Structure-function studies of the staphylococcal methicillin resistance anti-repressor, MecR2 **

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RUNNING TITLE: Molecular analysis of methicillin resistance factor MecR2

Background: PBP2a-based methicillin resistance in S. aureus is regulated by the MecR2.

Results: The structure of MecR2 shows a dimeric multi-domain ROK-family protein, which non-specifically binds oligonucleotides but not ligands.

Conclusion: MecR2 represents an evolution within ROK proteins to give rise to a protein-binding anti-repressor.

Significance: The present results pave the way for the design of new antimicrobials.

SUMMARY

Methicillin resistance in Staphylococcus aureus is elicited by the Mec1-MecR1-MecA axis encoded by the mec locus. Recently, MecR2 was also identified as the third regulator of mec through binding of the methicillin repressor, MecI. Here we show that plasmid-encoded full-length MecR2 restores resistance in a sensitive S. aureus mecR2 deletion mutant of the resistant strain N315. The crystal structure of MecR2 reveals an N-terminal DNA-binding domain (NDD), an intermediate scaffold domain (ISD), and a C-terminal dimerization domain (CDD) that contributes to oligomerization. The protein shows structural similarity to ROK (from repressors, open-reading frames and kinases) family proteins, which bind DNA and/or sugar molecules. We found that functional cell-based assays of three point mutants affecting residues participating in sugar binding in ROK proteins had no effect on the resistance phenotype. By contrast, MecR2 bound short double-stranded DNA oligonucleotides non-specifically and a deletion mutant affecting the NDD showed a certain effect on activity, thus contributing to resistance less than the wild-type protein. Similarly, a deletion mutant, in which a flexible segment of ISD had been replaced by four glycines, significantly reduced MecR2 function, thus indicating that this domain may likewise be required for activity. Taken together, these results provide the structural basis for the activity of methicillin anti-repressor, MecR2, which would sequester MecI away from its cognate promoter region and facilitate its degradation.

Staphylococcus aureus is the most prevalent human infectious agent associated with nosocomial and community infections. It has an extraordinary capacity to become resistant to antibiotics: it was the first bacterial pathogen reported to have become insensitive to penicillin (1-4). Among the distinct strains is methicillin-resistant S. aureus (MRSA), which currently refers to strains that are generally resistant to β-lactam antibiotics (BLAs; penicillins and cephalosporins). Some strains are also resistant to other chemotherapeutics such as aminoglycosides, glycopeptides, macrolides, lincosamides, and fluoroquinolones (3,5-8). MRSA is characterized by its ability to thrive in the presence of BLAs due to the biosynthesis of a penicillin-binding protein with low susceptibility to BLAs, termed PBP2a, PBP2* or MecA. The latter is encoded by the gene mecA, which is contained in a transducible mobile element, staphylococcal chromosomal cassette mec (SCCmec) (9-11). SCCmec also includes two genes, mecI and mecR1, which encode a transcriptional DNA-binding repressor, MecI, and an integral-membrane zinc-dependent sensor/signal transducer metalloprotease, MecR1, respectively (12,13). This system is homologous to the blaI-blaR1-blaZ signal transduction system that triggers synthesis of a β-lactamase (BlaZ) in both MRSA and methicillin-susceptible S. aureus, as well as in Bacillus licheniformis (14-16). The currently accepted working model hypothesis for these systems predicts that MecI/BlaI constitutively represses its own biosynthesis and that of MecR1/BlaR1 and MecA/BlaZ through binding to
the mec/bla promoter (10,13,17). Once MecR1/BlaR1 detects the presence of BLAs through its extracellular sensor domain (18-22), a signal is transmitted across the membrane to the intracellular zinc-dependent metalloprotease domain, which becomes activated through proteolytic cleavage (23-25). This yields functional MecR1/BlaR1, which, in turn, would—directly or indirectly—cause cleavage of MecI/Blal (26,27). This cleavage would render the dimeric repressor inactive and release it from its DNA binding site. A similar effect of inhibitor inactivation, which was not based, however, on a proteolytic pathway but on the intracellular presence of a short dipeptidic peptidoglycan fragment induced by BLA stress, has been recently described for B. licheniformis (28). Finally, release of transcriptional repression would elicit biosynthesis of MecA/BlaZ.

However, some lines of evidence are not explained by this ternary model. Firstly, the reported cleavage sites of MecR1/Blal and MecI/Blal are not compatible with a single proteolytic substrate specificity (29). Secondly, the structure of MecI and Blal in complexes with target DNA revealed that the repressor cleavage site is found within an α-helix and is not surface accessible (29-32), as would be required for proper proteolytic processing. Thirdly, highly-resistant MRSA strains did not show significant variation in the phenotypic expression of resistance when wild-type MecI was overexpressed in trans (33). Lastly, the presence of functional MecR1-MecI did not correlate with the level of BLA resistance in a representative collection of epidemic MRSA strains (33). These and other findings led several authors to postulate the existence of a further regulatory element, MecR2/Blal2, although no candidate molecules were suggested (4,10,17,18,31,32,34-38).

