The Anti-Repessor MecR2 Promotes the Proteolysis of the mecA Repressor and Enables Optimal Expression of β-lactam Resistance in MRSA

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Abstract

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

Introduction

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

The MRSA characteristic phenotype is due to an extra penicillin-binding protein (PBP2a) coded by the mecA gene [5], which has a remarkable reduced affinity for many β-lactams [6]. In addition, >95% of MRSA strains have also a β-lactamase enzyme coded by blaZ that confers penicillin-resistance [7–9]. The mecA transcription can be controlled by the divergent mecR1-mecI regulatory genes, coding for a sensor-inducer and a repressor, respectively [10]. This genetic organization of the mecA locus is similar to that of the β-lactamase, which contains the structural gene blaZ and the homologous blaR1-blaI regulatory genes. In fact, there is a cross-talk between both systems [11–14], and the signal-transduction mechanisms are believed to be identical [15,16], involving two proteolytic steps, in contrast with the most common bacterial signal transduction mechanism that involves the phosphorylation of regulatory proteins [17]. Specifically, the currently accepted model of mecA regulation involves two main steps: (i) binding of the β-lactam antibiotic to the extracellular sensor domain of MecR1 leads to the autocatalytic cleavage of the sensor-inducer and activation of the cytoplasmatic inducer domain, which appears to be a prometalloprotease; (ii) the activated inducer domain of MecR1 either directly cleaves the prometallloprotease or promotes the repressor cleavage, which enables the ability of the repressor protein to dimerize and bind to
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Author Summary

Methicillin-resistance Staphylococcus aureus (MRSA) is an important human pathogen, causing a wide range of infections. MRSA strains are resistant to virtually all β-lactam antibiotics and often are also resistant to many other classes of antibiotics, leaving physicians with few therapeutic options. MRSA is defined by the presence of the mecA gene. The induction of mecA transcription in response to β-lactams involves a unique series of proteolytic steps and some critical details of this signal transduction mechanism are still illusive. For instance, it is not fully explained why the induction of mecA by its cognate regulatory genes mecR1-mecI appears to be very inefficient and it is not clear if the observed Mec1 repressor proteolysis is mediated directly by the activated MecR1 sensor-inducer. In this study, we demonstrate that the mecA regulatory locus is not a two-component system but instead it is a three-component system containing the previously unrecognized anti-repressor mecR2 gene. MecR2 disturbs the binding of the repressor Mec1 to the mecA promoter, which leads to its proteolytic inactivation independently from MecR1. Moreover, our data shows that in the presence of functional mecR1-mecI genes, mecR2 is essential for a robust induction of mecA transcription and, as consequence, for the optimal expression of β-lactam resistance.

The mecR2 gene, enabling the expression of the resistance gene. MecR1 once cleaved can no longer transmit signal but, since the expression of mecR1-mecI is also up-regulated, the mecA induction continues as long as the antibiotic is present in the environment.

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

Here, we identify the missing link that explains the puzzling observations described above. We demonstrate that the mecA regulatory locus is in fact a three-component system that contains, besides mecR1-mecI genes, the mecR2 gene, which is co-transcribed with mecR1-mecI. In vitro and in vivo assays show that MecR2 acts as an anti-repressor by interacting directly with MecI repressor, disturbing its binding to the mecA promoter, which results in its inactivation by proteolytic cleavage. In MRSA strains with functional mecR1-mecI genes, MecR2 is essential for the full induction of mecA transcription, compensating for the inefficient induction of mecA by mecR1 and enabling the optimal expression of β-lactam resistance. These findings suggest a need to revise the current model for the induction of mecA expression in clinical MRSA strains and open new avenues for the design of alternative therapeutic strategies targeting the regulatory pathway of mecA expression. In addition, this unusual combination of repressor, sensor-inducer and anti-repressor, together with the unique modulation of a series of proteolytic cleavage steps underlying the signal transduction mechanism, provides important insights into the evolution of antibiotic-resistance and transcriptional control of genes in bacteria.

Results/Discussion

The mecA cognate regulatory locus is a three-component system

Since the MRSA phenotype is not affected by the overexpression in trans of the mecA repressor [24], we hypothesized that a third regulator might be involved in the mecA transcriptional control. Taking into account that mecA gene is part of a large polymorphic exogenous DNA fragment (the so called SCCmec element), which has integrated in the chromosome [25], we reasoned that the putative additional regulator should be located within this chromosomal cassette, most likely upstream to the mecA gene; i.e., genetically linked to the mecR1-mecI region. Upon analysis of prototype sequences of SCCmec types II and III, which are characterized by complete mecR1-mecI coding sequences [26], we found a highly conserved region (99.9% homology) immediately downstream of mecI. This region contains the divergent small coding sequence for a phenol-soluble modulin, psm-mec, involved in S. aureus virulence and colony spreading [27,28], and a putative open-reading frame (ORF) that, due to a difference in four-tandem thymine residues, has a variable length (Figure 1A); 870 bp in SCCmec type II prototype strain N315 (accession no. D66934, positions 41794-40925) and 1149 bp in SCCmec type III prototype strain HU25 (accession no. AF422694, positions 4729-3861). Both variants are homologous to the repressor of the xylose operon of S. xylosus, XylR (accession no. X57599) with an amino-acid identity of 60–64%; the four-thymine deletion in strain N315 eliminates the N-terminal DNA-binding helix-turn-helix domain of XylR (Figure 1B). Available genomic and SCCmec sequence data demonstrate that both ORF variants are disseminated in S. aureus and in other staphylococcal species containing SCCmec sequences (Figure S1). We coined the name of mecR2 for this putative ORF.

Analysis of the upstream sequences of the putative mecR2 gene revealed no obvious promoter sequences. A putative terminator region, consisting of two perfect inverted repeats of 19 base pairs, was identified downstream the stop codon of mecR2. No terminator sequences were found in the mecI-mecR2 intergenic region, suggesting that the putative mecR2 gene might be co-transcribed together with mecR1-mecI from the mecR1 promoter. This hypothesis was confirmed by transcriptional analysis of mecR2 by reverse-transcriptase PCR (RT-PCR) in prototype strains N315 and HU25. Using internal primers for the putative coding region of mecR2 a positive signal was detected in both prototype strains. Moreover, using pairs of primers spanning the mecI-mecR2 and mecR1-mecI regions, positive signals were detected suggesting that mecR1-mecI-mecR2 genes are co-transcribed from the mecR1 promoter (Figure S2).

