

A counterselection method for *Lactococcus lactis* genome editing based on class IIa bacteriocin sensitivity

Xing Wan¹ · Anne M. Usvalampi^{1,2} · Per E. J. Saris¹ · Timo M. Takala¹

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Abstract In this paper, we present a new counterselection method for deleting fragments from *Lactococcus lactis* chromosome. The method uses a non-replicating plasmid vector, which integrates into the chromosome and makes the cell sensitive to bacteriocins. The integration vector carries pUC *ori* functional in *Escherichia coli* but not in *L. lactis*, an erythromycin resistance gene for selecting single crossover integrants, and two fragments from *L. lactis* chromosome for homologous recombinations. In addition, the integration vector is equipped with the *Listeria monocytogenes* gene *mptC* encoding the mannose-phosphotransferase system component IIC, the receptor for class IIa bacteriocins. Expression of *mptC* from the integration vector renders the naturally resistant *L. lactis* sensitive to class IIa bacteriocins. This sensitivity is then used to select the double crossover colonies on bacteriocin agar. Only the cells which have regained the endogenous bacteriocin resistance through the loss of the *mptC* plasmid will survive. The colonies carrying the desired deletion can then be distinguished from the wild-type revertants by PCR. By using the class IIa bacteriocins leucocin A, leucocin C or pediocin AcH as the counterselective agents, we deleted 22- and 33-kb chromosomal fragments from the wild-type nisin producing *L. lactis* strain N8. In conclusion, this

counterselection method presented here is a convenient, efficient and inexpensive technique to generate successive deletions in *L. lactis* chromosome.

Keywords Counterselection · Class IIa bacteriocin · Genome editing · Lactic acid bacteria

Introduction

Chromosomal deletions in bacteria are conventionally accomplished by two homologous recombinations using an integration vector with two chromosomal fragments neighbouring the area to be deleted. This approach usually involves two crossover steps. First, two chromosomal fragments flanking the target deletion area are ligated to each other in a vector not replicating in the target host. The construct is then integrated into the chromosome at either of the two homologous regions via single crossover (SCO) recombination. The selection of integrants is typically based on antibiotic resistance. Then, the integrants are screened for a second recombination, resulting in the deletion of the target region, or a reversion to the wild-type genotype. Since the frequency of the second recombination can be low and only part of the double crossover (DCO) colonies carry the deletion, finding the desired strain without selection often requires extensive screening. Therefore, a variety of systems have been developed to either select the DCO or increase its frequency. For instance, temperature-sensitive (TS) pG⁺Host vectors and Cre-*loxP* plasmids have been used for *Lactococcus lactis* genome editing (Biswas et al. 1993; Zhu et al. 2015b). The integration of a pG⁺Host vector into the chromosome takes place at 37 °C, after which the plasmid is excised by second crossover at 28 °C. TS vectors are good tools for creating single knockout strains. However, culturing *L. lactis* at 37 °C causes heat-induced mutations, which makes

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✉ Timo M. Takala
timo.takala@helsinki.fi

¹ Department of Food and Environmental Sciences, University of Helsinki, P.O. Box 56, Helsinki, Finland

² Department of Biotechnology and Chemical Technology, School of Chemical Technology, Aalto University, P.O. Box 16100, Espoo, Finland

TS vectors less suitable for successive deletions in the same strain (Smith et al. 2012). In the Cre-*loxP* system, the chromosomal region to be deleted is first replaced by the chloramphenicol resistance gene *cat* with flanking *lox* recognition sites for the Cre recombinase (Lambert et al. 2007). Then, via transient expression of the Cre recombinase, the *lox-cat-lox* cassette is removed. However, there is no actual selection for the gene replacement; instead, the second crossover can be found only by screening for erythromycin sensitivity. In addition, the method is not completely seamless, as it leaves short inactive *lox*-sites into the chromosome.

Other tools for generating chromosomal deletions are counterselectable vectors, which carry both positive and negative selection markers. The integration of the vector is selected with the positive marker, usually an antibiotic resistance gene, and the loss of the vector through second homologous recombination is selected with the negative marker causing sensitivity to a selective agent. Thus, only those strains, which have undergone the second recombination and lost the integrated vector, will survive. For *L. lactis*, the vector pCS1966 carrying the orotate transporter gene *oroP* as a counterselectable marker has been developed (Solem et al. 2008). Expression of *oroP* renders the cells sensitive to 5'-fluoroorotic acid (FOA), which is then used to select the second recombination. Even though the method seems effective, the FOA selection has some practical inconveniences. Firstly, FOA is rather expensive compound and poorly soluble in aqueous solutions. In addition, for selecting DCO colonies, *L. lactis* has to be cultured in chemically defined media supplemented with FOA. Moreover, *oroP* is a common gene among *L. lactis* strains (Siezen et al. 2011), which causes the risk of unwanted homologous recombinations between the native and the introduced *oroP* genes.

