# Characterization of a Sensory Complex Involved in Antimicrobial Peptide Resistance: Communication Between a Histidine Kinase and an ABC Transporter in *Bacillus subtilis*


## Dissertation

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# Characterization of a Sensory Complex Involved in Antimicrobial Peptide Resistance: Communication Between a Histidine Kinase and an ABC Transporter in *Bacillus subtilis*


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vorgelegt von

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# List of Publications

### Publications and manuscripts presented in this thesis:

### Chapter II

**Dintner S.**, Staroń A., Berchtold E., Petri T., Mascher T. and Gebhard S. (2011) Co-evolution of ABC-transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in Firmicutes bacteria. J. Bacteriol. 193(15):3851-62.

### Chapter III

Kallenberg F., **Dintner S.**, Schmitz R., and Gebhard S. (2013) Identification of regions important for resistance and signalling within the antimicrobial peptide transporter BceAB of *Bacillus subtilis*. J. Bacteriol. 2013, 195(14):3287.

### Chapter IV

**Dintner S.**, Heermann R., Fang C., Jung K. and Gebhard S. (2014) A Sensory Complex Consisting of an ATP-Binding-Cassette Transporter and a Two-Component Regulatory System Controls Bacitracin Resistance in *Bacillus subtilis*. J. Biol. Chem., published August 12, 2014 as doi:10.1074/jbc.M114.596221

### Publication not presented in this thesis:

Gebhard, S., **Dintner S.** (2014) Peptidantibiotika - Frühwarnsysteme und Katastrophenschutz bei Bakterien. BIOspektrum. doi: 10.1007/s12268-014-0437-y

# Contributions to the Publications Presented in this Thesis

## Chapter II

Both authors contributed equally to this work. **Sebastian Dintner** performed the data acquisition, the phylogenetic analyses, the analysis of coevolution and the secondary structure analyses of the extracellular domain. **Anna Staroń** supported the data acquisition and performed the identification of the response regulator binding sites and the identification of candidate histidine kinases. Evi Berchtold and Tobias Petri calculated the pairwise alignment correlation. Susanne Gebhard designed the study, coordinated the bioinformatic work and wrote the manuscript.
We declare that we both contributed equally to this publication above. We hereby also confirm the above statements.

Sebastian Dintner Anna Staroń

## Chapter III

Felix Kallenberg performed the site-directed mutagenesis, characterized the mutants, and participated in cloning of constructs; **Sebastian Dintner** performed the Western blot and two-hybrid analyses and participated in cloning of constructs and site-directed mutagenesis; Roland Schmitz performed the random mutagenesis; and Susanne Gebhard designed the study, coordinated the experimental work, and wrote the manuscript.

## Chapter IV

**Sebastian Dintner** performed the interaction studies, purification and characterization of BceAB and BceS, and *in vitro* phosphorylation assays. Ralf Heermann conducted and analyzed the SPR experiments. CF purified BceR and participated in the BACTH assays. Ralf Heermann and Kirsten Jung gave valuable input for the manuscript. Susanne Gebhard designed the study and coordinated the experimental work. **Sebastian Dintner** and Susanne Gebhard analyzed the data and wrote the manuscript.
We hereby also confirm the above statements.

Sebastian Dintner PD Dr. Susanne Gebhard

# Summary

In their habitats, microorganisms are often in competition for limited nutrients. In order to succeed, many Gram-positive bacteria resort to production of peptide antibiotics. Therefore, resistance mechanisms against these compounds are essential. The first step of ensuring survival is the perception of the harmful drugs and mediation of resistance against it. In recent years, a group of ABC-transporters have been recognized as important resistance determinate against antimicrobial peptides. The expression of these transporters is generally regulated by a two-component system, which in most cases is encoded next to the transporter. Together they are described as detoxification modules. The permeases of the transporters are characterized by a large extracellular domain, while the histidine kinases lack an obvious input domain. One of the best understood examples is the BceRS-BceAB system of *Bacillus subtilis*, which mediates resistance against bacitracin, mersacidin and actagardine. For this system it was shown that the histidine kinase is not able to detect the substrate directly and instead has an absolute requirement for the transporter in stimulus perception. This describes a novel mode of signal transduction in which the transporter is the actual sensor and therefore regulates its own expression. To date, mechanistic details for this unique mode of signal transduction remain unknown. Several other examples have been described for transport proteins that have acquired additional sensing or regulatory functions beyond solute transport, and these have been designated trigger transporters. For these bifunctional transporters a direct protein-protein interaction with membrane-integrated or soluble components of signal transduction relays has been postulated. However, for most sensor/co-sensor pairs, conclusive proof of such an interaction is lacking, and so far little is known about the sites that might mediate contacts between the putative protein interfaces and how communication is achieved.

Based on sequence and architectural similarities, we identified over 250 BceAB-like transporters in the protein database, which occurred almost exclusively in Firmicutes bacteria. To whether the regulatory interplay between the ABC transporter and the two-component system was a common theme in these antimicrobial peptide resistance modules, we carried out a phylogenetic study of these identified systems. We identified a clear coevolutionary relationship between transport permeases and histidine kinases. Furthermore, we identified conserved putative response regulator binding sites in the promoter regions of the transporter operons. Additionally, we were able to provide a tool to identify TCSs for transporters lacking a regulatory system in their genomic neighbourhood, which was based on the coclustering of histidine kinases and transporter permeases. These findings also suggested the existence of a sensory complex between BceAB-like transporters and BceS-like histidine kinases.

To further investigate the signaling mechanism, we performed a random mutagenesis of the transport permease BceB with the aim to identify regions or residues within the transporter that are involved in signaling and/or resistance. With this approach we were able to identify mutations that

affected either the ability for signaling or mediation of resistance. This showed a partial genetic separation of the two qualities, which could be achieved by single amino acid replacements. These results provide first insights into the signaling mechanism of the Bce system.

In order to analyse the proposed communication between two-component system and ABC transporter, we further characterized their interactions by *in vivo* and *in vitro* approaches. We could demonstrate that the transporter BceAB is indeed able to interact directly with the histidine kinase. Because it was unknown how the signal perception by BceAB-type transporters occurs, we next analyzed substrate binding by the transporter permease BceB and could show direct binding of bacitracin by BceB. Finally, *in vitro* signal transduction assays indicated that complex formation with the transporter influenced the activity of the histidine kinase.

In summary this thesis clearly shows the existence of a sensory complex comprised of BceRS-like two-component systems and BceAB-like ABC transporters and provides first functional insights into the mechanism of stimulus perception, signal transduction and antimicrobial resistance mechanism employed by these wide spread detoxification systems against antimicrobial peptides.

# Zusammenfassung

Um sich in solch hart umkämpften Habitaten wie dem Boden zu behaupten sind Bakterien dazu übergegangen Antibiotika zu produzieren, um das Wachstum der Konkurrenz einzudämmen. Eine Gruppe solcher Substanzen sind antimikrobielle Peptide, die von Gram-positiven Bakterien produziert werden. Zum Schutz vor Peptidantibiotika haben Gram-positive Bakterien eine Vielzahl verschiedener Resistenzmechanismen entwickelt. Den effizientesten Resistenzmechanismus gegen Peptidantibiotika stellt eine Gruppe ATP-abhängiger ABC-Transporter dar. Diese Transporter weisen einen besonderen Transmembranaufbau auf. Sie bestehen aus zehn Transmembranhelices und einer großen extrazellulären Domäne. Die Expression dieser Transportergruppe wird durch ein Zweikomponentensystem reguliert. Die Histidinkinase besitzt ebenfalls einen ungewöhnlichen Transmembranaufbau, da sie keine offensichtliche Bindedomäne besitzt. Zusammen bilden der Transporter und die Histidinkinase ein Resistenzmodul gegen Peptidantibiotika, das in Firmicutes weit verbreitet ist. Eines der am besten verstandenen Systeme ist das BceRS-BceAB System in *Bacillus subtilis*. Dieses System vermittelt Resistenz gegen Bacitracin, Actagardin und Mersacidin. Für dieses System konnte gezeigt werden, dass die Histidinkinase BceS alleine nicht in der Lage ist, auf Bacitracin zu reagieren, sondern stattdessen für die Reizwahrnehmung und die Vermittlung der Resistenz auf den Transporter BceAB angewiesen ist. Der Transporter reguliert somit eine eigene Produktion. Wie der Resistenzmechanismus in diesem System genau funktioniert konnte bisher aber noch nicht hinreichend geklärt werden. Dass Transporter neben ihrer Funktion Substrate über eine Zellmembran zu transportieren auch an der Reizwahrnehmung und der Antwortregulation beteiligt sein können, ist in unterschiedlichsten Beispielen beschrieben worden. Um die Signalweiterleitung an membranständige oder zytoplasmatische Komponenten des Signalwegs gewährleisten zu können, müssen diese miteinander interagieren, zum Beispiel durch direkte Protein-Protein Interaktionen. Bisher konnte jedoch für viele solcher Sensorkomplexe keine endgültige Erklärung für solch eine Interaktion dargestellt werden.

Basierend auf einer Datenbankanalyse konnten über 250 BceAB-artige Transporter identifiziert und ein Großteil davon einer BceS-artigen Histidinkinase zugeordnet werden. Durch eine phylogenetische Studie konnte weiterhin gezeigte werden, dass BceRS-artige Zweikomponentensysteme und BceAB-artige Transporter in Firmicutes Bakterien weit verbreitet sind und sich über Ko-Evolution gemeinsam zu Resistenzmodulen gegen Peptidantibiotika entwickelt haben. Dazu konnte eine konservierte Antwortregulator-Bindestelle in den Promoter Regionen der Transporteroperons bestimmt werden. Zudem war es möglich aufgrund dieser Klassifizierung für diejenigen Permeasen ohne ein benachbartes Zweikomponentensystem anhand der Genomsequenz ein mögliches Regulationssystem zuzuordnen. Diese Erkenntnisse unterstützten die Vermutung über einen sensorischen Komplex zwischen BceS-ähnlichen Histidinkinasen und BceAB-ähnlichen ABC Transportern.

