Signal Transduction Mechanisms and Wiring Specificity of Bce-type Antimicrobial Peptide Sensing and Detoxification Modules in Firmicutes

Dissertation

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München, 29.07.2015

Chong Fang

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Abbreviations

ABC	ATP-binding cassette
AMP	antimicrobial peptide
bp	base pair(s)
CM	cytoplasmic membrane
EMSA	electrophoretic mobility shift assay
HK	histidine kinase
IM-HK	intramembrane-sensing histidine kinase
IPTG	isopropyl-β-D-thiogalactopyranoside
MLS	macrolide-lincosamide-streptogramin B
OD	optical density
OM	outer membrane
PCR	polymerase chain reaction
PG	peptidoglycan
RR	response regulator
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
TCS	two-component system
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

List of publications

Publications and manuscripts presented in this thesis:

Chapter II

Fang C., Stiegeler E, Cook G.M., Mascher T., Gebhard S. (2014) *Bacillus subtilis* as a Platform for Molecular Characterisation of Regulatory Mechanisms of *Enterococcus faecalis* Resistance against Cell Wall Antibiotics. PLoS ONE 9(3): e93169.

Chapter III

Gebhard S., **Fang C.**, Shaaly A., Leslie D.J., Weimar M.R., Kalamorz F., Carne A. and Cook G.M. (2014) Identification and Characterisation of a Bacitracin Resistance Network in *Enterococcus faecalis*. Antimicrob. Agents Chemother. Vol. 58 no. 3 1425-1433.

Chapter IV

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Publication not presented in this thesis:

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. doi: 10.1074/jbc.M114.596221.

Contributions to publications presented in this thesis

Chapter II

Susanne Gebhard, Thorsten Mascher and Gregory M. Cook conceived and designed the experiments. Chong Fang and Emanuel Stiegeler performed the experiments. Chong Fang, Emanuel Stiegeler and Susanne Gebhard analyzed the data. Susanne Gebhard and Chong Fang wrote the manuscript.

Chapter III

David J. Leslie, Marion R. Weimar, and Falk Kalamorz characterized the resistance network in *E. faecalis*; Chong Fang performed all work with *B. subtilis*; Aishath Shaaly and Alan Carne performed the proteomic analysis; Susanne Gebhard and Gregory M. Cook designed the study and coordinated experimental work; Susanne Gebhard wrote the manuscript.

Chapter IV

Thorsten Mascher and Susanne Gebhard conceived and designed the experiments. Chong Fang, Anna Staroń and Martin Grafe performed the *in vivo* experiments. Chong Fang performed the *in vitro* BceR purification and EMSAs. Ralf Heermann conducted and analyzed the SPR experiments. Ralf Heermann and Kirsten Jung gave valuable input for the manuscript. Thorsten Mascher, Ralf Heermann and Chong Fang analyzed the data and wrote the manuscript.

Chong Fang Prof. Dr. Thorsten Mascher

Summary

The environment of many bacteria often contains antimicrobial peptides (AMPs) that are produced by competing microorganisms or the host immune defense systems. Most AMPs target the bacterial cell envelope. Among all the mechanisms exploited by bacteria to survive AMP challenge, the most efficient and significant way is the use of ABC transporters, which remove AMPs from their sites of action. A special type of BceAB-like ABC transporters is widely distributed in Firmicutes bacteria. The transporters are unique in their dual role as both mediators of resistance and sensors for the underling signal transduction. The ABC transporter binds and thereby senses the AMP, and then passes the signal onto the cognate histidine kinase, which harbors only a short extracellular loop and is by itself not capable of AMP sensing. Signaling from the histidine kinase to the cognate response regulator by phosphotransfer then strongly induces the transcription of the ABC transporter operon, thereby mediating AMP resistance. Since the ABC transporter is usually located in direct genomic neighborhood to its two-component system, both together form Bce-like AMP detoxification modules, which are widely conserved in Firmicutes bacteria.

In the first part of my thesis, I focused on studying AMP resistance signaling in *Enterococcus faecalis*. The knowledge of AMP resistance-related systems is limited by the challenge of genetic manipulation of *E. faecalis*. Therefore, we exploited *Bacillus subtilis* as a host for heterologous studies. Two previously studied *E. faecalis* AMP resistance systems were introduced and proved well functional in *B. subtilis*. We confirmed that *B. subtilis* is a suitable heterologous host for studying the *E. faecalis* cell wall-targeting antibiotic resistance module, with considerations being paid to the genomic background and the expression level. Previous studies identified two BceAB-like ABC transporters and one BceRS-like two-component system in the genome of *E. faecalis*, but these ABC-transporters are not located near the two-component system operon. Neither the function of nor the relationship between them is known. By using the established *B. subtilis* platform, we functionally characterized a bacitracin sensing and detoxification network comprised of these two ABC transporters and the one two-component system, and gained a deeper understanding of the Bce-type antibiotic resistance module of *E. faecalis*.

In the second part of my thesis, I then analyzed the determinants of wiring signaling specificity for Bce-like two-component systems of *B. subtilis*. The genome of *B. subtilis* encodes three paralogous Bce-like systems, which share significant sequence and structural similarity and are therefore predicted to have considerable cross-talk. However, previous studies demonstrated that these three systems are insulated quite well with only minor cross-regulation between the BceS histidine kinase and the PsdR response regulator. We first aimed at understanding the molecular mechanisms evolved by *B. subtilis* to maintain the intrasystem signaling fidelity and intersystem insulation with regards to RR-promoter. By performing *in vivo* chimeric promoter activity assays and *in vitro* response regulator

binding assays, we demonstrated that *B. subtilis* developed a hierarchical cooperative binding model, involving two binding sites and a linker region on the promoter, to maintain the regulatory specificity of Bce-like response regulator to their target promoters. Next we aimed at understanding the phosphotransfer specificity between Bce-like histidine kinases and their cognate response regulators. Towards that aim, we performed *in vivo* chimeric response regulator assays with either the cognate or the non-cognate histidine kinases. We were able to identify a novel specificity determinant — the α1-β2-α2 region — within the response regulator receiver domain that is necessary to determine the specific signaling with the cognate histidine kinase.

In summary, this thesis established *B. subtilis* as a platform for heterologous studying AMP responsive signaling systems of *E. faecalis*, which then provided a deeper understanding of the bacitracin sensing and resistance network in this organism. Moreover, it provides new insight into specificity determining mechanisms of two Bce-like systems of *B. subtilis*.

Zusammenfassung

Der Lebensraum vieler Bakterien enthält antimikrobielle Peptide (AMPs), welche von Konkurrenten oder dem Immunsystem des Wirtes produziert weden. Viele AMPs haben die bakterielle Zellhülle als Hauptangriffspunkt. Unter allen bakteriellen AMP-Resistenzmechanismen, stellt der effizienteste und bedeutendste Mechanismus das Verwenden von ABC-Transporter dar, welche AMPs von ihren Wirkorten entfernen. Ein spezieller Typ von BceAB-ähnlichen ABC-Transportern ist weitverbreitet in firmicuten Bakterien. Diese Transporter sind einzigartig in ihrer Doppelrolle als sowohl Vermittler von Resistenz, als auch Sensoren für die zugrundeliegende Signaltransduktion. Die ABC-Transporter binden und erkennen somit die AMPs. Danach geben sie das Signal an die zugehörigen Histidinkinasen weiter, welche nur über eine kleine extrazelluäre Domäne verfügen und selbst zur AMP-Erkennung nicht in der Lage sind. Die Signalweiterleitung von der Histidinkinase zum zugehörigen Antwortregulator mittels der Phosphatgruppenübertragung induziert dann die Transkription des ABC-Transporter-Operons stark und vermittelt so die AMP-Resistenz. Da die ABC-Transporter häufig genomisch in nächster Nähe zu ihren Zweikomponentensystemen liegen, bilden sie zusammen ein Bce-artiges Entgiftungsmodul gegen Peptidantibiotika, welches weitgehend konserviert in firmicuten Bakterien vorliegt.

Im ersten Teil meiner Arbeit lag das Hauptaugenmerk auf der Erforschung des Signalwegs der AMP-Resistenz in *Enterococcus faecalis*. Das Wissen hierüber ist in *E. faecalis* aufgrund der in dieser Bakterienart schwierigen Genmanipulation gering. Deshalb wollten wir *Bacillus subtilis* als Wirt für heterologe Studien etablieren. Zwei vormals untersuchte Resistenzsysteme von *E. faecalis* gegen Peptidantibiotika wurden in *B. subtilis* eingebracht und funktionierten dort einwandfrei. Wir konnten somit bestätigen, dass *B. subtilis* ein geeigneter heterologer Wirt zur Untersuchung von Resistenzmodulen aus *E. faecalis* ist. Frühere Studien identifizierten zwei BceAB-ähnliche ABC-Transporter und ein BceRS-artiges Zweikomponentensystem im Genom von *E. faecalis*. Die ABC-Transporter befanden sich aber genomisch nicht in der Nähe des Zweikomponentensystems und weder über ihre Funktion noch eine mögliche Interaktion zwischen ihnen war Näheres bekannt. Durch das Verwenden der etablierten *B. subtilis*-Plattform konnten wir die Funktionsweise eines Bacitracinerkennungs- und Entgiftungsnetzwerkes beschreiben, welches aus den oben erwähnten zwei ABC-Transportern und dem Zweikomponentensystem bestand. Somit konnten wir ein besseres Verständnis des Bce-ähnlichen Rsistenzmoduls gegen Antibiotika in *E. faecalis* erlangen.

Im zweiten Teil meiner Arbeit analysierte ich die Determinanten, welche für die Signalspezifizität von Bce-ähnlichen Zweikomponentensystemen in *B. subtilis* verantwortlich sind. Das Genom von *B. subtilis* codiert drei paraloge Bce-ähnliche Systeme, welche eine signifikante Ähnlichkeit in der Sequenz und Struktur besitzen, weshalb ihnen ein hohes Maß an *Crosstalk* vorhergesagt wurde. Frühere Studien konnten hingegen zeigen, dass diese drei Systeme ziemlich gut voneinander isoliert

sind und es nur zu einer geringen Kreuzregulation zwischen der BceS Histidinkinase und dem PsdR Antwortregulator kommt. Unser erstes Ziel war es, die molekularen Mechanismen zu verstehen, welche die systeminterne Signalspezifizität und die Isolierung zwischen den Systemen aufrechterhalten. Mittels *in vivo* Aktivitätsanalysen chimärer Promotoren und *in vitro* Bindungsstudien von Antwortregulatoren konnten wir zeigen, dass *B. subtilis* hierfür ein fein abgestimmtes hierarchisches und kooperatives Bindungsmodell entwickelte. Dieses beinhaltet zwei Bindestellen und eine *Linker*-Region auf dem Promotor und sorgt dafür, dass die Regulationsspezifität von Bce-ähnlichen Antwortregulatoren zu ihren Zielpromotoren erhalten bleibt. Als nächstes untersuchte ich die Spezifität der Phosphatgruppenübertragung zwischen der Bce-ähnlichen Histidinkinase und ihrem Antwortregulator. Um dieses Ziel zu erreichen, führten wir *in vivo* Bindungsstudien chimärer Antwortregulatoren mit entweder der zugehörigen oder nicht zugehörigen Histidinkinase durch. Wir konnten damit eine neue Spezifizitätsdeterminante — die α1-β2-α2-Region — auf der Empfängerdomäne des Antwortregulators identifizieren, welche für die Signalspezifizität mit der zugehörigen Histidinkinase verantwortlich ist.

Zusammenfassend etablierte diese Arbeit *B. subtilis* als eine Plattform für heterologe Studien von Signaltransduktionssystemen aus *E. faecalis*, welche auf Antibiotika reagieren. Diese heterologe Plattform ermöglichte uns ein tieferes Verständnis des Netzwerks, welches Bacitracin erkennt und die Resistenz ermöglicht. Zusätzlich erlangten wir neue Erkenntnisse über spezifitätsbestimmende Mechanismen zweier Bce-artiger Systeme in *B. subtilis*.

Chapter I

Introduction

1. Introduction

Survival in the competitive bacterial habitat demands both production of and defense against numerous antimicrobial peptides (AMPs). The bacterial cell envelope is the first and principal line to confront and protect the cell from antibiotics. It is therefore the target of a wide array of antibiotics. To cope with myriad AMPs and improve the chances of survival in harsh living environments, bacteria, like *Bacillus subtilis* and *Enterococcus faecalis*, have evolved a variety of direct and indirect resistance mechanisms.

