Regulation of multidrug resistance in Lactococcus lactis

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	: Prof. dr. J. M. van Dijl
	: Prof. dr. J. Kok

For Mama, Papa, Ryan, Fajri, Wouter, and everyone who got involved in the making of this book.

List of publications

1. **Agustiandari, H**., Peeters, E., de Wit, J.G., Charlier, D., Driessen, A. J. M. Distinct mechanism of LmrR mediated gene regulation of multidrug resistance in *Lactococcus lactis*. Submitted

2. Madoori, P. K., **Agustiandari, H**., Driessen, A. J. M., Thunnissen, A. M. 2009. Structure of the transcriptional regulator LmrR and its mechanism of multidrug recognition. EMBO, **28**, 156 – 166

3. Zaidi, A.H., Bakkes, P.J., Lubelski, J., **Agustiandari, H**., Kuipers, O. P., Driessen, A. J. M. 2008. The ABC-type multidrug resistance transporter LmrCD is responsible for an extrusion-based mechanism of bile acid resistance in *Lactococcus lactis*. J.Bacteriol., **190**, 7357-7366

4. **Agustiandari, H**., Lubelski, J., van den Berg van Saparoea, H. B., Kuipers, O. P., Driessens, A. J. M. 2008. LmrR is a transcriptional repressor of expression of the multidrug ABC transporter LmrCD in *Lactococcus lactis*. J. Bacteriol., **190**, 759-763

5. Lubelski, J., de Jong, A., van Merkerk, R., **Agustiandari, H**., Kuipers, O. P., Kok, J., Driessen, A. J. M. 2006. LmrCD is a major multidrug resistance transporter in *Lactococcus lactis*. Mol. Microbiol., **61**, 771-781

Content

Preface

It all started online when I accidently stumbled to this beautiful photo of the ancient-looking academic building belongs to the University of Groningen back in spring 2004. A couple of months later I arrived at Schipol airport with two big luggages, and a great amount of excitement!

Many summers passed so quickly each year in the Netherlands with so many memories I will not forget. As if it was just yesterday when I started pipetting as early as 6 o'clock in the morning, left the lab at midnight, running up and down to different floors, carrying 3 different timers at once, and sang personalized lullabies to the growing cultures or incubating reactions for good results. And finally, here it is, my book. Regardless I was quite disappointed to find out that the biological center in Haren is far from looking old and ancient however, I will be defending my PhD in the academic building of the University of Groningen!

I would like to thank my supervisor and promoter, Arnold Driessen for the opportunity to join the molecular microbiology group as your PhD student. Thank you for all of your help, motivation, critical comments, and stimulating discussions over those years. I still remember when we were doing FRET of Hoechst 33342/LmrR together, and I dropped the whole box full of ice on the floor at the beginning of the experiment. You said "What happened?" I answered "You made me nervous because now I cannot make any mistakes." I was surprised that you still can pippet quite fast after all of these years! And I promise you will not be seeing any pink poster from me anymore ;-)

I also would like to thank the members of the multidrug resistance (MDR) group: Bart (thank you for all of your help!), Ronald, Jacek, Abhishek, Patrick (for stimulating discussions), and Jacobien – my first and only student (for making a lot of mutants together). I will remember that fun "Bad Results Go Away" borreltje in our small MDR office, and constant supply of junk foods in the early years.

To my paranimfs Ilja and Davide, thank you! Ilja, thank you for those fun cocktail and techno parties, those "lekker" chinese take away in one of the offices whenever we stayed late in the lab, x-mas market and weekend trip to Dusseldorf, shopping for your suit, and helping me moving out from Groningen. Davide, thank you for those great chats, cozy dinners, delicious cannoli treats, brunch, and always cheering me up in the hallway whenever you saw me walking (or running). Albert,

thank you for your continuous availability for coffee breaks, music festivals, mud walking to Ameland, sneak previews at Pathe, and cheesy bars hoping.

Big thanks to the “fungus” group (Jeroen, Marta W, Andrij) for those emergency buffers and “dirty” tricks that work! Alexej (for fun discussions in all topics), Paolo and Carmen (for constant motivation in the beginning of my PhD period), Janny (for those nice results of qRT-PCR), Intan (for everything!), Bea (for helping me dealing with “constant mistakes” created by the persistent Dutch tax office), Moses (for calibrating all of my pipettes twice a year), and all members of the Molecular Microbiology group.

I also would like to thank our collaborators: Pramod and Andy (for the beautiful structure of LmrR); Eveline and Danny (for helping me a lot with the experimental works and critical analysis of my data during my 3 weeks visit in your lab), Veerle and Phu (for nice time at VUB).

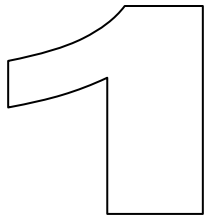
Fean, Sandra K, Wangsa, Paulien, Stephan, Fia, Jens, Goetz, Anja, Parisa, Dusko, Daniela, Francesca, Francisca, Arne, Okta, Saras, Elena, and to everyone that I cannot mention one by one; thank you for those good times in Groningen! Great parties, biking in the rain, casino, Images, laser game, cosmic bowling, sunbathing in the park, “spontaneous after midnight visit with a can of beer due to the excessive stress caused by thesis writing”, exotic drinks, Noorderzon festivals, Pink pop, asthanga yoga, street dance class, pasta cooked in vodka, KFC Thursday, “forever tourist” in Holland, Go Go, travelling to Cologne, Prague, and Lisbon, extensive amount of “dates” in the city center, and so much more! Elise, Jussi, David, Martin, and the rest of “Da Container C” people (x-mas market in Bremen, Hamburg trip, Bourtange, crazy student parties; that 6 months was one of the best times I had in Groningen!).

Bastiaan (thank you for everything; trips all over Holland, and sailing!), Sandra A, Lia, April, Dodi (always great time with you!), Feba (fun tour inside the Radio Nederland Wereldomroep, you can kidnap me anytime for more interview!). A super big thank to Hirwan and Gerard (I can always be myself around you! Thank you for refueling “life” in me, you two are just fabulous!).

And to Mama and Papa, I cannot be where I am standing right now without your love and help. Mama, thank you so much for sending me abroad after high school. You are giving me such a great opportunity that I am grateful for! Papa, thank you for teaching me what life is and great tips over these years. Terima kasih banyak Ma, Pa atas semua yang telah Mama dan Papa berikan ke Pipit selama ini. For my brothers Ryan and Fajri, remember our dreams?!

Last but not least, Wouter. Thank you my yellow head for being there during rainy and sunny days, painting nice colors in my life through out these times we spent together. You have showed me a different world full with exciting ideas, fun, and love. What more can I say? This is great!

Chapter



General introduction

THE RISE OF THE SUPERBUGS

Just about all organisms maintain close physical contact with other organisms in a changeable and dynamic ecosystem. Networking is important to cope with sudden environmental changes and it is beneficial for survival. Changing conditions and competition amongst organisms for essential nutrients, together with humans' interference in the global biosphere have lead to rapid evolutionary changes in all organisms involved. The first introduction of pesticides and antibiotics during the industrialization era in 1940's significantly increased the quality of humans' life as it provided protection against pests and infections caused by pathogenic bacteria (121). However, due to the extensive use of antibiotics and other toxic compounds, bacteria quickly developed numerous ways to gain resistance against these molecules. The ability of bacteria to be resistant to several chemically unrelated drugs is termed the multidrug resistance (MDR) phenomenon. Bacterial MDR was thought to arise via natural selection involving spontaneous mutation(s) in the genome to execute novel activities against a range of antibiotics. This resistant trait is generally passed on to the next generation and quickly leads to a fully resistant colony. It is important to note that bacteria did not evolve a specific response towards the presence of antibiotics. Rather, they became resistant via the mobilization and/or modification of pre-existing defense mechanisms that allow them to cope with the unfavorable environmental conditions (157).

One specific example of drug resistance is the re-emergence of the multidrug resistant tuberculosis bacterial strain (MDR-TB) which emphasizes the need for a new strategy and control of infectious disease since treatment with conventional drugs such as rifampicin and isoniazid have failed frequently (63). The first report on MDR-TB from 2005 showed that this organism is not only resistant to the previously used drugs but also to three out of six classes of second line antitubercular drugs like aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine and para-aminosalycilic (63). This new strain is also known as the extensively drug resistance tuberculosis (XDR-TB) that has emerged due to the improper use of antibiotics during treatments of tuberculosis (60). Another concerning example is the methicilin-resistant *Staphylococcus aureus* (MRSA), a major cause of nosocomial infections that can be acquired in hospitals. This organism readily takes up new plasmids, transposons, and its genome easily undergoes mutations. Its resistance to the blockbuster antibiotic vancomycin has lead to the emergence of a new strain known as VRSA (vancomycin resistant *S. aureus*) (1).

Molecular studies on multidrug resistance intensified after the discovery of P-glycoprotein (P-gp) in mammalian cancer cells (157). Cells that overexpressed

P-gp survived the cytotoxic effects of anti cancer drugs during therapy, and became cross resistant to a wide range of drugs from different classes (157). Shortly after the discovery of P-gp, bacterial resistance to antibiotics became apparent (75,105) as well as for instance the emergence of resistant insects to pesticides (12,41). Multidrug resistant bacteria were first observed in 1960s (176), followed by the discovery of cross resistant tumors and cell lines which occurred after the introduction of combined chemotherapy (19,67). P-gp is a transport protein that expels drugs from the cell thus, protects the cells against the adverse effects of these drugs.

MULTIDRUG RESISTANCE IN BACTERIA

Several mechanisms exist to gain resistance to toxic compounds. They are based on drug degradation via enzymatic reactions, an alteration of the drug target, the prevention of drug entry, and finally the active drug expulsions from the cell. For instance, some bacteria produce β-lactamases that catalyze the hydrolysis of the β-lactam ring of penicillin (162) while chloroamphenicol transferase modifies aminoglycosides into inactive entities (11). Drug target alteration prevents or reduces the binding of the drug. For instance, some amino acid mutations in penicillin binding proteins (PBPs) prevent the irreversible binding of penicillin which subsequently renders peptidoglycan biosynthesis penicillin-insensitive (162). In tetracycline resistant bacteria, the ribosomes often undergo a covalent modification that prevents the binding of tetracycline (160). However, these mechanisms are specific only to certain classes of antibiotics and usually do not results in multidrug resistance. Bacteria contain a cytoplasmic membrane that function as a barrier that separates the inner and the outer part of the cell. This barrier also prevents the rapid entry of small molecules like drug(s). Gram-negative bacteria contain an additional outer membrane that consists of phospholipids and lipopolysaccharides (LPS) which exhibits a reduced permeability towards lipophilic drugs. Gram-positive bacteria have no outer membrane but instead a thick peptidoglycan layer that allows diffusion of small molecules (108). Mycobacteria on the other hand, contain a thick extracellular layer of mycolic acids that is highly impermeable for drugs. Both Gram-negative bacteria and mycobacteria can decrease their membrane permeability via the loss of porins (107). However, these barriers do not counteract the toxicity effect of the drugs once they enter the cells. Therefore, cells need another line of defense and one of the most common mechanisms is the active drug extrusion from the cell by transporter proteins embedded in the cytoplasmic membrane. Drug extrusion is an

energy dependent phenomenon and a growing number of membrane proteins have been described to function as multidrug resistance efflux proteins. The active extrusion of drugs appears to be the main mechanism of MDR and SDR (specific drug resistance) in bacteria (132).

MULTIDRUG RESISTANCE TRANSPORTERS IN BACTERIA

Tetracycline resistance in both Gram-positive and -negative bacteria is due to the expression of transporters that mediate tetracycline efflux (28). These proteins belong to a large family of MDR transporters that are equipped with a broad substrate specificity of many chemically unrelated compounds (123,132,141). Based on their bioenergetic mechanisms and structural differences, bacterial MDR transporters can be divided into two major classes, i.e., primary and secondary transporters. Some bacteria mostly rely on the primary transporters for drug resistance, whereas others utilize the secondary transporters (125,126). Primary MDR transporters belong to the ABC superfamily and use the free energy derived from ATP hydrolysis to extrude toxic compounds from the cell (55). ABC transporters involved not only in drug export but also in a variety of other cellular activities such as peptide export, uptake of nutrients, and also in transport-unrelated functions such as DNA repair, translation, and regulation in gene expression (24,31,55). The proteins that are involved in the transport unrelated functions utilize the universally conserved ATP-binding cassette for their catalytic activities and lack the membrane domain. ABC transporters are membrane proteins with a typical organization, i.e., two nucleotide binding domains (NBD) where ATP hydrolysis take place, and two transmembrane domains (TMD) responsible for substrates recognition and transport (55). Each TMD consists of six membrane spanning α-helical domains with a low amino acid sequence identity among the group of ABC transporters. The two TMDs may exist as a single fused polypeptide chain that associates with a heterodimeric, homodimeric or even two fused NBDs. The MDR transporters are usually single polypeptides that each comprise of two TMDs and NBDs. Some MDR transports are homo- or heterdimeric proteins that built from subunits consisting of a TMD fused to the NBD. The secondary multidrug transporter mediate drugs extrusion in a coupled exchange with protons (or sodium ions) also known as drug/H+ or drug/Na+ antiporters (132). Secondary transporters form the largest group of known extrusion systems in bacteria comprising of four subdivisions, i.e., the major facilitator superfamily (MFS), the small multidrug resistance family (SMR), the resistance-nodulation-cell division family (RND), and the multidrug and toxic compound extrusion (MATE) family.

These MDR transporters share little homology amongst each other but they may recognize a similar range of substrates.

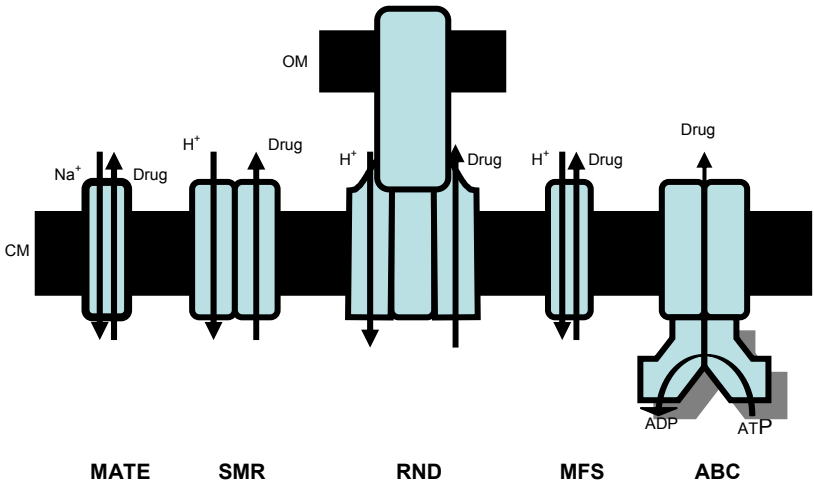


Figure 1 Distribution of MDR pumps in Gram positive and negative bacteria. Primary transporters utilize the energy derived from ATP hydrolysis to secrete drugs out of the cells. They belong to the ATP-Binding Cassette (ABC) superfamily and function either as a homo- or hetero dimeric transporters. To date, many of the ABC type transporters involved in MDR are mostly found in the Gram positive bacteria. Secondary transporters are driven by the proton motive force (PMF), and catalyse drug export by H^+/Na^+ antiporter: Transporters of the Major Facilitator Superfamily (MFS) function as monomers, while trasnporters of the Small Multidrug Resistance family (SMR) function as either a homo or heterodimers. The Resistance-Nodulation-cell Division (RND) transporters consist of a tripartite system and functions as trimers. RND transporters are mainly found in the Gram negative bacteria and require accessories protein such as an outer membrane protein (OMP) and membrane fusion protein (MFP). Another member of the secondary transporters belongs to the MATE (Multi Antimicrobial Extrusion) family that use the sodium motive force.

PHYSIOLOGICAL FUNCTIONS OF MULTIDRUG RESISTANCE IN BACTERIA

The exact physiological function of most bacterial MDR transporters is unknown, but often they are involved in the defense mechanisms against specific class of chemical compounds. The MDR phenotype is often associated with an enhanced expression level of these transporters or by mutations in the structural gene. For instance, the expression of the *B. subtilis* MDR transporter Blt results in an

increase efflux of spermidine in the medium (188). The *blt* gene is co transcribed with the *bltD* gene which encodes a spermidine acetyltransferase which catalyzes a key step in spermidine degradation. Reserpine, an antagonist of MDR transporters inhibits spermidine efflux by Blt (187) showing the close functional relationship of Blt with MDR transporters. The ability of some MDR transporters to recognize lipids or fluorescence lipid derivatives, and to transport detergent (133,185), bile salts (84,168), organic solvents (184), and ionophores (37,156), might indicate a natural functions in phospholipid transport or of lipid linked precursor of peptidoglycan (23,59,86,177,191). Various antibiotics that appear to be the substrates of the MexAB-OprM transporter of *Pseudomonas aeruginosa* are secondary metabolites that result from the aromatic amino acid biosynthesis pathway (129). For instance, the presence of iron in the medium significantly increased the expression of MexAB-OprM causing the efflux of peptide pyoverdine that is involved in iron uptake (130). This finding suggests a physiological role of MDR-like transporters in secondary metabolite excretion.

REGULATION OF MULTIDRUG RESISTANCE IN BACTERIA

Bacterial MDR can be either intrinsic or acquired. The basal expression of MDR pumps in the wild-type only suffices to provide a certain level of protection. At a higher drug concentration, resistance can be acquired via several mechanisms: (i) amplification and mutations of the MDR transporters causing changes in the expression level (106) or activity (70); (ii) mutations in the regulatory genes causing constitutive expression of the MDR transporters (2), and, (iii) resistance gene transfer among cells via transposons and plasmids (68*)* (For general review of mechanisms of bacterial resistance see refs (8,49)). Regulatory proteins respond to changes in the environment and trigger necessary cellular modifications at the transcriptional, translational or protein levels. Transcriptional regulators often comprise two-domains, i.e., the DNA binding domain and a ligand binding domain. Other regulatory mechanisms belong to the so-called two-component regulatory systems. Herein, the membrane-linked kinase acts as a sensor and in the presence of the inducer, the kinase will phosphorylate the DNA binding protein, which subsequently modulates the transcription of the gene(s) of interest by binding to the cognate promoter (135) (Table 1).

Although a lot of efforts have been made to understand the mechanisms of drug transporters in bacteria, the hydrophobic nature of these proteins make them intrinsically difficult to crystallize in order to obtain structural information. A few high resolution structures of MDR transporters are now available for instance

Sav1866 (32) however, the elucidation of the exact mechanism of action and the molecular basis of drug recognition and specificity remains a challenge. On the other hand, the structural and functional analysis of the regulatory pathways that govern the expression of these MDR transporters progresses rapidly. The expression level of many MDR transporters is closely controlled by inducers that are also substrates for the transporters. Many of the MDR transporters are subject for regulation by the transcriptional regulatory proteins. Gene regulation is crucial as an uncontrolled expression of the MDR transporter might be toxic to the cells. Transcriptional regulators are cytoplasmic proteins that can act as activators or repressors that influence transcription of target gene(s) at local or global level (46). Transcriptional regulators can also undergo autoregulation (2,56). A general mechanism for resistance development upon the exposure of cells to drugs or antibiotics is the immediate up-regulation of the low-expressed MDR transporters. For instance, the local transcriptional activator BmrR of *Bacillus sub*tilis (3) and the transcriptional repressor QacR of *Staphylococcus aureus* (43) enhance the expression of the MDR transporters Bmr and QacA through the binding of chemically unrelated cationic drugs like tetraphenylphosphonium (TPP^+) and Rhodamine 6G, respectively. The expression of *E. coli* RND transporter AcrAB is under the regulatory control by the global activators MarA, Rob, and SoxS (6). Derepression of *mexAB-oprM* operon in *nalB*-type mutant bearing mutation in *mexR* causes resistance phenotype to fluoroquinolones, chloroamphenicol, and β-lactams antibiotics (131,142). The expression level of *mexAB-oprM* during the exponential growth phase is being control by the local transcriptional repressor MexR (36). Interestingly, in the early stationary phase of growth, a higher expression of *mexAB-oprM* is being modulated by the accumulation of quorum sensing autoinducer C4-HSL in the medium, and not by MexR (143).

The structural studies of transcriptional regulators of MDR transporters are of particular interest as it may provide an insight in the molecular basis of drug recognition. They often interact with the similar panel of substrates as recognized by the MDR transporter. A typical DNA binding domain of bacterial transcriptional regulators comprises an α-helix-turn-helix (HTH) motif that may be organized in different structural environments such as three-helix bundles and winged helix motifs (120). About 95% of all bacterial transcriptional factors use the HTH motif to bind to their target DNA (25,38,50,51,127,140). MDR related transcriptional regulators belong to one of the following families: AraC, MarR, MerR, and TetR. The distinction is based on the high level of similarity of the N-terminus DNA binding domain that constitutes about one third of the polypeptide. Recent findings indicate some of the MDR-related transcriptional regulators belong to the PadR family for instance, the LadR of *Listeria monocytogenes* and LmrR of

Lactococcus lactis. PadR regulatory proteins are involved in the regulation of detoxification pathways in bacteria such as phenolic acid metabolism. The following section describes in detail mechanistic and functional aspects of MDR-related transcriptional regulators

QacR regulates the expression of the MDR transporter QacA/B in *Staphylococcus aureus*

QacA and QacB are MDR transporters of *S. aureus.* These two nearly homologous genes belong to the MFS family. In a proton motive force (PMF)-dependent manner, they extrude a wide range of mono- or bivalent cationic lipophilic substrate from the cell. The identified substrate range encompasses of 12 different chemical groups (79,102,103,122) which mostly concern positively charged lipophilic compounds that are able to pass the cytoplasmic membrane, and accumulate in response to the transmembrane electrical potential, $\Delta\psi$ (negative inside). A gene located upstream of *qacA* termed *qacR* specifies a trans-acting transcriptional repressor of the *qacAB* genes. These genes are found on the multi resistance plasmids such as pSK1 present in clinical isolates (73,124,166,167). The QacR protein is a product of a divergently transcribed ORF that binds specifically to the promoter region of *qacA* thereby repressing its transcription (43). QacR belongs to the TetR/CamR family of transcriptional regulators that share a high degree of homology in their N-terminal DNA binding domains (139). In the absence of QacA substrates, QacR binds to a region of dyad symmetry (IR1) consisting of 15 base-pairs with each half site separated by a 6 base pair spacer sequence (43). Binding of QacA substrates to QacR causes the derepression of *qacA* transcription. This event leads to the overexpression of the QacA transporter and thus, to MDR in *S. aureus*. The direct interaction between QacR to structurally unrelated QacA substrates like benzalkonium, dequalinium, ethidium bromide, chlorhexidine and rhodamine 6G induces conformational changes in QacR which results in a reduction of its binding affinity to the IR1 operator DNA (43). Interestingly, plant alkaloids such as berberine, an amphipathic cationic compound, induces both the expression of the plasmid encoded *qacA* and chromosomally encoded *norA* genes, which both encoded for MDR transporters (47). Therefore, the QacA-QacR system provides protection against compounds produced by plants and naturally derived cationic and hydrophobic antimicrobial agents. The presence of the *qacA-qacR* locus on multi resistance plasmids in clinical *S. aureus* strain suggests that the system also functions to protect cells against synthetic man-made drugs (73). QacA shows a much greater substrate range as compare to QacB. Thus

QacA may have evolved from QacB to provide resistance against clinical drugs (124).

DNA binding: QacR binds to the palindromic sequence at the IR1 DNA operator site as dimers. Upon addition of substrates, the four DNA bound QacR molecules dissociate as dimers (44). This observation shows that QacR does not self assemble into tetramers, while the two dimers appear to have no direct contact with each other in the DNA bound state (44). QacR adopts a somewhat different DNA binding mode than TetR (153). The structure of the QacR: DNA complex shows that the two QacR dimers bind to the extended 28 base pair IR1 operator on the opposite sites, in which the two HTH motifs from each dimer make a contact with the DNA major groove (153). QacR comprises nine α-helices (153). The first three helices form the three helix bundle DNA binding domain of the HTH motif (α2 and α3). DNA recognition occurs in the α3 helix. The DNA binding domain of QacR is highly homologous to TetR but the N-terminus of α1 in QacR makes contact with the phosphate backbone in a different orientation than the corresponding helix in TetR (153). The dimerization domain/substrate binding site of QacR comprises α-helices 4 to 9 that show a low homology to the corresponding domain of TetR. The C-terminal anti-parallel α-helices from each dimer form a four helix dimerization region (153).

The QacR dimers bind cooperatively to the operator DNA which is an exceptional feature among members of the TetR transcriptional regulators (153). Gel filtration and light scattering studies are consistent with a cooperative binding of two QacR dimers to the IR1 DNA (153). Because of the large distance of > 5 Å between each dimer, this cooperative binding mode does not seem to involve protein-protein interactions (153). Rather the structure and adopted conformational changes in the DNA inflicted by the bound QacR causes such a cooperative binding behavior (153). There are 16 bases and 44 phosphate contacts with the IR1 site generated by the binding of the two QacR dimers. Each monomer contacts the DNA differently; one distal and one more proximal to the dyad, each recognizing particular bases (153). The removal of two base pairs from the wild-type six base pair spacer region abolishes the binding of QacR, confirming that spacing is important for proper binding to the IR1 operator. A common feature of the TetR/CamR members of transcriptional regulators is their short recognition helix that insert deeply into the floor of the DNA major groove. One notable difference between TetR and QacR concerns their DNA binding mechanism. Upon binding to the DNA, TetR induces a bending of 17° of the DNA towards the protein that optimizes the HTH interaction within each DNA half-site including a widening of the DNA major groove. On the other hand, the entire IR1 operator widens upon binding of the two QacR dimers resulting in a minor bending of the DNA by only

3° (153). The HTH motif of TetR makes identical contacts to each symmetry DNA half site which is a common feature of dimer binding to a palindromic sequence in protein-DNA interactions. However, each HTH motif of the QacR dimer makes contacts at a non palindromic site on the operator DNA and thus it is not symmetrical.

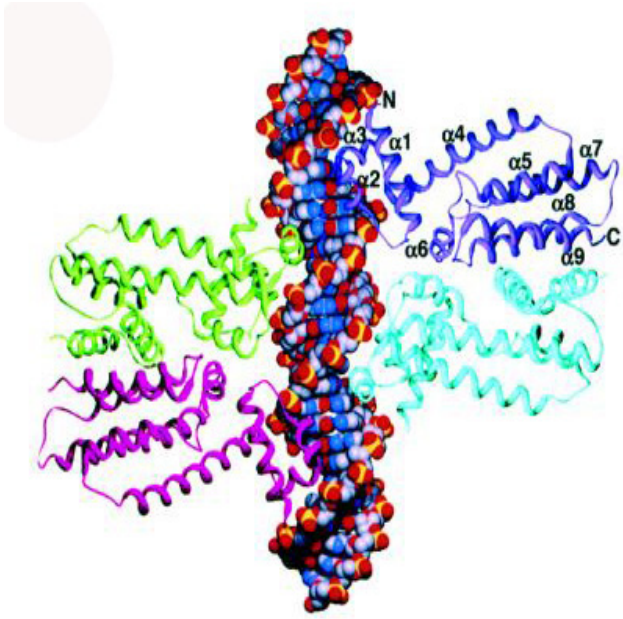


Figure 2. The crystal structure of QacR: DNA complex solved at 2.90 Å resolution. The four identical monomers of QacR form two functional dimers and bind to the symmetrical version of a 28 bp IR1 operator site of *qacA* in the absence of drug molecules. Reproduced from reference 153 with permission.

Drug binding: QacR shows an unusual feature of overlapping mini pockets within its large hydrophobic drug binding site (151). These mini pockets can bind different substrates, and serves as a model for multi-site binding allowing interactions with a range of structurally distinct drugs. Structural studies on both AcrB transporter that contain such mini pockets as well as the transcriptional regulator QacR revealed interactions between these mini pockets and displayed no clear boundaries (151). Moreover, a low resolution structure of the MDR transporter AcrB was shown to bind four different drugs simultaneously (151). TtgR of *Pseudomonas putida* is structurally related to QacR. Unlike QacR that

binds to a variety of positively charged substances, TtgR binds negatively charged toxins due to the presence of polar residues in its hydrophobic binding pocket. TtgR also bind a broad range of naturally occurring antimicrobial compounds such as phloretin, quercetin and naringenin (10). Interestingly, the QacR dimer always binds only one ligand whereas the TtgR dimer has been showed to bind up to three molecules of phloretin; two molecules at low- and one molecule at the high affinity binding sites. The multidrug binding pocket of TtgR is larger in comparison to QacR and posses two overlapping binding sites; a highly hydrophobic general site that binds compounds such as tetracycline, chloroamphenicol, quercetin, and naringenin (one ligand per TtgR dimer), and a polar site for high affinity phloretin binding pocket (10).

QacR exhibits positive or negative cooperativity between certain drug classes as well as non cooperative and uncooperative interaction with others (151). QacR binds both mono- and bivalent cationic drugs. Monovalent drugs like rhodamine 6G and ethidium bromide bind non simultaneously to different but overlapping regions of the extended QacR ligand binding pocket (152). Because of the presence of flexible carbon atom linker in the bivalent dequalinium, this molecule can interact with both binding sites of QacR. Rhodamine 6G and ethidium bromide bind through their ring system (152). Hence, it appears that QacR also can bind two (or more) drug simultaneously at overlapping binding sites.

Proflavin and rhodamine 6G bind QacR at overlapping sites. Upon binding, proflavin stacks with the side chains of Trp61 and Tyr93, whereas Glu57 and Glu58 help to neutralize the positive charge and sandwich the proflavin ring system in place (151). In the QacR dimer, proflavin binds at the same site as rhodamine 6G and both compete for binding (151). Rhodamine 6G and ethidium bromide bind to different but overlapping binding sites. These drugs do not bind to the QacR dimer simultaneously possibly because of steric hindrance (151). The structure of QacR in complex with both proflavin and ethidium bromide was solved at 2.96 Å resolution (151). Binding of both drugs simultaneously did not result in additional structural changes of the QacR dimer (151). Upon dual drug binding to the QacR dimer, structural comparison between the QacR: proflavin: ethidium bromide and QacR: ethidium bromide binary complexes showed the ethidium bromide molecule had shifted to another pocket to avoid clashing with proflavin. This adjustment offers a closest ring-to-ring approach of 4.0 Å engaging ethidium bromide in a favorable van der Waals interaction with proflavin while still preserving the hydrophobic and aromatic stacking interactions with the QacR protein (151). Aromatic residues play a critical role in drug binding. They are needed for the structural organization of the multidrug binding pocket and in the

drug-binding induced conformational transitions allowing the drug to slide into the binding pocket while still maintaining stacking interaction (151).

Regulation of drug resistance transporters Bmr and Blt in *Bacillus subtilis*

Bmr and Blt are two MDR transporters of *B. subtilis*. They shares up to 51% amino acids sequence homology and when overexpressed they are involved in the export of a similar range of drugs (4). Unlike *bmr*, *blt* is not transcribed in the wild-type cells. Likewise, disruption of the *bmr* gene but not of *blt* causes cells to be more susceptible to toxic drugs (4). Bmr is involved in the export of a diverse range of compounds that are lipophilic, monovalent and cationic (3,178). Most of the substrates are also substrates of P-gp and the transport activity of Bmr can be inhibited by reserpine and verapamil. Interestingly, cells challenged with rhodamine did not show neither the up regulation of *blt* transcription nor the interaction of this compound with the transcriptional activator BltR, whereas *in vitro*, rhodamine is a substrate for the Blt transporter (4). This suggests that the substrates binding domains of BmrR and BltR are functionally different.

DNA binding: The level of expression of *bmr* and *blt* is under a control of transcriptional activators BmrR and BltR, respectively (4). These proteins belong to the MerR family of transcriptional regulators. Although BmrR and BltR are very homologous in their N-terminal DNA binding domains, their C-terminus drug(s) binding site show little sequence homology (4). Unlike BmrR, the identity of the ligand(s) that bind BltR is still unclear although is it clear what substrates are transported by Blt. The *bmr* and *blt* genes localize in different operons. The *bmr* promoter exclusively controls the expression of *bmr* while the *blt* promoter controls the expression of two genes, *blt* and *bltD*. BltD is highly homologous to an acetyltransferase that could implicate a metabolism-associated function of the Blt transporter (4).

Transcriptional activation of both *bmr* and *blt* genes is subject for regulation by the global activator Mta (13). A truncate of Mta that comprises only the N-terminal DNA binding domain (MtaN) binds both the promoter regions of *bmr* and *blt* at exactly the same nucleotide sequences that are bound by BmrR and BltR, respectively (13). MtaN comprises the winged HTH motif that is structurally related to the same region of BmrR. However, the α5 dimerization helix and the first wing of the DNA binding domain of MtaN are in different orientations as compared to the corresponding regions in BmrR suggesting these two protein interact with DNA in a distinct manner (13). With respect to the drug binding domain, Mta is homologous to the global regulatory protein of multidrug resistance TipA in *Streptomyces lividans* (13,58). Therefore, *bmr* and *blt* expression by Mta

may be at the level of global control, and their expression can be effected by stresses induced by means of the presence of toxic compounds or peptides in the medium. The *S. aureus* NorA protein is homologous to the *B. subtilis* Bmr and Blt (4). Likewise, NorA provides resistance to the cells to a similar panel of drugs as transported by BmrE. However, little is known about the regulation of *norA* expression.

The BmrR dimer directly interacts with drugs. Ligand-bound BmrR binds to the inverted repeats located between the -35 and -10 region of *bmr* promoter region. The -35 and -10 consensus motifs are separated by a 19 base pair spacer sequence which is quite long as compared to the typically observed 16-17 base pairs (4). The long spacer sequence together with the location of the -35 and -10 region on the other side of the DNA helix are necessary to prevent activation of *bmr* transcription by RNA polymerase in the absence of drugs (4). The constitutive drug resistance phenotype of selected *B. subtilis* strains is caused by a 2 base pair deletion in the spacer region of *bmr* promoter causing continuous overexpression of Bmr (54). Constitutive expression of *blt* was observed in *B. subtilis* 168ACF that carries the *acfA* mutation, i.e., a 1 base pair deletion in the spacer region of the *blt* promoter (4).

BmrR belongs to the winged-helix superfamily of a four-helix-bundle and a three stranded antiparallel β sheets (39). The topology of BmrR HTH motif corresponds to β1, α1, α2 (recognition helix), W1 (sheets β2 and β3), W2 (α3 and α4) with α5 function as a linker connecting the DNA and substrate binding domains (54). The DNA binding elements of BmrR are responsible in making contacts with the *bmr* promoter while still promoting the favorable DNA conformation for binding of RNA polymerase (54). The residues Ser41, Tyr42, and Arg43 in W1 are necessary for DNA binding through van der Waals interactions and hydrogen bonding. Moreover, Arg43 together with a glutamate, aspartate or glutamine at position 26 are conserved amongst MerR family members indicating that W1 is an important DNA binding element (54). In contrast to other winged-helix proteins, α2 and α3 of W2 of BmrR are part of the HTH motif rather than forming a loop (54).

The structure of the ternary BmrR:TPP^{+}:DNA complex shows a remarkable twisting of the promoter region with a reorientation of the -35 and -10 region to allow RNA polymerase to bind and initiate transcription (54). This mechanism involves base pairing disruption of 2 residues in the center of the pseudodyad of the *bmr* promoter (54). The A-T base pair at +1 position breaks causing the adenine and thymine to slide away from each other. On the other hand, the A-T base pair at the -1 position is able to form miss-pairing interaction regardless of the misplacement (54). The resulting DNA distortion causes a

"bunches up" in the middle of the operator region and a space shortening equivalent to 2 base pairs (54). Residues Tyr24, Tyr25, Lys60, and Lys66 of BmrR stabilize the base pair distortion via contact with the DNA back bone (54).

Drug binding: The binding of ligands to BmrR was previously studied using the isolated C-terminal substrate binding domain (BRC) (87). This domain can be readily expressed, dimerized, and able to bind two drugs per dimer (87). This finding is consistent with the BmrR:drug:DNA complex which shows one drug molecule bound per BmrR monomer (54). There is no obvious binding pocket or cavity found in apo BRC structure (192). However, the BRC:TPP^+ structure revealed the presence of a charged residue (Glu134) buried in the core of the protein but positioned at the base of the binding site (192). Ligand binding occur via electrostatic interaction between the negative charge of the carboxylate group of Glu134 with the positively charged substrates like TPP^+ or Rhodamine 6G (192). Glu21 initiates the entry of TPP^+ into the internal binding site of BRC. This residue is located proximal to α2 and creates a negative patch that attracts cationic compounds (54). Binding of molecules will take place only via the unfolding of α2 (54). The BRC Glu134Ala mutant shows reduced binding affinity to five out of six BmrR ligands (178). This suggests the presence of a multi variant binding site that can accommodate a diverse range of chemically unrelated compounds. Structural comparison between the apo BRC and BRC:TPP+ showed no major conformational changes which suggests the unwinding of α6 in BmrR may act as a signal during drug induced transcriptional activation (192).

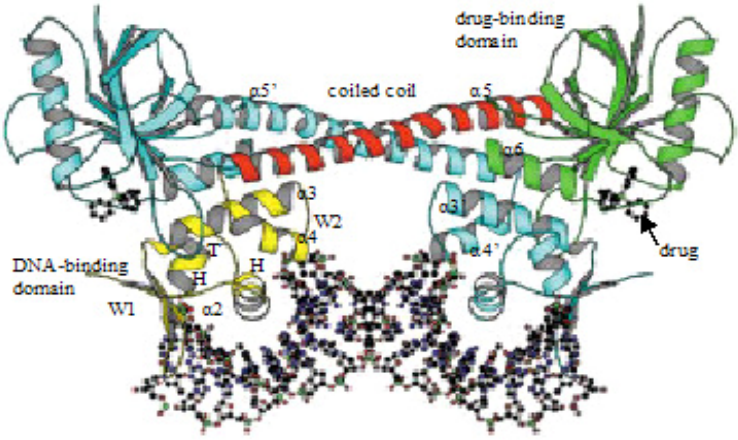


Figure 3. A structural overview of the BmrR dimer with bound DNA and drug TPP. The N-terminus is responsible for the DNA binding domain and it is directly linked to the C-terminus which comprised two-third of the protein responsible for the ligand binding domain indicated by a long helix α5. Reproduced from reference 54 with permission.

TetR regulates the expression of the specific tetracycline MDR pump: TetA

The overexpression of TetA transporter results in resistance to tetracycline. TetA belongs to the MFS and specifies an antiporter that mediates the extrusion of tetracycline in complex with a divalent metal cation e.g. Mg^{2+} in exchange for protons. Unlike Gram-positive bacteria, expression of *tetA* in Gram-negative bacteria is controlled by the product of a divergently transcribed gene, the *tetR* repressor (56). TetR proteins can be classified into 8 subfamilies, i.e., TetR A to E, G, H, and J (144). TetR proteins are transcriptional regulators that control the transcription of various genes associated with antibiotic biosynthesis, osmotic stress, multidrug resistance, and pathogenicity in both Gram-positive and -negative bacteria (135).

The best characterized TetR protein is encoded by the *Tn10* gene. TetR(B) binds to the overlapping sequences of *tetA*(B) and two divergent *tetR*(B) promoter regions (56). Homodimeric TetR binds non-cooperatively to the two adjacent inverted repeats known as the *tet* operators O_1 and O_2 that overlap with the *tetA*(B) and *tetR*(B) operator DNA region (98). Binding of TetR in the presence of the tetracycline-Mg^{2+} complex induces conformational changes in TetR, whereupon derepression of both *tetA* and *tetR* genes occur which expel tetracycline from the cell (57). TetR shows a higher binding affinity for tetracycline-Mg^{2+} complexes than the drug target, the ribosomes. This ensures a full protection to the cells via TetA overexpression (56). TetR binding affinity to O_2 is four folds higher than to the O_1 sequence (69).The binding of TetR to O_1 results in a complete block of transcription of both the *tetA* and *tetR* genes, whereas down regulation of *tetA*(B) but not *tetR*(B) gene is being observed when TetR binds to the O_2 site only (98). When the low amount of repressor-operator bound complex is detected, the synthesis of *tetR*(B) but not *tetA*(B) is being activated. In addition, the uncontrolled expression of the integral membrane protein TetA is lethal to the cells as nonspecific cation transport causes a collapse of the $\Delta\psi$ (35).

TetR(D) is organized as a dimer. The crystal structure of the TetR(D):*tetO* complex shows that each subunit comprises of 10 α-helices (117). α8 and α10 from each monomer form antiparallel helices that are part of the four helix bundle of the dimerization domain (57,146) and protein-protein interaction (145,150). The HTH motif of TetR comprises α2 and α3 while α1 stabilizes the HTH structure and α4 contributes to the formation of a hydrophobic center in the DNA binding domain that is critical for the binding of TetR to the *tet* operator (117). In addition, truncation at the N-terminal region of TetR abolished the binding to DNA (17). In the TetR(D) bound tetracycline-Mg^{2+} complex, both α3 and α3' were found to be apart by 39.6 Å. This space is sufficient to prevent binding of the repressor to the

DNA major grooves that are about 34 Å apart (57). TetR (D) binds to the 15 base pair fragment of the *tet* operator except for the central 3 base pairs. Under those conditions, α3 and α3' of the TetR dimer are separated by 36.6 Å while binding of the drug increases this gap by 3 Å (117). The TetR-DNA interface contains no water molecules unlike common protein-DNA interaction. Pro39 of the HTH motif plays an important role in establishing this particular contact in the TetR-DNA complex (53,117,180). The ligand induced conformational changes of TetR(B) and TetR(D) have been described in detail elsewhere (52,57,104,148,149,158). The TetR homodimer traps two tetracycline-Mg^{2+} molecules inside tunnels that are located in the center of the protein (57). Each of the binding tunnels consists of α-helices that are derived from both monomers; the first tunnel comprises α-helices 4 to 8 and 8' and 9' while the second one consists of α-helices 4' to 8' and 8 and 9. The structure of ligand-bound TetR shows a large opening adjacent to the C-terminus at α9 (and α9' from the other subunit) that was not observed with the apo TetR. This large opening functions as a drug entry gate (57,115). The tetracycline derivative glycylcycline contains a glycylamido substituent that causes steric hindrance thereby interfering with the TetR sliding door α9'. This ligand does not induce the depression of *tetA* (118). By means of a chelator, the Mg^{2+} can be removed from the TetR:tetracycline-Mg^{2+} complex, and the resulting TetR:tetracycline complex has almost a similar conformation as the apo TetR. This demonstrates that Mg^{2+} fulfils a critical role in the induction mechanism (116). Further details on the mechanism of TetR regulation can be found elsewhere (18,115-117,148,169-171,179).

The multiple antibiotic resistance (mar) regulon in *Escherichia coli*

E. coli is resistant to multiple compounds such as dyes, antimicrobial agents, detergents, fluoroquinolones, and numerous antibiotics including β-lactams, chloroamphenicol, erythromycin, and tetracycline. This resistance is caused by the overexpression of the tripartite drug efflux pump AcrAB-TolC (84,96,109,114,184). The physiological function of the AcrAB-TolC complex in *E. coli* is to transport stress related toxic compounds that are found in natural environments such as fatty acids and bile salts (84,109,168). The overexpression of the global regulators MarA, SoxS, and Rob modulates the expression of many of the *mar* regulon genes by binding to the marbox upstream of each promoter. The *mar* box is a 20 base pair nucleotide sequence motif located in the promoter region of the target genes such as the *acrAB* and *tolC* genes (6,113,182). Previous studies showed that MarA/SoxS/Rob autoregulate their own transcriptions and of each

other (91,99,100,111), suggesting the presence of additional binding sites within the *mar, sox* and *rob* promoters.

Mutations in AcrR, a divergently transcribed local repressor of *acrAB* operon cause the increased resistance to fluoroquinolones in clinical *E. coli* isolates (66,110,181). AcrR posses the typical HTH DNA binding motif and belongs to the TetR family of transcriptional regulators (83). AcrR represses both transcription of *acrR* and *acrAB* but it does not induce the expression of these genes under general stress conditions such as 4% ethanol, 0.5 M NaCl, or when cells enter the stationary phase (83). Transcription of these genes is linked to an unidentified regulatory protein (83). Thus, the expression of *acrAB* is primarily controlled by AcrR while its induction is modulated by MarA and other global regulators. Previous results indicate that the physiological function of AcrAB might relate to the export of non-freely diffusible quorum sensing molecules (134). *AcrAB* expression increases as the growth rate decreases and this might be linked to the accumulation of the quorum-sensing signals produced by the cells (83,136).

MarA belongs to the AraC family of transcriptional activators (64,65,91,137). The cellular level of MarA is controlled by the MarR repressor, the first gene of the *marRAB* operon. Both MarA and MarR bind to the DNA region *marO* that is located upstream of the *marR* gene and contains several regulatory binding sites surrounding the *marRAB* promoter (45). MarA binds to the marbox of a large number of *mar* regulon genes (6), and it also activates its own transcription. The MarA binding site is located 16 base pairs upstream of the -35 and -10 region of the *marRAB* operon (91,137). Over 60 chromosomal genes are differentially regulated in *E. coli* cells that constitutively express *marA* (14). In another study describing the inducible MarA expression system, an additional of 67 MarA-regulated genes were identified (128). MarA activates at least 40 different promoters of target genes (95), and also a gene with a marbox that diverges substantially from the consensus sequence (15). One example is the transcriptional activation of *micF* by MarA. This gene produces an antisense RNA that repress the expression of *ompF*, a gene codes for the outer membrane protein that allows passive diffusions of small molecules (34). Thus, reduced drug influx by OmpF combined with an active extrusion by AcrAB-TolC is a very efficient response mechanism controlled by MarA when cells are exposed to toxic molecules. A recent study also shows that MarA functions as the repressor of *rob* transcription in *E. c*oli (147).

MarA binds DNA as a monomeric protein. The DNA binding site lacks any inverted or direct repeats commonly characterized in bacterial regulatory sequences. Interestingly, MarA and its homolog SoxS posses no ligand binding site which make these proteins are considerably smaller in size as compared to the

members of the AraC family (94). The crystal structure of MarA:*marO* complex reveals an unusual feature of two HTH DNA binding motif that are connected by a long α-helix (90). A typical HTH binding motif can recognize only 6 base pairs out of the 11-12 base pairs of the operator sequence (161). This is the reason why most of the bacterial regulatory proteins need to dimerize to comprise two HTH motifs. Monomeric MarA is capable of binding to two successive DNA major grooves utilizing its two HTH domains. A previous study revealed that the N-terminal HTH domain of MarA contacts the marbox consensus sequences (42). A more detailed description of the MarA:DNA complex and the binding mechanism can be found elsewhere (30,42,89,90).

MarR repress the transcription of the *marRAB* operon by binding to *marO* at sequences different from the marbox that is recognized by MarA (91). MarR binds as a dimer to two site in *marO,* assigned as site I and II located downstream from the MarA binding site (92). To a certain extent, MarA and MarR compete for binding to *marO* (91). Site I overlaps the -35 and -10 region while site II is neither required for repression nor it is needed for binding of MarR to site I (92). The crystal structure of MarR has been solved at 2.3 Å showing that MarR posses the typical DNA binding domain of the winged-helix family (9). The α3 and α4 helices of MarR form the HTH motif with the β-sheets functioning as the "wings". Importance residues for DNA recognition by MarR are located in the α4 helix as mutations in this region abolish DNA binding (5). The structure of MarR is stabilized by a number of salt bridges (9,76). MarR binds antibiotics like tetracycline and chloroamphenicol, weak aromatic acids like salicylate, and other compounds such as the redox cycling molecules menadione and plumbagin (7,155). Salicylate binds to two sites on each subunit of the MarR dimer. These binding sites are located at the surface near to the DNA recognition helix α4 (9). Hence, the binding of the inducer disturbs MarR binding to *marO*, causing the depression of *marRAB,* and *mar* regulon activation via the synthesis of MarA (45). In addition, the MarA homologs SoxS and Rob bind to *marO* DNA and activate the expression of genes belong to the *mar* and *sox* regulon which contributed to the MDR phenotype of *E. coli*. In addition, the nucleoid-associated global regulatory protein known as FIS also bind to a region within *marO* upstream of the marbox. FIS stimulates the MarA, SoxS, and Rob mediated activation of transcription. Further information on SoxS, Rob and FIS can be found in references (64,65,83,93,94,101,112,114,137,138,165,172,184). Analogous *mar* regulatory networks are found in many bacterial species suggesting that these organisms carry closely related genes (29,62,74,97).