Most recently, comparative genomic sequence analysis revealed that, in some clinical MRSA strains, a putative gene is found upstream of mecA, which is co-transcribed with mecI and mecR1 (34). Its transcript could be detected by reverse-transcriptase PCR of cultures induced with oxacillin—a methicillin analogue that has replaced the latter in clinical use—from a set of prototype clinical MRSA strains, paralleling mecA induction. Furthermore, in the presence of fully functional MecR1 and MecI, this gene was essential for the optimal expression of BLA resistance. Finally, in vitro and in vivo assays showed that the encoded protein acted as an anti-repressor by disrupting MecI binding to the mecA promoter and fostering its MecR1-independent proteolytic inactivation (34). Collectively, these findings indicated that the long sought-for gene encoding MecR2/Blal2 had been found, and so it was termed mecR2 (34).

In order to shed light on the structural determinants of folding and function of MecR2, we developed an efficient protocol to produce and purify large quantities of the functional wild-type protein. Furthermore, we assayed activity of MecR2 in cell-based assays and in vitro, and determined its X-ray crystal structure. We report here the molecular determinants of its function, which were further validated by mutational studies.

**EXPERIMENTAL PROCEDURES**

**Recombinant overexpression and purification** — The mecR2 gene was amplified from genomic DNA from S. aureus strain HU25 (M-I-A<sup>376</sup>, see GenBank AF422694, protein sequence identical to UniProt entry Q99XE2) by PCR and cloned into expression vector pCri8a between NcoI and XhoI restriction sites, giving rise to plasmid pCri8a:mecR2 (for strains and plasmids used in this study, see Table 1). This construct added an N-terminal His<sub>6</sub>-tag and a tobacco-etch virus (TEV) protease cleavage site; the N-terminus of the mature protein was, thus, preceded by a twenty-residue segment (M<sup>20</sup>GSSHHHHHHSSGGGNYFQGG<sup>31</sup>); amino-acid one-letter-code; superscript numbers depict protein residue numbering with negative numbers referring to extra N-terminal residues preceding the mature N-terminus, which is M<sup>31</sup> according to Q99XE2). A mutant, in which segment T<sup>130</sup>→T<sup>133</sup> had been replaced by four glycine residues (termed MecR2-T<sup>130</sup>→T<sup>133</sup>→GGGG), was amplified from the pST181::spac::mecR2 recombinant plasmid by PCR and cloned into expression vector pCri8a at the NcoI and XhoI restriction sites. Expression vectors were transformed into Escherichia coli BL21 DE3 cells and 1 liter cultures of transformed bacteria were induced for protein expression with 0.1mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18°C for 24h when the optical density at <i>λ=600nm</i> (OD<sub>600</sub>) reached 0.6. Cultures were subsequently centrifuged at 7,000×g (4°C, 20 min) and pellets suspended in 70mL ice-cold buffer A (20mM Tris-HCl, 0.5M sodium chloride, pH8.0). Cells were lysed with a cell disruptor (Constant Cell Disruption Systems Ltd.) operated at 1.35KBar, and the lysate was subsequently centrifuged at 75,600×g in an Avanti J-25 centrifuge with a JA-25.50 rotor (4°C, 20 min). The soluble fraction containing His<sub>6</sub>-TEV-MecR2 was applied onto a His-trap FF crude column (GE Healthcare) attached to an ÄKTA Purifier UP<sup>®</sup>-10 FPLC system previously equilibrated with buffer A. The protein was eluted with an imidazole gradient (0 to 0.5M imidazole in buffer A) and fractions containing the protein were subjected to a final size-exclusion chromatography step in a Superdex 75 16/60 column (GE Healthcare), previously equilibrated with 20mM Tris-HCl, 0.2M sodium chloride, pH7.4. Protein purity was assessed by 10%-tricine SDS-PAGE. TEV protease digestion of the N-terminal His<sub>6</sub>-tag was assayed under different conditions but yields were not satisfactory. As such, crystallization trials were performed using tagged MecR2 (hereafter wild-type MecR2). The selenomethionine variant of MecR2 was obtained in the same manner, except that 30 min before induction the cells were added to 500mL of medium lacking methionine and containing 25mg of selenomethionine (Sigma-Aldrich) instead.

**Circular dichroism experiments** — The CD spectra of wild-type MecR2 and MecR2-T<sup>130</sup>→T<sup>133</sup>→GGGG were recorded with a JASCO J-815 CD spectrometer operated with the following parameters: response 1s; scan speed 50nm/min; data acquisition interval 0.1nm; accumulations 3; and bandwidth 1nm. Quartz cuvettes for far-UV (190–250nm) with path lengths of 1 mm were used. Samples contained 0.2 mg/mL protein in 10mM sodium phosphate; 40mM sodium chloride, pH7.0. The CD spectrum of cuvette and buffer alone was subtracted from the protein solution spectra.

**MecR2 DNA-binding assays in vitro** — Recombinant wild-type MecR2 was assayed for DNA-binding capacity by electrophoretic mobility shift analysis. The 25-bp oligonucleotides encompassing the Z-dyad sequence of the blu promoter sequence with an additional 1-bp overhang on either end (C/G), which had been employed in structure-function studies with MecI (15,29,30) (termed here MR2-
EMSA1 and 2, see Table 2), were purchased from Sigma and annealed as described to yield 200nmol of double-stranded (ds) DNA in buffer 20mM Tris HCl, 0.1M sodium chloride, pH7.4. Purified MecR2 (100µM and 200µM) in buffer 20mM Tris HCl, 0.2M sodium chloride, pH7.4 was mixed with DNA solution at 0.5:1, 1:1, 2:1, and 4:1 protein:dsDNA molar ratios and analyzed in a band-shift assay in an 18.5% PAGE gel using annealed oligonucleotides as negative control. The same experiment was repeated with 25-bp nucleotides of same nucleotide composition but scrambled sequence (MR2-EMSA3 and 4).

