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Large-scale chromosome flip-flop reversible inversion mediates phenotypic switching of expression of antibiotic resistance in lactococci

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ABSTRACT

Bacteria can gain resistance to antimicrobials by acquiring and expressing genetic elements that encode resistance determinants such as efflux pumps and drug-modifying enzymes, thus hampering treatment of infection. Previously we showed that acquisition of spectinomycin resistance in a lactococcal strain was correlated with a reversible genomic inversion, but the precise location and the genes affected were unknown. Here we use long-read whole-genome sequencing to precisely define the genomic inversion and we use quantitative PCR to identify associated changes in gene expression levels. The boundaries of the inversion fall within two identical copies of a prophage-like sequence, located on the left and right replichores; this suggests possible mechanisms for inversion through homologous recombination or prophage activity. The inversion is asymmetrical in respect of the axis between the origin and terminus of the replication and modulates the expression of a SAM-dependent methyltransferase, whose heterologous expression confers resistance to spectinomycin in lactococci and that is up-regulated on exposure to spectinomycin. This study provides one of the first examples of phase variation via large-scale chromosomal inversions that confers a switch in antimicrobial resistance in bacteria and the first outside of *Staphylococcus aureus*.

1. Introduction

Antibiotic resistance is among the greatest threats to global health, food security, and development facing mankind today (de Kraker et al., 2016). The health burden of infections caused by antibiotic resistant bacteria is as severe as that of influenza, tuberculosis and human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) combined (Cassini et al., 2019). Strategies by which bacteria can overcome antimicrobial drugs include use of efflux pumps, inactivating enzymes, alternative metabolic pathways, impermeable cell membranes and changes in cell structure and have been comprehensively reviewed elsewhere (Blair et al., 2015; Morar and Wright, 2010; Peterson and Kaur, 2018; Wright, 2007). Expression of resistance can be energetically costly and could impose a fitness penalty under laboratory conditions (Andersson, 2006; Kojic et al., 2008; Stickland et al., 2010). Inevitably, there is a trade-off between the advantage and the burden of carrying resistance. This is expected to lead to irreversible loss of resistance through replacement or mutation, with the former being the more probable (Andersson, 2003; Levin, 2001). However, complete and irreversible re-sensitization has not been widely observed (Baym et al., 2016), suggesting that some bacteria within a population are able to reversibly switch between resistance and susceptibility.

Studies of resistance acquisition have tended to emphasize point mutations in target molecules and horizontal gene transfer (Croucher et al., 2016). However, using pulsed-field electrophoresis we previously showed that spontaneous appearance of spectinomycin-resistant variants in a population of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis S50 was correlated with a large chromosomal inversion and that the resistant variants bore a significant fitness cost (Kojic et al., 2008); that cost might be escaped in the absence of antimicrobial selection by reversing the genomic inversion. However, at the time of the previous study, lack of whole-genome sequence data precluded the identification

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of the precise genomic location of and boundaries of the inversion.

This phenomenon in lactococci is reminiscent of the large reversible inversion ("flip-flop") previously described in *Staphylococcus aureus*, where it underlies phase variation between large- and small colony variants (Cui et al., 2012; Guérillot et al., 2019). Phase variation is a genetically regulated mechanism by which a bacterial population responds to a changing environment (Ahmad et al., 2017; Phillips et al., 2019). There are no other documented examples of large chromosomal inversions as the basis for phase switching between antimicrobial resistance and susceptibility, to the best of the authors' knowledge.

The main goal of the current study was to characterize in detail the chromosomal inversion that reversibly confers resistance to spectinomycin in *L. lactis* subsp. *lactis* bv. diacetylactis S50. Thereby, we shed light on the molecular mechanism of this little-studied single-step strategy by which bacteria can acquire resistance to an antimicrobial drug. Previous attempts to discover the ends of the inversion by short-read sequencing entire genomes were unsuccessful, but revealed that sequences repeated with high sequence identity are most likely involved. Therefore, in the present study we deployed long-read sequencing to resolve the repetitive sequences that could not be resolved using short sequence reads. An additional aim was to elucidate the changes in gene transcription and assess whether resistance is transmissible to other bacteria by cloning of selected genes involved in the genomic inversion and spectinomycin resistance.

2. Material and methods

2.1. Bacterial strains and culture conditions

All strains and derivatives used in this study are listed in Table 1. Lactococcal strains were grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (HiMedia Laboratories, Mumbai, India) (0.5 % w/v) (GM17) at 30 °C. *Escherichia coli* DH5α was grown aerobically in Luria-Bertani (LB) broth (HiMedia Laboratories, Mumbai, India) at 37 °C. Solid medium was made by adding 1.75 % (w/v) agar (Torlak, Belgrade, Serbia), to the liquid media. Antibiotics were used at the following concentrations: Erythromycin (SERVA Electrophoresis GmbH, Heidelberg, Germany) was used at 300 μg/mL for *E. coli* and 10 μg/mL for lactococci for selection and maintaining of transformants. Spectinomycin (Merck KGaA, Darmstadt, Germany) was used at 250 and 500 μg/mL for maintaining of invertants and 50, 100, 150, 200, 250, 300, 350, 400 and 500 μg/mL for determination of MIC values for spectinomycin of transformants in lactococci. For blue/white colour screening, 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) (Fermentas, Vilnius, Lithuania) was added to LB medium plates of colonies with cloned fragments at a final concentration of 40 μg/mL.

