

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Contact Killing of Bacterial Pathogens on Metallic Copper



MASSEY UNIVERSITY

A thesis presented in partial fulfillment of the requirements for the degree of

Master of Science

in

Microbiology

at Massey University, Auckland,

New Zealand

Sha Liu

2016

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.

Acknowledgements

First and foremost, I would like to express my sincere gratitude towards my supervisor, Dr. Xue-Xian Zhang, for his constant supports and patience. He has walked me through this project, given me the freedom to explore on my own, and meanwhile the professional guidance and illuminating suggestions. I am deeply grateful for his instructions and encouragements not only in lab work, but also in my future life. I have become increasingly interested in microbiology during working with him on this interesting and innovative research project.

I would like to take this opportunity to thank Dr. Gayle Ferguson for her critical comments and helpful discussions on the experiments. She has provided me with many valuable insights of this study. I am also indebted to Dr. Heather Hendrickson and Richard Yulo for offering me training and giving me advice on fluorescence microscopy. Thanks also to Dr. Philippe Remigi for kindly providing the $\Delta mvaT$ strain and for his efficient technical assistance.

My thanks would also go to Dr. Stephen Ritchie at Auckland City Hospital for helping provide his medical expertise and clinical bacterial isolates. I would like to thank Nikki Murray at the Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North for helping perform the SEM analysis.

I am grateful to Dr. Yunhao Liu, Naren and Elena Colombi for helping me with each small question and for all the fun we have had when we were working in the lab together. Thanks to every academic staffs and fellow students in Evolutionary Genetics and Microbial Ecology Laboratory not only for the prior observations and insights on *P. fluorescens* SBW25, but also for our friendship.

Finally, I own my deeply gratitude to my beloved family and friends for their endless considerations, unconditional love and great confidence in me all through these years, without which this thesis would have never been completed. Especially, thanks to my parents and Jun Wang for always backing me up, standing by me, and encouraging me in all of my pursuits and inspiring me to follow my dreams.

I could not have done this project without all your support. I have been amazingly fortunate to have all of you on the way.

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

My strength lies solely in my tenacity.”

- Louis Pasteur-

Table of Contents

| | |
|---|-------------|
| Abstract | I |
| Acknowledgements | III |
| Table of Contents | V |
| List of Figures | X |
| List of Tables | XII |
| List of Abbreviations | XIII |
| Chapter 1 Introduction | 1 |
| 1.1 Hospital-acquired infections — a serious public health concern | 1 |
| 1.2 Using copper-containing surface materials for HAIs prevention | 3 |
| 1.2.1 The antimicrobial properties of copper..... | 3 |
| 1.2.2 The potential of copper in reducing HAIs | 4 |
| 1.3 The mechanisms of copper-mediated contact killing..... | 7 |
| 1.3.1 Current understanding of copper leading to cell death..... | 7 |
| 1.3.2 Toxicity of ionic copper..... | 7 |
| 1.4 The copper ions burst releasing hypothesis | 10 |
| 1.5 Testing the copper ions burst releasing hypothesis | 11 |
| 1.6 Small colony variants (SCVs) of <i>S. aureus</i> and <i>P. aeruginosa</i> | 12 |
| 1.7 Potential of bacteria to develop resistance to metallic copper | 13 |
| 1.8 Specific aims of this project | 15 |

| | | |
|------------------|--|-----------|
| Chapter 2 | Materials and Methods | 18 |
| 2.1 | Bacterial strains and growth conditions | 18 |
| 2.2 | Laboratory Media | 21 |
| 2.3 | Copper materials | 22 |
| 2.4 | Isolation of small colony variants (SCVs) | 22 |
| 2.5 | Bacterial sensitivity to copper ions | 23 |
| 2.6 | Bacterial contact killing assay on copper | 24 |
| 2.7 | Fluorescent microscopic analysis | 26 |
| 2.8 | Scanning Electron Microscopy (SEM) analysis | 27 |
| 2.9 | Microtiter plate biofilm formation assay | 27 |
| 2.10 | Experimental evolution of bacterial resistance to metallic copper | 28 |
| 2.11 | PCR and agarose gel electrophoresis | 30 |
| Chapter 3 | Results | 32 |
| 3.1 | Isolation of small colony variants of nosocomial pathogenic bacteria | 32 |
| 3.1.1 | SCVs of <i>Staphylococcus aureus</i> | 32 |
| 3.1.2 | SCVs of <i>Pseudomonas aeruginosa</i> | 34 |
| 3.2 | Phenotypic characterization of the SCV mutants | 35 |
| 3.2.1 | Fluorescent microscopic analysis | 35 |
| 3.2.2 | Scanning Electron Microscopy (SEM) analysis | 37 |
| 3.2.3 | Quantifying biofilm formation | 38 |
| 3.3 | Bacterial contact killing assays on copper surfaces | 39 |
| 3.3.1 | Assaying the rates of bacterial contact killing on the surfaces of | |

