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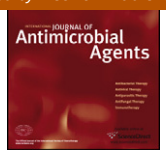

A novel resistance mechanism to triclosan that suggests horizontal gene transfer and demonstrates a potential selective pressure for reduced biocide susceptibility in clinical strains of *Staphylococcus aureus*

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ABSTRACT

The widely used biocide triclosan selectively targets FabI, the NADH-dependent trans-2-enoyl-acyl carrier protein reductase, which is an important target for narrow-spectrum antimicrobial drug development. In relation to the growing concern about biocide resistance, we compared in vitro mutants and clinical isolates of *Staphylococcus aureus* with reduced triclosan susceptibility. Clinical isolates of *S. aureus* as well as laboratory-generated mutants were assayed for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) phenotypes and genotypes related to reduced triclosan susceptibility. A potential epidemiological cut-off (ECOFF) MBC of >4 mg/L was observed for triclosan in clinical isolates of *S. aureus*. These showed significantly lower MICs and higher MBCs than laboratory mutants. These groups of strains also had few similarities in the triclosan resistance mechanism. Molecular analysis identified novel resistance mechanisms linked to the presence of an additional *sh-fabI* allele derived from *Staphylococcus haemolyticus*. The lack of predictive value of in-vitro-selected mutations for clinical isolates indicates that laboratory tests in the present form appear to be of limited value. More importantly, detection of *sh-fabI* as a novel resistance mechanism with high potential for horizontal gene transfer demonstrates for the first time that a biocide could exert a selective pressure able to drive the spread of a resistance determinant in a human pathogen.

1. Introduction

There is growing concern worldwide regarding the possible effect of biocides on antibiotic resistance. The Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) in the USA, the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and the Scientific Committee on Consumer Safety (SCCS) in the European Union (EU), and the Australian Microbiological Society have, amongst others, all expressed concern and have programmes running to investigate the impact of biocide use on antimicrobial resistance [1–5]. Bacterial resistance to biocides has been well studied in vitro, but concrete evidence of clinical resistance is lacking [6,7]. In view of the new licensing requirements, protocols are urgently needed to provide risk assessments on the use of biocidal products, especially as there is no consensus on the methodologies to be used to study bacterial resistance towards biocides.

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The biocide triclosan has received much attention because it is widely used and reports indicating emergence of triclosan resistance have been published [8–11]. Furthermore, in contrast to other biocides, triclosan at low concentrations acts similarly to antibiotics on a specific cellular target, the enoyl-acyl carrier protein reductase (FabI), an essential enzyme in bacterial fatty acid synthesis. Triclosan exhibits excellent activity against *Staphylococcus aureus* and is used to control the carriage of meticillin-resistant *S. aureus* (MRSA) in hospitals [shampoo or bath additive with 2% (20 g/L) triclosan] [12]. Laboratory studies with *Escherichia coli* and *S. aureus* have shown that mutations in FabI and its overexpression decrease bacterial susceptibility to triclosan [9,13,14]. The possible selective pressure exerted by triclosan raises some concern as FabI is a promising target for new narrow-spectrum antimicrobials against *Mycobacterium tuberculosis*, *Plasmodium falciparum* and drug-resistant *S. aureus* [15–17].

The aim of this study was to analyse the molecular nature and phenotypes of triclosan resistance in *S. aureus*, with particular focus on the relationship between in-vitro-selected mutants and clinical isolates.

2. Methods

2.1. Clinical strains

A collection of 1388 *S. aureus* strains collected in 2002–2003 from different geographical origins, representing hospital and community-acquired infections, were screened to ascertain triclosan susceptibility. *Staphylococcus haemolyticus* strains were from a collection of clinical isolates in Siena (Italy).

2.2. Bacterial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines, except for the way triclosan was added to the cultures [18]. Stock solutions of triclosan (Irgasan; Sigma, Steinheim, Germany) were prepared at 102 400 mg/L in methanol. Owing to the high hydrophobicity of triclosan, serial 16-fold diluted substocks in methanol where prepared from which to prepare subsets of three dilutions in the microtitre plate. This approach was taken to avoid serial two-fold dilutions in microplates in order to minimise absorption of triclosan to the plastic and to decrease the chances of triclosan precipitating out of solution when triclosan in methanol was added to water. Minimum bactericidal concentrations (MBCs) were determined by subculturing 10 μL from each well without visible bacterial growth on Mueller–Hinton agar plates (Biotec, Grosseto, Italy). After 24 h of incubation at 37 °C, the dilution yielding three colonies or less was scored as the MBC, as described by the CLSI for starting inocula of 1×10^5 CFU/mL [19]. No neutralisation step was included in the MBC assay as initial experiments verified that triclosan carry-over did not occur when 10 μL was inoculated onto agar (data not shown). The sensitivity to chemical compounds was tested by phenotype microarray utilising Biolog microtitre plates PM11 through PM20 as described (Biolog Inc., Hayward, CA) [20].

