Pedro Arêde Rei

Licenciado

On the track of β-lactam resistance: Studies on the regulation of methicillin-resistance in *Staphylococcus aureus*

Dissertação para obtenção do Grau de Doutor em Biologia

Orientador: Doutor Duarte Carvalho Oliveira, Assistant Researcher, CREM, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Co-orientador: Doutora Hermínia de Lencastre, Full Professor, ITQB, Universidade Nova de Lisboa

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Acknowledgments

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen, causing a wide range of infections. MRSA has not only developed an intrinsic resistance to all β-lactams, but has also acquired resistance to virtually all classes of antimicrobial agents. The characteristic MRSA phenotype is conferred by the presence of *mecA* gene which is regulated by a sensor-inducer (MecR1) and a repressor (MecI). However, *mecA* induction by its cognate sensor/inducer is very inefficient and, therefore, it is believed that optimal expression of β-lactam resistance in MRSA requires a non-functional MecR1-MecI system. Surprisingly, no correlation was found between the presence of functional MecR1-MecI and the level of β-lactam resistance in a representative collection of epidemic MRSA strains, suggesting the existence of other *mecA* regulators.

In these studies, we show that the *mecA* regulatory locus is not a two-component system but, actually, it is a three-component system containing besides *mecR1-mecI*, the previously unidentified anti-repressor *mecR2*. The crystal structure of MecR2 reveals a three-domain architecture, with an N-terminal DNA-binding-like domain, an intermediate scaffold domain, and a C-terminal dimerization domain, important to the functional dimeric oligomerization state. MecR2 disturbs the binding of the repressor MecI to the *mecA* promoter, which leads to its proteolytic inactivation independently from MecR1, presumably by non-specific cytoplasmatic proteases. Our data also demonstrates that in the presence of functional *mecR1-mecI* genes, *mecR2* is essential for a robust induction of *mecA* transcription and, consequently, for the optimal expression of resistance phenotype in MRSA. These observations point to a revision of the current model for the transcriptional control of the *mecA* gene.

Keywords: MRSA; β-lactam resistance; *mecA* regulation; anti-repressor MecR2; repressor MecI

Resumo

Os *Staphylococcus aureus* resistentes à meticilina (MRSA, do inglês "methicillin-resistant *Staphylococcus aureus*") são um importante agente patogénico, que em certas circunstâncias podem causar infecções. Para além de terem desenvolvido resistência a todos os antibióticos β-lactâmicos, os MRSA são também frequentemente resistentes a outras classes de agentes antimicrobianos. O fenótipo caracteristico dos MRSA deve-se à presença do gene *mecA*, que é regulado por um sensor/transdutor (MecR1) e um repressor (MecI). No entanto, como a indução do gene *mecA* através do MecR1 é muito ineficiente, pensa-se que os MRSA só conseguem expressar elevados níveis de resistência aos β-lactâmicos se o sistema MecR1-MecI não estiver funcional. Curiosamente, dados recentes demonstram ausência de relação entre a expressão das proteínas MecR1-MecI funcionais e o nível de resistência aos β-lactâmicos numa colecção de estirpes MRSA epidémicas.

Os resultados aqui apresentados mostram que o *locus* que regula a transcrição do gene *mecA* contém três genes reguladores (*mecR*1-*mecI*-mec*R2*). A resolução da estrutura tridimensional do MecR2 revela que esta proteína é constituída por três domínios, um semelhante a domínios com capacidade de ligação ao ADN, um intermédio e um de dimerização. Os nossos resultados indicam que o MecR2 desestabiliza a ligação do repressor à região operadora do gene *mecA*, e que a inativação do MecI ocorre independente do MecR1, sendo efectuada possivelmente por proteases nativas. Este estudo demostra ainda que nas estirpes MRSA, que contêm os genes *mecR1-mecI* funcionais, o gene *mecR2* é essencial para induzir eficazmente a transcrição do *mecA*. Em conjunto, estes dados revelam que o actual modelo que descreve o controlo da transcrição do gene *mecA*, deve ser repensado.

Palavras-chave: MRSA; resistência aos β-lactâmicos; regulação do gene *mecA*; Anti-repressor MecR2; repressor MecI

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Abbreviations

ACME	Arginine catabolic mobile element
aux	Auxiliary genes
agr	accessory gene regulator
BLAST	Basic local alignment search tool
BTH	Bacterial two-hybrid
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
cat	Chloramphenicol resistance gene
CC	Clonal complex
CDD	C-terminal dimerization domain
CoNS	Coagulase negative staphylococci
DBD	DNA-binding domain
DEPC	Diethyl pyrocarbonate-treated water
DHFA	Dihydrofolic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ery	Erythromycin resistance gene
fem	Factors essential for methicillin resistance
GlcNAc	N-Acetylglucosamine
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HMW	High-molecular weight
LMW	Low-molecular weight
HTH	Helix-turn-helix
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IS	Insertion sequence
ISD	Intermediate scaffold domain
KD	Dissociation constant
KDa	Kilodalton
LA	Luria agar
LB	Luria broth
MBP	Maltose-binding protein
MGT	Glycosyltransferases
MIC	Minimum inhibitory concentration
MOPS	Morpholine propanesulfonic acid
mRNA	Messenger ribonucleic acid

MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
MurNAc	N-Acetylmuramic acid
NDD	N-terminal DNA-binding-like domain
OD	Optical density
ORF	Open reading frame
PAP	Population analysis profiles
PBP	Penicillin binding protein
PMSF	Phenylmethanesulfonylfluoride
PVL	Panton-Valentine leukocidin
qRT-PCR	Quantitative Real-time RT-PCR
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNase	Ribonuclease
ROK	Repressors, open-reading frames, and kinases
RT-PCR	Reverse-transcriptase PCR
SCCmec	Staphylococcal cassette chromosome mec
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ST	Sequence type
TBE	Tris-borate EDTA buffer
TCRS	Two-component regulatory systems
TE	Tris-EDTA buffer
tet	Tetracycline resistance gene
TEV	Tobacco etch virus
THFA	Tetrahydrofolic acid
TM	Transmembrane segments
Tris	Tris(hydroxymethyl)aminomethane
TSA	Trypticase soy agar
TSB	Trypticase soy broth
van	Vancomycin resistance gene
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
YTH	Yeast two-hybrid

List of Publications

This thesis is based on two articles:

Arêde, P., C. Milheirico, H. de Lencastre, and D.C. Oliveira. The anti-repressor MecR2 promotes the proteolysis of the *mecA* repressor and enables optimal expression of β-lactam resistance in MRSA. PLoS Pathog, 2012. 8(7): p. e1002816.

Arêde, P. Botelho, T. Guevara, T. Usón, I, Oliveira, DC. Gomis-Rüth, FX. Structure-function studies of the staphylococcal methicillin resistance anti-repressor, MecR2. 2012. Submitted.

Thesis Outline

This Ph.D. Thesis is organized into four chapters. It includes a general introduction, one published manuscript and one manuscript in preparation.

Chapter I includes a general description of the mechanisms involved in methicillin resistance in *S. aureus.* In this Chapter, special attention was given to the mode of action of this class of antimicrobial agents, their targets, and the strategies developed by the bacteria to acquire resistance against these drugs, specially the resistance mechanism mediated by *mecA* gene.

Chapter II is entitled “The role of *mecR2* in the regulation of methicillin-resistance in MRSA.” and includes a published manuscript. This Chapter describes the genetic and biochemical studies carried out in order to elucidate the role of the previous unidentified *mecR2* gene on regulation *mecA* transcription in MRSA strains with a fully functional *mec* regulatory system.

Chapter III is entitled “Structure-function studies of the anti-repressor, MecR2” and includes a manuscript in preparation. This chapter describes the three-dimensional structure of the anti-repressor MecR2, as well as further evidences regarding MecR2::MecI interaction. It is also shown that the main function of the anti-repressor MecR2 is its binding to the MecI repressor, disrupting the interaction of MecI to the promoter *mecA*.

Chapter IV provides a general conclusion of results and includes a model, which illustrates the induction of *mecA* by its three cognate regulators (*mecR1-mecI-mecR2*) and an outlook for future research.

CHAPTER I

General Introduction

1.1 *Staphylococcus aureus* as a human pathogen

1.1.1 General features

Staphylococcus aureus are a gram-positive cocci and microscopically are observed as individual organisms, in pairs, in irregular, or in grapelike clusters. The term *Staphylococcus* is derived from the Greek term staphyle, meaning "a bunch of grapes." *S. aureus* are non-motile, non–spore-forming bacteria and their colonies are usually large (6-8 mm in diameter), smooth, and translucent (see Figure 1.1). *S. aureus* were first recognized by Koch in 1878 and Pasteur in 1879 [1] but their more detailed characterization only came some years later with Ogston [2] and Rosenbach [3], who described two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (golden-like) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus epidermidis*. The colonies of most strains of *S. aureus* are pigmented, ranging from cream-yellow to orange, which is due to the presence of triterpenoid (C_{30}) carotenoids, rather than the more typical C_{40} carotenoids [4, 5]. *S. aureus* is part of the human flora, mainly in the axillae, the inguinal and perineal areas, and the anterior nares, whereas, *S. epidermidis* is ubiquitous in skin [6].

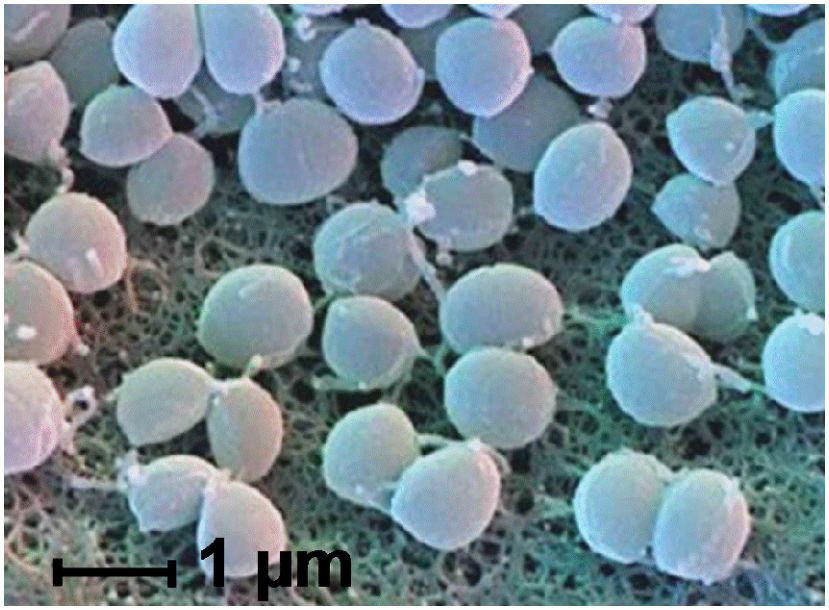

Figure 1.1 - Scanning Electron Microscopy of *S. aureus* N315. Adapted from [7].

At a biochemical level, *S. aureus* is a facultative anaerobe, which can grow by aerobic respiration or lactic acid fermentation of glucose. It is catalase positive, and can survive in NaCl concentrations of up to 15 percent. While most staphylococci are coagulase-negative, *S. aureus* is coagulase positive [8] and can grow between 10ºC and 45ºC. The G +C content of *S. aureus* DNA is within the range of 30 to 38

mol percent, being staphylococci one of the members of the low G +C group of the gram-positive bacterial phylogenetic group [9].

The staphylococcal cell is surrounded by a mesh-like structure 20-40 nm thick, called peptidoglycan, which is composed of a series of short glycan chains of c.a. 20 alternating N-acetyl-muramic-acid and β-1,4-N-acetylglucosamine residues [10]. Peptidoglycan is an essential and specific component of the bacterial cell wall found on the outside of the cytoplasmic membrane of almost all bacteria [11, 12]. Its main function is to preserve cell integrity and it also contributes to the maintenance of a defined cell shape and serves as a scaffold for anchoring other cell envelope components such as proteins and teichoic acids [13]. *S. aureus*-specific pentaglycine interpeptide cross-bridges are assembled in the cytoplasm by *auxiliary* genes *femX* [14], *femA* [15], and *femB* [16], which attach the glycine residues to the L-lysine residue of the stem peptide, in a sequential manner [17]: FemX adds the first glycine, FemA adds the 2^{nd} and 3^{rd}, whereas, the FemB adds the 4^{th} and 5^{th}. FemA and FemB are not interchangeable, meaning that inactivation of one of these genes results in cell walls that contain mono-or triglycine cross-bridges, respectively.

1.1.2 Colonization and infection

The capacity to asymptomatically colonize healthy individuals is a biological property of *S. aureus*, with approximately 30% of humans being asymptomatic nasal carriers of *S. aureus* [18]. Indeed, *S. aureus* carriers have a higher risk of acquiring *S. aureus* related infections and are an important dissemination vehicle of *S. aureus*, either by direct contact (i.e. skin-to-skin contact with a carrier or infected individual) or by contact with contaminated surfaces or objects [19-21].

Due to its capacity to cause opportunistic infections, *S. aureus* should always be considered a potential pathogen which, in certain circumstances, may cause a variety of suppurative (pus-forming) infections and toxinoses in humans [19]. Examples are superficial skin lesions such as boils, styes and furunculosis or more serious infections such as pneumonia, mastitis, phlebitis, meningitis, mastitis, phlebitis and urinary tract infections and even deep-seated infections, such as osteomyelitis and endocarditis. Moreover, *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices [18]. *S. aureus* is also responsible for food poisoning by releasing enterotoxins into food, and for the toxic shock syndrome by releasing superantigens into the blood stream [22-24].

The pathogenicity of *S. aureus* is a complex process involving a diverse array of extracellular and cell wall components that are coordinately expressed during different stages of infection; i.e. colonization, escape from the host defense, growth and cell division, and bacterial dispersion. The adaptive response is highly coordinated and is modulated by regulatory elements via signal transduction pathways [19].

Genomic analysis has revealed two major families of global regulators in *S. aureus*: two-component regulatory systems (TCRS) [25, 26] and the SarA homologs, a family of proteins homologous to SarA [25, 27], of which the best-characterized regulators of virulence factors are the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (SarA), respectively. The *agr* locus consists of two divergent transcripts, RNAII and RNAIII, driven by two distinct promoters, P2 and P3, respectively [28]. RNAIII is the effector of the *agr* response that involves up-regulation of genes involved in exoprotein synthesis and down-regulation of genes encoding surface proteins [29, 30]. Unlike *agr*, the *sar* locus activates the synthesis of both extracellular (e.g. hemolysins) and cell wall-associated proteins (e.g. fibronectin-binding protein) [31]. These extracellular proteins can be divided into two groups depending on when they are expressed in cells growing in rich medium: proteins that are expressed only when cell densities are low and proteins exclusively expressed at high cell densities. By virtue of their proteolytic activities and toxin effects on host cells, the exotoxins synthesized during the post-exponential phase facilitate the local invasion and hematogenous dissemination phases of *S. aureus* infections [32].

The virulence factors of S*. aureus* can be classified in three different groups: (i) those which are involved in the attachment to the host cells, such as collagen-binding protein, coagulase or fibronectin-binding proteins A and B; (ii) factors involved in the invasion of host cells and consequently tissue penetration, such as α-toxin and hemolysins; and (iii) virulence factors involved in evasion of host defences such as protein A, lipase and toxic shock syndrome toxin 1, [33-37]. The coordinated regulation of virulence determinants during the exponential and post exponential phases has a decisive contribution to the development of *S. aureus* infections.

1.1.3 Antibiotic resistance

S. aureus has been a stumbling block for anti-microbial chemotherapy able to develop resistance to all therapeutic agents deployed in clinical practice. Antibiotics, which literally means agents “against life”, are molecules that prevent microbes, both bacteria and fungi, from growing. One of the first documented identification of an antibiotic compound dates from 1929 by Alexander Fleming, who observed that a culture plate of *S. aureus* had been contaminated by a blue-green mould (*Penincillinium notatum*) and that colonies of *S. aureus* adjacent to the mould could not grow. Then, Fleming grew the mould in a pure culture and found that it produced a substance that killed a number of disease-causing bacteria. He named the substance penicillin and realised that his discovery might have therapeutic value if the antibiotic could be produced in large quantity [38].

Antibiotics can be classified according to their mode of action, cellular target, and main clinically relevant mechanism of resistance. According to the physiological cellular target, antibiotics are often grouped into four major classes (see Figure 1.2): cell wall synthesis, protein synthesis, nucleic acid synthesis, or folic acid metabolism inhibitors. The cell wall synthesis inhibitors, such as the β-lactams and the glycopeptides are the most representative and widely used class of antibiotics against *S. aureus*

infections. The β-lactams (e.g. penicillins and cephalosporins) bind covalently to PBPs, and also inhibit the reticulation of the pentapeptidic chains of the peptidoglycan precursors. The glycopeptides (vancomycin and teicoplanin), also target cell wall synthesis. These later antibiotics bind the D-Ala-D-Ala termini of peptidoglycan precursors, blocking the transpeptidation reaction carried out by PBPs. Although glycopeptides are the last resort to treat methicillin resistant *S. aureus* (MRSA) infections, strains with reduced susceptibility to these antibiotics have already been reported [39, 40]. Vancomycin intermediate *S. aureus* (VISA) strains are characterized by a thickening of the cell wall, which is believed to reduce the ability of vancomycin to diffuse into the division septum of the cell, whereas, the vancomycin resistant *S. aureus* (VRSA) modify peptidoglycan precursors ending in D-Ala-D-Ala to D-Ala-D-Lac instead, with low affinity for glycopeptides [41-43].

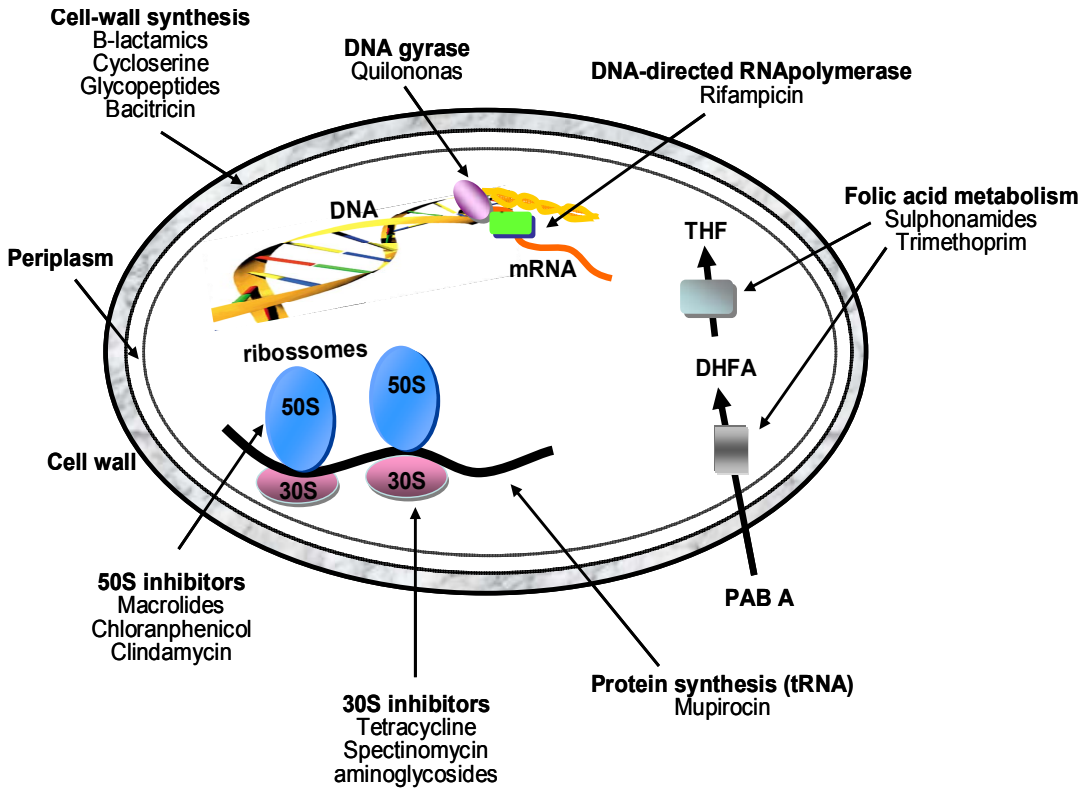

Figure 1.2 - The bacterial cell with the 4 main antibiotic targets; cell-wall synthesis, nucleic acid synthesis, protein synthesis, and folic acid metabolism. Examples of the most representative and most used antibiotics are shown, as well as their specific targets. mRNA, messenger RNA; tRNA, transfer RNA; PABA, p-aminobenzoic acid; DHFA, dihydrofolic acid; THFA, tetrahydrofolic acid. Adapted from [54].

Another class of antibiotics are the inhibitors of protein synthesis such as the macrolides, lincosamides and streptogramins, which target the 50S subunit of the ribosome. On the other hand, tetracyclines and aminoglycosides selectively block the 30S subunit of the ribosome. This antimicrobial class also inhibit the elongation step of the protein synthesis and prevent the association of aminoacyl-

trRNA to the receptor site on the mRNA-ribosome complex. Currently, most *S. aureus* strains are fully resistant to this antibiotic class, either by the active efflux of the macrolides and tetracyclines to outside of the cell, or by modification of the target (ribosomal methylation) [44-46].

The inhibition of the nucleic acids synthesis is accomplished by drugs belonging to the quinolones and fluoroquinolones family. This antibiotic class binds to enzymes involved in DNA coiling (e.g. topoisomerase IV and DNA girase), to DNA polymerase and inhibits the chromosomal replication. Mutations in *grlA*, the gene encoding topoisomerase IV subunit A, are the main resistance mechanism in *S. aureus* [47-50].

Finally the inhibitors of acid folic synthesis class, such as the sulfamides and diaminopyridines, selectively bind to enzymes involved in the synthesis of acid folic [51, 52]. Bacterial resistance to sulfamides reported in *S. aureus* is mainly due to chromosomal point mutations leading to an increased production of p-aminobenzoic acid [53].

1.1.3.1 Resistance to β-lactam antibiotics

β-lactam antibiotics are the most widely used class of antimicrobial agents, mainly because they have broad spectrum, have low toxicity and have low cost. These agents are characterized by a four-membered β-Lactam ring and target the bacterial enzymes involved in the last steps of cell wall synthesis, the so-called penicillin-binding proteins (PBPs) [55, 56] (see Figure 1.3). β-lactams mimic the D-Ala-D-Ala dipeptide, particularly regarding the distribution of three electrostatic-negative wells, and act as suicide inhibitors. The active site serine attacks the carbonyl of the β-lactam ring, resulting in the opening of the ring and formation of a covalent acyl-enzyme complex. This complex is hydrolysed very slowly, thus effectively preventing further reactions [57, 58]. This antibiotic class encloses a large number of drugs which can be divided into several groups according to their chemical structure, such as: penicillins derivatives (penicillin G, cloxacillin and ampicillin-like agents); cephalosporins (have a 3,6-dihydro-2H-1,3-thiazine ring fused to the β-lactam ring as in cefoxitin); carbapenems (contain a β-lactam ring fused to a five-membered ring as in imipinem); monobactams (with a second thiazole ring not fused to the β-lactam ring) and β-lactamase inhibitors (e.g. clavulanic acid combined with pecicillin) [59, 60]. The mechanism of penicillin resistance is due to the production of a plasmid borne β-lactamase enzyme encoded by the *blaZ* gene [61, 62]. Penicillinase-resistant penicillins, such as methicillin, were then developed to treat those infections, with apparently success, but shortly after MRSA strains began to arise and spread. MRSA have spread first in hospital settings and then, within community, in parallel to the earlier emergence and spread of penicillin-resistant *S. aureus* [54, 63]. Nowadays, most *S. aureus* strains are resistant to natural penicillins, as well as to aminopenicillins and antipseudomonal penicillins [54, 64, 65]. This mechanism of resistance is not due to β-lactamase production but rather to the expression of an

additional penicillin binding protein (PBP2a), which has an extremely low reactivity with β-lactam antibiotics [66].

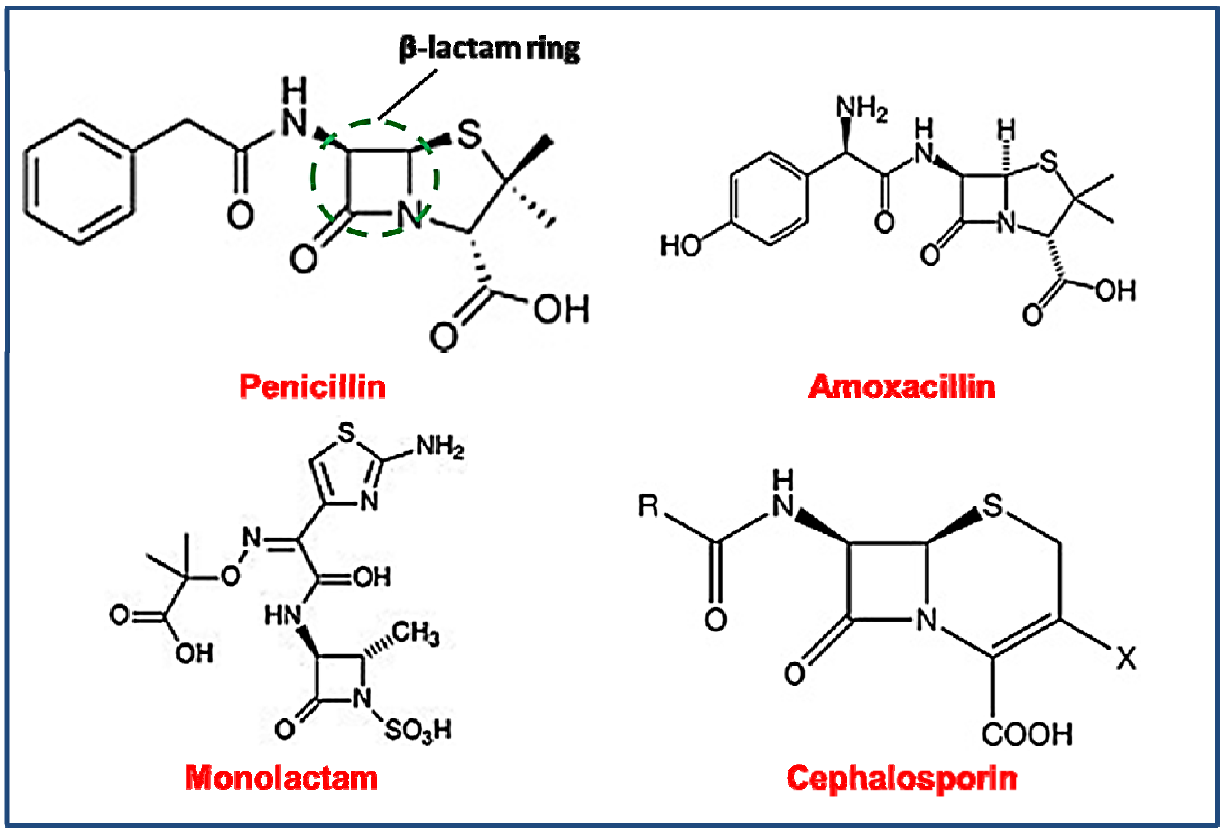

Figure 1.3 - Schematic representation of some β-lactam antibiotics. The characteristic structure of the β-lactam ring is marked. R represents the chemical substitute group that confers different properties to the antibiotics. Adapted from [67].

Like PBPs, β-lactamases also have the ability to bind β-lactams, but in this case the antibiotic is rapidly hydrolysed by these enzymes into biologically inactive metabolites [68].

1.1.4 Epidemiology and evolution of MRSA

Before the introduction of antibiotics into clinical practice, the rate of mortality due to infections caused by *S. aureus* exceeded 80% and over 70% of the infected patients developed metastatic infections [69]. Currently, this organism is a leading cause of infections in hospitals in many countries and has also become an important community- and livestock-associated pathogen [19, 70-73].

S. aureus strains were fully susceptible to penicillin G when it was initially introduced in early 1940s, until the appearance of the first penicillin-resistant *S. aureus*, in 1942 [74, 75]. Penicillin-resistant *S. aureus* were uncommon at the beginning, appearing only in healthcare settings, but in a very few years an increasing number of resistant isolates were detected and became increasingly prevalent in the community as well [76]. During the 1950s others antibiotics with a broad spectrum of activity like, tetracycline, streptomycin and chloramphenicol started to be widely used against a variety of different bacteria, and also against *S. aureus* isolates positive for the β-lactamase [77].

In 1961, methicillin, the first semi synthetic penicillinase-resistant penicillin, was introduced into clinical practice, specifically for the treatment of penicillin-resistant *S. aureus* infections. However, its introduction was rapidly followed by reports of methicillin-resistant isolates, and the outcome of infections caused by MRSA were worse than the outcome of those that resulted from methicillin-sensitive strains [78, 79]. In the early 1980s and 1990s methicillin-resistant clones started also to be reported in the community - Community-associated MRSA (CA-MRSA), in individuals with no prior hospital exposure [80, 81]. CA-MRSA isolates carry a distinct molecular makeup and lack the multidrug resistance pattern usually harboured by nosocomial MRSA strains [82-85].

Another landmark of antimicrobial chemotherapy was the emergence of *S. aureus* clinical isolates with reduced susceptibility to vancomycin (the so-called intermediate resistance or VISA) in 1997 in Japan [86]. Shortly after, other cases were reported in other countries [40, 87], as well as infections caused by highly vancomycin-resistant *S. aureus* (VRSA) that have remained relatively rare [88, 89].

1.1.4.1 Hospital-acquired MRSA (HA-MRSA)

MRSA has been recognized as a major problem both in hospital and community in many countries and data collected by the SENTRY Antimicrobial Surveillance Program indicates average prevalences of MRSA in hospitals from different regions worldwide, as follows: 23% in Europe, 36% in USA, 29% in Latin America, 23% in Australia and 67% in Japan [90-93]. Data from the European Antimicrobial Resistance Surveillance Network (EARSS) have shown that the prevalence of MRSA in European countries is not uniform, varying widely between the Northern and Southern countries [94]. While the MRSA prevalence either in Scandinavian countries or Netherlands is extremely low, (below 1% and 5%, respectively), the minimum rate of MRSA prevalence in Southern countries is 25% reaching 50% in some of these countries [95].

Nosocomial MRSA is remarkable for its pattern of spread, being associated with just a few genetic lineages [96]. Historically, two hypothesis for the evolution of MRSA have been proposed: (i) the single-clone theory described by Kreiswirth and colleagues, which suggests that all MRSA clones have the same MSSA ancestor that acquired the PBP2a [97], and (ii) the multi-clone theory, which suggests that PBP2a has been acquired several times by different MSSA lineages [96]. Recent data from several studies supports the multi-clone theory [96, 98-100].

Crisostomo *et al* [101] have studied a collection of MSSA and MRSA isolates recovered in Denmark and the U.K. in the 1960s, and showed a close relationship between the MRSA clones and the prevalent epidemic MSSA lineages in the 1960s, which suggests that those epidemic MSSA lineages were the first recipients of the SCC*mec* element, the polymorphic chromosomal cassette that harbours the *mecA* gene coding for PBP2a. MRSA and MSSA lineages shared phenotypic and genetic properties, including phage group, antibiotype, pulsed-field gel electrophoresis pattern, *spaA* type and multilocus sequence-type (ST). For instance, MSSA isolates belonging to ST250 group were proposed to represent the progeny of a strain that served as one of the first *S. aureus* recipients of the methicillin-resistance determinant in Europe [101, 102]. The early MRSA were referred to as Archaic clone. Two different studies carried out by Enright *et al* and Gomes *et al* have corroborated these findings and further clarified the origins of early MRSA clones [96, 100]. Robinson and colleagues have investigated the frequency of SCC*mec* transfer, using a collection of 147 MRSA strains from different countries, demonstrating that the acquisition of SCC*mec* element has occurred at least 20 times and that its acquisition was four times more common than its replacement [103]. Furthermore, SCC*mec* type IV is twice more predominant when compared with other SCC*mec*, which might mean that most of the MRSA clones emerged through the acquisition of this SCC*mec* element. This success could be related with its small size, facilitating its transfer among staphylococci [103, 104].

Interestingly, *S. aureus* strains associated with pandemic MRSA lineages were easier transformed by a *mecA*-containing plasmid than other *S. aureus* lineages, suggesting that the genetic background is important for the stability and maintenance of SCC*mec* [105]. Additionally, some *S. aureus* lineages are not able to integrate the SCC*mec* element into their chromosome, apparently due to their specific *attB*SCC sequence (designated *orfX*) which varies among the different *S. aureus* lineages [106]. However, the main *S. aureus* lineages share the ability to become MRSA [107, 108].

The prevalence of nosocomial MRSA clones can shift over time, either in one region or in a specific hospital [109-111]. For instance, in a Portuguese hospital between 1996 and 2005 the ST239-MRSA-III (Brazilian clone) was replaced by the ST22-MRSA-IV (EMRSA-15) clone [112]. Moreover, sporadic isolates (MRSA strains from single patients) and minor clones (MRSA strains from a single hospital) have been reported additionally to the major HA-MRSA clones [113].

1.1.4.2 Community-acquired MRSA (CA-MRSA)

Since the 1990s, the epidemiology of MRSA has changed because infections are no longer confined to the hospital settings, and have also started to appear in healthy community individuals without established risk factors for the acquisition of MRSA. Several CA-MRSA clones have spread worldwide, not only in the community but, in most recent years, also in healthcare facilities as well, replacing the classic HA-MRSA clones in some hospitals [64, 114, 115]. CA-MRSA was firstly reported in Western Australia in 1993 from indigenous populations with no previous contacts with the nosocomial setting [81].

CA-MRSA prevalence varies substantially among countries, with high prevalence rates in the USA (USA300 clone), Australia (mainly the ST30 clone) and Europe (mainly the ST80 clone) [116], whereas prevalence rates are low in Scandinavia countries, Switzerland or Netherlands [91, 117]. Outbreaks of CA-MRSA infections have been described in specific populations groups, such as prison inmates [118], military recruits [119], native Americans [120] and competitive sports participants [121]. CA-MRSA has mainly been isolated from skin and soft tissues, such as abcesses, cellulitis, folliculitis and impetigo [120, 122-124]. In addition, severe necrotising pneumonia caused by CA-MRSA has already been described [123] .

When comparing CA-MRSA with HA-MRSA, several differences have been found, mainly in predominant SCC*mec* types, growth rates, and distribution of antibiotic resistance and toxin genes [116, 125]. The majority of CA-MRSA isolates harbour the SCC*mec* type IV, type V or type VII [103, 126, 127]. However, SCC*mec* types I, II, and III can also be found in some CA-MRSA isolates [31, 128]. It has been proposed that CA-MRSA are associated with several specific lineages of *S. aureus*, by the acquisition of SCC*mec* elements, mainly SCC*mec* type IV, by MSSA strains [126, 129]. In fact, detailed molecular characterization of CA-MRSA has shown that the genetic background of CA-MRSA strains are distinct from the predominant HA-MRSA clones within defined geographic regions, suggesting that CA-MRSA did not emerge from local HA-MRSA [116]. Moreover, the larger clonal diversity of CA-MRSA compared to HA-MRSA suggests that more *S. aureus* lineages have the ability to evolve to CA-MRSA strains than to HA-MRSA [96, 126, 130]. Many studies have reported that specific genetic backgrounds together with PVL (which is a specific *S. aureus* exotoxin) and SCC*mec* types IV or V, are genetic markers for CA-MRSA [129, 131, 132]. The existence of *pvl* positive MSSA strains in the community which share the same genetic background than the *pvl* positive CA-MRSA corroborates this hypothesis [100, 133]. However, other studies reporting on PVL-positive CA-MRSA which harbour SCC*mec* elements other than type IV or V [134], and on PVL-negative CA-MRSA have contradict that those genetic markers are specific for CA-MRSA strains [135-137].

Until recently, it was believed that dissemination of PVL-positive CA-MRSA clones was restricted to continents, i.e. the ST1 and ST8 clones in USA, the ST80 clone in Europe and the ST30 clone in Australia [116]. However, at the present, the five major PVL-positive CA-MRSA clones appear to be disseminating worldwide: the ST1 clone has been also observed in Asia, USA and Europe; the ST8 clone in USA and Europe; the ST30 clone in South America and Europe; and the ST59 and ST80 clones in Asia, Europe, USA and Middle-East [71, 133, 136, 138-142]. In addition to these major CA-MRSA clones, several minor PVL-positive CA-MRSA clones have been also reported worldwide [143-145]. CA-MRSA clones, such as USA 300, are often more virulent than the HA-MRSA clones, and it has been proposed that not only PVL is involved in skin or soft-tissue infections, but others genes such as *hla* (which encodes for an α-hemolysine involved in pathogenesis of pneumonia) or the mobile genetic element found in USA300 (called arginine catabolic mobile element (ACME)), may be also involved. ACME seems to be essential and contributes to the growth and survival of this clone, playing an important role in virulence as

well [146, 147]. The observation that these CA-MRSA clones have already spread worldwide, even in healthcare facilities is worrying, since PVL-positivity is related to high mortality and morbidity.

