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Genome-Wide Enrichment Screening Reveals Multiple Targets and Resistance Genes for Triclosan in *Escherichia coli*

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Triclosan is a widely used biocide effective against different microorganisms. At bactericidal concentrations, triclosan appears to affect multiple targets, while at bacteriostatic concentrations, triclosan targets FabI. The site-specific antibiotic-like mode-of-action and a widespread use of triclosan in household products claimed to possibly induce cross-resistance to other antibiotics. Thus, we set out to define more systematically the genes conferring resistance to triclosan; A genomic library of *Escherichia coli* strain W3110 was constructed and enriched in a selective medium containing a lethal concentration of triclosan. The genes enabling growth in the presence of triclosan were identified by using a DNA microarray and confirmed consequently by ASKA clones overexpressing the selected 62 candidate genes. Among these, forty-seven genes were further confirmed to enhance the resistance to triclosan; these genes, including the FabI target, were involved in inner or outer membrane synthesis, cell-surface material synthesis, transcriptional activation, sugar phosphotransferase (PTS) systems, various transporter systems, cell division, and ATPase and reductase/dehydrogenase reactions. In particular, overexpression of *pgsA*, *rcsA*, or *gapC* conferred to *E. coli* cells a similar level of triclosan resistance induced by *fabI* overexpression. These results indicate that triclosan may have multiple targets other than well-known FabI and that there are several undefined novel mechanisms for the resistance development to triclosan, thus probably inducing cross antibiotic resistance.

Keywords: triclosan, multiple targets, genomic library, resistance, *E. coli*

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Introduction

Triclosan (5-chloro-2-(2,4,-dichlorophenoxy) phenol) is a broad spectrum antimicrobial agent and has been widely used as household biocide in hand-soaps, toothpastes, fabrics, plastics, and paints for more than 40 years (Jones *et al.*, 2000; Russell, 2004; von der Ohe *et al.*, 2012). Triclosan is reported to block lipid biosynthesis by specifically inhibiting enoyl-acyl carrier protein (ACP) reductase (FabI) in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, or its homologue, InhA, in *Mycobacerium smegmatis* and *Mycobacterium tuberculosis* (Heath *et al.*, 1998; McMurry *et al.*, 1998b; White *et al.*, 2005) However, at concentrations higher than the MIC (minimal inhibitory concentration), triclosan appears to act upon multiple non-specific targets, causing a disruption of bacterial cell membrane functions (Villalain *et al.*, 2001; Gomez Escalada *et al.*, 2005). Other studies have shown that overexpression of *marA*, *soxS* or *acrAB* results in reduced susceptibility to triclosan in *E. coli* (McMurry *et al.*, 1998a). Triclosan has also been found to be a multi-target inhibitor affecting F-ATPase, weak acid transmembrane proton carriers, glycolytic enzymes (pyruvate kinase, lactic dehydrogenase, aldolase and phosphotransferase system (PTS)), and biofilm formation in mutants of the oral pathogen *Streptococcus* that lack a triclosan-sensitive FabI (Phan and Marquis, 2006). Therefore, previous results indicate that triclosan may have multiple targets, primarily in the inner and outer membranes but also at the level of intracellular enzymes.

However, no systematic genome-scale analysis was made to define the additional targets and novel mechanisms of resistance for triclosan. In this study, we systematically screened triclosan targets by combining plasmid-based genomic library enrichment and DNA microarray analysis of enriched transformants (Lynch *et al.*, 2007). Using this approach, we screened an overexpressed genomic library in the presence of a lethal concentration of triclosan (62.5 ng/ml) and identified genes conferring resistance to triclosan. Furthermore, the selected genes were individually overexpressed and their minimal inhibitory concentrations (MICs) were measured, providing evidence for novel mechanisms of action of triclosan, i.e., multi-targeting.

