The enzymes essential for bacterial peptidoglycan biosynthesis are attractive targets for antimicrobial drug development. One of these is MurB, which contains FAD as a cofactor and catalyzes the NADPH-dependent reduction of UDP-N-acetylenolpyruvylglucosamine (UDP-GlcNacEP) to UDP-N-acetylmuramylcarboxamide. This study examined the roles of the conserved amino acid residues in Staphylococcus aureus MurB, which are located near the active site in x-ray crystal structures. Seven of 11 site-directed mutated murB genes lost the ability to complement a temperature-sensitive murB mutant. Biochemical characterization of the seven mutated MurB proteins revealed that they cannot carry out the reduction of UDP-GlcNacEP, although they can all catalyze the intramolecular reduction of FAD via NADPH. Spectrometric analyses of the oxidized form of the mutated proteins in the presence and absence of NADP+ or UDP-GlcNacEP revealed that these essential amino acid residues play four distinct roles in substrate interactions: Arg213 is essential for maintenance of the electronic state of FAD; Arg176 is required for interaction with UDP-GlcNacEP; His209 is required for interaction with both UDP-GlcNacEP and NADP+; and Asn71, Tyr175, Ser226, and Glu296 are not apparently required for interaction with either ligand. The results presented here identify for the first time the amino acid residues of MurB that are required for the interaction with UDP-GlcNacEP and NADP+.

The expansion of infectious diseases caused by multidrug-resistant Staphylococcus aureus and other pathogenic bacteria has become an increasing global problem. Thus, the development of antibacterial drugs that have novel mechanisms of action or targets is needed. Peptidoglycan is a component of the bacterial cell wall and prevents cell lysis caused by high intracellular pressure. Because the enzymes responsible for peptidoglycan biosynthesis are ubiquitous in bacteria but are not found in humans, they are expected to be good targets for antibacterial drug development.

Peptidoglycan consists of a saccharide backbone that is formed by alternating N-acetylglucosamine and N-acetylmuramic acid residues with tetra- or pentapeptides that branch from N-acetylmuramic acid (1–3). In S. aureus, 1-lysine of one of the tetrapeptides is cross-linked to D-alanine of the other by a pentaglycine chain. The initial step of peptidoglycan biosynthesis is the conversion of UDP-N-acetylmuramic acid and phosphoenolpyruvate to UDP-N-acetylenolpyruvylglucosamine (UDP-GlcNacEP) by MurA. Subsequently, UDP-GlcNacEP is reduced to UDP-N-acetylmuramic acid by MurB. Next, five amino acid residues (1-R-A, 2-D-Glu, 3-Lys, and 4-D-Ala-5-D-Ala) are sequentially added onto the D-lactyl group of UDP-N-acetylmuramic acid by MurC, MurD, MurE, and MurF enzymes in a ribosome-independent manner.

Because MurB is one of the essential enzymes for peptidoglycan biosynthesis (4–6), chemical compounds that block MurB function are candidate antimicrobial agents (7, 8). Several inhibitors have been designed based on the crystal structure of the UDP-GlcNacEP-MurB complex (9, 10). However, it has not yet been determined whether MurB-targeting drugs are clinically effective.

MurB is a flavoprotein that belongs to the FAD-binding protein superfamily, which shares a characteristic FAD binding fold (11). The MurB family is divided into at least two groups by amino acid sequence alignment: type I, which includes Escherichia coli MurB; and type II, which includes S. aureus MurB. This latter type lacks both a loop structure containing Tyr190 and a single split βββ fold, which are found in the substrate binding domain of type I E. coli MurB (12). X-ray structural analyses indicated that the E. coli type I and S. aureus type II MurB proteins have similar overall folds, although there are differences between the substrate binding regions due to amino acid deletions (12, 13).

The enzyme reaction catalyzed by MurB is performed by Ping Pong Bi Bi mechanism, alternatively Ping Pong Tetra Uni in the Cleland system of nomenclature; the first step is the reduction of FAD, which consumes one equivalent of NADPH, and the second is an electron transfer from FADH2 to UDP-GlcNacEP (14). In this mechanism, both NADPH and UDP-GlcNacEP bind to the same subdomain (15, 16) (Fig. 1). Ser226 in E. coli MurB, which corresponds to Ser236 in S. aureus MurB, is a catalytic residue and participates in the proton transfer to an enol intermediate that is formed during the second reduction step (17, 18).

Experimental evidence for essential amino acid residues and their
TABLE 1

Primers used for site-directed mutagenesis of MurB

| Mutation | Primer DNA sequence* |
|----------|----------------------|
| G67A     | 5’-GTTACATATTTAGGAAATGCTCAAAT-3’ |
| 5’-ATTTGAGGCCATTTCATTAAATGTTAAG-3’ |
| G69A     | 5’-ATTGAGAAATGCTCAAAATATTATTT-3’ |
| 5’-AAATTATTTGGGAGCATTTTCTAAAT-3’ |
| S70A     | 5’-TTAGAAGATGGGCAATTATTATTT-3’ |
| 5’-AAATATTTGCGCCATTTCCTAAT-3’ |
| N71A     | 5’-AGAGAAATGCTCAAAATATTATTT-3’ |
| 5’-CAGGCAATTAATAGCTGAGCATCTTTCT-3’ |
| Y175F    | 5’-TACGGTTAGATTTTGTAAGCAATT-3’ |
| 5’-ATTGCTTACGCAAATTCAATCTACA-3’ |
| R176A    | 5’-AGGATTAGATTTACCTAATGCTTTCT-3’ |
| 5’-GAATATTTGCTAACATCAATCTAAT-3’ |
| R230A    | 5’-TTAACAGAGGCTGGAATTTCAAACA-3’ |
| 5’-TGTTTAGAGCTGTCTGTTTAA-3’ |
| S226A    | 5’-CCTTTACAGGTGGTTATATCCAAAG-3’ |
| 5’-CTTTCAGATGGAACCCATGGAGG-3’ |
| R230A    | 5’-AGGTGATTCCACAGGCCTGTTCCC-3’ |
| 5’-GACCCGGCTGTTGAAATACACT-3’ |
| H259A    | 5’-GAAATTTACCAACCACGCTTTGTTTATGTT-3’ |
| 5’-ACCATAAAAACACGCGTTTGGATGAACTT-3’ |
| E296A    | 5’-GAATTTAATCTGCTGAAGCATTAC-3’ |
| 5’-AATGCAGATCCGATTATTTAC-3’ |

* The underlined bases indicate the nucleotide positions that were changed.