2.2. DNA manipulations

For plasmid isolation from *E. coli*, the QIAprep Spin Miniprep kit was used according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Total DNA from *Lactococcus lactis* subsp. *lactis* bv. diacetylactis S50 and spectinomycin-resistant derivative S50-1RSS were isolated by modified method described by Hopwood et al. (1985); the logarithmic-phase cells were pre-treated with lysozyme (4 mg/mL, for 15 min at 37 °C) prior to treatment with SDS. Standard heat-shock transformation was used for plasmid transfer into *E. coli* DH5α (Hanahan, 1983). *L. lactis* subsp. *cremoris* MG7284 and *L. lactis* subsp. *lactis* IL1403 were transformed with plasmid constructs by electroporation using the method described by Holo and Nes (1989) with the modifications specified by Miljkovic et al. (2018). Digestion with restriction enzymes was conducted according to the supplier's instructions (Thermo Fisher Scientific, Waltham, MA, USA). DNA was ligated with T4 DNA ligase (Agilent technologies, Santa Clara, CA, USA) according to the manufacturer's recommendations. Platinum™ *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific, Waltham, MA, USA) was used to

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmids	Relevant characteristics	Source or reference
***Lactococcus lactis* subsp. *lactis* bv. diacetylactis**		
S50	Natural isolate from butter Prt^+, Bac^+, Bac^r, Rif^r, Str^s, Spc^s	Kojic et al., 1991
S50-1RSS	Spectinomycin resistant derivative of S50 Prt–, Bac–, Bac^s, Rif^r, Str^r, Spc^r	Kojic et al., 2005
L. lactis* subsp. *lactis		
IL1403	Prt–, Bac^s	Chopin et al., 1984
IL1403/pAZIL-D8K17_05845-9		This study
IL1403/pAZIL-D8K17_05845-10		This study
IL1403/pAZIL-D8K17_07155-1		This study
IL1403/pAZIL-D8K17_07155-4		This study
IL1403/pAZIL-D8K17_06285-1		This study
IL1403/pAZIL-D8K17_06285-2		This study
IL1403/pAZIL-D8K17_06055-c-1		This study
IL1403/pAZIL-D8K17_06055-c-2		This study
Lactococcus lactis* subsp. *cremoris		
MG1363	Prt^-, Lac^-, Bac^s	Gasson, 1983
MG1363/pAZIL-D8K17_05845-9		This study
MG1363/pAZIL-D8K17_05845-10		This study
MG1363/pAZIL-D8K17_07155-1		This study
MG1363/pAZIL-D8K17_07155-4		This study
MG1363/pAZIL-D8K17_06285-1		This study
MG1363/pAZIL-D8K17_06285-2		This study
MG1363/pAZIL-D8K17_06055-c-1		This study
MG1363/pAZIL-D8K17_06055-c-2		This study
Escherichia coli		
DH5α	*supE44 ΔlacU169 (ø80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
Plasmids		
pAZIL	7109 bp, Em^r, shuttle cloning vector	Kojic et al., 2011
pAZIL-D8K17_05845-9 pAZIL-D8K17_05845-10	pAZIL vector carrying the gene encoding multi antimicrobial extrusion protein (Na(+)/drug antiporter)	This study
pAZIL-D8K17_07155-1 pAZIL-D8K17_07155-4	pAZIL vector carrying the gene encoding SAM-dependent 16S rRNK methyltransferase	This study
pAZIL-D8K17_06285-1 pAZIL-D8K17_06285-2	pAZIL vector carrying the gene encoding 16S rRNA (guanine(527)-N(7))-methyltransferase	This study
pAZIL-D8K17_06055-c-1 pAZIL-D8K17_06055-c-2	pAZIL vector carrying the gene encoding 16S rRNA (guanine(1207)-N(2))-methyltransferase	This study

Prt^+/Prt^- – proteinase positive/negative, Bac^+/Bac^- – bacteriocin positive/negative; Bac^s – bacteriocin sensitive; Lac^- – lactose utilization negative; Em^r – erythromycin resistance; Rif^r – rifampicin resistance; Str^r – streptomycin resistance; Spc^r – spectinomycin resistance.

amplify DNA fragments by PCR in GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). DNA fragments from agarose (Lonza Group Ltd, Basel, Switzerland) gels and PCR products were purified using QIAquick Gel extraction kit as described by the manufacturer (Qiagen, Hilden, Germany). The purity and quantity of extracted DNA were assessed using a NanoDrop spectrophotometer (GE Healthcare, Life Science) and agarose gel electrophoresis against a DNA standard of known fragment sizes and concentrations (1 Kb Plus DNA Ladder, Thermo Fisher Scientific, Waltham, MA, USA). DNA sequencing was performed by the Macrogen Sequencing Service (Macrogen Europe, Amsterdam, The Netherlands).

