Unraveling gene regulation of MDR and ABC-transporters in Gram-positive bacteria

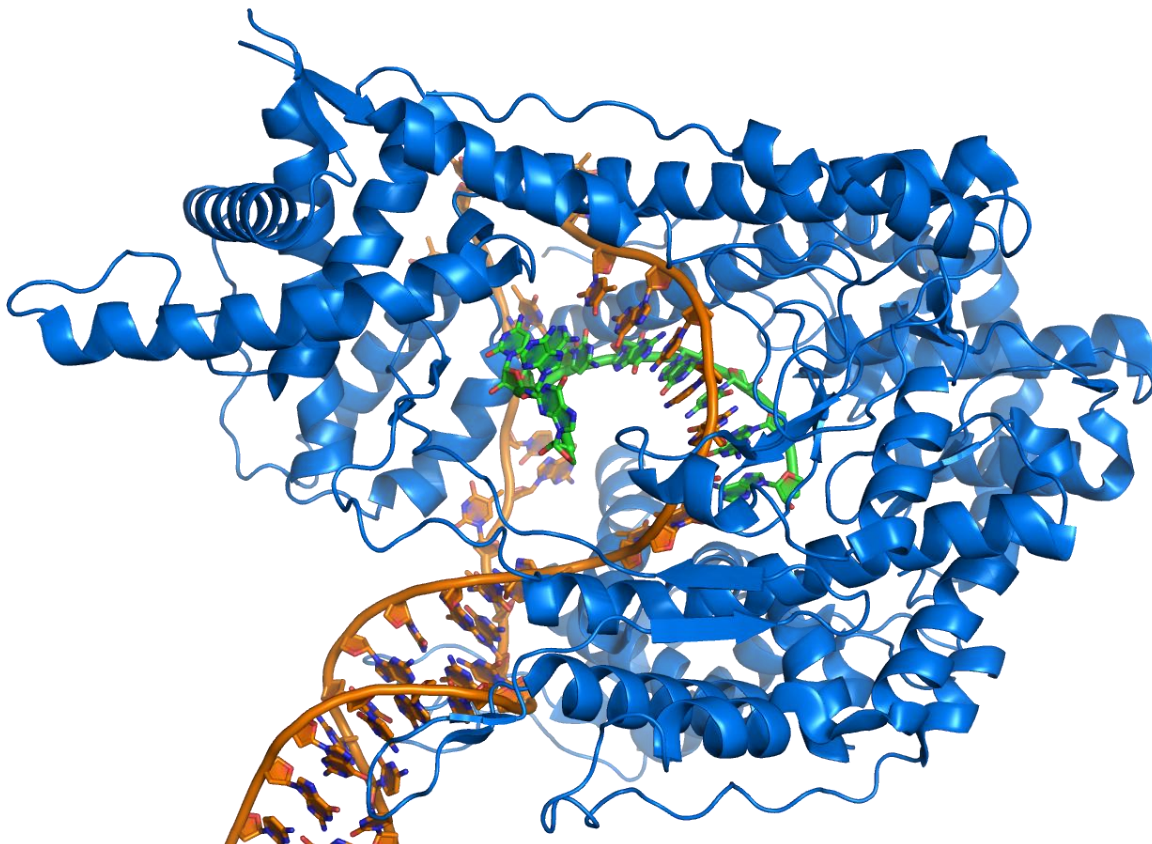

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Table of contents

Abstract 2
1. Introduction 2
2. Overview of different efflux transporters, their structure and mechanism 3
3. Regulatory genes and proteins 5
3.1. Regulation by repressing transcription 5
3.2. Induction of transcription 7
3.3. Regulation by global modulators 8
3.4. Upregulation by mRNA 10
4. Discussion and future perspectives 13
References 15

*Description figure on title page: 3D illustration of T7 RNA polymerase (blue) unwinding DNA (orange) and transcribing mRNA (green)

Abstract
Bacteria become more resistant to current antibiotics which means less antibiotics are available to treat bacterial infections. New antibiotics are needed and can be found by understanding the mechanism of resistance and developing antimicrobials that interfere with it. There are different mechanisms that confer antibiotic resistance and next to the protection of the cell wall, drug efflux is the most important mechanism in Gram-positive bacteria because it confers resistance to many structurally diverse antimicrobials. Fitness of cells is lowered if the efflux transporters are expressed unnecessary and, therefore, gene expression has to be regulated in accordance to changes in the environment. The most straightforward way to regulate drug efflux transporters is by direct induction or repression of its gene. Inhibitory proteins such as QacR and LmrR repress *qacA* and *lmrC/D*, respectively, in absence of antimicrobials and release the DNA once bacteria are challenged with antibiotics. Inducing proteins, on the other hand, induce gene transcription once it is bound to DNA and a substrate. Global modulator proteins MgrA and NorG regulate many processes, including the drug efflux genes *norA/B/C, tet38* and *abcA*. Mutations in DNA, and therefore in mRNA, can also cause upregulation of gene expression as well. Bmr3 and NorA are upregulated by an increased mRNA half-time caused by a point mutation. The mutation in *bmr3* and *norA* leads to a stabilization of their transcripts which upregulates translation. This thesis shows that genes can be regulated on different levels and in different ways which induce resistance to antimicrobials.

1. Introduction

Antibiotic resistance in bacteria forms an increasing problem in treating bacterial infections. Antibiotics become less effective because bacteria developed resistance to them over time and the number of effective antimicrobials is diminishing. (26) Understanding the mechanism of resistance could support the development of new antibiotics. Antibiotics have different methods to inhibit bacterial growth. The first method is to inhibit cell wall synthesis by interfering with the enzymes that are needed to form the peptidoglycan layer. (39) β-Lactam antibiotics and glycopeptides are responsible for this effect and on top of this, β-lactams induce expression of autolytic enzymes that is normally needed to restructure the cell wall during growth. (39) Teicoplanin and vancomycin can also prevent peptidoglycan cross-links from forming, which are needed for the cell wall, making the cell wall unstable. The second method to prevent the cell from growing is to inhibit protein synthesis by disrupting the ribosomes. Bacterial ribosomes differ from eukaryotic ribosomes and this can be utilized by prokaryotic-specific drugs, e.g. chloramphenicol, macrolides and tetracyclines. (44) The third method that is utilized by antibiotics is the interference with DNA synthesis. This can be done by inhibiting folic acid synthesis which is required for DNA synthesis, by creating double-stranded breaks in the DNA and preventing replication. Rifamycin antibiotics target RNA synthesis by binding to a subunit of a DNA-bound RNA polymerase which kills the cells. (23) These targets are being exploited by current antibiotics, since interfering with essential processes will kill the bacteria.