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

We next evaluated the role of mecR2 on the MRSA phenotype by constructing a series of recombinant strains using two parental strains with contrasting phenotypes. The first of these, strain COL is highly resistant to meticillin, has no mecI, has a partially deleted
Figure 1. The mecA regulatory locus is a three-component system. (A) Genetic organization of the mecA regulatory locus in major SCCmec types I–V and prototype MRSA strains used in this study. The magnified DNA sequence shows the two mecR2 start codons in SCCmec type III and III (boxed), the stop codon (boxed), the four-thymine deletion in SCCmec type II (underlined), and the putative terminator (inverted arrows). (B) Multiple...
mecR1, expresses mecA constitutively, is negative for β-lactamase [29], and is mecR2 negative. The second, strain N315 has a low-level methicillin-resistance phenotype, carries wild-type mecR1-mecI sequences, has an inducible expression of mecA, carries a β-lactamase plasmid [10,18], and is mecR2 positive. In previous studies, we have observed a sharp decrease in resistance to oxacillin in strain COL overexpressing in trans the repressor mecl (COL+mecl), whereas the great majority of other MRSA strains tested, including strain N315, did not show alterations in the oxacillin-resistance phenotype [24]; being oxacillin a methicillin analogue that has replaced methicillin in clinical use. In this study, we have cloned the mecI-mecR2 region from strain N315 in the same plasmid vector. When strain COL was transformed with this recombinant plasmid (COL+mecI-mecR2), the resistant-phenotype was completely restored and so was the constitutive expression of mecA (Figure 2).

To exclude possible artifacts due to the overexpression of genes from multi-copy plasmids, we reconstructed the mecR1-mecI-mecR2 regulatory locus of prototype strain N315 in the chromosome of strain COL, using an insertion-deletion strategy with a thermosensible plasmid (Figure S3). First, we inserted the wild-type sequences of mecR1-mecI (strain COL::R1), which caused a decrease of oxacillin-resistance when compared to the parental strain COL, in agreement with the poor induction of mecA by MecR1 alone (Figure 2). Compared to COL+mecl (Figure 2), the decrease of oxacillin-resistance in COL::R1 was less severe, most likely due to the presence of the inducer MecR1. Upon introduction of the complete mecA regulatory locus; i.e. mecR1-mecI-mecR2 (strain COL::R1-R2), the phenotype of parental strain COL was fully restored. As control experiments, we re-introduced the recombinant plasmid over-expressing mecl in recombinant strains COL::R1 and COL::R1-R2, originating strains COL::R1+mecl and COL::R1-R2+mecl, respectively. In both cases no effect was detected on the phenotypic expression of resistance, suggesting that the functions of mecR1 and mecR2 are not affected when mecl is overexpressed in trans, in line with our previously reported observations [24]. Altogether, these experiments suggest that mecR2 compensates for the poor induction of mecA by mecR1, enabling the optimal expression of resistance.

To further clarify the mecR2 function, the chromosomal copy of mecR2 gene from strain N315 was replaced by an antibiotic-resistance marker (N315::ΔmecR2), using a similar insertion-deletion strategy (Figure S3). Deletion of mecR2 caused a sharp decrease in the phenotypic expression of oxacillin-resistance in strain N315. We then cloned the mecR2 gene from strain N315 under the control of an inducible promoter (pBAD-mecR2), and, in the presence of the inducer (IPTG 100 μM), we succeeded in complementing the mecR2 null-mutant in strain N315 (Figure 4A) and restored the COL phenotype of recombinant strain COL::R1 (Figure 4B). Since in these experiments only the mecR2 gene was deleted from the chromosome of strain N315 and complemented in trans, it can be concluded that the intergenic mecI-mecR2 region has no role in the phenotypic expression of β-lactam resistance. Altogether, these observations demonstrated that mecR2 interferes with the mecI-mediated repression of mecA, compensates for the inefficient MecR1-mediated induction of mecA and enables the optimal expression of β-lactam resistance in the presence of functional mecR1-mecI sequences.

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

mecR2 is required for the full induction of mecA transcription

We next analyzed the effect of mecR2 on the induction profile of mecA transcription in parental strain N315, its null-mutant for mecR2, and in the complemented mutant, by Northern blotting (Figure 5A) and quantitative Real-time RT-PCR (qRT-PCR) analysis (Figure 5B). In relative terms, upon induction with sub-
The functions of mecR1 cause a decrease of the resistance level to oxacillin, which can be reverted by the reconstruction of the full mecA regulatory locus, mecR1-mecI-mecR2 (COL::RI-R2). Control experiments with mecI overexpressed in trans (COL::RI-mecI and COL::RI-R2-mecI) demonstrate that the functions of mecR1 and mecR2 are not affected by high levels of MecI. For comparative purposes the overexpression of mecI in parental strain COL (COL::RI-R2) also shown. The oxacillin-resistance levels were evaluated by diffusion disks containing 1 mg of oxacillin (left) or by population analysis profiles (PAP’s) (right).

**Role of Anti-Repressor MecR2 on the MRSA Phenotype**

MIC oxacillin, a much stronger induction of mecA transcription was observed in the parental strain than in the mecR2 null-mutant (N315::ΔmecR2), in which the amount of mecA transcript seems to be not sustained during the last two time-points. In the complemented mutant (N315::ΔmecR2+spac::mecR2) there was a sustained induction of mecA transcription throughout the time-course of the experiment. However, in the complemented strain, although the resistant phenotype of the parental strain was fully restored (as illustrated in Figure 4A), the amount of mecA transcript was substantially lower and virtually identical to the mecR2 null-mutant. Although this discordance is in agreement with previous studies reporting on the lack of a correlation between the cellular amounts of mecA transcript or protein and the phenotypic level of resistance [31,32], we cannot formally exclude other possible explanations, such as MecR2 having multiple targets that affect the resistance phenotype. Nevertheless, these data suggest that the mecR2 interferes with the induction of mecA transcription in response to β-lactams.

**mecR2 transcription analysis**

We have also analyzed by qRT-PCR the induction profile of mecR2 in parental strain N315 and in the complemented mecR2 null-mutant (N315::ΔmecR2+spac::mecR2) – Figure 6. qRT-PCR data for parental strain N315 showed that mecR2 transcription was induced in the presence of sub-MIC oxacillin, in agreement with data from RT-PCR that showed that mecR2 was co-transcribed with mecI-mecR1 from the inducible mecI promoter (Figure S2). In the complemented mutant, in the presence of the inducer (IPTG), mecR2 transcription levels were 10 fold higher than those of parental strain and, as such, the low levels of mecA transcription observed for this strain (Figure 5) cannot be attributable to an inefficient induction of mecR2 transcription from the Pα promoter.

