INTRODUCTION

Metallic copper is a self-sanitizing material that has recently received increasing research attention worldwide, as it has the potential to reduce the rate of healthcare-associated infections (HAIs) (Grass et al., 2011; O’Gorman & Humphreys, 2012; Alvarez et al., 2016; Muller et al., 2016). When applied to commonly touched places within hospital facilities, copper surfaces can provide extra protection by effectively killing pathogens during intervals between routine cleaning and human contacts. The efficiency of copper in reducing hospital environmental contamination has been demonstrated by a number of recent trials (Marais et al., 2010; Mikolay et al., 2010; Karpanen et al., 2012; Schmidt et al., 2016). For example, introduction of copper surfaces to objects previously covered with plastic, wood or stainless steel in intensive care units caused an 83 % reduction of average microbial burden (Schmidt et al., 2012). Patients staying in intensive care units equipped with copper surfaces had significantly lower rate of HAIs, including infections caused by the methicillin-resistant Staphylococcus aureus (Salgado et al., 2013).

Copper is a common metal with a long history of domestic use, but its broad spectrum of antimicrobial properties has largely been ignored until recently: new research shows that a wide range of microorganisms can be killed rapidly on copper surfaces within a few hours or even minutes of contact (Grass et al., 2011). However, the chemical and physical interactions between bacteria and copper are very complex, and the mechanisms of contact killing are not fully understood (Hans et al., 2016). More importantly, the potential of bacterial pathogens to develop resistance to metallic copper and possible impacts on the medical use of antibiotics have rarely been explored (Santo et al., 2010). Copper compounds have been used in agriculture for centuries as bactericides and fungicides. It has been shown that copper amendment in soil selects for antibiotic resistance in the field (Berg et al., 2005; Ji et al., 2012). In terms of molecular mechanisms, a recent study by Hao et al. (2014) revealed...
that antibiotic resistance and the cellular copper metabolism are metabolically linked in *Escherichia coli* via the global regulator MarR. Therefore, with the increased use of copper surfaces in the hospital environment, it is highly possible that copper-resistant strains will evolve and display reduced susceptibility to common antiseptics and antibiotics (Baker-Austin et al., 2006).

Copper in its ionic form is known to be an essential nutrient but, in excess, it is highly toxic (Rensing & Grass, 2003; Zhang & Rainey, 2008). Mounting evidence suggests that oxidative stress induced by ionic copper is the major factor causing cell death (Molteni et al., 2010). Copper ions damage the cell membrane (which could result in leakage of cellular contents) and further degrade genomic DNA in the cytoplasm (Warnes et al., 2012). All bacteria possess efflux systems that act to pump out any excess copper ions inside the cell. However, such systems can only help delay cell death, but cannot prevent it (Santo et al., 2008; Elguindi et al., 2009). This strongly indicates that bacterial cells continuously absorb and accumulate copper ions until the export systems are outpaced, resulting in cell death. However, the current paradigm shedd little light on the critical cellular components that trigger the release of copper ions from the metal surface.

Here, we hypothesize that bacterial cells are predominantly killed by a burst release of toxic copper ions, resulting from chemical reactions between surface components of a bacterium and the surface of metallic copper (Fig. S1, available in the online Supplementary Material). Cell surface substrates such as extracellular polymeric substances (EPS), LPS, pili and flagella contain nitrogen, sulphate or phosphate in their structures, which are potentially reactive with metallic copper, producing more and more toxic copper ions (Vu et al., 2009; Chen et al., 2011). This copper ion burst releasing (CIBR) model well explains a previous finding that contact killing is suppressed by a thin layer (~1.7 µm) of synthetic polymer with grid holes, which can prevent physical contact between bacterial cells and metallic copper without affecting the movement of copper ions (Mathews et al., 2013).

