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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Sensitivity to the two-peptide bacteriocin lactococcin G is dependent on UppP, an enzyme involved in cell-wall synthesis

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Summary

**Most bacterially produced antimicrobial peptides
(bacteriocins) are thought to kill target cells by a
receptor-mediated mechanism. However, for most
bacteriocins the receptor is unknown. For instance, no
target receptor has been identified for the two-peptide
bacteriocins (class IIb), whose activity requires the
combined action of two individual peptides. To identify
the receptor for the class IIb bacteriocin lactococcin
G, which targets strains of *Lactococcus lactis*, we
generated 12 lactococcin G-resistant mutants and per-
formed whole-genome sequencing to identify muta-
tions causing the resistant phenotype. Remarkably, all
had a mutation in or near the gene *uppP* (*bacA*), encod-
ing an undecaprenyl pyrophosphate phosphatase; a
membrane protein involved in peptidoglycan synthe-
sis. Nine mutants had stop codons or frameshifts in
the *uppP* gene, two had point mutations in putative
regulatory regions and one caused an amino acid
substitution in UppP. To verify the receptor function of
UppP, it was shown that growth of non-sensitive *Strep-
tococcus pneumoniae* could be inhibited by lactococ-
cin G when *L. lactis uppP* was expressed in this
bacterium. Furthermore, we show that the related**

**class IIb bacteriocin enterocin 1071 also uses UppP as
receptor. The approach used here should be broadly
applicable to identify receptors for other bacteriocins
as well.**

Introduction

Lactic acid bacteria (LAB) produce a large variety of bac-
teriocins (ribosomally synthesized antimicrobial peptides
from bacteria), which kill target bacteria by permeabilizing
their plasma membrane (Nissen-Meyer *et al.*, 2009; Cotter
et al., 2013). Many of these bacteriocins do not, however,
act directly on the membrane lipids, but rather on specific
membrane proteins, i.e. via a receptor-mediated process
(Ramnath *et al.*, 2000; Héchard and Sahl, 2002; Diep
et al., 2007; Gabrielsen *et al.*, 2012; Uzelac *et al.*, 2013).
Particularly the unmodified bacteriocins of class II [com-
prising the pediocin-like bacteriocins (class IIa), the two-
peptide bacteriocins (class IIb), the cyclic bacteriocins
(class IIc) and the linear non-pediocin-like bacteriocins
(class IId) (Cotter *et al.*, 2013)] seem to require such mem-
brane localized proteins for their activity. The nature of the
pore formed after bacteriocin-receptor binding is not char-
acterized, but in some cases circumstantial evidence sug-
gests that binding of the bacteriocin to the receptor may
change the conformation of the receptor, which then leads
to membrane leakage (Diep *et al.*, 2007; Kjos *et al.*, 2010).
Through this receptor-mediated mode of action, bacterioc-
ins show antimicrobial activity at very low – nanomolar to
picomolar – concentrations, and the specific receptor rec-
ognition also determines the inhibitory spectra of the dif-
ferent bacteriocins.

In order to protect themselves from self-killing,
bacteriocin-synthesizing cells produce immunity proteins.
In some cases, such immunity proteins have been
shown to prevent membrane leakage by binding to the
bacteriocin-receptor complex (Diep *et al.*, 2007). A detailed
understanding of the mode of action of bacteriocins
will thus involve delineating the molecular interactions
between a bacteriocin, its receptor and the immunity
protein. A bacteriocin's cognate immunity protein is gener-
ally encoded in the same operon as the bacteriocin (van

Belkum *et al.*, 1991; Cotter *et al.*, 2013) and can thus be easily identified by DNA sequencing and analysis of the operon region. Identification of the receptor on target cells is, on the other hand, not trivial, and only a few such bacteriocin receptors have been identified to date. The membrane-embedded IIC and IID subunits of the mannose phosphotransferase system have been shown to act as receptor for the pediocin-like (class IIa) bacteriocins, first circumstantially by genetic analysis (Ramnath *et al.*, 2000; 2004; Gravesen *et al.*, 2002; Héchard and Sahl, 2002) and then conclusively by immunoprecipitation where a physical association between these bacteriocins, their immunity proteins and the IIC and IID subunits of the mannose phosphotransferase was established (Diep *et al.*, 2007). Interestingly, the mannose phosphotransferase system also functions as the receptor for lactococcin A, a class IId LAB bacteriocin that is very different from the pediocin-like bacteriocins (Diep *et al.*, 2007), as well as for microcin E492, a bacteriocin targeting various *Enterobacteriaceae* species (Bieler *et al.*, 2006). Membrane-located target proteins have also been identified for the class IIc bacteriocin garvicin ML and the class IId bacteriocin LsbB; the former targets the maltose ABC transporter (Gabrielsen *et al.*, 2012) and the latter targets a metallopeptidase known as YvjB (Uzelac *et al.*, 2013). Due to their narrow inhibitory spectrum and high potency, it has been hypothesized that even the two-peptide (class IIb) bacteriocins target specific proteins in the membrane of sensitive bacteria (Nissen-Meyer *et al.*, 2010), but no receptor has yet been identified for any of these bacteriocins.