Materials and Methods

Bacterial strains, plasmid, enzymes, and chemicals

To construct the genomic library, we used *E. coli* strain W3110. We also obtained the ASKA overexpression clones, harboring the pET vector that encodes candidate genes,

from the National BioResource Project (Japan) (Kitagawa *et al.*, 2006). Plasmid pUC19 and all enzymes were purchased from New England BioLabs (USA). All ligates were electroporated by a Gene Pulser system (Bio-Rad, USA) and propagated in *E. coli* W3110. In all experiments we used Luria-Bertani (LB) liquid or LB agar medium containing the following antibiotics where appropriate: 50 μg/ml ampicillin (Ap) and 17 μg/ml chloramphenicol (Cm). Isopropyl β-D-1-thiogalactopyronoside (IPTG) and triclosan were added to final concentrations of 1 mM and 62.5 ng/ml, respectively.

Preparation of *E. coli* genomic library and growth conditions

E. coli W3110 genomic DNA was prepared using Genomic Tips (QIAGEN, Germany). Purified genomic DNA was partially digested with *Sau*3AI and the DNA fragments were separated on a 1% agarose gel. Fragments between 0.5 and 2 kb were extracted and cloned into the *Bam*HI site of pUC19. Ligation products were electroporated into electro-competent *E. coli* W3110 cells and plated on LB plates with Ap. Transformants were grown overnight at 37°C and harvested by adding LB directly to the plates and scraping the cells. The genomic library was cultivated in LB medium containing Ap in the absence and presence of triclosan (62.5 ng/ml) until the cells had grown to the late exponential phase (24 h).

Plasmid purification, labeling and hybridization

Plasmids were purified by a Mini Prep kit (QIAGEN). Purified plasmids were completely digested with *Sau3A*I. The digested plasmids were confirmed on 1% agarose gel; those derived from the triclosan-treated culture (samples) were labeled using the general random primed labeling method. Digested plasmid DNA (1 mg) was mixed with 25 nM dATP, 25 nM dCTP, 25 nM dGTP, 10 nM dTTP, 40 nM Cy3-dUTP, 1 ml of Klenow fragment (3′–5′) (50 unit/ml) and Klenow buffer (Amersham Pharmacia Biotech), and incubated for 1 h at 37°C. The Cy3-labeled DNAs were mixed with equal amounts of Cy5-labeled DNAs derived from digested plasmids purified from cultures grown identically and concurrently without triclosan (control). The labeled DNA mixtures were separated from unincorporated nucleotides using a QIAquick PCR purification kit (QIAGEN) and dried by Speed-Vac. The labeled pellets were resuspended in 30 ml hybridization solution (25% formamide, 5× SSC, 0.1% SDS, 10% dextran sulfate in double-distilled water), denatured for 5 min at 95°C, and loaded on to the DNA microarray (DigtalGenomics, Korea). The *E. coli* DNA chip was supplied by the Center for the 21C Frontier Program of Microbial Genomics and Applications (Korea). Hybridization was performed for 16 h at 42°C in a hybridization chamber in a water bath. The microarrays were washed with washing buffer I (2× SSC, 0.1% SDS in double-distilled water) for 5 min at 42°C in the dark. A second washing was performed with washing buffer II (0.1× SSC, 0.1% SDS in double-distilled water) at room temperature for 10 min in the dark. The final washing was carried out with washing buffer III (0.1× SSC in double-distilled water) at room temperature for 1 h.

Microarray data quantification and analysis

The microarray was analyzed using GenePix software (Axon Instruments, USA). Resistance genes were identified on the basis of the Cy5 intensities. The resistance rank of each gene was determined for the samples obtained after the growth in the presence of triclosan (62.5 ng/ml). These experiments were repeated 5 times. The 5 ranks were averaged, and the mean was used to rank the gene insert. This final rank value provides a measure of the degree of enrichment of a particular gene insert during the growth in the presence of triclosan (62.5 ng/ml).

Overexpression of candidate genes and determination of MICs and growth

To test selected candidates with overexpression clones, we used ASKA clones provided by the National BioResource Project (Japan) (Kitagawa *et al.*, 2006). ASKA clones are a set of overexpression clones carrying individual genes under the control of IPTG-inducible promoter and the overexpression is already confirmed. Individual strains were inoculated on 96 well plates containing 200 ml LB with 1 mM IPTG, Cm, and various concentrations of triclosan (0–8,000 ng/ml) and incubated in BioscreenC (Labsystems) at 37°C for 48 h.

