Biochemical and Structural Basis of Triclosan Resistance in a Novel Enoyl-Acyl Carrier Protein Reductase

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ABSTRACT  Enoyl-acyl carrier protein reductases (ENR), such as FabI, FabL, FabK, and FabV, catalyze the last reduction step in bacterial type II fatty acid biosynthesis. Previously, we reported metagenome-derived ENR homologs resistant to triclosan (TCL) and highly similar to 7-H9251 hydroxysteroid dehydrogenase (7-AHSDH). These homologs are commonly found in Epsilonproteobacteria, a class that contains several human-pathogenic bacteria, including the genera Helicobacter and Campylobacter. Here we report the biochemical and predicted structural basis of TCL resistance in a novel 7-AHSDH-like ENR. The purified protein exhibited NADPH-dependent ENR activity but no 7-AHSDH activity, despite its high homology with 7-AHSDH (69% to 96%). Because this ENR was similar to FabL (41%), we propose that this metagenome-derived ENR be referred to as FabL2. Homology modeling, molecular docking, and molecular dynamic simulation analyses revealed the presence of an extrapolated six-amino-acid loop specific to FabL2 ENR, which prevented the entry of TCL into the active site of FabL2 and was likely responsible for TCL resistance. Elimination of this extrapolated loop via site-directed mutagenesis resulted in the complete loss of TCL resistance but not enzyme activity. Phylogenetic analysis suggested that FabL, FabL2, and 7-AHSDH diverged from a common short-chain dehydrogenase reductase family. This study is the first to report the role of the extrapolated loop of FabL2-type ENRs in conferring TCL resistance. Thus, the FabL2 ENR represents a new drug target specific for pathogenic Epsilonproteobacteria.

KEYWORDS enoyl ACP reductase, extrapolated loop, metagenome, triclosan resistance

Enoyl-acyl carrier protein (ACP) reductase (ENR) catalyzes the last step of the bacterial type II fatty acid synthesis (FASII) cycle to reduce the enoyl-ACP to fully saturated acyl-ACP (Fig. 1). NADH, NADPH, and reduced flavin mononucleotide (FMNH2) function as coenzymes in this reduction reaction (1). A majority of the enzymes involved in the FASII cycle are relatively conserved among bacteria, except ENR (2). To date, four prototypic bacterial ENR isozymes have been reported, including FabI (3), FabL (4), FabV (5), and FabK (6). Except for FabK, which is an FMN-containing protein, all ENR isozymes belong to the short-chain dehydrogenase reductase (SDR) superfamily (7). These ENRs share low sequence similarity (15% to 30%), although their active sites and specific sequence motifs required for coenzyme binding are highly conserved (8–10).

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol] (TCL) is a broad-spectrum antimicrobial that targets ENR of various organisms, thereby blocking FASII and ultimately preventing microbial growth (11). For years, TCL has been incorporated in a variety of consumer and personal care products worldwide (12–14) because of its potential antimicrobial activity. However, TCL resistance is prevalent among bacteria, and various mechanisms have been proposed as the basis for this resistance, including high ENR activity and structural changes in the ENR active site (20).
expression (15), mutant ENR versions tolerant to TCL (16), cell membrane modifications (17), various efflux pumps (15, 18), TCL-degrading enzymes (19), novel ENRs, and other unknown TCL resistance determinants (20). Additionally, TCL has been known to impose selective pressure on bacterial pathogens, thereby inducing coresistance or cross-resistance to other antibiotics (16, 20–25). Furthermore, there are increasing concerns regarding the excessive use of TCL and its negative effects on the environment and public health (12, 13).

FabI is known as the only effective ENR target for TCL, although substitutions of various key amino acid residues in FabI result in significant TCL resistance (20, 26–28). FabK has been reported to confer either low (20) or high (6) resistance to TCL, whereas other ENRs, such as FabL, confer low resistance, and FabV (5), 7-α-hydroxysteroid dehydrogenase (7-AHSDH)-like ENR homologs, and FabG-like ENR homologs confer high resistance to TCL (20). Being pivotal for bacterial survival and growth, ENRs have been used as potential targets for various antimicrobials for decades, and various synthetic ENR inhibitors either have been marketed or are currently being developed or in trial (29).

