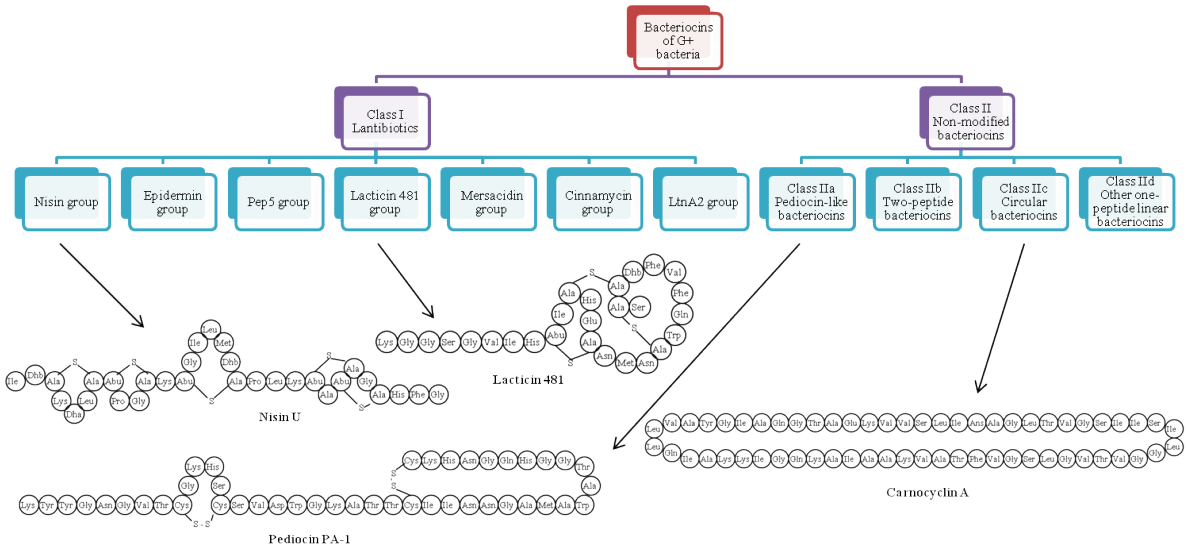

Figure 1: Classification scheme of the bacteriocins of Gram-positive bacteria, based on Cotter *et al.* (2005a). The main distinction is made between the class I lantibiotics which undergo extensive posttranslational modifications and the class II non-modified bacteriocins. The amino acid sequence and structural features of a few example bacteriocins of different classes are shown for reference.

1.1.2. Mode of action and resistance mechanisms

Class Ia bacteriocins generally have broad antimicrobial spectra, are mainly produced by and display antimicrobial activity against Gram-positive bacteria. The latter may be due to restricted access to the cytoplasmic membrane in Gram-negative bacteria, as compromising the Gram-negative outer membrane in some cases has been shown to sensitize cells to lantibiotics (Schved *et al.*, 1994; Stevens *et al.*, 1991). Although mechanistic studies have not been performed for all peptides of this class, it has become apparent that there are common features in the mode of action of lantibiotics. First and foremost, the requirement for a target- or docking molecule, which in many cases has been demonstrated to be the cell wall precursor lipid II (Brotz *et al.*, 1998a; Wiedemann *et al.*, 2001; Wiedemann *et al.*, 2006). Different lantibiotics have furthermore been demonstrated to specifically target different sites on the lipid II molecule, the model lantibiotic nisin for example binding to the pyrophosphate moiety of the lipid II molecule (Hsu *et al.*, 2004). The binding of nisin to lipid II in turn facilitates a dual effect on target cells (figure 2); (i) the inhibition of peptidoglycan biosynthesis by interaction with and sequestration of lipid II and (ii) the formation of pores in the cytoplasmic membrane (Wiedemann *et al.*, 2001). While the pore-forming activity of nisin occurs at micromolar concentrations *in vitro* (Brotz *et al.*, 1998b), the interaction with lipid II has been demonstrated to increase the activity by a factor of 10^3 (Breukink *et al.*, 1999).This dual mode of action thus explains the high potency of many lantibiotics, which often display specific antimicrobial activity in the nanomolar range *in vivo* (Breukink *et al.*, 1999). Nisin has a modular structure, where the two N-terminal ring structures (known as the "pyrophosphate cage") are essential for binding to the pyrophosphate moieties of lipid II, whereas the C-terminal region is able to insert into the cytoplasmic membrane, oligomerize and form a pore consisting of eight nisin molecules and four lipid II molecules (Hasper *et al.*, 2004). The hinge region separating these modules is important for movement of the modules relative each other, and thus also vital for pore formation (Yuan *et al.*, 2004). Similar structural modules are believed to form functional units also in other lantibiotics; the pyrophosphate cage is for instance conserved in the nisin- and epidermin group (Sahl and Bierbaum, 1998). Variations in these structures may thus account for observed differences in activity and spectrum of different types of lantibiotics. All lantibiotics do however not share the pore-forming mechanism of nisin, but instead target enzymatic reactions: Mersacidin and nukacin ISK-1 both bind lipid II and inhibit peptidoglycan biosynthesis in sensitive cells (Asaduzzaman *et al.*, 2009; Brotz *et al.*, 1998a), while lantibiotics of the cinnamycin-group have been shown to target phosphatidylethanolemine in the cell membrane and function by inhibiting the activity of phospholipase A2 (Marki *et al.*, 1991).

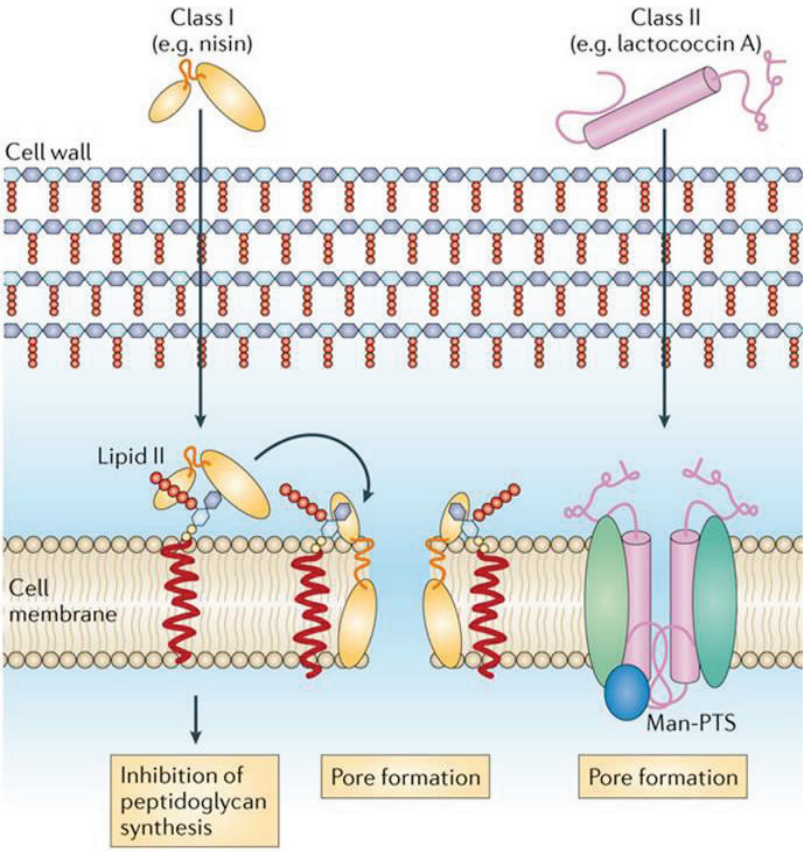

Figure 2: Mode of action of the class I lantibiotics and the class II pediocin-like bacteriocins, modified from Cotter *et al.*(2013). Class I bacteriocin shown targeting the lipid II cell wall precursor for dual antimicrobial effect (i) inhibition of cell wall synthesis and (ii) formation of pore s in the cytoplasmic membrane. Class IIa bacteriocin interacting with the transmembrane IID and IIE subunits of the mannose PTS system, rendering the permease open.

Class II bacteriocins generally have well-defined and narrow antimicrobial spectra. Furthermore, it has been established that certain class II bacteriocins kill target cells by pore formation or by interfering with the integrity of the cytoplasmic membrane (Drider *et al.*, 2006; Kjos *et al.*, 2011a). For most class II bacteriocins however, the detailed molecular mechanisms and potential target molecule(s) are as of yet unknown. The exception is the class IIa bacteriocins and lactococcin A, which have been demonstrated to specifically target the transmembrane IIC and IID proteins of the mannose phosphotransferase system (man-PTS), a broad specificity hexose uptake system, on sensitive cells (Diep *et al.*, 2007). Furthermore, it was shown that that an N-terminal extracellular loop-containing region in the IIC protein is the major determinant responsible for species-specificity, potentially serving as an interaction site for the class IIa bacteriocins (Kjos *et al.*, 2010). The interactions of the bacteriocin with the man-PTS components in turn is believed to cause structural changes in the complex which render the permease open as a pore (figure 2), leading to leakage of

solutes, disruption of cell membrane integrity and cell death (Kjos *et al.*, 2011a). The finding that the target molecule of a bacteriocin is in fact a permease, thus opens up the possibility that bacteriocins rather than themselves polymerizing into pores, instead can actually target the cells own uptake systems to produce the same lethal effect. Recent findings have shown that some class II bacteriocins, namely lactococcin 972 and garvicin A, can act by inhibiting septum formation (Maldonado-Barragan *et al.*, 2013; Martinez *et al.*, 2008), but whether or not this activity is receptor-mediated is not clear. However, as we learn more about the mode of action of bacteriocins, it becomes clear that the presumption that all bacteriocins act by forming pores in the cytoplasmic membrane is not always true. The mechanisms by which these antimicrobial peptides act appear to be nearly as diverse as the variance in their structures and physicochemical properties.

Similar to the situation for antibiotics, resistance development upon exposure to bacteriocins is a relatively common occurrence in bacteria. However, the frequency of resistance development varies greatly, depending on both the type of bacteriocin and the sensitive strain, likely reflecting the different modes of action for antimicrobial activity (Gravesen *et al.*, 2002). There are also a few examples of intrinsic resistance to bacteriocins (Collins *et al.*, 2010; McBride and Sonenshein, 2011). A number of different mechanisms have been implicated in acquired and intrinsic resistance towards bacteriocins, both specific and non-specific.

In bacteriocins where a target receptor mediates antimicrobial activity, loss or reduced expression of the receptor has been shown to result in increased resistance to the corresponding bacteriocin. This is the case for class IIa bacteriocins and lactococcin A, where loss or reduced expression of the man-PTS which functions as a target receptor causes resistance in *Listeria monocytogenes* and *Lactococcus lactis* accordingly (Kjos *et al.*, 2011b). For the class II bacteriocin lactococcin 972, which targets the cell wall precursor lipid II, resistance has been linked to peptidoglycan remodelling (Roces *et al.*, 2012). Cell surface changes that in some way affect the interactions taking place between bacteriocin and cell surface or receptor is another and more general mechanism for bacteriocin resistance. Resistance in *Li. monocytogenes* against the class I lantibiotic nisin has been correlated with changes in both altered fatty acid and phoshoplipid composition, which is thought to adversely affect how nisin interacts with the membrane (Crandall and Montville, 1998; Verheul *et al.*, 1997). Also in class IIa-resistant cells of *Li. monocytogenes* which express normal levels of the receptor man-PTS, resistance has been associated with changes in membrane phospholipid composition (Vadyvaloo *et al.*, 2002) and cell surface charge (Vadyvaloo *et al.*, 2004). A third known resistance mechanism is known as immune mimicry. Immune mimicry entails the expression of functional immunity homologues in non-producing strains, i.e. strains without a cognate bacteriocin gene cluster (Draper *et al.*, 2009; Fimland *et al.*, 2002). The immunity genes are thought to be remnants of inactivated or deleted bacteriocin operons, which nevertheless provide the non-producing strain with a competitive advantage. Immunity determinants providing resistance towards

the two-peptide lantibiotic lacticin 3147 are for example produced not only by lacticin 3147-producing strains, but also by *Bacillus licheniformis* and *Enterococcus faecium* (Draper *et al.*, 2009). Enzymatic activity which degrades peptide bacteriocins, consequently leading to loss or lowered antimicrobial activity, is another mechanism by which bacteria can acquire resistance towards bacteriocins. Extracellular proteases or peptidases of different specificities are likely candidates for such non-specific activity. Even so, there are not many reports of bacteriocin-resistance caused by enzymatic degradation. One known example is however the extracellular gelatinase in *E. faecalis*, which has been shown to degrade class IIa bacteriocins (Sedgley *et al.*, 2009). For the class I lantibiotic nisin, there are two known enzymes, nisin resistance protein (NRS)(Sun *et al.*, 2009) and nisinase (Jarvis, 1967), that specifically degrade or modify the C-terminal end of the peptide, leading to loss of activity and consequently resistance towards the bacteriocin. The presence of transporters which can export antimicrobial peptides out of the cell is yet another mechanism which can cause bacteriocin resistance. It is well known that transporters play a role in bacteriocin producer self immunity for different classes of bacteriocins, including lantibiotics (Draper *et al.*, 2008) and circular bacteriocins (Kemperman *et al.*, 2003a; Martinez-Bueno *et al.*, 1998). Different transporter complexes have also been shown to contribute to intrinsic resistance of *Li. monocytogenes* to nisin, and of *C. difficile* to nisin and gallidermin (Collins *et al.*, 2010; McBride and Sonenshein, 2011).

It has become clear that some bacteriocin resistance traits can provide cross-resistance to other bacteriocins or antimicrobial agents (Crandall and Montville, 1998; Mehla and Sood, 2011; Rekhif *et al.*, 1994), and that sequential exposure to a bacteriocin in some cases can provide increased or more stable resistance compared to single exposure (Gravesen *et al.*, 2002; Ming and Daeschel, 1993). Bacteriocin resistance mechanisms are therefore not always specific, clear-cut, on/off mechanisms, but can result from an interplay between several contributing factors, most of which are not yet well understood. It is most certainly an area of bacteriocin research that requires more attention in the future, seeing as it is crucial to be able to minimize resistance development for any practical application of bacteriocins, but especially so if bacteriocins are ever to be considered as viable options to antibiotics in medical therapeutics.

1.1.3. Applications and future perspectives

With recent years increasing development of resistance to classical antibiotics, there is an ever increasing need for novel antimicrobial agents, both for medical use, as well as for use in food and feed industry. However, the search for new antibiotics is slow, as there in time are fewer classes of antibiotics left to be discovered, but also due to cumbersome laws and regulations and large costs associated with research and development (Butler and Buss, 2006; Coates *et al.*, 2011). Bacteriocins have many favourable characteristics in this context, which make them some of the most promising candidates for novel antimicrobials. Firstly, bacteriocins target uniquely bacterial targets on sensitive cells, by differing mechanisms of action to classical antibiotics. Consequently, the toxicity of bacteriocins to eukaryotic cells is generally low, especially at the concentrations required for antimicrobial activity. In contrast to antibiotics which often act in micromolar concentrations, bacteriocins often act in the nanomolar range (Breukink *et al.*, 1999; Cotter *et al.*, 2005a), i.e. displaying extremely high specific activity, both in vitro and in vivo (Breukink *et al.*, 1999; Cotter *et al.*, 2013). Furthermore, it is possible that the activity of bacteriocins can be increased through combination with other antimicrobials or membrane-active substances (Brumfitt *et al.*, 2002; Giacometti *et al.*, 2000). The class IIa lantibiotic nisin is used as a food additive, and the toxicity of nisin and related peptides has been demonstrated to occur at concentrations 1000-fold higher than required for their antimicrobial activity (Maher and McClean, 2006). Bacteriocins can have broad or narrow antimicrobial spectra, but those produced by Gram-positive bacteria are generally most active against other Gram-positive bacteria. Broad spectrum antimicrobial agents can be useful for targeting infections of unknown origin. However, it has been established that use of broad spectrum antibiotics has a negative impact on the commensal human microbiota, and so the prospect of targeting specifically a pathogenic genus/species, without affecting the commensal population adversely, is highly beneficial (Cotter *et al.*, 2013). Importantly, the antimicrobial spectra of bacteriocins commonly include pathogenic and antibiotic resistant strains of *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Propoionibacterium*, *Listeria* and *Clostridium*, rendering the possible applications of such bacteriocins numerous. Staphylococci and enterococci are responsible for a large part of nosocomial infections, and instances where acquired resistance to antibiotics presents a serious problem in medical treatment (Rice, 2010). The LAB bacteriocins are a special case, in that many of the producing strains have generally recognized as safe (GRAS) status (US Food and Drug Administration, 2001) or Qualified Presumption of Safety (QPS) status (EFSA Panel on Biological Hazards (BIOHAZ), 2012). Especially many species of *Lactobacillus*, *Leuconostoc* and *Pediococcus* are approved and widely used as starter cultures. As a consequence, these bacteriocin producers are particularly suited for food applications and use as probiotics, but it also paves the way for their further development into other applications. Indeed, several commercial probiotic strains used today actually do produce bacteriocins and thus may provide added health benefits, but those bacteriocins

may not have been characterized (Cotter *et al.*, 2013). The physicochemical characteristics of bacteriocins also constitute an advantage of these antimicrobials: many bacteriocins are heat stable, tolerant of low pH and in a few cases also resistant to a range of proteolytic enzymes. Characteristics that make these bacteriocins or bacteriocins producers well suited for industrial applications, as probiotics or for therapeutic use through oral administration. Lastly and importantly, the bacteriocins due to their gene-encoded nature present excellent targets for bioengineering. Bioengineering could produce engineered peptides with a range of improved functional properties such as increased thermal and pH stability, resistance to proteases, solubility, potency and modified target specificity. Indeed, several of these traits have already been engineered for derivatives of the lantibiotic nisin (Molloy *et al.*, 2012), most notably the enhanced activity against both Gram-positive and Gram-negative pathogens (Field *et al.*, 2012), which demonstrates the real potential of bioengineering in creating "tailor-made" bacteriocins for specific applications.

The potential applications of bacteriocins in food are diverse: bacteriocins can be added to foods as concentrated preparations (as preservatives, additives, shelf-life extenders or ingredients), or bacterial starters cultures which produce bacteriocin in situ. Nisin produced by *L. lactis* ssp. is the only bacteriocin now widely in use as a food preservative, due to its efficacy and food-grade status. Nisin was approved for use as a preservative by the UN food and Agriculture Organization (FAO) as early as in 1969, is now approved in more than 80 countries worldwide, where it is mainly used for the inhibition of *Li. monocytogenes* in cheeses and prevent growth of *C. botulinum* in meat products (Gómez-López, 2012; Rai *et al.*, 2011). Pediocin PA-1, which is produced by different species of *Pediococcus*, is yet another promising bacteriocin for prevention of common pathogens, e.g. *L. monocytogenes*, in meats, cheeses and fermented vegetables. Pediocin PA-1 is not yet approved for use as a food additive, but is currently approved as a food ingredient or shelf-life extender (Gómez-López, 2012).

With respect to clinical applications of bacteriocins, there have been some promising recent advances, and several bacteriocins are now being developed for human application (Cotter *et al.*, 2013). The in vivo activity of bacteriocins against pathogens has been demonstrated for several bacteriocins of both class I and class II in animal models. The class IIa bacteriocin-producing strain *Lactobacillus salivarius,* which is a probiotic strain of human origin, has for example been shown to protect against infection by the food-borne pathogen *Li. monocytogenes in vivo* in a mouse model (Corr *et al.*, 2007), while several lantibiotics have been demonstrated to control *Staphylococci* and/or *Enterococci* in clinically relevant settings (Fontana *et al.*, 2006; Mota-Meira *et al.*, 2005; van Staden *et al.*, 2012). Hopefully, the development of such bacteriocins will go on to clinical trials, with a view to being approved for use in human therapeutics in the future.

As mentioned previously, resistance development is also a serious challenge with use of bacteriocins as antimicrobial agents. Some efforts have been made to lower resistance development by using bacteriocins in combination with other antimicrobial agents with differing target/mechanism

of action (Brumfitt *et al.*, 2002; Naghmouchi *et al.*, 2012). With a detailed understanding of the mode of action of bacteriocins, in addition to the possibility of bioengineering and combination use with other antimicrobials or synergistic compounds, bacteriocin resistance development can hopefully be minimized to rationalize the future use of bacteriocins in clinical settings.

1.2. Circular bacteriocins

1.2.1. General characteristics and classification

The circular bacteriocins constitute a group of ribosomally synthesized antimicrobial peptides characterized by their N- to C-terminus covalent linkage forming a circular backbone (Maqueda *et al.*, 2008). In this thesis we will consistently use the term circular instead of cyclic, to clearly distinguish the ribosomal head-to-tail ligated bacteriocins from non-ribosomal or intramolecularly cyclized peptides. Circular bacteriocins are synthesized as linear precursors, containing a leader sequence which is cleaved off during maturation (Martinez-Bueno *et al.*, 1998; Samyn *et al.*, 1994). Circular bacteriocins are produced by Gram-positive bacteria of the phylum Firmicutes, mainly lactic acid bacteria, but a few have also been isolated from other Bacilli and Clostridia. Isolation sources range from fermented foods, dairy products, mammalian faeces, exudate and meat (table 1). The circular bacteriocins are known for their pH and thermal stability, as well as their resistance to many proteolytic enzymes, properties which make this class of bacteriocins especially interesting for potential industrial applications.

To date, there have been characterized 9 circular bacteriocins, and these are subdivided into two classes based on physicochemical characteristics and level of sequence identity (Cotter *et al.*, 2005a; Martin-Visscher *et al.*, 2009). Subclass i includes the highly characterized circular bacteriocin enterocin AS-48, as well as carnocyclin A (Martin-Visscher *et al.*, 2008), circularin A (Kawai *et al.*, 2004b), uberolysin (Wirawan *et al.*, 2007), lactocyclicin Q (Sawa *et al.*, 2009) leucocyclicin (Masuda *et al.*, 2011) and garvicin ML (Borrero *et al.*, 2011). These peptides are characterized by having several positively charged amino acid residues (overall cationic charge) and a high isoelectric point (pI~10) (table 1). An alignment of these peptides is shown in figure 3. As the figure clearly illustrates, there is little sequence identity and few conserved residues within this subclass.

Subclass ii has currently only two members: gassericin A (Kawai *et al.*, 2004b) and butyrivibriocin AR10 (Kalmokoff *et al.*, 2003). These display a higher level of sequence identity (44.8 percent), and also differ from the subclass i peptides in that they have more negatively charged residues and a low isoelectric point (pI 4-7). Reutericin 6 was initially identified as a circular bacteriocin, early studies indicating that it was similar to gassericin A but containing different levels of D-ala. However, later analysis revealed that it is in fact identical to gassericin A (neither peptide actually containing D-ala) (Arakawa *et al.*, 2010).

Table 1: General properties of the circular bacteriocins: garvicin ML (GarML); carnocyclin A (CclA); circularin A(CirA); enterocin AS-48(AS-48); uberolysin A (UblA); lactocyclicin Q (LycQ); leucocyclicin Q (LcyQ) ; gassericin A (GaaA) and butyrivibriocin AR10 (BviA). Physicochemical properties were calculated using ProtParam (Wilkins *et al.*, 1999), where molecular weight (MW) is calculated for the circularized peptides, whereas the remaining parameters are calculated for the linear forms. The grand average of hydropathicity (GRAVY) is calculated as the sum of hydropathy values of all amino acids divided by the total number of residues, i.e. increasing positive score indicates greater hydrophobicity.

Sub class	Bacteriocin	GenBank Accession	Producer organism	Isolation source	Leader seq (aa)	Mature peptide (aa)	MW (Da) circular	pI	Net charge	GRAVY
i	GarML	EKF52513	*Lactococcus garvieae*	Duck intestines	3	60	6007.2	10.13	+5	0.89
i	CclA	ACC93994	*Carnobacterium maltaromaticum*	Fresh pork	4	60	5862.0	10.00	+4	1.06
i	CirA	CAD97580	*Clostridium beijerinckii*	Soil	3	69	6771.0	10.46	+4	1.01
i	AS-48	CAA72917	*Enterococcus faecalis*	Human wound exudate	35	70	7149.5	10.09	+6	0.54
i	UblA	ABG48503	*Streptococcus uberis*	Cow mammary secretion	6	70	7048.3	9.60	+3	0.94
i	LycQ	BAH29711	*Lactococcus sp.*	Cheese	2	61	6060.1	9.70	+2	0.83
i	LcyQ	BAL14584	*Leuconostoc mesenteroides*	Janese pickles	2	61	6115.2	9.53	+2	0.74
ii	GaaA	BAH08712	*Lactobacillus gasseri*	Human infant feces	33	58	5653.6	6.75	0	0.10
ii	BviA	AAC69560	*Butirivibrio fibrosolvens*	Rumen isolate	22	58	5981.9	4.03	-2	1.00

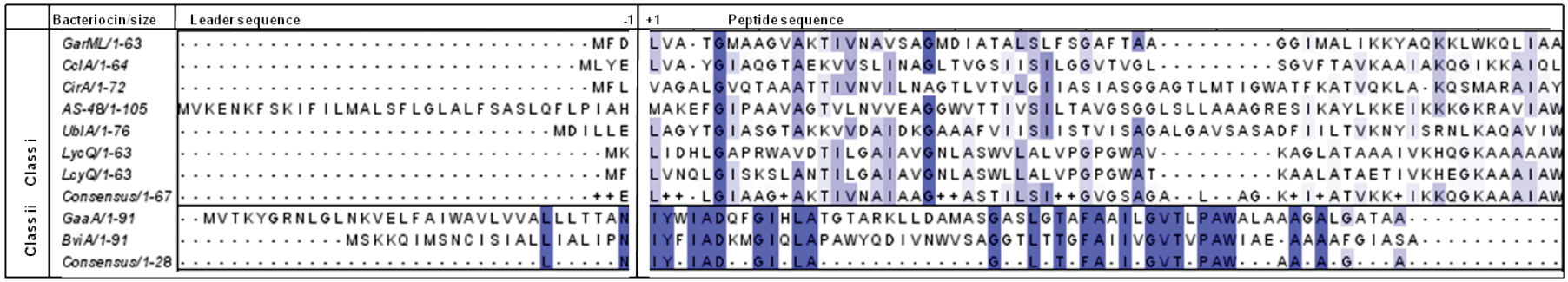

Figure 3: Multiple alignments of the class i and ii circular bacteriocins created by T-Coffee (Notredame *et al.*, 2000). The linear precursor peptides are displayed with indicated cleavage site for circularization, and the level of sequences identity is indicated by colour scale.

1.2.2. Genetics and biosynthesis

The genetic background of a majority of the circular bacteriocins has been described, and functional analysis of the gene clusters of enterocin AS-48 and a few other circular bacteriocins (CirA, GaaA and CclA) has also provided some insight into the roles of the genes in the biosynthesis of the peptides. Figure 4 displays the gene clusters of the genetically characterized circular bacteriocins to date, which are either chromosomally located or plasmid encoded. Despite limited sequence similarity (average 20-30 % pairwise reciprocal ortholog identity) of the encoded proteins, there are a number of shared traits between these gene clusters.

The majority of the encoded proteins in circular bacteriocin gene clusters are predicted to be hydrophobic, and thus believed to be membrane-associated. The minimal set of genes required for production and immunity comprises 5-7 genes/encoded proteins: The bacteriocin precursor; a short (49-88 residues), high pI, hydrophobic immunity protein with predicted α-helical structure (Kawai *et al.*, 2009) that provides a basal immunity level towards the cognate bacteriocin (Belkum *et al.*, 2010; Kemperman *et al.*, 2003a); an ATPase which is likely associated/complexed with one or more of the membrane proteins; an integral membrane protein with unknown function (DUF 95), and lastly one or more putative membrane protein(s). Subclass ii gene clusters contain a membrane protein (160-174 residues) that is similar to the ABC-2 subfamily of membrane transporters. Subclass i gene clusters however encode a very large (477-581 residues) protein which contains no known functional domains or homology, but with a predicted transmembrane topology (van Belkum *et al.*, 2011). Most of the subclass i gene clusters also have an accessory operon (3-4 genes) encoding an ABC transporter complex, consisting of a permease, an ATPase and an accessory (extracellular) protein. This ABC transporter has in several cases been shown not to be essential for bacteriocin production and immunity (Belkum *et al.*, 2010; Kemperman *et al.*, 2003a; Martinez-Bueno *et al.*, 1998), but may however provide an increased level of immunity and enhance the production of the bacteriocin (Diaz *et al.*, 2003).

The extensive common features of the circular bacteriocin gene clusters may indicate that there are commonalities in the biosynthesis of these peptides. The maturation of circular bacteriocins requires both cleavage of the leader sequence and a circularization reaction, and additionally the peptide must be exported out of the cell. However, the mechanisms involved or indeed the enzymes responsible have not yet been characterized. Furthermore, the sequence of events and potential coupling of these processes is unknown (Conlan *et al.*, 2010; Maqueda *et al.*, 2008). The localization of most of the biosynthetic machinery in the membrane may suggest that maturation takes place at the membrane interface possibly in proximal to externalisation. The biosynthesis of other ribosomally synthesized head-to-tail circularized peptides such as the bacterial pilin precursor TrbC in *E. coli*, the pilin-like peptide VirB2 in *Agrobacterium tumefaciens* and the plant cyclotides exemplified by kalata B1 may shed some light on how these processes may proceed.

The removal of the leader sequence is believed to be the first step in maturation and a requirement for further processing into the mature peptide. The leader sequences of the circular bacteriocins range from 2-35 residues (figure 3), containing mainly hydrophobic residues but often with a charged amino acid in the -1 position, i.e. the residue which is believed to affect specificity of the cleavage reaction (Cebrian *et al.*, 2010). As no peptidase functionality has been characterized in the encoded proteins of the gene clusters, it is possible that the leader sequences are cleaved by trans-acting chromosomally encoded signal peptidases, as is the case for TrbC (Eisenbrandt *et al.*, 2000) and VirB2 (Cebrian *et al.*, 2010). The leader sequences of the circular bacteriocins are however highly variable both in size and sequence (figure 3) and differ from the known recognition site of the bacterial SPase I (Paetzel *et al.*, 2002), which could imply a requirement for host specific enzymes with different specificities. Mutational analysis of the enterocin AS-48 precursor has shown that changing the residue at the -1 position (His-1Ile) abolishes production of the bacteriocin, thus suggesting that this residue is critical for cleavage site recognition and further processing (Cebrian *et al.*, 2010).

The circularization reaction is an intriguing aspect of circular bacteriocin biosynthesis. Other known head to tail ligated circular peptides, e.g. TrbC and kalata B1 type proteins, require both N- and C-terminal regions with more or less conserved motifs that are essential for circularization. TrbC maturation requires the coordinated action of three proteases, catalyzing cleavage of 27 residues from the C-terminal extension (unknown protease), cleavage of the N-terminal extension (LepB signal peptidase), and finally cleavage of the final 4-residue C-terminal motif with concurrent circularization (TraF protease) (Eisenbrandt *et al.*, 2000). Kalata B1 maturation proceeds through several steps preformed by an asparaginyl endopeptidase, which catalyzes cleavage of the N-terminal C-terminal extensions and subsequently circularization by a transpeptidation (Conlan *et al.*, 2012). The circular bacteriocins in this respect stand out, in most cases containing very short N-terminal sequences and lacking any C-terminal sequence which could assist the circularization process. It has therefore been proposed that the C-terminus requires some form of activation before circularization can occur. The VirB2 peptide is more similar to circular bacteriocins in that it also lacks a C-terminal extension. The VirB2 peptide is processed by removal of a 47 residue N-terminal extension by a general signal peptidase and further circularized either in rapid succession by an unknown enzyme or indeed by the same peptidase. Interestingly, circularization occurs in the absence of the plasmid-encoded *virB* operon, indicating that processing of VirB2 is entirely performed by chromosomally encoded factors (Lai *et al.*, 2002). It has been shown for enterocin AS-48 that heterologous produced active bacteriocin is not achievable outside the genus *Enterococcus* (Fernandez *et al.*, 2007), which could indicate that chromosomal factors are required for maturation of this bacteriocin.

The N- and C- terminal ends of the circular bacteriocins, which are involved in the circularization, consist mainly of stretches of hydrophobic residues (figure 3). Also, the circularization point is always located internal to an alpha helix in the structure of the peptide (figure 5), requiring the

cyclization process to occur in a largely hydrophobic region. It has however been suggested that the hydrophobic environment might in fact be essential for the circularization reaction to occur (Montalban-Lopez *et al.*, 2012). Mutational analysis of enterocin AS-48 has shown that substituting the first and last residues of the mature peptide affects the circularization reaction: Substitution Met_1Ala lowered the circularization efficiency significantly, whereas substitution of the C-terminal Trp to Ala resulted in production of both circular as well as small amount of a linear form of the peptide, thus establishing that the nature of both the N- and C-terminal residues are critical to the efficiency of the circularization process (Sawa *et al.*, 2009). Indeed, all half of the circular bacteriocins contain either an aromatic or a small hydrophobic residue in the ultimate position, which points to the hydrophobic nature of this residue potentially being important for the circularization mechanism.

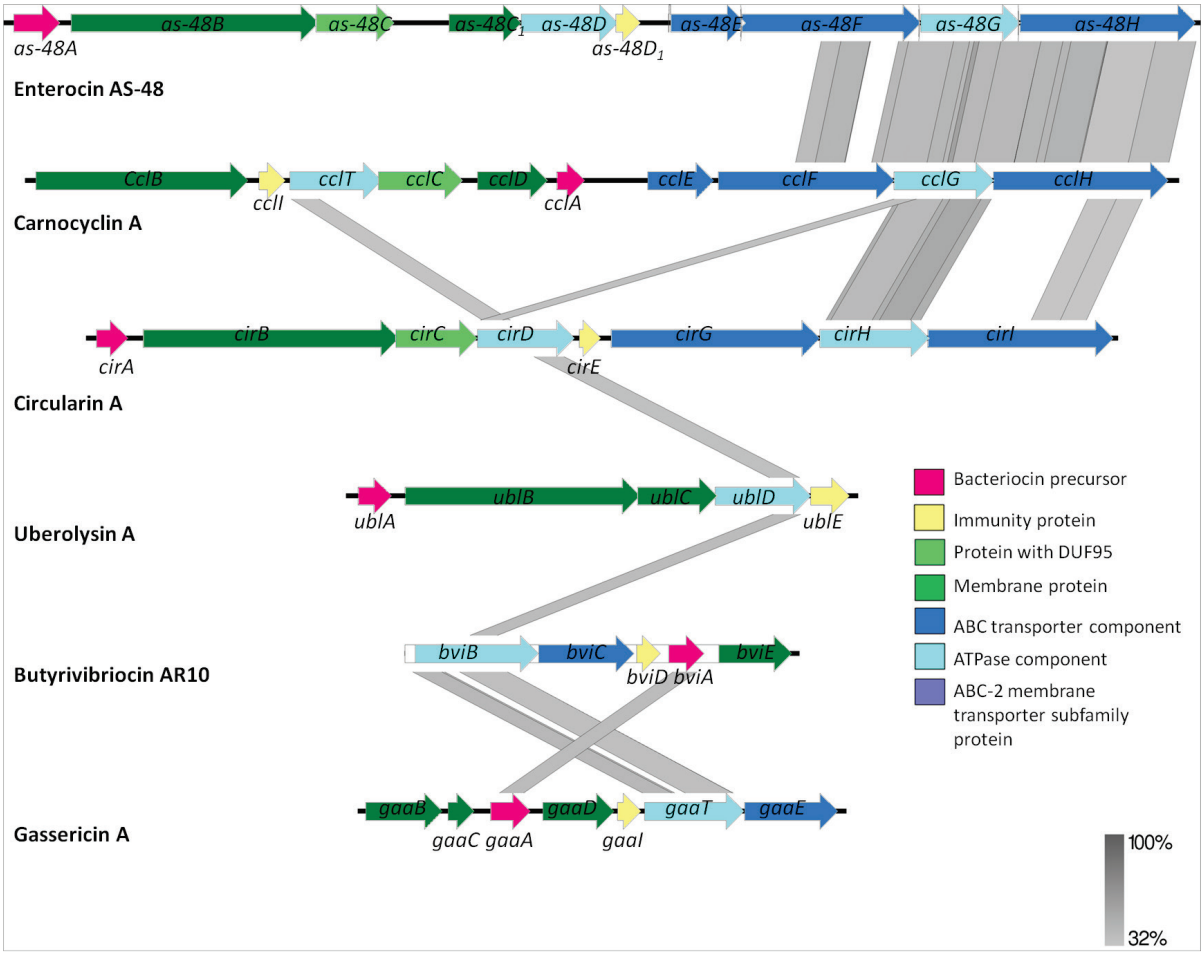

Figure 4: Genetic organization of the characterized circular bacteriocin gene clusters: enterocin AS-48 (AS-48ABCC$_1$DD$_1$EFGH) (Martinez-Bueno *et al.*, 1998), carnocyclin A (*cclBITCDAEFGH*) (Belkum *et al.*, 2010), circularin A (*cirABCDEGHI*) (Kemperman *et al.*, 2003a), uberolysin A (u*blABCDE*) (Wirawan *et al.*, 2007), butyrivibriocin AR10 (*bviBCDAE*) and gassericin A (*gaaBCADITE*) (Kawai *et al.*, 2009). The genes are coloured according to function, as indicated by the key, and homology at the protein sequence level is indicated by greyscale blocks.