Complementary internal primers that had 20-bp overlaps with the mutation site. The first round PCR (15 cycles) were carried out using the upper primer of each construction in Table 1 paired with themurB3 primer, 5’-CCGGAATTCATAATGAGTGCTGTTT-3’, corresponding to the 3’-end of the murB gene containing the EcoRI site (in boldface type) and the lower primer paired with themurB5 primer, 5’-TCAACTAATGTCTGTTTACCTGCAACGTTCC-3’, corresponding to the upstream sequence of the MurB open reading frame. Each group of two primary PCR products was purified, mixed, and used for the second round of PCR (15 cycles) using themurB5 and murB3 primers, and products were digested with EcoRI and cloned into the pND50 shuttle vector at the EcoRI and Smal sites. Site-specific mutations on cloned fragments were confirmed by DNA sequencing. The resulting plasmids were designated pNDmurBG67A, pNDmurBG69A, pNDmurBS70A, pNDmurBN71A, pNDmurBR175F, pNDmurBR313A, pNDmurBS226A, pNDmurBR230A, pNDmurBH259A, and pNDmurBE296A, respectively.

Complementation Assay—Complementation assays were performed as described previously (6). The S. aureus murB temperature-sensitive mutant TS2901 was transformed with 100 ng of plasmids described above, pNDmurBs, and its vector pND50 by electroporation in a 0.2-cm cuvette (Gene Pulser II; Bio-Rad). Transformants were grown on NaCl-free LB agar plates containing 12.5 μg/ml chloramphenicol at 30 or 43°C. The numbers of transformants were counted after 24 h.

Plasmids for Overexpression of MurB—To construct plasmid series of pET5XmurB for overexpression of S. aureus MurB in E. coli, the MurB open reading frame was amplified with PCR using the primers for murB3 described above and a primer for murBSN, 5’-GAAAGGATGTACCTCCATGTTGATATATAAAA-3’, that incorporated the Ncol site (in boldface type) with the initiation codon and using pSmurB, a plasmid harboring the wild-type S. aureus murB gene (6), as a template. The product was digested with Ncol and EcoRI and then ligated into the Ncol and EcoRI sites of pET21d (Novagen), resulting in the plasmid pET5XmurB. The HindIII site in its multiple cloning site was removed by digestion with Sall and Xhol and self-ligated. This resulting plasmid was designated pETSXmurB. With regard to the seven mutant murB genes that eliminated the ability to complement the temperature-sensitive S. aureus murB mutant in vivo, each DNA fragment harboring the mutation site was cut off by digestion with HindIII and EcoRI from the respective pND50-based plasmid and exchanged with the correspond-
ing region of pETSSmurB at the HindIII and EcoRI sites. The resulting plasmids were designated pETSSmurBN71A, pETSSmurBY715F, pETSSmurBR176A, pETSSmurBR213A, pETSSmurBS226A, pETSSmurBH529A, and pETSSmurBE296A, respectively.

Purification of *S. aureus* MurB—BL21 (DE3)/pLYsS (Novagen) were transformed with plasmids of the pETSSmurB series. Each strain was grown in 1 liter of LB medium at 37 °C until the OD₆₀₀ reached 0.5, after which the culture was adjusted to 0.5 mM isopropyl 1-thio-β-d-galactopyranoside. After a 2-h induction, cells were harvested by centrifugation, washed once with 30 ml of 0.9% NaCl and frozen in liquid N₂. The frozen cell paste (~3 g) was resuspended and lysed in 6 ml of lysing buffer (50 mM Hepes-KOH, pH 7.6, 1 mM dithiothreitol, 1 mM EDTA, 20% (v/v) glycerol, 0.25 M KCl, 0.3 mg/ml lysozyme (Sigma), and 20 mM spermidine (Sigma)) on ice for 20 min. Samples were again frozen and thawed and then sonicated four times for 30 s using a Branson Sonifier 450. The resulting homogenate was centrifuged at 145,000 × g for 30 min at 4 °C, and cleared lysates (Fraction I) were adjusted to 0.3 g/ml ammonium sulfate and stirred for 20 min at 4 °C. After centrifugation at 40,000 × g for 20 min, ammonium sulfate was added to the supernatant to a final concentration of 0.5 g/ml. The precipitate was collected, washed once with 30 ml of 0.9% NaCl and frozen in liquid N₂. The frozen cell paste (~3 g) was resuspended in 6 ml of lysing buffer (50 mM Hepes-KOH, pH 7.6, 1 mM dithiothreitol, 1 mM EDTA, 20% (v/v) glycerol) and dialyzed against the same buffer (Fraction II). Fraction II was loaded at a flow rate of 1 ml/min onto a DEAE-Sepharose CL6-B column (1.5 × 25 cm) equilibrated with buffer C and then eluted with a linear gradient from 0 to 0.8 M NaCl in buffer C. Active wild-type MurB was identified in the 0.2 M NaCl eluate. Purified MurB (Fraction III) was dialyzed against buffer C’, loaded onto a monoQ H5/5 column at a flow rate of 0.5 ml/min, and eluted with a linear gradient from 0 to 0.5 M NaCl in buffer C’. Active wild-type MurB again eluted at 0.2 M NaCl and was pooled (Fraction IV). The various mutated forms of MurB were prepared in a similar way, and MurB was followed using the absorbance of FAD at 460 nm. The purity of each Fraction IV exceeded 80% on SDS-PAGE. The protein concentration was determined by the Bradford method using bovine serum albumin as a standard. Proteins were stored at −80 °C.

