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Original scientific paper

OVER-EXPRESSED CmbT MULTIDRUG RESISTANCE TRANSPORTER IMPROVES THE FITNESS OF *Lactococcus lactis*

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The influence of the over-expression of CmbT multidrug resistance transporter on the growth rate of *Lactococcus lactis* NZ9000 was studied. *L. lactis* is a lactic acid bacteria (LAB) widely used as a starter culture in dairy industry. Recently characterized CmbT MDR transporter in *L. lactis* confers resistance to a wide variety of toxic compounds as well as to some clinically relevant antibiotics. In this study, the *cmbT* gene was over-expressed in the strain *L. lactis* NZ9000 in the presence of nisin inducer. Over-expression of the *cmbT* gene in *L. lactis* NZ9000 was followed by RT-PCR. The obtained results showed that the *cmbT* gene was successfully over-expressed by addition of sub-inhibitory amounts of nisin. Growth curves of *L. lactis* NZ9000/pCT50 over-expressing the *cmbT* gene and *L. lactis* NZ9000 control strain were followed in the rich medium as well as in the chemically defined medium in the presence solely of methionine (0.084 mM) or mix of methionine and cysteine (8.4 mM and 8.2 mM, respectively). Resulting doubling times revealed that *L. lactis* NZ9000/pCT50 had higher growth rate comparing to the control strain. This could be a consequence of the CmbT efflux activity, which improves the fitness of the host bacterium through the elimination of toxic compounds from the cell.

Key words: Lactococcus lactis, bacterial fitness, multidrug resistance

Abbrev. MDR – multi drug resistance

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INTRODUCTION

Lactococcus lactis is a lactic acid bacteria(LAB) widely used as a starter culture in dairy industry. Although lactococci have acquired the „Generaly Regarded As Safe“ (GRAS) status, many investigators have speculated that lactococci as well as other LAB may act as reservoirs of antibiotic resistance genes. In order to prevent the spread of these genes, the studies on the antibiotic resistance mechanisms and multidrug resistance (MDR) transporters within them, are of great importance for lactococci intended for use in the food industry.

Although lactococci can be isolated from various environments, *L. lactis* is predominantly studied because of its role in the dairy industry as the main component of many industrial starter cultures used for the manufacture of different dairy products like fermented milk, sour cream, soft and hard cheeses (WARD *et al*., 2002). Some recent studies have shown that commensal bacteria including LAB may be reservoirs of antibiotic resistance genes that could be transferred to diverse bacterial species including pathogens (SCOTT, 2002; MATHUR and SINGH, 2005). In order to prevent the spread of antibiotic resistance genes, the studies of multidrug resistance (MDR) transporters in lactococci that are used in the food industry is essential. Recently, we have described the CmbT, a novel major facilitator superfamily (MFS) MDR transporter in *L. lactis* which apart from the role in antibiotic resistance appeared to actively extrude wide variety of toxic drugs. Additionally, the physiological role of the CmbT was determined to be related to the transport of compounds involved in sulphur metabolism (FILIPIC *et al.*, 2013).

Various studies dealing with antibiotic resistance showed the linkage between the antibiotic resistance and decreased bacterial fitness in the absence of selective pressure (GAGNEUX *et al*., 2006; O'NEILL *et al*., 2006; ROZEN *et al*., 2007; MARCUSSON *et al*., 2009). It was noticed that the antibiotic resistant strains are competitively disadvantaged comparing to the antibiotic susceptible strains in the absence of antibiotic. In other words, some mutations, that decrease antibiotic susceptibility, simultaneously reduce the fitness costs. For example, *E. coli* clinical isolates with decreased susceptibility to fluoroquinolones suffered reduced growth competitiveness (KOMP LINDGREN *et al*., 2005).

The relationships between the resistance mutations, drug susceptibility, and growth fitness are complex and still are not completely understood. Although in some cases the addition of a resistance mutation was shown to improve bacterial fitness (MARCUSSON *et al*., 2009), drug resistance mostly influences bacterial fitness in two ways. Mutations either cause little or no fitness cost, leading to their high frequency among resistant isolates or the accumulation of mutations cause a progressive decrease in bacterial fitness (ANDERSSON, 2006; ANDERSSON and HUGHES, 2007).