1.2.3. Structure and physicochemical properties

The solution structures of enterocin AS-48 and carnocyclin A have been solved, revealing a common structural motif for the subclass i circular bacteriocins. Both peptides have a compact globular structure consisting of 4 (CclA) or 5 (AS-48) α-helices enclosing a hydrophobic core (Gonzalez *et al.*, 2000; Martin-Visscher *et al.*, 2009). The cyclization point is located internal to an α-helix, which has been demonstrated to have a pronounced effect on the stability of the structure (Gonzalez *et al.*, 2000). The presence of several basic amino acid residues imparts a localized positive charge on the surface of the structure, which is thought to promote attraction to the negatively charged surface of the target cell (figure 5). The overall structure is similar to the saposin fold (excluding intrachain disulfide bonds), found in the mammalian antimicrobial and cytotoxic peptide NK lysin (Gonzalez *et al.*, 2000). Protein structure homology modelling of the remaining peptides suggests that this structure is indeed shared among the subclass i bacteriocins (Martin-Visscher *et al.*, 2009) (figure 5). The 3D structures of BviA and GaaA have not yet been solved, but secondary structure prediction suggests that also the subclass ii peptides consist of predominantly α-helical regions, and could possibly form the same type of structure (Martin-Visscher *et al.*, 2009). These peptides are however dissimilar in their content of charged residues, and therefore do not display the localized charges seen in the 3D structures of class i peptides. The compact circular structure of circular bacteriocins is believed to be the reason why these bacteriocins in general exhibit very high thermal- and pH stability, and even renders the peptides resistant to degradation by many proteolytic enzymes (van Belkum *et al.*, 2011). Characterization of enterocin AS-48 linear forms show that the peptide retains antimicrobial activity to some extent (Montalban-Lopez *et al.*, 2008), indicating that the circular form is not essential for antimicrobial activity, but could be more important for stabilizing the structure (Montalban-Lopez *et al.*, 2008; Montalban-Lopez *et al.*, 2011).

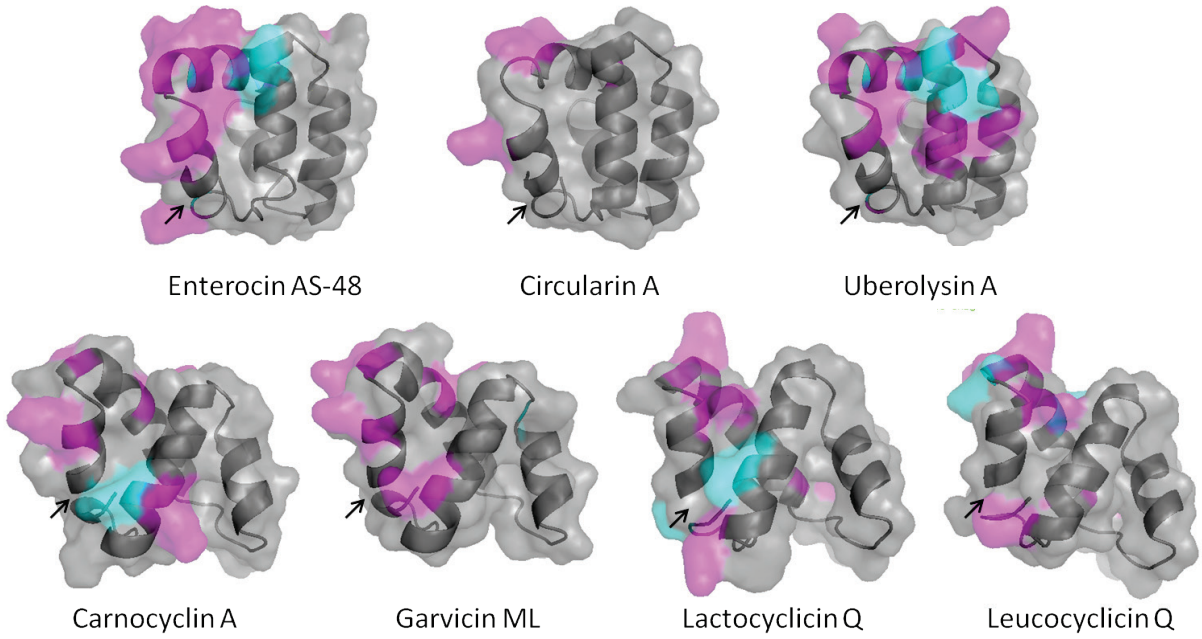

Figure 5: 3D structures of subclass i circular bacteriocins shown as cartoon rendering with transparent surface, where positively charged residues are coloured in magenta and negatively charged residues in cyan, and the circularization points are indicated by arrows. Structures were predicted by homology modelling (SWISS-MODEL) based on the alignment shown in figure 3. The solution structure of enterocin AS-48 (PDB ID 1E68) was used as template for homology modelling of circularin A and uberolysin A, and the solution structure of carnocyclin A (PDB ID 2KJF) was used as template for homology modelling of garvicin ML, lactocyclicin Q and leucocyclicin Q. While the longer peptides (circularin A and uberolysin A) are predicted to form 5 α-helices like enterocin AS-48, the shorter peptides (garvicin ML, lactocyclicin Q and leucocyclicin Q) are predicted to form 4 α-helices like carnocyclin A.

1.2.4. Activity spectrum and mode of action

Circular bacteriocins, in contrast to other class II bacteriocins, have broad antimicrobial activity spectra. Reflecting their producer organisms, the antimicrobial spectra generally include Gram-positive bacteria of the phylum *Firmicutes*, i.e. mainly lactic acid bacteria (order *Lactobacillales*), but also other *Bacilli* and *Clostridia* (table 2). Antimicrobial activity against other Gram-positive phyla has also been reported. A few circular bacteriocins (AS-48, LcyQ and LycQ) have been found to display antimicrobial activity against Gram-negative bacteria, namely *Escherichia coli*, although generally at significantly higher concentrations than are required for activity against Gram-positive bacteria (Galvez *et al.*, 1989; Masuda *et al.*, 2011; Sawa *et al.*, 2009). Interestingly, activity of carnocyclin A against Gram-negative species has been shown to increase upon disruption of the outer cell membrane by EDTA (Martin-Visscher *et al.*, 2011), which could indicate that restricted access to the membrane inhibits activity against Gram-negative bacteria.

Like other class II bacteriocins, the circular bacteriocins are generally believed to act by disruption of the integrity of the membrane of target cells (van Belkum *et al.*, 2011). The shared structural motif of subclass ii circular bacteriocins could suggest that these bacteriocins also have a common functional mechanism, at least in that the positively charged patches on the surface of the structures are thought to be the driving force behind initial attraction and subsequent insertion into the negatively charged phospholipid layer of the target cell membrane (Martin-Visscher *et al.*, 2009). However, the studies that have been carried out have so far highlighted distinct differences in the modes of action within this class of bacteriocins.

Enterocin AS-48 has been demonstrated to form non-selective pores in liposomes, leading to leakage of ions and low molecular weight compounds (Galvez *et al.*, 1991). Furthermore, enterocin AS-48 is oligomeric at physiological pH, and the suggested mechanism for molecular function of AS-48 involves a structural transition of this complex from a water-soluble form to a membrane-bound state (Sanchez-Barrena *et al.*, 2003).

Carnocyclin A was also shown to form pores in lipid membranes, but unlike AS-48 the pores formed by CclA are both anion-selective and voltage dependent (Gong *et al.*, 2009). Studies of the circular bacteriocins gassericin A and subtilosin A might indicate that also these bacteriocins do not require a target receptor (Kawai *et al.*, 2004a; Thennarasu *et al.*, 2005). The fact that several circular bacteriocins have been reported to act on lipid bilayers/liposomes may support the notion that a target receptor is not required for antimicrobial activity. However, some of these studies have been performed at bacteriocin concentrations significantly above that which is required for antimicrobial activity in vivo, which means it cannot be ruled out that a target receptor is involved in target recognition.

Table 2: Antimicrobial activity spectra of the circular bacteriocins: garvicin ML (Borrero *et al.*, 2011), carnocyclin A (Cebrian *et al.*, 2010), circularin A (Kemperman *et al.*, 2003b), enterocin AS-48 (Galvez *et al.*, 1989), uberolysin A (Wirawan *et al.*, 2007), lactococcin Q (Thennarasu *et al.*, 2005), leucocyclicin Q (Masuda *et al.*, 2011), gassericin A (Itoh *et al.*, 1995) and butyrivibriocin AR10 (Kalmokoff and Teather, 1997). Antimicrobial activity is denoted as +, weak activity as w and no activity as -, while blank cells are not determined. The values given here reflect the antimicrobial spectra of these bacteriocins as reported in the original papers, but in some cases only the high/moderately sensitive strains are included, and the values

			Bacteriocin	GarML	CclA	CirA	AS-48	UblA	LycQ	LcyQ	GaaA	BviA
			Subclass	i	i	i	i	i	i	i	ii	ii
			Producer organism	*Lactococcus garvieae*	*Carnobacterium maltaromaticum*	*Clostridium beijerinckii*	*Enterococcus faecalis*	*Streptococcus uberis*	*Lactococcus sp.*	*Leuconostoc mesenteroides*	*Lactobacillus gasseri*	*Butirivibrio fibrosolvens*
Activity against genus	*Firmicutes*	*Lactobacillales*	*Lactobacillus*	+	+	+			+	+	+	+
			Leuconostoc		+							
			Pediococcus	+	+				+	+		
			Lactococcus	+	+	+			+	+		
			Streptococcus	+				+				
			Enterococcus	+	+	+	+	+	+	+		
			Carnobacterium		+							
			Weisella							+		
		Bacillales	*Staphylococcus*				+	+			+	
			Bacillus				+		+	+	+	
			Listeria	+	+			+			+	+
		Clostridiales	*Clostridium*	+		+						+
			Ruminococcus									+
			Eubacterium									+
			Lachnospira									+
	Proteobacteria	*Entero-bacteriales*	*Escherichia*	-	-		+	-	w	w		
			Salmonella		-		+					
			Shigella				+					
			Klebsiella				+					
		Other	*Pseudomonas*		-		+					
			Agrobacterium				+					

1.3. *Lactococcus garvieae*

1.3.1. General characteristics

Lactococcus garvieae is a species of low GC, Gram-positive, facultatively anaerobic non-motile, non-spore-forming cocci (Vendrell *et al.*, 2006), closely related to the common dairy bacterium and model organism *L. lactis*. The lactococci were separated from the genus *Streptococcus* in 1985, and *L. garvieae* was established as the senior synonym of *Enterococcus seriolicida* in 1996 (Eldar *et al.*, 1996). Like all lactic acid bacteria, *L. garvieae* produces lactic acid as the major metabolic end product of carbohydrate fermentation, and is thus acid tolerant, but can also grow at high pH (9.6). The species is robust, tolerating temperatures from 4-45 °C, and can also grow on 6.5 % NaCl and in 40 % bile (Vendrell *et al.*, 2006). *L. garvieae* is part of the normal flora of many dairy products (Fernandez *et al.*, 2010; Walther *et al.*, 2008), especially in artisanal cheeses, but can also be found in a wide range of animal samples (Pot *et al.*, 1996; Sanchez *et al.*, 2007).

1.3.2. Genomics and pathogenicity

L. garvieae first became reputed as a major fish pathogen, after it was identified as the causative agent responsible for septicemia in rainbow trout in the 1980s (Vendrell *et al.*, 2006). Numerous outbreaks of fatal lactococcosis has since then been described in different fishes as well as in other marine and fresh-water species (Tsai *et al.*, 2012), causing significant economic losses in fish farming worldwide. Lactococcosis is generally a warm water infection, which peaks in summer when the temperature rises above 15 °C and becomes endemic (Vendrell *et al.*, 2006). *L. garvieae* has also been identified in clinical samples from water buffalos with mastitis, cows with mammary infection, and from human clinical samples (urinary tract, blood, skin) (Chan *et al.*, 2011; Teixeira *et al.*, 1996). An increasing number of reports on *L. garvieae* have thus implicated this bacterium in human infections, particularly in infective endocarditis, and consequently this bacterium has been labelled as a potential zoonotic agent (Russo *et al.*, 2012). Even so, the virulence of *L. garvieae* in human hosts is generally considered to be low, the role of *L. garvieae* as the causative agent in human infection has not been unequivocally demonstrated, and *L. garvieae* may therefore yet prove to be more of an opportunist than an overt pathogen in humans (Fihman *et al.*, 2006; Russo *et al.*, 2012).

Due to its status as a major fish pathogen and emerging zoonotic agent, there has been a recent increase in interest in *L. garvieae* genomics. In the last few years, two complete genomes and 11 draft genomes have been published, with at least 5 more soon to be released (NCBI Genome database). The genomes of sequenced *L. garvieae* range between 1.93-2.24 Mb, have GC contents between 37.7 and 38.8 %, and contain from 1874-2281 genes. Comparative genomic analyses of *L. garvieae* strains from different sources (dairy, fish, meat, vegetable and cereal) have shown that the

species displays high genetic and genomic heterogeneity, which are generally not consistent with ecological niche, and are clustered in two main genomic lineages (Ferrario *et al.*, 2012).

The main focus of genomic studies on *L. garvieae* has been to identify potential pathogenicity factors. Early epidemiological studies focused on two antigenic types designated as KG^+ and KG^- (based on agglutination with strain KG 7404 antiserum) which distinguishes non-capsulated from capsulated strains accordingly. Capsulation is a common defence strategy of pathogenic bacteria to protect against the host immune system (Peterson, 1996), and capsulated strains of *L. garvieae* have been found to be more virulent (lower LD_{50}) that their non-capsulated counterparts (Barnes *et al.*, 2002). A capsule gene cluster encoding 15 genes flanked by IS-elements was recently characterized and identified as a crucial virulence factor distinguishing a highly virulent *L. garvieae* strain from an non-virulent strain, both isolated from yellowtail (Morita *et al.*, 2011). In addition to the capsule gene cluster, a number of potential virulence factors have been identified in different *L. garvieae* genomes, but have yet to be demonstrated to be involved in the virulence process. Of these, exotoxins such as hemolysin and genes implicated in adhesion/colonization such as LPxTG-sortases, adhesins of different specificities (fibronectin-binding, collagen-binding, mucin-binding) and enzymes that confer tolerance to aerobic environments (superoxide dismutase, NADH oxidase) are common (Miyauchi *et al.*, 2012; Morita *et al.*, 2011). A putative ADP ribosylating toxin has also been identified in plasmid pGL5 of the human clinical strain *L. garvieae* 21881 (Aguado-Urda *et al.*, 2012).

1.3.3. Bacteriocin production

Bacteriocin production does not appear to be widespread in *L. garvieae*, with only four bacteriocins having been isolated from this species to date. However, as *L. garvieae* is increasingly being studied, we may see an increase in the discovery of new bacteriocins from this species. The four "garvicins" characterized thus far (table 3), have shown that there is indeed heterogeneity in the type of bacteriocins produced by *L. garvieae*, and so this species may serve as a possible reservoir of interesting new antimicrobial peptides.

The first discovered bacteriocin, garviecin L1-5 is a very low molecular weight (2.5 kDa) heat stable bacteriocin of unknown class, produced by *L. garvieae* L1-5 isolated from raw cow's milk. The bacteriocin has a broad antimicrobial spectrum, with activity against closely related species of lactococci as well as different genera like *Enterococcus*, *Listeria* and *Clostridium* (Villani *et al.*, 2001). The bacteriocin has not been further characterized, and so the peptide sequence and any other data about this peptide are unknown.

The second bacteriocin to be characterized from *L. garvieae* is Garvicin ML. Garvicin ML (GarML) is a 60 amino acid long bacteriocin produced by *L. garvieae* DCC43 (Borrero *et al.*, 2011), which was isolated from Mallard duck (*Anas platyrhynchos*) intestines (Sanchez *et al.*, 2007). GarML is synthesized as a 63 amino acid peptide precursor, which is processed between the N- terminal Asp^3 and Leu^4 and circularized with the C-terminal Ala^{63} to form the mature circular 60 amino acid peptide (Borrero *et al.*, 2011) (figure 3). Like other circular bacteriocins, GarML has a very broad spectrum of antimicrobial activity. As is often the case, the most sensitive species are those most closely related to the producer, i.e. *L. garvieae* and other lactococci. However, different strains of *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, food pathogens such as *Clostridium* and *Listeria* and even *Propionibacterium* (*Actinobacteria*) have been shown to be either highly or moderately sensitive to this bacteriocin (Borrero *et al.*, 2011).

Garvieacin Q (GarQ), produced by *L. garvieae* BCC 435778 isolated from a fermented pork sausage, is a class IId bacteriocin. Garvieacin Q is synthesized as a 70 amino acid residue precursor, containing no modified residues. It is cleaved at a double-glycine motif (like the class IIa peptides) upon export out of the cell to form a mature 50 amino acid peptide, but does however not contain the hallmark "pediocin box" motif of the class IIa peptides. The antimicrobial spectrum of GarQ comprises, in addition to *L. garvieae*, strains of *Enterococcus*, *Lactobacillus*, *Listeria* and *Pediococcus* (Villani *et al.*, 2001).

The most recently identified bacteriocin from *L. garvieae* is produced by the human clinical isolate *L. garvieae* 21881. Garvicin A (GarA) is a class IId bacteriocin, which has a leader sequence very similar to that of GarQ (95 % identity), and is equally cleaved at a double-glycine motif upon export, but has no similarity of the mature peptide to any other known bacteriocin. Interestingly, the antimicrobial spectrum of GarA is narrow, actually displaying only intraspecies activity. GarA has

potent bactericidal activity, rapidly killing almost 100 % of the cell population when added to exponentially growing target cells, and appears to act by inhibiting cell wall biosynthesis/septum formation (Maldonado-Barragan *et al.*, 2013).

Table 3: General characteristics of bacteriocins produced by *L.garvieae*.

Peptide full name and abbreviation		Producer strain	Isolation source	Size (aa) leader/mature		Mass (Da)	Class	Activity spectrum
Garviecin L1-5	-	L1-5	raw cow's milk	-	-	2500.0	-	broad
Garvicin ML	GarML	DCC43	mallard duck intestines	3	30	6004.2	IIc	broad
Garvieacin Q	GarQ	BCC 43578	fermented pork sausage	20	50	5339.0	IId	broad
Garvicin A	GarA	L1-5	human clinical isolate	20	43	4678.6	IId	narrow

2. Aims of the study

During the last few decades, there have been discovered a vast array of new antimicrobial peptides from bacteria. As outlined in chapter 1.1.3, many of the characteristics of these bacteriocins make them highly promising candidates as antimicrobial agents, for use in medicine as well as in food and feed industry, and the bacterial strains producing them as probiotics. There are however a few areas of bacteriocin research that are not yet sufficiently explored, that are important to illuminate if bacteriocins are to be considered as real alternatives to traditional antimicrobials. These issues include first and foremost a detailed understanding of the mode of action, where there is a clear need of identifying potential target molecules and mechanism of action on target cells for most class I and II bacteriocins. Secondly, mechanisms of resistance development and intrinsic immunity are poorly understood for many classes of bacteriocins, and understanding these processes is essential in order to be able to minimize resistance.

In this project, we have studied a specific bacteriocin, i.e. garvicin ML, a highly stable circular bacteriocin produced by the lactic acid bacterium *L. garvieae*, which has a broad spectrum of activity and is active against a range of pathogenic bacteria. The main objectives of this study, summarized below, were aimed at gaining a deeper understanding of exactly those aspects of bacteriocin biology that are vital for the potential future use of these as antimicrobial agents: biosynthesis, mode of action and resistance mechanisms.

- Study the genomic context and potential virulence traits of the *L. garvieae* producer strain.
- Investigate the functional genetic background for GarML biosynthesis and immunity
- Gain a detailed understanding of the target recognition and mode of action of this bacteriocin
- Gain insight into mechanisms which cause resistance to GarML in sensitive bacteria, and characterize the resistance frequency and –level of such mechanisms.

3. Main results

Paper I

Genome sequence of the bacteriocin-producing strain *Lactococcus garvieae* DCC43

Gabrielsen, C., Brede, D.A., Hernandez, P.E., Nes, I.F., and Diep, D.B. (2012) *Journal of bacteriology* **194**: 6976-6977.

The genome of the bacteriocin-producing strain *Lactococcus garvieae* DCC43 was sequenced and de novo assembled first and foremost in order to determine the genetic background of GarML production, but also to analyze this strain in context of *L. garvieae* genomics, with particular respect to known virulence traits. The assembled draft genome consists of 68 contigs, ranging in size from 227 to 178,922 bp. The contigs add up to a total of 2,244,387 bp, with a GC content of 37.8 %, which makes it the largest *L. garvieae* genome sequenced to date (GenBank, august 2013). Two contigs sized 35,101 and 52,437 bp, displayed significant homology to the *L. garvieae* 21881 plasmid pGL5 (Aguado-Urda *et al.*, 2012), indicating that this strain has at least two large plasmids. At least two or three complete prophages were also present in the genome, the exact number was difficult to determine due to the repetitive nature of these phages, which cause assembly to collapse.

Annotation of the draft genome yielded 2,227 protein-coding genes and 50 tRNAs, among which was identified the gene cluster encoding garvicin ML and its cognate biosynthetic machinery (paper II). Of potential virulence genes, we did not observe any similarity to the capsule gene cluster which has been established as crucial for virulence in fish (Morita *et al.*, 2011). Four genes were predicted to be hemolysins or hemolysin-like, but no true (β-hemolytic) activity was observed from this strain on blood agar. Nor were any potential antibiotic resistance genes identified. There were however two internalin like mucin-binding proteins. This type of proteins are commonly found in a wide variety of bacterial proteins, and they displayed little or no homology to InlA/InlB of virulent *L. monocytogenes*, which are required for entry into human cells (Dramsi *et al.*, 1995; Gaillard *et al.*, 1991). Five predicted LPXTG-specific sortases, two of which have no known counterparts in other *L. garvieae* strains, were also identified. Sortases are transpeptidase enzymes which cleave proteins at a specific signal sequence (commonly LPXTG) and covalently attach these proteins to the cell wall, some of which have a housekeeping role, while others have been implicated in bacterial pathogenesis (Spirig *et al.*, 2011) .

Comparative genomic analysis of *L. garvieae* DCC43 to other sequenced *L. garvieae* strains revealed that although DCC43 has 99 % sequence identity of the 16S rDNA, this strain varies greatly in both genome sequence and organization compared to the other strains (paper I). Furthermore,

genomic BLAST (Cummings *et al.*, 2002) of sequenced strains revealed that *L. garvieae* DCC43 belongs to a separate clade, which is significantly distanced from the other *L. garvieae* DCC43 strains (figure 7). These results could suggest that *L. garvieae* DCC43 may represent a novel *L. garvieae* subspecies.

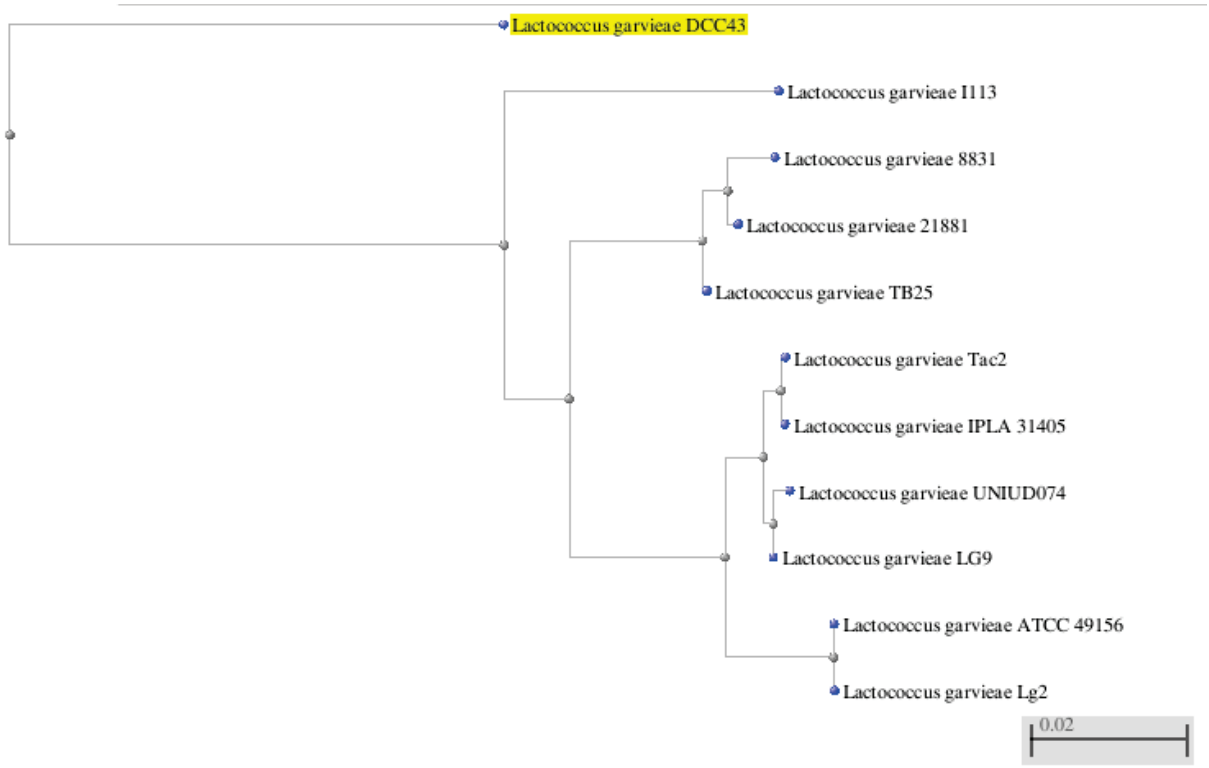

Figure 7: Phylogenetic tree of sequenced *L. garvieae* strains, based on genomic BLAST of the largest contig of the *L. garvieae* DCC43 draft genome (yellow).

Paper II

Functional genetic analysis of the garvicin ML gene cluster in *Lactococcus garvieae* DCC43 gives new insights into circular bacteriocin biosynthesis

Gabrielsen, C., Brede, D.A., Nes, I.F., Salehian, Z. and Diep, D.B. (manuscript)

To elucidate the genetic background for production and immunity to GarML, bioinformatic and functional analysis at gene and protein level of the producer strain *L. garvieae* DCC43 was performed.

Bioinformatic analysis identified nine open reading frames, organized in four operons, that were involved in GarML biosynthesis and immunity. Of these, *garA* encodes the bacteriocin precursor, *garBCDE* encode the putative immunity gene followed by an ATPase and two transmembrane proteins, *garFGH* encode an ABC transporter complex, and *garX* encodes a large transmembrane protein. The *garBCDE* and *garFGH* operons appear to be regulated by translational coupling, as some of the encoded ORFs overlap. While *garX* is located downstream on the same strand as *garA*, the other two operons are located upstream on the reverse strand, and this organization is unique in circular bacteriocins. Comparative analysis of the GarML gene cluster to other circular bacteriocin gene clusters furthermore revealed that although there is little sequence identity at protein level (average 20-30 % pairwise identity), there are many commonalities and shared characteristics of the encoded proteins.

Functional analysis revealed that while deletion of *garFGH* did not have any significant effect on either bacteriocin production or immunity, knocking-out *garBCDE* and *garX* had dramatic effects on both these phenotypes. The *garBCDE* deletion mutant displayed no observable production of active bacteriocin, and a 250-fold lowering of the sensitivity to GarML, in effect a complete loss of immunity. The *garX* knock-out mutant similarly did not produce any active bacteriocin, and also displayed an 8-16-fold reduction in immunity to GarML. The results thus suggested that the GarX and GarBCDE proteins were essential for biosynthesis and immunity to GarML.

Mass spectrometry allowed detection of trypsin fragments diagnostic to all three forms of the bacteriocin in the supernatant of wild-type producer strain; the linear full length peptide with leader sequence (MFD), linear peptide without the leader sequence, as well as the mature circular peptide. Interestingly, we detected the two linear forms in all of the knock-out mutants as well as in the controls, which indicates that cleavage of the leader sequence is a separate process from circularization, but also that it precedes circularization in time. Furthermore, finding these peptides in all our knock-outs, cleavage is likely performed by neither GarX, GarBCDE nor GarFGH. The circular bacteriocin was detected in the *garFGH* deletion mutant, but not in the *garBCDE* deletion mutant nor in the *garX* knock-out mutant, which is consistent with antimicrobial assays, and corroborates the involvement of the encoded proteins in GarML biosynthesis. Furthermore, these

results imply that the circular structure of GarML is essential for the antimicrobial activity of this peptide.

Paper III

The maltose ABC transporter in *Lactococcus lactis* facilitates high-level sensitivity to the circular bacteriocin garvicin ML.

Gabrielsen, C., Brede, D.A., Hernandez, P.E., Nes, I.F., and Diep, D.B. (2012) *Antimicrobial agents and chemotherapy* **56**: 2908-2915.

We exposed the GarML-sensitive strain *L. lactis* IL1403 to bacteriocin concentrations ranging from 150-300 BU mL^{-1}, to select for GarML-resistance. Colonies arose at frequencies from 10^{-7} to 10^{-8} at the lower concentrations, but above 250 BU mL^{-1} no growth was observed. Of the resistant colonies, six isolates were found to show an average 6-11-fold reduction of MIC_{50} compared to the wild-type, a phenotype which was stable for more than 50 generations of non-selective growth. The GarML resistant phenotype was specific, in that these isolates displayed no change in sensitivity towards other lactococcal bacteriocins.

Upon determination of carbohydrate fermentation patterns, it was discovered that all six GarML-resistant isolates had lost the ability to ferment starch and glucose, relative to the wild-type which grows on both carbohydrates. Furthermore, it was shown that in the wild-type sensitivity to GarML is correlated to carbon catabolite repression (CCR), the main mechanism responsible for hierarchical regulation of sugar utilization in *L. lactis*: Bacteriocin sensitivity increased up to 4-fold during growth on non-CCR-inducing sugars maltose and galactose (control) compared to growth on glucose (CCR-inducing), and the effect of the former was indeed alleviated upon addition of glucose. Thus, relief of CCR in general, and growth on maltose specifically, was demonstrated to increase sensitivity to GarML in *L. lactis*.

The maltose negative phenotype of the six GarML-resistant isolates was subsequently determined to be caused by a 13.5 kb genomic deletion, corresponding to nt 2733559-1747095 in the genome of *L. lactis* IL1403. The basis for the deletion event was likely two genes (*tra981I* and *yrdA*) flanking the deletion, which both encode transposon-related functions for insertion sequence (IS) element 981I. The deleted region mainly encodes carbohydrate metabolism-related functions, including an alpha-amylase (*amyY*), a maltose O-acetyltransferase (*maa*), an oligo-1,6-glucosidase (*dexA*), a neopullulanase (*dexC*) and a maltose ABC transporter complex (*malEFG*) in addition to several hypothetical proteins (*yreA*, *yreB*, *yreC*, *yreD*, *yreE* and L200065).

Based on these results, we hypothesized that loss of a potential receptor molecule or other mediator of GarML activity could account for the GarML-resistant phenotype of the isolates. Of the deleted genes, the membrane-bound and CCR-regulated maltose ABC transporter complex encoded by *malEFG* was considered the most likely candidate. We therefore complemented two of the resistant isolates with the *malEFG* genes, expressed from a nisin-inducible promoter.

Complementation rendered the resistant isolates 200B3 and 150G1 highly sensitive to GarML, on average displaying 22- and 58-fold reductions in the MIC_{50} accordingly, in effect not only restoring the wild-type level of sensitivity towards GarML (5 BU mL^{-1}) but reducing it even further (0.3 and 0.7 BU mL^{-1} accordingly). Furthermore, we were able to establish a dose-dependent relationship between sensitivity to the bacteriocin and induction of *malEFG*, by graded expression induced with 0-10 ng mL^{-1} nisin, where sensitivity to GarML ranged 50-fold (1-50 BU mL^{-1}) increasing with the expression level of *malEFG*. These results demonstrate that the maltose ABC transporter of *L. lactis* IL1403 plays an essential role in sensitivity to the circular bacteriocin GarML.

Paper IV

Functional genomic analysis of *Lactococcus lactis* ssp. *cremoris* reveals a novel resistance mechanism to the circular bacteriocin garvicin ML

Gabrielsen, C., Brede, D.A., Nes, I.F., Salehian, Z. and Diep, D.B. (manuscript)

The circular bacteriocin GarML is generally active against lactococci, but by screening different strains for sensitivity towards GarML, we discovered a genetic lineage of *L. lactis* ssp. *cremoris* strains which displayed unusually low sensitivity towards this bacteriocin (MIC_{50} values of ≥106 BU mL^{-1}). Interestingly however, another strain of the same lineage, the spontaneously rifampicin and streptomycin-resistant derivative MG1614, displayed high level sensitivity to GarML (5 BU mL^{-1}). We therefore hypothesized that *L. lactis* MG1363 and its progenitor strains have an intrinsic resistance mechanism towards GarML, which has been inactivated in *L. lactis* MG1614, and proceeded to investigate the basis for this phenotype.

As the maltose ABC transporter has previously been determined to facilitate sensitivity to GarML in *L. lactis*, we first confirmed the presence of the *malEFG* genes and the maltose metabolism in this strain, thus excluding the possibility that loss or reduced expression of these could account for GarML-resistance. Furthermore, the possible link between GarML and the streptomycin- and rifampicin resistance of *L. lactis* MG1614 was investigated by creating corresponding resistant strains of *L. lactis* MG1363, but neither of the resulting mutants were affected in sensitivity to GarML. Potential extracellular proteolytic activity of all the lactococcal strains was assayed on different substrates, but no such activity was observed that could account for the GarML resistance of the *L. lactis* ssp. *cremoris* lineage.

The genomes of *L. lactis* MG1363 and MG1614 were sequenced, reads were mapped, and SNPs and indels were called according to the complete genome sequence of *L. lactis* MG1363 (Linares *et al.*, 2010; Wegmann *et al.*, 2007). Comparing the two strains, only 13 identified SNPs, 10 of which were intragenic, in addition to two indels differed. Most noticeable was a genomic deletion of 27.7 kb (nt 675922-703641), encompassing 35 complete and one truncated ORF, in strain MG1614. This deletion had occurred in a region termed the “integration hot spot”, and adjacent to the deletion region were identified IS981-related genes which were likely responsible for causing this event.

To determine whether the genomic deletion was the cause of the GarML-resistant phenotype, an isogenic double crossover mutant of *L. lactis* MG1363 was created, containing an identical deletion. This strain, DC01, was rendered equally sensitive to GarML as *L. lactis* MG1614, thus establishing that loss of this region causes sensitivity to GarML, and implicating that the genetic basis of GarML-resistance in *L. lactis* MG1363 is located in this region.

The deletion region encodes, amongst other, an oligopeptide uptake system, an endopeptidase, two transcriptional regulator-like proteins, and 14 hypothetical proteins with unknown function. Interestingly, several of the hypothetical proteins displayed similarity to bacteriocin-associated genes. However, none of the ORFs display any significant similarity to the garvicin ML gene cluster (paper II). Based on bioinformatic analysis and known resistance mechanisms, several ORFs were cloned and expressed in *L. lactis* DC01, including the bacteriocin- and immunity-like ORFs (llmg_0687-88, llmg_0704-05 and llmg_0706), the *oppDFBCA* operon encoding an oligopeptide ABC transport system as well as the intracellular endopeptidase pepO. However, none of the candidate genes provided any significant change in sensitivity towards GarML.