To test the above-mentioned CIBR model of contact killing, we phenotypically characterized 13 small colony variants (SCVs) isolated in vitro from the two most common nosocomial pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. SCVs are known to overproduce various types of EPS with enhanced attachment to biotic and abiotic surfaces. The CIBR model predicts that SCVs will be more susceptible to contact killing on copper than wild-types. Having confirmed this prediction, we compared the rates of contact killing between *Pseudomonas fluorescens* SBW25 and its derived isogenic mutants with altered levels of expression of the major cell surface components, including EPS, LPS and colonic acid (CA) capsules. *P. fluorescens* SBW25 is a non-pathogenic model organism for studies of molecular genetics and experimental evolution (Zhang & Rainey, 2007b, 2013; Silby et al., 2009). Finally, we subjected *P. fluorescens* SBW25 to a daily transfer with and without copper contact killing, and monitored the dynamic changes of bacterial resistance to metallic copper over a period of 100 transfers. Our data highlight the importance of cell surface components for bacterial contact killing on copper, and implicate low bacterial evolvability for resistance to metallic copper.

**METHODS**

**Isolation of SCVs.** A summary of the bacterial strains used in this study is provided in Table 1. All bacteria were cultured in Luria–Bertani (LB) medium but at 28°C for *P. fluorescens*, and 37°C for *P. aeruginosa* and *S. aureus*. To isolate spontaneous SCV mutants from *S. aureus*, single colonies were inoculated into 5 ml of LB broth supplemented with varying concentrations of gentamycin or kanamycin from 5 to 25 µg ml⁻¹ in 5 µg ml⁻¹ increments. After 3 days of aerobic growth at 37°C, bacterial cells were subject to dilution plating for single colonies on LB agar containing 0.004 % Congo Red. SCVs were easily distinguished from the wild-type as they formed smaller colonies with darker red colour (Malone, 2015). The stability of the SCV phenotypes was then examined by streaking onto the same Congo Red plates three times, before they were frozen-stored at ~80°C. The SCVs of *P. aeruginosa* PA01 were obtained in a different way by growing the wild-type strain under static conditions for 7 days. The resultant cultures were mixed by vortexing, and subsequently diluted and plated onto LB agar containing 0.004 % Congo Red. Small dark red colonies were randomly picked up and their stability was checked via colony-to-colony transfer three times on LB agar with 0.004 % Congo Red.

**Assays for bacterial resistance to metallic copper and copper ions.** Contact killing was performed on two types of copper materials, pure copper (UNS number C11000, 99.9 % Cu) and brass (UNS number C27200, 63.5 % Cu plus 36.5 % Zn), purchased from Wakesfield Metals. They were approximately 1 mm thick and were cut into small coupons of 1 × 1 cm for assays of contact killing. The so-called wet inoculation method was used to measure bacterial resistance to metallic copper (Molteni et al., 2010). To do this, bacterial cells grown in LB medium were washed once with sterile water, and 20 µl was inoculated onto the surface of a copper coupon (1 ×1 cm). Coupons were placed beforehand in a Petri dish containing a few drops of sterile water to maintain moisture. After a given period of time at room temperature (~20°C), cells on the coupon were released into 2 ml H₂O in a 30 ml tube by vortexing, and colony forming units (c.f.u.) were subsequently determined by dilution plating onto LB agar plates. Treatment effects among bacterial strains were assessed by ANOVA (GraphPad Prism 5).

Bacterial sensitivity to copper ions was assessed on LB agar plates supplemented with varying concentrations of copper sulphate. Inoculants were prepared by making a 10-times dilution of an overnight culture, and then 10 µl (~10 000 cells) was dropped onto the copper-containing plates. The MIC is defined as the lowest CuSO₄ concentration at which no colonies were observed after 3 days of incubation.

