The Lactococcal ImrP Gene Encodes a Proton Motive Force-dependent Drug Transporter*

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To genetically dissect the drug extraction systems of Lactococcus lactis, a chromosomal DNA library was made in Escherichia coli and recombinant strains were selected for resistance to high concentrations of ethidium bromide. Recombinant strains were found to be resistant not only to ethidium bromide but also to daunomycin and tetraphenylphosphonium. The drug resistance is conferred by the ImrP gene, which encodes a hydrophobic polypeptide of 408 amino acid residues with 12 putative membrane-spanning segments. Some sequence elements in this novel membrane protein share similarity to regions in the transposon Tn10-encoded tetracycline resistance determinant TetA, the multidrug transporter Bmr from Bacillus subtilis, and the bicyclomycin resistance determinant Bcr from E. coli. Drug resistance associated with ImrP expression correlated with energy-dependent extrusion of the molecules. Drug extrusion was inhibited by ionophores that dissipate the proton motive force but not by the ATPase inhibitor ortho-vanadate. These observations are indicative for a drug-proton antiport system. A ImrP deletion mutant was constructed via homologous recombination using DNA fragments of the flanking region of the gene. The L. lactis (ΔImrP) strain exhibited residual ethidium extrusion activity, which in contrast to the parent strain was inhibited by ortho-vanadate. The results indicate that in the absence of the functional drug-proton antiport LmrP, L. lactis is able to overexpress another ATP-dependent, drug extrusion system. These findings substantiate earlier studies on the isolation and characterization of drug-resistant mutants of L. lactis (Bolhuis, H., Molenaar, D., Poelarends, G., van Veen, H. W., Poolman, B., Driessen, A. J. M., and Konings, W. N. (1994) J. Bacteriol. 176, 6957–6964).

For many years antibiotics have been found effective in the treatment of several infectious diseases caused by various pathogens. The occurrence of antibiotic resistance, however, transformed many of the up to now readily treatable diseases to a new threat to public health (Cullinton, 1992; Nikaido, 1994). One of the mechanisms underlying antibiotic resistance involves the extrusion of the compounds by an efflux pump or carrier (Tennet et al., 1989; Levi, 1992; Bolhuis et al., 1994; Midgley, 1987, 1989; Miyachi et al., 1992). For most (micro) organisms, it is not clear whether efflux is mediated by one multispecific system or by several, more or less specific systems (Hächler et al., 1991). The most intriguing mechanisms of drug extrusion are those that can handle a wide variety of structurally unrelated compounds (antibiotics, drugs, etc.), and those are often referred to as multidrug resistance (MDR)* transporters. The bacterial MDR type transporters as well as several closely related specific drug extrusion systems (SDR) can be divided into four groups on the basis of their relatedness in the primary sequences, similarity in the global molecular structure, and/or mechanism of energy coupling (Nikaido, 1994). The first and largest group consists of secondary transporters that are characterized by the presence of either 12 or 14 putative transmembrane spanning segments (Paulsen and Skurray, 1993; Lewis, 1994; Marger and Saier, 1993; Lomovskaya and Lewis, 1992; Rouch et al., 1990). The second group, often referred to as the Staphylococcal multidrug resistance (Smr) family, comprises drug-proton antiporters that are about 100 amino acids long and are composed most likely of four transmembrane α-helices (Yerushalmi et al., 1995; Grinius and Goldberg, 1994). The third group of secondary drug extrusion transporters is formed by the resistance-nodulation division (RND) family, found in Gram-negative bacteria (Saier et al., 1994). The resistance-nodulation division systems require an accessory protein that spans the periplasm and most likely interacts with an outer membrane pore. The accessory proteins are required for the transport of the substrates to the external medium. The fourth group of efflux systems consists of ATP binding cassette transporters (Fath and Kolter, 1993; Higgins, 1993; Molenaar et al., 1992) and is best exemplified by the mammalian P-glycoprotein (MDR1). Recently, also a bacterial homolog of MDR1 was identified in Lactococcus lactis.2

Although a number of MDR- and specific drug resistance-type of transporters have been identified in bacteria, their mechanism of action and energy coupling to transport have not been studied in great detail. The resistance to high concentrations of ethidium bromide (Eth*) daunomycin (Dau*), and rhodamine 6G (Rh*), of three independently isolated mutants of L. lactis MG1363, suggested that at least two different transport mechanisms are involved in multidrug resistance in this organism (Bolhuis et al., 1994). One of the mechanisms is dependent on the proton motive force (Δp), while the other is ATP-dependent. The Δp-dependent system, termed LmrP, is the first multidrug transporter for which both the membrane potential (Δψ), and the proton gradient (ΔpH), has been shown

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X89779.