In einer weiteren Studie konnten mittels zufälliger Mutagenese der Transporterpermease BceB Aminosäurereste identifizierte werden, die an der Signalweiterleitung und/oder Resistenzvermittlung beteiligt waren. Durch einige der eingefügten Mutationen wurde nur die Signalweiterleitung bzw. nur die Resistenz beeinträchtigt. Dies spricht dafür, dass eine partielle genetische Trennung der Aufgaben des Transporters möglich ist. Hierdurch konnten erste wichtige Einblicke in den Signalweiterleitungsmechanismus des Bce-Systems gewonnen werden.

Um die vorgeschlagene Kommunikation zwischen Zweikomponentensystem und ABC-Transporter weiterführend zu untersuchen, wurden Interaktionsstudien durchgeführt. Die auf *in vitro* und *in vivo* Studien basierenden Ergebnisse konnten eine direkte Interaktion zwischen BceS und BceAB darstellen. Darüber hinaus konnten wir in dieser Arbeit durch eine Oberflächenresonanz-Spektroskopie zum ersten Mal zeigen, dass die Transporterpermease Bacitracin direkt und spezifisch bindet. Außerdem konnte durch eine *in vitro* Rekonstruktion des Signalwegs im Bce-System gezeigt werden, dass die Aktivität der Histidinkinase durch die Anwesenheit des Transporters beeinflusst wird.

Zusammenfassend zeigt die vorliegende Arbeit direkte Hinweise, dass BceRS-artige Zweikomponentensysteme und BceAB-artige ABC-Transporter zusammen einen sensorischen Komplex für Peptidantibiotika bilden. Dies wird unterstützt durch erste funktionelle Einblicke in die Mechanismen der Reizwahrnehmung und Signalweiterleitung in diesen in *Firmicutes* Bakterien weit verbreiteten Resistenzsystemen.

# Chapter I

## Introduction

## Aims

# 1. Introduction

Bacterial life is characterized by continuous interaction between the bacterial cell and its environment. Bacteria live in harsh environmental conditions, in which they must compete for limited nutrients to survive. In order to gain competitive advantage over their rivals, bacterial species have evolved a variety of antimicrobial agents, e.g. antimicrobial peptides (AMPs). The main target of these peptides is the bacterial cell wall. On the other hand, bacteria have also evolved mechanisms to counteract such threatening conditions. Therefore bacteria possess several communication systems, which transduce the extracellular information over the cell membrane into the cell. These systems enable bacteria to monitor parameters such as osmotic activity, ionic strength, pH, temperature and the concentrations of nutrients and harmful components, including antimicrobial substances, facilitating adaptation and survival in the harsh conditions. The regulation of antibiotic production is attended by mediation of resistance in the bacterial world. As bacterial infections grow increasingly difficult to treat, a deeper understanding of the mechanisms behind the production of and resistance to antimicrobial substances is coming into scientific focus. Many different types of signal transduction systems have been shown to be involved in the biosynthesis of antibiotics and the regulation of resistance modules against them (Gebhard, 2012). One of the major signal transduction systems within bacterial resistance mechanisms is the two-component system (TCS), consisting of a membrane bound histidine kinase (HK) and its cognate response regulator (RR), which often mediates differential gene expression (Jung *et al.*, 2012; Capra & Laub, 2012). A deeper understanding of the underlying regulatory principles is urgently needed.

## 1.1 The cell envelope of Gram-positive bacteria

The bacterial cell envelope, which includes the membrane and other components such as peptidoglycan (PG) surrounding the cytoplasm, is an essential and complex multi-layered structure. Its integrity and functionality is crucial for survival of the cell and must be maintained at all times. The cell membrane forms a crucial communication interface between the cell and its environment. It contains a number of different sensory systems, allowing the cell to monitor and respond to environmental fluctuations (Dijkstra & Keck, 1996; Silhavy *et al.*, 2010).

The cytoplasmic membrane, in Gram-negatives as well as in Gram-positives bacteria, consists of a phospholipid bilayer containing integral membrane proteins, which are often involved in essential processes including energy production and transport. The cell envelopes of most bacteria fall into one of two major groups, defined by their ability to be Gram-stained. Gram-negative bacteria are surrounded by a thin PG layer, which itself is surrounded by an outer membrane containing lipopolysaccharide. In Gram-positive bacteria, the PG layer is many times thicker and an outer membrane is lacking. The cell wall also serves as a scaffold for anchoring other cell envelope

components such as proteins (Vollmer & Seligman, 2010). Additionally, long anionic polymers, called teichoic acids (TAs), are woven through these layers of PG (Neuhaus & Baddiley, 2003). The cell envelope counteracts the high internal osmotic pressure, gives the cell its shape and protects it from environmental threats, but it also serves as a selective barrier for nutrients and other molecules. However, the outer layers of the cell envelopes of Gram-positive and Gram-negative bacteria differ significantly. The following sections will focus on the composition of the Gram-positive cell wall in detail.

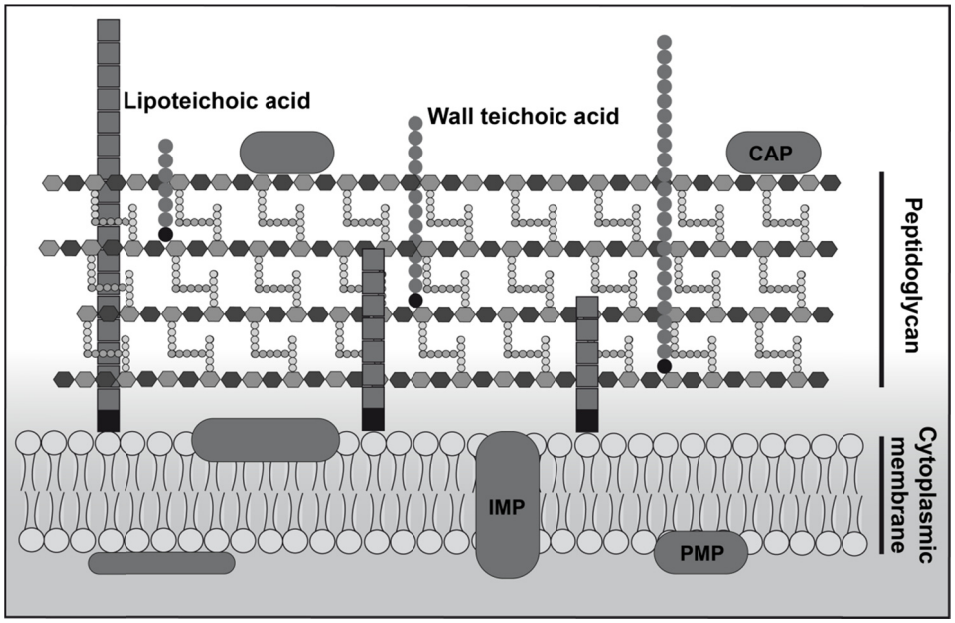


**Figure 1: Schematic view of the cell wall of a Gram-positive bacterium.** The cytoplasmic membrane with embedded proteins is covered by a multi-layered peptidoglycan shell decorated with lipoteichoic acids and wall teichoic acids. CAP, covalently attached protein; IMP, integral membrane protein; PMP, peripheral membrane protein. Modified from (Silhavy *et al.*, 2010; Delcour *et al.*, 1999).

The outer layer of the Gram-positive cell envelope consists of a multi-layered murein sacculus, also called cell wall, which is 10 - 20 nm thick. This allows the Gram-positive cell to sustain turgor pressures in the order of 20 atm (in contrast to 2 - 5 atm in the case of the single-layered PG of Gram-negative bacteria). The overall complex composition of PG builds a net-like structure, giving the sacculus both enormous strength and flexibility (Vollmer *et al.*, 2008; Delcour *et al.*, 1999) (Figure 1). The general chemical features of PG are linear glycan strands cross-linked by short peptides. The glycan strands are made up of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) residues. The glycan strands are generally cross-linked either directly or through a short peptide bridge. These bridges show a species-dependent variation either in the peptide stem, in the glycan strands or in the position or composition of the interpeptide bridge (Turner *et al.*, 2014; Vollmer, 2008; Vollmer *et al.*, 2008).

Surface proteins can be attached to the PG via noncovalent ionic interactions, but many are also covalently attached to stem peptides within the PG layer (Dramsi *et al.*, 2008; Navarre & Schneewind, 1999). The surface proteins enable the bacterial cell to adhere to host tissue, which is an important step

for invasive infections (Foster *et al.*, 2014). The bacterial envelope usually has a global negative net charge caused by another major component of most Gram-positive cell walls - the teichoic acids (TAs). TAs are polymers of glycerol- or ribitol-phosphate units. Two different types of TAs in the Gram-positive cell wall have been described. The wall teichoic acids (WTAs), which are covalently attached to the PG, or the lipoteichoic acids (LTAs), which are anchored in the cytoplasmic membrane (Neuhaus & Baddiley, 2003). The overall negative charge on TAs can be modified by introducing positive charges along the polymer backbone, and these modifications can have an effect on the interactions of bacteria with other cells and molecules. For example, in *Staphylococcus aureus*, a D-alanine transferase couples D-alanine moieties to free hydroxyls on the polyribitol phosphate backbone. This decreases the overall negative charge of the cell envelope and reduces the susceptibility to cationic AMPs (Collins *et al.*, 2002; Peschel *et al.*, 1999; Peschel *et al.*, 2000).