Results

Genome-wide screening by enrichment culture

In this study, we combined a plasmid-based genomic library screening system with DNA microarray analysis (Lynch *et al.*, 2007) for a systematic genome-wide screening for triclosan targets and resistance genes. In cultures with a lethal concentration of triclosan, cells bearing genomic inserts that confer resistance to triclosan will predominate in growth. Firstly, growth of *E. coli* W3110 was determined in LB media containing various concentrations of triclosan (0–240 ng/ml) with BioscreenC: no growth was observed at 62.5 ng/ml of triclosan. For the enrichment experiment, an *E. coli* W3110 genomic library was constructed and transformed into *E.*

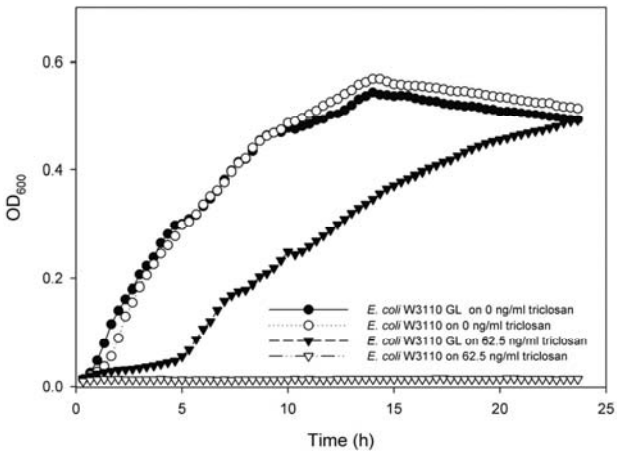

Fig. 1. Effect of triclosan on the growth of *E. coli* W3110 GL and *E. coli* W3110 (control). *E. coli* W3110 GL and its parent strain, *E. coli* W3110, were cultivated in LB medium containing ampicillin in the absence and presence of triclosan (62.5 ng/ml).

coli W3110, generating *E. coli* W3110 GL. When cultured in the presence of lethal concentration of triclosan (62.5 ng/ml), only *E. coli* W3110 GL was able to grow after a lag (Fig. 1), indicating that the overexpressed genes from the genomic library confer triclosan resistance to the clones. Therefore, plasmid DNAs isolated from enriched *E. coli* W3110 GL in the presence of triclosan were analyzed with a DNA microarray. Workflow is shown in Fig. 2.

Identification of multiple targets conferring triclosan resistance

After five repeat experiments, 74 genes which conferred growth advantage to their host cells in the presence of triclosan (62.5 ng/ml) were identified; the genes displaying 4-fold increases in expression level in DNA microarray analysis were selected and shown in Table 1. Obviously, many genes involved in lipid and cell wall biosynthesis were identified because the triclosan's original target is in FabI, fatty acid synthesis pathway. In particular, *fabI*, *pgsA*, *acpP*, and *accD* are all involved in lipid biosynthesis, and *rfe*/*rfaY* and *fimDFHI*/*htrE* are involved in LPS and fimbrial biosynthesis, respectively. In addition, *rscA* acts as an activator in colanic acids and capsule synthesis. Several genes encoding PTS systems such as the fructose-like transporter (*frvAB*/*frwC*), arbutin, cellobiose, and salicin transporter (*ascF*) and N-acetylgalactosamine transporter (*agaVW*) were identified. A putative ABC transporter (*uup*), putative drug efflux transporter (*emrK*), heme exporter (*ccmB*), and putative protein transporter (*fdrA*) were also identified. As genes related to carbohydrate metabolism, *gapC*, *glpA*, and *edd* were selected; *gapC* encodes glyceraldehyde 3-phosphate dehydrogenase C and is known to be a pseudogene: *glpA* is a glycerol-3-phosphate dehydrogenase involved in glycerol degradation and anaerobic respiration: *edd* is an Entner-Doudoroff pathway enzyme. Protein metabolism-related genes encoding chaperons (*dnaK* and *mopA*), ribosomal protein subunits (*rplI,* and *rpsN/M*) and tRNA synthetases (*tyrS* and *trpS*) were also identified. In addition, various regulators and cell division-related genes were found. As transcriptional regulators, *sdiA*, *asr*, *idnR*, *arcB*, *ascG*, *fnr*, *narL*, and *crp* were identified, and cell division genes (*ftsL*/*H*) were also identified. Finally, oxidoreductases and dehydrogenases including *fabI*, *hycB/D*, *gapC*, *fdhF*, *hisD*, *glpA*, and *hemG* were found in this DNA microarray analysis.