**Enzymatic Assays**—A typical aerobic MurB assay was performed as described previously (20) in a reaction mixture (0.1 ml) of 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 0.5 mM dithiothreitol, 0.1 mM UDP-GlcNAcEP, and 0.15 mM NADPH in an open cuvette. Anaerobic reactions were performed as described previously (14) with some modifications. Reactions (0.4 ml) contained 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 0.5 mM dithiothreitol, 0.1 mM UDP-GlcNAcEP, 0.15 mM NADPH, 10 mM d-glucose, 20 units/ml glucose oxidase, and 26 units/ml catalase. The reaction mixtures were added to semimicrowaves with Teflon caps (GL Sciences) and flushed with nitrogen gas. After maintaining the mixture at room temperature for 5 min, reactions were started by adding enzyme followed by both flushing with nitrogen gas and capping under a nitrogen atmosphere. Under these conditions, oxygen was scavenged immediately (21).

In each assay, the initial velocity of NADPH oxidation at room temperature was determined by monitoring the decrease in absorbance at 340 nm every 30 or 60 s for 5 min using a UV-160A or a UV-2200 spectrophotometer (Shimadzu) and with 3–30 pmol of MurB. To determine specific activities, an extinction coefficient of 6220 M⁻¹ cm⁻¹ at 340 nm was used for NADPH. It was confirmed that the absorbance was linear up to 0.27 mM. *Kₘ* values for NADPH and UDP-GlcNAcEP were determined as described previously (22).

**Determination of FAD Content of MurB**—Determination of the FAD content was performed as described previously (23). FAD was released from MurB (Fraction IV; 3 μg) by boiling for 5 min followed by the addition of 70% ethanol. After centrifugation at 10,000 × g at room temperature for 5 min to remove denatured proteins, flavin was recovered in the supernatant. The flavin was analyzed by HPLC using a TOSOH analytical C-18 column (TSK-gel ODS-80Ts QA; 4.6 × 250 mm) at a flow rate of 1 ml/min with a solvent of 35% (v/v) methanol, 100 mM formic acid, 100 mM ammonium formate (pH 3.5) using a Hitachi L-7480 fluorescence detector (excitation, 470 nm; emission, 530 nm) and with FAD (Yamasa) as a standard.

**Determination of *Kₘ* Values of MurB for UDP-GlcNAcEP or NADP⁺**—Determination of *Kₘ* values of wild-type and mutated MurB for either UDP-GlcNAcEP or NADP⁺ were carried out using difference spectrum analyses as described previously (22). Absorption spectra were recorded with a Shimadzu UV-2200 spectrophotometer at a scan rate of 50 nm/min. MurB at a final concentration of 15–20 nM was assayed in buffer C at room temperature. Difference spectra, which can improve both the sensitivity and selectivity to detect the spectral perturbation elicited by ligand binding, were computed from the spectra in the presence and absence of ligand and were corrected for dilution. The absorbance changes at 510 nm as a function of ligand concentration were fitted to the following theoretical equation for a 1:1 binding stoichiometry by nonlinear regression using the Kaleida Graph program (Synergy Software Ltd.), where *A* represents the measured absorption, *A₀* is the starting absorbance, Δ*A* is the total measured change in absorbance, *Eₐ* is the enzyme concentration, *Kₐ* is the ligand dissociation constant, and [S₀] is the concentration of ligand added to the enzyme.

\[
A = A₀ + (\Delta A/Eₐ)(Kₐ/[S₀] + [S₀]) - \left(\left(1 + [S₀]/Kₐ\right)^{-1} - 1\right)/2
\]  
(Eq. 1)

**Molecular Modeling of Active Site Residues**—The *S. aureus* MurB–UDP-GlcNAcEP complex was drawn using Swiss-PdbViewer 3.7, which automatically fitted UDP-GlcNAcEP from the *E. coli* MurB–UDP-GlcNAcEP complex (13) (Protein Data Bank code 2mbr) into the *S. aureus* MurB structure (12) (Protein Data Bank code 1hsk) using a three-dimensional match procedure. The graphic was rendered using POV-Ray 3.6.

**RESULTS AND DISCUSSION**

**Selection of Amino Acid Residues of MurB for Mutational Analyses**—Fig. 2 shows the amino acid sequences for several regions of MurB from a number of bacteria, with boxes enclosing highly conserved residues. X-ray crystal structure analyses of the *E. coli* MurB–UDP-GlcNAcEP complex and *S. aureus* MurB revealed that most of these conserved residues are located close to the cofactor FAD or substrate UDP-GlcNAcEP binding sites (12, 13). In this study, we selected 11 amino acid residues (Fig. 2) that are highly conserved among both type I and II enzymes and are located near the active sites as residues that may be essential for enzyme activity. We examined this by constructing mutants in which the targeted amino acid was replaced with alanine or, in the case of tyrosine residues, with phenylalanine. Gly67, Ser70, Ser226, and Arg213 are located close to the FAD binding site, whereas Tyr217, Arg176, Ser226, Arg230, His259, and Glu296 are located close to the UDP-GlcNAcEP binding site. Both Asn71 and Arg213 are near the enolpyruvyl group of UDP-GlcNAcEP. Ser226 corresponds to Ser229 of *E. coli* MurB, which was reported previously as a catalytic residue whose hydroxyl group is thought to transfer a proton for the reduction of UDP-GlcNAcEP (18).
In Vivo Complementation Test of the Mutated murB Genes with a Temperature-sensitive murB Mutant—We next examined the abilities of the mutated murB genes to complement the temperature-sensitive S. aureus murB strain TS2901. The TS2901 strain was electroporated with plasmids harboring the murB genes, and the transformants were counted (6). Wild-type murB gene formed a similar number of transformants at 30 and 43 °C, whereas the empty vector plasmid could not form transformants at 43 °C (Table 2). The number of transformants at 43 °C was less than 1% of that at 30 °C for the N71A, Y175F, R176A, R213A, S226A, H259A, and E296A mutated murB genes. On the other hand, similar numbers of transformants were formed at 30 and 43 °C by the G67A, G69A, S70A, and R230A mutants. Thus, it appears that the N71A, Y175F, R176A, R213A, S226A, H259A, and E296A mutants lose MurB activity, whereas G67A, G69A, S70A, and R230A retain the activity.