2.3. Cloning and expression of four candidate resistance genes from *Lactococcus lactis subsp. lactis bv. diacetylactis S50 genome*

In the present study, we cloned and expressed the genes encoding: multi antimicrobial extrusion protein (D8K17_05845), S-adenosylmethionine (SAM)-dependent 16S rRNA methyltransferase (D8K17_07155), 16S rRNA (guanine^{527}-N^{7})-methyltransferase (D8K17_06285) and 16S rRNA (guanine^{1207}-N^{2})-methyltransferase (D8K17_06055). The conditions and primers (Life Technologies Ltd, Paisley, UK) used in PCR for amplification of all the selected genes are listed in Table 2. Platinum™ *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific, Waltham, MA, USA) was used for PCR and amplified products were cloned into the pAZIL vector predigested with *Sma*I. The resulting plasmids were named: pAZIL-D8K17_05845-9, pAZIL-D8K17_05845-10, pAZIL-D8K17_07155-1, pAZIL-D8K17_07155-4, pAZIL-D8K17_06285-1, pAZIL-D8K17_06285-2, pAZIL-D8K17_06055-c-1 and pAZIL-D8K17_06055-c-2. After sequencing, these clones were used for transformation of strains MG1363 and IL1403. Transformants were selected on GM17 plates containing 10 μg/mL erythromycin; resulting transformants were tested for ability to grow on spectinomycin by streaking on GM17 Petri plates containing different concentrations of spectinomycin (50, 100, 150, 200, 250, 300, 350, 400 and 500 μg/mL). Strains S50, MG1363 and IL1363 were used as negative controls, while invertant S50-1RSS was used as a positive control. All obtained derivatives are listed in Table 1.

2.4. Real time quantitative PCR (RT-qPCR)

The total RNA from S50, S50-1RSS and S50-1RSS-Spc was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) from bacterial cultures grown in spectinomycin 500 μg/mL. Lysis of cells was performed by Protocol 2: Enzymatic Lysis and Mechanical Disruption of Bacteria from RNAprotect Bacteria Reagent Handbook (Qiagen, January 2015). Residual DNA was digested using an Ambion DNA *free*™ Kit (Thermo Fisher Scientific, MA, USA). Isolated RNA was quantified using a NanoDrop spectrophotometer (GE Healthcare, Life Science) and integrity was analyzed on a 1.2 % formaldehyde-agarose gel. The first strand of cDNA was synthesized with a RevertAid RT Reverse Transcription Kit according to the instructions of the enzyme manufacturer (Thermo Fisher Scientific, MA, USA), using 1 μg of isolated RNA as a template. Random hexamers (Applied Biosystems) and RiboLock RNase inhibitor (Thermo Fisher Scientific, MA, USA) were used in the reactions. The qPCR was carried out in triplicate using the KAPA SYBR Fast qPCR Kit (KAPA Biosystems, MA, USA) in a 7500 Fast Real-Time PCR System (Applied Biosystems) under the conditions and appropriate pairs of primers listed in Table 2. The results were normalized to transcription of reference housekeeping *rpoD* gene (Larsen et al., 2016).

2.5. Statistical analysis

Statistical analyses and visualization were performed using GraphPad Prism software and SPSS 20.0 for Windows. The results are shown as means ± standard errors. The differences between control and experimental groups were assessed using Student's *t*-test. A p value less than 0.05 was considered to be statistically significant.

2.6. Complete genome sequencing and annotation

Genome sequencing was performed on RSII SMRTBell 20 kb insert libraries, using a PacBio SMRT Cell Seq-RSII (P6-C4), by Macrogen (Macrogen Korea, Seoul, Republic of Korea). Genome assembly was performed using Canu (Koren et al., 2017). Complete genome sequences of *L. lactis* subsp. *lactis* bv. diacetylactis S50 and S50-1RSS have been

Table 2
PCR cycling condition and primers used in this study.