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The abbreviations used are: MDR, multidrug resistance; bp, base pairs; kb, kilobase pairs; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; ORF, open reading frame; TFP*, tetraphenylphosphonium.

2 H. W. van Veen and K. Venema, unpublished results.
to function as driving force for drug extrusion. In this paper, we describe the gene cloning, characterization, mutant construction, and functional analysis of LmrP.

MATERIALS AND METHODS

Growth of the Organisms—Bacterial strains and plasmids used in this study are listed in Table I. L. lactis strains were grown at 30 °C on M17 medium (Difco) supplemented with glucose (25 mM) and erythromycin (5 μg/ml) when appropriate. Escherichia coli was grown aerobically at 37 °C on Luria Broth (Sambrook et al., 1989) without further additions or with carbenicillin (50 μg/ml) or erythromycin (100 μg/ml). For the determination of growth rates in the presence of various toxic compounds, the E. coli strains were grown semianerobically in microplates; growth was monitored at 30 °C on Luria Broth containing 25 mM glucose. Growth rates were estimated as described previously (Bohlius et al., 1994).

DNA Manipulation and Cloning of the lmrP Gene—General procedures for cloning and DNA manipulations were performed essentially as described by Sambrook et al. (1989). For the cloning of lmrP, chromosomal DNA from L. lactis was isolated as described previously (Leenhouts et al., 1990) and partially digested with HindIII. DNA fragments ranging from 2 to 5 kb were isolated from a 1% agarose gel using the Qiaex DNA extraction kit (Qiagen) and, subsequently, ligated into the vector pBluescript SKII (Stratagene) and transformed into E. coli DH5α. Positive clones were isolated on the basis of ampicillin resistance and a positive phenotype using M17 X-gal agar plates. A number of the blue colonies were subsequently grown for about 30 generations under non-selective conditions in M17 medium lacking erythromycin. Non-selective growth allows a second recombination event to occur, which results in the deletion of the wild-type gene lmrP or pORILMRC. In both cases, the strains are erythromycin-sensitive and β-galactosidase-negative (white colonies on M17 X-gal agar plates). A number of clones were selected, and the ΔlmrP mutation was confirmed by the polymerase chain reaction (PCR) as well as Southern hybridization experiments.

Polymerase Chain Reaction—Chromosomal DNA and synthetic oligonucleotide primers were used at a concentration of 200 ng/10 μl of total PCR reaction mixture. The reactions were performed with Vent DNA-polymerase (New England Biolabs) using denaturation, annealing, and proliferation temperatures of 94, 45, and 73 °C, respectively. PCR products were analyzed by ethidium-stained agarose gel-electrophoresis.

Ethidium and Daunomycin Transport in Whole Cells—The ethidium and daunomycin transport assays are based on the fluorescence properties of the compounds upon interaction with DNA/RNA as described before (Bohlius et al., 1994). A washed cell-suspension (various buffers) with an A₆₅₀ of 0.5 was incubated with 10 μM of ethidium bromide or daunomycin, and the fluorescence was followed using excitation and emission wavelengths of 500 and 580 nm, respectively, for ethidium bromide, and 480 and 590 nm, respectively, for daunomycin. The fluorescence was measured with a Perkin Elmer LS 580 fluorimeter with computer-controlled data acquisition and storage.