**Site-directed mutagenesis of MecR2** — MecR2 mutant variants were obtained as previously described (39,40). Briefly, a round of two independent PCR reactions was performed on a PCR8::mecR2 with two complementary mutagenic primers and the two flanking mecR2 primers generating two intermediate PCR products with overlapping terminals (for primers and nucleotides, see Table 2). For the deletion mutant lacking segment S32-K62 (protein MecR2-A S35-K62), primer pairs MR2-3D F1/MR2-SDM2 and MR2-3D R1/MR2-SDM1 were used; for deletion mutant MecR2-T150-

I160—GGGG, primers MR2-SDM4 and MR2-SDM3 were used; and for point mutants E228A (MecR2-E228—A), N178A-E179A (MecR2-N178P-E179—AA), and E248A (MecR2-E248—A), the respective primers were MR2-SDM10 and MR2-SDM9, MR2-SDM14 and MR2-SDM15, and MR2-SDM12 and MR2-SDM11. Both intermediate PCR products were then diluted 50 times and mixed to form the DNA template of the second PCR, using primers spanning the entire mecR2 gene (MR2-3D F1/R1). All PCR reactions were performed with the Phusion High-Fidelity DNA Polymerase (New England Biolabs).

**Cell-based activity of recombinant MecR2** — In order to assess the activity of recombinant wild-type MecR2 expressed from vector pCri8::mecR2 and the aforementioned mutants generated by site-directed mutagenesis, the full respective inserts were cloned into a S. aureus expression vector containing the Pspl IPTG-inducible promoter (pSPT181::spac). Briefly, using flanking primers MR2-3D F1/R1, the insert sequence was amplified using the Phusion High-Fidelity DNA Polymerase (New England Biolabs) and, after digestion with Xmal (New England Biolabs), inserted into the Xmal linearized pSPT181::spac plasmid using the Rapid DNA Dephos & Ligation kit (Roche), according to the manufacturer’s recommendations. Ligation reactions were transformed into E. coli DH5α cells. Recombinant plasmid integrity was confirmed by restriction analysis and the correct insert orientation was confirmed by PCR using primer pairs spacF1/MR2-RT2 and spacR1/MR2-RT1. Insert sequences were also confirmed by DNA sequencing at STAB Vida (www.stabvida.com). After stabilization in E. coli, the recombinant plasmid was electroporated into S. aureus restriction-deficient strain RN4220 and finally transduced by the 80a phage to the knock-out mecR2 mutant strain N315 (N315::ΔmecR2), as previously described (41,42). The ability of the recombinant wild-type and mutant MecR2 expressed in trans to complement the N315::ΔmecR2 oxacillin-resistance phenotype was then evaluated, as previously described (34).

**Crosslinking experiments** — Recombinant MecR2 (45.0KD) was mixed with MecI (14.8KDa)—produced as previously described (30)—at a molar ratio of 1:2.8 in 50µl of 100mM HEPES, pH9.0. Paraformaldehyde was added as cross-linking agent at 0.1% (v/v) and the mixture was incubated at room temperature. The reaction was stopped at distinct time points by adding 10µl 5x Laemmlı buffer with β-mercaptoethanol. Samples were analyzed by 10%-tricine SDS-PAGE gels stained with Coomassie-blue. Control experiments were performed with both purified proteins alone under the same experimental conditions.

**Western blotting analysis** — The cross-linking reaction was performed as aforementioned and three different time points (0, 10, 30min) were analyzed in 15% Tris-Glycine SDS-PAGE. After electrophoresis, the proteins were transferred to 0.45-µm nitrocellulose membranes (Trans-Blot, Bio-Rad), which were blocked at room temperature for 1h with 20mL of blocking solution (137mM sodium chloride; 2.7mM potassium chloride; 4.3mM disodium hydrogen phosphate; 1.47mM potassium dihydrogen phosphate; 0.05% Tween-20) with 6% low-fat milk. MecI and MecR2 were detected by immunoblot analysis using custom polyclonal antibodies (from Eurogentec) at dilution 1:1000 and a secondary antibody (goat anti-rabbit IgG (H+L) peroxidase conjugated antibody, from Pierce) at dilution 1:50,000 in 10% blocking solution. The immune complexes were detected using an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent; Pierce) according to the manufacturer’s instruction. Membranes were exposed to hyperfilm ECL films (GE Healthcare).