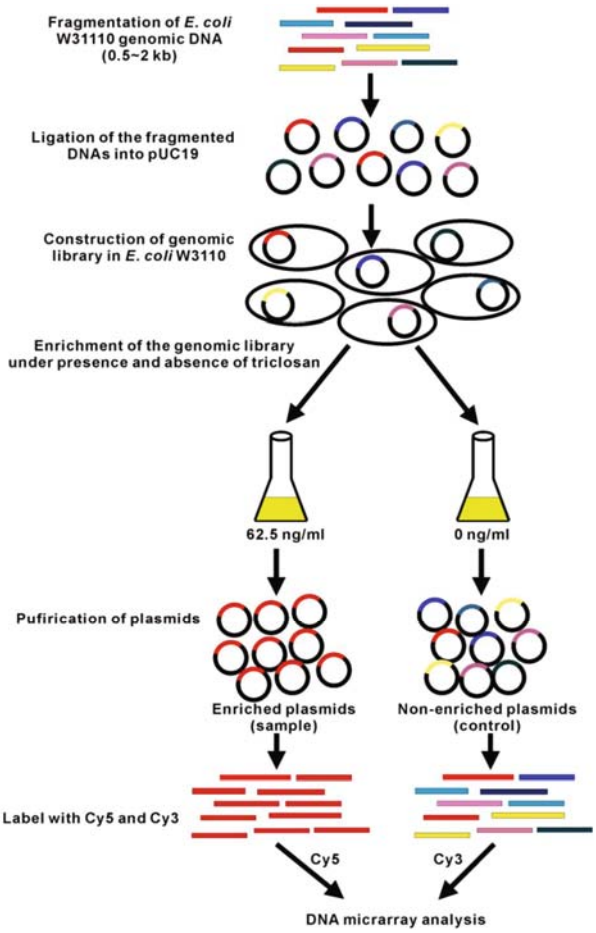

Fig. 2. An overall scheme of the genome-wide screening for multiple targets conferring triclosan resistance to *E. coli*. *E. coli* W3110 genomic DNA was fragmented with *Sau3A*I, size selected (0.5–2 kb), and cloned into the *Bam*HI site of the pUC19. *E. coli* W3110 competent cells were electroporated with ligation products, generating a genomic library, *E. coli* W3110 GL. *E. coli* W3110 GL cells were cultivated in LB medium containing ampicillin in the absence and presence of triclosan (62.5 ng/ml) until the cells reached the late exponential phase. All library pools were harvested and their plasmids were purified, completely digested with *Sau3A*I, and labeled with Cy5 (sample) and Cy3 (control), respectively. The mixtures were hybridized to an *E. coli* DNA microarray and specific genes conferring triclosan tolerance were identified.

MICs and growth curves of ASKA overexpression clones of candidate genes

From the earlier identified genes, sixty-two of them were further tested with ASKA overexpression clones. We tested MICs for each clone from ASKA, because we wanted to clarify the resistance level when those probable target genes were overexpressed. The resistance they conferred against various concentrations of triclosan (0–8,000 ng/ml) was assessed to determine MICs (Table 2). In particular, overexpression of *pgsA*, *rcsA* or *gapC* conferred almost the same level of triclosan resistance (2,000 ng/ml) when compared to the MIC of the *fabI* overexpression clone. In addition, overexpression of *ahpF* and *ascF* induced an approximately 10-fold increase in MIC compared to that of the wild type strain (62.5 ng/ml). The other 41 clones also showed increased MICs, from 2- to 4-fold. In order to further confirm the results we tested growths of *pgsA*, *rcsA* or *gapC* overexpression clones in the presence of triclosan and compared to that of *fabI* overexpression clone. As shown in Fig. 3, these three clones displayed growth at high concentrations of triclosan (2,000 ng/ml) but with some lags.