## 1.2 Cell wall biosynthesis

As mentioned above PG is an essential and specific component of the bacterial cell envelope of almost all bacteria. Any degradation of the cell wall or inhibition of its biosynthesis consequently results in cell lysis. Although the bacterial cell wall has been extensively studied for decades, even central questions regarding its architecture still remain mostly unanswered (Turner *et al.*, 2014). The cell wall is highly conserved in its chemical structure (Vollmer, 2008). The proteins responsible for assembly are also conserved (Lovering *et al.*, 2012). PG biosynthesis can be divided into three major stages according to the location of its biochemical reactions (Figure 2): (1) synthesis of cell wall precursors in the cytoplasm, (2) membrane-anchored assembly of these precursors and transport through the cytoplasmic membrane, and (3) incorporation of new PG units into the existing cell wall. The starting point of the cell wall biosynthesis is UDP-activated Glc*N*Ac, which comes from central carbon metabolism. Glc*N*Ac is converted to Mur*N*Ac in a two-step reaction catalysed by MurA and MurB. Thereafter, the first three amino acids of the pentapeptide are added successively by the ligases MurC, MurD and MurE (Barreteau *et al.*, 2008). The last two residues of the pentapeptide are first joined and then attached as a D-Ala-D-Ala dipeptide. The corresponding enzymes are the ligases Ddl and MurF. The next steps occur at the inner face of the cytoplasmic membrane (Bouhss *et al.*, 2008). The resulting Mur*N*Ac pentapeptide is coupled to the lipid carrier undecaprenol-monophosphate at the interior side of the cytoplasmic membrane by the translocase MraY. The resulting complex is called lipid I. Subsequent addition of Glc*N*Ac by the glycosyltransferase MurG results in lipid II, which is comprised of the complete PG subunit linked via a pyrophosphate to the lipid carrier. Many Gram-positive bacteria modify lipid II or nascent PG by the addition of amino acids to position 3 of the peptide by Fem-transferases (Fonvielle *et al.*, 2009). The cell wall precursor is then translocated by FtsW/RodA or MurJ flippases to the exterior side of the cytoplasmic membrane (Mohammadi *et al.*, 2011; Mohammadi *et al.*, 2014; Sham *et al.*, 2014) and incorporated into the existing PG through

transglycosylation and transpeptidation to produce new glycan strands or extend existing strands (Barrett *et al.*, 2007; Sauvage *et al.*, 2008). The remaining undecaprenol-pyrophosphate (UPP) is dephosphorylated and transferred to the interior side of the membrane to ensure a sustainable transfer of another cell wall precursor (Chang *et al.*, 2014). Synthesis of new PG and its incorporation into the sacculus is accompanied cleavage of existing material and removal of old material by hydrolases. The PG turnover products are transported into the cytoplasm, where they are recycled for *de novo* PG synthesis (Goodell & Schwarz, 1985; Park & Uehara, 2008).

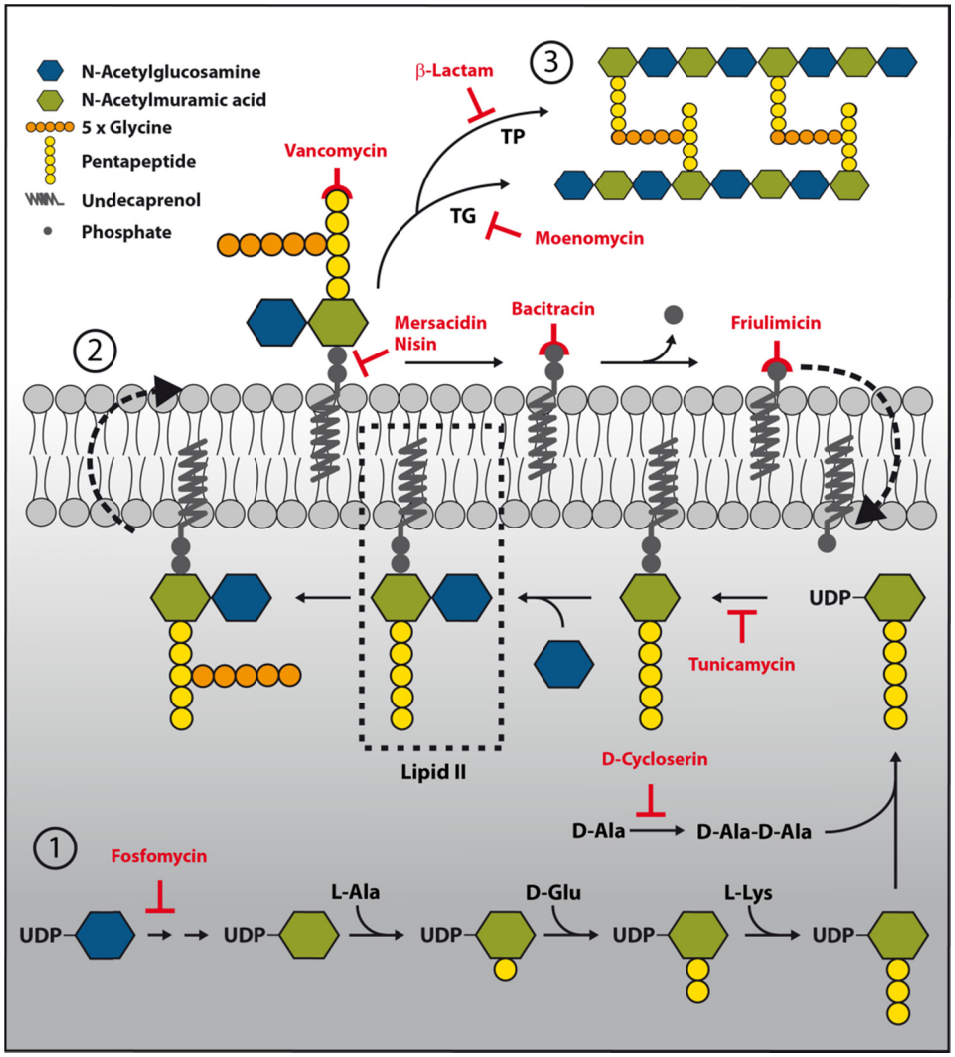


**Figure 2: Schematic representation of cell wall biosynthesis in *S. aureus*.** The synthesis of PG starts in the cytoplasm, where lipid II is generated by several enzymatic steps (Step 1). After translocation into the periplasm (Step 2), the cell wall precursors are incorporated into the growing cell wall (Step 3). The remaining undecaprenol-pyrophosphate is dephosphorylated and transferred to the interior side of the membrane to be available for binding and transfer of another cell wall precursor. The targets of the cell wall precursors are indicated in red. TP, transpeptidation; TG, transglycosylation (Gebhard & Dintner, 2014).

## 1.3 Cell wall active antimicrobial peptides

As described above, many enzymatic reactions are needed to ensure the successful biosynthesis of the cell wall. For this reason the enzymatic steps are favoured targets for antimicrobial substances (Figure 2). A diverse group of such compounds produced by bacteria as well as higher organisms, including animals and humans, are the AMPs. AMPs show an amphipathic and a cationic property, but they are variable in sequence, secondary structure, size and mode of action (Peschel & Sahl, 2006). Bacteriocins, generated by Gram-positive bacteria, are gene encoded, ribosomally produced AMPs that have been a major focus of scientific interest in recent years. Bacteriocins are small peptides that are active against other bacteria and against their producer strains, which have developed specific immunity mechanisms (Cotter, 2005). Bacteriocins are generally subdivided into two classes: those which are translationally modified (class I), and unmodified or minimally modified peptides (class II) (Cotter, 2014; Rea *et al.*, 2011).

One prominent example of bacterial AMPs are the heavily modified lantibiotics (class I), that were named after their characteristic lanthionine or methyllanthionine residues. Their structure can be either elongated, for example in nisin or subtilin, or more globular, as is the case for mersacidin or actargardine (Bierbaum & Sahl, 2009). Nisin is a 34-amino acid peptide with two well defined amphipathic domains. It is widely used in the food industry and has potent activity in the nanomolar range against a broad range of Gram-positive (Gross & Morell, 1971), and to a lesser extent Gram-negative organisms (Stevens *et al.*, 1991). A number of studies have demonstrated that nisin acts by a two-step mechanism in which the peptide first binds to lipid II and then inserts itself into the bacterial membrane to create a pore (Breukink *et al.*, 1999; Breukink & de Kruijff, 2006). In addition to interactions with lipid II, a recent study has demonstrated that nisin also binds to lipid III and lipid IV to interfere with biosynthesis of WTAs and/or LTAs (Muller *et al.*, 2012). It has been postulated that its interaction with lipid II may contribute to the very low resistance levels seen for nisin, as it would likely be very difficult for an organism to alter this highly conserved component of the cell wall synthesis pathway (Yount & Yeaman, 2013).

Another family of AMPs is comprised of cyclic or semi-cyclic lipopeptides and lipoglycopeptides. Members of this group include vancomycin, daptomycin, telavancin, and dalbavancin. These agents may also interfere with the lipid II pathway, and some share biophysical (e.g., $Ca^{2+}$-binding) and structural features with certain mersacidin-like lantibiotics. The most medically relevant compound in this group of antibiotics is vancomycin. It binds tightly to the terminal D-alanyl-D-alanine of the peptide chain of lipid II at the outside of the cell and thereby inhibits the crosslinking (Kahne *et al.*, 2005). By the modification of the dipeptide terminus of the lipid II to D-alanyl-D-lactate, the affinity of vancomycin is significantly reduced, and this results in vancomycin resistance (Barna & Williams, 1984).

Cyclic lipopeptides are produced via non-ribosomal synthesis on large, multifunctional peptide synthetases, generating a macrocyclic ring structure with an attached lipid adduct. Representative

cyclic lipopeptides in preclinical development and clinical use include ramoplanin, empedopeptin, and daptomycin. Ramoplanins, such as teicoplanin and ramoplanin A2, are a family of glycolipodepsipeptides produced by *Actinoplanes* ATCC 33076. Synthesis of ramoplanin is exceptionally complex, involving many enzymatic steps that produce a 49-member ring structure from an initial 17 amino acid template (Hamburger *et al.*, 2009). A number of studies have suggested that ramoplanins likely act through the binding and sequestration of lipid II, inhibiting subsequent biosynthesis of peptidoglycan.

