Role of β-lactamase operon on *mecA* expression in *Staphylococcus aureus*

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Dissertation for the Master Degree in Medical Microbiology

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen and is also emerging in the community. MRSA is cross-resistant to virtually all β-lactam antibiotics and has acquired two main resistance mechanisms: production of β-lactamase (*bla*), coded by *blaZ*, and production of penicillin binding protein 2a (PBP2a), coded by *mecA*. Both genes are regulated by homologous sensor-transducers (BlaR1 and MecR1) and repressors (BlaI and MecI), and coregulation of *mecA* and *blaZ* by both systems has been demonstrated, although with remarkable different efficiencies. In fact, induction of *mecA* by *mecI-mecR1* is so slow that it is believed it is not functional in most MRSA strains.

However, recent data from our laboratory has unexpectedly demonstrated that not only there is no correlation between the presence of *mecI* gene and the resistance level in epidemic MRSA strains, but also that for most strains there were no significant changes on the resistance phenotype upon the *mecI* overexpression in *trans*. Interestingly, the two strains in which *mecI* overexpression affected the resistance expression were negative for the *bla locus*, suggesting that this *locus* may interfere directly with the MecI-mediated repression of *mecA* and account for those puzzling observations.

In this master thesis we have explored this hypothesis using molecular biology strategies and phenotypic analysis of β-lactam resistance. The data obtained demonstrate that the presence of a wild-type plasmid containing the *bla locus* not only disrupts the MecI-mediated repression, but also significantly enhances the expression of resistance. Several preliminary hypotheses were formulated to explain these observations and preliminary data, together with published evidence, support the working model that BlaI forms functional hetero-dimers with MecI, which upon induction are readily inactivated by BlaR1. These results provide new insights into the regulatory mechanism(s) of *mecA* and open new perspectives for the role of β-lactamase operon in the MRSA phenotype.

RESUMO

Os *Staphylococcus aureus* resistentes à meticilina (MRSA, do inglês "methicillin-resistant *Staphylococcus aureus*") são um dos principais agentes responsáveis por infeções hospitalares. Os MRSA são resistentes a praticamente todos os antibióticos β-lactâmicos devido a dois mecanismos principais: produção de β-lactamase (*bla*), codificada pelo gene *blaZ*, e produção de uma proteína de ligação à penicilina (PBP2a, do inglês "penicillin binding protein 2"), codificada pelo gene *mecA*. Estes dois genes são regulados por sistemas homólogos, constituídos por um sensor-transdutor (BlaR1 e MecR1) e um repressor (BlaI e MecI), de tal modo que ambos os sistemas são capazes de co-regular os genes *mecA* e *blaZ*, embora com eficiências de indução muito diferentes. De facto, a indução mediada pelo sistema *mecI-mecR1* é tão lenta que se acredita que este sistema não está funcional na maioria das estirpes MRSA.

No entanto, dados recentes do nosso laboratório, demonstram a ausência de relação entre a presença do gene *mecI* e o nível de resistência à meticilina em estirpes MRSA epidémicas, e também que, o fenótipo de resistência da grande maioria das estirpes não é perturbado pela sobre-expressão em *trans* do repressor *mecI*. Curiosamente, as duas estirpes em que a expressão da resistência foi afetada pela sobre-expressão do *mecI* são negativas para o *locus* da β-lactamase, o que sugere que este *locus* pode interferir diretamente com a repressão do gene *mecA* mediada pelo MecI.

Nesta tese de mestrado esta hipótese foi explorada usando estratégias de biologia molecular e ensaios fenotípicos da resistência aos β-lactâmicos. Os resultados obtidos demonstram que a presença do plasmídeo nativo da β-lactamase não só anula a repressão mediada pelo MecI, como também aumenta o nível de resistência das estirpes parentais. Várias hipóteses foram então formuladas para explicar estas observações. Dados preliminares, em conjunto com evidências experimentais publicadas, sugerem que o BlaI forma hetero-dímeros com o MecI que, após a indução, são inativados eficientemente pelo BlaR1. Em conclusão, estes resultados apresentam novas perspetivas para o mecanismo de regulação do *mecA* e para uma nova importante função do operão da β-lactamase para o fenótipo das estirpes MRSA.

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ABBREVIATIONS

Amp: Ampicillin

ATc: Anidrotetracycline

bla: β-lactamase

bp: Base pairs

CA: Clavulanic acid

CA-MRSA: Community acquired methicillin-resistant *Staphylococcus aureus*

CC: Clonal complex

Cd: Cadmium

C_f: Final concentration

Cm: Chloramphenicol

dNTP's: Desoxiribonucleotides

GlcNAc: N-acetylglucosamine

IPTG: Isopropyl β-D-1-thiogalactopyranoside

IS: Insertion sequence

kb: Kilobase

LA: Luria-Bertani agar

LB: Luria-Bertani broth

MIC: Minimum inhibitory concentration

MLST: Multilocus sequence typing

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin-susceptible *Staphylococcus aureus*

MurNAc: N-acetylmuramic acid

Oxa: Oxacillin

PBP: Penicillin binding protein

PCR: Polymerase chain reaction

Pen: Penicillin

PFGE: Pulse field gel electrophoresis

SCC*mec*: Staphylococcal cassette chromosome *mec*

spa*A: Staphylococcus aureus* protein A

ST: Sequence type

Tc: Tetracycline

TSA: Triptic soy agar

TSB: Triptic soy broth

$\mathbf{V_f}$: Final Volume

VISA: Vancomycin-intermediate *Staphylococcus aureus*

VRSA: Vancomycin-resistant *Staphylococcus aureus*

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INTRODUCTION

1. The *Staphylococcus* genus

Staphylococci are gram positive cocci of about 0,8-1,0 µm in diameter that divide in perpendicular planes to form irregular clumps. They are facultative anaerobes, nonsporulating, but are resistant to drying and are readily dispersed in dust particles through the air and surfaces (77, 105).

Staphylococcus genus belongs to *Staphylococcaceae* family and so far 45 species and 24 subspecies have been identified (38). The genus contains common pathogens of humans and animals and commonly infects the skin and wounds, occasionally causing life-threatening diseases (92). In humans two major species are recognized, *Staphylococcus epidermidis*, a nonpigmented, comensal, nonpathogenic organism usually found on the skin or mucous membranes and *Staphylococcus aureus*, a yellow pigmented species that is often associated with pathological conditions (96).

2. *Staphylococcus aureus* as a human pathogen

2.1 Clinical relevance

Staphylococcus aureus is the leading cause of nosocomial infections ranging from minor skin diseases to severe infections, as endocarditis and septicemia (2). The primary reservoir for *S. aureus* is the nasal cavity and three carriage patterns have been proposed: persistent, intermittent and noncarriage (146). Based on surveillance studies, in the United States of America, the United Kingdom, Japan, and other countries, it is estimated that at any time one third of the population is colonized with *S. aureus*

asymptomatically (148). The nasal carriage can be a risk factor for endogenous infection as these commensal bacteria have the ability to disseminate and invade the host organism (111) but, normally, these individuals do not acquire disease, presumably because the other resident microorganisms compete successfully for resources and limit pathogen growth. In addition, in healthy individuals the innate immune system is particularly active at mucosal surfaces and may inhibit the microbial growth. Most infections result from a colonized individual that transmits to a weakened individual or infects a damaged tissue (25). The most common infections, impetigo, cellulitis and abscesses are the result of invasion and laceration of the skin or cellular tissues. The dissemination to adjacent tissues can originate bacteremia, endocarditis, osteomyelitis, arthritis and pneumonia. *S. aureus* infections are also associated with the presence of medical devices in the organism (78).

2.2 Virulence factors

The virulence of *S. aureus* is due to a combination of many virulent factors such as toxins, enzymes, cell wall components and antigens (92). *S. aureus* secrete several toxins responsible for different symptomatologies as food poisoning (enterotoxins A, B, C, D, E, G and H), scalded skin syndrome (exfoliating toxin A and B) and toxic shock syndrome (enterotoxin TSST-1). These toxins can work as superantigens stimulating large numbers of immune response cells, resulting in extended inflammatory reactions (91).

Along with toxins this bacteria secretes hemolisins (α, β, γ and δ), a leucocidin and a few enzymes (coagulase, hialurodinase, fibrinolisine, catalase, lipase and nucleases) that contribute to damage the host cell or stimulate a large number of lymphocytes and cause systemic inflammatory responses (32). The fibrin matrix produced as a result of coagulase activity protects the bacteria from attack by host cells and probably accounts for the extremely localized nature of many *S. aureus* infections as in boils and pimples (148, 150). This enzyme enables differentiation of *S. aureus* from many other staphylococcal species (26).

2.3 Antibiotic resistance

Antibiotic resistance is associated with the permanent change of the highly flexible bacterial genome under pressure (43). In fact, although *Staphylococcus aureus* is naturally susceptible to virtually every antibiotic developed so far (18), it is one of the pathogens of greatest concern because of its incredible facility to acquire antibiotic resistance traits along with the ability to cause life-threatening infections and to adapt to different conditions (89). Although chromosomal mutations are also important, resistance is often a consequence of horizontal gene transfer, mostly occurring in hospitals and healthcare institutions, where the selective pressures for resistance are greatest (27, 94).

As new antibiotics have emerged, such as, quinolones, aminoglycosides, oxalidiones, *S. aureus* has developed efficient mechanisms to neutralize them (88). But the increasing overall burden of staphylococcal disease in many countries in both healthcare and community settings is mainly caused by methicillin-resistant *S. aureus* strains (MRSA), which are virtually resistant to all classes of β-lactams (44). Infections caused by antibiotic-resistant strains of *S. aureus* have reached epidemic proportions in many parts of the world and resistant strains that are contained within hospitals temporarily, can eventually arise within the community (52).

2.4 Epidemiology of antibiotic resistance

Before the discovery of β-lactams the mortality rate of *S. aureus* invasive infection was about 80% (88). Introduction of penicillin into clinical practice, in 1940, allowed a drastic decrease in the mortality rate (103, 135). However, penicillin resistance has developed soon after as a natural evolutionary response of bacteria to this drug (30, 75). To combat penicillin-resistant strains, methicillin was introduced into clinical practice, in 1960. However, in 1961, the first MRSA strains were described (8). Over the years, new successful clones have arisen, giving rise to the on-going

worldwide pandemic of MRSA in hospitals, although the prevalence of infections may vary significantly in different countries (49, 136) (Fig. 1).

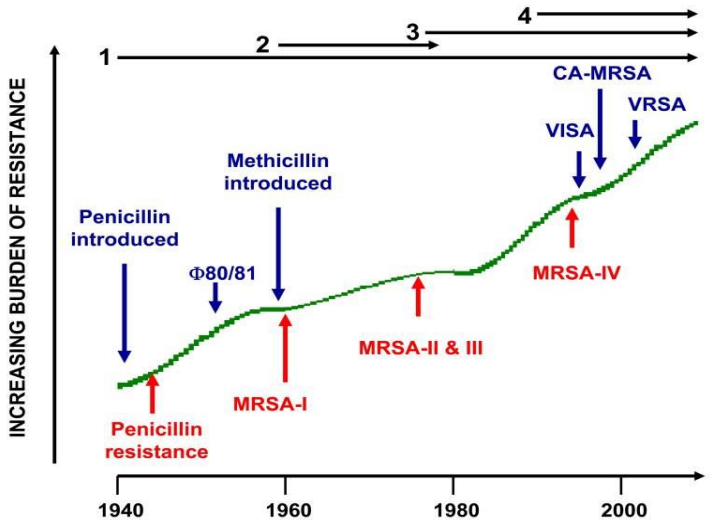


Figure 1 - Timeline of the four resistance waves in *S. aureus* (18). Wave 1 began after the introduction of penicillin into clinical practice and continues till today. Wave 2 had arisen after the introduction of methicillin into clinical practice (first MRSA strains). Wave 3 began with emergence of new MRSA strains, marking the on-going worldwide pandemic of MRSA. Wave 4 began with the emergence of MRSA strains in the community.

In response to β-lactams, *S. aureus* has acquired two main resistance mechanisms: production of β-lactamase, that hydrolyze the β-lactam ring of penicillin, and production of PBP2a, an extra penicillin binding protein (PBP) with low affinity to virtually all β-lactams (60, 95). This latter mechanism, characteristic of MRSA, together with the former, confers resistance to all β-lactams, including penicillins, cephalosporins and carbapenems. The β-lactam resistance genes were spread over time duo to horizontal transfer and clonal expansion in several waves (18).

Traditionally, MRSA strains have been recognized mainly as nosocomial pathogens. However, in recent years, its epidemiology has radically changed, being increasingly isolated in the community and affecting people without known risk factors (52). The first cases of community-acquired MRSA (CA-MRSA) infections were reported in indigenous populations in Australia, in the early 1990's (144), and soon after in the United States (113), which were followed by several reports worldwide (15, 123). Unlike hospital clones, CA-MRSA's were susceptible to most antibiotics but contained several virulence factors (22, 61, 106).

Most contemporary MRSA strains are resistant to many classes of antimicrobial agents leaving physicians with few therapeutic options. Glycopeptides are considered the last resort therapy against MRSA (76). However, in 1997, the first case of reduced susceptibility to vancomycin, designated VISA from vancomycin-intermediate *Staphylococcus aureus*, was described (63). Since then, several MRSA strains with reduced susceptibility to vancomycin have been found throughout the world (93, 133, 134). In 2002, for the first time, a strain fully resistant to vancomycin was identified, designated VRSA from vancomycin-resistant *Staphylococcus aureus* (33). In contrast to the chromosomally mediated resistance for VISA strains that result in a thickened cell wall (54, 55), the VRSA strains acquired the *vanA* operon from *Enterococcus faecalis*, which allows synthesis of the terminal peptide ended in D-Ala-D-Lac, rather than D-Ala-D-Ala. This new terminus has a remarkable reduced affinity for vancomycin (48, 129). Together with CA-MRSA, VISA and VRSA are the most recent waves of antimicrobial resistance in *S. aureus* (18).

