

# Genetic characterisation and heterologous expression of leucocin C, a class IIa bacteriocin from *Leuconostoc carnosum* 4010

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Received: 7 July 2012 / Revised: 31 August 2012 / Accepted: 4 September 2012 / Published online: 9 October 2012  
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**Abstract** *Leuconostoc carnosum* 4010 is a protective culture for meat products. It kills the foodborne pathogen *Listeria monocytogenes* by producing two class IIa (pediocin-like) bacteriocins, leucocin A and leucocin C. The genes for leucocin A production have previously been characterised from *Leuconostoc gelidum* UAL 187, whereas no genetic studies about leucocin C has been published. Here, we characterised the genes for the production of leucocins A and C in *L. carnosum* 4010. In this strain, leucocin A and leucocin C operons were localised in different plasmids. Unlike in *L. gelidum*, leucocin A operon in *L. carnosum* 4010 only contained the structural and the immunity genes *lcaAB* without transporter genes *lcaECD*. On the contrary, leucocin C cluster included two intact operons. Novel genes *lecCI* encode the leucocin C precursor and the 97-aa immunity protein LecI, respectively. LecI shares 48 % homology with the immunity proteins of sakacin P and listeriocin. Another leucocin C operon *lecXTS*, encoding an ABC transporter and an accessory protein, was 97 % identical with the leucocin A transporter operon *lcaECD* of *L. gelidum*. For heterologous expression of leucocin C in *Lactococcus lactis*, the mature part of the *lecC* gene was fused with the signal sequence of *usp45* in the secretion vector pLEB690. *L. lactis* secreted leucocin C efficiently, as shown by large halos on lawns of *L. monocytogenes* and *Leuconostoc mesenteroides* indicators. The function of LecI was then demonstrated by expressing the gene *lecI* in *L.*

*monocytogenes*. LecI-producing *Listeria* was less sensitive to leucocin C than the vector strain, thus corroborating the immunity function of LecI.

**Keywords** *Leuconostoc* · Class IIa bacteriocin · Leucocin C · Heterologous expression

## Introduction

Bacteriocins are ribosomally synthesised anti-microbial peptides produced by bacteria. Because of their activity against foodborne pathogens and food spoilage bacteria, bacteriocins and bacteriocin-producing cultures have been noticed as potential natural preservatives (Mills et al. 2011). Many lactic acid bacteria (LAB), for example, *Lactococcus*, *Lactobacillus* and *Leuconostoc*, have been reported to secrete bacteriocins. LAB bacteriocins are generally divided into two main groups, namely, class I (modified bacteriocins, mainly lantibiotics) and class II (unmodified bacteriocins) (Rea et al. 2011). Class II bacteriocins can further be divided into four subgroups, of which class IIa (pediocin-like) is comprised of bacteriocins sharing a highly conserved N-terminal sequence YGNGV/L and characterised by their high activity against the food pathogen *Listeria monocytogenes* (Nissen-Meyer et al. 2009).

Class II bacteriocins do not undergo post-translational modifications and are thereby structurally simple. Generally, only genes encoding the bacteriocin, immunity protein and transporters are required for the production of class II bacteriocins. Most bacteriocins are synthesised as a precursor carrying an N-terminal signal peptide, which is cleaved by the transporter during the exportation of bacteriocin. The most common type of Class II bacteriocin signal peptide is the double-glycine leader (Nes et al. 2007). Class IIa bacteriocins kill targeted cells by binding to mannose phosphotransferase

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permease, leading to a bacteriocin-permease pore, and thus disrupting the proton motive force of the cells and depleting the ATP pool. The self-protection system of class IIa bacteriocins requires an intracellular immunity protein, which binds to the bacteriocin-permease complex blocking the pore (Kjos et al. 2011). Synthesis of some class IIa bacteriocins is under the control of a three-component regulatory system, which include a sensor (histidine protein kinase), a response regulator and an induction factor (peptide pheromone) (Drider et al. 2006).

Many *Leuconostoc* strains produce one or more bacteriocins (Papathanasopoulos et al. 1997; Vaughan et al. 2001). *Leuconostoc carnosum* 4010 is a commercial bioprotective strain for vacuum packed meats (Budde et al. 2003). It produces two different class IIa bacteriocins, leucocin A and leucocin C. The genes for leucocin A have previously been characterised from *L. carnosum* Tal1a and *Leuconostoc gelidum* UAL 187 (Felix et al. 1994; van Belkum and Stiles 1995). The leucocin A gene cluster in *L. gelidum* UAL 187 is composed of the structural and the immunity genes *leaAB* and the ABC transporter genes *leaECD*. In contrast, the knowledge of leucocin C is fairly limited. Even though the amino acid sequence of the secreted 43-aa leucocin C peptide and its activity against *Listeria* have been published (Fimland et al. 2002b; Papathanasopoulos et al. 1997), there are no reports about genetics of leucocin C. A study about cross-immunity of class IIa bacteriocins showed that leucocin A immunity protein does not protect the cell against leucocin C, indicating that the two leucocins are independent bacteriocins and should have their own production-immunity systems (Fimland et al. 2002a). However, none of the genes typically needed for class IIa bacteriocin production, i.e., dedicated translocators for secretion, an immunity protein or a regulatory system, has been found for leucocin C production.

In this study, our aims were to localise the gene cluster for leucocin C production in *L. carnosum* 4010 genome, to characterise the genes and to demonstrate their explicit functions. This study also aimed to produce leucocin C in a heterologous host *Lactococcus lactis* for development of new strains with improved anti-listerial activity.

## Materials and methods

### Bacterial strains, plasmids and culture conditions

Strains and plasmids used in this study are listed in Table 1. *L. carnosum* 4010, *Leuconostoc mesenteroides* ATCC 8293 and *L. lactis* strains were grown in M17 (Oxoid Ltd. Basingstoke, UK) supplemented with 0.5 % (w/v) glucose (M17G) at 30 °C. M17G was supplemented with 1 µg nisin/ml for *L. lactis* transformant selection and 0.5 µg nisin/ml for plasmid maintenance and nisin induction. *E. coli* was

grown in Luria-Bertani (10 g/l Bacto™ Tryptone, 5 g/l Bacto™ Yeast Extract, 10 g/l NaCl) agar or in broth with shaking at 37 °C. *L. monocytogenes* strains were routinely grown in brain heart infusion (BHI; Lab M, Lancashire, UK) agar or in broth with shaking at 30 °C. When appropriate, erythromycin (Erm) was used in concentrations of 250 µg/ml for *E. coli* and 5 µg/ml for *L. monocytogenes*. *Lactobacillus rhamnosus* 1/6 was grown on MRS agar (Lab M) at 37 °C.

