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Attenuated Virulence and Biofilm Formation in *Staphylococcus aureus* following Sublethal Exposure to Triclosan

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Subeffective exposure of *Staphylococcus aureus* to the biocide triclosan can reportedly induce a small-colony variant (SCV) phenotype. *S. aureus* SCVs are characterized by low growth rates, reduced pigmentation, and lowered antimicrobial susceptibility. While they may exhibit enhanced intracellular survival, there are conflicting reports regarding their pathogenicity. The current study reports the characteristics of an SCV-like strain of *S. aureus* created by repeated passage on sublethal triclosan concentrations. *S. aureus* ATCC 6538 (the passage 0 [P0] strain) was serially exposed 10 times to concentration gradients of triclosan to generate strain P10. This strain was then further passaged 10 times on triclosan-free medium (designated strain ×10). The MICs and minimum bactericidal concentrations of triclosan for P0, P10, and ×10 were determined, and growth rates in biofilm and planktonic cultures were measured. Hemolysin, DNase, and coagulase activities were measured, and virulence was determined using a *Galleria mellonella* pathogenicity model. Strain P10 exhibited decreased susceptibility to triclosan and characteristics of an SCV phenotype, including a considerably reduced growth rate and the formation of pinpoint colonies. However, this strain also had delayed coagulase production, had impaired hemolysis ($P < 0.01$), was defective in biofilm formation and DNase activity, and displayed significantly attenuated virulence. Colony size, hemolysis, coagulase activity, and virulence were only partially restored in strain ×10, whereas the planktonic growth rate was fully restored. However, ×10 was at least as defective in biofilm formation and DNase production as P10. These data suggest that although repeated exposure to triclosan may result in an SCV-like phenotype, this is not necessarily associated with increased virulence and adapted bacteria may exhibit other functional deficiencies.

Staphylococcus aureus is an important human pathogen that is responsible for a range of hospital and community-acquired infections (10, 15). Successful treatment of such infections is complicated not only by the emergence of methicillin-resistant *S. aureus* (MRSA) strains (10) but also, it has been claimed, by the spontaneous generation of small-colony variants (SCVs) (55).

S. aureus SCVs are slow growing subpopulations that may be recovered from patients with persisting or relapsing infections (44), particularly those undergoing treatment with aminoglycoside antibiotics (13), which exhibit decreased susceptibility to antimicrobials. SCVs display a distinct phenotype, characterized by the formation of pinpoint colonies on agar, low growth rate, and reduced pigmentation. Such characteristics may lead to their misidentification in the hospital laboratory (28), potentially complicating diagnosis (43).

The SCV phenotype has commonly been attributed to defects in the electron transport chain due to mutations in *hemB*, *ctaA*, or *menD*, which inhibit hemin, hemin A, and menadione biosynthesis, respectively (14, 54). Some SCVs result from thymidine auxotrophy, due to mutations in genes encoding thymidine synthesis or transport (9). Such SCVs can survive on exogenous thymidine, for instance, in the airway secretions of patients with cystic fibrosis, which are rich in necrotic cells (9, 24, 25). Thymidine is also required for menadione biosynthesis, suggesting that menadione auxotrophy may be a common factor in SCV formation in menadione and thymidine mutants. However, SCVs isolated from infections can result from mechanisms other than menadione and thymidine auxotrophy (47), suggesting a diversity in SCV mechanisms that has not been systematically investigated and that SCVs which emerge from various sources are not necessarily functionally equivalent.

Reduced susceptibility to antibiotics in SCVs is likely to be multifactorial, depending on whether the particular SCV is attributable to aminoglycoside-selected auxotrophy or was generated by other means, since electron transport chain defects result in reduced electrochemical gradients which may impair the uptake of aminoglycosides and other cationic molecules (4, 37). Additionally, the lowered growth rate in SCVs could reduce antimicrobial susceptibility by nonspecific mechanisms associated with reduced expression of pharmacological targets. Alternatively, enhanced survival within the host cells could shield the bacteria from inhibitory concentrations of antibiotics and biocides. The clinical significance of this, however, depends on whether SCVs can revert to full virulence following cessation of treatment (37) and on whether the expression of SCV phenotypes influences the ability to form recalcitrant biofilms on surfaces.

The generation of the SCV phenotype in *S. aureus* has reportedly been induced *in vitro* by exposure to triclosan (7, 49), a trichlorinated diphenyl ether biocide. Effective concentrations of triclosan are highly bactericidal toward susceptible species, where the molecule causes membrane damage through direct interaction and by perturbing lipid biosynthesis (36, 46, 53). Triclosan is used as an antibacterial in a variety of consumer and clinical ap-

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plications (5, 12, 16), including triclosan wash solutions, which have been used to reduce carriage of MRSA in hospitals (2, 26, 45, 56). The enoyl-acyl carrier protein reductase FabI has been identified to be a primary pharmacological target (31, 36) for triclosan. Inhibition renders the cell unable to synthesize fatty acids and growth is therefore inhibited, and depending on concentration and time, bacteria can be permanently inactivated (21). Concern has been expressed that the use of triclosan and other antiseptics should be limited to applications where a clear health benefit can be demonstrated, due to the possibility of reduced susceptibility to the molecule and potentially also to chemically unrelated compounds (18).

Reduced susceptibility to triclosan has been shown in a number of *in vitro* studies but not, to date, in the environment (32, 38). In one study, SCVs generated by the *in vitro* exposure of bacteria to sublethal concentrations of triclosan impregnated in silicone disks exhibited reduced susceptibility to the molecule and altered colony morphology (7). Since triclosan-exposed *S. aureus* has the potential to display an SCV-like phenotype and some clinical SCV isolates appear to be able to cause disease (49), it has been inferred that triclosan use might generate SCVs, which could potentially be of clinical significance (7). However, these *in vitro* studies do not necessarily reflect the clinical impact of triclosan-selected SCVs (48). For example, in a previously reported study, SCVs generated using triclosan-impregnated disks demonstrated a low growth rate, reduced coagulase and DNase activity, and decreased hemolysis (7), which are suggestive of decreased virulence.