Gly67 and Gly69 comprise a Gly-X-Gly motif, which is a conserved sequence in the FAD-binding domain of FAD-binding proteins. X-ray structural analysis of S. aureus MurB indicates that Gly67 and Gly69

| murB allele | E. coli residue | Efficiency of transformation |
|------------|---------------|-----------------------------|
|            | 30 °C         | 43 °C | 43 °C/30 °C ratio |
| G67A       | Gly67         | 3.4   | 3.8   | 1.1 |
| G69A       | Gly69         | 4.1   | 1.9   | 0.94 |
| S70A       | Ser70         | 0.96  | <0.001 | <0.0002 |
| N71A       | Asn71         | 4.8   | <0.001 | <0.0002 |
| Y175F      | Tyr175        | 2.0   | 0.018 | 0.009 |
| R176A      | Arg176        | 3.8   | 0.001 | 0.0002 |
| R213A      | Arg213        | 3.9   | <0.001 | <0.0002 |
| S226A      | Ser226        | 3.9   | <0.001 | <0.0002 |
| R230A      | Asn233        | 4.0   | 3.9   | 0.97 |
| H259A      | Gln259        | 4.9   | <0.001 | <0.0002 |
| E296A      | Glu296        | 0.65  | <0.001 | <0.0001 |
| Wild-type  |               | 3.9   | 5.2   | 1.3 |

*The corresponding residues for the E. coli enzyme are shown.

In Vivo Complementation Test of the Mutated murB Genes with a Temperature-sensitive murB Mutant—We next examined the abilities of the mutated murB genes to complement the temperature-sensitive S. aureus murB strain TS2901. The TS2901 strain was electroporated with plasmids harboring the murB genes, and the transformants were counted (6). Wild-type murB gene formed a similar number of transformants at 30 and 43 °C, whereas the empty vector plasmid could not form transformants at 43 °C (Table 2). The number of transformants at 43 °C was less than 1% of that at 30 °C for the N71A, Y175F, R176A, R213A, S226A, H259A, and E296A mutated murB genes. On the other hand, similar numbers of transformants were formed at 30 and 43 °C by the G67A, G69A, S70A, and R230A mutants. Thus, it appears that the N71A, Y175F, R176A, R213A, S226A, H259A, and E296A mutants lose MurB activity, whereas G67A, G69A, S70A, and R230A retain the activity.

Gly67 and Gly69 comprise a Gly-X-Gly motif, which is a conserved sequence in the FAD-binding domain of FAD-binding proteins. X-ray structural analysis of S. aureus MurB indicates that Gly67 and Gly69...
interact with the α-phosphoryl moiety of FAD by the main-chain nitrogen, and the following residue, Ser271, interacts with the β-phosphoryl moiety of FAD via both its main-chain nitrogen and its side-chain hydroxyl group (12, 13). The fact that both G67A and G69A can complement the temperature-sensitive mutant suggests that the glycine residues of the GXG motif can be replaced with an alanine residue. This result is consistent with the finding that several FAD binding proteins have alanine at glycine positions in the GXG motif (24) and that the main-chain nitrogen of the glycine residues is responsible for the interaction with FAD (12, 13).

**Purification of Mutated MurB Proteins**—To determine the roles of the essential residues of the *S. aureus* MurB protein, each mutated MurB that was inactive for in vivo complementation was expressed in *E. coli* and purified to homogeneity. To follow the purification of wild-type MurB, we measured the specific activity for UDP-GlcNAcEP reductase under aerobic conditions (Table 3). On the last step of column chromatography (monoQ), the MurB proteins eluted with peaks of both $A_{280}$ and $A_{340}$. The final fraction (Fraction IV) of each mutated MurB protein contained a single 34-kDa band on SDS-polyacrylamide gel electrophoresis (Fig. 3), which is consistent with the molecular mass of *S. aureus* MurB. The purity of all of the isolated MurB proteins was estimated to be greater than 80%.

**UDP-GlcNAcEP Reductase Activity of Wild-type and Mutated MurB Proteins**—Purified wild-type MurB was examined for UDP-GlcNAcEP reductase activity under aerobic conditions. NADPH oxidation by wild-type MurB was dependent on the concentrations of both UDP-GlcNAcEP and NADPH (Fig. 4, A and B, respectively). The $K_m$ value for UDP-GlcNAcEP or NADPH was 53 or 4.1 μM, respectively, which is similar to that of *E. coli* MurB (14). Production of UDP-N-acetylmuramic acid in the reaction was confirmed by HPLC analysis with chemically characterized UDP-N-acetylmuramic acid as a standard (data not shown).

Next, we examined the ability of the mutant proteins to catalyze UDP-GlcNAcEP-dependent NADPH oxidation under anaerobic conditions, because the reoxidation of the reduced form of MurB is greatly reduced in the absence of oxygen. The wild-type MurB enhanced UDP-GlcNAcEP-dependent oxidation of NADPH, but the mutated forms of MurB lacked this increase (Fig. 5). Whereas the UDP-GlcNAcEP-dependent oxidation of NADPH by each mutated MurB protein was below the significant level, we could not deny the possibility that the background amount, that is the NADPH oxidation in the absence of UDP-GlcNAcEP, hid the small activities of any proteins to oxidize NADPH in a UDP-GlcNAcEP-dependent manner.