### Plasmid isolations

*L. carnosum* 4010 plasmid DNA was isolated according to Anderson and McKay's method for isolating large plasmids from *Lactococcus* with the following modifications (Anderson and McKay 1983). Cells of 50 ml culture were collected by centrifugation (10,000 rpm, 4 min, JA-14, Beckman Avanti™ J-251). Pelleted cells were resuspended in 3.8 ml sucrose-Tris-EDTA buffer and divided into two 15-ml Falcon tubes. Reagent volumes used for each tube were five times of that in Anderson and McKay's method for screening. For each tube, 485 µl of lysozyme (20 mg/ml) and 25 µl of mutanolysin (5,000 U/ml) were added before incubation at 37 °C for 1 h. After cell lysis by SDS, the tubes were vortexed for 1 min. Extraction by phenol (5 ml) was repeated. The clear aqueous layer was subsequently extracted with 5 ml of chloroform-isoamyl alcohol (24:1). DNA precipitation by isopropanol was carried out in a freezer for 1 h. Pelleted DNA was washed with 4 ml of 70 % ethanol and dissolved in 200 µl of sterile Milli-Q water. RNA in the obtained DNA sample was degraded by RNase (0.1 mg/ml).

Vectors and constructed plasmids from *E. coli*, *L. lactis* and *L. monocytogenes* were isolated with E.Z.N.A. plasmid mini kit (Omega Bio-Tek, Norcross, GA, USA). For plasmids from *L. lactis* or *Listeria*, lysozyme (20 mg/ml) was added to cell resuspension solution and incubated at 37 °C for 1 h before lysing the cells.

### DNA techniques

Fragments for screening, cloning or sequencing were amplified by standard PCR with either Taq DyNAzyme™ II DNA polymerase or Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) in Eppendorf Mastercycler (Hamburg, Germany). Due to the lack of available nucleotide sequence for leucocin C, the PCR primers for amplifying leucocin C gene were designed according to its amino acid sequence. PCR primers used are listed in Table 2. PCR products and plasmid constructs were sequenced by outsourced DNA sequencing service (Institute of Biotechnology, University of Helsinki). In order to obtain complete sequence of large unknown DNA fragments, primer walking technique was used. DNA was separated in agarose gel containing

**Table 1** Bacterial strains and plasmids used in this study

| Bacterial strains or plasmids           | Relevant properties   | Reference/source   |
|---|---|--|
| <b>Strains</b>                          |   |  |
| <i>Leuconostoc carnosum</i> 4010        | Wild type leucocin A and C producer; DMRICC 4010  | Chr. Hansen A/S, Hørsholm, Denmark; Budde et al. (2003)                              |
| <i>L. mesenteroides</i> ATCC 8293       | Leucocin C-sensitive indicator strain   | ATCC   |
| <i>Lactobacillus rhamnosus</i> 1/6      | Wild type strain, <i>pepR</i> promoter  | Valio Ltd., Helsinki, Finland; Varmanen et al. (1998)                                |
| <i>Escherichia coli</i> TG1             | Transformation host   | Sambrook and Russell (2001)  |
| <i>E. coli</i> ECO758                   | TG1 carrying <i>lecI</i> -plasmid pLEB732   | This study   |
| <i>E. coli</i> SAA594                   | TG1 carrying vector pTF1  | This study   |
| <i>Lactococcus lactis</i> MG1363        | Plasmid-free transformation host  | Gasson (1983)  |
| <i>L. lactis</i> NZ9000                 | MG1363 <i>pepN::nisRK</i> , transformation host for nisin-induced gene expression   | Kuipers et al. (1998)  |
| <i>L. lactis</i> LAC358                 | NZ9000 carrying vector pLEB688  | Li et al. (2011)   |
| <i>L. lactis</i> LAC360                 | NZ9000 carrying the secretion vector pLEB690  | Li et al. (2011)   |
| <i>L. lactis</i> LAC405                 | MG1363 carrying <i>lecC</i> -plasmid pLEB728  | This study   |
| <i>L. lactis</i> LAC406                 | MG1363 carrying <i>lecCI</i> -plasmid pLEB729   | This study   |
| <i>L. lactis</i> LAC407                 | MG1363 carrying <i>lecI</i> -plasmid pLEB730  | This study   |
| <i>L. lactis</i> LAC408                 | NZ9000 carrying <i>lecCI</i> -plasmid pLEB729   | This study   |
| <i>L. lactis</i> LAC409                 | NZ9000 carrying <i>lecC</i> -plasmid pLEB728  | This study   |
| <i>L. lactis</i> LAC411                 | MG1363 carrying vector pLEB690  | This study   |
| <i>Listeria monocytogenes</i> WSLC 1018 | Leucocin C-sensitive indicator strain, transformation host, ATCC 19118  | Prof. Martin Loessner, ETH Zürich, Switzerland                                       |
| <i>L. monocytogenes</i> Scott A         | Leucocin C-sensitive indicator strain, NRRL B-33013   | Prof. Martin Loessner, ETH Zürich, Switzerland                                       |
| <i>L. monocytogenes</i> MUU22           | WSLC 1018 carrying vector pTF1  | This study   |
| <i>L. monocytogenes</i> MUU23           | WSLC 1018 carrying <i>lecI</i> -plasmid pLEB732   | This study   |
| <b>Plasmids</b>                         |   |  |
| pLEB688                                 | <i>L. lactis</i> expression vector harbouring lactococcal promoter P45. 3466 bp, Nis <sup>R</sup>   | Li et al. (2011)   |
| pLEB690                                 | <i>L. lactis</i> secretion vector harbouring lactococcal promoters P45 and P <sub>nisz</sub> , and signal sequence <i>SSusp45</i> . 3746 bp, Nis <sup>R</sup> | Li et al. (2011)   |
| pLEB728                                 | Leucocin C gene <i>lecC</i> fused to <i>SSusp45</i> in pLEB690 ( <i>NaeI</i> )  | This study   |
| pLEB729                                 | Leucocin C and the immunity gene <i>lecCI</i> fused to <i>SSusp45</i> in pLEB690 ( <i>NaeI</i> )  | This study   |
| pLEB730                                 | Leucocin C immunity gene <i>lecI</i> in pLEB688 ( <i>NcoI/SmaI</i> )  | This study   |
| pLEB732                                 | <i>lecI</i> with <i>pepR</i> promoter from <i>L. rhamnosus</i> 1/6 in pTF1 ( <i>EcoRI/SmaI</i> )  | This study   |
| pTF1                                    | Gram <sup>-</sup> /Gram <sup>+</sup> shuttle vector, 3043 bp, Erm <sup>R</sup> ; pLEB579 carrying multiple cloning site from pBluescript                      | Gift from Dr. Lars Fieseler, ETH Zürich, Switzerland. pLEB579, Beasley et al. (2004) |