There is a clear need to determine the ability of triclosan-generated SCVs to initiate infection (48). To our knowledge, there are currently no published reports regarding the impact of triclosan exposure on the pathogenic capability of resulting SCV-like strains. Additionally, while antibiotic-selected SCVs may revert to wild-type phenotype *in vivo* following cessation of treatment, this has not been reported for triclosan-selected SCVs. The present study therefore investigates the growth and virulence of an SCV-like *S. aureus* strain created by serial exposure to subinhibitory concentrations of triclosan. The potential reversion of this strain following subsequent repeated passage on triclosan-free medium was also studied.

MATERIALS AND METHODS

Bacterial strains and growth media. *Staphylococcus aureus* ATCC 6538 was acquired from the American Type Culture Collection and cultured on Mueller-Hinton agar (MHA), Colombia blood agar (CBA), or mannitol salt agar (MSA) (Oxoid, United Kingdom) aerobically at 37°C. For supplementation experiments, hemin (final concentration, 1, 10, or 100 μM), menadione (final concentration, 0.4, 4, or 40 μM), or thymidine (final concentration, 0.62, 6.2, or 62 μM) was added to MHA. Cryogenic stocks of the bacteria were archived at −80°C.

Selection of isolates with reduced susceptibility to triclosan. Reproducible concentration gradients of triclosan were created on Mueller-Hinton agar by depositing stock solutions of triclosan (100 μg/ml or 1 mg/ml) with a Wasp II spiral plater (Don Whitley, Shipley, United Kingdom) (30, 35). Plates were dried for 1 h at room temperature prior to radial deposition of an overnight suspension of *S. aureus* and incubated for 4 days aerobically at 37°C. Growth observed at the highest triclosan concentration was aseptically removed and used to inoculate further gradient plates. This process was repeated for 10 passages. A further 10 passages were performed on triclosan-free MHA. Wild-type bacteria (the passage 0 [P0] strain), those passaged 10 times on triclosan (strain P10), and those passaged a further 10 times on triclosan-free MHA (strain ×10) were archived at −80°C for subsequent MIC and MBC determination.

Determination of bacterial MICs. Overnight cultures of *S. aureus* were diluted 1:100 in sterile Mueller-Hinton broth (MHB), and aliquots (120 μl) were delivered to wells of polystyrene 96-well plates (Corning Ltd., Corning, NY). Stock solutions (1.16 mg/ml) of triclosan were prepared in 25% ethanol, filter sterilized, and stored at −80°C. Doubling dilutions of triclosan were added to the diluted overnight cultures (final concentrations, 232 μg/ml to 0.23 μg/ml). Sterile and triclosan-free controls were also included. Plates were incubated for 24 h aerobically at 37°C with shaking at 100 rpm. MICs were determined as the lowest concentration of triclosan showing no turbidity in comparison to a sterile negative control. Three technical and two biological replicates were conducted. Previous validation studies with staphylococci and other genera showed that the triclosan solvent has no effect on the outcome of MIC or minimum bactericidal concentration (MBC) determinations (30).

Determination of bacterial MBCs. Aliquots (10 μl) taken from MIC plates (see above) were spot plated onto MHA in triplicate and incubated aerobically at 37°C. MBCs were determined as the lowest concentration of triclosan at which no growth was observed after 4 days of incubation.

Planktonic growth rate measurement. Overnight suspensions of *S. aureus* P0, P10, and ×10 were diluted 1:100 in MHB (30 ml) and incubated at 37°C with shaking at 150 rpm. Samples were regularly removed and diluted as appropriate. Optical density was measured at 630 nm using a Helios spectrophotometer (Pye Unicam Ltd., Cambridge, United Kingdom).

Determination of biofilm growth rates. Overnight suspensions of *S. aureus* P0, P10, and ×10 were diluted 1 in 40 in MHB, and aliquots (200 μl) were delivered to wells of polystyrene 96-well plates. Sterile controls were also included. Plates were incubated statically aerobically or in an anaerobic workstation (Don Whitley, Shipley, United Kingdom) at 37°C in humid containers to minimize evaporation. At 6 time points up to 50 h, planktonic and biofilm growth was measured. Planktonic suspensions were transferred into semi-micro cuvettes, and cell density was measured spectrophotometrically at 630 nm. The remaining biofilm was stained for 60 s with 1% (wt/vol) crystal violet (200 μl), which was then removed, and wells were washed three times with distilled water before allowing plates to air dry. Biofilm-bound crystal violet was eluted in 100% ethanol (200 μl), and absorbance was measured spectrophotometrically at 600 nm. Biofilm units (arbitrary units) were calculated by dividing biofilm absorbances by their corresponding planktonic OD.

Determination of biofilm architecture and viability. Overnight suspensions of *S. aureus* P0, P10, and x10 were diluted 1 in 40 in MHB (40 ml) in a Duran bottle containing a partially submerged sterile glass microscope slide. Bottles were incubated at 37°C statically for 24, 48, or 72 h before gentle rinsing in phosphate-buffered saline (PBS; 50 ml). Biofilm viability and structure were visualized by fluorescence microscopy. Briefly, a working solution of BacLight LIVE/DEAD stain (Invitrogen Ltd., Paisley, United Kingdom) was prepared by adding 1 μl each of SYTO 9 (component A) and propidium iodide (component B) to 98 μl distilled water. This solution (20 μl) was applied directly to the biofilm and the slide was covered with a glass coverslip. Slides were incubated at room temperature in the dark for 15 min, according to the standard BacLight staining protocol. Biofilms were visualized with an Axioskop 2 fluorescence microscope with a ×10 objective lens (Carl Zeiss Ltd., Rugby, United Kingdom), and images were captured using a digital microscope eyepiece (Cosmos Biomedical, Derbyshire, United Kingdom) and exported as JPEG files. Bacterial cells incubated in the presence of both stains fluoresce either green (viable) or red (dead). The excitation and emission maxima for these dyes are 480 and 500 nm for SYTO 9 and 490 and 635 nm for propidium iodide. The percent viable biomass was determined by calculating the proportion of red fluorescence as a percentage of total fluorescence. Biofilm three-dimensional structure was visualized with an LSM Confocor 2 confocal microscope with a ×10 objective lens (Carl Zeiss Ltd., Rugby, United Kingdom). Images were processed, surface rendered, and quantified using Imaris software (Bitplane AG, Zurich, Switzerland).