**Microscopic analyses.** Cells for fluorescence microscopic analysis were obtained from LB agar plates supplemented with 38 µM Calcofluor White (Fluorescent Brightener 28; Sigma-Aldrich) and 45 µM ferric sulphate. Calcofluor is a specific stain for β-glucans and chitin, which are commonly found in bacterial extracellular polymers such as cellulose (McDonald et al., 2009). Iron was added to suppress the production of pyoverdin, a fluorescent siderophore that will interfere with the Calcofluor fluorescence staining (Zhang & Rainey, 2013). Cells immobilized on a 1.5 % agarose pad were examined under an upright fluorescence microscope (Olympus BX61) with an emission and excitation wavelength of 477 and 436 nm, respectively. Cell images were analysed using the ImageJ program (Collins, 2007).
## Table 1. Bacterial strains and their resistance to ionic copper and metallic copper

MIC was determined in LB agar plates supplemented with varying concentrations of copper sulphate. Resistance to metallic copper is expressed as a percentage of cells surviving 10 min of contact with the surfaces of pure copper, and the data are means and standard errors of six replicates. Asterisks indicate significance at $P<0.05$ when compared with the respective wild-type.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Ionic copper (MIC, µM)</th>
<th>Metallic copper (survival %)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K40</td>
<td>Wild-type strain</td>
<td>3250</td>
<td>17.72±1.79</td>
<td>Stephen Ritchie</td>
</tr>
<tr>
<td>K40-SCV1</td>
<td>SCV derived from K40</td>
<td>3000</td>
<td>9.28±1.29*</td>
<td>This work</td>
</tr>
<tr>
<td>K40-SCV2</td>
<td>SCV derived from K40</td>
<td>3000</td>
<td>8.60±1.21*</td>
<td>This work</td>
</tr>
<tr>
<td>K40-SCV3</td>
<td>SCV derived from K40</td>
<td>3000</td>
<td>10.93±1.03*</td>
<td>This work</td>
</tr>
<tr>
<td>K40-SCV4</td>
<td>SCV derived from K40</td>
<td>3000</td>
<td>9.35±0.77*</td>
<td>This work</td>
</tr>
<tr>
<td>K40-SCV5</td>
<td>SCV derived from K40</td>
<td>3000</td>
<td>10.20±1.53*</td>
<td>This work</td>
</tr>
<tr>
<td>K40-SCV6</td>
<td>SCV derived from K40</td>
<td>3000</td>
<td>7.85±0.65*</td>
<td>This work</td>
</tr>
<tr>
<td>H59</td>
<td>Wild-type strain</td>
<td>3250</td>
<td>23.21±1.63</td>
<td>Stephen Ritchie</td>
</tr>
<tr>
<td>H59-SCV1</td>
<td>SCV derived from H59</td>
<td>3000</td>
<td>10.47±1.56*</td>
<td>This work</td>
</tr>
<tr>
<td>H59-SCV2</td>
<td>SCV derived from H59</td>
<td>3000</td>
<td>8.05±0.70*</td>
<td>This work</td>
</tr>
<tr>
<td>E182</td>
<td>Wild-type strain</td>
<td>3250</td>
<td>20.39±0.88</td>
<td>Stephen Ritchie</td>
</tr>
<tr>
<td>E182-SCV1</td>
<td>SCV derived from E182</td>
<td>3000</td>
<td>9.24±0.74*</td>
<td>This work</td>
</tr>
<tr>
<td>E182-SCV2</td>
<td>SCV derived from E182</td>
<td>3000</td>
<td>12.98±1.12*</td>