The problem with using antibiotics on a large scale is the rapid development of multi-drug resistance (MDR) in bacteria. (44) Resistance to antimicrobials can be acquired in different ways (Fig. 1). First, by producing enzymes that modify the drug targets and break down or modify the drugs itself, bacteria render them useless. Second, alteration of the cell walls can lead to a reduced permeability for the drugs, limiting the influx. Lastly, the bacteria can express drug efflux transporters on the membranes that actively drive the drugs out of the cell. These mechanisms differ slightly for Gram-positive and Gram-negative bacteria, because unlike the outer cell membrane of Gram-negative bacteria, the peptidoglycan layer of Gram-positive bacteria provides less protection, since it is more permeable for small molecules. (5) In Gram-positive bacteria the most important mechanism, next to the protection of

the cell wall, is the efflux of chemicals because it confers resistance to many structurally dissimilar antibiotics. (40) For that reason this thesis will lay focus on the efflux transporters of Gram-positive bacteria.

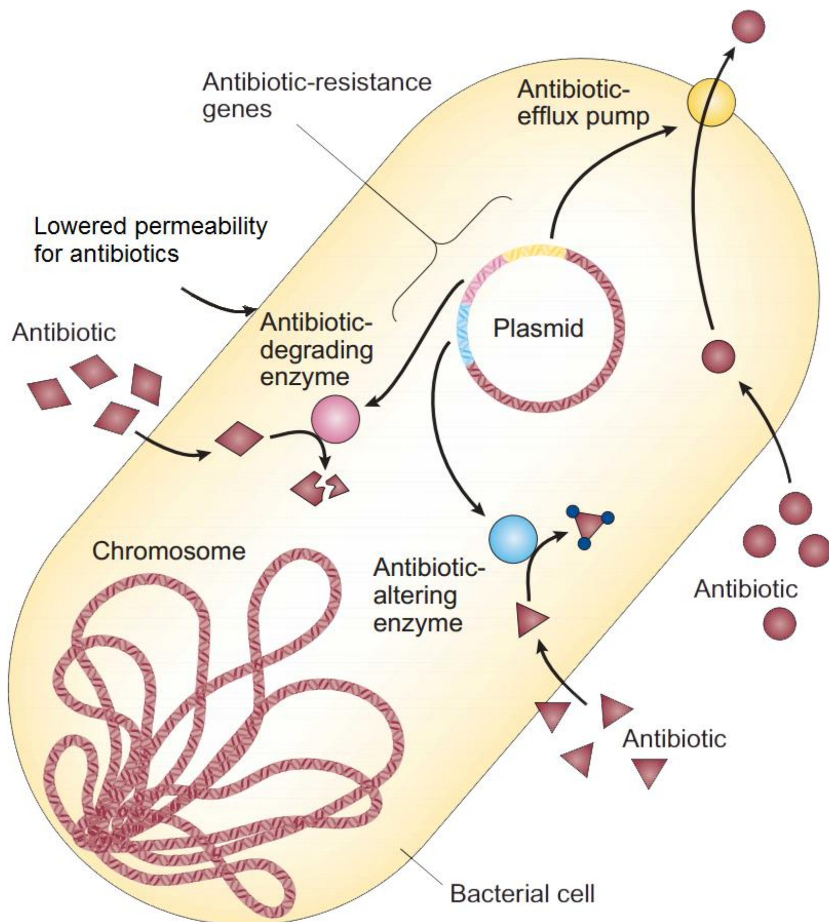

Figure 1. Modes of resistance against antibiotics. First, bacteria can become resistant by lowering the cell wall permeability to prevent drugs from entering the cell. Second, by breaking down or modifying the antibiotics, the deleterious effects of the drugs are nullified. The third way to prevent the drug from working, is by modifying the drug's target which makes it unrecognizable for the drug. The last method to become resistant to drugs is to transport the drugs out of the cell; this is done by the efflux transporters on the membrane. (Levi and Marshall, 2004, (25))

It is important for bacteria to regulate the expression of MDR transporters, for it will lower the fitness of the bacteria when proteins are unnecessarily expressed. (10) Because antibiotic resistance is only beneficial for a period of time, it only needs to be upregulated when it is needed. Research showed that gene expression of *Bacillus subtilis* and *Staphylococcus aureus* changes in a way that is favorable in an environment with toxic compounds. (3, 13) Changes in gene expression are a fact, but in what ways are drug efflux transporter genes regulated in Gram-positive bacteria? This thesis will first give an overview of several different efflux transporters and will discuss the different modes of drug efflux transporter gene regulation.

2. Overview of different efflux transporters, their structure and mechanism

There are five categories of efflux transporters, each transporting a wide variety of compounds out of the cell: the ATP-binding cassette (ABC) superfamily, the resistance-nodulation-division (RND) superfamily, the multidrug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), and the small multidrug resistance (SMR) family which is part of the drug/metabolite

superfamily. (26, 38) The ABC-transporter superfamily is the only family that uses ATP hydrolysis to actively drive antimicrobials and toxic chemicals out of the cell (Fig. 2).
The drug efflux transporters of the remaining superfamilies are secondary active transporters and are sodium/proton antiporters that effluxes drugs in exchange for sodium or protons. ABC-transporters have two subunits which bind and hydrolyze ATP and transport chemicals through a conformational change in the membrane-spanning domains. (9) RND transporters consist of three subunits with a periplasmic domain and are more common in Gram-negative bacteria. MATE transporters consist of 9 to 12 transmembrane domains. (56) SMR transporters function as dimers and the largest group of drug efflux transporters belong to the MFS.