Discussion

Triclosan blocks the enoyl-acyl carrier protein(ACP) reductase, preventing the bacterium from synthesizing fatty acids essential for building cell membranes and for other cellular functions (McMurry *et al.*, 1998a; Heath *et al.*, 1999).

Table 1. Enriched genes from *E. coli* W3110 GL in the presence of triclosan (62.5 ng/ml). Only more than 4-fold enriched genes are listed.

Gene	Function	Class[a]
hisD	Histidinol dehydrogenase	AAM
murI	Glutamate racemase	
ilvA	Threonine dehydratase	
hycB	Formate hydrogenlyase subunit 2	BEN
atpF	ATP synthase B chain	
cybC	Soluble cytochrome b562 precursor	
hycD	Formate hydrogenlyase subunit 4	
gapC	Glyceraldehyde 3-phosphate dehydrogenase C	CHM
edd	Phosphogluconate dehydratase	
glpA	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	
agaV	PTS system, N-acetylgalactosamine-specific IIB component 2	
frvA	PTS system, fructose-like-1 IIA component	
frwB	PTS system, fructose-like-2 IIB component 1	
frvB	PTS system, fructose-like-1 IIBC component	
fabI	Enoyl-[acyl-carrier-protein] reductase	LPC
pgsA	CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase	
acpP	Acyl carrier protein	
accD	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	
rfe	GlcNAc-1-phosphate transferase	
rfaY	Lipopolysaccharide core biosynthesis protein rfaY	
fimD	Type 1 fimbriae	
fimF	FimF protein precursor	
fimH	FimH protein precursor	
fimI	Fimbrin-like protein precursor	
htrE	Outer membrane usher protein, fimbrial assembly	
ahpF	Alkyl hydroperoxide reductase subunit F	MSM
ascF	PTS system, arbutin-, cellobiose-, and salicin-specific IIABC component	MTR
uup	ABC transporter ATP-binding protein uup	
frwC	PTS system, fructose-like-2 IIC component	
emrK	Multidrug resistance protein K	
ccmB	Heme exporter protein B	
oppA	Periplasmic oligopeptide-binding protein precursor	
agaW	PTS system, N-acetylgalactosamine-specific IIC component 2	
fdrA	Protein transporter	
rpoC	DNA-directed RNA polymerase beta' chain	NAM
fmt	Methionyl-tRNA formyltransferase	
rnb	Exoribonuclease II	
recT	Recombinase	
pncB	Nicotinate phosphoribosyltransferase	NCM
aceF	Dihydrolipoamide acetyltransferase component	
hemG	Protoporphyrinogen oxidase	
dut	Deoxyuridine 5′-triphosphate nucleotidohydrolase	
dnaK	Chaperone protein dnaK	PMS
rplI	50S ribosomal subunit protein L9	
rpsN	30S ribosomal protein S14	
rpsM	30S ribosomal protein S13	
tyrS	Tyrosine tRNA synthetase	
trpS	Tryptophan tRNA synthetase	
mopA	Chaperonin	
sdiA	Regulatory protein	RCD
asr	Acid shock protein	
ftsL	Cell division protein	
idnR	L-idonate regulatory protein	
arcB	Aerobic respiration control sensor protein	
ftsH	Cell division protein	
ascG	Cryptic asc operon repressor	
fnr	Fumarate regulatory protein	
crp	Catabolite gene activator	
rcsA	Colanic acid capsular biosynthesis activation protein A	SMC
fdhF	Formate dehydrogenase H	
narL	Nitrate/nitrite response regulator protein	
fimD	Outer membrane usher protein fimD precursor	
atoC	Acetoacetate metabolism regulatory protein atoC	
ydfB, ydgT, yqjI, yeaZ, ygiC, yhaI, yqjC, yieK, ygaR, ybjE, yodB, yjcQ		UNC

[a] AAM, Amino acid metabolism; BEN, Bioenergetics; CHM, Carbohydrate metabolism; LPC, Lipid and cell wall biosynthesis; MSM, Miscellaneous metabolism; MTR, Membrane transport; NAM, Nucleic acid metabolism; NCM, Nucleotide and cofactor metabolism; PMS, Protein metabolism and secretion; RCD, Regulation and cell cycle and division; SMC, signaling, motility and chemotaxis; UNC, Uncategorized.