**UDP-GlcNAcEP-independent Oxidation of NADPH by Mutated MurB Proteins**—MurB catalyzes the two reaction steps; the first is the reduction of FAD by NADPH, and the second is the electron transfer from FADH$_2$ to UDP-GlcNAcEP (Fig. 1). Under aerobic conditions, one can examine the first half of the reaction, because the reduced MurB is reoxidized by molecular oxygen, allowing UDP-GlcNAcEP-independent NADPH oxidation to proceed (18, 25). Hence, we examined the activity of the mutated *S. aureus* MurB proteins under aerobic conditions. We found that all of the mutated MurB proteins catalyzed the oxidation of NADPH under aerobic conditions (Fig. 6). The $K_m$ for NADPH was 38 μM for wild-type MurB and 3.3–36 μM for mutated proteins (Table 4), suggesting that the mutant MurB proteins do not show a loss of affinity for NADPH. Under these conditions, the rate of NADPH oxidation by the free form of FAD in the presence of 150 μM NADPH was 0.19 mmol/min/mol of FAD, which is much lower than the rates catalyzed by the mutant MurB proteins (Table 4).

When NADPH reduces MurB-bound FAD to FADH$_2$, the yellow color of the oxidized form of MurB is immediately bleached (23). We also found that the yellow color of the oxidized form of the mutant and wild-type MurB protein was reduced by the addition of NADPH under both aerobic and anaerobic conditions (data not shown). These results indicated that the mutant MurB proteins can carry out the first catalytic step, namely the reduction of bound FAD by NADPH (Fig. 1).

**UDP-GlcNAcEP Inhibition of NADPH Oxidation by the Mutant Enzymes**—Both NADPH and UDP-GlcNAcEP have been shown to bind to the same site on MurB, which prevents them from binding at the same time (14). Consistent with this, UDP-GlcNAcEP had a biphasic

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**TABLE 3**

| Fraction | Purification step | Total protein | Specific activity$^a$ | Total activity$^a$ | Yield | Purification |
|----------|------------------|---------------|----------------------|-------------------|-------|-------------|
| I        | Cell lysate      | 220           | 0.46                 | 101               | 100   | 1           |
| II       | (NH$_4$)$_2$SO$_4$ precipitate | 44           | 1.2                  | 51                | 50    | 2.6         |
| III      | DEAE-Sepharose   | 15            | 2.1                  | 32                | 32    | 4.6         |
| IV       | MonoQ            | 3.9$^b$       | 4.6                  | 18$^b$            | 18$^b$| 10          |

$^a$ 1 unit = 1 μmol of NADPH oxidized/min.

$^b$ A quarter of fraction III was applied to a monoQ column.

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**FIGURE 3. Purified fraction of wild-type and mutant MurB.** Each MurB (Fraction IV; 2 μg) was analyzed by SDS-polyacrylamide (12.5%) gel electrophoresis and stained with CBB R-250.
effect on the NADPH oxidation by the wild-type protein: stimulatory at low concentrations and inhibitory at high concentrations (Fig. 4A).

Since UDP-GlcNAcEP failed to stimulate the NADPH oxidation by the mutant enzymes (Fig. 5), the mutations might affect the ability of UDP-GlcNAcEP to inhibit NADPH oxidation. To examine this point, we assayed the mutant enzymes under aerobic conditions. As under anaerobic conditions (Fig. 5), the mutant MurB proteins did not stimulate the oxidation of NADPH by UDP-GlcNAcEP under aerobic conditions (Fig. 7). In these experiments, UDP-GlcNAcEP inhibited NADPH oxidation by the N71A, Y175F, S226A, and E296A proteins; however, the inhibitory effect of UDP-GlcNAcEP on NADPH oxidation was much less for the R176A, R213A, and H259A proteins (Fig. 7). These results suggest that the former four mutant proteins have normal affinity for UDP-GlcNAcEP, whereas the latter three have reduced affinity for this ligand. Additionally, the ability of UDP-GlcNAcEP to inhibit NADPH oxidation by the former four mutant proteins supports the idea that UDP-GlcNAcEP and NADPH share a binding pocket in S. aureus MurB.

Absorption Spectrum Analyses for Mutated MurB Proteins—To determine whether the mutant proteins have altered abilities to bind FAD, we evaluated their FAD content and the absorption spectra for bound FAD. Flavin removed from the mutant or wild-type MurB proteins showed fluorescence peaks with the same retention time on HPLC as the FAD standard (data not shown). The amount of FAD in each MurB was determined to be 0.8–1.1 mol/mol of MurB, suggesting that each mutated MurB contains an equivalent amount of noncovalently bound FAD.

Next, the absorption spectra of FAD bound to wild-type and mutant MurB proteins were compared under aerobic conditions, wherein MurB is in an oxidized form. Wild-type MurB showed two characteristic absorption maximums for FAD, one of which was at 462 nm. Other than R213A, all of the mutant MurB proteins had an absorption maximum at 461 or 462 nm. R213A, in contrast, had an absorption maximum at 454 nm, which is noticeably different from the wild type. This result indicates that the electronic state of flavin is significantly different in the R213A and wild-type forms of MurB. In other words, Arg213 may help maintain the electronic state of FAD. This characteristic differentiates R213A from the other mutant MurB proteins.