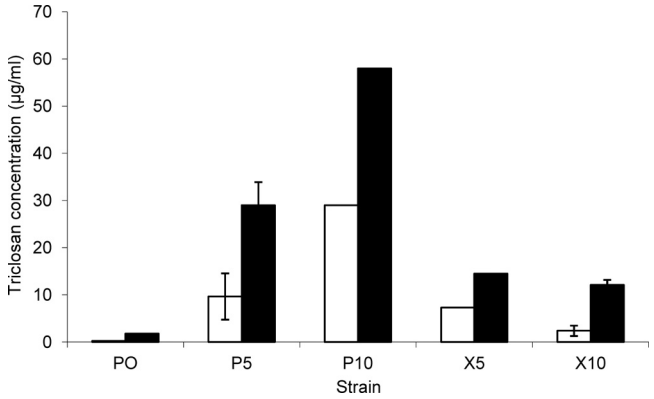

FIG 1 MICs (white bars) and MBCs (black bars) of triclosan to *S. aureus* ATCC 6538 before exposure (P0), after 5 or 10 passages in the presence of triclosan (P5 and P10, respectively), and after 5 or 10 subsequent passages on triclosan-free medium (×5 and ×10, respectively). Error bars show standard deviation ($n = 6$).

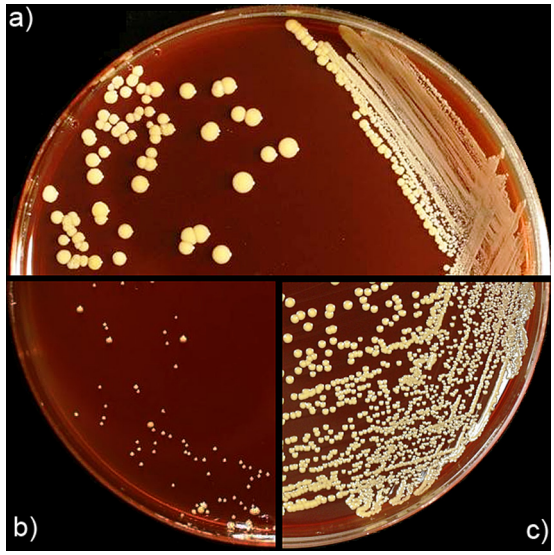

FIG 2 Colonies formed on Colombia blood agar by *S. aureus* parent strain (P0; a), triclosan-adapted strain (P10; b), and one passaged a further 10 times on triclosan-free medium (×10; c) after aerobic incubation at 37°C for 48 h. Colonies formed by the triclosan-adapted strain were markedly smaller and more heterogeneous than those formed by the parent strain.

Determination of hemolysin activity. The method used for determination of hemolysin activity was adapted from that of Hathaway and Marshall (19). Overnight suspensions of *S. aureus* P0, P10, and ×10 were diluted 1:50 in MHB (50 ml) in a conical flask and incubated with shaking at 37°C to an optical density at 600 nm of 0.3. Whole defibrinated horse blood (Oxoid, United Kingdom) was added to a final concentration of 5% (vol/vol), before incubating for a further 3 h. Negative and positive controls (sterile MHB and distilled water, respectively) were included. Aliquots (1 ml) were centrifuged at 16,000 × *g* (1-14 Microfuge; Sigma, United Kingdom) for 4 min at room temperature, and the absorbance of the supernatant was measured spectrophotometrically at 540 nm. Colony counts were performed by plating appropriate dilutions on MSA. To avoid variation caused by differences in cell number, specific hemolysis was calculated as the change in the A_{540} (ΔA_{540})/CFU.

Determination of DNase activity. The method used for determination of DNase activity was adapted from the Health Protection Agency National Standard thermonuclease test method (20). Overnight suspensions of *S. aureus* P0, P10, and ×10 were diluted 1 in 50 in MHB (50 ml) in a conical flask and incubated with shaking at 37°C to an optical density at 600 nm of 0.3. Aliquots (1 ml) were incubated at 100°C for 15 min and centrifuged at 13,000 rpm for 5 min, and supernatants were retained. Wells were cut into plates containing DNase test agar (Oxoid, United Kingdom) and filled with supernatant (40 μl). Plates were incubated overnight at 37°C and then overlaid with 1 M HCl. Polymerized DNA makes the agar opaque, and zones of clearing surrounding the wells indicate levels of DNase activity. Zone diameters were recorded, and colony counts were performed by plating appropriate dilutions of unheated cultures on MSA to account for variations caused by differences in cell number.

Coagulase assay. Suspensions of *S. aureus* P0, P10, and ×10 in Mueller-Hinton broth (1 ml, $OD_{600} = 0.4$) were added to rabbit plasma with EDTA (3 ml; Bactident coagulase; Merck, Darmstadt, Germany), in triplicate, and incubated at 37°C in a water bath. Tubes were monitored for signs of coagulation over 3 h and scored on a five-point scale according to the manufacturer's instructions.