In addition to the MRSA emergence in the community, MRSA has also emerged in the farm environment [148]. In 2003, a new MRSA clone isolated from pigs and pig farmers in The Netherlands, which was not related to HA-MRSA or CA-MRSA, was reported [149] and since then MRSA clones have been isolated from other animals, such as, pets and horses [150-152]. One interesting characteristic related with this clone, is the presence of a new DNA methylation enzyme, making these strains non-typeable by pulsed-field gel electrophoresis (PFGE) with restriction endonuclease SmaI. This clone is characterized by ST398 and carries a SCC*mec* type V [149, 153]. The same clone has also been reported in USA, in Asia and in other European countries, such as: Italy, Portugal, Germany or France [151, 154-157].

Transmission of MRSA between animals and humans has been previously described, mainly associated with colonized companion animals, horses, and persons who take care of them [150, 158]. Therefore, the surveillance of MRSA clones from animal origin should be considered in order to understand the transmission routes and reservoirs of these clones and to re-define control measures that favour the prevention of infections caused by them [150]. Currently, little information exists concerning the genetic determinants or metabolic changes responsible for the enhanced epidemicity of the farm-associated MRSA strains, and consequently, few strategies exist that might control the spread of these pathogens [149, 159].

1.1.4.3 SCC*mec* element

The acquisition of the SCC*mec* element, which harbours the central determinant of methicillin resistance, the *mecA* gene, is the genetic event required for *S. aureus* to become a MRSA. This mobile element inserts into the *S. aureus* chromosome at a specific site (*attB*SCC), which is located closely to the chromosomal replication origin, at the 3' end of an open reading frame (ORF) with unknown function (*orfX*) [160]. The genetic origin of SCC*mec* is still unknown. However, it has been speculated that SCC*mec* element was acquired by *S. aureus* from coagulase negative staphylococci (CoNS) [161, 162]. A study published by Wielders *et al* seems to support this idea, since from a neonate, who had not been previously in contact with MRSA, an epidemic MSSA and a *S. epidermidis* resistant strain and shortly after an isogenic MRSA strain were isolated. Moreover, the SCC*mec* element found in MRSA strain appeared to be identical to the one carried by the *S. epidermidis* isolate. This finding documents the *in vivo* transmission, by horizontal transfer, of the *mecA* gene between the two staphylococcal species [163]. In addition, Tsubakishita *et al* have recently shaded some light on this matter, reporting a plasmid in *Macrococcus caseolyticus* carrying a transposon containing an unusual form of the *mec* gene complex which is located in the same operon than *blaZ*, revealing a potential mechanism of the generation of a

new SCC*mec*-like element in those species [164]. Moreover, the same authors in another recent study observed a similar sequence between the *mecA* locus of the *Staphylococcus fleurettii* chromosome and the *mecA*-containing region (~12 kbp long) of SCC*mec* in *S. aureus*, which suggests that the *mec* gene complex found in MRSA might have been assembled in this species [165].

So far, eleven main types of SCC*mec* elements (SCC*mec* type I to type XI) and several variants, which range in size from 20.9 to 66.9 kb, have been described and characterized [166-168]. This classification is based on the *mec* complex class and type of *ccr* complex [126, 169-173]. The *mec* gene complex is composed of the *mecA* gene, its regulatory elements *mecI-mecR1*, and insertion sequences which can be located upstream or downstream of *mecA* [174]. Several SCC*mec* types carry insertion sequences upstream and downstream of *mecA* gene (for instance, SCC*mec* types I, and IV carry insertion sequence IS1272, whereas SCC*mec* V harbours the insertion sequence IS431), that truncate completely the *mecI* repressor as well as the most part of *mecR1,* leading to a de-repression of the structural *mecA* gene [169, 174, 175]. The cassette chromosome recombinase (*ccr*) genes, which belong to the invertase/resolvase class, and allow the integration and/or excision of SCC*mec* from the *S. aureus* genome [172]. The *ccr* gene complex can be constituted by two genes (*ccr*A and *ccr*B) or by a single gene (*ccr*C) [171, 176]. Besides the *mec* complex and the *ccr* genes, the remaining parts of the SCC*mec* are called the J ("joining") regions (J1 to J3). The J1 region is located between the chromosomal right junction and the *ccr* genes, while the J2 region corresponds to the region between *ccr* genes and the *mec* complex and the region spanning from the *mec* complex to *orfX* is called J3 [169, 171, 174, 177-180]. Several variants of the SCC*mec* type I to type IV are defined by differences in the J regions [178, 180-182]. The J regions are no essential components to the cassette, although in some cases they harbour additional antibiotic determinants, particularly in SCC*mec* type II and III. In fact, the SCC*mec* elements are important reservoirs of non-β-lactam resistant genes. SCC*mec* types I, IV, V, VI, VII only contain the resistant gene *mecA*, while SCC*mec* types II, III and VIII, contain other resistance determinants to multiple classes of antibiotics (e.g. aminoglycosides and macrolides) due to the presence of additional resistance genes integrated in genetic mobile elements like plasmids (e.g. pUB110, pI258 and pT181) and transposons (Tn554 and ΨTn554). Additionally, the SCC*mec* element also contain several ORFs with unknown function, as well as genes coding for virulence factors that are involved in infections (*pls* or *clf* genes).

Several characteristics are common at all types of SCC*mec* that had been describes so far, (i) the *ccr* gene complex [176]; (ii) the structural *mecA* gene and its regulatory locus; and (iii) the typical flanking nucleotide sequences, which are inverted and directed repeats located at both ends of the SCC*mec* [183].

1.1.4.4. *mecA* gene origin

The *mecA* gene is not exclusive to MRSA strains, and can be also found in methicillin-resistant coagulase-negative staphylococci [184, 185]. Based on DNA and amino acid sequences homologies, it has been proposed that the *mecA* gene of *S. aureus* may have evolved from a fusion event between a β-lactamase gene and a PBP gene [186].

Couto *et al* have shown that a close homologue of the *S. aureus mecA* gene was ubiquitous in epidemiologically unrelated isolates of *S. sciuri* [187]. The introduction of the *mecA* homologue from *S. sciuri* strain, which was methicillin susceptible, in a MSSA strain conferred resistance to β-lactams and allowed growth and continuous synthesis of peptidoglycan in the presence of high β-lactam antibiotic concentrations [188]. Furthermore, comparing the transpeptidase and transglycosylase domains of the *mecA* gene in *S. sciuri* and MRSA, they share 96% and 80% of similarity, respectively. Antignac *et al* have provided further evidence for the proposition that the genetic resistance determinant *mecA* present in MRSA strains has evolved from *S. sciuri*, by reconstructing the methicillin resistant phenotype in *S. aureus* strain COL (lacking the SCC*mec*), using the homologous *mecA S. sciuri* (*pbpD* gene) [189] . The authors demonstrated that *S. aureus* transductants were able to produce large amounts of the 84-KDa *S. sciuri* PBP 4 and exhibited properties typical of those of wild type strain, including broad-spectrum, high-level, and homogeneous resistance to structurally different β-lactams [187]. Recently another divergent *mecA* homologue was found out in a MRSA clone isolated from human and bovine populations and characterized by a novel staphylococcal SCC*mec* (SCC*mec* type-XI) [190].

1.2. Regulation of gene expression in Prokaryotes

Adaptation to predictable environmental changes is dependent to a large extent on the ability of an organism's proteins and RNAs to be regulated at the level of gene expression [191]. Changes in gene regulation might contribute to morphological diversity. Jacob and co-workers [192] in the 1970s, developed the concept that changes in patterns of gene expression (rather than evolution of new genes) have had a decisive role in generating much of the biological diversity. Since then, this concept has been extended and supported by the work of many authors as well as evolutionary studies [193-195].

Two steps of gene expression are essentially the same in all forms of life: a gene is transcribed into mRNA and consequently that mRNA is translated into protein. Moreover, all cells contain at least one form of RNA polymerase and the machinery which is responsible for the translation of the mRNA into protein [196]. At any given time, a cell – from a prokaryote or eukaryote organism – expresses only a subset of its genes to direct production of other molecules, ensuring the synthesis of only the necessary mRNAs and proteins, and in appropriate amounts, to accomplish the genetic programs, such as: the presence of other cells, sporulation, apoptosis, or a response to a specific environmental condition [193, 197].

Regulation of the gene expression in bacteria occurs primarily at the transcription level. However, a gene can be switch on or off by many mechanisms which can perturb different steps in its expression, from transcription initiation to protein degradation or modification [198-200]. Because this field of research became too wide, for the present purposes, this section will focus mainly on the mechanisms by which the transcription initiation of a bacterial gene can be regulated through the binding of a protein to the DNA.

1.2.1 Initiation of transcription

RNA polymerase (RNAP), which is the main enzyme involved in gene expression and is responsible for mRNA synthesis, comprises four subunits (α' α'' β β'). In bacteria, the RNAP core enzyme is typically found to be associated with another essential subunit, called sigma factor (σ), forming the holoenzyme (see Figure 1.4), which confers promoter-specific transcription initiation in RNAP and restricts transcription initiation to the promoter sequences [201]. Several different σ factors are known and each of which promotes the binding of RNAP to a specific set of promoters. However, the holoenzyme which carries the σ^{70} is the most common form, transcribing most of the genes [202-205]. The remaining σ factors are commonly referred to as alternative sigma factors and they can recognize different -10 and -35 regions, for instance -12 and -24 in the case of σ^{54}, directing transcription of specific groups of genes under specific cellular conditions [206].

The process of transcription initiation is a sophisticated multi-step process, and in a simplified way, it begins when RNAP holoenzyme binds to a specific promoter region forming a closed complex, in which DNA is completely double-stranded [204, 207, 208]. Melting of the DNA strands causes the formation of an open complex. This open complex when in the presence of the four nucleoside triphosphates, proceeds to an initiated complex which can be temporarily engaged in an iterative abortive transcription process, generating and releasing short new RNA chains [204, 209, 210].

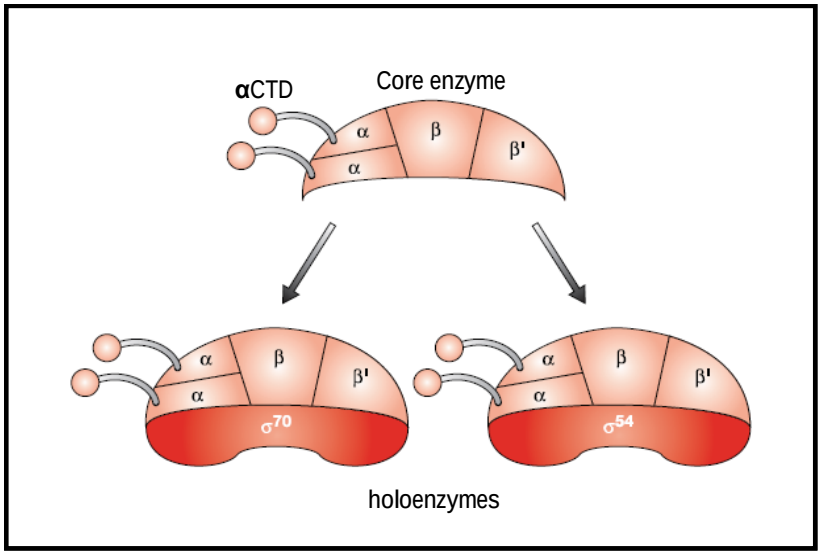

Figure 1.4 - Schematic representation of the RNA polymerase core with the isoforms present in E.coli. The core enzyme has four subunits, β and β' which are present in a single copy, whereas the α subunit is present in two copies and is attached at one carboxy-terminal domain by a flexible linker. The holoenzymes's name mirrors the σ subunit size. Adapted from [188].

1.2.2 Promoter recognition

A promoter is a sequence of DNA from which RNAP initiates transcription. The RNAP together with the σ^{70} subunit recognizes four different and important sequence of elements, such as: two hexamers centered at or near positions -10 and -35 upstream from the transcription start site (designated by their locations as the -10 and -35 regions, respectively), the spacer DNA separating them, and a region between -40 and -60 (the UP element), which is a very A+T-rich region recognized by the carboxyl domain of the α-subunit (see Figure 1.5) [203, 211, 212]. The canonical sequences of the -10 and -30 regions as read on the nontemplate strand are, TATAAT and TTGACA, respectively [213]. It has been shown that both sequences are directly recognized by the σ factor, although, the sequence in the -35 region is recognized by an helix-turn-helix (HTH) domain, whereas, the -10 sequence is recognized by a different domain [214, 215]. A consensus length of 17 bp has been established for the spacer between the -10 and -35 regions and promoters with such a spacer length have been found to be more active *in vitro*, as well as *in vivo*, than those with shorter or longer spacers [216, 217]. Some differences can be found in promoter recognition. For example some promoters apparently work quite well without a recognizable -35 region and any activating proteins, due to an extended -10 region with the sequence TGNTATAAT [204, 218].

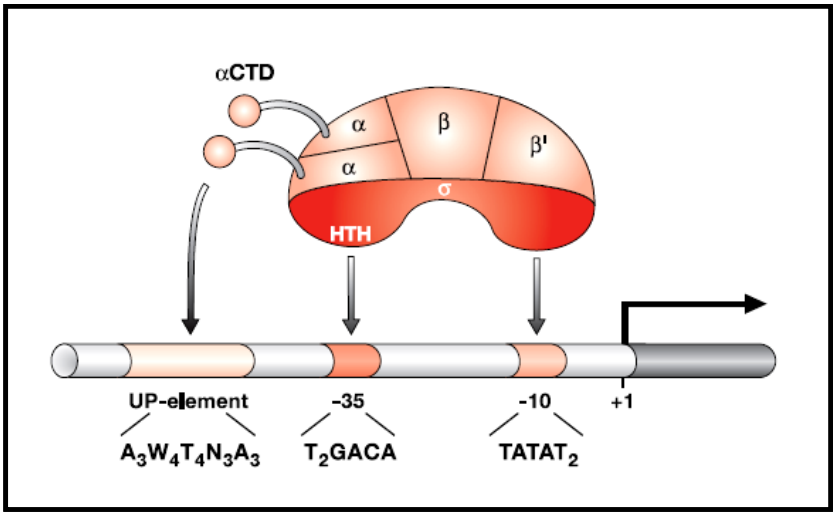

Figure 1.5 - The RNA polymerase-promoter interactions. A promoter with consensus sequences for the -35 and -10 regions as well as the UP-element (boxed) is shown. The σ subunit can simultaneously bind to -10 and -35 promoter regions, whereas the UP-element, if present, is recognized by the carboxi-terminal extensions of the α subunits. W represents adenine or thymine; N represents any base; and a subscript describes the number of reiteration of the respective base [188].

Different promoters in bacterial cell can bind to RNAP unequally. So far, three main strategies to direct RNAP to specific promoter have been described: (i) several parts of DNA that form each promoter may differ in terms of efficiency by which they are recognized by RNA polymerase; (ii) many bacteria have different σ subunits, and since the σ subunit of RNAP is responsible for promoter recognition, changes in the activity or concentration of a particular σ subunit can redirect RNA polymerase towards a specific set of promoters; and (iii) transcription factors (activators and repressors) are present in all bacteria and can bind to particular promoters activating or repressing the transcription initiation [219, 220]. The global response to the physiological requirements of the cell will be achieved depending on the combination of those strategies, as well as the overall topology of DNA, dictating the strength of the promoter. Intrinsic properties of the promoter can also be changed by specific regulatory proteins at any RNAP-promoter binding point. A weak promoter will be under a positive control whereas a strong promoter will be down-regulated and in case of promoters which are constitutively expressed, transcription will be initiated at a prefixed rate [209].

1.2.3 The molecular mechanisms regulating transcription initiation

1.2.3.1 Activators vs repressors

The modulation of the activity of proteins that control the initiation of transcription – activators and repressors - can occur by either intracellular or extracellular stimuli that signal for a particular regulatory pathway. These stimuli are sensed by the transcription factors, leading to their activation or inactivation. An activator is a factor that increases the affinity of the RNAP to the promoter, whereas, a repressor function is to block or to make difficult the access of the RNAP to the promoter or impeding the clearance process [221, 222].

Two types of activators can be described considering whether they directly bind or not to the RNA polymerase: (i) Activators that bind directly to the RNAP enzyme, either by small surface-exposed patches, referred as activating regions, which interact with specific targets in RNA polymerase, or by overlapping the -35 region of the target promoter. Consequently, this kind of activators can potentially contact different parts of RNAP or binding to promoter's target DNA site well upstream of the -35 region, leading to the contact between the promoter and the RNAP α subunit C-terminal domain. (ii) Activators that do not make direct contact with RNAP. In this case, the activator function is achieved by inducing a conformational change in the target promoter, for instance, as observed for the MecR protein encoded by the mercury resistance locus [223-226].

In both activators and repressors, alterations in the DNA structure and protein-protein interactions, either between DNA-binding regulatory proteins or between them and subunits of the RNAP are essential mechanisms of action. There are regulatory proteins responsible for the regulation of global cell response (for example, the regulation of carbon metabolism in *E.coli*) whereas, others control only a set of promoters involved in a more specific cell response (for example, the regulation of L-arabinose degradation by AraC in *E. coli*) [227, 228].

1.2.3.2 The helix-turn-helix domain

The helix-turn-helix (HTH) domain is a common denominator in basal and specific transcription factors among a large majority of bacterial regulatory proteins [229]. In structural terms, the HTH domains have evolved from the basic 3-helical cores: the tetra-helical bundle, the winged-helix and the ribbon-helix-helix type configurations [229, 230]. In functional terms, the HTH domains are present in most transcription factors of all prokaryotic genomes and some eukaryotic genomes. They have been recruited to a wide range of functions beyond transcription regulation, which include DNA repair and replication, RNA metabolism and protein-protein interactions in diverse signalling contexts. Beyond their basic role in

mediating macromolecular interactions, the HTH domains have also been incorporated into the catalytic domains of diverse enzymes [231, 232]. This domain consists in one α-helix, a turn, and a second α-helix, arranged perpendicularly in a very peculiar way that has the ability to insert into the major groove of DNA [233]. The side chains of amino acids exposed along the recognition helix make sequence-specific contacts with edges of base pairs. A second helix lies across the DNA, helping the recognition of the helix position and strengthening the binding. The differences in the residues along the outside of recognition helices largely account for differences in the DNA-binding specificities of regulators [234, 235] . Protein-DNA recognition trough the HTH motifs appears associated with several features; generally DNA-binding sites are at least partially palindromic, normally the proteins bind DNA as a homo-oligomers and, as mentioned above, the sequence specific recognition is usual made by the second α-helix of the HTH motif through the major groove of DNA. The local DNA configuration and conformation, as well as its curvature, bending and flexibility are also important to establish the specificity of protein-DNA interactions [236, 237]. In activator molecules or dual function regulators, the HTH motif is located either at NH_2 or COOH-terminal, whereas, in repressor proteins the same motif is exclusively present at the NH_2-terminus. Additionally, the HTH motif is normally bound to a larger response domain, which transmits the signal to the DNA-binding domain that allows or prevents the binding to the operator sequences [238, 239].

1.3. Regulation of β-lactam resistance in *S. aureus*

1.3.1 Cell-wall biosynthesis: the cellular target of β-lactams

The biosynthesis of peptidoglycan is a complex process involving many different cytoplasmic and membranes steps. The first stage consists in the formation of the soluble nucleotide precursors, from UDP-GlcNAc to UDP-MurNAc-pentapeptide. In particular, the synthesis of the peptide moiety is performed by a series of enzymes designated as the Mur ligases which are responsible for the additions of l-alanine, d-glutamic acid, meso-diaminopimelic acid (A2pm) or l-lysine, and d-alanyl-d-alanine to UDP-MurNAc, respectively [240-243].

The membrane stage of peptidoglycan biosynthesis is catalyzed by the MarY enzyme, which transfers the muramyl-pentapedtide from UDP-Mur-Nac-pentapedtide to the membrane acceptor localized on the cytoplasm. The UDP-GlcNac precursor is then linked to the muramyl residue of lipid I to form β-1,4 glycosidic bond [244, 245]. The last steep in peptidoglycan synthesis is the transpeptidation and transglycolysation reactions, which are responsible for the formation of peptide and glycosidic bonds and are catalysed by the Penicillin Binding Proteins (PBPs) and monofunctional glycosyltransferases (MGTs) [246] (see Figure 1.6). The penicillin-binding domains of PBPs (which are transpeptidases or

carboxypeptidases) are characterized by three specific motifs: SXXK, (S/Y) XN and (K/H) (S/T) G, which define the active-site serine of penicillin-recognizing enzyme family. The serine of the SXXK motif is central to the catalytic mechanism, attacking the carbonyl of the penultimate D-Ala amino acid of the stem peptide, which releases the last D-Ala amino acid from the 'donor' peptide and forms a covalent acyl–enzyme complex [247, 248]. In transpeptidases, the carbonyl of the D-Ala amino acid, now forming an ester linkage with the active site serine, then undergoes an attack from a primary amine linked in various ways to the third residue of a second 'acceptor' stem peptide. A peptide bridge is then created between two stem peptides, forming a link between glycan strands. In DD-carboxypeptidases, the acyl-enzyme intermediate is hydrolysed; this process eliminates 'donor' stem peptides from the peptidoglycan [249-252].The glycan chains are linked together via the last glycine residue of a pentaglycine cross-bridge attached to the L-Lys residue on one stem peptide and the D-Ala residue on another.

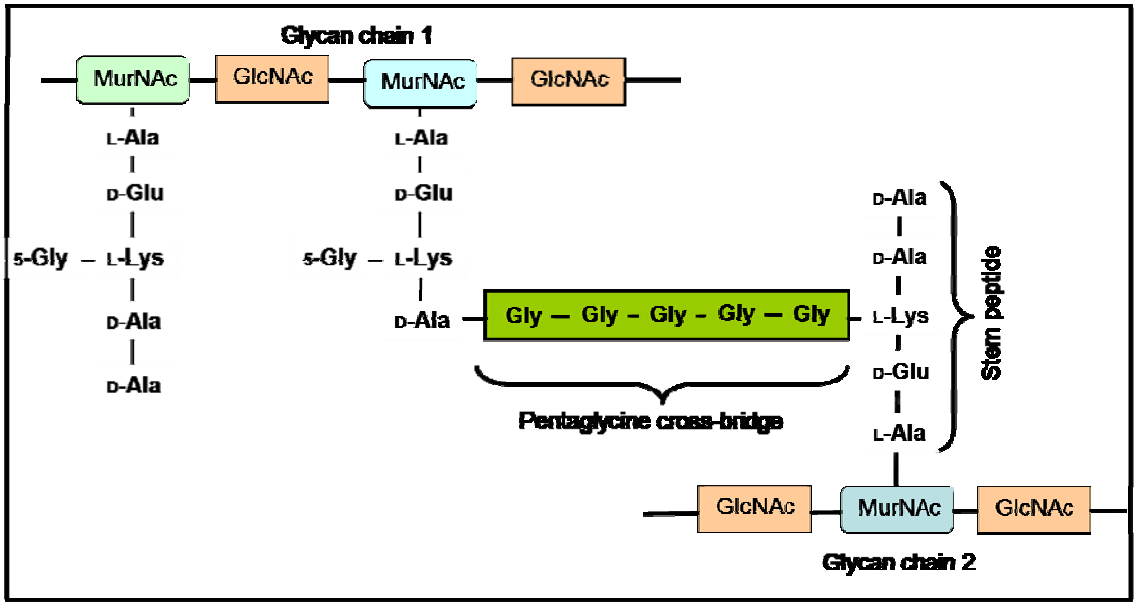

Figure 1.6 - The peptidoglycan structure of S. aureus. The glycan chains are composed by alternate and repeated disaccharide units, GlcNAc and MurNAc. Cross-linking of the peptides is mediated by an interpeptide pentaglycine bridge, which binds the ε-amino group of the L-lysine component of one muropeptide to the penultime D-Ala of another. Adapted from [250].

1.3.1.1 PBPs

The targets of β-lactam antibiotics are the cell wall-synthesizing enzymes (penicillin-binding enzymes), which are commonly detected by their ability to bind covalently radiolabeled penicillin (hence the name Penicillin binding proteins, PBPs) [254]. PBPs are present in almost all bacteria, and participate not only in peptidoglycan synthesis during cell growth but also in cell septation and, in some

species, sporulation [250, 255, 256]. In gram-positive bacteria, PBPs are exocellular being anchored through short hydrophobic carboxy – or amino-terminal sequences to the outer face of the cytoplasmatic membrane, whereas, in gram-negative they are pseudoperiplasmatic [257]. The enzymatic activities associated with PBPs are transpeptidation, endopeptidation, DD-carbopeptidation and also transglycosilation, in the case of bibunctional PBPs [258].

According to their structure, PBPs can be divided in two main groups: high-molecular weight (HMW) PBPs and low-molecular weight (LMW) PBPs. HMW PBPs have two characteristic domains and a membrane spanning non-cleavable signal peptide, which traps the enzyme to the external surface of the cell membrane. The C-terminal domain is responsible for transpeptidation activity and β-lactam antibiotics covalently bind to its catalytic centre [249, 259]. Based on catalytic activity of their N-terminal domain, HMW PBPs are divided in two classes (A and B). The class A of HMW PBPs are bifunctional enzymes capable of both transglycosylation and transpeptidation, because its N-terminal region has transglycosylase activity [256]. The class B of LMW PBPs are monofunctional DD-peptidases, involved in peptidoglycan trimming and have no penicillin-binding domain [255].

S. aureus has four native PBPs: PBP1 (85 KDa), PBP2 (81 KDa), PBP3 (75 KDa) and PBP4 (41 KDa). PBP1 and PBP3 are HMW class B and PBP2 is HMW class A, whereas PBP4 is LMW [255, 260, 261]. Contrary to PBP 1 and PBP 2, PBPs 3 and 4 are not essential for growth and survival of *S. aureus*. However all the native PBPs have high affinity for β-lactams [262-264]. The essential function of PBP1 is intimately integrated into the mechanism of cell division, playing an important role in septum's formation [265]. PBP2 is the major peptidoglycan transpeptidase and also the most abundant among the native PBPs, being the only bifunctional PBP present in *S. aureus* [266, 267]. It is believed that PBP3 is a transpeptidase functioning in no growing cells [268], and PBP4 is a DD-carboxipeptidase and transpeptidase involved in secondary cross-linking of the peptidoglycan [269].

1.3.1.2 PBP2a

Penicillin binding protein 2a (PBP2a) is the key determinant of the broad-spectrum β-lactam resistance in MRSA strains. Because of its low reactivity for β-lactams, PBP2a provides transpeptidase activity to allow cell wall synthesis at β-lactam concentrations that inhibit the β-lactam-sensitive PBPs normally produced by *S. aureus* [270-273]. PBP2a is a high molecular weight membrane bound transpeptidase with 668 amino acids, and 76.162 KDa [186, 270, 273], and is located on the extracellular surface of the cytoplasmic membrane, where it catalizes the final steps of cell wall assembly [256, 274]. Although PBP2a is the most abundant PBP in MRSA cells this abundance appears not to be correlated with the level of resistance [275, 276]. Even in strains where *mecA* gene is expressed constitutively, PBP2a appears not to be involved in cell wall synthesis, with exception of cells which have been treated with β-lactam antibiotics [277].

The action mechanism of PBP transpeptidation based on a serine-derived acyl-enzyme, is very similar to that present in β-lactamases, including the three-active site signature sequences [SXXK, (S/Y)XN and KTS/KTG] [278-280]. Lim and Strynadka [281] revealed the three soluble domain structures of the PBP2a, which are, the N-terminal lobe (corresponding to the anchor characteristic of the HMW PBPBs), a centralized non-penicillin binding domain of unknown function and a C-terminal transpeptidase domain, which has a folding pattern that is typical of the PBP transpeptidases and the serine β-lactamases. However, in the case of PBP2a, the active site motif of the nucleophilic serine which is located on a α-helix is sequestered within an extended narrow groove. This structural difference gives to PBP2a a uniqueness in comparison to the native PBPs, decreasing drastically the efficiency with which β-lactam antibiotics trap the enzyme as a penicilloyl-intermediate complex [281, 282].

Upon exposure to β-lactam antibiotics, the native PBP2 of MRSA strains loses its transpeptidase activity, which is taken over in those strains by PBP2a. However, as PBP2a has no transglycosylase activity, MRSA peptidoglycan biosynthesis depends on the functional cooperation between the transglycosylase domain of the native PBP2 and the transpeptidase domain of PBP2a [283]. Although MRSA strains are able to grow in the presence of β-lactams, striking changes in muropeptide composition can be observed, such as: severe decreasing of trimeric and higher oligomeric components (from almost 50% to less than 10%), while the proportion of monomeric and dimeric components drastically increases (from about 15% up to 50%) [277, 284]. The anomalous composition of the cell wall in those strains in the presence of the β-lactam antibiotics reflects the limited capacity of PBP2a for cross-linking more than single monomeric glycan chains [277].

1.3.1.3 Factors affecting methicillin resistance

Although PBP2a is essential to confer β-lactam resistance to MRSA, this resistance may be affected by any factor that interferes with PBP2a or with the *mecA* expression. Resistance to high levels of methicillin depends, in addition to PBP2a, on chromosomally encoded factors – the *aux* (auxiliary) or *fem* genes [285, 286]. The *aux* genes are located in the *S. aureus* genome, outside of the SCC*mec*, and are mainly involved in the synthesis and degradation of the peptidoglycan or cell wall turnover, although some appear to have putative regulatory functions, and others encode proteins with as yet unidentified functions [17, 287-289]. In this section, we will briefly discuss some of these factors as well as their impact on β-lactam resistance.

It has been shown that a different configuration of stem peptide influences methicillin resistance. For instance, the addition of glycine to the growth medium led to stem peptides of peptidoglycan ending in two glycine residues instead of two alanine residues. This alteration converts a highly resistant homogeneous strain to a heterogeneous phenotype [253, 290]. Additionally, inactivation of *murE* gene reduces the UDP-linked muramyl pentapeptides and accumulates of UDP-linked muramyl dipeptides in the cell wall precursor pool, also leading to reduction of methicillin resistance [291]. It has been

demonstrated that inactivation of *llm* gene (similar to the teichoic acid linkage unit synthesis gene *tagO* from *B. subtilis*) decreases the levels of β-lactam resistance, leading a homogeneous strain to a heterogeneous phenotype, and it is also involved with increased autolytic activity [292]. Alteration of the pentaglycine cross-bridge configuration has also a strong impact on the levels of methicillin resistance in MRSA. Inactivation of either *femA* or *femB* genes results in a reduction of methicillin resistance and also affects the secretion of virulence factors which could diminish the ability of cell to cause infection [293-295]. Genes such as: *fmtA-C, hmrA* and *hmrB*, *pbp2* (genes that are associated with peptidoglycan biosynthesis), *sigB, dtl (*involved in stress response and virulence), and *ctaA* (involved in stress response induced by starvation) also play a pivotal role on modulation of methicillin resistance [243, 287, 296].

Besides the *aux* genes, many authors have demonstrated that several chemical compounds also have the capacity to modulate methicillin resistance, such as: baicalin (which is a flavone compound) [297], Triton X-100, polidocanol (a dodecyl polyethylenneoxide ether) [298] and glycerol monolaurate [299]. It is thought that those compounds target the cytoplasmic membrane and may interfere with the signalling domain of MecR1, but not directly with PBP2a. However, compounds such as polyoxotungstates [300] ant totarol [301], lead to a decrease on PBP2a synthesis, but the mechanism of how this is achieved is still unknown.

1.3.1.4 Heterogeneity

MRSA strains appears to be consistent in that they all contain and express the *mecA* gene, although, the degree of antibiotic resistance varies widely from one strain to another and also within the progeny of a single MRSA isolate, revealing a surprisingly degree of heterogeneity in the phenotypic expression levels of antibiotic resistance (since a few micrograms per milliliter to several milligrams per millilitre) [302-304]. Some of these strains, which have this heterogeneous phenotype, display relatively low MIC ranging from 4-24 mg/L, and, at the clinical setting, are difficult to detect with conventional antimicrobial susceptibility tests and can be easily misinterpreted as sensitive (MSSA) [305]. This peculiar characteristic observed over the decades in most MRSA strains is the phenomenon referred to as heteroresistance in which, methicillin resistant strains show a basal resistance to low concentrations of β-lactam antibiotics and give rise to a few subclones able to grow at high concentrations of the antibiotic [306, 307]. However, the number of those highly resistant subclones, their resistance levels and the frequency with which they arise in a culture are strain-specific and reproducible, implying a strong genetic control in the population structure of these bacteria [308]. The genetic basis of heteroresistance is still not fully understood. One study proposed that, once these highly resistant subclones – referred to as Homo*- arose in a population, they are very stable and do not revert readily to a heterogeneous phenotype [305]. Chromosomal mutations (*chr**), involving genes other than those which are present in the SCC*mec* element, have been suggested to contribute to high level resistance in these subpopulations

[309]. Some genes such as, *hmrA*; *hmrB*; *lytA* and the *dlt* operon are putative candidates to undergo *chr** mutations which may contribute to the appearance of these more resistant subpopulations [310-312].

According to its heterogeneous resistance profiles, MRSA strains can be divided in four different classes, which range from diverse degrees of heterogeneous resistance to strains which have a homogeneous resistance profile [304]. Cultures of strains which belong to expression classes 1; 2 and 3 show heterogeneous resistance profile, whereas, cultures of strains which belong to expression class 4 have a resistance homogeneous profile (all cells are highly resistance with a methicillin MIC ≥ 800 µg/ml). The main difference among expression classes 1, 2 and 3 is the methicillin MIC for the majority of the cells, which is only slightly higher than MSSA strains in expression class 1, between 6 and 12 µg/ml in class 2, between 50 and 200 µg/ml in class 3, and higher than 800 µg/ml in class 4 [304].

Although heterogeneity has a molecular base and *chr** mutations, other factors have been found that also contribute to heterogeneity, such as: temperature, pH, osmolarity, growth medium, growth phase, trace metals, chelating agents and visible light have strong influence in methicillin resistance levels [309, 313]. Moreover, no mechanism or genetic model that explains the difference among these different classes has been proposed yet.

1.3.2 *bla*-system

The main mechanism of β-lactam resistance, particularly amongst Gram-negative bacteria, is the production of enzymes, called β-lactamases, which have hydrolytic activity and are able to disrupt the amide bond of the characteristic β-lactam ring [314]. β-lactamases can be considered as ancient enzymes that were quite rare until the introduction of the β-lactam antibiotics into medicine and agriculture around sixty years ago [315]. Described for the first time in *Escherichia coli* isolates, even before the clinical use of penicillin, these enzymes have since then been described in Gram-negative, Gram-positive bacteria and in mycobacteria [316, 317]. β-lactamase enzymes can be plasmid or chromosomally encoded and may also be associated with mobile genetic elements such as integrons and transposons [318, 319].

Most S*. aureus* isolates carry a plasmid-encoded β-lactamase, although the *bla* operon can be also found in the chromosome [320]. In β-lactamase-producing *S. aureus* isolates, the β-lactamase is usually inducible [321]. Four classes of *S. aureus* β-lactamase enzymes have been identified by serologic [322, 323] and kinetic [324] methods. Those enzymes can be organized into four classes (A to D) [324]. Classes A, C and D are usually located on plasmids and share a similar fold as well as a similar mechanism of action leading to β-lactam inactivation [325, 326]. This mechanism involves formation of a serine nucleophile by deprotonation of the active site serine with a general base, and nucleophile attack of the β-lactam ring to form an acyl-enzyme intermediate. Consequently, hydrolysis of the acyl-enzyme intermediate is carried out through a general base activated by a water molecule [317, 327]. The differences among the catalytic mechanism of the serine β-lactamases are related with the type of

residues involved in acylation and deacylation processes. β-lactamase enzymes belonging to class B require a metal cofactor (e.g. zinc) to function, and therefore are completely different from the serine β-lactamases, in terms of fold, sequence and mechanism of action [328]. The class B enzymes are usually located at the chromosome and can be divided into three sub-classes (B1 to B3). The β-lactamases belonging to the sub-class B2 are able to bind only one zinc ion whereas sub-classes B1 and B3 have the capacity to be binuclear (binding one or two zinc ions) [256, 328, 329].

