Triclosan (also know as Irgasan) is a broad-spectrum bio-
cide that has been used for more than 30 years in tooth-
paste, cosmetics, antiseptic soaps, carpets and even toys. For many years, the mode of antibacterial action of tri-
closan was thought to be via non-specific disruption of the bac-
terial cell membrane. However, McMurry \textit{et al.} \textsuperscript{1} dis-
covered that mutations in the enoyl-[acyl-carrier-protein]
(ACP) reductase gene (\textit{fabI}) of \textit{Escherichia coli}
gave rise to resistance to triclosan, while susceptibilities to a range of unrelated antibacterials were unaffected. There are approxi-
mately 12 000 molecules of FabI per cell in mid-log phase growth. This number increased by ap-
proximately three- to four-fold in the \textit{E. coli} FabI overexpressor. Triclosan selectively
inhibited the incorporation of \textsuperscript{14}C-acetate into TCA-precipitable product, an indicator of fatty
acid biosynthesis. Furthermore, it inhibited \textit{de novo} fatty acid biosynthesis in this organism. \textit{In vitro}, triclosan inhibited recombinant, purified \textit{S. aureus} FabI with an \textit{IC}_{50} of approximately 1 \textmu M. The combination of these biochemical and genetic data provide further evidence that the mode of action of triclosan in \textit{S. aureus} is via inhibition of FabI.

**Introduction**

Triclosan, a widely used antibacterial agent, possesses potent activity against \textit{Staphylococcus}
\textit{aureus}. This study reports on an investigation of the antibacterial target of triclosan in this patho-
gen. A strain of \textit{S. aureus} overexpressing the enoyl-[acyl-carrier-protein] reductase (FabI),
demonstrated by Western immunoblotting, gave rise to an increase in the MIC of triclosan, while susceptibilities to a range of unrelated antibacterials were unaffected. There are approxi-
mately 12 000 molecules of FabI per cell in mid-log phase growth. This number increased by ap-
proximately three- to four-fold in the \textit{S. aureus} FabI overexpressor. Triclosan selectively
inhibited the incorporation of \textsuperscript{14}C-acetate into TCA-precipitable product, an indicator of fatty
acid biosynthesis. Furthermore, it inhibited \textit{de novo} fatty acid biosynthesis in this organism. \textit{In vitro}, triclosan inhibited recombinant, purified \textit{S. aureus} FabI with an \textit{IC}_{50} of approximately 1 \textmu M. The combination of these biochemical and genetic data provide further evidence that the mode of action of triclosan in \textit{S. aureus} is via inhibition of FabI.

Further work on \textit{E. coli} by Heath \textit{et al.} \textsuperscript{4} illustrated that FabI was the target of both triclosan and a series of related
2-hydroxydiphenyl ethers. It was also shown that triclosan was a potent micromolar inhibitor of the wild-type FabI, whereas the mutated FabI protein that gave rise to tri-
closan resistance was less susceptible to inhibition by both
triclosan and the other 2-hydroxydiphenyl ethers tested. Crystal structures have been solved for the \textit{E. coli} FabI
complexed with triclosan in the active site; these have pro-
vided a molecular mechanism for inhibition of \textit{E. coli} FabI
by triclosan.\textsuperscript{4,5} McMurry \textit{et al.}\textsuperscript{6} have also shown that mutations in the gene encoding InhA from \textit{Mycobacterium
smegmatis} gives rise to triclosan resistance. InhA is the
mycobacterial homologue of \textit{E. coli} FabI and thus the
mode of antibacterial action of triclosan appears to be the
same in both \textit{M. smegmatis} and \textit{E. coli}.

One of the best attributes of triclosan is its exceptional
activity against staphylococci (MICs < 0.06 mg/L) including...
methicillin-resistant Staphylococcus aureus (unpublished data). More recently, Heath et al. have demonstrated that FabI is the target of triclosan in S. aureus. The focus of this current study is to further characterize the target–compound interactions.

Materials and methods

Bacterial strains and media

S. aureus RN4220 was propagated at 37°C in tryptic soy broth (TSB) unless otherwise indicated. This organism was used as the recipient strain for electroporation of recombinant plasmids. S. aureus RN4220 harbouring plasmid pCU1\(^{10}\), pCS06 or pCS06A was grown at 37°C in TSB containing chloramphenicol (10 mg/L) for plasmid maintenance. S. aureus WCUH29 is a clinical isolate from the NCIMB 40771 culture collection. S. aureus Oxford is a clinical isolate from Oxford University, UK.

