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

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To genetically dissect the drug extrusion 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 lmrP 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 lmrP 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 antiporter system. A lmrP deletion mutant was constructed via homologous recombination using DNA fragments of the flanking region of the gene. The L. lactis (ΔlmrP) strain exhibited residual ethidium bromide 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 antiporter 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., Moleneara, 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 (Tennent et al., 1989; Levi, 1992; Bolhuis et al., 1994; Midgley, 1987, 1989; Miyauchi 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; Moleneara 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 (Ethb), daunomycin (Daua), and rhodamine 6G (Rhoa), 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 (Δφ), and the proton gradient (ΔpH), has been shown

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1 The abbreviations used are: MDR, multidrug resistance; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; ORF, open reading frame; TPP+, tetraphenylphosphonium.

2 H. W. van Veen and K. Venema, unpublished results.

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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 (100 μg/mL) or with carbenicillin (50 μg/mL) or with carbenicillin (50 μg/mL). Growth rates were estimated as described previously (Bolhuis et al., 1994). For the cloning of LmrP on the chromosome, thereby allowing the pOR1280 derivative to replicate in E. coli (Leenhouts and Venema, 1993; Hagting et al., 1994). The resulting construct pOR1280 was isolated from E. coli EC1000 and transformed to L. lactis MG1363. As this strain does not contain the essential replication factor (RepA), selection for growth in the presence of erythromycin forces the plasmid to integrate into the chromosome by homologous recombination. Positive colonies were selected on the basis of erythromycin resistance and a β-galactosidase positive phenotype using M17 X-gal agar plates. A number of the blue colonies were subsequently grown for about 30 generations under nonselective conditions in M17 medium lacking erythromycin. Nonselective growth allows a second recombination event to occur, which results in the deletion of either the wild-type gene LmrP or pOR1280. 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 LmrP 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/100 μL of total PCR reaction mixture. The reactions were performed with Vent DNA-polymerase (New England Biolabs) using denaturing, 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 (Bolhuis et al., 1994). A washed cell-suspension (various buffers) with an A₅₆₀ of 0.5 was incubated with 10 μM of ethidium bromide or daunomycin, and the fluorescence was followed using excitation and emission wavelengths of 580 and 590 nm, respectively, for ethidium bromide, and 480 and 590 nm, respectively, for daunomycin. The fluorescence was measured with a Perkin Elmer LS 50B fluorometer 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 (Bolhuis et al., 1994). To genetically dissect the different efflux activities, strategies were developed to clone the transporter genes by complemen-

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**Table I**

**Bacterial strains and plasmids**

| Bacterium/plasmid | Relevant characteristics | Source/Refs. |
|-------------------|--------------------------|--------------|
| L. lactis ML3     | Wild-type                | Gasson (1983) |
| MG1363            | ML3, plasmid free, Lac-, Prt- | Gasson (1983) |
| MG1363∆lmrP       | MG1363, ∆lmrP            | This work    |
| E. coli BX2       | LE392, pCN300a::Tet     | François et al. (1987) |
| HB101             | pBR322( regulatory region of lacZ) | Sambrook et al. (1989) |
| DH5x              | supE44, supE44ΔlacI957, lacI957ΔlacZ1515 | BRL Inc., Bethesda |
| MC1061            | aradiA139, araI139-6969, lacI174, galU, galK, hsdR, strA (streptomycin) | Sambrook et al. (1989) |
| EC1000            | MC1000, ApR, Km, carrying a single copy of pWV01 repA in the gpb gene | K. Leenhouts, unpublished |
| CS1562            | F-, lacI, lacI100, attC, Tn10 | Bachmann |

**Plasmids**

| Plasmid          | Relevant characteristics | Source/Refs. |
|------------------|--------------------------|--------------|
| pBluescript SK1- | Ap', expression vector   | Stratagene   |
| pSKLKRNC.2       | pBluescript SK II, carrying lmrP of L. lactis on a 3.2-kb HindII fragment | This work |
| pKK223-3         | Ap', expression vector   | Pharmacia    |
| pGK13            | Em', Cm', E. coli-L. lactis shuttle vector | Kok et al. (1984) |
| pKKLMRNC.2       | pGK13, carrying lmrP of L. lactis on a 3.2-kb HindII fragment | This work |
| pOR1280          | Em', LacZ', integration vector lacking repA | Leenhouts and Venema (1993) |
| pORILMRNC        | pOR1280, containing flanking regions of lmrP | This work |
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 (<sup>−35</sup>) and TATAAA (<sup>−10</sup>) were found 190 bp upstream of an ATG initiation codon. The proposed translation initiation site is preceded by a ribosomal binding site at proper distance of the ATG. A putative terminator sequence is located 190 bp downstream of the ATG. 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 ImrP. 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 HindIII-EcoRI fragment (promoter region and 5' end of ImrP) totally abolished the ethidium resistance (data not shown). These experiments indicate that ImrP is essential and sufficient in conferring ethidium resistance to E. coli.