2.5 Treatment and prevention

Extensive use of antibiotics has promoted the selection of resistant *Staphylococcus aureus* strains and, as a result, the therapeutic options are often scarce (34, 88). Prevention of staphylococcal infections is problematic because many individuals are asymptomatic carriers, and diseases such as acne and impetigo can be transmitted by simple contact with contaminated hands. To control this transmission in

surgery wards and nurseries the carriers of known pathogenic strains must be isolated or treated to eradicate the carrier state (35, 101). Nowadays, the treatment of choice for *S. aureus* infection in most countries is a penicillin-resistant β-lactam antibiotic (for example, oxacillin or cloxacillin) or a lipopeptide (daptomycin) (9, 127). Combination therapy with gentamicin may be used to treat serious infections like endocarditis, but its use is controversial because of the high risk of damage to the kidneys (23).

3. β-lactam resistance mechanisms

3.1 Cell wall: the β-lactams target

In gram positive bacteria the peptidoglycan is the main constituent of cell wall. It is a polymer with a complex organization that confers mechanic resistance to the cell (125). The basic unit of the peptidoglycan is a disaccharide-pentapeptide composed of the amino sugars N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), which are linked together by β-1,4 glycosidic bonds. In *S. aureus* each MurNAc is attached to a short amino acid chain that can be cross-linked to an amino acid chain of another strand through a pentaglycine, allowing the formation of peptide cross bridges (126). The polymerization of the newly synthesized disaccharide-peptide and incorporation into the growing peptidoglycan are achieved through the action of four penicillin-binding proteins (PBP1, PBP2, PBP3 and PBP4), which catalyze the transpeptidation and transglycosylation reactions responsible for the formation of the peptidic and glycosidic bonds, respectively (47, 124, 132).

β-lactams inhibit the cell wall synthesis (Fig. 2). All β-lactams have in common a β-lactam ring, which has a similar structure to the natural substrate of PBP's, the D-Ala-D-Ala terminus of the amino acid chain. Thus, the β-lactam competes with the natural substrate for the active site of these enzymes, preventing the final stages of peptidoglycan synthesis. This process causes primarily a cell growth arrest and ultimately autolysis and cell death (90). Notably, this class of antimicrobial agents

stands up in clinical practice due to the low toxicity in eukaryotic organisms, since they act in an exclusively bacterial structure (145).

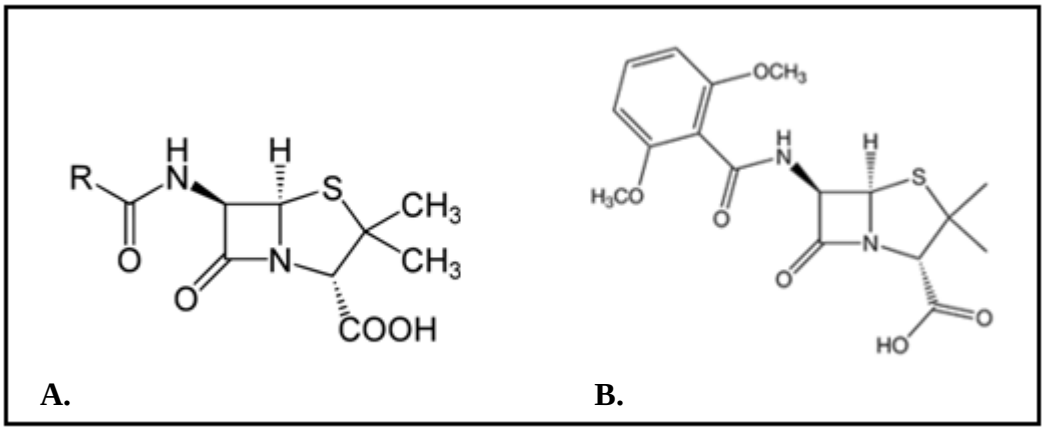


Figure 2 - Structure of Penicillin (A) and Methicillin (B).

3.2 Penicillin resistance

3.2.1 β-lactamase

In bacteria, four types of β-lactamases (A, B, C, D) have been distinguished by serotyping and differences in hydrolysis rates of selected β-lactam substrates (74). Types A, C and D are usually located on plasmids and are active-site serine enzymes, whereas type B enzymes typically reside in the chromosome and are zinc-dependent (97, 147). Structural evidences support the proposal that β-lactamases descended from the cell wall PBP's (97). The action of this group of enzymes consists on the interaction with the β-lactam antibiotic and subsequent disruption of the amide bond in the four-membered β-lactam ring, rendering the antibiotic inactive in an irreversibly manner (19, 86, 153).

Staphylococcal β-lactamases are from type A (115) and are large surface attached molecules that reduce the external level of active drug. Penicillin-resistant strains have acquired an exogenous plasmid coding for penicillinase, which confers resistance only to penicillin (86). When penicillin was introduced into clinical practice, only about 5% of *S. aureus* isolates acquired the plasmid but, since then, through horizontal transfer of the plasmid and strain selection, 80 to 90% of isolates carry the β-lactamase gene (82, 85, 86).

3.2.2 β-lactamase operon

In *S. aureus* the operon responsible for synthesis of β-lactamase is located in transposon Tn*552* (117) and contains the *blaZ* gene, which encodes for β-lactamase, and the regulatory genes *blaR1* and *blaI* (4, 139). These regulatory genes are divergently transcribed from *blaZ* (20) (Fig. 3).

BlaR1 is a high molecular weight sensor-transducer transmembrane protein and consists of two domains (97). One is a carboxyl-terminal domain of approximately 27-kDa, the sensor domain, extending to the extracellular medium and containing penicillin binding motifs, that have been shown to bind to β-lactam compounds, and an active site serine, which is involved in activation of the signaling cascade (73, 151). The other domain is an amino-terminal domain of approximately 38 kDa, the transducer, that is intracellular and contains four transmembrane α-helices (TM1, TM2, TM3, TMA4) (57). These transmembrane segments are interconnected by three loops (L1, L2, L3), where L1 and L3 connect to cytoplasm and L2 is exposed on the outside of the cell. The L3 segment has a zinc metalloprotease domain, defined by a histidine sequence and a glutamic acid, which is believed to interact in an unknown way with promoter-bound BlaI dimers (56, 155).

BlaI is a repressor that blocks transcription of both structural and regulatory genes and has been shown to bind specifically to two regions of dyad symmetry, the operators, which are located between *blaZ* and the regulator *blaR1* (R1 dyad and Z dyad) (20, 51). A dimeric BlaI, binds non cooperatively but with similar affinities to the two operators resulting in repression of *blaZ* along with the regulators (50). This

repressor has two functional domains. The amino-terminal domain of approximately 11 kDa is responsible for operator recognition, and the carboxyl-terminal domain of approximately 3 kDa for subunit dimerization (50). DNA-binding experiments demonstrate that formation of BlaI dimer, as well as intact amino and carboxyl termini, are absolutely required for the binding activity of the protein (20, 152). Proteolytic cleavage disrupts the dimer interface, causing its dissociation and releasing from the operator (155). In 1968, Cohen and Sweeney have speculated on the existence of another regulator, the chromosomal gene *blaR2*, involved in the induction of β-lactamase (21).

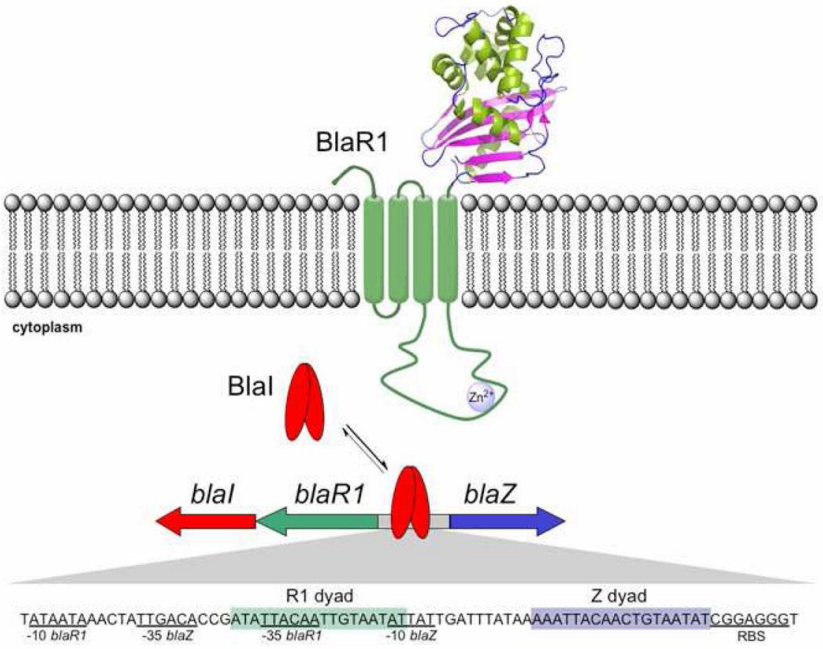


Figure 3 - β-lactamase operon (87).

3.3 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

3.3.1 *mecA* gene

The characteristic element of all methicillin-resistant *S. aureus* (MRSA) strains is a specific gene, *mecA*, which codes for PBP2a, an inducible 76 kDa PBP that is absent in susceptible strains (14, 60). In MRSA, PBP2a, which has low binding affinity to β-lactam antibiotics, can substitute for essential functions of high affinity PBP's and enables staphylococci to survive under exposure to high concentrations of these agents (60, 112). The organization of the *mec* operon is similar to the *bla* operon, containing the *mecA* gene and the respective regulators, *mecI* and *mecR1* (62). In fact, *bla* and *mec* regulatory genes have been shown to be interchangeable *in vivo* (83, 99).

Although clearly necessary, there are some evidences suggesting that *mecA* may not be sufficient to assure high-level resistance to methicillin. As a matter of fact, it has been shown that MRSA strains with virtually identical amounts of PBP2a, showed methicillin inhibitory concentrations (MIC) values spread over a range of several hundred fold (59, 119). Later it was demonstrated the importance of many chromosomal genes in defining resistance levels, namely the *fem* genes (for factor essential for methicillin resistance), that do not interfere with the transcription and transduction of *mecA* (10, 11). De Lencastre *et al.* proposed that the survival and growth of these bacteria in the presence of β-lactams require the cooperative functioning of a large number of genes, a process similar to the bacterial stress response mechanism (28).

3.3.2 SCC*mec*

The *mecA* gene is incorporated into a large mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*) (66). The emergence of MRSA lineages is due to the acquisition and insertion of SCC*mec* element into a chromosome gene of unknown function, the *orfX* gene, by susceptible strains (29, 68). SCC*mec*

contains the *mec* gene complex, which includes *mecA* and the regulatory genes *mecI* and *mecR1*, and the *ccr* gene complex, which encodes for recombinases that allow mobility of SCC*mec* (68). Besides these gene complexes, there are also three so-called J-regions (J1, J2 and J3), which constitute non-essential components of the cassette and may carry additional antimicrobial resistance determinants (65, 66).

SCC*mec* elements are highly diverse in their structural organization and genetic content (37) and have been classified into types and subtypes. Types are defined by the combination of the *ccr* gene complex allotype and the class of *mec* gene complex. Variations in the J regions within the same *mec*-*ccr* complex are used for defining subtypes. To date, eight major SCC*mec* types, designated I to VIII, have been recognized along with numerous subtypes (29, 68) and three new types, IX to XI, have been recently described (84, 131).

There are three classes for the *mec* gene complex in *S. aureus*. The class A *mec* gene complex, the prototype complex, contains intact *mecA*, *mecR1* and *mecI*, a hypervariable region (HVR) and insertion sequence (IS) IS431. The class B *mec* gene complex is composed of intact *mecA*, a truncated *mecR1* (N-terminal inducer domain only) that resulted from insertion of IS1272, a HVR and the same IS as class A. The class C *mec* is similar to class B but *mecR1* gene is truncated by IS431 (first 111 bp only) and is subdivided in two sub classes depending on the orientation of the IS's. The *ccr* complex consists of two adjacent genes, *ccrA* and *ccrB*, in SCC*mec* I-IV, VI and VIII, and *ccrC* in V and VII (65, 68).

3.3.3 Heterogeneous and homogeneous resistance

The phenotypic expression of methicillin resistance is highly dependent on growth conditions such as, temperature, medium salt concentration, growth phase and other external factors (128). In addition, many MRSA strains exhibit a heterogeneous expression profile, in which the majority of cells are susceptible to low concentrations of β-lactam antibiotic, with only a small proportion growing at high concentrations (119). Growth of a heterogeneous strain in the presence of β-lactam antibiotic alters the resistance phenotype by selecting for highly resistance clones. These clones produce a

homogeneous population of highly resistant cells that can grow at high concentration of antibiotic (59). In laboratory, with repeated subculture in antibiotic-free medium, the proportion of highly resistant cells gradually diminishes and the original heterogeneous pattern reemerges in most strains (141). There are some rare clinical isolates that consistently are homogeneous despite repeated subculture, with the COL strain of *S. aureus* being one of these (16, 59).

The phenomenon of heterogeneous and homogeneous resistance in wild-type strains is still unexplained. Heterogeneous strains may be deficient in a factor or lack a critical modification in a biochemical pathway, possibly for cell wall synthesis, that is important for functions of PBP2a. Homogeneous strains then arise from heterogeneous strains by antibiotic selective pressure favoring clones whose genetic background is well adapted for a fully functional PBP2a (16, 138).

In the clinical setting, the heterogeneous phenotypic expression of oxacillin resistance is a major problem, since it may originate false negatives in the phenotypic detection assays of resistance. Because these strains are in fact positive for *mecA*, prescription of β-lactam antibiotics may select for high-level β-lactam resistant MRSA subpopulations, causing treatment failures (42, 64).

Another type of methicillin resistance is the borderline (or low-level) resistance, exhibited by strains with a minimum inhibitory concentration (MIC) at or just above the susceptibility breakpoint (17, 102). Borderline strains can be divided in two types: strains with *mecA* gene and production of PBP2a, presenting a heterogeneous profile, and strains without *mecA* gene, which do not contain highly resistant subpopulations. These *mecA*-negative strains can result from modification of normal PBP genes or overproduction of staphylococcal β-lactamase (140). As this later mechanism led to low-level resistance it is not as clinically relevant as production of PBP2a (140).