<td>This work</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type</td>
<td>4500</td>
<td>0.81±0.12</td>
<td>Stover et al. (2000)</td>
</tr>
<tr>
<td>PAO1-SCV1</td>
<td>SCV derived from PAO1</td>
<td>4400</td>
<td>0.47±0.11*</td>
<td>This work</td>
</tr>
<tr>
<td>PAO1-SCV2</td>
<td>SCV derived from PAO1</td>
<td>4300</td>
<td>0.55±0.11*</td>
<td>This work</td>
</tr>
<tr>
<td>PAO1-SCV3</td>
<td>SCV derived from PAO1</td>
<td>4400</td>
<td>0.49±0.09*</td>
<td>This work</td>
</tr>
<tr>
<td>MU49-14</td>
<td>PAO1 ΔwssA ΔpelFΔpscA, defective in alginate, Pel and Psl production</td>
<td>4400</td>
<td>1.99±0.36*</td>
<td>Ghafour et al. (2011)</td>
</tr>
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<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SBW25</td>
<td>Wild-type strain</td>
<td>3200</td>
<td>10.19±1.66</td>
<td>Silby et al. (2009)</td>
</tr>
<tr>
<td>LSWS</td>
<td>Derivative of SBW25 overproducing cellulose polymers</td>
<td>3200</td>
<td>4.80±0.34*</td>
<td>McDonald et al. (2009)</td>
</tr>
<tr>
<td>MU49-17</td>
<td>SBW25 Δwss, defective in cellulose production</td>
<td>3200</td>
<td>12.94±1.96</td>
<td>McDonald et al. (2009)</td>
</tr>
<tr>
<td>CA</td>
<td>SBW25 ΔmntA, overproducing CA capsule</td>
<td>3300</td>
<td>27.14±4.31*</td>
<td>Philippe Remigi, unpublished</td>
</tr>
<tr>
<td>FS</td>
<td>Derivative of SBW25 with LPS overproduction</td>
<td>3200</td>
<td>5.39±1.79*</td>
<td>Ferguson et al. (2013)</td>
</tr>
<tr>
<td>MU49-21</td>
<td>SBW25 Δfuc defective in LPS production</td>
<td>3200</td>
<td>11.43±3.34</td>
<td>Ferguson et al. (2013)</td>
</tr>
<tr>
<td>MU49-22</td>
<td>SBW25 ΔflaA, no flagella</td>
<td>3200</td>
<td>12.93±2.23</td>
<td>Xue-Xian Zhang, unpublished</td>
</tr>
<tr>
<td>MU49-23</td>
<td>SBW25 ΔflaG, no type IV pili</td>
<td>3200</td>
<td>9.13±1.43</td>
<td>Xue-Xian Zhang, unpublished</td>
</tr>
<tr>
<td>MU49-24</td>
<td>SBW25 ΔflaA ΔpilG, no flagella and pili</td>
<td>3200</td>
<td>13.25±1.44</td>
<td>Xue-Xian Zhang, unpublished</td>
</tr>
</tbody>
</table>
Cells for scanning electron microscopy (SEM) analysis were fixed in a modified Karnovsky’s fixative (3 % glutaraldehyde, 2 % formaldehyde in phosphate buffer, pH 7.2) for at least 8 h. Cells collected by centrifugation at 4000 r.p.m. for 4 min were clamped between two membrane filters (0.4 µm, Isopore; Merck Millipore) in an aluminium clamp and processed three times in phosphate buffer washes (15 min each), followed by dehydration in a graded series of ethanol (25, 50, 75, 95 and 100 %) for 15 min each and a final 100 % for 1 h. The samples were then critical point dried using liquid CO2 as the transition fluid. Finally, the samples were mounted onto aluminium stubs and sputter coated with gold (BAL-TEC SCD 005 sputter coater) and viewed in an FEI Quanta 200 scanning electron microscope with an energy-dispersive X-ray spectroscopy (EDS) module.