Of note, in parental strain N315, mecR2 transcription levels appear to be residual when compared to those of mecA (10^4 fold less). Although this might be explained by an experimental artifact, one can also speculate that this may be due to different promoter strengths and/or to promoter blockage by RNA polymerase, since mecA and mecR1 promoters are divergent and overlap partially. In terms of signal-transduction mechanism, once the expression of mecA is induced in response to β-lactams, there is no need for high cellular levels of inducer, repressor or anti-repressor. Actually, the basal transcription of mecR1-mecI-mecR2 is only necessary to assure that the repressor protein is still present when the antibiotic induction stops, so that the transcription of the resistance gene is shutdown. The apparent very low transcription level of mecR2 in parental strain N315 may also explain the lack of complementation when mecR2 was overexpressed in trans. In fact, this artificial system, when compared to wild-type strains, presumably generates extremely high cellular amounts of MecR2, which may originate a loss of function by oligomerization or non-specific interactions with other cellular targets. Finally, the apparent residual mecR2 transcription levels may also explain our failed attempts to analyze by Northern blotting the transcription of mecR2 in prototype MRSA strains, even with large amounts of total RNA (10–30 μg) and long autoradiograph expositions (72 h). To our knowledge, Northern blotting analysis of mecR1-mecI-blaR1-blaI transcripts was described in only two studies and, in both cases, clear signals were obtained only when regulatory genes were overexpressed from recombinant plasmids [33,34].

**Figure 3. Reconstruction of the mecA regulatory locus in prototype strain COL.** Reconstruction of the mecR1-mecI locus in the chromosome of strain COL (COL::RI) causes a decrease of the resistance level to oxacillin, which can be reverted by the reconstruction of the full mecA regulatory locus, mecR1-mecI-mecR2 (COL::RI-R2). Control experiments with mecI overexpressed in trans (COL::RI-mecI and COL::RI-R2-mecI) demonstrate that the functions of mecR1 and mecR2 are not affected by high levels of MecI. For comparative purposes the overexpression of mecI in parental strain COL (COL::RI-R2) is also shown. The oxacillin-resistance levels were evaluated by diffusion disks containing 1 mg of oxacillin (left) or by population analysis profiles (PAP’s) (right).

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

Among the five major SCCmec types, only SCCmec types II and III are characterized by complete mecR1-mecI regulatory locus [26] – Figure 1A. SCCmec type III strains appear to have a conserved point mutation within mecI coding sequence resulting in a truncated non-functional repressor protein [24,35]. Concerning SCCmec type II strains, the accumulation of deleterious mutations has also been described in some strains [19–23]. However, data from our MRSA collections [24], as well as from available genomic and SCCmec type II sequences, suggest that many strains have wild-type sequences for mecI and mecR1. For instance, in a BLAST analysis against the mecI sequence from strain N315, c.a. 20 entries were found with 100% sequence identity, mostly from S. aureus strains but also from a few coagulase-negative staphylococci (S. epidermidis, S. saprophyticus, S. fleurettii, S. cohnii, etc.). These observations suggest that the mecR2 function may be required for the optimal expression of β-lactam resistance in those SCCmec type II strains with wild-type sequences for mecI and mecR1.

Figure 4. Role of mecR2 on the optimal expression of β-lactam resistance. (A) Deletion of mecR2 from the chromosome of strain N315 (N315::ΔmecR2) causes a decrease on the resistance level to oxacillin, which can be reverted upon complementation with mecR2 expressed from an inducible promoter (N315::ΔmecR2+spac::mecR2) in the presence of the inducer (IPTG 100 μM). (B) The poor expression of oxacillin resistance by recombinant strain COL::RI, can also be reverted upon complementation with mecR2 expressed from an inducible promoter (COL::RI+spac::mecR2) in the presence of the inducer (IPTG 100 μM). The oxacillin-resistance levels were evaluated by diffusion disks containing 1 mg of oxacillin (left) and by population analysis profiles (PAP’s) (right).

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Figure 5. Effect of mecR2 on the induction of mecA transcription. (A) Northern blot and (B) qRT-PCR analysis of the mecA induction profile in parental strain N315, mecR2 null-mutant (N315::ΔmecR2) and complemented mutant (N315::ΔmecR2::spac::mecR2, IPTG 100 μM). Cultures were induced with a sub-MIC concentration of oxacillin (0.05 mg/L) and samples were taken at 0’, 5’, 10’ 30’ and 60’.

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Figure 6. mecR2 transcription analysis. qRT-PCR analysis of the mecR2 induction profile in parental strain N315 and its complemented mecR2 mutant (N315::ΔmecR2::spac::mecR2, IPTG 100 μM). Cultures were induced with a sub-MIC concentration of oxacillin (0.05 mg/L).

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In order to explore that hypothesis, we sought to test the role of mecR2 in the phenotypic expression of β-lactam resistance in prototype strains of epidemic MRSA clones characterized by SCCmec type II. The MRSA population has a very strong clonal structure and only a few epidemic clones are responsible for the majority of infections worldwide [36,37]. Three epidemic MRSA clones characterized by SCCmec type II have been described [38]: clone ST5-II, “New York/Japan” or USA100; clone ST36-II, EMRSA-16 or USA200; and clone ST45-II or USA600. MRSA clones ST5-II and ST36-II are two of the most important nosocomial clones in the USA and UK, respectively. Prompted by this epidemiological data, we evaluated the role of mecR2 in three representative strains of those SCCmec type II clones selected from a large US collection of MRSA [39]: strains USA100, USA200 and USA600. For this purpose, the chromosomal mecR2 deletion of strain N315 (N315::ΔmecR2) was transduced into those strains originating the recombinant strains: USA100::ΔmecR2, USA200::ΔmecR2 and USA600::ΔmecR2. In the three prototype strains, deletion of mecR2 caused a sharp decrease of the phenotypic expression of β-lactam resistance, which could be complemented by expressing mecR2 in trans under the control of an inducible promoter (spac::mecR2) (Figure 7A). We have also analyzed the effect of mecR2 on the induction of mecA transcription in strains USA100, USA200 and USA600 by Northern blotting. Compared to N315, the three parental strains expressed mecA at higher levels and, in agreement with what was observed for strain N315 (Figure 5), deletion of mecR2 caused a sharp decrease on the mecA induction and transcription levels (Figure 7B).