Comparative analysis was undertaken to elucidate which ORFs in the deletion region did not have orthologs in other GarML-sensitive lactococci, i.e. *L. lactis* IL1403 and *L. lactis* SK11. Furthermore, available microarray data was analyzed to determine which of the ORFs in this region are expressed under similar growth conditions (Linares *et al.*, 2010). Excluding the involvement of ORFs based on these criteria, we were left with 6 hypothetical genes with unknown function, which are the most likely candidates responsible for resistance to GarML. Neither of these display similarity to any known resistance mechanism against bacteriocins. As exhaustive efforts to clone fragments containing these ORFs failed, we were however unable to pinpoint the exact gene or genes causing resistance to GarML in *L. lactis* MG1363 at this time. We proceeded to assay the MIC_{50} of the *L. lactis* ssp. *cremoris* lineage towards a range of other bacteriocins. The results show that GarML resistance is specific, as the differences in sensitivity are not observed for other bacteriocins targeting lactococci such as lactococcin A, lactococcin B, nisin and circularin A. These results thus provide evidence for a novel, inherent resistance mechanism, which is specific for GarML, in this lineage of *L. lactis* ssp. *cremoris* strains.

4. Discussion

4.1. A study of the circular bacteriocin Garvicin ML

In this work we have chosen to study garvicin ML, a representative of a class of circular antimicrobial peptides which are gaining interest due to their favourable characteristics, i.e. high thermal and pH stability in addition to resistance to a number of proteases, which make them promising candidates for potential industrial applications. Although there are only characterized 9 circular bacteriocins to date, head-to-tail ligated ribosomally synthesized peptides with different functions are widely disseminated in all kingdoms of life, and are still being discovered at high rate (CyBase, September 2013). The results presented in this thesis shed light on different aspects of the biology of the GarML, intending to gain a deeper understanding of exactly those aspects of bacteriocin biology that are vital for the potential future use of these as antimicrobial agents: biosynthesis, mode of action and resistance mechanisms.

GarML is produced by *L. garvieae* DCC43, a species which is gaining interest due to it being a known fish pathogen and considered an emerging zoonotic agent. Paper I therefore describes the genome sequence and genomic context of the GarML producing strain, with focus on potential virulence traits. Based on this genome sequence, paper II describes the identification and functional analysis of the gene cluster involved in GarML biosynthesis and immunity. Given the antimicrobial spectrum of GarML, we chose to use the common industrial bacterium *L. lactis* as a model organism for studies on GarML mode of action and resistance mechanisms. In Paper III the mode of action of GarML is investigated, wherein is presented evidence for a transport complex acting as a target molecule or mediator of GarML antimicrobial activity in *L. lactis*. Paper IV however describes the identification of a novel, specific and inherent resistance mechanism to GarML, which is conserved in a lineage of *L. lactis* ssp. *cremoris* strains.

4.2. *Lactococcus garvieae* DCC43 genomics

As part of the study of garvicin ML, we sequenced the genome of the producer strain *L. garvieae* DCC43. Genome sequencing did not reveal any evidence indicating that *L. garvieae* DCC43 is a pathogen, first and foremost because it did not contain the capsule gene cluster which is implicated in pathogenicity in fish, but also because no antibiotic resistance genes, no hemolytic activity nor any other unequivocal virulence factors were identified in this strain (paper I). Although one cannot conclude based on genomic data alone that *L. garvieae* DCC43 is not a pathogen, there is however no strong indication to the contrary based on these results. One concern is however that *L. garvieae* DCC43 contains two plasmids that display similarity to the pGL5, a plasmid which encodes a putative

ADP ribosylating toxin, from the human clinical strain *L. garvieae* 21881 (Aguado-Urda *et al.*, 2012), which could indicate that this strain has taken up putative virulence factor-containing plasmids. The status of this strain is important for the potential use of the bacterium and/or the bacteriocin itself in food, feed or medical applications. Given the high specific activity of this bacteriocin against *L. garvieae* strains (Borrero *et al.*, 2011), there is an obvious potential for using GarML to combat pathogenic *L. garvieae*, for example in fish farming where lactococcosis outbreaks causes significant economic losses worldwide.

Comparative genomics of *L. garvieae* DCC43 revealed that this strain has the largest known genome, with the most predicted protein-coding genes, of all the sequenced *L. garvieae* strains to date. Importantly, our results highlight large sequence variation and genomic organization of this strain, clearly distinguishing it phylogenetically from the other *L. garvieae* strains (paper I), which might imply that *L. garvieae* DCC43 in fact represents a novel subspecies. Thus, the genome-sequence of this strain contributes new knowledge of this ubiquitous species, which until now has been studied mainly on the basis of its pathogenicity.

4.3. Genetics and biosynthesis of GarML

Based on the draft genome sequence of *L. garvieae* DCC43 (paper I) and the known sequence of the GarML structural gene (Borrero *et al.*, 2011), we identified and characterized the gene cluster involved in GarML biosynthesis and immunity. This gene cluster consists of nine open reading frames organized in 4 operons: the *garA* gene encodes the bacteriocin precursor, *garX* codes for a large transmembrane protein, *garBCDE* encode a putative immunity protein (*garB*) followed by an ATPase and two transmembrane proteins, and *garFGH* codes for an ABC transporter complex (paper II). Despite limited sequence similarity of the encoded proteins, there are many similarities between the organization and encoded functions of the circular bacteriocin gene clusters, particularly of subclass i, which might reflect a common theme in the biosynthetic machinery and mechanisms for their production (paper II). But although the gene clusters of most circular bacteriocins have been identified, there are still several aspects of the biosynthesis of these peptides which have remained elusive. Functional analysis combined with mass spectroscopy of the GarML gene cluster has however provided new insights into the biosynthesis of this bacteriocin (paper II).

The maturation of circular bacteriocins is generally thought to consists of three events: removal of the leader peptide, circularization and export, which take place either in a defined order in a stepwise fashion or in a coordinated one-step reaction, however the sequence of events and potential coupling of the processes are not known (Conlan *et al.*, 2010; Maqueda *et al.*, 2008). In this work, we have however provided evidence for leader sequence cleavage occurring without subsequent circularization in two knock-out mutants (Δ*garBCDE* and *garX*::pCG47), which demonstrates not only that these processes are independent, but that leader sequence cleavage precedes circularization

in time (paper II). This may indicate that the leader is not required as a recognition signal for export out of the cell. It is however conceivable that the leader functions as a critical control checkpoint for the biosynthetic machinery, i.e. that export and circularization is only possible when the processed N-terminal end of the mature bacteriocin is accessible. Furthermore, the evidence suggests that leader sequence cleavage is not performed by any of the proteins encoded by the GarML gene cluster, i.e. *garX*, *garBCDE* or *garFGH*, because we still observe cleavage in their absence (paper II). These results thus explain the apparent lack of any protease/peptidase functional domains encoded in the gene clusters of circular bacteriocins, and furthermore may reflect a situation which is more similar to the mechanisms of other bacterial head-to-tail ligated circular peptides like TrbC from *E. coli* (Eisenbrandt *et al.*, 2000) and VirB2 from *A. tumefaciens* (Cebrian *et al.*, 2010) which are N-terminally cleaved by chromosomally encoded signal peptidases. The requirement for chromosomal determinants has been suggested previously for enterocin AS-48 (Fernandez *et al.*, 2007; Montalban-Lopez *et al.*, 2012), and may explain the apparent problems in expressing circular bacteriocins outside the genera or species of the original producer (Fernandez *et al.*, 2007; Kawai *et al.*, 2009) and in obtaining stable heterologous production (Kemperman *et al.*, 2003a).

While leader sequence cleavage is likely performed by a trans-acting unknown peptidase, the circularization reaction however appears to be encoded in the GarML gene cluster. In two knock-out mutants (*garBCDE* and *garX*), no antimicrobial activity or circular bacteriocin was detected in culture supernatants, suggesting that circularization was not taking place. Moreover, immunity was significantly impaired in both mutants. Interestingly, the functions of GarBCDE and GarX appeared to be non-overlapping, as neither compensated to any significant extent for inactivation of the other, which would be the expected outcome if these proteins either could perform circularization or provide immunity separately. Our results therefore demonstrate that circularization of GarML does not occur, and immunity is severely affected, without the presence of both GarBCDE and GarX (paper II). Consequently, it is likely that these proteins are linked, either functionally or by regulation, to provide both immunity and biosynthesis of GarML. In other circular bacteriocin clusters, the genes corresponding to *garX* and *garD* are encoded by the same polycistronic transcript (Kemperman *et al.*, 2003a; Martinez-Bueno *et al.*, 1998), thus supporting the possible link between the encoded proteins. Co-production and/or -regulation of the immunity and biosynthetic machinery is indeed common in bacteriocin production (Draper *et al.*, 2008), a strategy which is clearly advantageous for the producer organism. In the GarML gene cluster there are however no encoded proteins which display similarity to known regulatory domains. We therefore believe that GarX and GarBCDE are functionally linked, possibly by forming a multi-complex in the cell membrane, to perform circularization of the linear peptide into the mature circular form, most likely concomitant with export out of the cell (figure 5, paper II).

For most of the subclass i circular bacteriocins, an ABC transporter complex has been identified as belonging to the gene cluster, but this ABC transporter has in several cases been shown

not to be essential for bacteriocin production and immunity (Belkum *et al.*, 2010; Kemperman *et al.*, 2003a; Martinez-Bueno *et al.*, 1998). This is also the case for the *garFGH* operon of the GarML gene cluster, which can be deleted without any apparent effect on either production of mature bacteriocin or immunity to GarML (paper II). For enterocin AS-48 the corresponding ABC transporter has however been shown to provide an increased level of immunity and enhance the production of the bacteriocin (Diaz *et al.*, 2003). It is possible that GarFGH functions as a secondary transporter of GarML, to prevent extracellular bacteriocin from penetrating the cell envelope, in a manner similar to the immunity-providing ABC transporters of many lantibiotics (Draper *et al.*, 2008). However, our experimental results did not support this notion as the presence of the intact *garFGH* did not compensate for the loss of immunity resulting from deletion of the *garBCDE* operon or the *garX* gene (paper II), and thus we were unable to ascribe any function to GarFGH. However, based on the apparent conservation of these ABC transporters between circular bacteriocin gene clusters (paper II), in addition to the known function of the corresponding complex in enterocin AS-48 (Diaz *et al.*, 2003), it is possible that an independent function or accessory role of GarFGH will be elucidated at a later stage.

The 3D structures of class i circular bacteriocins share a common structural motif (Martin-Visscher *et al.*, 2009), which could imply a common mode of action. In particular, the localized positive charges on the surface of the structures are thought to be the driving force behind attraction to the negatively charged membrane on target cells (Martin-Visscher *et al.*, 2009). In two knock-out mutants (*garBCDE* and *garX*) that produced both linear forms of GarML but not the mature circular bacteriocin, we observed no antimicrobial activity, which suggested that the circular structure of GarML is essential for its antimicrobial activity (paper II). It is tempting to speculate that the head-to-tail peptide bond is important for retention of the 3D structure, and consequently that loss of structural integrity could affect the interaction of the bacteriocin with the target cell.

4.4. Mode of action of GarML

For circular bacteriocins it has been and remains a controversial issue whether these peptides require a target receptor or docking molecule like the class Ia lantibiotics and IIa pediocin-like bacteriocins (Breukink *et al.*, 1999; Diep *et al.*, 2007), or whether the peptides interact unspecifically with the target cell membrane to create pores. The mode of action of most circular bacteriocins has not yet been determined, but for two of the most studied cases, enterocin AS-48 and carnocyclin A, it has been shown that the bacteriocins can permeabilize liposomes and/or lipid bilayers (Galvez *et al.*, 1991; Gong *et al.*, 2009). Also studies of circular bacteriocins gassericin A and subtilosin A indicate that the bacteriocins do not require a target receptor (Kawai *et al.*, 1998; Thennarasu *et al.*, 2005). Thus, it has been suggested that circular bacteriocins in general exert their activity independently of any target molecule in the cell membrane of. However, several of these studies have been performed

at bacteriocin concentrations significantly above that which is required for antimicrobial activity *in vivo*. For GarML, we have however demonstrated that a maltose ABC transporter complex mediates sensitivity to this bacteriocin (paper III). Deletion of the transporter complex led to a 6-11-fold decrease in sensitivity to GarML, and by complementation high-level sensitivity to the bacteriocin was restored. However, consistent with other circular bacteriocins, we observed receptor-independent killing at higher concentrations of GarML. Our results therefore suggest that this class of bacteriocins may indeed require a specific interaction with a target receptor/mediator for antimicrobial activity at low concentrations. Such a dual concentration-dependent mode of action has been demonstrated for the lantibiotic nisin (Wiedemann *et al.*, 2001), and can also be supported by studies of pore formation with the circular peptide subtilosin A (Thennarasu *et al.*, 2005).

The finding that a sugar transporter acts as a mediator of antimicrobial activity is similar to the situation for class IIa pediocin-like bacteriocins and lactococcin A, which target the mannose PTS uptake system in sensitive strains (Diep *et al.*, 2007), and are thought to act by rendering the permease open for efflux of solutes and dissipation of the proton motive force, eventually leading to cell death (Kjos *et al.*, 2011a). A similar mechanism of killing could easily be visualized for GarML, and the requirement for the entire complex (MalEFG) for mediation of antimicrobial activity (unpublished results) may support this hypothesis.

The targeting of sugar uptake systems is furthermore interesting from an ecological perspective. In this context, bacteriocin production can be viewed as a competitive mechanism (Dobson *et al.*, 2012; Kjos *et al.*, 2011a) targeting competitors for the (primary) food source of the producer strain. In the case of GarML, it is feasible that the producer strain *L. garvieae* DCC43 antagonizes other lactococci, generally believed to be of plant origin (Kelly *et al.*, 2010), competing for the plant-derived sugar maltose by targeting the maltose uptake system. Maltose utilization is indeed a widespread trait among lactococci (Bounaix *et al.*, 1996; Elliott *et al.*, 1991), and a protein BLAST search reveals that the *L. lactis* IL1403 maltose ABC transporter subunits have high sequence identity to the homologous proteins in the producer strain *L. garvieae* DCC43 (72 %, 68 % and 76 % identity to MalE, MalF and MalG accordingly, with 100 % coverage for all) (paper I), lending further support to this notion.

4.5. Resistance mechanisms to GarML

The significance of understanding resistance mechanisms to bacteriocins is a prerequisite for safely employing such peptides in biotechnological applications, and high frequency of resistance will impede the efficacy of these peptides. In this work, we have observed examples of both adaptive and inherent mechanisms of resistance towards GarML. Firstly, resistance development by loss of the maltose ABC transporter was observed at average frequencies from 10^{-7} to 10^{-8} by exposure to GarML concentrations of 150-250 BU mL^{-1} (paper III). This frequency is low compared to adaptive resistance

to class Ia and IIa bacteriocins, which has been shown to range from 10^2-10^7 and from 10^4-10^6 accordingly in *Li. monocytogenes* (Gravesen *et al.*, 2002). Furthermore, above a threshold level of 250 BU mL^{-1} no resistance development was observed, indicating that at high concentrations of bacteriocin this adaptation is not sufficient for survival. Given that the maltose ABC transporter of *L. lactis* is a non-essential cell component, this low frequency of resistance development is surprising. However, the lack of resistance development above the threshold level is consistent with receptor-independent killing or the presence of a second cellular target molecule, at high concentrations of GarML.

In this work we have identified a novel inherent resistance mechanism which is conserved in a lineage of *L. lactis* ssp. *cremoris* strains (paper IV). The potential involvement of the maltose ABC transporter in this mechanism of resistance was tested and ruled out; the same was any known extracellular proteolytic activity. The determinant(s) responsible for the resistance phenotype were determined to be located in a 27.7 kb region termed the "integration hot spot" of the *L. lactis* ssp. *cremoris* MG1363 genome (paper IV), which is rich in IS- and plasmid-derived sequences (Wegmann *et al.*, 2007). Several of the ORFs in this region displayed similarity to bacteriocin and immunity genes of class II bacteriocins, which could point to this region constituting a "resistance" island which at some point has provided the strain resistance against different bacteriocins. The region also encodes an oligopeptide ABC transporter (*oppDFBCA*) and an endopeptidase (*pepO*), which are both required for growth in milk (Tynkkynen *et al.*, 1993), and could explain the conservation of this region in this *L. lactis* ssp. *cremoris* lineage (Le Bourgeois *et al.*, 2000).

Based on gene complementation and bioinformatic analysis, six ORFs encoding hypothetical proteins with unknown functions remain the most likely candidates for providing GarML-resistance (paper IV). As neither of the putative GarML-resistance genes display any similarity to the genes required for immunity in the GarML producer strain (paper II), we can exclude the possibility of an immunity-like mechanism causing the resistant phenotype. Furthermore, the mechanism appears to be specific towards GarML, as sensitivity towards other bacteriocins targeting lactococci, including another circular bacteriocin, is not affected in these strains (paper IV). Thus, we have evidence for a new, specific and inherent mechanism of resistance to GarML in this lineage of *L. lactis* ssp. *cremoris* strains. This finding might have implications for a wide range of bacteriocins, as similar intraspecies variance in sensitivity to certain bacteriocin is quite common (Eijsink *et al.*, 1998). It has been speculated that this variation could be related to cell envelope composition, like changes in the D-alanylation of LTA to reduce the negative charge of the cell surface (Fabretti *et al.*, 2006; Kramer *et al.*, 2006). Another described mechanism is known as immune mimicry, which implies a close co-evolutionary development between the producer and the resistant strains. In addition, ABC-transporters functioning as efflux pumps can render certain strains less sensitive to bacteriocins, as the AnrAB transporter complex in *Li. monocytogenes* confers innate resistance to nisin (Collins *et al.*, 2010). None of these mechanisms however comply with the resistance identified in *L.lactis* towards

GarML. The fact that this resistance in found within a genomic island flanked by functional IS-elements could have implications for the dissemination of this resistance to closely related species like *L. garvieae* or *E. faecalis*. This perspective prompts caution and should be investigated further.

As highlighted in the introduction, immunity-like mechanisms notwithstanding, there are not many examples of inherent resistance mechanisms towards bacteriocins of lactic acid bacteria. However, there is increasing evidence for this type of resistance playing an important role in bacteriocin biology, e.g. efflux transporters providing bacteria with intrinsic resistance towards bacteriocins, which in addition to the mechanism presented for GarML may go a long way in explaining the large intraspecies variations in sensitivity to bacteriocins (Eijsink *et al.*, 1998) that cannot easily be explained by sequence variations of the target receptor (Kjos *et al.*, 2009) and changes in the cell surface (Vadyvaloo *et al.*, 2002; Vadyvaloo *et al.*, 2004) of target cells.

5. Concluding remarks and future perspectives

The work presented in this thesis provides new insight into several aspects of the biology of the circular bacteriocin GarML. The genome sequence of the producer strain *L. garvieae* DCC43 allowed determination of the gene cluster involved in GarML biosynthesis and the genomic context of this strain (paper I). Functional analysis of the GarML gene cluster combined with mass spectrometry provided evidence for cleavage of the leader sequence of GarML preceding circularization in time, and indicated that a chromosomally encoded peptidase could be responsible for this step in maturation (paper II). Future investigation aimed at elucidating the enzyme(s) responsible for leader sequence cleavage could contribute to establishing stable heterologous expression systems for circular bacteriocins, which would not only aid in further elucidating the biosynthetic processes, but could also allow bacteriocin production in an industrially relevant bacterial species and scale. We demonstrated that two of the operons in the GarML gene cluster, namely *garX* and *garBCDE*, are implicated in biosynthesis of GarML, specifically in the circularization reaction, as well as providing immunity towards GarML, and that their functions appeared to be linked (paper II). Future investigations should thus be aimed at further characterizing the interaction between these proteins, to elucidate the functional domains involved in and to gain a detailed understanding of the circularization mechanism. A better understanding of these processes could in turn allow for bioengineering of circular bacteriocins with enhanced properties for specific purposes in the future.

In paper III, we provided evidence for the maltose ABC transporter in *L. lactis* mediating high-level sensitivity to GarML, potentially by acting as a receptor for this bacteriocin. It is therefore tempting to speculate that the bacteriocin kills sensitive cells in a manner similar to the proposed model for class IIa pediocin-like bacteriocins, which are believed to act by rendering the mannose PTS permease open for efflux of solutes and dissipation of the proton motive force, eventually leading to cell death, and future studies should thus aim at elucidating the receptor interaction and the exact mechanism of cell killing for GarML.

In this work, we have demonstrated examples of both adaptive and inherent resistance to GarML. In paper III, we have shown that *L. lactis* can develop resistance to GarML by loss of the maltose ABC transporter, which occurs at relatively low frequencies. In paper IV, we have however provided evidence for a new, specific and inherent mechanism of resistance to GarML in a lineage of *L. lactis* ssp. *cremoris* strains. Future efforts will hopefully not only pinpoint which gene or genes are actually responsible for GarML resistance, but further investigate the mechanism at play. Elucidating the mechanism could in turn allow for rational bioengineering of bacteriocins which are not targeted by this specific resistance mechanism. This knowledge is therefore important for the potential use of circular bacteriocins in general, and GarML specifically, in biotechnological or medical applications in the future.

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Paper I

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GENOME ANNOUNCEMENT

Genome Sequence of the Bacteriocin-Producing Strain *Lactococcus garvieae* DCC43

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This work describes the draft genome sequence of *Lactococcus garvieae* DCC43. The 2.2-Mb draft genome contains 2,227 predicted protein-coding genes, among which is a region encoding the bacteriocin garvicin ML. No antibiotic resistance genes or capsule-related virulence genes were identified. Two plasmid replication regions indicate that this strain likely contains plasmids. Comparative genomics suggests that this strain displays a high degree of sequence variation from the previously sequenced *L. garvieae* strains.

Lactococcus garvieae DCC43, a producer of the potent circular bacteriocin garvicin ML (11), was isolated from mallard duck intestines (*Anas platyrhynchos*) (16). This bacteriocin displays antagonistic activity against a range of food spoilage and pathogenic bacteria, including *Listeria* and *Clostridium*, as well as related *L. garvieae* strains (5). *L. garvieae* is a well-known pathogen responsible for lactococcosis in fish (18) but has also been associated with infections in animals and humans (6, 17). However, *L. garvieae* is also part of the bacterial flora of various dairy products (9, 19) and is commonly found in animal samples (13, 16). To date, eight strains of *L. garvieae* have been sequenced (two complete and six draft genomes) (2, 3, 10, 12, 14, 15), but little is known about their virulence traits and underlying mechanisms. This ubiquitous bacterium is therefore of interest from a comparative genomics perspective as an emerging pathogen and as the producer of a bacteriocin which could potentially be used to combat such pathogens.

Here we report the genome sequence of *L. garvieae* DCC43. Total genomic DNA was extracted from a bacterial culture using a genomic-tip kit (Qiagen). A genomic library was generated and pair end sequenced on a HiSeq 2000 system (Illumina). The sequencing service was provided by the Norwegian Sequencing Centre (University of Oslo). Reads were trimmed based on quality (limit, 0.01) and ambiguous bases (none allowed), and reads below 50 bp were discarded, before *de novo* assembly was done using the software program CLC Genomics workbench 5.5 (CLC Bio); 13,705,987 reads were used, resulting in an approximately 500-fold average genome coverage. The minimum contig size was 200 bp, and minimum coverage was 100-fold. Genome annotation was performed using the RAST (Rapid Annotation using Subsystem Technology) server (4).

The draft genome of *L. garvieae* DCC43 consists of 68 contigs, ranging in size from 227 to 178,922 bp, constituting a total of 2,244,387 bp with a GC content of 37.8%. It contains 2,227 predicted protein-coding genes and 50 predicted tRNAs. The coverage ratio indicates the presence of at least 4 rRNA operons containing predicted single copies of 23S, 16S, and 5S rRNA genes, as well as one additional 5S rRNA gene. Among the protein-coding genes is a locus encoding the bacteriocin garvicin ML (5). No evident antibiotic resistance genes were found, and no similarity was observed to the capsule gene cluster of *L. garvieae* Lg2, which has been implicated in virulence in fish (12). However, the genome contains two predicted internalins and five predicted LPXTG-specific sortases, two of the latter having no known counterparts in other *L. garvieae* strains. The genome contains 2 or 3 complete prophage regions, as well as two plasmid replication regions on contigs which both show a high degree of similarity to *L. garvieae* 21881 plasmid pGL5 (1).

Comparative genomics analysis of the sequenced *L. garvieae* strains by Mauve progressive alignment (8) and genomic BLAST (7) shows that *L. garvieae* DCC43 displays high sequence variation despite >99% identical 16S rRNA gene sequences, actually forming a separate clade from the other strains. This could suggest that DCC43 may represent a novel *L. garvieae* subspecies.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited in DDBJ/EMBL/GenBank under the accession no. AMQS00000000. The version described in this paper is the first version, AMQS01000000.

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Paper II

Functional genetic analysis of the GarML gene cluster in *Lactococcus garvieae* DCC43 gives new insights into circular bacteriocin biosynthesis

Running title: Functional genetics of GarML biosynthesis

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Abstract

Garvicin ML (GarML) is a circular bacteriocin produced by *Lactococcus garvieae* DCC43. The recently published draft genome of this strain allowed determination of the genetic background for bacteriocin production. Bioinformatic analysis identified a gene cluster consisting of nine open reading frames organized in 4 operons involved in the production of and immunity to GarML. The *garA* gene encodes the bacteriocin precursor, *garX* a large transmembrane protein, *garBCDE* a putative immunity protein (*garB*) followed by an ATPase and two transmembrane proteins, and *garFGH* encode an ABC transporter complex. Functional genetic analysis revealed that deletion of *garFGH* had little effect on sensitivity to or production of GarML. In contrast, deletion of *garBCDE* or inactivation of *garX* resulted in high level sensitivity to GarML, and completely abolished production of active bacteriocin.

Mass spectrometry of culture supernatants revealed that wild-type cells contain the mature circular form as well as the linear forms of the bacteriocin, both with and without the three amino acid leader sequence, while bacteriocin-negative mutants only contain the linear forms. These results indicate that the cleavage of the leader peptide precedes circularization, and is likely performed by a functional entity separate from the GarML gene cluster. To our knowledge, this is the first evidence for these processes being separated in time. Loss of immunity, antimicrobial activity and our inability to detect the circular bacteriocin in the Δ*garBCDE* and *garX*::pCG47 mutants demonstrates that both these units are indispensible for GarML biosynthesis, specifically in the circularization process, but also for immunity, and that their functions are interlinked.

Introduction

Bacteriocins are ribosomally encoded antimicrobial peptides produced by bacteria, which display activity against other bacterial strains (Klaenhammer 1993). The production of bacteriocins is a widespread trait, in both Gram-positive and Gram-negative bacteria, and particularly so in lactic acid bacteria, which produce numerous bacteriocins varying in size, structure and properties. These bacteriocins are classified into two main classes, the class I lantibiotics which contain posttranslationally modified amino acids and the class II heat-stable unmodified non-lantibiotics.

Garvicin ML is a potent bacteriocin produced by *Lactococcus garvieae* DCC43 (Borrero *et al.*, 2011; Sanchez *et al.*, 2007), and a member of the group known as the circular bacteriocins, characterized by their N- to C-terminal covalent link forming a circular backbone (van Belkum *et al.*, 2011). These antimicrobial peptides are attracting much interest because of their physicochemical characteristics, namely high pH and thermal stability as well as resistance to proteases, which make this class of bacteriocins highly promising as drugs or natural preservatives for diverse applications in medicine and food technology.

Circular bacteriocins are synthesized as linear precursors, with a leader peptide of variable size (2-35 amino acid residues) and sequence which is cleaved off at some point during the maturation process, which also includes cyclization of the peptide and export out of the producer cell (van Belkum *et al.*, 2011). Although several of the circular bacteriocins have now been genetically characterized, the enzymes responsible for the different steps of maturation, the mechanisms and potential coupling of the processes are still unknown (Conlan *et al.*, 2010).

To date there have been characterized 9 circular bacteriocins, which are subdivided into two classes (Cotter *et al.*, 2005): Subclass i are cationic peptides with a high isoelectric point (pI close to 10) that comprises the majority of the known circular bacteriocins: Garvicin ML (GarML) (Borrero *et al.*, 2011), enterocin AS-48 (Maqueda *et al.*, 2004), circularin A (Kawai *et al.*, 2004), carnocyclin A (Martin-Visscher *et al.*, 2008), uberolysin (Wirawan *et al.*, 2007), lactocyclicin Q (Sawa *et al.*, 2009) and leucocyclicin (Masuda *et al.*, 2011). These peptides display limited sequence identity, but share a conserved 3D structure consisting of 4 or 5 α-helices forming a compact globular bundle around a hydrophobic core, where several positively charged residues located on the surface of the structure are thought to attract the peptides to the membrane of the target cells (Martin-Visscher *et al.*, 2009).

Subclass ii circular bacteriocins are different in that they share high sequence identity, contain more acidic residues and hence have a lower isoelectric point (pI close to 5). These include gassericin A (Kawai *et al.*, 1998) and butyrivibriocin AR10 (Kalmokoff *et al.*, 2003). Previously the circular peptide subtilosin A (Babasaki *et al.*, 1985) was considered to belong to this subclass, however this and a few other circular peptides, including the sporulation killing factor (SKF) of *Bacillus thuringiensis* (Liu *et al.*,

2010), contain thioether linkages and are structurally and genetically distinct from the circular bacteriocins. These peptides have therefore been reclassified as members of a novel subclass of peptides known as the sactibiotics (Murphy *et al.*, 2011).

For most of the circular bacteriocins, the respective gene clusters have been described (Belkum *et al.*, 2010; Diaz *et al.*, 2003; Kawai *et al.*, 2009; Kemperman *et al.*, 2003; Martinez-Bueno *et al.*, 1998): A minimal set of 5-7 genes are generally required for production of and immunity to the bacteriocin, including the bacteriocin structural gene and a gene encoding a small hydrophobic high pI peptide which constitutes the cognate immunity protein that provides a basal immunity level (Martinez-Bueno *et al.*, 1998). Furthermore, 2-4 genes encoding putative membrane proteins, one of which contains the domain of unknown function 95 (DUF95) as well as an ATPase make up the minimal set of genes for biosynthesis and immunity. These membrane proteins may form a transporter complex responsible for exporting the peptide out of the producer cell. Additionally, most clusters contain 3-4 genes thought to constitute another ABC transporter, which has been shown to enhance production of the bacteriocin and contribute to an additional level of immunity towards the cognate bacteriocin (Diaz *et al.*, 2003). Although the sequence similarities between the encoded proteins of different circular bacteriocin gene clusters are generally low, there appears to be strong functional conservation in the biosynthetic machinery and -mechanisms of this class of bacteriocins. However, the precise roles of the encoded proteins in the biosynthesis, export and immunity to circular bacteriocins remain to be elucidated.

In this study we have identified the gene cluster responsible for production of the circular bacteriocin garvicin ML (GarML) in the producer strain *L. garvieae* DCC43 and functionally analyzed the production of this bacteriocin at gene and protein level, which has provided new insights into the biosynthesis of subclass i circular bacteriocins.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in table S1. Lactococci were routinely grown in M17 medium (Oxoid, Hampshire, U.K.) supplemented with 0.4 % (wt vol^{-1}) glucose at 30 °C. *E. coli* strains used for cloning purposes were grown in Lysogeny broth (LB) at 37 °C with 225 rpm shaking. Tetracycline was added at a final concentration of 5 μg mL^{-1} when required for selection of lactococcus transformants. Ampicillin was added at a final concentration of 100 μg mL^{-1} and kanamycin of 50 μg mL^{-1} when required for selection of *E. coli* transformants.

Bacteriocin purification and assays

The bacteriocin Garvicin ML was concentrated from the supernatant of the producing strain *L. garvieae* DCC43 grown in de Man, Rogosa and Sharpe (MRS) medium (Oxoid, Hampshire, U.K.) by precipitation with 45 % (wt vol^{-1}) ammonium sulfate. Bacteriocin activity was determined using microtiter plate assays (MPA) as described previously (Gabrielsen *et al.*, 2012a), where one bacteriocin unit (BU) was defined as the amount of bacteriocin required to produce 50 % growth inhibition in 200 μL of culture against the indicator strain *L. lactis* IL1403(Chopin *et al.*, 1984). Production of and immunity to bacteriocin was assayed by agar diffusion tests (ADT), where 1 μl mL^{-1} of the overnight culture of the indicator strain was added to GM17 softagar and poured into plates. After solidification and drying, circular wells were punched out and bacteriocin solution added. The plates were incubated overnight at the appropriate temperature and the area of growth inhibition subsequently measured.

Bioinformatic analysis

The entire genome of the bacteriocin-producer *L. garvieae* DCC43 has previously been sequenced and de novo assembled by Gabrielsen *et al*. (2012b). From the known nucleotide sequence of the GarML structural gene (GenBank accession no GU205098) (Borrero *et al.*, 2011), the GarML encoding gene (*garA*) was localized to contig 1 of the *L. garvieae* DCC43 draft genome (GenBank accession no AMQS00000000). ORFs in the draft genome located close to the GarML precursor gene were predicted using CLC DNA workbench 6.0.2, allowing for all possible start codons, using the bacterial genetic code (11), and including small (>50 codons) ORFs.

Similarity searches of the putative proteins encoded by the ORFs of the GarML cluster to proteins of other circular bacteriocin clusters (known sequences) or to other known proteins (non-redundant protein database) were performed by protein BLAST alignment (Altschul *et al.*, 1990), with E-value cutoff at 0.01. Conserved domains were identified similarly through the NCBI Conserved Domain Database (Marchler-Bauer *et al.*, 2013), with a E-value threshold of 0.01.

The physicochemical properties of the putative proteins were predicted using ProtParam (Wilkins *et al.*, 1999), including theoretical isoelectric point (pI), average molecular weight, aliphatic index (AI) and grand average of hydropathicity (GRAVY). The putative cellular localizations of the proteins were predicted using PSORTb v3.0.2 (Yu *et al.*, 2010).

For the proteins with significant structural homologues in the Protein Data Bank (PDB), i.e. GarML and GarG, the 3D structure of the protein was modeled based on Tcoffee multiple alignment (Notredame *et al.*, 2000) and SWISS-model homology modeling (Arnold *et al.*, 2006) using the closest homolog as the template, which for GarML is the solution structure of carnocyclin A (accession no. 2KJF), and for GarG the structure of ATP-binding protein MJ0796 (accession no. 1L2T). For GarF and GarH , the MacB-like PCD and the RND MFP domains were aligned and homology modeled to the most similar templates (3FTJA and 3LNNB, LXE8A accordingly) separately. For the proteins which had no close sequence or structural homologues, the secondary structure was predicted using either MEMSAT3/-SVM (Nugent and Jones, 2009) for the putative transmembrane proteins or the Jpred server (Cole *et al.*, 2008) for the soluble proteins.

Cloning and functional analysis of the GarML gene cluster

Cloning techniques were in general performed according to Sambrook and Russell (2001). Cloning was mainly performed in *E. coli* before introduction of plasmids into Lactococci. Transformations of Lactococci were performed as described by Holo And Nes (1989). Plasmid isolation was carried out using E.Z.N.A.® Plasmid Miniprep Kit (Omega Bio-tek, Inc.) or QIAGEN Plasmid Midi Kit (QIAGEN). Restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase and T4 DNA ligase (New England Biolabs Inc.) were used according to the supplier's instructions. Oligonucleotides were supplied by Invitrogen Life Technologies. PCR was performed using Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and PCR products were purified using NucleoSpin® Extract II (MACHEREY-NAGEL).

Vectors used for deletion of the two operons *garBCDE* and *garFGH* were created by PCR amplification of approximately 1 kb fragments flanking the respective operons using primers listed in table S1. The flanking sequences of each operon were spliced by overlap extension PCR using the outer primer pairs, and the resulting fragments were subsequently ligated into pCR®-Blunt IITOPO. A SnaBI/SmaI fragment of pAS222 (Jonsson *et al.*, 2009) containing the lactococcal thermosensitive replicon repA and a tetracycline resistance marker was then blunt-cloned in to the SmaI site, creating deletion-vectors pCG44 and pCG45. The deletion vectors were introduced into *L. garvieae* DCC43 and selection for deletion of *garBCDE* and *garFGH* by double crossover carried out as described previously (Biswas *et al.*, 1993), PCR and sequencing used to confirm the deletion of each operon.

To inactive *garX*, a 600 bp internal fragment of this gene (primer pair garX-F/garX-R) was cloned into pAS222, creating vector pCG47. As a control, a similar sized fragment of an intergenic region (primer pair IGR-F/IGR-R) was cloned into pAS222 to create pCG48. The vectors were transformed into *L. garvieae* DCC43 and selection for integration performed as described previously. The correct integration of the vectors at either locus was confirmed by PCR and sequencing, while incubation at 37 °C combined with antibiotic selection was used for all subsequent assays.

The *L. garvieae* DCC43 Δ*garFGH*, Δ*garBCDE* and *garX*::pCG47 mutants were functionally characterized with respect to growth, immunity and production of bacteriocin. Growth was evaluated by 24 hour kinetic measurement of growth (OD_{600}) in GM17 medium of 100-fold diluted cultures using SPECTROstar Nano microplate reader (BMG LABTECH). Immunity to GarML was quantified by MPA and ADT against concentrated GarML, while production of bacteriocin was measured by ADT using *L. lactis* IL1403 as the indicator strain.

