Triclosan targets lipid synthesis

Triclosan is a broad-spectrum antibacterial and antifungal agent, which acts by previously unidentified mechanisms, that is used in products such as antiseptic soaps, toothpastes, fabrics and plastics. Here we show that triclosan blocks lipid synthesis in *Escherichia coli*, and that mutations in, or overexpression of, the gene *fabI* (which encodes enoyl reductase, involved in fatty acid synthesis) prevents this blockage. This is, to our knowledge, the first evidence that triclosan acts on a specific bacterial target, rather than as a nonspecific 'biocide'.

Five independent triclosan-resistant mutants of *E. coli* K12 strain AG100 (ref. 3) with different levels of resistance were isolated on LB agar plates containing 0.2 μg ml⁻¹ triclosan, a trichlorinated diphenyl ether (Irgasan, a gift from Ciba–Geigy) (Table 1). We prepared a genomic *Sau*AlI library from strain AGT11 (mutant AGT11 deleted for *acrAB*) in plasmid pBR322. Ten clones with inserts of various sizes all expressed the same level of triclosan resistance.

From the partial sequence of one clone, pLYT8, and from the *E. coli* genomic database, we identified two genes, *ycjD* and *fabI* (Fig. 1), in the insert. A deletion of *ycjD* (in clone pLYT12) or of most of *fabI* (in clone pLYT11) showed that triclosan resistance was associated with the intact *fabI* gene on pLYT12; the *tet* promoter from the vector was not required (Fig. 1).

The *fabI* gene encodes enoyl-acyl carrier protein reductase, an essential enzyme of relative molecular mass 27,900 that is protein reductase, an essential enzyme of *Escherichia coli*, pLYT12; the base, we identified two genes, was associated with the intact pLYT11) showed that triclosan resistance in the original mutant AGT11.

The sequence of a *fabI* PCR product prepared from strain AGT23 (covering the same DNA region as was sequenced for pLYT8) revealed a single mutation (ATG became ACG), resulting in the replacement of methionine 159 by threonine in the FabI protein. Strain AGT25 also had a single mutation: phenylalanine 203 was replaced by leucine (TTC became CTC).

P1 transduction of *sci-3118::Tn10kan* (ref. 7) together with wild-type *fabI*, both at position min 29 of the *E. coli* chromosome, into the two remaining (unsequenced) triclosan-resistant mutants, AGT8 and AGT9 (and into the sequenced mutant AGT11 as a control), led to loss of the triclosan-resistant phenotype at the same frequency for all of the mutants. Therefore, in the mutants whose DNA we have not yet sequenced, altered *fabI* is again probably responsible for triclosan resistance.

If FabI activity were inhibited by triclosan, fatty acid synthesis and consequently lipid synthesis should be reduced. We preincubated logarithmically growing cells of strain AG100 in LB broth with triclosan (or with other drugs as controls) for 8 minutes, and then added [1-14C]-acetate (sodium acetate, 59.5 mCi mmol⁻¹ ICN Pharmaceuticals, added at 5 μCi ml⁻¹) to assay lipid synthesis. Eight minutes later, we pipetted 0.08 ml of the culture onto a Whatman 3MM paper disc. We determined radioactivity levels after treating the discs in cold 10% trichloroacetic acid (TCA) and washing them in TCA and 10% cold ethanol. All drugs were tested at concentrations just sufficient to stop the growth of AG100 an hour after their addition.

Ethanol (0.5%), present from the solvent used for most of the drugs, had little effect by itself. Diazaborine (see below), a specific inhibitor of FabI (8 μg ml⁻¹; a gift from G. Hoeugenauer), reduced acetate incorporation into lipids by 93%. Triclosan (0.24 μg ml⁻¹) inhibited 92% of acetate incorporation, and the effect occurred within 3 minutes. In contrast, chloramphenicol (13 μg ml⁻¹), a protein-synthesis inhibitor, and ciprofloxacin (0.045 μg ml⁻¹), an inhibitor of DNA synthesis, reduced incorporation by only 19% and 2%, respectively. In the resistant mutant AGT11, lipid synthesis was blocked by only 2% with 0.24 μg ml⁻¹ triclosan and by 75% with 26 μg ml⁻¹ triclosan. These results are consistent with the target of triclosan being FabI.