Lactococcin G was the first two-peptide bacteriocin to be isolated (Nissen-Meyer *et al.*, 1992) and is presently the best-characterized bacteriocin in class IIb. The class now includes at least 15 other bacteriocins, among these are enterocin 1071 (Balla *et al.*, 2000; Franz *et al.*, 2002; Balla and Dicks, 2005) and lactococcin Q (Zendo *et al.*, 2006) that have, respectively, 57% and 88% sequence identity with lactococcin G (Oppegård *et al.*, 2007). As the name indicates, all two-peptide (class IIb) bacteriocins consist of two different peptides whose genes are located next to each other in the same operon, and both peptides must be present in approximately equal molar amounts to obtain optimal antibacterial activity (Moll *et al.*, 1996; 1998; 1999; reviewed in Oppegård *et al.*, 2007; Nissen-Meyer *et al.*, 2010). Lactococcin G consists of the 39-residue alpha-peptide and the 35-residue beta-peptide (Nissen-Meyer *et al.*, 1992). These two peptides interact upon exposure to membrane-like entities (Hildeng-Hauge *et al.*, 1998) and appear to form a membrane-penetrating helix–helix structure stabilized by helix–helix-interacting GxxxG-motifs upon contact with sensitive bacteria (Oppegård *et al.*, 2008; 2010; Rogne *et al.*, 2008). This motif is found in all two-peptide bacteriocins that have currently

been sequenced (Nissen-Meyer *et al.*, 2010), and it has been proposed that the two peptides of many, if not all, two-peptide bacteriocins form a membrane-penetrating helix–helix structure that interacts with an integrated membrane protein in sensitive bacteria. Attempts to identify such a target protein for lactococcin G by utilizing a similar immunoprecipitation approach as used to identify the receptors for lactococcin A and the pediocin-like bacteriocins have, however, not succeeded (D.B. Diep *et al.*, unpubl. results).

Here we present an alternative way of identifying putative bacteriocin receptors by the use of whole-genome sequencing of spontaneous mutants of sensitive cells that have gained resistance to lactococcin G and enterocin 1071. Genome comparisons of wild-type *L. lactis* with 12 independent mutants resulted in an unequivocal identification of UppP/BacA, a undecaprenyl pyrophosphate phosphatase involved in cell-wall synthesis, as the putative receptor for these two bacteriocins. Heterologous expression of *uppP* in the non-sensitive *Streptococcus pneumoniae* turned this bacterium sensitive to both lactococcin G and enterocin 1071. The approach of whole-genome sequencing of bacteriocin-resistant mutants combined with heterologous expression should be a widely applicable method for identification of receptors also for other bacteriocins.

Results

Generation of lactococcin G-resistant mutants

An approach which previously has been successful for identification of bacteriocin receptors involves generation of resistant mutants and subsequent phenotypic (sugar fermentation profiling) and genotypic (PCR and DNA sequencing) analyses to identify the alterations responsible for the acquired resistance (Gabrielsen *et al.*, 2012). Therefore, we generated lactococcin G-resistant strains by exposing sensitive strains of *Lactococcus lactis* (IL1403 and MG1363) to lactococcin G on GM17 agar plates. Ten lactococcin G-resistant mutants of *L. lactis* IL1403 and two lactococcin G-resistant mutants of *L. lactis* subsp. *cremoris* MG1363 were isolated (see *Experimental procedures* and Table 1). Seven out of the 10 mutant IL1403 strains were resistant to the highest lactococcin G concentration tested (Table 1). The remaining three strains (IL3, IL5 and IL7) were less resistant, but still highly resistant compared to the wild-type strain. The two MG1363-derived mutant strains were both resistant to the highest lactococcin G concentration tested (Table 1).

All the mutant strains were also assayed against the lactococcin G-like two-peptide bacteriocin enterocin 1071, which has 57% sequence identity with lactococcin G.

Table 1. Spontaneous mutants resistant to lactococcin G (LcnG) and enterocin 1071 (Ent1071).

Strain	MIC (nM) ± st.dev		Fold increase in resistance (MIC$_{mut}$/MIC$_{wt}$)		Position of mutation in Genbank sequence	Genetic consequence of mutation
	LcnG	Ent1071	LcnG	Ent1071		
IL1403 (wt)	0.01 ± 0.01	0.05 ± 0.01	1	1	–	–
IL1	> 25	> 25	> 2500	> 500	2 275 477	GAA to TAA: E191Stop in *uppP*
IL2	> 25	> 25	> 2500	> 500	2 275 894	GGA to TGA: G53Stop in *uppP*
IL3	20 ± 2	> 25	2000	> 500	2 276 047	GAT to AAT: D2N in *uppP*
IL4	> 25	> 25	> 2500	> 500	2 275 631	TGG to TGA: W140Stop in *uppP*
IL5	7 ± 3	3 ± 1	700	60	2 276 128	T to G 77 nucleotides upstream of translation start in *uppP*
IL6	> 25	> 25	> 2500	> 500	2 276 059	G to T 9 nucleotides upstream of translation start in *uppP*
IL7	18 ± 1	> 25	1800	> 500	2 275 292	TAT to TAG: Y253Stop in *uppP*
IL8	> 25	> 25	> 2500	> 500	2 275 369	CAA to TAA: Q228Stop in *uppP*
IL11	> 25	> 25	> 2500	> 500	2 275 812	ACC to A-C: frameshift at T80 in *uppP* leading to loss of 199 amino acids
IL12	> 25	> 25	> 2500	> 500	2 275 997	TGG to TGA: W18Stop in *uppP*
MG1363 (wt)	0.02 ± 0.01	0.11 ± 0.03	1	1	–	–
MG1	> 25	> 25	> 1250	> 225	2 432 943	CAA to C-A: frameshift at Q85 in *bacA* leading to loss of 199 amino acids
MG2	> 25	> 25	> 1250	> 225	2 432 965 684 550	CAA to TAA: Q78Stop in *bacA* GAA to AAA: E338K in *oppD*

MIC values, fold increase of bacteriocin resistance, position of differences relative to the Genbank reference genome (NC_002662) and genetic consequence of mutation are indicated.

Overall, the degree of resistance to enterocin 1071 was similar to what was observed for lactococcin G (Table 1); all the mutants were resistant to the highest enterocin 1071 concentration tested, except the strain IL5. These results suggest that the same genetic determinant(s) are involved in the sensitivity to lactococcin G and the related bacteriocin enterocin 1071.

