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LmrR is a transcriptional repressor of the expression of the Multidrug ABC transporter LmrCD in *Lactococcus lactis*

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Regulation of lmrCD expression in Lactococcus lactis

SUMMARY

LmrCD is an ABC-type multidrug transporter of Lactococcus lactis. LmrR encodes a putative transcriptional regulator. In a ΔlmrR strain, lmrCD is up-regulated. LmrR binds the promoter region of lmrCD and interacts with drugs that cause lmrCD up-regulation. This suggests that LmrR is a drug-dependent transcriptional regulator of lmrCD expression.
In recent years, the exposure of human pathogenic bacteria to antibiotics and toxic drugs has led to a major boost in the emergence of (multi) drug resistant pathogens (24), which now becomes a serious problem in public health causing millions of death worldwide (6,22). The overexpression of multidrug efflux pump(s) is one of the causes of the resistance phenotype observed in bacteria (17,19,27). Bacteria possess various genes that encode putative multidrug resistance (MDR) transporters, but for most of these systems the exact physiological function is unclear (25). Resistance readily develops when cells are exposed to drugs or antibiotics, and the immediate response usually involves the up-regulation of low-expressed MDR transporters through local or global transcriptional regulators (1,11,12,26). The Gram-positive bacterium Lactococcus lactis is widely used in fermented food production. The genomes of L. lactis IL1403 (5) and MG1363 (31) contain about 40 genes that encode putative MDR-transporters. LmrA and LmrP of L. lactis have been implicated in the MDR phenotype but gene inactivation analysis of a number of putative MDR transporter genes suggests that the intrinsic multidrug resistance of L. lactis is due to the expression of the heterodimeric ATP-binding cassette (ABC) transporter LmrCD (20). Exposure of L. lactis cells to the compounds Daunomycin, Ethidium bromide and Rhodamine 6G readily resulted in the development of a MDR phenotype (4). DNA-microarray analysis revealed that in these strains the expression of lmrCD is strongly increased (4 to 8-fold), whereas several other genes are up- or down-regulated in a strain-specific manner (18). This suggests that LmrCD is also a major determinant of acquired drug resistance. The DNA region upstream of the lmrCD genes specifies a putative regulatory protein LmrR (formerly YdaF) that by homology belongs to the PadR family of transcriptional regulators (Pfam PF03551). PadR proteins are involved in the regulation of the expression of the phenolic acid decarboxylase (pad) gene(s) that are required for the detoxification (13) and metabolism (10,23,29) of phenolic acid compounds. In lactic acid bacteria, phenolic acids are converted to 4-vinyl derivatives that are further reduced to 4-ethyl derivatives (3). The PadR family is related to the bacterial and archaeal MarR family of transcriptional regulators of multiple antibiotic resistance. These proteins share a common domain organization which comprises a N-terminal winged helix-turn-helix DNA binding motif that via a conserved hinge region is connected to a C-terminal highly divergent domain (2). The latter region has been postulated to be involved in substrate(s) binding. Interestingly, in the L. lactis MDR strains, the lmrR gene either contains a frame-shift mutation or a point mutation (T82I in the hinge region) (18). This suggests that the up-regulation of lmrCD observed in these strains is related to a defective LmrR protein.
Regulation of \emph{lmrCD} expression in \emph{Lactococcus lactis}

