Mode of action study for a novel class of antimicrobial polymers against methicillin-resistant *Staphylococcus aureus*

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**MODE OF ACTION STUDY FOR A NOVEL
CLASS OF ANTIMICROBIAL POLYMERS
AGAINST METHICILLIN-RESISTANT
*STAPHYLOCOCCUS AUREUS***

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A thesis submitted to Nanyang Technological University in
partial fulfilment of the requirement for the degree of Master
of Engineering

2019

Supervisor:

Professor Chan Bee Eng, Mary

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Abstract

The development of antibiotics has been hampered by the rapid evolution of antimicrobial resistance and lack of new mechanisms discovered. Membrane-polarizing cationic polymers have attracted attention due to their low propensity to induce resistance. A cationic polymer (labelled as PIM1) has been found to exert excellent antimicrobial efficacy with low cell toxicity. To understand the mechanism of action of PIM1, investigations were carried out in methicillin-resistant *Staphylococcus aureus* (MRSA). It was found that PIM1 had a distinctively different mechanism from common cationic polymers, since it did not physically disrupt and perforate the cytoplasmic membrane. Instead, the polymer blocked aerobic respiration, leading to pleiotropic effects on multiple biosynthetic pathways in the bacteria. Whole genome sequencing of PIM1-resistant MRSA strains revealed that genes associated with bacteria surface charge and the electron transport chain (ETC) were crucial for the activity of PIM1. Studies with respiratory mutants implied a strong relationship between the level of aerobic respiration and the efficacy of PIM1. Moreover, PIM1 was found to decrease the intracellular menaquinone content at sub-inhibitory concentrations, suggesting a direct interaction between PIM1 and the ETC component menaquinone. The results suggest that PIM1 binds to the bacteria surface through electrostatic attraction, and interact with the membrane-bound ETC which leads to cell death.

Declaration: Due to patent filing, the exact structure of the cationic polymer PIM is not disclosed here. I apologise for the inconvenience caused.

Chapter 1: Introduction

1.1 Literature review

1.2.1 Antibiotic targets and resistance in *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive round-shaped bacterium that can be both commensal and pathogenic. It is a leading cause of clinical infections including bacteraemia, infective endocarditis, skin and soft tissue, osteoarticular, and hospital-acquired infections [1]. Moreover, *S. aureus* is a major foodborne pathogen, which is capable of producing enterotoxins responsible for food poisoning [2]. Major intracellular targets for antibiotics developed against *S. aureus* are the cell envelope, protein synthesis, and nucleic acid synthesis which are absent or significantly different from human cells [3]. Nonetheless, antibiotic resistance can develop through either horizontal transfer of mobile genetic elements or acquisition of mutations. Horizontally acquired resistance can result in enzymatic modification of drug or target site, drug efflux, or bypassing the drug target [4]. Acquisition of mutation usually lead to modification of drug target, derepression of drug efflux pumps, or accumulation of mutations that alter the bacteria surface to reduce drug access [5]. One examples of cell envelope targeting drug is β -lactams, which were the earliest generation of *S. aureus* antibiotics. Penicillin, methicillin and oxacillin all belong to this category. The inhibitory target of β -lactams is the penicillin binding protein 2a (PBP2a) responsible for crosslinking peptidoglycan in the cell wall [6]. Methicillin-resistant *S. aureus* (MRSA) was observed shortly after the first clinical use of β -lactams. The resistance is acquired by horizontal transfer of staphylococcal cassette chromosome to the PBP2a gene *mecA*, which led to lower binding

affinity of PBP2a to β -lactams [7, 8]. Since then MRSA has become a formidable clinical threat, causing high morbidity and mortality. Vancomycin, a glycopeptide that blocks peptidoglycan synthesis, has been used to treat serious infections by MRSA [9]. However, genetic adaptation has given MRSA the most feared vancomycin resistance. The vancomycin-intermediate *S. aureus* emerged after prolonged vancomycin treatment. Another category of high level vancomycin-resistant *S. aureus* accepted plasmid transfer of *vanA* operon from vancomycin-resistant *Enterococcus faecalis* [10].

S. aureus, like most bacterial pathogens, has extraordinary ability to develop resistance to any antibiotic it is exposed to. There has been a lack of new chemical classes of antibiotics since the 1980s [11]. The organism has at least one resistance mechanism towards all clinically available antibiotics. The current clinical solution to treat multidrug resistant MRSA is to switch drugs or employ different drug combinations. Recently, there has been some discovery of new antimicrobial targets. One promising molecule named triclosan targets the fatty acid biosynthesis protein FabI [12]. The latest development was teixobactin, which inhibits peptidoclycan and wall teichoic acid biosynthesis by binding to lipid II [13]. Due to the structure and composition of lipid II, the binding site cannot be easily modified by point mutations. Nonetheless, there remains an obvious need for development of newer antibiotic drugs and discovery of antibiotic targets that are difficult to acquire resistance.

1.2.2 Cationic antimicrobial polymers

With the intensive development of polymer science and characterisation methods since 1980s, various macromolecular structures have been devised for

antimicrobial applications. These polymer structures often show enhanced bactericidal activities and low cytotoxicity compared to small amphiphilic molecules. Moreover, antimicrobial polymers have the advantage of being tethered to surfaces without losing their biological activities, delivering a variety of applications like wound dressing, antifouling and food packaging [14]. Many different types of antimicrobial polymers have been designed, and the main categories include cationic antimicrobial polymer [15], and biocide-releasing polymers [16]. The cationic antimicrobial polymers have been described to contain two major functional components: the cationic group and the hydrophobic group [17]. The fundamental mode of action of synthetic cationic antimicrobial polymers is based on membrane activity. One important feature of the bacterial cell envelope is the net negative charge provided by teichoic acid in Gram-positive bacteria cell wall, lipopolysaccharide and phospholipids in Gram-negative outer membrane, and the phospholipid bilayer in the cytoplasmic membrane [18]. Cationic antimicrobial polymers take advantage of the negative charge and target the cytoplasmic membrane. The widely accepted antimicrobial mechanism of cationic polymers consists of multiple steps: (i) the polymers are first adsorbed onto the bacterial surface with the aid of cationic groups; (ii) the high binding affinity enhanced by hydrophobic groups cause cytoplasmic membrane disorganisation; (iii) cytoplasmic contents leak out, eventually leading to cell lysis [19, 20]. It is believed that this membrane-active mechanism has a low potential of inducing microbial drug resistance [21].

Ever since the first cationic antimicrobial polymer polyhexamethylene biguanide chloride (PHMB) was introduced in 1983 [22, 23], there has been a constant exploration of structural design to optimize functionality of the polymer.

Examples of the cationic centre are ammonium groups (including primary, secondary, tertiary and quaternary ammonium) [24], and iminium structures (pyridinium, imidazolium, and guanidinium) [25-27]. The unique feature of iminium groups is that the positive charge is delocalized evenly through the conjugated system, which give rise to better microbial efficacy compared with quaternary ammonium groups [28]. The hydrophobic groups have also been studied extensively to facilitate the penetration into the cytoplasmic membrane. Common strategies involve alteration of alkyl chain length, and adopting linear versus cyclic structures [17]. Although many polymer systems have been developed to date, the understanding towards the antimicrobial mechanism is still limited. There has been a lack of new potential mechanisms explored for the past decade. In-depth mode of action studies will establish fundamentals for improving structure-activity relationships.

1.2 Objectives and scope of project

A series of PIM salts has been synthesized, out of which PIM1 exhibited low cell toxicity and good antimicrobial activity against a wide range of microorganisms, including both Gram-positive and Gram-negative bacteria. The objective of this project is to investigate the mechanism of action of PIM1 through microbiological and biochemical approaches. The model organism is MRSA, which has been well characterized and has high clinical relevance and significance. The investigation began with studying the impact of PIM1 on the physiology of MRSA. In the second stage, a resistance evolution study was carried out and the genomic variations associated with resistant strains were critically analysed. Based on findings from the resistance study, further characterizations were carried out to determine the specificity of the activity of PIM1. All results were discussed and interpreted after data presentation. Therefore, no separate discussion section was included. Conclusions from this study would provide a deeper understanding into the field of antimicrobial polymers, and lay foundation for future work.

Chapter 2: Materials and Methods

2.1 Strains and culture conditions

Bacteria strains used in this study are listed in Table 1. MRSA strains were cultured in TSB (tryptic soy broth) or on TSA (tryptic soy agar). Freezer stocks of strains were made with TSB and 30% glycerol. Transposon mutants were constructed from the Nebraska library [29]. For transposon mutants, 10 µg/mL Erythromycin (Erm) was added to the culture media.