2.3. Biocide activity testing

Biocide activity was tested according to the standards defined by the European standard EN 1276 [21]. In brief, $1.5–5 \times 10^8$ CFU of bacteria in 1 mL were mixed with 1 mL of bovine serum albumin (BSA) (Sigma) at 0.03 g/L (clean conditions) as interference substance. Afterwards, this bacterial suspension was mixed with 8 mL of a triclosan dilution containing 1.25 times the desired test concentration. For the activity assay, preparation of triclosan stock was performed as follows: 300 mg of triclosan was diluted in 1 mL of dimethyl sulphoxide (DMSO) and this mixture was diluted in 200 mL of hard water (composition defined in EN 1276) [21]. Subsequent dilutions of triclosan were undertaken in hard water. A solution of hard water containing 0.5% DMSO was tested according to EN 1276 against *S. aureus* to ensure that a solution with 0.5% DMSO does not have bactericidal activity. The concentrations of triclosan utilised for the assay were 100, 600 and 1000 mg/L. After 5 min of contact time between triclosan, BSA and bacteria at 20 °C, 1 mL of the test solution was mixed with 8 mL of neutraliser (3 g/L lecithin, 30 g/L polysorbate 80, 5 g/L sodium thiosulfate, 1 g/L L-histidine and 30 g/L saponin) and 1 mL of water. After 5 min of the neutralisation step, 1 mL of the neutralisation mix and 1 mL of ten-fold dilutions were cultured onto tryptic soy agar (TSA) (Liofilchem, Roseto degli Abruzzi, Italy) plates in duplicate and were incubated at 37 °C for 48 h. CFU/mL were determined and log CFU/mL reduction was calculated for each strain against each of the three triclosan concentrations tested. The concentration of 600 mg/L was determined as the lowest concentration tested that produced a 5 log reduction in CFU/mL with reference strain *S. aureus* ATCC 6538.

2.4. In vitro selection of triclosan-resistant mutants

Triclosan-resistant mutants were selected from *S. aureus* reference strains, including the standard laboratory strain RN4220, the reference strain for biocide testing ATCC 6538, and three MRSA clinical isolates (MW2, Mu50 and COL) for which the genome sequences were available. Single-step mutants were selected by culturing ca. 1×10^{11} CFU of *S. aureus* cells, harvested from 30 mL of liquid culture, on TSA with 0.5 mg/L triclosan (plates contained <0.1% methanol from the biocide stock). Multistep mutants were selected by serial passage of strains in liquid tryptic soy broth (Liofilchem) containing two-fold increasing concentrations of triclosan (0.25 mg/L to 4 mg/L). Single colonies were randomly selected from each assay and were subcultured for further analysis.

2.5. Statistical correlation test

Three different statistical tests were performed to assess potential correlations between phenotypes and genotypes of clinical isolates and laboratory mutants. Fisher's exact test was used as a statistical test applied to contingency tables to determine whether there were non-random associations between two categorical variables. Spearman's correlation coefficient was chosen because we had unknown sample distributions and the tested variables did not show a linear relationship [22]. Two-sample Kolmogorov–Smirnov test was used to compare the fold change distribution of the two types of strains (clinical isolates and in vitro mutants) [22].

2.6. Molecular analysis

The central part of the *fabI* gene was amplified in isolates showing reduced susceptibility to triclosan. DNA was amplified with primers TAGCCGTAAAGAGCTTGAA and ATATTTTCACCTGTAACGCCA (Eurofins MWG Operon, Germany), controlled with Vector NTI- software v.6 (Informax Inc., Bethesda, MD), using standard PCR conditions and were sequenced by the Sanger method (BMR Genomics, University of Padova, Italy). For some selected strains, without mutations in the central part of *S. aureus fabI* (*sa-fabI*), primers GATACAGAAAGGACTAAATCAAA and TTTCCATCAGTCCGATTATTATA were used to amplify and sequence the whole gene. A selection of *fabI* allele sequences has been deposited in GenBank (accession nos. JF797286 through JF797303). Whole-genome sequencing of the *S. aureus* clinical isolate QBR-102278-1619 was performed by the Institute of Applied Genomics (University of Udine, Italy) using an Illumina Genome Analyzer