RESULTS

Cloning of the Lactococcal Drug Resistance Determinant lmrP—It has been shown that L. lactis MG1363 contains at least two different transport systems that are involved in efflux-mediated multidrug resistance (Bohlius et al., 1994). To genetically dissect the different efflux activities, strategies were developed to clone the transporter genes by complemen-
A library of HindIII-digested chromosomal DNA of *L. lactis* ML3 Eth<sup>+</sup> was made in the expression vector pKK223–3. The DNA library was transformed to *E. coli* HB101, which does not grow on solid media containing ethidium bromide at concentrations above 75 μg/ml. Transformants were plated on Luria Broth agar plates supplemented with ethidium bromide (100 μg/ml) plus carbenicillin (50 μg/ml). Five putative ethidium-resistant colonies were obtained, of which one contained the expression vector with an insert of approximately 3200 base pairs (pKKLMR3.2). For further analysis and DNA sequencing, the 3.2-kb HindIII fragment was ligated into the HindIII site of pBluescript SK II<sup>+</sup>, giving pSKLMR3.2. A restriction map of the 3.2-kb HindIII fragment and multiple cloning site of the vector are shown in Fig. 1A. Southern hybridization using the 3.2-kb HindIII fragment of pKKLMR3.2 as probe confirmed the lactococcal origin of the cloned DNA fragment (data not shown). The digoxigenin-11-dUTP labeled probe hybridized with chromosomal DNA from the Eth<sup>+</sup> strain as well as with DNA from the wild-type strain ML3, its plasmid-free derivative MG1363, and the MDR mutants Rho<sup>+</sup> and Dau<sup>+</sup>, but not with chromosomal DNA from *E. coli* HB101. Although the Eth<sup>+</sup>, Rho<sup>+</sup>, and Dau<sup>+</sup> mutants of *L. lactis* MG1363 have increased resistance toward ethidium bromide, the hybridization experiments provided no indications that the increased resistance was due to amplification of a gene contained by the 3.2-kb HindIII fragment.

Nucleotide Sequence and Identification of the *lmrP* Gene—Nucleotide sequencing of the 3.2-kb chromosomal DNA fragment revealed an open reading frame (ORF) of 1224 bp (position 634-1858) (Fig. 1B). This ORF designated *lmrP* encodes a polypeptide of 408 amino acids, corresponding with a molecular mass of 45,033 Da. Putative promoter sequences TTGACT (−235) and TATAAA (−10) with a spacing of 16 bp were found 190 bp upstream of an ATG initiation codon. The proposed translation initiation side is preceded by a ribosomal binding site at proper distance of the ATG. A putative terminator

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**Fig. 1.** A, restriction map and open reading frames (arrows) of the 3.2-kb HindIII fragment of *L. lactis*. Flanking regions corresponding to the multiple cloning site of pBluescript SK II<sup>+</sup> are also shown. B, nucleotide sequence of the multidrug transporter (*lmrP*) and the flanking regions. Putative ribosomal binding site (rbs), promoter elements (−10/−35), and terminator sequence (−⋯−) are indicated. The deduced amino acid sequence of LmrP is shown below the DNA sequence (residue numbers between brackets). The predicted membrane spanning α-helices are underlined and shown in boldface.
sequence with a calculated free energy $\Delta G$ of $-7.6$ kcal was identified 28 bp downstream of the TAA stop codon. A second putative ORF (ORF2) was found downstream of $\text{lmrP}$. However, deletion of up to 200 bp 5' of the NruI site (Fig. 1A) did not affect the ethidium resistance in $E. coli$ HB101, whereas removal of the 800-bp HindII-II-EcorI fragment (promoter region and 5' end of $\text{lmrP}$) totally abolished the ethidium resistance (data not shown). These experiments indicate that $\text{lmrP}$ is essential and sufficient in conferring ethidium resistance to $E. coli$.

Properties of the $\text{lmrP}$ Gene Product—The amino acid composition of the LmrP protein corresponds with that of a membrane protein being rich in hydrophobic residues (Val, Leu, Ile, Phe, Met, and Ala), constituting 53% of the total number of residues. The hydropathy profile of LmrP, according to the method of Kyte and Doolittle (1982), classifies the polypeptide sequence with a calculated free energy $\Delta G$ of $-7.6$ kcal was identified 28 bp downstream of the TAA stop codon. A second putative ORF (ORF2) was found downstream of $\text{lmrP}$. However, deletion of up to 200 bp 5' of the NruI site (Fig. 1A) did not affect the ethidium resistance in $E. coli$ HB101, whereas removal of the 800-bp HindII-II-EcorI fragment (promoter region and 5' end of $\text{lmrP}$) totally abolished the ethidium resistance (data not shown). These experiments indicate that $\text{lmrP}$ is essential and sufficient in conferring ethidium resistance to $E. coli$.