Since *L. lactis* has been used as a model organism in numerous studies due to the efficiency of *L. lactis* as a cell factory, the advancement in the genetics of LAB including gene expression systems was made (DE VOS, 1999; DORRIAN *et al*., 2011). Moreover, the importance of *L. lactis* in dairy industry cannot be neglected. Hence, the aim of this work was to study the fitness of *L. lactis* in the presence of over-expressed CmbT MDR protein. To our knowledge, this paper for the first time reports the ability of the over-expressed MDR CmbT protein to increase *L. lactis* fitness.

MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, media

The bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* subsp. *lactis* NZ9000 was used as a host for the nisin controlled gene expression (NICE) vector pNZ8113 and its *cmbT* – containing derivative (pCT50). *L. lactis* strains were grown at 30°C in M17 broth (Merck, Germany) supplemented with 0.5% glucose (GM17), or chemically defined medium (CDM) (JENSEN and HAMMER, 1998; POOLMAN and KONINGS, 1988) enriched with 0.1 x methionine or 10 x methionine/ 10 x cysteine (1 x methionine = 0.84 mM, 1 x cysteine = 0.82 mM) (LOZO *et al*., 2008). Chloramphenicol was used at a final concentration of 5 μg ml^{-1} when culturing *L. lactis* NZ9000 harboring pCT50. When necessary, media were solidified by adding agar (1.5% w/v) (Torlak, Serbia).

2.2. Protein sequence analysis

TMHMM was used to predict transmembrane helices in CmbT protein using the web server: http://www.cbs.dtu.dk/services/TMHMM (SONNHAMMER *et al*., 1998).The program reads a fasta-formatted protein sequence and predicts locations of transmembrane, intracellular and extracellular regions.

2.3. Reverse-transcription polymerase chain reaction (RT-PCR)

Reverse-transcription polymerase chain reaction (RT-PCR) was used to assess the expression levels of the *cmbT* gene induced by different concentrations of nisin. After induction of *L. lactis* NZ9000/pCT50 with various nisin concentrations, total RNA was isolated from the cells using SV Total RNA Isolation kit (Promega, WI USA). The concentrations of isolated RNAs were measured by NanoVue Spectrophotometer (GE Healthcare, UK). Reverse transcription was performed by RevertAid reverse transcriptase (Fermentas, Lithuania) following the manufacturer's instructions by using 1 μg of total RNA as template and primer complementary to six His residues in the Histag (HISTAG: 5'-GTGATGGTGATGGTGATG-3'). The obtained cDNA was used as template for PCR amplifications in 25 successive cycles following the manufacturer's instructions with primers FcmbT-NcoI (5'-CATGCCATGGAGGAGATTTTATTGCATG-3') and HISTAG.

2.4 Growth curve determination

Overnight cultures of *L. lactis* NZ9000 and *L. lactis* NZ9000/pCT50 were diluted into GM17 fresh medium, CDM enriched with 0.1 x methionine or CDM with 10 x methionine/ 10 x cysteine (with or without chloramphenicol) and were grown at 30°C until mid-exponential growth phase. Subsequently, expression of the *cmbT* gene was induced by adding nisin (Sigma, Germany) at a final concentration of 1 ng ml^{-1} shown previously to induce synthesis of the highest amount of CmbT protein (FILIPIC *et al*., 2013). In order to achieve the same experimental conditions the nisin was added to the control strain in the same concentration. Aliquots (100 μl) of the cultures was used for serial dilutions and the cells were spread onto GM17 agar plates containing chloramphenicol when necessary and incubated at 30°C for 24 h. This procedure was repeated hourly. Growth curves of *L. lactis* NZ9000 and *L. lactis* NZ9000/pCT50 in the appropriate medium were determined and the doubling times (g) were calculated ($g = (\log_{10} N_t - \log_{10} N_0) / \log_{10} 2$).

RESULTS

3.1. Properties of the CmbT protein.

The *cmbT* gene was annotated in the genome of *L. lactis* subsp. *cremoris* MG1363 as *llmg*1104, and was predicted to encode an integral membrane protein (454 amino acids). Analysis of the CmbT obtained by using TMHMM software showed the presence of 14 hydrophobic transmembrane spanning (TMS) domains (Figures 1a and 1b).

