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Triclosan Can Select for an AdeIJK-Overexpressing Mutant of *Acinetobacter baumannii* ATCC 17978 That Displays Reduced Susceptibility to Multiple Antibiotics

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In order to determine if triclosan can select for mutants of *Acinetobacter baumannii* ATCC 17978 that display reduced susceptibilities to antibiotics, we isolated a triclosan-resistant mutant, *A. baumannii* AB042, by serial passaging of *A. baumannii* ATCC 17978 in growth medium supplemented with triclosan. The antimicrobial susceptibility of AB042 was analyzed by the 2-fold serial dilution method. Expression of five different resistance-nodulation-division (RND) pump-encoding genes (*adeB*, *adeG*, *adeJ*, *A1S_2818*, and *A1S_3217*), two outer membrane porin-encoding genes (*carO* and *oprD*), and the MATE family pump-encoding gene *abeM* was analyzed using quantitative reverse transcriptase (qRT) PCR. *A. baumannii* AB042 exhibited elevated resistance to multiple antibiotics, including piperacillin-tazobactam, doxycycline, moxifloxacin, ceftriaxone, cefepime, meropenem, doripenem, ertapenem, ciprofloxacin, aztreonam, tigecycline, and trimethoprim-sulfamethoxazole, in addition to triclosan. Genome sequencing of *A. baumannii* AB042 revealed a ^{116}G→V mutation in *fabI*, the gene encoding the target enzyme for triclosan. Expression analysis of efflux pumps showed overexpression of the AdeIJK pump, and sequencing of *adeN*, the gene that encodes the repressor of the *adeIJK* operon, revealed a 73-bp deletion which would cause a premature termination of translation, resulting in an inactive truncated AdeN protein. This work shows that triclosan can select for mutants of *A. baumannii* that display reduced susceptibilities to multiple antibiotics from chemically distinct classes in addition to triclosan resistance. This multidrug resistance can be explained by the overexpression of the AdeIJK efflux pump.

Triclosan is a bisphenolic biocide widely used in various domestic cleaning products, such as toothpaste, soaps, and cosmetics, and hospital equipment (1). The increasing use of triclosan in domestic products has raised concerns about its role in selecting for triclosan-resistant bacterial strains that exhibit cross-resistance to clinically relevant antibiotics. Resistance to triclosan can result from target site (fatty acid biosynthetic enzyme, enoyl-[acyl-carrier protein] reductase, *fabI*) modification, active efflux, or enzymatic degradation (2). Of these mechanisms, active efflux has the most important role in imparting cross-resistance to chemically distinct classes of antibiotics.

In Gram-negative bacteria, efflux pumps belonging to the resistance-nodulation-division (RND) family are considered one of the most important contributors of intrinsic resistance to clinically relevant antibiotics (3). These pumps are known for their broad specificities, and triclosan is a substrate of several of these pumps. In addition, triclosan has been shown to select for RND pump-overexpressing mutants in several organisms, including *Escherichia coli* (4), *Pseudomonas aeruginosa* (5, 6), and *Salmonella enterica* (7).

In this study, we analyzed the ability of triclosan to select for mutants of *Acinetobacter baumannii* (an organism notorious for causing infections in immunocompromised individuals [8]) that display elevated resistance to chemically unrelated antibiotic classes. Infections caused by *A. baumannii* are becoming increasingly difficult to treat because of its widespread resistance to almost every class of antibiotic in clinical use (9, 10). Due to the ability of *A. baumannii* to cause multidrug-resistant infections, the Centers for Disease Control and Prevention (CDC) recently categorized it as an organism that poses a serious threat to human health (http://www.cdc.gov/drugresistance/threat-report-2013/). While the resistance of *A. baumannii* to various antibiotics can be attributed in part to its impressive ability to acquire various resistance genes (9), it also displays high intrinsic resistance mediated by reduced outer membrane permeability and the activity of energy-dependent efflux proteins (8). To date, three RND pumps in *A. baumannii* have been characterized, namely, AdeABC (11), AdeFGH (12), and AdeIJK (13). In addition, a multidrug and toxic compound extrusion (MATE) family pump in *A. baumannii*, AbeM, has been characterized (14). While none of the RND pumps characterized has been shown to efflux triclosan, it is a substrate of the AbeM pump.

Although triclosan resistance has been reported in clinical isolates of *A. baumannii* as a result of mutations in *fabI* (15), its role in *A. baumannii* cross-resistance to antibiotics has never been reported. In this paper, we report that triclosan can indeed select for AdeIJK-overexpressing mutants of *A. baumannii* with reduced susceptibilities to various classes of chemically unrelated antibiotics.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *A. baumannii* ATCC 17978