Lactococcin G-resistance does not cause changes in carbohydrate fermentation

Several class II bacteriocins have been shown to target sugar transporters in the membrane of sensitive cells and mutants resistant to such bacteriocins show an altered sugar fermentation pattern compared to their wild-type counterparts (Kjos *et al.*, 2011; Gabrielsen *et al.*, 2012). In the regular growth medium (M17 supplemented with glucose), the growth rate of the resistant mutants was similar to that of the wild-type cells, except for a slightly extended lag phase for the mutants (Fig. 1). In order to examine if the mutants differed in growth on other sugars, we compared the carbohydrate fermentation profiles of the wild-type and two resistant mutants (IL11 and IL12) using the API fermentation kit, which allows us to test 49 different carbohydrates simultaneously. However, no differences were observed between the wild-type and the resistant mutants, suggesting that, unlike several other class II bacteriocins (pediocin-like, lactococcin A, garvicin ML), the target receptor for lactococcin G is probably not involved in any sugar metabolism pathway.

Lactococcin G-resistant mutants contain mutations in a gene coding for a membrane protein involved in peptidoglycan synthesis

Since we did not observe any clear phenotype of our lactococcin G-resistant mutants, we performed whole-genome sequencing of the resistant mutants to see if we could identify bacteriocin receptors based on mutations in the genome. As a proof of principle, we first tested this method on an already characterized bacteriocin system; lactococcin A is one of the class II bacteriocins for which the receptor proteins in the membrane of target cells have already been identified, namely the IIC and IID subunits of the mannose phosphotransferase system (Diep *et al.*, 2007; Kjos *et al.*, 2009). Two mutants of *L. lactis* IL1403 that are partially resistant to lactococcin A (30-fold increased resistance) were isolated and whole-genome sequencing was performed. Remarkably, the only change from the wild-type was that each of the mutants contained one mutation in the receptor genes (one in *ptnC* resulting in the substitution G188A in the mannose phosphotransferase IIC subunit and the other in *ptnD* resulting in P115H in the IID subunit, see *Supporting information* for details), thus clearly demonstrating that whole-genome sequencing of resistant mutants can be used to pinpoint bacteriocin receptors.

Knowing that bacteriocin receptors can be identified using this method, we sequenced all the 10 lactococcin G-resistant mutants derived from the sensitive wild-type strain *L. lactis* IL1403 as well as the two mutants derived
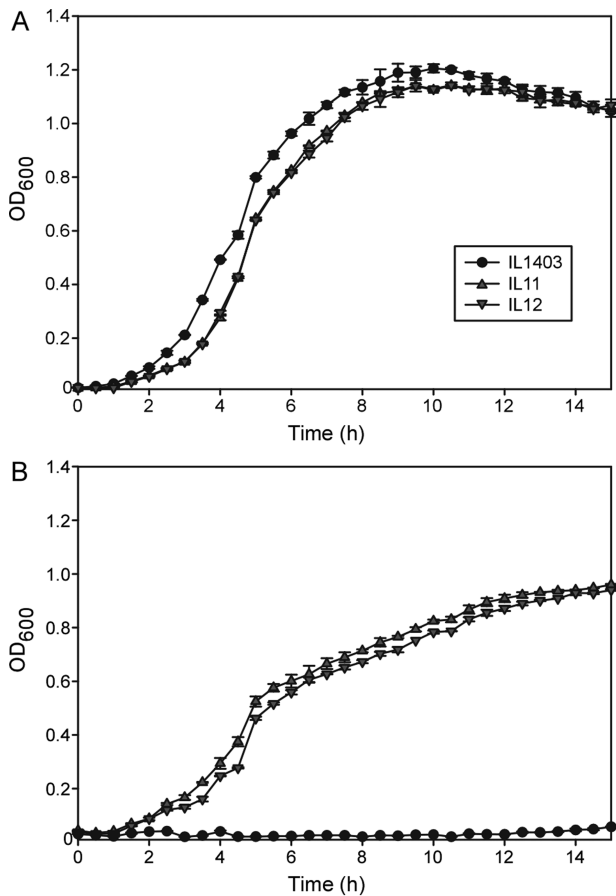
Fig. 1. Growth of *L. lactis* IL1403 and the derived lactococcin G-resistant mutants IL11 and IL12 in normal growth medium GM17 (A) and in GM17 containing lactococcin G (amount corresponding to 20× wild-type MIC) (B).

from the sensitive strain *L. lactis* MG1363 (about 18 million reads per genome, 2×100 nt paired-end reads). Both of the wild-type strains (IL1403 and MG1363) were also sequenced to identify differences between our lab strains and the sequences in the databases. Indeed, our sequence analysis detected 170 differences between our wild-type IL1403 strain and the Genbank sequence NC_002662 (Table S3). Our MG1363 wild-type strain differed from the Genbank reference sequence (NC_009004) in 75 positions (Table S4). Most of these differences probably represent real changes that have accumulated in the strains during the time they have spent in different labs (Fig. S3), although some differences may represent sequencing errors in the database sequence (see *Supporting information* for details).

The genome sequences of the lactococcin G-resistant strains showed that each of the 10 resistant strains derived from IL1403 contained one single point mutation in their genomes compared to the wild-type reference. Most notably, all of these point mutations were located in or near the gene *uppP* (locus tag L58643) encoding undecaprenyl

pyrophosphate phosphatase, UppP, a membrane located protein involved in peptidoglycan synthesis (El Ghachi *et al.*, 2004). Furthermore, each of the two lactococcin G-resistant mutants derived from strain MG1363 (MG1 and MG2) also contained a single point mutation in the orthologous gene encoding undecaprenyl pyrophosphate phosphatase (named *bacA* in the annotated MG1363 genome sequence, locus tag llmg_2476). The proteins from IL1403 (UppP) and MG1363 (BacA) are 96.8% identical. In addition to the mutations in *bacA*, one of the resistant strains (MG2) had a codon change in the penultimate position of *oppD* (locus-tag llmg_0697), the ATPase component of an ABC-type dipeptide/oligopeptide/nickel transport system. However, it is very unlikely that this mutation is linked with lactococcin G resistance, since this gene was intact in the other resistant mutants. The mutations in the resistant strains are summarized in Table 1. In nine out of the 12 mutants, the *uppP/bacA* gene was truncated, due to either insertions/deletions causing frameshifts or introduction of stop codons within the gene. One of the strains contained a single point mutation inside *uppP* (IL3), while two strains (IL5 and IL6) contained mutations in the putative regulatory region of *uppP*. The position of the truncations relative to the predicted transmembrane helices and intra- and extracellular regions of UppP are shown in Fig. 2A, and the positions of the latter three mutations in the *uppP* upstream sequence are shown in Fig. 2B.