In addition, many bacteria also produce non-ribosomally synthesized peptides, such as the small circular metallo-peptide bacitracin (Johnson *et al.*, 1945; Economou *et al.*, 2013) which is primarily active against Gram-positive bacteria. Bacitracin is produced by strains of *Bacillus subtilis* and *Bacillus licheniformis* as a mixture of closely related dodecapeptides and requires a divalent metal ion for its biological activity (Johnson *et al.*, 1945; Ming & Epperson, 2002). The most potent form is bacitracin A (Azevedo *et al.*, 1993; Ikai *et al.*, 1995). Bacitracin interferes with bacterial cell-wall biosynthesis by binding UPP. This mode of action weakens cell wall biosynthesis by preventing the recycling of the lipid carrier and ultimately leads to bacterial death (Stone & Strominger, 1971; Storm & Strominger, 1973; Economou *et al.*, 2013).

## 1.4 Resistance mechanisms against antimicrobial peptides

To counteract AMP action, bacteria have developed a broad range of resistance mechanisms, which include drug-specific responses such as proteolytic degradation (Sun *et al.*, 2009) or increased production of the inhibited enzyme (Cao & Helmann, 2002), as well as less specific strategies such as biofilm formation (Otto, 2006). One widely distributed resistance mechanism is the alteration of the global net negative charge of the cell envelope to hinder binding of AMPs. Due to the positively charged peptides and the negatively charged bacterial cell envelope, it comes to electrostatic attraction between cationic AMPs and bacterial cell envelopes (Peschel & Sahl, 2006). Bacteria can utilize a number of mechanisms to modulate their cell envelope charge. The mechanism best understood for achieving changing charge of the cell envelope is the D-alanylation of TAs, catalysed by the DltABCD system (Perego *et al.*, 1995; Neuhaus & Baddiley, 2003; Reichmann *et al.*, 2013). For example, a *dltA* mutant shows increased sensitivity to several cationic AMPs in *Lactobacillus casei* BL23, relative to the wild type strain. D-alanylation TAs is limited to Firmicutes (Revilla-Guarinos *et al.*, 2013; Neuhaus & Baddiley, 2003). Recently, an alternative effect of the Dlt-system was proposed, based on steric hindrance of cationic AMP passage through the cell wall due to an increased density of the PG sacculus (Saar-Dover *et al.*, 2012).

Another mechanism to change the net negative charge of the bacterial cell is the lysinylation of membrane phospholipids by MprF. Usually, the phospholipid phosphatidylglycerol is modified by transferring lysyl or alanyl groups of aminoacyl tRNAs to glycerol moiety of phosphatidylglycerol.

Inactivation of MprF in *S. aureus* Sa113 resulted in an increased sensitivity to nisin, gallidermin or protegrin 3 and other AMPs, which shows that *mprF* involved in mediation of resistance against a wide range of AMPs (Peschel *et al.*, 2001). MprF was shown to be involved in the synthesis of Lys-phosphatidylglycerol and Ala- phosphatidylglycerol. In addition to this synthetic function, MprF is also involved in the inner-to-outer translocation of Lys- phosphatidylglycerol. Both mechanisms are thought to reduce the net negative charge of the cell envelope, thus decreasing electrostatic interactions between AMPs and the cell. MprF homologues are common in most bacterial species, but appear to be most abundant in Firmicutes and actionobacteria (Ernst & Peschel, 2011).

While changes in charge provide a protection against a wide range of AMPs a further more specific mechanism of AMP resistance, are described in bacteria for example against bacitracin. Resistance can be mediated by *de novo* synthesis of undecaprenol-phosphate (Cain *et al.*, 1993; Chalker *et al.*, 2000), the expression of alternative UPP phosphatases (UppPs) (Bernard *et al.*, 2005; Cao & Helmann, 2002) and the removal of the antibiotic by specific transporters (Mascher *et al.*, 2003, Ohki, 2003 #242; Neumüller *et al.*, 2001). The latter mechanism is mediated by antibiotic-specific adenosine triphosphate-binding cassette (ABC) transporters and is not restricted to the detoxification of bacitracin. A number of different types of transporters have been described as self-resistance mechanisms in AMP-producing strains as well as for protection against foreign AMPs (Gebhard, 2012). One type of transporters, which mediates self-protection of lantibiotic producing strains, is the so-called LanFEG-type transporter: its expression leads to a specific resistance against self-produced lantibiotics. Most transporters of this group act in a very narrow substrate range, providing resistance only against the produced lantibiotic or structurally very similar peptides (Otto *et al.*, 1998). One example for this transporter family is the NisFEG transporter of *Lactococcus lactis*. To mediate resistance against Nisin, the transporter is thought to move the lantibiotic from the cytoplasmic membrane to the culture supernatant (Otto *et al.*, 1998; Stein *et al.*, 2005; Stein *et al.*, 2003).

BcrAB-type transporters are also involved in resistance against AMPs, but they are described to act specifically against the cyclic peptide bacitracin. One system possessing such a transporter is the biosynthetic locus for bacitracin in *Bacillus licheniformis* ATCC10716 (Podlesek *et al.*, 1995; Neumüller *et al.*, 2001). In this system, BcrAB mediates self-resistance together with a co-transcribed UppP BcrC. Another homolog of this type of transporter is described in *Enterococcus faecalis*. Here the resistance is mediated against foreign bacitracin (Manson *et al.*, 2004). To date, no information is available on the mechanism of AMP transport by BcrAB-type transporters. Some indirect evidence is supported by an earlier report that all three genes (*bcrABC*) of *B. licheniformis* are necessary to confer resistance against bacitracin when heterologously expressed in *B. subtilis* (Podlesek *et al.*, 1995), suggesting the following mode of resistance. Bacitracin is removed by the transporter from the site of action, which in turn enables BcrC to dephosphorylate the cellular target UPP.

A further group comprises a very unique type of transporter. The permease domain of this transporter shows unusual domain architecture with 10 transmembrane helices a large extracellular domain of about 200 amino acids between helices 7 and 8. They have been classified as the peptide-7 exporter (Pep7E) family in the Transporter Classification Database (Saier *et al.*, 2009). One of the best understood systems, harbouring such a type of transporter, is the BceRS-BceAB module in *B. subtilis* (Rietkötter *et al.*, 2008). Here the transporter BceAB is part of a detoxification module together with a TCS consisting of a so called intramembrane-sensing HK, without any extracellular domain, and an OmpR-like RR (Mascher, 2006). A very characteristic feature of this system is the absolute requirement of the transporter in signal perception and induction of its own promoter (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008), which will be described later in detail. A co-occurrence of such a TCS and this type of transporter has been described earlier (Joseph, 2002). For most BceAB-type transporters several substrates have been identified, and often one transporter will recognize structurally very different AMPs, like nisin, bacitracin, ramoplanin and actargardine (Gebhard & Mascher, 2011). In this regard, BceAB-type transporters differ from the other resistance transporters, which are more restricted in their substrate range and transport either bacitracin (BcrAB group) or lantibiotics (LanFEG group). How these type of transporter mediate resistance is still not clear.

## 1.5 Regulation of transporter-mediated resistance against AMPs

Mediation of resistance against AMPs in Firmicutes bacteria is of particular importance to facilitate survival in the harsh competition of the microbial world. To ensure that the different mechanisms of resistance are expressed at the right time, the harmful AMPs have to be recognised as early as possible. For this reason, bacteria have evolved a multitude of signal transduction systems that perceive the presence of AMPs and initiate and modulate the expression of specific resistance determinates. Generally, they consist of transmembrane proteins that link the input and the intracellular response. In the case of TCSs, these transmembrane signaling systems consisting of a HK and its cognate RR (Mascher *et al.*, 2006). The N-terminal input domain of HKs from different systems shows variations in membrane topology, composition and domain arrangement (Mascher *et al.*, 2006). Overall, these kinases can be divided into two groups with respect to their mode of signal perception. One type is able to perceive the signal directly and the other type requires an accessory transporter for stimulus perception.

### 1.5.1 Histidine kinases with direct stimulus perception

HK, which regulate the expression of transporter by direct stimulus perception fall into the largest group of extracellular sensing HKs (Mascher *et al.*, 2006). Sequence analysis of the NisK and SpaK HKs, which regulate biosynthesis and immunity for nisin and subtilin, respectively (Kleerebezem, 2004), shows that both belong to the EnvZ-like subgroup of HK (Parkinson & Kofoid, 1992), and are

characterized by a large extracytoplasmic input domain (around 100 amino acids) flanked by two transmembrane helices (Mascher *et al.*, 2006). Production of nisin is auto-induced in response to extracellular nisin (Kuipers *et al.*, 1995). NisK is suggested to be induced by direct interaction with nisin and regulates the expression of the NisFEG transporter, which mediates the self-protection of *L. lactis* (de Ruyter *et al.*, 1996; Kleerebezem, 2004).

### 1.5.2 Histidine kinases with indirect signal perception

BceS-like HKs are also involved in the regulation detoxification transporter of AMPs. This type of HKs share striking similarities in their overall domain organization: they are small HKs of less than 400 amino acids total length. The cytoplasmic transmitter domain harbors only the standard features characteristic for all HK (HisKA, HATPase_c for kinase activity). The N-terminal input domain consists of two transmembrane helices with a small periplasmic linker (around 25 amino acids) in between thus lacking an obvious input domain. This minimalistic type of HK is described to be involved in the regulation of self-protection of AMP producer strains. This is shown for the bacitracin biosynthesis locus of *Bacillus licheniformis*, in which BacRS regulates the expression of *bcrABC*, but not the bacitracin biosynthesis itself (Neumüller *et al.*, 2001). The sensing mechanism of bacitracin by BacS is not known.

This type of HK is further involved in the regulation of BceAB-type transporter. In a phylogenetic analysis, these so-called intramembrane-sensing HKs formed a distinct group and were found almost exclusively in Firmicutes bacteria (Mascher, 2006; Mascher, 2014), where they form part of the cell envelope stress response (Mascher *et al.*, 2003). Most of the TCSs containing such a HK are encoded next to an ABC transporter (Joseph, 2002). Here the expression of the transporter is induced by sublethal concentrations of its substrate AMPs and requires the presence of both the HK and the transporter. This unique mode of signal transduction is described and characterized very well in the BceRS-BceAB resistance module of *B. subtilis* and will be described in more detail in the following paragraph.