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
A. baumannii strains		
ATCC 17978	Wild type	ATCC
AB042	Triclosan-resistant mutant of ATCC 17978	This study
P. aeruginosa strains		
PAO750	PAO1: Δ*mexAB-oprM* Δ*mexCD-oprJ* Δ*mexEF-oprN* Δ*mexJK* Δ*mexXY* Δ*opmH* Δ*pscC*	34
PA005	PA0750 with chromosomally integrated mini-Tn*7*-LAC-*adeIJK* containing the Gen^r marker	This study
PA006	PA0750 with chromosomally integrated mini-Tn*7*-LAC-*adeIJK* with Gen^r marker removed	This study
E. coli strains		
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ*-thi-1 gyrA96 relA1*	Invitrogen
Plasmids		
pGEM-T Easy	Amp^r, PCR cloning vector	Promega
pPLS001	Amp^r, pGEM-T Easy-*adeIJ′*	This study
pPLS002	Amp^r, pGEM-T Easy-*adeJ′K*	This study
pPLS007	Amp^r, pGEM-T Easy-*adeIJK*	This study
pUC18T-mini-Tn*7*T-Gm-LAC	Amp^r Gen^r; mini-Tn*7* expression vector containing *lacI*^{q} and *tac* promoter	24
pPLS009	Amp^r Gen^r, pUC18T-mini-Tn*7*T-Gm-LAC-*adeIJK*	This study
pPLS097	Amp^r Gm^r, *E. coli-A. baumannii* shuttle vector	This study
pPLS093	Amp^r, pGEM-T Easy:*adeN*	This study
pPLS145	Amp^r Gm^r, pPLS097:*adeN*	This study
pPLS147	pGEM-T Easy:*fabI*	This study
pPLS148	Amp^r, pGEM-T Easy:*fabI*$^{116G\rightarrow V}$ Amp^r	This study
pPLS150	Amp^r Gm^r, pPLS097:*fabI*$^{116G\rightarrow V}$	This study
pPLS151	Amp^r Gm^r, pPLS097:*fabI*	This study
pTNS2	Amp^r, helper plasmid encoding the site-specific TnsABCD Tn*7* transposition pathway	24
pFLP2	Amp^r; source of FLP recombinase	24

was used to generate the triclosan-resistant mutant. Lennox broth (LB) (BioShop, Inc., Burlington, ON, Canada) was used as the growth medium. Bacterial cultures were incubated at 37°C with shaking (200 rpm).

Isolation of triclosan-resistant mutant. The triclosan-resistant mutant *A. baumannii* AB042 was isolated by serial transfer of *A. baumannii* ATCC 17978 in LB supplemented with increasing concentrations (2-fold for each transfer) of triclosan (KIC Chemicals, Inc., New Paltz, NY, USA), starting at 4 mg/liter. A 1:100 inoculum was transferred to the LB supplemented with increasing concentrations of triclosan. *A. baumannii* AB042 was isolated by streaking cells from LB supplemented with 128 mg/liter of triclosan on LB agar.

Antimicrobial susceptibility assays. Susceptibility testing for triclosan and antibiotics was carried out using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (16). Susceptibility assays were performed on three biological replicates.

RNA extraction and cDNA synthesis. Overnight cultures of bacterial strains were subcultured in LB (1:100 dilution) and allowed to grow to an A_{600} of 0.6 to 0.8. Then, 1 ml of this culture was centrifuged, and the cell pellet was frozen at −80°C for 1 h to facilitate cell lysis. RNA extraction was performed using the RNeasy kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instructions. To remove any genomic DNA carryover, the samples were treated with DNase I (Qiagen) for 30 min at 37°C, followed by heat inactivation at 65°C for 5 min. Then, 1 μg of total RNA was used to synthesize cDNA using the Bio-Rad iScript reverse transcriptase kit (Bio-Rad, Mississauga, ON, Canada) following the manufacturer's instructions. The control reaction (with no reverse transcriptase [NRT]) was set up using all components of the reaction mixture but without the reverse transcriptase enzyme.

Reverse transcriptase quantitative PCR (qRT-PCR). Analysis was carried out on five RND pump-encoding genes (*adeB*, *adeG*, *adeJ*, *A1S_2818*, and *A1S_3217*), two outer membrane porins (*carO* and *oprD*), the MATE family pump *abeM*, the triclosan target-encoding gene *fabI*, and the TetR regulator-encoding gene of the *adeIJK* operon, *adeN*. The primers used for qRT-PCR analysis are listed in Table 2. The efficiency of each primer was tested by using a 10-fold serial dilution of cDNA mix, and only primers with an efficiency between 95% and 105% were used for the analysis. The expression of genes was determined by quantitative PCR using SsoFast Evagreen Supermix (Bio-Rad, Mississauga, ON, Canada). Two different control reactions were included in the analysis, a no-template control (NTC) and an NRT control. We used 16S rRNA as the reference gene. Relative expression was determined using the cycle threshold ($\Delta\Delta C_t$) method on the Bio-Rad C1000 CFX96 real-time system (Bio-Rad). Reactions were set up using 300 nM primers and 5 μl of the cDNA template (diluted 1:10). All reactions were carried out in triplicate with at least two biological replicates. Target gene expression was measured using expression relative to that of the 16S reference gene, and *A. baumannii* ATCC 17978 was used as the control strain. Data analysis was carried out using the Bio-Rad CFX 2.0 software.

Sanger sequencing. Genomic DNA from *A. baumannii* ATCC 17978 and *A. baumannii* AB042 was extracted from overnight cultures using a genomic DNA extraction kit (Bio Basic, Markham, ON, Canada). Sequencing of *fabI*, *adeN*, and the promoter region of *adeIJK* was carried out at the McGill University Innovation Centre for sequencing (Montreal, QC, Canada).