1.1. The bacterial cell envelope — the first defense system

The cell envelope is an essential and complex structure of the bacterial cell with sophisticated layers. It is crucial for maintaining cell integrity, cell shape, surface properties, solute permeability, and self-defense. It keeps the bacterial cell as a separate individual while also enabling bacterial communication (Braun *et al.*, 2014). The Gram-negative bacterial cell envelope has three layers including the outer membrane, the peptidoglycan cell wall and the cytoplasmic membrane (Fig. 1.1). The outer membrane plays an important role in separating the cell from toxic molecules and stabilizing the cytoplasmic membrane. Compared to the Gram-negative bacteria, the Gram-positive cell envelope has only two functional layers: the peptidoglycan cell wall and the cytoplasmic membrane (Fig. 1.1). For Gram-positive bacteria, lacking the protective outer membrane necessitates a peptidoglycan cell wall thicker and more complex than Gram-negative bacteria to tolerate the harsh environmental challenges and support the cell membrane (Silhavy *et al.*, 2010).

1.1.1. The composition of the Gram-positive bacterial cell wall

The cell wall of Gram-positive bacteria varies among different species, but can be described in general as a three dimensional net-like structure comprised of many peptidoglycan layers, teichoic acids (TAs) and surface proteins (Silhavy *et al.*, 2010) (Fig. 1.1).

The peptidoglycan of Gram-positive bacteria is around 30-100 nm thick with up to 40 layers consisting of glycan chains cross-linked by cell wall peptides, while the Gram-negative bacterial peptidoglycan has only one to a few layers (Bertsche *et al.*, 2014). Every glycan strand is made up of repeating *N*-acetylglucosamine-(β1-4)-*N*-acetylmuramic acid (GlcNAc-MurNAc) disaccharide units. The penta-peptide moiety with a common sequence L-Ala-D-Glu-DAA (dibasic amino acid)-D-Ala-D-Ala is linked to the lactic acid of *N*-acetylmuramic acid via an amide bond with the first amino acid (L-alanine). DAA is the dibasic amino acid that differs between bacteria. Most Gram-negative species, as well as some Gram-positives such as Bacilli and Mycobacteria, use mDAP (*meso*-diaminopimelate), while most Gram-positives use L-Lys (Scheffers & Pinho, 2005, Bertsche *et al.*, 2014, Wheeler *et al.*, 2014). The glycan strands and the peptide stems together form the peptidoglycan chains, which are connected by cross-bridges (Vollmer *et al.*, 2008). The length of the peptidoglycan chains and the

cross-links vary among Gram-positive bacteria. *Staphylococcus aureus* has short peptidoglycan strands but a high degree of cross-links, whereas *B. subtilis* has longer strands but a lower degree of cross-links (Vollmer, 2008, Vollmer & Seligman, 2010, De Pedro & Cava, 2015). The three dimensional mesh-like peptidoglycan plays an important role in the tolerance of turgor and in maintaining the shape and viability of the cell (Desmarais *et al.*, 2013).

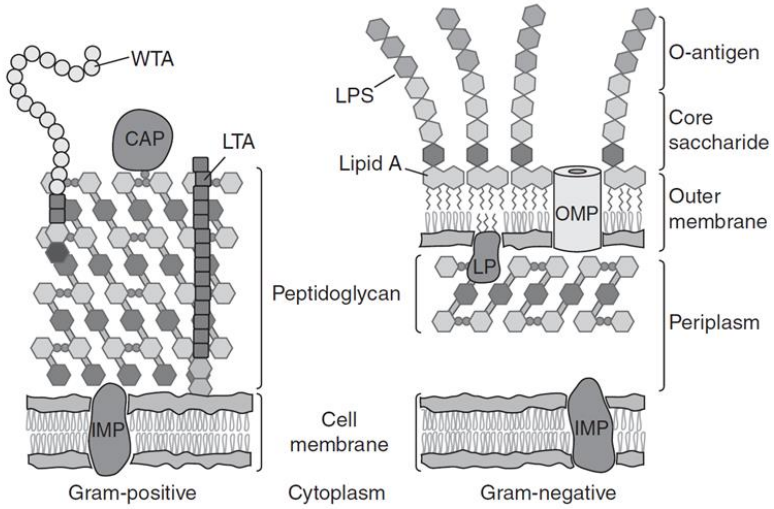

Figure 1.1. Structure and composition of the Gram-positive (left) and Gram-negative (right) cell envelopes. CAP, covalently attached protein; IMP, integral membrane protein; OMP, outer membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; WTA, wall teichoic acid (Silhavy *et al.*, 2010).

Another important and widespread constituent of the Gram-positive bacterial cell wall is TA. It can be tethered either to the cell wall by phosphodiester bond to the C6 hydroxyl of MurNAc (wall teichoic acids, WTA) or to the glycolipids that are embedded in the outer layer of the cytoplasmic membrane (lipoteichoic acids, LTA) (Perego *et al.*, 1995, Silhavy *et al.*, 2010). The structure and composition of WTA and LTA vary among different Gram-positive bacteria, but the common feature is that they are phosphate-rich polymers comprised of a continuum of anionic charge. This feature can significantly affect the synthesis and degradation of the cell wall, cation homeostasis, the antimicrobial resistance, and the interaction of bacterial cells with various surfaces (Hughes *et al.*, 1973, Neuhaus & Baddiley, 2003, Bhavsar *et al.*, 2004, Brown *et al.*, 2013).

Surface proteins are attached to the peptidoglycan, TAs or the cytoplasmic membrane by non-covalent interactions or covalent bonds. They are responsible for peptidoglycan synthesis and turnover during cell growth and division, recognition and interaction with their host for Gram-positive pathogens and nutrient utilization (Navarre & Schneewind, 1999, Ton-That *et al.*, 2004, Scott & Barnett, 2006).

1.1.2. The biosynthesis of the Gram-positive bacterial cell wall

The bacterial cell wall has a dynamic structure and undergoes a constant remodeling process: it is synthesized, modified and hydrolyzed to allow cell growth, cell division, and AMP resistance. The synthesis process can be generally divided into three stages that occur respectively in cytoplasm,

membrane and extracellular cell wall compartment, including peptidoglycan assembly, TAs and proteins attachment involved with a variety of enzymes and substrates (Shockman & Barren, 1983, Scheffers & Pinho, 2005, Johnson *et al.*, 2013) (Fig. 1.2).

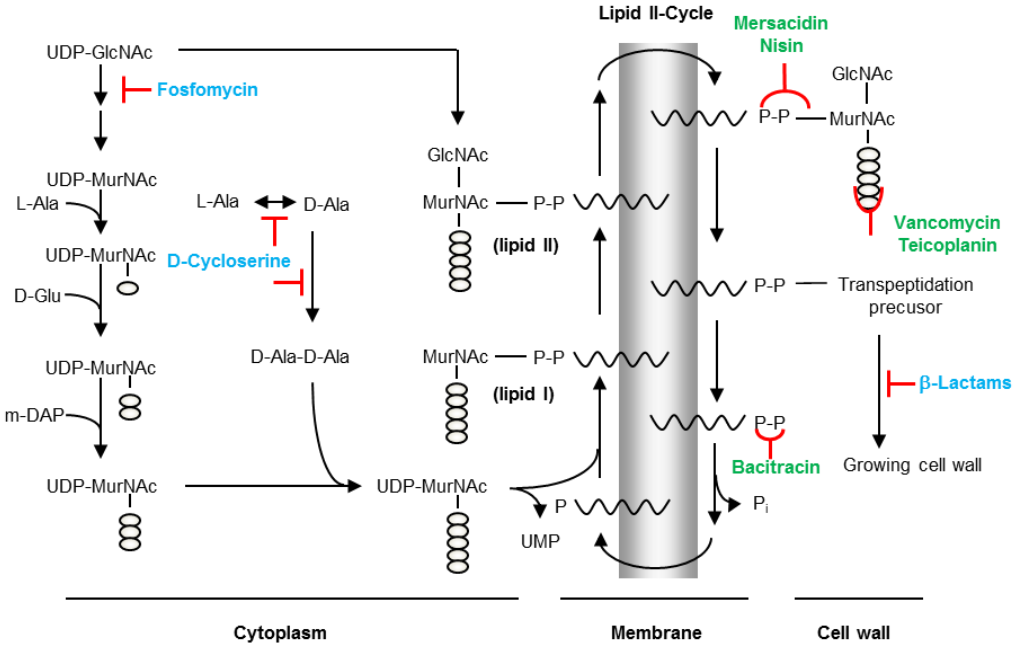

Figure 1.2. Cell wall biosynthesis of the Gram-positive bacteria and its inhibition by antibiotics. Important steps in cell wall biosynthesis are schematically depicted together with their cellular locations. GlcNAc, N-acetyl-glucosamine; MurNAc, N-acetyl-muramic acid. Amino acids are symbolized by small grey circles. Undecaprenyl is showed as waved lines. Some cell wall antibiotics relevant for this thesis are given and placed next to the steps they inhibit. Antibiotics in green sequester the substrate of the given step; those in blue inhibit the corresponding enzymatic function. See text for details on their actions. This figure was originally based in parts on (Jordan *et al.*, 2008), with modifications.

In the cytoplasm, peptidoglycan synthesis starts with the conversion of fructose-6-phosphate into UDP-GlcNAc by sequential reactions involving different enzymes. UDP-GlcNAc can be converted into UDP-MurNAc penta-peptide, which is further transferred to a membrane undecaprenyl phosphate lipid carrier to form lipid I. At the next step, GlcNAc is linked to MurNAc from lipid I via β1-4 glycoside bond to generate lipid II. Amino acids involved in peptide cross-bridges are also linked to the DAA of the penta-peptide. Translocation of lipid II to the exterior face of the membrane is followed by polymerization and cross-linking catalyzed by penicillin-binding proteins (PBPs). These two functions of PBPs are executed by different domains: transglycosylase domain and transpeptidase domain (Ton-That *et al.*, 2004, Bugg *et al.*, 2011, Typas *et al.*, 2012, Johnson *et al.*, 2013). PBPs work cooperatively with autolysins as well as WTA and LTA synthesis enzymes for cell wall growth. In most bacteria, the insertion of new cell wall material for cell elongation is guided by actin-like protein MreB and for cell division is organized by tubulin-like protein FtsZ (Shockman & Barren, 1983, Jones *et al.*, 2001, Carballido-López & Errington, 2003, Scheffers & Pinho, 2005, Kawai *et al.*, 2009, Domínguez-Cuevas *et al.*, 2013). After adding the peptidoglycan precursor into the cell wall, the membrane

lipid carrier remains in the pyrophosphate form and will further be dephosphorylated and flipped back to the cytoplasmic side of the membrane for recycling. The steps of the cell wall biosynthesis linked to the cytoplasmic membrane via undecaprenyl are referred to as the "Lipid II cycle". The cell wall synthesis process, especially the lipid II cycle, is the target of numerous AMPs.

1.2. Antimicrobial peptides — the inhibitors of bacterial cell wall synthesis

AMPs are secondary metabolites produced for self-defense by a variety of organisms like bacteria, fungi, plants, insects, and animals. They are small (usually 6 to 100 amino acids) and usually positively charged amphipathic molecules with different lengths, sequences, secondary structures, and antimicrobial spectrum (Berdy, 2005, Nakatsuji & Gallo, 2012, Bahar & Ren, 2013). They can be separated into four groups based on the secondary structure they mainly harbor: β-strands, α-helices, loop structures, and extended structures (Davies & Webb, 1998, Lee *et al.*, 2015). AMPs can be synthesized either nonribosomally or ribosomally. Nonribosomally synthesized AMPs like bacitracin, gramicidin, and glycopeptides are drastically modified and mainly produced by bacteria. They are synthesized according to the multiple-carrier thiotemplate mechanism by a series of very large and multifunctional peptide synthetases in an ordered fashion. Ribosomally synthesized AMPs can be produced by a wide range of organisms as major defense molecules against microorganisms (Stein *et al.*, 1996, Hancock & Chapple, 1999, Papagianni, 2003). Lantibiotics, a large family of AMPs, are ribosomally synthesized and post-translationally modified with unusual amino acids such as lanthionine and methyllanthionine (McAuliffe *et al.*, 2001, Chatterjee *et al.*, 2005). One of the most famous members is Nisin, a type A lantibiotic containing five lanthionine rings and three dehydrated amino acids produced by *Lactococcus lactis* during stationary growth phase (Hsu *et al.*, 2004).

The modes of action AMPs exert against bacteria include inhibition of the cell wall synthesis, membrane dysfunction by channels/pores formation, and repression of intracellular functions like DNA, RNA or proteins synthesis (Yeaman & Yount, 2003). Cell wall targeting AMPs implement their functions either by disrupting the activity of enzymes involved in cell wall synthesis or isolating substrates/precursors of corresponding enzymes (Jordan *et al.*, 2008).