**Crystallization and structure analysis** — Crystallization assays were performed by the sitting-drop vapor diffusion method. Reservoir solutions were prepared by a Tecan robot and 100mL crystallization drops were dispensed on 96x2-well MRC plates (Innovadyne) by a Cartesian nanodrop robot (Genomic Solutions) at the High-Throughput Crystallography Platform at Barcelona Science Park for initial screenings both at 20 and 4°C in Bruker steady-temperature crystal farms and using initial protein concentrations of 5.4 and 2.7mg/ml. Preliminary crystallization hits were improved and best conditions were scaled up to the microliter range in 24-well Cryschem crystallization dishes (Hampton Research). Crystals suitable for structure analysis were obtained at 5.4mg/ml protein concentration in 20 mM Tris-HCl, 200 mM sodium chloride, pH7.4 by using 0.2M sodium chloride, 20% PEG 1000, 0.1M potassium dihydrogen phosphate/ disodium hydrogen phosphate, pH6.2 as reservoir solution. Crystals were cryo-protected with reservoir solution supplemented with 30% glycerol. Crystallization conditions for the selenomethionine-derivatized protein were similar to the native ones. Complete diffraction datasets were collected from liquid-N2 flash-cryo-cooled crystals at 100K (provided by an Oxford Cryosystems 700 series cryostream) at beam lines ID23-1 on an ADSC Quantum Q315r CCD detector and ID29 on a Dectris PILATUS 6M pixel detector, respectively, of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) within the “Block Allocation Group Barcelona.” Crystals were orthorhombic, with two molecules per asymmetric unit. Diffraction data were integrated, scaled, merged, and reduced with programs XDS (43) and SCALA (44) within the CCP4 suite of programs (45) (see Table 3).

The structure of MecR2 was solved by a combination of multiple-wavelength anomalous diffraction with SHIELXE/D (46) and fragment search and density modification with ARClMBOLDO (47) by using two native datasets and two datasets from a selenomethionine-derivatized
crystal collected at the selenium absorption peak and the inflection point as determined by a previous XANES scan (Table 3). The resulting electron density map enabled straightforward tracing of the entire polypeptide chain on a Silicon Graphics Octane2 workstation with program TURBO-Frodo (48). Subsequent crystallographic refinement with BUSTER/TNT (49), which included Translation Libration Screw-motion refinement and non-crystallographic symmetry restraints, alternated with manual model building until completion of the model. The latter comprised residues M\textsuperscript{3} to A\textsuperscript{376} according to UniProt entry Q99XE2 plus an N-terminal proline resulting from the cloning strategy (termed P\textsuperscript{5}; see above) of molecule A, and D\textsuperscript{3} to A\textsuperscript{376} of molecule B. Three loop segments were disordered and were thus omitted from the final model: E\textsuperscript{52}-S\textsuperscript{58} and L\textsuperscript{152}-E\textsuperscript{158} of molecule A, and G\textsuperscript{51}-P\textsuperscript{63} of molecule B. In addition, one phosphate anion, four potassium cations, six glycerol molecules, and 278 solvent molecules were tentatively assigned (Table 3).

Miscellaneous — Figures were prepared with SETOR (50), CHIMERA (51), and TURBO-Frodo. Structure similarities were determined with DALI (52). Experimental model validation was performed with MOLPROBITY (53) and WHATIF (54). Close contacts (<4Å) and interaction surfaces (taken as half of the surface area buried at the complex interface) were calculated with CNS (55), and interface shape complementarity was computed with SC (56) within CCP4 (45). In all cases, a probe radius of 1.4Å was used. Inter-domain flexibility was ascertainment with HINGEPROT employing standard settings ((57); see http://bioinfo3d.cs.tau.ac.il/HingeProt). The final coordinates are available from the Protein Data Bank (PDB) at www.pdb.org (access code 4IJA).

RESULTS AND DISCUSSION

Recombinant overexpression and purification of MecR2 — Previously, the effects of MecR2 on oxacillin-resistance had been studied with a short variant present in the S. aureus prototype strain N315 lacking the first 87 residues of the full-length protein (34). Preliminary recombinant overexpression assays in E. coli revealed that this short variant was unstable and, as such, an efficient recombinant overexpression system was developed for full-length MecR2 of S. aureus strain HU25 (GenBank AF422694) containing an additional 20-residue N-terminal tag for purification (45.0KDa; hereafter wild-type MecR2) by means of expression vector pCri8::mecR2. The protein was folded correctly, eluted as a dimer (Fig. 2c), and proved suitable for structural and functional studies.

Cell-based activity of recombinant MecR2 — In order to assess the activity of full-length wild-type MecR2 (including the tag), the insert of vector pCri8::mecR2 was transferred into S. aureus expression vector pSPT181::spac (containing the IPTG-inducible Pspac promoter) to give recombinant plasmid pSPT::spac-mecR2-3D. This plasmid was then transduced into the S. aureus strain N315 mecR2 deletion mutant (N315::ΔmecR2) and its ability to restore the oxacillin-resistance phenotype of parental strain N315 was evaluated. As illustrated in Fig. 1, the phenotype of N315 was fully restored in the presence of the inducer (IPTG), demonstrating that the present full-length wild-type MecR2 variant is biologically active.

MecR2 binds MecI in cross-linking experiments — Previous studies had suggested a direct interaction between MecR2 and MecI in a bacterial two-hybrid system and in electrophoretic shift assays of the binding of MecI to the mecA promoter in the presence of MecR2 (34). In this study, we sought to evaluate the binding of recombinant MecI and wild-type MecR2 proteins in vitro. Control cross-linking experiments with each protein alone indicated concentration-dependent dimerization of both proteins (data not shown), in accordance with the dimeric behaviour of each of both proteins in solution (see above for MecR2 and (30) for MecI) When performing SDS-PAGE of cross-linking reactions of a mixture of MecI and MecR2 with paraformaldehyde, a time-dependent transition leading to a band migrating as ~120KDa was observed (Fig. 2a). Western blotting analysis with polyclonal antibodies against both proteins at three different time points of the cross-linking reaction confirmed in the ~120-KDa band (Fig. 2a, black frames) the presence of both MecI and MecR2 (Fig. 2b), which is consistent with a MecR2 dimer (2x45.0KDa) binding to a MecI dimer (2x14.8KDa).

