Anti-virulence approaches and novel peptidomimetics for combating resistant and biofilm associated bacteria

Liu, Yang

Publication date:
2012

Document Version
Publisher's PDF, also known as Version of record

Anti-virulence approaches and novel peptidomimetics for combating resistant and biofilm associated bacteria

Ph.D. Thesis

by

Yang Liu

Infection Microbiology Group
Center for Systems Microbiology
Department of Systems Biology
Technical University of Denmark

June 2012

Preface

This thesis is submitted as a partial fulfillment of the requirements to obtain the Ph.D. degree in Technical University of Denmark (DTU). The work presented was carried out in the Infection Microbiology Group, Centre of Systems Microbiology, Department of Systems Biology in DTU under the supervision of Prof. Dr. Søren Molin and Associate Prof. Dr. Anders Folkesson. The work was performed from January 2009 to June 2012. This Ph.D. project was co-financed by DTU and Danish Research Council.

Yang Liu 刘洋

Kgs. Lyngby

June 2012

Acknowledgements

First and foremost, I would like to thank my supervisor Professor Søren Molin. I appreciate Søren to give me the chance to perform my PhD study in his group. Without his guidance and support I would not have been able to complete this thesis. Thank you for creating a great scientific environment with an international atmosphere and plenty of visitors for new inspiration.

I would also like to thank my mentor Associate Professor Anders Folkesson, who has shown an enormous support for me during this thesis.

I enjoyed working with Rasmus, Søren Damkiær, María, Lei, Vinoth, Anna, Nicholas, Martin Holm Rau, Martin Weiss Nielsen, Susse, Fatima and Juliane from Søren's group and appreciate for your helps. Thanks Associate Professor Claus Sternberg for your supporting of the equipment training and Associate Professor Lars Jelsbak for your creative discussion about sciences.

I would like to thank Tove Johansen, Susanne, Lisser St. Clair-Norton for all your efforts to keep the lab clean and tidy. Thanks to our secretaries Mette Munk, Pernille Winther and Hanne Christiansen for helping me with the administrative issues.

The project was started by the creative work from Associate Professor Henrik Franzyk and his colleagues at University of Copenhagen. Thanks for providing large amount of compounds for my lab work. It is glad to hear that Sara, Christian and Associate Professor Mogens Kilstrup are going to continue to find more information of the peptidomimetic project.

I would like to acknowledge the neighbour groups in the building, led by Prof. Peter R. Jensen for interesting discussions during seminars. I also would like to thank our collaborators at Panum Institute and Rigshospitalet from University of Copenhagen. Especially, I would like to thank Hong Wu, Zhijun Song and Wang Hengzhuang for their guidance about science and social life in Denmark.

I want to express my gratitude to my parents for their endless support and my husband Liang Yang for being a role model of enthusiastic scientist.

Singapore, June 2012, Yang Liu

Abstract

Anti-virulence approaches and novel peptidomimetics for combating resistant and biofilm associated bacteria

The misuse and overuse of antibiotics has a broad impact on the environment. Antibiotic resistance has become a major threat for modern medical treatment of infectious diseases. There are multiple mechanisms leading to antibiotic resistance such as expression of cell membrane efflux pumps and antibiotic-degrading enzymes. Moreover, bacterial biofilm communities are widely accepted as a major resistance mechanism in infection sites.

Biofilms are surface-associated microbial communities consisting of microcolonies embedded in self-produced extracellular polymer substances (EPS). EPS can contribute to cell-cell adhesion and restrict antibiotic penetration. Biofilm cells show much greater resistance to stressful conditions than their free-living counterparts. Conventional treatment strategies could not eradicate biofilm-related infections, such as biofilm infections related to medical implants and chronic wounds. There is a need for developing anti-biofilm therapeutics.

Biofilm formation is a dynamic and complicated process which requires cell surface structures (e.g. type IV pili), motility, chemotaxis, subpopulation differentiation, iron signaling and quorum sensing and so on. Targeting these mechanisms might provide alternative strategies to conventional antimicrobial treatment. Microbial cells inside biofilms resemble planktonic cells in stationary phase such as their slow growth rate. Thus development of antimicrobial compounds which are effective against slow growing cells might also be another useful strategy.

This Ph.D. project aimed at developing effective treatment strategies against biofilms formed by two model organisms, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. *P.aeruginosa* is a gram-negative opportunistic pathogen which causes a variety of severe human infections and diseases, including colonization of the lungs of cystic fibrosis (CF) patients and infection of burns and immunecompromised patients. *S.epidermidis* is a gram-positive nosocomial pathogen which frequently causes infections associated with implanted foreign materials. In this study, quorum-sensing interfering compounds, iron chelators and efflux pump inhibitors (EPI) have been used for controlling *P. aeruginosa* biofilms. A series of novel peptidomimetics (α-peptide/β-peptoid chimeras) have been tested against cells from stationary growth phase and biofilms of *S. epidermidis*. Structure-activity relationship and cytotoxicity features of these peptidomimetics were explored. Transcriptomic, genomic and adaptive evolution approaches have been applied to address the potential risk of resistance of peptidomimetics, which in turn provide useful information for designing the next generation peptide based anti-biofilm compounds.

Publication list

1. **Liu Y**, Marvig R, Jendresen C, Dühring S, Yang L, Franzyk H, Kilstrup M, Jelsbak L, Molin S, Folkesson A. Stress response and resistant development to antimicrobial peptidomimetics by *Staphylococcus epidermidis* (Manuscript in preparation.)

2. Liu Y, Knapp K, Yang L, Molin S, Franzyk H, Folkesson A. α-peptide/β-peptoidchimeraswith low cytotoxicity exhibit in vitro antimicrobial and antibiofilm activity (Submitted.)

3. Jochumsen N, **Liu Y**, Molin S, Folkesson A. A Mig-14-like protein (PA5003) affects antimicrobial peptide recognition in *Pseudomonas aeruginosa*. Microbiology. 2011, 157 (Pt 9): 2647-2657.

4. Yang L, **Liu Y**, Markussen T, Høiby N, Tolker-Nielsen T, Molin S. Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. FEMS Immunology and Medical Microbiology. 2011, 62(3): 339-347.

5. Yang L, Hu Y, **Liu Y**, Zhang J, Ulstrup J, Molin S. Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. Environmental Microbiology. 2011, 13(7): 1705-1717.

6. Yang L, **Liu Y**, Wu H, Hoiby N, Molin S, Song Z. Current understanding of multi-species biofilms. International Journal of Oral Science. 2011, 3(2): 74-81.

7. Liu Y, Yang L, Molin S. Synergistic activities of an efflux pump inhibitor and iron chelators against *Pseudomonas aeruginosa* growth and biofilm formation. Antimicrobial Agents and Chemotherapy. 2010, 54(9):3960-3963.

8. Yang L, **Liu Y**, Sternberg C, Molin S. Evaluation of Enoyl-Acyl Carrier Protein Reductase Inhibitors as *Pseudomonas aeruginosa* Quorum-Quenching Reagents. Molecules. 2010, 15: 780-792.

9. Yang L, **Liu Y**, *etc*. Combating biofilms. FEMS Immunol Med Microbiol. 2011 Aug 24. doi: 10.1111

Table of Contents

1 Introduction

This initial chapter aims at introducing the problems caused by bacterial antibiotic resistance and biofilms.

1.1 Bacterial antibiotic resistance

The discovery of penicillin has initiated a period of drug innovation in the treatment of bacterial infectious diseases. Many types of antibiotics have been discovered based on their capability to either kill bacteria (bactericidal) or inhibit their growth (bacteriostatic). **Figure 1** shows the action model of three commonly used antibiotics: ciprofloxacin, tobramycin and colistin. Ciprofloxacin is a fluoroquinolone and exhibits broad-spectrum antimicrobial activity by inhibiting the function of DNA-gyrase, which resulting in inhibition of DNA replication in bacteria. Tobramycin is an aminoglycoside and exhibits its antimicrobial activity by binding to the bacterial 30S and 50S ribosome, thus preventing protein synthesis in bacteria. The bactericidal colistin (also called polymyxin E) belongs to the polymyxin group of antibiotics that binds to lipopolysaccharides and phospholipids of the outer cell membrane of Gram-negative bacteria and cause membrane permeabilization.

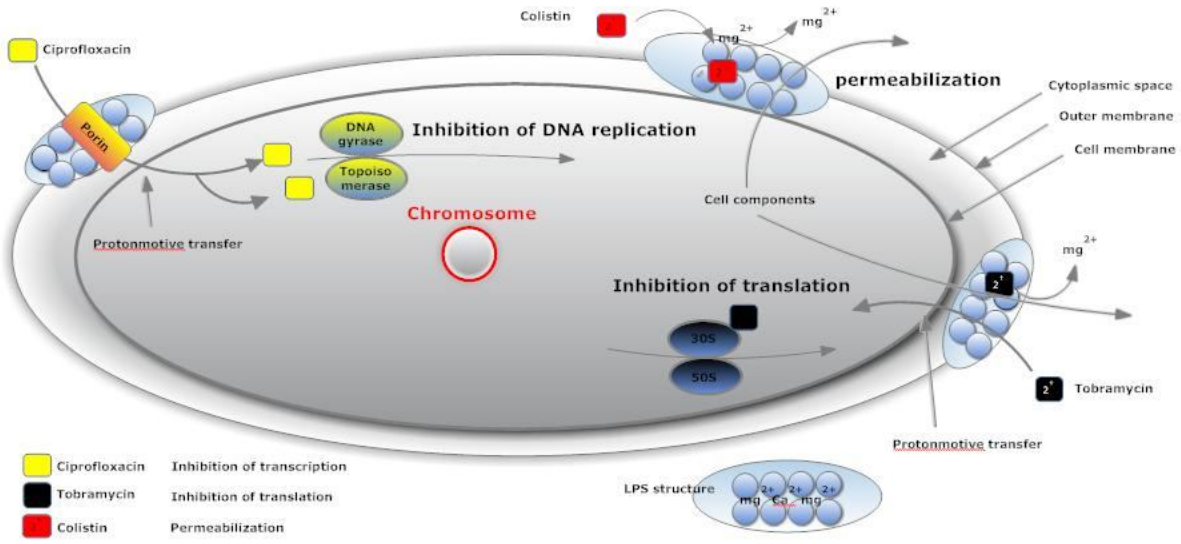


Figure 1.Modes of action for ciprofloxacin, tobramycin and colistin. (Drawn by Frederik and Morten)

However, the high turnover rate of bacteria has made it able to evolve resistant mutants to almost any classes of regular antibiotics in a short time (Maki and Schuna 1978; Martinez and Baquero 2000).

The inappropriate usage of antibiotics has led to the emergence of antibiotic-resistant bacteria in modern society. **Figure 2** outlines the history of antibiotic development and the rise of antibiotic resistance.

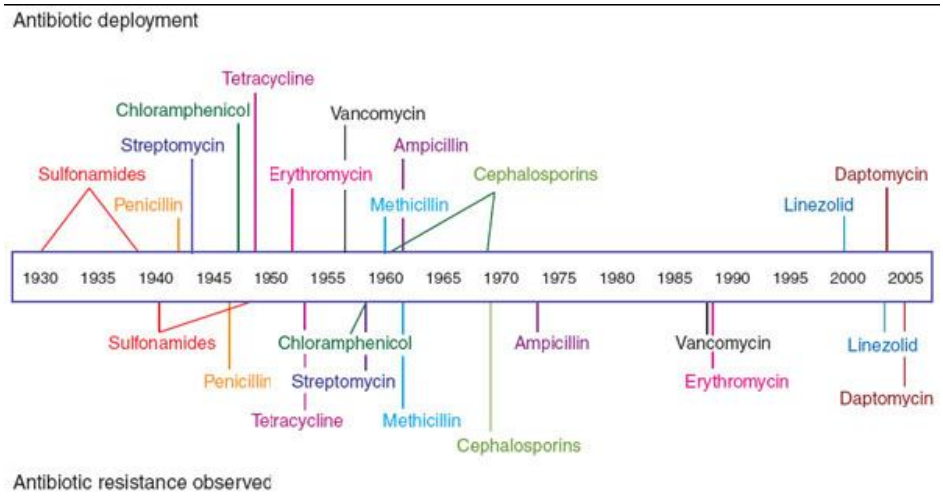


Figure 2.Schedule of antibiotic deployment and the rise of antibiotic resistance. The year each antibiotic was deployed is depicted above the timeline, and the year resistance to each antibiotic was observed is depicted below the timeline(Clatworthy, Pierson et al. 2007).

Several different molecular mechanisms contribute to antibiotic resistance, including enzymatic deactivation of antibiotics, reduction of cell wall permeability to antibiotics, alteration of target sites of antibiotic, induction of efflux mechanisms to remove antibiotics (**Figure 3**) (Hawkey 1998).

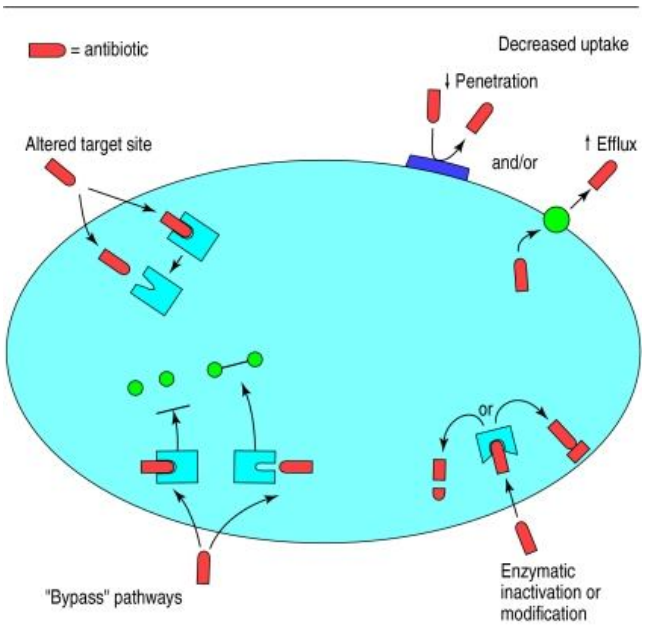


Figure 3.Four major biochemical mechanisms of antibiotic resistance(Hawkey 1998).

1.2 Biofilm mediated antibiotic resistance

In recent years, microbial biofilms are recognized to contribute significantly to medical infections and cause tremendous problems for their treatment. Biofilms are microbial matrix-enclosed aggregations of cells adhering to biological and non-biological surfaces (Hoyle and Costerton 1991; Costerton, Lewandowski et al. 1995; Hall-Stoodley, Costerton et al. 2004). Bacteria grown in biofilms can become 10 -1,000 times more resistant to antimicrobial agents than their planktonic counterparts(Hoyle and Costerton 1991).Biofilms are proposed to be responsible for 65% of all bacterial infections (Potera 1999). Figure 4 shows biofilms formed by *P. aeruginosa* from a scratch sample of airway surface taken from a patient with persistent *Pseudomonas* infection (Kobayashi 2001).

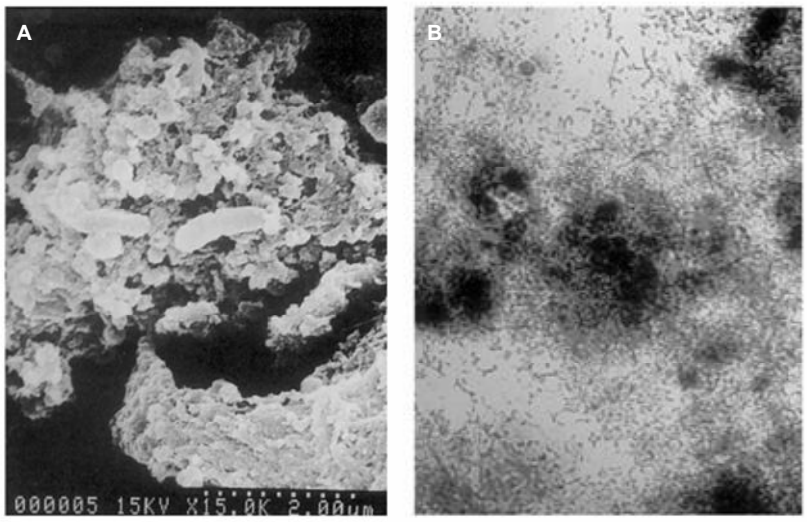


Figure 4.Biofilm formed from *P. aeruginosa*. A: scratch sample of airway surface taken from a patient with persistent *Pseudomonas* infection. B: Sputum from exacerbated infection showing planktonic bacteria released from a piece of biofilm(Kobayashi 2001).

There are numerous mechanisms underlying biofilm-associated antibiotic resistance. The EPS materials can efficiently reduce the diffusion of antimicrobial agent to the deeper part of biofilms (Gulot, Georges et al. 2002). Subpopulations can be differentiated during biofilm growth which have distinct physiology and confer tolerance towards different classes of antibiotics (Pamp, Gjermansen et al. 2008). Persistent cells are frequently found in antibiotic treated biofilms which can spread and form new biofilms (Pamp, Gjermansen et al. 2008). Thus, a better understanding of biofilm physiology can facilitate development of anti-biofilm strategies.

1.2.1 Genetic regulation of biofilm formation

The formation of a heterogeneous biofilm structure is a dynamic process which requires the expression of different gene products (Sauer, Camper et al. 2002; Stoodley, Sauer et al. 2002; Klausen, Aaes-Jorgensen et al. 2003) There are many different mechanisms that contribute to biofilm development (**Figure 5**). For example, Davey *et al*. reported that rhamnolipid surfactant production may be able to maintain open channels and affect architecture in *P.aeruginosa* biofilm (Davey, Caiazza et al. 2003). Klausen *et al*. showed that twitching ability could determine the mature biofilm structure by forming the motile and non-motile subpopulations (Klausen, Heydorn et al. 2003). Greenberg's group proved that iron signaling is required for the formation of mushroom-like biofilm structures (Banin, Vasil et al. 2005).

Figure 5 outlines the development of the *P. aeruginosa* biofilm. The biofilm formation of this organism is characterized as five stages of development: (i) reversible attachment, (ii) irreversible attachment, (iii) maturation-1, (iv) maturation-2, and (v) dispersion(Sauer, Camper et al. 2002).

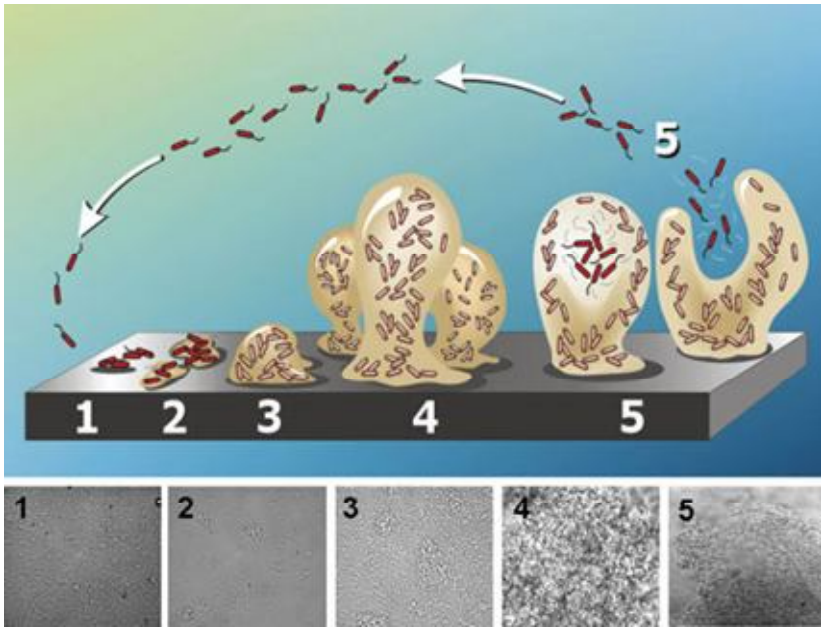


Figure 5.Diagram showing the development of a biofilm as a five-stage process. Stage 1: initial attachment of cells to the surface. Stage 2: production of EPS resulting in more firmly adhered "irreversible" attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. The *bottom panels* (a-e) show each of the five stages of development represented by a photomicrograph of *P. aeruginosa* when grown under continuous-flow conditions on a glass substratum.(Stoodley, Sauer et al. 2002)

1.2.2 Biofilm model organism – *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous Gram-negative bacterium responsible for frequent nosocomial and burn infections (Lyczak, Cannon et al. 2000). It is also the most frequent cause of pulmonary infection in patients suffering from cystic fibrosis (CF) (Deretic, Schurr et al. 1995). The chronic CF infection is impossible to eradicate and can ultimately lead to pulmonary failure and death (Frederiksen, Koch et al. 1997) (Hoiby and Koch 1990). It is hard to control *P. aeruginosa* infections because it is naturally resistant to many antibiotics. One more important reason is its tendency to colonize surfaces in a biofilm form which makes the cells impervious to therapeutic concentrations of antibiotics (Hill, Rose et al. 2005).
Biofilm formation by *P. aeruginosa* requires surface pili, flagellum, extracellular polysaccharides, extracellular DNA (eDNA) and surface proteins (Harmsen, Yang et al. 2010). These components are crucial for the initial attachment of *P. aeruginosa* to surface.

P. aeruginosa type IV piliare thin polar filaments 50-60 Å in diameter and up to ≈ 5 μm long. Type IV pili are dynamic filaments that can be extended and retracted independently of each other from the pole (Skerker and Berg 2001). Pili are able to attach via the tip to abiotic and biotic surfaces. The extension and retraction of type IV pili are the driving force of the surface-associated so-called twitching motility (Skerker and Berg 2001). *P. aeruginosa* is able to swim in liquid by means of a single polar flagellum. Using the microtiter plate assay, O'toole and Kolter showed that flagella or flagellum-driven motility is required for biofilm formation by *P. aeruginosa* PA14(O'Toole and Kolter 1998). It was speculated that in static cultures, the swimming motility might enable the bacteria to overcome repulsive forces at the surface and that the microcolonies may be formed by twitching motility-driven cell aggregation.

*P. aeruginosa*is known to produce at least three secreted polysaccharides: Pel, Psl and alginate (**Figure 6**). These polysaccharides differ in chemical structure and biosynthetic mechanisms (Friedman and Kolter 2004; Stapper, Narasimhan et al. 2004; Ryder, Byrd et al. 2007). **Figure 6** shows the alginate, Psl and Pel polysaccharides in *P. aeruginosa* biofilms revealed by using Atomic Force Microscopy (AFM), Confocal laser scanning microscopy (CLSM) and Scanning Electron Microscopy (SEM) respectively (Franklin, Nivens et al. 2011).

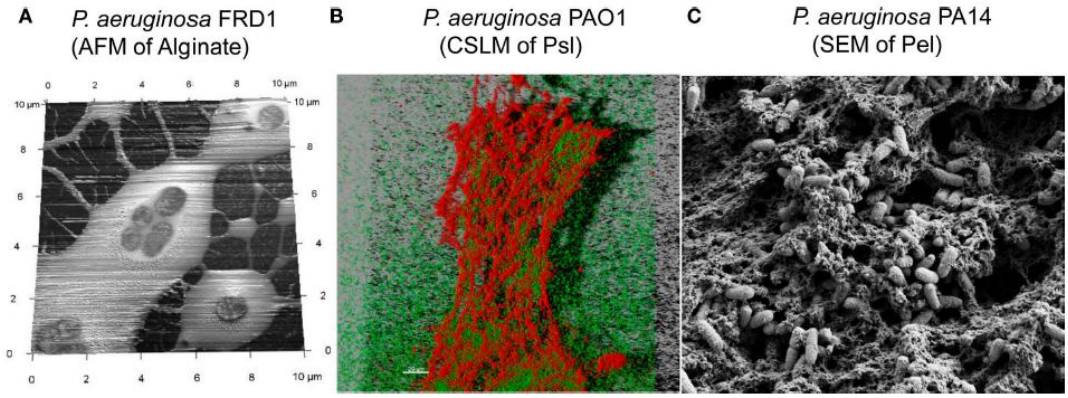


Figure 6.Extracellular polysaccharidealginate (A), Psl (B) and Pel (C) of *P. aeruginosa*, visualized using three approaches. (A) Atomic Force Microscopy image of *P.aeruginosa* FRD1, showing alginate as a soft loosely adhered polymer that surrounds the cells. (B) Confocal laser scanning microscopy (CLSM) image of hydrated *P. aeruginosa* PAO1, cultured as a pellicle. The CLSM image shows a three-dimensional reconstruction of the pellicle, with the *P. aeruginosa* cells expressing the green fluorescent protein and the Psl polysaccharide counterstained with Cell Mask Orange (Invitrogen Corp.). (C) Scanning Electron Microscopy (SEM) image of *P. aeruginosa* PA14, cultured as a pellicle. The image shows the extracellular matrix, which includes Pel, as a fabric-like matrix that surrounds and connects the cells that form a microbial mat at the air-water interface (Franklin, Nivens et al. 2011).

1.2.3 Biofilm model organism – *Staphylococcus epidermidis*

Staphylococcus epidermidis is a Gram-positive, coagulase-negative cocci that belongs to our normal flora. It causes catheter-associated biofilm infections and remains an important cause of nosocomial infection (Donlan 2001).

Biofilm formation by *S.epidermidis* is proposed to be a four-step process involving initial attachment, accumulation, maturation, and detachment by Otto (Otto 2004). Several EPS components are required for the attachment and accumulation phases and are noted on the bottom of the **Figure 7**. The polysaccharide intercellular adhesin (PIA) is one of the most important EPS component involved in *S.epidermidis* biofilm accumulation(Mack, Fischer et al. 1996). PIA is a linear beta-1,6-linked glucosaminoglycan which can also protect *S. epidermidis* against major components of human innate host defense, such as antimicrobial peptides(Vuong, Voyich et al. 2004).

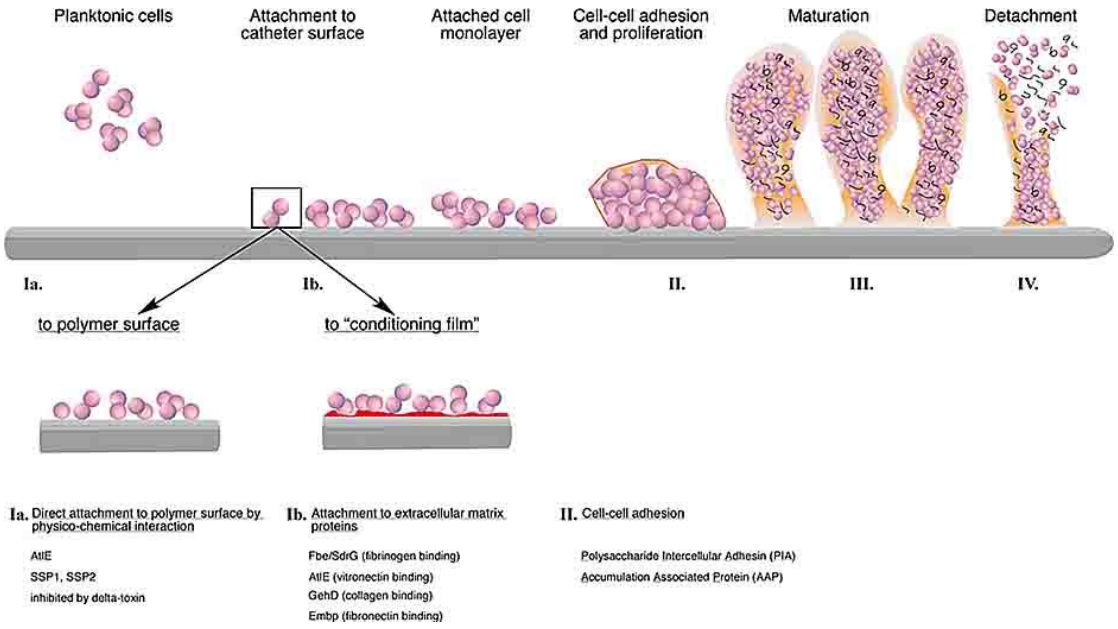


Figure 7.Model of biofilm formation on a catheter surface in *Staphylococcus epidermidis*.(Otto 2004)

2. Anti-virulence targets and approaches

The emergence of antibiotic resistance and biofilm-associated implant infections cause many problems for the current medical treatment for bacterial infection diseases (Costerton, Stewart et al. 1999). We are now entering the post-antibiotic era, with limited treatment options for many bacterial infections, such as the now widely disseminated multidrug-resistant *P. aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA). Thus, many alternative treatment strategies are currently being investigated that are based on the inhibition of bacterial virulence rather than of bacterial growth. Such strategies might reduce the selection pressure for antibiotic resistance since virulence traits are not essential for bacterial survival. **Table 1** shows examples of some reported anti-virulence strategies, including inhibiting toxin function, toxin delivery, virulence expression regulator (quorum sensing), and adhesion.

Table 1. Some of the strategies that target various pathways related to virulence (Clatworthy, Pierson et al. 2007)

Targets	Inhibitors (examples)	Modes of action *in vitro*	Effect on *in vivo* (or *ex vivo*) infection
Toxin function			
B. anthracis lethal factor (LF)	(2*R*)-2-[(4-fluoro-3-methylphenyl) sulfonylamino]-*N*-hydroxy-2-(tetrahydro-2*H*-pyran-4-yl) acetamide (LFI)	Binds LF active site and inhibits LF protease activity	Protects mice from spore infection when used in combination with ciprofloxacin; provides complete protection in mice immunized with LF and PA
	Peptide analogs derived from the optimal peptide substrate sequence	Inhibit LF cleavage of MKKs *in vitro*	Protect macrophages from LF-induced cytolysis *ex vivo*
B. anthracis protective antigen (PA)	Cisplatin	Inhibits LF translocation into the host cytosol	Protective when administered simultaneously with anthrax lethal toxin in murine models
	Hexa-D-arginine	Inhibits PA processing	Delays lethal toxin-induced toxemia in rodent models and cultured macrophages
Toxin delivery			
Type III secretion system (T3SS)	Acylated hydrazones of different salicylaldehydes (for example, INP0400)	Prevent effector molecule translocation	Attenuate *Y. pseudotuberculosis* infection and inhibit intracellular replication of *C. trachomatis* and *C. pneumoniae ex vivo*; preincubation of compound with bacteria before infection suppresses secretory and inflammatory responses in a bovine intestinal loop model of *S. enterica* serovar Typhimurium infection
Regulators of virulence expression			
V. cholerae ToxT	Virstatin	Prevents expression of the toxin-coregulated pilus and cholera toxin in *V. cholerae*	Protects infant mice from intestinal colonization with *V. cholerae*
Quorum sensing: LuxR homologs	Structural analogs of AHLs (for example, halogenated furanones)	Accelerate turnover of LuxR homologs; inhibit expression of quorum sensing regulated genes; inhibit the production of carbapenem in *E. carotovora* and virulence factors in *P. aeruginosa*	Promote clearance of *P. aeruginosa* from the lungs of mice in a pulmonary infection model; increase the survival time of mice in a lethal *P. aeruginosa* lung infection model
Quorum sensing: *S. aureus* AgrC	Inhibitory autoinducing peptides (AIPs)	Inhibits *agr* locus activation	Administration of inhibitory AIP to mice during *S. aureus* infection inhibits abscess formation
Adhesion			
Pilin chaperones (for example, PapD in UPEC)	Pilicides: bicyclic 2-pyridones and N-substituted amino acid derivatives	Inhibit pilus assembly	Bicyclic 2-pyridones inhibit adhesion of *E. coli* to bladder carcinoma cells *ex vivo*

2.1 Efflux pump inhibitor

Most bacteria have a series of efflux pump systems which are involved in the extrusion of substrates from within cells into the external environment (Webber and Piddock 2003). So far, several different families of efflux pump systems are present in bacteria, including SMR (small multidrug resistance), RND (resistance-nodulation-division), MFS (major facilitator superfamily) and ABC (ATP binding cassette) (**Figure 8**). The SMR, RND and MFS efflux systems use the proton motive force as an energy source, while the ABC efflux system utilizes ATP hydrolysis to drive the export of substrates(Van Bambeke, Balzi et al. 2000).

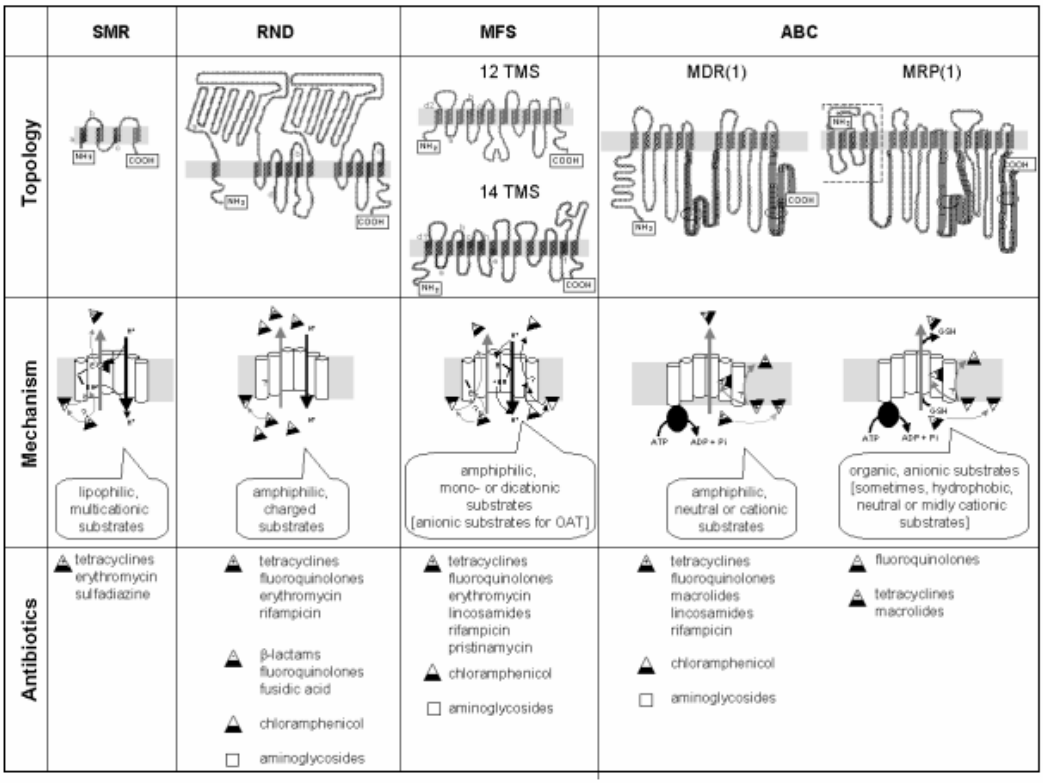


Figure 8.Main classes of efflux pumps acting on antibiotics (Van Bambeke, Balzi et al. 2000).

Many efflux pumps are activated only after exposure of bacterial cells to toxic substrates such as antibiotics (Aendekerk, Ghysels et al. 2002). Long term exposure of bacterial cells to antibiotics can often lead to multiple drug resistant (MDR) bacteria which consistently express certain efflux pumps (Poole 2000).

Besides extrusion of antibiotics, some of the signal molecules which are involved in bacterial virulence as well as biofilm formation also require an active efflux pump system. In *Burkholderia pseudomallei* BpeAB-OprB efflux pump is required for optimal production of quorum-sensing-controlled virulence factors such as siderophore and phospholipase C as well as for biofilm formation(Chan and Chua 2005).Thus, targeting efflux pumps using efflux pump inhibitors (EPI) might be a potential strategy to control multiple drug resistant bacteria. A wide range of EPIs have been identified in recent years (**Figure 9**) (Pages and Amaral 2009). EPIs

have been shown to reduce antibiotic resistance, quorum sensing, virulence and biofilm formation (Hannula and Hanninen 2008; Kvist, Hancock et al. 2008; Varga, Armada et al. 2012).

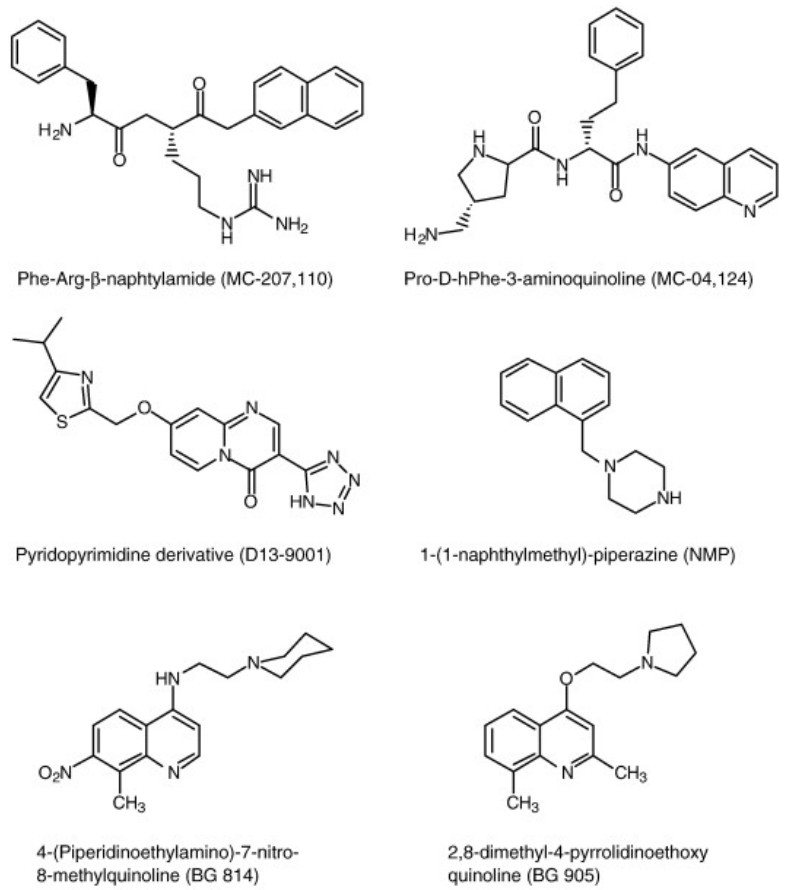


Figure 9.Some of the well-described inhibitors targeting the Gram-negative bacteria efflux pumps (Pages and Amaral 2009).

2.2 Iron chelator

Iron source is limited under both environmental conditions and host. Bacteria have evolved multiple iron acquisition systems to scavenge iron from minerals as well as host iron storage proteins (such as hemoglobin) (Wandersman and Delepelaire 2004).

One of the most common mechanisms for bacterial iron acquisition is release of small, high-affinity iron chelating compound-siderophores (Bagg and Neilands 1987). Siderophores are the strongest binders to Fe^{3+}in nature and usually form a stable, hexadentate, octahedral complex with Fe^{3+}. Bacteria synthesize siderophores and release them through ABC efflux systems. After binding with Fe^{3+}, siderophores can be recognized by their specific receptors at the membrane and transported into cells (**Figure 10**).

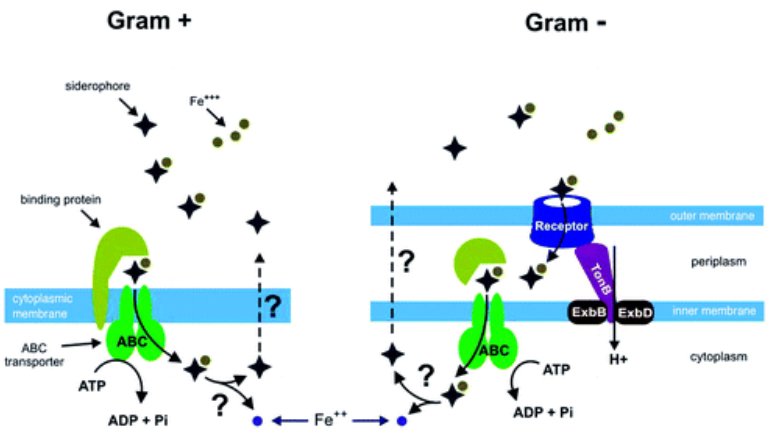


Figure 10. Iron acquisition systems of Gram-positive and Gram-negative species (Wandersman and Delepelaire 2004).

Iron signalling plays an important role in bacterial virulence and biofilm formation. In addition to its role as an iron-scavenger, the major *P. aeruginosa* siderophore pyoverdine regulates the production of several virulence factors which are major contributors to infection (Lamont, Beare et al. 2002). Pyoverdine also contributes to subpopulation interactions and structure development of *P. aeruginosa* biofilms (Yang, Nilsson et al. 2009).

Interference of iron uptake can be used to combat bacterial infections. Lactoferrin was shown to repress *P. aeruginosa* biofilm formation by enhancing twitching motility and inhibiting micro-colony formation (Singh, Parsek et al. 2002). Iron chelator deferoxamine and deferasirox were reported to enhance the activity of tobramycin against *P. aeruginosa* biofilm cells (Moreau-Marquis, O'Toole et al. 2009). Siderophore-antibiotic conjugates are shown to effectively inhibit bacterial growth (Miller, McKee et al. 1991; Kline, Fromhold et al. 2000).

2.3 Quorum sensing inhibitor

It has been known that a wide range of extracellular products are regulated in a cell density-dependent manner via cell-to-cell communication or 'quorum sensing' in bacteria (Miller and Bassler 2001). Bacteria secrete small molecules such as oligopeptides for Gram-positive bacteria and N-Acyl Homoserine Lactones (AHL) for Gram-negative bacteria as signaling molecules. *P. aeruginosa* possesses two AHL-dependent quorum sensing systems. These are termed the *las* and *rhl* systems, comprising of the LuxRI homologues, LasRI and RhlRI respectively (Swift, Downie et al. 2001). LasI directs the synthesis of primarily *N*- (3-oxododecanoyl)-L -homoserine lactone (3-oxo-C12-HSL) and together with the transcriptional regulator LasR regulates the production of virulence factors including elastase, the LasA protease, alkaline protease and exotoxin A (Pearson, Pesci et al. 1997). RhlI directs the synthesis of *N*- butanoyl- L -homoserine lactone (C4-HSL) which activates RhlR and in turn RhlR/C4-HSL induces the production of rhamnolipid, elastase, LasA protease, hydrogen cyanide, pyocyanin, siderophores

and the cytotoxic lectins PA-I and PA-II (Toder, Gambello et al. 1991). The *las* and the *rhl* systems are considered to be organized in a hierarchical manner such that the *las* system exerts transcriptional control over both *rhlR* and *rhlI*(Latifi, Foglino et al. 1996).The regulation of the *P. aeruginosa* quorum sensing network could be shown in **Figure 11** (Welch, Mikkelsen et al. 2005).

