Triclosan resistance in clinical isolates of *Acinetobacter baumannii*

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The susceptibility to triclosan of 732 clinical *Acinetobacter baumannii* isolates obtained from 25 hospitals in 16 cities in China from December 2004 to December 2005 was screened by using an agar dilution method. Triclosan MICs ranged between 0.015 and 16 mg l⁻¹, and the MIC₉₀ was 0.5 mg l⁻¹, lower than the actual in-use concentration of triclosan. Twenty triclosan-resistant isolates (MICs ≥ 1 mg l⁻¹) were characterized by antibiotic susceptibility, clonal relatedness, *fabI* mutation, *fabI* expression, and efflux pump phenotype and expression to elucidate the resistance mechanism of *A. baumannii* to triclosan. The resistance rates of triclosan-resistant isolates to imipenem, levofloxacin, amikacin and tetracycline were higher than those of triclosan-sensitive isolates. Triclosan resistance was artificially classified as low level (MICs 1–2 mg l⁻¹) or high level (MICs ≥ 4 mg l⁻¹). High-level triclosan resistance could be explained by a Gly95Ser mutation of *FabI*, whilst wild-type *fabI* was observed to be overexpressed in low-level resistant isolates. Active efflux did not appear to be a major reason for acquired triclosan resistance, but acquisition of resistance appeared to be dependent on a background of intrinsic triclosan efflux.

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INTRODUCTION

Acinetobacter baumannii is a clinically significant pathogen that is characteristic of multidrug resistance, and has a great survival ability in environmental and human reservoirs (Peleg *et al.*, 2008). Efflux is an important mechanism for the emergence of multidrug-resistant pathogens. Three multidrug efflux pumps have been identified in *A. baumannii* so far, namely AdeABC, AbeM and AdeIJK, which display broad substrate specificity including antibacterial agents, biocides and dyes (Damier-Piolle *et al.*, 2008; Magnet *et al.*, 2001; Su *et al.*, 2005).

Triclosan is a broad-spectrum biocide that has been used for over 30 years. In addition to health-care settings, it is widely used in hand soaps, toothpastes, shower gels, hand creams and underarm deodorants, as well as in fabrics and plastics (Yazdankhah *et al.*, 2006). Like other biocides, triclosan has been thought to act as a non-specific biocide by affecting membrane structure and function. Recently, a defined target in the fatty acid biosynthetic pathway, enoyl-acyl carrier protein reductase (*FabI*), has been shown to be inhibited by triclosan in several studies (Heath *et al.*, 1999; Hoang & Schweizer, 1999; McMurry *et al.*, 1998b). *fabI* mutations in active-site residues and its overexpression confer resistance to triclosan to varying degrees (Fan *et al.*, 2002; Heath *et al.*, 1999; McMurry *et al.*, 1998b; Webber *et al.*, 2008). In addition, triclosan is a substrate of multidrug efflux pumps, such as AcrAB–TolC in *Escherichia coli*, and MexAB–OprM,

MexCD–OprJ and MexEF–OprN in *Pseudomonas aeruginosa*. Active effluxes are responsible for intrinsic and acquired resistance to triclosan in many bacteria (Chuanchuen *et al.*, 2001; McMurry *et al.*, 1998a; Mima *et al.*, 2007).

Triclosan resistance has been studied in several bacteria, such as *E. coli*, *Staphylococcus aureus*, *P. aeruginosa* and *Salmonella enterica* (Chuanchuen *et al.*, 2001; Fan *et al.*, 2002; Hoang & Schweizer, 1999; McMurry *et al.*, 1998a, b; Webber *et al.*, 2008). However, characterization of triclosan resistance in *A. baumannii* has not yet been reported. In this study, we screened the susceptibility of 732 clinically derived *A. baumannii* isolates to triclosan. Further investigation of *fabI* mutation, *fabI* expression and efflux pump expression was performed to elucidate the resistance mechanism of *A. baumannii* to triclosan.

METHODS

Bacteria. A total of 732 *A. baumannii* isolates was obtained from 25 hospitals in 16 cities in China from December 2004 to December 2005. All isolates were identified as members of the *Acinetobacter calcoaceticus*–*A. baumannii* complex by using a Vitek GNI⁺ card (bioMérieux). Species identification was confirmed by PCR amplification of the intrinsic *bla*_{OXA-51} allele (Turton *et al.*, 2006). *A. baumannii* ATCC 19606^T was used as the reference strain.

