HmrA is an antibiotic resistance factor of methicillin-resistant Staphylococcus aureus. Molecular analysis of this protein revealed that it is not a muramidase or β-lactamase but a non-specific double-zinc endopeptidase consisting of a catalytic domain and an inserted oligomerization domain, which probably undergo a relative interdomain hinge rotation upon substrate binding. The active-site cleft is located at the domain interface. Four HmrA protomers assemble to a large ~170-kDa homotetrameric complex of 125 Å. All four active sites are fully accessible and ~50–70 Å apart, far enough apart to act on a large meshwork substrate independently but simultaneously. In vivo studies with four S. aureus strains of variable resistance levels revealed that the extracellular addition of HmrA protects against loss of viability in the presence of oxacillin and that this protection depends on proteolytic activity. All of these results indicate that HmrA is a peptidase that participates in resistance mechanisms in vivo in the presence of β-lactams. Furthermore, our results have implications for most S. aureus strains of known genomic sequences and several other cocci and bacilli, which harbor close orthologs. This suggests that HmrA may be a new widespread antibiotic resistance factor in bacteria.

β-Lactam antibiotics (BLAs), such as penicillin, methicillin, and oxacillin, interfere with bacterial cell wall homeostasis by targeting penicillin-binding proteins, which participate in peptidoglycan cross-linking (1). In response to BLAs and other threats, bacteria have developed a series of strategies, collectively referred to as antibiotic resistance. Among such bacteria is methicillin-resistant Staphylococcus aureus, the most prevalent human pathogen associated with hospital-borne and community infections (2–4). Methicillin-resistant S. aureus resistance is mainly exerted through a signal transduction system that triggers the synthesis of a penicillin-binding protein termed MecA (also known as PBP2a and PBP2a). In contrast to housekeeping penicillin-binding proteins, MecA exerts basal peptidoglycan remodeling functions even in the presence of BLAs (5). The signaling system is composed of at least two elements: an integral membrane metalloprotease, MecR1, which senses the presence of environmental BLAs through an extracellular penicillin-binding domain (6), and a transcriptional repressor, MecI, which represses MecA synthesis in the dormant ground state (7–10). In addition to the MecI-MecR1-MecA axis, there are other factors governing staphylococcal methicillin resistance (11–14). The genes responsible are native constituents of the S. aureus genome, and the proteins that they encode participate mostly in cell wall biosynthesis and turnover. Genes mupR and -F, fmtA to -C, sigB, dlt, pbp2, and ctaA as well as the auxiliary genes (aux) also known as factors essential for methicillin resistance (genes or mutants femA to -F, femR, and femX) have been described (11, 15). Staphylococcal murein hydrolases, such as the gene product of lytH, are further required as lytic enzymes in peptidoglycan processing. Other factors have only a supporting role in BLA resistance, such as those encoded by the global regulators sar and agr. Functions for several other required genes and gene products, however, remain elusive (12, 14, 16). Some years ago, Hiramatsu and co-workers (17) reported two new genes, hmrA and hmrB, which were engaged in methicillin resistance in the pre-methicillin-resistant S. aureus strain N315. In order to gain more insight into BLA antibiotic resistance mechanisms, we studied the gene product of hmrA,
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the 394-residue protein HmrA, at the molecular level in vitro and at the cellular level in vivo. We aimed to obtain structural and functional information to help understand how this protein contributes to tolerance to BLAs and to provide a structural scaffold for the design of specific inhibitors that may lead to novel treatment for staphylococcal infections.

EXPERIMENTAL PROCEDURES

Cloning Strategies—The hmrA gene was amplified from vector pHMR-A (17) by PCR and cloned into expression vector pET-28a between Ncol and XhoI restriction sites (Novagen). This strategy entailed that the second amino acid was a glycine instead of a serine (see UniProtKB sequence data base entry Q99Q45). The expression vector obtained was termed pET-hmrA. Two point mutants affecting putative active-site residues, C93A and E128A, and a double mutant, C93A/E128A, were introduced into pET-hmrA by means of the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The resulting vectors were termed pET-hmrA-C93A, pET-hmrA-E128A, and pET-hmrA-C93A/E128A, respectively.

Protein Expression and Purification—Expression vectors were transformed into Escherichia coli BL21(DE3) cells, and 1-liter cultures of transformed bacteria were induced for protein expression with 1 mM isopropyl-β-D-1-thiogalactopyranoside at 37 °C for 90 min 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 (50 mM HEPES, pH 7.5). Cells were lysed by cell disruption with a cell disruptor (Constant Cell Disruption Systems) operated at 1.35 kilobars, and the lysate was subsequently centrifuged at 75,600 × g in a Beckman Avanti J-25 centrifuge with a JA-25.50 rotor (4 °C, 20 min). The soluble fraction containing HmrA was applied onto a previously equilibrated HiLoad Sepharose Q 16/10 column (GE Healthcare) attached to an ÄKTApurifier UPC-10 FPLC system. The protein was eluted with a linear gradient of NaCl (0–1 M), and fractions containing the protein were subjected to a final size exclusion chromatography step in a Superdex 200 16/60 column (GE Healthcare), previously equilibrated with 50 mM HEPES, 400 mM sodium chloride, 100 mM zinc chloride, pH 7.5. Protein purity was assessed by 10% Tricine SDS-PAGE. The selenomethionine variant of HmrA was obtained in the same way, except that 30 min before induction, the cells were added to 500 ml of minimal medium lacking methionine and containing 25 mg of selenomethionine (Sigma-Aldrich) instead. Protein expression and purification of HmrA mutants C93A, E128A and C93A/E128A proceeded as for the wild-type protein. Apo-HmrA was generated by dialysis of the wild-type protein against 10 mM o-phenanthroline for 24 h at 4 °C to remove the catalytic zinc ions.