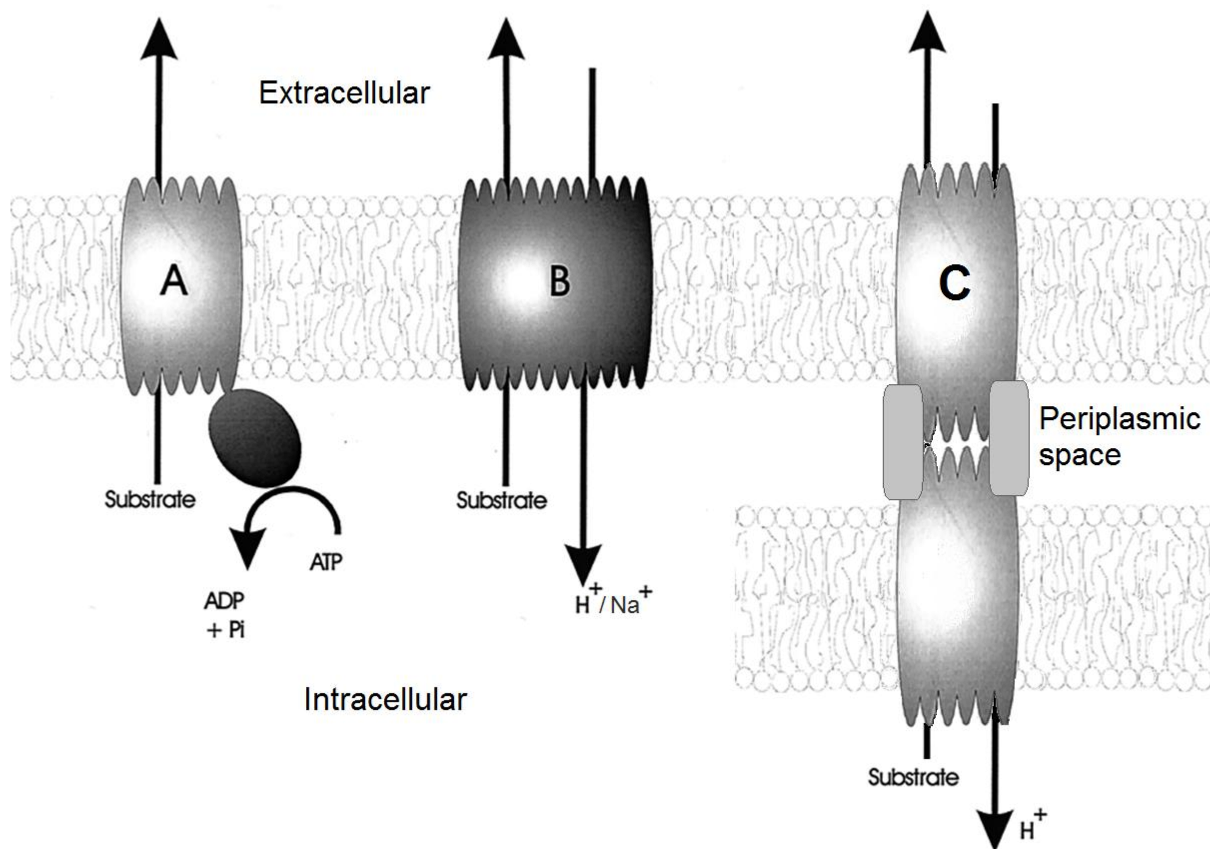

Figure 2. The two different types of drug efflux transporters. (A) ABC-transporters are primary active transporters that need ATP hydrolysis for energy to drive the drugs out of the cell. (B) Secondary active efflux transporters of the MFS, MATE and SMR family are antiporters and use sodium or proton gradient to pump the substrates. (C) Transporters from the RND superfamily are more common in Gram-negative bacteria. It consists usually of three parts and contains a periplasmic domain. (Putman *et al.*, 2000)

Some of the transporters are specific for one type of substrates, but most transporters recognize and transport structurally dissimilar substrates (40) All the MDR transporters have a drug-recognition pocket where the substrates 'enter' the transporter. After being recognized by the transporter, the substrates will be transported across the membrane. This can be achieved in multiple ways. The first method is by flipping a part of the transporter protein, a flippase, the substrate can be moved outside the cell. (21) Flippase activity normally applies for translocating phospholipids from one side of the membrane to the

other, but research showed that some drug efflux transporters also use this kind of mechanism to efflux drugs. (36, 43) Another way drugs can be transported is through a conformational change of the transporter protein that facilitates the efflux of the substrate. By doing so, the substrate will be gated or pushed out of the cell. Most substrates seem to be amphipathic, since it is easier to be transported across the membrane, but some are highly hydrophilic or hydrophobic. This depends on the structure of the transporter and is one of the reasons why so many different drugs are being pumped out by efflux transporters.

3. Regulatory genes and proteins

Since it is unfavorable for bacteria to continuously produce proteins that are not needed, expression of proteins need to be upregulated when needed and downregulated when it is not. This can be done in multiple ways and a combination of multiple mechanisms is also possible. Early studies to elucidate efflux transporter gene regulation were done with QacR in *Staphylococcus aureus* and BmrR in *B. subtilis*. (54)

3.1. Regulation by repressing transcription

One of the first bacterial drug efflux gene reported was *qacA* in *S. aureus*. (13, 54) QacA is an MFS transporter and is regulated negatively by the trans-acting QacR, a member of the TetR regulatory protein family that represses transcription. QacR binds downstream from the *qacA* promoter as a set of two dimers in absence of QacA substrates as shown with electrophoretic gel-mobility shift assay (EMSA), DNase I protection experiments and dynamic light scattering studies. (13, 14) Binding downstream from the promoter does not prevent the binding of RNA polymerase to the promoter, but it does inhibit the functional transcription of *qacA* by blocking RNA polymerase. It does not bind to its own gene; *qacR* transcription was not changed after fusing the gene to a reporter gene and introducing a plasmid with a trans-acting *qacR*. Therefore, it is not directly autoregulated. *In vitro* experiments show that in presence of different structurally dissimilar substrates, including ethidium bromide, profavlin, rhodamine 6G and benzalkonium chloride, *qacA* expression was upregulated caused by a release of QacR from the operator. *In vivo* expression experiments confirm this. When a substrate is bound to QacR, it changes conformation which is responsible for the derepression. (35) Binding assays show that the conformational change favors binding to the substrate over the IR1 operator and releases the operator.

All of the experiments show that QacR does not bind as a single dimer to DNA, but rather as a complex of two dimers, indicating QacR binds cooperatively to the DNA. This cannot be done by cross-dimer protein-protein interactions, since the dimers are too far apart. (42) This suggests that a conformational change in the DNA could be responsible for the cooperative binding. DNA with bound QacR is slightly twisted and bent and shows widening of the major groove throughout the binding site with two especially widened regions. The exact QacR-DNA complex structure was determined by crystalizing it using multiple isomorphous replacement where thymines were replaced with 5-iodouracil. (42) A pair of QacR dimers binds with the C-terminals to each other to form dimers and the N-terminal which has a DNA binding domain bind to IR1 in the two widenings in the major grooves. The C-terminal is also responsible for drug binding and when a drug binds, it triggers a conformational change in QacR and will release the DNA which unblocks RNA polymerase and promotes transcription.

A different regulatory protein is LmrR in *Lactococcus lactis*. LmrR is a member of the PadR transcription regulatory protein family and was formerly called YdaF. It is an inhibitor that represses transcription of the ABC transporter *lmrC/D* and, unlike QacR, its own gene in absence of toxic compounds. (1, 28) The

transporter is expressed constitutively but resistance can be further induced by Hoechst 33342, rhodamine 6G, cholate and daunomycin. (2) EMSA and DNase I protection assays showed that LmrR forms multiple complexes with promoter regions of both *lmrR* and *lmrC/D* which indicates that LmrR is autoregulated and functions as dimers. A closer look reveals two binding sites on the *lmrC/D* operator sequence that forms a dimer of LmrR; site I on the -35 and -10 regions which LmrR forms a high affinity bond with and site II 29 bps further on which is a low-affinity binding site. (1) When substrates for LmrR are present intracellular, they will bind to the protein and it will release the DNA, making it accessible again for RNA polymerase. Because the genes are in the vicinity of each other, *lmrR* and *lmrC/D* are partly co-transcribed. This results in a longer transcript when RNA polymerase starts at *lmrR's* promoter and a shorter transcript when transcription starts at l*mrC/D* (Fig. 3).