DMRICC Danish Meat Research Institute Culture Collection, Roskilde, Denmark; WSLC Weihenstephan *Listeria* Collection, Technische Universität München, Freising-Weihenstephan, Germany

ethidium bromide (0.5 µg/ml) by electrophoresis and visualised under UV light.

In Southern hybridisation, DNA was first transferred from gel to nylon membrane by vacuum blotting. Probes were labelled with digoxigenin (DIG) by standard PCR supplemented with DIG-labelled dUTP (Boehringer Mannheim, Germany). DNA on membrane was then hybridised and detected according to Boehringer Mannheim DIG labelling and detection kit.

Calf intestinal phosphatase, DNA ligase, polynucleotide kinase and restriction enzymes were used as recommended by the suppliers (Fermentas, Vilnius, Lithuania; New England Biolabs, Ipswich, MA, USA; Promega, Madison, WI, USA). Plasmids were transferred into *E. coli*, *L. lactis* and *L. monocytogenes* by electroporation with a Bio-Rad Gene Pulser device (Bio-Rad Laboratories, Richmond, CA, USA) essentially as described previously (Holo and Nes 1989; Park and Stewart 1990; Zabarovsky and Winberg 1990).

**Table 2** PCR primers used in this study. Restriction sites used in clonings are underlined

| Primer name            | Use, restriction site                   | Sequence 5' → 3'                         |
|------------------------|---|--|
| LecC forw              | <i>lecC</i> probe, <i>lecC</i> cloning  | AAAAACTACGGTAAACGGTGTTCCTG               |
| LecC rev               | <i>lecC</i> probe                       | ACTGCATGCTTACTTGTGCCAACCAGCGTTACC        |
| LecC new rev           | <i>lecC</i> cloning                     | TTAGTTATGCCATCCAGCATTGC                  |
| LecC inverse forw      | <i>AcII</i> , <i>HpaI</i> inverse PCR   | GTTATGAATGGCCTAACCGGC                    |
| LecC inverse rev       | <i>AcII</i> , <i>HpaI</i> inverse PCR   | GTTTGCATGTTGGTCCACGC                     |
| LecI down forw         | <i>HindIII</i> inverse PCR              | TAGTCCGTGAGCGGTTTATGAGAC                 |
| LecS mid forw          | <i>HindIII</i> inverse PCR              | CTAAAGGCACAACCATTGCG                     |
| LecI forw              | <i>lecI</i> , <i>BspHI</i>              | ACTTCATGAAAATAAGATGGTTTTCTGGTG           |
| LecI rev               | <i>lecI</i>                             | TCAGTAGCCATATCTAATACTAGA                 |
| LeuA forw              | <i>lcnAI</i> probe                      | AAGTATTATGGTAACGAGTTC                    |
| LeuA-im rev            | <i>lcnAI</i> probe, <i>lcnA</i> operons | ACTGAATTCGCGCCGCTATCTTTCAAAGATACTATAAAAC |
| LeuA operon rev        | <i>lcnA</i> operons                     | AGTTGAACTCCCTTCAGTAGC                    |
| P <sub>pepR</sub> forw | P <sub>pepR</sub> , <i>EcoRI</i>        | ATGTGGAATTCTGCTTTGATACTCACCA             |
| P <sub>pepR</sub> rev  | P <sub>pepR</sub>                       | AGCCGGATCCTTAGGTCAGGATCGTTGTTC           |

### Clonings and gene expression constructs

To produce leucocin C and its immunity protein LecI in *L. lactis*, the mature part of the leucocin C gene *lecC*, with and without the immunity gene *lecI*, were amplified by PCR using Phusion High-Fidelity DNA polymerase (Finnzymes) with primer pairs LecC forw-LecI rev and LecC forw-LecC new rev, respectively. The amplicons were cloned as blunt-end fragments into the *NaeI* site in the secretion vector pLEB690 (Li et al. 2011), resulting in plasmids pLEB728 (*lecC*) and pLEB729 (*lecCI*). Blunt-end cloning into the *NaeI* site in pLEB690 results in an *SSusp45* gene fusion, which enables secretion of a native mature protein with no extra amino acids in its N-terminus. As a control, the leucocin C immunity gene was amplified with the primers LecI forw-LecI rev and cloned as a *BspHI*-blunt fragment into the *NcoI*-*SmaI* cut expression vector pLEB688 (Li et al. 2011), resulting in the plasmid pLEB730. Ligation mixtures of the above-mentioned plasmid constructs were electroporated into *L. lactis* MG1363 (Gasson 1983) and the transformant colonies were selected with nisin. Plasmids were isolated from the transformants, and the correct constructions were verified by sequencing. The *lecC* and *lecCI* secretion/expression plasmids were then electroporated into *L. lactis* NZ9000 (Kuipers et al. 1998).

