ABSTRACT: The wide use of the antimicrobial agent/biocide, triclosan, promotes triclosan-resistant bacterial strains, including *Staphylococcus aureus*, as well as leads to accumulation in the aquatic and terrestrial environments. Knowledge of the molecular actions of triclosan on *S. aureus* is needed to understand the consequence of triclosan resistance and environmental accumulation of triclosan on *S. aureus* resistant strains, as well as to develop biphenyl ether analogs as antibiotic candidates. Triclosan inhibits an essential enzyme in the fatty acid biosynthetic pathway, the reduced nicotinamide adenine dinucleotide (NADH)/reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enoyl-acyl carrier protein (enoyl-ACP) reductase, or FabI. In this study, we used error-prone polymerase chain reaction (epPCR) to generate mutations in the *S. aureus* FabI enzyme. Instead of using an elaborate FabI enzyme activity assay that involves ACP-linked substrates to determine whether triclosan inhibits the enzyme activities of individual FabI mutants, we used an efficient and economical assay that we developed, based on thermal shift principles, to screen for triclosan binding to FabI mutants in cells. We identified four active-site mutations. More interestingly, we also identified nine triclosan-resistant mutations distant from the active site (G113V, Y123H, S166N, N220I, G227C, A230T, V241I, F252I, and H253P) but located in disparate positions in the monomer and dimer interface regions in *S. aureus* FabI. We suggest that these sites may serve as potential allosteric sites for designing potential therapeutic inhibitors that offer advantages in selectivity since allosteric sites are less evolutionarily conserved.

INTRODUCTION

Up to 30% of the human population is colonized with nasal *Staphylococcus aureus*, and nasal colonization can cause opportunistic and sometimes life-threatening infections. The perturbation of cutaneous and mucosal barriers enables *S. aureus* to migrate into underlying tissues and/or the bloodstream to cause infection. Clinical manifestations of *S. aureus* infections include bacteremia, endocarditis, osteomyelitis, and skin and soft tissue infections. Triclosan (5-chloro-2-(2,4-dichloro-phenoxy) phenol; C12H7Cl3O2), an antimicrobial agent/biocide, was originally used in hospital settings as a disinfectant to remove bacterial colonies, including *S. aureus*, on skin and often used on surfaces in the 1970s, and was incorporated into a variety of consumer products including soaps, hand sanitizers, toothpaste, and mouthwash. The ubiquitous use of triclosan induces bacterial resistance to triclosan, leading to the ban of triclosan in household cleansing products by the FDA in 2016. The physicochemical properties lead to the bioaccumulation and persistence potential of triclosan in the aquatic and terrestrial environment (10^{-6}−10^{-8} g/L in soil and municipal sewage) as well as in body fluids (e.g., a mean value of 13 μg/L in urine). Since triclosan and its biphenyl ether analogs have broad-spectrum activity against Gram-positive and Gram-negative bacteria and some fungi—it is still considered to be an attractive lead for antibiotic development. It provides a potential scaffold for the development of antibiotics targeting the fatty acid biosynthetic pathway, via inhibition of enoyl-acyl carrier protein (enoyl-ACP) reductase. Consequent to the wide variety of resistance mechanisms that *S. aureus* employ to resist antibiotics, new therapies are continuously under development.

The knowledge of the molecular actions of triclosan on *S. aureus* is needed to understand the consequence of environmental accumulation of triclosan on *S. aureus* resistant strains as well as to develop biphenyl ether analogs as antibiotic candidates. The cell growth-inhibitory activities of triclosan are attributed to its inhibition of lipid synthesis by specifically inhibiting an essential enzyme in the fatty acid biosynthetic pathway, the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enoyl-acyl carrier protein reductase (NADH)/reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enoyl-acyl carrier protein reductase.
tase, or FabI. S. aureus cells can develop triclosan resistance through multiple means including active-site mutations in FabI, promoter region mutations to increase FabI expression, horizontal gene transfer, and formation of small-colony variants. S. aureus FabI (SaFabI) is a dimer with two identical chains in the apo form, and upon binding triclosan (with NADP+), the two dimers interact to form a tetramer.

In this study, error-prone polymerase chain reaction (epPCR) was used to provide random mutations in SaFabI, and mutants with point mutations throughout SaFabI were selected for triclosan binding studies. A cellular thermal shift assay, originally developed to monitor drug target engagement in cells and tissues, was used to monitor triclosan binding to SaFabI (WT and mutants) in cells by monitoring SaFabI in cells quantitatively under different conditions.