Overall structure of MecR2 — The crystal structure of MecR2 was determined by a combination of multiple-wavelength anomalous diffraction and ab initio approaches, and two molecules are present in the asymmetric unit of the crystal, monomers A and B (see Table 3 for crystallographic data). The monomer structure reveals an elongated shape of roughly 45x60x80Å that is subdivided into three domains: an N-terminal DNA-binding domain (NDD), an intermediate scaffold domain (ISD), and a C-terminal dimerization domain (CDD)(Fig. 3a; the orientation of the left panel is hereafter taken as a reference). NDD (residues P\textsuperscript{1}/D\textsuperscript{1}-H\textsuperscript{70}) starts at the front surface, close to the top of the molecule, and enters a small αβ domain. It consists of three α-helices (α1-α3) followed by a β-ribbon (β1β2) whose tip—the loop connecting β1 and β2 (Lβ1β2)—is disordered (Fig. 3a,b). These elements conform to the architecture of a winged helix-turn-helix domain as observed in DNA-binding transcriptional repressors, which generally show disordered β-ribbon tips when not bound to operator DNA (58). In the latter, α1 and α2 contribute to creating a scaffold for correct positioning of helix α3. This is the recognition helix that penetrates the major groove of double-stranded DNA, as found in e.g. the DNA-binding domains (DBDs) of MecI and Blal (29–32).

After strand β2, the polypeptide chain enters ISD (residues L\textsuperscript{71}-N\textsuperscript{193} + S\textsuperscript{446}-A\textsuperscript{376}), which contains a central twisted five-stranded β-sheet (β3-β6 plus β9) that is parallel for all but one of its strands and shows connectivity -1,-1+3x,+1x (Fig. 3b,c). On its right, the sheet accommodates two helices (α4 and α5) and a short β-hairpin (βF8β), which is inserted between β6 and α5 and is folded back towards the sheet (Fig. 3a,b); on its left, two perpendicular helices (α6 and α12) are found. In monomer A, segment L\textsuperscript{152}-E\textsuperscript{158} within Lβ8α5 on the front surface of the molecule was disordered (Fig. 3a,b). Inserted between the latter helices is the CDD (residues L\textsuperscript{184}-T\textsuperscript{345}), which starts with a five-stranded β-sheet (β10-β12 plus β15-β16) that is equivalent to the one found in ISD, both in connectivity and topology (Fig. 3b,c). On its bottom side, this sheet is decorated with helices inserted between β12 and β15 (α7-α9) and between β15 and β16 (α10 and α11). In addition, a long β-ribbon (β13β14) is inserted
between β12 and α7; it contributes to oligomerization (see below). The overall architecture of ISD and CDD is such that the two respective β-sheets trap helices α6 and α12 in between so that a pseudo-twofold axis is generated which matches one sheet plus its helix with the other sheet-helix pair (Fig. 3b). Thus, α6 could be formally assigned to either ISD or CDD. The interface between these two domains contributes to an apparent ligand-binding cleft (Figs. 3b and 4a). It is framed by Lβ6β7 and β7 at its top; β10, Lβ10β11, β11 plus Lβ12β13 and Lβ14α7 at its bottom; Lβ9α6 and α6 at its back; residue Y46 of Lβ3β4 on its right; and R200 of β10 and E177 of β9 on its left (Fig. 4a). The cleft accommodates two (potential) potassium cations and a (tentative) phosphate anion in monomer A; in monomer B only one cation-binding site is found, which is created by atoms N199 Oδ1, A180 O, and A228 O, all 2.6–2.9 Å apart from the metal. A further three (monomer B) and four (monomer A) solvent molecules 3.0–3.6 Å apart from the metal complete the ligand sphere of this site. The rightmost potassium of monomer A is much more loosely bound, with just two protein atoms at <3.5 Å, N326 and S345 Oγ. Finally, the phosphate anion of monomer A is bound by N178 Nδ2, H140 Oδ1, E179 Oε1 and a solvent molecule (Fig. 4a).

Oligomeric state of MecR2 — MecR2 eluted as a dimer in calibrated size-exclusion chromatography and a dimer was also found to bind a Mec1 dimer in cross-linking experiments (see above). Consistent with this, the two molecules found in the crystal asymmetric unit give rise to a dimer with a large interaction surface (1,465 Å²; ~8% of the total surface of a monomer) with complementarity (0.72) that is in the range reported for protein oligomers and protein/protein inhibitor interfaces (0.70–0.76; (56)). This interaction includes 83 contacts (<4 Å), among them roughly symmetric hydrophobic contacts between nine residues of either monomer, and 34 hydrogen bonds and polar interactions. Altogether, these findings point to biological relevance for the dimeric arrangement. Protein segments involved in dimerization are provided by each CDD: Lα6β10, Lβ11β12, the second half of α9 and Lα9β15, and β-ribbon β13β14. The two monomers are not completely equivalent, and this gives rise to an rmsd value upon superposition of 0.97 Å for 353 Ca-atoms deviating less than 3 Å of out 361 common residues. Analysis of inter-domain flexibility based on the elastic network model revealed potential hinge motions at the two domain junctions of each monomer, which increase on going from the CDDs to the NDDs (Fig. 4c).