Whole-genome sequencing. Whole-genome sequencing of *A. baumannii* ATCC 17978 and *A. baumannii* AB042 was carried out at the next-generation sequencing (NGS) facility of the Manitoba Institute of Child Health (MICH) using the Illumina MiSeq platform. In total, 1,764,672 and 3,669,973 pairs of 150-base sequence reads were generated for AB042 and ATCC 17978, respectively. These sequence runs passed the quality check with 90% passed-filter (PF) reads, and 87% had a Q score of ≥30. Assembly of the genomes was carried out using MIRA (v4.0.2) (17) and Velvet (v1.2.10) (18) software. Comparison of the genomes with those of the published *A. baumannii* ATCC 17978 (GenBank accession no

TABLE 2 Primers used in this study

Primer	Sequence	Target gene and purpose	Reference
16S_RT_F	ACATCTCACGACACGAGCTG	16S rRNA, gene expression	38
16S_RT_R	CGTAAGGGCCATGATGACTT		
adeB_RT_F	GGATTATGGCGACTGAAGGA	*adeB*, gene expression	38
adeB_RT_R	AATACTGCCGCCAATACCAG		
adeG_RT_F	CGTAACTATGCGGTGCTCAA	*adeG*, gene expression	38
adeG_RT_R	ATCGCGTAGTCACCAGAACC		
adeJ_RT_F	CATCGGCTGAAACAGTTGAA	*adeJ*, gene expression	38
adeJ_RT_R	GCCTGACCATTACCAGCACT		
A1S_3217_RT_F	ACCGCTTTAGAGGTCGAACA	*A1S_3217*, gene expression	38
A1S_3217_RT_R	GTGACTTGGGAAAGCCCATA		
A1S_2818_RT_F	AATTGAGCCAAGCTCATGCT	*A1S_2818*, gene expression	38
A1S_2818_RT_R	TCCGCGATGAAATTGATACA		
carO_RT_F	AGCAGTTCGTGGTCAAGAGG	*carO*, gene expression	38
carO_RT_R	TTGGAGCAAAACCAAAACCT		
oprD_RT_F	CCAGCTCAGTTGCTCAATCA	*oprD*, gene expression	38
oprD_RT_R	AACAACGCCTACACCGAAAC		
abeM_RT_F	TGCCAATTGGTTTAGCTGTG	*abeM*, gene expression	This study
abeM_RT_R	TACTTGGTGTGCGGCAATAA		
adeN_RT_F	CAACCTGAACACATTGCCTTT	*adeN*, gene expression	This study
adeN_RT_R	TTTTGGACATCCAGAGCACA		
fabI_RT_F	TTTAGAAGCTGGCGTTCGTT	*fabI*, gene expression	This study
fabI_RT_R	AGCAGCCAAAGTACGGATTG		
fabI_FL_F	GTGAGATCGGCATGACACAA	*fabI*, cloning and sequencing of *fabI*[a]	This study
fabI_FL_R	ATAACGGTAGCGGAGTTCAG		
adeN_FL_R	AGTCTACTATACTATAAGCATTTC	*adeN*, cloning and sequencing of *adeN*[b]	This study
adeN_ FL_F	GATAAGCAGTGTTAGCCGTCG		
adeIJK_PR_F	CTTCAGAAATTTGATATGCT	*adeIJK* promoter, sequencing[c]	This study
adeIJK_PR_R	GATTATGTTATGCCATAAGC		
adeI_For_Sp	TT**ACTAGT**TATCTAAACGAGGTG[d]	*adeI*, *adeJ*, cloning of *adeIJ′* fragment (4,051 bp)	This study
adeJ_Rev_Kp	TCAATACGATTGCACCAATGAC		
adeJ_For_Kp	TATATGAAAGCTGGTCAATTCCG	*adeJ*, *adeK*, cloning of *adeJ′K* fragment (1,983 bp)	This study
adeK_Rev_Xh	CCCACCGA**CTCGAG**CTTTTATAAG[e]		
PaglmS_Dn	GCACATCGGCGACGTGCTCTC	*glmS* and pUC18T-miniTn*7*T-Gm-LAC, confirmation of the insertion of mini-Tn*7* element in *P. aeruginosa*	24
Tn*7*R	CACAGCATAACTGGACTGATTTC		

[a] Primers bind 233 bp upstream of the start and 11 bp downstream of stop codons, respectively.
[b] Primers bind 32 bp upstream of the start and 38 bp downstream of stop codons, respectively.
[c] Promoter/sequencing of the promoter region 244 bp upstream and 220 bp downstream of the start codon of the *adeI* gene.
[d] Engineered SpeI site is underlined, and introduced base changes are shown in bold type.
[e] Engineered XhoI site is underlined, and introduced base changes shown are bold.

NC_009085) was carried out using BWA version 0.6.2 (19), with the default three-mismatch penalty setting. Before the sequence variation analysis, the duplicate sequence reads were also removed from the mapped bam files. We used the Genome Analysis Toolkit (GATK) package (20) for sequence variation analysis, including single-nucleotide variants (SNV) and insertions/deletions (indels). The variation calls were ensured by filtering against variant confidence and mapping qualities. We used the ANNOVAR program (21) for sequence variation annotations.

To screen for large deletions/insertions, we developed a program based on the coverage distribution model for detection (our unpublished data). The deletions with a P value of <0.001 were selected. The large deletions/insertions of the two strains and those of the parent strain were compared and visualized by the Integrated Genome Browser (IGV version 2.3.23) (22).