Examples of AMPs acting on bacterial cell wall are wide-ranging (Fig. 1.2). Fosfomycin and D-cycloserine can target and hinder the cytoplasmic steps of the bacterial cell wall synthesis (Nikolaidis *et al.*, 2014). Most lantibiotics can target the lipid II and impede the cell wall synthesis (Breukink & de Kruijff, 2006). Nisin has antimicrobial function against a wide range of Gram-positive bacteria and the outgrowth of spores of Bacilli and Clostridia (Héchard & Sahl, 2002, de Arauz *et al.*, 2009). It can bind to lipid II and use it as an anchor molecule to further insert itself into the lipid bilayers. Thus it presents a dual mode of antimicrobial activity causing inhibition of the cell wall biosynthesis and pore formation on the membrane, which ultimately result in cell lysis (Nagao *et al.*, 2006). Mersacidin, a type B lantibiotic with a more globular structure, can complex lipid II and prevent the cell wall

synthesis (Stein, 2005, Willey & van Der Donk, 2007). Lipid II is also the target of glycopeptides like teicoplanin and vancomycin. They can inhibit polymerization and cross-linking by binding to the D-Ala-D-Ala dipeptide terminus of the lipid II and block the cell wall synthesis, which eventually leads to cell death (Marshall *et al.*, 1998, Silver, 2003). Bacitracin, a branched cyclic nonribosomally synthesized dodecylpeptide AMP mainly produced by *Bacillus licheniformis* and some strains of *B. subtilis*, binds tightly to the undecaprenyl pyrophosphate and prevents its dephosphorylation and recycling (Bernlohr & Novelli, 1963, Katz & Fisher, 1987, Azevedo *et al.*, 1993, Konz *et al.*, 1997).

1.3. Mechanisms of antimicrobial peptide resistance in Gram-positive bacteria

To survive in a competitive environment, bacteria have developed different strategies either via spontaneous mutations or acquisition of additional genes to acquire AMP resistance. Some bacteria can form biofilm to confer resistance (Otto, 2006). Resistance can also be achieved by synthesizing proteases to degrade the AMPs (Sun *et al.*, 2009). Resistance against cell wall acting AMPs can also be mediated by reducing the access of the drugs to the cell envelope by changing the cell's surface charge ─ possible in both Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, the negative charge of the cell surface can be reduced by incorporating D-Ala to the highly negatively charged TAs. This is accomplished by gene products of the *dlt* operon. Bacteria are more sensitive to cationic AMPs if this operon is inactivated (Neuhaus & Baddiley, 2003, McBride & Sonenshein, 2011, Reichmann *et al.*, 2013). The reduced negative charge of TAs was postulated to diminish the electrostatic attraction between the AMPs and the cell envelope (Peschel & Sahl, 2006). However, an alternative model was proposed: the D-alanylation of TAs modifies the electrostatic interaction between TAs themselves thereby making the cell envelope more compact and less permeable for AMPs to reach their cell wall targets (Saar-Dover *et al.*, 2012, Revilla-Guarinos *et al.*, 2014).

Specific resistance against AMPs includes modifying their cell wall targets. In enterococci, resistance against vancomycin is conferred by altering the binding target D-Ala-D-Ala on the C-terminal of lipid II into D-Ala-D-Lac or D-Ala-D-Ser (Bugg *et al.*, 1991). This switch leads to a reduced number of hydrogen bonds from five to four between AMPs and their target ─ lipid II, which decreases the binding affinity by 1000-fold (Bugg *et al.*, 1991, Kahne *et al.*, 2005). Two types of vancomycin resistance were found in *E. faecalis* and will be described in detail in **Section 1.5** (Walsh *et al.*, 1996).

The most efficient mechanism against AMPs is mediated by the ATP-binding cassette (ABC) transporters. These transporters usually contain one or two permease domains with variable number of transmembrane (TM) helices, and each permease domain is associated with an ATPase (Gebhard, 2012). ATP hydrolysis provides energy for resistance against AMPs. Three different types of ABC transporters, the LanFEG-type, the BceAB-type, and the BcrAB-type, have been found widespread in Firmicutes bacteria for AMP resistance (Gebhard, 2012). The BcrAB-type and the LanFEG-type transporters are mainly responsible for sensing and resistance against self-produced AMPs and most of

them have a very narrow substrate range. Some of them were found to be associated with AMP biosynthesis genes. For example, the ABC transporter BcrAB together with the undecaprenyl pyrophosphate phosphatase BcrC are encoded in the bacitracin biosynthesis locus and confer self-resistance in *B. licheniformis* (Podlesek *et al*., 1995); the NisFEG system in *L. lactis* is responsible for mediating resistance to the self-produced nisin (Stein *et al.*, 2003).

The BceAB-type transporters are hardly ever associated with AMP biosynthetic genes. The range of resistance is quite broad including lantibiotics, cyclic AMPs like bacitracin, glycopeptides, and peptides from the innate immune systems of higher organisms like defensins and cathelicidins (summarized in (Gebhard & Mascher, 2011)). The permeases of these transporters have 10 TM helices and a large extracellular loop between helices 7 and 8. TM helices 2 to 4 and TM helices 8 to 10 form two FtsX-domains (Dintner *et al.*, 2011, Dintner *et al.*, 2014). Furthermore, this kind of transporters are not only responsible for AMP resistance but also indispensable for AMP perception (Rietkötter *et al.*, 2008, Staroń *et al.*, 2011). However, the molecular mechanisms of substrate detection, signaling and resistance are not fully understood (more details see **Section 1.7**).

1.4. Regulatory network orchestrating antimicrobial peptide resistance in *Bacillus subtilis*

B. subtilis is the best-characterized member of the Gram-positive bacteria and can be isolated from diverse environments, e.g., soil, water source, and plant root surfaces. It is a rod-shaped bacterium that can form highly resistant dormant endospores in response to nutrient limitation (Earl *et al.*, 2008, van Dijl & Hecker, 2013). The genome of *B. subtilis* contains around 4.2×10^6 bp with 4,100 protein-coding genes, and about 4-5% of the genome is devoted to antibiotics production (Kunst *et al.*, 1997). Antibiotics such as subtilosin, surfactin, bacilysin, lantibiotics including subtilin, ericin and mersacidin have been reported to be synthesized ribosomally or nonribosomally by a wide array of *B. subtilis* strains to inhibit competitors in the same environment (Stein, 2005). In addition to the ability of AMP production, AMP resistance is also crucial and orchestrated by a complex regulatory network, which is shown in Figure 1.3A. One of the specific and most efficient defense mechanisms is the Bce-type ABC transporter that are mainly found in Firmicutes bacteria (Dintner *et al.*, 2011). As mentioned above, the Bce-type ABC transporter is responsible for both AMP perception and resistance. The expression of the ABC transporter operon is regulated by a Bce-type two-component system (TCS), which is comprised of a membrane-anchored histidine kinase (HK) and a cytoplasmic response regulator (RR) (Joseph *et al.*, 2002) (details of TCSs will be introduced in **Section 1.6**). The sensor domain of the Bce-type HK harbors a short extracellular loop (<10 amino acids for most) between the two transmembrane helices and is not able to detect the AMP (Mascher, 2006, Mascher, 2014). The ABC transporter and TCS are genetically and functionally linked, and together they form the Bce-type AMP sensing and detoxification module (Dintner *et al.*, 2011). The signal transduction circuit starts

when the ABC transporter detects the AMP and passes the signal to the TCS to activate the HK. The phosphotransfer from the HK to the RR will in turn trigger the upregulation of the ABC transporter operon for AMP resistance. The TCS operon is under the control of a constitutive promoter, while the ABC transporter operon is expressed under the control of an AMP inducible, RR-dependent promoter (Ohki *et al.*, 2003, Staroń *et al.*, 2011). The genome of *B. subtilis* encodes three such systems to cope with the challenges from different kinds of AMPs (Joseph *et al.*, 2002) (Fig. 1.3A blue systems). The BceRS-BceAB system can sense and confer resistance against bacitracin, actagardine and mersacidin. It has also been reported to respond to a fungal defensing plectasin (Staroń *et al.*, 2011). The PsdRS-PsdAB system shares the same inducer actagardine with the Bce system but cannot confer resistance against it. Other antibiotics that can be detected and detoxified by the Psd system are nisin, enduracidin, gallidermin and subtilin (Staroń *et al.*, 2011). The only known inducer for the YxdJK-YxdLM-YxeA system is a human neutrophil peptide, LL-37 (Pietiäinen *et al.*, 2005). This system is assumed to be involved in resistance against an unknown group of antibiotics. The gene locus harbors an extra *yxeA* gene encoding a long peptide that is conserved in many Gram-positive bacteria. It might be an immune protein participating in the proposed AMP resistance by interacting with and neutralizing the antibiotic (Joseph *et al.*, 2004).

B. subtilis also developed other response systems to counteract cell envelope damage caused by AMPs. The LiaRS TCS, which is widespread in most Firmicutes bacteria, is a damage-sensing signal transduction system (Wolf *et al.*, 2012) (Fig. 1.3A red system). It can strongly respond to a wide range of cell wall antibiotics, such as bacitracin, nisin, ramoplanin, and vancomycin (Mascher *et al.*, 2004, Pietiäinen *et al.*, 2005, Hachmann *et al.*, 2009). In the presence of a stimulus, the phosphorylated LiaR can strongly induce the expression of the *liaIH-liaGFSR* operons. While in the absence of stimulus, the transcription of the *liaIH* operon is switched off and the *liaGFSR* operon is under the control of a weak constitutive promoter, P_{liaG} (Jordan *et al.*, 2006). The LiaRS TCS has a strong inhibitor, LiaF, and deletion of *liaF* led to a constitutive active system in the absence of cell envelope stress (Jordan *et al.*, 2006). However, the functions of most gene products of the *lia* operon are not clearly known. The LiaG is a putative membrane anchored hypothetical protein with unknown function. The LiaH is a member of phage shock protein family, and it is homologous to the *Escherichia coli* phage shock protein PspA, which suggests that the Lia system harbors a PspA-like response to maintain the membrane integrity (Model *et al.*, 1997, Darwin, 2005, Wolf *et al.*, 2010). The LiaH is anchored to the membrane by the small membrane protein LiaI (Domínguez-Escobar *et al.*, 2014).

Another important signal transduction system that can regulate AMP resistance involves the extracytoplasmic function (ECF) σ factors. They are small proteins containing only two of the four conserved regions of the primary σ factor. Additionally, they are usually co-transcribed with corresponding anti-σ factors (Heimann, 2002). The anti-σ factor often harbors an extracytoplasmic

sensor domain that can sense the stimuli and an intracellular inhibitory domain that can regulate the activity of its σ factor. The genome of *B. subtilis* encodes at least three ECF σ factors related to cell wall-targeting AMP resistance (reviewed in (Jordan *et al.*, 2008). The best understood one is the σ^W. A variety of cell wall active antibiotics, such as vancomycin, cephalosporin, and LL-37, can trigger the anti-σ factor RsiW releasing the σ^W, which then regulates around 60 genes for AMP resistance (Helmann, 2006) (Fig. 1.3A green system). Additionally, σ^M and σ^X also play important role in cell wall active antibiotics resistance by regulating gene operons such as undecaprenyl pyrophosphate phosphatase (Cao & Helmann, 2002, Jordan *et al.*, 2008).