Structural similarities — Sequence similarity searches suggested that MecR2 groups with the ROK family of proteins (from repressors, open-reading frames, and kinases), which includes transcriptional repressors and sugar kinases (59–61). One archetypal ROK protein is xylose transcriptional repressor (XylR), which regulates xylose utilization as a carbon source in bacteria (62–66). However, there is no structural data on XylR available. E. coli protein Mlc is the only functionally and structurally characterized ROK-family protein with DNA-repressor function (60,67). Mlc is a dimeric/tetrameric transcriptional repressor that controls the utilization of glucose in E. coli (68). It shows overall fold similarity and quaternary arrangement with MecR2 and is likewise subdivided into three domains equivalent to NDD, ISD, and CDD. In addition, two unpublished structures corresponding to proteins of unknown function from Thermotoga maritima (PDB code 2HOE) and Vibrio cholerae (PDB 1Z05), deposited with the PDB by structural genomics consortia, also displayed high structural similarity scores with MecR2. These are the only three-domain ROK proteins structurally reported, which form part of a large group of mostly two-domain (ISD+CDD) ROK proteins, generally dimeric or tetrameric sugar kinases that bind and phosphorylate sugars (59–61,69).

MecR2 has a non-functional ligand-binding cleft — As for MecR2, Mlc has a ligand-binding cleft that sits at the interface between ISD and CDD. It further has an adjacent regulatory zinc-binding site, which is required for repressor activity (60) and is provided by the segment topologically equivalent to the protruding β-ribbon β13β14 in MecR2. In contrast to the latter, however, this segment is folded back towards the body of the molecule in Mlc, in a similar fashion to that in ROK glucokinase from E. coli (70) and glucomannokinase from Arthrobacter sp. (71), where it contributes to shaping the floor of a sugar-binding cleft. This segment encompasses a widely conserved consensus sequence among ROK proteins, CXCGXXGCXE (60,69), which contains three zinc-binding cysteine residues. A similar site is also found in Bacillus subtilis fructokinase YdhR (72), an undescribed putative glucokinase from Enterococcus faecalis (PDB 2QM1), an undescribed putative regulatory protein from Salmonella typhimurium (PDB 2AP1), and the aforementioned protein from V. cholerae, so that ROK family members containing this consensus sequence share a conserved metal-binding site. By contrast, MecR2 lacks these cysteine residues, and its chain trace is completely different, giving rise to an extended β-ribbon that engages in dimerization (see above). This β-ribbon is similar in the aforementioned protein from T. maritima, although in this case the ribbon is four residues shorter than that in MecR2. Only the last glutamate of the consensus sequence is found in the latter two proteins — E248 in MecR2 and it contributes to the hypothetical ligand-binding cleft (see above).

Another ROK-signature motif is found in several ROK proteins comprising the C-terminal residues EXGH, about ten residues upstream of the previous consensus sequence (see Fig. 4 in (69)). The histidine—missing in MecR2—is engaged in zinc binding in Mlc and the V. cholerae protein, while the glutamate—equivalent to E228 in MecR2—is engaged in sugar binding in E. coli glucokinase and Arthrobacter sp. glucomannokinase, together with the conserved residues at positions equivalent to E248, N178, and E179 in MecR2. The latter two residues are engaged in phosphate anion binding (see above). Although these residues are likewise conserved in Mlc, this protein does not bind glucose, i.e. its regulation does not depend on allosteric changes induced by sugar binding (73): inactivation is exerted through recruitment by the glucose transporter protein EIICB25c of the phosphotransferase system (74–76). By contrast, XylR binds xylose, glucose, and glucose-6-phosphate in vitro (66), i.e. it is a three-domain transcriptional repressor with a functional regulatory sugar-binding cleft. Overall, these findings indicate that ROK proteins include members that bind sugars such as the sugar kinases and XylR but also some that do not such as Mlc. Accordingly, we set out to assess if MecR2 has a functional sugar-binding ligand-binding site despite lacking the zinc-binding site, and three mutants affecting participating residues (MecR2-N178E179→AA, MecR2-E248→A, and MecR2-E248→A) were
constructed and assayed for their functional roles in the mec locus. These studies revealed that the mutants showed anti-repressor activity in cell-based assays that was indistinguishable from the wild-type protein (Fig. 1). We therefore conclude that ligand binding is not required for function in MecR2, i.e. that there is a non-functional ligand-binding cleft.

MecR2 has non-specific DNA-binding capacity — Three-domain ROK transcriptional repressors such as Mlc and XylR possess N-terminal DBDs that engage in DNA-operator binding and, thus, in the regulation of the transcription of the respective effector genes (60,65). MecR2 NDD likewise conforms to the structural determinants of such a DBD (see above). The reported structures of Mlc, T. maritima and V. cholerae are DNA-unbound, and they display the two recognition helices of a dimer in a relative spatial arrangement that is not adequate for binding to two successive turn of the major groove of double-stranded (ds) DNA (60,67). This is consistent with the finding that structural flexibility—which allows for major structural rearrangement—of Mlc was identified as essential for DNA-binding activity and regulatory function (67).