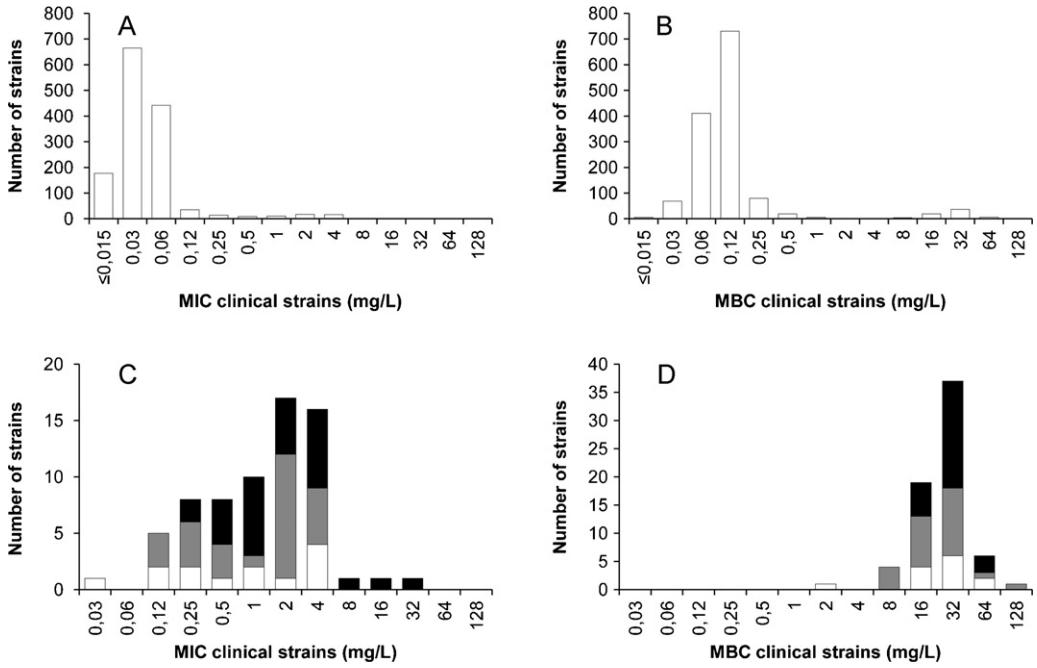

Fig. 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) distribution and *fabI* genotypes of clinical *Staphylococcus aureus* isolates. Triclosan susceptibility of 1388 clinical isolates is reported according to their (A) MIC and (B) MBC. The genotype of those clinical isolates with reduced susceptibility to triclosan (high MBC) is shown in panels (C) and (D) by sorting strains according to their MIC and MBC, respectively. Shading differentiates triclosan-resistant strains with a mutated *sa-fabI* (grey), wild-type *sa-fabI* (open bars) and those heterodiploid for the *sh-fabI* gene (black).

II platform (Illumina, San Diego, CA). Open-reading frame (ORF) prediction was carried out using Prodigal software (Oak Ridge National Laboratory, Oak Ridge, TN). Detection of *S. haemolyticus fabI* (*sh-fabI*) was performed by real-time PCR using primers TGGCGAAGAAGTAGGCAATAT and GCAACAATACTACCACCGTT. The *sh-fabI* insert in QBR-102278-1619 was deposited in GenBank with accession no. JQ712986.

3. Results

Analysis of 1388 clinical isolates of *S. aureus* revealed a continuous distribution of triclosan MICs from ≤0.015 mg/L to 32 mg/L, with a single modal MIC of 0.03 mg/L (Fig. 1A). In contrast, triclosan MBCs presented a discontinuous distribution (Fig. 1B). After performing sampling of the MBC data set in order to balance the scale of observations, we can fit a mixture of normal distributions showing that we have two different populations, suggesting a potential epidemiological cut-off (ECOFF) [23] MBC of ≤2 mg/L for the susceptible population and >4 mg/L for 'resistant' strains (Fig. 1B). Although statistical analysis showed that MIC and MBC values of triclosan of clinical strains were moderately correlated ($\rho = 0.73$; $P < 0.001$), it would appear that the MBC is better able to separate triclosan-non-susceptible strains than the MIC. Sixty-eight strains presenting reduced susceptibility for this biocide (MBC > 4 mg/L) were chosen for further characterisation. The biocide activity assay according to EN 1276 confirms a decreased activity of triclosan for strains with reduced susceptibility to the biocide (Table 1).

To assess the molecular basis of resistance to triclosan, mutant strains were selected in vitro from five *S. aureus* reference strains. Single-step mutants were selected in four of them with frequencies of 2.4×10^{-9} for MW2, 3.4×10^{-10} for Mu50, 3.4×10^{-9} for COL and 1.4×10^{-9} for ATCC 6538. From strain RN4220, which presented intermediate susceptibility (MBC = 2 mg/L), only multistep mutants could be selected. Irrespective of the strains from which they were selected, the mutants showed triclosan MICs of 1–8 mg/L (modal MIC = 4 mg/L) and MBCs of 4–32 mg/L (modal MBC = 8 mg/L) (Fig. 2A and B). Unlike the clinical isolates, MICs and MBCs of triclosan for in vitro mutants present a strong statistically significant non-linear correlation ($\rho = 0.90$; $P < 0.001$). The difference between the MIC and MBC of laboratory mutants was usually of one or two dilutions, whilst for clinical strains these differences were generally much higher (Fig. 2C). This was the case even when the in vitro mutants and the clinical isolates presented the same *sa-fabI* mutation (Tables 2 and 3). This was found to be significantly different using a two-sample Kolmogorov–Smirnov test ($P < 0.001$). Phenotype microarray for chemical sensitivity to over 300 compounds [20] confirmed that the in-vitro-selected triclosan-resistant mutants did not acquire any further resistance phenotype in addition to triclosan (data not shown).

To identify the genotypes conferring reduced triclosan susceptibility, the *fabI* gene was sequenced. Among the 68 clinical isolates with reduced susceptibility to triclosan, 30 presented a mutation in *sa-fabI*, whilst 38 strains had a wild-type *sa-fabI* allele (Table 2; Fig. 1C and D). Of the 30 strains with a mutated *sa-fabI*, 22 carried previously described mutations, whilst 8 strains showed four novel mutations, which is in accordance with other published data [9,10] (Table 2; Fig. 3A). Clustering was observed for the TTC611TGC mutation, only found in strains from Italy (4 of 5) and France (2 of 7) and the four GCA593GGA-CTT622TTT double mutants, which were isolated at different cities in the USA and Canada. Most in-vitro-selected mutants had previously characterised *fabI* mutations [9–11,17], with the exception of RN4220 mutants, which all showed a GAC301TAC mutation, and one ATCC 6538 derivative, which had a TTC611TCC change (Table 3; Fig. 3A). Only two of six mutations selected in vitro (GCA593GGA and TTC611TGC) matched mutations detected in clinical isolates (Fig. 3A). Two clones (MO035 and MO079) showed no variation in the *sa-fabI* gene despite high MICs and MBCs to triclosan (Table 3).

To identify further the molecular basis of reduced triclosan susceptibility of clinical isolates with a wild-type *fabI* allele, the whole genome of one strain with a triclosan MIC of 4 mg/L and MBC of 32 mg/L (QBR-102278-1619) was sequenced. A 3016 bp chromosomal insert carrying an additional *fabI* gene, showing 84% nucleotide and 91% amino acid identity to *sa-fabI*, and an insertion sequence

Table 1
Testing of triclosan activity on *Staphylococcus aureus* strains following Clinical and Laboratory Standard Institute (CLSI) and European standard EN 1276 guidelines.