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Construction and Analysis of the ImrP Deletion Mutant—To establish the in vivo role of L. lactis lmrP, a deletion mutant was constructed via homologous recombination as described 
under “Materials and Methods.” The integration event was 
confirmed via PCR analysis, using two different sets of primers 
(LMR28/LMR2) specific to sequences outside of the deleted fragment, and these 
yielded a 1340-bp PCR product with template DNA from 
the wild-type strain DNA and a 560-bp product with DNA from 
ΔlmrP (Fig. 5, A and B, lanes 4 and 2). The second set of 
oligonucleotide primers (LMR28/LMR29) complementary to 
sequences outside of the deleted fragment and inside (LMR2) the deleted fragment, 
gave a 730-bp PCR product with wild-type DNA while, as 
eXpected, no product was obtained with DNA from the ΔImrP strain (Fig. 5, A and B, lanes 3 and 1).

To analyze the drug resistance phenotype of L. lactis 
MG1363(ΔlmrP), the mutant strain was studied further in 
growth and transport assays. Within the ethidium bromide 
concentration range tested (0–25 μM), the L. lactis deletion 
mutant MG1363(ΔlmrP) was only slightly more sensitive than 
L. lactis MG1363(wild-type), whereas L. lactis MG1363/ 
pGKLMR3.2 was more resistant to ethidium bromide than the 
wild-type (data not shown). Since L. lactis possesses an ATP-
deleted efflux activity in addition to the Δp-driven efflux activity, it is possible that alterations in the expression of the 
ATP-dependent system could have masked the drug 
resistance phenotype of L. lactis MG1363(ΔImrP) and MG1363/
pGKLMR3.2. Indeed, in the presence of ortho-vanadate, the 
Inhibitor of the ATP-dependent efflux activity, the ΔImrP 
strain accumulated more ethidium than the wild-type strain 
(Fig. 6, +). Overexpression of LmrP from pGKLMR3.2 reduced 
the intracellular ethidium levels to the same extent irrespective of whether or not the cells were preincubated with 
ortho-vanadate. (Fig. 6; ±).

Comparison of the ImrP Nucleotide Sequence from Wild-type, 
EthR, DauR, and RhoR Strains of L. lactis—In order to establish 
whether the MDR phenotypes of EthR, DauR, and RhoR were 
due to mutation(s) in the promoter region and/or the structural 
gene for ImrP, the corresponding DNA sequences were compared with that of the wild-type. The genes from L. lactis 
MG1363 (wild-type), DauR, and RhoR were amplified by PCR 
using Vent DNA polymerase. The 1400-bp product was di-
gested with ClaI, and the two products (850 and 550 bp) were 
ligated into pSKN, a derivative of pBluescript SKII + containing 
a unique NcoI site in the multiple cloning site. The nucleo-
tide sequence of ImrP from L. lactis MG1363 (wild-type) and 
the MDR strains EthR, DauR and RhoR appeared to be identical. In addition, the amplified and sequenced 490-bp PCR 
product from the chromosome of these strains, containing the pro-
moter region and ribosomal binding site, did not reveal any 
differences in the DNA region that could explain the differ-
ences in drug resistance of the various strains.

**DISCUSSION**

In this paper, we report the isolation of a chromosomal DNA 
fragment from L. lactis, which effects an increased resistance 
to ethidium bromide, daunomycin, and TPP + when expressed 
in E. coli. This DNA fragment contains an ORF that specifies 
an integral membrane protein (LmrP) with 12 putative α-helical 
membrane spanning segments. When the amino acid se-
were preincubated for 10 min in a phosphate-free buffer (50 mM

aspartate (Asp66) and the positively charged arginine (Arg70)

motif A is identical to the consensus sequence G\(_{\text{pil}}\)PGVlGG

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between transmembrane segment 2 and 3. It has been suggested that this loop is of structural importance to the polypeptide, but it might also be involved in the conformational change required for the opening and closing of the transport channel (Paulsen and Skurray, 1993). Functional studies of site-directed mu-

aspartate (Asp\(_{286}\)) and a lysine (Lys\(_{291}\)) in the cytoplasmic loop

between transmembrane segment 8 and 9, which align with Asp\(_{286}\) and Lys\(_{291}\) of the G\(_{\text{XXDRXR}}\)(K/R) consensus sequence.