Table 1. List of primers used

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5’ to 3’); endonuclease sites underlined</th>
<th>endonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{lmrR} FW1</td>
<td>GGC\textbf{CCATGG}GGGCAGAATACCAAAAGAAATG</td>
<td>NcoI</td>
</tr>
<tr>
<td>\textit{lmrR} RV1</td>
<td>GCG\textbf{TCTAGA}TTTAATCGCTTTCATCTTTTATT</td>
<td>XbaI</td>
</tr>
<tr>
<td>\textit{lmrR} FW2</td>
<td>TAT\textbf{AGATCT}GCAATTCGAAATCCAAATAG</td>
<td>BglII</td>
</tr>
<tr>
<td>\textit{lmrR} RV2</td>
<td>TAT\textbf{GGATCC}GTAAGTTGCTTCAGAAGTCG</td>
<td>BamHI</td>
</tr>
<tr>
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<td>TAAATTCAGATTCTCATTCTTACTT</td>
<td></td>
</tr>
<tr>
<td>\textit{lmrR} RV3</td>
<td>TCTTTTTCCCTTTCTATCATTITAAAAAACA</td>
<td></td>
</tr>
<tr>
<td>\textit{lmrCDpmtr} FW1</td>
<td>ATTGTAATCTTTAAACACGATTAC</td>
<td></td>
</tr>
<tr>
<td>\textit{lmrCDpmtr} FW2</td>
<td>ACAAAATAACGTCGTAAATCG</td>
<td></td>
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<tr>
<td>\textit{lmrCDpmtr} RV1</td>
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<tr>
<td>\textit{lmrRpmtr} FW1</td>
<td>TGTCGCAAACGCAATTGTC</td>
<td></td>
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<tr>
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<td>TCAAGGAAAGTTGTCTTCCAGCGCTAA</td>
<td></td>
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<tr>
<td>\textit{lmrRpmtr} RV1</td>
<td>CTGCCATTCTTTTTCCTTTC</td>
<td></td>
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<tr>
<td>\textit{lmrRpmtr} RV2</td>
<td>GGGCTCGTAACATTTCTTTTGGTATTCTG</td>
<td></td>
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<tr>
<td>\textit{lmrC} RT-PCR FW</td>
<td>GTTGAAGACGTTGGGAATTATTTCTCAGGTGG</td>
<td></td>
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<tr>
<td>\textit{lmrC} RT-PCR RV</td>
<td>CACCGTTCTGCTTTTCTGTGATCGATTTTC</td>
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</tr>
<tr>
<td>\textit{lmrD} RT-PCR FW</td>
<td>CTTTCTGATGATGACTCAGCTTCTCAGTGG</td>
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<tr>
<td>\textit{lmrD} RT-PCR RV</td>
<td>CAAAGCAGAATTGATGATAAGTTAGAAGA</td>
<td></td>
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<tr>
<td>\textit{lmrR} RT-PCR FW</td>
<td>ATGGCAGAAATACCAAAAGAAATG</td>
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<tr>
<td>\textit{lmrR} RT-PCR RV</td>
<td>TATATTAATCGCTTCTACTCATTCTTAT</td>
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<tr>
<td>\textit{secY} RT-PCR FW</td>
<td>TACAACCTGCTCCAGCTACGA</td>
<td></td>
</tr>
<tr>
<td>\textit{secY} RT-PCR RV</td>
<td>GTTCCTCAGAGCGAGACAAAT</td>
<td></td>
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</table>

Since the previously characterized MDR strains of \emph{L. lactis} were obtained by long-term drug challenge, repeated transfer, and growth experiments (4), there is a risk that other factors contribute to the MDR phenotype besides LmrR. To evaluate the exact role of LmrR in \emph{lmrCD} expression, the \textit{lmrR} gene was deleted by chromosomal replacement (15,16). A PCR fragment containing the complete \textit{lmrR} gene and the flanking regions was amplified from genomic DNA of \emph{L. lactis} NZ9000 (7) using the primer pair \textit{lmrR} FW2/RV2 (Table 1). The PCR product and
the plasmid pORI280 were digested with BglII/BamHI and ligated resulting in pORIYdaF. Subsequently, the complete \( lmR \) gene was removed from this plasmid by a PCR method using phosphorylated primers facing back to back i.e., \( lmR \) FW3/RV3 (Table 1). The obtained linear PCR product was self-ligated resulting in pORIYdaFDel that was introduced into \( L. \text{ lactis} \) NZ9000 cells containing the temperature-sensitive pVE6007 plasmid which bears the \( repA \) gene necessary for the replication of pORIYdaFDel. Single transformants were grown overnight at elevated growth temperature (37°C) to induce the loss of pVE6007. Integrants were selected by growth in M17 medium (Difco) containing 0.5% glucose (w/v) (GM17) and erythromycin (5 \( \mu g/ml \)), and grown further for 30 to 40 generations in medium without the antibiotic to allow excision of the integrated structure. The deletion was confirmed by PCR and nucleotide sequencing of the corresponding region of the chromosome. \( L. \text{ lactis} \) NZ9000 parental and \( \Delta lmR \) cells were grown at 30°C in GM17, harvested at an OD_{660} of 1 (late log phase) and their transcriptomes were compared by DNA microarray analysis (8,30).