Diazaborine inhibits FabI in *E. coli* and *Salmonella typhimurium*; its binding depends on the presence of the co-factor NAD⁺. In a similar way, activated isoniazid inhibits InhA, the *Mycobacterium tuberculosis* enoyl reductase, which shares significant sequence identity with *E. coli* FabI. A Gly 93→Ser mutation in *E. coli* FabI causes diazaborine resistance, reduces the binding of diazaborine to the enzyme, and

Table 1 Characteristics of triclosan-resistant *E. coli* mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>fabI</em> mutation</th>
<th>MIC (ratio to wild-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Triclosan</td>
</tr>
<tr>
<td>AG100</td>
<td>None (wild-type)</td>
<td>1.0</td>
</tr>
<tr>
<td>AGT8</td>
<td>ND</td>
<td>5.8</td>
</tr>
<tr>
<td>AGT9</td>
<td>ND</td>
<td>3.1</td>
</tr>
<tr>
<td>AGT11</td>
<td>Gly 93→Val</td>
<td>95</td>
</tr>
<tr>
<td>AGT23</td>
<td>Met 159→Thr</td>
<td>12.2</td>
</tr>
<tr>
<td>AGT25</td>
<td>Phe 203→Leu</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Minimal inhibitory concentration (MIC) values, determined by broth dilution with twofold steps, are the average of three experiments and are expressed as ratios to the MIC for the wild-type strain AG100. For triclosan, 0.8 μg ml⁻¹; diazaborine, 6 μg ml⁻¹; ND, not determined.

Figure 1 Restriction map (partial) and triclosan resistance of pLYT8 and its deletion mutants. In plasmid pLYT8 (Fig. 1), in the insert. A deletion of *ycjD* (in clone pLYT12) or of most of *fabI* (in clone pLYT11) showed that triclosan resistance was associated with the intact *fabI* gene on pLYT12; the *tet* promoter from the vector was not required (Fig. 1).

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![Figure 1 Restriction map (partial) and triclosan resistance of pLYT8 and its deletion mutants.](image-url)
lowers the specific activity of the enzyme\textsuperscript{11}. Our most triclosan-resistant mutant, AGT11, had a Gly 93→Val mutation. Its growth rate in LB broth was about 40% less than that of the wild-type parent, indicating that it may have a less active FabI enzyme.

The finding that mutations at residues 93, 159 and 203 lead to triclosan resistance correlates strikingly with the crystal structure of wild-type \textit{E. coli} FabI protein\textsuperscript{12}; all three of these residues line the cleft at which NADH binds. At this site, diazaborine is mutagenic to AGT11, requiring higher drug concentrations than those needed to stop growth, judging by a 30–50% loss in the absorbance \((A_{450})\) of susceptible cultures within two hours of the addition of triclosan, accompanied by a 4–5 log decrease in viability. Parental strain AG100 needed 0.15 \(\mu\)g ml\(^{-1}\) triclosan to inhibit the growth rate by 50% but 8 \(\mu\)g ml\(^{-1}\) for lysis, and the Gly 93→Val mutant AGT11 required 13 \(\mu\)g ml\(^{-1}\) triclosan for 50% inhibition of the growth rate but did not lyse even when 256 \(\mu\)g ml\(^{-1}\) was added; this amount far exceeds the solubility of the drug. These results indicate that the fabI mutants also offer protection against triclosan-mediated lysis.

Our results show that organisms that are intrinsically resistant to triclosan may contain triclosan-insensitive enoyl reductases. Like triclosan, other drugs that are at present thought to be non-specific ‘biocides’ might actually have specific targets.