The transcription of uppP is reduced in the mutant strain IL5

The expression of *uppP* in the two mutants harbouring mutations in putative regulatory regions of *uppP* (IL5 and IL6) and the mutant with the point mutation leading to a D2N substitution in UppP (IL3) was analysed by quantitative PCR (qPCR). When compared to the wild-type strain, no significant change in the transcription level of *uppP* was observed in IL3 and IL6. In IL5, however, a 20-fold reduction in *uppP* expression was observed (Fig. 3). Presence (0.5 nM) or absence of lactococcin G had no significant effect on *uppP* expression in any of the strains (results not shown).

Inactivation of uppP does not have a major impact on cell growth and sensitivity to bacitracin and nisin

As mentioned above, the growth rate of UppP-inactivated mutants was similar to that of wild-type cells, except for a slightly extended lag phase for the mutants (Fig. 1). Also, no differences between wild-type and mutants were observed with phase-contrast microscopy (data not shown). Moreover, inactivation of UppP did not have a major impact on the sensitivity of the cells to bacitracin, an antibiotic that targets the substrate of UppP (undecapre-

A

Amino acid position
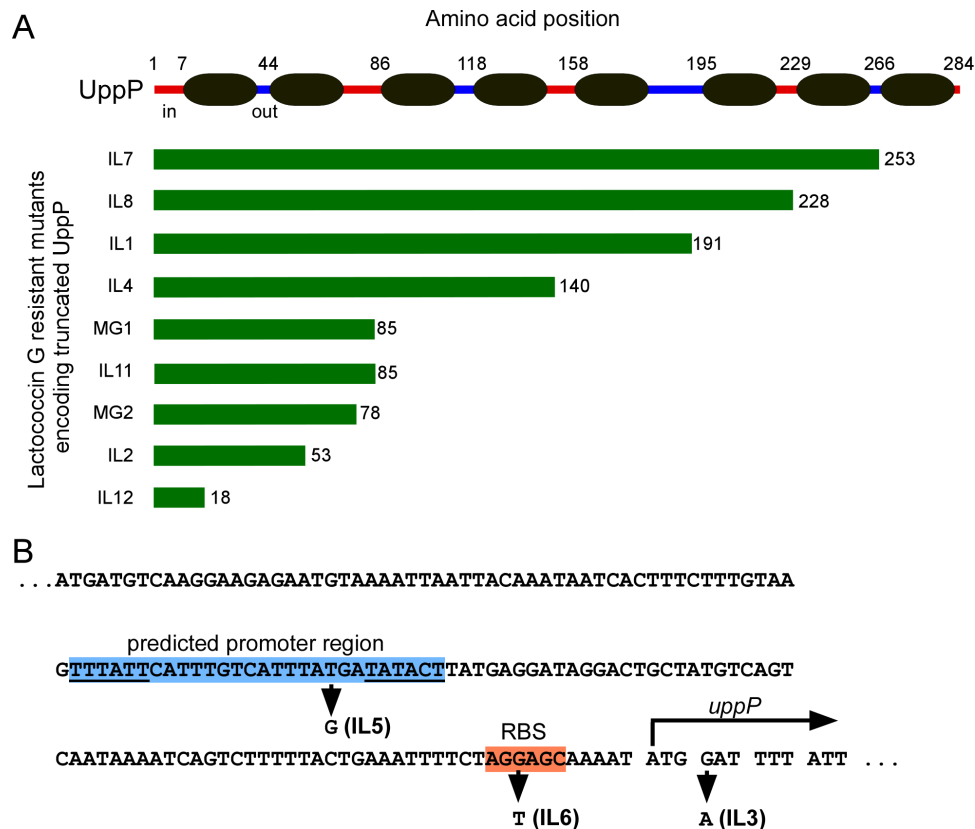
Fig. 2. Position of mutations in or near *uppP*.
A. Schematic overview of the mutants containing truncated *uppP* gene products due to frameshift mutations or introduction of stop-codons. The top panel shows an outline of UppP with transmembrane helices (black boxes), intracellular loops (red) and extracellular loops (blue) as predicted by TMHMM v 2.0 (Krogh *et al.*, 2001), and the green line show the length of the truncated UppP variants.
B. Two of the mutations mapped in the putative regulatory region of *uppP* (IL5 and IL6). The mutation in IL3 mapped to the second codon of *uppP*, causing an amino acid substitution (D2N). The promoter region was predicted using PPP (E-value = 0.056) (Zomer *et al.*, 2007) and is marked in blue, with the putative −35 and −10 regions underlined. The putative ribosome binding site (RBS) is marked in red.

nyl pyrophosphate) (Storm and Strominger, 1973). Nor did it alter the sensitivity to nisin, a bacteriocin that targets the lipid II component of the cell-wall (Breukink *et al.*, 1999) (data not shown). Taken together, this suggests that inactivation of UppP does not have a detrimental effect on cell-wall synthesis and the viability of *L. lactis*.