Susceptibility test. MICs were determined by an agar dilution method according to Clinical and Laboratory Standards Institute guidelines. The drugs tested were triclosan (Sigma), imipenem (Merck), levofloxacin (Bayer), amikacin (Sigma) and tetracycline (Sigma). *P. aeruginosa* ATCC 27853 was used as a control. The MICs

Abbreviations: CCCP, carbonylcyanide-3-chlorophenyl hydrazone; PAβN, phenylarginine-β-naphthylamide.

of triclosan and the four other antimicrobial agents were also determined in the presence of the efflux pump inhibitors phenyl-arginine- β -naphthylamide (PA β N) (Sigma) at a concentration of 100 mg l⁻¹ and carbonylcyanide-3-chlorophenyl hydrazone (CCCP) (Sigma) at a concentration of 100 μ M.

PFGE. Genomic DNA was prepared from isolates and digested with *Apa*I (Sangon) as described by Seifert & Gerner-Smidt (1995). DNA fragments were separated by electrophoresis in 1% agarose gels (Sangon) using a CHEF Mapper XA (Bio-Rad) at 14 °C and 6 V cm⁻¹ with alternating pulses at a 120° angle in a 5–20 s pulse time gradient for 22 h. PFGE results were interpreted by using the criteria of Tenover *et al.* (1995).

PCR amplification and sequencing. The primers *fabI*_up and *fabI*_dw (Table 1) were used to amplify the complete *fabI* gene, in accordance with the genomic sequence of *A. baumannii* ATCC 17978 (GenBank accession no. NC_009085). The amplicons were sequenced using an ABI 3730 sequence analyser (Applied Biosystems). The nucleotide sequences and deduced amino acid sequences were analysed using MEGA software (version 4.0).

Real-time RT-PCR. Expression of the genes *fabI*, *adeB*, *abeM* and *adeJ* was measured by using real-time RT-PCR. The primers used are described in Table 1. Total RNA was prepared using an E.Z.N.A. bacterial RNA kit (Omega Bio-Tek) according to the manufacturer's instructions. The concentration and quality of RNA were determined using a spectrophotometer (Beckman Instruments). Reverse transcription was performed by using a PrimeScript RT reagent kit (Takara Bio) with 300 ng RNA in a 10 μ l reaction mixture containing 2 μ l PrimeScript buffer, 0.5 μ l PrimeScript reverse transcriptase enzyme mix I, 0.5 μ l oligo(dT) primer (50 μ M) and 0.5 μ l random hexamers (100 μ M). The mixtures were incubated at 37 °C for 40 min to synthesize cDNA and heated to 85 °C for 10 s to inactivate the reverse transcriptase. A DNA engine Opticon 2 real-time PCR detection system (Bio-Rad) was used for real-time PCR. PCRs were performed by using a SYBR PrimeScript RT-PCR kit (Takara Bio) with 1 μ l cDNA and 0.5 μ l each primer (10 μ M) in a total volume of 25 μ l. Each sample was run in duplicate. The 16S rRNA gene was used as a housekeeping gene to normalize the expression of target genes. Results were given as the relative expression of the mRNA compared with that of *A. baumannii* ATCC 19606^T, which was set at 1.

RESULTS

Triclosan MICs against 732 clinical *A. baumannii* isolates ranged from 0.015 to 16 mg l⁻¹ with a MIC₅₀ of 0.25 mg

l⁻¹ and a MIC₉₀ of 0.5 mg l⁻¹, which are lower than the actual in-use concentrations of triclosan (2000–20 000 mg l⁻¹). However, triclosan is stable in the environment and may remain on surfaces at low concentrations. Materials that release triclosan slowly, such as plastics and fabrics, are also used at low concentrations. For example, the final concentration of triclosan in triclosan baths is approximately 4–5 mg l⁻¹, lower than the MICs of some clinical isolates (Bamber & Neal, 1999). In this study, we found 20 isolates with elevated triclosan MICs (MICs >MIC₉₀; MICs $\geq$ 1 mg l⁻¹), and the least susceptible isolates had MICs of up to 16 mg l⁻¹. The clinical implication of this reduced susceptibility to triclosan is unknown.