We set out to assess the DNA-binding capacity of MecR2 in an electrophoretic mobility shift assay in the presence of a 25-base pair (bp) dsDNA encompassing the Z-dyad sequence of the bla promoter sequence (see MR2-EMSA1 and 2 in Table 2), which had been used for structural and functional studies of MecI (29,30). We found that MecR2 strongly bound and completely retarded this DNA at a protein:dsDNA ratio of 4:1 (Fig. 5, left). Similar effects were observed on another 25-bp dsDNA of scrambled sequence (see MR2-EMSA 3 and 4 in Table 2; Fig. 5, right). These results indicate strong but unspecific DNA binding in vitro. To assess the potential biological importance of this function, a deletion mutant was constructed, MecR2-AS<sup>55</sup>-<sup>62</sup>, in which seven residues of Lβ1β2 within the winged-helix NDD had been deleted. This variant showed significantly diminished anti-repressor activity when compared with the wild-type protein (Fig. 1). We conclude that MecR2 possesses a functional DBD that may be required for exerting oxacillin resistance.

ISD may be relevant for function — In the search for biologically-relevant regions of the structure of MecR2, we noticed that the flexible segment contained within Lβ8α5 of ISD was located on the surface of one of the two monomers within the dimer (see above and Fig. 4b). We constructed a mutant, in which an eleven-residue stretch was replaced by four glycines (MecR2-T<sup>150</sup>-I<sup>160</sup>→GGGG) to maintain the overall structure of the domain, and assayed its cell-based activity (Fig. 1). Similarly to MecR2-AS<sup>55</sup>-<sup>62</sup>, this variant was not capable of restoring the oxacillin-resistance phenotype in the presence of inducer.

To verify that this effect was not due to an unfolded protein variant, MecR2-T<sup>150</sup>-I<sup>160</sup>→GGGG was recombinantly overexpressed under the same conditions as wild-type MecR2. Both proteins evinced comparable elution profiles in calibrated size-exclusion chromatography, which revealed that both proteins were well folded and dimeric (Fig. 2c). In addition, circular dichroism experiments showed indistinguishable spectra for both protein variants, which likewise correspond to properly folded proteins (Fig. 2d). Accordingly, the phenotype observed for the mutant is actually due to the missing ISD fragment and we conclude that this region may be implicated in biological activity.

Functional implications of MecR2 — The similarity of MecR2 with ROK-family bacterial sugar kinases and transcriptional repressors, both in the overall monomeric structures and the general dimeric quaternary arrangements have evolutionary and functional implications. Accordingly, XylR would represent the first step in evolution from an ancient two-domain ROK sugar-binding kinase—putatively evolved from a common ancestral hexokinase (69,77)—refurbished to produce a three-domain DNA-binding transcriptional repressor through N-terminal fusion with a winged helix-turn-helix DBD. XylR still binds and is allosterically regulated by sugar. Mlc would represent the next step—as already anticipated in (60)—to a three-domain DNA-binding transcriptional repressor that does not bind sugar and is not regulated by binding to an inducer or by proteolytic cleavage but through sequestration by a glucose transporter, i.e. through a protein-protein interaction (67,76). Finally, MecR2 would represent a last step in the evolutionary process, in which a three-domain ligand-independent Mlc-like repressor would have kept an unspecific DNA-binding ability putatively required for biological function as an anti-repressor. In addition, the dimeric protein would have developed a region within its ISD that potentially would likewise be required for antibiotic resistance. This anti-repressor activity would entail binding and sequestering away from its cognate promoter a dimeric transcriptional repressor, MecI. Finally, MecI sequestering would suppress its repressor activity of the mec locus by promoting its proteolytic cleavage, presumably by native proteinases, and enhancing the signal transduction mediated by the cognate integral membrane sensor-transducer, MecR1. This, in turn, would trigger the methicillin-resistance response.
REFERENCES

A database named Dali, which contains a vast collection of protein structures, can be a valuable tool for predicting hinge points and investigating the activity of protein-protein interactions. It is particularly useful for exploring the structural basis of ligand binding. The Dali database utilizes a sophisticated algorithm to compare protein structures, allowing researchers to identify regions of structural similarity and infer functional relationships. This information can be crucial for understanding the mechanisms of protein-protein interactions and for predicting the effects of mutations or structural changes on protein function.

ACKNOWLEDGMENTS

We are grateful to the Automated Crystallography Platform at Barcelona Science Park for assistance during crystallization experiments and to Raquel Garcia-Castellanos for important contributions to the project.

FOOTNOTES


We acknowledge the help provided by ESRF synchrotron local contacts.

ABBREVIATIONS

BLA, β-lactam antibiotic; BlaZ, β-lactamase; bp, base pair; CDD, C-terminal dimerization domain; DBD, DNA-binding domain; ds, double stranded; IPTG, isopropyl-β-D-1-thiogalactopyranoside; ISD, intermediate scaffold domain; MRSA, methicillin-resistant Staphylococcus aureus; NDD, N-terminal DNA-binding domain; PDB, Protein Data Bank at www.pdb.org, SCCmeC, staphylococcal chromosomal cassette mec; ROK, repressor, open-reading frame and kinase; and TEV, tobacco-etch virus.
Figure 1. Biological activity of wild-type and mutant MecR2 variants. The genes encoding wild-type full-length MecR2 (WT), as well as point and replacement mutants of the protein, were cloned into the S. aureus expression vector pSPT181::spac under the control of the IPTG-inducible Pspac promoter. The biological activity of the MecR2 variants was then evaluated by the inhibition halos produced by transformed S. aureus strains on oxacillin disks (containing 1 mg/mL of oxacillin), thus testing the complementation of the phenotype of the mecR2 null-mutant in prototype strain N315 (N315::ΔmecR2). Top panel, resistant N315 strain (left) and susceptible N315::ΔmecR2 strain (right). Panels two to seven depict the effect of distinct MecR2 variants after induction with IPTG (left dishes) and without induction (right dishes): WT, MecR2-T150I-166GGGG, MecR2-ΔS55-K62, and point mutants MecR2-N178E179AA, MecR2-E228A, and MecR2-E248A. Variants MecR2-T150I-166GGGG and MecR2-ΔS55-K62 (framed) cannot reconstitute the resistant phenotype while the three latter point mutants can, i.e. they are functionally not relevant.