Table 1. List of MRSA strains used in this study

Strains	Relevant characteristics	Source
LAC	CA-MRSA USA300 strain	[30]
LAC $\Delta menD$	Deletion of <i>menD</i> from LAC	[31]
LAC $\Delta hemB$	Deletion of <i>hemB</i> from LAC	[31]
LAC <i>mprf::tn</i>	<i>mprf</i> transposon mutant, Erm-resistant	[29]
LAC*	Erm-sensitive derivative of LAC	[32]
LAC* <i>qoxB::tn</i>	<i>qoxB</i> transposon mutant, Erm-resistant	[33]
LAC* <i>cydA::tn</i>	<i>cydA</i> transposon mutant, Erm-resistant	[33]
LAC* $\Delta dltD$	Deletion of <i>dltD</i> from LAC	[29]
LAC* Δdlt operon	Deletion of <i>dlt</i> operon from LAC	[29]

2.2 Determination of minimum inhibitory concentrations (MICs)

MICs were determined in TSB by the broth microdilution method. A single bacteria colony was picked and grown overnight. Next day the cultures were diluted to an OD of 0.01 and grown to exponential phase (OD of approximately 1). Next, the cultures were adjusted to approximately 5×10^5 cells per mL (1000-fold dilution of OD=1 culture) and 50 μ L of these cultures were added to 50 μ L of serial dilutions of the test compound in TSB medium. After 18 h of incubation at 37°C with constant shaking at 500 rpm, the absorbance at 600nm (OD₆₀₀) was measured using a SPECTROstar Nano microplate reader (BMG Labtech). The MIC₉₀ is defined as the concentration at which $\geq 90\%$ of the isolates in a test population are inhibited [34]. Experiments were performed with 3 independent biological samples.

2.3 Membrane permeability assay

Permeability of cytoplasmic membrane was monitored with nucleic acid dye SYTOX green (ThermoFisher). The method was adapted from previous studies on *S. aureus* membrane permeability [35, 36]. The bacterial strains were grown overnight in TSB medium. Next day the cultures were diluted to an OD of 0.01 and grown to exponential phase (OD of approximately 1). Next, the cells were pelleted by centrifugation at 7,000 rpm for 10 min, and resuspended to OD of 2 in 0.85% NaCl solution. The dye SYTOX green (1 μ M) was added together with compounds of interest (PIM1, nisin at 8 x MIC). The cell suspension was incubated in dark for 1 h. Next, the mixture was washed by pelleting and resuspending to OD of 2 in 0.85% NaCl solution. 2 μ l of the washed cell suspension was spotted on a slide with a thin 1.5% agarose gel patch and covered with a coverslip. The observation was performed at x1000 magnification using an Axio Imager.A2 Zeiss microscope equipped with a GFP filter set. The images were taken with the ZEN 2012 (blue edition) software. Cells were counted using the ImageJ software. Experiments were performed with 3 independent biological samples.

2.4 Membrane potential assay

The measurement of membrane potential was performed with the voltage-sensitive fluorescent dye 3,3'-Dipropylthiadicarbocyanine iodide DiSC₃(5) (Sigma-Aldrich). Bacterial strains were grown in the same condition as for membrane permeability assay. The exponential phase bacteria cells from a culture aliquot were pelleted by centrifugation at 7,000 rpm for 10 min, and resuspended to OD of 0.2 in PBS supplemented with 0.5 mg/mL BSA. BSA was added to reduce the absorption of the DiSC₃(5) dye to the polystyrene surfaces of the plates [37]. 200 µl of these cell suspensions were aliquoted into black-wall transparent bottom 96-well microplate (Greiner) and the fluorescence signal was measured at 622 nm excitation and 670 nm emission using a Tecan Infinite M200Pro plate reader. After measuring baseline fluorescence for 2 minutes, DiSC₃(5) was added to a final concentration of 1 µM. The fluorescence quenching was monitored until signal the intensity stabilized. The compounds of interest (PIM1, nisin, DI water) were then added. The final concentration in the medium was 8 µg/mL for PIM1 and 64 µg/mL for nisin. The fluorescence signal was subsequently followed for 60 min. Experiments were performed with 3 independent biological samples.

2.5 Macromolecular synthesis

Protocol for measuring incorporation rate of radioactive-labelled precursors was adapted from Ling *et al.* [38] and Bowman *et al.* [39]. MRSA strain LAC* cells were grown in minimal medium (0.02 M HEPES, 0.002M MgSO₄, 0.0001M CaCl₂, 0.4% succinic acid, 0.043M NaCl, 0.5% (NH₄)₂SO₄, 5% TSB) to exponential phase. Cells were pelleted by centrifugation for 10 min at 8,000 rpm and 4°C. The supernatant was removed, and the pellet was resuspended in fresh minimal medium to an OD of 0.2 (10⁸ cells per mL). 350 µl of this cell suspension was aliquoted to 50 mL conical tubes, and 100 µl was removed for background measurement. Antibiotics were added to the cell suspension, immediately followed by addition of radioactive precursors. The precursors used were N-acetyl-glucosamine, D-[6-³H] (1 mCi mL⁻¹), thymidine, [methyl-³H] (0.25 mCi mL⁻¹), uridine, [5-³H] (1 mCi mL⁻¹), L-glutamine, ¹⁴C(U) (0.1 mCi mL⁻¹) (Hartmann Analytics). 100 µl cell suspension was removed and added to 500 µl ice cold 25% trichloroacetic acid (TCA) and 1% casamino acid (CA) solution. The mixture was filtered through nitrocellulose filter (PerkinElmer Life Sciences), followed by washing with 2 mL ice cold 25% TCA + 1% CA solution, and 16 mL ice cold water. Measurements were taken immediately upon addition of precursor, and after 20 min incubation at 37°C and 500 rpm. Afterwards, the filters were dissolved in 9 mL of scintillation liquid Filter Count (PerkinElmer Life Sciences). Incorporated radioactivity was measured with Wallac 1409 DSA liquid scintillation counter. The count/min (cpm) values were normalized to the OD of cell suspension, and experiments were performed with 3 independent biological samples.

2.6 Bacterial respiration

The oxygen consumption rate (OCR) of LAC* and resistant mutants were measured using a Seahorse XFp Analyzer (Agilent). Overnight culture was diluted to OD of 0.01 in fresh TSB, and grown to early exponential phase (OD of 0.2 to 0.5). Next, the cultures were diluted to a final OD of 0.01, and 100 μ l of the diluted cells were added to a XFp Tissue Culture Microplate precoated with poly-D-lysine (PDL). Cells were centrifuged at 1400 x g for 10 min to attach them to the PDL-coated surfaces. After centrifugation, 100 μ l of fresh TSB was added in each well. For LAC*, baseline OCR were measured for 3 cycles (9 min) before the antibiotics were injected. Readings were followed for 10 measurements after injection. For the PIM1 resistant mutants, baseline respiration was measured for 10 cycles. Each data point plotted was the average and standard deviation from 3 biological replicates.

2.7 Resistance study

In order to obtain PIM1-resistant mutants, the MRSA strain LAC* was serially passed with increasing PIM1 concentrations. On day one, exponentially growing bacteria cells were seeded in 100 μ l of TSB containing different concentrations (0.25xMIC, 0.5xMIC, 1xMIC, 2xMIC, 4xMIC) of PIM1 in a 96-well microplate. The plate was incubated at 37°C and 500 rpm. At 24 hr intervals, growth was checked by measuring OD₆₀₀. Cultures from the highest PIM1 concentration that allowed 50% growth was used to inoculate the next passage. The concentration of PIM1 was increased accordingly as resistance developed (0.25x, 0.5x, 1x, 2x, 4x of the new MIC). The sequential passaging was repeated until cultures reached 128x the original MIC of wildtype strain. 5 μ l of the liquid culture was streaked on a TSA plate. Single colonies were picked from the plates and their resistance was confirmed by MIC measurements. As a control, ciprofloxacin resistant mutants were selected using the same procedure [40]. 3 independent experiment of sequential passaging were done, all yielding cultures with high resistance.