The 20 isolates with reduced susceptibility to triclosan were artificially classified into two groups, low-level resistance (MICs 1–2 mg l⁻¹, *n*=8) and high-level resistance (MICs $\geq$ 4 mg l⁻¹, *n*=12) (Table 2). Twenty randomly selected sensitive isolates and ATCC 19606^T were used as triclosan-sensitive control isolates to test for common features found only among the resistant isolates. The resistant isolates showed two distinct PFGE patterns, of which three isolates belonged to pulsotype A and 17 isolates belonged to pulsotype B (data not shown). The susceptibility to four different antimicrobial agents (amikacin, tetracycline, levofloxacin and imipenem) was compared between the triclosan-resistant and -sensitive isolates (Table 2). The MICs of the four antimicrobial agents were interpreted using Clinical and Laboratory Standards Institute criteria. All triclosan-resistant isolates were also resistant to all four antimicrobial agents, whilst the rates of resistance to amikacin, tetracycline, levofloxacin and imipenem amongst the triclosan-sensitive isolates were 55, 55, 40 and 40 %, respectively. There were statistically significant differences in antimicrobial susceptibility between the two groups of isolates by χ^2 test (*P*<0.05, two-tailed test). Imipenem MICs were reduced by PA β N in imipenem-resistant isolates; however, no or few inhibitory effects were observed on the MICs of amikacin, tetracycline and levofloxacin in the presence of PA β N or CCCP. Previous

Table 1. Sequences of primers used for this study

Gene	Primer	Sequence (5'→3')	Reference
<i>fabI</i>	<i>fabI</i> _up	GTGAGATCGGCATGACACAA	This study
	<i>fabI</i> _dw	CTGAAGTCCGCTACCGTTAT	
<i>fabI</i> (quantitative)	<i>fabI</i> _Q_up	AGGCATAACGCGCTCAGAAC	This study
	<i>fabI</i> _Q_dw	GCACACGCTTGATGGTGACT	
<i>adeB</i> (quantitative)	<i>adeB</i> _Q_up	AACGGACGACCATCTTTGAGTATT	Peleg <i>et al.</i> (2007)
	<i>adeB</i> _Q_dw	CAGTTGTTCCATTTACGCATT	
<i>abeM</i> (quantitative)	<i>abeM</i> _Q_up	GGTAGGTGTAGGCTTATGGA	This study
	<i>abeM</i> _Q_dw	CTTCGGCAACTAATGGTGT	
<i>adeJ</i> (quantitative)	<i>adeJ</i> _Q_up	AGCGTATTGACAGCGGTATT	This study
	<i>adeJ</i> _Q_dw	ATAGAGCACGCCAGAGAAGA	
16S rRNA (quantitative)	16S RNA_Q_up	CAGCTCGTGTCTGAGATGT	Peleg <i>et al.</i> (2007)
	16S RNA_Q_dw	CGTAAGGGCCATGATGACTT	

Table 2. Characteristics of the triclosan-resistant and -sensitive isolates of *A. baumannii*

Numbers in parentheses refer to fold changes in triclosan susceptibility.