**Biofilm quantification.** The ability of *P. aeruginosa* and *S. aureus* strains to form biofilm on microtitre plates was quantitatively assessed using a standard procedure as previously described (O’Toole, 2011). Briefly, bacterial cells in fresh LB medium were inoculated into a 96-well microtitre plate (100 µl per well), and incubated at 37 °C for 18 h. Biofilms formed on the inner wall of a well were stained with 125 µl of 0.1 % Crystal Violet for 15 min. The dye was then dissolved using 125 µl of 30 % acetic acid, and spectrophotometrically measured at a wavelength of 550 nm (A550).

**Evolution experiment.** The neutrally marked strain of *P. fluorescens* SBW25-lacZ was selected as the ancestor for experimental evolution (Zhang & Rainey, 2007a), as it forms distinctive blue colonies on LB agar plates supplemented with X-Gal (60 µg ml−1). Strain identity was additionally controlled by strain-specific PCR amplification of xut genes using primers xutA-compF and xutR-lacZF (Liu et al., 2015). The experimental procedures are outlined in Fig. S2. The work involved two treatments with and without copper contact killing, and each was performed with eight independent evolutionary lines. For the copper treatment, cells from overnight culture were washed once with sterile water, and then 20 µl was dropped onto a brass coupon placed in a Petri dish. After 60 min at room temperature, the brass coupon was transferred into a 30 ml plastic tube containing 2 ml of LB medium. Surviving cells on the coupon (~10 000) were released into the fresh medium by vigorous vortexing, and the coupon was subsequently removed with the help of a sterile inoculation loop. In the control line without the copper treatment, bacterial cultures were subject to serial daily transfer by 1000-fold dilution (thus, ten generations per transfer). The evolved bacterial populations were stored at −80 °C every ten transfers for assays of contact killing on copper (and other future analyses such as genome sequencing).

**RESULTS**

**Characterization of SCVs isolated in vitro**

Thirteen spontaneous SCVs (three from *P. aeruginosa* PAO1 and ten derived from three wild-type strains of *S. aureus*) were subjected to phenotypic characterization. All SCVs formed distinctive smaller colonies with darker red colour on LB agar plates supplemented with 0.004 % Congo Red (Fig. 1a). SEM analysis showed that SCVs had a similar cell shape and size, but produced more EPS when compared with wild-type strains (Fig. 1b). Overproduction of EPS was further visualized under fluorescence microscopy, whereby Calcofluor White, a fluorochrome dye, was used to specifically stain the cell surface-located EPS (Fig. 1c).

SCVs normally have an enhanced ability to form biofilms as a result of EPS overproduction (Malone, 2015). To test if this holds for the SCVs evolved in vitro in this work, we quantitatively compared the biofilms formed by wild-type strains and their derived SCVs, and the data are shown in Fig. S3. Parallel to our expectation, the SCVs formed three to four times more biofilms in microtitre plates after growth at 37 °C for 18 h compared with the wild-type strains. However, no significant differences were found among SCVs derived from the same wild-type strain (P<0.05).

**Contact killing of SCVs on pure copper and brass**

A standard wet inoculation method was used to compare the rates of contact killing between wild-type and its derived SCVs on the surfaces of pure copper (99.9 % Cu) and brass (63.5 % Cu). An a priori test of the methodology was performed with two wild-type strains, *P. aeruginosa* PAO1 and *S. aureus* K40. Both strains were more rapidly killed on pure copper than on brass (Fig. S4). For example, within 10 min of exposure on pure copper and brass the rate of contact killing for *P. aeruginosa* was 99.2 and 62.5 %, respectively (Fig. S4a). Similar results were obtained for *S. aureus*, with 82.3 and 37.1 % of cells being killed by pure copper and brass, respectively (Fig. S4b). These results are consistent with previous findings that the antimicrobial activities of copper alloys are proportional to the copper content of the surface materials (Grass et al., 2011). Interestingly, a cross comparison of data presented in Fig. S4 (a, b) suggest that the Gram-negative *P. aeruginosa* cells were more susceptible to copper-mediated contact killing than the Gram-positive *S. aureus* cells. This was further confirmed by a parallel contact-killing assay of the two strains on the surfaces of brass (Fig. S5).