2.8 Whole genome sequencing

Genomic DNA of wildtype LAC* and a select number of PIM1-resistant mutant strains was isolated with the Wizard Genomic DNA purification kit (Promega). Concentration of isolated DNA was measured with a NanoDrop spectrophotometer (ThermoFisher), and adjusted to 50 ng/μl. The DNA was cleaned up further with a DNA Clean up and Concentration Kit (Zymogen). The gDNA concentration was measured using a Qubit dsDNA HS Assay Kit (ThermoFisher), and diluted to 0.5 ng/μl in buffer (5 mM Tris pH8.5, 0.1% Tween 20). Next the samples were prepared for Illumina sequencing. To this end, tagmentation reaction were performed using the Nextera DNA Library Preparation Kit (Illumina). The tagmented DNA samples were amplified and barcoded by PCR using indexing primers. Tagmented and barcoded DNA samples were run on a 1.5% agarose gel at 100V for 20 min. Band intensity was analysed by ImageJ to normalize DNA concentrations, and samples were pooled. Large DNA fragments were removed by running the pooled DNA sample on a 1.5% agarose and excising the DNA bands ranging from 250 to 800 bp. The DNA was subsequently extracted from the gel with MinElute Gel Extraction Kit (QIAGEN) using a standard protocol, with one modification. The step requiring incubation at 50°C for 10 min to dissolve the gel was performed at room temperature using 2x the amount of buffer until the gel slice dissolved. As further DNA quality control, the purified sample was loaded on a High Sensitivity DNA Chip (Agilent) and the size distribution of DNA fragments was analysed using a Agilent 2100 Bioanalyzer instrument. The sample was sequenced on an Illumina Miseq instrument using a paired end 150 kit MRC LMS Genomics lab (Imperial College London Hammersmith Campus).

The CLC Genomics Workbench Software was used to identify genomic alterations in the PIM1-resistant mutants. Briefly, the Illumina reads of the WT LAC* strains were mapped to the USA300 FPR3757 genome sequence and annotations were transferred. The consensus sequence was generated for the LAC* strain and used as reference genome. Illumina reads for the PIM1-resistant mutants were mapped onto the consensus, and genomic variations (single nucleotide variations, small deletions and insertions) with a frequency at or above 65% were detected. Large deletions were found by manually searching the whole genome for zero coverage regions.

2.9 Menaquinone extraction and quantification

Intracellular menaquinone was extracted following a modified Bligh and Dyer protocol [41, 42]. Briefly, bacteria day cultures were grown to exponential phase (OD of 1). The cultures were diluted to 10^8 cfu/mL and treated with corresponding MIC concentrations of PIM1 (32 μ g/mL), PIM5 (128 μ g/mL), or daptomycin (32 μ g/mL) for 15 or 30 min. Treatment by the same volume of DI water was used as control. At the end of treatment period, bacteria cells were pelleted by centrifugation at 7000 rpm for 10 min. The cells were washed twice in PBS. The washed bacteria cells were pelleted again and the supernatant was removed. The bacteria pellets were frozen at -80°C and lyophilized in the FreeZone 2.5 Plus Benchtop Freeze Dryer (Labconco). To extract menaquinone from the dry biomass, 1 mL methanol-water (10:1) mixture and 1 mL hexane were added to 10 mg dry mass. The tubes were mixed on tube rotator for 15 min, and the upper layer was transferred to a small vial. Another 1 mL of hexane was added and the above-mentioned procedure was repeated. The extracted menaquinone was obtained by evaporating the combined upper layer in a stream of nitrogen gas ($<37^{\circ}\text{C}$). Menaquinone samples were analysed with high performance liquid chromatography (HPLC) at the Antimicrobial Resistance Interdisciplinary Research Group (AMR IRG) in Singapore-MIT Alliance for Research and Technology (SMART). The analysis was performed with Agilent 1290 ultrahigh pressure liquid chromatography system (Agilent), equipped with ZORBAX 300SB-C18 2.1x50mm 1.8 μ m RRHD column (Agilent). The major forms of menaquinone in MRSA contains seven (MK-7) or eight (MK-8) isoprene units [43]. Therefore, MK-7 and MK-8 were used as standards in the HPLC analysis. The relative abundance of menaquinone were calculated by

normalizing the peak intensity in the UV-vis spectra (254 nm) to bacterial dry mass.

2.10 Rhodamine-conjugated polymer binding assay

PIM1 was conjugated with the fluorescent dye rhodamine B (Sigma-Aldrich), to quantify the binding of polymers to the bacterial surface. The idea of using rhodamine B as a fluorescent label to track the interaction between PIM1 and *S. aureus* came from previous works where rhodamine B was conjugated to either peptides or synthetic polymers to investigate biochemical interactions [44, 45]. A single colony of bacteria was picked and used to inoculate the overnight culture in 5 mL TSB. On the next day, overnight culture was backdiluted to OD of 0.01 in fresh TSB, and grown to exponential phase (OD of 0.2 to 0.5). Bacteria cells were harvested by centrifuging 20 min day culture at 7,000 rpm for 10 min. The harvested cells were washed twice in PBS, and the final OD was adjusted to 0.4. Next, 4 mL aliquots of the bacteria suspension were transferred to Falcon tubes. Cells were pelleted and the supernatant was removed. The pellets were resuspended in 4 mL PBS containing 20 $\mu\text{g/mL}$ PIM1-rhodamine B conjugate, and incubated at room temperature. At 0, 30, 60 min time points, 1 mL bacteria suspension was removed and pelleted by centrifugation at the top speed for 2 min. The supernatant was removed and cells were resuspended in fresh PBS. The washing procedure was repeated to reduce background signal. The fluorescent output of bacteria suspension was measured at 553 nm excitation and 627 nm emission. Experiments were performed with 3 independent biological samples.

Chapter 3: Results and Discussions

3.1 Impact of PIM1 on the physiology of the MRSA

3.1.1 PIM1 has minimal membrane damaging effect

Due to the fact that bacteria surfaces are negatively charged [46], most cationic antibiotic polymers exert their antimicrobial action by electrostatic interactions with the cell envelope and physically disrupting the cytoplasmic membrane [47, 48]. To evaluate if PIM1 falls within the category of membrane-disrupting cationic polymers, membrane depolarization assay was performed with the fluorescent dye SYTOX green. Wildtype LAC* cells were incubated with SYTOX green after treatment by PIM1 (Fig. 1). Nisin, a known pore-forming antibiotic [49, 50], was used as a control. SYTOX green is a nucleic acid dye which cannot penetrate the cytoplasmic membrane under normal physiological conditions. The dye can only diffuse inside the bacteria when the physical integrity of the membrane is compromised. Therefore, a positive signal from the dye indicate a permeablized cytoplasmic membrane. After 1 h of treatment, only 11% of bacteria cells were permeablized by PIM1, while the membrane depolarizing antibiotic caused more than 30% depolarized cells with much stronger fluorescent signal (Table 1). This strongly suggests that, unlike conventional cationic antimicrobial polymers, physical disruption to the bacterial cell envelope is not the major mechanism for cell death caused by PIM1.

Figure 1. Cytoplasmic membrane permeability was assessed by nucleic acid dye SYTOX green. Intact cytoplasmic membrane is impermeable to SYTOX green. Microscopic pictures of LAC* WT treated 1 h with (A) control (DI water), (B) 8x MIC PIM1, (C) 8x MIC nisin are shown. The number of cells were counted with ImageJ software. The percentage of bacteria cells permeabilized by PIM1 is significantly lower than the percentage caused by nisin. Images are representative of 3 independent biological samples.

Table 2. Percentage of permeabilized cells after treatment

Treatment	% of permeable cells
No treatment	2.67%
PIM1 8x MIC	10.56%
Nisin 8x MIC	33.47%

The other criteria to look at membrane damage is proton motive force (PMF) or (trans)membrane potential. Many antibiotics target the cell membrane and cause dissipation of membrane potential, leading to dysregulation of a multitude of cellular functions [51, 52]. The impact of PIM1 on transmembrane potential was investigated with the fluorescent dye DiSC₃(5) (Fig. 2). DiSC₃(5) is a cationic membrane-permeable dye that can act as a potentiometric probe to reflect the PMF. Upon addition to energized cells, the dye penetrates the lipid

bilayer and accumulates inside the cell, as reflected by the initial decrease in fluorescent signal. The dye is rapidly released upon membrane depolarization, and the increase in fluorescence intensity is proportional to the extent of membrane potential dissipation. All agents were added at sub-lethal concentrations, and the data shown is representative of three biological replicates. As predicted from the SYTOX green assay, PIM1 induced minimal membrane potential dissipation compared to nisin during the 2 h measurement period. This observation, together with the SYTOX green assay, lead to the conclusion that the bacterial cytoplasmic membrane remained intact under the exposure to PIM1. Nisin is a representative membrane-perforating agent, and biosynthetic cationic antimicrobial polymers (e.g. ϵ -polylysine, α -poly-L-lysine, α -poly-D-lysine) have been reported to elicit the same concentration-dependent depolarization profile as nisin [53]. Since the response profile induced by PIM1 is distinctively different from that of nisin, it follows that PIM1 is not a membrane-active compound, despite its positive charges. It is likely that PIM1 has a different mode of action compared to common types of cationic polymers. The distinctive mechanism could be the reason for PIM1's superior broad spectrum antimicrobial activity against both Gram-positive and Gram-negative organisms.

