Contact Killing of Bacterial Pathogens on Metallic Copper

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Abstract

Hospital-acquired infections (HAIs) are a serious health concern worldwide. Currently in New Zealand, about one in ten patients admitted to hospitals will acquire an infection while receiving treatments for other medical or surgical conditions. An emerging strategy for HAIs prevention is to use self-sanitising copper surfaces on items commonly touched in hospitals, which can provide sustained protection against microbial contamination. This is due to the fact that a wide range of microorganisms can be rapidly killed on copper in a process termed "contact killing". However, the mechanisms of copper-mediated contact killing are not fully understood; and moreover, the potential of bacterial pathogens to develop resistance to metallic copper has so far not been examined.

Here we hypothesize that bacteria are predominantly killed by a burst release of toxic copper ions resulted from chemical reactions between surface components of bacterial cell and metallic copper. To test this copper ion burst release hypothesis, we isolated and phenotypically characterized small colony variants (SCVs) derived from the two most common nosocomial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Consistent to our expectation, SCV mutants overproducing exopolysaccharides (EPS) are more rapidly killed than wild type on the surfaces of pure copper (99.9% Cu) and brass (63.5% Cu). Similar results were obtained with a panel of mutants with altered production of cell surface components (EPS, lipopolysaccharides,

capsules, flagella and pili) in a non-pathogenic model organism of *Pseudomonas fluorescens* SBW25.

Next, a unique approach of experimental evolution was used to assess the potential emergence of bacterial resistance to metallic copper. Specifically, *P. fluorescens* SBW25 was subjected to daily passage of sub-lethal conditions on the surfaces of brass. After 100 daily transfers, the evolved strains had a slight increase of survival rate on brass; but importantly, ~97% of cells can still be killed on brass within one hour.

Taken together, our results clearly indicate that the rate of bacterial killing on copper is largely determined by surface components of a bacterial cell, providing support for the copper ion burst release hypothesis. Our primary data of experimental evolution showed that bacteria have limited ability to evolve resistance to metallic copper.

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"Let me tell you the secret that has led me to my goal.

My strength lies solely in my tenacity."

- Louis Pasteur-

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List of Abbreviations

ATP   adenosine triphosphate

A_{550} absorbance measured at 550 nm

bp   base pairs

CA   colanic acid

CF   cystic fibrosis

cfu   colony forming unit

CFW   calcofluor white

CFP   cyan fluorescent protein

cm   centimeter

CV   crystal violet

DF   dilution factor

DNA   deoxyribonucleic acid

dNTP   deoxyribo nucleotide tri-phosphate

DR   death rate

EDAX   energy dispersive x-ray spectroscopy

EPA   environmental protection agency

EPS   exopolysaccharides or extracellular polysaccharides

FS   fuzzy spreader

FTIR   synchrotron fourier-transform infrared spectromicroscopy

g   gram

Gem   gentamicin

h   hour

HAIs   hospital-acquired infections

ICP   inductively coupled plasma mass spectrometry

ICU   intensive care units

Km   kanamycin

kV   kilovolt

LB   luria-bertani

LSWS   large spreading wrinkly spreader

LPS lipopolysaccharide

MIC minimal inhibitory concentration

min minutes

ml milliliter

mM millimolar

MRSA methicillin-resistant *Staphylococcus aureus*

MS mass spectrometry

MSSA methicillin sensitive *Staphylococcus aureus*

nm nanometer

OD optical density

PCR polymerase chain reaction

pmol picomole

ROS reactive oxygen species

rpm revolutions per minute

s seconds

SCVs	small colony variants
SD	standard deviation
SEM	scanning electronic microscope
SiC	silicon carbide
TAC	tricarboxylic-acid cycle
TBE	tris-borate-ethylenediamine tetraacetic acid
UV	ultraviolet
VRE	vancomycin resistant *Enterococcus*
WHO	world health organization
WT	wild type
ºC	degrees celsius
μg	microgramme
μl	microliter
μm	micrometre
μM	micromolar

CHAPTER ONE: INTRODUCTION

1.1   Hospital-acquired infections — a serious public health concern

Hospital-acquired infections (HAIs) are a major and growing health problem worldwide. Every year, over two million hospitalized patients will be affected by HAIs in the United States alone, causing more than one hundred thousand deaths (Hall et al., 2009). The rate of HAIs is nearly 25% for patients admitted to the intensive care units (ICU) (Klevens et al., 2007). Globally, as estimated by the World Health Organization (WHO), hundreds of millions of people acquire an infection while receiving treatments for other diseases in hospitals every year. Additionally, HAIs also pose a serious risk to the safety of hospital visitors and staff.

HAIs are an important cause of excess morbidity, mortality and cost. Vulnerable people (e.g, the very young or old patients, and those suffering from chronic disease with weaken immune system) are more likely to acquire HAIs while staying in hospital. HAIs usually result in longer hospital stay, thus producing substantial excess costs to patient's family and the public healthcare system (Graves et al., 2007).

Currently in New Zealand, HAIs affect ~10% of hospitalized patients with an estimated excess cost of $136.2 million per annum (Casey et al., 2010). Consequently, Office of the Auditor-General has stated that reducing HAIs is a high priority for New Zealand healthcare institutions. It has been estimated that at least one-third of HAIs can be

prevented through optimal hand hygiene, judicious use of medical devices and maintaining high standard of hospital environmental hygiene (Graves et al., 2007).

Commonly touched items, such as doorknobs, push plates, toilet seats and bed rails, in hospitals are usually made of surface materials devoid of antimicrobial properties - such as aluminum, stainless steel, plastics or wood. Microbial pathogens present on these commonly touched surfaces can be transmitted via contact between patients, visitors and healthcare workers, thus these surfaces are considered to be the major source of HAIs. Therefore, the current infection control protocols involve frequent cleaning, hand washing in particular. However, despite considerable efforts to improve hygiene compliance by healthcare staff, the average compliance rate with hand hygiene procedures is only 40% (Pittet et al., 2000). Therefore, new strategies are urgently required to complement the current HAIs prevention practices.

An emerging strategy for HAIs prevention is to apply antimicrobial copper-containing surfaces to commonly touched places within hospital facilities. Metallic copper is a self-sanitizing material that can kill a wide range of microorganisms within minutes of contact (Grass et al., 2011). This rapid "contact killing" demonstrates the great potential of copper material in reducing HAIs, as copper surfaces can provide sustained protection by effectively killing pathogens during intervals between routine cleanings and human contact.

1.2 Using copper-containing surface materials for HAIs prevention

1.2.1 The antimicrobial properties of copper

Copper is element number 29 on the Periodic Table of Elements. It is a malleable metal with a long history of domestic use. The use of copper by human can be dated back to between 2600 and 2200 B.C.. The oldest described medical use of copper was mentioned in the Smith Papyrus, an Egyptian medical text, in which Roman used copper to sterilize wounds and make water drinkable (Dollwet & Sorenson, 1985). The medical efficiency of copper was evidenced by the fact that copper workers appeared to be immune to cholera, which broke out in Paris in 1832 (Moore & Kellerman, 1905). Later, the therapeutic capacities of copper were recognized for the treatment of skin infectious diseases (Hymes et al., 2006).

Copper is one of the most common coinage metals in many countries, including New Zealand. The current New Zealand $1 and $2 coins were introduced on 11 February 1991, and both are made of aluminum bronze containing Cu 92%, Al 6% and Ni 2%. Copper has been selected as the main constituent of coins mainly because of its excellent wear resistance and anti-corrosion properties, as well as its anti-microbial activities (Fournier & Govers, 2003). A previous study performed in 1982 showed that increase of copper concentration in United States penny from 2.6% to 95% resulted in a significant reduction of microbial infections caused by daily use of the coin (Data were adapted from http://www.copper.org).

The antimicrobial properties of copper and copper alloys have recently received increasing attention due to the widespread of resistance to antibiotics among nosocomial bacterial pathogens (S. L. Warnes, 2014). In 2008, the United States Environmental Protection Agency (EPA) has officially approved the registration of antimicrobial copper alloys, which can efficiently kill many disease-causing bacteria, including the Methicillin-resistant *Staphylococcus aureus* (MRSA) "superbugs" (Noyce et al., 2006).

1.2.2 The potential of copper in reducing HAIs

The fact that a wide range of microorganisms can be killed on copper surfaces within minutes of contact , termed "contact killing", indicates a great potential of copper in reducing the risk of HAIs. This has prompted several hospital trials overseas in order to evaluate the roles of copper in maintaining hospital environmental hygiene, and also reducing the rates of HAIs. Figure 1.1 shows countries wherein the clinical trials have taken place, including the United States, United Kingdom, Chile, Germany, Finland, and Japan (Figure was adapted from http://www.antimicrobialcopper.com). However, similar hospital trials have not performed in New Zealand yet.

Figure 1.1   Hospital trials investigating the antimicrobial copper's potential around the world. This figure is adapted from the Antimicrobial Copper Association website (http://www.antimicrobialcopper.com).

Data available so far from these clinical trials consistently indicate that use of copper-containing surfaces can significantly reduce bacterial burden by more than 80% and decrease the risk of infection by nearly 60% when compared with control surfaces (i.e.: glass, plastic, wood, and stainless steel) (Grass et al., 2011). One of the initial studies of applying copper items in hospitals at the United Kingdom showed that pure copper or copper alloys were both effective against the follow bacterial strains: *Enterobacter aerogenes*, *Escherichia coli* O157:H7, MRSA, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Vancomycin-resistant *Enterococcus faecalis* (VRE). More than 99.9% of those pathogens were killed within a time frame of two hours at room temperature. Importantly, as shown in Figure 1.2, under typical indoor conditions copper exhibited efficacy against the MRSA superbug, whereas two silver-containing materials from two different manufacturers and triclosan showed few antimicrobial activities, and behaved like the negative control of stainless steel (Figure was adapted from http://www.antimicrobialcopper.com).
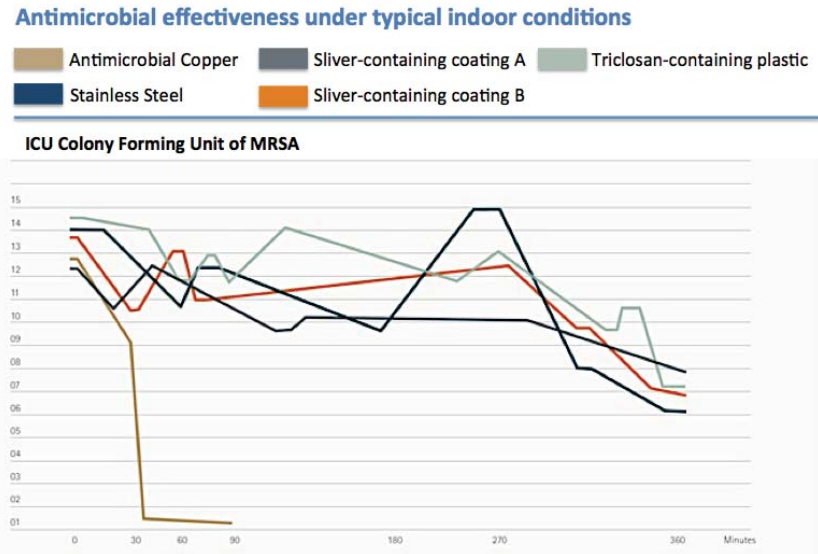
Figure 1.2 A comparison of antimicrobial properties of four different materials under typical indoor conditions. This figure was adapted from the Antimicrobial Copper Association website (http://www.antimicrobialcopper.com), showing the changes of colony forming units (cfu) over time: (copper, brown line; sliver-containing coating A, dark blue line; sliver-containing coating B, orange line; stainless steel, black line; triclosan-containing plastic, green line).

A recent clinical trial in the US assessed the antimicrobial efficacy of copper in ICU. The results indicate that use of antimicrobial copper surfaces in ICU could reduce the number of HAIs by 58% compared with control surfaces (non-copper) (Salgado et al., 2013). A ten-weeks trial performed at the Selly Oak Hospital in Birmingham showed that bacterial contaminations in copper-coated toilet seats, brass tap handles, and brass door push plates were 90% to 100% lower than that of the control surfaces (i.e. plastic or aluminum surfaces). Impressively, methicillin sensitive *Staphylococcus aureus* (MSSA), VRE, and *E. coli* were found on control surfaces only (Casey et al., 2010). Taken together, those hospital trials around the world suggested a great potential of copper in reducing the risk of pathogen transmission via physical contact.

1.3　The mechanisms of copper-mediated contact killing

1.3.1　Current understanding of copper-mediated cell death

The physical and chemical interactions between bacterial cells and the surfaces of metallic copper are very complex, and consequently, the mechanisms of copper-mediated contact killing are not fully understood (Hans et al., 2016). Current evidence suggests that oxidative stress induced by ionic copper is the major factor causing cell death. Damage of cell membrane, deoxyribonucleic acid (DNA) degradation, inhibition of cellular respiration, and inducing of cellular reactive oxygen species (ROS) stress have been observed (Stohs & Bagchi, 1995). However, the source of the toxic copper ions and the order of the detected cell damages and also their relative importance remains elusive. It is highly possible that bacterial death is multifactorial rather than a result of single mechanism.

1.3.2　Toxicity of ionic copper

Copper in its ionic form is known to be an essential nutrient for all organisms. It functions as a co-factor for many cellular enzymes owing to its capacity to change its oxidative states between Cu^+ and Cu^{2+}. However, copper ions can be very toxic when absorbed in excess (Zhang & Rainey, 2008). Reactive hydroxyl radicals are generated by copper ions via a Fenton-type reaction:

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + 2OH^-$$
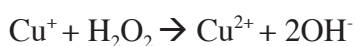
Bacterial cells have evolved mechanisms whereby H_2O_2 concentrations are kept at very low levels. Therefore, production of reactive oxygen species may not be the primary cause of cell damages. It is also possible that copper ions may lead to depletion of sulfhydryls, such as in cysteines or glutathiones, in a cycle between the following two reactions:

$$2Cu^{2+} + 2RSH \rightarrow 2Cu^+ + RSSR + 2H^+$$

$$2Cu^+ + 2H^+ + O_2 \rightarrow 2Cu^{2+} + H_2O_2$$
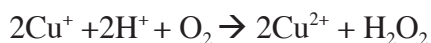
Then the hydrogen peroxide can participate in the abovementioned reaction, leading to further generation of toxic free hydroxyl radicals. Those hydroxyl radicals can efficiently damage many kinds of biomolecules, such as DNA, proteins, and lipids (Espirito Santo et al., 2008). Therefore, the toxicity of copper ions is attributable to the tendency of alternation between cuprous Cu^+ (the reduction state), and cupric Cu^{2+} (the oxidation state) (Espirito Santo et al., 2008).

However, as a common metal, copper is well known to be safe for regular contacts. It has been reported that the concentrations of free copper ions on the surfaces of copper are ~0.3 μM at 25°C, which is not sufficient to kill bacterial cells (Espirito Santo et al., 2008). Mathews *et al*. have recently placed a thin synthetic polymer between bacterial cells and copper metal, which prevented the cell-metal physical contacts without affecting the passage of copper ions (Figure 1.3). Interestingly, their results indicate that rapid killing was eliminated: even after 3.5 h, bacterial survival rate was only reduced

by 1 log, whereas on uncoated copper coupons, more than 10^6 cells were dead after 30 min contact (Mathews et al., 2015).

Taken together, the toxicity of copper ions can only partially explained the observed rapid killing of bacterial cells upon contact with metallic copper (Rensing & Grass, 2003). A current model of bacterial contact killing on copper is proposed by Grass *et al*. (2011) and it is shown in Figure 1.4. It highlights the importance of cellular damages caused by toxic copper ions, but the source of copper ions remains elusive.
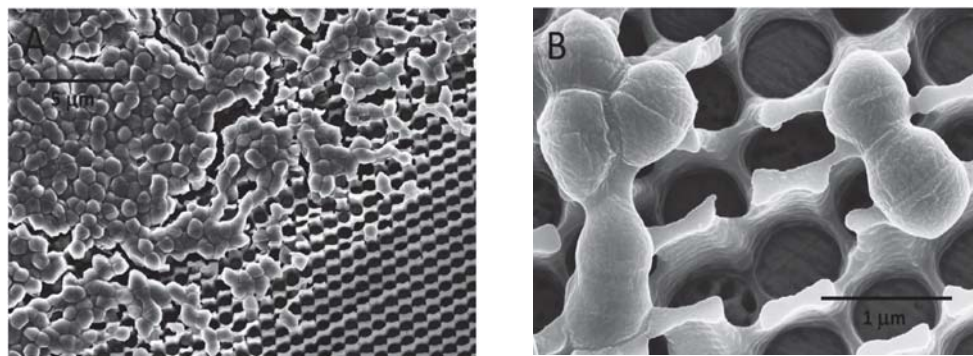
Figure 1.3   **SEM image of *E. hirae* on honeycomb-like grids with holes.** Cells were wet plated. **(A)** Image at low magnification. **(B)** Image at high magnification. Figure was adapted from Mathews, *et al* (2013).
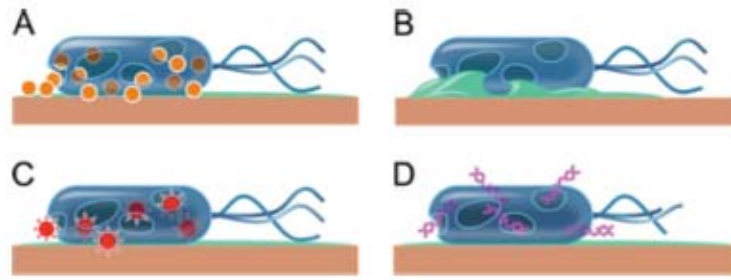
Figure 1.4   Current model of the tentative events in contact killing. (A) Copper dissolves from the copper surface and causes cell damage. **(B)** The cell membrane ruptures because of copper and other stress phenomena, leading to loss of membrane potential and cytoplasmic content. **(C)** Copper ions induce the generation of reactive oxygen species, which cause further cell damage. **(D)** Genomic and plasmid DNA becomes degraded. Figure was adapted from Grass *et al.* (2011).