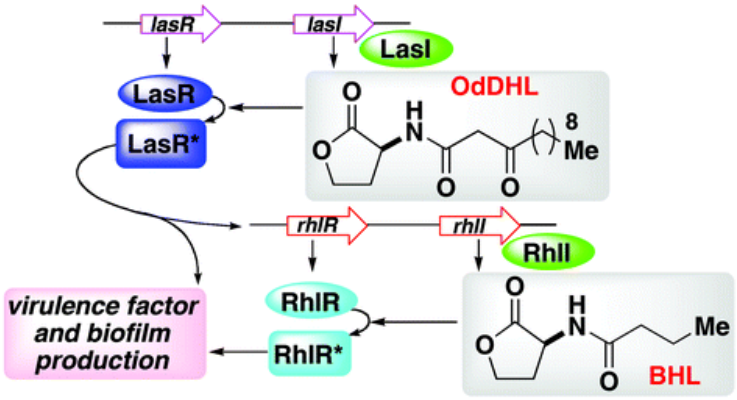


Figure 11.The quorum sensing network in *P. aeruginosa* (Welch, Mikkelsen et al. 2005).

It has been found that quorum sensing regulated genes are involved in biofilm maturation and survival. A PAO1 *lasI* mutant defective in quorum sensing signal OdDHL production was unable to structurally mature into mushroom shaped SDS-resistant microcolonies as in the wild type but developed a flat SDS-susceptible biofilm (Davies, Parsek et al. 1998). Another study found that a*rhlI* mutant formed pillar like structures compared to more mushroom shaped wilt type structure (Lequette and Greenberg 2005). The quorum sensing double mutant *lasRrhlR* showed a similar pattern of changes in formation of looser biofilm structures as well as increased sensitivity to antimicrobial factors tobramycin, hydrogen peroxide and polymorphonuclear leukocytes (PMN) (**Figure 12**) (Bjarnsholt, Jensen et al. 2005).

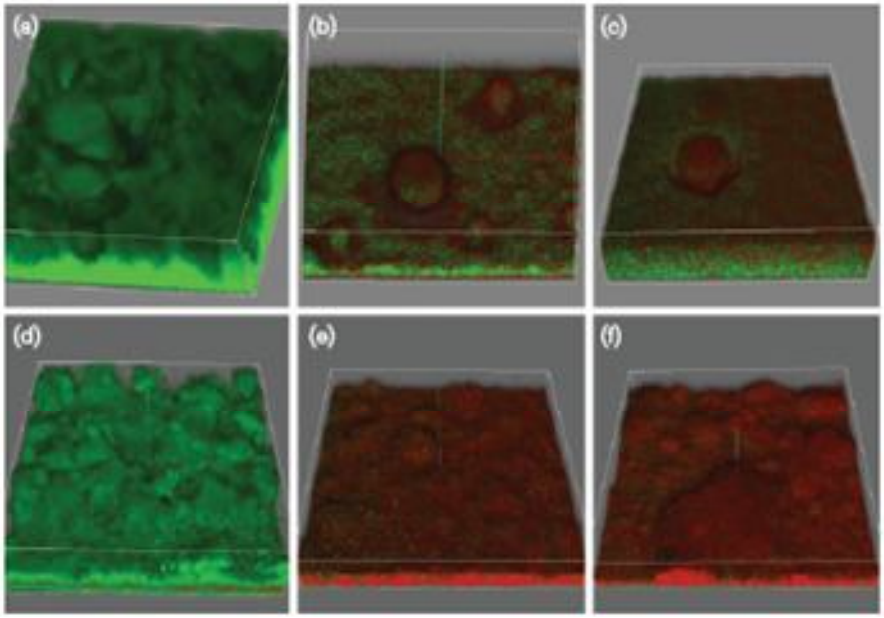


Figure 12.Tobramycin treatment of *P. aeruginosa* biofilms. Wild type *P. aeruginosa* and the *lasRrhlR* mutant, both expressing GFP as a tag, were grown as biofilms in flow chambers for 3 days. On day 3, 10 mg tobramycin ml^{-1} or 20 mg tobramycin ml^{-1} was added to the biofilm flow medium. Propidium iodide was added to the media after day 3 to continuously monitor the killing of the tobramycin-treated biofilms and the control. The present pictures show biofilms after 48 h of tobramycin treatment. (a) Untreated wild type; (b) 10 mg tobramycin ml^{-1}, wild type; (c) 20 mg tobramycin ml^{-1}, wild type; (d) untreated *lasRrhlR*; (e) 10 mg tobramycin ml^{-1}, *lasRrhlR*; (f) 20 mg tobramycin ml^{-1}, *lasRrhlR*.(Bjarnsholt, Jensen et al. 2005)

3 Peptidomimetics

Antimicrobial peptides (AMPs) have been considered an alternative to conventional antibiotics, due to their rapid-action, broad antimicrobial activity, immunomodulatory capability, and potency against slow-growing cells and biofilm cells. However, the application of natural AMPs has been limited by some properties, such as protease susceptibility, intrinsic toxicity, and high cost of production. Therefore, great efforts have been made on the *de novo* design of synthetic mimics of antimicrobial peptides (peptidomimetics) with high therapeutic potential and low clinical setbacks.

In order to facilitate the rational design of novel peptidomimetics, it is necessary to better understand the features, structure–activity relationships and the mechanisms of resistance development of AMPs. In addition, efforts should be spent on structure optimization and mode-of action study of peptidomimetics.

3.1 Antimicrobial peptides

Several hundreds of AMPs have been identified from almost all living organisms such as bacteria, fungi, plants, invertebrate and vertebrates species (Hancock and Chapple 1999).AMPs play an important role in our innate immune system for elimination of invading pathogenic microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and viruses(Hancock 2001; Brown and Hancock 2006; Hancock and Sahl 2006; Diamond, Beckloff et al. 2009). AMPs can directly kill microbial cells by damaging their cell envelopes. They can also recruit and promote other elements of innate immune response in higher organisms (Scott and Hancock 2000; Yang, Biragyn et al. 2004; Bowdish, Davidson et al. 2005; Hancock and Sahl 2006)(**Figure 13**). Regarding their functions and features, they have generally been termed ‘cationic antimicrobial peptides’, ‘host-defense peptides’, or ‘cationic amphiphilic peptides’.For clarity, the term cationic antimicrobial peptides, abbreviated to AMPs, is used in this thesis.

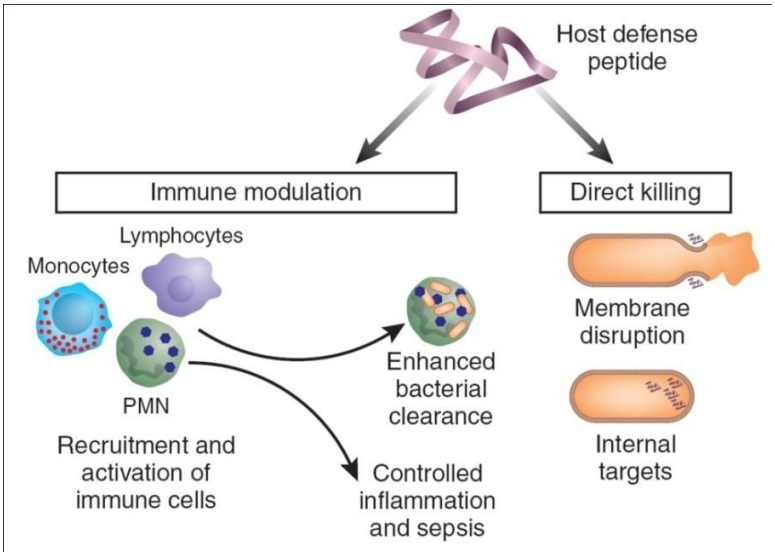


Figure 13.Biological function of host defense AMPs. Both direct antimicrobial killing and innate immune modulation occur with such peptides although certain peptides have one or the other activity preferentially. (Hancock and Sahl 2006)

3.1.1 Natural properties of AMPs

Individual AMPs are extremely diverse in their primary sequences and have a wide range of secondary structures. The four most prominent structures include α-helices, β-sheets (stabilized by two or three disulphide bonds), looped structures (due to a single disulphide bond) and extended structures rich in certain amino acids. The β-sheet and α-helical molecules are by far the most common AMPs in nature. Somemajor structural classes and examples of AMPs are showed in **Figure 14**.Such asα-helical magainin-2, β-sheeted polyphemusin, extended indolicidin, looped thanatin, and human β-defensin-2 of mixed structure (Hancock 2001; Hancock and Sahl 2006).

Despite the various primary sequences and different secondary structures, AMPs show similar general physical features. They are normally short (12-50 amino acids), positively charged (net charge of +2 to +9), and fold into amphiphilic secondary structures upon binding to biomembranes. They often contain the basic amino acids lysine orarginine, giving a net positive charge, anda substantial proportion (≈50%) of the hydrophobic residues alanine, leucine, phenylalanineor tryptophan (Hancock 2001; Hancock and Sahl 2006).

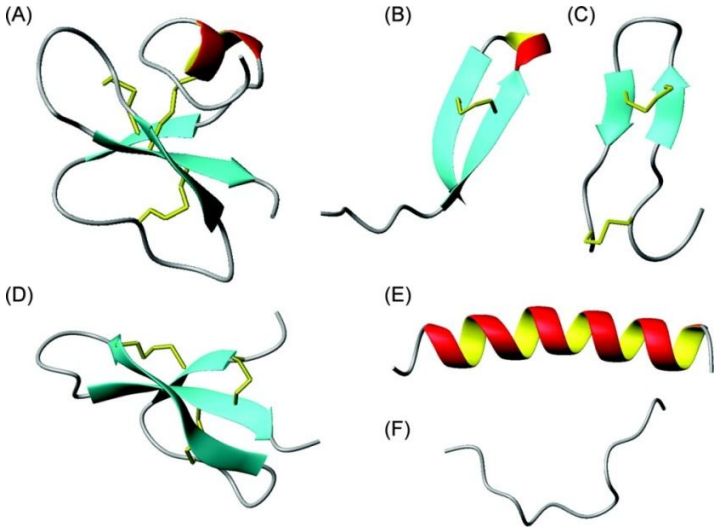


Figure 14. Major structural classes of antimicrobial peptides.(A) Mixed structure of human β-defensin-2 (PDB code 1FQQ) (216); (B) looped thanatin (PDB code 8TFV); (C) β -sheeted polyphemusin (PDB code 1RKK) (202);(D) rabbit kidney defensin-1 (PDB code 1EWS) (165);(E) α -helical magainin-2 (PDB code 2MAG) (76); (F) extended indolicidin (PDB code 1G89) (212). The disulfide bonds are indicated in yellow.(Jenssen, Hamill et al. 2006)

3.1.2 Mode of action of antimicrobial peptides

The most important features of AMPs concerning their activity are their positive charge and amphiphilicity. The overall positive charge ensures accumulation at negatively charged microbial cell surfaces that contain acidicpolymers, such as lipopolysaccharide(LPS), and wall-associated teichoic acids, of Gram-negative and Gram-positive bacteria respectively. Owing to the presence of disulphide bridges or interaction with target membranes, nearly all AMPs fold into amphiphilic structures. The positively charged polar face helps the peptide to reach the target membrane through electrostatic bonding, and the nonpolar face of the peptides allows insertion into the lipid bilayer through hydrophobic interactions (Powers and Hancock 2003; Yeaman and Yount 2003).

After insertion into the membrane, antimicrobial peptides act by either disrupting the physical integrity of the membrane, or translocation across the membrane and act on internal targets, mediate cell killing by inhibition of nucleic acid synthesis, protein synthesis, enzymatic activity, or cell wall synthesis(Brogden 2005; Jenssen, Hamill et al. 2006)(**Figure 15**).
Four models have been proposed for the membrane disruption mechanisms of AMPs. In the first model, peptides reorient to span the membrane as an aggregate with micelle-like complexes of peptides and lipids, but without adopting any particular orientation (**Figure 15A**). In the second and third models, peptide molecules are inserted into the membrane, forming either toroidal pore (**Figure 15B**) or barrel-stave channels (**Figure 15C**). For the fourth model, peptides cover the membrane surface in a carpet-like manner, act through the detergent-like dissolution of membrane, and subsequently leading to membrane disruption (**Figure 15D**).

Indeed, besides the membrane disruption mechanism, AMPs have been shown to have

alternative ways of causing cell death. Some may interact with internal anionic targets such as nucleic acids, cellular enzymes. For example, buforin II, pleurocidin, and dermaseptin have all been shown to penetrate the cell membrane and inhibit DNA and RNA synthesis (Park, Kim et al. 1998; Patrzykat, Friedrich et al. 2002; Rotem and Mor 2009) (**Figure 15E**). Some AMPs can interfere with protein synthesis, folding or function. For instance, indolicidin and the pig cathelicidin PR-39 can reduce the rate of protein synthesis in target bacterial cells (Boman, Agerberth et al. 1993; Sitaram, Subbalakshmi et al. 2003) (**Figure 15F**). The insect antimicrobial peptides pyrrhocoricin, drosocin and apidaecin have been shown to inhibit correct folding of proteins by targeting the bacterial chaperone DnaK (Otvos, O et al. 2000; Kragol, Lovas et al. 2001) (**Figure 15G**). Some AMPs can specifically inhibit the activity of aminoglycoside-modifying enzymes, which contain an anionic binding pocket (Boehr, Draker et al. 2003) (**Figure 15H**). Some AMPs can also target the formation of cell wall structure (**Figure 15I**). For instance, lantibiotics such as nisin and mersacidin can interfere with the transglycosylation of lipid II, which is necessary for the synthesis of peptidoglycan (Brotz, Bierbaum et al. 1997; Brumfitt, Salton et al. 2002; Kruszewska, Sahl et al. 2004).

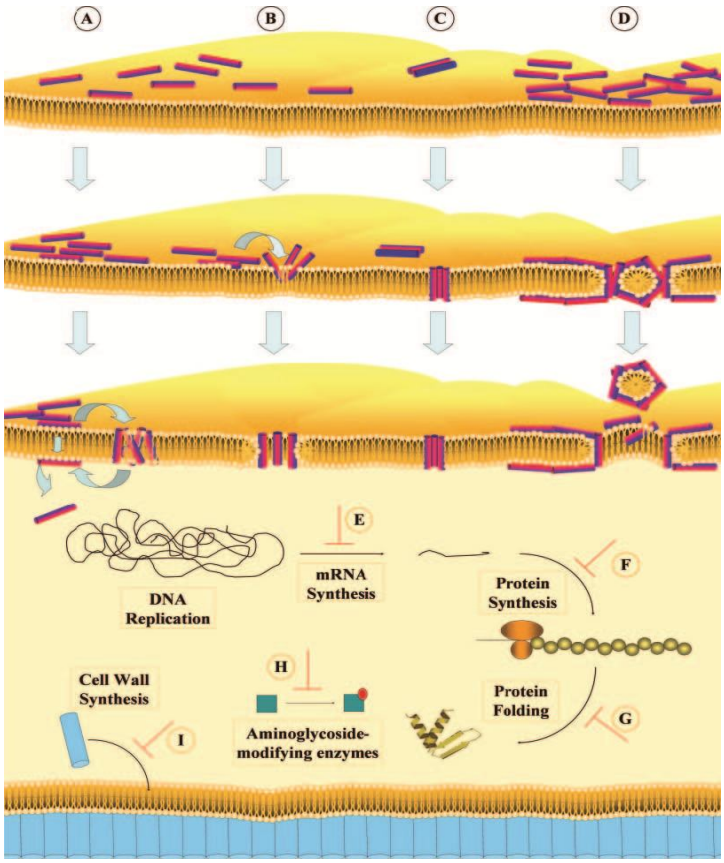


Figure 15.Mechanisms of action of antimicrobial peptides. The bacterial membrane is represented as a yellow lipid bilayer with the peptides shown as cylinders, where the hydrophilic regions are colored red and the hydrophobic regions are blue. Cell wall-associated peptidoglycan molecules are depicted as purple cylinders. Models to explain mechanisms of membrane permeabilization are "aggregate" model (A), "toroidal pore" model (B), "barrel-stave" model (C), and "carpet" model (D). The mechanisms of action of peptides which do not act by permeabilizing the bacterial membrane are depicted in panels (E) inhibition of DNA and RNA synthesis; (F) decrease the rate of protein synthesis; (G) inhibition of correct protein folding; (H) inhibition of enzymes involved in the modification of aminoglycosides; (I) interfere with cell wall synthesis. (Jenssen, Hamill et al. 2006)

3.1.3 AMPs as antibiotics (advantages and limits)

Due to their distinct bactericidal activity, APMs have been in now used to treat infections caused by multidrug resistant bacteria. For example, colistin, a polymyxin class of AMP, was used for the treatment of infections caused by multidrug-resistant (MDR) gram-negative bacteria such as *P. aeruginosa* and *Acinetobacter baumannii* (Falagas and Kasiakou 2005).

The activities of many conventional antibiotics are often hindered by slow growing cells with low level of metabolic rate (Brown, Allison et al. 1988). In contrast, AMPs effectively kill slow

growing microbial cells such as cells from biofilms (Batoni, Maisetta et al. 2011). Thus, a combination of AMPs and conventional antibiotics has been recently shown to be a feasible strategy to treat biofilms both *in vitro* and *in vivo*. **Figure 16** shows that colistin-tobramycin combinations are better at killing of biofilm *P. aeruginosa* than their single usage (Herrmann, Yang et al. 2010).

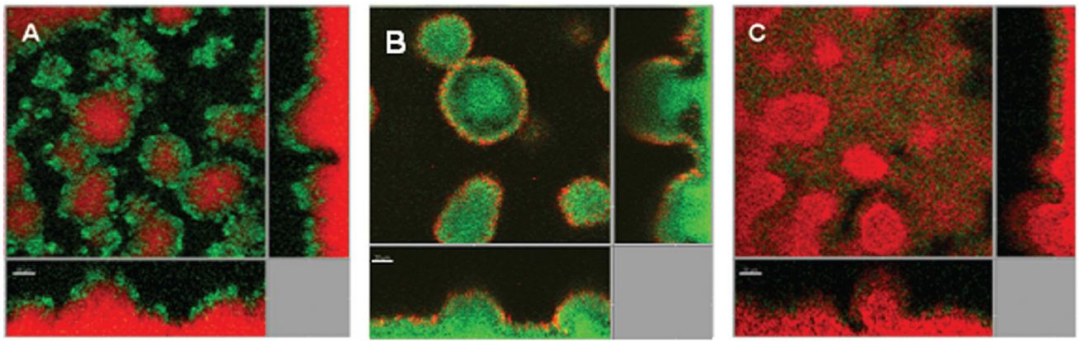


Figure 16.Distribution of live (green) and dead(red) cells in *Pseudomonas aeruginosa* biofilms. Biofilms were grown in laminar flow for 4 days and then were continuously exposed to 10 x minimum inhibitory concentrations of colistin, tobramycin, and the combination of colistin sulphate and tobramycin for 24 h(A–C) (Herrmann, Yang et al. 2010).

Despite many attractive features, AMPs still have many unfavorable characteristics which limited their applications. **Table 2**summarizes both the advantages and disadvantages of AMPs (Gordon, Romanowski et al. 2005).

Table 2.Advantages and disadvantages of AMPs as antibiotics (Gordon, Romanowski et al. 2005)

Advantages
Broad-spectrum activity (antibacterial, antiviral, antifungal)
Rapid onset of killing
Cidal activity
Potentially low levels of induced resistance
Concomitant broad anti-inflammatory activities
Disadvantages
Discovery costs of synthesis and screening
Patent exclusivity for economic viability
Systemic and local toxicity
Reduced activity based on salt, serum, and pH sensitivity
Susceptibility to proteolysis
Pharmacokinetic (PK) and pharmacodynamic (PD) issues
Sensitization and allergy after repeated application
Natural resistance (e.g., *Serratia marcescens*)
Confounding biological functions (e.g., angiogenesis)
High manufacturing costs

3.2 AMPs resistance

Several important pathogens such as *S. aureus, S. epidermidis, Salmonella enterica, and Paeruginosa* have evolved multiple strategies for conferring intrinsic or inducible resistance towards AMPs. When exposed to AMPs, those bacteria have specific sensing systems which activate those AMP resistance mechanisms.

3.2.1 Bacterial mechanisms of AMP resistance

The most common bacterial mechanisms of AMP resistance include modifications of the cell envelope, the inactivation of AMPs and active extrusion of harmful peptides.

As the initial interactions between bacteria and AMPs are driven by electrostatic forces, resistance can be achieved by increase of the positive charge of the cell surface to repel cationic AMPs. As shown in **Figure 17**, the mechanisms are often different between Gram-positive and Gram-negative bacteria owing to the different composition of their cell envelope surfaces (Nizet 2006). In *Salmonella typhimurium*, AMP resistance is mainly achieved by modifications of lipid A in the outer membrane, either by acylation (PagP) (Guo, Lim et al. 1998) or addition of an aminoarabinose moiety (Pmr system) (Gunn, Ryan et al. 2000). In many Gram-positive bacteria such as *S. aureus*, *Streptococcus pyogenes*, D-alanylation of the cell wall teichoic acids (*Dlt* operon) contributes to AMP resistance (Abachin, Poyart et al. 2002; Kristian, Datta et al. 2005). Another strategy exploited by *S. aureus* is the MprF-mediated lysinylation of the major membrane lipid phosphatidylglycerol, leading to the formation of positively charged lysylphosphatidylglycerol (LPG)(Peschel, Jack et al. 2001; Nishi, Komatsuzawa et al. 2004).

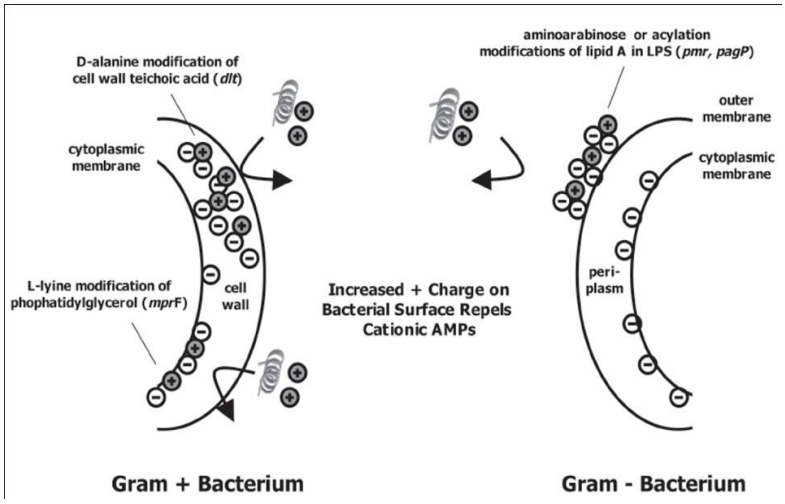


Figure 17.Bacterial resistance to cationic antimicrobial peptides mediated by alterations in surface charge.(Nizet 2006)

Besides charge, other physico-chemical properties of the cell membrane also influence the membrane integration of AMPs. Bacterial resistance can be achieved by alteration of those properties. For example, it confers AMP resistance for *S.aureus*, by increasing membrane fluidity via elevated levels of longer-chain and unsaturated lipids (Bayer, Prasad et al. 2000). Some so-called small colony variants (SCVs) of *S. aureus* are more resistant to AMPs due to their reduced membrane potential (Yeaman and Bayer 2006).

Many bacteria produce AMP-degrading proteases, such as protease V8 secreted by *S. aureus* and the protease SepA of *S. epidermidis* (Sieprawska-Lupa, Mydel et al. 2004; Lai, Villaruz et al. 2007). Moreover, AMPs-trapping molecules were produced to bind or neutralize AMPs, such as staphylokinase secreted by *S.aureus* and the M1 surface protein of Group A *Streptococcus* (GAS) (Jin, Bokarewa et al. 2004; Lauth, von Kockritz-Blickwede et al. 2009).

The extrusion of harmful AMPs by efflux pumps is another important mechanism of bacterial resistance against AMPs. For example, the MtrCE system of *Neisseria gonorrhoeae* and QacA of *S. aureus* export toxic substances out of the cell and away from the membrane by utilizing proton motive force (PMF)(Shafer, Qu et al. 1998; Kupferwasser, Skurray et al. 1999). The extrusion of lantibiotic epidermidin out from *S.epidermidis* cytoplasmis mediated by the EpiEFG ABC (ATP binding cassette) transporter (Peschel and Gotz 1996; Otto, Peschel et al. 1998).

3.2.2 AMP sensing systems

For the survival of bacteria it is crucial that they can sense and rapidly react to environmental threats. Two-component sensor/regulator systems (TCSs) play essential roles in bacteria for obtaining environmental information. TCSs are composed of two functional units, a membrane-anchored sensor histidine kinase (HK) sensing extracellular signals and a cytoplasm response regulator (RR) mediating differential gene expression (Hoch 2000).

The first described AMP sensing systems are the Gram-negative PhoP/PhoQ and the Gram-positive Aps systems which are based on investigations done in *Salmonella typhimurium* and *Staphylococcus epidermidis*, respectively. Both possess Two-component sensor/regulator systems (TCSs). Notably, the Aps system has a third component ApsX with unknown function. Another significant difference between the two systems is that, while the *staphylococcal* Aps system predominantly up-regulates AMP resistance mechanisms, the PhoP/PhoQ regulon controls various genes not necessarily limited to AMP resistance mechanisms, but apparently involved in abroad scale innate host defense(Otto 2009).

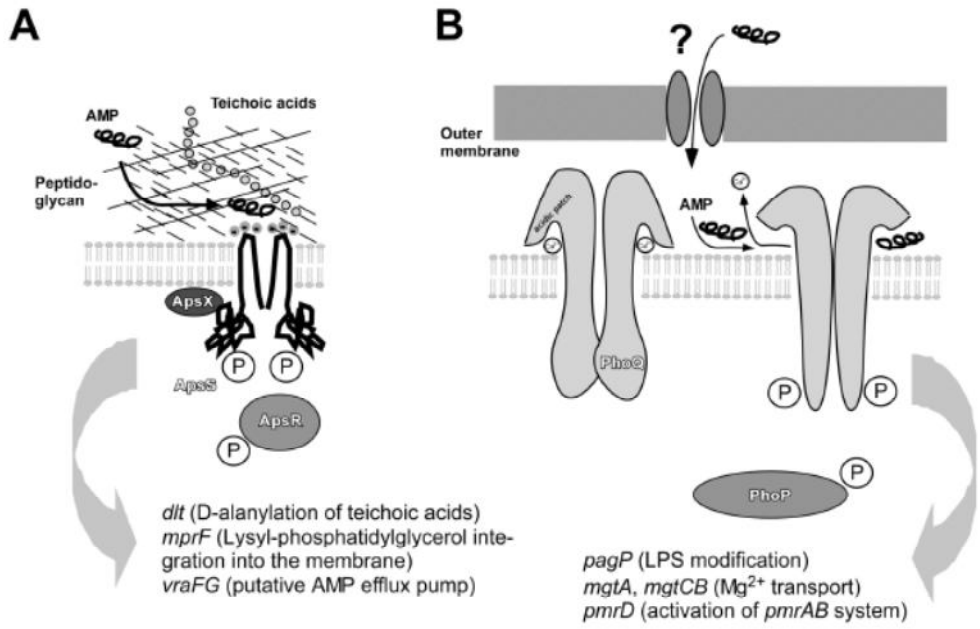


Figure 18.The Gram-positive and Gram-negative AMP sensors respectively. A, The Staphylococcus Aps system. B, The Salmonella PhoP/PhoQ system. The main regulatory targets of the systems are shown at thebottom.(Otto 2009)

Figure 18A shows the model of Aps sensor and regulated mechanisms in *S.epidermidis and S.aureus* (Li, Cha et al. 2007; Li, Lai et al. 2007; Otto 2009). ApsS is the membrane-anchored histidine kinase sensor part, ApsR is the cytoplasmic response regulator DNA-binding protein that interacts with target gene promoters, while the role of ApsX in the signaling transduction is unknown. Cationic AMPs bind to the anionic loop of ApsS and trigger activation of the Aps system, leading to activation of mechanisms involved in cationic AMP resistance:(*i*) D-alanylation of teichoic acids by the *dlt* system, (*ii*) lysylination of phospholipids by MprF, and (*iii*) putative transport of AMPs through an ABC transporter (VraFG).The VraFG transporter is previously described to confer resistance to the glycopeptide antibiotic vancomycin. The transporter may work either by expelling the pore-forming peptides from the membrane or by importing them into the cell for proteolytic inactivation.

The Gram-negative PhoP/PhoQ system (**Figure 18B**) is a intensively studied bacterial two-component signal transduction system(Groisman 2001). Stimulants such as low concentration of divalent cations, low pH, and cationic AMPs, activate the PhoQ sensor, which phosphorylates

PhoP. The activated PhoP regulates a series of genes and cellular activities, such as modification of lipopolysaccharide (LPS), synthesis of AMP-degrading proteases, and also activates a further two-component regulatory system *pmrA*/*pmrB*, which also up-regulates genes involved in LPS modification and resistance to polymyxin B (Gunn and Miller 1996). Besides, the PhoP/PhoQ system also activates the genes of Mg^{2+} transport(Garcia Vescovi, Soncini et al. 1996), and survival in macrophages (Miller, Kukral et al. 1989).

Importantly, besides the specific AMP resistance mechanisms, there are some general stress response systems which are capable of recognizing AMPs and to respond to their presenceThe alternative sigma (σ) factors, which activated by adequate triggers, can alter the promoter selectivity of RNA polymerase, therefore regulate gene expression and only optimal sets of genes are transcribed under the specific conditions(Paget and Helmann 2003).For example, the SigW and SigM extracytoplasmic sigma factors were activated when *Bacillus subtilis* was exposed to AMPs(Pietiainen, Gardemeister et al. 2005).

Finally, **Table 3** summarizes the most comment AMP resistance mechanism from different bacteria.

Table 3. Mechanisms of bacterial antimicrobial peptide resistance(Nizet 2006)

AMP Resistance Phenotype	Gene(s)	AMPs Affected	Organism(s)	Selected Reference
Cell Surface Alterations				
D-alanylation of teichoic acid in bacterial cell wall	*dlt* operon	Defensins, Caths *	*Staphylococcus aureus Group B Streptococcus*	(Collins, Kristian et al. 2002); (Poyart, Pellegrini et al. 2003)
L-lysine to phosphatidylg-lycerol	*mprF lysS*	Defensins, Caths	*Staphylococcus aureus*	(Peschel, Jack et al. 2001);(Nishi, Komatsuzawa et al. 2004)
Addition of aminoarabinose to lipid A in LPS	*pmr* genes	Defensins, Caths	*Salmonella enterica Pseudomonas aeruginosa*	(Gunn, Ernst et al. 2000); (Moskowitz, Ernst et al. 2004)
Acylation of lipid A in LPS	*pagP*	Defensins, Caths	*Salmonella enterica e*	(Guo, Lim et al. 1998)
Proteolytic Degradation of AMPs				
Elastase	*lasB*	Caths	*Pseudomonas aeruginosa*	(Schmidtchen, Frick et al. 2002)
Metalloproteinase (aureolysin)	*aur*	Caths	*Staphylococcus aureus*	(Sieprawska-Lupa, Mydel et al. 2004)
Surface protease	*pgtE*	Caths	*Salmonella enterica*	(Guina, Yi et al. 2000)

AMP Trapping Mechanisms				
Staphylokinase	*sak*	Defensins	*Staphylococcus aureus*	(Jin, Bokarewa et al. 2004)
M1 surface protein	*emm1*	Caths	*Group A Streptococcus*	(Lauth, von Kockritz-Blickwede et al. 2009)
SIC protein	*sic*	Defensins, Caths	*Group A Streptococcus*	(Frick, Akesson et al. 2003)
Shedding of host proteoglycans that in turn neutralize AMPs	*lasA*	Defensins, Caths	*Pseudomonas aeruginosa Group A Streptococcus*	(Park, Pier et al. 2001; Schmidtchen, Frick et al. 2001)
Active Efflux of AMPs				
ATP-dependent efflux system	*mtr* genes	Caths	*Neisseria gonorrhoeae*	(Jerse, Sharma et al. 2003)
K+-linked efflux pump	*sap* operon *sapA*	Protamine Defensins	*Salmonella enterica Haemophilus influenzae*	(Parra-Lopez, Lin et al. 1994; Mason, Munson et al. 2005)
Plasmid-encoded efflux pump	*qacA*	Rabbit tPMP	*Staphylococcus aureus*	(Kupferwasser, Skurray et al. 1999)
Alteration of Host Processes				
Downregulate AMP transcription	Unknown	Caths	*Shigella dysenteriae*	(Islam, Bandholtz et al. 2001)
Stimulation of host cathepsins	Unknown	Defensins	*Pseudomonas aeruginosa*	(Taggart, Greene et al. 2003)
Regulatory Networks				
Two-component regulator	*phoP/ phoQ*	Defensins, protamine	*Salmonella enterica Pseudomonas aeruginosa*	(Macfarlane, Kwasnicka et al. 2000; Ernst, Guina et al. 2001)
Two-component regulator	*pmrA/ pmrB*	Defensins, Polymyxin B	*Salmonella enterica Pseudomonas aeruginosa*	(Gunn, Ryan et al. 2000; McPhee, Lewenza et al. 2003)
Three-component regulator	*apsS/apsR/apsX*	Defensins	*S.epidermidis Staphylococcus aureus*	(Li, Lai et al. 2007) (Li, Cha et al. 2007)
Extracytoplasmic sigma factor	*rpoE*	Defensins	*Salmonella enterica*	(Crouch, Becker et al. 2005)
Thermoregulated transcription factor	*prfA*	Defensins	*Listeria monocytogenes*	(Lopez-Solanilla, Gonzalez-Zorn et al. 2003)

* Abbreviation: Cath = cathelicidin.

3.2.3 Experimental evolution of bacterial resistance towards AMP antibiotics

Even throughout million years' co-evolution, few bacterial species have developed highly resistance against AMPs (Peschel and Sahl 2006). AMPs operate through non-specific mechanisms, targeting the cell membranes and other macromolecules, and therefore have been considered to be less likely to induce bacterial resistance than conventional antibiotics(Zasloff 2002).

Experimental evolution studies have been used in the study of bacterial resistance development toward AMP antibiotics. The studies indicated that resistance towards some specific AMP antibiotics, such as pexiganan derived from magainin and a AMP mimic based on phenylene ethynylene (SMAMP**1**), was much harder to obtain than other conventional antibiotics (Perron, Zasloff et al. 2006; Tew, Clements et al. 2006).

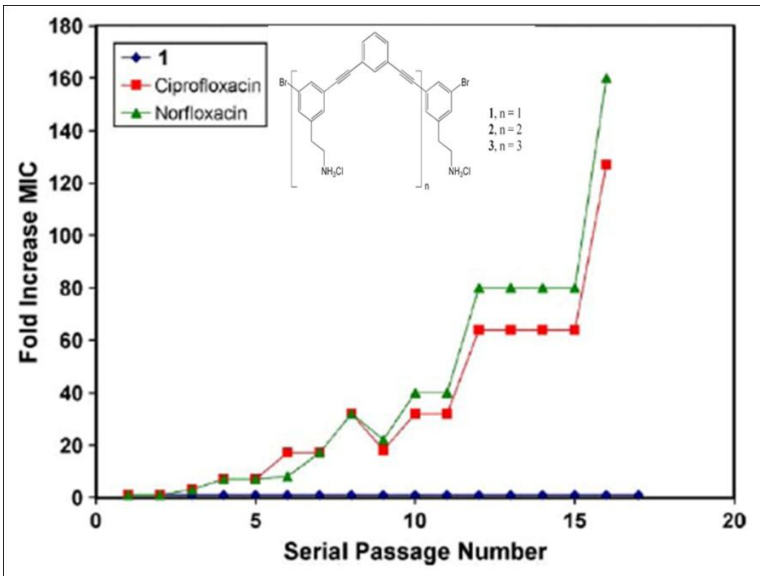


Figure 19. Susceptibility of *S. aureus* to develop resistance against SMAMP**1**, as well as two conventional antibiotics, ciprofloxacin and norfloxacin. Modified from(Tew, Clements et al. 2006)

A more thorough understanding of the mechanisms of AMP mode of action as well as bacteria resistance may provide novel strategies or templates for designing novel AMP antibiotics, especially for prevention or treatment of infections caused by pathogens resistant to conventional antibiotics.

3.3 Peptidomimetics and peptide-mimetic polymers

To date, great efforts have been dedicated on developing AMP mimics (peptidomimetics) with high therapeutic potential and low clinical setbacks, either through the modification of existing peptides, or the *de novo* synthesis of structure mimics of AMPs.

Several strategies are used for *de novo* synthesis of peptidomimetics. Among which, the peptide-mimetic polymer approach draws broad interest due to its relatively low cost of production as well as the ease of tuning key structural parameters. The design principle combines the biological features of antimicrobial peptides and the facile and inexpensive polymer synthesis methods, which have led to a new class of affordable and biocompatible antimicrobials(Palermo and Kuroda 2010). Many kinds of peptidomimetics have been developed using this method, such as synthetic oligomers (Tew, Scott et al. 2010), dendrimers (Chen, Beck-Tan et al. 2000), and linear polymers (Ilker, Nusslein et al. 2004; Mowery, Lee et al. 2007; Kuroda, Caputo et al. 2009).

3.3.1 Design principles of peptide-mimetic polymers

The most important concerns of peptidomimetic development are the *in vivo* stability, intrinsic toxicity and suitable formulation considering the cost of production.

Recently, several strategies have been used to design novel AMP polymers. In these systems, extensive optimization has led to a select few compounds that displayed potent antimicrobial activity combined with high protease stability and low cytotoxicity. Despite the diversity in molecular size, sequence, and conformation, these lead compounds all showed potential activity. Interestingly, promising antimicrobial efficacy of α/β-peptides was achieved even when the peptides were not able to form amphiphilic helices (Schmitt, Weisblum et al. 2004).This indicates that the activity of AMP polymers seems to depend on their physiochemical properties or global properties of polymer chains instead of defined sequence and secondary structure(Palermo and Kuroda 2010).

The principle of designing peptide-mimetic polymers is to achieve better properties while retaining the basic properties of membrane-active natural AMPs such as positive charge and amphiphilicity. In addition, the length (molecular weight) is optimized to avoid hemolysis and retain antimicrobial activity (**Table 3**).

Table 3 Design principles which enable favorable activity profiles in peptide-mimetic polymers(Palermo and Kuroda 2010)

Parameter	Rationale	Design strategies	References
Amphiphilic balance	Cationic groups confer selectivity	Random copolymers (fHB ~0.4)	(Kuroda, Caputo et al. 2009); (Mowery, Lee et al. 2007)
	Tune hydrophobicity for non-hemolytic, antimicrobial activity	Amphiphilic homopolymers	(Ilker, Nusslein et al. 2004); (Lienkamp, Madkour et al. 2008)
		PEGylated polymer disinfectants	(Sellenet, Allison et al. 2007)
Type of cationic groups	Cationic groups with high affinity and selectivity for bacterial membranes	Primary amines	(Palermo and Kuroda 2009)
		Guanidine	(Gabriel, Madkour et al.

			2008)
		Quaternized pyridines with "same center" arrangement	(Sellenet, Allison et al. 2007; Sambhy, Peterson et al. 2008)
Typeof hydrophobic groups	Minimize overall hydrophobicity to avoid hemolytic activity	Short alkyl (methyl, ethyl)	(Kuroda, Caputo et al. 2009); (Lienkamp, Madkour et al. 2008)
		Cyclic groups (cyclohexene)	(Mowery, Lee et al. 2007)
MW	Optimal length to avoid hemolysis and retain antimicrobial activity	<10 kDa for low hemolysis and antimicrobial potency	(Kuroda and DeGrado 2005); (Mowery, Lindner et al. 2009)

3.3.2 α-peptide/β-peptoid chimeras

Several approaches are employed in designing AMP mimicry: Incorporation of unnatural α-amino acids, partial or complete replacement of α-amino acids with β-amino acids (Cheng, Gellman et al. 2001; Seebach, Beck et al. 2004)or oligomers of N-alkylglycines *(i.e,*. α-peptoids)(Zuckermann, Kerr et al. 1992)(**Figure 21**).

Peptoids (*N*-alkylated oligoglycines) are isomers of peptides, with the side chains attached to the backbone nitrogen rather than to the α–carbon(Simon, Kania et al. 1992). Peptoids are extensively used for achieving AMP mimicry, since they are easily synthesized on solid phase with diverse sequences at relatively low cost(Zuckermann, Kerr et al. 1992).

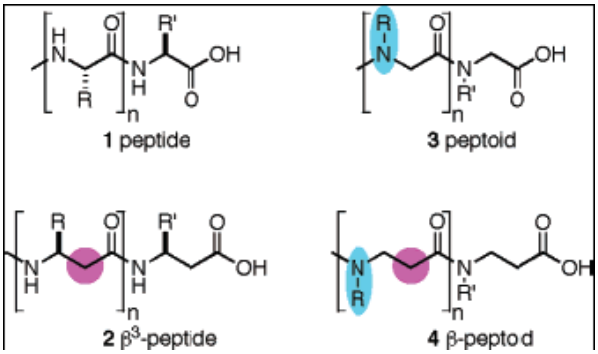


Figure 21. Backbone structures of natural R-peptides and mainpeptidomimetic designs (peptoid)(Zuckermann, Kerr et al. 1992).