Figure 2. Membrane potential assay using the fluorescent dye DiSC₃(5). Fluorescence intensity changes of DiSC₃(5) in LAC* cell suspensions treated with PIM1 (blue), nisin (red) and DI water (green) are plotted. Time points of dye and test compound addition are indicated with arrows. DiSC₃(5) is quenched upon accumulation inside the cell, leading to initial decrease in signal. Subsequent membrane potential dissipation results in increase in fluorescence. The data shown are representative of 3 biological samples.

3.1.2 Impact of PIM1 on macromolecular synthesis

Since PIM1 was found to be distinctively different from common types of membrane active cationic polymers, the question arises whether PIM1 behaves like small molecule antibiotics that target specific intracellular enzyme or protein. Therefore, the impact of PIM1 on major macromolecular synthesis pathways was investigated. In order to gain insight into the cellular pathways targeted by PIM1, DNA, RNA, peptidoglycan and protein synthesis was assessed in MRSA strain LAC* in the absence or presence of PIM1 (1x MIC or 4x MIC) and control antibiotics ciprofloxacin, rifampicin, vancomycin, erythromycin (4x MIC) known to inhibit one of the four specific macromolecular synthesis pathways [54-57]. This was done by following the incorporation of the radioactive-labelled precursors (³H-thymidine, ³H-uridine, ³H-N-acetyl-glucosamine, ¹⁴C-L-

glutamine) to assess DNA, RNA, peptidoglycan and protein synthesis, respectively. Ciprofloxacin, rifampicin, vancomycin and erythromycin inhibited as expected DNA, RNA, peptidoglycan and protein synthesis in LAC*. During the 20 min labelling period, PIM1 had virtually no effect on any of the biosynthesis pathways at 1x MIC (Fig. 3). DNA, RNA and protein synthesis (but not peptidoglycan synthesis) were inhibited when cells were treated with PIM1 at 4x MIC. However, the inhibition was weaker as compared to the control antibiotics used at 4x the MIC. Taken together, these data highlight that PIM1 affects multiple macromolecular biosynthesis pathways, since inhibition of DNA, RNA and protein synthesis was all observed. Therefore, PIM1 has pleiotropic effects on the cell and the inhibition of multiple cellular pathways is likely a downstream effect of the main action of PIM1.

Figure 3. Impact of PIM1 on major macromolecular biosynthesis pathways. ^3H -thymidine, ^3H -uridine, ^3H -N-glucosamine, ^{14}C -glutamine were used as precursors for DNA, RNA, peptidoglycan, and protein synthesis. PIM1 was tested at 1x and 4x MIC. Samples treated with ciprofloxacin, rifampicin, vancomycin, and erythromycin (4x MIC) were used as controls. The average and standard deviation from 3 biological replicates are plotted.

3.1.3 PIM1 blocks bacterial respiration

The impact of PIM1 on bacterial respiration was assessed by measuring the real-time oxygen consumption rate (OCR) with a Seahorse XFp Analyzer, where changes in oxygen concentration just above a bacteria monolayer is monitored using a fluorophore on a sensor chip [58, 59]. The platform measures OCR at picomole resolution, which can be used as a proxy to bacterial respiration rate [60]. Wildtype LAC* cells were tested in a standard rich medium (TSB), and the logarithmic increase in OCR for untreated sample is consistent with the doubling time of exponential growth phase. Treatment of LAC* with PIM1 induced rapid decrease in OCR (Fig. 4). A sub-MIC dose partially suppressed respiration, and doses above MIC concentration caused the OCR to decrease to near base-line level for the 60-min measurement period. The inhibition was dose-dependent and instantaneous, which suggests that the respiration suppression is an event prior to bacteria death. It was evidenced that PIM1 causes a block in the respiration chain. However, the mechanism leading to respiration inhibition by PIM1 and what role this plays in the overall mode of action remains unclear.

Figure 4. Antibiotics suppress bacterial respiration. Real-time changes in oxygen consumption rate (OCR) were measured on a Seahorse XFp Extracellular Flux Analyser. The first three data points are basal respiration rates, and the time point of compound injection is indicated an by arrow. Changes in OCR of wildtype LAC* treated with PIM1

at 0.25x, 1x, 4x MIC was compared against cells treated with rifampicin and erythromycin at 4x MIC. The data shown are representative of 3 biological replicates.

3.2 Development of resistant mutants and whole genome sequencing analysis

3.2.1 *S. aureus* develops resistance to PIM1 upon serial passage

To test if *S. aureus* can develop resistance to PIM1, 20 independent cultures of MRSA strain LAC* were passed in TSB containing increasing concentrations of PIM1 (for experimental details refer to the methodology section). Acquisition of resistance to ciprofloxacin was carried out as a comparison. To screen for potential strains of LAC* that became resistant to PIM1 by acquiring mutations in their genome, wild type bacteria cells were challenged with sub-inhibitory concentrations of PIM1 in liquid medium (Fig. 5). By the 15th day of the serial passage, all 20 cultures acquired resistance to PIM1. The speed of resistance acquisition was comparable to that of ciprofloxacin. The resistant cultures were able to withstand PIM1 treatment as high as 128 times the MIC observed for the wildtype LAC* strain. The liquid cultures were plated and single colonies were picked. After confirmation of resistance by MIC assays, the genomic DNA of 21 selected PIM1-resistant strains was isolated and variations in the genome were analysed by whole genome sequencing (Table 3).

Figure 5. Resistance acquisition during sequential passage in the presence of sub-inhibitory concentrations of antibiotics. The y-axis represents the highest concentration in which the cells grew to more than half of the untreated control population. The speed of resistance acquisition for PIM1 was comparable to ciprofloxacin. The highest concentration tested was 128x MIC for PIM1 and 64x MIC for ciprofloxacin. The figure is representative of 20 independent cultures treated by PIM1 and 4 independent cultures treated by ciprofloxacin.

Table 3. PIM1-resistant strains analysed by whole genome sequencing

Strain	Day of evolution	Resistance (# x MIC)
LAC*	-	-
ANG4944	13	64
ANG4945	12	>128
ANG4946	15	>128
ANG4947	12	>128
ANG4948	15	>128
ANG4949	13	>128
ANG4950	16	>128
ANG4951	15	>128
ANG4952	8	128
ANG5103	15	>128
ANG5104	15	>128
ANG5105	15	>128
ANG5106	15	>128
ANG5107	15	128
ANG5108	15	128
ANG5109	15	128
ANG5110	15	128
ANG5111	15	>128
ANG5112	15	>128
ANG5113	15	>128
ANG5114	15	>128

3.2.2 Genomic variations in PIM1-resistant strains

The whole genome sequencing analysis revealed that most PIM1-resistant strains had gene mutations belonging to 2 major pathways (Table 4). The full list of genomic variations can be found in the supplementary information (Table S1). Largest number of mutations were found related to the aerobic respiration. Every mutant strain sequenced contained at least one mutation related to the electron transport chain (ETC). Reoccurring mutations were also found on the cationic antimicrobial peptide (CAMP) resistance pathway, which is an innate mechanism bacteria developed to evade the activity of cationic antimicrobial peptides. These mutations provided valuable insights into the mode of action of PIM1.

Table 4. Common genetic variations found on multiple PIM1-resistant strains

Pathway	Gene ID	Annotation	Strain	AA change*
Cationic-antimicrobial peptide (CAMP) resistance	SAUSA300_0645 (<i>graR</i>)	DNA-binding response regulator	ANG4947, 4948	Phe13Val
			ANG4951	Thr11Ala
			ANG5107	Asp182Gly
			ANG5108	Glu184Lys
			ANG5111	Glu184Val
	SAUSA300_0646 (<i>graS</i>)	Signal transduction histidine kinase	ANG5108	Leu169Val
	SAUSA300_0648 (<i>vraG</i>)	ABC transporter permease	ANG4952	Gln454Pro
	SAUSA300_0647 (<i>vraF</i>)	ABC transporter ATP-binding protein	ANG5112	Ser195Pro
	SAUSA300_1255 (<i>fmtC</i>)	Oxacillin/methicillin resistance-related FmtC protein, controls lysylation of phosphatidylglycerol	ANG5104	Ala96Val
			ANG5105	Arg50Cys
			ANG5106	Gly61Glu
			ANG5109	Ser295Leu
			ANG5111	Arg50Cys
			ANG5112	Ser295Leu

Aerobic respiration	SAUSA300_0945 (<i>menF</i>)	Isochorismate synthases	ANG5103	Gly222Val
	SAUSA300_0946 (<i>menD</i>)	2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	ANG5104	Ser287Tyr
			ANG5105	Thr525fs
			ANG5109	Lys289fs
	SAUSA300_1737 (<i>menE</i>)	O-succinylbenzoate-CoA ligase	ANG4947, 4948	Ala34fs
			ANG4944, 4951	Thr314Lys
			ANG5106	Ser59Leu
	SAUSA300_0944 (<i>menA</i>)	1,4-dihydroxy-2 naphthoate octaprenyltransferase	ANG5111	Trp205*
			ANG5107	Ala288del
	SAUSA300_0948 (<i>menB</i>)	Naphthoate synthase	ANG5113	Arg19Ser
			ANG4949, 4950	Gly122Asp
	SAUSA300_1359 (<i>hepT</i>)	Polyprenyl synthase	ANG5110	Arg39Ser
	SAUSA300_0249 (<i>ispD</i>)	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	ANG4945, 4946	Ile49fs
			ANG4949, 4950	Gly16Asp
				Ile49fs
	SAUSA300_0844 (<i>ndh2</i>)	NADH dehydrogenase	ANG5112	Arg207*
			ANG5108	Trp49*
	SAUSA300_0962 (<i>qoxB</i>)	Cytochrome _{aa3}	ANG5108	Trp277*

* Amino acid change: fs refers to frameshift. * indicates stop codon.