Not surprisingly, we found four active-site mutants that exhibited a reduced affinity for triclosan binding at 0.1 mM, a concentration that showed triclosan binding to WT SaFabI. However, we also found 10 nonactive-site mutants that exhibited a reduced affinity for triclosan binding. Nine of these mutations were at either the monomer–monomer or the dimer–dimer interface regions of the SaFabI molecule. The results of this study provide further understanding at a molecular level to enhance the design of inhibitors, including allosteric inhibitors, that may overcome triclosan-resistant S. aureus strains and possibly resistance in other species that share similar FabI structures. The results further provide under-

Table 1. Ratio (%) of Band Intensities of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Results of SaFabI in E. coli Cells at 65 °C to that at 37 °C with Different Triclosan Concentrations

| triclosan | intensity at 65 °C/intensity at 37 °C (%) | mutation location | predicted mutation effect |
|-----------|-----------------------------------------|------------------|-------------------------|
|           | 0X^c                                  |                  |                         |
|           | 1X^d                                  |                  |                         |
|           | 10X^e                                 |                  |                         |
| WT        | avg SD                                | avg SD           | avg SD                  |
|           | 2.6 1.6                               | 84.6 17.7        | 123.5 15.0              |
| 1         | group mutants                         |                  |                         |
| M1L       | 2.5 0.1                               | 72.1 9.8         |                        |
| A33V      | 3.7 0.5                               | 98.7 0.4         |                        |
| L35F      | 3.9                                   | 92.7             |                        |
| E42D      | 2.0 0.4                               | 85.7 0.9         |                        |
| K46N      | 3.9 3.2                               | 91.3 9.5         |                        |
| E49R      | 4.0 0.6                               | 96.8 1.9         |                        |
| N56H      | 3.3 3.4                               | 96.9 3.1         |                        |
| N56K      | 2.6 1.6                               | 74.6 0.3         |                        |
| P58Q      | 4.0 0.2                               | 67.3 8.2         |                        |
| A60V      | 3.9 2.4                               | 88.1 8.2         |                        |
| E71D      | 2.4 0.3                               | 79.3 6.0         |                        |
| F77L      | 2.4 0.6                               | 83.5 15.1        |                        |
| Q79R      | 4.1 1.1                               | 97.8 1.1         |                        |
| V64L      | 3.9 1.0                               | 90.1 13.0        |                        |
| N98Y      | 2.2 0.1                               | 89.2 8.8         |                        |
| M99E      | 4.1 0.4                               | 92.4 5.2         |                        |
| V171A     | 2.6 1.2                               | 93.9 111.3       | QR-4^g                  |
| N182T     | 3.1 1.3                               | 98.4 13.8        |                        |
| K199R     | 3.5 0.5                               | 79.8 0.9         |                        |
| G200D     | 3.9 1.3                               | 88.4 135.4       |                        |
| E212G     | 2.9 1.7                               | 73.1 6.5         |                        |
| 2A        | R45P                                  | 3.5 1.0          | 4.7 1.5                 |
|           | D66Y                                  | 3.4 2.2          | 3.2 21.2                |
|           | N220I                                 | 3.2 2.3          | 6.2 132.0               |
|           | A230T                                 | 3.5 0.3          | 11.2 2.7               |
|           | V241I                                 | 3.6 1.6          | 24.3 13.0              |
| 2B        | F252I                                 | 4.1 1.2          | 10.1 1.9               |
|           | A95V                                  | 2.7 1.0          | 4.1 3.8                |
|           | G113V                                 | 3.8 2.7          | 6.6 3.1                |
|           | Y123H                                 | 3.7 1.1          | 6.3 4.3                |
|           | K164R                                 | 4.1 2.8          | 8.3 5.7               |
|           | S166N                                 | 3.2 0.6          | 2.9 0.6                |
|           | F204S                                 | 2.9 0.0          | 2.3 5.5                |
|           | G227C                                 | 4.2 1.1          | 5.8 1.7               |
|           | H253P                                 | 4.0 1.7          | 5.5 5.5               |

Note: No triclosan. 0.1 mM triclosan + 0.2 mM NADP+. 1 mM triclosan + 2 mM NADP+. A residue not in the active site or interface. No SD, n = 1. Values >0.1 not listed. Dimer–Dimer interface residue. Active-site residue. Monomer–Monomer interface residue.
standing of the consequence of environmentally accumulated triclosan on *S. aureus* resistant strains.