Preparation of cell fraction and supernatant

Cell cultures were grown to stationary phase before separation of supernatant and cells by centrifugation at 6500 x g for 10 minutes. Supernatants were concentrated by ammonium sulphate precipitation as described previously, and resuspended in 1 ml of 10 mM tris-HCl buffer (pH 7.5). Cell pellets were resuspended in 70 % isopropanol and 0.1 % trifluoroascetic acid and shaken on ice for 4 hours, to remove any bacteriocin attached to on the extracellular side of the cell surface. After re-centrifugation, cell pellets were then washed twice in 10 mM tris-HCl (pH 7.5) and resuspended in 1 ml volume of the same buffer. A volume of 500 µl cell suspension was mechanically disrupted with 500 mg glass beads (acid-washed <106 microns, Sigma-Aldrich) using a Thermo Savant FastPrep® FP120 Cell Disrupter (Qbiogene Inc, CA, USA) for 4x20 s runs at 6 m/s with 1 minute intervals on ice. Non-lysed cells and large cellular debris were removed by centrifugation at 10 000 x g for 30 minutes at 4°C. Supernatant samples were filtered, desalted and concentrated using columns with 10 kDa weight limit (Amicon Ultra-2 Centrifugal Filter Unit with Ultracel-10 membrane, Millipore), by centrifugation at 6000 x g for 10 minutes and washed twice with 10 mM tris-HCl (pH 7.5) before elution.

Digestion in tip (DIT)

Before use, STAGE columns were prepared, conditioned and equilibrated as described by (Rappsilber *et al.*, 2003). A volue of 2.5 µl immobilized trypsin slurry (Poroszyme, Applied Biosystems) was added, and the trypsin-part of the column was packed/equilibrated by pressing through 2 x 10 µl 50 mM $(NH_4)HCO_3$. The protein solution was mixed with 0.1 x volume 500 mM $(NH_4)HCO_3$ and pressed through. The column (STAGE+immobilized trypsin) was rinsed with 2 x 10 µl 0.1 % TFA, which simultaneously

inactivates the enzyme. Tryptic peptides were eluted from the column using 70 % acetonitrile/0.03 % TFA. Prior to MS analysis, the eluate was evaporated and the peptides dissolved in 10 µl loading buffer.

LC-MS analysis

The peptides were separated on a 50 cm, 75 µm i.d. Acclaim PepMap column using a linear 3-40 % ACN gradient over 90 minutes, with a flow rate of 300 nl/min. The nano-LC (Dionex Ultimate 3000 UPLC) system was interfaced with a Q Exactive quadrupole orbitrap mass spectrometer (Thermo Scientific), acquiring positive ion mass spectra in data dependent (dd) mode: For every full scan at resolution 70000 10 MS/MS scans at resolution 17500 were recorded. The dd settings were as follows: automatic gain control (AGC) target 1x 10^5 ions, max injection time 50 ms, normalized collision energy 30, isolation width 2.0 m/z.

Nucleotide sequence accession numbers

The new ORFs and updated annotation of the GarML gene cluster was appended to the draft genome of *L. garvieae* DCC43 (GenBank accession no AMQS00000000).

Results

Sequence and bioinformatic analysis of the GarML gene cluster

The genome of the GarML producer strain *L. garvieae* DCC43 was previously sequenced and de novo assembled to allow determination of the genetic determinants behind GarML production and immunity (Gabrielsen et al 2012b). A 119 bp fragment encompassing the structural gene of the GarML precursor and some surrounding sequence has previously been determined by reverse genetics (Borrero *et al.*, 2011), and from this known nucleotide sequence (GenBank accession no GU205098), the GarML precursor gene was localized to contig 1 of the *L. garvieae* DCC43 draft genome (GenBank accession no AMQS00000000). This contig is 175.6 kb in size and contains chromosomal genes (e.g. a ribosomal protein cluster, tRNA synthetase, and RNA polymerase alpha subunit), thus establishing that the GarML gene cluster is chromosomally encoded.

In the vicinity of the GarML precursor gene, henceforth referred to as *garA* in accordance with suggested nomenclature (Arnison *et al.*, 2013), we identified eight ORFs that appeared to be involved in bacteriocin production and immunity of GarML, based on similarity to gene clusters of other circular bacteriocins (figures 1 and 2). A single ORF named *garX* is located 61 bp downstream and on the same strand as *garA*, while the remaining operons *garBCDE* and *garFGH* are located upstream on the opposite strand. The predicted physicochemical characteristics, secondary structure, subcellular localization and conserved domains of the putative proteins encoded by the *gar* operons are summarized in table 1, the main features of which are illustrated in figure 1.

Promoter and terminator prediction suggests that *garA* and *garX* form independent transcriptional units. However, *garBCDE* and *garFGH* constitute operons that are probably regulated by translational coupling, as the end of *garC* overlaps (by 11 bp) with the start of *garD*, as do *garF*, *garG* and *garH* (by 1 and 4 bp accordingly) (figure 1).

The putative protein encoded by *garB* was predicted to be a small (6514.12 Da), hydrophobic, high pI protein with a secondary structure of two alpha-helices, likely to be membrane-located. These characteristics concur with those of immunity proteins of other circular bacteriocins (Belkum *et al.*, 2010; Kemperman *et al.*, 2003; Martinez-Bueno *et al.*, 1998), and we therefore predicted *garB* to encode the specific immunity protein to GarML.

Using BLAST to search for homologues of the putative proteins encoded by the GarML gene cluster (table 1), specifically among other circular bacteriocins, we found that GarC is similar to CclT, CirD and AS-48D of the carnocyclin A, circularin A and enterocin AS-48 gene clusters accordingly, which are all predicted soluble ATP-binding proteins. The protein displays high degree of homology to members of the P-loop NTPase domain superfamily, containing a nucleotide binding site (formed by Walker A, Walker

B, Q loop, D-loop and H-loop motifs), as well as an ABC transporter signature motif (LSGGQ). The evidence thus suggests that *garC* encodes the ATPase subunit of an ABC transporter complex.

GarD displays homology to CclC of the carnocyclin A gene cluster. The protein was predicted to consist of four transmembrane helices, indicating that it is a membrane protein. Interestingly, *garD* was found to contain a conserved domain of unknown function, DUF95, which has been found in the gene clusters of almost all genetically characterized circular bacteriocins to date. DUF95 proteins share a similar overall structure, with several predicted transmembrane proteins, and are believed to constitute integral membrane proteins, but the function of this family of proteins is unknown (Pfam database accession PF01944).

The proteins encoded by the *garFGH* operon display high degree of similarity to AS-48FGH and CclFGH of the enterocin AS-48 and carnocyclin A gene clusters, which are thought to encode ABC transporter complexes involved in bacteriocin immunity (Diaz *et al.*, 2003). GarH displays high degree of homology to the SalY multi-domain (permease component of ABC-type antimicrobial peptide transport system) and is predicted to have a total of four transmembrane helices, three of which are constituted by the C-terminal FtsX domain found in many permease proteins. The protein also contains a MacB-like PCD, i.e. a periplasmic core domain found in a variety of ABC transporters. GarG (like GarC) displays high degree of homology to members of the P-loop NTPase domain superfamily, contains the same motifs, and is therefore thought to encode the ATPase component of this ABC transporter complex. GarF on the other hand is predicted to contain a leader sequence, and displays similarity to the RND MFP multi-domain, which is a membrane fusion protein component of the RND (Resistance, Nodulation, and cell Division) family of transporters. These proteins, including GarF, contain biotinyl/lipoyl-like domains. Based on these results, we propose that *garFGH* encodes an ABC transporter in which *garG* encodes a soluble ATP-binding protein; *garF* forms the transmembrane permease component and *garF* an accessory periplasmic protein.

The perhaps most enigmatic putative protein located in the GarML gene cluster is encoded by *garX*. This is the only ORF encoded on the same strand as the GarML precursor, with a predicted promoter overlapping the predicted terminator of *garA*. It is a relatively large gene, 1434 bp, encoding a 56 564 Da protein which is likely membrane-associated, as the secondary structure prediction suggested a structure of 8-12 transmembrane helices separated by intracellular and extracellular segments of varying size. This putative protein has no significant sequence similarity to any known proteins or conserved domains, including those of other circular bacteriocin clusters, and as such bioinformatic analysis cannot give any more clues as to the possible function of this protein.

Comparative analysis of the GarML gene cluster

Comparative analysis of the GarML gene cluster to the gene clusters of other circular bacteriocins reveals that although there is limited sequence identity of the encoded proteins (average 20-30 % pairwise identity), there are many shared traits and commonalities between the encoded proteins (figure 3). The GarML gene cluster appears to be most similar to the enterocin AS-48 (GenBank accession Y12234 and AJ438950) and carnocyclin A (GenBank accession EU624394) gene clusters, which are the peptides with which GarML shares most sequence homology. However, the inverted organization of the *garBCDE* and *garFGH* versus *garA* and *garX* units in *L. garvieae* DCC43 appears to be unique.

Functional analysis of the GarML gene cluster

To investigate the biological roles of the different genes of the GarML cluster, we then proceeded to make deletion mutants of the individual operons. For the Δ*garBCDE* and Δ*garFGH* mutants this was readily achieved by a classical double crossover approach. For *garX* we failed to delete this gene after several attempts. Instead, a gene inactivation approach was used to generate *garX*::pCG47. We then determined the impact of these genotypes on the production, immunity and growth of *L. garvieae* DCC43, the results of which are displayed in figure 3 and summarized in table 2.

The deletion of the first operon in the GarML gene cluster, *garFGH*, encoding a putative ABC transporter complex, appeared to have no major impact on either growth, immunity to or extracellular production of GarML. The immunity was determined to be at wild-type level (MIC_{50} value of 320 BU mL^{-1}), as was the extracellular production of GarML. These results suggest that this ABC transporter is not required for either immunity or production of bacteriocin.

The second operon in the GarML gene cluster, *garBCDE*, encodes the putative immunity protein as well as two putative membrane proteins and an ATPase. Deletion of this operon had dramatic effects on the phenotype of the producer strain. Firstly, production of GarML was not detected in the Δ*garBCDE* mutant (figure 3). Furthermore, it was shown that the immunity of this deletion mutant was lowered 250-fold (MIC_{50} =1.25 BU $mL^{-}1$ compared to the wild-type producer strain 320 BU mL^{-1}), which is 4-fold lower than the sensitivity level of the indicator strain *L. lactis* IL1403 (5 BU mL^{-1}). Together, these results provide strong evidence that the *garBCDE* operon is not only vital for biosynthesis but also for immunity to GarML.

Lastly, the *garX*::pCG47 mutant also displayed a total loss of GarML production and a weakened immunity with an approximate 8-16-fold reduction in immunity towards GarML compared to the wild-type producer (figure 3). This mutant was less sensitive compared to the Δ*garBCDE* mutant, but even so, it is evident that also GarX in some manner contributes to immunity towards extracellularly added GarML.

Proteomic analysis of GarML biosynthesis

To explore the roles of the *garBCDE* and *garX* operons in biosynthesis of GarML at the protein level, we performed mass spectrometry analysis of culture supernatants of the wild-type producer *L. garvieae* DCC43 and the deletion/knock-out mutants.

For wild type producer cells and the bacteriocin positive Δg*arFGH* mutant, we were able to detect specific trypsinated fragments diagnostic to the circular mature bacteriocin as well as the full-length linear precursor peptide (FLPP) and the processed linear precursor peptide (PLPP) (i.e., without the leader sequence) (figure 3 and table 2). By contrast, only the linear forms FLPP and PLPP were detected for the Δ*garBCDE* and *garX*::pCG47 mutants, which is consistent with the fact that these mutants produce no active bacteriocin (figure 1). Together, these results confirm that the circular structure of the bacteriocin is essential for the antimicrobial activity and that both GarBCDE and GarX are involved in the circularization step that eventually gives rise to the mature and active GarML.

Discussion

In this work, we have identified and characterized the gene cluster responsible for biosynthesis and immunity to the circular bacteriocin GarML, which is chromosomally encoded in the producer strain *L. garvieae* DCC43 (figure 1A). Bioinformatic analysis suggests that the gene cluster contains four transcriptional units: the monocistronic *garA* (bacteriocin precursor) and *garX* (transmembrane protein), and the polycistronic *garBCDE* (immunity protein and putative transport complex) and *garFGH* (putative ABC transporter) (figure 1B). The gene cluster shares several traits, both in genetic organization and in the putative functions of the encoded proteins, with other circular bacteriocin gene clusters (figure 2). Functional analysis combined with mass spectrometry of deletion mutants of these operons revealed new insights into biosynthesis of GarML, which may thus apply to circular bacteriocins in general.

Deletion of the *garFGH* operon did not have any significant effect on either the production of mature GarML or immunity (figure 3), and thus this operon is evidently non-essential to GarML biosynthesis. Similarly, the corresponding genes *AS-48FGH* in the producer strain of enterocin AS-48 was not at first identified as part of the gene cluster encoding this bacteriocin, however it was later shown to provide a small increase in both production of and immunity to enterocin AS-48 (Diaz *et al.*, 2003). It is possible that GarFGH may function as a secondary transporter of GarML, to prevent extracellular GarML from penetrating the cell envelope, in a manner similar to the immunity-providing ABC transporters of many lantibiotics (Draper *et al.*, 2008). However, our experimental results did not support this notion as the presence of the intact *garFGH* did not compensate for the loss of immunity resulting from deletion of the *garBCDE* operon or the *garX* gene. Thus, we are unable to ascribe any function to this transport complex at this time, and whether this operon plays a role or not in bacteriocin biosynthesis remains to be seen.

By mass spectrometry, we were able to detect all three forms of the GarML peptide in the wild-type producer strain and the bacteriocin-positive mutant *ΔgarFGH*; the mature circular peptide as well as the full length linear peptide with the three amino acid residue leader sequence (MFD) and the linear peptide without the leader sequence (figure 4). As expected, we were not able to detect the circular form of GarML in the bacteriocin-negative mutants Δ*garBCD*E and *garX*::pCG47 although both these mutants contain the linear forms of GarML. Based on these observations, it can be concluded that both Δ*garBCD*E and *garX*::pCG47 mutants have lost the ability to perform the circularization step which is essential for the antimicrobial activity of GarML.

For most class II linear bacteriocins including lactococcin G (class IIb) and lactococcin A (class IId), a dedicated membrane-located ABC-transporter is responsible for the removal of the leader sequence from the prepeptide and the concurrent export of the mature peptide to the exterior (Franke *et al.*, 1999; Havarstein *et al.*, 1995; Stoddard *et al.*, 1992). For class II circular bacteriocins like GarML, however,

little is known about the process behind their maturation. Nevertheless, it is generally believed that the maturation of circular bacteriocins consists of three events: removal of the leader peptide, circularization and export, and that these three events take place either in a defined order in a stepwise fashion or in a coordinated one-step reaction, however the sequence of events and potential coupling of the processes are not known (Conlan *et al.*, 2010; Maqueda *et al.*, 2008). In the present study, or at least for garvicin ML, removal of the leader and circularization are separate processes, as we could detect the linear processed form of GarML (without leader) in the two bacteriocin-negative mutants (and also in the bacteriocin-positive cells). This is to our knowledge the first time it has conclusively been demonstrated that these processes are independent, and furthermore that leader sequence cleavage takes place before circularization.

Furthermore, as the linear peptides are detected in all three mutants and wild type cells, this observation suggests that leader sequence cleavage is most likely executed by a separate functional entity, i.e. one being not encoded by *garX*, *garFGH* or *garBCDE*. However, no candidate protease or peptidase genes are located in the vicinity of the GarML gene cluster. The cleavage of the leader sequence must therefore be performed by a yet unidentified, specific or non-specific, peptidase. Similar ideas have been suggested previously for enterocin AS-48 (Fernandez *et al.*, 2007; Montalban-Lopez *et al.*, 2012), and may explain the apparent problems in expressing circular bacteriocins outside the genera or species of the original producer (Fernandez *et al.*, 2007; Kawai *et al.*, 2009) and in obtaining stable heterologous production (Kemperman *et al.*, 2003).

The detection of linear forms of circular bacteriocins in the supernatant of the producing strain has previously been taken into account for the circularization reaction taking place on the extracellular side of the cytoplasmic membrane (Cebrian *et al.*, 2010). However, in the case of GarML the results indicate that cell autolysis can explain detection of these species in the culture supernatant, as the linear forms of GarML were in a few cases not detectable in supernatants from cells grown in sucrose-stabilized media (results not shown).

Functional analysis of the Δ*garBCD*E and *garX*::pCG47 mutants revealed that production of functional bacteriocin and immunity were impaired in both strains. This is analogous to the situation for enterocin AS-48, where *AS-48B* and *AS-48C* (similar to *garX* and *garD* accordingly) have been implicated in biogenesis and immunity (Fernandez *et al.*, 2008). Moreover, mass spectrometry confirmed that neither the Δ*garBCD*E nor the *garX*::pCG47 mutant produced the mature circular bacteriocin. The results thus show that both GarBCDE and GarX are involved specifically in the circularization step of GarML biosynthesis, which has not been demonstrated for any other circular bacteriocin previously. The functions of GarBCDE and GarX appear to be non-overlapping, as neither compensates to any significant extent for inactivation of the other, which would be the expected outcome if these proteins either could

perform circularization or provide immunity separately. If one operon were responsible for circularization and the other for immunity, one would expect to observe immunity without production in one strain, and detect circular bacteriocin and/or observe detrimental growth effects in the strain without the immunity determinant(s), which is not the case. Our results therefore demonstrate that circularization does not occur, and immunity is severely affected, without the presence of both GarBCDE and GarX. Consequently, it is likely that these proteins are linked, either functionally or by regulation, to provide both immunity and biosynthesis of GarML. In other circular bacteriocin clusters, the genes corresponding to *garX* and *garD* are encoded by the same polycistronic transcript (Kemperman *et al.*, 2003; Martinez-Bueno *et al.*, 1998), thus supporting the possible link between the encoded proteins. Co-production and/or -regulation of the immunity and biosynthetic machinery is indeed common in bacteriocin production (Draper *et al.*, 2008; Nes *et al.*, 1996), a strategy which is clearly advantageous for the producer organism. In the GarML gene cluster there are however no encoded proteins which display similarity to known regulatory domains. We therefore believe that GarX and GarBCDE are functionally linked, possibly by forming a multi-complex in the cell membrane, to perform circularization of the linear peptide into the mature circular form, most likely concomitant with export out of the cell.

In conclusion, based on these results, a possible model for GarML biosynthesis emerges (figure 5): The N-terminal three amino acid residues (MFD) of the precursor peptide are cleaved off by an unknown peptidase. The linear peptide is subsequently exported out of the cell with concomitant circularization by a GarCDE and GarX multi-complex. The mature and active bacteriocin is then released into the extracellular space. GarB by analogy to other circular bacteriocin immunity proteins (Belkum *et al.*, 2010; Kemperman *et al.*, 2003; Martinez-Bueno *et al.*, 1998) likely constitutes the primary immunity determinant, but full immunity requires the entire multi-complex.

Acknowledgements

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Figures and tables

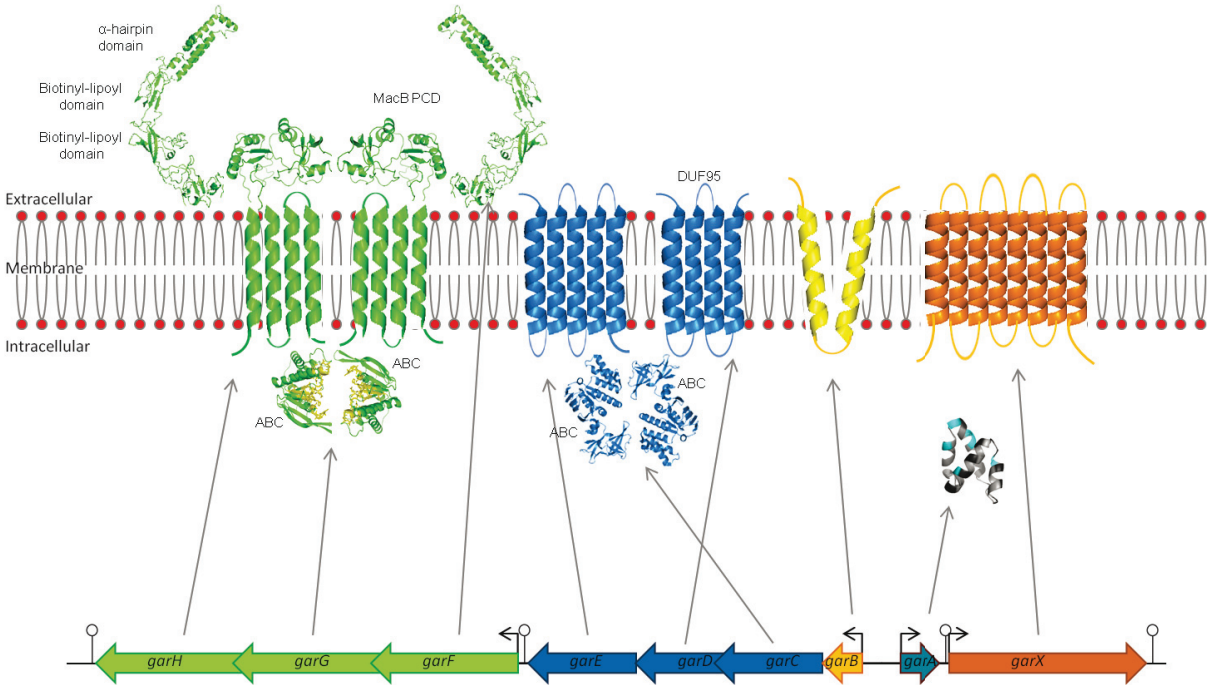


Figure 1: A) Genetic organization of the GarML gene cluster, with predicted promoter and terminator sequences indicated by arrows and circles accordingly. The GarML precursor is encoded by *garA* and the putative immunity protein by *garB*. B) Predicted localization, secondary/tertiary structure and functional domains of the putative gene products of the GarML gene cluster, based on the characteristics of table 1. ATPase subunits are displayed as dimers, whereas other proteins are shown as monomers.

Table 1: Bioinformatic analysis of the putative proteins encoded by the GarML gene cluster.

Gene	Locus tag/nt	Protein id	aa	Homologs	Structure prediction	Localization (confidence)	pI	Mw	G[a]	AI[b]	Domains/ superfam.	Putative function[c]
				BLASTp	MEMSAT/JPRED	Psortb	ProtParam				NCBI CDD	
garH	C426_0084	EKF52509.1	398	AS-48H, CclH, CirI	4 TM helices, periplasmic domain	Membrane (10.0)	8.7	43901	0.1	109.9	MacB PCD, FtsX, SalY	ATCC
garG	C426_0085	EKF52510.1	227	AS-48G, CclG, CirH	-	Membrane (10.0)	5.2	25788	-0.3	100.4	P-loop NTPase domain, SalX	ATCC ATPase
garF	C426_0086	EKF52511.1	399	AS-48F, CclF	-	Unknown	5.9	44142	-0.4	97.6	RND MFP, Biotinyl lipoyl domain	ATCC
garE	c91715-92197	-	160	low/no similarity	5 TM helices	Membrane (10.0)	9.7	18119	1.2	166.3	-	Membrane protein
garD	c92200-92703	-	167	CclC	4 TM helices	Membrane (10.0)	9.5	18948	1.0	141.8	DUF95	Membrane protein DUF95
garC	C426_0087	EKF52512.1	213	CclT, CirD, AS-48D	-	Cytoplasmic (7.5)	5.7	24476	-0.2	105.2	P-loop NTPase domain, SalX	ATCC ATPase
garB	c93335-93502	-	55	low/no similarity	2 helices	Membrane (9.55)	10.2	6514	1.3	166.6	-	Immunity protein
garA	C426_0088	EKF52513.1	63	GarML	-	Membrane (9.55)	10.1	6025	0.9	115.8	-	Bacteriocin precursor
garX	C426_0089	EKF52514.1	477	low/no similarity	8-12 TM helices	Membrane (10.0)	9.6	56564	0.7	138.8	-	Membrane protein

[a] G is GRAVY index, i.e. the grand average of hydropathicity index

[b] AI is aliphatic index

[c] ATCC is ABC transporter complex component

Figure 2: Comparative analysis of the GarML gene cluster to other circular bacteriocin gene clusters. Grayscale blocks indicate sequence identity at protein level.

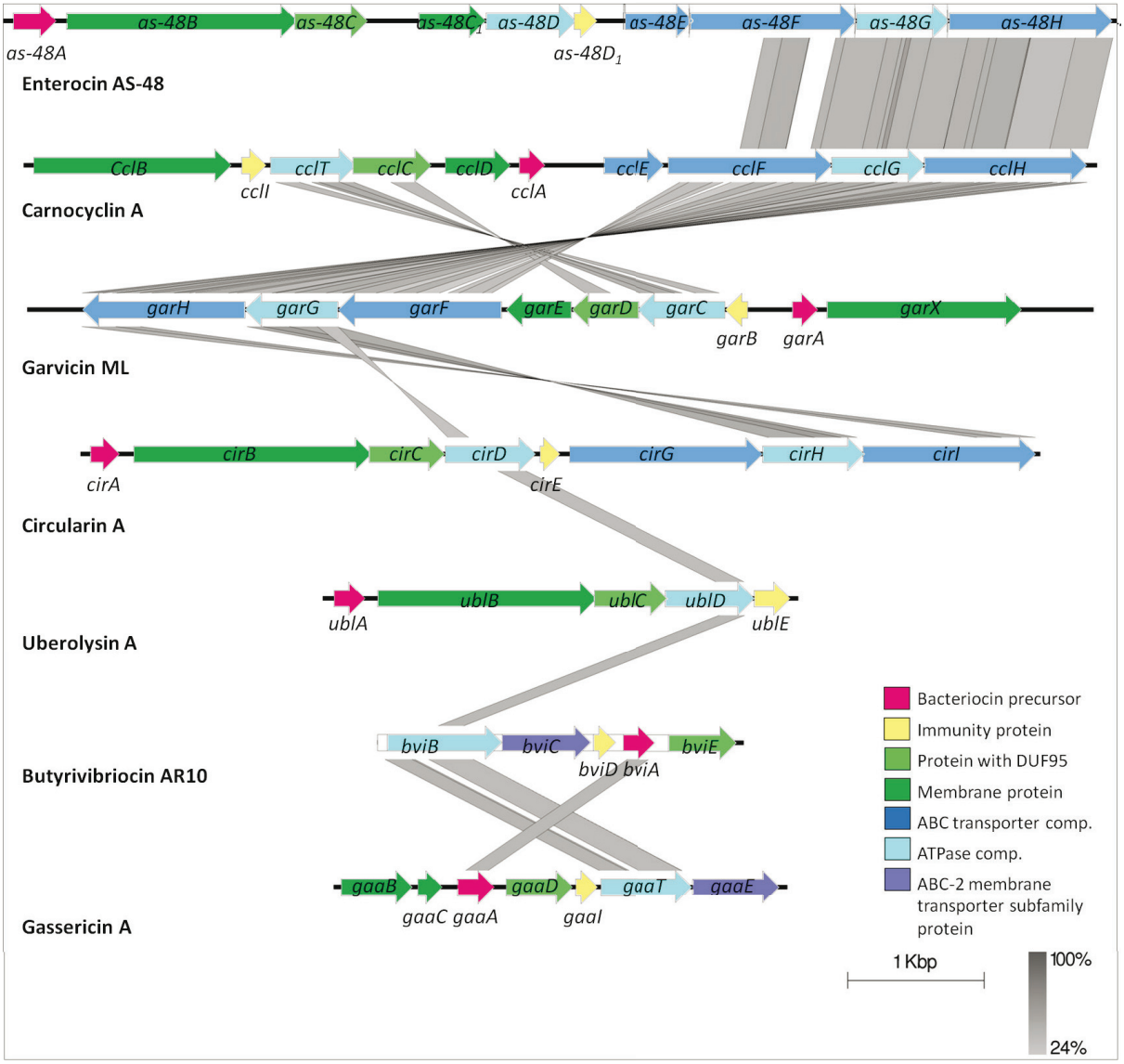


Figure 3: Immunity and antimicrobial activity of *L. garvieae* DCC43 wild-type and deletion/KO mutants.

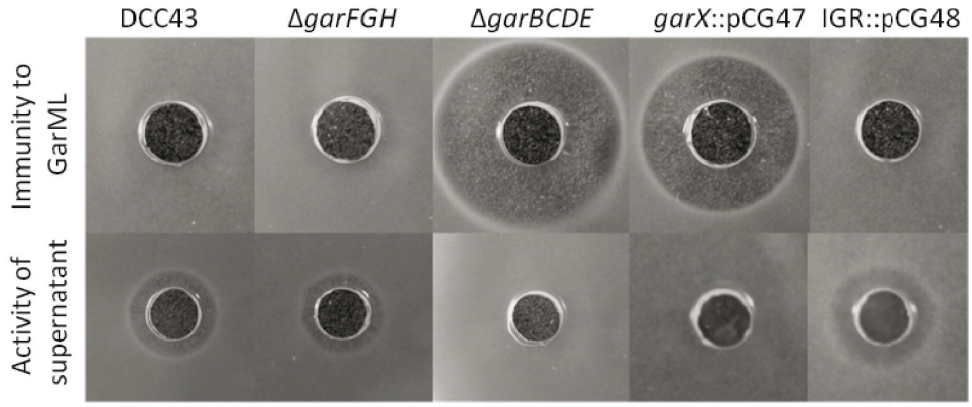


Table 2: Effect of deletions/insertions in the of GarML gene cluster on growth, immunity and production of GarML.

Strain	Genotype	Growth[a]	Sensitivity[b]	Antimicrobial activity[c]	Peptide species[d]
DCC43	wt	+	-	+	Pre, lin, cir
DCC43	Δ*garFGH*	+	-	+	Pre, lin, cir
DCC43	Δ*garBCDE*	+	+	-	Pre, lin
DCC43	*garX*::pCG47	+	+	-	Pre, lin
DCC43	IGR::pCG48	+	-	+	Pre, lin, cir

[a] Growth was measured by OD_{600} over time, denoted as + if no significant difference in growth rate and maximum cell density compared to the control strain was observed.

[b] Sensitivity was measured by ADT using the strains as indicators against concentrated GarML.

[c] Antimicrobial activity was determined by ADT of 20-fold concentrated supernatant against the indicator strain *L. lactis* IL1403 or IL1403 (pAS222).

[d] Peptide species detected by LC-MS in the supernatant of the strain: Precursor peptide with leader sequence (Pre), Linear peptide without leader sequence (Lin) and circular mature peptide (Cir).

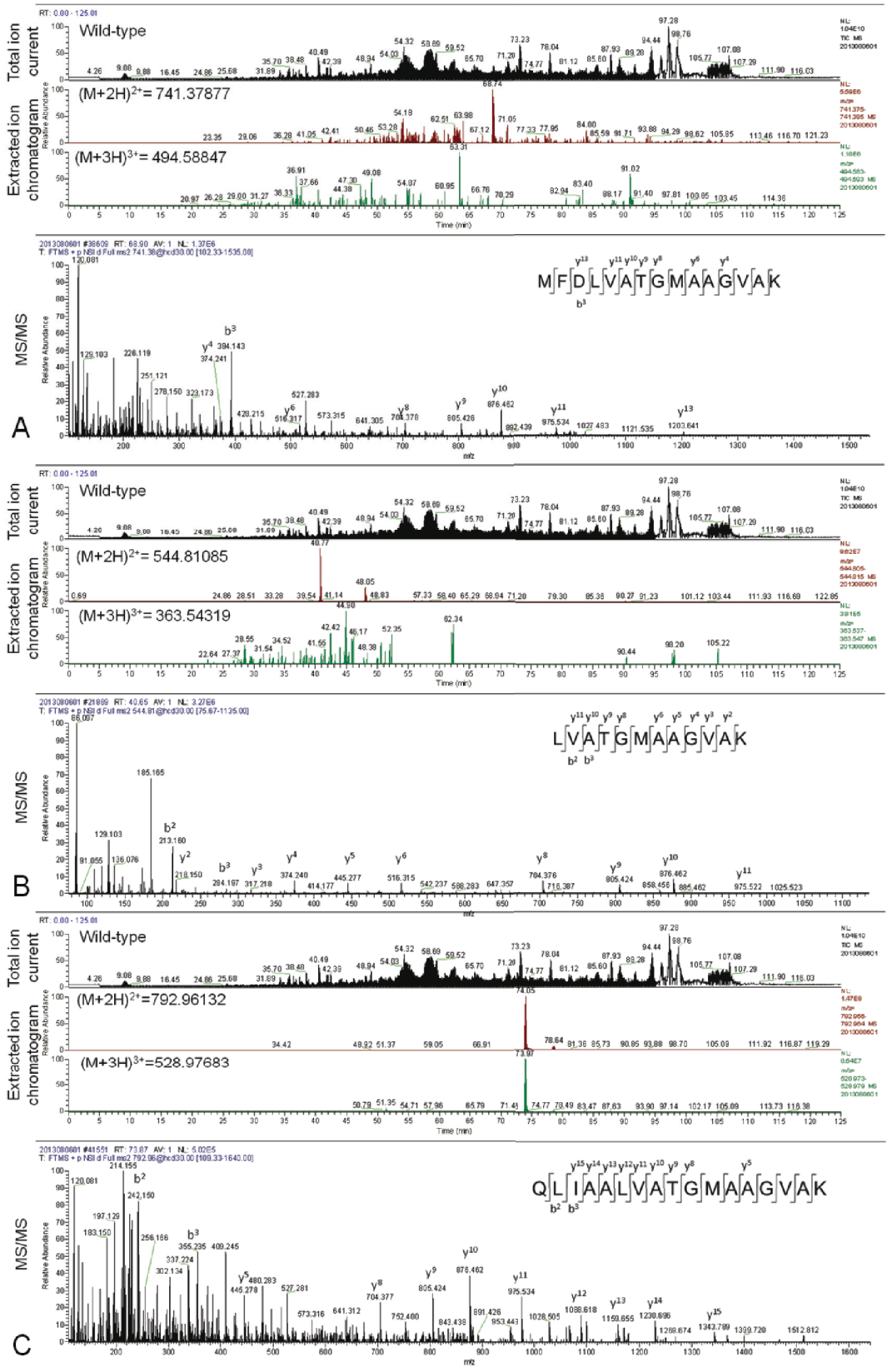

Total ion current
Wild-type
Extracted ion chromatogram
(M+2H)2+= 741.37877
(M+3H)3+= 494.58847
Time (min)
MS/MS
MFDLVATGMAAGVAK
A
(M+2H)2+= 544.81085
(M+3H)3+= 363.54319
LVATGMAAGVAK
B
(M+2H)2+=792.96132
(M+3H)3+=528.97683
QLIAALVATGMAAGVAK
C

Figure 4: Detected trypsin fragments of full length linear (A), linear peptide without leader sequence (B) and circular (C) forms of GarML in wild-type producer strain *L. garvieae* DCC43 supernatant by LC- MS. The upper panel displays the total ions current (TIC) of the analyzed sample, the second and third panels display the extracted ion chromatograms (EIC) of the $(M+2H)^{2+}$ and $(M+3H)^{3+}$ ions correspondingly, and the lower panel show the MS/MS spectra marked with the identified y- and b- ions of the corresponding sequence.

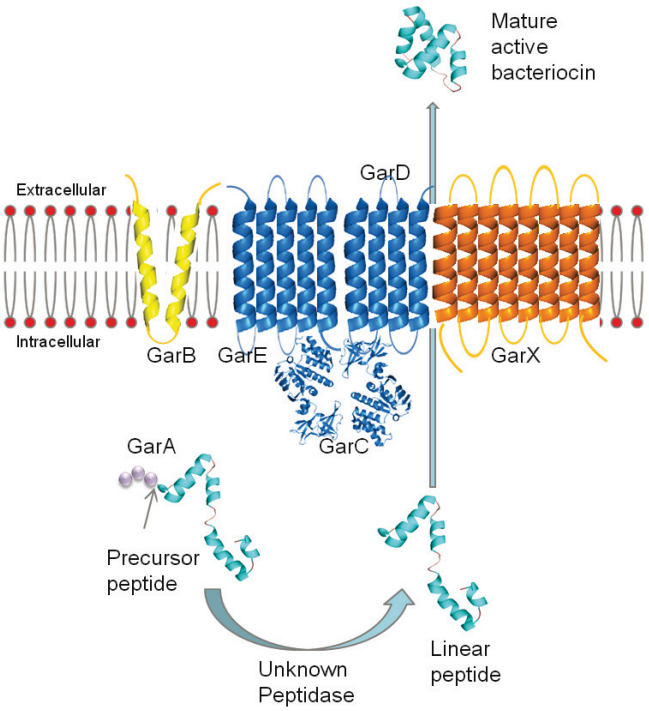


Figure 5: Proposed model for biosynthesis of GarML in *L. garvieae* DCC43. The N-terminal three amino acid residues (represented by mauve spheres) of the precursor peptide are cleaved off by an unknown peptidase. The linear peptide is subsequently exported out of the cell with concomitant circularization by a GarCDE and GarX multi-complex. The mature and active bacteriocin is then released into the extracellular space. GarB is likely the primary immunity determinant providing host self-protection towards GarML, however full immunity requires the entire multi-complex.