To detect cellular damage on copper surfaces and also the expected absorption of copper ions, bacterial cells with and without copper treatment were subjected to SEM analysis coupled with EDS. Representative cell images and EDS spectra are presented in Fig. S6. Bacterial cells of irregular shapes, including shrunken cells, were clearly visible under SEM. This suggests that copper causes damage to the cell envelope structure. Significantly, accumulation of copper ions was not detected by SEM/EDS in cells without the copper treatment, whereas peaks of copper ions were present in most copper-treated cells (Fig. S4b, c). However, the EDS data were not quantitative; there were huge variations among cells with regard to the amount of intracellular copper. Thus, we were unable to use SEM/EDS to compare levels of accumulated copper ions between different bacterial strains.

Armed with the contact killing methodology described above, we compared the rates between wild-type strains and their derived SCV mutants. The proposed CIBR hypothesis predicts that SCVs produce more extracellular polymers; consequently, they will be more rapidly killed than the wild-type strains. Indeed, all SCVs had significantly more cells (~10 %) killed within 10 min of contact on pure
**Fig. 1.** Morphological analysis of wild-type *P. aeruginosa* and *S. aureus* and their derived SCVs. (a) Colonies formed on LB agar supplemented with 0.004 % Congo Red dye. (b) Representative SEM images at 20 000× magnification. (c) Fluorescence microscope images of bacterial cells grown on LB agar containing 38 μM Calcofluor White M2R and 45 μM ferric sulfate.

**Fig. 2.** Contact killing of wild-type *S. aureus* strains K40, E182 and H59 (a) and *P. aeruginosa* PAO1 (b) versus their derived SCVs on the surfaces of pure copper. The rate of contact killing was expressed as the percentage of cells killed within 10 min for *S. aureus* and 5 min for *P. aeruginosa*. The line in the middle of the box represents the median, whereas the bottom and top boxes represent the 25th and 75th percentiles, respectively. The lower and upper whiskers indicate the smallest and the largest values, respectively.
copper for *S. aureus* and 5 min for *P. aeruginosa* (*P*<0.05; Fig. 2). Similar results were obtained on the surfaces of brass (Fig. S7). The differences were smaller on brass when compared with data on the surfaces of pure copper (Fig. 2), but they remained significant at least for SCVs of *P. aeruginosa* (*P*<0.05).

Finally, it should be noted that a *P. aeruginosa* mutant, MU49-14, defective in the production of all three major types of EPS (namely alginate, Pel and Psl) was more resistant to contact killing (Table 1). Specifically, after 5 min of contact on pure copper, 1.99±0.36 % of MU49-14 cells survived, which is significantly higher than the survival rate of 0.81±0.12 % of wild-type *P. aeruginosa* PAO1 (*P*<0.05). This result is also consistent with the expectation made from the CIBR hypothesis, as this mutant does not produce any of the three types of EPS, and thus is less reactive to metallic copper.

**Assessing the roles of cell surface polymers in copper-mediated contact killing**

Next we sought to determine the roles of specific cell surface polymers in copper-mediated contact killing, including EPS, LPS, pili, flagella and CA capsules. The work was performed using a non-pathogenic model organism of *P. fluorescens* SBW25, which belongs to the same genus as the nosocomial pathogen *P. aeruginosa*. Eight mutants were tested in this work, which overproduce (or are defective in producing) one of these cell surface components. The results are summarized in Fig. 3. Mutants overproducing EPS and LPS were more sensitive to copper-mediated contact killing, whereas the opposite was found for mutants overproducing the CA capsule (*P*<0.05). No significant difference was detected for mutants defective in the production of flagella and/or pili (Fig. 3).