The three genes involved in β-lactamase synthesis are clustered together and are frequently located on the transposon Tn552: *blaZ* (codes for the extracellular β-lactamase), *blaR1* (codes for the transmembrane signal transducer, BlaR1) and *blaI* (codes for a repressor protein, BlaI) [320, 330, 331]. The β-lactamase regulatory genes are co-transcribed as a single mRNA in the opposite direction to the transcription of *blaZ* [332]. The synthesis of β-lactamase is regulated by a mechanism similar to that present in the *pen* system (which has the structural gene *penP* and the pen regulatory genes *penI* and *penR1*) of *Bacillus licheniformis* [333]. Both systems share high level of homology: *penP* and *blaZ* genes specify Class A β-lactamases; *penR1* and *blaR1* encode hydrophobic transmembrane proteins that show 51% amino acid identity, and both intracellular domains have a sequence signature of a neutral zinc metallopeptidase [334]; *penI* and *blaI* code for repressor proteins with 59% of identity [335, 336].

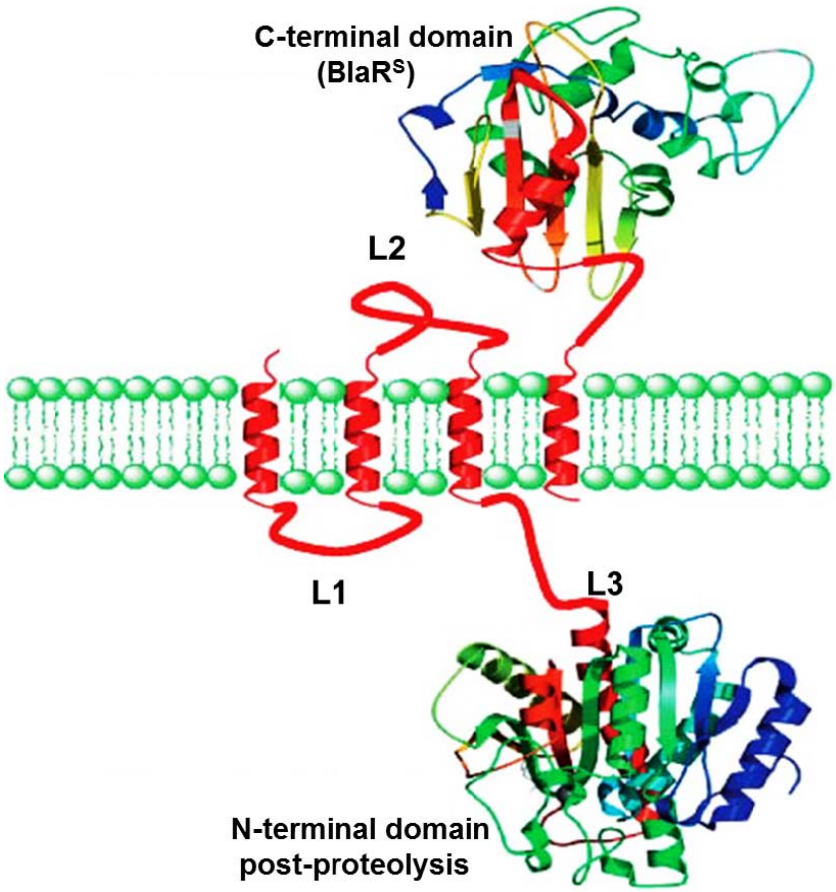

Figure 1.7 - Schematic representation of BlaR1 membrane protein. Adapted from [278].

BlaR1 is a high molecular weight sensor-transducer transmembrane protein with two domains [256]. The amino-terminal domain, with approximately 38-KDa - the transducer - exhibits four transmembrane segments (TM1-TM4) that form a four-α-helix bundle embedded in the plasma bilayer [334]. These transmembrane segments are interconnected by three loops (L1-L3), the loop L1 and L3 are facing the cytoplasm, whereas the L2 loop is exposed in the outside of the cell (see Figure 1.7). The L3 segment has a zinc metalloprotease domain, harbouring a histidine sequence and a glutamic acid, which is believed to cleave the repressor BlaI [338, 339]. The carboxyl-terminal domain of BlaR1 with approximately 27- KDa, is fused at the carboxyl end of the TM4 extending to the extracellular medium. This domain possesses the amino acid sequences signature of penicillin binding proteins [340, 341]. BlaI is present as a dimmer in solution and its DNA-binding function is located at the N-terminus, whereas, the dimerization function is located in the C-terminal region [332, 342].

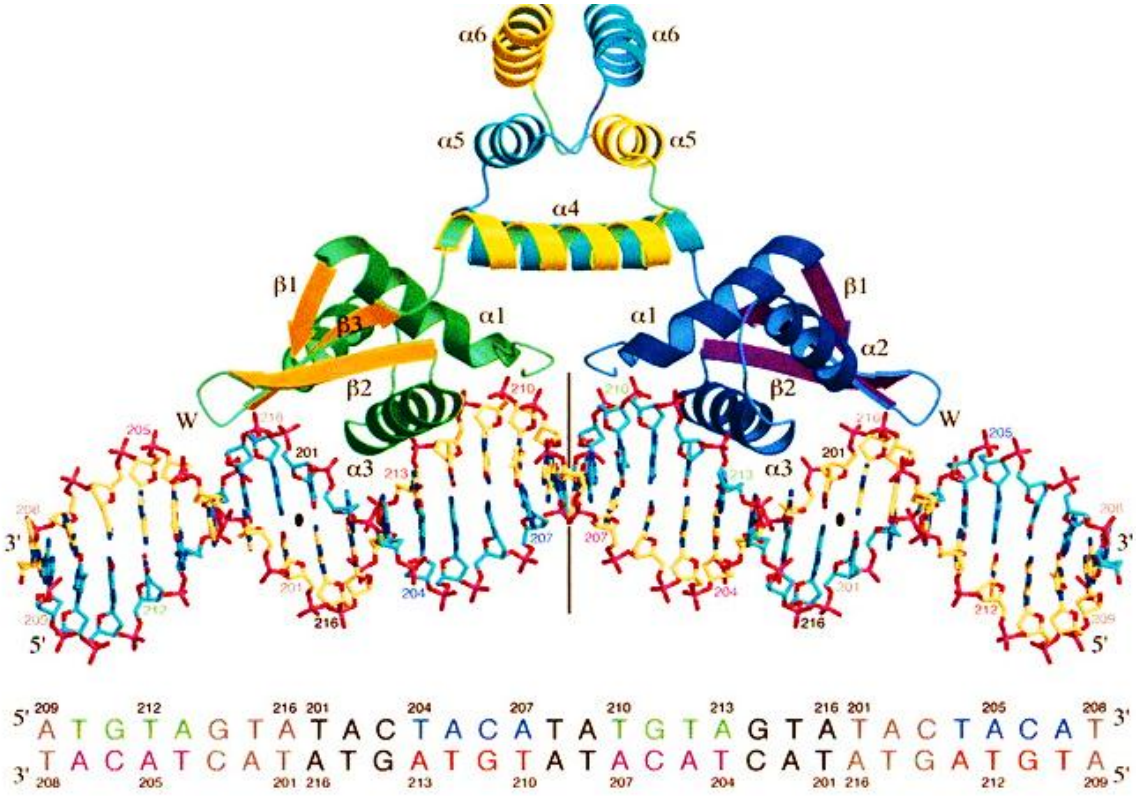

Figure 1.8 - Overall view of BlaI bound to DNA. The DNA sequence is shown in colors and the model is labeled with the same colors. The dyad axes of DNA are indicated by a line and a bullet. Adapted from [335].

In the absence of penicillin, the DNA-binding protein BlaI bind in dimers to the operator region, thus repressing mRNA transcription from both *blaZ* and *blaR1-blaI*, whereas a paired proline PXXP motif in L2 loop and the C-terminal of BlaR1 are bound non-covalently (see Figure 1.8) [341]. The induction of the *blaZ* transcription mechanism begins with the binding of the β-lactam to the serine active site of the extracellular sensor domain of BlaR1 [341]. The serine acylation event disturbs the L2 loop::C-terminal domain binding, leading to a conformational alteration of the extracellular L2 Loop. This event generates a signal transduction that is propagated trough the transmembrane α-helices [334, 341], leading to the conversion of the intracellular zinc metalloprotease domain of BlaR1 from an inactive proenzyme into an active protease, presumably by autoproteolysis [332, 334, 338, 343, 344]. The activated zinc metalloprotease directly or indirectly cleaves BlaI, allowing the synthesis of *blaZ* mRNA and *blaR1-blaI* mRNA. Actually, the molecular mechanism that promotes the BlaI cleavage by the zinc metalloprotease domain of BlaR1 is not fully understood. Thus, Filée and colleagues [345] have proposed an alternative theory, suggesting that in *B. licheniformis* a coactivator (BlaR2), dependent of the activated BlaR1 receptor, combined with the cellular penicillin stress are required for BlaI inactivation. Moreover, the induction process does not promote a complete BlaI cleavage, and about 40-50% of the intact dimer is still present [339]. However, recent studies carried out by Llarrull and colleagues [346], using a heterologous expression system, have shown that *S. aureus* BlaR1 directly cleaves the repressor BlaI.

1.3.3 *mec*-system

Methicillin resistance in staphylococci is mediated by the chromosomally localized *mecA* gene, which is responsible for the synthesis of the PBP2a [296, 347]. This extra PBP, which has reduced β-lactam affinity, is able to mediate the cell wall synthesis, after native PBPs have been inactivated by the β-lactam antibiotics [270, 348]. *mecA* transcription can be regulated by the *mecR1-mecI* regulators, located immediately upstream from *mecA* promoter and transcribed in opposite direction [79, 349, 350]. MecI repressor was found to be a homodimer, both unbound and in complex with the double-stranded DNA promoter *mecA*, consisting in an 11-KDa DNA binding domain and a 3-KDa dimerization domain [351, 352]. Whereas, MecR1 is a high-molecular weight class C PBP, consisting of a ~38-KDa N-terminal (putative) integral-membrane metalloprotease and a ~27-KDa C-terminal extracellular β-lactam sensor connected by a linker [353]. The genetic organization of the *mec* locus is similar to the β-lactamase operon, and there is a good homology between the sensor inducers (the identity of the sensor domains is 43%, of the protease domains is 33%, and of the full length proteins is 34%) and the repressors (sharing 61% identity to each other) (see Figure 1.9) [276, 321, 332, 341, 349, 354, 355], and conformational analysis has shown a conservation of the structural motifs [31, 334, 356]. Furthermore, several studies have shown that proteolytic cleavage in both repressors occurs at the same two amino acids in the same relative position [332, 350]. Due to their similarity, both regulatory systems have been shown to regulate the *mecA* expression. Both purified MecI and BlaI, as well as the heterodimers MecI-BlaI are able to

protect a 30bp palindrome spanning sequence the predicted *mecA* – 10 and the *mecR1* - 35 promoter sequences [350, 357, 358].

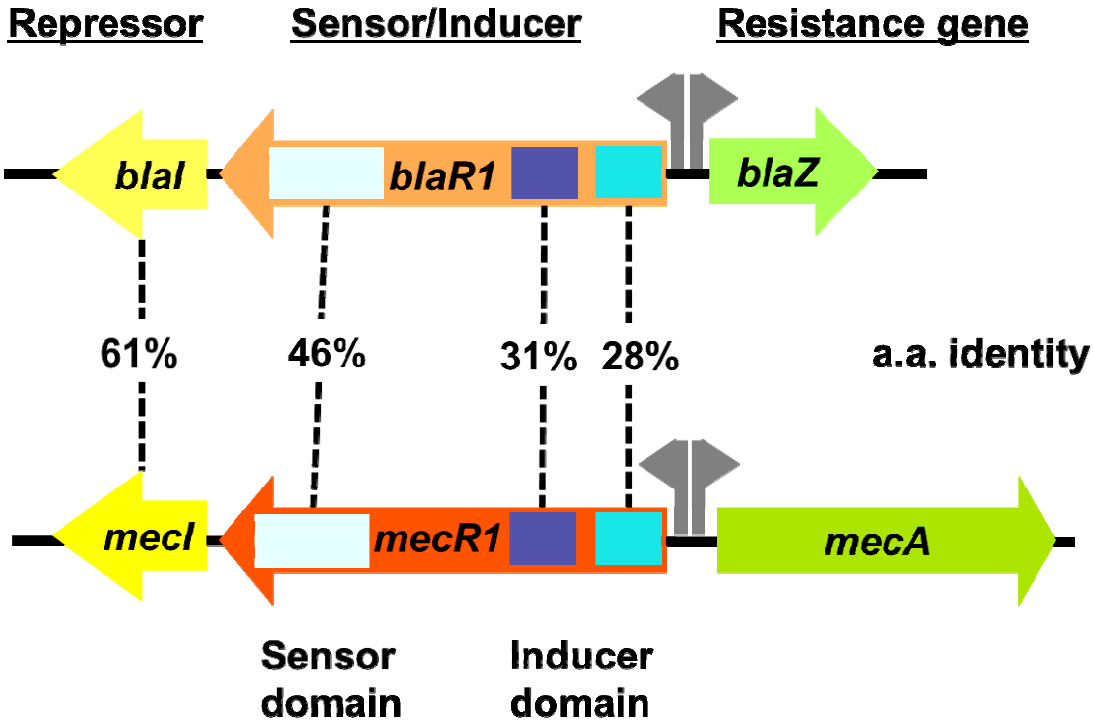

Figure 1.9 - Schematic representation of the β-lactamase and *mecA* operons. The levels of the aminoacid identity of the regulatory proteins are shown.

Based on the high homology between the *bla* and *mec* systems, it is believed that the signal-transduction mechanisms leading to β-lactam resistance are very similar [359, 360]. Although none of the following regulatory steps are still fully understood, the current model of *mecA* regulation is based in three main events (see Figure1.10): (i) the mechanism leading to MecR1 acylation; (ii) the events involved in the signal transduction pathway; and (iii) the proteolytic cleavage of MecI repressor [339, 344]. The induction of *mecA* gene starts when a serine residue present in the PBP-like penicillin sensor domain of MecR1 senses the β-lactam, leading the sensor domain of MecR1 to undergo an irreversible acylation by the β-lactam [361]. The acylation event triggers a conformational change in the MecR1 sensor domain, transmitting the signal to the cytoplasmic domain of MecR1. The signal might be propagated through the transmembrane α-helices, enabling the activation of the cytoplasmic domain which has a sequence signature of a zinc metalloprotease [361]. Once activated, the MecR1 cytoplasmic domain directly or indirectly promotes the cleavage of the cytosolic MecI within its dimerization domain, preventing its binding to the *mecA* operator allowing PBP2a expression [339, 344]. MecR1 once cleaved can no longer transmit signal but, since the expression of *mecR1-mecI* is also up-regulated, the *mecA* induction continues as long as the antibiotic is present in the environment. When the β-lactam antibiotic is no longer

detected in the extracellular space, MecR1 is no longer activated, and a steady state is established consisting of MecI-dimers bound to the *mecA* promoter and residual copies of MecR1 at the cell membrane.

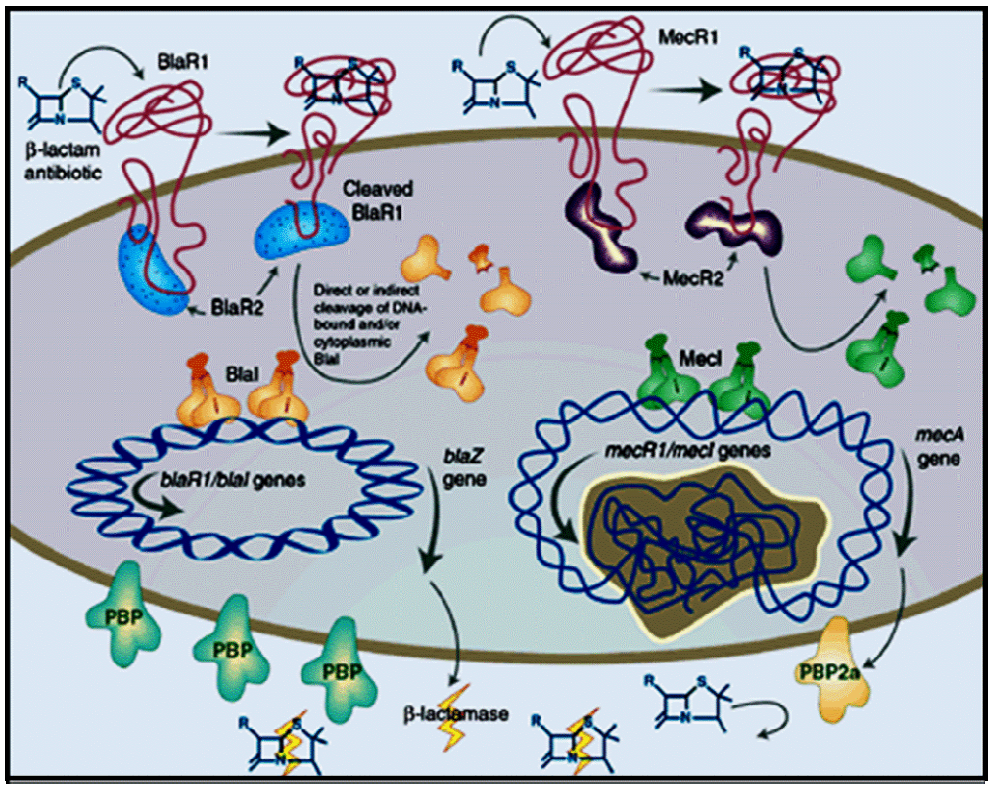

Figure 1.10 - Schematic representation of the mechanisms involved in the regulation of the *mecA* and *blaZ* genes expression in S. aureus. On the left - The binding of the β-lactam antibiotic to the extracellular domain of the sensor transducer BlaR1 which stimulates BlaR1 autocatalytic activation. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI protein (the repressor), allowing transcription of both *blaZ* and *blaR1-blaI*. β-lactamase, the extracellular enzyme encoded by *blaZ* hydrolyzes the β-lactam ring of penicillin, thereby rendering it inactive. On the right - The gene product of *mecA*, PBP2a, has a low affinity to β-lactam antibiotics. The regulation of *mecA* expression is similar to the one of *blaZ*. The repressors BlaI and MecI regulate production of the β-lactamase and PBP2a genes in similar ways, but their sensor-transducers are not interchangeable. BlaR2 and MecR2 are the hypothetical accessory proteins that may be required for the cytoplasmatic signal transduction of both mechanisms. Adapted from [342].

Of note, there is still no evidence supporting a direct interaction between the inducer (MecR1) and the repressor (MecI) and, as such the signal transduction mechanism remains to be clarified. Remarkably, the MecI cleavage site is located in one region involved in dimers formation, at one end of the MecI repressor, and not within the DNA dimerization domains [339, 351]. Garcia-Castellanos and co-workers [351, 362], based on analysis of the MecI structure, found out that the (putative) cleavage

consensus sequence encountered within the repressor is protected and different from the MecR1 (auto) lytic cleavage site, so much so it is difficult to conceive that MecR1 is able to directly cleave MecI.

Despite the similarities in terms of structure and function, the *bla* and *mec* systems still retain distinct identities. For instance, both sensor-transducers (MecR1 and BlaR1) are specific for their cognate repressors and are not functionally interchangeable [357], i.e. the MecI repression may only be released when induced by MecR1 and the same happens with the BlaI and BlaR1 [357]. Additionally, the kinetics of the signal transduction, which leads to *mecA* induction, is quite different: some minutes if mediated by *blaR1-blaI*, versus, several hours when *mecA* is induced by its cognate regulatory genes [276, 363]. The poor induction of PBP2a expression in isolates carrying the *mec* regulatory locus fully functional may be explained by the fact that the repressor MecI is a tight regulator of *mecA* transcription and also because most β-lactam antibiotics do not activate MecR1 efficiently [364-366]. Consequently, some isolates, referred to as "pre-MRSA" are, in clinical terms, methicillin-sensitive despite carrying the *mecA* gene [349, 364]. Nevertheless, selective pressure by antibiotic usage has apparently promoted the appearance of MRSA isolates with deletions or mutations within the *mecA* promoter or *mecI* coding region, giving rise to an inactive repressor and constitutive PBP2a expression or, as most strains are β-lactamase positive, *blaI-blaR1* mediated inducible PBP2a expression [367-370]. In agreement with this observation, studies carried out *in vitro* have shown that inactivation of the *mecI* gene leads to increased levels of β-lactam resistance [349, 364]. Therefore, it has been proposed that MRSA strains with high MIC (minimal inhibitory concentration) to β-lactams, as many contemporary MRSA isolates, must have no functional *mecI-mecR1* genes [364].

However, in a recent study a correlation between *mecI-mecR1* functionality, and β-lactam resistance could not be established in a collection of pandemic MRSA clones [371]. On the other hand, in spite of the clear negative effect of the presence of MecI on the phenotypic expression of resistance, and in an apparent contradiction with what was previously described, Rosato *et al* have found that either *mecI* or *blaI* must be functional in all MRSA, suggesting that this may be a protective mechanism preventing overproduction of a toxic protein [372]. Moreover, Oliveira *et al* have overexpressed a wild-type copy of MecI in *trans* in a large collection of prototype MRSA strains, and surprisingly, MecI overexpression did not affect the phenotypic expression of β-lactam resistance in the majority of those strains, suggesting the existence of other *mecA* regulators [371]. As a matter of fact, several authors based on disparate observations have postulated the existence of a third element mediating the signal transduction between the MecR1 and MecI proteins [344, 345, 352, 371, 373]

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CHAPTER II

The role of *mecR2* in the regulation of methicillin-resistance in MRSA

Based on results published in: Arêde, P. Milheiriço, C. de Lencastre, H. and Oliveira, D.C. The anti-repressor MecR2 promotes the proteolysis of the *mecA* repressor and enables optimal expression of β-lactam resistance in MRSA. PloS Pathog, 2012. 8(7): p. e1002816.

2.1 Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen, which is cross-resistant to virtually all β-lactam antibiotics. MRSA strains are defined by the presence of *mecA* gene. The transcription of *mecA* can be regulated by a sensor-inducer (MecR1) and a repressor (MecI), involving a unique series of proteolytic steps. The induction of *mecA* by MecR1 has been described as very inefficient and, as such, it is believed that optimal expression of β-lactam resistance by MRSA requires a non-functional MecR1-MecI system. However, in a recent study, no correlation was found between the presence of functional MecR1-MecI and the level of β-lactam resistance in a representative collection of epidemic MRSA strains. Here, we demonstrate that the *mecA* regulatory locus consists, in fact, of an unusual three-component arrangement containing, in addition to *mecR1-mecI*, the up to now unrecognized *mecR2* gene coding for an anti-repressor. The MecR2 function is essential for the full induction of *mecA* expression, compensating for the inefficient induction of *mecA* by MecR1 and enabling optimal expression of β-lactam resistance in MRSA strains with functional *mecR1-mecI* regulatory genes. Our data shows that MecR2 interacts directly with MecI, destabilizing its binding to the *mecA* promoter, which results in the repressor inactivation by proteolytic cleavage, presumably mediated by native cytoplasmatic proteases. These observations point to a revision of the current model for the transcriptional control of *mecA* and open new avenues for the design of alternative therapeutic strategies for the treatment of MRSA infections. Moreover, these findings also provide important insights into the complex evolutionary pathways of antibiotic resistance and molecular mechanisms of transcriptional regulation in bacteria.

2.2 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of infections in hospitals in many countries and has also become an important community- and livestock-associated pathogen [1-3]. Recently, a report from CDC has reassessed the burden of MRSA infections in the USA, putting the number of deaths attributable to MRSA in front of those related to HIV-AIDS, Alzheimer disease or homicide [4]. MRSA are resistant to virtually all β-lactam antibiotics, one of the most clinically relevant class of antimicrobial agents. In addition, contemporary MRSA strains are frequently resistant to many other antimicrobial classes leaving clinicians with few therapeutic options.

The MRSA characteristic phenotype is due to an extra penicillin-binding protein (PBP2A) coded by the *mecA* gene [5], which has a remarkable reduced reactivity for many β-lactams [6]. In addition, >95% of MRSA strains have also a β-lactamase enzyme coded by *blaZ* that confers penicillin-resistance [7-9]. The *mecA* transcription can be controlled by the divergent *mecR1-mecI* regulatory genes, coding for a sensor-inducer and a repressor, respectively [10]. This genetic organization of the *mecA* locus is similar to that of the β-lactamase, which contains the structural gene *blaZ* and the homologous *blaR1-blaI* regulatory genes. In fact, there is a cross-talk between both systems [11-14], and the signal-transduction mechanisms are believed to be identical [15, 16], involving two proteolytic steps, in contrast with the most common bacterial signal transduction mechanism that involves the phosphorylation of regulatory proteins [17]. Specifically, the currently accepted model of *mecA* regulation involves two main steps: (i) binding of the β-lactam antibiotic to the extracellular sensor domain of MecR1 leads to the autocatalytic cleavage of the sensor-inducer and activation of the cytoplasmatic inducer domain, which appears to be a prometalloprotease; (ii) the activated inducer domain of MecR1 either directly cleaves the promoter-bound MecI dimers or promotes the repressor cleavage, which disables the ability of the repressor protein to dimerize and bind to the *mecA* promoter, enabling the expression of the resistance gene. MecR1 once cleaved can no longer transmit signal but, since the expression of *mecR1-mecI* is also up-regulated, the *mecA* induction continues as long as the antibiotic is present in the environment.

Some details of the signaling mechanism involved in the transcriptional control of *mecA* are still elusive. For instance, induction of *mecA* by MecR1 has been described as extremely slow [12, 13], so that cells with intact *mecR1-mecI* regulatory system appear phenotypically susceptible in spite of the presence of *mecA* – the so-called "pre-MRSA" phenotype [10, 18]. Based on these observations, it has been postulated that high-level resistance to β-lactams, characteristic of many contemporary MRSA clinical strains, implies a non-functional *mecR1-mecI* regulatory system. In agreement with this hypothesis, several studies have described the accumulation of point mutations and/or gene deletions in the *mecR1-mecI* coding sequences [19-23]. Still, in some of these studies [19, 21, 23], based on contradictory observations, the existence of alternative *mecA* regulatory mechanisms has also been proposed. In fact, in a recent study, we could not establish any correlation between the *mecR1-mecI* integrity and the β-lactam resistance phenotype in a representative collection of epidemic MRSA strains and, unexpectedly, overexpression *in trans* of a wild-type copy of MecI had no effect on the phenotypic expression of resistance in most strains [24].

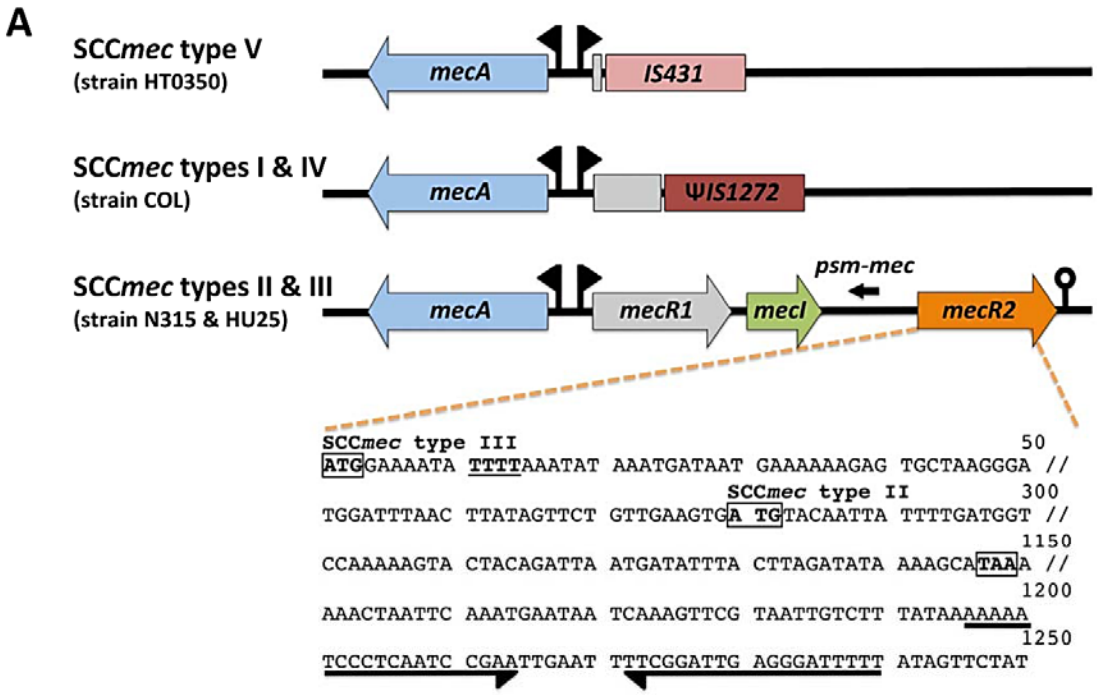
Here, we identify the missing link that explains the puzzling observations described above. We demonstrate that the *mecA* regulatory locus is in fact a three-component system that contains, besides *mecR1-mecI* genes, the *mecR2* gene, which is co-transcribed with *mecR1-mecI*. *In vitro* and *in vivo* assays show that MecR2 acts as an anti-repressor by interacting directly with MecI repressor, disturbing its binding to the *mecA* promoter, which results in its inactivation by proteolytic cleavage. In MRSA strains with functional *mecR1-mecI* genes, MecR2 is essential for the full induction of *mecA* transcription, compensating for the inefficient induction of *mecA* by *mecR1* and enabling the optimal expression of β-

lactam resistance. These findings suggest a need to revise the current model for the induction of *mecA* expression in clinical MRSA strains and open new avenues for the design of alternative therapeutic strategies targeting the regulatory pathway of *mecA* expression. In addition, this unusual combination of repressor, sensor-inducer and anti-repressor, together with the unique modulation of a series of proteolytic cleavage steps underlying the signal transduction mechanism, provides important insights into the evolution of antibiotic-resistance and transcriptional control of genes in bacteria.

2.3 Results and Discussion

2.3.1 The *mecA* cognate regulatory locus is a three-component system

Since the MRSA phenotype is not affected by the overexpression *in trans* of the *mecA* repressor [24], we hypothesized that a third regulator might be involved in the *mecA* transcriptional control. Taking into account that *mecA* gene is part of a large polymorphic exogenous DNA fragment (the so called SCC*mec* element), which has integrated in the chromosome [25], we reasoned that the putative additional regulator should be located within this chromosomal cassette, most likely upstream to the *mecA* gene; i.e. genetically linked to the *mecR1-mecI* region. Upon analysis of prototype sequences of SCC*mec* types II and III, which are characterized by complete *mecR1-mecI* coding sequences [26], we found a highly conserved region (99.9% homology) immediately downstream of *mecI*. This region contains the divergent small coding sequence for a phenol-soluble modulin, *psm-mec*, involved in *S. aureus* virulence and colony spreading [27, 28], and a putative open-reading frame (ORF) that, due to a difference in four-tandem thymine residues, has a variable length (Figure 2.1A): 870bp in SCC*mec* type II prototype strain N315 (accession no. D86934, positions 41794-40925) and 1149bp in SCC*mec* type III prototype strain HU25 (accession no. AF422694, positions 4729-3861). Both variants are homologous to the repressor of the xylose operon of *S. xylosus*, XylR (accession no. X57599) with an amino-acid identity of 60-64%; the four-thymine deletion in strain N315 eliminates the N-terminal DNA-binding helix-turn-helix domain of XylR (Figure 2.1B). Available genomic and SCC*mec* sequence data demonstrate that both ORF variants are disseminated in *S. aureus* and in other staphylococcal species containing SCC*mec* sequences (Figure 2.2). We coined the name of *mecR2* for this putative ORF.



B

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              XylR  MENNFIVNENEKRVLKQIFNNSNISRTQISKNLELNKATISNILNNLKHK  50
MecR2 SCCmec III  MENILNINDNEKRVLREIYNHHNISRTQISKNLEINKATISSILNKLKYK  50
 MecR2 SCCmec II  --------------------------------------------------  0

              XylR  SLVNEVGEGNSTKSGGRKPILLEINQKYGYYISMDLTYDSVELMYNYFDA 100
MecR2 SCCmec III  SLVNEVGEGDSTKSGGRKPILLKVNHLYGYFISLDLTYSSVEVMYNYFDG 100
 MecR2 SCCmec II  ----------------------------------------MYNYFDG  7

              XylR  TILKQDSYELNDKNVSSILQILKSNINVSEKYDTLYGLLGISISIHGIVD 150
MecR2 SCCmec III  NVIKHESYDLPDEKVSSILSIIKKHIDIQEKLDTYNGLLGVSVSIHGVVD 150
 MecR2 SCCmec II  NVIKHESYDLPDEKVSSILSIIKKHIDIQEKLDTYNGLLGVSVSIHGVVD 57

              XylR  DEQNIINLPFHKNEKRTFTDELKSFTNVPVVIENEANLSALYEKSLYINS 200
MecR2 SCCmec III  NEQHVTYLPFHETEGISIAKKIKEITNVPVVVENEANLSALYERNFNHNL 200
 MecR2 SCCmec II  NEQHVTYLPFHETEGISIAKKIKEITNVPVVVENEANLSALYERNFNHNL 107

              XylR  NINNLITLSIHKGIGAGIIINKKLYRGSNGEAGEIGKTLVLESINNNDNK 250
MecR2 SCCmec III  SYNNLIALSIHKGIGAGLIINNQLYRGANGEAGEIGKTLV-SKVSDNVEI 249
 MecR2 SCCmec II  SYNNLIALSIHKGIGAGLIINNQLYRGANGEAGEIGKTLV-SKVSDNVEI 156

              XylR  YYKIEDICSQDALIQKINNRLGVTLTFTELIQYYNEGNSIVAHEIKQFIN 300
MecR2 SCCmec III  FHKIEDIFSQEALLHNLSNQLNEKMTLSKLIQFYNEKNPVVVEEMEQFIN 299
 MecR2 SCCmec II  FHKIEDIFSQEALLHNLSNQLNEKMTLSKLIQFYNEKNPVVVEEMEQFIN 206

              XylR  KMTVLIHNLNTQFNPDAIYINCPLINELPNILNEIKEQFSCFSQGSPIQL 350
MecR2 SCCmec III  KIAVLIHNLNTQFNPNAIYINCPLFNEMPEILEAIKNQFKQYSRNE-IQI 348
 MecR2 SCCmec II  KIAVLIHNLNTQFNPNAIYINCPLFNEMPEILEAIKNQFKQYSRNE-IQI 255

              XylR  HLTTNVKQATLLGGTLAIMQKTLNINNIQMNIK--                383
MecR2 SCCmec III  KLTSNVKFATLLGGTLAIIQKVLQINDIYLDIKAX                 383
 MecR2 SCCmec II  KLTSNVKFATLLGGTLAIIQKVLQINDIYLDIKA-                 289
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Figure 2.1 - The *mecA* regulatory locus is a three-component system. **(A)** Genetic organization of the *mecA* regulatory locus in major SCC*mec* types I-V and prototype MRSA strains used in this study. The magnified DNA sequence shows the two *mecR2* start codons in SCC*mec* type III and III (boxed), the stop codon (boxed), the four-thymine deletion in SCC*mec* type II (underlined), and the putative terminator (inverted arrows). **(B)** Multiple sequence alignment between the repressor of the xylose operon (XylR) and MecR2 from prototype SCC*mec* types II and III strains. Green – identical residues; red – similar residues; white – divergent residues. The figure was prepared using "The Sequence Manipulation Suite" freely available at http://www.bioinformatics.org.