1.4   The copper ions burst releasing hypothesis

Research in Xue-Xian's laboratory aims to test a new hypothesis that bacterial cells are predominantly killed by a burst release of toxic copper ions (Cu^+/Cu^{2+}), which result from chemical reactions between surface components of bacterial cell and metallic copper (Figure 1.5). Cell surface components, including exopolysaccharides (EPS), lipopolysaccharide (LPS), pili and flagella, contain nitrogen, sulphate or phosphate in their structure, which are potentially reactive with copper. Bacteria possess efflux systems to pump excess copper ions outside of the cell. However, such systems can only help delay cell death, but cannot prevent it (Elguindi et al., 2009). This strongly suggests that bacterial cells continuously absorb and accumulate copper ions until the export system cannot handle, causing cell death. Together, our new model predicts

important roles of cell surface components in determining the rate of bacterial killing on copper.
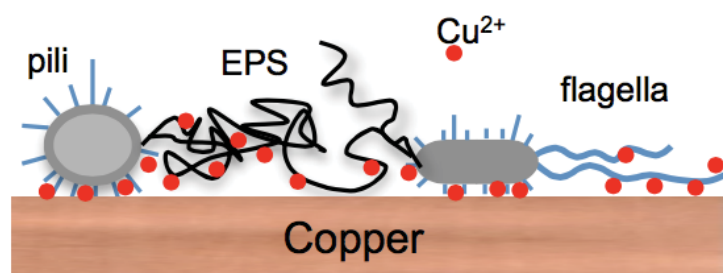
Figure 1.5   The copper ions burst releasing model. Cell surface components, including exopolysaccharides (EPS), lipopolysaccharide (LPS), pili and flagella, can react with copper ions and play critical roles in copper-mediated contact killing.

1.5   Testing the copper ions burst releasing hypothesis

One way to test the above-mentioned copper ion burst-releasing hypothesis is to compare the rate of copper-mediated contact killing between wild-type cells and their derived mutants with altered production of cell surface structures. In this work, we specifically targeted small colony variants (SCVs) of S*taphylococcus aureus* and *Pseudomonas aeruginosa*, which represent two most common nosocomial pathogens. *S. aureus* strains are Gram-positive, spherical cells, whereas strains of *P. aeruginosa* are Gram-negative rod-shaped cells. SCVs over-produce extracellular polymers such as EPS; thus, our hypothesis predicts that they are more rapidly killed on copper than the wild-type strains.

Extracellular polysaccharides are the most important determining factors of the physical property of a bacterial cell, and importantly, they are directly involved in the bacteria-metal contact (Schmidt et al., 2012). On metallic copper surfaces, polysaccharides likely play a destructive role: they can potentially react with metallic copper and help to release more and more copper ions from the metal, which will be subsequently absorbed into the bacterial cell, resulting in cell death.

1.6 Small colony variants (SCVs) of *S. aureus* and *P. aeruginosa*

This project involves the isolation and characterization of SCVs derived from *S. aureus* and *P. aeruginosa* under laboratory conditions. SCVs are naturally occurring slow growing subpopulations, which are characterized by their auxotrophic growth deficiency and depressed α-cytotoxin activity (Massey et al., 2001). Slow growth of SCVs is likely due to mutations of genes involved in the biosynthesis of thiamine, menadione, hemin or thymidine (Melter & Radojevič, 2010). Those mutations result in decreased function of the corresponding metabolic pathways, especially the electron transport chains or the tricarboxylic-acid cycle (TAC), leading to reduced production of adenosine triphosphate (ATP) (Massey et al., 2001).

These variants are not particularly virulent but are able to persist viable inside host cells (Melter & Radojevič, 2010). SCVs usually cause chronic and relapsing infections, which are a serious problem for patients of cystic fibrosis (CF, a genetic disorder caused

by a mutation in *cftr* gene). It has been found that more than 70 % of CF patients were colonized or infected with SCVs during their lifetime (Kahl et al., 2003).

A remarkable characteristic of SCVs is that they are resistant to aminoglycoside antibiotics such as gentamicin (Gem) and kanamycin (Km). Notably, this property can be used to induce and select for SCV mutants in vitro (Massey et al., 2001). Of particular note is that SCVs overproduce a mixture of extracellular polysaccharides. These include alignate and the Pel and Psl EPS for *P. aeruginosa*. Consequently, SCVs possess enhanced ability to adhere various biotic and abiotic surfaces and form biofilms (Giddens et al., 2007).

1.7 Potential of bacteria to develop resistance to metallic copper

Given that the mechanisms of copper-mediated contact killing are poorly understood, there are concerns with regard to the probable emergence and spread of metallic copper resistant strains, as a consequence of increased use of copper in hospital environments.

Copper is an important trace element essentially required for bacterial growth, but it is also highly toxic when excess. Therefore, copper homeostasis must be carefully regulated to ensure efficient supply of copper ions and avoid toxicity (Kozlowski et al., 2009). For example, in the model organism of *Pseudomonas fluorescens* SBW25, two copper-specific transporter systems (Cop and Cue) have been described, whose expression is induced by elevated levels of copper ions in the medium (Figure 1.6). At

low-copper environments, Cop systems are activated to facilitate uptake of copper ions into the cell. This system can offer cells with essential copper ions for cellular function. Under high-copper environments, the Cue systems are activated, which play significant roles in pumping excess copper ions outside of the cell (Zhang & Rainey, 2008).
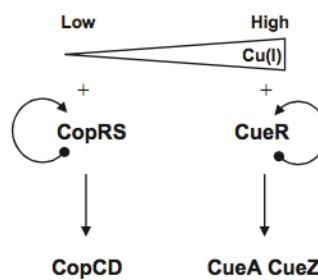
Figure 1.6   A proposed model of copper homeostasis in *P. fluorescens* SBW25. Expression of *copCD* is controlled by CopRS. At low levels of copper concentration, Cop system is activated, playing a role in absorbing copper into a cell. Transcription of *cueA* and *cueZ* is controlled by CueR. At high levels of copper concentration, Cue system is activated, pumping excess copper out of a cell. CopRS and CueR are subject to auto-regulation. Figure was adapted from Zhang, X. X., & Rainey, P. B. (2008).

Bacteria have evolved various mechanisms to counteract the toxic effects of excess copper ions. Mechanisms reported so far include extracellular sequestration of copper ions, relative impermeability of bacterial membranes to copper ions, copper-scavenging proteins, and active extrusion of copper from the cell (Solioz & Stoyanov, 2003). Taking *E. coli* for an example, it possesses three major systems for the protection of cells against copper toxicity: CopA and Cus systems efflux Cu^+, along with CueO system oxidizes peri-plasmic Cu^+ to Cu^{2+} (Outten et al., 2001).

The precise roles of those copper homeostasis systems in bacterial survival on metallic copper remain poorly understood. However, it is interesting to note that those mechanisms can only slightly improve survival of bacteria on metallic copper, but they cannot help prevent contact killing on copper (Espirito Santo et al., 2008). Along with the accumulation of toxic copper ions, the efflux systems will be outpaced, resulting in cell death (Peña et al., 1999). Thus, we would normally think that bacteria have very limited ability to develop resistance to metallic copper. Copper items installed within hospital facilities will have long lasting antimicrobial activities. However, these have not been subjected to any experimental tests yet.

1.8   Specific aims of this project

The primary goal of this project is to elucidate the mechanisms of copper-mediated contact killing, which are currently not fully understood, and also explore the potential of bacterial pathogens to develop resistance to metallic copper. More specifically, we test a new hypothesis, termed the copper ion burst release hypothesis, which is described above and outlined in Figure 1.5. It posits that bacteria are predominantly killed by a burst release of toxic copper ions, which result from complex chemical reactions between cell surface components and metallic copper.

The specific tasks are listed below:

1) **Isolation of SCVs from the two most common nosocomial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.** SCVs can be induced under laboratory conditions by the treatment of antibiotics, i.e. kanamycin (Km) and gentamicin (Gem), or growth under static conditions.

2) **Phenotypic characterization of SCV mutants.** These include scanning electron microscopy (SEM) analysis of bacterial cells **(a)**, visualization of EPS production by fluorescence microscopy **(b)**, minimum inhibitory concentrations of copper ions **(c)** as well as assays for biofilm formation **(d)**.

3) **Comparison of wild-type strains and their derived SCV mutants in terms of their abilities to survive on metallic copper.** The copper contact-killing assays will be performed on the surfaces of two copper materials, pure copper (99.9% Cu) and brass (63.5% Cu).

   SCV mutants overproduce cell surface polymers such as EPS. Thus, our copper ions burst release model (outlined in Figure 1.5) predicts that SCVs ought to be more rapidly killed on copper than wild-type strains on copper.

4) **Assessing the role of various cell surface components in determining the rate of bacterial contact killing on copper.** This work will be performed with a non-pathogenic model strain of *Pseudomonas fluorescens* SBW25, and involve a panel of mutants with altered cell surface properties.

5) **Experimental evolution of bacterial resistance to metallic copper.** A non-pathogenic strain of *P. fluorescens* SBW25 was subjected to daily passage of sub-lethal conditions on the surface of brass (Cu 63.5%). Dynamic changes of bacterial resistance to copper will be determined every 10 days for a period of 100 days in total.

CHAPTER TWO: MATERIALS AND METHODS

2.1   Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 2.1. Bacteria were grown routinely in Lysogeny broth, which is a nutrient-rich medium also known as Luria-Bertani (LB) medium. *P. fluorescens* strains were grown at 28°C, whereas *S. aureus* and *P. aeruginosa* strains at 37°C. If necessary, antibiotics and other supplements were added at the following concentrations except where specifically indicated: X-Gal (60 µg/ml), gentamicin (Gem), kanamycin (Km), Congo Red (0.004%), Fluorescent Brightener 28 (35 µg/ml), ferric sulfate (18 µg/ml).

Table 2.1.   List of bacterial strains used in this study

Strain	Genotypes and relevant characteristics	Source/reference
S. aureus		
K40	Wild-type strain isolated from patient in Auckland City Hospital	Stephen Ritchie, unpublished
K40-SCV1	SCV mutant derived from K40 with the induction of 20 µg/ml kanamycin, colony 1	This work
K40-SCV2	SCV mutant derived from K40 with the induction of 20 µg/ml kanamycin, colony 2	This work
K40-SCV3	SCV mutant derived from K40 with the induction of 20 µg/ml kanamycin, colony 3	This work

Strain	Genotypes and relevant characteristics	Source/reference
K40-SCV4	SCV mutant derived from K40 with the induction of 10 μg/ml gentamycin, colony 1	This work
K40-SCV5	SCV mutant derived from K40 with the induction of 10 μg/ml gentamycin, colony 2	This work
K40-SCV6	SCV mutant derived from K40 with the induction of 10 μg/ml gentamycin, colony 3	This work
H59	Wild-type strain isolated from patient in Auckland City Hospital	Stephen Ritchie, unpublished
H59-SCV1	SCV mutant derived from H59 with the induction of 20 μg/ml kanamycin	This work
H59-SCV2	SCV mutant derived from H59 with the induction of 5 μg/ml gentamycin	This work
E182	Wild-type strain isolated from patient in Auckland City Hospital	Stephen Ritchie, unpublished
E182-SCV1	SCV mutant derived from E182 with the induction of 20 μg/ml kanamycin	This work
E182-SCV2	SCV mutant derived from E182 with the induction of 5 μg/ml gentamycin	This work
P. aeruginosa		
PAO1	A derivative of the original Australian PAO isolate	Stover et al. 2000
PAO1-SCV1	SCV mutant isolated from a 7-days static culture of PAO1, colony 1	This work
PAO1-SCV2	SCV mutant isolated from a 7-days static culture of PAO1, colony 2	This work

Strain	Genotypes and relevant characteristics	Source/reference
PAO1-SCV3	SCV mutant isolated from a 7-days static culture of PAO1, colony 3	This work
MU49-14	PAO1 Δ*wg8*Δ*pelF*Δ*pscA*, defective in alginate, Pel and Psl biosynthesis	Ghafoor et al. 2011
P. fluorescens		
SBW25	Wild-type strain isolated from phyllosphere of sugar beet at Oxford University farm	Bailey et al. 1995
LSWS	Spontaneous mutant of SBW25 with the overproduction of cellulose	Spiers et al. 2003
MU49-25	LSWS containing a neutral, chromosomal *lacZ* marker	Zhang and Rainey, 2007
MU49-17	SBW25 with deletion of the *wss* operon (Δ*wss*), defective in cellulose production	Rainey et al. 2003
CA	SBW25 Δ*mvaT* with overproduction of the colanic acid (CA) capsule	Philippe Remigi, unpublished
FS	Spontaneous mutant of SBW25 with the overproduction of lipopolysaccharide (LPS)	Rainey and Travisano, 1998
MU49-21	SBW25 Δ*fuz* defective in LPS production	Ferguson et al. 2013
MU49-22	SBW25 Δ*fliA*, a flagella nonproducing mutant	Xue-Xian Zhang, unpublished
MU49-23	SBW25 Δ*pilG*, a type IV pili nonproducing mutant	Rainey and Bailey, 1996
MU49-24	SBW25 carrying deletion of *fliA* and *pilG* (Δ*fliA*Δ*pilG*), a flagella and Type IV pili nonproducing mutant	Xue-Xian Zhang, unpublished

(SCV= Small Colony Variant; LSWS = Large Spreading Wrinkly Spreader; CA = Colanic Acid capsulated strain; FS = Fuzzy Spreader)

2.2 Laboratory Media

The LB media were prepared according to the recipes shown in Table 2.2. They were sterilized by autoclaving at 121°C for 20 min. Medium supplements such as ferrous sulphate and copper sulphate were filter-sterilized and added when the LB agars were cooled to 55°C in a water bath. Bacterial strains were permanently stored at -80°C freezer in a 2-ml plastic tube containing 1 ml of bacterial culture plus 0.8 ml of glycerol saline. The glycerol saline is composed of NaCl (8.5 g) and Glycerol (700 ml) per liter.

Table 2.2   Laboratory media used in this study

LB broth	Supplements	Application
Tryptone (10 g/L) Yeast Extract (5 g/L) Sodium Chloride (10 g/L)	Agar (18 g/L)	Agar plates
	Congo Red (0.004%)	SCV selection and identification
	Fluorescent Brightener 28 (35 µg/ml) Ferric Sulfate (18 µg/ml)	Fluorescent staining
	X-Gal (60 µg /ml)	Identification of strains marked with *lacZ*

2.3 Copper materials

Pure copper (99.9% Cu) and brass (C27200, half hard) sheets were purchased from Wakefield Metals Ltd, Auckland, New Zealand. Brass is a yellowish alloy containing 63.5% copper and 36.5% zinc. It possesses higher strength and antioxidant properties when compared with pure copper. In terms of corrosion resistance, brass has high resistance to atmospheric corrosion as well as brackish water, seawater, and non-oxidizing acid corrosion. Additionally, brass is much cheaper than pure copper. Thus, brass can potentially be used within facilities of public hospitals.

The pure copper and brass sheets were cut into 1 cm x 1 cm coupons and stored in ethanol (>99.8%) at room temperature. When needed, the surfaces were mechanically polished using 1200-grit silicon carbide (SiC) paper, and degreased ultrasonically in acetone before use.

2.4 Isolation of small colony variants (SCVs)

The SCVs of *S. aureus* can arise *in vivo* and *in vitro* following exposure to aminoglycoside antibiotics (Von Eiff et al., 2000). In this work, spontaneous SCV mutants were isolated by treatments of Gem and Km. Briefly, single colonies of wild-type *S. aureus* were inoculated into 5 ml of LB broth containing either Gem or Km at the following final concentrations: 5, 10, 15, 20, 25 µg/ml. Bacterial growth was checked by visual inspection over a period of 3 days after inoculation. Bacterial cultures

were then subjected to dilute-plating for single colonies on LB agar plates supplemented with 0.004% Congo Red (Table 2.2). SCV mutants can be easily distinguished from the wild-type strain as they form small dark red colonies. To check stability of the SCV phenotype, few SCV mutants were randomly picked up and streaked onto LB plus Congo Red plates for 3 times before they were permanently stored at glycerol saline in -80°C freezer.

The SCV mutants of *P. aeruginosa* PAO1 were obtained by growing the wild-type strain under static conditions for 7 days. The resultant cultures were mixed by vortexing and subsequent dilute-plating onto LB agar plates with 0.004% Congo Red. SCVs formed intense dark red colonies, whereas the ancestral wild-type strain gave rise to brownish colonies. Moreover, colonies of SCVs are relatively larger than those of wild type. Three candidate SCVs were randomly picked up and their stability was tested by transferred three times on LB agar plates plus 0.004% Congo Red.

2.5 Bacterial sensitivity to copper ions

Sensitivity of *Pseudomonas* and *S. aureus* strains to ionic copper was determined for cells grown in LB agar plates supplemented with varying concentrations of $CuSO_4$. A stock solution of 100 mM was prepared by adding 2 g of $CuSO_4$ into 80 ml of water, which was sterilized by passing through a 0.22 μm pore filter membrane. Appropriate amount of 100 mM $CuSO_4$ solution was added into a Petri dish and then mixed through with 20 ml LB agar.

To prepare the bacterial inoculants, 1 ml overnight culture of each strain in LB broth was spun down and then re-suspended into the same amount of sterile water. After diluting by 10-times, 10 μl cell solutions (~10,000 cells) were dropped onto LB agar plates containing $CuSO_4$. Of note, two agar plates were tested for each copper concentration in order to minimize experimental errors. Bacterial growth was monitored daily by photographing over a period of 5 days at 28°C for *P. fluorescens* or 37°C for *P. aeruginosa* and *S. aureus*. The minimal inhibitory concentration (MIC) was considered to be the minimum concentration tested at which no colony formation was observed after 3 days incubation.

2.6 Bacterial contact killing assay on copper

Contact killing assay was performed using a so-called wet inoculation method (outlined in Figure 2.1). Briefly, strains stored in -80°C freezer were inoculated onto LB plates with 0.004% Congo Red for single colonies. Then, a single colony was inoculated into 5 ml LB broth for each bacterial strain and grew overnight at either 28°C or 37°C. The inoculant cells were then prepared by spinning down 1 ml culture and re-suspending into the same amount of sterile water.
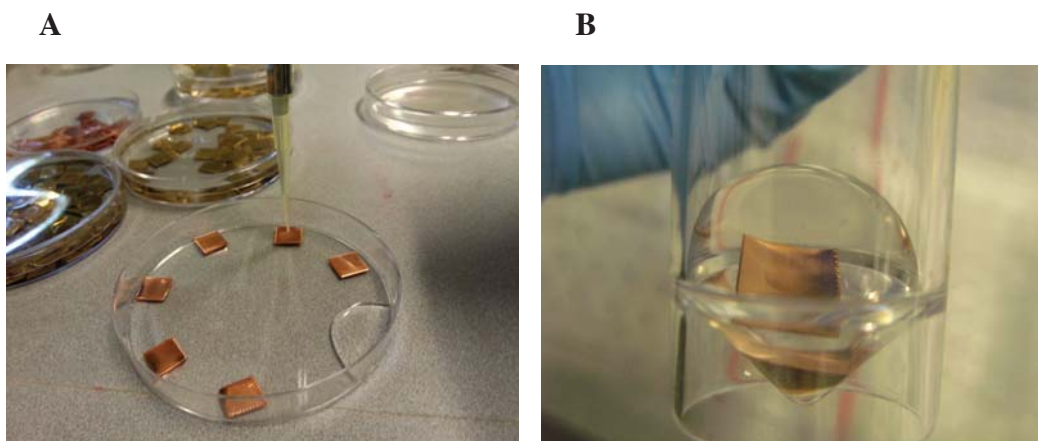
Figure 2.1   Demonstration of the wet incubation method. A. Twenty microliter of bacterial solution were added to the surface of each copper coupon. **B.** A copper coupon with bacteria was put into a plastic tube containing 2 ml of sterilized water. After cells were released by vortexing, the coupon was removed with a sterile inoculation loop.