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**Figure 1. Sensitivity of \( L. \text{ lactis} \) NZ9000, \( \Delta lmR \) and \( \Delta lmR CD \) to Hoechst 33342.** Cells of \( L. \text{ lactis} \) NZ9000 (●), \( \Delta lmR CD \) (○) and \( \Delta lmR \) (□) were grown in GM17 medium in the presence of increasing concentrations of Hoechst 33342. The specific growth rates are plotted as a function of the drug concentration.
Expression of a gene was considered to be significantly altered when the Cyber T
baysian $p$ value $\leq 1 \times 10^{-5}$. All transcriptome data discussed here have been deposited
and are accessible through GEO Series accession number GSE9168. LmrC and
LmrD are highly up-regulated (> 4.5-fold) upon deletion of lmrR confirming our
previous assumption that LmrR is a transcriptional repressor of lmrCD. A limited
number of other genes are significantly and strongly (~2-fold) transcribed
differentially. These are mostly related to the intracellular redox state such as trxA,
thioredoxin and superoxide dismutase. Genes that were more than 2-fold down-
regulated are: glnR, glutamine synthetase repressor, cysK, cysteine synthase, and
rplD, 50S ribosomal protein L4. The *L. lactis* NZ9000 $\Delta$lmrR (□) strain showed a
similar growth and resistance against Hoechst 33342 (Fig. 1) and Daunomycin
(data not shown) as compared to *L. lactis* NZ9000 cells (●), but was significantly
more resistant than the $\Delta$lmrCD strain (○) as expected for the de-repression of
lmrCD. Interestingly, we have previously shown that overexpression of lmrCD
restores the drug sensitive phenotype of the $\Delta$lmrCD strain to parental levels only,
despite the increased drug extrusion activity relative to the parental strain (18).

To determine the function of LmrR, the lmrR gene (351 bp) was PCR
amplified from *L. lactis* MG1363 (9) genomic DNA using the primers lmrR
FW1/RV1 (Table 1). The lmrR gene was inserted between the Ncol/XbaI sites of
the pNSC8048 expression vector (encoding a C-terminal Strep-tag) yielding
pNSC8048-lmrR. Following a similar method, the lmrR gene was amplified from
the Rhodamine-resistant *L. lactis* MG1363 strain (4) that contains a point mutation
(T82I), yielding pNSC8048-lmrRRho. *L. lactis* NZ9000 cells, a MG1363 derivative
containing pepN::nisR/K (7) were transformed with these plasmids, and grown at
30°C in GM17 and 5 μg/ml chloroamphenicol to the mid log phase (OD$_{660}$ 0.7-0.8)
whereupon expression was induced by the addition of nisin to 5 ng/ml (7). Growth
was continued for 1 hr, cells were harvested by centrifugation, resuspended in
TrisCl pH 7.0, and lysed by incubation with 10 mg/ml freshly prepared lysozyme
for 45 minutes at 30°C, followed by the addition of 10 mM MgSO$_4$, 100 μg/ml
DNase I and complete protease inhibitor (Roche), and subsequent French Pressure
treatment at 15,000 psi. Cellular debris and membranes were removed by low
speed and ultra centrifugation, and LmrR was purified to homogeneity via
Streptactin Sepharose column chromatography (IBA GmbH) according to the
manufacturer’s protocol. To remove associated DNA, LmrR was further purified
by HiTrap Heparin HP column chromatography (Amersham) in a buffer containing
20 mM TrisCl pH 8.0, 0.2 mM EDTA, and 0.5 mM DTT. The protein was eluted
using a linear gradient of 0.15-1.5 M NaCl in the same buffer. LmrR containing
fractions were pooled and concentrated using a Microcon centrifugal 10 kD cut-off
filter (Millipore Corporation, Bedford, MA, USA). Purified LmrR migrates as a 13.5 kDa protein on SDS-PAGE and mostly as a dimer in gel filtration (data not shown). The ability of LmrR to bind the promoter region of *lmrCD* was assessed by an Electrophoretic Mobility Shift Assay (EMSA) (14). DNA fragments of 205 and 387 bp containing the predicted promoter regions of *lmrCD* and *lmrR*, respectively, were amplified with Pwo DNA Polymerase (Roche) using the PCR primer pairs *lmrCD*pmtr FW1/RV1 and *lmrR*pmtr FW1/RV1 (Table 1). After [γ-32P]ATP end-labeling, the probes were purified and mixed with LmrR (0-50 µg/ml). After 10 minutes incubation at 30°C, the samples were subjected to 6 % PAGE to separate the LmrR bound from the free DNA probe. LmrR causes a mobility shift of the DNA fragment containing the *lmrCD* promoter with an apparent Kd of 0.45 µM (Fig. 2A). The observed shift was efficiently prevented by the addition of an excess unlabeled DNA probe of the *lmrCD* promoter. Interestingly, the LmrR(T82I) mutant failed to induce a DNA mobility shift (Fig. 2B) demonstrating that this mutant is deficient in promoter binding. Since earlier microarray studies suggested that *lmrR* expression is under the control of an auto-regulatory mechanism (18), the ability of LmrR to bind to its own promoter region was also analyzed by EMSA. LmrR effectively binds to the DNA fragment containing the *lmrR* promoter region, while the LmrR(T82I) mutant fails to bind (Fig. 2B). We conclude that LmrR binds specifically to both the *lmrR* and *lmrCD* promoter regions consistent with its proposed role as a transcriptional regulator.