Indeed, the protease-resistant compounds β-peptides (Porter, Wang et al. 2000; Arvidsson, Ryder et al. 2005) and peptoids (Chongsiriwatana, Patch et al. 2008)are being intensively investigated as antibacterials.

Also, oligomers composed of two different types of residues are capable of imitating the antibiotic activity of natural AMPs (Schmitt, Weisblum et al. 2007; Horne and Gellman 2008).

An approach for the design and synthesis of a series of α-peptide/β-peptoid chimeras has been reported previously. This kind of peptidomimetics consists of alternating repeats of α-peptide and β-peptoid residues. In this design, lysine (Lys) and/or homoarginine (hArg) contribute to strongly cationic properties and intramolecular hydrogen-bonding capacity, while β-peptoid residues seem to improve lipophilicity, structure-promoting effects, and protease resistance (Foged, Franzyk et al. 2008). They were synthesized using standard solid-phase procedures, and the method is suitable for large-scale, cost-efficient manufacturing(Olsen, Bonke et al. 2007; Olsen, Ziegler et al. 2010; Hein-Kristensen, Knapp et al. 2011).These chimeras exhibit a broad spectrum of activities being potent against several Gram-negative bacteria, Gram-positive bacteria, fungi, and parasites. In addition, they are non-hemolytic and highly resistant to degradation by proteases (Olsen, Bonke et al. 2007; Olsen, Lambert et al. 2008; Olsen, Ziegler et al. 2010).

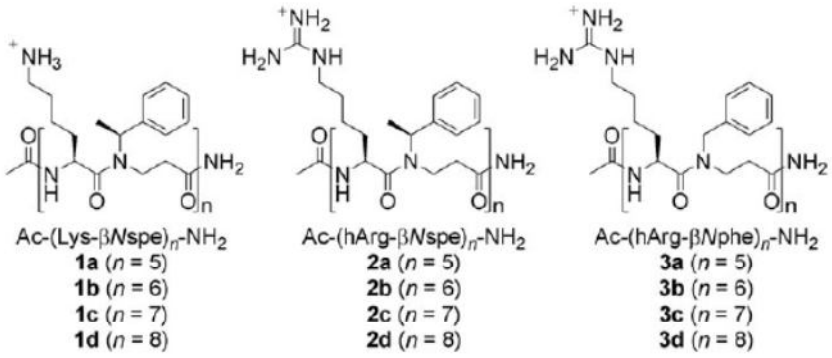


Figure 22.Chemical structures of the α-peptide/β-peptoid hybrid oligomers. The abbreviations used for the β-peptoid units were adapted from the abbreviations commonly used for peptoids (i.e., N-alkylglycines), by adding the β-prefix. βNspe=N-(S)-1-phenylethyl-balanine, βNphe=β-N-phenylalanine, hArg=homoarginine.

Some structure-activity relationship (SAR) studies have been performed. Circular dichroism (CD) spectroscopy demonstrates the presence of some degree of secondary structure(Olsen, Bonke et al. 2007; Olsen, Lambert et al. 2008; Olsen, Ziegler et al. 2010).Notably, a higher degree of secondary structure was found for analogues containing chiral side chains in the β-peptoid units as compared to chimeras with achiral β-peptoid residues (Olsen, Bonke et al. 2007), but the effect of this on antibacterial activity remains largely unresolved (Olsen, Ziegler et al. 2010).

The membrane-destabilizing effects of the chimeras have been investigated in model liposomes prepared from phosphatidylcholine, and several of the chimeras permeabilized such liposomal membranes (Foged, Franzyk et al. 2008). In addition, the effects of some of the chimeras on cell membranes of viable bacteria and the effects of membrane permeabilization on killing of bacteria were tested. The synthetic chimeras exert their killing effect by permeabilization of the bacterial cell envelope. The chain length of the chimeras largely influences the antibacterial activity. The outer membrane of Gram-negative bacteria may act as a barrier against chimeras. The tolerance of *S. marcescens* to chimeras may be owing to differences in the composition of

the lipopolysaccharide (LPS) layer also responsible for its resistance to polymyxin B (Hein-Kristensen, Knapp et al. 2011).

Efforts should be conducted to obtain intensive SAR and cytotoxicity data. Furthermore, the mode of action, the bacterial resistance mechanisms, and the effects of these chimeras on biofilm culture should be explored. This information will help us to derive useful trends for the future design of antimicrobial peptidomimetic constructs with potential for enhanced selectivity.

Concluding remarks

Antibiotic resistance caused by multidrug resistant bacteria remains a major threat to our health. We are gradually entering into the Post-Antibiotic Era, when antibiotics no longer work. Meanwhile, more and more medical implants are used in hospitals which are often colonized by biofilm-forming bacteria such as *S. epidermidis*. In this thesis' work, different approaches were used to combat biofilms formed by *P. aeruginosa* and *S. epidermidis*.

Firstly, we targeted quorum sensing signaling pathways of *P. aeruginosa*. We have used molecular dynamics simulation and experimental studies to elucidate the efficiencies of two potential quorum-quenching reagents, triclosan and green tea epigallocatechin gallate (EGCG), which both function as inhibitors of the enoyl-acyl carrier protein (ACP) reductase (ENR) from the bacterial type II fatty acid synthesis pathway. Our studies suggest that EGCG has a higher binding affinity towards ENR of *P. aeruginosa* and is an efficient quorum-quenching reagent. EGCG treatment was further shown to be able to attenuate the production of virulence factors and biofilm resistance of *P. aeruginosa*(**paper I**).

Secondly, we interfered with the iron signaling pathway of *P. aeruginosa*. The efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN) was paired with iron chelators 2,2′ -dipyridyl, acetohydroxamic acid, and EDTA to assess synergistic activities against *P aeruginosa* growth and biofilm formation. All of the tested iron chelators synergistically inhibited *P. aeruginosa* growth and biofilm formation with PAβN. PAβN-EDTA showed the most promising activity against *P. aeruginosa* growth and biofilm formation (**paper II).**

To combat another biofilm model organisms *S. epidermidis*, we tested the effects of novel α-peptide/β-peptoid chimeras against the slow growing stationary growth phase and biofilm cells. All tested peptidomimetics were bactericidal against both exponentially growing and stationary-phase *S. epidermidis* cells with similar killing kinetics. At the Minimum Inhibitory Concentration (MIC) all peptidomimetics inhibited biofilm formation, while peptidomimetics at concentrations above MIC (80-160 μg/ml) eradicated young (6-h-old) biofilms, even higher concentrations were needed to eradicate mature (24-h-old) biofilms completely. Cytotoxicity assays showed a clear correlation between oligomer length and cell toxicity within each subclass of peptides, but all possessed a high differential toxicity favoring killing of bacterial cells. The study suggests this class of peptidomimetics may constitute promising antimicrobial alternatives for the prevention and treatment of MDR *S. epidermidis* infections. (**paper III**).

To better understand the potential resistance mechanisms of novel α-peptide/β-peptoid chimeras, we used transcriptomics, adaptive evolution combined with genome sequencing to identify stress response and resistant development after exposure of *S. epidermidis* to α-peptide/β-peptoid chimeras. Transcriptomic profiles of *S.epidermidis* exposed to peptidomimetics and vancomycin suggests that peptidomimetics target the cell membrane in a similar but distinct mode as vancomycin. Comparative genomic and transcriptomic analysis of evolved resistant populations showed that general stress response especially the stringent response is an important mechanism for *S. epidermidis* to respond to peptidomimetics. In addition, some specific mechanisms are involved to the defense of peptidomiemtics, such as the *VraRS* and *Aps* sensor systems, various

efflux pumps and membrane modification enzymes. These results suggest that peptidomimetics could constitute a potential additive to conventional antibiotic treatment. In addition, this study addressed the potential risk of resistance development of peptidomimetics, which provided useful information for designing the next generation peptide based antimicrobial compounds. (**paper IV**).

References

Abachin, E., C. Poyart, et al. (2002). "Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of Listeria monocytogenes." Mol Microbiol **43**(1): 1-14.

Aendekerk, S., B. Ghysels, et al. (2002). "Characterization of a new efflux pump, MexGHI-OpmD, from Pseudomonas aeruginosa that confers resistance to vanadium." Microbiology **148**(Pt 8): 2371-2381.

Arvidsson, P. I., N. S. Ryder, et al. (2005). "Exploring the antibacterial and hemolytic activity of shorter- and longer-chain beta-, alpha,beta-, and gamma-peptides, and of beta-peptides from beta2-3-aza- and beta3-2-methylidene-amino acids bearing proteinogenic side chains--a survey." Chem Biodivers **2**(3): 401-420.

Bagg, A. and J. B. Neilands (1987). "Molecular mechanism of regulation of siderophore-mediated iron assimilation." Microbiol Rev **51**(4): 509-518.

Banin, E., M. L. Vasil, et al. (2005). "Iron and Pseudomonas aeruginosa biofilm formation." Proc Natl Acad Sci U S A **102**(31): 11076-11081.

Batoni, G., G. Maisetta, et al. (2011). "Use of antimicrobial peptides against microbial biofilms: advantages and limits." Curr Med Chem **18**(2): 256-279.

Bayer, A. S., R. Prasad, et al. (2000). "In vitro resistance of Staphylococcus aureus to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity." Infect Immun **68**(6): 3548-3553.

Bjarnsholt, T., P. O. Jensen, et al. (2005). "Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent." Microbiology **151**(Pt 2): 373-383.

Boehr, D. D., K. A. Draker, et al. (2003). "Broad-spectrum peptide inhibitors of aminoglycoside antibiotic resistance enzymes." Chem Biol **10**(2): 189-196.

Boman, H. G., B. Agerberth, et al. (1993). "Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine." Infect Immun **61**(7): 2978-2984.

Bowdish, D. M., D. J. Davidson, et al. (2005). "A re-evaluation of the role of host defence peptides in mammalian immunity." Curr Protein Pept Sci **6**(1): 35-51.

Brogden, K. A. (2005). "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?" Nat Rev Microbiol **3**(3): 238-250.

Brotz, H., G. Bierbaum, et al. (1997). "The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation." Eur J Biochem **246**(1): 193-199.

Brown, K. L. and R. E. Hancock (2006). "Cationic host defense (antimicrobial) peptides." Curr Opin Immunol **18**(1): 24-30.

Brown, M. R., D. G. Allison, et al. (1988). "Resistance of bacterial biofilms to antibiotics: a growth-rate related effect?" J Antimicrob Chemother **22**(6): 777-780.

Brumfitt, W., M. R. Salton, et al. (2002). "Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci." J Antimicrob Chemother **50**(5): 731-734.

Chan, Y. Y. and K. L. Chua (2005). "The Burkholderia pseudomallei BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence." J Bacteriol **187**(14): 4707-4719.

Chen, C. Z., N. C. Beck-Tan, et al. (2000). "Quaternary ammonium functionalized poly(propylene imine) dendrimers as effective antimicrobials: structure-activity studies." Biomacromolecules **1**(3): 473-480.

Cheng, R. P., S. H. Gellman, et al. (2001). "beta-Peptides: from structure to function." Chem Rev **101**(10): 3219-3232.
Chongsiriwatana, N. P., J. A. Patch, et al. (2008). "Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides." Proc Natl Acad Sci U S A **105**(8): 2794-2799.
Clatworthy, A. E., E. Pierson, et al. (2007). "Targeting virulence: a new paradigm for antimicrobial therapy." Nat Chem Biol **3**(9): 541-548.
Collins, L. V., S. A. Kristian, et al. (2002). "Staphylococcus aureus strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice." J Infect Dis **186**(2): 214-219.
Costerton, J. W., Z. Lewandowski, et al. (1995). "Microbial biofilms." Annu Rev Microbiol **49**: 711-745.
Costerton, J. W., P. S. Stewart, et al. (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-1322.
Crouch, M. L., L. A. Becker, et al. (2005). "The alternative sigma factor sigma is required for resistance of Salmonella enterica serovar Typhimurium to anti-microbial peptides." Mol Microbiol **56**(3): 789-799.
Davey, M. E., N. C. Caiazza, et al. (2003). "Rhamnolipid surfactant production affects biofilm architecture in Pseudomonas aeruginosa PAO1." J Bacteriol **185**(3): 1027-1036.
Davies, D. G., M. R. Parsek, et al. (1998). "The involvement of cell-to-cell signals in the development of a bacterial biofilm." Science **280**(5361): 295-298.
Deretic, V., M. J. Schurr, et al. (1995). "Pseudomonas aeruginosa, mucoidy and the chronic infection phenotype in cystic fibrosis." Trends Microbiol **3**(9): 351-356.
Diamond, G., N. Beckloff, et al. (2009). "The roles of antimicrobial peptides in innate host defense." Curr Pharm Des **15**(21): 2377-2392.
Donlan, R. M. (2001). "Biofilms and device-associated infections." Emerg Infect Dis **7**(2): 277-281.
Ernst, R. K., T. Guina, et al. (2001). "Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity." Microbes Infect **3**(14-15): 1327-1334.
Falagas, M. E. and S. K. Kasiakou (2005). "Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections." Clin Infect Dis **40**(9): 1333-1341.
Foged, C., H. Franzyk, et al. (2008). "Cellular uptake and membrane-destabilising properties of alpha-peptide/beta-peptoid chimeras: lessons for the design of new cell-penetrating peptides." Biochim Biophys Acta **1778**(11): 2487-2495.
Franklin, M. J., D. E. Nivens, et al. (2011). "Biosynthesis of the Pseudomonas aeruginosa Extracellular Polysaccharides, Alginate, Pel, and Psl." Frontiers in microbiology **2**: 167.
Frederiksen, B., C. Koch, et al. (1997). "Antibiotic treatment of initial colonization with Pseudomonas aeruginosa postpones chronic infection and prevents deterioration of pulmonary function in cystic fibrosis." Pediatr Pulmonol **23**(5): 330-335.
Frick, I. M., P. Akesson, et al. (2003). "SIC, a secreted protein of Streptococcus pyogenes that inactivates antibacterial peptides." J Biol Chem **278**(19): 16561-16566.
Friedman, L. and R. Kolter (2004). "Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix." J Bacteriol **186**(14): 4457-4465.

Gabriel, G. J., A. E. Madkour, et al. (2008). "Synthetic mimic of antimicrobial peptide with nonmembrane-disrupting antibacterial properties." Biomacromolecules **9**(11): 2980-2983.
Garcia Vescovi, E., F. C. Soncini, et al. (1996). "Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence." Cell **84**(1): 165-174.
Gordon, Y. J., E. G. Romanowski, et al. (2005). "A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs." Curr Eye Res **30**(7): 505-515.
Groisman, E. A. (2001). "The pleiotropic two-component regulatory system PhoP-PhoQ." J Bacteriol **183**(6): 1835-1842.
Guina, T., E. C. Yi, et al. (2000). "A PhoP-regulated outer membrane protease of Salmonella enterica serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides." J Bacteriol **182**(14): 4077-4086.
Gulot, E., P. Georges, et al. (2002). "Heterogeneity of diffusion inside microbial biofilms determined by fluorescence correlation spectroscopy under two-photon excitation." Photochem Photobiol **75**(6): 570-578.
Gunn, J. S., R. K. Ernst, et al. (2000). "Constitutive mutations of the Salmonella enterica serovar Typhimurium transcriptional virulence regulator phoP." Infect Immun **68**(6): 3758-3762.
Gunn, J. S. and S. I. Miller (1996). "PhoP-PhoQ activates transcription of pmrAB, encoding a two-component regulatory system involved in Salmonella typhimurium antimicrobial peptide resistance." J Bacteriol **178**(23): 6857-6864.
Gunn, J. S., S. S. Ryan, et al. (2000). "Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of Salmonella enterica serovar typhimurium." Infect Immun **68**(11): 6139-6146.
Guo, L., K. B. Lim, et al. (1998). "Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides." Cell **95**(2): 189-198.
Hall-Stoodley, L., J. W. Costerton, et al. (2004). "Bacterial biofilms: from the natural environment to infectious diseases." Nat Rev Microbiol **2**(2): 95-108.
Hancock, R. E. (2001). "Cationic peptides: effectors in innate immunity and novel antimicrobials." Lancet Infect Dis **1**(3): 156-164.
Hancock, R. E. and D. S. Chapple (1999). "Peptide antibiotics." Antimicrob Agents Chemother **43**(6): 1317-1323.
Hancock, R. E. and H. G. Sahl (2006). "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies." Nat Biotechnol **24**(12): 1551-1557.
Hannula, M. and M. L. Hanninen (2008). "Effect of putative efflux pump inhibitors and inducers on the antimicrobial susceptibility of Campylobacter jejuni and Campylobacter coli." J Med Microbiol **57**(Pt 7): 851-855.
Harmsen, M., L. Yang, et al. (2010). "An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal." FEMS Immunol Med Microbiol **59**(3): 253-268.
Hawkey, P. M. (1998). "The origins and molecular basis of antibiotic resistance." BMJ **317**(7159): 657-660.
Hein-Kristensen, L., K. M. Knapp, et al. (2011). "Bacterial membrane activity of alpha-peptide/beta-peptoid chimeras: influence of amino acid composition and chain length on the activity against different bacterial strains." BMC Microbiol **11**: 144.
Herrmann, G., L. Yang, et al. (2010). "Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm Pseudomonas aeruginosa." J Infect Dis **202**(10): 1585-1592.

Hill, D., B. Rose, et al. (2005). "Antibiotic susceptabilities of Pseudomonas aeruginosa isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions." J Clin Microbiol **43**(10): 5085-5090.
Hoch, J. A. (2000). "Two-component and phosphorelay signal transduction." Curr Opin Microbiol **3**(2): 165-170.
Hoiby, N. and C. Koch (1990). "Cystic fibrosis. 1. Pseudomonas aeruginosa infection in cystic fibrosis and its management." Thorax **45**(11): 881-884.
Horne, W. S. and S. H. Gellman (2008). "Foldamers with heterogeneous backbones." Acc Chem Res **41**(10): 1399-1408.
Hoyle, B. D. and J. W. Costerton (1991). "Bacterial resistance to antibiotics: the role of biofilms." Prog Drug Res **37**: 91-105.
Ilker, M. F., K. Nusslein, et al. (2004). "Tuning the hemolytic and antibacterial activities of amphiphilic polynorbornene derivatives." J Am Chem Soc **126**(48): 15870-15875.
Islam, D., L. Bandholtz, et al. (2001). "Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator." Nat Med **7**(2): 180-185.
Jenssen, H., P. Hamill, et al. (2006). "Peptide antimicrobial agents." Clin Microbiol Rev **19**(3): 491-511.
Jerse, A. E., N. D. Sharma, et al. (2003). "A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection." Infect Immun **71**(10): 5576-5582.
Jin, T., M. Bokarewa, et al. (2004). "Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism." J Immunol **172**(2): 1169-1176.
Klausen, M., A. Aaes-Jorgensen, et al. (2003). "Involvement of bacterial migration in the development of complex multicellular structures in Pseudomonas aeruginosa biofilms." Mol Microbiol **50**(1): 61-68.
Klausen, M., A. Heydorn, et al. (2003). "Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants." Mol Microbiol **48**(6): 1511-1524.
Kline, T., M. Fromhold, et al. (2000). "Antimicrobial effects of novel siderophores linked to beta-lactam antibiotics." Bioorg Med Chem **8**(1): 73-93.
Kobayashi, H. (2001). "Airway biofilm disease." Int J Antimicrob Agents **17**(5): 351-356.
Kragol, G., S. Lovas, et al. (2001). "The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding." Biochemistry **40**(10): 3016-3026.
Kristian, S. A., V. Datta, et al. (2005). "D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion." J Bacteriol **187**(19): 6719-6725.
Kruszewska, D., H. G. Sahl, et al. (2004). "Mersacidin eradicates methicillin-resistant Staphylococcus aureus (MRSA) in a mouse rhinitis model." J Antimicrob Chemother **54**(3): 648-653.
Kupferwasser, L. I., R. A. Skurray, et al. (1999). "Plasmid-mediated resistance to thrombin-induced platelet microbicidal protein in staphylococci: role of the qacA locus." Antimicrob Agents Chemother **43**(10): 2395-2399.
Kuroda, K., G. A. Caputo, et al. (2009). "The role of hydrophobicity in the antimicrobial and hemolytic activities of polymethacrylate derivatives." Chemistry **15**(5): 1123-1133.

Kuroda, K. and W. F. DeGrado (2005). "Amphiphilic polymethacrylate derivatives as antimicrobial agents." J Am Chem Soc **127**(12): 4128-4129.
Kvist, M., V. Hancock, et al. (2008). "Inactivation of efflux pumps abolishes bacterial biofilm formation." Appl Environ Microbiol **74**(23): 7376-7382.
Lai, Y., A. E. Villaruz, et al. (2007). "The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci." Mol Microbiol **63**(2): 497-506.
Lamont, I. L., P. A. Beare, et al. (2002). "Siderophore-mediated signaling regulates virulence factor production in Pseudomonasaeruginosa." Proc Natl Acad Sci U S A **99**(10): 7072-7077.
Latifi, A., M. Foglino, et al. (1996). "A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS." Mol Microbiol **21**(6): 1137-1146.
Lauth, X., M. von Kockritz-Blickwede, et al. (2009). "M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition." J Innate Immun **1**(3): 202-214.
Lequette, Y. and E. P. Greenberg (2005). "Timing and localization of rhamnolipid synthesis gene expression in Pseudomonas aeruginosa biofilms." J Bacteriol **187**(1): 37-44.
Li, M., D. J. Cha, et al. (2007). "The antimicrobial peptide-sensing system aps of Staphylococcus aureus." Mol Microbiol **66**(5): 1136-1147.
Li, M., Y. Lai, et al. (2007). "Gram-positive three-component antimicrobial peptide-sensing system." Proc Natl Acad Sci U S A **104**(22): 9469-9474.
Lienkamp, K., A. E. Madkour, et al. (2008). "Antimicrobial polymers prepared by ROMP with unprecedented selectivity: a molecular construction kit approach." J Am Chem Soc **130**(30): 9836-9843.
Lopez-Solanilla, E., B. Gonzalez-Zorn, et al. (2003). "Susceptibility of Listeria monocytogenes to antimicrobial peptides." FEMS Microbiol Lett **226**(1): 101-105.
Lyczak, J. B., C. L. Cannon, et al. (2000). "Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist." Microbes Infect **2**(9): 1051-1060.
Macfarlane, E. L., A. Kwasnicka, et al. (2000). "Role of Pseudomonas aeruginosa PhoP-phoQ in resistance to antimicrobial cationic peptides and aminoglycosides." Microbiology **146 (Pt 10)**: 2543-2554.
Mack, D., W. Fischer, et al. (1996). "The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis." J Bacteriol **178**(1): 175-183.
Maki, D. G. and A. A. Schuna (1978). "A study of antimicrobial misuse in a university hospital." Am J Med Sci **275**(3): 271-282.
Martinez, J. L. and F. Baquero (2000). "Mutation frequencies and antibiotic resistance." Antimicrob Agents Chemother **44**(7): 1771-1777.
Mason, K. M., R. S. Munson, Jr., et al. (2005). "A mutation in the sap operon attenuates survival of nontypeable Haemophilus influenzae in a chinchilla model of otitis media." Infect Immun **73**(1): 599-608.
McPhee, J. B., S. Lewenza, et al. (2003). "Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa." Mol Microbiol **50**(1): 205-217.

Miller, M. B. and B. L. Bassler (2001). "Quorum sensing in bacteria." Annu Rev Microbiol **55**: 165-199.
Miller, M. J., J. A. McKee, et al. (1991). "The design, synthesis and study of siderophore-antibiotic conjugates. Siderophore mediated drug transport." Biol Met **4**(1): 62-69.
Miller, S. I., A. M. Kukral, et al. (1989). "A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence." Proc Natl Acad Sci U S A **86**(13): 5054-5058.
Moreau-Marquis, S., G. A. O'Toole, et al. (2009). "Tobramycin and FDA-approved iron chelators eliminate Pseudomonas aeruginosa biofilms on cystic fibrosis cells." Am J Respir Cell Mol Biol **41**(3): 305-313.
Moskowitz, S. M., R. K. Ernst, et al. (2004). "PmrAB, a two-component regulatory system of Pseudomonas aeruginosa that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A." J Bacteriol **186**(2): 575-579.
Mowery, B. P., S. E. Lee, et al. (2007). "Mimicry of antimicrobial host-defense peptides by random copolymers." J Am Chem Soc **129**(50): 15474-15476.
Mowery, B. P., A. H. Lindner, et al. (2009). "Structure-activity relationships among random nylon-3 copolymers that mimic antibacterial host-defense peptides." J Am Chem Soc **131**(28): 9735-9745.
Nishi, H., H. Komatsuzawa, et al. (2004). "Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in Staphylococcus aureus." Antimicrob Agents Chemother **48**(12): 4800-4807.
Nizet, V. (2006). "Antimicrobial peptide resistance mechanisms of human bacterial pathogens." Curr Issues Mol Biol **8**(1): 11-26.
O'Toole, G. A. and R. Kolter (1998). "Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development." Mol Microbiol **30**(2): 295-304.
Olsen, C. A., G. Bonke, et al. (2007). "Alpha-peptide/beta-peptoid chimeras." Org Lett **9**(8): 1549-1552.
Olsen, C. A., M. Lambert, et al. (2008). "Solid-phase peptide synthesis and circular dichroism study of chiral beta-peptoid homooligomers." Amino Acids **34**(3): 465-471.
Olsen, C. A., H. L. Ziegler, et al. (2010). "Antimicrobial, hemolytic, and cytotoxic activities of beta-peptoid-peptide hybrid oligomers: improved properties compared to natural AMPs." Chembiochem **11**(10): 1356-1360.
Otto, M. (2004). "Virulence factors of the coagulase-negative staphylococci." Front Biosci **9**: 841-863.
Otto, M. (2009). "Bacterial sensing of antimicrobial peptides." Contrib Microbiol **16**: 136-149.
Otto, M., A. Peschel, et al. (1998). "Producer self-protection against the lantibiotic epidermin by the ABC transporter EpiFEG of Staphylococcus epidermidis Tu3298." FEMS Microbiol Lett **166**(2): 203-211.
Otvos, L., Jr., I. O, et al. (2000). "Interaction between heat shock proteins and antimicrobial peptides." Biochemistry **39**(46): 14150-14159.
Pages, J. M. and L. Amaral (2009). "Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria." Biochim Biophys Acta **1794**(5): 826-833.
Paget, M. S. and J. D. Helmann (2003). "The sigma70 family of sigma factors." Genome Biol **4**(1): 203.

Palermo, E. F. and K. Kuroda (2009). "Chemical structure of cationic groups in amphiphilic polymethacrylates modulates the antimicrobial and hemolytic activities." Biomacromolecules **10**(6): 1416-1428.
Palermo, E. F. and K. Kuroda (2010). "Structural determinants of antimicrobial activity in polymers which mimic host defense peptides." Appl Microbiol Biotechnol **87**(5): 1605-1615.
Pamp, S. J., M. Gjermansen, et al. (2008). "Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes." Mol Microbiol **68**(1): 223-240.
Park, C. B., H. S. Kim, et al. (1998). "Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions." Biochem Biophys Res Commun **244**(1): 253-257.
Park, P. W., G. B. Pier, et al. (2001). "Exploitation of syndecan-1 shedding by Pseudomonas aeruginosa enhances virulence." Nature **411**(6833): 98-102.
Parra-Lopez, C., R. Lin, et al. (1994). "A Salmonella protein that is required for resistance to antimicrobial peptides and transport of potassium." EMBO J **13**(17): 3964-3972.
Patrzykat, A., C. L. Friedrich, et al. (2002). "Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in Escherichia coli." Antimicrob Agents Chemother **46**(3): 605-614.
Pearson, J. P., E. C. Pesci, et al. (1997). "Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes." J Bacteriol **179**(18): 5756-5767.
Perron, G. G., M. Zasloff, et al. (2006). "Experimental evolution of resistance to an antimicrobial peptide." Proc Biol Sci **273**(1583): 251-256.
Peschel, A. and F. Gotz (1996). "Analysis of the Staphylococcus epidermidis genes epiF, -E, and -G involved in epidermin immunity." J Bacteriol **178**(2): 531-536.
Peschel, A., R. W. Jack, et al. (2001). "Staphylococcus aureus resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine." J Exp Med **193**(9): 1067-1076.
Peschel, A. and H. G. Sahl (2006). "The co-evolution of host cationic antimicrobial peptides and microbial resistance." Nat Rev Microbiol **4**(7): 529-536.
Pietiainen, M., M. Gardemeister, et al. (2005). "Cationic antimicrobial peptides elicit a complex stress response in Bacillus subtilis that involves ECF-type sigma factors and two-component signal transduction systems." Microbiology **151**(Pt 5): 1577-1592.
Poole, K. (2000). "Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria." Antimicrob Agents Chemother **44**(9): 2233-2241.
Porter, E. A., X. Wang, et al. (2000). "Non-haemolytic beta-amino-acid oligomers." Nature **404**(6778): 565.
Potera, C. (1999). "Forging a link between biofilms and disease." Science **283**(5409): 1837, 1839.
Powers, J. P. and R. E. Hancock (2003). "The relationship between peptide structure and antibacterial activity." Peptides **24**(11): 1681-1691.
Poyart, C., E. Pellegrini, et al. (2003). "Attenuated virulence of Streptococcus agalactiae deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells." Mol Microbiol **49**(6): 1615-1625.
Rotem, S. and A. Mor (2009). "Antimicrobial peptide mimics for improved therapeutic properties." Biochim Biophys Acta **1788**(8): 1582-1592.

Ryder, C., M. Byrd, et al. (2007). "Role of polysaccharides in Pseudomonas aeruginosa biofilm development." Curr Opin Microbiol **10**(6): 644-648.
Sambhy, V., B. R. Peterson, et al. (2008). "Antibacterial and hemolytic activities of pyridinium polymers as a function of the spatial relationship between the positive charge and the pendant alkyl tail." Angew Chem Int Ed Engl **47**(7): 1250-1254.
Sauer, K., A. K. Camper, et al. (2002). "Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm." J Bacteriol **184**(4): 1140-1154.
Schmidtchen, A., I. M. Frick, et al. (2002). "Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37." Mol Microbiol **46**(1): 157-168.
Schmidtchen, A., I. M. Frick, et al. (2001). "Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin." Mol Microbiol **39**(3): 708-713.
Schmitt, M. A., B. Weisblum, et al. (2004). "Unexpected relationships between structure and function in alpha,beta-peptides: antimicrobial foldamers with heterogeneous backbones." J Am Chem Soc **126**(22): 6848-6849.
Schmitt, M. A., B. Weisblum, et al. (2007). "Interplay among folding, sequence, and lipophilicity in the antibacterial and hemolytic activities of alpha/beta-peptides." J Am Chem Soc **129**(2): 417-428.
Scott, M. G. and R. E. Hancock (2000). "Cationic antimicrobial peptides and their multifunctional role in the immune system." Crit Rev Immunol **20**(5): 407-431.
Seebach, D., A. K. Beck, et al. (2004). "The world of beta- and gamma-peptides comprised of homologated proteinogenic amino acids and other components." Chem Biodivers **1**(8): 1111-1239.
Sellenet, P. H., B. Allison, et al. (2007). "Synergistic activity of hydrophilic modification in antibiotic polymers." Biomacromolecules **8**(1): 19-23.
Shafer, W. M., X. Qu, et al. (1998). "Modulation of Neisseria gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family." Proc Natl Acad Sci U S A **95**(4): 1829-1833.
Sieprawska-Lupa, M., P. Mydel, et al. (2004). "Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases." Antimicrob Agents Chemother **48**(12): 4673-4679.
Simon, R. J., R. S. Kania, et al. (1992). "Peptoids: a modular approach to drug discovery." Proceedings of the National Academy of Sciences of the United States of America **89**(20): 9367-9371.
Singh, P. K., M. R. Parsek, et al. (2002). "A component of innate immunity prevents bacterial biofilm development." Nature **417**(6888): 552-555.
Sitaram, N., C. Subbalakshmi, et al. (2003). "Indolicidin, a 13-residue basic antimicrobial peptide rich in tryptophan and proline, interacts with Ca(2+)-calmodulin." Biochem Biophys Res Commun **309**(4): 879-884.
Skerker, J. M. and H. C. Berg (2001). "Direct observation of extension and retraction of type IV pili." Proc Natl Acad Sci U S A **98**(12): 6901-6904.
Stapper, A. P., G. Narasimhan, et al. (2004). "Alginate production affects Pseudomonas aeruginosa biofilm development and architecture, but is not essential for biofilm formation." J Med Microbiol **53**(Pt 7): 679-690.
Stoodley, P., K. Sauer, et al. (2002). "Biofilms as complex differentiated communities." Annu Rev Microbiol **56**: 187-209.

Swift, S., J. A. Downie, et al. (2001). "Quorum sensing as a population-density-dependent determinant of bacterial physiology." Adv Microb Physiol **45**: 199-270.
Taggart, C. C., C. M. Greene, et al. (2003). "Inactivation of human beta-defensins 2 and 3 by elastolytic cathepsins." J Immunol **171**(2): 931-937.
Tew, G. N., D. Clements, et al. (2006). "Antimicrobial activity of an abiotic host defense peptide mimic." Biochim Biophys Acta **1758**(9): 1387-1392.
Tew, G. N., R. W. Scott, et al. (2010). "De novo design of antimicrobial polymers, foldamers, and small molecules: from discovery to practical applications." Acc Chem Res **43**(1): 30-39.
Toder, D. S., M. J. Gambello, et al. (1991). "Pseudomonas aeruginosa LasA: a second elastase under the transcriptional control of lasR." Mol Microbiol **5**(8): 2003-2010.
Van Bambeke, F., E. Balzi, et al. (2000). "Antibiotic efflux pumps." Biochem Pharmacol **60**(4): 457-470.
Varga, Z. G., A. Armada, et al. (2012). "Inhibition of quorum sensing and efflux pump system by trifluoromethyl ketone proton pump inhibitors." In Vivo **26**(2): 277-285.
Vuong, C., J. M. Voyich, et al. (2004). "Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system." Cell Microbiol **6**(3): 269-275.
Wandersman, C. and P. Delepelaire (2004). "Bacterial iron sources: from siderophores to hemophores." Annu Rev Microbiol **58**: 611-647.
Webber, M. A. and L. J. Piddock (2003). "The importance of efflux pumps in bacterial antibiotic resistance." J Antimicrob Chemother **51**(1): 9-11.
Welch, M., H. Mikkelsen, et al. (2005). "Cell-cell communication in Gram-negative bacteria." Mol Biosyst **1**(3): 196-202.
Yang, D., A. Biragyn, et al. (2004). "Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense." Annu Rev Immunol **22**: 181-215.
Yang, L., M. Nilsson, et al. (2009). "Pyoverdine and PQS mediated subpopulation interactions involved in Pseudomonas aeruginosa biofilm formation." Mol Microbiol **74**(6): 1380-1392.
Yeaman, M. R. and A. S. Bayer (2006). "Antimicrobial peptides versus invasive infections." Curr Top Microbiol Immunol **306**: 111-152.
Yeaman, M. R. and N. Y. Yount (2003). "Mechanisms of antimicrobial peptide action and resistance." Pharmacol Rev **55**(1): 27-55.
Zasloff, M. (2002). "Antimicrobial peptides of multicellular organisms." Nature **415**(6870): 389-395.
Zuckermann, R. N., J. M. Kerr, et al. (1992). "Efficient Method for the Preparation of Peptoids [Oligo(N-substituted glycines)] by Submonomer Solid Phase Synthesis." J. Am. Chem. Soc. **114**: 10646-10647.

Paper I

Molecules **2010**, *15*, 780-792; doi:10.3390/molecules15020780

OPEN ACCESS

molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Article

Evaluation of Enoyl-Acyl Carrier Protein Reductase Inhibitors as *Pseudomonas aeruginosa* Quorum-Quenching Reagents

Liang Yang *, Yang Liu, Claus Sternberg and Søren Molin

Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, 2800, Denmark; E-Mails: yali@bio.dtu.dk (Y.L.); cst@bio.dtu.dk (C.S.); sm@bio.dtu.dk (S.M.)

* Author to whom correspondence should be addressed: E-Mail: ly@bio.dtu.dk; Tel.: +45-452-52-526; Fax: +45-45-887-328.

Received: 7 December 2009; in revised form: 21 January 2010 / Accepted: 27 January 2010 / Published: 3 February 2010

Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen which is responsible for a wide range of infections. Production of virulence factors and biofilm formation by *P. aeruginosa* are partly regulated by cell-to-cell communication quorum-sensing systems. Identification of quorum-quenching reagents which block the quorum-sensing process can facilitate development of novel treatment strategies for *P. aeruginosa* infections. We have used molecular dynamics simulation and experimental studies to elucidate the efficiencies of two potential quorum-quenching reagents, triclosan and green tea epigallocatechin gallate (EGCG), which both function as inhibitors of the enoyl-acyl carrier protein (ACP) reductase (ENR) from the bacterial type II fatty acid synthesis pathway. Our studies suggest that EGCG has a higher binding affinity towards ENR of *P. aeruginosa* and is an efficient quorum-quenching reagent. EGCG treatment was further shown to be able to attenuate the production of virulence factors and biofilm formation of *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*; quorum-quenching; enoyl-acyl carrier protein reductase; molecular dynamics simulation

---

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen which causes a wide range of infective diseases such as pulmonary infections, medical-device-related infections, urinary tract infections,

wound infections as well as potentially fatal cystic fibrosis lung infections [1,2]. *P. aeruginosa* produces a large number of virulence factors and is notorious for its tolerance to many antimicrobial agents [3,4]. Another important feature of *P. aeruginosa* infections is the formation of surface attached complex multicellular communities, often referred to as biofilms [5,6]. Biofilm cells display multiple phenotypes and are surrounded by resistant extracellular polymeric substance (EPS) materials [7], which are often major causes for persistent infections [6]. Both production of virulence factors and biofilm formation are partly regulated by bacterial cell-to-cell communication (quorum-sensing) system in *P. aeruginosa* [3,8,9].

Quorum sensing (QS) is a widespread prokaryotic intercellular communication system which is based on the production of extracellular signal molecules (autoinducers) in relation to cell density [10]. Once the autoinducers reach their critical threshod concentrations, they can adjust the conformation of autoinducer receptors and together they can affect the expression profiles of a large number of genes [10]. There are three interconnected QS systems, *las*, *rhl* and *pqs* systems, in *P. aeruginosa* [3,11,12]. The major signal molecules involved in *las*, *rhl* and *pqs* QS systems are 3-oxo-C12-HSL, C4-HSL, and 2-heptyl-3-hydroxy-4-quinolone (PQS) respectively [12,13]. Among them, the *las* QS system is at the top of the QS hierarchy regulating the *rhl* and *pqs* QS systems [14].

Targeting pathways which are essential for the synthesis of QS molecules might be an approach for identifying quorum-quenching reagents. Recently, type II fatty acid synthesis intermediates were shown to be substrates for the LuxI family of autoinducer synthases [15]. The type II fatty acid synthesis pathway is present in most prokaryotes, plants, and several protozoans and has a different architectural organization from the type I fatty acid synthesis pathway of animals and human beings. In *P. aeruginosa*, mutations in the type II fatty acid synthesis *fabI* gene, which encodes enoyl-acyl carrier protein (ACP) reductase (ENR), lead to significant reduction of the 3-oxo-C12-HSL molecule of the *las* QS system [16]. High-throughput screening previously identified a large number of type II fatty acid synthesis inhibitors for different organisms [17]. These identified compounds have been served as scaffolds for structure based design of novel type II fatty acid synthesis inhibitors. Studying the effects of different type II fatty acid synthesis inhibitors on *P. aeruginosa* QS can provide valuable information for designing novel classes of *P. aeruginosa* quorum-quenching reagents.

In this study, we compared the effects of two reported broad spectrum type II fatty acid inhibitors, 5-chloro-2-(2,4-dichlorophenoxy)phenol (triclosan) and green tea (−)-epigallocatechin gallate (EGCG), on *P. aeruginosa* QS. These two inhibitors were reported be able to specifically bind with and inhibit the *Escherichia coli* ENR (EcENR) [18,19]. Since the complete three-dimensional structure of *P. aeruginosa* ENR (PaENR) is not available yet, we built a PaENR structure model through homology modeling. Then we used molecular dynamics (MD) simulations to analyze the binding affinities of triclosan and EGCG to PaENR. The MD results suggested that EGCG had a higher binding affinity to PaENR than triclosan. In agreement with the MD analysis, experiments showed that EGCG was a more efficient inhibitor of *P. aeruginosa* QS regulated virulence and biofilm formation than triclosan.

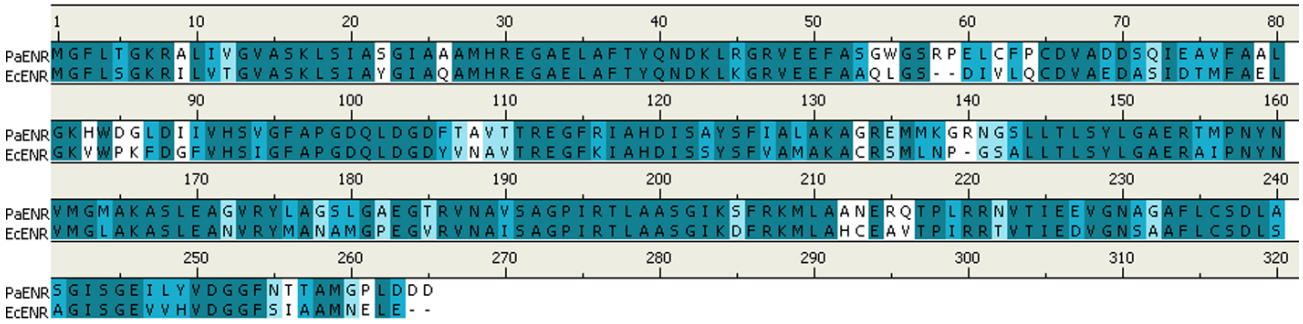
2. Results and Discussion

The *P. aeruginosa* QS system is a model system for studies of the *N*-Acyl Homoserine Lactone (AHLs)-mediated QS in Gram-negative bacteria. The QS systems are widely used by pathogenic bacteria to coordinate expression of virulence products as well as biofilm formation, which cause a variety of persistent infections for human beings, animals and plants [20,21]. Recently, QS systems were proposed as a target for the development of anti-pathogenic drugs [22]. A number of quorum-quenching reagents were identified based on the structure of the QS signal molecules [22]. However, several of the QS molecule analogs are chemically unstable or toxic for animals and human beings [23–25]. Thus, alternative methods are needed to search for novel classes of quorum-quenching reagents with different structures from the QS molecules. In this work, we investigated a novel approach to identify quorum-quenching reagents through targeting the type II fatty acid synthesis pathway.