DNA manipulations

Standard DNA manipulations were performed as described previously.\(^3\) Plasmid DNAs were purified from bacterial hosts using plasmid purification kits (Qiagen, Valencia, CA, USA) as directed by the manufacturer. For S. aureus, the protocol was modified by addition of lysostaphin (AMBI Technologies, Purchase, NY, USA) to a final concentration of 100 mg/L to the P1 buffer and incubating for 45 min at 37°C before the addition of the P2 buffer.

S. aureus RN4220 genomic DNA was isolated by pelleting 100 mL of an overnight culture and resuspending in 10 mL of GET (0.1 M glucose, 0.01 M EDTA and 0.05 M Tris–HCl pH 8) buffer containing 300 µL of a 1 mg/mL stock of lysostaphin and 10 µL of a 100 mg/mL stock of RNase A. Following lysostaphin/RNase A treatment for 1 h at 37°C, 2 mL of a 20% SDS solution were added and incubation was continued for 30 min at 37°C until the solution became viscous. Sodium perchlorate (5 M) was added to a final concentration of 1 M, and the sample was extracted twice with phenol/chloroform/isooamyl alcohol (Gibco-BRL, Rockville, MD, USA). Precipitation of DNA from the aqueous phase was achieved by adding 2 volumes of 95% ethanol, mixing and centrifugation at 14 000 rpm for 30 min. The ethanol was decanted, the nucleic acid pellet was washed with 70% ethanol and the DNA collected by centrifugation. The DNA pellet was resuspended finally in 1 mL distilled, deionized water and stored at 4°C.

Cloning of the S. aureus RN4220 fabI gene

The oligonucleotide primers Fab1 (5’-GGCGGATCCCTTCCGCGATGGAGATACAC-3’) and Fab2B (5’-GGCGGATCTGATAATCTCGTGG-3’) were used to amplify the fabI gene, including the putative fabI promoter region, from S. aureus RN4220 genomic DNA. These primers were constructed with BamHI genomic DNA. The resulting 1388 bp DNA fragment was PCR purified using the Qiagen PCR cleanup kit as directed by the manufacturer. The purified product was digested with BamHI (Gibco-BRL) and cloned into pCU1 at the BamHI site. Following introduction into E. coli DH10B, the resulting recombinant plasmids were screened for orientation of insert by diagnostic PCR and restriction enzyme digestion. The construct containing fabI in the sense orientation was denoted pCS06. Double-stranded sequencing of the insert using an ABI automated sequencer (PE Biosystems, Foster City, CA, USA) confirmed the expected nucleotide sequence.

The primers Fab3 (5’-GGCCCGGGATAAGGAGTTATCTTTACATG-3’) and Fab2S (5’-GGCCCGGGTGTAAGGAGTATCTTTACATG-3’) were used to amplify the fabI gene lacking a promoter sequence from S. aureus RN4220 genomic DNA as described above. SmaI restriction sites (CCCGGG) were incorporated at the 5’ ends of the primer sequences for later manipulations. The resulting 1018 bp DNA fragment was purified, digested with SmaI and cloned into pCU1 at the SmaI site. Following diagnostic PCR, a construct containing the fabI coding sequence in the sense orientation was identified and denoted pCS06A. Sequence confirmation was obtained as above.

Introduction of recombinant plasmids into S. aureus RN4220

pCU1, pCS06 and pCS06A were introduced into S. aureus RN4220 via electroporation as described previously by Kraemer & Iandolo.\(^{10}\)

Overexpression assay

S. aureus strains RN4220, RN4220(pCU1), RN4220(pCS06) and RN4220(pCS06A) were used in the overexpression assay. A 96-well microtitre MIC format was used. Serial dilutions of antibiotics were made in TSB such that the range of concentrations in a final assay volume of 100 µL was 8–0.006 mg/L. Antibiotics tested included triclosan (Ciba Geigy, Chapel Hill, NC, USA), tetracycline, penicillin G, erythromycin, neomycin (Sigma, St Louis, MO, USA), mupirocin (SmithKline Beecham Pharmaceuticals, Philadelphia, PA, USA) and ciprofloxacin (US Pharmacia, Peapack, NJ, USA). An overnight culture grown in TSB was diluted in fresh TSB to achieve a cell density of 100 mL at 37°C. After incubation was continued for 30 min at 37°C, the solution became viscous. Sodium perchlorate (5 M) was added to a final concentration of 1 M, and the sample was extracted twice with phenol/chloroform/isooamyl alcohol (Gibco-BRL, Rockville, MD, USA). Precipitation of DNA from the aqueous phase was achieved by adding 2 volumes of 95% ethanol, mixing and centrifugation at 14 000 rpm for 30 min. The ethanol was decanted, the nucleic acid pellet was washed with 70% ethanol and the DNA collected by centrifugation. The DNA pellet was resuspended finally in 1 mL distilled, deionized water and stored at 4°C.