## 1.6 The BceRS-BceAB module of *Bacillus subtilis*

The BceRS-BceAB module of *B. subtilis* confers resistance against bacitracin, actagardine, and mersacidin (Mascher *et al.*, 2003; Ohki *et al.*, 2003; Rietkötter *et al.*, 2008; Staron *et al.*, 2011). The module is comprised of a TCS and an ABC transporter. The TCS consists of a HK BceS belonging to the so called intramembrane sensing HKs and an OmpR-like RR BceR. They regulate together the expression of *bceAB* encoding for an ABC transporter in the adjacent operon (Figure 3). This transporter consists of an ATPase domain and a permease domain, which shows very unusual domain architecture with ten transmembrane helices and a characteristic, large extracellular domain between transmembrane helix seven and eight. Upregulation of the transporter is induced in the presence of

sublethal concentrations of its substrate AMP, and this requires the presence of both the TCS and the transporter itself. Due to the architecture of BceS it was further described, that the HK has an absolute requirement for BceAB in signal perception and mediating resistance (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008). Furthermore it has been shown that the extracellular domain of the permease BceB is indispensable for signaling and contributes to the specificity of the transporter, proposing to be the signal perception domain of the module (Rietkötter *et al.*, 2008; Falord *et al.*, 2012; Hiron *et al.*, 2011). Importantly both, the signal transduction and the mediation of resistance are dependent on ATP-hydrolysis (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011). In *B. subtilis* there are two further paralog Bce systems described, the PsdRS-PsdAB and the YxdJK-YxdLM module. These systems are very similar in sequence, genomic context organization and topology of their proteins, but they differ in their substrate range. The Psd system is induced by a lipid II-binding lipopeptide, enduracidin, and the lipid II binding lantibiotics nisin, subtilin, actagardine, and gallidermin. At the same time the transporter also recognizes substrates that differ considerably to that of actagardine like the lipodepsipeptide, enduracidin, but not the similar ramoplanin (Staron *et al.*, 2011; Joseph *et al.*, 2004).

In the recent years, several BceAB-type transporters have been identified as resistance determinates against AMPs in nonproducing strains (Becker *et al.*, 2009; Collins *et al.*, 2010; Meehl *et al.*, 2007; Ohki *et al.*, 2003). The regulating TCS of these transporters is generally encoded in an operon adjacent to that of the transporter. (Collins *et al.*, 2010; Joseph *et al.*, 2004; Li *et al.*, 2007; Meehl *et al.*, 2007; Ohki *et al.*, 2003; Ouyang *et al.*, 2010). This widespread co-occurrence of BceS-type HKs and BceAB-type transporters have been described in Firmicutes bacteria earlier (Joseph, 2002).

Recently, the functional relationship between BceRS-type TCS and BceAB-type transporter for signal transduction and resistance was also observed in several other detoxification modules against AMPs in *S. aureus*, *L. casei* and *E. faecalis* (Hiron *et al.*, 2011; Falord *et al.*, 2012; Revilla-Guarinos *et al.*, 2013; Gebhard *et al.*, 2014). In some cases a separation of the two functions, sensing and detoxification, of the transporter were observed. A transporter is present, which ensures the signal perception to maintain the induction of a second transporter that in turn mediates the actual resistance. Such a scenario is found for example in *S. aureus*, where the TCS BraRS together with its sensing transporter BraDE induces expression of the transporter VraDE, which provides resistance against bacitracin (Hiron *et al.*, 2011).

Taken together, these results proposing an actual model for the mechanism of signal transduction in the Bce module of *B. subtilis* and the current working model assume the existence of a sensory complex between transporter and TCS (Figure 3). Bacitracin is first detected by BceAB this leads to a proposed conformational change due to the ATP-hydrolysis. The signal perception is then transmitted to BceS, which in turn is autophosphorylated and transfers the phosphate to BceR. This leads to binding of BceR to the $P_{bceA}$ promoter and an up regulation of *bceAB* expression. The elevated amount

of BceAB leads to removal of bacitracin. With this unique signal transduction pathway the transporter regulates its own expression.

At the same time, several important questions remain to be answered. First, how does BceAB sense bacitracin? The BceAB-type transporters are reported to provide different ranges of AMPs (Gebhard & Mascher, 2011), but it is still unclear how the different substrates are recognized and distinguished? The membrane topology of BceB with its large extracellular domain has been suggested to be involved in bacitracin detection (Rietkötter *et al.*, 2008). Second question is how BceS and BceAB interact to maintain the signal transduction? One possibility is that BceS activity is triggered by a conformational change of BceAB. Another question is how the transporter confers bacitracin resistance? The direction of transport remains unknown and is still a matter of debate. There are several possibilities: BceAB is proposed to be an importer removing the AMP from its site of action followed by intracellular inactivation, an exporter removing the AMP from the membrane into extracellular space or acts like a flippase to mediate resistance (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011; Bernard *et al.*, 2007; Kingston *et al.*, 2014).

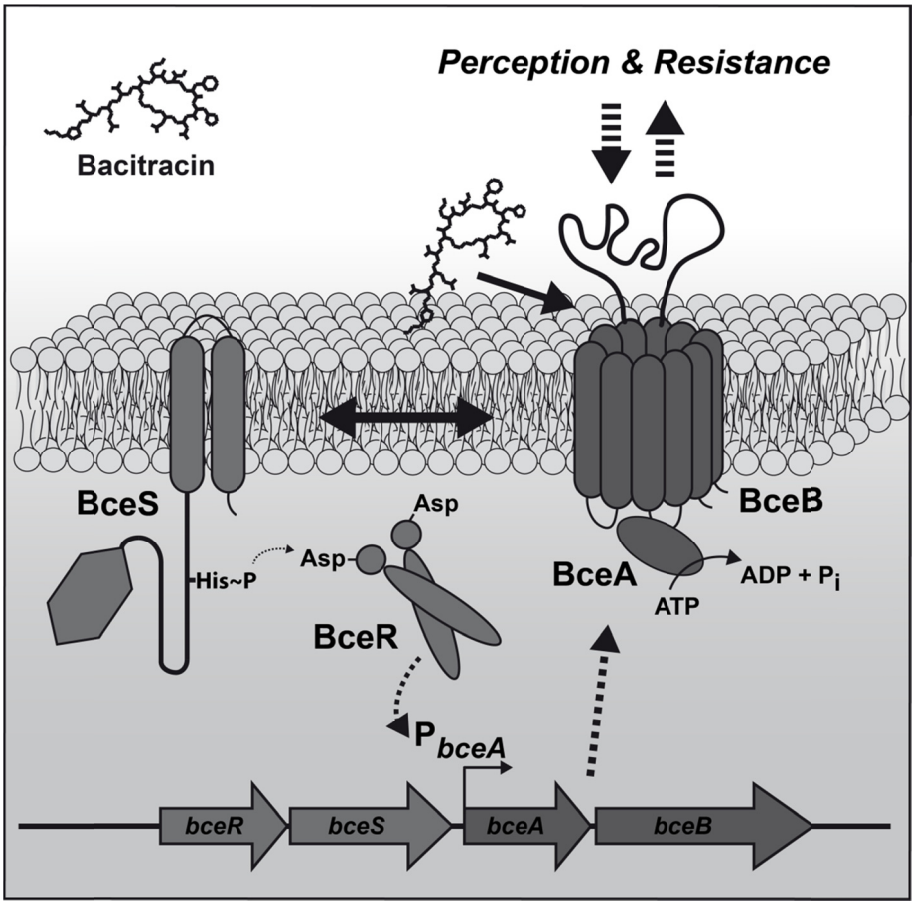


**Figure 3: Working model for the BceRS-BceAB bacitracin resistance module of *B. subtilis*.** Bacitracin is bound by the transporter BceAB. BceAB and BceS interact to form a sensory complex in the membrane. ATP-hydrolysis by the transporter triggers the activation of BceS, which in turn leads to phosphorylation of BceR. Activation of the target promoter ($P_{bceA}$) by BceR then induces increased production of BceAB to ensure resistance. Events relating to transcription are labelled with dotted arrows; the potential interactions of the components of sensory complex are indicated with a double headed arrow.

# 2. Aims of this thesis

Recent studies of BceS-like HKs, which lack an obvious input domain, have raised the question of the function of such HKs. As described in several examples, these minimalistic HKs require accessory ABC transporter to act as their sensors. Together they are thought to build a sensory complex against AMPs. The aim of this thesis was to analyse the functional relationship between BceS-type HKs and BceAB-type ABC transporters. Further we wanted to characterize the functional role of BceAB-like transporter concerning the signal transduction and the mechanism of resistance. Therefore we combined different approaches to emblaze this unique signal transduction pathway in more detail.

**Chapter II**

A comprehensive phylogenetic study of these systems was carried out to obtain insights into the distribution and potential conservation of such resistance modules. Additionally, we had a closer look on the extracytoplasmic domain of BceAB with regard to their primary sequences and secondary structures to get valuable insights of the substrate specificity. Moreover, we wanted to identify a conserved putative RR binding motif for BceR-like regulators.

**Chapter III**

In this study, we performed a random mutagenesis analysis of the transport permease BceB of *B. subtilis* with the aim of identifying regions or residues within the transporter that are involved in signaling and/or resistance. A central question in this was whether transport and signalling are genetically separable processes.

**Chapter IV**

To gain a deeper understanding of the molecular mechanisms underpinning these unusual resistance modules, we set out to characterize the protein-protein and protein-substrate interactions within these modules in more detail. Therefore we investigated the formation of protein contacts with an *in vivo* approach using bacterial two-hybrid assay and *in vitro* using a pull-down assay. Further, we tested whether BceB is able to bind its substrates directly, as an understanding of the transporter's affinity for its substrates would provide valuable first insights into resistance mechanism. Finally, we reconstructed the entire Bce-system *in vitro* to test for the effect of the transporter of HK activity.