For each treatment, 20 μl of the above-prepared bacterial solution was dropped onto the surface of a copper coupon (1 cm × 1 cm) in a Petri dish and left at room temperature (~22°C) for given period of time. A total of 6 coupons (i.e. 6 repeats) were inoculated for each strain at each time point. To enumerate viable cells on copper, each coupon was transferred into a 30 ml sterile plastic tube containing 2 ml sterile water. After vigorously vortexing for 30 s, the copper coupon was removed with the help of a sterile inoculation loop, and the resultant bacterial solution was then subjected to serial 10-fold dilution in sterile water. Finally, 100 μl of each dilution was inoculated in a LB agar plate and colonies were counted after 2-days incubation.

The rate of contact killing is expressed as death rate (DR), which is calculated using the following equations:

- Death Rate (DR) = (A - B) / A × 100%

- A= $N_0 \times$ DF $\times$ 10 $\times$ 0.02 (original, i.e.: time zero)

- B= $N_t \times$ DF $\times$ 10 $\times$ 2 (experimental time point)

Where, N_0 is the number of colonies counted in plates at time zero (original); N_t is the number of colonies counted in plates at desired time point; DF is dilution factor.

2.7   Fluorescent microscopic analysis

Bacterial cells subjected to fluorescent microscopic analysis were treated with Calcofluor White (CFW) staining, using Fluorescent Brightener 28 purchased from Sigma-Aldrich. CFW can bind to β(1-3) and β(1-4) polysaccharides, which are commonly found in extracellular polymers such as cellulose. Bacterial cells were prepared by growing on LB plates containing Fluorescent Brightener 28 (35 μl /ml) and ferric sulfate (18 μl /ml). Ferric sulfate was added in order to inhibit the production of pyoverdin, a fluorescent iron-chelating siderophore. Pyoverdin will interfere the fluorescence signals produced by CFW-stained EPS. Cells in a single colony were immobilized in ~ 1 ml 1.5% agarose pad on a microscope slide. Bacterial cells were then examined under an upright fluorescence microscope (Olympus BX61) with an emission wavelength of 477 nm and excitation wavelength of 436 nm. The images were analysed using the Image J program.

2.8   Scanning Electron Microscopy (SEM) analysis

SEM analysis of *P. aeruginosa* and *S. aureus* cells were performed using standard procedures by Ms. Niki Murray at the Manawatu Microscopy and Imaging Centre, Massey University, Palmerton North. Briefly, bacterial cells from LB agar plates were fixed in modified Karnovsky's fixative (3% glutaraldehyde, 2% formaldehyde in phosphate buffer, pH 7.2) for at least 8 hours. The samples were spun down at 4000 rpm for 4 min. Each pelleted solution was clamped between two membrane filters (0.4μm, Isopore, Merck Millipore LTD) in an aluminium clamp and processed with three times in phosphate buffer washes (15 min each) then dehydrated in a graded series of ethanol (25%, 50%, 75%, 95%, and 100%) for 15 min each and a final 100% for 1 hour. Next, the samples were subject to critical point during using liquid CO_2 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 a FEI Quanta 200 scanning electron microscope at an accelerating voltage of 20kV.

2.9   Microtiter plate biofilm formation assay

Quantifying biofilm formation of *P. aeruginosa* and *S. aureus* cells were performed by using standard procedures from George A. O'Toole, Microbiology and Immunology, Dartmouth Medical School (George, 2011). Briefly, strains stored in -80°C freezer were inoculated onto LB agar plates. After two-days incubation, cells were gathered into a 1.5 ml tube containing 1 ml LB broth. The original cell density of each tube was

standardized by adjusting to an OD_{600} value ~2.5, which was similar to cell density of overnight culture. Then, 100 µl of bacterial solution of each strain was added into a 96-cell microtiter plate and incubated for 18 hours at 37°C, allowing biofilm to form. Eight replicates were preformed for each treatment.

After incubation, cells were dumped out then gently washed with water. This could help to remove those unattached cells and to lower the background straining. Next, 125 µl of 0.1% Crystal Violet (CV) were added into each cell well of microtiter plate. After 15 min, the excess cells and dye were ridded by rinsing the plate 3 times with water. Then plates were allowed to dry overnight. To be followed, 125 µl of 30% acetic acid were added to each cell well of microtiter plate to solubilize CV. After incubated at room temperature (~22°C) for 15 min, solutions were transferred to a new microtiter plate. Absorbance at 550 nm was measured using a plate reader. 30% acetic acid was used as the blank.

2.10   Experimental evolution of bacterial resistance to metallic copper

This work was performed using LSWS marked with *lacZ* (MU49-25, LSWS-*lacZ*). LSWS is a naturally occurring derivative of non-pathogenic *P. fluorescens* SBW25, a model organism used in many experimental evolution studies. The LSWS mutant rather than wild-type SBW25 was selected as the ancestral strain of the evolutionary experiment. This was because LSWS over-produces an acetylated form of cellulose, thus expressing the similar phenotype as the SCV mutants. The LSWS mutant was

marked with a promoterless *lacZ*, which forms blue colonies on LB agar plates supplanted with X-Gal (60 μg /ml), which allows for rapid identification of the evolving bacterial strains, ensuring no contamination.

As outlined in Figure 3.12A (see Results), eight independent evolutionary cell lines were set up for copper treatment. Bacterial cells from overnight culture were washed once with sterile water, and 20 μl was added onto the surface of a brass coupon (1 cm × 1 cm) in a Petri dish and left at room temperature (~22°C) for 1 hour. The brass coupon was then transferred into a 30 ml plastic tube containing 2 ml LB broth. Survived cells (~10,000 in total) were released into the medium by vortxing (~30 seconds at maximum speed) and grown overnight for the next round of contact killing treatment. As a control, the LSWS-*lacZ* strain has been subjected to the same treatment but without the brass-mediated contact killing (Figure 3.12B, see Results).

During the process of experimental evolution, bacterial strains were examined every 10 daily transfers. First, bacteria were streaked onto LB plus X-Gal plates to check if there are any possible microbial contaminations. Second, identity of the *P. fluorescens* strains were further confirmed by polymerase chain reaction (PCR) using a pair of strain-specific primers, xutA-compF and xutR-lacZF, which amplify a 300 bp DNA fragment of *xutA* gene for xylose utilization in *P. fluorescens* SBW25. Third, resistance to metallic copper was determined on the surface of brass for 1 hour, using the method described above. Fourth, the bacterial cultures were stored in glycerol saline at -80°C for further genotypic and phenotypic characterizations.

2.11 PCR and agarose gel electrophoresis

A 300 bp DNA fragment of *xutA* gene was amplified by PCR using primers xutA-compF and xutR-lacZF (Liu et al., 2015), with Taq DNA polymerase purchased from Invitrogen (Auckland). PCR was performed in a final volume of 50 μl in a reaction containing 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 pmol/μl of each primer, and 1 unit of Taq DNA polymerase. Bacterial cells in overnight culture of LB broth were spun down and washed once using sterile water, and then 5 μl was used as template DNA for PCR. The PCR reaction was prepared using stock solutions shown in Table 2.3. The 10 mM dNTP stock solution was prepared by mixing each of the four dNTP purchased from Bioline at the concentration of 100 mM. PCR reactions were carried out in a gradient thermal Palm-Cycler™ (Corbett Life Science) and the temperature profiles are shown in Table 2.4.

Table 2.3   Reagents in 50μl of PCR reaction

Reagent	Volume (μl)	Final Concentration
10x Buffer	5.0	1x
$MgCl_2$ (50 mM)	1.5	1.5 mM
dNTP (10 mM)	1.0	0.2 mM
Forward Primer "xutA-compF" (10 pmol/μl)	1.0	0.2 pmol/μl
Reverse Primer "xutR-lacZF" (10 pmol/μl)	1.0	0.2 pmol/μl
Taq DNA Polymerase (5 U/μl)	0.2	1U
Template DNA	5.0	1-5 ng per 50μl
MilliQ H_2O	35.3	-

Table 2.4   Typical PCR reaction conditions

Stage	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1x
Denaturation	94°C	45 s	30x
Annealing	56°C	45 s	
Elongation	72°C	1 min per kb	
Final Elongation	72°C	10 min	1x
Hold	4°C	-	-

Amplified DNAs were separated in 1% agarose gel containing 1x SYBR Safe™ DNA gel stain (Invitrogen). Gel electrophoresis was performed in 1x TBE buffer at 140 volts using the ready-to-use Lambda DNA/EcoRI plus HindIII molecular weight marker (Thermo Fisher Scientific New Zealand Ltd). DNA was visualized under UV lights generated from a High Performance Ultraviolet Transilluminator, and photographed using the DigiDoc-It™ Imaging System equipped with the Doc-It LS Analysis Software.

CHAPTER THREE: RESULTS

3.1 Isolation of small colony variants (SCVs) of nosocomial pathogenic bacteria

A primary goal of this project is to test the copper ion burst release hypothesis (outlined in Figure 1.5), which posits that cell surface components are responsible for rapid bacterial killing on metallic copper. To this end, we isolated and characterized SCVs derived from the two most common nosocomial pathogenic bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. SCV mutants overproduce cell surface polymers such as exopolysaccharides (EPS), which can potentially react with metallic copper releasing more and more toxic copper ions. If true, the SCV mutants will be more rapidly killed than their respective isogenic strains on copper surfaces.

3.1.1 SCVs of *Staphylococcus aureus*

S. aureus is a Gram-positive coccal bacterium responsible for a wide range of hospital-acquired infections (HAIs), including skin and soft tissue infections, bacteremia, sepsis, endocarditis, and pneumonia (Lowy, 1998). SCVs are naturally occurring slow-growing subpopulations with increased resistance to antibiotics and also an enhanced ability to form biofilms. Under laboratory conditions, SCVs can be isolated by growing *S. aureus* strains in the presence of antibiotics such as gentamicin (Gem)

and kanamycin (Km). They form small dark red colonies on LB agar plates supplemented with 0.004% Congo Red, which can be easily distinguished from colonies of wild-type cells (Von Eiff et al., 2000).

Three *S. aureus* clinical isolates - namely K40, H59 and E182 - have been subjected to SCV isolation using the method of Gem and Km induction (Table 2.1). For strain K40, three SCVs were randomly picked up from cells grown in LB containing 10 μg/ml Gem, and another three were isolated from LB with 20 μg/ml Km. Thus, a total of six K40-derived SCVs were obtained and further characterized in this work. For each of the *S. aureus* strains H59 and E182, one representative SCV has been selected from LB cultures treated with 5 μg/ml Gem and 20 μg/ml Km, respectively.

Figure 3.1 shows the colony morphology of SCV mutant "K40-SCV1" in comparison with the wild-type strain K40 (of note, plate photos for all the ten SCV mutants are available in Appendix 1-3). In LB plate with 0.004% Congo Red, it took three days for the SCV mutant K40-SCV1 to form visible colonies with smaller size and darker red color when compared with wild-type strain. This indicates that K40-SCV1 grows more slowly and also produces more cell surface polymers than the wild type.
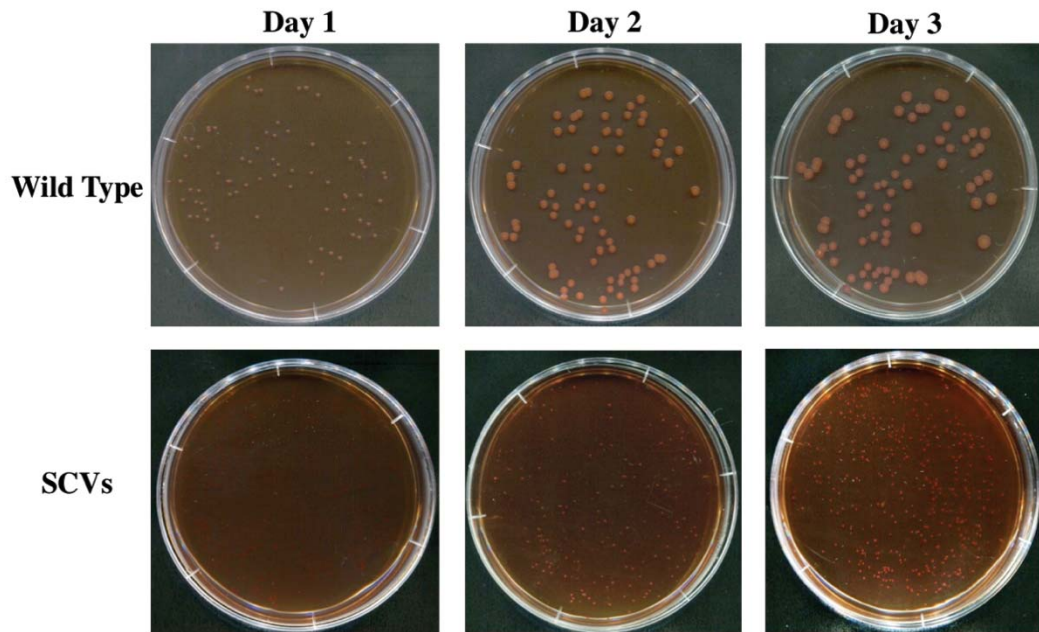
Figure 3.1   Colonies formed by wild type and SCV mutants of *S. aureus*. Wild-type *S. aureus* K40 and its derived small colony variant K40-SCV1 were incubated in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C.

3.1.2   SCVs of *Pseudomonas aeruginosa*

P. aeruginosa is a rod-shaped Gram-negative bacterium ubiquitously found in environment such as soil and water, but it can cause serious infections in immunocompromised patients (Bodey et al., 1983). It is one of most common nosocomial pathogens responsible for a wide range of HAIs. *P. aeruginosa* is the main causative agent of pulmonary infections in people with chronic lung diseases, particularly cystic fibrosis (CF). SCVs are a naturally evolved antibiotic-resistant subpopulation during the process of chronic infection (Malone, 2015).

In this work we isolated SCVs of *P. aeruginosa* PAO1 by growing the bacteria under

static conditions for 7 days at 37°C. Three SCVs were randomly picked up from LB agar plates supplemented 0.004% Congo Red. They formed distinct small colonies with dark red color as reported in literature (Malone, 2015). A representative plate image is shown in Figure 3.2, and plate images for all three SCVs are available in Appendix 4-6.
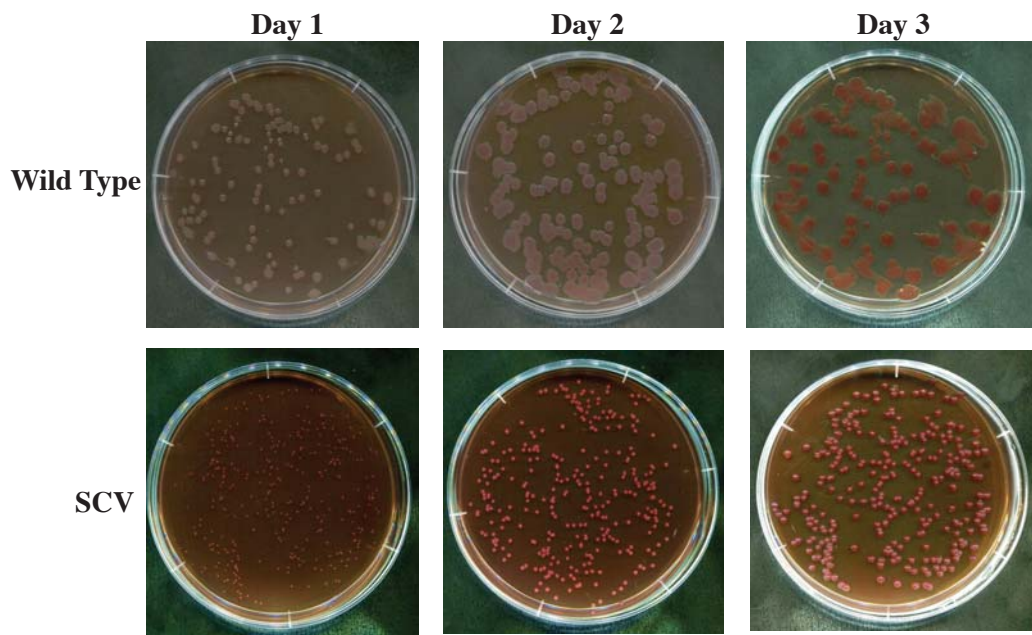

Figure 3.2   Colonies formed by wild-type *P. aeruginosa* and a derived SCVmutant.
Wild-type *P. aeruginosa* PAO1 and the small colony variant SCV1 were grown in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C.

3.2   Phenotypic characterization of the SCV mutants

3.2.1   Fluorescent microscopic analysis

The SCV mutants formed dark red colonies on LB plates with 0.004% Congo Red, suggesting that they overproduce extracellular surface polymers. To further confirm this

phenotype, we stained bacterial cells with Fluorescent Brighter (specifically, Calcofluor White) making them visible under fluorescent microscope. A representative image of SCV cells is shown in Figure 3.3, and results for all *S. aureus* and *P. aeruginosa* SCVs are available in Appendix 7 and Appendix 8, respectively. Consistent with our expectation, these SCV mutants had a much stronger fluorescent intensity than the related wild-type cells, as a result of EPS overproduction.