PadR, a new family of transcriptional regulator involved in multidrug resistance

LadR is a transcriptional regulator that controls the expression of the MDR transporter MdrL in *Listeria monocytogenes* (61). Overexpression of MdrL results in the rapid efflux of rhodamine 6G out of the cells. Interestingly, LadR belongs to the PadR family of transcriptional regulators, while most of the MDR specific transcriptional regulators belong to the MerR, AraC, or LysR family of activators or are members of the TetR, MarR, or LacI family of transcriptional repressors (46). PadR regulators are involved in regulating the expression of the phenolic acid decarboxylase (*pad*) gene(s) that detoxifies derivatives of phenolic acids such as ρ-coumaric, ferulic, and caffeic acids (48). Some organisms such as *Pseudomonas* strains (40,119) and *Acetinobacter calcoaceticus* (154) use these compounds as a sole source of carbon for growth. Based on phylogenetic analysis of related firmicutes, LadR forms an independent group in a large family of PadR regulators (PF03551). There are two distinct families of the *padR*-related genes: subfamily I of longer sequence of about 176 amino acids, and subfamily II of shorter sequence of about 110 amino acids (61). LadR is the product of divergently transcribed gene *ladR* that is located upstream of *mdrL*, and it is conserved in all sequenced *Listeria* genomes. LadR proteins of *L. monocytogenes, L. innocua, L. ivanovii,* and *L. grayi* are closely related to the PadR protein of *Pediococcus pentosaceus* (61). The intergenic region of *ladR-mdrL* is 166 base pairs long and contains two non overlapping putative -35 and -10 regions (61). The -35 region of the *mdrL* promoter contains an inverted repeat with a sequence that fits with the consensus motif of the PadR binding site, i.e., ATGT-8N-ACAT (61). LadR is a 176 amino acids long protein with a low sequence identity (24-29%) with PadR regulators from *Pediococcus pentosaceus* (16) and *Lactobacillus plantarum* (48). The PadR proteins from *P. pentosaceus* and *L. plantarum* negatively regulate the *padA* gene specifies the phenolic acid decarboxylase (16,48). LadR shares 25.5% homology to the repressor and co-activator AphA of *Vibrio cholerae* (33,71,72). AphA is a quorum sensing regulator that activates the virulence cascade of *V. cholerae* and works in conjunction with another regulator AphB. AphA is a repressor of the penicillin amidase activity coded by *pva* gene (71,72). The crystal structure of dimeric AphA (33) reveals a number of conserved residues that are also found in LadR (61). AphA has two domains; a globular N-terminal domain and a distinct C-terminal domain. The N-terminal domain of AphA adopts the winged-HTH motif similar to MarR (33). MdrL, QacA/B, and Bmr have a common ability to bind rhodamine 6G. A LadR-dependent regulation model for the expression of *mdrL* has been proposed. In the absence of rhodamine, apo LadR binds to the *mdrL* promoter

at the PadR box and repress the transcription of *mdrL* gene. In the presence of rhodamine, deactivation of LadR takes place, and the transcription of *mdrL* gene is initiated resulting in the overexpression of MdrL which in turn mediates the efflux of rhodamine from the cells. Since LadR is a newly identified member of PadR family, its exact physiological functions in both non pathogenic and pathogenic *Listeria* species remains to be determined.

LmrR regulates the expression of the major MDR ABC transporter LmrCD of *Lactococcus lactis*

The Gram-positive bacterium *Lactococcus lactis* is a non-pathogenic bacterium and widely used in the fermented food production. The genome of *L. lactis* contains about 40 genes that encode putative MDR transporters (21,183). By heterologous expression in drug-sensitive *E. coli* strains, the MDR transporters LmrA and LmrP have been implicated in the drug resistance phenotype of *L. lactis*. However, recent gene inactivation analysis suggests that the intrinsic multidrug resistance of *L. lactis* is due to the expression of the ABC transporter LmrCD. LmrC and LmrD are half-transporters that heterodimerize to form a functional MDR transporter (82). Overexpression of LmrCD is sufficient to protect cells against several hydrophobic drugs e.g. daunomycin, ethidium bromide, Hoechst 33342, and the fluorescence dye BCECF-AM (82). Moreover, LmrCD was recently being shown to transport bile acids as one of its natural substrates (190). Deletion of *lmrCD* in *L. lactis* NZ9000 renders the cells to be hyper sensitive to drugs such as Hoechst 33342, daunomycin, ethidium bromide, rhodamine 6G, and cholate. The drug resistant phenotype can be restored by the expression of *lmrCD* but not *lmrA* (81). Transcriptome analysis of four drug resistant strains of *L. lactis* adapted to increasing concentration of daunomycin, ethidium bromide, cholate and rhodamine 6G (20) revealed a similar response of the up regulation of *lmrC* and *lmrD*, and another gene termed *lmrR* (formerly *ydaF*) (81). The *lmrR* gene (*l*actococcal *m*ultidrug *r*esistance *r*egulator) is located upstream of the *lmrCD* genes. Nucleotide sequencing on *lmrR* in the four MDR strains showed frame shift and point mutations resulting in the production of non functional LmrR variants. The up regulation of *lmrR* in these MDR strains suggested an autoregulation mechanism of *lmrR* expression. Moreover, the constitutive expression of *lmrC*, *lmrD*, and *lmrR* in the MDR strains indicates that LmrR functions as transcriptional repressor for both *lmrCD* and for its own transcriptions. Transcriptome analysis of the Δ*lmrR* strain showed only the up regulation of the *lmrC* and *lmrD* genes, implying that LmrR is a specific local transcriptional regulator of the expression of *lmrCD*. LmrR binds to two distinct sites in the promoter region of *lmrCD* which

are about 29 base-pairs apart. This separation is longer compared with the other known bacterial repressor DNA binding sites e.g. MarR, a 13 base-pairs separation (92). The *lmrCD* promoter contains two sites for LmrR binding, i.e., site I is located between the -35 and -10 region, whereas site II consists of short imperfect palindrome sequences. Interestingly, *in vitro* site II is not essential for LmrR binding.

Based on homology, LmrR belongs to the PadR family of transcriptional regulators (Pfam PF03551). The palindromic motif of ATGT-8N-ACAT is conserved among the PadR-like regulators, and LmrR binds to a slightly modified but highly homologous motif, i.e. ATGT-10N-ACAT. LmrR also bind to a long stretch of DNA on its own promoter region with no apparent structural features. Interestingly, many of the PadR regulators are involved in the detoxification and enzymatic degradation of phenolic acid, whereas LmrR regulates the expression of an MDR transporter that expels toxic molecules from the cell.

LmrR, like other PadR regulators shares the typical winged-helix turn helix motif in its N-terminal DNA-binding while the C-terminal domain specifies the substrate recognition. LmrR of *L. lactis* and PadR-related proteins of *Staphylococcus*, *Enterococcus*, and *Streptococcus* belong to the subfamily I of PadR protein that are smaller (~110 amino acids long) than the subfamily II proteins to which PadR of *L. lactis* belongs (~ 176 amino acids long) (61). Sequence alignment of LmrR with the members of the PadR (and the more distantly MarR family) indicates the presence of highly conserved amino acyl residues that are located in the hinge region which connects the DNA- and substrate binding domains (9,85), that are critical for DNA binding ability.

The crystal structures of apo LmrR, LmrR bound Hoechst 33342, and LmrR bound daunomycin were solved at 2.0 Å and 2.2 Å, respectively (85). LmrR follows α1-α2-α3-β1-β2-α4 topology with two defined domains. The first domain of the typical winged-helix turn helix DNA binding consists of helices α1, α2, and the DNA recognition helix α3 together with strands β1 and β2 to form the wing. The LmrR protein derived from the rhodamine challenged MDR strain is non functional due to a point mutation of a highly conserved threonine located in the hinge (wing) region of β2. This mutant is unable to bind to the promoter regions of both *lmrCD* and *lmrR* and hence, constitutive transcriptions of these genes take place (2).

Table 1 Transcriptional regulator that control the expression of the MDR pumps in bacteria

Organism	Regulatory protein	Regulatory family	Regulatory function	Regulatory substrates	Regulated gene of MDR pump	Class of MDR pump	References
Eschericia coli	MarA/SoxS/Rob	AraC	Global regulators (activator/repressor)	MarA/SoxS/Rob*	*acrAB* and *tolC*	RND	(88,91,99,100,111)
	MarR	MarR	Repressor of *marA*	DNP, Sa, Md, Pg	*acrAB* and *tolC* via M*arA*	RND	(7,9,92)
	EmrR	MarR	Repressor	Eb, CCCP, DNP, FCCP, Na, Sa, TCS	*emrAB*	MFS	(26,80,189)
	AcrR	TetR	Repressor	Eb, Pf, R6G	*acrAB*	RND	(164)
Staphylococcus aureus	QacR	TetR/CamR	Repressor	Bc, Be, Ch, Cv, Dc, Eb, Mg, Pf, R6G	*qacA/qacB*	MFS	(43,44,152,153)
	MgrA (formerly NorR)	MarR	Global regulators (activator/repressor)	Sf, Cf, Mf, Nf, Ct, Eb, Tc	*norA, norB, norC, tet38*	MFS	(173-175)
Bacillus subtilis	BmrR	MerR	Activator	ABM, ADCP, Ao, DEC, DDPB, R6G, TPP	*bmr*	MFS	(3,178,192)
	BltR	MerR	Activator	unknown	*blt*	MFS	(4)
	Mta	MerR	Global activators	unknown	*bmr* and *blt*	MFS	(13)
Vibrio cholerae	VceR	TetR	Repressor	CCCP, Sa, Dc	*vceCAB*	MFS	(22,186)
	BreR	TetR	Repressor	Ch, Dc, Cc	*breAB*	RND	(27)

Pseudomonas aeruginosa	MexR	MarR	Repressor	unknown	*mexAB-oprM*	RND	(76,163)
Campylobacter jejuni	CmeR	TetR	Repressor	Tch, Gch, Tdc,Ch, Dc,Cc	*cmeABC*	RND	(77,78)
Listeria monocytogenes	LadR	PadR	Repressor	R6G	*mdrL*	MFS	(61)
Lactococcus lactis	LmrR	PadR	Repressor	H33342, Da, Na-Ch	*lmrCD*	ABC	(2)

1). Drugs abbreviation: DNP = 2.4-dinitrophenol, Sa = Salicylate, Md = Menadione, Pg = Plumbagin, Eb = Ethidium bromide, CCCP = carbonyl cyanide *m*-cholorphenylhydrazone, FCCP = carbonyl cyanide *p*-(trifluoro-methoxy), Na = Nalidixic acid, TCS = tetrachlorosalycilamilide, Pf = Proflavine, Bc = Benzalkonium, Be = Berberine, Ch = Chlorhexidine, Cv = Crystal violet, Dc = Dequalinium, R6G = Rhodamine 6G, ABM = 5-(1-adamanthylcarboxyethyl)-3-benzyl-4-methylthiazolium, ADCP = 4-amino-3,6-dimethylbenzo[b]cycloheptano[e]pyridinium, Ao = astrazon orange, DEC = diethyl-2,4'-cyanine, DDPB = 5,6-dichloro-1,3-diethyl-2-(phenylaminovinyl)benzoimidazolium, TPP = tetraphenylphosphonium, Sf = sparfloxacin, Cf = ciprofloxacin, Tc = tetracycline, Dc = deoxycholate, Cdc = Chenodeoxycholate, Tch = taurocholate, Gch = Glycocholate, Ch = cholate, H33342 = Hoechst 33342, Da = daunomycin. **2).** MarA/SoxS/Rob* = MarA and SoxS proteins do not have ligand binding domains

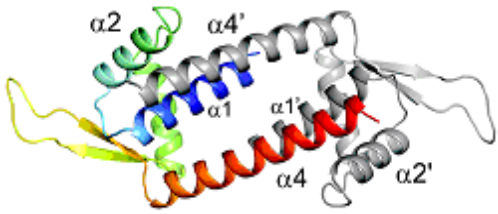


Figure 4. The crystal structure of apo LmrR was solved at 2.0 Å resolution. The N-terminal domain of LmrR is responsible for DNA binding and adopts a winged-HTH motif. The unique architecture of LmrR is marked by the presence of a symmetric central pore formed by the C-terminal domains of the two monomers that is responsible for ligand binding. From reference (85) with permission.

The substrate binding domain of LmrR is made up of a long α4 dimerization helix. The dimerization helix of α4 resembles a protruding arm that intersects with the wHTH domain of the dyad-related subunit with the anti parallel orientation against α1' and interacting with the C-terminal region of α2', and the loop connecting helix α2' and α3'. Interestingly, neither the C-terminal helices of α4 and α4' nor the N-terminal of α1 and α1' interact with each other. A striking structural feature of LmrR is the presence of a large flat-shape central pore for ligand(s) binding. This organization is unique because none of the MarR/PadR related transcriptional regulators possess a central pore at their dimer interface. Moreover, both drug binding sites of BmrR from *B. subtilis* and QacR from *S. aureus* are asymmetric that formed within a single subunit whereas LmrR posses a symmetric binding pocket where both subunits contribute equally to this structure (85). Hoechst 33342 and daunomycin show a common mode of binding: their flat ring systems are wedged in between the W96 and W96' side chains forming aromatic stacking interactions with each of the two indole systems with no hydrogen bonds were observed between drug(s) and LmrR.

A difference in LmrR binding mechanism to the *lmrCD* promoter and its own promoter was demonstrated by Atomic Force Microscopy (AFM) analysis. With a DNA fragment corresponding to the *lmrR* promoter, a severe deformation and supercoiling of the DNA strands occurred upon LmrR binding. This likely to ensures a tight repression of *lmrR* transcription. On the other hand, the binding of LmrR to the *lmrCD* control region introduced DNA bending with the visualization

of two “blobs.” This data suggest the transcription initation by RNA polymerase is possibly being prevented by the binding of two LmrR dimers.

CONCLUDING REMARKS

The emergence of the resistant pathogenic bacterial strains towards a wide range of antibiotics from different classes is progressing in an alarming speed causing a serious threat to public health worldwide. This phenomenon is based on selection for organisms that gained the ability to survive the lethal doses of antibiotics over time. For examples are the *Mycobacterium tuberculosis* (XDR-TB), Methicillin-resistant *Staphylococcus aureus* (MRSA), and Vancomycin-resistant *Enterococcus* (VRE) strains that are highly resistant to different classes of antibiotics available nowadays (1,159). The observed antibiotic resistance in bacteria can be either intrinsic or acquired. Intrinsic resistance is based on the natural mechanisms present in these cells and dependent on the genetic content of the cell. Acquired resistance may relate to different mechanisms such as the occurrence of mutations in the transporter and/or regulatory genes that changed expression and/or selectivity of the MDR transporters, or was obtained by horizontal transfer of the mobile genetic elements that carry genes encoded for resistance to antibiotics (132). Bacteria do not posses specific defense mechanisms to extrude the synthetically introduced antibiotics. Active secretion of drug molecules from the cell results in a multidrug resistance (MDR) phenotype that is often reinforced by the (over)expression of membrane bound MDR transporters. Sequence analysis of a number of bacterial genomes revealed that these transporters appear ubiquitous in nature. One of the known physiological functions of the MDR pumps is to provide an extensive protection against a diverse range of toxic molecules found in their natural environment. The employment and modification of these existing transport systems is subject to regulation by transcriptional regulators that ensure expression of the membrane transporter only when needed in response to the environmental stimuli. Possibly, an excessive production of these proteins is lethal or disadvantageous to the cells.

The study of the mechanism of drug extrusion and recognition by MDR transports is hampered by the difficulty to obtain structural information as these membrane proteins often resist crystallization. On the other hand, the MDR related regulatory proteins are soluble, relatively easy to over express and often can successfully be transformed in high diffracting crystals. These proteins have been showed to contain multiple ligands binding sites that recognized the drugs that are also transported by the MDR transporter that is regulated by these proteins. Thus

they can provide detailed information on the mechanisms of multiple drug recognition. The next stage of analysis is to understand how these drugs affect the ability of these regulators to interact with DNA and how this promotes expression of the designated MDR transporters. Molecular mechanisms causing antibiotic resistance are diverse and often unique for the organism under study. Therefore, a complete, understanding of the mechanisms of regulation and expression of MDR transporter may full future design of novel inhibitors that prevent such resistance mechanisms and thereby increase the life span of currently used antibiotics.

SCOPE OF THE THESIS

The aim of this thesis is to elucidate the molecular basis by which the transcriptional regulator LmrR regulates the expression of the major multidrug resistance ATP-binding cassette transporter LmrCD of *Lactococcus lactis*. Chapter 1 provides an introduction in multidrug resistance transporter and describes the various regulatory mechanism involved in bacterial MDR. Chapter 2 describes the functional and transcriptomic study that identifies LmrCD, a heterodimeric ATP-binding cassette transporter, as the major determinant for both intrinsic and acquired MDR phenotype in *L. lactis*. Cells lacking *lmrCD* genes are hyper sensitive to the presence of several hydrophobic drugs such as Hoechst 33342, daunomycin and ethidium bromide. Conversantly, the overexpression of LmrCD resulted in increased resistances of the cells against the toxic effects of these compounds. Transcriptome analysis on four drug resistance mutant strains of *L. lactis* obtained after a challenge with increasing concentration of daunomycin, ethidium bromide, rhodamine, and cholate revealed the up regulation of *lmrC* and *lmrD* genes. In addition, a common response of these cells is the up regulation of a gene located upstream of *lmrCD* termed *lmrR* (formerly *ydaF*). Sequence analysis suggests that LmrR belongs to the PadR family of transcriptional regulators. A function of LmrR as a transcriptional regulator was confirmed experimentally by its ability to bind to the promoter region of *lmrCD*. The drug resistant phenotype of the selected strains was caused by the constitutive up regulation of *lmrCD* via the premature termination of the *lmrR* gene (Chapter 2) or the expression of a non-functional mutant of LmrR. Chapter 3 describes a further characterization of LmrR. Inactivation of the *lmrR* gene results in a constitutively high expression of the *lmrCD* genes, and does not affect the expression of other genes in *L. lactis* demonstrating that its functions as repressor of *lmrCD* expression only. LmrR binds to its own promoter and to the promoter region of *lmrCD* in two distinct manners. LmrR was found to directly bind drugs suggesting a regulatory

mechanism in which drug binding to LmrR causes the derepression of *lmrCD*. Chapter 4 describes a primer extension analysis showing the presence of multiple transcripts for the *lmrCD* genes and only a single transcript for *lmrR*. Atomic Force Microscopy (AFM) analysis further confirmed major differences in the LmrR binding mode for the *lmrR* and *lmrCD* promoter regions. The binding of LmrR to its own promoter results in a severe DNA deformation and super coiling whereas binding of LmrR to the promoter region of *lmrCD* only causes a distinct DNA bending. Chapter 5 describes a structural analysis of LmrR. The crystal structure of apo LmrR and LmrR bound to either Hoechst 33342 or daunomycin was solved to atomic resolution. The N-terminal domain of LmrR comprises the typical DNA binding winged-helix turn helix motif as found in previously characterized bacterial transcriptional regulators. The LmrR structure also show some unusual features. The two C-terminal regions of the LmrR dimer form a flat-shape hydrophobic central pore that is responsible for drug binding. Hoechst 33342 or daunomycin bind in a similar fashion with their aromatic rings sandwiched in between the indole groups of two dimer-related tryptophan residues. Further functional analysis of site-directed mutants of LmrR suggest an allosteric coupling between the multidrug and DNA binding sites of LmrR that likely plays a role in the induction mechanism. Finally, Chapter 6 summarizes the findings of this thesis and provides an outlook for further research.

REFERENCES

1. Adhikari, R. P., G. C. Scales, K. Kobayashi, J. M. Smith, B. Berger-Bachi, and G. M. Cook. 2004. Vancomycin-induced deletion of the methicillin resistance gene mecA in Staphylococcus aureus. J. Antimicrob. Chemother. 54:360-363.
2. Agustiandari, H., J. Lubelski, van den Berg van Saparoea HB, O. P. Kuipers, and A. J. Driessen. 2008. LmrR is a transcriptional repressor of expression of the multidrug ABC transporter LmrCD in Lactococcus lactis. J. Bacteriol. 190:759-763.
3. Ahmed, M., C. M. Borsch, S. S. Taylor, N. Vazquez-Laslop, and A. A. Neyfakh. 1994. A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. J. Biol. Chem. 269:28506-28513.
4. Ahmed, M., L. Lyass, P. N. Markham, S. S. Taylor, N. Vazquez-Laslop, and A. A. Neyfakh. 1995. Two highly similar multidrug transporters of Bacillus subtilis whose expression is differentially regulated. J. Bacteriol. 177:3904-3910.
5. Alekshun, M. N., Y. S. Kim, and S. B. Levy. 2000. Mutational analysis of MarR, the negative regulator of marRAB expression in Escherichia coli, suggests the presence of two regions required for DNA binding. Mol. Microbiol. 35:1394-1404.
6. Alekshun, M. N. and S. B. Levy. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. Antimicrob. Agents Chemother. 41:2067-2075.

7. Alekshun, M. N. and S. B. Levy. 1999. Alteration of the repressor activity of MarR, the negative regulator of the Escherichia coli marRAB locus, by multiple chemicals in vitro. J. Bacteriol. 181:4669-4672.
8. Alekshun, M. N. and S. B. Levy. 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell 128:1037-1050.
9. Alekshun, M. N., S. B. Levy, T. R. Mealy, B. A. Seaton, and J. F. Head. 2001. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. Nat. Struct. Biol. 8:710-714.
10. Alguel, Y., C. Meng, W. Teran, T. Krell, J. L. Ramos, M. T. Gallegos, and X. Zhang. 2007. Crystal structures of multidrug binding protein TtgR in complex with antibiotics and plant antimicrobials. J. Mol. Biol. 369:829-840.
11. Alton, N. K. and D. Vapnek. 1979. Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. Nature 282:864-869.
12. Ariaratnam, V. and G. P. Georghiou. 1974. Carbamate resistance in Anopheles albimanus. Cross resistance spectrum and stability of resistance. Bull. World Health Organ 51:655-659.
13. Baranova, N. N., A. Danchin, and A. A. Neyfakh. 1999. Mta, a global MerR-type regulator of the Bacillus subtilis multidrug-efflux transporters. Mol. Microbiol. 31:1549-1559.
14. Barbosa, T. M. and S. B. Levy. 2000. Differential expression of over 60 chromosomal genes in Escherichia coli by constitutive expression of MarA. J. Bacteriol. 182:3467-3474.
15. Barbosa, T. M. and S. B. Levy. 2002. Activation of the Escherichia coli nfnB gene by MarA through a highly divergent marbox in a class II promoter. Mol. Microbiol. 45:191-202.
16. Barthelmebs, L., C. Divies, and J. F. Cavin. 2000. Knockout of the p-coumarate decarboxylase gene from Lactobacillus plantarum reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. Appl. Environ. Microbiol. 66:3368-3375.
17. Berens, C., L. Altschmied, and W. Hillen. 1992. The role of the N terminus in Tet repressor for tet operator binding determined by a mutational analysis. J. Biol. Chem. 267:1945-1952.
18. Berens, C., D. Schnappinger, and W. Hillen. 1997. The role of the variable region in Tet repressor for inducibility by tetracycline. J. Biol. Chem. 272:6936-6942.
19. Biedler, J. L. and H. Riehm. 1970. Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies. Cancer Res. 30:1174-1184.
20. Bolhuis, H., D. Molenaar, G. Poelarends, H. W. van Veen, B. Poolman, A. J. Driessen, and W. N. Konings. 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant Lactococcus lactis. J. Bacteriol. 176:6957-6964.
21. Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium Lactococcus lactis ssp. lactis IL1403. Genome Res. 11:731-753.
22. Borges-Walmsley, M. I., D. Du, K. S. McKeegan, G. J. Sharples, and A. R. Walmsley. 2005. VceR regulates the vceCAB drug efflux pump operon of Vibrio cholerae by alternating between mutually exclusive conformations that bind either drugs or promoter DNA. J. Mol. Biol. 349:387-400.
23. Bosch, I., K. Dunussi-Joannopoulos, R. L. Wu, S. T. Furlong, and J. Croop. 1997. Phosphatidylcholine and phosphatidylethanolamine behave as substrates of the human MDR1 P-glycoprotein. Biochemistry 36:5685-5694.
24. Bouige, P., D. Laurent, L. Piloyan, and E. Dassa. 2002. Phylogenetic and functional classification of ATP-binding cassette (ABC) systems. Curr. Protein Pept. Sci. 3:541-559.
25. Brennan, R. G. and B. W. Matthews. 1989. Structural basis of DNA-protein recognition. Trends Biochem. Sci. 14:286-290.

26. Brooun, A., J. J. Tomashek, and K. Lewis. 1999. Purification and ligand binding of EmrR, a regulator of a multidrug transporter. J. Bacteriol. 181:5131-5133.
27. Cerda-Maira, F. A., C. S. Ringelberg, and R. K. Taylor. 2008. The bile response repressor BreR regulates expression of the Vibrio cholerae breAB efflux system operon. J. Bacteriol. 190:7441-7452.
28. Chopra, I. and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65:232-260.
29. Cohen, S. P., W. Yan, and S. B. Levy. 1993. A multidrug resistance regulatory chromosomal locus is widespread among enteric bacteria. J. Infect. Dis. 168:484-488.
30. Dangi, B., P. Pelupessey, R. G. Martin, J. L. Rosner, J. M. Louis, and A. M. Gronenborn. 2001. Structure and dynamics of MarA-DNA complexes: an NMR investigation. J. Mol. Biol. 314:113-127.
31. Dassa, E. and P. Bouige. 2001. The ABC of ABCS: a phylogenetic and functional classification of ABC systems in living organisms. Res. Microbiol. 152:211-229.
32. Dawson, R. J. and K. P. Locher. 2006. Structure of a bacterial multidrug ABC transporter. Nature 443:180-185.
33. De Silva, R. S., G. Kovacikova, W. Lin, R. K. Taylor, K. Skorupski, and F. J. Kull. 2005. Crystal structure of the virulence gene activator AphA from Vibrio cholerae reveals it is a novel member of the winged helix transcription factor superfamily. J. Biol. Chem. 280:13779-13783.
34. Delihas, N. and S. Forst. 2001. MicF: an antisense RNA gene involved in response of Escherichia coli to global stress factors. J. Mol. Biol. 313:1-12.
35. Eckert, B. and C. F. Beck. 1989. Overproduction of transposon Tn10-encoded tetracycline resistance protein results in cell death and loss of membrane potential. J. Bacteriol. 171:3557-3559.
36. Evans, K., L. Adewoye, and K. Poole. 2001. MexR repressor of the mexAB-oprM multidrug efflux operon of Pseudomonas aeruginosa: identification of MexR binding sites in the mexA-mexR intergenic region. J. Bacteriol. 183:807-812.
37. Eytan, G. D., M. J. Borgnia, R. Regev, and Y. G. Assaraf. 1994. Transport of polypeptide ionophores into proteoliposomes reconstituted with rat liver P-glycoprotein. J. Biol. Chem. 269:26058-26065.
38. Fromknecht, K., P. D. Vogel, and J. G. Wise. 2003. Combinatorial redesign of the DNA binding specificity of a prokaryotic helix-turn-helix repressor. J. Bacteriol. 185:475-481.
39. Gajiwala, K. S. and S. K. Burley. 2000. Winged helix proteins. Curr. Opin. Struct. Biol. 10:110-116.
40. Gasson, M. J., Y. Kitamura, W. R. McLauchlan, A. Narbad, A. J. Parr, E. L. Parsons, J. Payne, M. J. Rhodes, and N. J. Walton. 1998. Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. J. Biol. Chem. 273:4163-4170.
41. Georghiou, G. P. 1965. Genetic studies on insecticide resistance. Adv. Pest. Control Res. 6:171-230.
42. Gillette, W. K., R. G. Martin, and J. L. Rosner. 2000. Probing the Escherichia coli transcriptional activator MarA using alanine-scanning mutagenesis: residues important for DNA binding and activation. J. Mol. Biol. 299:1245-1255.
43. Grkovic, S., M. H. Brown, N. J. Roberts, I. T. Paulsen, and R. A. Skurray. 1998. QacR is a repressor protein that regulates expression of the Staphylococcus aureus multidrug efflux pump QacA. J. Biol. Chem. 273:18665-18673.

44. Grkovic, S., M. H. Brown, M. A. Schumacher, R. G. Brennan, and R. A. Skurray. 2001. The staphylococcal QacR multidrug regulator binds a correctly spaced operator as a pair of dimers. J. Bacteriol. 183:7102-7109.
45. Grkovic, S., M. H. Brown, and R. A. Skurray. 2001. Transcriptional regulation of multidrug efflux pumps in bacteria. Semin. Cell Dev. Biol. 12:225-237.
46. Grkovic, S., M. H. Brown, and R. A. Skurray. 2002. Regulation of bacterial drug export systems. Microbiol. Mol. Biol. Rev. 66:671-701, table.
47. Grkovic, S., K. M. Hardie, M. H. Brown, and R. A. Skurray. 2003. Interactions of the QacR multidrug-binding protein with structurally diverse ligands: implications for the evolution of the binding pocket. Biochemistry 42:15226-15236.
48. Gury, J., L. Barthelmebs, N. P. Tran, C. Divies, and J. F. Cavin. 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding padA gene of Lactobacillus plantarum. Appl. Environ. Microbiol. 70:2146-2153.
49. Harbottle, H., S. Thakur, S. Zhao, and D. G. White. 2006. Genetics of antimicrobial resistance. Anim Biotechnol. 17:111-124.
50. Harrison, S. C. 1991. A structural taxonomy of DNA-binding domains. Nature 353:715-719.
51. Harrison, S. C. and A. K. Aggarwal. 1990. DNA recognition by proteins with the helix-turn-helix motif. Annu. Rev. Biochem. 59:933-969.
52. Hecht, B., G. Muller, and W. Hillen. 1993. Noninducible Tet repressor mutations map from the operator binding motif to the C terminus. J. Bacteriol. 175:1206-1210.
53. Helbl, V., C. Berens, and W. Hillen. 1995. Proximity probing of Tet repressor to tet operator by dimethylsulfate reveals protected and accessible functions for each recognized base-pair in the major groove. J. Mol. Biol. 245:538-548.
54. Heldwein, E. E. and R. G. Brennan. 2001. Crystal structure of the transcription activator BmrR bound to DNA and a drug. Nature 409:378-382.
55. Higgins, C. F. 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8:67-113.
56. Hillen, W. and C. Berens. 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu. Rev. Microbiol. 48:345-369.
57. Hinrichs, W., C. Kisker, M. Duvel, A. Muller, K. Tovar, W. Hillen, and W. Saenger. 1994. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. Science 264:418-420.
58. Holmes, D. J., J. L. Caso, and C. J. Thompson. 1993. Autogenous transcriptional activation of a thiostrepton-induced gene in Streptomyces lividans. EMBO J. 12:3183-3191.
59. Holtje, J. V. 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli. Microbiol. Mol. Biol. Rev. 62:181-203.
60. Holtz, T. H. and J. P. Cegielski. 2007. Origin of the term XDR-TB. Eur. Respir. J. 30:396.
61. Huillet, E., P. Velge, T. Vallaeys, and P. Pardon. 2006. LadR, a new PadR-related transcriptional regulator from Listeria monocytogenes, negatively regulates the expression of the multidrug efflux pump MdrL. FEMS Microbiol. Lett. 254:87-94.
62. Ishida, H., H. Fuziwara, Y. Kaibori, T. Horiuchi, K. Sato, and Y. Osada. 1995. Cloning of multidrug resistance gene pqrA from Proteus vulgaris. Antimicrob. Agents Chemother. 39:453-457.
63. Jain, A. and R. Mondal. 2008. Extensively drug-resistant tuberculosis: current challenges and threats. FEMS Immunol. Med. Microbiol. 53:145-150.
64. Jair, K. W., W. P. Fawcett, N. Fujita, A. Ishihama, and R. E. Wolf, Jr. 1996. Ambidextrous transcriptional activation by SoxS: requirement for the C-terminal domain of the RNA polymerase alpha subunit in a subset of Escherichia coli superoxide-inducible genes. Mol. Microbiol. 19:307-317.

65. Jair, K. W., X. Yu, K. Skarstad, B. Thony, N. Fujita, A. Ishihama, and R. E. Wolf, Jr. 1996. Transcriptional activation of promoters of the superoxide and multiple antibiotic resistance regulons by Rob, a binding protein of the Escherichia coli origin of chromosomal replication. J. Bacteriol. 178:2507-2513.
66. Jellen-Ritter, A. S. and W. V. Kern. 2001. Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in Escherichia coli mutants Selected with a fluoroquinolone. Antimicrob. Agents Chemother. 45:1467-1472.
67. Johnson, I. S., J. G. Armstrong, M. Gorman, and J. P. Burnett, Jr. 1963. The Vincalkaloids: A new class of oncolytic agents. Cancer Res. 23:1390-1427.
68. Kazama, H., H. Hamashima, M. Sasatsu, and T. Arai. 1998. Distribution of the antiseptic-resistance gene qacE delta 1 in gram-positive bacteria. FEMS Microbiol. Lett. 165:295-299.
69. Kleinschmidt, C., K. Tovar, and W. Hillen. 1991. Computer simulations and experimental studies of gel mobility patterns for weak and strong non-cooperative protein binding to two targets on the same DNA: application to binding of tet repressor variants to multiple and single tet operator sites. Nucleic Acids Res. 19:1021-1028.
70. Knol, J., L. Veenhoff, W. J. Liang, P. J. Henderson, G. Leblanc, and B. Poolman. 1996. Unidirectional reconstitution into detergent-destabilized liposomes of the purified lactose transport system of Streptococcus thermophilus. J. Biol. Chem. 271:15358-15366.
71. Kovacikova, G., W. Lin, and K. Skorupski. 2003. The virulence activator AphA links quorum sensing to pathogenesis and physiology in Vibrio cholerae by repressing the expression of a penicillin amidase gene on the small chromosome. J. Bacteriol. 185:4825-4836.
72. Kovacikova, G., W. Lin, and K. Skorupski. 2004. Vibrio cholerae AphA uses a novel mechanism for virulence gene activation that involves interaction with the LysR-type regulator AphB at the tcpPH promoter. Mol. Microbiol. 53:129-142.
73. Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu. 2001. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet 357:1225-1240.
74. Lee, E. H., E. Collatz, I. Podglajen, and L. Gutmann. 1996. A rob-like gene of Enterobacter cloacae affecting porin synthesis and susceptibility to multiple antibiotics. Antimicrob. Agents Chemother. 40:2029-2033.
75. Levy, S. B. and B. Marshall. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10:S122-S129.
76. Lim, D., K. Poole, and N. C. Strynadka. 2002. Crystal structure of the MexR repressor of the mexRAB-oprM multidrug efflux operon of Pseudomonas aeruginosa. J. Biol. Chem. 277:29253-29259.
77. Lin, J., M. Akiba, O. Sahin, and Q. Zhang. 2005. CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in Campylobacter jejuni. Antimicrob. Agents Chemother. 49:1067-1075.
78. Lin, J., C. Cagliero, B. Guo, Y. W. Barton, M. C. Maurel, S. Payot, and Q. Zhang. 2005. Bile salts modulate expression of the CmeABC multidrug efflux pump in Campylobacter jejuni. J. Bacteriol. 187:7417-7424.
79. Littlejohn, T. G., I. T. Paulsen, M. T. Gillespie, J. M. Tennent, M. Midgley, I. G. Jones, A. S. Purewal, and R. A. Skurray. 1992. Substrate specificity and energetics of antiseptic and disinfectant resistance in Staphylococcus aureus. FEMS Microbiol. Lett. 74:259-265.

80. Lomovskaya, O., K. Lewis, and A. Matin. 1995. EmrR is a negative regulator of the Escherichia coli multidrug resistance pump EmrAB. J. Bacteriol. 177:2328-2334.
81. Lubelski, J., J. A. de, M. R. van, H. Agustiandari, O. P. Kuipers, J. Kok, and A. J. Driessen. 2006. LmrCD is a major multidrug resistance transporter in Lactococcus lactis. Mol. Microbiol. 61:771-781.
82. Lubelski, J., P. Mazurkiewicz, M. R. van, W. N. Konings, and A. J. Driessen. 2004. ydaG and ydbA of Lactococcus lactis encode a heterodimeric ATP-binding cassette-type multidrug transporter. J. Biol. Chem. 279:34449-34455.
83. Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst. 1996. The local repressor AcrR plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals. Mol. Microbiol. 19:101-112.
84. Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst. 1995. Genes acrA and acrB encode a stress-induced efflux system of Escherichia coli. Mol. Microbiol. 16:45-55.
85. Madoori, P. K., H. Agustiandari, A. J. Driessen, and A. M. Thunnissen. 2008. Structure of the transcriptional regulator LmrR and its mechanism of multidrug recognition. EMBO J.
86. Margolles, A., M. Putman, H. W. van Veen, and W. N. Konings. 1999. The purified and functionally reconstituted multidrug transporter LmrA of Lactococcus lactis mediates the transbilayer movement of specific fluorescent phospholipids. Biochemistry 38:16298-16306.
87. Markham, P. N., M. Ahmed, and A. A. Neyfakh. 1996. The drug-binding activity of the multidrug-responding transcriptional regulator BmrR resides in its C-terminal domain. J. Bacteriol. 178:1473-1475.
88. Martin, R. G., E. S. Bartlett, J. L. Rosner, and M. E. Wall. 2008. Activation of the Escherichia coli marA/soxS/rob regulon in response to transcriptional activator concentration. J. Mol. Biol. 380:278-284.
89. Martin, R. G., W. K. Gillette, N. I. Martin, and J. L. Rosner. 2002. Complex formation between activator and RNA polymerase as the basis for transcriptional activation by MarA and SoxS in Escherichia coli. Mol. Microbiol. 43:355-370.
90. Martin, R. G., W. K. Gillette, S. Rhee, and J. L. Rosner. 1999. Structural requirements for marbox function in transcriptional activation of mar/sox/rob regulon promoters in Escherichia coli: sequence, orientation and spatial relationship to the core promoter. Mol. Microbiol. 34:431-441.
91. Martin, R. G., K. W. Jair, R. E. Wolf, Jr., and J. L. Rosner. 1996. Autoactivation of the marRAB multiple antibiotic resistance operon by the MarA transcriptional activator in Escherichia coli. J. Bacteriol. 178:2216-2223.
92. Martin, R. G. and J. L. Rosner. 1995. Binding of purified multiple antibiotic-resistance repressor protein (MarR) to mar operator sequences. Proc. Natl. Acad. Sci. U. S. A 92:5456-5460.
93. Martin, R. G. and J. L. Rosner. 1997. Fis, an accessorial factor for transcriptional activation of the mar (multiple antibiotic resistance) promoter of Escherichia coli in the presence of the activator MarA, SoxS, or Rob. J. Bacteriol. 179:7410-7419.
94. Martin, R. G. and J. L. Rosner. 2001. The AraC transcriptional activators. Curr. Opin. Microbiol. 4:132-137.
95. Martin, R. G. and J. L. Rosner. 2002. Genomics of the marA/soxS/rob regulon of Escherichia coli: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. Mol. Microbiol. 44:1611-1624.
96. Mazzariol, A., Y. Tokue, T. M. Kanegawa, G. Cornaglia, and H. Nikaido. 2000. High-level fluoroquinolone-resistant clinical isolates of Escherichia coli overproduce multidrug efflux protein AcrA. Antimicrob. Agents Chemother. 44:3441-3443.

97. McDermott, P. F., D. G. White, I. Podglajen, M. N. Alekshun, and S. B. Levy. 1998. Multidrug resistance following expression of the Escherichia coli marA gene in Mycobacterium smegmatis. J. Bacteriol. 180:2995-2998.
98. Meier, I., L. V. Wray, and W. Hillen. 1988. Differential regulation of the Tn10-encoded tetracycline resistance genes tetA and tetR by the tandem tet operators O1 and O2. EMBO J. 7:567-572.
99. Michan, C., M. Manchado, and C. Pueyo. 2002. SoxRS down-regulation of rob transcription. J. Bacteriol. 184:4733-4738.
100. Miller, P. F., L. F. Gambino, M. C. Sulavik, and S. J. Gracheck. 1994. Genetic relationship between soxRS and mar loci in promoting multiple antibiotic resistance in Escherichia coli. Antimicrob. Agents Chemother. 38:1773-1779.
101. Miller, P. F. and M. C. Sulavik. 1996. Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in Escherichia coli. Mol. Microbiol. 21:441-448.
102. Mitchell, B. A., M. H. Brown, and R. A. Skurray. 1998. QacA multidrug efflux pump from Staphylococcus aureus: comparative analysis of resistance to diamidines, biguanidines, and guanylhydrazones. Antimicrob. Agents Chemother. 42:475-477.
103. Mitchell, B. A., I. T. Paulsen, M. H. Brown, and R. A. Skurray. 1999. Bioenergetics of the staphylococcal multidrug export protein QacA. Identification of distinct binding sites for monovalent and divalent cations. J. Biol. Chem. 274:3541-3548.
104. Muller, G., B. Hecht, V. Helbl, W. Hinrichs, W. Saenger, and W. Hillen. 1995. Characterization of non-inducible Tet repressor mutants suggests conformational changes necessary for induction. Nat. Struct. Biol. 2:693-703.
105. Neu, H. C. 1992. The crisis in antibiotic resistance. Science 257:1064-1073.
106. Ng, E. Y., M. Trucksis, and D. C. Hooper. 1994. Quinolone resistance mediated by norA: physiologic characterization and relationship to flqB, a quinolone resistance locus on the Staphylococcus aureus chromosome. Antimicrob. Agents Chemother. 38:1345-1355.
107. Nikaido, H. 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264:382-388.
108. Nikaido, H. and D. G. Thanassi. 1993. Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. Antimicrob. Agents Chemother. 37:1393-1399.
109. Nikaido, H. and H. I. Zgurskaya. 2001. AcrAB and related multidrug efflux pumps of Escherichia coli. J. Mol. Microbiol. Biotechnol. 3:215-218.
110. Notka, F., H. J. Linde, A. Dankesreiter, H. H. Niller, and N. Lehn. 2002. A C-terminal 18 amino acid deletion in MarR in a clinical isolate of Escherichia coli reduces MarR binding properties and increases the MIC of ciprofloxacin. J. Antimicrob. Chemother. 49:41-47.
111. Nunoshiba, T., E. Hidalgo, Z. Li, and B. Demple. 1993. Negative autoregulation by the Escherichia coli SoxS protein: a dampening mechanism for the soxRS redox stress response. J. Bacteriol. 175:7492-7494.
112. Nunoshiba, T., E. Hidalgo, C. F. mabile Cuevas, and B. Demple. 1992. Two-stage control of an oxidative stress regulon: the Escherichia coli SoxR protein triggers redox-inducible expression of the soxS regulatory gene. J. Bacteriol. 174:6054-6060.
113. Oethinger, M., I. Podglajen, W. V. Kern, and S. B. Levy. 1998. Overexpression of the marA or soxS regulatory gene in clinical topoisomerase mutants of Escherichia coli. Antimicrob. Agents Chemother. 42:2089-2094.
114. Okusu, H., D. Ma, and H. Nikaido. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. 178:306-308.

115. Orth, P., F. Cordes, D. Schnappinger, W. Hillen, W. Saenger, and W. Hinrichs. 1998. Conformational changes of the Tet repressor induced by tetracycline trapping. J. Mol. Biol. 279:439-447.
116. Orth, P., W. Saenger, and W. Hinrichs. 1999. Tetracycline-chelated Mg2+ ion initiates helix unwinding in Tet repressor induction. Biochemistry 38:191-198.
117. Orth, P., D. Schnappinger, W. Hillen, W. Saenger, and W. Hinrichs. 2000. Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. Nat. Struct. Biol. 7:215-219.
118. Orth, P., D. Schnappinger, P. E. Sum, G. A. Ellestad, W. Hillen, W. Saenger, and W. Hinrichs. 1999. Crystal structure of the tet repressor in complex with a novel tetracycline, 9-(N,N-dimethylglycylamido)- 6-demethyl-6-deoxy-tetracycline. J. Mol. Biol. 285:455-461.
119. Overhage, J., H. Priefert, and A. Steinbuchel. 1999. Biochemical and genetic analyses of ferulic acid catabolism in Pseudomonas sp. Strain HR199. Appl. Environ. Microbiol. 65:4837-4847.
120. Pabo, C. O. and R. T. Sauer. 1992. Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. 61:1053-1095.
121. Palumbi, S. R. 2001. Humans as the world's greatest evolutionary force. Science 293:1786-1790.
122. Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell, and R. A. Skurray. 1996. Multidrug resistance proteins QacA and QacB from Staphylococcus aureus: membrane topology and identification of residues involved in substrate specificity. Proc. Natl. Acad. Sci. U. S. A 93:3630-3635.
123. Paulsen, I. T., M. H. Brown, and R. A. Skurray. 1996. Proton-dependent multidrug efflux systems. Microbiol. Rev. 60:575-608.
124. Paulsen, I. T., M. H. Brown, and R. A. Skurray. 1998. Characterization of the earliest known Staphylococcus aureus plasmid encoding a multidrug efflux system. J. Bacteriol. 180:3477-3479.
125. Paulsen, I. T., L. Nguyen, M. K. Sliwinski, R. Rabus, and M. H. Saier, Jr. 2000. Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. J. Mol. Biol. 301:75-100.
126. Paulsen, I. T., M. K. Sliwinski, and M. H. Saier, Jr. 1998. Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. J. Mol. Biol. 277:573-592.
127. Perez-Rueda, E. and J. Collado-Vides. 2001. Common history at the origin of the position-function correlation in transcriptional regulators in archaea and bacteria. J. Mol. Evol. 53:172-179.
128. Pomposiello, P. J., M. H. Bennik, and B. Demple. 2001. Genome-wide transcriptional profiling of the Escherichia coli responses to superoxide stress and sodium salicylate. J. Bacteriol. 183:3890-3902.
129. Poole, K. 1994. Bacterial multidrug resistance--emphasis on efflux mechanisms and Pseudomonas aeruginosa. J. Antimicrob. Chemother. 34:453-456.
130. Poole, K., D. E. Heinrichs, and S. Neshat. 1993. Cloning and sequence analysis of an EnvCD homologue in Pseudomonas aeruginosa: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. 10:529-544.
131. Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco. 1996. Expression of the multidrug resistance operon mexA-mexB-oprM in Pseudomonas aeruginosa: mexR encodes a regulator of operon expression. Antimicrob. Agents Chemother. 40:2021-2028.
132. Putman, M., H. W. van Veen, and W. N. Konings. 2000. Molecular properties of bacterial multidrug transporters. Microbiol. Mol. Biol. Rev. 64:672-693.

133. Putman, M., H. W. van Veen, B. Poolman, and W. N. Konings. 1999. Restrictive use of detergents in the functional reconstitution of the secondary multidrug transporter LmrP. Biochemistry 38:1002-1008.
134. Rahmati, S., S. Yang, A. L. Davidson, and E. L. Zechiedrich. 2002. Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. Mol. Microbiol. 43:677-685.
135. Ramos, J. L., M. Martinez-Bueno, A. J. Molina-Henares, W. Teran, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes. 2005. The TetR family of transcriptional repressors. Microbiol. Mol. Biol. Rev. 69:326-356.
136. Rand, J. D., S. G. Danby, D. L. Greenway, and R. R. England. 2002. Increased expression of the multidrug efflux genes acrAB occurs during slow growth of Escherichia coli. FEMS Microbiol. Lett. 207:91-95.
137. Rhee, S., R. G. Martin, J. L. Rosner, and D. R. Davies. 1998. A novel DNA-binding motif in MarA: the first structure for an AraC family transcriptional activator. Proc. Natl. Acad. Sci. U. S. A 95:10413-10418.
138. Rosner, J. L., B. Dangi, A. M. Gronenborn, and R. G. Martin. 2002. Posttranscriptional activation of the transcriptional activator Rob by dipyridyl in Escherichia coli. J. Bacteriol. 184:1407-1416.
139. Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray. 1990. Efflux-mediated antiseptic resistance gene qacA from Staphylococcus aureus: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. 4:2051-2062.
140. Rousseau, P., E. Gueguen, G. Duval-Valentin, and M. Chandler. 2004. The helix-turn-helix motif of bacterial insertion sequence IS911 transposase is required for DNA binding. Nucleic Acids Res. 32:1335-1344.
141. Saier, M. H., Jr. and I. T. Paulsen. 2001. Phylogeny of multidrug transporters. Semin. Cell Dev. Biol. 12:205-213.
142. Saito, K., H. Yoneyama, and T. Nakae. 1999. nalB-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the mexR gene of the Pseudomonas aeruginosa chromosome. FEMS Microbiol. Lett. 179:67-72.
143. Sawada, I., H. Maseda, T. Nakae, H. Uchiyama, and N. Nomura. 2004. A quorum-sensing autoinducer enhances the mexAB-oprM efflux-pump expression without the MexR-mediated regulation in Pseudomonas aeruginosa. Microbiol. Immunol. 48:435-439.
144. Schnappinger, D. and W. Hillen. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch. Microbiol. 165:359-369.
145. Schnappinger, D., P. Schubert, C. Berens, K. Pfleiderer, and W. Hillen. 1999. Solvent-exposed residues in the Tet repressor (TetR) four-helix bundle contribute to subunit recognition and dimer stability. J. Biol. Chem. 274:6405-6410.
146. Schnappinger, D., P. Schubert, K. Pfleiderer, and W. Hillen. 1998. Determinants of protein-protein recognition by four helix bundles: changing the dimerization specificity of Tet repressor. EMBO J. 17:535-543.
147. Schneiders, T. and S. B. Levy. 2006. MarA-mediated transcriptional repression of the rob promoter. J. Biol. Chem. 281:10049-10055.
148. Scholz, O., M. Kintrup, M. Reich, and W. Hillen. 2001. Mechanism of Tet repressor induction by tetracyclines: length compensates for sequence in the alpha8-alpha9 loop. J. Mol. Biol. 310:979-986.
149. Scholz, O., P. Schubert, M. Kintrup, and W. Hillen. 2000. Tet repressor induction without Mg2+. Biochemistry 39:10914-10920.
150. Schubert, P., D. Schnappinger, K. Pfleiderer, and W. Hillen. 2001. Identification of a stability determinant on the edge of the Tet repressor four-helix bundle dimerization motif. Biochemistry 40:3257-3263.