Name of primers	Sequence (5′ - 3′)	Template	Cycling conditions	Volume (μL)
Primers used for cloning of genes under following conditions:				
MATE-EcoRI-Fw	GAATTCTCCTCTGCCCTTGACTAATCTCCC	Chromosomal DNA of S50	95 °C/30 s 61 °C/30 s 68 °C/60 s [30 cycles]	50
MATE-SalI-Rev	GTCGACTGGAAAATAATATATAAAAAAACG			
SAM-SacI-Fw	GAGCTCATTCATGCAAGTGCTCAACG		95 °C/30 s 62 °C/30 s 68 °C/3 min [30 cycles]	50
SAM-PstI-Rev	CTGCAGATATTGTTCAAAGTCATTTAG			
16SMet-SacI-Fw	GAGCTCAGGCCGAACTAAGAATTAGTGG		95 °C/30 s 65 °C/30 s 68 °C/2 min [30 cycles]	50
16SMet-SalI-Rev	GTCGACGGCACCTGCAATGGTCATGAATAG			
16SMet-c-EcoRI-Fw	GAATTCATTCATGAAAAATATTTTATC		95 °C/30 s 56 °C/30 s 68 °C/60 s [30 cycles]	50
16SMet-c-PstI-Rev	CTGCAGCTTGGCTAAATTCACCAC			
Primers used in RT-qPCR under following conditions:				
rpoD-Fw	CCAAGTCCTTTGGCTCTTGTC	cDNA from S50 and 50-1RSS (with and without spectinomycin selection)	95 °C/3 min 95 °C/15 s 60 °C/60 s [40 cycles]	10
rpoD-Rev	CTGCCCCTTCACCACCAGC			
MATE-Fw	GCTCACTTATCAATTCCC			10
MATE-Rev	GATAAAGGTTCCACCTCCGACACC			
SAM-Fw	CACTGCTCAAAAAATTCCC			10
SAM-Rev	CCAATTTACCCTCTGGTGCC			
16SMet-Fw	GATATTGGTGCTGGAGCTGG			10
16SMet-Rev	GTAAAGTCACATTTTCGAGG			
16SMet-c-Fw	TCGCATGACTTCCAGACC			10
16SMet-c-Rev	TGTTCCATAACCACACCC			

deposited in GenBank under accession numbers RBVM01000001.1 (GCA_003627395.1) and RBVN01000001.1 (GCA_003627415.1), respectively.

3. Results

3.1. Genomic sequences of wild-type and spectinomycin-resistant variants

The wild-type strain *L. lactis* subsp. *lactis* bv. diacetylactis S50 and its spectinomycin-resistant derivative S50-1RSS were described in our previous study (Kojic et al., 2008). Minimum inhibitory concentration (MIC) of spectinomycin for S50 (70 μg/mL) and S50-1RSS (2 mg/mL) were determined previously using the microdilution method (Kojic et al., 2008). Complete genome sequences of both strains were determined using the long-read PacBio RSII sequencing platform. The genome of strain S50 consisted of one chromosome (2,461,759 bp) and seven plasmids: pS8 (8.2 kbp), pS9 (9.3 kbp), pS12 (11.8 kbp), pS13 (13.3 kbp), pS38 (37.8 kbp), pS100 (99.7 kbp) and pS156 (156 kbp). Plasmids of strain S50 represent 13 % of the total genome size, which is the largest lactococcal plasmidome described so far.

Strain S50-1RSS represents a spectinomycin-resistant plasmid-cured derivative of S50 and contains only three small plasmids (pS9, pS38 and a third that shares > 98 % identity with pS7a and pS7b (Strahinic et al., 2009)).

3.2. Characterization of the large chromosomal inversion in strain S50-1RSS

Comparison of the sequences from strains S50 and S50-1RSS revealed a large chromosomal inversion of about 600 kb (1/4 of chromosome, encompassing 528 chromosomal ORFs) (Fig. 1), falling between two prophages that are oriented in opposite directions to each other. The prophages are located at positions 942,997-977,322 and 1,564,113-1,531,198 in the S50 chromosome (GenBank: RBVM01000001.1) and they share 99.73 % nucleotide identity over 64 % of the length with each other. Both prophages showed similarity to the 35,538-bp bacteriophage bIL285 (GenBank: AF323668.1) (Chopin et al., 2001): respectively the two prophages showed 99.99 % identity over 77 % of the length and 99.83 % identity over 89 % of the length. The endpoints of the inversion fall within the two identical copies of a 18,722-bp sequence that occurs in both prophages (located at positions 954,379 - 973,100 and 1,554,733 - 1,536,012).

The replication terminus in lactococci can be identified by the presence of a dif_{SL} nucleotide sequence (ATCTTTCCGAAAAACTGTAATTTTCTTGACA) immediately upstream of a gene encoding a XerS-like recombinase (Le Bourgeois et al., 2007). This site appears at positions 1320838 – 1320868 in S50 (GenBank: RBVM01000001.1) and 1195893 – 1195923 in S50-1RSS (GenBank: RBVN01000001.1). Thus, the inversion causes the the replication terminus to move by about 125 kb with respect to the origin of replication. Similarly, inversion alters the distances from *oriC* for genes within the inverted region, but it does not move genes between the leading and lagging strand. Comparison of S50 and S50-1RSS chromosomes didn't reveal any other genetic change(s) such as SNPs, insertions or deletions that could generate difference in spectinomycin resistance.

3.3. Four genes (three methyltransferases and one MDR efflux pump) that could be involved in spectinomycin resistance are located in the inverted region

Among the genes located in inverted region were four genes with predicted products that could plausibly be involved in spectinomycin resistance: three encoding for methyltransferases and one for a MATE-family efflux pump. Previous studies (Peterson and Kaur, 2018) have implicated 16S rRNA methyltransferases and efflux pumps as determinants of resistance to aminoglycoside antibiotics in Gram-negative bacteria (Shakil et al., 2008), though mechanisms of spectinomycin resistance in Gram-positives are largely unknown. Positions and orientations of these candidate genes are presented in Table 3.