A. *S. aureus*

Wild Type   **SCV**
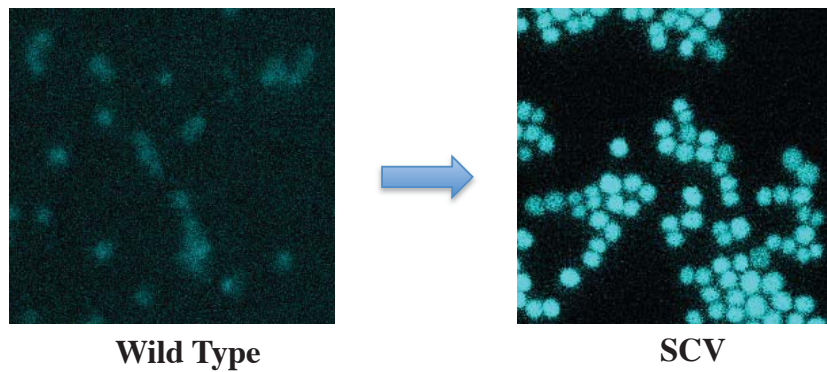
B. *P. aeruginosa*

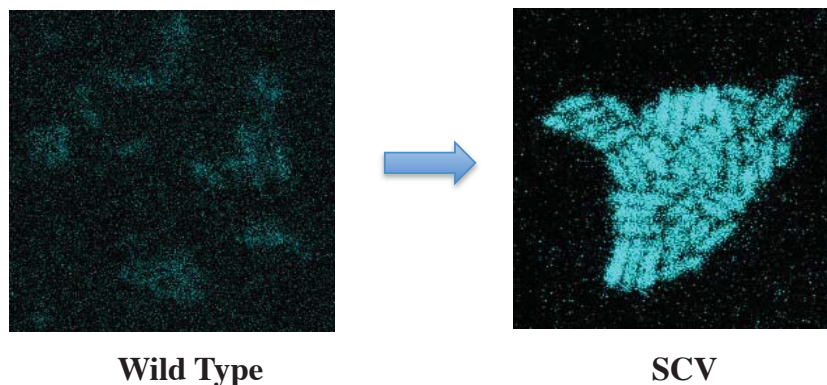
Wild Type   **SCV**

Figure 3.3 Fluorescent microscope images of *S. aureus* (A) and *P. aeruginosa* (B). Wild type and SCV mutants were grown on LB plates containing Calcofluor White (CFW) Fluorescent Brightener 28 (35 µl /ml) and Ferric Sulfate (18 µl /ml). Cells were then examined under an upright fluorescence microscope with an emission wavelength of 477 nm and excitation wavelength of 436 nm.

3.2.2 Scanning Electron Microscopy (SEM) analysis

To further analyze cell surfaces of the SCV mutants, both *S. aureus* and *P. aeruginosa* wild-type strains and their derived SCVs were subjected to scanning electron microscope (SEM) analysis. Representative SEM images were shown in Figure 3.4 (and images for all strains are available in Appendix 9 and Appendix 10 for *S. aureus* and *P. aeruginosa*, respectively). Those SEM images clearly indicate that SCV mutants had the similar cell shapes as their wild-type ancestral strains, but there were some extracellular polymers or cell debris found only in SCV cell clusters.
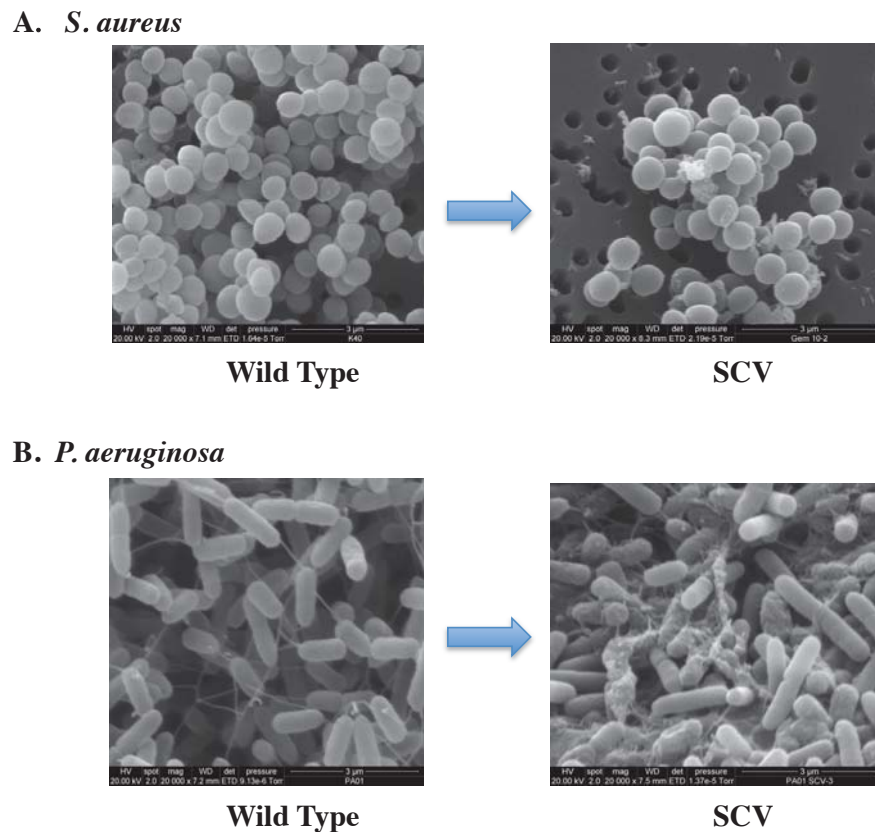
A. *S. aureus*

Wild Type   **SCV**

B. *P. aeruginosa*

Wild Type   **SCV**

Figure 3.4   Representative scanning electron microscope images of *S. aureus* (A) and *P. aeruginosa* (B). Wild type and SCVs were prepared from colonies grown on LB agar plates for three days at 37℃.

3.2.3 Quantifying biofilm formation

Many studies have shown that SCVs have an enhanced ability to form biofilms owing to the overproduction of EPS (Häußler et al., 2003; Kirisits et al., 2005). To test if this holds for the SCV mutants evolved in vitro in this work, we compared biofilm formation capacities between wild type and SCVs using the method of crystal violet (CV) staining (see details in the Materials and Methods). Data of absorbance at the wavelength of 550 nm with a representative image of the microtiter plate are shown in Figure 3.5. Images of the CV-stained microtiter plates for all *S. aureus* and *P. aeruginosa* strains are available in Appendix 11. The results clearly show that SCV mutants formed more biofilms than related wild-type strains as indicated by the A_{550} values. No significant difference was observed among individual SCVs (significance level: 0.05).
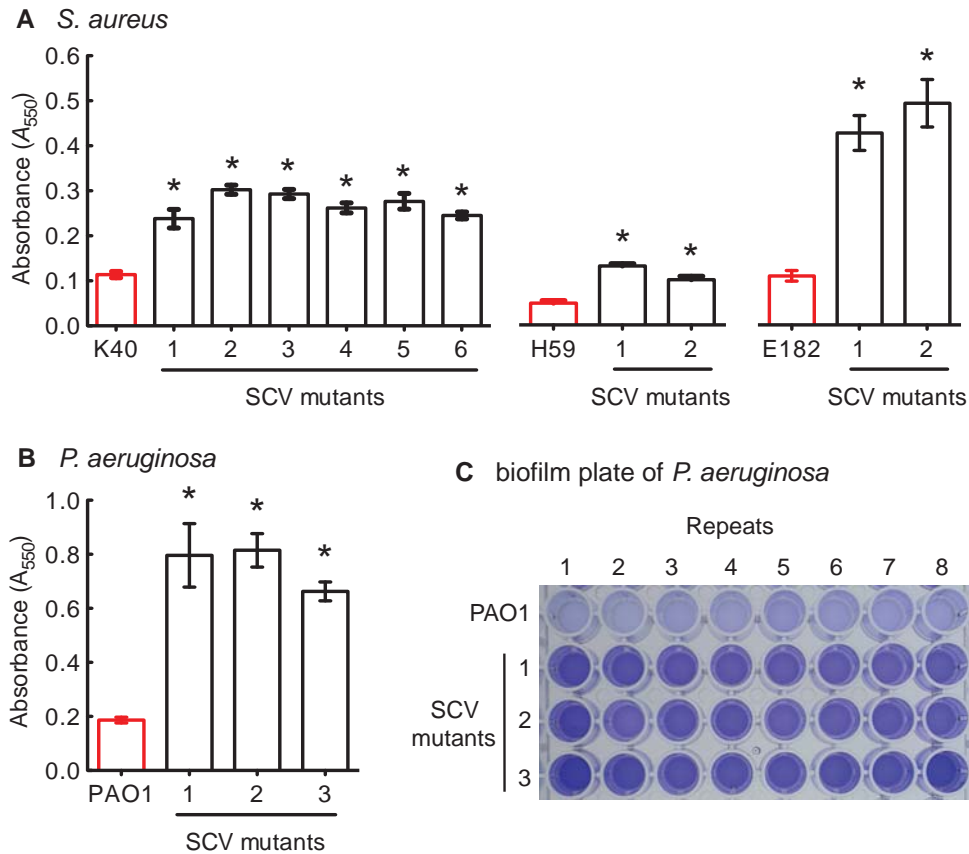
Figure 3.5   Quantification of biofilms formed by SCV mutants of *S. aureus* and *P. aeruginosa*. Biofilms were stained with 0.1% crystal violet (CV), and the dye was solubilized with 30% acetic acid. The absorbance was measured at the wavelength of 550 nm for both *S. aureus* (**A**) and *P. aeruginosa* (**B**), and wild-type strains are indicated by red bars. Data are means and standard errors of eight replicates. Asterisks denote significant difference ($P < 0.05$). **C.** A CV-stained microtitre plate used for absorbance measurement.

3.3   Bacterial contact killing assays on copper surfaces

3.3.1   Assaying the rates of bacterial contact killing on the surfaces of pure copper versus brass

Previous works have shown that both pure copper and copper alloys have intrinsic antimicrobial activities and the efficiency of contact killing is proportional to copper

content of the surface materials (S. Warnes & Keevil, 2011). To confirm these findings, we compared the rates of bacterial contact killing on two copper materials: pure copper (99.9% Cu) and brass (63.5% Cu); and the assays were performed with wild-type *S. aureus* K40 and *P. aeruginosa* PAO1. Results are shown in Figure 3.6. Consistent with expectation, both strains were more rapidly killed on pure copper than that on brass. More specifically, at 10 min after exposure 82.28±1.79% and 37.09±6.88% *S. aureus* K40 cells were killed on the surfaces of pure copper and brass, respectively (Figure 3.6A). Similar for *P. aeruginosa* PAO1 at the 10-min time point, 99.19±0.12% and 62.47±4.36% cells were killed on the surfaces of pure copper and brass, respectively (Figure 3.6B).
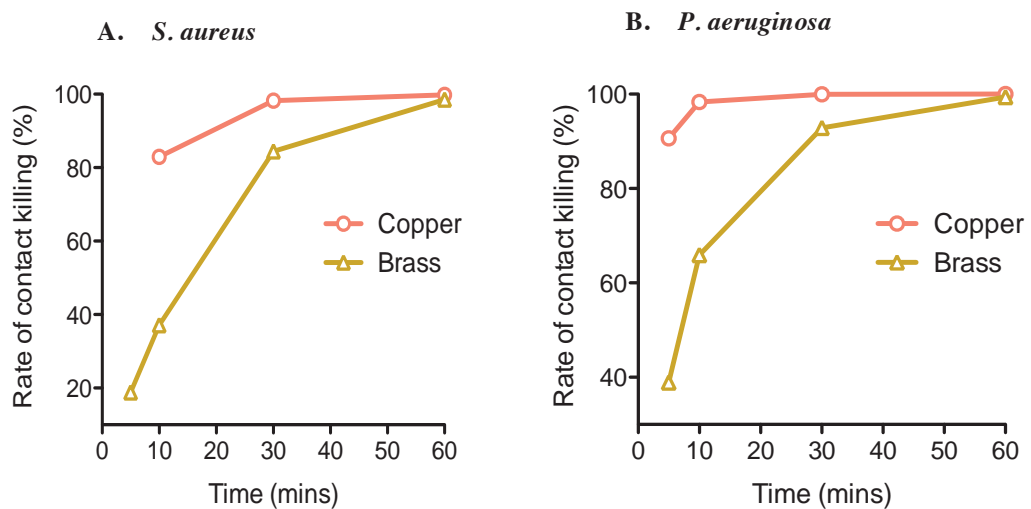

Figure 3.6 A comparison of antimicrobial properties between pure copper and brass.
Contact killing assays for *S. aureus* K40 (**A**) and *P. aeruginosa* PAO1 (**B**) were performed using the wet inoculation method as described in Materials and Methods. Rate of contact killing is expressed as percentage of cells killed within a given period of time. Data are means and standard errors of six replicates.

3.3.2 Comparing copper susceptibilities between wild-type *S. aureus* and *P. aeruginosa*

Data presented above in Figure 3.6 suggested that the Gram-positive *S. aureus* K40 was more resistant to copper-mediated contact killing than the Gram-negative bacterium *P. aeruginosa* PAO1. For example, after 10 min contacts with the surfaces of pure copper, 82.28±1.79% *S. aureus* K40 cells were killed, whereas the death rate was 99.19±0.12% for *P. aeruginosa* PAO1. However, the experiment was designed to test the effects of copper materials; thus, the assays for *S. aureus* K40 (Figure 3.6A) and *P. aeruginosa* PAO1 (Figure 3.6B) were performed separately.

To more precisely compare the rates of contact killing between *S. aureus* K40 and *P. aeruginosa* PAO1, we assayed the two strains in parallel on the surfaces of brass. Results shown in Figure 3.7 clearly indicate that *P. aeruginosa* PAO1 was more rapidly killed than *S. aureus* K40. Significant differences were found at the time points of 5, 10 and 30 min of contact on brass ($P < 0.05$).
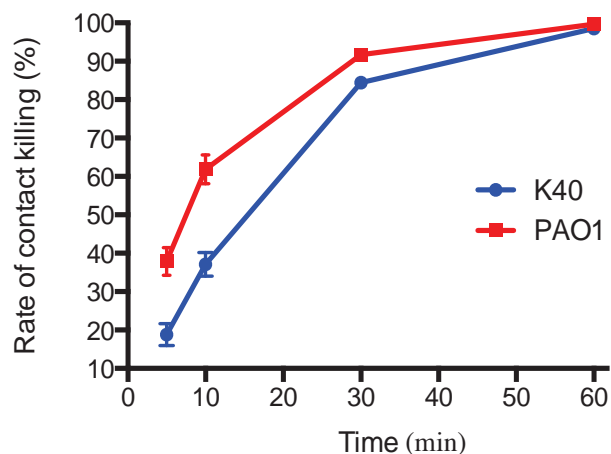
Figure 3.7   A comparison of copper-mediated contact killing between Gram-positive *S. aureus* K40 and Gram-negative *P. aeruginosa* PAO1. The contact killing assays were performed using the wet inoculation method and the rate of contact killing was calculated as percentage of cells killed within a given period of time. Data are means and standard errors of six replicates.

3.3.3   Comparing the rates of bacterial contact killing between wild type and SCV mutants on the surfaces of pure copper

Armed with the knowledge of how wild-type *Pseudomonas* and *Staphylococcus* strains are killed on copper surfaces, we proceeded to compare the rates of bacterial contact killing between wild-type strains and their derived SCV mutants. The assays were first performed on the surfaces of pure copper (99.9% Cu) during a one-hour period of contact. The full dataset is available in Appendix 12-13 for *S. aureus* and Appendix 14-15 for *P. aeruginosa*. In general, the SCV mutants are more rapidly killed by copper when compared with their respective wild-type strains. This is what we expected from the copper ion burst release hypothesis (outlined in Figure 1.5). For *S. aureus* strains, significant differences were found between wild type and SCVs at the time point of 10

min ($P < 0.05$): ~ 80% of wild-type cells were killed whereas the rate of killing for SCVs were approximately 90% (Figure 3.8A). Similar results were obtained for *P. aeruginosa* PAO1 and its three derived SCVs (Figure 3.8B). After 5 min exposure on copper ~ 97% SCVs were killed, which was significantly higher than the ~90% killing rate of the wild-type strain ($P < 0.05$).

It should be noted that stainless steel (74% Fe, 18% Cr, 8% Ni) was included as a negative control in this experiment, as it has no antimicrobial activities. Results showed that the number of bacterial cells remained at similar levels during the process of copper-mediated contact killing (Appendix 13, Appendix 15, and Appendix 18).

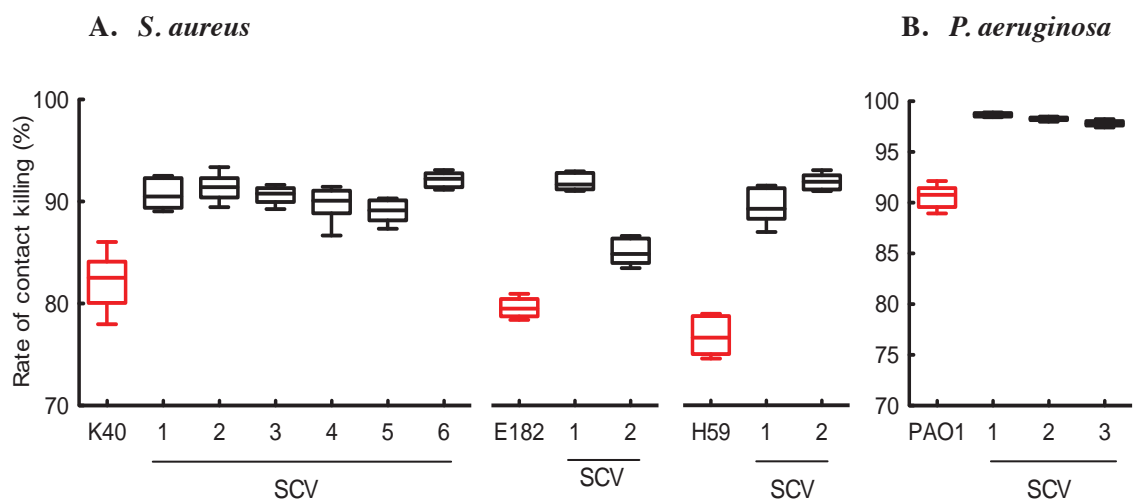**A. *S. aureus***　　　　　　　　　　　　　　　　　**B. *P. aeruginosa***

Figure 3.8　Rates of contact killing on the surfaces of pure copper between wild-type *S. aureus* (A) and *P. aeruginosa* (B) and their derived SCV mutants. The contact killing assays were performed with six replicates using the wet inoculation method and the rate of contact killing was calculated as percentage of cells killed after 10 and 5 minutes contact for *S. aureus* and *P. aeruginosa*, respectively. The line in the middle of the box represents the median; the bottom and top boxes represent the 25th and 75th percentiles, respectively; and the lower and upper whiskers indicate the smallest and the largest values, respectively.

3.3.4 Comparing the rates of bacterial contact killing between wild type and SCV mutants on the surfaces of brass

Contact killing assays were also performed on the surfaces of brass which contains 63.5% Cu (and 37.5% Zn). The data of 5 min are shown in Figure 3.9 and details are in Appendix 16-19. Significant but smaller difference was found between wild type and SCVs when compared with that on pure copper. The killing rate of *S. aureus* was ~18% and 30% for wild type and SCVs, respectively. The values were increased to ~37% and 50% for the wild-type and SCVs of *P. aeruginosa*, respectively. No significant difference was found among SCVs evolved from the same wild-type ancestor for both *S. aureus* and *P. aeruginosa*.