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

Since strains N315, USA100, USA200, and USA60 have complete mecR1 genes and strain COL has a truncated mecR1 gene but with a complete N-terminal inducer domain, with the previous experiments we could not formally exclude that MecR2 function is dependent of at least the N-terminal inducer domain of MecR1. Therefore, we sought to test the effect of mecR2 in a prototype SCCmec type V MRSA strain, characterized by an extensive deletion of mecR1 spanning both N- and C-terminal domains [40] – Figure 1A. Among our collections, we selected strain HT0350 [41], since it was the only strain also negative for the β-lactamase locus [24], similar to what was observed for strain COL, overexpression of MecI in strain HT0350 (HT0350+mecI) caused a sharp decrease of resistance level, which was fully reverted with the co-overexpression of MecI and MecR2 (HT0350+mecI-mecR2) – Figure 8A. These data suggest that the effect of mecR2 on the expression of β-lactam resistance in S. aureus is not dependent of mecR1, and as such MecR2 may act as an anti-repressor.

Since mecA transcription can be co-regulated by the regulators of the β-lactamase (bla) locus, blaR1-blaI, and parental strains N315, USA100, USA200 and USA600 are bla positive, we sought to evaluate the effect of bla genes on the observed mecR2-induced phenotypes. For this purpose, we took advantage of the fact that the experimental strategy used to construct the mecR2 knockout in prototype strain N315 generated an intermediate mutant strain which lost the β-lactamase plasmid, probably due to the multiple passages, many of which at 45°C. As in all other chromosomal manipulations, the mecR2 genetic deletion was transduced back to the parental β-lactamase positive strain N315 to generate the final deletion mutant (N315::ΔmecR2) tested in all previous experiments. As illustrated in Figure 8B, in both variants of the mecR2 chromosomal deletion, there was a sharp decrease of the β-lactam resistance. Together with the experimental data for strains COL and HT0350, both bla negative, this assay indicated that the mecR2 function on the phenotypic expression of β-lactam resistance is not dependent on the presence of the β-lactamase plasmid.

In addition, in order to exclude an interaction of MecR2 with bla regulators, we sought to evaluate the phenotype of a mecR2 deletion mutant in prototype strain HU25, a highly resistant MRSA strain which is positive for the bla locus and has a truncated non-functional MecI protein due to a premature stop codon [24]. Previous studies have shown that in the presence of oxacillin, the transcription of mecA is readily induced in strain HU25, presumably by the bla system [24]. As illustrated in Figure 8C, the absence of mecR2 in strain HU25 (strain HU25::ΔmecR2) had no effect on the phenotypic expression of oxacillin resistance, suggesting that MecR2 is not required for the mecA induction mediated by the BlaR1-BlaI system.

MecR2 interacts directly with MecI

The MecR2 is homologous to the transcriptional repressor of the xylosine operon, XylR [42], which has a N-terminal DNA-binding domain and a C-terminal dimerization domain. The mecR2 gene in the prototype strain N315 has no DNA binding domain due to a deletion of four tandem-thymine residues, which, together with the genetic experiments done with this variant (Figures 4, 7 and 8), suggests that only the dimerization domain is involved in the MRSA phenotype. Therefore, we reasoned that the mode of action of MecR2 might involve a direct interaction with the MecI dimer, through its dimerization domain, which eventually would interfere with its binding to the mecA promoter. To test this hypothesis, we evaluated the MecR2-MecI interaction using a bacterial two-hybrid in vivo strategy [43]. In these experiments, we used the small mecR2 variant present in prototype strain N315. As in-house controls, the MecI::MecR2 interaction, previously demonstrated using the yeast two-hybrid strategy [44], as well as the MecR2::MecR2 interaction were also evaluated. Positive results were observed in 4 out of the 8 MecE::MecR2 combinations and in 1 of 4 MecE::MecI combinations (Figure 9A). No MecR2::MecR2 interaction was detected in the four combinations tested (data not shown) and, as such, the assay was not conclusive in this case. Altogether, these observations provide evidence for a MecR2::MecI direct interaction.

MecR2 interferes with the binding of MecI to the mecA promoter

Next, we evaluated the interference of purified MecR2 protein with the binding of MecI to the promoter of mecA (PmecA) at several molar ratios by the electrophoretic mobility shift assay (EMSA), a strategy previously used to study the binding of purified MecI protein to PmecA [14;24]. In these experiments, we expressed in E. coli the full MecR2 protein from prototype strain HU25, since the shorter variant of strain N315 could not be expressed and purified in a soluble form at high concentrations. As illustrated in Figure 9B, MecR2 interferes with the binding of MecI to PmecA in a concentration-dependent manner: the heavier band presumably reflecting the binding of MecI dimers to PmecA decreases in intensity, whereas the intermediate band reflecting the binding of MecI monomers to PmecA and the lighter free DNA band increase in intensity. In line with the genetic experiments, this effect was optimal for a MecR2::MecI molecular ratio below one; in the presence of excess MecR2 the binding of MecI to PmecA was restored. This in vitro loss of effect at higher concentrations of MecR2 suggests that under these conditions MecR2 may be trapped in a non-active conformation; e.g. MecR2 may oligomerize in a concentration-dependent manner and stop interacting with MecI. It should be noted that in wild-type strains, mec and mecR2 are co-transcribed from the mecR1 promoter and, as such,
the cellular amounts of both proteins are likely to be similar. Since in these experiments we used the full MecR2 variant containing a putative N-terminal DNA binding, control EMSA experiments with MecR2 alone were performed to verify that purified MecR2 did not bind to \( \text{P}_{\text{mecA}} \) alone (Figure S4A). In addition, control experiments with mixtures of MecI and MBP (maltose-binding protein, which has an identical molecular weight to MecR2) were performed to demonstrate that inhibition of MecI binding to \( \text{P}_{\text{mecA}} \) is specific for MecR2 (Figure S4B). Finally, in order to exclude the hypothesis that at higher concentrations MecR2 binds (not specifically) to secondary sites in \( \text{P}_{\text{mecA}} \) DNA in a MecI-dependent manner, EMSA assays with MecI-MecR2 mixtures

**Figure 7.** \( \text{mecR2} \) is essential for the optimal expression of \( \beta \)-lactam resistance in strains with functional \( \text{mecI-mecR1} \) regulatory locus. (A) Deletion of \( \text{mecR2} \) from the chromosome of prototype epidemic strains USA100, USA200 and USA600 harboring SCCmec type II causes a decrease on the resistance level to oxacillin, which can be reverted upon complementation with mecR2 expressed from an inducible promoter (\( \text{spac::mecR2} \)) in the presence of the inducer (IPTG 100 \( \mu \)M). (B) Northern blot analysis of the \( \text{mecA} \) induction profile in parental strains USA100, USA200 and USA600 and respective \( \text{mecR2} \) null-mutants. Cultures were induced with a sub-MIC concentration of oxacillin (0.05 mg/L) and samples were taken at 0’, 10’ and 60’. For comparative purposes the profile of parental strain N315 and \( \text{mecR2} \) null-mutant were also repeated. Note that film was exposed for 4 h whereas in Figure 5A it was exposed for 48 h.