3.3.4 Origin and evolution

In the search for the possible origin of *mecA*, some authors identified a genetic element closely related to the *S. aureus mecA* gene in the animal commensal species *Staphylococcus sciuri* (24). This element is the *pbpD* gene, which is the genetic

determinant of penicillin binding protein 4 (PBP4) of *S. sciuri* that was shown to share several properties with *S. aureus* PBP2a (1). However, this homologue was not identified as part of the *mec* gene complex or of the SCC*mec* element. In 2010 Tsubakishita *et al.* observed a potential mechanism of the generation of a new SCC*mec*-like element in *Macrococcus caseolyticus* (143), and, more recently, a divergent *mecA* homologue was discovered in human and bovine populations located in a novel staphylococcal cassette chromosome *mec* element (SCC*mec* type-XI) (45).

3.3.5 Molecular epidemiology of MRSA

Currently, the characterization of methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA clones is mostly based on three molecular methods.

Pulse field gel electrophoresis (PFGE) is a technique based on the resolution of large restriction fragments in an agarose gel, resulting from the digestion of total DNA with a rare cutter enzyme (e.g. *SmaI*) (98). The groups defined by PFGE are clustered into types, with a similarity coefficient of 80%, and subtypes, with a similarity coefficient of 95% (39).

Multilocus sequence typing (MLST) is a genotyping method based on sequence analysis of approximately 450 base pairs (bp) of seven housekeeping genes. Isolates with identical sequences at the seven genetic loci are grouped in the same sequence type (ST). *S. aureus* isolates that differ in less than three loci are assigned into the same clonal complex (CC) (36, 40).

Spa typing is a method designed for investigation of *S. aureus* outbreaks and relies upon analysis of variable numbers of tandem repeats in *spa* gene, which codes for protein A, a constituent of the cell wall. This method takes into account the number of repeats as well as point mutations (130).

Molecular typing of large international strain collections using these techniques has shown that 88% of the MSSA strains can be assigned to one of eleven clonal complexes, whereas MRSA strains are generally concentrated into one of six. This means that MSSA strains have a more diverse genetic structure and this is in agreement

with the hypothesis that MRSA derived recently from a limited number of MSSA lineages by acquisition of SCC*mec* (39, 66).

4. Regulation of β-lactam resistance

The signaling pathway that regulates β-lactam resistance has been studied for many years. Although β-lactamase and PBP2a are genetically and biochemically diverse, they are both dependent on a series of proteolytic cleavages of signaling components and are regulated by similar proteins (155). When a β-lactam antibiotic binds to the sensor domain of BlaR1 it reacts with the serine active site causing serine acylation, which is presumable, the initial event (151). Recently, it was hypothesized that signal propagation is mediated by an altered interaction between the BlaR1 sensor domain and the L2 extracellular loop, which might induce a conformational change in the transducer domain, leading to activation of the zinc-metalloprotease through a series of proteolytic steps (56, 57). Zhang *et al* proposed an autocatalytic cleavage of the transducer domain of BlaR1, metalloprotease dependent, which promotes, directly or indirectly, cleavage of the dimeric BlaI (155). Probably, other molecules are also required because it has not yet been demonstrated a direct association between the sensor-transducer and the repressor (3). The BlaI cleavage occurs near the carboxyl terminal and generates an 11-kDa and a 3-kDa fragment (155). C-terminal cleavage renders the repressor unable to dimerize, a necessary condition for repressor activity. Thus, proteolytic cleavage causes dimer dissociation and consequently, its releasing from DNA (87). However, the induction process does not promotes a completely cleavage of the repressor, and about 40-50 % of the intact dimer is still present (155). Fileé *et al* suggested the presence of a ligand acting as a co-activator that could displace the repressor from its respective operator, inducing a conformational change in the repressor. Thus, the BlaI proteolysis might be a secondary phenomenon resulting from the activity of cellular proteases (41). Following proteolysis, BlaI dissociates from its divergon binding site, enabling transcription of *blaZ*, *blaR1* and *blaI*, and, consequently

production of β-lactamase and the respective regulators (50). When BlaR1 is cleaved it can no longer be functional (12). Thus, this protein must be continually produced in order to sense the β-lactam and keep the signal-transduction active. This explains why BlaR1 production is linked with β-lactamase production (155). When the extracellular antibiotic concentration decreases, BlaR1 is no longer auto-activated, and in consequence the BlaI proteolytic cleavage stops. The repressors gain conditions to dimerize again and to bind DNA, suppressing *blaZ*, *blaR1* and *blaI* expression (99, 155).

This regulatory system is unique among bacteria. In fact, a signal transmitted by proteolytic events had not been described among these microorganisms so far (3, 155). Among the unknown questions concerning this signaling transduction pathway are the mechanism of BlaR1 acylation, the proteolytic cleavage events and the repressor mechanisms once the threat of the β-lactams has passed (43).

Expression of *mecA* is believed to be regulated by a similar pathway, with the respective regulators, the sensor-transducer, MecR1, and the repressor, MecI (3) (Fig. 4). Interestingly, the two repressors are virtually interchangeable, which is a consequence of their similarity (99). BlaI and MecI are almost identical, sharing 60% of amino acid identity and the proteolytic cleavage takes place at the same two amino acids in the same position (46). In fact, there is a cross-talk between both regulatory systems, which means that both can control the transcription of *blaZ* and *mecA* (53, 83, 99). However, this homology does not extend to the sensor-traducers, BlaR1 and MecR1, which share 34% amino acid identity only, and as such are not interchangeable (99).

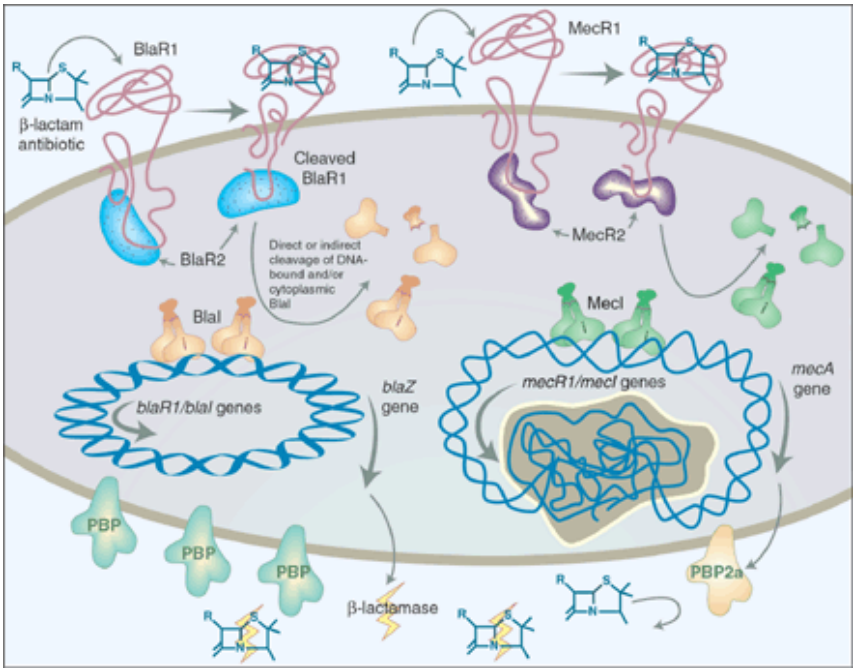


Figure 4 - Regulation of β-lactam resistance in *S. aureus* (3).

The main difference between these two systems is the kinetics of the signal-transduction. BlaR1 takes only a few minutes to induce *blaZ*/*mecA* expression, whereas MecR1 takes several hours (53, 99, 118). This might happen because MecI is a much stronger repressor than BlaI or because the poor response of MecR1 to some penicillins, such as methicillin and oxacillin (10). Moreover, the putative chromosomal encoded factor, BlaR2 may have a role in BlaI cleavage (21, 121). Actually, due to this slow induction most contemporary MRSA strains had lost or have mutations in the *mec* regulatory genes (138). Others, called pre-MRSA, that carry *mecA* and fully functional *mecI* and *mecR1* genes are, in clinical terms, phenotypically susceptible to methicillin, precisely due to the strong repression of MecI (81). Some studies that corroborate this observation showed that, the in vitro deletion of *mecI* caused the increase in the resistance levels of β-lactams (31, 81). In fact, in 1992, Hiramatsu *et al* stated that the high level of resistance to β-lactam antibiotics acquired by MRSA was explained by some genetic alterations in the regulatory genes *mecR1* and *mecI* (81, 138).

Interestingly, the great majority of clinical MRSA strains is positive for the β-lactamase *locus* (86). Therefore, in clinical isolates, the regulation of PBP2a is accomplished mainly by *bla* regulatory genes because of deletions and mutations in the *mec* regulatory genes (72). All these facts lead to the proposal of the current model for the transcriptional expression of *mecA* in contemporary MRSA strains: high-level resistance to β-lactams, implies non-functional *mecI-mecR1* regulatory system, and, strains possessing wild type regulatory genes present low resistance to β-lactams (81). Despite this fact, other studies have shown no correlation between the presence of MecI and the MIC level, as some strains negative for *mec* regulatory genes have a low resistance level, while other strains with intact *locus* are highly resistant (61, 103). Furthermore, two major pandemic nosocomial MRSA clones have a complete *mecI-mecR1 locus*, suggesting no correlation between the epidemicity of MRSA and regulators functionality (37, 109). Based on these contradictory observations, it has been postulated the existence of other unknown determinants controlling the *mecA* expression (79, 149).

5. Role of β-lactamase operon in the stabilization and expression of methicillin resistance in *S. aureus*

The restricted distribution of the SCC*mec* element within *S. aureus* population may be partly determined by strain properties that contribute to transformation efficiency and the stability of PBP2a, suggesting that some genetic backgrounds are better adapted than others to SCC*mec* and *mecA* acquisition (72). Chambers *et al.* observed that the major MRSA lineages might be favored recipients while MSSA strains can only tolerate *mecA* to some extent. This factor could account for the relatively limited clonal distribution of *mecA* in nature in addition to the fitness cost associated to SCC*mec* and the low antibiotic selective pressure (71).

Regulatory genes seem to have an important permissive role that allows a restrictive host genome to become transformed by *mecA* (13). Particularly interesting is

the ability of *mec* or *bla* regulatory genes to stabilize *mecA,* a finding that suggests a role of these elements in facilitating the dissemination of this gene. As *mecR1-mecI* genes strongly repress *mecA* expression, which is a survival disadvantage in the presence of a β-lactam antibiotic, it is likely that *bla* regulatory genes have played this role (72). In 1980 Stewart and Rosenblum observed that β-lactamase plasmid is a critical determinant for transduction of the methicillin resistance and reported that methicillin resistance tends to be unstable in clinical isolates when this plasmid is absent (137). These observations are understandable in view of the ability of the β-lactamase operon to stabilize *mecA* in some genetic backgrounds. The maintenance of a functional *blaZ* gene might be also useful for bacteria as a "first line defense" against first generation β-lactams (i.e. penicillins) or because it may be linked to other positively selected genes, as the cadmium resistance genes present in some β-lactamase plasmids. Moreover, β-lactamase likely causes little fitness cost as it is a secreted enzyme and much smaller than PBP2a, which is a transpeptidase with poor cross-link activity that has to be integrated into the cell-wall machinery. Therefore, one can speculate that there is a major advantage for MRSA strains to keep the β-lactamase *locus* (100), and in fact more than 95% of MRSA strains are still positive for *bla* genes (86), despite the fact that *mecA* can provide resistance to virtually all β-lactams. In short, β-lactamase regulatory genes seem to provide a compromise solution to the need for some control over PBP2a production to minimize the cost of maintaining *mecA* while also being able to express the protein in the presence of an antibiotic.

A recent study by Oliveira and Lencastre has challenged the current model for the transcriptional control of *mecA* in clinical MRSA strains (107). The authors overexpressed in *trans* the wild-type *mecI* gene in a collection of prototype MRSA clinical strains. These strains came from different clonal types and some have wild-type *mecA* regulatory genes, while others have mutations in these genes. According to the current model, it was expected a significant decrease in the oxacillin resistance phenotype, particularly for those strains with SCC*mec* types I and IV-VII, which do not have *mecI* gene. However, for virtually all strains, there was no significant decrease in the resistance phenotype, suggesting the presence of other yet unidentified elements that might contribute to the control of the expression of β-lactam resistance. Interestingly, the only two strains showing a decrease in the resistance phenotype were negative for

the β-lactamase operon. This observation suggests that the other strains containing the β-lactamase operon are, in some way, protected by the negative effect of the overexpressed MecI repressor in terms of resistance expression. Previous studies showing that the *bla* regulatory genes can efficiently control the *mecA* gene along with the *mec* regulators favor this hypothesis (53, 99), although the disruption of MecI-mediated repression directly by *bla* regulators has never been described.

This thesis came in the wake of these studies that suggest new perspectives for the role of *bla* genes in the stabilization and regulation of *mecA* gene, and, consequently, on the phenotypic expression of methicillin resistance in clinical MRSA strains.

MATERIALS AND METHODS

1. Bacterial strains and plasmids

Culture media, reagents, buffer solutions and antibiotics are listed in ANNEX. The strains and plasmids used in this study are listed in Table 1. *S. aureus* cultures stored at -80ºC were routinely grown on TSB or TSA (Difco) with aeration at 37ºC. *E. coli* strains were grown on LB or LA (Roth) with aeration at 37 ºC. Culture media were supplemented with antibiotics, when appropriate, at the following concentrations: chloramphenicol at 10 µg/mL, ampicillin at 100 µg/mL, tetracycline at 10 µg/mL, $CdCl_2$ at 50 µM, anidrotetracycline at 1 µg/mL and 2 µg/mL, and Isopropyl β-D-1-thiogalactopyranoside (IPTG) ranging from 1 mM to 1000 mM.

Table 1 - Strains and plasmids

Strain/plasmid	Relevant characteristics	Reference
Strains		
S. aureus		
COL	Homogeneous MRSA SCC*mec* I; β-lactamase negative	(109)
VNG17	Heterogeneous MRSA SCC*mec* IV; β-lactamase negative	(108, 120)
RJP17	Heterogeneous MRSA SCC*mec* IV; β-lactamase negative	(108, 120)
HT0350	Heterogeneous MRSA SCC*mec* V; β-lactamase negative	(142)
MW2	Heterogeneous MRSA SCC*mec* IV; β-lactamase positive	(6)
RN4220	MSSA strain, restriction-deficient mutagenized RN450	(80)

Table 1 – Cont.