2.1. MD Simulation of Binding Affinities of Compounds to PaENR

The structure and functions of *E. coli* ENR (EcENR) are well studied by many different research groups. EcENR has high sequence similarity to the *P. aeruginosa* ENR (PaENR) based on blast searching of PaENR sequence in the RCSB protein data bank [26]. Pairwise protein sequence alignment shows that the essential amino acids of EcENR which are involved in binding of NADH to EcENR [27] are conserved between these two ENRs: the residues in PaENR corresponding to Gly-93, Met-159 and Phe-203 of EcENR are Gly-95, Met-162 and Phe-206 (Figure 1).

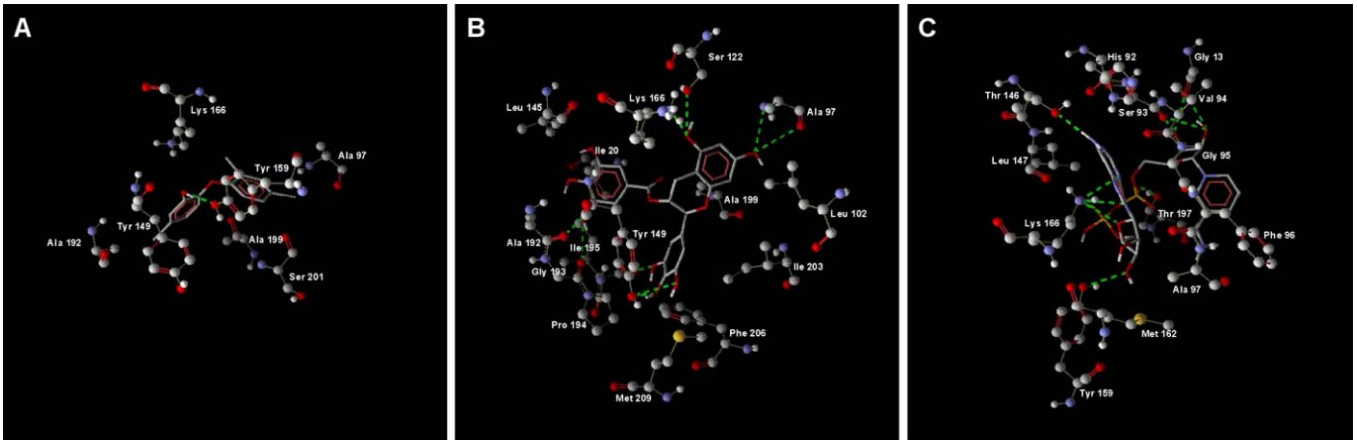
Figure 1. Pairwise protein sequence alignment of ENR from *P. aeruginosa* (PaENR) with ENR from *E. coli* (EcENR). Alignment was performed by Discovery Studio Visualizer 2.0 (Accelrys) and conserved residues are shown in dark blue with a white background.



We have used the EcENR structures to build a *P. aeruginosa* ENR (PaENR) model by homology modeling with Modeller. Then we used molecular dynamics simulation to investigate the binding affinities of two reported EcENR inhibitors, triclosan and EGCG, to PaENR. Since bacterial ENR activity is NADH dependent, and both triclosan and EGCG were reported to competitively bind with ENR against NADH [18,19], we also simulated the binding affinity and mode of NADH with PaENR. The RMSD values of all of the three systems became stable after 2.4 ns simulation (data not shown). After MD simulation, we examined the final structures of the PaENR-triclosan, PaENR-EGCG and PaENR-NADH complexes by binding score analysis and hydrogen bond interaction analysis with Molegro Molecular Viewer. The estimated binding scores of triclosan, EGCG and NADH to PaENR

were -84.9197, -157.744 and -194.123 respectively and the hydrogen bond interactions of triclosan, EGCG and NADH to PaENR are shown in Figure 2. Based on this analysis, triclosan has fewer hydrogen bond interactions with PaENR than EGCG and NADH. The hydroxyl groups from EGCG enable it to form several hydrogen bonds with PaENR (Figure 2B). Most of the hydrogen bond interactions of PaENR-triclosan, PaENR-EGCG and PaENR-NADH complexes are mediated by amino acid residues close to residue 95, residue 150 and residue 200 (Figure 2), which is in accordance with the experimental crystal structure analysis of ENRs from other species [27]. Based on the MD simulation analysis, EGCG has a higher binding affinity to PaENR than triclosan and this result suggests that EGCG may have better inhibition effects towards QS activity via interfering PaENR than triclosan. By employing another approach, Sharma *et al*. used cluster analysis to group docked confirmations generated through 100 independent docking runs of triclosan and EGCG against Enoyl-ACP Reductase from Plasmodium falciparum (PfENR) [28]. Similar to our results, they reported that triclosan and EGCG tend to occupy different binding pockets of the PfENR. Our method using Molegro Molecular Viewer to calculate the binding affinities of triclosan, EGCG and NADH to PaENR is based on simple energy functions, which is fast but provides relatively low accuracy. Rigorous free energy perturbation methods after MD simulation could obtain more accurate protein–ligand binding energy [29].

Figure 2. Hydrogen bond interaction analysis by Molegro Molecular Viewer. A: PaENR-triclosan; B: PaENR-EGCG; C: PaENR-NADH.

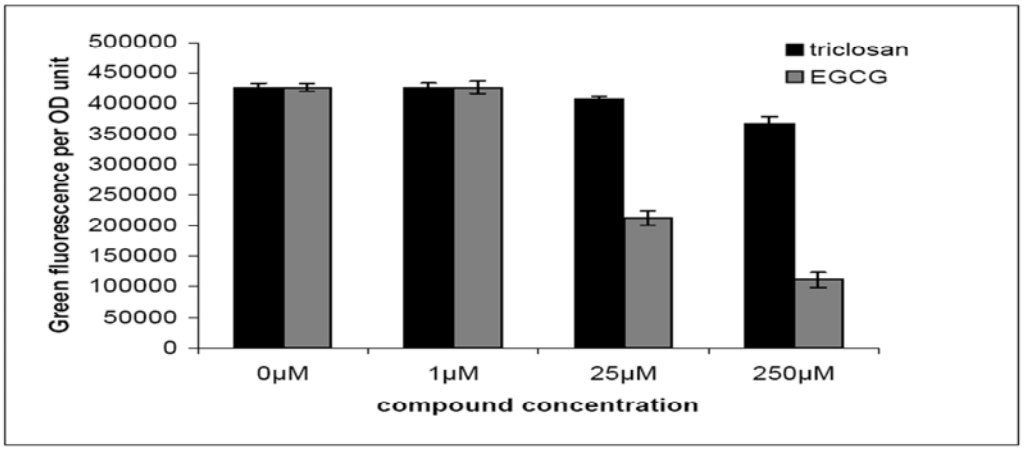


2.2. Quorum-Sensing, Virulence and Biofilm Attenuation by PaENR Inhibitors

It was shown previously that a high binding affinity to EcENR is very critical for EcENR inhibitors to act as antibiotics, since the AcrAB efflux system of *E. coli* can efficiently pump EcENR inhibitors out of the cells [30]. *P. aeruginosa* has an efflux system highly homologous to the *E. coli* AcrAB system, which was shown to be essential for its resistance to the ENR inhibitor triclosan [16]. Triclosan is, however, not an efficient PaENR inhibitor, and it is actually widely used in the *Pseudomonas isolation agar* (PIA; Difco) (whose formulation contains 25 μg/mL of triclosan) to isolate *Pseudomonas* species from other bacterial species (such as *E. coli*). We propose here that a high binding affinity of compounds to PaENR is necessary for them to efficiently interfere with the *P. aeruginosa* type II fatty acid synthesis and attenuate QS signaling.

To test our proposal we examined the quorum-quenching capabilities of triclosan and EGCG by means of a quorum-quenching assay based on a *lasB*::*gfp(ASV)* translational fusion in *P. aeruginosa* wild-type PAO1 strain [31]. In this assay an unstable version of Gfp (ASV) was fused with the QS regulated LasB protein, by which the *P. aeruginosa* QS activity can be indicated by measuring green fluorescence per OD_{600} unit. As shown in Figure 3, triclosan showed a slight reduction in green fluorescence per OD unit, while EGCG showed significant reduction in green fluorescence per OD unit. Both triclosan and EGCG showed no growth inhibition of *P. aeruginosa* PAO1 strain under our tested concentrations. This suggests that EGCG is an active quorum-quenching reagent.

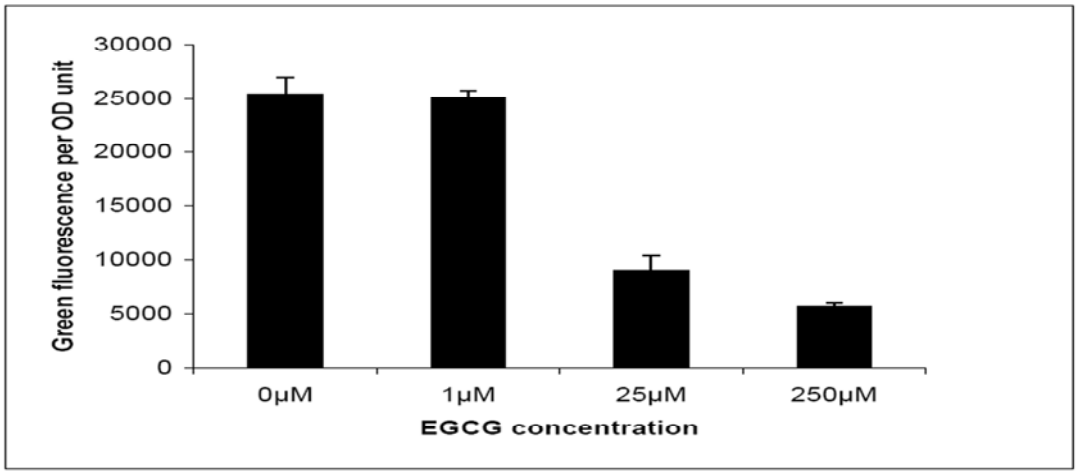
Figure 3. Expression of *lasB*:*gfp*(*ASV*) in wild-type *P. aeruginosa* treated with triclosan (black bar) and EGCG (gray bar). Results are average values of green fluorescence divided by OD_{600} taken from a single time point measurement corresponding to maximal induction of the reporters in the late log phase of growth. Inhibitors were added at concentrations of 0 μM, 1 μM, 25 μM and 250 μM. Averages and SDs of three replicates are shown.



We also investigated whether attenuation of *P. aeruginosa* QS by EGCG might lead to reduction of QS regulated virulence factors and activities *via* measuring transcription of *pqsABCDE* operon and swarming motility of *P. aeruginosa* PAO1 strain.

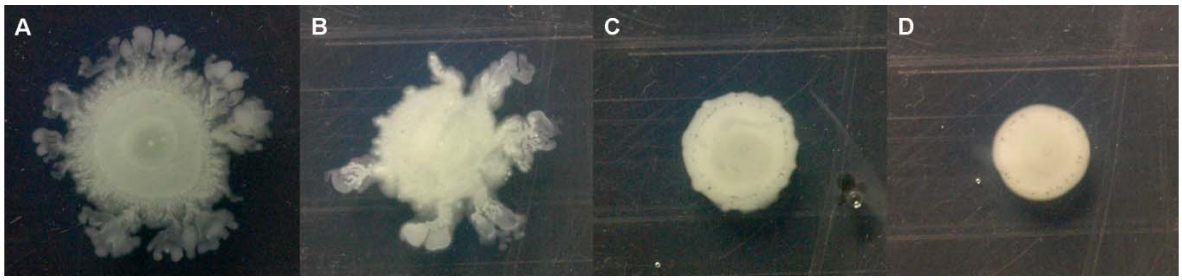
The *pqsABCDE* operon is involved in synthesis of *Pseudomonas* quinolone signal (PQS), which functions as a signal molecule as well as an iron chelator [32]. The transcription of the *pqsABCDE* operon was shown to be under stringent regulation of the *las* QS system [33]. In the *pqsABCDE* transcription assay, an unstable version of Gfp (ASV) was fused with a QS regulated *pqsABCDE* promoter, by which the transcription of the *pqsABCDE* operon can be estimated by measuring green fluorescence per OD unit [34]. As shown in Figure 4, transcription of the *pqsABCDE* operon in the *P. aeruginosa* PAO1 was reduced by EGCG in a dose dependent manner. The swarming motility is characterized by a coordinated translocation of a bacterial population across solid or semi-solid surfaces. By dropping a cell pellet on the surface of a solid agar plate, bacterial cells are able to move away from the initial location leading to the formation of arm-like or dendritic fractal-like patterns.

Figure 4. Expression of *pqsA*:*gfp*(*ASV*) in wild-type *P. aeruginosa* treated with EGCG. Results are average values of green fluorescence divided by OD_{600} taken from a single time point measurement corresponding to maximal induction of the reporters in the late log phase of growth. Inhibitors were added at concentrations of 0 μM, 1 μM, 25 μM and 250 μM. Averages and SDs of three replicates are shown.



Since it was reported that both type II fatty acid synthesis [35] and PQS regulation [36] were involved in production of the biosurfactant rhamnolipid, which is required for *P. aeruginosa* swarming motility, we investigated whether EGCG could attenuate swarming motility of *P. aeruginosa* PAO1 strain. As expected, our results showed that swarming motility by *P. aeruginosa* PAO1 strain was reduced by EGCG in a dose dependent manner (Figure 5).

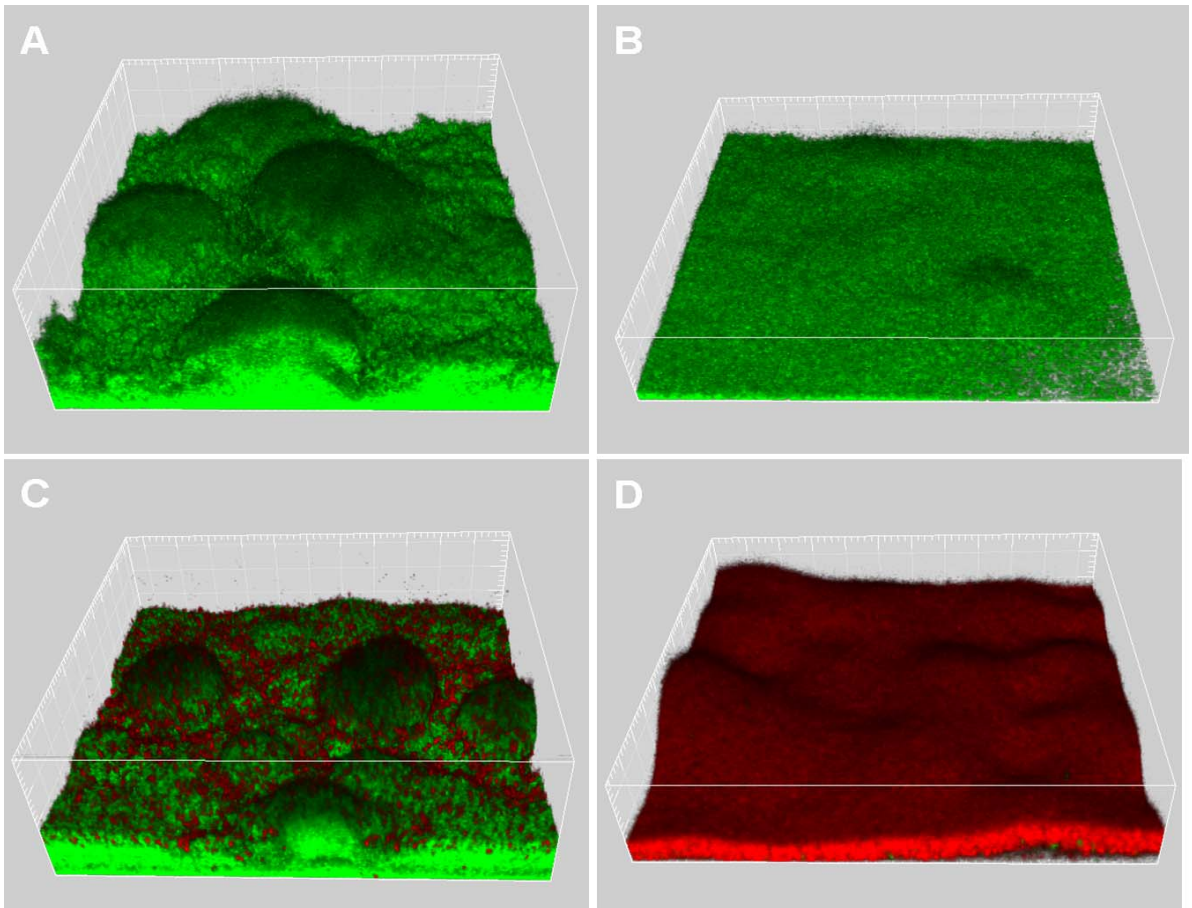
Figure 5. Swarming motility of wild-type *P. aeruginosa* strain on agar plates containing 0 μM (A), 1 μM (B), 25 μM (C) and 250 μM EGCG (D).



P. aeruginosa QS and swarming motility have been shown to play important roles in antibiotic resistance and biofilm formation [37–39]. Thus we further investigated the effects of EGCG on *P. aeruginosa* biofilm formation and its antibiotic resistance. In order to examine the effects of EGCG on *P. aeruginosa* biofilm formation, we grew Gfp tagged *P. aeruginosa* PAO1 in flow-chambers irrigated with normal FAB medium, with or without 25 μM EGCG. After 4 days of growth, the biofilms were observed by confocal laser scanning microscopy (CLSM), which can acquire in-focus images from selected depths based on fluorescence of the samples. Biofilm images were acquired point-by-point and three-dimensional structures of biofilms were reconstructed using the IMARIS software package (Bitplane AG). The *P. aeruginosa* PAO1 grown in normal FAB medium without EGCG formed heterogeneous biofilms containing mushroom-shaped multicellular structures (Figure 6A), while *P.*

aeruginosa PAO1 grown in FAB medium with 25 μM EGCG formed biofilms displaying less spatially organized multicellular structures (Figure 6B). After 4 days of growth, the biofilms were treated with 50 μg/mL ciprofloxacin for 24 hours. The dead bacteria in the biofilms were visualized by CLSM after staining with propidium iodide. The results showed that ciprofloxacin treatment caused killing of the bacteria located in the outer part of the cap-portion of the mushroom-shaped structures in 4-days-old *P. aeruginosa* PAO1 biofilms grown in normal FAB medium (Figure 6C), while ciprofloxacin treatment caused killing of most of bacteria in *P. aeruginosa* PAO1 biofilms grown in FAB medium with 25 μM EGCG (Figure 6D). These results are in accordance with previous studies showing that quorum-quenching reagent treated biofilms are more sensitive to treatments by antimicrobial agents [34,40,41].

Figure 6. 4-days-old biofilms of Gfp-tagged wild-type grown in FAB medium without (A) or with 25 μM EGCG (B). Biofilms were further treated with 50 μg/ml ciprofloxacin for 24 h (C: biofilm grown in FAB without EGCG; D: biofilm grown in FAB with 25 μM EGCG), after which they were stained with propidium iodide and images were acquired by CLSM. Live cells appear green and dead cells appear red.



3. Experimental

3.1. Homology Modeling

The sequence of the PaENR protein is highly homologous to the EcENR protein (sequence similarity = 84.0%, sequence identity = 68.8%). We have built a three-dimensional structure model of

PaENR based on the 1.75 Å crystal structure of EcENR (accession code: 1QSG) [42]. Homology modeling was performed with program Modeller 9V6 [43]. The model was assessed by VARIFY 3D [44,45]. Out of the few predicted iterative models, the best model having the lowest value of the MODELLER objective function was selected for the further analysis. The 3D structures of triclosan, EGCG and NADH ligands were obtained from the PDBeChem database [46]. The ligands were then inserted into the PaENR with Molegro Molecular Viewer v1.2.0 (http://www.molegro.com/) and saved as protein-ligand complexes respectively.

3.2. Molecular Dynamics Simulations

The molecular dynamics (MD) package Desmond 2.2 (D.E. Shaw Research & Schrödinger) and OPLS-AA 2005 force field [47] were used for MD simulations of the three computationally built PaENR-ligand complexes. Hydrogen atoms were added using the protein preparation wizard in Maestro 9.0 (Schrödinger). Each structure was embedded in a box of TIP3P water molecules [48] with the buffering distance set to 10 Å. For long range electrostatics, the smooth particle mesh Ewald method [49] was used along with periodic boundary conditions. By assuming normal charge states of ionizable groups corresponding to pH 7, sodium (Na^+) and chloride (Cl^-) counter-ions at physiological concentration of 0.015 mol/L were added in the box in random positions to ensure the global charge neutrality. The starting structures were relaxed by performing minimization of the model system using a hybrid method of the steepest descent and the limited-memory Broyden-Fletcher-Goldfarb-Shanno (LBFGS) algorithms [50] with a convergence threshold for the gradient in units of 1.0 kcal Å/mol. The minimization was followed by equilibration dynamics at constant temperature (T 310K) and constant pressure (P 1 bar). 2.4 ns of production MD simulations were performed for each PaENR-ligand complex with an integration time step of 2 fs. The constant pressure and temperature were controlled via Langevin dynamics method [51]. Snapshot structures were extracted for every 0.6 ps, resulting in 4000 structures from each trajectory. Thermodynamic properties (temperature, pressure, volume and energy) were monitored during MD simulations to check their convergence to stable values. Analysis of the trajectories was performed using the VMD 1.8.7 [52]. The hydrogen bond interactions and binding scores of PaENR-ligand complexes were analyzed by using Molegro Molecular Viewer.

3.3. Bacterial Cell Based Quorum-Quenching Assay

5-Chloro-2-(2,4-dichlorophenoxy)phenol (triclosan; ≥97.0%) and (−)-epigallocatechin gallate (EGCG; ≥ 97.0%) were purchased from Sigma-Aldrich. The quorum-quenching assay was carried out by growing the *P. aeruginosa lasB*::*gfp (ASV)* QS reporter strain [30] in a 96-well microtiter tray (black polystyrene; Nunc) in AB medium [53] together with 0 μM, 1 μM, 25 μM and 250 μM of triclosan and EGCG. The microtiter tray was incubated at 34 °C over night without shaking and then measured in a Wallac 1420 VICTOR3 plate reader (Perkin-Elmer, MA). The instrument was set to measure green fluorescent protein (Gfp) expression by means of the protein's fluorescence at 535 nm upon excitation at 485 nm and the growth in the wells as the OD_{600}.

3.4. Virulence Inhibition Assays

PQS synthesis assay and swarming motility assay were used as previously reported [54] with modification to measure the production of QS regulated virulence factors. For the PQS synthesis assay, the expression of a plasmid-borne *pqsA*::*gfp(ASV)* fusion in the microtitre tray cultures of the PAO1 wild-type grown in AB medium with 0 μM, 1 μM, 25 μM and 250 μM of EGCG was measured in the same manner as the above *P. aeruginosa lasB*::*gfp* QS reporter strain quorum-quenching assay. For the swarming motility assay, swarming plates consisted of AB minimal medium supplemented with glucose, casamino acids, and 0.5% Bacto agar. EGCG was mixed into the medium immediately before casting at concentrations of 0 μM, 1 μM, 25 μM and 250 μM. The plates were left for drying without lids in a fume hood for 1 h at room temperature. Five-microliter drops of overnight cultures of *P. aeruginosa* wild-type PAO1 strain were placed on the prepared plates and incubated for 18 hours at room temperature before pictures of each plate were taken.

3.5. Biofilm Assay

Biofilms were grown for 4 days in flow chambers with individual channel dimensions of 1 × 4 × 40 mm. The flow system was assembled and prepared as described previously [55]. Inoculation of the system was carried out by injecting 300 μL 1:1,000 diluted over night culture of a Gfp tagged *P. aeruginosa* strain [34] into flow channel with a small syringe. After inoculation, the flow chambers were left upside down without flow for 1 h to allow bacterial attachment to the glass cover, after which medium flow was started with a Watson Marlow 205S peristaltic pump. The flow chambers were irrigated with medium with or without 25 μM EGCG. The mean flow velocity in the flow chambers was 0.2 mm s/L, corresponding to laminar flow with a Reynolds number of 0.02. The biofilms were grown at 30 °C. Biofilm tolerance to ciprofloxacin was assessed by irrigating 4-day-old flow-chamber-grown *P. aeruginosa* biofilms with medium containing 50 μg/mL ciprofloxacin (MIC = 1 μg/mL) for 24 h, followed by staining of the dead cells with propidium iodide, and CLSM image acquisition. All microscopy observations and image acquisitions were done with a Zeiss LSM510 confocal laser scanning microscope (CLSM) equipped with detectors and filter sets for monitoring of Gfp and propidium iodide fluorescence. Images were obtained using a 40x/1.3 objective. Simulated three-dimensional images and sections were generated using the IMARIS software package (Bitplane AG).

4. Conclusions

Our study suggests that the type II fatty acid synthesis pathway is an alternative target for development of anti-virulence drug lead compounds. Type II fatty acid synthesis pathway inhibitors can be used synergistically with conventional antibiotics to treat bacterial biofilm related infections. Our study also shows that molecular dynamics simulation can be used as a tool to predict the binding mode and the affinity of compounds to target proteins in a relatively accurate manner. Future work of screening novel type II fatty acid synthesis pathway inhibitors could be done by molecular docking based virtual screening followed by molecular dynamics simulations.

Acknowledgements

This work was supported by a research grant from Lundbeck Foundation and a postdoctoral grant from the Danish Council for Independent Research.

References

1. Bodey, G.P.; Bolivar, R.; Fainstein, V.; Jadeja, L. Infections caused by Pseudomonas aeruginosa. *Rev. Infect Dis.* **1983**, *5*, 279–313.
2. Gibson, R.L.; Burns, J.L.; Ramsey, B.W. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **2003**, *168*, 918–951.
3. Passador, L.; Cook, J.M.; Gambello, M.J.; Rust, L.; Iglewski, B.H. Expression of Pseudomonas aeruginosa virulence genes requires cell-to-cell communication. *Science* **1993**, *260*, 1127–1130.
4. Carmeli, Y.; Troillet, N.; Karchmer, A.W.; Samore, M.H. Health and economic outcomes of antibiotic resistance in Pseudomonas aeruginosa. *Arch. Intern. Med.* **1999**, *159*, 1127–1132.
5. Hoiby, N.; Krogh Johansen, H.; Moser, C.; Song, Z.; Ciofu, O.; Kharazmi, A. Pseudomonas aeruginosa and the *in vitro* and *in vivo* biofilm mode of growth. *Microbes Infect.* **2001**, *3*, 23–35.
6. Costerton, J.W.; Stewart, P.S.; Greenberg, E.P. Bacterial biofilms: A common cause of persistent infections. *Science* **1999**, *284*, 1318–1322.
7. Sauer, K.; Camper, A.K.; Ehrlich, G.D.; Costerton, J.W.; Davies, D.G. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* **2002**, *184*, 1140–1154.
8. Parsek, M.R.; Greenberg, E.P. Quorum sensing signals in development of Pseudomonas aeruginosa biofilms. *Meth. Enzymol.* **1999**, *310*, 43–55.
9. Kjelleberg, S.; Molin, S. Is there a role for quorum sensing signals in bacterial biofilms? *Curr. Opin. Microbiol.* **2002**, *5*, 254–258.
10. Williams, P.; Camara, M.; Hardman, A.; Swift, S.; Milton, D.; Hope, V.J.; Winzer, K.; Middleton, B.; Pritchard, D.I.; Bycroft, B.W. Quorum sensing and the population-dependent control of virulence. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **2000**, *355*, 667–680.
11. Latifi, A.; Winson, M.K.; Foglino, M.; Bycroft, B.W.; Stewart, G.S.; Lazdunski, A.; Williams, P. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in Pseudomonas aeruginosa PAO1. *Mol. Microbiol.* **1995**, *17*, 333–343.
12. Pesci, E.C.; Milbank, J.B.; Pearson, J.P.; McKnight, S.; Kende, A.S.; Greenberg, E.P.; Iglewski, B.H. Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. USA* **1999**, 96, 11229–11234.
13. Latifi, A.; Foglino, M.; Tanaka, K.; Williams, P.; Lazdunski, A. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol. Microbiol.* **1996**, *21*, 1137–1146.

14. Wagner, V.E.; Bushnell, D.; Passador, L.; Brooks, A.I.; Iglewski, B.H. Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.* **2003**, *185*, 2080–2095.
15. Val, D.L.; Cronan, J.E. *In vivo* evidence that S-adenosylmethionine and fatty acid synthesis intermediates are the substrates for the LuxI family of autoinducer synthases. *J. Bacteriol.* **1998**, *180*, 2644–2651.
16. Hoang, T.T.; Schweizer, H P. Characterization of Pseudomonas aeruginosa enoyl-acyl carrier protein reductase (FabI): A target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. *J. Bacteriol.* **1999**, *181*, 5489–5497.
17. Campbell, J.W.; Cronan, J.E. Bacterial fatty acid biosynthesis: Targets for antibacterial drug discovery. *Annu. Rev. Microbiol.* **2001**, *55*, 305–332.
18. Heath, R.J.; Rubin, JR.; Holland, D.R.; Zhang, E.; Snow, M.E.; Rock, C.O. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *J. Biol. Chem.* **1999**, *274*, 11110–11114.
19. Zhang, Y.M.; Rock, C.O. Evaluation of epigallocatechin gallate and related plant polyphenols as inhibitors of the FabG and FabI reductases of bacterial type II fatty-acid synthase. *J. Biol. Chem.* **2004**, *279*, 30994–31001.
20. Singh, P.K.; Schaefer, A.L.; Parsek, M.R.; Moninger, T.O.; Welsh, M.J.; Greenberg, E.P. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **2000**, *407*, 762–764.
21. De Kievit, T.R.; Iglewski, B.H. Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **2000**, *68*, 4839–4849.
22. Rasmussen, T.B.; Givskov, M. Quorum-sensing inhibitors as anti-pathogenic drugs. *Int. J. Med. Microbiol.* **2006**, *296*, 149–161.
23. Glansdorp, F.G.; Thomas, G.L.; Lee, J.K.; Dutton, J.M.; Salmond, G.P.; Welch, M.; Spring, D.R. Synthesis and stability of small molecule probes for Pseudomonas aeruginosa quorum sensing modulation. *Org. Biomol. Chem.* **2004**, *2*, 3329–3336.
24. Hentzer, M.; Givskov, M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J. Clin. Invest* **2003**, *112*, 1300–1307.
25. Telford, G.; Wheeler, D.; Williams, P.; Tomkins, P.T.; Appleby, P.; Sewell, H.; Stewart, G.S.; Bycroft, B.W.; Pritchard, D.I. The Pseudomonas aeruginosa quorum-sensing signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. *Infect. Immun.* **1998**, *66*, 36–42.
26. Deshpande, N.; Addess, K.J.; Bluhm, W.F.; Merino-Ott, J.C.; Townsend-Merino, W.; Zhang, Q.; Knezevich, C.; Xie, L.; Chen, L.; Feng, Z.; Green, R.K.; Flippen-Anderson, J.L.; Westbrook, J.; Berman, H.M.; Bourne, P.E. The RCSB Protein Data Bank: A redesigned query system and relational database based on the mmCIF schema. *Nucleic Acids Res.* **2005**, *33*, 233–237.
27. Baldock, C.; Rafferty, J.B.; Stuitje, A.R.; Slabas, A.R.; Rice, D.W. The X-ray structure of Escherichia coli enoyl reductase with bound NAD+ at 2.1 A resolution. *J. Mol. Biol.* **1998**, *284*, 1529–1546.
28. Kumar, S.S.; Prasanna, P.; Gyanendra, K.; Namita, S.; Avadhesha, S. Green tea catechins potentiate triclosan binding to enoyl-ACP reductase from Plasmodium falciparum (PfENR). *J. Med. Chem.* **2007**, *50*, 765–775.

29. Woo, H.J. Calculation of absolute protein-ligand binding constants with the molecular dynamics free energy perturbation method. *Methods Mol. Biol.* **2008**, *443*, 109–120.
30. Chuanchuen, R.; Beinlich, K.; Hoang, T.T.; Becher, A.; Karkhoff-Schweizer, R.R.; Schweizer, H.P. Cross-resistance between triclosan and antibiotics in Pseudomonas aeruginosa is mediated by multidrug efflux pumps: Exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. *Antimicrob. Agents Chemother. (Bethesda)* **2001**, *45*, 428–432.
31. Rasmussen, T.B.; Bjarnsholt, T.; Skindersoe, M.E.; Hentzer, M.; Kristoffersen, P.; Kote, M.; Nielsen, J.; Eberl, L.; Givskov, M. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *J. Bacteriol.* **2005**, *187*, 1799–1814.
32. Bredenbruch, F.; Geffers, R.; Nimtz, M.; Buer, J.; Haussler, S. The Pseudomonas aeruginosa quinolone signal (PQS) has an iron-chelating activity. *Environ. Microbiol.* **2006**, *8*, 1318–1329.
33. McGrath, S.; Wade, D.S.; Pesci, E.C. Dueling quorum sensing systems in Pseudomonas aeruginosa control the production of the Pseudomonas quinolone signal (PQS). *FEMS Microbiol. Lett.* **2004**, *230*, 27–34.
34. Yang, L.; Barken, K.B.; Skindersoe, M.E.; Christensen, A.B.; Givskov, M.; Tolker-Nielsen, T. Effects of iron on DNA release and biofilm development by Pseudomonas aeruginosa. *Microbiology* **2007**, *153*, 1318–1328.
35. Rehm, B.H.; Mitsky, T.A.; Steinbuchel, A. Role of fatty acid de novo biosynthesis in polyhydroxyalkanoic acid (PHA) and rhamnolipid synthesis by pseudomonads: Establishment of the transacylase (PhaG)-mediated pathway for PHA biosynthesis in Escherichia coli. *Appl. Environ. Microbiol.* **2001**, *67*, 3102–3109.
36. Diggle, S.P.; Winzer, K.; Chhabra, S.R.; Worrall, K.E.; Camara, M.; Williams, P. The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol. Microbiol.* **2003**, *50*, 29–43.
37. Overhage, J.; Bains, M.; Brazas, M.D.; Hancock, R.E. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J. Bacteriol.* **2008**, *190*, 2671–2679.
38. Shrout, J.D.; Chopp, D.L.; Just, C.L.; Hentzer, M.; Givskov, M.; Parsek, M.R. The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. *Mol. Microbiol.* **2006**, *62*, 1264–1277.
39. Davies, D.G.; Parsek, M.R.; Pearson, J.P.; Iglewski, B.H.; Costerton, J.W.; Greenberg, E.P. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **1998**, *280*, 295–298.
40. Bjarnsholt, T.; Jensen, P.O.; Burmolle, M.; Hentzer, M.; Haagensen, J.A.; Hougen, H.P.; Calum, H.; Madsen, K.G.; Moser, C.; Molin, S.; Hoiby, N.; Givskov, M. Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. *Microbiology* **2005**, *151*, 373–383.
41. Hentzer, M.; Wu, H.; Andersen, J.B.; Riedel, K.; Rasmussen, T.B.; Bagge, N.; Kumar, N.; Schembri, M.A.; Song, Z.; Kristoffersen, P.; Manefield, M.; Costerton, J.W.; Molin, S.; Eberl, L.; Steinberg, P.; Kjelleberg, S.; Hoiby, N.; Givskov, M. Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. *EMBO J.* **2003**, *22*, 3803–3815.

42. Stewart, M.J.; Parikh, S.; Xiao, G.; Tonge, P.J.; Kisker, C. Structural basis and mechanism of enoyl reductase inhibition by triclosan. *J. Mol. Biol.* **1999**, *290*, 859–865.
43. Eswar, N.; Eramian, D.; Webb, B.; Shen, M.Y.; Sali, A. Protein structure modeling with MODELLER. *Methods Mol. Biol.* **2008**, *426*, 145–159.
44. Bowie, J.U.; Luthy, R.; Eisenberg, D. A method to identify protein sequences that fold into a known three-dimensional structure. *Science* **1991**, *253*, 164–170.
45. Luthy, R.; Bowie, J.U.; Eisenberg, D. Assessment of protein models with three-dimensional profiles. *Nature* **1992**, *356*, 83–85.
46. Dimitropoulos, D.; Ionides, J.; Henrick, K. Using MSDchem to search the PDB ligand dictionary. *Curr. Protoc. Bioinform.* **2006**, DOI:10.1002/0471250953.bi1403s15.
47. Jorgensen, W.L.; Maxwell, D.S.; Tirado-Rives, J. Development and testing of the opls all-atom force field on conformational energetics and properties of organic liquids. *J. Am. Chem. Soc.* **1996**, *118*, 11225–31126.
48. Jorgensen, W.L.; Chandrasekhar, J.; Madura, J.D.; Impey, R.W.; Klein, M.L. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **1983**, *79*, 926–935.
49. Essmann, U.; Perera, L.; Berkowitz, M.L. A smooth particle mesh Ewald method. *J. Chem. Phys.* **1995**, *103*, 8577–8593.
50. Broyden, C.G. The convergence of a class of double-rank minimization algorithms. *IMA J. Math. Appl.* **1970**, *6*, 76–90.
51. Paterlini, M.G.; Ferguson, D.M. Constant temperature simulations using the Langevin equation with velocity Verlet integration. *Chem. Phys.* **1998**, *236*, 243–252.
52. Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual molecular dynamics. *J. Mol. Graph.* **1996**, *14*, 33–38.
53. Clark, D.J.; Maaløe, O. DNA replication and the division cycle in Escherichia coli. *J. Mol. Biol.* **1967**, *23*, 99–112.
54. Yang, L.; Rybtke, M.T.; Jakobsen, T.H.; Hentzer, M.; Bjarnsholt, T.; Givskov, M.; Tolker-Nielsen, T. Computer-aided identification of recognized drugs as Pseudomonas aeruginosa quorum-sensing inhibitors. *Antimicrob. Agents Chemother. (Bethesda)* **2009**, *53*, 2432–2443.
55. Sternberg, C.; Tolker-Nielsen, T. Growing and analyzing biofilms in flow cells. *Curr. Protoc. Microbiol.* **2006**, DOI: 10.1002/9780471729259.mc01b02s00.

Paper II

Antimicrobial Agents and Chemotherapy

Synergistic Activities of an Efflux Pump Inhibitor and Iron Chelators against *Pseudomonas aeruginosa* Growth and Biofilm Formation

Yang Liu, Liang Yang and Søren Molin

Antimicrob. Agents Chemother. **2010, 54(9):3960. DOI: 10.1128/AAC.00463-10.**
Published Ahead of Print 21 June 2010.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2010, p. 3960–3963 Vol. 54, No. 9
0066-4804/10/$12.00 doi:10.1128/AAC.00463-10

Synergistic Activities of an Efflux Pump Inhibitor and Iron Chelators against *Pseudomonas aeruginosa* Growth and Biofilm Formation▽

Yang Liu, Liang Yang,* and Søren Molin

Center for Systems Microbiology, Department of Systems Biology, Technical University of Denmark, 2800 Lyngby, Denmark

Received 6 April 2010/Returned for modification 11 May 2010/Accepted 9 June 2010

The efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN) was paired with iron chelators 2,2′-dipyridyl, acetohydroxamic acid, and EDTA to assess synergistic activities against *Pseudomonas aeruginosa* growth and biofilm formation. All of the tested iron chelators synergistically inhibited *P. aeruginosa* growth and biofilm formation with PAβN. PAβN-EDTA showed the most promising activity against *P. aeruginosa* growth and biofilm formation.

Pseudomonas aeruginosa is an important opportunistic pathogen that can cause a wide range of human infections (4). *P. aeruginosa* is notorious for its tolerance to antimicrobial agents and continues to cause a serious public health problem worldwide (1). The intrinsic multidrug resistance of *P. aeruginosa* is due, to a large extent, to the expression of efflux pump systems, which include MexAB-oprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN (14). These efflux systems are reported to promote the export of antibiotics, organic solvents, biocides, and dyes (14). Recently, efflux pump inhibitors (EPIs), which specifically target the efflux activity and pump components, have been identified and proposed as novel agents to combat drug efflux mechanisms of pathogen (10). For example, phenyl-arginine-β-naphthylamide (PAβN) has been reported to be an efficient EPI for *P. aeruginosa*, as it can potentiate fluoroquinolone activity in resistant *P. aeruginosa* strains (9).

Among all of the reported *P. aeruginosa* Mex efflux systems, MexAB-OprM is the only system that is expressed constitutively in cells grown in standard laboratory media (15). This suggests that the export of antimicrobial agents is not the primary function of the MexAB-OprM efflux system. The MexAB-OprM system was identified initially by growing *P. aeruginosa* in iron-depleted minimal medium containing 2,2′-dipyridyl (Dipy) (13). In that study, Poole and colleagues reported that MexAB-OprM is overexpressed under severe iron limitation conditions, suggesting that the MexAB-OprM system plays an essential role for *P. aeruginosa* survival under iron limitation conditions (13). The study thus suggests that the combination of efflux inhibitors and iron chelators synergistically inhibits *P. aeruginosa* growth. In the present study, we evaluated the synergistic activities of EPI with iron chelators against *P. aeruginosa* growth and biofilm formation.