Another counterselection method developed for lactic acid bacteria is based on uracil phosphoribosyltransferase (*upp*) for *Lactobacillus acidophilus*, *Lactobacillus casei* and *L. lactis* (Goh et al. 2009; Song et al. 2014). The expression of *upp* from integration vector causes sensitivity to 5-fluorouracil, which can be used to select the DCO colonies. As *upp* gene is commonly existing and expressed in lactic acid bacteria, the deletion of *upp* from the host is a prerequisite for its use in counterselection. Another possible inconvenience with the *upp* system is that regardless of the *upp* mutation, 5-fluorouracil may still be metabolised and thus be toxic to the mutants (Martinussen and Hammer 1994).

Class IIa (pediocin-like) bacteriocins are *Listeria*-active peptides, which form a membrane pore leading to cell leakage (Drider et al. 2006). On sensitive cells, the component IIC (MptC) of the mannose-phosphotransferase system (Man-PTS) is responsible for the specific binding of the class IIa bacteriocins (Kjos et al. 2010; Ramnath et al. 2004). *L. lactis* is naturally resistant to class IIa bacteriocins, as the *Lactococcus* Man-PTS component IIC (PtnC) and MptC in

Listeria are different. However, heterologous expression of listerial *mptC* gene in *L. lactis* renders the lactococcal cells sensitive to class IIa bacteriocins, as MptC can form a functional Man-PTS permease complex with the lactococcal component IID (Kjos et al. 2010).

In this study, we describe a counterselection system for genome editing in *L. lactis*. The integration vector carries erythromycin resistance gene for selecting SCO integrants and the listerial *mptC* gene for selecting the loss of the vector through the second crossover with class IIa bacteriocin. We demonstrated the efficiency of the bacteriocin counterselection by deleting chromosomal fragments over 30 kb from a wild-type *L. lactis* strain.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus* strains were grown in M17 (Oxoid Ltd. Basingstoke, UK) supplemented with 0.5 % (w/v) glucose (M17G) at 30 °C. For *L. lactis* strains LAC360, LAC409 and LAC428, 0.5 µg nisin/ml was added for plasmid maintenance. *Escherichia coli* was grown in LB at 37 °C. *Listeria monocytogenes* WSLC 1033 was grown in brain heart infusion (BHI, Lab M, Lancashire, UK) broth with shaking at 37 °C. Pediocin AcH (Ped) producer *Lactobacillus plantarum* WHE 92 was grown in MRS (Merck, New Jersey, USA) at 30 °C. For transformant selection, erythromycin (Erm) was used at final concentrations of 250 µg/ml for *E. coli* and 10 µg/ml for *L. lactis*. For selecting DCO colonies, leucocin A (LcnA) from *L. lactis* LAC428 and leucocin C (LecC) from LAC409 were used at the concentrations of 3 and 0.3 µg/ml. Alternatively, LecC and Ped were used as pasteurised cell-free culture supernatants at the concentration of 20 µl/ml.

Nucleic acid techniques

Plasmids from *E. coli* and *L. lactis* were isolated with GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA). In addition to supplier's procedure, lysozyme (10 mg/ml) was added to *L. lactis* cell resuspension solution and incubated at 37 °C for 1 h before lysing the cells.

Fragments for screening, cloning and sequencing were amplified by standard PCR with either Taq DyNAzyme™ II DNA polymerase or Phusion High-Fidelity DNA polymerase (Thermo Scientific) in Eppendorf Mastercycler (Hamburg, Germany). PCR primers used are listed in Table 2. Overlap extension PCR (OE-PCR) was used to fuse the two lactococcal chromosomal fragments for homologous recombinations with the pUC *ori* from pBluescript (Agilent Technologies, Santa Clara, CA, USA). PCR products were

Table 1 Strains and plasmids used in this study

Bacterial strain or plasmid	Relevant properties	Reference/source
Strains		
<i>Escherichia coli</i> TG1	Host for constructing integration vectors	Sambrook and Russell (2001)
<i>E. coli</i> EMIN18	TG1 carrying replicative <i>mptC</i> plasmid pWUST25	This study
<i>E. coli</i> EMIN20	TG1 carrying integration vector pWUST27	This study
<i>E. coli</i> EMIN21	TG1 carrying integration vector pWUST28	This study
<i>Lactobacillus plantarum</i> WHE 92	Wild-type pediocin AcH producer	Ennahar et al. (1996)
<i>Lactococcus lactis</i> N8	Wild-type nisin producer, target for chromosomal deletions	Valio Ltd. Helsinki, Finland
<i>L. lactis</i> LAC360	Negative control strain for MIC determinations	Li et al. (2011)
<i>L. lactis</i> LAC409	Heterologous leucocin C producer	Wan et al. (2013)
<i>L. lactis</i> LAC428	Heterologous leucocin A producer	Wan et al. (2015)
<i>L. lactis</i> MINL18	N8 carrying replicative <i>mptC</i> expression plasmid pWUST25	This study
<i>L. lactis</i> MINL20	N8 carrying integrated plasmid pWUST27	This study
<i>L. lactis</i> MINL21	N8 carrying integrated plasmid pWUST28	This study
<i>L. lactis</i> MINL25	N8 with 22-kb deletion in Tn5481	This study
<i>L. lactis</i> MINL26	N8 with 33-kb deletion including <i>xyl</i> operon	This study
<i>L. lactis</i> MINL27	MINL25 carrying integrated plasmid pWUST28	This study
<i>L. lactis</i> MINL28	N8 with 22-kb deletion in Tn5481, and 33-kb deletion including <i>xyl</i> operon	This study
<i>Listeria monocytogenes</i> WSLC 1033	ATCC 19117, Source of <i>mptC</i> gene	A gift from prof. Martin Loessner, ETH Zürich, Switzerland
Plasmids		
pLEB579	<i>L. lactis</i> cloning vector containing pSH71 <i>repAC</i> and <i>ermC</i> , <i>Erm^R</i>	Beasley et al. (2004)
pWUST25	pLEB579 carrying nisin-inducible promoter <i>P_{nisZ}</i> from <i>L. lactis</i> N8, fused with <i>mptC</i> from <i>L. monocytogenes</i> WSLC 1033, <i>Erm^R</i>	This study
pWUST27	Integration vector for <i>L. lactis</i> with pUC <i>ori</i> , <i>ermC</i> , <i>P_{nisZ-mptC}</i> , and two ~1-kb fragments for 22-kb chromosomal deletion	This study
pWUST28	Integration vector for <i>L. lactis</i> with pUC <i>ori</i> , <i>ermC</i> , <i>P_{nisZ-mptC}</i> , and two ~1-kb fragments for 33-kb chromosomal deletion	This study

