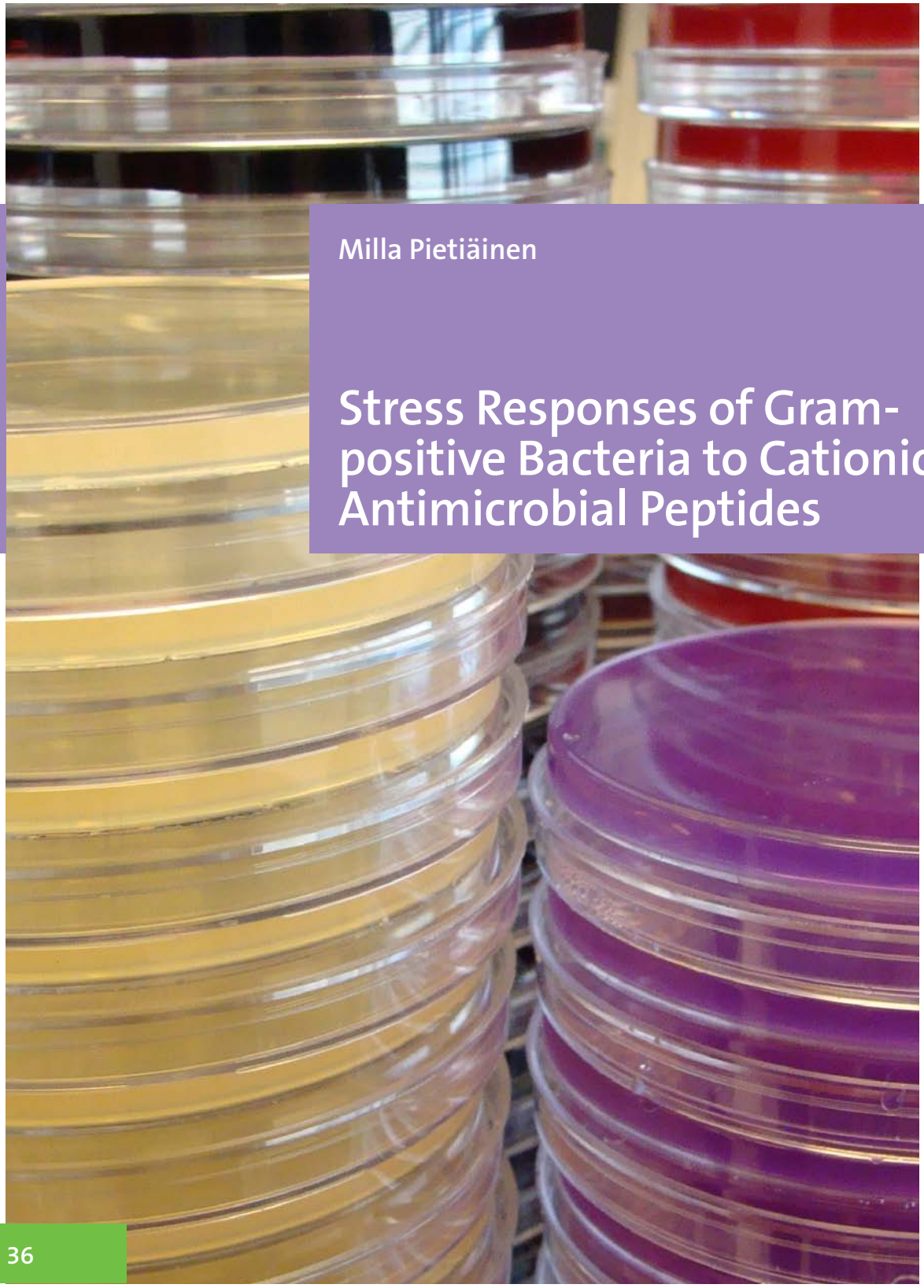
NATIONAL INSTITUTE
FOR HEALTH AND WELFARE



RESEARCH

Milla Pietiäinen

Stress Responses of Gram-positive Bacteria to Cationic Antimicrobial Peptides

RESEARCH 36

Milla Pietiäinen

Stress Responses of Gram-Positive Bacteria to Cationic Antimicrobial Peptides

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in Auditorium 1041, Biocenter 2, Viikinkaari 5, on September 17th, 2010, at 12 noon

National Institute for Health and Welfare, Helsinki, Finland
and
Faculty of Biological and Environmental Sciences, Department of Biosciences, Division of Genetics, University of Helsinki, Finland

NATIONAL INSTITUTE
FOR HEALTH AND WELFARE

Cover Photo: Different bacterial growth media on Petri dishes
(photographed by Lotta Siira)

ISBN 978-952-245-311-2 (printed)
ISSN 1798-0054 (printed)
ISBN 978-952-245-312-9 (pdf)
ISSN 1798-0062 (pdf)

Helsinki University Print
Helsinki, Finland 2010

Supervised by
Professor Matti Sarvas
National Institute for Health and Welfare
Helsinki, Finland

Docent Vesa P. Kontinen
National Institute for Health and Welfare
Helsinki, Finland

Rewieved by
Professor Per Saris
Department of Food and Environmental Sciences
University of Helsinki, Finland

Docent Hannu Saarilahti
Department of Biosciences
University of Helsinki, Finland

Opponent
Professor Jan Maarten van Dijl
University of Groningen
The Netherlands

To my Parents

Abstract

Milla Pietiäinen. Stress Responses of Gram-Positive Bacteria to Cationic Antimicrobial Peptides. National Institute for Health and Welfare (THL), Research 36. 137 pages. Helsinki 2010.
ISBN 978-952-245-311-2 (printed), ISBN 978-952-245-312-9 (pdf)

As the resistance of bacteria to conventional antibiotics has become an increasing problem, new antimicrobial drugs are urgently needed. One possible source of new antibacterial agents is a group of cationic antimicrobial peptides (CAMPs) produced by practically all living organisms. These peptides are typically small, amphipathic and positively charged and contain well defined α-helical or β-sheet secondary structures. The main antibacterial action mechanism of CAMPs is considered to be disruption of the cell membrane, but other targets of CAMPs also exist. Some bacterial species have evolved defence mechanisms against the harmful effects of CAMPs. One of the most effective defence mechanisms is reduction of the net negative charge of bacterial cell surfaces.

Global analysis of gene expression of two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, was used to further study the stress responses induced by different types of CAMPs. *B. subtilis* cells were treated with sublethal concentrations of α-helical peptide LL-37, β-sheet peptide protegrin 1 or synthetic analogue poly-L-lysine, and the changes in gene expression were studied using DNA macroarrays. In the case of *S. aureus*, three different α-helical peptides were selected for the transcriptome analyses: temporin L, ovispirin-1 and dermaseptin K4-S4(1-16). Transcriptional changes caused by peptide stress were examined using oligo DNA microarrays.

The transcriptome analysis revealed two main cell signalling mechanisms mediating CAMP stress responses in Gram-positive bacteria: extracytoplasmic function (ECF) sigma factors and two-component systems (TCSs). In *B. subtilis*, ECF sigma factors σ^W and σ^M as well as TCS LiaRS responded to the cell membrane disruption caused by CAMPs. In *S. aureus*, CAMPs caused a similar stress response to antibiotics interfering in cell wall synthesis, and TCS VraSR was strongly activated. All of these transcriptional regulators are known to respond to several compounds other than CAMPs interfering with cell envelope integrity, suggesting that they sense cell envelope stress in general.

Among the most strongly induced genes were *yxdLM* (in *B. subtilis*) and *vraDE* (in *S. aureus*) encoding homologous ABC transporters. Transcription of *yxdLM* and *vraDE* operons is controlled by TCSs YxdJK and ApsRS, respectively. These TCSs seemed to be responsible for the direct recognition of CAMPs. The *yxdLM* operon was specifically induced by LL-37, but its role in CAMP resistance remained unclear. VraDE was proven to be a bacitracin transporter.

We also showed that the net positive charge of the cell wall affects the signal recognition of different TCSs responding to cell envelope stress. Inactivation of the

Dlt system responsible for the D-alanylation of teichoic acids had a strong and differential effect on the activity of the studied TCSs, depending on their functional role in cells and the stimuli they sense.

Keywords: cationic antimicrobial peptide, stress response, two-component system, sigma factor, transcriptome analysis, *Bacillus subtilis*, *Staphylococcus aureus*

Tiivistelmä

Milla Pietiäinen. Stress Responses of Gram-Positive Bacteria to Cationic Antimicrobial Peptides [Kationisten antimikrobipeptidien aiheuttamat stressivasteet Gram-positiivisissa bakteereissa]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 36. 137 sivua. Helsinki 2010.
ISBN 978-952-245-311-2 (painettu), ISBN 978-952-245-312-9 (pdf)

Bakteerien vastustuskyvystä perinteisiä antibiootteja vastaan on tulossa kasvava ongelma ja uusia antimikrobilääkkeitä tarvitaan pikaisesti. Kationiset antimikrobipeptidit (CAMP) ovat eräs mahdollinen vaihtoehto uusiksi lääkkeiksi. Näitä peptideitä tuottavat lähes kaikki elävät organismit ja niillä on useita tyypillisiä piirteitä kuten pieni koko, amfipaattisuus sekä positiviinen varaus. Lisäksi kyseiset peptidit sisältävät usein niille tyypillisiä α-helikaalisia tai β-levy sekundäärirakenteita. Kationiset peptidit tuhoavat bakteereita pääasiallisesti häiritsemällä niiden solukalvon rakennetta, mutta niillä on myös muita vaikutuskohteita. Jotkin bakteerilajit ovat pystyneet kehittämään puolustumekanismeja antimikrobipeptidejä vastaan. Eräs tärkeimmistä tällaisista mekanismeista on bakteerin pintarakenteiden positiivisen varauksen kasvattaminen.

Erilaisten kationisten antimikrobipeptidien aiheuttamaa stressivastetta ja muutosta geeniekspressiossa tutkittiin kahdessa Gram-positiivisessa mikrobissa, *Bacillus subtilis*- ja *Staphylococcus aureus*- bakteerissa. *B. subtilis* -bakteerisolut käsiteltiin joko LL-37:llä (α-helikaalinen peptidi), protegriini 1:llä (β-levy peptidi) tai synteettisellä analogilla, poly-L-lysiinillä ja niiden aiheuttamia muutoksia geenien ilmenemiseen tutkittiin DNA-makroarray-tekniikkalla. *S. aureus*-bakteerin kohdalla transkriptomianalyysiin valittiin kolme erilaista α-helikaalista peptidiä: temporin L, ovispirin-1 ja dermaseptin K4-S4(1-16). Peptidien aiheuttamia muutoksia transkriptiossa tutkittiin oligo-DNA-mikroarray tekniikkalla.

Transkriptomianalyysit paljastivat kaksi pääasiallista peptidistressiin reagoivaa solun signaalivälityssysteemiä, ECF-sigmafaktorit sekä kaksikomponenttisysteemit (TCS). *B. subtiliksessa* ECF-sigmafaktorit σ^W ja σ^M sekä kaksikomponentti-systeemi LiaRS reagoivat peptidien aiheuttamiin häiriöihin solumembraanissa. *S. aureus*-bakteerissa peptidit aiheuttivat hyvin samankaltaisen stressivasteen kuin soluseinän synteesiä häiritsevät antibiootit aiheuttaen kaksikomponenttisysteemi VraSR:n voimakkaan aktivoitumisen. Kaikki nämä transkription säätelijät reagoivat useisiin erilaisiin solun seinämää häiritseviin yhdisteisiin eli ne todennäköisesti aistivat yleisesti soluseinämän stressitilaa.

Voimakkaimmin indusoituneita geenejä olivat *yxdLM* (*B. subtilis*) ja *vraDE* (*S. aureus*), jotka koodaavat toistensa kaltaisia ABC-kuljettajaproteiineja. *yxdLM* ja *vraDE* operonien transkriptiota säätelee kaksikomponettisysteemit YxdJK ja ApsRS ja ne mitä ilmeisimmin reagoivat spesifisesti antimikrobipeptidien läsnäoloon. *yxdLM* aktivoitui spesifisesti LL-37:n vaikutuksesta, mutta sen rooli peptidi-

resistenssisä jäi epäselväksi. Tulokset osoittivat, että VraDE on basitrasiinin kuljettaja.

Osoitimme myös, että soluseinän positiivinen varaus vaikuttaa soluseinämän stressiä aistivien kaksikomponenttisysteemien signaalin tunnistukseen. Dlt-systeemi on vastuussa teikkohappojen D-alanylaatiosta ja tämän systeemin inaktivaatiolla oli suuri vaikutus tutkittavien kaksikomponenttisysteemien aktiivisuuteen. Vaikutus vaihteli riippuen siitä mikä kunkin kaksikomponenttisysteemin funktio on solussa ja minkälaisia aktivoivia signaaleja ne tunnistavat.

Avainsanat: kationinen antimikrobipeptidi, transkriptomianalyysi, stressivaste, kaksikomponenttisysteemi, sigmafaktori, *Bacillus subtilis*, *Staphylococcus aureus*

Contents

List of original papers

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I **Milla Pietiäinen**, Marika Gardemeister, Maria Mecklin, Soile Leskelä, Matti Sarvas and Vesa P. Kontinen (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: 1577–1592

II Hanne-Leena Hyyryläinen, **Milla Pietiäinen**, Tuula Lunden, Anna Ekman, Marika Gardemeister, Sanna Murtomäki-Repo, Haike Antelmann, Michael Hecker, Leena Valmu, Matti Sarvas and Vesa P. Kontinen (2007): The density of negative charge in the cell wall influences two-component signal transduction in *Bacillus subtilis*. Microbiology 153: 2126–2136

III **Milla Pietiäinen**, Patrice François, Hanne-Leena Hyyryläinen, Manuela Tangomo, Vera Sass, Hans-Georg Sahl, Jacques Schrenzel and Vesa P. Kontinen (2009): 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 10:429

Abbreviations

aa	amino acid
ABC	ATP-binding cassette
AmyQ	α-amylase of *Bacillus amyloliquefaciens*
ATP	adenosine triphosphate
CAMP	cationic antimicrobial peptide
D-ala	D-alanyl ester
ECF σ factor	Extracytoplasmic function sigma factor
EGTA	ethylene glycol tetraacetic acid
GlcNAc	N-acetylglucosamine
HK	histidine kinase
IPTG	isopropyl-β-D-1-thiogalactopyranoside
LPG	lysylphosphatidylglycerol
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MATE	multidrug and toxic compound extrusion
MDR	multidrug resistance
MFS	major facilitator
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MurNAc	N-acetylmuramic acid
ORF	open reading frame
PBP	penicillin binding protein
PCR	polymerase chain reaction
PG	peptidoglycan
PG-1	protegrin 1
PLL	poly-L-lysine
PMF	proton motive force
qRT-PCR	quantitative real-time reverse transcription PCR
RND	resistance-nodulation-cell division
RR	response regulator
SMR	small MDR family
TA	teichoic acid
TCS	two-component system
VISA	vancomycin intermediate-resistant *S. aureus*
VRSA	vancomycin-resistant *S. aureus*
WTA	wall teichoic acid

.

1 Introduction

Cationic antimicrobial peptides (CAMPs) are produced by practically all living organisms, from the unicellular bacteria to the higher organisms in plant and animal kingdoms, and they act as an important part of innate immunity. The ever-growing problem of bacterial resistance to antibiotics used in health care has raised CAMPs as a subject of great scientific interest and it is hoped that they will provide potential new antimicrobial drugs. So far, over 1000 natural CAMPs have been identified and extensive research has also been performed to produce *de novo* designed peptides (Hale & Hancock, 2007).

Considering the long co-existence of pathogenic bacteria and CAMPs produced by host cells, CAMPs have retained their antimicrobial effectiveness remarkably well. However, some specific bacterial counter-measures against CAMPs have evolved as a result of dynamic co-evolutionary processes (Peschel & Sahl, 2006). One of the generally accepted killing mechanisms of CAMPs is that they attack the cell membrane of bacterial cells. The close monitoring and maintenance of cell envelope integrity is crucial for the survival of bacteria in changing environmental conditions, and especially in the presence of substances that interfere with the cell envelope (Jordan *et al.*, 2008).

In this thesis, two bacterial species belonging to the *Firmicutes* branch of Gram-positive bacteria were exposed to stress caused by CAMPs of human and animal origin, and the effects on gene expression were analyzed. Despite the phylogenetic relationship between the selected bacterial species, *Bacillus subtilis* and *Staphylococcus aureus*, they represent microbes living in completely different ecological niches. This gives an opportunity to identify the universal stress responses against CAMPs among Gram-positive bacteria, as well as determine the special features reflecting the demands of different habitats for the bacterial species. Understanding the bacterial mechanisms of resistance against compounds interfering with the cell envelope might help in identifying new targets for future antimicrobials.

B. subtilis is an apathogenic endospore-forming rod-shaped bacterium living in the soil. The majority of classical antibiotics are produced by microorganisms of the soil biosphere (Berdy, 2005), making soil an especially challenging habitat. The production of antimicrobial compounds is thought to give a competitive advantage against other microorganisms struggling for the limited resources. For example, more than two dozen antimicrobial agents with variable structures are produced by different *B. subtilis* species (Stein, 2005). Bacterial species of the genus *Bacillus* are also important industrial enzyme producers. Following the development of advanced molecular genetic tools, *B. subtilis* has become the best characterized species of the genus *Bacillus* and is often referred as the model Gram-positive bacterium in the field of molecular bacteriology.

S. aureus is a facultative anaerobic coccus belonging to the normal microbial flora of humans and animals. However, as an opportunistic pathogen, *S. aureus* is one of the most important bacteria causing diseases in humans. The anterior nares are the primary ecological niches of *S. aureus*, and nasal carriage has been identified as a major risk for infections, especially in clinical settings (Corbella *et al.*, 1997; Kluytmans *et al.*, 1995; von Eiff *et al.*, 2001). Resistance against several host-produced CAMPs is suggested to be a prerequisite for the ability of *S. aureus* to colonize the skin and other epithelial tissues (Foster, 2005; Peschel & Sahl, 2006). *S. aureus* can cause a variety of diseases, including skin and soft tissue infections, endocarditis, osteomyelitis, septic arthritis and pneumonia. In addition, *S. aureus* is often associated with catheter-related infections and postoperative wound infections. A wide range of virulence factors produced by *S. aureus* have been identified. These include both cell surface-associated proteins important in bacterial adhesion to host cells as well as secreted proteins such as proteases, lipases and several exo- and enterotoxins (Dinges *et al.*, 2000; Foster & Hook, 1998).

The emergence of antibiotic resistant strains of *S. aureus* has become an increasing problem. Practically all *S. aureus* strains isolated today produce β-lactamase and are therefore resistant to β-lactam antibiotics such as penicillin. A number of semisynthetic penicillins able to withstand bacterial β-lactamase, referred to as staphylococcal penicillins, were developed at the beginning of the 1960s. Soon after the first staphylococcal penicillin, methicillin, was introduced into clinical practice, the first methicillin-resistant *S. aureus* isolates (MRSA) were described (Jevons, 1961). Today, MRSA is the most commonly identified antibiotic-resistant pathogen worldwide. Methicillin resistance is provided by the mobile chromosomal cassette SCC*mec* carrying the *mecA* gene encoding an altered penicillin-binding protein called PBP2´or PBP2A (Hartman & Tomasz, 1984; Matsuhashi *et al.*, 1986; Ubukata *et al.*, 1985). Different types of SCC*mec* cassettes can also contain genes providing resistance against several antibiotics other than β-lactams. The glycopeptide antibiotic vancomycin has been the primary drug used in the treatment of MRSA infections. However, the first vancomycin-intermediate MRSA strain (VISA) with reduced susceptibility to vancomycin due to the thickened cell wall was isolated in 1996 (Hiramatsu *et al.*, 1997; Sieradzki & Tomasz, 2003). The feared transfer of the plasmid-encoded vancomycin resistance gene *vanA* from *Enterococcus feacalis* took place, as the first clinical vancomycin-resistant MRSA strain (VRSA) was isolated in 2002 in the United States (Chang *et al.*, 2003).

2 Review of the literature

2.1 Cationic antimicrobial peptides – Host defence peptides

The existence of antimicrobial compounds in blood, secretions and various tissues was discovered at the beginning of the last century. The isolated compounds included bacteriolytic substances such as lysozyme, basic antimicrobial proteins and basic linear polypeptides. Even though the identity of the peptides was unclear, the idea of the interaction of these peptides with negatively charged cell surfaces and the disruption of important cell functions was soon established (reviewed by Brogden, 2005). The first isolated and purified cationic antimicrobial peptides included insect cecropins (Steiner *et al.*, 1981), amphibian magainins (Zasloff, 1987) and mammalian defensins (Ganz *et al.*, 1990), and several hundreds of antimicrobial peptides have since been identified. For long time, membrane disruption was thought to be the only or at least the major mechanism of action of these peptides, but recent studies have clearly shown that many of these peptides have alternative ways of causing cell death, for example through internal targets (Brogden, 2005; Hale & Hancock, 2007; Yeaman & Yount, 2003; Yount *et al.*, 2006). Several further roles of these peptides in addition to killing microbes have been discovered, such as in the immune response, wound healing and angiogenesis (Mookherjee & Hancock, 2007).

It has been suggested that the term 'cationic antimicrobial peptide' should only be used in cases where microbial killing has been proven to be the main function of the particular peptide. Otherwise, the term 'host defence peptide' would be more accurate (Hale & Hancock, 2007). For clarity, the term cationic antimicrobial peptide (CAMP) is used throughout the text in this thesis.

2.1.1 Diversity of antimicrobial peptides

Cationic antimicrobial peptides play a crucial role in innate immunity in all biological kingdoms and are produced in many tissues and cell types. CAMPs have a very broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, protozoa as well as some enveloped viruses such as influenza A and human immunodeficiency virus (HIV-1) (Jenssen *et al.*, 2006). CAMPs are an extremely diverse group of peptides having very little homology in their primary sequences, but they share some common features important to their function. CAMPs are generally 12-50 amino acids long and despite their small size they are usually gene-encoded and expressed either constitutively or inducibly. All CAMPs are proteolytically spliced from larger precursors including a signal sequence for secretion, and may go through post-translational modifications such as glycosylation, carboxy-terminal amidation, halogenization or cyclization (Zasloff, 2002). Their amino acid sequences often contain the basic amino acids lysine or arginine, giving the net positive charge characteristic of antimicrobial peptides, and

a substantial proportion of the hydrophobic residues alanine, leucine, phenylalanine or tryptophan. CAMPs are usually divided into subgroups on the basis of their secondary structure. These structural groups include α-helical linear peptides, β-sheet peptides, peptides containing extended structures rich in certain amino acids and looped peptides (Hancock, 2001) (Figure 1). The α-helical and β-sheet molecules are by far the most common antimicrobial peptides in nature. In addition to these "classical" groups of small peptides, polypeptides or larger proteins have also been shown to have antimicrobial activity. In addition, bacterial cells can produce CAMPs, often referred as bacteriocins. It is notable that anionic antimicrobial peptides also exist, as exemplified by human dermcidin (Schittek *et al.*, 2001) and maximin H5 from amphibians (Lai *et al.*, 2002).

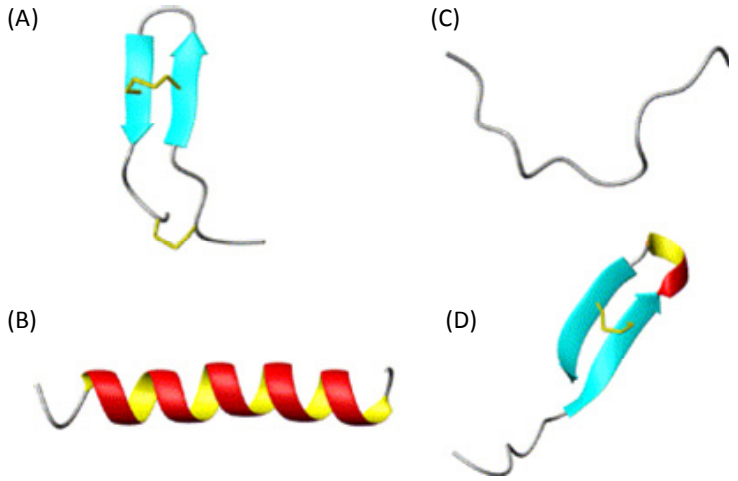


***Figure 1. Structural classes of CAMPs*:** (A) β-sheet peptides, tachyplesin I; (B) α-helical peptides, magainin 2; (C) peptides with extended structures, indolicidin; (D) looped peptides, thanatin. Adapted from Powers and Hancock (2003).

Linear α-helical peptides

Linear α-helical peptides comprise one of the two largest groups of antimicrobial peptides and include several hundreds of peptides present in both invertebrates and vertebrates (Bulet *et al.*, 2004). The skin of a variety of frog species is an especially rich source of α-helical peptides. For example, 50 and 76 peptides belonging to the dermaseptins and temporins, respectively, have been characterized to date (Mahalka & Kinnunen, 2009; Nicolas & El Amri, 2009). Typical for the peptides of both families is a highly variable antimicrobial region and a conserved preproregion of the precursors. Although β-sheet peptides are the most abundant peptides in mammals, several α-helical peptides belonging to cathelicidin family have also been found (Bals & Wilson, 2003; Bulet *et al.*, 2004; Gennaro & Zanetti, 2000; Zanetti,

2004). Cathelicidins are derived from prepropeptides, having a well-conserved N-terminal propeptide segment. Several different cathelicidins are usually produced by an individual mammalian species, the exception being humans, which express only one cathelicidin, LL-37. LL-37 has broad antimicrobial and immunomodulatory activity and is primarily produced by phagocytic leukocytes and epithelial cells (Durr *et al.*, 2006).

α-Helical peptides are usually 12-25 residues long and the α-helices often contain a slight bend in the center of the molecule that might have a role in suppressing haemolytic activity (Zhang *et al.*, 1999). Crucial for their function seems to be the lack of structure in aqueous solutions, but an inducible structure when interacting with the hydrophobic environment of membranes (Chen *et al.*, 2005b; Lee *et al.*, 2003). Another important factor is the ability to self-associate (i.e. the ability to oligomerize/dimerize). Several studies have shown that self-association of α-helical peptides in the membrane-bound state correlates with antimicrobial activity (Strahilevitz *et al.*, 1994), but self-association in the aqueous environment may in fact interfere with antimicrobial activity (Chen *et al.*, 2005b; Lee *et al.*, 2003).

β-sheet peptides

The second class of antimicrobial peptides consists of a highly diverse group of peptides at the level of primary structure. β-Sheet peptides contain cysteine residues and form more restrained conformations characterized by the presence of a variable number of antiparallel β-strands stabilised by a series of intramolecular disulphide bonds. Depending on the quantity of cysteine residues and the overall length of the peptide, they can adopt either a β-sheet conformation with triple strands, as in the case of most verterbrate defensins, or a β-hairpin structure seen, for example, in porcine protegrins. Larger peptides may also contain minor helical segments, as in invertebrate defensins and mammalian β-defensins (Bulet *et al.*, 2004). The number of disulphide bridges usually varies from one to four; for example, bactenecin from bovine neutrophils contains one, protegrin-1 from porcine leukocytes and tachyplesins from horseshoe crabs two, mammalian and insect defensins three and plant defensins up to four disulphide bonds (Bulet *et al.*, 2004; Carvalho Ade & Gomes, 2009; Ganz & Lehrer, 1995; Kokryakov *et al.*, 1993; Lehrer, 2004; Romeo *et al.*, 1988). The anti-fungal peptide mytimicin from the blue mussel contains as many as 12 cysteine residues forming intramolecular disulphide bridges (Charlet *et al.*, 1996). Recently, a common motif present in all cysteine-stabilized antimicrobial peptides was discovered. Characteristic of this γ-motif is two cysteine-stabilized antiparallel β-sheets and the distribution of basic residues in the poles of the motif (Yeaman & Yount, 2007; Yount & Yeaman, 2004).

Defensins are one of the most important groups of CAMPs in mammals. They are divided into three sub-groups, α-, β- and θ–defensins. The classification of α- and β- defensins is based on the peptide precursor and gene structures, as well as the placement and disulphide pairings of the six conserved cysteine residues. α-Defensins are mainly produced constitutively by neutrophils, macrophages and intestinal Paneth cells, whereas β-defensins are inducibly expressed in epithelial

tissues (Selsted & Ouellette, 2005). The structure of θ-defensins is exceptional among mammalian CAMPs, resulting from the head-to-tail cyclization of the peptide backbone. θ-Defensins are produced from mutated α-defensin genes (Leonova *et al.*, 2001; Tang *et al.*, 1999), and they are present in several species of Old World monkeys and orangutans, but not in humans (Nguyen *et al.*, 2003).

Like α-helical peptides, β-sheet peptides are also amphipathic, but in contrast to α-helical peptides they often exist as dimers in aqueous solutions. The mechanisms by which these kinds of peptides interact and cause membrane disruption are still poorly understood. It was assumed that intramolecular disulphide bridges are essential for the antimicrobial activitity, but it seems that their main function is to protect peptides from proteolysis (Selsted & Ouellette, 2005).