Protein Characterization, Cross-linking, and Analytical Ultracentrifugation Studies—Protein quaternary arrangement was analyzed by size-exclusion chromatography in a calibrated Superdex 200 26/60 column. Inductively coupled plasma mass spectrometry was performed with an Elan-6000 spectrometer to determine the metal contents of pure protein samples (PerkinElmer Life Sciences). Cross-linking experiments were conducted with protein (at 0.09 mg ml⁻¹) in 50 μl of 100 mM HEPES, pH 9.0, 0.4% (v/v) paraformaldehyde (Sigma-Aldrich). Reactions were stopped with 10 μl of 5× Laemmli buffer (with β-mercaptoethanol) at distinct time points. Samples were analyzed by 10% Tricine SDS-polyacrylamide gels stained with Coomassie Blue. Analytical ultracentrifugation experiments were carried out at the Institute of Biochemistry and Biotechnology of the Martin-Luther University at Halle-Wittenberg by using HmrA (at 20, 40, 70, 100, 250, and 400 μg ml⁻¹) dialyzed against 50 mM HEPES, 100 mM sodium chloride, 50 μM zinc chloride, pH 7.5 (hereafter buffer A) for 24 h at 4 °C. This dialysis buffer was further used for protein dilution and for the base-line/blank experiments. Experiments were conducted in a Beckman Optima XL-A centrifuge with a 50Ti rotor. Sedimentation equilibrium measurements (absorption at 230 and 280 nm) were carried out in double sector cells at 48,400 × g at 20 °C. The apparent molecular mass was calculated using the software provided by Beckman Instruments (Palo Alto, CA).

Thermal Stability Assays—Response to thermal denaturation was assayed according to the thermofluor method (18) by using Sypro® Orange dye (Invitrogen) in a LightCycler480 real-time PCR apparatus (Roche Applied Science). Initial assays were performed in 50–μl volumes by using buffers (50 mM) at different pH values (sodium acetate and cacodylate for pH 5.0, MES and BisTris for pH 6.0, 6.5, and 7.0; Tris-HCl and HEPES for pH 7.5 and 8.0; and Bicine and CHES for pH 8.5 and 9.0), as well as 0, 100, and 200 mM sodium chloride. A subsequent test with chloride salts of divalent metals (manganese, zinc, cobalt, and calcium; all from Sigma-Aldrich) at 50, 100, 500, and 1000 μM was conducted in two buffers (buffer A without zinc and 50 mM CHES, 100 mM sodium chloride, pH 8.5). In a third round of assays, a fine screen of concentrations of zinc chloride was performed (0, 0.01, 0.1, 0.5, 1, 10, 50, and 100 μM).

Hydrolitic Activity Assays—All assays were carried out at 37 °C in buffer A with an HmrA concentration of 0.09 mg ml⁻¹ (2.1 μM) unless otherwise stated. Measurements were performed in an Infinite M200 plate reader (Tecan), a Powerwave XS plate reader (Bio-Tek), a FL-800 fluorimeter plate reader (Bio-Tek), and a Cary 400 UV-visible spectrophotometer (Varian), and continuous readings were performed over 12–72 h. General endopeptidase activity was assessed by incubating HmrA solutions (0–0.093 μM) overnight with fluorescein-labeled DQ™ gelatin, casein, or albumin (all from Molecular Probes) at 50–100 μg ml⁻¹ according to the manufacturer’s instructions and also with non-fluorescent albumin (New England Biolabs) at 20 mM. Specific peptidolytic activity was tested by using a peptide containing the fluorescent donor 2-aminoethylamino-1-naphthalene sulfonate (EDANS) and the quencher (p-dimethylaminophenyl)azobenzoate (DABCYL), DABCYL-Leu-Ala-Arg-Val-Glu-EDANS (Bachem). In addition, a fluorogenic hexapeptide library with an N-terminal o-aminobenzyl (Abz) group and a C-terminal 2,4-dinitrophenyl (dnp) lysine (K(dnp)) (a further development of the method of Ref. 19), which included compounds Abz-Lys-Asp-Glu-Ser-Tyr-Arg-K(dnp), Abz-Thr-Val-Leu-Glu-Arg-Ser-K(dnp), Abz-Asp-Tyr-Val-Ala-Ser-Glu-K(dnp), Abz-Tyr-Gly-Lys-Arg-Val-Phe-K(dnp), and Abz-Val-Lys-Phe-Tyr-Asp-Ile-K(dnp), was further assayed for cleavage, as well as probe Abz-Gly-Ile-Val—
Arg-Ala-k(dnp) (Bachem). Activity was also tested against the specific 7-methoxycoumarin-4-acetate (Mca)-containing matrix metalloproteinase substrates, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-k(dnp)-NH2 and Mca-Pro-Leu-Gly-Leu-Dap(dnp)-Ala-Arg-NH2 (where Dap represents L-di-aminopropionyl and Nva represents norvaline; Bachem). Substrate concentrations varied from 0.0125 to 1 mM in all cases. Cleaved fragments were analyzed by HPLC and mass spectrometry in order to assess substrate specificity. A search for optimal pH for activity was performed with fluoropeptide Abz-Asp-Tyr-Val-Ala-Ser-Glu-K(dnp) by using sodium acetate as buffer for pH 5.5; MES for pH 6.0, 6.5, and 7.0; HEPES for pH 7.5 and 8.0; and CHES for pH 8.5 and 9.0, respectively. Activity measurements for HmrA mutants C93A, E128A, and C93A/E128A proceeded with substrate Abz-Gly-Ile-Val-Ala-Aka-k(dnp) as for the wild-type protein.