To produce the leucocin C immunity protein LecI in *L. monocytogenes*, *lecI* gene was cloned with the promoter of *pepR* gene from *L. rhamnosus* strain 1/6 (Varmanen et al. 1998) into a cloning vector pTF1. Vector pTF1 harbours lactococcal pSH71 replicon (Gasson 1983), an Erm resistance gene, and multiple cloning sites from pBluescript. The promoter of *pepR* gene was chosen because we previously found it functional in various bacteria (unpublished results). The *pepR* promoter region was amplified by PCR with primer pair P<sub>pepR</sub> forw-P<sub>pepR</sub> rev and restricted with *BclI*

(cleavage site immediately after the RBS). The resulting 135-bp fragment was ligated with the *BgIII*-cut *lecI* plasmid pLEB730 (*BgIII* cleavage site 7 nt before *lecI* ATG). Then, from the P<sub>pepR</sub>-pLEB730 ligation mixture, the P<sub>pepR</sub>-*lecI* fragment was amplified by PCR with primers P<sub>pepR</sub> forw-LecI rev, restricted with *EcoRI* and ligated with *EcoRI*-*SmaI*-cut pTF1. The ligation mixture was electroporated into *E. coli* TG1. The resulting *lecI* expression plasmid pLEB732 was isolated and further electroporated into *L. monocytogenes* WSLC 1018. The plasmid was isolated from *Listeria*, and its correct construction was confirmed by sequencing. As a negative control, the vector pTF1 was electroporated into *L. monocytogenes* WSLC 1018.

### Anti-bacterial activity

Bacteriocin activities were determined by conventional spot-on-lawn method. The indicator strains *L. mesenteroides* ATCC 8293, *L. monocytogenes* Scott A and *L. monocytogenes* WSLC 1018 were first grown overnight in M17G or BHI broth. Two hundred microlitres of the indicator cultures were added to 5 ml of M17G soft agar, poured onto a M17G agar plate and let dried. Ten-microlitre droplets of pasteurised supernatants (75 °C, 10 min) from *L. lactis* and *L. carnosum* overnight cultures were spotted onto the surface of the indicators, and the plates were incubated overnight at 28 °C.

### SDS-PAGE and identification of the activity band

Proteins from 1-ml of the pasteurised cell-free overnight culture supernatants of the *L. lactis* strains LAC 409 (leucocin C) and LAC 360 (vector pLEB690) were concentrated with 10 µl of StrataClean resin (Agilent Technologies, Santa Clara, CA, USA). The resin beads were washed with sterile



water, and the proteins were released in 10 µl of Bio-Rad Tricine sample buffer (Bio-Rad, Hercules, CA, USA). The concentrated supernatant samples containing the beads were analysed by tricine-SDS-PAGE essentially as described by Schägger (2006). Ten microlitres of samples were loaded into gels, except 2 µl of leucocin C sample that was loaded for inhibition test. Prestained Spectra Multicolor Low Range Protein Ladder (Thermo Scientific, Rockford, IL, USA) and 3.4 kDa pure nisin (Aplin and Barrett Limited, Bristol, UK) were used as protein size markers. Two identical gels were prepared of 16.5 % acrylamide resolving gel and 1 cm of 10 % acrylamide spacer gel. The electrophoresis was run at 4 °C with 30 V (about 20 mA) for the first 30 min and at 150 V for about 2 h (90 mA in the beginning, 55 mA in the end). After electrophoresis, the gels were fixed. One gel was stained with Silver Stain Plus kit (Bio-Rad) and the other with Coomassie Brilliant Blue R-250. The Coomassie-stained gel was used for identification of the bacteriocin band. After staining and destaining, the gel was washed for 2 h in distilled water and placed onto a BHI agar plate. Ten millilitres of BHI soft agar containing 200 µl of overnight *L. monocytogenes* WSLC 1018 culture was poured onto the plate. The plate was incubated overnight at 30 °C.

#### Leucocin C immunity assay

To determine the function of leucocin C immunity protein LecI, 3 µl of overnight *Listeria* carrying the *lecI* plasmid pLEB732 or the vector pTF1 was added to 300 µl BHI + Erm<sup>S</sup> broth containing different concentrations (0, 5, 10, 20, 50, and 100 µl/ml) of leucocin C-producing *L. lactis* culture supernatant in Bioscreen microtiter plates (100 wells). The plates were incubated in Bioscreen C (Labsystems, Helsinki, Finland) at 30 °C with constant shaking for 24 h. The optical density at 600 nm wavelength was measured every 2 h.

#### GenBank accession number

The sequence of the complete leucocin C gene cluster has been submitted to GenBank with accession number JQ061256.

## Results

#### Localisation of leucocin A and C genes in *L. carnosum* 4010 genome

Since *L. carnosum* 4010 carries plasmids and class IIa bacteriocin genes are often plasmid-associated, the first aim was to study the loci of the leucocin A and leucocin C genes with Southern blotting by using leucocin A and C genes as probes. The probes were produced and labelled

with PCR. Leucocin A gene *lcaA* was amplified with its immunity gene *lcaB*. The amplified fragments were confirmed to be the leucocin A and C genes by sequencing and translating to amino acids. The novel leucocin C gene was named *lecC*. Plasmid DNA of *L. carnosum* 4010 was isolated, run in agarose gel, transferred to nylon membrane and hybridised with DIG-labelled probes. *L. carnosum* 4010 was found to carry several plasmids (Fig. 1). The *lecC* probe hybridised with the largest of the plasmids and with the chromosomal band (Fig. 1a). The latter was most likely due to a partial degradation of large plasmid during plasmid isolation, resulting in plasmid fragments migrating with the chromosomal band in agarose gel. The *lcaAB* probe hybridised with two plasmid bands, presumably representing different forms of the second largest plasmid (Fig. 1b). Hence, the genes for the two leucocins were shown to be located in different plasmids, indicating that they are two independent bacteriocins in *L. carnosum* 4010.