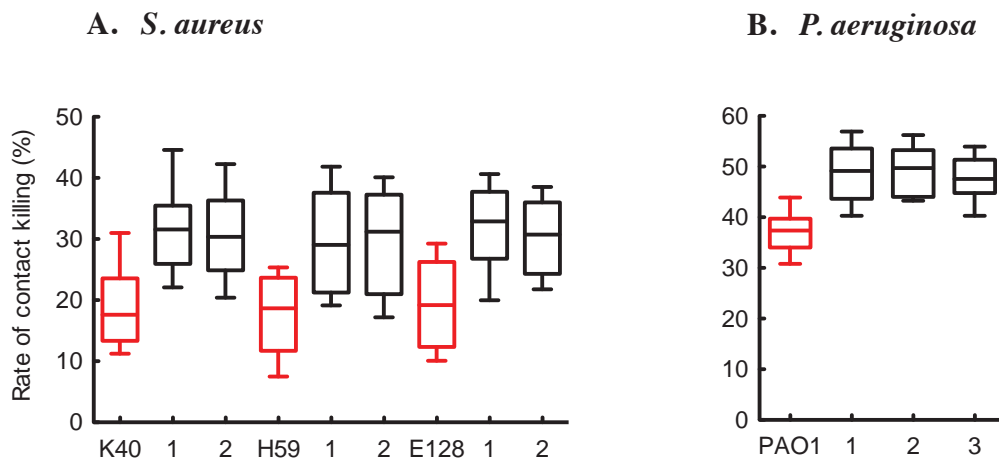**A. *S. aureus***

B. *P. aeruginosa*

Figure 3.9   Rates of contact killing on the surfaces of brass between wild-type *S. aureus* (A) and *P. aeruginosa* (B) and their derived SCV mutants. The contact killing assays were performed with six replicates using the wet inoculation method and the rate of contact killing was calculated as percentage of cells killed after 5 minutes contact. The line in the middle of the box represents the median; the bottom and top boxes represent the 25th and 75th percentiles, respectively; and the lower and upper whiskers indicate the smallest and the largest values, respectively.

Taken together all the contact killing data presented above, we can conclude that SCV mutants are more susceptible to contact killing on the surfaces of pure copper and brass. This is consistent with predictions made from the copper ion burst release hypothesis.

3.4   Assessing the roles of specific surface polymers in copper-mediated contact killing

Next, we sought to determine the roles of specific surface polymers in copper-mediated contact killing, including EPS, lipopolysaccharide (LPS), pili, flagella and colanic acid (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*. A total of eight mutants were tested in this work, which over-produce (or defective in producing) one of these cell surface polymers. Results on the surfaces of copper and brass are shown in Figure 3.10 and details are available in Appendix 20.

It is interesting to note that mutants overproducing LPS and the extracellular cellulose polymers are more sensitive to copper-mediated contact killings, whereas mutant cells overproducing the CA capsule are more resistant to contacting killing on copper. After 10 min contact on the surfaces of pure copper 89.81±1.66% wild-type cells were killed; however, the killing rates for mutants overproducing CA capsule, cellulose and LPS were 72.81±4.31%, 95.10±0.34% and 94.61±1.79%, respectively, which are significantly different from that of wild-type cells ($P < 0.05$). No significant difference

was detected for mutants defective in the production of these extracellular polymers as well as flagella and pili.
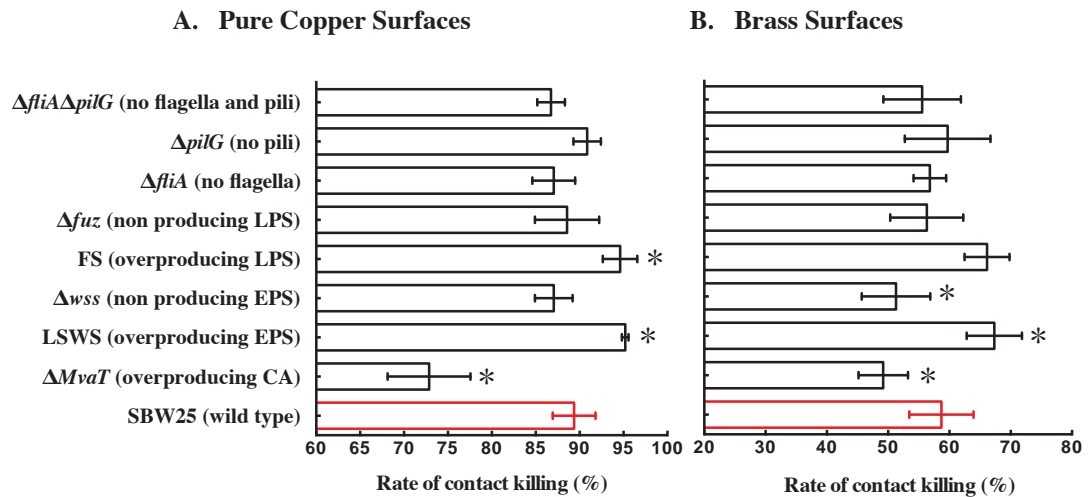
A.   Pure Copper Surfaces   **B.   Brass Surfaces**

Figure 3.10   Rates of contact killing between SBW25 WT and mutants on pure copper (A) and brass (B). Contact killing assays were preformed on *P. fluorescens* SBW25 WT (red bar) and mutants (black bar) respectively. Overnight cultures were wet inoculated on pure copper coupons (left hand side) and brass coupons (right hand side), after contact 10 min, demonstrating the changes of death rates (%) over time (min). Asterisks denote significant difference compared with wild-type SBW25 ($P < 0.05$). Strains included in the assays: wild-type SBW25; SBW25 Δ*mvaT* with overproduction of the colanic acid (CA) capsule; LSWS (Large Spreading Wrinkly Spreader), spontaneous mutant of SBW25 with the overproduction of exopolysaccharides (EPS), especially cellulose; SBW25 with deletion of the *wss* operon (Δ*wss*), defective in EPS, especially cellulose production; FS (Fuzzy Spreader), spontaneous mutant of SBW25 with the overproduction of lipopolysaccharide (LPS); SBW25 Δ*fuz* defective in LPS production; SBW25 Δ*fliA*, a flagella nonproducing mutant; SBW25 Δ*pilG*, a type IV pili nonproducing mutant; SBW25 carrying deletion of *fliA* and *pilG* (Δ*fliA*Δ*pilG*), a flagella and Type IV pili nonproducing mutant.
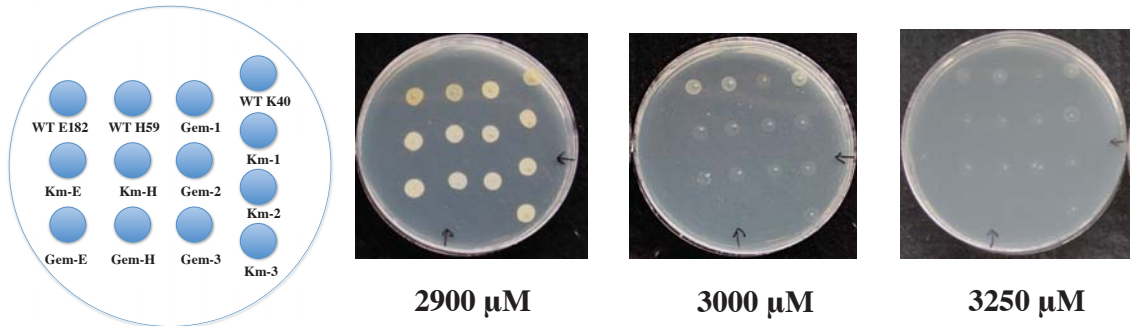
3.5   Bacterial sensitivity to copper ions

To estimate the contribution of copper toxicity in bacterial contact killing on copper, we determined the minimal inhibitory concentration (MIC) of all *S. aureus*, *P. aeruginosa* and *P. fluorescens* strains described above. The results are summarized in Table 3.2 with representative plate photos shown in Figure 3.11. For both *S. aureus* and *P. aeruginosa*, the SCV mutants were more sensitive to copper ions when compared with their wild-type ancestors.   However, all *P. fluorescens* mutants displayed the similar levels of resistance to copper ions with a MIC value of 3200 μM, except the CA capsulated cells (MIC, 3300 μM).

Table 3.1   Sensitivity of wild-type bacteria and the derived mutants to copper ions when grown in LB agar plates. Asterisks denote significant difference compared with its original wild-type strain ($P < 0.05$).
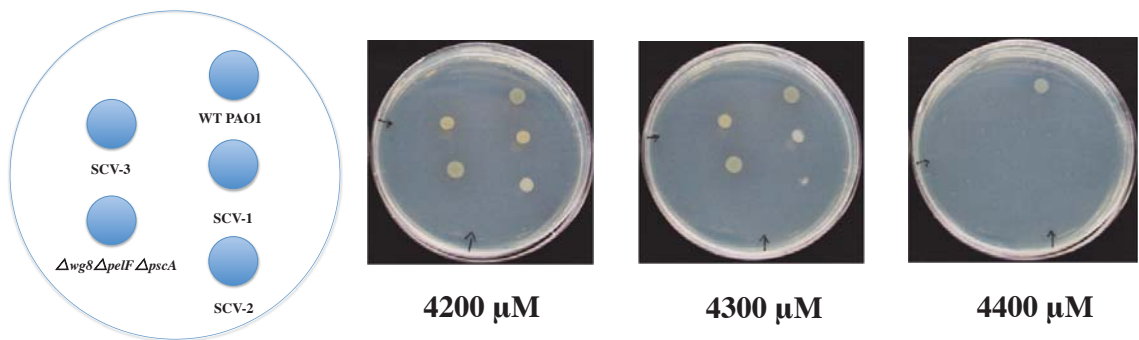
Strain	Genotype and relevant Characteristics	Resistance to ionic copper (MIC, μM)	Resistance to metallic copper (10-min survival rate, %)
S. aureus			
K40	Wild type	3250	17.72±1.79
K40-SCV1	SCV of K40	3000	9.28±1.28*
K40-SCV2	SCV of K40	3000	8.60±1.21*
K40-SCV3	SCV of K40	3000	10.93±1.03*
K40-SCV4	SCV of K40	3000	9.35±0.77*
K40-SCV5	SCV of K40	3000	10.20±1.53*
K40-SCV6	SCV of K40	3000	7.85±0.65*
H59	Wild type	3250	23.21±1.63
H59-SCV1	SCV of H59	3000	10.47±1.56*
H59-SCV2	SCV of H59	3000	8.05±0.70*
E128	Wild type	3250	20.39±0.88
E128-SCV1	SCV of E128	3000	9.24±0.74*
E128-SCV2	SCV of E128	3000	12.98±1.12*
P. aeruginosa			
PAO1	Wild type	4500	0.81±0.12
PAO1-SCV1	SCV of PAO1	4400	0.47±0.11*
PAO1-SCV2	SCV of PAO1	4300	0.55±0.11*
PAO1-SCV3	SCV of PAO1	4400	0.49±0.09*
MU49-14	$\Delta wg8\Delta pelF\Delta pscA$, defective in alginate, Pel and Psl biosynthesis mutant derived from wild-type PAO1	4400	1.99±0.36*

Strain	Genotype and relevant Characteristics	Resistance to ionic copper (MIC, μM)	Resistance to metallic copper (10-min survival rate, %)
P. fluorescens			
SBW25	Wild type	3200	10.19±1.66
LSWS	Spontaneous mutant overproducing cellulose	3200	4.80±0.34*
MU49-17	Δwss, cellulose non-producing mutant derived from wild-type SBW25	3200	12.94±1.96
CA	ΔmvaT, a colanic acid over-producing mutant of SBW25	3300	27.14±4.31*
FS	Fuzzy spreader, a spontaneous mutant of SBW25 overproducing LPS	3200	5.39±1.79*
MU49-21	ΔfuZ, a LPS nonproducing mutant of SBW25	3200	11.43±3.34
MU49-22	ΔfliA, a SBW25 mutant defective in flagella production	3200	12.93±2.23
MU49-23	ΔpilG, a SBW25 mutant defective in pili production	3200	9.13±1.43
MU49-24	ΔfliAΔpilG, a SBW25 mutant defective in flagella and pili production	3200	13.25±1.44

A. *S.aureus*

			WT K40
WT E182	WT H59	Gem-1	
			Km-1
Km-E	Km-H	Gem-2	
			Km-2
Gem-E	Gem-H	Gem-3	
			Km-3

2900 μM   3000 μM   3250 μM

B. *P. aeruginosa*

WT PAO1

SCV-3

SCV-1

△wg8 △pelF △pscA

SCV-2

4200 μM   4300 μM   4400 μM

C. *P. fluorescens*

WT SBW25

LSWS

△wss

△MvaT

3100 μM   3200 μM   3300 μM
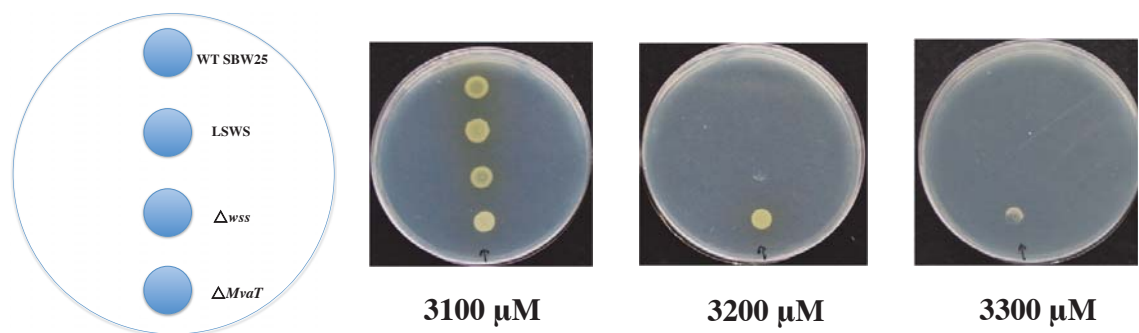
Figure 3.11   Typical photos for the sensitivity of *Pseudomonas* and *S. aureus* strains to ionic copper. Minimal inhibitory concentration (MIC) units were determined on LB agar plates supplemented with varying concentrations of CuSO$_4$ (μM) after incubated three days.

3.6   Exploring the potential of bacterial pathogens to develop resistance to metallic copper

To assess the potential of bacterial pathogens to develop resistance to copper, we subjected a non-pathogenic strain of *P. fluorescens* to a long-term experimental evolution experiment. The ancestor MU49-25 was naturally evolved from *P. fluorescens* SBW25 because of its ability to over produce extracellular polymers, (specifically, cellulose) (Spiers et al., 2003). It was marked with a promoterless *lacZ*, and thus, it forms blue colonies on LB agar plates supplanted with X-Gal. This character was used for strain identification during the process of experimental evolution, ensuring that there was no contamination (Figure 3.13). Additionally, PCR amplification of a strain specific gene *xutA* for xylose utilization to verify the strain identity (Figure 3.14).

As outlined in Figure 3.12, the experiment started by growing the ancestral *P. fluorescens* strain in 2 ml Luria-Bertani (LB) medium in 8 replicates, and 20 μl of the washed cells will be applied to brass (63.5% Cu, Treatment A) for 60 min. According to our results from a proof-of-concept study, this initial copper treatment regime will kill 99.96% of cells. Next, the viable cells (~10,000) will then be transferred into 2 ml fresh LB medium for next round of contact killing on the following day. Treatment B was set up as a control for bacterial adaptation in the LB medium, which will be preformed in parallel with Treatment A but without selection on metallic copper.

Bacterial cultures of every 10 transfers were stored in glycerol stock at -80°C freezer, and their ability to survive from bacterial contact killing on brass for 60 min was

measured. Results are shown in Figure 3.15 (Graph for Whiskers analysis is available in Appendix 21). Survival rates of strains from the control line (without copper treatment) remained the similar levels during the whole process of evolution. In the contrast, the survival rate of cells in the copper treatment line increased from 0.035±0.001% to 0.968±0.305% during the first 30 transfers, and further increased to 2.793±0.194% from the 40th transfer. Surprisingly, no further increase was observed over the course of 40th to 100th daily transfers. The fact that ~97% cells can still be killed within 10 min contact after 100 rounds of contact killing on brass strongly suggests that bacteria have very limited ability to develop resistance to metallic copper.
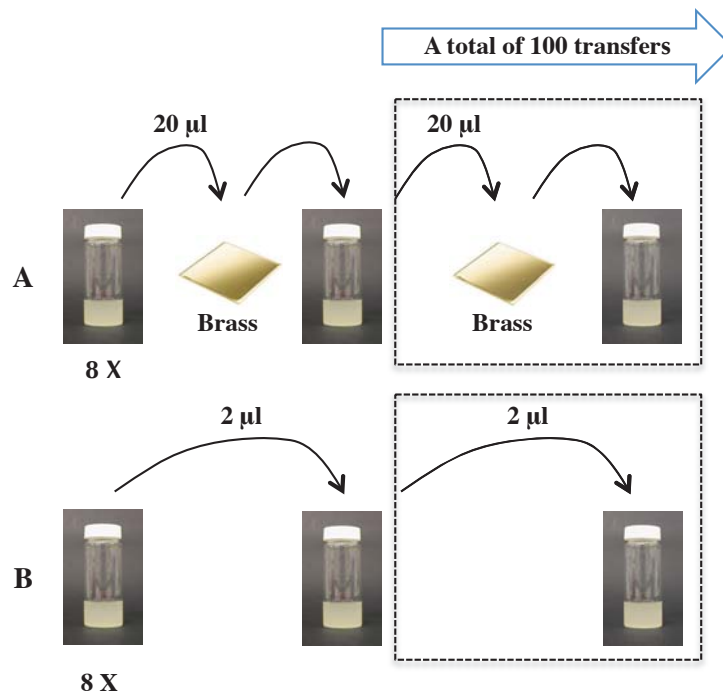
Figure 3.12   Outline of the proposed mutation accumulation experiment.   A. For each transfer, bacterial cells grown in 5 ml LB medium will be washed once with sterile water, and then 20 μl added onto a brass coupon. After 60 min treatment at room temperature, all cells on the coupon will be suspended into fresh LB medium (2 ml). The survived cells will be able to re-grow and subject to the round of treatment on the following day.   **B.** A control without the treatment of contact killing on copper.