Strain/plasmid	Relevant characteristics	Reference
COL-I	COL + pGC2::*mecI*	(107)
COL-I + p*bla*	COL + pGC2::*mecI* + p*bla*	This study
COL-I + *blaIblaR1*	COL + pGC2::*mecI* + pSPT181::P*spac*::*blaIblaR1*	This study
COL-I + O*blaIblaR1*	COL + pGC2::*mecI* + pSPT181::P*spac*::O*blaIblaR1*	This study
COL-I + *blaR1*	COL + pGC2::*mecI* + pSPT181::P*spac*::*blaR1*	This study
COL-I + O*blaR1*	COL + pGC2::*mecI* + pSPT181::P*spac*::O*blaR1*	This study
COL-I + *blaI*	COL + pGC2::*mecI* + pSPT181::*blaI*	This study
COL-I + NTD*blaR1*	COL + pGC2::*mecI* + pSPT181::P*spac*::NTD*blaR1*	This study
COL-I + ΔNTD*blaR1*	COL + pGC2::*mecI* + pSPT181::P*spac*::ΔNTD*blaR1*	This study
COL-I + L3*blaR1*	COL + pGC2::L3*blaR1*	This study
VNG17-I	VNG17 + pGC2::*mecI*	(107)
VNG17 + p*bla*	VNG17 + p*bla*	This study
VNG17-I + p*bla*	VNG17 + pGC2::*mecI* + p*bla*	This study
RJP17-I	RJP17 + pGC2::*mecI*	(107)
RJP17 + p*bla*	RJP17 + p*bla*	This study
RJP17-I + p*bla*	RJP17 + pGC2::*mecI* + p*bla*	This study
HT0350-I	HT0350 + pSPT181::*mecI*	This study
HT0350 + p*bla*	HT0350 + p*bla*	This study
HT0350-I + p*bla*	HT0350 + pSPT181::*mecI* + p*bla*	This study

Table 1 – Cont.

Strain/plasmid	Relevant characteristics	Reference
MW2-I	MW2 + pGC2::*mecI*	(107)
E. coli		
NEB 10-beta Competent *E. coli*	*araD*139 Δ(*ara-leu*)7697 *fhuA lacX74galK* (Φ80 Δ(*lacZ*)*M15*) *mcrA galU recA1 endA1 nupG rpsL* (Str^R) Δ(*mrr-hsdRMS-mcrBC*)	New England BioLabs
Plasmids		
p*bla*	Native β-lactamase plasmid from MW2 strain, Amp^r, Cd^r	(6)
pGC2	High-copy number *E. coli-S. aureus* shuttle plasmid, Cm^r	(104)
pSPT181	High-copy number *E. coli-S. aureus* shuttle plasmid, Tc^r	(69)
pKOR1	Thermosensitive *E. coli-S. aureus* shuttle plasmid, Cm^r	(7)
pSPT181::P*spac*	pSPT181 with P*spac* promoter and repressor *lacI* cloned from pDH88 (154)	Arêde, P.
pGC2::*mecI*	*mecI* gene from N315 strain cloned into pGC2	(107)
pSPT181::P*spac*::*blaIblaR1*	*blaR1blaI* fragment from MW2 cloned into pSPT181 under control of P*spac* promoter	Arêde, P.
pSPT181::P*spac*::O*blaIblaR1*	*blaR1blaI* fragment with operator region from MW2 cloned into pSPT181 under control of P*spac* promoter	Arêde, P.
pSPT181::P*spac*::*blaR1*	*blaR1* gene from MW2 cloned into pSPT181	Arêde, P.
pSPT181::P*spac*::O*blaR1*	*blaR1* gene with operator region from MW2 cloned into pSPT181	Arêde, P.
pSPT181::*blaI*	*blaI* gene from MW2 cloned into pSPT181	This study
pSPT181::P*spac*::NTD*blaR1*	N-terminal cytoplasmic sensor domain of *blaR1* cloned into pSPT181	This study

Table 1 – Cont.

Strain/plasmid	Relevant characteristics	Reference
pSPT181::P*spac*::ΔNTD*blaR1*	Truncated N-terminal domain of *blaR1* gene (without loop 3 metalloprotease) cloned into pSPT181	This study
pGC2::L3*blaR1*	504 bp of *blaR1* N-terminal domain containing the loop 3 metalloprotease cloned into pGC2	This study
pKOR1::Δ*blaR1*	1.0 kb upstream and downstream *blaR1* vicinities cloned into pKOR1	This study

2. Molecular methods

2.1 DNA isolation

Total DNA from *S. aureus* was isolated from bacterial cultures with the Wizard Genomic DNA purification Kit (Promega) according to the manufacturer's recommendation and using lysostaphin (10 µg/mL) and RNAse (10 µg/mL) in the lysis step (5). Alternatively, genomic DNA of *S. aureus* was isolated by a boiling prep with a lysis step at 37ºC for 30-60 minutes with 10 µg/mL of lysostaphin. Plasmid DNA was isolated from bacterial cultures with the High Pure Plasmid Isolation Kit (Roche). For *S. aureus* plasmid DNA isolation the cultures were incubated at 37ºC for 30-60 minutes with 10 µg/mL of lysostaphin in cell suspension buffer for an efficient cell-wall lysis.

2.2 DNA purification and manipulation

Restriction endonuclease digestions (New England Biolabs) were performed according to the manufacturer's directions. 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 Go Taq Flexi DNA polymerase (Promega). PCR primers and reagents are listed in ANNEX. PCR amplification of cloning inserts was obtained with the proof reading *Pfu* Turbo DNA Polymerase (Agilent). Recombination between PCR products (containing *att*B sites) and a donor vector (containing *att*P sites) were performed with Gateway BP Clonase II enzyme (Invitrogen) according to the manufacturer's directions.

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.

Transformation of recombinant plasmids into NEB 10-beta Competent *E. coli* cells (New England Biolabs) was performed in accordance with the manufacturer's recommendations. Selection of transformants was performed with ampicillin at 100 µg/mL.

2.3 Electrophoresis analysis of PCR and DNA restriction reactions

Routine electrophoresis analysis of PCR products and restriction reactions was analyzed by agarose gel electrophoresis at 0,8% (p/v) (Invitrogen) with Tris-Acetate-EDTA (TAE) at 85 V. 25 µL of a 1:10 dilution of Gel Red (Biotium) was added to each 100 mL of agarose. For band visualization, gel images were acquired in a transilluminator (Gel Doc, Quantity One 4.5.0, BioRad). Molecular weight estimations of the DNA fragments were made against 1 kb (kilobase) Plus DNA Ladder

(Invitrogen). DNA was quantified by U.V. spectroscopy with NanoDrop ND-1000 instrument (Thermo Scientific).

2.4 Electroporation of recombinant plasmids into *S. aureus*

Recombinant plasmids were introduced into electrocompetent restriction minus *S. aureus* strain RN4220 as previously described (122). Briefly, DNA and competent-cells were mixed in an electroporation cuvette with 0.2 cm electrode and submitted to electroporation in a Gene Pulser (BioRad) at the following settings: resistance 200 Ω, capacitance 25 µF, and voltage 2,5 kV. Immediately after the electric shock 1 mL of TSB was added to the cuvette. The mixture was transferred to an eppendorff tube and placed in a rotating device at 37ºC for one hour. Aliquots of 200 and 20 µL were spread onto TSA supplemented with antibiotic and incubated overnight at 37ºC.

2.5 Preparation of transducing lysates and transduction

Recombinant plasmids were transferred from strain RN4220 to other *S. aureus* strains by bacteriophage-mediated transduction as previously described (110). Briefly, for preparation of transducing lysates, donor strains were grown on BHI slants (Difco) overnight at 37ºC. Cells were collected with 1 mL of TSB and calcium chloride was added to a final concentration of 5mM. Phage 80α lysate was serial 10-fold diluted (10^{-2} to 10^{-7}) in phage buffer. 10 µL of cell suspension was mixed with 10 µL of each phage dilution and 3 mL of phage top agar, equilibrated at 45ºC and supplemented with 5mM of $CaCl_2$. The mixture was poured into plates containing phage bottom agar and incubated overnight at 30ºC. On the following day 2 mL of phage buffer were added to plates showing confluent lysis. Plates were kept at 4ºC for one hour. The phage top agar and the phage buffer were collected into a 15 mL falcon tube and vigorously vortexed in order to disrupt the agar. The tubes were kept for one more hour at 4ºC. The suspension

was centrifuged at 4500 rpm for 20 minutes at 4ºC. The supernatant was collected and filtered through a 0,45 µL sterile filter.

For transduction, recipient strains were grown overnight in a BHI slant. 100 µL of cell suspension supplemented with 5mM of $CaCl_2$ were added to 10 µL and 100 µL of the phage lysate from donor strain and phage buffer was added to a final volume of 300 µL. The mixture was incubated for 20 minutes at 37ºC and 3 mL of 0,3GL top agar, equilibrated at 45ºC, were added. The mixture was then poured onto plates of 0,3GL agar with a gradient of selective antibiotic. Plates were incubated overnight at 37ºC.

3. Overexpression of β-lactamase regulatory genes

A DNA fragment containing the wild-type *blaI* coding region and the putative ribosomal binding site from the prototype strain MW2 was amplified by PCR with the high-fidelity *Pfu* Turbo DNA polymerase (Agilent) with primers blaF5 and blaR9 (ANNEX) containing the recognition sequences for endonucleases *PstI* and *BamHI*, respectively. After double digestion and purification, the inserts were directionally cloned into the multiple cloning site of pSPT181. pSPT181 is a high-copy number *E. coli/ S. aureus* shuttle plasmid with resistance determinants to ampicillin (*E. coli*) and tetracycline (*S. aureus*), with a T6 promoter upstream to the multiple cloning site. The integrity of the insert was verified by DNA restriction and PCR analysis. Two recombinant plasmids were then introduced in parallel into the restriction-deficient *S. aureus* strain RN4220 by electroporation and then transduced into the COL + pGC2::*mecI* strain using phage 80α. Using the same strategy, a PCR fragment containing the metalloprotease loop 3 (L3) domain, obtained from strain MW2, was amplified with the *Pfu* Turbo DNA polymerase with primers blaL3F1/ *EcoR1* and blaL3R1/ *BamHI* (ANNEX), cloned into pGC2 plasmid and introduced into strain COL + pSPT181::*mecI* in two experimental replicas. pGC2 is a high-copy number *E. coli/ S. aureus* shuttle plasmid with resistance determinants to ampicillin (*E. coli*) and

chloramphenicol (*S. aureus*), in which the multiple cloning site is flanked by the strong SP6 and T7 bacteriophage promoters.

The N-terminal cytoplasmic domain of *blaR1* with or without the metalloprotease L3 domain were also PCR amplified with the *Pfu* polymerase along with the putative ribosomal binding site, from the prototype strain MW2, with primers blaR1F2, blaR1F3 and blaR10 (ANNEX) containing recognition sequences for endonuclease *XmaI*. After digestion of PCR fragments, linearization of expression vector pSPT181::P*spac* and dephosphorylation of vector arms, fragments were ligated to the multiple cloning site of the vector. The recombinant plasmids were then transformed and propagated in NEB 10-beta Competent *E. coli* cells. After the verification of insert integrity and orientation, the recombinant plasmids were electroporated into RN4220 and subsequently transduced to COL + pGC2::*mecI* in two experimental replicas.

4. Genetic knock-out of β-lactamase regulatory genes (Work in progress)

Genetic knock out's were done by allelic replacement with inducible counter-selection using pKOR1 plasmid (7). This plasmid is an *E. coli/ S. aureus* shuttle vector with specific characteristics. It contains a lambda recombination cassette allowing efficient cloning without the use of restriction enzymes and ligases. This cassette encodes for *ccdB*, an *E. coli* gyrase inhibitor that suppresses the growth of cells containing pKOR1 without insert. pKOR1 has a thermosensitive origin of replication in *S. aureus* which facilitates the chromosomal integration at non-permissive temperature (43ºC). Moreover, in *S. aureus* the plasmid allows selection for chromosomal excision and plasmid segregation via inducible antisense expression of the essential gene *secY* that is controlled by an anydrotetracycline (ATc) inducible promoter (Fig. 5).

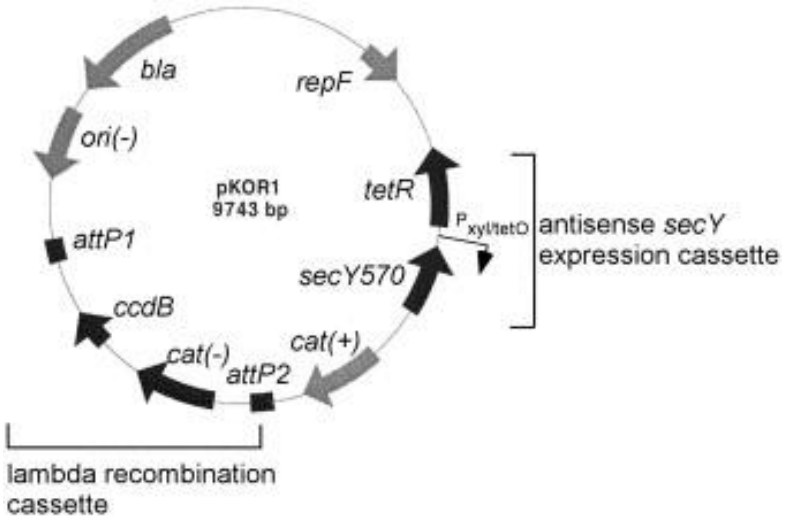


Figure 5 - Map of pKOR1. *repF* (Replication gene of pE194ts), *secY570* (N-terminal 570 nucleotides of *secY* including ribosome binding site), *cat* (chloramphenicol acetyltransferase), attP (phage lambda attachment site), *ori*(-) (ColE1 plasmid replication origin), *bla* (β-lactamase). (+) or (-) indicates gram-positive (+) and gram-negative (-) bacteria (7).