151. Schumacher, M. A., M. C. Miller, and R. G. Brennan. 2004. Structural mechanism of the simultaneous binding of two drugs to a multidrug-binding protein. EMBO J. 23:2923-2930.
152. Schumacher, M. A., M. C. Miller, S. Grkovic, M. H. Brown, R. A. Skurray, and R. G. Brennan. 2001. Structural mechanisms of QacR induction and multidrug recognition. Science 294:2158-2163.
153. Schumacher, M. A., M. C. Miller, S. Grkovic, M. H. Brown, R. A. Skurray, and R. G. Brennan. 2002. Structural basis for cooperative DNA binding by two dimers of the multidrug-binding protein QacR. EMBO J. 21:1210-1218.
154. Segura, A., P. V. Bunz, D. A. D'Argenio, and L. N. Ornston. 1999. Genetic analysis of a chromosomal region containing vanA and vanB, genes required for conversion of either ferulate or vanillate to protocatechuate in Acinetobacter. J. Bacteriol. 181:3494-3504.
155. Seoane, A. S. and S. B. Levy. 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance (mar) operon in Escherichia coli. J. Bacteriol. 177:3414-3419.
156. Sharom, F. J., G. Diodato, X. Yu, and K. J. Ashbourne. 1995. Interaction of the P-glycoprotein multidrug transporter with peptides and ionophores. J. Biol. Chem. 270:10334-10341.
157. Sheps, J. A. and V. Ling. 2007. Preface: the concept and consequences of multidrug resistance. Pflugers Arch. 453:545-553.
158. Smith, L. D. and K. P. Bertrand. 1988. Mutations in the Tn10 tet repressor that interfere with induction. Location of the tetracycline-binding domain. J. Mol. Biol. 203:949-959.
159. Sood, S., M. Malhotra, B. K. Das, and A. Kapil. 2008. Enterococcal infections & antimicrobial resistance. Indian J. Med. Res. 128:111-121.
160. Speer, B. S., N. B. Shoemaker, and A. A. Salyers. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin. Microbiol. Rev. 5:387-399.
161. Spott, S., F. Dong, B. Kisters-Woike, and B. Muller-Hill. 2000. Dimerisation mutants of Lac repressor. II. A single amino acid substitution, D278L, changes the specificity of dimerisation. J. Mol. Biol. 296:673-684.
162. Spratt, B. G. 1994. Resistance to antibiotics mediated by target alterations. Science 264:388-393.
163. Srikumar, R., C. J. Paul, and K. Poole. 2000. Influence of mutations in the mexR repressor gene on expression of the MexA-MexB-oprM multidrug efflux system of Pseudomonas aeruginosa. J. Bacteriol. 182:1410-1414.
164. Su, C. C., D. J. Rutherford, and E. W. Yu. 2007. Characterization of the multidrug efflux regulator AcrR from Escherichia coli. Biochem. Biophys. Res. Commun. 361:85-90.
165. Tanaka, T., T. Horii, K. Shibayama, K. Sato, S. Ohsuka, Y. Arakawa, K. Yamaki, K. Takagi, and M. Ohta. 1997. RobA-induced multiple antibiotic resistance largely depends on the activation of the AcrAB efflux. Microbiol. Immunol. 41:697-702.
166. Tennent, J. M., B. R. Lyon, M. Midgley, I. G. Jones, A. S. Purewal, and R. A. Skurray. 1989. Physical and biochemical characterization of the qacA gene encoding antiseptic and disinfectant resistance in Staphylococcus aureus. J. Gen. Microbiol. 135:1-10.
167. Tennent, J. M., J. W. May, and R. A. Skurray. 1984. Multiple antibiotic resistance in Staphylococcus aureus and Staphylococcus epidermidis: plasmids in strains associated with nosocomial infection. Pathology 16:250-255.
168. Thanassi, D. G., L. W. Cheng, and H. Nikaido. 1997. Active efflux of bile salts by Escherichia coli. J. Bacteriol. 179:2512-2518.
169. Tiebel, B., K. Garke, and W. Hillen. 2000. Observing conformational and activity changes of tet repressor in vivo. Nat. Struct. Biol. 7:479-481.

170. Tiebel, B., N. Radzwill, L. M. ung-Hilbrich, V. Helbl, H. J. Steinhoff, and W. Hillen. 1999. Domain motions accompanying Tet repressor induction defined by changes of interspin distances at selectively labeled sites. J. Mol. Biol. 290:229-240.
171. Tiebel, B., L. M. ung-Hilbrich, D. Schnappinger, and W. Hillen. 1998. Conformational changes necessary for gene regulation by Tet repressor assayed by reversible disulfide bond formation. EMBO J. 17:5112-5119.
172. Travers, A., R. Schneider, and G. Muskhelishvili. 2001. DNA supercoiling and transcription in Escherichia coli: The FIS connection. Biochimie 83:213-217.
173. Truong-Bolduc, Q. C., P. M. Dunman, J. Strahilevitz, S. J. Projan, and D. C. Hooper. 2005. MgrA is a multiple regulator of two new efflux pumps in Staphylococcus aureus. J. Bacteriol. 187:2395-2405.
174. Truong-Bolduc, Q. C., J. Strahilevitz, and D. C. Hooper. 2006. NorC, a new efflux pump regulated by MgrA of Staphylococcus aureus. Antimicrob. Agents Chemother. 50:1104-1107.
175. Truong-Bolduc, Q. C., X. Zhang, and D. C. Hooper. 2003. Characterization of NorR protein, a multifunctional regulator of norA expression in Staphylococcus aureus. J. Bacteriol. 185:3127-3138.
176. Ubukata, K., N. Itoh-Yamashita, and M. Konno. 1989. Cloning and expression of the norA gene for fluoroquinolone resistance in Staphylococcus aureus. Antimicrob. Agents Chemother. 33:1535-1539.
177. van, H. A., A. J. Smith, H. Sprong, I. Fritzsche, A. H. Schinkel, P. Borst, and M. G. van. 1996. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 87:507-517.
178. Vazquez-Laslop, N., P. N. Markham, and A. A. Neyfakh. 1999. Mechanism of ligand recognition by BmrR, the multidrug-responding transcriptional regulator: mutational analysis of the ligand-binding site. Biochemistry 38:16925-16931.
179. Vergani, B., M. Kintrup, W. Hillen, H. Lami, E. Piemont, E. Bombarda, P. Alberti, S. M. Doglia, and M. Chabbert. 2000. Backbone dynamics of Tet repressor alpha8intersectionalpha9 loop. Biochemistry 39:2759-2768.
180. von, H. G. 1991. Proline kinks in transmembrane alpha-helices. J. Mol. Biol. 218:499-503.
181. Wang, H., J. L. Dzink-Fox, M. Chen, and S. B. Levy. 2001. Genetic characterization of highly fluoroquinolone-resistant clinical Escherichia coli strains from China: role of acrR mutations. Antimicrob. Agents Chemother. 45:1515-1521.
182. Webber, M. A. and L. J. Piddock. 2001. Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli. Antimicrob. Agents Chemother. 45:1550-1552.
183. Wegmann, U., M. O'Connell-Motherway, A. Zomer, G. Buist, C. Shearman, C. Canchaya, M. Ventura, A. Goesmann, M. J. Gasson, O. P. Kuipers, D. van Sinderen, and J. Kok. 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J. Bacteriol. 189:3256-3270.
184. White, D. G., J. D. Goldman, B. Demple, and S. B. Levy. 1997. Role of the acrAB locus in organic solvent tolerance mediated by expression of marA, soxS, or robA in Escherichia coli. J. Bacteriol. 179:6122-6126.
185. Woodcock, D. M., M. E. Linsenmeyer, G. Chojnowski, A. B. Kriegler, V. Nink, L. K. Webster, and W. H. Sawyer. 1992. Reversal of multidrug resistance by surfactants. Br. J. Cancer 66:62-68.
186. Woolley, R. C., G. Vediyappan, M. Anderson, M. Lackey, B. Ramasubramanian, B. Jiangping, T. Borisova, J. A. Colmer, A. N. Hamood, C. S. McVay, and J. A. Fralick. 2005.

Characterization of the Vibrio cholerae vceCAB multiple-drug resistance efflux operon in Escherichia coli. J. Bacteriol. 187:5500-5503.

187. Woolridge, D. P., J. D. Martinez, D. E. Stringer, and E. W. Gerner. 1999. Characterization of a novel spermidine/spermine acetyltransferase, BltD, from Bacillus subtilis. Biochem. J. 340 (Pt 3):753-758.
188. Woolridge, D. P., N. Vazquez-Laslop, P. N. Markham, M. S. Chevalier, E. W. Gerner, and A. A. Neyfakh. 1997. Efflux of the natural polyamine spermidine facilitated by the Bacillus subtilis multidrug transporter Blt. J. Biol. Chem. 272:8864-8866.
189. Xiong, A., A. Gottman, C. Park, M. Baetens, S. Pandza, and A. Matin. 2000. The EmrR protein represses the Escherichia coli emrRAB multidrug resistance operon by directly binding to its promoter region. Antimicrob. Agents Chemother. 44:2905-2907.
190. Zaidi, A. H., P. J. Bakkes, J. Lubelski, H. Agustiandari, O. P. Kuipers, and A. J. Driessen. 2008. The ABC-type multidrug resistance transporter LmrCD is responsible for an extrusion-based mechanism of bile acid resistance in Lactococcus lactis. J. Bacteriol. 190:7357-7366.
191. Zgurskaya, H. I. and H. Nikaido. 1999. Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A 96:7190-7195.
192. Zheleznova, E. E., P. N. Markham, A. A. Neyfakh, and R. G. Brennan. 1999. Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. Cell 96:353-362.

Chapter

LmrCD is a major MDR transporter in *Lactococcus lactis*

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SUMMARY

When *Lactococcus lactis* is challenged with drugs it displays a multidrug resistance phenotype. *In silico* analysis of the genome of *L. lactis* indicates the presence of at least 40 putative multidrug resistance (MDR) transporters, of which only four, i.e. the ABC transporters LmrA, LmrC and LmrD, and the major facilitator LmrP, have been experimentally associated with the MDR. To understand the molecular basis of the MDR phenotype in *L. lactis*, we have performed a global transcriptome analysis comparing four independently isolated drug-resistant strains of *L. lactis* with the wild type strain. The results show a strong and consistent up-regulation of the *lmrC* and *lmrD* genes in all four strains, while the mRNA levels of other putative MDR transporters were not significantly altered. Deletion of *lmrCD* renders *L. lactis* sensitive to several toxic compounds, and this phenotype is associated with a reduced ability to secrete these compounds. Another gene, which is strongly up-regulated in all mutant strains, specifies LmrR (YdaF), a local transcriptional repressor of *lmrCD* that belongs to the PadR family of transcriptional regulators and that binds to the promoter region of *lmrCD*. These results demonstrate that the heterodimeric MDR ABC-transporter LmrCD is a major determinant of both acquired and intrinsic drug resistance of *L. lactis*.

INTRODUCTION

Multidrug resistance (MDR) has been described first as the ability of cancer cells to develop cross-resistance to a multitude of cytotoxic agents. The factors responsible for this phenomenon include a group of integral membrane proteins that extrude a variety of chemically unrelated compounds (23). More than 25 years ago, Levy and colleagues reported that the efflux of tetracycline in *Escherichia coli* is mediated by a transport protein (27). Subsequently, an increasing number of (multi)drug resistance (MDR) proteins have been reported and it became evident that, in analogy to eukaryotes, bacteria utilize MDR pumps for protection against toxic compounds.

The availability of microbial genomic sequences readily allowed the prediction and classification of membrane transport systems with a putative role in multidrug resistance (36). Multidrug resistance efflux pumps appear to be highly ubiquitous among bacteria (33,39,40), and an analysis of more than 100 genomic sequences revealed that transporters of this class constitute on average 10 % of all the transporters in an organism (30). Multidrug transporters can be grouped into five distinct families, namely the Major Facilitator Superfamily (MFS), the Small Multidrug Resistance family (SMR), the Multidrug and Toxic compounds Extrusion family (MATE), the Resistance-Nodulation-cell Division family (RND), all secondary transporters, and the ATP-binding cassette superfamily (ABC), which is a group of primary transporters (6,9,22,34,35,45,50). Most of multidrug transporters described in eukaryotes including the well-characterized human P-glycoprotein (Pgp), MRP1, and ABCG2 fall in the latter family, and are ATP driven (15,17,23,29,31,43). In contrast, the vast majority of multidrug efflux systems described in bacteria belong to the four families of proteins that are driven by the proton or sodium motive force (see reviews: 8,14,22,26,28,48). The ubiquitous nature of these systems also raises the following questions as to which putative multidrug resistance pumps do actually contribute to drug protection? What is the common mode of energization of these systems? Why are these putative MDR pumps so redundant?

In Gram-negative bacteria a major role in resistance to drugs has been attributed to the action of members of the RND family, such as AcrAB-TolC from *E. coli* or MexAB-OprM from *Pseudomonas aeruginosa* (22,34). These proteins form tripartite complexes that consist of an RND transporter in the cytoplasmic membrane, an outer membrane pore and an adaptor protein that connects the proteins in both membranes. These systems transport drugs across two membranes but also bind drugs in the periplasm to extrude them across the outer membrane.

Due to the organization of the cell envelope, the tripartite systems transporters are not found in Gram-positive bacteria (22). The best studied examples of MDR pumps in these bacteria include MFS transporters such as NorA from *Staphylococcus aureus* or its homolog PmrA from *S. pneumoniae*. Although putative ABC-type multidrug resistance transporters are relatively common in bacteria (30,40) (also see: www.membranetransport.org), only a small number of them were shown to be associated with multidrug resistance (39). In the non-pathogenic Gram positive organism *Lactococcus lactis*, the multidrug resistance phenomenon has been studied in great detail. Both secondary and primary active transporters have been implicated in the MDR phenotype: the MFS transporter LmrP (4), the ABC homodimeric half-transporter LmrA (49), and the recently described heterodimeric ABC transporter which consists of two half-transporters, LmrC and LmrD (24). *L. lactis* cells which are challenged for a prolonged period of time with drugs display a multidrug resistance phenotype. Therein, *L. lactis* cells were stepwise selected to grow in the presence of increasing concentration of different drugs (3). Cells grown in the presence of increasing amounts of ethidium, daunomycin or rhodamine 6G were found not only to become resistant to the drug used in the challenge, but also to unrelated drugs (3). This phenomenon is associated with an increased ability of the cells to extrude the drugs, but the exact identity of the transport system(s) responsible for the acquired drug resistance has not been determined. Although LmrA and LmrP have been suggested to be main determinants of drug resistance in *L. lactis*, inactivation of their genes has no effect on the intrinsic drug resistance of the cells (4,10). This has been taken to suggest that regulatory mechanisms that drive the expression of thus far uncharacterized drug transporters compensate for the loss of LmrA or LmrP.

To elucidate the molecular basis of the multidrug resistance phenotype of *L. lactis*, we have investigated the gene expression profiles in the previously isolated drug-resistant strains of *L. lactis* (3) using DNA-microarray analysis. Remarkably, the results indicate that in all drug-resistant *L. lactis* strains tested, the *lmrCD* genes are strongly up-regulated, while the expression of genes of other (putative) MDR transporters such as *lmrA* and *lmrP* remained unaltered. Deletion of the *lmrCD* genes in *L. lactis* resulted in a strain that is hypersensitive to several toxic compounds, a phenomenon associated with a reduced ability to extrude these compounds from the cell. These data demonstrate that LmrCD is a major drug transporter responsible for the intrinsic and acquired drug resistance in *L. lactis* for the panel of drugs tested.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Lactococcus lactis NZ9000, a derivative of the plasmid free strain MG1363 containing *pepN::nisRK*, was used as a host for the gene disruption procedure. Cells were grown as described previously to an OD_{660} of 0.8 in M17 medium (Difco) supplemented with 0.5 % (w/v) glucose and 5 μg/ml chloramphenicol. The *L. lactis* MG1363 MDR mutants, selected by growth in the presence of ethidium, rhodamine, daunomycin (3) or the drug specific mutant, resistant to cholate (51) were described before.

Recombinant DNA techniques

DNA manipulations were performed essentially as described by Sambrook *et al* (42). The *lmrCD* genes were isolated from genomic DNA of *L. lactis* MG1363 and cloned into pNS8048E, which resulted in pNSCD (24). For Southern blot analysis, chromosomal DNA of *L. lactis* NZ9000 was digested with XbaI and SalI, resolved on 1 % agarose gel and transferred to Zeta-probe positively charged membrane using 0.4 M NaOH and 0.6 M NaCl. The DNA probe was prepared with PCR DIG labelling mix^{Plus} (Roche) and primers: YdaG1F – 5'-cgttcgtgaagcgacttaca-3'; YdaG2R – 5'-tggcgttaatacggtccatc-3'. Detection was performed with an alkaline phosphatase-labelled anti-DIG antibody. Expected sizes of the target DNA sequences were: 4038 bp in case of *L. lactis* NZ9000 and 2035 bp *L. lactis* NZ9000 Δ*lmrCD*.

Construction of lmrCD *deletion strain*

A deletion of *lmrCD* was introduced in the genome of *L. lactis* NZ9000 by a gene replacement method described before (18,21). In order to insert *lmrCD* into pNS8048E using restriction sites NcoI and XbaI, primers: YdaG/YdbA-F (cgcccatggggaagcataaatgggttgccttatt); GA-R MG (gcgtctagattcaaaaacgaattgattatg) were used to amplify both genes together from genomic DNA of *L. lactis* MG1363. The construct was digested with ScaI and MvaI, treated with Klenow and subsequently ligated. Truncated *lmrCD* was inserted using NcoI and XbaI into pORI280 yielding pORIGAMGKO. Since the pORI plasmid and the *L. lactis* do not contain *repA* for plasmid replication, the plasmid ws maintained in *L. lactis* LL302, which contains a chromosomal copy of the *repA* gene and allows for pORI plasmid replication. Subsequently, pORIGAMGKO was introduced in *L. lactis*

NZ9000 cells containing the temperature sensitive pVE6007 plasmid which contains the *repA* gene. After losing the pVE6007 plasmid by raising the temperature to 42°C, the pORIGAMGKO was integrated into the genome. Excision of integrated compound and detection of resolution structure was facilitated by reporter gene *lacZ* (21). Strains *L. lactis* NZ9000 Δ*lmrA* (13) and *L. lactis* NZ9000 Δ*lmrACD* (46) are described elsewhere.

Growth studies

The specific growth rates of *L. lactis* MG1363, *L. lactis* NZ9000 and *L. lactis* NZ9000 Δ*lmrCD* in the presence of drugs was determined by growth in 96-well microtitre plates as described previously (41). In order to complement the multidrug sensitive phenotype of *L. lactis* NZ9000 Δ*lmrCD* cells were transformed with the control plasmid pIL252 or with pILLmrCD which contains *lmrCD* and its upstream putative promoter region covering *ydaF*. Additionally, to preclude any interference of the *lmrCD* upstream sequences pNS8048E or pNSCD (formerly known as pNSGA) which encodes the *lmrCD* genes under control of the *nisA* promoter (24), were introduced in *L. lactis* NZ9000 Δ*lmrCD*. In addition, plasmids were used that encode LmrCD mutants, i.e., pNSLmrC(D495N)/LmrD, pNSLmrC/LmrD(E587Q), pNSLmrC(D495N)/LmrD(E587Q), pNSLmrC(D495N) or pNSLmrD(E587Q) (25) under control of the *PnisA*. Overnight cultures of *L. lactis* were diluted into fresh GM17 medium supplemented with chloramphenicol when appropriate and grown at 30 °C until the mid-exponential phase (OD_{660} of 0.5). Subsequently, cells were diluted into fresh medium to an OD_{660} of 0.05 and 150 µl aliquots of the cell suspensions were transferred to 96-well microplates (Garnier, Germany) containing 50 µl of various concentrations of different toxic compounds in GM17 medium supplemented with chloramphenicol and 100 pg/ml of nisin when necessary. Growth of cells was monitored at 690 nm every 10 min for 16 h using a multiscan photometer (Molecular Devices, Spectra Max 340). The drug concentration that caused inhibition of the growth rate by 50 % (IC_{50} values) was determined.

DNA Microarray analysis

DNA microarray experiments were essentially performed as described (11,47). In short, RNA was isolated from five separately grown replicate cultures of *L. lactis* MG1363 and the four *L. lactis* MG1363 strains that were resistant to daunomycin, rhodamine, ethidium (3) or cholate (51). All of these strains were grown in GM17 medium and harvested at the mid exponential phase. Subsequently, single-strand

reverse transcription (amplification) and indirect labelling of 20 μg of total RNA, with either Cy3 or Cy5 dye, were performed (including a sample in which the dyes were swapped to correct for dye-specific effects). Labelled cDNA samples were hybridized to probes representing 2496 open reading frames of *L. lactis* MG1363 spotted in duplicate as described (11,47). After overnight hybridization, slides were washed for 5 min at 37°C in 2xSSC (Standard Saline Citrate), 0.5% SDS and 5 min in 1xSSC, 0.25% SDS to remove nonspecifically hybridized cDNAs. Slides were scanned using a GeneTac LS IV laser scanner (Genomic Solutions Ltd.). Subsequently, individual spot intensities were determined using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Slide data were processed and normalized using MicroPrep, which yielded average ratios of gene expression with mutant over the wild type strain. Expression of a gene was considered to be significantly altered when the Cyber T baysian p value ≤ 0.001. All DNA microarray data obtained in this study are available at http://molgen.biol.rug.nl/publication/mdr_data/supplementary.html

Protein expression and purification

A 351 bp PCR fragment which contained the complete open reading frame of *ydaF* was amplified from *L. lactis* MG1363 strain genomic DNA using a pair of primers of ydaF forward 5' GGC**CCATGG**GGGCAGAAATACCAAAAGAAATG and reverse GCG**TCTAGA**TTTAATCGCTTCACTCTTCTTAT, which were designed to create Nco I/Xba I digestion sites at the head and tail of the fragment (indicated as underlined and bold letters). The properly digested *ydaF* fragment was inserted into a multiple cloning site of pNSC8048 with a C-terminal strep-tag fused plasmid and transformed into *L. lactis* NZ9000 cells, which is a derivative of strain MG1363 harbouring the *nisR/nisK* genes important for the induction and overexpression of YdaF (LmrR) under the Nisin inducible system yielding pNSC8048-*ydaF*. YdaF was overexpressed by growing NZ9000 cells transformed with pNSC8048-*ydaF* in a rich medium (M17, 0.5% glucose and 5 μg/ml chloroamphenicol) at 30ºC. When cells were in the mid log phase (OD_{660} 0.7-0.8), 5 ng/ml of nisin A was added and growth was continued for one hour. Cells were collected, lysed with 20 μg/ml freshly prepared lysozyme solution, followed by the addition of 10 mM $MgSO_4$ and 100 μg/ml Dnase I (Sigma). Cells disrupted by French Pressure treatment (15400 psi), and remaining debris was removed by low speed centrifugation (14,000 rpm for 15 minutes at 4°C; Sorvall SS34 rotor). YdaF was purified from the supernatant using Streptactin Sepharose column chromatography according to the manufacture's protocol (IBA biotagnology

GmbH). The purified protein was stored at -80°C in elution buffer (100 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA, and 2.5 mM desthiobiotin).

In vitro DNA binding assay

The specific interaction of purified YdaF with a 205bp DNA fragment containing the putative promoter region of *lmrCD* was carried out using the Electrophoresis Mobility Shift Assay (EMSA) as described (12). The putative promoter region was amplified using the PCR primers: 5' ATTGTAATCTTTAACAGCATTAAC (forward) and GGCAACCCATTTATGCTTCA (reverse). The purified PCR product was end labelled with ^{32}P-γ-ATP and T4 Polynucleotide Kinase (New England Biolabs) at 37 °C for 20 minutes, and further purified using the PCR clean up kit (Sigma) to remove the associated proteins. The EMSA assay was performed in a total volume of 20 µl containing 80 mM Tris-HCl (pH 8.0), 4 mM EDTA, 20 mM $MgCl_2$, 2 mM DTT, 400 mM KCl, 40% (v/v) glycerol, the labelled promoter DNA fragment (4000 cpm), BSA (1 µg) and poly(dI-dC) (Roche) to compete for any unspecific binding. Where indicated purified Strep-tagged YdaF (1 µg) and/or an excess of unlabelled promoter DNA fragment was added. After 10 minutes incubation at 30°C, samples were loaded on 6 % polyacrylamide gel, and electrophoresis was performed in TBE buffer at 100 V for 1 hour. Gel were dried and analysed by autoradiography.

Transport measurements

Fluorescent assays of transport were performed essentially as described previously (24).

RESULTS

Transcriptome analysis of multidrug resistant strains of L. lactis

Previously, it has been demonstrated that cells of *L. lactis* challenged with increasing concentrations of toxic drugs develop an MDR phenotype (3,51). This phenomenon is associated with an increased ability to excrete the toxic compounds from the cells. As predicted by Paulsen et al. *L. lactis* IL1403 contains 40 genes that specify putative MDR systems (See Table 1 and www.membranetransport.org).

Table 1. Transcriptome analysis of *L. lactis* MG1363 and four drug resistant mutants.

Gene name[a]	Expression ratio[b]			
	Dau^r	Eth^r	Rho^r	Chol^r
ABC superfamily:				
lmrC (ydaG)	5.1	7.3	4.2	9.3
lmrD (ydbA)	4.3	8.3	7.2	8.0
ynaD, ynaC, lmrA, yhcA, yfcA, yfcB, ypgD, ysdA, ysdB, yjjC, yjjD, ycfB, ycfC, ecsA, ecsB, ycgA, ycfI	-	-	-	-
Major Facilitator Superfamily (MFS):				
yxbD	-	-	3.4	3.5
napC, blt, pmrA, yjdE, pmrB, yddA, yjdA, ypfE, ybfD, ypiB, ydiC, yniG, yfjF, yweA, lmrP, ycdH, yqiA	-	-	-	-
Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily:				
ypbC, yvhA	-	-	-	-

[a] Tabulation of genes that encode putative multidrug resistant (see www.membranetransport.org) transporters belonging to various MDR families (IL1403 nomenclature was used)
[b] Mutant over the wild type, Cyber T baysian *p* value ≤0.001
*only significant changes in expression (with *p* value ≤ 0.001) are shown.
- indicates expression ratio *p* value above 0.001

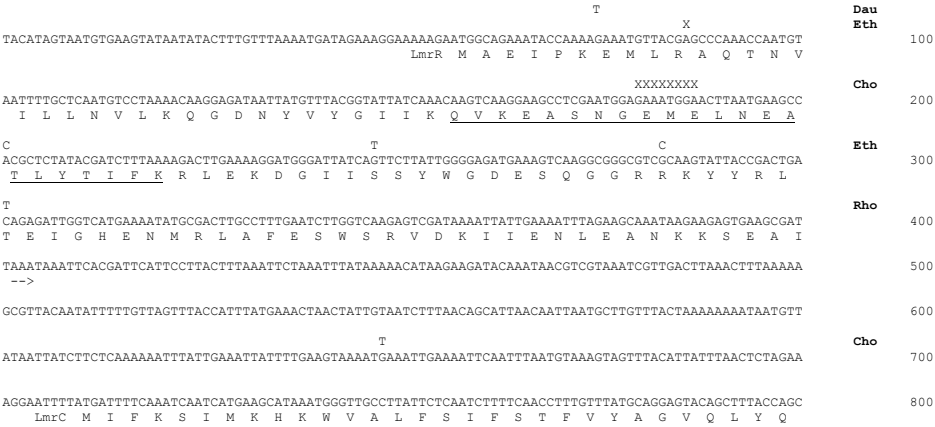
These genes are also present in *L. lactis* MG1363. In our analysis (Table 1), one gene, *ycgB,* was not included because its amplicon was not present on the microarray slides. The ABC (20 genes) and MFS transporters (18 genes) are the most abundant types of drug transporters, while only two genes specify proteins that belong to the Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily of a MATE subtype. So far, only four of these gene products have been functionally characterized and shown to encode MDR transporters, namely the homodimeric ABC half-transporter LmrA (49), the MFS transporter LmrP (4), and LmrCD, a heterodimer ABC half-transporter (24). To identify which system(s) are responsible for the observed MDR phenotype in *L. lactis*, we performed transcriptome analysis on four MDR strains. The MDR strains were

obtained previously by challenging *L. lactis* with increasing concentrations of daunomycin, ethidium, rhodamine 6G (3) or cholate (51). Their MDR phenotype is a stable attribute, and is maintained even when cells are grown in the absence of added drugs (data not shown). Therefore, in our analysis, cells of the wild type strains and derived drug-resistant mutants were grown in GM17 medium in the absence of added drugs. Table 1 shows the changes in the expression levels of the genes of all putative MDR systems of *L. lactis* MG1363 in the various drug resistant strains relative to the parental wild type strain. Strikingly, only the *lmrCD* genes are significantly (Cyber T baysian *p* value ≤ 0.001) and strongly (6.7 fold on average) up-regulated in all four drug resistant strains whereas the expression levels of *lmrA*, *lmrP* and the genes of the other putative MDR transporters were not significantly affected. Only *yxbD,* a gene encoding a putative MFS MDR transporter, was significantly up-regulated in the cholate and rhodamine resistant strains. These data suggest that the elevated expression of *lmrCD* is the major cause of the acquired MDR phenotype of the drug-challenged *L. lactis* strains.

The lmrR (ydaF) *gene encodes a negative transcriptional regulator of the* lmrCD *genes*

The microarray analysis demonstrated that an additional gene, *lmrR (ydaF)*, which is located next to the *lmrCD* genes, is significantly and strongly (9.4 fold on average) up-regulated in all four drug resistant strains of *L. lactis*. LmrR (Lactococcal multidrug resistance Regulation) is a protein, which, based on amino acid homology can be annotated as putative transcriptional regulator that belongs to the PadR family (pfam03551). The high level of up-regulation (see supplementary data) of *ydaF* in the MDR mutants of *L. lactis* suggests an involvement of this protein in the regulation of the adjacent *lmrCD* genes. Thus, the upstream region of *lmrCD,* including the *lmrCD* promoter area and *lmrR* of all four mutants were sequenced and compared to the wild type. Fig. 1 shows the DNA sequences indicate that in the Dau^r, Eth^r, Cho^r mutants full length LmrR is not produced. The *lmrR* gene in Dau^r strain contains a point mutation that changes the glutamate codon at position 7 into a stop codon, while in the Eth^r and Cho^r strains out of frame deletion early in the gene prevents the expression of the full length protein. Interestingly, *lmrR* in the Rho^r strain contains only a single point mutation that changes a threonine residue at position 82 in LmrR to an isoleucine. This mutation took place in a conserved part of the proteins of the PadR family but is clearly outside of the Helix-Turn-Helix motif (underlined in Fig.1), which suggests that Thr-82 is important for LmrR regulatory activity.

A



B

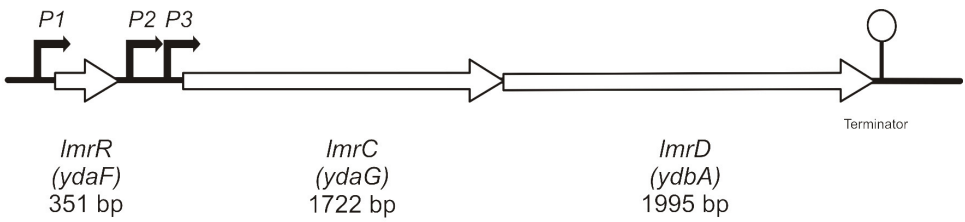


Figure 1. (A) Sequence of the upstream region of *lmrCD* including the *lmrR (ydaF)* gene in *L. lactis* MG1363. Above the sequence shown are mutations in the upstream regions of the same region in the *L. lactis* MDR mutants (X stands for deletion of one nucleotide). Specified in the right margin: (Dau) daunomycin, (Eth) ethidium bromide, (Cho) cholate and (Rho) Rhodamine 6G resistant strains, respectively. The underlined amino acid residues represent a putative Helix-Turn-Helix DNA-binding motif (as predicted by the program available at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hth.html). **(B)** Schematic representation of localization of *lmrRCD* operon in genome of *L. lactis* MG1363 with indicated putative promoters of *lmrR* (P1) and *lmrCD* (P2, P3) and the terminator.

A lack of full LmrR protein production in 3 out of 4 MDR mutants of *L. lactis* and the fact that all four strains show highly elevated levels of mRNA of the three genes *lmrC*, *lmrD* and *lmrR* leads us to propose that LmrR is a repressor that regulates the transcription of *lmrCD* while it autoregulates the expression of *lmrR*. The lack of functional LmrR would relieve the repression of both the *lmrCD* and

lmrR genes. The point mutation in LmrR in the Rho^r strain most probably affects the functionality of the protein.

To investigate the function of LmrR, the protein was overexpressed in *L. lactis* with a C-terminal Strep-tag and purified to homogeneity with a streptactin column. Purified LmrR was subjected to an electrophoretic mobility shift (EMSA) assay with a radiolabelled DNA fragment corresponding to a 205 bp upstream region of *lmrC* which comprises the putative *lmrCD* promoter. Addition of LmrR resulted a major shift in the mobility of the radiolabelled DNA fragment while no shift was observed when the assay was performed in the presence of an excess of unlabelled DNA fragment (Fig. 2). This result indicates that LmrR (YdaF) binds specifically to the promoter region of *lmrCD* which is consistent with its predicted function as a transcriptional regulator.

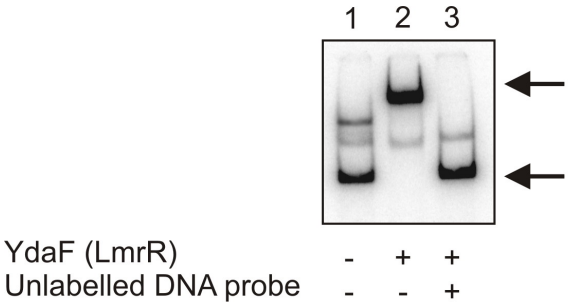


Figure 2. Gel retardation assay using the purified YdaF and the promoter region of *lmrCD*. A ^{32}P-labelled DNA fragment corresponds to a 205 bp upstream region of *lmrC* comprising the putative *lmrCD* promoter region was incubated in the absence (lane 1) and presence of purified YdaF (lane 2), and in the presence of both purified YdaF and an excess unlabelled promoter DNA fragment (lane 3). Arrows indicate the retarded and non-retarded bands.

Other changes in gene expression patterns of L. lactis *MDR mutants*

The whole transcriptome data is provided in the supplementary materials. Among the other genes that show significant (Cyber T baysian p value ≤ 0.001) deviations in expression in the MDR mutants relative to the wild type, three genes showed significant changes in 3 of the mutants, 43 genes showed different expression levels in 2 of the mutants and 214 genes showed change in only one of the mutants. Among those genes sets of genes that are organized in operon-like structures where upregulated, such as the genes involved in purin ribonucleotide biosynthesis, a cholin ABC transport system, an oligopeptide ABC transport system, a phosphate

ABC transport system, a cytochrome d ABC transport system, genes involved in riboflavin biosynthesis, lipoprotein precursors, and a number of genes involved in amino acid and amine metabolism (7). These genes were not studied further because none of them specifies a (putative) MDR transporter, nor were they up-regulated in all four drug resistant *L. lactis* strains, suggesting they are indirectly affected, e.g. as a result of various stress responses

The L. lactis *Δ*lmrCD *is hypersensitive to drugs*

The two ABC half-transporters LmrC and LmrD form a stable heterodimeric complex that functions as an MDR transporter (24). In order to further investigate the physiological role of LmrCD in *L. lactis*, a large internal deletion of both contiguous genes *lmrC* and *lmrD* was created using the method described by Leenhouts et al (21) for marker-free gene replacement. Briefly, plasmid pORIGAMGKO, which contains a truncated version of *lmrCD,* with a large 2.1 kb deletion, was used to replace wild-type *lmrCD* by homologous recombination. Southern blot hybridization confirmed the deletion (Fig. 3).

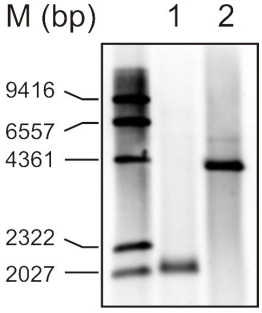


Figure 3. Southern hybridization analysis of chromosomal DNAs of *L. lactis.* Genomic DNAs of *L. lactis* NZ9000 Δ*lmrCD* (1) and *L. lactis* NZ9000 (2) were digested with XbaI and SalI, and analyzed for the presence of *lmrCD* using an *lmrCD*-selective probe prepared with the PCR DIG labelling mix^{plus} and the primers specified in the Experimental procedure section. Lane M represents the molecular weight DIG-labelled marker II.

L. lactis NZ9000 Δ*lmrCD* and the parental strain *L. lactis* NZ9000 were grown in the presence of several toxic drugs and the half-maximal growth inhibitory drug concentration (IC_{50}) was determined. The Δ*lmrCD* strain was found to be significantly more sensitive to a series of MDR substrates, i.e., Hoechst 33342, Daunomycin, Rhodamine 6G, and Cholate (Table 2) that were used to obtain the drug resistant variants of *L. lactis*. On the other hand, the strain shows an unaltered sensitivity to a series of antibiotics. The observed drug susceptibility of *L. lactis* NZ9000 Δ*lmrCD* could be completely rescued by the introduction of a low copy number plasmid bearing the *lmrCD* genes under control of their endogenous

promoter (Fig. 4, Hoechst 33342 and Daunomycin) or the nisin-inducible promoter *PnisA* (Fig. 5).

Table 2. Susceptibility of *L. lactis* NZ9000 parental and Δ*lmrCD* strain for toxic compounds.

Drug	IC_{50} (μM)[a]		Relative resistance wild-type/ Δ*lmrCD*[b]
	wild type	Δ*lmrCD*	
Hoechst	5.55	1.55	3.6
Daunomycin	55	4.5	12.2
Cholate	5000	2500	2
Rhodamine 6G	6.9	4.1	1.7
Ethidium	4.8	3.7	1.3
Quinine	2.5	2.5	1
Tetracycline	0.124	0.124	1
Erythromycine	0.079	0.074	1
Kanamycin	57.9	54.8	1
Chloramphenicol	3.50	3.50	1
Puromycin	24.2	24.2	1

[a]Results shown for two independent experiments.

However, cells were not rescued by introduction of a plasmid encoding the LmrCD complex with a mutation in the putative catalytic base, E587Q, of LmrD which is essential for ATP hydrolysis and functionality (Fig. 5) (25). When LmrCD was expressed with the corresponding mutation in the putative catalytic base of LmrC, D495N, which only partially interferes with the ATPase and drug extrusion activity (25), the drug resistant phenotype of *L. lactis* NZ9000 Δ*lmrCD* was partially rescued. These data demonstrate that LmrCD is a major determinant of the intrinsic drug resistance of *L. lactis*.

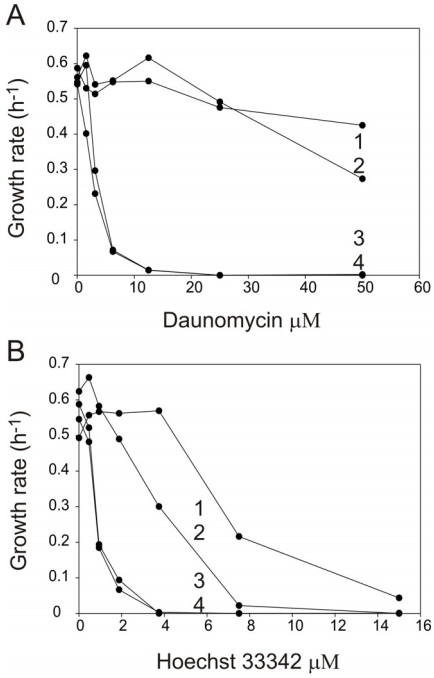


Figure 4. Sensitivity of *L. lactis* strain NZ9000 and Δ*lmrCD* to daunomycin and Hoechst 33342. Cells of *L. lactis* NZ9000 Δ*lmrCD* harboring pILLmrCD (1), *L. lactis* NZ9000 (2), *L. lactis* NZ9000 Δ*lmrCD* harboring the control plasmid pIL252 (3) and *L. lactis* NZ9000 Δ*lmrCD* (4) were grown in GM17 medium in the presence of increasing concentrations of daunomycin (Panel A) or Hoechst 33342 (Panel B). The growth rates are plotted as a function of the drug concentration.

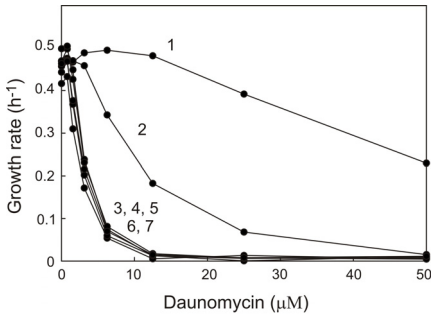


Figure 5. Expression of LmrCD restores the daunomycin sensitive phenotype of *L. lactis* NZ9000 Δ*lmrCD*. Cells of *L. lactis* NZ9000 Δ*lmrCD* harboring pNS8048E vector bearing the wild type LmrCD (1), LmrC(D495N)/LmrD (2), LmrC(D495N) (3), LmrD(E587Q) (4); LmrC/LmrD(E587Q) (5), LmrC(D495N)/LmrD(E587Q) (6) or no insert (7) were grown in GM17 medium supplemented with 5 μg/ml chloramphenicol, and increasing concentrations of daunomycin. The growth rates are plotted as a function of the drug concentration.

The L. lactis *Δ*lmrCD *shows an increased accumulation of toxic compounds*

Overexpression of LmrCD in wild type *L. lactis* results in a decreased accumulation of several drugs (24). Since the deletion of *lmrCD* renders cells hypersensitive to a range of drugs, while the drug sensitivity can be restored by expression of *lmrCD*, drug export assays were performed with *L. lactis* NZ9000 Δ*lmrCD* cells. The accumulation of the DNA binding dye Hoechst 33342 and the pH-sensitive fluorophore BCECF was monitored in the parental and the Δ*lmrCD* strain. Hoechst 33342 is a membrane permeable substrate of LmrCD (24) that is essentially non-fluorescent in an aqueous environment, whereas it becomes fluorescent upon membrane- and DNA-binding.

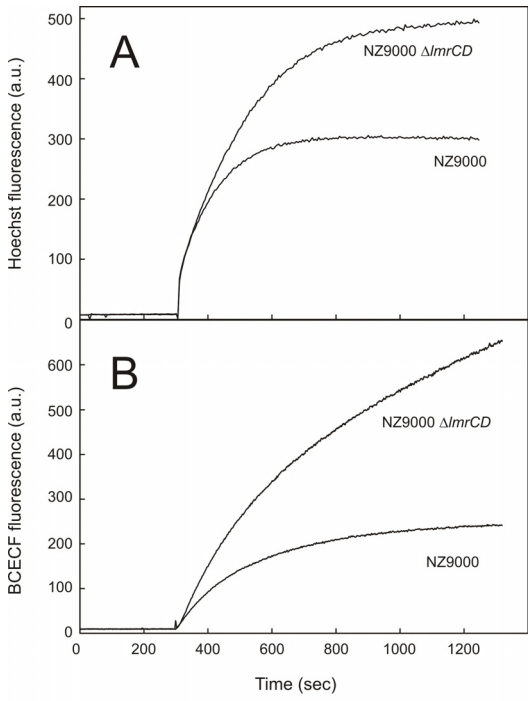


Figure 6. *L. lactis* NZ9000 Δ*lmrCD* exhibits an increased level of drug accumulation. The influx of Hoechst 33342 (A) and BCECF-AM (B) into *L. lactis* NZ9000 and *L. lactis* NZ9000 Δ*lmrCD* was followed fluorometrically in time. Cells were energized with glucose after approximately 5 min.

This feature of Hoechst 33342 allows a rapid monitoring of its intracellular accumulation. BCECF-AM is another substrate of LmrCD (24) that can permeate the cell. Upon entry, an intracellular esterase cleaves the non-fluorescent BCECF-AM ester to liberate the membrane- impermeable, fluorescent acid. Since the fluorescence of BCECF depends on the pH, the presence of a pH difference across the membrane was prevented by the inclusion of the ionophores nigericin and valinomycin to the reaction buffer. As shown in Fig. 6, both Hoechst and BCECF are accumulated more rapidly and to higher levels in glucose energized NZ9000 Δ*lmrCD* cells as compared to the parental strain. The increased drug accumulation in *L. lactis* NZ9000 Δ*lmrCD* cells could be reversed by the expression of *lmrCD*, demonstrating that LmrCD is responsible for efflux of these toxic compounds.

DISCUSSION

In order to determine which gene(s) are responsible for the acquired MDR phenotype of a set of multidrug resistant strains of *L. lactis* that were individually selected to grow in the presence of increasing concentrations of rhodamine, ethidium, daunomycine or cholate (3), we have performed a combined transcriptome/gene disruption analysis. To our surprise, the transcriptome analyses of the four MDR strains revealed only the significant up-regulation of the *lmrCD* genes within this large set of putative MDR transporter genes. *The lmrCD* genes encode two ABC half-transporters that have previously been identified to form a heterodimeric active MDR pump (24). Sequence analysis of the *lmrCD* genes from the MDR strains did not reveal any mutations that could further add to the drug resistance phenotype (van Merkerk, Lubelski and Driessen, unpublished). However, the transcriptome analyses showed that another gene, *lmrR* (*ydaF*), was found to be consistently up-regulated in all of these strains. The gene *lmrR* is located immediately upstream of the *lmrCD* genes and encodes a putative transcriptional regulator of the PadR family. Sequence analysis of *lmrR* in all four strains suggests that the molecular basis of the elevated expression of *lmrCD* in the MDR mutant strains lies in the mutational inactivation of LmrR. Furthermore, the specific binding of the purified LmrR to a 205 bp upstream region of the *lmrC* gene comprising the putative promoter region of *lmrCD* further suggests that LmrR is involved in controlling the expression of *lmrCD*. Based on our transcriptome data, LmrR appears to be a negative regulator of *lmrCD* expression but also of its own gene. Future studies should more specifically address the mechanism of regulation of drug resistance in *L. lactis* and reveal the sensing mechanism involved. Another putative drug transporter, YxbD, is up-regulated only in the Rho^r and Cho^r strains.

YxbD belongs to the MFS of transporters and is homologous to quinolone resistance protein NorA from *Bacillus thuringiensis* (25 % identical amino acids). Since this protein was not up-regulated in all four MDR strains it was not studied further. The data presented in this study demonstrate that LmrCD is a major drug resistance pump involved in acquired drug resistance in *L. lactis* rather than LmrA and LmrP which have previously been characterized in great detail. It should be stressed that our current analysis only involved drug resistant strains that were challenged with four small molecule toxic compounds. It is well possible that other MDR transporters are up-regulated when *L. lactis* is selected to grow on drugs and/or antibiotics different from the ones tested here.