## RESULTS

**SaFabI Mutations from Error-Prone PCR.** A total of 219 colonies were obtained from the error-prone PCR (epPCR) product (mutated genes of SaFabI), ligated to a pET-15b plasmid and transformed into *Escherichia coli* cells. In these colonies, DNA sequence analysis results indicated that there were 22 A→C, 91 A→T, and 125 A→G mutations to give a total of 238 A mutations. Similarly, there were a total of 122 G, 187 T, and 67 C mutations to give a total of 614 nucleotide mutations. Out of the 219 colonies, 42 colonies consisted of single amino acid mutation in SaFabI protein, while 33 colonies had double-residue mutations. The remaining colonies consisted of SaFabI with multiple, up to six, mutations, frame shifts, and multiple-residue deletions, as well as silent mutations. We found 326 mutations in 191 residues in SaFabI throughout the length of this 256-amino-acid-residue protein. We focused on the 35 of these 42 single-mutation SaFabI mutants (See Materials and Methods section for not studying 7 of the 42 mutants).

The results of mass spectrometry analysis of 14 randomly selected mutants (A33V, L35F, R45P, F77L, A95V, N98Y, M99I, N182T, K199R, G200D, F204S, E212G, V241I, and F227C) from the 35 mutants listed in Table 1 shown each mass to be within 1 Da of the expected mass calculated from its sequence information. Although this work was done in *E. coli* cells, we also demonstrated that the WT and a mutant (A33V) protein not only was expressed in *E. coli* cells but also in *S. aureus* cells (see Materials and Methods section), as demonstrated by gel results (data not shown).

**Quantitation of WT SaFabI in Cells without and with Triclosan at Different Temperatures.** For the samples of WT SaFabI in cells at 37 °C (see Materials and Methods section), the overexpressed SaFabI at ∼34 kDa was a dark band, as shown in a representative sample (Figure 1, panel A, lane 1) with an intensity of 0.9 (see Materials and Methods section for the intensity unit), indicating that a specific amount of WT SaFabI was extracted from the cells as a soluble protein at 37 °C. The average value of the ratio for repeated runs (n = 13) was 1.1 ± 0.4, with individual values ranging from 0.6 to 2.0.

The gel band of the same representative cell sample, but heated to 65 °C, was hardly visible (Figure 1, panel A, lane 3), with an intensity of only 0.02 (or 2.2% of that at 37 °C) for this particular sample, indicating that SaFabI in cells was hardly soluble at 65 °C, as expected.28 The average intensity value of 13 runs at 65 °C was 0.03 ± 0.01 (or 2.6 ± 1.6% of that at 37 °C, with values ranging from 0.5 to 5.9%) (Table 1, WT, 0 °C).

In contrast, for WT samples treated with triclosan at 1× concentration (0.1 mM), bands were observed for samples at both 37 and 65 °C. At 37 °C, the intensity value of the dark band for a representative sample was 0.7 (Figure 1, panel A, lane 4), with an average value of 0.6 ± 0.2 (individual values ranging from 0.3 to 0.9; n = 11). At 65 °C, the intensity value was 0.5 for the representative sample (Figure 1, panel A, lane 4), with an average value of 0.5 ± 0.2. As reported earlier,28 WT SaFabI pure protein was stabilized by triclosan binding, and the temperature for 50% unfolding was increased from 40 to 60 °C. The average band intensity at 65 °C was 84.6 ± 17.7% (n = 11) of that at 37 °C (Table 1, WT, 1× triclosan). These values for the WT (control) samples set the standard for the mutant samples under the same sample conditions.

**SaFabI Mutants in Cells without and with Triclosan at Different Temperatures.** At 37 °C, all cells with SaFabI mutants, without and with triclosan, exhibited dark bands in gels, similar to those of WT under the same condition, as shown in gels of representative samples (Figure 1) of F77L (panel B), V241I (panel C), and G227C (panel D) with lane 1 without triclosan (0×) and lane 2 with triclosan (1×).

