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Triclosan Resistance in a Bacterial Fish Pathogen, *Aeromonas salmonicida* subsp. *salmonicida*, is Mediated by an Enoyl Reductase, FabV

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Triclosan, the widely used biocide, specifically targets enoyl-acyl carrier protein reductase (ENR) in the bacterial fatty acid synthesis system. Although the fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* exhibits triclosan resistance, the nature of this resistance has not been elucidated. Here, we aimed to characterize the triclosan resistance of *A. salmonicida* subsp. *salmonicida* causing furunculosis. The fosmid library of triclosan-resistant *A. salmonicida* subsp. *salmonicida* was constructed to select a fosmid clone showing triclosan resistance. With the fosmid clone showing triclosan resistance, a subsequent secondary library search resulted in the selection of subclone pTSR-1. DNA sequence analysis of pTSR-1 revealed the presence of a chromosomal-borne *fabV*-encoding ENR homolog. The ENR of *A. salmonicida* (FabVas) exhibited significant homology with previously known FabV, including the catalytic domain $YX_{(8)}K$. *fabVas* introduction into *E. coli* dramatically increased its resistance to triclosan. Heterologous expression of FabVas might functionally replace the triclosan-sensitive FabI *in vivo* to confer *E. coli* with triclosan resistance. A genome-wide search for *fabVas* homologs revealed the presence of an additional *fabV* gene (*fabVas2*) paralog in *A. salmonicida* strains and the *fabVas* orthologs from other gram-negative fish pathogens. Both of the potential FabV ENRs expressed similarly with or without triclosan supplement. This is the first report about the presence of two potential FabV ENRs in a single pathogenic bacterium. Our result suggests that triclosan-resistant ENRs are widely distributed in various bacteria in nature, and the wide use of this biocide can spread these triclosan-tolerant ENRs among fish pathogens and other pathogenic bacteria.

Keywords: *Aeromonas salmonicida*, enoyl reductase, FabV, triclosan resistance

Introduction

Antibiotic resistance and the growing number of multidrug-resistant bacteria has become a global public health concern [43]. One of the major concerns about antibiotic resistance is its rapid development over time, from resistance to single class of antibiotics to multidrug resistance, and finally to extreme drug resistance, raising a challenge for the development of more effective antibiotics [41]. Anthropogenic activities such as the use of biocides [12] and agricultural practices have significantly increased the emergence of antibiotic-resistant bacteria in the environment, with recent examples of multidrug-resistant genes from marine environments [1, 23], farms [49], soils [5], and wastewater treatment plants [34].

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a biocide used in hand soaps, toothpaste, body washes, household plastics, deodorants, fabrics, chopping boards, shampoos, and a variety of various personal care and other commercial products. It exhibits broad-spectrum activity against many microorganisms, including gram-positive and gram-negative bacteria, some fungi, and mycobacteria (to some extent) [36]. Triclosan specifically inhibits the bacterial fatty acid biosynthesis system (FAS) by binding to

the enoyl substrate site of enoyl-acyl carrier protein (ACP) reductase (ENR). The detailed mechanism for ENR inhibition by triclosan includes ternary complex formation by accelerated binding of NAD^+ and triclosan to the enoyl substrate site of FabI, the triclosan target ENR [14]. However, triclosan-resistant bacteria are abundant in nature, and many mechanisms for triclosan resistance are known, namely (i) overexpression of ENR [46]; (ii) presence of mutated and/or triclosan-tolerant ENR [26]; (iii) the moderation of outer membrane [35]; and (iv) the upregulation of efflux pumps [27, 46]. More recently a number of other target genes from *Escherichia coli* (*pgsA*, *rcsA*, *gapC*, *ahpF*, *ascF*) conferring resistance to triclosan have been identified as potential targets for triclosan [47]. Triclosan has also been known to induce cross-resistance in various microorganisms such as *Pseudomonas aeruginosa* [7], *Salmonella enterica* serovar Typhimurium [2], *Staphylococcus aureus* [38], *Mycobacterium smegmatis* [26], *Acinetobacter baumannii* [6], and *E. coli* [47].