***Galleria mellonella* pathogenesis assay.** The pathogenesis model was adapted from that of Peleg et al. (42). Final larval-stage *G. mellonella* (Live Foods Direct, Sheffield, United Kingdom) was stored in the dark at 4°C for less than 7 days, before randomly assigning 16 to each treatment group and incubating at 37°C for 30 min. Overnight suspensions of *S. aureus* strains P0, P10, and ×10 were washed twice in PBS and diluted appropriately in PBS to achieve an optical density at 600 nm of 0.1 (5×10^5 to 8×10^5 CFU/ml, as confirmed by colony counts on MSA). Aliquots of each suspension (5 μl) were injected into the hemocele of each larva via the last left proleg using a Hamilton syringe (2.5×10^3 to 4×10^3 CFU per individual). Larvae were incubated in plastic petri dishes at 37°C, and the number of surviving individuals was recorded daily (dead larvae were unresponsive to touch and appeared black). An untreated group and a group injected with sterile PBS were used as negative controls. The experiments were terminated when at least 2 individuals in a control group had died. Three independent replicates were performed, and significance was calculated using the log-rank test. Data were plotted as survival curves, and representative data are presented. Dead individuals were homogenized in sterile PBS and diluted appropriately, and aliquots were spread on MSA to calculate bacterial load per individual at death.

RESULTS

Triclosan exposure selects for isolates with reduced triclosan susceptibility. The MIC of triclosan to *Staphylococcus aureus* ATCC 6538 (P0) was 0.23 μg/ml. After 5 passages (P5), this increased to 9.67 μg/ml, and after 10 exposures (P10) it increased to 29 μg/ml (a 126-fold increase compared to that for the parent strain; Fig. 1). Colonies of P10 were markedly smaller than those of its parent strain (Fig. 2). Further passaging on triclosan-free medium resulted in a partial reversion of susceptibility and colony size; following 5 passages on MHA, the MIC was 7.3 μg/ml, and after 10 passages it was 1.1 μg/ml. MBC data showed a similar trend (Fig. 1).

Triclosan-adapted *S. aureus* grows more slowly than its parent strain in planktonic and biofilm modes. The growth kinetics of triclosan-adapted *S. aureus* (P10), its parent strain (P0), and the strain further passaged on triclosan-free medium (×10) were compared in shaken broth cultures (planktonic growth). As shown in Fig. 3, P10 underwent an extended lag phase and entered stationary phase later than P0 and ×10. The planktonic growth kinetics of P0 and ×10 were not significantly different. The exponential-phase growth rate of P10 (0.19/h) was less than half that of P0 (0.43/h) and ×10 (0.42/h). Biofilm growth was measured over 50 h using the well-characterized microplate assay (41). Formation of biofilm by P10 and ×10, relative to planktonic growth, was significantly lower than that by P0 (Fig. 4). Strain ×10 formed less

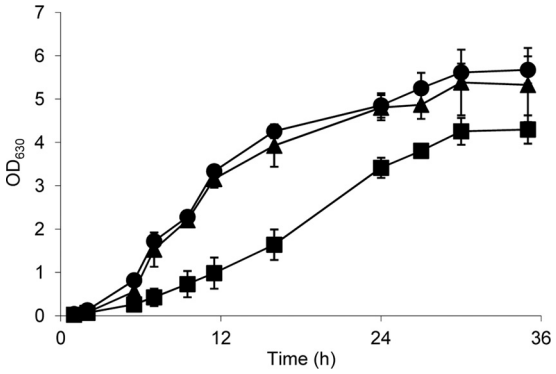

FIG 3 Planktonic growth of *S. aureus* parent strain (P0; triangles), triclosan-exposed strain (P10; squares), and one passaged a further 10 times on triclosan-free medium (×10; circles). P10 exhibited an extended lag phase, slower growth, and a delayed stationary phase compared to P0. Error bars show standard deviation ($n = 3$).

biofilm mass than P10 for the first 15 h of growth, after which levels were not significantly different. Strains P10 and ×10 were also significantly biofilm defective when grown anaerobically (data not shown). Biofilms formed on glass slides by P0, P10, and ×10 differed markedly with respect to aerial coverage (Fig. 5). Additionally, epifluorescence and confocal microscopy showed that P0 formed relatively thick three-dimensional microcolony structures, whereas strains P10 and ×10 formed thinner, less structurally complex biofilms (Fig. 6a). Analysis of representative images indicated that the density of biomass was markedly higher in P0 biofilms than in those of P10 or ×10 over 72 h (Fig. 6b).

The *in vitro* hemolytic activity of triclosan-adapted *S. aureus* is lower than that of the parent strain. The ability of planktonic P0, P10, and ×10 populations to lyse erythrocytes was investigated. Higher absorbance readings are indicative of lysis and release of hemoglobin into the supernatant. Hemolysis by P10 was, on average, 2% of the P0 value, a statistically significant difference ($P < 0.01$; Fig. 7). Although ×10 showed a partial reversion of hemolytic activity, it was still significantly lower than that of the parent strain (5% of the P0 value).

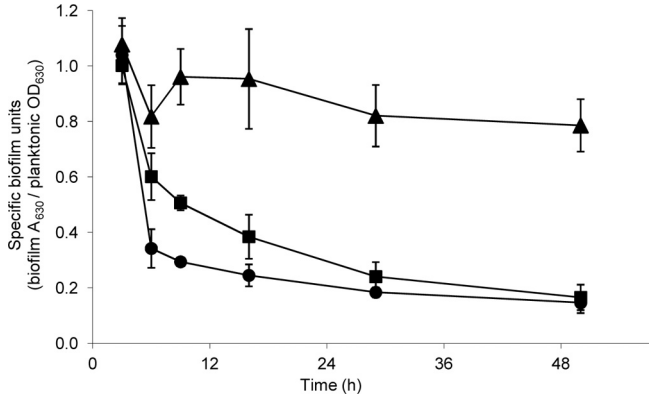

FIG 4 Biofilm growth of *S. aureus* parent strain (P0; triangles), triclosan-exposed strain (P10; squares), and one passaged a further 10 times on triclosan-free medium (×10; circles) on polystyrene. Data are shown as biofilm units. A biofilm unit is defined as the absorbance of the biofilm-bound crystal violet divided by the corresponding planktonic OD and corrects the data to give a representation of biofilm formation irrespective of planktonic mass. Biofilm formation by P10 was markedly lower than that by P0. This difference was significant at all time points from 6 h ($P < 0.001$; $n = 12$).