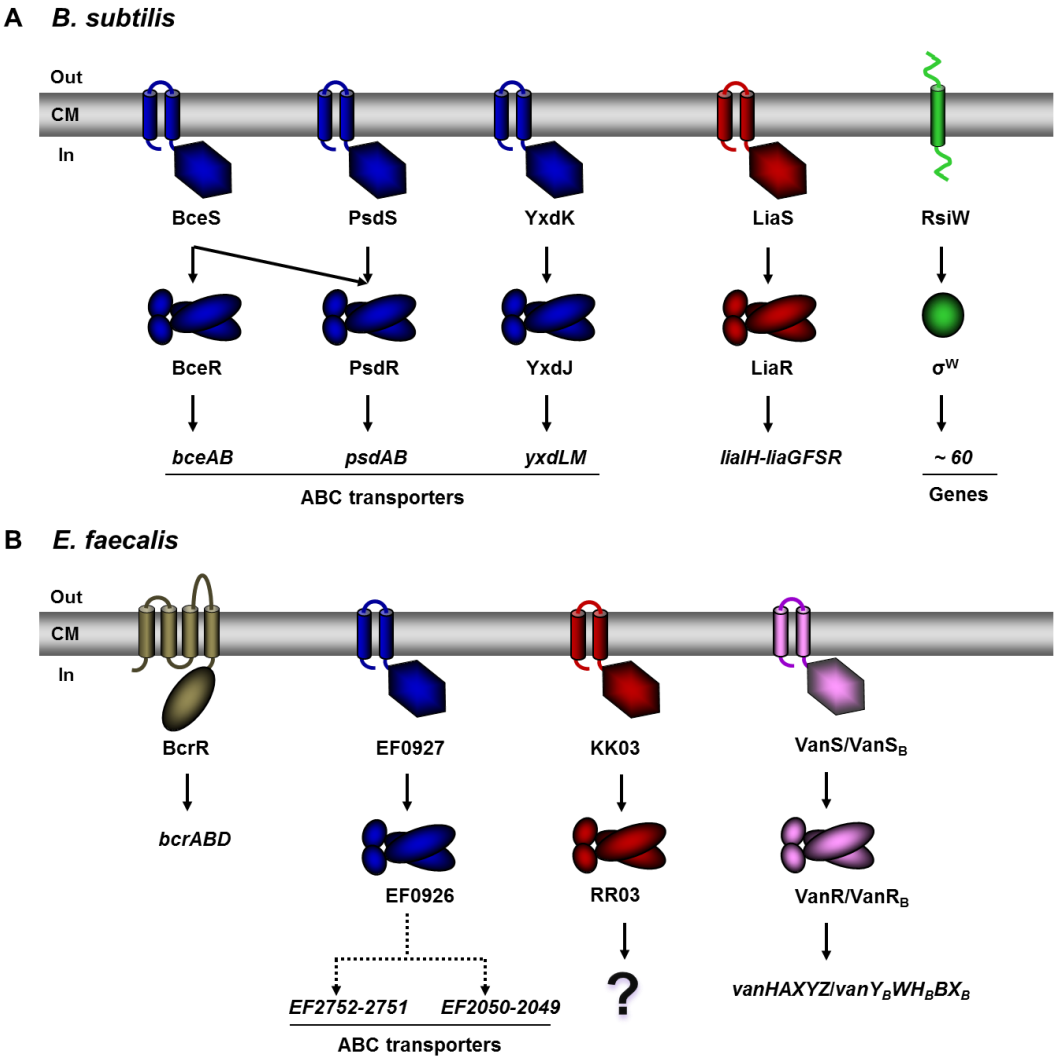

Figure 1.3. The regulatory networks orchestrating AMP resistance in *B. subtilis* (A) and *E. faecalis* (B). The Bce-type AMP sensing and detoxification models are colored in blue and the Lia systems are colored in red in both *B. subtilis* and *E. faecalis*. The σ^W and its anti-σ factor RsiW in *B. subtilis* are colored in green. The one-component system BcrR and the two-component system VanRS in *E. faecalis* are colored in brown and pink, respectively. The known signal transduction is shown by solid arrow, while the unknown signal transduction is shown by dotted arrow. CM, cytoplasmic membrane. This figure was originally based in parts on (Jordan *et al.*, 2008), with modifications.

1.5. Regulatory network orchestrating antimicrobial peptide resistance in *Enterococcus faecalis*

E. faecalis, another low-GC Gram-positive bacterium, is a core member of the normal intestinal microflora in humans and animals. It is mostly a harmless commensal, but opportunistically pathogenic and can cause life-threatening infections especially in hospital settings. *E. faecalis* strain V583, the first vancomycin resistant clinical isolate reported in the U.S., contains four DNA molecules: the main chromosome (the size is 3.2×10^6 bp, the G+C content is 37.5%) with a total of 3337 predicted protein-encoding open reading frames and three circular plasmids (Paulsen *et al.*, 2003). In addition to vancomycin, *E. faecalis* V583 can also resist to several antibiotics, such as bacitracin and teicoplanin, which leads to the difficulty of clinical treatment (Sahm *et al.*, 1989, McBride *et al.*, 2007). A deeper understanding of the AMP resistance network in *E. faecalis* will therefore provide useful information for clinical research.

E. faecalis has high-level of bacitracin resistance, which is mediated by an ABC transporter BcrAB (Manson *et al.*, 2004). The *bcrAB* genes together with *bcrD* form the *bcrABD* operon. BcrD is suggested to be able to increase the amount of undercaprenyl phosphate as an undercaprenyl pyrophosphate phosphatase for bacitracin resistance. The expression of the *bcrABD* operon is regulated by a constitutively transcribed one-component system, BcrR (Gauntlett *et al.*, 2008) (Fig. 1.3B brown system). BcrR, a membrane-bound transcriptional regulator, can perceive bacitracin directly and bind to P_{bcrA} to induce the expression of the *bcrABD* operon for bacitracin resistance (Gebhard *et al.*, 2009).

Two BceAB-like ABC transporters: EF2050-EF2049 and EF2752-2751, and one BceRS-like TCS EF0926-EF0927 were found in the genome of *E. faecalis* by comparative genomic analysis (Dintner *et al.*, 2011) (Fig. 1.3B blue system). However, neither of the ABC transporter operons was located adjacent to the operon of the BceRS-like TCS. The functions of these two ABC transporters have not been described so far. The functional analysis of these two ABC transporters and one TCS is described in **Chapter III**.

A LiaR highly conserved ortholog in *E. faecalis*, RR03, was demonstrated to be up-regulated in response to bacitracin and the RR03 mutant in *E. faecalis* showed increased bacitracin sensitivity (Hancock & Perego, 2004). A RR03 ortholog from *S. aureus*, VraR, was demonstrated to play an important role in cell wall-targeting antibiotics, which suggests a similar function of RR03 from *E. faecalis* (Kuroda *et al.*, 2003) (Fig. 1.3B red system).

Two major types of inducible glycopeptide resistance have been identified in *E. faecalis*, which were demonstrated to be regulated by two TCSs ─ the VanRS (in VanA type *E. faecalis*) and the $VanR_BS_B$ (in VanB type *E. faecalis*) (Arthur *et al.*, 1997, Arthur & Quintiliani, 2001) (Fig. 1.3B pink system).

The VanA type strain has high level of resistance against both vancomycin and teicoplanin, which are also the inducers. The resistance is mediated by products of *vanHAXYZ* operon, of which the expression is regulated by VanRS TCS, by altering the binding target (D-Ala-D-Ala) on lipid II of glycopeptide into D-Ala-D-Lac or D-Ala-D-Ser. VanH is a D-lactate dehydrogenase and can reduce pyruvate to D-lactate. VanA, an ATP-dependent D-Ala-D-Lac ligase, is able to add D-lactate to D-Ala and form D-Ala-D-Lac. The remaining D-Ala-D-Ala is then hydrolyzed by VanX (a D-Ala-D-Ala dipeptidase) (Arthur *et al.*, 1992, Marshall & Wright, 1998). The VanB type strain confers resistance against vancomycin and teicoplanin, but it is only capable of vancomycin perception. The functions of products of the $vanY_BWH_BBX_B$ operon are similar to the VanA type (Evers & Courvalin, 1996).

1.6. Two-component signal transduction systems

TCSs play an important role in regulating the resistance against cell wall targeting AMPs. A typical TCS consists of a membrane-anchored HK that detects the signal input and a cytoplasmic RR that mediates corresponding cellular output. Signal transduction between these two proteins is accomplished by transferring a phosphoryl group from the HK to the cognate RR. TCSs are widely distributed in bacteria, archaea, some lower eukaryotes, and plants. The absence of TCS in mammals makes it a potential and promising target for antibiotic development (Wuichet *et al.*, 2010).

1.6.1. Histidine kinases (HKs)

HKs are the first protein in the TCS signal transduction pathways. They receive the input stimulus and subsequently transfer the signal to the RRs. Most HKs harbor a diverse sensing (input) domain and a highly conserved kinase core (Stock *et al.*, 2000).

There are three types of input domains for HKs. The periplasmic (or extracellular)-sensing (the largest group) domain contains an extracellular sensory region framed by at least two transmembrane helices. The membrane-spanning sensing domain usually harbors 2 to 20 transmembrane regions responsible for signal perception. The cytoplasmic sensing domain, either in membrane anchored HK or soluble HK, senses the input inside the cytoplasm (Mascher *et al.*, 2006). The *E. coli* HK PhoQ of the PhoQP TCS, which is responsible for bacterial virulence and cationic antimicrobial peptide resistance, possesses a sensing domain comprised of two membrane-spanning antiparallel helices and a periplasmic sensor region (Lemmin *et al.*, 2013). The periplasmic region adopts a mixed α/β-fold containing a central five-stranded anti-parallel β-sheet flanked by α-helices and additional loops on each side for direct signal detection (Cheung *et al.*, 2008).

The cytoplasmic kinase core is connected to the input domain via a linker region that contains a number of amino acids (Fabret *et al.*, 1999). The linker region such as the HAMP or the PAS domain are critical for signal transduction (Stock *et al.*, 2000). The cytoplasmic kinase core consists of a dimerization and histidine phosphotransfer (DHp) domain and a catalytic and ATP binding (CA)-

domain (Krell *et al.*, 2010) (Fig. 1.4). The DHp domain with a long α-hairpin structure is responsible for dimerization (Marina *et al.*, 2005). HK catalyzes autophosphorylation on the conserved histidine residue (located on the first α-helix) in the presence of ATP by the CA domain (West & Stock, 2001). The phosphoryl group is subsequently transferred to the RR for mediation cellular response.

1.6.2. Response regulators (RRs)

Most RRs contain two domains: a conserved N-terminal receiver (regulatory) domain and a diverse C-terminal output (effector) domain (Stock *et al.*, 2000). A flexible linker joins the two domains together (Fig. 1.4). The receiver domain has a modular secondary structure with alternating β-strands and α-helices adopting a topology with a central five-stranded paralleled β-sheet surrounded by two α-helices on one side and three on the other (Fig. 1.5) (Bourret, 2010). The highly conserved aspartate residue, which is responsible for receiving the phosphoryl group from the histidine kinase, is located at the end of the β3 strand (Lukat *et al.*, 1991, Appleby & Bourret, 1998).

Bacterial RRs have a great variety of output domains to elicit the specific cellular response according to the input obtained by the HK. They can be assigned into five groups by their functions: DNA-binding, RNA-binding, ligand-binding, protein-binding, and enzyme (Galperin, 2010). A majority of RR receiver domains are connected to a DNA-binding output domain and have the function of gene-transcriptional regulation. The OmpR subfamily is the largest RR group possessing a winged helix-turn-helix (wHTH) DNA binding output domain (Galperin, 2006). The secondary structure of the OmpR output domain is β1-β2-β3-β4-α1-β5-α2-α3-β6-β7. The α2-loop-α3 builds up the helix-turn-helix motif and the loop connecting β6 and β7 is referred as a wing. OmpR can bind to the region upstream of the -35 element on promoters of two porin genes: *ompF* and *ompC*, and regulate the transcription by interacting with the α subunit of RNA polymerase to adjust to changes in osmolarity in *E. coli* (Slauch *et al.*, 1988, Forst *et al.*, 1989, Slauch *et al.*, 1991).

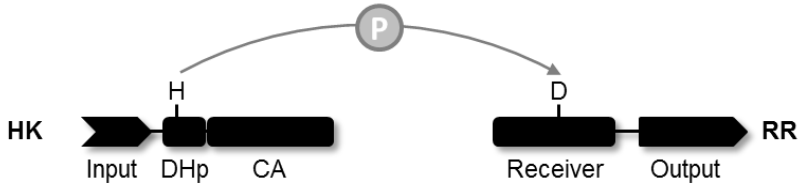

Figure 1.4. Schematic overview of the two-component signal transduction paradigm and the domain structure of each component. HK, histidine kinase. RR, response regulator. The name of each domain is given below the corresponding domain structure. The signal transduction between HK and RR is represented as transferring a phosphoryl group from the histidine residue (H) on the HK DHp domain to the aspartate residue (D) on the RR receiver domain.

1.6.3. Phosphotransfer between histidine kinase and response regulator

Three phosphotransfer reactions and two phosphoprotein intermediates are involved in the basic two-component signal transduction pathways. In the first step, the HK executes autophosphorylation of the

histidine residue by the CA domain in the presence of ATP, creating phosphoramidate. In the second step, the RR catalyzes the transfer of the phosphoryl group from phospho-His (HK) to Asp (RR), resulting in a high-energy acyl phosphate. In the final step, the RR can also catalyze dephosphorylation of phospho-Asp (RR) by transferring the phosphoryl group to a water molecule. A divalent metal ion (usually Mg^{2+} *in vivo*) is required for every step (Stock *et al.*, 2000). The phosphotransfer between the HK and the RR is mediated by protein-protein interaction via the cytoplasmic domain of the HK and the receiver domain of the RR (Casino *et al.*, 2010). Phosphorylation-mediated conformational change of the RR, especially the α4-β5-α5 face on the receiver domain, passes the signal from the receiver domain to the output domain for further regulation (Hoch & Silhavy, 1995, Gao *et al.*, 2007, Bourret, 2010, Gao & Stock, 2010).