MNKNNHFHLL GAVIATGILS FSGVLIETAM NVTFPTLINE FGLSTSKIQW VTTIYLLVIA TIPLSSYFN
ERFSARKLFL VANLIFLVGV LTNCFSPNFA MLLFGRLLQG VGTGIGLPLM FHLILTKAPL KRGMMMGIG
TLTTSIAPAI GPTYGGIISN SLDWRYIYIF LLPLVVISLV LGLACLPREG EKTPKKLALR VIALSIMFF
AFISALSAEH LMTFALLFIV GLVGAFLFVQ FNRKESLIDL GILKNHRFVA LIFSLLVYQA LLGLSFVLP
SFIQVSAGFS SSVAGLFMFS GALIGAVLAP ISGKVLDQIG ARKPITTGLI LAALGLALIF FLPTKSLAL
LLGAHIVLML GLGISYSNLM TCSLSTLATD QLSDGNALVN TLQQFIGAVA TAVVATTLSI QGLNGFKVG
TSHGTSIILA LFFILIIVSL IVSFPNLKKI RANH

Figure 1a. Amino acid sequence of the CmbT protein. The transmembrane spanning domains revealed by TMHMM programme are highlighted.

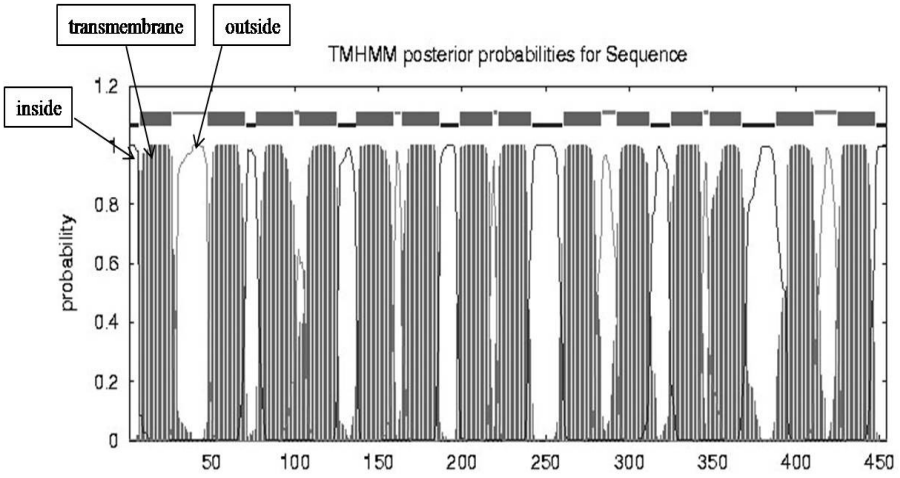

Figure 1b. Grafical presentation of the transmembrane spanning domain in the CmbT protein analysed by TMHMM software.

3.2. Over-expression evaluation of the cmbT in L. lactis

The *cmbT* gene was cloned previously into the pNZ8113 expression vector under control of a tightly regulated, inducible *nisA* promoter (FILIPIC *et al*., 2013). Cells were grown in the presence of nisin (0, 0.1 and 1 ng ml^{-1}) in order to induce the expression of the *cmbT* gene, and subsequently total RNA was isolated for expression analysis. RT-PCR analysis revealed that the *cmbT* transcript was successfully over-expressed upon induction by 1 ng ml^{-1} of nisin (Figure 2).

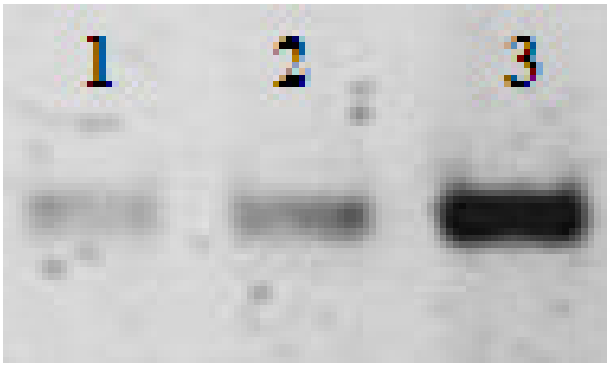

Figure 2. Nisin-induced over-expression of the *cmbT* gene in *L. lactis* NZ9000/pCT50 measured by RT-PCR using specific Histag primer: Lane 1. 0 ng/ml; Lane 2. 0.1 ng/ml; Lane 3. 1 ng/ml.