Although ImrP was originally cloned from an ethidium-resis-
tant strain of \(L.\) lactis ML3, the nucleotide sequence was identical to that of the wild-type and the other multidrug-resistant mutants of \(L.\) lactis MG1363. Also, the promoter regions upstream of the structural gene were identical, and DNA hybridization experiments indicated that the observed increase in drug resistance and drug efflux is not due to amplification of the ImrP gene on the chromosome. As shown previously (Bolhuis et al., 1994), the MDR phenotype of the drug-resistant mutants Eth\(_R\), Dau\(_R\), and Rho\(_R\) resulted from increased drug extrusion from the cell. These mutant strains have a comparable drug resistance spectrum, but differ with respect to their mode of energy coupling to active drug efflux. Whereas ethidium extrusion from the Eth\(_R\) strain is mainly driven by an ATP-dependent transport activity, a significant contribution of both the ATP-dependent and the \(\Delta\p\)-driven transport systems was observed in the ethidium extrusion from Dau\(_R\) and Rho\(_R\) (Bolhuis et al., 1994). Since the nucleotide sequence of ImrP in the MDR strains is identical, the increased resistance in these strains must be due either to an increased expression of ImrP, possible affected by mutation of a transcription factor, or to an alternative drug extrusion activity.

The ImrP gene was cloned in \(E.\) coli HB101 by selection for resistance to high concentrations of ethidium bromide. In the hypersensitive \(E.\) coli CS1562, expression of ImrP not only increased resistance to ethidium but also to unrelated hydrophobic compounds like TPP\(_p\) and daunomycin. Increased ethidium resistance was also observed when ImrP was present on a plasmid in \(L.\) lactis MG1363, but the effect was less pronounced. The lower LmrP-dependent ethidium resistance in \(L.\) lactis is most likely the result of the relatively low copy number of the pGK13 vector in \(L.\) lactis as compared with the vector used in \(E.\) coli (Kok et al., 1984). Transport studies in \(L.\) lactis showed that LmrP catalyzes the energy-dependent efflux of ethidium and daunomycin. The energy-dependent efflux of ethidium from the wild-type strain and \(L.\) lactis MG1363(pGKLMR3.2) was completely inhibited upon dissipation of the \(\Delta\p\) but unaffected by the ATPase inhibitor ortho-

vanadate.

These observations are consistent with a drug-pro-

ton antiporter mechanism for LmrP. Deletion of part of the ImrP gene from the chromosome of \(L.\) lactis MG1363 (wild-type strain) affected drug extrusion when the cells were pretreated with ortho-vanadate. However, the \(\Delta\)ImrP mutation did not result in significant changes in the drug resistance phenotype. These results suggest an increased expression (or activity) of the ATP-dependent drug transporter (Bolhuis et al., 1994). It is possible that this increased expression is essential to survive the otherwise lethal effect of the deletion of ImrP. The occurrence of efflux-mediated resistance to various hydrophobic toxic compounds and antibiotics in both pathogens and non-

pathogens implicates a general mechanism and an important physiological function. This function could involve a general detoxification mechanism against naturally occurring hydro-

![Fig. 6. Ethidium uptake in \(L.\) lactis MG1363 (wild-type), \(L.\) lactis MG1363/pGKL3R3.2, and \(L.\) lactis MG1363/ΔlmrP. Cells were preincubated for 10 min in a phosphate-free buffer (50 mM HEPES, 25 mM \(K_2\)SO\(_4\), 5 mM MgSO\(_4\), pH 7.4) in the presence (+) or absence (-) of 0.5 mM ortho-vanadate. L-Arginine (10 mM) was added as source of metabolic energy to allow ortho-vanadate to be taken up. The assay was started upon addition of 10 \(\mu\)M of ethidium bromide to the cell suspension.](image-url)
phobic compounds (Gottesman and Pastan, 1993; Higgins, 1993).

REFERENCES