Strain	MIC (mg l ⁻¹)															<i>FabI</i>		Relative efflux pump expression		
	TRI	TRI + PAβN	TRI + CCCP	IMP	IMP + PAβN	IMP + CCCP	LEV	LEV + PAβN	LEV + CCCP	AMK	AMK + PAβN	AMK + CCCP	TET	TET + PAβN	TET + CCCP	Mutation	Relative expression	<i>adeB</i>	<i>abeM</i>	<i>adeJ</i>
H6	16	4 (4)	8 (2)	>256	>128 (-)	>128 (-)	32	8 (4)	8 (4)	256	128 (2)	128 (2)	1024	512 (2)	1024 (1)	Gly95Ser	0.60	0.01	0.22	1.40
F6	4	2 (2)	4 (1)	64	16 (4)	32 (2)	16	16 (1)	16 (1)	512	512 (1)	512 (1)	1024	1024 (1)	1024 (1)	Gly95Ser	4.35	0.72	4.15	3.13
W398	4	2 (2)	4 (1)	64	16 (4)	32 (2)	16	16 (1)	16 (1)	1024	1024 (1)	1024 (1)	1024	512 (2)	512 (2)	Gly95Ser	1.46	0.92	1.42	2.00
F1	4	1 (4)	2 (2)	64	16 (4)	32 (2)	32	16 (2)	16 (2)	512	512 (1)	512 (1)	1024	1024 (1)	1024 (1)	Gly95Ser	3.34	0.21	4.68	1.41
F2	4	1 (4)	4 (1)	64	16 (4)	32 (2)	32	16 (2)	16 (2)	512	512 (1)	512 (1)	1024	512 (2)	1024 (1)	Gly95Ser	4.48	0.62	0.77	1.07
F30	4	1 (4)	2 (2)	32	8 (4)	16 (2)	8	8 (1)	8 (1)	256	128 (2)	128 (2)	512	128 (4)	256 (2)	Gly95Ser	1.45	0.00	0.96	1.32
H1	4	1 (4)	4 (1)	64	16 (4)	32 (2)	8	8 (1)	8 (1)	128	64 (2)	128 (1)	1024	1024 (1)	256 (4)	Gly95Ser	2.06	0.00	4.91	1.44
H7	4	1 (4)	4 (1)	128	16 (8)	32 (4)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	Gly95Ser	1.40	0.99	2.06	2.37
W391	4	1 (4)	4 (1)	128	32 (4)	64 (2)	16	16 (1)	16 (1)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	Gly95Ser	0.94	0.50	0.77	1.78
W396	4	1 (4)	4 (1)	64	16 (4)	32 (2)	16	16 (1)	16 (1)	1024	1024 (1)	1024 (1)	1024	512 (2)	1024 (1)	Gly95Ser	0.63	1.12	2.37	2.28
W378	4	2 (2)	2 (1)	128	32 (4)	64 (2)	16	16 (1)	16 (1)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	Gly95Ser	0.86	0.23	0.89	1.73
NB207	4	1 (4)	2 (1)	128	64 (2)	64 (2)	8	8 (1)	8 (1)	512	512 (1)	512 (1)	1024	512 (2)	1024 (1)	Gly95Ser	2.24	0.88	6.30	2.42
F27	2	0.25 (8)	1 (2)	64	8 (8)	8 (8)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	-	6.88	0.29	3.93	1.04
F29	2	0.25 (8)	1 (2)	64	8 (8)	16 (4)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	1024 (1)	512 (2)	-	8.00	0.73	2.44	2.91
F13	1	0.125 (8)	1 (1)	128	32 (4)	32 (4)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	-	7.09	0.62	2.82	2.10
F16	1	0.125 (8)	1 (1)	64	32 (2)	32 (2)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	-	8.46	0.61	11.25	2.64
F17	1	0.125 (8)	1 (1)	64	32 (2)	32 (2)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	512 (2)	1024 (1)	-	7.77	1.08	2.02	2.28
F22	1	0.125 (8)	1 (1)	128	32 (4)	64 (2)	16	16 (1)	16 (1)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	-	9.72	1.24	1.46	2.11
F12	1	0.06 (16)	1 (1)	256	64 (4)	128 (2)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	-	11.46	0.31	6.19	1.89
F23	1	0.06 (16)	1 (1)	128	32 (4)	64 (2)	32	16 (2)	16 (2)	1024	1024 (1)	1024 (1)	1024	1024 (1)	1024 (1)	-	9.35	1.34	1.56	2.08
ZY2	0.5	0.25 (2)	0.5 (1)	128	32 (4)	64 (2)	16	8 (2)	8 (2)	512	512 (1)	256 (2)	512	256 (2)	512 (1)	-	0.80	0.28	0.69	0.31
F15	0.5	0.06 (8)	0.125 (4)	128	16 (8)	32 (4)	16	4 (4)	4 (4)	1024	1024 (1)	1024 (1)	512	512 (1)	512 (1)	-	0.96	0.35	2.69	0.40
ZY43	0.25	0.03 (8)	0.25 (1)	128	32 (4)	64 (2)	8	8 (1)	8 (1)	1024	1024 (1)	1024 (1)	512	256 (2)	512 (1)	-	0.71	0.43	3.65	0.97
ZY42	0.25	0.015 (16)	0.125 (2)	128	32 (4)	64 (2)	8	4 (2)	4 (2)	512	256 (1)	512 (1)	512	512 (1)	512 (1)	-	0.95	0.00	1.57	0.28
ZY47	0.25	0.004 (64)	0.125 (2)	0.25	0.25 (1)	0.25 (1)	0.06	0.06 (1)	0.06 (1)	256	256 (1)	256 (1)	2	2 (1)	2 (1)	-	0.97	0.24	0.74	0.68
CQ5	0.25	0.03 (8)	0.125 (2)	4	1 (4)	2 (2)	8	4 (2)	4 (2)	512	512 (1)	512 (1)	512	512 (1)	512 (1)	-	1.47	1.00	3.12	0.40
CQ6	0.25	0.03 (8)	0.25 (1)	4	4 (1)	4 (1)	8	4 (2)	4 (2)	1024	1024 (1)	1024 (1)	1024	512 (2)	512 (2)	-	0.95	3.41	1.59	0.83
SY206	0.25	0.03 (8)	0.25 (1)	128	16 (8)	64 (2)	8	8 (1)	8 (1)	1024	1024 (1)	1024 (1)	1024	512 (2)	512 (2)	-	2.48	1.00	3.10	0.84
W1657	0.125	0.03 (4)	0.06 (2)	0.5	0.25 (2)	0.25 (2)	0.25	0.25 (1)	0.125 (2)	2	2 (1)	2 (1)	2	1 (2)	2 (1)	-	1.26	0.04	0.39	0.84
L9924	0.125	0.03 (4)	0.06 (2)	0.5	0.25 (2)	0.25 (2)	0.125	0.125 (1)	0.06 (2)	2	2 (1)	2 (1)	2	2 (1)	1 (2)	-	0.24	0.18	3.31	0.81
JX16	0.125	0.03 (4)	0.125 (1)	0.5	0.25 (2)	0.25 (2)	0.5	0.25 (2)	0.25 (2)	2	2 (1)	2 (1)	2	2 (1)	2 (1)	-	0.47	0.42	0.01	0.43
B30	0.125	0.03 (4)	0.125 (1)	0.5	0.25 (2)	0.25 (2)	0.25	0.125 (2)	0.125 (2)	4	4 (1)	4 (1)	2	2 (1)	1 (2)	-	1.47	0.26	4.40	4.15
SH46	0.125	0.03 (4)	0.125 (1)	0.25	0.25 (1)	0.25 (1)	0.125	0.125 (1)	0.125 (1)	1	1 (1)	1 (1)	2	2 (1)	1 (2)	-	1.32	2.38	1.57	0.35
F11	0.125	0.008 (16)	0.125 (1)	64	16 (4)	32 (2)	4	4 (1)	4 (1)	1024	1024 (1)	1024 (1)	512	512 (1)	512 (1)	-	1.48	1.31	4.99	0.51
H18	0.125	0.015 (8)	0.125 (1)	128	32 (4)	64 (2)	8	4 (2)	4 (2)	1024	1024 (1)	1024 (1)	512	512 (1)	512 (1)	-	1.15	0.44	4.92	2.66