1.7. Signaling specificity of Bce-type two-component systems in *Bacillus subtilis*

Harboring numerous highly related TCSs in one genome, such as the three homologous Bce-like TCSs in *B. subtilis*, increases the possibility of cross-talk, which can be deleterious. Direct-coupling analysis, which is based on the co-evolution of inter-protein contact residues, previously predicted a considerable potential for cross-talk among these three systems (Szurmant & Hoch, 2010, Procaccini *et al.*, 2011). Instead, a previous *in vivo* study showed that these systems are generally well insulated from each other: Only some minor degree of cross-regulation was observed between BceS and PsdR in the presence of high concentrations of bacitracin (Rietkötter *et al.*, 2008) (Fig. 1.3A). This raises the questions: How do bacteria simultaneously coordinate the activity of so many highly related signaling systems to maintain the signal transduction specificity and prevent unwanted cross-talk? How does the HK discriminate its cognate RR from the non-cognate ones in the pool of homologous RRs? How is the RR able to discriminate the cognate promoter region from non-cognate ones?

1.7.1. Signaling specificity between the histidine kinase and the response regulator

Myriad mechanisms have been employed by bacterial cell to maintain the intrasystem signal transduction fidelity and intersystem insulation. Specificity can be achieved by different cellular localizations as well as by differentiation of temporal expression of different systems (Ubersax & Ferrell Jr, 2007). At the phosphotransfer level, three mechanisms are applied to maintain the specificity of TCS. Most HKs are bifunctional, that is, they exhibit both kinase and phosphatase activities, and can thereby tightly control the activity of the cognate RR by preventing unspecific phosphorylation through noncognate HKs or small phosphodonors (Boll & Hendrixson, 2011). The competition between the cognate RR and noncognate ones can also avoid cross-talk (Laub & Goulian, 2007). The molecular recognition between cognate partners, which is the most important mechanism, enables the HK and the RR of one system to interact specifically in order to avoid accidental interactions with components from other systems (Podgornaia & Laub, 2013).

Specificity of TCSs is dictated primarily at the level of molecular recognition, requiring proper paring of amino acids located on the interaction surface of both HK and RR. These amino acids have co-evolved, which means the change in a residue of one protein over the course of evolution will cause the compensatory change of another residue on the partner protein to maintain a functional interaction between these two proteins (Szurmant & Hoch, 2010). The co-crystal structure of HK853-RR468 from *Thermotoga maritima* provides a clear view of the HK/RR interaction surfaces and implies the possible positions of those amino acids (Fig. 1.5). The interaction surfaces involving in forming the HK853-RR468 pair are: (1) the α1 helix and the β5-α5 loop of the RR468 receiver domain with the two α helices of the HK853 DHp domain; (2) the β3-α3 loop of the RR468 with the ATP lid and the β4-α4 loop of the HK853 CA domain; and (3) the RR468 β4-α4 loop with the DHp-CA interdomain linker of HK853 (Casino *et al.*, 2009). The importance of these amino acids in TCS specificity has been proved by experiments. For instance, three amino acids on the α1-helix of the HK EnvZ DHp domain were demonstrated to play a significant role in specificity determination between EnvZ/OmpR TCS in *E. coli* (Bourret, 2010).

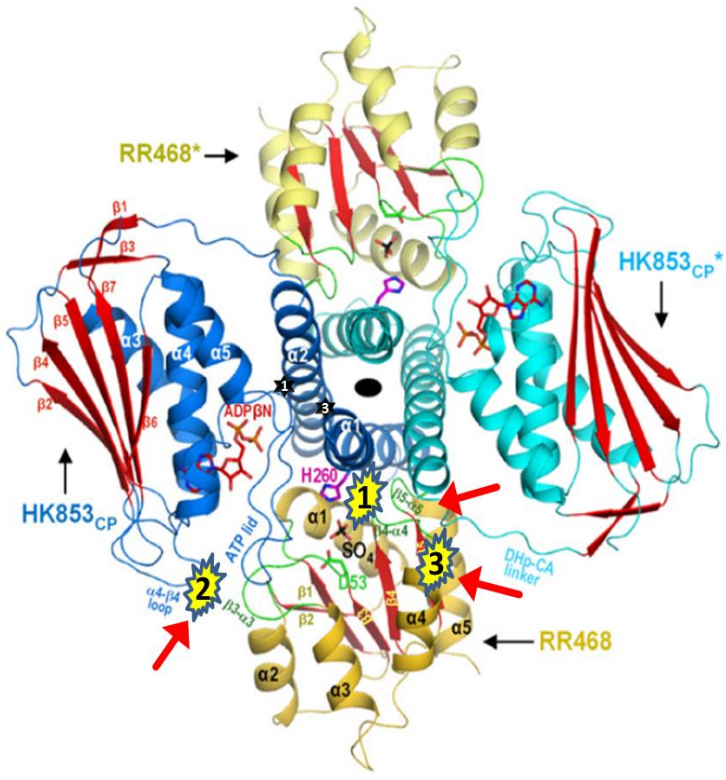

Figure 1.5. The co-crystal structure of the $HK853_C$-RR468 complex. C, cytoplasmic domain of the HK853. The structure of the complex is viewed from the cell membrane along the two-fold axis (indicated with a black ellipse) with the cell membrane and the cell interior at the top and bottom, respectively. α helices of the left and right $HK853_C$ protomer is colored in blue and and cyan, respectively. The two RR468 molecules are shown in gold and light yellow, respectively. β strands are colored red in all cases. The side chains of the phosphoacceptor H260 (pink) and D53 (green) residues, and the bound sulfate (black) and ADPβN (red) molecules are illustrated in stick representation. In one protomer of each $HK853_C$ and RR468, secondary structure elements and relevant loops have been labeled. Three interaction surfaces in this complex are labeled with yellow stars pointed by red arrows. The numbers of contacts are in consistence with in the text. This figure is based on (Casino *et al.*, 2009), with modifications.

Signal transduction fidelity is equally important for the three high homologous Bce-type TCSs in *B. subtilis* for proper antibiotic resistance. These three systems share significant sequence and structural similarity. The HK BceS is 30% and 29% identical to PsdS and YxdK respectively, while the RR BceR is 40% and 35% identical to PsdR and YxdJ (Joseph *et al.*, 2002). Instead of high level of cross-talk, only a minor level of cross-phosphorylation between BceS and PsdR has been demonstrated *in*

vivo at high concentrations of bacitracin (Rietkötter *et al.*, 2008). However, the residues on BceRS and PsdRS TCSs that dictate intrasystem specificity and minimize intersystem cross-talk remain unclear. The nature and localization of these amino acids still needs to be unraveled. A first insight into this question is provided by the data described in **Chapter V**.

1.7.2. Specificity on the response regulator transcriptional regulation level

In bacteria, transcription initiation starts with promoter recognition by the σ subunit of holo RNA polymerase on the -35 promoter element followed by discerning and unwinding of the DNA double helix at the -10 promoter element (Lee *et al.*, 2012). For promoters lacking a -35 element or deviating significantly from the consensus sequence at the appropriate position, the σ subunit can still be recruited to the promoter by interaction with activators like RRs binding to the upstream region (Jarmer *et al.*, 2001, Paget & Helmann, 2003).

Specific interaction between a regulator and its target is important for bacteria to trigger the desired response to the right stimulus, which is primarily determined via molecular recognition between amino acids of the output domain and nucleotides within the RR binding site. The output domain structure of OmpR indicates that the α3 helix (recognition helix on the output domain) is responsible for specific interaction with the DNA major groove, and the β6-β7 loop (wing on the output domain) is responsible for specific interaction with the DNA minor groove (Martínez-Hackert & Stock, 1997).

In *B. subtilis*, the transcription of the Bce-type ABC transporter genes is upregulated by binding of the Bce-like RRs to the promoter. BceR and PsdR belong to the OmpR subfamily (Fabret *et al.*, 1999) with a winged helix-turn-helix output domain. The known binding sites on P_{bceA} and P_{psdA} have eleven out of fourteen identical base pairs. This indicates a considerable potential of cross-regulation at the RR/promoter level between these two systems. *In vivo*, however, the regulation is highly specific between BceR/P_{bceA} and PsdR/P_{psdA}. This raises the question of how Bce-like RRs specifically regulate the transcription of the cognate ABC transporters. A clear understanding of the specificity determinants on *bceA* and *psdA* promoters that determine exclusive binding of BceR and PsdR, respectively, is currently lacking. This question is addressed comprehensively in **Chapter IV**.

1.8. Aims of this thesis

This thesis aimed to investigate the cell wall-targeting AMP sensing and resistance modules in two Firmicutes bacteria: *E. faecalis* and *B. subtilis*. We aimed at gaining a deeper understanding on the signal transduction mechanisms and the determinants of wiring specificity of the underling TCSs-dependent regulation.

Chapter II

The technical challenges of molecular genetic studies in *E. faecalis* hinder a deeper understanding of the molecular mechanism in antibiotic detection, signal transduction, and gene regulation. The genetically highly tractable Gram-positive model organism *B. subtilis* on the other hand might be a suitable candidate as a heterologous host. In this chapter, two fundamentally different regulators of *E. faecalis*, the bacitracin sensor BcrR and the vancomycin-sensing two component system $VanS_B$-$VanR_B$, were introduced into *B. subtilis* and their functions were monitored using target promoters fused to reporter genes (*lacZ* and *luxABCDE*). We explored and validated *B. subtilis* as a platform for studying the regulatory mechanisms of cell wall antibiotic resistance of *E. faecalis*.

Chapter III

In this chapter, the established *B. subtilis* platform was subsequently used for an in-depth heterologous functional analysis of two Bce-type ABC transporters and one Bce-type TCS of *E. faecalis*. Combined with studies in the native host, we analyzed the bacitracin sensing and resistance network of *E. faecalis*.

Chapter IV

Both the output domains of BceR and PsdR as well as their known binding sites are highly homologous in *B. subtilis*. The aim of this chapter was to gain a full comprehension of the mechanism that dictates specific binding of RR to its cognate promoter (BceR-P_{bceA}, PsdR-P_{psdA}). *In vivo* experiments were used to first dissect the promoter and later identify the specificity dictating elements. *In vitro* assays were then performed to further corroborate the specificity determining mechanism.

Chapter V

Due to the high sequence and structure similarity of BceRS and PsdRS TCSs in *B. subtilis*, the question of what determines signal transduction specificity between a HK and its cognate RR was raised. In this chapter, different regions on the receiver domain were exchanged between BceR and PsdR to rewire the signal transduction *in vivo* and thereby identify the specificity determinants for Bce-type TCSs.

Chapter II

Bacillus subtilis as a Platform for Molecular Characterisation of Regulatory Mechanisms of *Enterococcus faecalis* Resistance against Cell Wall Antibiotics

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Bacillus subtilis as a Platform for Molecular Characterisation of Regulatory Mechanisms of *Enterococcus faecalis* Resistance against Cell Wall Antibiotics

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Abstract

To combat antibiotic resistance of *Enterococcus faecalis*, a better understanding of the molecular mechanisms, particularly of antibiotic detection, signal transduction and gene regulation is needed. Because molecular studies in this bacterium can be challenging, we aimed at exploiting the genetically highly tractable Gram-positive model organism *Bacillus subtilis* as a heterologous host. Two fundamentally different regulators of *E. faecalis* resistance against cell wall antibiotics, the bacitracin sensor BcrR and the vancomycin-sensing two-component system $VanS_B$-$VanR_B$, were produced in *B. subtilis* and their functions were monitored using target promoters fused to reporter genes (*lacZ* and *luxABCDE*). The bacitracin resistance system BcrR-BcrAB of *E. faecalis* was fully functional in *B. subtilis*, both regarding regulation of *bcrAB* expression and resistance mediated by the transporter BcrAB. Removal of intrinsic bacitracin resistance of *B. subtilis* increased the sensitivity of the system. The *lacZ* and *luxABCDE* reporters were found to both offer sensitive detection of promoter induction on solid media, which is useful for screening of large mutant libraries. The $VanS_B$-$VanR_B$ system displayed a gradual dose-response behaviour to vancomycin, but only when produced at low levels in the cell. Taken together, our data show that *B. subtilis* is a well-suited host for the molecular characterization of regulatory systems controlling resistance against cell wall active compounds in *E. faecalis*. Importantly, *B. subtilis* facilitates the careful adjustment of expression levels and genetic background required for full functionality of the introduced regulators.