Strain	MIC (mg/L)	MBC (mg/L)	EN 1276 (log reduction CFU/mL)[a]			Note
			100 mg/L	600 mg/L	1000 mg/L	
ATCC 6538	0.12	0.25	0.33	5.45	>5.48	Wild-type
QBR-102278-1177	4	32	0.18	4.04	5.48	Mutated *sa-fabI*
QBR-102278-1219	4	32	0.27	3.96	4.01	Mutated *sa-fabI*
QBR-102278-1619	4	32	0.41	4.67	5.45	*sh-fabI*

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.
[a] Values report logarithmic reduction (R) of bacterial counts within 5 min contact time and subsequent neutralisation (product is considered active if log R > 5).

IS*1272* (Fig. 3B) was found in an intergenic region of the *S. aureus* chromosome (MW2 position 141825) (Fig. 3B). The integration occurred in the loop of a hairpin with an 18 bp inverted repeat stem, which determined an insert between two short direct repeats. Database searches with this additional *fabI* gene showed its presence, with 100% identity, in the chromosome of *S. haemolyticus* (Fig. 3B), which does not have any further *fabI* gene. This strongly suggests that the *sh-fabI* allele most likely belongs to the core genome of *S. haemolyticus*. Supporting this statement, PCR analysis demonstrated the presence of *sh-fabI* in a selection of five *S. haemolyticus* clinical strains, irrespective of their susceptibility to triclosan (MBC range 1–32 mg/L). Further searches for *sh-fabI* showed multiple hits in different staphylococci, including *S. aureus* and *Staphylococcus epidermidis*, where *sh-fabI* was located on plasmids that also carry the multidrug resistance (MDR) efflux pump for quaternary ammonium compounds QacA (GenBank accession nos. FR821778 and GQ900465) [24,25]. The fact that these plasmids carry the 3016 bp insert bordered by parts of the inverted repeat of the *S. aureus* chromosome indicates the direction of horizontal transfer.

PCR assays of the 68 clinical isolates with reduced susceptibility to triclosan identified *sh-fabI* in 24 of the 38 strains with wild-type *fabI* and in 4 of the 30 strains with mutated *fabI* (Table 2). Distribution of *sh-fabI* in *S. aureus* strains with reduced triclosan susceptibility showed geographical clustering, with positivity in 9/10 isolates from Mexico, 7/10 from Canada, 5/10 from Brazil and 4/8 from Japan, with no strains from other countries including the USA, Italy, Spain and Germany. Only one of the *sh-fabI*-positive clinical isolates was positive for the MDR efflux determinant *qacA* (data not shown). Clinical strains with decreased susceptibility to triclosan had a strong association with the presence of a mutated *fabI* gene or the alternative *sh-fabI* gene (Fisher's exact test, $P < 0.001$).

4. Discussion

FabI is the target of isoniazid, an important agent for the treatment of tuberculosis, and is one of the drug targets that has been rediscovered in recent years for rational antimicrobial drug development [17,26]. In this context, careful analysis of the effect of triclosan, a widely utilised biocide and disinfectant, which also targets FabI, on the susceptibility of staphylococci is of prime interest.

To address the molecular basis of triclosan resistance in *S. aureus*, 68 strains with reduced susceptibility to the biocide selected from a worldwide collection of clinical and community-acquired *S. aureus* were analysed. As FabI is the only known target of triclosan [9,13,14], attention was focused on the nucleotide sequence of *fabI*. Surprisingly, only approximately one-half of the strains showing high MBC values to triclosan had detectable mutations in the