Supplementary information

Table S1: Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Lactococcus lactis ssp. *lactis*		
IL1403	Indicator strain for GarML	Chopin *et al.*(1984)
Lactococcus garvieae		
DCC43	Producer strain of GarML.	Sanchez *et al.(2007)*
DCC43 Δ*garFGH*	Deletion mutant of DCC43	This study
DCC43 Δ*garBCDE*	Deletion mutant of DCC43	This study
DCC43 *garX*::pCG47	Knock-out mutant of DCC43	This study
DCC43 IGR::pCG48	Knock-out mutant of DCC43	This study
Plasmids		
pAS222	Lactococcal vector with thermosensitive replicon repA, *Tet*^{r}.	Jonsson *et al.* (2009)
pCG44	pAS222-based deletion vector of *garFGH*	This study
pCG45	pAS222-based deletion vector of *garBCDE*	This study
pCG46	pAS222-based deletion vector of *garX*	This study
pCG47	pAS222-based knock-out vector of *garX*	This study
pCG48	pAS222-based knock-out vector of intergenic region (IGR)	This study

[a] *Tet*^{r}, tetracycline resistance

Figures S1-S15: Detected trypsin fragments of circular (S1-S5), full length linear (S6-S10) and linear peptide without leader sequence (S11-S15) forms of GarML in supernatant of *L. garvieae* DCC43 wild-type, Δ*garFGH*, Δ*garBCDE*, *garX*::pCG47 and *IGR*::pCG48 mutants accordingly by LC- MS. The upper panel displays the total ions current (TIC) of the analyzed sample, the second and third panels display the extracted ion chromatograms (EIC) of the $(M+2H)^{2+}$ and $(M+3H)^{3+}$ ions correspondingly, and the lower panel show the MS/MS spectra marked with the identified y- and b- ions of the corresponding sequence.

Figure S1

Circular peptide trypsin fragment

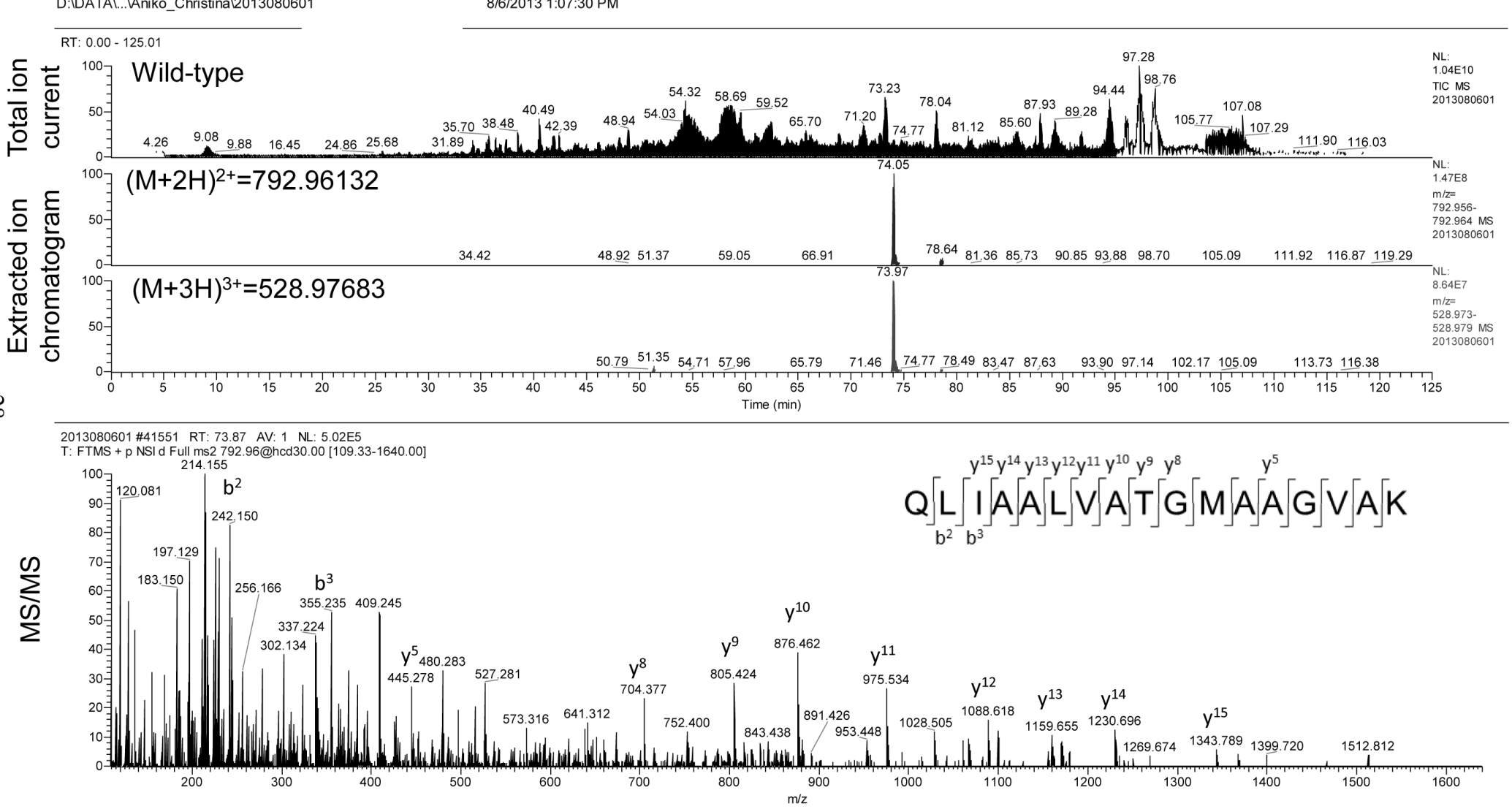


Figure S2

Circular peptide trypsin fragment

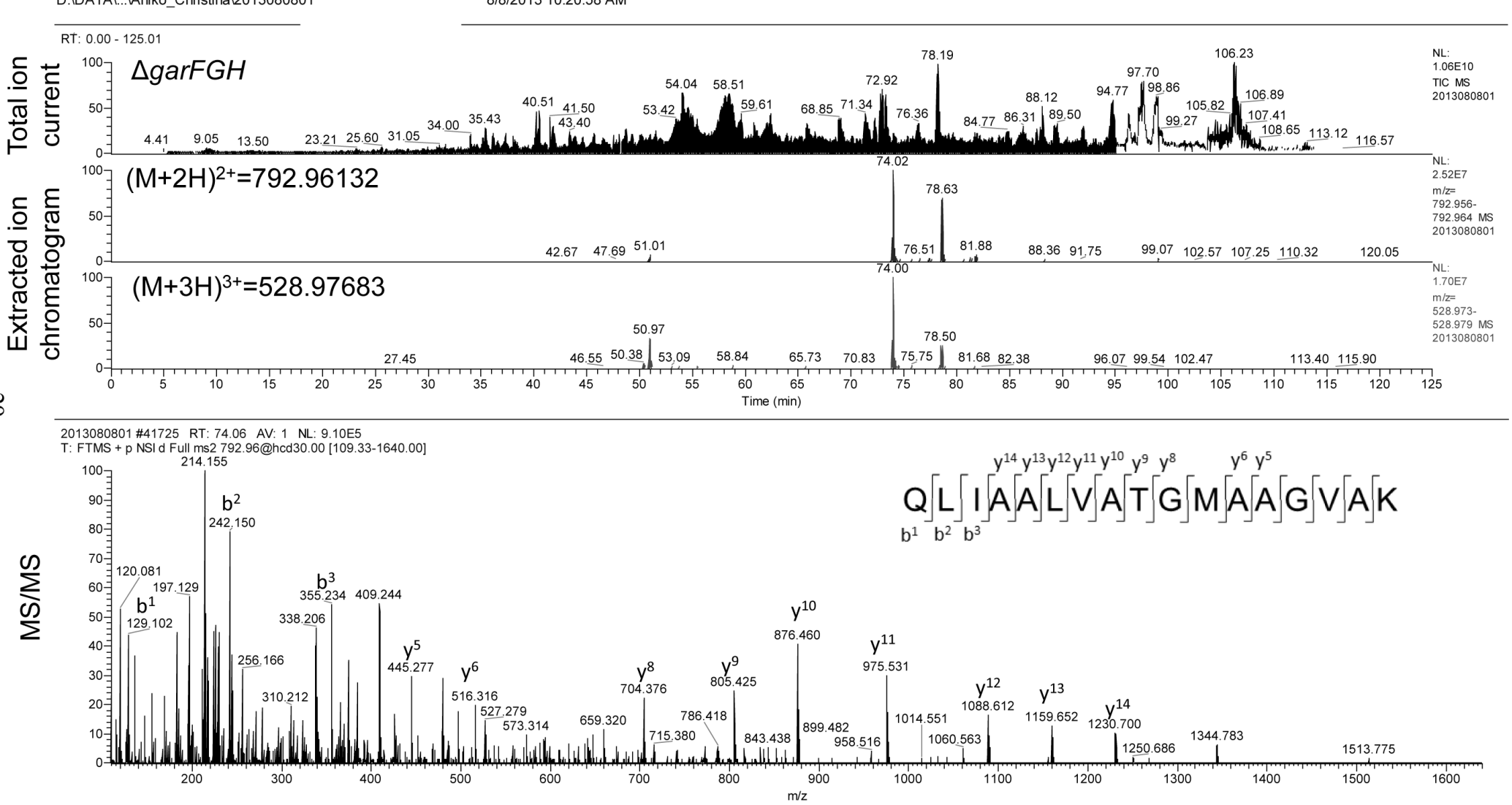
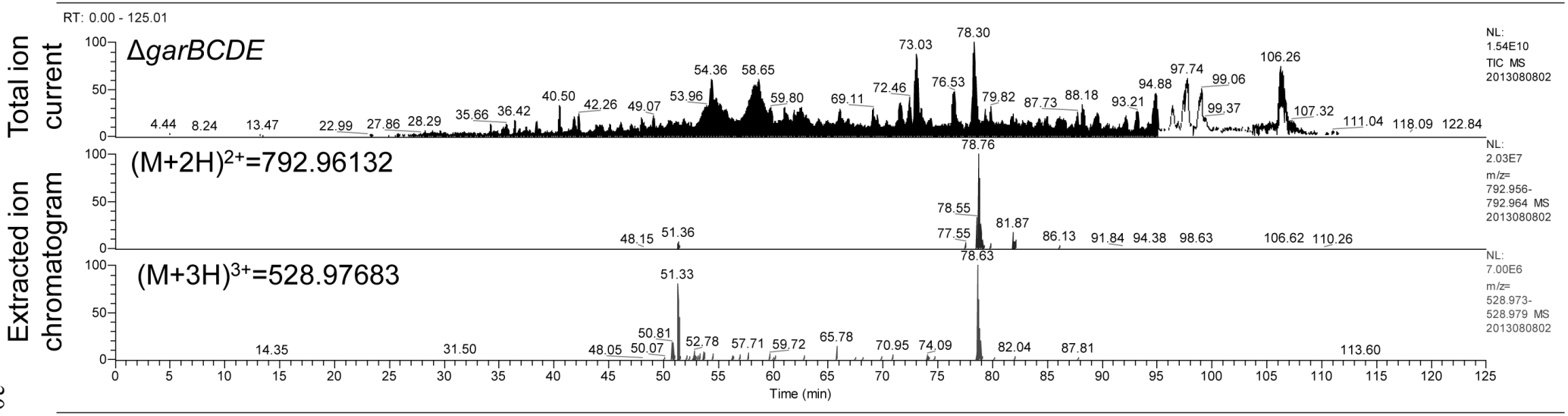
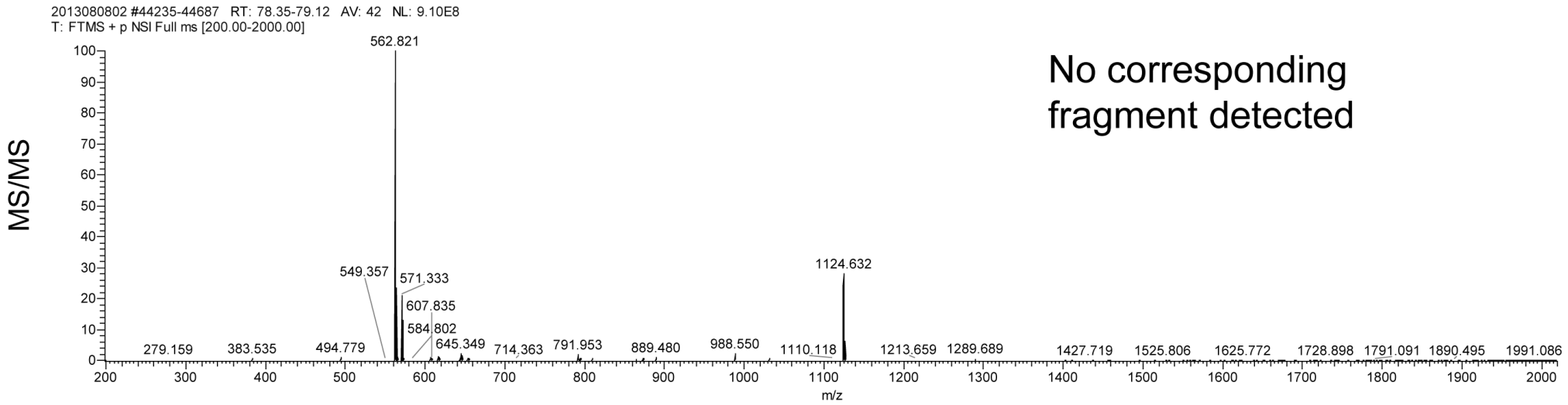