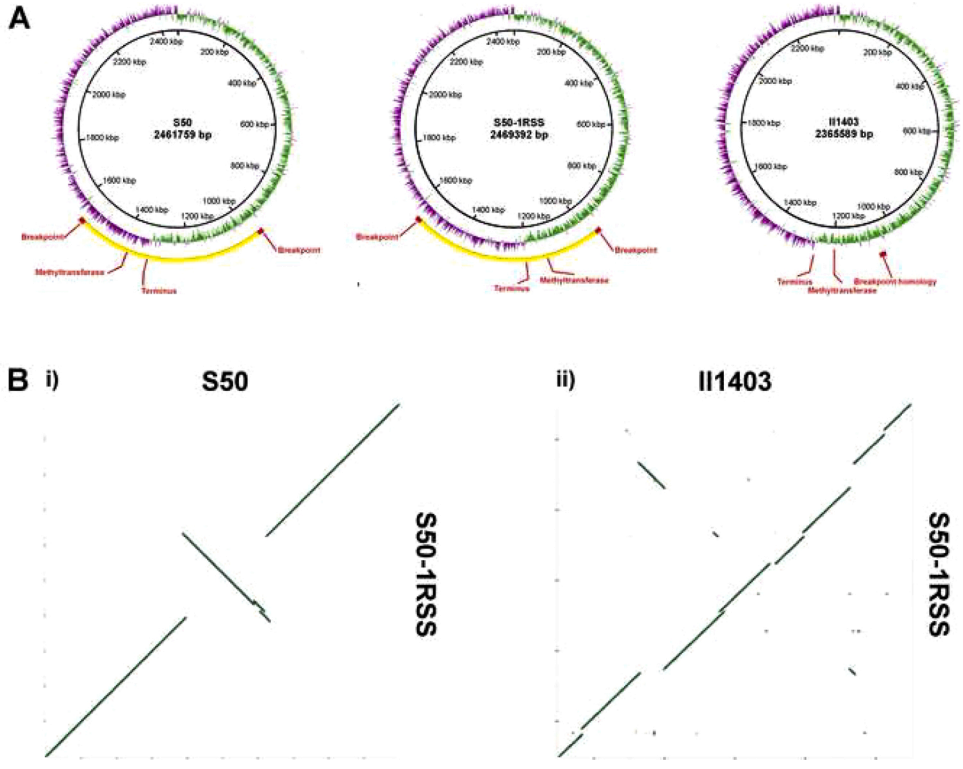

Fig. 1. Comparison of the complete-chromosome sequences of *L. lactis* subsp. *lactis* S50, S50-1RSS and IL1403. A) Circular maps and GC skew of the chromosomal sequences of S50 (GenBank: RBVM01000001.1; 2461759 bp), S50-1RSS (RBVN01000001.1; 2469392 bp) and IL1403 (NC_002662.1; 2365589 bp) were constructed using BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011). The inner circle illustrates nucleotide position in kilobase pairs (kbp), the outer circle illustrates the GC skew of (-) strand (-) and (+) strand (-). Flip-flop breakpoints (prophage sequences) are represented by red squares at the ends of the inverted region (yellow quarter circle) in strains S50 and S50-1RSS, while strain IL1403 contains only one sequence sharing similarity with break-point sequences. Position of terminus (Terminus) and gene for class-I SAM-dependent methyltransferase (Methyltransferase) are indicated (S50 locus tag: D8K17_07155, S50-1RSS locus tag: D8M10_06065). Terminus of replication in invertant S50-1RSS is displaced/moved by about 125 kb and the methyltransferase gene by about 252 kb. B) Dotplots between i) S50 (x-axis) and S50-1RSS (y-axis) showing the presence of a large inversion of 600 kb located around the middle of the chromosome, and between ii) IL1403 (x-axis) and S50-1RSS (y-axis) pointing to a more similar organization without large chromosomal rearrangements obtained using SynMap (https://genomevolution.org/CoGe/SynMap.pl).

Table 3
Position and orientation of genes for methyltransferases and efflux pump located in inverted region of strains S50 and S50-1RSS.