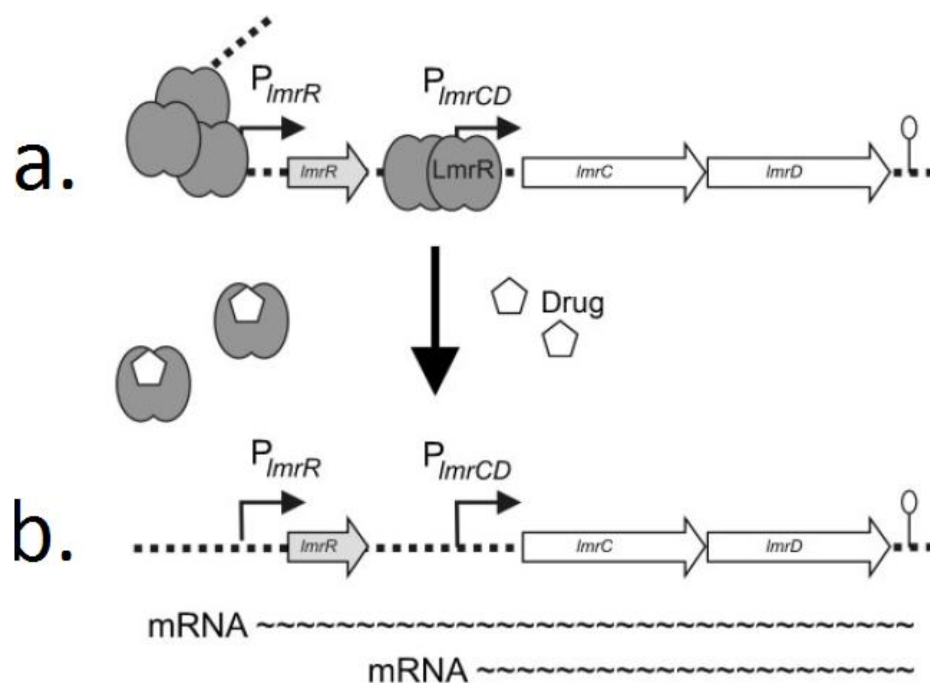

Figure 3. Two states of *lmrR* and *lmrCD* transcription sites. (a) In absence of drugs, multiple dimers (three depicted) and two dimers of LmrR repress *lmrR* and *lmrCD* operators, respectively. (b) When drugs are present, LmrR binds to the drug and releases the DNA which makes transcription possible. The genes are partly co-transcribed and share the same stop site which results in two transcripts: a longer one when transcription starts at the promoter of *lmrR* and a shorter one when it starts at the promoter of *lmrCD*. (Augustiandari *et al*., 2011)

Using an EMSA, Agustiandari *et al*. (2011) showed that when LmrR was bound to site I, the affinity for site II increased and promoted the formation of LmrR dimers. The affinity of LmrR for *lmrR* promoter is two- to fourfold higher than for *lmrC/D* and because of this difference a low concentration of toxins causes only *lmrC/D* to be upregulated. A higher concentration is needed to induce transcription of both *lmrC/D* and *lmrR* which will in turn repress *lmrC/D* and *lmrR* transcription. This negative feedback is needed, for LmrC/D can secrete hydrophobic metabolites that are essential for the cell. On top of that, too high concentrations of LmrR can interfere with the response to toxins by insufficiently derepressing efflux transporter transcription. *In vivo* experiments with *L. lactis* showed that transcription of these genes were increased, as predicted, by using daunomycin and Hoechst 33342. (1, 2) Moreover, Agustriandari *et al*. (2011) used an *L. lactis* NZ9000 Δ*lmrR* knock-out strain and observed an increased

expression of *lmrC/D* compared to the wild type and concluded that LmrR regulated *lmrC/D* negatively. Overexpression of *lmrR* confirmed the repressing role of LmrR. Similar regulatory mechanisms are present in the regulation of the MATE-family transporter MepA in *S. aureus* and the ABC-transporter LmrA/B in *L. lactis* by MepR and LmrA, respectively. (24, 57)

To summarize, transcriptional inhibitors of drug efflux transporters repress gene transcription, until a substrate is present intracellular. At that point, when the substrate binds to the repressor, it releases the DNA which makes transcription possible. A conformational change of the regulatory protein or a shift in affinity towards the substrate facilitates the release. One difference between QacR and LmrR is that the latter is autoregulatory. Furthermore, QacR binds upstream of *qacA* which blocks transcription while LmrR binds to the promoter of *lmrCD* to prevent RNA polymerase from initializing.

3.2. Induction of transcription

Next to negative regulation, positive gene regulation is also possible. Another early discovered and well-studied regulatory protein is BmrR in *B. subtilis* which regulates the efflux transporter Bmr. (3, 58) BmrR belongs to the MerR family transcriptional regulators, which regulates response to stress such as oxidative stress and exposure to toxic compounds and heavy metals. It is a direct inducer of *bmr* and its gene is located directly downstream of *bmr*. Bmr transports various antimicrobials, including fluoquinolone antibiotics, ethidium bromide, puromycin and chloramphenicol, as shown with *in vivo* experiments. Bmr shares similarities with other MDR efflux transporters. It shares 51% sequence homology with Blt (Bmr-like transporter) in *B. subtilis* and it recognizes and transports roughly the same substrates. (50) The sequences of Bmr and NorA, which will be discussed later on, is 44% identical and also transport similar substrates. (18) The regulator protein consists of a homodimer which has an N-terminal DNA-binding domain and a C-terminal drug-binding pocket and is activated by diverse lipophilic positively charged molecules. Several among these molecules are the substrates for the Bmr transporter. (41)

Sequence analyses indicated that an ORF, that later was called *bmrR*, was located immediately downstream of *bmr.* This ORF contained an N-terminal helix-to-helix motif which was similar to known N-terminal DNA binding domains of bacterial regulatory proteins SoxR (*E. coli*) and TipA (*Streptomyces lividans)*. (3) SoxR is responsible for the induction of superoxide resistance by inducing DNA repair and transcription of proteins that reduce oxidative stress. (37) SoxR binds to DNA, but it does not do anything in its own, which is common in MerR regulatory proteins. (31) It has been proposed that SoxR undergoes a transformational change when it is bound to superoxides. This change alters the structure of DNA and induces transcription. TipA regulates resistance to toxins and antibiotics, such as thiostrepton, promothiocin and nosiheptide by upregulating transcription of MDR genes that prevent antibiotics from binding to ribosomes. (19) Upregulation of transcription is achieved by twisting the DNA, thereby aligning two recognition sequences which induces transcription. Despite the fact that the regulatory proteins induce different responses, the mechanism by which it upregulates transcription is the same: the (activated) regulator changes the structure of DNA by binding to it.