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\section*{Oldest known fossils of monocotyledons}

The monocotyledonous angiosperm clade (class Liliopsida) includes roughly 50,000 species\textsuperscript{2} of diverse forms. The group comprises such economically noticeable plants as palms, orchids, most of the horticultural bulbs, and grasses, which include some of the most important food crops, such as maize, rice and other grains. Modern monocotyledons are diverse and dominate many habitats, but the fossil record of these plants is meagre, fossils of monocotyledonous flowers are rare, and the earliest putative monocotyledonous fossils (pollen and leaves) are all equivocal\textsuperscript{10}. Here we describe the oldest known fossil flowers that can be definitely assigned to the Liliopsida.

Our understanding of the diversification of dicotyledonous angiosperms in the Late Cretaceous period has increased given fresh evidence of fossilized flowers from North America, Sweden and Portugal\textsuperscript{11,12}. These fossils include taxa with affinities to several major clades of dicotyledons. Molecular and morphological analyses\textsuperscript{12,13} suggest that the monocotyledon clade is an early offshoot from relatively primitive dicotyledons, and one might therefore expect to find evidence for concurrent monocotyledon and early dicotyledon diversification. However, monocotyledonous fossil flowers have not been reported previously from these mid and late Cretaceous deposits.

We have now recovered fossil flowers from fluvial sediments of the Turonian Raritan Formation, which is exposed in New Jersey, United States (Upper Cretaceous stage, approximately 90 million years before present)\textsuperscript{14}. The nature of the associated palaecoflora (leaves, flowers and pollen) indicates that there was a subtropical to tropical palaecoclimate at the time of deposition. The monocotyledonous flowers are associated with abundant angiosperm dicotyledonous flowers and fruits representing at least 100 separate species (see, for example, refs 7–11), fern vegetative and reproductive structures\textsuperscript{5}, and various leaves and reproductive structures of conifers\textsuperscript{10}. The exquisite preservation of floral morphology and the often perfect retention of cell-by-cell anatomical details in these fossils allow us to study an array of characters and to compare them closely with those of modern flowers.

The fossil monocotyledonous flowers are minute, unisexual (only staminate flowers are known), trimerous, radially symmetrical and pedicellate. They are 0.06–0.1 mm across and 0.04–0.08 mm high. The perianth comprises six basally fused tepals. The tepals are triangular in shape and valvate in bud; they are glabrous and lack stomata; and their apices curved toward their adaxial sides (Fig. 1a, b).

The androecium consists of three stamens arranged in the centre of the receptacle. The anthers are sessile, two-celled and extrorse, and they each dehisce to produce a longitudinal slit exposing a one-layered endothecium with U-type thickenings. The connective extensions are thick and well developed and extend beyond the anther sacs (Fig. 1c). Clusters of pollen grains are preserved within the open anther sacs. Pollen is boat-shaped (polar/equatorial diameter = 5 \(\mu\)m/12 \(\mu\)m) and monosulcate with a fine granulose (Fig. 1d) and tectate exine, and the columellae are apparent in some broken grains. There is no indication of a gyroecium or pistiloid(s).

The distinctive combination of features and organization of the floral organs indicates a close relationship of the fossils with members of the modern monocotyledon family Triuridaceae. We tested this relationship by including the new fossil taxon in a morphological cladistic analysis that includes 103 monocotyledon taxa (comprising families and genera), the fossil taxon, and 101 morphological characters\textsuperscript{13}.

Figure 1 Scanning electron micrographs of fossil flowers from the Upper Cretaceous stage of New Jersey, \textbf{a}, Top view of a mature and open flower. The tepals show the apex curved toward the centre of the flower. Note the central position of the androecium, which is formed by three stamens, in a single whorl. Original magnification \(\times 20\); scale bar, 600 \(\mu\)m. \textbf{b}, Side view of a bud, showing the tepals basally fused and the stout pedicell. Original magnification \(\times 30\); scale bar, 300 \(\mu\)m. \textbf{c}, Dissection of a bud; note the sunken anthers in the receptacle (re), extrorsely opened and with prolonged connective extensions (cn). Original magnification \(\times 50\); scale bar, 300 \(\mu\)m. \textbf{d}, Monosulcate pollen found in one another, showing the fine granulose exine. Original magnification \(\times 4,000\); scale bar, 3 \(\mu\)m.