Figure S3
Circular peptide trypsin fragment





No corresponding fragment detected

Figure S4

Circular peptide trypsin fragment

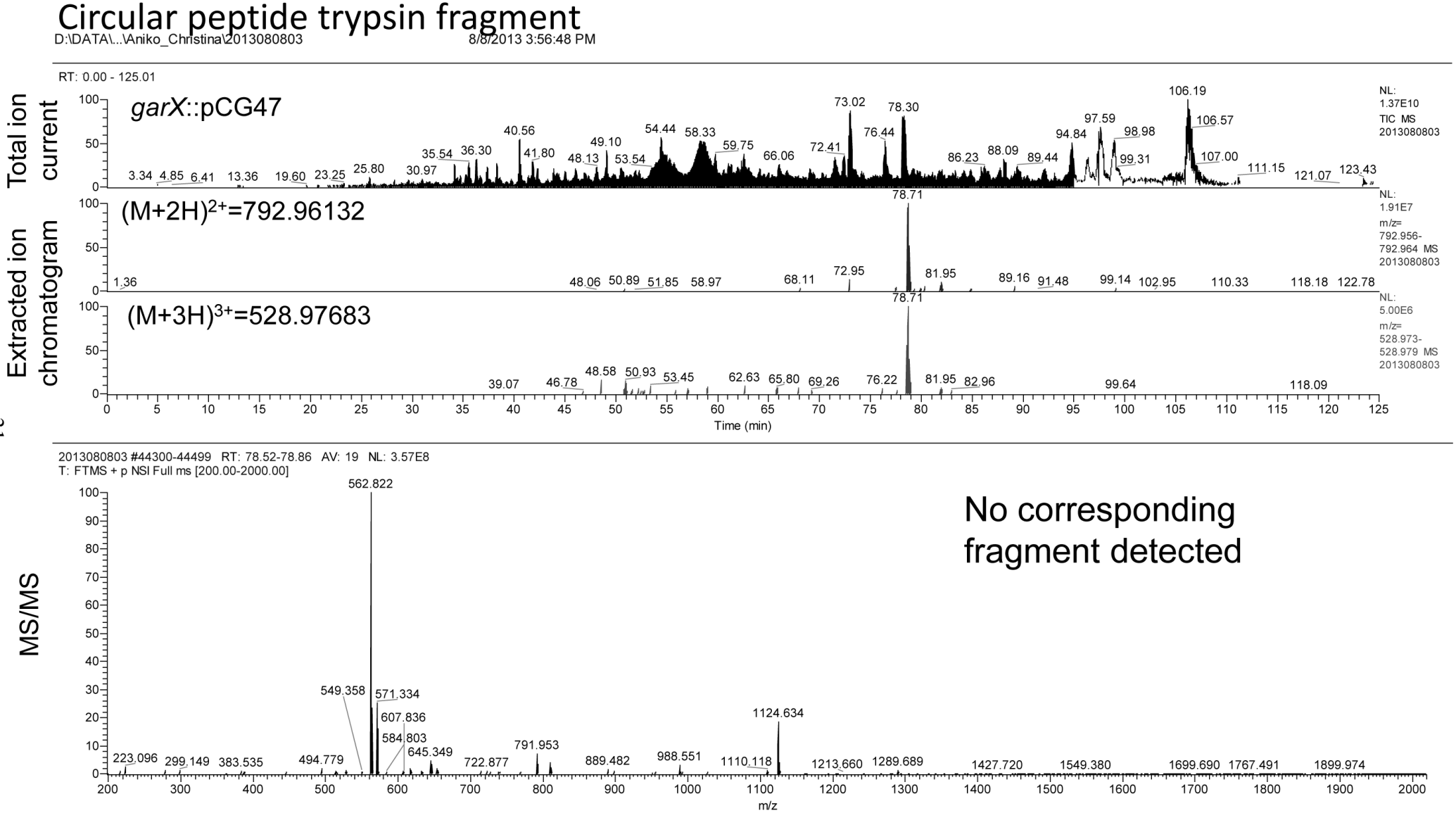


Figure S5
Circular peptide trypsin fragment

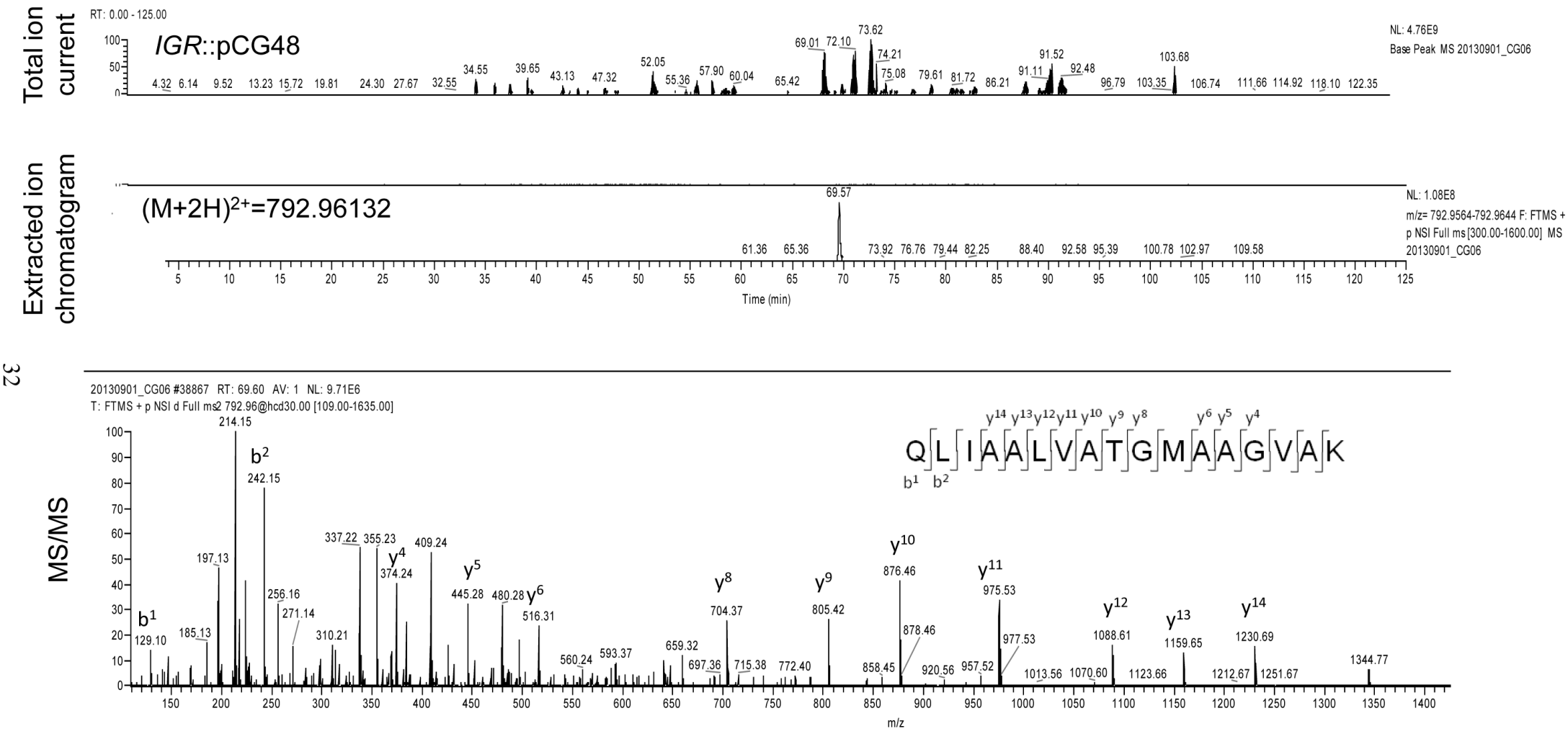


Figure S6

Linear full length peptide trypsin fragment

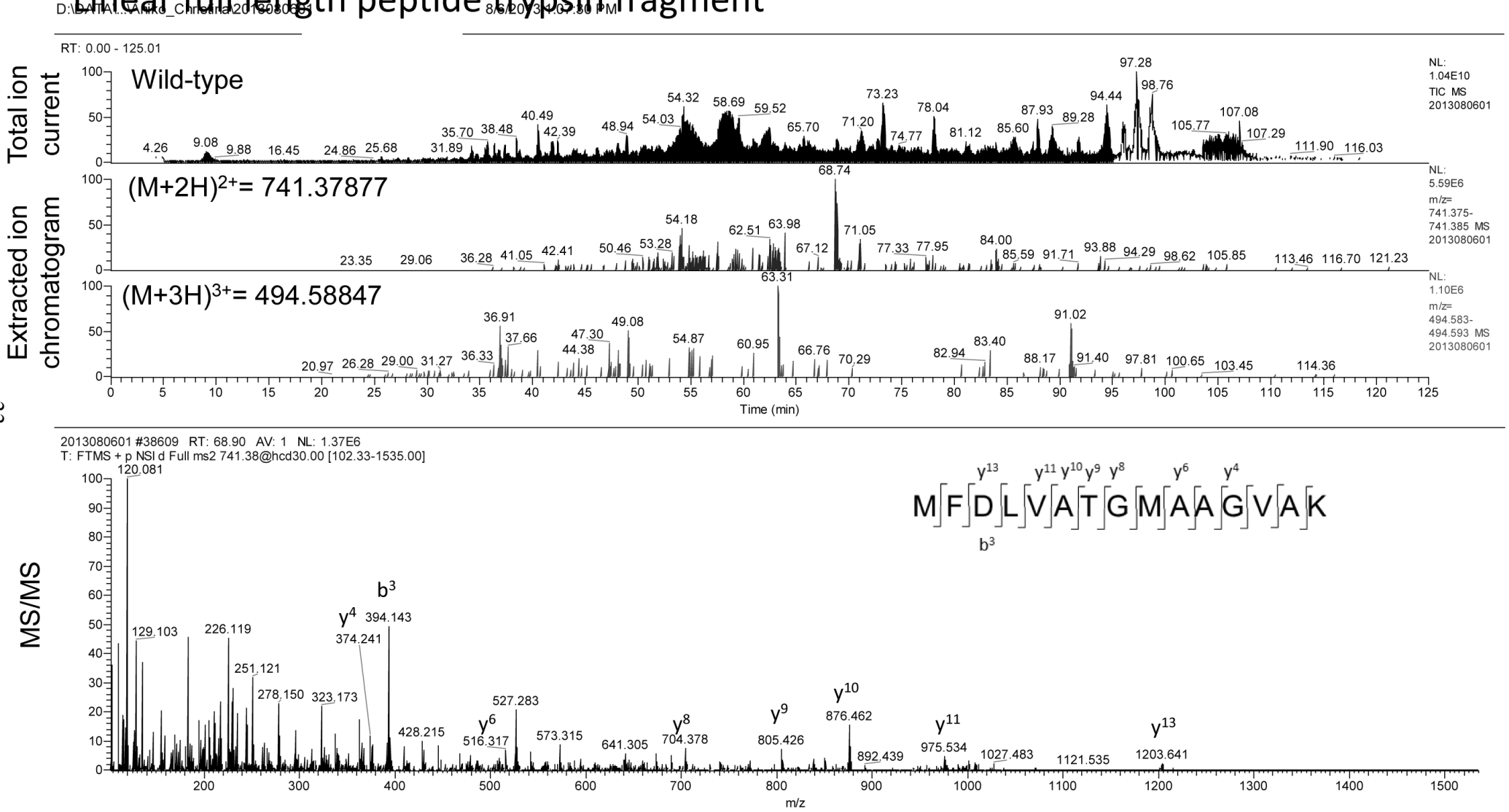


Figure S7

Linear full length peptide trypsin fragment

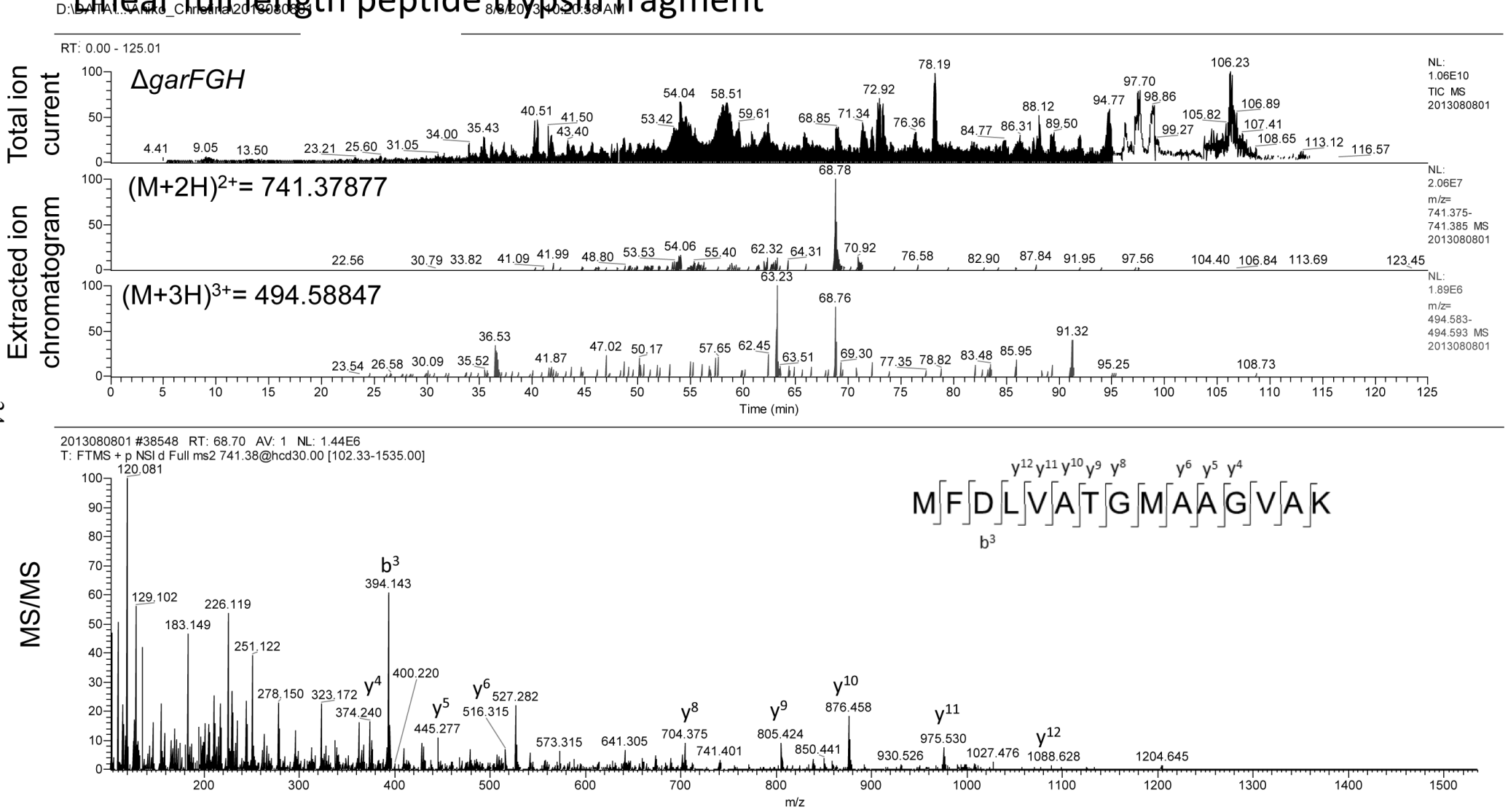


Figure S8

Linear full length peptide trypsin fragment

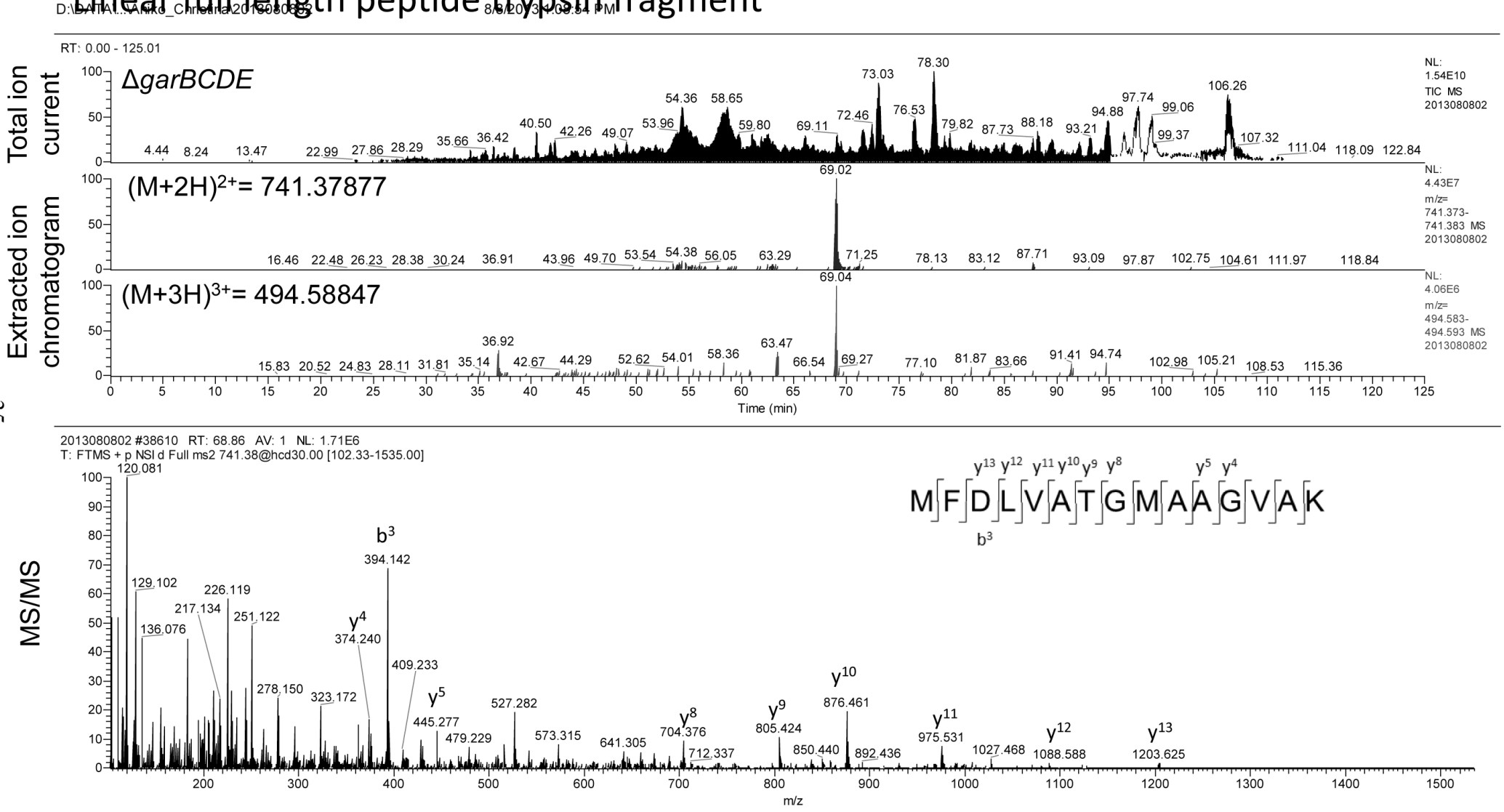


Figure S9

Linear full length peptide trypsin fragment

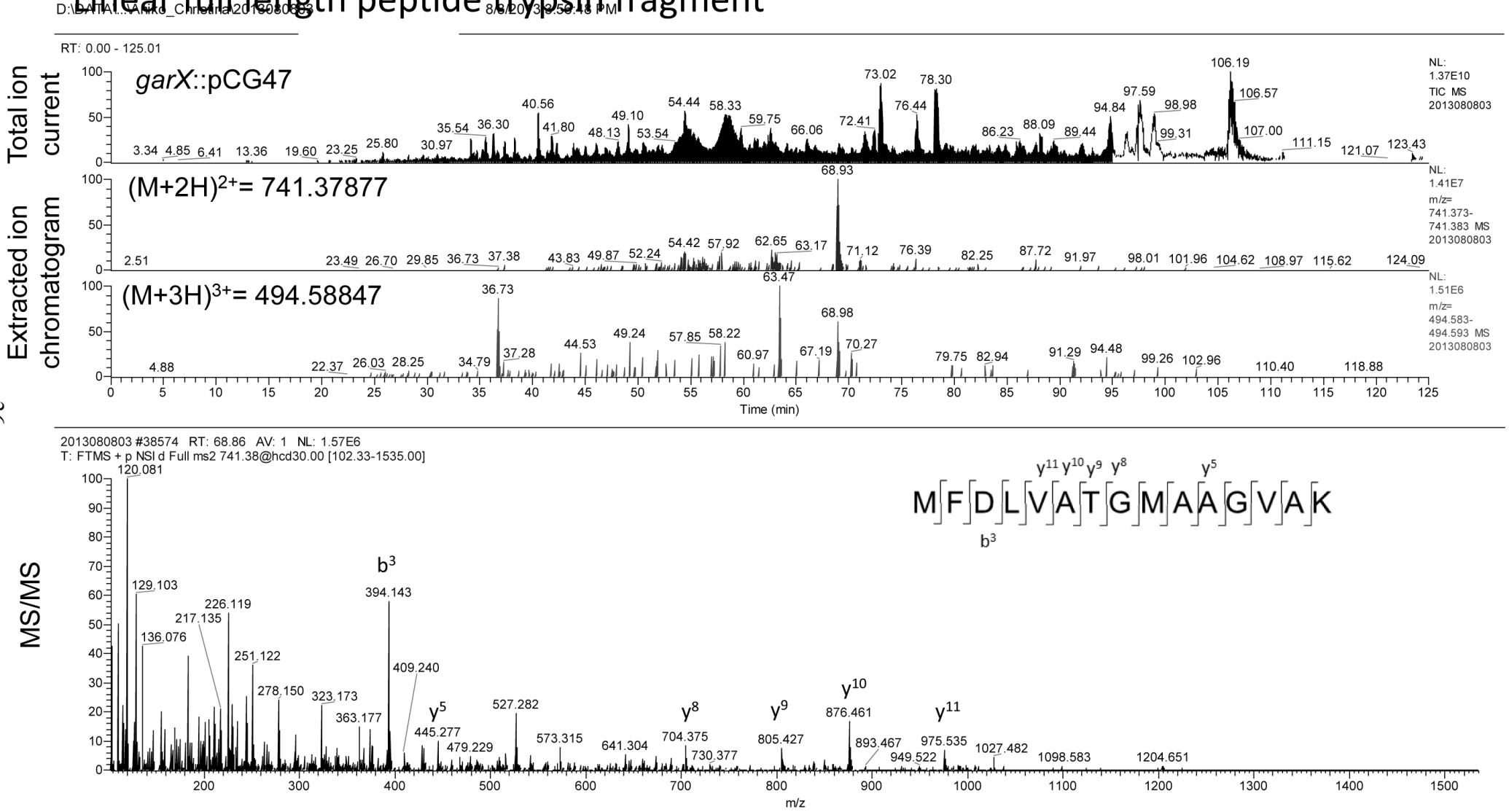


Figure S10

Linear full length peptide trypsin fragment

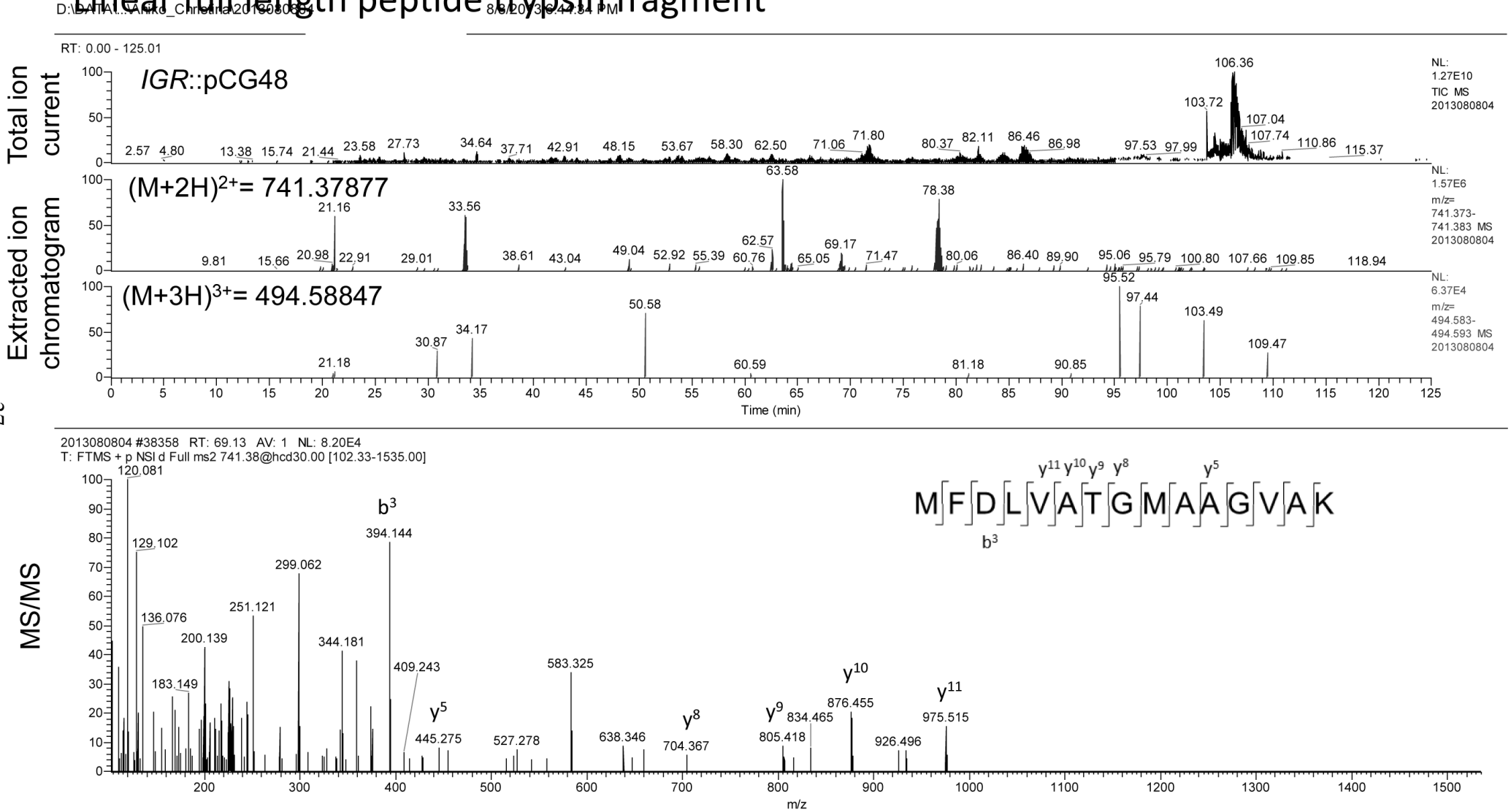


Figure S11

Linear peptide without leader trypsin fragment

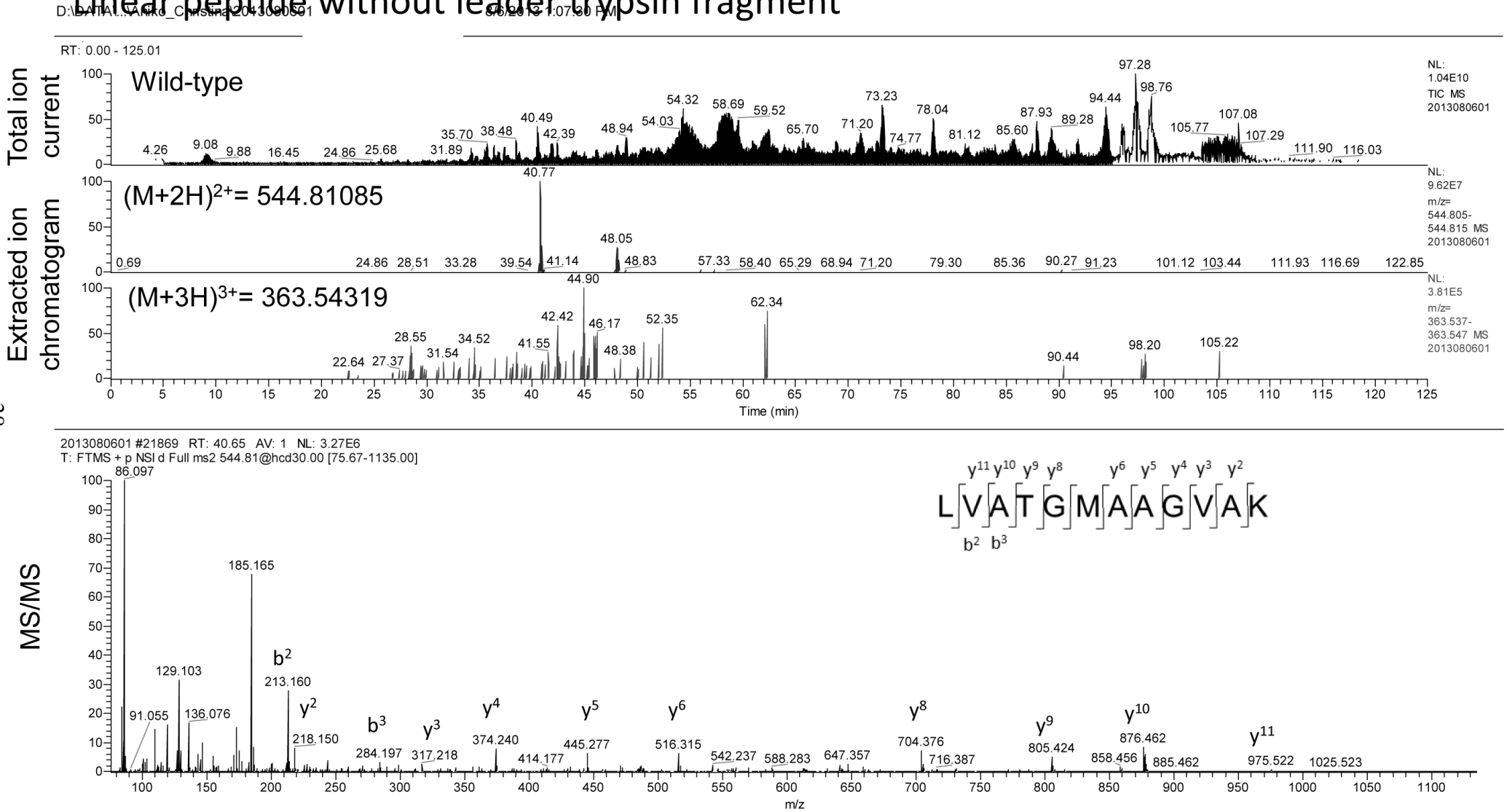


Figure S12

Linear peptide without leader trypsin fragment

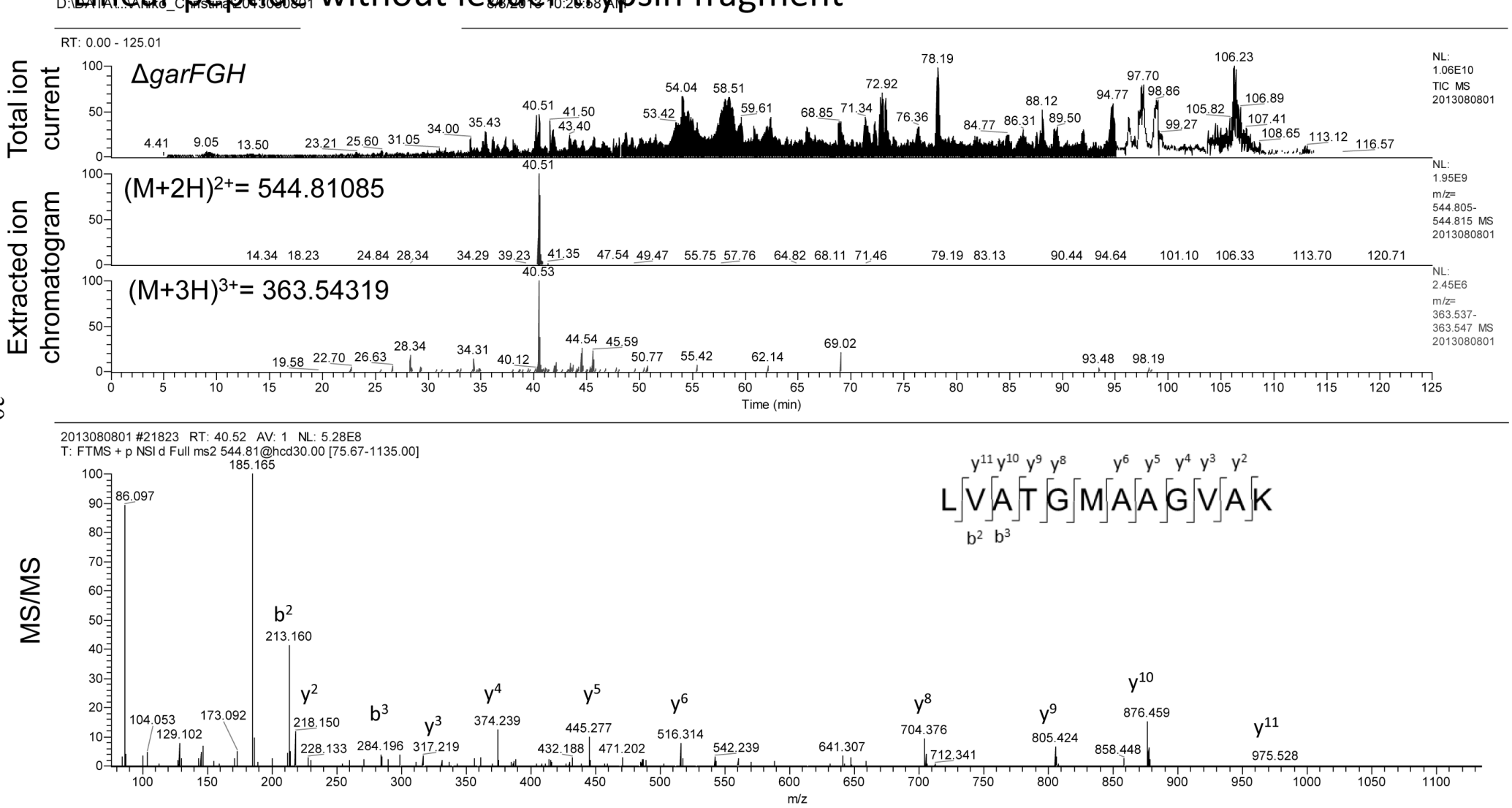


Figure S13

Linear peptide without leader trypsin fragment

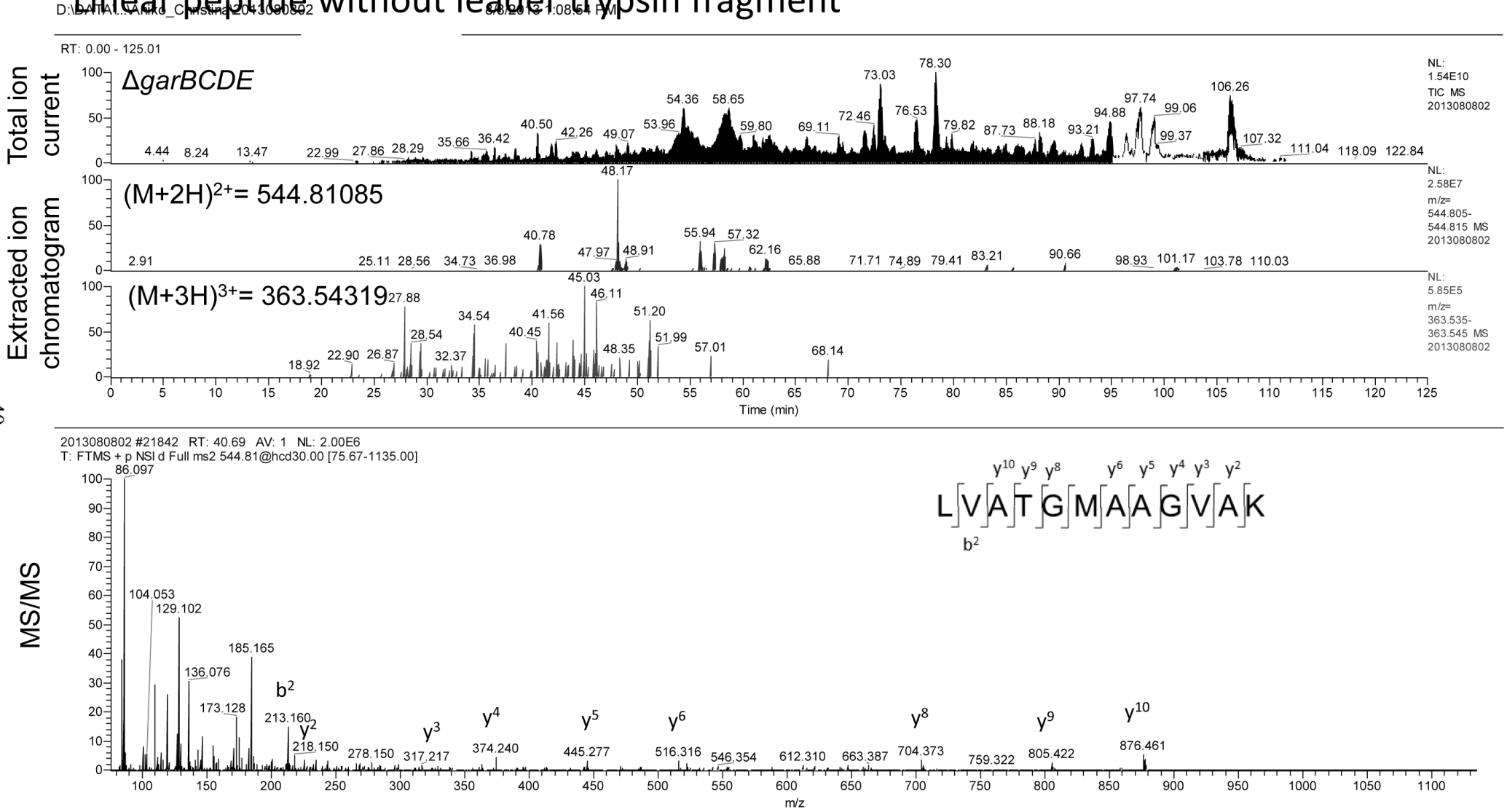


Figure S14

Linear peptide without leader trypsin fragment

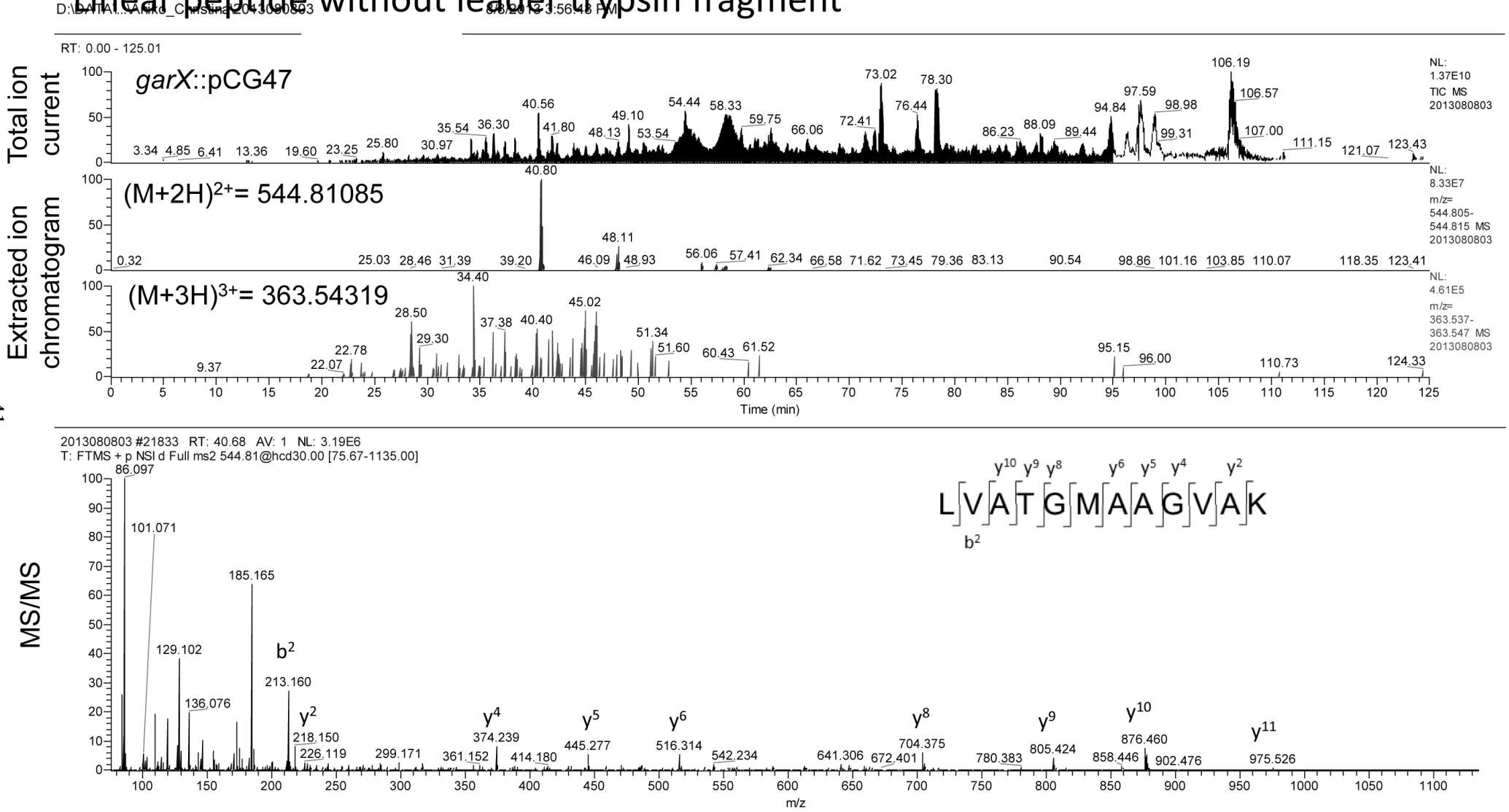
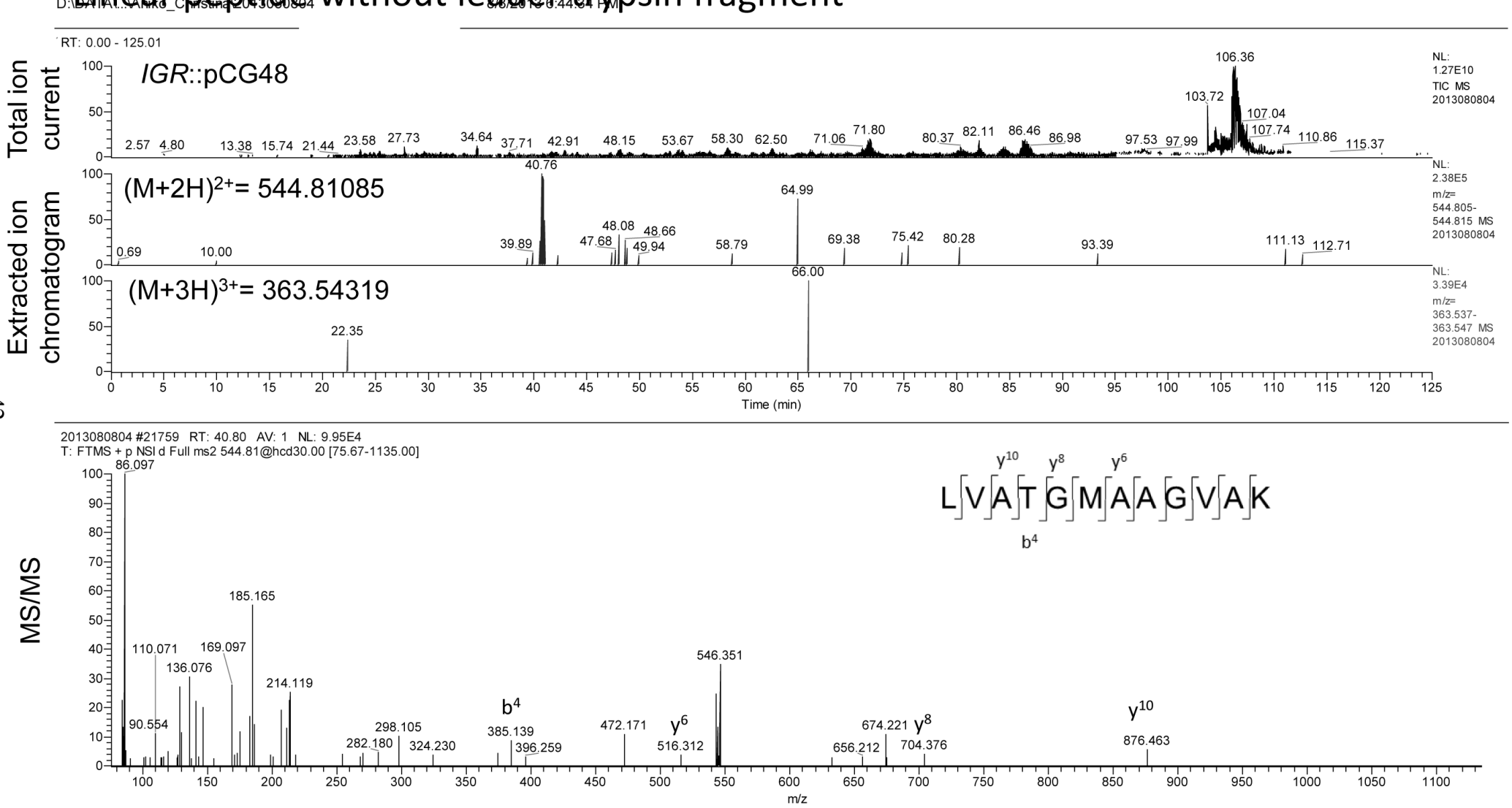


Figure S15

Linear peptide without leader trypsin fragment



Paper III

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The Maltose ABC Transporter in *Lactococcus lactis* Facilitates High-Level Sensitivity to the Circular Bacteriocin Garvicin ML

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We generated and characterized a series of spontaneous mutants of *Lactococcus lactis* IL1403 with average 6- to 11-fold-lowered sensitivities to the circular bacteriocin garvicin ML (GarML). Carbohydrate fermentation assays highlighted changes in carbohydrate metabolism, specifically loss of the ability to metabolize starch and maltose, in these mutants. PCR and sequencing showed that a 13.5-kb chromosomal deletion encompassing 12 open reading frames, mainly involved in starch and maltose utilization, had spontaneously occurred in the GarML-resistant mutants. Growth experiments revealed a correlation between sensitivity to GarML and carbon catabolite repression (CCR); i.e., sensitivity to GarML increased significantly when wild-type cells were grown on maltose and galactose as sole carbohydrates, an effect which was alleviated by the presence of glucose. Among the genes deleted in the mutants were *malEFG*, which encode a CCR-regulated membrane-bound maltose ABC transporter. The complementation of mutants with these three genes recovered normal sensitivity to the bacteriocin, suggesting an essential role of the maltose ABC transporter in the antimicrobial activity of GarML. This notion was supported by the fact that the level of sensitivity to GarML was dose dependent, increasing with higher expression levels of *malEFG* over a 50-fold range. To our knowledge, this is the first time a specific protein complex has been demonstrated to be involved in sensitivity to a circular bacteriocin.

Bacteriocins are ribosomally synthesized peptides or proteins of bacterial origin which display antimicrobial activity, most often against strains closely related to the producer (45, 54). Bacteriocins are frequently active at nanomolar concentrations and in general act by pore formation or disruption of the integrity of the target cell membrane (31). The bacteriocin-producing strain as a rule has one or, occasionally among lantibiotics, two immunity determinants, which render(s) the producer immune to the deleterious effects of the respective bacteriocin (17, 20). Specific bacteriocins generally display well-defined (broad or narrow) inhibitory spectra; i.e., they are active only against selected genera or species while having no antagonistic effects on others. This phenomenon is consistent with the theory that bacteriocins utilize a specific receptor molecules on target cells to exert their effects (18, 24, 45). However, only two target molecules are hitherto known. The class I bacteriocin nisin and some closely related lantibiotics have all been shown to employ lipid II, a cell wall precursor molecule, as a docking site. Dependent on their concentration, these bacteriocins can either inhibit peptidoglycan biosynthesis (at low bacteriocin concentrations) or form lethal pores in the cytoplasmic membrane (at high bacteriocin concentrations) (8, 57, 58). Among class II bacteriocins, the pediocin-like bacteriocins (class IIa) and lactococcin A have been demonstrated to target the membrane-located components of the mannose phosphotransferase system (man-PTS) of sensitive cells (15). The efficiency of the man-PTS as a receptor for class IIa bacteriocins was also shown to depend on specific sequence regions of these man-PTS subunits (32, 33).

Circular bacteriocins form a separate class of bacteriocins (56), characterized by their N- to C-terminal covalent link forming a circular backbone. Circular bacteriocins are synthesized as linear precursor proteins, containing a signal peptide (2 to 35 amino acid residues) which is cleaved off during the maturation process. The linear peptides (58 to 70 amino acid residues) are cyclized by the formation of an amide bond between the N- and C-terminal residues, before being exported out of the cell. The details of these mechanisms, the potential coupling of the three processes, and the enzymes responsible are still unclear (10, 39). Circular bacteriocins are subdivided into two classes (11, 41): subclass i includes cationic peptides with limited sequence identity and a high isoelectric point (pI ~10), whereas subclass ii circular bacteriocins share high sequence identity, with more acidic residues, and have a lower isoelectric point (pI ~5). Characterizations of the three-dimensional structures of several circular bacteriocins have revealed that they share a compact globular structure consisting of repeated α-helical motifs surrounding a hydrophobic core (41). This highly stable circular structure makes the circular bacteriocins particularly resilient, with characteristic traits such as high thermo-, pH-, and proteolytic stability. These traits make the circular bacteriocins especially interesting for potential industrial applications. For in-depth reviews on circular bacteriocins, we refer the reader to van Belkum et al. (56) and Maqueda et al. (39).

Enterocin AS-48, produced by *Enterococcus faecalis* subsp. *liquefaciens*, is the first-discovered and most studied circular bacteriocin (38). Other circular bacteriocins include gassericin A (3,

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference
Strains		
Lactococcus lactis subsp. *lactis*		
IL1403	Indicator strain for GarML	9a
IL1403 200B1	GarML-resistant isolate	This study
IL1403 200C1	GarML-resistant isolate	This study
IL1403 150G1	GarML-resistant isolate	This study
IL1403 150G2	GarML-resistant isolate	This study
IL1403 150H3	GarML-resistant isolate	This study
IL1403 150H4	GarML-resistant isolate	This study
Lactococcus garvieae DCC43	Producer strain of GarML	51
Plasmids		
pNZ8037	Expression vector containing the nisin-inducible promoter P_{nisA}; MCS; Cam^r	12
pNZ9530	Lactococcal expression vector of *nisRK*; Erm^r	34
pCG11	Expression vector with *malEFG* fusion under the nisin-inducible promoter P_{nisA}; Cam^r	This study

[a] Cam^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Erm^r, erythromycin resistance; MCS, multiple-cloning site.

27, 28), circularin A (27), carnocyclin A (42), subtilosin (4, 29), butyrivibriocin AR10 (26), uberolysin (59), lactocyclicin Q (52), and leucocyclicin (43). A recently identified circular bacteriocin is garvicin ML (GarML) (6). GarML is a 60-amino-acid subclass i circular bacteriocin produced by *Lactococcus garvieae* DCC43, which was isolated from mallard ducks (*Anas platyrhynchos*) (51). Protein structure modeling suggested that GarML, similar to other circular bacteriocins, folds into a compact globular bundle comprised of conserved α-helices enclosing a hydrophobic core. A cluster of basic amino acid residues imparts a positive charge on the surface of the peptide, which is thought to attract the peptide to the negatively charged surface of the target cell (6). GarML also shares the common characteristics of circular bacteriocins, being resistant to high temperatures, alkaline or acidic pH, and proteolytic enzymes. The antimicrobial spectrum of GarML is relatively broad; the most sensitive species are those most closely related to the producer, *Lactococcus garvieae* and *Lactococcus lactis*. However, strains of *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Propionibacterium*, *Clostridium*, and *Listeria* have all been shown to be highly or moderately sensitive (6). In a continued effort to unveil the mode of action of bacteriocins, an important prerequisite for the use of bacteriocins in potential future food safety or medical applications (11), in the present study, we present the identification a maltose ATP binding cassette (ABC) transporter which is required for the antimicrobial activity of this novel circular bacteriocin.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and their derivatives used in this study are listed in Table 1. Lactococcal strains were routinely grown at 30°C in M17 medium (Oxoid, Hampshire, United Kingdom) supplemented with 0.4% (wt/vol) glucose, galactose, or maltose. *Escherichia coli* strains were grown in lysogeny broth (LB) at 37°C with shaking at 225 rpm. Erythromycin or tetracycline was added at a final concentration of 5 μg ml^{-1}, and chloramphenicol was added at a final concentration of 10 μg ml^{-1} for selection in lactococci. Ampicillin was added at a final concentration of 100 μg ml^{-1}, chloramphenicol was added at a final concentration of 30 μg ml^{-1}, and tetracycline was added at a final concentration of 12.5 μg ml^{-1} for selection in *E. coli*.

Bacteriocin preparation and assays. The bacteriocin GarML was concentrated from the supernatant of the producing strain *L. garvieae* DCC43 by precipitation with 45% (wt/vol) ammonium sulfate. Bacteriocin sensitivity was determined by using microtiter plate assays (MPAs), where 100-fold dilutions of the indicator strains were exposed to 2-fold serial dilutions of bacteriocin. Bacteriocin sensitivity was assessed by the minimal concentration of bacteriocin producing 50% growth inhibition, i.e., the MIC_{50}, of the indicator strain. One bacteriocin unit (BU) was defined as the amount of bacteriocin required to produce 50% growth inhibition in 200 μl of culture. Alternatively, bacteriocin sensitivity was determined by a spot-on-lawn soft-agar assay, where the concentrated supernatant was spotted directly onto a soft-agar plate containing a 100-fold dilution of the indicator strain. Bacteriocin activity was seen as clear zones of growth inhibition of the indicator strain. The proteinaceous nature of the antimicrobial substance was confirmed by the addition of proteinase K.

Isolation of *L. lactis* IL1403 GarML-resistant mutants. Bacterial cultures grown overnight were 100-fold diluted and added to soft agar containing bacteriocin at concentrations ranging from 150 to 300 BU ml^{-1}. The solution was plated onto agar, and incubation was carried out until colonies appeared. The bacteriocin sensitivity phenotypes of the produced mutants were confirmed and quantified by microtiter plate assays. The clonal identity of the mutant isolates as *L. lactis* IL1403 was confirmed by multilocus sequence typing (MLST) of four partial genes: *pheS*, encoding phenylalanine tRNA synthetase; *rpoA*, encoding the RNA polymerase alpha chain; *pepX*, encoding X-prolyl dipeptidyl peptidase); as well as the 16S rRNA gene (L200142), according to the scheme described by Rademaker et al. (49). The primers used for MLST are given in Table S1 in the supplemental material.

Growth and phenotypic assays. Growth assays were performed in microtiter plates with 100-fold-diluted cultures grown overnight using Bioscreen C (Oy Growth Curves Ab Ltd., Helsinki, Finland), measuring the optical density at 600 nm (OD_{600}) in intervals. An API 50 CH kit (bioMérieux, Marcy l'Etoile, France) was used for determining carbohydrate metabolism patterns of bacterial cultures. The stability of the bacteriocin-resistant phenotype was assessed during >50 generations of growth without selective pressure (bacteriocin) in liquid cultures. At regular intervals, 10 colonies from each culture were replica plated onto plates containing 0, 25, 50, and 100 BU ml^{-1} bacteriocin to determine the sensitivity level of the cell population.

Genetic techniques. Transformation of lactococci was performed as described previously (25). Isolation of total DNA was performed using a Thermo Savant FastPrep FP120 cell disrupter (Qbiogene Inc., CA) and an E.Z.N.A. plasmid miniprep kit (Omega Bio-tek Inc., GA). Plasmid isolation was carried out by using an E.Z.N.A. plasmid miniprep kit or a Qiagen plasmid midi kit (Qiagen, Düsseldorf, Germany) following enzymatic lysis with lysozyme (4 mg ml^{-1}) and mutanolysin (100 U ml^{-1}) at 37°C for 30 min when required. Cloning techniques were in general performed according to methods described by Sambrook and Russell (50). Restriction enzymes, calf intestinal alkaline phosphatase, and T4 DNA ligase (New England BioLabs Inc., MA) were used according to the supplier's instructions. Oligonucleotides were supplied by Invitrogen Life Technologies (Scotland, United Kingdom). PCR was performed using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, MA), and PCR products were purified using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). Sequencing was performed by using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA). The primers used in this study are given in Table S1 in the supplemental material.

Complementation of GarML-resistant mutants with the *L. lactis* maltose ABC transporter. The open reading frames (ORFs) of *malE* and *malFG* were amplified from *L. lactis* IL1403 genomic DNA by PCR, using primer pairs malEFG-F/malE-R2 and malFG-F2/malEFG-R, respectively. The PCR products were then spliced by overlap extension (SOE) PCR using primers malEFG-F and malEFG-R. The *malEFG* fusion product was confirmed by sequencing and subsequently ligated as a transcriptional fusion into the NcoI/SmaI site of pNZ8037 (12), creating pCG11. In this expression vector, the transcription of *malEFG* is under the control of the nisin-inducible promoter P_{nisA}. The vectors pCG11and pNZ9530 (34) were introduced into wild-type *L. lactis* IL1403 and GarML-resistant mutant isolates 200B3, 200C1, and 150G1 by electroporation. The transcription of *malEFG* was induced by the addition of nisin at concentrations ranging from 0.1 to 10 ng ml^{-1}, and sensitivity to bacteriocin was assayed by MPAs with standard GM17 medium as described above. The ability of the *malEFG*-complemented GarML-resistant mutants to ferment maltose was assessed by growth assays with M17 medium supplemented with maltose (0.4%, wt vol^{-1}).