Extended peptides rich in certain amino acids

The class of peptides with extended structures contains peptides lacking classical secondary structures due to their unusual amino acid composition, rich in one or more specific amino acids. For example, in the case of human salivary histatins, the peptides are rich in histidin residues, whereas indolicin from bovine neutrophils has a high content of tryptophan and proline (Oppenheim *et al.*, 1988; Selsted *et al.*, 1992). The role of membrane disruption as a mechanism of action in the case of these peptides has not been completely elucidated. Model membrane studies with indolicidin have shown that it is not effectively translocated across membranes, and it was also unable to completely depolarize the cytoplasmic membrane of *E. coli* and *S. aureus* (Friedrich *et al.*, 2000; Wu *et al.*, 1999). Histatins seem to have intracellular targets (Helmerhorst *et al.*, 1999).

Looped peptides

In contrast to other antimicrobial peptides, looped peptides cannot form amphipathic structures due to their proline-arginine rich sequence. The looped structure is formed by a single disulphide, amide or isopeptide bond (Powers & Hancock, 2003). It is thought that members of this group might also have other targets than the cell membrane. For example, the looped peptide thanatin does not induce changes in membrane permeability and D- and L- enantiomers possess different kinds of antimicrobial activity, suggesting an involment of specific receptor molecules (Fehlbaum *et al.*, 1996). Lantibiotics produced by Gram-positive bacteria are also often considered to belong to the class of looped peptides. These peptides contain small ring structures enclosed by a thioether bond (see below).

Protein-derived peptides

In addition to the classical small antimicrobial peptides, considerably larger polypeptides and proteins have been demonstrated to have antimicrobial activity. These proteins are usually proteolytically cleaved into several smaller fragments, some having a similar composition and structure to CAMPs. For example, lactoferricin derived from iron-binding glycoprotein lactoferrin present in mammalian milk and other fluid secretions has been reported to have direct antimicrobial activity (Gifford *et al.*, 2005). Another example is buforin I from the Asian toad, which is produced proteolytically from the histone protein H2A

normally associated with the nucleosome (Kim *et al.*, 1996). Peptides derived from proteins of the complement cascade also appear to have antibacterial activity (Nordahl *et al.*, 2004).

Chemokines are important immunomodulatory signalling molecules, but there is clear evidence indicating that they can also have a direct antimicrobial function. More than 30 chemokines have been reported to have either direct antibacterial or antifungal activity. These chemokines, often referred to as kinocidins, contain three structurally and functionally different domains: an N-terminal unstructured region responsible for the chemotactic trait, a central domain constructed by a triple-stranded β-sheet and a C-terminal α-helical domain contributing to antimicrobial action (Yount *et al.*, 2006). A body of evidence suggests that the α-helical domain may be cleaved from the parent protein in order to form a functional microbicide (Bjorstad *et al.*, 2005). Moreover, kinocidins contain the γ-core motif present in variety of disulphide-stabilized antimicrobial peptides, suggesting a close evolutionary relationship between kinocidins and antimicrobial peptides (Yount *et al.*, 2006).

Bacteriocins

Bacteriocins are antimicrobial peptides or proteins mainly synthesised by Gram-positive bacteria. The fundamental difference between bacteriocins and conventional antibiotics is that bacteriocins are gene-encoded and ribosomally synthesised. Based on their primary structure, bacteriocins are classified into three groups: class I, class II and class III bacteriocins (reviewed by Hechard & Sahl, 2002).

The class I bacteriocins include peptides that undergo extensive post-translational modifications such as the dehydration of serines and threonines to didehydroalanine and didehydrobutyrine, respectively, and (methyl) lanthione ring formation. Due to the presence of lanthione rings, peptides belonging to class I bacteriocins are referred to as lantibiotics. Lantibiotics can be further divided into group A and group B lantibiotics. Group A lantibiotics are linear, amphipathic and positively charged peptides, whereas group B consists of globular peptides. Nisin is the best characterized group A lantibiotic (Fig. 2), but this group also includes other extensively studied peptides such as subtilin, epidermin, gallidermin, Pep5 and lacticin481 (reviewed by Hechard & Sahl, 2002; Lubelski *et al.*, 2008). Lantibiotics are effective against a wide range of bacterial species and it seems that the killing of bacterial cells is often achieved via specific receptors. For example, nisin interacts with lipid II, which is a precursor in bacterial cell wall synthesis (Breukink & de Kruijff, 1999; Breukink *et al.*, 1999).

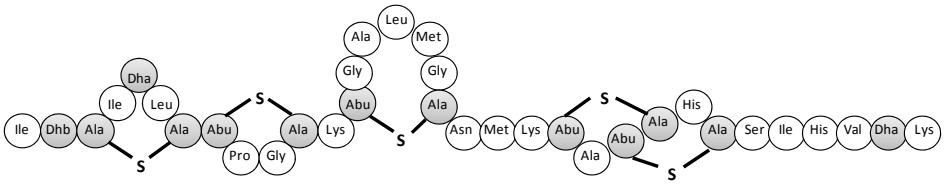


Figure 2. Structure of lantibiotic nisin. Dha = dehydroalanine, Dhb = dehydrobutyrine, Abu = aminobutyric acid, Ala-S-Ala = lanthionine, Abu-S-Ala = 3-methyllanthionine. Adapted from Hechard and Sahl (2002).

The class II bacteriocins are unmodified, cationic and hydrophobic peptides causing membrane permeabilisation in sensitive bacteria. In contrast to the class I bacteriocins, the class II bacteriocins are only effective against a narrow range of bacterial species that is limited to related species or strains of the producing bacteria. Pediocin PA-1 is one of the most extensively studied class II bacteriocins and it is postulated to cause the leakage of K+ cations from bacterial cells (Bhunia 1991). Class III includes large bacteriocins with poorly characterized mechanisms of action.

2.1.2 Antibacterial mechanism of action

The exact action mechanism of CAMPs has still not been established. For a long time, the sole mechanism was thought to be the interactions of CAMPs with the cytoplasmic membrane, causing increased membrane permeability and leading to the leakage of cytoplasmic components. However, increasing evidence suggests that alternative or additional targets also exist (Hale & Hancock, 2007; Yeaman & Yount, 2003). Studies supporting this theory have shown that some peptides can cause cell death without significant membrane perturbation. Based on this knowledge, it has even been proposed that CAMPs should be divided into two functional groups: membrane-disruptive and membrane-nondisruptive peptides (Powers & Hancock, 2003). This distinction is not completely clear, since peptides that attack membranes of some species may be membrane-nondisruptive in other species. Furthermore, many peptides have a multifunctional role, affecting both cell membranes as well as internal targets. However, whatever the actual target of peptides, the ability to interact with lipid bilayers is crucial for their action.

Structural determinants of antimicrobial activity

The most important features of CAMPs concerning their action against microbes are charge, amphipathicity and hydrophobicity. For simplicity, they are often considered as individual features, but *in vivo* they cannot be separated from each other.

As the target of CAMPs is the negatively charged bacterial membrane, it is not surprising that an increased positive net charge strongly correlates with the antimicrobial activity of CAMPs to a certain extent (Yeaman & Yount, 2003). On the other hand, cationicity also has a strong correlation with the toxic effects of CAMPs. For example, Ovispirin-1 is a peptide with a positive charge of +8 and is

highly toxic to mammalian cells (Sawai *et al.*, 2002). The majority of native CAMPs have a net charge ranging from +4 to +6, which may represent an optimal charge for activity (Tossi *et al.*, 2000).

Studies conducted with modified peptides have shown that within a certain range, the addition of positively charged residues can increase the efficacy of peptides (Tossi *et al.*, 2000). Studies with maganin 2 analogues demonstrated that an increase in the charge from +3 to +5 resulted in increased antibacterial activity against both Gram-positive and negative bacteria. A further increase in the charge to +6 and +7 caused a loss of antimicrobial activity and led to increased haemolytic activity (Dathe *et al.*, 2001). A similar pattern was observed in a study on the α-helical amphipathic peptide L-V13K (Chen *et al.*, 2007), in which a systematic increase in the charge from +4 to +8 made V13K analogues more active against bacteria, while having a low level of haemolytic activity. A further increase in the positive charge to +9 and +10 had a dramatic effect on the haemolytic activity. Contradictory results have also been presented, in which modulation of the net charge of cecropin/melittin analogues from +5 to +9 had no significant effect on antimicrobial activity (Scott *et al.*, 1999).

Nearly all CAMPs form amphipathic structures upon interaction with target membranes. In these structures, the positively charged residues are segregated on the hydrophilic face and the hydrophobic residues lie on the opposite face of the peptides. This kind of amphipathic structure can be achieved through different protein conformations. It is clear that both amphipathicity and hydrophobicity are necessary for the function of CAMPs. The positively charged polar face helps the peptide to reach the target membrane through electrostatic bonding between CAMPs and structures on the bacterial surface, and the nonpolar face of peptides then allows insertion into the lipid bilayer through hydrophobic interactions (Powers & Hancock, 2003; Yeaman & Yount, 2003).

Both amphipathicity and hydrophobicity influence peptide activity towards negatively charged membranes of bacterial cells, but they may have an even more profound effect on neutral membranes of eukaryotic cells. A high degree of amphipathicity is correlated with increased toxicity toward cells composed of neutral phospholipids (Dathe & Wieprecht, 1999). Peptide hydrophobicity is approximately 50% for most antimicrobial peptides. Hydrophobicity is required for effective membrane permeabilization, but increased hydrophobicity is also strongly correlated with eukaryotic cell toxicity and the loss of antimicrobial activity (Chen *et al.*, 2007; Yeaman & Yount, 2003).

Initial peptide interactions with the cell envelope

Whether the final target of a peptide is the cell membrane or some intracellular component, it has to find its way through the cell envelope. Some bacterial species may form a dense outermost layer under certain environmental conditions. This capsular layer or glycocalyx has an overall net negative charge due to its acidic constituents, including polysaccharides such as polyribitol phosphate or dextran sulphate (Yount *et al.*, 2006). It has been suggested that one function of capsular

polysaccharides may be the segregation and inactivation of CAMPs before they reach the cell membrane (Campos *et al.*, 2004; Yeaman & Yount, 2003).

It is generally accepted that peptides are first attracted to bacterial surfaces via electrostatic bonding. Gram-positive organisms are surrounded by a relatively thick peptidoglycan (PG) layer containing teichoic or teichuronic acids. In Gram-negative bacteria, the cell wall consists of a thin peptidoglycan layer surrounded by an outer membrane layer in which acidic lipopolysaccharide (LPS) predominates. In both cases, the anionic outer layer presents a strong attractant to negatively charged peptides. In fact, there is increasing evidence that the cell wall itself could be the primary target of some peptides, and the manner in which they interfere with the assembly or integrity of the cell wall may even resemble the mechanism of cell wall-active β-lactam antibiotics (Ginsburg, 2004; Yount *et al.*, 2006). The best characterized examples of peptides interfering with peptidoglycan assembly are the lantibiotics nisin and mersacidin, which directly target lipid II (Brotz *et al.*, 1998a; Brotz *et al.*, 1998b). Inhibition of peptidoglycan synthesis is also found in Gram-negative microbes. For example, seminial plasmin from bovine seminal plasma inhibits *E. coli* cell wall assembly (Chitnis & Prasad, 1990).

A strong correlation between the net positive charge of peptides and membrane binding activity has been demonstrated in several studies (Bessalle *et al.*, 1992; Dathe *et al.*, 2001; Matsuzaki *et al.*, 1997; Vaz Gomes *et al.*, 1993). These electrostatic forces act over relatively long distances, and it has been demonstrated that lysine and arginine form particularly strong electrostatic bonds with phosphate groups in the lipid bilayer (Mavri & Vogel, 1996). Cationic peptides are suggested to traverse through the outer cell membrane of Gram-negative bacteria by a mechanism termed "self-promoted uptake" (Hale & Hancock, 2007; Hancock, 1997). According to this model, the initial action of the peptide involves the competitive replacement of LPS-associated divalent cations stabilizing the outer membrane. This is due to the higher affinity of peptides for negatively charged LPS than native divalent cations such as Mg^{2+} and Ca^{2+} (Jenssen *et al.*, 2006). This causes instability in the outer membrane, allowing the translocation of peptides through the lipid bilayer.

Studies with D- and L-enantiomers of native and model peptides have shown that both enantiomers exhibit equivalent antimicrobial activities, leading to the conclusion that most peptides interact with membranes without any specific receptors being involved (Bland *et al.*, 2001; Chen *et al.*, 2006; Wade *et al.*, 1990). On the other hand, there are several important exceptions to this generalization. One of the best studied examples is the lantibiotic nisin. It recognizes and specifically binds to Lipid II, a membrane-anchored cell wall precursor essential for bacterial PG synthesis, and uses it as a "docking molecule" to form pores in membranes in a targeted manner with high efficiency (Breukink *et al.*, 1999; Brotz *et al.*, 1998b). In the absence of Lipid II, nisin can also bind to anionic lipids and insert itself between the phospholipid groups, but this is only observed in model systems with micromolar concentrations compared to the nanomolar concentrations needed *in vivo* (Breukink & de Kruijff, 2006). Some other specific peptide binding sites might also exist; for example, tachyplesin has been shown to have a specific affinity for

LPS (Hirakura *et al.*, 2002). Peptides with non-equivalent activities for native all-L peptides versus their all-D enantiomers have also been observed, suggesting receptor-type interactions (Fehlbaum *et al.*, 1996; Vunnam *et al.*, 1997).

Membrane-disruptive mechanisms of cationic antimicrobial peptides

The initial binding events between CAMPs and the cell membrane are driven by electrostatic forces, followed by conformational phase transition and the insertion of peptides into the membrane. The amphipathic nature of peptides facilitates insertion at the interface of the hydrophilic head groups and hydrophilic fatty acyl chains of the membrane phopholipids (Powers & Hancock, 2003; Yeaman & Yount, 2003). Membrane disruption only follows if a sufficient amount of peptide accumulates in the cell membrane, a phenomenon referred to as the "threshold concentration". The concentration needed for membrane dysfunction depends on several factors, including the biochemical properties of a particular peptide and the phospholipid composition, fluidity or size of the head groups (Lee *et al.*, 2005; Yang *et al.*, 2000). At low peptide-to-lipid ratios, inactive peptides are embedded parallel to the membrane surface, causing stretching of the membrane. After the critical peptide concentration is reached, peptides shift to the active state and are orientated perpendicular to the membrane, causing the pore formation (Chen *et al.*, 2003; Heller *et al.*, 1998; Ludtke *et al.*, 1994; Sharon *et al.*, 1999; Yang *et al.*, 2000).

Based on studies with artificial membranes, three separate permeabilization models have been proposed (Fig. 3). In the barrel-stave model, a variable number of helical peptide molecules are inserted into the membrane, forming a barrel-like bundle around a central pore (Ehrenstein & Lecar, 1977). The hydrophilic surfaces of aggregated peptides form the inner pore lining, while the hydrophobic peptide regions face towards the acyl chains of the membrane. Although the barrel-stave mechanim is the oldest model presented, only a few peptides, such as alamethicin, have been shown to cause pore formation in this manner (Beven *et al.*, 1999; Sansom, 1991; Zhang *et al.*, 2001).

In the toroidal pore model, inserted peptides cause bending of the lipid monolayers, leading to the formation of pores lined with both the inserted peptides and the phospholipid head groups (Matsuzaki *et al.*, 1996). This type of action has been proposed for magainin, protegrins and melittin (Hallock *et al.*, 2003; Matsuzaki *et al.*, 1996; Yang *et al.*, 2001). The main difference between the barrel-stave and toroidal pore mechanisms is that in the latter, CAMPs are always associated with the lipid head groups, also when forming pores.

The third commonly supported model is the carpet mechanism, in which peptides act like detergents, covering the membrane surface in a carpet-like manner and subsequently leading to membrane disruption without long-lasting channel formation (Pouny *et al.*, 1992). Numerous peptides, including cecropins, dermaseptins and ovispirin (Amiche *et al.*, 1999; Gazit *et al.*, 1995; Pouny *et al.*, 1992; Yamaguchi *et al.*, 2001), are predicted to act via the carpet mechanism.

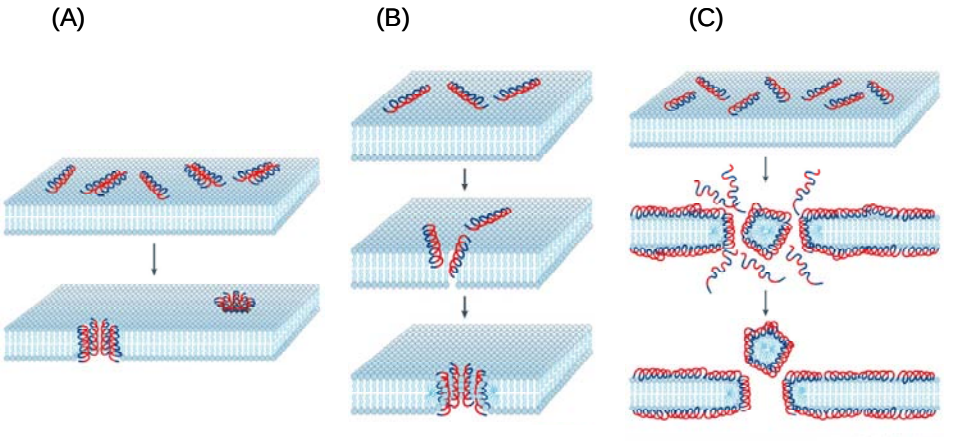


Figure 3. Models of membrane disruption mechanisms of CAMPs: (A) Barrel-stave mechanism; (B) Toroidal pore mechanism; (C) Carpet mechanism. Adapted from Brogden (2005).

It should be taken into consideration that none of the models presented above has been proven to be the actual mechanism acting *in vivo* (Brogden, 2005). It is possible that the actual membrane permeabilization of CAMPs involves elements from each of the models, and that the different models are in fact different stages of the same membrane rupture event (Dathe & Wieprecht, 1999; Papo & Shai, 2003). Recently, a new model termed "leaky slit" has also been presented, in which oligomerized peptides are suggested to form amphipathic membrane-spanning ribbons (Mahalka & Kinnunen, 2009; Zhao *et al.*, 2006). In this model, the hydrophobic side of the peptide ribbon faces towards the hydrocarbon chains of the lipid bilayer, while the hydrophilic side causes the formation of a positive curvature. This kind of action would be similar to that of amyloid-forming peptides and proteins in general. Cationic peptides predicted to act in this manner include temporin B and L, plantaricin A, magainin-2, sakacin P and dermaseptin S9 (Auvynet *et al.*, 2008; Zhao *et al.*, 2005; Zhao *et al.*, 2006)

Interestingly, the mode of membrane permeabilization might differ between mammalian and bacterial cells. For example, magainin forms small pores in the cell membrane of *Bacillus megaterium*, but causes more dramatic membrane disruption in ovary cells of the Chinese hamster (Imura *et al.*, 2008). Temporins 1Ta and Tl act in the barrel-stave manner when causing haemolysis, whereas their antimicrobial activity is achieved by the carpet mechanism (Carotenuto *et al.*, 2008).

Membrane-nondisruptive mechanisms of antimicrobial peptides

The clear dissociation between membrane permeabilization and cell death shown in several studies implies that alternative mechanisms to membrane dysfunction exist (Brogden, 2005; Hale & Hancock, 2007). CAMPs have even been suggested to have a "multitarget" mechanism of action (Powers & Hancock, 2003), describing their ability to interact with multiple anionic targets such as nucleic acids, cellular enzymes or constituents of the cell wall and membrane.

Due to the polyanionic nature of nucleic acids, it is not surprising that most CAMPs can bind to DNA and RNA with great avidity *in vitro*. One of the best characterized examples of CAMPs having microbicidal activity via DNA binding is buforin II, first isolated from the Asian toad (Park *et al.*, 1996). Buforin II is an α-helical linear peptide derived proteolytically from histone H2A and it has an ability to penetrate the cell membrane and kill bacterial cells by binding to DNA and RNA (Kim *et al.*, 2000; Park *et al.*, 1998a). Other histones reported to have antimicrobial activity are hipposin from shrimps (Birkemo *et al.*, 2003) and parasin I from catfish (Park *et al.*, 1998b). Another extensively studied example of DNA-binding CAMP is indolicin, a member of the cathelicidin family found in bovine neutrophils. For a long time it was thought to act through membrane permeabilization without cell lysis (Falla *et al.*, 1996), until its DNA-binding ability was discovered (Hsu *et al.*, 2005).

Many CAMPs can interfere in protein synthesis, folding or function. Direct inhibition of protein synthesis has been reported in studies on the pig cathelicidin PR39 and *E. coli* (Boman *et al.*, 1993), and a pleurocidin derivative from winter salmon restraining histidine incorporation in *E. coli* (Patrzykat *et al.*, 2002). Other peptides affecting protein synthesis include the α-helical peptides dermaseptin and pleurocidin and the human defensin HPN-1 (Jenssen *et al.*, 2006). Several studies have suggested that CAMPs cause the arrest of certain essential biosynthetic processes by binding and inactivating intracellular proteins. For example, correct folding of many proteins in *E. coli* is inhibited by the insect antimicrobial peptides pyrrhocoricin, drosocin and apidaecin, as the peptides bind to the bacterial chaperone DnaK (Kragol *et al.*, 2001; Otvos *et al.*, 2000). Another peptide, microsin B17, interferes in DNA replication by inactivating DNA gyrase of *E. coli* (del Castillo *et al.*, 2001).

Not only intracellular but also extracytoplasmic proteins such as autolysins can be targets for certain CAMPs. For instance, the lantibiotics nisin and Pep5 activates otherwise inert autolysins in cell wall extracts of staphylococci, which may be the explanation for cell lysis after peptide treatment (Bierbaum & Sahl, 1985; Bierbaum & Sahl, 1987). Inhibition of septum formation by CAMPs has also been postulated in studies with the lantibiotic microsin 25 interacting with membrane-associated septum-forming molecules in *E. coli* (Salomon & Farias, 1992). Inhibition of septum formation leads to filamentous cell formation, which is also seen in *E. coli* cells treated with indolicidin (Subbalakshmi & Sitaram, 1998) and *Salmonella typhimurium* cells treated with PR-39 and its truncated form PR-26 (Shi *et al.*, 1994).

Synergistic activity of CAMPs

Virtually all higher organisms produce more than one CAMP at a particular site, leading to the assumption that peptides may act co-operatively to kill bacteria. The enhanced antimicrobial activity of combinations of peptides compared to single peptides was first discovered in the dermaseptin family (Mor *et al.*, 1994). Recent studies have implied that the production of numerous structurally similar peptides within the same organism is a way to increase the antimicrobial spectrum by combining different peptide isoforms (Mangoni & Shai, 2009). For example, Gram-negative bacteria are resistant to the temporins 1Ta and Tb, but when either of them is combined with temporin 1Tl at sub-inhibitory concentrations, resistance is overcome (Rosenfeld *et al.*, 2006). Two out of three chicken gallinacins have also been demonstrated to act in synergy against *Salmonella enteriditis* (Milona *et al.*, 2007). More distinct peptides can also work together. For example, pig protegrin 1 has been shown to work in synergy with indolicidin, bactenesin or LL-37 (Yan & Hancock, 2001). Furthermore, human α- and β-defensins have been reported to act synergistically with LL-37 to kill *S. aureus* and *E. coli* cells (Chen *et al.*, 2005a; Nagaoka *et al.*, 2000).

CAMPs can assist in the uptake or enhance the antimicrobial effects of other microbicidal compounds such as traditional antibiotics or other host-derived antimicrobial proteins. For example, tachyplesin III combined with β-lactams or colistins inhibits the growth of multidrug-resistant *Pseudomonas aeruginosa* (Cirioni *et al.*, 2007). Synergistic interactions between CAMPs and enzymes are observed in some cases. Host-derived secretory phopholipase A of lacrimal fluid is an enzyme catalyzing the hydrolysis of the sn-2-ester-bond in phospholipids. In addition to this enzymatic function, it has antimicrobial activity of its own, which is strongly enhanced in the presence of magainin 2, indolicidin and temporins L and B (Zhao & Kinnunen, 2003).

Selective toxicity of CAMPs

It is widely accepted that at least some CAMPs exhibit selective toxicity, meaning that they can distinguish their target microbial cells from the host cells. The main features defining this selectivity are the composition of the cell membrane and membrane potential of the target cell (Yeaman & Yount, 2003). Bacterial cell membranes are rich in acidic phospholipids such as phosphatidylglycerol, cardiolipin and phosphatidylserine, giving a strong net negative charge to bacterial membranes. On the contrary, the outer leaflet of the plasma membrane in mammalian cells is mainly composed of zwitterionic phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. Due to these differences, CAMPs with a positive charge are more prone to binding to bacterial cell membranes. However, to a lesser extent there are also some negatively charged molecules on mammalian cell surfaces, such as gangliosides, which have been shown to have an essential role in the cellular entry of buforin IIb (Lee *et al.*, 2008). Not only the charge but also the composition of the cell membrane affects selectivity. For example, cholesterol is only present in mammalian membranes and stabilizes and protects the plasma membrane from attack by CAMPs. Another significant

distinction between prokaryotic and eukaryotic cells is the differences in their membrane potential. This electrochemical gradient results from proton flux across the membrane, and in normal mammalian cells it ranges from -90 to -110 mV, while in the case of bacterial cells it varies from -130 to -150 mV (Yeaman & Yount, 2003). The strong electronegative membrane potential may draw cationic peptides deeper into the microbial cell.

Recently, the issue of cell selectivity has been challenged and it has been suggested that all CAMPs should be considered as potentially toxic to mammalian cells in the absence of microbial targets (Matsuzaki, 2009). There is some clear evidence that CAMPs prefer microbial cells, as was shown in an experiment where dye-labelled magainin selectively bound to *S. aureus* but not to epithelial cells (Zasloff, unpublished work in Matsuzaki, 2009). It might be that CAMPs are less harmful to host cells simply by restricting their production in relatively inert host tissues such as epithelial cells, or inside the phogolysosomes of leukocytes (Matsuzaki, 2009; Yeaman & Yount, 2003).

2.1.3 Other roles of antimicrobial peptides

Many mammalian CAMPs lose their ability to kill bacterial cells under physiological conditions. It is known that antimicrobial activity is often antagonized by divalent and monovalent cations, glycosaminoglycans or mucins. However, CAMPs have been shown to cause an antimicrobial response *in vivo*, indicating that they may function as modulators of innate immunity. CAMPs have been reported to participate in the inactivation of endotoxin (LPS), they induce the production of several cytokines and chemokines and can also serve as chemokines (Mookherjee *et al.*, 2006a; Mookherjee & Hancock, 2007; Mookherjee *et al.*, 2007). They also play a role in cellular differentiation and proliferation, the suppression of apoptosis, wound repair and the stimulation of angiogenesis (Mookherjee & Hancock, 2007; Steinstraesser *et al.*, 2008). Furthermore, CAMPs can directly affect the regulation and expression of genes related to innate immunity (Mookherjee *et al.*, 2006b). A body of evidence suggests that CAMPs operate at the interface of innate and adaptive responses, serving as signals influencing the initiation, polarisation and amplification of adaptive immune responses (Bowdish *et al.*, 2005; Mookherjee & Hancock, 2007; Oppenheim *et al.*, 2003). A new interesting area of peptide research concerns the anticancer activities of CAMPs (Hoskin & Ramamoorthy, 2008).

2.1.4 Pharmaseutical use of antimicrobial peptides

As bacterial resistance to conventional antibiotics is an ever-growing problem, there is an urgent need to find or develop new antimicrobial drugs. Despite the great expectations of CAMPs being a potential new source of antibiotics, few CAMPs have been tested in clinical trials and their use has been restricted to topical applications (Hancock & Sahl, 2006; Oyston *et al.*, 2009). Four cationic antimicrobial peptides have been tested in phase 3 clinical efficacy trials. These include the magainin derivative Pexiganan for treating foot ulcers, a pig protegrin derivative Iseganan for oral mucosaitis, the human bactericidal permeability protein derivative Neuprex for sepsis and the indolicidin variant Omiganan for the treatment

of catheter-associated infections (Hancock & Sahl, 2006). However, only Omiganan has been approved for clinical use and is now pending licensing (Oyston *et al.*, 2009).

In addition to adequate efficacy, several problems concerning the drug usage of CAMPs exist. Possible toxicity to host cells, lability to proteases and high costs of production are factors limiting the clinical use of CAMPs. To overcome these challenges, considerable resources have been targeted at designing novel peptides with improved qualities. One potential source of new microbicidal compounds could be the antimicrobial peptides produced by Gram-positive bacteria. The nonribosomally synthesized peptides polymyxin B and gramicidin S have been in medical use for a long time and the lantibiotic nisin is used as a food additive to restrict bacterial growth. Nisin and lacticin 3147 are also used in veterinary medicine to prevent bovine mastitis (Ryan *et al.*, 1998). New candidates for medical use include the lantibiotic mutacin 1140 (Smith & Hillman, 2008).