Most of the FAS-II enzymes are relatively conserved among bacteria [18], except those catalyzing the last step of the fatty acid elongation cycle, namely, the ENR, resulting in the formation of a saturated acyl-ACP by an NAD(P)H-dependent reduction of the double bond of enoyl-ACP. Initially, this reaction was known to be catalyzed by the product *fabI*, considered a potent target of diazaborine compounds. This enzyme was later shown to be the target for triclosan [28]. A number of missense mutations were also reported from *fabI*, leading to triclosan resistance. So far, four ENR isozymes have been reported from bacteria, namely FabI, which is the only ENR in *E. coli* [4], FabL [16], FabV [25], and FabK [15]. Except for FabK, which is a TIM barrel flavoprotein, all other ENRs are members of the short-chain dehydrogenase/reductase (SDR) superfamily [24]. Most of the bacterial groups contain a chromosomal *fabI* gene, whose products contain a conserved Tyr-156–$(Xaa)_6$–Lys-163 (according to amino acids numbering in FabI of *E. coli*) catalytic dyad, sharing about 40% similarity to *E. coli* FabI [25]. FabL, a moderately triclosan-resistant NADPH-dependent ENR, was identified in *Bacillus subtilis.* Although FabL shares a degree of sequence homology and a similar catalytic dyad with FabI, it is not a member of the FabI family [16]. Recently, a third class of ENR, FabV, was reported from *Vibrio cholerae,* which is completely refractory to triclosan inhibition. FabV is well conserved among a variety of organisms, including several clinically important pathogens such as *P. aeruginosa*, *Yersinia pestis*, and *Burkholderia* species [25]. This enzyme has a different catalytic domain (Tyr-Xaa_8-Lys). Unlike FabI and FabL, which have a space of six amino acid residues between the active site tyrosine and lysine, this enzyme has a space of eight residues in the catalytic dyad. This catalytic domain differs from the other SDR superfamily, enclosing seven amino acid residues between tyrosine and lysine [25]; moreover, this enzyme is 60% larger than the typical SDR family members [31].

The genus *Aeromonas* comprises 14 bacterial species, mostly human pathogens and several fish pathogens, and is widespread in aquatic environments [32]. *A. hydrophila*, *A. veronii* biovar *sobria*, *A. caviae*, *A. jandaei*, *A. veronii* biovar *veronii*, *A. schubertii*, and *A. trota* have been associated with various human infections, including gastroenteritis, wound infections, and septicemia [11]. *A. salmonicida* is a non-motile aeromonad, the etiological agent of furunculosis, a condition of bacterial septicemia in fish, and specifically salmonid fish [8]; however, the atypical type of *A. salmonicida* subsp. *salmonicida* has a broad host range, including even non-salmonid fish [43].

Genome analysis of *A. salmonicida* subsp. *salmonicida* A449 chromosome and two large plasmids [32] has revealed that this organism carries more than 25 genes for multidrug resistance and major facilitator efflux family proteins to counteract antimicrobials [9, 33]. As triclosan from all sources finally makes it way to water (ultimately sea water), marine bacteria must have evolved certain mechanisms to tolerate this toxic biocide in their corresponding environment. In this study, we aimed to investigate the triclosan resistance mechanism of *A. salmonicida*. To our best knowledge, this is the first study describing the triclosan resistance mechanism specifically in *A. salmonicida* subsp. *salmonicida,* and possibly in most of the other known fish pathogenic bacteria.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains DH5α and EPI-300 were routinely grown at 37°C in Luria-Bertani (LB) broth or LB agar, while *A. salmonicida* subsp. *salmonicida* strain 14791 (*AS* 14791) was grown at 25°C on tryptone soya medium (Oxoid, UK) supplemented with the desired antibiotics. The antibiotic concentration used for susceptibility testing was as follows: ampicillin 100 μg/ml; tetracycline 20 μg/ml; chloramphenicol 30 μg/ml; and triclosan 1–100 μg/ml. Triclosan was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fosmid pCC1FOS (Epicentre, USA) was used to construct the genomic library, whereas pUC119 and pRK415 were used for further subcloning experiments. The *AS* 14791 strain was obtained from the Korean Agricultural Culture Collection. In order to examine its resistance to triclosan, this strain was grown on tryptone soya medium supplemented with 20 μg/ml of triclosan.

Table 1. Strains and plasmids used in this work.

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	F-, *Δ(argF-lac)169*, φ80d*lacZ58*(M15), *ΔphoA8*, *glnX44*(AS), *λ-*, *deoR481*, *rfbC1*?, *gyrA96*(NalR), *recA1*, *endA1*, *thiE1*, *hsdR17*	[37]
EPI-300	F-, *mcrA Δ(mrr-hsd RMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (StrR) nupG trfA tonA*	Epicentre
A. salmonicida subsp. *salmonicida*		
KACC 14791	Triclosan resistance	Korean Agricultural Culture Collection
Plasmids		
pCC1FOS	Cp^r, copy control fosmid vector	Epicentre
pUC119	Ap^r, cloning vector	[45]
pRK415	Tc^r, RK2-derived broad-host-range vector	[19]
pTSR	Cp^r, triclosan-resistant fosmid clone carrying 30 kb insert DNA of *A. salmonicida* in pCC1FOS	This study
pTSR-1	Ap^r, pUC119 carrying a 1.6 kb *Bam*HI fragment of pTSR with triclosan resistance	This study
pTSRK415	Tc^r, pRK415 containing a 1.6 kb *Bam*HI fragment from pTSR-1	This study

[a]Ap^r, ampicillin resistance; Cp^r, chloramphenicol resistance; Tc^r, tetracycline resistance.