# Chapter II

## Coevolution of ABC Transporters and Two-Component Regulatory Systems as Resistance Modules against Antimicrobial Peptides in Firmicutes Bacteria

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*J. Bacteriol.*, Aug. 2011, p. 3851–3862, Vol. 193, No. 15

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# Chapter III

## Identification of Regions Important for Resistance and Signalling within the Antimicrobial Peptide Transporter BceAB of *Bacillus subtilis*

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*J. Bacteriol.*, July 2013, p. 3287–3297, Vol.195, No. 14

# Chapter IV

## A Sensory Complex Consisting of an ATP-Binding-Cassette Transporter and a Two-Component Regulatory System Controls Bacitracin Resistance in *Bacillus subtilis*

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# Chapter V

**Discussion**

**Outlook**

# 1. Concluding Discussion

There is increasing evidence that certain transport proteins have acquired additional sensing or regulatory functions beyond solute transport, and these have been designated as trigger transporters. For these bifunctional transporters a direct protein-protein interaction with membrane-integrated or soluble components of signal transduction relays has been postulated (Tetsch & Jung, 2009). However, for most sensor/co-sensor pairs, conclusive proof of such an interaction is lacking, and so far little is known about the sites that might mediate contacts between the putative hetero-oligomeric interfaces and how communication is achieved.

One type of such transporters, the BceAB-like ABC transporter, was the focus of this thesis. Together with BceRS-like TCSs, this type of transporters forms detoxification modules against peptide antibiotics (Mascher, 2006). The aim of this thesis was to gain a better understanding of the distribution and function of these systems, using a range of approaches from comparative genomics to molecular genetics and biochemical investigation.

## 1.1 Phyletic distribution and co-evolution

Most compounds of the class of lantibiotics, cyclic peptides like bacitracin, glycopeptides (vancomycin) and lipodepsipeptides (ramoplanin) are produced mainly by Firmicutes bacteria and target closely related species. Several ABC transporters of the BceAB-type have been identified as resistance determinants against AMPs, but they are not described to be related to biosynthetic loci (Becker *et al.*, 2009; Collins *et al.*, 2010; Meehl *et al.*, 2007; Ohki *et al.*, 2003). In order to investigate the phyletic distribution of resistance modules possessing such a BceAB-type transporter, we applied a database analysis based on the typical domain architecture of BceB-like permeases (**Chapter II**). By analysing the genetic neighbourhood of the identified ABC transporters, we observed a conserved co-occurrence of the encoding genes with operons encoding BceRS-like TCSs. We investigated the evolutionary and regulatory relationship between BceS-like HKs and BceAB-like ABC transporters. They are mostly found in *Bacillales*, *Lactobacillales* and *Clostridiales*. The number of systems per organism showed a great disparity. For examples species of the *Bacillales* harboured up to six BceAB-type transporters, while others like the *Lactobacillales*, possessed only one or two. This reflects the strong need for soil living bacterial species for such detoxification modules in order to respond to the great variety of AMPs in this environment (**Chapter II**). Since the study of 2011, described in **Chapter II**, additional resistance modules of this type were described in *E. faecalis*, *L. casei* BL23 and *S. aureus* (Gebhard *et al.*, 2014; Revilla-Guarinos *et al.*, 2013; Falord *et al.*, 2012).

By calculating phylogenetic trees of BceS-like HKs and BceB-like permeases we were able to show a clear coevolutionary relationship between the two protein families in a qualitative (comparison of the phylogenetic trees) and quantitative way (correlation coefficient) (**Chapter II**). Coevolution of

the two protein families could, for example, be due to direct physical interaction between BceS-like HKs and BceB-like permeases. This case is highly likely, because BceS was shown to have an absolute requirement for the ABC transporter BceAB in *B. subtilis* (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008). In the meantime several other studies showed this conserved functional link between HK and ABC transporter to cooperate in signaling and detoxification of AMPs (Falord *et al.*, 2012; Hiron *et al.*, 2011; Gebhard *et al.*, 2014). Together, these data show the widespread distribution of a unique resistance module against AMPs, in which the transporter plays an important role in signaling and mediation of resistance (**Chapter II**).

## 1.2 Sensing Process – the extracellular binding domain and its specificity

One of the known inducing compounds of the BceRS-BceAB system of *B. subtilis* is bacitracin. Until now it is still not clear what kind of stimulus is sensed by the resistance module. For example Bacitracin could be detected directly as the free peptide, or as the target:peptide complex (UPP-bacitracin). Alternatively, it could be detected indirectly by sensing the caused damage. For the Bce system it was shown that the BceRS-dependent promoter $P_{bceA}$ responds to bacitracin in L-forms (cell wall-deficient bacteria) of *B. subtilis* (Wolf *et al.*, 2012). This response, irrespective of the presence or absence of the cell wall, suggests a direct drug-sensing mechanism of stimulus perception by Bce-like detoxification modules. Based on the architecture of the N-terminal input domain of BceS-like HKs it was not clear if this type of HKs are able to sense the signal directly. Instead, these HKs were shown to require BceAB-like transporters for sensing (Joseph, 2002; Rietkötter *et al.*, 2008; Hiron *et al.*, 2011). The membrane topology of BceB-like permeases is, characterized by the presence of a large extracellular loop, which was shown to be essential for bacitracin perception by BceB (Rietkötter *et al.*, 2008). Additionally Msadek and colleagues showed recently, that the extracellular domain indeed is involved in signal perception as it was shown to determine the specificity of the transporter (Hiron *et al.*, 2011). The analysis of the phylogenetic groups could not find any correlation between the group assignment of BceB-like permeases and their substrate range. In addition the sequence conservation the extracellular domain is very low (less than 10 %) and a detailed investigation of the predicted secondary structure also did not reveal any correlations with substrate range. This high degree of variability is probably required for adaptation to the large variety of substrates recognized by these modules (**Chapter II**). Moreover, we were not able to identify any mutation by a random mutagenesis of BceB in the extracellular domain of the transporter, despite its large size of around 200 amino acids, (**Chapter III**). Form this results it is still not predictable how the substrate perception of the transporter occurs or whether the extracellular domain is involved in perception of AMPs at all. Most of the Bce-like detoxification modules analyzed are not specific for a single substrate but instead distinguish between often structurally very diverse peptides (Gebhard, 2012). For example substrate spectra of the paralog systems Psd and Bce in *B. subtilis* are different. The Psd system distinguishes

between very similar actargardine and mersacidin, and reacts to enduracidin but not to ramoplanin. *B. subtilis* provides two different systems to detoxify bacitracin (Bce) and nisin (Psd) (Staron *et al.*, 2011), whereas *S. aureus* utilises only one system to detoxify both substances (Hiron *et al.*, 2011). By testing the binding of bacitracin and nisin to BceB via a surface plasmon resonance spectroscopy in the present thesis, we were able to clearly show that BceB binds its substrate bacitracin directly and specific (**Chapter IV**). The affinity to the transporter is high (60 nM) and consistent with the *in vivo* threshold of the target promoter, $P_{bceA}$, of 70 nM (own unpublished data). This is coherent with the observation, that bacitracin is sensed at the same time that detoxification starts, at very low concentration (Rietkötter *et al.*, 2008). Here is the first time shown, that the direct binding of BceB-like permeases is the likely mechanism for signal perception in these resistance modules.

## 1.3 Conserved regulation mechanism in Bce-like systems

The ABC transporter BceAB is shown to be required for signal perception and detoxification of bacitracin. Furthermore, both processes are functionally linked to transport activity of BceAB (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011). Conformational changes in the transporter are proposed to trigger the autophosphorylation of BceS, which in turn leads to a phosphotransfer to the RR BceR. This is followed by the binding of BceR to the $P_{bceA}$ promoter and to an up regulation of the expression level of *bceAB* (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008). To proof if a regulatory link between BceRS like TCSs and expression of BceAB-like transporters is common in this type of resistance module, we analysed regulatory elements of the ABC transporter expression, especially RR binding sites of BceAB-like systems (**Chapter II**). Several binding sites have already been identified in the promoter of the transporter operon (Joseph *et al.*, 2004; Ohki *et al.*, 2003; Ouyang *et al.*, 2010). Similar RR binding sites were also identified in AMP detoxification modules of *S. aureus* (Hiron *et al.*, 2011) and *L. casei* BL23 (Revilla-Guarinos *et al.*, 2013), where the authors could also show a regulatory relationship between TCS and ABC transporter to maintain resistance against AMPs. We were able to identify a putative binding site in about 70 % of all promoter regions analysed. With these findings we could clearly show that the transporters are indeed likely to be regulated by their adjacent TCS (**Chapter II**).

Furthermore we used the phylogenetic analysis to identify putatively corresponding TCS for ABC transporters whose genes were not directly associated with a TCS operon. These candidate TCSs can be identified by genome analysis for BceS-like HKs and by the phylogenetic analysis it is possible to match these kinases to orphan transporters (**Chapter II**). Several predictions were successful and that the regulatory relationship is true was shown recently for example in *E. faecalis*. Here two putative ABC-transporters, EF2050-2049 and EF2752-2751 were shown to be involved in bacitracin resistance. The transporter EF2752-2751 was previously identified by our phylogenetic analysis as an orphan transporter and indeed the genome of *E. faecalis* encoded for a single BceRS-like TCS,

EF0926/0927 (**Chapter II**). The transporter (EF2752-2751) was described to be responsible for sensing bacitracin and regulating the expression, together with the HK EF0927, of the second transporter EF2050-2049, which was shown to be responsible for detoxification (Gebhard *et al.*, 2014). A similar complex regulatory relationship between HK and ABC transporters have also been described in *S. aureus* and in *L. casei* (Gebhard & Mascher, 2011; Hiron *et al.*, 2011; Revilla-Guarinos *et al.*, 2013).

As shown previously, the BceAB transporter in *B. subtilis* is required for both sensing and detoxification. By a random mutagenesis of the transporter permease BceB in **Chapter III** we identified residues, which affected only one function of the transporter but not the other. With this we were able to show for BceAB a partial genetic separability of signaling and resistance. This leads to a speculation, that BceAB-like transporters at first possessed both functions and in *S. aureus* and *E. faecalis* a duplication event occurred by which the transporters specialised in either resistance or signaling.