Difference Spectrum Analysis for Interaction with NADP+—Positively charged residues of MurB are thought to be involved in the interaction with NADP+ (22). We next examined NADP+ binding by the mutant MurB proteins by following the increase in absorbance at 510 nm upon NADP+ binding (22). Difference spectrum for NADP+ binding to wild-type MurB showed a characteristic peak at 510 nm under aerobic conditions (Fig. 8C). The extent of the increase in absorbance increase was dependent on the concentration of NADP+ (Fig. 8A), which is consistent with the characteristics of E. coli MurB. The Kd value for NADP+ for wild-type MurB was calculated to be 16 μM (Table 4). MurB mutants N71A, Y175F, R176A, S226A, and E296A had absorbance increases at 510 nm due to the addition of NADP+ (Fig. 8A). There was less than a 5-fold difference in Kd values for these mutated forms MurB for NADP+ compared with the wild-type enzyme (Table 4). Therefore, oxidized forms of the N71A, Y175F, R176A,
S226A, and E296A mutants retain their affinity for NADPH/H11001. Similarly, E. coli MurB S229A, which corresponds to S. aureus MurB S226A, was previously shown to have an affinity for NADPH/H11001 that is comparable with wild-type MurB (18).

For H259A and R213A, the difference spectra due to the NADPH/H11001 addition were significantly different from that of the wild-type enzyme (Fig. 8, D and E). These mutant MurB proteins did not show difference spectrum peaks at 510 nm (Fig. 8A). This suggests that these mutated MurBs have altered interactions between FAD and NADPH/H11001; specifically, both His259 and Arg213 are required for proper changes in the electronic state of the oxidized form of the MurB upon interaction with NADPH/H11001. Because the R213A mutant has a deficiency in the maintenance of the electronic state of FAD (see "Absorption Spectrum Analyses for Mutated MurB Proteins"), the absence of a difference spectrum peak at 510 nm with NADPH/H11001 is probably due to a secondary effect.

Difference Spectrum Analysis for Interaction with UDP-GlNAcEP—Next, we measured the difference spectra with UDP-GlNAcEP for mutant MurB proteins under aerobic conditions. The difference spectrum for the wild-type protein with UDP-GlNAcEP showed a peak at 510 nm (Fig. 8F). The height of this difference peak depended on the concentration of UDP-GlNAcEP (Fig. 8B). The difference spectrum peak at 510 nm upon UDP-GlNAcEP binding was also observed for N71A, Y175F, S226A, and E296A (Fig. 8B). The \(K_d\) values for UDP-GlNAcEP of these mutated forms of MurB were within 5-fold of the wild-type value (Table 4), suggesting that these mutated MurBs retain their affinity for UDP-GlNAcEP.

In contrast, the difference spectrum for the R176A, R213A, and H259A mutants with UDP-GlNAcEP was significantly different from that of the wild type; these mutants did not show the characteristic peak at 510 nm found with the wild-type enzyme (Fig. 8, G–I). The results suggest that either R176A, R213A, and H259A lose the affinity for UDP-GlNAcEP or they lose the ability to cause a spectrum change for the FAD cofactor upon binding of UDP-GlNAcEP. The former possibility appears to agree with the results shown in Fig. 7, that NADPH oxidation by R176A, R213A, and H259A was poorly inhibited by UDP-GlNAcEP under aerobic conditions. Again, for R213A, the secondary effect caused by a loss of the ability to maintain the electronic state of the FAD might explain the lack of a difference spectrum peak at 510 nm upon the addition of UDP-GlNAcEP.

Identification of Four Groups of Active Site Essential Amino Acids—Based on absorption and difference spectrum analyses, we were able to

### TABLE 4

| MurB   | \(K_m\) NADPH | \(V_{max}\) | \(V_{max}/K_m\) | \(K_m\) NADPH | \(K_d\) UDP-GlNAcEP |
|--------|----------------|-------------|----------------|--------------|------------------|
| Wild type | 38             | 40.5        | 1.0            | 16           | 41               |
| N71A   | 8.0            | 91.8        | 11.4           | 20           | 12               |
| Y175F  | 9.5            | 15.7        | 1.6            | 27           | 173              |
| R176A  | 23             | 16.7        | 0.7            | 43           | —                |
| R213A  | 36             | 13.3        | 0.3            | 73           | 180              |
| S226A  | 3.3            | 34.8        | 10.5           | 73           | 180              |
| H259A  | 24             | 36.8        | 1.5            | —            | —                |
| E296A  | 14             | 77.5        | 5.5            | —            | —                |

\(a\) \(K_m\), \(V_{max}\), and \(V_{max}/K_m\) for the NADPH oxidase activity in the absence of UDP-GlNAcEP under aerobic conditions have been calculated from Fig. 6.

\(b\) \(K_d\) values for NADPH/H11001 and UDP-GlNAcEP were calculated from Fig. 8.

\(c\) The \(K_d\) value could not be determined by a difference spectrum.
separate the mutant MurB proteins into four categories. The first includes R213A. In this study, the absorption maximum at 462 nm for FAD in the wild-type MurB protein shifted to 454 nm in R213A. Furthermore, R213A did not show a characteristic increase at 510 nm in the difference spectrum upon binding either NADP\(^+\) or UDP-GlcNAcEP. These results suggest that the electronic state of the isoalloxazine ring of FAD is disturbed in R213A. This means that Arg\(^{213}\) is important for maintaining the electronic state of the isoalloxazine ring of FAD, which...
Four Groups of Active Site MurB Mutants

is essential for the UDP-GlcNAcEP reducing activity of MurB. This finding is consistent with suggestion that the side-chain guanidinium of Arg213 makes a hydrogen bond with the isoalloxazine ring of FAD (12, 13) (Figs. 9 and 10). Alternatively, the loss of the interaction between Arg213 and the isoalloxazine ring of FAD might change the intramolecular location of FAD in MurB, which makes hydride transfer from FADH₂ to the enolpyruvyl moiety of UDP-GlcNAcEP impossible.