Previously, we identified a novel, TCL-tolerant, 7-AHSDH-like protein homolog (GenBank accession number KT982367.1; NCBI protein database number AOR51268.1) from soil metagenome, which complements ENR activity in a conditional Escherichia coli mutant JP1111 [fabI(Ts)] and renders the bacteria completely tolerant to TCL (20). The gene encoding this 7-AHSDH-like protein is of special interest, as it is present in Epsilonproteobacteria, a class that contains many pathogens of humans, including Helicobacter pylori and Campylobacter jejuni (20). The gene encoding the 7-AHSDH-like homolog from H. pylori and C. jejuni has been shown to confer TCL resistance in E. coli (20). In the present study, we aimed to determine whether the 7-AHSDH-like protein possesses dual enzymatic activities of ENR and 7-AHSDH and its role in TCL resistance. Based on phylogenetic analysis, biochemical characterization, and molecular simulation, the 7-AHSDH-like enzyme showed divergent evolution from the SDR family, with a unique TCL resistance mechanism. We propose that this 7-AHSDH-like enzyme be referred to as FabL2. Because the FabL2-type ENRs are commonly found along with FabI-type ENRs (30) in the pathogenic bacterial group of Epsilonproteobacteria, FabL2 has significant implications in health care and drug discovery.

RESULTS AND DISCUSSION

Phylogenetic analysis of FabL2, 7-AHSDH-like protein, and prototypic ENRs. ENR catalyzes the final reduction step in the bacterial FASII cycle and is indispensable.
for establishing and maintaining the rate of fatty acid biosynthesis (31, 32). Amino acid sequence analysis revealed that FabL2 shared significant homology (69% to 96%) with the 7-AHSDH homologs of Epsilonproteobacteria and relatively less similarity to FabL (41%), FabI (27%), and prototypic 7-AHSDH (34%) (20). Moreover, FabL2 ENR shared similar structural features, such as highly conserved tyrosine and lysine residues of the active site, with prototypic FabL and FabL ENRs. Similarly, key residues of the enzyme, such as Ser146, Lys163, Thr193 and RINA-like sequences, were strictly conserved among the FabL2 ENR and prototypic 7-AHSDH (20).

Additionally, the FabL2 protein conferred complete TCL tolerance when expressed in E. coli and complemented the ENR activity in the E. coli mutant JPP1111, carrying the fabI(Ts) mutation (20). Phylogenetic analysis of the FabL2 protein with other prototypic ENRs and 7-AHSDH proteins and their homologs revealed that FabL2-type ENRs clustered as a separate clade (Fig. 2), suggesting that FabL2 diverged either from closely related Fabl and Fabl ENRs or from 7-AHSDH during evolution. Therefore, we designated this enzyme FabL2. Consequently, distinguishing these types of ENRs based only on sequence comparison/annotation is not always ideal (20).

**NADPH-dependent ENR activity of the FabL2 protein.** A fusion protein of FabL2 was purified and confirmed to be of expected size (33.56 kDa) using SDS-PAGE (see Fig. S2A and B in the supplemental material). The conversion of NADH/NADPH cofactors to NAD/NADP at 340 nm was monitored to assess the ENR activity. Because the purified protein exhibited maximum activity in 100 mM sodium phosphate buffer (pH 7.0) (Fig. S2C), all subsequent assays were performed using this buffer. No enzymatic activity was observed in 100 mM sodium citrate buffer regardless of the pH (data not shown). The purified protein catalyzed the turnover of NADPH (Michaelis constant $K_m = 27.64 \mu M$) (Fig. 3A) into NADP in the presence of crotonyl coenzyme A (crotonyl-CoA) ($K_m = 9.627 \mu M$) as a substrate (Fig. 3B). These $K_m$ values for the metagenomic ENR were equivalent to those reported for Chlamydia trachomatis and E. coli ENRs (4, 33), although these values were slightly lower than those reported by Ward et al. and Basso et al. (34, 35). The purified protein did not utilize NADH as a cofactor, thus exhibiting ENR activity only with NADPH.

Among other prototypic ENRs, FabL from Bacillus subtilis showed high similarity to FabL2 ENR (41%), which uses NADPH as a cofactor (4), whereas FabV using NADPH as a cofactor (1) did not show any similarity to FabL2. The $k_{cat}$ values for FabL2 with NADPH (1.09 $\mu M$/min) and crotonyl-ACP (0.18283 $\mu M$/min) (Table S1) were within the range reported previously (36) but were lower than those for the ENR from C. trachomatis (33). These variations might be due to the use of different substrates. Overall, biochemical analyses confirmed that FabL2 enzyme possesses NADPH-dependent ENR activity. However, despite its high similarity to 7-AHSDH from Epsilonproteobacteria, FabL2 did not exhibit 7-AHSDH activity when tested with cholic acid substrate and NADH and NADPH cofactors (data not shown). These data suggest that the enzyme FabL2 from soil metagenome is a bona fide ENR similar to FabL.