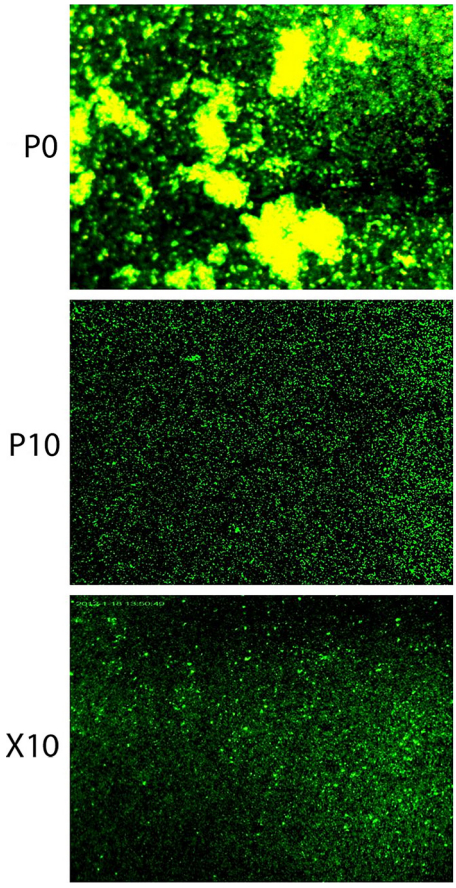

FIG 5 Biofilm growth on glass of *S. aureus* parent strain (P0), triclosan-exposed strain (P10), and one passaged a further 10 times on triclosan-free medium (×10) as viewed by epifluorescence microscopy. Green fluorescence represents viable cells, and red fluorescence represents nonviable cells. Representative fields of view at 24 h are shown.

The *in vitro* DNase activity of triclosan-adapted *S. aureus* is lower than that of the parent strain. The levels of thermostable DNase produced by planktonic P0, P10, and ×10 populations were investigated with an agar diffusion test. Zones of clearing after addition of heated culture supernatant indicate DNase activity. Activity (mean zone diameter, 9.47 ± 0.53 cm) was observed only surrounding wells filled with culture supernatant from the P0 strain. No DNase activity was observed surrounding wells filled with culture supernatants from the P10 or ×10 strain (see Table 2). Cell density in the P0 cultures (4.6×10^8 CFU/ml) was not significantly different from that in the P10 or ×10 populations ($P = 0.2$).

The *in vitro* coagulase activity of triclosan-adapted *S. aureus* is lower than that of the parent strain. The levels of coagulase produced by planktonic P0, P10, and ×10 populations were investigated using a standard tube coagulase test. Coagulase activity was observed in all strains. Although strain P0 displayed a positive result after 30 min, significant coagulation was delayed in strains P10 and ×10, with both showing a positive result after 2 h (Table 1).

Supplementation with hemin, menadione, or thymidine does not restore a non-SCV phenotype. To ascertain whether the