Aminopeptidase activity was assayed with the p-nitroanilide derivatives (pNA) of a representative set of natural L-amino acids and dipeptides (Bachem) and two non-natural alanine variants (p-Ala-pNA and k-Ala-pNA; Bachem). Reaction mixtures of 150 μl contained 6.5 μg of HmrA, 50 μM zinc chloride, 100 mM sodium chloride, and 33 mM substrate in 50 mM HEPES, pH 7.5, or 50 mM CHES, pH 8.5. In addition, 7-amido-4-methylcoumarin derivatives of a subset of natural L-amino acids (Bachem) were tested under the same conditions. Carboxypeptidase activity was tested with N-(4-methoxyphenylazoforinyl)-Phe, N-(4-methoxyphenylazoforinyl)-Arg (Bachem), N-(3-[2-furyl]acylloyl)-Ala-Lys, and N-(3-[2-furyl]acylloyl)-Ala-Phe-NH2 (Sigma-Aldrich) and benzoyl-Ala-NHmrA with 10 mM EDTA (Roche Applied Science) were used as a positive control at the same concentration. Sample 2 was used as a positive control at the same concentration. Activity was assayed with DQ peptidoglycan from S. aureus (Sigma-Aldrich) was used as a positive control. Hydrolytic activity against specific S. aureus stem peptide components was assayed by using the following peptides: (Gly)5 and H-Ala-d-Glu(Lys-d-Ala-d-Ala-ΔOH)OH (both from Bachem) and N-acetyl muramyl-l-Ala-d-isoGln (Sigma-Aldrich) at 0.1 mM. Lysostaphin or ahronopeptidase (both from Sigma-Aldrich) was used as a positive control. Hydrolytic activity against specific S. aureus stem peptide components was assayed by using the following peptides: (Gly)5 and H-Ala-d-Glu(Lys-d-Ala-d-Ala-ΔOH)OH (both from Bachem) and N-acetyl muramyl-l-Ala-d-isoGln (Sigma-Aldrich) at 0.1 mM.

Kinetic parameters for hydrolytic reactions were obtained assuming simple hyperbolic kinetics following procedures described in the “Assay Guidelines Manual” from the National Institutes of Health Chemical Genomics Center (available on the NCGC, National Institutes of Health, Web site).

Inhibition Studies—The effect of standard peptidase inhibitors on the peptidolytic activity of HmrA was tested with substrate DABCYL-Leu-Ala-Arg-Val-Glu-EDANS as aforementioned (see Table 2).

Crystallization and Structure Analysis—Crystallization assays were performed by the hanging drop vapor diffusion method. Reservoir solutions were prepared manually and dispensed on 24-well Cryscem crystallization dishes (Hampton Research). Crystallization trials with wild-type HmrA protein did not render crystals that diffracted to better than 3.5 Å and were not further pursued. Crystals of selenomethionine-derivatized HmrA suitable for structure analysis were obtained from drops containing 2 μl of protein solution (at 7.5 mg ml-1 in 50 mM HEPES, 100 mM sodium chloride, 100 μM zinc chloride, pH 7.5, 0.75 μl of 0.1 M ATP disodium salt, and 1 μl of reservoir solution (49% 2-methyl-2,4-pentanediol, 0.1 M Tris-HCl, pH 8.0) at 4°C. Crystals were cryoprotected with 50% 2-methyl-2,4-pentanediol, 30% glycerol, 0.1 M Tris-HCl, pH 8.0. Suitable diffraction data were collected from a single liquid-N2 flash-cryocooled crystal at 100 K (provided by an Oxford Cryosystems 700 series cryostream) at beam line ID23-1 (equipped with an ADSC Q315R CCD detector) of the European Synchrotron Radiation Facility (Grenoble, France) within the Block Allocation Group “BAG Barcelona.” The wavelength chosen corresponds to the selenium absorption peak as determined from a previous XANES fluorescence scan. The crystal was tetragonal and contained a tetramer in the crystallographic asymmetric unit, with a solvent content of 72% and a Matthews coefficient of 4.3 Å3/Da. Diffraction data were integrated, scaled, merged, and reduced with the programs XDS (20) and SCALA (21), the latter within the CCP4 suite of programs (22).