Triclosan was widely considered to be a non-specific biocide that attacks the bacterial membrane until the missense mutation in FabI (G93V), which confers resistance by preventing the formation of the FabI-NAD^+-triclosan ternary complex, was found (McMurry *et al*., 1998b; Heath and Rock, 2000). *E. coli* cells possess only a single NADH-dependent enoyl-ACP reductase encoded by *fabI* that utilizes all chain lengths. In the inhibition of FabI, the bicyclic ring of triclosan stacks with the nicotinamide ring of NAD^+. However, recent studies have suggested that triclosan may act upon multiple targets (Villalain *et al*., 2001; Gomez Escalada *et al*., 2005; Phan and Marquis, 2006). In this study, we analyzed the *E. coli* genes responsible for increased triclosan resistance using genomic library enrichment followed by microarray analysis.

Among the selected candidates showing a high resistance to triclosan, it is natural that several genes are involved in cell defense against triclosan. RcsA is a short-lived protein that interacts with RcsB to optimize transcription of genes involved in the synthesis of colanic acids. It has been reported that the level of RcsA is limiting for colanic acid synthesis and is kept very low by its sensitivity to Lon protease (Costa and Antón, 2001). Therefore, overexpression of *rcsA* will accelerate the synthesis of colanic acids, forming a barrier against triclosan by generating mucoid colonies. It has been reported that cells covered with colanic acids were more resistant to acid and heat treatment and to osmotic and oxidative stress. Taken together, these results strongly suggest that overexpression of *rcsA* contributes to high tolerance to triclosan by overproducing colanic acids. Overexpression of *sdiA* is known to change the cell morphology to rounder and shortened forms (Wei *et al*., 2001) and leads to resistance to

Table 2. MIC values of triclosan in ASKA clones overexpressing candidate genes conferring resistance to triclosan

Gene	Function	Class[a]	MIC (ng/ml)
fabI	Enoyl-[acyl-carrier-protein] reductase	LPC	2000
pgsA	CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase	LPC	2000
rcsA	Colanic acid capsular biosynthesis activation protein A	SMC	2000
gapC	Glyceraldehyde 3-phosphate dehydrogenase C	CHM	2000
ahpF	Alkyl hydroperoxide reductase subunit F	MSM	500
ascF	PTS system, arbutin-, cellobiose-, and salicin-specific IIABC component	MTR	500
sdiA	Regulatory protein SdiA	RCD	270
fdhF	Formate dehydrogenase H	SMC	270
fdrA	Protein transporter	MTR	250
pncB	Nicotinate phosphoribosyltransferase	NCM	250
uup	ABC transporter ATP-binding protein	MTR	250
narL	Nitrate/nitrite response regulator protein	SMC	250
ydfB	Hypothetical protein	UNC	250
ydgT	Hypothetical protein	UNC	250
hycB	Formate hydrogenlyase subunit 2	BEN	250
yqjI	Hypothetical protein	UNC	250
frwC	PTS system, fructose-like-2 IIC component	MTR	250
atpF	ATP synthase B chain	BEN	250
asr	Acid shock protein	RCD	250
dnaK	Chaperone protein	PMS	125
aceF	Dihydrolipoamide acetyltransferase component	NCM	140
htrE	Outer membrane usher protein htrE precursor	UNC	140
hisD	Histidinol dehydrogenase	AAM	140
fimD	Outer membrane usher protein fimD precursor	SMC	140
ftsL	Cell division protein	RCD	125
edd	Phosphogluconate dehydratase	CHM	125
yeaZ	Hypothetical protease	UNC	125
acpP	Acyl carrier protein	LPC	125
glpA	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	CHM	125
atoC	Acetoacetate metabolism regulatory protein	SMC	125
emrK	Multidrug resistance protein K	MTR	125
ygiC	Hypothetical protein	UNC	125
ccmB	Heme exporter protein B	MTR	125
accD	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	LPC	125
yhaI	Hypothetical protein	UNC	125
rpoC	DNA-directed RNA polymerase beta' chain	NAM	125
idnR	L-idonate regulatory protein	RCD	125
	Protoporphyrinogen oxidase	NCM	125
agaV	PTS system, N-acetylgalactosamine-specific IIB component 2	CHM	125
dut	Deoxyuridine 5′-triphosphate nucleotidohydrolase	NCM	125
frvA	PTS system, fructose-like-1 IIA component	CHM	125
frwB	PTS system, fructose-like-2 IIB component 1	CHM	125
cybC	Soluble cytochrome b562 precursor	BEN	125
yqjC	Protein yqjC precursor	UNC	125
yieK	Hypothetical protein	UNC	125
murI	Glutamate racemase	AAM	125
ygaR	Hypothetical	UNC	125
imp	Organic solvent tolerance protein precursor	UNC	0
ybjE	Hypothetical protein	UNC	0
oppA	Periplasmic oligopeptide-binding protein precursor	MTR	0
hycD	Formate hydrogenlyase subunit 4	BEN	0
arcB	Aerobic respiration control sensor protein	RCD	0
rfe	GlcNAc-1-phosphate transferase	LPC	0
fmt	Methionyl-tRNA formyltransferase	NAM	0
rfaY	Lipopolysaccharide core biosynthesis protein	LPC	0
fimF	FimF protein precursor	LPC	0
fimH	FimH protein precursor	LPC	0
agaW	PTS system, N-acetylgalactosamine-specific IIC component 2	MTR	0
yodB	Cytochrome b561 homolog 1	UNC	0
fimI	Fimbrin-like protein fimI precursor	SMC	0
frvB	PTS system, fructose-like-1 IIBC component	CHM	0
yjcQ	Hypothetical transporter	UNC	0