purified with GeneJET PCR Purification Kit, or extracted from agarose gel with GeneJET Gel Extraction Kit (Thermo Scientific). Plasmid constructs and PCR products were sequenced by DNA sequencing service in the Institute of Biotechnology (University of Helsinki, Finland).

T4 DNA ligase, T4 polynucleotide kinase and restriction enzymes were used as recommended by the suppliers (Thermo Scientific; New England Biolabs, Ipswich, MA, USA; Promega, Madison, WI, USA). Plasmids were transferred into *E. coli* and *L. lactis* by electroporation with a Bio-Rad Gene Pulser device (Bio-Rad Laboratories, Richmond, CA, USA) essentially as described previously (Holo and Nes 1989; Zabarovsky and Winberg 1990). Two micrograms of each integration vector and 20 ng of transformation control plasmid pLEB579 were used in *L. lactis* electroporation.

Bacteriocins and MICs

LcnA and LecC were obtained from heterologous producers *L. lactis* LAC428 and LAC409 by precipitating overnight culture supernatants with ammonium sulphate, as described previously (Wan et al. 2013, 2015). The LcnA and LecC concentrations in the crude preparations were estimated to be about 150 and 100 µg/ml, respectively, by comparing band intensities with standard protein samples in SDS-PAGE, as described previously (Wan et al. 2015). *L. lactis* strain LAC360, which carries the empty cloning vector pLEB690 (Li et al. 2011), was treated in the same way as LAC409 and LAC428 and used as a negative control in minimum inhibitory concentration (MIC) determinations.

To determine the MICs, *mptC* host *L. monocytogenes* WSLC 1033, *L. lactis* N8, the strain MINL18 with

Table 2 PCR primers used in this study

Primer name	Use	Sequence 5' → 3', <u>restriction site</u>
Erm F	<i>ermC-repAC</i> , <i>Sall</i>	ACTGGT <u>CGAC</u> CGTTAAACCGTGTGCTCTACG
Rep R	<i>ermC-repAC</i> , <i>ApaI</i>	ATAGGGCCCAAATAAAAAGCCCCCTTCGACT
Erm R	<i>ermC-P_{nisZ}mptC</i>	CGCTAAAACGTCTCAGAAACG
Rep down F	<i>ermC-P_{nisZ}mptC</i>	GAGGGCAATTATCAGTGTGG
Pnis F	<i>nisZ</i> promoter, <i>XhoI</i>	ACTGCTCGAGAGTCTTATAACTATACTGAC
Pnis R	<i>nisZ</i> promoter, <i>NcoI</i>	TATGCGCGCCCATGGTGAGTGCCTCCTATAATTTATTTTG
MptC F	<i>mptC</i> , <i>PciI</i>	GCTACATGTCTGTCAATCAATAATTTTAG
MptC R	<i>mptC</i> , <i>ApaI</i>	ACTGGGCCCTTAATAGTCGTTAATATATCGC
22H1 F	Homologous region 1 for 22-kb deletion, OE-PCR, DCO screening, sequencing	GCAGTCCTTACTTCTGAGAC
22H1 OE-H2 R	Homologous region 1 for 22-kb deletion	TAATCTAAAGACGATTTGGACTTTGTGGAAGCAG
22H2 OE-H1 F	Homologous region 2 for 22-kb deletion	AAAGTCCAAATCGTCTTTAGATTAGAGTTCTGTG
22H2 OE-ori R	Homologous region 2 for 22-kb deletion, DCO screening, sequencing	GTTTCCCGACTGATTGGTACAGGTATTGGAGG
33H1 F	Homologous region 1 for 33-kb deletion, OE-PCR, DCO screening, sequencing	TCAGCAGCTAAATCACTGAC
33H1 OE-H2 R	Homologous region 1 for 33-kb deletion	TGCCGAACCATTGTGTACTCTTCCCAAATCTC
33H2 OE-H1 F	Homologous region 2 for 33-kb deletion	GGAAGAGTACACAATGGTTCGGCAACGATTGG
33H2 OE-ori R	Homologous region 2 for 33-kb deletion, DCO screening, sequencing	GTTTCCCGACTGACACCACCTGAAAAGTCCAAG
Ori F OE22	pUC <i>ori</i> , overlap extension to 22H2	ACCTGTACCAATCAGTCGGGAAACCTGTCGTG
Ori F OE33	pUC <i>ori</i> , overlap extension to 33H2	TTCAGGTGGTGTGTCAGTCGGGAAACCTGTCGTG
Ori R	pUC <i>ori</i> , OE-PCR	GTCCACTGAGCGTCAGACC