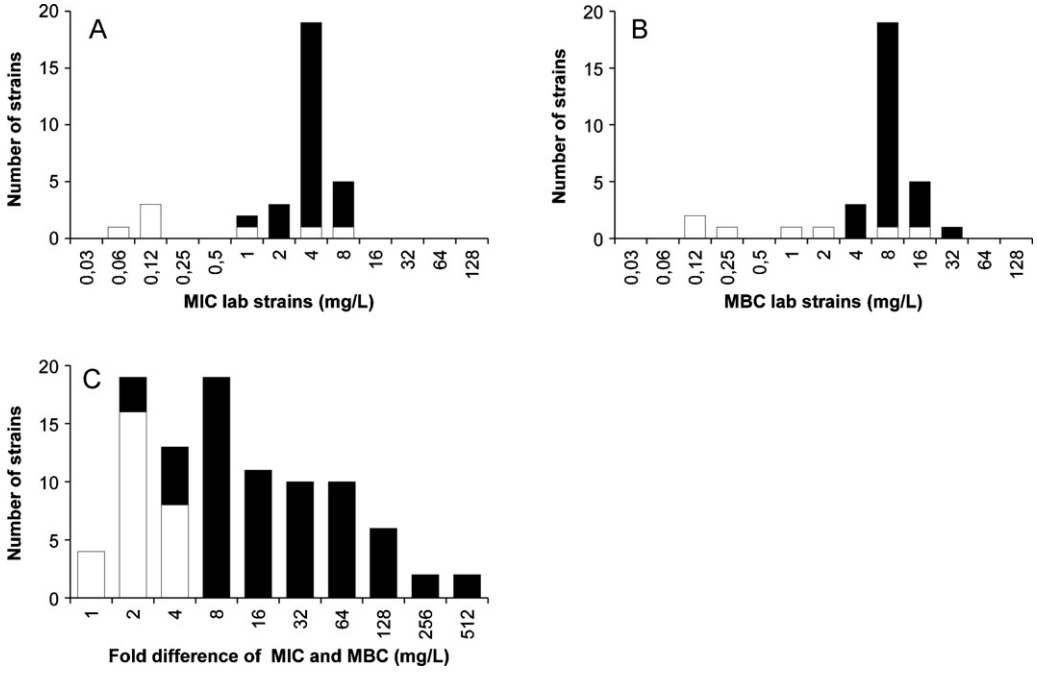

Fig. 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) distribution and *fabI* genotypes of laboratory mutants. Triclosan susceptibility of laboratory strains, including reference strains and mutants, is reported according to their (A) MIC and (B) MBC. Genotypic data are shown by shading of the columns differentiating susceptible reference stains (wild-type *sa-fabI*, open bars) and triclosan-resistant mutants with mutated *sa-fabI* (black) and wild-type *sa-fabI* (open bars). (C) Distribution of the MBC/MIC fold change of strains with reduced susceptibility to triclosan selected in vitro ($n = 28$) (open bars) and isolated from the clinical strain collections ($n = 68$) (black).

Table 2
fabI gene sequences of *Staphylococcus aureus* clinical isolates and reference strains.

Isolate	Polymorphic sites in *fabI*[a] 1222222233333333344444555566666773801256780133677883567947891126802 3446651241589338149081801330120783	*sh–fabI*	*sa-fabI*	MIC (mg/L)	MBC (mg/L)	Comment [b]
COL	CTAGGCTACGCGCTTATGTCCTCAGACTTCTTTT	–	wt	0.25	1	Reference strain
QBR-102278-1619	.....	+	wt	4	32	wt allele in 16 sequenced genomes
QBR-102278-2351	.....	+	wt	8	32	wt allele in 16 sequenced genomes
QBR-102278-1888	.....	–	wt	0.03	16	wt allele in 16 sequenced genomes
QBR-102278-2376	.....	+	wt	4	32	wt allele in 16 sequenced genomes
QBR-102278-2175	.....	+	wt	0.25	16	wt allele in 16 sequenced genomes
QBR-102278-2138	.....	+	wt	4	32	wt allele in 16 sequenced genomes
QBR-102278-2365	.....	+	wt	2	32	wt allele in 16 sequenced genomes
QBR-102278-2305	.....	–	wt	4	64	wt allele in 16 sequenced genomes
QBR-102278-2321	.....	–	wt	4	32	wt allele in 16 sequenced genomes
QBR-102278-2092	.....	+	wt	4	32	wt allele in 16 sequenced genomes
QBR-102278-1219	.....G.	–	Mutated	4	32	TTC611TGC known mutation
QBR-102278-1192	.....G.	–	Mutated	4	32	TTC611TGC known mutation
QBR-102278-1177	.....G.	–	Mutated	4	32	TTC611TGC known mutation
QBR-102278-1522	.....G.	–	Mutated	4	32	TTC611TGC known mutation
QBR-102278-1503	.....G.	–	Mutated	4	32	TTC611TGC known mutation
QBR-102278-1505	.....G.	–	Mutated	2	16	TTC611TGC known mutation
QBR-102278-1508	.....G.	–	Mutated	2	8	TTC611TGC known mutation
QBR-102278-1865	.....G...	–	Mutated	0.5	16	GCA593GGA known mutation
QBR-102278-1970	.....G...	–	Mutated	0.5	32	GCA593GGA known mutation
QBR-102278-1917	.....G..T	–	Mutated	2	16	GCA593GGA, CTT622TTT known mutations
QBR-102278-1207	......C..T.T.CTCT...C...T....	–	Mutated	0.12	8	ACA583TCA new allele
QBR-102278-1353	......C..T.T.CTCT...C...T....	–	Mutated	0.12	16	ACA583TCA new allele

Table 2 (*continued*)