**Predicted structure of FabL2.** Sequence analysis of metagenomic TCL-resistant FabL2 showed 41.0% sequence similarity with FabL from B. subtilis. Sequence alignment revealed Gly102, Tyr160, Lys163, and Phe204 as the catalytic residues of FabL2 (Fig. 4A) and an extrapolated loop to be tested for TCL resistance (Fig. 4B) and ENR function (Fig. 4C). Therefore, the structure of Fabl from B. subtilis (PDB code 3OID; chain A) was considered the template for homology modeling of FabL2. Among 10 predicted models, the best model of FabL2 was selected based on the lowest molecular probability density function (MOLPDF) score of 1,414.23 and discrete optimized protein energy (DOPE) score of $-26,020.47$. The representative structure of FabL2 was extracted after molecular dynamic (MD) simulation refinement. The stereochemical quality of the refined FabL2 structure revealed that 88.6% of the residues occupied the most favored region of the Ramachandran plot (37) (Fig. S3A). These results suggest that phi and psi backbone dihedral angles in the modeled structure are reasonably accurate.
FIG 2 Phylogenetic analysis of FabL2 ENR and its homologs. Maximum likelihood analysis was performed with well-characterized 7-AHSDH, FabL, FabV, FabI, and FabK (in bold) and their homologs, with sequence identity >50% using the Uniref50 database. Bootstrap values are shown for each node with >50% support in a bootstrap analysis of 500 replicates. The scale bar represents 0.2 estimated amino acid substitution per residue.
Analysis of FabL2 with ProSA-web (38) revealed a Z-score of −7.31, which was within the range of Z-scores of experimentally determined structures (Fig. S3B).

The MD-refined structure of FabL2 has an architecture similar to that reported for FabL proteins (39). Briefly, the overall structure of FabL2 comprises a central 7-stranded parallel β-sheet (β1 to β7) sandwich-like structure flanked on both sides by three α-helices, forming an NADPH-binding Rossmann-like fold (40) (Fig. 5A). Our modeled FabL2 structure also exhibited the same folding pattern in the substrate-binding region (α8 and α9) located near the carboxyl end of β6 and β7 as previously described for different ENRs and other members of the SDR family (4, 41, 42). Despite the high similarity of FabL2 with FabL from B. subtilis, FabL2 contained an extrapolated region extending between Tyr96 and Val101 (Fig. 4A and 5A and B). The structural superimposition of FabL2 and FabL affirmed that the extrapolated six amino acid residues formed a loop (Fig. 5A, orange). The role of this loop in substrate specificity and TCL resistance of FabL2 was subsequently validated.

Interaction of TCL with FabL2. The best docking pose of TCL with FabL2 revealed a Genetic Optimization of Ligand Docking (GOLD) fitness score of 53.00. Despite the high docking score, TCL was flipped away (~5.7 Å) from the catalytic site of FabL2 and was bound at the rim region of the tunnel leading to the substrate binding site (Fig. 5B). Furthermore, despite the phenol moiety, the phenoxy group of TCL was oriented toward the catalytic pocket of FabL2. Molecular interactions between FabL2 and TCL implied that the Arg98 residue of the extrapolated loop formed two H bonds with the backbone oxygen and phenolic oxygen of TCL (Fig. 5C). Moreover, other nonpolar interactions confirmed the binding of TCL at the rim region of the tunnel leading to the catalytic site of FabL2 (Fig. 5C). Our rational approach concluded that TCL could not reach the catalytic site of FabL2 due to H bonding with Arg98 and other nonpolar interactions (Fig. 5A and B). This flexible loop may determine the shape and size of the tunnel. We further speculate that the extrapolated loop is highly flexible and plays a key role in TCL resistance of FabL2.