The second category includes R176A, for which the inhibitory effect of UDP-GlcNAcEP on NADPH oxidation was greatly reduced and whose interaction with UDP-GlcNAcEP could not be detected by difference spectrum analysis. These results suggest that Arg176 plays an important role in the binding of the substrate, UDP-GlcNAcEP. It should be emphasized that Arg176 is critical for interaction with UDP-GlcNAcEP but not with NADP⁺, although the two ligands are thought to share the same binding pocket. X-ray structural analysis of E. coli MurB revealed that the side-chain guanidinium of Arg159, which corresponds to Arg176 in S. aureus MurB, forms a hydrogen bond with the substrate’s enolpyruvyl moiety. This interaction is thought to contribute to the formation and stabilization of the reaction intermediate (13) (Figs. 9 and 10). Therefore, the inability to make this hydrogen bond in R176A might explain its loss of affinity for and ability to reduce UDP-GlcNAcEP.

The third category includes H259A. Difference spectrum analyses of H259A showed an abnormality in the interaction with both UDP-GlcNAcEP and NADP⁺. In addition, H259A diminished the inhibitory effect of UDP-GlcNAcEP on NADPH oxidation under aerobic conditions. Thus, it appears that His259 is important for interaction with both UDP-GlcNAcEP and NADP⁺. This is consistent with the previous idea that UDP-GlcNAcEP and NADP⁺ share the same binding pocket on MurB. These results are the first to identify residues that are crucial for the interaction of MurB and its two substrates, UDP-GlcNAcEP and NADP⁺. His259 of S. aureus MurB corresponds to Gln288 of E. coli MurB, which forms a hydrogen bond with a water molecule in the active site. This water, in turn, interacts with the catalytic Ser229 of E. coli

FIGURE 9. Essential residues in the active site of S. aureus MurB. Essential residues and FAD were mapped on the x-ray structure of S. aureus MurB, which was superimposed with the structure of UDP-GlcNAcEP that was obtained from the structure of the E. coli MurB-UDP-GlcNAcEP complex.

FIGURE 10. Schematic representation of the second half of the reaction mediated by MurB. Shown are the interactions of FAD or substrate UDP-GlcNAcEP with essential residues of MurB as determined in this study. The number in parentheses represents the amino acid residue of E. coli MurB. Predicted hydrogen bonds are indicated by dotted lines. M⁺ indicates the location of the cationic ion based on a previous report (13) and assumes the presence of a water molecule.
MurB (Ser226 in S. aureus MurB) and provides it with a proton after it donates a proton to the enolpyruvyl moiety of the substrate during the second half of the reaction (13, 17) (Fig. 10). Thus, it is likely that H259A is unable to reduce UDP-GlcNAcEP due to loss or altered location of this important water molecule.

The fourth category includes N71A, Y175F, S226A, and E296A, which appear to retain the affinity for both NADP$^+$ and UDP-GlcNAcEP because difference spectrum analyses show that the $K_d$ values for each ligand do not differ much from the wild-type values. These four mutant MurB proteins were able to carry out the first step of the MurB reaction because they had the ability to oxidize NADPH and mediate NADPH-dependent intramolecular reduction of FAD to FADH$_2$. However, although the mutated proteins could bind UDP-GlcNAcEP, they could not reduce it. The hydroxyl group of Ser226 is thought to transfer a proton to reduce UDP-GlcNAcEP (17, 18). Our finding that S. aureus S226A MurB loses the activity to reduce UDP-GlcNAcEP without a severe effect on the substrate interaction is similar to the effects of the S229A mutation on E. coli MurB and suggests that these two residues play the same role in MurB (Fig. 10).

The side-chain oxygen of Asn71 has been shown to make a hydrogen bond with a water molecule that is critically involved in stabilization of the reaction intermediate during proton transfer from FADH$_2$ (13) (Fig. 10). Therefore, the defect in the second half-reaction in N71A might be due to dislocation of the water molecule. This agrees with previous structural analysis of E. coli S226A (17), which also loses an active site water molecule that is critical for supporting the protonation of the enolpyruvyl moiety of UDP-GlcNAcEP. Also, x-ray structural analysis suggested that the main-chain nitrogen of Asn71 of S. aureus MurB makes a hydrogen bond with the α-phosphate of FAD (12). Our finding that N71A contains a stoichiometric amount of FAD and has an absorption spectrum that is indistinguishable from the wild-type enzyme can be explained by the presence of a hydrogen bond between the main-chain nitrogen of the substituted alanine residue and the α-phosphate of FAD.

Structural analysis has suggested that the proton from the protonated site direction as the substrate, UDP-GlcNAcEP. This is due to the conformation of MurB and dynamic changes upon substrate binding, especially in the substrate binding domain, have been suggested by both x-ray and NMR analyses (12, 15, 16).

Structural analysis has shown that Tyr175 of S. aureus MurB forms a hydrogen bond with the N-acetyl moiety of UDP-GlcNAcEP (12, 13) (Figs. 9 and 10). Thus, our present results, in which Y175F lost the ability to reduce UDP-GlcNAcEP, suggest that the hydrogen bond between Tyr175 and the N-acetyl moiety of the substrate is important for the suitable positioning of the enolpyruvyl moiety so that the proton can be transferred from FADH$_2$. Structural analysis of the crystal of the Y175F-UDP-GlcNAcEP complex is needed to verify this prediction.