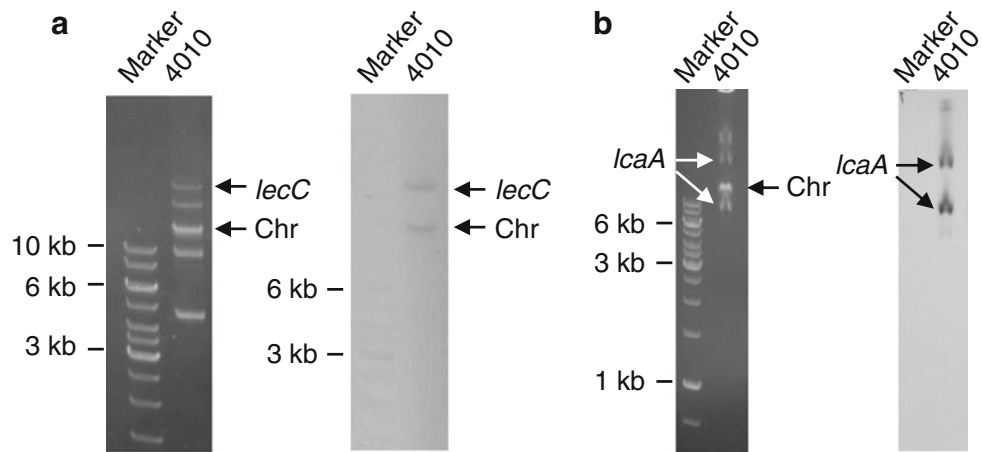
#### Characterisation of leucocin C operons

Since leucocin C gene was shown to be located independently of leucocin A, the large leucocin C plasmid could also carry other leucocin C-dedicated genes. Thus, next aim was to characterise the flanking region of *lecC* gene by sequencing. Different approaches, including arbitrary primed PCR, inverse PCR and restriction Southern cloning strategy, were used to obtain *lecC*-flanking fragments for sequencing (data not shown).

The best working strategies were restriction of the isolated plasmid DNA with *AclI*, *HindIII* or *HpaI*; self-ligation and inverse-PCR. First, *HpaI*-inverse PCR produced about 1-kb fragment containing an operon with two genes, the *lecC* structural gene and a putative immunity gene *lecI* downstream of *lecC*, and about 400 bp downstream of *lecI* including a putative transcription terminator. *AclI*-inverse PCR produced about 8-kb fragment containing the whole 5-kb *lecC* region: the *lecCI* operon and a complementary operon with three ABC-transporter genes *lecXTS* upstream of *lecC* (Fig. 2). The 8-kb *AclI* fragment also included a region about 3 kb downstream of *lecS*. No more *lecC*-related genes existed in this region. The closest gene to leucocin C cluster, about 540 bp downstream of *lecS*, was a 282-bp *orf* 74 % homologous to *yail* gene of *L. lactis* IL1403. To investigate the downstream region of *lecI*, *HindIII*-inverse PCR was used. In the 2.5-kb downstream area, only a plasmid mobilisation gene *mob* and some hypothetical genes were found. These genes matched to several *Leuconostoc* plasmids, e.g., *L. mesenteroides* J18 plasmid pKLE03 (accession number: NC\_016821), which indicates that the sequencing of the entire *lecC* cluster was completed.

The five genes organised in two operons, *lecXTS* and *lecCI*, were identified to be class IIa-related genes by

**Fig. 1** *L. carnosum* 4010 plasmids in agarose gels and Southern blots hybridised with DIG-labelled *lecC* (a) or *lcaAB* (b) probes. Leucocin A and C genes were located in different plasmids. *Chr* chromosomal band, *Marker* GeneRuler™ 1 kb DNA ladder (Fermentas)



sequence homology. The mature part of *lecC* and the immunity gene *lecI* were novel genes without significant homology to any known genes. However, the protein sequence of LecI matched with other class IIa immunity proteins, with the best scores with listeriolysin and sakacin P immunity proteins (48 % identity, 73 % similarity) (Fig. 3). Still, experimental evidence would be needed to confirm its functionality as an immunity protein. Interestingly, the organisations of the leucocin C operons, the 72-bp double-glycine type signal sequence of *lecC* and the sequence of the ABC transporter operon *lecXTS* were practically identical with those of leucocin A in *L. gelidum* (97 % identity). Therefore, the next aim was to determine whether the leucocin A operons in *L. carnosum* 4010 would be the same, i.e., whether the strain carries two sets of practically identical bacteriocin transporters.

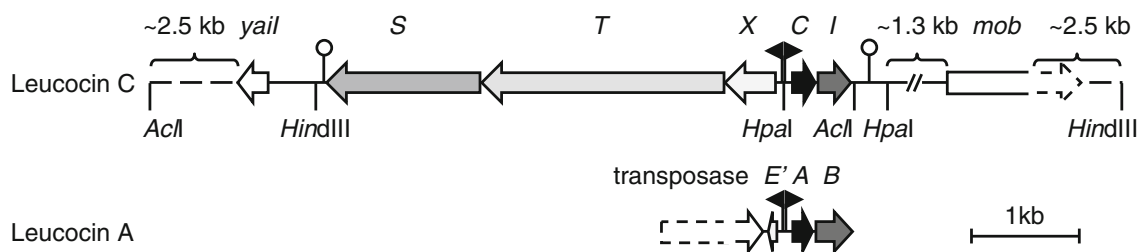
#### Characterisation of leucocin A operons

Assumed leucocin A gene cluster was amplified by PCR with primers designed according to the leucocin A gene sequences from *L. gelidum* UAL 187. Surprisingly, the obtained 4.5 kb amplicon was somewhat smaller than the expected size (5 kb) of *lcaA* region in *L. gelidum* (result not shown). Sequencing of the PCR product showed that the

amplicon contained identical leucocin A and its immunity genes *lcaAB* as in *L. carnosum* Ta11a (Fig. 2). However, the transporter operon *lcaECD* was missing due to a disruption by anIS30 family transposase in the accessory gene *lcaE*. The DNA sequence upstream of the transposase gene was not leucocin A-related but matched to a non-coding region of *L. mesenteroides* J18 plasmid pKLE03 (accession number: NC\_016821). Therefore, the leucocin A gene cluster in *L. carnosum* 4010 only included the structural and immunity genes, but not transporters.