replicative *mptC* plasmid and the strain MINL20 with integrated *mptC* plasmid were cultured with serial dilutions of LcnA or LecC. Honeycomb microtiter plate wells were filled with 200 μ l of appropriate media inoculated with 0.2 % of overnight culture of each indicator strain. Leucocins (LcnA 0, 0.5, 1 μ g/ml; LecC 0, 0.01, 0.02, 0.05 μ g/ml) and the negative control were added to the wells. The plates were incubated in Bioscreen C (Labsystems, Helsinki, Finland) at 30 °C with constant shaking for *Listeria* and at 30 °C with 30-s shaking before measurement for *L. lactis*. The optical density was measured every hour with a wideband filter (420–580 nm). All MIC determinations were performed in triplicate. The MIC was defined as the lowest bacteriocin concentration for which OD value of all three parallel cultures was below 0.1 in 12 h.

Construction of plasmids

Construction of the replicative *mptC* plasmid pWUST25 and the integration vectors pWUST27 and pWUST28 is shown in Fig. 1. The *mptC* from *L. monocytogenes* WSLC1033 was fused with the nisin-inducible promoter *P_{nisZ}* from *L. lactis* N8 to create *P_{nisZ}-mptC* fragment as follows: Individual fragments of *mptC* and *P_{nisZ}* were amplified from *L. monocytogenes* and *L. lactis* genome with primers MptC

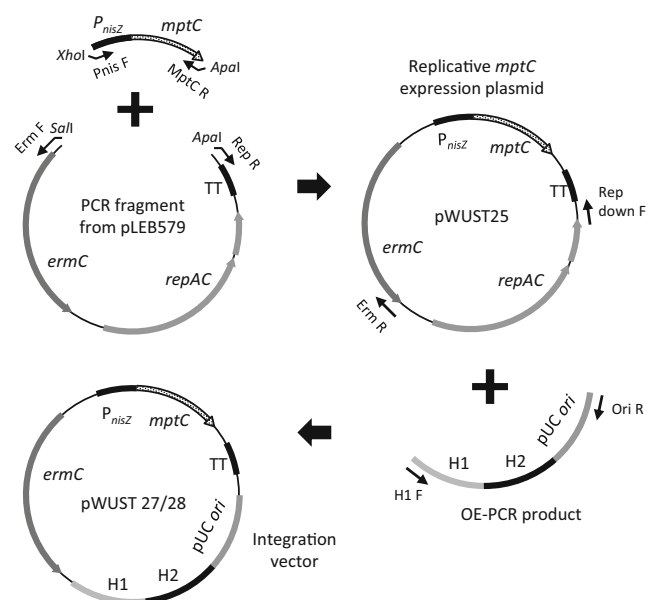


Fig. 1 Construction of replicative *mptC* expression plasmid pWUST25 and integrative plasmids pWUST27 and pWUST28 (not in scale). The replicative plasmid was made of a *P_{nisZ}-mptC* fragment ligated with *repAC* + *ermC* fragment. For integration vectors, *repAC* was replaced by the OE-PCR fusion of pUC *ori* and two chromosomal fragments (H1 and H2, about 1 kb each) for homologous recombinations. PCR primers used are shown with short arrows. Restriction sites *ApaI* and *Sall/XhoI* used for constructing pWUST25 are marked in the end of the primers. *TT* transcription terminator

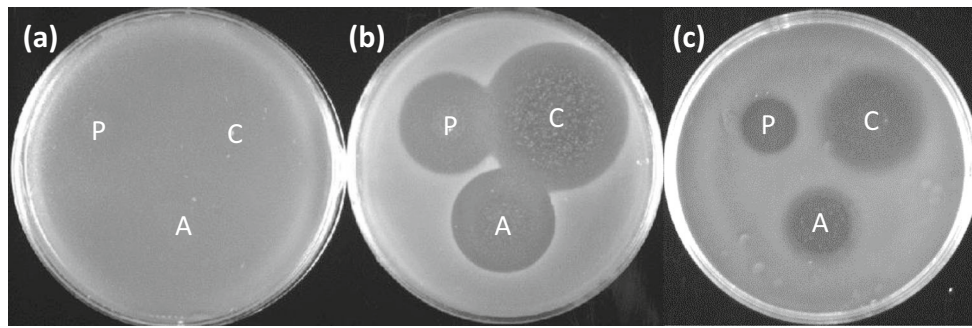


Fig. 2 Sensitivity to class IIa bacteriocins LcnA, LecC and pediocin of a wild-type *Lactococcus lactis* N8, **b** *Listeria monocytogenes* WSLC 1033 and **c** *L. lactis* N8 derivative MINL18 carrying replicative *mptC* expression plasmid. Leucococins A and C were used as crude preparations,

pediocin as cell-free culture supernatant. Three microliters of leucococins and 10 μ l of pediocin were spotted on indicator lawns. A LcnA, C LecC, P pediocin