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Introduction

Enterococcus faecalis is one of the most common causes of nosocomial infections. Increasing incidences of infections with antibiotic resistant strains, particularly with vancomycin resistant enterococci (VREs), therefore pose a major health risk [1,2]. Vancomycin is a glycopeptide antibiotic that targets the lipid II cycle of cell wall biosynthesis by binding to the terminal D-alanyl-D-alanine (D-Ala-D-Ala) moiety of peptidoglycan precursors on the surface of the cell, thus inhibiting their incorporation into the cell wall [3]. Many other antimicrobial substances also target the lipid II cycle [4], including bacteriocins and mammalian defensins [5,6], both of which will likely be encountered by *E. faecalis* in its natural gut habitat. Furthermore, many enterococcal isolates were found to be highly resistant against bacitracin [7,8], yet another inhibitor of cell wall biosynthesis [9].

The molecular mechanisms leading to resistance are often well known. In the case of vancomycin, high-level resistance is for example ensured by target alteration through replacement of the terminal D-Ala-D-Ala by D-Ala-D-lactate. In VanA-type strains, this is accomplished through the action of the VanHAX system, while in VanB-type strains the $VanH_BBX_B$ proteins mediate resistance [10,11]. High-level bacitracin resistance of *E. faecalis* is conferred by the ATP-binding cassette (ABC) transporter BcrAB, which presumably removes the antibiotic from its site of action (i.e. the cytoplasmic membrane) [7]. The precise mechanism of bacitracin resistance by ABC-transporters is not yet fully understood [12].

The expression of most resistance genes is induced in the presence of the respective antibiotic. For example, the *van* operons are induced in the presence of vancomycin by the two-component systems VanS-VanR or $VanS_B$-$VanR_B$ for VanA- and VanB-type resistance, respectively [11,13]. Bacitracin-dependent induction of *bcrAB* is mediated by the one-component transmembrane regulator BcrR [7,14]. While the regulators and target promoters, as well as the conditions leading to induction are known, we lack in-depth understanding of the molecular mechanisms of regulation. For example, while both VanS and $VanS_B$ respond to vancomycin,

their sensory domains differ considerably in size with 37 amino acids for VanS and 103 residues for $VanS_B$, and share only low sequence similarity [15]. It is therefore difficult to envisage the same sensing mechanism for both proteins. It is similarly unclear how BcrR detects bacitracin, because the protein lacks any obvious extracellular domains but is nevertheless able to directly interact with its substrate [14,16]. Additionally, it is not known how a membrane-bound transcriptional regulator like BcrR activates transcription from its target promoter. While a direct interaction with RNA-polymerase has been proposed [16], experimental evidence is lacking to date.

Sensory perception of antimicrobial substances by bacteria is a first and essential step in antibiotic resistance, and a thorough understanding of the mechanisms involved would provide an important basis for the development of new drugs to combat resistance. However, in many genera, e.g. the enterococci, investigations are hampered by the difficulty to manipulate these bacteria genetically. Although more and more genetic tools are becoming available for enterococci, poor transformability of many strains, including clinical isolates, still impedes studies involving, for example, high-throughput or detailed mutagenic approaches. To circumvent these problems, heterologous hosts have been chosen, often using *E. coli* [17], or electro-transformable laboratory strains of *E. faecalis* [7,14]. The latter provide improved transformability, but no additional genetic tools, while the former host does not appear well suited to study resistance against cell wall active compounds, due to the major differences between the Gram-positive and Gram-negative cell envelope. Alternatively, *Bacillus subtilis* has been used successfully for the functional expression of the VanS-VanR two-component system of *E. faecalis*, as well as of the VanB-type resistance proteins [1,18]. Like *E. coli*, *B. subtilis* is easy to manipulate and a large number of genetic tools are available. The G+C contents of *B. subtilis* (43.5%) and of *E. faecalis* (37.5%) are comparable, which is of great advantage for heterologous gene expression. Furthermore, the transcription machinery in both organisms is sufficiently similar to facilitate the interaction of heterologous transcriptional regulators with the native machinery, as has been shown *in vitro* for activation of *B. subtilis* RNA polymerase by *E. faecalis* BcrR [16]. Importantly for the present application, the intrinsic resistance mechanisms of *B. subtilis* against cell wall antibiotics are well understood [19,20], allowing directed deletion of genes to create a clean genetic background.

In the present study, we have used two well-understood examples from *E. faecalis* to develop and validate *B. subtilis* as a platform for studying the regulatory mechanisms leading to resistance against cell wall-active antibiotics. To test the feasibility of our approach and determine the optimal genetic background of the host, we chose the one-component regulator BcrR and could show full functionality with highly similar behaviour to its native context. This set-up was then applied to the $VanS_B$-$VanR_B$ two-component system. A previous attempt at heterologous expression of this system in *B. subtilis* had resulted in a constitutively active behaviour [18]. Optimization of expression levels and growth conditions now resulted in vancomycin-dependent induction of the target promoter, further supporting the suitability of *B. subtilis* as host organism.

Materials and Methods

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. *E. coli* DH5αand XL1-blue were used for cloning. *E. coli* and *B. subtilis* were grown routinely in Luria-Bertani (LB) medium at 37°C with agitation (200 rpm). *B. subtilis* was transformed by natural competence as previously described [21]. Selective media contained ampicillin (100 μg ml^{-1} for *E. coli*), chloramphenicol (5 μg ml^{-1} for *B. subtilis*), kanamycin (10 μg ml^{-1} for *B. subtilis*), erythromycin 1 μg ml^{-1} with lincomycin 25 μg ml^{-1} (for macrolide-lincosamide-streptogramin B (mls) resistance in *B. subtilis*) or spectinomycin (100 μg ml^{-1} for *B. subtilis*). Bacitracin was supplied as the Zn^{2+}-salt. Unless otherwise stated, media for strains carrying pXT-derived constructs contained 0.2% (w/v) xylose for target gene expression. Solid media contained 1.5% (w/v) agar. Growth was measured as optical density at 600 nm wavelength (OD_{600}).

Construction of plasmids and genetic techniques

All primer sequences used for this study are listed in Table 2; all plasmid constructs are listed in Table 1.

Transcriptional promoter fusions of P_{bcrA} to *lacZ* or bacterial luciferase (*luxABCDE*) were constructed in vectors pAC6 [22] or pAH328 [23] by the sites of EcoRI/BamHI and EcoRI/SpeI, respectively, obtaining plasmids pES601and pNTlux101, respectively. The transcriptional promoter fusion of P_{vanYB} to bacterial luciferase was cloned into the EcoRI and SpeI sites of vector pAH328 creating plasmid pCF133. The exact regions contained in the constructs are given in Table 1.

For heterologous, xylose-inducible expression of *bcrR* or *bcrR-bcrAB* in *B. subtilis* (pES701 and pES702) the respective DNA fragments were amplified from the plasmid pAMbcr1 [7] and cloned in the vector pXT [24] using the BamHI and EcoRI restriction sites, placing the genes under the control of the vector's *xylA*-promoter. Plasmid pCF132 was constructed by inserting $vanR_BS_B$ from *E. faecalis* V583 into the BamHI and HindIII sites of vector pXT for heterologous, xylose-inducible expression in *B. subtilis*.

Constructs for unmarked gene deletions in *B. subtilis* were cloned into the vector pMAD [25]. For each operon to be deleted, 800–1000 bp fragments located immediately before the start codon of the first gene (''up'' fragment) and after the stop codon of the last gene (''down'' fragment) were amplified. The primers were designed to create a 17–20 bp overlap between the PCR-products (Table 2), facilitating fusion of the fragments by PCR overlap extension and subsequent cloning into pMAD. Gene deletions were performed as previously described [25].

All constructs were checked for PCR-fidelity by sequencing, and all created strains were verified by PCR using appropriate primers.

Antimicrobial susceptibility assays

All cultures were grown in Mueller-Hinton (MH) medium for antibiotic susceptibility assays [26]. Minimal inhibitory concentration (MIC) of bacitracin and vancomycin were determined by broth-dilution assays. Freshly grown overnight cultures of *B. subtilis* in MH medium were used as inoculum at a dilution of 1:500. After 24 h incubation in the presence of two-fold serial dilutions of the antibiotic the MIC was scored as the lowest concentration where no growth was observed.

β-Galactosidase assays

Cells were inoculated from fresh overnight cultures and grown in LB medium at 37°C with aeration until they reached an OD_{600} between 0.4 and 0.5. The cultures were split into 2 mL aliquots and challenged with different concentrations of bacitracin with one aliquot left untreated. After incubation for an additional 30 min at 37°C with aeration, the cultures were harvested and the cell pellets were frozen at −20°C. β-galactosidase activities were determined as described, with normalization to cell density [27].

Table 1. Plasmids and strains used in this study.

Name	Description[a]	Source
Vectors		
pAC6	Vector for transcriptional promoter fusions to *lacZ* in *B. subtilis*, integrates in *amyE*; cm^r	[22]
pAH328	Vector for transcriptional promoter fusions to *luxABCDE* in *B. subtilis*; integrates in *sacA*; cm^r	[23]
pMAD	Vector for construction of unmarked deletions in *B. subtilis*, temperature sensitive replicon; mls^r	[25]
pXT	Vector for xylose-inducible gene expression in *B. subtilis*; integrates in *thrC*; spc^r	[24]
Plasmids		
pAMbcr1	*E. coli-E. faecalis* shuttle vector containing a 4.7 kb EcoRI-fragment encompassing the *bcrR-bcrABD* locus of *E. faecalis* AR01/DGVS	[7]
pCF102	pMAD containing the joined "up" and "down" fragments for unmarked deletion of *bceRS-bceAB*	This study
pCF104	pMAD containing the joined "up" and "down" fragments for unmarked deletion of *psdRS-psdAB*	This study
pCF119	pMAD containing the joined "up" and "down" fragments for unmarked deletion of *yxdJK-yxdLM-yxeA*	This study
pCF132	pXT containing the $vanR_BS_B$ operon of *E. faecalis* V583	This study
pCF133	pAH328 containing P_{vanYB} of *E. faecalis* V583 from -215 to +65 relative to the $vanY_B$ start codon	This study
pES601	pAC6 containing P_{bcrA} of *E. faecalis* AR01/DGVS from -219 to +170 relative to the *bcrA* start codon	This study
pES701	pXT containing *bcrR* of *E. faecalis* AR01/DGVS	This study
pES702	pXT containing the *bcrR-bcrAB* region of *E. faecalis* AR01/DGVS	This study
pNTlux101	pAH328 containing P_{bcrA} of *E. faecalis* AR01/DGVS from -219 to +170 relative to the *bcrA* start codon	This study
E. coli		
DH5α	*supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[39]
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F'*::Tn*10* *proAB* $lacI^q$ Δ(*lacZ*)M15]	Stratagene
E. faecalis		
AR01/DGVS	Plasmid-cured clinical isolate; bac^r	[7]
V583	Sequenced clinical strain containing plasmids pTEF1, pTEF2, pTEF3; van^r	[40]
B. subtilis		
W168	Wild-type, *trpC2*	Laboratory stock
SGB34	W168 *thrC*::pES702	This study
SGB35	TMB035 *thrC*::pES702	This study
SGB36	TMB035 *thrC*::pES702 *amyE*::pES601; kan^r, spc^r, cm^r	This study
SGB40	W168 *thrC*::pES701 *amyE*::pES601; spc^r, cm^r	This study
SGB42	W168 *thrC*::pES702 *amyE*::pES601; spc^r, cm^r	This study
SGB43	TMB035 *thrC*::pES701 *amyE*::pES601; kan^r, spc^r, cm^r	This study
SGB273	TMB1518 *sacA*::pNTlux101; cm^r	This study
SGB274	TMB1518 *thrC*::pES701 *sacA*::pNTlux101; spc^r, cm^r	This study
TMB035	W168 *bceAB*::kan; kan^r	This study
TMB1518	W168 with unmarked deletions of the *bceRS-bceAB*, *psdRS-psdAB*, *yxdJK-yxdLM-yxeA* loci	This study
TMB1560	TMB1518 *sacA*::pCF133; cm^r	This study
TMB1562	TMB1518 *thrC*::pCF132 *sacA*::pCF133; spc^r, cm^r	This study

[a]Bac, bacitracin; cm, chloramphenicol; fs, fusidic acid; kan, kanamycin; mls, macrolide-lincosamide-streptogramin B group antibiotics; rif, rifampin; spc, spectinomycin; van, vancomycin; r, resistant.