[a] AAM, Amino acid metabolism; BEN, Bioenergetics; CHM, Carbohydrate metabolism; LPC, Lipid and cell wall biosynthesis; MSM, Miscellaneous metabolism; MTR, Membrane transport; NAM, Nucleic acid metabolism; NCM, Nucleotide and cofactor metabolism; PMS, Protein metabolism and secretion; RCD, Regulation and cell cycle and division; SMC, signaling, motility and chemotaxis; UNC, Uncategorized.

the DNA-damaging agent mitomycin C as well as to other drugs (Rahmati *et al.*, 2002). In particular, its overexpression affects the expression of a number of genes including the *acrAB* multidrug efflux pump proteins. NarL is also a dual transcriptional regulator in *E. coli*, and over-expression of *narL* also causes a drug-resistant phenotype (Hirakawa *et al.*, 2003). Therefore, overexpression of *sdiA* or *narL* could confer triclosan resistance to *E. coli* cells by changing cell morphology and stimulating multidrug efflux pumps. *ascF* is known as a cryptic PTS family gene in *E. coli*. The cryptcity of this and other *E. coli* β-glucoside metabolic operons presumably serve as a protective device against toxic β-glucosides found in nature (Hall and Xu, 1992).

In summary, several defense mechanisms against triclosan may be suggested from this study, which is concomitant with the our previous genomic signature studies with *E. coli* triclosan-resistant mutant IFN4 (Yu *et al.*, 2010); First, cells can protect themselves by overproducing cell-surface material, especially colanic acids and membrane lipids, resulting in a stronger cell wall barrier: Second, increased multidrug efflux and transporters may enhance more efficient triclosan efflux: Third, cells may decrease the effective internal concentration of triclosan by producing more dehydrogenases and reductases, which can bind and capture triclosan. In this connection, it is notable that resistance development to triclosan via diverse mechanisms may contribute to the cross-resistance to clinically important antimicrobials (Yazdankhah *et al.*, 2006). A proteomic analysis of these overexpression clones would reveal more conclusively a novel triclosan resistance network in *E. coli* (Webber *et al.*, 2008; Condell *et*