2.2 Bacterial mechanisms for resisting antimicrobial peptides

Considering the ancient origin of CAMPs, surprisingly few bacterial species have developed highly effective resistance mechanisms against CAMPs. However, it seems that the co-evolution of CAMPs and bacterial resistance mechanisms has led to the emergence of a diverse repertoire of CAMPs (Peschel & Sahl, 2006; Zasloff, 2002). It has also been shown that resistance against a particular CAMP emerges through continued selection under laboratory conditions, although resistance is clearly more difficult to obtain than in the case of conventional antibiotics (Perron *et al.*, 2006). Certain bacterial species have exceptionally broad resistance to different kinds of CAMPs, including species belonging to genera such as *Serratia*, *Burkholderia*, *Morganella* and *Proteus* (Yeaman & Yount, 2003; Zasloff, 2002). These bacteria are protected against CAMPs by either stable structural or functional properties. Several important pathogens such as *Staphylococcus aureus* and *Salmonella enterica* have also evolved several strategies to avoid the toxicity of CAMPs. The most common bacterial resistance mechanisms include modifications of the cell envelope, the inactivation of CAMPs and active extrusion of harmful peptides.

2.2.1 Modifications of the cell envelope

Reduction of the net negative charge of the cell envelope

The initial interactions between bacteria and CAMPs are driven by electrostatic forces. From this point of view, it is not surprising that many of the resistance mechanisms used by bacteria are based on the reduction of the negative charge of the cell envelope. The essential molecules of the cell envelope can hardly be replaced without serious defects, but some bacteria are able to modify the charge of these molecules in order to prevent the attachment of CAMPs to the cell surface. Many Gram-positive bacteria such as *S. aureus*, *Streptococcus pyogenes*,

Streptococcus agalactiae and *Listeria monocytogens* are able to partially neutralize the negative charge of the cell wall by modifying teichoic acids with D-alanine (Abachin *et al.*, 2002; Kristian *et al.*, 2005; Peschel *et al.*, 1999; Poyart *et al.*, 2003). D-alanylation of teichoic acids is carried out by proteins encoded by the *dltABCD* operon found in many genomes of Gram-positive bacteria (Neuhaus & Baddiley, 2003; Perego *et al.*, 1995; Peschel *et al.*, 1999). It has been shown that *dlt*-deficient mutants are more susceptible to a broad variety of CAMPs and other cationic host defence factors (Koprivnjak *et al.*, 2002; Koprivnjak *et al.*, 2008; Kristian *et al.*, 2005; Peschel *et al.*, 1999). *S. aureus* also exploits another strategy to modify the charge of the cell envelope. The MprF catalyses the addition of L-lysine residues to the major membrane lipid phosphatidylglycerol, leading to the formation of positively charged lysyl-phosphatidylglycerol (LPG). Mutation of the *mprF* gene causes the same kind of phenotype as inactivation of the *dlt* operon (Peschel *et al.*, 2001). However, there appear to be certain limits after which modulation of the cell envelope charge is not sufficient to maintan resistance against CAMPs. Resistance may be overcome at high concentrations of CAMPs or by a high positive charge of a certain peptide, as in the case of human β-defensin hbD3 with a net positive charge of +10 (Harder *et al.*, 2001; Midorikawa *et al.*, 2003; Weidenmaier *et al.*, 2004). Reduction of the cell envelope charge is also a common mechanism in Gram-negative bacteria and is mainly achieved by modifications of lipid A in the outer membrane. The best characterised example is the incorporation of aminoarabinose into lipid A of *Salmonella enterica* and *Pseudomonas aeruginosa* (Ernst *et al.*, 2001; Gunn *et al.*, 1998).

Alterations in membrane structure and fluidity and reduction of the membrane potential

The susceptibility of bacteria to CAMPs is also influenced by physico-chemical properties of the cell membrane other than charge. It has been shown that the cell membranes of *S. aureus* strains resistant to tPMP-1 (a human kinocidin derived from platelets) contain elevated levels of longer-chain and unsaturated membrane lipids compared to tPMP-1-susceptible strains. This leads to higher membrane fluidity in resistant strains (Bayer *et al.*, 2000). It is postulated that the increased fluidity causes proton leakage out of the cells and consequently leads to a reduced membrane potential, interfering with the permeabilization of the peptides (Bayer *et al.*, 2006). On the other hand, reduced membrane fluidity due to an increased cardiolipin and lysyl-phosphatidylglycerol content in the membranes of *S. aureus* protoplasts is reported to associate with a reduced susceptibility to HNP-1, gramicidin D and tPMP-1 (Xiong *et al.*, 2005). It has been shown that in addition to the membrane stabilizing effect, cardiolipin also functions as a proton reservoir affecting the membrane potential (Kates *et al.*, 1993). The importance of membrane potential for the action of CAMPs has also been demonstrated with so-called small colony variants of *S. aureus* having mutations in the respiratory chain that cause a loss of membrane potential. A reduced membrane potential renders these *S. aureus* mutants more resistant to CAMPs (Yeaman & Bayer, 2006). The overall phospholipid composition and asymmetry between the two lipid layers in the bacterial cell membrane influences the susceptibility towards CAMPs. The outer leaflet of *S.*

aureus strains resistant to cationic molecules such as poly-L-lysine, cytocrome C and tPMP-1 contains significantly higher amounts of positively charged phospholipids compared to susceptible strains (Mukhopadhyay *et al.*, 2007).

2.2.2 Inactivation of CAMPs

α-Helical peptides are especially susceptible to proteolysis caused by bacterial peptidases and proteases. For example, the metalloprotease aureolysin and the serine protease V8 secreted by *S. aureus* are able to cleave LL-37. It has been shown that the production of these proteases is correlated with CAMP resistance *in vitro* (Sieprawska-Lupa *et al.*, 2004). In addition, the protease SepA of *S. epidermidis* has a similar function (Lai *et al.*, 2007). Many other species such as *Streptococcus pyogenes*, *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis*, *Porphyromonas gingivalis* and *Prevotella* spp. produce CAMP-degrading proteases (Guina *et al.*, 2000; Nyberg *et al.*, 2004; Schmidtchen *et al.*, 2002).

CAMPs containing disulphide bridges or bacteriocins containing lanthionine amino acids are more resistant to proteolytic degradation. The presence of proline residues and amidation of the C-terminus also protects CAMPs from proteolysis. However, several bacterial species produce membrane bound metalloproteases involved in bacteriocin self-immunity, referred as Abi proteins (Kjos *et al.*, 2010). Non-nisin-producing *Lactococcus lactis* strains produce NSR protein which inactivates nisin by removing six amino acids from its carboxyl end (Sun *et al.*, 2009). Additional strategies to inactivate more stable CAMPs have also evolved among bacterial species. In contrast to peptidases and proteases, these mechanisms are usually specific for certain peptides. For example, the exoprotein staphylokinase produced by *S. aureus* can bind and inactivate α-defensins. In addition to its function in fibrinolysis, it also contributes to resistance to bacterial killing by α-defensins, and virulence (Jin *et al.*, 2004). *Streptococcus pyogenes* secretes SIC protein (**s**treptococcal **i**nhibitors of **c**omplement) and cell wall-bound M1 protein capable of binding LL-37 and other CAMPs with high affinity (Frick *et al.*, 2003; Peschel & Sahl, 2006). NisI immunity protein of *L. lactis* confers resistance to nisin and functions probably by intercepting nisin on the cell membrane (Qiao *et al.*, 1995; Stein *et al.*, 2003).

2.2.3 Extrusion of CAMPs

A general strategy of bacterial cells to avoid drugs or other toxic agents is their active extrusion from the cell or the cytoplasmic membrane. This active drug efflux is conducted against a concentration gradient via integral membrane proteins utilizing metabolic energy. Most efflux systems are specific for a narrow range of structurally related substrates, but systems exporting a broad spectrum of structurally dissimilar compounds also exist. These systems are referred to as multidrug resistance (MDR) transporters, and they are classified into two main groups based on the source of energy utilized in the translocation of toxic agents (Lubelski *et al.*, 2007). Primary transporters belong to the ATP binding cassette (ABC) superfamily hydrolysing ATP to provide free energy. Secondary exporters are drug/H+ or drug/Na+ antiporters utilizing proton motive force (PMF). Secondary

transporters can be further classified into several families according to their amino acid homology and secondary structure. These subclasses are the major facilitator superfamily (MFS), the small MDR family (SMR), the resistance-nodulation-cell division family (RND) and the multidrug and toxic compound extrusion family (MATE) (Fig. 4).

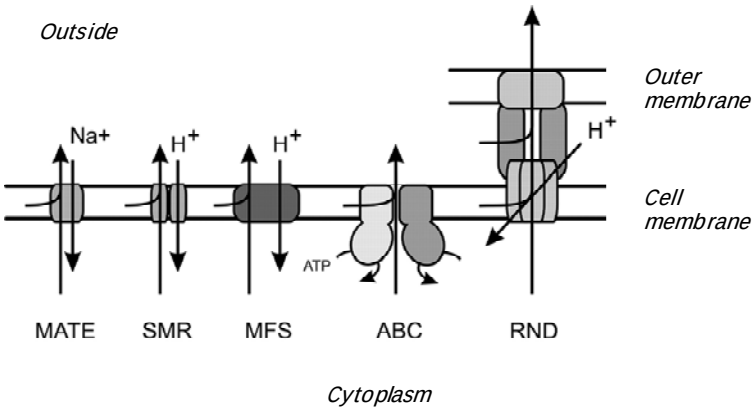


Figure 4. Major families of MDR transporters. Adapted from Lubelski (2007)

There is increasing evidence that secondary transporters are also important factors in bacterial resistance against CAMPs. One of the best characterised examples is the RND multidrug resistance transporter MtrCDE in *Neisseria gonorrhoeae* and *Neisseria meningitides*, conferring resistance to LL-37 and protegrins and various cell envelope stress-causing agents such as antibiotics, organic dyes and detergents (Shafer *et al.*, 1998; Tzeng *et al.*, 2005). In *Yersinia enterocolitica*, MFS transporter RosAB is also involved in CAMP resistance and it is believed to affect bacterial survival in neutrophils (Bengoechea & Skurnik, 2000). In *S. aureus*, the plasmid-encoded MFS exporter QacA is associated with resistance to tPMP-1, a human kinocidin derived from platelets (Kupferwasser *et al.*, 1999). However, it seems that this resistance phenotype is not caused by the efflux itself but rather results from the changes in the membrane fluidity and membrane potential caused by QacA (Bayer *et al.*, 2006).

Primary transporters of antimicrobial peptides have mainly been reported among Gram-positive bacteria producing bacteriocins themselves. For instance, *S. epidermidis* strains producing the lantibiotic epidermidin have evolved an additional protective system removing harmful peptides from the membrane after they have reached the lipid bilayer. This epidermidin resistance is mediated by the EpiEFG ABC transporter, which specifically binds only epidermidin and its very close relative, gallidermin (Otto *et al.*, 1998; Peschel & Gotz, 1996). Similar systems have been identified to export nisin from *L. lactis* and subtilin and mersacidin from *B. subtilis* (Altena *et al.*, 2000; Klein & Entian, 1994; Siegers & Entian, 1995).

2.3 How do Gram-positive bacteria sense cell envelope stress?

The cell envelope is an indispensable barrier protecting bacterial cells from environmental threats. The cell envelope gives cells their shape, withstands the high internal turgor pressure and mediates the controlled trafficking of proteins, ions and nutrients. It is also the target for numerous antibiotics, antimicrobial peptides and other antimicrobial compounds. For the survival of bacteria it is crucial that they can sense and rapidly react to environmental changes threathening cell envelope assembly, maintenance or function. In Gram-positive bacteria, two main cell signal transduction systems contribute to the sensing of cell envelope stress: two-component systems (TCSs) and alternative sigma (σ) factors (Jordan *et al.*, 2008).

2.3.1 Cell envelope of Gram-positive bacteria

The fundamental difference between Gram-positive and Gram-negative bacteria is the structure of their cell envelope. The cell envelope of Gram-negative bacteria consists of two lipid bilayers and a thin peptidoglycan sacculus between the membranes. In contrast, Gram-positive bacteria lack the outer membrane but have a substantially thicker cell wall with multiple peptidoglycan layers. In addition, many Gram-positive bacteria have other protective envelope structures such as extracellular polysaccharide capsules, S-layer proteins and mycolic acids (Weidenmaier & Peschel, 2008). Compared to Gram-negative bacteria, the cell envelope of Gram-positive bacteria comprises only two functional layers: a cell wall and cell membrane. Therefore, Gram-positive bacteria are generally considered to lack the periplasmic space present in the Gram-negative cell envelope between the peptidoglycan and inner membrane. However, this idea has recently been challenged by electron microscopy studies also showing the presence of a periplasmic-like space between the membrane and cell wall in Gram-postive bacteria (Matias & Beveridge, 2005; Matias & Beveridge, 2006). Either way, the cell wall-membrane interface of Gram-positive bacteria is the cell compartment in which several cellular processes take place.

Cell wall

The cell wall of Gram-positive bacteria is an approximately 20-50 nm thick, three dimensional net-like structure mainly composed of multiple layers of peptidoglycan, additional poly-anionic teichoic acids and substantial proportions of proteins. Together, they form a negatively charged matrix maintaining an optimal metal cation homeostasis essential to membrane-bound enzyme systems. Peptidoglycan strands of varying lengths consist of disaccharide subunits of N-acetyl glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) with short stem peptides linked to carboxyl group of muramyl residues (Foster & Popham, 2002). The lipid linked disaccharide-pentapeptide precursors of peptidoglycan, called lipid II, are synthesized in the cytoplasm by a great number of enzymes. Lipid II precursors are polymerized into glycan strands and cross-linked to each other in enzymatic reactions carried out by penicillin-binding proteins (PBSs) present in the cell wall (Ghuysen, 1991). Rigid peptidoglycan strands are cross-linked to each other via flexible peptide bridges, thus forming a strong but nevertheless elastic structure.

Peptidoglycan and poly-anionic glycopolymers are present in the cell wall in almost equal amounts. For example, in *B. subtilis* glycopolymers make up 35-60% of the cell wall dry weight (Neuhaus & Baddiley, 2003). The structures of glycopolymers are often specific to a particular species or even strain, and are highly variable in their sugar content, net charge and "side group decorations". The most important group of glycopolymers is teichoic acids, which are either covalently linked to sugar residues of the peptidoglycan (wall teichoic acids, WTAs) or anchored to the cell membrane via a glycolipid anchor (lipoteichoic acids, LTAs) (Delcour *et al.*, 1999; Foster & Popham, 2002). The cell wall of Gram-positive bacteria usually contains one type of both teichoic acids, but there are also exceptions such as *B. subtilis*, which has one LTA, two kinds of WTAs and in addition can produce teichuronic acid under low-phosphate conditions.

Teichoic acids are linear polymers formed of sugar monomers linked via anionic phosphodiester linkages, which are responsible for the overall net negative charge of the cell surface. The sugar backbones of WTAs often contain glycerol or ribitol, but tetroses, hexoses and complex sugar combinations also exist. LTAs are usually less variable than WTAs and are mainly composed of glycerol-phophate repeating units (Archibald *et al.*, 1993; Neuhaus & Baddiley, 2003; Weidenmaier & Peschel, 2008). Most teichoic acids contain additional sugars or amino acids, such as D-alanine. The D-alanine content of teichoic acids is controlled by the *dlt* operon discussed in earlier sections. Teichuronic acids differ from classical teichoic acids in having a backbone lacking the phosphate groups. However, they are still anionic due to the presence of uronic acid, for example glucuronic acid in *B. subtilis* (Neuhaus & Baddiley, 2003; Weidenmaier & Peschel, 2008).

The function of teichoic acids has not been completely elucidated. They are considered to be essential for the viability of Gram-positive bacteria and proposed to have important roles in cell wall function, maintenance and turnover. However, studies with mutants lacking or having reduced amounts of certain teichoic acids have revealed that WTAs are dispensable, at least under laboratory conditions, in both *S. aureus* and *B. subtilis* (D'Elia *et al.*, 2006a; D'Elia *et al.*, 2006b). On the contrary, LTAs seem to be essential for *S. aureus*, but mutants with as much as a 90% reduction in the LTA content are still viable and do not show any major phenotypic changes (Fedtke *et al.*, 2007; Grundling & Schneewind, 2007). This does not exclude the fact that TAs have a fundamental role in maintaining overall cell integrity. In addition to their crucial role in protecting the cell envelope from harmful substances such as antibiotics, surfactants, phages, CAMPs and antimicrobial fatty acids of human skin, TAs have recently been shown to contribute to host cell adhesion and colonization, inflammation and immune activation caused by Gram-positive pathogens (Kohler *et al.*, 2009; Weidenmaier & Peschel, 2008; Xia *et al.*, 2010). Furthermore, it was recently shown that WTAs have a role in targeting the major autolysin Atl of *S. aureus* to the septal region of the cell surface leading to cell division (Schlag *et al.*, 2010)

Cell membrane

The cell membrane controls the permeability of the bacterial cell and the transportation of various kinds of molecules. In addition, it is a place where functions related to respiration, the replication of DNA and synthesis of cell wall components occur.

The composition of the prokaryotic cell membrane differs from that of eukaryotic cells. Bacterial membranes are typically enriched in negatively charged phospholipids such as phosphatidylglycerol, cardiolipin and phosphatidylserine, whereas the zwitterionic phospholipids phosphatidylcholine, phosphatidylethanolamine and sphingomyelin common in eukaryotic cells are rarely present in prokaryotic membranes. Sterols such as cholesterol and ergesterol are also absent from bacterial membranes (Yeaman & Yount, 2003). Due to these differences, bacterial membranes tend to be generally much more electronegative than eukaryotic membranes. However, Gram-positive bacteria can reduce the overall negative charge of cell membrane by producing positively charged lysine esters of phosphatidylglycerol, lysyl-PG (Peschel *et al.*, 2001). As in eukaryotic cells, the membrane lipids are asymmetrically distributed between the two lipid layers of the bacterial membrane, influencing membrane stability, intercellular recognition and signal transduction (Pomorski *et al.*, 2004; Tannert *et al.*, 2003). The lipid content varies between bacterial species. For example, in *B. subtilis*, phosphatidylglycerol accounts for 75% of the total membrane lipid content, the rest being mainly lysyl-PG and cardiolipin, whereas in *S. aureus* up to 38% of membrane lipids are lysyl-PG (Peschel *et al.*, 2001).

2.3.2 Alternative sigma factors

Adaptation to environmental changes demands timely regulation of gene expression and only optimal sets of genes are transcribed under specific conditions. The binding of a sigma factor to the core RNA polymerase is essential for the recognition of the appropriate promoter sequence and the initiaton of transcription. The promoter selectivity of RNA polymerase can be altered by alternative σ factors that are activated by adequate triggers (Paget & Helmann, 2003). All bacteria have one primary σ factor similar to σ^{70} in *E. coli* and σ^{A} in *B. subtilis* mediating most of the transcription in exponentially growing cells. The number of σ factors varies between bacterial species; for example, *E. coli* posses six σ factors, *B. subtilis* at least 16 σ factors and *Streptomyces coelicolor* over 60 σ factors, while only four σ factors are found from the genome of *S. aureus* (Helmann, 2006; Shaw *et al.*, 2008). One of the best characterized alternative σ factors is σ^{B}, which orchestrates the general stress responses of Gram-positive bacteria such as *B. subtilis* and *S. aureus* (Hecker & Volker, 2001; Morikawa *et al.*, 2003).

Sigma factors are classified into two major families known as the σ^{70} and σ^{54} families (Gross *et al.*, 1998; Gruber & Gross, 2003; Helmann & Chamberlin, 1988). Most σ factors belong to the σ^{70} family, which is further divided into groups based on sequence similarity and domain topology. These groups include essential primary σ factors and their non-essential paralogues, σ factors contributing to motility,

sporulation and heat shock resistance and the **e**xtra**c**ytoplasmic **f**unction (ECF) σ factors, which are the largest group in the σ^{70} family (Gruber & Gross, 2003; Staron *et al.*, 2009).

ECF σ factors are often involved in controlling cell envelope stress responses or transport processes (Helmann, 2002; Lonetto *et al.*, 1994). Comparative genomic analyses have revealed that ECF σ factors are ubiquitously present in bacterial species. Although there is an average of six ECF σ factors per bacterial genome (Staron *et al.*, 2009), the number of ECF σ factors varies greatly between species. For example, *Streptomyces coelicolor* is predicted to encode more than 50 ECF σ factors, the other extreme being *S. aureus*, with only a single putative ECF σ factor (σ^S) so far characterized (Helmann, 2006; Shaw *et al.*, 2008).

ECF σ factors share several common features. Unlike the primary σ factors binding the classical consensus sequences in the -10 ('TATAAT') and -35 ('TTGACA') regions (Helmann, 1995; Travers, 1987), many of the ECF σ factors recognize a highly conservative 'AAC' motif in the -35 region and 'CGT' clusters in the -10 region (Helmann, 2002; Lane & Darst, 2006). ECF σ factors also lack two of the four conserved domains present in the primary σ factors. In addition, ECF σ factors can autoregulate their own expression and are usually cotranscribed with their cognate anti-σ factors. Anti-σ factors are transmembrane proteins that bind and inactivate σ factors in the absence of an appropriate stimulus. After receiving an adequate signal, anti-σ factor is released from the σ factor, enabling the activation of RNA polymerase by ECF σ factor (Helmann, 2002; Jordan *et al.*, 2008; Staron *et al.*, 2009).

ECF sigma factors of *B. subtilis*

The *B. subtilis* genome comprises seven ECF σ factors, σ^W, σ^M, σ^X, σ^Y, σ^V, σ^Z and σ^{YlaC} (Helmann, 2002; Kunst *et al.*, 1997). At least σ^W, σ^M, σ^X have been shown to contribute to cell envelope stress responses.

The best characterized ECF σ factor in *B. subtilis* is σ^W. Based on promoter consensus searches, about 30 target promoters of σ^W controlling the expression of about 60 genes have been identified (Cao *et al.*, 2002a; Huang *et al.*, 1999). The σ^W regulon is induced by numerous stress conditions, including cell wall active antibiotics such as vancomysin, cephalosporin, fosfomycin and D-cycloserine, as well as membrane active compounds, alkali shock and bacteriocins (Butcher & Helmann, 2006; Cao *et al.*, 2002b; Wiegert *et al.*, 2001). It is postulated that σ^W regulates genes involved in the inactivation or extrusion of toxic compounds from the cell (Helmann, 2006). The role of σ^W in antibiotic resistance was confirmed in studies demonstrating that σ^W deletion causes increased sensitivity to fosfomycin and antimicrobial compounds synthesized by other *Bacillus* species (Butcher & Helmann, 2006; Cao *et al.*, 2001).

The σ^W-encoding gene (*sigW*) is cotranscribed with its cognate anti-σ factor gene *rsiW*. In the absence of a proper signal, the membrane-anchored RsiW directly binds

to σ^W. Activation of σ^W is achieved through a proteolytic cascade releasing σ^W from RsiW. The first step of the cascade is degradation of the extracytoplasmic domain of RsiW by a membrane-bound protease, PrsW (Ellermeier & Losick, 2006; Heinrich & Wiegert, 2006). This is followed by the RasP-mediated cleavage of the membrane spanning region of RsiW, leading to the formation of a soluble N-terminal fragment of RsiW (Schobel *et al.*, 2004). This fragment is further degraded by the cytoplasmic ClpX proteolytic complex (Zellmeier *et al.*, 2006).

ECF σ factor σ^M directly controls the expression of at least 57 genes. In addition, σ^M activates several other gene regulators. The functions of these σ^M regulated genes include cell wall synthesis, cell division and the determination of cell shape, DNA repair and detoxification (Eiamphungporn & Helmann, 2008). The σ^M regulon is activated by several stress conditions such as an acidic pH, high salinity, heat, superoxide, paraquat and cell wall active antibiotics (Cao & Helmann, 2002; Horsburgh & Moir, 1999; Mascher *et al.*, 2003; Minnig *et al.*, 2003; Thackray & Moir, 2003). Mutants with a σ^M deletion display increased sensitivity to certain cell envelope active compounds such as bacitracin, moenomycin, SDS and some β-lactams (Cao & Helmann, 2002; Mascher *et al.*, 2007). σ^M is also activated under phosphate deprivation and involved in TA synthesis in the *B. subtilis* strain W23 (Minnig *et al.*, 2005). σ^M is cotranscribed with its anti-σ complex composed of two separate proteins, YhdL and YhdK. The direct binding of σ^M to the N-terminal part of YhdL has been demonstrated by using a yeast two-hybrid system (Yoshimura *et al.*, 2004).

The first ECF σ factor of *B. subtilis* characterized in detail was σ^X. It is cotranscribed with the gene *rsiX* encoding the anti-σ factor of σ^X (Huang & Helmann, 1998). The σ^X regulon comprises ~30 genes and the main role of σ^X is predicted to be in modulation of the net negative charge of the cell envelope (Cao & Helmann, 2004). This is carried out by two σ^X regulated operons, *dltABCDE* and *pssA-ybfM-psd*. The former operon is responsible for the D-alanylation of wall TAs (Perego *et al.*, 1995) and the latter is involved in the synthesis of the neutral lipid phosphatidylethanolamine, resulting in a lower net negative charge of the cell envelope (Cao & Helmann, 2004).

The role of the other ECF σ factors, σ^Y, σ^V, σ^Z and σ^{YlaC}, remains unclear. The σ^Y regulon consists of six genes organized into one cistron. This cistron contains a gene predicted to encode a toxic peptide and another gene encoding a putative immunity protein against toxic peptide (Cao *et al.*, 2003; Tojo *et al.*, 2003). It was also demonstrated that disruption of σ^Y might have an effect on sporulation induced by nitrogen depletion (Tojo *et al.*, 2003). DNA macroarray analysis of genes regulated by σ^V revealed 13 genes belonging to the σ^V regulon, although an extensive overlap between other ECF σ factors complicates the defining of the regulons (Zellmeier *et al.*, 2005).

Although the role of ECF σ factors in the maintenance of cell envelope integrity under stress conditions seems to be indisputable, little is known about the exact signals activating the σ factors and how these signals are received. Studies with *E.*

coli have revealed that unfolded proteins in the periplasm trigger the proteolytic degradation of the anti-σ factor of σ^E, leading to the activation of σ^E (Rowley *et al.*, 2006). These observations suggest that the primary candidate for the sensory module of the ECF σ factors is the protease starting the proteolytic cascade of anti-σ factors, e.g. PrsW in the case of σ^W of *B. subtilis* (Helmann, 2006; Jordan *et al.*, 2008). Furthermore, an extensive regulatory overlap between the different ECF σ factors has been demonstrated in several studies both *in vitro* and *in vivo*. Many of the operons regulated by ECF σ factors contain multiple promoter elements recognized by several ECF σ factors (Cao & Helmann, 2002; Huang *et al.*, 1998; Mascher *et al.*, 2007; Qiu & Helmann, 2001).

2.3.3 Two-component systems

Two-component signal transduction systems are one of the most important bacterial sensory systems for obtaining information from the world outside. TCSs consist of two functional units, a membrane-anchored sensor histidine kinase (HK) sensing extracellular signals and a cytoplasmic response regulator (RR) responsible for mediating differential gene expression (Fig. 5). HKs are typically integral membrane proteins containing two distinct domains: a highly variable N-terminal input domain capable of binding a specific signalling molecule or reacting to a physical stimulus and a conserved C-terminal cytoplasmic transmitter domain interacting with the cognate RR. The transmitter domain is responsible for autokinase and ATP-binding activities of HK. The activation of TCS includes three phosphoryl-transfer reactions. First, binding of the signal ligand causes an ATP-dependent autophosphorylation reaction of a conserved histidine residue in the transmitter domain of HK. Secondly, a phosphoryl group is transmitted further to an acceptor aspartic acid residue in the N-terminal receiver domain of RR, leading to activation of the C-terminal output domain with DNA-binding properties (Parkinson, 1993; Stephenson & Hoch, 2002). Finally, RR is dephosphorylated in order to set the system back to an inactive state (Parkinson, 1993).

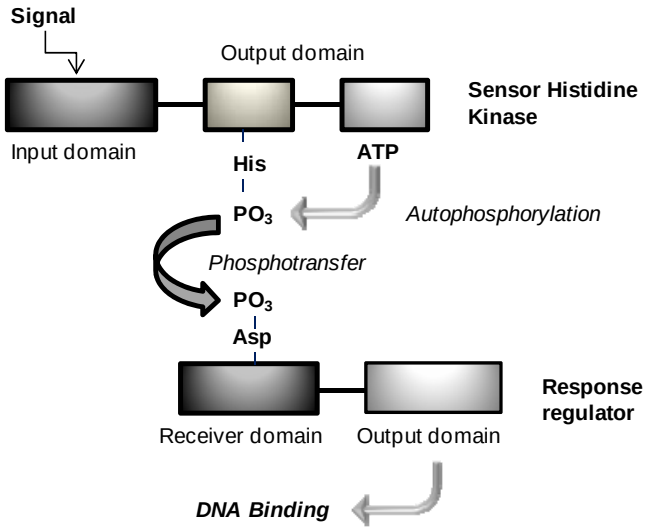


Figure 5. Schematic representation of the two-component signal transduction system. Adapted from Stephenson and Hoch (2002).