At 65 °C, all mutant samples without triclosan also exhibited results that were similar to those of WT, with little or no visible bands observed (Representative gels in Figure 1, lane 3 in panels B, C, and D). For samples with triclosan at 0.1 mM (1×), most of the samples were similar to those of the WT

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**Figure 1.** SDS polyacrylamide (12%) gel electrophoresis (PAGE) results for soluble SaFabI, as a band at ~34 kDa and indicated by an arrow in the left-hand side of panel A, in different *E. coli* cells, each with an overexpressed SaFabI WT or mutant protein. Controlled (equal) amount of cells in lysogeny broth (LB) medium with dimethyl sulfoxide (DMSO) (1% final concentration) were treated without (0×) or with (1×) 0.1 mM triclosan plus 0.2 mM NADP+ and incubated at 37 or 65 °C for 3 min. For cells incubated at 37 °C, lane 1 (0×) and lane 2 (1×) of all four panels, WT (panel A), F77L (panel B), V241I (panel C), and G227C (panel D), showed similar patterns, both in mobility and in intensity (quantitative intensity data in Table 1). These results suggested that all cells at 37 °C contained soluble/stable SaFabI protein. However, for cells incubated at 65 °C, the patterns varied. In the WT samples, no band at 34 kDa was observed in the sample without triclosan (0×) (panel A, lane 3), suggesting that the previously soluble WT protein had become insoluble/unstable at 65 °C. However, in the sample with 1× triclosan (panel A, lane 4), a sizable band was observed, suggesting that triclosan stabilized the WT SaFabI protein to give a soluble form in the cells. For F77L (panel B), lanes 3 and 4 were each similar to the corresponding ones in the WT samples (panel A). For V241I (panel C), no band was observed in the sample without triclosan (lane 3), and only a faint band was observed in the sample with 1× triclosan (lane 4), suggesting that only a small amount of V241I was stabilized by triclosan. For G227C (panel D), no band was observed in both samples without (Lane 3) and with (Lane 4) triclosan, suggesting that G227C was insoluble/unstable at 65 °C even with the addition of 0.1 mM triclosan.
under the same condition. The average band intensity value for these mutant samples, relative to those at 37 °C, was around 85%, the value reported above for WT samples (Table 1). The values for individual mutants ranged from 72.1 to 98.4% (Table 1). We designated these mutants as group 1 mutants, with triclosan binding to SaFabI mutants at 0.1 mM, similar to those of WT.

However, at 65 °C with triclosan at 0.1 mM (1×), 14 SaFabI mutants exhibited gel band intensity less than that of WT under the same condition (Table 1). For example, only faint or hardly visible bands were observed for mutants R45P (Figure 2, panel C, lane 2), G227C (Figure 1, panel D, lane 4 and D66Y exhibited the lowest intensity value (21.2, Table 1) in group 2A but higher than those in group 2B, discussed below.

The remaining eight mutants (A95V, G113V, Y123H, K164R, S166N, F204S, G227C, and H253P) exhibited hardly visible bands even in the presence of a higher concentration of triclosan (1 mM; 10×) at 65 °C, with the band intensity being only about 2−7% of those at 37 °C (Table 1), with the gel of G227C shown in Figure 2, panel D, lane 3. This group was designated as group 2B, with the intensity value at 65 °C relative to 37 °C ranging from 2.3 to 7.0%. These mutants did not show significant binding of triclosan even at 1 mM.

**SaFabI Mutation Sites and Triclosan Association.** Our results showed that the 21 mutants that exhibited triclosan affinity similar to that of WT were all non-active-site mutations, but eight were conserved-residue mutations (M1L, A33V, L35F, M99I, V171A, K199R, G200D, and E212G), as predicted by SIFT20 (Table 1).

For the 14 triclosan-resistant mutants (groups 2A and 2B), all but one (V241I) were of conserved residues that scored below 0.1 by Sorting Intolerant from Tolerant (SIFT), predicting deleterious function. We identified four active-site mutations (D66Y in group 2A and A95V, K164R, and F204S in group 2B). The mutation site of R45P was near the active site (Figure 3A). Interestingly, the remaining nine triclosan-resistant mutants (G113V, Y123H, S166N, N220I, G227C, A230T, V241I, F252I, and H253P) were distant from the active site. Seven of these mutations were either at the QR or the PR interface, with the interface residues defined previously22 (Table 1 and Figure 3A). The remaining two mutations, N220I and V241I, appeared to be near the PR interface, as shown in the apo-SaFabI structure (PDB code: 3GNT) (Figure 3B).