Table 2. cont.

Strain	MIC (mg l ⁻¹)												<i>FabI</i>		Relative efflux pump expression														
	TRI		TRI + PAβN		TRI + CCCP		IMP + PAβN		IMP + CCCP		LEV + PAβN		LEV + CCCP		AMK + PAβN		AMK + CCCP		TET + PAβN		TET + CCCP		Mutation		Relative expression				
	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	PAβN	CCCP	+	-	+	-			
P19	0.125	0.008 (16)	0.06 (2)	0.03 (2)	0.125	0.008 (16)	0.06 (2)	0.03 (2)	0.125	0.008 (16)	0.06 (2)	0.03 (2)	0.125	0.008 (16)	0.06 (2)	0.03 (2)	0.125	0.008 (16)	0.06 (2)	0.03 (2)	0.125	0.008 (16)	+	-	1.39	0.06	9.85	2.98	
NB317	0.06	0.03 (2)	0.03 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	1 (2)	1 (2)	+	-	0.98	0.09	1.45	0.64
JH318	0.06	0.03 (2)	0.03 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	2 (1)	2 (1)	+	-	1.44	3.94	1.09	0.95
SY319	0.03	0.015 (2)	0.03 (1)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	32 (1)	32 (1)	+	-	1.27	0.14	0.45	0.73
GZ320	0.03	0.015 (2)	0.03 (1)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	1 (2)	1 (2)	+	-	1.89	0.21	0.03	0.45
19606 ^T	0.25	0.03 (8)	0.25 (1)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	0.5 (2)	0.5 (2)	1	0.5 (2)	2 (1)	2 (1)	+	-	1.00	1.00	1.00	1.00