For the genetic knock out of *blaR1*, DNA fragments of 1 kb were PCR amplified upstream and downstream of the regulator, using the primers attB1-blaF6 and blaR6-*BamHI*, for the upstream fragment, and *BamHI*-blaF8 and attB2-blaR7, for the downstream fragment (ANNEX). The PCR products were digested with *BamHI* and ligated with T4 ligase. The ligation product was used for recombination with pKOR1 and the recombinant products were transferred to NEB 10-beta Competent *E. coli* cells. The resulting plasmid, pKOR1::Δ*blaR1* was transferred via electroporation to the *S. aureus* restriction minus strain RN4220 and then transduced into the parental strain, using phage 80α and selection with 10 µg/ mL of chloramphenicol.

Attempts to insert pKOR1::Δ*blaR1* in strains with *bla* locus integrated into the chromosome failed due to the resistance of those strains to bacteriophage infection. Therefore we set up a strategy to generate genetic knock-out's in the β-lactamase plasmid which is not single-copy. For the integration of recombinant pKOR1::Δ*blaR1*

into β-lactamase plasmid, COL strain transformed with both plasmids was grown at 43ºC on TSB supplemented with 10 µg/mL of chloramphenicol. A transducing-lysate of this culture was prepared and transduced back to strain COL with chloramphenicol and ampicillin selection, in order to select p*bla*-pKOR1::Δ*blaR1* co-integrates only. Next, one colony was picked and inoculated in TSB supplemented with 10 µg/mL of chloramphenicol at 30ºC, a permissive temperature, which enables co-integrate resolution. A transducing-lysate of this culture was prepared and transduced back to strain COL with ampicillin selection and anydrotetracyclin counter selection at 1 and 2 µg/mL. Control experiments were made without anydrotetracyclin. Plates were incubated at 30ºC for 2 days. Deletion of *blaR1* was confirmed by PCR amplification with primers blaF9 and blaR8 (ANNEX).

5. Phenotypic analysis

Susceptibility to oxacillin was routinely analyzed by disc diffusion method with 1 µg oxacillin discs prepared in-house. The cultures were homogeneously spread in a TSA plate with a swab and the antibiotic discs were carefully placed. The plate was incubated at 30ºC for 48 hours. The growth inhibition area was measured with a scale and compared with parental strains.

To infer the contribution of *blaZ* gene, TSA plates were supplemented with 2 µg/mL of clavulanic acid, a β-lactamase inhibitor, and susceptibility to oxacillin and penicillin was evaluated with 1 µg and 10 U diffusion discs, respectively. IPTG was added into TSA plates in a concentration range of 1 - 1000 mM when appropriate.

The parental and recombinant strains were tested by population analysis profiles (PAPs), as previously described (70). In short, 10 mL drops of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions of the overnight cultures were plated on TSA plates containing 0, 0.75, 1.5, 3, 6, 12.5, 25, 50, 100, 200, 400 and 800 mg/mL of oxacillin. The plates were tipped onto a 90° angle, and the drops migrated in parallel ways across

the plate to the opposite side. The plates were then incubated at 30°C. For each oxacillin concentration, colonies were counted for the first dilution with non-confluent growth after 24 h and 48 h of incubation at 30ºC.

RESULTS

According to recent data from our laboratory, the oxacillin resistance phenotype of most MRSA strains is not affected by the overexpression *in trans* of the *mecA* repressor (107). This surprising observation contradicts the current model and suggests the presence of other elements involved in the transcriptional control of *mecA* gene. The only two strains for which a decrease of the oxacillin-resistance phenotype was observed were negative for the β-lactamase *locus*, suggesting that this *locus* might be involved in that phenomenon. In order to explore the putative effect of the β-lactamase operon in the protection against the repressive effect of *mecI*, several exploratory experiments were performed, as described below.

1. Introduction of native β-lactamase plasmid into prototype strains

1.1 Introduction of native β-lactamase plasmid into strain COL-I

The prototype strain COL has a high and homogeneous level of oxacillin resistance, is negative for *mecI* and has a partially deleted *mecR1*, is naturally negative for the β-lactamase *locus* and has been used in many studies addressing the oxacillin-resistance mechanisms. Recombinant strain COL-I, overexpressing *in trans* the *mecA* repressor, pGC2::*mecI*, is characterized by a massive decrease in the resistance level. In order to evaluate the role of β-lactamase operon in the observed "MecI-protection effect", we have first introduced the native β-lactamase plasmid into strain COL-I. Strain MW2, similarly to COL, has no *mecI* and a partially deleted *mecR1*, but is β-lactamase positive and its oxacillin resistance phenotype was not affected by the *mecI* overexpression. Moreover, MW2 genome and plasmid sequence have been determined (6). The native β-lactamase plasmid from MW2 strain (p*bla*) was introduced into COL-I

strain via 80α bacteriophage-mediated horizontal gene transfer with selection for ampicillin resistance. The strategy turned out successful with the introduction of the large β-lactamase plasmid into strain COL first, followed by the introduction of the recombinant pGC2::*mecI*. The presence of both plasmids was confirmed by restriction analysis and PCR detection of the β-lactamase operon and *mecI* gene. This experiment was done in two independent replicas.

When oxacillin susceptibility was tested, it was observed that the transformant COL-I in the presence of β-lactamase plasmid restored the resistance phenotype of strain COL in spite of the overexpression of *mecI* gene. These observations were also confirmed by population analysis profile (PAP) assays (Fig. 6 and Table 2).

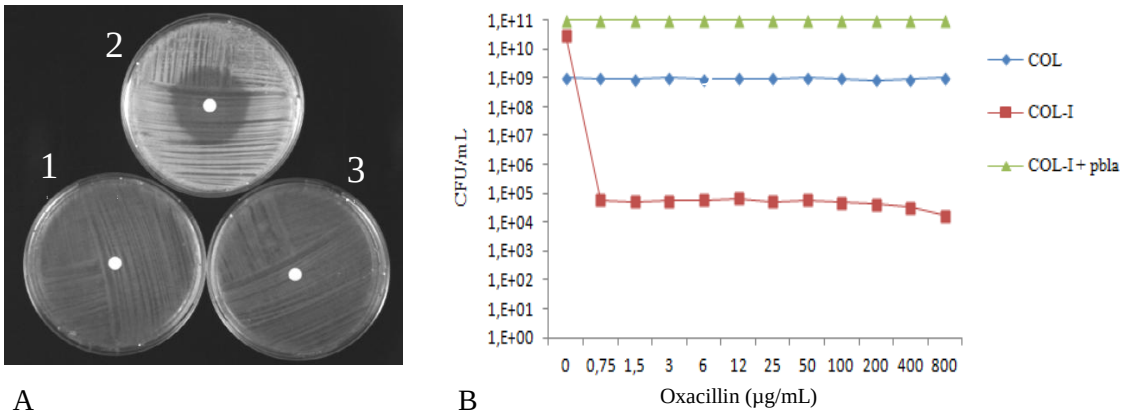


Figure 6. Phenotypic expression of oxacillin resistance in COL and recombinant strains.
A. Disc diffusion test 1: COL; 2: COL-I; 3: COL-I + p*bla*; B. Population analysis profile.

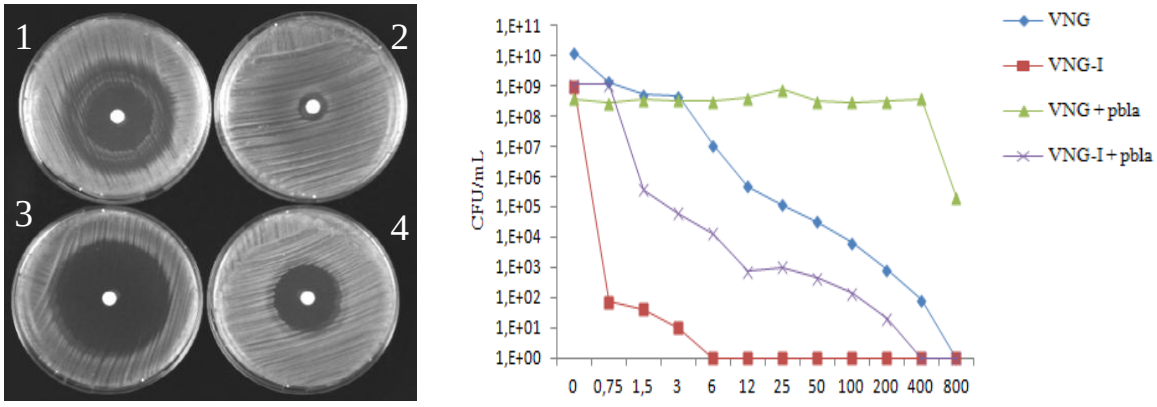
Table 2 - Oxacillin-resistance of parental strain COL and recombinant strains

Strain	Relevant genotype	Oxacillin-disc inhibition halo (mm)
COL	*mecI* $^{-}$ Δ*mecR1 bla* $^{-}$	8
COL-I	*mecI* Δ*mecR1 bla* $^{-}$	32
COL-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	7

1.2 Introduction of native β-lactamase plasmid into VNG17 and VNG17-I strains

Strain VNG17 and VNG17-I (overexpressing *mecI*) were transformed with the β-lactamase plasmid of strain MW2 (p*bla*). Given that strain COL-I showed a revertable phenotype in the presence of the β-lactamase plasmid we aimed to confirm these observations in other strains also negative for the β-lactamase *locus*. Strain VNG17 was the only other strain in which the overexpression of *mecI* caused a decrease in the oxacillin-resistance. Similarly to COL, strain VNG17 has no *mecI* and partially deleted *mecR1* but has a low-level resistance to oxacillin. The procedure was the same for introduction of the native β-lactamase plasmid into COL-I strain. The plasmid from prototype MW2 strain was introduced into VNG17 and VNG17-I strains via 80α bacteriophage-mediated horizontal gene transfer. For strain VNG17-I the protocol was also more successful with introduction of the β-lactamase plasmid into strain VNG17 first, following introduction of the pGC2::*mecI*. The presence of the plasmids was confirmed by restriction analysis and PCR detection of the β-lactamase operon and *mecI* gene.

As shown in Fig. 7 and Table 3, the introduction of the β-lactamase plasmid either in the parental or recombinant strain with *mecI* overexpression, caused a massive decrease in the susceptibility to oxacillin. In addition, the parental strain transformed with p*bla* showed a remarkable shift from low-level and heterogeneous to high-level and homogeneous expression of oxacillin resistance.



A B

Figure 7 - Phenotypic expression of oxacillin resistance in VNG17 and recombinant strains.
A. Disc diffusion test 1: VNG17; 2: VNG17 + p*bla*; 3: VNG17-I; 4: VNG17-I + p*bla*; B. Population analysis profile.

Table 3 - Oxacillin-resistance of parental strain VNG17 and recombinant strains

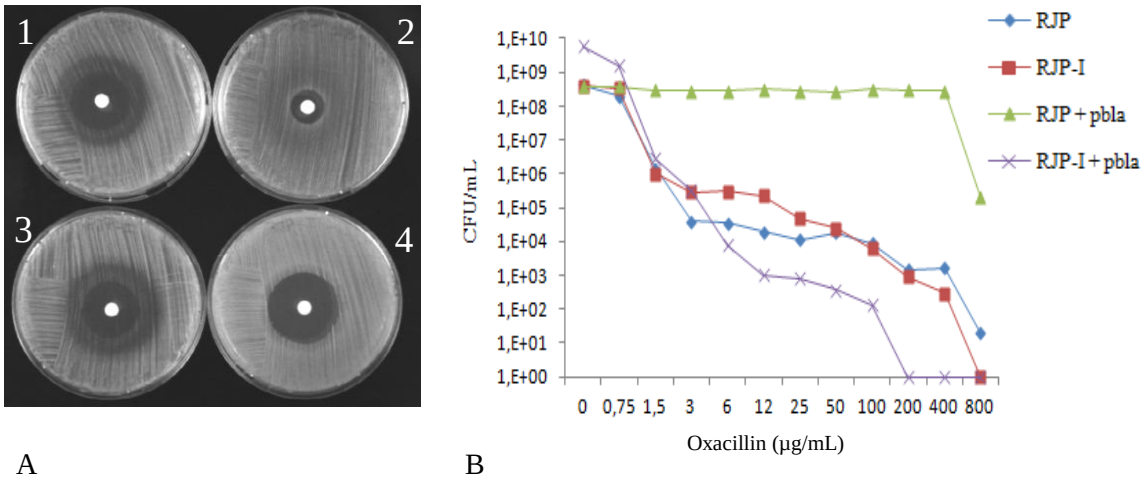
Strain	Relevant genotype	Oxacillin-disc inhibition halo (mm)
VNG17	*mecI* $^{-}$ Δ*mecR1 bla* $^{-}$	33/53[a]
VNG17 + p*bla*	*mecI* $^{-}$ Δ*mecR1 blaZ blaI blaR1*	13
VNG17-I	*mecI* Δ*mecR1 bla* $^{-}$	57
VNG17-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	33/38[a]

[a] Heterogeneous population

1.3 Introduction of native β-lactamase plasmid into RJP17 and RJP17-I strains

RJP17 strain is also a β-lactamase negative strain, which belongs to the same clone of VNG17 and was isolated in the same country and time period. Similarly to VNG17, RJP17 is *mecI* negative, has a partial deleted *mecR1* and expresses low level resistance to oxacillin. However, it was not detected any alteration in the oxacillin resistance phenotype upon the overexpression of *mecI* (strain RJP17-I) (107). Nevertheless, we have also evaluated the effect of the β-lactamase *locus* in this strain. Introduction of the β-lactamase plasmid in the recombinant strain overexpressing the *mecI* gene, RJP17-I, was also tested. The procedure was exactly the same as performed for VNG17 and VNG17-I strains. Results are summarized in Fig. 8 and Table 4.

Since the resistant phenotype was not affected by the overexpression of MecI, the presence of p*bla* in strain RJP17-I did not cause any significant alterations. However, similarly to what was observed for strain VNG17, the parental strain transformed with p*bla* shifted to a high-level and homogeneous expression profile of oxacillin resistance.



A B

Figure 8 - Phenotypic expression of oxacillin resistance in RJP17 and recombinant strains.
A. Disc diffusion test 1: RJP17; 2: RJP17 + p*bla*; 3: RJP17-I; 4: RJP17-I + p*bla*; B. Population analysis profile.