**DISCUSSION**

**Direct Detection of SaFabI and Triclosan Association in Cells.** To determine the SaFabI mutation effect on triclosan binding, many studies monitor the triclosan minimum inhibitory concentration (MIC) of cells with SaFabI mutants.18 However, as indicated earlier, S. aureus cells develop triclosan resistance through multiple means. Having lowered triclosan susceptibility (higher MIC values) is not necessarily related to mutation in SaFabI. Recent studies have shown that when S. aureus cells are treated with sublethal concentrations of triclosan, certain populations transform into small-colony variants, with upregulated expression of FabI and/or efflux pumps.30−33 A method directly measuring SaFabI binding of triclosan in cells provides more specific molecular information about the interactions between the protein and the inhibitor. Generally, this is done with the FabI enzyme activity assays with and without triclosan.19,34,35 Since the substrates are linked to acyl carrier protein (ACP), the assays are challenging due to the need to prepare the ACP and link it to the substrates. Consequently, screening for triclosan-resistant mutants is challenging. Based on cellular thermal shift assay method,37 we used the simple thermal shift assay that we developed,38 which does not use ACP-linked substrates, to screen for triclosan-resistant mutants in this study. Since each individual protein exhibits a characteristic thermal unfolding profile in solution, the unfolded proteins at a specific elevated temperature form aggregates and are no longer soluble in the solution. Ligand binding stabilizes proteins thermodynamically, shifts the thermal unfolding profiles to a higher temperature to allow proteins to remain in the solution at the elevated
temperatures. In this study, the gel band intensity of well-controlled samples provided semiquantitative information on triclosan affinity toward SaFabI mutants.

**Mutation Effect on SaFabI Stability.** In general, mutations often affect the propagation of structural/dynamical information throughout the protein backbone, which sometimes translate into significant conformational changes, and sometimes into subtle variations of vibrational patterns. Both can seriously affect ligand binding. In this study, all 35 singly mutated SaFabI mutants generated by the error-prone PCR method, regardless of their ability to associate with triclosan, showed similar thermal denaturation behavior (gel band intensity) when compared with WT SaFabI under the same experimental conditions at both 37 and 65 °C, suggesting that these mutations did not affect the gross, overall structures of the SaFabI mutants to cause different unfolding behavior. We assumed that some mutants obtained from the error-prone PCR runs that were not included in the study (such as ESK, see Materials and Methods section) might have structures more drastically altered to give unstable proteins in cells even at 37 °C.

**Mutation Effect on SaFabI and Triclosan Association.** In the presence of triclosan (with NADP⁺), we found 21 mutants (group 1; Table 1) with protein stability similar to WT SaFabI when compared under the same experimental conditions. Thus, these mutants were associated with triclosan, at 0.1 mM, with affinities similar to that of WT SaFabI. Thus, they were not triclosan-resistant mutants. Some of these 21 mutations involved conserved residues, such as A33V, L35F, M99I, V171A, K199R, G200D, and E212G, and were predicted by SIFT to be functionally deleterious but exhibit triclosan association similar to that of the WT. Not surprisingly, none of the mutation sites of these mutants were in the active site, defined previously as the residues involved in triclosan and/or NADP⁺ binding.

Among the 14 triclosan-resistant mutants identified, four were at the active site: D66Y, A95V, K164R, and F204S. At 10-fold higher triclosan concentration (1 mM), the mutant D66Y exhibited some triclosan association whereas A95V, K164R, and F204S remained triclosan resistant. Mutations of A95 and F204 residues are well known to cause triclosan resistance (for example, refs 18, 19, 21, 33, 36). It has been shown that the F204C mutant from clinical isolates negates the ability of triclosan to form a stable NADP⁺-FabI-triclosan complex. D66Y and K164R have not previously been reported to be triclosan-resistant mutations. Other active-site mutations, such as T195A, A198G, and F204V/C, are found in clinical isolates.