#### Heterologous expression of leucocin C in *L. lactis*

In order to study the function of the newly found leucocin C immunity gene *lecI*, leucocin C peptide would be needed. A way to get the bacteriocin would be to produce it in a heterologous non-bacteriocinogenic host *L. lactis*. The mature part of the *lecC* gene was fused with the lactococcal signal sequence of *usp45* in the plasmid pLEB690, under the control of the constitutive promoter P45 and the nisin-inducible promoter  $P_{nisZ}$ . The *lecC* gene was cloned with and without the putative immunity gene *lecI*. To control that the immunity protein alone does not cause any inhibition, the *lecI* gene was cloned into pLEB688 with no *SSusp45*. The constructed plasmids were transferred into *L. lactis* MG1363 for constitutive



**Fig. 2** Organisations of the leucocin A and C gene clusters in *L. carnosum* 4010. Promoters are shown as black flags. Transcriptional terminators are shown as lollipop symbols. *yail* *L. lactis* gene with unknown function; *S* secretion gene *lecS* for ABC transporter; *T* translocator gene *lecT* for ABC transporter; *X* accessory gene *lecX*; *C*

leucocin C precursor gene *lecC*; *I* immunity gene *lecI*; *mob* plasmid mobilisation gene; *E'* truncated accessory gene *lcaE*; *A* leucocin A precursor gene *lcaA*; *B* immunity gene *lcaB*. Restriction sites for *AclII*, *HindIII* and *HpaI* used in inverse PCR are shown. *Solid line* sequence in GenBank, *dashed line* unpublished/unknown sequences

**Fig. 3** Alignment of leucocin C immunity protein LecI with its closest relatives listeriocin (LisB), enterocin CRL 35 (EntA) and sakacin P (SakP) immunity proteins

|      |  |
|------|--|
| LecI | MK-IRWFSGGEBRKNSAVPLIDDLISELNKKDDNEPVIIVVLNKKYKDELI-   |
| LisB | --IKKVKWYSGGDBRGEKAIGLILELLKBLNTNSDSQLLQEVVLNKKYKEELE- |
| EntA | MSNLKWFSGGDDRRKKAQEVIIITELLDDLEIDLGNESLRKVLGVSYLEKLEK- |
| SakP | MAGLNWFSGGNERQQQASLLIKDLVADLKQSGGKEPLQKVLVLSFDEELK-    |
|      |  |
| LecI | KKETSVPFILSRNLNVDSNVVVRDNNIIMTDKESDILRDIRKLSSTRYGY     |
| LisB | NKGSVPLVLSRMLAI SHAIRKNGVILSDTQS-TIKELTSLSSIRYGYF      |
| EntA | NEGTSVPLVLSRMLNIEISNAIKKDGVSLENENQSKKLKELISISNIRYGY    |
| SakP | RGESVIPFILSRMTVAVSTVTLKEHQITLSAKESDQLDDLYALLQIRYGY     |

expression and NZ9000 for nisin-inducible expression. The production of bacteriocin was determined by anti-bacterial assay on a plate. Leucocin C was effectively secreted by *L. lactis* strains, as shown by large halos on *Listeria* and *Leuconostoc* indicator plates (Fig. 4). Nisin-induced expression of leucocin C in NZ9000 was much better than constitutive expression from the promoter P45 and comparable with the wild type leucocin producer *L. carnosum* 4010. The halos of LecC and LecCI strains were of the same size, showing that co-expression of the putative immunity gene *lecI* had no significant effect on bacteriocin production. This efficient leucocin C-producing *L. lactis* could be used as the source of the bacteriocin for studying the function of the putative immunity gene *lecI*.

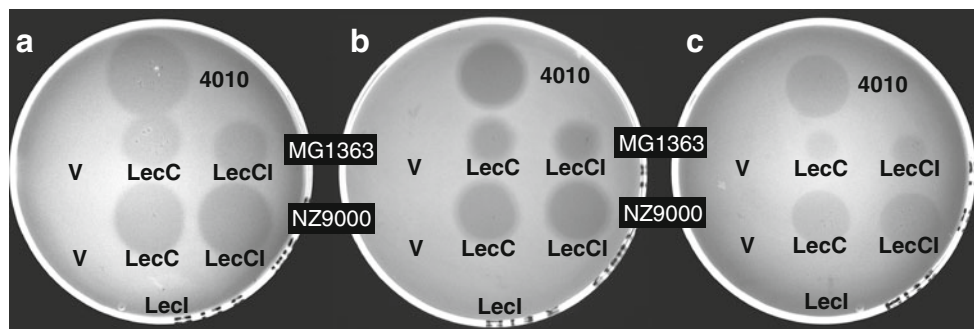
#### Identification of the bacteriocin band

To identify that the killing of the indicator strains by *L. lactis* LecC strains was due to a bacteriocin peptide, the proteins from culture supernatants were concentrated and analysed with SDS-PAGE. With silver staining, potential leucocin C band migrating between the 3.4 kDa nisin and the 10 kDa marker bands was observed (Fig. 5a). Even though the actual size of leucocin C is 4.6 kDa, the putative leucocin C band migrated slower than the 4.6 kDa marker band. However, as the 3.4 kDa nisin migrated slower than the 4.6 kDa band in the marker, the exact size determination according to the marker is not fully reliable. As expected, the putative leucocin C band could not be seen in Coomassie gel, since the loaded sample

volume was only 2  $\mu$ l instead of 10  $\mu$ l in silver-stained gel. The lower sample volume was used because preliminary tests showed that 10  $\mu$ l of leucocin C sample in gel causes too large inhibition for size identification (results not shown). The Coomassie gel was then placed into a *Listeria* soft agar plate for inhibition test. A large halo between the 4.6 and 10 kDa marker bands appeared in leucocin C lane (Fig. 5b), confirming the anti-bacterial activity of the visible leucocin C band in the corresponding silver-stained gel (Fig. 5a).