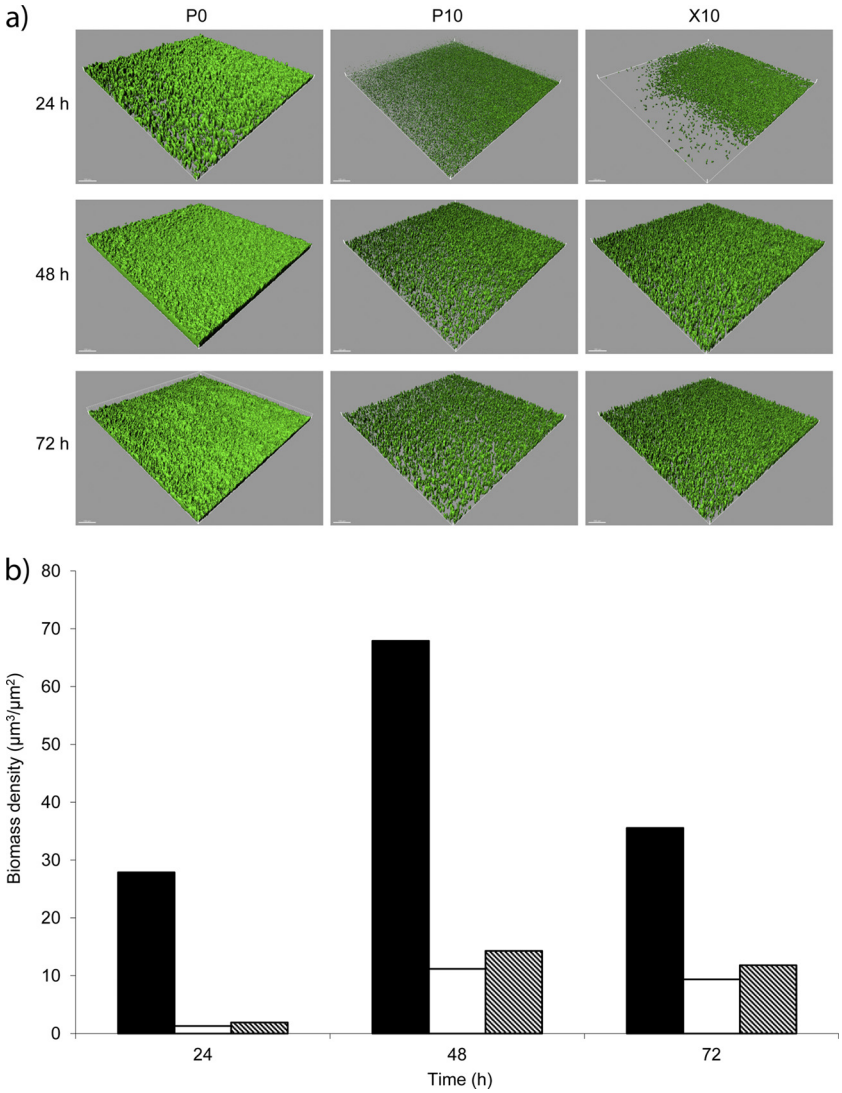

FIG 6 Biofilm growth on glass of *S. aureus* parent strain (P0), triclosan-exposed strain (P10), and one passaged a further 10 times on triclosan-free medium (×10) as viewed by confocal microscopy. Specialist software was used to surface render the images, allowing comparison of relative density and structure (a) and measurement of biomass density (b). Representative images and data are shown (P0, black bars; P10, white bars; ×10, striped bars).

SCV phenotype was caused by mutations affecting biosynthesis of hemin, menadione, or thymidine, MHA containing these molecules was inoculated with P10. The concentrations used were based on the previous reports that maximal restoration of normal phenotypes occurred with 1.0 μM hemin, 0.375 μM menadione, or 6.2 μM thymidine (9, 56). Further plates were inoculated with P0 and ×10 to control any growth-inhibitory effects of medium supplementation. No supplementation affected the colony morphologies of any strain (Table 2), suggesting that the phenotype of P10 is not attributable to defects in hemin, menadione, or thymidine biosynthesis.

Triclosan-adapted *S. aureus* is less virulent in an invertebrate model. The *Galleria mellonella* pathogenesis assay was used to assess the relative virulence of the test strains. Groups of larvae were injected with P0, P10, or ×10 and incubated at 37°C. Dead individuals were recorded daily, and data were plotted as survival curves (Fig. 8a). Over 6 days of incubation, the P10 treatment group suffered the fewest deaths, whereas survival in the group inoculated with the parent strain P0 fell rapidly, leveling out at 5 individuals in the example shown. In comparison, ×10 displayed moderate virulence, suggesting a partial reversion in phenotype. A log-rank test including all replicates showed that all three treatment groups were significantly different ($n = 48$). Within 24 h of death, the bacterial load of each individual was measured. Although numbers varied markedly between individuals (10^7 to 10^{11}), there was no significant difference between treatment groups (Fig. 8b). Colony morphologies of bacteria recovered from dead larvae were similar to the morphology of the inoculum in all groups, suggesting that the SCV-like strain did not revert to wild type, or vice versa, during infection. Results have been summarized in Table 2.

DISCUSSION

S. aureus is known to generate phenotypically distinct subpopulations termed small-colony variants (SCVs), which have occasionally been isolated from patients with chronic and relapsing infec-

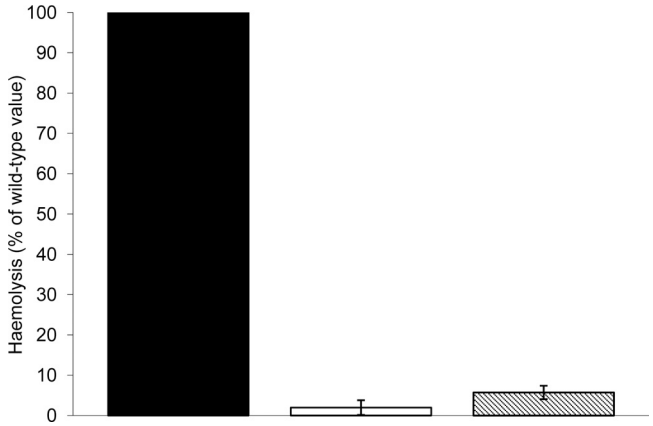

FIG 7 Hemolytic activity of *S. aureus* parent strain (P0; black bar), triclosan-exposed strain (P10; white bar), and one passaged a further 10 times on triclosan-free medium (×10; striped bar). Whole blood (final concentration, 5%, vol/vol) was added to pre-stationary-phase cultures, and free hemoglobin from lysed erythrocytes was measured spectrophotometrically at 450 nm. Data are expressed as mean percentages of the mean P0 value. Error bars show standard deviation ($n = 3$).

tions. Clinically occurring SCVs commonly result from hemin, menadione, or thymidine auxotrophy but may also result from mutations in other genes (47). SCV-like variants can arise in response to sublethal exposure to aminoglycoside antibiotics (13) and the biocide triclosan. Thus, concerns have been expressed that exposure of *S. aureus* to triclosan might lead to the emergence of *S. aureus* SCVs with reduced susceptibility to antimicrobials (7). However, care must be taken when extrapolating clinical relevance from *in vitro* data. The fact that triclosan is normally applied topically at concentrations considerably higher than those used to generate SCVs should be taken into account (48), as should the fact that although elevated (0.0029%, wt/vol), the MICs for triclosan in the adapted strains described in the current study remained orders of magnitude lower than the triclosan concentrations in washes used to control MRSA in hospitals (0.3 to 1.0%, wt/vol) (11, 57). Little is known about the physiology and pathogenicity of triclosan-induced SCVs, and doubts concerning their ability to initiate infection in healthy individuals have been raised (48).