Determination of Minimum Inhibitory Concentration (MIC)

In order to determine the MIC of triclosan, *E. coli* EPI-300 cells were first grown to an OD_{600} of 1.0, and this bacterial suspension was serially diluted up to 1×10^5 CFU/ml. The cell suspension (1×10^5 CFU/ml) was spread onto the LB agar medium containing triclosan in a range of 0.1–10 μg/ml. LB plates were incubated at 37°C for 3 days and bacterial colony formation was examined at regular intervals of 24 h. The lowest concentration of triclosan that prevented the bacterial growth of *E. coli* EPI-300 was considered as the MIC for triclosan. This experiment included triplicates for each concentration of triclosan.

General DNA Manipulations

Standard recombinant DNA techniques were followed using the method previously described [37]. DNA sequencing and primer synthesis were performed commercially at the DNA sequencing facility of MacroGen (Seoul, Korea). Sequence comparisons (nucleotides/amino acids) were performed using the BLAST and ORF finder online service provided by the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov). Multiple alignment analysis was performed using BioEdit software in combination with GeneDoc (http://www.cris.com/~Ketchup/genedoc.shtml).

Construction of Genomic Library and Selection of Clones Showing Triclosan Resistance

The genomic library of *AS* 14791 was constructed in *E. coli* EPI-300 using the fosmid pCC1FOS (Epicentre). Genomic DNA was isolated with a Dokdo-prep bacterial genomic DNA purification kit according to the manufacturer's instructions. The genomic DNA was size-fractionated in a 0.5% low-melting-point agarose gel and DNA fragments over 30 kb were collected for library construction. The purified genomic DNA was ligated into a linearized pCC1FOS vector. The ligation mixture was then packaged into lambda phages using packaging extracts, and subsequently introduced into *E. coli* EPI-300 to select transformants on LB agar supplemented with chloramphenicol. These genomic libraries in *E. coli* were stored at −80°C in cryotubes as clone pools with over 500 clones in each pool.

To select triclosan-resistant clones from the fosmid library, the library pool stocks were diluted in a buffer (per liter: NaCl, 8.5 g; KH_2PO_4, 0.3 g; Na_2HPO_4, 0.6 g; $MgSO_4$, 0.2 g; gelatin, 0.1 g), and genomic clones of *AS* 14791 were spread on LB agar containing 30 μg/ml of chloramphenicol and 10 μg/ml of triclosan. The number of clones per plate was adjusted to approximately 500 by dilution of the library stock, and at least 5-fold the number per initial stock was used to select triclosan-resistant clones. Growing *E. coli* colonies were picked on LB with triclosan and further tested at higher concentrations of triclosan up to 100 μg/ml. Pure cultures of triclosan-resistant clones were processed for fosmid isolation followed by *Bam*HI restriction digestion, and the unique clones were finally selected based on *Bam*HI restriction profiles.

Since all selected clones showed a similar restriction pattern, we chose one of the selected fosmid library clones carrying pTSR with a 30 kb insert; the pTSR was partially digested with *Sau*3AI to subclone the triclosan-resistant gene. DNA fragments of 1–10 kb were gel purified, ligated into the *Bam*HI site of pUC119, and a shotgun library was transformed into *E. coli*. Plasmid restriction

profiling of transformants showing elevated levels of triclosan resistance was compared to select the subclone carrying the smallest DNA insert. In order to eliminate the high copy number effect of pUC119, the insert DNA was also transferred into the low-copy-number vector pRK415, resulting in pTSRK415. Triclosan resistance of *E. coli* carrying pTSRK415 was also investigated.

RNA Extraction from *Aeromonas salmonicida* subsp. *salmonicida* and Reverse-Transcription Quantitative PCR (RT-qPCR)