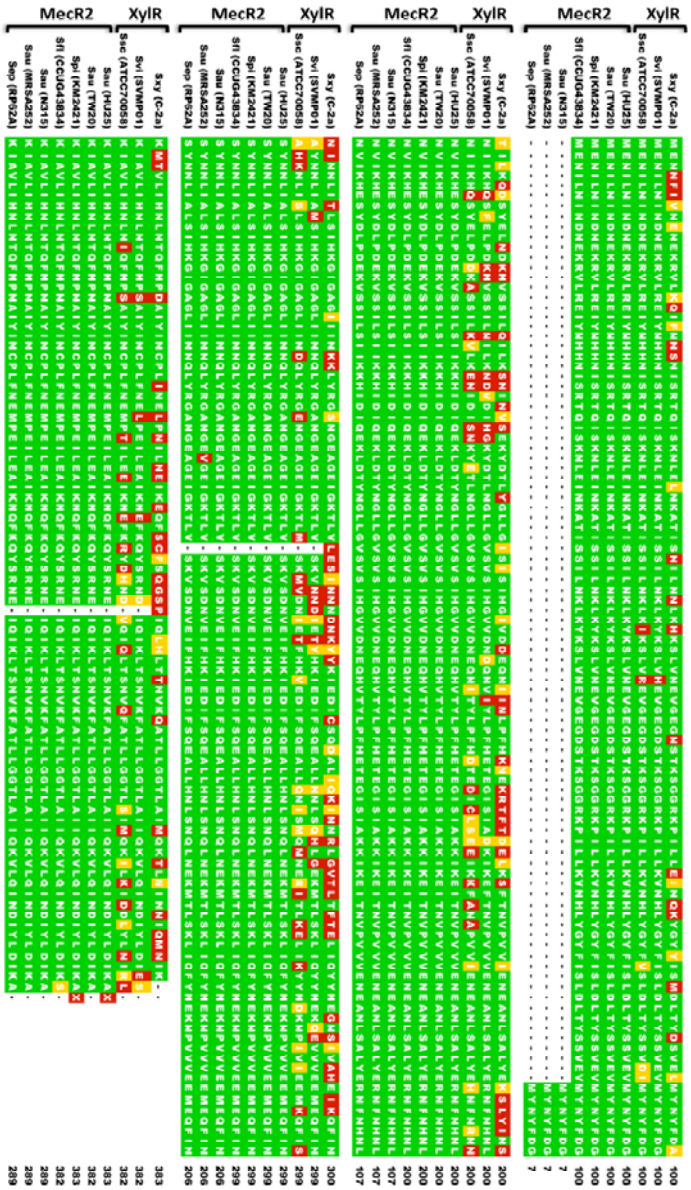

Figure 2.2 - Multiple sequence alignment between the repressor of the xylose operon (XylR) and the anti-repressor MecR2 found in staphylococci. Species names (strain code in parenthesis): Sxy – *S. xylosus*, Svi – *S. viridians,* Ssc – *S. sciuri*, Sau – *S. aureus,* Spi – *S. pseudintermedius*, Sfl – *S. fleuretti*, Sep – *S. epidermidis*. Green – identical residues; red – similar residues; white – divergent residues. The figure was prepared using "The Sequence Manipulation Suite" freely available at http://www.bioinformatics.org.

Analysis of the upstream sequences of the putative *mecR2* gene revealed no obvious promotersequences. A putative terminator region, consisting of two perfect inverted repeats of 19 base pairs, was identified downstream the stop codon of *mecR2*. No terminator sequences were found in the *mecI-mecR2* intergenic region, suggesting that the putative *mecR2* gene might be co-transcribed together with *mecR1-mecI* from the *mecR1* promoter. This hypothesis was confirmed by transcriptional analysis of *mecR2* by reverse-transcriptase PCR (RT-PCR) in prototype strains N315 and HU25. Using internal primers for the putative coding region of *mecR2* a positive signal was detected in both prototype strains. Moreover, using pairs of primers spanning the *mecI-mecR2* and *mecR1-mecI* regions, positive signals were detected suggesting that *mecR1-mecI-mecR2* genes are co-transcribed from the *mecR1* promoter (Figure 2.3).

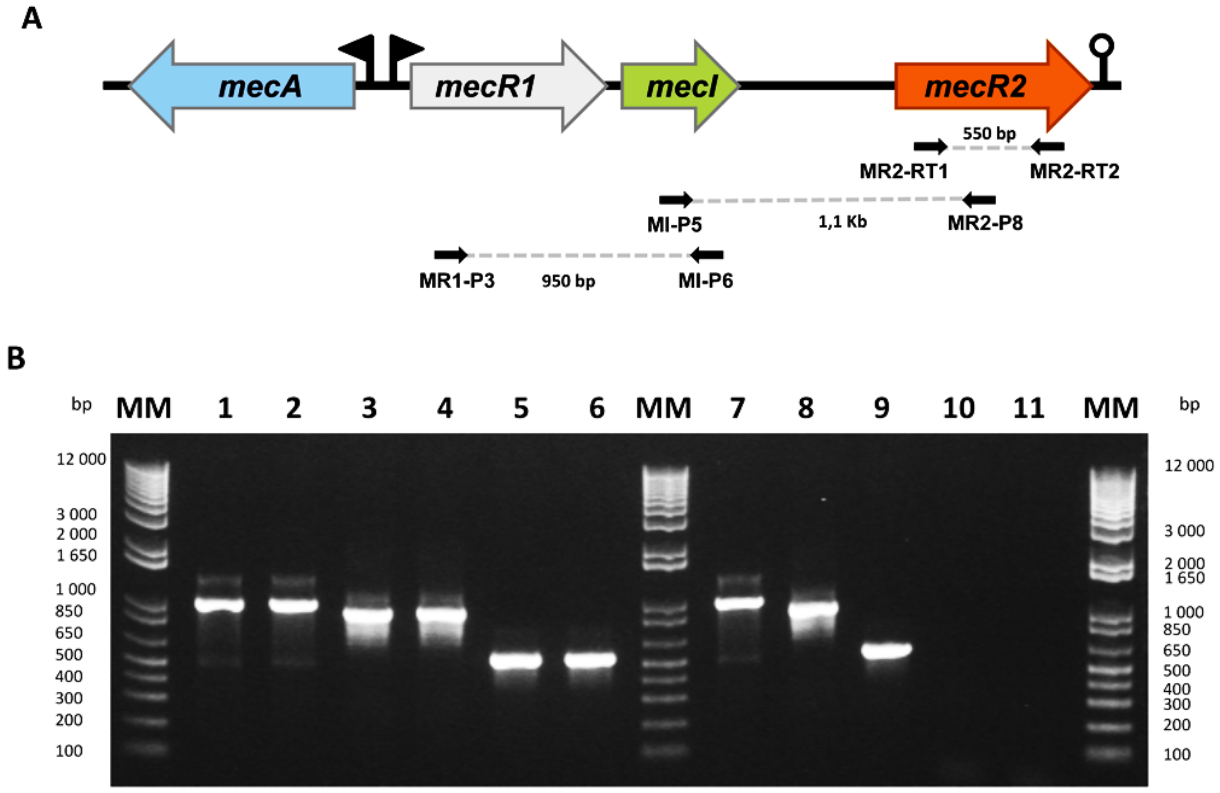

Figure 2.3 - Transcriptional analysis of *mecR2*. **(A)** Genetic organization of the *mecA* regulatory locus and location of primers used in the RT-PCR assays. **(B)** Gel electrophoresis of the RT-PCR products obtained with total RNA from strains N315 and HU25, respectively. MM, molecular weight marker (1Kb DNA ladder); lanes 1-2, *mecI-mecR2* co-transcript; lanes 3-4, *mecR1-mecI* co-transcript; lanes 5-6, *mecR2* transcript; lanes 7-9, positive controls, PCR reactions using the same primer pairs and chromosomal DNA from strain N315 as template; lanes 10-11, negative control, RT-PCR reactions without the reverse-transcription step for total RNA preparations of strains N315 and HU25, respectively, using the primer pair MR2-RT1/MR2-RT2 (i.e. the one originating the smallest amplicon).

2.3.2 *mecR2* is involved in the optimal expression of β-lactam resistance

We next evaluated the role of *mecR2* on the MRSA phenotype by constructing a series of recombinant strains using two parental strains with contrasting phenotypes. The first of these, strain COL is highly resistant to methicillin, has no *mecI,* has a partially deleted *mecR1*, expresses *mecA* constitutively, is negative for β-lactamase [29], and is *mecR2* negative. The second, strain N315 has a low-level methicillin-resistance phenotype, carries wild-type *mecR1-mecI* sequences, has an inducible expression of *mecA*, carries a β-lactamase plasmid [10, 18], and is *mecR2* positive. In previous studies, we have observed a sharp decrease in resistance to oxacillin in strain COL overexpressing *in trans* the repressor *mecI* (COL+*mecI*), whereas the great majority of other MRSA strains tested, including strain N315, did not show alterations in the oxacillin-resistance phenotype [24]; being oxacillin a methicillin analogue that has replaced methicillin in clinical use. In this study, we have cloned the *mecI-mecR2* region from strain N315 in the same plasmid vector. When strain COL was transformed with this recombinant plasmid (COL+*mecI-mecR2*) the resistant-phenotype was completely restored and so was the constitutive expression of *mecA* (Figure 2.4).

To exclude possible artifacts due to the overexpression of genes from multi-copy plasmids, we reconstructed the *mecR1-mecI-mecR2* regulatory locus of prototype strain N315 in the chromosome of strain COL, using an insertion-deletion strategy with a thermosensible plasmid (Figure 2.5). First, we inserted the wild-type sequences of *mecR1-mecI* (strain COL::RI), which caused a decrease of oxacillin-resistance when compared to the parental strain COL, in agreement with the poor induction of *mecA* by MecR1 alone (Figure 2.6). Compared to COL+*mecI* (Figure 2.4), the decrease of oxacillin-resistance in COL::RI was less severe, most likely due to the lower levels of MecI repressor when expressed from the chromosome and to the presence of the inducer MecR1. Upon introduction of the complete *mecA* regulatory locus; i.e. *mecR1-mecI-mecR2* (strain COL::RI-R2), the phenotype of parental strain COL was fully restored, suggesting that in the presence of functional *mecR1-mecI*, *mecR2* is required for the optimal expression of resistance. When we re-introduced the recombinant plasmid over-expressing *mecI* in recombinant strain COL::RI-R2 (strain COL::RI-R2+*mecI*) no effect was detected on the phenotypic expression of resistance, suggesting that *mecR2* expressed from its "native" chromosomal location is able

to disrupt the *mecI*-mediated repression, even when *mecI* is overexpressed *in trans*, which explains our previously reported observations [24]. As control experiment, overexpression of *mecI* in recombinant strain COL::RI, COL::RI+*mecI*, had the same effect observed in strain COL, COL+*mecI*.

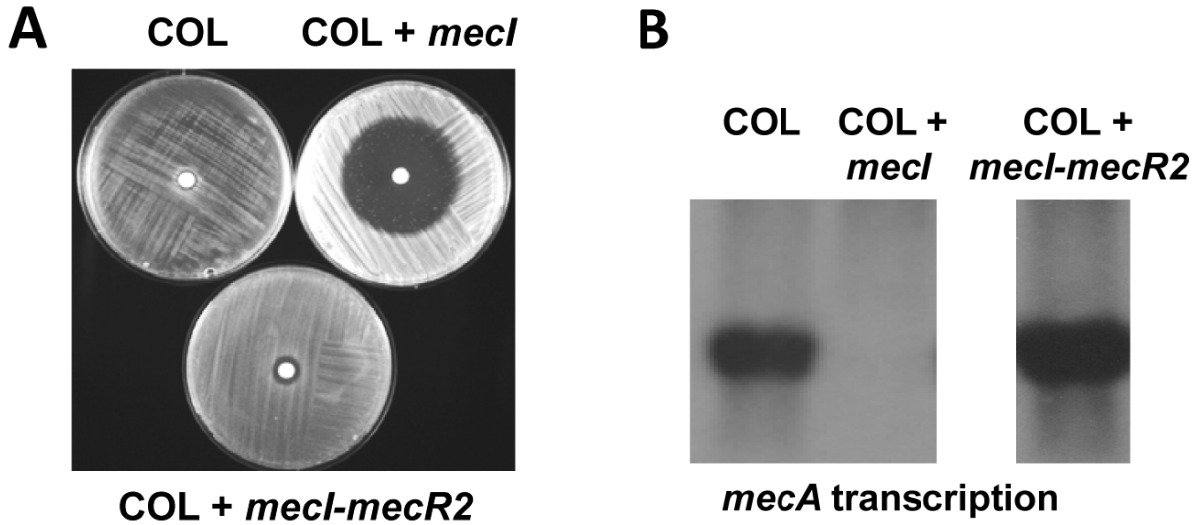

Figure 2.4 - *mecR2* interferes with the *mecI*-mediated repression of β-lactam resistance. **(A)** Co-overexpression of *mecI* and *mecR2* region (COL+*mecI-mecR2*) reverted the effect of *mecI* overexpression (COL+*mecI*) on the oxacillin-resistance phenotype in strain COL, as evaluated with diffusion disks containing 1 mg of oxacillin. **(B)** Northern blotting analysis of *mecA* transcription shows that in the presence of *mecR2* locus the repressor effect of *mecI* is reverted.

To further clarify the *mecR2* function, the chromosomal copy of *mecR2* gene from strain N315 was replaced by an antibiotic-resistance marker (N315::Δ*mecR2*), using a similar insertion deletion strategy (Figure 2.5). Deletion of *mecR2* caused a sharp decrease in the phenotypic expression of oxacillin-resistance in strain N315. We then cloned the *mecR2* gene from strain N315 under the control of an inducible promoter (*spac*::*mecR2*) and, in the presence of the inducer (IPTG 100 μM), we succeeded in complementing the *mecR2* null-mutant in strain N315 (Figure 2.7A) and restored the COL phenotype of recombinant strain COL::RI (Figure 2.7B). Since in these experiments only the *mecR2* gene was deleted from the chromosome of strain N315 and complemented *in trans*, it can be concluded that the intergenic *mecI-mecR2* region has no role in the phenotypic expression of β-lactam resistance. Altogether, these observations demonstrated that *mecR2* interferes with the *mecI*-mediated repression of *mecA* and is essential for the optimal expression of β-lactam resistance in the presence of functional *mecR1-mecI* sequences.

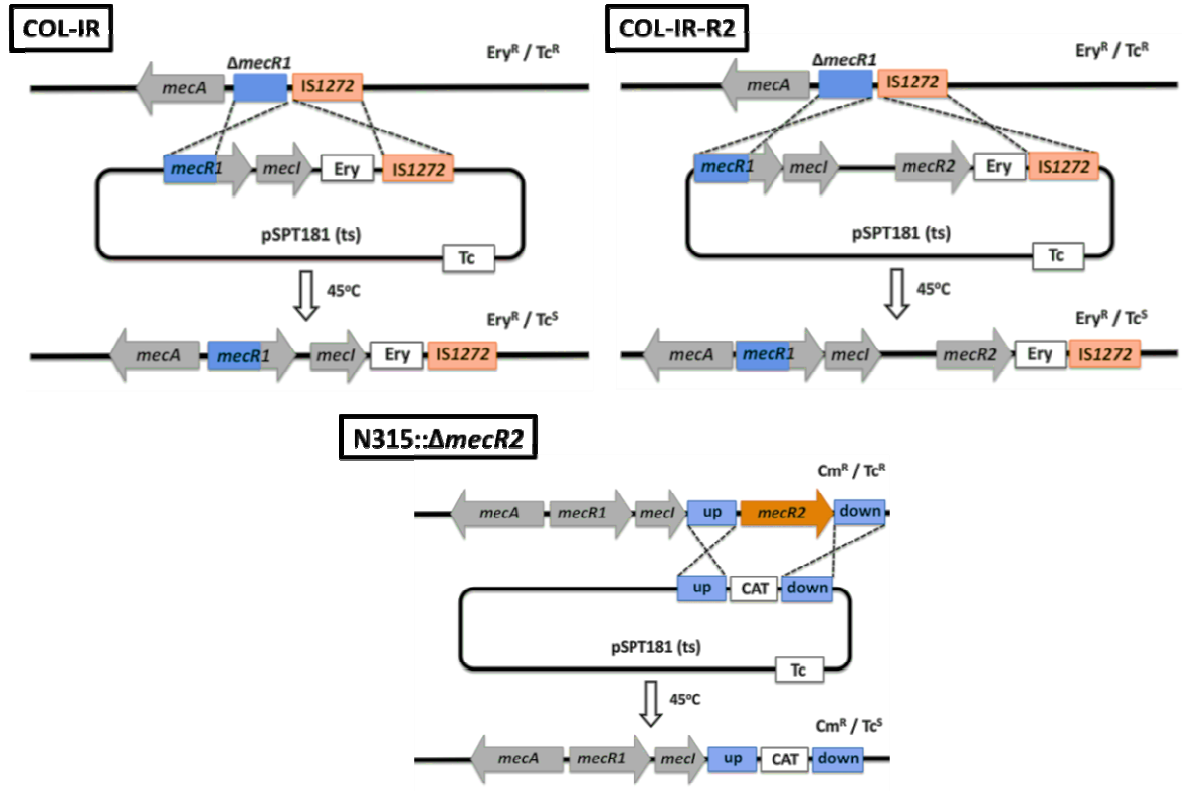

Figure 2.5 - Insertion-deletion strategies used for the reconstruction of the *mecA* regulatory locus in the chromosome of strain COL and deletion of *mecR2* from the chromosome of strain N315. Integration through homologous recombination of recombinant thermosensible plasmids was promoted at a non-permissive temperature (45^{O}C) and with selection for tetracycline resistance (Tc^{r}). Resolution of co-integrates was promoted at a permissive temperature (30^{O}C) with selection for tetracycline resistance and segregation of the excised plasmids was promoted at 45^{O}C without antibiotic selection. Colonies susceptible to tetracycline (Tc^{s}) and resistant to erythromycin (Ery^{r}) or chloramphenicol (Cm^{r}) were selected for further analysis.

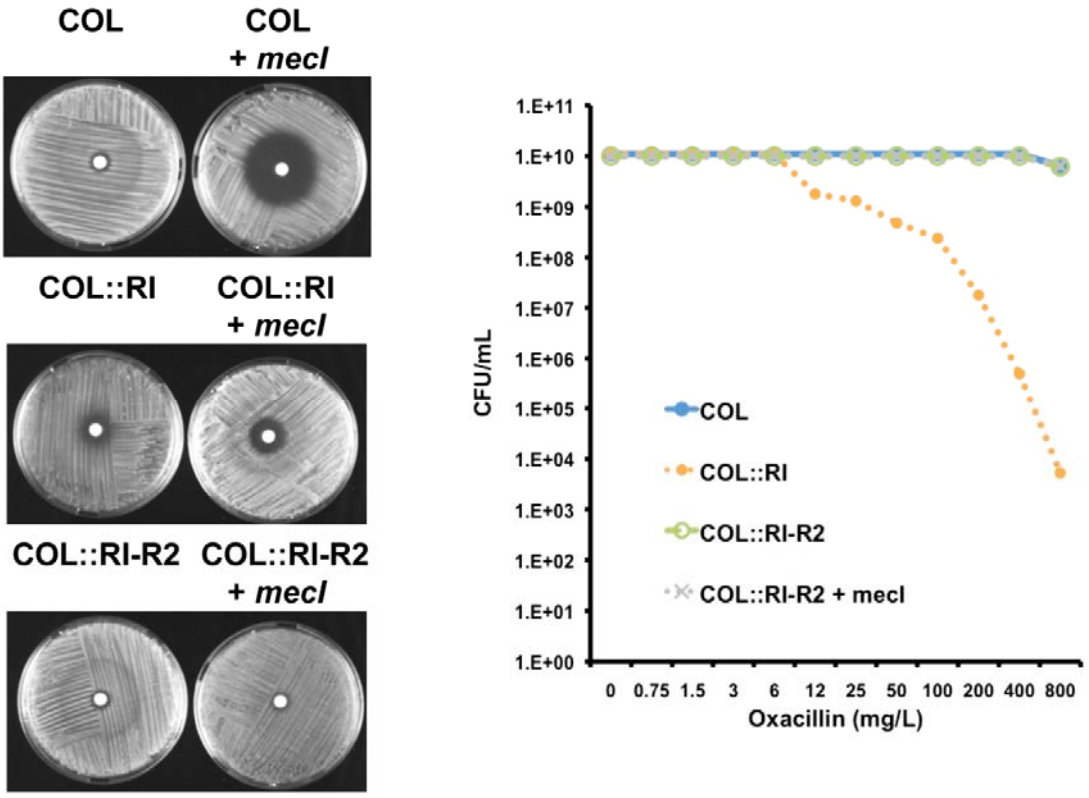

Figure 2.6 - Reconstruction of the *mecA* regulatory locus in prototype strain COL. Reconstruction of the *mecR1-mecI* locus in the chromosome of strain COL (COL::RI) causes a decrease of the resistance level to oxacillin, which can be reverted by the reconstruction of the full *mecA* regulatory locus, *mecR1-mecI-mecR2* (COL::RI-R2). Overexpression of *mecI* in COL::RI-R2 no longer cause a decrease in the resistance level to oxacillin (COL::RI-R2 + *mecI*). As control, the overexpression of *mecI* in strain COL::RI, originated a phenotype similar to COL+*mecI* (Fig. 2). The oxacillin-resistance levels were evaluated by diffusion disks containing 1 mg of oxacillin (left) or by population analysis profiles (PAP's) (right).

Of note, first attempts to complement the *mecR2* null-mutant by overexpression it *in trans* only succeeded if *mecR2* was co-overexpressed together with *mecI* (data not shown). This requirement for low MecR2 cellular amounts and/or equimolar cellular amounts of MecI and MecR2, suggests that at high cellular concentrations MecR2 function may be lost, either due to oligomerization or (non-specific) interference with essential cellular targets. A classical example of the requirement for equimolar ratios between interacting proteins is the *Escherichia coli* helicase DnaB / replication factor DnaC complex, in which the replication is inhibited when DnaC is in excess [30].

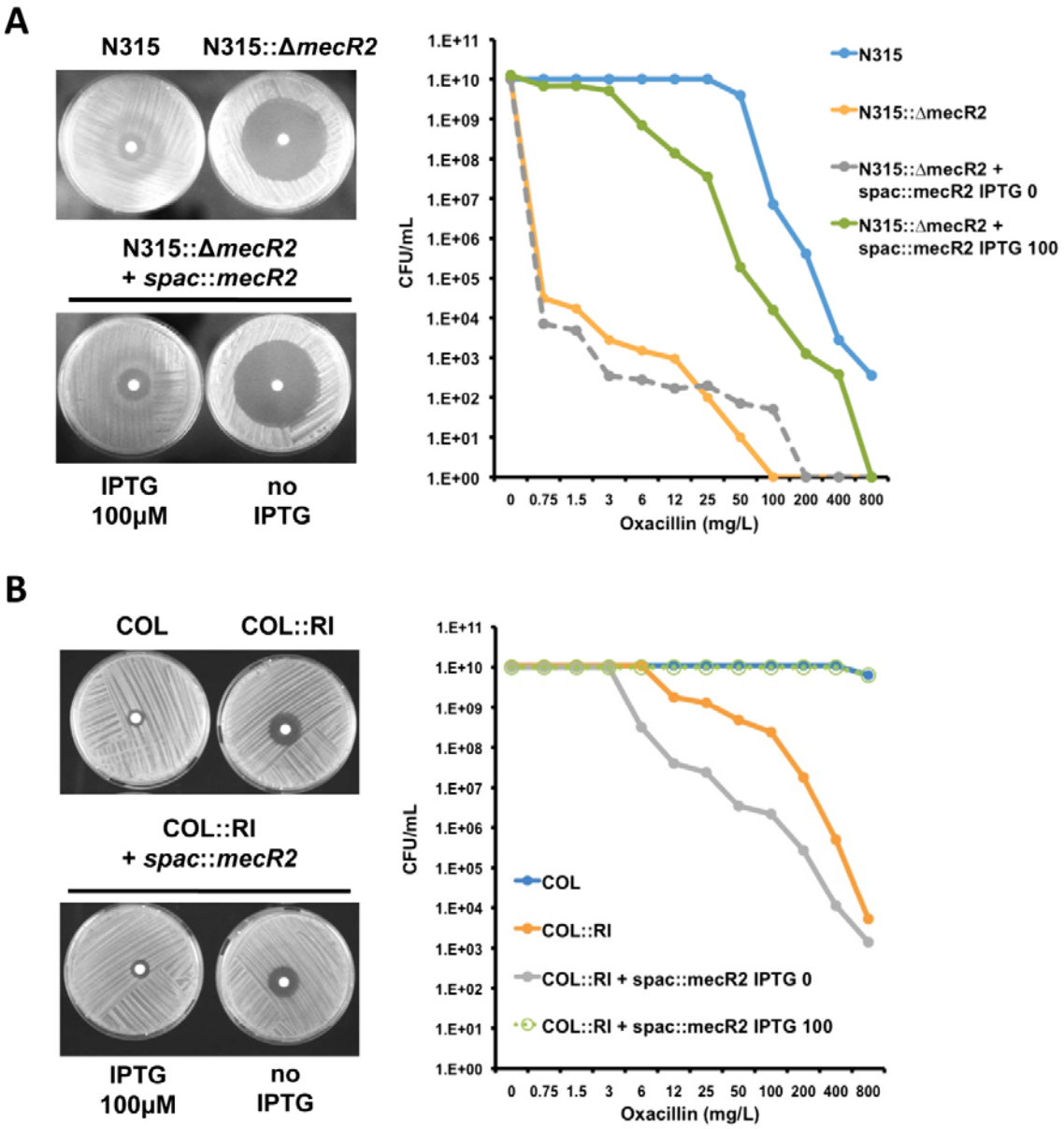

Figure 2.7 - Role of *mecR2* on the optimal expression of β-lactam resistance. **(A)** Deletion of *mecR2* from the chromosome of strain N315 (N315::Δ*mecR2*) causes a decrease on the resistance level to oxacillin, which can be reverted upon complementation with *mecR2* expressed from an inducible promoter (N315::Δ*mecR2*+*spac*::*mecR2*) in the presence of the inducer (IPTG 100μM). **(B)** The poor expression of oxacillin resistance by recombinant strain COL::RI, can also be reverted upon complementation with *mecR2* expressed from an inducible promoter (COL::RI+*spac*::*mecR2*) in the presence of the inducer (IPTG 100μM). The oxacillin-resistance levels were evaluated by diffusion disks containing 1 mg of oxacillin (left) and by population analysis profiles (PAP's) (right).

2.3.3 *mecR2* is required for the full induction of *mecA* transcription

We next analyzed the effect of *mecR2* on the induction profile of *mecA* transcription in parental strain N315, its null-mutant for *mecR2*, and in the complemented mutant, by Northern blotting (Figure 8A) and quantitative Real-time RT-PCR (qRT-PCR) analysis (Figure 2.8B). In relative terms, upon induction with sub-MIC oxacillin, a much stronger induction of *mecA* transcription was observed in the parental strain than in the *mecR2* null-mutant (N315::Δ*mecR2*), in which the amount of *mecA* transcript seems to be not sustained during the last two time-points. In the complemented mutant (N315::Δ*mecR2*+spac::*mecR2*) there was a sustained induction of *mecA* transcription throughout the time-course of the experiment. However, in the complemented strain, although the resistant phenotype of the parental strain was fully restored (as illustrated in Figure 2.7A), the amount of *mecA* transcript was substantially lower and virtually identical to the *mecR2* null-mutant. Although this discordance is in agreement with previous studies reporting on the lack of a correlation between the cellular amounts of *mecA* transcript or protein and the phenotypic level of resistance [31, 32], we cannot formally exclude other possible explanations, such as MecR2 having multiple targets that affect the resistance phenotype. Nevertheless, these data suggest that the *mecR2* interferes with the induction of *mecA* transcription in response to β-lactams.

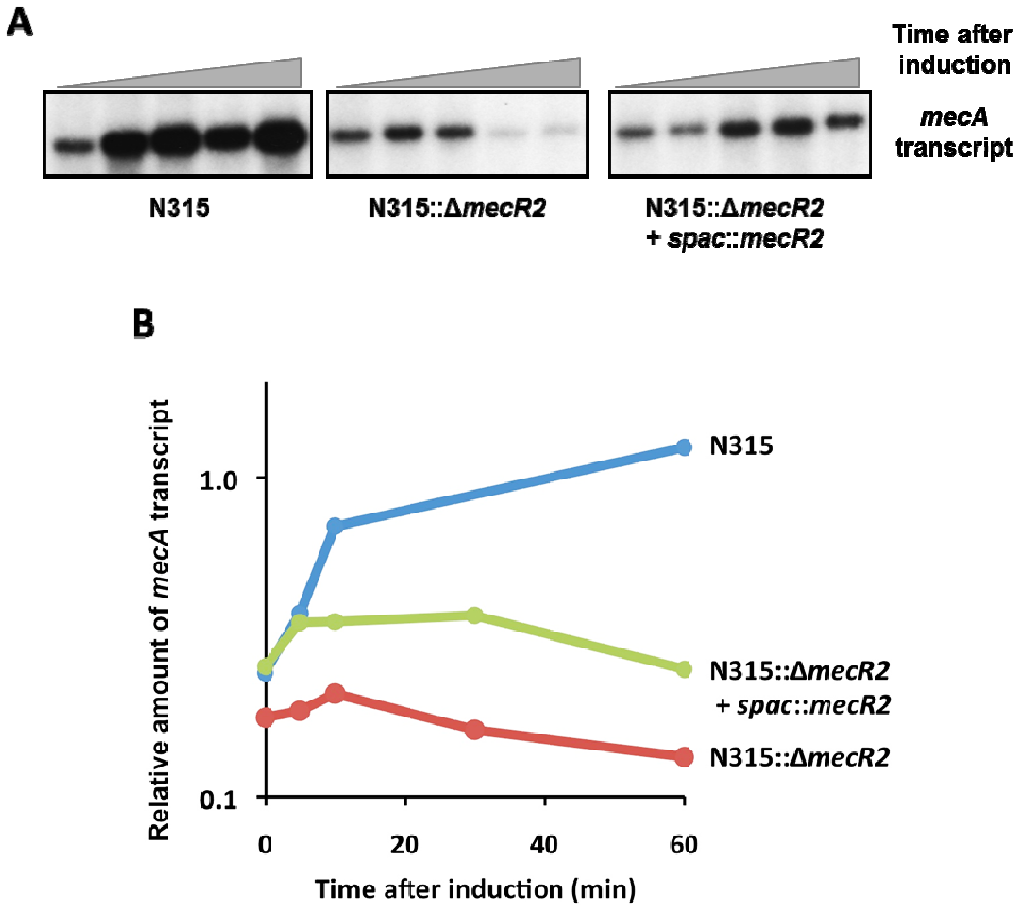

Figure 2.8 - Effect of *mecR2* on the induction of mecA transcription. **(A)** Northern blot and **(B)** qRT-PCR analysis of the *mecA* induction profile in parental strain N315, *mecR2* null-mutant (N315::Δ*mecR2*) and complemented mutant (N315::Δ*mecR2*+*spac*::*mecR2*, IPTG 100μM). Cultures were induced with a sub-MIC concentration of oxacillin (0.05mg/L) and samples were taken at 0', 5', 10' 30' and 60'.

The effect of *mecR2* on the induction profile of *mecA* transcription was also analysed by qRT-PCR in parental COL strain and in both COL::RI and COL::RI-R2 recombinant strains (Figure 2.9). Upon challenge with sub-MIC oxacillin, a stronger induction of *mecA* transcription was observed in the COL::RI-R2 strain, especially into the last two time-points. As expectable, the amounts of *mecA* transcript were not affected in the strain COL::RI, being extremely low when compared with both COL and COL::RI-R2 strains. This observations are in accordance with the Figure 2.7B in which the sensor transducer MecR1 poorly induces the transcription of *mecA* gene. However when the *mecR2* gene is present (strain COL::RI-R2), the regulatory system which triggers the *mecA* transcription initiation is much faster, even the basal amounts of *mecA* transcript are higher than those observed to the strain COL::RI.

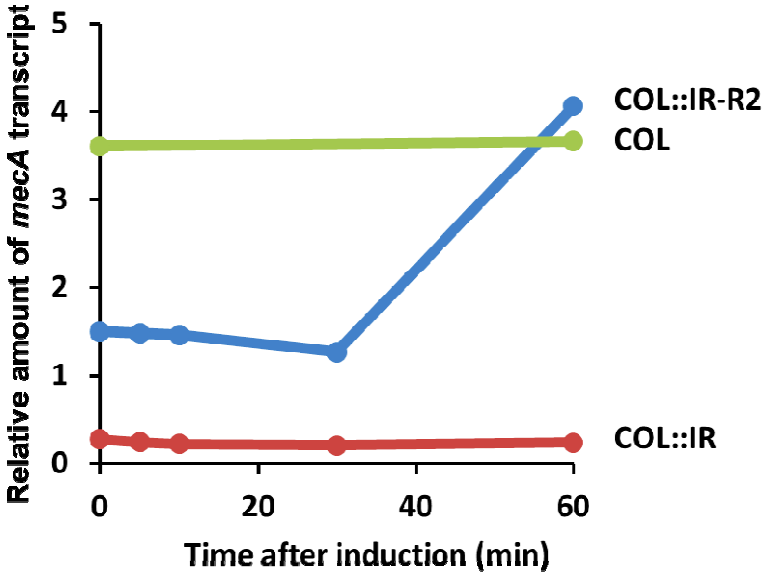

Figure 2.9 - Effect of *mecR2* on the induction of *mecA* transcription in strain COL. qRT-PCR analysis of the *mecA* induction profile in parental strain COL and in the recombinant strains COL::*RI and* COL::*RI-R2*. Cultures were induced with a sub-MIC concentration of oxacillin (0.05mg/L) and samples were taken at 0', 5', 10' 30' and 60'.

2.3.4 *mecR2* transcription analysis

We have also analyzed by qRT-PCR the induction profile of *mecR2* in parental strain N315 and in the complemented *mecR2* null-mutant (N315::Δ*mecR2*+spac::*mecR2*) – Figure 2.10. qRT-PCR data for parental strain N315 showed that *mecR2* transcription was induced in the presence of sub-MIC oxacillin, in agreement with data from RT-PCR (Figure 2.3) that showed that *mecR2* was co-transcribed with *mecR1-mecI* from the inducible *mecR1* promoter. In the complemented mutant, in the presence of the inducer (IPTG), *mecR2* transcription levels were 10 fold higher than those of parental strain and, as such, the low levels of *mecA* transcription observed for this strain (Figure 2.8) cannot be attributable to an inefficient induction of *mecR2* transcription from the P*spac* promoter.

Of note, in parental strain N315, *mecR2* transcription levels appear to be residual when compared to those of *mecA* (10^9 fold less). Although this might be explained by an experimental artifact, one can also speculate that this may be due to different promoter strengths and/or to promoter blockage by RNA polymerase, since *mecA* and *mecR1* promoters are divergent and overlap partially. In terms of signal-transduction mechanism, once the expression of *mecA* is induced in response to β-lactams, there

is no need for high cellular levels of inducer, repressor or anti-repressor. Actually, the basal transcription of *mecR1-mecI-mecR2* is only necessary to assure that the repressor protein is still present when the antibiotic induction stops, so that the transcription of the resistance gene is shutdown. The apparent very low transcription level of *mecR2* in parental strain N315 may also explain the lack of complementation when *mecR2* was over-expressed *in trans*. In fact, this artificial system, when compared to wild-type strains, presumably generates extremely high cellular amounts of MecR2, which may originate a loss of function by oligomerization or non-specific interactions with other cellular targets. Finally, the apparent residual *mecR2* transcription levels may also explain our failed attempts to analyze by Northern blotting the transcription of *mecR2* in prototype MRSA strains, even with large amounts of total RNA (10–30 μg) and long autoradiograph expositions (72h). To our knowledge, Northern blotting analysis of *mecR1-mecI*/*blaR1-blaI* transcripts was described in only two studies and, in both cases, clear signals were obtained only when regulatory genes were overexpressed from recombinant plasmids [33, 34].

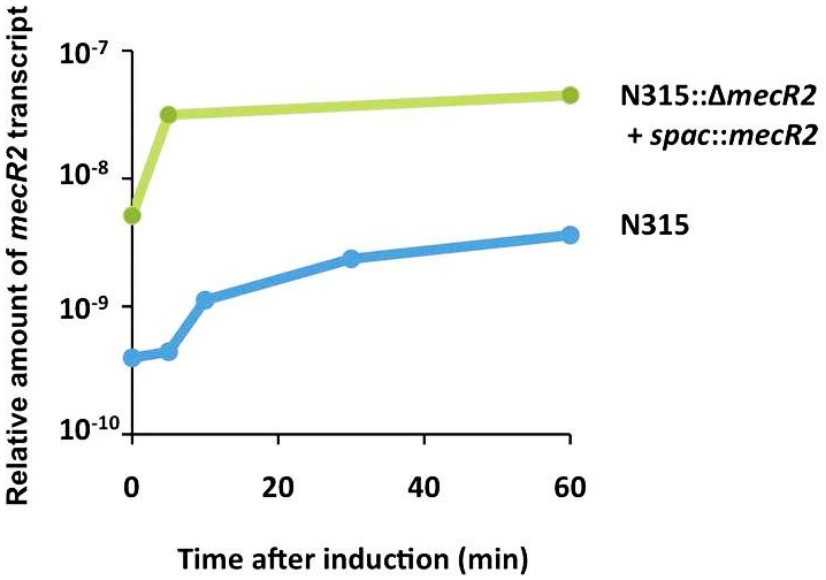

Figure 2.10 - *mecR2* transcription analysis. qRT-PCR analysis of the *mecR2* induction profile in parental strain N315 and its complemented *mecR2* mutant (N315::Δ*mecR2*+*spac*::*mecR2*, IPTG 100μM). Cultures were induced with a sub-MIC concentration of oxacillin (0.05mg/L). For comparative purposes with qRT-PCR for *mecA* and graphic legibility, relative amounts of *mecR2* transcripts were multiplied by a factor of 10^9 for parental strain N315 and 10^8 for the complemented *mecR2* mutant.