AMK, Amikacin; IMP, imipenem; LEV, levofloxacin; TET, tetracycline; TRI, triclosan.

studies have also demonstrated that the inhibitive effects of PAβN are limited to nalidixic acid, clarithromycin, rifampicin and linezolid in *A. baumannii* (Pannek *et al.*, 2006; Ribera *et al.*, 2002).

Twelve high-level resistant isolates had substitutions of glycine (GGC) to serine (AGC) at aa 95 in *FabI*. This missense mutation was found in all of the high-level resistant isolates but not in any of the low-level resistant ones (Table 2). Gly95 is located at a signature motif for the FAD-dependent pyridine nucleotide reductase family [D..(FILMV)..(IV)G..P...L], which is equivalent to the glycine found at aa 93 of *E. coli* and that at aa 95 of *P. aeruginosa* (Heath *et al.*, 1999; Hoang & Schweizer, 1999). Gly93Ser and Gly93Val substitutions in *E. coli* and a Gly95Val substitution in *P. aeruginosa* significantly elevate triclosan MICs and confer resistance to triclosan by preventing the formation of the *FabI*-NAD⁺-triclosan ternary complex (Bergler *et al.*, 1992; Heath *et al.*, 1999; Hoang & Schweizer, 1999). Heath *et al.* (1999) found that Gly93 in *E. coli* lies on one side of the substrate-binding pocket; when Val replaces Gly93, it occupies the space normally occupied by the dichlorophenyl ring of triclosan; they suggested that the Gly93Val mutation thus hampered formation of the ternary complex due to steric interference. We hypothesized that the Gly95Ser substitution in *FabI* of *A. baumannii* might result in triclosan resistance by the same mechanism. Because the mutant *fabI* gene was expressed normally or was slightly increased, high levels of triclosan resistance can be attributed mainly to *fabI* mutation rather than *fabI* overexpression. However, for the low-level resistant isolates, expression of wild-type *fabI* was increased significantly and was higher than that in the high-level resistant isolates and the sensitive isolates, indicating that low-level resistance was achieved via overexpression of wild-type *fabI* (Fig. 1). *FabI* overexpression has also been observed in an intermediate resistant clinical isolate of *Staphylococcus aureus* (Fan *et al.*, 2002). Moreover, low-level triclosan resistance has been observed when wild-type *fabI* was overexpressed artificially in *Salmonella enterica* serovar Typhimurium (Webber *et al.*, 2008).

Efflux pump overexpression has been shown to account for triclosan resistance in micro-organisms such as *P. aeruginosa* and *E. coli* (Chuanchuen *et al.*, 2001; McMurry *et al.*, 1998a; Mima *et al.*, 2007; Webber *et al.*, 2008). Su *et al.* (2005) confirmed that triclosan was a substrate for the efflux pump AbeM in *A. baumannii*, which increased the triclosan MIC fourfold (from 0.5 to 2 mg l⁻¹).

In this study, efflux pump inhibition tests and expression studies were performed to demonstrate the relationship between active efflux and triclosan resistance. Triclosan MICs were determined in the presence or absence of two types of efflux pump inhibitor, PAβN and CCCP (Table 2). CCCP exhibited few or no inhibitory effects on triclosan MICs at a concentration of 100 μM. In contrast, PAβN inhibited triclosan MICs in both triclosan-sensitive and -resistant isolates. Triclosan MICs were reduced more than

Fig. 1. Comparison of the relative *fabI* expression of triclosan-resistant and -sensitive isolates. From left to right, the 12 high-level resistant isolates, 8 low-level resistant isolates and 20 sensitive isolates represented are (respectively): H6, F6, W398, F1, F2, F30, H1, H7, W391, W396, W378 and NB207; F27, F29, F13, F16, F17, F22, F12 and F23; and ZY2, F15, ZY43, ZY42, ZY47, CQ5, CQ6, SY206, W1657, L9924, JX16, B30, SH46, F11, H18, P19, NB317, JH318, SY319 and GZ320. ■, High-level resistant isolates; □, low-level resistant isolates; ▲, sensitive isolates.