TCSs are ubiquitously distributed among bacterial species and average bacterial genome harbors 50 genes encoding TCSs (Whitworth, 2008). Early attempts to classify histidine kinases have mainly been based on sequence analysis of the conserved regions of the transmitter domains, providing only scarce information on the functional role of the different HKs (Fabret *et al.*, 1999; Grebe & Stock, 1999). Comparisons of the input domains of HKs have been problematic due to the great sequence variation in these domains, reflecting the huge variety of environmental stimuli sensed by bacterial cells (Hoch, 2000). It seems that more precise predictions about the physiological roles of individual TCS can be gained by comparing the domain architecture and genomic context of input domains of HKs instead of mere similarities in sequences (Mascher, 2006; Mascher *et al.*, 2006).

Based on HK topology, most TCSs can be classified into three major groups (Mascher *et al.*, 2006). The largest group comprises TCSs with extracellular sensing HKs having an extracellular sensory domain flanked by at least two transmembrane helices. This kind of extracellular domain stucture is typical for HKs sensing solutes and nutrients such as nitrate, nitrite or citrate. In these cases, signal detection occurs by direct interaction between the input domain and the molecule (Janausch *et al.*, 2002; Stewart, 2003; Stewart & Bledsoe, 2003). The group of extracellular sensing HKs also includes two-component systems mediating resistance to cell envelope stress, the best characterized example being PhoQ and its cognate RR PhoP in the Gram-negative bacterium *Salmonella typhimurium* (Groisman *et al.*, 1989; Miller *et al.*, 1989). The PhoQP TCS confers resistance to CAMPs and is activated by direct binding of antimicrobial peptides to the extracellular sensor domain, leading to modifications of lipopolysaccharides and lipid A on the cell surface (Bader *et al.*,

2005). This group of HKs comprises several kinases associated with β-lactam resistance in Gram-positive bacteria such as CroS in *Enterococcus faecalis*, CesK and LisK in *Listeria monocytogenes* and CiaH in *Streptococcus pneumoniae* (Jordan *et al.*, 2008).

The second largest group of HKs consists of cytoplasmic-sensing HKs, either being membrane anchored or soluble proteins. The input domain of these HKs locates in the cytoplasm and therefore senses changes in intracellular conditions. The third diverse group of HKs includes sensor kinases with 2 to 20 transmembrane regions connected by very short extracellular linkers buried in the cell membrane (Mascher, 2006; Mascher *et al.*, 2006). In other words, they lack almost the entire extracellular sensing domain, leading to an assumption that they sense stimuli present in the membrane or in the membrane interface. The transmembrane helices seem to be important for signal recognition. These TCSs participate in various physiological processes such as thermosensing, quorum sensing, energy metabolism and solute transport. This group of HKs includes the subgroup of intramembrane-sensing HKs playing a key role in the sensing of cell envelope stress in Gram-positive bacteria (Jordan *et al.*, 2008). The sensory domain organization of these small HKs is strikingly similar, comprising two transmembrane helices connected by short extracytoplasmic loops less than 25 aa in length. Two types of genomic organization can be distinguished among these HKs: kinases that are parts of three-component systems and kinases that are linked to ABC transporters (such as LiaS and BceS, respectively, in *B. subtilis*) (Mascher, 2006). A similar domain composition of the input domain is also found in other phylogenetically distinct cell wall stress-related TCSs, such as VanRS responsible for inducible resistance to vancomycin (Hong *et al.*, 2004; Hutchings *et al.*, 2004) and other closely related glycopeptides in *Streptomyces coelicolor*, and PmrB/BasS-like HKs contributing to CAMP resistance in *E. coli* and *Salmonella* spp. (Gunn *et al.*, 1998; Gunn *et al.*, 2000; Wosten & Groisman, 1999).

TCSs in *B. subtilis*

The genome of *B. subtilis* harbors 36 HKs and 34 RRs (Fabret & Hoch, 1998; Kunst *et al.*, 1997) listed in Table 1. The TCSs involved in cell envelope stress tolerance include LiaRS, BceRS and its homologues, WalKR (YycFG) and CssRS.

Table 1. Two-component systems in B. subtilis.

Kinase	Response regulator	Function
BceS	BceR	Resistance to antibiotics affecting the cell envelope
CheA	CheY	Chemotaxis and motility
CitS	CitT	Mg^2/citrate transport
ComP	ComA	Early competence
CssS	CssR	Protein quality control
DctS	DctR	Transport of fumarate and succinate
DegS	DegU	Regulation of production of degradative enzymes and competence
DesK	DesR	Adaption to cold temperature
GlnJ	GlnL	Glutamine degradation
KinA	Spo0F	Initiation of sporulation
KinB	Spo0F	Initiation of sporulation
KinC		Initiation of sporulation
KinD	Spo0A-P	Initiation of sporulation
KinE	Spo0F-P	Initiation of sporulation
LiaR	LiaR	Unknown
LytS	LytT	Rate of autolysis
NatK	NatR	Sodium ion extrusion
PhoR	PhoP	Phosphate starvation
ResE	ResD	Aerobic and anaerobic respiration
YbdK	YbdJ	Unknown
YcbM	YcbL	Unknown
YclK	YclJ	Anaerobic growth
YdfH	YdfI	Unknown
YesM	YesN	Unknown
YfiJ	YfiK	Regulation of amino acid biosynthesis and uptake
YhcY	YhcZ	Unknown
YkoH	YkoG	Oxidative stress
YrkQ	YrkP	Unknown
YufL	YufM	Transport of malate
YvcQ	YvcP	Involved in bacitracin resistance
YvfT	YvfU	Unknown
YvrG	YvrH	Involved in membrane conformation and lipid composition
YwpD		Unknown
YxdK	YxdJ	Responds to CAMPs
YxjM	YxjL	Unknown
YycG (WalK)	YycF (WalR)	Essential functions, cell wall processes

LiaRS in *B. subtilis* has been reported to strongly respond to several cell wall active antibiotics such as bacitracin, ramoplanin and vancomycin (Mascher *et al.*, 2003; Mascher *et al.*, 2004). It is also induced by other stress-causing conditions including alkaline shock, secretion stress, ethanol, phenol and organic solvents, although clearly to a lesser extent (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Tam le *et al.*, 2006; Wiegert *et al.*, 2001). LiaRS is also activated by the intrinsic peptide produced by the *yydFGHIJ* operon of *B. subtilis* (Butcher *et al.*, 2007). The LiaRS locus comprises 6 genes, *liaIH-liaGFSR* with two promoter regions upstream from *liaI* and *liaG*. *liaGFSR* encodes the histidine kinase LiaS and its cognate response regulator LiaR, whereas LiaF is an inhibitor of LiaRS, binding directly to LiaR (Jordan *et al.*, 2006; Mascher *et al.*, 2003). This is why LiaRSF is often referred to as a three-component system. The gene *liaG* encodes a putative membrane-anchored protein with unknown function. The *liaGFSR* genes are constitutively expressed at a low level from the promoter upstream from *liaG*. In

contrast, *liaIH* genes are completely silent in the absence of LiaR. LiaRS probably controls the expression of only two operons, *liaIH-GFSR* itself and *yhcYZ-yhdA*, the primary target being *liaIH-GFSR* (Jordan *et al.*, 2006). The function of proteins encoded by LiaIH is largely unknown; LiaI is a small hydrophobic protein with two putative transmembrane helices and LiaH belongs to the phage shock protein family. The expression of *liaIH-GFSR* operon is strictly controlled by at least five regulators, and without any extracellular signal it is kept inactive during the exponential growth phase (Jordan *et al.*, 2006; Jordan *et al.*, 2007). However, it is activated at the onset of the stationary phase, when bacterial cells are adapting to worsening living conditions, finally leading to the formation of dormant endospores (Jordan *et al.*, 2007).

Three intramembrane sensing TCSs are found from the genome of *B. subtilis*: *bceRS-bceAB* (formerly *ytsAB-ytsCD*), *yvcPQ-yvcRS* and *yxdJK-yxdLM* (Joseph *et al.*, 2002; Joseph *et al.*, 2004). Typical of them all is that TCS encoding genes are located in the immediate upstream region of the genes encoding ABC transporters. Both TCS genes and ABC transporter genes are organized into separate operons with their own promoter regions. TCS genes are expressed constitutively, whereas the genes encoding ABC transporters are under the control of the cognate TCS (Mascher, 2006). The BceRS-BceAB system seems to play a key role in bacitracin resistance in *B. subtilis*. The recognition of sublethal concentrations of bacitracin by BceRS causes the expression of the detoxification unit encoded by *bceAB* responsible for the removal of bacitracin from the cells (Mascher *et al.*, 2003; Ohki *et al.*, 2003). The two other systems, YxdJK-YxdLM and YvcPQ-YvcRS, are poorly characterized, but it seems that YvcRS might have a role in lantibiotic resistance; hence, it is activated by nisin and to a lesser extent by bacitracin (Jordan *et al.*, 2008; Mascher *et al.*, 2003). Despite genetic analysis, the function of the YxdJK-YxdLM system has remained unclear (Joseph *et al.*, 2004).

The YycFG system is the only TCS essential for the viability of *B. subtilis* (Fabret & Hoch, 1998). YycFG is a part of the operon *yycFGHIJ*, in which the *yycH* and *yycI* encode inhibitors of sensor kinase YycG (Szurmant *et al.*, 2005; Szurmant *et al.*, 2007). The YycFG regulon consists of several genes involved in cell wall metabolism and homeostasis (Bisicchia *et al.*, 2007; Dubrac & Msadek, 2004). It seems that YycFG senses some signal derived from normal cell wall metabolism, since the TCS is active under normal growth conditions. The activating signal may be the D-ala-D-ala moiety of lipid II (Dubrac *et al.*, 2008). This hypothesis is further supported by the finding that the YycFG regulon is not differentially expressed under cell envelope stress caused by vancomycin (Bisicchia *et al.*, 2007).

CssRS responds to cell envelope stress caused by the accumulation of misfolded proteins at the membrane-cell wall interface (Darmon *et al.*, 2002; Hyyryläinen *et al.*, 2001). CssRS is a functional orthologue of the Cpx system of *E. coli* (Connolly *et al.*, 1997; Danese & Silhavy, 1997; Missiakas & Raina, 1997; Pogliano *et al.*, 1997) and it determines the level of proteolytic activity in the cell envelope by controlling the expression of *htrA* and *htrB* genes encoding serine-type surface proteases involved in protein quality control (Darmon *et al.*, 2002; Hyyryläinen *et al.*, 2001).

TCSs in *S. aureus*

The *S. aureus* genome consists of 17 potential TCSs (Kuroda *et al.*, 2001) listed in Table 2. TCSs reported to participate in cell envelope stress responses are VraSR, GraRS, YycFG and LytSR.

Table 2. Two-component systems in S. aureus

Kinase	Response regulator	Function
AgrC (SA1843)	AgrA (SA1844)	Quorum sensing, regulation of virulence factors
ArlS (SA1246)	ArlR (SA1247)	Virulence, regulation of exoprotein production
GraS (SA0614)	GraR (SA0614)	CAMP resistance, vancomycin resistance
KdpD (SA1882)	KdpE (SA1883)	Potassium transport
LytS (SA0250)	LytR (SA0251)	Regulation of autolysis
NreB (SA2180)	NreC (SA2179)	Regulation of nitrite/nitrate reduction
PhoR (SA1515)	PhoP (SA1516)	Regulation of phosphatase synthesis
SA0216	SA0215	Unknown
SA1158	SA1159	DesK homologue, adaptation to low temperature
SA1666	SA1667	Unknown
SA2152	SA2151	Heme sensor system
SA2417	SA2418	Unknown
SaeS (SA0660)	SaeR (SA0661)	Virulence, regulation of toxin production
SrrB (SA1322)	SrrA (SA1323)	Respiratory response regulator, virulence
VraS (SA1701)	VraR (SA1700)	Antibiotic resistance, PG synthesis
WalK (SA0017)	WalR (SA0018)	Cell wall and membrane composition, essential

VraSR is a LiaRS homologue originally discovered to be upregulated in a vancomycin-resistant strain of *S. aureus*, and later on also in a vancomycin intermediate-resistant strain (Kuroda *et al.*, 2000; McAleese *et al.*, 2006). The *vraSR* genes are located in the same operon with two upstream genes, one encoding a LiaF homologue and another one encoding a putative protein with unknown function. Unlike LiaRS, the VraSR regulon comprises several genes having a role in cell envelope synthesis and maintenance. The VraSR regulon is induced by cell wall interfering antibiotics such as vancomycin, β-lactams, bacitracin, teicoplanin and D-cycloserine. Deletion mutants of VraSR display increased sensitivity to all inducing antibiotics, excluding fosfomycin and D-cycloserine (Kuroda *et al.*, 2003). VraSR is also activated by perturbations in cell wall assembly (Gardete *et al.*, 2006; Sobral *et al.*, 2007), but not by general sress conditions such as osmotic shock, heat or pH changes (Kuroda *et al.*, 2003).

GraRS is homologous to BceRS in *B. subtilis* and it controls the expression of its downstream genes encoding an ABC transporter, VraFG. In addition, GraRS, also referred as Aps, controls the expression of *dlt*, *mprF* and *vraDE* operons (Li *et al.*, 2007a). GraRS mediates resistance to several CAMPs, including LL-37, gallidermin, polymyxin B and the LP9 peptide derived from human lysozyme (Herbert *et al.*, 2007; Kraus & Peschel, 2008; Meehl *et al.*, 2007). In addition, GraRS mediates vancomycin-intermediate resistance in *S. aureus* (Herbert *et al.*, 2007; Howden *et al.*, 2008). Deletion of *graRS* leads to alterations in the charge of the cell envelope and attenuated virulence (Kraus & Peschel, 2008; Li *et al.*, 2007a).

Like its homologue in *B. subtilis*, YycFG of *S. aureus* is essential for the viability of bacterial cells (Martin *et al.*, 1999). YycFG seems to be a positive regulator of

autolytic activity. YycG-depleted cells have shown changes in their cell wall structure, including an increased glycan chain length and peptidoglycan cross-linking. Due to the importance of YycFG in cell wall metabolism, it was renamed as WalKR (Dubrac *et al.*, 2007). It has also been demonstrated that up-regulation of YycFG confers decreased susceptibility to vancomycin and daptomycin (Friedman *et al.*, 2006; Jansen *et al.*, 2007).

LytSR has been identified to contribute to the regulation of autolysis and murein hydrolase activity in the cell wall (Brunskill & Bayles, 1996). It has been suggested that LytRS senses changes in the membrane potential caused by proton motive force (Patton *et al.*, 2006).

3 Aims of the study

Bacterial cells are amazingly adaptable to changing environmental conditions. The aim of this thesis was to learn more about the stress responses caused by cationic antimicrobial peptides attacking the cell membrane essential for the survival of bacterial cells. These toxic peptides are commonly present in various bacterial habitats and are produced either by other microorganisms competing for the living space or by host cells defending themselves against bacterial invasion. Understanding the mechanisms by which bacteria can resist toxic compounds may help in the development of new antimicrobial drugs in the future.

The specific aims of the study were:

1) To investigate the stress responses caused by different types of cationic antimicrobial peptides in two different Gram-positive bacteria, *B. subtilis* and *S. aureus*.

2) To further characterize role of the highly induced genes in CAMP stress. In particular, the genes participating in cell signalling, ECF σ factors and two-component systems, were subjects of interest.

3) To study the effect of the cell envelope charge on the function of TCSs mediating cell envelope stress.

4) To determine whether transcriptome analysis of bacterial cells treated with CAMPs could reveal something about the action mechanism of peptides.

4 Materials and methods

The bacterial strains and plasmids used in this study are listed in the original articles. Methods and CAMPs used in this study are listed in Tables 3 and 4, respectively.

Table 3. Methods used in this study

Method	Described and used in
Antimicrobial susceptibility tests	I, III
β-Lactamase assay	II
cDNA synthesis	I, II, III
2D electrophoresis	I, II
DNA macroarray	I
Molecular cloning techniques	I, II, III
Oligo DNA microarray	III
Phenotype array	III
Quantitative real-time RT-PCR	I, II, III
RNA isolation	I, II, III
Site-directed mutagenesis	I, II, III

Table 4. Cationic antimicrobial peptides used in transcriptome analyses

Peptide	Sequence	Charge	Origin
Dermaseptin K4-S4(1-16)	ALWKTLLKKVLKAAK-NH_2	+5	Amphibian
LL-37	LLGDFFRKSKEKIGKEFKRI VQRIKDFLLRNLVPRTES-NH_2	+6	Human
Ovispirin-1	KNLRRIIRKIIHIIKKYG-NH_2	+8	Ovine
Protegrin 1	RGGRLCYCRRRFCVCVGR-NH_2	+6	Porcine
Temporin L	FVQWFSKFLGRIL-NH_2	+2	Amphibian
Poly-L-lysine	L-lys_n	+n	Synthetic

5 Results and discussion

5.1 CAMPs induce a strong stress response in bacterial cells (I, III)

5.1.1 CAMP stress response in *Bacillus subtilis* (I)

In order to investigate the stress responses caused by CAMPs in *B. subtilis*, three different peptides were chosen for the transcriptome analysis. Human cathelicidin LL-37 is a linear α-helical peptide, whereas porcine protegrin PG-1 is a cysteine rich peptide forming a β-sheet hairpin structure stabilized by two disulphide bridges (Johansson *et al.*, 1998; Kokryakov *et al.*, 1993). A synthethic peptide, poly-L-lysine (PLL), was selected as a synthetic analogue of cationic peptides (Vaara & Vaara, 1983), although it differs from natural peptides in being a non-amphipathic molecule. LL-37 is predicted to disrupt membranes either by a carpet mechanism or by forming toroidal pores (Henzler-Wildman *et al.*, 2004; Henzler Wildman *et al.*, 2003; Oren *et al.*, 1999). However, it has also recently been suggested that LL-37 may exert its antimicrobial effects by compromising the membrane barrier properties of the target microbes by a mechanism involving cytotoxic oligomers forming amyloid-like fibres (Sood *et al.*, 2008). PG-1 causes rapid lysis of bacterial cells by forming pore-like oligomeric structures known as β-barrels (Mani *et al.*, 2006; Tang & Hong, 2009). The mechanism of action of PLL on membranes is still unclear.

Samples for transcriptome analysis were collected from bacterial cultures treated with sublethal concentrations of CAMPs and were compared with control samples without peptide treatment. The peptides were added at the exponential growth phase and the samples were collected 20 min after the addition of peptides. The amount of peptide needed to cause a slight growth arrest in cell cultures without killing the bacterial cells varied between peptides from a nanomolar concentration of PG-1 to a millimolar concentration of PLL. Both the CAMP-treated and control samples were treated similarly. The purified total RNA was converted to cDNA labelled with P^{33} and hybridized on DNA macroarrays containing all ~4000 ORFs of the *B. subtilis* genome. qRT-PCR was also used to verify some of the most interesting gene expression responses.

As expected, peptides had a strong impact on the gene expression of treated cells. Only the induced genes were analysed further. All genes induced at least two-fold in two independent array experiments were considered as upregulated ones. Altogether, LL-37 activated 96 genes, the induction ratios varying from 2-fold to 60-fold. LL-37 seemed to cause a more severe stress reaction than the other natural peptide PG-1, which caused activation of 58 genes with clearly lower induction

ratios (from 2-fold to 15-fold) than LL-37. The synthetic analogue PLL caused the upregulation of 86 genes (from 2- to 18-fold induction) (I).

The fact that most of the genes responding to CAMP stress were genes with an unknown function made interpretation of the results difficult, but some common features could be identified from the induction patterns of all three peptides. One of the most prominent findings was the activation of genes belonging to the σ^W and σ^M regulons (I, Tables 5 and 6). These two ECF σ factors are predicted to have a role in the maintenance of cell envelope integrity and are activated by several stress conditions such as alkaline shock and antibiotics, interfering peptidoglycan synthesis (Cao *et al.*, 2002b; Wiegert *et al.*, 2001). Genes that were induced by all three peptide treatments belonged to at least one of the ECF σ factor regulons, and many of these genes participate in interactions with cell envelope-interfering compounds (I). Such genes included the *dlt* operon responsible for the D-alanylation of lipoteichoic and wall teichoic acids (Perego *et al.*, 1995) contributing to increased resistance to antimicrobial peptides (Cao & Helmann, 2004; Peschel *et al.*, 1999), and *bcrC* encoding an alternative undecaprenyl pyrophosphate phosphatase related to bacitracin resistance (Bernard *et al.*, 2005; Cao & Helmann, 2002). Interestingly, only some of the genes regulated by these σ factors were induced. Of the 30 verified promoters controlling the expression of about 60 genes of the σ^W regulon (Cao *et al.*, 2002a; Huang *et al.*, 1999), about one third were induced by the natural peptides LL-37 and PG-1. PLL was the most effective activator of the σ^W regulon and induced 23 out of the 30 verified promoters known to belong to this regulon (I). The same phenomenon was also seen in the case of σ^M-regulated genes, although LL-37 seemed to be a more effective activator of σ^M than the other two peptides tested (I).

Table 5. Induction of the σ^W regulon by CAMPs

Category/Operons*	Function or Homology	Upregulation by LL-37	PG-1	PLL
Regulation				
sigW rsiW	SigW and its anti-σ factor		+	+
ysdB	Negative regulator of SigW			
Cell envelope synthesis				
pbpE	Penicillin binding protein	+	+	+
racX	Amino acid racemase	+	+	+
yuaFGI (?)	YuaG: similar to flotillin	+	+	+
Resistance to toxic peptides				
fosB	Fosfomycin resistance			
yfhLM	SdpC resistance		+	+
yknWXYZ	SdpC resistance		+	+
YqeZyqfAB	Sublancin resistance	+	+	+
ydbST	Resistance to *B. amyloliquefaciens*		+	+
Predicted proteases				
yjoB	ATPase possibly involved in protein degradation			+
yteIJ	Putative integral inner membrane protein			
Detoxification (?)				
ybfO	Putative exported hydrolase			
yceC	Putative stress adaptation protein	+	+	+
ydjP	Putative peroxydase			
ythPQ	Putative ABC transporter			+
Small peptides (bacteriocins?)				
ydjO				
yvlC				
yxzE				
yoaF				+
yoaG				

*Possible functional roles based on sequence similarities are indicated by question marks.
σ^W-dependent genes with an unknown function are not listed. Categories of genes adapted from Helmann 2006.

Table 6. Induction of the SigM regulon by CAMPs

Category/Operon	Function or Homology	Upregulation by LL-37	PG-1	PLL
Regulation				
sigM *yhdLK**	SigM and its anti-σ factor	+		+
yjbCspx	Spx transcription factor	+	+	+
abh	Transition state regulator		+	
ywaC	Putative ppGpp synthase			+
rapD	Response regulator aspartate phosphatase			
ywtF	Transcription factor			
Cell division/shape				
divIC	Component of septosome	+		
*(**murG**)B divIB ylxXW spb**	Cell division	+	+	
rodA	Cell division membrane protein			
*(maf)**ysxA**mreBCD**minCD**	Cell division and shape determination	+		
Cell envelope synthesis				
yfnI	Similar to lipoteichoic acid synthase			
bcrC	Undecaprenyl pyrophosphate (UPP) Phosphatase	+	+	+
*(**ydbO**-ydbP(as))-ddlmurF**	MurF: D-ala-D-ala ligase	+		
*recU**ponA**	PG synthesis, ponA: penicillin-binding protein	+		+
dltABCDE	D-alanylation of teichoic acids	+	+	
pbpX	Low MW penicillin-binding protein		+	
DNA monitoring and repair				
(sms)disA yacLM	DisA: DNA integrity monitoring protein			
Detoxification				
yrhHIJ	YrhH:putative methyltransferase, YrhJ:cytochrome P450	+		+
yqjL	Hydrolase, paraquat resistance			
yceCDEFG	Tellurium resistance operon	+	+	+

*The whole operon was not induced. The induced genes are marked with bold letters.
σ^M-dependent genes with an unknown function are not listed. Categories of genes adapted from Eiamphungporn and Helmann 2008.

On the other hand, several induced genes did not belong to any of the ECF sigma factor regulons (I). These results suggested that other regulators of transcription also mediate the stress response caused by CAMPs. The most strongly induced genes included *liaI* (58-fold induction by LL-37 and 15-fold induction by PG-1) and other genes of the *lia* operon and *yxdL* (22-fold induction by LL-37), which was strongly and specifically induced by LL-37 (I). Both *liaI* and *yxdL* encode proteins with an unknown function (see below). Neither of these operons is regulated by ECF σ factors. However, both operons are controlled by two-component systems adjacent to genes they are regulating. Taken together, these observations suggest that antimicrobial peptides elicit a complex stress response in *B. subtilis*, with several cell signalling systems involved.

Differences also existed in the stress responses activated by different peptides, in particular between natural peptides and PLL. For example, genes involved in purine, pyrimidine and ribosomal protein synthesis were strongly induced in cells treated with PLL, a phenomenon not seen with the natural peptides. Furthermore, some genes that were induced at high levels by either or both of the natural peptides were not activated by PLL as it was the case with the *lia* operon (I).

5.1.2 CAMP stress response in *Staphylococcus aureus* (III)

S. aureus is relatively resistant to CAMPs produced by mammalian cells. In this study, transcriptome analysis was used to determine whether certain special features exist in the CAMP stress response due to the pathogenic nature of *S. aureus*. The experimental setting was similar to that used in the transcriptome analysis of *B. subtilis*. All peptides used in the experiments were well-characterized linear α-helical peptides. This allowed us to determine whether strong peptide-specific responses or more general stress responses are triggered by all α-helical peptides in general. Ovispirin-1 is a derivative of ovine cathelicidin SMAP-29, whereas temporin L and dermaseptin S4 are expressed on the skin of amphibians (Navon-Venezia *et al.*, 2002; Sawai *et al.*, 2002; Simmaco *et al.*, 1996). A truncated derivative of S4 known as dermaseptin K4-S4(1-16) was used in this study (Navon-Venezia *et al.*, 2002). Temporin L is predicted to be a pore-forming peptide, whereas the other two peptides are predicted to act in a carpet-like manner (Brogden, 2005; Rinaldi *et al.*, 2002; Zhao & Kinnunen, 2002; Zhao *et al.*, 2002). All three peptides were C-terminally amidated in order to prevent possible proteolytic degradation. In addition, the effect of human cathelicidin LL-37 on gene expression of *S. aureus* was studied.

In order to capture the early stages of the stress response, samples were collected 10 min after peptide treatment. Peptides were added at sublethal concentrations causing a slight growth arrest, the exception being LL-37, as it turned out that *S. aureus* strain Newman was highly resistant to LL-37. The peptide concentrations used in array experiments were: 3 μM temporin L, 4 μM ovispirin-1, 3 μM dermaseptin K4-S4(1-16) and 3 μM LL-37. The changes in gene expression were analyzed using whole genome oligoarrays with Cy5- and Cy3-labelled cDNA (Charbonnier *et al.*, 2005). Three independent array experiments were performed. The results were verified by qRT-PCR in the case of the most interesting genes.

All three α-helical peptides caused induction of a large number of genes, most of which were upregulated by more than one peptide. Temporin L and ovispirin-1 caused the strongest effects on gene expression and upregulated 247 and 241 genes, respectively. Up to 40-fold induction ratios were detected with these two peptides. Dermaseptin K4-S4(1-16) induced 63 genes (from 2-fold to 17-fold induction) (III). LL-37 induced the expression of only 27 genes with low induction ratios, possibly reflecting lower stress in the LL-37-treated cells compared to cells treated with other peptides (Table 7). All α-helical peptides studied seemed to cause a similar kind of transcriptional response, regardless of the origin of the peptides. No significant differences were detected between the pore-forming and carpet-forming peptides, indicating that the mechanism of membrane dysfunction does not play a major role in the stress response. The induction patterns of CAMPs were strikingly similar to those observed in transcription profiling studies with vancomycin-treated cells (Kuroda *et al.*, 2003). A prominent feature was the induction of the VraSR two-component system, and consequently almost the whole VraSR regulon by CAMPs (III). In addition to vancomycin, VraSR is activated by other cell envelope interfering antimicrobial peptides, including bacitracin, mercacidin and daptomycin (Muthaiyan *et al.*, 2008; Sass *et al.*, 2008a; Utaida *et al.*, 2003). Surprisingly, the

results indicated that human cathelicidin LL-37 does not induce genes belonging to the VraSR regulon, the only exception being *prsA* (Table 7).