Heterologous expression of wild-type uppP gene from L. lactis *makes* S. pneumoniae *sensitive to lactococcin G*

We repeatedly attempted to ectopically express *uppP* in the *L. lactis* wild-types and in the resistant mutants (using different cloning vectors) in order to further verify the involvement of UppP in sensitivity to lactococcin G. However, for unknown reasons, we were not able to reintroduce this gene. Therefore, we tried a different approach and wondered if expression of *L. lactis uppP* in a heterologous host that is otherwise resistant to lactococcin G could render this strain sensitive. To test this, we introduced the lactococcal *uppP* in *S. pneumoniae*, another lactic acid

bacterium that is insensitive to lactococcin G. Indeed, the *S. pneumoniae* strain used, D39, was resistant to the highest lactococcin G concentration tested (3 μM, which is 300 000-fold higher than the MIC value for *L. lactis* IL1403). A single copy of *uppP* downstream of a Zn^{2+}-inducible promoter was integrated into the genome of *S. pneumoniae* D39, resulting in strain *S. pneumoniae* MK194. In line with our hypothesis, expression of the lactococcal *uppP* in *S. pneumoniae* D39 conferred lactococcin G sensitivity, and the level of sensitivity correlated with the *uppP* expression level (Fig. 4); *S. pneumoniae* MK194 was only inhibited by lactococcin G when expression of *uppP* was induced by the addition of increasing amounts of Zn^{2+} to the growth medium (Fig. 4). The presence of the inducer (Zn^{2+}) at the concentrations used did not alter the growth of *S. pneumoniae* (Fig. 4B). Both wild-type *S. pneumoniae* D39 and uninduced strain MK194 were resistant to the highest lactococcin G concentrations tested (MIC > 3000 nM, Table S1), but induction of MK194 with 0.2 mM Zn^{2+} made this strain more than 50-fold more
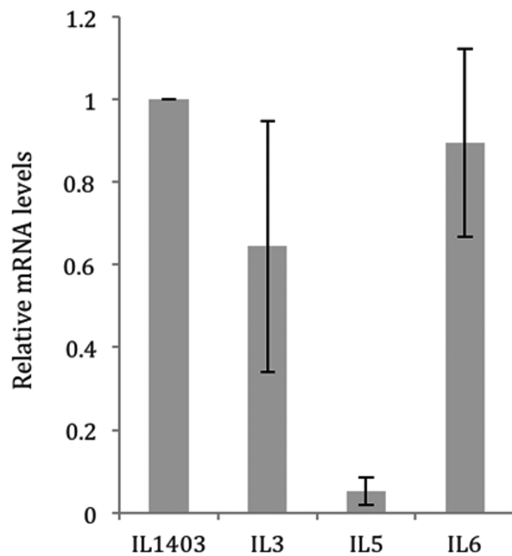
Fig. 3. Quantitative PCR analysis of the *uppP* expression in IL1403 and the mutant strains IL3, IL5 and IL6. The *uppP* levels are shown as relative values compared to the *uppP* level in the wild-type strain IL1403. The values are the average of three independent runs and the standard deviations are represented by error bars.

sensitive to lactococcin G (MIC = 56 nM). To further verify that the induced sensitivity to lactococcin G was specifically due to expression of *uppP*, and not a general response to membrane protein overexpression, we also expressed the membrane-associated protein GFP-Pbp2x (Peters *et al.*, 2014) from the same Zn²⁺-inducible promoter, and showed that this did not influence the lactococcin G sensitivity of *S. pneumoniae* (Fig. S1). Moreover, expression of *uppP* increased the sensitivity of the recombinant *S. pneumoniae* towards the related bacteriocin enterocin 1071 approximately fourfold (MIC = 1850 nM without induction to MIC = 475 nM with induction). Together, these results clearly demonstrate that *uppP* expression confers sensitivity to lactococcin G and the related bacteriocin enterocin 1071.

Discussion

In the present work, whole-genome sequencing of spontaneous bacteriocin-resistant mutants has been used to identify a putative membrane receptor for the two-peptide bacteriocin lactococcin G. Results from altogether 12 lactococcin G-resistant mutants derived from two separate sensitive strains unequivocally pointed to UppP/BacA, an undecaprenyl pyrophosphate phosphatase, as a putative receptor for this bacteriocin. UppP/BacA participates in the dephosphorylation of *de novo* synthesized undecaprenyl pyrophosphate to undecaprenyl phosphate, the immediate precursor of lipid I and lipid II in peptidoglycan biosynthesis

(Bickford and Nick, 2013). UppP/BacA is a membrane-spanning protein with eight putative transmembrane helices. Most of the detected mutations (9 of 12) resulted in the production of truncated versions of UppP/BacA (Table 1 and Fig. 2A). The positions of the truncation sites in the various mutants span most of the *uppP/bacA* coding sequence (Fig. 2A) and lead to the removal of one, two, three, four, six and seven of the 8 predicted transmembrane regions. In two of the lactococcin G-resistant strains (IL5 and IL6), the *uppP* open reading frame remained unaltered, but these strains instead contained changes in the upstream region of *uppP* 9 and 77 nucleotides from the start codon (Fig. 2B). The mutation in IL5, positioned 77 nucleotides upstream from the start codon, is situated
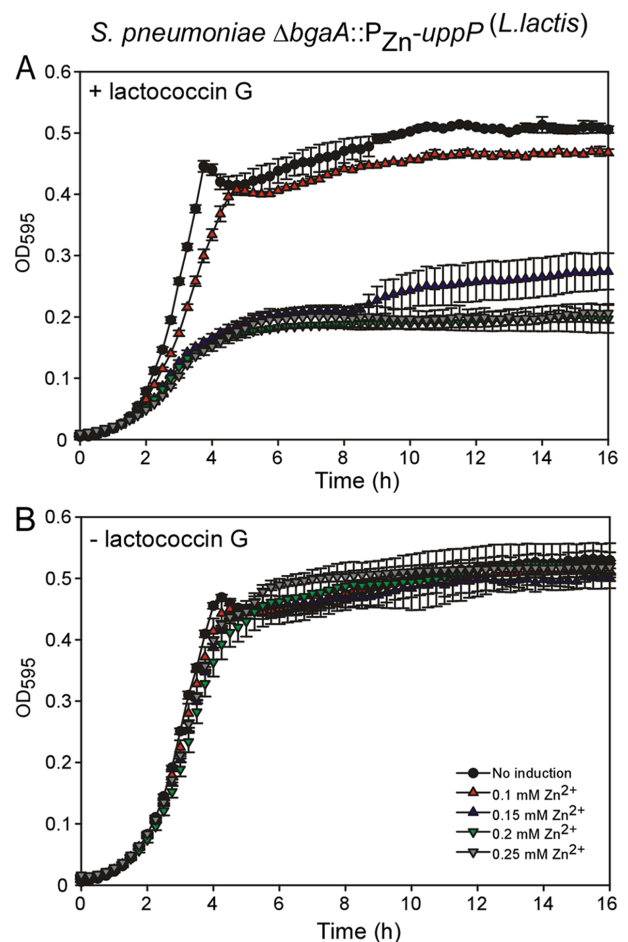
Fig. 4. A. Growth of *S. pneumoniae* MK194 (encoding a Zn²⁺-inducible *uppP* from *L. lactis*) in C + Y medium containing 450 nM lactococcin G. Expression of *uppP* is induced by different concentrations of Zn²⁺ in the growth medium. *S. pneumoniae* becomes sensitive to lactococcin G when expression of *L. lactis* *uppP* is induced.
B. Control showing the inducing Zn²⁺ concentrations used did not significantly alter the growth of *S. pneumoniae*.