The wild-type *P. aeruginosa* strain PAO1 (7) and two isogenic mutants, *pvdA* (deficient in the synthesis of the iron siderophore pyoverdine [18]) and *mexAB-oprM* (deficient in the synthesis of the entire MexAB-OprM efflux pump [11]) were used in this study. Bacterial strains were cultivated in AB minimal medium supplemented with 30 mg/liter glucose (5). The AB minimal medium is an iron-restricted medium that promotes the production of the iron siderophore pyoverdine in *P. aeruginosa* (17). Stock solutions of 100 mg/ml Dipy (Sigma-Aldrich) in ethanol, 100 mg/ml acetohydroxamic acid (Sigma-Aldrich) in water, and 100 mg/ml EDTA (Sigma-Aldrich) in water were kept at 4°C until use. A stock solution of 10 mg/ml PAβN (Sigma-Aldrich) in water was kept at −20°C until use. The growth-inhibitory assay was performed by growing *P. aeruginosa* strains in 96-well microtiter dishes. Overnight cultures of *P. aeruginosa* strains were diluted 100 times in freshly prepared medium containing an appropriate concentration of PAβN. Diluted cultures (100 μl) were added to each well of the 96-well microtiter dishes. Iron chelators (Dipy, acetohydroxamic acid, and EDTA) were added to cultures in the 96-well microtiter dishes in 2-fold dilution series. Culture dishes were incubated at 37°C for 24 h, and the optical densities of the cultures were recorded at 600 nm using a VICTOR3 plate reader (Perkin-Elmer). The evaluation of the growth-inhibitory effect at each drug concentration was monitored in triplicate assays. Biofilms were cultivated as previously described (3) by partially immersing coverslips in green fluorescent protein (GFP)-tagged PAO1 (17) cultures in Falcon tubes in the presence of different compounds. The ciprofloxacin MICs for *P. aeruginosa* in the absence or presence of PAβN and/or iron chelators were determined by a serial dilution assay.

The growth of *P. aeruginosa* PAO1 was reduced significantly at concentrations of more than 200 μg/ml PAβN and 80 μg/ml Dipy, respectively (Fig. 1, first row). We observed clear synergistic effects from the combination of PAβN and Dipy. A concentration of 100 μg/ml PAβN significantly inhibited the growth of PAO1 in the presence of 20 μg/ml Dipy, and similar inhibition was obtained with a combination of 50 μg/ml PAβN and 40 μg/ml Dipy (Fig. 1, first row).

The synergistic effects of PAβN and Dipy on an *mexAB-oprM* mutant also were tested. The *mexAB-oprM* mutant was more sensitive than the wild-type PAO1 strain to Dipy and PAβN individually as well as to the combination of the two (Fig. 1, second row). Dipy at 40 μg/ml completely inhibited the

* Corresponding author. Mailing address: Infection Microbiology Group, Center for Systems Microbiology, Department of Systems Biology, Building 301, Technical University of Denmark, 2800 Lyngby, Denmark. Phone: 45 4525 2526. Fax: 45 4588 7328. E-mail: ly@bio.dtu.dk.

▽ Published ahead of print on 21 June 2010.

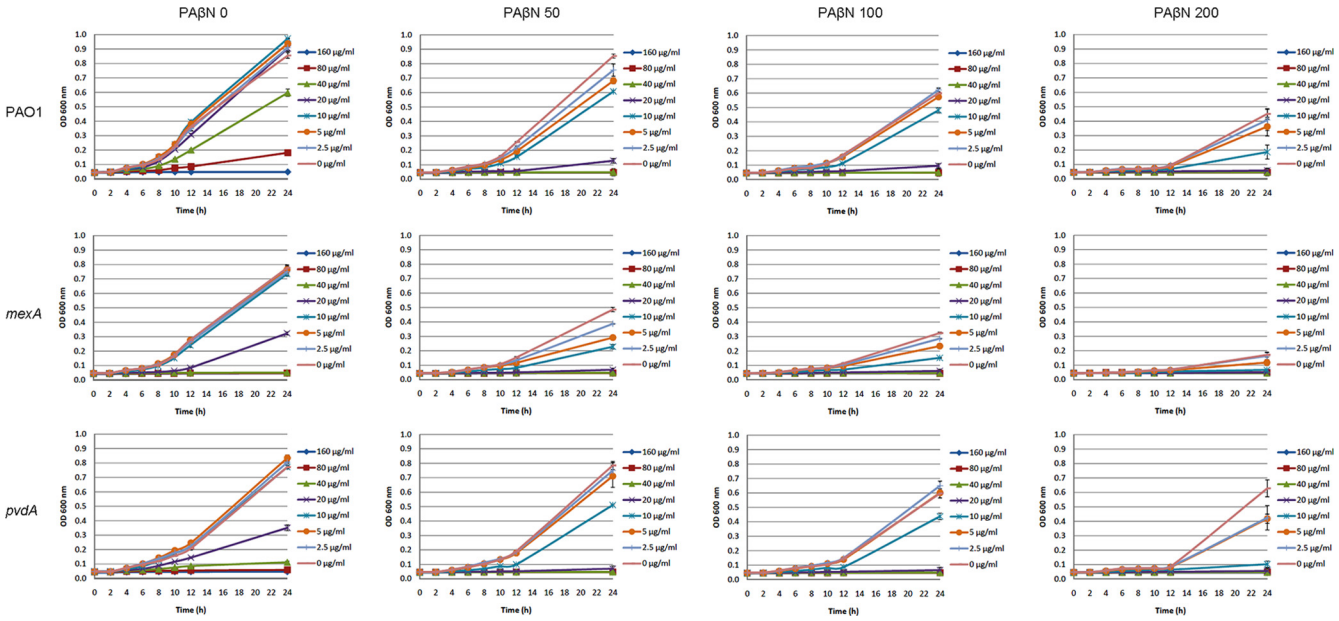


FIG. 1. Growth of *P. aeruginosa* wild-type PAO1, *mexAB-oprM* mutant, and *pvdA* mutant in the presence of different PAβN-Dipy combinations. The optical densities of the cultures at 600 nm (OD 600) were recorded after 0, 2, 4, 6, 8, 10, 12, and 24 h of incubation at 37°C. Each value represents the means and standard deviations from three wells of the microtiter dishes.

growth of *mexAB-oprM* mutant (Fig. 1, second row) but only partially inhibited the growth of wild-type PAO1 (Fig. 1, first row); 200 μg/ml PAβN reduced the growth of the *mexAB-oprM* mutant to a very low level (Fig. 1, second row), while it only partially inhibited the growth of wild-type PAO1 (Fig. 1, first row). It also was noticed that PAO1 in the presence of more than 100 μg/ml PAβN was more sensitive to Dipy than the *mexAB-oprM* mutant in the presence of no PAβN (Fig. 1). This result suggests that the synergistic activities between PAβN and Dipy is only partly associated with PAβN being a MexAB-OprM efflux pump inhibitor. We also tested whether the MexAB-OprM efflux pump is involved in pyoverdine synthesis, since pyoverdine can facilitate the growth of *P. aeruginosa* under iron-limited conditions (12). The *mexAB-oprM* mutant was found to produce normal amounts of pyoverdine, and PAβN did not reduce pyoverdine synthesis in the wild-type PAO1 strain (data not shown). The synergistic activities between PAβN and Dipy might partially be due to the ability of PAβN to affect membrane integrity when added at high concentrations (9).

The synergistic action by PAβN and Dipy on a *pvdA* mutant also was tested. As expected, the *pvdA* mutant is more sensitive to the iron chelator Dipy (Fig. 1, third row) than the wild-type PAO1 strain (Fig. 1, first row). However, the PAβN and Dipy combination inhibited the growth of the *pvdA* mutant in the same manner as wild-type PAO1 (Fig. 1), which indicated that the synergistic activities between PAβN and Dipy do not depend on the production of the iron siderophore pyoverdine.

In a further investigation of synergistic actions by PAβN and iron chelators, the effects of acetohydroxamic acid and EDTA combined with PAβN were tested. Weak synergistic activities from PAβN and acetohydroxamic acid added at high concentrations (e.g., 100 μg/ml of PAβN and 80 μg/ml of acetohydroxamic acid) were observed for both *P. aeruginosa* PAO1 and the *pvdA* mutant (Fig. 2A). In contrast, EDTA added at low concentrations (such as 2.5 μg/ml) significantly reduced the growth of *P. aeruginosa* PAO1 and the *pvdA* mutant when combined with 50 μg/ml PAβN (Fig. 2B). This possibly is due to the fact that EDTA can cause the release of protein-lipopolysaccharide complexes of *P. aeruginosa* (16). The *pvdA* mutant is more sensitive to the iron chelator acetohydroxamic acid and EDTA than wild-type PAO1 (Fig. 2). However, combinations of PAβN, acetohydroxamic acid, and EDTA inhibited the growth of the *pvdA* mutant as well as wild-type PAO1 (Fig. 2).

Since both iron uptake and efflux pump activities are reported to play important roles in *P. aeruginosa* biofilm formation (2, 11, 17, 18), we further tested whether PAβN and iron chelators could synergistically reduce *P. aeruginosa* biofilm formation. Biofilms of *P. aeruginosa* strain PAO1 were cultivated for 24 h and analyzed by confocal laser-scanning microscopy (CLSM). To gain statistical significance, three independent biofilm images of each biofilm were acquired by CLSM and then analyzed by the COMSTAT program (6). All three tested iron chelators were found to synergistically reduce *P. aeruginosa* biofilm formation when added in combination with PAβN (Fig. 3 and 4). However, EDTA was found to increase *P. aeruginosa* biofilm formation when used alone at the tested concentration (Fig. 3 and 4). This might be due to the fact that EDTA can cause the release of lipopolysaccharide from the cell wall, and the lipopolysaccharide released from the cells might serve as matrix material for the attachment of biofilm cells (8).

Finally, we also tested the effects of combinations of PAβN and iron chelators on the antibiotic sensitivity of *P. aeruginosa*. We found that PAβN increased the activity of ciprofloxacin 4-fold (MIC = 0.05 μg/ml, down from 0.2 μg/ml) at a concentration of 50 μg/ml in our assay. None of the three tested iron chelators (Dipy at a concentration of 10 μg/ml, acetohydrox-

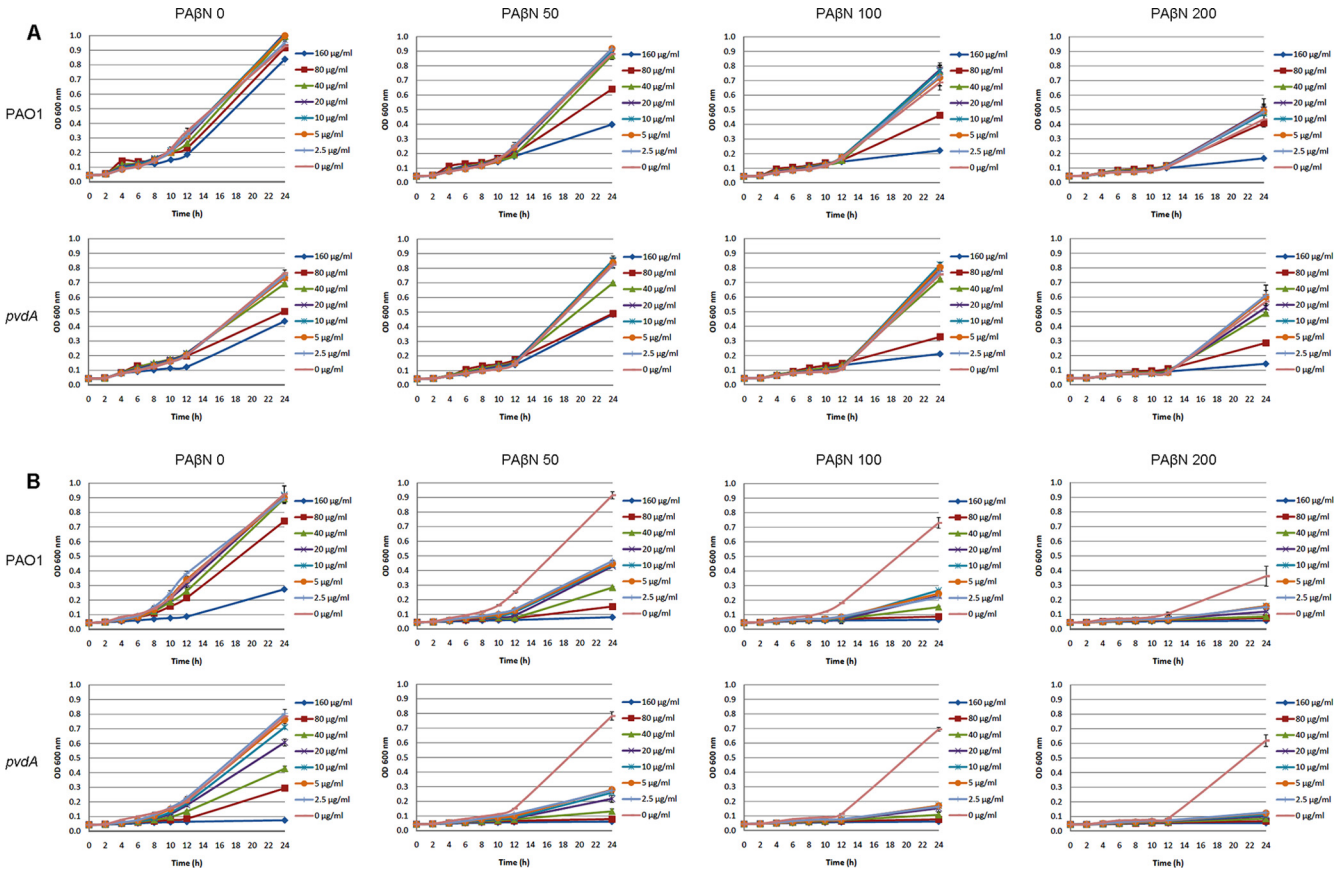


FIG. 2. Growth of *P. aeruginosa* wild-type PAO1 and *pvdA* mutant in the presence of different PAβN-acetohydroxamic acid combinations (A) and PAβN-EDTA combinations (B). The optical densities of the cultures were recorded after 0, 2, 4, 6, 8, 10, 12, and 24 h of incubation at 37°C. Each value represents the means and standard deviations from three wells of the microtiter dishes.

amic acid at a concentration of 80 μg/ml, and EDTA at a concentration of 5 μg/ml) affected the activity of ciprofloxacin. The combination of PAβN and the iron chelators at the concentrations mentioned above had the same effect on the activity of ciprofloxacin as PAβN alone, i.e., reducing the MIC from 0.2 μg/ml to 0.05 μg/ml. However, considering the synergistic effects of PAβN and iron chelators on inhibiting the growth and biofilm formation of *P. aeruginosa*, it seems that combina-

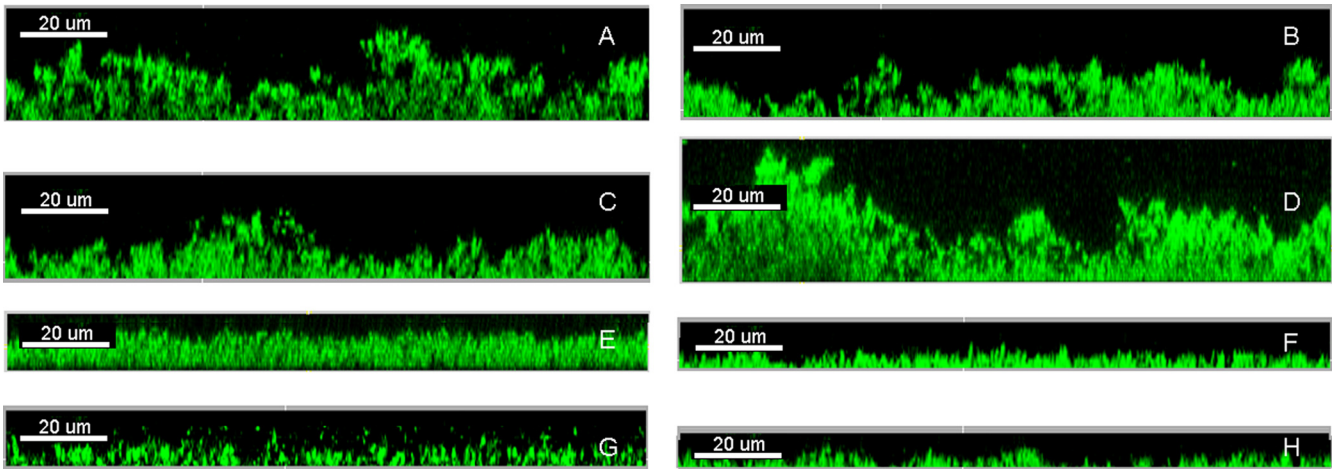


FIG. 3. Biofilm formation at the air-liquid interface of glass slides immersed in culture medium containing medium alone (A), 20 μg/ml Dipy (B), 80 μg/ml acetohydroxamic acid (C), 5 μg/ml EDTA (D), 50 μg/ml PAβN (E), 50 μg/ml PAβN and 20 μg/ml Dipy (F), 50 μg/ml PAβN and 80 μg/ml acetohydroxamic acid (G), and 50 μg/ml PAβN and 5 μg/ml EDTA (H) was observed by confocal laser-scanning microscopy and analyzed by IMARIS (Bitplane AG).

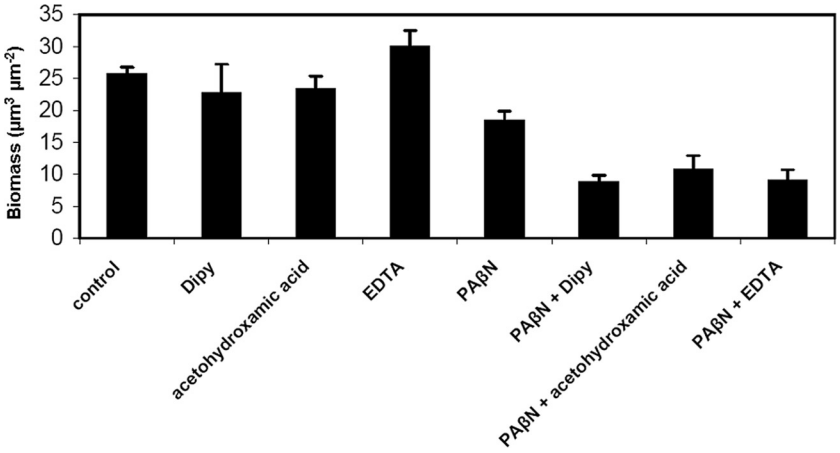


FIG. 4. Quantification of biofilms by COMSTAT. The results are means of datasets obtained from the analysis of three CLSM images acquired at random positions in each of the biofilms. Standard deviations are shown in parentheses.

tion treatment with PAβN, iron chelators, and conventional antibiotics reduces the risk of the development of antibiotic resistance and tolerance.

In conclusion, we have shown here that synergistic effects from combinations of PAβN and different iron chelators could be obtained against *P. aeruginosa* growth and biofilm formation. Our study suggests that combinations of EPIs and iron chelators constitute promising therapeutic interventions against *P. aeruginosa* infections. Further studies of synergies from combining EPIs and iron chelators against *P. aeruginosa* growth *in vivo* should be carried out.

This work was supported by a grant from the Lundbeck Foundation and a grant from the Danish Council for Independent Research.

REFERENCES

1. **Arruda, E. A., I. S. Marinho, M. Boulos, S. I. Sinto, H. H. Caiaffa, C. M. Mendes, C. P. Oplustil, H. Sader, C. E. Levy, and A. S. Levin.** 1999. Nosocomial infections caused by multiresistant Pseudomonas aeruginosa. Infect. Control Hosp. Epidemiol. **20:**620–623.
2. **Banin, E., M. L. Vasil, and E. P. Greenberg.** 2005. Iron and Pseudomonas aeruginosa biofilm formation. Proc. Natl. Acad. Sci. U. S. A. **102:**11076–11081.
3. **Bernard, C. S., C. Bordi, E. Termine, A. Filloux, and S. de Bentzmann.** 2009. Organization and PprB-dependent control of the Pseudomonas aeruginosa tad Locus, involved in Flp pilus biology. J. Bacteriol. **191:**1961–1973.
4. **Bodey, G. P., R. Bolivar, V. Fainstein, and L. Jadeja.** 1983. Infections caused by Pseudomonas aeruginosa. Rev. Infect. Dis. **5:**279–313.
5. **Clark, D. J., and O. Maaløe.** 1967. DNA replication and the division cycle in Escherichia coli. J. Mol. Biol. **23:**99–112.
6. **Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll, and S. Molin.** 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology **146:**2395–2407.
7. **Holloway, B. W., and A. F. Morgan.** 1986. Genome organization in Pseudomonas. Annu. Rev. Microbiol. **40:**79–105.
8. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of E. coli. Biochem. Biophys. Res. Commun. **21:**290–296.
9. **Lomovskaya, O., M. S. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, D. Cho, S. Chamberland, T. Renau, R. Leger, S. Hecker, W. Watkins, K. Hoshino, H. Ishida, and V. J. Lee.** 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy. Antimicrob. Agents Chemother. **45:**105–116.
10. **Pagès, J. M., M. Masi, and J. Barbe.** 2005. Inhibitors of efflux pumps in Gram-negative bacteria. Trends Mol. Med. **11:**382–389.
11. **Pamp, S. J., M. Gjermansen, H. K. Johansen, and T. Tolker-Nielsen.** 2008. Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol. Microbiol. **68:**223–240.
12. **Poole, K., D. E. Heinrichs, and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in Pseudomonas aeruginosa: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. **10:**529–544.
13. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in Pseudomonas aeruginosa: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
14. **Poole, K., and R. Srikumar.** 2001. Multidrug efflux in Pseudomonas aeruginosa: components, mechanisms and clinical significance. Curr. Top. Med. Chem. **1:**59–71.
15. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon mexA-mexB-oprM in Pseudomonas aeruginosa: mexR encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
16. **Watt, S. R., and A. J. Clarke.** 1994. Role of autolysins in the EDTA-induced lysis of Pseudomonas aeruginosa. FEMS Microbiol. Lett. **124:**113–119.
17. **Yang, L., K. B. Barken, M. E. Skindersoe, A. B. Christensen, M. Givskov, and T. Tolker-Nielsen.** 2007. Effects of iron on DNA release and biofilm development by Pseudomonas aeruginosa. Microbiology **153:**1318–1328.
18. **Yang, L., M. Nilsson, M. Gjermansen, M. Givskov, and T. Tolker-Nielsen.** 2009. Pyoverdine and PQS mediated subpopulation interactions involved in Pseudomonas aeruginosa biofilm formation. Mol. Microbiol. **74:**1380–1392.

Paper III

High *in vitro* antimicrobial activity of β-peptoid-peptide hybrid oligomers against planktonic and biofilm cultures of *Staphylococcus epidermidis*

Yang Liu[1], Kolja M. Knapp[2], Liang Yang[1], Søren Molin[1], Henrik Franzyk[2] and Anders Folkesson[1]*

[1]Department of Systems Biology, Technical University of Denmark, DK-2800, Kgs.Lyngby, Denmark.

[2] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100, Copenhagen, Denmark

Keywords: Peptidomimetic, Biofilm, Cytotoxicity, Staphylococcus.

***Corresponding author:** Anders Folkesson
Mailing address: anf@bio.dtu.dk
Phone: +45 4525 2767

Abstract

We have investigated the antimicrobial effect of a set of peptidomimetics on *Staphylococcus epidermidis* planktonic and biofilm cultures.

An array of β-peptoid-peptide hybrid oligomers, displaying differences in amino acid/peptoid composition and chain length, was studied with respect to antimicrobial activity against *S. epidermidis* in both planktonic and biofilm cultures comparing the effects to those of the common antibiotic, vancomycin. Susceptibility and time-kill assays were performed to investigate the activity against planktonic cells, while confocal laser-scanning microscopy was used to investigate the dynamics of the activity against cells within biofilms.

All tested peptidomimetics were bactericidal against both exponentially growing and stationary-phase *S. epidermidis* cells with similar killing kinetics. At the Minimum Inhibitory Concentration (MIC) all peptidomimetics inhibited biofilm formation, while peptidomimetics at concentrations above MIC (80-160 μg/ml) eradicated young (6-h-old) biofilms, while even higher concentrations were needed to eradicate mature (24-h-old) biofilms completely. Chiral and guanidinylated hybrids exhibited the fastest killing effects against slow-growing cells, and had more favorable antibiofilm properties than analogues only containing lysine or lacking chirality in the β-peptoid residues. However, the results of the mature biofilm killing assay indicated more complex structure-activity relationships. Cytotoxicity assays showed a clear correlation between oligomer length and cell toxicity within each subclass of peptides, but all possessed a high differential toxicity favoring killing of bacterial cells.

This class of peptidomimetics may constitute promising antimicrobial alternatives for the prevention and treatment of multidrug-resistant (MDR) *S. epidermidis* infections.

---

1. Introduction

The opportunistic human pathogen *Staphylococcus epidermidis* (*S. epidermidis*) infections frequently affect immunocompromised and immunosuppressed patients, especially when subjected to prolonged use of indwelling medical devices [1]. The prevalent antibiotic resistance and the capability to form biofilms have led to an increase in complications associated with the treatment of *S. epidermidis* infections [2]. Formation of *S. epidermidis* biofilms on the surface of indwelling devices such as catheters and prosthetic heart valves are difficult to treat with conventional antibiotics [3]. The increased antibiotic tolerance of biofilm-associated cells has been correlated to slow growth rate, protection mediated by extracellular polymeric substances (EPS), and the development of tolerant subpopulations [1, 4]. Discovery and development of novel agents for prevention and treatment of *S. epidermidis* biofilm infections are therefore urgently needed.

Natural host-defense antimicrobial peptides (AMPs) are produced by most living organisms, and due to their evolved properties and alternative modes of action they are considered to be effective against multidrug-resistant (MDR) bacteria [5]. AMPs have also been reported to efficiently kill slow-growing cells from planktonic and biofilm cultures, and thus they have been proposed as promising alternative agents in the treatment of MDR infections [6]. Peptidomimetics are structural analogues of peptides containing amide bond isosteres or altered peptide backbones that result in higher stability as well as improved pharmacological profiles. Typically, peptidomimetics arise either from modification of an existing active peptide, or from the design of structurally similar compounds that mimic peptides e.g., β-peptides or peptoids. Appropriately designed peptidomimetics have been shown capable of maintaining a broad-spectrum antimicrobial activity while possessing advantageous properties over natural AMPs such as stability against proteolytic enzymes and low toxicity towards mammalian cells [7].

Previously, we have described a synthetic approach for the design of peptidomimetics consisting of alternating repeats of α-amino acids and β-peptoid residues [8-10]. These studies suggested that one strategy to design peptidomimetics with a favorable balance between potency and cytotoxicity involves incorporation of chiral hydrophobic β-peptoids and guanidinylated amino acid side chains while keeping the length relatively short.

In the present study, we investigate the antimicrobial activity of the simple alternating β-peptoid-peptide hybrid oligomers (i.e., **1a-3d)** and the mixed amino/guanidino subtype of peptidomimetics (i.e., **4a-4d**) against the biofilm-forming and methicillin-resistant *S. epidermidis* (MRSE) strain RP62A (ATCC 35984) in both planktonic and biofilm cultures. Their effect is compared with that of vancomycin, which is commonly used for the treatment of resistant or severe Gram-positive organisms, and thus constitutes the current antibiotic of "last resort" [11].

2. Materials and Methods

Synthesis of β-peptoid-peptide hybrid oligomers. The four mixed amino- and guanidino-functionalized oligomers **4a-d[12]** were synthesized by using our previously described solid-

phase procedure [8, 10]. The β-peptoid-peptide hybrid oligomers were dissolved in sterile deionized water (5 mg/ml) and aliquots were stored at −20 °C.

2.1. Hemolysis assay

The hemolysis was performed as described earlier [13], detecting hemoglobin by measuring the OD at 405 nm; melittin (400 mg/ml) and Tris buffer (TBS; pH 7.2, 150 mM NaCl) defined 100% hemolysis and 0% hemolysis, respectively.

2.2. Cytotoxicity assay

The cytotoxicity assay was performed essentially as reported previously using HeLa cells [10]. Briefly, HeLa cells were incubated (37 °C, at 50 rpm) for 1 h with peptidomimetics. The tested concentration range was 0.1 μM to 1000 μM. The dehydrogenase activity was determined as a result of the amount of formazan produced as measured by absorbance at 492 nm.

2.3. Bacterial strains and growth media

The *S. epidermidis* RP62A (ATCC 35984) strain was selected as the model organism for our study, as it is considered a benchmark strain among the biofilm-producing *S. epidermidis* strains [14]. Tryptic soy broth (TSB; Oxoid) medium containing 0.25 % glucose was used for biofilm cultivation in a static chamber system. Susceptibility assays were carried out in Mueller-Hinton broth (MHB; Fluka). SYTO9 and propidium iodide (PI) (LIVE/DEAD reagents, Molecular Probes) were used at a concentration of 1 μM for staining live or dead bacteria in biofilms.

2.4. Bacterial susceptibility assay

Minimum inhibitory concentrations (MICs) were measured by using methods described previously by Wiegand *et al.*[15]. The MBC was determined as the lowest concentration that resulted in less than 0.1% survival of the subculture. All MIC and MBC determinations were made in triplicate. For selected compounds the MICs were also measured by using the same method in the biofilm media (TSB with 0.25% glucose).

2.5. Time-kill assay in fresh MHB

S. epidermidis cells (1×10^7 CFU/ml) were separately treated with β-peptoid-peptide hybrid oligomers at 4 μg/ml or vancomycin at 4 and 20 μg/ml in fresh MHB. Time-kill experiments were performed at 37 °C with shaking at 220 rpm under aerobic conditions. Culture aliquots (50 μl) were taken at different time points (0, 1, 3, 5, 8 and 24 h), serially diluted, plated onto tryptic soy agar, and then incubated at 37 °C for 24 h followed by counting of CFU. Time–kill curves were constructed by plotting the $\log_{10}$ CFU/ml versus time over a 24-h time period. Assays were performed in duplicate on at least two occasions, and similar results were obtained. The detection limit for these assays was 5×10^2 CFU/ml.

2.6. Time-kill assay of stationary-phase cells in nutrient-depleted MHB

Nutrient-depleted MHB (depMHB) was prepared by the method described previously with modifications [16]. Briefly, *S. epidermidis* was cultivated in MHB at 37 °C for 46 h. The cultures were centrifuged at 8000 g for 30 min at 4 °C, the supernatants were collected and adjusted to pH 7.0, then filtered through a 0.22 μm pore size Syringe filter (TPP, Switzerland). Stationary-phase cells (1×10^7 CFU/ml) were separately incubated in depMHB in the presence of β-peptoid-peptide hybrid oligomers or vancomycin at 4 and 20μg/ml. Time-kill assay was performed as above.

2.7. Biofilm susceptibility assay

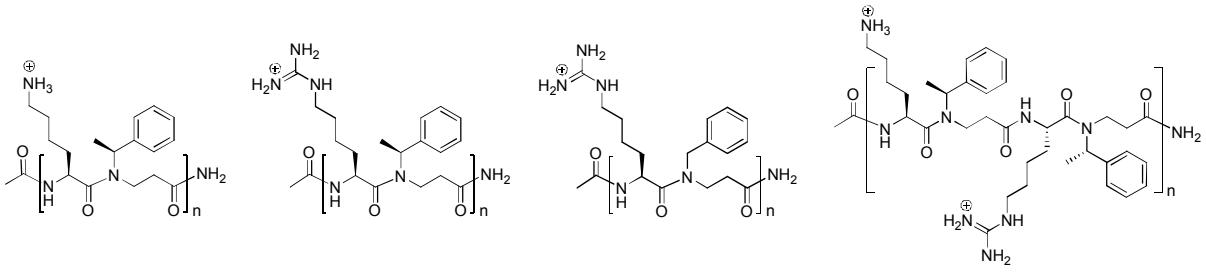
Static chamber *S. epidermidis* biofilms were cultivated in a cover-glass cell culture chamber (Nunc, Roskilde, Denmark) as described previously [17]. Briefly, the chamber wells were inoculated with 1.5 ml diluted overnight cultures (5×10^5CFU/ml). After incubation at 37 °C, the 0, 6 and 24 h preformed biofilms in the chambers were washed gently twice with sterile PBS (1 ml) to remove planktonic cells. Then fresh medium containing antimicrobial agents were added, and then the biofilm cultures were incubated at 37 °C for 24 h. After removal of the medium, the biofilm cells were stained with the LIVE/DEAD reagents, and were then observed by confocal laser-scanning microscopy (CLSM). This assay was repeated three times, and similar results were obtained.

2.8. Microscopy image acquisition and analysis

All microscopy observations and image acquisitions were performed by using methods described previouslyby Qin *et al.* [17]. For the quantification of biofilms, at least six CLSM images from each sample were analysed by using the computer program COMSTAT. Error bars represent the mean + standard deviation. Statistical differences in comparison to control (without added antimicrobial agent) were determined by one-way analysis of variance (ANOVA). Differences were considered statistically significant with $p < 0.05$.

3. RESULTS

In the present study, sixteen β-peptoid-peptide hybrid oligomers (**1a-4d**) (**Table 1, Figure 1**), were investigated for their antimicrobial activity against both planktonic and biofilm-associated *S. epidermidis* cells as well as for their cytotoxicity towards human cells. Peptidomimetics **1a-4d** are oligomers consisting of alternating repeats of α-amino acids and N-alkyl-*β*-alanine (*β*-peptoid) residues**.** The four examined subclasses differ in the nature of cationic side chains (amino or guanidino functionalized, i.e. Lys or hArg), and presence of α-chirality in the β-peptoid residues (β*N*spe or β*N*phe).The representative compounds of this subclass have chain lengths corresponding to 8-16 residues.



Ac-[Lys-β*N*spe]$_n$-NH$_2$ | Ac-[hArg-β*N*spe]$_n$-NH$_2$ | Ac-[hArg-β*N*phe]$_n$-NH$_2$ | Ac-[Lys-β*N*spe-hArg-β*N*spe]$_n$-NH$_2$

1a (n = 5) **2a** (n = 5) **3a** (n = 5) **4a** (n = 1)
1b (n = 6) **2b** (n = 6) **3b** (n = 6) **4b** (n = 2)
1c (n = 7) **2c** (n = 7) **3c** (n = 7) **4c** (n = 3)
1d (n = 8) **2d** (n = 8) **3d** (n = 8) **4d** (n = 4)

Figure 1. Chemical structures of the β-peptoid-peptide hybrids. The abbreviations used for the β-peptoid units were adapted from those used for α-peptoids [27], by adding the β-prefix: βNspe = N-[(*S*)-1-phenylethyl]-β-alanine, βNphe = N-phenyl-β-alanine, hArg = homoarginine.

Table 1. Antimicrobial activity of peptidomimetics towards *S. epidermidis* and cytotoxicity against Hela cells.

Subclass	Compound no.	Mw (g/mol)	MIC in MHB		MIC in biofilm medium[a]	Hela (IC_{50})
			μg/ml (μM)	MBC/ MIC	μg/ml(μM)	μg/ml(μM)
1 : all-amino; α-chiral	**1a**	1624.81	32 (19.7)	2	>128 (>78.8)	
	1b	1937.74	4 (2.06)	2	>128 (>66)	>1938 (>1000)
	1c	2250.67	2 (0.89)	2	64 (28.4)	
	1d	2563.60	2 (0.78)	1	16 (4.8)	92 (36)
2 : all-guanidino; α-chiral	**2a**	2356.38	2 (0.85)	1	ND	
	2b	2815.84	1 (0.36)	1	4 (1.42)	90 (32)
	2c	3275.30	2 (0.61)	1	8 (2.44)	
	2d	3734.76	4 (1.07)	1	ND	27 (7.2)
3 : All-guanidino; no α-chirality	**3a**	2286.25	4 (1.75)	2	32 (14.0)	
	3b	2732.69	1 (0.37)	1	8 (2.92)	262 (96)
	3c	3179.13	2 (0.63)	1	16 (5.02)	
	3d	3625.57	2 (0.55)	1	16 (4.41)	40 (11)
4 : amino-guanidino 1:1; α.chiral	**4a**	935.95	>256 (>273)	ND	ND	
	4b	1812.84	32 (17.7)	2	>128 (>70.6)	
	4c	2689.72	2 (0.74)	1	16(5.96)	794 (295)
	4d	3566.61	1 (0.28)	1	4 (1.12)	46 (13)
Vancomycin		1485.71	1 (0.67)	1	2 (1.35)	

[a] biofilm media, TSB with 0.25% glucose. MIC, minimum inhibitory concentration. MBC, minimum bactericidal concentration. $IC_{50,}$ the half maximal inhibitory concentration against Hela cells. MIC, MBC and IC_{50} values represent the mean of three individual experiments. ND, not determined. The unit of μg/ml is generally used, with μM (micromolar) shown in parentheses.

3.1. The hemolytic and cytotoxic properties of β-peptoid-peptide hybrids

Peptidomimetics **1b**, **1d**, **2b**, **2d**, **3b**, **3d** had previously been found to exhibit minimal hemolysis (< 10%) even at a concentration of 512 μg/ml, and similarly low hemolytic activity was determined in the present work for compounds **4c** and **4d** (at 512 μg/ml: <5% and 10% hemolysis, respectively) by using the same conditions [6].

From each subclass of peptidomimetics the oligomers consisting of 12 or 16 residues were investigated for their cytotoxicity against human HeLa cells (Table 1; IC_{50} is the half maximal inhibitory concentration), and the sequence length and content of guanidinium side chains clearly turned out to be the most significant structural features influencing this pharmacological property. Thus, potency in killing human cells increased ~20-fold when extending the sequences from 12 to 16 residues within the Lys-containing subclasses (**1** and **4**), whereas this effect was less pronounced

(3-7 fold increase) for the all-hArg analogues (i.e., **2b**, **2d**, **3b**, and **3d**). Noticeably, all 16-meric peptidomimetics exhibited relatively high cytotoxicity (IC_{50}<< 100 μM) as compared to the 12-mers containing lysine (i.e. **1b** and **4c**). Similarly, the lack of α-chirality in the β-peptoid residues in **3b** was accompanied by a 3-fold diminished cytotoxicity relative to the corresponding fully chiral oligomer (**2b**).

3.2. Susceptibility testing in MHB and biofilm media

The concentrations ofβ-peptoid-peptide hybrid oligomers required for inhibition or killing of planktonic *S. epidermidis* cells in MHB or biofilm media (TSB with 0.25% glucose) are summarized in Table 1. For subclass **1** and **4**, MIC values decreased significantly with increasing oligomer length. Thus, chimeras **1d** and **4d** ranked the most potent in their subclasses, had MICs of 2 μg/ml (0.78 μM) and 1 μg/ml (0.28 μM), respectively. In contrast, less pronounced differences in the MICs were found for members among subclasses**2** and **3**, which all were active in the range of 1-4 μg/ml (0.36-1.75 μM). Hybrids **2b** and **3b**, which are dodecamers, proved most effective within their subclass exhibiting MICs of 1 μg/ml (0.36 μM) and 1 μg/ml (0.37 μM), respectively. The MIC of the reference antibiotic vancomycin was 1 μg/ml (0.67 μM). MBCs of the compounds were all equal to or the double of their MICs clearly indicating a bactericidal mode of action. However, it was found that *S. epidermidis* cells were less susceptible to the peptidomimetics when challenged in biofilm media where **2b** and **4d** exhibited the lowest MIC (4 μg/ml) while the MIC of vancomycin was 2 μg/ml. The peptidomimetics **1d**, **2b**, **3b**, **4c** and **4d** all exhibited high antimicrobial activity and selectivity (i.e. IC_{50}>>MIC; Table 1) in the killing of bacteria over human cells, and therefore these five chimeras were further investigated.

3.3. β-peptoid-peptide hybrids efficiently kill fast-growing *S. epidermidis* cells

The time-kill kinetics was investigated in fresh MHB to determine whether β-peptoid-peptide hybrid oligomers and vancomycin are effective against fast-growing *S. epidermidis* cells (**Figure 2**). Five β-peptoid-peptide hybrid oligomers (**1d**, **2b**, **3b**,**4c**, **4d**) representing all subclasses or vancomycin, at a concentration of 4 μg/ml (equal to 2-4 times their MICs), were separately added to the cultures in order to determine their effect on cell viability as compared with untreated control cells. The starting inoculum was 1×10^7 CFU/ml. Untreated control cells grew fast (increasing ~$2\log_{10}$ CFU/ml over a 24-h period). Compound 4d (4 μg/ml) exhibited the fastest bactericidal activity in growing cultures of *S. epidermidis* as it produced a 3-log reduction in CFU/ml within 1 hour. Oligomers 1d and 2b required a longer time (3 hours) to achieve a similar bactericidal effect, while compounds 3b and 4c needed 5 hours to achieve a 3-log reduction in CFU/ml. Notably, vancomycin exhibited a more protracted bactericidal activity than the five peptidomimetics as more than 5 hours were required to achieve a bactericidal effect. Increasing the vancomycin concentration to 20 μg/ml did not improve the killing curve, which suggests that it has a concentration-independent activity against fast-growing *S. epidermidis* cells.

3.4. β-peptoid-peptide hybrids efficiently kill slow-growing stationary-phase *S. epidermidis* cells

In order to determine whether the peptidomimetics also possessed bactericidal activity against stationary-phase cells time-kill kinetics was performed by using nutrient-depleted MHB (depMHB). Stationary-phase *S. epidermidis* cultures were left untreated or were treated separately with compounds 1d, 2b, 3b, 4c, 4d, or vancomycin at 4 μg/ml (**Figure 2B**). It took around 3 hours for

compound 4d, 6 hours for compound 2b and 1d, and 24 hours for compound 4c, to produce a bactericidal effect (**Figure 2B**). Oligomer 3b caused a ~ 2-log decrease in viability after 24 hours, whereas vancomycin (4 μg/ml) did not exhibit a killing effect under these growth conditions.

