Structure-Function Studies of the Staphylococcal Methicillin Resistance Antirepressor MecR2*

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

Results: The structure of MecR2 shows a dimeric multidomain ROK family protein, which nonspecifically binds oligonucleotides but not sugar ligands.

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

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

Methicillin resistance in Staphylococcus aureus is elicited by the MecI-MecR1-MecA axis encoded by the mec locus. Recently, MecR2 was also identified as a 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, an intermediate scaffold domain, and a C-terminal dimerization domain that contributes to oligomerization. The protein shows structural similarity to ROK (repressors, open reading frames, and θιnasases) 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 nonspecifically, and a deletion mutant affecting the N-terminal DNA-binding domain 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 Similarly, a deletion mutant, in which a flexible segment of intermediate scaffold domain had been replaced by four glycines, significantly reduced MecR2 function, thus indicating that this domain may likewise be required for activity.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 a methicillin antirepressor, 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), 5 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 because of 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 (9–11). Staphylococcal chromosomal cassette mec also includes two genes, mecL and mecR1, which encode a transcriptional DNA-binding repressor, MecL, and an integral membrane zinc-dependent sensor/signal transducer metalloprotease, MecR1, respectively (12, 13). This system is homologous to the bla-blaI-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 MecL/BlaI constitutively represses its own biosyn-
thesis 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 metalloproteinase 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/BlaI (26, 27). This cleavage would render the dimeric repressor inactive and directly or indirectly—cause cleavage of MecI/BlaI (26, 27).

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 antirepressor by disrupting MecI-dependent proteolytic inactivation (34). Collectively, these findings indicated that the long sought-for gene encoding MecR2/BlaR2 had been found, and so it was termed mecR2 (34).

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, 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 (Met1–Ala376; see GenBank access number 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 pCRI8::mecR2. For strains and plasmids used in this study, see Table 1. This construct added an N-terminal His6 tag and a tobacco etch virus (TEV) protease cleavage site, so that the N terminus of the mature protein was preceded by a 20-residue segment (−21MGSSHHHHHHSSGGNLVQPG+1); negative superscript numbers refer to extra N-terminal residues preceding the