Table 4 - Oxacillin-resistance of parental strain RJP17 and recombinant strains

Strain	Relevant genotype	Oxacillin-disc inhibition halo (mm)
RJP17	*mecI* $^{-}$ Δ*mecR1 bla* $^{-}$	27/42[a]
RJP17 + p*bla*	*mecI* $^{-}$ Δ*mecR1 blaZ blaI blaR1*	15
RJP17-I	*mecI* Δ*mecR1 bla* $^{-}$	27/44[a]
RJP17-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	38/40[a]

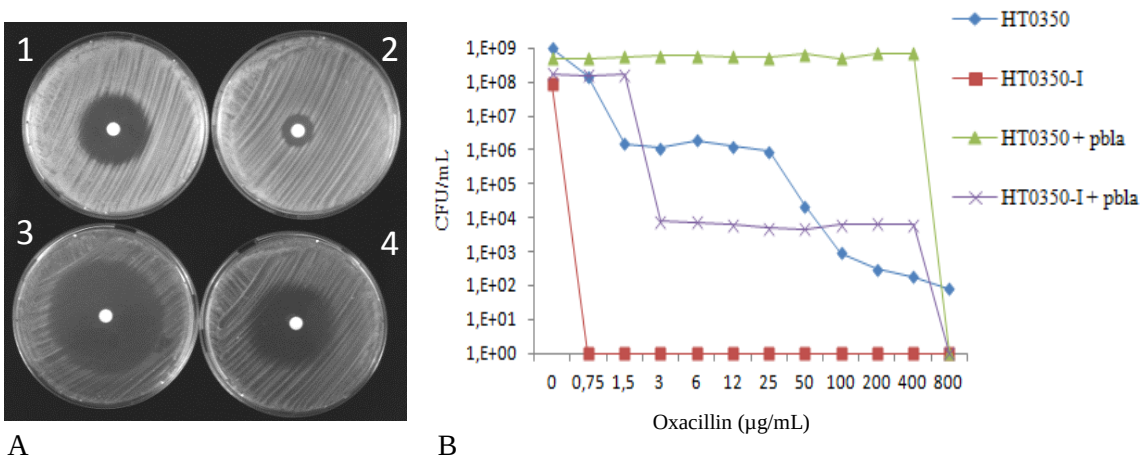
[a] Heterogeneous population

1.4 Introduction of native β-lactamase plasmid into HT0350 strain

HT0350 strain is other β-lactamase negative strain but with a more extensive deletion of *mecR1* due to the presence of IS431, a typical characteristic from SCC*mec* type V strains. While strains COL, VNG17, RJP17 still have a complete N-terminal domain of *mecR1* with the four-transmembrane segments (960 amino acids), strain HT0350 has only the first 36 amino acids of the MecR1. Similarly to VNG17 and RJP17, HT0350 is negative for *mecI* gene and expresses low-level resistance to oxacillin. The plasmid from prototype MW2 strain was introduced into this strain, via 80α bacteriophage-mediated horizontal gene transfer, in order to test, the effect of the β-lactamase *locus*. As we can see in Fig. 9 and Table 5 the β-lactamase plasmid promoted a significant increase in the oxacillin resistance phenotype.

Since strain HT0350 is intrinsically resistant to chloramphenicol, it was not included in previous *mecI* overexpression studies with plasmid pGC2, which carries a chloramphenicol resistance marker. Therefore, *mecI* was cloned in pSPT181, which carries a tetracycline resistance marker, and HT0350 phenotypic expression of oxacillin-resistance was evaluated in the presence of *mecI* overexpression in *trans*.

Thereafter, the β-lactamase plasmid was introduced in the HT0350-I strain, similarly to the above experiments. The results of the *mecI* overexpression and the later introduction of p*bla* are shown in Fig. 9 and Table 5. The presence of the β-lactamase plasmid in the parental strain promoted a shift from low-level and heterogeneous to high-level and homogeneous phenotypic expression of oxacillin resistance, and the recombinant strain HT0350-I restored the phenotype of the parental strain.



A B

Figure 9 - Oxacillin susceptibility test of parental strain HT0350 and recombinant strains.
A. Disc diffusion test. 1: HT0350; 2: HT0350 + p*bla*; 3: HT0350-I; 4: HT0350-I + p*bla*; B. Population analysis profile.

Table 5 - Oxacillin-resistance of parental strain HT0350 and recombinant strains

Strain	Relevant genotype	Oxacillin-disc inhibition halo (mm)
HT0350	*mecI* ⁻ Δ*mecR1 bla* ⁻	35
HT0350 + p*bla*	*mecI* ⁻ Δ*mecR1 blaZ blaI blaR1*	16
HT0350-I	*mecI* Δ*mecR1 bla* ⁻	56
HT0350-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	40

Once we have confirmed the role of β-lactamase plasmid in the phenotypic expression of oxacillin-resistance and the interference with the *mecI*-mediated expression of *mecA*, we aimed to further experiments to investigate which element(s) of the β-lactamase operon would be, specifically, involved.

2. Phenotypic effect of clavulanic acid

Clavulanic acid is an inhibitor of the β-lactamase (114) and, as such, its incorporation in the oxacillin susceptibility testing allows to infer the specific contribution of *blaZ* gene in the phenotypic expression of oxacillin resistance. For this purpose, the oxacillin-resistance phenotype was re-evaluated for all parental and recombinant strains in TSA plates supplemented with 2 µg/mL of clavulanic acid. Table 6 shows the phenotypic effect of clavulanic acid in all parental and recombinant strains. As a control, we tested the susceptibility to penicillin (coded by *blaZ*) in the presence and absence of clavulanic acid. The addition of clavulanic acid caused an increase in the susceptibility to penicillin in all strains positive for p*bla*. Regarding the oxacillin-resistance phenotype, all strains presented a similar resistance phenotype in the presence and absence of clavulanic acid.

Table 6 - Clavulanic acid effect on oxacillin and penicillin-resistance phenotype

	Inhibition halo diameter (mm)			
	Oxa disc		Pen disc	
Clavulanic acid (µg/mL)	0	2	0	2
MW2	30/39[a]	29/39[a]	8	11
MW2-I	30/40[a]	30/40[a]	7	11
COL	11	12	7	7
COL-I	15/40[a]	19/39[a]	28	27
COL-I + p*bla*	13	13	7	7
VNG17	34/52[a]	31/51[a]	15/45[a]	15/46[a]
VNG17 + p*bla*	17/20[a]	12/17[a]	7	10
VNG17-I	58	57	50	50
VNG17-I + p*bla*	35	35	7	10
RJP17	29/39[a]	27/38[a]	15/35[a]	14/34[a]
RJP17 + p*bla*	16	16	7	7
RJP17-I	29	28	16	15
RJP17-I + p*bla*	34	35	7	11
HT0350	35	35	7	11
HT0350 + p*bla*	16	16	7	22
HT0350-I	50	50	43	43
HT0350-I + p*bla*	38	37	7	7

[a] Heterogeneous population

3. Introduction of β-lactamase regulators into COL-I strain

In order to explore the mechanisms by which the *bla* regulators interfere with MecI repression and "boost" the phenotypic expression of oxacillin resistance, recombinant plasmids containing only the regulators were introduced into COL-I strain via bacteriophage-mediated horizontal gene transfer.

3.1 Introduction of *blaIblaR1* into COL-I strain

Previously, the *blaIblaR1* coding region has been cloned in pSPT181 plasmid under the control of the inducible promoter P*spac* (Arêde, P., unpublished data). At the same time, a similar construct was performed cloning this segment with the operator region, O*blaIblaR1*. These constructs were introduced into COL-I strain and oxacillin susceptibility was tested in the presence of several concentrations of IPTG. As shown in Table 7, the resistance phenotype of parental strain COL could not be restored in strain COL-I transformed with the β-lactamase regulators. Furthermore, the expression of these genes caused an increase in the susceptibility. The phenotype was not influenced by the presence or absence of the operator region, since it was not observed any significant difference between the two sets of experiments.

3.2 Introduction of *blaR1* and *blaI* genes into COL-I strain

Since introduction of both regulators simultaneously did not reproduce the effect of the whole β-lactamase plasmid, showing an even more susceptible phenotype, the two regulators were tested separately.

The *blaR1* gene has been previously cloned into pSPT181 plasmid under the control of the inducible promoter, P*spac*, as well as the same sequence plus the operator region, O*blaR1* (Arêde, P., unpublished data). The *blaI* gene was cloned in the pSPT181 plasmid under the control of the constitutive promoter T6. The constructs were introduced into strain COL-I and the susceptibility to oxacillin was tested. The presence of either β-lactamase regulator did not restore the oxacillin-resistance phenotype, as previously seen in the presence of p*bla*. Indeed, introduction of *blaR1* gene originates a phenotype even more susceptible than COL-I strain, similar to the phenotype upon introduction of *blaR1blaI*. The presence of the operator region did not seem to influence the resistance phenotype, since the halo diameters were similar. The presence of only *blaI* gene had no effect in the resistance phenotype of strain COL-I, presenting a similar halo diameter (Table 7).

3.3 Introduction of *blaR1* domains into COL-I strain

In order to overcome what seem to be a toxic effect from BlaR1, two short BlaR1 domains were cloned into the expression vector pSPT181, under the control of the inducible promoter P*spac*. The first constructed, called NTD*blaR1*, contains the initial 817 bp of *blaR1* and includes the zinc metalloproteases domain, thought to be involved in the signal transducing. The second construct, called ΔNTD*blaR1*, contains the initial 511 bp of *blaR1* and do not include the zinc metalloprotease domain. The constructs were introduced into COL-I strain and susceptibility to oxacillin was tested under the presence of different IPTG concentrations. The results are shown in Table 7. Neither construct showed an influence in the increased resistant phenotype seen above with introduction of the whole plasmid. Moreover, the overexpression of these BlaR1 domains causes an increase in the susceptibility.

Since even the smaller *blaR1* constructs seemed to increase, as well, the oxacillin susceptibility of COL-I strain, we designed a construct containing the coding region for L3 metalloprotease domain only, which was cloned into pGC2 plasmid and introduced into COL + pSPT181::*mecI*. This construct is the smallest possible in order

to include the metalloprotease domain, with 504 bp only, corresponding to the cytoplasmic loop 3. However, the presence of this element, did not restore the resistance phenotype of COL strain, originating a similar oxacillin-resistance phenotype to strain COL-I.

Table 7 - Oxacillin-resistance of strain COL transformed with β-lactamase regulators

Strain	Relevant genotype	IPTG (mM)	Oxacillin-disc inhibition halo (mm)
COL	*mecI*$^-$ Δ*mecR1 bla*$^-$	-	8
COL-I	*mecI* Δ*mecR1 bla*$^-$	-	33
COL-I +pSPT181::P*spac*:: *blaR1blaI*	*mecI* Δ*mecR1 blaR1 blaI*	0	32/45[a]
		100	40
COL-I +pSPT181::P*spac*:: O*blaR1blaI*	*mecI* Δ*mecR1 blaR1 blaI*	0	39
		100	40
COL-I +pSPT181::P*spac*:: *blaR1*	*mecI* Δ*mecR1 blaR1 blaI-*	0	37/15[a]
		100	41/19[a]
COL-I +pST1P81::P*spac*:: O*blaR1*	*mecI* Δ*mecR1 blaR1 blaI-*	0	40
		100	39
COL-I +pSPT181::P*spac*:: NTD*blaR1*	*mecI* Δ*mecR1* Δ*blaR1 blaI-*	0	46/24[a]
		100	40/23[a]
COL-I +pSPT181::P*spac*:: ΔNTD*blaR1*	*mecI* Δ*mecR1* Δ*blaR1blaI-*	0	41/20[a]
		100	44/21[a]
COL-I + pGC2::*blaR1*L3	*mecI* Δ*mecR1* Δ*blaR1blaI-*	-	32
COL-I + pSPT181::*blaI*	*mecI* Δ*mecR1 blaR1*$^-$ *blaI*	-	37

[a] Heterogeneous population

4. Deletion of *bla* regulators from native β-lactamase plasmid

The previous attempts to introduce the *bla* regulators into COL-I strain turned out to be unsuccessful, since the introduction of *blaR1* gene and its domains caused an increase in the oxacillin susceptibility, suggesting a toxic effect. We set-up a knock out experiment by allelic replacement, with inducible counter-selection (7), to delete the β-lactamase regulators. Attempts to perform this experiment in strains with *bla* genes integrated into the chromosome (i.e. in single copy) were not successful because these strains were not amenable to genetic manipulations (*S. aureus* is not transformable and these strains were resistant to bacteriophage infection). Therefore, we decided to construct the genetic knock-outs in p*bla* plasmid which is not single-copy and, as such, our strategy became more complex with many purifying steps by bacteriophage transduction. These experiments are still in progress by the time this thesis is being prepared.

DISCUSSION AND CONCLUSION

Induction of β-lactam resistance in *S. aureus* is triggered by the binding of β-lactam molecules to the extracellular domain of the sensor-transducer protein. This binding promotes a conformational change, which is propagated intramolecularly and leads to the activation of the cytoplasmic inducer domain by auto-proteolysis. Soon after these events, there is a direct or indirect inactivation of the promoter-bound repressor, which allows the expression of the resistance genes (155). Despite the fact that the β-lactamase genes confer resistance to penicillins only, its regulatory genes can efficiently induce the expression of *mecA* gene, which codes for broad-spectrum β-lactam resistance. This happens because the β-lactamase repressor, BlaI, has high similarity to the *mecA* repressor, MecI, and can bind to the operator region and repress *mecA* transcription (46). This cross-talk is not valid for the sensor-transducers, which are not interchangeable and are only active against the cognate repressors (99).

In the so-called "pre-MRSA" strains (138), the presence of *mecI-mecR1* system, results in a tight repression of PBP2a synthesis and full induction of methicillin resistance takes up to 48 hours. In these strains the production of PBP2a is not altered whether or not the strains also contain the *blaI-blaR1* system (83). Probably, this happens because MecI is a very strong repressor of *mecA* expression (10). As a consequence of these observations, it has been assumed that high-level resistance, as seen in many contemporary MRSA strains, requires a non-functional *mecI-mecR1* regulatory locus (62, 81).