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Luciferase assays

Luciferase activities of *B. subtilis* strains were assayed using a Synergy 2 multi-mode microplate reader from BioTek controlled by the software Gen5. LB medium was inoculated 1:500 from overnight cultures, and each strain was grown in 100 µl volumes in a 96-well plate. Cultures were incubated at 37°C with shaking (intensity: medium), and the OD_{600} was monitored every 10 min. At an OD_{600} of 0.02 (4–5 doublings since inoculation; corresponding to $OD_{600} = 0.1$ in cuvettes of 1 cm light-path length), either bacitracin was added to final concentrations of 0.03, 0.1, 0.3, 1 µg ml^{-1}, or vancomycin to final concentrations of 0.01, 0.025, 0.05, 0.25 µg ml^{-1}; in all cases one well was left untreated. Cultures were further incubated for 2 h, and the OD_{600} and luminescence (endpoint-reads; 1 s integration time; sensitivity: 200) were monitored every 5 min. OD_{600} values were corrected using wells containing 100 µl LB medium as blanks. Raw luminescence output (relative luminescence units, RLU) was normalized to cell density by dividing each data-point by its corresponding corrected OD_{600} value (RLU/OD).

Table 2. Primers used in this study.

Primer name	Sequence (5′-3′)[a]	Use
TM1569	AGTGGATCCTAGGAACGTTTTTACCAAC	*bcrAB* rev
TM1798	TTAAGGATCCGAAAAACCCGTTGATGGACG	*bcrR* fwd
TM1800	TTAAGAATTCTTTTATTTCATTCCCATCTGC	*bcrR* rev
TM1801	TTAAGAATTCTTTTGCTGTTAATCGGCAAG	P_{bcrA}-*lacZ* fwd
TM1802	TTAAGGATCCCAAGCTGCAACATCATTTTC	P_{bcrA}-*lacZ* rev
TM2450	AAATTGGATCCGGAAACTACAGACTGTTATG	$vanR_B$ fwd
TM2451	AAATTAAGCTTTATACCTGTCGGTCAAAATC	$vanS_B$ rev
TM2550	AATTTGAATTCTTTGTTCTGGCTGGATTTAC	P_{vanYB} fwd
TM2551	AATTTACTAGTTCCCCAGATTGTTTCATATG	P_{vanYB} rev
TM2813	TTAAACTAGTCAAGCTGCAACATCATTTTC	P_{bcrA}-*lux* rev
TM2347	AATTTGGATCCAGTTTAATATCAACGGCCTG	*yxdJK-yxdLM-yxeA* deletion up fwd
TM2348	AGGTAATTCTGCAATAGTCC	*yxdJK-yxdLM-yxeA* deletion up rev
TM2349	ctattgcagaattacctGGAAGAAGTCAAGTTTGAAG	*yxdJK-yxdLM-yxeA* deletion down fwd
TM2350	AATTTGGATCCTTCTGCTTCCGAAAAAACAG	*yxdJK-yxdLM-yxeA* deletion down rev
TM2351	AATTTGGATCCGAGGAAGCAAAAGGAAATC	*bceRS-bceAB* deletion up fwd
TM2352	CTTGATTTCATGAAACAGCG	*bceRS-bceAB* deletion up rev
TM2355	ctgtttcatgaaatcaagATGGTGTTATATACTGCGC	*bceRS-bceAB* deletion down fwd
TM2356	AATTCCATGGACGAATCCAGTTATCATAGC	*bceRS-bceAB* deletion down rev
TM2357	AATTTGGATCCCTACGATCTAAATGGTTTCC	*psdRS-psdAB* deletion up fwd
TM2358	ATTTTTGAAGATGACCGCCC	*psdRS-psdAB* deletion up rev
TM2361	cggtcatcttcaaaaatGTCATATTTATAAGCGTGCTG	*psdRS-psdAB* deletion down fwd
TM2362	AATTCCATGGAGAGATTGAAGCATTCATCG	*psdRS-psdAB* deletion down rev

[a]Restriction sites are underlined; overlaps to other primers for PCR fusions are shown by lower case letters.
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Results and Discussion

Functional transfer of the BcrR-BcrAB bacitracin resistance system to *B. subtilis*

In *E. faecalis*, expression of the genes *bcrAB* that encode the bacitracin resistance transporter BcrAB is controlled solely by the one-component regulator BcrR [14]. This regulator is encoded by a gene directly upstream of the transporter operon, but as an independent transcriptional unit [7]. To test if BcrR could be functionally produced in *B. subtilis*, we introduced a transcriptional fusion of its target promoter, P_{bcrA}, to *lacZ* (pES601), together with an expression construct of *bcrR* controlled by a xylose-inducible promoter (pES701), into the wild-type strain. Addition of increasing concentrations of bacitracin led to a strong upregulation (approximately 80-fold) of promoter activities with a threshold concentration for induction of 0.3 μg ml^{-1} (Fig. 1A). No promoter activities above background (ca. 1 Miller Unit (MU)) could be detected in a strain lacking BcrR (data not shown), demonstrating that the observed induction was indeed due to BcrR activity. It was shown previously that the sensitivity of BcrR is increased in a strain of *E. faecalis* lacking BcrAB, and this was attributed to competition between the transporter and BcrR in bacitracin binding [14]. While *B. subtilis* itself does not contain a BcrAB-like transporter, it nevertheless possesses a transport system for bacitracin resistance, BceAB, belonging to a different family of transporters [28]. To test if this unrelated transporter could also influence the sensitivity of BcrR, we next introduced the expression and reporter constructs into a strain carrying a *bceAB*::kan deletion (TMB035). Here, the threshold for induction was ten-fold lower at 0.03 μg ml^{-1} bacitracin, with 0.1 μg ml^{-1} leading to full induction. Furthermore, the maximal amplitude of induction was significantly increased ($p = 0.006$) to more than 200-fold (Fig. 1B). Therefore, the BceAB transporter of *B. subtilis* appeared to decrease the availability of bacitracin for detection by BcrR, similar to the effect of BcrAB in *E. faecalis*.

We next introduced a construct containing *bcrR* under control of the xylose-inducible promoter followed by *bcrAB* under BcrR-dependent control of its native promoter (pES702) into TMB035 (*bceAB*::kan). In this strain, the induction behaviour was comparable to that of wild-type *B. subtilis* carrying BcrR alone (Fig. 1C). Introduction of the same construct into the wild-type background produced a strain harbouring both transporters, BceAB and BcrAB. While the induction threshold was not significantly altered compared to strains possessing only one transporter, the amplitude of induction was lowered to approximately 50-fold (Fig. 1D). These data clearly show that both BceAB and BcrAB are able to compete with BcrR for bacitracin binding and closely reflect the behaviour of the system in *E. faecalis*. As stated above, this competition is most likely due to removal of bacitracin by the transporters.

The decreased sensitivity of P_{bcrA} induction in strains harbouring the construct of *bcrR* together with *bcrAB*, with the latter being controlled by its native promoter (Fig. 1C and D), further implied that *bcrAB* was expressed in a BcrR-dependent manner in *B. subtilis*. We therefore wanted to test if this construct was also able to impart bacitracin resistance to the *B. subtilis* host. The minimal inhibitory concentration (MIC) of bacitracin was strongly reduced from 128 μg ml^{-1} in the wild-type to 2–4 μg ml^{-1} in the *bceAB*-deleted strain TMB035 (Table 3), consistent with earlier reports [20,29]. Introduction of the *bcrR-bcrAB* construct increased the

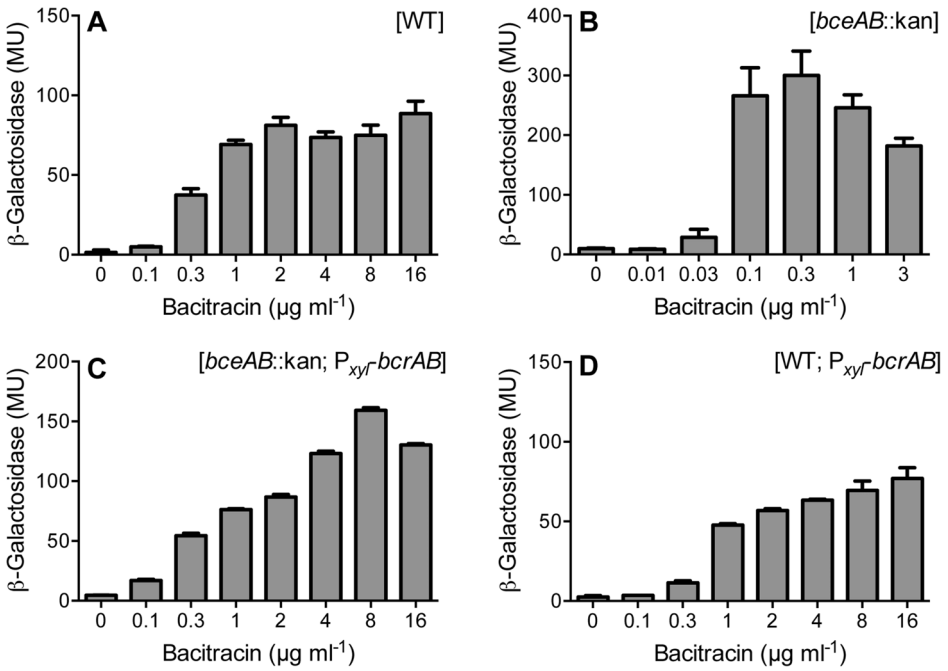

Figure 1. BcrR-dependent induction of P_{bcrA} by bacitracin in *B. subtilis*. The P_{bcrA}-*lacZ* reporter construct pES601 was introduced into different strains of *B. subtilis* producing either BcrR or BcrR and BcrAB. The relevant genes for bacitracin transporters in each strain are given at the top right of each graph. (A) SGB40; wild-type (WT) *B. subtilis* with BcrR. (B) SGB43; *bceAB*::kan mutant with BcrR. (C) SGB36; *bceAB*::kan mutant with BcrR and BcrAB. (D) SGB42; wild-type *B. subtilis* with BcrR and BcrAB. Cultures growing exponentially in the presence of 0.2% (w/v) xylose were challenged with different concentrations of bacitracin as indicated for 30 min, and β-galactosidase activities, expressed in Miller Units (MU), were determined. Results are shown as the mean plus standard deviation of three to four biological replicates.
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resistance of the *bceAB*-deleted strain to 32 μg ml^{-1} (Table 3). This degree of protection conferred to *B. subtilis* (i.e. 8- to 16-fold increase in MIC) is the same as that conferred to *E. faecalis* itself, where BcrAB raises the MIC from 32 μg ml^{-1} to >256 μg ml^{-1} [7]. The difference in final resistance reached is due to the differing degrees of intrinsic bacitracin resistance between the two hosts. Additional expression of the *E. faecalis* transporter in wild-type *B. subtilis* could not further increase its resistance (Table 3). In fact we have to date been unable to raise the MIC of the wild-type strain, even with overproduction of its native BceAB transporter (own unpublished observation), suggesting that the level of resistance is not limited by transport capacity.

Taken together, our results demonstrate full functionality of the *E. faecalis* Bcr-system in *B. subtilis*, both regarding gene regulation and bacitracin resistance. Importantly, however, the native resistance determinants of the *B. subtilis* host were shown to interfere with the sensitivity and amplitude of promoter induction and masked the resistance imparted by the introduced system. This observation is addressed in the following section.

Development of a sensitive recipient strain

When employing a heterologous host for functional studies of resistance and associated regulatory systems, it is of vital importance to consider any potential interference from intrinsic resistance determinants. One advantage of using *B. subtilis* as the heterologous host is that its resistance determinants against cell wall antibiotics are very well known. Several proteins were shown to contribute to broad-spectrum protection from charged antimicrobial peptides, for example by modification of teichoic acids in the cell envelope [30], but most of these mechanisms are not drug-specific. In contrast, antimicrobial peptide transporters such as the BceAB system described above, are thought to function by removal of the antibiotic from its site of action [12,20,31–33], and are thus likely to interfere with heterologously introduced

Table 3. Antibiotic susceptibility of *B. subtilis* strains.

Strain	Relevant resistance proteins	Bacitracin MIC[a] (μg ml^{-1})	Vancomycin MIC[a] (μg ml^{-1})
W168	BceAB$^+$	128	0.25
TMB035	BceAB$^-$	2–4	0.25
TMB1518	BceAB$^-$	4	0.25
SGB34	BceAB$^+$, BcrR-BcrAB$^+$	128	0.25
SGB35	BceAB$^-$, BcrR-BcrAB$^+$	32	0.25

[a]Minimal inhibitory concentrations (MIC) determined from three biological replicates; where a range of concentrations is given, results varied between replicates.
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resistance determinants. *B. subtilis* possesses three paralogous systems of differing substrate specificities: BceAB mediates resistance against bacitracin, mersacidin, actagardine and plectasin [20,31]; PsdAB confers resistance against a broad-range of lipid II-binding lantibiotics such as nisin or gallidermin [31]; for YxdLM no role in resistance has been identified to date, but it's expression is induced in response to the human cathelicidin LL-37 [34]. All three transporters are encoded together with an operon for a two-component regulatory system, BceRS, PsdRS and YxdJK, respectively, which controls expression of its corresponding transporter operon [28,31,35].