**Comparative analysis of bacterial resistance to metallic copper and ionic copper**

The MIC of copper ions was determined on LB agar supplemented with varying concentrations of copper sulphate. The obtained MIC data for 27 bacterial strains used in this study are shown in Table 1. The MIC data were compared with the corresponding data of bacterial resistance to metallic copper expressed as the survival rate (%) after 10 min of contact with pure copper. For both *S. aureus* and *P. aeruginosa*, SCVs were more susceptible to copper ions and less resistant to copper-mediated contact killing compared with the wild-type (Table 1). Moreover, the capsulated cells of *P. fluorescens* showed higher levels of resistance to both ionic and metallic copper than the wild-type. These data suggest a seemingly positive correlation between bacterial resistance to ionic and metallic copper. However, this finding was not supported by data from isogenic mutants of *P. fluorescens* and *P. aeruginosa*. The triple deletion mutant of *P. aeruginosa* PAO1 defective in the production of alginate, Pel and Psl was less resistant to copper ions, but more resistant to contact killing. The LPS- and EPS-overproducing mutants of *P. fluorescens* SBW25 were more susceptible to contact killing but they showed the same level of resistance to copper ions.

**Experimental evolution of bacterial resistance to metallic copper**

To assess the potential of bacterial pathogens to develop resistance to metallic copper, a *lacZ*-marked strain of *P. fluorescens* SBW25 was subjected to daily passage of sub-lethal conditions on the surface of brass. The work was performed in a non-pathogenic strain of *P. fluorescens* to minimize health and safety concerns. Importantly, *P. fluorescens* SBW25 is one of the most commonly used model organisms for studies of experimental evolution (Rainey et al., 2014). As outlined in Fig. S2, cells surviving the contact killing (~10 000 cells at the beginning of the experiment) were inoculated into fresh LB broth for the next round of contact killing. A control of eight independent evolutionary lines was set up whereby the same strain of *P. fluorescens* SBW25 was propagated by serial transfer in LB medium without the copper treatment. Dynamic changes of bacterial resistance to metallic copper were monitored every ten transfers, and preliminary data for the first 100 transfers are shown in Fig. 4. Parallel to our expectation, survival rates of cells in the control lines (i.e. those without copper treatment) remained at similar levels (~0.035 %) during the whole process of evolution. In contrast, the survival rate of cells from the copper treatment lines steadily increased from 0.035±0.001 to 0.968±0.305 % during the first 30 transfers, and further increased to 2.551±0.331 % at the end of 100 transfers.

**DISCUSSION**

HAI s are a serious public health problem worldwide (Graves et al., 2003; Simoes et al., 2016). A novel strategy for HAI prevention is to use antimicrobial copper-containing surface materials within hospital facilities (Michels et al., 2015). The data presented here address two important questions with regard to our current understanding of bacterial contact killing on copper, i.e. how bacteria are killed by toxic copper ions derived from metallic copper, and whether bacteria can evolve resistance to metallic copper. Specifically, we examined the rates of contact killing for a panel of bacterial strains with altered synthesis of cell surface components. The results showed that all 13 SCVs of *P. aeruginosa* and *S. aureus* as well as the related isogenic mutants of *P. fluorescens* SBW25 (i.e. those overproducing EPS and LPS) are more susceptible to contact killing on copper, when compared with their respective wild-type strains. These results are consistent with expectation made from the proposed CIBR hypothesis (Fig. S1), highlighting the important roles of cell surface structure in bacterial contact killing on copper. However, the complex chemical reactions between cell surface polymers and the copper metal have not yet been investigated, as this was beyond the scope...
of the current study. Data from further chemical analysis will complement the biological evidence presented here, together providing a strong empirical testing of the CIBR hypothesis.

The slow-growing auto-aggregative SCVs are of great clinical importance, as they are associated with many difficult-to-treat chronic infections (Biswas et al., 2009; Johns et al., 2015). SCVs have enhanced ability to attach to biotic (and abiotic) surfaces and form biofilms (Latimer et al., 2012). More importantly, they often show increased resistance to antibiotics as well as other environmental stresses such as heavy metals and oxidants (Malone, 2015). It is well known that SCVs can rapidly evolve both in vivo and in vitro following exposure to aminoglycosides, β-lactam and other antibiotics (Schmitz et al., 1999; Latimer et al., 2012). In this study, we isolated ten SCVs derived from three clinical strains of S. aureus with the treatment of kanamycin and gentamycin. Like the large spread wrinkly spreader morphotype of P. fluorescens SBW25 (McDonald et al., 2009), SCVs were supposed to be selected for colonization at the air–liquid interface. Thus, the static incubation method was employed to select for SCVs of P. aeruginosa PAO1. We show that those SCVs isolated in vitro have similar phenotypes to SCVs evolved in vivo, in terms of cell and colony morphology as well as an increased level of biofilm formation. Therefore, it is very important to note that SCVs are more susceptible to contact killing on copper than their wild-type ancestors. This suggests a potential significant role of copper materials in preventing the spread of SCVs and biofilm formation in hospital environments. The results also have practical implications, as purified EPS of bacterial origin may be capable of enhancing the antimicrobial activities of copper materials when added to surface-cleaning agents.