The most interesting findings in this study were the 10 non-active-site mutants—R45P, G113V, Y123H, S166N, N220I, G227C, A230T, V241I, F252I, and H253P—that resisted triclosan association at 0.1 mM. Furthermore, among these 10 mutants, seven of them—G113V, Y123H, S166N, G227C, A230T, F252I, and H253P—were in the PR (monomer—monomer) and QR (dimer—dimer) interface regions, as defined by published work. These regions have not previously been reported to directly affect triclosan association. Structural studies have indicated that some residues in the interface regions undergo local movements upon binding triclosan, with helices α4/α5 and α6 moving and/or becoming ordered to form a four-helix bundle to define the QR interface during the tetramer formation that occurs with triclosan association. We noted that residue G113 is in the α4 helix, Y123 is in the α5 helix, and S166 is in the α6 helix. G113T21 and G113C53 have been found in triclosan-resistant clinical isolates.

Two of the remaining three triclosan-resistant mutations—N220I and V241I—are not defined as interface residues, but appeared to be near the PR interface (Figure 3B). The only non-active-site and noninterface mutant—R45P—is located near an active-site residue (S44).

We noted that the V171A mutant, with a mutation at the QR interface, was not a triclosan-resistant mutant (Table 1). This mutation, though at the interface, was from V to A and probably did not affect specific interactions between residues in the PR and QR interface regions, whereas the nine triclosan-resistant mutations in the interface regions changed specific
interactions. In the PR interface region, A230 and G227 residues are in a hydrophobic pocket, as shown in the X-ray structure (PDB code: 4ALI).

The mutations A230T and G227C would introduce "hydrophobic pocket clash" (introducing bulky and/or polar side chains) near the mutation sites. The other PR interface mutations F252I and H253P introduced changes in electrostatic interactions, with the F252I mutation causing the loss of charge–dipole interaction with E210/R214/K256, and the H253P mutation causing the loss of salt bridges. The N220 and V241 residues are not identified as interface residues but appeared to be near the PR interface (Figure 3B). The V241 residue is involved in interchain interactions. In the PR interface region, A230 and G227 are likely affected by a bulkyside-chain probably interfering with this interaction. Deteriorated molecular studies of triclosan and each SaFabI mutant will provide quantitative information on the effect of the mutation toward triclosan association.

These triclosan-resistant mutations that were distant from the active site were not clustered in one or two regions in the interface but were spread out in residues in or near the monomer–monomer and dimer–dimer interface regions in the SaFabI molecule. These allosteric sites, of which some might even be "hidden allosteric sites," presumably exert allosteric control over enzyme activity and provide potential sites for designing druglike molecules or pharmaceuticals. The presence of druggable, topographically distinct allosteric sites offers new paradigms for small molecules to inhibit the essential FabI enzyme in S. aureus. It has been stated that molecules that target allosteric sites may offer advantages in selectivity since allosteric sites are less evolutionarily conserved.

Allosteric inhibitors targeting the interface regions may effectively abolish FabI function in S. aureus, as well as those pathogens with FabI structures similar to that of SaFabI.

## CONCLUSIONS

Using error-prone PCR methods, we have identified nine mutations distant from the active site but spread out in monomer–monomer and dimer–dimer interfaces (G113V, Y123H, S166N, N220I, G227C, A230T, V241I, F252I, and H253P) in the FabI enzyme of S. aureus and induce triclosan resistance. We suggest that these sites may serve as potential allosteric sites for designing potential therapeutic inhibitors.

## MATERIALS AND METHODS

### SaFabI Gene Mutation

Error-prone PCR (epPCR) techniques were used to randomly introduce mutations to the WT SaFabI gene, using a commercially available kit with Mutazyme II DNA polymerase (GeneMorph II, Agilent Technology; Santa Clara, CA) and a thermal cycler (Eppendorf AG 22331; Hamburg, Germany). To first determine the number of cycles needed to introduce a sufficient number of mutations in the SaFabI gene, the amount of amplified DNA in each doubling cycle was monitored by gel electrophoresis for epPCR products obtained from various numbers of cycles. Their gel intensities were obtained from a calibration curve constructed with the intensities on gels of known amounts of WT SaFabI gene. Based on the results and the GeneMorph II kit instruction, the epPCR with 25 cycles was used, with 100 ng templates, to give a medium mutation frequency (one to six mutations per individual gene) and 10 μg product.