Figure 2. In vitro studies of MecR2 and MecI proteins. (a) SDS-PAGE analysis of the time-dependent transition due to the action of paraformaldehyde from a mixture of purified MecI and MecR2 (14.8 and 45.0KDa, respectively) to MecR2-MecI heterotetramers. Due to the excess of MecI over MecR2 in the reaction mixture (2.8:1 molar ratio), MecR2 progresses directly from the monomer to the heterotetrameric complex. In contrast, MecI proceeds from a monomer over a homodimer to the heterotetramer. (b) Western-blot analysis of three different time points of the reaction shown in (a) (0, 10, 30min), confirming the presence of both MecI and MecR2 in the ~120-KDa heterotetramer-complex bands (framed in (a)). (c) Elution profile of recombinant wild-type MecR2 (blue curve) and MecR2-T150I-166GGGG mutant (red curve) proteins on a calibrated Superdex 75 size-exclusion chromatography column operated at 0.5mL/min using 20mM Tris-HCl, 0.2M sodium chloride, pH 7.4 as buffer. Cytochrome C (13.08mL;12KDa) was used as an internal marker for reference. 9.60mL elution volume correspond to ~90KDa. (d) Far-UV CD spectra of wild-type MecR2 (green curve) and MecR2-T150I-166GGGG (red curve) proteins in the native state in solution.

Figure 3. Molecular structure of MecR2. (a) Ribbon-type plot of MecR2 in three orthogonal views depicting the NDD (cyan helices and blue strands), ISD (yellow helices and orange strands), and CDD (pink helices and magenta strands). The bound tentative potassium and phosphate ions (monomer A) are depicted as green and orange/red spheres, respectively. The flanking residues of the disordered regions within NDD and ISD are indicated by black and gray arrows, respectively. (b) Topology scheme of MecR2 showing the regular secondary structure elements of MecR2 with their labels and delimiting amino-acid positions. A twofold axis relating the β-sheets of ISD and CDD is depicted in discontinuous trace. The position of the ligand-binding cleft is further indicated. (c) Cartoon depicting the topology of the main building elements of ISD and CDD, the five-stranded β-sheet and its three flanking helices. Each structural element carries the labels as found in the two domains. The arrows hallmark insertion points of distinct secondary structure elements within each domain: ① for ISD and ② for CDD.

Figure 4. Ligand-binding cleft and quaternary structure of MecR2. (a) Detail of the apparent ligand-binding cleft of MecR2 monomer A in stereo. Selected residues, the phosphate anion and the two potassium cations are labeled. (b) MecR2 dimer made up of monomer A (right; similar orientation to Fig.3a, left) and monomer B (left). The three domains are depicted in cyan/blue (NDD), salmon/orange (ISD), and purple/magenta (CDD). Potassium and phosphate ions are shown as green and orange/red spheres, respectively. The flanking residues of the flexible region within β8α5 of ISD are indicated by black arrows. (c) Same as (b) showing the result of the analysis of inter-domain flexibility based on the elastic network model.

Figure 5. Electrophoretic mobility shift assays with MecR2. Electrophoretic mobility shift assays with a 25-bp dsDNA encompassing the recognition sequence of MecI/BlaI within the blaZ promoter (left) and a 25-bp dsDNA with the same nucleotide composition but scrambled sequence (right). In each case, the left lanes depict controls with DNA alone and lanes 2 to 5 show the effect of increasing amounts of MecR2 protein (protein:dsDNA ratios of 0.5:1, 1:1, 2:1, and 4:1).
Table 1 — Strains and plasmids

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<td>R. Novick</td>
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* Restriction sites are underlined.
### Table 3 — Crystallographic data

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<td>0.110 (0.465) / 0.035 (0.194)</td>
<td>0.065 (0.568) / 0.035 (0.336)0.106 (0.843) / 0.057 (0.451)</td>
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</tr>
<tr>
<td>No. of protein atoms / solvent molecules / ligands / ions</td>
<td>5,813 / 278 / 6 (CH₂OH)₂CHOH / 4 K⁺, 1 PO₄&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;rmsd&lt;/sub&gt; from target values</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bonds (Å) / angles (°)</td>
<td>0.010 / 1.04</td>
<td>0.010 / 1.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average B-factors for protein atoms (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>49.2</td>
<td>49.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main-chain conformational angle analysis&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residues in favored regions/outliers/all residues</td>
<td>705 / 0 / 714</td>
<td>705 / 0 / 714</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values in parentheses refer to the outermost resolution shell.  
<sup>b</sup> Friedel mates were treated separately during processing of selenomethionine-derivative data.  
<sup>c</sup> For definitions, see Table 1 in (79).  
<sup>d</sup> According to MOLPROBITY (53).
Fig. 1

IPTG 100µM

no IPTG