**Conclusion**—Here, we selected 11 residues that are possibly essential for S. aureus MurB activity. These residues were selected because sequence alignment showed they are highly conserved in other bacterial MurB proteins and because x-ray structural analyses have shown that they interact with the substrate UDP-GlcNAcEP or the cofactor FAD. In the present study, each residue was replaced with alanine or phenylalanine, and the resulting mutant genes were examined for their ability to complement the temperature-sensitive growth of a murB mutant of S. aureus. Seven of the 11 mutated proteins were found to be essential for in vivo activity and were further purified and biochemically characterized. These studies identified six residues that are essential for S. aureus MurB both in vivo and in vitro: Asn7$^1$, Tyr175, Arg176, Arg213, His259, and Glu296, in addition to Ser226, which was previously reported as essential for the activity of E. coli MurB. The results described here support the molecular reaction mechanism predicted by x-ray structure analyses (Fig. 10). This study also identified amino acid residues crucial for the interaction with NADP$^+$ and UDP-GlcNAcEP and further classified them into four categories; the first has a defect in the FAD-bound state, the second has a defect in UDP-GlcNAcEP binding, the third has a defect in the interaction with both UDP-GlcNAcEP and NADP$^+$, and the last is essential for catalysis of the second half of the reaction but does not affect the interactions with substrates. Because MurB is an attractive target for antibiotics, the enzymological data obtained here is expected to contribute to development of antibacterial drugs.

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**REFERENCES**

1. van Heijenoort, J. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magruder, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) 2nd ed., pp. 1025–1034, American Society for Microbiology, Washington, D. C.

2. Hölte, J. V. (1998) *Microbiol. Mol. Biol. Rev.* 62, 181–203.

3. Navarre, W. W., and Schneewind, O. (1999) *Microbiol. Mol. Biol. Rev.* 63, 174–229.

4. Miyakawa, T., Matsuzawa, H., Matsuhashi, M., and Sugino, Y. (1972) *J. Bacteriol.* 112, 950–958.

5. Rowland, S. L., Erington, J., and Wake, R. G. (1995) *Gene* (Amst.) 164, 113–116.

6. Matsuo, M., Kurokawa, K., Nishida, S., Li, Y., Takimura, H., Kato, C., Fukuhara, N., Maki, H., Miura, K., Murakami, K., and Sekizumi, K. (2003) *FEBS Microbiol. Lett.* 222, 107–113.

7. El Zoehly, A., Sanschagrin, F., and Levesque, R. C. (2003) *Mol. Microbiol.* 47, 1–12.

8. Gotz, F. (2004) *Curr. Opin. Microbiol.* 7, 477–487.

9. Anderson, C. J., Bronson, J. J., D’Andrea, S. V., Deshpande, M. S., Falk, P. J., Grant, Young-K. A., Harte, W. E., Ho, H. T., Misco, P. F., Robertson, J. G., Stock, D., Sun, Y., and Walsh, A. W. (2000) *Bioorg. Med. Chem. Lett.* 10, 715–717.

10. Bronson, J. J., DenBleyker, K. L., Falk, P. J., Mate, R. A., Ho, H. T., Pucci, M. J., and Snyder, L. B. (2003) *Bioorg. Med. Chem. Lett.* 13, 873–875.

11. Murzin, A. G. (1996) *Curr. Opin. Struct. Biol.* 6, 386–394.

12. Benson, T. E., Harris, M. S., Choi, G. H., Cialdella, J. I., Herberg, J. T., Martin, J. P., Jr., and Baldwin, E. T. (2001) *Biotechnology* 40, 2340–2350.

13. Benson, T. E., Filman, D. J., Walsh, C. T., and Hogle, J. M. (1995) *Nat. Struct. Biol.* 2, 644–653.

14. Dhalla, A. M., Yanchunas, J. Jr., Ho, H. T., Falk, P. J., Villafranca, J. J., and Robertson, J. G. (1995) *Biochemistry* 34, 5390–5402.

15. Farmer, B. T., II, Constantine, K. L., Goldenfarb, V., Friedrichs, M. S., Wittekind, M., Yanchunas, J. Jr., Robertson, J. G., and Mueller, L. (1996) *Nat. Struct. Biol.* 3, 995–997.

16. Constantine, K. L., Mueller, L., Goldenfarb, V., Wittekind, M., Metzler, W., J., Yanchunas, J,
Four Groups of Active Site MurB Mutants

Jr., Robertson, J. G., Malley, M. F., Friedrichs, M. S., and Farmer, B. T., II (1997) J. Mol. Biol. 267, 1223–1246
17. Benson, T. E., Walsh, C. T., and Hogle, J. M. (1997) Biochemistry 36, 806–811
18. Benson, T. E., Walsh, C. T., and Massey, V. (1997) Biochemistry 36, 796–805
19. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., pp. 13.36–13.39, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Anwar, R. A., and Vlaovic, M. (1979) Can. J. Biochem. 57, 188–196
21. Englander, S. W., Calhoun, D. B., and Englander, J. J. (1987) Anal. Biochem. 161, 300–306
22. Axley, M. J., Fairman, R., Yanchunas, J., Jr., Villafranca, J. J., and Robertson, J. G. (1997) Biochemistry 36, 812–822
23. Benson, T. E., Marquardt, J. L., Marquardt, A. C., Etzkorn, F. A., and Walsh, C. T. (1993) Biochemistry 32, 2024–2030
24. Fraaije, M. W., Van Berkel, W. J., Benen, J. A., Visser, J., and Mattevi, A. (1998) Trends Biochem. Sci. 23, 206–207
25. Taku, A., Gunetileke, K. G., and Anwar, R. A. (1970) J. Biol. Chem. 245, 5012–5016
26. Bross, P., Engst, S., Strauss, A. W., Kelly, D. P., Rasched, I., and Ghisla, S. (1990) J. Biol. Chem. 265, 7116–7119
27. Becker, D. F., Fuchs, J. A., Banfield, D. K., Funk, W. D., MacGillivray, R. T., and Stankovich, M. T. (1993) Biochemistry 32, 10736–10742
28. Dwyer, T. M., Rao, K. S., Goodman, S. I., and Freeman, F. E. (2000) Biochemistry 39, 11488–11499
29. van den Heuvel, R. H., Fraaije, M. W., Mattevi, A., and van Berkel, W. J. (2000) J. Biol. Chem. 275, 14799–14808