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positive mecR2

Prototype strain HU25 is positive for mecR2 and has a truncated non-functional MecI and, as such, the mecA expression is under the exclusive control of bla regulatory genes. Deletion of mecR2 in strain HU25 (HU25::ΔmecR2) has no effect on the phenotypic expression of oxacillin resistance.

MecR2 promotes the proteolytic cleavage of MecI

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

First, we demonstrated that the cognate mecA regulatory locus contains, besides MecR1-MecI, the anti-repressor MecR2. MecR2 compensates for the inefficient MecR1-mediated induction of mecA, being essential for the optimal expression of β-lactam resistance (Figures 3, 4 and 7A), and enabling the full induction of mecA transcription (Figures 5 and 7B). These findings explain the puzzling observation of the poor mecA induction by MecR1, reported in studies analysing the effects of mecR1-mecI only (without mecR2) on mecA expression in recombinant strains [12,13]; an experimental artefact also observed in this study with recombinant strain COL::RI (artificially made positive for mecR1-mecI) and in the mecR2 null mutant strains (Figures 4 and 7). Because wild-type MRSA strains positive for mecR1-mecI are also positive for mecR2, these strains are in fact able to express optimal levels of β-lactam resistance and, as such, mecA is efficiently induced upon exposure to β-lactams by its cognate three-component regulatory system.

Second, the findings herein described also clarify the relevance and specificity of MecI proteolysis observed upon induction with β-lactams [16,45–48]. Our data demonstrates that MecI proteolysis is mediated by unspecific cytoplasmatic proteases. First, we demonstrated that the cognate mecA regulatory locus contains, besides MecR1-MecI, the anti-repressor MecR2. MecR2 compensates for the inefficient MecR1-mediated induction of mecA, being essential for the optimal expression of β-lactam resistance (Figures 3, 4 and 7A), and enabling the full induction of mecA transcription (Figures 5 and 7B). These findings explain the puzzling observation of the poor mecA induction by MecR1, reported in studies analysing the effects of mecR1-mecI only (without mecR2) on mecA expression in recombinant strains [12,13]; an experimental artefact also observed in this study with recombinant strain COL::RI (artificially made positive for mecR1-mecI) and in the mecR2 null mutant strains (Figures 4 and 7). Because wild-type MRSA strains positive for mecR1-mecI are also positive for mecR2, these strains are in fact able to express optimal levels of β-lactam resistance and, as such, mecA is efficiently induced upon exposure to β-lactams by its cognate three-component regulatory system.

Second, the findings herein described also clarify the relevance and specificity of MecI proteolysis observed upon induction with β-lactams [16,45–48]. Our data demonstrates that MecI proteolysis is required for optimal expression of resistance and that MecR2 alone (i.e. without MecR1, Figure 8A) interferes specifically with the MecI function and promotes its inactivation by proteolytic cleavage, presumably mediated by (non-specific) native cytoplasmatic proteases (Figure 9). Our findings contrast with
published observations for the \textit{blaR1-blaI} system of \textit{Bacillus licheniformis}, demonstrating that the proteolysis of the repressor is a secondary event not required for induction of resistance [48], and also for the \textit{blaR1-blaI} system of \textit{S. aureus} demonstrating that BlaR1 directly promotes the BlaI cleavage [15]. Altogether, these observations suggest the existence of subtle differences between the \textit{mecR1-mecI-mecR2} and the \textit{blaR1-blaI} regulatory systems and that, in \textit{S. aureus}, BlaR1 may accumulate the MecR1 and MecR2 functions.

Our findings lead us to propose a revised model for the induction of \textit{mecA} expression in wild-type MRSA strains (Figure 10):

(i) in the presence of \beta-lactams, MecR1 is activated and induces the transcription of \textit{mecA} and \textit{mecR1-mecI-mecR2}; (ii) the anti-repressor MecR2, destabilizes MecI-dimers, disturbing their binding to the \textit{mecA} promoter and fostering their proteolytic inactivation, resulting in a sustained induction of \textit{mecA} transcription; (iii) when depletion of \beta-lactam occurs, MecR1 is no longer activated and a steady state is established consisting of stable MecI-dimers bound to the \textit{mecA} promoter (and protected from proteolysis) and residual copies of MecR1 at the cell membrane; the remaining free MecR2 molecules are most likely degraded by

Figure 9. MecR2 interacts directly with MecI, interfering with the binding of MecI to the \textit{mecA} promoter and fostering the proteolysis of MecI. (A) \textit{In vivo} analysis of the MecR2::MecI interaction using the bacterial two-hybrid strategy. This strategy is based on the restoration of the adenylate cyclase (CyaA) activity of \textit{E. coli}, which activates a specific reporter gene, \textit{lacZ}. Interactions between protein fusions were evaluated in liquid cultures through the hydrolysis of the chromogenic X-gal substrate by the activated \text{\beta}-galactosidase. The MecR2::MecI interaction was evaluated using the eight possible combinations: fusions either with T25 or T18 fragments of CyaA at either the N’ or C’ terminals. Tube 1, T18-MecR2::MecI-T25; tube 2, T18-MecR2::T25-MecI; tube 3, MecR2-T18::MecI-T25; tube 4, MecR2-T18::T25-MecI; tube 5, MecR2-T25::T18-MecI; tube 6, T25-MecR2::T18-MecI; tube 7, MecR2-T25::MecI-T18; tube 8, T25-MecR2::MecI-T18; tube 9, positive control provided by the manufacturer (Zip-T25::Zip-T18); tube 10, negative control (T25::T18); tube 11, “in-house” positive control testing the MecI-MecI interaction T18-MecI::T25-MecI. (B) Electrophoretic mobility shift assay (EMSA) of the binding of purified MecI to a labeled 212 bp DNA fragment encompassing the \textit{mecA} promoter in the presence of purified MecR2. MecI concentration was constant in all binding reactions (0.05 \mu g). Lane 1, negative control, labeled DNA only; lane 2, 8-fold excess of MecI; lane 3, 4-fold excess of MecI; lane 4, binding control, MecI only; lane 5, 2-fold excess of MecI; lane 6, equimolar amounts of MecI and MecR2; lane 7, 2-fold excess of MecR2; lane 8, control for specific binding, MecI with a 125 molar excess of unlabelled DNA. (C) Western blotting analysis of MecI cleavage in total protein extracts (60–80 mg/lane). Lane 1, prototype strain N315; lane 2, mecR2 null-mutant (N315::\textit{DmecR2}); lane 3, strain HT0350 co-overexpressing MecI and MecR2 (HT0350::\textit{mecImecR2}); lane 4, strain HT0350 overexpressing MecI (HT0350::\textit{mecI}). Cultures of N315 and N315::\textit{mecR2} cultures were induced with a sub-MIC concentration of oxacillin (0.05 mg/L).