The structure was solved by single-wavelength anomalous diffraction with SHELXD (23), which identified 28 sites corresponding to five of six possible selenium sites and two zinc sites for each of the four protein molecules. Subsequent phasing with SHELXE (23) revealed that P412 2 was the correct space group, due to significant difference in the pseudo-free correlation coefficient with the other possible space groups. Visual inspection of the heavy atom/ion sites enabled us to divide them into four sets of seven and to derive non-crystallographic symmetry operators with LSQKAB in CCP4. These operators were used in a subsequent density modification step with 4-fold averaging by the DM program (24). These calculations rendered an electron density map that enabled straightforward tracing of the entire polypeptide chain of each protomer with TURBO-FRODO (25) on a Silicon Graphics Octane2 Work station. Subsequently, crystallographic refinement with REFMAC5 (26), which included TLS refinement, alternated with manual model building until the final refined model was obtained. It included residues Met1–Gly391 of molecules A and D, Gly392–Lys390 of molecule B, and Met1–Lys390 of molecule C and two zinc ions per protein chain. Furthermore, 32 additional elongated density blobs throughout the structure were tentatively interpreted as...
glycerol molecules. Finally, 366 solvent molecules and one tentative zinc ion engaged in crystal contacts were also assigned.

In Vivo Growth and Killing Assays—To study the effect of exogenous addition of HmrA in the phenotypic response to BLAs, four S. aureus strains with a wide range of phenotypic responses to oxacillin were used: strain RN4220 (fully susceptible (27)), strain CDC-1 (very low level resistance (28)), strain N315 (heterogeneous resistance (29)), and strain COL (high level homogeneous resistance (30)). Liquid culture assays were performed in 200 μl of fresh tryptic soy broth without dextrose (from Bacto) incubated in 96-well cell culture plates (BD Biosciences) at 30°C, and A600 was monitored in a Power-wave XS microplate reader (Bio-Tek). Previously, precultures had been grown overnight in tryptic soy broth at 37°C and diluted with fresh tryptic soy broth as stated below. A range of oxacillin (Sigma-Aldrich) concentrations (0, 0.75, 1.5, 3, 6, 12, 25, 50, 100, 200, 400, and 800 μg ml⁻¹) and purified wild-type HmrA, mutant C93A/E128A-HmrA, or buffer A were tested. For strain COL, oxacillin concentrations of 1,600 and 3,200 μg ml⁻¹ were also tested because this strain has a minimum inhibitory concentration (MIC) of 800 μg ml⁻¹ for oxacillin at 30°C. For killing assays, cultures were diluted until A600 was 2.4, as measured in a SHIMADZU UV-mini1240 spectrophotometer. Aliquots of 84 μl were then added to 100 μl of tryptic soy broth containing twice the aforementioned oxacillin concentrations (0–1,600 μg ml⁻¹ for RN4220, CDC-1, and N315; 0–6,400 μg ml⁻¹ for COL) and 16 μl of either purified wild-type HmrA, C93A/E128A-HmrA (both at 5.1 mg ml⁻¹), or buffer A. A600 was recorded for the next 23 h at 30-min intervals. Cell viability was checked by plating serial dilutions of each culture on tryptic soy agar plates (Difco), which were incubated overnight at 37°C. Growing assays were carried out in the same way as the killing
assays except that the overnight precultures were initially diluted 10 times more (\(A_{600} \sim 0.24\)).

In order to study the potential effect of preincubation of antibiotic with HmrA, 1 ml of tryptic soy broth containing 200 \(\mu\)g ml\(^{-1}\) oxacillin was incubated overnight at 30 °C with either purified HmrA, apo-HmrA, or buffer A. After the incubation, reaction mixtures were filtered through a 100-kDa filter membrane (Millipore). In parallel, an overnight culture of RN4220 in tryptic soy broth was diluted until \(A_{600} = 2\) and \(A_{600} = 0.2\). 100-\(\mu\)l aliquots of these diluted cultures were incubated, respectively, with 100 \(\mu\)l of each of the filtered pretreated antibiotic solutions or with fresh tryptic soy broth supplemented with 200 \(\mu\)g ml\(^{-1}\) oxacillin. \(A_{600}\) was recorded for the next 23 h at 30-min intervals in each case.

**RESULTS AND DISCUSSION**

**Biophysical and Functional Assays in Vitro—**Wild-type HmrA protein and a selenomethionine-derivatized form were obtained in large amounts by recombinant overexpression and purified to homogeneity. Inductively coupled plasma mass spectrometry analysis of the recombinant wild-type enzyme revealed the presence of zinc but no other cations in significant amounts, thus indicating that HmrA is a zinc-binding protein. In order to confirm this, stability against thermally induced protein unfolding was analyzed following the thermofluor approach (18). Several divalent cations were assayed, but only zinc significantly stabilized the apoprotein, giving rise to a shift of melting temperature from 48 °C (in the absence of metal) to 56–58 °C, whereas melting temperatures of 45, 45, and 48 °C were recorded for cobalt, calcium, and manganese, respectively. This calorimetric technique was also used to identify optimal buffer conditions for HmrA stability.