close to a putative −10 promoter box [predicted by PPP (Zomer *et al.*, 2007), with an E-value of 0.056], suggesting that this mutation might have an effect on the *uppP* transcription level. Indeed, gene-expression analysis by qPCR verified that the transcription of the *uppP* gene was reduced by approximately 20-fold in IL5 compared to the wild-type strain (Fig. 3). The upstream mutation in IL6 (9 nt from the start codon) leads to a change in a putative ribosome binding site for this gene (AGGAGC in the wild-type changed to AG<u>T</u>AGC in the mutant), which may cause problems for translation. In addition, one strain (IL3) contained a mutation that leads to an amino acid substitution in UppP: the exchange of aspartic acid in position 2 with asparagine. The effect of this substitution on UppP is not clear. It might interfere with the structure or stability of UppP, thereby making it less efficient as bacteriocin receptor. However, effects on mRNA secondary structures, translation regulation, or other unknown effects might also occur (Keller *et al.*, 2012). No significant change in the transcription level of *uppP* was observed in the two mutant strains IL3 and IL6 (Fig. 3).

Most of the mutations in UppP/BacA gave rise to a very high level of lactococcin G resistance (Table 1). For example, all the truncations conferred high degree of resistance, albeit removal of only the C-terminal transmembrane region (IL7) had slightly less effect than truncation of larger parts of the protein. Furthermore, the mutation in the putative ribosomal binding site (IL6) conferred full resistance, while the mutation adjacent to the putative −10 promoter region (IL5) only led to partial resistance. Finally, the change of amino acid in position 2 from aspartic acid to asparagine (IL3) gave partial resistance. Thus, as expected, the mutations with putatively less severe effect on UppP (promoter point mutation, single amino acid substitution or deletion of a single transmembrane segment) conferred less resistance to lactococcin G.

For unknown reasons, we were not able to clone full-length *uppP* in the *L. lactis* mutants nor in the wild-type. We also encountered the same problems when trying to use *Escherichia coli* as an intermediate host for the lactococcal vectors. Possibly, these cells are not viable due to the extra copy of *uppP* causing imbalance in the cell-wall synthesis machinery, but this remains to be determined. By heterologous expression of *uppP* from *L. lactis* IL1403 in the non-sensitive *S. pneumoniae*, we were able to render *S. pneumoniae* sensitive to lactococcin G and enterocin 1071, verifying that the expression of UppP is directly linked to the sensitivity to lactococcin G and the related bacteriocin enterocin 1071. The level of sensitivity observed in *S. pneumoniae* (MIC = 56 nM) is not comparable to the high sensitivity observed for wild-type *L. lactis* (MIC = 0.01 nM). This difference can probably be attributed to intrinsic resistance mechanisms of *S. pneumoniae*

(e.g. membrane composition, cell surface properties, capsule or proteolysis) that makes it more difficult for lactococcin G to function. Alternatively, there might be a second, and yet unidentified, factor required for optimal lactococcin G activity, which is present in *L. lactis* but not in *S. pneumoniae*. It should be noted that *S. pneumoniae* also contains an *uppP*-like gene (locus tag SPD_0417), whose product is 63% identical to the UppP of *L. lactis* IL1403 (Fig. S2), but the streptococcal protein does not confer sensitivity to lactococcin G. Probably, there are specific regions/residues in the lactococcal UppP/BacA that are important for the specific recognition of lactococcin G, also explaining the high specificity of lactococcin G. Future work should aim at identifying the specific interaction sites between lactococcin G and UppP/BacA. Importantly, UppP was also shown to be the target for another class IIb bacteriocin, namely enterocin 1071 which show 57% identity to lactococcin G. It should be noted that while lactococcin G sensitivity of *S. pneumoniae* increased > 50-fold upon induction of *uppP* expression, the enterocin 1071 sensitivity increased only fourfold (Table S1). This result is in line with the observation that enterocin 1071 is also less effective against *L. lactis* compared to lactococcin G, possibly reflecting that UppP from *L. lactis* is a better target for lactococcin G than enterocin 1071. Furthermore, it is also highly likely that lactococcin Q uses UppP as a target, since this bacteriocin is very similar to lactococcin G (88% identity) (Zendo *et al.*, 2006).