It was thought that BmrR would regulate the nearby *bmr* gene in the same way. EMSA showed that, indeed, BmrR bound to the *bmr* promoter in *B. subtilis*. (3) Experiments showed that *B. subtilis* Δ*bmrR* strains were unable to induce *bmr* transcription when challenged with antibiotics and dyes. Binding assays did also not show any signs of proteins binding to *bmr* promoter in the knock-out strain, supporting the evidence that BmrR regulates *bmr* expression positively. The affinity of BmrR for DNA changes when it binds to a substrate. When a substrate is bound to the dimer, the affinity for DNA

increases and will bind to the *bmr* promoter as shown by Ahmed *et al*. (1994) with an *in vitro* binding assay. DNA-bound BmrR changes the conformation of DNA, just as SocR and TipA does, which induce transcription. In the B-DNA conformation, the conformation that DNA assumes in its physiological condition, *bmr* is inaccessible for RNA polymerase, because the promoter is placed on opposite sides of the DNA, separated by a 19-bp spacer as shown by X-ray crystallography (Fig. 4). A spacer of 17 bps would bring the two promoter parts to the same side which makes transcription possible. When a dimer of BmrR binds to the -35 and -10 *bmr* promoter region, it induces base unpairing and slides the DNA to shorten it by approximately 5 Å, which is about 2 bps, aligning the two promoter parts. (58) Moreover, binding of BmrR untwists and bends the DNA away from the protein, making it more accessible for RNA polymerase. *In vivo* experiments showed that *bmr* transcription was increased significantly after exposing *B. subtilis* cultures to rhodamine and tetraphenylphosphonium. This was done by fusing the *bmr* gene to the reporter gene lacZ which encodes for β-galactosidase and by measuring the β-galactosidase activity which correlates with transcription. This shows that genes can be regulated not only by changing the affinity of the regulatory protein for RNA polymerase, DNA or other proteins, but by changing the conformation of DNA.

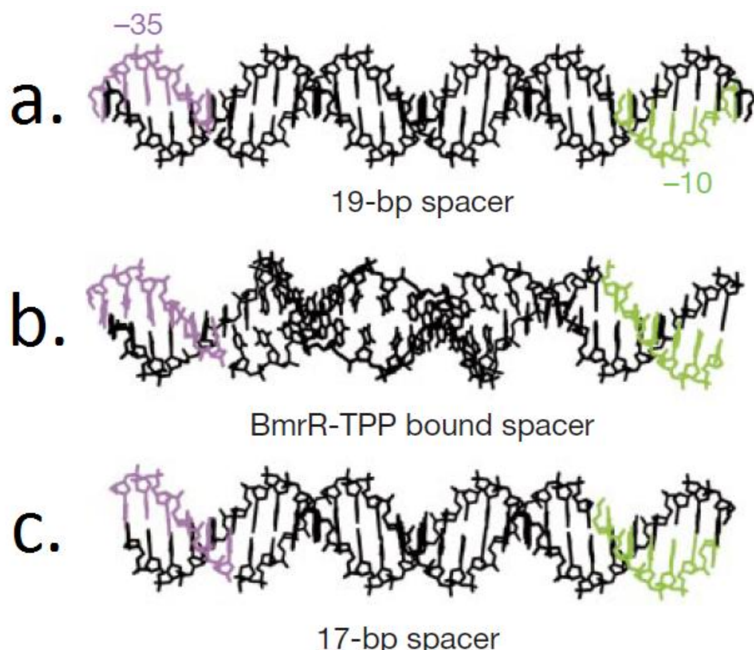

Figure 4. A representation of *bmr* promoter region. (a) The *bmr* promoter is on opposite directions in its B-DNA state. (b) BmrR protein bound to the substrate tetraphenylphosphonium and the promoter causes a reduction of the spacer between the -35 and -10 region, aligning the promoter parts to the same side of the DNA. (c) A 17-basepair spacer would bring the two promoter parts to the same side of the DNA strand. (Zheleznova *et al*., 2001)

3.3. Regulation by global modulators

Besides gene-specific regulation, there are proteins that regulate many processes. MgrA (SarA subfamily, formally known as NorR) protein in *S. aureus* is a regulator of various processes, including virulence factors, autolytic activity and drug efflux pumps. (17) The SarA family regulatory proteins directly regulate virulence genes by binding to the promoters with the helix-turn-helix N-ternimal. MgrA was first known to be involved in regulation of the MFS transporter NorA. (51) NorA consists of 12 transmembrane segments and transports various lipophilic cations. Truong-Bolduc *et al*. (2003) conducted a study with *mgrA* overexpression in *S. aureus* and showed the strain was four times more resistant to quinolones and ethidium bromide than the control strain. An increased transcription of *norA*

was also observed. An EMSA showed that MgrA bound to *norA* promoter. A control experiment was performed to verify the increased resistance was due to an increase in *norA* transcription. In this experiment, *mgrA* was overexpressed and *norA* was knocked out. Only a slight decrease in susceptibility was observed which suggests the effect of *mgrA* overexpression was largely due to an increase in NorA.

Because global regulators interact with multiple systems and proteins, two other closely related global regulators, *agr* and *sarA*, were taken into account. These are, among other things, involved in regulation of gene transcription of virulence factors and biofilm formation. (27) Since *arg* and *sarA* play important roles in regulation of various processes, the authors presumed that these two genes could play a role in regulating *norA* expression as well. An *agr* and a *sarA* mutant strains were used to test the presumption and showed that the sensitivity for quinolones and ethidium bromide did not change when *mgrA* was overexpressed. This suggests that these two regulatory systems are needed for MgrA to regulate *norA* expression, though it is not clear in what way they are involved. Next to that, EMSA was performed to check where MgrA bound to the DNA and showed that it only binds to *norA* promoter and not to that of itself, indicating that *mgrA* is not directly autoregulatory. Later studies using microarrays showed that several other open reading frames (ORFs) were involved in response to MgrA. (49, 50) Among the potential genes, the MFS efflux transporters NorB, NorC and Tet38 were identified. By using overexpression and knock-out techniques as described before, the regulatory role of MgrA was confirmed. EMSA showed that MgrA bound to *norA* and *norC* promoter, but only limited to that of *norB* and did not bind at all to the *tet38* promoter. This indicates that MgrA regulates *norA* and *norC* directly, *norB* to a lesser extent directly, and *tet38* indirectly.