F-MptC R and Pnis F-Pnis R, respectively. The two fragments were then restricted with *Pci*I and *Nco*I separately, and ligated together. The ligated product was used as the template in PCR with Pnis F-MptC R primers. The *P_{nisZ}-mptC* fragment was restricted with *Xho*I and *Apa*I, ligated with *Sall*-*Apa*I digested *repAC-ermC* fragment amplified from the *L. lactis* cloning vector pLEB579. The replicon *repAC* originating from the lactococcal plasmid pSH71 (Gasson 1983) is also functional in *E. coli*. The ligation mixture was electroporated into *E. coli* TG1, the right clone was found by PCR screening, and the resulting *mptC* expression plasmid pWUST25 was isolated and electroporated into *L. lactis* N8 for testing the MptC function from replicative multicopy plasmid.

For the integrative plasmids, the *repAC* genes in pWUST25 were replaced with the fusion of pUC *ori* from pBluescript and two chromosomal fragments (H1, H2, about 1 kb each) from *L. lactis* N8. The genome sequence of *L. lactis* N8 was kindly provided by Professor M. Qiao (Nankai University, China). First, the three pieces of H1, H2 and pUC *ori* were joined by OE-PCR, and the resulting fragment was then ligated blunt-ended with *ermC-P_{nisZ}-mptC* amplified from pWUST25. The transcription terminator of *repAC* was included in the *ermC-P_{nisZ}-mptC* fragment to prevent transcriptional elongation downstream of *mptC*. The ligation mixtures were electroporated into *E. coli* TG1, right clones were screened by PCR, and the resulting integration vectors were isolated and electroporated into *L. lactis* N8. Two integration vectors were constructed: for chromosomal deletions of 22,286 bp from the transposon Tn5481 and a 33,256-bp region containing genes for xylose utilisation. These fragments were chosen, because they are large regions not containing any genes from the core genome of *L. lactis* (Siezen et al. 2011; M. Qiao, unpublished). Transposon Tn5481 is also known as the nisin-sucrose transposon, but the aimed 22-kb deletion included neither nisin nor sucrose genes. The putative genes included in the two regions to be deleted are listed in Table S1.

Generation of deletion mutants

After electroporation into *L. lactis* N8, transformants with integrated plasmid were selected with erythromycin, and the correct SCO integration was confirmed by PCR and bacteriocin sensitivity on M17G + Erm + LecC plate. For the second crossover, integrants were grown o/n in M17G + Erm broth, followed by five transfers (1 % inoculum) in M17G broth without Erm (about 35 generations). The cultures were serially diluted to 10^{-4} , from which 0.1 ml were spread onto M17G agar plates supplemented with LcnA, LecC, or Ped. For comparison, the o/n M17G + Erm broth culture was diluted to 10^{-2} , and spread similarly onto M17G + bacteriocin agar plates. The plates were incubated overnight, and the obtained colonies were confirmed not to carry integration vectors by Erm sensitivity on M17G + Erm agar plate. The bacteriocin resistant, Erm-sensitive DCO colonies were screened by PCR with corresponding H1 F – H2 R primers, and the correct chromosomal deletions were confirmed by sequencing. Growth of the deletion strains in M17 broth supplemented with 0.5 % glucose or 1 % xylose were examined using Bioscreen C (Labsystems) with 1 % inoculums and measuring the optical density every hour with a wideband filter (420–580 nm).

For generating double deletion mutant, the Δ 22-kb *L. lactis* strain MINL25 was used as the host in transformation to uptake the integration vector for the 33-kb deletion. Consequent SCO and DCO strains were obtained and screened by the same above-mentioned method.

Results

Expression of *mptC* in *L. lactis* N8

Previously, it has been demonstrated that expression of listerial *mptC* in a nonsensitive *L. lactis* strain without Man-PTS resulted in sensitivity to class IIa bacteriocins (Kjos et al.

2010). Since *L. lactis* N8 carries the *ptn* genes encoding Man-PTS, the first aim here was to test whether the expression of listerial *mptC* makes the wild-type *L. lactis* N8 sensitive to class IIa bacteriocins. As listerial component IIC can form a functional Man-PTS permease complex with lactococcal component IID, expressing only the IIC component from *Listeria* could be sufficient to make *L. lactis* N8 sensitive to class IIa bacteriocins. The gene *mptC* from *Listeria* was cloned into a replicative plasmid under the control of a nisin promoter, and the construct was introduced into *L. lactis* N8, resulting in the *mptC* expression strain MINL18. Sensitivity to bacteriocins was first tested by spot-on-lawn method on plates, and compared with *Listeria* and the wild-type *L. lactis* N8. *L. monocytogenes* WSLC 1033 and the strain MINL18 showed high sensitivity to class IIa bacteriocins leucocins A and C and pediocin AcH, as large halos were obtained on indicator plates, whereas the host strain N8 was completely resistant (Fig. 2). On both *Listeria* and MINL18 plates, small colonies grew inside the bacteriocin halos, indicating that *L. lactis* can develop bacteriocin resistance similarly as *Listeria*. Even though the inhibition zones were bigger on *Listeria* plate, the MICs of both *L. monocytogenes* WSLC 1033 and *L. lactis* MINL18 were determined to be 1 µg/ml for LcnA and 0.02 µg/ml for LecC. In conclusion, the expression of listerial *mptC* made the wild-type *L. lactis* sensitive to class IIa bacteriocins.