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the cellular protein turnover pathway. This model implies that in the absence of MecR2, functional MecI-dimers are more resilient to proteolytic inactivation and outcompete the MecR1 signalling, a hypothesis supported by the Western blotting experiments (Figure 9C).

Concluding remarks

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

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

In short, this study points to a revision of the model for the transcriptional control of *mecA* by its cognate regulatory locus, which may pave the way for the design of alternative therapeutic strategies targeting the induction mechanism of the resistance gene [52,53]. If successful, these strategies may extend the clinical utility of β-lactams for the treatment of MRSA infections. Recycling β-lactams is particularly relevant given that MRSA pose a substantial burden for the public health, are often multi-drug resistant and, in the past 40 years, very few new classes of antibiotics have reached the clinic.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Tables S1 and S2, respectively. *S. aureus* strains were routinely grown at 37°C with aeration in tryptic soy broth (TSB, Difco) or on tryptic soy agar plates (TSA, Difco). *E. coli* strains were grown with aeration at 37°C in Luria-Bertani broth (LB, Difco) or in Luria-Bertani agar (LA, Difco). Recombinant *E. coli* strains were selected and maintained with ampicillin at 100 μg/mL. Recombinant *S. aureus* strains were selected and maintained either with tetracycline at 5 or 40 μg/mL, chloramphenicol at 20 μg/mL, or erythromycin at 10 μg/mL, as appropriate. Phenotypic analysis of β-lactam resistance in *S. aureus* parental and recombinant strains was performed by diffusion-disks containing 1 mg of oxacillin, Oxacillin E-test (AB Biodisk), or by population analysis profiles (PAPS) at 30°C for 24–48 h, as previously described [24,54,55].
Oxacillin is a methicillin analogue and has replaced methicillin in clinical use.

DNA manipulations

DNA manipulations were performed by standard methods [56,57]. Total DNA from S. aureus was isolated from bacterial cultures with the Wizard Genomic DNA purification Kit (Promega) according to the manufacturer’s recommendations and using lysostaphin (0.3 mg/mL) and RNase (0.3 mg/mL) in the lysis step. Plasmid DNA was isolated from bacterial cultures with the High Pure Plasmid Isolation Kit (Roche). For plasmid DNA isolation from S. aureus strains the culture pellets were resuspended in “Suspension Buffer” supplemented with 0.1 mg/mL of lysostaphin and incubated at 37°C for 30-60 minutes. Restriction enzymes were used as recommended by the manufacturer (New England Biolabs). Dephosphorylation of vector arms and insert ligation was performed with Rapid DNA Dephos & Ligation kit (Roche), according to the manufacturer’s recommendations. Routine PCR was performed with GoTaq Flexi DNA polymerase (Promega). PCR amplification of cloning inserts was performed by high-fidelity PCR (Pfu Turbo DNA polymerase, Stratagene). 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 Purified Gene Clean Turbo Kit (MP Biomedicals), following the manufacturer’s recommendations. DNA sequencing was performed by Macrogen (www.macrogen.com) or STAB Vida (www.stabvida.com). All primers used in this study are listed in Table S3.

Construction of recombinant S. aureus strains

As control, we constructed a plasmid pGC::mecI-mecR2 (strain COL::erm), containing mecI-mecR2 regulatory locus in the chromosome of strain COL, we first construct pSPT::IS-erm-mecR2-mecI-mecR1. The integration into COL chromosome of the three recombinant plasmids (pSPT::IS-erm-mecR2-mecI-mecR1, pSPT::IS-erm-mecR2-mecI-mecR1 and pST::IS-erm-A mecR1) was performed by an insertion-deletion strategy by homologous recombination (Figure S3). First, insertion into the chromosome was promoted by growing transductants in TSB at a non-permissive temperature (45°C) without antibiotic selection for 2-3 days, with daily re-inoculum in fresh medium. Serial dilutions were plated onto TSA plates supplemented with erythromycin (Ery) and tetracycline (Tc). Single-colonies Erm’-Tc’ were screened for chromosomal insertion of the plasmids by PCR and the absence of plasmid DNA was confirmed. Resolution of integrates by homologous-recombination was promoted by growing selected single colonies in TSB supplemented with tetracycline at 40 μg/mL at the permissive temperature of 30°C for 4-5 days, with daily re-inoculum in fresh medium. Finally segregation of the excised plasmids was promoted by growing cultures at 45°C without antibiotic selection for 2-3 days, with daily re-inoculum in fresh medium. Culture aliquots were plated onto TSA plates supplemented with erythromycin and single colonies Erm’-Tc’ were selected by replica plating onto TSA plates supplemented with erythromycin or tetracycline.

To construct the mecR2 gene null mutant in strain N315, two DNA fragments of 1000 bp corresponding to the 5’ and 3’ vicinities of the mecR2 gene were amplified by PCR from strain N315 DNA using primers MR2-P2/MR2-P3 and MR2-P4/MR2-P5, respectively. The cat gene coding for chloramphenicol resistance was also amplified by PCR from pGC2 plasmid with primers CAT-P1/CAT-P2. The three fragments were double-digested with Sall/PstI, BamH1/Xhol and Xhol/Sall, respectively, and then sequentially cloned into pSPT181, originating the pSPT::cat-A mecR2 recombinant plasmid. Following the same insertion-deletion strategy described above, but selecting for chloramphenicol resistance instead of erythromycin resistance, we obtained the recombinant strain N315::A mecR2 in which the chromosomal copy of mecR2 was replaced by the cat gene (Figure S3). To complement the N315::A mecR2 null-mutant three recombinant plasmids were constructed: (i) pSPT::mecR2, pSPT181 vector containing at the XmaI site the mecR2 gene from strain N315, obtained by PCR with primers MR2-P6/MR2-P7 (the proper insert orientation was selected by restriction analysis using the HindIII site within mecR2 gene); (ii) pSPT::mecR2, pSPT181 containing the mecR2-mecR2 genes of strain N315, constructed by sequential cloning, first, at the BamH1/PstI, the mecR2 gene site obtained with primers MI-P3/MI-P4 and then, at the XmaI site, the mecR2 gene obtained with primers MR2-P6/MR2-P7 (iii) pSPT::spac-mecR2, pSPT181 with the mecR2 gene under the control of Ppac promoter, constructed by sequential cloning the 1.6 kb EcoR1-BamH1 fragment from plasmid pHB8 containing the spac locus (Ppac-polylinker-lacI repressor) and then, at the XmaI site of the spac polylinker, the mecR2 gene from strain N315 obtained with primers MR2-P6/MR2-P7. As control, N315::A mecR2 was transformed with a pST181 derivative containing the spac locus only (pSPT::spac).
To generate the mecR2 gene null mutant in prototype strains USA100, USA200, USA600, and HU25 the chromosomal deletion of strain N315:ΔmecR2 was transduced by bacteriophage infection with selection for chloramphenicol resistance, originating recombinant strains USA100::ΔmecR2, USA200::ΔmecR2, USA600::ΔmecR2 and HU25::ΔmecR2. Mutant strains USA100::ΔmecR2, USA200::ΔmecR2 and USA600::ΔmecR2 were then complemented with recombinant plasmid pSPT::pta-mecR2.