QBR-102278-1935	.....C..T.T.CTCT...C...T....	–	Mutated	0.25	16	ACA583TCA new allele
QBR-102278-1277	.....C..T.T.CTCT...C...T....	–	Mutated	0.25	128	ACA583TCA new allele
QBR-102278-1919	.....C..T.T.CTCT...C...T....	–	Mutated	0.12	16	ACA583TCA new allele
QBR-102278-1883	.....C...T.T.CTCT...C....G..T	–	Mutated	2	8	GCA593GGA CTT622TTT known mutations
QBR-102278-2345	.....C...T.T.CTCT...C........	–	wt	1	2	wt allele in 4 sequenced genomes
QBR-102278-2363	..........T.CTCT............	+	wt	16	32	wt allele in 23 sequenced genomes
QBR-102278-1878	.....C..T.T.CTCT...C....G..T	–	Mutated	2	16	GCA593GGA, CTT622TTT known mutations
QBR-102278-2069	.....C..T.T.CTCT...C....G..T	–	Mutated	2	32	GCA593GGA, CTT622TTT known mutations
QBR-102278-1894	GT....C..T.T.CTCT...C....G..T	–	Mutated	2	16	GCA593GGA, CTT622TTT known mutations
QBR-102278-1651	.....C..T.T.CTCT........G...	–	Mutated	2	32	GCA593GGA known mutation
QBR-102278-1653	.....C..T.T.CTCT........G...	–	Mutated	2	32	GCA593GGA known mutation
QBR-102278-2019	.....C..T.TCCTCT.........G..	–	Mutated	0.25	16	TTC610GTC new allele
ATCC25923	...A...C..T.T.CTCT....T........A..	–	wt	0.06	1	Reference strain
QBR-102278-1097	....TTC.....T.CTCT............	–	Mutated	0.25	32	GGT226TGT,GGC255GGT new allele
QBR-102278-1203	T..........T.CTCT..............	+	wt	2	16	wt allele in 4 sequenced genomes
QBR-102278-2105	..........T.CTCT............	+	wt	2	32	wt allele in 4 sequenced genomes
QBR-102278-1091	..........T.CTCT............	+	wt	4	32	wt allele in 4 sequenced genomes
QBR-102278-1107	T..........T.CTCT..............	+	wt	4	32	wt allele in 4 sequenced genomes
QBR-102278-1052	T..........T.CTCT............C...	+	wt	0.5	64	wt allele in 4 sequenced genomes
QBR-102278-1544	..........T.CTCT........G...	–	Mutated	2	64	GCA593GGA known mutation
QBR-102278-1144	..........T.CTCT.........G.	–	Mutated	1	32	TTC611TGC known mutation, new allele
MW2	..........T.TTCT............	–	wt	0.5	1	Reference strain
QBR-102278-2311	..........T.C..............	–	wt	1	64	wt allele in 4 sequenced genomes
QBR-102278-2212	..........T.C..............	+	wt	2	32	wt allele in 4 sequenced genomes
QBR-102278-2221	..........T.C..............	+	wt	0.5	16	wt allele in 4 sequenced genomes
QBR-102278-2605	.....C...T.T.C......C........	+	wt	32	64	wt allele in 4 sequenced genomes
QBR-102278-2546	....TC....CT.C..............	+	Mutated	1	64	GGC255GGT, GGC338GCT new allele
QBR-102278-2342	................T..T..G...	+	Mutated	2	32	GCA593GGA known mutation
QBR-102278-2348	................T..T..G...	+	Mutated	0.5	32	GCA593GGA known mutation
QBR-102278-2254	................T.....G...	+	Mutated	1	32	GCA593GGA known mutation
QBR-102278-2194	................T.....G...	–	Mutated	0.5	32	GCA593GGA known mutation

Table 2 (*continued*).

Mu50	.....T..T.....	–	wt	0.25	0·5	Reference strain
QBR-102278-2346	.....T..T.....	–	wt	2	32	wt allele in 23 sequenced genomes
QBR-102278-2222	.....T..T.....	+	wt	1	32	wt allele in 23 sequenced genomes
QBR-102278-2210	.....T..T.....	+	wt	1	32	wt allele in 23 sequenced genomes
QBR-102278-1889	.....T..T.....	–	wt	8	16	wt allele in 23 sequenced genomes
QBR-102278-2269	.....T..T.....	+	wt	1	32	wt allele in 23 sequenced genomes
QBR-102278-2207	.....T..T.....	+	wt	4	32	wt allele in 23 sequenced genomes
QBR-102278-1730	.....T..T.....	–	wt	4	32	wt allele in 23 sequenced genomes
QBR-102278-2205	.....T.....	+	wt	1	16	wt allele in 19 sequenced genomes
QBR-102278-2204	.....T.....	+	wt	1	16	wt allele in 19 sequenced genomes
ATCC6538	.....T..T.....CAC.	–	wt	0.12	0·25	wt new allele
QBR-102278-1236	.....T..T.....CAC.	–	wt	4	16	wt allele in 23 sequenced genomes
QBR-102278-1607	.....T..T.....CAC.	–	wt	0.12	32	wt allele in 23 sequenced genomes
QBR-102278-2072	.....T..T.....CAC.	+	wt	0.25	32	wt allele in 23 sequenced genomes
QBR-102278-1210	.....T..T.....	–	wt	0.25	16	wt allele in 23 sequenced genomes
QBR-102278-2070	.....T..T.....	–	wt	0.12	32	wt allele in 23 sequenced genomes
QBR-102278-1158	G.....GT.....	–	wt	2	8	wt new allele
QBR-102278-1969	.....A.....	–	wt	0.25	32	wt new allele
QBR-102278-2018	.....A.....	+	wt	0.5	16	wt new allele
RN4220	.....A.....	–	wt	1	2	Reference strain

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; wt, wild-type.
[a] Polymorphic sites are indicated with respect to the *fabI* sequence of *S. aureus* COL.
[b] GenBank last accessed in December 2011.

Table 3
Genotype and phenotype of in vitro multistep and single-step exposure mutants.

ID	Polymorphic sites in *fabI* [a] 1222222233333333344444555566666773801256780133677883567947891126802 3446651241589338149081801330120783	FabI	*sa-fabI*	MIC (mg/L)	MBC (mg/L)	Comment
COL	CTAGGCTACGCGCTTATGTCCTCAGACTTCTTTT		wt	0.12	1	Reference strain
MO082	........T..........................	Ala95Val	Mutated	8	16	SSM
MO083	........T..........................	Ala95Val	Mutated	4	16	SSM
MO084	........T..........................	Ala95Val	Mutated	4	8	SSM
MW2	........T...T.TTCT.................		wt	0.12	0.12	Reference strain
MO075	........T...T.TTCT.................	Ala95Val	Mutated	4	16	SSM
MO076	........T...T.TTCT.................	Ala95Val	Mutated	4	8	SSM
MO077	........T...T.TTCT.................	Ala95Val	Mutated	8	32	SSM
Mu50	....................T..T..........		wt	0.06	0.12	Reference strain
MO079	....................T..T..........		wt	4	16	SSM
MO080	........T...........T..T..........	Ala95Val	Mutated	4	4	SSM
ATCC6538	....................T..T......CAC.		wt	0.12	0.25	Reference strain
CR001	....................T..T..G...CAC.	Ala198Gly	Mutated	4	8	SSM
CR002	....................T..T....G.CAC.	Phe204Cys	Mutated	4	8	SSM
CR003	....................T..T....G.CAC.	Phe204Cys	Mutated	2	8	SSM
CR004	....................T..T....G.CAC.	Phe204Cys	Mutated	2	8	SSM