**TABLE 1**

| Strains and plasmids | Relevant characteristics | Source |
|----------------------|-------------------------|--------|
| **Strains** | | |
| *E. coli* DH5α | Recipient strain for recombinant plasmids | Stratagen |
| *E. coli* BL21 (DE3) | Recipient strain for expression vector pCRI8a | Novagen |
| BL21 + pCRI8a::mecR2 | *E. coli* BL21 (DE3) overexpressing mecR2 with an N-terminal His6 tag and a TEV protease cleavage site | This study |
| **Plasmids** | | |
| pCRI8a | | |
| pSPT181::spac | pET30 (Invitrogen) derivative for recombinant overexpression containing His6-GST-TEV fragment, Kan’ | Ref. 78 |
| pCRI8a::mecR2 | pCRI8a expression vector with mecR2 gene from strain HU25 | This study |
| pSPT181::mecR2 3D (wild-type) | pSPT181 with 1.6-kb EcoRI-BamHI fragment containing the IPTG-inducible Pac promoter and the transcriptional repressor LacI from pDH88, Apr, Tcr | Ref. 34 |
| pCRI8a::mecR2 Thr150–Ile160 → GGGG | pCRI8a expression vector containing the mecR2 gene from strain HU25 with an N-terminal His tag and a TEV protease cleavage site from pCRI8a::mecR2 | This study |
| pCRI8a::mecR2 mutant variant Thr150–Ile160 → GGGG | pCRI8a expression vector containing the mecR2 mutant variant Thr150–Ile160 → GGGG | This study |
| pSPT181::mecR2 ΔSer55–Lys62 | pSPT181 vector containing the mecR2 mutant variant ΔSer55–Lys62 | This study |
| pSPT181::mecR2 ΔSer55–Lys62 | pSPT181 vector containing the mecR2 mutant variant ΔSer55–Lys62 | This study |
| pCRI8a::mecR2 N178A,E179A | pSPT181 vector containing the mecR2 mutant variant ΔSer55–Lys62 | This study |
| pCRI8a::mecR2 N178A,E179A | pSPT181 vector containing the mecR2 mutant variant ΔSer55–Lys62 | This study |
| pCRI8a::mecR2 E228A | pSPT181 vector containing the mecR2 mutant variant E228A | This study |
| pCRI8a::mecR2 E248A | pSPT181 vector containing the mecR2 mutant variant E248A | This study |
mature N terminus, which is Met¹ according to Q99XE2). A mutant, in which segment Thr¹⁵⁰–Ile¹⁶⁰ had been replaced by four glycine residues (termed MecR2 Thr¹⁵⁰–Ile¹⁶⁰ → GGGG), was amplified from the pSPT181::spac::mecR2 recombinant plasmid by PCR and cloned into expression vector pCrib8a at the Ncol and Xhol 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 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 18 °C for 24 h when the optical density at λ = 600 nm (A₆₀₀) reached 0.6. Cultures were subsequently centrifuged at 7,000 × g (4 °C, 20 min), and pellets were resuspended in 70 ml of ice-cold buffer A (20 mM Tris·HCl, 0.5 M sodium chloride, pH 8.0). The cells were lysed with a cell disruptor (Constant Cell Disruption Systems Ltd.) operated at 1.35 Kbp, 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 His₆-TEV-MecR2 was applied onto a His-trap FF column (GE Healthcare), previously equilibrated with buffer A. The protein was eluted with imidazole gradient (0–0.5 M) in buffer 2 (20 mM Tris·HCl, 0.1 M sodium chloride, pH 7.4–8.0). Protein purity was assessed by 10% Tricine SDS-PAGE. TEV proteinase activity of recombinant wild-type MecR2 expressed from vector pCri8a (MecR2野生型) was measured using annealed oligonucleotides as negative control. The same protocol was followed for point mutants, except that the second PCR round of two independent PCRs was performed on primers generating two intermediate PCR products with overlapping terminals (for primers and nucleotides; Table 2). For the deletion mutant lacking segment Ser⁵⁵–Lys⁶⁵ (protein MecR2 ΔSer⁵⁵–Lys⁶⁵), primer pairs MR2–3F1/MR2–3R1 and MR2–3F2/MR2–3R2 were used; for point mutation MecR2-Thr¹⁵⁰–Ile¹⁶⁰ → GGGG, primers MR2–3F4 and MR2–3R4 were used; and for point mutants E228A (MecR2-Glu²²⁸→Ala), N178A,E179A (MecR2-Asn¹⁷⁸,Glu¹⁷⁹→Ala-Ala), and E248A (MecR2-Glu²⁴⁸→Ala), the respective primers were MR2–3F1/MR2–3R1 and MR2–3F2/MR2–3R2. 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 (MR2–3F1/R1). All PCRs were performed with the Phusion high fidelity DNA polymerase (New England Biolabs).

**Circular Dichroism Experiments**—The CD spectra of wild-type MecR2 and MecR2-Thr¹⁵⁰–Ile¹⁶⁰ → GGGG were recorded with a JASCO J-815 CD spectrometer operated with the following parameters: response, 1 s; scan speed, 50 nm/min; data acquisition interval, 0.1 nm; accumulations, 3; and bandwidth, 1 nm. Quartz cuvettes for far UV (190–250 nm) with path lengths of 1 mm were used. The samples contained 0.2 mg/ml protein in 10 mM sodium phosphate, 40 mM sodium chloride, pH 7.0. 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 bla promoter sequence with an additional 1-bp overhang on either end (C/G), which had been employed in structure-function studies with Mec1 (15, 29, 30) (termed here MR2-EMSA1 and 2; Table 2), were purchased from Sigma and annealed as described to yield 200 nmol of dsDNA in buffer 20 mM Tris·HCl, 0.1 M sodium chloride, pH 7.4. Purified MecR2 (100 and 200 µM) in buffer 20 mM Tris·HCl, 0.2 M sodium chloride, pH 7.4, 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 the same nucleotide composition but scrambled sequence (MR2-EMSA3 and 4).