Predicted gene product	Genome of strain S50		Genome of strain S50-1RSS	
	Position	Orientation	Position	Orientation
MATE family efflux transporter	RBVM01000001.1: 1107636 - 1109015 (Locus tag: D8K17_05845)	complement	RBVN01000001.1: 1407746 -1409122 (Locus tag: D8M10_07375)	
16S rRNA (guanine^{1207}-N^{2})-methyltransferase	RBVM01000001.1: 1155485 - 1156087 (Locus tag: D8K17_06055)		RBVN01000001.1: 1360675-1361277 (Locus tag: D8M10_07165)	complement
16S rRNA (guanine^{527}-N^{7})-methyltransferase	RBVM01000001.1: 1198687 - 1199403 (Locus tag: D8K17_06285)	complement	RBVN01000001.1: 1317359-1318075 (Locus tag D8M10_06935)	
Class-I SAM-dependent methyltransferase	RBVM01000001.1: 1383435 - 1384055 (Locus tag: D8K17_07155)	complement	RBVN01000001.1: 1132706 - 1133326 (Locus tag D8M10_06065)	

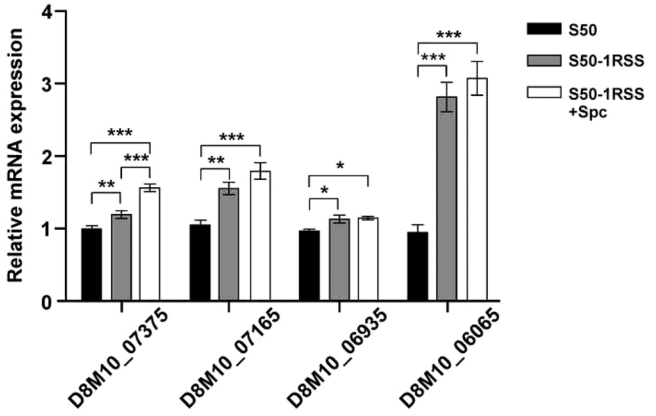

Fig. 2. The changes of relative mRNA levels of four genes located in inverted region of S50/S50-1RSS chromosomes that could plausibly be involved in spectinomycin resistance: D8M10_07375 – locus tag of MATE family efflux pump in S50-1RSS, corresponding to locus tag D8K17_05845 in S50, D8M10_07165 – locus tag of 16S rRNA (guanine(527)-N(7))-methyltransferase in S50-1RSS, corresponding to locus tag D8K17_06285 in S50, D8M10_06935 – locus tag 16S rRNA (guanine(1207)-N(2))-methyltransferase in S50-1RSS, corresponding to locus tag D8K17_06055 in S50 and D8M10_06065 – locus tag of Class-I SAM-dependent methyltransferase in S50-1RSS, corresponding to locus tag D8K17_07155 in S50. RT-qPCR data were normalized against the sigma 70 gene *rpoD* as an internal control. Student's *t*- test was used to compare the differences of gene transcription between the WT strain S50, invertant S50-1RSS and invertant grown in medium containing spectinomycin (250 μg/mL) – S50-1RSS + Spc (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.4. Spectinomycin induces expression of three candidate resistance genes

To determine the influence of the 600 kb chromosomal inversion on transcription of candidate genes that could be involved in spectinomycin resistance, expression was quantified with RT-qPCR using primers specific for each selected gene. The relative expression levels of each gene were measured for strain S50 and invertant S50-1RSS during logarithmic growth phase in GM17 medium. In addition, spectinomycin resistant strain S50-1RSS was grown in presence of spectinomycin (250 μg/mL) to check the effect of spectinomycin upon transcription. This showed that three of the four selected genes have significantly increased transcription in the invertant S50-1RSS, namely those encoding the MATE family efflux pump (S50-1RSS locus tag: D8M10_07375, S50 locus tag: D8K17_05845), and two predicted methyltransferases (S50-1RSS locus tags D8M10_06935 and D8M10_06065, S50 locus tags: D8K17_06285 and D8K17_06055). Additionally, transcription of all three genes was further increased when the invertant S50-1RSS was grown in the presence of spectinomycin, with the highest increase for the efflux pump gene (Fig. 2). These results indicate that spectinomycin induces transcription of all three genes.

3.5. Heterologous expression of SAM-dependent methyltransferase from strain S50 confers resistance to spectinomycin in heterologous host

Genes encoding the efflux pump (D8K17_05845) and a methyltransferases (D8K17_07155, D8K17_06285 and D8K17_06055) were amplified (including their promoter regions) using high-fidelity *Taq* polymerase, cloned into pAZIL vector pre-digested by *Sma*I restriction enzyme and expressed in heterologous hosts *Lactococcus lactis* subsp. *lactis* IL1403 and *Lactococcus lactis* subsp. *cremoris* MG1363. Transformants were selected for growth on different concentrations of spectinomycin. Transformants of MG1363 and IL1403 carrying clones pAZIL-D8K17_07155-1 and pAZIL-D8K17_07155-4 were able to grow on GM17 Petri dishes containing spectinomycin at concentrations up to 250 μg/mL, revealing that the gene encoding this methyltransferase confers resistance of strain S50-1RSS to spectinomycin. No transformants could grow in the maximal concentration (500 μg/mL) of spectinomycin, indicating that heterologous expression of methyltransferase D8K17_07155 on the multi-copy plasmid did not confer as high a level of resistance as seen in the invertant strain S50-1RSS, which can grow at 500 μg/mL drug concentration (Kojic et al., 2008). This suggests that either the level of expression is lower or that additional factors contribute to resistance. Other transformants (carrying clones for D8K17_05845, D8K17_06285 and D8K17_06055) were not able to grow even on 100 μg/mL of spectinomycin.