Properties of the ImrP 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 as an integral membrane protein with 12 hydrophobic regions large enough to span the plasma membrane (Fig. 1B, underlined and boldface). A secondary structure model of LmrP based on the hydropathy profile and the positive-inside rule of von Heijne (1986) is shown in Fig. 2. Both the carboxyl- and the amino-terminal ends as well as a large central hydrophilic loop between helix 6 and 7 are predicted to be on the cytoplasmic side of the membrane.

Multidrug Resistance Linked to ImrP Expression in E. coli—The ImrP gene was isolated for its ability to confer ethidium bromide resistance to E. coli HB101. To check whether the ImrP gene product is involved in the efflux of multiple drugs, growth of E. coli was monitored in the presence of various concentrations of drugs. For these experiments pSKLMR3.2 was transferred to E. coli CS1562, containing a TolC integration mutation (tolC::Tn10), which makes the strain hypersusceptible to various (hydrophobic) toxic compounds due to an impaired barrier function of the outer membrane. Overnight cultures of E. coli CS1562/pSKLMR3.2 and the control strain CS1562/pBluescript SKII+ grown on Luria Broth, were transferred to 96-well microplates containing 250 $\mu$L of LB medium supplemented with glucose (25 mM), chloramphenicol (50 $\mu$g/mL), ampicillin (50 $\mu$g/mL), and ethidium (A), daunomycin (B), or TPP$^+$ (C) at various concentrations. The relative growth rate is plotted as a function of the drug concentration. The growth rates in the absence of added drug corresponded to 0.54 and 0.63 h$^{-1}$ for E. coli CS1562/pSKLMR3.2 and E. coli CS1562(pBluescript SKII+), respectively.

ImrP mediates pmf-dependent drug extrusion, inhibited ethidium efflux in both the wild-type and the strain expressing LmrP (Fig. 4; Nig). Active ethidium efflux from...
both strains was not affected by the ATPase inhibitor ortho-vanadate (see below, Fig. 6). Comparable results were obtained with the fluorescent chemotherapeutic agent daunomycin as transport substrate (data not shown), confirming that ImrP encodes a transport protein catalyzing Δp-dependent extrusion of multiple drugs.

Construction and Analysis of the lmrP Deletion Mutant—To establish the in vivo role of LmrP in L. lactis, 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 to distinguish between the wild-type and deleted gene. Chromosomal DNA isolated from wild-type and putative deletion mutants was used as template DNA for the PCR reaction. One set of oligonucleotide primers (LMR28/LMR29) is complementary to sequences outside of the deleted fragment, and these yielded a 1340-bp PCR product with template DNA from the chromosome of these strains, containing the promoter region and ribosomal binding site, did not reveal any differences in the DNA region that could explain the differences in drug resistance of the various strains.

To analyze the drug resistance phenotype of L. lactis MG1363ΔlmrP, 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 MG1363ΔlmrP 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-dependent efflux activity in addition to the Δp-driven efflux activity, it is possible that alterations in the expression of the ATP-dependent system could (partially) have masked the drug resistance phenotype of L. lactis MG1363ΔlmrP and MG1363/pGKLMR3.2. Indeed, in the presence of ortho-vanadate, the inhibitor of the ATP-dependent efflux activity, the ΔlmrP 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 digested 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 nucleotide 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 promoter region and ribosomal binding site, did not reveal any differences in the DNA region that could explain the differences 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-
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<sup>+</sup> 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 ImrP-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 ImrP 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 Δp but unaffected by the ATPase inhibitor orthovanadate. These observations are consistent with a drug-proton antiporter mechanism for ImrP. 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 ΔImrP 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 (Bohuis et al., 1994). It is possible that this increased expression is essential to survive the otherwise lethal effect of the deletion of ImrP.
phobic compounds (Gottesman and Pastan, 1993; Higgins, 1993).

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