2.3.5 *mecR2* is essential for the optimal expression of β-lactam resistance in strains with functional *mecI-mecR1* regulatory locus.

Among the five major SCC*mec* types, only SCC*mec* types II and III are characterized by complete *mecR1-mecI* regulatory locus [26] – Figure 2.1A. SCC*mec* type III strains appear to have a conserved point mutation within *mecI* coding sequence resulting in a truncated non-functional repressor protein [24, 35]. Concerning SCC*mec* type II strains, the accumulation of deleterious mutations has also been described in some strains [19-23]. However, data from our MRSA collections [24], as well as from available genomic and SCC*mec* type II sequences, suggest that many strains have wild-type sequences for *mecI* (and *mecR1*). For instance, in a BLAST analysis against the *mecI* sequence from strain N315, c.a. 20 entries were found with 100% sequence identity, mostly from *S. aureus* strains but also from a few coagulase-negative staphylococci (*S. epidermidis*, *S. saprophyticus, S. fleurettii, S. cohinii*, etc.). These observations suggest that the *mecR2* function may be required for the optimal expression of β-lactam resistance in those SCC*mec* type II strains with wild-type sequences for *mecI* and *mecR1*.

In order to explore that hypothesis, we sought to test the role of *mecR2* in the phenotypic expression of β-lactam resistance in prototype strains of epidemic MRSA clones characterized by SCC*mec* type II. The MRSA population has a very strong clonal structure and only a few epidemic clones are responsible for the majority of infections worldwide [36, 37]. Three epidemic MRSA clones characterized by SCC*mec* type II have been described [38]: clone ST5-II, "New York/Japan" or USA100; clone ST36-II, EMRSA-16 or USA200; and clone ST45-II or USA600. MRSA clones ST5-II and ST36-II are two of the most important nosocomial clones in the USA and UK, respectively. Prompted by this epidemiological data, we evaluated the role of *mecR2* in three representative strains of those SCC*mec* type II clones selected from a large US collection of MRSA [39]: strains USA100, USA200 and USA600. For this purpose, the chromosomal *mecR2* deletion of strain N315 (N315::Δ*mecR2*) was transduced into those strains originating the recombinant strains: USA100::Δ*mecR2*, USA200::Δ*mecR2* and USA600::Δ*mecR2*. In the three prototype strains, deletion of *mecR2* caused a sharp decrease of the phenotypic expression of β-lactam resistance, which could be complemented by expressing *mecR2 in trans* under the control of an inducible promoter (spac::*mecR2*) (Figure 2.11A). We have also analyzed the effect of *mecR2* on the induction of *mecA* transcription in strains USA100, USA200 and USA600 by Northern blotting. Compared to N315, the three parental strains expressed *mecA* at higher levels and, in agreement with what was observed for strain N315 (Figure 2.8), deletion of *mecR2* caused a sharp decrease on the *mecA* induction and transcription levels (Figure 2.11B).

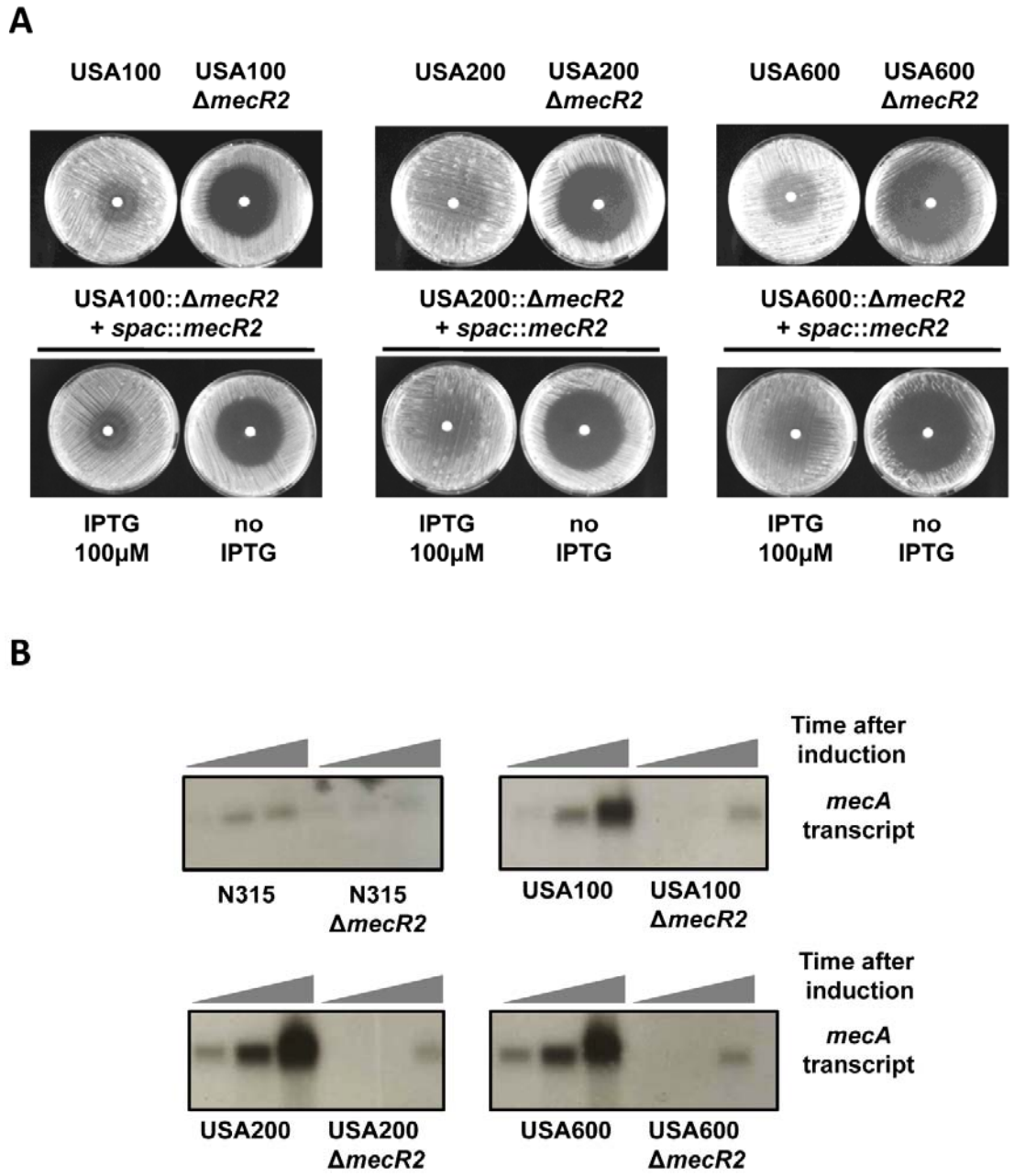

Figure 2.11 - *mecR2* is essential for the optimal expression of β-lactam resistance in strains with functional *mecI-mecR1* regulatory locus. **(A)** Deletion of *mecR2* from the chromosome of prototype epidemic strains USA100, USA200 and USA600 harboring SCC*mec* type II causes a decrease on the resistance level to oxacillin, which can be reverted upon complementation with *mecR2* expressed from an inducible promoter (*spac*::*mecR2*) in the presence of the inducer (IPTG 100μM). **(B)** Northern blot analysis of the *mecA* induction profile in parental strains USA100, USA200 and USA600 and respective *mecR2* null-mutants. Cultures were induced with a sub-MIC concentration of oxacillin (0.05mg/L) and samples were taken at 0', 10' and 60'. For comparative purposes the profile of parental strain N315 was also repeated. Note that film was exposed for 4h whereas in Figure 2.8A it was exposed for 48h.

2.3.6 *mecR2* function is not dependent on *mecR1* neither on the β-lactamase locus

Since strains N315, USA100, USA200, and USA600 have complete *mecR1-mecI* genes and strain COL has a truncated *mecR1* gene but with a complete N-terminal inducer domain, with the previous experiments we could not formally exclude that MecR2 function is dependent of at least the N-terminal inducer domain of MecR1. Therefore, we sought to test the effect of *mecR2* in a prototype SCC*mec* type V MRSA strain, characterized by an extensive deletion of *mecR1* spanning both N- and C-terminal domains [40] – Figure 2.1A. Among our collections, we selected strain HT0350 [41], since it was the only strain also negative for the β-lactamase locus [24]. Similar to what was observed for strain COL, overexpression of MecI in strain HT0350 (HT0350+*mecI*) caused a sharp decrease of resistance level, which was fully reverted with the co-overexpression of MecI and MecR2 (HT0350+*mecI-mecR2*) – Figure 2.12A. These data suggests that the effect of *mecR2* on the expression of β-lactam resistance in *S. aureus* is not dependent of *mecR1*, and as such MecR2 may act as an anti-repressor.

Since *mecA* transcription can be co-regulated by the regulators of the β-lactamase (*bla)* locus, *blaR1-blaI*, and parental strains N315, USA100, USA200 and USA600 are *bla* positive, we sought to evaluate the effect of *bla* genes on the observed *mecR2*-induced phenotypes. For this purpose, we took advantage of the fact that the experimental strategy used to construct the *mecR2* knockout in prototype strain N315 generated an intermediate mutant strain which lost the β-lactamase plasmid, probably due to the multiple passages, many of which at 45ºC. As in all other chromosomal manipulations, the *mecR2* genetic deletion was transduced back to the parental β-lactamase positive strain N315 to generate the final deletion mutant (N315::Δ*mecR2*) tested in all previous experiments. As illustrated in Figure 2.12B, in both variants of the *mecR2* chromosomal deletion, there was a sharp decrease of the β-lactam resistance. Together with the experimental data for strains COL and HT0350, both *bla* negative, this assay indicated that the *mecR2* function on the phenotypic expression of β-lactam resistance is not dependent on the presence of the β-lactamase plasmid.

In addition, in order to exclude an interaction of MecR2 with *bla* regulators, we sought to evaluate the phenotype of a *mecR2* deletion mutant in prototype strain HU25, a highly resistant MRSA strain which is positive for the *bla* locus and has a truncated non-functional MecI protein due to a premature stop codon [24]. Previous studies have shown that in the presence of oxacillin, the transcription of *mecA* is readily induced in strain HU25, presumably by the *bla* system [24]. As illustrated in Figure 2.12C, the absence of *mecR2* in strain HU25 (strain HU25::Δ*mecR2*) had no effect on the phenotypic expression of oxacillin resistance, suggesting that MecR2 is not required for the *mecA* induction mediated by the BlaR1-BlaI system.

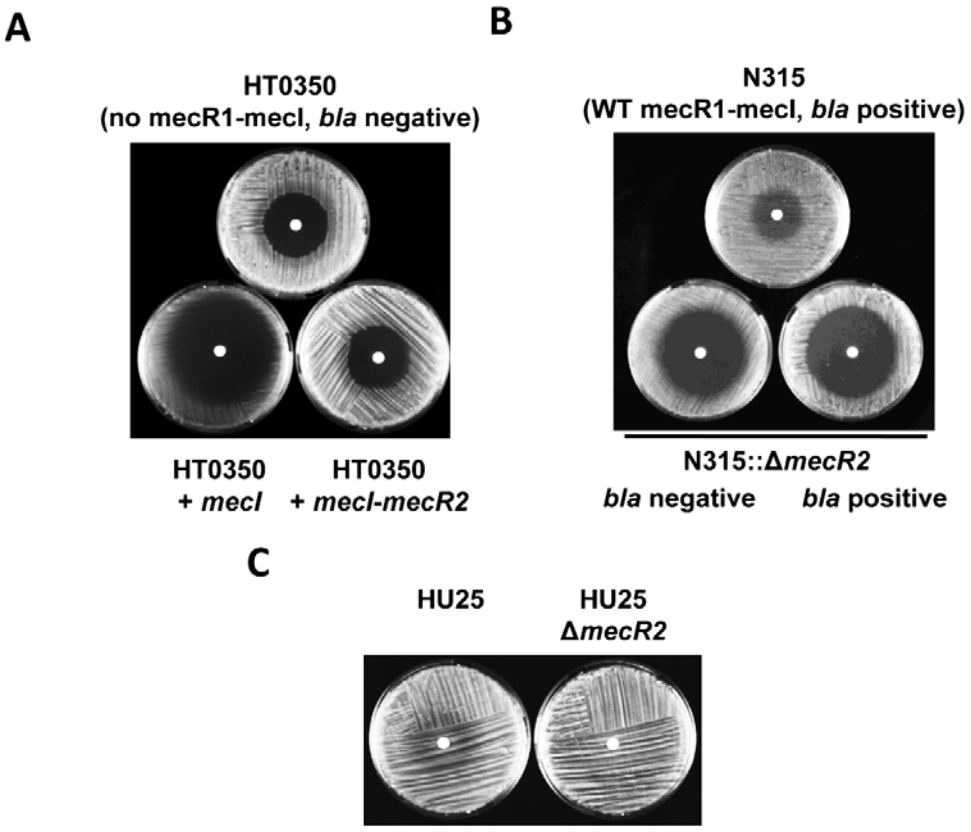

Figure 2.12 - The *mecR2* function is not dependent of *mecR1* neither of the β-lactamase locus and does not interfere with the function of β-lactamase regulatory genes. **(A)** Prototype strain HT0350 is negative for *mecR1-mecI* and for the β-lactamase locus. Co-overexpression of *mecI* and *mecR2* in strain HT0350 (HT0350+*mecI-mecR2*), reverts the effect of *mecI* overexpression (HT0350+*mecI*). **(B)** The strategy used to delete *mecR2* in prototype strain N315 generated an intermediate mutant that has lost the β-lactamase plasmid. The chromosomal *mecR2* deletion was then transduced back to the parental strain generating a β-lactamase positive *mecR2* null mutant. In both variants, the deletion of *mecR2* caused a sharp decrease of the resistance level to oxacillin. **(C)** Prototype strain HU25 is positive for *mecR2* and the β-lactamase locus and has a truncated non-functional MecI. Deletion of *mecR2* in strain HU25 (HU25::Δ*mecR2*) has no effect on the phenotypic expression of oxacillin resistance.

2.3.7 MecR2 interacts directly with MecI

The MecR2 is homologous to the transcriptional repressor of the xylose operon, XylR [42], which has an N-terminal DNA-binding domain and a C-terminal dimerization domain. The *mecR2* gene in the prototype strain N315 has no DNA binding domain due to a deletion of four tandem-thymine residues,

which, together with the genetic experiments done with this variant (Figures 2.7, 2.11 and 2.12), suggests that only the dimerization domain is involved in the MRSA phenotype. Therefore, we reasoned that the mode of action of MecR2 might involve a direct interaction with the MecI dimer, through its dimerization domain, which eventually would interfere with its binding to the *mecA* promoter.

As a first attempted to characterize the hypothetic interaction between MecI and MecR2 proteins *in vivo*, we used the yeast two-hybrid (YTH) strategy (Matchmaker GAL4 Two-Hybrid System, Clontech Laboratories, Inc), in which the coding regions of *mecI* and *mecR2* genes were obtained through high-fidelity PCR amplification using the chromosomal DNA of N315 strain as a template, and MI-YTH / MIP5 (to amplified the *mecI* gene) and MR2-YTH / MR2 P1 (to amplified the *mecR2* gene) as a primers (see Table 2.3). Both PCR products were digested with NcoI and BamHI restriction enzymes and fused to the binding and activation domains of transcription factor GAL4, into the two-hybrid pAS-21 and pACT-1 plasmids. The ligation products were consequently transformed into the *E. coli* strain DH5α, $Amp^{R.}$ Mating of *S. cerevisiae* strains and colony lift assays for detection of β-galactosidase activity were performed according to the manufacture's recommendations. Unfortunately, after many attempts we were not able to get a positive signal supporting the interaction between the MecI and MecR2.

As the YTH has failed, we evaluated the MecR2-MecI interaction using a bacterial two-hybrid (BTH) *in vivo* strategy [43]. In these experiments, we used the small *mecR2* variant present in prototype strain N315. As in-house controls, the MecI::MecI interaction, previously demonstrated using the yeast two-hybrid strategy [44], as well as the MecR2::MecR2 interaction were also evaluated. Positive results were observed in 4 out of the 8 MecI::MecR2 combinations and in 1 of 4 MecI::MecI combinations (Figure 2.13A). No MecR2::MecR2 interaction was detected in the four combinations tested (data not shown) and, as such, the assay was not conclusive in this case. Altogether, these observations provide evidence for a MecR2::MecI direct interaction.

2.3.8 MecR2 interferes with the binding of MecI to the *mecA* promoter

Next, we evaluated the interference of purified MecR2 protein with the binding of MecI to the promoter of *mecA* (P*mecA*) at several molar ratios by the electrophoretic mobility shift assay (EMSA), a strategy previously used to study the binding of purified MecI protein to P*mecA* [14, 24]. In these experiments, we expressed in *E. coli* the full MecR2 protein from prototype strain HU25, since the shorter variant of strain N315 could not be expressed and purified in a soluble form at high concentrations. As illustrated in Figure 2.13B, MecR2 interferes with the binding of MecI to P*mecA* in a concentration-dependent manner: the heavier band presumably reflecting the binding of MecI dimers to P*mecA* decreases in intensity, whereas the intermediate band reflecting the binding of MecI monomers to P*mecA* and the lighter free DNA band increase in intensity. In line with the genetic experiments, this effect was

optimal for a MecR2::MecI molecular ratio below one; in the presence of excess MecR2 the binding of MecI to P*mecA* was restored. This *in vitro* loss of effect at higher concentrations of MecR2 suggests that under these conditions MecR2 may be trapped in a non-active conformation; e.g. MecR2 may oligomerize in a concentration-dependent manner and stop interacting with MecI. It should be noted that in wild-type strains, *mecI* and *mecR2* are co-transcribed from the *mecR1* promoter and, as such, the cellular amounts of both proteins are likely to be similar. Since in these experiments we used the full MecR2 variant containing a putative N-terminal DNA binding, control EMSA experiments with MecR2 alone were performed to verify that purified MecR2 did not bind to P*mecA* alone (Figure 2.14A). In addition, control experiments with mixtures of MecI and MBP (maltose-binding protein), which has an identical molecular weight to MecR2 were performed to demonstrate that inhibition of MecI binding to P*mecA* is specific for MecR2 (Figure 2.14B). Finally, in order to exclude the hypothesis that at higher concentrations MecR2 binds (not specifically) to secondary sites in P*mecA* DNA in a MecI-dependent manner, EMSA assays with MecI-MecR2 mixtures were also performed with a much smaller DNA fragment (39bp instead of 212bp) containing the MecI protected sequences and the same results were obtained (data not shown). Altogether, these assays demonstrate that MecR2 acts as an anti-repressor disturbing the binding of MecI to the *mecA* promoter.

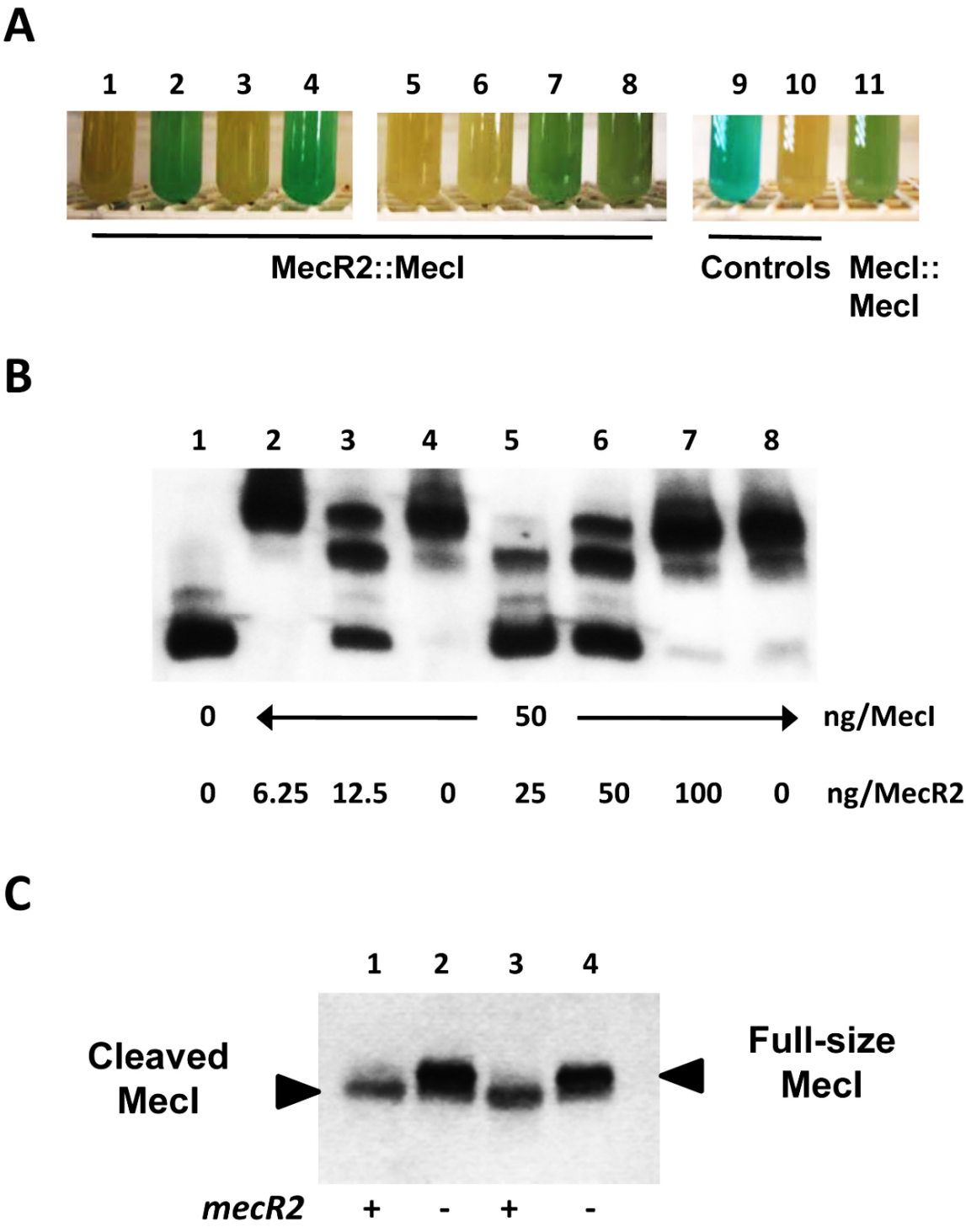
A
1 2 3 4 5 6 7 8 9 10 11
MecR2::MecI
Controls
MecI::
MecI
B
1 2 3 4 5 6 7 8
0
50
ng/MecI
0 6.25 12.5 0 25 50 100 0 ng/MecR2
C
1 2 3 4
Cleaved
MecI
Full-size
MecI
mecR2 + - + -

Figure 2.13 - MecR2 interacts directly with MecI, interfering with the binding of MecI to the *mecA* promoter and fostering the proteolysis of MecI. **(A)** *In vivo* analysis of the MecR2::MecI interaction using the bacterial two-hybrid strategy. This strategy is based on the restoration of the adenylate cyclase (CyaA) activity of *E. coli*, which activates a specific reporter gene, *lacZ.* Interactions between protein fusions were evaluated in liquid cultures through the hydrolysis of the chromogenic X-gal substrate by the activated β-galoctasidase. The MecR2::MecI interaction was evaluated using the eight possible combinations: fusions either with T25 or T18 fragments of CyaA at either the N' or C' terminals. Tube 1, T18-MecR2::MecI-T25; tube 2, T18-MecR2::T25-MecI; tube 3, MecR2-T18::MecI-T25; tube 4, MecR2-T18::T25-MecI; tube 5, MecR2-T25::T18-MecI; tube 6, T25-MecR2::T18-MecI; tube 7, MecR2-T25::MecI-T18; tube 8, T25-MecR2::MecI-T18; tube 9, positive control provided by the manufacturer (Zip-T25::Zip-T18); tube 10, negative control (T25::T18); tube 11, "in-house" positive control testing the MecI-MecI interaction T18-MecI::T25-MecI**. (B)** Electrophoretic mobility shift assay (EMSA) of the binding of purified MecI to a labeled 212bp DNA fragment encompassing the *mecA* promoter in the presence of purified MecR2. MecI concentration was constant in all binding reactions (0.05 μg). Lane 1, negative control, labeled DNA only; lane 2, 8-fold excess of MecI; lane 3, 4-fold excess of MecI; lane 4, binding control, MecI only; lane 5, 2-fold excess of MecI; lane 6, equimolar amounts of MecI and MecR2; lane 7, 2-fold excess of MecR2; lane 8, control for specific binding, MecI with a 125 molar excess of unlabelled DNA. **(C)** Western blotting analysis of MecI cleavage in total protein extracts (60-80 mg/lane). Lane 1, prototype strain N315; lane 2, *mecR2* null-mutant (N315::Δ*mecR*2); lane 3, strain HT0350 co-overexpressing MecI and MecR2 (HT0350+*mecImecR2*); lane 4, strain HT0350 overexpressing MecI (HT0350+*mecI*). Cultures of N315 and N315::Δ*mecR2* cultures were induced with a sub-MIC concentration of oxacillin (0.05mg/L).

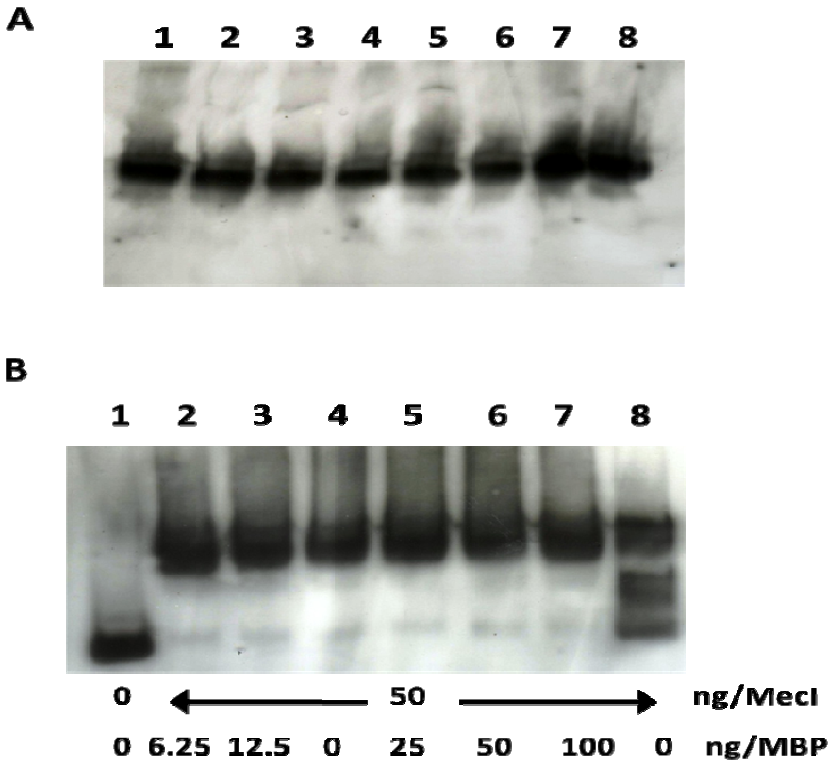

Figure 2.14 - Control experiments for the electrophoretic mobility shift assays. **(A)** Binding of purified MecR2 to *mecA* promoter. Lane 1, negative control, labeled DNA only; lane 2, 0.001 µg of MecR2; lane 3, 0.01 µg of MecR2; lane 4, 0.05 µg of MecR2; lane 5, 0.1 µg of MecR2; lane 6, 0.25 µg of MecR2; lane 7, 0.5 µg of MecR2; lane 8, 1 ug of MecR2. **(B)** Binding of purified MecI to the labeled *mecA* promoter DNA sequence in the presence of MBP (Maltose-binding protein) at several molar ratios. MecI concentration was constant in all binding reactions (0.05 µg). Lane 1, negative control, labeled DNA only; lane 2, 8-fold excess of MecI; lane 3, 4-fold excess of MecI; lane 4, binding control, MecI only; lane 5, 2-fold excess of MecI; lane 6, equimolar amounts of MecI and MBP; lane 7, 2-fold excess of MBP; lane 8, control for specific binding, MecI with a 125 molar excess of unlabelled DNA.

2.3.9 MecR2 promotes the proteolytic cleavage of MecI

Based on structural data, it has been suggested that the proteolysis of MecI observed during *mecA* induction is not mediated by the activated MecR1 inducer domain and instead, is a secondary event not required for induction [45-47]. This is in agreement with what has been found for the β-lactamase system of *Bacillus licheniformis* [48]. We speculated that MecR2, by interacting with MecI and disturbing its binding to the *mecA* promoter, could foster a local melting of MecI-dimers, making the

scissile bonds more accessible to proteolytic inactivation. To test this hypothesis, we compared by Western blotting the MecI proteolysis in total protein extracts from prototype strain N315 *versus* its *mecR2* null-mutant under induction conditions, and from strain HT0350 overexpressing MecI-MecR2 *versus* HT0350 overexpressing MecI only. As illustrated in Figure 2.13C, in the absence of MecR2, intact MecI accumulates in both pairs of strains analyzed. Because parental strain HT0350 is negative for all *mecA* regulators and its derivatives used in these experiments overexpress constitutively MecI-MecR2 or MecI, the observed MecR2-induced proteolysis of MecI does not involve MecR1 (neither BlaR1) and, most likely, is mediated by unspecific cytoplasmatic proteases.

2.4 MecR2, the missing link in the signal-transduction mechanism of *mecA* expression

The findings described in this report clarify some critical aspects of the unique signal transduction mechanism underlying the induction of *mecA* gene.

First, we demonstrated that the cognate *mecA* regulatory locus contains, besides MecR1-MecI, the anti-repressor MecR2. MecR2 compensates for the inefficient MecR1-mediated induction of *mecA*, being essential for the optimal expression of β-lactam resistance (Figures 2.6, 2.7 and 2.11A), and enabling the full induction of *mecA* transcription (Figures 2.8 and 2.11B). These findings explain the puzzling observation of the poor *mecA* induction by MecR1, reported in studies analysing the effects of *mecR1-mecI* only (without *mecR2*) on *mecA* expression in recombinant strains [12, 13]; an experimental artefact also observed in this study with recombinant strain COL::RI (artificially made positive for *mecR1-mecI* only) and in the *mecR2* null mutant strains (Figures 2.7 and 2.11). Because wild-type MRSA strains positive for *mecR1-mecI* are also positive for *mecR2*, these strains are in fact able to express optimal levels of β-lactam resistance and, as such, *mecA* is efficiently induced upon exposure to β-lactams by its cognate three-component regulatory system.

Second, the findings herein described also clarify the relevance and specificity of MecI proteolysis observed upon induction with β-lactams [16, 45-48]. Our data demonstrates that MecI proteolysis is required for optimal expression of resistance and that MecR2 alone (i.e. without MecR1, Figure 2.12A) interferes specifically with the MecI function and promotes its inactivation by proteolytic cleavage, presumably mediated by (non-specific) native cytoplasmatic proteases (Figure 2.13C). Our findings contrast with published observations for the *blaR1-blaI* system of *Bacillus licheniformis,* demonstrating that the proteolysis of the repressor is a secondary event not required for induction of resistance [48], and also for the *blaR1-blaI* system of *S. aureus* demonstrating that BlaR1 directly promotes the BlaI cleavage [15]. Altogether, these observations suggest the existence of subtle differences between the *mecR1-*

mecI-mecR2 and the *blaR1-blaI* regulatory systems and that, in *S. aureus*, BlaR1 may accumulate the MecR1 and MecR2 functions.

Our findings lead us to propose a revised model for the induction of *mecA* expression in wild-type MRSA strains (Figure 2.15): (i) in the presence of β-lactams, MecR1 is activated and induces the transcription of *mecA* and *mecR1-mecI-mecR2*; (ii) the anti-repressor MecR2, destabilizes MecI-dimers, disturbing their binding to the *mecA* promoter and fostering their proteolytic inactivation, resulting in a sustained induction of *mecA* transcription; (iii) when depletion of β-lactam occurs, MecR1 is no longer activated and a steady state is established consisting of stable MecI-dimers bound to the *mecA* promoter (and protected from proteolysis) and residual copies of MecR1 at the cell membrane; the remaining free MecR2 molecules are most likely degraded by the cellular protein turnover pathway. This model implies that in the absence of MecR2, functional MecI-dimers are more resilient to proteolytic inactivation and outcompete the MecR1 signalling, a hypothesis supported by the Western blotting experiments (Figure 2.13C).

2.5 Concluding remarks

This study demonstrates that the central element of methicillin-resistance in *S. aureus*, the *mecA* gene, can be regulated by a three-component system consisting of a transcriptional repressor, a sensor-inducer and an anti-repressor, a very unusual arrangement for the transcriptional control of genes in bacteria. In addition, the induction of the resistance gene expression involves a unique series of proteolytic steps, being the proteolytic cleavage of the repressor modulated by the anti-repressor.

This study also sheds light on the evolution of antibiotic-resistance genes. The *mecA* gene itself is probably ancient and predates the use of antibiotics in clinical practice [49, 50]. Before its recent acquisition by MRSA, *mecA* was assembled into a gene complex containing its transcriptional regulators and incorporated into a mobile genetic element. Tsubakishita *et al*. have proposed that the *mecA* gene complex found in MRSA has been assembled in the animal-related *Staphylococcus fleurettii* species [51]. Remarkably, in this species the *mecA-mecR1-mecI* locus was found immediately upstream to the complete and functional xylose operon, containing the XylR repressor homologous to MecR2. This suggests that a specific selection acted on XylR, a transcriptional repressor of sugar metabolism, to originate the MecR2 function, an anti-repressor of an antibiotic-resistance gene, and that the three-component *mecA* regulatory locus was assembled in *S. fleuretti* before being transferred to *S. aureus.*

In short, this study points to a revision of the model for the transcriptional control of *mecA* by its cognate regulatory locus, which may pave the way for the design of alternative therapeutic strategies targeting the induction mechanism of the resistance gene [52, 53]. If successful, these strategies may extend the clinical utility of β-lactams for the treatment of MRSA infections. Recycling β-lactams is

particularly relevant given that MRSA pose a substantial burden for the public health, are often multi-drug resistant and, in the past 40 years, very few new classes of antibiotics have reached the clinic.