3.2.2.1 Mutations related to bacterial surface charge

The CAMP resistance pathway is an innate defence mechanism bacteria developed to evade the activity of antimicrobial peptides. The main function of CAMP resistance pathway is repulsion of cationic polymers and peptides via modification to cell wall and membrane surface charges [61]. There are multiple components in the CAMP resistance mechanism (Fig. 6). The two-component GraRS regulatory system senses the presence of CAMP and upregulates the expression of the *dltABCD*, *mprF*, and *vraFG* operons [62]. In Gram-positive bacteria, the DltABCD enzymes mediate the D-alanylation of wall teichoic acid

(WTA) and lipoteichoic acid (LTA), and MprF converts the membrane lipid phosphatidylglycerol to lysyl-phosphatidylglycerol. Both processes contribute to repulsion, or reduced binding of CAMP, by neutralizing the bacterial surface charge. Moreover, GraRS also activates VraFG, which is in charge of exporting CAMP from the cell. The repulsion and exporting mechanisms together confer resistance to positively-charged membrane-active agents.

Several PIM1-resistant strains contained base substitution mutations in *graR,S*, *vraG,F*, and *fmtC* genes (*fmtC* directly controls the lysylation of phosphatidylglycerol). Notably, no frameshift or nonsense mutations were detected in these genes, and the base substitutions only led to single amino acid variations. Previously, it was shown that a mutant deficient in *graR* became more susceptible to CAMP [63]. Taking into consideration the functions of CAMP resistance genes, the mutations found in the PIM1-resistant strains most likely resulted in gain-of-function changes. These mutations likely increased the activity of CAMP resistance pathway, leading to a less negatively charged cell envelope, which reduced the electrostatic binding of PIM1. Notably, the gain-of-function changes were also observed for daptomycin-resistant strains [64], indicating that CAMP resistance related mutations are a common response to cationic antimicrobial agents.

Figure 6. Overview of the CAMP resistance pathway in *S. aureus*. CAMP induces the GraRS system, resulting in increased expression of Dlt operons, which D-alanylates teichoic acids, and MprF, which lysylates phosphatidylglycerol. Both processes lead to a less positively charged bacterial surface and reduces CAMP attraction. GraRS also activates VraGF which exports CAMP. Green annotations mark the mutations related to the CAMP resistance pathway as revealed by WGS analysis, and how the mutations likely contribute to the increased resistance to PIM1. Adapted from [61].

3.2.2.2 Mutations on the aerobic respiration chain

S. aureus has a branched ETC and NADH dehydrogenase is the first complex on the chain [65]. NADH dehydrogenase extracts electrons from NADH and pass them to menaquinone (MK, also known as vitamin K₂) by reducing MK to the quinol form. The electron shuttle MK then diffuses to proximity of the terminal oxidases. MRSA has two terminal oxidases, cytochrome aa₃ and cytochrome bd, each requiring a heme group as cofactor. Cytochrome aa₃ consists of 4 protein subunits, namely Qox-A, B, C, and D, and is the main terminal oxidase active under normal aerobic conditions. The other cytochrome bd has 2 components, Cyd-A and B, and is activated under micro-aerobic environment. A dual function of the two cytochromes was described [66], meaning that when one of the cytochromes is shut down, the other cytochrome can act as the alternative oxidase. As a result, the aerobic growth of *cydB* or *qoxB* single mutant bacteria was minimally affected. When both *cydB* and *qoxB* were deleted, however, the mutant was completely incapable of aerobic respiration.

Figure 7. Schematic showing aerobic respiration in *S. aureus*. The ETC is branched, with two terminal oxidases, namely cytochrome aa₃ and bd complexes. The dominant terminal oxidase, cytochrome aa₃, is composed of QoxA-D proteins, and requires heme A and heme B as cofactors. The other terminal oxidase, cytochrome bd, consists of CydA and CydB, and has heme B and heme O as cofactors [66]. Red annotations indicate how ETC mutations found in the PIM1-resistant strains affect aerobic respiration. Mutations include NADH dehydrogenase gene *ndh2*, menaquinone synthesis genes *ispD*, *hepT*, *menA*, *menB*, *menD*, *menE*, *menF*, and cytochrome aa₃ gene *qoxB*. Adapted from [33, 67].

Mutations in genes coding for ETC components were found in all mutant strains (Fig. 7). The mutations included NADH dehydrogenase gene *ndh2* and cytochrome aa₃ gene *qoxB*. Nonetheless, majority of mutations were related to the MK biosynthesis. Genomic variations were detected in *ispD*, *hepT*, *menA*, *menB*, *menD*, *menE*, *menF*, which all code for enzymes on the MK biosynthesis pathway. Synthesis of MK in bacteria is a well-established process [68], which includes formation of 1,4-dihydroxy-2-naphthoate (DHNA) from chorismate, and attachment of a polyprenyl chain to DHNA (Fig. 8). Frameshift and nonsense mutations were found in *ispD*, which codes for the 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) synthase in the non-mevalonate pathway [69]. As a result, the amount of MK precursors, isopentenyl diphosphate (IPP) and its

isomer dimethylallyl diphosphate (DMAPP), were likely to be greatly reduced. Moreover, a great number of *men* genes were inactivated, leading to less conversion of chorismate to DHNA. The overall consequence of these gene mutations was reduced production of menaquinone. Therefore, the PIM1-resistant strains likely have a less active aerobic respiration.

Figure 8. Isoprenoid production from the non-mevalonate pathway and production of MK. Red annotations show how inactivating mutations in the MK synthesis pathway reduce overall MK production. Adapted from [68, 69].

To confirm that the ETC mutations in PIM1-resistant strains were inactivating mutations, the basal respiration rates of the resistant strains in TSB were measured and compared against the wildtype and $\Delta hemB$ mutant (Fig. 9). Since the heme group is a necessary cofactor for both cytochromes, the $\Delta hemB$ mutant was completely unable to respire, as reflected by the flat OCR curve. Although the basal respiration rates of PIM1-resistant strains (ANG4944-4952)

varied depending on the exact mutations, they were all much slower than the exponentially increasing rate of the wildtype bacteria. This observation strongly suggests that aerobic respiration was reduced/blocked in the PIM1-resistant strains as part of their effort to escape the toxicity of PIM1.

Figure 9. Basal respiration rate of PIM1-resistance mutants. OCR assays were conducted on a Seahorse XFp Extracellular Flux Analyser. The bacteria strains were tested in TSB. The basal respiration rate was monitored for wildtype LAC*, LAC $\Delta hemB$, PIM1-resistant strains ANG4944-4952 for 1 h. The data shown are representative of 3 biological replicates.

3.3 Binding affinity to bacterial surface influences PIM1's activity

3.3.1 Inactivation of CAMP resistance pathway confers sensitivity to PIM1

Since mutations in several CAMP resistance genes were observed in PIM1-resistant strains, the effect of bacterial surface charge on antimicrobial activity of PIM1 was further investigated. Mutants with increased sensitivity to CAMP was obtained, namely $\Delta dltD$, Δdlt operon and $mprF::tn$ (Table 5). The dlt mutants lack D-alanine in their teichoic acid [70], and the $mprF$ mutant is unable to transfer L-lysine residue to membrane lipid phosphatidylglycerol [71]. As a result, both dlt and $mprF$ mutants are in particular vulnerable to cationic polymers. Despite that PIM1 does not depolarize cytoplasmic membrane like common CAMPs as discussed in section 4.1.2, the mutants $\Delta dltD$, Δdlt operon and $mprF::tn$ are still more sensitive to PIM1 (Table 5). It seems that although PIM1 does not kill bacteria by membrane disruption, the electrostatic interaction with bacteria surface is nonetheless a critical condition for PIM1 to bind.