Table 3 (*continued*).

d2	.....T..T....G.CAC.	Phe204Cys	Mutated	1	4	MSM
d7	.....C.T..T......CAC.	Tyr147His	Mutated	2	8	MSM
MO051	........T.....T..T......CAC.	Ala95Val	Mutated	4	8	MSM
MO052	.....T..T....C.CAC.	Phe204Ser	Mutated	8	16	MSM
MO053	........T.....T..T......CAC.	Ala95Val	Mutated	4	8	MSM
MO054	........T.....T..T......CAC.	Ala95Val	Mutated	4	8	MSM
MO055	........T.....T..T......CAC.	Ala95Val	Mutated	4	8	MSM
MO056	........T.....T..T......CAC.	Ala95Val	Mutated	4	8	MSM
MO057	........T.....T..T......CAC.	Ala95Val	Mutated	4	8	MSM
RN4220	.....A.....		wt	1	2	Reference strain
MO034	.........T.....A.....	Asp101Tyr	Mutated	8	8	MSM
MO035	.....A.....		wt	8	8	MSM
MO036	.........T.....A.....	Asp101Tyr	Mutated	4	8	MSM
MO047	.........T.....A.....	Asp101Tyr	Mutated	4	8	MSM
MO048	.........T.....A.....	Asp101Tyr	Mutated	4	4	MSM
MO049	.........T.....A.....	Asp101Tyr	Mutated	4	8	MSM
MO050	.........T.....A.....	Asp101Tyr	Mutated	4	8	MSM

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SSM, single-step mutant; MSM, multistep mutant.
[a] Polymorphic sites are indicated with respect to the *fabI* sequence of *Staphylococcus aureus* COL.