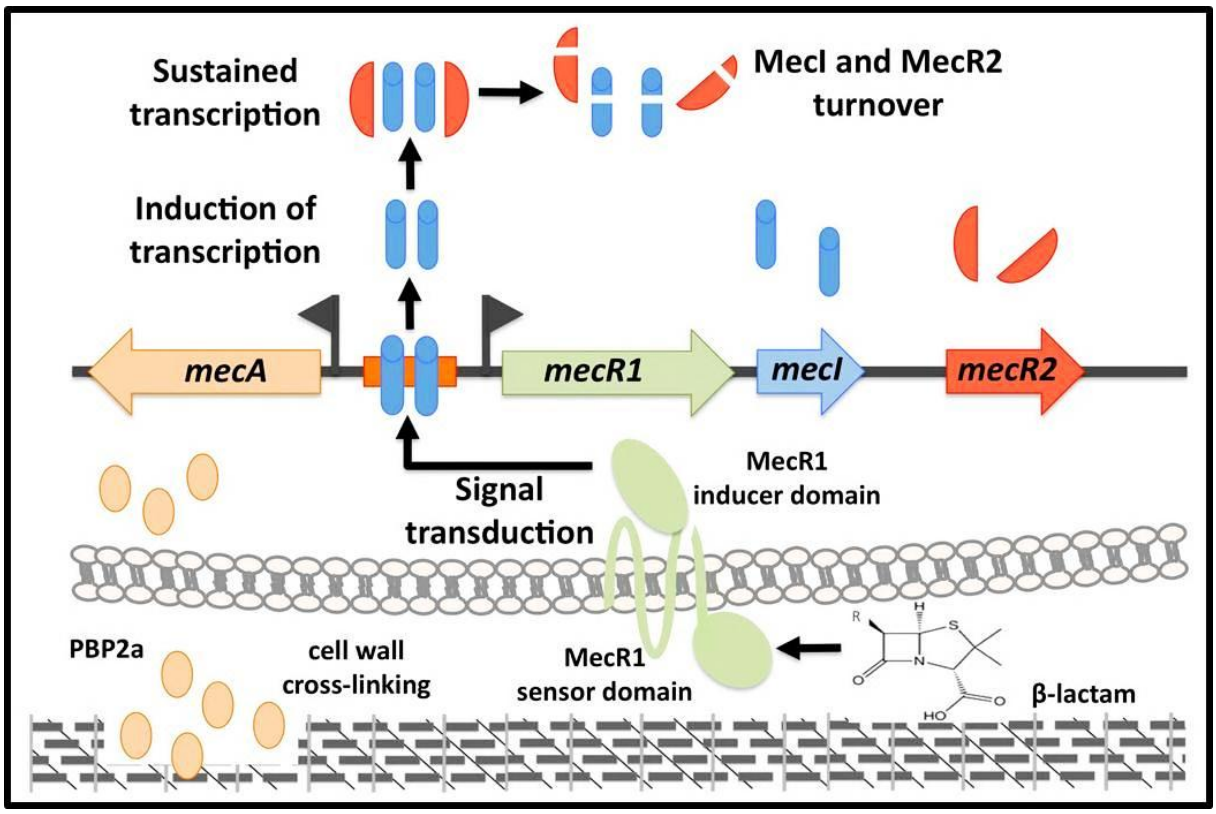

Figure 2.15 - Model for the *mecA* induction by MecR1-MecI-MecR2. In the presence of a β-lactam antibiotic, MecR1 is activated and rapidly induces the expression of *mecA* and *mecR1-mecI-mecR2*. The anti-repressor activity of *MecR2* is essential to sustain the *mecA* induction since it promotes the inactivation of MecI by proteolytic cleavage. In the absence of β-lactams, MecR1 is not activated and a steady state is established with stable MecI-dimers bound to the *mecA* promoter and residual copies of MecR1 at the cell membrane.

2.6 Materials and Methods

2.6.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2, respectively. *S. aureus* strains were routinely grown at 37°C with aeration in tryptic soy broth (TSB, Difco) or on tryptic soy agar plates (TSA, Difco). *E. coli* strains were grown with aeration at 37°C in Luria-Bertani broth (LB, Difco) or in Luria-Bertani agar (LA, Difco). Recombinant *E. coli* strains were selected and maintained with ampicillin at 100μg/mL. Recombinant *S. aureus* strains were selected and maintained either with tetracycline at 5 or 40μg/mL, chloramphenicol at 20μg/mL, or erythromycin at 10μg/mL, as appropriate. Phenotypic analysis of β-lactam resistance in *S. aureus* parental and recombinant strains was performed by diffusion-disks containing 1mg of oxacillin, oxacillin E-test (AB Biodisk), or by population analysis profiles (PAPS) at 30°C for 24-48h, as previously described [24, 54, 55]. Oxacillin is a methicillin analogue and has replaced methicillin in clinical use.

2.6.2 DNA manipulations

DNA manipulations were performed by standard methods [56, 57]. Total DNA from *S. aureus* was isolated from bacterial cultures with the Wizard Genomic DNA purification Kit (Promega) according to the manufacturer's recommendations and using lysostaphin (0.5 mg/mL) and RNAse (0.3 mg/mL) in the lysis step. Plasmid DNA was isolated from bacterial cultures with the High Pure Plasmid Isolation Kit (Roche). For plasmid DNA isolation from *S. aureus* strains the culture pellets were resuspended in "Suspension Buffer" supplemented with 0.1 mg/mL of lysostaphin and incubated at 37°C for 30-60 minutes. Restriction enzymes were used as recommended by the manufacturer (New England Biolabs). Dephosphorylation of vector arms and insert ligation was performed with Rapid DNA Dephos & Ligation kit (Roche), according to the manufacturer's recommendations. Routine PCR was performed with GoTaq Flexi DNA polymerase (Promega). PCR amplification of cloning inserts was performed by high-fidelity PCR (*Pfu* Turbo DNA polymerase, Strategene). DNA purification from PCR and digestion reactions was performed with High Pure PCR Product Purification Kit (Roche). For ligation protocols, the inserts and linearized plasmids were resolved in a low melting agarose gel (1%) (Invitrogen) and DNA bands were purified with Gene Clean Turbo kit (MP Biomedicals), following the manufacturer's recommendations. DNA sequencing was performed by Macrogen (www.macrogen.com) or STAB Vida (www.stabvida.com). All primers used in this study are listed in Table 2.3.

Table 2.1 - Strains used in this study

Strain	Relevant characteristics	Source /Reference
E. coli DH5α	Recipient strain for recombinant plasmids	
E. coli Bl21 (DE3)	Recipient strain for expression vector pCri8a	Novagene
S. aureus RN4220	Restriction-deficient derivative of reference strain NCTC8325-4	R. Novick
S. aureus COL	Prototype MRSA strain, homogeneous Oxa^r, Δ*mecR1* (no C-terminal inducer domain, ΨIS*1272* insertion), *mecI* negative, *mecR2* negative, β-lactamase negative, clone ST250-I	Stratagene A. Tomasz [29]
S. aureus N315	Prototype MRSA strain, heterogeneous Oxa^r, wild-type *mecR1-mecI*, *mecR2* positive, β-lactamase positive, clone ST5-II	K. Hiramatsu [18, 24]
S. aureus HU25	Homogeneous Oxa^r, wild-type *mecR1*, truncated *mecI*, *mecR2* positive, β-lactamase positive, clone ST239-III	[24, 58]
S. aureus USA100	Epidemic MRSA strain NRS382, complete *mecR1-mecI* locus, *mecR2* positive, β-lactamase positive, clone ST5-II	[39]
S. aureus USA200	Epidemic MRSA strain NRS383, complete *mecR1-mecI* locus, *mecR2* positive, β-lactamase positive, clone ST36-II	[39]
S. aureus USA600	Epidemic MRSA strain NRS387, complete *mecR1-mecI* locus, *mecR2* positive, β-lactamase positive, clone ST45-II	[39]
S. aureus HT0350	Heterogeneous Oxa^r, deleted *mecR1-mecI* (IS*431* insertion), β-lactamase negative, clone ST377-V	J. Étienne [41]
COL + pGC::*mecI*	COL overexpressing *mecI in trans*	[24]
COL + pGC::*mecI-mecR2*	COL co-overexpressing the *mecI-mecR2* locus *in trans*	This study
COL::*erm*	COL with *erm* gene inserted into the chromosome upstream to the *mecI* gene (control)	This study
COL::RI	COL with the *mecR1-mecI* locus inserted into the chromosome upstream to *mecI* gene	This study
COL::RI-R2	COL with the *mecR1-mecI-mecR2* locus inserted into the chromosome upstream to *mecI* gene	This study
COL::RI-R2 + pGC::*mecI*	COL::RI-R2 overexpressing *mecI in trans*	This study
N315::Δ*mecR2* mut	N315 *mecR2* null mutant intermediate, β-lactamase negative	This study
N315::Δ*mecR2*	N315 *mecR2* deletion backcross, β-lactamase positive	This study

Table 2.1 Strains used in this study (cont.)

N315::Δ*mecR2* + pSPT::*spac*	N315::Δ*mecR2* transformed with pSPT181 containing the P*spac* inducible promoter (control)	This study
N315::Δ*mecR2* + pSPT::*mecR2*	N315::Δ*mecR2* overexpressing *mecR2 in trans*	This study
N315::Δ*mecR2* + pSPT::*mecI-mecR2*	N315::Δ*mecR2* overexpressing *mecI* and *mecR2 in trans*	This study
N315::Δ*mecR2* + pSPT::*spac-mecR2*	N315::Δ*mecR2* expressing *mecR2 in trans* from the inducible *Pspac* promoter	This study
COL::RI + pSPT::*spac-mecR2*	COL-IR expressing *mecR2 in trans* from the inducible *Pspac* promoter	This study
USA100::Δ*mecR2*	USA100 *mecR2* null mutant, β-lactamase positive	This study
USA100::Δ*mecR2* pSPT::*spac-mecR2*	USA100::Δ*mecR2* expressing *mecR2 in trans* from the inducible *Pspac* promoter	This study
USA200::Δ*mecR2*	USA200 *mecR2* null mutant, β-lactamase positive	This study
USA200::Δ*mecR2* pSPT::*spac-mecR2*	USA200::Δ*mecR2* expressing *mecR2 in trans* from the inducible *Pspac* promoter	This study
USA600::Δ*mecR2*	USA600 *mecR2* null mutant, β-lactamase positive	This study
USA600::Δ*mecR2* pSPT::*spac-mecR2*	USA600::Δ*mecR2* expressing *mecR2 in trans* from the inducible *Pspac* promoter	This study
HT0350 + pSPT::*mecI*	HT0350 overexpressing *mecI in trans*	This study
HT0350 + pSPT::*mecI-mecR2*	HT0350 co-overexpressing *mecI* and *mecR2 in trans*	This study
DH5α + pProEX::*mecI*	*E. coli* DH5α overexpressing *mecI*	This study
BL21 + pCri8a::*mecR2*	*E. coli* BL21 (DE3) overexpressing *mecR2*	This study

Table 2.2 - Plasmids used in this study

Strain	Relevant characteristics	Source/Reference
pGC-2	*E. coli - S.aureus* shuttle vector, high-copy number, insert expression driven by bacteriophage promoters SP6 and T7, Ap^r, Cm^r	P. Matthes
pSPT181 (ts)	*E. coli - S. aureus* shuttle vector, thermosensible, insert expression driven by bacteriophage promoter SP6, Ap^r, Tc^r	[59]
pSP64E	*E. coli* pSP64 vector with a 1.2 kb *Bam*H1-*Sal*I fragment containing the *erm* gene from Tn551 (integrative vector in *S. aureus*), Ap^r, Ery^r	[60]
pDH88	*E. coli - B. subtilis* shuttle vector containing the IPTG inducible P*spac* promoter and the transcriptional repressor LacI, Ap^r, Cm^r	[61]
pSPT::*spac*	pSPT181 with 1.6 kb EcoR1-BamH1 fragment containing the IPTG inducible P*spac* promoter and the transcriptional repressor LacI from pDH88, Ap^r, Tc^r	This study
pCri8a	pET30 (Invitrogen) derivative containing His6-GST-Tev fragment, Kan^r	[62]
pGC::*mecI*	pGC2 with *mecI* gene from strain N315	[24]
pGC::*mecI-mecR2*	pGC2 with mecI gene and the *mecR2* locus from strain N315	This study
pSPT::IS-*erm*	pSPT181 with a 0.6 kb fragment of IS*1272* and a 1.2 kb *Bam*H1-*Sal*I fragment containing the *erm* gene from pSP64E	This study
pSPT::IS-*erm*-Δ*mecR1*	pSPT::IS-*erm* with a 0.5 kb fragment of the N-terminal domain of *mecR1*	This study
pSPT::IS-*erm*-*mecI-mecR1*	pSPT::IS-*erm* with a 1.9 kb fragment containing *mecI-mecR1* from strain N315	This study
pSPT::IS-*erm*-*mecR2-mecI-mecR1*	pSPT::IS-*erm* with a 3.5 kb fragment containing *mecR2-mecI-mecR1* from strain N315	This study
pSPT::*cat*-Δ*mecR2*	pSPT181 vector containing the chloramphenicol acetyl transferase (Cm^r) from pGC-2 flanked by 1.0 kb up and downstream vicinities of *mecR2*	This study
pSPT::*mecR2*	pSPT181 vector containing the *mecR2* gene from strain N315	This study
pSPT::*mecI*	pSPT181 vector containing the *mecI* gene from strain N315	This study
pSPT::*mecI-mecR2*	pSPT181 vector containing the *mecI* and *mecR2* genes from strain N315	This study
pSPT::*spac-mecR2*	pSPT181 vector containing the *mecR2* gene from strain N315 under control of the P*spac* inducible promoter	This study
pProEX::*mecI*	Expression vector $pP_{RO}EX^{TM}$ Hta (Invitrogen) with His_6 tag N-terminal fusion to *mecI* gene from strain N315, Ap^r	[63]
pCri8a::*mecR2*	pCri8a with *mecR2* gene from strain HU25	This study

Table 2.3 - Primers used in this study

Name	Sequence (5' → 3') *
MA-P1	AAATCGATGTAAAGGTTGGC
MA-P2	GTTCTG CAG TAC CGGATT TG
MA-RT1	AACATTGATCGCAACGTTCAAT
MA-RT2	TGGTCTTTCTGCATTCCTGGA
MA-PF1	ATA TCG TGA GCA ATG AAC TG
MA-PR1	TAT ATA CCA AAC CCG ACA AC
MI-P1	TATAGAATTCGCACAACAAATTTCTGAGCG
MI-P2	GATCGGATCCATGCATATGGATTTCACTGG
MI-P3	TCTAGGATCCTCAACGACTTGATTGTTTC
MI-P4	TAATCTGCAGCACAACAATTTTCTCAG
MI-P5	GCGGTTTCAATTCACTTGTC
MI-P6	TGGTTTTTGGACTCCAGTCC
MI-BTH1	TATATCTAGAGGATAATAAAACGTATGAAATATCATCTGC
MI-BTH2	TCTAGGTACCCGTTTATTCAATATATTTCTCAATTCTTCTATTTC
MI-Box1	TTGACATAAATACTACATTTGTAATATACTACAAATGTA
MI-Box2	AGACTACATTTGTAG TATATTACAAATGTAGTATTT ATG
MR-P1	TATACCCGGGAAAGTTCGTCATTGGAATCG
MR-P2	GATCGGATCCATACGCTTGTTTCGATTAGG
MR-P3	GCACTTTATGATTCAATGCC
MR2-P1	GTTAGGATCCGCTATCAACATTTACCAGCA
MR2-P2	TATAGTCGACCAAAATACTAGAAATCGTTGCC
MR2-P3	TAATCTGCAGCACAACAATTTTCTCAG
MR2-P4	TATAGGATCCTGCTGGTAAATGTTGATAGC
MR2-P5	TAATCTCGAGTTAGAAGTCTTACACACTCC
MR2-P6	ATTACCCGGGCTATCAACATTTACCAGCA
MR2-P7	TATACCCGGGTATGGGGTAGGCAATTATGG
MR2-P8	CTACTAACCTTTTCATCAGG
MR2-RT1	AATGAAGCGAATCTTTCAGC
MR2-RT2	AATTGCTAATGTACCACCTAGC
MR2-RT3	CCATTCCATGAAACTGAAGG
MR2-RT4	AACGCTGAAAGATTCGCTTC
MR2-BTH1	TCTATCTAGAGTACAATTATTTTGATGGTAATGTC
MR2-BTH2	TCTAGGTACCCGTGCTTTTATATCTAAGTAAATATCATTAATCG
MR2-Cri1	TTAATACGCCATGGTTGATAAAAAAGAGTGCTAAGG
MR2-Cri2	GGATCCTCGAGCTATTATGCTTTTATATCTAAG
IS1272-P1	TATACTGCAGATGATTGTTCAGAATGTCC
IS1272-P2	TATAGTCGACAAGAGTTAAGAGCCATTGC
CAT-P1	TAATCTGCAGAAGAAAGCAGACAAGTAAGC
CAT-P2	TGTAGTCGACAAACCTTCTTCAACTAACGG
pta-RT1	AGAAGCAATCATTGATGGCGA
pta-RT2	ACCTGGCGCTTTTTTCTCAG
MI-YTH	TGGACCATGGATAATAAAACGTATGAAATATCATCTCC
MR2-YTH	TCGACCATGGCATACAATTATTTTGATGGTAATGTC
GAL4-BD	GAAGAGAGTAGTAACAAAGG
GAL4-AD	TATTCGATGATGAAGATACC

* Restriction sites are underlined. For primers MI-Box1/MI-Box2 the MecI protected sequences are underlined.

2.6.3 Construction of recombinant *S. aureus* strains

All recombinant plasmids used in this work were firstly constructed and stabilized in *E. coli* DH5α, electroporated into *S. aureus* restriction-deficient strain RN4220 and finally transduced by the 80α phage to the desired parental strain, as previously described [64, 65]. The Integrity of plasmid inserts was confirmed by restriction analysis, PCR and DNA sequencing. The integrity of chromosomal insertion-deletions was confirmed by PCR, DNA sequencing and Southern blotting of pulsed-field gel electrophoresis of chromosomal DNA. Chromosomal insertion-deletions were backcrossed by phage transduction to the original parental strains.

To co-overexpress *mecI* and *mecR2* in strain COL, a fragment containing the *mecI-mecR2* region from strain N315 was amplified using primers MI-P1/MR2-P1, double-digested with EcoR1/BamH1 and cloned into pGC2, originating the recombinant plasmid pGC::*mecI-mecR2*. To reconstruct the *mecA* regulatory locus in the chromosome of strain COL, we first construct pSPT::IS-*erm*, a pSPT181 derivative containing the terminal fragment from IS*1272* located in the upstream vicinity of *mecA* in strain COL and the erythromycin (*erm*) resistance cassette gene from Tn*551*. The 0.6 kb terminal fragment of IS*1272* was amplified from strain COL using primers IS1272-P1/IS1272-P2, double-digested with PstI/SalI and cloned into pSPT181, originating pSPT::IS. The *erm* cassette was recovered from the pSP64E plasmid by BamHI/SalI double-digestion and was cloned into pSPT::IS, originating pSPT::IS::*erm.* To reconstruct the *mecR1-mecI* locus in strain COL (strain COL::RI), we amplified by high-fidelity PCR a 1.9 kb DNA fragment from strain N315 chromossomal DNA, containing the wild-type coding sequences of *mecR1* and *mecI* genes, using primers MI-P2/MR1-P1. The fragment was double-digested with BamH1/AvaI and directionally cloned into pSPT::IS-*erm*, originating pSPT::IS-*erm-mecI-mecR1*. To reconstruct the full *mecA* regulatory locus in strain COL (strain COL::RI-R2), we amplified by high-fidelity PCR a 3.5 kb DNA fragment from strain N315 chromossomal DNA, containing the *mecR1-mecI-mecR2* locus, using primers MR2-P1/MR1-P1. The fragment was double-digested with BamH1/AvaI and directionally cloned into pSPT::IS-*erm*, originating pSPT::IS-*erm-mecR2-mecI-mecR1*. As control, we constructed a strain with a integrated *erm* gene in the *mecA* upstream vicinity (strain COL::*erm*): a 0.5 kb DNA fragment containing the terminal fragment of the N-terminal cytoplasmatic domain of *mecR1*, was amplified using primers MR1-P1/MR1-P2, double-digested with BamH1/AvaI and cloned in pSPT::IS-*erm*, originating pSPT::IS-*erm*-Δ*mecR1*. The integration into COL chromosome of the three recombinant plasmids (pSPT::IS-*erm-mecI-mecR1,* pSPT::IS-*erm-mecR2-mecI-mecR1* and pST::IS-*erm*-Δ*mecR1*) was performed by an insertion-deletion strategy by homologous recombination (Figure 2.5). First, insertion into the chromosome was promoted by growing transductants in TSB at a non-permissive temperature (45°C) without antibiotic selection for 2-3 days, with daily re-inoculum in fresh medium. Serial dilutions were plated onto TSA plates supplemented with erythromycin (Ery) and tetracycline (Tc). Single-colonies Erm^r-

Tc^r were screened for chromosomal insertion of the plasmids by PCR and the absence of plasmid DNA was confirmed. Resolution of integrates by homologous-recombination was promoted by growing selected single colonies in TSB supplemented with tetracycline at 40μg/mL at the permissive temperature of 30°C for 4-5 days, with daily re-inoculum in fresh medium. Finally segregation of the excised plasmids was promoted by growing cultures at 45°C without antibiotic selection for 2-3 days, with daily re-inoculum in fresh medium. Culture aliquots were plated onto TSA plates supplemented with erythromycin and single colonies Erm^r-Tc^s were selected by replica plating onto TSA plates supplemented with erythromycin or tetracycline.

To construct the *mecR2* gene null mutant in strain N315, two DNA fragments of 1000bp corresponding to the 5' and 3' vicinities of the *mecR2* gene were amplified by PCR from strain N315 DNA using primers MR2-P2/MR2-P3 and MR2-P4/MR2-P5, respectively. The *cat* gene coding for chloramphenicol resistance was also amplified by PCR from pGC2 plasmid with primers CAT-P1/CAT-P2. The three fragments were double-digested with SalI/PstI, BamH1/XhoI and XhoI/SalI, respectively, and then sequentially cloned into pSPT181, originating the pSPT::*cat*-Δ*mecR2* recombinant plasmid. Following the same insertion-deletion strategy described above, but selecting for chloramphenicol resistance instead of erythromycin resistance, we obtained the recombinant strain N315::Δ*mecR2* in which the chromosomal copy of *mecR2* was replaced by the *cat* gene (Figure 2.5). To complement the N315Δ*mecR2* null-mutant three recombinant plasmids were constructed: (i) pSPT::*mecR2*, pSPT181 vector containing at the XmaI site the *mecR2* gene from strain N315, obtained by PCR with primers MR2-P6/MR2-P7 (the proper insert orientation was selected by restriction analysis using the HindIII site within *mecR2* gene); (ii) pSPT::*mecImecR2*, pSPT181 containing the *mecI-mecR2* genes of strain N315, constructed by sequential cloning, first, at the BamH1/PstI, the *mecI* gene site obtained with primers MI-P3/MI-P4 and then, at the XmaI site, the *mecR2* gene obtained with primers MR2-P6/MR2-P7; (iii) pSPT::*spac-mecR2*, pSPT181 with the *mecR2* gene under the control of *Pspac* promoter, constructed by sequential cloning the 1.6kb EcoR1-BamH1 fragment from plasmid pDH88 containing the *spac* locus (P*spac*-polylinker-*lacI* repressor) and then, at the XmaI site of the *spac* polylinker, the *mecR2* gene from strain N315 obtained with primers MR2-P6/MR2-P7. As control, N315Δ*mecR2* was transformed with a pST181 derivative containing the *spac* locus only (pSPT::*spac*).

To generate the *mecR2* gene null mutant in prototype strains USA100, USA200, USA600, and HU25 the chromosomal deletion of strain N315::Δ*mecR2* was transduced by bacteriophage infection with selection for chloramphenicol resistance, originating recombinant strains USA100::Δ*mecR2*, USA200::Δ*mecR2,* USA600::Δ*mecR2* and HU25::Δ*mecR2*. Mutant strains USA100::Δ*mecR2*, USA200::Δ*mecR2* and USA600::Δ*mecR2* were then complemented with recombinant plasmid pSPT::*spac-mecR2*.

2.6.4 Transcription analysis

Total RNA extraction and purification was performed as previously described [66]. Briefly overnight cultures were grown in TSB, supplemented with antibiotics when appropriate, and then diluted 1:50 in fresh TSB and grown to the mid-log phase (OD_{620}~0.7). Cultures were stabilized with two volumes of RNAprotect Bacteria Reagent (Qiagen), according to the manufacturer's recommendations. The cells were centrifuged and pellets were ressuspended in 1 mL of Trizol reagent (Invitrogen). The resuspended cells were transferred to a new tube with silica beads (Lysing Matrix B tubes, Bio101) and cell lysis was performed in the FastPrep FP120 apparatus (Bio 101). RNA was extracted with chloroform, precipitated with isopropanol, washed twice with ethanol at 80% and rssuspended in diethyl pyrocarbonate (DEPC)-treated water. For the analysis of the *mecA* and *mecR2* induction profiles, after cultures were grown to OD_{620}~0.7, oxacillin at 0.05 μg/mL was added and cultures were incubated for an additional 60 minutes. Samples were taken either at 0, 5, 15, 30, and 60 or at 0, 10 and 60 minutes, stabilized, pelleted and kept on ice until being simultaneously processed. For RT-PCR and qReal-time RT-PCR experiments (see below), total RNA preps were treated twice with DNAse (RNase-Free DNase Set I, Qiagen) and purified with RNeasy Mini Kit (Qiagen), according to the manufacture's recommendations. Control PCR reactions were performed to test the absence of DNA contamination in total RNA preps.

Transcription analysis of *mecR1-mecI-mecR2* was performed by RT-PCR for mid-log phase induced cultures (oxacillin at 0.05μg/mL) of strains N315 and HU25 with primer pairs MR2-RT1/MR2-RT2 (*mecR2* transcript), MI-P5/MR2-P8 (*mecI-mecR2* co-transcript), MR1-P3/MI-P6 (*mecR1-mecI* co-transcript), and MA-P1/MA-P2 (*mecA* transcript, inducible positive control). RT-PCR reactions were set-up using the One-Step RT-PCR kit (Qiagen), according to the manufacture's recommendations. To control the absence of DNA contamination, all samples were tested in a parallel reaction without the reverse-transcription step. To control the size of the amplified transcripts, PCR reactions with chromosomal DNA were also performed in parallel. The *mecA* transcript was detected in both induced and non-induced samples suggesting that the RT-PCR assay was too sensitive to discriminate between basal and induced transcription levels.

The induction profiles of *mecA* and *mecR2* were determined by quantitative Real-time RT-PCR (qRT-PCR) and/or Northern blotting. For the qRT-PCR data analysis, relative gene expression was expressed as a ratio to the transcript of *pta*, a housekeeping gene with constitutive expression [67]. Standard curves were generated using serial dilutions (0.4-40 ng/reaction) of genomic DNA and primers MR2-RT3/MR2-RT4, MA-RT1/MA-RT2 and pta-RT1/pta-RT2 for amplification internal fragments of *mecR2*, *mecA*, and *pta*, respectively. qRT-PCR reactions were performed with QuantiTect SYBR Green RT-PCR Kit (Qiagen); each 25μl reaction containing 12.5μl SybrGreen mix, 0.25μl RT enzyme mix, 12.5 pmol of each primer and 40 ng of purified RNA. Amplification consisted of an initial RT step at 50°C for 30 min, followed by a denaturation step at 95°C for 15 min, then by 45 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C. For each RNA sample three independent qReal-Time RT-PCR experiments were

carried out. Fluorescence was measured at the end of the annealing-extension phase of each cycle. A threshold value for the fluorescence of all samples was set manually. The reaction cycle at which the PCR product exceeds this fluorescence threshold was identified as the threshold cycle (C_T). The C_T was then converted to relative quantity of mRNA by using a standard curve. To verify the specificity of the PCR amplification products, melting curve analysis was performed between 60 – 95°C.

For Northern blot analysis, total RNA (5 µg) was resolved through a 1.2% agarose-0.66M formaldehyde gel in MOPS (morpholine propanesulfonic acid) running buffer (Sigma). Blotting of RNA onto Hybond N+ membranes (Amersham) was performed with Turboblotter alkaline transfer systems (Schleicher & Schuell). For detection of *mecA* specific transcripts, a DNA probe was constructed by PCR amplification with primers MA-P1 and MA-P2. After purification the probe was labeled with a Ready To Go labeling kit (Amersham) by using [a-^{32}P]dCTP (Amersham) and was hybridized under high-stringency conditions. The blots were subsequently washed and autoradiographed.

2.6.5 Bacterial two-hybrid assays

This strategy is based on the restoration of the adenylate cyclase (CyaA) activity by heterodimerization of protein fusions containing the T25 and T18 fragments, which form the catalytic domain of CyaA. CyaA is involved on cAMP synthesis, which binds to CAP forming the cAMP/CAP complex that activates a specific reporter gene, *lacZ* [43]. All strains and plasmids used in the bacterial two-hybrid studies are described in Table 2.4. Both genes, *mecI* and *mecR2*, were amplified from the chromosomal DNA of strain N315 by high-fidelity PCR, using primers MI-BTH1/MI-BTH2 for *mecI* and MR2-BTH1/MR2-BTH2 for *mecR2*. PCR products were double-digested with KpnI/XbaI and fused to T25 or T18 fragments either at the N' or C' terminals, using plasmids pUT18, pUT18c, pKNT25 and pKT25, originating the following fusion proteins: T18-MecI, MecI-T18, T25-MecI, MecI-T25, T18-MecR2, MecR2-T18, T25-MecR2 and MecR2-T25. The eight MecI::MecR2 recombinant plasmid combinations were co-transformed into the reporter strain *Escherichia coli* BTH101 and grown on Luria-Bertani (LB) and LA agar supplemented with 8 µg/mL 5-bromo-4-choro-3-indolyl-β-D-galactopyranoside (X-gal), 50 µg/mL kanamycin, 100 µg/mL ampicillin, 100 µg/mL streptomycin, 500 µM (IPTG) and 2 % glucose. As a positive control, plasmids p25Zip and p18Zip, containing two leucine zipper domains, were also co-transformed into *E.coli* BTH101 strain. Additionally, as in-house controls, the previously reported MecI::MecI interaction based on the yeast two-hybrid strategy [44] was evaluated, as well the MecR2-MecR2 interaction, by co-transforming the four combinations of *mecI*-containing plasmids and the four combinations of *mecR2*-containing plasmids, respectively.

Table 2.4 - Strains and plasmids used in the bacterial two-hybrid assays

Strains/ Plasmids	Relevant characteristics	Source
E. coli BTH 101	Reporter strain, cya^-	[43]
pUT18	BTH plasmid; N-terminal *cyaA*-T18 fusion; Amp^r	[43]
pUT18c	BTH plasmid; C-terminal *cyaA*-T18 fusion; Amp^r	[43]
pKT25	BTH plasmid; C-terminal *cyaA*-T25 fusion; Kan^r	[43]
pKNT25	BTH plasmid; N-terminal *cyaA*-T25 fusion; Kan^r	[43]
p18Zip	BTH control plasmid; Amp^r	[43]
p25Zip	BTH control plasmid; Kan^r	[43]
pUT18::*mecI*	BTH plasmid containing *mecI*::*cyaA*-T18 fusion	This study
pUT18c::*mecI*	BTH plasmid containing *cyaA*-T18::*mecI* fusion	This study
pKT25::*mecI*	BTH plasmid containing *cyaA*-T25::*mecI* fusion	This study
pKNT25::*mecI*	BTH plasmid containing *mecI*::cyaA-T25 fusion	This study
pUT18::*mecR2*	BTH plasmid containing *mecR2*::cyaA-T18 fusion	This study
pUT18c::*mecR2*	BTH plasmid containing cyaA-T18::*mecR2* fusion	This study
pKT25::*mecR2*	BTH plasmid containing cyaA-T25::*mecR2* fusion	This study
pKNT25::*mecR2*	BTH plasmid containing *mecR2*::cyaA-T25 fusion	This study

2.6.6 Electrophoretic mobility shift assays (EMSA)

To overexpress and purify MecR2 protein, a *mecR2* gene insert was obtained from the chromosome of strain HU25 by high-fidelity PCR amplification with primers MR2-cri1/MR2-cri2 and double-digestion with NcoI/XhoI. The *mecR2* insert was then cloned in frame into the expression vector pCri8a, generating the recombinant plasmid pCri8a::*mecR2*, expressing *mecR2* with a N' terminal His_6 tag. pCri8a::*mecR2* was stabilized in *E. coli* DH5α and then transformed to *E. coli* Bl21 (DE3). MecR2 protein overexpression was carried out in LB medium supplemented with 50 mg/mL kanamycin, at 18°C, and induced at an OD_{600}~0.5 with 1 μM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5h. MecI protein was overexpressed from recombinant strain DH5α+pP_{RO}EX::*mecI* [63], using LB medium supplemented with 100 µg/mL ampicillin, at 37°C and induced at an OD_{600}~0.5 with 1 μM IPTG for 3h. Protein extracts were purified as previously described [63]. The purity of the proteins was assessed by 10% tricine SDS-PAGE analysis and mass-spectroscopy. The concentrations of purified MecR2 and MecI were estimated using the Protein Assay Kit II (BioRad), as recommended by the manufacturer. For the electrophoretic mobility shift assay we used the chemiluminescent-based DIG Gel Shift Kit (Roche), following the manufacturer's recommendations. As DNA target we used a 212bp fragment encompassing the *mecA*

promoter and operator sequences from prototype strain N315 obtained by PCR amplification with primers MA-PF1/MA-PR1. The binding of each purified protein to the *mecA* promoter (P*mecA*) was first evaluated and then MecI-MecR2 mixtures were tested. As control the binding to P*mecA* of MecI-MBP (Maltose-binding protein, MBP2*, New England Biolabs) mixtures were also evaluated. EMSA assays with MecI-MecR2 mixtures were also performed with a smaller 39bp DNA fragment containing the MecI protected sequences, obtained by annealing primers MI-Box1/MI-Box2.

2.6.7 Western blotting

To prepare protein extracts of *S. aureus*, parental and recombinant strains were grown in TSB supplemented with oxacillin at sub-MIC concentration (0.05μg/mL) until mid-log phase (OD_{620}~0.7). Cell pellets were frozen in liquid nitrogen, thawed and resuspended in Buffer A (50mM Tris-HCl; 10mM MgCl2; 0.5mM PMSF) containing 10μg/mL DNase I. Cells were broken mechanically in a French press followed by centrifugation (22,000 × g, 20 min, 4°C) to remove unbroken cells and cell debris. The supernatants containing the cytoplasmic proteins were recovered and filtered through 0.45-μm-pore-size membrane filters. Protein extracts (60-80 μg) were resolved in a 18% Tris-Glycine SDS-PAGE.

After electrophoresis, the proteins were transferred to a 0.45 μm nitrocellulose membrane (Trans-Blot, Bio-Rad). The membranes were blocked at room temperature for 1 hour, in 20 mL of Blocking solution - Tween- Phosphate Buffered Saline (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4; 1.47 mM KH2PO4; 0.05% Tween-20) with 6% low-fat milk. MecI protein was detected by imunoblot analysis using a custom polyclonal antibody (Eurogentec) raised against purified MecI (1/1.000 dilution) and a 1/50.000 dilution of secondary antibody (Goat Anti-Rabbit IgG (H+L) Peroxidase Conjugated Antibody, Pierce) in 10% Blocking solution. The immune complexes were detected using an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent; Pierce), according to the manufacturer's instruction. Membranes were exposed to Amersham Hyperfilm ECL film (GE Healthcare).

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CHAPTER III

Structure-function studies of the anti-repressor MecR2

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3.1 Abstract

Methicillin resistance in *Staphylococcus aureus* is elicited by a signal-transduction system consisting of the MecI-MecR1-MecA axis encoded by the mec divergon. Recently, MecR2 was further identified as the long-sought gene of mec-mediated antibiotic resistance. Here we show that MecR2 tightly but transiently binds the *mecA* repressor, MecI. The crystal structure of MecR2 reveals a three-domain architecture, with an N-terminal DNA-binding-like domain, an intermediate scaffold domain, and a C-terminal dimerization domain, which contributes to a functional dimeric oligomerization state. This architecture positions the two DNA-binding-like domains on the same face of the dimer, at distances a priori suited for recognition of the major groove of double-stranded DNA. The protein shows structural similarity to transcriptional repressors of the ROK (repressors, open reading frames, and kinases) family of proteins, which bind DNA and/or sugar molecules. However, purified MecR2 did not bind DNA in vitro and functional analysis in vivo of three mutants affecting residues participating in sugar binding in related ROK proteins, $E^{228} \rightarrow A$, $N^{178}E \rightarrow AA$, and $E^{248} \rightarrow A$, revealed no loss of function in an *in vivo* assay. Accordingly, MecR2 function in methicillin resistance solely consists in sequestering MecI away from its cognate promoter region, and this facilitates proteolytic inactivation by non-specific cytoplasmatic proteases.