Transcription analysis

Total RNA extraction and purification was performed as previously described [60]. Briefly overnight cultures were grown in TSB, supplemented with antibiotics when appropriate, and then diluted 1:50 in fresh TSB and grown to the mid-log phase (OD600~0.7). Cultures were stabilized with two volumes of RNAprotect Bacterial Reagent (Qiagen), according to the manufacturer’s recommendations. The cells were centrifuged and pellets were resuspended in 1 mL of Trizol reagent (Invitrogen). The resuspended cells were transferred to a new tube with silica beads (Lysing Matrix B tubes, Bio101) and cell lysis was performed in the FastPrep FP120 apparatus (Bio 101). RNA was extracted with chloroform, precipitated with isopropanol, washed twice with ethanol at 80% and resuspended in diethyl pyrocarbonate (DEPC)-treated water. For the analysis of the meca and mecR2 induction profiles, after cultures were grown to OD600~0.7, oxacillin at 0.05 μg/mL was added and cultures were incubated for an additional 60 minutes. Samples were taken either at 0, 5, 15, 30, and 60 or at 0, 10 and 60 minutes, stabilized, pelleted and kept on ice until being simultaneously processed. For RT-PCR and qReal-time RT-PCR experiments (see below), total RNA preps were treated twice with DNase (RNase-Free DNase Set I, Qiagen) and purified with RNeasy Mini Kit (Qiagen), according to the manufacturer’s recommendations. Control PCR reactions were performed to test the absence of DNA contamination in total RNA preps.

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

The induction profiles of meca and mecR2 were determined by quantitative Real-time RT-PCR (qRT-PCR) and/or Northern blotting. For the qRT-PCR data analysis, relative gene expression was expressed as a ratio to the transcript of pta, a housekeeping gene with constitutive expression [61]. Standard curves were generated using serial dilutions (0.4–40 ng/reaction) of genomic DNA and primers MR2-RT3/MR2-RT4, MA-RT1/MA-RT2 and pta-RT1/pta-RT2 for amplification internal fragments of mecR2, meca, and pta, respectively. qRT-PCR reactions were performed with QuantiTect SYBR Green RT-PCR Kit (Qiagen); each 25 μl reaction containing 12.5 μl SybrGreen mix, 0.25 μl RT enzyme mix, 12.5 pmol of each primer and 40 ng of purified RNA. Amplification consisted of an initial RT step at 50°C for 30 min, followed by a denaturation step at 95°C for 15 min, then by 45 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C. For each RNA sample three independent qReal-Time RT-PCR experiments were carried out. Fluorescence was measured at the end of the annealing-extension phase of each cycle. A threshold value for the fluorescence of all samples was set manually. The reaction cycle at which the PCR product exceeds this fluorescence threshold was identified as the threshold cycle (Ct). The Ct was then converted to relative quantity of mRNA by using a standard curve. To verify the specificity of the PCR amplification products, melting curve analysis was performed between 60–95°C.

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

Bacterial two-hybrid assays

This strategy is based on the restoration of the adenylate cyclase (CyaA) activity by heterodimerization of protein fusions containing the T25 and T18 fragments, which form the catalytic domain of CyaA. CyaA is involved on cAMP synthesis, which binds to CAP forming the cAMP/CAP complex that activates a specific reporter gene, lacZ [43]. All strains and plasmids used in the bacterial two-hybrid studies are described in Table S4. Both genes, meca and mecR2, were amplified from the chromosomal DNA of strain N315 by high-fidelity PCR, using primers MI-BTH1/MI-BTH2 for meca and MR2-BTH1/MR2-BTH2 for mecR2. PCR products were double-digested with KpnI/XbaI and fused to T25 or T18 fragments either at the N’ or C’ terminals, using plasmids pUT18, pUT18c, pKNT25 and pKT25, originating the following fusion proteins: T18-MecI, MecI-T18, T25-MecI, MecI-T25, T18-MecR2, MecR2-T18, T25-MecR2 and MecR2-T25. The eight MecI::MecR2 recombinant plasmid combinations were co-transformed into the reporter strain Escherichia coli BTH101 and grown on Luria-Bertani (LB) and LA agar supplemented with 8 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 50 μg/mL kanamycin, 100 μg/mL ampicillin, 100 μg/mL streptomycin, 500 μM (IPTG) and 2% glucose. As a positive control, plasmids p25Zip and p18Zip, containing two leucine zipper domains, were also co-transformed into E. coli BTH101 strain. Additionally, as in-house controls, the previously reported MecI::MecI interaction based on the yeast two-hybrid strategy [44] was evaluated, as well the MecR2-MecR2 interaction, by co-transforming the four combinations of meca-containing plasmids and the four combinations of mecR2-containing plasmids, respectively.