Copper Treatment   **Non-Copper Treatment**
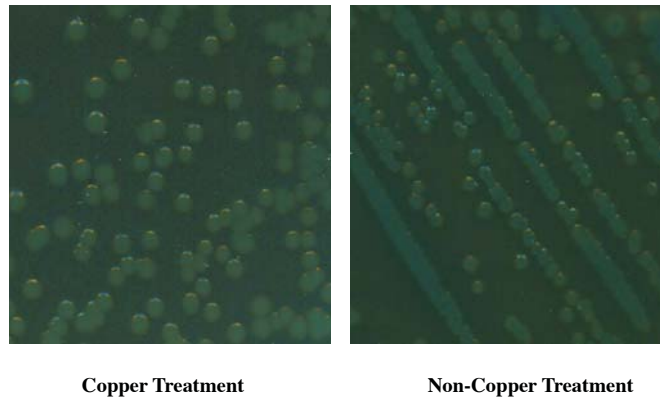
Figure 3.13   Blue colonies formed by the 100th evolutional MU49-25 (LSWS-*lacZ*) strain used in the evolutionary experiment. Serial dilutions were performed with copper treatment cultures up to 10^{-6} then inoculated onto LB plus X-Gal (60 µg /ml) plates. Incubated at 37°C for two days. Non-copper treatment cultures were inoculated onto X-Gal plates by streaking.
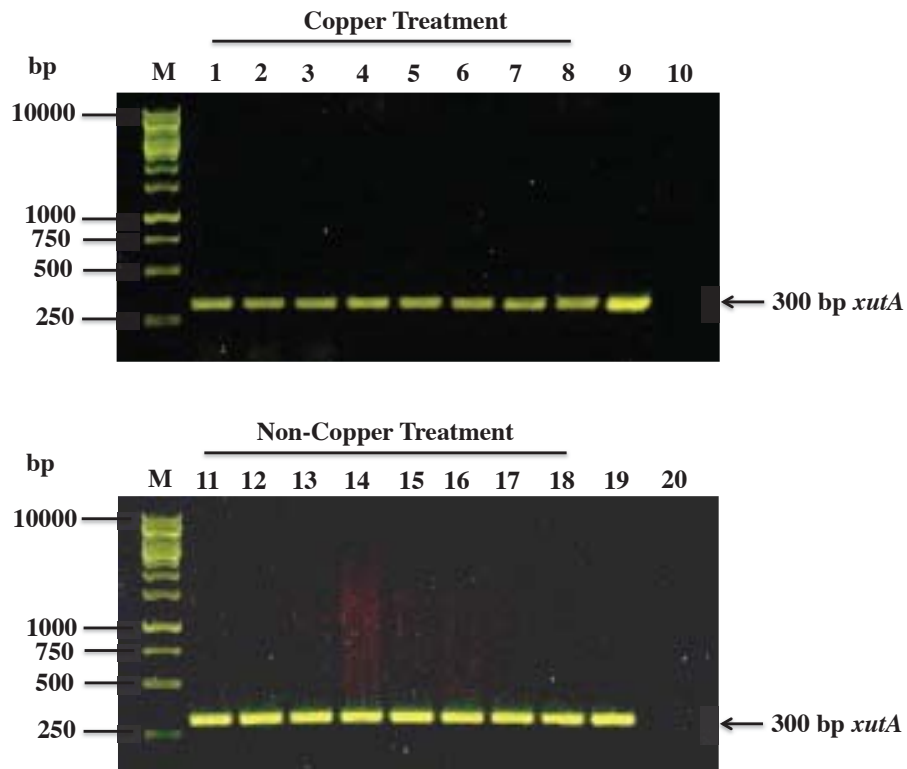

Figure 3.14   PCR verification of *P. fluorescens* SBW25-specific *xutA* gene for xylose utilization.   The *xutA* gene was amplified by PCR using primers xutA-compF and xutR-lacZF. Gel electrophoresis was performed in 1x TBE buffer at 130 volts using Lambda DNA/EcoRI plus HindIII molecular weight marker (1kb). DNA was visualized under UV lights. **Lane M**, DNA molecular weight marker. **Lane 1-8**, copper-treatment cultures 1 to 8. **Lane 11-18**, non-copper-treatment cultures (control group) 1 to 8. **Lane 9 and Lane 19**, wild-type SBW25 genome DNA. **Lane 10 and Lane 20**, negative control.
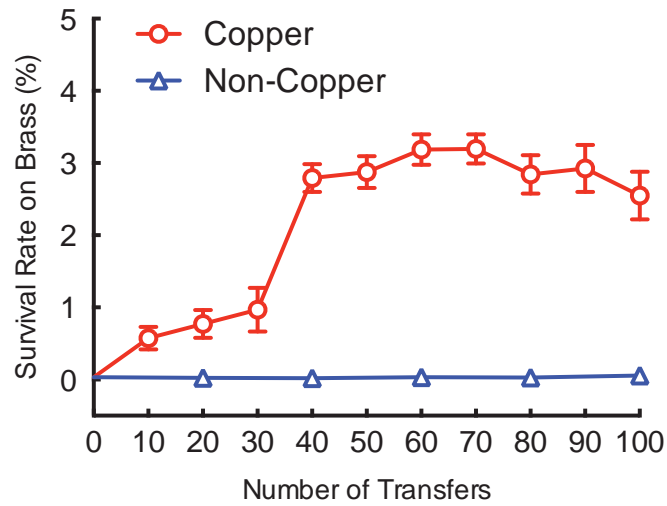
Figure 3.15   Dynamic changes in bacterial resistance to copper. A non-pathogenic strain of *P. fluorescens* SBW25 was subjected to daily passage of sub-lethal (1-h copper treatment) conditions on the surface of brass, and changes of survival rates were monitored every 10 days. As a control, the LSWS-*lacZ* strain was subjected to the same treatment but without the brass-mediated contact killing. The changes of survival rates were monitored every 20 days.

CHAPTER FOUR: DISCUSSION

4.1 Isolation and characterization of small colony variants (SCVs)

SCVs are commonly found in chronic infections caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the two most common opportunistic nosocomial pathogens. They grow slowly and show high resistance to antibiotics. Morphologically, SCVs over produce extracellular polysaccharides (or exopolysaccharides, EPS), which differ in chemical and structural composition among different species (Straight & Kolter, 2009) as well as environmental conditions. The EPS produced by *P. aeruginosa* SCVs is typically composed of three major polymeric substances, namely Pel, Psl and alginate (Franklin et al., 2011). Pel and Psl contain glucose, mannose and rhamnose in their structures.

SCVs can be generated in vitro using different methods, including prolonged growth (Bui et al., 2015), induction by aminoglycoside antibiotics (Von Eiff et al., 2000), growth under static conditions (Hall-Stoodley & Stoodley, 2005) as well as site-directed mutagenesis of *hem* genes for hemin biosynthesis (Vaudaux et al., 2011). SCVs isolated independently using different methods may differ greatly in terms of EPS composition, and they do not necessarily express the same phenotypes as those naturally evolved SCVs.

In this work, at least two SCVs were isolated for each of the *S. aureus* and *P. aeruginosa* wild-type strains. While the *S. aureus* SCVs were induced by gentamycin (Gem) and kanamycin (Km) treatment, SCVs of *P. aeruginosa* were obtained via growth under static conditions. We showed that these SCVs overproduce EPS, as indicated by data of colony morphology in agar plate containing 0.004% Congo Red and fluorescent staining with Calcofluor White (CFW), and an enhance ability to form biofilms. However, the chemical nature of EPS produced by those SCV mutants has not been determined. Such information would be very helpful for a comprehensive understanding of SCVs, but it is not essentially required for interpretation of our contact killing data. The copper ion burst release hypothesis is not limited to specific types of EPS, rather than EPS in general plus many other cell surface components.

4.2   Antimicrobial properties of copper-containing surface materials

Copper is often used in combination with other elements, such as zinc and tin, in order to increase its hardness and corrosion resistance. Both copper and copper alloys possess intrinsic antimicrobial activities, and in general, their antimicrobial efficiencies are proportional to their copper contents (Champagne & Helfritch, 2013). In this work, we have performed the contact killing assays in two copper materials: pure copper (99.9% Cu) and brass. Brass is one of the most commonly used copper alloys, which contains 63.5% Cu and 36.5% Zn. Given the lower copper content in brass, it was not surprising to notice that brass has lower antibacterial activities than pure copper. The rate of

bacterial contact killing was ~45% and ~37% lower on brass than on pure copper for *S. aureus* and *P. aeruginosa,* respectively. However, the difference was found only at the time point of 10 min. After 60 min, no significant difference was observed between pure copper and brass. All bacterial cells were killed after 24 h on the surfaces of both pure copper and copper alloys. Finally, it should be noted that oxidized copper also possesses antimicrobial activities, but the rate of bacterial killing is lower compared with non-oxidized copper and copper alloys (Karpanen et al., 2012). We have not specifically designed any experiments to examine the precise effects of surface oxidation on bacterial killing. However, it is very important to emphasize that we have carefully prepared the copper surfaces for contact killing assays in order to minimize the effects caused by surface oxidization (see details in Materials and Methods).

In addition to copper content, environmental factors such as temperature and moisture also affect the antimicrobial efficiency of copper materials (Michels et al., 2009). In general, bacteria are more rapidly killed under dry conditions when compared with wet conditions (Espirito Santo et al., 2008). Therefore, there are two general inoculation methods reported in literature for assaying bacterial contact killing on copper: wet inoculation and dry inoculation method (S. Warnes & Keevil, 2011). Using the dry inoculation method, bacterial cells can be killed within a timeframe of few seconds (Espirito Santo et al., 2008). In this work, we needed to compare the rates of bacterial killing between wild type and its derived mutants; thus, it is not appropriate to use the dry inoculation method, and it would be technically very difficult to perform the contact killing assays within seconds. We choose the wet inoculation method whereby ~90%

cells were killed with 5 to 10 min.

Gram-positive bacterial cells have a thick cell wall structure with multiple layers of peptidoglycan, whereas the cell wall of Gram-negative cells is relatively thin and possess few layers of peptidoglycan. Consequently, Gram-positive bacterial cells have higher resistance to environmental stress than Gram-negative cells. Previous work showed that it took a longer time to kill Gram-positive bacteria than Gram-negative bacteria on copper surfaces (Grass et al., 2011). Our own data show that the bacterial killing rate of *S. aureus* K40 and *P. aeruginosa* PAO1 on the surfaces of pure copper for 10 min were 82±1.79% and 99±0.12%, respectively. Thus, our data support the previous finding that Gram-negative bacteria are more susceptible to copper surfaces than Gram-positive bacterial cells.

4.3   Mechanisms of copper-mediated contact killing

Cell surface components are the first line of defense when bacterial cells contact with the metal surface, but their roles in copper-mediated contact killing have not been elucidated before (Espirito Santo et al., 2008). Data presented in this thesis clearly show that mutants over producing extracellular polymeric substances are more rapidly killed on the surfaces of pure copper and brass. The results provide genuine support for the proposed copper ion burst release hypothesis. These cell surface substrates contain nitrogen, sulphate and phosphate in their structure, which can potentially react with

copper releasing more and more toxic copper ions that will eventually kill bacterial cells (Vu et al., 2009). Some polysaccharides have been shown to be capable of binding copper ions (Chen et al., 2011).

There has been no doubt that copper ions play a major role in the process of bacterial contact killing on copper; but the still unresolved questions are (1) where the copper ions are originated from, and furthermore, (2) to what extent bacterial resistance to ionic copper help bacterial cell survive on metallic copper. The first question has been answered here by the copper ion burst release hypothesis, which implicates that the accumulated copper ions are originated from chemical reaction between EPS and metallic copper. To answer the second question, we compared the data of minimal inhibitory concentration (MIC) and the rates of bacterial contact killing on pure copper (Table 3.1). In most cases, strains with higher MIC have higher survival rate on copper. However, for some strains the MIC scores of copper ions are not positively correlated with resistance to metallic copper. For example, the triple deletion mutant defective in Pel, Psl and alginate production has a significantly higher resistance to metallic copper, but it has lower MIC to copper ions than the wild-type strain (Table 3.1). The LSWS mutant overproducing cellulose has a higher resistance to metallic copper, but it does not differ from the wild-type strain in terms of MIC for copper ions. These data strongly suggest that bacteria were not fully killed by toxic copper ions. Other damaging factors should be considered, particularly the cellular damages caused by physical interactions between bacterial cells and copper surfaces.

4.4   Evolution of bacterial resistance to metallic copper

Copper is a precious metal that has been domestically used for thousands years. However, there is a low chance that resistant pathogenic strains could be evolved with increased use of copper in hospital environments; and in a worst-case scenario resistance to metallic copper is associated with resistance to antibiotics and antiseptics, causing failure of current antibiotic treatments and hospital hygiene controls. Additionally, introducing copper surfaces to items commonly touched in public healthcare systems will certainly involve a significant investment. Therefore, it is critically important to assess the potential of bacterial pathogens to develop resistance to metallic copper. In this work, we have taken a unique approach to examining the evolutionary effects of long-term contacts with copper surfaces on bacterial resistance to metallic copper as well as resistance to antibiotics and antiseptics. While this work is currently ongoing, our primary data from a total of 100 daily transfers indicate that bacteria have very limited ability to evolve resistance to metallic copper.

CHAPTER FIVE: CONCLUSION AND FUTURE RESEARCH

Cell surface components play critical roles in copper-mediated contact killing of bacterial pathogens

Data of contact killing presented in this thesis indicate that the rate of bacterial killing on copper is largely determined by surface components of a bacterial cell. Specifically, bacterial cells with enhanced production extracellular surface polymers were more rapidly killed on the surfaces of pure copper and brass. Thus, our data support the copper ion burst release hypothesis that bacterial cells are predominantly killed by a burst release of toxic copper ions, resulting from chemical reactions between surface components of a bacterial cell and metallic copper.

Small colony variants (SCVs) can be isolated *in vitro*

While testing the copper ion burst release hypothesis, we have successfully isolated SCVs of both *S. aureus* (K40, H59, and E182) and *P. aeruginosa* (PAO1), with the former being isolated by gentamicin (Gem) and kanamycin (Km) treatments, the later from 7-day static cultures. Phenotypic characterization showed that all SCVs are slow growing cell overproducing cell surfaces polymers. All SCVs have enhanced abilities to form biofilms on microtiter plates. It is very likely (but not experimentally tested here)

that these SCVs isolated *in vitro* have the similar levels of virulence as the naturally evolved SCVs.

Bacteria have limited ability to evolve resistance to metallic copper

Copper and copper alloys possess intrinsic antimicrobial activities and thus, have a great potential in reducing the risk of hospital-acquired infections (HAIs) when used in commonly touched places of hospital facilities such as door handle, pushing plate and toilet seats. However, the mechanisms of copper-mediated contact killing are currently not fully understood (Hans et al., 2016). Consequently, there is a serious concern regarding the emergence of copper-resistant pathogenic strains and the possible link between bacterial resistance to copper and resistance to antibiotics and antiseptics currently used in hospitals.

In this work, we have subjected the non-pathogenic model bacterium – *P. fluorescens* SBW25 – to a long-term experimental evolution, whereby cells were daily treated with metallic copper for a period of 100 days. Our primary data show that the evolved strains have a slight increase of resistance to metallic copper; however, ~97% of evolved cells can still be effectively killed after exposure on the surfaces of brass. Therefore, our data strongly suggest that bacterial pathogen have limited ability to develop resistance to metallic copper surfaces.

Future research:

The copper ion burst release hypothesis highlights the importance of bacterial surface materials in bacterial contact killing on copper. They react with metallic copper releasing more and more toxic copper ions, which eventually kill bacteria. In this thesis, we have tested this new hypothesis using bacterial strains with altered production of extracellular substrates such as exopolysaccharides (EPS), lipopolysaccharide (LPS) and flagella and pili, including SCV mutants of *P. aeruginosa* and *S. aureus*. Our data of contact killing on the surfaces of both pure copper and brass are consistent to expectation made from the copper ion burst release hypothesis. However, direct evidence on chemical reactions between bacterial surface materials (EPS, LPS, flagella and pili) and metallic copper are still lacking. Therefore, future work will first involve the monitoring of copper ions after bacterial cells or purified bacterial surface materials are added to copper surfaces. Concentration of ionic copper can be measured by using the standard method of inductively coupled plasma mass spectrometry (ICP-MS) (Mathews et al., 2013).

Secondly, it will be extremely helpful to visualize the cellular damages caused by metallic copper, and examine the cellular distribution of copper ions in the copper damaged cells. This can potentially be achieved using the scanning electron microscope coupled with an energy dispersive x-ray spectroscopy (EDAX) module, which is available in the Manawatu Microscopy and Imaging Centre of Massey University.

Finally, in this work we have used a unique approach of long-term experimental evolution to examine the capability of bacterial pathogens to develop resistance to metallic copper. While promising results have been obtained in this thesis, the experimental evolution work has been performed only for 100 days in total due to time constraint of my Master's study. It would certainly be helpful that this experiment be extended to a longer period of at least one year (for example, a total of 500 days) Therefore, it is important to note that the long-term experimental evolution work initiated in this study is currently ongoing in Xue-Xian's lab. According to our original design, the evolved strains at the end of this experiment (e.g., 500 daily transfer) will be subjected to extensive genotypic and phenotypic characterizations. More specifically, genome re-sequencing will be used to identify the mutations accumulated during the course of evolution, following by characterization of their functionalities. It is anticipated that such genomic and genetic data will provide valuable clues how bacteria are rapidly killed on copper, in addition to comments on the potential emergence of copper-resistant strains. More importantly, we will examine resistance to antibiotics and antiseptics of the evolved strains and explore their possible correlations with resistance to metallic copper. Together, the obtained data will greatly help increase our understanding of the mechanisms of bacterial contact killing and the potentials of bacterial resistance to antimicrobial copper surfaces.
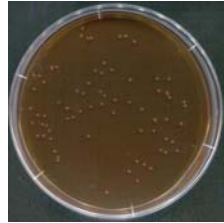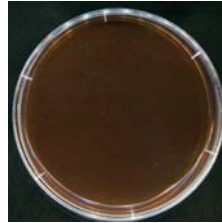
Day 1

K40 (Wild Type)   **K40-SCV1**   **K40-SCV2**
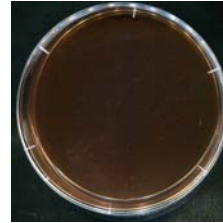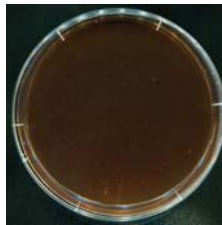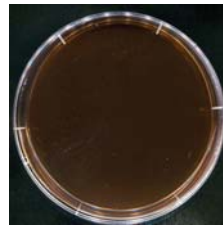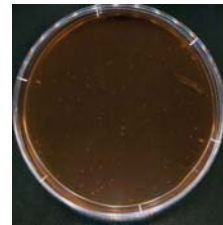
K40-SCV3   **K40-SCV4**   **K40-SCV5**   **K40-SCV6**
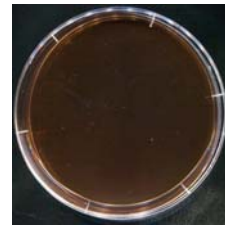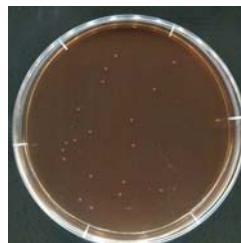
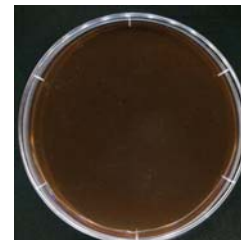**H59 (Wild Type)**   **H59-SCV1**   **H59-SCV2**
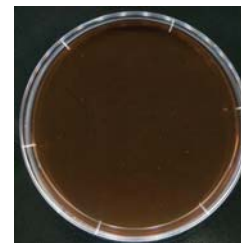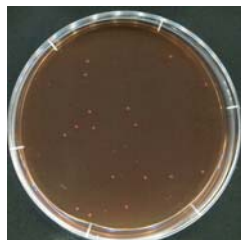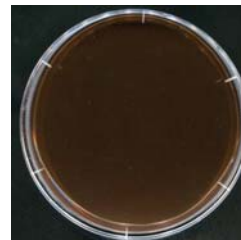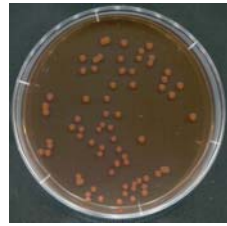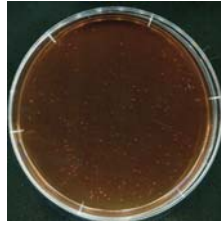
E128 (Wild Type)   **E128-SCV1**   **E128-SCV1**

Appendix 1   Colonies formed by wild type and SCV mutants of *S. aureus* after one day. Wild-type *S. aureus* K40 and its derived small colony variants were incubated in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C.