Based on previous studies that claimed the influence of unknown elements in the control and regulation of *mecA* gene, the ability of *bla* genes to control *mecA* expression efficiently, and recent data from our laboratory, suggesting that the *bla locus* might be responsible for the stability of the oxacillin-resistance phenotype upon overexpression of *mecI*, the purpose of this work was to clarify the role of the β-lactamase operon in the phenotypic expression of β-lactam resistance in clinical MRSA strains.

First, we designed a strategy to demonstrate the role of the β-lactamase *locus* in the expression of oxacillin resistance and its ability to interfere with *mecI*-mediated repression of *mecA*. In order to do so, a native β-lactamase plasmid was purified from

the prototype strain MW2 and introduced into the prototype β-lactamase negative strain COL. MW2 was isolated in USA in 1998. It belongs to the MLST sequence type 1, carries the SCC*mec* type IV and has non-functional *mecI-mecR1* system. It expresses a heterogeneous level of resistance to oxacillin. MW2 genome and native plasmid sequences are available (6), which facilitates the genetic manipulations. Strain COL was isolated in UK in 1961. It belongs to the sequence type 250, carries the SCC*mec* type I, has non-functional *mecI-mecR1* system and is highly and homogenous resistant to oxacillin (109). It has a constitutive expression of *mecA* and is naturally negative for β-lactamase. Strain COL is a reference strain used in many studies addressing the mechanisms of β-lactam resistance (28) and its genomic sequence is also available (109). Moreover, upon overexpression of *mecI* in this strain (COL-I strain), a dramatic decrease in the oxacillin-resistance phenotype was observed (107). Therefore, the COL/COL-I pair of parental/recombinant strains is an excellent "reporter" system to probe the effect of genetic determinants in the phenotypic expression of oxacillin-resistance.

The introduction of the intact native β-lactamase plasmid (p*bla*) from strain MW2 into COL-I caused a remarkable reversion in the resistant phenotype, which was virtually the same of parental strain COL. Moreover, in strain COL-I + p*bla* the population analysis profile of oxacillin-resistance expression was identical to the parental strain. These observations demonstrate that the presence of the β-lactamase plasmid influences, in fact, the expression of oxacillin-resistance, by interfering with the MecI function, and point to a revision of the current model for the transcriptional control of *mecA*, defending that high level of resistance implies non-functional *mecI* gene. Here, we demonstrate that a recombinant strain overexpressing the repressor *mecI* could fully restore the high and homogenous level of resistance in the presence of the β-lactamase plasmid.

To further explore these observations, the native plasmid from prototype strain MW2 was also introduced into three other β-lactamase negative strains. Two of them, VNG17 and RJP17, were isolated in the same country and time period. They belong to the MLST sequence type 5, carry SCC*mec* type IV, and have non-functional *mecI-mecR1* system (108, 120). Interestingly, despite the fact that both strains are β-lactamase negative and have a heterogeneous expression profile of resistance, only strain VNG17

phenotype was affected by the *mecI*-overexpression experiments (106), which challenges our hypothesis about the influence of the β-lactamase plasmid. The β-lactamase plasmid was introduced in the recombinant strains overexpressing the *mecI* gene (VNG17-I and RJP17-I), and also in the parental strains, since they present, unlike strain COL, a low-level resistance that allows the detection of positive effects on the phenotypic expression of resistance. Upon introduction of the β-lactamase plasmid, the effect of *mecI* overexpression in strain VNG17 was disrupted and the resistance level of the parental strain was restored, demonstrating, once again, the interference of β-lactamase with the MecI-mediated repression of oxacillin resistance. Moreover, both parental strains transformed with the *bla* plasmid shifted from a low-level and heterogeneous to a high-level and homogenous resistance phenotype. These observations suggest that β-lactamase genes not only interfere with the MecI-mediated repression but are also "boosters" of the phenotypic expression of oxacillin-resistance in MRSA strains. Strains COL, VNG17 and RJP17 have no *mecI* and a partial-deleted *mecR1* but still with the full N-terminal inducer domain. In order to evaluate the effect of the truncated *mecR1* on the previous observations, we have tested the effect of p*bla* into a fourth β-lactamase negative strain – strain HT0350. This strain belongs to MLST sequence type 377 and carries SCC*mec* type V, which is characterized by a non functional *mecI* and a virtually full deletion of *mecR1* gene (67, 142). In fact, strain HT0350 has only the first 111 bp of the coding sequence of *mecR1*, which corresponds to a few 36 amino acids of the N-terminal domain attached to the membrane. By testing the effect of the β-lactamase plasmid in this strain, we expected to clarify the role of *mecR1* gene on those puzzling observations concerning the effect of p*bla* on oxacillin resistance. Similarity to the previous strains, upon introduction of the β-lactamase plasmid, HT0350 turned from a low-level and heterogeneous to a high-level and homogeneous oxacillin-resistant strain. Furthermore, the *mecI* repressor was overexpressed in *trans* in HT0350 (strain HT0350-I) and the β-lactamase plasmid was introduced in that recombinant strain. The overexpression of *mecI* gene in strain HT0350 caused a massive decrease in the oxacillin-resistance phenotype, which was fully reverted upon introduction of p*bla*. Altogether, this set of experiments with the native *bla* plasmid from strain MW2 provides new important insights into the regulatory mechanism of *mecA* expression: the β-lactamase *locus* enhances the phenotypic

expression of oxacillin resistance in heterogeneous MRSA strains and disrupts the strong effect of MecI, which are two new unexpected functions for the *bla* locus.

Prompted by these observations, we designed a series of experiments to clarify which determinant(s) of the β-lactamase plasmid were involved. We reasoned that the *bla* operon, more specifically, the *bla* regulators, would be the most plausible candidates. However, formally it cannot be excluded the possible influence of other elements present in the β-lactamase plasmid.

Given that *blaZ* gene has no regulatory functions described so far, it was expected that, when alone, it would not influence the expression of oxacillin-resistance mediated by *mecA* gene. In order to exclude the putative effect of *blaZ* gene, we re-evaluated the oxacillin-resistance in the presence of clavulanic acid, which is a specific β-lactamase inhibitor (114). Addition of clavulanic acid had no significant effect in the oxacillin-resistance phenotypes of MRSA strains transformed with *pbla*, with or without *mecI*-overexpression in *trans*. Therefore, the structural gene of the *bla* operon, *blaZ*, coding for penicillin-resistance is not involved in the enhancer effect of p*bla* on the phenotypic expression of oxacillin-resistance.

We came up with a few working hypotheses for the mechanism of action of *bla* regulators on the expression of oxacillin resistance. First, we may assume that BlaI form heterodimers with MecI, with each monomer binding to a different dyad in the *mecA* operator region. In fact, the formation of functional BlaI-MecI heterodimers has already been described by McKinney *et al* (99). As BlaI-BlaR1 system is much more efficient than MecI-MecR1, which strongly represses *mecA*, we may speculate that promoter-bound MecI-BlaI heterodimers are efficiently delocalized, upon induction by β-lactams, either by BlaR1 alone or in partnership with MecR1. This functional partnership would require an interaction between the activated BlaR1 and the N-terminal inducer domain of MecR1 (present in strains COL, VNG17 and RJP17 but not on strain HT0350), turning out the latter more efficient, in such a way that MecI dimers or MecI-BlaI hetero-dimers would delocalize easily from the operator region. However, no data in the literature provides any evidence to support this model and, data from strain HT0350, which has a virtual fully deleted *mecR1*, also refutes this hypothesis. Finally, but also against all published evidences, BlaR1 could act on MecI directly, inducing the *mecA* expression.

The MecI-BlaI heterodimer model may also explain the "enhancer" effect of p*bla* on the phenotypic expression of oxacillin resistance in the tested parental strains with low-level and heterogeneous oxacillin-resistance. Since those strains are *mecI* and *bla* negative, the constitutive expression of the truncated MecR1 may impose some fitness cost or toxic effect to the cell that interferes with the optimal phenotypic expression of resistance. This makes sense if one takes into account that truncated MecR1 (NTD-MecR1) has a complete N-terminal inducer domain with four transmembrane regions, and as such, overstated cellular amounts of this domain are likely to interfere with cell viability (107, 116). The introduction of the β-lactamase plasmid would bring a protective effect through the action of the repressor BlaI, since it is able to bind to the operator region of *mecA* and thus, control the production of both PBP2a and truncated MecR1. In the absence of β-lactams, BlaI would protect the cells from a cellular excess of NTD-MecR1, and in the presence of antibiotic *mecA* would be rapidly induced by the activated BlaR1, allowing the expression of high-level oxacillin-resistance. Nevertheless, other factors are involved in the heterogeneous expression profile of oxacillin resistance, as strain COL with NTD-MecR1 is highly resistant and strain HT0350 virtually without MecR1 is low-level resistant.

With these interesting but puzzling observations in our hands we aimed to clarify the mechanism underlying the effect of *bla* regulators on the expression of oxacillin resistance in MRSA strains. In order to do so, the subsequent step was to test the influence of each *bla* regulator or functional domain in the oxacillin-resistance phenotype. For this purpose, we constructed a series of recombinant plasmids containing, under the control of P*spac* inducible promoter, the following inserts: *blaI-blaR1* with or without its operator sequences; *blaR1* with or without its operator sequences and the N-terminal inducer domain of *blaR1* with (NTD-*blaR1*) or without (ΔNTD-*blaR1)* the zinc metalloprotease domain present in loop 3 (L3) (Fig. 10).

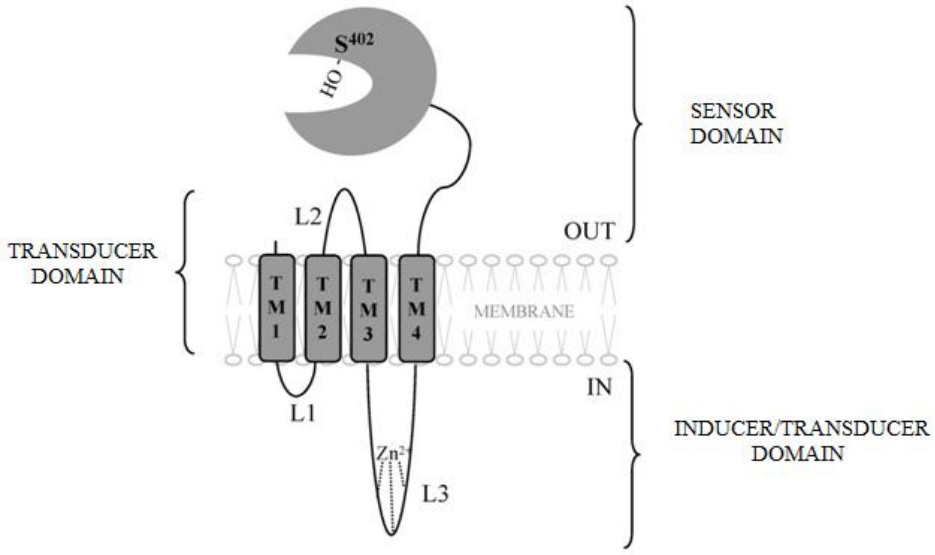


Fig 10 - Membrane topology of the penicillin-sensory transducer, BlaR1(56).

These recombinant plasmids were introduced into COL-I strain to evaluate the effect on the oxacillin-resistance phenotype. Unexpectedly, for all cases, using different concentrations of the inducer, it was not observed any increase of resistance levels as occurred in strain COL-I transformed with the intact β-lactamase plasmid. This result could be an indication that these genes might not be involved in the oxacillin-resistance expression. However, the phenotype revealed to be even more susceptible than COL-I strain, suggesting that the overexpression of the β-lactamase regulators might be toxic for the cell, as previously reported for MecR1 (107, 116). Although P*spac* is an inducible promoter it presents a leaky activity, which together with the high-copy number of pSPT181 plasmid might account for this unexpected phenotype. To overcome this effect, we will co-transform these strains with a second plasmid overexpressing the repressor of P*spac* promoter (112).

We have also tested the phenotypic response of strain COL-I to the over-expression of the cytoplasmic components of *blaI-blaR1* system – *blaI* and the L3

domain of *bla*R1. The overexpression of *blaI* caused no significant alterations in the resistance phenotype: a slightly large halo could be noticed but the same effect was observed in the control experiment with the empty plasmid vector. Nevertheless, contrary to what was observed with *blaR1*, the *blaI* overexpression did not originate a hyper-sensitive phenotype, suggesting that this latter gene is not toxic to the cell. It is reasonable to assume that the overexpression of *blaI* alone would not reproduce the phenotype observed with the introduction of the complete *bla* plasmid, since the inducer is not present. Concerning the overexpression of L3 domain of BlaR1 in COL-I, no significant effect on the phenotypic expression of resistance was observed as well. BlaR1 has two functional domains, the sensor domain that extends to the extracellular medium where β-lactams can bind, and the transducer domain that lies in the cytoplasm and has four transmembrane α-helices interconnected by three loops (Fig. 10). Importantly, the zinc metalloprotease domain, present in loop 3 (L3), is cleaved during the induction process and is thought to be the connective element between BlaR1 and BlaI (57). Although no toxic effect was observed in these experiments, the lack of effect on COL-I phenotype suggests that the overexpressed L3 domain is not functional and that other parts of BlaR1 are required for its activation.

In parallel with the above experiments, we set up a strategy to generate genetic knock-out's of the *bla* regulator genes by allelic replacement with inducible counter-selection, which allows a much rapid and efficient gene deletion in *S. aureus* than the traditional methods (7). As any genetic knock-out strategy, this strategy was designed for chromosomal (i.e single copy) genes. We found a MRSA clone fully characterized in a comparative genomic study (58) with a chromosomal cassette containing the β-lactamase locus. We have tested four prototype strains of this lineage but in all cases we could not succeed because the strains were resistant to bacteriophage infection or at least to the three phages routinely used for the genetic manipulation of *S. aureus*. Therefore, we decided to perform these experiments in strain COL-I with p*bla* and, as such, all steps required a "purifying" transduction step to assure that all copies of p*bla* were equal. These experiments are still in progress but eventually will clarify about the role of *blaR1* and *blaI* on the phenotypic expression of oxacillin resistance.