Table 7. Genes induced in S. aureus cells treated with LL-37

		Fold induction*			
Gene	**LL-37**	**Tem L**	**Ovi-1**	**Derm**	**Protein/Similarity**
clpB	2.2	3.9	4.1		ClpB chaperone homologue
ctsR	2.1	4.2	4.2		Transcription repressor of class III stress gene homologue
dnaJ	2.0	2.2	2.6		DnaJ protein
dnaK	2.1	2.5	2.9		DnaK protein
fhuG	2.0				Ferrichrome transport permease
groEL	2.3	3.8	4.3	2.2	GroEL protein
groES	2.0	3.2	3.6	2.4	GroES protein
lysC	2.0	5.8	8.3		Aspartokinase II
prsA	2.7	3.2	4.5	2.9	Peptidyl-prolyl cis/trans isomerase homologue
pyrE	2.1				Orotate phosphoribosyltransferase
pyrF	2.2				Orotidine-5-phosphate decarboxylase
ribA	2.1				Riboflavin biosynthesis protein
SA0205	2.1	17.2	11.2	6.2	Similar to lysostaphin precursor
SA0335	2.1	2.1	2.1		Component of twin-arginine translocation pathway
SA0336	2.2	2.2	2.1	2.1	Hypothetical protein
SA0481	2.0	4.1	4.0		Conserved hypothetical protein
SA0482	2.0	4.2	4.3		Putative ATP:guanido phosphotransferase
SA1406	2.0	2.4	2.9	2.1	Conserved hypothetical protein
SA1407	2.1	2.6	3.3		Conserved hypothetical protein
SA1599	2.1	3.2	2.4		Similar to transaldolase
SA1683	2.2				ABC transporter (ATP-binding protein) homologue
SA1820	2.1	3.6	3.0	2.2	Similar to bacteriophage terminase small subunit
SA1821	2.0	3.1	2.8	2.0	Hypothetical protein
SA2162	2.0	2.4	2.7		Similar to thioredoxin reductase
SAS016	2.5	5.0	7.4	5.4	Hypothetical protein
vraD	5.0	8.2	32.4	17.2	Similar to ABC transporter
vraE	2.0	3.4	11.8	6.2	Similar to ABC transporter

* For comparison, the induction ratios from DNA microarray experiments with other CAMPs are also listed if detected. tem L = temporin L, ovi-1= ovispirin-1, derm = dermaseptin K4-S4(1-16)

Strong induction of three operons emerged in all transcription profiles of CAMP-treated bacterial cells (III). These operons were *vraDE*, SA0205 and SAS016 encoding an ABC-type transporter similar to BceAB-like transporters in *B. subtilis* (Mascher *et al.*, 2003; Ohki *et al.*, 2003), a lysostaphin-like cell wall peptidase and a functionally unknown peptide of 55 amino acids, respectively. *vraDE* was also the most strongly induced operon in cells treated with LL-37 (Table 7). Surprisingly, no significant induction of the previously described genes contributing to CAMP resistance, such as *dlt* operon or *mprF* (Peschel *et al.*, 1999; Peschel *et al.*, 2001), was detected (III).

Antimicrobial peptide treatment also caused a response in general stress genes such as the *ctsR-clpC* operon, *groELS* and *dnaJK*, indicating that *S. aureus* cells try to adapt to the stress caused by CAMPs (III). Some of these target genes showed similar induction in *S. aureus* during phagocytosis by neutrophils or when surviving within epithelial or phagocytic cells (Garzoni *et al.*, 2007; Voyich *et al.*, 2005). Notably, several amino acid biosynthesis operons and genes encoding enzymes of the citric acid cycle were also induced (III). Simultaneous repression of genes

involved in anaerobic metabolism (Fuchs *et al.*, 2007) indicates that the cells were metabolically active and aerobically respiring. Another interesting phenomenon resulting from the CAMP treatments was the down-regulation of several virulence factors and their regulators, such as *saeRS* and *agr* (III).

5.2 ECF sigma factors and LiaRS-like TCSs mediates stress responses caused by damages in the cell envelope (I, III)

Transcription analysis revealed that CAMPs cause the induction of several signal transduction pathways. *In B. subtilis*, cell envelope stress caused by CAMPs was mainly mediated through ECF σ factors σ^W and σ^M and TCSs such as LiaRS (I). In *S. aureus*, strong activation of TCS VraSR was detected (III). All these regulatory systems have been reported to respond to disturbances of the cell envelope caused by a variety of cell envelope-interfering conditions.

5.2.1 SigW and SigM in *B. subtilis* (I)

A prominent finding in the transcriptome analysis was the activation of σ^W and σ^M ECF σ factors under CAMP stress (I, see also Tables 5 and 6 in section 5.1.1). σ^W also responds to a large number of antimicrobial compounds other than CAMPs, such as cephalosporin, vancomycin, D-cycloserine, cell membrane-active nigericin and Triton X-100, and more mildly to fosfomycin, bacitracin and tunicamycin (Cao *et al.*, 2002a). σ^M responds to several stress conditions interfering with the cell envelope, such as antibiotics, acid, ethanol, heat and superoxide stresses (Thackray & Moir, 2003).

The DNA macroarray results indicated that σ^W and σ^M have an important role in CAMP stress resistance in *B. subtilis*. However, neither σ^W, σ^M, nor the σ^W/σ^M double mutant showed increased sensitivity to any of the peptides used in this study (I). One possible explanation for this could be cross-regulation between different ECF σ factors. It has been demonstrated that regulons of different σ factors are partially overlapping, as in the case of σ^W, σ^M and σ^X (Cao *et al.*, 2002a; Huang *et al.*, 1998; Mascher *et al.*, 2007). Sigma factor σ^X also contributes to the maintenance of cell envelope integrity (Cao & Helmann, 2004), and it was induced by one peptide studied here, PG-1 (I). In order to find out more about the cross-regulation between different ECF σ factors, we carried out DNA array analysis with *sigM* and *sigW* knockout mutants using LL-37 for the induction. LL-37 treatment elicited a significantly lower number of induced genes in both σ mutants than in the wild type (I). As expected, the inactivation of one individual ECF σ factor silenced the genes it regulates, but the genes under the control of the other ECF σ factor were also induced at a decreased level. Surprisingly, the array data also showed that some genes that are expressed independently of σ^M and σ^W were also attenuated (I). These results suggest significant cross-talk between the σ^M and σ^W regulons, although it is apparent that not all the affected genes are directly regulated by σ factors. The functional overlap of several σ factors may be one possible explanation why the inactivation of σ^W, σ^M or both of them did not cause increased sensitivity to

antimicrobial peptides. This idea is further supported by the fact that the level of transcription of σ^W is elevated in cells lacking σ^X (Helmann, 2006; Huang *et al.*, 1998). The redundancy of σ^W, σ^M and σ^X regulons was confirmed in a recent study where inactivation of all three ECF σ factors made cells significantly more sensitive to several antibiotics compared to the wild type (Mascher *et al.*, 2007).

But what is the role of σ^W and σ^M in CAMP resistance, if any? Previously, it has been demonstrated that ECF σ factors are not active under normal laboratory growth conditions but are activated in specialized conditions such as cell envelope stress caused by cell envelope-interfering compounds (Helmann, 2002; Helmann, 2006). In fact, cells without any functional ECF σ factor present are still viable, at least under laboratory conditions (Asai *et al.*, 2008).

σ^W is the most thoroughly studied of the seven ECF σ factors found in *B. subtilis*. It is activated by several antibiotics and other cell envelope-interfering compounds. However, σ^W mutants only show increased sensitivity to fosfomycin (Cao *et al.*, 2002a). Thus, it seems that most of the σ^W-inducing agents are so-called gratuitous inducers: the activation of σ^W does not provide any advantage against the inducers (Helmann, 2006). This phenomenon is also seen in alkaline shock, where the σ^W regulon is strongly induced but inactivation of σ^W does not cause any significant changes in the sensitivity (Wiegert *et al.*, 2001). This might also be true in the case of CAMPs.

Recent studies have shed some light on the functional roles of the genes regulated by σ^W. Many of these genes were also induced by LL-37, PG-1 and/or PLL (Table 5). At least six operons controlled by σ^W have a verified role in resistance against antimicrobials, especially toxic peptides. SdpC is a toxic peptide produced by sporulating *B. subtilis* cells in order to lyse nonsporulating siblings in a process known as cannibalism (Ellermeier *et al.*, 2006; Gonzalez-Pastor *et al.*, 2003). Cells producing SdpC are resistant to its toxic effects, since they also simultaneously express immunity protein SdpI (Ellermeier *et al.*, 2006). In the absence of immunity protein SdpI, resistance to SdpC is mediated through σ^W. Two operons controlled by σ^W are associated with SdpC resistance: the *yknWXYZ* operon encoding ABC transporter and *yfhLM*, in which *yfhL* encodes a membrane protein homologous to SdpI immunity protein (Butcher & Helmann, 2006). Genes regulated by σ^W also contribute to resistance against the bacteriocin sublancin, which is encoded by the SPB prophage of *B. subtilis* (Butcher & Helmann, 2006).

B. subtilis strains are able to produce more than 20 antimicrobial compounds. The genes needed for the synthesis of toxic compounds as well as specific immunity genes are usually located on mobile genetic elements only present in some strains of *B. subtilis* (Stein, 2005). It has been proposed that σ^W provides broad-based intrinsic immunity to different kinds of antimicrobial compounds produced by *B. subtilis* or other closely related microbes (Helmann, 2006). From this perspective, it is not surprising that σ^W also responds to stress caused by CAMPs (I). CAMPs may cause similar disruption of the cell envelope than antimicrobials produced by bacterial

cells, leading to countermeasures by σ^W, even though they may not be efficient against CAMPs.

At the time the transcriptome analysis of *B. subtilis* cells treated with CAMPs was performed (I), little was known about the σ^M regulon and its function. Only a few genes had been verified to belong to the σ^M regulon (Horsburgh & Moir, 1999; Thackray & Moir, 2003). Today, knowledge of the σ^M regulon is more focused and ~57 genes in 30 operons have been identified to be regulated by σ^M (Eiamphungporn & Helmann, 2008; Jervis *et al.*, 2007). Many of these genes were previously assigned to σ^W and σ^X regulons. Due to the redundancy of antibiotic-inducible ECF σ sigma factors, σ^M does not usually cause increased sensitivity to cell envelope active compounds with the exception of bacitracin, moenomycin, SDS and some β-lactam antibiotics (Cao *et al.*, 2002b; Mascher *et al.*, 2007).

The σ^M regulon contributes to several central functions in the maintenance of cell envelope integrity, and its induction is referred to as a good reporter for interference in cell envelope synthesis and function (Eiamphungporn & Helmann, 2008). This is in agreement with our results (I), showing that CAMPs also activate many genes belonging to the σ^M regulon (Table 6). It has been demonstrated that σ^M regulates several other regulatory proteins, one of the most interesting being Spx, which controls several genes contributing to the maintenance of reducing conditions inside bacterial cells (Nakano *et al.*, 2005; Zuber, 2004). The gene encoding this transcription regulator, *spx* (formerly *yjbD*), was upregulated by all three CAMP treatments (I). It has been postulated that some bactericidal antibiotics generate reactive oxygen species (Kohanski *et al.*, 2007) and Spx-dependent genes may have a role in preventing cell death. Another interesting finding was the strong induction of *yrhH* by LL-37, and also to a lesser extent by PLL (I). *yrhH* encodes a putative methyltransferase and it has been suggested that the *yrhHIJ* operon might contribute to the regulation of membrane fluidity or in the defence against toxic fatty acids (Eiamphungporn & Helmann, 2008). The possible role of *yrhHIJ* genes in the CAMP resistance remains to be elucidated.

5.2.2 LiaRS and VraSR have different roles in the defence against antimicrobial agents (I, III)

LiaRS of *B. subtilis* and its homologue in *S. aureus*, VraSR, responded strongly to CAMPs (I, III). LiaRS (**l**ipid II-**i**nteracting **a**ntibiotics response **r**egulator and **s**ensor) controls the expression of *liaIH* genes with an unknown function and responds to several stress conditions. VraSR (**v**ancomycin **r**esistance **a**ssociated **s**ensor and **r**egulator) has been reported to react to several cell wall-interfering antibiotics. The genomic context of the *lia/vra* loci of *B. subtilis* and *S. aureus* differs from each other (Fig. 6), probably reflecting the differences in their physiological functions. The *liaIHGFSR* locus of *B. subtilis* contains six genes, while the *vra* locus of *S. aureus* contains only four genes. Homology can be found between the response regulators, sensor kinases and the inhibitory proteins (LiaF and SA1702), but not between the other proteins. The major difference between these two regulatory

systems seems to be the number of the genes they regulate. Only two operons, *liaIHGFSR* and *yhcYZ-yhdA*, are known to be under the regulation of LiaRS TCS, the *lia* operon being the main target (Jordan *et al.*, 2006). On the contrary, the large VraSR regulon in *S. aureus* comprises ~46 genes involved in cell wall associated functions such as protein quality control, protein folding and the modulation of cell wall biosynthesis (Kuroda *et al.*, 2003).

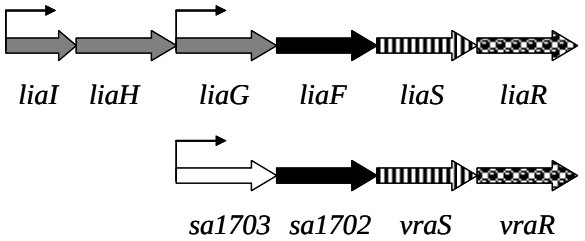


Figure 6. The genomic context of lia and vra operons. Genes are not drawn to scale. Dotted arrows represent response regulators and arrows with vertical lines represent histidine kinases. Gene encoding inhibitory protein LiaF and its homologue SA1702 are marked as black arrows. The functions of the other genes in the operons are unknown.

The *liaIH* genes were among the most strongly induced genes in *B. subtilis* cells treated with LL-37 (58-fold induction) and PG-1 (15-fold induction) (I). The *liaIH* genes reacted to CAMPs in a very rapid and transient way, as was shown in the qRT-PCR measurements; the expression of *liaI* was at the highest level after 10 minutes of exposure to LL-7 and declined to the basal level after two hours (I). The increase in *liaIH* expression was also confirmed at the level of translation, as LiaH was identified from the proteome analysis of cytoplasmic proteins (I). The function of LiaIH proteins is still a mystery. LiaI is a small hydrophobic protein with two putative transmembrane helices, while cytoplasmic LiaH shares some similarity with phage-shock protein PspA of *E. coli* possibly involved in the maintenance of cell envelope integrity (Darwin, 2005; Jordan *et al.*, 2007; Kleerebezem *et al.*, 1996). Even though the *lia* operon is strongly activated by cell wall-active antibiotics such as vancomycin, ramoplanin, bacitracin and nisin, the deletion of this operon did not cause increased sensitivity to any of the inducers tested (Jordan *et al.*, 2007; Mascher *et al.*, 2003; Mascher *et al.*, 2004). It is also activated to a lesser extent by various stress-causing compounds and conditions such as fosfomycin, tunicamycin, detergents, ethanol, phenol, alkaline shock and secretion stress, indicating that LiaRS TCS broadly senses various stress conditions (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Tam le *et al.*, 2006). In this study, both the natural CAMPs as well as Triton X-100 induced the *lia* operon, but somewhat surprisingly LiaRS did not respond to the synthetic peptide PLL (I).

The *lia* operon is also induced at the onset of the stationary phase without any exogenous stimuli (Jordan *et al.*, 2007). Cells entering the stationary phase have to adapt to the worsening living conditions, which may eventually lead to endospore formation (Errington, 2003; Msadek, 1999; Phillips & Strauch, 2002). The transition state regulator AbrB prevents P_{liaI} activity during logarithmic growth by directly

binding to the promoter region of *liaI*. In the stationary growth phase, AbrB repression is released by the Spo0A sporulation regulator and the *liaI* promoter is induced by some intrinsic stimulus (Jordan *et al.*, 2007). At least one intrinsic peptide encoded by *yydFGHIJ* operon activating LiaRS has been identified (Butcher *et al.*, 2007). Interestingly, a small peptide with 49 amino acids encoded by the *yydF* gene is predicted to be positively charged and able to adopt an α-helical conformation, both properties associated with CAMPs. It seems that *yydF* is a signalling molecule rather than an antimicrobial peptide (Butcher *et al.*, 2007).

Similarly to its homologue in *B. subtilis*, VraSR TCS in *S. aureus* was activated by the α-helical CAMPs temporin-L, dermaseptin and ovispirin-1 (III). The induction pattern of the VraSR-regulated genes was very similar to that observed with vancomycin-treated cells (Kuroda *et al.*, 2003). In contrast to LiaRS, the inactivation of *vraSR* leads to increased sensitivity to most of its inducers, as observed in previous studies with several cell wall-active antibiotics (Gardete *et al.*, 2006; Kuroda *et al.*, 2003). This was also seen in this study, as the *vraSR* deletion mutant was shown to be more sensitive to teicoplanin, bacitracin and a wide range of β-lactams, as well as to more unusual compounds with antimicrobial activity such as EGTA, phenothiazines and sodium tungstate (III). As CAMPs were strong inducers of the VraSR regulon, we expected to see increased sensitivity to CAMPs in *vraSR* deletion mutants. However, only slightly increased susceptibility to ovispirin-1 was observed. The inactivation of v*raSR* did not affect the sensitivity to other bacteriocins or CAMPs tested, including nisin, daptomycin, Pep5, LL-37 and hBD3 (III).

When the functional roles of the two homologous TCSs, LiaRS and VraSR, are compared, it seems that LiaRS is a general sensor for worsening living conditions and is activated by a broad spectrum of stress conditions, while VraSR protects the cells from various cell wall-disturbing agents. This is further supported by the fact that VraSR is activated by a variety of cell wall-active antibiotics, but is not induced by general stress conditions such as heat, osmotic shock or changes in pH (Kuroda *et al.*, 2003). The exact signal that these TCSs sense is still under investigation. It has been postulated that VraSR senses some byproduct formed when peptidoglycan sythesis is disturbed (Belcheva & Golemi-Kotra, 2008; Boyle-Vavra *et al.*, 2006). In support of this concept, VraSR is not activated in LL-37-treated cells (Table 7). The *S. aureus* strain Newman was found to be highly resistant to LL-37. Disturbances in the cell wall do not therefore probably take place and VraSR remains in inactivated stage.

5.3 BceRS-like TCSs specifically respond to antimicrobial peptides (I, III)

As LiaRS and VraSR seem to react to cell envelope stress in general, the specific recognition of toxic peptides is mediated through BceRS-like TCSs in both *B. subtilis* and in *S. aureus* (I, III). Characteristic of TCSs of this kind is that they control the expression of the ABC transporter encoding genes adjacent to them. The genome of *B. subtilis* harbors three TCS-ABC transporter systems of this kind: *yxdJK-yxdLM*, *bseRS-bceAB* and *yvcPQ-yvcRS* (Joseph *et al.*, 2002). The *yxdLM* operon was strongly and specifically induced by LL-37 (I). Two homologues of *yxdLM* can be found from the *S. aureus* genome: *vraDE* and *vraFG*. The former was strongly activated by all CAMPs tested, while only slight induction of *vraFG* was detected in the qRT-PCR measurements (III). The genomic context of these two ABC transporter operons of *S. aureus* differs somewhat from that found in *B. subtilis*. An operon encoding a three-component regulatory system known as Aps (also referred to as GraRS) (Li *et al.*, 2007a; Meehl *et al.*, 2007) is located adjacent to *vraFG* operon, whereas no other operon can be identified from the immediate proximity of *vraD* (Fig. 7).

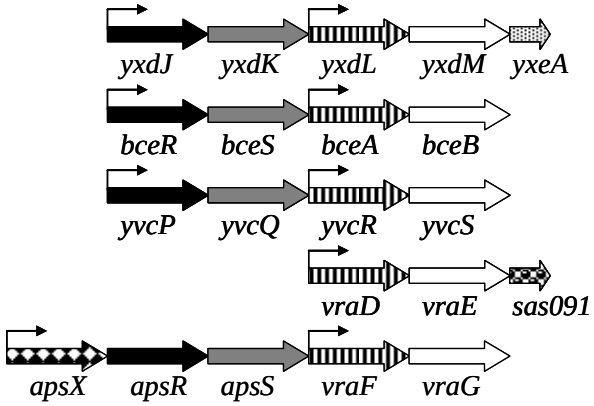


Figure 7. Comparison of the genomic context of yxdJK-yxdLM operons and their homologues in B. subtilis and S. aureus. Genes are not drawn to scale. Black arrows represent response regulators and grey arrows sensor kinases of two-component systems. Arrows with vertical lines represent the ATP-binding proteins and white arrows the permease proteins of ABC transporters. The function of ApsX, YxeA and SAS091 is unknown.

5.3.1 LL-37 is a specific inducer of the YxdLM ABC transporter (I)

LL-37 was revealed to be a strong and specific inducer of the *yxdLM* operon in *B. subtilis* (I). Both *yxdLM* homologues, *bceAB* and *yvcRS*, were also slightly induced in cells treated with LL-37, but clearly to a lesser extent compared to *yxdLM* (I). Transription of *yxdLM, bceAB* and *yvcRS* operons is controlled by the adjacent TCS responding to the extracellular presence of antimicrobial compounds (Joseph *et al.*, 2002; Joseph *et al.*, 2004; Mascher *et al.*, 2003). The regulation of these three operons was further studied by determining their expression in the wild-type strain and mutants of the upstream histidine kinase genes (*yxdK*, *yvcQ* and *bceS*) treated with LL-37 by using qRT-PCR (I, Table 8). The results suggest that the expression of *yxdLM* is tightly regulated by YxdJK and not affected by the two other sensory systems (I). The slight downregulation of *yxdL* gene observed in the *yvcQ* and *bceS* mutants probably resulted from the high variation between individual qRT-PCR measurements. On the contrary, it seems probable that expression of the *yvcRS* operon is partly regulated by YxdJK, whereas expression of the *bceAB* operon is not affected either by YxdJK or YvcQP (I). The cross-regulation between these TCS ABC transporter systems is further supported by a recent study in which it was demonstrated that the YvcPQ-YvcRS system indirectly responds to bacitracin only at high concentrations in a BceRs-BceAB-dependent manner (Rietkötter *et al.*, 2008).

Table 8. Cross regulation of the BceRS-like TCSs

TCS-mutation	**Transcription of operon**		
	yxdLM	*yvcPQ*	*bceAB*
yxdJK	0	↓	no effect
yvcQP	(↓)	↓	no effect
bceSR	(↓)	no effect	↓

The effect of inactivation of TCS on the transcription of ABC transporter encoding genes: 0 no transcription, ↓ decreased transcription, no effect = transcription level same as in WT, parentheses = decreased transcription is probably due to the high variation between separate experiments

Studies with *B. subtilis* have revealed that ABC transporters encoded by *yxdLM* and its homologues have a role in the removal of harmful constituents. The BceRS-BceAB system is the best characterized of these detoxification modules and it has been shown to be the primary and most efficient factor in bacitracin resistance (Joseph *et al.*, 2002; Joseph *et al.*, 2004; Mascher *et al.*, 2003; Ohki *et al.*, 2003). Bacitracin is a cyclic nonribosomally synthesized dodecylpeptide produced by *B. licheniformis* and some *B. subtilis* strains (Azevedo *et al.*, 1993; Ishihara *et al.*, 2002). The direction of bacitracin transport by BceAB is still a matter of debate (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008). The YvcPQ-yvcRS system is also

induced by bacitracin and nisin, but its exact function is still unclear (Jordan *et al.*, 2008; Mascher *et al.*, 2003).

Despite the strong induction of the *yxdLM* operon by LL-37, it seems that LL-37 is not the substrate for the YxdLM ABC transporter. Neither the *yxdL* mutant nor the *yxdL/yvcR* double mutant showed increased sensitivity to LL-37 (I). It is possible that LL-37 resembles some as yet unidentified molecule and is therefore able to activate the YxdJK sensory system. The specific activation of YxdJK TCS only by LL-37 may indicate direct binding of activating ligand to the sensor kinase. Direct sensing of a signal molecule has been postulated for the BceRS-BceAB system (Rietkötter *et al.*, 2008). Interestingly, it seems that bacitracin is sensed during its transport through BceAB, and the large periplasmic loop of the BceA permease component of the transporter may be involved in mediating the signal to the BceRS TCS (Rietkötter *et al.*, 2008). The function of YxdJK-YxdLM remains to be elucidated, but it is still tempting to speculate that it plays some role in resistance against toxic peptides.

5.3.2 VraDE confers resistance to bacitracin (III)

All CAMP treatments caused strong induction of the ABC transporter encoding the *vraDE* operon in *S. aureus*. In addition to CAMPs, bacitracin also caused strong, almost 500-fold induction of gene expression (III). VraDE induction has also been demonstrated with other antimicrobial peptides such as human β-defensins and the lantibiotic mersacidin (Sass *et al.*, 2008a; Sass *et al.*, 2008b). In order to determine whether *vraDE* has any role in resistance against antimicrobial compounds, susceptibility tests with *vraDE* mutants were performed (III). The CAMPs tested included ovispirin-1-NH_2, temporin L-NH_2, two lantibiotics (nisin and Pep5), human cathelicidin LL-37, and hBD3 defensin. In addition, MICs for vancomycin, teicoplanin, bacitracin and daptomycin were determined. In contrast to the results of a previous study postulating the possible role of VraDE in CAMP resistance (Sass *et al.*, 2008b), the *vraDE* mutant showed increased sensitivity to bacitracin (about 10-fold difference in MIC), but no other differences were observed in antimicrobial sensitivities (III). These results strongly indicate that VraDE is a bacitracin-specific detoxification module, similarly to BceAB in *B. subtilis*.

As mentioned above, no TCS-encoding operon is located adjacent to *vraDE* genes (Fig. 7). Expression of the *vraDE* operon is controlled by the Aps (also referred to as GraRS) system. Aps also regulates the expression of the neighbouring *vraFG* operon encoding an ABC transporter homologous to *vraDE*. In addition, Aps controls the expression of *mprF* and *dlt* operons responsible for the overall charge of the cell envelope (Kraus *et al.*, 2008; Li *et al.*, 2007a). It seems that Aps is one of the major mediators in CAMP-induced stress responses in both *S. aureus* and *S. epidermidis* (Li *et al.*, 2007a; Li *et al.*, 2007b). This is further supported by the observation that natural mutation in the ApsS sensor of the *S. aureus* strain SG511 leads to increased susceptibility to CAMPs (Sass & Bierbaum, 2009). The structure of the sensor component ApsS of *S. aureus* differs from that of *S. epidermidis*, leading to the differential CAMP recognition in these two bacterial species. Furthermore, it seems that the short extracellular loop of ApsS directly interacts with CAMPs, activating

the sensor (Li *et al.*, 2007a). Our results also indicated that the C-terminal amide group in linear peptides is an important element for the activation of the Aps sensory system, as high-level induction of *vraDE* by ovispirin-1 was shown to be dependent on the amide modification of the C-terminus (III).

It has also been demonstrated that the VraFG transporter confers resistance to several CAMPs, including hBD3, nisin, indolicidin and LL-37 (Li *et al.*, 2007a). It is, however, quite surprising that only slight induction of the *vraF* gene by ovispirin-1-NH2 and no induction at all by other peptides was detected (III). Considering the simultaneous strong induction of *vraDE*, it is obvious that other regulatory elements than Aps must also be involved in the regulation of *vraDE* expression (III). The existence of an unidentified regulator of *vraDE* was also suggested in a recent study by Li *et al*. (2007a).

5.4 D-alanylation of teichoic acids affects the signal sensing by TCSs (II)

The Dlt system seems to play a crucial role in resistance against CAMPs in *S. aureus* and other Gram-positive bacteria (Fabretti *et al.*, 2006; Kovacs *et al.*, 2006; Peschel *et al.*, 1999). In *B. subtilis*, DltA, DltB, DltC and DltD comprise the Dlt system responsible for the D-alanylation of both lipoteichoic and wall teichoic acids, leading to a decreased net negative charge in the cell wall matrix (Perego *et al.*, 1995). In this study we wanted to investigate how the charge of the cell envelope affects signal sensing through TCSs. The TCSs of interest were CssRS, LiaRS and YxdJK. CssRS was initially found out to participate in the maintenance of cell envelope integrity under secretion stress. It responds to the accumulation of misfolded proteins and controls the expression of *htrA* and *htrB* genes encoding serine-type surface proteases involved in quality control (Darmon *et al.*, 2002; Hyyryläinen *et al.*, 2001). Interestingly, inactivation of either CssS or DltD seems to cause similar effects on the quality control of secreted proteins, as the stability and secretion of some misfolded or heterologous proteins is improved in both of the mutants (Hyyryläinen *et al.*, 2000; Hyyryläinen *et al.*, 2001; Vitikainen *et al.*, 2005).