**Site-directed Mutagenesis of MecR2—**MecR2 mutant variants were obtained as described previously (39, 40). Briefly, a round of two independent PCRs was performed on pCrio8::mecR2 with two complementary mutagenic primers and the two flanking mecR2 primers generating two intermediate PCR products with overlapping terminals (for primers and nucleotides; Table 2). For the deletion mutant lacking segment Ser⁵⁵–Lys⁶⁵ (protein MecR2 ΔSer⁵⁵–Lys⁶⁵), primer pairs MR2–3F1/MR2–3R1 and MR2–3F2/MR2–3R2 were used; for deletion mutant MecR2-Thr¹⁵⁰–Ile¹⁶⁰ → GGGG, primers MR2–3F4 and MR2–3R4 were used; and for point mutants E228A (MecR2-Glu²²⁸→Ala), N178A,E179A (MecR2-Asn¹⁷⁸,Glu¹⁷⁹→Ala-Ala), and E248A (MecR2-Glu²⁴⁸→Ala), the respective primers were MR2–3F1/MR2–3R1 and MR2–3F2/MR2–3R2. 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 (MR2–3F1/R1). All PCRs were performed with the Phusion high fidelity DNA polymerase (New England Biolabs).

**Cell-based Activity of Recombinant MecR2—**To assess the activity of recombinant wild-type MecR2 expressed from vector pCrib8a::mecR2 and the aforementioned mutants generated by site-directed mutagenesis, the full respective inserts were cloned into a S. aureus expression vector containing the Pspac IPTG-inducible promoter (pSPT181::spac). Briefly, using flanking primers MR2–3F1/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 dephosphorylation and ligation kit (Roche Applied Science), according to the manufacturer’s recommendations. Ligation reactions were transformed into {Escherichia coli} DH5α cells. Recombinant plasmid

### Table 2

| Name                | Sequence (5’ → 3’) |
|---------------------|--------------------|
| MR2–3F1             | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–3D R1           | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| Spac F1             | QAAGATTTTATTGAGTCG |
| Spac R1             | TTATGCGGCTTGAACACTGAC |
| MR2–RT1             | AATGAGCAGAATCCTTCTCAG |
| MR2–RT2             | AATGAGCAGAATCCTTCTCAG |
| MR2–SDM1            | ATGGGGAAGCGGGTGCAATTGGAAAAACACT |
| MR2–SDM2            | ACCCTTCACAGAGAATGTTTTATACCTCACCACACCTCAT |
| MR2–SDM3            | TGAGAAATGACCCAGAATGCTTGAGCTGAGATTTCAAAT |
| MR2–SDM4            | TCTTTGCAAAATTTAATCCACTCACCACACCTCAT |
| MR2–SDM5            | CACGCGGCAAAATTTAATCCACTCACCACACCTCAT |
| MR2–SDM6            | GCTACGGGGAATCCACTCACCACACCTCAT |
| MR2–SDM7            | GCTAAGCAGAATCCTTCTCAG |
| MR2–SDM8            | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–SDM9            | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–SDM10           | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–SDM11           | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–SDM12           | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–SDM13           | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–SDM14           | TATACCCGGGAAACAGGATAATACCTGCGGCA |
| MR2–SDM15           | TATACCCGGGAAACAGGATAATACCTGCGGCA |

* Restriction sites are underlined.

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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. After stabilization in E. coli, the recombinant plasmid was electroplated into S. aureus restriction-deficient strain RN4220 and finally transduced by the 80s phage to the knock-out mecR2 mutant strain N315 (N315::ΔmecR2), as described previously (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 described previously (34).