Based on our results, we hypothesized that removing this extrapolated flexible loop (Tyr96 to Val101) from FabL2 renders it sensitive to TCL. To test this hypothesis, we created mutant FabL2 (mFabL2) lacking the extrapolated loop and predicted its structure as previously described for FabL2 (Fig. 4B). The best model had the lowest PDF and DOPE scores, 1,353.46 and −24,680.74, respectively. PROCHECK analysis of mFabL2 revealed that 92.6% of amino acid residues occupied the most favored region of the Ramachandran plot (Fig. S3C). The Z-score of mFabL2 model was −6.74, which followed the Z-score pattern of wild-type FabL2 (Fig. S3D). FabL2 and mFabL2 showed a similar overall topologies and model qualities, except for the tunnel leading to catalytic cavity (Fig. 5D). Based on these data, we speculate that the native function of mFabL2 was unaffected by the deletion of the flexible loop.

Although the docking score of mFabL2 was the same as that of wild-type FabL2 (53.00), TCL occupied the catalytic active site of mFabL2. The binding of TCL with
**FIG 4** Sequence alignment of FabL2, mutant FabL2 (mFabL2), and template structure. (A) Sequence alignment of FabL2 and the template structure (PDB code 3OID), which is the crystallographic structure of *Bacillus subtilis* FabL. The extrapolated mismatched six amino acids comprising the highly flexible loop of FabL2 are shown in red boxes, and the highly conserved catalytic active-site residues are shown in magenta boxes. (B) Sequence alignment of mFabL2 and template structure. (C) Complementation analysis of m-FabL2 ENR. Each plate has been divided into three sections: 1, JP111 with pGEM-T Easy only; 2, JP1111 carrying *E. coli* FabI in pGEM-T Easy; and 3, JP1111 carrying m-FabL2 in pGEM-T Easy. Plates were incubated at 30°C and 42°C for 48 h.
FIG 5 Homology model and docking of FabL2, mFabL2, and triclosan (TCL). (A) Molecular dynamic optimized model of FabL2. The α-helices and β-strands are labeled and the extrapolated loop of 7-AHSDH is shown in orange. NDAP is shown as a stick model. (B) FabL2-TCL complex after docking. TCL is shown as a stick model in magenta; it is bound at the rim region of the tunnel leading to the catalytic site. The size of the tunnel opening is determined by the loop (orange). The putative catalytic site of FabL2 is shown as a sphere (light blue). (C) Two-dimensional representation of molecular interactions between FabL2 and TCL. Two H bonds between TCL and the Arg98 residue of FabL2 are shown. (D) Molecular dynamic optimized model of mFabL2. NADPH is shown as a stick (Continued on next page)
mFabL2 was similar to that observed with other ENR family members with respect to the orientation of the phenol moiety toward the substrate binding site (Fig. 5E). Moreover, the H bonding between TCL and Tyr154 of mFabL2 stabilized its orientation in the active site of mFabL2 (Fig. 5F). Further, the H bonding between amino acid residues of mFabL2 and NADPH generated a stable enzyme complex of mFabL2 (Fig. 5G). This binding pattern of TCL is conserved across all ENR family members (28, 39, 43, 44).

**MD simulation of the mFabL2-TCL complex.** Detailed analysis of the binding mode of TCL with the active site of mFabL2 was conducted via 20-ns MD simulation. The root mean square deviation (RMSD) of the Cα atoms (Fig. 5H), the simulation of the mFabL2-TCL complex (Fig. 5I), and analysis of molecular interactions (Fig. 5J; see also Fig. S4A and B) indicated that the system remained stable during the entire simulation period. This mechanism of targeting the catalytic Tyr by TCL is well documented among other ENR family members (28, 39, 44). Our analysis also revealed an additional interaction between NADPH and TCL via H bonding (Fig. S4A and B), which may strengthen the binding of TCL with mFabL2. Moreover, other molecular interactions, including π-π, alkyl-alkyl, π-alkyl, and van der Waals interactions, were observed between the catalytic site residues of mFabL2 and TCL (Fig. 5G; see also Table S2). Taken together, these data validated our hypothesis and revealed that the deletion of the extrapolated residues did not disturb the native folding of mFabL2 and restore its sensitivity to TCL. Experimental validation confirmed the native function of mFabL2 as well as its inhibition by TCL.