Cloning and single-copy expression for *adeIJK* operon. For cloning of the *adeIJK* operon, primers were designed (Table 2) to amplify 4,051- and 1,983-bp regions of the *adeIJK* operon, both of which were cloned in the PCR cloning vector pGEM-T Easy (Promega, Madison, WI, USA) separately to construct plasmids pPLS001 and pPLS002, respectively. In order to assemble the entire operon, pPLS001 was digested using AatII and KpnI (New England BioLabs, Pickering, ON, Canada), and a resulting 4,061-bp fragment (containing *adeI* and the partial *adeJ* gene) was gel purified (BioBasic, Markham, ON, Canada). This fragment was ligated with pPLS002, which itself was digested with the same enzymes, yielding the plasmid pPLS007. The entire *adeIJK* operon in pPLS007 was sequenced and subcloned by digesting pPLS007 with SpeI and XhoI (New England BioLabs) and subsequently ligating the gel-purified fragment to pUC18T-mini-Tn*7*T-Gm-LAC, digested with the same enzymes to yield the recombinant plasmid pPLS009. Therefore, pPLS009 contains the *adeIJK* operon, the expression of which is driven from the *tac* promoter and controlled by the *lacI*^{q}-encoded Lac repressor, and it can be induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (BioBasic) to the growth medium.

Insertion of the *adeIJK* operon in the surrogate *P. aeruginosa* strain PAO750 in a single copy was carried out by a method previously described (23). Briefly, 50 ng each of pPLS009 and the helper plasmid pTNS2 (24) was electroporated in *P. aeruginosa* PAO750 (25). Transformants were selected on LB agar supplemented with gentamicin (30 mg/liter) (BioBasic, Markham, ON, Canada). The gentamicin resistance marker was subsequently removed using the FLP recombinase to obtain *P. aeruginosa* PA006. PCR was used to confirm the insertion of the mini-Tn*7* element in *P. aeruginosa* PA006 as described previously (24).

Cloning of *adeN*, *fabI*, and *fabI*$^{116G\rightarrow V}$. A PCR-based strategy was used to clone wild-type (WT) and mutant *fabI* and the wild-type *adeN*.

TABLE 3 Antimicrobial susceptibilities of *A. baumannii* isolates

Strain	MIC (mg/liter)[a]																
	TRI	TZP	CRO	FEP	CAZ	IPM	MEM	DOR	ETP	CIP	MXF	AMC	ATM	TGC	DOX	CST	SXT
ATCC 17978	8	4	16	4	8	0.25	0.5	0.25	8	0.25	≤0.06	32	16	0.12	≤0.12	2	2
AB042	256	32	32	8	8	0.25	1	0.5	16	0.5	0.25	32	32	0.5	0.5	2	4
AB042/pPLS097	256	ND	ND	ND	ND	ND	ND	ND	ND	0.5	0.125	ND	ND	ND	ND	ND	ND
AB042/pPLS145(*adeN*)	256	ND	ND	ND	ND	ND	ND	ND	ND	0.25	≤0.06	ND	ND	ND	ND	ND	ND
PA006	32	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PA006 plus IPTG[s]	64	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 17978/pPLS097	8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 17978/pPLS151(*fabI*)	32	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 17978/pPLS150 (*fabI*^{116GàV})	>256	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] TRI, triclosan; TZP, piperacillin-tazobactam; CRO, ceftriaxone; FEP, cefepime; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; DOR, doripenem; ETP, ertapenem; CIP, ciprofloxacin; MXF, moxifloxacin; AMC, amoxicillin-clavulanic acid; ATM, aztreonam; TGC, tigecycline; DOX, doxycycline; CST, colistin; SXT, trimethoprim-sulfamethoxazole; IPTG, isopropyl β-D-1-thiogalactopyranoside; ND, not determined.

Gene-specific primers (listed in Table 2) were used to amplify the respective genes and the PCR products cloned into the cloning vector pGEM-T Easy (Promega, Madison, WI, USA). For subcloning *adeN*, pPLS093 (pGEM-T Easy:*adeN*) was digested with PstI and SphI (New England BioLabs, Pickering, ON, Canada), and the gene was gel purified and cloned into pPLS097, which itself was digested with the same enzyme. Subcloning of the wild-type and mutant *fabI* was achieved by digesting pPLS148 (pGEM-T Easy:*fabI*) and pPLS147 (pGEM-T Easy:*fabI*$^{116G\rightarrow V}$) with SalI and SphI (New England BioLabs), respectively, gel purifying the respective genes, and cloning in pPLS097, digested with the same enzymes. Recombinant plasmids were sequenced to confirm the presence of target genes.

Plasmids were introduced into *A. baumannii* by using a method previously described (25), with some modifications. Briefly, an overnight culture of *A. baumannii* was subcultured in 15 ml of LB, and cells were grown to mid-log phase. The culture was distributed in 1-ml aliquots into microcentrifuge tubes, and cells were harvested by centrifugation (10,000 rpm) at room temperature for 2 min. Cells were then washed twice with ice-cold water, and pellets in each microcentrifuge tube were resuspended in 20 μl of ice-cold double-distilled water (dH_2O) and pooled. Then, 500 ng of plasmid was added to 100 μl of cells and incubated on ice for 15 min. Cells were then electroporated at 2,500 V, following which, 1 ml of LB was added immediately and cells were incubated at 37°C for 1 h with shaking for recovery. Cells were plated on LB agar plates supplemented with carbenicillin (200 μg/ml) and incubated at 37°C overnight.

