Triclosan targets lipid synthesis

Nature v.394 6aug98

scientific correspondence

Laura M. McMurry, Margret Oethinger, Stuart B. Levy
Center for Adaptation Genetics and Drug Resistance, and Department of Molecular Biology and Microbiology,
Tufts University School of Medicine,
Boston, Massachusetts 02111, USA
e-mail: slevy@opal.tufts.edu

Triclosan is a broad-spectrum antibacterial T and antifungal agent[1,2], which acts by previously undetermined 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*3AI library from strain AGT11K (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 involved in the synthesis of fatty acids[4]. Sequencing of the entire *fabI* gene of pLYT8 (residues 190–1,265 (numbering according to Bergler[5]), including the upstream 'BoxC' region) uncovered a single mutation, GGT to GTT, at codon 93 of the *fabI* gene. This mutation resulted in the conversion of glycine 93 to valine in the protein.
We replaced the mutation-carrying 606- base-pair *Ssp*I-*Hin*dIII fragment of mutant pLYT12 with its wild-type counterpart (derived from a KlenTaq polymerase (Clontech) polymerase chain reaction (PCR) product from parental strain AG100). The resulting sequence-confirmed plasmid, pLYT27, increased triclosan resistance in the host strain by 16- to 32-fold, showing that the wild-type gene has a clear multicopy effect, as would be expected if *FabI* were the target for the drug. As cells containing plasmid pLYT8 or pLYT12, each of which contains the Gly 93-->Val mutation, were even more resistant (about 300-fold more resistant than wild type; Fig. 1), this mutation must indeed be responsible for the triclosan resistance in the original mutant AGT11.

Table 7 Characteristics of triclosan-resistant E cob mutants

Strain	*fabI* mutation	MIC (ratio to wild type) Triclosan	Diazaborine
AG100	None (wild-type)	1.0	1.0
ACT8	ND	5.8	7.1
AGT9	ND	3.1	3.6
AGT11	Gly 93-->Val	95	37
AGT23	Met 159-->Thr	12.2	0.6
AGT25	Pre 203-->Leu	61	4.3

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 (MIC for triclosan, 0.8 µg ml^{-1}; diazaborine,, 6 µg ml^{-1}). ND, not determined.

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[6] of *zci-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-^{14}C]-acetate (sodium acetate, 59.5 µCi 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. Hoegenauer), 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*.

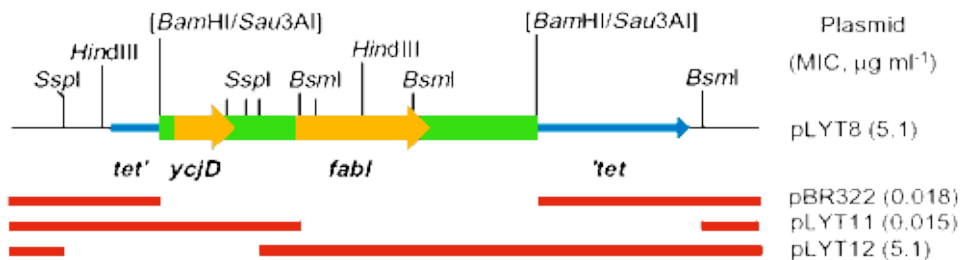

Figure 1 Restriction map (partial) and triclosan resistance of pLYT8 and its deletion mutants. In plasmid pLYT8 the thick (green and orange) horizontal region represents chromosomal DNA (*Sau*3AI-digested) inserted into the *Bam*HI site of the *tet* gene (blue) of the pBR322 vector. The deletion mutant pLYT11 was created using *Bsm*I, and deletion mutant pLYT12 was created using *Ssp*I. The minimal inhibitory concentration (MIC) of triclosan (in parentheses) was measured by agar dilution for each plasmid in hypersusceptible host AG100A (AG100 *acrAB*::kan)

Diazaborine inhibits *FabI* in *E. coli* and *Salmonella typhimurium*[8]; its binding depends on the presence of the co-factor NADH[8,9]. In a similar way, activated isoniazid inhibits InhA10, 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[8], and lowers the specific activity of the enzyme[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 *E. coli FabI* protein[12]: all three of these residues line the cleft at which NADH binds. At this site, diazaborine is covalently linked to NAD+ (ref. 12). Four of the five triclosan-resistant mutants were also resistant to diazaborine (Table 1), further indicating that *FabI* is the actual target of triclosan.

Triclosan lyses *E. coli*[13] and *Porphyromonas gingivalis*[14] cells. We found that such lysis needed higher drug concentrations than those needed to stop growth, judging by a 30–50% loss in the absorbance (A_{530}) 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* mutation also offers 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 nonspecific ‘biocides’ might actually have specific targets.

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