**The extrapolated loop of FabL2 is involved in TCL resistance.** Bioinformatics analysis revealed that FabL2 has an extra six-residue loop (Tyr96 to Val101), which was specific to and supposed to be involved in TCL tolerance. These six residues extend the loop dramatically, which pushes Gly102 (conserved catalytic residue) away, creating a new topology of the TCL binding site of FabL2. We speculate that Arg98, which is sequestered between Gly102 and TCL, interferes with their binding. Moreover, docking analysis of mFabL2 revealed that TCL is able to access the active site of mFabL2. Therefore, we conclude that the loop from Tyr96 to Val101 is responsible for the observed TCL tolerance of FabL2; its removal may result in the loss or reduction of TCL resistance. As expected, deletion of the loop from Tyr96 to Val101 via site-directed mutagenesis resulted in the loss of TCL resistance in mFabL2 (MIC, 2.5 μg/ml), whereas wild-type FabL2 was capable of conferring resistance to TCL even at concentrations as high as 600 μg/ml (Fig. S5A and B). Moreover, complementation analysis revealed that mFabL2 retained its ENR activity (Fig. 4C). This result indicates that the loop from Tyr96 to Val101 is involved in TCL tolerance but not in ENR activity. The strict amino acid conservation of this extrapolated loop (Fig. S6A) suggests that the loop was recently introduced into FabL2 of Epsilonproteobacteria.

The extrapolated loop is highly unique and is present only in FabL2-type ENR and its homologs in Epsilonproteobacteria; it is absent in the closely related prototypic FabL-type ENRs and prototypic 7-AHSDH homologs (Fig. S6A and B and Table S3). It is unclear how and why these enzymes have evolved to contain this extrapolated loop. However, this extrapolated loop (Tyr96 to Gly102) in FabL2 is involved in the topology of tunnel leading to the enzyme active site. The residues of the target loop are not considered catalytic moieties of ENR, since removal of the loop did not affect ENR activity. Although we have not tested specific point mutation of the extrapolated loop,

![FIG 5 Legend](Continued)

model. (E) mFabL2-TCL complex after docking. TCL is shown as a stick model (magenta), and the putative catalytic site is shown as a sphere (light blue). (F) Two-dimensional representation of molecular interactions between mFabL2 and TCL after docking. The absence of the extrapolated loop in mFabL2 significantly widened the opening of the tunnel leading to the catalytic site of mFabL2. The hydrogen bond is shown as green dashed lines, while the corresponding residue is depicted as green closed circles. (G) Two-dimensional representation of mFabL2 and NADPH interactions. All interactions are indicated with dashed lines: H bonds, black; salt bridge interactions, orange; and other hydrophobic interactions, light magenta. Amino acid residues connected via H bonds are depicted as green closed circles. (H) Root mean square deviation of Cα atoms of mFabL2, representing its stability during the simulation. (I) Potential energy of the system, indicating the stability of the mFabL2-TCL complex. (J) Total number of H bonds between NADPH bound mFabL2 and TCL during the entire simulation period.
we speculate that specific point mutation of the loop would not alter the overall structure of the loop. Our rational approach suggested that extrapolated loop is highly flexible and fluctuates back and forth to open and close the opening of the tunnel leading to the active site of FabL2 (Fig. 5A). However, this prediction still awaits further biochemical investigation.

Our analysis of docking of TCL into the active site of FabL2 suggested that TCL is flipped away from docking site by ~5.7 Å, and hence, TCL could not reach the catalytic site. If we delete this loop, the main entrance of the tunnel will remain open and TCL would be able to reach the active site of FabL2. In fact, deletion of extrapolated loop could successfully abolish TCL resistance in mFabL2. Finally, we perceived that extrapolated loop of FabL2 serves as a checkpoint to selectively allow the substrate(s) to reach the active site, which needs to be further verified by structural characterization. Taken together, the findings of this study showed that minor changes in the structure of bacterial proteins due to small-scale structural variations in the coding sequence can render the bacteria resistant to antibiotics.