In contrast to this described positive effect of BceAB-like transporters on the activity of HKs, there are also cases described, where transporters show repressing function on the activity of the cognate HKs. In recent years, a number of systems were described in which transport proteins are used as co-sensors for the signal transduction machinery. They are able to interfere with signal transduction processes by either transporting effector molecules into the cytoplasm or by interacting directly with sensory components (Tetsch & Jung, 2009).

One well-known example is the widespread Pst/Pho system, which senses environmental phosphate. Transcription of the genes for bacterial high-affinity phosphate transport systems is usually regulated by a TCS, PhoBR in Gram-negative bacteria (van Veen, 1997), PhoPR in Gram-positive bacteria (Qi *et al.*, 1997; Sola-Landa *et al.*, 2005) and SenX3-RegX3 in *Mycobacteria* (Glover *et al.*, 2007), where PhoR/SenX3 acts as the sensor kinase and PhoB, PhoP or RegX3 as the cognate RR. Mutations in Pst have been shown to lead to constitutive activation of the Pho regulon genes in a number of bacteria such as *Escherichia coli* (Wanner, 1996), *Sinorhizobium meliloti* (Yuan *et al.*, 2006), and *Mycobacterium smegmatis* (Gebhard & Cook, 2008). The PstSCAB ABC transporter and the peripheral membrane protein PhoU are required for signal transduction (Steed & Wanner, 1993) and, together with PhoR/SenX3, are thought to form a membrane-bound repressor complex under phosphate-replete conditions (van Veen, 1997). An additional example is represented by a sensory complex consisting of a one-component system and a secondary transporter, the CadC/LysP system in *E. coli*. The central component of the Cad system is the membrane-integrated pH sensor and transcriptional activator CadC, which regulates induction of the *cadBA* operon under low pH. CadC activity is also lysine-dependent (Haneburger *et al.*, 2012). Lysine-dependent activation of CadC requires the co-sensor LysP, a lysine-specific permease (Tetsch *et al.*, 2008). Together these example show a repressing regulatory effect of the transporter on the signal transduction system, which stands in contrast to the BceAB-like systems described above.

One system, where the transporter also acts as an activator or a HK is the UhpABC/UhpT system for glucose 6-phosphat (G6P) sensing and transport in *E. coli*. Here two homolog transporters UhpT and UhpC are able to transport G6P, but the latter nearly lost transport activity. UhpC works as a receptor and shows a high affinity for G6P and a low rate of transport relative to UhpT. By signal perception UhpC transfers the signal to and activates by direct interaction UhpB HK that up regulates the expression of *uhpT*, which in turn transports G6P (Island & Kadner, 1993). This example shows that the transport activity of UhpC is not necessary for signal transfer to the cognate HK. One similar situation is described in BraRS-BraDE/VraDE system of *S. aureus*. Here the BraDE transporter is shown to be a signal transporter and VraDE is the detoxification transporter controlling bacitracin and nisin resistance. It is shown that both distinct roles are dependent on ATP-hydrolysis. This stands in contrast to situation in the Bce-system where the transport activity is mediating the signal transfer and the resistance in one transporter (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011). Despite the striking similarities between BceAB and other transporters in signal transfer, the Bce-systems show a very unique mode of regulatory mechanism.

## 1.4 Interaction between histidine kinase and ABC transporter

Coevolution of two protein families can be due to a number of reasons, such as participation of both proteins in the same process, interaction of both proteins with the same ligand, or direct physical interaction between two proteins. Because of the described absolute requirement of the ABC transporter in stimulus detection and detoxification in Bce-like resistance modules, such a direct interaction between HK and transporter is conceivable to ensure the information transfer between both proteins and was proposed based on the observed coevolution of both proteins (**Chapter II**). A direct functional link between HK and transporter permease is further likely, considering the architecture of the N-terminal input domain of BceS-like kinases. Based on the lack of any obvious extracellular input domain this type of HK is thought to detect the stimulus at or within the cytoplasmic membrane (Mascher, 2006). Furthermore, they lack any cytoplasmic domains with which they could directly perceive an intracellular stimulus. Together with the observation that no BceS-like kinase was found without an ABC transporter we proposed that BceS-like kinases are not able to sense the stimulus directly but instead transfer the information coming from the transporter into the cell (**Chapter II**).

An important question in such a model that remains unanswered is how do BceS-like HKs and BceAB-like transporters communicate. One possibility is that the perception of AMPs leads to a conformational change of the permease domain which leads to an interaction with and an activation of the cognate HK (Hiron *et al.*, 2011), assuming a direct interaction between both proteins. First experimental evidence for a direct interaction was provided in *S. aureus*, where the interaction between the HK GraS and the permease VraG was shown by a bacterial two-hybrid assay (BACTH). Beside the transporter and the TCS, this system requires an additional cytosolic component, the GraX,

in order to function. Here GraX is also shown to interact with the HK and the ATPase domain of the transporter (Falord *et al.*, 2012). However, the Bce system in *B.subtilis* possesses no GraX-like protein but also observed an interaction between BceS and BceB. Importantly, we provided first direct experimental confirmation by an *in vitro* pull-down assay, which showed a direct and specific interaction between BceS and BceAB. Furthermore we applied an *in vitro* phosphorylation assay, where we observed a basal activity of BceS, which was significantly reduced by the presence of the ABC transporter BceAB. This further supports a direct interaction of both proteins. In none of the described assays we observed an effect of bacitracin on the interaction, concluding that the complex between HK and ABC transporter is formed constitutively (**Chapter IV**). Further, in *S. aureus* the RR GraR was shown to interact with the ATPase VraF and the cytosolic GraX, and is thought to be a part of the multicomponent complex (Falord *et al.*, 2012). We could also observe such an involvement of BceR in the sensory complex, based on our BACTH analysis, but only when BceS and BceB were simultaneously expressed in the cells (**Chapter IV**). This strengthens the role of the HK and the transporter as the central components of the sensory complex, giving the other components a scaffold to interact for maintaining signal transduction.

Following demonstration of complex formation, other central questions that remain to be answered are where the interaction occurs and how this interaction influences signal transduction. There are three possibilities: (I) interaction between extracytoplasmic regions (II) interaction within the membrane or (III) cytoplasmic interaction. The latter two options appear more likely because BceS-like HKs lack an extracellular domain (Mascher, 2006). To begin to address this question experimentally we carried out a random mutagenesis on the permease BceB. With this, we were able to identify residues that are involved in signaling and resistance by the Bce system and are thus interesting candidates for further investigations of the interaction (**Chapter III**). This approach showed an accumulation of mutations in the C-terminal part of BceB, especially in transmembrane helix VIII. Most of the residues of transmembrane VIII were affected in both signalling and resistance. The transmembrane helix VIII is directly connected to the large extracellular domain of the permease, which is thought to be the binding domain of the transporter and plays a role in substrate specificity (Hiron *et al.*, 2011; Rietkötter *et al.*, 2008). It is conceivable that the transmembrane helix VIII serves as transmitter to communicate extracellular bacitracin binding to the cytoplasmic face of the membrane (**Chapter III**). In the N-terminal part of BceB we also identified mutations affecting highly conserved residues. One interesting mutation of residue G215 is located in transmembrane helix V and abolished interaction between BceS and BceB in the BACTH assay. However, this mutation is still able to mediate resistance similar to wild type and significant signaling activity was observed in *B. subtilis*. This suggests that the mutation weakens the interaction to give negative results in the two-hybrid assay, but that the signal can still be passed to the HK. There are also other mutations described, which inhibit the signaling but allow a physical interaction between BceS and BceB in the BACTH (**Chapter III**).

One could imagine, that these residues are involved in serving as an interaction surface for the HK BceS to ensure the central scaffold of BceS and BceAB in the sensory complex mentioned above. To further investigate the region of interaction between BceS and BceAB we choose a genetic approach. *B. subtilis* possess a homolog Psd system, which is very similar in sequence and topology of the proteins to the Bce system but is induced by nisin, not by bacitracin. We constructed a chimeric HK by fusing the transmembrane region of BceS and the cytoplasmic domain of PsdS. The chimeric HK showed signaling activity by induction with nisin, but not with bacitracin (C. Fang and T. Mascher, personal communication). This suggests that the transmembrane region of the HK is not involved in the specificity because the transmembrane region of BceS enabled the communication of cytoplasmic domain of PsdS with the transporter PsdAB to ensure the signal transduction. In summary, these results propose that BceS and BceB interact in the cell membrane by several residues to ensure the above discussed constitutive sensory complex. This probably enables the communication of bacitracin binding to the extracellular domain of BceB to the cytoplasmic domain of BceS.

## 1.5 Working model for the BceRS-BceAB system of *B. subtilis*

Based on the results obtained in this thesis using comparative genomics, molecular genetics and biochemical approaches (**Chapter II, III and IV**), we propose the following model for the signal transduction in Bce-type detoxification modules in Firmicutes bacteria, exemplified by the Bce system of *B. subtilis* (Figure 4). The membrane-bound signaling complex consists of HK BceS and the ABC transporter BceAB, which is comprised of a permease and two ATPase subunits. This signaling complex is proposed to be formed consistently both in the presence an absence of its substrate AMPs. The module perceives its signal AMP by direct binding to the transporter, which results in ATP dependent transport activity and the caused conformational changes of the transporter are suggested to trigger the activation of its cognate HK. The autophosphorylation of the HK in turn leads to a phosphorylation of the RR and an elevated expression of *bceAB*. The following increased amount of ABC transporter in the membrane leads to resistance against AMPs most likely by removal of the peptide from its site of action. The response threshold of $P_{bceA}$ promoter is at very low substrate concentrations in the nanomolar range (**Chapter IV**). This promoter activity is gradually increased over 3 – 4 orders of magnitude bacitracin concentration (Rietkötter *et al.*, 2008). The careful adjustment of amount of transporter to the increasing amount of bacitracin makes the BceRS-BceAB system to a very efficient detoxification module against AMPs.