Together, our results clearly show that the presence of an intact UppP/BacA is a prerequisite for lactococcin G sensitivity. As discussed above, it is likely that UppP/BacA functions as a docking molecule or a receptor for lactococcin G, in which binding of lactococcin G to UppP/BacA induces pore formation. It could be speculated that one of the products of UppP/BacA (and not UppP/BacA itself) has this receptor function, in a similar manner as lipid II which functions as the receptor for a number of lantibiotic bacteriocins, including nisin (Breukink *et al.*, 1999; Wiedemann *et al.*, 2001; Breukink and de Kruijff, 2006; Bierbaum and Sahl, 2009). It is improbable, however, that a narrow-spectrum bacteriocin like lactococcin G uses ubiquitous membrane molecules, such as undecaprenyl phosphate, lipid I or lipid II, as receptors or docking molecules. Moreover, it seems that the products of UppP/BacA are also present in the lactococcin G-resistant cells in which UppP/BacA is inactivated, since the lactococcin G-resistant mutants and the wild-type strains were equally sensitive to both bacitracin and nisin. The antibiotic bacitracin uses undecaprenyl pyrophosphate, the substrate molecule for UppP/BacA, as its membrane receptor (Storm and Stromniger, 1973), while nisin targets lipid II (Breukink *et al.*, 1999). A drastic reduction in UppP/BacA activity might therefore be expected to increase the sensitivity to bacitracin (due to accumulation of undecaprenyl pyroph-

osphate) (Bernard *et al.*, 2005) and decrease the sensitivity to nisin (reduced production of lipid II). The facts that this did not occur and that the growth rates of UppP-inactivated mutant and wild-type cells were similar indicate that UppP/ BacA is not essential. *L. lactis* thus probably contain multiple genes encoding undecaprenyl pyrophosphate phosphatase activity, similar to what has been shown in *E. coli* (El Ghachi *et al.*, 2005). One candidate for such activity in *L. lactis* is *ykdB* (locus tag L32731), which is homologous to the *E. coli* proteins YbjG and PgpB, which both have undecaprenyl pyrophosphate phosphatase activity (El Ghachi *et al.*, 2005). It could be speculated that the lactococcin G activity towards UppP has been a driving force for the evolution of redundant undecaprenyl pyrophosphate phosphatase activity in *L. lactis*, and more generally, that protein-targeting bacteriocins is a driver for different gene duplication events in bacterial evolution. Nevertheless, the role of these proteins in lactococci needs to be further investigated since recent results from *E. coli* suggest that UppP/BacA has a different physiological role than YbjG and PgpB; while the former is important for *de novo* synthesis of undecaprenyl pyrophosphate on the inside of the cell, the latter proteins rather act on the outside of the cellular membrane to recycle the lipid carrier (Bickford and Nick, 2013).

Our results also demonstrate that when used with a suitable reference genome, the VAAL algorithm (Nusbaum *et al.*, 2009) was remarkably consistent in calling differences between the reference genome and Illumina reads. VAAL called 170 differences between the NC_002662 reference genome and sequence reads from our IL1403 wild-type strain. The same differences were all detected in each of the IL1403 mutants that we analysed. Besides these 170 differences, each of the 10 different IL1403 mutants harboured one single point mutation causing the bacteriocin-resistant phenotype.

In conclusion, we have shown that genome sequencing and polymorphism detection are sufficient to pinpoint mutations that are responsible for the bacteriocin-resistant phenotype and in that way identify the putative bacteriocin receptor proteins. While lipid II is known as the docking molecule or receptor for many different lantibiotics (Breukink and de Kruijff, 2006), the receptors for the class II non-lantibiotic bacteriocins appear more diverse: the mannose phosphotransferase system for lactococcin A (member of class IId) and pediocin-like bacteriocins (members of class IIa), the maltose-ABC transporter for garvicin ML (member of class IIc), a membrane-bound Zn-dependent peptidase for the bacteriocin LsbB (member of class IId), and here UppP/BacA for lactococcin G and enterocin 1071 and most probably also lactococcin Q (members of class IIb). Using the genomics approach described in this work will probably allow identification of the receptor for many other bacteriocins in the coming years and similar approaches can also be used to identify mutations involved in antibiotic resistance (Wozniak *et al.*, 2012). The search for novel bacteriocin receptors is an important research field, not only because it is a critical aspect in drug development, but also to unravel the little-understood biological role of bacteriocins in nature, especially with regard to the ecological impact of their characteristic, narrow inhibition spectra. One way to shed light on this important issue is to examine the diversity of receptors for different bacteriocins, and how the bacteriocin/receptor/immunity protein interactions affect the producers, the sensitive target cells and resistant cells in complex communities, such as in the mammalian gut.

Experimental procedures

Bacterial strains and growth conditions

Lactococcus lactis was grown in GM17-medium [M17-medium supplemented with 0.4% (w/v) glucose] without shaking at 30°C. *Lactobacillus sakei* Lb790 (containing the two-plasmid system for production of lactococcin G- and enterocin 1071-peptides) was grown in MRS medium containing 10 μg ml^{-1} erythromycin and 10 μg ml^{-1} chloramphenicol, without shaking at 30°C. *S. pneumoniae* was grown in C + Y medium (Martin *et al.*, 1995) pH 6.8 at 37°C without shaking. *E. coli* was grown in LB medium at 37°C with shaking.

Production and purification of bacteriocins

The alpha and beta peptides of lactococcin G and enterocin 1071 were purified from a two-plasmid expression system in *L. sakei* Lb790 as previously described (Oppegård *et al.*, 2007; 2010).

Bacteriocin activity assays

The antimicrobial activity of lactococcin G and enterocin 1071 against sensitive and resistant target cells was tested using a 96-well microtitre plate-based activity assay similarly as previously described (Oppegård *et al.*, 2007; 2008; 2010). When measuring the antimicrobial activity of lactococcin G and enterocin 1071 against wild-type *L. lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1363 and mutants of these, overnight (stationary phase) cultures of these cells were diluted about 1:50 in GM17-medium containing 0.1% (v/v) Tween 80, and 200 μl of this cell suspension were added to each well together with twofold dilutions of the bacteriocin. The microtitre plates were then incubated for 4–5 h at 30°C before the growth inhibition was measured spectrophotometrically at 600 nm. The minimum inhibitory concentration (MIC) is defined as the peptide concentration [the sum of both peptides (in a 1:1 ratio)] that inhibited growth by 50%.