Conclusions. We conclude that FabL2 ENR confers complete TCL tolerance via a unique extrapolated loop in its protein structure. This study is the first to show TCL tolerance conferred by residues other than those directly interacting with the substrate or cofactor. Furthermore, the presence of TCL-resistant FabL2 ENR homologs among the human-pathogenic bacteria of the *Epsilonproteobacteria* class indicates that these bacteria may be unaffected by TCL treatment. Additionally, although the amino acid sequence of FabL2 was highly similar to that of 7-AHSDH, the lack of 7-AHSDH activity in FabL2 indicates that sequence alignments alone are not sufficient for determining protein function.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and general DNA manipulation. *E. coli* strains DH5α, EPI300, and BL21(DE3) were grown at 37°C in Luria-Bertani (LB) broth or on LB agar media containing appropriate antibiotics: TCL (1 to 600 μg/ml), Sigma-Aldrich Co., St. Louis, MO), chloramphenicol (50 μg/ml), ampicillin (100 μg/ml), or kanamycin (50 μg/ml). Recombinant DNA manipulation was performed as previously described (43). Oligonucleotide synthesis and DNA sequencing were conducted at the DNA sequencing facility of Macrogen (Seoul, Republic of Korea). Nucleotide and amino acid sequences were compared using the online version of BLAST and ORF finder, publicly available at the National Center for Biotechnology Information (NCBI) portal (http://blast.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using BioEdit v7.2.5 and GeneDoc v2.7 software.

Phylogenetic analysis. Phylogenetic analysis was performed as previously described (20) for metagenomic FabL2 ENR using amino acid sequences of FabL2 and its homologs, prototypic FabL, FabI, FabV, FabK ENRs, and prototypic 7-AHSDH from *Comamonas testosteroni* and its homologs retrieved from the UniRef50 database (updated on 19 September 2017). Top 10 entries were selected from each homology search. All identified sequences compiled together with the closely related prototypic ENRs and metagenomic FabL2, and redundant sequences were removed using the online Decrease Redundancy search. All identified sequences compiled together with the closely related prototypic ENRs were used to construct the phylogenetic tree using the MUSCLE algorithm (48). To analyze the alignment output in MEGA 6, the maximum likelihood method was used in combination with the nearest-neighbor-interchange strategy, resulting in the deletion of gaps present in less than 50% of the sequences and generating 500 bootstrapped replicates resampling data sets to evaluate the confidence.

Expression and purification of FabL2 ENR. A gene encoding FabL2 ENR was PCR amplified from pBEF1-4 (20) using gene-specific forward primer (5′-ATCAGATTTAGACTACAAAATATGGAAAGGC AA-3′) and reverse primer (5′-TTACATCTTTATTTACATATAGATGGTCGACTTTCA-3′) containing BamHI and SalI restriction sites (underlined), respectively. The amplified PCR product was digested with BamHI and SalI restriction endonucleases and cloned into pET-30b (+) expression vector to generate the recombinant vector pEBF1-4.

To express the FabL2 protein, pEBF1-4 was transformed into *E. coli* BL21(DE3) cells, and recombinant cells were selected on LB agar medium containing kanamycin. *E. coli* cells carrying pEBF1-4 were grown in 200 ml of LB supplemented with kanamycin at 37°C until reaching an optical density of 0.5 at 600 nm (OD₆₀₀). To induce protein expression, isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added to the bacterial culture during the late exponential phase. For protein purification, *E. coli* cells were harvested, resuspended in 5 ml of binding buffer (20 mM Tris-Cl, 0.5 M NaCl, 40 mM imidazole [pH 8.0]), and subjected to sonication (sonic dismembrator model 500; Fisher Scientific) for 2 min (pulse ON, 5 s; pulse OFF, 10 s). This mixture was then centrifuged at 3,500 × g for 6 min at 25°C. The supernatant was collected and recentrifuged at 17,000 × g for 10 min at 25°C and filtered using a 0.45-μm membrane filter. The fusion protein was purified using AKTA prime liquid chromatography system (GE Healthcare, Buckinghamshire, UK) with a His Trap HP affinity column (1-ml bed volume; GE Healthcare). The identity of the purified fusion protein was confirmed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
Analysis of FabL2 enzymatic activity. While the deduced amino acid sequence of FabL2 showed high sequence similarity to that of 7-AHSDH, fabL2 has previously been shown to complement ENR mutant E. coli (20). To test if FabL2 possesses dual enzymatic activities, enzyme assays were performed using the purified fusion protein. Biochemical characterization of the purified fusion protein was performed to determine the optimum reaction conditions and Michaelis-Menten kinetics for ENR activity. All enzyme assays were performed as previously described (33), with slight modifications. Briefly, ENR activity was measured in a 100-μL volume containing NADH/NADPH cofactors (250 μM), crotonyl-coenzyme A (crotonyl-CoA) substrate (200 μM), FabL2 protein (450 nM), and sodium phosphate buffer (100 mM; pH 7.0) at 25°C. Crotonyl-CoA, NADH, and NADPH were purchased from Sigma-Aldrich. Enzymatic reactions were monitored using a UV-visible (UV-Vis) spectrophotometer (DU730 Life Science; Beckman Coulter Inc., Fullerton, CA) at 340 nm and 30-s intervals for a total of 3 min. The protein did not exhibit ENR activity with NADH and preferred NADPH as a cofactor, the latter was used in all subsequent enzyme assays. To determine the value of the Michaelis constant (K_m) of the protein, 100 nM purified protein was added to 100 μL of the reaction mixture containing 200 μM NADPH and various concentrations of crotonyl-CoA (3, 6, 12, 24, 36, and 48 μM). To determine the K_m value of NADPH, 100 nM protein was added to 60 μM crotonyl-CoA and various concentrations of NADPH (5, 10, 15, 20, 30, 50, and 75 μM). The oxidation of NADPH cofactor was spectrophotometrically measured at 340 nm. The reaction mixtures were incubated at 25°C for 10 min. To determine the optimal buffer composition and pH for enzymatic activity of the protein, reactions were conducted using different buffers over a wide pH range: 100 mM sodium citrate buffer (pH 3.2, 4.2, 5.2, and 6.2) and 100 mM sodium phosphate buffer (pH 6.5, 7, 7.5, and 8.0). To determine whether FabL2 possessed 7-AHSDH activity, enzyme assays were performed using cholic acid (Sigma-Aldrich) as the substrate and NADH and discrete optimized protein energy (DOPE) score for further analysis.