The relative RNA expression levels of *fabVas* and *fabVas2* were determined by RT-qPCR. Bacterial total RNA was isolated using the Hybrid-R RNA extraction kit (GeneAll Bio Inc., Seoul, Korea) from bacterial culture at the late exponential growth stage after 48 h incubation in the absence and presence of triclosan (5 μg/ml) at 25°C. The RNA was eluted in RNase-free water, and was directly used as template for cDNA synthesis by the Prime Script RT Master Mix kit (TaKaRa Biotech. Co., Japan), by following the manufacturer's instructions. Potential contamination of DNA in the RNA was excluded by DNaseI treatment prior to cDNA synthesis. The cDNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Quantitative PCR (qPCR) using the cDNA was conducted using a CFX384 Real Time System (Bio-Rad, Hercules, CA, USA). The qPCR components contained SYBR Premix Ex Taq mix (TaKaRa Biotech. Co., Japan), 1 μl of diluted cDNA template, 10 μM both forward and reverse primers (Table 2), and RNase free water. Thermal cycling included two reaction steps; an initial preheat for 3 min at 95°C, followed by 39 cycles at 95°C for 5 sec, 55°C for 10 sec, and 72°C for 35 sec. The qPCR data were displayed using the CFX Manager software ver. 3.1. The oligonucleotide primers were designed based on the *A. salmonicida* subsp. *salmonicida* A449 geonome sequence (Table 2). RT-qPCR results of individual genes were evaluated using the iCycler iQ Real-Time PCR Detection System. The C(t) values of qPCR products of each gene were used to determine the target cDNA concentration based on the relative comparison with *gyrA* gene expression of *A. salmonicida* subsp. *salmonicida* as a reference gene. The cDNA copies for *fabVas* and *fabVas2* of *A. salmonicida* subsp. *salmonicida* were compared with the number of cDNA copies of the *gyrA* gene. The RT-qPCR experiments included three replicates.

Table 2. Oligonucleotides used for RT-qPCR.

Gene	Forward (5′- 3′) Reverse (5′- 3′)	Tm[a] (°C)	Amplicon size (bp)
$fabV_{as}$	F: TGCTTCCACCGGTTATGGTC	55	192
	R: GCATCGCCGTTGACACTTTT		
$fabV_{as2}$	F: GTGCCTCCTCTGGCTTCG	55	266
	R: GATCACCAGATCCACCTGCC		
gyrA	F: CATCATCGTTCACGAGCTGC	55	268
	R: AAGGTTCATCACCTTGGGCT		

[a]Annealing temperature for PCR amplification.

Results

Candidate of Triclosan Resistance Gene for *Aeromonas salmonicida* A449

A. salmonicida subsp. *salmonicida* 14791 showed elevated levels of triclosan resistance when tested on tryptone soya medium containing 20 μg/ml of triclosan. In order to have an initial understanding of the mechanism of triclosan resistance in the *A. salmonicida* subsp. *salmonicida*, we searched for homologs of four already known ENR genes from the genome of *A. salmonicida* subsp. *salmonicida* A449, which was completely sequenced. The closest candidate was ABO89763, annotated as *trans*-2-enoyl-CoA reductase, exhibiting 70% similarity with FabV, a triclosan-tolerant ENR. Moreover, the FabV homolog from *A. salmonicida* subsp. *salmonicida* A449 contains the Tyr-$(Xaa)_8$-Lys motif, first seen in *V. cholerae* FabV, indicating that this protein among aeromonad organisms might have ENR activity and triclosan tolerance.

Selection of a Triclosan-Resistant Clone

In order to select a triclosan-resistant clone from the fosmid library of *AS* 14791 by functional screening, we first determined the MIC of triclosan for the host *E. coli*. The MIC of triclosan for *E. coli* EPI-300 was 0.2 μg/ml. The fosmid library construction of *AS* 14791 generated approximately 1,300 clones with 35 kb of average insert DNA (data not shown). The number of clones in the genomic library was able to cover the whole genome of *AS* 14791, which is approximately 4.7 Mb [32]. Genomic library screening of *AS* 14791 for triclosan resistance resulted in the selection of several candidate fosmid clones. All of the selected clones showed a similar restriction pattern by *Bam*HI, indicating that all of the clones might be overlapped clones spanning the same genomic DNA to carry the gene for triclosan resistance (data not shown). Fosmid clone pTSR, which showed elevated levels of resistance to triclosan (Table 3), was selected and further used to isolate a gene for triclosan

Table 3. Triclosan susceptibility of *E. coli* strains carrying various plasmids.

Strain	Triclosan MIC (μg/ml)
E. coli	
DH5α	0.2
EPI-300	0.2
EPI-300 (pTSR)	100
DH5α (pTSR-1)	100
DH5α (pTSRK415)	100

resistance. This fosmid clone, carrying a 30 kb insert, consistently complemented the growth of *E. coli* carrying FabI, the triclosan-sensitive target enzyme for fatty acid biosynthesis.