HmrA did not show aminopeptidase or carboxypeptidase activity; nor did it hydrolyze a representative set of five BLAs or a chromogenic cefoxitin derivative, nitrocefin (data not shown). In contrast, HmrA proved to be an endopeptidase against several fluorescent oligopeptide substrates (Table 1). These experiments further revealed that HmrA has no clear substrate specificity and a pH optimum for activity of 7.5–8.5 (Fig. 1A), which is consistent with the results obtained in the thermofluor assays. In addition, HmrA cleaved fluorescein-la-
FIGURE 2. Structure of HmrA. A, Richardson plot (67) of HmrA in stereo depicting the CD and the OD approximately in standard orientation (68). The regular secondary structure elements are shown as orange ribbons (helices α1–α12) and red arrows (strands β1–β12) and labeled. The two zinc ions are displayed as magenta spheres, and the glycerol molecules lining the crevice are shown as green stick models. B, close-up view of the co-catalytic zinc binding site (magenta spheres) and the zinc-binding residues plus the general base/acid glutamate, which are shown as sticks and labeled. The organometallic bonds are shown in green. C, close-up view of the active-site cleft approximately in the same orientation as A. Protein residues delimiting the crevice are shown (carbons in green, oxygens in red, sulfurs in yellow, and nitrogens in blue) and labeled. Residues participating in zinc binding that are shown in B have not been displayed for clarity. The glycerol molecules found in the cleft are shown as red stick models. Segments Lβ8β9 and Lβ7α6 from a neighboring molecule (in purple) also contribute to shaping the active-site cleft through residues Ala229 and Asn230 and His183, Ala184, Ser185, and Glu186, respectively (not labeled). D, SDS-PAGE depicting a cross-linking experiment of wild-type HmrA, which reproduces the time-dependent transition from a monomer (~43 kDa) to a tetramer (~172 kDa) due to the action of paraformaldehyde. The flanking lanes show molecular weight markers. E, quaternary arrangement of HmrA as found in the crystal asymmetric unit. Oligomerization occurs mainly through the ODs, and the CDs (shown with their respective zinc ions) protrude away from the tetrameric central stalk. F, close-up view of E illustrating the interaction of one tetramerization domain (green coil depicted from Leu168 to Glu283) with the other three (shown for their semitransparent Connolly surface). Residue side chains engaged in close contacts are displayed as sticks colored according to their overall chemical nature (red, acidic; blue, basic; orange, non-polar; lilac, polar) and labeled.
beled albumin and casein but not gelatin (Fig. 1B), possibly indicating that the latter is not a good general protease substrate due to its limited variety in amino acids. HmrA also digested standard albumin, although nonspecifically and with low efficiency (Fig. 1E). In addition, HmrA showed activity in a fluorescein-derived peptidoglycan assay, whereas apo-HmrA and holo-HmrA in the presence of EDTA were inactive. This activity was roughly 20% of that of lysozyme (Fig. 1C). However, the sugar backbone of peptidoglycan is not the target of HmrA as it was inactive in a muramidase assay (Fig. 1D). In order to assess the capacity to process the linear stem peptides that are responsible for cross-linking the chains of N-acetyl glucosamine and N-acetyl muramic acid in the bacterial cell wall of S. aureus (see Fig. 1 in Ref. 6), three peptides were assayed: (Gly)₅, H-Ala-d-Glu(Lys-d-Ala-d-Ala-OH)OH, and N-acetyl-muramyl-l-Ala-d-isoGln. These were not cleaved under the conditions assayed in vitro, so that we cannot conclude that HmrA is a peptidoglycanase.

A set of standard peptidase inhibitors targeting different classes of peptidases was tested against HmrA in a fluorogenic peptide assay. Serine and cysteine peptidase inhibitors exerted no relevant inhibitory activity. In contrast, HmrA was efficiently inhibited by general zinc-chelating agents, such as EDTA, α-phenanthroline, and tetraethlenepentamine as well as by excess zinc but also, unexpectedly, by ATP, ADP, and AMP (at 1 mM) (Table 2). The latter compounds were assayed due to the beneficial effect of ATP on crystallization experiments (see “Experimental Procedures”), but because this molecule was not found in the crystal structure, we cannot explain this inhibitory effect at the molecular level. However, a possible functional explanation could be that HmrA, which does not possess a signal peptide and is thus probably synthesized intracellularly, needs to be inhibited until it reaches its final destination, putatively the peptidoglycan or extracellular milieu (see below). In this sense, common cellular components, such as nucleoside phosphates, may provide a simple means for efficient inhibition. In summary, biophysical, hydrolytic, and inhibitory assays indicate that HmrA is a nonspecific zinc-dependent endopeptidase but not a muramidase or β-lactamase, which is inhibited by an abundant intracellular component.