A year later, Truong-Bolduc *et al*. (2007) extracted proteins that were bound to the *norA* promoter and after separating them with an SDS-PAGE, they noticed another protein was bound to the promoter in addition to MgrA. After comparing the protein's sequence to ORFs, an ORF was found that was predicted to encode a protein involved in regulation of quinolone and β-lactam resistance. It was then called NorG. (48) It belongs to the GntR-like transcriptional regulators which regulate diverse processes, for example fatty acid degradation, rhizopine metabolism and gluconate utilization. Microarray and real-time RT-PCRs showed that NorG was involved in many diverse processes, including energy production, cell wall and membrane synthesis, and various transport and metabolism systems which indicates NorG is also a global regulator just like MgrA. (46) EMSA showed NorG bound to the promoters of *norA*, *norB*, *norC, tet38* and the ABC-transporter *abcA* and by using reverse DNA footprinting, the exact binding sites of NorG were determined. It has two binding sites on *norA*/*B*/*C* and *abcA* promoters that contain an imperfect palindrome and a common nucleotide sequence AAA which is located in the loop formed by the palindrome. Among these MDR transporters, NorG induced the expression of *norB* the most and repressed the *abcA* expression the most (Fig. 5). (46, 48) However, overexpression of *norG* did not increase *norA* mRNA levels. Peculiarly, by knocking out the *norG* gene, expression of *norA, -B,* and -*C* did not change, indicating other proteins play a part in the regulation as well. *abcA* transcription, on the other hand, was decreased by overexpressing *norG*, suggesting NorG is a direct repressor of *abcA*. Finally, NorG can be directly downregulated by MgrA. To determine the mechanism of MgrA on *norB* expression, binding assays were performed and showed that MgrA bound to *norG*, the direct promoter of *norB*. Although the upregulation of MgrA indirectly downregulates NorB, it increases overall antibiotic resistance as it upregulates many other efflux transporters, including AbcA, NorA, BcrA-like drug efflux transporter SACOL2525 and SACOL2566, as shown with a microarray analysis. (46)

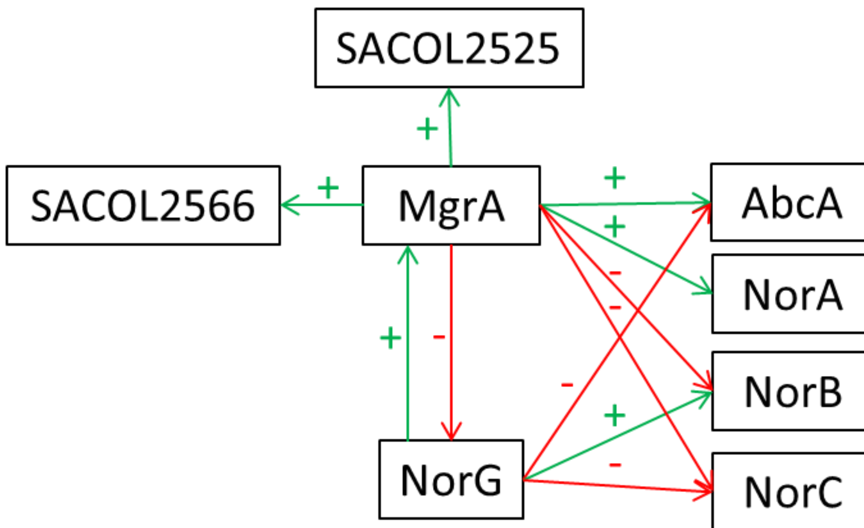

Figure 5. Induction and repression of MgrA and NorG on different drug efflux transporters. MgrA and NorG induce and repress different genes, as shown with microarray analyses. MgrA induces more drug efflux transporters than it represses and it also represses NorG, which leads to a derepression. Other proteins (both known and uncharacterized) that are omitted play a part in the regulation as well.

To summarize, MgrA binds to *norG* promoter and inhibits NorG which in turn indirectly inhibits NorB production. *norB* is also presumable repressed directly by MgrA. AbcA is upregulated directly by MgrA and downregulated directly by NorG. Regulation of NorA is still unclear, since both MgrA and NorG bind to its promoter, but NorG does not seem to have any effect on *norA* expression. The exact mechanism of regulation is still unknown and, furthermore, multiple other proteins and regulatory systems play a part in the regulation which makes understanding the mechanisms more difficult, as will be discussed in the discussion section.

3.4. Upregulation by mRNA

Regulation on a transcriptional level is not the only method to increase or decrease protein expression. Translational regulation is another way to regulate gene expression. The translation from mRNA to proteins depends, among other things, on the amount of mRNA, degradation rate and its half-life. More proteins may be synthesized when the amount of mRNA is higher, when its half-life is longer, or when the rate of degradation is lower. The ABC-transporter Bmr3 in *B. subtilis* is regulated by stabilizing the mRNA transcript, which increases its half-life. (33) The efflux transporter transports roughly the same substrates as Bmr and Blt, even though they show relatively low homology. (32) Overexpression of the hypothetical MDR protein Bmr3 in *B. subtilis* 168 led to an increased resistance to puromycin, tosufloxacin and norfloxacin. Ohki and Tateno (2004) added high concentrations of puromycin to *B. subtilis* 168 cultures and observed two groups of spontaneous mutants; one group was highly resistant to lincomycin and puromycin (PLR mutants), and the other group to ethidium bromide, tosufloxacin, norfloxacin, daunomycin, and puromycin (PR mutants). PLR mutants showed a mutation was present in *lmrA* which represses the drug efflux transporter LmrB. (30) This causes a derepression of LmrB, increasing antibiotic resistance. PR mutants acquired resistance in a different way. Southern hybridization and sequencing showed that the protein, which was responsible for the increased resistance in PR mutants, was a known MDR transporter in *B. subtilis*: Bmr3. Two mutations in the *bmr*3 gene were found in the 5' UTR. Two changes in amino acids were also found, but protein function and

drug specificity were not altered. (33) Northern blotting was used to determine *bmr3* mRNA levels, and compared to the wild type, mRNA level of PR mutants was 20-fold higher.

A closer look at the mRNA structure revealed why the mutation lead to higher mRNA levels, and thus to a higher protein expression. The mRNA forms a stem-loop at the 5'-proximal region which is a factor for mRNA stabilization (Fig. 6). (15) One mutation, that changed a T to an A in the mRNA, resulted in an unmatched T-T pair to be changed to a matched A-T pair. This stabilizes the loop. The other mutation changed a C to T. The half-life of *bmr3* mRNA measured in PR mutants was four times higher than the wild-type strain mRNA, and by fusing the mRNA that formed the loop to the *lacZ* gene, the contributions of the mutations to the increased resistance was determined. (33) The T to A mutation increased β-galactosidase activity by 50-fold, the C to T mutation only two-fold and both of the mutations led to an activity of 200-fold compared to that of the wild-type strain. *lacZ* mRNA half-life measurements show the T to A mutation increased half-life by approximately four times compared to the control strain (4 and 15 minutes, respectively) but the C to T mutation did not increase half-life at all. Therefore, the *lacZ* T to A mutation is probably responsible for the increased stability. The C to T mutation fusion strain that show increased β-galactosidase activity may be due to an increase in mRNA synthesis rate. It should be noted, however, that these mutations occur in presence of a very high concentration of puromycin (100μg/ml) and even though a (known) feedback for this mutation is lacking, this spontaneously developed phenotype is beneficial for the bacteria in this environment. Further research needs to be done to learn about possible regulation mechanisms and about how the mutation is induced.