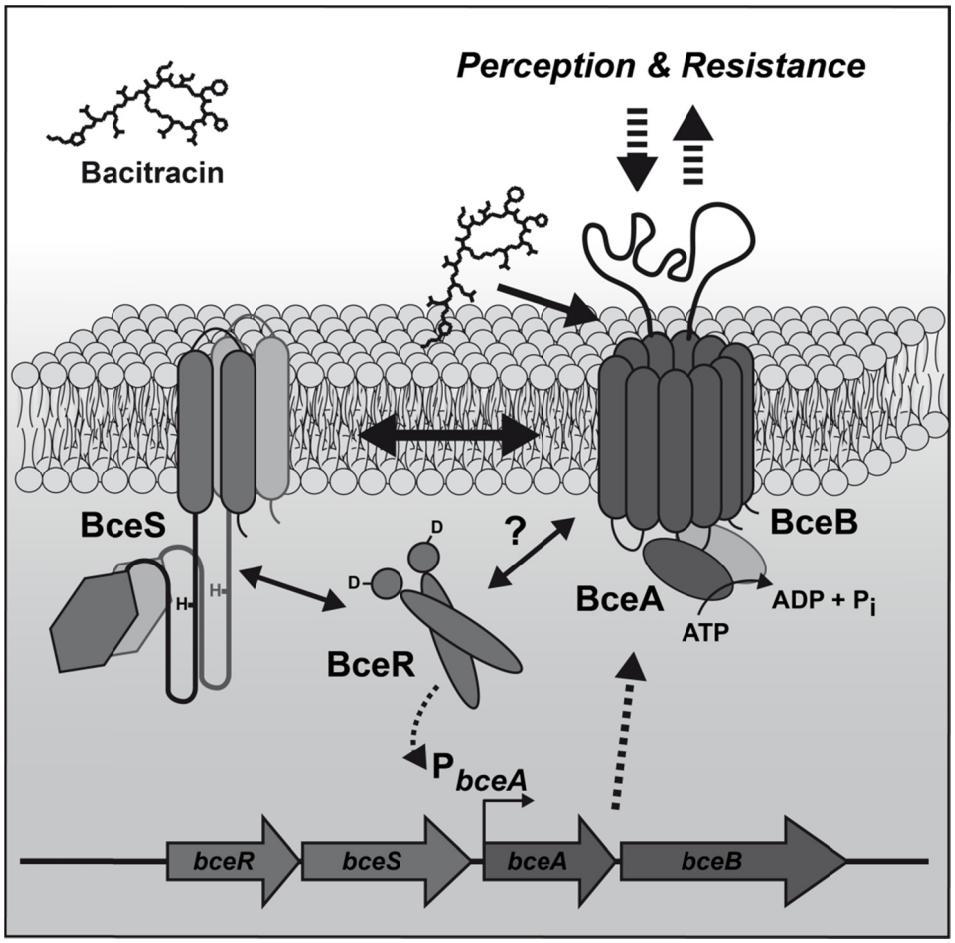


**Figure 4: Working model for the BceRS-BceAB bacitracin resistance system of *B. subtilis*.** Bacitracin is bound directly by the transporter BceAB. BceAB and BceS interact to form a sensory complex in the membrane. ATP-hydrolysis by the transporter triggers the activation of BceS, which in turn leads to phosphorylation of BceR. Activation of the target promoter ($P_{bceA}$) by BceR then induces increased production of BceAB to ensure resistance. Interactions between proteins are marked by double headed arrows; transcription events are labelled with dotted arrows; the potential interaction of BceR with the sensory complex of BceS and BceAB is indicated with a question mark.

In summary the thesis gives a phylogenetic distribution of Bce-like detoxification modules, a tool to identify RR binding sites and TCS for orphan ABC transporters (**Chapter II**). Furthermore it gives first valuable insights into functionally important residues of BceB, which open the way to further investigations into the signaling mechanism **(Chapter III**). Additionally, in **Chapter IV** we were able to gain functional insights into the mechanism of signal perception and signal transduction of these wide spread resistance module in Firmicutes bacteria against AMPs.

# 2. Outlook

Clearly, in this working model several crucial questions remain unanswered concerning the sensing process, the direction of transport and the mediation of resistance in this unique signal transduction pathway. In the next few paragraphs some ideas are discussed which could generate further valuable information on the functionality of these modules.

## 2.1 Signal perception in BceAB-like transporters

The membrane topology of BceB-like permeases is very characteristic, and the amino acid sequence of transporters is well conserved (25 – 40 %) among Firmicutes bacteria. However, the large extracellular domain of the permease shows a great variability in its sequence (less than 10 %) (**Chapter II**). As it was shown that the extracellular domain is essential for signaling and for substrate specificity, the extracellular domain is thought to be the binding site of the transporter (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011). While we could show the first time, that BceB binds bacitracin directly, it is still not clear where exactly the binding of bacitracin occurs (**Chapter IV**). Further work will be required to solve this fundamental mechanism of resistance. In the future, truncation experiments would be helpful to narrow down regions of the transporter which contribute to the signal perception as was for example applied in the characterisation of the lipid II flippase FtsW (Mohammadi *et al.*, 2014) and of the Lys-PG flippase MprF (Ernst *et al.*, 2009). Further, it would be interesting to produce the extracellular domain in isolation as purified soluble protein and test the interaction with different AMPs by a binding assay to gain more information about signal perception in these systems. This would be a definite test to determine if extracellular domain is the actual binding site. Due to the low sequence similarity mentioned above, it is also unsure how binding occurs. By assuming that the extracellular domain is the true binding site it would be also interesting to analyse the crystal structure of extracellular domain:bacitracin complex.

## 2.2 Direction of transport

Another important open question is how this type of transporter confers resistance against AMPs. Until now the mechanism of transport remains unknown and is still a matter of speculation. Some studies favour the transporter to be an exporter or a flippase, expecting the complex of UPP:bacitracin to freely diffuse within the cytoplasmic membrane. In this scenario the transporter would work as a flippase, accumulating the complex of UPP:bacitracin in the outer leaflet of the membrane (Bernard *et al.*, 2007). Alternatively, BceAB may flip UPP to the inner face of the membrane where it is now protected from bacitracin and can be dephosphorylated by a cytosolic acting UPP phophatase (UppP) (Kingston *et al.*, 2014). It is also possible that the transport mechanism is more similar to that of LanFEG-type transporters, which mediate self-resistance in lantibiotic producer strain. These

transporters act by removing the AMP from the cell membrane to the extracellular space (Stein *et al.*, 2003; Okuda *et al.*, 2010). Others suggest an import function due to the general architecture of the ABC transporter, where the ECD might act as a substrate binding domain (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011). To address the direction of the transport mechanism in the future, it would be interesting to investigate the transport activities of cells with fluorescently labelled substrate as was shown for the NukFEG transporter (Okuda *et al.*, 2010) Alternatively, direction of transport could be analyzed by quantitative HPLC analyses of the cells supernatant after incubating them with substrate AMP as was applied for the NisFEG transporter (Stein *et al.*, 2003).

## 2.3 Is the response regulator part of the complex?

As mentioned above, there initial observations indicate that the RR GraR of *S. aureus* and BceR of *B. subtilis* might interact with the ATPase domain of their cognate ABC-transporters, suggesting that the regulators could contribute to the signal transduction complex (Falord *et al.*, 2012) (**Chapter IV**). Does this interaction have a physiological role in the cell? In order to answer this question, it is important to investigate the localization of the RR in its native surrounding for example by a western blot analysis of the cell fractions (cytoplasm, cell membrane), and to test if localisation is changed in the absence or presence of the inducing AMP. A similar study was done, for the transcriptional activator MalT of *E. coli*, which was shown to be sequestered by the maltose transporter $MalFGK_2$. MalT was shown to be localized to the membrane in the absence but not in the presence of maltose. This approach provided evidence that MalT interacts with the resting transporter, thereby preventing induction of MalT by endogenous maltotriose. These results suggest a coupling between transport and regulation by this type of ABC transporter (Richet *et al.*, 2012).

In the case of BceR, interaction with BceAB might prevent dimerization of BceR, which is a pre-requisite for BceR activity. It could be also possible to prevent an unspecific phosphorylation of the RR or the interaction brings the regulator and the HK in close proximity to support the phosphotransfer between them. The physiological meaning of such a shuttling of BceR between the membrane and the cytoplasm is less obvious. Possibly, direct sensing of the transport activities by BceS and BceR is increasing the regulatory speed and allow for a large dynamic range in the regulation of *bceAB* expression. The dynamic interactions of transport proteins with other cellular proteins are difficult, but will give us valuable insights into this unique mechanism of signal transduction.

# Supplementary Material

The following supplementary material can be found under following link:

http://jb.asm.org/content/early/2011/06/10/JB.05175-11/suppl/DC1

## Chapter II

**Supplemental file 1** - Fig. S1, multiple-sequence alignments of permease ECDs (PDF file, 142K)
**Supplemental file 2** - Table S1, dataset for phylogenetic analysis of BceRS-BceAB-like resistance modules (MS Excel file, 810K)
**Supplemental file 3** - Table S2, dataset of YycG- and OppB homologs from 26 genomes containing BceRS-BceAB-like modules (MS Excel file, 76K)
**Supplemental file 4** - Table S3, identification of putative response regulator binding sites in promoters of BceAB-like transport operons (MS Excel file, 74K)

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Gebhard, S., Dintner S. (2014) Peptidantibiotika - Frühwarnsysteme und Katastrophenschutz bei Bakterien. BIOspektrum. doi: 10.1007/s12268-014-0437-y

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Dintner S., Staroń A., Berchtold E., Petri T., Mascher T. and Gebhard S. (2011) Co-evolution of ABC-transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in Firmicutes bacteria. J. Bacteriol. 193(15):3851-62.

## Conferences

### Poster

Sensory Transduction in Microorganisms, Ventura, Kalifornien, USA (2014)

"Powerful collaboration between sensor kinase and ABC-transporter in antimicrobial peptide detoxification modules in Firmicutes bacteria." (Postersession II, 8)

7th Conference on Functional Genomics of Gram-positive Microorganisms, Montecatini Terme, Italien. (2013)

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