Overall Structure of the Monomer—Because no properly diffraction wild-type protein crystals could be obtained, the crystal structure of the selenomethionine-derivatized variant of HmrA was solved by single-wavelength anomalous diffraction (Table 3). This revealed four equivalent molecules (A–D) in the asymmetric unit, which have a bilobal structure consisting of a catalytic zinc-containing domain (CD; residues Met⁵/Gly⁷–Thr¹⁶⁷ and Glu²⁸³–Lys³⁹⁰/Gly³⁹¹) and an inserted oligomerization domain (OD; Leu¹⁶⁸–Asn²⁸², Fig. 2A). The CD shows a three-layer (αβα)-sandwich architecture with seven Β-strands (Β₁–Β₆ and Β₁₂) arranged in a mixed parallel/antiparallel twisted β-sheet. The β-sheet harbors eight helices (α₁–α₄, α₈, and α₁₀–α₁₂) on the side that is distal to the OD and two helices (α₅ and α₉) on the opposite side (Fig. 2A). The sheet is bifurcated at its C-terminal border between strands Β₃ and Β₅ through the action of residues His⁹⁵ and Ile⁹⁸ of helix α₄. As a result, the latter helix approaches the active-site cleft and contributes to zinc binding (see below).

The OD is inserted between strand Β₆ and helix α₈ of the CD. It consists of two intercalated β-α-β segments (β⁷–α₆–β₈ and β₉–α₇–β₁₀–β₁₁) with typical right-handed crossover connectivity, which are related by a roughly vertical 2-fold axis (Fig. 2A). This gives rise to an overall open-faced α/β sandwich architecture with α/β-plaits topology, whose two layers are created, respectively, by a slightly curved β-sheet (β⁷, β₈, β₉, and β₁₀–β₁₁), which is twisted by ~60°, and two helices (α₆ and α₇), which nestle into the concave side of the sheet. The helix axes roughly parallel the strands. The last strand of the OD sheet is subdivided into two, Β₁₀ and Β₁₁, by a bulge (loop Lβ₁₀β₁₁; Pro²⁷⁶–Gly²⁸₀), which contributes to the active-site crevice (see below). The two OD layers are joined by a large hydrophobic core that traverses the entire domain. Overall, the OD fold topology is equivalent to that of the pro-domains of...
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A/B-type metallocarboxypeptidases of the funnelin tribe and of subtilisin-like serine peptidases (38–41).

**Active-site Cleft**—The active-site cleft lies at the interface between the CD and OD domains, at the C-terminal end of the CD β-sheet, as often found in open twisted α/β domains (42). In the crystal structure, a series of four glycerol molecules were tentatively modeled into this cleft, based on discontinuous electron density, mimicking a bound substrate or product because neither the resolution of the diffraction data nor the occupancy within the crystal allowed for a more accurate assignment (Fig. 2, A and C). The cleft is carved into the front surface of the CD and is elongated, traversing the moiety from left to right, thus providing enough space to accommodate oligopeptides and perhaps larger molecules in both the non-primed and the primed regions of the cleft. This provides a structural explanation for the endopeptidolytic activity found in vitro (see above). The cleft is top-framed by a segment in extended conformation, Asp308–Asp316, whose central part around Gly313 may bind the main chain of a substrate around the scissile bond. The bottom of the cleft is delimited on its non-primed side by Lβ12α10, the end of β3, Lβ3α4, and the beginning of α4 below the zinc site. On the primed side, the cleft is delimited by Lβ8α5 and segment Ala127–Lys137. The convex face of the four-stranded β-sheet of the OD further delineates the top of the cleft on its primed side. The bulge segment preceding β11 also contributes to the cleft as well as segments Lβ7α6 and Lβ8β9 at the tip of the OD of an adjacent protein molecule (Fig. 2C). A central double zinc-binding site resides at the bottom of the cleft, approximately at half-width (Fig. 2, A and B). The co-catalytic zinc ions (3.3–3.5 Å apart) are jointly bound by Cys93 S, Cys93 N, and Cys93 C. In addition, one zinc ion is further bound by the Ne2 atoms of His156 and His95 and the other by His341 Ne2 and by both Glu128 Oe1 and Oe2. The latter residue is adjacent to the general base/acid glutamate, Glu128, believed to participate in water-mediated hydrolysis in structurally related hydrolyses (see below), which is held in place by a hydrogen bond of one of its carboxylic atoms with Thr315 Oγ1. This active-site environment led us to design three mutants, C93A, E128A, and C93A/E128A, which were obtained through recombinant overexpression and purified to homogeneity in amounts comparable with the wild-type protein, and assay them for activity. Consistent with their key role in zinc binding and catalysis, all three mutants displayed only residual activity against a fluorogenic peptide when compared with the wild-type enzyme (2.3 ± 0.2, 4.5 ± 0.1, and 1.5 ± 0.2%, respectively).