The epPCR product (mutated SaFabI genes) was ligated to a pET-15b plasmid, an expression vector consisting of a hexahistidine segment at the N-terminal end to give pET-15b-SaFabI mutant plasmids. These plasmids were transformed into DH5α Z-Competent E. coli cells (Zymo; Irvine, CA), and the mixture was plated on ampicillin containing agar plates. Individual colonies were harvested and labeled. Each colony was prepared and stored as freeze-down samples.

### SaFabI Mutation Identification

The pET-15b-SaFabI mutant plasmid was extracted from individual colonies, and the SaFabI mutant gene was sequenced (Sanger sequencing at the University of Illinois at Chicago (UIC) Research Resources Center (RRC)) and compared with the sequence of the WT SaFabI gene. Some of the mutated cells were randomly selected, and their gene products were purified with affinity column chromatography methods for high-resolution mass spectrometry analysis by the UIC RRC to confirm the mutation identities.

### SaFabI-Plasmid in S. aureus Cells

To demonstrate that the WT and mutated SaFabI genes in the plasmid were expressed not only in E. coli cells but also in S. aureus cells, we introduced a shuttle vector pHT370 containing SaFabI WT or A33V genes to give pHT370-SaFabI-WT or -A33V plasmid. The A33V mutation was randomly selected from our mutation list (Table 1). This plasmid pHT370-SaFabI-A33V was introduced to S. aureus RN4220 cells via electroporation.

Cell culture of a single colony was used for overnight culture, and the overnight culture was diluted in fresh Brain heart infusion growth medium and incubated at 37 °C. Gel electrophoresis (see below) was used to monitor SaFabI WT and A33V proteins expressed in S. aureus cells.

### Cellular Thermal Shift Assay

Chemicals. Triclosan (Millipore Sigma, St. Louis, MO) was weighed out and dissolved in DMSO to give 100 mM stock solutions. NADP⁺ (Millipore Sigma; St. Louis, MO) was weighed out and dissolved in 50 mM Tris buffer at pH 8 with 100 mM NaCl to give 100 mM stock solutions. Both triclosan and NADP⁺ stock solutions were stored as frozen aliquots at −80 °C, and each aliquot was discarded after a single use to avoid multiple freeze-thaw cycles.

Cells. pET-15b plasmids harboring the mutated SaFabI genes were transformed into E. coli competent cells—BL21 CodonPlus(DE3)-RIL (Zymo, Irvine, CA)—and were used to express the WT FabI proteins from S. aureus (SaFabI) as well as SaFabI mutant proteins. Cells were grown overnight at 37 °C in lysogeny broth (LB) medium with Ampicillin (Fischer Scientific, Hanover Park, Illinois) (100 μg/mL). This overnight culture (50 μL) was transferred to fresh LB medium (4 mL) for growth for ∼2.5 h with an optical density at 600 nm (OD₆₀₀) ∼0.3 to 0.5. Isopropl β-D-1-thiogalacto-pyranoside was added (1 mM final concentration) to induce protein expression at 19 °C to express SaFabI protein in a soluble form for ∼16 h (overnight growth with the OD₆₀₀ generally at ~1.4 to 1.8). The protein expression in cells was switched to 19 °C since the amount of SaFabI in a few mutant cells, such as D66Y and L35F, in a soluble form were reduced when the induced expression was carried out at 37 °C. Cells were centrifuged, and the supernatant (mostly LB) was removed leaving only the cell pellet, which was either used fresh or stored at −80 °C for later use. Each cell pellet was resuspended with LB (400 μL) for cellular thermal shift assay and/or gel electrophoresis run. Cells were discarded when the gel electrophoresis runs showed that the amounts of SaFabI
expressed in some mutant cells were less than 80% of that in the WT cells. Repeated runs were carried out and only those with a similar amount of proteins as the WT were used, consisting of cells with 35 different single mutations. A few SaFabI mutant cells, such as E5K, were not used since no or little protein was found either in soluble form or insoluble form in repeated gel electrophoresis runs.

Cellular Thermal Shift Cell Samples. The thermal shift method is based on the principle that each soluble protein exhibits a characteristic thermal unfolding temperature in solution, beyond which the protein unfolds to become insoluble. When a molecule binds to the protein, the protein is stabilized and remains soluble and stable up to a higher unfolding temperature.