RESULTS AND DISCUSSION

While the benefits of using antibacterial cleaning products in domestic settings continue to be a topic of debate, the exponential growth in their use over the past few decades has led to increasing concerns regarding their ability to select for multidrug-resistant bacteria (2). Indeed, the phenomenon of cross-resistance has been demonstrated in studies on *E. coli* (4) and *P. aeruginosa* (26), among other pathogens. In this study, we analyzed the potential of triclosan to select for mutants of *A. baumannii* that display elevated resistance to antibiotics from chemically distinct classes.

A. baumannii AB042 was isolated from the serial passaging of *A. baumannii* ATCC 17978 in LB supplemented with triclosan. The MIC of *A. baumannii* AB042 for triclosan was 256 mg/liter (Table 3). This represents a 32-fold increase in resistance to triclosan compared to that of the parent strain. Next, we analyzed the susceptibility of *A. baumannii* AB042 to various clinically relevant antibiotics. We observed an ≥8-fold increase in resistance to piperacillin-tazobactam, a ≥4-fold increase in resistance to doxycycline, moxifloxacin, and tigecycline, and a 2-fold increase in resistance to ceftriaxone, cefepime, meropenem, doripenem, ertapenem, ciprofloxacin, aztreonam, and trimethoprim-sulfamethoxazole (Table 3). The MICs of ceftazidime, imipenem, amoxicillin-clavulanic acid, and colistin (polymyxin E) were unchanged.

In order to understand the mechanism of triclosan resistance in *A. baumannii* AB042, we analyzed the expression of *fabI* and also sequenced the gene in this strain. The FabI enzyme is the target for triclosan, and mutations in *fabI* have been known to result in high triclosan resistance. Although we did not observe any changes in the expression of *fabI* in *A. baumannii* AB042 compared to that of the parent strain, we observed that *fabI* from AB042 contained a single base pair change, leading to a $^{116}G\rightarrow V$ mutation. A similar mutation was shown to cause triclosan resistance in other organisms as well, including *E. coli* (27) and *P. aeruginosa* (28) (Fig. 1). However, in *S. enterica*, this mutation was not found to contribute to the high triclosan resistance (7). Therefore, in order to confirm the role of the $^{116}G\rightarrow V$ *fabI* mutation in triclosan resistance of *A. baumannii* AB042, we introduced *fabI*^{WT} and *fabI*$^{116G\rightarrow V}$ separately into *A. baumannii* ATCC 17978, and their susceptibilities to triclosan were determined. We found that the introduction of *fabI*$^{116G\rightarrow V}$ in *A. baumannii* ATCC 17978 elevated its resistance to triclosan by 32-fold (Table 3). This shows that the $^{116}G\rightarrow V$ mutation in *fabI* of *A. baumannii* AB042 is indeed responsible for its reduced susceptibility to triclosan. While we also observed slightly reduced susceptibility of *A. baumannii* ATCC 17978 upon the introduction of wild-type *fabI* (4-fold), it is most likely due to the overexpression of the triclosan target gene, as no change in the susceptibility was observed when the empty vector was introduced into the host strain (Table 3).

Since *A. baumannii* AB042 displayed reduced susceptibility to multiple chemically unrelated antibiotics compared to that of the parent *A. baumannii* ATCC 17978, we analyzed the expression of efflux pumps in this strain (Fig. 2). We analyzed the expression of

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E. coli          81 VWPKFDGFVHSIGFAPGDQLDGDYVNAVTREGFKIAHDIS 120
S. enterica      81 VWPKFDGFVHSIGFAPGDQLDGDYVNAVTREGFKVAHDIS 120
P. aeruginosa    83 HWDGLDIIVHSVGFAPGDQLDGDFTAVTTREGFRIAHDIS 122
A. baumannii    104 HWDGVDGVVHSIGFAPAHTLDGDFTEVTDRDGFKIAHDIS 143
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FIG 1 Alignment of *fabI* region containing the G→V mutation in *Acinetobacter baumannii* with *E. coli*, *P. aeruginosa*, and *S. enterica*. The ^{116}G residue of *A. baumannii* and the corresponding glycine residues from *E. coli*, *P. aeruginosa*, and *S. enterica* are shown in bold. Only partial protein sequences are shown.