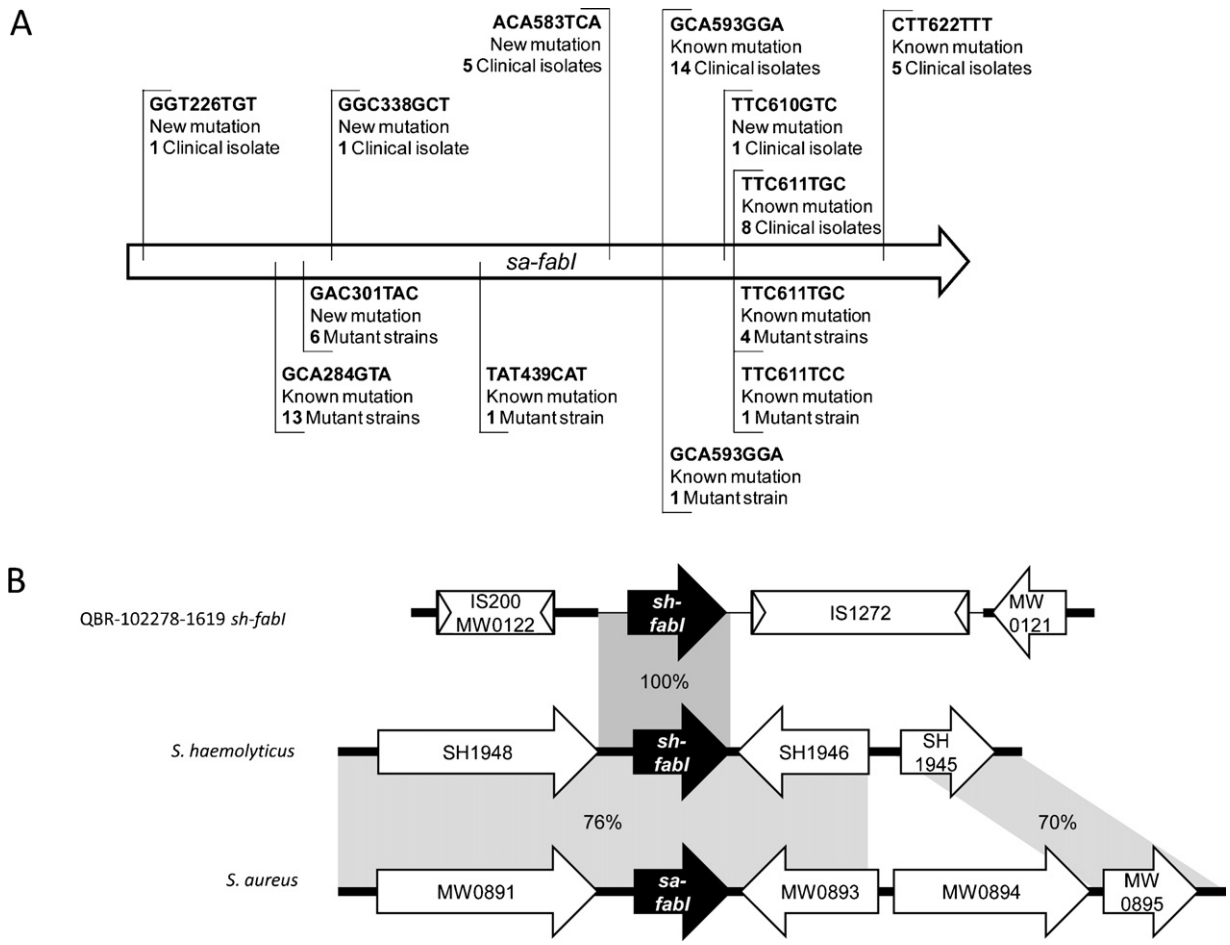

Fig. 3. Schematic map of mutations in the *Staphylococcus aureus fabI* (*sa-fabI*) and of *Staphylococcus haemolyticus fabI* (*sh-fabI*) genes. (A) Mutations in *sa-fabI* are reported on a schematic map. Mutations detected in clinical isolates are mapped above the sequence, whilst mutations selected in vitro are shown below the sequence. (B) Schematic alignment of the *sh-fabI* gene region of strain QBR-102278-1619 to *S. haemolyticus* (NC_007168) and *S. aureus* MW2 (NC_003923). Gene numbering of the QBR-102278-1619 open-reading frame (ORF) is as for MW2. The alignments have been reproduced from an alignment performed with the web version of the Artemis Comparison Tool (Sanger Centre). The thin line represents the 3016 bp fragment inserted in the *S. aureus* chromosome in strain QBR-102278-1619. Overall nucleotide identity in the shaded areas is given in percent.

coding region of *sa-fabI*. Whole-genome sequencing of one of these strains showed the presence a 3 kb genomic islet carrying an additional *fabI* gene identical to that belonging to the core genome of *S. haemolyticus sh-fabI*. By cloning *sa-fabI* onto a plasmid vector, it has been demonstrated that triclosan resistance can be achieved by increasing the amount of target [14]. In a similar way, the presence of *sh-fabI* together with *sa-fabI* constitutes a completely novel resistance mechanism, acting by increasing the target amount through heterologous target duplication. The only known mechanisms of triclosan resistance at the time of writing this article were due to chromosomal mutations. One of the most important observations in this work is the identification of likely horizontal transfer of this novel biocide resistance mechanism.

Detection of the inverted repeat sequences gained by insertion in the *S. aureus* genome indicates that the direction of transfer is from *S. haemolyticus* to *S. aureus* and from the *S. aureus* chromosome to plasmids [24,25]. Further identification of *sh-fabI* in numerous staphylococci in metagenome and microbiome databases indicates that the gene is actively spreading.

It is difficult to unequivocally establish the selective forces that cause selection of a specific mechanism of resistance, especially when determinants can confer simultaneous resistance to different drugs or when several different resistance elements are associated in the same gene transfer element [27]. For biocides that can produce cross-resistance to antibiotics, it is difficult to know whether the selective agent has been the biocide or the antibiotic itself. In the case of FabI, this enzyme is targeted only by triclosan in *S. aureus*. Identification of a resistance mechanism to triclosan acting by heterologous target duplication excludes other antimicrobials as being selective forces. This finding is a direct demonstration that the biocide triclosan produces a selective pressure on *S. aureus* and other staphylococci and is the first clear evidence that utilisation of biocides can drive development of biocide resistance in clinical isolates.

Agencies such as the FDA request a risk–benefit assessment for human antibiotics that includes evaluating the risks of resistance generation. For antibiotics used in animals, these resistance risks are an important safety issue that is addressed in all antibiotic submissions. Recently, the need for such requirements has been raised for biocides. For instance, a recent EU proposal for licensing of biocides asks that 'compounds should have no unacceptable effects on the target organisms, in particular unacceptable resistance or cross-resistance' [28]. In view of the requirements posed, the possibility of devising an in vitro assay for testing bacterial resistance to the biocide triclosan was evaluated. It is known that triclosan-resistant *fabI* mutants can be selected in vitro [9–11]. The aim was to assess whether such mutants have any predictive value for resistance observed in clinical isolates [29]. Mutants were selected by two distinct procedures in five different reference strains, but a mutation that was also detected in clinical

isolates was found in only 5 of 28 mutants, albeit the most prevalent one. A second very important aspect is that all in-vitro-generated mutant strains show similar MIC and MBC values, indicating that triclosan remained bactericidal for these strains. This is in contrast to clinical isolates where MICs were much lower than MBCs, indicating a more bacteriostatic action of triclosan in these resistant strains. This difference was also observed in the in vitro mutants and clinical isolates carrying the same mutation and suggests that clinical isolates might have accumulated compensating mutations that modify the phenotype and allow a reduction in the probable fitness cost given by the mutations generated in vitro [27]. Thus, both the phenotypic profile and the genotype of mutations differed in vitro from those detected in clinical isolates. With respect to the request by current legislation to run in vitro tests before placing an active compound on the market, we can conclude that such a test is feasible for triclosan, but that such a test does not yield results of clinical relevance if performed according to a standard experimental set-up. However, the data from this study suggest that an ECOFF MBC of >4 mg/L may be a good indicator of triclosan 'resistance'. We plan to undertake further studies to assess this.

Summarising, a novel resistance mechanism was identified in clinical isolates based on 'heterodiploidy' due to an additional copy of *sh-fabI* from *S. haemolyticus*. Detection of the same *sh-fabI* islet in staphylococcal plasmids indicates that this novel resistance element is being actively transferred, most likely due to positive selection by triclosan.

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