Moreover, to determine whether the killing effects are concentration-dependent, stationary-phase *S. epidermidis* cultures were left untreated or were treated separately with the compounds at 20 μg/ml which corresponds to approximately 10 times the MIC concentration (**Figure 2C**). Compounds 2b and 4d exhibited rapid bactericidal activities with more than a 3-log reduction after 1hour, while compounds 1d, 4c and 3b gave bactericidal effects after 3 hours, 8 hours, and 24 hours, respectively. Notably, vancomycin at high concentration (20 μg/ml) caused a decrease of ~ 2-log after 24 hours.

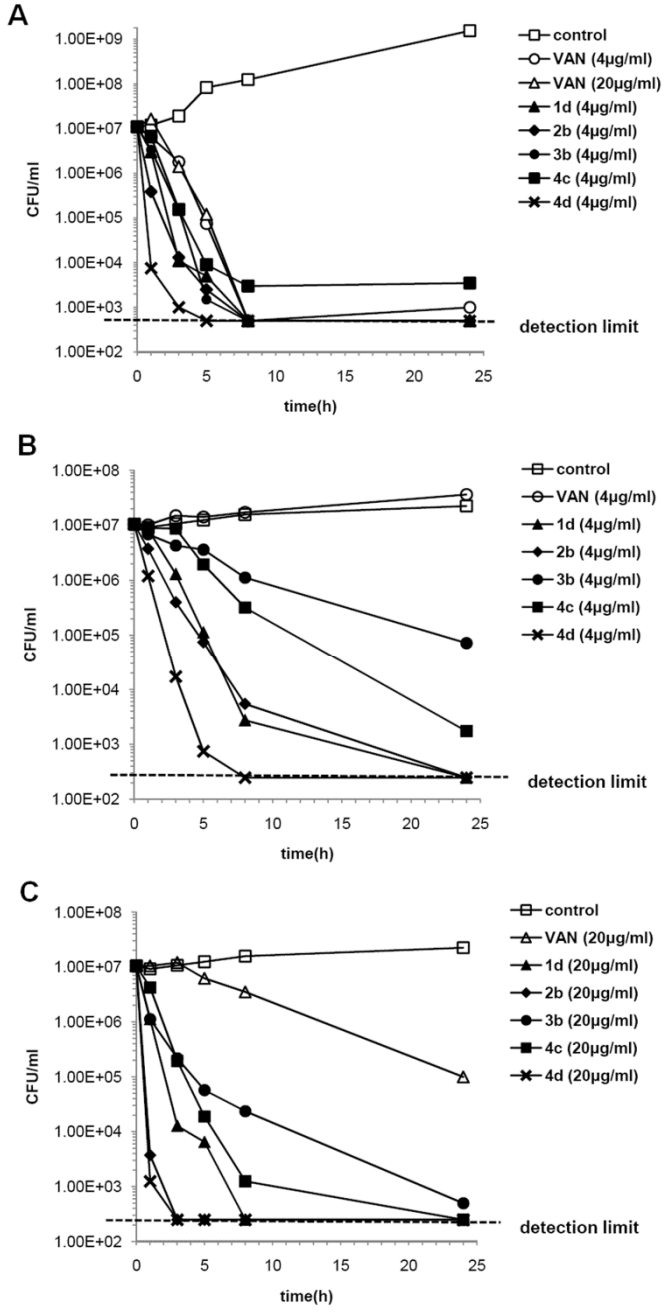


Figure 2. Time-kill curves for *S. epidermidis* RP62A upon exposure to β-peptoid-peptide hybrids and vancomycin. Cells were treated with compounds at low and high concentrations (4 and 20 μg/ml) in depleted MHB (B and C)as well as at low concentration (4 μg/ml) in fresh MHB (A). Viability was counted at the indicated time points by serial dilution plating. Values are the mean of independent tests performed in duplicate. CFU: colony-forming units. VAN: vancomycin.

3.5. β-peptoid-peptide hybrids inhibit *S. epidermidis* biofilm formation

Next the potential of β-peptoid-peptide hybrid oligomers as prophylactic agents for preventing *S. epidermidis* biofilm formation was investigated. Thus we tested the selected hybrids at low concentration (≤ 1×MIC) in an assay allowing for quantification of biofilm formation by using fluorescence microscopy and LIVE-DEAD staining (**Figure 3**). At the MICs, all five peptidomimetics as well as vancomycin prevented formation of *S. epidermidis* biofilms. In particular, compound 2b (4 μg/ml) inhibited biofilm formation corresponding to a 100% reduction of biomass. Furthermore, we tested whether subMICs (1-2 μg/ml) of such peptidomimetics were able to prevent biofilm formation. For oligomers 1d and 4c, a concentration of 2 μg/ml was used due to their relatively high MICs (16 μg/ml). At these subMICs, oligomers 4d (1 μg/ml) and 1d (2 μg/ml) caused significant reductions of biomass (~ 65% and ~ 40%, respectively), while vancomycin (1 μg/ml) caused 65% reduction. However, the killing effect of vancomycin appeared to be very heterogeneous since some parts of the biofilm were reduced, while other parts were not. Consequently, the remaining amount of biomass at different positions of the static chamber varied a lot, as reflected in a large standard deviation (**Figure 3**).

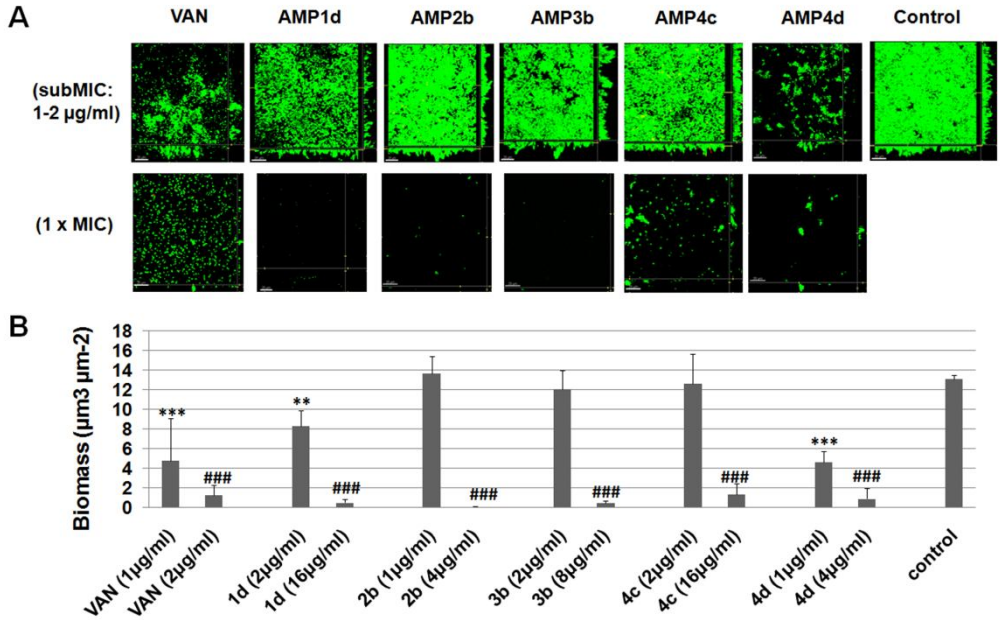


Figure 3. Effects of antimicrobials in preventing formation of *S. epidermidis* (RP62A) biofilm, when tested at subMIC and at 1×MIC. (A): Confocal laser-scanning microscopy images. The biofilms were stained with SYTO9 (green fluorescent for live cells) and PI (red fluorescent for dead cells). The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. Bars represent 20 μm. The assays were repeated three times and similar results were obtained. (B): COMSTAT analysis of biomass. Symbols (*, #) indicate a statistically significant difference ($p < 0.05$) with respect to the control (no antimicrobial added). The absence of a symbol indicates no statistically significant difference with respect to the control.

3.6. Activity of β-peptoid-peptide hybrids towards young *S. epidermidis* biofilms

To determine whether the selected peptidomimetics were effective also for the killing of bacterial cells within biofilms, CLSM in combination with SYTO9 DNA viability staining were

used to evaluate the produced amount of biomass as a measure of cell viability. The differences in bacterial survival in the biofilms were quantified by analyzing the CLSM data with the computer program COMSTAT [18]. For the young (6 hours) *S. epidermidis* biofilms, the peptidomimetics were added at low and high concentrations (**Figure 4**). At low concentrations (1-4 × MIC, 8-16 µg/ml), peptidomimetics 1d, 2b, 4c and 4d all reduced cell viability by 80-85%, whereas oligomer 3b had no significant effect. The reference antibiotic vancomycin (8 µg/ml) gave rise to ~50% reduction in live biomass, however, the killing effects of both vancomycin and oligomer 3b appeared to be very heterogeneously distributed within the biofilm. Nevertheless, at high concentrations (10-40×MIC, 80-160 µg/ml), all tested compounds almost completely disrupted preformed *S. epidermidis* biofilms (95-100% reduction of biomass) (**Figure 4**).

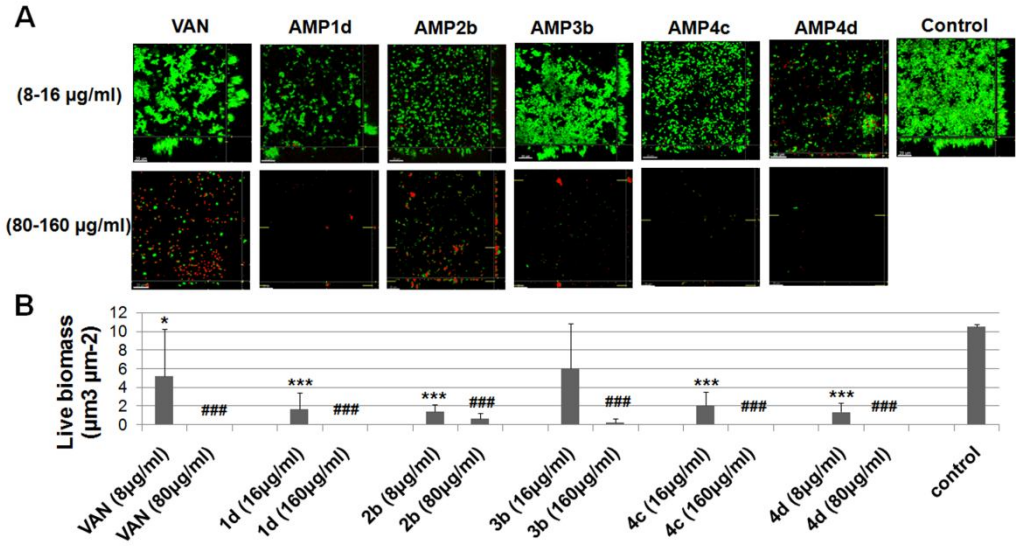


Figure 4. Effects of antimicrobials against established *S. epidermidis* (RP62A) young (6-h-old) biofilm when tested at 8-16 and 80-160 µg/ml. (A): Confocal laser-scanning microscopy images. (B): COMSTAT analysis of live biomass. All symbols (* and #) indicate a statistically significant difference ($p < 0.05$) with respect to the control (no antimicrobial added). The absence of a symbol indicates no statistically significant difference with respect to the control. Bars represent 20 µm.

3.7. Activity of β-peptoid-peptide hybrids towards mature *S. epidermidis* biofilms

In the study of the killing effect towards mature (24-h-old) *S. epidermidis* biofilms, the selected peptidomimetics were tested at three different concentration levels (**Figure 5**). At low concentrations (8-16 µg/ml) only oligomer 4d (8 µg/ml) were able to cause ~43% reduction of viable biomass. At high concentration (160 µg/ml), oligomers 4d and 4c, as well as vancomycin reduced live biomass by 71%, 46%, and 50%, respectively. At 400 µg/ml, peptidomimetics 4d, 2b, 4c and vancomycin caused significant reductions in living biomass (78%, 77%, 45% and 69%, respectively). Hybrid oligomers 4d and 2b were most potent in killing of mature biofilm in accordance with their low MICs in biofilm media and high activities against stationary-phase *S. epidermidis* cells (**Table 1, Figure 3**). Although, less effective than oligomer 1d in the time-kill study, oligomer 4c exhibited better killing effect on mature biofilm than compound 1d. Surprisingly,

although vancomycin was not as potent as the tested peptidomimetics in killing slow-growing stationary-phase cells, it exhibited a significant activity against cells in mature biofilms. The observed distinct heterogeneity of survival in the vancomycin-treated biofilms suggests that the mode of action for vancomycin is different from that of the peptidomimetics.

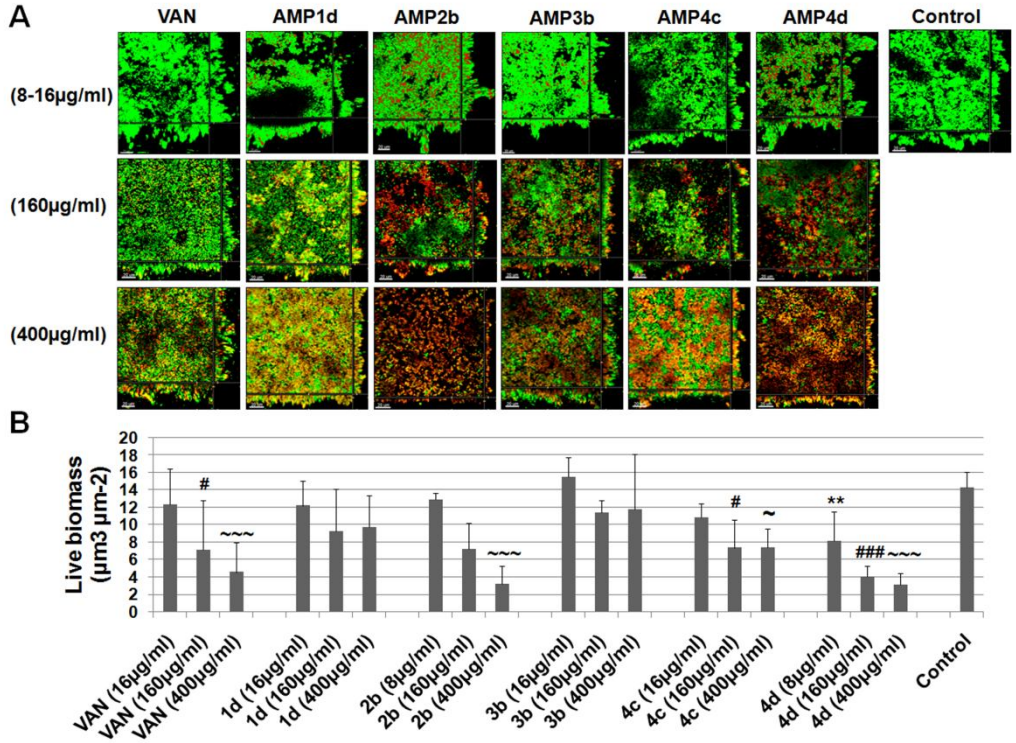


Figure 5. Effects of antimicrobials against established *S. epidermidis* (RP62A) mature (24-h-old) biofilm, when tested at 8-16, 160, and 400 µg/ml. (A): Confocal laser-scanning microscopy images. (B): COMSTAT analysis of live biomass. Symbols (*, # and ~) indicate a statistically significant difference ($p < 0.05$) with respect to the control (no antimicrobial added). The absence of a symbol indicates no statistically significant difference with respect to the control. Bars represent 20 µm.

4. DISCUSSION

We set out to probe the relationship between structure (sequence composition and chain length) and activities (antimicrobial, antibiofilm, and cytotoxic) for a series of β-peptoid-peptide hybrid oligomers using the biofilm-forming and methicillin-resistant strain *S. epidermidis* RP62A as model organism.

The susceptibility of *S. epidermidis* planktonic cells exhibited similar trends in structure-activity relationships as found in previous studies [8, 19]. Thus, a longer chain length was often correlated with an increased antimicrobial activity within each subclass. This tendency was more pronounced in lysine-containing subclasses (1 and 4) than in homoarginine-rich subclasses (2 and 3) (**Table 1**).

S. epidermidis cells were found to be less susceptible to the peptidomimetics when tested in biofilm media (TSB+0.25% glucose) as compared to MHB (**Figure 2, Table 1**). This difference is

most likely due to the fact that *S. epidermidis* grow faster and reach a higher cell density in biofilm media than in MHB (data not shown).

In the present study, peptidomimetics 1b and 4c were found to possess the most favorable cytotoxicity profiles towards HeLa cells (IC_{50} > 1900 μg/ml and 794 μg/ml, respectively), suggesting that a design of alternating 12-meric oligomers displaying only amino or guanidino/amino functional groups in a 1:1 ratio may be a promising strategy to keep the cytotoxicity at an acceptable level. However, the all-Lys compound 1b did not show any significant antimicrobial activity in biofilm medium indicating that some content of guanidino side chains is required for antibiofilm activity. Likewise, the hemolytic activity of 4c proved favorably low as no hemolysis was detected at a concentration of 256 μg/ml and less than 5% at 512 μg/ml.

The finding that hArg-rich peptidomimetics belonging to subclasses 2 and 3 exhibited significant cytotoxicity towards human cells may be ascribed to their improved interaction with human membranes promoted by the ability of guanidinium groups to form bidentate hydrogen bonds with the phospholipids that constitute the major part of eukaryotic membranes.

Chirality appears essential for efficient killing of both slow-growing planktonic cells and biofilm cultures, which corroborates preliminary studies showing that the presence of chiral β-peptoid residues results in higher antimicrobial potency against *S. aureus*, which may be due to their higher degree of secondary structure in compound 1d/2b *vs* compound 3b [8, 10]. In comparison to their lysine-containing counterparts (*e.g.*, 1d, 4c), the fully guanidinylated (i.e. hArg-rich) oligomers (*e.g.*, 2b) exhibited faster killing of slow-growing cells and possessed enhanced antibiofilm capacity in accordance with our previous studies showing that the presence of hArg residues in this type of peptidomimetics strongly promote activity against *S. aureus*. There was little correlation between the killing ability towards slow- growing cells compared to mature biofilms. Hybrid 4c was much less potent towards slow growing cells compared to compound 2b , however , 4c exhibited higher killing activity of mature biofilm cells than 2b.

In particular, there is a need for studies aimed at elucidating the mode of action for these peptidomimetics against *S. epidermidis*, as well as estimating the potential resistance mechanisms of *S. epidermidis* planktonic and biofilm cells against these oligomers. The efficient killing of slow-growing bacterial cells exerted by both AMPs and antimicrobial peptidomimetics may be due to their distinct killing mechanisms. Unlike conventional antibiotics that penetrate into the bacterial cells and interfere with intracellular targets, most AMPs and antimicrobial peptidomimetics appear mainly to act via pore formation or mechanisms involving transient disruption of the integrity of bacterial cell membranes [20]. However, there is growing evidence that several highly potent AMPs may in fact exert their antibacterial effect via intracellular targets [21, 22].

The tolerance of *S. epidermidis* biofilm cells towards β-peptoid-peptide hybrid oligomers may be due to the induction of the AMP-sensing (Aps) regulatory system [1], which is well conserved among staphylococci. In *Staphylococcus aureus*, the Aps system activates transcription of three genomic sequences involved in AMP resistance including *mprF*, *dlt* operon and *vraFG* [1]. The *S. aureus mprF* gene encodes LPG synthetase that transfers L-lysine from lysyl-tRNA to phosphatidylglycerol (PG) [23]. The *S. aureus dlt* operon is responsible for the D-alanylation of teichoic acid, which is a highly negatively charged component of the cell wall polymer lipoteichoic acid (LTA) [24]. The aminoacylation of PG and LTA results in a partial neutralization of the

negative charge of the *S. aureu*s cell envelope, and thereby reduces its susceptibility towards AMPs [25]. The *vraFG* operon encodes an ABC transporter involved in resistance of *S. aureus* to AMPs and vancomycin. These resistance systems of *S. aureus* have homologues in *S. epidermidis*, however, further studies need to be performed in order to establish whether they are involved in tolerance of *S. epidermidis* cells towards β-peptoid-peptide hybrid oligomers [1].

Our results obtained for the treatment of mature *S. epidermidis* biofilms with selected β-peptoid-peptide hybrid oligomers and vancomycin indicate the presence of a physiological differentiation in mature *S. epidermidis* biofilms conferring overall increased antibiotic tolerance to the cell cultures (Figure 5). The slow-growing cells in the biofilm are not only more tolerant towards vancomycin but also towards the peptidomimetics. This tolerance might be due to the substantial amount of extracellular polymeric substances (EPS) such as poly-N-acetylglucosamine (PNAG, also known as PIA) and poly-γ-glutamic acid (PGA) present in surface layers of *S. epidermidis* biofilms [26]. Both PNAG and PGA protect *S. epidermidis* from AMPs and phagocytosis [26]. However, resistant *S. epidermidis* cells were found to be distributed randomly upon treatment with peptidomimetics suggesting that other mechanisms might be involved in the tolerance observed for β-peptoid-peptide hybrid oligomers.

Our experiments clearly demonstrate that the present class of peptidomimetics is able to kill both fast-growing and slow-growing bacterial cells. Furthermore, the tested hybrid oligomers inhibited formation of *S. epidermidis* biofilm and displayed antibiofilm activity against preformed *S. epidermidis* biofilms. Also, the combined structure-activity relationships concerning their overall pharmacological profiles (i.e. antimicrobial activity versus cytotoxicity) indicate that these peptidomimetics constitute promising lead compounds towards *S.epidermidis* biofilm due to their high antibiofilm activity combined with low cytotoxicity, and negligible hemolytic activity. We have shown that these peptidomimetics possess bactericidal activity that is comparable, if not higher than the current “last resort” antibiotic vancomycin. This class of compounds should therefore be considered relevant alternatives in the search for novel treatment options for severe nosocomial Gram-positive infections.

5. ACKNOWLEDGMENTS

Y.L. was funded by a PhD grant from the Technical University of Denmark and the Danish Research Council for Technology and Production (grant number 09-065902/FTP). We thank, the Brødrene Hartmanns Fond (Copenhagen) for a materials grant supporting the synthesis work.

REFERENCES

[1] Otto M. Staphylococcus epidermidis--the 'accidental' pathogen. Nat Rev Microbiol. 2009;7:555-67.
[2] Raad I, Alrahwan A, Rolston K. Staphylococcus epidermidis: emerging resistance and need for alternative agents. Clin Infect Dis. 1998;26:1182-7.
[3] Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001;358:135-8.
[4] Folkesson A, Haagensen JA, Zampaloni C, Sternberg C, Molin S. Biofilm induced tolerance towards antimicrobial peptides. PLoS ONE. 2008;3:e1891.
[5] Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis. 2001;1:156-64.

[6] Parisien A, Allain B, Zhang J, Mandeville R, Lan CQ. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. J Appl Microbiol. 2008;104:1-13.
[7] Chongsiriwatana NP, Patch JA, Czyzewski AM, Dohm MT, Ivankin A, Gidalevitz D, et al. Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. Proc Natl Acad Sci U S A. 2008;105:2794-9.
[8] Olsen CA, Bonke G, Vedel L, Adsersen A, Witt M, Franzyk H, et al. Alpha-peptide/beta-peptoid chimeras. Org Lett. 2007;9:1549-52.
[9] Olsen CA, Lambert M, Witt M, Franzyk H, Jaroszewski JW. Solid-phase peptide synthesis and circular dichroism study of chiral beta-peptoid homooligomers. Amino Acids. 2008;34:465-71.
[10] Olsen CA. Peptoid-Peptide hybrid backbone architectures. Chembiochem. 2010;11:152-60.
[11] Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, et al. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. J Bacteriol. 2005;187:2426-38.
[12] Hein-Kristensen L, Knapp KM, Franzyk H, Gram L. Bacterial membrane activity of alpha-peptide/beta-peptoid chimeras: influence of amino acid composition and chain length on the activity against different bacterial strains. BMC Microbiol. 2011;11:144.
[13] Schmitt MA, Weisblum B, Gellman SH. Interplay among folding, sequence, and lipophilicity in the antibacterial and hemolytic activities of alpha/beta-peptides. J Am Chem Soc. 2007;129:417-28.
[14] Rachid S, Ohlsen K, Witte W, Hacker J, Ziebuhr W. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming Staphylococcus epidermidis. Antimicrob Agents Chemother. 2000;44:3357-63.
[15] Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3:163-75.
[16] Mascio CT, Alder JD, Silverman JA. Bactericidal action of daptomycin against stationary-phase and nondividing Staphylococcus aureus cells. Antimicrob Agents Chemother. 2007;51:4255-60.
[17] Qin Z, Yang X, Yang L, Jiang J, Ou Y, Molin S, et al. Formation and properties of in vitro biofilms of ica-negative Staphylococcus epidermidis clinical isolates. J Med Microbiol. 2007;56:83-93.
[18] Heydorn A, Ersboll B, Kato J, Hentzer M, Parsek MR, Tolker-Nielsen T, et al. Statistical analysis of Pseudomonas aeruginosa biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. Appl Environ Microbiol. 2002;68:2008-17.
[19] Olsen CA, Ziegler HL, Nielsen HM, Frimodt-Moller N, Jaroszewski JW, Franzyk H. Antimicrobial, Hemolytic, and Cytotoxic Activities of beta-Peptoid-Peptide Hybrid Oligomers: Improved Properties Compared to Natural AMPs. Chembiochem. 2010;11:1356-60.
[20] Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 2005;3:238-50.
[21] Patrzykat A, Friedrich CL, Zhang L, Mendoza V, Hancock RE. Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in Escherichia coli. Antimicrob Agents Chemother. 2002;46:605-14.
[22] Park CB, Kim HS, Kim SC. Mechanism of action of the antimicrobial peptide buforin II: Buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochemical and Biophysical Research Communications. 1998;244:253-7.
[23] Oku Y, Kurokawa K, Ichihashi N, Sekimizu K. Characterization of the Staphylococcus aureus mprF gene, involved in lysinylation of phosphatidylglycerol. Microbiology. 2004;150:45-51.
[24] Peschel A, Vuong C, Otto M, Gotz F. The D-alanine residues of Staphylococcus aureus teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. Antimicrob Agents Chemother. 2000;44:2845-7.
[25] Kristian SA, Durr M, Van Strijp JA, Neumeister B, Peschel A. MprF-mediated lysinylation of phospholipids in Staphylococcus aureus leads to protection against oxygen-independent neutrophil killing. Infect Immun. 2003;71:546-9.
[26] Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, et al. Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system. Cell Microbiol. 2004;6:269-75.
[27] Porter EA, Wang X, Lee HS, Weisblum B, Gellman SH. Non-haemolytic beta-amino-acid oligomers. Nature. 2000;404:565.

Paper IV

Stress response and resistant development to antimicrobial peptidomimetics by *Staphylococcus epidermidis*

Yang Liu[1], Rasmus Lykke Marvig[1], Christian Bille Jendresen[1,2], Sara Bayard Dühring[1], Liang Yang[1], Henrik Franzyk[3], Mogens Kilstrup[1], Lars Jelsbak[1], Søren Molin[1,2], Anders Folkesson [1]*.

[1]Department of Systems Biology, Technical University of Denmark, DK-2800, Kgs.Lyngby, Denmark.

[2]Novo Nordisk Foundation Center for Biosustainability, DK-2970 Hørsholm, Denmark

[3] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100, Copenhagen, Denmark

Keywords: Peptidomimetic, Stress response, Resistant delelopment, Staphylococcus.

***Corresponding author:** Anders Folkesson

Mailing address: anf@bio.dtu.dk; Phone: +45 4525 2767

Abstract

Antimicrobial peptidomimetics are proposed as alternatives to conventional antimicrobials for treatment of infectious diseases caused by multiple-resistant pathogens. It is unclear how pathogens invoke their stress response and further develop resistance against antimicrobial peptidomimetics. Understanding these mechanisms is crucial for optimizing the application of antimicrobial peptidomimetics in the hospital. In the present study, we used *Staphylococcus epidermidis*as as a model organism to study bacterial response and resistance to a novel group of antimicrobial peptidomimetics which were recently developed [1]. These peptidomimetics are β-peptoid-peptide hybrid oligomers which showed great antimicrobial and anti-biofilm activities compared to vancomycin against *S. epidermidis*.

Transcriptomic profiles of *S. epidermidis* exposed to peptidomimetics and vancomycin suggest that peptidomimetics target the cell membrane in a similar but distinct mode as vancomycin. Defense against peptidomimetics involves not only general signaling pathways but also specific mechanisms. The VraRS regulon, ABC transporters and efflux pumps are differentially expressed in the response toward peptidomimetics.

Furthermore, populations which are resistant to peptidomimetics were experimentally evolved by continuous passage of *S. epidermidis* in the presence of sub-lethal concentration of peptidomimetics. Comparative genomic analysis of evolved resistant populations showed

convergent mutations in several regulatory systems and AMP-specific targets. Among them, the RNAP and *relA* regulatory systems are thought to regulate the balance of the cells between self-protection and nutritional capability (the SPANC balance), therefore regulating the stress resistance. In addition, cation transporters, efflux pumps and cell membrane modification enzymes were also hot spots of mutation. Transcriptomic profile of the evolved resistant populations reveals the importance of efflux pumps in the resistant feature. Furthermore, our study showed special metabolic adaptation is involved in resistance to antimicrobial peptidomimetics.

To conclude, defense and resistance against peptidomimetics involves not only general stress system but also AMP-specific mechanisms. These results suggest that peptidomimetics may be alternative or additive to standard antibiotic therapy.

Introduction

Clinically significant antibiotic resistance has arisen against virtually every antibiotic developed due to the extensive usage of antibiotics[2]. Cationic antimicrobial peptides have gained a lot of attention as "old" but effective antimicrobial agents. Antimicrobial peptides (AMPs) play important roles in the innate immune system of multicellular organisms[3]. AMPs have a broad spectrum of antimicrobial activity against bacteria, fungi and virus[4]. In addition to directly killing microorganisms, AMPs can also modulate immune response such as enhancing antibody response, recruiting phagocytes to the site of infection[4].

AMPs are usually consisting of two or more positively charged amino acids (e.g. arginine, lysine, histidine) and a large proportion (generally >50%) of hydrophobic amino acids[3]. The amphipathicity of APMs allows them to easily insert into lipid bilayer of microbial membranes and behave as microbicide[3]. However, AMPs, as peptides, are sensitive to proteases, which are secreted by a wide range of microorganisms[5]. Also, many AMPs have hemolytic activity which further limits their application in human [6]. To overcome these drawbacks, antimicrobial peptidomimetics are designed based on the natural AMPs by using synthetic peptide analogues such as β -peptide and peptoid as the building blocks[7]. Antimicrobial peptidomimetics have been shown to have great potential for treatment of infections caused by multi-resistant microorganisms and have already entered clinical evaluation [8].

Unlike many of the other conventional antibiotics which inhibit specific targets (e.g. DNA or proteins), most of the cationic AMPs directly interact with the negative-charged microbial cell membranes and disrupt cellular integrity [9]. AMPs can efficiently eradicate biofilms which contain persist dormant bacteria [10]. However, as AMPs belong to the ancient host defense effector molecules and widely exist among all high organisms, AMPs and AMP-resistance mechanisms are believed to be co-evolved and lead to a transient host–pathogen balance that further shapes the existing AMP repertoire[11].

Bacteria have evolved specific sensing systems that activate AMP resistance mechanisms when exposed to AMPs[12]. The two component sensor systems PhoPQ and Aps are capable of sensing AMPs and regulating expression of various genes in Gram-negative and Gram-positive

bacteria, respectively [13-14] [15-16]. Bacteria are able to alter their cell surface charge, active certain efflux systems and produce proteases to become resistant towards AMPs [17]. In the presence of AMPs, *Staphylococcus epidermidis* uses its Aps AMP-sensing systems to upregulate AMP resistance mechanisms, including the D-alanylation of teichoic acids, addition of lysyl-phosphati-dylglycerolto the cytoplasmic membrane, and induction of the putative VraFG AMP efflux pump [15]. These AMP sensing systems are usually composed by classical bacterial two-component sensor/regulator system with structural, mechanistic, and functional differences[12]. How different structure groups from AMPs evoke AMP sensing systems is very complicated and understanding this can facilitate regional design of novel AMPs and antimicrobial peptidomimetics. Besides APM specific sensing systems, bacteria are also able to sense and respond to AMPs by general stress response systems. For example, several sensor systems were activated when *Bacillus subtilis* was exposed to AMPs, including the SigW and SigM extracytoplasmic sigma factors as well as the YxdJK and LiaRS two-component systems [18].

In the present study, we used *S. epidermidis* as a model organism to study stress response and resistance development against a novel class of antimicrobial peptidomimetics. *S. epidermidis*is is a Gram-positive and coagulase-negative *staphylococci*, which is part of human skin flora (commensal) and frequently causes infections on catheters and implants[19]. *S. epidermidis* cells were treated with sub-lethal concentrations of two of our recently developed peptidomimetics **4d**, **2b,** with α-peptide/β-peptoid chimeras structures [20], and glycopeptides vancomycin (VAN). The stress response of *S. epidermidis* towards these compounds was studied by use of DNA microarrays.

Since the development of resistance to AMPs occurs slower than conventional antibiotics as AMPs usually attack multiple hydrophobic and/or polyanionic targets [21], it is difficult to obtain mutants that are resistant to AMPs. Thus experimental evolution training methods [22] by multiple passages with sub-lethal concentrations of **4d**, **2b** and VAN were used for obtaining resistant populations. Whole-genome re-sequencing has been used to find the beneficial mutations in the resistant populations gained at the end of bacterial evolution experiments. Transcriptomic and phenotypic analysis was also applied to some populations or specific clones.

Our purpose is to identify the stimulons of the peptidomimetics and to determine whether the differentially expressed genes play a role in the resistance. Moreover, we are interested in finding out whether there are strong peptide-specific responses or a more general stress response triggered by peptidomimetic exposure. Furthermore, through the investigations of the changes of the evolved resistant populations and clones on genomic, transcriptomic and phenotypic levels, we aim to explore the mode of action of peptidomimetics and improve our understanding of the microbial resistance mechanisms against the peptidomimetics.

Results

Transcriptomic analysis of stress responses of *S. epidermidis* RP62A to antimicrobial peptidomimetics

To access the response of RP62A to **4d**, **2b** and VAN, RP62A during log phase growth was exposed to the compounds at sub-lethal concentrations (see Methods) which slightly inhibited RP62A growth. Samples were collected after 10 min's exposure and mRNA was isolated and subjected to microarray analysis. We have used the custom-made Roche-NimbleGen *S. epidermidis* GeneChip in this study, which includes probes that monitor the expression of virtually all ORFs from *S. epidermidis* RP62A genomes.

All of the three antimicrobial compounds up-regulated a large number of genes (71, 59 or 23 respectively). The peptides **4d** and **2b** induced a similar sub set of genes, and substantially different from induced genes induced by VAN. **Table 1** shows a set of the genes induced by at least one of the tested compounds for at least 3-fold and represents a list of "marker" genes for the stress responses against these compounds. The complete list of the induced genes (higher than 2-fold induction) is shown in **Table S1**.

4d, **2b** and VAN all strongly induced the expression of the VraRS two-component system (SERP1422- SERP1423) and a putative cell wall-active antibiotics response protein SERP1424. The two-component system VraRS positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus* [23]. The up-regulation of VraRS system suggests that the peptidomimetics act through targeting cell wall.

4d and 2b treatments induced general stress response genes such as the *ctsR-clpC* operon, *clpB*. CtsR is the master regulator which regulates the expression of some stress response genes, such as Clp ATPases components (clpC, clpB). **4d** treatment also induced *hrcA* and *dnaJ* [24]. HrcA- and CtsR-regulated genes, which encoding classical chaperones and Clp ATPases components, were over expressed substantially when expose *S. aureus* to heat shock situation [25].

An obvious feature of transcriptome of peptidomimetics-treated samples was the over expression of efflux pumps. **4d**, **2b** and VAN all induced *vraD* (ABC transporter, ATP-binding protein) and SERP2131 (cation transporter E1-E2 family ATPase). In addition, **4d** and **2b** induced some other ABC transporter operons, such as *oppBCDFA* (oligopeptide ABC transporter), *vraFG*, SERP0097-SERP0099, *sitBC*, SERP0340 (ABC transporter ATP-binding protein), SERP0886-0889, SERP0953 (putative peptide ABC transporter, permease protein). **4d** and **2b** also induced the expression of arsenical pump (*arsD*, *arsA*), as well as some monovalent cation/H^+ antiporters, such as SERP0281, SERP0289. The data suggests the extrusion of AMPs out of the cell by differential efflux pumps is a major response of *S.epidermidis* cells towards peptidomimetics.

Furthermore, **4d** and **2b** largely induced the expression of several amino acid biosynthesis operons (*dap*, *lys*, *ilv-leu*, and *thr* operons), which are genes of intermediary metabolism. *lysC* and *asd*, which encode the "common-pathway" enzymes of the synthesis of all aspartate family amino acids, aspartokinase II and aspartate semialdehyde dehydrogenase, respectively, were

induced around 20 times by both **4d** and **2b**. The other genes encode enzymes involved in the biosynthesis of the aspartate family amino acids, lysine (*dapA*, *dapB*, *dapD*), threonine (*thrC*, *thrB*, *hom*), and isoleucine (*ilvABCD*,*leuABCD*), separately. SERP0968 and SERP0969, encoding an amidohydrolase family protein and an alanine racemase family protein, separately, which locate next to the *dap* genes, were also up-regulated. Notably, lysine is a particularly important amino acid in *Staphylococcus*, being required not only as a building block for proteins but also as a component of the cell wall peptidoglycan. Besides, *gltD*, which encodes glutamate synthase subunit beta, was also upregulated by the two peptidomimetics. Notably, **4d** caused a much stronger induction of amino acids production than **2b**.

Moreover, **4d** and **2b** and VAN treatment induced the expression of *htrA* (serine protease HtrA-like), SERP0294 (Peptidase M50B-like; pfam13398), SERP0974 (acylphosphatase), SERP0224 (hypothetical protein). **4d** and **2b** induced the expression of SERP2469 (alcohol dehydrogenase, zinc-containing), SERP0617 (hypothetical protein). Some genes were only induced by **4d**, such as SERP0419 (ribosomal subunit interface protein), *prsA* (putative protein export protein), SERP0620 (hypothetical protein). There are some genes were only induced by VAN, such as *betA* and *betB* which are involved in osmotic balance, SERP0932 (putative transcriptional regulator), and SERP0551 (hypothetical protein).

Genes down-regulated by **4d**, **2b**, VAN

4d, **2b** and VAN reduced the expression of 60, 42 and 20 genes, respectively. Most of the genes were down-regulated by more than one compound (**Table S2**).A striking phenomenon is that treatment of **4d, 2b** and VAN caused down-regulation of the *SaeRS* operon, which is the regulator for several virulence factors.

Experimental evolution of antimicrobial resistance in *S. epidermidis* RP62A

10 independent lineages of *S. epidermidis* RP62A were transferred in LB media with increasing concentrations of **4d**, **2b** or VAN, respectively. As controls, 3 independent lineages of RP62A were transferred in LB media without addition of antimicrobial agent. In the end, after 40 transfers (theoretically corresponds to 200 – 240 generations), 5, 4, and 3 highly resistant populations were evolved for **4d**, **2b**, or VAN, respectively (**Figure S1**). The experiment process of the development of resistance to the compounds (lineage RD14, RD22, RD33 as examples) is illustrated in **Figure S2**.

In order to evaluate stability of the resistant phenotypes, all of the evolved resistant populations were cultivated in LB without antimicrobial compounds for 10 passages and their antimicrobial resistance were tested by micro dilution method. All the resistant populations were able to maintain their resistance (data not shown). The MICs of populations in LB media (MillQ) for **4d**, **2b**, and VAN were around 32-64 μg/ml, 8-16μg/ml or 8 μg/ml respectively (**Table 2**). Notably, the resistance was not detected in LB media prepared by DD H2O (Double distilled water), which suggests the resistance may be related to cationic concentration. In the control selection lineages (referred to as SET_RD0), grown in LB media without antibiotics, no

significant change in ABs resistance was observed compared to RP62A, indicating that antibiotic resistance developed due to the selection protocol (**Table 2**).

There was a high degree of cross-resistance between the **4d** and **2b**. The populations resistant to one peptidomimetic are also resistant to the other at a similar degree. The peptidomimetic resistant populations have around 2 times higher MIC towards VAN. However, no resistance towards **4d** or **2b** was found in VAN resistant populations (**Table 2**).

Comparative genomics analysis of the resistant lineages

To investigate the genetic changes underlying adaptation, we sequenced the genomes of 12 resistant populations belonging to 3 sets (Set RD1: RD11_P40 - RD15_P40 developed from **4d**; Set RD2: RD21_P40 - RD24_P40 developed from **2b**; Set RD3: RD31_P40 -RD33_P40 developed from VAN), and 3 control populations belong to Set RD0 (RD01_P40-RD0_P40 developed from non-antibiotic adding media). Notably, instead of sequence a single clone of each line, we sequenced the endpoint population of each line. The reference genome (RP62A, also named RD00 in this study) was re-sequenced to exclude polymorphisms caused by errors in reference assembly.

All the 16 genomes were sequenced to an average of 114 fold coverage, generating 90 base paired-end reads and a total data set of 50 gigabase pairs (Gb).We identified high-quality SNPs in the non repetitive parts of the genomes by mapping sequence reads for each line against RP62A(CP000029 and CP000028).

A total of 83 mutations were detected in the 15 populations (1-10 events per population), include 78 SNPs, 4 microindels and 1 large deletion (~60 kb). Number of different mutations for each population was summarized in **Table 3.**

Since we sequenced population instead of single clone, the fraction of the sequenced population to contain a given polymorphism was calculated (see the **Table S3**). This gives a very detailed view of the heterogeneity of the populations and showed a very high incidence of parallel mutations (**Table 4**). Specially, RD14_P40 is a pure clone which has a 100% detection of G34A conversion in gene relA-2 (SERP1196).

Convergent mutations provide evidence of adaptive events. Among point mutations, 33 of the 78 nonsynonymous (NS) mutations were shared among two or more lines, but some were shared extensively. Focusing on convergent mutations, we clustered them into several functional units (**Table 5** and **Table S3**).