3.3. Effect of the cmbT over-expression on L. lactis NZ9000 growth rate

Comparative analysis of the growth rate for strains *L. lactis* NZ9000 and *L. lactis* NZ9000/pCT50 in the exponential phase of growth in GM17 and CDM (with methionine or methionine and cysteine) showed that *L. lactis* NZ9000 had lower growth rate than *L. lactis* NZ9000/pCT50 (Figures 3a, 3b and 3c). In GM17 medium, the doubling time calculated for *L. lactis* NZ9000/pCT50 was 49 min (specific growth rate = 1.22/h), while the doubling time for *L. lactis* NZ9000 was 55.8 min (specific growth rate = 1.08/h). In CDM medium, doubling time for *L. lactis* NZ9000/pCT50 was 222 min (specific growth rate = 0.27/h) in the presence of 0.1 x methionine and 109.8 min (specific growth rate = 0.55/h) in the medium containing 10 x methionine and 10 x cysteine. For *L. lactis* NZ9000 doubling times were 230 min (specific growth rate = 0.26/h) in the CDM with 0.1 x methionine and 114 min (specific growth rate = 0.53/h) in the 10 x methionine and 10 x cysteine.

DISCUSSION

The aim of the study was to determine whether the accumulation of overexpressed MDR CmbT protein, shown previously to decrease the susceptibility of *L. lactis* to various drugs, would influence their biological fitness.

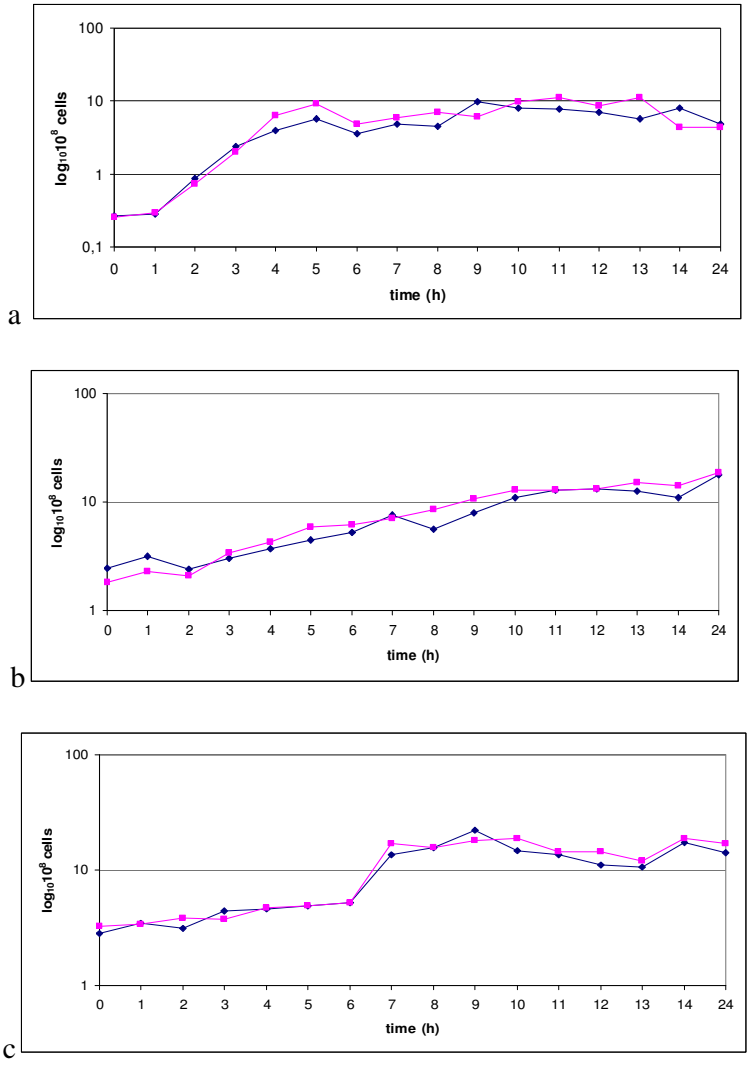

Figure 3. Growth curves of control strain *L. lactis* NZ9000 (♦) and CmbT expressing recombinant *L. lactis* NZ9000/pCT50 (■) in: GM17 reach medium (a), CDM medium with 0.1 x methionine (b) and CDM medium with 10 x methionine and 10 x cysteine (c).

One of the important aspects of the *L. lactis* fitness is the ability to survive and persist in a variety of environments, including varied human and animal anatomical niches, food, and acidic conditions. Hence, the understanding the mechanisms of lactococci fitness and survival in extreme and changing environments could in final extent contribute to the strategies for their use as starter cultures in dairy industry.