Our data show that deletion of *lmrCD* renders *L. lactis* sensitive to daunomycin, hoechst, cholate and rhodamine 6G, and that the increased susceptibility is due to a reduced capacity to secrete these compounds from the cell. This observation demonstrates that LmrCD is also a major determinant of the intrinsic drug resistance in *L. lactis*. Our analysis is one of the few reports showing a distinct multidrug sensitive phenotype upon deletion of a bacterial ABC-type MDR system. Phenotypic effects of the disruption of well-characterized ABC-type MDR transporters, such as LmrA from *L. lactis* (10) or BmrA from *B. subtilis* (44) are only minor, while most studies in other bacteria show effects only with proton motive force-dependent MDR transporters. A gene disruption approach to unravel the contribution of all 34 potential MDR pumps of the Gram-positive bacterium *Enterococcus faecalis* identified several genes as being involved in resistance to known antimicrobials (10). Interestingly, 4 of the genes identified in that study were ABC transporters.

The drug sensitive phenotype of *L. lactis* NZ9000 Δ*lmrCD* can be restored by providing *lmrCD in trans* on a low copy number plasmid, and not by LmrCD mutants defective in ATPase and transport activity (46). To determine whether the drug sensitive phenotype of *L. lactis* NZ9000 Δ*lmrCD* can be reversed by the expression of other, possibly cryptic, MDR transporters, cells were re-selected for growth in the presence of increasing concentrations of daunomycin by the method that was described previously by Bolhuis *et al* (3). These attempts did not result in any appreciable increase in the drug resistance of *L. lactis* NZ9000 Δ*lmrCD* (unpublished results). This strongly indicates that LmrCD is a key determinant in drug resistance in *L. lactis* and suggests that ABC MDR transporters play a more prominent role in drug resistance of bacteria than previously anticipated.

Other genes up-regulated in at least one MDR mutant of *L. lactis* could not be directly linked with the MDR phenotype(s) and the observed changes in their expression patterns may be secondary and related to stress imposed by the drug used for the challenge. Of special interest is the up-regulation of genes (*pst*)

encoding the phosphate-specific transporter in the Dau^r strain. Pst was reported previously to be associated with a multidrug resistance phenotype in *Mycobacterium smegmatis* (1,2) However, up-regulation of *pst* genes may also be of secondary origin, possibly as a response for higher phosphate requirement of Dau^r strain as a result of higher energy requirement.

Because of the stable phenotype of *L. lactis* NZ9000 Δ*lmrCD* it will be particularly suitable to screen for MDR transporters from other Gram-positive bacteria. Further strain engineering, as demonstrated for MDR deletion strains of *Saccharomyces cereviseae* (38), may lead to hypersensitivity to a greater number of noxious agents, but this will first require the identification of other systems that contribute to drug resistance in *L. lactis*. In this respect, a triple knockout strain lacking *lmrA* and *lmrC* and *lmrD* exhibits the same drug susceptibility as that of the Δ*lmrCD* strain to: Hoechst 33342, ethidium, daunomycin and cholate (unpublished results, 46). A hypersensitive *L. lactis* strain could be a sensitive indicator for the genome-wide screening of novel MDR determinants involved in resistance development against novel antimicrobials. Currently, for heterologous screening, expression and characterization of bacterial MDR systems, drug-hypersensitive *E. coli* strains are used as hosts (16,19,20). Because of the unpredictability of stable expression, *E. coli* seems less suited for screening of MDR transporters of Gram-positive organisms including many human pathogens such as *Ent. faecalis, S. pneumoniae, S. pyogenes* and *Listeria monocytogenes*. These bacteria all contain homologs of LmrCD, with up to 50-60% identity and, remarkably, these homologs exhibit similar genetic organizations. A recent analysis of predicted MDR transporters in *S. pneumoniae* (37) indeed revealed that the LmrCD homologs SP2073 and SP2075 encode an MDR transporter. Finally, a hypersensitive *L. lactis* strain would also be beneficial for developing cell-based screening systems for novel antimicrobials against Gram-positive organisms.

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REFERENCES

1. Banerjee,S.K., Misra,P., Bhatt,K., Mande,S.C., and Chakraborti,P.K. 1998. Identification of an ABC transporter gene that exhibits mRNA level overexpression in fluoroquinolone-resistant *Mycobacterium smegmatis*. *FEBS Lett* 425: 151-156.
2. Bhatt,K., Banerjee,S.K., and Chakraborti,P.K. 2000. Evidence that phosphate specific transporter is amplified in a fluoroquinolone resistant *Mycobacterium smegmatis*. *Eur J Biochem* 267: 4028-4032.
3. Bolhuis,H., Molenaar,D., Poelarends,G., van Veen,H.W., Poolman,B., Driessen,A.J.M., and Konings,W.N. 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. *J Bacteriol* 176: 6957-6964.
4. Bolhuis,H., Poelarends,G., van Veen,H.W., Poolman,B., Driessen,A.J.M., and Konings,W.N. 1995. The Lactococcal *lmrP* gene encodes a proton motive force-dependent drug transporter. *J Biol Chem* 270: 26092-26098.
6. Bolhuis,H., van Veen,H.W., Poolman,B., Driessen,A.J.M., and Konings,W.N. 1997. Mechanisms of multidrug transporters. *FEMS Microbiol Rev* 21: 55-84.
7. Bolotin,A., Wincker,P., Mauger,S., Jaillon,O., Malarme,K., Weissenbach, S. D. Ehrlich., and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res* 11: 731-753.
8. Borges-Walmsley,M.I., McKeegan,K.S., and Walmsley,A.R. 2003. Structure and function of efflux pumps that confer resistance to drugs. *Biochem J* 376: 313-338.
9. Borges-Walmsley,M.I., and Walmsley,A.R. 2001. The structure and function of drug pumps. *Trends Microbiol* 9: 71-79.
10. Davis,D.R., McAlpine,J.B., Pazoles,C.J., Talbot,M.K., Alder,E.A., White,C. *et al.* 2001. *Enterococcus faecalis* multi-drug resistance transporters: application for antibiotic discovery. *J Mol Microbiol Biotechnol* 3: 179-184.
11. den Hengst,C.D., van Hijum,S.A., Geurts,J.M., Nauta,A., Kok,J., and Kuipers,O.P. 2005. The Lactococcus lactis CodY regulon: identification of a conserved cis-regulatory element. *J Biol Chem* 280: 34332-34342.
12. Ebbole,D.J., and Zalkin,H. 1988. Detection of pur operon-attenuated mRNA and accumulated degradation intermediates in Bacillus subtilis. *J Biol Chem* 263: 10894-10902.
13. Gajic,O., Buist,G., Kojic,M., Topisirovic,L., Kuipers,O.P., and Kok,J. 2003. Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal multidrug resistance proteins. *J Biol Chem* 278: 34291-34298.
14. George,A.M. 1996. Multidrug resistance in enteric and other gram-negative bacteria. *FEMS Microbiol Lett* 139: 1-10.
15. Gottesman,M.M., Fojo,T., and Bates,S.E. 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2: 48-58.
16. Huda,N., Lee,E.W., Chen,J., Morita,Y., Kuroda,T., Mizushima,T., and Tsuchiya,T. 2003. Molecular cloning and characterization of an ABC multidrug efflux pump, VcaM, in Non-O1 *Vibrio cholerae*. *Antimicrob Agents Chemother* 47: 2413-2417.
17. Lage,H. 2003. ABC-transporters: implications on drug resistance from microorganisms to human cancers. *Int J Antimicrob Agents* 22: 188-199.
18. Law,J., Buist,G., Haandrikman,A., Kok,J., Venema,G., and Leenhouts,K. 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J Bacteriol* 177: 7011-7018.

19. Lee,E.W., Chen,J., Huda,M.N., Kuroda,T., Mizushima,T., and Tsuchiya,T. 2003a. Functional cloning and expression of emeA, and characterization of EmeA, a multidrug efflux pump from *Enterococcus faecalis*. *Biol Pharm Bull* 26: 266-270.
20. Lee,E.W., Huda,M.N., Kuroda,T., Mizushima,T., and Tsuchiya,T. 2003b. EfrAB, an ABC multidrug efflux pump in *Enterococcus faecalis*. *Antimicrob Agents Chemother* 47: 3733-3738.
21. Leenhouts,K., Buist,G., Bolhuis,A., ten Berge,A., Kiel,J., Mierau, M. Dabrowska, G. Venema, and J. Kok. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet* 253: 217-224.
22. Li,X.Z., and Nikaido,H. 2004. Efflux-mediated drug resistance in bacteria. *Drugs* 64: 159-204.
23. Litman,T., Druley,T.E., Stein,W.D., and Bates,S.E. 2001. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* 58: 931-959.
24. Lubelski,J., Mazurkiewicz,P., van Merkerk R., Konings,W.N., and Driessen,A.J.M. 2004. *ydaG* and *ydbA* of *Lactococcus lactis* encode a heterodimeric ATP-binding cassette-type multidrug transporter. *J Biol Chem* 279: 34449-34455.
25. Lubelski,J., van Merkerk,R., Konings,W.N., and Driessen,A.J.M. 2006. Nucleotide-binding sites of the heterodimeric LmrCD ABC-Multidrug transporter of *Lactococcus lactis* are asymmetric. *Biochemistry* 45: 648-656.
26. Markham,P.N., and Neyfakh,A.A. 2001. Efflux-mediated drug resistance in Gram-positive bacteria. *Curr Opin Microbiol* 4: 509-514.
27. McMurry,L., Petrucci,R.E., Jr., and Levy,S.B. 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli. *Proc Natl Acad Sci U S A* 77: 3974-3977.
28. Nikaido,H. 1996. Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 178: 5853-5859.
29. Ouellette,M., Legare,D., and Papadopoulou,B. 2001. Multidrug resistance and ABC transporters in parasitic protozoa. *J Mol Microbiol Biotechnol* 3: 201-206.
30. Paulsen,I.T. 2003. Multidrug efflux pumps and resistance: regulation and evolution. *Curr Opin Microbiol* 6: 446-451.
31. Paulsen,I.T., Chen,J., Nelson,K.E., and Saier,M.H., Jr. 2001. Comparative genomics of microbial drug efflux systems. *J Mol Microbiol Biotechnol* 3: 145-150.
32. Paulsen,I.T., Nguyen,L., Sliwinski,M.K., Rabus,R., and Saier,M.H., Jr. 2000. Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. *J Mol Biol* 301: 75-100.
33. Paulsen,I.T., Sliwinski,M.K., and Saier,M.H., Jr. 1998. Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. *J Mol Biol* 277: 573-592.
34. Poole,K. 2004. Efflux-mediated multiresistance in Gram-negative bacteria. *Clin Microbiol Infect* 10: 12-26.
35. Putman,M., van Veen,H.W., and Konings,W.N. 2000. Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* 64: 672-693.
36. Ren,Q., Kang,K.H., and Paulsen,I.T. 2004. TransportDB: a relational database of cellular membrane transport systems. *Nucleic Acids Res* 32: D284-D288.
37. Robertson,G.T., Doyle,T.B., and Lynch,A.S. 2005. Use of an efflux-deficient *Streptococcus pneumoniae* strain panel to identify ABC-class multidrug transporters involved in intrinsic resistance to antimicrobial agents. *Antimicrob Agents Chemother* 49: 4781-4783.

38. Rogers,B., Decottignies,A., Kolaczkowski,M., Carvajal,E., Balzi,E., and Goffeau,A. 2001. The pleitropic drug ABC transporters from *Saccharomyces cerevisiae*. *J Mol Microbiol Biotechnol* 3: 207-214.
39. Saier,M.H., Jr., and Paulsen,I.T. 2001. Phylogeny of multidrug transporters. *Semin Cell Dev Biol* 12: 205-213.
40. Saier,M.H., Jr., Paulsen,I.T., Sliwinski,M.K., Pao,S.S., Skurray,R.A., and Nikaido,H. 1998. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. *FASEB J* 12: 265-274.
41. Sakamoto,K., Margolles,A., van Veen,H.W., and Konings,W.N. 2001. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA. *J Bacteriol* 183: 5371-5375.
42. Sambrook,J., Fritsh,E., and Maniatis,T. 1989. Molecular Cloning: A laboratory manual, 2nd Ed.
43. Schinkel,A.H., and Jonker,J.W. 2003. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* 55: 3-29.
44. Steinfels,E., Orelle,C., Fantino,J.R., Dalmas,O., Rigaud,J.L., Denizot,A. Di Pietro, and J. M. Jault. 2004. Characterization of YvcC (BmrA), a multidrug ABC transporter constitutively expressed in *Bacillus subtilis*. *Biochemistry* 43: 7491-7502.
45. van Bambeke F., Balzi,E., and Tulkens,P.M. 2000. Antibiotic efflux pumps. *Biochem Pharmacol* 60: 457-470.
46. van den Berg van Saparoea,H., Lubelski,J., van Merkerk,R., Mazurkiewicz,P.S., and Driessen,A.J.M. 2005. Proton motive force-dependent Hoechst 33342 transport by the ABC transporter LmrA of *Lactococcus lactis*. *Biochemistry* 44: 16931-16938.
47. van Hijum,S.A., de,J.A., Baerends,R.J., Karsens,H.A., Kramer,N.E., Larsen, C. D. den Hengst, C. J. Albers, J. Kok, and O. P. Kuipers. 2005. A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. *BMC Genomics* 6: 77.
48. van Veen,H.W., Putman,M., Margolles,A., Sakamoto,K., and Konings,W.N. 2000. Molecular pharmacological characterization of two multidrug transporters in Lactococcus lactis. *Pharmacol Ther* 85: 245-249.
49. van Veen,H.W., Venema,K., Bolhuis,H., Oussenko,I., Kok,J., Poolman,B., A. J. M. Driessen, and W. N. Konings. 1996. Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. *Proc Natl Acad Sci U S A* 93: 10668-10672.
50. Webber,M.A., and Piddock,L.J. 2003. The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* 51: 9-11.
51. Yokota,A., Veenstra,M., Kurdi,P., van Veen,H.W., and Konings,W.N. 2000. Cholate resistance in *Lactococcus lactis* is mediated by an ATP-dependent multispecific organic anion transporter. *J Bacteriol* 182: 5196-5201.

Chapter

3

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

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SUMMARY

LmrCD is an ABC-type multidrug transporter of *Lactococcus lactis*. LmrR encodes a putative transcriptional regulator. In a Δ*lmrR* 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.

Table 1. List of primers used

Name	Primer sequence (5' to 3'); endonuclease sites underlined	endonuclease
lmrR FW1	GGC**CCATGG**GGGCAGAAATACCAAAAGAAATG	NcoI
lmrR RV1	GCG**TCTAGA**TTTAATCGCTTTCACTCTTCTTAT	XbaI
lmrR FW2	TAT**AGATCT**GCAATTCGAAGTCCAATTAAG	BglII
lmrR RV2	TAT**GGATCC**GTAAGTTGCTTCACGAACGTC	BamHI
lmrR FW3	TAAATTCACGATTCATTCCTTACTT	-
lmrR RV3	TCTTTTTCCTTTCTATCATTTTAAACA	-
*lmrCD*pmtr FW1	ATTGTAATCTTTAACAGCATTAAC	-
*lmrCD*pmtr FW2	ACAAATAACGTCGTAAATCG	-
*lmrCD*pmtr RV1	GGCAACCCATTTATGCTTCA	-
*lmrR*pmtr FW1	TGTCGCAAACGCAATTTGTC	-
*lmrR*pmtr FW2	TCAAGGAAAGTTGTCTTCCACCGCTAA	-
*lmrR*pmtr RV1	CTGCCATTCTTTTTCCTTTC	-
*lmrR*pmtr RV2	GGGCTCGTAACATTTCTTTTGGTATTTCTG	-
lmrC RT-PCR FW	GTTGAAGAACGTGGGAATAATTTCTCAGGTGG	-
lmrC RT-PCR RV	CCTCCTGTGCTTTCTGTGTATCGTAGATTTC	-
lmrD RT-PCR FW	CGTTTCTGATGATGAATCAGTCTTCTCAGTTGG	-
lmrD RT-PCR RV	CAAAAACGAATTGATTATGATAAAGTTCAGAG	-
lmrR RT-PCR FW	ATGGCAGAAATACCAAAAGAAATG	-
lmrR RT-PCR RV	TTATTTAATCGCTTCACTCTTCTTAT	-
secY RT-PCR FW	TACAACTGCTCCAGCTACGA	-
secY RT-PCR RV	GTTCCTCCAAGAGCGACAAT	-

Since the previously characterized MDR strains of *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 *lmrCD* expression, the *lmrR* gene was deleted by chromosomal replacement (15,16). A PCR fragment containing the complete *lmrR* gene and the flanking regions was amplified from genomic DNA of *L. lactis* NZ9000 (7) using the primer pair *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 *lmrR* gene was removed from this plasmid by a PCR method using phosphorylated primers facing back to back i.e., *lmrR* FW3/RV3 (Table 1). The obtained linear PCR product was self-ligated resulting in pORIYdaFDel that was introduced into *L. 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 μg/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. lactis* NZ9000 parental and Δ*lmrR* cells were grown at 30ºC in GM17, harvested at an OD_{660} of 1 (late log phase) and their transcriptomes were compared by DNA microarray analysis (8,30).

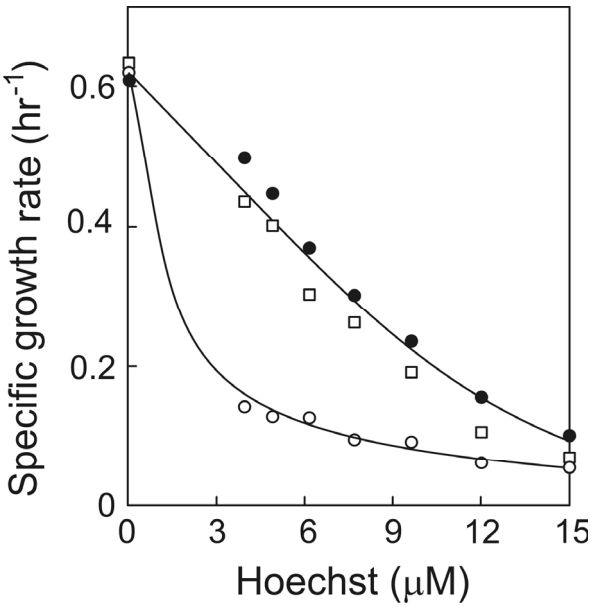

Figure 1. **Sensitivity of *L. lactis* NZ9000, Δ*lmrR* and Δ*lmrCD* to Hoechst 33342**. Cells of *L. lactis* NZ9000 (●), Δ*lmrCD* (○) and Δ*lmrR* (□) were grown in GM17 medium in the presence of increasing concentrations of Hoechst 33342. The specific growth rates are plotted as a function of the drug concentration.

Expression of a gene was considered to be significantly altered when the Cyber T baysian *p* value ≤1E-05. All transcriptome data discussed here have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) 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 Δ*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 Δ*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 Δ*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 NcoI/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-$lmrR^{Rho}$. *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 gelfiltration (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-^{32}P]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 K_d 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.

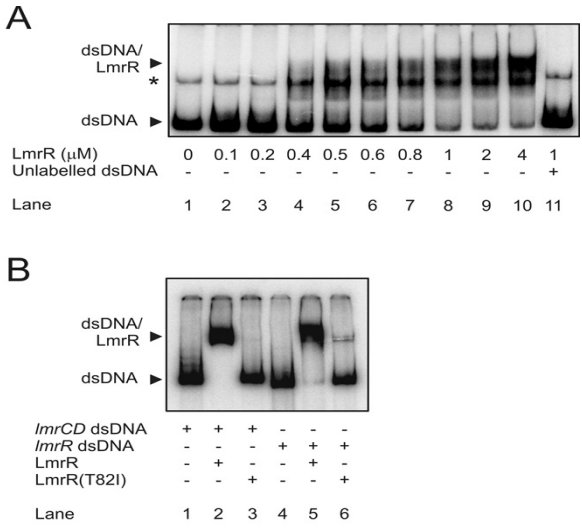

Figure 2. Interaction of LmrR with the *lmrCD* and *lmrR* promoter regions. EMSA was performed with increasing amounts of purified LmrR incubated with 0.7 μM of [^{32}P]-labeled 370 bp and 387 bp dsDNA probes comprising the promoter regions of **(A)** *lmrCD* and **(B)** *lmrR*, respectively. Where indicated, 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.

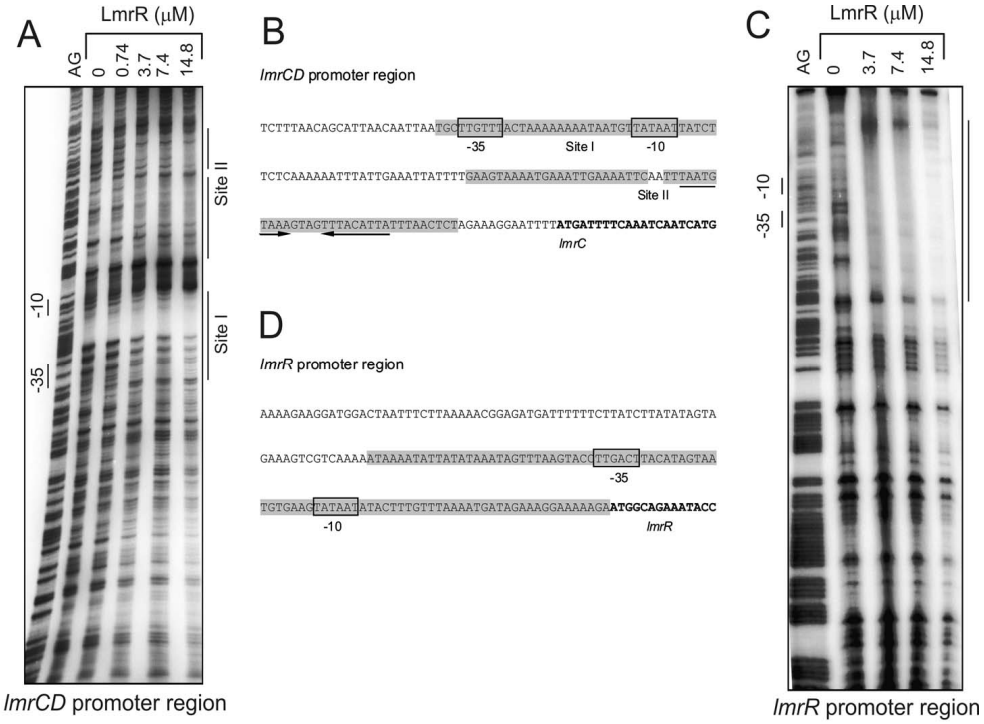

Figure 3. DNase I protection of the *lmrCD* and *lmrR* promotor regions by LmrR. Site-specificity of binding of LmrR to the *lmrCD* **(A, B)** and *lmrR* **(B, D)** promoter regions. The DNase I digested promoter fragments (**A,C**) are flanked by the Maxam-Gilbert ladder on the left (AG). Poly(dI-dC) was present to suppress unspecific binding. Nucleotide sequences of the *lmrC* (**B**) and *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 *lmrCD* and *lmrR* promoters, DNase I protection assays were performed (14). DNA fragments containing the promoter regions of *lmrCD* and *lmrR* were amplified by PCR using the primer sets *lmrCD*pmtr FW2/RV1 and *lmrR*pmtr 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 *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).

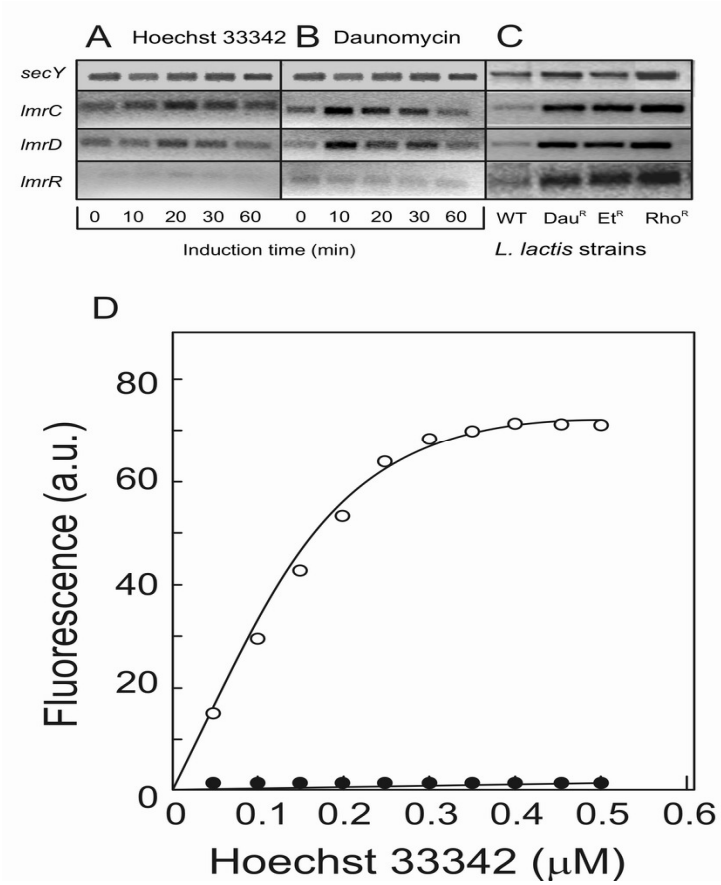

Figure 4. Expression of *lmrC*, *lmrD* and *lmrR* in *L. lactis* MG1363 and MDR strains and binding of Hoechst 33342 to LmrR. The expression of *lmrC*, *lmrD* and *lmrR* and the control gene *secY* was measured by RT-PCR using specific primer pairs and total RNA isolated from the parental *L. lactis* MG1363 incubated in the presence of Hoechst 33342 (5 μM) **(A)** or Daunomycin (50 μM) **(B)** and from the drug resistant derivatives Dau^r, Eth^r, and Rho^r 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 *lmrCD* and *lmrR* genes was further investigated by RT-PCR using the primer sets listed in Table 1. Transcript levels were followed upon a challenge of *L. lactis* MG1363 cells with the chemically unrelated drugs Daunomycin (50 μM) (Fig. 4A) and Hoechst 33342 (5 μM) (Fig. 4B), both substrates of LmrCD (20). The expression of *lmrC* and *lmrD* transiently increased up to 2-fold within 10 minutes. Unlike in the MDR strains (Fig. 4C), no detectable change in *lmrR* expression was detected in the drug challenged cells (Fig. 4AB). This shows that the *lmrCD* genes are up-regulated in response to the challenge with toxic drugs, and suggest that the auto-regulatory mechanism of *lmrR* differs, at least in timing, from that of the structural genes *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 μg/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 ~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 *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, *L. lactis* NZ9000, Δ*lmrCD* and Δ*lmrR* 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 *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 *lmrCD* promoter region is weakened, allowing the RNA polymerase to initiate transcription. This results in a de-repression of the *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 *lmrCD* and prevent further expression. The phenotype of the MDR strains of *L. lactis* can now be partially explained as a constitutive de-regulation of *lmrCD* expression due to a defective LmrR that is unable to bind the *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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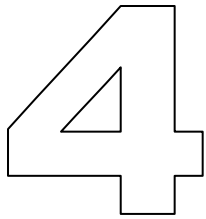
REFERENCES

1. Ahmed, M., C. M. Borsch, S. S. Taylor, N. Vazquez-Laslop, and A. A. Neyfakh. 1994. A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. J. Biol. Chem. 269:28506-28513.
2. Alekshun, M. N., S. B. Levy, T. R. Mealy, B. A. Seaton, and J. F. Head. 2001. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. Nat. Struct. Biol. 8:710-714.
3. Barthelmebs, L., C. Divies, and J. F. Cavin. 2000. Knockout of the p-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. Appl. Environ. Microbiol. 66:3368-3375.
4. Bolhuis, H., D. Molenaar, G. Poelarends, H. W. van Veen, B. Poolman, A. J. M. Driessen, and W. N. Konings. 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. J. Bacteriol. 176:6957-6964.
5. Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. 11:731-753.
6. Cohen, M. L. 2000. Changing patterns of infectious disease. Nature 406:762-767.

7. de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos. 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62:3662-3667.
8. den Hengst, C. D., S. A. van Hijum, J. M. Geurts, A. Nauta, J. Kok, and O. P. Kuipers. 2005. The *Lactococcus lactis* CodY regulon: identification of a conserved cis-regulatory element. J. Biol. Chem. 280:34332-34342.
9. Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. 154:1-9.
10. Gasson, M. J., Y. Kitamura, W. R. McLauchlan, A. Narbad, A. J. Parr, E. L. Parsons, J. Payne, M. J. Rhodes, and N. J. Walton. 1998. Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. J. Biol. Chem. 273:4163-4170.
11. Grkovic, S., M. H. Brown, N. J. Roberts, I. T. Paulsen, and R. A. Skurray. 1998. QacR is a repressor protein that regulates expression of the *Staphylococcus aureus* multidrug efflux pump QacA. J. Biol. Chem. 273:18665-18673.
12. Grkovic, S., M. H. Brown, and R. A. Skurray. 2002. Regulation of bacterial drug export systems. Microbiol. Mol. Biol. Rev. 66:671-701.
13. Gury, J., L. Barthelmebs, N. P. Tran, C. Divies, and J. F. Cavin. 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding *padA* gene of *Lactobacillus plantarum*. Appl. Environ. Microbiol. 70:2146-2153.
14. Kloosterman, T. G., W. T. Hendriksen, J. J. Bijlsma, H. J. Bootsma, S. A. van Hijum, J. Kok, P. W. Hermans, and O. P. Kuipers. 2006. Regulation of glutamine and glutamate metabolism by GlnR and GlnA in *Streptococcus pneumoniae*. J. Biol. Chem. 281:25097-25109.
15. Law, J., G. Buist, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts. 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J. Bacteriol. 177:7011-7018.
16. Leenhouts, K., G. Buist, A. Bolhuis, B. A. ten, J. Kiel, I. Mierau, M. Dabrowska, G. Venema, and J. Kok. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. 253:217-224.
17. Lewis, K. 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. Sci. 19:119-123.
18. Lubelski, J., A. de Jong, R. van Merkerk, H. Agustiandari, O. P. Kuipers, J. Kok, and A. J. M. Driessen. 2006. LmrCD is a major multidrug resistance transporter in *Lactococcus lactis*. Mol. Microbiol. 61:771-781.
19. Lubelski, J., W. N. Konings, and A. J. Driessen. 2007. Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. Microbiol. Mol. Biol. Rev. 71:463-476.
20. Lubelski, J., P. Mazurkiewicz, R. van Merkerk, W. N. Konings, and A. J. M. Driessen. 2004. *ydaG* and *ydbA* of *Lactococcus lactis* encode a heterodimeric ATP-binding cassette-type multidrug transporter. J. Biol. Chem. 279:34449-34455.
21. Mazurkiewicz, P., A. J. M. Driessen, and W. N. Konings. 2004. Energetics of wild-type and mutant multidrug resistance secondary transporter LmrP of *Lactococcus lactis*. Biochim. Biophys. Acta 1658:252-261.
22. Neu, H. C. 1992. The crisis in antibiotic resistance. Science 257:1064-1073.

23. Overhage, J., H. Priefert, and A. Steinbuchel. 1999. Biochemical and genetic analyses of ferulic acid catabolism in *Pseudomonas* sp. Strain HR199. Appl. Environ. Microbiol. 65:4837-4847.
24. Palumbi, S. R. 2001. Humans as the world's greatest evolutionary force. Science 293:1786-1790.
25. Paulsen, I. T. 2003. Multidrug efflux pumps and resistance: regulation and evolution. Curr. Opin. Microbiol. 6:446-451.
26. Poole, K. 1994. Bacterial multidrug resistance--emphasis on efflux mechanisms and *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 34:453-456.
27. Saier, M. H. Jr., I. T. Paulsen, M. K. Sliwinski, S. S. Pao, R. A. Skurray, and H. Nikaido. 1998. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. FASEB J. 12:265-274.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. Segura, A., P. V. Bunz, D. A. D'Argenio, and L. N. Ornston. 1999. Genetic analysis of a chromosomal region containing *vanA* and *vanB*, genes required for conversion of either ferulate or vanillate to protocatechuate in *Acinetobacter*. J. Bacteriol. 181:3494-3504.
30. van Hijum, S. A., J. A. de, R. J. Baerends, H. A. Karsens, N. E. Kramer, R. Larsen, C. D. den Hengst, C. J. Albers, J. Kok, and O. P. Kuipers. 2005. A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. BMC. Genomics 6:77.
31. Wegmann, U., M. O'Connell-Motherway, A. Zomer, G. Buist, C. Shearman, C. Canchaya, M. Ventura, A. Goesmann, M. J. Gasson, O. P. Kuipers, D. van Sinderen, and J. Kok. 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J. Bacteriol. 189:3256-3270.

Chapter



Distinct mechanisms of LmrR mediated gene regulation of multidrug resistance in *Lactococcus lactis*

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Submitted

SUMMARY

Multidrug resistance (MDR) in *Lactococcus lactis* is due to the expression of the membrane ATP-binding cassette (ABC) transporter LmrCD. In the absence of drugs, the transcriptional regulator LmrR prevents expression of the *lmrCD* operon by binding to its operator site. Through an autoregulatory mechanism LmrR also suppresses its own expression. Although the *lmrR* and *lmrCD* genes have their own promoters, primer extension analysis showed the presence of a long transcript spanning the entire *lmrR-lmrCD* cluster, in addition to various shorter transcripts harboring the *lmrCD* genes only. "In-gel" Cu-phenantroline footprinting analysis indicated an extensive interaction between LmrR and the *lmrR* promoter/operator region. Atomic Force Microscopy imaging of the binding of LmrR to the control region of *lmrR* DNA showed severe deformations indicative of DNA wrapping and looping, while LmrR binding to a fragment containing the *lmrCD* control region induced DNA bending. The results further suggest a drug-dependent regulation mechanism in which the *lmrCD* genes are co-transcribed with *lmrR* as a polycistronic messenger. This leads to an LmrR-mediated regulation of *lmrCD* expression that is being exerted from two different locations and by distinct regulatory mechanisms.

INTRODUCTION

In their living environment, bacteria have to cope with naturally occurring toxic molecules (plant alkaloids, bile salts), harmful metabolic end products, antimicrobial peptides, and secondary metabolites such as antibiotics. A widespread mechanism to counteract the inhibitory action of such molecules is their secretion from the cell by membrane bound multidrug resistance (MDR) transporters (5,25,37). For instance, the cationic compound Berberine alkaloids produced by many plants are substrates for MDR pumps such as QacA and NorA of *Staphylococcus aureus* (15,26,27,35). Soil- or plant-associated organisms display the highest abundance of chromosomally encoded MDR efflux systems (29,34). MDR transporters are often subject to regulatory control (11) as their expression at a high level might be critical to the cells (6,12). The expression of most MDR transporters is either positively or negatively controlled by local regulatory proteins (6,13) and/or globally by stress related regulators. For example, the overexpression of the *acrAB* MDR locus in *Escherichia coli* is regulated by the global regulators MarA, Rob and SoxS, the local repressor AcrR (3,19), and the quorum sensor regulator SdiA (32).

The Gram positive bacterium *Lactococcus lactis* plays a major role in fermented dairy food production. *L. lactis* readily develops a MDR phenotype upon a long term exposure to structurally unrelated compounds such as daunomycin, Hoechst 33342, ethidium bromide, rhodamine 6G, or cholate (4,18,22). This MDR phenotype is due to the constitutive expression of the *lmrCD* genes that encode a heterodimeric ATP-Binding Cassette (ABC) MDR transporter that secretes these compounds from the cell (17). Expression of the *lmrCD* genes is controlled by a local transcriptional regulator termed LmrR (2). LmrR acts as a drug-sensitive repressor of the expression of the *lmrCD* genes. Most of the transcriptional regulators involved in MDR belong to the AraC, MarR, MerR, or TetR family of transcriptional regulators. However, LmrR belongs to PadR, a family of mostly poorly characterized regulatory proteins that are involved in the regulation of detoxification mechanisms such as phenolic acid metabolism (10,28,36). In addition to LmrR, LadR from *Listeria monocytogenes* is the only characterized member of MDR-related PadR regulators (2,16). In this family of regulators, the expression of the detoxification genes is typically induced by the presence of the toxic compounds in the medium via a direct interaction with the PadR-like regulator. Indeed, LmrR has been shown to bind several of the LmrCD substrates such as Hoechst 33342, daunomycin, and Na-cholate. On the other hand, LmrR does not bind ρ-coumaric acid and ferulic acid (unpublished data) which are the phenolic acid derivatives that have been shown to bind to PadR (12). Recently, we

have solved the structure of the LmrR dimer in the apo form and in two drug bound forms, i.e., with Hoechst 33342 and Daunomycin (20). The dimer contains two N-terminal DNA binding domains with a typical winged helix-turn-helix (HTH) motif while the C-terminal regions form a large flat-shaped central pore at the subunit interface. The latter constitutes the drug binding pocket of LmrR that is symmetric with equal contributions of both monomers to the overall structure.

The *lmrR* gene is located upstream of the *lmrCD* genes (2). In independently isolated drug resistance strains of *L. lactis* that are cross-resistant against a series of drugs, the *lmrCD* genes are constitutively expressed because of the presence of defective forms of LmrR that are no longer able to bind to the promoter/operator ($^{p}/_{o}$) region of the *lmrCD* genes (17). In these strains, the *lmrR* gene is also up-regulated suggesting that in wild-type cells, LmrR represses its own expression. Biochemical data demonstrate that LmrR indeed binds to its own promoter region (2). Here, we have analyzed the interaction between LmrR and the control regions of the *lmrCD* and *lmrR* genes using "in-gel" Cu-phenantroline (OP-Cu) footprinting analysis and Atomic Force Microscopy (AFM) imaging. The data suggest distinct modes of binding of LmrR to the *lmrR* and *lmrCD* control regions resulting in the formation of different transcripts that encode the structural genes either with or without the *lmrR* transcriptional regulator gene. Expression of both *lmrR* and *lmrCD* is elevated when cells are grown in the presence of drugs, suggesting a mechanism in which the regulator gene and the functional genes are induced and co-transcribed from a polycistronic messenger.

EXPERIMENTAL PROCEDURES

Protein purification

Strep-tagged LmrR protein was overexpressed in *L. lactis* NZ9000 and purified by strep-tag affinity chromatography followed by chromatography with a heparin column as described before (2).

Primer extension and RT-PCR analysis

RNA was extracted from *L. lactis* MG1363 using Trizol® reagent (Invitrogen). To prevent genomic DNA contamination, RNA samples were treated on-column with DNase I using the RNeasy mini kit (Qiagen). Genomic DNA was extracted from *L. lactis* MG1363 using the GenElute Bacterial Genomic isolation kit (Sigma-Aldrich). Primer extension analysis was performed as described previously (7)

using AMV Reverse Transcriptase (Roche Applied Science). 5' end labeled primers DC620r or DC621r were used for transcription start determination of *lmrR* or *lmrC*, respectively. Labeling was done using [γ-^{32}P]-ATP (GE Healthcare). Reference ladders were generated by chemical sequencing methods (21). cDNA was prepared from about 2 μg of *L. lactis* RNA by using Superscript II Reverse Transcriptase (Invitrogen) and 200 ng random primers. The reaction was followed by RNase H treatment (Fermentas). Transcript analysis was done by PCR with primers Cdprmf/DC621r or DC636f/DC621r, using cDNA as template. Primer sequences are shown in Supplementary Table 1.

Electrophoretic mobility shift assays and 'in-gel' Cu-phenantroline footprinting

Labeled DNA fragments were produced by PCR (ReadyMix Taq PCR Reaction Mix; Sigma-Aldrich) using a pair of primers, of which one was 5' end labeled with [γ-^{32}P]-ATP (GE Healthcare). For the promoter regions of *lmrR* and *lmrCD*, the primer pairs DC634f/DC620r and DC635f/DC621r, respectively, were used with *L. lactis* MG1363 genomic DNA as template. Labeled fragments were purified by polyacrylamide gel electrophoresis. The truncated fragments of the promoter regions of *lmrR* and *lmrCD* were prepared similarly using the set of primers listed in Supplementary Table 2. EMSAs were performed as described previously (8). Binding reactions were performed in LmrR binding buffer (20 mM Tris pH 8.0, 1 mM $MgCl_2$, 20 mM KCl, 0.1 mM DTT, 0.4 mM EDTA, 12.5 % glycerol) by incubating at 37°C during 30 minutes in the presence of 25 μg/ml sonicated herring sperm DNA as a non-specific competitor. K_Ds were estimated based on these EMSAs, as the protein concentration at which about 50% of the DNA is bound (expressed in dimer equivalents). 'In gel' OP-Cu footprinting was performed as described previously (30). Reference ladders were generated by chemical sequencing methods (21).

Atomic Force Microscopy

For AFM experiments, the DNA fragments were prepared by PCR with ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich). The $^{p}/_{o}$ region of *lmrR* was amplified as a 997 bp fragment with the primer pair AFM *lmrR* pmtr FW/AFM *lmrR* pmtr RV, and *L. lactis* MG1363 genomic DNA as template. A 1016 bp fragment containing the $^{p}/_{o}$ region of *lmrCD* was amplified with the primer pair AFM *lmrCD* pmtr FW/AFM *lmrCD* pmtr RV. Following PCR amplification, all fragments were purified by agarose gel electrophoresis using a GenElute gel extraction kit (Sigma-Aldrich). A number of trials were performed to find the best concentration for both

DNA and LmrR with final concentrations of 1.86 and 0.04 µM for *lmrR* DNA and LmrR protein, respectively; and 0.16 and 0.018 µM for *lmrCD* DNA and LmrR protein, respectively. These binding reactions were diluted in LmrR binding buffer in a total volume of 15 µl. The mixture was then diluted 2-fold in adsorption buffer (40 mM Hepes, pH 6.9, 10 mM $NiCl_2$) and 15 µl of the suspension was deposited on freshly cleaved mica. This was incubated during 5 min to allow adsorption of the nucleoprotein complexes. Subsequently, samples were rinsed with deionized ultrapure water and excess water was blotted off with absorbing paper. The mica surface was blown dry in a stream of filtered air. The NanoScope IIIa atomic force microscope (Digital Instruments/Veeco) was operated in the tapping mode, in air. Images of 512 x 512 pixels were acquired by using Nanoprobe SPM tips, type RTESP7 (Veeco) with a 115-135-µm cantilever, a nominal spring constant of 50 N/m and resonance frequencies in the range from 244 to 295 kHz. The scan size was 1.5 µm x 1.5 µm and the scan rate was 2 Hz. The Nanoscope 6.11r1 software (Digital Instruments/Veeco) was used to flatten the images and to make zoomed 3D surface plots. The contour lengths of DNA molecules or DNA arms of complexes were measured by manual tracing with ImageJ (1). DNA molecules or complexes with overlapping parts or having visible anomalies were omitted from the analysis.

Quantitative PCR

Cultures of *L. lactis* NZ9000 and NZ9000(Δ*lmrR*) were grown overnight on M17 supplemented with 0.5% glucose at 30°C. Cultures were diluted 1:100 to an OD_{660} of 0.07-0.08 in the same medium without or with 1 µM Hoechst 33342 (Sigma-Aldrich) or 20 µM daunomycin (Calbiochem, VWR). These subinhibitory drug concentrations ensured near to identical growth rates of the different types of cells. Cells were further grown at 30°C and during the early exponential-, late exponential- and stationary growth phase, samples of 5 ml were collected and flash frozen in liquid nitrogen. Total RNA was isolated using Trizol® reagent (38). Residual chromosomal DNA was removed by using the TURBO DNA-*free*™ kit (Ambion®, Applied Biosystems) according to the manufacturer's instructions. Purified RNA was quantified by measuring absorption at 260 nm using a Nanodrop ND1000 spectrophotometer. The quality of the RNA preparations was checked by visualizing the integrity of 16S and 23S rRNA on an agarose gel, and by verifying the absence of DNA contamination by PCR. The cDNA molecules were synthesized using iScript™ cDNA synthesis kit (Bio-Rad) as recommended. Total RNA was isolated from at least two separately grown replicate cultures**.**

For the qPCR experiments, the primers were designed in order to have a length of 22-23 nucleotides, a G/C content of 45-47% (See Supplementary Table 3) and a T_m of about 60-65 ^oC. The length of the primer products ranged between 200 and 230 bp. qPCR was carried out on a MiniOpticon Real-Time PCR System (Bio-Rad). After dilution of the cDNA, 4 µl was added to 21 µl of the PCR mixture (12.5 µl of iQ SYBR Green Supermix and 0.5 µl of each primer of 10 pmol/µl). Thermal cycling conditions were set as follows: initial denaturation at 95^{o}C for 3 min followed by 40 cycles of 95^{o}C for 20 sec, 55^{o}C for 20 sec and 72^{o}C for 30 sec. An additional step starting from 65^{o}C to 95^{o}C was performed to establish a melting curve. This was used to verify the specificity of the PCR reaction for each primer set. qPCR measurements were performed in duplicate for each sample. The *tufA* gene was used as an internal control and for normalization of the results (9).

RESULTS

Mapping of the transcription start sites of lmrCD *and* lmrR

Primer extension analysis was performed to map the transcription initiation sites of the *lmrCD* and *lmrR* genes using RNA extracted from *L. lactis* MG1363 cells (Fig. 1). Transcription of *lmrR* is initiated at a single G residue located 26 nucleotides upstream of the ATG start codon (Figs. 1A and 2A). In contrast, *lmrCD*-specific reverse transcription resulted in at least four different cDNA molecules (Figs. 1B and 2B). Transcripts C and D start at an A residue 55 and 61 nucleotides upstream of the ATG start codon of *lmrC*, respectively. Transcript B starts at a T residue that is located 100 bp upstream of the ATG start codon of LmrC. A fourth cDNA molecule represents a transcript that is larger than the labeled fragment used for the Maxam-Gilbert (MG) sequencing ladder, which was prepared by PCR amplification using primers CDprmf and DC621r (Fig. 1D, Supplementary Table 1). Therefore, this transcript must also contain at least part of the open reading frame (ORF) of *lmrR*. To test whether or not this transcript corresponds to transcript A as detected with *lmrR*-specific primer extension, RT-PCR analysis was performed using primer pairs CDprmf/DC621r and DC636f/DC621r (Figs. 1C and D). These reactions resulted in amplification, confirming the existence of an mRNA molecule that spans both the *lmrR* and *lmrCD* genes. It thus appears that an RNA polymerase initiated at the *lmrR* promoter may proceed till the end of *lmrD*. Indeed, using the program TransTerm, intrinsic terminators were predicted to occur neither in the *lmrR* and *lmrCD* genes nor in the intergenic region between *lmrR* and

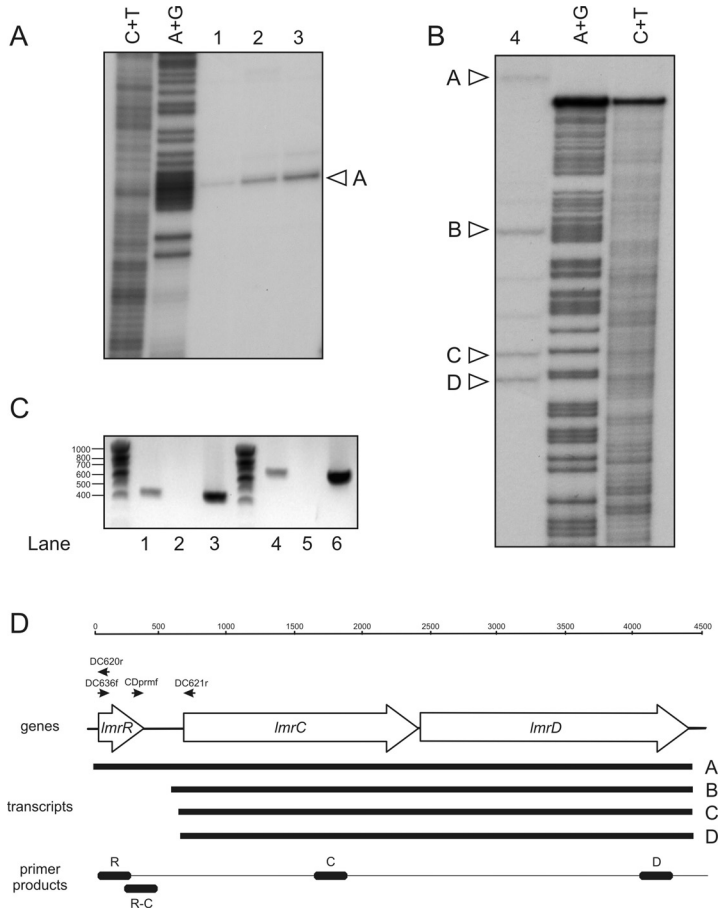

Figure 1. Primer extension analysis of the transcripts showing the transcription start sites of (**A**) *lmrR* using primer DC620r and (**B**) *lmrC* using primer DC621r. The amounts of total RNA used were 12.5 µg (lane 1), 25 µg (lane 2), 50 µg (lane 3) and 100 µg (lane 4). The main primer extension products are indicated with an arrow and are designated A-D. A+G and C+T represent the corresponding MG sequencing ladders. A systematic correction in the alignment of the cDNA product with the sequencing ladders has been performed to take into account the difference in migration velocity of the cDNA and the reference ladders due to different ends generated by the AMV reverse transcriptase and the chemical modification and cleavage reactions. (**C**) RT-PCR analysis with cDNA as template with primers CDprmf and DC621r (Lane 1); as lane 1, without addition of RT (negative control) (Lane 2); with primers CDprmf and DC621r with genomic DNA as template (Lane 3); with primers DC636f and DC621r and with cDNA as template (Lane 4); as lane 4, but without addition of RT (negative control) (Lane 5); and with primers DC636f and DC621r with genomic DNA as template (Lane 6). (**D**) Schematic overview of the transcripts A, B, C and D, with respect to the ORFs (indicated with open arrows) and primer products used for qPCR. The location of the primers used for primer extension and RT-PCR analysis is also indicated.