Cross-linking Experiments—Recombinant MecR2 (45.0 kDa) was mixed with Mecl (14.8 kDa)—produced as described previously (30)—at a molar ration of 1:2.8 in 50 μl of 100 mM HEPES, pH 9.0. Parafomaldehyde 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 10 μl of 5× Laemmli 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 described above, and three different time points (0, 10, and 30 min) 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 1 h with 20 ml of blocking solution (137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium hydrogen phosphate, 1.47 mM potassium dihydrogen phosphate, 0.05% Tween 20) with 6% low fat milk. Mecl 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; 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 instructions. The 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 100-nl crystallization drops were dispensed on 96-well MRC plates (Innovadyne) by a Cartesian nanodrop robot, respectively, of the European Synchrotron Radiation Facility (Grenoble, France) within the “Block Allocation Group Barcelona.” Crystals were orthorhombic, with two molecules per asymmetric unit. The diffraction data were integrated, scaled, merged, and reduced with programs XDS (43) and SCALA (44) within the CCP4 suite of programs (45) (Table 3).

The structure of MecR2 was solved by a combination of multiple-wavelength anomalous diffraction with SHELXE/D (46) and fragment search and density modification with ARCIAMBOLO (47) by using two native data sets and two data sets from a selenomethionine-derivated 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 work station with the program TURBO-Frodo (48). Subsequent crystallographic refinement with BUSTER/TNT (49), which included translation libration screw motion refinement and noncrystallographic symmetry restraints, alternated with manual model building until completion of the model. The latter comprised residues Met1–Ala376, according to UniProt entry Q99XE2 plus an N-terminal proline resulting from the cloning strategy (termed Pro2; see above) of molecule A and Asp3–Ala376 of molecule B. Three loop segments were disordered and were thus omitted from the final model: Glu52–Ser58 and Leu152–Glu158 of molecule A and Gly51–Pro60 of molecule B. In addition, 1 phosphate anion, 4 potassium cations, 6 glycerol molecules, and 278 solvent molecules were tentatively assigned (Table 3).

Miscellaneous—The 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 (<4 Å) 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 1.4 Å was used. Interdomain flexibility was ascertained with HingeProt employing standard settings (Ref. 57). The final coordinates are available from the Protein Data Bank (PDB; 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 N-terminally truncated variant of the protein as present in the S. aureus prototype strain N315 (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™
accession number AF422694) containing an additional 20-residue N-terminal tag for purification (45.0 kDa; hereafter referred to as wild-type MecR2) by means of expression vector pCt8::mecR2. The protein was folded correctly, eluted as a dimer (see Fig. 2C), and proved suitable for structural and functional studies.

**Cell-based Activity of Recombinant MecR2**—To assess the activity of full-length wild-type MecR2 (including the tag), the insert of vector pCt8::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 their dimeric behavior 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 at ~120 kDa 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 boxes) the presence of both MecI and MecR2 (Fig. 2B), which is consistent with a MecR2 dimer (2 × 45.0 kDa) binding to a MecI dimer (2 × 14.8 kDa).

**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 monomeric structure reveals an elongated shape of roughly 45 × 60 × 80 Å 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 Pro1–Asp3–His70) 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. 3, A and B). These elements con-
form 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 the DNA-binding domains (DBDs) of MecI and Blal, for example (29–32).