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Triclosan mode of action in *S. aureus*

**Macromolecular synthesis assay**

Exponentially growing *S. aureus* WCUH29, RN4220 or Oxford were incubated at 37°C with 0.5 μCi/mL [14C]acetate, [14C]thymidine, [14C]uridine, [14C]N-acetylglucosamine and 1.0 μCi/mL [14C]isoleucine in Luria Broth. Growth was independently measured spectrophotometrically, and at various time points biomass was precipitated in TCA, filtered and washed on glass fibre. The precursors are soluble in trichloroacetic acid (TCA) while the macromolecules they give rise to are not. Drug and radiolabel were added to start the experiment. Incorporated radioactivity was assayed in the form of TCA-precipitable counts per minute and data were expressed as percentage inhibition of incorporation compared with a drug-free control.

To confirm that fatty acid biosynthesis was inhibited, experiments were performed in parallel to extract total lipid into chloroform/methanol, and fatty acid methyl esters (FAMEs) were prepared using a standard BF3-methanol derivatization, extracted with hexane and purified by TLC.

**Enzymic inhibition of *S. aureus* FabI by triclosan**

Purified FabI was obtained as described previously. Assays were carried out in half-area, 96-well microtitre plates using 50 μL assay mixtures containing 100 mM sodium N-[2-acetamido]-iminodiacetic acid (NaADA), pH 6.5, 4% glycerol, 0.25 mM crotonoyl CoA, 1 mM NADH and an appropriate dilution of *S. aureus* FabI. The IC50 of triclosan was measured using a concentration range of 0.01–10 μM. The consumption of NADH was monitored for 20 min at 30°C by following the change in absorbance at 340 nm. Initial velocities were estimated from an exponential fit of the non-linear progress curves represented by the slope of the tangent at t0. The IC50 was estimated from a non-linear fit of the initial velocities to a standard, four-parameter model using GraFit 4.0 (Erithacus Software, Surrey, UK) and is reported as the mean ± s.d. of seven determinations.

**Western immunoblotting and estimation of FabI abundance in *S. aureus* cells**

A procedure described previously by Zhang *et al.* was followed. Polyclonal rabbit antibody was generated using purified *S. aureus* FabI as antigen.

**Results and discussion**

**Inhibition of FabI by triclosan**

Heath *et al.* recently reported the inhibitory activity, or IC50, of triclosan for *S. aureus* FabI to be c. 3 μM. Independently and concurrently, these experiments were carried out as described in Materials and methods. The IC50 of triclosan for *S. aureus* FabI was 0.976 ± 0.162 μM, indicating specific, targeted inhibition of FabI by triclosan in *S. aureus*, and providing further confirmation of the reported FabI activity in *S. aureus*.

**Overexpression assay**

The genetic characterization for the mode of action of triclosan was initiated by examining the MICs of triclosan for *S. aureus* that overexpressed the presumed target, FabI. The *S. aureus* RN4220 fabI gene including the putative promoter was amplified using the specific oligonucleotide primers Fab1 and Fab2, and cloned on the 4.6 kb multi-copy, *E. coli*–*S. aureus* shuttle plasmid pCU1. The complementing amplification product encompassing the fabI gene product and lacking the upstream promoter region was prepared using the primers Fab3 and Fab2, and also cloned into pCU1. The recombinant plasmids were introduced into *S. aureus* RN4220 by electroporation. MICs of triclosan for the recombinant strains are shown in the Table.

<table>
<thead>
<tr>
<th>Agent</th>
<th>pCS06b</th>
<th>pCS06A</th>
<th>pCU1</th>
<th>Wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triclosan</td>
<td>2</td>
<td>0.125</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>Neomycin</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Mupirocin</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
</tbody>
</table>

*a* The MICs were determined by a serial dilution method in brain–heart infusion or TSB media and were performed a minimum of three times.