fourfold in the presence of PA β N in 15 triclosan-sensitive isolates and 17 triclosan-resistant isolates, which was taken as a sign of efflux pump overexpression. The most significant reduction in triclosan MICs was 64-fold. The susceptibility to triclosan of 12 isolates with a Gly95Ser mutation in the presence of PA β N was not restored to the level of sensitive isolates – they were still resistant to triclosan (MICs ≥ 1 mg l⁻¹). PA β N affects the MICs of antimicrobial agents by two different mechanisms, inhibiting efflux pumps and enhancing membrane permeability (Pannek *et al.*, 2006). If PA β N reduced the triclosan MICs by inhibiting the efflux pumps, active efflux may be responsible for the intrinsic MIC of triclosan against *A. baumannii*, and low-level and high-level resistance to triclosan might be acquired by *fabI* overexpression and *fabI* mutation, respectively, against a background of intrinsic triclosan efflux.

The genes *adeB*, *abeM* and *adeJ* encode the resistance-nodulation-cell division (RND) protein of the efflux pumps AdeABC, AbeM and AdeIJK, respectively. As *adeABC* genes are co-transcribed, the real-time RT-PCR results for *adeB* represented expression of the AdeABC pump (Marchand *et al.*, 2004), as for AdeIJK pumps (Damier-Piollé *et al.*, 2008). Detailed information on the relative expression of *adeB*, *abeM* and *adeJ* genes is given in Table 2. No overexpression of the *adeB* gene was found in triclosan-resistant isolates; however, the *abeM* gene was found to be overexpressed in both triclosan-sensitive and -resistant isolates relative to the type strain ATCC 19606^T. No statistically significant differences in mean expression of *adeB* and *abeM* were found between triclosan-sensitive and -resistant isolates by one-way ANOVA ($P=0.491$ and 0.471 , respectively). Thus, it appeared that expression of the *adeB* and *abeM* genes did not correlate with triclosan

Fig. 2. Comparison of the relative *adeJ* expression of triclosan-resistant and -sensitive isolates. From left to right, the 20 resistant isolates and 20 sensitive isolates represented are (respectively): H6, F6, W398, F1, F2, F30, H1, H7, W391, W396, W378, 207, F27, F29, F13, F16, F17, F22, F12 and F23; and ZY2, F15, ZY43, ZY42, ZY47, CQ5, CQ6, SY206, W1657, L9924, JX16, B30, SH46, F11, H18, P19, NB317, JH318, SY319 and GZ320. □, Triclosan-resistant isolates; ▲, triclosan-sensitive isolates.

resistance. Comparison of *adeJ* expression between triclosan-sensitive and -resistant isolates is shown in Fig. 2. Although the mean expression level of *adeJ* in triclosan-resistant isolates was higher than that in triclosan-sensitive isolates by one-way ANOVA ($P=0.001$), the highest level of expression of the *adeJ* gene was found in the triclosan-sensitive isolate B30. No tight correlation could be found between expression of the three known efflux pumps (AdeABC, AbeM and AdeIJK) and triclosan resistance. However, we could not exclude the possibility that active efflux is one of the mechanisms for triclosan resistance, as a number of other putative drug transporters have been identified by whole-genome sequencing of *A. baumannii* strains AYE and SDF and their function remains to be verified (Fournier *et al.*, 2006).

In summary, the majority of our clinical *A. baumannii* isolates from China were susceptible to triclosan, and only 3% exhibited reduced susceptibility. The resistance rates of the triclosan-resistant isolates to imipenem, levofloxacin, amikacin and tetracycline were higher than those of triclosan-sensitive isolates. High-level triclosan resistance could be explained by a Gly95Ser mutation of FabI, whilst overexpression of wild-type *fabI* was responsible for the low-level resistance. Active efflux did not appear to be a major reason for acquired triclosan resistance. As clinical isolates could acquire resistance to triclosan by *fabI* mutation and *fabI* overexpression, it is necessary to rationalize triclosan usage and monitor the susceptibility of *A. baumannii* to this drug.