**Figure 2. Interaction of LmrR with the *lmrCD* and *lmrR* promoter regions.** EMSA was performed with increasing amounts of purified LmrR incubated with 0.7 µM of [32P]-labeled 370 bp and 387 bp dsDNA probes comprising the promoter regions of (A) *lmrCD* and (B) *lmrR*, respectively. Where indicated, wild-type LmrR was substituted for the LmrR(T82I) mutant. * single stranded probe DNA. Unless indicated otherwise, LmrR was used at a concentration of 3.7 µM.
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**Figure 3.** DNase I protection of the \textit{lmrCD} and \textit{lmrR} promoter regions by LmrR. Site-specificity of binding of LmrR to the \textit{lmrCD} (A, B) and \textit{lmrR} (B, D) promoter regions. The DNase I digested promoter fragments (A, C) are flanked by the Maxam-Gilbert ladder on the left (AG). Poly(dI-dC) was present to suppress unspecific binding. Nucleotide sequences of the \textit{lmrC} (B) and \textit{lmrR} (D) promoter regions indicating the LmrR protected regions (shaded gray), the putative -35 and -10 regions (boxed), the inverted repeats (arrows) and the structural genes (bold).

To map the binding regions on the \textit{lmrCD} and \textit{lmrR} promoters, DNase I protection assays were performed (14). DNA fragments containing the promoter regions of \textit{lmrCD} and \textit{lmrR} were amplified by PCR using the primer sets \textit{lmrCDpmtr FW2/RV1} and \textit{lmrRpmtr FW2/RV2}, respectively. Various amounts of purified LmrR (0-200 µg/ml) were added, whereupon LmrR protected DNA sequences were determined by the Maxam-Gilbert DNA sequencing method (28). LmrR protects two sites on the \textit{lmrCD} promoter that are separated by 29-base pairs: site I corresponds to the putative -10 and -35 regions and site II contains two direct inverted repeats, i.e., ATGT-10N-ACAT (Fig. 3AB). Interestingly, a similar motif of ATGT-8N-ACAT is conserved among PadR-like regulators (13). A screen
of the *L. lactis* genome for potential binding sites using the site II motif yielded only the promoter region of *lmrCD* consistent with our transcriptome analysis results which show that LmrR is a local transcriptional regulator. LmrR protected a much longer stretch of DNA on its own promoter region with no apparent structural features (Fig. 3CD).