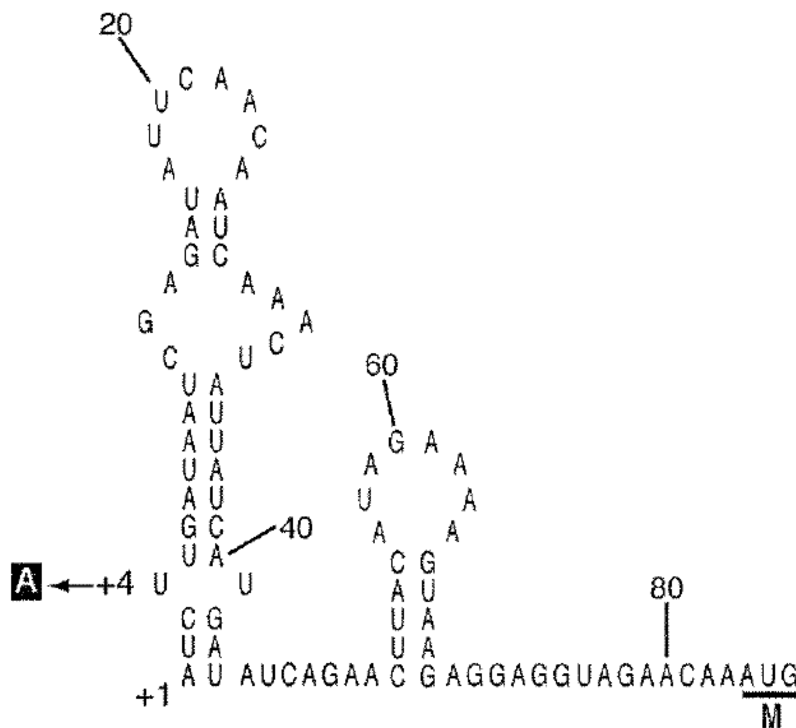

Figure 6. Point mutation in the 5' UTR of *bmr3* causes the hair pin loop to be stabilized. At the +4 location to the transcription initiation start site, the U changes into an A which pairs the unpaired U-U mRNA. This stabilizes the mRNA. (Ohki and Tateno, 2004)

Point mutations in other drug efflux transporter genes also result in a more stable mRNA which can cause an upregulation of proteins. A mutation in *flqB* which is upstream in the 5' UTR of *norA* caused an upregulated expression of NorA. (12) The point mutation changed a T to G. Researchers observed that mRNA levels of *flqB* mutant strain (MT) *S. aureus norA* were higher than in the wild-type strain. At first

they thought the mutation altered the affinity of DNA for a regulatory protein, but further research showed that this specific site does not bind any proteins. (11) The number of plasmid copies was not different between the mutant and wilde-type strain and the mutation also did not create an extra transcription site nor did it induce affinity for RNA polymerase. After assessing *norA* mRNA stability of the wild-type and the MT, it was clear that the half-life of MT mRNA was increased by almost five-fold compared to the WT (7 minutes versus 34 minutes, respectively). (12) A Northern blot verified the increased mRNA. An mRNA computer-prediction showed the mRNA formed a stem-loop in the WT, as in the case of Bmr3 mRNA. The mutation in MT, however, created an additional loop instead of stabilizing the existing loop. Multiple other types of mutations in this region such as deletions and insertions also influences the stability of *norA* mRNA in presence of antibiotics. (8) Interestingly enough, another research group did not observe an increased mRNA stability in an *S. aureus* strain with the same mutation and suggested the increased *norA* mRNA was due to an increase in transcription. (18) The authors did mention that there were technical and methodological differences between their work and previous work which could explain the differences in results. Nevertheless, a decrease in MgrA inhibition was observed which suggests the 5' UTR interacts with MgrA.

Events that lead to a different mRNA sequence or structure can have other effects as well, next to stabilization. The ABC-transporter VmlR, formerly known as ExpZ, in *B. subtilis* confers resistance to lincomycin and virginiamycin M, and it seems that *vmlR* expression could be regulated on a transcriptional level by antisense mRNA. (34) Increased antimicrobial resistance was shown by performing a disk diffusion test with *B. subtilis* Δ*vmlR*. The knock-out strain was 40 times more sensitive to virginiamycin M than the control strain and 17 times more sensitive to lincomysin. Resistance to these antibiotics was increased after inducing bacteria cultures with virginiamycin M and lincomycin. Northern hybridization showed a thickened band at 1.9kb after induction and confirmed the upregulation of *vmlR*. This indicates that the ABC-transporter is inducible by drugs and is regulated on a transcriptional level. A second probe was used that targeted the 5' UTR, in contrast to the first probe that targeted the start of *vmlRí*'s ORF. Without the induction of *B. subtilis* culture with antibiotics, a thick band below 300bp was clear. After inducing the culture with virginiamycin M, a new band next to the existing one appeared at 1.9kb; VmlR. When zooming in on the band at approximately 200bp, smaller bands that ranged from 95 to 200kbs were visible. Ohki *et al.* (2005) used antisense DIG-labeled RNA for the same stretch of 5' UTR and observed the same band patterns, which indicates that the transcripts are transcribed from the antisense DNA. Because the first probe that targeted *vmlR* RNA could not detect these smaller transcripts, they may be prematurely terminated or be posttranscriptionally modified.

By using primer extension analysis, two start sites in the 5' UTR could be derived. The first site belongs to *vmlR* at 225bp upstream of *vlmR*'s first codon. The second one belongs to the mRNA detected by the second probe at 89bs upstream. Alongside to that, several inverted repeats were detected and a computer prediction showed a typical terminator loop was formed in the 5' UTR. Ohki *et al.* assumed the loop terminated *vmlR* transcription prematurely and posttranscriptional modification resulted in the different smaller transcripts detected by the second probe. Strains with different mutations were fused with the *lacZ* gene to test this assumption. By deleting the terminator region, an increase in β-galactose activity was observed that could not be upregulated by adding virginiamycin M. This indicates the loop represses transcription and is necessary for the response to the antibiotic. To assess whether the predicted terminator loop truly is a transcriptional terminator, an *in vitro* transcription analysis was performed with the highly specific Sp6 RNA polymerase. The two products that were transcribed matched the transcript sizes of the complete transcript and the incomplete transcript caused by the terminator. A deletion of the loop resulted in the transcription of one full-length transcript. To exclude

other factors, the stability of *vmlR* mRNA was measured and no change was observed after inducing *B. subtilis* cultures with virginamycin M. This is a strong indication that the loop in the 5' UTR terminates transcription and is possibly regulated by antisense RNA in the presence of virginiamycin M and lincomycin. When these antibiotics are present, transcription will not stop at the 5' UTR terminator, but will somehow partially read through the loop and produce the entire transcript. The exact mechanism, however, is not known.

Just like regulatory proteins, mRNA is important in gene expression. It can induce translation by stabilization the mRNA or by unblocking ribosomes which makes translation possible. The regulatory role of mRNA is typically determined by the 5' UTR, where the mutations occur. (16) This could mean that the mutations alter the regulation of MDR efflux transporters as well, even though sometimes there is no direct link found between the mutation and the effect on gene expression.