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REFERENCES

- Bamber, A. I. & Neal, T. J. (1999). An assessment of triclosan susceptibility in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*. *J Hosp Infect* **41**, 107–109.
- Bergler, H., Hogenauer, G. & Turnowsky, F. (1992). Sequences of the *envM* gene and of two mutated alleles in *Escherichia coli*. *J Gen Microbiol* **138**, 2093–2100.
- Chuanchuen, R., Beinlich, K., Hoang, T. T., Becher, A., Karkhoff-Schweizer, R. R. & Schweizer, H. P. (2001). Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrob Agents Chemother* **45**, 428–432.
- Damier-Piolle, L., Magnet, S., Brémont, S., Lambert, T. & Courvalin, P. (2008). AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **52**, 557–562.
- Fan, F., Yan, K., Wallis, N. G., Reed, S., Moore, T. D., Rittenhouse, S. F., DeWolf, W. E., Jr, Huang, J., McDevitt, D. & other authors (2002). Defining and combating the mechanisms of triclosan resistance in clinical isolates of *Staphylococcus aureus*. *Antimicrob Agents Chemother* **46**, 3343–3347.
- Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangelot, S. & other authors (2006). Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet* **2**, e7.
- Heath, R. J., Rubin, J. R., Holland, D. R., Zhang, E., Snow, M. E. & Rock, C. O. (1999). Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *J Biol Chem* **274**, 11110–11114.
- Hoang, T. T. & Schweizer, H. P. (1999). Characterization of *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. *J Bacteriol* **181**, 5489–5497.
- Magnet, S., Courvalin, P. & Lambert, T. (2001). Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob Agents Chemother* **45**, 3375–3380.
- Marchand, I., Damier-Piolle, L., Courvalin, P. & Lambert, T. (2004). Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. *Antimicrob Agents Chemother* **48**, 3298–3304.
- McMurry, L. M., Oethinger, M. & Levy, S. B. (1998a). Overexpression of *marA*, *soxS*, or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiol Lett* **166**, 305–309.
- McMurry, L. M., Oethinger, M. & Levy, S. B. (1998b). Triclosan targets lipid synthesis. *Nature* **394**, 531–532.
- Mima, T., Joshi, S., Gomez-Escalada, M. & Schweizer, H. P. (2007). Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. *J Bacteriol* **189**, 7600–7609.
- Pannek, S., Higgins, P. G., Steinke, P., Jonas, D., Akova, M., Bohnert, J. A., Seifert, H. & Kern, W. V. (2006). Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine- β -naphthylamide. *J Antimicrob Chemother* **57**, 970–974.
- Peleg, A. Y., Adams, J. & Paterson, D. L. (2007). Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **51**, 2065–2069.
- Peleg, A. Y., Seifert, H. & Paterson, D. L. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin Microbiol Rev* **21**, 538–582.
- Ribera, A., Ruiz, J., Jimenez de Anta, M. T. & Vila, J. (2002). Effect of an efflux pump inhibitor on the MIC of nalidixic acid for *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* clinical isolates. *J Antimicrob Chemother* **49**, 697–698.
- Seifert, H. & Gerner-Smidt, P. (1995). Comparison of ribotyping and pulsed-field gel electrophoresis for molecular typing of *Acinetobacter* isolates. *J Clin Microbiol* **33**, 1402–1407.
- Su, X. Z., Chen, J., Mizushima, T., Kuroda, T. & Tsuchiya, T. (2005). AbeM, an H⁺-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob Agents Chemother* **49**, 4362–4364.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.
- Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E. & Pitt, T. L. (2006). Identification of *Acinetobacter baumannii* by detection of the *bla*_{OXA-51-like} carbapenemase gene intrinsic to this species. *J Clin Microbiol* **44**, 2974–2976.
- Webber, M. A., Randall, L. P., Cooles, S., Woodward, M. J. & Piddock, L. J. (2008). Triclosan resistance in *Salmonella enterica* serovar Typhimurium. *J Antimicrob Chemother* **62**, 83–91.
- Yazdankhah, S. P., Scheie, A. A., Hoiby, E. A., Lunestad, B. T., Heir, E., Fotland, T. O., Naterstad, K. & Kruse, H. (2006). Triclosan and antimicrobial resistance in bacteria: an overview. *Microb Drug Resist* **12**, 83–90.