After strand β2, the polypeptide chain enters ISD (residues Leu71–Asn193 + Ser346–Ala376), 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. 3, B and C). On its right, the sheet accommodates two helices (α6 and α5) and a short β-hairpin (β7/β8), which is inserted between β6 and α5 and is folded back toward the sheet (Fig. 3, A and B); on its left, two perpendicular helices (α6 and α12) are found. In monomer A, segment Leu152–Glu158 within L88a5 on the front surface of the molecule is disordered (Fig. 3, A and B). Inserted between the latter helices is the CDD (residues Leu194–Thr345), which starts with a five-stranded β-sheet (β10–β12 plus β15 and β16) that is equivalent to the one found in ISD, both in connectivity and in topology (Fig. 3, B and C). On its bottom side, this sheet is decorated with helices inserted between β12 and β15 (α7–α9) and between β15 and
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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-2-fold axis is generated that 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 Tyr82 of Lβ3β4 on its right; and Arg200 of β10 and Glu177 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 Asn181O61, Ala210O, and Ala226O, all 2.6–2.9Å 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: Asn314O and Ser203O. Finally, the phosphate anion of monomer A is bound by Asn178N62, His140Nε1, Glu179Oε1, 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 MecI dimer in cross-linking experiments (see
Consistent with this, the two molecules found in the crystal asymmetric unit give rise to a dimer with a large interaction surface (1,465 Å$^2$; $\sim$8% of the total surface of a monomer) with complementarity (0.72) that is in the range reported for protein oligomers and protein/protein inhibitor interfaces (0.70–0.76) (56). This interaction includes 83 contacts (<4 Å), among them roughly symmetric hydrophobic contacts between nine residues of either monomer and 34 hydrogen bonds and polar interactions. Altogether, these findings point to biological relevance for the dimeric arrangement. Protein segments involved in dimerization are provided by each CDD: L$_{\text{ISD}}$ 6–10, L$_{\text{ISD}}$ 11–12, the second half of L$_{\text{NDD}}$ 9 and L$_{\text{NDD}}$ 15, and -ribbon 13–14. The two monomers are not completely equivalent, and this gives rise to a root mean square deviation value upon superposition of 0.97 Å for 353 C$_\alpha$ atoms deviating less than 3 Å out of 361 common residues. Analysis of interdomain flexibility based on the elastic network model revealed potential hinge motions at the two domain junctions of each monomer, which increase upon 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 glucides (59–61, 69).

**MecR2 Has a Nonfunctional 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 upper).
protruding β-ribbon β13β14 in MecR2. In contrast to the latter, however, this segment is folded back toward the body of the molecule in Mlc, in a fashion similar 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, CXXGXXGCXE (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 code 2QM1), an undescribed putative regulatory protein from *Salmonella typhimurium* (PDB code 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 in the corresponding region, 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—Glu²⁴⁸ 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 EXG/H, about 10 residues upstream of the previous consensus sequence (see Fig. 4 in Ref. 69). The histidine—missing in MecR2—is engaged in zinc binding in Mlc and the *V. cholerae* protein, whereas the glutamate—equivalent to Glu²⁴⁸ in MecR2—is engaged in sugar binding in *E. coli* glucokinase and *Arthrobacter* sp. glucomannokinase, together with the conserved residues at positions equivalent to Glu²⁴⁸, Asn¹⁷⁸, and Glu¹⁷⁹ 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 ElIC⁶Glc 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 whether MecR2 has a functional sugar-binding ligand-binding site despite lacking the zinc-binding site, and three mutants affecting participating residues (MecR2-Asn¹⁷⁸-Glu¹⁷⁹→Ala-Ala, MecR2-Glu²⁴⁸→Ala, and MecR2-Glu²⁴⁸→Ala) were constructed and assayed for their functional roles in the mec locus. These studies revealed that the mutants showed antirepressor 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 it has a nonfunctional ligand-binding cleft.

**MecR2 Has Nonspecific 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 turns of the major groove of dsDNA (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-bp dsDNA encompassing the Z-dyad sequence of the *bla* promoter sequence (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 panel). Similiar effects were observed on another 25-bp dsDNA of scrambled sequence (see MR2-EMSA 3 and 4 in Table 2; Fig. 5, right panel). 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-ΔSer⁵⁵–Lys⁶², in which seven residues of LB1B2 within the winged helix NDD had been deleted. This variant showed significantly diminished antirepressor 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 LB8α5 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 11-residue stretch was replaced by 4 glycines (MecR2-Thr¹⁵⁰–Ile¹⁶⁰→GGGG) to maintain the overall structure of the domain and assayed its cell-based activity (Fig. 1). Similarly to MecR2-ΔSer⁵⁵–Lys⁶², this variant was not capable of restor-
ing the oxacillin resistance phenotype in the presence of inducer.

To verify that this effect was not due to an unfolded protein variant, MecR2-Thr<sup>150</sup>–Ile<sup>160</sup> → GGGG was recombiantly 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 in 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 antirepressor. In addition, the dimeric protein would have developed a region within its ISD that potentially would likewise be required for antibiotic resistance. This antirepressor activity would entail binding and sequestering away from its cognate promoter a dimeric transcriptional repressor, Mecf. Finally, Mecf 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.

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