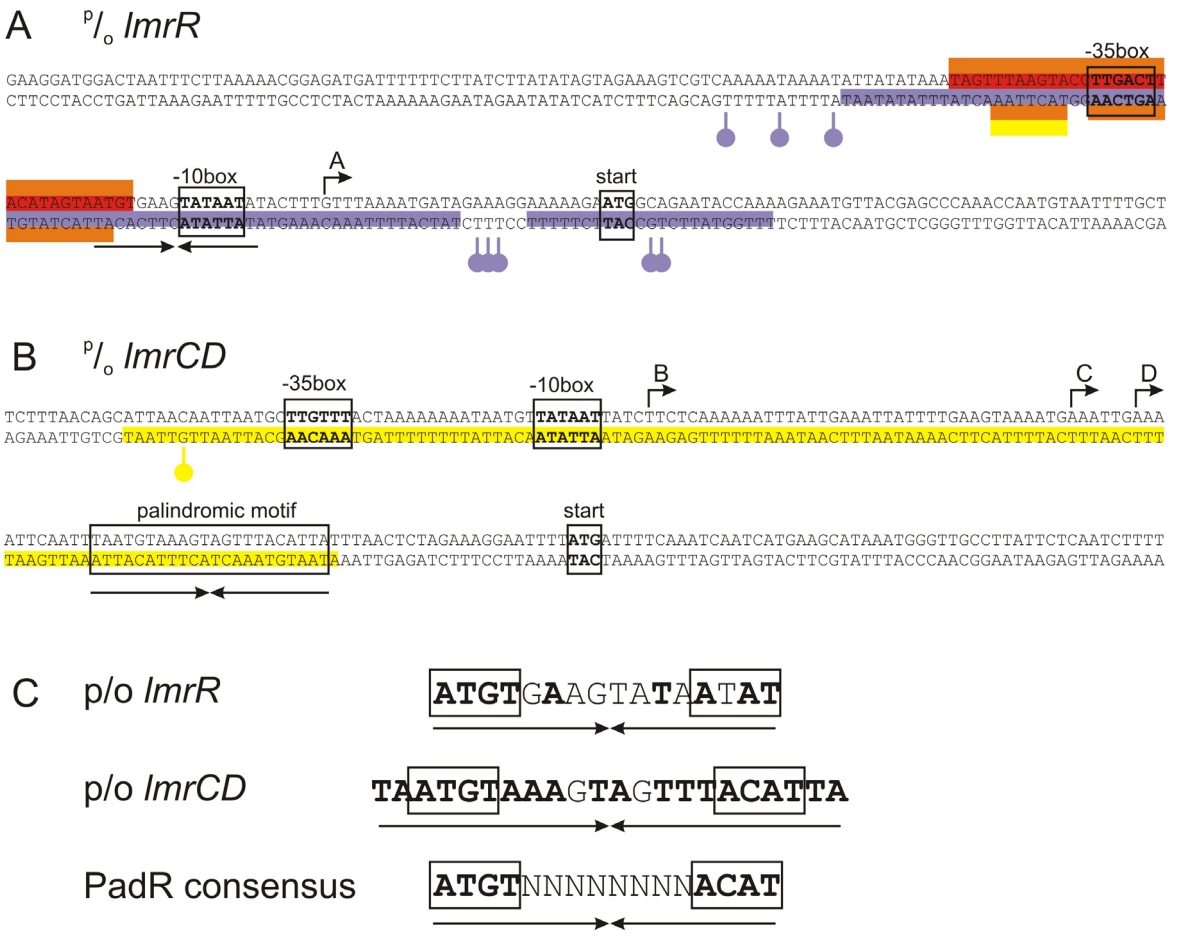

Figure 2. Schematic representation of the transcriptional elements on (**A**) *lmrR* and (**B**) *lmrCD* control region DNA including the position of the -35 and -10 regions, the transcription initiation sites, and the translation start codon (boxed). For the promoter region of *lmrCD*, the promoter elements are only predicted for transcript B. The letters A to D represent the 5' end of the major transcripts observed in primer extension analysis. In addition, this figure also represents the protected areas observed in the footprinting assays of LmrR binding to $^{p}/_{o}$ *lmrR* (**A**) and to $^{p}/_{o}$ *lmrCD* (**B**). For $^{p}/_{o}$ *lmrR*, protection zones are indicated for the complexes B1 (yellow), B2 (orange), B3 (red), and B4 (purple). For $^{p}/_{o}$ *lmrCD*, the protection zone is indicated in yellow. The ball-and-stick symbols represent the positions of the hyperreactivity sites. The identified imperfect palindromes are shown in the sequences with double arrows. (**C**) Representation of the imperfect IRs as identified in $^{p}/_{o}$ *lmrR*, $^{p}/_{o}$ *lmrCD*, and the PadR consensus IR. Palindromic residues are in bold, and the conserved PadR motif is boxed.

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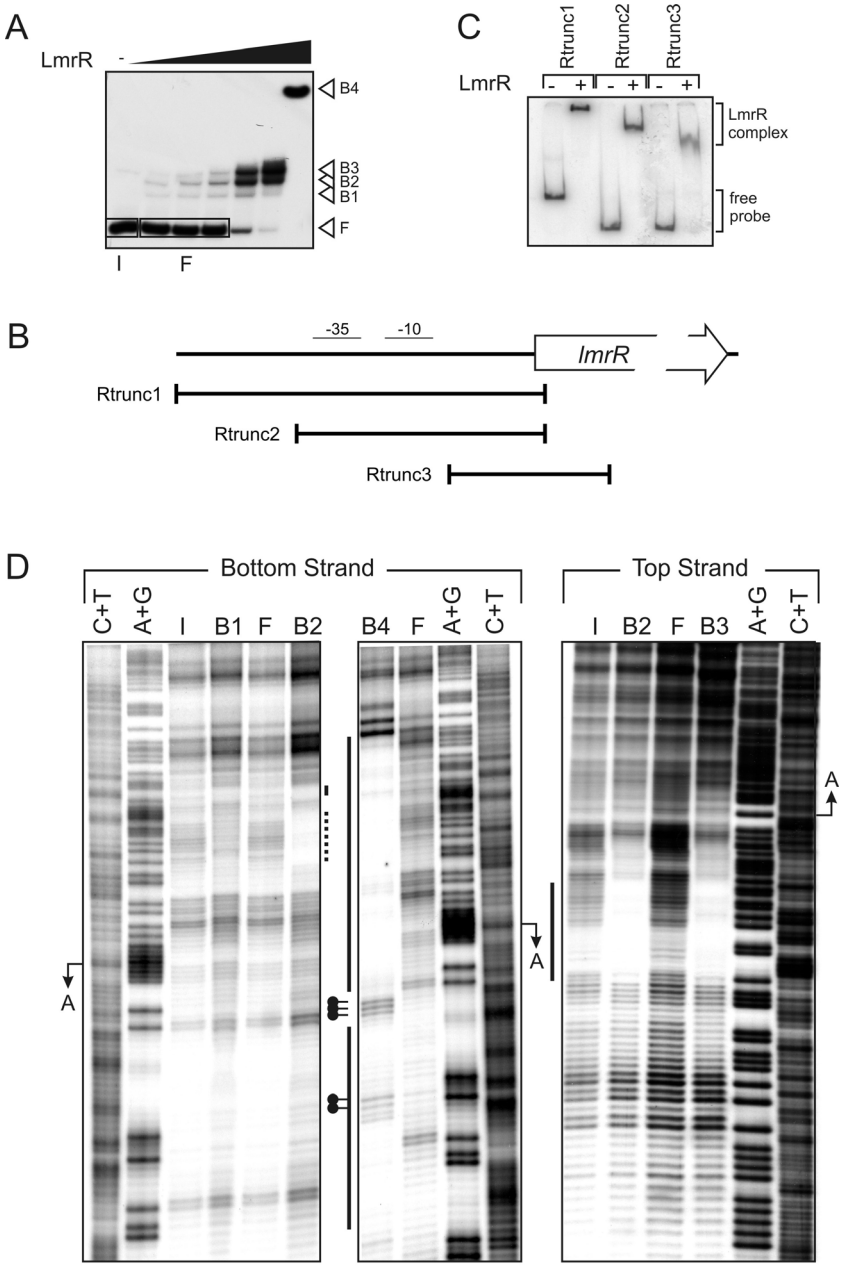
lmrC, but a terminator was detected downstream of *lmrD*. Putative Shine-Dalgarno (SD) sequences for both *lmrR* and *lmrC* were detected upstream of the respective start codons. Regions that show sequence conservation with the consensus -35 and -10 promoter elements could be identified slightly upstream of the start of transcripts A and B. (Figs. 2A and B). Both promoters show a putative Pribnow box with a perfect match to the consensus, a good matching -35 sequence, the two being separated by a linker of ideal length (17 bp). However, due to the multiple transcripts observed for *lmrCD*, additional promoter element(s) might be involved in *lmrCD* expression although it cannot be excluded that these transcripts have arisen by degradation of the longer transcripts.

Identification of the LmrR binding sites in the control regions of lmrR *and* lmrCD

Previously, it has been shown that LmrR protects a long stretch of DNA in the control region of its own gene against DNase I (1). Here, we show that LmrR forms multiple complexes with $^{P}/_{o}$ *lmrR* DNA as observed in an electrophoretic mobility shift assay (EMSA) (Fig. 3A). This result suggests the presence of multiple binding sites that likely involve several copies of LmrR. Three complexes (B1, B2 and B3) showed a slightly different migration velocity whereas complex B4, which was detected only at a higher LmrR concentration, was strongly retarded in its mobility.

Figure 3. Binding of LmrR to the *lmrR* promoter/operator region. (**A**) EMSA of the binding of purified LmrR to a 210 bp labeled DNA fragment containing the *lmrR* $^{P}/_{o}$ region. The LmrR stock concentration was 81.5 μM (dimer) and further diluted. There was no LmrR added in lane 1, and LmrR was added at concentrations of 0.01 μM (lane 2), 0.02 μM (lane 3), 0.03 μM (lane 4), 0.1 μM (lane 5), 0.2 μM (lane 6), and 0.8 μM (lane 7), respectively. The positions of the free DNA (F) and of the different LmrR-bound DNA complexes (B1, B2, B3 and B4) are indicated. These different complexes and the boxes named I (input DNA) and F (free DNA) were excised for 'in gel' footprinting analysis. (**B**) Scheme of the coverage of the $^{P}/_{o}$ *lmrR* truncated fragments Rtrunc1, Rtrunc2 and Rtrunc3 relative to the *lmrR* promoter elements and ORF. (**C**) EMSA of the binding of purified LmrR to truncated DNA fragments, Rtrunc1 (266 bp), Rtrunc2 (170 bp) and Rtrunc3 (152 bp) corresponding to the regions of the *lmrR* operator site indicated in Figure 2. LmrR was added at a final concentration of 1.85 μM (dimer). (**D**) 'In gel' OP-Cu footprinting of LmrR binding to the $^{P}/_{o}$ region of *lmrR* with the bottom strand labeled (left two panels) or with the top strand labeled (right panel). The EMSA that was used for the experiment with the bottom strand labeled is shown in Fig. 3A. Next to each autoradiograph, protected regions are indicated with a vertical line. Hyperreactivity sites are also indicated with ball-and-stick symbols. For the outer left panel, a full line corresponds to protection observed in complex B1 and B2 whereas a dashed line corresponds to an additional protection observed in complex B2 only. A+G and C+T represent the MG sequencing ladders. Next to the ladder, the position of the transcription start is shown. A schematic representation of the protection region is displayed in Fig. 2.


A
LmrR
-
B4
B3
B2
B1
F
I
F
C
Rtrunc1
Rtrunc2
Rtrunc3
LmrR
- + - + - +
LmrR complex
free probe
B
-35
-10
lmrR
Rtrunc1
Rtrunc2
Rtrunc3
D
Bottom Strand
Top Strand
C+T
A+G
I
B1
F
B2
B4
F
A+G
C+T
I
B2
F
B3
A+G
C+T
A
A
A

The average apparent binding dissociation constant (K_D) of the LmrR-$^{p}/_{o}$ *lmrR* interaction is between 25 and 50 nM. There was a rapid transition in the formation of the different complexes, especially in the formation of complex B2 at the expense of complex B1, which indicates a binding cooperativity. EMSAs were also performed with truncated DNA fragments containing only a part of the *lmrR* control region or ORF (Fig. 3B and 3C). Interestingly, LmrR was able to bind DNA probes consisting of the control region alone (Rtrunc1 and Rtrunc2), but also a DNA probe consisting mainly of the *lmrR* gene, starting only 4 bp upstream of the transcription start (Fig. 3B) (Rtrunc3).

To further determine which regions in the DNA are recognized by LmrR in each of the multiple complexes observed in the EMSA, "in-gel" Cu-phenantroline (OP-Cu) footprinting was performed with the various complexes (Fig. 3D). The fastest migrating complex B1 exhibited protection at a site located between 2 and 8 bp upstream of the -35 promoter element (Fig. 2A, yellow bar, and Fig. 3D). This site might be considered as a 'core' binding site from which LmrR binding is nucleated. The slower migrating complexes B2 and B3 both showed a downstream extension of this initially protected region, including the -35 Box (Fig. 2A, orange and red bars). Footprinting with a DNA fragment having the top strand labeled revealed no clear-cut differences in the protected regions of complexes B2 and B3. Here, a difference in migration velocity could also be caused by conformational changes of the protein-DNA complex, rather than by stoichiometrical differences. The highly retarded complex B4 showed an extensive protection encompassing about 102 bp, including the entire promoter and transcription start site (Fig. 2A, purple bar and Fig. 3D). In this protected region, an imperfect inverted repeat (IR) is apparent (Fig. 2A and C). This IR exhibits one mismatch as compared to the PadR consensus sequence, but has the optimal spacing of 8 nucleotides between the palindromic halfsites (Fig. 2C) (16). Several hyperreactivity signals were observed for complex B4 indicating local DNA deformations upon LmrR binding (Fig. 2A and 3D).

The binding of LmrR to DNA fragments covering the $^{p}/_{o}$ region of the *lmrCD* genes showed a distinctively different signature. Previous footprinting results indicated that LmrR binds to two different sites on the *lmrCD* promoter (2): site I comprising the -35 and -10 region and site II which harbors an imperfect IR similar to the PadR consensus sequence but with a spacing of 10 bp (Fig. 2C) (16). EMSAs were performed with shortened probes corresponding to either site I or site II (Supplementary Fig. 1). It appears that LmrR binds DNA probes containing the -35 and -10 region (site I) stronger than the probes comprising site II containing the palindromic sequence. With the full-length *lmrCD* $^{p}/_{o}$ DNA a single complex was observed upon binding of LmrR (Fig. 4A). The overall binding affinity of this

interaction appears 2- to 4-fold lower as compared to the affinity for $^{p}/_{o}$ *lmrR* DNA, with an average apparent K_D between 75 and 100 nM. "In-gel" OP-Cu footprinting of LmrR binding to the *lmrCD* control region showed a single extended protected region of about 126 bp (Fig. 4B) that overlaps all the transcription initiation sites (Site I) in the *lmrCD* control region and their cognate promoter elements, and the previous identified imperfect IR (Site II; Fig. 2B). At the promoter-distal side of the protected region, a hyperreactivity site was observed, again indicating LmrR-induced DNA deformations. These results demonstrate different modes of binding of LmrR to the *lmrR* and *lmrCD* promoter/operator regions.

Atomic Force Microscopy of the binding of LmrR to lmrR and lmrCD promoter DNA

AFM was used to visualize the DNA conformational changes that occur upon the binding of LmrR to the $^{p}/_{o}$ regions of *lmrR* and *lmrCD*, respectively (Figs. 4 and 5). Tapping-mode AFM in air was used to allow for a high resolution topographic imaging of the soft protein/DNA sample surfaces without creating any destructive frictional forces. With the *lmrR* $^{p}/_{o}$ DNA, 22 unbound 997 bp-long DNA molecules and 41 DNA-LmrR complexes were analyzed. The contour length of unbound DNA molecules was manually traced using the ImageJ software, resulting in an average contour length of 313 nm (standard deviation 29 nm; Fig. 5C). This yields an axial bp rise of 0.31 nm/bp, which is lower than the theoretical rise of B-form DNA (i.e. 0.34 nm/bp), but in good agreement with other AFM studies. This difference can be explained by the limited resolution of the microscope and the smoothing procedure that rounds sharp bends (33). Based on DNA persistence length analysis of other DNA molecules measured in similar experimental conditions, it can be assumed that the molecules are able to freely equilibrate on the surface before capturing (23,31). A heterogenous population of LmrR-*lmrR* nucleoprotein complexes was observed, ranging from having apparently a single site bound, possibly the 'core' nucleation site, or having apparently two sites bound (data not shown), to the most notably complexes with a large complexed region as shown in Fig. 5A. Here, several LmrR molecules seem to be involved in the condensation of the binding site area. This type of complexes most probably corresponds to the B4 population observed in the EMSA (Fig. 3A). It is clear that LmrR binding induces severe DNA deformations including sharp DNA bending, DNA condensation and possibly even DNA wrapping around the protein (local DNA supercoiling) or DNA looping (Fig. 5A). The contour length of the naked DNA arms of all complexes was measured without

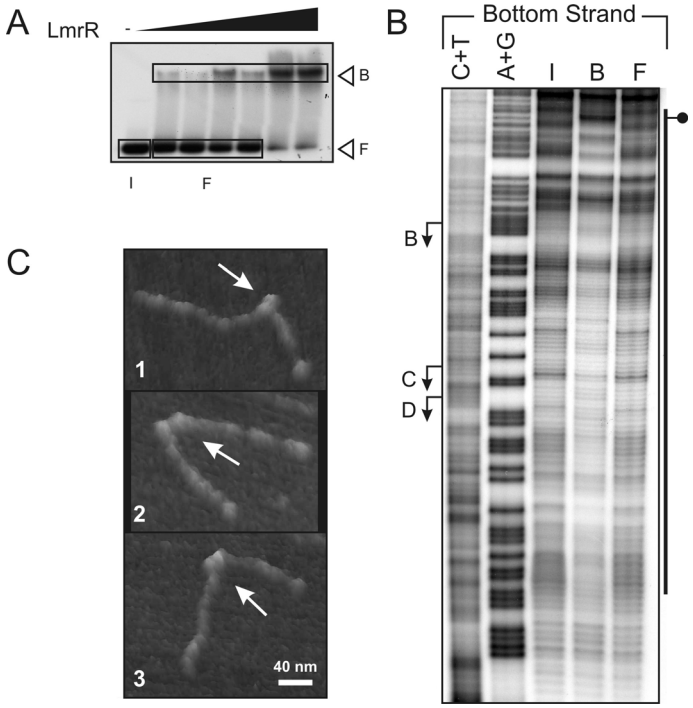


Figure 4. Binding of LmrR to the *lmrCD* promoter/operator region. (**A**) EMSA of the binding of LmrR to a DNA fragment corresponding to the *lmrCD* $^{p}/_{o}$ region. LmrR was added at the same concentrations as in the EMSA shown in Fig. 3A. The positions of the free DNA (F) and of the bound complexes (B) are indicated. (**B**) 'In gel' OP-Cu footprinting analysis of the LmrR-*lmrCD* promoter region complex that was excised from the gel shown in (**B**). Next to the autoradiograph, the protected regions are indicated with a vertical line and the hyperreactivity sites as a ball-and-stick symbol. A+G and C+T represent the MG sequencing ladders. Next to the ladder, the positions of the transcription starts are shown. A schematic representation of the protected region is displayed in Fig. 2. (**C**) A selection of three AFM images of LmrR-$^{p}/_{o}$ *lmrCD* protein-DNA complexes, as typically observed.

making a distinction between the different types of complexes (depending on the degree of binding; Fig. 5B). These measurements resulted in an average length of 79 nm (st. dev. 39 nm) for the short DNA arm and 191 nm (st. dev. 40 nm) for the long DNA arm. Therefore, the total average visible contour length of the complexes (short + long arms) is 270 nm (standard deviation 52 nm; Fig. 5C). This is a difference of 43 nm with the average length of the unbound DNA molecules and taking a bp rise of 0.31 nm/bp into account, this corresponds to about 139 bp

that are condensed inside the DNA-LmrR complex. Due to the heterogeneity of the complexes, the distributions are broad. These observations demonstrate that the binding mechanism of LmrR to the *lmrR* promoter/operator DNA involves interactions with multiple LmrR molecules that are likely bound in a cooperative fashion.

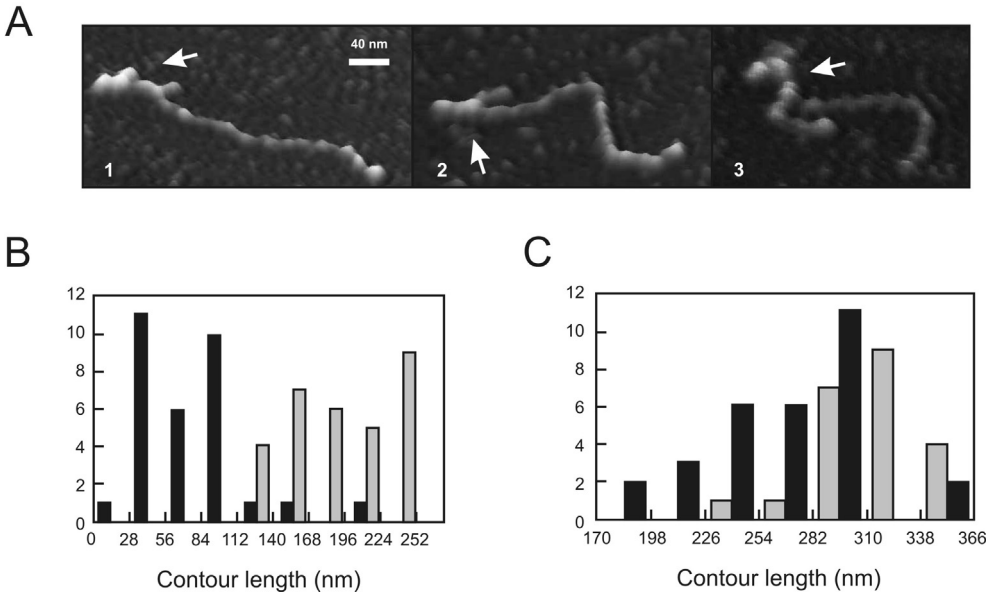


Figure 5. AFM analysis of the binding of LmrR to the promoter/operator site of *lmrR*. (**A**) A selection of three AFM images of LmrR-$^{P}/_{o}$ *lmrCD* protein-DNA complexes. (**B)** Contour length measurements of the long (grey bars) and short (black bars) arms of LmrR complexed with the DNA fragment. (**C**) Contour lengths of the sum of the long and short arm of the LmrR-complexed DNA fragments (black bars) and of the free DNA fragments (grey bars).

AFM experiments with the *lmrCD* control region DNA resulted in LmrR/DNA complexes with a more homogenous architecture as compared to the LmrR / $^{P}/_{o}$ *lmrR* complexes (Fig. 4C). In the observed complexes, LmrR induces a significant DNA bending. Typically, the complexed region had a bi-lobed structure. These two ”blobs” present in the AFM images may represent the binding of two LmrR dimers to the DNA (Fig. 4C) (20). Taken together, the AFM results support the notion that LmrR binds the *lmrR* and *lmrCD* operator regions by

different mechanisms and indicate higher order interactions of LmrR with the operator region of its own gene.

Expression analysis of the lmrCD *and* lmrR *genes in* L. lactis

Our analysis indicates the presence of a long transcript harboring both the *lmrR* and *lmrCD* genes. To assess the expression levels of *lmrR* in growing cell cultures, qPCR was employed on RNA extracted from *L. lactis* cells growing on M17 medium with glucose in the absence and presence of subinhibitory concentrations of the drugs daunomycin and Hoechst 33342. As a control, *L. lactis* NZ9000(Δ*lmrR*) was used that expresses the *lmrCD* genes constitutively (2).

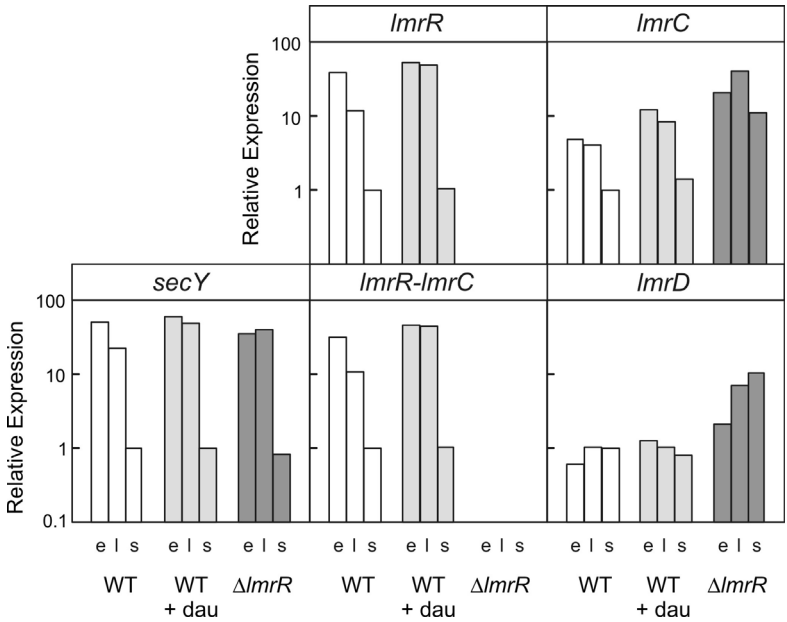


Figure 6. qPCR expression analysis of *lmrCD*, *lmrR* and the intergenic region that separates the *lmrR* and *lmrC* genes in *L. lactis* NZ9000 (WT) cells grown to different growth stages in the absence and presence of daunomycin (dau). *L. lactis* NZ9000(Δ*lmrR*) cells were included as a control. Expression levels were related to elongation factor Tu (*tufA*), and for each gene normalized for the expression in the stationary phase of *L. lactis* NZ9000 (WT) cells in the absence of daunomycin. *SecY* was used as an additional house-keeping gene. The efficiency of amplification reactions was determined by running a standard curve with serial dilutions of cDNA. PCR efficiencies were similar for the various primer sets and above 95%. Growth stages: e, early exponential; l, late exponential; and s, stationary growth phase.

Primer sets were designed to monitor the transcript levels of *lmrR*, *lmrC* and *lmrD* each, and in addition, a set was designed that detects the intergenic region that separates the *lmrR* and *lmrCD* genes in the long polycistronic *lmrR-lmrCD* transcript (Fig. 1D). Expression levels were related to that of the house-keeping gene *tufA* that encodes the translation elongation factor Tu (9). In addition, the *secY* transcript encoding the major subunit of the preprotein translocase was monitored. The expression level of these control genes was constant during exponential growth, but unlike *tufA*, the expression of *secY* dropped when cells entered the stationary phase (Fig. 6). As expected, the *lmrC* and *lmrD* genes are highly expressed in *L. lactis* NZ9000(Δ*lmrR)* cells during the exponential and stationary growth phase. With *L. lactis* NZ9000 wild type cells, lower levels of *lmrCD* expression were observed that dropped dramatically when the cells entered the stationary phase. A similar response was observed with the transcript containing the *lmrR* gene and the *lmrR-lmrC* intergenic region, suggesting that the long transcript is present during the entire exponential growth phase. When cells were exposed to daunomycin (Fig. 6) or Hoechst 33342 (data not shown), expression levels of *lmrC* and *lmrD* increased. Since Hoechst 33342 also caused an increase in the *tufA* expression, the corresponding data could not be quantified. Remarkably, exposure to the drugs also resulted in increased levels of the transcript harboring the *lmrR* gene and the *lmrR-lmrC* intergenic region. Summarizing, these data demonstrate that both the regulatory *lmrR* gene and the structural *lmrCD* genes are expressed in exponentially growing wild-type cells and that their expression increases upon an exposure to toxic drugs.

DISCUSSION

The ABC transporter LmrCD was previously shown to be a major determinant of the MDR phenotype in *L. lactis* (17). Transcription of *lmrCD* is controlled by LmrR, a local regulatory repressor whose gene is located upstream of *lmrCD* (2). The *lmrR* and *lmrCD* genes are transcribed in the same direction. LmrR has previously been shown to function as a drug-controlled negative transcriptional regulator of the expression of the *lmrCD* genes. Our current primer extensions analysis now revealed the presence of three major transcripts of *lmrCD* and one longer transcript spanning the *lmrR* and *lmrCD* genes. The occurrence of multiple transcripts of *lmrCD* might indicate the presence of alternative promoters. Alternative promoters are quite frequent in bacteria and may be used to cope with changes in the environment as for instance altered nutritional requirements that

result in changes in the expression of a particular gene. In most cases, however, one promoter is responsible for the constitutive expression whereas the others are inducible by different stimuli (24) that may even function with another alternative σ-factor. At this stage it is unclear whether the presence of these multiple transcripts indeed reflects functional differences in the regulation and/or expression mechanism. Possibly, additional global or local regulators might be involved in the regulation of the different promoters. However, qPCR analysis of the expression of *lmrR* (from the long transcript) and of the *lmrCD* genes (likely both from the long and shorter transcripts) indicated that these genes are expressed throughout the exponentially grown cells, and that their expression is further elevated when cells are exposed to toxic drugs. For *lmrCD*, the drug-induced expression levels are lower than observed in the deregulated strain that lacks the *lmrR* gene indicating that the drug-induced de-repression is not maximized in such cells.

LmrR binds to two regions in the *lmrCD* operator sequence. Site I, comprising the -35 and -10 region leading to initiation at transcription start site B, appears to be a high affinity binding site for LmrR. Site II harbors an imperfect IR that is similar to the PadR consensus binding site, but the two half-sites are separated by 10 instead of 8 bp (16). EMSA experiments suggested that the palindromic sequence on its own is only weakly recognized by LmrR (Supplementary Fig. 1) and that the binding of LmrR to the entire control region of the *lmrCD* genes results in one dominant species of DNA-protein complex. Footprinting analysis supports the notion that in this complex both site I and site II are protected by LmrR. Visualization of these protein-DNA complexes revealed a significant DNA bending with two protein 'blobs' being present on the DNA, wherein each "blob"likely corresponds to a LmrR dimers. Taken together, these results suggest that site I and site II are each bound by an LmrR dimer in a highly cooperative manner since it was not possible to detect a complex having only the higher-affinity site I bound. On the other hand, LmrR binds to a more extended and less distinct region in the *lmrR* operator site. In this control region, multiple copies of the LmrR protein bind and this is a sequential event, nucleated by binding to a site just upstream of the -35 box and extending further downstream, overlapping the promoter and transcription initiation site and spreading into the *lmrR* ORF. A PadR-like imperfect IR is located in the middle of this large protected zone, which might be recognized by LmrR. This binding seems to involve a cooperative mechanism in which protein-protein interactions between adjacently bound LmrR dimers and DNA conformational changes play an important role. It yields a higher order multimeric LmrR-DNA complex in which the DNA is condensed, looped or even wrapped around the protein as suggested by the AFM observations. Previous observations describing the cooperative binding of two dimers of λ repressor to

different and adjacent operator sites in the same DNA molecule have shown that this is mediated by the interactions between the carboxyl domains of the repressors which promote the DNA to twist and bend due to its flexibility (14). Moreover, the binding of the repressor to a strong binding site will enhance the binding affinity of a weaker site thus promoting cooperative binding between repressor molecules as described above. The same mechanism may apply for the binding of LmrR to the two different sites in the *lmrCD* operator region. Overall, our data suggest different LmrR binding mechanisms to the control regions of the *lmrCD* and *lmrR* genes with binding to $^{p}/_{o}$ *lmrR* occurring in a tighter fashion and with a higher binding affinity.

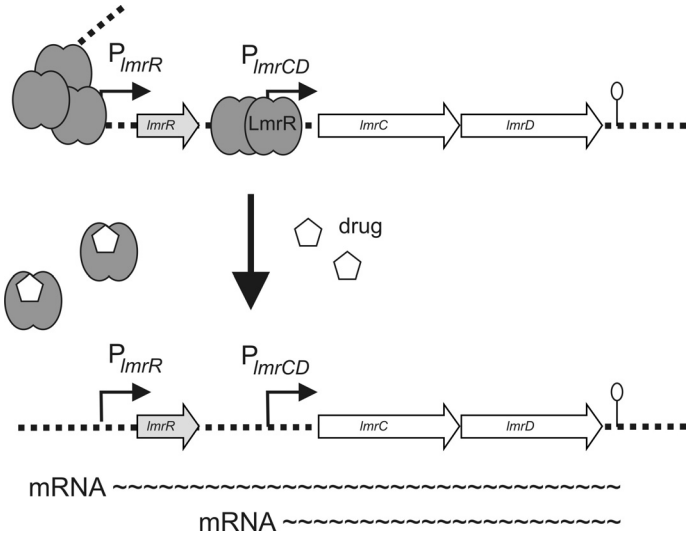


Figure 7. Schematic representation of the regulation of *lmrR* and *lmrCD* expression by LmrR in *L. lactis*. In the wild-type cells growing in drug-free media, LmrR binds and represses the transcription of both the *lmrCD* and *lmrR* genes. Binding to the *lmrR* operator sequences involves cooperative binding of multiple copies of the LmrR dimer, while LmrR binds as two dimers to the *lmrCD* operator sequences. When cells are challenged with a drug, the LmrR dimer binds a drug molecule and this causes the release of the LmrR-drug complex from the *lmrCD* and *lmrR* operator sequences allowing the initiation of transcription with the formation of a polycystronic mRNA that supports the translation of both the *lmrR* and *lmrCD* genes, and mRNAs harboring the *lmrCD* genes only.

Based on our new insights, the following two-step mechanism of *lmrCD* regulation is envisaged (Fig. 7): Binding of two LmrR dimers to the *lmrCD* promoter region will result in a repression of *lmrCD* expression. Simultaneously,

extensive binding of multiple LmrR dimers to the *lmrR* control region leads to a strong auto-repression. When cells are challenged with toxic compounds, the drugs will enter the cell and bind to LmrR. At first, this likely only causes a reduced binding of LmrR for the *lmrCD* operator binding sites. Consequently, there is a de-repression of *lmrCD* transcription. At higher drug concentrations, the repression at the *lmrR* operator site might be relieved as well, since this is a higher affinity binding involving more LmrR dimers that are tightly interacting with each other and with the strongly deformed DNA. This de-repression yields a polycistronic messenger containing the information for the regulator and for the transporter, resulting in an even higher production of LmrCD. Therefore, LmrR-mediated regulation of *lmrCD* expression is being exerted from two different locations and by different mechanisms. Meanwhile, LmrR is also involved in an autorepression that is modulated by drugs. Only upon release of LmrR from the *lmrR* operator site (at high intracellular drug concentrations), additional LmrR regulatory protein is being produced. These additional regulatory proteins could assure a fast response to re-repress *lmrR* and *lmrCD* expression as most LmrR dimers were already saturated with the drug effector molecule. Newly synthesized LmrCD will insert into the membrane and mediate the export of the drugs from the cell. Due to the decreased cellular drug levels, LmrR will return to its apo form and re-associate first with $^{p}/_{o}$ *lmrR* and then with the *lmrCD* operator site and again inhibit the expression. This drug dependent regulatory phenomenon results in a fine-tuned demand-depending expression of the LmrCD transporter.

In the previously selected MDR strains, the *lmrR* gene harbors mutation(s) that lead to the production of nonfunctional LmrR variants that are unable to repress the expression of both *lmrR* and *lmrCD* (2). This not only causes the up-regulation of *lmrCD* but also in increased levels of the *lmrR* transcript. Strikingly, microarray analysis on all four drug resistant strains of *L. lactis* demonstrated that *lmrR* is significantly and more strongly (9.4-fold on average) up-regulated than *lmrCD* (6.7-fold on average) (17) consistent with the notion that LmrR binds the *lmrR* promoter region more strongly than the *lmrCD* promoter region. Consequently, expression of *lmrR* is controlled by a well-tuned and damped feedback autoregulatory loop. This tightly controlled *lmrR* expression may serve to ensure a highly sensitive drug-sensing regulatory mechanism of *lmrCD* expression. High cellular levels of LmrR would render this mechanism less sensitive to drugs as increased intracellular drug concentrations will be needed to achieve derepression of *lmrCD* expression. In contrast, direct transcription from the *lmrCD* operator sites is likely more responsive to drugs because of a less extensive LmrR binding mechanism. Since *lmrR* and *lmrCD* are at least partially co-transcribed, expression of high levels of LmrCD will be prevented as the newly synthesized

LmrR will readily repress further transcription. This will for instance minimize the risk that also important hydrophobic metabolites in the cell are lost due to uncontrolled and unwanted secretion. Indeed, in the *L. lactis* NZ9000(Δ*lmrR*) strain, higher *lmrCD* transcript levels are observed than in wild-type cells challenged with drugs. Future studies will focus on the structural basis of the LmrR-DNA interaction, and the exact stoichiometry of LmrR binding to the various operator sequences.

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REFERENCES

1. Abramoff,M.D.,Magelhaes,P.J., and Ram,S.J. 2004. Image Processing with ImageJ. Biophotonics International 11: 36-42.
2. Agustiandari,H.,Lubelski,J., van den Berg van Saparoea,H.B., Kuipers,O.P., and Driessen,A.J.M. 2008. LmrR is a transcriptional repressor of expression of the multidrug ABC transporter LmrCD in Lactococcus lactis. J Bacteriol 190: 759-763.
3. Alekshun,M.N., and Levy,S.B. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. Antimicrob Agents Chemother 41: 2067-2075.
4. Bolhuis,H., Molenaar,D., Poelarends,G., van Veen,H.W., Poolman,B., Driessen,A.J.M., and Konings,W.N. 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant Lactococcus lactis. J Bacteriol 176: 6957-6964.
5. Chopra,I., and Roberts,M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65: 232-260.
6. Eckert,B., and Beck,C.F. 1989. Overproduction of transposon Tn10-encoded tetracycline resistance protein results in cell death and loss of membrane potential. J Bacteriol 171: 3557-3559.
7. Enoru-Eta,J., Gigot,D., Glansdorff,N., and Charlier,D. 2002. High resolution contact probing of the Lrp-like DNA-binding protein Ss-Lrp from the hyperthermoacidophilic crenarchaeote Sulfolobus solfataricus P2. Mol Microbiol 45: 1541-1555.
8. Enoru-Eta,J., Gigot,D., Thia-Toong,T.L., Glansdorff,N., and Charlier,D. 2000. Purification and characterization of Sa-lrp, a DNA-binding protein from the extreme thermoacidophilic archaeon Sulfolobus acidocaldarius homologous to the bacterial global transcriptional regulator Lrp. J Bacteriol 182: 3661-3672.
9. Friedrich,U., and Lenke,J. 2006. Improved enumeration of lactic acid bacteria in mesophilic dairy starter cultures by using multiplex quantitative real-time PCR and flow cytometry-fluorescence in situ hybridization. Appl Environ Microbiol 72: 4163-4171.

10. Gasson,M.J., Kitamura,Y., McLauchlan,W.R., Narbad,A., Parr,A.J., Parsons, E. L., J. Payne, M. J. Rhodes, and N. J. Walton. 1998. Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. J Biol Chem 273: 4163-4170.
11. Grkovic,S., Brown,M.H., and Skurray,R.A. 2002. Regulation of bacterial drug export systems. Microbiol Mol Biol Rev 66: 671-701, table.
12. Gury,J., Barthelmebs,L., Tran,N.P., Divies,C., and Cavin,J.F. 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding padA gene of Lactobacillus plantarum. Appl Environ Microbiol 70: 2146-2153.
13. Hickman,R.K., McMurry,L.M., and Levy,S.B. 1990. Overproduction and purification of the Tn10-specified inner membrane tetracycline resistance protein Tet using fusions to beta-galactosidase. Mol Microbiol 4: 1241-1251.
14. Hochschild,A., and Ptashne,M. 1986. Cooperative binding of lambda repressors to sites separated by integral turns of the DNA helix. Cell 44: 681-687.
15. Hsieh,P.C., Siegel,S.A., Rogers,B., Davis,D., and Lewis,K. 1998. Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. Proc Natl Acad Sci U S A 95: 6602-6606.
16. Huillet,E., Velge,P., Vallaeys,T., and Pardon,P. 2006. LadR, a new PadR-related transcriptional regulator from Listeria monocytogenes, negatively regulates the expression of the multidrug efflux pump MdrL. FEMS Microbiol Lett 254: 87-94.
17. Lubelski,J., de Jong,A., van Merkerk,R., Agustiandari,H., Kuipers,O.P., Kok,J., and Driessen,A.J.M. 2006. LmrCD is a major multidrug resistance transporter in Lactococcus lactis. Mol Microbiol 61: 771-781.
18. Lubelski,J., Mazurkiewicz,P., van Merkerk,R., Konings,W.N., and Driessen,A.J.M. 2004. ydaG and ydbA of Lactococcus lactis encode a heterodimeric ATP-binding cassette-type multidrug transporter. J Biol Chem 279: 34449-34455.
19. Ma,D., Alberti,M., Lynch,C., Nikaido,H., and Hearst,J.E. 1996. The local repressor AcrR plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals. Mol Microbiol 19: 101-112.
20. Madoori,P.K., Agustiandari,H., Driessen,A.J.M., and Thunnissen,A.M. 2009. Structure of the transcriptional regulator LmrR and its mechanism of multidrug recognition. EMBO J 28: 156-166.
21. Maxam,A.M., and Gilbert,W. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol 65: 499-560.
22. Mazurkiewicz,P., Driessen,A.J.M., and Konings,W.N. 2004. Energetics of wild-type and mutant multidrug resistance secondary transporter LmrP of Lactococcus lactis. Biochim Biophys Acta 1658: 252-261.
23. Minh,P.N.,Devroede,N., Massant,J., Maes,D., and Charlier,D. 2009. Insights into the architecture and stoichiometry of Escherichia coli PepA*DNA complexes involved in transcriptional control and site-specific DNA recombination by atomic force microscopy. Nucleic Acids Res 37: 1463-1476.
24. Musso,R.E., Di,L.R., Adhya,S., and de Crombrugghe,B. 1977. Dual control for transcription of the galactose operon by cyclic AMP and its receptor protein at two interspersed promoters. Cell 12: 847-854.
25. Neyfakh,A.A., Bidnenko,V.E., and Chen,L.B. 1991. Efflux-mediated multidrug resistance in Bacillus subtilis: similarities and dissimilarities with the mammalian system. Proc Natl Acad Sci U S A 88: 4781-4785.
26. Neyfakh,A.A., Borsch,C.M., and Kaatz,G.W. 1993. Fluoroquinolone resistance protein NorA of Staphylococcus aureus is a multidrug efflux transporter. Antimicrob Agents Chemother 37: 128-129.

27. Ng,E.Y., Trucksis,M., and Hooper,D.C. 1994. Quinolone resistance mediated by norA: physiologic characterization and relationship to flqB, a quinolone resistance locus on the Staphylococcus aureus chromosome. Antimicrob Agents Chemother 38: 1345-1355.
28. Overhage,J., Priefert,H., and Steinbuchel,A. 1999. Biochemical and genetic analyses of ferulic acid catabolism in Pseudomonas sp. Strain HR199. Appl Environ Microbiol 65: 4837-4847.
29. Paulsen,I.T., Nguyen,L., Sliwinski,M.K., Rabus,R., and Saier,M.H., Jr. 2000. Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. J Mol Biol 301: 75-100.
30. Peeters,E., Thia-Toong,T.L., Gigot,D., Maes,D., and Charlier,D. 2004. Ss-LrpB, a novel Lrp-like regulator of Sulfolobus solfataricus P2, binds cooperatively to three conserved targets in its own control region. Mol Microbiol 54: 321-336.
31. Peeters,E., Willaert,R., Maes,D., and Charlier,D. 2006. Ss-LrpB from Sulfolobus solfataricus condenses about 100 base pairs of its own operator DNA into globular nucleoprotein complexes. J Biol Chem 281: 11721-11728.
32. Rahmati,S.,Yang,S., Davidson,A.L., and Zechiedrich,E.L. 2002. Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. Mol Microbiol 43: 677-685.
33. Rivetti,C., Guthold,M., and Bustamante,C. 1996. Scanning force microscopy of DNA deposited onto mica: equilibration versus kinetic trapping studied by statistical polymer chain analysis. J Mol Biol 264: 919-932.
34. Saier,M.H., Jr., Paulsen,I.T., Sliwinski,M.K., Pao,S.S., Skurray,R.A., and Nikaido,H. 1998. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. FASEB J 12: 265-274.
35. Schumacher,M.A., and Brennan,R.G. 2003. Deciphering the molecular basis of multidrug recognition: crystal structures of the Staphylococcus aureus multidrug binding transcription regulator QacR. Res Microbiol 154: 69-77.
36. Segura,A., Bunz,P.V., D'Argenio,D.A., and Ornston,L.N. 1999. Genetic analysis of a chromosomal region containing vanA and vanB, genes required for conversion of either ferulate or vanillate to protocatechuate in Acinetobacter. J Bacteriol 181: 3494-3504.
37. Tennent,J.M., Lyon,B.R., Gillespie,M.T., May,J.W., and Skurray,R.A. 1985. Cloning and expression of Staphylococcus aureus plasmid-mediated quaternary ammonium resistance in Escherichia coli. Antimicrob Agents Chemother 27: 79-83.
38. Zaidi,A.H., Bakkes,P.J., Lubelski,J., Agustiandari,H., Kuipers,O.P., and Driessen,A.J.M. 2008. The ABC-type multidrug resistance transporter LmrCD is responsible for an extrusion-based mechanism of bile acid resistance in Lactococcus lactis. J Bacteriol 190: 7357-7366.