RESULTS

Generation and characterization of GarML-resistant mutants. Upon exposure to GarML at concentrations ranging from 150 to 300 BU ml^{-1}, *L. lactis* IL1403 colonies arose at an average frequency from 10^{-7} at the lower concentrations (150 BU ml^{-1}) to 10^{-8} at the higher concentrations (200 to 250 BU ml^{-1}) of bacteriocin. No colonies were obtained at concentrations above 250 BU ml^{-1}. Colonies were subcultured in nonselective medium, and the sensitivity level of these cultures was determined by microtiter plate assay (MPA). Three phenotypes were recognized: (i) the majority of colonies (68% from three biological replicas) showed wild-type sensitivity (results not shown), indicating that tolerance to the bacteriocin was due to a transient adaptation; (ii) a few colonies (approximately 20%) showed a slight increase in the MIC_{50} (2 to 3 times more tolerant to GarML than the wild type), and we defined these isolates as low-level-tolerant isolates; and (iii) a total of six isolates from four different biological replicates showed a consistent average 6- to 11-fold increase in the MIC_{50} against GarML, corresponding to 30 to 55 BU ml^{-1}, compared to wild-type *L. lactis* IL1403 (5 BU ml^{-1}). High-level resistance to the bacteriocin, as is often observed for class IIa bacteriocins (23, 47) and lantibiotics (35), was not observed. The isolates showed slight variations in MIC_{50} values between measurements but no consistent internal variation; i.e., all six isolates appeared to be within the same level of sensitivity to GarML. These isolates, denoted 200B3, 200C1, 150G1, 150G2, 150H3, and 150H4 (with the main number indicating the concentration of bacteriocin exposure and the letter identifying the biological replicate), were defined as being GarML resistant and were selected for further studies. The GarML-resistant phenotype was found to be specific; i.e., the isolates were not affected in their sensitivity to other bacteriocins targeting lactococci: lactococcin A, lactococcin G, and nisin (data not shown). The GarML-resistant phenotype was shown to persist for more than 50 generations of growth without selective pressure in liquid medium (data not shown), thus indicating that that the phenotype resulted from a stable genetic change. The clonal identity of these isolates as *Lactococcus lactis* IL1403 was confirmed by multilocus sequence typing (MLST) of four genetic loci: *pheS*, *rpoA*, *pepX*, and the 16S rRNA gene (data not shown).

GarML-resistant mutants are defective in maltose and starch catabolism. The *L. lactis* IL1403 GarML-resistant mutants were further phenotypically characterized. Carbohydrate fermentation assays highlighted two specific differences that distinguished all six mutant isolates from the wild type: while wild-type *L. lactis* IL1403 ferments starch and maltose, the mutant isolates did not grow on either carbohydrate. Growth experiments confirmed these observations (Fig. 1). Growth of the wild type and mutants in standard GM17 medium (containing 0.4% glucose) did not show any significant differences in the growth rate or in the maximal optical density at the stationary growth phase of the cultures, indicating that the GarML-resistant phenotype does not cause changes in the growth performance with glucose as the main energy source. However, clear differences were observed when maltose was used as the sole carbohydrate source. Consistent with data from previous reports, wild-type *L. lactis* IL1403 grows at a low rate to a maximum absorbance (OD_{600}) of approximately 0.5 in maltose-containing M17 medium (53). The GarML-resistant mutants, however, grew only to an absorbance of 0.2, corresponding to the residual growth in M17 medium without added sugar. These findings thus confirmed that the GarML-resistant mutants had lost the ability to utilize maltose.

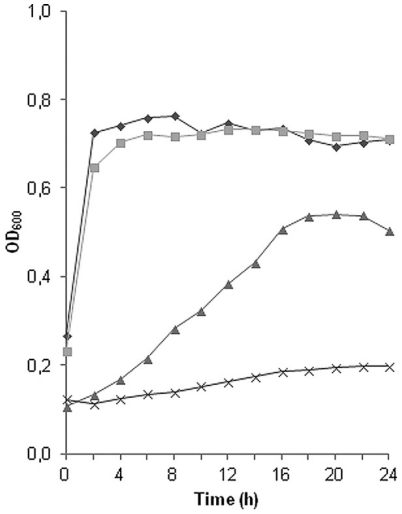

FIG 1 Growth of wild-type *L. lactis* IL1403 and spontaneous GarML-resistant mutants in glucose- and maltose-containing medium. Growth was measured by OD_{600} in M17 medium with glucose (0.4%) or maltose (0.4%). Graphs show the growth of wild-type *L. lactis* IL1403 in glucose (♦) and maltose (▲) and the growth of *L. lactis* IL1403 GarML-resistant mutants in glucose (■) and maltose (×). The average growth for all six mutant isolates 200B3, 200C1, 150G1, 150G2, 150H3, and 150H4 is shown as single graphs for simplification.

Carbon catabolite repression has an effect on sensitivity to GarML. Sugar metabolism in most bacteria is normally regulated through a hierarchical mechanism known as carbon catabolite repression (CCR), in which the presence of a preferred sugar (e.g., glucose) represses the expression of genes involved in the metabolism of sugars with less energy output (e.g., galactose and maltose) (13). In *L. lactis*, several genes involved in maltose utilization (encoding maltose phosphorylase, the maltose ATP binding cassette [ABC] transporter, and β-phosphoglucomutase) are regulated by CCR (1, 46, 48) via catabolite-responsive elements (*cre*) (Fig. 2) that are bound by the serine-phosphorylated HPr/CcpA repression protein complex in response to high glucose availability in the cell (44). To examine whether sensitivity to GarML is linked to CCR regulation, bacteriocin sensitivity assays were performed on wild-type and GarML-resistant mutant isolates grown

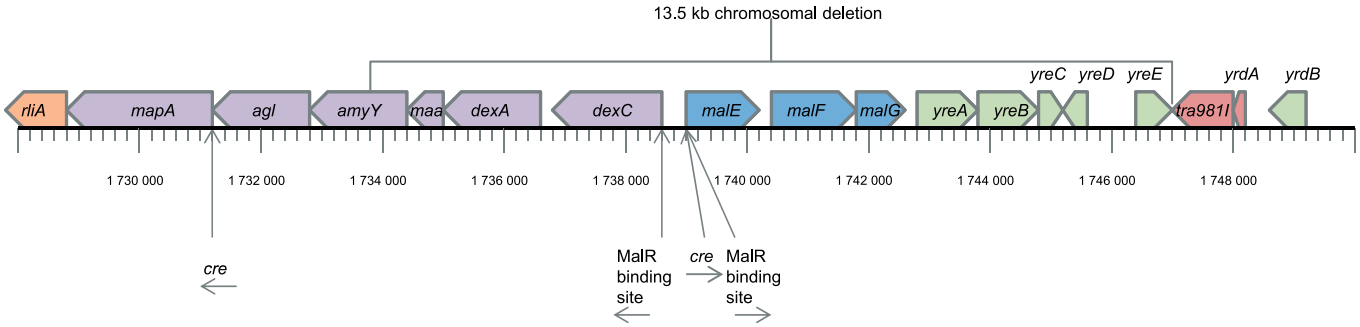

FIG 2 Chromosomal region encoding functions involved in starch/maltose breakdown, uptake, and conversion in *L. lactis* IL1403. The *rliA* gene encodes a transcriptional regulator; *mapA* encodes maltose phosphorylase; *agl* encodes α-glucosidase; *amyY* encodes α-amylase; *maa* encodes maltose *O*-acetyltransferase; *dexA* encodes oligo-1,6-glucosidase; *dexC* encodes neopullulanase; *malEFG* encode the maltose ABC transporter substrate binding protein and two permease proteins, respectively; *yreABCDE* encode hypothetical proteins; *L200065* encodes a hypothetical protein; *tra981I* encodes a transposase of IS*981*I; *yrdA* encodes a transposase; and *yrdB* encodes a hypothetical protein. The 13.3-kb deletion characterized for GarML-resistant mutants is indicated. Genes shown in purple are involved in sugar utilization, blue genes are components of the maltose ABC transporter, green genes are hypothetical proteins with unknown functions, and IS*981*I-related genes are marked in red. The MalR transcriptional activator (encoded by *rliA*) is indicated in orange, and its putative binding sites are indicated with arrows (2). Catabolite-responsive elements (*cre*) subject to regulation by carbon catabolite repression (CCR) are also indicated by arrows (1).

in medium with a single sugar or a mixture of different sugars; glucose (CCR inducing), maltose (non-CCR inducing), and galactose (non-CCR inducing). Galactose was selected as a control, as genes involved in the utilization of this carbohydrate are also regulated by CCR in *L. lactis* (37). The results showed that wild-type *L. lactis* IL1403 indeed became more sensitive to GarML when grown on maltose and/or galactose than when grown on glucose (Fig. 3). The effect was most pronounced on maltose, where the wild type displayed an average 4-fold reduction in the MIC_{50} (increased sensitivity to GarML) relative to growth on glucose, corresponding to approximately 1.25 BU ml^{-1}. Growth on glucose alone or in combination with maltose or galactose, however, alleviated the effect and restored a normal level of sensitivity to GarML, which is consistent with a response to a carbon catabolite repressive situation. These results demonstrate that relief of CCR in general, and growth on maltose specifically, has a distinct effect on the sensitivity of *L. lactis* IL1403 to GarML. The *L. lactis* IL1403 GarML-resistant mutants, however, did not display any significant changes in sensitivity to the bacteriocin regardless of the type of sugar(s) added to the growth medium (data not shown).

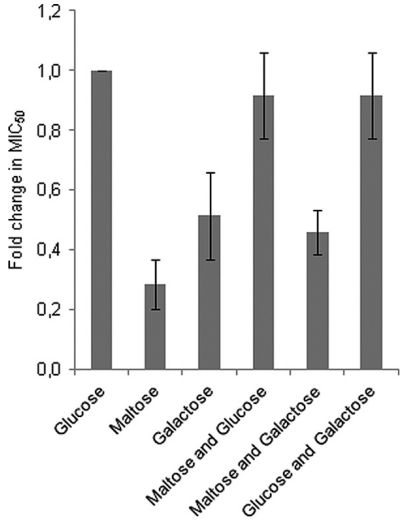

FIG 3 Sensitivity of wild-type *L. lactis* IL1403 to garvicin ML in different media. Sensitivity to garvicin ML is given as average fold change in the MIC_{50} in M17 medium containing 0.4% sugar (glucose, maltose, galactose, glucose and maltose, and glucose and galactose) relative to growth on glucose (set to 1). Error bars show standard deviations from three biological replicates.

GarML-resistant mutants have a large deletion in a chromosomal region involved in carbohydrate metabolism. In *L. lactis* IL1403, a chromosomal region of approximately 15 kb located at kb 1727 to 1742 in the genome (GenBank accession no. AE005176) contains most of the genes involved in the breakdown, uptake, and conversion of starch and maltose (Fig. 2). An α-amylase (encoded by *amyY*) located in this region is responsible for the hydrolysis of starch into maltose, and a dedicated ABC transporter (encoded by *malEFG*) is the sole uptake system for maltose in *L. lactis* (36). By PCR analysis, it was found that the *malE*, *malF*, and *malG* structural genes were present in wild-type *L. lactis* strain IL1403, as expected from the genome sequence, but to our surprise, these genes were absent in all six of the GarML-resistant mutant isolates. Further investigation revealed that a minimum 12.6-kb region encompassing 12 ORFs was absent in all six GarML-resistant mutants, indicating that a chromosomal deletion had occurred in these mutants. The putative deletion was subsequently confirmed by sequencing of mutant isolates 150G2 and 150H4, showing an identical 13.5-kb deletion (region from nucleotide [nt] 1733559 to nt 1747095) in both mutants (Fig. 2). The fact that these two independently selected mutants contain the same deletion in their genomes indicates that a sequence-specific DNA rearrangement had occurred. The chromosomal deletion, comprising an α-amylase (*amyY*), a maltose *O*-acetyltransferase (*maa*), an oligo-1,6-glucosidase (*dexA*), a neopullulanase (*dexC*), and the maltose ABC transporter substrate binding and permease components (*malEFG*) as well as additional hypothetical proteins (*yreA*, *yreB*, *yreC*, *yreD*, *yreE*, and L200065), thus explains the inability of the GarML-resistant mutants to ferment starch and maltose.

Directly adjacent to the deleted chromosomal region are two genes (*tra981I* and *yrdA*) encoding transposon-related functions for insertion sequence (IS) element IS*981*I (Fig. 2), which provide the most likely explanation for this deletion event. This multicopy IS element was previously shown to be active under laboratory

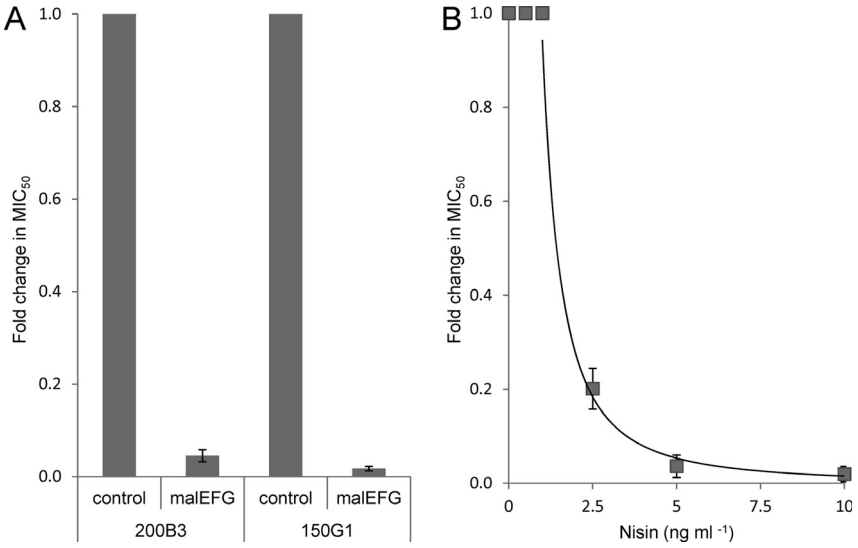

FIG 4 Sensitivity of mutant isolates complemented with the maltose ABC transporter (*malEFG*) to GarML. Sensitivity is given as average fold changes in the MIC_{50} relative to the respective controls (set to 1). The expression of *malEFG* was induced by the addition of nisin. Error bars show standard deviations from three biological replicates. (A) Sensitivity of GarML-resistant mutant isolates 200B1 and 150G1 complemented with the maltose ABC transporter (*malEFG*) at an induction level of 1 ng ml^{-1} nisin relative to controls (empty vector). (B) Correlation between sensitivity to GarML and expression levels of the maltose ABC transporter in GarML-resistant mutant strain 200B3, with the induction level ranging from 0.1 to 10 ng ml^{-1} relative to the control (no added nisin). No change in the MIC_{50} was observed at concentrations below 1 ng ml^{-1} nisin. A sharp decrease in the MIC_{50} from 1 to 10 ng ml^{-1} nisin is indicated by a power trend line ($y = 0.942\ x^{-1.783}$; $R^2 = 0.978$).

conditions and to have caused similar large chromosomal deletions by replicative transposition in *L. lactis* IL1403 (14).

The presence of *msmK* (encoding the multiple-sugar ABC transporter ATP-binding protein) and *pgmB* (encoding β-phosphoglucomutase), involved in maltose transport and breakdown, respectively, was also assessed by PCR. These genes are implicated in the metabolism of other sugars and are located separately from the starch/maltose gene cluster. However, none of these genes were found to be affected in the six GarML-resistant mutants.

Complementation of mutants with the maltose ABC transporter restores sensitivity to GarML. The deletion characterized for GarML-resistant mutants led us to hypothesize that the deletion of a potential receptor molecule for the bacteriocin could link the observed genotype to the bacteriocin-resistant phenotype. Most bacteriocins of lactic acid bacteria (LAB) affect the permeability of the membrane, and therefore, it is generally believed that potential bacteriocin receptors on target cells are likely to be membrane located in order to allow a specific interaction. This is true for the receptor of class IIa bacteriocins, lactococcin A, and lipid II-targeting lantibiotics (8, 15, 57). Genes within the deleted region of the GarML-resistant mutants, however, code for either hypothetical proteins with an unknown function or secreted or intracellular enzymes (Fig. 2). The only exception is the maltose ABC transporter encoded by an operon comprising three genes, *malE*, *malF*, and *malG*, encoding the substrate binding and two permease components of the permease, respectively. As indicated previously, the *malEFG* operon is regulated at the transcriptional level by CCR but also by the transcriptional activator MalR (encoded by the proximately located *rliA* gene) in the presence of maltose (Fig. 2) (2, 44). This corresponded very well with our observation that growth on maltose and non-CCR-inducing sugars affected sensitivity to GarML. Together, these findings suggested that the maltose ABC transporter is implicated in the sensitivity to GarML.

To test whether this membrane-located protein complex is involved in sensitivity to GarML, the *L. lactis* IL1403 GarML-resistant mutants (lacking *malEFG* as well as other genes) were complemented with *malEFG* using a two-plasmid system comprising pNZ9530 and pCG11, with the latter expressing the *malEFG* genes from a nisin-inducible promoter. Indeed, the complementation of the resistant mutants with these three genes rendered resistant cells highly sensitive to GarML. The complemented mutant isolates 200B3 and 150G1 showed average 22- and 58-fold reductions in the MIC_{50}, respectively (Fig. 4A), relative to that of the control (empty vector). This corresponds not only to a restoration of the wild-type level of sensitivity (5 BU ml^{-1}) but to an even further reduction in the MIC_{50}: 0.3 BU ml^{-1} for mutant isolate 200B3 and 0.7 BU ml^{-1} for mutant isolate 150G1. Furthermore, it was shown that the sensitivity of the complemented clones responded to the inducer nisin in a dose-dependent manner. The graded expression of *malEFG* by induction with 0.1 to 10 ng ml^{-1} nisin yielded a power correlation ($y = 0.942\ x^{-1.783}$; $R^2 = 0.978$) between the expression level and sensitivity to GarML for the complemented mutant *L. lactis* IL1403 200B3 (Fig. 4B). The level of sensitivity to GarML for mutant isolate 200B3 thus ranges approximately 50-fold (1 to 50 BU ml^{-1}), increasing with the expression level of *malEFG*. The sensitivity level of the control (empty vector) remained unchanged irrespective of the induction level. At these induction levels ($<$10 ng ml^{-1} nisin), no detrimental effects on the growth of the clones were observed. However, at a higher concentration of the inducer ($\geq$10 ng ml^{-1} nisin), in a few cases, we observed lethality for the complemented mutant but not for the control, even in the absence of bacteriocin. It should be noted that at 10 ng ml^{-1} nisin, the concentration of nisin should be sublethal to *L. lactis* (34), and the observed lethality is more likely caused by detrimental effects commonly resulting from the overexpression of membrane proteins (40). Growth assays of the complemented mutants on medium containing maltose showed that

functional complementation was not achieved, as the mutants did not regain the capacity to metabolize maltose (data not shown). This could reflect a possible requirement for one or several of the additional deleted genes and/or regulatory sequences for maltose utilization in *L. lactis*.

DISCUSSION

Most bacteriocins act by disruption of the integrity of the target cell membrane (24, 31). The mechanisms of action of most circular bacteriocins have not yet been determined, but for two of the most studied cases, enterocin AS-48 and carnocyclin A, it was previously shown that the bacteriocins can permeabilize liposomes and/or lipid bilayers (21, 22). Also, previous studies of the circular bacteriocins gassericin A and subtilosin A indicated that the bacteriocins do not require a target receptor (28, 55). Thus, it has been suggested that circular bacteriocins in general exert their activity independently of any target molecule in the cell membrane of sensitive cells. For the circular bacteriocin GarML, we observed a relatively broad but defined inhibition spectrum and large interspecies variation in sensitivity to GarML, which led us to hypothesize that a specific target molecule might be involved in the recognition of this bacteriocin. As detailed further below, this study provides evidence that GarML requires the maltose ABC transporter on target cells for antimicrobial activity.

Spontaneous GarML-resistant mutants with 6- to 11-fold-increased sensitivities to the bacteriocin were shown to be defective in maltose utilization (Fig. 1), caused by a large deletion of the chromosomal region encoding these functions (Fig. 2). Furthermore, growth experiments showed a clear correlation between sensitivity to GarML and carbon catabolite repression (CCR) (Fig. 3), where growth on the non-CCR-inducing sugars maltose and/or galactose (in the absence of glucose) increased the sensitivity to GarML markedly (Fig. 3). Growth in maltose-containing medium thus renders wild-type *L. lactis* IL1403 even more sensitive to GarML, up to approximately 44-fold compared to the resistant mutants (grown on glucose). These findings thus pointed to a possible role of the maltose- and CCR-regulated maltose ABC transporter in sensitivity to GarML, and the subsequent complementation of GarML-resistant mutants with this permease confirmed this hypothesis by effectively restoring the sensitivity to the bacteriocin (Fig. 4A). Moreover, the response to the expression of the maltose ABC transporter appeared to be dose dependent, as higher expression levels of these genes increased the sensitivity of cells to GarML (Fig. 4B), implying a direct correlation between the potency of the bacteriocin and the expression level of the maltose transporter. These results support the notion that the maltose ABC transporter plays an essential role in sensitivity to this bacteriocin. The maltose ABC transporter may potentially function as a target receptor for the bacteriocin, rendering the permease open for the efflux of intracellular solutes, eventually leading to cell death. However, we cannot rule out the possibility that the ABC transporter may be used as a docking molecule, in a manner similar to that of lipid II for the class I lantibiotic nisin (8, 58), or that it transports the bacteriocin to another intracellular target. Continued research efforts may shed further light on the details of the mode of action of GarML.

To our knowledge, this is the first time that a specific protein complex has been demonstrated to be involved in the sensitivity to a circular bacteriocin and also the first example of an ABC transporter acting as a cellular target for bacteriocins in Gram-positive bacteria. However, the finding corroborates recent discoveries in the bacteriocin field. For class IIa bacteriocins and lactococcin A, the sugar transporter man-PTS has been shown to function as a target receptor on sensitive cells (15), and for another bacteriocin, lactococcin 972, the sugar transporter CelB has been implicated in sensitivity (9). Pending future discoveries, it is therefore tempting to predict that sugar uptake systems may emerge as a common theme in bacteriocin target recognition among class II bacteriocins of Gram-positive bacteria and probably also among some microcins of Gram-negative bacteria (5). In this context, bacteriocin production can be viewed as a competitive mechanism (16, 31) targeting competitors for the (primary) food source of the producer strain. In the case of GarML, it is feasible that the producer strain *L. garvieae* DCC43 antagonizes other lactococci, generally believed to be of plant origin (30), competing for the plant-derived sugar maltose by targeting the maltose uptake system. Maltose utilization is indeed a widespread trait among lactococci (7, 19), and a protein BLAST search revealed that the *L. lactis* IL1403 maltose ABC transporter subunits have high sequence identity to the homologous proteins in the sequenced strain *L. garvieae* ATCC 49156 (71%, 67%, and 77% identities to MalE, MalF, and MalG, respectively, with 100% coverage for all), lending further support to this notion. Future work should therefore be directed at elucidating any potential common mechanisms in related strains containing homologous maltose ABC transporters and whether this may reflect the activity spectrum of GarML.

GarML has a predicted structure homologous to other class ii circular bacteriocins, where basic amino acid residues in patches on the surface of the compact hydrophobic globular structure are thought to play a role in interactions with the negatively charged membrane on target cells (6, 41). The fact that several circular bacteriocins have been reported to act on lipid bilayers/liposomes may support the notion that such unspecific surface interactions are sufficient for antimicrobial activity. However, several of these studies were performed with bacteriocin concentrations significantly above the levels required for antimicrobial activity *in vivo*. For GarML, we have demonstrated that the maltose ABC transporter facilitates high-level sensitivity to this bacteriocin. However, consistent with other circular bacteriocins, we observed receptor-independent killing at higher concentrations of GarML. Our results therefore suggest that this class of bacteriocins may indeed require a specific interaction with a target receptor/mediator for antimicrobial activity at low concentrations. Such a dual concentration-dependent mode of action was demonstrated previously for the lantibiotic nisin (58) and can also be supported by data from previous studies of pore formation with the circular bacteriocin subtilosin A (55). The finding that an ABC transporter is involved in bacteriocin sensitivity is unprecedented for Gram-positive bacteria, and the identification of a novel type of target molecule for antimicrobial peptides could potentially be of importance both for future applications of circular bacteriocins specifically and for drug design and delivery in general.

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Supporting information

Table S1: Primers used in this study.

Primer	Amplified locus	Sequence (5'-3') [a]
pheS -F	*pheS*	CAYCCNGCHCGYGAYATGC
pheS -R	*pheS*	CCWARVCCRAARGCAAARCC
rpoA-F	*rpoA*	ATGATYGARTTTGAAAAACC
rpoA-R	*rpoA*	ACHGTRTTRATDCCDGCRCG
atpA-F	*atpA*	TAYRTYGGKGAYGGDATYGC
atpA-R	*atpA*	CCRCGRTTHARYTTHGCYTG
bcaT-F	*bcaT*	TTTKSHRTGCCDGTWGG
bcaT-R	*bcaT*	GGWCCHACTTCYGTYTC
pepN-F	*pepN*	ATKTCTTAYGCWGAYRTYGT
pepN-R	*pepN*	TTKCTTCAAGSMAWGSCC
pepX-F	*pepX*	TTTGGGTTGAAAGTCCAGT
pepX-R	*pepX*	CCAAGAAGAAATTCCAGC
SSU-F	16S rRNA	GCGGCGTGCCTAATACATGC
SSU-R	16S rRNA	ATCTACGCATTTCACCGCTAC
malE-F	*malE*	TGGAAAAAAGTTGCTCTTGG
malE-R	*malE*	GTCATATTGTGAAGCAGGAG
malF-F	*malF*	TTCTTTACCACCTGAAGCCA
malF-R	*malF*	GCCCTCCTTAAATGCATTAG
malG-F	*malG*	CTCTAACATTCCGTTACCTT
malG-R	*malG*	GCTGTGATTCCATTGACGTA
pip-F	*pip*	AAATGACTCTTGCTTCCGA
pip-R	*pip*	TACATGGAAGTTGTTTGCCC
arb-F	*arb*	GTTAAATCTTCACCGTTGCT
arb-R	*arb*	ACTGCAGCTAATCAATGTGA
yrcB-F	*yrcB*	TTCTTTTTCTGGTTCTGCCC
yrcB-R	*yrcB*	GCCTGTCGCTATTATCCTAC
dsxB-F	*dxsB*	TAAATTCACGTTTCGCACCA
dsxB-R	*dxsB*	AGAAAAAATAGACAGCCCAGC
kinF-F	*kinF*	TGCTATCCACACGAATTCTAAC
kinF-R	*kinF*	TGTTGCTTTAGCCGTGATT
IlrF-F	*IlrF*	CTGTCATAACCCCAAATCCG
IlrF-R	*IlrF*	AGGTTTATGATGGAGTTGAAGG
rliA-F	*rliA*	TTGCACTTGCCCCTAATTG
rliA-R	*rliA*	CGGTCAGTCGGGTCATTA
mapA-F	*mapA*	TGTGGTAAAAATGGGGCAAA
mapA-R	*mapA*	ATTGAAAAAGAAGACCGTCG

Primer	Amplified locus	Sequence (5'-3') [a]
agl-F	*agl*	CGATATTCCCAGGCTGAC
agl-R	*agl*	CTACGTGAATTGGGGATTG
amyY-F	*amyY*	GTGCTGTAAGCTGGAATTG
amyY-R	*amyY*	CAGTAAAAAAAGCGGCTG
maa-F	*maa*	CGTTCTCTTTTTCTTCCCAG
maa-R	*maa*	AGAATGGTTGCTGGCGAA
dexA-F	*dexA*	CTATTCCACTTCGCAAGCA
dexA-R	*dexA*	ATTGGTGGCAAAAAACAGTC
dexC-F	*dexC*	GCTTTCAAAGATCCACGTT
dexC-R	*dexC*	GCAATTTATCATCGTCCTGAG
yreA-F	*yreA*	AATCTTCCGAAAACAACGAG
yreA-R	*yreA*	CCAGAAATTTGAACCCCTTC
yreB-F	*yreB*	AATAAAGGGTCAGTGGGAAC
yreB-R	*yreB*	GGTTTCTACGGGGCTTAAAT
yreC-F	*yreC*	AGGCTGACGAGATTGAAG
yreC-R	*yreC*	CCCACTTTTGTACGTCTTTT
yreD-F	*yreD*	CCAGACGATGTGTCCAAAAA
yreD-R	*yreD*	TCGTTGGATTGGTTTTTGGG
hyp-F	*hyp*	CCACGTTGACCTCTTTCA
hyp-R	*hyp*	ATGGTCAAAAAAGCGAGTGA
yreE-F	*yreE*	AATTTCACCGCTCGTACT
yreE-R	*yreE*	ATCTAAAGAAGCGGCACAG
tra981I-F	*tra981I*	AGGCTTCGATGTACCAGA
tra981I-R	*tra981I*	GGCTCAAACCATACAAACTT
yrdA-F	*yrdA*	TCACTTCTTTTTCTCGGCGA
yrdA-R	*yrdA*	CGCTTTCTATCATTCTGGTC
yrdB-F	*yrdB*	AAACCCTTATCTCGTCACTAC
yrdB-R	*yrdB*	ATTCTGTACCATCTTCCGTT
malEFG-F	*malEFG*	GGAAACACA<u>TCATGA</u>AATCATGG
malEFG-R	*malEFG*	AGAAGAGATAAAAA<u>GCATGC</u>CG
malE-R2	*malE*	TCTGAACATATAACTTTGTCCTCCTCTATTATTTTAGTTGCTTTAGAGATAG
malFG-F2	*malFG*	CTATCTCTAAAGCAACTAAATAATAGAGGAGGACAAAGTTATATGTTCAGA

[a] Y, R, H, D, K, Y, W, M, and N are degenerate nucleotides: R for A or G; M for A or C; Y for C or T; K for G or T; W for A or T; N for A, G, C or T; S for G or C; H for A, C or T; and D for A or G or T. Inserted restriction sites are indicated by underlined sequence.

Paper IV

Functional genomic analysis of *Lactococcus lactis* ssp. *cremoris* reveals a novel resistance mechanism to the circular bacteriocin garvicin ML.

Running title: Resistance mechanisms to GarML.

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Abstract

In this work, we have investigated the genetic basis of inherent resistance to the circular bacteriocin garvicin ML (GarML) in a lineage of *Lactococcus lactis* ssp. *cremoris* strains. Sequencing and comparative genomic analysis of *L. lactis* ssp. *cremoris* MG1363 and the spontaneously streptomycin- and rifampicin-resistant derivative strain MG1614, which are GarML-resistant and GarML-sensitive accordingly, revealed a 27.7 kb genomic deletion flanked by IS elements in the latter strain, which was subsequently established to be the cause of the GarML-resistant phenotype. The deleted region contains several bacteriocin-like and immunity-like ORFs, which could indicate that this region is a "resistance island" which at some point has provided the strain with resistance towards also other bacteriocins. We cloned and expressed several genes present in this region which were considered likely resistance factors, but were unable to restore resistance in the GarML-sensitive strain. Only six hypothetical proteins encoded in this region do not have functional orthologs in other bacteriocin-sensitive lactococcal strains, and at the same time have been demonstrably expressed in MG1363 under similar growth conditions. Neither of these potential resistance-genes have any known function. The results therefore indicate that a new, not yet described, mechanism is responsible for resistance to GarML in this lineage of *L. lactis* ssp. *cremoris* strains.

Introduction

Bacteriocins are ribosomally synthesized peptides or proteins of bacterial origin which display antimicrobial activity, most often against strains closely related to the producer (Nes *et al.*, 2007; Tagg *et al.*, 1976). Bacteriocins are frequently active at nanomolar concentrations (Breukink *et al.*, 1999) and in general act by pore formation or disruption of the integrity of the target cell membrane (Kjos *et al.*, 2011a).

Similar to the situation for antibiotics, resistance development upon exposure to bacteriocins is a relatively common occurrence in bacteria. However, the frequency of resistance development varies greatly, depending on both the type of bacteriocin and the sensitive strain, likely reflecting the different modes of action for antimicrobial activity (Gravesen *et al.*, 2002). There are also a few examples of intrinsic resistance to bacteriocins (Collins *et al.*, 2010; McBride and Sonenshein, 2011). A number of different mechanisms have been implicated in acquired and intrinsic resistance towards bacteriocins, both specific and non-specific.

In bacteriocins where a target receptor mediates antimicrobial activity, loss or reduced expression of the specific receptor has been shown to yield increased resistance to the corresponding bacteriocin. This is the case for class IIa bacteriocins and Lactococcin A, where loss or reduced expression of the man-PTS which functions as a target receptor causes resistance in *Li. monocytogenes* and *L. lactis* accordingly (Kjos *et al.*, 2011b).

Cell surface changes that in some way affect the interactions taking place between bacteriocin and cell surface or receptor may be another and more general mechanism causing resistance to bacteriocins. This has been shown to be the case for resistant cells of *Li. monocytogenes* against the class I lantibiotic nisin, where acquired resistance towards the bacteriocin has been correlated with changes in both altered fatty acid and phoshoplipid composition of the target cell membrane, which is thought to adversely affect how nisin interacts with the membrane (Crandall and Montville, 1998; Verheul *et al.*, 1997).

In some cases, bacteriocin immunity genes are found in non-producing strains (Fimland *et al.*, 2002), i.e. without a cognate bacteriocin operon. The presence and expression of functional immunity homologues in non-producing strains is called immune mimicry (Draper *et al.*, 2009). Immunity determinants providing resistance towards the two-peptide lantibiotic lacticin 3147 have for example been found not only in lacticin 3147-producing strains, but also in *Bacillus licheniformis* and *Enterococcus faecium* (Draper *et al.*, 2009).

Enzymatic activity degrading peptide bacteriocins and consequently leading to loss or decrease of activity is another mechanism by which bacteria could acquire resistance towards bacteriocins. Even so, there are not many reports of bacteriocin-resistance caused by enzymatic degradation. One known

example is however the extracellular gelatinase in *Enterococcus faecalis*, which has been shown to degrade class IIa bacteriocins. For the class I lantibiotic nisin, there are two known enzymes, nisin resistance protein (NRS)(Sun *et al.*, 2009) and nisinase (Jarvis, 1967), that specifically degrade/modify the C-terminal end of the peptide, leading to loss of activity and thus resistance towards the bacteriocin.

The presence of transporters which can export antimicrobial peptides out of the cell is yet another mechanism which can cause bacteriocin resistance. It is well known that transporters play a role in bacteriocin producer self-immunity for different classes of bacteriocins, including lantibiotics (Draper *et al.*, 2008) and circular bacteriocins (Kemperman *et al.*, 2003; Martinez-Bueno *et al.*, 1998). Different transporter complexes have also been shown to contribute to innate resistance of *Li. monocytogenes* to nisin, and of *C. difficile* to nisin and gallidermin (Collins *et al.*, 2010; McBride and Sonenshein, 2011).

In this work, we have investigated the genetic basis of inherent resistance to the circular bacteriocin garvicin ML (GarML) in a lineage of *Lactococcus lactis* ssp. *cremoris* strains. The resistance trait originates from a genomic region enriched in IS elements and putative plasmid-derived sequences, of which none of the implicated genes show any similarity to known bacteriocin resistance factors. We believe this to constitute a novel resistance mechanism, which contributes to the understanding of how dissemination of resistance factors leads to intraspecies variations in sensitivity to bacteriocins.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and their derivatives used in this study are listed in table 1. Lactococcal strains were routinely grown in M17 medium (Oxoid, Hampshire, U.K.) supplemented with 0.4 % (wt vol^{-1}) glucose at 30 °C. *E. coli* strains were grown in Lysogeny broth (LB) at 37 °C with 225 rpm shaking. Erythromycin or tetracycline was added at a final concentration of 5 μg ml^{-1} and chloramphenicol at a concentration of 10 μg ml^{-1} when required for selection in *Lactococcus*. Ampicillin was added at a final concentration of 100 μg ml^{-1}, kanamycin of 50 μg ml^{-1}, chloramphenicol of 30 μg ml^{-1} and tetracycline of 12.5 μg ml^{-1} when required for selection in *E. coli.*

Bacteriocin purification and assays

The bacteriocin Garvicin ML was concentrated from the supernatant of the producing strain *Lactococcus garvieae* DCC43 by precipitation with 45 % (wt vol^{-1}) ammonium sulfate. Bacteriocin sensitivity was determined using microtiter plate assays (MPA) as described previously (Gabrielsen *et al.*, 2012a), where one bacteriocin unit (BU) was defined as the amount of bacteriocin required to produce 50 % growth inhibition in 200 μl of culture. The agar well diffusion test (ADT) was also used for determination of antimicrobial activity. GM17 soft agar plates were prepared with the respective indicator strain and antibiotic (if required) added, and circular wells stamped out after solidification. 50 μl of the bacteriocin stock solution was then added to each well in the soft agar plate. The plates were incubated overnight at 30 °C and area of growth inhibition measured.