| | |
|---|-----------|
| pure copper versus brass | 39 |
| 3.3.2 Comparing copper susceptibilities between wild-type <i>S. aureus</i> and <i>P. aeruginosa</i> | 41 |
| 3.3.3 Comparing the rates of bacterial contact killing between wild type and SCV mutants on the surfaces of pure copper..... | 42 |
| 3.3.4 Comparing the rates of bacterial contact killing between wild type and SCV mutants on the surfaces of brass | 44 |
| 3.4 Assessing the roles of specific surface polymers in copper- mediated contact killing..... | 45 |
| 3.5 Bacterial sensitivity to copper ions..... | 47 |
| 3.6 Exploring the potential of bacterial pathogens to develop resistance to metallic copper | 51 |
| Chapter 4 Discussion | 55 |
| 4.1 Isolation and characterization of small colony variants (SCVs) | 55 |
| 4.2 Antimicrobial properties of copper-containing surface materials | 56 |
| 4.3 Mechanisms of copper-mediated contact killing..... | 58 |
| 4.4 Evolution of bacterial resistance to metallic copper..... | 60 |
| Chapter 5 Conclusion and Future Research | 61 |
| Appendices | 65 |
| Appendix 1 Colonies formed by wild type and SCV mutants of <i>S. aureus</i> after one day | 65 |
| Appendix 2 Colonies formed by wild type and SCV mutants of <i>S. aureus</i> | |

| | | |
|-------------|--|----|
| | after two days | 66 |
| Appendix 3 | Colonies formed by wild type and SCV mutants of <i>S. aureus</i> | |
| | after three days | 67 |
| Appendix 4 | Colonies formed by wild type and SCV mutants of <i>P. aeruginosa</i> | |
| | after one day | 68 |
| Appendix 5 | Colonies formed by wild type and SCV mutants of <i>P. aeruginosa</i> | |
| | after two days | 68 |
| Appendix 6 | Colonies formed by wild type and SCV mutants of <i>P. aeruginosa</i> | |
| | after three days | 68 |
| Appendix 7 | Fluorescent microscope images of <i>S. aureus</i> | 69 |
| Appendix 8 | Fluorescent microscope images of <i>P. aeruginosa</i> | 70 |
| Appendix 9 | Scanning electron microscope images of <i>S. aureus</i> | 70 |
| Appendix 10 | Scanning electron microscope images of <i>P. aeruginosa</i> | 71 |
| Appendix 11 | Quantification of biofilms formed by wild-type strains and SCV mutants of <i>S. aureus</i> and <i>P. aeruginosa</i> | 71 |
| Appendix 12 | Death rate (average $\bar{x} \pm SD\%$) of <i>S. aureus</i> strains in different time points on pure copper surfaces | 72 |
| Appendix 13 | Rates of contact killing on the surfaces of pure copper among wild-type K40 (A), E128 (B) and H59 (C) and derived SCVs..... | 73 |
| Appendix 14 | Death rate (average $\bar{x} \pm SD\%$) of <i>P. aeruginosa</i> strains in different time points on pure copper surfaces | 73 |
| Appendix 15 | Rates of contact killing on the surfaces of pure copper among wild-type PAO1 and derived SCVs..... | 74 |

| | | |
|-------------------|--|-----------|
| Appendix 16 | Death rate (average $\bar{x} \pm SD\%$) of <i>S. aureus</i> strains in different time points on brass surfaces | 74 |
| Appendix 17 | Rates of contact killing on the surfaces of brass among wild-type K40 (A), E128 (B) and H59 (C) and derived SCVs..... | 75 |
| Appendix 18 | Death rate (average $\bar{x} \pm SD\%$) of <i>P. aeruginosa</i> strains in different time points on brass surfaces | 75 |
| Appendix 19 | Rates of contact killing on the surfaces of brass among wild-type PAO1 and derived SCVs | 76 |
| Appendix 20 | Death rate (average $\bar{x} \pm SD\%$) of <i>P. fluorescens</i> strains in different time points on pure copper and brass surfaces | 77 |
| Appendix 21 | Dynamic changes in bacterial resistance to copper | 78 |
| References | | 79 |