Day 2

K40 (Wild Type)   **K40-SCV1**   **K40-SCV2**
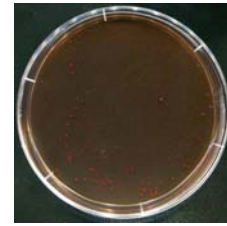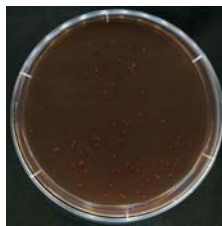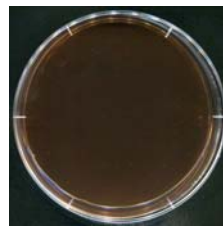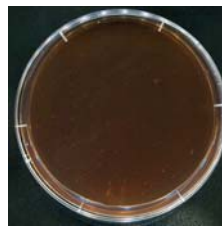
K40-SCV3   **K40-SCV4**   **K40-SCV5**   **K40-SCV6**
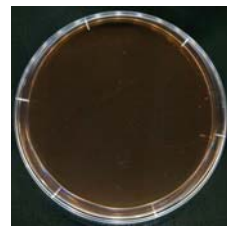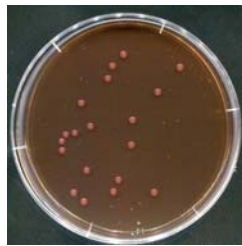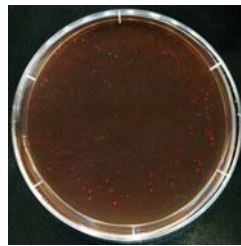
H59 (Wild Type)   **H59-SCV1**   **H59-SCV2**

E128 (Wild Type)   **E128-SCV1**   **E128-SCV1**

Appendix 2   Colonies formed by wild type and SCV mutants of *S. aureus* after two days. Wild-type *S. aureus* K40 and its derived small colony variants were incubated in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C**.**

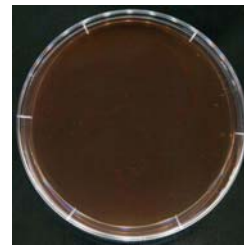**Day 3**

K40 (Wild Type)   K40-SCV1   K40-SCV2
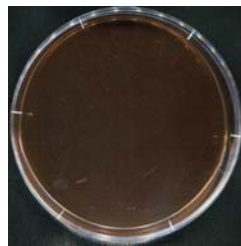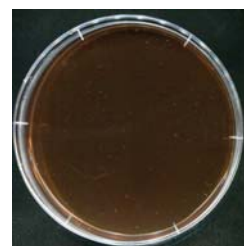
K40-SCV3   K40-SCV4   K40-SCV5   K40-SCV6

H59 (Wild Type)   H59-SCV1   H59-SCV2
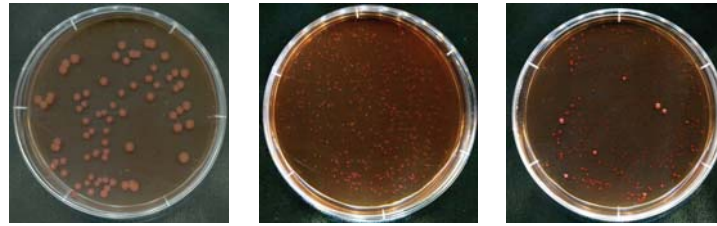
E128 Wild Type   E128-SCV1   E128-SCV1

Appendix 3   Colonies formed by wild type and SCV mutants of *S. aureus* after three days. Wild-type *S. aureus* K40 and its derived small colony variants were incubated in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C.

PAO1 (Wild Type) PAO1-SCV1 PAO1-SCV2 PAO1-SCV3

Appendix 4   Colonies formed by wild type and SCV mutants of *P. aeruginosa* after one day. Wild-type *P. aeruginosa* PAO1 and its derived small colony variants were incubated in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C.
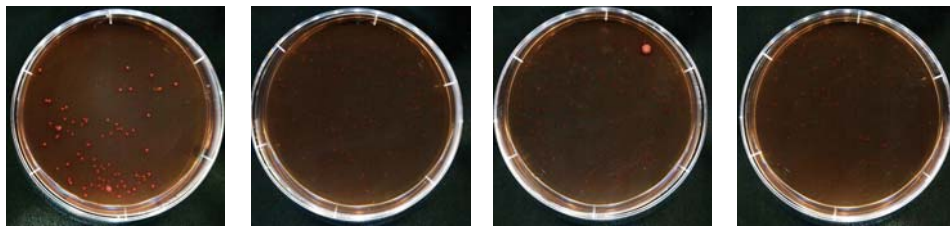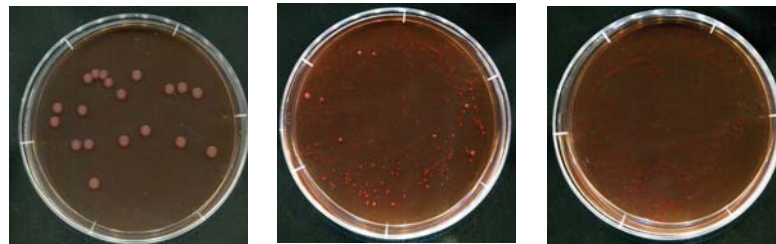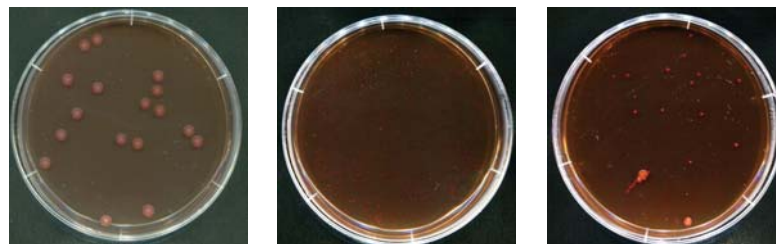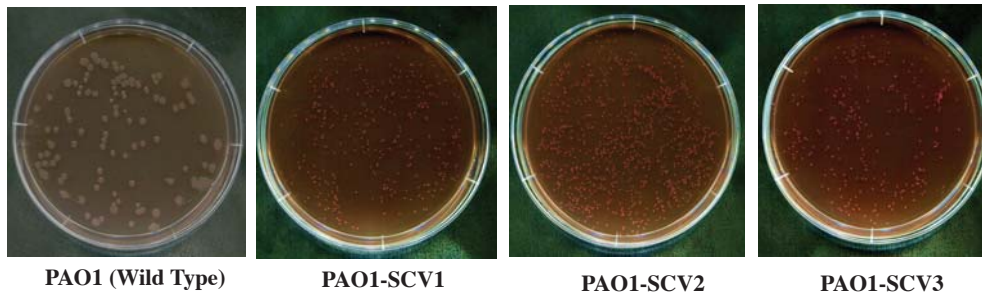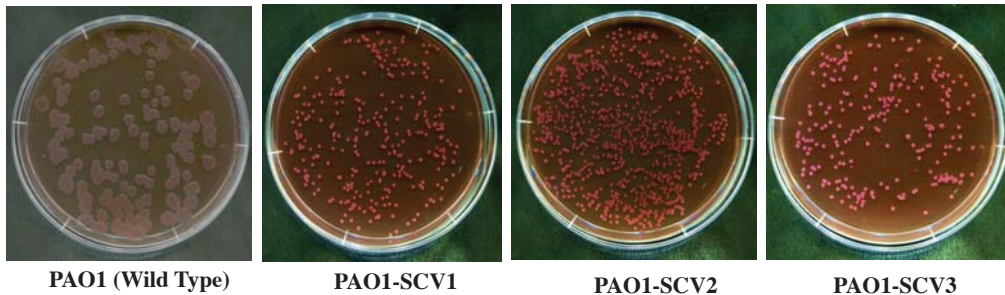
Day 2

PAO1 (Wild Type) PAO1-SCV1 PAO1-SCV2 PAO1-SCV3

Appendix 5   Colonies formed by wild type and SCV mutants of *P. aeruginosa* after two days. Wild-type *P. aeruginosa* PAO1 and its derived small colony variants were incubated in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C.

Day 3

PAO1 (Wild Type) PAO1-SCV1 PAO1-SCV2 PAO1-SCV3

Appendix 6   Colonies formed by wild type and SCV mutants of *P. aeruginosa* after three days. Wild-type *P. aeruginosa* PAO1 and its derived small colony variants were incubated in LB agar plates supplemented with 0.004% Congo Red. The growth statuses were recorded from day one to day three at 37°C.
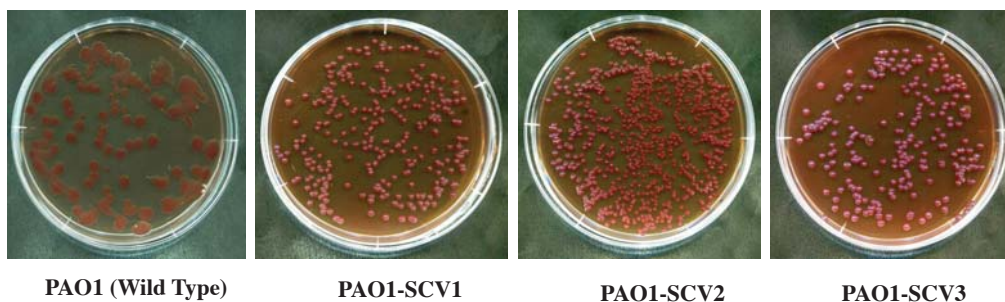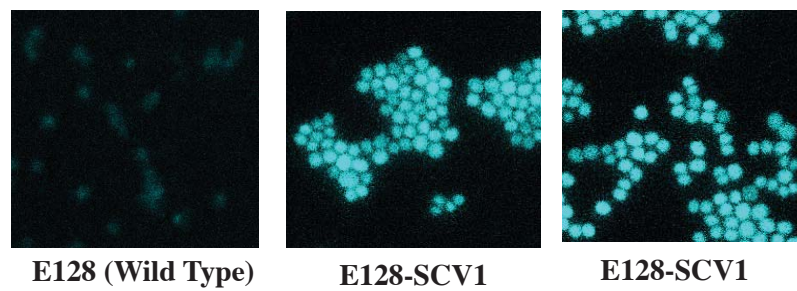
K40 (Wild Type)   **K40-SCV1**   **K40-SCV2**

K40-SCV3   **K40-SCV4**   **K40-SCV5**   **K40-SCV6**

H59 (Wild Type)   **H59-SCV1**   **H59-SCV2**

E128 (Wild Type)   **E128-SCV1**   **E128-SCV1**
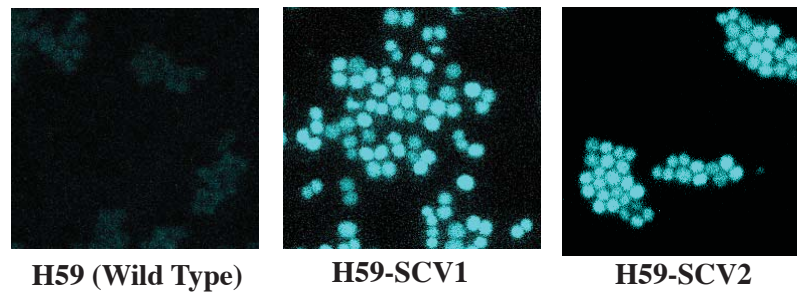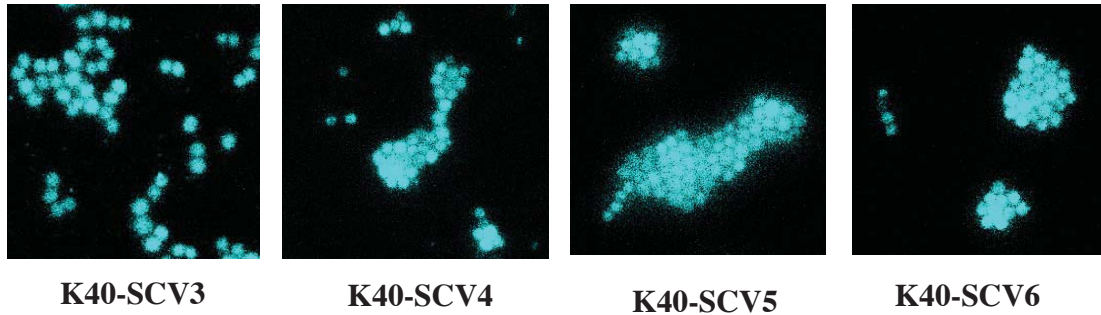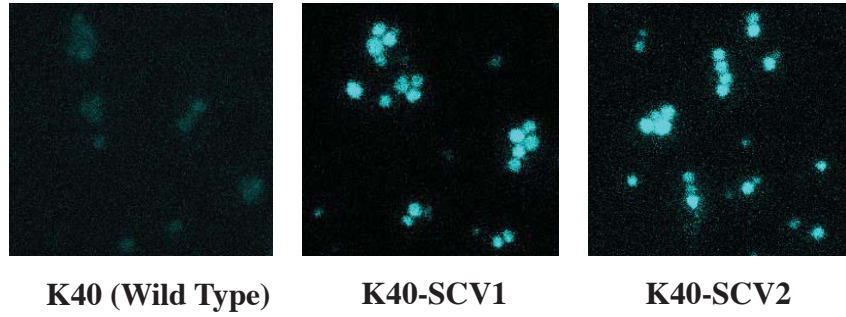
Appendix 7   Fluorescent microscope images of *S. aureus*. Wild type and SCV mutants were grown on LB agar plates containing Calcofluor White (CFW) Fluorescent Brightener 28 (35 μl /ml) and ferric sulfate (18 μl /ml). Cells were then examined under an upright fluorescence microscope with an emission wavelength of 477 nm and excitation wavelength of 436 nm.

| PAO1 (Wild Type) | PAO1-SCV1 | PAO1-SCV2 | PAO1-SCV3 |
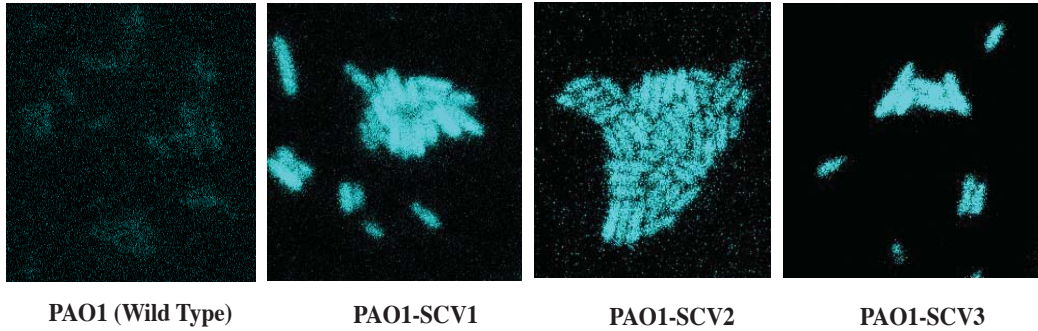
Appendix 8   Fluorescent microscope images of *P. aeruginosa.* Wild type and SCV mutants were grown on LB agar plates containing Calcofluor White (CFW) Fluorescent Brightener 28 (35 µl /ml) and ferric sulfate (18 µl /ml). Cells were then examined under an upright fluorescence microscope with an emission wavelength of 477 nm and excitation wavelength of 436 nm.