Table 5. PIM1 activity against surface charge mutants

Strain	PIM1 MIC ₉₀ (μg/mL)
LAC*	2
LAC* $\Delta dltD$	0.25
LAC* Δdlt operon	0.25
LAC	2
LAC $mprF::tn$	0.5

3.3.2 Electrostatic adsorption is the first step of PIM1-bacteria interaction

To test the effect of surface charge mutations on PIM1's antimicrobial efficacy, the binding affinity of PIM1 to PIM1-resistant strain was evaluated. In order to visualize the accumulation of polymer on bacterial surface, PIM1 was labelled with the fluorescent dye rhodamine B. Wildtype LAC* and a PIM1-

resistant strain ANG4951 were exposed to a sub-inhibitory concentration of PIM1-rhodamine. The treated bacteria were washed thoroughly to make sure the fluorescence signal was proportional to the amount of polymer attached to the bacteria surface. The fluorescent intensity was normalized to OD₆₀₀ of bacterial suspension. From the binding assay (Fig. 10A), PIM1 binds instantaneously to wildtype bacteria, as reflected by the high fluorescent signal at 1 min. The polymer gradually accumulates on the surface, thus the increase in signal intensity over the 60 min period. In contrast, PIM1 had a hard time binding to the PIM1-resistant strain, the amount of PIM1 accumulated on the surface of ANG4951 was only half of the amount on wildtype cells. The reduced binding, likely caused by the less negatively charged bacteria surface, partly contributes to the resistance of ANG4951 (Fig. 10B). This strongly suggests that electrostatic attraction is the first step dominating PIM1-bacteria interaction.

Figure 10. (A) PIM1-rhodamine binding assay. Wildtype MRSA LAC* and PIM1-resistant strain AND4951 was treated with rhodamine-B labelled PIM1 during a 60 min period. The fluorescent signal was normalized to OD₆₀₀ of the bacterial suspension, and was directly proportional to the amount of PIM1-rhodamine bound to the surface of bacteria. (B) Dose-response curves of PIM1 against LAC* and ANG4951. The data shown is average and standard deviation from 3 biological replicates.

3.4 PIM's activity is dependent on respiration level

3.4.1 Respiratory-deficient mutants are less susceptible to PIM

The WGS analysis revealed that MRSA are less susceptible to PIM1 when aerobic respiration is inhibited. To investigate the specific relationship between respiration level and the activity of PIM1, the antimicrobial efficacy of PIM1 against several respiratory-deficient mutants was measured. A structural analog of PIM1 named PIM5 was also tested for comparison. PIM5 contains two extra oxygen atoms on the alkyl chain and thus exhibits higher hydrophilicity than PIM1. The list of respiratory-deficient mutants tested is shown in Table 6. Silencing *qoxB* inactivates the main terminal oxidase cytochrome aa₃ on the ETC, while silencing *cydA* effectively shuts down the alternative terminal oxidase cytochrome bd. It should be noted that the respiration and growth rates of *qoxB::tn* and *cydA::tn* were minimally affected due to the dual function of the cytochromes. When any one of the cytochromes was inhibited, bacteria could respire through the alternative cytochrome, hence the MIC is similar to wild type. In the case of $\Delta hemB$ and $\Delta menD$ where aerobic respiration was completely disabled, the bacteria was markedly more resistant to PIM1. Interestingly, the lack of heme and MK increased PIM1 resistance to different extents. Although both mutants have a completely inactive respiration chain, the resistance of $\Delta menD$ is higher than the resistance of $\Delta hemB$. The difference is especially pronounced for PIM5. This suggests that MK could potentially have direct interactions with the PIM series polymers.

Table 6. PIM1 MIC against respiratory-deficient MRSA mutants

Strain	MIC ₉₀ (μg/mL)	
	PIM1	PIM5
LAC/LAC*	2	8
LAC $\Delta menD$	16	≥ 2048
LAC $\Delta hemB$	4	128
LAC* <i>qoxB::tn</i>	2	32
LAC* <i>cydA::tn</i>	1	8

When exogenous MK and heme was supplemented in the growth medium for $\Delta menD$ and $\Delta hemB$, the resistance was partially reversed (Fig. 11A and B). Addition of MK and heme restored the ETC of MK- and heme-deficient mutants, and promoted bacterial growth. The untreated $\Delta menD$ grew to OD₆₀₀ 0.3 overnight without MK supplementation. In contrast, the overnight OD₆₀₀ of untreated sample was 0.5 in the presence of 10 μg/mL MK or 0.6 in 100 μg/mL MK. Similarly, addition of heme increased the overnight OD₆₀₀ of untreated sample from 0.3 to 0.6. This strengthened the observation that PIM's antimicrobial activity is closely linked to the level of aerobic respiration.

Interestingly, heme sensitized facultative anaerobe *Enterococcus faecalis* to PIM1 and PIM5. *E. faecalis* shares some phenotypic properties of respiratory-deficient MRSA strains because it lacks a functional ETC. Despite that genes encoding for type a and b cytochromes are present in *E. faecalis*, the strain is naturally defective for heme production [72, 73]. However, *E. faecalis* contains a heme uptake system, and therefore aerobic respiration can be activated in the presence of exogenous heme. The OD₆₀₀ of untreated *E. faecalis* increased from 0.3 to 0.55 with heme supplemented to growth medium. For both PIM1 and PIM5, the activation of aerobic respiration led to higher susceptibility (Fig. 11C).

Therefore, the relationship between PIM1's antimicrobial activity and aerobic respiration is not limited to *S. aureus*, but also applicable to other organism.

Figure 11. Dose-response curves of PIM1 and PIM5 against (A) LAC $\Delta menD$ with and without supplementation of 10 or 100 $\mu\text{g/ml}$ exogenous MK, (B) LAC $\Delta hemB$ and (C) *E. faecalis* VR583 with and without supplementation of 8 $\mu\text{g/ml}$ heme. The data shown are average and standard deviation from 3 biological replicates.

3.4.2 Intracellular menaquinone content decreases under exposure to PIM

The significantly higher resistance of $\Delta menD$ compared to $\Delta hemB$ raised the possibility that MK has direct interactions with PIM1 which might contribute to the antimicrobial activity. To investigate this further, the intracellular MK content in PIM-treated MRSA was measured by high performance liquid chromatography (HPLC). The major forms of MK in MRSA contains seven (MK-7) or eight (MK-8) isoprene units [43]. Therefore, MK-7 and MK-8 were used as standards in the HPLC analysis (Fig. 12A). The relative abundance of MK was calculated by normalizing the peak intensity in the LC-MS spectra (254 nm) to bacterial dry mass from which MK was extracted.

Changes in MK content after exposure to PIM1 and PIM5 are shown in Fig. 12B and C. Compared to control sample treated by DI water, both PIM1 and PIM5 induced significant decreases in the intracellular MK level. The decrease in MK-8 is more pronounced, mostly like because it is the more prominent form of MK in MRSA. It should be noted that the polymers were dosed at MIC concentrations and did not cause cell-death over the period of measurement (15 and 30 min). Therefore, the drop in MK content is most likely the cause for impaired bacterial fitness, instead of the result of cell death. Furthermore, samples treated with daptomycin did not lead to reduction in MK content (Fig. 12D), corroborating that the changes in MK content was not caused by membrane disruption. This observation suggests that MK not only affects the level aerobic respiration, but also has direct interactions with PIM1 and PIM5. There was a report of MK-targeting antibiotic lysocin E with cyclic peptide structure [28]. However, the interaction of lysocin E with MK was through non-covalent binding and subsequent membrane depolarization, which differs from the

mechanism observed for PIM1. Nonetheless, further biochemical investigations are needed to discover the detailed reaction mechanism between PIM and MK.

Figure 12. (A) UV-vis spectrum (254 nm) of MK extract from MRSA dry mass. The elution peak for MK-7 was observed at 10.4 min, and the peak for MK-8 was eluted at 12 min. Changes in relative MK content after 15 and 30 min exposure to MIC concentration of (B) PIM1, (C) PIM5 and (D) daptomycin were observed. The LC-MS peak intensity were normalized to dry biomass. The data shown are average and standard deviation from 3 independent biological samples.

3.5 Relationship between the 2 categories of genomic variations

From the whole genome sequencing analysis discussed in section 3.2, 2 major pathways have been identified to be responsible for the resistance mechanism, namely the CAMP resistance pathway and aerobic respiration. Section 3.3 and 3.4 have been dedicated to understanding the role these 2 pathways play in the resistance mechanism.