Site-directed mutagenesis. Sequence comparison, homology modeling, and docking analysis revealed that FabL2-type ENR carries an extraplated highly flexible loop comprising six amino acid residues (Y96 to V101); this was specific to FabL2 ENR only, as other known ENRs lack this loop. To test if this extrapolated flexible loop was involved in TCL resistance and enzymatic activity, overlap extension PCR was used to delete the 18-bp loop (Fig. S1, region b). Briefly, two PCRs were performed to amplify the overlapping fragments A and C of the FabL2 gene (Fig. S1) from pBF1-4 using the primer pairs 7A-1/7A-2 (7A-1, 5'-GCCAAAAGCTGTCAGGTCG-3', and 7A-2, 5'-AATGACGCGATTGCTGACAGAAG-3') and 7A-3/7A-4 (7A-3, 5'-TCTGAAACATGCGATGTCGCGGAGTACGGATAATTAT-3', and 7A-4, 5'-CGCGCTCATA TTATGCTGTCACA-3'), respectively. The underlined sequences of 7A-2 and 7A-3 are complementary to each other to anneal for overlap extension. The PCR conditions were as follows: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at variable temperatures (55°C or 63°C) or the amplifying fragment A or C, respectively) for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The amplified PCR products A and C were gel purified. This was followed by a fusion PCR using fragments A and C as templates in equimolar concentrations without any primers and the following conditions: initial denaturation at 95°C for 3 min, 10 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min 35 s, followed by a final extension at 72°C for 5 min. The fusion product was subsequently amplified using primers 7A-1 and 7A-4 (Fig. S1) and the following conditions: initial denaturation at 95°C for 3 min, 20 cycles of denaturation at 95°C for 1 min, annealing at 63°C for 30 s, and extension at 72°C for 1 min 35 s, followed by a final extension at 72°C for 5 min. The purified fusion product was cloned into the pGEM-T Easy vector and transformed into E. coli DH5α to confirm the deletion of the loop and test for TCL resistance as previously described (20). The mutated version of FabL2 was designated mFabL2.

Complementation. To investigate the ENR activity of mFabL2, complementation studies were performed. The recombinant pGEM-T Easy plasmid carrying mFabL2 was transformed into a conditional temperature-sensitive fabl mutant of E. coli, JP1111, which is unable to grow at the high temperature of 42°C (50). E. coli JP1111 organisms containing the mFabL2 vector were grown in triplicates on LB agar medium supplemented with ampicillin (100 μg/ml) and IPTG at 30°C and 42°C. The growth of E. coli JP1111 at 42°C for 48 h indicated complementation of FabL ENR activity.