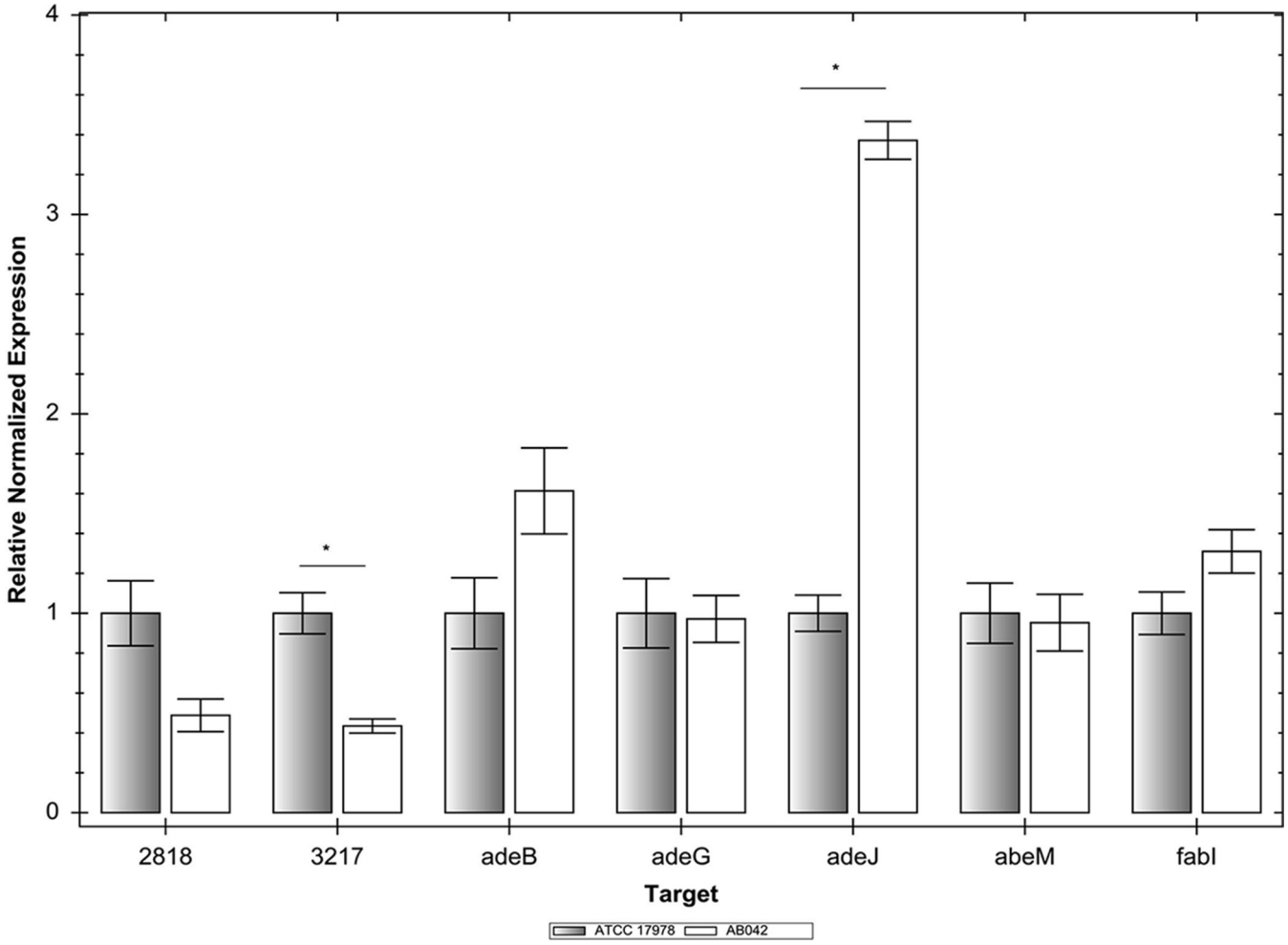

FIG 2 Expression of efflux pump-encoding genes and *fabI* in *Acinetobacter baumannii* AB042. Expression of five RND pump-encoding genes (*adeB*, *adeG*, *adeJ*, *A1S_2818*, and *A1S_3217*), MATE pump-encoding *abeM*, and the enoyl-[acyl-carrier protein] reductase gene *fabI* as measured by qRT-PCR compared with that in the parent strain, *A. baumannii* ATCC 17978. We used 16S rRNA as the housekeeping gene control. Error bars represent the standard errors of the mean. Significant change in expression is shown by an asterisk.

four different efflux pump-encoding genes characterized to date in *A. baumannii*, namely, *adeB* (RND pump-encoding gene of the AdeABC pump), *adeG* (RND pump-encoding gene of the AdeFGH pump), *adeJ* (RND pump-encoding gene of the AdeIJK pump), and *abeM* (the MATE family pump). In addition, we also analyzed the expression of two yet-uncharacterized RND pump-encoding genes, namely, *A1S_2818* and *A1S_3217*, which are a part of the *A1S_2817* to *A1S_2818* and *A1S_3219*, *A1S_3218*, *A1S_3217*, and *A1S_3214* operons, respectively. AdeABC, AdeFGH, and AdeIJK, the three RND family efflux pumps characterized in *A. baumannii*, have been shown to efflux various clinically relevant antibiotics, including fluoroquinolones, β-lactams, tetracycline, tigecycline, and trimethoprim-sulfamethoxazole (12, 13, 23, 29), and are commonly found to be overexpressed in clinical isolates of *A. baumannii* (30). To our knowledge, none of these three RND pumps has been shown to efflux triclosan. However, AbeM, a MATE family pump, effluxes triclosan in addition to antibiotics, such as norfloxacin, ofloxacin, ciprofloxacin, gentamicin, kanamycin, erythromycin, chloramphenicol, and trimethoprim (14).

We observed that the only efflux pump-encoding gene that showed an increased expression (3.5-fold) in *A. baumannii* AB042 was *adeJ*, which is part of the *adeIJK* operon (Fig. 2). None of the other pumps analyzed (with the exception of *A1S_3219*, whose expression was reduced in *A. baumannii* AB042) showed any altered expression in *A. baumannii* AB042, including the AbeM pump that effluxes triclosan. Antibiotics to which *A. baumannii* AB042 showed reduced susceptibility—namely, moxifloxacin, cefepime, meropenem, ertapenem, ciprofloxacin, tigecycline, and trimethoprim-sulfamethoxazole (Table 3)—are known substrates of the AdeIJK pump (13, 31). While doxycycline, doripenem, and ceftriaxone have not been recognized as substrates of the AdeIJK pump, their structural similarities to its established substrates (tetracycline, meropenem-ertapenem, and cefepime, respectively) suggest that these three antibiotics are also effluxed by AdeIJK. In addition to the expression of efflux pumps, we also analyzed the expression of two porin-encoding genes, *carO* (32) and *oprD* (33), shown to play a role in the carbapenem resistance of *A. baumannii*. Their expression remained unaltered in AB042 (data not shown). Altogether, the antibiotic susceptibility profile of *A. baumannii* AB042 and the expression analysis of efflux pumps suggest that the overexpression of AdeIJK is responsible for its reduced susceptibility to various antibiotics.