Among these, an obvious target was the RNA polymerase complex, one of the most central transcriptional regulatory hubs. 2 lines in set RD2 and 2 lines in set RD3 contained a NS mutation in codon 508 of the RNA polymerase (RNApol) beta subunit (*rpoB*), and 1line in set RD3 contained NS mutations in codon 99 and codon 110 of the RNA polymerase (RNApol) beta' subunit (*rpoC*).

Another key target was GTP pyrophosphokinase *relA*, which regulates the concentration of the stringent response alarmone ppGpp. 4 lines in set RD1 contained NS mutations in 6 different codons (12, 49, 58, 111, 534, 686) of the GTP pyrophosphokinase relA-2, RD12 in set RD1

contained a NS mutation in codon 179 in relA-1. GTP pyrophosphokinases are bifunctional enzymes which catalyze the synthesis and degradation of (p)ppGpp. The C-terminal domain is probably involved in the reciprocal regulation of the two catalytic activities, since truncation of the C-terminal domain enhances the synthesis activity of the enzyme [26]. For RD14_P40, only a mutation was found in *relA-2*. ppGpp can interact with differential targets, decrease the production of ribosomes, rRNAs and tRNAs, arrest the cell cycle, but will increase the production of genes involved in amino acid biosynthesis and metabolisms involved in famine.

Another target involved in stringent response is the prolyl-tRNA synthetase (*proS*). 2 lines in set RD1 and 1 line in set RD2 contained a NS mutation in codon 127 of the prolyl-tRNA synthetase (*proS*). The mutations caused decrease of prolyl-tRNA gene transcription.

Furthermore, a striking target was the transporters and efflux pumps. 3 lines in set RD1 and 3 lines in set RD2 contained NS mutations in 5 different codons (12, 76, 79, 82 and 102) of the potassium uptake protein trkA. 1 line in set RD1 and 3 lines in set RD2 contained NS mutations in 3 different codons (301, 298, 86) of the sodium transport family protein SERP0458. Besides, a NS mutation in SERP0014 (RND family efflux transporter) and a NS mutation in SERP0016 (ABC transporter permease) were found in RD11_P40 in set RD1.

Moreover, enzymes involve in membrane modification were also targets, such as FmtC, NagB and TagA. 2 lines in set RD2 contained a NS mutation in codon 176 of the lysylphosphatidylglycerol (LPG) synthase FmtC, which also called multiple peptide resistance factor (MprF). Mutation in FmtC caused decrease of negative charge of cell membrane; therefore weaken the ability of AMP to bind to cell memebrane. RD13_P40 and RD23_P40 contained a NS mutation in codon 71 of the glucosamine-6-phosphate (GlcN6P) isomerase (NagB), which catalyzes the reversible conversion of GlcN6P to D-fructose-6-phosphate. RD13_P40 also contained a NS mutation in codon 238 of UDP-N-acetyl-D-mannosamine transferase TagA.

One nucleotide mutation was found in the intergenic area of SERP2477//SERP2478 in RD22_P40 (153 upstream hypothetical protein//226 downstream hypothetical protein). Remarkably, a large deletion (~60 kp) was found in this intergenic area in RD24_P40. Coexpression of the hypothetical proteins SERP2477, SERP2478 and sdrH were found in *S.epidermidis* (http://string-db.org/). sdrH is a cell-surface-associated protein, a member of serine-aspartate repeat (Sdr) protein family in *S.epidermidis* [27]. Conserved domains blast shows that SERP2477 belongs to the TIR_2 super family. This is a family of bacterial Toll-like receptors. Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors that recognize structurally conserved molecules derived from microbes.

NS mutations were found in translation initiation factor IF-2 (InfB) in populations RD02_P40, RD11_P40, RD22_P40, and RD32_P40, in 4 different codons (230, 264, 309, 646) respectively. Previous studies proved that mutations in the *metY-nusA-infB* operon suppress the slow growth

of a ΔrimM mutant in *E.coli*, by increasing the synthesis of the ribosome binding factor RbfA [28]. Mutations in *infB* may suppress the slow growth rate caused by other mutations.

One nucleotide mutation in the intergenic region of SERP2096//SERP2097 were found in 3 lines in set RD0, 1 line in set RD1 (RD13_P40), and 1 line in set RD2 (RD23_P40). The intergenic region is the upstream of both genes. SERP2096 is a putative perfringolysin O regulator protein, a phosphotransferase system IIC component, glucose/maltose/N-acetylglucosamine-specific which involved in carbohydrate transport and metabolism. No conserved domain was found in SERP2097.

One indel in RD01_P40, one NS mutation in RD03_P40, and one nonsense mutation in RD33_P40 were found in gene encode SERP0927. A mutation of C->T was found at the intergenic region of *parC*//SERP0927 (10 downstream DNA topoisomerase IV, A subunit//202 upstream amino acid carrier protein) for RD32_P40 and RD33_P40. SERP0927 is an amino acid carrier protein, Na+/alanine symporter, which involved in amino acid transport and metabolism.

A NS mutation of either G666T or G666A was found in all the 3 populations of set RD3, which caused M222I change (Met changed to Ile) in the sensory box histidine kinase YycG. The YycF/YycG two-component regulatory system (TCS) of *Staphylococcus aureus* represents the only essential TCS that is almost ubiquitously distributed in low G+C Gram-positive bacteria. YycG (WalK/VicK) is a sensor histidine-kinase and YycF (WalR/VicR) is the cognate response regulator. Both proteins play an important role in the biosynthesis of the cell envelope. Mutations in YycG have been involved in development of resistance against vancomycin [29] and daptomycin [30]. Notably, a mutation prevalence study showed that the mutation of YycGF was most frequently carried by Vancomycin-intermediate *Staphylococcus aureus* (VISA) strains [29].

Gene expression changes of the evolved populations

To study the mechanisms involved in adaptive evolution, mRNA transcriptional profiling was performed at the endpoint of evolution for evolution populations RD11, RD14, RD21 and RD31.

Gene expression changes of RD14 and RD11
The genes induced both in RD14 and RD11 were SERP0014- SERP0016 (encode RND and ABC transporters), SERP0915 (encodes an IS256-like transposase), SERP0061 (encodes a putative 2-keto-4-pentenoate hydratase) and SERP0224 (encodes a hypothetical protein). Notably, SERP0014, SERP0015 and SERP0016 were up regulated for around 10 times in both populations.

The most strongly induced genes in RD14 were the virulence genes *ica* operon (*icaBCD* which encode intercellular adhesion proteins) which have been induced for around 15 times. Other genes induced in RD14 were the SERP0311-0313 (which encode the Aps (antimicrobial peptide

sensing) system), *vraFG* (encode an ABC transporter), genes encode various dehydrogenases (asd, ald, serA, SERP2469), sdhA and adhB (L-serine dehydratase), and so on.

betA and *betB*, which involved in osmotic balance, were induced in RD11. SERP2279, a gene locates next to *secY* (encodes preprotein translocase subunit SecY), was also up regulated in RD11.
Genes down regulated in both RD14 and RD11 were the *ure* operon (encode urease), SERP1972 (encodes an AraC family transcriptional regulator), geh-2 (encodes a lipase).

Besides, some other genes were down regulated in RD14, such as SERP0736-0739 (phenol soluble modulin beta 1); *glpFK* (glycerol uptake facilitator protein and glycerol kinase); *hld* (delta-hemolysin); sarV (accessory regulator V); SERP2161 (putative lipoprotein); SERP1968 (PTS system, sucrose-specific IIBC components) and geh-1(encodes a lipase).
SERP2417 and SERP1627, which encode a putative lipoprotein and a hypothetical protein respectively, were down regulated in RD11. Notably, SERP1972 and SERP2161 were also down regulated when *S. epiderdimis* cells exposed to sub-lethal concentration of **4d** (**Table S4**).

Gene expression changes of RD21 and RD31
Remarkably, for RD21 and RD31, the expression pattern of a large fraction of genes (21% and 15% respectively) was changed (**Table S5**). A single mutation to a regulatory hub can result in large-scale changes to the expression state of cells in the mutated population [31]. Studies showed that a single mutation in *rpoC* changed the expression pattern of 20–27% of genes in the genome of the mutated population [32]. Therefore the mutation of RNA polymerase beta subunit (*rpoB*) may result in the broad-scale changes to the expression in RD31. It's surprising that even only one point mutation in a sodium transport family protein (SERP0458) was found in 95% of the RD21 population, the expression pattern has been change strongly.

RD14, relA-2 and ppGpp

According to the genome sequence data, there is only one point mutation in *relA-2* in 100% of the RD14 population, which means RD14 it's a pure clone. We streaked out RD14 population on LB plate and the colonies were obvious smaller than RP62A wide type colonies. Some colonies were picked out and tested for MIC values. All of them have the same MICs towards **4d** as the RD14 population. A colony was picked randomly and named RD14-1.

The mutation (V12M) in the RD14 chromosome relative to that of RP62A locates to the N-terminal ppGpp regulatory domain of RelA, replacing a valine that is conserved in Gram-positive species (not shown).

In order to examine whether the *relA* mutation caused any perturbation of the ppGpp pool, the RD14-1 and its ancestor RP62A were grown in chemically defined media with amino acid and radioactive phosphate, labelling its nucleotides. The generation times of RP62A and RD14-1 were 48 minutes and 83 minutes respectively. The ppGpp pool was 24.5 pmol/OD600 in RD14, while the compound was virtually undetectable in the ancestral strain (**Figure S3**).

In Gram-positive bacteria, the transcriptional basis of the stringent response is largely mediated through altered balance between the adenine and guanine branches of the purine interconversion pathways as IMP dehydrogenase is inhibited by ppGpp, thus giving lower flux towards GTP. Therefore, we determined the pool sizes of the remaining nucleotides. The ATP pool sizes were of equal size in the two strains (1.41±0.36 nmol/OD_{600} in RP62A and 1.31±0.17 nmol/OD_{600} in RD14-1), while the other nucleoside triphosphate pools were lower in RD14-1. Especially the GTP pool was less than half the size: 0.41±0.12 nmol/OD_{600} in RP62A and 0.17±0.01 nmol/OD_{600} in RD14-1. The ATP/GTP ratios were thus 3.4 and 7.7. The difference was also seen on the nucleoside monophosphate level as there were 9.7±0.4 pmol/OD_{600} and 8.9±0.1 pmol/OD_{600} AMP and 2.3±0.8 and 1.4±0.4 pmol/OD_{600} GMP in RP62A and RD14-1, respectively (**Figure S4**).

According to the array data of RD14 (**Table7, Table S4**), the transcriptomic response to the relA mutation: Many genes of diverse function had altered expression in RD14 relative to RP62A, suggesting that the effects of the mutation are driven by changes relating to altered growth rate or pleiotropic small molecule-mediated regulation.

Discussion

Stress response against peptidomimetics

The strongly induction of the *VraRS* regulon suggests that the peptidomimetics **4d** and **2b** work through the interaction with cell wall. Besides targeting membranes, several AMPs such as bacitracin and nisin inhibit cell wall biosynthesis [33-34]. A transcriptomic profiling study with daptomycin, a lipopeptide antibiotic, also showed induction of the *VraRS* regulon, which is probably caused by the effects on the cell wall biosynthesis inhibition as well as membrane depolarization [35-36]. The humanβ-defensin 3 has also shown a similar dual effect [37]. Our previous killing kinetic studies have shown that our peptidomimetics kill bacterial cells much faster than Vancomycin (data not shown). Therefore, these peptidomimetics may also have both cell wall biosynthesis inhibition and membrane depolarization effects.

Over expression of a lot of ABC transporters and putive efflux pumps suggests extrusion of peptidomimetics out from cell cytoplasm is a prominent mechanism. The expression of *vraFG* ABC transporter is under the control of the *Aps* (*GraRS*) sensor system, which also controls the expression of *dlt* system, encoding components which catalyze the D-alanylation of teichoic acids, and *mprF*, encoding the MprF enzyme responsible for the lysinylation of phospholipids of

the membrane [15]. However, the *dlt* system and *mprF* genes were not induced upon exposure to peptidodomimetics.
Furthermore*, S. epidermidis* cells try to adapt to the stress and harmful effects of peptidomimetics by inducing some general stress response genes. The highly increased production of several amino acids in the peptidomimetics treated cells is probably a consequence of stringent response cause by amino acid starvation.

Experimental resistant development against peptidomimetics
Adaptive laboratory evolution (ALE) can be used to address fundamental questions about adaptation to selection pressures, and ultimately, the process of evolution. High-throughput technologies, systems biology and microbial genetics have provided insights into bacterial adaptation on phenotypic and genotypic levels [38]. ALE studies have been performed under various selection pressures, such as nutrition-limited medium, high temperature and antibiotic treatment. What we have learnt from the previous ALE studies were: First, the complete set of mutations in either clonal or population samples can be determined by next-generation sequencing technologies. Second, adaptive mutations frequently target regulatory mechanisms, such as RNA polymerase. Third, systems-level optimization principles underlie the genetic changes seen in adaptive evolution. Fourth, diversity in evolving populations is related to the mutation rate, the fitness effects of beneficial mutations, and the size of bottleneck involved [38-39].

In our study, a very high incidence of parallel mutations was found in the experimental developed resistant populations. The convergent mutations can be clustered into several functional units, *i.e.*, RNA polymerase (RNAP) complex, GTP pyrophosphokinase RelA, transporters and efflux pumps and enzymes responsible for membrane modification. Mutations in the RNAP and RelA regulatory systems have been frequently detected in experimental evolution studies. RelA regulates the concentration of stringent response alarmone ppGpp. ppGpp affects the replication, transcription, and translation of the resource-consuming cell processes, through the interaction with various targets, such as RNA Polymerase, translational GTPases, DnaG primase and IMP dehydrogenase [40-42]. Differently, mutations in transporters, efflux pumps and cell membrane modification enzymes are responsible to more specific AMP resistant mechanisms. Notably, the membrane modification mechanism was not detected in the transcriptional profiling of the stress response assay.

Regarding to the transcriptomic profiling of the RD14 and RD11 population, the most prominent feature is the strong induction of SERP0014- SERP0016 (RND and ABC transporters) and an IS256-like transposase. The mutation of *relA-2* in RD14 even caused induction of *Aps* system and induced the expression of *VraFG* ABC transporter. The *Aps* system may play an important role in sensing and respond to the presence of peptidomimetics. The *S. epidermidis* 1457 *apsS* and *apsX* mutants become much susceptible toward **4d** and **2b** compared to wild-type strain (data not shown). Moreover, the pleiotropic effects of *relA-2* mutation have changed the expression of various genes.

relA-2 and ppGpp

Since the RD14 is a pure clone containing a 100% point mutation in *relA-2*. It becomes a good sample for studying the regulation of *relA* and stringent response in *S. epiderimidis*.

The nucleotide pool determinations clearly demonstrate that the mutation in RD14 causes an increased level of ppGpp. The regulatory responses of ppGpp are highly diverse among bacteria and are linked to many traits [43]. The ppGpp pool might determine for the balance between adenine and guanine nucleotide pools, which influences the rate of transcription initiation of some promoters, especially those of ribosomal RNA. In *E. coli*, ppGpp is essential for regulation of the rRNA content [44]. Gram-positive bacteria do not rely on stringent sequences in the promoter, nor ppGpp and DksA in the stringent response [45]. Rather the ppGpp inhibits IMP dehydrogenase, which in turn changes the balance between the ATP and GTP nucleotide pools and subsequently influences transcription initiation. The size of the GTP pool also influences transcription as a co-repressor of the CodY repressor in some firmicutes – in addition to the regulation by branched chain amino acids. A lower GTP pool will release repression of the CodY targeting genes. The transcriptomic analysis we have conducted here reveals that there is some overlap between genes induced in RD14 and those most up-regulated in a *S. aureus codY* null mutant such as the *ica* operon and binds to CodY [46-47].

A ppGpp-up mutation causing a growth inhibition as accompanied by a high counter-selective pressure, but nevertheless this experiment has shown that it can even be selected for under treatment with a sub-lethal concentration of an antibiotic.

Recently, small single-domain (p)ppGpp synthetases have been identified in firmicutes by homology to the C-terminal domain of Rsh (RelA-SpotT Homologs) proteins. The genes are present in *S. aureus*, named *relP* and *relQ* (4), and by homology search we find homologs in the RP62A genome as well: SERP2065 (*relP*, 79% identical on amino acid level) and SERP0586/relA-1 (*relQ*, 92% identical). These enzymes carry only the synthesis function and are thus not capable of hydrolysis, suggesting the hydrolysis function relies on functional Rsh protein, and in line with this a we note that a full deletion of *rsh* has not been possible to obtain in *S. aureus* [48].

The mutation isolated here can be explained by a partial loss of ppGpp hydrolysis caused by either an enzyme with less hydrolytic activity or by altered expression of the gene. The V12M mutation is due to a GTG to ATG codon substitution. An analysis with the RBS calculator software (https://salis.psu.edu/software/reverse) [49] reveals that the nucleotide substitution at the twelfth codon rises the probability of a higher translation start rate here, but not matching that of the annotated start codon, which is a GTG.

Conclusion

S. epidermidis cells respond to the stress caused by peptidomimetics through either general stress response systems or specific mechanisms. The *VraRS* regulon and *Aps* (*GraRS*) system are responsible for sensing and reacting to the peptidomimetics. Differential ABC transporters and efflux pumps are involved in the repulse of harmful peptides from cells.

Adaptive evolution of the *S. epidermidis* cells toward the peptidomimetics caused mutations in regulatory systems and genes of simple specific roles. RNAP and *relA* are the most frequently mutated regulatory targets. To some extent, both of them regulate the balance of the cells between self-protection and nutritional capability (the SPANC balance), therefore regulating the stress resistance. Cation transporters, efflux pumps and cell membrane modification enzymes were also hot spots of mutation. Notably, the membrane modification mechanism seems only detected in the adaptive resistant but not in the stress response studies.

Furthermore, the phenotypic and transcriptomic studies of the *relA-2* mutant RD14 has provided detailed information about the *relA* regulated stringent response in *S. epiderimids*. Briefly, the V12M mutation of *relA-2* causes an increased pool of ppGpp. The accumulated ppGpp inhibites IMP dehydrogenase, which in turn lowers the GTP/ATP ratios and subsequently influence transcription initiation of various genes, including stress response genes and importantly, the genes encoding the *Aps* system and *VraFG* transporter.

To summary, general stress response especially the stringent response is an important mechanism for *S. epidermidis* to respond to peptidomimetic **4d** and **2b**. In addition, some specific mechanisms are involved to the defense of peptidomiemtics, such as the *VraRS* and *Aps* sensor systems, various efflux pumps and membrane modification enzymes. These results suggest that peptidomimetics could constitute a potential additive to conventional antibiotic treatment.

Material and methods

Bacterial strains, chemicals, and growth conditions. *Staphylococcusepidermidis* RP62A was used as a reference strain. Peptidomimetics were synthesized as previously described [50-52] .Vancomycin was purchased from Sigma. LB broth (prepared in MillQ water) was used for the resistance develop assay and the MIC testing.TSB media with 0.25% glucose was used as growth medium for all the microarray study.

Determination of minimum inhibitory concentration. The minimum inhibitory concentration (MIC) was determined using a standard broth microdilution technique as previously described [53]. The MIC was determined as the lowest concentration where no visible growth occurred.

Resistant bacteria were taken directly from the frozen culture to minimise a possible loss of induced resistance by incubation without the presence of ABs. To exclude differences in the MIC due to testing directly from frozen culture the wild-type RP62A strain was tested directly from frozen culture and from overnight culture. No difference in MIC was observed between the two testing methods. All assays were performed in duplicate and on at least two separate occasions.

Develop of resistant mutants/populations. Serial passage was initiated from overnight cultures and added to LB medium containing antibiotics (1:100 dilutions; Final bacterial concentration of approximately 1.0×10^7 CFU/ml). The bacteria were incubated overnight at 37℃ and an aliquot was transferred to a new tube with a higher concentration of relative antibiotic. No antibiotic was added to the LB media for the first transfer. From passage no. 2, a non-inhibitory concentration of antibiotic of 0.5 μg/ml was used (The RP62A strain has MICs of around 2 μg/ml for all the three tested antimicrobial agents before the passages). The primary passages in the presence of sublethal MICs resulted in induced resistance at various levels at all tested conditions. Several resistant strains were further passed in medium with increasing concentrations of different compounds. At some transfers such as transfer no. 9 for lineage RD14, the experiment was started from frozen culture sample (**Figure S2**). Concentration was doubled after every 3 stable passages under a concentration. Each mixed-population sample was periodically frozen in 25% (w/v) glycerol at -80℃. The stability of the induced resistance was determined by serial passages in medium without antibiotics.

Sample preparation for genome sequencing. We revived mixed populations from frozen cultures of the end of the evolution experiments, by growing them overnight at 37℃ with shaking at 200 rpm in LB media. Overnight culture was centrifuged. Bacteria pellet was resuspended in 100 μl lysis buffer (500 μg/ml lysostaphin in 50 mM EDTA (pH 7.5)) for 30 min at 37°C. Whole genome DNA was purified using Wizard genomic DNA purification kit (Promega).Whole genome DNA of 12 resistant populations(5 lines developed from **4d**, 4 lines developed from **2b**, 3 lines developed from VAN), 3 control populations together with the reference RP62A, in total 16 genomes, were prepared for sequencing.

Genome sequence and mutation analysis. Samples were sequenced on an Illumina Hiseq2000 platform generating 90 base paired-end reads.Reads were mapped against the genome of RP62A (Genbank accession numbers CP000029 and CP000028) using Novoalign (Novocraft Technologies) [54]. Average insert sizes were 468 nucleotides, and the average genomic coverage depths were 112-116 fold. Pileups of the read alignments were produced by SAMtools release 0.1.7 [55]. Single nucleotide polymorphisms were called by the varFilter algorithm in SAMtools in which minimum SNP coverage was set to 3 (samtools.pl varFilter-d 3 -D 10000). Only SNP calls with quality scores (Phred-scaled probability of sample reads being homozygous reference) at least 20 (*i.e.*$P\leq0.01$) were retained. Microindels were extracted from the read pileup by the following criteria; (1) quality scores of at least 500, (2) root-mean-square (RMS) mapping qualities of at least 25, and (3) support from at least one fifth of the covering reads. To avoid

false-positives, the reference genome was re-sequenced to exclude polymorphisms caused by errors in reference assembly. The fraction of the sequenced population to contain a given polymorphism was calculated as the number of reads supporting the polymorphism divided by the total number of reads.

Determine the sub-lethal concentrations. To determine the sub-lethal concentrations of **4d**, **2b** and VAN, 50 ml of TSB+0.25%glc medium was inoculated with approximately $5x10^6$ *S.epidermidis* RP62A cells (OD600 = 0.01) in 250ml flasks, the cultures were incubated with shaking at 250rpm at 37°C. Antibiotics were added in the early exponential phase (OD600 = 0.3) in several concentration levels. OD600 value was measured every 30min and the growth kinetics were drawn. The addition of the ABs at these sub-lethal concentrations (2 μg/ml of **4d**, 2 μg/ml of **2b,** and 5 μg/ml of VAN) slowed down the growth slightly.

Sample preparation for microarray

For testing the stress response of wide type RP62A towards antibiotics, bacteria were grown in TSB medium with 0.25% glucose to the early exponential phase (OD600 = 0.3) and antibiotics were added at the sub-lethal concentrations described above. Samples were taken for RNA isolations after treating the cultures with the compounds for 10 minutes. Control cultures without antibiotic additions were treated similarly and in parallel. While for the resistant populations, samples were harvested at early exponential (OD600 = 0.3) without being exposed to any antibiotics.

The cells from 4 ml of the cultures were immediately mixed with RNAprotect Bacteria Reagent (Qiagen) and stored at -80°C. RNA isolation was performed by use of a FastPrep BLUE (Q-BioGene) kit and following the protocol described before [56]. Purified RNA samples were analyzed using the RNA NanoLab chip on the 2100 Bioanalyser (Agilent, Palo Alto, CA). Triplicate experiments were performed for each treatment. For all experiments, the same amounts of RNA were used.

Microarray experiments and data analysis. DNA microarrays with probes for RP62A strain and plasmid pSERP were designed and manufactured on 12x135K slides by Roche-NimbleGen (http://www.nimblegen.com) from the GENBANK entries. cRNA synthesis and labelling was performed with a modification of the low-Input Quick Amp labelling kit from Agilent (Gautier et al., unpublished protocol), using only Cy3 as the only dye. After washing according to the NimbleGen protocol for expression arrays, the slides were scanned with a Roche 2-micron scanner and the images processed with Nimble Scan v.2.5.The data obtained in the XYS format were pre-processed with the help of the bioconductor suite of tools [57] and differential expression patterns identified with the LIMMA package and the method TREAT [58], using a log2 fold change of 1 as a prior. Genes showing significant changes ($P < 0.05$) and at least a 2-fold induction/reduction of expression, in the antibiotic-treated cells as compared to the control cells (non-treated) were accepted as differentially expressed.

Nucleotide pools assay. RP62A and the single-colony isolate RD14-1 were grown in the chemically defined SA-media [59] supplemented with glucose to 1% at 30°C. Cultures growing exponentially were diluted in preheated media to an optical density at 600 nm (OD_{600}) of less than 0.05. At OD_{600} = 0.2, 150 µL was transferred to 1 µL of [33P]-labelled orthophosphate treated with inorganic pyrophosphates as previously described [60]. At OD_{600} around 0.8 to 0.9, 100 µL of the radioactively labelled culture was transferred to 20 µL of 10 M HCOOH stored in an Eppendorf tube at -20°C, vigorously mixed and transferred to dry ice. Nucleotides were extracted [60], separated and quantified as described [61-62], except for ppGpp which was quantified by one-dimensional TLC of the raw nucleotide [63]. The compounds contributing to the intensity of the ppGpp spot was found to bind charcoal. The experiments were done in duplicate, and each pool size is reported as average ± standard deviation.

Tables

Table 1. Genes induced by 4d, 2b, VAN

Gene ID	Gene Name*	Down regulated with**			Known Regulation in SA***	Protein/Similarity
		4d	**2b**	VAN		
SERP0019				7.9		hypothetical protein
SERP0021	*vraD*	2.9	4.4	19.8	AMP, VCM	ABC transporter, ATP-binding protein
SERP0099		4.7	3.5			ABC transporter, substrate-binding protein
SERP0109	*gltD*	5.5	2.9		AMP, VCM	glutamate synthase subunit beta
SERP0162	*ctsR*[#]	7.8	4.4		AMP	transcriptional regulator CtsR
SERP0224		5.3	3.4	3.5	AMP, VCM	hypothetical protein
SERP0294		3.9	3.2	5.9	AMP, VCM	hypothetical protein
SERP0314	*vraF*	2.7	3			ABC transporter, ATP-binding protein
SERP0419		7.2	2.9			ribosomal subunit interface protein
SERP0564	*clpB*	10.4	9		AMP, VCM	ATP-dependent Clp protease, ATP-binding sub
SERP0570	*oppB*[#]	5.8	2.8		AMP, VCM	oligopeptide ABC transporter, permease prot
SERP0617		6.9	4			hypothetical protein
SERP0620		4.7				hypothetical protein
SERP0887		4.7	7.7			ABC transporter, permease protein
SERP0896		3.7				aspartate kinase
SERP0897	*hom*	9.7	4.6		AMP, VCM	homoserine dehydrogenase
SERP0898	*thrC*[#]	9.5	4.7		AMP, VCM	threonine synthase
SERP0963	*lysC*	25.1	21.5		AMP	aspartate kinase
SERP0964	*asd*	22.6	17.6		AMP, VCM	aspartate-semialdehyde dehydrogenase
SERP0965	*dapA*[#]	12.8	7.4		AMP, VCM	dihydrodipicolinate synthase

SERP0968		12	7.1		AMP, VCM	amidohydrolase family protein
SERP0969		7.5	6.8		AMP	alanine racemase family protein
SERP1292	*htrA*	9.7	4.6	3.4	AMP, VCM	serine protease HtrA, putative
SERP1422	*vraR*[#]	9.7	6.4	6.1	AMP, VCM	DNA-binding response regulator VraR
SERP1424		10.1	6.8	6.7	AMP, VCM	hypothetical protein
SERP1425		5.5		2.9	AMP, VCM	hypothetical protein
SERP1667	*ilvI*	8.4	3.7		AMP	acetolactate synthase 1 regulatory subunit
SERP1668	*ilvC*[#]	30.9	6		AMP	ketol-acid reductoisomerase
SERP1669	*leuA*[#]	23.2	4.2		AMP	2-isopropylmalate synthase
SERP2017			3.2			amino acid permease family protein
SERP2131		8.5	6.1	7.7	AMP, VCM	cation transporter E1-E2 family ATPase
SERP2159		4	3.7		AMP, VCM	4-aminobutyrate aminotransferase
SERP2428	*arsA*[#]	4.1	3.3			arsenical pump-driving ATPase
SERP2469		8.9	6.6			alcohol dehydrogenase, zinc-containing
SERP2541		4.2	5.1		AMP	homoserine O-acetyltransferase, putative

*: Only the most strongly induced genes, the marker genes, are shown in this table. The complete list of genes induced at least 2-fold by several ABs is shown in **Table S1**.

[#]: In the case of operons, only the first gene or the most strongly induced gene is shown.

: **4d, **2b**, VAN: RP62A exposed to sub-lethal concentration of **4d**, **2b** or VAN respectively.

***: VCM, vancomycin inducible; AMP, AMP inducible. Data are from a microarray study on *S. aureus* against linear CAMPs [64]

Table 2. MICs (µg/ml) of the resistant populations in LB (MillQ) *

		4d	**2b**	**VAN**
original strain	RP62A	2	2	2
populations developed from **4d**	RD11_P40**	64	32	2
	RD12_P40	32	16	2
	RD13_P40	64	64	4
	RD14_P40	64	32	4
	RD15_P40	64	16	2
populations developed from **2b**	RD21_P40	8	16	2-4
	RD22_P40	8	16	2

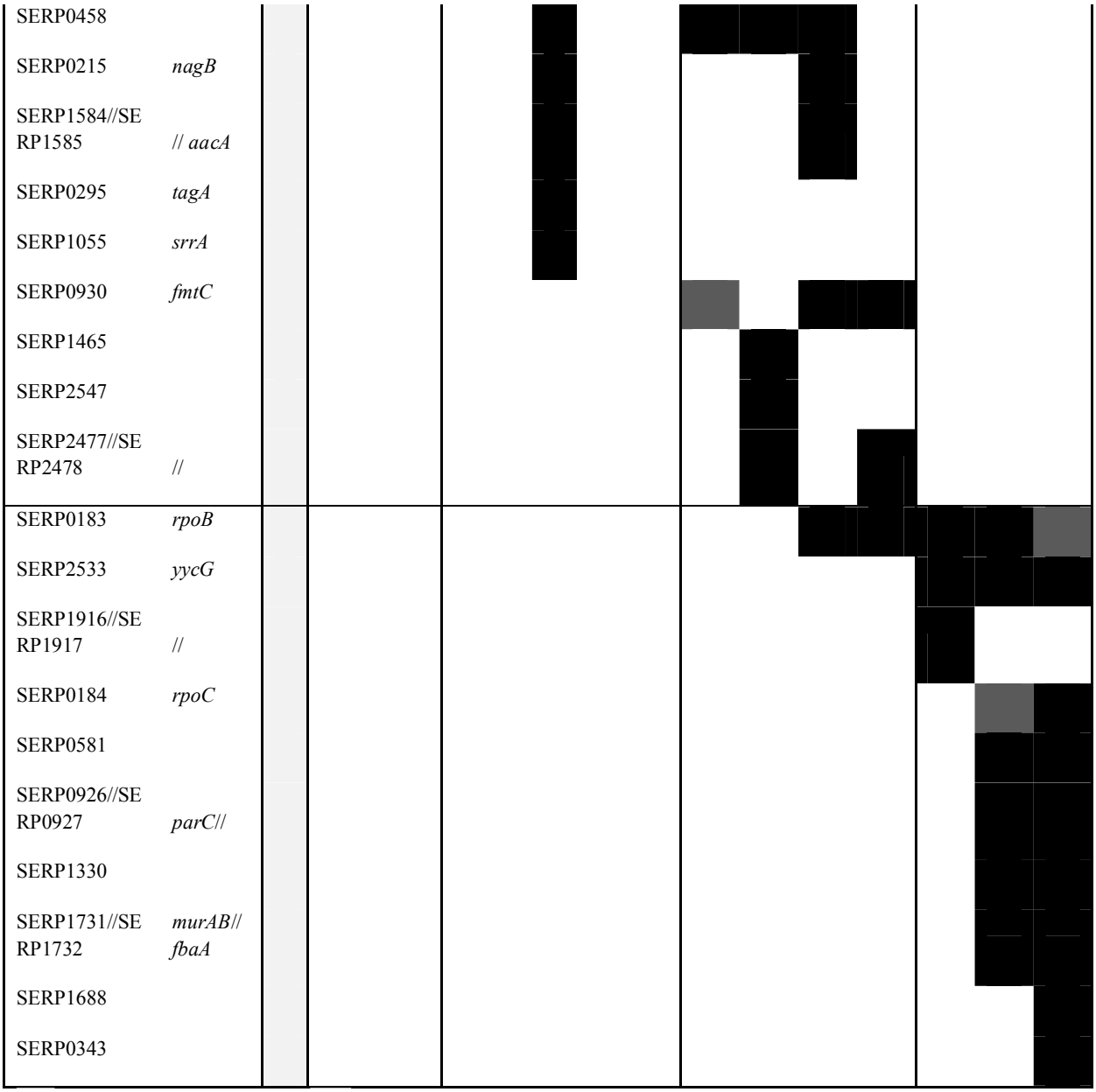
	RD23_P40	16	8	2-4
	RD24_P40	8	16	4
populations developed from VAN	RD31_P40	2	2	8
	RD32_P40	2	2	8
	RD33_P40	2	2	8
controls	RD01_P40	2	2	2
	RD02_P40	2	2	2
	RD03_P40	2	2	2

* Populations were cultivated in LB without antibiotics for 10 passages and they were still resistant

** RD11_P40 stands for the population after passage no. 40 in lineage RD11.

Table 3. List of mutations during adaptive evolution (parallel evolution)

			Set RD0 (CK)			Set RD1 (4d)					Set RD2 (2b)				Set RD3 (VAN)		
Locus tag	Gene name	RD00	RD01	RD02	RD03	RD11	RD12	RD13	RD14	RD15	RD21	RD22	RD23	RD24	RD31	RD32	RD33
SERP2096//SERP2097	//																
SERP0927																	
SERP0836	*infB*																
SERP1272//SERP1273	*ald*//																
SERP1126//SERP1127	// *rpoD*																
SERP1312																	
SERP1680	*rsbU*																
SERP1849//SERP1850	*sarV*// *moaA*																
SERP1159																	
SERP1820	*rplX*																
SERP1955	*mqo-1*																
SERP1382																	
SERP2163//SERP2164	//																
SERP0674																	
SERP0014																	
SERP0016																	
SERP1196	*relA-2*																
SERP0830	*proS*																
SERP0586	*relA-1*																
SERP0588																	



■ :SNP called by SAMtools; ▬ SNP may be present but not called by SAMtools. // : the mutation locates between the two genes.

Table 4. Number of different mutation events for each population

Number of events	RD 01	RD 02	RD 03	RD 11	RD 12	RD 13	RD 14	RD 15	RD 21	RD 22	RD 23	RD 24	RD 31	RD 32	RD 33	In total
SNPs	4	7	6	4	5	10	1	2	1	7	9	3	3	7	9	78
short indels	2												1		1	4
large deletion*												1				1
In total:	6	7	6	4	5	10	1	2	1	7	9	4	4	7	10	83

*, large deletion (>30bp)

Table 5. Convergent mutational events in functional unit level

Function unit	Fraction of mutated lineages (No. of mutation events)*					Mutational type#	NOTE
	Gene name	Set RD0	Set RD1	Set RD2	Set RD3		
Extended RNApol complex	*rpoB*			2/4 (1)**	2/3(1)	a	DNA-directed RNA polymerase subunit beta
	rpoC				1/3(2)	a	DNA-directed RNA polymerase subunit beta'
	//rpoD	1/3 (1)				e	conserved HP***//RNA polymerase sigma factor RpoD
General stress response regulation	*relA-1*		1/5 (1)			a	GTP pyrophosphokinase
	relA-2		4/5 (6)			a,b	GTP pyrophosphokinase
	ald//SERP1273	3/3 (1)				e	alanine dehydrogenase//universal stress protein
	proS		2/5 (1)	1/4 (1)		a	prolyl-tRNA synthetase
Transpoter,efflux pump	*trkA*		3/5 (2)	3/4 (3)		a	potassium uptake protein
	SERP0458		1/5 (1)	3/4 (3)		a	sodium transport family protein
	SERP0014		1/5 (1)			a	RND family efflux transporter, MFP component
	SERP0016		1/5 (1)			a	ABC transporter permease
Membrane modification	*fmtC*(*mprF*)			2/4 (1)		a	LPG synthase. lysylation of phospholipids
	tagA(SERP0295)		1/5 (1)			a	UDP-N-acetyl-D-mannosamine transferase

	nagB		1/5 (1)	1/4 (1)		a	glucosamine-6-phosphate isomerase
VAN	*yycG*				3/3 (2)	a	sensory box histidine kinase YycG

*, Fraction of mutated lineages means how many lineages out of each experiment set contain mutations in a specific gene or site. The number in brackets means how many different mutation events were shared in total.

**,for example, in set RD2, 2 out of 4 lineages contain mutation in rpoB, while only 1 mutation event was found, *i.e.*, all the mutations were the same.

***, HP, hypothetical protein.

#, mutational types: a, missense mutations; b, nonsense mutations; c, insertions and deletions (indels); d, large deletion(≈60kb);e, intergenic mutations.

Table 6. Number of genes with changed expression in evolved populations

	Differently expressed	Up regulated	Down regulated	Replicates of array samples	Mutations
RD14	57	39	18	2	*relA-2* / GTP pyrophosphokinase (100%)
RD11	21	11	10	3	SERP0014 / RND family efflux transporter, MFP component (75%) SERP0016 / ABC transporter, permease protein(25%); *trkA* / potassium uptake protein TrkA(83%) *infB* / translation initiation factor IF-2(99%)
RD21	532	373	159	2	SERP0458 / sodium transport family protein (95%)
RD31	371	239	63	3	*rpoB* / DNA-directed RNA polymerase, beta subunit (59%); *yycG* / sensory box histidine kinase YycG (45%) SERP1916//SERP1917 (40%); SERP0927 / amino acid carrier protein (57%)

The number of genes with changed expression at least 2-fold in evolved populations ($P \leq 0.05$)

Table 7. **Genes up regulated in evolved resistant mutant RD14 or RD11**

Gene_ID	Gene_NAME*	RD14	RD11	Protein/Similarity
SERP0014		8.8	9.1	RND efflux transporter
SERP0015		12.4	13.6	ABC transporter, ATP-binding protein
SERP0016		11.2	10.9	ABC transporter, permease protein
SERP0019		4.0		hypothetical protein
SERP0061		3.4	3.4	2-keto-4-pentenoate hydratase, putative
SERP0224		3.0	3.4	hypothetical protein
SERP0311	*apsX*	6.0		hypothetical protein
SERP0312	*apsR/graR*	4.7		DNA-binding response regulator

SERP0313	*apsS/graS*	4.3		sensor histidine kinase
SERP0314	*vraF*	3.2		ABC transporter, ATP-binding protein
SERP0315	*vraG*	2.8		ABC transporter, permease protein
SERP0663		4.9		myosin-cross-reactive antigen
SERP0915		7.1	4.1	IS256-like transposase
SERP0964	*asd*	2.9		aspartate-semialdehyde dehydrogenase
SERP1271	*pepQ*	4.1		proline dipeptidase
SERP1282		2.5		GAF domain-containing protein
SERP1286		4.8		OsmC/Ohr family protein
SERP1288	*serA*	4.5		D-3-phosphoglycerate dehydrogenase
SERP2091		6.8		hypothetical protein
SERP2094	*sdhA*[#]	4.9		L-serine dehydratase, iron-sulfur-dependent, alpha subunit
SERP2096		4.3		perfringolysin O regulator protein, putative
SERP2098		5.5		hypothetical protein
SERP2099	*xylB*	4.0		D-xylulose kinase
SERP2102		3.3		hypothetical protein
SERP2103	*dep*	4.0		gamma-glutamyltranspeptidase
SERP2105	*capA*	4.4		capA protein
SERP2175		6.1		amidohydrolase family protein
SERP2176	*betA*[#]		4.4	choline dehydrogenase
SERP2212		4.6		hypothetical protein
SERP2279			3.8	hypothetical protein
SERP2295	*icaB*[#]	14.3		intercellular adhesion protein B
SERP2469		3.4		alcohol dehydrogenase, zinc-containing
SERP2541		3.6		homoserine O-acetyltransferase, putative

*: The genes up regulated for no less than 2 fold are shown in this table.

[#]: In the case of operons, only the first gene or the most strongly induced gene is shown.

References

1. Olsen, C.A., *Peptoid-Peptide hybrid backbone architectures.* Chembiochem, 2010. **11**(2): p. 152-60.
2. Neu, H.C., *The crisis in antibiotic resistance.* Science, 1992. **257**(5073): p. 1064-73.
3. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* Nature, 2002. **415**(6870): p. 389-95.
4. Bulet, P., R. Stocklin, and L. Menin, *Anti-microbial peptides: from invertebrates to vertebrates.* Immunological reviews, 2004. **198**: p. 169-84.
5. Peschel, A. and H.G. Sahl, *The co-evolution of host cationic antimicrobial peptides and microbial resistance.* Nature reviews. Microbiology, 2006. **4**(7): p. 529-36.
6. Polyansky, A.A., et al., *N-terminal amphipathic helix as a trigger of hemolytic activity in antimicrobial peptides: a case study in latarcins.* FEBS letters, 2009. **583**(14): p. 2425-8.
7. Scott, R.W., W.F. DeGrado, and G.N. Tew, *De novo designed synthetic mimics of antimicrobial peptides_Curr Opin Biotechnol.* Curr Opin Biotechnol, 2008. **19**(6): p. 620-7.
8. Giuliani, A. and A.C. Rinaldi, *VIP_Beyond natural antimicrobial peptides: multimeric peptides and other peptidomimetic _Cell Mol Life Sci*

Cell Mol Life Sci, 2011. **68**(13): p. 2255-66.