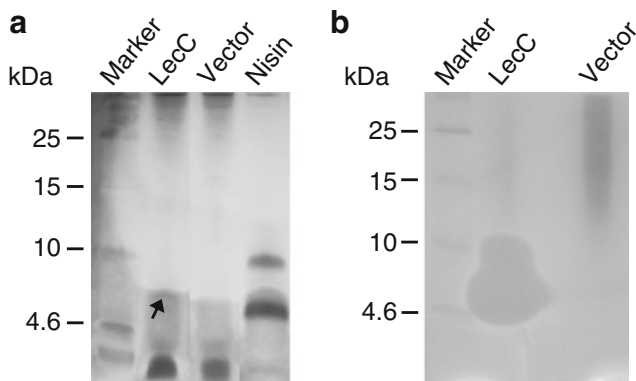
#### LecI protects the cell against leucocin C

The last aim in this work was to confirm by experimental evidence, whether LecI was actually a functional immunity protein of leucocin C. The *lecI* gene was cloned with the constitutive lactobacillar promoter of *pepR* gene into the plasmid vector pTF1 in *E. coli*. The constructed plasmid and the empty vector were introduced into *L. monocytogenes*. The *Listeria* transformants were then grown with different concentrations of leucocin C-producing *L. lactis* culture supernatant. The growth curves are shown in Fig. 6. With no leucocin C in media, the vector and *lecI* strains grew equally. When leucocin C supernatant was added into the media, the growth of both strains retarded, but the retardation of the vector strain was significantly stronger than the LecI strain. With higher leucocin C concentrations (20, 50, and 100  $\mu$ l supernatant per millilitre), the difference in growth of the two strains was less pronounced (data not shown), showing that the immunity capacity of LecI was



**Fig. 4** Anti-bacterial assay of *L. lactis*-producing leucocin C (LecC, LecCI) compared to the wild type leucocin A and C producer *L. carnosum* 4010. Indicator strains: **a** *L. monocytogenes* WSLC 1018, **b** *L. monocytogenes* Scott A, **c** *L. mesenteroides* ATCC 8293. Onto indicator lawn, 10  $\mu$ l of cell-free overnight culture supernatants were

spotted. All host strains were cultured with nisin (0.5  $\mu$ g/ml) as selective agent for the plasmids and/or for nisin induction. *Second row from top* *L. lactis* MG1363 as the host. *Third row from top* nisin-inducible *L. lactis* NZ9000 as the host. V, plasmid vector pLEB690. *LecI* *L. lactis* MG1363 carrying *lecI* plasmid pLEB730

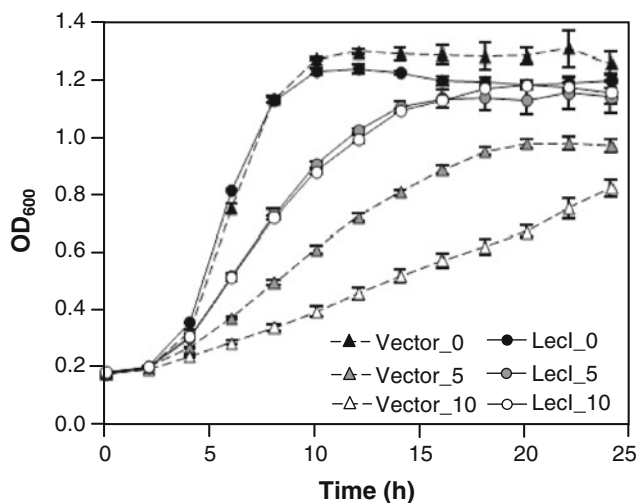


**Fig. 5** SDS-PAGE of supernatant concentrations from overnight cultures of leucocin C-producing *L. lactis* LAC409 (LecC) and the vector pLEB690 strain LAC360 (Vector). **a** Silver-stained gel. **b** Coomassie-stained gel on *L. monocytogenes* WSLC 1018 agar. Supernatant proteins adsorbed onto 10  $\mu$ l StrataClean resin were released with 10  $\mu$ l of SDS buffer and loaded into 16.5 % acrylamide gels. Ten microlitres of samples were loaded, except LecC for Coomassie/inhibition gel, 2  $\mu$ l was loaded. A putative leucocin C band (black arrow) in **a** and corresponding inhibition zone in **b** appeared between 4.6 and 10 kDa. As bacteriocins may migrate somewhat differently in gel than marker bands, 1  $\mu$ g of pure nisin (3.4 kDa) was used as a bacteriocin size marker. The actual size of leucocin C is about 4.6 kDa. Double bands of bacteriocins may indicate that different forms of bacteriocin, e.g., dimers or precursor peptides are present

limited. Yet, production of LecI protected *Listeria* cells against leucocin C, showing the immunity function of LecI.

## Discussion

Lactic acid bacterium *L. carnosum* 4010 produces two class IIa bacteriocins, leucocin A and leucocin C. In this study, we



**Fig. 6** Growth of LecI-producing *Listeria* in different leucocin C concentrations (0, 5, 10  $\mu$ l of leucocin C-producing *L. lactis* supernatant per millilitre). LecI-producing cells tolerated leucocin C better than the vector strain. Vector *Listeria*-carrying pTF1. Error bars SD of the mean from three parallel cultures

have shown that leucocin C has its own genes for the production-immunity system, independently from the well characterised bacteriocin leucocin A. Until now, the leucocin C genes have remained unknown, even though the secreted leucocin C has been purified from *L. mesenteroides* strain 6, and the amino acid sequence of mature leucocin C has been determined (Fimland et al. 2002b). Most class IIa bacteriocins are encoded by plasmid-associated genes (Ennahar et al. 2000). Likewise, the leucocin C genes were shown to be plasmid-encoded in *L. carnosum* 4010. Leucocin A genes were found from another plasmid. However, the leucocin A region differed from the previously characterised leucocin A operon in *L. gelidum* UAL 187 (van Belkum and Stiles 1995). The signal peptide of leucocin A in the strain 4010 was identical with that of leucocin A in *L. carnosum* Ta11a, differing by 7 aa of LcaA leader in *L. gelidum* UAL 187 (Felix et al. 1994). In addition, in *L. carnosum* 4010, the transporter operon was almost entirely missing, and thus, the leucocin A region only contained the structural and the immunity genes, with 87 bp of the 5' end of the accessory gene *lcaE*. As leucocin A is secreted by the strain (Budde et al. 2003), either the genes for leucocin A transporter are located somewhere else in the genome or leucocin A is secreted by some other transporter.

In most cases with class IIa bacteriocin genes, the bacteriocin structural genes are followed by an immunity gene in the same operon, and another operon(s) contains genes for secretion proteins (Ennahar et al. 2000). The leucocin C operons in *L. carnosum* 4010 followed this typical bacteriocin gene organisation. Two operons containing five genes were characterised. The organisations of *lca* operons in *L. gelidum* and *lec* operons in *L. carnosum* were the same. Moreover, the *lecC* signal sequence and the *lecXTS* operon-encoding accessory protein, translocator and secretion protein for leucocin C ABC transporter were virtually identical with the *lca* operon of leucocin A (Hastings et al. 1991; van Belkum and Stiles 1995). Since leucocin A operon in the strain 4010 does not contain dedicated transporter genes, leucocin A could possibly be secreted by the leucocin C transporter. However, further experiments are required to identify the actual leucocin A transporter.