The TCSs of interest were induced either by secretion stress caused by α-amylase (AmyQ) hypersecretion from the plasmid pKTH10 or by treating cell cultures with LL-37 or other cell envelope-interfering substances. The effects of inactivation of the Dlt system on the activity of three different TCSs of *B. subtilis* were analyzed by β-galactosidase assays and qRT-PCR measurements.

In order to determine how the *dlt* mutation affects the expression of *htrA* and *htrB* genes, a *lacZ* reporter gene was inserted into *htrA* and *htrB* genes, enabling the measurement of transcription activity from the promoters P_{htrA} and P_{htrB} using β-galactosidase assays. AmyQ hypersecretion resulted in an approximately 10-fold induction of expression in the wild type strain, and similar induction of expression from the P_{htrB} promoter was also detected (II). On the contrary, hardly any induction of P_{htrA} and P_{htrB} was observed in the *dltD* mutant (II). These results were also

verified by qRT-PCR measurements suggesting that the D-alanylation of teichoic acids modulates signal transduction via CssRS. We were also able to demonstrate a decreased level of HtrA protein in the culture medium of the *dlt* mutant compared to the wild type (II).

Another TCS responding to severe secretion stress in *B. subtilis* is LiaRS (Hyyryläinen *et al.*, 2005). Interestingly, the effect of the *dlt* inactivation was quite opposite to that seen in the case of CssRS. LiaRS was activated about five-fold higher in the *dlt* mutant than in the wild type strain under secretion stress (II). When LL-37 was used as an inducer of LiaRS, the increased activity of LiaRS in the *dlt* mutant was even more clearly seen. The enhanced induction was observed with both a P_{liaI}–*lacZ* reporter and by measuring P_{liaI} expression with qRT-PCR. YxdJK was the third sensory system used to study the Dlt dependence of TCS induction. Similarly to LiaRS, YxdJK activity was enhanced in the *dlt* mutant (II).

Whether the density of the negative charge in the cell wall also modulates the response of LiaRS to other cell envelope-interfering compounds with non-ionic nature was also determined. For this purpose, *B. subtilis* cells were treated with the detergent Triton X-100 or vancomycin. Vancomycin is known to be a strong inducer of LiaRS (Mascher *et al.*, 2004), and the activity of LiaRS was enhanced hundreds-fold in the wild-type strain compared to the expression level in non-treated cells (II). Interestingly, the response to vancomycin was significantly lower in the *dltD* mutant in contrast to the results obtained from the experiments with LL-37. Triton X-100 caused similar induction of LiaRS in both the wild type strain and *dlt* mutant (II).

The effect of the Dlt system on the induction patterns of CssRS, LiaRS and YxdJK may indicate that *B. subtilis* can modulate cell signalling via TCSs by controlling the density of the negative charge of the cell envelope, but other possible explanations also exist. The decreased activity of CssRS in the *dlt* mutant may result from improved post-translocational protein folding due to the binding of divalent cations to the cell wall with an increased net negative charge (Hyyryläinen *et al.*, 2000). Similarly, the increased negative charge of the cell wall in cells lacking the Dlt system facilitates the access of cationic peptides to the cell surface (Peschel *et al.*, 1999), which might explain the enhanced activity of LiaRS and YxdJK in LL-37-treated cells. This is further supported by the observation that non-ionic Triton X-100 did not cause differential activity of LiaRS in wild type or *dlt*-mutant strains (II). The decreased expression of genes regulated by LiaRS in the vancomycin-treated cells probably reflects the different action mechanims of CAMPs and vancomycin. The altered charge of the cell wall due to *dlt* mutation probably does not affect the action of uncharged vancomycin. It is still puzzling why the activity of LiaRS was decreased in the *dlt* mutant compared to the wild type after vancomycin treatment. The glycopeptide antibiotic vancomycin inhibits PG synthesis by binding to the lipid II precursor, but in addition it can bind to free D-ala-D-ala termini present in the cell wall (Daniel & Errington, 2003; Sheldrick *et al.*, 1978). As mentioned before, LiaRS does not sense vancomycin directly, but rather the disturbances it causes in the cell envelope. However, it is possible that when D-alanyl esters are absent from teichoic acids, the binding of vancomycin to the cell wall is diminished and the overall cell envelope stress is alleviated.

The importance of the cell wall in protection against CAMPs is emphasized by the observation that the charge of the cell membrane did not affect to the signal sensing through TCSs of interest (II). This was demonstrated by two mutant strains with an altered membrane charge. The inactivated components were MprF, which modifies membrane lipids with L-lysine (Peschel & Collins, 2001; Staubitz & Peschel, 2002), and *psd*, which encodes phosphatidylserine decarboxylase and is involved in the biosynthesis of phosphatidylethanolamine (Cao & Helmann, 2004; Matsumoto *et al.*, 1998). Both of these mutations lead to an increased negative charge of the cell membrane. Compared to the wild type, the mutations had no significant effects on the activity of LiaRS or YxdJK after LL-37 treatment. These results suggest that the membrane surface charge does not modulate signalling via the TCSs (II).

5.5 Is cell wall synthesis also a target for CAMPs? (I, III)

One of the purposes of this study was to determine whether transcriptome profiles could tell something about the mechanims of action of different types of CAMPs. The overall impression from DNA array experiments was that all CAMPs studied here appeared to elicit similar kinds of stress responses in bacterial cells, with only a few undisputable exceptions such as the *yxdLM* operon in *B. subtilis* (I, III). As membrane disruption is considered to be the primary mechanism of the CAMP-mediated killing of bacterial cells, the changes in gene expression were surprisingly similar to those seen in the transcriptome analysis with cell wall-interfering antibiotics such as vancomycin (Cao *et al.*, 2002b; Kuroda *et al.*, 2003; Utaida *et al.*, 2003). This naturally raises the question of whether cell wall synthesis is an additional target for CAMPs. In addition to the effects on membranes, several lantibiotics such as nisin and mersacidin produced by Gram-positive bacteria inhibit cell wall biosynthesis (Breukink *et al.*, 1999; Brotz *et al.*, 1998a; Brotz *et al.*, 1998b). The target of both peptides is Lipid II, the basic peptidoglycan building block. Nisin uses Lipid II as a docking molecule to form pores in the membrane, whereas mersacidin inhibits the transglycosylation reaction of cell wall biosynthesis (Breukink & de Kruijff, 2006; Brotz *et al.*, 1997). On the contrary, specific receptors for CAMPs of animal origin have rarely been described (Yeaman & Yount, 2003). Thus, instead of binding to a specific target, it seems more likely that the possible cell wall defect caused by CAMPs results from the unspecific binding of positively charged peptides to the negatively charged components of the cell wall. Another possible explanation could be that CAMPs reactivate otherwise inert autolysins attached to the peptidoglycan, leading to increased cell wall turnover, as has been reported in the case of the lantibiotics nisin and Pep5 (Bierbaum & Sahl, 1985; Bierbaum & Sahl, 1987). One of the most strongly induced genes in CAMP-treated *S. aureus* cells was SA0205, encoding a lysostaphin-like cell wall peptidase (III). On the other hand, it should be remembered that most of the components of the cell wall are synthesised by integral membrane proteins. Thus, even if the main target of CAMPs is the cell membrane, as generally suggested, it seems reasonable that severe membrane disruption caused by CAMPs also reflects cell wall biosynthesis. The possible role of CAMPs in causing defects in the cell wall must be verified in future studies.

5.6 General challenges in transcriptome analysis of bacterial cells (I,III)

The DNA arrays used in this study are powerful tools in providing insights into the changes in gene expression of bacterial cells under varying living conditions. They may also help to characterise the possible roles of the genes of unknown function. The great advantage of the DNA array technique is that it enables the analysis of the whole genome at the same time. Nonetheless, there are notable challenges when the DNA array technique is used to identify genes responding to a particular stress condition, and especially in defining the role of different regulatory elements in the response. The genes are often controlled by multiple regulatory proteins and the contribution of one regulator can be easily masked by other regulators. In addition, it is difficult to distinguish the indirect effects caused by the activation of a certain regulatory element (Helmann, 2006). It is also debated that the sublethal concentrations of antimicrobial peptides used in many transcriptome analysis represent a sudden antibiotic shock rarely reflecting the natural situation, at least in the case of *B. subtilis* (Rietkötter *et al.*, 2008).

In this study, a two-fold change in gene expression was considered as a significant change (I,III). However, it is obvious that the collected data included several genes that were false positives or had no significant role in the stress response. On the other hand, some relevant genes may have been discarded from the final results. The outcome of the analysis could be improved by using more biological replicates and more accurate statistical methods. The high variation between experiments, especially in the case of the highly induced genes, complicated the analysis of results. This was particularly problematic in the data analysis of the qRT-PCR results. The changes in expression of the induced genes were clearly higher in the qRT-PCR measurements than in the DNA microarray, but overall consistency was found in the results (I, III). It should also be remembered that the high induction of a particular gene does not automatically mean that the gene in question has a direct role in stress tolerance. This was seen, for example, in the case of the LiaRS system in *B. subtilis* (I).

In addition, the possible differences in culture conditions, sample handling and in performing the DNA array or qRT-PCR experiments may have caused variation in the results. One of the most important factors affecting the final results was the quantity of peptides used in the experiments, and especially the definition of the sublethal concentrations for each peptide. The antibacterial activity of a particular peptide may vary depending on the culture medium used in experiments, as well as the peptide batch in use. In addition, the possible proteolytic degradation of peptides during storage or sample preparation could not been monitored. To avoid these problems, all the samples compared to each other within an experiment were simultaneously cultured and similarly handled.

6 Conclusions

The bacterial responses to stress caused by cationic antimicrobial peptides were investigated in the present study. Two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, were selected for transcriptome analyses examining the changes in transcription under peptide stress. This study contributes to the understanding of how bacteria can resist and adapt to the effects of toxic peptides, which may give new insights into future drug development.

Two main mechanisms mediating the stress responses were revealed in the transcriptome analyses: ECF σ factors and two-component systems (TCSs). In *B. subtilis*, σ^W, σ^M and LiaRS were demonstrated to play an important role in sensing general stress in the cell envelope caused by CAMPs. As the *S. aureus* genome harbors only one putative ECF σ factor, TCSs seem to be the major sensor system in CAMP stress in *S. aureus*. VraSR, homologous to LiaRS of *B. subtilis*, has previously been associated with resistance to antibiotics that interfere with the cell wall, and it was also strongly activated by CAMPs in the present study. However, inactivation of any of the regulatory systems mentioned above did not cause significantly increased sensitivity to CAMPs. In the case of ECF σ factors, one reason for this might be the redundancy of different ECF σ regulons. Significant cross-regulation between σ^W and σ^M was also demonstrated in our study. LiaRS seems to be a general sensor for worsening living conditions and might have a role in the process leading to spore formation rather than directly in stress tolerance. VraSR does not respond to general stress conditions, but instead confers resistance to vancomycin and other glycopeptide antibiotics. It propably senses some byproduct resulting from disturbance in the cell wall, indicating that CAMPs might also interfere in cell wall assembly.

The BceRS-like TCSs seem to be responsible for the direct recognition of antimicrobial peptides. Typical for these TCSs is that they regulate the expression of adjacent genes encoding ABC transporters such as YxdLM in *B. subtilis*. *yxdLM* and its homologue *vraDE* in *S. aureus* were the most highly induced operons in the transcriptome analyses. *yxdLM* was only induced by human cathelicidin LL-37, whereas *vraDE* was strongly activated by all CAMPs tested and also by bacitracin. The exact role of YxdLM remains to be elucidated, as it seemed likely that LL-37 is not the substrate for it. Our results strongly suggest that the VraDE ABC transporter confers resistance to bacitracin, like its homologue BceAB in *B. subtilis*.

Transcriptome analysis did not reveal any specific extrusion or inactivation mechanism against CAMPs of animal or human origin in either *B. subtilis* or *S. aureus*. It seems that, for example, BceAB-like transporters have primarily evolved to serve in the active extrusion of antimicrobial peptides produced by competiting bacterial species living in the same habitat. In addition, bacterial species producing toxic peptides themselves have to have mechanisms against the harmful effects of

the produced peptide, as is proposed for the function of σ^W in *B. subtilis* (Helmann, 2006).

An increase in the net positive charge of the cell wall is proposed to be the main defence mechanism of bacterial cells against CAMPs. This is mainly achieved by the Dlt system responsible for the D-alanylation of teichoic acids, leading to an increased positive charge of the cell wall (Perego *et al.*, 1995; Peschel *et al.*, 1999). In the research for this thesis, we also demonstrated that the regulation of transcription by TCSs is influenced by the Dlt system in *B. subtilis*. Inactivation of the Dlt system differentially affects the activity of different TCSs, depending on their functional role in the cells and the stimuli they sense. The activity of LiaRS and YxdJK was increased in *dlt* mutants under CAMP stress, indicating that access of CAMPs to the cell membrane was enhanced. Interestingly, the alterations in the charge of the cell membrane did not cause differential transcription via LiaRS or YxdJK. This observation further supports the importance of the cell wall in protecting bacterial cells from positively charged toxic peptides.

Taken together, CAMPs elicit complex stress responses in both *B. subtilis* and *S. aureus*, involving several sensory systems and also some as yet unidentified regulatory elements. This does not mean that all the signalling pathways activated in CAMP stress specifically act in CAMP defence, but rather that they respond to the diversity of environmental changes depending on the lifestyle of the particular bacterial species.

7 Acknowledgements

This study was performed at the Bacteriology Unit, National Institute for Health and Welfare (formerly National Public Health Institute). The Director General Pekka Puska and the former Head of Institute Jussi Huttunen are thanked for providing excellent working facilities. I want to express my gratitude also to my former executives, Professor Matti Sarvas for giving me an opportunity to work at his laboratory, Professor Ilkka Julkunen for appreciating the work made in our group, and Docent Mirja Puolakkainen for her positive and fair attitude. Docent Jaana Vuopio, Professor Anja Siitonen and Professor Petri Ruutu are acknowledged for their support at the final stages of this thesis project. This work was financially supported by European Commission and the Academy of Finland.

My warmest thanks go to my supervisors, Professor Matti Sarvas and Docent Vesa P. Kontinen. I am most grateful to Matti for all his help and invaluable comments concerning my thesis. His wealth of knowledge, encouragement and genuine interest in my project were essential for the finishing of this work. Vesa is sincerely acknowledged for his persistent struggle for the continuation of molecular bacteriology research at THL. Vesa is also thanked for all the hard work he made for my articles, especially during my maternity leave.

Professor Per Saris and Docent Hannu Saarilahti are warmly acknowledged for the careful review of this thesis. I am most grateful for their instructive comments concerning my thesis and their understanding attitude to my tight schedule.

I sincerely thank all the co-authors of the original articles for the fruitful collaboration. I am deeply grateful to Hanne-Leena Hyyryläinen, without her contribution and admirable expertise in molecular biology this thesis would not have been completed. Patrice François, Manuela Tangomo and Jacques Schrenzel are greatly acknowledged for their expertise in DNA microarray experiments. Marika Gardemeister is warmly thanked for teaching me the secrets of qRT-PCR, Soile Leskelä for her guidance with DNA macroarray experiments, Tuula Lunden for the excellent work with proteomics and Maria Mecklin for starting the experiments with CAMPs. Anna Ekman, Sanna Murtomäki-Repo and Leena Valmu are thanked for their contributions. Haike Antelmann and Michael Hecker are thanked for proteome analysis and Vera Sass and Hans-Georg Sahl for the CAMP collaboration. I wish to thank all the members of European *Bacillus* -consortium for nice collaboration and memorable meetings.

This thesis study has given me an opportunity to work with many talented people. I wish to thank all those who have worked with me in Molecular Bacteriology -group, many of them are still good friends of mine. Once again I want to thank Hanne-Leena Hyyryläinen for close collaboration and most of all for her friendship and invaluable support during these years. Tuula Lunden and Marika Vitikainen are thanked for all the years together, especially the time in the Infection Pathogenesis Laboratory. Both Tuula and Marika were great role models for how to become a

good scientist. Sanna Laaksonen is thanked for being such a good roommate in the recent years. Stefan Weber and Rana Al Majidi are thanked for their contribution to *S. aureus* research and for refreshing my English skills. Marika Gardemeister, Emilia Lindberg, Soile Leskelä, Raili Seppälä-Lehto, Taina Turunen, Anna Ekman and Tiina Petänen are thanked for the collaboration during the years at Vaccine Development Laboratory. My special thanks go to Marika and Emilia for their great company, humour and friendship.

I express my warm thanks to the members of *Chlamydia* –group, especially Outi Rautio, Anu Haveri and Eveliina Markkula for sharing the lab facilities but also for their support and friendship.

The people working in the Bacteriology Unit are thanked for being such a nice company and all the laughs during the coffee breaks. I want to thank especially Lotta Siira for her advice and help with numerous things including the cover picture of this thesis, Salha Ibrahem for sharing the worries of thesis process, Tuula Siljander for the good advice and encouragement, Anni Vainio and Taru Kauko for their friendship and Kirsi Mäkisalo for her help with practical matters.

I thank Kirsi-Mari Härkönen, Tarja Alander and Kirsi-Marja Leskelä for their help with official matters and Pia Korkeamäki and Carina Bergsten for IT-help. Jukka Lindeman is warmly thanked for saving me from the catastrophe with the Endnote –program. Sanna Koivumäki and Christine Strid are thanked for the excellent lay out of this thesis and always so friendly service.

Maarit Haataja from the Faculty of Biological and Environmental Sciences is warmly acknowledged for answering patiently to all my questions consernig thesis process.

I warmly thank all my friends and relatives for all the good moments together. I am most grateful to my mother Marja-Liisa and father Viljo for their love and encouragement throughout my life. I have always been able to count on their help if needed. My sister Mari and her husband Marko are very dear to me and I am deeply thankful for their support. My special thanks go to my niece Emma and nephew Joni for bringing so much joy into my life.

Finally, I want to thank the two most important persons in my life, my husband Aki and my daughter Aurora. I am deeply grateful to Aki for being so incredibly understanding and flexible during this writing process. Most of all I want to thank him for his love and for sharing all these years together. Aurora is my little sunshine; she makes me happy when the sky is gray. Like Aki's and Aurora's mutual favourite song says; nothing else matters.

Helsinki, June 2010

Milla Pietiäinen

8 References

Abachin, E., Poyart, C., Pellegrini, E., Milohanic, E., Fiedler, F., Berche, P. & Trieu-Cuot, P. (2002). Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. *Mol Microbiol* **43**, 1-14.

Altena, K., Guder, A., Cramer, C. & Bierbaum, G. (2000). Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. *Appl Environ Microbiol* **66**, 2565-2571.

Amiche, M., Seon, A. A., Pierre, T. N. & Nicolas, P. (1999). The dermaseptin precursors: a protein family with a common preproregion and a variable C-terminal antimicrobial domain. *FEBS Lett* **456**, 352-356.

Archibald, A. R., Hancock, I. C. & Harwood, C. R. (1993). Cell wall structure, Synthesis, and Turnover. In *Bacillus subtilis and other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington DC: American Society for Microbiology: 381-410.

Asai, K., Ishiwata, K., Matsuzaki, K. & Sadaie, Y. (2008). A viable *Bacillus subtilis* strain without functional extracytoplasmic function sigma genes. *J Bacteriol* **190**, 2633-2636.

Auvynet, C., El Amri, C., Lacombe, C., Bruston, F., Bourdais, J., Nicolas, P. & Rosenstein, Y. (2008). Structural requirements for antimicrobial versus chemoattractant activities for dermaseptin S9. *FEBS J* **275**, 4134-4151.

Azevedo, E. C., Rios, E. M., Fukushima, K. & Campos-Takaki, G. M. (1993). Bacitracin production by a new strain of *Bacillus subtilis*. Extraction, purification, and characterization. *Appl Biochem Biotechnol* **42**, 1-7.

Bader, M. W., Sanowar, S., Daley, M. E., Schneider, A. R., Cho, U., Xu, W., Klevit, R. E., Le Moual, H. & Miller, S. I. (2005). Recognition of antimicrobial peptides by a bacterial sensor kinase. *Cell* **122**, 461-472.

Bals, R. & Wilson, J. M. (2003). Cathelicidins--a family of multifunctional antimicrobial peptides. *Cell Mol Life Sci* **60**, 711-720.

Bayer, A. S., Prasad, R., Chandra, J. & other authors (2000). In vitro resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity. *Infect Immun* **68**, 3548-3553.

Bayer, A. S., Kupferwasser, L. I., Brown, M. H., Skurray, R. A., Grkovic, S., Jones, T., Mukhopadhay, K. & Yeaman, M. R. (2006). Low-level resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein 1 in vitro associated with qacA gene carriage is independent of multidrug efflux pump activity. *Antimicrob Agents Chemother* **50**, 2448-2454.

Belcheva, A. & Golemi-Kotra, D. (2008). A close-up view of the VraSR two-component system. A mediator of *Staphylococcus aureus* response to cell wall damage. *J Biol Chem* **283**, 12354-12364.

Bengoechea, J. A. & Skurnik, M. (2000). Temperature-regulated efflux pump/potassium antiporter system mediates resistance to cationic antimicrobial peptides in *Yersinia*. *Mol Microbiol* **37**, 67-80.

Berdy, J. (2005). Bioactive microbial metabolites. *J Antibiot (Tokyo)* **58**, 1-26.

Bernard, R., El Ghachi, M., Mengin-Lecreulx, D., Chippaux, M. & Denizot, F. (2005). BcrC from *Bacillus subtilis* acts as an undecaprenyl pyrophosphate phosphatase in bacitracin resistance. *J Biol Chem* **280**, 28852-28857.

Bernard, R., Guiseppi, A., Chippaux, M., Foglino, M. & Denizot, F. (2007). Resistance to Bacitracin in *Bacillus subtilis*: Unexpected Requirement of the BceAB ABC Transporter in the Control of Expression of Its Own Structural Genes. *J Bacteriol* **189**, 8636-8642.

Bessalle, R., Haas, H., Goria, A., Shalit, I. & Fridkin, M. (1992). Augmentation of the antibacterial activity of magainin by positive-charge chain extension. *Antimicrob Agents Chemother* **36**, 313-317.

Beven, L., Helluin, O., Molle, G., Duclohier, H. & Wroblewski, H. (1999). Correlation between antibacterial activity and pore sizes of two classes of voltage-dependent channel-forming peptides. *Biochim Biophys Acta* **1421**, 53-63.

Bierbaum, G. & Sahl, H. G. (1985). Induction of autolysis of staphylococci by the basic peptide antibiotics Pep 5 and nisin and their influence on the activity of autolytic enzymes. *Arch Microbiol* **141**, 249-254.

Bierbaum, G. & Sahl, H. G. (1987). Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. *J Bacteriol* **169**, 5452-5458.

Birkemo, G. A., Luders, T., Andersen, O., Nes, I. F. & Nissen-Meyer, J. (2003). Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus L.*). *Biochim Biophys Acta* **1646**, 207-215.

Bisicchia, P., Noone, D., Lioliou, E., Howell, A., Quigley, S., Jensen, T., Jarmer, H. & Devine, K. M. (2007). The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Mol Microbiol* **65**, 180-200.

Bjorstad, A., Fu, H., Karlsson, A., Dahlgren, C. & Bylund, J. (2005). Interleukin-8-derived peptide has antibacterial activity. *Antimicrob Agents Chemother* **49**, 3889-3895.

Bland, J. M., De Lucca, A. J., Jacks, T. J. & Vigo, C. B. (2001). All-D-cecropin B: synthesis, conformation, lipopolysaccharide binding, and antibacterial activity. *Mol Cell Biochem* **218**, 105-111.

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

Bowdish, D. M., Davidson, D. J., Scott, M. G. & Hancock, R. E. (2005). Immunomodulatory activities of small host defense peptides. *Antimicrob Agents Chemother* **49**, 1727-1732.

Boyle-Vavra, S., Yin, S. & Daum, R. S. (2006). The VraS/VraR two-component regulatory system required for oxacillin resistance in community-acquired methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiol Lett* **262**, 163-171.

Breukink, E. & de Kruijff, B. (1999). The lantibiotic nisin, a special case or not? *Biochim Biophys Acta* **1462**, 223-234.

Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H. & de Kruijff, B. (1999). Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**, 2361-2364.

Breukink, E. & de Kruijff, B. (2006). Lipid II as a target for antibiotics. *Nat Rev Drug Discov* **5**, 321-332.

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

Brotz, H., Bierbaum, G., Reynolds, P. E. & Sahl, H. G. (1997). The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation. *Eur J Biochem* **246**, 193 - 199.

Brotz, H., Bierbaum, G., Leopold, K., Reynolds, P. E. & Sahl, H. G. (1998a). The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. *Antimicrob Agents Chemother* **42**, 154 - 160.

Brotz, H., Josten, M., Wiedemann, I., Schneider, U., Gotz, F., Bierbaum, G. & Sahl, H. G. (1998b). Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol Microbiol* **30**, 317-327.

Brunskill, E. W. & Bayles, K. W. (1996). Identification of LytSR-regulated genes from *Staphylococcus aureus*. *J Bacteriol* **178**, 5810-5812.

Bulet, P., Stocklin, R. & Menin, L. (2004). Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev* **198**, 169-184.

Butcher, B. G. & Helmann, J. D. (2006). Identification of *Bacillus subtilis* sigma-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. *Mol Microbiol* **60**, 765-782.

Butcher, B. G., Lin, Y. P. & Helmann, J. D. (2007). The yydFGHIJ operon of *Bacillus subtilis* encodes a peptide that induces the LiaRS two-component system. *J Bacteriol* **189**, 8616-8625.

Campos, M. A., Vargas, M. A., Regueiro, V., Llompart, C. M., Alberti, S. & Bengoechea, J. A. (2004). Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect Immun* **72**, 7107-7114.

Cao, M., Bernat, B. A., Wang, Z., Armstrong, R. N. & Helmann, J. D. (2001). FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in *Bacillus subtilis*. *J Bacteriol* **183**, 2380-2383.

Cao, M. & Helmann, J. D. (2002). Regulation of the *Bacillus subtilis* bcrC bacitracin resistance gene by two extracytoplasmic function sigma factors. *J Bacteriol* **184**, 6123-6129.

Cao, M., Kobel, P. A., Morshedi, M. M., Wu, M. F., Paddon, C. & Helmann, J. D. (2002a). Defining the *Bacillus subtilis* sigma(W) regulon: a comparative analysis of promoter consensus search, run-off transcription/macroarray analysis (ROMA), and transcriptional profiling approaches. *J Mol Biol* **316**, 443-457.

Cao, M., Wang, T., Ye, R. & Helmann, J. D. (2002b). Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* sigma(W) and sigma(M) regulons. *Mol Microbiol* **45**, 1267-1276.

Cao, M., Salzberg, L., Tsai, C. S., Mascher, T., Bonilla, C., Wang, T., Ye, R. W., Marquez-Magana, L. & Helmann, J. D. (2003). Regulation of the *Bacillus subtilis* extracytoplasmic function protein sigma(Y) and its target promoters. *J Bacteriol* **185**, 4883-4890.

Cao, M. & Helmann, J. D. (2004). The *Bacillus subtilis* extracytoplasmic-function sigmaX factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. *J Bacteriol* **186**, 1136-1146.

Carotenuto, A., Malfi, S., Saviello, M. R., Campiglia, P., Gomez-Monterrey, I., Mangoni, M. L., Gaddi, L. M., Novellino, E. & Grieco, P. (2008). A different molecular mechanism underlying antimicrobial and hemolytic actions of temporins A and L. *J Med Chem* **51**, 2354-2362.

Carvalho Ade, O. & Gomes, V. M. (2009). Plant defensins--prospects for the biological functions and biotechnological properties. *Peptides* **30**, 1007-1020.

Chang, S., Sievert, D. M., Hageman, J. C. & other authors (2003). Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *N Engl J Med* **348**, 1342-1347.

Charbonnier, Y., Gettler, B., Francois, P., Bento, M., Renzoni, A., Vaudaux, P., Schlegel, W. & Schrenzel, J. (2005). A generic approach for the design of whole-genome oligoarrays, validated for genomotyping, deletion mapping and gene expression analysis on *Staphylococcus aureus*. *BMC Genomics* **6**, 95.

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J. A. & Bulet, P. (1996). Innate immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, Mytilus edulis. *J Biol Chem* **271**, 21808-21813.

Chen, F. Y., Lee, M. T. & Huang, H. W. (2003). Evidence for membrane thinning effect as the mechanism for peptide-induced pore formation. *Biophys J* **84**, 3751-3758.

Chen, X., Niyonsaba, F., Ushio, H., Okuda, D., Nagaoka, I., Ikeda, S., Okumura, K. & Ogawa, H. (2005a). Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against *Staphylococcus aureus* and *Escherichia coli*. *J Dermatol Sci* **40**, 123-132.

Chen, Y., Mant, C. T., Farmer, S. W., Hancock, R. E., Vasil, M. L. & Hodges, R. S. (2005b). Rational design of alpha-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. *J Biol Chem* **280**, 12316-12329.