WT or mutant cells in the LB medium (400 μL) were divided into two sets, with one set for samples without triclosan (0X) and the other set for samples with triclosan, either at 0.1 mM with 0.2 mM NADP⁺ (1X) or at 1 mM with 2 mM NADP⁺ (10X). Initially, ~10 different mutant cells were processed at a time with 1X. In these samples, triclosan (1.5 μL of 10 mM solution in DMSO) with NADP⁺ (3 μL of a 10 mM solution) was added to the cells (145.5 μL). In the control samples (0X), DMSO was added to give 1% final concentration, the same as in the 1X sample. After the initial experiments, a separate set of selected mutants were subsequently treated with 10X. All samples were incubated at 37 °C for 30 min. The samples (0X and 1X) were each split into 50 μL each (set 1 and set 2). Set 1 samples were labeling as the “37 °C samples.” Set 2 samples were further incubated at 65 °C for 3 min and labeled as the “65 °C samples.”

Gel Electrophoresis Samples and Analysis. All thermal shift cell samples (with or without triclosan, heated to 37 or 65 °C) of mutants and their paired controls (WT) were subjected to three cycles of freezing (in liquid nitrogen) and thawing (at 20–23 °C), followed by centrifugation at 23,420g for 45 min. Soluble supernatants (30 μL), mixed with a solution containing SDS, glycerol, mercaptoethanol, and dye (15 μL) were heated at 95 °C for 10 min and loaded onto polyacrylamide (12%) gels for electrophoresis runs. Gels were stained with the Colloquium Blue dye (AquaStain Protein Gel Stain, Bulldog Bio; Portsmouth, NH) and gel images were captured with a camera. The bands for SaFabI proteins (WT and mutants) were at ~34 kDa. Gel band intensities/density were measured by AlphaView SA software (Protein Simple; San Jose, California) via the Multiplex Band Analysis Tool. Within this software, the SaFabI band at about 34 kDa was enclosed with a rectangular box. A white background was assigned as 0 pixel and a black band as 65,535. For simplicity, from here on, the pixel/intensity unit was 10⁶ pixels. Thus, the black band intensity was 0.66 (10⁶ pixels). The gel background intensity was found to be close to zero, or about 1 × 10⁻⁵ (10⁵ pixels). These intensity values of SaFabI bands were recorded.

For each sample without or with triclosan, the ratio (%) of the band intensity at 65 °C to that at 37 °C was determined. This was done since the amount of SaFabI in different cell samples varied slightly, despite careful controls throughout the process for quantitative measurements.

Mutation Sites in SaFabI Mutants. SaFabI is a homodimer with the monomer–monomer interface referred to as PR interface, and upon binding triclosan, two dimers associate to give a tetramer with a QR interface. The mutated residues that were in the active site or in both interfaces of SaFabI were identified, according to published work (PDB: 4ALI). The SaFabI mutants were also analyzed by a web-based software Sorting Intolerant from Tolerant (SIFT) to predict amino acid changes that affect protein function. SIFT predicts whether an amino acid substitution affects protein function, with scores less than 0.05 as deleterious. Some SIFT users have found that substitutions with scores less than 0.1 provide better sensitivity for detecting deleterious SNPs. SIFT presumes that important amino acids will be conserved in the protein family and changes at well-conserved positions tend to be predicted as deleterious. For prediction, SaFabI sequences and amino acid substitutions were submitted to SIFT, and the program, using default settings, selected FabI proteins from 19 species, including E. coli, Bacillus subtilis, etc., for sequence alignment to determine conserved residues for analysis.

UCSF Chimera was used to visualize the structure and to analyze potential structural conflicts in mutants, based on the structures of SaFabI without (PDB: 3GNT) and with triclosan (PDB: 4ALI).

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Author Contributions
R.D. performed the protein purification, cells of mutant SaFabI preparation, the triclosan binding experiments, data analysis, and manuscript preparation. P.K. performed the error-prone PCR experiments, analyzed the DNA mutations in SaFabI gene, and WT and A33V SaFabI plasmids in S. aureus cell experiments. L.W.-M.F. designed and supervised the project and participated in manuscript preparation.

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED

0X, no triclosan added; 1X, 0.1 mM triclosan with 0.2 mM NADP⁺; 10X, 1 mM triclosan with 2 mM NADP⁺; ACP, acyl carrier protein; epPCR, error-prone PCR; MIC, minimum inhibitory concentration; Sa, Staphylococcus aureus
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