![Figure 4](image)

**Figure 4. Expression of *lmrC*, *lmrD* and *lmrR* in *L. lactis* MG1363 and MDR strains and binding of Hoechst 33342 to LmrR.** The expression of *lmrC*, *lmrD* and *lmrR* and the control gene *secY* was measured by RT-PCR using specific primer pairs and total RNA isolated from the parental *L. lactis* MG1363 incubated in the presence of Hoechst 33342 (5 µM) (A) or Daunomycin (50 µM) (B) and from the drug resistant derivatives Dau<sup>+</sup>, Eth<sup>+</sup>, and Rho<sup>+</sup> in the absence of drugs (C). Binding of Hoechst 33342 to LmrR (○) and LmrR(T82I) mutant (●) (D). Binding was measured as an increase in Hoechst 33342 fluorescence (a.u. = arbitrary units).
The expression of the \textit{lmrCD} and \textit{lmrR} genes was further investigated by RT-PCR using the primer sets listed in Table 1. Transcript levels were followed upon a challenge of \textit{L. lactis} MG1363 cells with the chemically unrelated drugs Daunomycin (50 \(\mu\)M) (Fig. 4A) and Hoechst 33342 (5 \(\mu\)M) (Fig. 4B), both substrates of LmrCD (20). The expression of \textit{lmrC} and \textit{lmrD} transiently increased up to 2-fold within 10 minutes. Unlike in the MDR strains (Fig. 4C), no detectable change in \textit{lmrR} expression was detected in the drug challenged cells (Fig. 4AB). This shows that the \textit{lmrCD} genes are up-regulated in response to the challenge with toxic drugs, and suggest that the auto-regulatory mechanism of \textit{lmrR} differs, at least in timing, from that of the structural genes \textit{lmrCD}.

To determine whether LmrR interacts directly with drugs, binding studies were performed with Hoechst 33342. This drug is essentially non-fluorescent in aqueous medium but becomes highly fluorescent when bound to DNA or protein (21). Addition of increasing amounts of Hoechst 33342 to purified LmrR (5.7 \(\mu\)g/ml) in 50 mM TrisCl pH 7.0, results in a saturable increase in fluorescence (excitation and emission wavelengths of 355 and 457 nm, respectively) (Fig. 4D). Binding saturates at \(\sim\)1 mole of Hoechst 33342 per 1.7 moles of LmrR. In contrast, no fluorescence increase was observed upon Hoechst 33342 addition to the LmrR(T82I) mutant. Therefore, these data suggest that LmrR interacts directly with drugs and that it acts as a drug-regulated local transcriptional regulator of \textit{lmrCD}. Interestingly, many of the PadR regulators are involved in the regulation of the expression of enzymes involved in phenolic acid degradation and detoxification, whereas LmrR regulates the expression of an MDR transporter that expels toxic molecules from the cell. However, \textit{L. lactis} NZ9000, \textit{ΔlmrCD} and \textit{ΔlmrR} cells showed a similar sensitivity to phenolic acid derivatives (data not shown) which excludes a role of LmrR in the regulation of phenolic acid metabolism.

Based on the current findings, we propose that the regulation of the MDR phenotype in \textit{L. lactis} occurs according to the following mechanism: When cells are exposed to toxic compounds in the medium, these compounds may permeate the cell membrane and bind LmrR. This binding event likely alters the LmrR conformation whereupon its interaction with the \textit{lmrCD} promoter region is weakened, allowing the RNA polymerase to initiate transcription. This results in a de-repression of the \textit{lmrCD} genes and hence initiates the expression of a MDR transporter that expels the drugs from the cell. In due course, drug-free LmrR will rebind to the promoter region of \textit{lmrCD} and prevent further expression. The phenotype of the MDR strains of \textit{L. lactis} can now be partially explained as a constitutive de-regulation of \textit{lmrCD} expression due to a defective LmrR that is unable to bind the \textit{lmrCD} promoter region. However, since the MDR strains show
an increased resistance to drugs as compared to the parental strain, other possibly strain-specific mechanisms seem to contribute to the phenotype as well. The previous transcriptome analysis of these MDR strains (18) showed a significant increase in transcript levels of the lmrR gene suggesting that LmrR is under control of auto-regulation. Consistent with this hypothesis, LmrR was found to protect a long stretch of DNA on its own promoter but this region is less defined as that on the lmrCD promoter region. Since no significant increase in the levels of the lmrR mRNA was observed upon a drug challenge, we hypothesize that the binding is either more extensive or tighter, allowing only a low level of lmrR expression. Auto-regulation may be necessary for subtle tuning of the LmrR levels in the cell as an excess of LmrR will interfere with a rapid response of cells towards toxic compounds entering the cells. Also the de-repression of lmrR might only be weakly influenced by, or even be independent of, drug binding to LmrR. This will be a subject for future studies.

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