Supplementary Table 1. Primer sets used for RT-PCR analysis

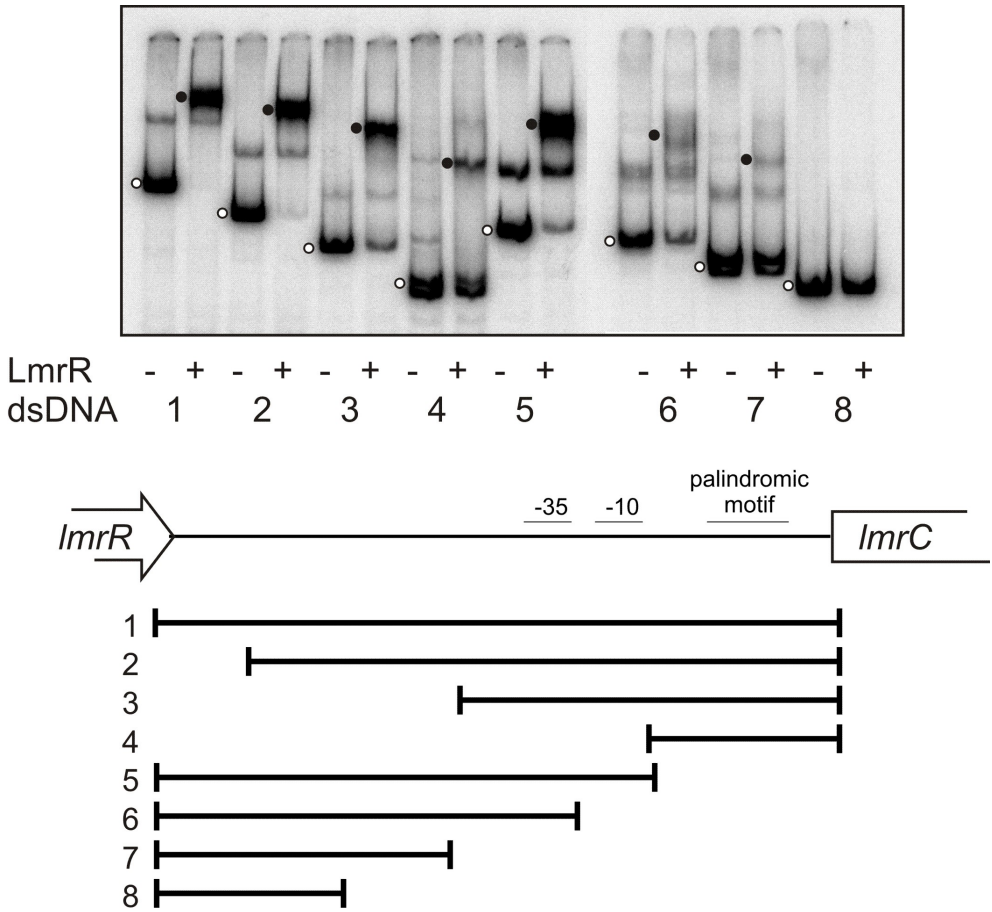
Primer name	5'→ 3' sequence
DC620r	CTCCTTGTTTTAGGACATTGAGC
DC621r	AAGATTGAGAATAAGGCAACCC
DC634f	CGGAGATGATTTTTTCTTATCTTATATAG
DC635f	CTATTGTAATCTTTAACAGCATTAAC
DC636f	ATGGCAGAAATACCAAAAGAATG
CDprmf	GTATTACCGACTGACAGAGATTGG

Supplementary Table 2. Primer sets used for extended EMSA analysis.

Primer name	5'→ 3' sequence
Region 1f	CAAATAAGAAGAGTGAAGCG
Region 1r	GGCAACCCATTTATGCTTCA
Region 2f	ACAAATAACGTCGTAAATCG
Region 2r	GGCAACCCATTTATGCTTCA
Region 3f	ATTGTAATCTTTAACAGCATTAAC
Region 3r	GGCAACCCATTTATGCTTCA
Region 4f	TTCTCAAAAAATTTATTGAAATTA
Region 4r	GGCAACCCATTTATGCTTCA
Region 5f	CAAATAAGAAGAGTGAAGCG
Region 5r	AAATTTTTTGAGAAGATAAT
Region 6f	CAAATAAGAAGAGTGAAGCG
Region 6r	GCATTAACAATTAATGCTTGTTTACT
Region 7f	CAAATAAGAAGAGTGAAGCG
Region 7r	GTTTACCATTTATGAAACTAACTATTG
Region 8f	CAAATAAGAAGAGTGAAGCG
Region 8r	CGTTGACTTAAACTTTAAAAAG

Supplementary Table 3. Primer sets used for qPCR analysis.

Primer name	5'→ 3' sequence	Length	GC content (%)
TufAf	TGACGAAATCGAACGTGGTCAAG	23	47
TufAr	GTCACCAGGCATTACCATTTCAG	23	47
SecYf	GCTTGCTATGGCACAATCTATCG	23	47
SecYr	ATGGCTGATGGAATACCAGAGAC	23	47
LmrRf	ATGTTACGAGCCCAAACCAATG	22	45
LmrRr	TCTGTCAGTCGGTAATACTTGC	22	45
lmrR-Cf	GTATTACCGACTGACAGAGATTG	23	43
lmrR-Cr	GTTTAAGTCAACGATTTACGACG	23	39
LmrCf	GCGAAAGACGAAGAACTTTCTGG	23	47
LmrCr	ACTGAAACAGTCCCTTCTGTTGG	23	47
LmrDf	CGAAAGCTTGCCTGACAAGTATG	23	47
LmrDr	CGAATGAAGTTCGTCCAGCAATG	23	47



Supplementary Fig. 1. EMSA of the binding of LmrR to truncated DNA fragments corresponding to different parts of the *lmrCD* control region. The concentration of wild-type LmrR was kept constant (1.85 µM dimer) in all lanes. The -35 and -10 boxes shown belong to the promoter of transcript B. Double-stranded DNA fragments (○) were obtained by PCR using the primers indicated in Supplementary Table 2. The shifted DNA is indicated by a ●.

Chapter

Structure of the transcriptional regulator LmrR and its mechanism of multi-drug recognition

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ABSTRACT

LmrR is a PadR-related transcriptional repressor that regulates the production of LmrCD, a major multidrug ABC transporter in *Lactococcus lactis*. Transcriptional regulation is presumed to follow a drug-sensitive induction mechanism involving the direct binding of transporter ligands to LmrR. Here we present crystal structures of LmrR in an apo state and in two drug-bound states complexed with Hoechst 33342 and daunomycin. LmrR shows a common topology containing a typical β-winged helix-turn-helix domain with an additional C-terminal helix involved in dimerization. Its dimeric organization is highly unusual with a flat-shaped hydrophobic pore at the dimer centre serving as a multi-drug binding site. The drugs bind in a similar fashion with their aromatic rings sandwiched in between the indole groups of two dimer-related tryptophan residues. Multi-drug recognition is facilitated by conformational plasticity and the absence of drug-specific hydrogen bonds. Combined analyses using site-directed mutagenesis, fluorescence-based drug binding and protein-DNA gel shift assays reveal an allosteric coupling between the multidrug and DNA binding sites of LmrR that likely plays a role in the induction mechanism.

INTRODUCTION

Multidrug resistance is frequently caused by the action of specialized membrane-bound pumps that posses or have acquired the ability to extrude a wide variety of chemically and structurally different compounds from the cell (13,28). The molecular mechanisms of substrate recognition by these multi-drug transporters are poorly understood, mainly because the proteins involved are very recalcitrant towards crystallization, a prerequisite for a detailed structural analysis by X-ray crystallography. Instead, general features explaining multi-drug binding specificity have been derived from structural studies of the transcriptional regulators of multi-drug transporters, which are soluble proteins and often bind many of the same diverse drugs that are substrates of the pumps (3,12,13,30,34).

LmrR is a recently identified transcription factor that controls the expression of the heterodimeric ABC transporter LmrCD, which is a major multidrug transporter in *Lactococcus lactis* (22). It is encoded in the immediate vicinity of the *lmrCD* genes and was shown to specifically bind to the *lmrCD* and *lmrR* promoters where it acts as a transcriptional repressor and autoregulator, respectively (1). Toxic compounds that form substrates of the LmrCD transporter include the DNA-binding drugs Hoechst 33342, daunomycin, ethidium bromide and rhodamine 6G. One of these drugs, Hoechst 33342, was shown to directly interact with LmrR, and its presence in the growth medium induced a significant up-regulation of the *lmrCD* genes (1). This strongly suggests that the transcription factor may act as a drug-sensor causing stimulation of LmrCD production in the presence of toxic compounds, thus promoting their extrusion from the cell. By homology LmrR belongs to the PadR family of transcriptional regulators found in bacteria and archaea (10,16). Only a few PadR family members have been functionally characterized, showing that these proteins play important roles in the regulation of distinct cellular pathways leading, for example, to MDR, virulence and detoxification. Crystal structures of two PadR-like proteins, AphA (8) and Pex (5), revealed the characteristics of the PadR fold, which includes a conserved N-terminal winged helix-turn-helix (wHTH) DNA binding domain (4) that is architecturally similar to that of the multiple antibiotic resistance repressor MarR (2), and a highly diverse C-terminal helical domain which serves as a dimerization module.

No structural data is available on multi-drug binding transcriptional regulators of the PadR family. To understand the structural basis for multi-drug recognition by LmrR, as well as to provide insights in the molecular mechanisms involved in the regulation of *lmrCD* expression, we have determined the crystal

structure of LmrR in an apo state, as well as in two drug-bound states, complexed with Hoechst 33342 and daunomycin.

EXPERIMENTAL PROCEDURES

Chemicals

H33342 (Molecular Probes) and daunomycin (Calbiochem) were purchased and used without further purifications.

Protein production, crystallization and X-ray data collection

Tagged LmrR, comprising full length LmrR (from *L. lactis* strain MG1363) and a C-terminal streptactin-tag (117-SRWSHPQFEK-126), was obtained by nisin-induced overexpression in *L. lactis* using the expression vector pNSC8048-*lmrR*, and initially purified via strep-tag affinity and heparin column chromatography as described elsewhere (1). Final purification to homogeneity was achieved by size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) with a running buffer containing 20 mM Tris-HCl, pH 8.0, 280 mM NaCl and 1 mM EDTA. To anticipate possible negative effects of the streptactin-tag on protein crystal growth or protein conformation, also untagged full length LmrR was produced using a modified version of the overexpression plasmid in which the strep-tag encoding sequence was deleted. Untagged LmrR was first purified on a heparin column with conditions similar to those used for tagged LmrR, followed by purification on a Mono S HR 5/5 cation exchange column (GE Healthcare) with a linear gradient of 0.03-1 M NaCl (20 mM Hepes, pH 8, 1 mM EDTA, 0.5 mM DTT). Finally, the protein was loaded on a Superdex 200 10/300 GL column and eluted using the same running buffer as for tagged LmrR.

Purified LmrR, with or without streptactin-tag, was concentrated in the gel filtration running buffer and either used immediately for crystallization or frozen in liquid nitrogen and stored at -80 ºC. Crystallization trials were set up with the aid of an Oryx6 crystallization robot (Douglas Instruments) using the PACT and JCSG crystallization screens (26). Lead conditions were optimized manually using the sitting drop vapor diffusion method with crystallization drops containing 1 µl of the protein solution (8 mg/ml) and 1 µl of the reservoir solution. Crystals of drug-bound LmrR complexes were obtained by co-crystallization using a LmrR solution (8 mg/ml) preincubated for 30 minutes with 2 mM H33342 or daunomycin.

Table 1. Selected Crystallographic Data and Statistics

		LmrR-drug complexes	
	Apo-LmrR	H33342	Daunomycin
Data analysis			
Space group	$C222_1$	$P4_32_12$	$P2_12_12_1$
Unit cell (Å)			
a	46.6	34.9	35.4
b	52.6	34.9	53.0
c	174.9	197.0	147.1
Resolution (Å)	35 - 2.0	65 - 2.2	70 - 2.2
Rmerge a	0.04 (0.5)[b]	0.05 (0.206)	0.036 (0.273)
Mean I/σI	46.6 (2.0)	21.1 (4.8)	28.9 (3.8)
Completeness (%)	100 (99.3)	99.8 (99.0)	96.0 (99.9)
Unique reflections	15006	6948	17020
Redundancy	6.7 (7.8)	3.9 (2.9)	3.0 (3.5)
Refinement statistics			
Resolution (Å)	23 - 2.0	50 - 2.2	50 - 2.2
Rwork/Rfreec	0.21/0.26	0.20 / 0.25	0.23/0.27
No. of non-H atoms			
Protein	1679	850	1859
Ligand	-	34	38
Waters	83	27	116
Root-mean-square deviations in			
bond length (Å)	0.011	0.015	0.019
bond angles (°)	1.4	1.0	0.8
Average B-values (Å2)			
Protein	15	36	27
Ligand	-	42	74
Ramachandran analysis			
Most favoured (%)	99.0	98.0	98.0
Additional allowed (%)	1.0	2.0	2.0

[a] $R_{merge} = \Sigma\Sigma|I_{hkl} - I_{hkl}(j)|/ \Sigma I_{hkl}$, where $I_{hkl}(j)$ is the observed intensity and I_{hkl} is the final average intensity value.
[b] Values in parentheses are for the highest-resolution shell.
[c] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$ and $R_{free} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where all reflections belong to a test set of 10% randomly selected data.

Diffracting crystals of LmrR in drug-free conditions were obtained with both tagged and untagged protein, while in the drug-bound form only streptactin-tagged LmrR yielded well diffracting crystals.

At drug-free conditions tagged LmrR crystals were obtained with 20% PEG 3350 in 0.1 M Bis-Tris propane, pH 8.5 and 0.2 M $NaNO_3$, while drug-free untagged LmrR crystals grew in 30% PEG 1500, 0.1 M propionic acid/cacodylate/Bis-Tris propane (PCB) cocktail buffer, pH 8.5. Crystals of H33342-bound LmrR grew against a well solution containing 25% PEG 1500, 0.1 M succinic acid/phosphate/glycine (SPG) buffer, pH 9.0, while crystals of daunomycin-bound LmrR-strep were obtained with 25% PEG 1500, 0.1 M malonic acid/imidazol/boric (MIB) buffer, pH 7.0. All crystals grew overnight at room temperature.

X-ray diffraction data were collected at cryogenic temperatures by using the MX beam lines of the European Synchrotron Research Facility (ESRF) at Grenoble. Prior to data collection, crystals were flash cooled in a cryoprotectant solution of mother liquor with 20% glycerol. The data were processed with MOSFLM (21) and merged using SCALA as implemented in CCP4. Relevant data statistics are shown in Table 1. Data on the tagged LmrR crystal grown in the absence of drugs, and its derived structure, will not be presented here, as they were merely used to verify that the C-terminal streptactin-tag did not affect the overall LmrR structure.

Structure determination of apo-LmrR

In the absence of drugs untagged LmrR crystallized in the space group $C222_1$ with two subunits of two different dimers in the asymmetric unit. The crystals diffracted up to 2.0 Å resolution. The untagged apo-LmrR structure was solved by molecular replacement using PHASER with the automated search process (23). Various search models were prepared and tried using the structures of homologous proteins from the PDB, as identified by the FFAS server (18). Molecular replacement succeeded with a search ensemble containing the structures of three hypothetical transcription factors (PDB entries 2ESH, 1YYV and 1XMA) having sequence identities with LmrR ranging from 15% to 34%. Phase improvement and construction of an initial protein model was performed by using the automatic map improvement and model building routines in RESOLVE (31). The final model was obtained by carrying out various cycles of refinement using REFMAC (25) interspersed with cycles of rebuilding and placement of water molecules using COOT (9). TLS refinement was used in the last refinement cycles to model anisotropic displacements (32,33). The final model of the apo-LmrR structure

contains two polypeptide chains: one discontinuous chain covering residues 5-70 and 75-109, and one continuous chain covering residues 5-109. In both polypeptides residues 1-4 and 110-116 are missing due to weak or absent electron density. Each polypeptide is one subunit of different biological dimers that are formed by crystallographic 2-fold axes.

Structure determination of drug-bound LmrR

H33342-bound LmrR crystals belong to space group $P4_32_12$ with one molecule per asymmetric unit, while daunomycin-bound LmrR crystallized in a different space group ($P2_12_12_1$) with a dimer in the asymmetric unit. The structures of the drug-bound complexes were solved by molecular replacement using the apo-LmrR monomer as a search model. Clear density in $2F_o$-F_c and F_o-F_c Fourier maps, calculated at the initial stages of refinement, indicated the location and binding mode of the drugs. The model building and refinement were done with COOT and REFMAC5. In both cases TLS refinement was used in the last refinement cycles. For the LmrR-H33342 complex the final protein model contains residues 3-108. No electron density is observed for residues 1-2, 109-126 (including the strep-tag) and 71-73 (β-wing) loop. The final protein model of LmrR-daunomycin contains residues 2-116 for chain A, and residues 5-115 for chain B.

Structure analysis

Relevant crystallographic statistics of the refined models are shown in Table 1. Stereochemistry of the models was validated with the programs Procheck (20) and MolProbity (7). 3D structural superpositions and assessment of conformational differences was carried out with the programs Lsqman (19) and Dyndom (11). Electrostatic surface potentials were calculated using APBS, and visualized using PyMOL (Delano Scientific). The molecular dipole moment of the LmrR dimer was calculated using the protein dipole moments server at http://bioportal.weizmann.ac.il/dipol. Additional analyses, like calculation of surface areas, were performed with various programs from the CCP4 program suite.

Site directed mutagenesis

Mutations of W67 and W96 in the *lmrR* gene were performed via round PCR using the pNSC8048-lmrR plasmid as the template together with synthetic primers containing the designated mutations. PCR products were ligated at 4˚C overnight

before being transformed to the *L. lactis* NZ9000 competent cells via electroporation. Selected colonies were inoculated at 30˚C in M17 media (Difco) supplemented with 0.5% glucose (w/v) and 5 µg/ml chloroamphenicol. Plasmid isolation was performed using GenElute Plasmid miniprep kit (Sigma-Aldrich) and the correct mutations verified via nucleotide sequencing.

Drug binding assays

Binding of H33342 to the DNA-free purified LmrR variants was monitored by the increase of H33342 fluorescence upon binding as described previously (1). Binding of daunomycin to LmrR mutant W67Y was monitored by tryptophan fluorescence quenching titration experiments using an Aminco Bowman Series 2 spectrofluorometer (excitation wavelength of 295 nm, emission spectra obtained from 300 to 450 nm). A detailed description of the drug binding assays, additional control experiments, and the procedure that was followed to derive the apparent dissociation constants (K_d) of the two drugs is included in the Supplementary Information.

DNA binding assay

The ability of the LmrR variants to bind to a 287 bp fragment corresponding to the promoter region of *lmrCD* was studied by means of an electrophoretic mobility shift assay (EMSA) as described (1).

Circular dichroism

Circular dichroism spectra were obtained at 25 ºC by using an Aviv 62ADS spectropolarimeter (Aviv Associates, Lakewood, NJ). The protein samples contained 0.24 mg/ml protein in 20 mM Tris-HCl, pH 8.0 and 50 mM NaCl.

Accession Numbers

The atomic coordinates and structure factors for apo-LmrR (entry 3F8B), H33342-bound LmrR (entry 3F8C), and daunomycin-bound LmrR (entry 3F8F) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatices, Rutgers University (http://www.rcsb.org).

RESULTS

Structure Determination of LmrR In Apo and Drug-Bound States

Full length LmrR was purified both as a fusion protein containing a C-terminal streptactin-tag and as untagged protein. The crystal structure of untagged LmrR was determined to 2.0 Å resolution, by the molecular replacement method using an ensemble of three structurally homologous, but functionally uncharacterized proteins from the Protein Data Bank (PDB) as a search model. The untagged LmrR structure was solved in the absence of bound drugs and thus represents an apo form. Tagged LmrR was also crystallized in the absence of drugs, and its structure determined at 2.5 Å resolution. Since the overall structural features of drug-free tagged LmrR are identical to that of drug-free untagged LmrR, we will describe the latter structure only and refer to it as apo-LmrR.

Crystals of drug-bound LmrR were obtained by co-crystallization using tagged and untagged LmrR preincubated with different lipophilic cationic drugs. Co-crystals of tagged LmrR complexed with Hoechst 33342 (H33342) and with daunomycin diffracted both to 2.2 Å resolution. Co-crystals obtained with untagged LmrR, or in the presence of ethidium bromide and rhodamine 6G, could not be used for structure determination because of their poor X-ray diffraction quality. In all cases, however, the presence of bound drug was indicated by a drug-specific coloring of the crystals. The structures of LmrR bound to H33342 and daunomycin were determined by molecular replacement using the apo-LmrR subunit structure as a search model.

Each of the three LmrR structures (apo, H33342-bound and daunomycin-bound) represents a different crystal form with one or two LmrR subunits in the asymmetric unit. In all crystals LmrR is present as dimers formed via either crystallographic or non-crystallographic symmetry. The dimeric nature of LmrR is consistent with the results of gel filtration chromatography and dynamic light scattering experiments (not shown), and agrees with the general oligomeric preference of other MarR/PadR family members. The overall fold of the LmrR dimer is the same in the different structures, but structural superpositions reveal some notable differences, highlighting a significant inherent conformational plasticity (further explained below). The stereochemical quality of the models is excellent with no Ramachandran outliers. The electron density for the polypeptide chains is generally well defined, except for the N- and C-termini, including the strep-tag, which are disordered and not included in the final models. Another flexible region that is disordered in most of the LmrR structures comprises the β-wing loop (residues 70-75). The drug binding site is located at the dyad axis of the

LmrR dimer. In the LmrR-H33342 complex structure, where the dimer is formed by crystallographic symmetry, the Hoechst compound binds in two mutually exclusive orientations related by the crystallographic dyad symmetry, resulting in an averaged electron density at the drug binding site (Supplementary Figure S1A). However, electron density for H33342 is well defined, allowing straightforward deconvolution of the two symmetry-related binding modes. Electron density for the daunomycin molecule in the LmrR-daunomycin complex is less well defined (Supplementary Figure S1B), indicating some disorder in binding. A summary of the data collection and model refinement statistics is presented in Table 1.

Overall Structure of Apo-LmrR

The crystal of apo-LmrR contains two independent copies of a subunit in the asymmetric unit, which each, through a crystallographic dyad rotation axis, forms a biologically relevant dimer with approximate overall dimensions of 100 Å × 38 Å × 38 Å (Figure 1). Each LmrR subunit has an (α + β) structure with topology α1 (residues 6-23), α2 (residues 28-39), α3 (residues 47-60), β1 (residues 63-67), β2 (residues 77-81), α4 (residues 83-108), and is divided into two functional domains: a typical wHTH DNA binding domain, which consists of helices α1, α2, the DNA recognition helix α3 and strands β1 and β2 (together forming the wing), and a dimerization domain containing the C-terminal helix α4 (Figure 1A). Helix α4 forms a protruding arm, which in the dimer crosses over to the wHTH domain of the dyad-related subunit, packing in a nearly antiparallel orientation against helix α1' (the prime indicates the other subunit), as well as interacting with the C-terminal region of helix α2' and the loop connecting helices α2' and α3' (Figure 1B). Although facing each other at the centre of the dimer, there is no interaction between the C-terminal helices α4 and α4', nor between N-terminal helices α1 and α1'. Remarkably, this dimeric arrangement results in the formation of a large flat-shaped pore (approximately 22 Å in width and 6 Å in height) running through the dimer centered around the dyad. The pore entrances are formed by helices α4 and α4' on one side (hereafter named the front entrance), and by helices α1, α1', and the two DNA recognition helices α3 and α3' on the other side (back entrance). The inside of the pore is largely hydrophobic and formed by residues from the N- and C-terminal helices of both subunits. The pore centre is constricted by a dyad-related pair of tryptophan residues (W96 in α4 and α4'), whose indole rings are oriented face-to-face at a distance of about 7 Å (as calculated from the centres of mass of the indole groups). Clusters of arginine and lysine residues surround the back entrance (K6, R10, K55, R59, R75 and K77 from each subunit) resulting in a

net positive surface charge, which is consistent with that side of the dimer forming the binding site for DNA.

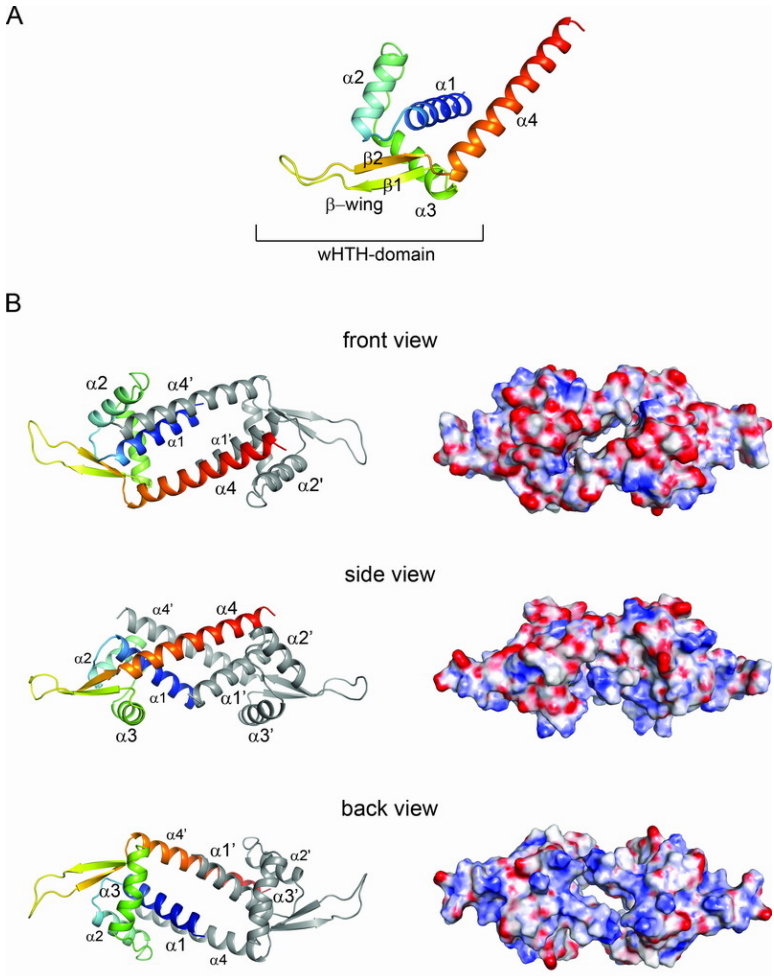

Figure 1. Overall structure of apo-LmrR (A) Ribbon representation of a single LmrR subunit with a rainbow color gradient from the N-terminus (blue) to the C-terminus (red). Secondary structure elements are indicated with labels. (B) The apo-LmrR dimer is shown in three orientations, related by 90º rotations, resulting in a front view (along the 2-fold rotation axis facing the α4 helices), a side view (perpendicular to the 2-fold axis) and a back view (along the 2-fold axis facing the α1 and α3 helices). Helices are indicated with labels. The left panel shows the LmrR dimer in a ribbon presentations, the right panel in electrostatic surface representations. The red and blue in the surface representations indicate strength of electrostatic surface potential (red, negative charge; blue, positive charge).

By contrast, the surface around the front entrance is largely negatively charged due to the presence of twelve Glu and Asp residues, six from each C-terminal helix in the dimer (E83, E87, E94, D100, E104 and E107). The opposite electrostatic surface charges around the front and back entrances (Figure 1B) create a small but significant overall molecular dipole moment (1192 Debye) running through the pore coinciding with the dyad axis.

The dimer interface

A striking feature of the LmrR structure is its unusual dimeric arrangement leading to the formation of a large central pore. As pointed out above the dimer interface is formed by interactions of the C-terminal helix of each subunit with the DNA-binding domain of its dimer mate. The surface area of one subunit that becomes buried upon dimerization is ~1160 Å^2, which is within the expected range for a stable dimer considering the 13.5 kDa size of the LmrR subunit (17). However, the buried surface area in the LmrR dimer is substantially smaller than the buried surface areas in other MarR/PadR dimers (*e.g.* the buried surface area in the MarR dimer is 3700 Å^2), in which dimerization usually involves more inter-helical interactions and the formation of a central compact core. Stabilization of the LmrR dimer mainly occurs via hydrophobic interactions. Residues in helix α4 that participate in forming the dimer interface are L91, A92, W96, R98, V99, I102, I103, N105 and L106. In the wHTH domain of the dimer-related subunit the residues important for dimerization are M8', A11', Q12', V15', I16' and V20' (from helix α1'), and V35', A38', N40', and M43' (from helix α2' and the loop connecting helices α2' and α3'). A few interactions are of polar or ionic nature. For example the side chain of N105 forms a hydrogen bond with the main chain oxygen of A38', and a salt bridge is formed between R98 and E42'. Another notable residue at the dimer interface is Q12' in helix α1'. The side chain of this residue forms a hydrogen bond with S95 in helix α4, and makes a π-cation interaction with W96 at the back face of the indole rings (assigning the one exposed towards the pore as their front face), thus stabilizing the conformation of this central residue in the pore.

Comparison with other winged helix proteins

A search of the PDB using the Dali server (Holm & Sander, 1996) showed that the LmrR subunit has significant structural homology with various DNA binding proteins containing helix-turn-helix or winged helix-turn-helix domains. Among these are the structurally and functionally characterized MarR/PadR family

members MarR of *E. coli* (PDB accession code 1JGS, 87 equivalent Cα-atoms were superimposed with a root-mean-square-deviation (RMSD) of 2.3 Å), OhrR of *B. subtilis* (1Z9C, 86 equivalent Cα-atoms were superimposed with a RMSD of 2.8 Å), AphA of *Vibrio cholerae* (1YG2, 82 equivalent Cα-atoms were superimposed with a RMSD of 3.7 Å) and Pex of *Synechococcus sp.* (2E1N, 78 equivalent Cα-atoms were superimposed with a RMSD of 1.8 Å). The structural similarities of LmrR with the MarR/PadR proteins are mainly confined to its wHTH domain and the sequence identities are rather low (ranging from 11% for MarR to 28% for Pex). Interestingly, structural similarity was also detected with a number of hypothetical transcriptional regulators in the PDB, for which structures and function have not been published. One such protein, from *Clostridium thermocellum,* showed a particularly high structural homology with LmrR (PDB accession code 1XMA, 99 equivalent Cα-atoms were superimposed with a RMSD of 2.7 Å and a sequence identity of 35%). A structure-based sequence alignment of LmrR with 1XMA, AphA, MarR and OhrR is presented in Figure 2A. Most of the conserved residues are hydrophobic and appear to be important in stabilization of the overall fold of the DNA binding domain. Currently, the only MarR/PadR protein for which a DNA-bound structure is known is OhrR (15). The regions in LmrR that are equivalent to the DNA binding site in OhrR, *i.e.* helix α3 and the β-wing, show the highest degree of conservation. Nevertheless, among the conserved residues only a few have a role in specific DNA binding in OhrR, suggesting that LmrR and OhrR recognize different DNA sequences. Interestingly, the putative DNA-binding sites of LmrR and 1XMA are highly conserved, which may indicate that these proteins bind similar DNA sequences. Unfortunately, the specific DNA binding sequence(s) of LmrR within the *lmrR* and *lmrCD* promoters have not yet been delineated (Agustiandari et al, 2008), and for the *C. thermocellum* homolog the target promoters are unknown. The comparison of LmrR with the four MarR/PadR transcription factors further reveals two invariant residues, G61 and G85, which are found in regions that connect the β-wing to helices α3 and α4 and appear to have crucial structural roles in stabilizing the conformation of the β-wing relative to the DNA recognition helix and the dimerization domain. Another invariant residue is T82, which is located at the proximal end of the wing between β2 and helix α4. Previously, it was shown that a T82I mutation in LmrR is associated with drug-resistant phenotypes of *L. lactis* (22), and that the LmrR-T82I mutant is deficient in both drug and DNA binding (1). In the LmrR structure the side chain hydroxyl group of T82 is making three hydrogen bonds, with the backbone carbonyl of G61 at the N-terminal end of the β-wing and with the backbone amides of I84 and G85 at the N-terminus of helix α4, while the main

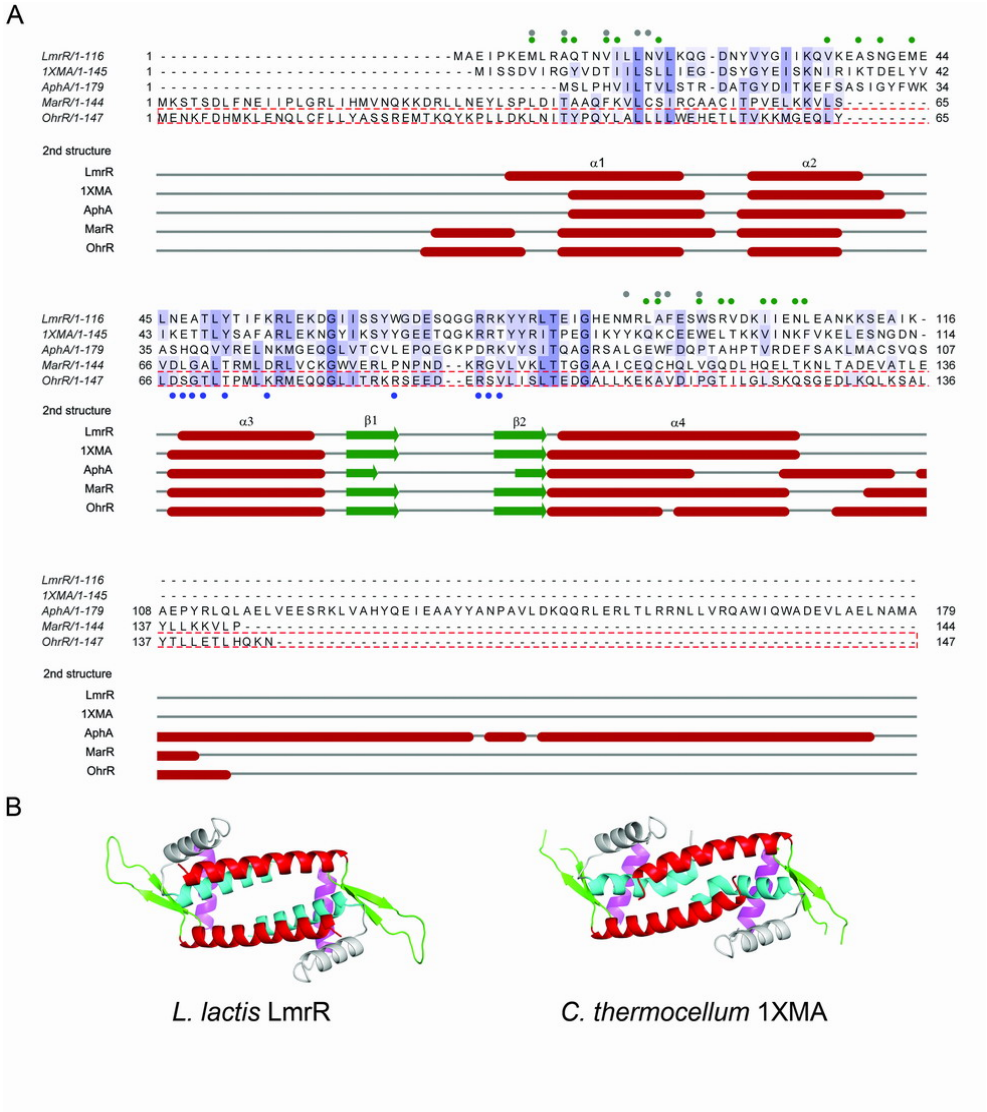

Figure 2. Comparison with other wHTH proteins (A) Structure-based multiple sequence alignment computed with the Tcoffee web server (27) and visualized with JalView (6). Structures were taken from the PDB using the following accession numbers: 1XMA, 1YG2 (AphA), 1JGS (MarR) and 1Z9C (OhrR. Residues of LmrR involved in dimerization are indicated with green dots, residues involved in drug binding with grey dots, and residues in OhrR involved in DNA binding are indicated with blue dots. (B) Ribbon representations of the LmrR and 1XMA dimers. Equivalent secondary structure elements are indicated in specific colors: α1, blue; α2, grey α3, magenta, α4, red and the β-wing, green.

chain amide of T82 forms a hydrogen bond with the backbone carbonyl of I62 (Supplementary Figure S2). These interactions are conserved in the other MarR/PadR structures, and appear to clamp the β-wing in place and stabilize the C-terminal helix. Replacement of T82 by an isoleucine residue will result in the loss of at least three of the four hydrogen bonds, as well as in the introduction of significant steric strain due to the presence of a bulkier and more hydrophobic side chain. The T82I mutation thus is predicted to induce a structural perturbation of LmrR, which probably corresponds to a detachment of the β-wing and a disordering of its C-terminal helix. Such a structural perturbation would explain the deleterious effect of the T82I mutation on overall LmrR function.

The structural organization of the LmrR dimer, with the two DNA-binding domains adjacent to one another and their DNA recognition helices facing the same side, is like in other MarR/PadR family members, pointing to a similar mode of DNA binding with the recognition helices fitting into two successive major grooves on one side of the DNA double helix. However, the dimeric architecture of LmrR is unique: none of the other structurally characterized MarR/PadR family members shows a central pore at the dimer interface. It should be noted, though, that the dimerization modules in MarR/PadR proteins are highly diverse, consisting of different numbers of α-helices packed together in various ways. Of the four proteins that were compared with LmrR, only 1XMA has an identical overall topology containing a single C-terminal dimerization helix. Interestingly, the sequence homology of LmrR with 1XMA extends into the C-terminal helix and among the conserved residues are W96, as well as residues near the C-terminus that in LmrR participate in dimer formation. Nevertheless, unlike in LmrR, in the *C. thermocellum* transcription factor the N- and C-terminal helices of the two subunits form a compact core at the dimeric interface that is completely closed. This difference at the dimer interface is coupled to a difference in the relative orientation of the C-terminal helices with respect to the wHTH domains and to significant bending of the N- and C-terminal helices (Figure 2B), which allow the subunits in the dimer of the *C. thermocellum* transcription factor to approach each other more closely than the subunits in the LmrR dimer. To predict whether the "closed" conformation of the 1XMA dimer would be accessible to LmrR, a 1XMA-based homology model was prepared of LmrR using the SWISS model server (http://swissmodel.expasy.org/). The results indicate that there are no major steric clashes that would prevent LmrR to adopt a "closed" conformation. However, in comparison with the 1XMA dimer, the conserved tryptophan pair and their surrounding residues in LmrR seem unsuitable to provide the necessary hydrophobic packing and interactions to stabilize a closure of the central pore (Supplementary Figure S3). On the other hand, additional overall conformation

changes and rearrangements of side chains could perhaps create a well-packed hydrophobic core. Clearly, homology modeling alone is not sufficient to assess the likelihood of LmrR also adopting a "closed" conformation. More sophisticated methods, like molecular dynamic simulations or NMR, could perhaps provide an answer, but these are beyond the scope of the present study.

Binding of Hoechst 33342 and Daunomycin

The structures of LmrR bound with H33342 and daunomycin reveal that the central pore in the LmrR dimer serves as a multi-drug binding site (Figure 3). In both complexes the pore accommodates a single drug molecule, consistent with the 1:2 (drug:LmrR subunit) stoichiometry of drug-binding obtained previously from fluorescent titrations with H33342 (1). The two drugs show a common mode of binding: their flat ring systems are wedged in between the W96 and W96' side chains forming aromatic stacking interactions with each of the two indole systems (Figures 3C-D). Further stabilization of the bound drugs is provided by various hydrophobic contacts in the pore. Remarkably, no hydrogen bonds are observed between the protein and the drugs. Rather, the orientation of the drugs is such that most of their polar atoms are in a solvent exposed position facing the front or back entrances of the pore. Several bound water molecules are present in the pore to act as hydrogen bond partners of the drugs.

The two drug-bound LmrR structures also show substantial differences. The elongated crescent-shaped Hoechst compound deeply penetrates the pore and stretches out over its entire width with the ethoxy-phenolic and N-methyl-piperazine groups extending towards the sidewalls and facing the back entrance of the pore. The area of the drug-protein interaction surface is substantial (~215 $Å^2$) and shows good shape-complementarity. W96 and W96' clamp down one of the central benzimidazole ring systems, which is aligned in an off-centered parallel orientation with respect to each of the two indole rings. The inter-ring distances are ~3.5 Å, optimal for allowing the formation of strong van der Waals interactions (24).

Less extensive interactions are formed with daunomycin. The presence of the bulky amino sugar substituent prohibits a deep penetration of the drug into the flat pore (Figure 5C). Only the aglycon chromophore interacts with the protein, while the amino sugar is exposed to the solvent at the front entrance of the pore. As a consequence the area of the drug-protein interaction surface in the LmrR-daunomycin complex is much smaller (~82 $Å^2$) than in the LmrR-H33342 complex. No electron density could be observed for the amino sugar of daunomycin, indicating that this substituent is highly flexible.

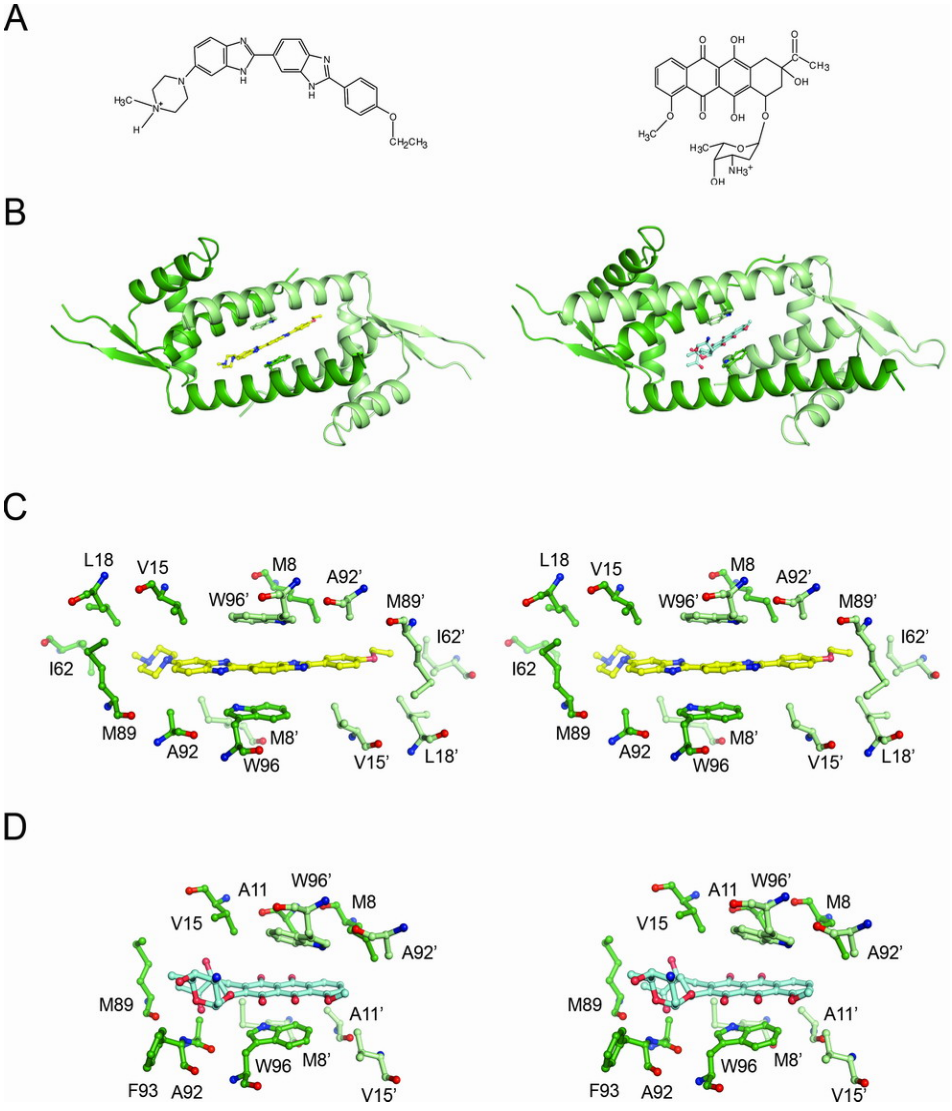

Figure 3. Crystallographic analysis of drug binding to LmrR (A) Chemical structures of H33342 (left) and daunomycin (right). (B) Ribbon diagrams of the LmrR-H33342 (left) and LmrR-daunomycin (right) complex, showing the drug molecule (sticks) bound inside the central pore of the dimer in between the two tryptophan residues W96 and W96′ (sticks). The two subunits of the LmrR dimer, as well as W96 and W96′ are colored with different shades of green. (C) Close-up stereo view of the drug-binding pore in the LmrR-H33342 complex depicting the drug and residues that contact the drug (maximum contact distance defined as 4 Å). (D) Similar stereo view as in C of the drug-binding pore in the LmrR-daunomycin complex.

The stacking interactions of the aromatic rings with the W96/W96' pair form the main contribution to drug binding stabilization, but the stacking geometry is less optimal than in the LmrR-H33342 complex. The weaker interactions between LmrR and daunomycin, as compared to the interactions between LmrR and H33342, are further illustrated by the relatively high atomic B-factors of daunomycin (Table 1).

Drug Binding Affinities and Importance of W96 for Drug and DNA Binding

To quantify the difference in binding affinity of LmrR for H33342 and daunomycin the dissociation constants of the two drugs were approximated from binding curves obtained from two different fluorescence based drug-binding assays (Figure 4A). Binding of H33342 to untagged LmrR was monitored by recording the increase in drug fluorescence when H33342 moves from an aqueous to a hydrophobic environment, *i.e.* when it binds to the drug binding site of LmrR. The apparent dissociation constant of H33342 obtained from fitting the binding curve was ~20 nM, showing that this compound has a strong affinity for the drug binding site of LmrR. Unfortunately, the spectral properties of daunomycin did not allow the use of a similar binding assay. Instead for daunomycin, we obtained a binding curve by measuring the fluorescence quenching of W96 upon titration of the drug. LmrR contains two tryptophan residues, W67 and W96, the former of which is located in the β-wing of the DNA binding domain. To avoid unwanted disturbances of the fluorescence signal, W67 was mutated to either alanine or tyrosine. The W67Y and W67A mutations had no significant effect on the binding of H33342 by LmrR, nor on the binding of *lmrCD* promoter DNA (Figures 4B and 4C). The dissociation constant of daunomycin, determined from W96-fluorescence quenching using the LmrR-W67Y mutant, was ~0.25 μM, confirming that its binding affinity for LmrR is weaker than the Hoechst compound.

To confirm its importance for drug binding, W96 was also mutated to alanine or tyrosine. Both these LmrR mutants, W96A and W96Y, lost the ability to bind H33342 (Figure 4B). Interestingly, although the W96Y mutant was still able to bind to the *lmrCD* promotor, the W96A mutant was not, nor was a W67Y/W96Y double mutant (Figure 4C). The impaired DNA binding of the W67Y/W96Y mutant is not due to a loss of structural integrity of the protein, as was assessed by circular dichroism (Supplementary Figure S4A). These results thus point to some indirect role in DNA binding for residue 96, in addition to a direct role in drug binding.

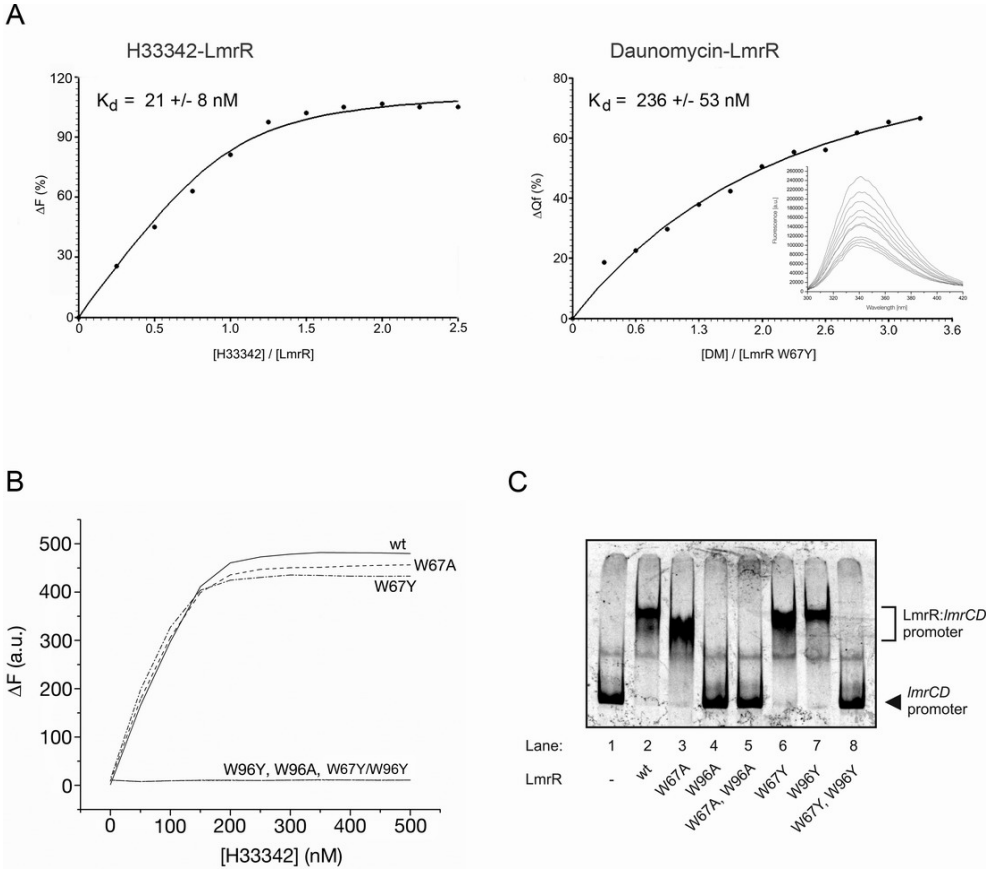

Figure 4. Spectroscopic and mutational analysis of drug and DNA binding by LmrR (A) Fluorescence titration curves measuring H33342 and daunomycin binding to LmrR. Binding of H33342 was monitored by recording the increase in drug fluorescence when titrating a solution of wild-type LmrR with increasing concentrations of H33342, using experimental conditions are described (1). Binding of daunomycin was monitored by performing tryptophan fluorescence quenching titration of the LmrR mutant W67Y with increasing concentrations of daunomycin. The apparent K_D values are calculated from the fitting of the data using non-linear regression analysis (r^2=0.993 and 0.996 for H33342 and daunomycin, respectively). Inset, tryptophan fluorescence emission spectra in the presence of increasing concentrations of daunomycin. (B) Fluorescence titration curves measuring H33342 binding to the W67 and W97 single mutants and the W67Y/W96Y double mutant of LmrR. The binding curves for the W96 single mutants, as well as the W67A/W96Y double mutant, are flat, thus revealing that these mutants lack drug binding capability. (C) Electrophoretic mobility shift assay (EMSA) of the LmrR mutants analyzing their binding to the *lmrCD* promoter DNA.