Generation of lactococcin G-resistant mutants

The lactococcin G-resistant mutants of *L. lactis* IL1403 were generated by slightly different methods. (i) The lactococcin

G-resistant *L. lactis* IL1403 mutants termed IL1-IL8 were generated as follows: a single colony was re-streaked and several resulting colonies were picked and incubated at 30°C overnight with 0.05 nM lactococcin G (~ 5 times MIC). The overnight cultures were plated on GM17 plates containing 5 nM lactococcin G. After incubation at 30°C overnight, one single colony was picked from the different plates, resulting in eight independent lactococcin G-resistant strains (IL1, IL2, IL3, IL4, IL5, IL6, IL7, IL8). (ii) The lactococcin G-resistant mutants of *L. lactis* IL1403 termed IL11 and IL12 were selected by plating IL1403 on GM17 agar plates containing 10–50 times MIC of lactococcin G (crude extract). After overnight incubation, resistant colonies were picked. (iii) For the lactococcin G-resistant *L. lactis* subsp. *cremoris* MG1363 strain, overnight cultures from two different colonies were diluted 1:50 and incubated at 30°C for 5 h in a 96-well microtitre plate with lactococcin G in twofold dilutions. Cells from the well containing lactococcin G with a concentration corresponding to two times the MIC-value (~ 0.05 nM) were harvested. Cultures of these cells were incubated overnight in GM17 containing 5 nM lactococcin G for selection of resistance. The overnight cultures were plated on GM17 plates containing 5 nM lactococcin G, and two independent lactococcin G-resistant colonies were picked.

Growth and phenotypic assays

For *L. lactis*, overnight cultures were diluted 100-fold in fresh medium and growth curves were monitored spectrophotometrically at 600 nm in 96-well microtitre plates using Bioscreen C (Oy Growth Curves Ab Ltd., Helsinki, Finland). For *S. pneumoniae*, cells were grown until $OD_{600} = 0.4$, and diluted 100-fold in fresh medium. Growth curves were monitored spectrophotometrically at 600 nm using Tecan Genios (Tecan Group, Männedorf, Switzerland).

For sugar fermentation profiles, an API 50 CH kit (bioMérieux, Marcy l'Etoile, France) was used according to the manufacturer's protocol.

Isolation of genomic DNA and whole-genome sequencing

DNA was isolated from 1.5–10 ml overnight cultures of the sensitive wild-type strains and spontaneous, resistant mutants using the Qiagen Blood and Tissue Kit (Qiagen N.V., the Netherlands) according to the producer's recommendations.

DNA samples were submitted to the Norwegian Sequencing Centre (sequencing.uio.no). Sequencing was performed using an Illumina HiSeq instrument, paired-end reads of 2×100 – nucleotides and 12× multiplexing according to the instructions of the manufacturer. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under Accession No. PRJEB5709.

Sequence analysis

The sequence reads were received from the Norwegian Sequencing Centre as FASTQ files containing the forward and reverse reads respectively. Due to the high sequence cover-age, we found it sufficient to use only the forward reads in the subsequent search for mutations. Before the reads could be aligned to and compared to the reference genome, the FASTQ files had to be split into sequence-containing FASTA files and QUAL files with quality information. The splitting was performed using a simple Biopython script. Then, reads from each separate resistant mutant and the corresponding sensitive wild-type strain were compared to the fully annotated database reference genome using the polymorphism discovery algorithm VAAL from Broad Institute (Nusbaum *et al.*, 2009), installed on the Abel Computing Cluster at the University of Oslo. The output from VAAL is a file containing a list of all observed differences (SNPs or indels) between the sample genome and the reference genome. Using this list, the variable sites were identified in the reference genome and assigned to genes using the genome browsing capabilities of the software platform Geneious version 6 (created by Biomatters and available from http://www.geneious.com/).

The upstream region of *uppP* was scanned for a putative promoter site using PPP (http://bioinformatics.biol.rug.nl/websoftware/ppp), in which Hidden Markov models are used to search for (lactococcal-like) Sigma A binding sites allowing 15 to 19 bp space between the −35 and −10 promoter elements (Zomer *et al.*, 2007).

Heterologous expression of L. lactis *uppP* in S. pneumoniae

The *uppP* gene was amplified with primers uppP-RBS-F-BamHI and uppP-R-XbaI (Table S2) using genomic DNA from *L. lactis* IL1403 as template. The product was digested with BamHI and XbaI and ligated into the corresponding sites, downstream of a Zn^{2+}-inducible promoter in plasmid pJWV102 (J.-W. Veening, unpublished). *E. coli* was transformed with the ligation mixture and transformants were selected on LA plates containing 100 μg ml^{-1} ampicillin to construct plasmid pJWV102-uppP. Plasmids were isolated from *E. coli* using High Pure Plasmid Isolation Kit (Roche) and checked by PCR and sequencing. The pJWV102 plasmid contains a tetracycline resistance cassette as well as sequences (flanking the insert) for integration in the *bgaA* locus of *S. pneumoniae* D39. *S. pneumoniae* D39 was transformed with pJWV102-uppP and transformants were selected on Columbia agar supplemented with 2% sheep blood and 1 μg ml^{-1} tetracycline for selection. Correct insert was verified by PCR. Different concentrations of $ZnCl_2$ were added to the growth medium (C + Y) to induce expression of *uppP*.

Quantitative PCR

Total RNA was isolated from fresh mid-log cultures of *L. lactis* IL1403 and mutants IL3, IL5 and IL6 using the RNeasy kit (Qiagen), according to the producer's recommendations, including the optional DNase treatment. The RNA was stabilized by the addition of RNAprotect Bacteria Reagent (Qiagen). RNA concentrations were measured using a Nano-Drop ND2000 spectrophotometer. cDNA was reverse transcribed from 2 μg of total RNA, using the Affinity Script QPCR cDNA Synthesis kit (Agilent Technologies). Quantitative PCR (qPCR) was performed on a Lightcycler 96 (Roche), using

gene-specific primer sets and FASTStart SYBR Green master (Roche). All measurements were made in triplicates. As an internal control, 16S rRNA was used (Magnani *et al.*, 2008). The primers used are listed in Table S2. The following qPCR-parameters were run: one cycle at 95°C for 600 s followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s and elongation at 72°C for 10 s. The three-step amplification was followed by a melting cycle (95°C for 10 s, 65°C for 60 s and 97°C for 1 s).

Acknowledgements

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.