Identification of a Gene Encoding FabV Enoyl Acyl Reductase Isoform

The pTSR fosmid clone carrying the 30 kb DNA fragment was digested with *Bam*HI, and resulting fragments were subcloned into pUC119 in order to obtain smaller high-copy-number complementing plasmids. One of these subclones was able to restore the growth of triclosan-sensitive EPI-300 in the medium containing triclosan. DNA sequence analysis and its comparison with the genome of the *A. salmonicida* subsp. *salmonicida* A449 strain revealed that this subclone carries a DNA fragment containing two open reading frames (ORFs), a similar genomic region of *A. salmonicida* subsp. *salmonicida* A449. One of these two ORFs was annotated as encoding *trans*-2-enoyl-CoA reductase (YP_001141511), whereas the second partial ORF (YP_001141513) was annotated as alanine aminotransferase.

In order to eliminate the multicopy effect of pUC119, we further subcloned the ORF encoding *trans*-2-enoyl-CoA reductase into a broad-host-range low-copy-number plasmid, pRK415. The originally triclosan-sensitive *E. coli* EPI-300 cells, harboring the ORF encoding *trans*-2-enoyl-CoA reductase in pRK415 (pTSRK415), showed significantly elevated levels of triclosan resistance (Table 3).

FabVas Shares an Identical Catalytic Domain with Other FabV ENRs

Multiple alignments of FabVas were performed with all previously known ENRs (FabI, FabL, FabV, and FabK), which revealed significant homology with FabV. This analysis was further extended to multiple alignment and comparisons of deduced amino acid sequences of FabVas with the two detailed studies of FabV ENRs of *V. cholerae* O1 biovar El Tor. N16961 [25] and *P. aeruginosa* PAO1 [48]. *fabVas* encodes a 398 amino acid protein, and the deduced amino acid sequence analysis showed that FabVas was 74% identical to FabV of *V. cholerae* O1 biovar El Tor. N16961 and 60% identical to the FabV of *P. aeruginosa* PAO1. From multiple alignment analysis, FabVas was found to contain a Tyr-$(Xaa)_8$-Lys motif (Fig. 1), reported as the active site of the FabV ENR first seen in *V. cholerae* [25] and then in *P. aeruginosa* [48]. This suggests that the protein might have ENR activity.

Genome-Wide Analysis Revealed a Secondary FabV in *A. salmonicida* Genome

Genome-wide searches of *A. salmonicida* subsp. *salmonicida* A449 for the presence of other SDRs and ENRs revealed an

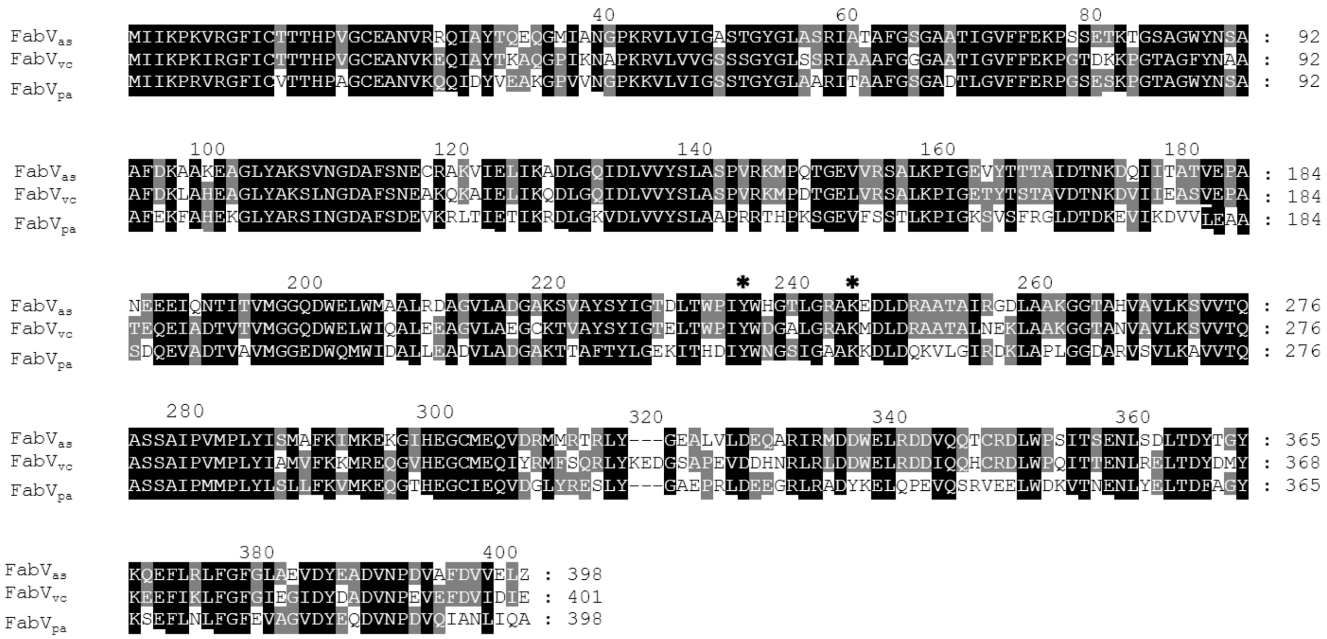

Fig. 1. Multiple alignment of *A. salmonicida* FabV (FabVas) with *V. cholerae* (FabVvc) and *P. aeruginosa* (FabVpa) FabV.
A. salmonicida FabV (top line) has 74% identity with FabV of *V. cholerae*, and 60% identity to that of *P. aeruginosa*. The active-site tyrosine and lysine residues are asterisked.