There have been conflicting reports concerning the virulence of SCVs, whether of clinical origin or created in the laboratory. For example, a *hemB* mutant was more virulent than its parent strain in a murine septic arthritis model (23) but demonstrated no significant difference in virulence in a rabbit endocarditis model (6). Another report suggested that *hemB* and *menD* mutants and SCVs of clinical origin were reduced in virulence (50). Clinically isolated SCVs may, however, be physiologically distinct from *hem* and *men* mutants generated in the laboratory, and in this respect, recent proteomic analyses revealed significant differences in protein expression between a *hemB* mutant, a gentamicin-induced SCV, and a clinical SCV isolate (29). Markedly increased expression of glycolytic and pyruvate dehydrogenase enzymes was detected in the laboratory mutant and gentamicin-induced strains compared to the clinical isolate. However, there has previously been little information available to indicate whether strains selected by triclosan exposure vary in virulence compared to the progenitor strains.

TABLE 1 Coagulase activity of *S. aureus* parent strain (P0), a triclosan-adapted strain (P10), and one passaged a further 10 times on triclosan-free medium (×10)

Time (h)	Activity for strain[a]:		
	P0	P10	×10
0.5	++++	−	−
1	++++	−	+
2	++++	+++	+++
3	++++	++++	++++

[a] Tubes were monitored for signs of coagulation over 3 h and scored on a five-point scale according to the manufacturer's instructions. −, no coagulase detected; +, small separate clots (negative); ++, small joined clots (negative); +++, extensively coagulated clots (positive); ++++, complete coagulation (positive) (Bactident coagulase; Merck, Darmstadt, Germany).

TABLE 2 Summary indicating presence or absence of tested characteristics in strains P0, P10, and ×10

Characteristic	Result for strain:		
	P0	P10	×10
MIC (μg/ml) to triclosan	0.23	29	1.1
Slow planktonic growth	−	+	−
Impaired biofilm growth	−	+	+
Impaired DNase activity	−	+	+
Impaired hemolytic activity	−	+	+
Delayed coagulase activity	−	+	+
Impaired virulence	−	+	+
Reversal by hemin supplementation	−	−	−
Reversal by menadione supplementation	−	−	−
Reversal by thymidine supplementation	−	−	−

The current investigation has demonstrated that repeated exposure of *S. aureus* to triclosan can result in a phenotype with marked similarities to SCVs but that is attenuated in biofilm formation capacity, hemolysis, DNase, coagulase activities, and pathogenesis in an animal model (Table 2). This suggests that while repeated exposure to triclosan may result in an SCV-like phenotype, this is not necessarily associated with increases in variables associated with pathogenicity.

Serial exposure of bacteria to concentration gradients of antimicrobials in agar is a highly selective procedure that has been validated as an effective method for selecting isolates with decreased susceptibility (35). The *G. mellonella* infection model has previously been used to study bacterial pathogenesis (5, 22, 39), including antimicrobial insusceptibility in *S. aureus* (42). Data generated with this model in the current study showed a clear distinction between the virulence of strains P0, P10, and ×10. Benefits of *G. mellonella* over other nonmammalian systems such as *Caenorhabditis elegans* include the fact that individual animals may be injected with a defined inoculum, individual animals can be maintained at human body temperature, and the system has been shown to broadly reflect virulence patterns seen in mammalian systems (22).

With respect to specific virulence factors, DNase activity was markedly reduced following triclosan exposure. It has been suggested that staphylococcal DNase interferes with the antimicrobial activity of neutrophil-produced extracellular traps (NETs) through the breakdown of the chromatin backbone. DNase-mediated NET degradation has been demonstrated for *S. aureus* in a murine model, in which mice infected with wild-type *S. aureus*