In summary, the results presented in this thesis demonstrate, doubtless, that the β-lactamase plasmid can increase the oxacillin-resistance phenotype in epidemic MRSA

strains, turning them from low-level and heterogeneous to high-level and homogeneous oxacillin-resistant strains; i.e. *bla* locus is a potent "enhancer" of the MRSA phenotype. Also, this work shows the contribution of the β-lactamase plasmid for a high level of oxacillin resistance in MRSA strains with either functional or non-functional *mecI* repressor; i.e. *bla* locus disrupts the *mecA* repression mediated by its cognate repressor. These are new and unexpected roles for the *bla* locus on the phenotypic expression of oxacillin resistance. Therefore, these results, contribute to the study of the regulation of *mecA* expression and point to a revision of the current model for the *mecA* regulation in clinical MRSA strains. Although we could not determine the exact mechanism underlying these observations, we have some evidences suggesting that *bla* regulators, *blaI* and *blaR1*, are involved. In fact, this is in perfect agreement with all evidence in the literature demonstrating that *bla* genes regulate efficiently *mecA* transcription and stabilize its acquisition. Hopefully, the experiments in progress will soon provide some insights into this important question.

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ANNEX

I – Culture Media, Reagents, Buffer Solutions and Antibiotics

Table 8 - Culture Media

Medium[a]	Constituents (per Liter)
Triptic soy agar (TSA)	15.0 g Tryptone Peptone; 5.0 g Soytone Peptone; 5.0 g Sodium Chloride; 15.0 g Agar.
Triptic soy broth (TSB)	15.0 g Tryptone Peptone; 5.0 g Soytone Peptone; 5.0 g Sodium Chloride.
Brain heart infusion (BHI)	200 g Calf Brains; 250 g Beef Heart; 10 g Peptone; 2 g Dextrose; 5 g Sodium Chloride; 2,5 g Disodium Phosphate.
Luria-Bertani broth (LB)	10,0 g Tryptone; 5 g Yeast Extract; 10 g Sodium chloride.
Luria-Bertani agar (LA)	10,0 g Tryptone; 5 g Yeast Extract; 10 g Sodium chloride; 15 g Agar.

[a] TSA, TSB and BHI were purchased from Difco; LA and LB were purchased from Roth.

Table 9 - Buffer solutions

Buffer solutions[a]	Composition
Tris-acetate-EDTA (TAE) 1X	40 mM Tris-acetate; 1 mM EDTA, pH 8,0. Stock solution: 50x
Tris EDTA (TE) 1X	10 mM Tris pH 8,0; 1 mM EDTA, pH 8,0.

[a] Buffer solutions were prepared in house.

Table 10 – Media and solutions for transduction experiments

Transduction solutions[a]	Components (per Liter)
Bottom phage agar	3 g casamino acids; 3 g yeast extract; 5,9 g NaCl; 12 g agar.
Top phage agar	3 g casamino acids; 3 g yeast extract; 5,9 g NaCl; 6 g agar.
Bottom 0,3 GL agar	3 g casamino acids; 3 g yeast extract; 5,9 g NaCl; 3,3 mL DL-lactic acid; 2 mL Glicerol 50%; 0,5 g Tri-sodium citrate; pH: 7,8; 6 g agar.
Top 0,3 GL agar	NaCl; 3,3 mL DL-lactic acid; 2 mL Glicerol 50%; 0,5 g Tri-sodium citrate; pH: 7,8; 12 g agar.
Phage buffer	1 Mm $MgSO_4$; 4 Mm $CaCl_2$; 50 mM TrisHCl pH: 7,8; 5,9 g NaCl; 1 g gelatin.
$CaCl_2$	Calcium (1x); Chloride (2x).

[a] Transduction solutions were prepared in-house.

Table 11 – Cell-wall lysis enzymes

Enzymes[a]	Stock Solution
Lysostaphin	10 µg/mL
RNAse	10 µg/mL

[a] Lysostaphin was purchased from Ambi Products; RNAse was purchased from Quiagen.

Table 12 – Antibiotic solutions

Antibiotics[a]	Stock Solution
Chloramphenicol	10 mg/mL
Tetracycline	10 mg/mL
Clavulanic acid	500 µg/mL
Oxacillin	100 mg/mL
Ampicillin	100 mg/mL
Anidrotetracycline	100 µg/mL
IPTG	1 M
Penicillin	1000 U

[a] Antibiotics were purchased from Sigma-Aldrich.

Table 13 – Restriction endonucleases

Restriction endonucleases[a]	Stock Solution
BamHI	20,000 U/mL
EcoR1	20,000 U/mL
PstI	20,000 U/mL
XmaI	10,000 U/mL
NdeI	20,000 U/mL

[a] Restriction endonucleases were purchased from New England Biolabs.

II – PCR Primers, Reagents and Thermocycling Conditions

Table 14 - Primers list

Target	Forward primer sequence[a] 5' → 3' (name/sequence)	Reverse primer sequence[a] 5' → 3' (name/sequence)
blaZ	blaZ F1/ GATAAGAGATTTGCCTAT GC	blaZ R1/ GCATATGTTATTGCTTG ACC
blaR1	blaR1 F1/ CTTATGATTCCATGACA TACG	blaR1 R1/ CATGACAATGAAGTA GAAGC
blaZ-blaI-blaR1	blaF1/ ATTTTCTGTACACTCTCATC	blaR1/ TTTTCGATTGATGAACAC CT
NTD*blaR1*	blaR1F2/ TATA CCCGGG GAACAT TTAATACGGAGTCC	blaR10/ ATAT CCCGGG AAAGA AGGTGTTGAAATGG
ΔNTD*blaR1*	blaR1F3/ TATA CCCGGG TTGGAG ATTGAATAGTCTCTGC	blaR10/ ATAT CCCGGG AAAGA AGGTGTTGAAATGG
*blaR1*L3	blaL3F1/ TATA GAATTC TGGAGT TTAATATATGGAAAGCCTTATTATA TCTTAAATA	blaL3R1/ TATA GGATCC TTTGA CTGCTTTTTCAGATCG
blaI	blaF5/ TATA GGATCC ATTTTCTG TACACTCTCATC	blaR9/ TATA CTGCAG ACATTTA TCAGATGGAAAGCC
Upstream *blaR1*	blaF6/ GGGGACAAGTTTGTACAA AAAGCAGGCTTTACACCTCGTTCGT TAAGC	blaR6/ TATA GGATCC ACATTTA TCAG ATGGAAAGCC
Downstream *blaR1*	blaF8/ CACA GGATCC GCATGTA ATTCA AACAGTTCACATGCC	blaR7/ GGGGACCACTTTGTACA AGAGTGGGTTTTCGATTGATGAA CACC
blaR1 knock out	blaF9/ TTCATAACATCCCATTCA GC	blaR8/ ATTGAAGCCTCAATAAGT GC
mecA	mecA P1/ AAATCGATGGTAAAGG TTGGC	mecA P2/ AGTTCTGCAGTACCGG ATTTGC
mecI	mecI P4/ GCGGGTTTCAATTCACTT GTC	mecI P5/ ATGGGAATTCAGCACA ACAAATTTCTGAGC

[a] Underline segments represent recognition sites for restriction endonucleases.

GoTaq DNA Polymerase PCR

Table 15 - Routine PCR reaction mix

Reagent	Volume (µL)[a]
Go Taq buffer 5x	5
MgCl 25 Mm	1,5
dNTP's 10 mM	1
Forward primer	0,5
Reverse primer	0,5
GoTaq polymerase	0,2
Template	2
H_2O milliQ	14,3

[a] for a final volume of 25 µL.

Pfu Turbo DNA Polymerase PCR

Table 16 - High-fidelity PCR mix

Reagent	Volume (µL)[a]
Pfu buffer 10x	5
Template	5
dNTP's 10 mM	4
Forward primer	1
Reverse primer	1
Pfu Turbo polymerase	1
H_2O milliQ	33

[a] for a final volume of 50 µL.

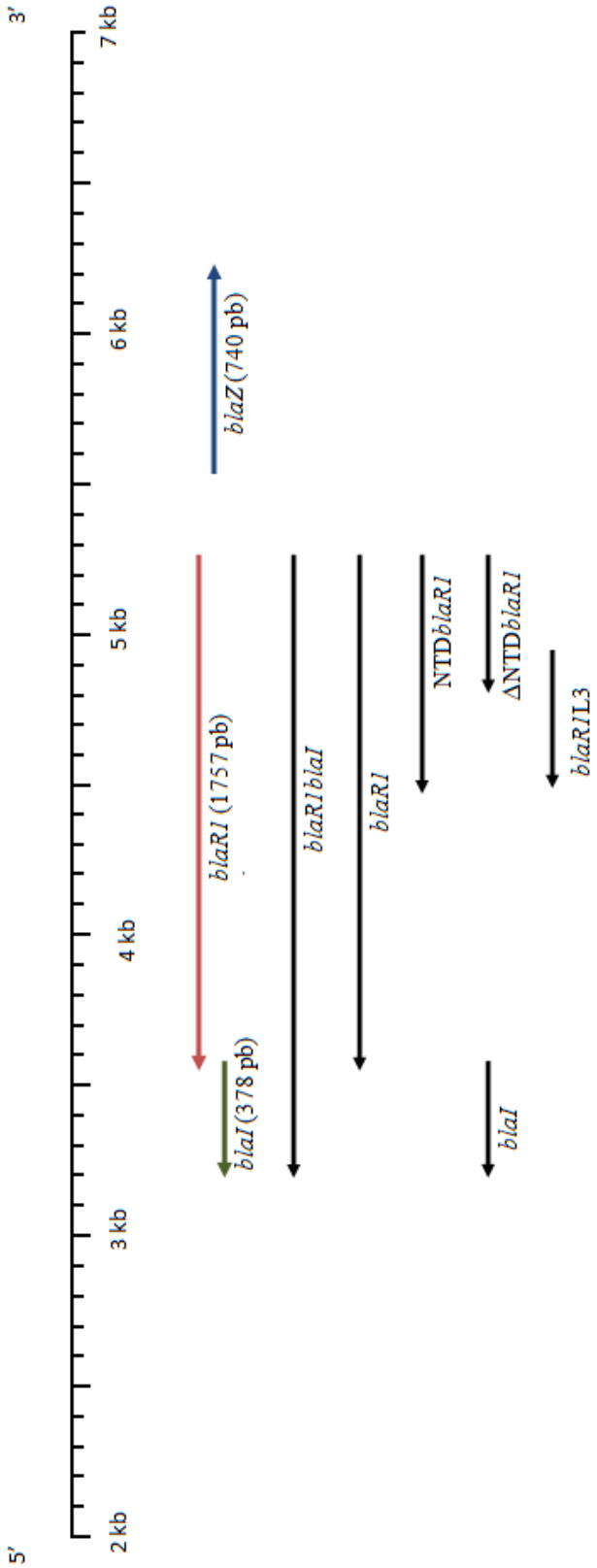
Thermocycling conditions:

Table 17 - Thermocycling conditions

Target	Annealing temperature (ºC)	Extension time (Minutes)
blaZ	55	0,5
blaR1	51	3,5
blaZ-blaI-blaR1	51	3,5
NTD*blaR1*	49	1
ΔNTD*blaR1*	55	1
*blaR1*L3	55	0,5
blaI	55	0,5
Upstream *blaR1*	55	1
Downstream *blaR1*	51	1
blaR1 knock out	55	3
mecA	55	1
mecI	55	0,5

III – *bla* Operon and Cloning Fragments

Fig. 11 - *bla* operon and cloning fragments. Colorful arrows represent *bla* operon genes; black arrows represent cloning fragments.


3'
7 kb
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blaZ (740 pb)
blaR1 (1757 pb)
blaI (378 pb)
blaR1blaI
blaR1
NTDblaR1
ΔNTDblaR1
blaR1L3
blaI

IV – Summary of Experimental Phenotypic Data

Table 18 - Summary of experimental phenotypic data

Strain	Relevant genotype	Oxacillin susceptibility	
		Halo diameter[a] (mm)	MIC (µg/mL)[b]
COL	*mecI*⁻ Δ*mecR1 bla*⁻	8	> 800
COL-I	*mecI* Δ*mecR1 bla*⁻	32	0,75
COL-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	7	> 800
COL-I + *blaIblaR1*	*mecI* Δ*mecR1 blaI blaR1*	40	-
COL-I+ O*blaR1blaI*	*mecI* Δ*mecR1 blaI blaR1*	40	-
COL-I + *blaR1*	*mecI* Δ*mecR1 blaR1*	41/19[a]	-
COL-I + O*blaR1*	*mecI* Δ*mecR1 blaR1*	39	-
COL-I + NTD*blaR1*	*mecI* Δ*mecR1* Δ*blaR1*	40/23[a]	-
COL-I + ΔNTD*blaR1*	*mecI* Δ*mecR1* Δ*blaR1*	44/21[a]	-
COL-I + *blaR1*L3	*mecI* Δ*mecR1* Δ*blaR1*	32	-
COL-I + *blaI*	*mecI* Δ*mecR1 blaI*	37[a]	-
VNG17	*mecI*⁻ Δ*mecR1 bla*⁻	33/53[a]	6
VNG17 + p*bla*	*mecI*⁻ Δ*mecR1 blaZ blaI blaR1*	13	800
VNG17-I	*mecI* Δ*mecR1 bla*⁻	57	0,75
VNG17-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	33/38[a]	1,5
RJP17	*mecI*⁻ Δ*mecR1 bla*⁻	27/42[a]	1,5
RJP17 + p*bla*	*mecI*⁻ Δ*mecR1 blaZ blaI blaR1*	15	800

Table 18 – Cont.

Strain	Relevant genotype	Oxacillin susceptibility	
		Halo diameter[a] (mm)	MIC (µg/mL)[b]
RJP17-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	38/40[a]	1,5
HT0350	*mecI*⁻ Δ*mecR1 bla*⁻	35	1,5
HT0350 + p*bla*	*mecI*⁻ Δ*mecR1 blaZ blaI blaR1*	16	800
HT0350-I	*mecI* Δ*mecR1 bla*⁻	56	0,75
HT0350-I + p*bla*	*mecI* Δ*mecR1 blaZ blaI blaR1*	40	3

[a] Heterogeneous population
[b] MIC's were determined based on PAP's profile; first oxacillin concentration causing a three-log decrease of CFU's (99,9 % of viability loss)