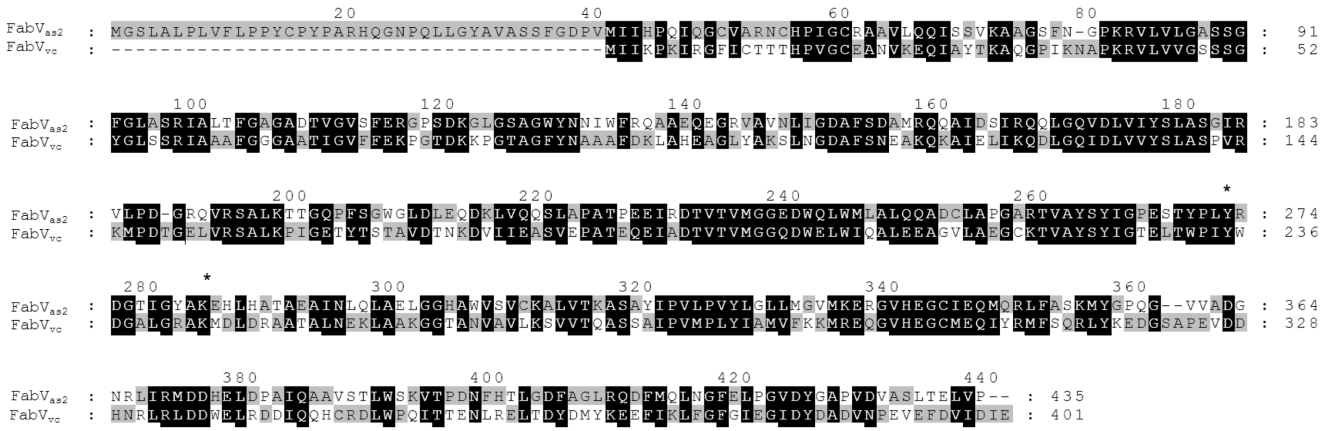

Fig. 2. Multiple alignment of *A. salmonicida* secondary FabV (FabVas2) ORF with *V. cholerae* FabV (FabVvc).
FabVas2 (top line) has 49% identity and 69% similarity with *V. cholerae*. The active-site tyrosine and lysine residues are asterisked.

additional ENR (YP_001140005) termed as FabVas2. Like the YP_001141511 ORF, multiple alignment analysis of YP_001140005 with the available ENRs showed significant homology with FabV (Fig. 2), indicating that the genome of this organism carries two paralogs of FabV. In addition, genome-wide searches revealed the presence of nine other different kinds of SDRs (YP_001140191, YP_001140383, YP_001140963, YP_001141579, YP_001141848, YP_001142856, YP_001142967, YP_001143327, and YP_001143116).

Relative Gene Expression of FabVas and FabVas2 at Transcriptional Level

Gene expression for FabVas and FabVas2 of *A. salmonicida* subsp. *salmonicida* in the presence and absence of triclosan (5 μg/ml) was investigated by RT-qPCR. The *gyrA* gene of *A. salmonicida* subsp. *salmonicida* was used as a reference gene. The mRNA expression of *fabVas* and *fabVas2* differed slightly for the control and treated samples (Fig. 3). The *fabVas2* gene was higly expressed relative to triclosan treatment as compared with the *fabVas* gene at the mRNA level. However, in the absence of triclosan, *fabVas* expression was found to be higher than that of *fabVas2*.

Most of the Known Fish Pathogens Carry Similar ENR Homologs to FabVas

In order to extend a secondary hypothesis that most of the fish pathogens might share the same mechanism to tolerate triclosan, a similarity search of FabVas was performed against 21 different fish pathogens whose complete genome sequence data are available. These were as follows: *V. anguillarum* 775 serotype O1, *V. anguillarum* 96F serotype O1, *V. anguillarum* RV22 serotype O2β, *V. ordalii* ATCC 33509, *V. vulnificus* YJ016 biotype 1, *V. splendidus* strain LGP32, *Aliivibrio salmonicida* strain LFI1238, *Flavobacterium psychrophilum* JIP02/86, *F. branchiophilum* FL-15, *Edwardsiella tarda* EIB202, *V. harveyi* ATCC BAA-1116, *F. columnare* ATCC 49512, *E. ictaluri* 93–146, *A. hydrophila* ATCC 7966, *A. salmonicida* A449, *A. veronii* strain B565, *A. caviae* Ae398, *Renibacterium salmoninarum* ATCC 33209, *Streptococcus parauberis*, *Lactococcus garvieae* UNIUD074, and *M. marinum* M. The analysis showed that most of these fish pathogens have FabVas homologs showing significant identity (56–97%) with FabVas enoyl reductase (Table 4). This result