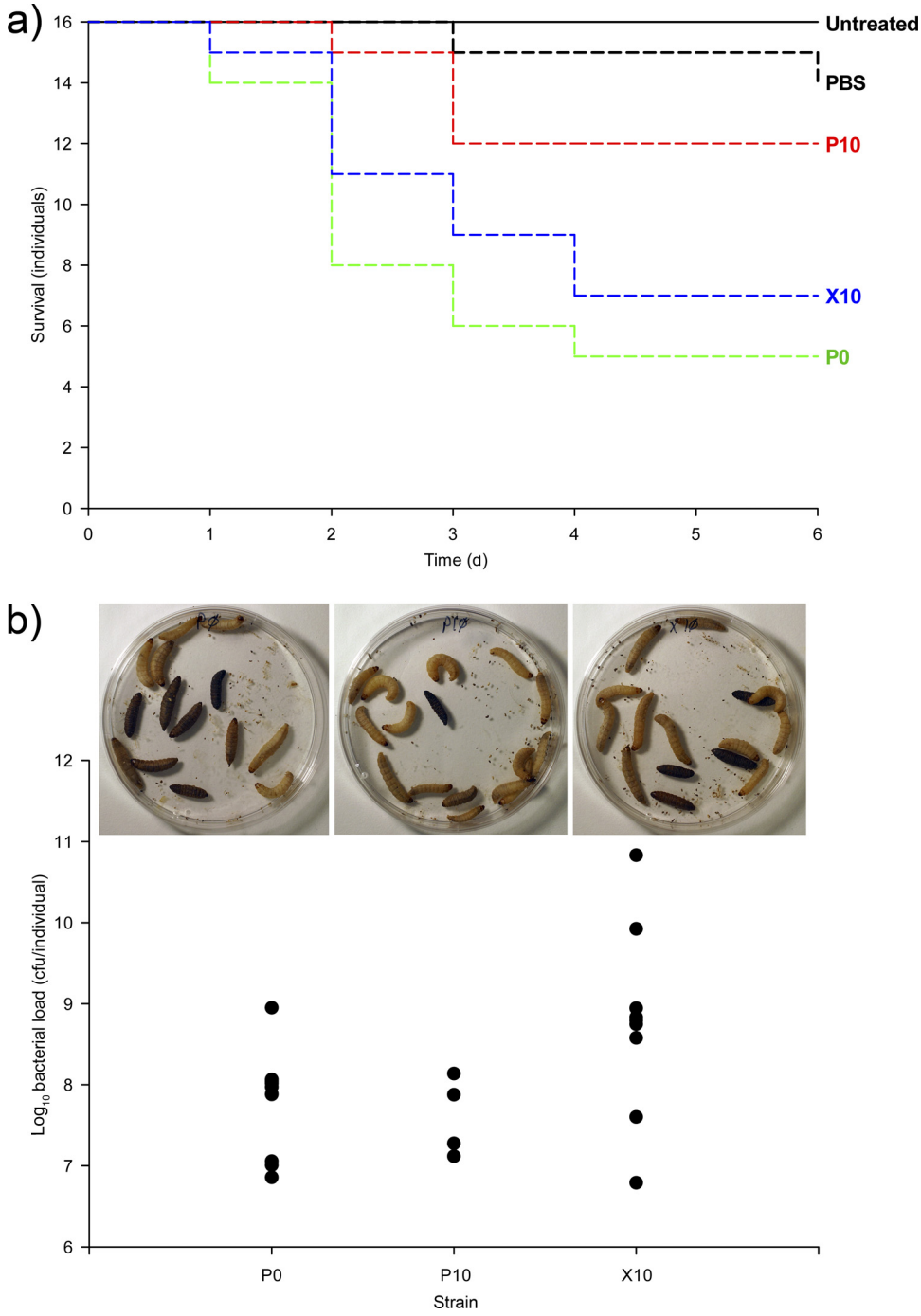

FIG 8 Example survival curve showing virulence of *S. aureus* parent strain (P0), triclosan-exposed strain (P10), and one passaged a further 10 times on triclosan-free medium (×10) in a *G. mellonella* survival assay (a; $n = 16$) and bacterial load at death (b; each point corresponds to the bacterial load in an individual animal). P0 caused significantly higher death rates than both other strains, whereas the virulence of ×10 was not fully restored. Untreated and PBS-treated control data are also shown. Although the bacterial load recovered from dead larvae varied, there was no significant difference between treatment groups. Example images of treatment groups are also shown.

exhibited a significantly higher mortality rate than those infected with the nuclease-deficient mutant (8). Host extracellular DNA (eDNA) in *G. mellonella* reportedly plays a similar role in the immune response as it does in humans (3), suggesting that a reduction in DNase activity might partially account for the reduced virulence of the *S. aureus* SCV observed in our *G. mellonella* assay.

Importantly, *S. aureus* became markedly defective in its ability to form biofilms following triclosan adaptation. Strains P10 and ×10 formed biofilm less readily than the parent strain on polystyrene over 50-h incubations, and biofilms grown on glass were less dense, exhibiting simpler architecture than the parent strain. To our knowledge, there are currently no data in the literature con-

cerning the ability of triclosan-selected SCVs to form biofilms, and data on other SCVs otherwise generated have not been conclusive; for example, *S. aureus* SCVs created by exposure to amikacin formed greater biofilm mass on polystyrene (52) but not on glass (51), and a *Staphylococcus epidermidis* SCV formed by a mutation in *hemB* was almost entirely biofilm defective at 24 h but, contrary to findings presented in the current study, reverted to levels approximating those of the wild type at 48 h (1). However, since the authors did not measure biofilm relative to planktonic growth, this may be a reflection of the inherently slow growth of the SCV phenotype, rather than a specific measure of biofilm-forming capability. In the current study, biofilm formation on polystyrene was measured quantitatively over 50 h, and these data were then converted to relative biofilm-forming units (absorbance of the biofilm-bound crystal violet divided by the corresponding planktonic OD) and therefore reflect biofilm formation, irrespective of planktonic bacterial productivity. Since initial attachment does not appear to be impaired and biofilm deficiency is not due solely to reduced growth rate, strains P10 and ×10 appear to be specifically deficient in biofilm maturation. This is potentially a consequence of repression of the intercellular polysaccharides or protein PIA or Aap, respectively. The staphylococcal accessory regulator gene *sarA*, which controls several virulence determinants, including biofilm formation, hemolysins, and DNase, may also be implicated. Furthermore, recent analysis has shown that an increase in DNase production resulting from a mutation in the stress response factor *sigB* inhibited biofilm growth by breaking down eDNA (27). Although this appears to be in contrast to data presented in the current investigation, there is much to learn about the role of eDNA in such a multifactorial process as biofilm formation. Importantly, since *S. aureus* infections related to medical devices are strongly associated with biofilm formation (17), the selection for biofilm-deficient strains may be clinically advantageous since biofilms are markedly less susceptible to antimicrobial therapy than their planktonic counterparts (34, 40), a phenomenon that is due to biofilm-specific physiology as well as diffusion resistance (33). Therefore, the inability to form biofilm could potentially outweigh changes in susceptibility related to the SCV phenotype. Further, despite a raised MIC to a single biocide, an inability to form biofilm may leave such SCVs vulnerable to a wide range of antimicrobial and immunological challenges.

Although the phenotype of the triclosan-adapted strain (P10) was practically identical to that of the previously described SCVs in terms of colony size and planktonic growth, initial supplementation data indicated that P10 was not an electron transport chain-defective SCV. It is, however, possible that mechanisms other than auxotrophy to hemin, menadione, or thymidine were responsible for the induction of an SCV-like response in this strain.

S. aureus generated from 10 further passages on triclosan-free medium (×10) showed reversion in planktonic growth rate and, to a lesser extent, in colony diameter. Only minor reversions in hemolytic activity and biofilm formation were observed, and like P10, no DNase activity was detected. Strain ×10 was significantly less pathogenic than the parent strain in the *G. mellonella* model and exhibited a marked increase in triclosan susceptibility. Furthermore, P10 did not revert to normal colony size *in vivo* during the course of the infection model (data not shown).

In conclusion, we have shown that while repeated exposure to triclosan might select for *S. aureus* strains with decreased susceptibility to triclosan, this may be associated with deficiencies in growth and virulence.

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