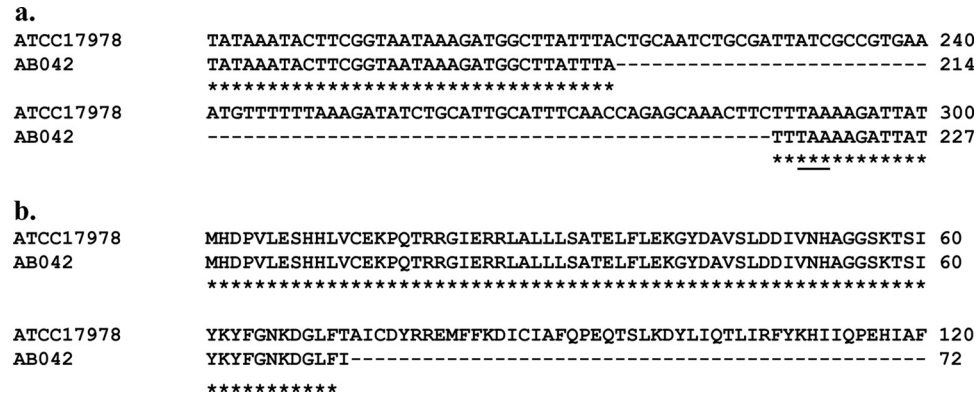

FIG 3 Nucleotide deletion in *adeN* resulting in the premature truncation of the protein in *Acinetobacter baumannii* AB042. A 73-bp deletion in *adeN* (a) causes the generation of a stop codon (underlined), leading to the translation of truncated protein (b). Only partial sequences are shown.

Since the AdeIJK pump had not been shown previously to efflux triclosan, we tested its specificity for triclosan using the single-copy gene expression system in the surrogate *P. aeruginosa* PAO750 (34), which lacks five different native RND pumps. This system allows us to achieve biologically relevant levels of gene expression and also prevents any interference from native RND pumps. We previously demonstrated the usefulness of this system in studying RND pumps of *A. baumannii* (23). We observed a small but reproducible 2-fold increase in the resistance of *P. aeruginosa* PA006 to triclosan upon the induction of expression of the *adeIJK* operon (Table 3). It should be noted that we confirmed the expression of the AdeIJK pump and its functionality using RT-PCR and measuring the susceptibility of PA006 (with and without the addition of IPTG) to tetracycline, trimethoprim, SDS, and ciprofloxacin (data not shown). This change in the MICs upon induction of AdeIJK shows that it is indeed capable of effluxing triclosan, although it is most likely responsible for a low level of triclosan resistance (as evident from a 2-fold increase in resistance to triclosan in *P. aeruginosa* PA006 upon the induction of the AdeIJK operon).

Triclosan has been shown to select for RND pump-overexpressing mutants in different organisms. This overexpression can result from mutations in the regulator protein-encoding gene (e.g., mutation in *nfxB* [26, 35] or *mexL* [36]) that lead to the overexpression of the MexCD-OprJ and MexJK pumps, respectively, of *P. aeruginosa*. The overexpression of pumps can also result from the altered expression of global regulators of efflux pumps, such as *marA*, *soxS*, or *ramA*, as shown in *E. coli* and *S. enterica* serovar Typhimurium (4, 7). Since *adeIJK* was the only efflux pump-encoding operon overexpressed in *A. baumannii* AB042, we investigated the mechanism of its upregulation. Expression of the AdeIJK efflux pump has been shown to be controlled by AdeN, a regulator protein belonging to the TetR family, the gene for which is located at a distant location from the operon (31). In order to see if the change in the expression of *adeIJK* was a result of the altered expression of *adeN* in *A. baumannii* AB042, we analyzed its expression using qRT-PCR but observed no changes in expression compared to that of the parent strain (data not shown). We then sequenced *adeN* and the promoter region of the *adeIJK* operon to look for mutations that may be responsible for the increased expression of *adeIJK* in *A. baumannii* AB042. While we did not find any changes in the promoter of *adeIJK*, the sequence of *adeN* revealed a large 73-bp deletion at position 224. This would result in a truncated protein due to the appearance of a premature stop codon after the 72nd amino acid residue in the protein (Fig. 3a and b). This truncation would result in the loss of six out of nine predicted α-helices (31) toward the C-terminal end of the protein, rendering it inactive, which would explain the overexpression of the AdeIJK pump.

We then carried out whole-genome sequencing of AB042 and the parent strain, ATCC 17978, to rule out the presence of any other mutation that may be responsible for the reduced susceptibility of AB042 to triclosan and antibiotics. We found a single base pair change (^{91}G→A) leading to a ^{31}D→N mutation in *A1S_0863*, a gene that encodes a beta-ketoacyl-acyl carrier protein synthase I or FabB. FabB is involved in the condensation reaction in fatty acid biosynthesis (37) but is not known to play a role in triclosan resistance; therefore, the significance of this mutation in AB042 is not yet clear.