*b* Plasmid designations are as follows: pCU1, vector; pCS06, pCU1 containing fabI with promoter; pCS06A, pCU1 containing a promoterless fabI. The wild-type strain is plasmid free.
The MIC of triclosan for *S. aureus* RN4220, expressing wild-type levels of FabI, was 0.06 mg/L. The MIC of triclosan for *S. aureus* RN4220 (pCS06), in which the copy number of FabI has been elevated via introduction of a multicopy plasmid leading to additional expression of wild-type FabI, was increased c. 30-fold over wild type, to 2 mg/L. Cells harbouring either pCU1 alone or the recombinant plasmid containing the amplicon lacking a promoter for *fabI* (pCS06A) did not show increased resistance to triclosan. These results are consistent with the observations of McMurry et al.\(^1\) for *E. coli*, in which wild-type *fabI* expressed on a multicopy plasmid in *E. coli* increased the triclosan resistance of the host strain 16- to 32-fold.

In some Gram-negative organisms such as *Pseudomonas aeruginosa* and *E. coli*, resistance to triclosan can be attributed to efflux by the MexAB-OprM or AcrAB systems rather than to overexpression of FabI.\(^{14,17}\) The *S. aureus* plasmid-containing strains were exposed to other classes of antibacterial agents to rule out any general mechanism of resistance such as efflux. No significant differences in susceptibility were observed between the strains overexpressing FabI and the corresponding wild-type strain for a range of antibiotics of different modes of action (Table). Thus, an increased MIC of triclosan was not due to an efflux mechanism in these strains. This also demonstrates that an overexpression system is a valid and selective method of testing a bacterial target’s susceptibility to a drug, as the FabI-overexpressing strain did not show any alteration in susceptibilities to a panel of unrelated antibiotics with distinct modes of action.

**Confirmation of protein levels**

Expression levels of FabI in the wild-type and FabI overexpression strains were examined by quantitative Western immunoblotting (Figure 1). The abundance of FabI in *S. aureus* strain RN4220 grown to mid-log phase was determined to be c. 12 000 copies per cell (Figure 1, lane 3). RN4220 containing the plasmid vector pCU1 had the same FabI levels as the host strain (Figure 1, lane 4), whereas the FabI-overexpressing strain RN4220(pCS06) produces approximately three- to four-fold more FabI (lane 5).

**Macromolecular synthesis**

To demonstrate that the primary mode of action of triclosan against *S. aureus* is via inhibition of fatty acid biosynthesis, exponentially growing *S. aureus* was incubated with radiolabelled macromolecular precursors of fatty acid biosynthesis, DNA replication, transcription, cell wall biosynthesis and translation, plus triclosan (Figure 2). As expected from an inhibitor of fatty acid biosynthesis, inhibition of acetate incorporation was observed early (20 min post-labelling) and increased over time; the remainder of the macromolecular profiles remained unaffected (\(\square\) 20 min; \(\square\) 40 min; \(\square\) 60 min).

FAB levels as the host strain (Figure 1, lane 4), whereas the FabI-overexpressing strain RN4220 (pCS06) produces approximately three- to four-fold more FabI (lane 5).

![Figure 1. Western immunoblotting analysis using anti-FabI antibody. Lanes 1 and 2, purified *S. aureus* FabI at 10 ng and 1 ng, respectively; lane 3, RN4220; lane 4, RN4220(pCU1); lane 5, RN4220(pCS06). Cells were grown to mid-log phase and samples were collected and assayed. Polyclonal rabbit anti-FabI antibody and HRP-conjugated goat anti-rabbit IgG (Sigma) were used at 1:1000 and 1:20 000 dilutions, respectively.](image)

![Figure 2. Macromolecular synthesis profile of *S. aureus* Oxford treated with 2 ng/mL triclosan. As expected from an inhibitor of fatty acid biosynthesis, inhibition of acetate incorporation was observed early (20 min post-labelling) and increased over time; the remainder of the macromolecular profiles remained unaffected (\(\square\) 20 min; \(\square\) 40 min; \(\square\) 60 min).](image)
In conclusion, the combination of these data provide further biochemical and genetic characterization of both the mode of action of triclosan against \textit{S. aureus} and the enoyl ACP reductase from this organism.

Acknowledgements

We thank Jeremiah Mitchell for technical assistance and the Genetic Technologies Group for sequencing support.

References


Figure 3. Macromolecular synthesis was carried out as described in Materials and methods in \textit{S. aureus} RN4220 at 20 min using sub-inhibitory levels of (a) triclosan, (b) ciprofloxacin and (c) tetracycline. Per cent inhibition is determined by comparison with non-drug-treated control. Experiments are an average of two independent measurements and the error bars indicate standard deviation from the mean.


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