4. Discussion and future perspectives

To conclude, expression of MDR efflux transporters can be regulated in different ways in presence of antimicrobials, often in ways that are known for other genes. This includes, but is not limited to, direct and indirect transcriptional regulation, regulation through global modulators, and by mRNA or RNA modification. Altering protein affinity for promoters appears to be a common method. Although this thesis discussed several ways to regulate gene expression, other ways are possible. The affinity of RNA polymerase for DNA or ribosomes for RNA, for instance, could be altered to change gene expression. Methylation and acetylation can also occur that silences and induces genes, respectively. These processes occur in the regulation a few ABC-transporters in human lung and white blood cells. (31, 52) Even though they are eukaryotic cells, methylation in prokaryotes also occurs and can apply to efflux transporter genes as well. (55) Post-translational modifications to system component, such as phosphorylation or protein modification, can also induce or repress MDR transporter expression. Furthermore, microRNAs can alter the gene expression by breaking down mRNA or blocking translation which can cause an up- or downregulation of efflux transporters. Regulation through microRNAs has been observed in both eukaryotic and prokaryotic cells, though it is currently only known that ABC-transporters are regulated this way in humans. (16, 45) All other known methods, that have not been discussed, that influence prokaryotic gene expression could also be possible regarding MDR efflux transporter gene expression. This includes modification of protein/DNA/RNA, mRNA turnover and translation/transcription rate. Bacteria can utilize these methods to regulate non-MDR transporter genes, and as such can also apply to drug efflux transporters.

It is evident that gene regulation is not always that simple, as seen in section 3.4. A whole network of multiple genes is playing a role in MDR efflux transporter gene regulation and not all of them are discussed in this thesis to prevent it from being too extensive. Other global regulator genes, for example *mta, arlRS* and *sarZ*, also play a role in gene expression of different MDR genes, including *norA* and *bmr*. (4, 10, 46, 47) Moreover, knock-out experiments show that other not-specified proteins are part of the regulation. Induction of MDR gene transcription can occur due to direct induction or as a consequence of another process that is activated by a global modulator, making drug efflux transporter genes even harder to characterize.

With regard to the studies that were discussed, the work of Ohki *et al.* (2004) showed an interesting fact. Two changes in amino acids were found, while the two mutations occurred in the 5' UTR. Perhaps other factors played a role or their sequencing failed, as the changes could not have been caused by the mutations and they did not mention the cause either. A noteworthy fact is that the authors argued with

other research groups about the correct nucleotide sequence and for this reason, other sequencing techniques might be used to verify the correct sequence. Besides this issue, the regulation of the mutations in the 5' UTR regions that have a regulatory role should be further investigated. The mutations are induced by drugs, which means it is regulated in some way, though it is not known whether they are maintained or reversed when drugs are removed. Various other studies showed that in presence of antibiotics, insertions and deletions in the same DNA region occurred. Altered protein expression and antimicrobial resistance was observed, supporting the idea that the 5' UTR is involved in gene regulation. Unfortunately, not all study groups agree with each other; two conflicting articles were found. In contrast to Fournier *et al*. (2001), Kaatz *et al*. (2005) found no evidence for *norA* mRNA stabilization in *S. aureus* after inducing mutagenesis that altered a basepair. MICs were for several substrates twice as high in strains with different point mutations (T to G, A and C) and *norA* expression was also higher, but mRNA stability was similar between the strains. Only the *flqB* T to C mutation increased *norA* mRNA half-life compared to the control (151 and 68 seconds, respectively). The differences in outcomes of the studies can be explained by analyzing their methods. The two research groups used different *S. aureus* strains to start with; Kaatz *et al*. used strain SH1000 whereas Fournier *et al*. used MT23142. Both of the research groups used computer-simulated folding, but ended up having different structures and binding strengths, indicating their sequences were not entirely the same. Computer predictions of Kaatz *et al.* showed all of the mutations had less favorable free energy and the study did not make any remarks about an additional hairpin or about changes in general. Kaatz *et al*. also froze the cell cultures at -70°C before extracting mRNA. On top of that, different plasmids were used; not use a single common plasmid was used. These differences could explain the different results.

Regardless of the differences between the studies, mutations leading to an increased MDR efflux transporter expression could be an important factor in antibiotic resistance, since stressing the bacteria will induce horizontal gene transfer and an SOS response that will increase chances of mutations. (53) Studies showed an SOS response can be induced in bacteria, including *E. coli, B. subtilis, S. aureus* and *L. lactis* by various stimuli, such as heat, UV-radiation and antibiotics. (7, 22) Reactive oxygen species that are produced by stimulating bacteria with antimicrobials, such as quinolones and β-lactams, damage DNA and together with the induced error-prone DNA repair system, there is even a higher chance of mutations. Antibiotic resistance can be induced by an SOS response in *E. coli* and *S. aureus* among other things. (20, 22). This is why encountering SOS response might be important in sensitizing bacteria for antibiotics. In addition, more research should be done with small RNAs (sRNAs) as well, since they also have the ability to alter gene expression. Studies show there are many transcription regulating sRNAs that are involved in various processes in bacteria, such as sporulation, toxin production, arginine metabolism and cell maintenance. (16) sRNAs are discovered earlier in humans than in bacteria, and they prove to be important in gene regulation. However, research about sRNA in prokaryotes is lagging behind the research and knowledge about sRNA in eukaryotes, and thefore, we probably only see the tip of the ice berg. Although the role of sRNAs in Gram-positive bacteria is not fully characterized yet, more and more sRNAs are being discovered and different studies show it could play a big role in gene regulation of different processes, just like in eukaryotic cells.

Taken together, there is no doubt that MDR transporter gene regulation can be complicated in some cases, especially when multiple regulators of different systems are involved, but it is important to understand the regulatory mechanism. By doing so, new agents can be developed that can that interfere with the regulation and provide us antimicrobials that are badly needed. Researchers are already looking at novel drug targets, but most of them are not feasible for different reasons, e.g. the drugs cannot reach the target. (39) Unfortunately, for the same reason, it is not guaranteed that new antimicrobials will be found by understanding the gene regulation. Next to that, newly synthesized or

modified antimicrobials could be too toxic to be used in a clinical setting or they are not administrable due to poor pharmacokinetics. (29) Nevertheless, it will give us more options for treating infections caused by bacteria that are resistant to current antibiotics. On top of that, research showed a combination of different antibiotics can cause conflicts in regulatory pathways. (6) With insight on the regulatory mechanisms, it is possible to predict and select the best combinations of existing drugs that cause conflicts in gene regulation, which can make bacteria more susceptible to antimicrobials. All in all, there is still much to learn about gene regulation of drug efflux transporters, moreover, more genes and regulatory pathways that confer antibiotic resistance are being discovered. Therefore, unraveling the gene regulation of drug efflux transporters could be a long way, but it will help us to understand and combat antibiotic resistant bacteria.

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