Construction of the integration vectors

The integration vectors for chromosomal deletions were constructed by replacing the *repAC* from the replicative *mptC* plasmid pWUST25 with pUC *ori* and two fragments from *L. lactis* N8 chromosome for homologous recombinations (Fig. 1). The strategy to obtain chromosomal deletion through two homologous recombinations is presented in Fig. 3.

Two integration vectors were constructed, aiming to delete a 22-kb region in the transposon Tn5481, and a 33-kb region containing xylose utilisation genes from *L. lactis* N8 chromosome.

Single crossover

The constructed integration vectors and the transformation control plasmid pLEB579 were electroporated into *L. lactis* N8, and the transformants were selected with erythromycin. Number of colonies obtained from 2 µg of electroporated integration vectors aiming at 22- and 33-kb deletions was 5 and 7, respectively, while transformation frequency was about 10⁶ colonies per microgram of pLEB579. Correct integrants were verified by PCR, and their sensitivity to class IIa bacteriocins was tested on M17G + Erm agar by spot-on-lawn method. All three bacteriocins, leucocin A, leucocin C and pediocin AcH, caused large inhibition zones, showing that *L. lactis* with integrated *mptC* renders the cells sensitive to the bacteriocins (Fig. 4). The MIC for *L. lactis* integrant was

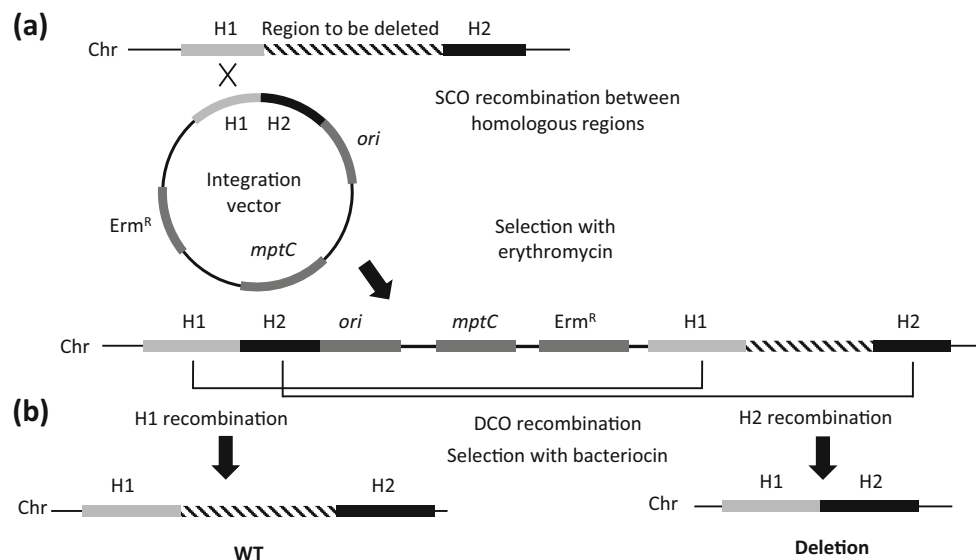


Fig. 3 Scheme of using *mptC*-based selection/counterselection integration vector for constructing gene knockout in *L. lactis* (not in scale). **a** Plasmid carrying two homologous regions (H1, H2) flanking the region to be deleted in *L. lactis* N8 genome is integrated into the chromosome at either of the homologous regions, and the integrant is selected by erythromycin resistance. The resulting integrant becomes sensitive to class IIa bacteriocins. **b** A second recombination event at H1 or H2 leads to the

excision of the integrated plasmid. Only the cells which have lost the integration vector can survive in the presence of class IIa bacteriocin. The second recombination at the same homologous region (H1) as in the integration event reverts the integrant to the wild type. If the second recombination takes place at H2, the event results in a chromosomal deletion between H1 and H2. Chr chromosomal DNA; diagonal stripes the region to be deleted

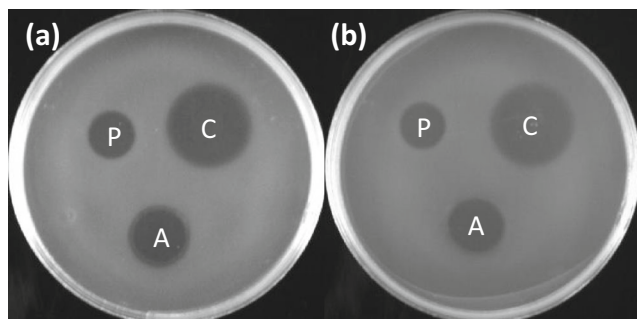


Fig. 4 Class IIa bacteriocin sensitivity of *Lactococcus lactis* N8 carrying integrated *mptC*-vectors. Indicators: **a** MINL25 for 22-kb deletion. **b** MINL26 for 33-kb deletion. LcnA and LecC were used as crude preparations, pediocin as cell-free culture supernatant. Three microliters of leucocins and 10 μ l of pediocin were spotted on indicator lawns. A LcnA, C LecC, P pediocin

the same as those of the replicative multicopy *mptC* plasmid (1 μ g/ml for LcnA, 0.02 μ g/ml for LecC).