3.2 Introduction

Staphylococcus aureus is the most prevalent human infective agent associated with hospital-borne and community infections due to its extraordinary capacity to become resistant to antibiotics: it was the first bacterial pathogen reported to become insensitive to penicillin [1-4]. Among the distinct strains is methicillin-resistant *Staphylococcus aureus* (MRSA), which currently refers to multidrug-resistant strains that are generally resistant to β-lactam antibiotics - penicillins and cephalosporins - but also to other chemotherapeutics such as aminoglycosides, glycopeptides, macrolides, lincosamides, and fluoroquinolones [3, 5-7]. MRSA is characterized by its ability to thrive due to the biosynthesis of a penicillin-binding protein with low susceptibility towards β-lactam antibiotics, termed PBP2a, PBP2' or MecA. The latter is encoded by gene *mecA*, which is contained in a transducible mobile element, staphylococcal chromosomal cassette *mec* (SCC*mec*) [8, 9]. SCC*mec* types II, III and VIII further includes two genes, *mecI* and *mecR1*, which encode, respectively, a transcriptional repressor, MecI, and an integral-membrane zinc-dependent sensor/signal transducer metalloproteinase, MecR1 [10]. This system is homologous to the *blaI-blaR1-blaZ* signal transduction system that triggers synthesis of a β-lactamase (BlaZ) in both MRSA and methicillin-susceptible *S. aureus*, as well as in *Bacillus licheniformis* [11-13]. The currently accepted working model hypothesis for these systems foresees that MecI/BlaI constitutively

represses its own biosynthesis and that of MecR1/BlaR1 and MecA/BlaZ through binding to the *mec* promoter [9, 14, 15]. Once MecR1/BlaR1 detects the presence of extracellular β-lactam antibiotic through its sensor domain, a signal is transmitted across the membrane to the intracellular zinc-dependent metalloproteinase domain, which becomes autoactivated through proteolytic cleavage [16]. This renders functional MecR1/BlaR1, which, in turn, would cleave MecI/BlaI. This cleavage would render the dimeric repressor inactive and release it from its DNA binding site. Finally, release of transcriptional repression would enable biosynthesis of MecA/BlaZ.

However, some lines of evidence are not explained by this ternary model: (i) the reported cleavage sites of MecR1/BlaR1 and MecI/BlaI are not compatible with a single proteolytic substrate specificity [17]; (ii) the structure of MecI and BlaI in complexes with target DNA revealed that the repressor cleavage site is found within an α-helix and is not surface accessible [17-19], as would be required for proper proteolytic processing; and (iii) highly-resistant MRSA strains did not show significant variation in the phenotypic expression of resistance when wild-type MecI was overexpressed *in trans* [20]. These and other findings led several authors to postulate the existence of a further regulatory element, MecR2/BlaR2, although no candidate molecules were suggested [4, 9, 15, 19, 21-25].

Most recently, comparative genomic sequence analysis revealed that, in some clinical MRSA strains, a putative gene is found upstream of *mecA*, which is co-transcribed with *mecI* and *mecR1* [26]. Its transcript could be detected by reverse-transcriptase PCR in oxacillin-induced cultures of two prototype clinical MRSA strains. Furthermore, in the presence of fully functional MecR1-MecI, this gene was essential to sustain the *mecA* induction readily induced by MecR1, enabling the optimal expression of β-lactam antibiotic resistance. Finally, *in vitro* and *in vivo* assays showed that the encoded protein acts as an anti-repressor by disturbing MecI binding to the *mecA* promoter and promoting its MecR1-independent inactivation proteolysis. Collectively, these findings indicated that the long-sought gene encoding MecR2/BlaR2 had been found, and so it was termed *mecR2* [26]. In order to shed light on the structural determinants of fold and function of MecR2, we developed an efficient protocol to produce large quantities of purified protein, assayed its binding capacity to MecI by bio-layer interferometry, solved its X-ray crystal structure, and report here the essential structural features, which were further cross-checked by mutational studies.

3.3 Results and Discussion

3.3.1 Large-scale recombinant heterologous overexpression and purification of MecR2

An efficient recombinant overexpression system for MecR2 in *Escherichia coli* was developed, and subsequent two-step chromatographic purification enabled to obtain large amounts of pure protein.

The protein was well folded as suggested size-exclusion chromatography and proved suitable for structural and functional studies.

3.3.2 Biological activity of purified MecR2

Previously, the effects of *mecR2* on the optimal expression of oxacillin-resistance were studied using the short variant present in prototype strain N315 [26]. This variant lacks the putative N-terminal DNA binding domain present in the MecR2 variant of prototype strain HU25, used here for the resolution of the MecR2 tri-dimensional structure. In order, to confirm the biological activity of this larger MecR2 variant together with the additional N-terminal sequences containing the His_6-tag and the TEV protease cleavage site, the insert of pCri8a::*mecR2* was inserted into the *S. aureus* expression vector pSPT181::*spac* containing the IPTG-inducible P*spac* promoter, resulting in recombinant plasmid pSPT::*spac-mecR2*-3D. This recombinant plasmid was then transduced into the *mecR2* deletion N315 mutant (N315::Δ*mecR2*) and its ability to restore the oxacillin-resistance phenotype of parental strain N315 was evaluated. As illustrated in Figure 3.5, in the presence of the inducer (IPTG 100μM) the phenotype of N315 was fully restored, demonstrating that the MecR2 variant used for the crystallization experiments is biologically active.

3.3.3 MecR2 establishes a non-obligate triggered transient interaction with MecI

Interaction between recombinant MecR2 and MecI proteins - the latter was obtained as previously reported [17,18] - was assayed by bio-layer interferometry. This is a label-free technique in solution that measures the shift in wavelength of the interference pattern of light reflecting from a protein layer immobilized on a biosensor tip (stationaty phase) here MecI due to binding by a partner kept in solution (mobile phase) here MecR2 [27, 28]. This technique allows for real-time measurements and enables to accurately and precisely assess binding association and dissociation rates by determining the variation in thickness of the bio-layer due to protein binding. The response in the steady state correlates with the dissociation constant (K_D) according to the equation:

$$\text{Response (R)} = (R_{max} \times [\text{ligand}]) / (K_D + [\text{ligand}])$$

Figure 3.1A depicts the association and dissociation curves for the different concentrations assayed (colored lines) and the corresponding theoretical simulations (red lines). Representation of the experimental response (R) in steady state against ligand concentration (Figure 3.1B) and fitting to the theoretical aforementioned equation allowed to estimate a K_D value of 1.2±0.3nM. The magnitude of this value reveals a strong binding affinity between the two proteins, proving that these proteins interact, at

least, *in vitro*. Complementary studies by size-exclusion chromatography revealed, however, that both proteins eluted separately (data not shown), i.e. this interaction is competed out by interaction of either protein with the resin. Consistently, trials to crystallize the complex would render only MecR2 crystals (data not shown). We conclude that MecI and MecR2 establish a strong but transient interaction as found in non-obligate triggered transient interactions [29].

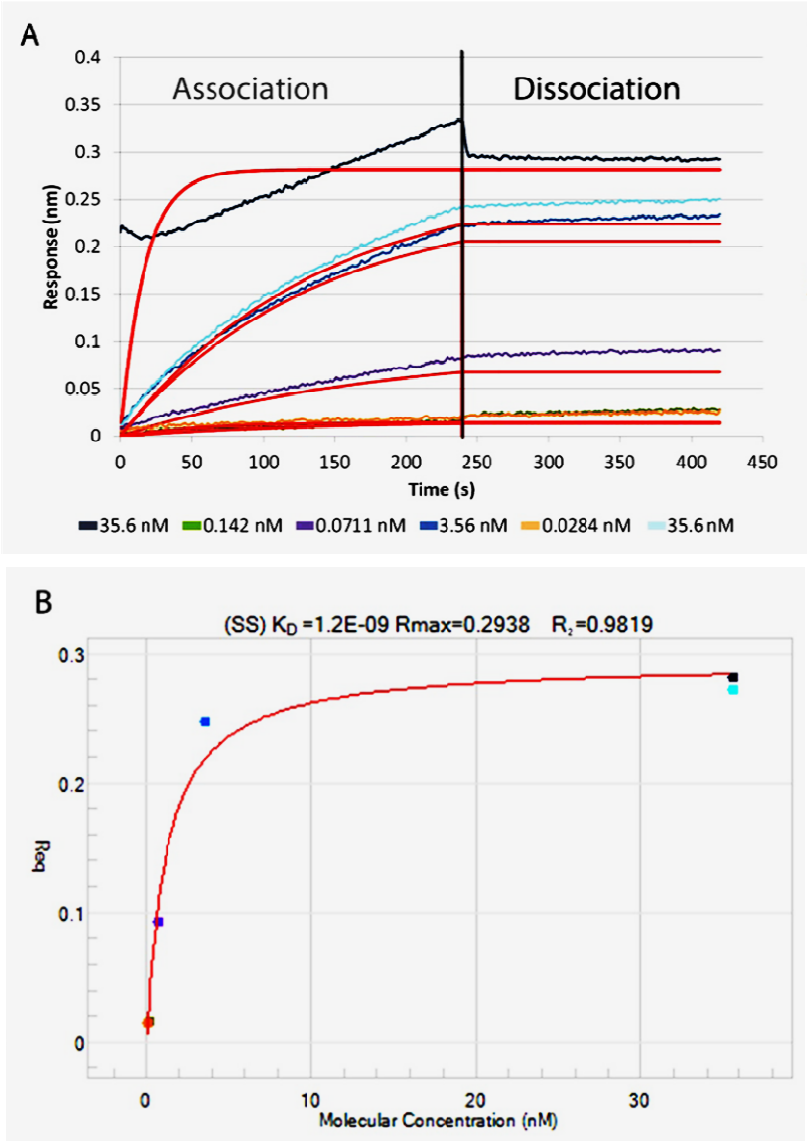

Figure 3.1 - Binding of MecR2 to MecI. **(A)** Experimental association and dissociation curves (colored) of a bio-layer interferometry experiment together with the corresponding theoretical simulations (red curves). **(B)** Steady-state analysis of the response for the same concentrations displayed in (A). The red curve depicts the hyperbolic regression employed to calculate the K_D value.

3.3.4 Overall structure of MecR2

The crystal structure of MecR2 was solved by a combination of multiple-wavelength anomalous diffraction and *ab initio* approaches, and two molecules are present in the asymmetric unit of the crystal, monomers A and B (see Table 3.1 for crystallographic data). The monomer structure reveals an elongated shape of roughly 45x60x80Å that is subdivided into three domains: an N-terminal DNA-binding-like domain (NDD), an intermediate scaffold domain (ISD), and a C-terminal dimerization domain (CDD) (Figure 3. 2A; the orientation of the left panel is hereafter taken as a reference). NDD (residues P^0/D^3-H^{70}) starts at the front surface, close to the top of the molecule, and enters a small $\alpha\beta$ domain. It consists of three α-helices (α1-α3) followed by a β-ribbon (β1β2) whose tip the loop connecting β1 and β2 (Lβ1β2) is disordered (Figure 3.2B). These elements conform to the architecture of a winged helix-turn-helix domain as observed in DNA-binding transcriptional repressors, which generally evince disordered β-ribbon tips when not bound to operator DNA [30]. In the latter, α1 and α2 contribute to creating a scaffold for adequate positioning of helix α3. This is the recognition helix that penetrates the major groove of double-stranded DNA, as found e.g. in the DNA-binding domains (DBDs) of MecI and BlaI [17-19].

After strand β2, the polypeptide chain enters ISD (residues L^{71}-N^{193} + S^{346}-A^{376}), which contains a central twisted five-stranded β-sheet (β3-β9 *plus* β9) that is parallel for all but one of its strands and shows connectivity -1,-1,+3x,+1x (Figures 3.2B,C). On its right, the sheet accommodates two helices (α4 and α5) and a short β-hairpin (β7β8), which is inserted between β6 and β5 and is folded back towards the sheet (Figure 3.2A, B); on its left, two perpendicular helices (α6 and α12) are found. Inserted between the latter helices is the CDD (residues L^{194}-T^{345}), which starts with a five-stranded β-sheet (β10-β12 *plus* β15-β16), which is equivalent to the one found in ISD both in connectivity and topology (Figures 3.2B, C). On its bottom side, this sheet is decorated with helices inserted between β12 and β15 (α7-α9) and between β15 and β16 (α10 and α11). In addition, a long β-ribbon (β13β14) is inserted between β12 and α7; it contributes to oligomerization (see below).

The overall architecture of ISD and CDD is such that the two respective β-sheets trap helices α6 and α12 in between in such a manner that a pseudo-twofold axis is generated relating, respectively, one sheet plus one helix with the other sheet-helix pair (Figure 3.2B). This entails that α6 could be formally assigned either to ISD or CDD. The interface between these two domains contributes to a ligand-binding cleft (Figures 3.2B and 3.3A). It is framed by Lβ6β7 and β7 at its top; β10, Lβ10β11, β11 *plus* Lβ12β13 and Lβ14α7 at its bottom; Lβ9α6 and α6 at its back; residue Y^{82} of Lβ3β4 on its right; and R^{200} of β10 and E^{177} of β9 on its left (Fig. 3.A). The cleft accommodates two potassium cations and a phosphate anion in monomer A; in monomer B only the leftmost cation site (see Figure 3.3A) is conserved, which is coordinated by atoms N^{181} Oδ1, A^{210} O, and A^{226} O at distances of 2.6-2.9Å. Further three (monomer B) and four (monomer A) solvent molecules at distances of 3.0-3.6Å complete the ligand sphere of this site. The rightmost potassium of monomer A is much more loosely bound, with just two protein atoms at

<3.5Å: N^{314} O and S^{203} Oγ. Finally, the phosphate anion of monomer A is bound by N^{178} Nδ2, H^{140} Nδ1, E^{179} Oε1 and a solvent molecule (Figure 3.3A).

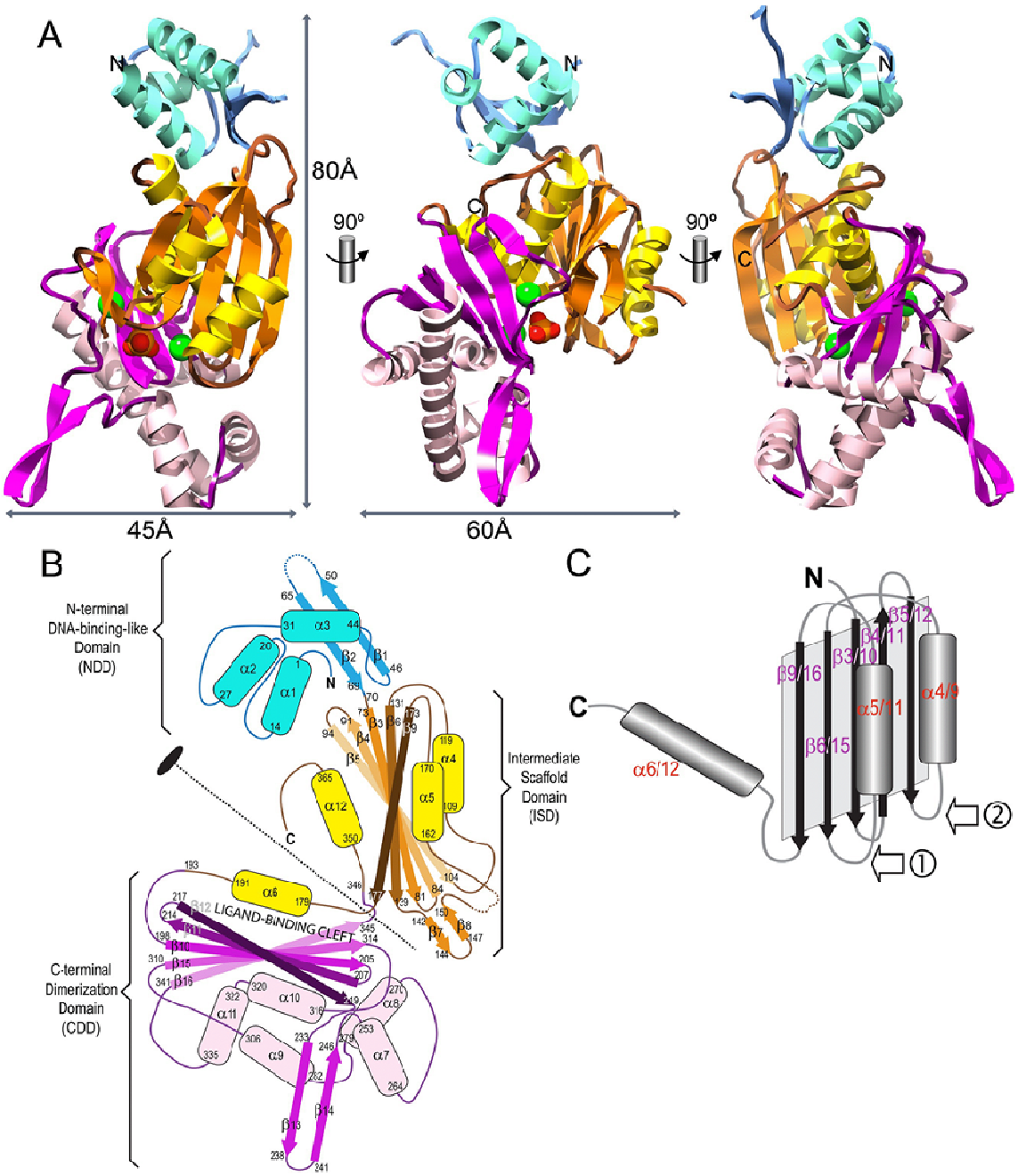

Figure 3.2 - Monomer structure of MecR2. **(A)** Ribbon-type plot of MecR2 in three orthogonal views depicting the NDD (cyan helices and blue strands), ISD (yellow helices and orange strands), and CDD (pink helices and magenta strands). The bound potassium and phosphate ions (monomer A) are depicted as green and orange/red CPK models, respectively. **(B)** Topology scheme of MecR2 showing the regular secondary structure elements of MecR2 with their labels and delimiting amino-acid positions. A twofold axis relating the b-sheets of ISD and CDD is depicted in discountinuous trace. The position of the ligand-binding cleft is further indicated. **(C)** Cartoon depicting the topology of the main building element of ISD and CDD, the five-stranded b-sheet and its three flanking helices. Each structural element carries the labels as found in the two domains. The arrows hallmark insertion points of distinct secondary structure elements within each domain: j for ISD and k for CDD.

3.3.5 MecR2 is a functional dimer

MecR2 eluted as a dimer in a calibrated size-exclusion chromatography assay. Consistently, the two molecules found in the crystal asymmetric unit give rise to a dimer with a surface of interaction of 1465Å^2 (~8% of the total surface of a monomer), which is in the range generally described for protein-protein complexes (1600 ±350Å^2; [31]) and much larger than generally observed for artificial crystal packing contacts. It evinces a surface complementarity (0.72) that is likewise in the range reported for protein oligomers and protein/protein inhibitor interfaces (0.70-0.76; [32]). This interaction includes 83 contacts (<4Å), among them hydrophobic contacts between nine residues of either monomer and 34 hydrogen bonds and polar interactions. Protein segments involved are provided by the CDD of each monomer: Lα6β10, Lβ11β12, the second half of α9 and Lα9β15, and β-ribbon β13β14. The two monomers are not completely equivalent, and this gives rise to an *rmsd* value upon superposition of 0.97Å for 353 Cα-atoms deviating less than 3Å of out 361 common residues. Analysis of inter-domain flexibility based on the elastic network model revealed potential hinge motions at the two domain junctions of each monomer, which increase on going from the CDDs to the NDDs (Figure 3.3 C).

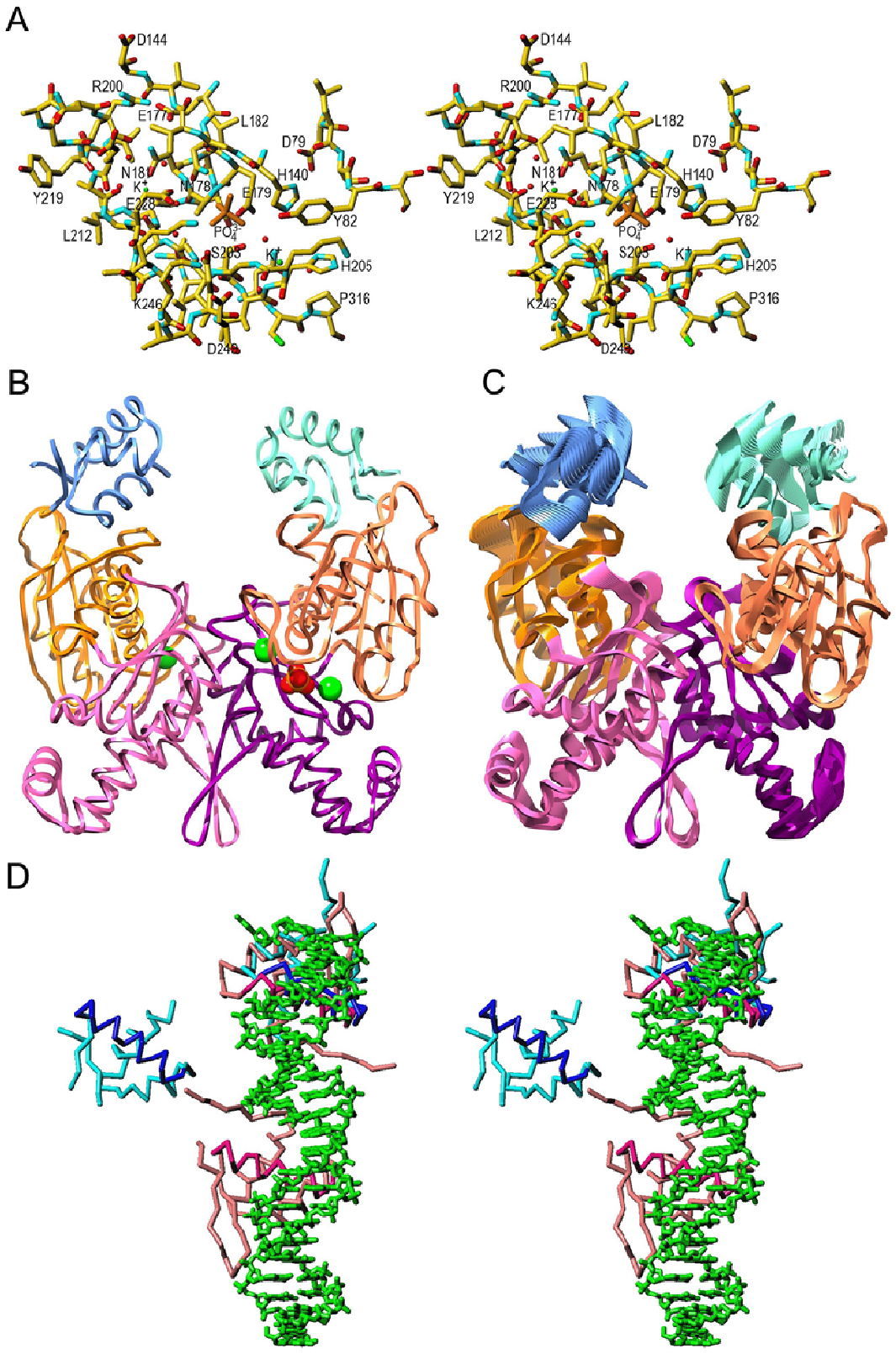
A
D144
R200
E177
L182
D79
N181
K+
N178
H140
Y219
E228
E179
Y82
L212
PO4 3-
S203
K+
H205
K246
P316
D248
B
C
D

Figure 3.3 - Ligand-binding cleft and quaternary structure of MecR2. **(A)** Detail of the ligand-binding cleft of MecR2 monomer A in stereo. Selected residues, the phosphate anion and the two potassium cations are labeled. **(B)** MecR2 dimer made up of monomer A (right; in the same orientation as in Figure 3.2A, left) and monomer B (left). The three domains are depicted in cyan/blue (NDD), salmon/orange (ISD), and purple/magenta (CDD). Potassium and phosphate ions are shown as green and orange/red CPK models, respectively. **(C)** Same as (B) showing the result of the analysis of inter-domain flexibility based on the elastic network model. **(D)** Superposition in stereo of MecR2 NDDs as found within the dimer (cyan Cα-model; recognition helices in dark blue) onto the MecI DBDs (pink Cα-model; recognition helices in magenta) as found within its complex with target DNA (green stick model; [17]).

3.3.6 Structural similarities

Sequence similarity searches suggested that MecR2 be grouped with the ROK family of proteins (from repressors, open-reading frames, and kinases), which includes transcriptional repressors and sugar kinases [33-35]. One archetypal well studied ROK protein is xylose transcriptional repressor (XylR), which regulates xylose utilization as a carbon source in bacteria [36-40], but there are no structural data on XylR available. *Escherichia coli* protein Mlc is the only functionally and structurally characterized ROK-family protein with DNA-repressor function [34, 41]. Mlc is a dimeric/tetrameric transcriptional repressor that controls the utilization of glucose in *E. coli* [42]. It evinces overall fold similarity and quaternary arrangement with MecR2 and is likewise subdivided into three domains equivalent to NDD, ISD, and CDD. In addition, two unpublished structures corresponding to proteins of unknown function deposited with the Protein Data Bank (PDB) by structural genomics consortia from *Thermotoga maritime* (PDB code 2HOE) and *Vibrio cholerae* (PDB code 1Z05) further displayed high structural similarity scores with MecR2. These are the only three-domain ROK proteins structurally reported, which add up to a large group of mostly dimeric or tetrameric two-domain ROK proteins, sugar kinases that bind and phosphorylated glucides and mostly possess only ISDs and CDDs [33-35, 43].

Table 3.1 - Crystallographic data

Dataset	Native 1	Native 2	Selenomethionine absorption peak	Selenomethionine inflection point
Cell constants (P212121; a, b, c, in Å)	66.47, 73.22, 157.38	67.39,73.14,157.22	66.62, 73.25, 157.82	66.62, 73.25, 157.82
Wavelength (Å)	0.97626	0.97919	0.97881	0.97908
No. of measurements / unique reflections	402,549 / 45,488	356,882 / 39,911	213,853 / 34,902	154,830 / 24,460
Resolution range (Å) (outermost shell) [a]	49.2 – 2.10 (2.21 – 2.10)	49.6 – 2.20 (2.32 – 2.20)	49.3 – 2.30 (2.42 – 2.30)	49.3 – 2.57 (2.75 – 2.57)
Completeness (%)	99.6 (98.7)	99.1 (95.0)	99.5 (97.2)	99.9 (100.0)
R_{merge} [b,c]	0.087 (0.716)	0.104 (0.420)	0.054 (0.454)	0.089 (0.708)
$R_{r.i.m.}$ (= R_{meas}) [b,c] / $R_{p.i.m.}$ [b,c]	0.092 (0.777) / 0.030 (0.299)	0.110 (0.465) /0.035 (0.194)	0.065 (0.568) / 0.035 (0.336)	0.106 (0.843) / 0.057 (0.451)
Average intensity ($<[<I> / \sigma(<I>)]>$)	19.8 (2.7)	17.0 (3.6)	22.9 (3.1)	19.8 (2.6)
B-Factor (Wilson) (Å^2) / Average multiplicity	32.4 / 8.8 (6.4)	27.4 / 8.9 (5.2)	45.7 / 6.1 (4.7)	55.8 / 6.3 (6.4)
Overall anomalous completeness (%) / multiplicity	-	-	98.3 / 3.2	99.4 / 3.3
Resolution range used for refinement (Å)	49.2 – 2.10			
No. of reflections used (among these, test set)	45,433 (762)			
Crystallographic R_{factor} (free R_{factor}) [c]	0.190 (0.229)			
No. of protein atoms / solvent molecules /	5,813 / 278 /			
ligands / ions	6 glycerol / 4 K^+, 1 $PO_4$3-			
Rmsd from target values bonds (Å) / angles (°)	0.010 / 1.04			
Average B-factors for protein atoms (Å^2)	49.2			
Main-chain conformational angle analysis [d] Residues in favored regions / outliers / all residues	705 / 0 / 714			

[a] Values in parentheses refer to the outermost resolution shell. [b] Friedel mates were treated separately during processing of selenomethionine-derivative data. [c] For definitions, see Table 1 in (66). [d] According to MOLPROBITY (62).

3.3.7 MecR2 has a non-functional ligand-binding cleft

As MecR2, Mlc has a ligand-binding cleft carved into the molecule at the interface between ISD and CDD. It further has an adjacent regulatory zinc-binding site, which is required for repressor activity [34] and is provided by the segment topologically equivalent to the protruding β-ribbon β13 β14 in MecR2. In contrast to the latter, however, this segment is folded back towards the body of the molecule in Mlc, in a similar fashion as in the ROK sugar kinases glucokinase from *E. coli* [46] and glucomannokinase from *Arthrobacter* sp. [47], where it contributes to shaping the floor of a sugar-binding cleft. This segment encompasses a widely conserved consensus sequence among ROK proteins, CXCGXXGCXE [34, 43], which contains three zinc-binding cysteine residues. A similar site is also found in *Bacillus subtilis* fructokinase YdhR [48], an undescribed putative glucokinase from *Enterococcus faecalis* (PDB 2QM1), an undescribed putative regulatory protein from *Salmonella typhimurium* (PDB 2AP1), and the aforementioned protein from *V. cholerae*, so that ROK family members containing this consensus sequence share a conserved metal-binding site. In contrast, MecR2 lacks these cysteine residues, and its chain trace is completely different, giving rise to an extended β-ribbon engaged in dimerization (see above). The latter is similar in the aforementioned protein from *T. maritima*, although in this case the ribbon is four residues shorter and contributes less to dimerization than in MecR2. Merely the last glutamate of the consensus sequence is found in the latter two proteins - E^{248} in MecR2 - and it contributes to the ligand-binding cleft (see above). Another ROK-signature motif comprising the C-terminal residues EXGH is found in several ROK proteins about ten residues upstream of the previous consensus sequence (see Figure 4 in [43]). The histidine - missing in MecR2 - is engaged in zinc binding in Mlc and the *V. cholerae* protein, while the glutamate - equivalent to E^{228} in MecR2 - is engaged in sugar binding in *E. coli* glucokinase and *Arthrobacter* sp. glucomannokinase, together with the conserved residues at positions equivalent to E^{248}, N^{178}, and E^{179} in MecR2. The latter two residues are engaged in phosphate anion binding (see above). Although these residues are likewise conserved in Mlc, this protein does not bind glucose, i.e. its inactivation does not depend on allosteric-changes induced by sugar binding [49]: inactivation is exerted through recruitment by the glucose transporter protein $EIICB^{Glc}$ of the phosphotransferase system [50-52]. In contrast, XylR binds xylose, glucose, and glucose-6-phosphate *in vitro* [40], i.e. it is a three-domain transcriptional repressor with a functional regulatory sugar-binding cleft. Overall, these findings entail that ROK proteins include members that bind sugars such as the sugar kinases and XylR but also some that do not such as Mlc.

3.3.8 MecR2 has a non-functional DNA-binding domain

Three-domain ROK transcriptional repressors like Mlc and XylR possess N-terminal DBDs engaged in DNA-operator binding and, thus, in the regulation of the transcription of the respective effector genes [33, 38]. MecR2 NDD likewise conforms to the structural determinants of such a DBD (see above). The reported structures of Mlc, *T. maritima* and *V. cholerae* are DNA-unbound, and they display the two recognition helices of a dimer in a relative spatial arrangement that is not adequate for binding to two successive turns of the major groove of dsDNA [33, 40]. This is consistent with the finding that structural flexibility of Mlc was identified as essential for its DNA-binding activity and for its regulation [40]. Therefore, we performed a structural superposition of MecR2 NDD onto the DBD of MecI in complex with its cognate operator sequence [17], which showed good overall fit of the monomers of both structures. However, detailed inspection revealed that MecR2 helix α3 is one turn shorter than in MecI (Figure 3.3D), thus precluding some of the interactions observed between MecI and DNA. In addition, the relative arrangement of the two domains of each protein is completely different: the two NDDs in MecR2 could not recognize DNA in the present conformation (Figure 3.3D). These findings led us to assess the DNA-binding capacity of MecR2, which revealed that the latter does not bind to a c.a. 200-bp dsDNA encompassing the *mec* promoter region [26]. In addition, a naturally occurring N-terminally truncated variant of MecR2 (residues M1-M94 according to UniProt Q99XE2) showed anti-repressor activity *in vivo*. Accordingly, MecR2 possesses a structurally conserved but functionally irrelevant NDD.

3.3.9 Functional implications of MecR2

The similarity of MecR2 with ROK-family bacterial sugar kinases and transcriptional repressors, both in the overall monomeric structures and the general dimeric quaternary arrangements, on one side, and its strong but transient MecI-binding capacity, on the other, have evolutionary and functional implications. Accordingly, XylR would represent the first step in evolution from a former two-domain ROK sugar-binding kinase - putatively evolved from a common ancestral hexokinase (42,50) - refurbished to produce a three-domain DNA-binding transcriptional repressor. The latter still binds and is thus allosterically regulated by sugar through N-terminal fusion with a winged helix-turn-helix DBD. Mlc would represent the next step - as already anticipated in (33) - to a three-domain DNA-binding transcriptional repressor that does not bind sugar nor is regulated by binding to an inducer or by proteolytic cleavage but through sequestration by a glucose transporter, i.e. through a protein-protein interaction (40,49). Finally, MecR2 would represent a last step in such an evolution, in which a three-domain Mlc-like repressor would have also lost its DNA-binding ability to render a dimeric protein sequester of a dimeric transcriptional repressor, MecI. The inter-domain flexibility of the anti-repressor MecR2 would be consistent with the

adaptability required for such a protein-binding protein. Finally, MecI sequestering would suppress its repressor activity and facilitate its cleavage by non-specific proteolysis.

3.3.10 Characterization of the MecR2::MecI interaction

Evidence for a direct interaction between MecR2 and MecI proteins was previously obtained using a bacterial two hybrid strategy and electrophoretic shift assays of the binding of MecI to the *mecA* promoter in the presence of MecR2 [26]. In this study, we sought to characterize in more detail this protein interaction. First, we first evaluated the *in vitro* cross-linking of purified MecI and MecR2 proteins. The SDS-PAGE analysis of the cross-linking reactions revealed a time-dependent transition from MecI and MecR2 monomers to a mixture of MecI dimers, MecR2 dimers and MecI::MecR2 tetramers (Figure 3.4A). Control cross-linking experiments with each protein alone also showed a time-dependent dimerization of MecI, whereas for MecR2, dimerization was much less effective and not time-dependent (Figure 3.4B and 3.4C, respectively). These data suggest binding of two MecR2 monomers to the MecI dimer (which is its active form) and that MecI may induce MecR2 dimerization.

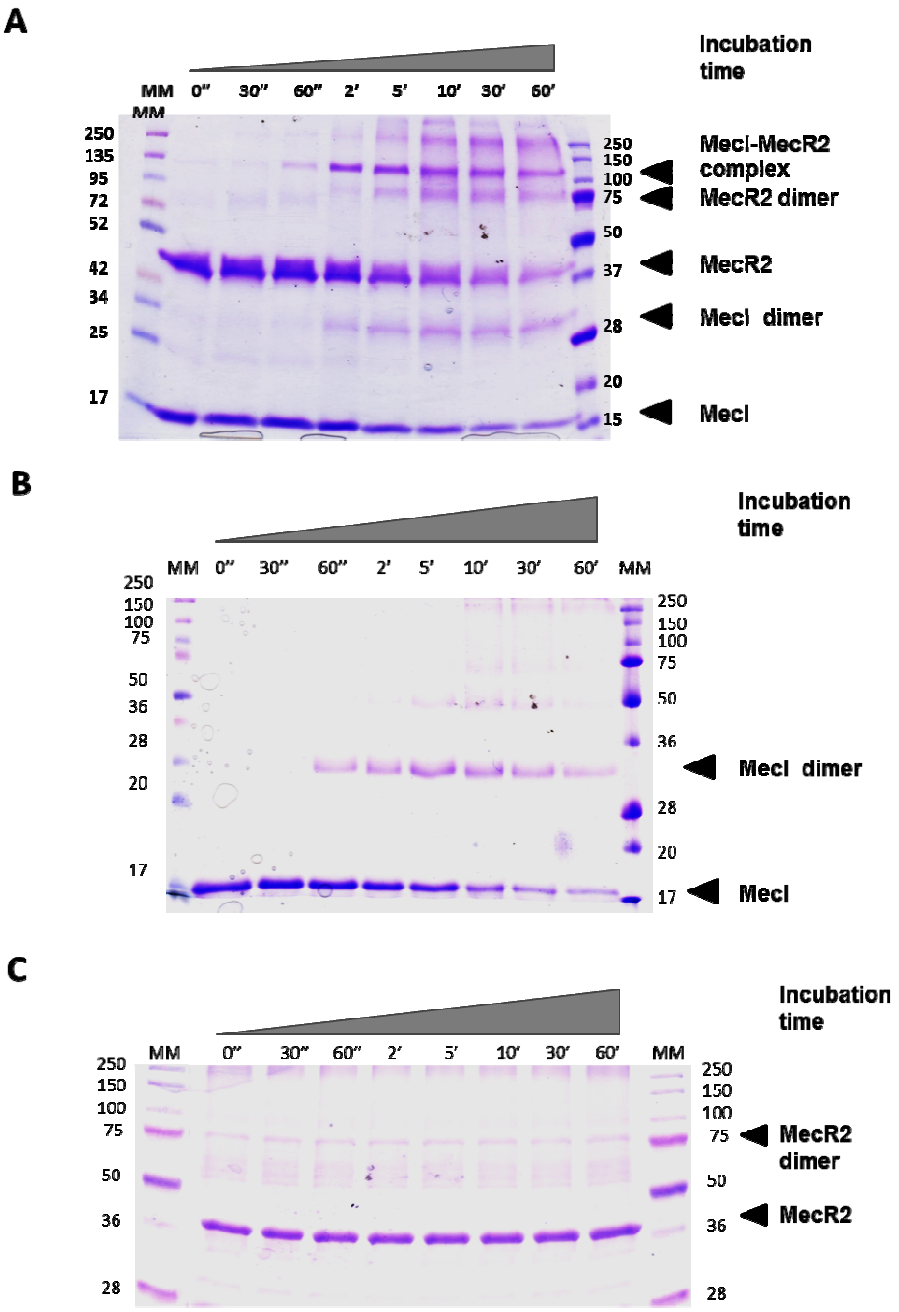

Figure 3.4 - MecR2 interacts with MecI. **(A)** *In vitro* chemical cross-linking of purified MecR2 and MecI proteins. SDS-PAGE analysis of the time-dependent transition due to the action of paraformaldehyde from a mixture of purified MecI and MecR2 and monomers (c.a. 17 and 45 kDa, respectively) to MecI dimers, MecR2 dimers and MecR2-MecI tetramers. MM, molecular weight marker (kDa). Control experiments for the in vitro MecR2-MecI cross-linking assay. SDS-PAGE analysis of the time-dependent cross-linking by paraformaldehyde of purified MecI **(B)** and purified MecR2 **(C)**.