4. Discussion

The development and spread of antibiotic resistance among bacteria present a universal threat to human, animal and environmental health. The origins of resistance are intensively studied and many mechanisms involved in resistance have been identified, including exogenous gene acquisition by horizontal gene transfer, alteration of the antibiotic target, and more recently, antibiotic tolerance through persistence (Baharoglu et al., 2013).

Spectinomycin is an aminoglycoside antibiotic that binds to the ribosome beneath the helix 34 of 16S rRNA thus preventing the translocation of peptidyl-tRNA within ribosome during translation (Borovinskaya et al., 2007; Carter et al., 2000). The most common mechanisms of resistance to aminoglycosides are: i) decreased intracellular concentration, ii) modifications of target and iii) enzymatic modification of the antibiotics. Post-transcriptional methylation of rRNA by methyltransferases is used by some aminoglycoside producers (Beauclerk and Cundliffe, 1987), and is an example of target-modification, whereby they methylate the aminoglycoside's binding site on the 16S RNA (Shakil et al., 2008).

Previously, we demonstrated that acquisition of spectinomycin resistance in *L. lactis* is associated with inversion of a large genomic region (Kojic et al., 2008), but the underlying molecular mechanisms and the precise location of the inversion were unknown. Here we show that the inverted region includes the replication terminus and a gene encoding a methyltransferase (S50-1RSS locus tag: D8M10_06065, GenBank: RKO32069.1) that could potentially modify the drug target. We demonstrated that heterologous expression of this gene conferred

increased resistance to spectinomycin, but not as high as seen in the invertant strain S50-1RSS, indicating either that level of expression is lower or that additional factors contribute to resistance. Also, possible additive or synergistic effect between functions (processes) encoded by selected genes may take place. This would explain the lower level of resistance when individual genes were heterologously expressed.

Genomic inversions are known in lactococci as differentiating different species and subspecies (Le Burgeois et al. 1995; Merrikh and Merrikh, 2018), but apart from our work, their role in acquisition of antimicrobial resistance is novel. Furthermore, inversions are found in different bacterial taxa where they contribute to phase variation, colony morphology, antibiotic resistance, regulation of virulence, immune evasion, host adaptation, hemolytic activity, and expression of dozens of genes (Iguchi et al., 2006; Jiang et al., 2019; Mackiewicz et al., 2001). However, to date the only documented contribution of genomic inversions to antimicrobial resistance come from the species *Staphylococcus aureus* (Cui et al., 2012). In that species, levels of expression of a large number of genes are affected, likely due to the asymmetry of the inversion altering their distances from the origin of replication. Furthermore, genome sequencing of phenotypically distinct sub-populations of *S. aureus* revealed that the inverted copies of *hsdMS* loci represented a conserved recombination hotspot promoting the generation of subpopulations of small-colony variants in *S. aureus*, which seems to be associated with activity of the prophage (Guérillot et al., 2019). Inversion in strain S50 is also associated with a prophage and therefore it is possible that it involves a similar mechanism leading to altered expression of a potentially large number of genes.

Creation of new head-on collision genes (genes in lagging strand) and operons through inversions is widespread in the evolutionary history of bacteria (Merrikh and Merrikh, 2018). This seemed to contradict prevailing opinion that genes in lagging strand are under negative selection pressure due to replication stress (Merrikh and Merrikh, 2018). It is generally accepted that more conserved genes are located on the leading strand due to the protection from mutations and transfer to the lagging strand causes a higher mutation rate resulting in selection that enhances probability of elimination. On the contrary, genes located on the lagging strand are thought to be more tolerant to mutations. Thus, these genes switch their position with a lower probability of being eliminated (Mackiewicz et al., 2001). However, it is postulated that additional replication-transcription conflicts within new head-on collision genes might cause higher gene-specific mutation rate and accelerate evolution of antibiotic resistance genes (Merrikh and Merrikh, 2018). Thus, gene inversion events are thought to be capable of driving increased antibiotic resistance. Inversion in strain S50-1RSS that confers spectinomycin resistance causes movement of the replication terminus by about 125 kb with respect to the origin (*oriC*) of replication, but it does not move genes between the leading and lagging strands, underlining that strain S50-1RSS harbours another mechanism of resistance.

The inverted region in resistant variant S50-1RSS shares the same orientation as its equivalent region in *Lactococcus lactis* subsp. *lactis* IL1403, while the sensitive wild-type strain S50 shows the opposite orientation. Since strain IL1403 is very sensitive to spectinomycin it seems that the constellation of the genes on a chromosome is not the only factor providing resistance, and other factors might be involved. Interestingly, the inversion that brings strain S50 genetically closer to IL1403 reduces its fitness under nonselective conditions.

Small-scale genetic inversions involving one or a few promoters and/or genes are common in a broad range of bacterial taxa (Jiang et al., 2019). However, observations of large-scale inversions involving hundreds of kilobases are few (Cui et al., 2012; Guérillot et al., 2019; Le Bourgeois et al., 1995).