The main function of CAMP resistance pathway is to add positive charges to the bacteria envelope and decrease binding of cationic agents. In section 3.3, strains lacking CAMP resistant genes ($\Delta dltD$, Δdlt operon and *mprF::tn* mutants) were found to be more susceptible to PIM1. Therefore, electrostatic adsorption was one of the determinants of PIM1's antimicrobial activity. This was confirmed by the assay with rhodamine B-conjugated polymer, where PIM1-resistant strains attracted less polymer to the surface comparing to the wildtype strain.

In section 3.4, a series of experiments were conducted to show that PIM1's activity was dependent on the level of aerobic respiration. When components of the aerobic respiration chain were knocked out, the bacteria show decreased susceptibility to PIM1. Moreover, menaquinone was found to be a potential target of PIM1, since deletion of menaquinone synthesis gene had the most impact on resistance, and PIM1 treatment induced a reduction in intracellular menaquinone content.

It is hard to judge which pathway plays a more important role in the resistance mechanism. The 2 pathways affect different stages of PIM1's activity. Firstly, as a cationic agent, PIM1 adsorbs onto the negatively-charged bacteria envelope by electrostatic attraction, a process influenced by the activity

of CAMP resistance pathway. When CAMP resistance genes acquire gain-of-function mutations, bacteria have more positive charges on the surface, leading to decreased adsorption of PIM1 and increased resistance. Secondly, perturbation of aerobic respiration occurs after the electrostatic adsorption of PIM1. Strains with a less active aerobic respiration chain show less susceptibility to PIM1. It was no surprise to observe genomic variations in both pathways for the PIM1-resistant strains. In fact, it is plausible to conclude that the activity of both pathways need to be altered in order to achieve a high resistance.

Chapter 4: Conclusion

With rapid development of antimicrobial resistance, there is an ever-growing need for new strategies to combat bacterial infections. However, the lack of new antimicrobial mechanisms and targets has hindered the discovery of antibiotics. The PIM-series antimicrobial polymers also carry positive charges. Nonetheless, they have been found to exert a distinctively different mechanism from cationic polymers previously characterized. PIM1 did not physically disrupt the membrane or cause membrane potential dissipation, which sets it apart from common membrane active compounds. Moreover, PIM1 had pleiotropic effects on multiple biosynthesis pathways in MRSA, including DNA, RNA, protein synthesis. This indicated that PIM1 did not have a specific enzyme target. Real-time monitoring of OCR revealed that PIM1 instantaneously blocked aerobic respiration, preceding cell death.

WGS analysis of PIM1-resistant MRSA strains developed by resistance evolution revealed 2 major pathways crucial for PIM1's activity. Firstly, many genomic variations were related to the CAMP-resistance pathway. These mutations likely resulted in a less negatively charged bacteria surface, reducing the electrostatic adsorption of PIM1. By labelling PIM1 with fluorescent dye rhodamine B, it was found that PIM1 binds significantly less to PIM1-resistant strains with mutations in CAMP-resistance pathway. This evidenced that despite PIM1 does not cause membrane perturbations like other cationic polymers, the electrostatic interaction still dominates the initial binding stage. Secondly, several genes related to synthesis of ETC components were found, which validates the postulation that PIM1 blocks aerobic respiration. Above all, the synthesis of electron shuttle MK was found to be most disabled in response to

PIM1-treatment. Indeed, studies with respiratory-deficient MRSA mutants revealed that the activity of PIM1 is strongly linked to the level of aerobic respiration, and more importantly, that PIM1 potentially has direct interactions with MK. By isolating the isoprenoid fraction from PIM1-treated bacteria and detecting with HPLC, PIM1 and its derivative PIM5 were found to greatly reduce the MK content.

This study discovered a new antimicrobial mechanism, which has not been reported before. It adds a new dimension to the development of cationic antimicrobial polymers. The action of PIM1 likely takes place in a 2-step process. The cationic polymer first binds to the negatively charged bacteria surface via electrostatic adsorption. The polymer then perturbs aerobic respiration, possibly by direct interactions with ETC component MK. More in-depth characterization and investigations are needed to find out the detailed interaction mechanism between PIM1 and MK. Moreover, it is important to understand how the perturbation of aerobic respiration leads to cell death.

The fact that *S. aureus* resistant cultures evolved within a period of 2 weeks and were able to withstand PIM1 treatment of 128x the original MIC did not align with the low propensity to elicit resistance for common cationic antimicrobial polymers. Nonetheless, this is very likely caused by PIM1 having a distinctively different antimicrobial mechanism than other cationic polymers. The PIM1-resistant strains were useful in understanding the mechanism of PIM1, and the knowledge obtained for *S. aureus* can provide inspiration for studies in other organisms.

Supplementary information

Table S 1. Full list of genomic alterations in PIM1-resistant mutants

Strain no.	Type ¹	Ref ²	Allele	Freq.	Annotation	AA change ³
ANG4945, ANG4946	INS	-	A	100	SAUSA300_0249 (<i>ispD</i>), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Ile49fs
	DEL	G	-	100	SAUSA300_0993 (<i>pdhA</i>), pyruvate dehydrogenase E1 component	Ala159fs
	SNV	C	T	100	SAUSA300_1499 (<i>aroK</i>), Shikimate kinase	Gly17Asp
ANG4947, ANG4948	SNV	G	A	100	SAUSA300_0250 (<i>tarJ</i>), Ribitol-5-phosphate dehydrogenase	Gly333Asp
	SNV	T	G	100	SAUSA300_0645 (<i>graR</i>), DNA-binding response regulator, involved in CAMP resistance	Phe13Val
	INS	-	A	100	SAUSA300_1252 (<i>alsT</i>), Na ⁺ /alanine symporter	Glu455fs
	DEL	G	-	100	SAUSA300_1737 (<i>menE</i>), O-succinylbenzoate-CoA ligase	Ala34fs
ANG4949, ANG4950	SNV	G	A	100	SAUSA300_0249 (<i>ispD</i>), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Gly16Asp
	INS	-	A	98.3		Ile49fs
	SNV	T	C	100	SAUSA300_0844 (<i>ndh2</i>), NADH dehydrogenase	Leu270Ser
	DEL [‡]	-	-	-		fs
	SNV	G	A	100	SAUSA300_0948 (<i>menB</i>), naphthoate synthase	Gly122Asp
	SNV	T	G	100	SAUSA300_0955 (<i>atl</i>), Autolysin.	Asp343Ala
	SNV	G	A	97.7	SAUSA300_1733, Dipeptidyl aminopeptidases	Pro228Ser
	SNV	G	A	100	SAUSA300_2025 (<i>rsbU</i>), Serine phosphatase	Thr66Ile
	INS	-	T	100	SAUSA300_2323 (<i>cobI</i>), Mg ²⁺ and Co ²⁺ transporters	Lys173fs

	SNV	G	A	100	SAUSA300_2386 (<i>flp</i>), beta-lactamase	Trp260*
ANG4944, ANG4951	SNV	C	G	100	SAUSA300_0415 (<i>lpl3</i>), tandem lipoprotein.	Asp152Glu
	SNV	A	G	100	SAUSA300_0645 (<i>graR</i>), DNA-binding response regulator, involved in CAMP resistance	Thr11Ala
	SNV	A	G	100	SAUSA300_1252 (<i>alsT</i>), Na ⁺ /alanine symporter	Ile251Val
	SNV	A	G	100	SAUSA300_0971 (<i>purI</i>), Phosphoribosylformylglycinamide synthase II	Thr314Lys
	SNV	G	T	100	SAUSA300_1737 (<i>menE</i>), O-succinylbenzoate-CoA ligase	Thr314Lys
ANG4952	SNV	A	C	100	SAUSA300_0648 (<i>vraG</i>), ABC transporter permease. Involved in CAMP resistance.	Gln454Pro
	SNV	C	A	100	SAUSA300_1442 (<i>srrA</i>), Respiratory response protein SrrA	Glu31*
ANG5103	SNV	G	T	100	SAUSA300_0945 (<i>menF</i>), isochorismate synthases	Gly222Val
	SNV	T	A	100	SAUSA300_1077 (<i>murD</i>), peptidoglycan biosynthesis	Leu2His
	SNV	G	A	100	SAUSA300_2025 (<i>rsbU</i>), serine phosphatase	Ala127Val
	SNV	G	A	100	SAUSA300_2230 (<i>modA</i>) ABC-type molybdate transporter	Thr150Ile
	SNV	C	T	100	SAUSA300_2323 (<i>cobI</i>), Mg ²⁺ and Co ²⁺ transporters	Gln232*
ANG5104	SNV	C	A	100	SAUSA300_0946 (<i>menD</i>), 2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	Ser287Tyr
	SNV	T	A	100	SAUSA300_1167 (<i>pnpA</i>), polynucleotide phosphorylase/polyadenylase	Leu338Ile
	SNV	C	T	100	SAUSA300_1255 (<i>fmtC</i>), oxacillin/methicillin resistance-related FmtC protein	Ala96Val
	SNV	C	A	100	SAUSA300_1370 (<i>ebpS</i>), cell surface elastin binding protein	Gly471Val
	SNV	A	T	100	SAUSA300_2025 (<i>rsbU</i>), serine phosphatase	Ile304Asn
	SNV	G	C	100	SAUSA300_2323 (<i>cobI</i>), Mg ²⁺ and Co ²⁺ transporters	Trp26Ser
ANG5105	SNV	G	A	100	SAUSA300_0022 (<i>walH</i>), uncharacterized protein	Ala173Thr