It has been proposed that antibiotic and drug resistance could be associated with a fitness cost in bacteria. The mutation-driven drug resistance usually provoke a reduction in biological fitness since antibiotics target basic bacterial functions such as RNA transcription, protein synthesis and the cell wall synthesis (MARCUSSON *et al*., 2009).

Moreover, while a number of studies point out the influence of the mutation-driven resistance to bacterial fitness, recent attentions have been shifted to inducible MDR efflux proteins. Efflux is a widespread MDR mechanism in bacteria. It has been shown that MDR efflux pumps apart from the efflux of a broad range of toxic agents could be beneficial to cells in the term of bacterial fitness (PEREZ *et al*., 2012, WOOD and CLUZEL, 2012). Consequently, MDRs may prove beneficial to cells in the presence of multiple stressors. In addition, some MDR efflux pumps not only confer resistance to drugs but have also been shown to have a role in the colonization of a variety of host organisms, and in their survival therein (PÉREZ *et al*., 2012). For example, the highest host drug resistance conferred by CmbT was observed for cholate, one of the most frequently encountered free bile acids in the human intestinal tract that strongly inhibits the growth of intestinal bacteria (FILIPIC *et al*., 2013).

Since our previous results revealed that CmbT may play a signal role in sulphur metabolism in *L. lactis* (FILIPIC *et al*., 2013), we have tested the hypothesis that acquisition of antibiotic resistance by overproduction of the CmbT may not produce a general burden to the host, but rather lead to specific changes in bacterial physiology.

In this work we have studied the effect of overproducing the multidrug efflux pump CmbT on *L. lactis* fitness. We found that overexpression of CmbT does not cause a decrease in *L. lactis* fitness, indicating the absence of a large metabolic burden even under specific conditions such as the growth in CDM. It has been shown that expression of the membrane proteins can be achieved only in a small percentage (about 3% of total proteins), which means that it does not cause significant damage of the membrane structure and does not lead to the detrimental effect of the host cells (BERNAUDAT *et al.,* 2011). Moreover, slightly higher growth rate of *L. lactis* NZ9000 overexpressing *cmbT* gene in rich (1.13 times) and CDM containing either 0.1 x methionine or 10 x methionine and 10 x cysteine (1.04 times) could be the consequence of the CmbT efflux activity that contributes to the toxin extrusion from the cell.

Finally, this study showed that *L. lactis* fitness is influenced by overexpression of the CmbT MDR protein. It could be concluded that the increased efflux activity by overproduced CmbT transporter could improve the fitness of the host *L. lactis* cells through the elimination of toxic compounds and the specific changes in bacterial physiology.

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UTICAJ POVEĆANE EKSPRESIJE CmbT MDR TRANSPORTERA NA RAST *Lactococcus lactis*

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Izvod

U ovom radu je izučavan uticaj povećane ekspresije *cmbT* gena, odgovornog za sintezu CmbT MDR transportera, na rast *Lactococcus lactis. L. lactis* pripada grupi bakterija mlečne kiseline (BMK) i ima veliku primenu u prehrambenoj industriji kao starter kultura. CmbT transporter je nedavno okarakterisan MDR protein soja *L. lactis*, koji doprinosi rezistenciji na različite toksične agense kao i na neke klinički značajne antibiotike. U ovom radu je *cmbT* gen višestruko eksprimiran u soju *L. lactis* NZ9000 dodavanjem nizina kao inducera. Povećana ekspresija *cmbT* gena je praćena metodom reverzne transkripcije (RT-PCR). Pokazano je da se nakon dodatka subinhibitornih koncentracija nizina u medijum za rast povećava količina sintetisane informacione RNK specifične za *cmbT* gen. Rast soja *L. lactis* NZ9000/pCT50, u kome je višestruko eksprimiran *cmbT* gen i *L. lactis* NZ9000 kontrolnog soja praćen je u bogatom i hemijski definisanom medijumu u prisustvu samo metionina (0.084 mM) ili kombinacije metionina i cisteina (8.4 mM i 8.2 mM). Praćene su krive rasta oba soja, a nakon izračunavanja odgovarajućih vremena generacije, rezultati su pokazali da *L. lactis* NZ9000/pCT50, brže raste u odnosu na kontrolni soj. Uočena razlika je najverovatnije posledica aktivnosti CmbT transportera koji doprinosi izbacivanju toksičnih agenasa iz ćelije i na taj način poboljšava adaptivne sposobnosti bakterije koja ga eksprimira i daje joj selektivnu prednost.

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