Chen, Y., Vasil, A. I., Rehaume, L., Mant, C. T., Burns, J. L., Vasil, M. L., Hancock, R. E. & Hodges, R. S. (2006). Comparison of biophysical and biologic properties of alpha-helical enantiomeric antimicrobial peptides. *Chem Biol Drug Des* **67**, 162-173.

Chen, Y., Guarnieri, M. T., Vasil, A. I., Vasil, M. L., Mant, C. T. & Hodges, R. S. (2007). Role of Peptide Hydrophobicity in the Mechanism of Action of {alpha}-Helical Antimicrobial Peptides. *Antimicrob Agents Chemother* **51**, 1398-1406.

Chitnis, S. N. & Prasad, K. S. (1990). Seminalplasmin, an antimicrobial protein from bovine seminal plasma, inhibits peptidoglycan synthesis in *Escherichia coli*. *FEMS Microbiol Lett* **60**, 281-284.

Cirioni, O., Ghiselli, R., Silvestri, C. & other authors (2007). Efficacy of tachyplesin III, colistin, and imipenem against a multiresistant *Pseudomonas aeruginosa* strain. *Antimicrob Agents Chemother* **51**, 2005-2010.

Connolly, L., De Las Penas, A., Alba, B. M. & Gross, C. A. (1997). The response to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways. *Genes Dev* **11**, 2012-2021.

Corbella, X., Dominguez, M. A., Pujol, M., Ayats, J., Sendra, M., Pallares, R., Ariza, J. & Gudiol, F. (1997). *Staphylococcus aureus* nasal carriage as a marker for subsequent staphylococcal infections in intensive care unit patients. *Eur J Clin Microbiol Infect Dis* **16**, 351-357.

D'Elia, M. A., Millar, K. E., Beveridge, T. J. & Brown, E. D. (2006a). Wall teichoic acid polymers are dispensable for cell viability in *Bacillus subtilis*. *J Bacteriol* **188**, 8313-8316.

D'Elia, M. A., Pereira, M. P., Chung, Y. S., Zhao, W., Chau, A., Kenney, T. J., Sulavik, M. C., Black, T. A. & Brown, E. D. (2006b). Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. *J Bacteriol* **188**, 4183-4189.

Danese, P. N. & Silhavy, T. J. (1997). The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*. *Genes Dev* **11**, 1183-1193.

Daniel, R. A. & Errington, J. (2003). Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. *Cell* **113**, 767-776.

Darmon, E., Noone, D., Masson, A., Bron, S., Kuipers, O. P., Devine, K. M. & van Dijl, J. M. (2002). A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*. *J Bacteriol* **184**, 5661-5671.

Darwin, A. J. (2005). The phage-shock-protein response. *Mol Microbiol* **57**, 621-628.

Dathe, M. & Wieprecht, T. (1999). Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. *Biochim Biophys Acta* **1462**, 71-87.

Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M. & Bienert, M. (2001). Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS Lett* **501**, 146-150.

del Castillo, F. J., del Castillo, I. & Moreno, F. (2001). Construction and characterization of mutations at codon 751 of the *Escherichia coli* gyrB gene that confer resistance to the antimicrobial peptide microcin B17 and alter the activity of DNA gyrase. *J Bacteriol* **183**, 2137-2140.

Delcour, J., Ferain, T., Deghorain, M., Palumbo, E. & Hols, P. (1999). The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek* **76**, 159-184.

Dinges, M. M., Orwin, P. M. & Schlievert, P. M. (2000). Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* **13**, 16-34, table of contents.

Dubrac, S. & Msadek, T. (2004). Identification of genes controlled by the essential YycG/YycF two-component system of *Staphylococcus aureus*. *J Bacteriol* **186**, 1175-1181.

Dubrac, S., Boneca, I. G., Poupel, O. & Msadek, T. (2007). New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J Bacteriol* **189**, 8257-8269.

Dubrac, S., Bisicchia, P., Devine, K. M. & Msadek, T. (2008). A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Mol Microbiol* **70**, 1307-1322.

Durr, U. H., Sudheendra, U. S. & Ramamoorthy, A. (2006). LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim Biophys Acta* **1758**, 1408-1425.

Ehrenstein, G. & Lecar, H. (1977). Electrically gated ionic channels in lipid bilayers. *Q Rev Biophys* **10**, 1-34.

Eiamphungporn, W. & Helmann, J. D. (2008). The *Bacillus subtilis* sigma^{M} regulon and its contribution to cell envelope stress responses. *Mol Microbiol* **67**, 830-848.

Ellermeier, C. D., Hobbs, E. C., Gonzalez-Pastor, J. E. & Losick, R. (2006). A three-protein signaling pathway governing immunity to a bacterial cannibalism toxin. *Cell* **124**, 549-559.

Ellermeier, C. D. & Losick, R. (2006). Evidence for a novel protease governing regulated intramembrane proteolysis and resistance to antimicrobial peptides in *Bacillus subtilis*. *Genes Dev* **20**, 1911-1922.

Ernst, R. K., Guina, T. & Miller, S. I. (2001). *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. *Microbes Infect* **3**, 1327-1334.

Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*. *Nat Rev Microbiol* **1**, 117-126.

Fabret, C. & Hoch, J. A. (1998). A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. *J Bacteriol* **180**, 6375-6383.

Fabret, C., Feher, V. A. & Hoch, J. A. (1999). Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *J Bacteriol* **181**, 1975-1983.

Fabretti, F., Theilacker, C., Baldassarri, L., Kaczynski, Z., Kropec, A., Holst, O. & Huebner, J. (2006). Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. *Infect Immun* **74**, 4164-4171.

Falla, T. J., Karunaratne, D. N. & Hancock, R. E. (1996). Mode of action of the antimicrobial peptide indolicidin. *J Biol Chem* **271**, 19298-19303.

Fedtke, I., Mader, D., Kohler, T. & other authors (2007). A *Staphylococcus aureus* ypfP mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity. *Mol Microbiol* **65**, 1078-1091.

Fehlbaum, P., Bulet, P., Chernysh, S., Briand, J. P., Roussel, J. P., Letellier, L., Hetru, C. & Hoffmann, J. A. (1996). Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. *Proc Natl Acad Sci U S A* **93**, 1221-1225.

Foster, S. J. & Popham, D. L. (2002). Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers and capsules. In *Bacillus subtilis and its closest relatives:From genes to cells*. Edited by J. A. Sonenschein & J. A. Hoch. Washington D.C.: American Society for Microbiology:21-41.

Foster, T. J. & Hook, M. (1998). Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* **6**, 484-488.

Foster, T. J. (2005). Immune evasion by staphylococci. *Nat Rev Microbiol* **3**, 948-958.

Frick, I. M., Akesson, P., Rasmussen, M., Schmidtchen, A. & Bjorck, L. (2003). SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J Biol Chem* **278**, 16561-16566.

Friedman, D. B., Stauff, D. L., Pishchany, G., Whitwell, C. W., Torres, V. J. & Skaar, E. P. (2006). *Staphylococcus aureus* Redirects Central Metabolism to Increase Iron Availability. **2**, e87.

Friedrich, C. L., Moyles, D., Beveridge, T. J. & Hancock, R. E. (2000). Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria. *Antimicrob Agents Chemother* **44**, 2086-2092.

Fuchs, S., Pane-Farre, J., Kohler, C., Hecker, M. & Engelmann, S. (2007). Anaerobic Gene Expression in *Staphylococcus aureus*. *J Bacteriol* **189**, 4275-4289.

Ganz, T., Selsted, M. E. & Lehrer, R. I. (1990). Defensins. *Eur J Haematol* **44**, 1-8.

Ganz, T. & Lehrer, R. I. (1995). Defensins. *Pharmacol Ther* **66**, 191-205.

Gardete, S., Wu, S. W., Gill, S. & Tomasz, A. (2006). Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **50**, 3424-3434.

Garzoni, C., Francois, P., Huyghe, A. & other authors (2007). A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells. *BMC Genomics* **8**, 171.

Gazit, E., Boman, A., Boman, H. G. & Shai, Y. (1995). Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. *Biochemistry (Mosc)* **34**, 11479-11488.

Gennaro, R. & Zanetti, M. (2000). Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers* **55**, 31-49.

Ghuysen, J. M. (1991). Serine beta-lactamases and penicillin-binding proteins. *Annu Rev Microbiol* **45**, 37-67.

Gifford, J. L., Hunter, H. N. & Vogel, H. J. (2005). Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell Mol Life Sci* **62**, 2588-2598.

Ginsburg, I. (2004). Bactericidal cationic peptides can also function as bacteriolysis-inducing agents mimicking beta-lactam antibiotics?; it is enigmatic why this concept is consistently disregarded. *Med Hypotheses* **62**, 367-374.

Gonzalez-Pastor, J. E., Hobbs, E. C. & Losick, R. (2003). Cannibalism by sporulating bacteria. *Science* **301**, 510-513.

Grebe, T. W. & Stock, J. B. (1999). The histidine protein kinase superfamily. *Adv Microb Physiol* **41**, 139-227.

Groisman, E. A., Chiao, E., Lipps, C. J. & Heffron, F. (1989). *Salmonella typhimurium* phoP virulence gene is a transcriptional regulator. *Proc Natl Acad Sci U S A* **86**, 7077-7081.

Gross, C. A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J. & Young, B. (1998). The functional and regulatory roles of sigma factors in transcription. *Cold Spring Harb Symp Quant Biol* **63**, 141-155.

Gruber, T. M. & Gross, C. A. (2003). Assay of *Escherichia coli* RNA polymerase: sigma-core interactions. *Methods Enzymol* **370**, 206-212.

Grundling, A. & Schneewind, O. (2007). Genes Required for Glycolipid Synthesis and Lipoteichoic Acid Anchoring in *Staphylococcus aureus*. *J Bacteriol* **189**, 2521-2530.

Guina, T., Yi, E. C., Wang, H., Hackett, M. & Miller, S. I. (2000). A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides. *J Bacteriol* **182**, 4077-4086.

Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S. I. (1998). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27**, 1171-1182.

Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K. & Miller, S. I. (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**, 6139-6146.

Hale, J. D. & Hancock, R. E. (2007). Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Rev Anti Infect Ther* **5**, 951-959.

Hallock, K. J., Lee, D. K. & Ramamoorthy, A. (2003). MSI-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain. *Biophys J* **84**, 3052-3060.

Hancock, R. E. (1997). Peptide antibiotics. *Lancet* **349**, 418-422.

Hancock, R. E. (2001). Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect Dis* **1**, 156-164.

Hancock, R. E. & Sahl, H. G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* **24**, 1551-1557.

Harder, J., Bartels, J., Christophers, E. & Schroder, J. M. (2001). Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* **276**, 5707-5713.

Hartman, B. J. & Tomasz, A. (1984). Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J Bacteriol* **158**, 513-516.

Hechard, Y. & Sahl, H. G. (2002). Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie* **84**, 545-557.

Hecker, M. & Volker, U. (2001). General stress response of *Bacillus subtilis* and other bacteria. *Adv Microb Physiol* **44**, 35-91.

Heinrich, J. & Wiegert, T. (2006). YpdC determines site-1 degradation in regulated intramembrane proteolysis of the RsiW anti-sigma factor of *Bacillus subtilis*. *Mol Microbiol* **62**, 566-579.

Heller, W. T., Waring, A. J., Lehrer, R. I. & Huang, H. W. (1998). Multiple states of beta-sheet peptide protegrin in lipid bilayers. *Biochemistry (Mosc)* **37**, 17331-17338.

Helmann, J. D. & Chamberlin, M. J. (1988). Structure and function of bacterial sigma factors. *Annu Rev Biochem* **57**, 839-872.

Helmann, J. D. (1995). Compilation and analysis of Bacillus subtilis sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res* **23**, 2351-2360.

Helmann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**, 47-110.

Helmann, J. D. (2006). Deciphering a complex genetic regulatory network: the *Bacillus subtilis* sigmaW protein and intrinsic resistance to antimicrobial compounds. *Sci Prog* **89**, 243-266.

Helmerhorst, E. J., Reijnders, I. M., van 't Hof, W., Veerman, E. C. & Nieuw Amerongen, A. V. (1999). A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. *FEBS Lett* **449**, 105-110.

Henzler-Wildman, K. A., Martinez, G. V., Brown, M. F. & Ramamoorthy, A. (2004). Perturbation of the hydrophobic core of lipid bilayers by the human antimicrobial peptide LL-37. *Biochemistry (Mosc)* **43**, 8459-8469.

Henzler Wildman, K. A., Lee, D. K. & Ramamoorthy, A. (2003). Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37. *Biochemistry (Mosc)* **42**, 6545-6558.

Herbert, S., Bera, A., Nerz, C., Kraus, D., Peschel, A., Goerke, C., Meehl, M., Cheung, A. & Gotz, F. (2007). Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. *PLoS Pathog* **3**, e102.

Hirakura, Y., Kobayashi, S. & Matsuzaki, K. (2002). Specific interactions of the antimicrobial peptide cyclic beta-sheet tachyplesin I with lipopolysaccharides. *Biochim Biophys Acta* **1562**, 32-36.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. & Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother* **40**, 135-136.

Hoch, J. A. (2000). Two-component and phosphorelay signal transduction. *Curr Opin Microbiol* **3**, 165-170.

Hong, H. J., Hutchings, M. I., Neu, J. M., Wright, G. D., Paget, M. S. & Buttner, M. J. (2004). Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (vanK) required for drug resistance. *Mol Microbiol* **52**, 1107-1121.

Horsburgh, M. J. & Moir, A. (1999). Sigma M, an ECF RNA polymerase sigma factor of Bacillus subtilis 168, is essential for growth and survival in high concentrations of salt. *Mol Microbiol* **32**, 41-50.

Hoskin, D. W. & Ramamoorthy, A. (2008). Studies on anticancer activities of antimicrobial peptides. *Biochim Biophys Acta* **1778**, 357-375.

Howden, B. P., Stinear, T. P., Allen, D. L., Johnson, P. D., Ward, P. B. & Davies, J. K. (2008). Genomic analysis reveals a point mutation in the two-component sensor gene graS that leads to intermediate vancomycin resistance in clinical *Staphylococcus aureus*. *Antimicrob Agents Chemother* **52**, 3755-3762.

Hsu, C. H., Chen, C., Jou, M. L., Lee, A. Y., Lin, Y. C., Yu, Y. P., Huang, W. T. & Wu, S. H. (2005). Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. *Nucleic Acids Res* **33**, 4053-4064.

Huang, X., Fredrick, K. L. & Helmann, J. D. (1998). Promoter recognition by *Bacillus subtilis* sigmaW: autoregulation and partial overlap with the sigmaX regulon. *J Bacteriol* **180**, 3765-3770.

Huang, X. & Helmann, J. D. (1998). Identification of target promoters for the *Bacillus subtilis* sigma X factor using a consensus-directed search. *J Mol Biol* **279**, 165-173.

Huang, X., Gaballa, A., Cao, M. & Helmann, J. D. (1999). Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function sigma factor, sigma W. *Mol Microbiol* **31**, 361-371.

Hutchings, M. I., Hoskisson, P. A., Chandra, G. & Buttner, M. J. (2004). Sensing and responding to diverse extracellular signals? Analysis of the sensor kinases and response regulators of *Streptomyces coelicolor* A3(2). *Microbiology* **150**, 2795-2806.

Hyyryläinen, H. L., Vitikainen, M., Thwaite, J., Wu, H., Sarvas, M., Harwood, C. R., Kontinen, V. P. & Stephenson, K. (2000). D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *Bacillus subtilis*. *J Biol Chem* **275**, 26696-26703.

Hyyryläinen, H. L., Bolhuis, A., Darmon, E. & other authors (2001). A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. *Mol Microbiol* **41**, 1159-1172.

Hyyryläinen, H. L., Sarvas, M. & Kontinen, V. P. (2005). Transcriptome analysis of the secretion stress response of *Bacillus subtilis*. *Appl Microbiol Biotechnol* **67**, 389-396.

Imura, Y., Choda, N. & Matsuzaki, K. (2008). Magainin 2 in action: distinct modes of membrane permeabilization in living bacterial and mammalian cells. *Biophys J* **95**, 5757-5765.

Ishihara, H., Takoh, M., Nishibayashi, R. & Sato, A. (2002). Distribution and variation of bacitracin synthetase gene sequences in laboratory stock strains of *Bacillus licheniformis*. *Curr Microbiol* **45**, 18-23.

Janausch, I. G., Zientz, E., Tran, Q. H., Kroger, A. & Unden, G. (2002). C4-dicarboxylate carriers and sensors in bacteria. *Biochim Biophys Acta* **1553**, 39-56.

Jansen, A., Turck, M., Szekat, C., Nagel, M., Clever, I. & Bierbaum, G. (2007). Role of insertion elements and yycFG in the development of decreased susceptibility to vancomycin in *Staphylococcus aureus*. *Int J Med Microbiol* **297**, 205 - 215.

Jenssen, H., Hamill, P. & Hancock, R. E. (2006). Peptide antimicrobial agents. *Clin Microbiol Rev* **19**, 491-511.

Jervis, A. J., Thackray, P. D., Houston, C. W., Horsburgh, M. J. & Moir, A. (2007). SigM-responsive genes of *Bacillus subtilis* and their promoters. *J Bacteriol* **189**, 4534-4538.

Jevons, M. P. (1961). "Celbenin"-resistant staphylococci. *BMJ* **1**, 124-125.

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J. & Tarkowski, A. (2004). *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J Immunol* **172**, 1169-1176.

Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D. & Agerberth, B. (1998). Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem* **273**, 3718-3724.

Jordan, S., Junker, A., Helmann, J. D. & Mascher, T. (2006). Regulation of LiaRS-dependent gene expression in bacillus subtilis: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. *J Bacteriol* **188**, 5153-5166.

Jordan, S., Rietkotter, E., Strauch, M. A., Kalamorz, F., Butcher, B. G., Helmann, J. D. & Mascher, T. (2007). LiaRS-dependent gene expression is embedded in transition state regulation in *Bacillus subtilis*. *Microbiology* **153**, 2530-2540.

Jordan, S., Hutchings, M. I. & Mascher, T. (2008). Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiol Rev* **32**, 107-146.

Joseph, P., Fichant, G., Quentin, Y. & Denizot, F. (2002). Regulatory relationship of two-component and ABC transport systems and clustering of their genes in the *Bacillus/Clostridium* group, suggest a functional link between them. *J Mol Microbiol Biotechnol* **4**, 503-513.

Joseph, P., Guiseppi, A., Sorokin, A. & Denizot, F. (2004). Characterization of the *Bacillus subtilis* YxdJ response regulator as the inducer of expression for the cognate ABC transporter YxdLM. *Microbiology* **150**, 2609-2617.

Kates, M., Syz, J. Y., Gosser, D. & Haines, T. H. (1993). pH-dissociation characteristics of cardiolipin and its 2'-deoxy analogue. *Lipids* **28**, 877-882.

Kim, H. S., Park, C. B., Kim, M. S. & Kim, S. C. (1996). cDNA cloning and characterization of buforin I, an antimicrobial peptide: a cleavage product of histone H2A. *Biochem Biophys Res Commun* **229**, 381-387.

Kim, H. S., Yoon, H., Minn, I., Park, C. B., Lee, W. T., Zasloff, M. & Kim, S. C. (2000). Pepsin-mediated processing of the cytoplasmic histone H2A to strong antimicrobial peptide buforin I. *J Immunol* **165**, 3268-3274.

Kjos, M., Snipen, L., Salehian, Z., Nes, I. F. & Diep, D. B. (2010). The abi proteins and their involvement in bacteriocin self-immunity. *J Bacteriol* **192**, 2068-2076.

Kleerebezem, M., Crielaard, W. & Tommassen, J. (1996). Involvement of stress protein PspA (phage shock protein A) of *Escherichia coli* in maintenance of the protonmotive force under stress conditions. *EMBO J* **15**, 162-171.

Klein, C. & Entian, K. D. (1994). Genes involved in self-protection against the lantibiotic subtilin produced by *Bacillus subtilis* ATCC 6633. *Appl Environ Microbiol* **60**, 2793-2801.

Kluytmans, J. A., Mouton, J. W., Ijzerman, E. P., Vandenbroucke-Grauls, C. M., Maat, A. W., Wagenvoort, J. H. & Verbrugh, H. A. (1995). Nasal carriage of *Staphylococcus aureus* as a major risk factor for wound infections after cardiac surgery. *J Infect Dis* **171**, 216-219.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**, 797-810.

Kohler, T., Weidenmaier, C. & Peschel, A. (2009). Wall teichoic acid protects Staphylococcus aureus against antimicrobial fatty acids from human skin. *J Bacteriol* **191**, 4482-4484.

Kokryakov, V. N., Harwig, S. S., Panyutich, E. A., Shevchenko, A. A., Aleshina, G. M., Shamova, O. V., Korneva, H. A. & Lehrer, R. I. (1993). Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS Lett* **327**, 231-236.

Koprivnjak, T., Peschel, A., Gelb, M. H., Liang, N. S. & Weiss, J. P. (2002). Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against *Staphylococcus aureus*. *J Biol Chem* **277**, 47636-47644.

Koprivnjak, T., Weidenmaier, C., Peschel, A. & Weiss, J. P. (2008). Wall Teichoic Acid Deficiency in *Staphylococcus aureus* Confers Selective Resistance to Mammalian Group IIA Phospholipase A2 and Human beta-Defensin 3. *Infect Immun* **76**, 2169-2176.

Kovacs, M., Halfmann, A., Fedtke, I., Heintz, M., Peschel, A., Vollmer, W., Hakenbeck, R. & Bruckner, R. (2006). A functional dlt operon, encoding proteins required for incorporation of d-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. *J Bacteriol* **188**, 5797-5805.

Kragol, G., Lovas, S., Varadi, G., Condie, B. A., Hoffmann, R. & Otvos, L., Jr. (2001). The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. *Biochemistry (Mosc)* **40**, 3016-3026.

Kraus, D., Herbert, S., Kristian, S. A., Khosravi, A., Nizet, V., Gotz, F. & Peschel, A. (2008). The GraRS regulatory system controls *Staphylococcus aureus* susceptibility to antimicrobial host defenses. *BMC Microbiol* **8**, 85.

Kraus, D. & Peschel, A. (2008). Staphylococcus aureus evasion of innate antimicrobial defense. *Future Microbiol* **3**, 437-451.

Kristian, S. A., Datta, V., Weidenmaier, C., Kansal, R., Fedtke, I., Peschel, A., Gallo, R. L. & Nizet, V. (2005). D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. *J Bacteriol* **187**, 6719-6725.

Kunst, F., Ogasawara, N., Moszer, I. & other authors (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249-256.

Kupferwasser, L. I., Skurray, R. A., Brown, M. H., Firth, N., Yeaman, M. R. & Bayer, A. S. (1999). Plasmid-mediated resistance to thrombin-induced platelet microbicidal protein in staphylococci: role of the qacA locus. *Antimicrob Agents Chemother* **43**, 2395-2399.

Kuroda, M., Kuwahara-Arai, K. & Hiramatsu, K. (2000). Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. *Biochem Biophys Res Commun* **269**, 485-490.

Kuroda, M., Ohta, T., Uchiyama, I. & other authors (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225-1240.

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H. & Hiramatsu, K. (2003). Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol Microbiol* **49**, 807-821.

Lai, R., Liu, H., Hui Lee, W. & Zhang, Y. (2002). An anionic antimicrobial peptide from toad *Bombina maxima*. *Biochem Biophys Res Commun* **295**, 796-799.

Lai, Y., Villaruz, A. E., Li, M., Cha, D. J., Sturdevant, D. E. & Otto, M. (2007). The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. *Mol Microbiol* **63**, 497-506.

Lane, W. J. & Darst, S. A. (2006). The structural basis for promoter -35 element recognition by the group IV sigma factors. *PLoS Biol* **4**, e269.

Lee, D. L., Ivaninskii, S., Burkhard, P. & Hodges, R. S. (2003). Unique stabilizing interactions identified in the two-stranded alpha-helical coiled-coil: crystal structure of a cortexillin I/GCN4 hybrid coiled-coil peptide. *Protein Sci* **12**, 1395-1405.

Lee, H. S., Park, C. B., Kim, J. M., Jang, S. A., Park, I. Y., Kim, M. S., Cho, J. H. & Kim, S. C. (2008). Mechanism of anticancer activity of buforin IIb, a histone H2A-derived peptide. *Cancer Lett* **271**, 47-55.

Lee, M. T., Hung, W. C., Chen, F. Y. & Huang, H. W. (2005). Many-body effect of antimicrobial peptides: on the correlation between lipid's spontaneous curvature and pore formation. *Biophys J* **89**, 4006-4016.

Lehrer, R. I. (2004). Primate defensins. *Nat Rev Microbiol* **2**, 727-738.

Leonova, L., Kokryakov, V. N., Aleshina, G., Hong, T., Nguyen, T., Zhao, C., Waring, A. J. & Lehrer, R. I. (2001). Circular minidefensins and posttranslational generation of molecular diversity. *J Leukoc Biol* **70**, 461-464.

Li, M., Cha, D. J., Lai, Y., Villaruz, A. E., Sturdevant, D. E. & Otto, M. (2007a). The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Mol Microbiol* **66**, 1136-1147.

Li, M., Lai, Y., Villaruz, A. E., Cha, D. J., Sturdevant, D. E. & Otto, M. (2007b). Gram-positive three-component antimicrobial peptide-sensing system. *Proc Natl Acad Sci U S A* **104**, 9469-9474.

Lonetto, M. A., Brown, K. L., Rudd, K. E. & Buttner, M. J. (1994). Analysis of the *Streptomyces coelicolor* sigE gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci U S A* **91**, 7573-7577.

Lubelski, J., Konings, W. N. & Driessen, A. J. (2007). Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. *Microbiol Mol Biol Rev* **71**, 463-476.

Lubelski, J., Rink, R., Khusainov, R., Moll, G. N. & Kuipers, O. P. (2008). Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cell Mol Life Sci* **65**, 455-476.

Ludtke, S. J., He, K., Wu, Y. & Huang, H. W. (1994). Cooperative membrane insertion of magainin correlated with its cytolytic activity. *Biochim Biophys Acta* **1190**, 181-184.

Mahalka, A. K. & Kinnunen, P. K. (2009). Binding of amphipathic alpha-helical antimicrobial peptides to lipid membranes: lessons from temporins B and L. *Biochim Biophys Acta* **1788**, 1600-1609.

Mangoni, M. L. & Shai, Y. (2009). Temporins and their synergism against Gram-negative bacteria and in lipopolysaccharide detoxification. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1788**, 1610-1619.

Mani, R., Cady, S. D., Tang, M., Waring, A. J., Lehrer, R. I. & Hong, M. (2006). Membrane-dependent oligomeric structure and pore formation of a beta-hairpin antimicrobial peptide in lipid bilayers from solid-state NMR. *Proc Natl Acad Sci U S A* **103**, 16242-16247.

Martin, P. K., Li, T., Sun, D., Biek, D. P. & Schmid, M. B. (1999). Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. *J Bacteriol* **181**, 3666-3673.

Mascher, T., Margulis, N. G., Wang, T., Ye, R. W. & Helmann, J. D. (2003). Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Mol Microbiol* **50**, 1591-1604.

Mascher, T., Zimmer, S. L., Smith, T. A. & Helmann, J. D. (2004). Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. *Antimicrob Agents Chemother* **48**, 2888-2896.

Mascher, T. (2006). Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in *Firmicutes* bacteria. *FEMS Microbiol Lett* **264**, 133-144.

Mascher, T., Helmann, J. D. & Unden, G. (2006). Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **70**, 910-938.

Mascher, T., Hachmann, A. B. & Helmann, J. D. (2007). Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. *J Bacteriol* **189**, 6919-6927.

Matias, V. R. & Beveridge, T. J. (2005). Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space. *Mol Microbiol* **56**, 240-251.

Matias, V. R. & Beveridge, T. J. (2006). Native cell wall organization shown by cryo-electron microscopy confirms the existence of a periplasmic space in *Staphylococcus aureus*. *J Bacteriol* **188**, 1011-1021.

Matsuhashi, M., Song, M. D., Ishino, F., Wachi, M., Doi, M., Inoue, M., Ubukata, K., Yamashita, N. & Konno, M. (1986). Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *J Bacteriol* **167**, 975-980.

Matsumoto, K., Okada, M., Horikoshi, Y., Matsuzaki, H., Kishi, T., Itaya, M. & Shibuya, I. (1998). Cloning, sequencing, and disruption of the *Bacillus subtilis* psd gene coding for phosphatidylserine decarboxylase. *J Bacteriol* **180**, 100-106.

Matsuzaki, K., Murase, O., Fujii, N. & Miyajima, K. (1996). An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry (Mosc)* **35**, 11361-11368.

Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N. & Miyajima, K. (1997). Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. *Biochim Biophys Acta* **1327**, 119-130.

Matsuzaki, K. (2009). Control of cell selectivity of antimicrobial peptides. *Biochim Biophys Acta* **1788**, 1687-1692.

Mavri, J. & Vogel, H. J. (1996). Ion pair formation of phosphorylated amino acids and lysine and arginine side chains: a theoretical study. *Proteins* **24**, 495-501.