Selecting the DCO colonies and finding the deletions

To find the clones, which has lost the integration plasmid through second recombination, two strategies were tested: first, the integrants were grown in M17G broth without Erm for about 35 generations. Alternatively, the integrants were cultured in M17G with Erm overnight. Both cultures were then serially diluted and plated onto bacteriocin agar. Twenty microliters per milliliter of LecC or Ped producer culture supernatant was shown to be sufficient to select DCO colonies, virtually without background or bacteriocin resistant mutants. From Erm-free pre-culture, about 1 % of the plated cells grew on bacteriocin plates, whereas from antibiotic pre-culture, about 0.01 % of the cells were resistant to bacteriocins. Nearly all (about 99 %) of the obtained colonies were confirmed to be Erm-sensitive, showing the excision of the integration vector through the second recombination. These colonies either contained the desired chromosomal deletion, or had reverted to the wild-type, as presented in Fig. 3.

The deletion strains were distinguished from the wild-type revertants by PCR and confirmed by sequencing. The proportion of the 22- and 33-kb deletions was about 10 and 40 %. The growth of the deletion strains on glucose and xylose was then examined. Compared to the wild-type, the deletions did not significantly affect the strains' growth on glucose (Fig. 5). As expected, the 33-kb deletion strain did not grow on xylose, showing that the deletion altered the strain's phenotype.

Double deletion

To test if the presented counterselection method can be used to generate multiple deletions in the same strain, 2 μ g of the integration vector pWUST28 aiming at deleting the 33-kb xylose region was introduced into the Δ 22-kb strain

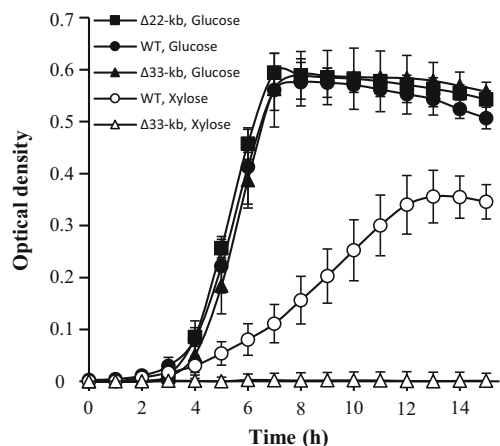


Fig. 5 Growth of *L. lactis* N8 (WT) and the deletion strains Δ 22- and Δ 33-kb on M17 supplemented with glucose or xylose. The optical density was measured with a wideband filter (420–580 nm) in Bioscreen C. Error bars standard deviation of the mean from three parallel cultures. The optical density of the growth in M17 without sugar is subtracted from the values in the graph

MINL25 yielding four SCO colonies. The same selection-counterselection procedure was followed, and the deletion of 33-kb region was confirmed by PCR, sequencing and xylose negative phenotype. About 8 % of the colonies on bacteriocin selection plates contained the correct double deletion. In conclusion, the method described in this paper can be used to create successive chromosomal deletions in *L. lactis* strain.

Discussion

In this study, we present a new counterselection method for generating markerless chromosomal deletions in wild-type nisin producing *L. lactis*. The method is based on bacteriocin sensitivity by expressing the *L. monocytogenes* gene *mptC* encoding Man-PTS component IIC, the receptor for class IIa bacteriocins. This is the first time when bacteriocin sensitivity marker is used for counterselection. First step in the method is to integrate *mptC* plasmid vector into the chromosome by homologous recombination. As a result, the integrant becomes resistant to erythromycin and sensitive to class IIa bacteriocins. Second step is to plate the integrant onto bacteriocin agar to select the cells which have lost the integrated vector through second homologous recombination. Thus, the bacteriocin selection completely eliminates the screening of DCO colonies using replica plates with and without antibiotic. The position of the second crossover determines if the DCO colony contains the desired chromosomal deletion or if it has reverted to wild-type. These two genome types can be differentiated for example by PCR. It is noticeable that the ratio between the deletion and wild-type may not be 1 to 1, but it can vary considerably depending on the genes to be deleted. In this study, the proportion of the colonies carrying the deletion

varied from about 8 to 40 %. Considering the large sizes of the deletions (22 and 33 kb), the frequencies are reasonably high, possibly because these regions did not contain any *L. lactis* core genes and their deletion did not affect the growth in M17G. In addition to the two deletions presented in this paper, we have used the method for other non-essential knockouts, of which the biggest was about 35 kb (data not shown). Also in these cases, the deletion frequencies have varied from about 10 to 40 %.