	SNV	C	T	100	SAUSA300_0279 (<i>esaA</i>), membrane protein, involved in protein secretion	Gln372*
	SNV	G	T	100	SAUSA_0910 (<i>mgtE</i>), magnesium transporter MgtE	Asp237Tyr
	DEL	A	-	91.7	SAUSA300_0946 (<i>menD</i>), 2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	Thr525fs
	SNV	C	T	100	SAUSA300_1255 (<i>fmtC</i>), oxacillin/methicillin resistance-related FmtC protein	Arg50Cys
	SNV	A	T	100	SAUSA300_1267 (<i>trpB</i>), tryptophan synthase beta subunit	Leu195Phe
	INS	-	T	95.2	SAUSA300_2025 (<i>rsbU</i>), serine phosphatase	Cys28fs
	INS	-	T	95.2	SAUSA300_2025 (<i>rsbU</i>), serine phosphatase	Cys28fs
ANG5106	SNV	C	G	100	SAUSA300_0869 (<i>rexB</i>), exonuclease RxB, DNA metabolism	Ala489Gly
	SNV	G	T	100	SAUSA_0910 (<i>mgtE</i>), magnesium transporter MgtE	Met102Ile
	SNV	C	G	100	SAUSA300_1154 (<i>cdsA</i>), cytidyltransferase	Ala113Gly
	INS	-	A	95.2	SAUSA300_1252 (<i>alsT</i>) Na ⁺ /alanine symporter	Glu455fs
	SNV	G	A	100	SAUSA300_1255 (<i>fmtC</i>), oxacillin/methicillin resistance-related FmtC protein	Gly61Glu
	SNV	G	A	100	SAUSA300_1737 (<i>menE</i>), o-succinylbenzoate-CoA ligase	Ser59Leu
	SNV	G	A	100	SAUSA300_2025 (<i>rsbU</i>), serine phosphatase	Gln6*
ANG5107	SNV	A	G	100	SAUSA300_0645 (<i>graR</i>), DNA-binding response regulator, CAMP resistance	Asp182Gly
	DEL	CAG	-	87.5	SAUSA300_0944 (<i>menA</i>), 1,4-dihydroxy-2-naphthoate octaprenyltransferase	Ala288del
	DEK	CTT	-	83.9	SAUSA300_2022 (<i>rpoF</i>), RNA polymerase sigma factor SigB	Glu149del
	SNV	C	G	100	SAUSA300_2323 (<i>cobI</i>), Mg ²⁺ and Co ²⁺ transporters	His129Asp
ANG5108	SNV	G	A	95.8	SAUSA300_0623 (<i>tagA</i>), teichoic acid biosynthesis proteins	Trp227*
	SNV	G	A	100	SAUSA300_0645 (<i>graR</i>), DNA-binding response regulator, CAMP resistance	Glu184Lys
	SNV	C	G	100	SAUSA300_0646 (<i>graS</i>), signal transduction histidine kinase, CAMP resistance	Leu169Val

ANG5109	INS	-	T	100	SAUSA300_0668, recombination and DNA strand exchange inhibitor protein	Ser139fs
	SNV	G	A	100	SAUSA300_0844 (<i>ndh2</i>), NADH dehydrogenase	Trp49*
	SNV	C	T	100	SAUSA300_0962 (<i>qoxB</i>), heme/copper-type cytochrome/quinol oxidases	Trp277*
	DEL	A	-	100	SAUSA300_1193 (<i>glpD</i>), glycerol-3-phosphate dehydrogenase, energy metabolism	Met139fs
	SNV	A	C	100	SAUSA300_0279 (<i>esaA</i>), membrane protein, involved in protein secretion	Lys379Gln
	SNV	C	T	100	SAUSA300_0800 (<i>sek</i>), staphylococcal enterotoxin K	Val136Ile
	DEL	A	-	93.75	SAUSA300_0946 (<i>menD</i>), 2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	Lys289fs
	SNV	T	A	100	SAUSA300_1193 (<i>glpD</i>), glycerol-3-phosphate dehydrogenase, energy metabolism	Leu146Ile
	SNV	C	T	100	SAUSA300_1255 (<i>fmtC</i>), oxacillin/methicillin resistance-related FmtC protein	Ser295Leu
	SNV	C	T	100	SAUSA300_1483, hypothetical protein	Arg47His
	SNV	G	T	100	SAUSA300_1604 (<i>mreD</i>), rod shape-determining protein, cell envelope biogenesis	Pro156Thr
	SNV	C	A	100	SAUSA300_2359 (<i>tcyA</i>), amino acid ABC transporter	Gly91Trp
	DEL	T	-	100	SAUSA300_2454, membrane spanning protein	Cys184fs
	DEL	T	-	100	SAUSA300_2498 (<i>crtN</i>), squalene synthase	Asn32fs
ANG5110	SNV	C	A	100	SAUSA300_0027, hypothetical protein	Ala389Glu
	SNV	T	C	100	SAUSA300_1255 (<i>fmtC</i>), oxacillin/methicillin resistance-related FmtC protein	Leu341Ser
	SNV	G	T	100	SAUSA300_1359 (<i>hepT</i>), polyprenyl synthase, involved in isoprenoid synthesis	Arg39Ser
	INS	-	A	96.2	SAUSA300_2127 (<i>sepA</i>), multidrug resistance efflux pump	Leu31fs
ANG5111	SNV	A	G	100	SAUSA300_0369, hypothetical protein	Tyr281His
	SNV	A	T	100	SAUSA300_0645 (<i>graR</i>), DNA-binding response regulator, CAMP resistance	Glu184Val

ANG5112	SNV	C	T	100	SAUSA300_0989 (<i>mjA</i>), mRNA degradation ribonucleases	Arg209His
	SNV	C	T	100	SAUSA300_1255 (<i>fmtC</i>), oxacillin/methicillin resistance-related FmtC protein	Arg50Cys
	SNV	C	T	100	SAUSA300_1737 (<i>menE</i>), o-succinylbenzoate-CoA ligase	Trp205*
	SNV	C	T	100	SAUSA300_0249 (<i>ispD</i>), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Arg207*
	SNV	T	C	100	SAUSA300_0647 (<i>vraF</i>), ABC transporter ATP-binding protein	Ser195Pro
ANG5113	SNV	C	T	100	SAUSA300_1255 (<i>fmtC</i>), oxacillin/methicillin resistance-related FmtC protein	Ser295Leu
	SNV	G	T	100	SAUSA300_1481, Gene: SAUSA300_1481	Thr224Lys
	SNV	G	T	100	SAUSA300_1541 (<i>grpE</i>), heat shock protein GrpE	Pro112Thr
	SNV	G	T	100	SAUSA300_0251 (<i>tarL</i>), putative teichoic acid biosynthesis protein, cell envelope biogenesis	Gly314Val
	SNV	C	T	100	SAUSA300_0821 (<i>sufU</i>), NifU family SUF system FeS assembly protein	Ala142Val
	SNV	G	T	100	SAUSA300_0944 (<i>menA</i>), 1,4-dihydroxy-2-naphthoate octaprenyltransferase	Arg19Ser
	DEL	TATC	-	94.6	SAUSA300_2025 (<i>rsbU</i>), serine phosphatase	Asp303fs
	SNV	G	A	100	SAUSA300_2323 (<i>cobI</i>), Mg ²⁺ and Co ²⁺ transporters, inorganic ion transport and metabolism	Val262Ile

¹ Type of mutation: INS stands for insertion, DEL stands for deletion. DEL[‡] denotes a multiple deletion of 54 bp. SNV is short for single nucleotide variant.

² Base in reference genome

³ Amino acid change: fs refers to frameshift. * indicates stop codon.

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