When S50-1RSS was grown under nonselective conditions in culture, we were able to detect a mixture of both genomic variants. The "flip" event (inversion) is induced and/or selected by spectinomycin pressure, while the "flop" events (reversion) most likely occur stochastically, via foreign (bacteriophage) DNA. Since the chromosome of S50 contains many repetitive sequences, most probably these two genomic variants could form stable cell subpopulations. According to this assumption it could be postulated that strain S50 never exists as a homogenous population but always includes a small sub-population of the inverted variant.

Spectinomycin resistance in strain S50-1RSS presents a specific novel mechanism of resistance to antibiotics. It is interesting that inversion of 600 kb of DNA does not generate any mutation and transfer of genes involved in resistance from lagging to leading strand, like in *S. aureus* (Guérillot et al., 2019). However, it did cause changes in levels of transcription, including gene(s) that confers resistance to this drug. It is not yet clear whether this effect is a direct consequence of the inversion or alteration in transcription is mediated via prophage or regulation driven by transcription factors. It was previously found that besides the development of direct antibiotic resistance, changes in regulation of gene expression contribute to the ability of bacteria to survive presence of antibiotics and other stresses by acquiring functional mutations and specific resistance mechanisms (Palmer et al., 2018). In addition, it was observed that methylation status (epigenetic regulation) in the cell can also influence expression (can cause phenotypic change) of other genes through DNA-protein interactions in the absence of mutation (Seib et al., 2017; Sanchez-Romero and Casadesus, 2020). SAM-dependent methyl transferase is able to methylate different substrates (Cooke et al., 2009), so it could indirectly influence (epigenetically) the expression of multiple genes, some of which likely contribute to spectinomycin resistance. Results obtained in this study in combination with previous literature indicate that inversion can have an effect on the expression of multiple genes in the S50-1RSS strain on different levels (Naseeb et al., 2016; Guérillot et al., 2019).

It is important to note that several attempts to determine the existence of inversion in the invertant S50-1RSS (which was unequivocally confirmed by the PFGE analysis) by the sequencing of the complete genomes of parental strain S50 and invertant S50-1RSS (using GS-FLX Titanium and Illumina HiSeq platforms combined with mate-pair 6−8 kb library) were unsuccessful. This problem was also reported by other groups and indicated that short sequencing reads can not cover long repeatable sequences in the genome (Guérillot et al., 2019; Koren et al., 2017). Nevertheless, assembling the complete chromosome as one contig was successfully achieved by applying long read sequencing, using PacBio SMRT sequencing of a 20 kb insert library.

5. Conclusions

Long-read sequencing confirmed the presence and enabled definition of a large chromosomal inversion in the strain S50, which provides spectinomycin resistance in the invertant by altering gene transcription. We mapped the endpoints of this inversion and determined that this 600-kb inversion occurred between two nearly identical prophages, shifting the replication termination site by 125 kb, indicating homologous recombination as the most likely mechanism. Among cloned and heterologously expressed genes that were hypothesized to be involved in resistance, one encoding a SAM-dependent methyltransferase increased resistance to spectinomycin (250 μg/mL), which suggests that methylation as an epigenetic signal is crucial for the difference in the expression of genes involved in adaptation to high concentrations of spectinomycin. The resistance was still not at the level as observed in the invertant, indicating involvement of other factor(s) (cumulative or synergistic effects of several alternations in gene expression) within the invertant, beyond simply altered expression of this gene. This manuscript aims to point out that the development of resistance can also occur in bacteria other than pathogens, which may be reservoirs for the spread of resistance, since lactococci, as constituents of the human microbiota, can interact with other bacteria of the gastrointestinal tract as well as with pathogens. Since beneficial, commensal, and pathogenic bacteria could use the same mechanism as the tool for adaptation, generation of intra-strain diversity and immune evasion, unveiling the molecular basis of this aspect of bacterial evolution is vital in achieving significant

implications to understand health and diseases. Further studies (transcriptome RNA-seq analysis, construction of selected gene mutants and promoter fusions) are needed to deeply elucidate this novel mechanism of regulation/expression spectinomycin resistance caused by inversion.

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Author statement

Submission declaration and verification: All authors declare that the work described in the manuscript entitled "**Large-scale chromosome flip-flop reversible inversion mediates phenotypic switching of expression of antibiotic resistance in lactococci**", or one with substantially the same content, was not published previously, is not being considered or published elsewhere and it is not under simultaneous consideration or in press by another journal. In addition, we declare that there is no conflict of interest and that all previously published work in this manuscript has been fully acknowledged.

Author contributions

The study was conceptualized by MK and BJ. Laboratory work was done by MM, KN and MK. Whole-genome analysis was carried out by DJS and MK. Supervision, Project administration and Funding were carried out by MK and BJ. The manuscript was drafted by MM and BJ, reviewed by MK and edited by JB, MK and DJS. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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