| K40 (Wild Type) | K40-SCV1 | K40-SCV2 |

| H59 (Wild Type) | H59-SCV1 |

| E128 (Wild Type) | E128-SCV1 |
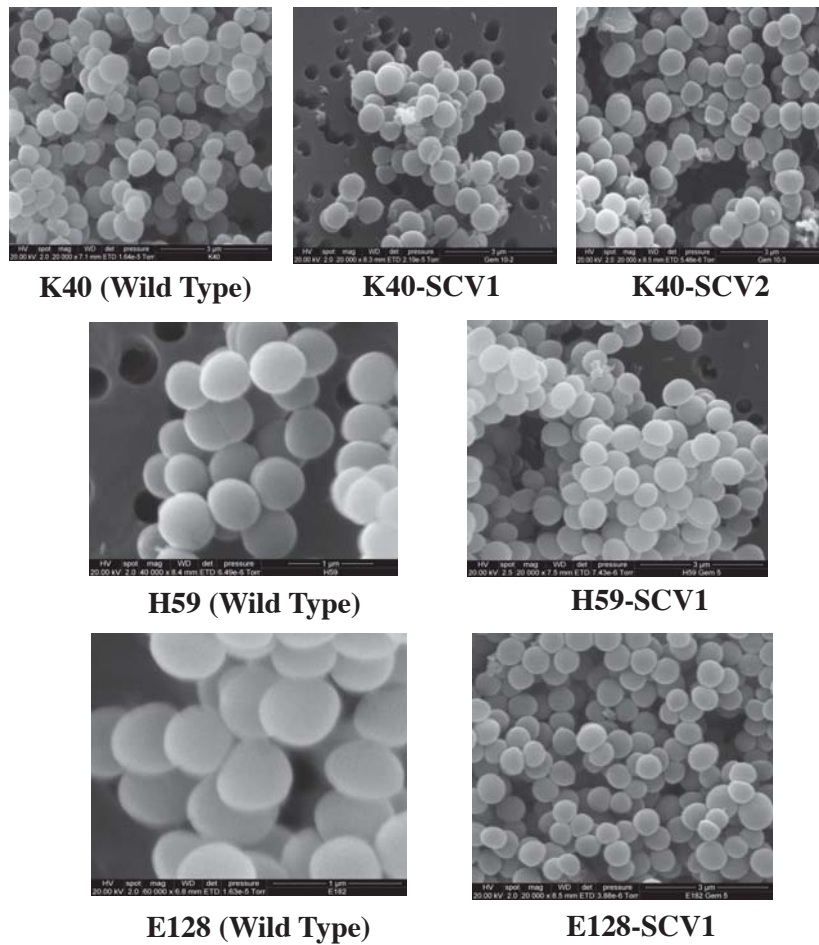
Appendix 9   Scanning electron microscope images of *S. aureus* . Wild type and SCVs were prepared from colonies grown on LB agar plates for 3 days at 37°C.
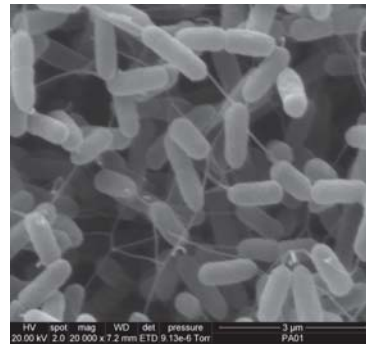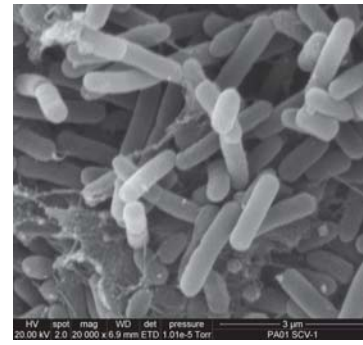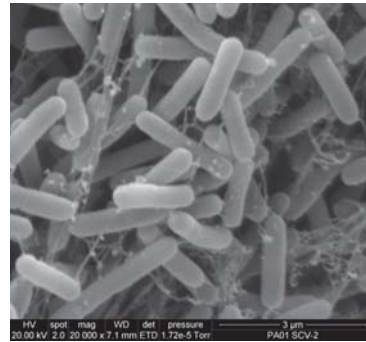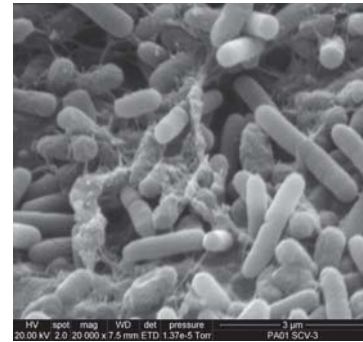
| PAO1 (Wild Type) | PAO-SCV1 |
| PAO1-SCV2 | PAO1-SCV3 |

Appendix 10   Scanning electron microscope images of *P. aeruginosa*. Wild type and SCVs were prepared from colonies grown on LB agar plates for 3 days at 37°C.
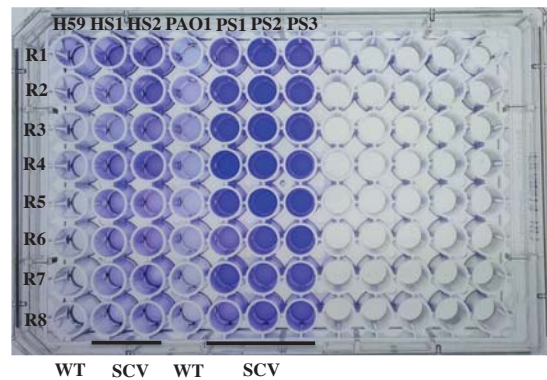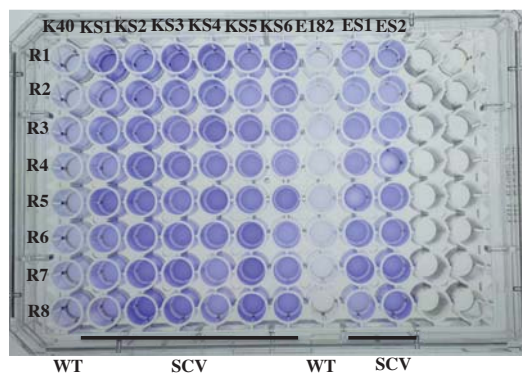
Appendix 11   Quantification of biofilms formed by wild-type strains and SCV mutants of *S. aureus* and *P. aeruginosa*. Biofilms were stained with 0.1% crystal violet (CV), and the dye was solubilized with 30% acetic acid. The absorbance was measured at the wavelength of 550 nm. Data are means and standard errors of eight replicates.

Appendix 12 Death rate (average x ± SD%) of *S. aureus* strains in different time points on pure copper surfaces. Asterisks denote significant difference compared with its original wild-type strain (P < 0.05).

	10 min	30 min	60 min	120 min	4 h	24 h
K40	82.28±1.79	98.19±0.22	99.77±0.03	99.98±0	-	100±0
K40-SCV1	90.72±1.28*	98.74±0.22	99.87±0.02	99.98±0	-	100±0
K40-SCV2	91.40±1.21*	99.26±0.11	99.88±0.02	99.98±0	-	100±0
K40-SCV3	89.07±1.03*	98.70±0.08	99.88±0.01	99.99±0	-	100±0
K40-SCV4	90.65±0.77*	98.65±0.10	99.87±0.01	99.99±0	-	100±0
K40-SCV5	89.80±1.53*	98.04±0.14	99.81±0.01	99.99±0	-	100±0
K40-SCV6	92.15±0.65*	99.11±0.11	99.93±0.01	99.99±0	-	100±0
E182	79.61±0.88	95.41±0.27	99.69±0.03	-	100±0	-
E182-SCV1	90.76±0.74*	98.40±0.08	99.78±0.02	-	100±0	-
E182-SCV2	87.02±1.12*	98.20±0.14	99.75±0.01	-	100±0	-
H59	76.79±1.63	93.58±0.61	99.13±0.11	-	100±0	-
H59-SCV1	89.53±1.56*	97.96±0.15	99.64±0.02	-	100±0	-
H59-SCV2	91.95±0.70*	99.04±0.09	99.86±0.01	-	100±0	-
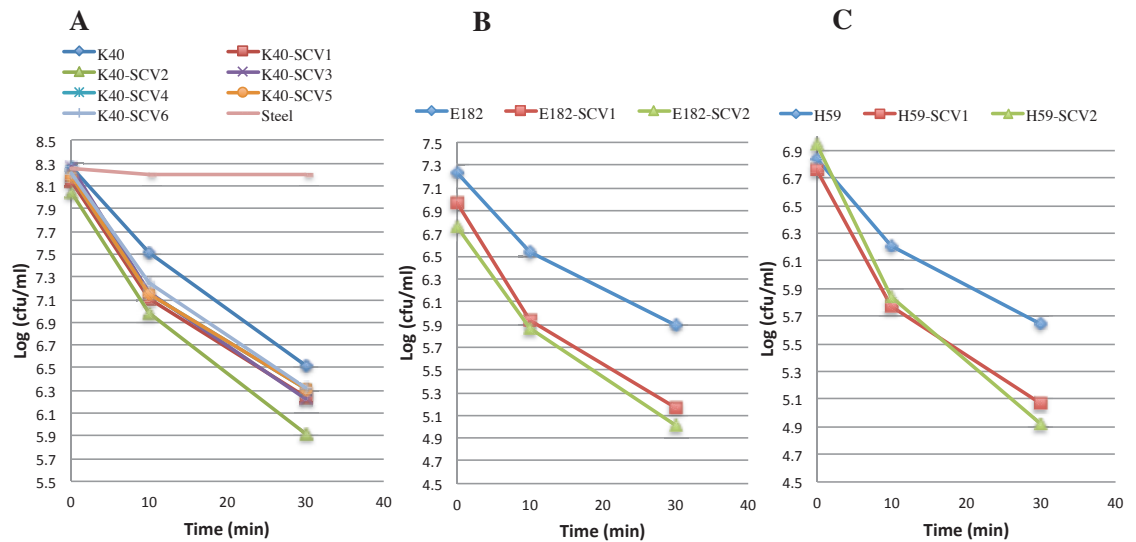
Appendix 13 **Rates of contact killing on the surfaces of pure copper among wild-type K40 (A), E128 (B) and H59 (C) and their derived SCV mutants.** The contact killing assays were performed with 6 replicates using the wet inoculation method. The rate of contact killing was calculated as percentage of cells killed after 10 min and 30 min.

Appendix 14 **Death rate (average x% ± SD%) of *P. aeruginosa* strains in different time points on pure copper surfaces.** Asterisks denote significant difference compared with wild-type PAO1 ($P < 0.05$).

	5 min	10 min	30 min	60 min
PAO1	90.60±0.92	99.19±0.12	99.99±0	100±0
SCV-1	98.68±0.18*	99.53±0.11	100±0	100±0
SCV-2	98.27±0.16*	99.45±0.11	100±0	100±0
SCV-3	97.83±0.26*	99.51±0.09	100±0	100±0
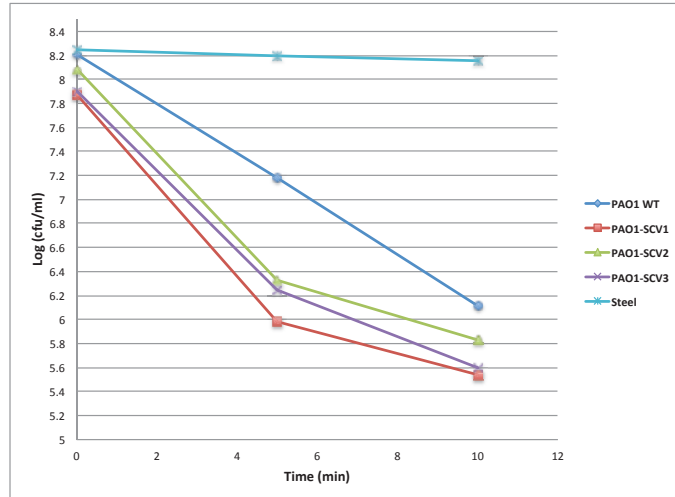
Appendix 15   Rates of contact killing on the surfaces of pure copper for wild-type PAO1 and its derived SCV mutants. The contact killing assays were performed with six replicates using the wet inoculation method. The rate of contact killing was calculated as percentage of cells killed after 5 min and 10 min.

Appendix 16   Death rate (average x % ± SD%) of *S. aureus* strains in different time points on brass surfaces. Asterisks denote significant difference compared with its original wild-type strain ($P < 0.05$).

	5 min	10 min	30 min	60 min	120 min	24 h
K40	18.78±6.39	37.09±6.88	84.41±1.01	98.57±0.08	99.99±0	100±0
K40-SCV1	30.68±6.84*	42.95±8.42*	84.94±1.09	98.77±0.11	99.99±0	100±0
K40-SCV2	31.06±6.84*	44.88±5.42*	85.80±0.99	98.81±0.11	99.99±0	100±0
E182	19.36±6.66	36.20±6.27	84.01±0.93	98.59±0.06	99.99±0	100±0
E182-SCV1	32.04±6.52*	46.24±4.19*	86.47±0.97	98.85±0.07	99.99±0	100±0
E182-SCV2	30.35±5.79*	44.88±6.97*	85.74±0.71	98.79±0.08	99.99±0	100±0
H59	17.73±6.12	35.75±7.30	83.73±0.86	98.48±0.06	99.99±0	100±0
H59-SCV1	29.55±7.93*	43.97±6.28*	84.11±1.33	98.57±0.07	99.99±0	100±0
H59-SCV2	29.72±7.97*	45.01±6.13*	85.46±0.95	98.89±0.06	99.99±0	100±0
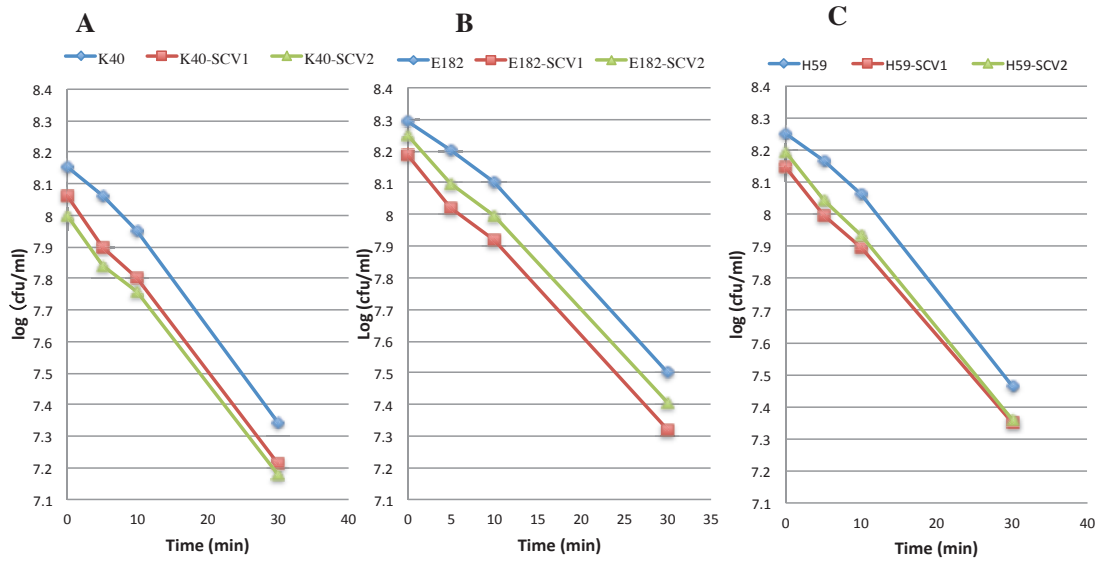
Appendix 17   Rates of contact killing on the surfaces of brass among wild-type K40 (A), E128 (B) and H59 (C) and their derived SCV mutants. The contact killing assays were performed with six replicates using the wet inoculation method. The rate of contact killing was calculated as percentage of cells killed after 5 min, 10 min and 30 min.

Appendix 18   Death rate (average x% ± SD%) of *P. aeruginosa* strains in different time points on brass surfaces. Asterisks denote significant difference compared with wild-type PAO1 ($P < 0.05$).

	5 min	**10 min**	**30 min**	**60 min**	**120 min**	**24 h**
PAO1	37.41±3.80	62.47±4.36	91.29±0.63	99.62±0.06	100±0	100±0
SCV1	48.80±5.32*	73.48±5.18*	92.52±0.65	99.75±0.05	100±0	100±0
SCV2	49.25±4.45*	75.29±4.82*	92.11±0.47	99.77±0.05	100±0	100±0
SCV3	47.69±4.17*	73.424.22*	92.96±0.47	99.73±0.05	100±0	100±0
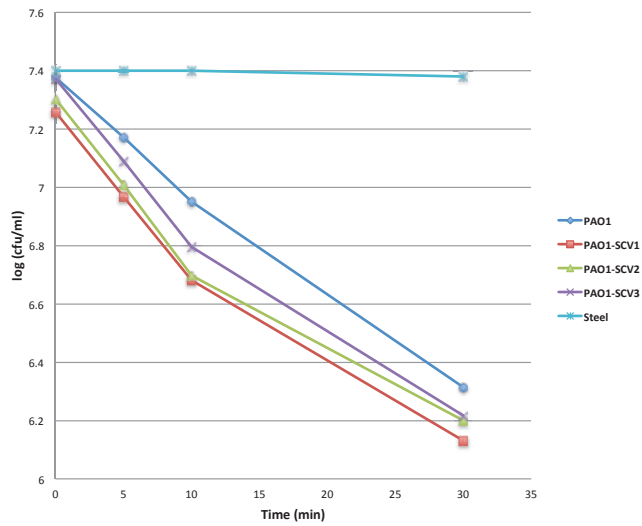
Appendix 19   Rates of contact killing on the surfaces of brass for wild-type PAO1 and its derived SCV mutants. The contact killing assays were performed with six replicates using the wet inoculation method. The rate of contact killing was calculated as percentage of cells killed after 5 min, 10 min and 30 min.

Appendix 20 Death rate (average x% ± SD%) of *P. fluorescens* strains in different time points on pure copper and brass surfaces. Asterisks denote significant difference compared with wild-type SBW25 (P < 0.05).

Strain	Characters	Copper		Brass	
		5 min	10 min	5 min	10 min
SBW25	Wild type	56.12±5.46	89.81±1.66	32.81±3.60	57.30±3.54
CA	Δ*MvaT*, a colanic acid overproducing mutant	51.23±5.84	72.86±4.31*	28.53±3.80*	49.21±3.69*
LSWS	Overproducing cellulose	64.56±4.61*	95.20±0.34*	39.94±3.42*	67.33±4.11*
MU49-17	Δ*wss*, cellulose non-producing mutant	53.05±4.43*	87.06±1.96	31.13±4.30	51.29±5.10*
FS	Overproducing lipopolysaccharide	69.50±3.99*	94.61±1.79*	41.72±8.17	66.15±3.35
MU49-21	Δ*fuZ*, a LPS nonproducing mutant	58.81±6.22	88.57±3.34	33.39±7.05	56.31±5.43
MU49-22	Δ*fliA*, a SBW25 mutant defective in flagella	50.85±2.65	87.07±2.23	34.00±7.91	56.82±2.43
MU49-23	Δ*pilG*, a SBW25 mutant defective in pili	53.11±7.33	90.87±1.43	36.75±7.05	59.71±6.37
MU49-24	Δ*fliA*Δ*pilG*, a SBW25 mutant defective in flagella and pili	50.13±7.27	86.75±1.44	33.58±6.48	55.56±5.78
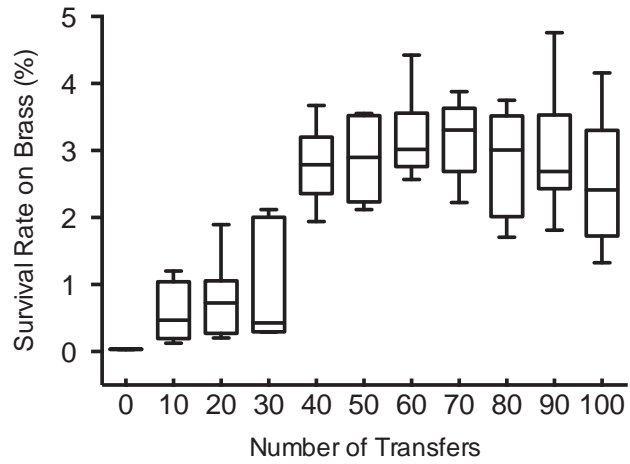
Appendix 21 Dynamic changes in bacterial resistance to copper. A non-pathogenic strain of *P. fluorescens* SBW25 was subjected to daily passage of sub-lethal (1-h copper treatment) conditions on the surface of brass, and changes of survival rates were monitored every 10 days.

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