Genetic techniques

Transformations of *Lactococci* were performed as described by Holo And Nes (1989). Isolation of total DNA was performed using FP120 FastPrep bead-beater (BIO101/Savent) and E.Z.N.A.® Plasmid Miniprep Kit (Omega Bio-tek, Inc.). Plasmid isolation was carried out using E.Z.N.A.® Plasmid Miniprep Kit (Omega Bio-tek, Inc.) or QIAGEN Plasmid Midi Kit (QIAGEN) following enzymatic lysis with lysozyme (4 mg ml^{-1}) and mutanolysin (100 U ml^{-1}) at 37 °C for 30 minutes when required.

Cloning techniques were in general performed according to Sambrook and Russell (2001). Restriction enzymes, calf intestinal alkaline phosphatase and T4 DNA ligase (New England Biolabs Inc.) were used according to the supplier's instructions. Oligonucleotides were supplied by Invitrogen Life Technologies (Scotland, UK). PCR was performed using Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and PCR products were purified using NucleoSpin® Extract II (MACHEREY-NAGEL).

Creating spontaneously resistant mutants against Rifampicin and Streptomycin

L. lactis MG1363 was exposed to Streptomycin (200 μg ml^{-1}) and Rifampicin (100 μg ml^{-1}) sequentially to create spontaneously Rif^R and Str^R mutants. The resulting strains, S200, R100, S200R and R100S, were resistant to streptomycin, rifampicin, or both rifampicin and streptomycin accordingly.

Assaying extracellular protease activity

To assess extracellular protease activity from lactococcal strains, over night culture was spotted onto Todd-Hewitt, GM17 with 3% (w/v) gelatin and skim milk plates. After incubating for 30 °C over night, clear zones indicative of protease activity were measured around the colonies.

Genome sequencing and analysis

Genomic DNA was prepared from bacterial cultures using a Genomic-tip Kit (Quiagen) and used to generate libraries which were indexed and run on a single lane of an Illumina HiSeq 2000, with paired end 100 bp reads. The sequencing service was provided by the Norwegian Sequencing Centre (UiO). Sequence reads were mapped to the previously assembled *L. lactis* MG1363 genome sequence (GenBank accession no. AM406671) and filtered using the Galaxy (Blankenberg *et al.*, 2010) tools bowtie for illumina (Version 1.1.2) (Langmead *et al.*, 2009) and filter SAM (Version 1.0.0) accordingly. Artemis (Carver *et al.*, 2012) was used for visualization and analysis of read alignments, coverage and SNPs.

Creating GarML resistant deletionmutant of *L. lactis MG1363*

The flanking regions of the 27.7 Kb chromosomal deletion in *L. lactis* MG1614 were amplified by PCR using the primer pair Pse19-F1/0719-R1. The resulting 2 kb fragment was subsequently ligated into the SnaB1 site of pAS222 (Jonsson *et al.*, 2009), a shuttle vector based on the thermosensitive replicon of pG^+host4 (Maguin *et al.*, 1996), creating vector pCG18. The vector was subsequently transformed into *L. lactis* MG1363 and used for double-crossover homologous recombination. Selection for double-crossover events was performed as described previously (Biswas *et al.*, 1993). The double crossover was confirmed by PCR and sequencing with primers Pse19-F2 and Pse19-R1.

Cloning of pepO and putative bacteriocin immunity genes

The operons, i.e. the ORFs including native regulatory sequences, of llmg_0687-88, llmg_0704-05, llmg_0706 and *pepO* were amplified using primer pairs 0687-F/0688-R, 0704-F/0705-R, 0706-F/0706-R and pepO-F/pepO-R accordingly. PCR products were subcloned into the *E. coli* cloning vector pCR®-Blunt II-TOPO® (Invitrogen). The plasmids were cut with SmaI and subsequently fused with SmaI-cut and CIP-treated pNZ8037 (de Ruyter *et al.*, 1996), resulting in the *E. coli-L.lactis* fusion shuttle vectors pCG26, pCG27, pCG28 and pCG30. The empty control vector pCG22 was constructed similarly, but without the insert in pCR®-Blunt II-TOPO®.

Comparative genomic analysis

Comparative genomic analysis and assignment of orthologs of *Lactococcus* genomes was performed using BioCyc Pathway/Genome Databases and Pathway Tools Software (Karp *et al.*, 2005), specifically the comparative analysis tool.

Results

Discovery of a *L. lactis* genetic lineage that is resistant to the circular bacteriocin GarML

The circular bacteriocin GarML, produced from *Lactococcus garvieae* DCC43, has previously been shown to be highly active against lactococci (Borrero *et al.*, 2011). We have also shown that it is the maltose ABC transporter in *L. lactis* IL1403 that is involved in mediating sensitivity to this bacteriocin (Gabrielsen *et al.*, 2012a). By virtue of serendipity, upon screening different lactococcal strains for sensitivity towards GarML, we realized that strains originating from one genetic lineage showed an unexpectedly high MIC_{50} against this bacteriocin (table 1). These strains included *L. lactis* NCDO712, *L. lactis* MG1363 (a prophage and plasmid-cured derivative of NCDO712) and *L. lactis* NZ9000 (a MG1363 derivative with *nisRK* integrated into the *pepN* gene) (figure 1). Interestingly though, another strain of the same lineage, the spontaneously streptomycin- and rifampicin resistant strain *L. lactis* MG1614, displayed high level sensitivity to GarML (table 1). The difference in sensitivity was substantial, with strain MG1363 (and the strains from which it descends) displaying a MIC_{50} of ≥106.7 BU ml^{-1}, whereas MG1614 has a MIC_{50} of 5 BU ml^{-1}, constituting a 21-fold difference in sensitivity to this bacteriocin. We therefore hypothesized that *L. lactis* MG1363 and its progenitor strains have an intrinsic resistance mechanism towards GarML, which has been lost or inactivated in *L. lactis* MG1614, and proceeded to investigate the basis of this phenotype.

Resistance to GarML is not linked to spontaneous antibiotic resistance, loss of malABC or extracellular proteolytic activity

L. lactis MG1614 is a Rifampicin and Streptomycin resistant derivative of strain MG1363 (figure 1) (Gasson, 1983).To investigate whether the GarML-sensitivity of this strain is linked to the acquired antibiotic resistance, we exposed *L. lactis* MG1363 to Streptomycin (200 μg ml^{-1}) and Rifampicin (100 μg ml^{-1}) sequentially to create spontaneously Rif^R and/or Str^R mutants. These mutants (R100, S200, R100S, S200R) did however not show any significant change in sensitivity towards GarML (results not shown), indicating that the antibiotic resistance phenotype of *L. lactis* MG1614 is not directly linked to the GarML-sensitive phenotype.

In *L. lactis* IL1403, the maltose ABC transporter has been shown to facilitate high level sensitivity to GarML (Gabrielsen *et al.*, 2012a). The genes encoding the homologous maltose ABC transporter in MG1363 and MG1614 are however intact and the maltose metabolism functional in both strains (results not shown), excluding the possibility that loss or reduced expression of the putative receptor/mediator molecule could account for the GarML-resistant phenotype.

We also measured extracellular proteolytic activity from all the lactococcal strains on different substrates, as this could potentially account for resistance towards an antimicrobial peptide. However,

only low levels of protease activity was observed, for the two strains 712 and SK11 (results not shown) which are known to have plasmids encoding protease functions (Gasson, 1983; Liu *et al.*, 2010). No protease activity was observed for either of the plasmid-free strains MG1363, MG1614, NZ9000 or IL1403 (results not shown). Also, introduction of a protease-encoding plasmid, pGKV500 encoding the extracellular protease PrtP/PrtM, into *L. lactis* MG1614 did not have any effect on susceptibility to GarML (results not shown). We therefore concluded that any known extracellular proteolytic activity could not explain the observed resistance to GarML.

Resequencing the genomes of *L. lactis* MG1363 and MG1614 reveals a 27.7 kb chromosomal deletion

The genomes of *L. lactis* MG1363 and MG1614 were sequenced, to be able to pinpoint any genetic differences that could account for the observed GarML-resistant and –sensitive phenotypes of these strains accordingly. The sequence reads were mapped to the previously assembled *L. lactis* MG1363 genome sequence (Bolotin *et al.*, 2001), and SNPs and indels were called according to this sequence and to the later revised genome sequence (Linares *et al.*, 2010). There were only 23 new SNPs identified in our strains compared to the revised genome sequence (table 2), and of these 19 were intergenic. Comparing the two strains, only 13 SNPs differ between MG1363 and MG1614, and of these 10 are intragenic. Mutations identified in the *rpsL* and *rpoB* genes of *L. lactis* MG1614 are most likely the genetic basis for the acquired resistance to streptomycin and rifampicin accordingly (Jin and Gross, 1988; Springer *et al.*, 2001). In addition, a total of 12 insertions or deletions were found in the genomes of *L. lactis* MG1363 and MG1614 compared to the revised genome (table 2). Of these, 10 were shared between the two strains, and two were unique in MG1614. The first is a single nucleotide deletion in a gene encoding a conserved uncharacterized protein (llmg_0972). The most noticeable feature is however a chromosomal deletion having occurred at nt 675922-703641 in *L. lactis* MG1614. This is a 27.7 Kb large deletion (figure 2) encompassing no less than 35 complete and one truncated ORFs, the putative functions of which are listed in table 2. As reported previously, large chromosomal rearrangements in *Lactococcus* are common, often resulting from active multicopy insertion sequences, found widely distributed around the genomes (de Visser *et al.*, 2004). In *L. lactis* MG1363, a 59-kb region termed the “integration hot spot” is particularly enriched in IS elements and putative plasmid-derived sequences (Wegmann *et al.*, 2007), and it is precisely in the corresponding region that we find this chromosomal deletion in strain MG1614. The two IS981-related transposon elements (llmg_0684, llmg_0721) and associated helper protein (llmg_0720) (figure 2) immediately adjacent to the chromosomal deletion thus lead us to believe that this event is IS-mediated. This particular IS element has been shown to be active in *L. lactis* MG1363 (Bongers *et al.*, 2003), and we have previously reported a similar 13.5 Kb deletion in *L. lactis* IL1403

likely caused by the same type of element (Gabrielsen *et al.*, 2012a). However, this is as far as we know the largest deletion mediated by this type of IS element reported to date.

The chromosomal deletion in *L. lactis* MG1614 mediates sensitivity to GarML.

To determine if the large chromosomal deletion in *L. lactis* MG1614 was the cause of the GarML-sensitive phenotype, we created an isogenic double-crossover mutant of *L. lactis* MG1363, containing an identical deletion to that found in strain MG1614. This strain, DC01, was rendered equally sensitive to GarML as MG1614 (figure 3), thus establishing that the chromosomal deletion causes sensitivity to GarML, and implicating that the genetic basis of GarML-resistance in strain MG1363 is located in this region.

Of the 35 complete ORFs located in the deleted region, 14 are annotated as hypothetical proteins with no known function, 4 are transposon- or IS-related, and 2 are pseudogenes. Using BLAST (Altschul *et al.*, 1990) we were able to assign putative functions or domains to a few more of these (table 3). The region encodes, amongst other, an oligopeptide uptake system, an endopeptidase, two transcriptional regulator-like proteins, a putative heavy metal transport system and various other putative proteins with different functional domains. Interestingly, several of the hypothetical proteins displayed similarity to bacteriocin-associated genes. However, none of the ORFs display any significant similarity to the garvicin ML gene cluster (Gabrielsen *et al.*, 2012b).

Based on the bioinformatic analysis, we then proceeded to clone candidate genes and operons which were considered most likely to be involved in GarML resistance and express these in *L. lactis* DC01. The candidate genes/operons included llmg_0687-88, an operon consisting of a hypothetical protein containing a bacteriocin-type signal sequence and enterocin A immunity-like protein; llmg_0704-05, an operon containing a lactococcin-like protein and enterocin A immunity-like protein; llmg_0706, a enterocin A immunity-like protein. Furthermore, the *oppDFBCA* operon encoding an oligopeptide ABC transport system as well as the intracellular endopeptidase pepO was considered to be of interest. However, introducing these constructs into *L. lactis* DC01 did not yield any significant increase in resistance against GarML for any of the candidate genes/operons (figure 3).

Comparative analysis of the genes located in the deletion region of *L. lactis* MG1614 to other genome-sequenced *L. lactis* strains, i.e. *L. lactis* ssp. *lactis* IL1403 and *L. lactis* ssp. *cremoris* SK11, was undertaken to elucidate which of the genes did not have functional orthologs in bacteriocin-sensitive strains. The results show that 15 of the genes have orthologs in either one or both strains (table 3). Available microarray data of *L. lactis* MG1363 grown in GM17 (GEO accession: GSM542388) was then analyzed to determine which of these genes were being expressed under similar growth conditions. The data show that only 11 genes in this region are actually expressed during growth in GM17 medium

(Linares *et al.*, 2010). Of these, llmg_0687, llmg_0704 and llmg_0706 have already been excluded by complementation. This leaves 6 hypothetical proteins, which do not have functional orthologs in sensitive strains and are expressed in MG1363, that are the most likely candidates responsible for resistance to GarML (highlighted in table 3). Cloning fragments containing these genes however proved to be difficult, and as exhaustive attempts to produce these constructs failed, we are unable to pinpoint the exact gene or genes causing resistance to GarML in *L. lactis* MG1363.

Resistance to GarML in MG1614 does not confer resistance to other bacteriocins

In order to investigate whether the GarML-resistant phenotype was specific, we preceded to assay the MIC_{50} of the *L. lactis* ssp. *cremoris* lineage towards a range of other bacteriocins. The results show that GarML resistance is indeed specific, as the differences in sensitivity are not observed for other bacteriocins targeting lactococci such as lactococcin A, lactococcin B, nisin and circularin A (table 1).

Discussion

The investigation of resistance mechanism development is an important aspect of bacteriocin research. Only by studying these mechanisms can we in turn learn how to minimize development of resistance, which is essential to the applications of these peptides in both food industry and the potential future use in medical therapeutics. For many classes of bacteriocins, including the circular bacteriocins, there is however much we do not yet know about mechanisms of resistance.

The discovery that a lineage of *Lactococcus lactis* ssp. *cremoris* strains displayed resistance towards the circular bacteriocin GarML, but that a spontaneously streptomycin- and rifampicin resistant strain of the same lineage (figure 1) was highly sensitive, provided an excellent opportunity to investigate a putative resistance mechanism against GarML. We approached this question by testing the hypotheses that resistance was either caused by loss of the maltose ABC transporter which causes sensitivity to GarML in *L. lactis* (Gabrielsen *et al.*, 2012a) or by extracellular proteolytic activity. Based on the results, these hypotheses were however quickly discarded. Sequencing then revealed that, amongst a few other SNPs and indels (table 2), distinguishing these two strains were a 27.7 kb chromosomal deletion in the genome of *L. lactis* MG1614 (figure 2), and by recreating this deletion in MG1363, we were able to establish that this was indeed the cause of resistance (figure 3).

The bacteriocin-like and immunity-like ORFs (table 3) located in this "integration hot spot" in the genome are most likely plasmid-derived remnants of bacteriocin operons. The presence of several apparent bacteriocin-related traits could however indicate that this region constitutes a "resistance island", which at some point could have provided the strain resistance against multiple bacteriocins, and thus provided the strain with a competitive advantage. Indeed, we find putative orthologs of several of these ORFs also in other lactococci (table 3). The region also encodes an oligopeptide ABC transporter (*oppDFBCA*) and an endopeptidase (*pepO*), which are both required for growth in milk (Tynkkynen *et al.*, 1993a), and could explain the conservation of this region in this *L. lactis* ssp. *cremoris* lineage (Le Bourgeois *et al.*, 2000). Based on current knowledge of resistance mechanisms, these ORF immediately stood out as potential resistance factors. However, by complementation we eliminated the possible involvement of the bacteriocin-like and immunity-like ORFs (llmg_0687, llmg_0688, llmg_0704, llmg_0705 and llmg_0706) in addition to the oligopeptide ABC transporter and the endopeptidase in GarML resistance (figure 3).

Based on bioinformatic analysis we were able to rule out the involvement of even more ORFs in the deletion region (figure 3), and are left with six hypothetical proteins (highlighted in table 3) as the most likely candidates for providing GarML-resistance. Furthermore, as neither of the putative GarML-resistance genes display similarity to the genes required for immunity in the GarML producer strain (Gabrielsen *et al*. 2013), we can exclude the possibility of an immunity-like mechanism causing the

resistant phenotype. Thus, it is very likely that we are dealing with a new mechanism of resistance to this bacteriocin. Furthermore, this mechanism appears to be specific towards GarML, as sensitivity towards other bacteriocins targeting lactococci, including another circular bacteriocin, is not affected in these strains (table 1). For circular bacteriocins, very little is known about resistance mechanisms on the whole, which makes this an important contribution to the field. In addition, this is to our knowledge the first time a specific resistance mechanism has been identified which is transmitted in a lineage of strains in a different species from the producer.

This finding might have implications for a wide range of bacteriocins, as similar intraspecies variance in sensitivity to certain bacteriocin is quite common (Eijsink *et al.*, 1998). It has been speculated that this variation could be related to cell envelope composition, like changes in the D-alanylation of LTA to reduce the negative charge of the cell surface (Fabretti *et al.*, 2006; Kramer *et al.*, 2006). Another described mechanism is known as immune mimicry, which implies a close co-evolutionary development between the producer and the resistant strains. In addition, ABC-transporters functioning as efflux pumps can render certain strains less sensitive to bacteriocins, as the AnrAB transporter complex in *Li. monocytogenes* confers innate resistance to nisin (Collins *et al.*, 2010). None of these mechanisms however comply with the resistance identified in *L.lactis* towards GarML.

We have yet been unable to clone and express the putative resistance-genes, but ongoing efforts will hopefully not only pinpoint which gene or genes are actually responsible for GarML resistance, but further investigate the mechanism at play. Elucidating the mechanism could in turn allow for rational bioengineering of bacteriocins which are not targeted by this specific resistance mechanism. This knowledge is therefore important for the potential use of circular bacteriocins in general, and GarML specifically, in biotechnological or medical applications in the future.

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Figures and Tables

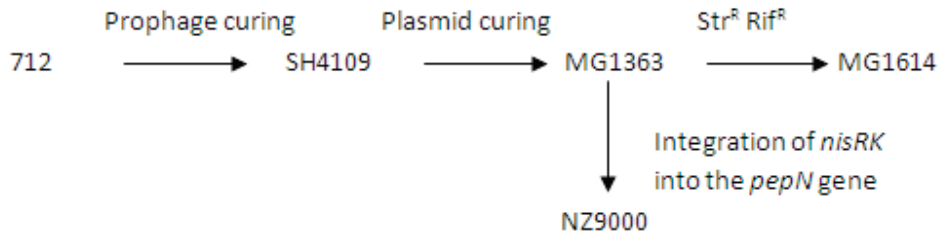

Figure 1: Lineage of *Lactococcus lactis* ssp. *cremoris* strains. The "mother" strain *L. lactis* ssp. *cremoris* NCDO 712 was cured of prophage φT712 resulting in strain SH4109. The latter was subsequently cured of plasmids by protoplast induction, creating strain MG1363. *L. lactis* MG1363 is a spontaneously resistant derivate of MG1363, while integration of *nisRK* into the *pepN* gene led to strain NZ9000.

Table 1: MIC50 of lactococci against different classes of bacteriocins: Nisin, bacteriocin RC714 (RC714), pediocin PA-1 (PA-1), lactococcin G (LcnG), lactococcin A (LcnA), garvicin ML (GarML) and circularin A (CirA).

Class		I	IIa	IIa	IIb	IId	IIc	
Bacteriocin		Nisin	RC714	PA-1	LcnG	LcnA	GarML	CirA
L. lactis	MG1363	10.0	0.0	0.0	6.7	1.7	106.7	6.7
L. lactis	MG1614	5.0	0.0	0.0	5.0	5.0	5.0	5.0
L. lactis	MG1614V	5.0	0.0	0.0	6.7	5.0	6.7	5.0
L. lactis	NCDO712	10.0	0.0	0.0	6.7	3.3	106.7	6.7
L. lactis	NZ9000	10.0	0.0	0.0	3.3	5.0	213.3	6.7
L. lactis	IL1403	6.7	0.0	0.0	1.7	0.8	3.3	6.7
L. lactis	SK11	5.0	0.0	0.0	ND	ND	5.0	5.0

Table 2: SNPs and indels identified in genomes of *L. lactis* MG1363 and MG1614, compared to the revised reference genome of *L. lactis* MG1363.

Region and function					Change		Presence in genome		
Ref pos (start/end)		Var type	Locus tag or gene	Putative Function	Nt	AA/FS	Rev ref	MG 1363	MG 1614
174861	-	SNP	intergenic	-	G->A	-	-	-	+
446867	-	SNP	intergenic	-	G->A	-	-	+	-
674549	-	SNP	llmg_0683	DNA replication protein	C->T	syn	-	+	+
678000	-	SNP	llmg_0687	Hyp. Protein with bacteriocin-type signal sequence	A->G	Ile->Val	-	+	+
678021	-	SNP	llmg_0687	Hyp. Protein with bacteriocin-type signal sequence	G->A	Glu->Lys	-	+	+
848833	-	SNP	llmg_0877	Similar to Immunoglobulin A1 protease precursor	C->T	syn	-	+	+
848837	-	SNP	llmg_0877	Similar to Immunoglobulin A1 protease precursor	C->T	Ser->Pro	-	+	+
946308	-	SNP	llmg_0983	Conserved hypothetical protein	G->A	Gly->Arg	-	+	+
946323	-	SNP	llmg_0983	Conserved hypothetical protein	C->T	Arg->Trp	-	+	+
970845	-	SNP	llmg_1005	Transc. reg/sugar kinase	C->A	Ala->Asp	-	+	-
1140716	-	SNP	llmg_1174	Hypothetical membrane protein	T->C	syn	-	+	+
1140746	-	SNP	llmg_1174	Hypothetical membrane protein	G->C	Glu->Gln	-	+	+
1167414	-	SNP	intergenic	-	T->G	-	+	-	+
1250809	-	SNP	ilvD	Dihydroxyacid dehydratase/phosphogluconate dehydratase	A->G	Lys->Glu	-	-	+
1791986	-	SNP	trmB	S-adenosylmethionine-dependent methyltransferase	T->G	Asp->Ala	-	-	+
1801013	-	SNP	thrA	Aspartokinases	A->G	syn	-	-	+
1968674	-	SNP	rpoB	DNA-directed RNA polymerase beta subunit	G->A	Ser->Phe	-	-	+
2000518	-	SNP	thiN	Thiamine pyrophosphokinase	C->A	Ala->Ser	-	-	+
2191553	-	SNP	recF	Recombinational DNA repair ATPase	G->A	Ala->Thr	-	-	+
2220880	-	SNP	intergenic	-	T->G	-	-	+	+
2456031	-	SNP	llmg_2500	Predicted hydrolase of the HAD superfamily	T->C	Lys->Arg	-	-	+
2493398	-	SNP	gapB	glyceraldehyde 3-phosphate dehydrogenase	A->C	Leu->Arg	-	-	+
2517498	-	SNP	rpsL	Ribosomal protein S12	T->C	Lys->Arg	-	-	+
636586	-	Del	llmg_0642	Conserved hypothetical protein	A->-	FS	-	+	+
670704	670705	Del	llmg_pseudo_18	-	GA->--	-	-	+	+
675922	703641	Del	llmg_0685	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0686	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0687	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0688	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0689	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0690	Cation transport ATPase	-	Loss	-	-	+
675922	703641	Del	llmg_0691	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0692	Contains Heavy-Metal-Associated (HMA) domain	-	Loss	-	-	+
675922	703641	Del	llmg_0693	Ferritin/ribonucleotide reductase-like	-	Loss	-	-	+

Region and function					Change		Presence in genome		
Ref pos (start/end)		Var type	Locus tag or gene	Putative Function	Nt	AA/FS	Rev ref	MG 1363	MG 1614
675922	703641	Del	llmg_0695	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0696	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0697	oligopeptide transport ATP-binding protein oppD	-	Loss	-	-	+
675922	703641	Del	llmg_0698	oligopeptide transport ATP-binding protein oppF	-	Loss	-	-	+
675922	703641	Del	llmg_0699	peptide transport system permease oppB	-	Loss	-	-	+
675922	703641	Del	llmg_0700	peptide transport system permease oppC	-	Loss	-	-	+
675922	703641	Del	llmg_0701	oligopeptide-binding protein oppA	-	Loss	-	-	+
675922	703641	Del	llmg_0702	endopeptidase O	-	Loss	-	-	+
675922	703641	Del	llmg_0703	tnp1675	-	Loss	-	-	+
675922	703641	Del	llmg_0704	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0705	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0706	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0707	tmp6	-	Loss	-	-	+
675922	703641	Del	llmg_0708	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0709	PadR-like family transcriptional regulator	-	Loss	-	-	+
675922	703641	Del	llmg_0710	Conserved hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0711	DNA-invertase/resolvase tnpR	-	Loss	-	-	+
675922	703641	Del	llmg_0712	Conserved hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0713	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0714	tnp981	-	Loss	-	-	+
675922	703641	Del	llmg_0715	pseudo_20	-	Loss	-	-	+
675922	703641	Del	llmg_0716	Hypothetical protein	-	Loss	-	-	+
675922	703641	Del	llmg_0717	tnp946	-	Loss	-	-	+
675922	703641	Del	pseudo_21	tnp712	-	Loss	-	-	+
675922	703641	Del	llmg_0718	nucleotide binding protein	-	Loss	-	-	+
675922	703641	Del	llmg_0719	ABC transporter substrate-binding protein oppA	-	Trunk	-	-	+
936740	-	Del	llmg_0972	Uncharacterized conserved protein	-->A	FS	-	-	+
1223555	1223558	Del	intergenic	-	GTGA->----	-	-	+	+
1318542	-	Del	llmg_1344	Hypothetical protein	A->-	FS	-	+	+
1660057	-	Del	intergenic	-	-->T	-	-	+	+
1826459	-	Del	intergenic	-	-->T	-	-	+	+
1921437	1921523	Del	llmg_1941	dedA family membrane-associated protein	-	trunc	-	+	+
1921643	1921785	Del	intergenic	-	-	-	-	+	+
2112675	-	Del	intergenic	-	-->T	-	-	+	+
2183058	-	Del	llmg_2218	Transcriptional activator Rgg/GadR/MutR	A->-	FS	-	+	+

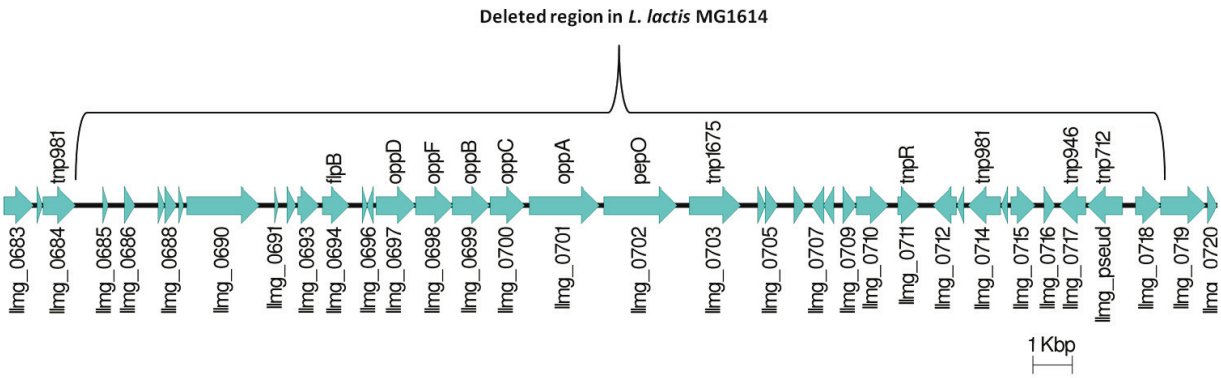

Figure 2: The genomic deletion in *L. lactis* MG1614, corresponding to nt 675922-703641 in the genome of *L. lactis* MG1363 (GenBank accession AM406671), is indicated by the brace. Gene names of annotated genes are given above the sequence and locus tags below.

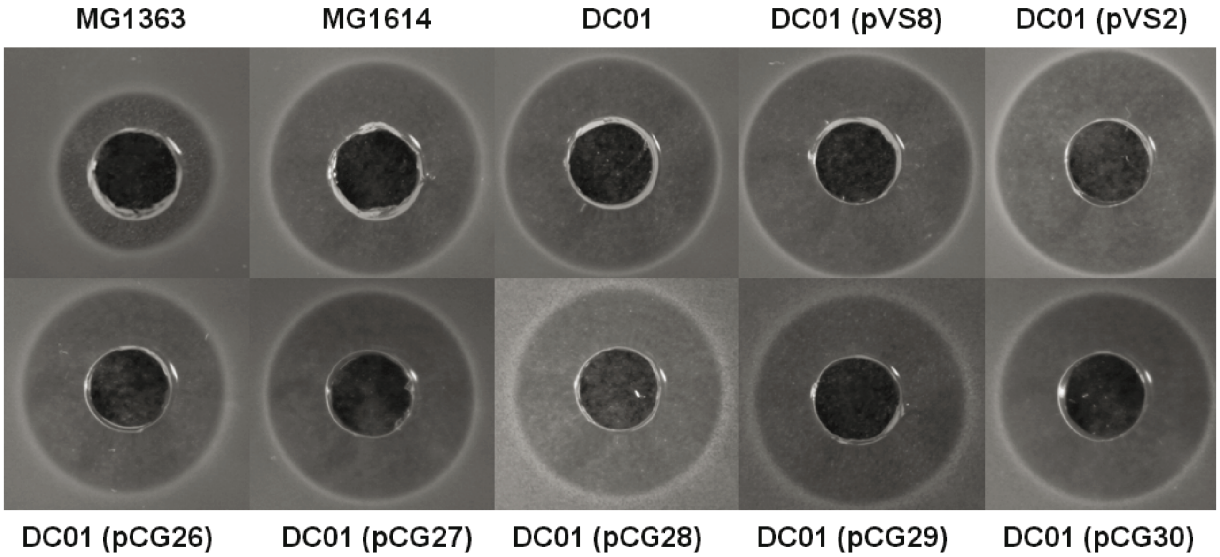

Figure 3: Agar diffusion tests of wild type strains MG1363 and MG1614, isogenic deletion mutant DC01 (Δ27.7 Kb) and DC01 complemented with plasmid constructs pVS8 (encoding *oppDFBCA* and *pepO*) pVS2 (empty vector control), pCG26 (encoding llmg_0687 and llmg_0688), pCG27 (encoding llmg_0704 and llmg_0705), pCG28 (encoding llmg_0706), pCG30 (encoding *pepO*) and pCG22 (empty control vector) against 50x concentrated GarML.

Table 3: Annotation, domains, orthologs and expression of ORFs encoded in the deletion region in *L. lactis* MG1363

Locus tag	Annotation and functional/domain prediction by similarity		Functional orthologs		Expression in *L. lactis* MG1363
	Annotation	Function (BLAST)	*L. lactis* SK11	*L. lactis* Il1403	
llmg_0685	Hypothetical protein	-			
llmg_0686	Hypothetical protein	-	Inferred gene	yaiI	+
llmg_0687	Hypothetical protein	Contains bacteriocin-type signal sequence			+
llmg_0688	Hypothetical protein	EntA immunity (Similarity to 0705/0706)		yajH	
llmg_0689	Hypothetical protein	-			
llmg_0690	Cation transport ATPase	Heavy metal-translocating P-type ATPase		yuiA	
llmg_0691	Hypothetical protein	-			
llmg_0692	Contains Heavy-Metal-Associated (HMA) domain	Heavy metal transport or detoxification protein	LACR_1043	yvfA	+
llmg_0693	Ferritin/ribonucleotide reductase-like	Stress induced DNA binding protein	LACR_1044		
llmg_0694	Transcriptional regulator FNR like protein B (flpB)	Activator and regulatory subunit of cAMP-dependent protein kinases			
llmg_0695	Hypothetical protein	-			+
llmg_0696	Hypothetical protein	-			+
llmg_0697	oligopeptide transport ATP-binding protein oppD	ABC-type transport system, ATPase	LACR_2030	oppD	
llmg_0698	oligopeptide transport ATP-binding protein oppF	ABC-type transport system, ATPase	LACR_2029	oppF	
llmg_0699	peptide transport system permease oppB	ABC-type transport systems, permease	LACR_2028	oppB	
llmg_0700	peptide transport system permease oppC	ABC-type transport systems, permease	LACR_2027	oppC	
llmg_0701	oligopeptide-binding protein oppA	ABC-type transport system,periplasmic	LACR_2026	oppA	
llmg_0702	endopeptidase O	Metalloendopeptidase	LACR_1984	pepO	
llmg_0703	tnp1675	Transposase DDE domain			
llmg_0704	Hypothetical protein	Lactococcin-like	LACR_1834		+
llmg_0705	Hypothetical protein	EntA immunity			
llmg_0706	Hypothetical protein	EntA immunity		L200065	+
llmg_0707	tmp6	L. lactis partial transmembrane protein Tmp6			+
llmg_0708	Hypothetical protein	Protein with DUF2089			+
llmg_0709	PadR-like transcriptional regulator	Predicted transcriptional regulator			
llmg_0710	Conserved hypothetical protein	Protein with DUF1129			
llmg_0711	DNA-invertase/resolvase tnpR	Site-specific recombinase	Inferred gene		
llmg_0712	Conserved hypothetical protein	-			
llmg_0713	Hypothetical protein	-			+
llmg_0714	tnp981	transposase for IS981F			
llmg_0715	pseudo_20				
llmg_0716	Hypothetical protein	-			+
llmg_0717	tnp946	transposase for IS946			
pseudo_21	tnp712	-			
llmg_0718	nucleotide binding protein	Dinucleotide-utilizing enzyme	LACR_0354		
llmg_0719	ABC transporter substrate-binding protein oppA	ABC transporter Substrate-binding			

Supplementary Information

Table S1: Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Relevant characteristics[b]	Source or reference
Lactococcus lactis ssp. cremoris		
NCDO 712	Starter culture, full plasmid complement	NCDO[c]
MG1363	Prophage and plasmid-cured derivative of NCDO 712	Gasson (1983)
R100	Rif^R derivative of MG1363	This work
S200	Str^R derivative of MG1363	This work
R100S	Rif^R derivative of R100	This work
S200R	Str^R derivative of S200	This work
DC01	Double-crossover deletion mutant (27.7 Kb) of MG1363	This work
NZ9000	MG1363 derivative with *nisRK* integrated into the *pepN* gene	Kuipers *et al.*(1998)
MG1614	Spontaneous Rif^R and Str^R derivative of MG1363	Gasson (1983)
MG1614V	Spontaneous oligopeptide transport deficient mutant of MG1614	Tynkkynen *et al*. (1993b)
SK11	Phage-resistant derivative of AM1	Jarvis and Wolff (1979)
Lactococcus lactis ssp. lactis IL1403	Plasmid-free derivative of IL594	Chopin *et al.*(1984)
Lactococcus garvieae DCC43	Producer strain of GarML	Sanchez *et al.*(2007)
Plasmids		
pAS222	Shuttle vector based on the thermo-sensitive replicon of pG^+host4. *Tet*^{r}	Jonsson *et al.*(2009)
pCR-Blunt II-TOPO	*E. coli* cloning vector. *Kan*^{r}	Invitrogen Life Technologies
pNZ8037	Lactococcus expression vector. *Cam*^{r}	de Ruyter *et al*.(1996)
pGKV500	Protease-encoding plasmid. *Erm*^{r}	Kok *et al.*(1985)
pCG18	pAS222 derived deletion vector	This work
pCG22	Fusion vector consisting of pCR-Blunt II-TOPO and pNZ8037. *Cam*^{r} *Kan*^{r}	This work
pCG26	Fusion vector containing llmg_0687 and llmg_0688.	This work
pCG27	Fusion vector containing llmg_0704 and llmg_0705.	This work
pCG28	Fusion vector containing llmg_0706.	This work
pCG30	Fusion vector containing *pepO*.	This work
pVS2	Lactococcus cloning vector. *Cam*^{r}	von Wright *et al.*(1987)
pVS8	pVS2-derived vector containing *oppDFBCA*and *pepO*	von Wright *et al.*(1987)

[a] The subspecies (*ssp*.) designations are based on the genomic lineages of the respective strains, not the phenotypic classification.
[b] *Cam*^{r}, chloramphenicol resistance; *Tet*^{r}, tetracycline resistance; *Amp*^{r}, ampicillin resistance; *Erm*^{r}, erythromycin resistance; Rif^R, rifampicin resistance; Str^R, streptomycin resistance; *Kan*^{r}, kanamycin resistance; MCS, multiple cloning site.
[C] National Collection of Industrial, food and Marine Bacteria (NCIMB), Aberdeen, Scotland, transferred from the National Collection of Food Bacteria (NCFB), previously the National Collection of Dairy Organisms (NCDO).