**Quaternary Structure**—Wild-type HmrA eluted as a tetramer of ~170 kDa in a calibrated size-exclusion chromatography assay. Analytical ultracentrifugation studies likewise revealed that the protein is a tetramer in the concentration range assayed (estimated molecular mass 166–169 kDa; sedimentation velocity constant s_app = 6.6 S). Finally, cross-linking experiments also pointed to a tetrameric arrangement (Fig. 2D). These findings in solution were corroborated by the crystal structure, which showed four HmrA protomers that assembled into a ~170-kDa homotetrameric complex of 105 × 125 × 90 Å maximal dimensions and 222-point symmetry (Fig. 2F). The protomers are essentially indistinguishable, and the assembly occurs mainly through the ODs. The interaction is nearly symmetric and mixed hydrophobic and hydrophilic, including close contacts made by up to 43 residues of each protomer (Fig. 2F). Oligomerization entails that two ODs join laterally through a dyad, so that their β-sheets interact to give rise to a continuous eight-stranded curved and arched antiparallel sheet, with two strands interrupted by a bulge (strands β10+β11; see above). The four helices (α6 and α7 of each monomer) nestle into the cavity formed on the concave side of this sheet, with their helix axes roughly antiparallel to each other and to the strands of the sheet. This gives rise to a large open dimeric α/β sandwich. Tetramerization is achieved by two such dimers contacting each other through the four helices, which results in an overall four-layer (p6mm)-sandwich (Fig. 2E). This tetrameric arrangement entails that all four active-site crevices are fully accessible and ~48, ~52, and ~70 Å away from each other, respectively (i.e. enough to act independently but simultaneously on potentially large and complex substrates, such as the peptidoglycan meshwork). In addition, the ODs contribute to delimiting the crevice (see above) (i.e. they have a double function in oligomerization and substrate binding).

**Structural Similarities**—Searches for structural relatives of HmrA identified several resolved proteins, including indole-3-acetic acid amino acid hydrolase (Protein Data Bank (PDB) code 1xmb (43)), carboxypeptidase G2 (PDB code 1cg2 (38)), β-alanine synthase (PDB codes 2v8d and 2v8h (44)), peptidase T (PDB code 1fno (45)), allantao amido hydrolase (PDB code 121I (46)), succinlydiaminopimelate desuccinylase (PDB codes 1gyv and 3ic1 (47, 48)), carninosinase (PDB code 2zog (49)), and acetylctitrulline deacyltase (PDB code 277v (50)). These enzymes cleave amide bonds in a large variety of substrates, but HmrA is unique in being an endopeptidase. The aforementioned enzymes form a subgroup within the M20 family of metallohydrolases according to the MEROPS data base (51) (available on the World Wide Web). All of these molecules contain a three-layer (αβα)-sandwich CD centered on a (mostly) eight-stranded β-sheet and an OD, which consists of an open α/β-sandwich with four antiparallel β-strands and two α-helices, inserted between the fifth and sixth β-strand. As to quaternary structures, all aforementioned HmrA relatives form dimers. Indole-3-acetic acid amino acid hydrolase crystallized as monomers, but dimerization in solution has not been ruled out for this amido hydrolase (52). Dimerization involves the same region of the ODs in all cases, and it occurs through formation of an overall open sandwich of antiparallel strands and four helices as found in HmrA dimers (see above). Accordingly, the only proven exception among these amido hydrolases to a dimeric arrangement is HmrA, which is the first case reported to render tetramers.

HmrA and its dimeric relatives harbor a co-catalytic dual metal-binding site, in which the two ions are bridged by a cat-

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**FIGURE 4. Effect of exogenous HmrA addition on growing assays.** Shown are growth curves in growing assays for *S. aureus* strains (from top to bottom): RN4220 (A), CDC-1 (B), N315 (C), and COL (D), with different oxacillin concentrations (0–800 µg ml⁻¹ for A–C; 0–3,200 µg ml⁻¹ for D). In each case, the results obtained after the addition of buffer (left) or HmrA solution (right) are depicted.
Structure and Function of S. aureus HmrA

A

B

C

D
analytic solvent molecule that ultimately performs the nucleophilic attack on the scissile bond assisted by a general base/acid glutamate (53). Although it has been postulated that the two ions participate, respectively, in stabilization of the tetrahedral reaction intermediate and in the activation of the catalytic water molecule in a more distantly related enzyme (54), the overall mechanism of hydrolysis is probably very similar to that of monometalated peptidases, such as gluzincins, metzincins, funnellins, and cowrins (38, 39, 55–57). In addition to the catalytic solvent, a total of five protein ligands participate in binding of the two zinc ions, which adopt a (distorted) tetrahedral coordination sphere each (Fig. 3A). Structural similarity of the M20 family amidohydrolase CD is also found with other dinuclear enzymes of the MEROPS families M17, M18, M28, and M42. All present the basic fold found in monometalated funnellins (39), which is distinct from the classic α/β-hydrolase fold (58), and mostly share two vicinal glutamate residues engaged in zinc binding and catalysis. They can be grouped in a common family termed EEM2-metallohydrolases.