According to their structures, leucocins A and C belong to different subgroups of class IIa bacteriocins (A in subgroup 2, C in subgroup 1) (Nissen-Meyer et al. 2009). Other class IIa bacteriocins of subgroup 1, e.g., sakacin P and listeriocin 743A, share high sequence similarity with leucocin C. Regardless of the sequence similarities, the organisations of these bacteriocin operons are different (Ennahar et al. 2000). Listeriocin 734A does not possess its own transporter genes and is secreted by the *sec* pathway (Kalmokoff et al. 2001). In the case of sakacin P, two genes *sppK* and *sppR* encode the regulatory proteins (Hühne et al. 1996). In *L. carnosum* 4010, regulatory genes were not present in the *lecC*-flanking regions.



In order to secrete class IIa bacteriocins in heterologous bacteria, naturally resistant *L. lactis* is a potential host candidate (Henderson et al. 1992; Kjos et al. 2009). Lactococci are resistant because class IIa bacteriocins do not bind to lactococcal mannose phosphotransferase permease, which serves as the target in sensitive cells (Kjos et al. 2009). The lactococcal *sec*-dependent secretion signal of *usp45* has been used to replace the native signal sequences of enterocin P or pediocin genes (Borrero et al. 2011; Li et al. 2011). Here, in our study, *lecC* gene was fused with the *SSusp45* in the secretion vector pLEB690, and leucocin C was secreted in *L. lactis* strains MG1363 and NZ9000. Large halos on indicator plates showed that leucocin C-producing *L. lactis* strains killed the indicators *L. monocytogenes* and *L. mesenteroides* (Fig. 4). Even though lactococci are naturally resistant to class IIa bacteriocins and hence their production would not require immunity factors, in some cases the co-expression of the cognate immunity gene has been reported to improve bacteriocin production. Co-expression of pediocin immunity gene has significantly increased the yields of heterologous pediocin in host *L. lactis* (Arqués et al. 2008). However, here in this study with leucocin C, there was no noticeable increase in LecC production with the co-expression of *lecI* (Fig. 4). As nisin promoter is known to be stronger than the P45 promoter (Li et al. 2011), leucocin C was also produced more with  $P_{nisZ}$  in nisin-inducible strain NZ9000 than with the constitutive P45 promoter in the strain MG1363. Nisin-induced secretion of active leucocin C in *L. lactis* NZ9000 with leucocin C expression plasmids was comparable with the wild type leucocin producer *L. carnosum* 4010. To correspond the inhibition of the indicators with the bacteriocin-sized peptide, i.e., leucocin C, the proteins from the *L. lactis* cultures were concentrated, separated in SDS-PAGE and the gel was placed onto a *Listeria* plate. Putative leucocin C band was observed with silver staining, and the peptide was shown to be highly active in SDS gel after fixation, Coomassie staining and destaining (Fig. 5). The inhibition zone from leucocin C was somewhat smeared or doubled, suggesting that different forms, e.g., dimer or precursor peptide may also be present. Determining sizes of bacteriocins by SDS-PAGE is often inexact. Sizes of many bacteriocins, e.g., pediocin have been inaccurately estimated by SDS-PAGE (Bhunia et al. 1987; Yang et al. 1992). Therefore, the determined size between 3.4 and 10 kDa is sufficient to confirm the role of leucocin C peptide in the inhibition caused by heterologous *L. lactis* (Fig. 5). In conclusion, this study shows that *L. lactis* as a heterologous host is able to efficiently produce highly active leucocin C for potential use in food industry.

The function of a bacteriocin immunity protein can be demonstrated by expressing the putative immunity gene in a bacteriocin-sensitive host and to examine the increase of

bacteriocin resistance. Previously, the functions of bacteriocin immunity genes of, e.g., enterocin A, leucocin A and sakacin P, have been investigated by expressing them in heterologous hosts (Fimland et al. 2002a). However, even though *Listeria* has been used as the model organism in proposing the mode of action of class IIa bacteriocins (Kjos et al. 2010), no immunity genes have been expressed and shown to function in *Listeria*. The gene *lecI* identified in this study was shown to confer immunity by expressing *lecI* in leucocin C-sensitive *Listeria* (Fig. 6). The sensitivity of LecI-producing *Listeria* towards leucocin C decreased, and this strain could grow in leucocin C-containing medium better than the vector strain. The immunity capacity was rather limited, as higher concentrations of leucocin C killed the LecI-*Listeria* as efficiently as the vector strain (data not shown). Limited immunity could be a consequence of a low expression level of *lecI* (not determined). Stronger expression could possibly have resulted in higher resistance, but the aim here was not to create *Listeria* strain with maximum bacteriocin resistance but to demonstrate the function of leucocin C immunity protein LecI.

Based on the common motifs in class IIa immunity proteins, they can be divided into three subgroups (Fimland et al. 2002a). The subgrouping of bacteriocins does not necessarily match with the subgrouping of the immunity proteins. For instance, pediocin and sakacin P bacteriocins are sorted into the same class IIa subgroup 1 (Nissen-Meyer et al. 2009), but their cognate immunity proteins belong to different subgroups: Ped-im in subgroup A and SakP-im in subgroup B. Immunity proteins in the subgroup B contain certain consensus motifs, e.g., the C-terminal SN/SIRYGY, which are also found in the amino acid sequence of LecI (Fig. 3) (Fimland et al. 2005). Therefore, based on the common consensus motifs and the highest identity, 48 %, with listeriocin and sakacin P immunity proteins, LecI is now suggested to be a member of subgroup B immunity proteins of class IIa bacteriocins. Leucocin A immunity protein is structurally different than LecI, and it belongs to subgroup A (Fimland et al. 2005). Therefore, it is not surprising that in the previous study leucocin A immunity protein did not protect the host against leucocin C (Fimland et al. 2002a). *Leuconostoc* strains producing the two leucocins need both immunity proteins.

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