In order to further study the role of the AdeIJK overexpression in the efflux of triclosan, we attempted to create a gene deletion of the *adeIJK* operon in AB042. However, despite numerous attempts and for reasons that are currently unclear to us, we were unable to isolate the gene deletion. We therefore employed an alternative strategy. We introduced the *adeN* gene into AB042 to repress the expression of *adeIJK*, which was confirmed by qRT-PCR (Fig. 4). We did not observe a change in the susceptibility of AB042 (Table 3) to triclosan upon the introduction of *adeN*, even when expression of the AdeIJK pump was repressed, indicating that this pump is likely to be responsible for a low level of resistance to triclosan in *A. baumannii*, as shown by data obtained from the experiment with the surrogate *P. aeruginosa* strain above. We also measured the susceptibility of AB042 complemented with *adeN* to ciprofloxacin and moxifloxacin as controls. The susceptibility of AB042 to these two antibiotics, which are substrates of the AdeIJK pump, is much lower than that of the parent, ATCC 17978. The introduction of *adeN* into AB042 increased its susceptibility to ciprofloxacin and moxifloxacin to the same levels as those of the parent strain (Table 3), therefore validating our approach of repressing the expression of *adeIJK* in lieu of gene knockouts to study its role in triclosan efflux. Altogether, these results suggest that while high resistance of AB042 to triclosan is the result of a mutation in *fabI*, triclosan exposure can result in the upregulation of AdeIJK, leading to multidrug resistance in *A. baumannii*.

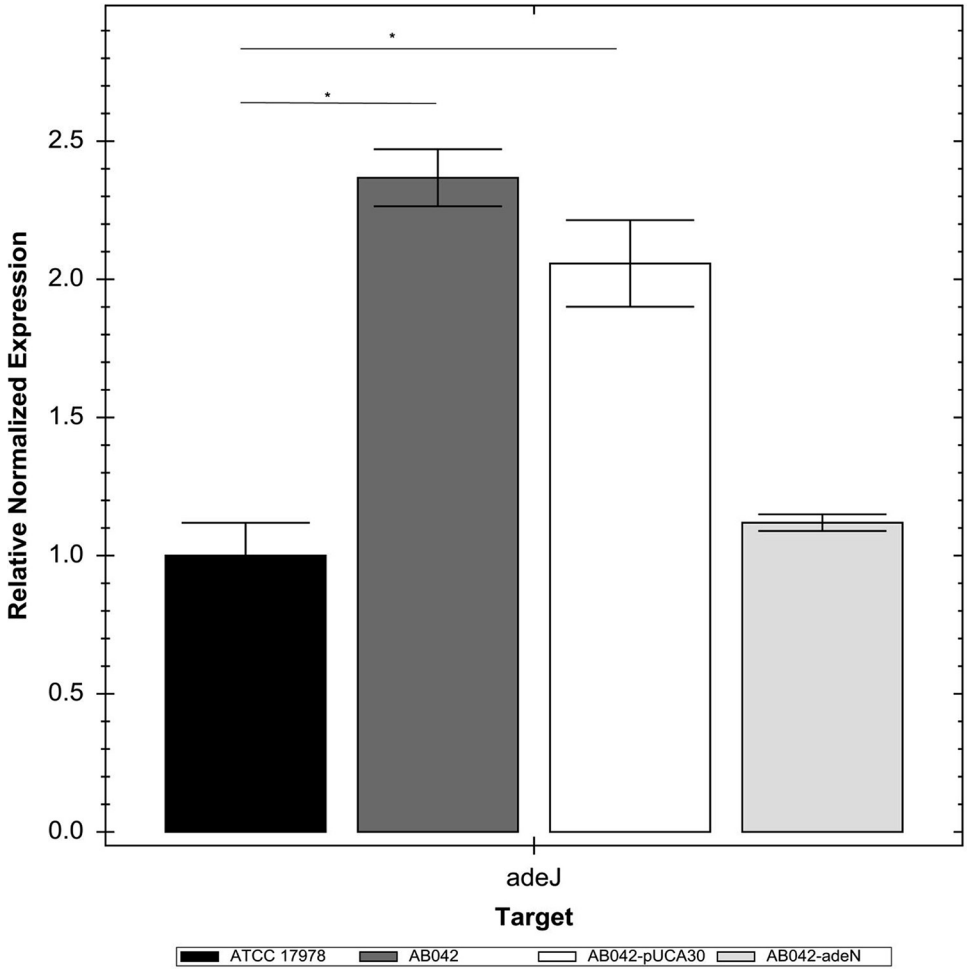

FIG 4 Expression of *adeJ* in *Acinetobacter baumannii* ATCC 17978 complemented with *adeN*. Expression of the *adeJ* was measured by qRT-PCR in *Acinetobacter baumannii* ATCC 17978 complemented with the wild-type *adeN*. We used 16S rRNA as the housekeeping gene control. Error bars represent the standard errors of the mean. Significant change in expression is shown by an asterisk.

In summary, it is evident from our work that the triclosan-resistant mutant *A. baumannii* AB042 exhibits higher resistance to clinically relevant antibiotics as a result of the overexpression of the AdeIJK multidrug resistance efflux pumps, resulting from the deletion in *adeN*.

This is the first study to show that triclosan can select for mutants of *A. baumannii* that overexpress the AdeIJK multidrug resistance efflux pump and display increased resistance to various unrelated classes of antibiotics. This study also further supports concerns about the potential of biocides such as triclosan in selecting for antibiotic-resistant mutants of clinically relevant bacterial pathogens.

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