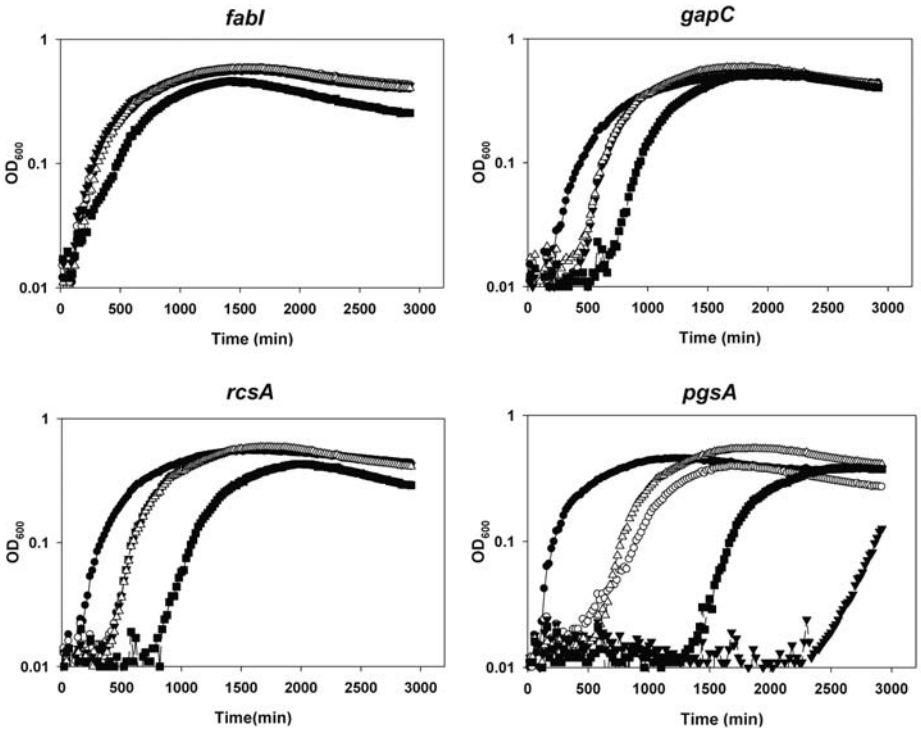

Fig. 3. Growth curves of *fabI*, *gapC*, *rcsA*, and *pgsA* overexpressed *E. coli* ASKA clones. Growths were automatically monitored in BioscreenC. Triclosan concentrations; 0 ng/ml (●), 125 ng/ml (○), 250 ng/ml (▼), 500 ng/ml (▽), 2000 ng/ml (■)

al., 2012).

Is triclosan a multi-targeting biocide? Of the genes conferring resistance to triclosan, dehydrogenase/oxidoreductase group is of particular interest; GapC conferred a comparable level of triclosan resistance to FabI, the defined triclosan target. So it is presumably not surprising that among these dehydrogenases/oxidoreductases, additional target of triclosan may exist. If triclosan target can be defined just simply by overexpression experiments, identified dehydrogenases/oxidoreductases are certainly 'multiple' targets of triclosan. Thus, one of the most critical findings of this study is in that there are other targets of triclosan, apart from well-established FabI; Some of the candidates identified are dehydrogenases (*gapC*, *hycB/D*, *glpA*, *edd*, and *hidD*), oxidoreductases (*fabI*, *ahpF*, *fdrA*, and *fdhF*) and an NAD salvage pathway related gene (*pncB*), which respectively use NAD^+ as a cofactor, contain an NAD/FAD domain or are involved in producing NAD^+. Because the bicyclic ring of triclosan stacks with the nicotinamide ring of NAD^+ (Heath and Rock, 2000), these enzymes might bind to triclosan when overexpressed. So, it is reasonable to claim that triclosan display multiple targets especially among dehydrogenases and oxidoreductases including *gapC* and others. Ii is particularly notable that *gapC*-overexpression clone grows almost like *fabI*-overexpression clones (Fig. 3). This suggestion of triclosan as a multi-targeting antimicrobial remains to be checked by a molecular modeling and by the site directed mutagenesis experiment.

These results, in short, strongly suggest that triclosan affect diverse and multiple targets in addition to FabI. Interesting question may arise here: Is this multi-targeting mode-of-action make triclosan a successful biocide? It is notable that, compared to its long and wide history of usage, there has been no serious resistance problem around triclosan (Russell, 2004; Saleh *et al.*, 2011). Thus, it is quite intuitive and tempting to suggest that developing multi-targeting antimicrobials like triclosan would be more effective in treatment of infectious disease and prevention of resistance development (Silver, 2007; Brötz-Oesterhelt and Brunner, 2008).

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