Bacterial cells produce various forms of extracellular substrates, which in general provide protection against environmental stresses (Kleanthous & Armitage, 2015). In the
present study, we assessed the contribution of EPS, LPS, flagella and pili as well as CA capsule to the rate of contact killing on copper. This was achieved using a panel of isogenic mutants derived from a model organism of *P. fluorescens* SBW25. No significant difference was detected for flagella and pili but, surprisingly, EPS and LPS stimulated the rates of contact killing instead of protecting cells to survive on copper surfaces. Only the CA capsules were shown to be able to enhance bacterial survival on metallic copper. Intriguingly, capsulated cells are more resistant to copper as well, when compared with the non-capsulated wild-type cells (Table 1). In contrast, all the EPS-overproducing SCVs were less resistant to both metal copper and ionic copper (Table 1). There seems to be a possible correlation between resistance to metallic and ionic copper. However, this was not noted for the EPS- and LPS-overproducing mutants of *P. fluorescens* SBW25, which showed similar levels of resistance to copper sulphate as the wild-type. More significantly, the *P. aeruginosa* mutant defective in alginate, Pel and Psl synthesis displayed increased resistance to metallic copper, but reduced resistance to copper ions. Together, the data suggest different but overlapping mechanisms for metallic and ionic copper resistances.

Questions pertaining to the evolution of bacterial resistance to metallic copper can be powerfully addressed through frequent exposure of bacterial cells to sub-lethal copper conditions in a continuous manner. To this end, and for the first time, we have monitored the dynamic changes of bacterial susceptibility to copper during the course of experimental evolution with (and without, as a control) contact killing on the surfaces of brass. Preliminary data from the first 100 daily transfers presented here suggest that bacteria have very limited ability to evolve resistance to metallic copper. A significant increase in copper resistance was detected, but more than 97% of bacterial cells were still killed within 60 min on brass. Of particular note is that copper has long been used as a common coinage metal. Previous studies have shown that viable bacterial cells can be isolated from copper coins with antimicrobial properties, although they have similar levels of resistance to metallic copper as the control strains, when re-examined under laboratory conditions with the wet inoculation method (Pachtet *et al.*, 1997; Santo *et al.*, 2010). These ‘sensitive’ strains had probably survived from contact killing due to soiling by organic matter (de Carvalho & Caramujo, 2014). Finally, it should be noted that the experimental evolution work on copper contact killing is currently ongoing in our lab. It is anticipated that new data from longer-term studies will be able to provide much more reliable assessments for the evolution of bacterial resistance to copper, and further identify any responsible mechanisms by use of genome re-sequencing and subsequent genetic characterization.

**ACKNOWLEDGEMENTS**

We are very grateful to Niki Murray at the Manawatu Microscopy and Imaging Centre of Massey University, who helped perform the SEM analysis in this work. We thank Stephen Ritchie, Philippe Remigi, Gayle Ferguson and Bernd Rehm for providing bacterial strains, Heather Hendrickson for assistance with fluorescence microscopy, Yunhao Liu for technical support and Jacob Malone for advice on SCV isolation for *P. aeruginosa*. This work was financially supported by the Massey University Research Foundation (MURF).

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