Conformational flexibility

As pointed out earlier notable conformational differences were identified between the different LmrR structures (Figure 5). Pair-wise structural superpositions of the isolated subunits in the different dimers, using all Cα atoms, result in root-mean-square deviations ranging from 1.0 to 2.0 Å. These deviations primarily result from differences in the orientation of the C-terminal helix α4 relative to the wHTH domain. The reorientations of helix α4 may be described as lever-arm rotations with the residues that attach the N-terminus of the helix to the wHTH domain serving as a hinge (Figure 5A). Even though the rotations are small (varying between 9° and 16°), the lever-like movement results in substantial translational shifts of residues near the C-terminus of helix α4 (up to ~8 Å as calculated from Cα-Cα distances). Except for small shifts of helix α1 and α2 no significant conformational changes are observed in the wHTH domains. Via the dimeric interface the conformational differences in the subunits are coupled to two pronounced differences in the LmrR dimeric structures. Firstly, when comparing the H33342-bound and daunomycin-bound LmrR dimers with the apo-LmrR dimer, one of the wHTH domains is rotated relative to the wHTH domain of the other subunit. The amounts of rotation are about equal for both drug-bound complexes (~14°), but the rotations are in opposite directions (Figure 5B). In fact, the largest conformational change (~28° rotation) is observed when mutually comparing the H33342-bound and daunomycin-bound LmrR dimers. The rotations of the wHTH domains are coupled to changes in the spacing between the two DNA recognition helices. The shortest spacing is observed in the daunomycin bound complex with a distance of ~26 Å (measured from the centroids of the helices), while in the H33342 bound complex the spacing is the largest with a distance of ~32 Å. Secondly, inside the pore differences are observed in the orientations of residues from one subunit relative to those of the other subunit. These differences are caused by shifts of the α1-α4' helix pair relatively to the α1'-α4 helix pair in the different LmrR structures, and directly affect the geometry of the central drug binding site (Figure 5C).

Unfortunately, since each crystal structure of LmrR represents a different crystal form, it is not possible to distinguish whether the conformational changes are drug-induced, or whether they are caused by differences in crystal packing. However, the observed structural differences point to a remarkable plasticity of LmrR. As the conformational rearrangements affect both the drug binding and DNA binding sites in the LmrR dimer, similar conformational changes likely play an important role in the induction mechanism of LmrR.

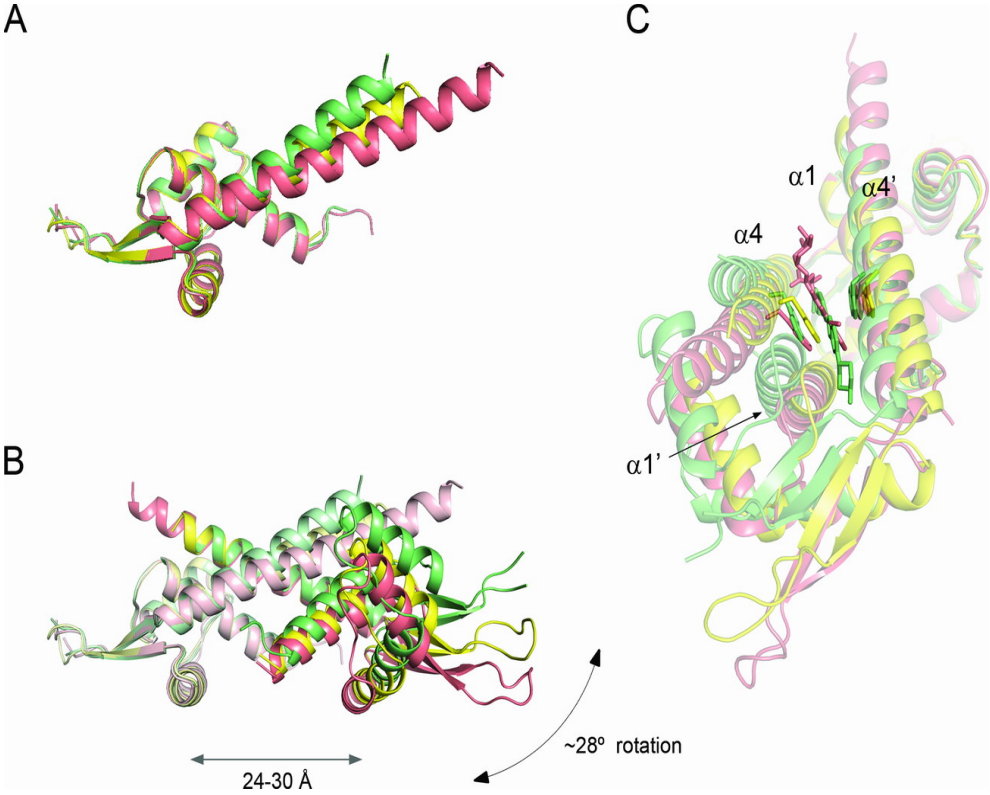

Figure 5. Conformational differences between the apo and two drug-bound structures of LmrR. (A) Superposition (in ribbon representation) of the apo-LmrR subunit structure (yellow) and the subunit structures of H33342-bound (green) and daunomycin-bound LmrR (salmon). The superposition was carried out using the Cα-atoms of the wHTH domain. (B) Superposition of the three LmrR dimers, showing the difference in relative position of the two wHTH domains. Only one of the two subunits (light colors) was used for the superposition (identical to the superposition in Figure 5A). Indicated are the range of distances between the two DNA recognition helices in the different dimers, and the largest rotational shift of the wHTH domains (based on comparing the H33342-bound and daunomycin-bound dimers). (C) The same superposition as in Figure 5B, but from a different view, showing the relative shifts of helix pair α1-α4' with respect to helix pair α1'-α4. Also shown in sticks are H33342 (green) and daunomycin (salmon), as well as the W96/W96' tryptophan pair. The sugar moiety of daunomycin is shown in a solvent exposed position at the front entrance of the pore, but it should be noted that its binding is highly disordered, as evident from the weak electron density associated with this subsituent.

DISCUSSION

The crystal structure of LmrR is the first structure of a transcription factor regulating the expression of a multi-drug ABC transporter. It is also the first time that the structural basis of multi-drug recognition has been studied for a PadR transcriptional regulator. Current knowledge of the mechanism of multi-drug recognition by transcriptional regulators of multi-drug transporters is largely based on crystallographic studies with the transcription factors BmrR from *Bacillus subtilis* (34), QacR from *Staphyloccoccus aureus* (29,30), and TtgR from *Pseudomonas putida* (3). The structures of LmrR bound to H33342 and daunomycin confirm the importance of several of the general structural features that seem important in multi-drug recognition, *e.g.* the availability of a large drug-binding pocket that can accommodate a large spectrum of drug ligands, the importance of aromatic and hydrophobic residues for providing van der Waals interactions to stabilize the bound ligands, the importance of water molecules for occupying regions of the pocket not occupied by the ligand and for solvating hydrophilic groups of the ligand that do not interact with the protein, and a flexible pocket wall that can change conformation upon ligand binding. However, there are also some striking differences in the way multi-drug binding is accomplished by LmrR as compared to BmrR and QacR. The foremost difference is that in LmrR the multi-drug binding pocket is formed by a symmetric pore located at the dimer centre with both subunits contributing equally to its architecture. In QacR and BmrR the drug-binding pockets are asymmetric and primarily formed within a single subunit. Also, in the drug-bound LmrR complexes the binding modes of the two different drugs are very similar and involve a common and strong aromatic stacking interaction with the W96/W96' tryptophan pair. In the other transcription factors the architecture of the drug-binding pockets allows different ligands to adopt different orientations within the pocket and to interact with different sets of amino acids. In BmrR and QacR, the binding affinity for cationic drugs is further augmented by electrostatic attraction between the positively charged ligand and buried or partially buried negatively charged glutamates or aspartate residues of the protein. No such interactions were observed in the H33342- and daunomycin-bound LmrR structures. However, in LmrR positive charges in the drugs may be stabilized by long range electrostatic interactions with the cluster of glutamate and aspartate residues that surround the front entrance of the pore. Also the apparent molecular dipole moment that was found running through the pore of LmrR may assist in attracting and binding cationic drugs. The relevance of this latter feature of the LmrR structure for drug specificity is however very unclear: it could be likewise important for directing LmrR towards the DNA substrate.

It is evident that the homology of LmrR with other members of the PadR protein family that have so far been functionally and structurally characterized, *i.e.* AphA and Pex, is relatively low. Our results thus confirm the classification of two PadR-subfamilies (16): subfamily I with longer sequences (~180 amino acids) to which AphA and Pex belong, and a more distant subfamily II with shorter sequences (~110 amino acids) to which LmrR belongs. To the best of our knowledge LmrR is so far the only member of PadR subfamily II that has been characterized both functionally and structurally. Interestingly, a BLAST search against translated nucleotide sequence databases (see Supplementary Figure S5) yields a large number of close homologs of LmrR that exist in various bacterial species, in particular those belonging to the Firmicutes (*Listeria, Bacillus, Staphylococcus, Enterococcus, Lactobacillus, Streptococcus, Clostridium*). Both the N- and C-terminal domain of LmrR are significantly conserved in these proteins, including W96, thus pointing to a high similarity in overall structure and dimeric organization. Likely, some of these proteins also have a similar role as LmrR in regulating multi-drug resistance, but lack of functional data prohibits such assessment for the moment. Furthermore, the sequence conservation in the C-terminal helix of these LmrR-like proteins is no guarantee for the existence of a central multi-drug binding pore, as is evident from the structural comparison of LmrR with the *C. thermocellum* homolog. Future studies should therefore reveal whether the multi-drug binding characteristics of LmrR are applicable to a larger set of proteins.

In the absence of a DNA-bound structure, the induction mechanism of LmrR remains to be determined. Comparison of the different LmrR structures, and the effects of the W96 mutations, reveals a possible allosteric coupling between the drug and DNA binding sites. Most likely the binding of a drug to LmrR locks the dimer in a conformational state that is incompatible with DNA binding, due to a relative positioning of the DNA recognition helices that is unsuitable for their simultaneous insertion in the successive major grooves of the DNA. Such an induction mechanism would be similar to the induction mechanism of various wHTH-domain containing transcription factors, although the origin and nature of the structural changes involved in this mechanism are likely to be different for LmrR. To explore this further we tested the suitability of the different LmrR structures to bind B-form DNA using model building. None of the three LmrR structures has a conformation that allows a good fit with DNA (Supplementary Figure S6). In daunomycin-bound LmrR the DNA recognition helices are spaced too close together and would sterically clash with the DNA. In contrast, in the apo form and H33342-bound complex of LmrR they are positioned too far apart, and one of the two wHTH DNA binding domains is shifted away from the DNA. It

should be noted, though, that such modeling does not take into account the possibility of DNA distortion, thus limiting its significance. Further studies are underway to better define the DNA binding characteristics of LmrR and unequivocally identify its operator DNA sequence. This knowledge will be crucial to allow crystallization of a DNA-bound LmrR complex.

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REFERENCES

1. Agustiandari H, Lubelski J, van den Berg van Saparoea HB, Kuipers OP, Driessen AJ. 2008. LmrR is a transcriptional repressor of expression of the multidrug ABC transporter LmrCD in Lactococcus lactis. J Bacteriol 190: 759-763
2. Alekshun MN, Levy SB, Mealy TR, Seaton BA, Head JF. 2001. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. Nat Struct Biol 8: 710-714
3. Alguel Y, Meng C, Terán W, Krell T, Ramos JL, Gallegos M-T, Zhang X .2007. Crystal structures of multidrug binding protein TtgR in complex with antibiotics and plant antimicrobials. J Mol Biol 369: 829-840
4. Aravind L, Anantharaman V, Balaji S, Babu MM, Iyer LM. 2005. The many faces of the helix-turn-helix domain: transcription regulation and beyond. FEMS Microbiol Rev 29: 231-262
5. Arita K, Hashimoto H, Igari K, Akaboshi M, Kutsuna S, Sato M, Shimizu T. 2007. Structural and biochemical characterization of a cyanobacterium circadian clock-modifier protein. J Biol Chem 282: 1128-1135
6. Clamp M, Cuff J, Searle SM, Barton GJ. 2004. The Jalview Java alignment editor. Bioinformatics 20: 426-427
7. Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB, 3rd, Snoeyink J, Richardson JS, Richardson DC. 2007. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res 35: W375-383
8. De Silva RS, Kovacikova G, Lin W, Taylor RK, Skorupski K, Kull FJ. 2005. Crystal structure of the virulence gene activator AphA from Vibrio cholerae reveals it is a novel member of the winged helix transcription factor superfamily. J Biol Chem 280: 13779-13783
9. Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 60: 2126-2132
10. Gury J, Barthelmebs L, Tran NP, Divies C, Cavin JF. 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding padA gene of Lactobacillus plantarum. Appl Environ Microbiol 70: 2146-2153

11. Hayward S, Berendsen HJ. 1998. Systematic analysis of domain motions in proteins from conformational change: new results on citrate synthase and T4 lysozyme. Proteins 30: 144-154
12. Heldwein EE, Brennan RG. 2001. Crystal structure of the transcription activator BmrR bound to DNA and a drug. Nature 409: 378-382
13. Higgins CF. 2007. Multiple molecular mechanisms for multidrug resistance transporters. Nature 446: 749-757
14. Holm L, Sander C. 1996).Mapping the protein universe. Science 273: 595-603
15. Hong M, Fuangthong M, Helmann JD, Brennan RG. 2005. Structure of an OhrR-ohrA operator complex reveals the DNA binding mechanism of the MarR family. Mol Cell 20: 131-141
16. Huillet E, Velge P, Vallaeys T, Pardon P. 2006. LadR, a new PadR-related transcriptional regulator from Listeria monocytogenes, negatively regulates the expression of the multidrug efflux pump MdrL. FEMS Microbiol Lett 254: 87-94
17. Janin J, Miller S, Chothia C. 1988. Surface, subunit interfaces and interior of oligomeric proteins. J Mol Biol 204: 155-164
18. Jaroszewski L, Rychlewski L, Li Z, Li W, Godzik A. 2005. FFAS03: a server for profile--profile sequence alignments. Nucleic Acids Res 33: W284-288
19. Kleywegt GJ. 1999. Experimental assessment of differences between related protein crystal structures. Acta Crystallogr D Biol Crystallogr 55: 1878-1884
20. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 26: 283-291
21. Leslie AG. 2006. The integration of macromolecular diffraction data. Acta Crystallogr D Biol Crystallogr 62: 48-57
22. Lubelski J, de Jong A, van Merkerk R, Agustiandari H, Kuipers OP, Kok J, Driessen AJ. 2006. LmrCD is a major multidrug resistance transporter in Lactococcus lactis. Mol Microbiol 61: 771-781
23. McCoy AJ. 2007. Solving structures of protein complexes by molecular replacement with Phaser. Acta Crystallogr D Biol Crystallogr 63: 32-41
24. McGaughey GB, Gagne M, Rappe AK. 1998. pi-Stacking interactions. Alive and well in proteins. J Biol Chem 273: 15458-15463
25. Murshudov GN, Vagin AA, Dodson EJ. 1997. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr D Biol Crystallogr 53: 240-255
26. Newman J, Egan D, Walter TS, Meged R, Berry I, Ben Jelloul M, Sussman JL, Stuart DI, Perrakis A. 2005. Towards rationalization of crystallization screening for small- to medium-sized academic laboratories: the PACT/JCSG+ strategy. Acta Crystallogr D Biol Crystallogr 61: 1426-1431
27. Poirot O, O'Toole E, Notredame C. 2003. Tcoffee@igs: A web server for computing, evaluating and combining multiple sequence alignments. Nucleic Acids Res 31: 3503-3506
28. Saier MH, Jr., Paulsen IT, Sliwinski MK, Pao SS, Skurray RA, Nikaido H. 1998. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. FASEB J 12: 265-274
29. Schumacher MA, Brennan RG. 2003. Deciphering the molecular basis of multidrug recognition: crystal structures of the Staphylococcus aureus multidrug binding transcription regulator QacR. Res Microbiol 154: 69-77
30. Schumacher MA, Miller MC, Grkovic S, Brown MH, Skurray RA, Brennan RG. 2001. Structural mechanisms of QacR induction and multidrug recognition. Science 294: 2158-2163

31. Terwilliger TC. 2003. SOLVE and RESOLVE: automated structure solution and density modification. Methods Enzymol 374: 22-37
32. Winn MD, Isupov MN, Murshudov GN. 2001. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. Acta Crystallogr D Biol Crystallogr 57: 122-133
33. Winn MD, Murshudov GN, Papiz MZ. 2003. Macromolecular TLS refinement in REFMAC at moderate resolutions. Methods Enzymol 374: 300-321
34. Zheleznova EE, Markham PN, Neyfakh AA, Brennan RG. 1999. Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. Cell 96: 353-362

Supplementary materials and methods

Drug binding assays

Protein concentrations were calculated from the absorbance at 280 nm (A280). The H33342-binding assay was performed as follows: to a solution of purified LmrR (0.2 μM as dimer) in 50 mM Tris-HCl, pH 7.0, increasing amounts of H33342 were added and the fluorescence was followed at excitation and emission wavelengths of 355 and 457 nm, respectively, with slit widths of 5 nm using a Perkin-Elmer LS 50B spectrofluorometer at room temperature. Fluorescence data were corrected as described previously [1]. To monitor the binding of daunomycin to LmrR the following procedure was employed. To a solution of LmrR (0.15 μM of dimeric species, 120 μl) in 50 mM Tris-HCl, pH 8.0, small volumes (1.2 μl) of a daunomycin stock solution were added in sequential steps. After each titration step the solution was mixed thoroughly and incubated for 2 minutes before measurements were made. Tryptophan fluorescence was recorded at room temperature with an Aminco Bowman Series 2 spectrofluorometer using a 0.2 ml cell and an excitation wavelength of 295 nm. Emission spectra were obtained from 300 to 450 nm. The excitation and emission slit widths were 4 nm. Each spectrum was scanned twice to obtain the final fluorescence emission spectra. Spectra were corrected for background emission and dilution effects.

Apparent dissociation constants (K_d) of the two drugs were obtained by data fitting using non-linear regression analysis (program LabFit), employing the following equation [2]:

$$RF = \frac{k}{2}\left(C_P + C_L + K_d - \sqrt{(C_P + C_L + K_d)^2 - 4C_P C_L}\right)$$

where RF is either the relative increase in fluorescence intensity (ΔF of H33342 upon binding to LmrR (expressed as $(I-I_0)/(I_{max}-I_0)$, with I_0 the intensity of fluorescence in the absence of ligand, I the intensity of fluorescence upon addition of ligand and I_{max} the maximum intensity of fluorescence at saturation) or the tryptophan fluorescence quenching (ΔQ_F) by daunomycin at 340 nm (expressed as $(I_0-I)/I_0$, with I_0 the intensity of fluorescence in the absence of quencher and I the

intensity of fluorescence upon addition of quencher). Cp and C_L are the total concentrations of protein and ligand (drug), respectively, and *k* is a constant.

Control experiments

As a control the drugs were titrated to a solution of the neutral tryptophan analog N-acetyl-tryptophanamide (0.4 mM) in 20 mM Tris-HCl, pH 8.0, 270 mM NaCl, and the fluorescence data were recorded using an identical setup as with the LmrR protein solution. No significant changes in fluorescence were recorded (Supplementary Figures S4B-C), thus showing that the changes in fluorescence recorded for drug titration in the presence of LmrR
are protein-driven, and not merely the result of an intrinsic affinity of the drugs for tryptophan.

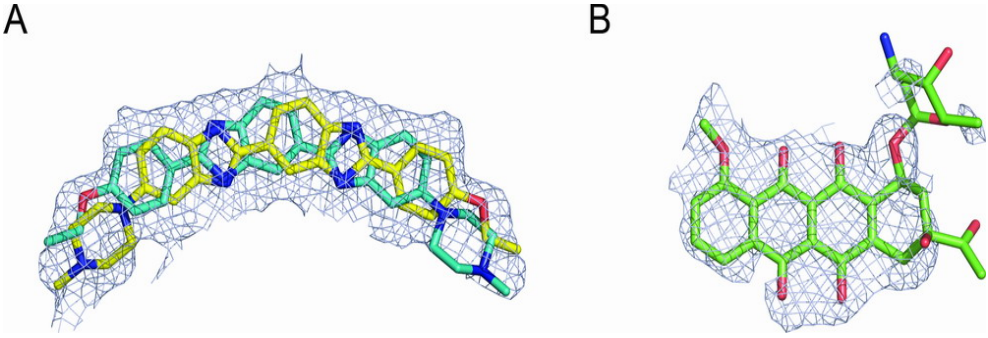


Figure S1. The binding of H33342 and daunomycin to LmrR. (A) σ_A-weighted *Fo-Fc* omit electron-density for bound H33342 (in sticks, showing the two binding modes that are related by the crystallographic dyad). Carbon atoms are shown in yellow or cyan (to distinguish the two binding modes), oxygen in red and nitrogen in blue. (**B**) σ_A-weighted *Fo-Fc* omit electron density for bound daunomycin. To remove model bias, the protein models (after removing the drugs) were subjected to 30 rounds of refinement by REFMAC [3], prior to map calculation. The contour level of the maps is 3s (H33342) and 2.5s (daunomycin) and the resolution is 2.2 Å.

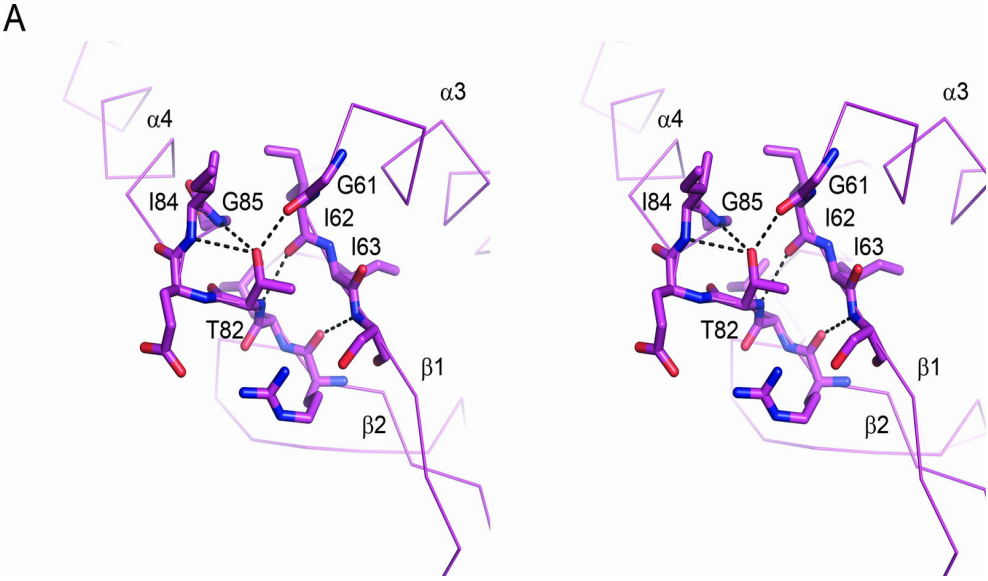


Figure S2. Stereo view showing the environment and interactions of T82 in the apo LmrR structure. Hydrogen bonds are indicated with dashed lines.

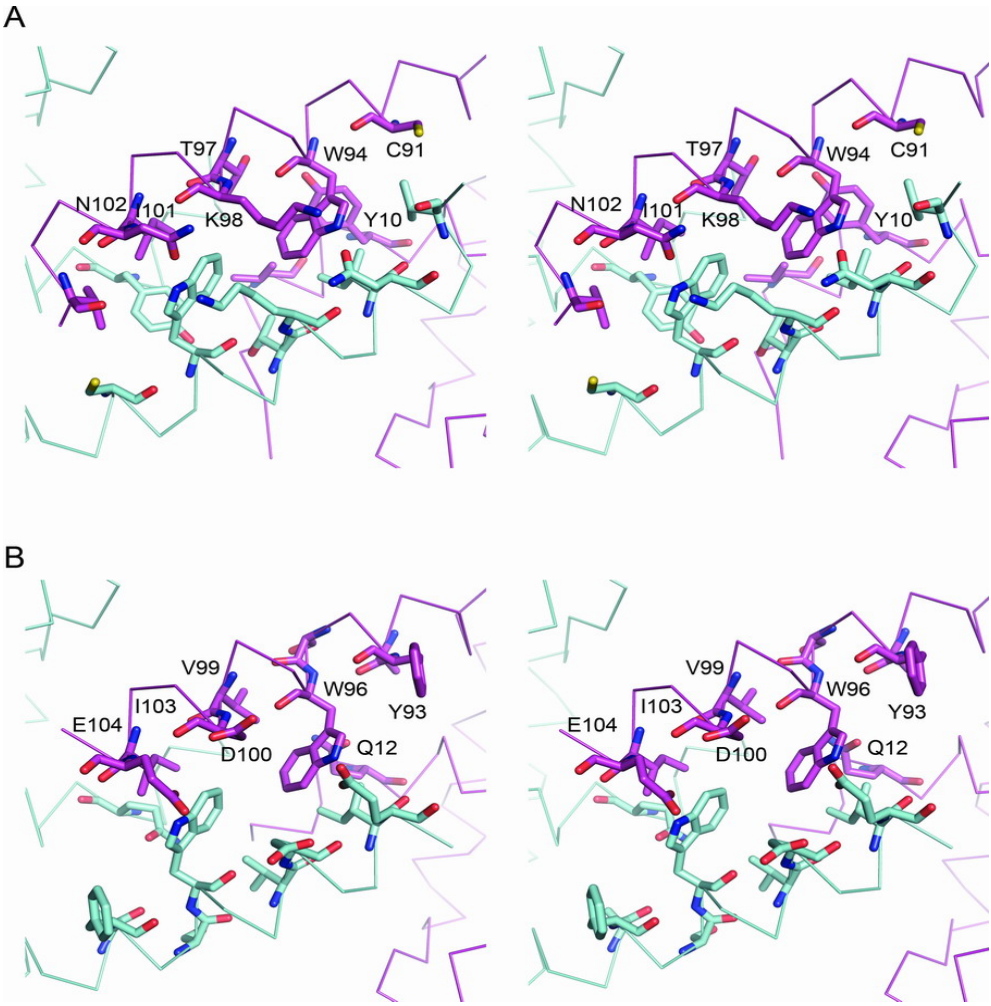


Figure S3. Assessment of LmrR adopting a 1XMA-like "closed" conformation. (**A**) Stereo view of the centre of the 1XMA dimer, along the 2-fold rotation axis, showing the environment of the conserved tryptophan pair (residues W94 and W94') and their close-packing with neighbouring residues. The two subunits in the dimer are differently colored in magenta and cyan (oxygen atoms in red, nitrogen in blue, sulphur in yellow. Only residues of one subunit are labeled. (**B**) Similar stereo view as in A of the centre of a "closed" LmrR dimer, obtained via homology-modeling using the 1XMA dimer as a template. Labels refer to the residues that are equivalent to those in 1XMA.

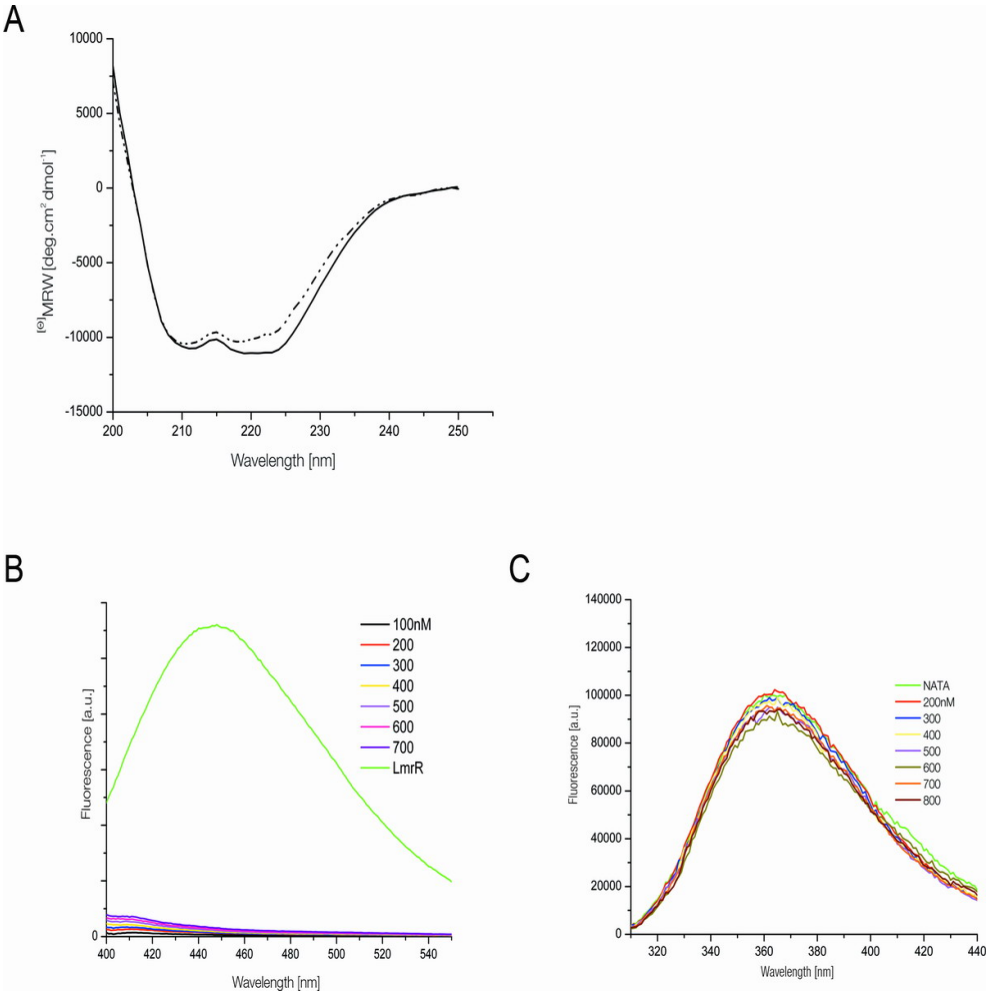


Figure S4. Spectroscopic control experiments (**A**) Circular dichroism spectra of wild-type LmrR (solid line) and the LmrR-W67Y/W96Y double mutant (dashed line). (**B**) Fluorescence titration experiment of H33342 to a 0.4 μM solution of N-acetyltryptophanamide (NATA). The flat lines show the fluorescence spectra of the NATA solution after adding increasing amounts of H33342 (final concentrations ranging from 100 to 700 nM). The upper green line shows the fluorescence spectrum after adding LmrR (to a final concentration of 0.2 μM as dimer) to the H33342/NATA solution at the end of the titration series. (**C**) Tryptophan fluorescence spectra recorded after titrating increasing amounts of daunomycin (final concentrations 200-800 nM) to a 0.4 μM
NATA solution.

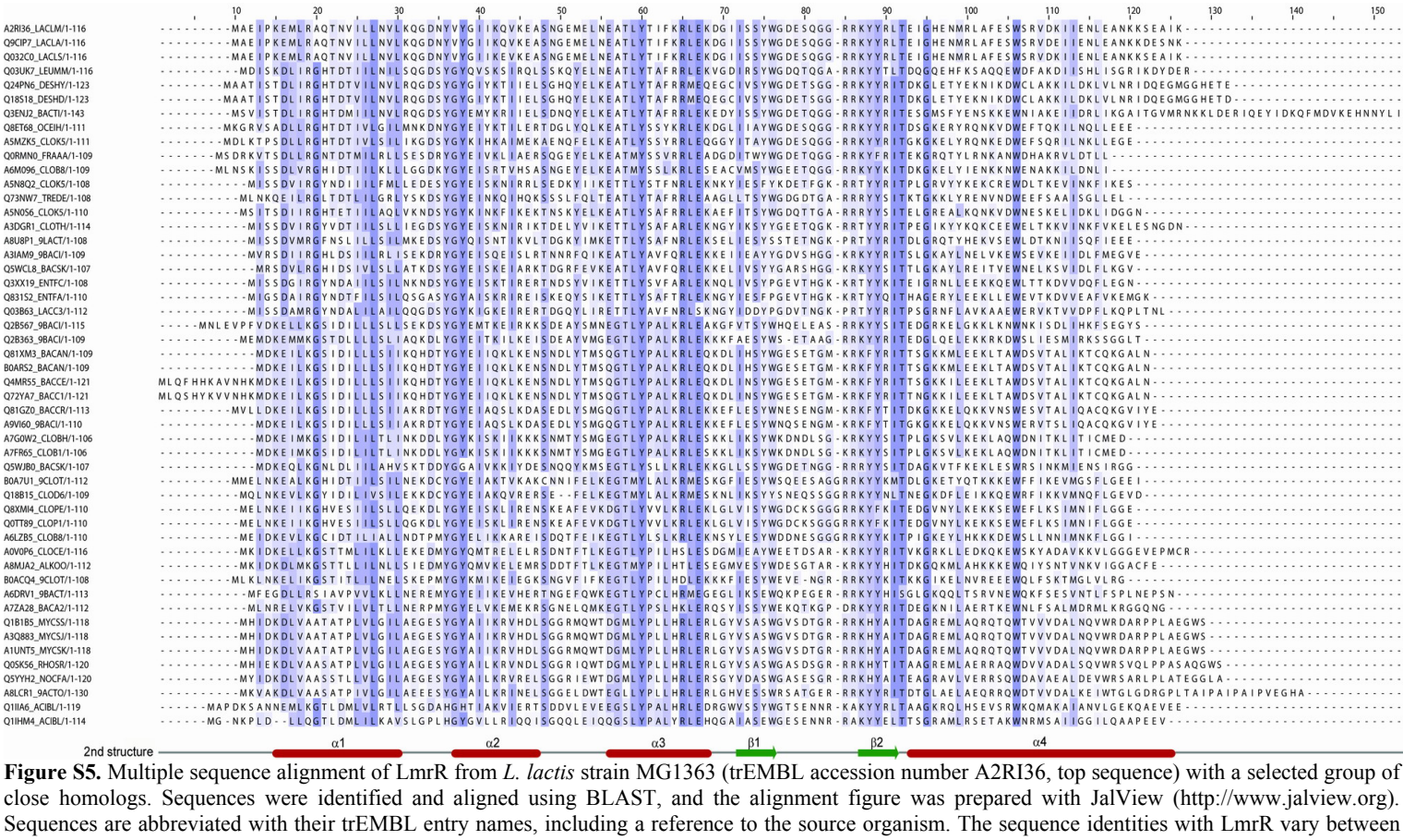


Figure S5. Multiple sequence alignment of LmrR from *L. lactis* strain MG1363 (trEMBL accession number A2RI36, top sequence) with a selected group of close homologs. Sequences were identified and aligned using BLAST, and the alignment figure was prepared with JalView (http://www.jalview.org). Sequences are abbreviated with their trEMBL entry names, including a reference to the source organism. The sequence identities with LmrR vary between 25-99% (the highest identity is with LmrR from *L. lactis* strain SK11) with 30% of the sequences having a sequence identity above 35%. The secondary structure assignment is based on the LmrR crystal structure. In blue boxes are the conserved residues (based on identities), with the more highly conserved residues indicated by darker shades.

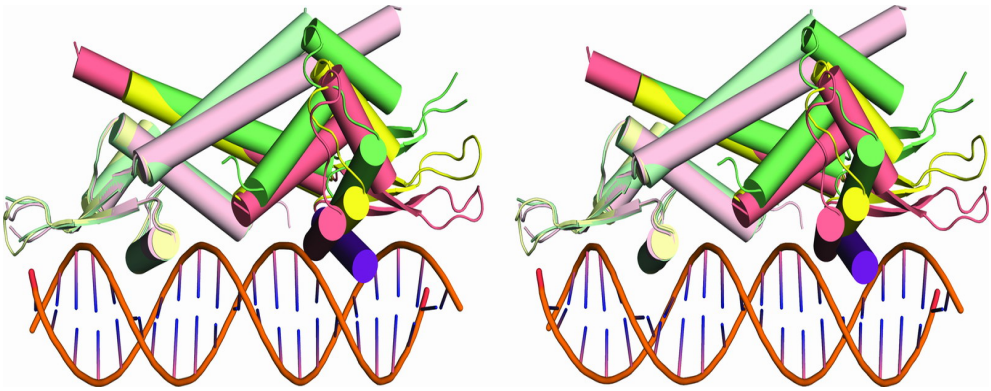


Figure S6. Stereo view of the three superimposed LmrR dimers (apo, H33342-bound and daunomycin-bound) docked to a 21 base-pair fragment of double-stranded B-form DNA. Docking was performed manually using the programs Coot [4] and PyMOL (Delano Scientific). One of the two DNA recognition helices in each dimer (in the figure defined as the left one) could be optimally positioned in one of the two neighbouring major grooves of the DNA, but this resulted in a significant misalignment of the second DNA recognition helix with respect to the other major groove. The optimal location for the second DNA recognition helix that would allow the formation of binding interactions is indicated in dark magenta. Colors for the different LmrR structures are as follows: yellow, apo; green, H33342-bound; salmon, daunomycin-bound. The light and dark colors distinguish the two subunits in each dimer.

Chapter

Summary and concluding remarks

The multidrug resistance (MDR) phenomenon is the ability of cells to develop resistance against a wide range of structurally unrelated toxic molecules. MDR is caused by the (over) expression of transporters that expel these compounds from the cell. Multidrug resistance has lead to multiple complications in the treatment of for instance cancer or infectious diseases. Examples of the latter are the extensively drug resistance tuberculosis (XDR-TB) and methicillin-resistant *Staphylococcus aureus* (MRSA) which have caused death of millions of people worldwide. The introduction and improper use of antibiotics have led to a selection of bacteria that by mutation(s) in their genome developed resistance mechanisms which allowed them to survive under this severe antibiotics pressure. Several different types of resistance mechanisms can be identified: 1) the enzymatic degradation and inactivation of dugs, 2) the alteration of the drug targets, 3) the prevention of drug entry, and finally 4) the active drug extrusion via the overexpression of membrane protein transporters (MDR transporters) (Chapter 1). Sequence analysis conducted on several bacterial genomes revealed that the presence of MDR-like transporters is ubiquitous in nature. Many efforts have been directed to elucidate the physiological function(s) of MDR transporters including their mechanisms of transport, substrate recognition, and their regulation of expression (Chapter 1).

The regulation of bacterial MDR transporter is an important aspect of multidrug resistance as deregulation is often the cause for the MDR phenotype. The majority of bacterial MDR transporters are subject to regulation at the transcriptional level by local or global regulatory proteins. In general, bacteria harbor resistance mechanisms which allow them to respond to the exposure to toxic molecules in the environment. This condition resulted in the up-regulation of low-expressed MDR transporters in the cells. For example, the well characterized local transcriptional activator BmrR of *Bacillus subtilis* and transcriptional repressor QacR of *Staphylococcus aureu*s regulate the expression of the MDR efflux pumps Bmr and QacA, respectively (Chapter 1). A typical MDR regulator comprises of two domains; the N-terminal DNA binding domain and the C-terminal ligand binding domain. A common feature of the DNA binding domain is the presence of the helix-turn-helix (HTH) motif. Further classification of MDR regulators is based on their high sequence homology of the DNA binding domain, and on the basis of this feature four different families can be assigned: AraC, MarR, MerR, and TetR. Interestingly, recent findings indicate that the MDR regulators LadR of *Listeria monocytogenes* and LmrR of *Lactococcus lacti*s belong to the PadR family of transcriptional regulators that have previously not been implicated in MDR. Unlike the membrane bound MDR transporters, transcriptional regulators are soluble proteins and can be overexpressed relatively straight forward to a higher amount. A structural and functional characterization of these MDR transcriptional regulators

is therefore important because these proteins often recognize a similar range of substrates as observed for the transporter that they regulate. This feature makes transcriptional regulators as ideal candidates to study the molecular basis of multidrug recognition. These new insights will be useful for the further development of the new antimicrobial compounds (Chapter 1).

*Lactococcus lacti*s is a Gram positive lactic acid bacterium and widely used in the production of fermented food products. Its genome contains about 40 putative MDR transporter genes of which some have been implicated in the energy dependent efflux of unrelated lipophilic and toxic compounds. For example the secondary transporter LmrP depends on the proton motive force to expel a range of drugs, while the ATP-binding cassette (ABC) transporters LmrA (3) and LmrCD (2) use the free energy derived from ATP hydrolysis to fulfill similar functions. Recent gene inactivation studies suggest that LmrC and LmrD are responsible for the intrinsic multidrug resistance in *L. lactis*. These are ABC half-transporter that heterodimerize to form a functional MDR transporter. The overexpression of LmrCD resulted in the cellular protection against the toxic effects of several drugs e.g. daunomycin, ethidium bromide, and Hoechst 33342 (Chapter 2), while deletion of the *lmrCD* genes from the genome of *L. lactis* NZ9000 strain renders cells hyper sensitive to these drugs. Moreover, the resistance phenotype of the *ΔlmrCD* strain can be restored to the wild-type level by the overexpression of *lmrCD* in trans. Interestingly, the overexpression of *lmrA* gene showed no effect and rendered the cells even more susceptible to the drugs (Chapter 2). DNA array analysis on four MDR strains of *L. lactis* that were previously grown in the presence of increasing concentrations of daunomycin, ethidium bromide, cholate, and rhodamine 6G revealed a significant up regulation of the *lmr*C and *lmrD* genes, and of a gene located upstream of *lmrCD* termed *lmrR* (*l*actococcal *m*ultidrug *r*esistance *r*egulator) formerly known as *ydaF*. Nucleotide sequencing analysis showed the presence of frame shifts and point mutations in the *lmr*R gene in these four MDR strains resulting in the production of non functional LmrR variants. These results suggested a possible role of LmrR in the down regulation of the expression of *lmrCD* and *lmrR* in *L. lactis* (Chapter 2).

By homology, LmrR belongs to the PadR family of transcriptional regulators. PadR regulators are involved in regulating the expression of the phenolic acid decarboxylase (*pad*) gene(s) that detoxifies derivatives of phenolic acids such as ρ-coumaric, ferulic, and caffeic acids (1). Many members of the PadR family are closely related to the MarR family of multiple antibiotic resistances found in bacteria and archaea. Transcriptome analysis of a Δ*lmrR* strain showed the significant up regulation of the *lmrC* and *lmrD* genes and not of other genes (Chapter 3). This result demonstrated that LmrR is a local transcriptional regulator

for the expression of *lmrCD* genes only, and confirmed the previous notion that LmrCD is the main transporter responsible for the acquired MDR phenotype of the various selected MDR strains of *L. lactis*. Further characterization showed that wild-type LmrR binds to two distinct sites on the promoter region of *lmrCD*; site 1 is located between the -35 and -10 region whereas site II contains two direct imperfect inverted repeats with a motif almost similar to the PadR binding site on the padA promoter (Chapter 3). Direct drug binding assays showed LmrR to interact with these molecules, while RT-PCR studies suggested that this drug binding event signals the induction of the expression of *lmrCD* genes. A simple model for the regulation of *lmrCD* gene expression by LmrR would be as follows: when the hydrophobic drug molecules are present in the environment they will permeate the cells, the DNA-bound LmrR binds the drug and undergoes a conformational changes that causes its release from the operator sites of the *lmrCD* genes whereupon RNA polymerase can initiate transcription of *lmrCD*. LmrCD will extrude the drug from the cells, and because of the lowered intracellular drug concentration, LmrR will return in its apo state and rebind to the operator sites and repress the *lmrCD* genes (Chapter 3). The transcriptome analysis of the independently isolated MDR strains also showed a significant increase in transcript levels of *lmrR* gene. This observation suggested that LmrR is a subject for autoregulation. LmrR binds to a long stretch of DNA of its own promoter region with no apparent binding motif. One of the LmrR variants containing a point mutation (T82I) failed to bind both the promoter regions of *lmrCD* and *lmrR* genes, and also is unable to bind drug as shown for Hoechst 33342. Sequence alignment of LmrR with other PadR/MarR regulators showed that this residue is highly conserved (Chapter 5) in this family of proteins. Also, the up regulation of the *lmrR* gene was observed in the wild-type cells when grown in the presence of drugs. RT-PCR analysis of all four MDR strains previously grown in the drug-free medium showed the constitutive expression of the *lmrCD* and *lmrR* genes since the cells lack of functional LmrR proteins. Overall, these results strongly indicate that the expression of *lmrCD* and *lmrR* genes follows two different mechanism of regulation in *L. lactis*. Interestingly, many of the PadR regulators are involved in the enzymatic phenolic acid degradation and detoxification, whereas LmrR regulates the expression of an MDR transporter that expels toxic molecules from the cell (Fig. 1 and Chapter 3).

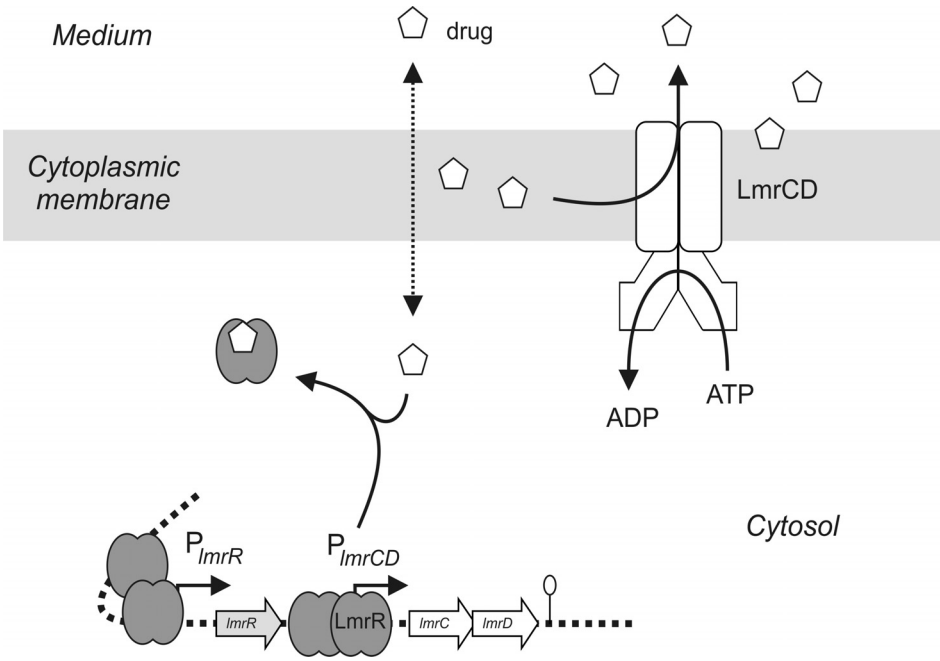


Figure 1. Drug resistance mechanism in *L. lactis*. In the absence of drugs or other toxic molecules, LmrR in different oligomeric states binds to the control DNA of *lmrCD* and *lmrR*, respectively. When the cells encounter a toxic compound in their growing environment, this compound will enter the cells and bind to the LmrR dimer in a stochiometric fashion. This condition will likely to result in a release of LmrR from the *lmrCD* and *lmrR* operator sequences and consequently in a derepression of *lmrCD.* The newly synthesized ABC transporter LmrCD in the membrane will expel the drugs from the cell and thus relieve the *lmrCD* depression mechanism. . Multiple LmrR proteins bind and deform the *lmrR* operator region, and cause a repression of *lmrR* expression by a mechanism that so far is poorly understood

Most of the synthetic compounds that are recognized by MDR transporters share similar characteristic to natural molecules commonly encountered by bacteria in their environment. For example, molecules involved in quorum sensing or antimicrobial compounds secreted by plants could be substrates for MDR-like transporters. MDR transporters might have emerged from transporters that are involved in the secretion of natural occurring molecules and when overexpressed they can contribute to the self defense mechanisms. The mapping of the transcription starting sites on *lmrCD* and *lmrR* genes revealed a different regulation mechanisms involved for these two genes. The *lmrCD* and *lmrR* genes are

organized in different operons. However, a read through transcript containing all genes was observed as well as three major transcripts with different lengths but all containing the complete *lmrCD* genes *(*Chapter 4). In-silica analysis did not identify any intrinsic terminator(s) downstream of *lmrR*. The multiple transcripts found for *lmrCD* may represent different responses to different inducers and conditions in the cells although this hypothesis requires validation by further studies. Alternatively, the longer transcripts are unstable and prone to degradation from the 3'end. A significant difference in the binding mechanism by LmrR to the *lmrCD* and *lmrR* promoter regions was revealed and visualized via the Atomic Force Microscopy. The binding of LmrR to the *lmrR* promoter induced a severe DNA deformation by means of DNA looping and wrapping due to extensive protein-protein and DNA interactions. The "in gel" footprinting confirmed an extensive protection of the *lmrR* promoter upon LmrR binding. These data indeed suggest a tight regulation of *lmrR* expression. On the other hand, the binding of LmrR to the control region of *lmrCD* resulted only in a clear bending of DNA (Chapter 4). Cells need to response very quick to changes they encountered in their environment. Therefore, this "uncomplicated" repression of *lmrCD* by LmrR might be an effective means to regulate the expression of *lmrCD* while the regulatory network is further tuned and damped by a strict regulation of *lmrR* expression Moreover, the uncontrolled expression of *lmrR* will desensitize the sensory mechanism that governs the drug-dependent regulation of *lmrCD* expression (Chapter 4).