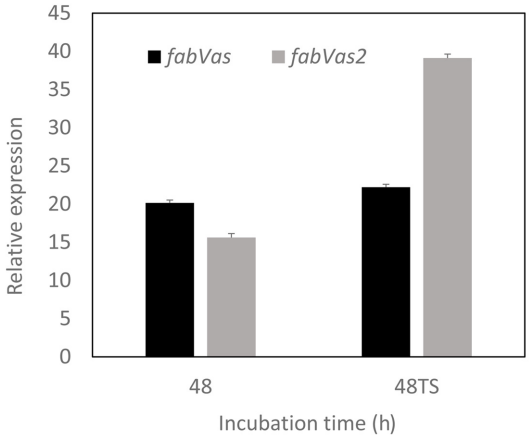

Fig. 3. Relative gene expression of *fabVas* and *fabVas2* in *Aeromonas salmonicida* subsp. *salmonicida* in the presence and absence of triclosan (5 μg/ml) by RT-qPCR.
Total RNA from *Aeromonas salmonicida* subsp. *salmonicida* was isolated at 48 h incubation (late exponential growth stage). Expression levels of *fabVas* and *fabVas2* for triclosan treated (48TS) and untreated samples (48) were estimated in comparison with *gyrA* as a reference gene. Vertical bars represent standard deviations of three replicates.

Table 4. Fish pathogens and FabV ENR homologs in their genome.

Accession No.	Causative agent/species	Disease	Main host	Identity to FabVas (%)	References
YP_001141511	*Aeromonas salmonicida* subsp. *salmonicida* A449	Furunculosis	Salmon, trout, goldfish	97	[39]
YP_004391941	*Aeromonas veronii* B565	Motile aeromonas septicemia (MAS)	Salmonid and non-salmonid fish	93	[39]
YP_857138	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	Septicemia	Amphibians and many others	92	[39]
YP_003295924	*Edwardsiella tarda* EIB202	Edwardsiellosis	Salmon and many others	90	[39]
YP_002933235	*Edwardsiella ictaluri* 93-146	Enteric septicemia	Catfish and tilapia	90	[39]
YP_004565967	*Vibrio anguillarum* 775	Vibriosis	Salmonids	71	[39]
YP_001445825	*Vibrio campbellii* ATCC BAA-1116	Vibriosis	Salmonids	72	[13]
YP_002417447	*Vibrio splendidus* LGP32	Vibriosis	Oysters	72	[39]
YP_002263407	*Aliivibrio salmonicida* LFI1238	Vibriosis	Atlantic salmon	72	[39]
YP_001296711	*Flavobacterium psychrophilum* JIP02/86	Coldwater disease	Salmonids and others	56	[39]
YP_004844142	*Flavobacterium branchiophilum* FL-15	Bacterial gill disease	Salmonid and non-salmonid fish	56	[39]
YP_004942027	*Flavobacterium columnare* ATCC 49512	Columnaris disease	Cyprinids and others	57	[39]

indicated that these organisms might share the same FabV ENR for enoyl reductase activity in fatty acid biosynthesis.

Discussion

The mechanism of triclosan resistance has been extensively studied in human pathogens; however, the resistance mechanisms of triclosan in fish pathogenic bacteria are largely uncharacterized. Several studies have reported toxic levels of triclosan (0.001–2,210 ng/l) from marine water around the globe [10, 30, 44]. Therefore, there must be a resistance mechanism among marine organisms to overcome the toxicity of triclosan. The discovery of a new class of ENRs from *V. cholerae*, denoted as FabV [25], led to the conclusion that FabI [3], FabL [16], and FabK [15] are not the only types of ENRs catalyzing the last step of type II fatty acid biosynthesis. Moreover, homology searches against available databases for the presence of ENR homologs is not always the best option, the reason being that ENR annotation is sometimes problematic [22]. For example, many proteins that align well and share significant homology with *S. pneumoniae* FabK have been reported not to exhibit any ENR activity [24, 28, 22]. Another example is that of *Enterococcus faecalis* carrying two different ENRs, namely FabI and FabK, where the latter has been proved to play a minor role in fatty acid biosynthesis in *E. faecalis* [20]. Therefore, functional analysis of bacterial ENR homologs by forward and reverse genetics seems to be the proper option to confirm its authentic activity.