3.3.11 Site-directed mutagenesis of MecR2

Based on MecR2 structural data analysis, two disordered regions were identified that may be involved in the interaction with MecI: regions S55-K62 and the T150-I160. Two MecR2 mutant variants were generated to evaluate this hypothesis: a deletion of S55-K62 (ΔS55-K62) and a substitution of T150-I160 by four glycines (T150-I160→GGGG). In addition, based on the MecR2 similarity to the transcriptional repressor of the xylose operon (XylR), the three putative ligand-binding cleft mutants were mutagenized, as follows: $N^{178}E \rightarrow AA$, $E^{228} \rightarrow A$, and $E^{248} \rightarrow A$. The biological activity of MecR2 mutagenized variants was evaluated using the complementation assay of deletion mutant N315::Δ*mecR2* with *mecR2* variants expressed from pSPT181::*spac* vector, containing the IPTG-inducible P*spac* promoter. Figure 3.5 summarizes all data. Concerning the two disordered regions, the T150-I160→GGGG variant failed to complement the N315::Δ*mecR2* phenotype, suggesting that this region is important for MecR2 function. The S55-K62 segment belongs to the putative N-terminal DNA binding domain and, as such, the lack of effect of its deletion is in agreement with previous data suggesting that this domain is not involved on the MecR2 function. Concerning the mutant variants for the three putative ligand-binding clefts, no loss of MecR2 function was observed, suggesting that MecR2 function is not modulated by ligand-binding. In comparison to XylR, which binds to xylose, glucose and glucose-6-phospate [40], MecR2 acting as an anti-repressor of *mecA* transcription seems to have lost this activity. Most likely this functional divergence has evolved in *S. fleuretti*, the species in which the *mecA* three-component regulatory system, *mecR1-mecI* and *mecR2*, appears to have been assembled [26] .In fact, in *S. fleuretti mecA* positive stains, the *mecR1-mecI* locus is linked to the full xylose operon and these strains, contrary to MRSA strains, do ferment xylose [53].

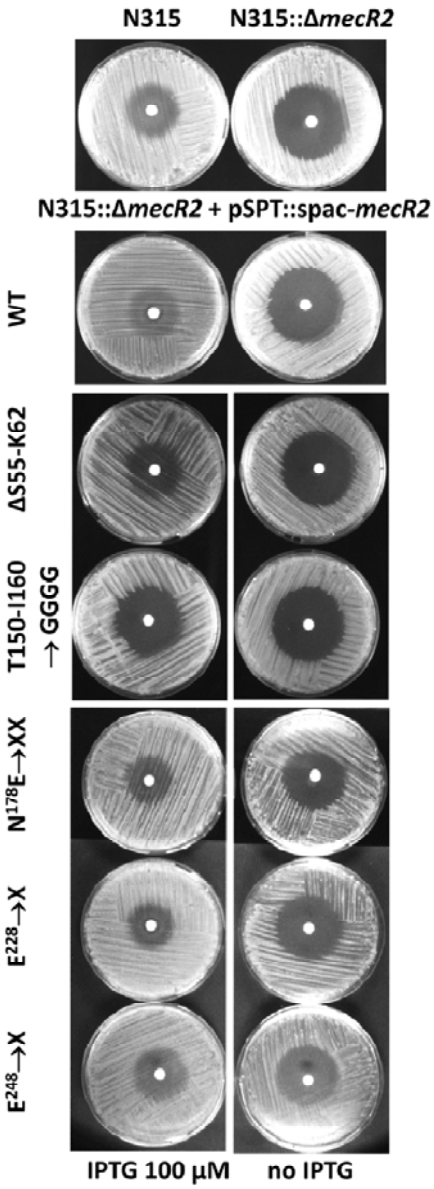

Figure 3.5 - Biological activity of purified MecR2 and mutagenized MecR2 variants. The purified MecR2 variant used in the crystallization assays (marked as WT here) and the site-directed MecR2 mutants were cloned into the *S. aureus* expression vector pSPT181::*spac* under the control of the IPTG-inducible P*spac* promoter. The biological activity of MecR2 variants was then evaluated by testing the complementation of the phenotype of the *mecR2* null-mutant in prototype strain N315 (N315::Δ*mecR2*) transformed with the pSP181::*spac* recombinant vectors expressing the *mecR2* variants.

3.4 Materials and Methods

3.4.1 Recombinant overexpression and purification

The bacterial strains and plasmids used in this study are listed in Table 3.2. The *mecR2* gene was amplified from genomic DNA from *S. aureus* strain HU25 by PCR and cloned into expression vector pCri8a (between NcoI and XhoI restriction sites) that adds an N-terminal His6-tag and a TEV protease cleavage site. This cloning strategy entailed that the N-terminus of the protein (according to UniProt entry Q99XE2) was preceded by the twenty-residue segment $M^{-20}GSSHH^{-15}HHHHS^{-10}SGENL^{-5}YFQG^{-1}P^{0}$ (amino-acid one-letter-code; upper-case numbers depict numbering of selected residues within this segment). The expression vector was transformed into *Escherichia coli* BL21 DE3 cells and 1 liter cultures of transformed bacteria were induced for protein expression with 0.1mM isopropyl-β-D-1-thiogalactopyranoside at 18ºC for 24h when the optical density at λ=600nm (OD_{600}) reached 0.6. Cultures were subsequently centrifuged at 7,000×*g* (4ºC, 20min) and pellets were resuspended in 70ml ice-cold buffer A (20mM Tris-HCl, 0.5M NaCl, pH8.0). Cells were lysed by cell disruption with a cell disruptor (Constant Cell Disruption Systems) operated at 1.35kBar and the lysate was subsequently centrifuged at 75,600×*g* in an Avanti J-25 centrifuge with a JA-25.50 rotor (4ºC, 20min). The soluble fraction containing His_6-TEV-MecR2 was applied onto a His-trap FF crude column (GE Helthcare) attached to an ÄKTA Purifier UPC-10 FPLC system and previously equilibrated with buffer A. The protein was eluted with an imidazole gradient (0 to 0.5M imidazole in buffer A) and fractions containing the protein were subjected to a final size-exclusion chromatography step in a Superdex 75 16/60 column (GE Healthcare), previously equilibrated with 20mM Tris-HCl, 0.2M NaCl, pH7.4. Protein purity was assessed by 10%-tricine SDS-PAGE. TEV protease digestion of the N-terminal His_6-tag was assayed under different conditions but yields were not satisfactory, so that crystallization trials were performed using tagged MecR2. The selenomethionine variant of MecR2 was obtained in the same way, except that 30min before induction the cells were added to 500ml of medium lacking methionine and containing 25mg of selenomethionine (Sigma-Aldrich) instead.

3.4.2 Biological activity of purified MecR2

In order to confirm the biological activity of recombinant MecR2 protein expressed from pCri8a::*mecR2*, the insert containing the full *mecR2* coding sequence plus the additional N-terminal sequence containing the His_6-tag and the TEV protease cleavage site was cloned into the *S. aureus* expression vector containing the P*spac* IPTG-inducible promoter (pSPT181::*spac*). Briefly, using flanking

primers MR2-3D F1/R1 the insert sequence was amplified with the Phusion High-Fidelity DNA Polymerase (New England Biolabs) and, after digestion with *XmaI* (New England Biolabs), was inserted into the *Xma*I linearized pSPT181::*spac* plasmid using the Rapid DNA Dephos & Ligation kit (Roche), according to the manufacturer's recommendations. Ligation reactions were transformed to *E. coli* DH5α. Recombinant plasmid integrity was confirmed by restriction analysis and the correct insert direction was confirmed by PCR using primer pairs spacF1/MR2-RT2 and spacR1/MR2-RT1. Insert sequences were also confirmed by DNA sequencing at STAB Vida (www.stabvida.com). After stabilization in *E. coli*, the recombinant plasmid was electroporated into *S. aureus* restriction-deficient strain RN4220 and finally transduced by the 80α phage to the knock-out *mecR2* mutant strain N315 (N315::Δ*mecR2*), as previously described ([55, 56] The ability of the recombinant *mecR2* expressed in trans to complement the N315::Δ*mecR2* oxacillin-resistance phenotype was then evaluated, as previously described [26].

3.4.3 Site-directed mutagenesis of MecR2

MecR2 mutant variants were obtained by two-step overlap extension PCR, as previously described [57, 58]. Briefly, a round of two independent PCR reactions was performed with two complementary mutagenic primers and the two flanking *mecR2* primers generating two intermediate PCR products with overlapping terminals (see Table 3.3). As an example, for S55-K62 deletion primer pairs MR2-3D F1/MR2-SDM2 and MR2-3D R1/MR2-SDM1 were used. The template for these PCR reactions was the recombinant plasmid pCri8a::*mecR2* used for the expression and purification of MecR2 protein. Both intermediate PCR products were then 50-fold diluted and mixed to form the DNA template of the second PCR, using primers spanning the entire *mecR2* gene (MR2–3D F1/R1). All PCR reactions were performed with the Phusion High-Fidelity DNA Polymerase (New England Biolabs). The mutagenized *mecR2* amplicons were digested with *Xma*I and ligated to pSPT181::*spac* as described above for the "wild-type purified" MecR2.

3.4.4 *In vitro* MecR2::MecI crosslinking

MecR2 protein was overexpressed and purified as described above. MecI was overexperssed and purified as previously described [59]. For the cross-linking experiments, MecI and MecR2 proteins were mixed at 0.09mg/mL each (a low concentration to minimize intermolecular cross-linking) in 50µl of 100mM HEPES, pH9.0, containing the paraformaldehyde (PFA) cross-linking agent at 0.1% (v/v). The cross-linking reactions occurred at room temperature and were stopped with 10µl 5x Laemmli buffer (with β-mercaptoethanol) at distinct time points. Samples were analyzed by 10%-tricine SDS-PAGE gels stained with Coomassie-blue. Control experiments were performed with both purified proteins alone at the same experimental conditions.

Table 3.2 - Strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source
Strains		
E. coli DH5α	Recipient strain for recombinant plasmids	Stratagene
E. coli Bl21 (DE3)	Recipient strain for expression vector pCri8a	Novagene
BL21 + pCri8a::*mecR2*	*E. coli* BL21 (DE3) overexpressing *mecR2* with a N-terminal His_6-tag and a TEV protease cleavage site	This study
S. aureus RN4220	Restriction-deficient derivative of reference strain NCTC8325-4	R. Novick
S. aureus N315	Prototype MRSA strain, heterogeneous Oxa^r, wild-type *mecR1-mecI*, *mecR2* positive, β-lactamase positive, clone ST5-II	K. Hiramatsu [60]
S. aureus HU25	Homogeneous Oxa^r, wild-type *mecR1*, truncated *mecI*, *mecR2* positive, β-lactamase positive, clone ST239-III	[61]
N315::Δ*mecR2*	N315 *mecR2* deletion backcross, β-lactamase positive	[26]
N315::Δ*mecR2* + pSPT::*spac-mecR2*	N315::Δ*mecR2* expressing *mecR2 in trans* from the inducible *Pspac* promoter	This study
Plasmids		
pCri8a	pET30 (Invitrogen) derivative containing His6-GST-Tev fragment, Kan^r	[62]
pSPT181::*spac*	pSPT181 with 1.6 kb EcoR1-BamH1 fragment containing the IPTG inducible P*spac* promoter and the transcriptional repressor LacI from pDH88, Ap^r, Tc^r	[26]
pCri8a::*mecR2*	pCri8a with *mecR2* gene from strain HU25	This study
pSPT181::*spac-mecR2* 3D (wild-type)	pSPT181 vector containing the *mecR2* gene with a N-terminal His_6-tag and a TEV protease cleavage site from pCri8a::*mecR2*	This study
pSPT181::*spac-mecR2* ΔS55-K623	pSPT181 vector containing the *mecR2* mutant variant ΔS55-K623	This study
pSPT181::*spac-mecR2* T150-I160→GGGG	pSPT181 vector containing the *mecR2* mutant variant T150-I160→GGGG	This study
pSPT181::*spac-mecR2* N178E→XX	pSPT181 vector containing the *mecR2* mutant variant N178E→XX	This study
pSPT181::*spac*-mecR2 E228→X	pSPT181 vector containing the *mecR2* mutant variant E228→X	This study
pSPT181::*spac-mecR2* E248→X	pSPT181 vector containing the *mecR2* mutant variant E248→X	This study

3.4.5 Bio-layer interferometry

Binding studies were performed with a FortéBio Octet Red96 biosensor at 25°C in 96-well microplates by using 200µl-reaction volumes and amine-reactive biosensor tips (Menlo Park, CA), which were incubated for 5min in coupling buffer (100mM 2-[N-morpholino]ethanesulfonic acid, pH4.0) immediately before usage. Recombinant purified MecI protein [18] was immobilized through covalent attachment to the biosensor by employing 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide and N-hydroxysuccinimide essentially as described [63]. MecR2 was assayed as soluble ligand at concentrations ranging between 0.028nM and 35.6nM in 0.01% phosphate-buffered saline with Tween 20 to avoid unspecific interactions. The binding reaction was blocked with ethanolamine according to manufacturer's instructions. A regeneration step with 0.05% sodium dodecyl sulfate in 1M NaCl solution was intercalated between successive assays to optimize resolution. Results were analyzed using dedicated software from FortéBio.

Table 3.3 - Primers used in this study

Name	**Sequence (5' → 3') ***
MR2-3D F1	TATACCCGGGAAGGAGATATACCATGGGCA
MR2-3D R1	TATACCCGGGGCTATTATGCTTTTATATCTA
Spac F1	GAAGATTTATTTGAGGTAGC
Spac R1	TTATGGCTTGAACAATCACG
MR2-RT1	AATGAAGCGAATCTTTCAGC
MR2-RT2	AATTGCTAATGTACCACCTAGC
MR2-SDM 1	ATGAGGTTGGTGAGGGTGATAAACCTATTCTTCTGAAGGT
MR2-SDM 2	ACCTTCAGAAGAATAGGTTTATCACCCTCACCAACCTCAT
MR2-SDM 3	TGGATAATGAGCAGCATGTGGGTGGAGGTGGAATTTCAATTCCA
MR2-SDM 4	TTCTTAGCAATTGAAATTCCACCTCCACCCACATGGTGCTCATTATC
MR2-SDM 9	ATGGGGAAGCGGGTGCAATTGGAAAAACACT
MR2-SDM 10	AGTGTTTTTCCAATTGCACCCGCTTCCCCAT
MR2-SDM 11	TCTTTCATAAGATTGCAGATATTTTTTCACA
MR2-SDM 12	TGTGAAAAAATATCTGCAATCTTATGAAAGA
MR2-SDM 13	CCAGTCGTAGTTGAAGCTGCAGCGAATCTTTCAGC
MR2-SDM 14	GCTGAAAGATTGGCTGCAGCTTCAACTACGACTCC

* Restriction sites are underlined.

3.4.6 Crystallization and structure analysis

Crystallization assays were performed by the sitting-drop vapor diffusion method. Reservoir solutions were prepared by a Tecan robot and 100nL crystallization drops were dispensed on 96x2-well MRC plates (Innovadyne) by a Cartesian (Genomic Solutions) nanodrop robot at the High-Throughput Crystallography Platform (PAC) at Barcelona Science Park for initial screenings both at 20 and 4ºC in a Bruker steady-temperature crystal farm and using initial protein concentrations of 5.4 and 2.7mg ml^{-1}. Preliminary crystallization hits were improved and best conditions were scaled up to the microliter range in 24-well Cryschem crystallization dishes (Hampton Research). Crystals suitable for structure analysis were obtained at 5.4mg ml^{-1} in 20 mM Tris-HCl, 200 mM NaCl, pH7.4 by using 0.2M NaCl, 20% PEG 1000, 0.1M KH_2PO_4/Na_2HPO_4, pH6.2 as reservoir solution. Crystals were cryo-protected with reservoir solution implemented with 30% glycerol. Crystallization conditions for the selenomethione-derivatized protein were similar to the native ones. Complete diffraction datasets were collected from liquid-N_2 flash-cryo-cooled crystals at 100K (provided by an Oxford Cryosystems 700 series cryostream) at beam lines ID23-1 on an ADSC Quantum Q315r CCD detector and ID29 on a Dectris PILATUS 6M pixel detector, respectively, of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) within the Block Allocation Group "BAG Barcelona." Crystals were orthorhombic, with two molecules per asymmetric unit. Diffraction data were integrated, scaled, merged, and reduced with programs XDS [64] and SCALA [65] within the CCP4 suite of programs [66] (see Table 3.1).

The structure of MecR2 was solved by a combination of multiple-wavelength anomalous diffraction with SHELXE/D [67] and *ab initio* approaches with ARCIMBOLDO [68] by using two native datasets and two datasets from a selenomethionine-derivatized crystal collected at the selenium absorption peak and the inflection point as determined by a previous XANES scan (Table 3.1). The resulting electron density map enabled straightforward tracing of the entire polypeptide chain on a Silicon Graphics Octane2 workstation with TURBO-Frodo program [69]. Subsequent crystallographic refinement with BUSTER/TNT [70], which included TLS and non-crystallographic refinement, alternated with manual model building until completion of the model. The latter comprised residues M^1 to A^{376} according to UniProt entry Q99XE2 *plus* an N-terminal proline resulting from the cloning strategy (termed P^0; see above) of molecule A, and D^3 to A^{376} of molecule B. Three loop segments were disordered and were thus omitted from the final model: E^{52}-S^{58} and L^{152}-E^{158} of molecule A, and G^{51}-P^{63} of molecule B. In addition, one phosphate anion, four potassium cations, six glycerol molecules, and 278 solvent molecules were assigned (Table 3.1).

3.5 Miscellaneous

Figures were prepared with SETOR [71], CHIMERA [72], and TURBO-Frodo. Structure similarities were determined with DALI [73]. Experimental model validation was performed with MOLPROBITY [45] and WHATIF [74]. Close contacts (<4Å) and interaction surfaces (taken as half of the surface area buried at the complex interface) were calculated with CNS [75], and interface shape complementarity was computed with SC [32] within CCP4 [66]. In all cases, a probe radius of 1.4Å was used. Inter-domain flexibility was ascertained with HINGEPROT employing standard settings ([76]; see http://bioinfo3d.cs.tau.ac.il/HingeProt). The final coordinates available from the PDB at www.pdb.org (access code XXXX).

3.6 Acknowledgments

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CHAPTER IV

Concluding Remarks & Future Perspectives

4.1 Conclusion

Infections caused by *Staphylococcus aureus* in the pre-antibiotic era were associated to a significant mortality rate of about 80%. The introduction of benzylpenicillin in the early 1940s caused a dramatic decrease in death rates from bacterial pneumonia and meningitis, leading to a spectacular success in curing formerly untreatable staphylococcal diseases [1]. However, an enzyme capable of inactivating the active β-lactam ring of penicillin was described firstly in *E.coli* [2], and shortly after, penicillinase production was also reported in *S. aureus* [3]. Four years later, over 50% of *S. aureus* nosocomial isolates recovered from London patients were resistant to penicillin [4, 5]. The introduction of penicillinase-stable penicillins (such as methicillin), provided temporary relief for clinicians, but methicillin resistant *Staphylococcus aureus* (MRSA) strains rapidly emerged [6]. At the same time, important achievements were made concerning the development and characterization of new antimicrobial classes, such as: the sulfonamides, streptomycin, tetracyclines, macrolides, cephalosporins and glycopeptides [7, 8]. However, once again, shortly after these antibiotics were introduced into clinical practice, resistance mechanisms rapidly emerged [9-11]. Antibiotic resistance is now widespread and has become a major problem in the treatment of clinical infections caused by *S. aureus* [12-14]. The spread of MRSA poses serious therapeutic problems within the hospital environment [15, 16]. Every year about two million patients acquire nosocomial infections in US hospitals [17], and about 60% of those infections involve antibiotic resistant bacteria [18]. Since 2000, only two new antibiotic classes have been introduced to treat MRSA infections, and, clinical resistance to both has already been described [19, 20]. Currently, glycopeptides (e.g. vancomycin and teicoplanin) have been the last-resort antibiotics against MRSA. Nevertheless, the large-scale use of glycopeptides has led to the emergence of resistance. The current prevalence of MRSA in most countries and the emergence of vancomycin intermediate *Staphylococcus aureus* (VISA) and vancomycin resistant *Staphylococcus aureus* (VRSA) [21, 22], demonstrates that the clinical options for treating these infections may become very limited.

Several strategies are being developed in order to overcome the health threat caused by the spreading of multi-resistant pathogens. First, new antibiotic compounds are being developed, such as the protein synthesis inhibitor – tigecycline [23], or the new anti-MRSA cephalosporins and carbapenems, which are stable against hydrolysis and show affinity to PBPs, including PBP2a, [24-26]. Second, synergistic combinations of different antibiotics are used, such as the combination of clavulanic acid which has poor antibacterial activity, with another β-lactam (e.g. amoxicillin), originating drugs for clinical use [27]. Third, new target-inhibitor combinations are being explored, using modern biochemical and genomic tools, combined with powerful synthetic-chemical-library methods, which enables a rapid identification of the target as well as a possible design of new drugs [28, 29].

MRSA isolates are broadly resistant to penicillins and cephalosporins [30]. β-lactams are rendered inactive against *S. aureus* by two primary mechanisms of resistance: (i) the expression of a β-

lactamase encoded by *blaZ* gene, that hydrolyses penicillins before it can reach the cellular targets, and (ii) the production of a single additional penicillin binding protein, PBP2a, encoded by *mecA* gene, with a remarkably low affinity to β-lactams [31]. The regulatory proteins that mediate *mecA* and *blaZ* transcription, *(*MecR1-Mec1 *and* BlaR1-BlaI, respectively) share high homology in terms of structure and function, so much so that there is a cross-talk between both systems [32, 33], and the signal-transduction mechanisms are believed to be similar [34, 35]. The currently accepted model for the induction of *mecA* transcription is based on two main proteolytic steps, as follows: (i) the binding of the β-lactam antibiotic to the extracellular sensor domain of MecR1, generates a transmembrane signal that leads to a conformational change and consequent activation of the intracellular (auto)-proteolytic domain, and (ii) the proteolytic cleavage of the cytosolic MecI within its dimerization domain, by the activated MecR1 intracellular domain, directly or indirectly, leading to *mecA* transcription and consequent expression of the resistance phenotype [34, 35].

Understanding the molecular pathways that lead to the optimal expression of *mecA* might be crucial to develop complementary therapeutic strategies and possibly new drugs against MRSA. To achieve this goal, it is important to clarify some molecular details involved in the control of *mecA.* The induction of *mecA* by MecR1 has been described as extremely slow, so that cells carrying the *mec* locus fully functional appear phenotypically susceptible in spite of the presence of *mecA* [33, 36, 37]. Moreover, there is still no evidence supporting a direct interaction between the inducer MecR1 and the repressor MecI. As a matter of fact, several authors based on contradictory observations have postulated the existence of a third element mediating the signal transduction between the MecR1 and MecI proteins [38-40].

We demonstrated that the *mecA* regulatory locus has a three-component arrangement, harboring besides the previously well described *mecR1-mecI* regulatory genes, an anti-repressor - the *mecR2* gene, which is co-transcribed together with *mecR1-mecI* from the *mecR1* promoter. The *mecR2* gene is highly conserved among MRSA strains carrying the SCC*mec* Type II and III, as well as, in other staphylococcal species containing SCC*mec* sequences. In order to access the impact of the *mecR2* gene on the MRSA phenotype several *mecR2* mutants were constructed in prototype MRSA strains with high variety in terms of phenotype. The deletion of *mecR2* gene caused a sharply decrease in the phenotypic levels of β-lactam resistance, demonstrating that *mecR2* is essential for the optimal expression of β-lactam resistance in strains with functional *mecI-mecR1* regulatory locus.

Altogether, our data shades some light on some open questions of the *mecA* regulatory mechanism. First, the effect of *mecR1-mecI* overexpression (without *mecR2*) on *mecA* induction profile in MRSA recombinant strains was previously evaluated, showing that the induction of *mecA* is very inefficient [33, 36, 37]. However, since all MRSA strains carrying *mecR1-mecI* regulatory genes fully functional are also positive to *mecR2*; indeed, those MRSA strains are able to fully induce the transcription of *mecA* by its cognate regulators (*mecR1-mecI-mecR2*) in the presence of β-lactams.

Second, due to the high similarity between the *bla* and *mec* systems, it is believed that the regulatory mechanisms that control the induction of *mecA* and *blaZ* are identical [34, 41, 42]. However the role and specificity of the repressor proteolysis appears to be different in the *mec* and *bla* systems. For instance, it has been proposed that in *B. licheniforms* the proteolysis of BlaI is not required for the induction of β-lactam resistance [38]. However, overexpression of the *bla* regulatory proteins of *S. aureus* in recombinant *E. coli* strains showed that BlaR1 directly cleaves the repressor BlaI [43], in agreement with previous studies in *S. aureus* [34]. Actually, our findings suggest subtle differences in terms of the proteolytic pathway leading to the induction of both structural genes (*mecA* and *blaZ*), corroborating also the hypothesis that the MecI repressor is not cleaved by the activated MecR1 [39, 40]. Our studies have shown that in the *mec* system, MecI proteolysis appears to be essential for induction of *mecA* transcription but that it occurs independently from the activated MecR1 and it is promoted by MecR2.

Third, the induction of *mecA* when mediated by its cognate sensor-inducer appears to be much less efficient than when mediated by BlaR1 [33, 42]. According to our results, we can speculate that the assembling of the anti-repressor *mecR2* to the *mecA* gene complex found in MRSA might have been an evolutionary strategy to compensate the inefficient induction mediated by MecR1. Most likely, since BlaR1 has high affinity to β-lactams and consequently induces *blaZ* transcription [42], we can infer that in *bla* system, BlaR1 accomplishes the same functions performed by MecR1 and MecR2.

The MecR2 crystal structure suggests that the anti-repressor MecR2 is a functional dimer, containing an N-terminal DNA-binding-like domain, an intermediate scaffold domain and C-terminal dimerization domain. The MecR2 protein shows structural similarity with xylose repressor (XylR) of *Staphylococcus xylosus,* which belongs to the ROK transcriptional repressors family. In order to evaluate the specific domains and residues identified from the MecR2 structural data analysis that might be important to MecR2 function, several *mecR2* mutant variants were generated. Complementation studies have shown that only the *mecR2* mutagenized variant lacking the disordered region T150-I160 located within the dimerization domain (that might be involved in the interaction with MecI), fails to complement the N315::Δ*mecR2* phenotype. These observations suggest that the dimerization domain is critical for the MecR2 function and might be directly involved in the interaction with MecI.

Based on the results reported on this thesis we propose a revision of the current model for the control of *mecA* transcription in MRSA (see Figure 4.1), as follows. Upon exposure to β-lactams the extracellular domain of MecR1 becomes active, triggering rearrangements which transmits a conformational change across the membrane, leading to the activation of the intracellular metalloprotease domain. Shortly after, the activated MecR1 cytoplasmic domain induces the cleavage of the repressor MecI, allowing the transcription of *mecA* along with *mecR1-mecI-mecR2*. The increasing amount of MecR2 molecules promote the local melting of MecI scissile bound, leaving it accessible to proteolytic cleavage presumably by native proteases. Although *mecA* induction can be achieved solely by MecR1-MecI, MecR2 is required for a fully *mecA* transcription, meaning that, MecR2 is essential to compensate the poor induction mediated by MecR1; i.e. the disruption of MecI mediated by the anti-repressor MecR2

is essential to a sustained induction of *mecA* transcription as long as the β-lactam is present. In the absence of β-lactams, the extracellular sensor domain of MecR1 is no longer activated, and the signal transduction is interrupted. This leads to the binding of MecI-dimers to the *mecA* promoter, remaining the residual copies of MecR1 anchored at the cell membrane. This model assumes that, in the absence of β-lactams, MecR2 is readily degraded by the cellular turnover pathway.

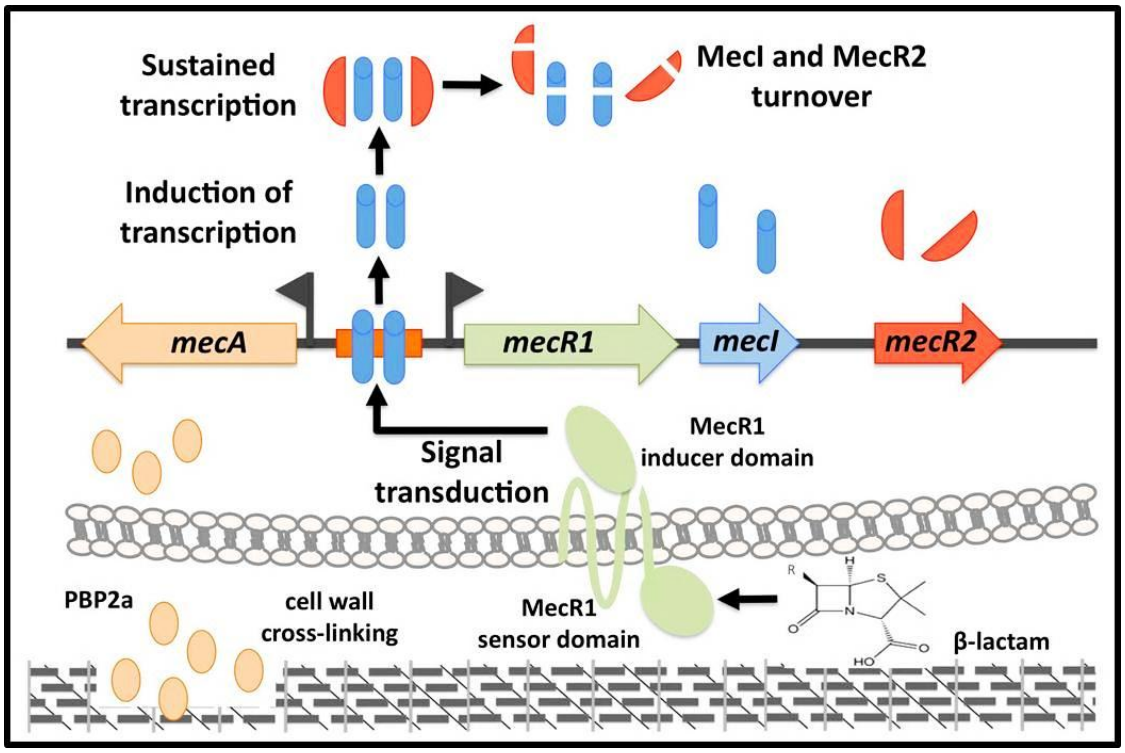

Figure 4.1 - Model depicting the transcriptional induction of *mecA* in the presence of β-lactams mediated by the regulator proteins MecR1-MecI-MecR2. In the presence of a β-lactam antibiotic, MecR1 is activated and rapidly induces the expression of *mecA* and *mecR1-mecI-mecR2*. The anti-repressor activity of *MecR2* is essential to sustain the *mecA* induction since it promotes the inactivation of MecI by proteolytic cleavage. In the absence of β-lactams, MecR1 is not activated and a steady state is established with stable MecI-dimers bound to the *mecA* promoter and residual copies of MecR1 at the cell membrane.

4.2 Future Perspectives

The work present on this thesis highlights important details concerning the “players” that efficiently regulate the expression of methicillin resistance in MRSA. We found that the *mec* regulatory locus consists, in an unusual three-component arrangement enclosing, in addition to *mecR1-mecI*, the *mecR2* gene coding for an anti-repressor. The anti-repressor MecR2 is essential to optimal expression of β-lactam resistance in MRSA that carry the *mec* regulatory system fully functional. The main function of MecR2 in methicillin resistance is to bind directly to *mecA* repressor (MecI) leaving it accessible to proteolytic cleavage, presumably by native proteases. However, others aspects mainly regarding the MecI proteolysis, remain to be clarified. Thus, the experiments that will be done in order to clarify the MecI proteolysis event could be divided in three different themes, as follows:

(i) MecR2-MecI complex tridimensional structure

Since the X-ray 3D structure of the PBP2A (*mecA),* MecI, MecR1 [44] and MecR2 have already been resolved, we will aim to resolve the structure of the MecI-MecR2 complex. Both proteins will be expressed and purified according to the methods previously optimized [45] (see chapter III). MecR2 protein crystallizes preferentially with polyethylene glycol (PEG) and salts (sodium chloride or ammonium sulphate), as precipitants and buffers at pH 7-8, both at 4 and 20ºC, while MecI protein crystallizes spontaneously in the chromatography collection tube. The structural data will be then used to modulate the molecular details of the interactions involved in the control of *mecA* expression.

(ii) Validation of the key molecular interactions between MecI::MecR2 revealed by the 3D-struture

Functional analysis *in vivo* using MecR2 mutagenized variants revealed loss of function only when the disordered region T150-I160 (which may be involved in the interaction with MecI, based on MecR2 3D structural data analysis), was substituted by four glycine (see chapter III). In order to evaluate MecI key interactions identified by the 3-D structures using site directed mutagenesis, single point mutations will be introduced, both in residues engaged in *mecA*-binding promoter, and in residues involved in the transient interaction with the anti-repressor MecR2. The mutagenized *mecI* variants will be expressed in *trans* using the prototype strain COL (which expresses *mecA* constitutively, has no *mecI* and is β-lactamase negative) in order to evaluate its biological activity.

(iii) Identification of native proteases presumably involved in the proteolytic cleavage of the *mecA* repressor (MecI)

It has been proposed that the proteolysis of MecI observed during *mecA* induction is not dependent of the activated MecR1 inducer domain [38-40]. We have also corroborated this hypothesis, showing that MecR2-induced proteolysis of MecI does not involve MecR1 and, most likely, is mediated by unspecific cytoplasmic proteases. Since the MecR1 inducer domain was previously described as a putative protometalloprotease, in this task we will evaluate the role of native metalloproteases in the observed MecI cleavage. So far two metalloproteases have been described in *S. aureus*: the Aureolysin [46] and the FtsH metalloproteases [47]. Additionally, the role of a global protease, ClpP protease [48] on MecI preteolysis will be also evaluated. The chromosomal protease deletions will be transduced to our prototype MRSA strains and mutants for the *mecA* regulators in order to evaluate the effect on the phenotype expression of β-lactam resistance. Preliminary experiments conducted with the *aur* mutant have shown a decrease on the levels of oxacillin resistance when compared with the wild type strain. This may suggest that the Zinc-dependent aureolysin metalloprotease might be involved in the MecI cleavage. Western blotting analysis will be done to compare the MecI proteolysis from total proteins extracts of wild type strains *versus* protease mutants after induction with a β-lactam. The impact of these three protease mutants on the optimal expression of β-lactam resistance will be also evaluated. Eventually, the same strategy will be used to evaluate the role of other native proteases.

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