Electrophoretic mobility shift assays (EMSA)

To overexpress and purify MecR2 protein, a mecR2 gene insert was obtained from the chromosome of strain HU25 by high-fidelity PCR amplification with primers MR2-crl1/MR2-crl2 and double-digestion with Ncol/Xhol. The mecR2 insert was then cloned in frame into the expression vector pCR.II, generating the recombinant plasmid pCR.II::mecR2, expressing mecR2 with a N’ terminal His6 tag. pCR.II::mecR2 was stabilized in E. coli DH5α and then transformed to E. coli B21 (DE3). MecR2 protein overexpression was carried out in LB medium supplemented with 50 mg/mL kanamycin, at 18°C, and induced at an OD600~0.5 with 1 μM Isopropyl β-D-thiogalactopyranoside (IPTG) for 5 h.
MecI protein was overexpressed from recombinant strain DH5α/pP25-Ec:mecI [62], using LB medium supplemented with 100 μg/mL ampicillin, at 37°C and induced at an OD₆₀₀=0.5 with 1 μM IPTG for 3 h. Protein extracts were purified as previously described [62]. The purity of the proteins was assessed by 10% tricine SDS-PAGE analysis and mass-spectrometry. The concentrations of purified MecR2 and MecI were estimated using the Protein Assay Kit II (BioRad), as recommended by the manufacturer. For the electrophoretic mobility shift assay we used the chemiluminescent-based DIG Gel Shift Kit (Roche), following the manufacturer’s recommendations. As DNA target we used a 212 bp fragment encompassing the mecA promoter and operator sequences from prototype strain N315 obtained by PCR amplification with primers MA-PF1/MA-PR1. The binding of each purified protein to the mecA promoter (PMecA) was first evaluated and then MecI-MecR2 mixtures were tested. As control the binding to PMecA of MecI-MBP (Maltose-binding protein, MBP2, New England Biolabs) mixtures were also evaluated. EMSA assays with MecI-MecR2 mixtures were also performed with a smaller 39 bp DNA fragment containing the MecI protected sequences, obtained by annealing primers MI-Box1/MI-Box2.

Western blotting

To prepare protein extracts of S. aureus, parental and recombinant strains were grown in TSB supplemented with oxacillin at sub-MIC concentration (0.05 μg/mL) until mid-log phase (OD₆₀₀=0.7). Cell pellets were frozen in liquid nitrogen, thawed and resuspended in Buffer A (50 mM Tris-HCl; 10 mM MgCl₂; 0.5 mM PMSF) containing 10 μg/mL DNase I. Cells were broken mechanically in a French press followed by centrifugation (22,000 × g, 20 min, 4°C) to remove unbroken cells and cell debris. The supernatants containing the cytoplasmic proteins were recovered and filtered through 0.45-μm-pore-size membrane filters. Protein extracts (60-80 μg) were resolved in a 18% Tris-Glycine SDS-PAGE.

After electrophoresis, the proteins were transferred to a 0.45 μm nitrocellulose membrane (Trans-Blot, Bio-Rad). The membranes were blocked at room temperature for 1 hour, in 20 mL of Blocking solution - Tween- Phosphate Buffered Saline (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na₂HPO₄; 1.47 mM KH₂PO₄; 0.05% Tween-20) with 6% low-fat milk. MecI protein was detected by immunoblot analysis using a custom polyclonal antibody (1/50.000 dilution of secondary antibody (Goat Anti-Rabbit IgG (Eurogentec) raised against purified MecI (1/1.000 dilution) and a detected by immunoblot analysis using the same primer pairs and chromosomal DNA from strain N315 as template; lanes 10–11, negative control, RT-PCR reactions without the reverse-transcription step for total RNA preparations of strains N315 and HU25, respectively, using the primer pair MR2-RT1/MR2-RT2 (i.e. the one originating the smallest amplicon).

Supporting Information

Figure S1 Multiple sequence alignment between the repressor of the xylose operon (XylR) and the anti-repressor MecR2 found in staphylococci. Species names (strain code in parenthesis): Sxy – S. xylosus, Svi – S. viridians, Scs – S. sciu, Sau – S. aureus, Spri – S. pseudintermedius, Sfl – S. flavescens, Sep – S. epidermidis, Green – identical residues; red – similar residues; white – divergent residues. The figure was prepared using “The Sequence Manipulation Suite” freely available at http://www.bioinformatics.org. (TIF)

Figure S2 Transcriptional analysis of mecR2. (A) Genetic organization of the mecA regulatory locus and location of primers used in the RT-PCR assays. (B) Gel electrophoresis of the RT-PCR products obtained with total RNA from strains N315 and HU25, respectively. MM, molecular weight marker (1 kb DNA ladder); lanes 1–2, mecI-mecR2 co-transcript; lanes 3–4, mecR1-mecI co-transcript; lanes 5–6, mecR2 transcript; lanes 7–9, positive controls, PCR reactions using the same primer pairs and chromosomal DNA from strain N315 as template; lanes 10–11, negative control, RT-PCR reactions without the reverse-transcription step for total RNA preparations of strains N315 and HU25, respectively, using the primer pair MR2-RT1/MR2-RT2 (i.e. the one originating the smallest amplicon). (TIF)

Figure S3 Insertion-deletion strategies used for the reconstruction of the mecA regulatory locus in the chromosome of strain COL and deletion of mecR2 from the chromosome of strain N315. Integration through homologous recombination of recombinant thermosensitive plasmids was promoted at a non-permissive temperature (45°C) and with selection for tetracycline resistance (Tc'). Resolution of co-integrates was promoted at a permissive temperature (30°C) with selection for tetracycline resistance and segregation of the excised plasmids was promoted at 45°C without antibiotic selection. Colonies susceptible to tetracycline (Tc') and resistant to erythromycin (Ery') or chloramphenicol (Cm') were selected for further analysis. (TIF)

Figure S4 Control experiments for the electrophoretic mobility shift assays. (A) Binding of purified MecR2 to mecA promoter. Lane 1, negative control, labeled DNA only; lane 2, 0.001 μg of MecR2; lane 3, 0.01 μg of MecR2; lane 4, 0.05 μg of MecR2; lane 5, 0.1 μg of MecR2; lane 6, 0.25 μg of MecR2; lane 7, 0.5 μg of MecR2; lane 8, 1 μg of MecR2. (B) Binding of purified MecI to the labeled mecA promoter DNA sequence in the presence of MBP (Maltose-binding protein) at several molar ratios. MecI concentration was constant in all binding reactions (0.05 μg). Lane 1, negative control, labeled DNA only; lane 2, 8-fold excess of MecI; lane 3, 4-fold excess of MecI; lane 4, binding control, MecI only; lane 5, 2-fold excess of MecI; lane 6, equimolar amounts of MecI and MBP; lane 7, 2-fold excess of MBP; lane 8, control for specific binding, MecI with a 125 molar excess of unlabelled DNA. (TIF)

Table S1 Strains used in this study. (DOC)

Table S2 Plasmids used in this study. (DOC)

Table S3 Primers used in this study. (DOC)

Table S4 Strains and plasmids used in the bacterial two-hybrid assays. (DOC)

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Author Contributions
Conceived and designed the experiments: DCO. Performed the experiments: PA CM DCO. Analyzed the data: DCO PA HdL. Wrote the paper: DCO.

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