Genome analysis of previously known ENRs in *A. salmonicida* A449 and fosmid library screening of *AS* 14791 revealed the presence of a FabV homolog sharing the same catalytic domain Tyr-$(Xaa)_8$-Lys with FabV of *V. cholerae*. This result is in perfect agreement with previous data from other gram-negative bacteria that are known to contain a triclosan-tolerant FabV ENR [17, 21, 25, 48]. The triclosan resistance of recombinant *E. coli* EPI-300 containing pTSRK415 eliminated the possibility of resistance occurrence due to overexpression of genes in a high-copy-number plasmid, showing that the *fabVas* gene is capable of conferring resistance to triclosan when expressed normally. This result indicates that the triclosan resistance in *A. salmonicida* is due to the insensitivity of FabVas to the biocide in question. These results are in accordance with those of previous studies [21] reporting that triclosan is a rapid reversible inhibitor of *Burkholderia mallei* FabV (FabVbm), with a K_i value of only 0.4 mM. At such a low concentration, triclosan would be almost unable to compete with the natural substrate of the enzyme in question [17]. It has been demonstrated that triclosan has an even lower (100-fold) affinity for *Y. pestis* FabV (FabVyp), indicating that FabV ENR is naturally insensitive to triclosan.

From the multiple alignment analysis, the ENR of this study that renders *A. salmonicida* subsp. *salmonicida* 14791

resistant to triclosan seems to be FabV, as it shares significant identity (74%) and a similar catalytic domain (Tyr-$(Xaa)_8$-Lys) [31] with FabV enoyl ACP reductase of *V. cholerae* [25]. It has been extensively reported that FabV is the main cause of triclosan tolerance in four human pathogenic bacteria, namely *P. aeruginosa* [48], *V. cholerae* [25], *B. mallei* [21], and *Y. pestis* [17]. However, this is the first report describing the presence of the same triclosan-tolerant enzyme from a fish pathogen.

Genome-wide searches of *A. salmonicida* subsp. *salmonicida* A449 revealed the presence of a secondary ENR that was presumably FabV (Fig. 2). Moreover, the presence of nine different SDRs suggests that this organism might have two or more ENRs catalyzing the enoyl reduction step during fatty acid biosynthesis. Our result is in agreement with that of *P. aeruginosa* [48] with two different ENRs, one of which is triclosan-tolerant. *A. salmonicida* was known to have a number of plasmid-borne antibiotic resistance genes; namely streptomycin/spectinomycin, quaternary ammonia compounds, sulfonamides, tetracycline, and chloramphenicol [32]. Moreover, three chromosomal β-lactamase genes (*amp*C, *amp*S, *cph*A) and major facilitator efflux family proteins have been recently reported from this organism [32]. Thus, the presence of these genes, along with triclosan-resistant *fabVas* and an additional FabV homolog in this study, indicates that this organism carries an array of genes to counteract antimicrobials and biocides.

Expression analysis in this study revealed that both the *fabVas* and *fabVas2* genes were actively transcribed in the absence and presence of triclosan, indicating that these ENRs might be mutually involved in the fatty acid biosynthesis in *A. salmonicida* subsp. *salmonicida*. We do not have any clue if FabVas2 will play a certain role for triclosan resistance. Although *fabVas*2 was transcribed in the presence of triclosan, it is not clear if the mRNA will be translated into an active ENR to confer triclosan resistance to *A. salmonicida* subsp. *salmonicida*. In fact, FabK of *E. faecalis* carrying two different ENRs, FabI and FabK, was found to play a minor role in triclosan resistance [20]. Similarly, FabL, not FabI, was responsible to confer triclosan resistance in *Bacillus subtilis* with two different FabL and FabI ENRs [16]. To our best knowledge, this report is the first of the presence of two potential FabV ENRs, *fabVas* and *fabVas2*, in a single pathogenic bacteria.

Genome-wide comparison of FabVas against most of the known fish pathogens revealed that most of these organisms might confer resistance to triclosan by sharing the same triclosan-resistant FabVas enzyme (Table 4). This might be due to lateral gene transfer among bacterial strains with diverse FabI homologs [28]. In addition, the transfer of antibiotic-resistant genes, such as genes encoding resistance to oxytetracycline, trimethoprim, and sulfonamides, from fish pathogens to other bacteria has been recognized [33, 38]. However, the lack of FabV in other fish pathogens indicates that those organisms might be susceptible to triclosan or have a different resistance mechanism to this biocide. Based on our results, we propose that triclosan resistance mediated by an alternative ENR may be widely distributed in bacterial systems in nature.

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