Interdomain Hinge Motion—Multidomain proteins, in which the constituting domains are connected by flexible linkers or hinge regions, alternate between open and closed states in response to the functional requirements of, respectively, substrate binding/product release and formation of a Michaelis complex for catalysis (59). Accordingly, no specific molecular mechanism, such as allostery, etc., is required to explain the transition, and examples of such hinge motions include metallopeptidases, such as thermolysin (60), astacin (61), and angiotensin-I-converting-enzyme-related carboxypeptidase (62). This hinge model further entails that, in principle, open unbound forms are more flexible than closed bound forms because the former can freely oscillate around the hinge, whereas the latter trap the substrate. The aforementioned HmrA relatives have been crystallized in their open, intermediate, and closed states (38, 44–46, 49, 63). In HmrA, all four monomers in the asymmetric unit present the same interdomain angle. Analysis of the molecular flexibility of HmrA based on the elastic network model unambiguously indicated the presence of two independent rigid bodies (scores of 0.97 and 0.92, respectively, for the CD and the OD) and Thr157 and Asn282 as hinge residues, around which major rearrangement though rigid body rotation may occur (Fig. 3B). As to which state of HmrA was captured in the present studies, the residual electron density found in the four active sites (interpreted as glycerol molecules mimicking a bound substrate or product; see above) and the observation that the ODs contribute to delimiting the active site of a vicinal molecule point to a closed bound conformation.

In Vivo Studies—To obtain insight into the function of HmrA, the influence of the exogenous addition of the protein on bacterial growth was studied in four S. aureus strains with different levels of response to the antibiotic: RN4220 (susceptible), CDC-1 (low level resistance), N315 (heterogeneous resistance), and COL (homogeneous resistance). Two types of assays were performed: growing assays, which started from diluted overnight cultures and assessed growth capacity in the presence of antibiotic stress (Fig. 4), and killing assays, which started from a 10-fold higher culture concentration (Fig. 5). In both cases, the optical density was monitored over time, and cell viability was confirmed by plating serial dilutions of culture aliquots on rich agar medium. These two assays are complementary because BLAs in general exert a bacteriostatic effect on S. aureus, soysis and, thus, cell death are an indirect consequence of the inhibition of cell wall synthesis over successive cell divisions (64). Both assays revealed that exogenous HmrA improved cellular viability in the presence of oxacillin concentrations well above the MIC of the respective strains. This effect was stronger for the susceptible and low MIC strains RN4220 and CDC-1, respectively, but even for the highly resistant strain COL, the protective effect was clear despite the extremely long lag phase caused by the very high supra-MIC antibiotic concentrations used. Similar experiments were performed using the C93A/E128A HmrA double mutant. The protective effect was not observed, indicating that the proteolytic activity of HmrA is indeed necessary for the protective effect against oxacillin. Furthermore, the observation that the protective effect is only observed after ~10 h may be explained by the existence of other players in this mechanism that likewise have an important role and are activated late, similar to what happens with the Mcel/MecR1/MecA resistance mechanism (65, 66).

In order to rule out the possibility that the protective effect of exogeneous HmrA was due to an intrinsic β-lactamase activity, which could reduce the effective concentrations of oxacillin, the growing assays for susceptible strain RN4220 were repeated with an overnight preincubation of exogenous HmrA with oxacillin in tryptic soy broth. We did not detect significant differences with the previous experiment and concluded that the effect of HmrA in the increased resistance to oxacillin was not due to degradation of the latter. This is consistent with a lack of significant β-lactamase activity against several BLAs in vitro (see above). Taken together, these observations point to a protective function of HmrA on S. aureus, in particular on sensitive or low level resistance strains (i.e. it functions as a true resistance factor against BLAs).

Outlook—The present study shows that HmrA is a nonspecific dimetatalated zinc-dependent hydrolyase, which is inhibited by metallohydrolyase inhibitors and can hydrolyze peptide bonds in extended peptides. Its role in resistance is linked to its hydrolytic activity. The protein might contribute to bacterial cell wall turnover and in this way contribute to resistance of S. aureus against cell wall active antibiotics, such as BLAs. In these duties, it hypothetically would assist the intrinsic methicillin-resistant S. aureus-specific penicillin-binding protein, MecA, which is a transpeptidase that can contribute to cell wall synthesis even in the presence of BLAs. This speculative role for HmrA is supported by structural studies, which identified a novel large tetramer that could process a large substrate meshwork through four distal active sites simultaneously. Moreover,

FIGURE 5. Effect of exogenous HmrA addition on killing assays. Shown are growth curves in killing assays for S. aureus strains (top to bottom): RN4220 (A), CDC-1 (B), N315 (C), and COL (D), with different oxacillin concentrations (0–800 μg ml⁻¹ for A–C; 0–3,200 μg ml⁻¹ for D). In each case, the results obtained after the addition of buffer (left) or HmrA solution (right) are depicted.
the enzyme is inhibited by adenosine phosphates, which are abundant intracellular but not extracellular components, thus supporting an extracellular proteolytic role for HmrA.

Finally, we found that the hmrA gene is present in over 65 S. aureus strains, suggesting it is a native constituent of the S. aureus core genome. Moreover, highly similar genes (>40% sequence identity; E-values of < 10−76) are also present in a number of other staphylococci, macrococci, bacilli, clostridials, enterococci, and even Gram-negative proteobacteria, such as Erwinia carotovora. Although these gene products await their functional characterization, the striking similarity with HmrA strongly points to a novel yet widespread mechanism of resistance against bacteria that may be the result of horizontal gene transfer.

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