McAleese, F., Wu, S. W., Sieradzki, K., Dunman, P., Murphy, E., Projan, S. & Tomasz, A. (2006). Overexpression of genes of the cell wall stimulon in clinical isolates of *Staphylococcus aureus* exhibiting vancomycin-intermediate- *S. aureus*-type resistance to vancomycin. *J Bacteriol* **188**, 1120 - 1133.

Meehl, M., Herbert, S., Gotz, F. & Cheung, A. (2007). Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **51**, 2679-2689.

Midorikawa, K., Ouhara, K., Komatsuzawa, H. & other authors (2003). *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. *Infect Immun* **71**, 3730-3739.

Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989). A two-component regulatory system (phoP phoQ) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci U S A* **86**, 5054-5058.

Milona, P., Townes, C. L., Bevan, R. M. & Hall, J. (2007). The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against *Salmonella* serovars. *Biochem Biophys Res Commun* **356**, 169-174.

Minnig, K., Barblan, J. L., Kehl, S., Moller, S. B. & Mauel, C. (2003). In *Bacillus subtilis* W23, the duet sigmaXsigmaM, two sigma factors of the extracytoplasmic function subfamily, are required for septum and wall synthesis under batch culture conditions. *Mol Microbiol* **49**, 1435-1447.

Minnig, K., Lazarevic, V., Soldo, B. & Mauel, C. (2005). Analysis of teichoic acid biosynthesis regulation reveals that the extracytoplasmic function sigma factor sigmaM is induced by phosphate depletion in *Bacillus subtilis* W23. *Microbiology* **151**, 3041-3049.

Missiakas, D. & Raina, S. (1997). Protein misfolding in the cell envelope of *Escherichia coli*: new signaling pathways. *Trends Biochem Sci* **22**, 59-63.

Mookherjee, N., Brown, K. L., Bowdish, D. M. & other authors (2006a). Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J Immunol* **176**, 2455-2464.

Mookherjee, N., Wilson, H. L., Doria, S. & other authors (2006b). Bovine and human cathelicidin cationic host defense peptides similarly suppress transcriptional responses to bacterial lipopolysaccharide. *J Leukoc Biol* **80**, 1563-1574.

Mookherjee, N. & Hancock, R. E. (2007). Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. *Cell Mol Life Sci* **64**, 922-933.

Mookherjee, N., Rehaume, L. M. & Hancock, R. E. (2007). Cathelicidins and functional analogues as antisepsis molecules. *Expert Opin Ther Targets* **11**, 993-1004.

Mor, A., Hani, K. & Nicolas, P. (1994). The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. *J Biol Chem* **269**, 31635-31641.

Morikawa, K., Inose, Y., Okamura, H., Maruyama, A., Hayashi, H., Takeyasu, K. & Ohta, T. (2003). A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes Cells* **8**, 699-712.

Msadek, T. (1999). When the going gets tough: survival strategies and environmental signaling networks in *Bacillus subtilis*. *Trends Microbiol* **7**, 201-207.

Mukhopadhyay, K., Whitmire, W., Xiong, Y. Q. & other authors (2007). In vitro susceptibility of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein-1 (tPMP-1) is influenced by cell membrane phospholipid composition and asymmetry. *Microbiology* **153**, 1187-1197.

Muthaiyan, A., Silverman, J. A., Jayaswal, R. K. & Wilkinson, B. J. (2008). Transcriptional Profiling Reveals that Daptomycin Induces the *Staphylococcus aureus* Cell Wall Stress Stimulon and Genes Responsive to Membrane Depolarization. *Antimicrob Agents Chemother* **52**, 980-990.

Nagaoka, I., Hirota, S., Yomogida, S., Ohwada, A. & Hirata, M. (2000). Synergistic actions of antibacterial neutrophil defensins and cathelicidins. *Inflamm Res* **49**, 73-79.

Nakano, S., Erwin, K. N., Ralle, M. & Zuber, P. (2005). Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Mol Microbiol* **55**, 498-510.

Navon-Venezia, S., Feder, R., Gaidukov, L., Carmeli, Y. & Mor, A. (2002). Antibacterial properties of dermaseptin S4 derivatives with in vivo activity. *Antimicrob Agents Chemother* **46**, 689-694.

Neuhaus, F. C. & Baddiley, J. (2003). A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiol Mol Biol Rev* **67**, 686-723.

Nguyen, T. X., Cole, A. M. & Lehrer, R. I. (2003). Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* **24**, 1647-1654.

Nicolas, P. & El Amri, C. (2009). The dermaseptin superfamily: a gene-based combinatorial library of antimicrobial peptides. *Biochim Biophys Acta* **1788**, 1537-1550.

Nordahl, E. A., Rydengard, V., Nyberg, P., Nitsche, D. P., Morgelin, M., Malmsten, M., Bjorck, L. & Schmidtchen, A. (2004). Activation of the complement system generates antibacterial peptides. *Proc Natl Acad Sci U S A* **101**, 16879-16884.

Nyberg, P., Rasmussen, M. & Bjorck, L. (2004). alpha2-Macroglobulin-proteinase complexes protect *Streptococcus pyogenes* from killing by the antimicrobial peptide LL-37. *J Biol Chem* **279**, 52820-52823.

Ohki, R., Giyanto, Tateno, K., Masuyama, W., Moriya, S., Kobayashi, K. & Ogasawara, N. (2003). The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. *Mol Microbiol* **49**, 1135-1144.

Oppenheim, F. G., Xu, T., McMillian, F. M., Levitz, S. M., Diamond, R. D., Offner, G. D. & Troxler, R. F. (1988). Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. *J Biol Chem* **263**, 7472-7477.

Oppenheim, J. J., Biragyn, A., Kwak, L. W. & Yang, D. (2003). Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Ann Rheum Dis* **62 Suppl 2**, ii17-21.

Oren, Z., Lerman, J. C., Gudmundsson, G. H., Agerberth, B. & Shai, Y. (1999). Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. *Biochem J* **341 (Pt 3)**, 501-513.

Otto, M., Peschel, A. & Gotz, F. (1998). Producer self-protection against the lantibiotic epidermin by the ABC transporter EpiFEG of *Staphylococcus epidermidis* Tu3298. *FEMS Microbiol Lett* **166**, 203-211.

Otvos, L., Jr., O, I., Rogers, M. E., Consolvo, P. J., Condie, B. A., Lovas, S., Bulet, P. & Blaszczyk-Thurin, M. (2000). Interaction between heat shock proteins and antimicrobial peptides. *Biochemistry (Mosc)* **39**, 14150-14159.

Oyston, P. C., Fox, M. A., Richards, S. J. & Clark, G. C. (2009). Novel peptide therapeutics for treatment of infections. *J Med Microbiol* **58**, 977-987.

Paget, M. S. & Helmann, J. D. (2003). The sigma70 family of sigma factors. *Genome Biol* **4**, 203.

Papo, N. & Shai, Y. (2003). Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? *Peptides* **24**, 1693-1703.

Park, C. B., Kim, M. S. & Kim, S. C. (1996). A novel antimicrobial peptide from *Bufo bufo gargarizans*. *Biochem Biophys Res Commun* **218**, 408-413.

Park, C. B., Kim, H. S. & Kim, S. C. (1998a). 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**, 253-257.

Park, I. Y., Park, C. B., Kim, M. S. & Kim, S. C. (1998b). Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. *FEBS Lett* **437**, 258-262.

Parkinson, J. S. (1993). Signal transduction schemes of bacteria. *Cell* **73**, 857-871.

Patrzykat, A., Friedrich, C. L., Zhang, L., Mendoza, V. & Hancock, R. E. (2002). Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. *Antimicrob Agents Chemother* **46**, 605-614.

Patton, T. G., Yang, S. J. & Bayles, K. W. (2006). The role of proton motive force in expression of the *Staphylococcus aureus* cid and lrg operons. *Mol Microbiol* **59**, 1395-1404.

Perego, M., Glaser, P., Minutello, A., Strauch, M. A., Leopold, K. & Fischer, W. (1995). Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation. *J Biol Chem* **270**, 15598-15606.

Perron, G. G., Zasloff, M. & Bell, G. (2006). Experimental evolution of resistance to an antimicrobial peptide. *Proc Biol Sci* **273**, 251-256.

Peschel, A. & Gotz, F. (1996). Analysis of the *Staphylococcus epidermidis* genes epiF, -E, and -G involved in epidermin immunity. *J Bacteriol* **178**, 531-536.

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G. & Gotz, F. (1999). Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem* **274**, 8405-8410.

Peschel, A. & Collins, L. V. (2001). Staphylococcal resistance to antimicrobial peptides of mammalian and bacterial origin. *Peptides* **22**, 1651-1659.

Peschel, A., Jack, R. W., Otto, M. & other authors (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**, 1067-1076.

Peschel, A. & Sahl, H. G. (2006). The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol* **4**, 529-536.

Petersohn, A., Brigulla, M., Haas, S., Hoheisel, J. D., Volker, U. & Hecker, M. (2001). Global analysis of the general stress response of *Bacillus subtilis*. *J Bacteriol* **183**, 5617-5631.

Phillips, Z. E. & Strauch, M. A. (2002). *Bacillus subtilis* sporulation and stationary phase gene expression. *Cell Mol Life Sci* **59**, 392-402.

Pogliano, J., Lynch, A. S., Belin, D., Lin, E. C. & Beckwith, J. (1997). Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. *Genes Dev* **11**, 1169-1182.

Pomorski, T., Holthuis, J. C., Herrmann, A. & van Meer, G. (2004). Tracking down lipid flippases and their biological functions. *J Cell Sci* **117**, 805-813.

Pouny, Y., Rapaport, D., Mor, A., Nicolas, P. & Shai, Y. (1992). Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. *Biochemistry (Mosc)* **31**, 12416-12423.

Powers, J. P. & Hancock, R. E. (2003). The relationship between peptide structure and antibacterial activity. *Peptides* **24**, 1681-1691.

Poyart, C., Pellegrini, E., Marceau, M., Baptista, M., Jaubert, F., Lamy, M. C. & Trieu-Cuot, P. (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**, 1615-1625.

Qiao, M., Immonen, T., Koponen, O. & Saris, P. E. (1995). The cellular location and effect on nisin immunity of the NisI protein from Lactococcus lactis N8 expressed in Escherichia coli and L. lactis. *FEMS Microbiol Lett* **131**, 75-80.

Qiu, J. & Helmann, J. D. (2001). The -10 region is a key promoter specificity determinant for the *Bacillus subtilis* extracytoplasmic-function sigma factors sigma(X) and sigma(W). *J Bacteriol* **183**, 1921-1927.

Rietkötter, E., Hoyer, D. & Mascher, T. (2008). Bacitracin sensing in *Bacillus subtilis*. *Mol Microbiol* **68**, 768-785.

Rinaldi, A. C., Mangoni, M. L., Rufo, A. & other authors (2002). Temporin L: antimicrobial, haemolytic and cytotoxic activities, and effects on membrane permeabilization in lipid vesicles. *Biochem J* **368**, 91-100.

Romeo, D., Skerlavaj, B., Bolognesi, M. & Gennaro, R. (1988). Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. *J Biol Chem* **263**, 9573-9575.

Rosenfeld, Y., Barra, D., Simmaco, M., Shai, Y. & Mangoni, M. L. (2006). A synergism between temporins toward Gram-negative bacteria overcomes resistance imposed by the lipopolysaccharide protective layer. *J Biol Chem* **281**, 28565-28574.

Rowley, G., Spector, M., Kormanec, J. & Roberts, M. (2006). Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nat Rev Microbiol* **4**, 383-394.

Ryan, M. P., Meaney, W. J., Ross, R. P. & Hill, C. (1998). Evaluation of lacticin 3147 and a teat seal containing this bacteriocin for inhibition of mastitis pathogens. *Appl Environ Microbiol* **64**, 2287-2290.

Salomon, R. A. & Farias, R. N. (1992). Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. *J Bacteriol* **174**, 7428-7435.

Sansom, M. S. (1991). The biophysics of peptide models of ion channels. *Prog Biophys Mol Biol* **55**, 139-235.

Sass, P., Jansen, A., Szekat, C., Sass, V., Sahl, H.-G. & Bierbaum, G. (2008a). The lantibiotic mersacidin is a strong inducer of the cell wall stress response of *Staphylococcus aureus*. *BMC Microbiol* **8**, 186.

Sass, P. & Bierbaum, G. (2009). Native graS mutation supports the susceptibility of *Staphylococcus aureus* strain SG511 to antimicrobial peptides. *Int J Med Microbiol* **299**, 313-322.

Sass, V., Pag, U., Tossi, A., Bierbaum, G. & Sahl, H.-G. (2008b). Mode of action of human beta-defensin 3 against *Staphylococcus aureus* and transcriptional analysis of responses to defensin challenge. *Int J Med Microbiol* **298**, 619-633.

Sawai, M. V., Waring, A. J., Kearney, W. R., McCray, P. B., Jr., Forsyth, W. R., Lehrer, R. I. & Tack, B. F. (2002). Impact of single-residue mutations on the structure and function of ovispirin/novispirin antimicrobial peptides. *Protein Eng* **15**, 225-232.

Schittek, B., Hipfel, R., Sauer, B. & other authors (2001). Dermcidin: a novel human antibiotic peptide secreted by sweat glands. *Nat Immunol* **2**, 1133-1137.

Schlag, M., Biswas, R., Krismer, B., Kohler, T., Zoll, S., Yu, W., Schwarz, H., Peschel, A. & Gotz, F. (2010). Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. *Mol Microbiol* **75**, 864-873.

Schmidtchen, A., Frick, I. M., Andersson, E., Tapper, H. & Bjorck, L. (2002). Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* **46**, 157-168.

Schobel, S., Zellmeier, S., Schumann, W. & Wiegert, T. (2004). The *Bacillus subtilis* sigmaW anti-sigma factor RsiW is degraded by intramembrane proteolysis through YluC. *Mol Microbiol* **52**, 1091-1105.

Scott, M. G., Yan, H. & Hancock, R. E. (1999). Biological properties of structurally related alpha-helical cationic antimicrobial peptides. *Infect Immun* **67**, 2005-2009.

Selsted, M. E., Novotny, M. J., Morris, W. L., Tang, Y. Q., Smith, W. & Cullor, J. S. (1992). Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J Biol Chem* **267**, 4292-4295.

Selsted, M. E. & Ouellette, A. J. (2005). Mammalian defensins in the antimicrobial immune response. *Nat Immunol* **6**, 551-557.

Shafer, W. M., Qu, X., Waring, A. J. & Lehrer, R. I. (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**, 1829-1833.

Sharon, M., Oren, Z., Shai, Y. & Anglister, J. (1999). 2D-NMR and ATR-FTIR study of the structure of a cell-selective diastereomer of melittin and its orientation in phospholipids. *Biochemistry (Mosc)* **38**, 15305-15316.

Shaw, L. N., Lindholm, C., Prajsnar, T. K., Miller, H. K., Brown, M. C., Golonka, E., Stewart, G. C., Tarkowski, A. & Potempa, J. (2008). Identification and characterization of sigma, a novel component of the *Staphylococcus aureus* stress and virulence responses. *PLoS One* **3**, e3844.

Sheldrick, G. M., Jones, P. G., Kennard, O., Williams, D. H. & Smith, G. A. (1978). Structure of vancomycin and its complex with acetyl-D-alanyl-D-alanine. *Nature* **271**, 223-225.

Shi, J., Ross, C. R., Chengappa, M. M. & Blecha, F. (1994). Identification of a proline-arginine-rich antibacterial peptide from neutrophils that is analogous to PR-39, an antibacterial peptide from the small intestine. *J Leukoc Biol* **56**, 807-811.

Siegers, K. & Entian, K. D. (1995). Genes involved in immunity to the lantibiotic nisin produced by *Lactococcus lactis* 6F3. *Appl Environ Microbiol* **61**, 1082-1089.

Sieprawska-Lupa, M., Mydel, P., Krawczyk, K. & other authors (2004). Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob Agents Chemother* **48**, 4673-4679.

Sieradzki, K. & Tomasz, A. (2003). Alterations of cell wall structure and metabolism accompany reduced susceptibility to vancomycin in an isogenic series of clinical isolates of *Staphylococcus aureus*. *J Bacteriol* **185**, 7103-7110.

Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangoni, M. L. & Barra, D. (1996). Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. *Eur J Biochem* **242**, 788-792.

Smith, L. & Hillman, J. (2008). Therapeutic potential of type A (I) lantibiotics, a group of cationic peptide antibiotics. *Curr Opin Microbiol* **11**, 401-408.

Sobral, R. G., Jones, A. E., Des Etages, S. G., Dougherty, T. J., Peitzsch, R. M., Gaasterland, T., Ludovice, A. M., de Lencastre, H. & Tomasz, A. (2007). Extensive and genome-wide changes in the transcription profile of *Staphylococcus aureus* induced by modulating the transcription of the cell wall synthesis gene murF. *J Bacteriol* **189**, 2376-2391.

Sood, R., Domanov, Y., Pietiäinen, M., Kontinen, V. P. & Kinnunen, P. K. (2008). Binding of LL-37 to model biomembranes: insight into target vs host cell recognition. *Biochim Biophys Acta* **1778**, 983-996.

Staron, A., Sofia, H. J., Dietrich, S., Ulrich, L. E., Liesegang, H. & Mascher, T. (2009). The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. *Mol Microbiol* **74**, 557-581.

Staubitz, P. & Peschel, A. (2002). MprF-mediated lysinylation of phospholipids in *Bacillus subtilis*--protection against bacteriocins in terrestrial habitats? *Microbiology* **148**, 3331-3332.

Stein, T., Heinzmann, S., Solovieva, I. & Entian, K. D. (2003). Function of Lactococcus lactis nisin immunity genes nisI and nisFEG after coordinated expression in the surrogate host Bacillus subtilis. *J Biol Chem* **278**, 89-94.

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol Microbiol* **56**, 845-857.

Steiner, H., Hultmark, D., Engstrom, A., Bennich, H. & Boman, H. G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**, 246-248.

Steinstraesser, L., Koehler, T., Jacobsen, F. & other authors (2008). Host defense peptides in wound healing. *Mol Med* **14**, 528-537.

Stephenson, K. & Hoch, J. A. (2002). Virulence- and antibiotic resistance-associated two-component signal transduction systems of Gram-positive pathogenic bacteria as targets for antimicrobial therapy. *Pharmacol Ther* **93**, 293-305.

Stewart, V. (2003). Biochemical Society Special Lecture. Nitrate- and nitrite-responsive sensors NarX and NarQ of proteobacteria. *Biochem Soc Trans* **31**, 1-10.

Stewart, V. & Bledsoe, P. J. (2003). Synthetic lac operator substitutions for studying the nitrate- and nitrite-responsive NarX-NarL and NarQ-NarP two-component regulatory systems of *Escherichia coli* K-12. *J Bacteriol* **185**, 2104-2111.

Strahilevitz, J., Mor, A., Nicolas, P. & Shai, Y. (1994). Spectrum of antimicrobial activity and assembly of dermaseptin-b and its precursor form in phospholipid membranes. *Biochemistry (Mosc)* **33**, 10951-10960.

Subbalakshmi, C. & Sitaram, N. (1998). Mechanism of antimicrobial action of indolicidin. *FEMS Microbiol Lett* **160**, 91-96.

Sun, Z., Zhong, J., Liang, X., Liu, J., Chen, X. & Huan, L. (2009). Novel mechanism for nisin resistance via proteolytic degradation of nisin by the nisin resistance protein NSR. *Antimicrob Agents Chemother* **53**, 1964-1973.

Szurmant, H., Nelson, K., Kim, E. J., Perego, M. & Hoch, J. A. (2005). YycH regulates the activity of the essential YycFG two-component system in *Bacillus subtilis*. *J Bacteriol* **187**, 5419-5426.

Szurmant, H., Mohan, M. A., Imus, P. M. & Hoch, J. A. (2007). YycH and YycI interact to regulate the essential YycFG two-component system in *Bacillus subtilis*. *J Bacteriol* **189**, 3280-3289.

Tam le, T., Eymann, C., Albrecht, D., Sietmann, R., Schauer, F., Hecker, M. & Antelmann, H. (2006). Differential gene expression in response to phenol and catechol reveals different metabolic activities for the degradation of aromatic compounds in *Bacillus subtilis*. *Environ Microbiol* **8**, 1408-1427.

Tang, M. & Hong, M. (2009). Structure and mechanism of beta-hairpin antimicrobial peptides in lipid bilayers from solid-state NMR spectroscopy. *Mol Biosyst* **5**, 317-322.

Tang, Y. Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C. J., Ouellette, A. J. & Selsted, M. E. (1999). A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **286**, 498-502.

Tannert, A., Pohl, A., Pomorski, T. & Herrmann, A. (2003). Protein-mediated transbilayer movement of lipids in eukaryotes and prokaryotes: the relevance of ABC transporters. *Int J Antimicrob Agents* **22**, 177-187.

Thackray, P. D. & Moir, A. (2003). SigM, an extracytoplasmic function sigma factor of *Bacillus subtilis*, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. *J Bacteriol* **185**, 3491-3498.

Tojo, S., Matsunaga, M., Matsumoto, T., Kang, C. M., Yamaguchi, H., Asai, K., Sadaie, Y., Yoshida, K. & Fujita, Y. (2003). Organization and expression of the *Bacillus subtilis* sigY operon. *J Biochem* **134**, 935-946.

Tossi, A., Sandri, L. & Giangaspero, A. (2000). Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers* **55**, 4-30.

Travers, A. A. (1987). Structure and function of *E. coli* promoter DNA. *CRC Crit Rev Biochem* **22**, 181-219.

Tzeng, Y. L., Ambrose, K. D., Zughaier, S., Zhou, X., Miller, Y. K., Shafer, W. M. & Stephens, D. S. (2005). Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. *J Bacteriol* **187**, 5387-5396.

Ubukata, K., Yamashita, N. & Konno, M. (1985). Occurrence of a beta-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. *Antimicrob Agents Chemother* **27**, 851-857.

Utaida, S., Dunman, P. M., Macapagal, D., Murphy, E., Projan, S. J., Singh, V. K., Jayaswal, R. K. & Wilkinson, B. J. (2003). Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology* **149**, 2719-2732.

Vaara, M. & Vaara, T. (1983). Polycations as outer membrane-disorganizing agents. *Antimicrob Agents Chemother* **24**, 114-122.

Wade, D., Boman, A., Wahlin, B., Drain, C. M., Andreu, D., Boman, H. G. & Merrifield, R. B. (1990). All-D amino acid-containing channel-forming antibiotic peptides. *Proc Natl Acad Sci U S A* **87**, 4761-4765.

Vaz Gomes, A., de Waal, A., Berden, J. A. & Westerhoff, H. V. (1993). Electric potentiation, cooperativity, and synergism of magainin peptides in protein-free liposomes. *Biochemistry (Mosc)* **32**, 5365-5372.

Weidenmaier, C., Kokai-Kun, J. F., Kristian, S. A. & other authors (2004). Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat Med* **10**, 243-245.

Weidenmaier, C. & Peschel, A. (2008). Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nat Rev Micro* **6**, 276-287.

Whitworth, D. E. (2008). Genomes and knowledge - a questionable relationship? *Trends Microbiol* **16**, 512-519.

Wiegert, T., Homuth, G., Versteeg, S. & Schumann, W. (2001). Alkaline shock induces the *Bacillus subtilis* sigma(W) regulon. *Mol Microbiol* **41**, 59-71.

Vitikainen, M., Hyyryläinen, H. L., Kivimäki, A., Kontinen, V. P. & Sarvas, M. (2005). Secretion of heterologous proteins in *Bacillus subtilis* can be improved by engineering cell components affecting post-translocational protein folding and degradation. *J Appl Microbiol* **99**, 363-375.

von Eiff, C., Becker, K., Machka, K., Stammer, H. & Peters, G. (2001). Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. *N Engl J Med* **344**, 11-16.

Wosten, M. M. & Groisman, E. A. (1999). Molecular characterization of the PmrA regulon. *J Biol Chem* **274**, 27185-27190.

Voyich, J. M., Braughton, K. R., Sturdevant, D. E. & other authors (2005). Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* **175**, 3907-3919.

Wu, M., Maier, E., Benz, R. & Hancock, R. E. (1999). Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry (Mosc)* **38**, 7235-7242.

Vunnam, S., Juvvadi, P. & Merrifield, R. B. (1997). Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine. *J Pept Res* **49**, 59-66.

Xia, G., Kohler, T. & Peschel, A. (2010). The wall teichoic acid and lipoteichoic acid polymers of Staphylococcus aureus. *Int J Med Microbiol* **300**, 148-154.

Xiong, Y. Q., Mukhopadhyay, K., Yeaman, M. R., Adler-Moore, J. & Bayer, A. S. (2005). Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of *Staphylococcus aureus*. *Antimicrob Agents Chemother* **49**, 3114-3121.

Yamaguchi, S., Huster, D., Waring, A., Lehrer, R. I., Kearney, W., Tack, B. F. & Hong, M. (2001). Orientation and dynamics of an antimicrobial peptide in the lipid bilayer by solid-state NMR spectroscopy. *Biophys J* **81**, 2203-2214.

Yan, H. & Hancock, R. E. (2001). Synergistic interactions between mammalian antimicrobial defense peptides. *Antimicrob Agents Chemother* **45**, 1558-1560.

Yang, L., Weiss, T. M., Lehrer, R. I. & Huang, H. W. (2000). Crystallization of antimicrobial pores in membranes: magainin and protegrin. *Biophys J* **79**, 2002-2009.

Yang, L., Harroun, T. A., Weiss, T. M., Ding, L. & Huang, H. W. (2001). Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys J* **81**, 1475-1485.

Yeaman, M. R. & Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* **55**, 27-55.

Yeaman, M. R. & Bayer, A. S. (2006). Antimicrobial peptides versus invasive infections. *Curr Top Microbiol Immunol* **306**, 111-152.

Yeaman, M. R. & Yount, N. Y. (2007). Unifying themes in host defence effector polypeptides. *Nat Rev Microbiol* **5**, 727-740.

Yoshimura, M., Asai, K., Sadaie, Y. & Yoshikawa, H. (2004). Interaction of *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors with the N-terminal regions of their potential anti-sigma factors. *Microbiology* **150**, 591-599.

Yount, N. Y. & Yeaman, M. R. (2004). Multidimensional signatures in antimicrobial peptides. *Proc Natl Acad Sci U S A* **101**, 7363-7368.

Yount, N. Y., Bayer, A. S., Xiong, Y. Q. & Yeaman, M. R. (2006). Advances in antimicrobial peptide immunobiology. *Biopolymers* **84**, 435-458.

Zanetti, M. (2004). Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* **75**, 39-48.

Zasloff, M. (1987). Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci U S A* **84**, 5449-5453.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395.

Zellmeier, S., Hofmann, C., Thomas, S., Wiegert, T. & Schumann, W. (2005). Identification of sigma(V)-dependent genes of *Bacillus subtilis*. *FEMS Microbiol Lett* **253**, 221-229.

Zellmeier, S., Schumann, W. & Wiegert, T. (2006). Involvement of Clp protease activity in modulating the sigma W stress response. *Mol Microbiol* **61**, 1569-1582.

Zhang, L., Benz, R. & Hancock, R. E. (1999). Influence of proline residues on the antibacterial and synergistic activities of alpha-helical peptides. *Biochemistry (Mosc)* **38**, 8102-8111.

Zhang, L., Rozek, A. & Hancock, R. E. (2001). Interaction of cationic antimicrobial peptides with model membranes. *J Biol Chem* **276**, 35714-35722.

Zhao, H. & Kinnunen, P. K. (2002). Binding of the antimicrobial peptide temporin L to liposomes assessed by Trp fluorescence. *J Biol Chem* **277**, 25170-25177.

Zhao, H., Rinaldi, A. C., Di Giulio, A., Simmaco, M. & Kinnunen, P. K. (2002). Interactions of the antimicrobial peptides temporins with model biomembranes. Comparison of temporins B and L. *Biochemistry (Mosc)* **41**, 4425-4436.

Zhao, H. & Kinnunen, P. K. (2003). Modulation of the activity of secretory phospholipase A2 by antimicrobial peptides. *Antimicrob Agents Chemother* **47**, 965-971.

Zhao, H., Jutila, A., Nurminen, T., Wickstrom, S. A., Keski-Oja, J. & Kinnunen, P. K. (2005). Binding of endostatin to phosphatidylserine-containing membranes and formation of amyloid-like fibers. *Biochemistry (Mosc)* **44**, 2857-2863.

Zhao, H., Sood, R., Jutila, A., Bose, S., Fimland, G., Nissen-Meyer, J. & Kinnunen, P. K. (2006). Interaction of the antimicrobial peptide pheromone Plantaricin A with model membranes: implications for a novel mechanism of action. *Biochim Biophys Acta* **1758**, 1461-1474.

Zuber, P. (2004). Spx-RNA polymerase interaction and global transcriptional control during oxidative stress. *J Bacteriol* **186**, 1911-1918.