In the conventional gene knockout procedure by two cross-over events, the integrant is pre-cultured in non-selective media for many generations before plating and searching of second crossover colonies. Here, we showed that this pre-culturing step in bacteriocin counterselection was unnecessary, as second crossover colonies could be obtained by plating the integrant directly from the selective media onto bacteriocin plate. The second crossover in the cells could have happened already in the antibiotic broth culture before plating. Because Erm is a bacteriostatic antibiotic, these Erm-sensitive cells may have survived overnight with the antibiotic. About 0.01 % of cells from Erm containing culture were bacteriocin resistant, thus giving the second crossover frequency 10^{-4} .

The integrants showed high sensitivity to the bacteriocins. According to the MIC determinations, the bacteriocin sensitivity was in the same range as in the *mptC* host *L. monocytogenes* WSLC 1033. We have noticed that for high bacteriocin sensitivity and efficient DCO selection, strong expression of *mptC* is needed (unpublished results). The expression of *mptC* in the integration vector was controlled by the strong nisin-inducible promoter P_{nisZ} (Li et al. 2011). However, nisin promoter is a suitable choice only for strains carrying the nisin-specific two-component signal transduction system (Kuipers et al. 1995). To apply the method in non-nisin *L. lactis* strains, other promoter should be used. Many strong promoters have been characterised for *L. lactis*, some of which could replace the P_{nisZ} used here. For constitutive gene expression, the phosphotransferase promoter P8 has been shown to be even stronger than the nisin promoter (Zhu et al. 2015a). However, high expression of *mptC* may cause stability problems in *E. coli*. In that case, inducible expression system, such as the agmatine-controlled expression system ACE (Linares et al. 2015) or the zinc-regulated expression system Zirex (Mu et al. 2013) could be applied. Yet, the suitability of a promoter should be tested for each *L. lactis* strain.

Currently, there is an increasing interest of using the CRISPR/Cas9 system for genomic deletions in bacteria, including lactic acid bacteria (Oh and van Pijkeren 2014; van Pijkeren and Britton 2014). However, so far, there are no reports about its use in *Lactococcus* genome editing. Instead, chromosomal deletions in *L. lactis* have often been generated by using pG⁺Host thermosensitive vectors (Biswas et al. 1993). These vectors are useful tools for single knockouts, but since culturing *L. lactis* at 37 °C induces spontaneous heat

resistant mutations, consecutive uses of the thermosensitive vector in the same strain seems impossible (Smith et al. 2012). The bacteriocin counterselection presented here did not have such a limitation, as a new *mptC* integration vector could be introduced into the $\Delta 22$ -kb strain, and the loss of the vector could be selected with bacteriocin resulting in the strain with two chromosomal deletions.

In addition to the thermosensitive vectors, two counterselectable markers have been published for genome editing of lactic acid bacteria: the gene *oroP* encoding orotate transporter in *L. lactis* (Solem et al. 2008), and the gene *upp* encoding uracil phosphoribosyltransferase in *L. acidophilus*, *L. casei* and *L. lactis* (Goh et al. 2009; Song et al. 2014). Expression of the genes caused sensitivity to 5-fluoroorotate or 5-fluorouracil, which were then used to select the loss of the integration vector through the second recombination event. The bacteriocin selection presented in this paper has several practical advantages over those two counterselection methods. First of all, selection of the DCO colonies with bacteriocin does not require chemically defined or semi-defined media, but simply commercial M17 agar supplemented with glucose and class IIa bacteriocin. Moreover, the chromosomal deletions could be made in a wild-type *L. lactis* without pre-treatments, e.g. deletion of endogenous Man-PTS. In fact, the Man-PTS permease component IID (PtnD) from the *L. lactis* host is probably needed for a functional bacteriocin receptor. As shown by Kjos et al. (2010), PtnD can form an active permease with listerial MptC. Like most *L. lactis* strains (Siezen et al. 2011), also N8 carries *ptnABCD* genes for Man-PTS (M. Qiao, unpublished). Hence, presumably because of the native *ptnD* gene, the introduction of *mptC* alone was enough to make *L. lactis* sensitive to bacteriocins.

For selection, purification of bacteriocins was not needed, but they could be applied as crude precipitates or pasteurised cell-free supernatants from bacteriocin producer cultures. Only 20 μ l/ml of leucocin C or pediocin containing supernatant could select the loss of the integrated plasmid. When tested on M17G + Erm agar, the majority of the bacteriocin resistant colonies were Erm-sensitive, confirming the excision of the integration vector. Only about 1 % of the colonies obtained on bacteriocin agar turned out to be resistant to Erm, indicating a development of spontaneous resistance to bacteriocins. As the recombination frequency here was shown to be approximately 10^{-4} , the frequency of developing resistance was thus 10^{-6} , similar as what has been reported with *L. monocytogenes* (Gravesen et al. 2002).

In conclusion, the bacteriocin counterselection presented here is a convenient method to generate chromosomal deletions in *L. lactis*. The method was efficient, simple, inexpensive, and it could be used successively in the same wild-type strain.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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