TCL resistance test. To determine and compare the growth and TCL resistance of E. coli DH5α expressing either metagenomic FabL2 or mFabL2, growth assays were performed in LB broth supplemented with ampicillin and various concentrations of TCL (0 to 600 μg/ml). E. coli DH5α expressing the Bacillus velezensis FabL homolog (WP_003155478.1) was used as a positive control at similar TCL concentrations, and E. coli DH5α carrying empty pGEM-T Easy vector was used as a negative control. Bacterial growth was monitored using a UV-Vis spectrophotometer (DU730 Life Science; Beckman Coulter Inc., Fullerton, CA) by measuring OD600 over 96 h.

Homology modeling of FabL2. Homology modeling is the construction of an atomic model of the target protein utilizing experimentally determined structures of evolutionarily related proteins (51). First, the FabL2 protein sequence (target) was analyzed against the Protein Data Bank (PDB) using the BLAST tool in NCBI to identify the suitable protein structure (template) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM =blastp&PAGE_TYPE=BlastSearch). Subsequently, the target-template alignment was subjected to MODELLER program implemented in Discovery Studio v4.5. Ten iterative models of FabL2 were generated, and the best model was selected based on the lowest probability density function (PDF) total energy and discrete optimized protein energy (DOPE) score for further analysis.

It is noteworthy that the homology model does not reflect the conformation or orientation of amino acids comprising side chains in their physiological state. To obtain the native conformation of FabL2, an unrestrained molecular dynamic (MD) simulation was performed with CHARMM36 force field in GROMACS v5.0.7.
(52). Briefly, the system was solvated in an octahedral box of transferable intermolecular potential three position (TIP3P) water model. Counterions (Na\(^+\)) were added to neutralize the system. The steepest descent minimization with a maximum tolerance of 10 kJ/mol/nm was employed to avoid any unfavorable interactions. The system was equilibrated in two phases. In the first phase, NVT (number of particles at constant volume and temperature) equilibration was conducted for 100 ps at 300 K. The temperature was maintained with a V-rescale thermostat. In the second phase, heavy atoms were restrained, and solvent molecules with counterions were allowed to move during the 100-ps simulation at 300 K and 10\(^5\) Pa pressure using the Parrinello-Rahman barostat. The final production step was conducted for 10 ns under periodic boundary conditions with NPT (number of particles at constant pressure and temperature) ensemble and bond constraint algorithm, linear constraint solver (LINCS). The representative structure of FabL2 was extracted from the last 6-ns trajectory using the clustering method. The stereochemical quality of the MD-refined model of FabL2 was verified using PROCHECK implemented in SAVES web server (http://services.mbi.ucla.edu/PROCHECK). The MD-refined model of FabL2 was also validated by ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) for its accuracy of potential errors. The Z-score of ProSA measures the deviation of the total energy of the structure with respect to an energy distribution derived from random conformations.

**Molecular docking simulation of TCL into FabL2.** Molecular docking is a computational technique used to predict the binding affinity and orientation of a ligand in the binding site of a protein. The two-dimensional (2D) structure of TCL was drawn in Accelrys Draw v4.2 and converted into three-dimensional (3D) structure in Discovery Studio v4.5. The MD-refined model of FabL2 and TCL was used as input data in the Genetic Optimization of Ligand Docking (GOLD) v5.2.2 program. The binding site of FabL2 was traced from its catalytic residues using Define and Edit Binding Site tools implemented in Discovery Studio v4.5. Docking results were analyzed with the GOLD fitness score, which includes hydrogen bond (H bond) energy, van der Waals energy, and ligand torsion strains. The best docking pose was selected based on the GOLD fitness score and H bonding with catalytic residues.

**Accession number(s).** The nucleotide sequence of pBF1 harboring the FabL2 gene has been deposited in the GenBank database under accession number KT982367. The FabL2 ENR protein sequence has been deposited in the NCBI protein database under accession number AOR51268.1.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00648-18.

**SUPPLEMENTAL FILE 1, PDF file, 2.7 MB.**

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We have no conflicts of interest to declare.

S.-W.L., R.K., K.W.L., and A.Z. developed the framework for evaluating TCL resistance of the novel FabL2-type ENR. S.-W.L. and K.W.L. conceived, organized, and supervised the project. R.K., A.Z., K.W.L., and S.-W.L. interpreted the results and prepared the manuscript. R.K., N.R., R.T.M., and H.J.K. performed experiments and analyzed the data. All authors contributed to the final version of the manuscript.

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Mechanism of triclosan inhibition of bacterial fatty acid synthetic pathway