9. Shai, Y., *Mode of action of membrane active antimicrobial peptides.* Biopolymers, 2002. **66**(4): p. 236-48.
10. Hurdle, J.G., et al., *Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections.* Nat Rev Microbiol, 2011. **9**(1): p. 62-75.
11. Peschel, A. and H.G. Sahl, *The co-evolution of host cationic antimicrobial peptides and microbial resistance.* Nat Rev Microbiol, 2006. **4**(7): p. 529-36.
12. Otto, M., *Bacterial sensing of antimicrobial peptides.* Contrib Microbiol, 2009. **16**: p. 136-49.
13. Ernst, R.K., T. Guina, and S.I. Miller, *Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity.* Microbes Infect, 2001. **3**(14-15): p. 1327-34.
14. Macfarlane, E.L., A. Kwasnicka, and R.E. Hancock, *Role of Pseudomonas aeruginosa PhoP-phoQ in resistance to antimicrobial cationic peptides and aminoglycosides.* Microbiology, 2000. **146 (Pt 10)**: p. 2543-54.
15. Li, M., et al., *Gram-positive three-component antimicrobial peptide-sensing system.* Proc Natl Acad Sci U S A, 2007. **104**(22): p. 9469-74.
16. Li, M., et al., *The antimicrobial peptide-sensing system aps of Staphylococcus aureus.* Mol Microbiol, 2007. **66**(5): p. 1136-47.
17. Nizet, V., *VIP_Antimicrobial peptide resistance mechanisms of human bacterial pathogens_Curr Issues Mol Biol.* Curr Issues Mol Biol, 2006. **8**(1): p. 11-26.
18. Pietiainen, M., et al., *Cationic antimicrobial peptides elicit a complex stress response in Bacillus subtilis that involves ECF-type sigma factors and two-component signal transduction systems.* Microbiology, 2005. **151**(Pt 5): p. 1577-92.
19. O'Gara, J.P. and H. Humphreys, *Staphylococcus epidermidis biofilms: importance and implications.* J Med Microbiol, 2001. **50**(7): p. 582-7.

20. Olsen, C.A., et al., *Antimicrobial, Hemolytic, and Cytotoxic Activities of beta-Peptoid-Peptide Hybrid Oligomers: Improved Properties Compared to Natural AMPs.* Chembiochem, 2010. **11**(10): p. 1356-1360.
21. Friedrich, C.L., et al., *J Biol Chem_Hancock_Structure and mechanism of action of an indolicidin peptide derivative with improved activity against gram-positive bacteria.* J Biol Chem, 2001. **276**(26): p. 24015-22.
22. Perron, G.G., M. Zasloff, and G. Bell, *Experimental evolution of resistance to an antimicrobial peptide.* Proc Biol Sci, 2006. **273**(1583): p. 251-6.
23. Kuroda, M., et al., *Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus.* Mol Microbiol, 2003. **49**(3): p. 807-21.
24. Chastanet, A., J. Fert, and T. Msadek, *Comparative genomics reveal novel heat shock regulatory mechanisms in Staphylococcus aureus and other Gram-positive bacteria.* Mol Microbiol, 2003. **47**(4): p. 1061-73.
25. Fleury, B., et al., *Transcriptomic and metabolic responses of Staphylococcus aureus exposed to supra-physiological temperatures.* BMC Microbiol, 2009. **9**: p. 76.
26. Geiger, T., et al., *Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of Staphylococcus aureus.* Infect Immun, 2010. **78**(5): p. 1873-83.
27. McCrea, K.W., et al., *The serine-aspartate repeat (Sdr) protein family in Staphylococcus epidermidis.* Microbiology, 2000. **146 (Pt 7)**: p. 1535-46.
28. Bylund, G.O., J.M. Lovgren, and P.M. Wikstrom, *Characterization of mutations in the metY-nusA-infB operon that suppress the slow growth of a DeltarimM mutant.* J Bacteriol, 2001. **183**(20): p. 6095-106.
29. Shoji, M., et al., *walK and clpP mutations confer reduced vancomycin susceptibility in Staphylococcus aureus.* Antimicrob Agents Chemother, 2011. **55**(8): p. 3870-81.
30. Friedman, L., J.D. Alder, and J.A. Silverman, *Genetic changes that correlate with reduced susceptibility to daptomycin in Staphylococcus aureus.* Antimicrob Agents Chemother, 2006. **50**(6): p. 2137-45.
31. Philippe, N., et al., *Evolution of global regulatory networks during a long-term experiment with Escherichia coli.* Bioessays, 2007. **29**(9): p. 846-60.
32. Conrad, T.M., et al., *RNA polymerase mutants found through adaptive evolution reprogram Escherichia coli for optimal growth in minimal media.* Proc Natl Acad Sci U S A, 2010. **107**(47): p. 20500-5.
33. Breukink, E., et al., *Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic.* Science, 1999. **286**(5448): p. 2361-4.
34. Brotz, H., et al., *The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II.* Antimicrob Agents Chemother, 1998. **42**(1): p. 154-60.
35. Muthaiyan, A., et al., *Transcriptional profiling reveals that daptomycin induces the Staphylococcus aureus cell wall stress stimulon and genes responsive to membrane depolarization.* Antimicrob Agents Chemother, 2008. **52**(3): p. 980-90.
36. Allen, N.E., J.N. Hobbs, and W.E. Alborn, Jr., *Inhibition of peptidoglycan biosynthesis in gram-positive bacteria by LY146032.* Antimicrob Agents Chemother, 1987. **31**(7): p. 1093-9.

37. Sass, V., et al., *Mode of action of human beta-defensin 3 against Staphylococcus aureus and transcriptional analysis of responses to defensin challenge.* Int J Med Microbiol, 2008. **298**(7-8): p. 619-33.
38. Hindre, T., et al., *New insights into bacterial adaptation through in vivo and in silico experimental evolution.* Nat Rev Microbiol, 2012. **10**(5): p. 352-65.
39. Conrad, T.M., N.E. Lewis, and B.O. Palsson, *Microbial laboratory evolution in the era of genome-scale science.* Mol Syst Biol, 2011. **7**: p. 509.
40. Traxler, M.F., et al., *The global, ppGpp-mediated stringent response to amino acid starvation in Escherichia coli.* Mol Microbiol, 2008. **68**(5): p. 1128-48.
41. Srivatsan, A. and J.D. Wang, *Control of bacterial transcription, translation and replication by (p)ppGpp.* Curr Opin Microbiol, 2008. **11**(2): p. 100-5.
42. Mitkevich, V.A., et al., *Thermodynamic characterization of ppGpp binding to EF-G or IF2 and of initiator tRNA binding to free IF2 in the presence of GDP, GTP, or ppGpp.* J Mol Biol, 2010. **402**(5): p. 838-46.
43. Dalebroux, Z.D. and M.S. Swanson, *ppGpp: magic beyond RNA polymerase.* Nat Rev Microbiol, 2012. **10**(3): p. 203-12.
44. Potrykus, K., et al., *ppGpp is the major source of growth rate control in E. coli.* Environ Microbiol, 2011. **13**(3): p. 563-75.
45. Wolz, C., T. Geiger, and C. Goerke, *The synthesis and function of the alarmone (p)ppGpp in firmicutes.* Int J Med Microbiol, 2010. **300**(2-3): p. 142-7.
46. Majerczyk, C.D., et al., *Direct targets of CodY in Staphylococcus aureus.* J Bacteriol, 2010. **192**(11): p. 2861-77.
47. Majerczyk, C.D., et al., *Staphylococcus aureus CodY negatively regulates virulence gene expression*. J Bacteriol, 2008. **190**(7): p. 2257-65.
48. Gentry, D., et al., *The rel gene is essential for in vitro growth of Staphylococcus aureus.* J Bacteriol, 2000. **182**(17): p. 4995-7.
49. Salis, H.M., E.A. Mirsky, and C.A. Voigt, *Automated design of synthetic ribosome binding sites to control protein expression.* Nat Biotechnol, 2009. **27**(10): p. 946-50.
50. Olsen, C.A., et al., *Alpha-peptide/beta-peptoid chimeras.* Org Lett, 2007. **9**(8): p. 1549-52.
51. Olsen, C.A., et al., *Antimicrobial, hemolytic, and cytotoxic activities of beta-peptoid-peptide hybrid oligomers: improved properties compared to natural AMPs.* Chembiochem, 2010. **11**(10): p. 1356-60.
52. Hein-Kristensen, L., et al., *Bacterial membrane activity of alpha-peptide/beta-peptoid chimeras: influence of amino acid composition and chain length on the activity against different bacterial strains.* BMC Microbiol, 2011. **11**: p. 144.
53. Wiegand, I., K. Hilpert, and R.E. Hancock, *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances.* Nat Protoc, 2008. **3**(2): p. 163-75.
54. Krawitz, P., et al., *Microindel detection in short-read sequence data.* Bioinformatics, 2010. **26**(6): p. 722-9.
55. Li, H., et al., *The Sequence Alignment/Map format and SAMtools.* Bioinformatics, 2009. **25**(16): p. 2078-9.

56. Yao, Y., D.E. Sturdevant, and M. Otto, *Genomewide analysis of gene expression in Staphylococcus epidermidis biofilms: insights into the pathophysiology of S. epidermidis biofilms and the role of phenol-soluble modulins in formation of biofilms.* J Infect Dis, 2005. **191**(2): p. 289-98.
57. Gentleman, R.C., et al., *Bioconductor: open software development for computational biology and bioinformatics.* Genome Biol, 2004. **5**(10): p. R80.
58. McCarthy, D.J. and G.K. Smyth, *Testing significance relative to a fold-change threshold is a TREAT.* Bioinformatics, 2009. **25**(6): p. 765-71.
59. Jensen, P.R. and K. Hammer, *Minimal Requirements for Exponential Growth of Lactococcus lactis.* Appl Environ Microbiol, 1993. **59**(12): p. 4363-6.
60. Jendresen, C.B., M. Kilstrup, and J. Martinussen, *A simplified method for rapid quantification of intracellular nucleoside triphosphates by one-dimensional thin-layer chromatography.* Anal Biochem, 2011. **409**(2): p. 249-59.
61. Bochner, B.R. and B.N. Ames, *Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography.* J Biol Chem, 1982. **257**(16): p. 9759-69.
62. Jendresen, C.B., J. Martinussen, and M. Kilstrup, *The PurR regulon in Lactococcus lactis - transcriptional regulation of the purine nucleotide metabolism and translational machinery.* Microbiology, 2012.
63. Cashel, M., *The control of ribonucleic acid synthesis in Escherichia coli. IV. Relevance of unusual phosphorylated compounds from amino acid-starved stringent strains.* J Biol Chem, 1969. **244**(12): p. 3133-41.
64. Pietiainen, M., et al., *Transcriptome analysis of the responses of Staphylococcus aureus to antimicrobial peptides and characterization of the roles of vraDE and vraSR in antimicrobial resistance.* BMC Genomics, 2009. **10**: p. 429.

Supplementary data

Table S1. Full list of genes up regulated by AMP4d, AMP2b, VAN

Gene_ID	Gene_ NAME*	**4d**	**2b**	VAN	PROTEIN
SERP0019				7.9	hypothetical protein
SERP0020		2.7			ABC transporter, permease protein
SERP0021		2.9	4.4	19.8	ABC transporter, ATP-binding protein
SERP0061					2-keto-4-pentenoate hydratase, putative
SERP0099		4.7	3.5		ABC transporter, substrate-binding protein
SERP0109	*gltD*	5.5	2.9		glutamate synthase subunit beta
SERP0162	*ctsR*	7.8	4.4		transcriptional regulator CtsR
SERP0163		7.2	7.4	3.0	UvrB/UvrC domain-containing protein
SERP0164		5.8	5.4		ATP:guanido phosphotransferase
SERP0165	*clpC*	5.2	3.7		ATP-dependent Clp protease, ATP-binding subunit ClpC
SERP0224		5.3	3.4	3.5	hypothetical protein
SERP0276		3.1	2.9		hypothetical protein
SERP0281		2.7	2.7		putative monovalent cation/H+ antiporter subunit A
SERP0282		2.8			putative monovalent cation/H+ antiporter subunit B
SERP0288		3.4			hypothetical protein
SERP0289		3.6	3.4		Na+/H+ antiporter, putative
SERP0291	*sitB*	3.0	2.7		ABC transporter, permease protein
SERP0294		3.9	3.2	5.9	hypothetical protein
SERP0314		2.7	3.0		ABC transporter, ATP-binding protein
SERP0315		2.7			ABC transporter, permease protein
SERP0319		2.9			hypothetical protein
SERP0339	*uppP*	2.6	2.7	2.9	undecaprenyl pyrophosphate phosphatase
SERP0340			3.0		ABC transporter ATP-binding protein
SERP0419		7.2	2.9		ribosomal subunit interface protein
SERP0542		2.9			Oye family NADH-dependent flavin oxidoreductase
SERP0551				3.1	hypothetical protein
SERP0564	*clpB*	10.4	9.0		ATP-dependent Clp protease, ATP-binding subunit ClpB
SERP0570	*oppB*	5.8	2.8		oligopeptide ABC transporter, permease protein
SERP0617		6.9	4.0		hypothetical protein
SERP0620		4.7			hypothetical protein
SERP0641		2.8			fmt protein
SERP0742		2.8			hypothetical protein
SERP0765			2.5		uracil permease
SERP0883		3.8	3.0		hypothetical protein
SERP0887		4.7	7.7		ABC transporter, permease protein
SERP0888		3.0	3.5		sensor histidine kinase, putative
SERP0889		2.9	4.1		LuxR family DNA-binding response regulator
SERP0896		3.7			aspartate kinase
SERP0897	*hom*	9.7	4.6		homoserine dehydrogenase

SERP0898	*thrC*	9.5	4.7		threonine synthase
SERP0932				2.9	transcriptional regulator, putative
SERP0954		2.3	2.2	2.9	hypothetical protein
SERP0963	*lysC*	25.1	21.5		aspartate kinase
SERP0964	*asd*	22.6	17.6		aspartate-semialdehyde dehydrogenase
SERP0965	*dapA*	12.8	7.4		dihydrodipicolinate synthase
SERP0968		12.0	7.1		amidohydrolase family protein
SERP0969		7.5	6.8		alanine racemase family protein
lysA	*lysA*	3.1			diaminopimelate decarboxylase
SERP0973			3.0	3.4	hypothetical protein
SERP0974		2.7	3.6	3.1	acylphosphatase
SERP0975		3.0	2.8		xpaC protein, putative
SERP0996		3.0			carboxyl-terminal protease
SERP1187		3.2	2.4		luciferase family protein
SERP1215		3.0		2.6	hypothetical protein
SERP1245			2.7		amino acid permease family protein
SERP1292		9.7	4.6	3.4	serine protease HtrA, putative
SERP1376	*prsA*	3.5	3.1	2.9	protein export protein PrsA, putative
vraR	*vraR*	9.7	6.4	6.1	DNA-binding response regulator VraR
vraS	*vraS*	8.2	4.7	4.2	sensor histidine kinase VraS
SERP1424		10.1	6.8	6.7	hypothetical protein
SERP1425		5.5		2.9	hypothetical protein
SERP1667		8.4	3.7		acetolactate synthase 1 regulatory subunit
SERP1668	*ilvC*	30.9	6.0		ketol-acid reductoisomerase
SERP1669	*leuA*	23.2	4.2		2-isopropylmalate synthase
SERP1701			3.3		TenA family transcription regulator
SERP1790	*lacE*	2.7			PTS system, lactose-specific IIBC components
SERP1908		4.1	2.8	4.1	hypothetical protein
SERP2109		2.7			esterase, putative
SERP2130		2.9		2.8	hypothetical protein
SERP2131		8.5	6.1	7.7	cation transporter E1-E2 family ATPase
SERP2159		4.0	3.7		4-aminobutyrate aminotransferase
SERP2176	*betA*			4.0	choline dehydrogenase
betB	*betB*			4.0	betaine aldehyde dehydrogenase
gpxA-2	*gpxA-2*	3.0			glutathione peroxidase
SERP2195		2.9	2.6		alpha keto acid dehydrogenase complex, E3 component, lipoamide dehydrogenase, putative
SERP2196		3.2			MarR family transcriptional regulator
SERP2314		3.2			hypothetical protein
SERP2327			3.1		acetoin dehydrogenase, E3 component, dihydrolipoamide dehydrogenase
SERP2333			3.0		hypothetical protein
SERP2345			3.5		dihydroxyacetone kinase subunit DhaK
gldA	*gldA*		2.9		glycerol dehydrogenase
SERP2425		3.3	3.1		hypothetical protein
SERP2426		2.8			ArsR family transcriptional regulator
SERP2428	*arsA*	4.1	3.3		arsenical pump-driving ATPase

SERP2469		8.9	6.6	alcohol dehydrogenase, zinc-containing
SERP2541		4.2	5.1	homoserine O-acetyltransferase, putative

*: The complete list of genes up regulated at least 2-fold by several antibiotics.

: **4d, **2b**, VAN: RP62A exposed to sub-lethal concentration of AMP4d, AMP2b or VAN respectively.

Table S2. Full list of genes down regulated by AMP4d, AMP2b, VAN

Gene ID	Gene Name*	Down regulated with**			Protein/Similarity
		4d	**2b**	VAN	
SERP0207	*sdrG*	3.1	2.8		sdrG protein
SERP0318		2.8	3.0		LysM domain-containing protein
SERP1737		3.8	2.4		pantothenate kinase
SERP1835		4.5	2.8		xanthine/uracil permease family protein
SERP1880		4.0	2.6		secretory antigen precursor SsaA
SERP2054		3.4	2.3		glycosyltransferase, group 1 family protein
SERP2408		21.3	10.3		putative phospholipid methyltransferase
SERP0190		3.3			amidohydrolase family protein
SERP0505		3.4			hypothetical protein
SERP0902		3.3			amino acid permease family protein
SERP1421		3.0			ribonuclease BN, putative
SERP1944		3.0			EmrB/QacA family drug resistance transporter
SERP1945		3.4			drug transporter, putative
SERP2034		3.2			amino acid permease family protein
SERP2316		3.1			hypothetical protein
SERP0081			3.2		hypothetical protein
SERP0776	*gmk*		3.0		guanylate kinase
SERP0364	*saeS*[#]	4.1	4.2	2.4	sensor histidine kinase SaeS
SERP0366		4.9	6.3	3.3	hypothetical protein
SERP0367		5.1	5.9	3.8	putative lipoprotein
SERP2127				3.3	FeoA family protein
SERP2149				3.7	hypothetical protein

SERP1972	3.5	2.7	AraC family transcriptional regulator
SERP2161	3.0		putative lipoprotein

*: List of genes induced at least 3-fold by several antibiotics.

#: In the case of operons, only the first gene or the most strongly induced gene is shown.

: **4d, **2b**, VAN: RP62A exposed to sub-lethal concentration of AMP4d, AMP2b or VAN respectively.

Table S4. **Genes down regulated in evolved resistant population RD14 and RD11**

PROTEIN_ID	GENE_ID	GENE_NAME	RD14	RD11	Protein/Similarity
YP_188319.1	SERP0736		5.3		phenol soluble modulin beta 1
YP_188320.1	SERP0737		4.3		phenol soluble modulin beta 1
YP_188321.1	SERP0738		5.3		phenol soluble modulin beta 1
YP_188322.1	SERP0739		4.9		phenol soluble modulin beta 1
YP_188448.1	glpF	glpF	9.2		glycerol uptake facilitator protein
YP_188449.1	glpK	glpK	3.2		glycerol kinase
YP_189055.1	hld	hld	4.9		delta-hemolysin
YP_189192.1	SERP1627			2.3	hypothetical protein
YP_189411.1	sarV	sarV	3.2		accessory regulator V
YP_189430.1	ureA	ureA	4.6	3.2	urease subunit gamma
YP_189431.1	ureB	ureB	4.0		urease subunit beta
YP_189432.1	ureC	ureC	4.0	2.6	urease subunit alpha
YP_189433.1	ureE	ureE	4.0	2.5	urease accessory protein UreE
YP_189434.1	ureF	ureF	4.6	3.2	urease accessory protein UreF
YP_189509.1	SERP1951			3.0	ABC transporter, ATP-binding protein
YP_189524.1	SERP1968		4.6		PTS system, sucrose-specific IIBC components
YP_189528.1	SERP1972		3.5	3.2	AraC family transcriptional regulator
YP_189717.1	SERP2161		3.2		putative lipoprotein
YP_189847.1	geh-1	geh-1	3.7		lipase
YP_189935.1	geh-2	geh-2	6.1	6.5	lipase
YP_189962.1	SERP2417			4.0	putative lipoprotein

The complete list of genes down regulated at least 2-fold in RD14 and RD11.

Table S5. Gene expression changes of RD21 and RD31

Additional excel table.

Figure S1. Schematic diagram of resistant develop experiment design.

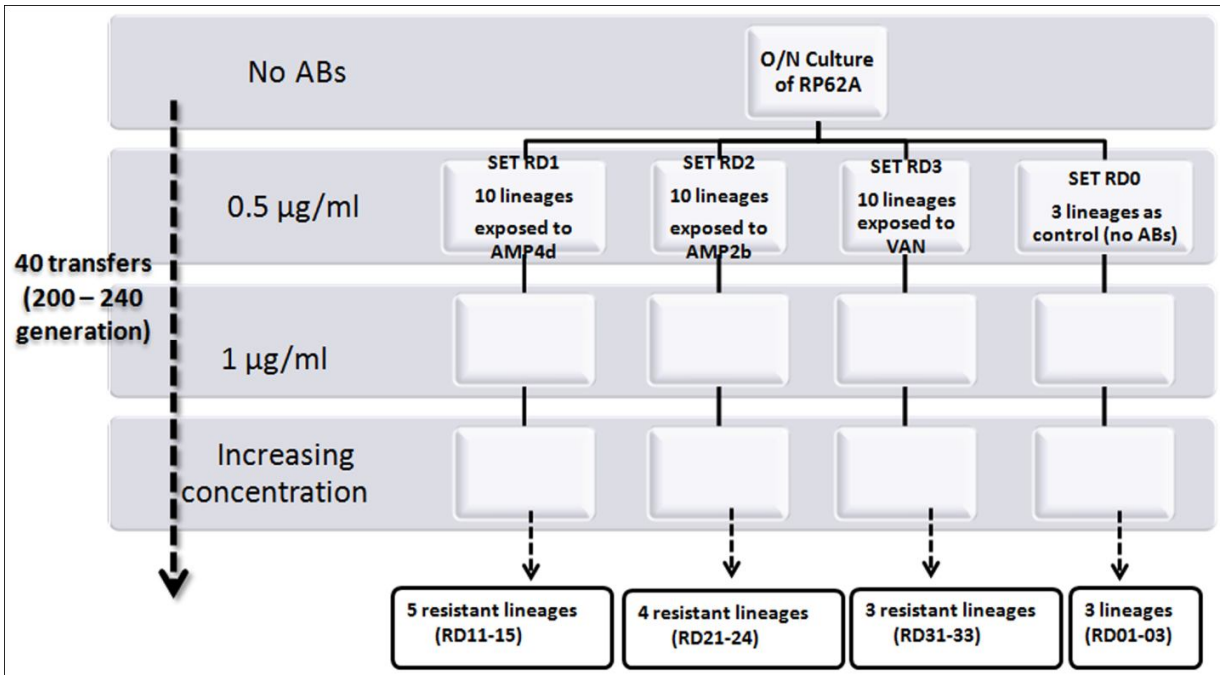


Figure S1. 10 independent lineages of *S.epidermidis*RP62A were transferred in LB media with increasing concentrations of AMP4d**,** AMP2b, or VAN, respectively, for 40 transfers. In the end, 5, 4 or 3 resistant populations were evolved for SET_RD1 (developed from AMP4d), SET_RD2 (developed from AMP2b), or SET_RD 3 (developed from VAN), respectively. As controls, 3 independent lineages were transferred in LB media without antibiotics (SET_RD0).

Figure S2. Overview of the selection experiments (linelage RD14, RD22, RD33 as examples).

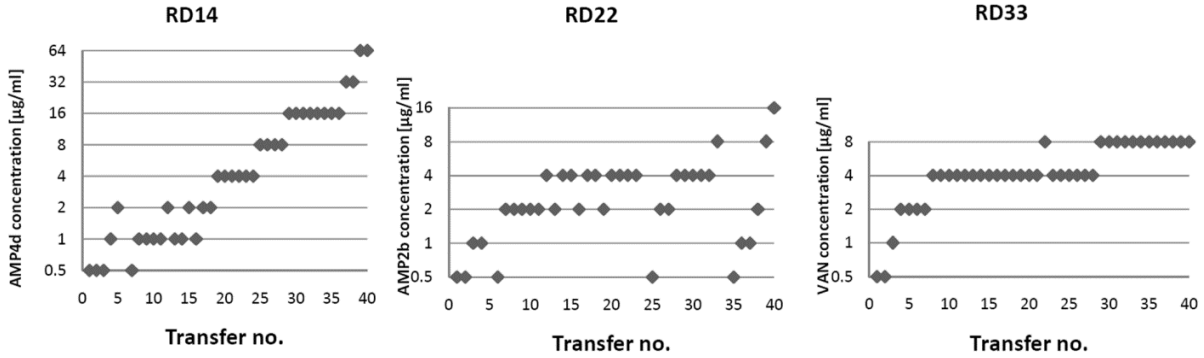
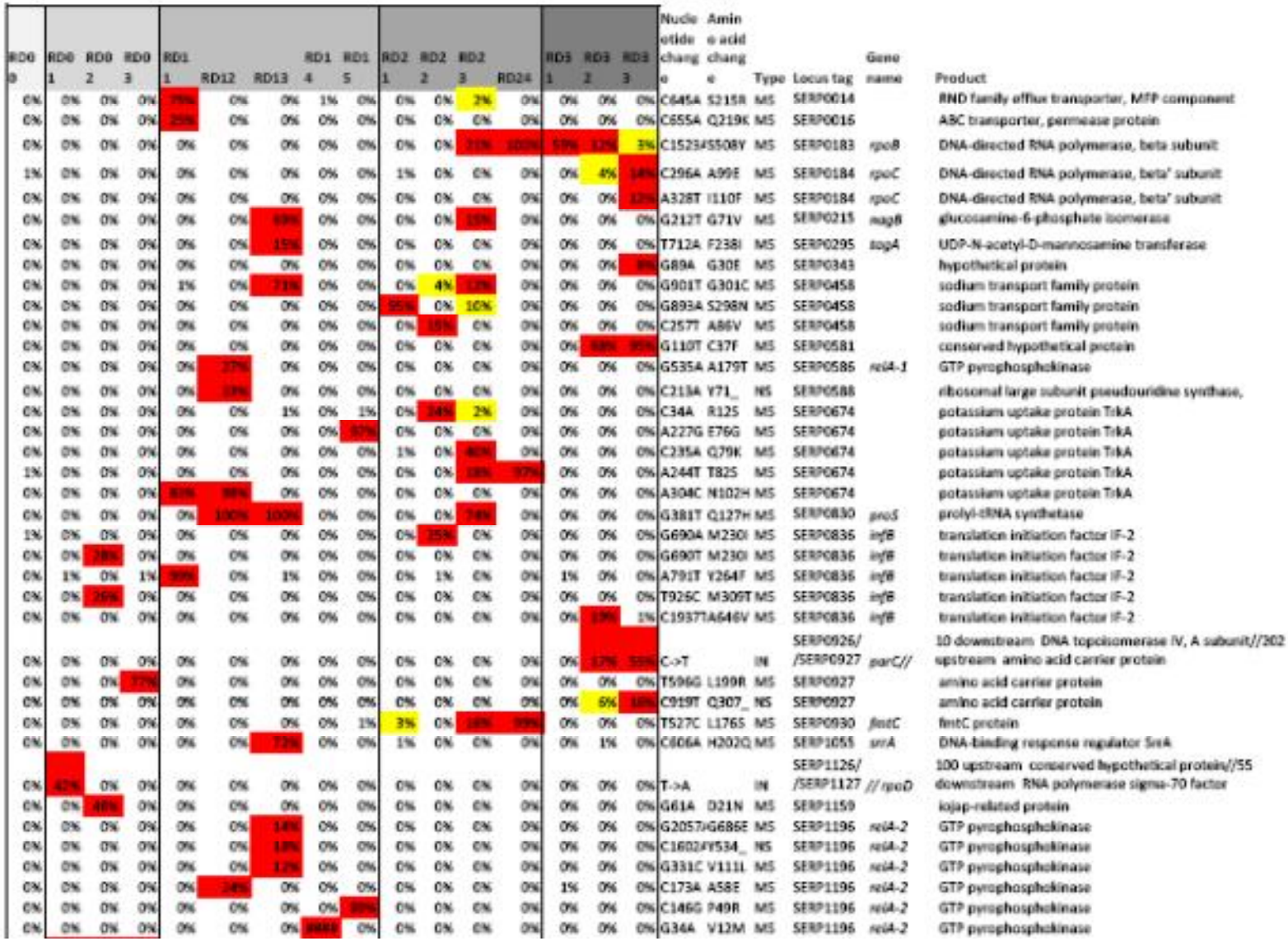


Figure S2. Overview of the selection experiment. No antibiotic was added to the LB media for the first transfer. From passage number 2, a non-inhibitory concentration of antibiotic of 0.5 µg/ml was used (MICs for RP62A with these antibiotics were 2 µg/ml). Then the concentrations were slowly increased. At some transfers such as transfer no. 9 for lineage RD14, the experiment was started from frozen culture sample.

Figure S3. Mutations and parallel evolution



RD0 0	RD0 1	RD0 2	RD0 3	RD1 1	RD12	RD13	RD1 4	RD1 5	RD2 1	RD2 2	RD2 3	RD24	RD3 1	RD3 2	RD3 3	Nucleotide change	Amino acid change	Type	Locus tag	Gene name	Product
0%	0%	0%	0%	75%	0%	0%	1%	0%	0%	0%	2%	0%	0%	0%	0%	C645A	S215R	MS	SERP0014		RND family efflux transporter, MFP component
0%	0%	0%	0%	25%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	C655A	Q219K	MS	SERP0015		ABC transporter, permease protein
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	21%	100%	59%	12%	3%	C1523/5508Y		MS	SERP0183	*rpoB*	DNA-directed RNA polymerase, beta subunit
1%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%	0%	4%	14%	C296A	A99E	MS	SERP0184	*rpoC*	DNA-directed RNA polymerase, beta' subunit
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	12%	A328T	I110F	MS	SERP0184	*rpoC*	DNA-directed RNA polymerase, beta' subunit
0%	0%	0%	0%	0%	0%	69%	0%	0%	0%	0%	15%	0%	0%	0%	0%	G212T	G71V	MS	SERP0215	*nagB*	glucosamine-6-phosphate isomerase
0%	0%	0%	0%	0%	0%	15%	0%	0%	0%	0%	0%	0%	0%	0%	0%	T712A	F238I	MS	SERP0295	*tagA*	UDP-N-acetyl-D-mannosamine transferase
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	8%	G89A	G30E	MS	SERP0343		hypothetical protein
0%	0%	0%	0%	1%	0%	71%	0%	0%	0%	4%	13%	0%	0%	0%	0%	G901T	G301C	MS	SERP0458		sodium transport family protein
0%	0%	0%	0%	0%	0%	0%	0%	0%	95%	0%	10%	0%	0%	0%	0%	G893A	S298N	MS	SERP0458		sodium transport family protein
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	15%	0%	0%	0%	0%	0%	C257T	A86V	MS	SERP0458		sodium transport family protein
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	69%	95%	G110T	C37F	MS	SERP0581		conserved hypothetical protein
0%	0%	0%	0%	0%	27%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	G535A	A179T	MS	SERP0586	*relA-1*	GTP pyrophosphokinase
0%	0%	0%	0%	0%	19%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	C213A	Y71_	NS	SERP0588		ribosomal large subunit pseudouridine synthase,
0%	0%	0%	0%	0%	0%	1%	0%	1%	0%	24%	2%	0%	0%	0%	0%	C34A	R12S	MS	SERP0674		potassium uptake protein TrkA
0%	0%	0%	0%	0%	0%	0%	0%	87%	0%	0%	0%	0%	0%	0%	0%	A227G	E76G	MS	SERP0674		potassium uptake protein TrkA
0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	46%	0%	0%	0%	0%	C235A	Q79K	MS	SERP0674		potassium uptake protein TrkA
1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	18%	37%	0%	0%	0%	A244T	T82S	MS	SERP0674		potassium uptake protein TrkA
0%	0%	0%	0%	83%	88%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	A304C	N102H	MS	SERP0674		potassium uptake protein TrkA
0%	0%	0%	0%	0%	100%	100%	0%	0%	0%	0%	74%	0%	0%	0%	0%	G381T	Q127H	MS	SERP0830	*proS*	prolyl-tRNA synthetase
1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	35%	0%	0%	0%	0%	0%	G690A	M230I	MS	SERP0836	*infB*	translation initiation factor IF-2
0%	0%	28%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	G690T	M230I	MS	SERP0836	*infB*	translation initiation factor IF-2
0%	1%	0%	1%	99%	0%	1%	0%	0%	0%	1%	0%	0%	1%	0%	0%	A791T	Y264F	MS	SERP0836	*infB*	translation initiation factor IF-2
0%	0%	29%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	T926C	M309T	MS	SERP0836	*infB*	translation initiation factor IF-2
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	19%	1%	C1937A	A646V	MS	SERP0836	*infB*	translation initiation factor IF-2
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	17%	55%	C->T		IN	SERP0926/ /SERP0927	*parC//*	10 downstream DNA topoisomerase IV, A subunit//202 upstream amino acid carrier protein
0%	0%	0%	37%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	T596G	L199R	MS	SERP0927		amino acid carrier protein
0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	10%	C919T	Q307_	NS	SERP0927		amino acid carrier protein
0%	0%	0%	0%	0%	0%	0%	0%	1%	3%	0%	18%	39%	0%	0%	0%	T527C	L176S	MS	SERP0930	*fmtC*	fmtC protein
0%	0%	0%	0%	0%	0%	72%	0%	0%	1%	0%	0%	0%	0%	1%	0%	C606A	H202Q	MS	SERP1055	*srrA*	DNA-binding response regulator SrrA
0%	42%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	T->A		IN	SERP1126/ /SERP1127	*//rpoD*	100 upstream conserved hypothetical protein//55 downstream RNA polymerase sigma-70 factor
0%	0%	48%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	G61A	D21N	MS	SERP1159		kojap-related protein
0%	0%	0%	0%	0%	0%	14%	0%	0%	0%	0%	0%	0%	0%	0%	0%	G2057A	G686E	MS	SERP1196	*relA-2*	GTP pyrophosphokinase
0%	0%	0%	0%	0%	0%	18%	0%	0%	0%	0%	0%	0%	0%	0%	0%	C1602A	Y534_	NS	SERP1196	*relA-2*	GTP pyrophosphokinase
0%	0%	0%	0%	0%	0%	12%	0%	0%	0%	0%	0%	0%	0%	0%	0%	G331C	V111L	MS	SERP1196	*relA-2*	GTP pyrophosphokinase
0%	0%	0%	0%	0%	24%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	C173A	A58E	MS	SERP1196	*relA-2*	GTP pyrophosphokinase
0%	0%	0%	0%	0%	0%	0%	0%	35%	0%	0%	0%	0%	0%	0%	0%	C146G	P49R	MS	SERP1196	*relA-2*	GTP pyrophosphokinase
0%	0%	0%	0%	0%	0%	0%	99%	0%	0%	0%	0%	0%	0%	0%	0%	G34A	V12M	MS	SERP1196	*relA-2*	GTP pyrophosphokinase

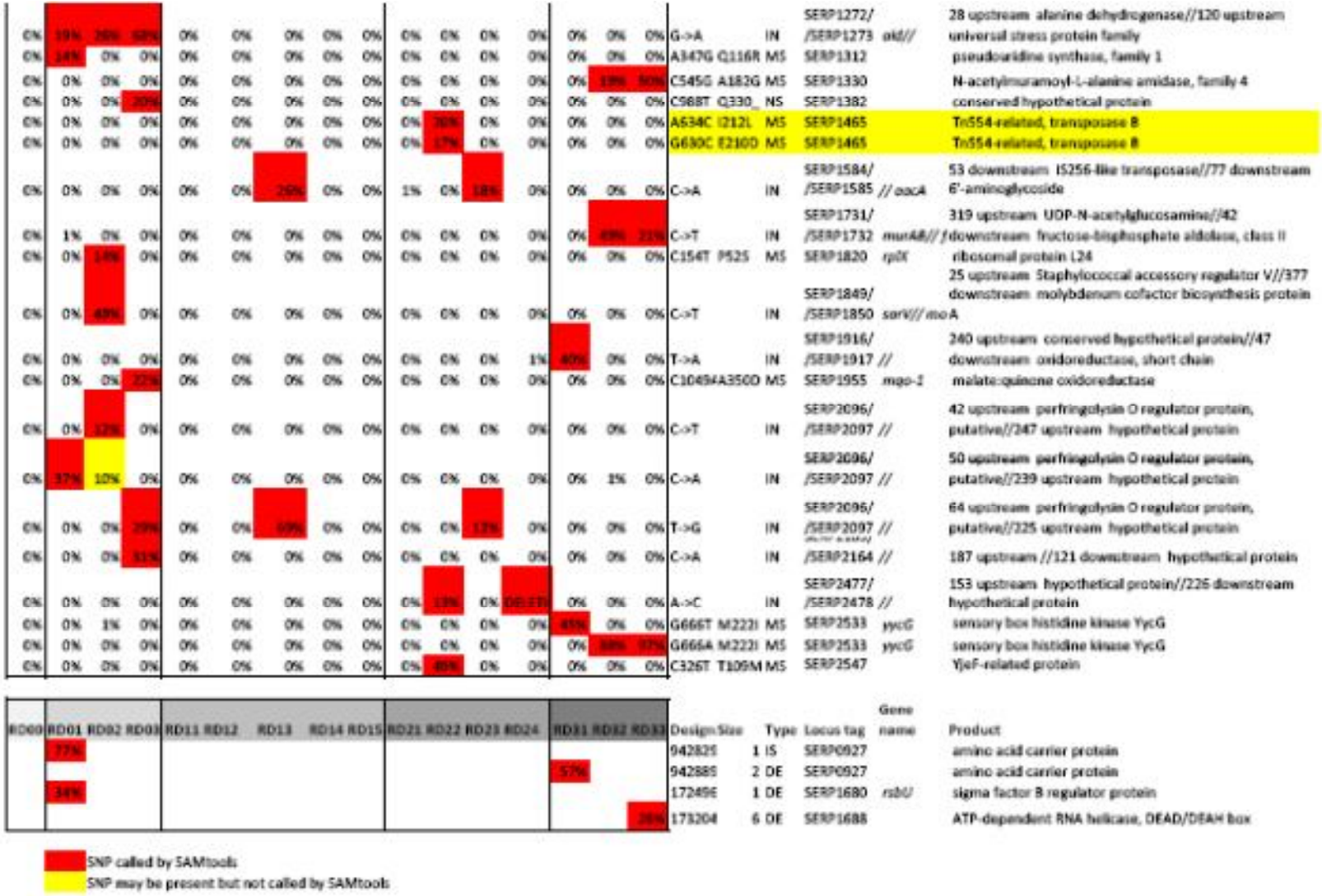


Figure S3. Mutations and parallel evolution. Mutation type: MS, Missense; NS, Nonsense; IN, Intergenic; IS, Insertion; DE, Deletion. The SNP call with with only 8% is in a population with THREE alleles (two mutations); Only RD24 have a 100% deletion(s)

Figure S4. S.epidermidis_nuc_pools (ppGpp study)

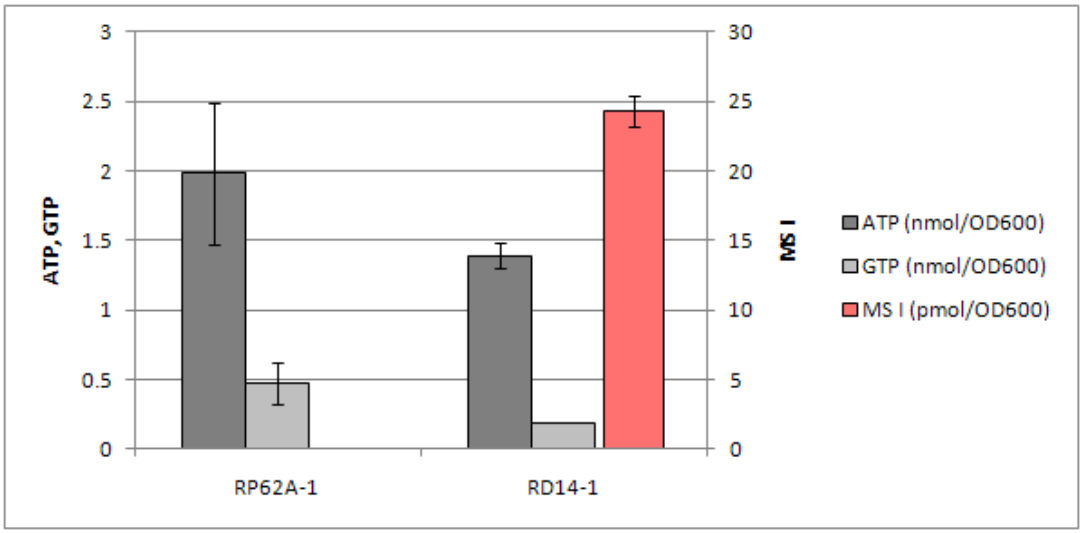


Figure S5. *S.epidermidis* nuc pools-2D