List of Figures

| | | |
|------------|---|----|
| Figure 1.1 | Hospital trials investigating the antimicrobial copper's potential around the world | 5 |
| Figure 1.2 | A comparison of antimicrobial properties of four different materials under typical indoor conditions..... | 6 |
| Figure 1.3 | SEM image of <i>E. hirae</i> on honeycomb-like grids with holes | 9 |
| Figure 1.4 | Current model of the tentative events in contact killing | 10 |
| Figure 1.5 | The copper ions burst releasing model | 11 |
| Figure 1.6 | A proposed model of copper homeostasis in <i>P. fluorescens</i> SBW25 | 14 |
| Figure 2.1 | Demonstration of the wet incubation method | 25 |
| Figure 3.1 | Colonies formed by wild type and SCV mutants of <i>S. aureus</i> | 34 |
| Figure 3.2 | Colonies formed by wild-type <i>P. aeruginosa</i> and a derived SCV | 35 |
| Figure 3.3 | Fluorescent microscope images of <i>S. aureus</i> (A) and <i>P. aeruginosa</i> (B) | 36 |
| Figure 3.4 | Representative scanning electron microscope images of <i>S. aureus</i> (A) and <i>P. aeruginosa</i> (B)..... | 37 |
| Figure 3.5 | Quantification of biofilms formed by SCV mutants of <i>S. aureus</i> and <i>P. aeruginosa</i> | 39 |
| Figure 3.6 | A comparison of antimicrobial properties between pure copper and brass | 40 |
| Figure 3.7 | A comparison of copper-mediated contact killing between | |

| | | |
|-------------|---|----|
| | Gram-positive <i>S. aureus</i> and Gram-negative <i>P. aeruginosa</i> | 42 |
| Figure 3.8 | Rates of contact killing on the surfaces of pure copper between wild-type <i>S. aureus</i> (A) and <i>P. aeruginosa</i> (B) and derived SCVs | 43 |
| Figure 3.9 | Rates of contact killing on the surfaces of brass between wild-type <i>S. aureus</i> (A) and <i>P. aeruginosa</i> (B) and their derived SCV mutants .. | 44 |
| Figure 3.10 | Rates of contact killing between SBW25 WT and mutants on pure copper (A) and brass (B) | 46 |
| Figure 3.11 | Typical photos for the sensitivity of <i>Pseudomonas</i> and <i>S. aureus</i> strains to ionic copper | 50 |
| Figure 3.12 | Outline of the proposed mutation accumulation experiment | 52 |
| Figure 3.13 | Blue colonies formed by the 100 th evolutionary MU49-25 (LSWS- <i>lacZ</i>) strain used in the evolutionary experiment..... | 52 |
| Figure 3.14 | PCR verification of <i>P. fluorescens</i> SBW25-specific <i>xutA</i> gene for xylose utilization | 53 |
| Figure 3.15 | Dynamic changes in bacterial resistance to copper | 54 |

List of Tables

| | | |
|-----------|--|----|
| Table 2.1 | List of bacterial strains used in this study | 18 |
| Table 2.2 | Laboratory media used in this study | 22 |
| Table 2.3 | Reagents in 50µl of PCR reaction | 30 |
| Table 2.4 | Typical PCR reaction conditions | 31 |
| Table 3.1 | Sensitivity of wild-type bacteria and the derived mutants to copper ions when grown in LB agar plates | 48 |

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 <i>Staphylococcus aureus</i> |
| MS | mass spectrometry |
| MSSA | methicillin sensitive <i>Staphylococcus aureus</i> |
| 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 <i>Enterococcus</i> |
| WHO | world health organization |
| WT | wild type |
| °C | degrees celsius |
| µg | microgramme |
| µl | microliter |
| µm | micrometre |
| µM | micromolar |