MDR transporters can bind and excrete many unrelated compounds thus, serving as a prominent defense mechanism in bacteria. The overexpression of these transporters in many cases is responsible for the emergence of the MDR strains. Therefore, an understanding of their structures can provide valuable information on the mechanisms of drug recognition and transport, and will provide new approaches towards the development of new drugs. However, membrane proteins are difficult to crystallize because they are not water soluble and prone to aggregation. On the other hand, a structural and functional analysis of the MDR regulatory proteins has provided many insights in one of the most intriguing aspects of MDR, namely multidrug recognition. The crystal structures of LmrR unbound and bound to Hoechst 33342 or daunomycin were solved at 2.0 Å and 2.2 Å, respectively (Chapter 5). LmrR follows α1-α2-α3-β1-β2-α4 topology with two defined domains. The N-terminus DNA binding domain of LmrR comprised the typical winged helix-turn-helix (HTH) motif of bacterial transcriptional regulators MDR related, and the C-terminus consisted of a large flat-shape central pore for ligand(s) binding (Fig.2)

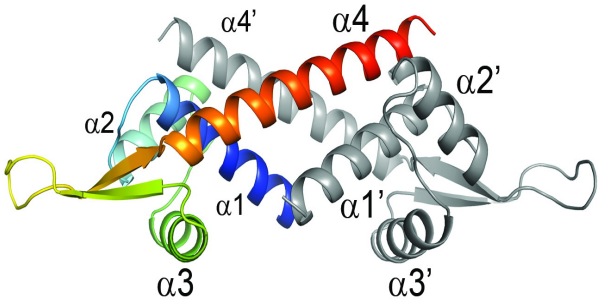


Figure 2. The crystal structure of apo LmrR solved at 2.0 Å resolution by molecular replacement. LmrR posses a large central pore of drug binding sites.

This feature is unique because none of the previously characterized MarR/PadR related transcriptional regulators have a central pore at their dimer interface. In addition, the binding pocket of LmrR is a symmetric where both subunits contributed equally to this structure unlike the drug binding sites of BmrR from *B. subtilis* and QacR from *S. aureus* which are asymmetric and formed within a single subunit. Upon drug binding, the flat ring system of either Hoechst 33342 or daunomycin molecule is wedged in between the W96 and W96' side chains forming the aromatic stacking interactions with each of the two indole systems with no hydrogen bonds were observed between drug(s) and LmrR. In addition, site directed mutagenesis analysis confirmed the important role of W96 in both drug and DNA binding (Chapter 5). By comparing the unbound and drug bound of LmrR, a number of changes in structural orientation were observed. When a dimer of LmrR bound to Hoechst 33342 or daunomycin was compared with the unbound form, one of wHTH domains is rotated in the opposite direction and relative to the wHTH domain of the other subunit. This rotation is coupled with changes in the spacing area between the two DNA recognition helices. The other differences were the orientation of residues from one subunit relative to those of the other subunit observed inside the binding pore that influenced the structure of the drug binding site. These changes have been postulated to induce the loss of binding of LmrR to the control DNA causing the upregulation of the MDR transporter LmrCD. The crystal structure of LmrR bound DNA will be a major challenge and is needed to reveal the molecular basis of the derepression mechanism of *lmrCD* expression by the transcriptional repressor LmrR in *L. lactis*.

REFERENCES

1. Gury, J., L. Barthelmebs, N. P. Tran, C. Divies, and J. F. Cavin. 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding padA gene of Lactobacillus plantarum. Appl. Environ. Microbiol. 70:2146-2153.
2. Lubelski, J., P. Mazurkiewicz, M. R. van, W. N. Konings, and A. J. Driessen. 2004. ydaG and ydbA of Lactococcus lactis encode a heterodimeric ATP-binding cassette-type multidrug transporter. J. Biol. Chem. 279:34449-34455.
3. Poelarends, G. J. and W. N. Konings. 2002. The transmembrane domains of the ABC multidrug transporter LmrA form a cytoplasmic exposed, aqueous chamber within the membrane 6. J. Biol. Chem. 277:42891-42898.

Nederlandse Samenvatting

Het verschijnsel van multidrug resistentie (MDR) is het vermogen van cellen om resistentie te ontwikkelen tegen een wijd bereik aan giftige moleculen die in hun structuur niet aan elkaar verwant zijn. MDR wordt veroorzaakt door de (over) expressie van transporteiwitten die deze verbindingen de cel uitdrijven. Multidrug resistentie heeft bijvoorbeeld geleid tot meervoudige complicaties bij de behandeling van kanker of infectieziekten. Voorbeelden van het laatste is de uitgebreide medicijn resistente *Mycobacterium tuberculosis* (XDR-TB) en de methicilline resistente *Staphylococcus aureus* (MRSA) die wereldwijd de dood van miljoenen mensen hebben veroorzaakt. De introductie en het oneigenlijke gebruik van antibiotica hebben geleid tot de selectie van bacteriën die door het veranderen van hun genoom resistentiemechanismen hebben ontwikkeld die het mogelijk maken onder zware antibioticadruk te overleven.

Verschillende typen resistentie mechanismen kunnen worden geïdentificeerd: 1) enzymen die de giftige verbindingen afbreken en buiten werking stellen, 2) het aanbrengen van veranderingen in de aangrijppunten van het medicijn, 3) het voorkomen dat de giftige verbinding de cel kan binnendringen, en tenslotte 4) de actieve uitstoot van giftige verbindingen dankzij de overexpressie van transporteiwitten in het membraan (MDR-transporters) (Hoofdstuk 1). Sequentieanalyse uitgevoerd op meerdere bacteriegenomen heeft onthuld dat in de natuur MDR transporter alom vertegenwoordigd zijn. Veel onderzoeksinspanningen zijn gericht op het ophelderen van de fysiologische functie(s) van MDR-transporters en op het transportmechanisme, de substraatherkenning en de expressie van MDR-transporters (Hoofdstuk 1).

Een belangrijk aspect is de expressie van bacteriële MDR-transporters aangezien de ontregeling van de regulatie van expressie vaak de oorzaak van het MDR fenotype is. De meerderheid van bacteriële MDR-transporters zijn onderworpen aan regulatie door plaatselijke of globale regulatoreiwitten die functioneren op het niveau van transcriptie. Over het algemeen herbergen bacteriën resistentiemechanismen die het hen mogelijk maken te reageren op blootstelling aan giftige verbindingen. Een dergelijke omstandigheid leidt in cellen tot het opreguleren van laag tot expressie komende MDR-transporteiwitten. De goed beschreven trancriptionele activator BmrR van *Bacillus subtilis* en de transcriptionele repressor QacR van *Staphylococcus aureus* reguleren bijvoorbeeld

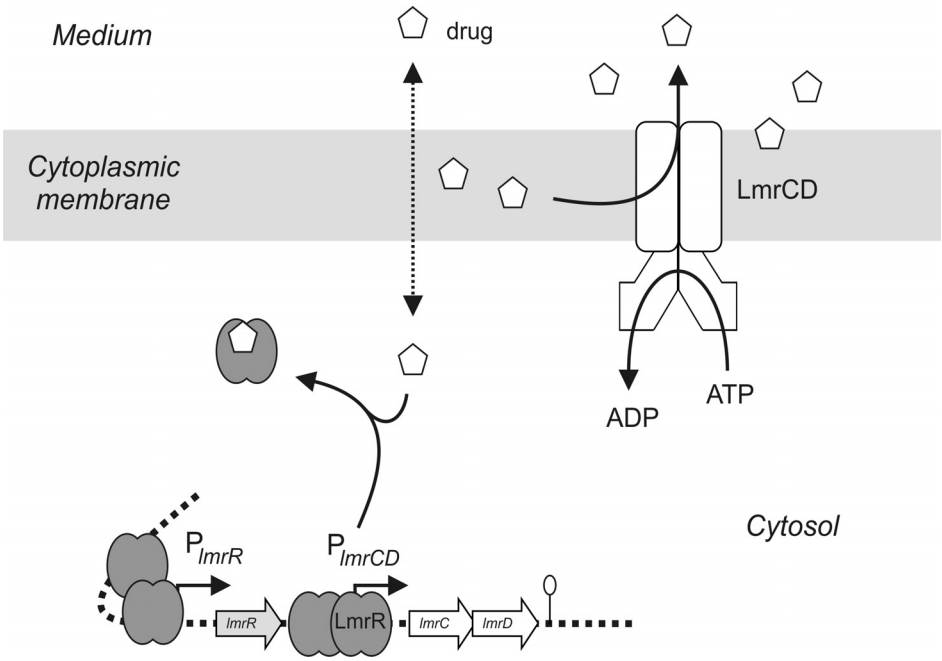
de expressie van respectievelijk de MDR transporteiwitten Bmr en QacA (Hoofdstuk 1). Typerend bestaat een MDR-regulatoreiwit uit twee domeinen; het DNA-bindende domein aan het N-uiteinde en het ligand-bindende domein aan het C-uiteinde. Een gemeenschappelijk kenmerk van het DNA-bindende domein is de aanwezigheid van een "helix-turn-helix" (HTH) motief. Verdere classificatie is gebaseerd op de hoge sequentiehomologie van het DNA-bindingsdomein en op basis van dit kenmerk kunnen vier verschillende families worden aangewezen: AraC, MarR, MerR en TetR. Interessant daarbij is dat recente studies aangeven dat de MDR-regulator LadR van *Listeria monocytogenes* en LmrR van *Lactococcus lactis* behoren tot de transcriptiefactor familie PadR die niet eerder betrokken waren bij MDR. In tegenstelling tot de membraangebonden MDR-transporteiwitten zijn transcriptiefactoren oplosbare eiwitten die relatief "rechttoe rechtaan" in grotere hoeveelheden tot overexpressie kunnen worden gebracht. Het ophelderen van de structuur en functie van deze MDR-transcriptiefactoren is belangrijk aangezien deze eiwitten vaak een vergelijkbaar bereik aan substraten herkennen als de transporteiwitten waarvan de expressie gereguleerd wordt. Dit kenmerk maakt de transcriptiefactoren ideale kandidaten voor het bestuderen van de moleculaire grondslag van multidrug-herkenning. Deze nieuwe inzichten zullen bruikbaar zijn voor de verdere ontwikkeling van nieuwe verbindingen die het MDR fenotype kunnen onderdrukken waardoor de werkzaamheid van medicijnen verhoogd kan worden (Hoofdstuk 1).

Lactococcus lactis is een Gram-positief melkzuurbacterie en wordt wijdverspreid toegepast voor het maken van gefermenteerde voedselproducten. Het genoom van *L. lactis* bevat ongeveer 40 vermeende MDR transporters waarvan sommige betrokken zijn bij de energieafhankelijke uitstoot van niet aan elkaar verwante vetoplosbare en giftige verbindingen. Het secondaire transporteiwit LmrP bijvoorbeeld gebruikt de protonengradiënt over het membraan om allerlei verbindingen uit te scheiden, terwijl de ATP-bindingscassette (ABC) transporteiwitten LmrA (3) en LmrCD (2) voor het vervullen van een vergelijkbare functie de vrije energie gebruiken afkomstig van de hydrolyse van ATP. Door de genen van deze transporteiwitten uit te schakelen kan een inzicht verkregen worden welke transporteiwitten verantwoordelijk zijn voor MDR in *L. lactis*. LmrC en LmrD blijken verantwoordelijk te zijn voor de inherente multidrug resistentie in *L. lactis*. Deze twee eiwitten zijn zogenaamde ABC half-transporters die tezamen als heterodimeer een werkzame MDR transporter vormen. De overexpressie van LmrCD leidde tot cellulaire bescherming tegen de giftige uitwerkingen van verscheidene verbindingen, b.v. daunomycin, ethidiumbromide en Hoechst 3334 (Hoofdstuk 2), terwijl verwijdering van de *lmrCD* genen uit het genoom van *L. lactis* stam NZ9000 de cellen overgevoelig maakt voor deze verbindingen.

Bovendien kan het resistente fenotype van de Δ*lmrCD* stam worden hersteld tot het wilde typen expressie niveau van LmrCD te herstellen. Het is daarbij interessant dat de overexpressie van het *lmrA*-gen geen effect liet zien en dat dit de cellen zelfs meer vatbaar maakte voor de giftige verbindingen (Hoofdstuk 2). In het verleden zijn er vier MDR-stammen van *L. lactis* ontwikkeld door de cellen bloot te stellen aan toenemende concentraties daunomycine, ethidiumbromide, cholaat en rhodamine 6G. Deze stammen bleken multidrug resistent en vertoonde niet alleen een hoge resistentie tegen de verbinding waaraan ze waren blootgesteld maar ook tegen allerlei ongerelateerde giftige verbindingen. DNA-array analyse van deze stammen heeft aangetoond dat de expressie van de *lmrC*- en *lmrD*-genen fors omhoog is gegaan. Tevens was er nog een opgereguleerd gen, genaamd *lmrR* (lactococcal multidrug resistance regulator), dat stroomopwaarts van *lmrCD* is gelegen, en dat voorheen bekend stond als *ydaF*. Analyse van de nucleotidenvolgorde liet zien dat er frameverschuivingen en puntmutaties plaatsgevonden hebben in het *lmrR* gen van al deze vier MDR-stammen waardoor er niet-werkzame LmrR varianten ontstaan. Deze resultaten suggereren een mogelijke rol van LmrR in het omlaag regelen van de expressie van *lmrCD* en *lmrR* in *L. lactis* (Hoofdstuk 2).

Op basis van aminozuurvolgorde homologie behoort LmrR tot de PadR-familie van transcriptieregulatoren. PadR transcriptionele regulatoren zijn betrokken bij het regelen van de expressie van fenolzuurdecarboxylase-genen (*pad*) die derivaten van fenolzuur, zoals ρ-coumarine-, feruline- en cafeïnezuren ontgiften (1). Veel leden van de PadR-familie zijn nauw verwant aan de MarR-familie voor meervoudige antibioticaresistentie die in bacteriën en archaea is aangetroffen. Analyse van het transcriptoom van een Δ*lmrR*-stam liet de verhoogde expressie zien van de *lmrC* en *lmrD* genen en niet van andere genen (Hoofdstuk 3). Dit resultaat toonde aan dat LmrR verantwoordelijk is voor de regulatie van de expressie van enkel de *lmrCD* genen, en bevestigde de eerdere waarneming dat LmrCD de hoofdtransporter is die verantwoordelijk is voor het verkregen fenotype van de verschillende geselecteerde MDR-stammen van *L. lactis*. Vervolgtypering liet zien dat het wilde type LmrR bindt aan twee afzonderlijke plekken op het promotorgebied van *lmrCD*; plek 1 is gelegen in het -35 en -10 gebied terwijl plek II overeenkomt met een rechtstreeks omgekeerde nucleotide volgorde herhalingen, waarbij de herhalingen niet perfect zijn. Deze regio bevat een motief dat bijna gelijk is dat van de PadR-bindingsplek op de *padA*-promotor (Hoofdstuk 3). Testen met rechtstreeks gebonden verbindingen lieten zien dat LmrR een interactie aangaat met deze moleculen, terwijl verdere expressie studies suggereren dat de binding van de giftige verbindingen aan LmrR leid tot een verhoogde expressie van de *lmrCD* genen. Een eenvoudig model voor de regulatie van de expressie van de

lmrCD genen door LmrR is als volgt: indien de vetoplosbare giftige moleculen in het milieu aanwezig zijn dan zullen zij de cel indringen, om vervolgens te binden aan het DNA-gebonden LmrR. LrmR ondergaat hierbij een structuurverandering waardoor het vrijkomt van de promotor regio van de *lmrCD* genen waarna het RNA-polymerase met de transcriptie van *lmrCD* kan beginnen. LmrCD zal de giftige verbinding vervolgens uit de cel stoten, en dit zal leiden tot een verlaagde concentratie van de verbinding in de cel. Hierdoor zal LmrR terugkeren tot zijn apo-toestand en opnieuw binden aan de promotor regio en opnieuw de expressie van de *lmrCD* genen onderdrukken (Hoofdstuk 3). Analyse van het transcriptoom van de onafhankelijk geïsoleerde MDR-stammen liet eveneens een toename van de transcriptieniveaus van het *lmrR* gen zien. Deze waarneming suggereerde dat LmrR is onderworpen aan zelfregulatie. LmrR bind aan een groot stuk DNA van zijn eigen promotorgebied waarbij er geen duidelijk bindingsmotief is. Een van de LmrR varianten die een puntmutatie (T82I) bevat bind niet langer aan beide promotorgebieden van de *lmrCD* en *lmrR* genen en is ook niet in staat giftige verbindingen te binden zoals voor Hoechst 33342 zichtbaar gemaakt. Het naast elkaar op één lijn brengen van de sequentie van LmrR met ander PadR/MarR regulators liet zien dat dit aminozuur residu sterk geconserveerd is (Hoofdstuk 5) in deze eiwitfamilie. Wanneer er gegroeid wordt in de aanwezigheid van giftige verbindingen werd geen verhoogde expressie van het *lmrR* gen waargenomen in wilde typen cellen. Echter de *lmrCD* en *lmrR* genen komen constitutief tot expressie in de cellen die een werkzaam LmrR gen missen. Deze resultaten tonen aan dat de expressie van de *lmrCD* en *lmrR* genen verloopt doormiddel van twee verschillende mechanismen. Daarbij is het interessant dat veel van de PadR regulatoren betrokken zijn bij de regulatie van processen waarbij giftige verbindingen worden afgebroken, terwijl LmrR de expressie van een MDR-transporter reguleert dat degelijke giftige moleculen de cel uitstoot (Fig. 1 en Hoofdstuk 3).



Figuur 1. **Mechanisme voor resistentie tegen giftige verbindingen in *L. lactis***. Bij afwezigheid van giftige moleculen bindt LmrR in verschillende oligomere vormen aan de DNA promotor regio van respectievelijk de *lmrCD* en *lmrR* genen. Indien de cellen een giftige verbinding in hun groeimilieu tegenkomen dan zal deze verbinding de cel binnengaan en op een stochiometrische manier binden aan de LmrR-dimeer. Deze toestand zal waarschijnlijk leiden tot het vrijkomen van LmrR van de *lmrCD* promotor regio waarna expressie van *lmrCD* mogelijk is. In het membraan zal de nieuw gesynthetiseerde ABC-transporter LmrCD de giftige verbinding de cel uitstoten en daarom zal bij een voldoende lage concentratie in de cel, het LmrR opnieuw binden aan de *lmrCD* promotor regio en de transcriptie remmen. Echter de initiatie van transcriptie van *lmrR* lijkt niet te worden beïnvloed door de aanwezigheid van giftige verbindingen. Meerdere LmrR-eiwitten binden aan de *lmrR* promotor regio, en dit veroorzaakt de onderdrukking van de *lmrR* expressie middels een mechanisme dat nog niet doorgrond is.

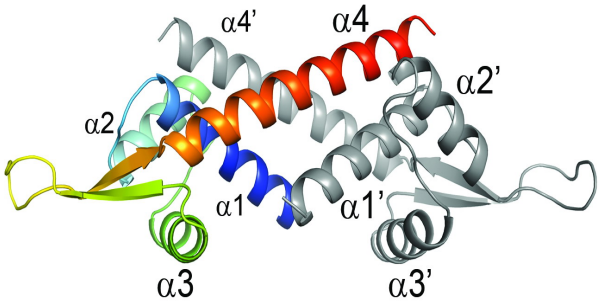
De meeste van de synthetische verbindingen die worden herkend door MDR-transporters delen een aantal kenmerken met natuurlijke moleculen die bacteriën aantreffen in hun leefmilieu. Moleculen die betrokken zijn bij "quorum sensing" of antimicrobiële verbindingen die door planten worden uitgescheiden kunnen bijvoorbeeld substraten zijn voor MDR-achtige transporters. MDR-transporters zijn misschien ontstaan uit transporters die betrokken zijn bij het uitscheiden van in de natuur voorkomende verbindingen en die, wanneer zij tot

overexpressie komen, kunnen bijdragen aan het eigen verdedigingsmechanisme. Het in kaart brengen van de startplaatsen van de transcriptie op de *lmrCD* en *lmrR* genen onthulde met betrekking tot deze genen een verschillend mechanisme voor regulatie. De *lmrCD* en *lmrR* genen zijn georganiseerd in verschillende "operons". Echter, een "read-through" transcript dat alle genen bevat werd waargenomen evenals drie transcripten met verschillende lengtes maar waarbij ieder de complete *lmrCD* genen bevat (hoofdstuk 4). Doormiddel van computer voorspeling werd stroomafwaarts van *lmrR* geen enkele wezenlijke terminatie sequentie geïdentificeerd. De meerdere transcripten die voor *lmrCD* zijn gevonden kunnen verschillende reacties op verschillende opwekkers en omstandigheden in de cellen vertegenwoordigen, hoewel deze hypothese bevestiging in vervolgstudies vereist. Als alternatief zijn de langere transcripten instabiel en gevoelig voor afbraak vanaf het 3' uiteinde. Een verschil tussen het bindingsmechanisme van LmrR aan de promotorgebieden van *lmrCD* en *lmrR* werd met "Atomic Force Microscopy" onthuld en zichtbaar gemaakt. Het binden van LmrR aan de *lmrR* promotor veroorzaakt een hevige vervorming van het DNA door middel van het vormen van DNA-lussen en windingen die toe te schrijven zijn aan uitgebreide eiwit-eiwit en DNA interacties. Met "in-gel footprinting" werd bij het binden van LmrR een uitgebreide bescherming van de *lmrR* promotor waargenomen. Deze gegevens suggereren inderdaad een nauwe regulatie van de expressie van *lmrR*. Anderzijds leidde het binden van LmrR aan het controlegebied van *lmrCD* enkel in een duidelijke buiging van het DNA (Hoofdstuk 4).

Cellen moeten zeer snel reageren op veranderingen die zij in hun milieu tegenkomen. Daarom kan het "ongecompliceerde" repressie mechanisme van de *lmrCD* genen door LmrR een doelmatig middel zijn voor de regulatie van de expressie van *lmrCD*. Daarentegen lijkt een meer complex netwerk van regulatie de expressie van *lmrR* af te stellen. Bovendien zal de onbeheerste expressie van *lmrR* het regulatie mechanisme waarmee giftige verbinding de expressie van *lmrCD* besturen minder gevoelig maken (Hoofdstuk 4).

MDR transporters kunnen vele niet aan elkaar verwante verbindingen binden en uitscheiden en dus als een uitstekend verdedigingsmechanisme in bacteriën dienen. In veel gevallen is de "overexpressie"van deze transporteiwitten verantwoordelijk voor de opkomst van de MDR-stammen. Begrip van hun structuren kan daarom waardevolle informatie over het mechanisme van herkenning en transport van giftige verbindingen opleveren en zal nieuwe benaderingen voor de ontwikkeling van medicijnen opleveren. Membraaneiwitten zijn echter moeilijk te kristalliseren aangezien zij niet in water oplosbaar zijn en daardoor snel aggregatie vertonen. Anderzijds heeft de analyse van de structuur en werking van de MDR regulatie-eiwitten inzichten in één van de meest intrigerende

aspecten van MDR, namelijk "multidrug"-herkenning, opgeleverd. De kristalstructuren van het vrije en het aan Hoechst 33342 of daunomycine gebonden LmrR werden opgelost bij respectievelijk 2.0 Å en 2.2 Å (Hoofdstuk 5). LmrR volgt een α1-α2-α3-β1-β2-α4-topologie met twee afgebakende domeinen. Het DNA-bindende domein aan het N-uiteinde van LmrR omvat het typische vleugelvormige "helix-turn-helix" motief van de bacteriële transcriptionele regulator die met MDR in verband staat, en het C-uiteinde dat bestaat uit een platte centrale porie voor het binden van het ligand (Fig. 2).



Figuur 2. De kristalstructuur van apo LmrR met een resolutie van 2.0 Å. LmrR bezit een grote centrale porie met plaatsen voor het binden van vlakke hydrofobe verbindingen.

Dit kenmerk is uniek aangezien geen enkele van de eerdere omschreven aan MarR/PadR verwante transcriptieregulators een centrale porie op het raakvlak van hun dimeer bezit. Aanvullend is de bindingsplaats van LmrR symmetrisch waarbij beide eenheden op gelijke wijze bijdragen aan deze constructie anders dan de plaatsen voor het binden van verbindingen aan BmrR van *B. subtilis* en QacR van *S. aureus* die asymmetrisch zijn en binnen één enkele eenheid gevormd zijn. Bij het binden van een enkel molecuul vormt het platte ringsysteem van Hoechst 33342 of daunomycin een wig tussen de W96 en W96' zijketen waarbij de aromatische stapelinteractie met beide indolsystemen wordt gevormd zonder dat waterstofbruggen tussen het gebonden molecuul en LmrR werden waargenomen. Aanvullende plaatsspecifieke mutatie experimenten bevestigde de belangrijke rol van W96 in het binden van zowel substraten als DNA (Hoofdstuk 5). Door het vrije en het substraat gebonden LmrR te vergelijken werden een aantal veranderingen in structurele oriëntatie waargenomen. In vergelijking met een

dimeer van LmrR dat gebonden is aan Hoechst 3342 of daunomycine is in de vrije vorm één van de wHTH domeinen gedraaid in de tegenovergestelde richting ten opzichte van het wHTH domein van de andere eenheid. Deze draaiing is gekoppeld aan veranderingen in het ruimtegebied tussen de twee helices voor het herkennen van DNA. Het andere verschil is de oriëntatie van residuen van één eenheid ten opzichte van diegene van de andere eenheid binnenin de bindingsporie waardoor de structuur van de substraat bindingsplaats wordt beïnvloedt. Deze veranderingen worden verondersteld het verlies van binding van LmrR aan het DNA voort te brengen dat de verhoogde expressie van de LmrCD MDR transporter veroorzaakt. De kristalstructuur van LmrR dat gebonden is aan DNA zal een grote uitdaging zijn en is nodig om de moleculaire basis van het mechanisme LmrR afhankelijke regulatie van de *lmrCD* expressie in *L. lactis* op te helderen.

Ringkasan dalam bahasa Indonesia

Fenomena ketahanan atas berbagai macam obat (MDR) ialah kemampuan dari sel untuk membangkitkan ketahanan terhadap berbagai molekul-molekul beracun, yang secara struktur tidak saling terkait. MDR disebabkan oleh di(over)ekspresikannya transporter yang membawa molekul-molekul tersebut ke luar dari sel. MDR menyebabkan terjadi berbagai komplikasi pada penanganan, misalnya, kanker atau penyakit-penyakit infeksi. Contoh pengaruh MDR pada penyakit infeksi antara lain adalah pada tuberkolosis (ketahanan berlebih akan obat, XDR-TB) dan *Staphylococcus aureus* (ketahanan akan metisilin, MRSA), yang telah menyebabkan kematian jutaan orang di seluruh dunia. Penggunaan dan penyalahgunaan antibiotik ditenggarai sebagai penyebab timbulnya sejumlah bakteri dengan genom yang termutasi sehingga mengembangkan mekanisme ketahanan, yang memungkinkan mereka untuk bertahan hidup dalam lingkungan yang mengandung antibiotik. Beberapa macam mekanisme ketahanan dapat diidentifikasikan sebagai: 1) degradasi dan inaktifasi obat secara enzimatik, 2) perubahan target obat, 3) pencegahan masuknya obat, dan yang terakhir 4) dikeluarkannya obat secara aktif dengan dioverekspresikannya transporter protein membran (MDR transporter) (BAB 1). Analisis sekuensi terhadap beberapa genom bakteri mengungkapkan bahwa transporter yang menyerupai MDR banyak ditemui di alam. Berbagai upaya telah dilakukan untuk menjabarkan fungsi fisiologis dari transporter MDR, termasuk mekanisme transport, pengenalan substrat, dan regulasi ekspresinya (BAB 1).

Regulasi transporter MDR dari bakteri merupakan salah satu aspek yang penting dalam ketahanan atas berbagai obat karena deregulasinya sering kali merupakan penyebab dari fenotip MDR. Regulasi dari sebagian besar transporter MDR pada bakteri terjadi pada tingkat transkripsi, oleh protein-protein regulator lokal maupun global. Secara umum, bakteri memiliki mekanisme ketahanan yang memungkinkannya untuk memberikan respon terhadap keberadaan molekul-molekul beracun disekitarnya. Keberadaan molekul-molekul beracun ini menyebabkan teraktivasinya regulasi transpoter MDR di dalam sel, yang sebelumnya diekspresikan secara terbatas. Contohnya adalah dua protein yang telah terkarakterisasi secara lengkap, yaitu aktivator transkripsi BmrR dari *Bacillus subtilis* dan represor transkripsi QacR dari *Staphylococcus aureus* yang masing-masing meregulasi ekspresi pompa efluks MDR Bmr dan QacA (BAB 1). Regulator MDR secara khas mempunyai dua domain; pada ujung N- adalah

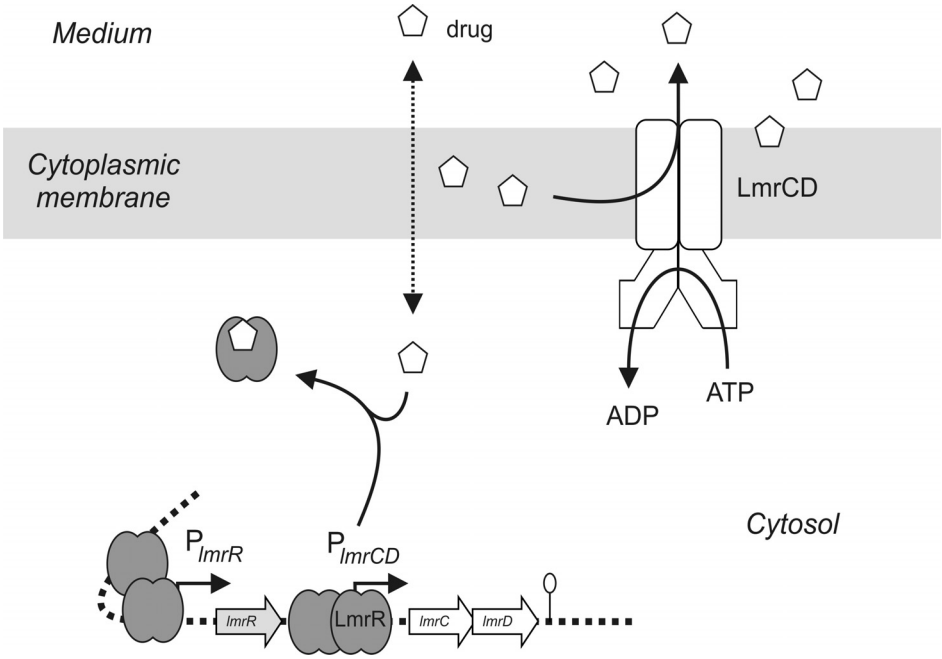
domain untuk pengikatan DNA dan pada ujung C- adalah domain untuk pengikatan ligan. Fitur umum dari domain untuk pengikatan DNA adalah adanya motif heliks-putar-heliks (HTH). Klasifikasi lebih lanjut dari regulator-regulator MDR didasarkan pada keterdekatan sekuen pada domain untuk pengikatan DNA, dan atas dasar inilah ada empat kelompok regulator MDR: AraC, MarR, MerR, dan TetR. Yang menarik adalah, baru-baru ini ditemukan suatu indikasi bahwa LadR dari *Listeria monocytogenes* dan LmrR dari *Lactococcus lactis* termasuk ke dalam keluarga regulator transkripsi PadR, yang sebelumnya tidak termasuk ke dalam MDR. Tidak seperti halnya transporter MDR yang terikat pada membran, regulator transkripsi merupakan protein terlarut dan secara relatif dapat dioverekspresikan secara langsung dalam jumlah besar. Karakterisasi struktur dan fungsi dari regulator transkripsi MDR ini menjadi penting karena protein ini sering kali mengenali substrat yang sama jumlahnya dengan transporter yang mereka regulasikan. Fitur ini menjadikan regulator transkripsi sebagai kandidat ideal untuk mempelajari dasar-dasar molekular dalam pengenalan berbagai macam obat oleh bakteri. Wawasan baru yang didapatkan akan sangat berguna untuk pengembangan senyawa-senyawa antimikroba.

Lactococcus lactis merupakan bakteri asam laktat yang gram positif dan banyak digunakan dalam produksi makanan-makanan hasil fermentasi. Dalam genomnya terdapat sekitar 40 gen putatif transporter MDR, yang beberapa diantaranya terlibat dalam proses, yang bergantung pada energi, pengeluaran senyawa-senyawa lipofilik yang tak terkait dan beracun. Sebagai contoh, transporter LmrP sekunder bergantung pada kekuatan motif proton untuk mengeluarkan sekelompok jenis obat, sementara transporter-transporter LmrA (3) dan LmrCD (2) yang memiliki kaset pengikatan ATP (ABC) memanfaat kan energi yang terbebaskan dalam proses hidrolisis ATP untuk menjalankan fungsi yang setara. Penelaahan inaktivasi gen akhir-akhir ini menyarankan bahwa LmrC dan LmrD bertanggung jawab atas sifat resistensi akan berbagai macam obta yang intrinsik pada *L. lactis*. Keduanya tergolong pada setengah transporter ABC yang mengalami heterodimerisasi untuk membentuk transporter MDR yang fungsional. Overekspresi LmrCD menimbulkan perlindungan selular terhadap pengaruh racun dari berbagai macam obat yang dicobakan, sebagai contoh daunomisin, etidium bromida, dan Hoechst 33342 (BAB 2), sedangkan penghilangan gen *lmrCD* dari genom *L. lactis* galur NZ9000 menghasilkan sel yang sangat sensitif terhadap obat-obatan tersebut. Lebih lanjut, fenotip yang memiliki ketahanan dari galur Δ*lmrCD* dapat dikembalikan keasalnya dengan overekspresi *lmrCD* secara trans. Menariknya, overekspresi gen lmrA tidak memberikan pengaruh dan menghasilkan sel yang lebih rentan terhadap obat (BAB 2) Analisis susunan DNA dari keempat galur MDR dari *L. lactis* yang ditumbuhkan dalam keberadaan daunomisin,

etidium bromida, kolat, dan rodamin 6G dengan konsentasi yang meningkat mengungkap regulasi naik yang terjadi secara signifikan dari gen *lmrC* dan *lmrD*, juga dari sebuah gen pada hulu *lmrCD* yang disebut *lmrR* (regulator ketahanan ganda atas obat pada lactococus) yang sebelumnya dikenal sebagai *ydaF*. Analisis urutan nukleotida menunjukkan terjadinya pergeseran kerangka dan mutasi tertentu pada gen *lmrR* di ke empat galur MDR yang menyebabkan diproduksinya varian LmrR yang tidak fungsional. Hasil-hasil tersebut menyarankan suatu kemungkinan peran LmrR dalam regulasi turun pada ekspresi *lmrCD* dan *lmrR* di *L. lactis* (BAB 2).

Secara homologi LmrR termasuk ke dalam keluarga regulator transkripsional PadR. Regulator PadR berperan dalam meregulasi pengekspresian gen asam fenolik dekarboksilase (*pad*) untuk menghilangkan pengaruh racun dari turunan asam-asam fenolik, seperti asam p-kumarat, asam ferulik, dan asam kafeik (1). Banyak anggota keluarga PadR yang berkerabat secara dekat dengan keluarga ketahanan akan berbagai antibiotik MarR yang ditemukan pada bakteri dan arkea. Analisis transkriptom dari galur Δ*lmrR* menunjukkan terjadinya secara siginifikan regulasi naik dari gen *lmrC* dan *lmrD* namun tidak dari gen lainnya (BAB 3). Hasil ini menunjukkan bahwa LmrR adalah regulator transkripsi local yang khusus untuk gen *lmrCD* dan mengkonfirmasi catatan sebelumnya bahwa LmrCD merupakan transporter utama yang bertanggung jawab atas fenotip MDR yang didapatkan dari berbagai galur MDR *L. lactis* terpilih. Karakterisasi lanjutan menunjukkan bahwa LmrR asalan berikatan dengan dua sis yang berbeda pada daerah promoter *lmrCD*; sisi 1 terletak diantara daerah -35 dan -10 dimana sisi II memiliki motif dua perulangan terbalik yang tidak sempurna, yang hampir serupa dengan sisi pengikatan PadR pada promoter *padA* (BAB 3). Uji pengikatan obat secara langsung menunjukkan bahwa terjadinya pengikatan obat ini memberikan sinyal induksi untuk ekspresi gen *lmrCD*. Suatu model sederhana mengenai regulasi ekspresi gen *lmrCD* oleh LmrR diuraikan sebagai berikut: ketika molekul obat yang bersifat hidrofobik hadir di lingkungan dan memasuki sel, LmrR yang terikat pada DNA mengikat molekul obat tersebut dan mengalami perubahan konformasi yang menyebabkannya terlepas dari sisi operator pada gen *lmrCD* dan saat itu juga RNA polymerase dapat memulai transkripsi *lmrCD*. LmrCD akan mengeluarkan molekul obat dari sel dan, karena konsentrasi obat dalam sel menurun, kembali ke kondisi apo dan berikatan kembali dengan sisi operator diikuti dengan menekan gen *lmrCD*. Analisis transkriptom dari berbagai galur MDR yang diisolasi secara independen juga menunjukkan peningkatan tingkat transkripsi gen *lmrR* yang signifikan. Salah satu varian LmrR yang memiliki mutasi tertentu (T82I) tidak mampu berikatan dengan daerah promoter pada kedua gen *lmrCD* dan *lmrR*, dan juga tidak mampu mengikat obat Hoechst 33342. Pencocokan sekuen LmrR

dengan regulator PadR/MarR lainnya menunjukkan bahwa residuini sangat terlestarikan (BAB 5) dalam keluarga protein ini. Analisis reaksi polymerase berantai – waktu berbalik (RT-PCR) dari keempat galur MDR yang sebelumnya ditumbuhkan dalam media yang bebas dari obat menunjukkan ekspresi gen *lmrCD* dan *lmrR* secara konstitutif karena sel-selnya kekurangan protein LmrR yang fungsional. Hasil hasil ini mengindikasikan secara kuat bahwa ekspresi gen *lmrCD* dan *lmrR* melalui dua mekanisme regulasi yang berbeda pada *L. lactis*. Yang menarik, banyak regulator PadR terlibat dalam degradasi dan detoksifikasi asam fenolik secara enzimatik, dimana LmrR mengergulasi ekspresi tranporter MDR yang mengeluarkan molekul beracun dari dalam sel (gambar 1 dan BAB 3).

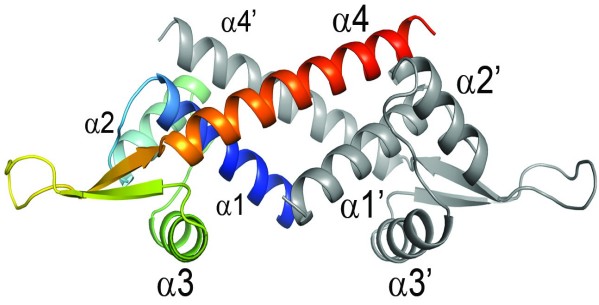


Gambar 1. Mekanisme ketahanan akan obat di *L. lactis*. Saat obat atau molekul beracun tidak ada, LmrR dalam berbagai tingkat oligomerisasi berturut-turut berikatan dengan DNA kontrol dari *lmrCD* dan lmrR. Ketika sel bertemu dengan senyawa beracun dalam lingkungan tumbuhnya, senyawa ini masuk ke dalam sel dan berikatan dengan dimer LmrR secara stoikhiometri. Keadaan ini kemungkinan besar akan menyebabkan terlepasnya LmrR dari urutan operator *lmrCD* dan akibatnya *lmrCD* akan tertekan. ABC transporter LmrCD yang kemudian terbentuk pada membran sel akan mengeluarkan obat dari dalam sel dan dengan demikian meringankan mekanisme penekanan *lmrCD*. Sebaliknya, awal transkripsi *lmrR* nampaknya tidak dipengaruhi oleh keberadaan obat. Berbagai protein LmrR berikatan dan mengubah daerah operator *lmrR*, dan menyebabkan penekanan ekspresi *lmrR* melalui suatu mekanisme yang belum jelas.

Sebagian besar senyawa sintetik yang dikenali ole transporter MDR memiliki karakter yang serupa dengan molekul alami yang ditemui oleh bacteria dilingkungannya. Sebagai contoh, molekul yang terlibat dalam pengenalan lingkungan akan senyawa antimikroba yang dikeluarkan oleh tanaman dapat menjadi substrat bagi transporter-transporter yang menyerupai MDR. Transporter MDR mungkin berasal dari transporter-transporter yang terlibat dalam sekresi molekul alami dan pada saat dioverekspresikan, mereka dapat ikut serta dalam mekanisme pertahanan diri. Pemetaan sisi pemulaian transkripsi pada gen *lmrCD* dan *lmrR* mengungkapkan adanya perbedaan mekanisme regulasi yang terlibat bagis kedua gen. Gen *lmrCD* dan *lmrR* tersusun dalam operon yang berbeda. Namun demikian, pintasan transkrip yang mengandung semua gen maupun tiga transkrip utama dengan panjang berbeda yang diamati, kesemuanya memiliki gen *lmrCD* yang lengkap. Analisis in-silico tidak mengidentifikasi adanya terminator internal hilir *lmrR*. Beragam transkrip *lmrCD* yang ditemui kemungkinan mewakili perbedaan respon terhadap induser dan kondisi yang berbeda dalam sel, walaupun hipotesis ini masih memerlukan validasi melalui penelaahan lebih lanjut. Alternatifnya, transkrip yang lebih panjang tidak stabil dan cenderung mengalami degradasi dari ujung 3'. Perbedaan signifikan pada mekanisme pengikatan oleh LmrR ke daerah promoter *lmrCD* dan *lmrR* tersingkap dan ditampilkan oleh mikroskop berkekuatan atomic. Pengikatan LmrR ke promoter *lmrR* mengakibatkan perubahan besar-besaran DNA dalam arti melingkungan dan pelingkupan DNA karena interaksi protein dan DNA secara berlebihan. Penandaan "dalam gel" mengkonfirmasi perlindungan berlebih promoter *lmrR* pada pengikatan LmrR. Data ini menyarankan ekspresi *lmrR* yang diregulasi dengna ketat.. Di sisi lain, pengikatan LmrR pada daerah kontral *lmrCD* hanya menghasilkan pelengkungan DNA dengan jelas (BAB 4). Sel perlu untuk merespon dengan cepat perubahan-perubahan yang ditemui dalam lingkungan. Oleh sebab itu, penekanan *lmrCD* oleh LmrR yang tidak rumit ini mungkin merupakan cara yang efektif untuk meregulasi ekspresi *lmrCD* sementara jaringan regulasinya diselaraskan dan dilengkapkan melalui ekspresi *lmrR* secara ketat. Lebih jauh, ekspresi *lmrR* yang tidak terkontrol akan mengurangi kesensitifan mekanisme sensor yang akan mengatur regulasi ekspresi *lmrCD* yang bergantung pada obat (BAB 4).

Transporter MDR mampu mengikat dan mengeluarkan berbagai macam senyawaan yang tidak saling terkait, dengan demikian menjadikannya mekanisme pertahanan utama pada bakteri. Overekspresi transporter ini dalam banyak hal bertanggung jawab atas munculnya galur-galur MDR. Oleh sebab itulah, pemahaman akan struktur dari MDR dapat menyediakan informasi berharga tentang mekanisme pengenalan dan transport obat, dan membuka pendekatan baru

untuk pengembangan obat baru. Namun demikian, protein-protein membran sulit untuk dikristalkan karena mereka tidak larut dalam air dan cenderung mengagregasi. Di lain pihak, analisis struktur dan fungsi dari protein regulator MDR memperluas wawasan mengenai salah satu aspek MDR yang paling menarik, yaitu pengenalan akan berbagai macam obat. Struktur kristal LmrR yang bebas dan berikatan dengan Hoechst 33342 atau daunomisin dielusidasi dengan resolusi 2.0 Å dan 2.2 Å. LmrR memiliki topologi α1-α2-α3-β1-β2-α4 dalam dua domain berbeda. Domain pengikatan DNA pada ujung N dari LmrR terbangun dari motif heliks-putar-heliks yang khas dari regulator transkripsi yang terkait dengan MDR pada bakteri, sedang ujung C terdiri dari sebuah pori tengah berbentuk rata berukuran besar untuk pengikatan ligan (Gambar 2).



Gambar 2. Struktur protein LmrR dielusidasikan dengan resolusi 2.0 Å. LmrR mempunyai pori tengah yang besar dimana reaksi pengikatan obat terjadi.

Fitur ini unik karena tidak ada satupun regulator transkripsi yang terkait dengan MarR/PadR yang telah dikarakterisasi sebelumnya memiliki pori tengah pada daerah antar muka dimernya. Terlebih lagi, sisi pengikatan LmrR berbentuk simetris dimana kedua sub unit memberikan kontribusi yang setara kepada struktur ini, tidak seperti pada sisi pengikatan obat pada BmrR dari *B. substilis* atau QacR dari *S. aureus* yang tidak simetris dan terbentuk dari sebuah sub unit tunggal. Ketika pengkatan obat terjadi, system cincin dari molekul Hoechts 33342 atau daunomisin yang rata terjepit diantara W96 dan rantai samping W96 membentuk tumpukan interaksi aromatis dimana setiap cincin pada kedua system indol tanpa adanya ikatan hydrogen antara obat dan LmrR teramati. Lebih jauh, analisis mutagenesis terarah mengkonfirmasi pentingnya peran W96 dalam pengikatan obat

dan DNA (BAB 5). Dengan membandingkan LmrR dalam keadaan bebas dan mengikat obat, beberapa perubahan orientasi dalam struktur teramati. Ketika dimer LmrR yang terikat kepada Hoechst 33342 atau daunomisin dibandingkan dengan bentuk bebasnya, salah satu domain wHTH berputar berlawanan arah relatif terhadap wHTH dari sub unit yang lain. Perputaran ini diikuti dengan perubahan ruang antara kedua heliks pengenal DNA. Perbedaan lainnya adalah arah dari residu-residu dari satu sub unit relatif terhadap residu-residu dari sub unit lain teramati di dalam pori pengikatan yang mempengaruhi struktur dari sisi pengikatan obat. Perubahan-perubahan ini diperkirakan memancing hilangnya kemampuan pengikatan LmrR untuk mengatur DNA menyebabkan terjadinya regulasi naik transporter MDR LmrCD. Struktur kristal LmrR yang berikatan dengan DNA merupakan tantangan dan diperlukan untuk menyibak dasar molecular dari mekanisme penekanan ekspresi *lmrCD* oleh transkripsi penekan LmrR di *L. lactis*.