To obtain a recipient strain that is well suited for the study of resistance mechanisms against cell wall antibiotics from *E. faecalis* and potentially also other genetically intractable Gram-positive bacteria, we therefore created unmarked deletions of all three entire genetic loci, *bceRS-bceAB*, *psdRS-psdAB* and *yxdJK-yxdLM-yxeA*. *yxeA* is a small gene of unknown function that may form a transcriptional unit with *yxdLM* and was therefore included in the deletion. To test for the absence of interference, we then introduced the *bcrR* expression construct pES701 used above into the triple deletion strain, TMB1518. While our study was in progress, the Losick-laboratory developed a new reporter system for *B. subtilis*, based on the bacterial luciferase operon *luxABCDE*, which allows time-resolved, semi-automated analyses of transcriptional promoter fusions [23,36]. To test the applicability of this reporter for our purposes, we inserted the BcrR target promoter P_{bcrA} upstream of the *lux* operon and introduced this construct into the triple deletion strain harbouring BcrR. At high expression levels of BcrR due to induction by xylose, addition of bacitracin to growing cultures of this strain resulted in a rapid response, with a more than ten-fold increase of promoter activity within 5 min after addition of 1 μg ml^{-1} bacitracin (Fig. 2A). Only background luminescence (ca. 10^3 relative luminescence units (RLU) per OD) was observed in the absence of bacitracin or in a strain lacking BcrR (Fig. 2A and data not shown). Analysis of promoter activities 30 min post-induction showed a similar dose-response behaviour (Fig. 2B) compared to the corresponding *lacZ* reporter strain shown above (Fig. 1B). While the threshold concentration for induction appeared slightly increased for the P_{bcrA}-*lux* construct, possibly due to the different growth conditions in 96-well plates compared to test-tubes, the maximal amplitude of induction was approximately doubled to over 500-fold, which can most likely be attributed to the very low background luminescence obtained with luciferase assays. Therefore both the *lacZ* and *lux* reporters are equally suitable to determine dose-response behaviours of regulatory systems, while the *lux* reporter offers higher sensitivity and additionally allows time-resolved analyses for dynamic studies.

To test if the cellular protein levels of a one-component regulator like BcrR affected the promoter induction behaviour, the same experiments were also carried out in the absence of xylose, relying on the basal activities of the P_{xylA}-promoter for *bcrR* expression (Fig. 2C and D). Under these conditions, the maximal promoter activities were reduced approximately eight-fold ($p = 0.0003$). Considering that the difference in P_{xylA} activity in the presence and absence of xylose is ten-fold under the conditions used here [36], this difference in BcrR-activity is likely directly due to a reduced copy number of BcrR in the cell. However, the dose-response behaviour was again similar to previous results, with a threshold concentration for induction in the range of 0.03 to 0.1 μg ml^{-1} bacitracin. Thus the overall function of BcrR was robust to changes in expression, with differences in protein levels merely affecting the amplitude of induction but not the response to the stimulus.

Qualitative activity assays on solid media for screening applications

To elucidate the molecular mechanisms of stimulus perception and signal transduction in regulatory systems, random or site-directed mutagenesis is often used. Particularly in the case of random mutagenesis approaches, but also for (synthetic) DNA-libraries, assays performed on solid media greatly facilitate screening of large numbers of clones. To evaluate the *lacZ* and *lux* reporters for such applications, the derived BcrR/P_{bcrA} reporter strains were streaked onto agar plates in the absence or presence of bacitracin. Strains harbouring the P_{bcrA}-*lacZ* fusions showed a blue colouration on XGal-containing agar plates in the presence of inducing concentrations of bacitracin, but remained white in its absence (Fig. 3A and B). As observed before in the quantitative assays, presence of the transporters BceAB or BcrAB diminished the intensity of colouration (Fig. 3B, sectors 1 and 2). In the strain possessing both transporters, bacitracin concentrations of at least 10 μg ml^{-1} were required to produce blue colonies (data not shown), consistent with the low promoter activities reported above for this strain. The reporter strain harbouring BcrR and the P_{bcrA}-*lux* construct showed strong luminescence when grown on agar plates containing 0.3 μg ml^{-1} bacitracin, and no detectable luminescence in its absence (Fig. 3C and D).

Both reporter constructs are therefore suitable for screening libraries of clones for promoter induction and are applicable for high-throughput approaches. In principle, screens for loss-of-function as well as gain-of-function mutations can be performed, depending on experimental design. This set-up offers a great advantage over studies performed directly in *E. faecalis*, where it is much more difficult to obtain large numbers of transformants than in the naturally competent *B. subtilis*. Importantly, the output of both promoters is sufficiently sensitive to allow assays to be performed at sub-lethal concentrations of the antibiotic, at least in the case of the Bcr-system. The feasibility of this approach was recently demonstrated in a study that identified essential residues in the *B. subtilis* bacitracin resistance transporter BceAB [29], and the same strategy should be applicable to the heterologous set-up described here.

Functional transfer of the $VanS_B$-$VanR_B$ two-component system to *B. subtilis*

Following successful transfer of the Bcr-system of *E. faecalis* to *B. subtilis*, we next wanted to test if our set-up could be applied to other regulatory systems. The two-component system VanS-VanR regulating VanA-type vancomycin resistance had previously been shown to be functional in *B. subtilis* [1]. However, heterologous expression of $vanR_BvanS_B$ encoding the regulatory system for VanB-type resistance had resulted in constitutive expression of the target promoter, P_{vanYB}, and the authors could show that this was due to constitutive activity of the sensor kinase $VanS_B$ under the conditions chosen [18]. To test if vancomycin-dependent modulation of $VanS_B$ activity could be obtained by optimization of conditions, we introduced an expression construct of the $vanR_BvanS_B$ operon under control of the xylose-inducible promoter P_{xylA} into TMB1518. The activity of the two-component system was monitored as activation of a P_{vanYB}-*luxABCDE* transcriptional fusion. In the absence of xylose, only low levels of the two-component systems will be produced in the cell, due to basal promoter activity of P_{xylA}. Under these conditions, addition of increasing concentrations of vancomycin to growing cultures of the reporter strain led to a gradual up-regulation of promoter activity (Fig. 4A). Importantly, and in contrast to previous data, only background activity was observed in the absence of vancomycin

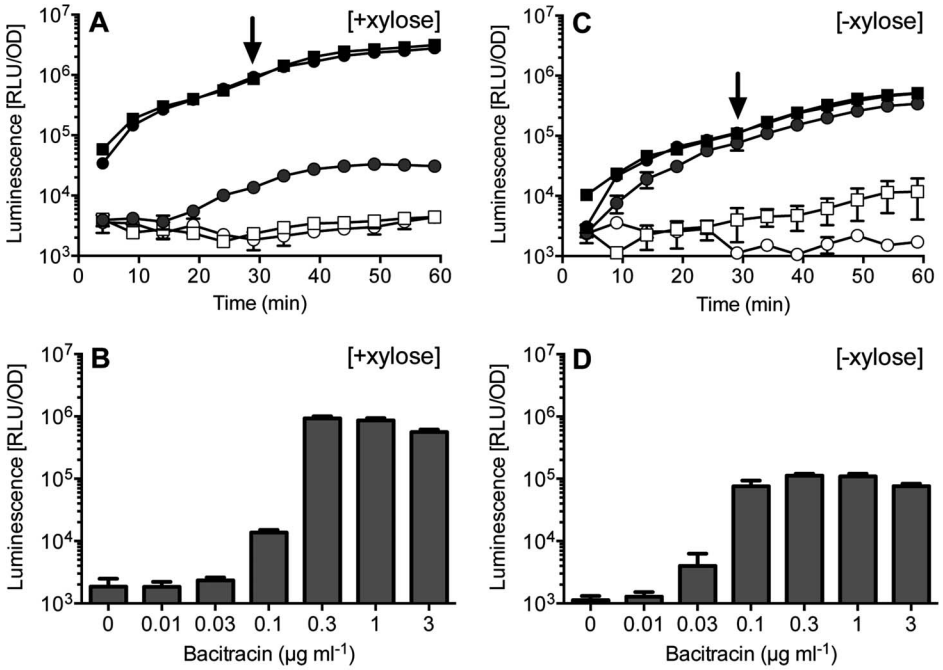

Figure 2. Time-resolved induction of P_{bcrA} by bacitracin in an unmarked, sensitive *B. subtilis* recipient strain. SGB274, carrying unmarked deletion of *bceRS-bceAB*, *psdRS-psdAB*, *yxdJK-yxdLM-yxeA* and harbouring the P_{bcrA}-*lux* reporter construct pNTlux101 and *bcrR* expression construct was grown in the presence of 0.2% (w/v) xylose (panels A and B), or in the absence of xylose (panels C and D). In early exponential phase (t = 0 min), bacitracin was added to final concentrations of 0 (open circles) 0.03 µg ml^{-1} (open squares), 0.1 µg ml^{-1} (grey circles), 0.3 µg ml^{-1} (solid circles) or 1 µg ml^{-1} (solid squares), and luminescence normalized to optical density (RLU/OD) was monitored. (A, C) Time-course of promoter induction over 60 min after bacitracin-challenge. (B, D) Dose-response at 30 min post-induction; the time point is labelled with the arrow in the panels above. Results are shown as the mean and standard deviation of three biological replicates.
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(Fig. 4A, open circles). The threshold concentration for induction was 0.01 µg ml^{-1}, and a maximum induction of ca. 500-fold was observed in the presence of 0.05–0.25 µg ml^{-1} vancomycin. The MIC of *B. subtilis* for vancomycin was determined as 0.25 µg ml^{-1} for both the wild-type and TMB1518 (Table 3), and therefore higher concentrations were not tested. In the previous study, promoter activities were analysed only in the presence of xylose to ensure high expression levels of the two-component system [18], which may have led to the high basal activities observed. We therefore next repeated the induction experiments, but in the presence of 0.2% xylose, and indeed found ten-fold increased promoter activities in the absence of vancomycin (Fig. 4B). Vancomycin-dependent induction was still observed, but only to a maximum of ten-fold over the uninduced control, due to the higher basal activity.

Together with previously published reports [1,18], our data show that the regulators of vancomycin resistance in *E. faecalis* can be functionally produced in *B. subtilis*, although the expression levels have to be adjusted for optimal signal-to-background ratios. The full functionality of the VanRS two-component systems, both of VanA-type resistance described previously [1] and VanB-type resistance shown here, validates the biological relevance of the heterologous set-up and paves the way for detailed mechanistic investigations into the respective modes of vancomycin detection. The high degree of competence of *B. subtilis*, for example, allows high-throughput screening of random mutants, synthetic DNA libraries, or chimeric protein fusions, which may lead to discovery of ligand binding sites and thus to elucidation of sensory mechanisms. Promising results can then be validated in a more targeted fashion in *E. faecalis*.

Additionally, Bisicchia and colleagues had reported that vancomycin resistance could be imparted on *B. subtilis* by expression of the VanB-type resistance operon $vanY_BWH_BBX_B$, further extending the applicability of this host organism.

Conclusions

In summary we here show that *B. subtilis* is well suited to the use as a host for functional production of regulatory systems that control resistance against cell wall active compounds in *E. faecalis*. Our data also show that care has to be taken regarding the genetic background of the host strain and that appropriate expression levels of the regulator genes have to be experimentally determined. Due to the availability of a range of inducible and constitutive promoters, for which strength and dynamic behaviour are very well characterized [36], *B. subtilis* offers a vast potential for optimization of expression levels, again supporting its suitability as a versatile heterologous host. Full functionality of any newly introduced system should of course be validated by comparison of its behaviour between *B. subtilis* and the native host before detailed mechanistic investigations are commenced.

To minimize interference from intrinsic resistance determinants against antimicrobial peptides, we have constructed a *B. subtilis* strain devoid of the most efficient systems. This strain should provide a clean genetic background for the study of a broad range of resistance mechanisms against cell wall active substances, particularly regarding their regulation. In addition to one-component regulation of bacitracin resistance and two-component regulation of vancomycin resistance implemented here, we have successfully applied this set-up to the functional reconstitution of a more complex regulatory and resistance network [37]. It should be

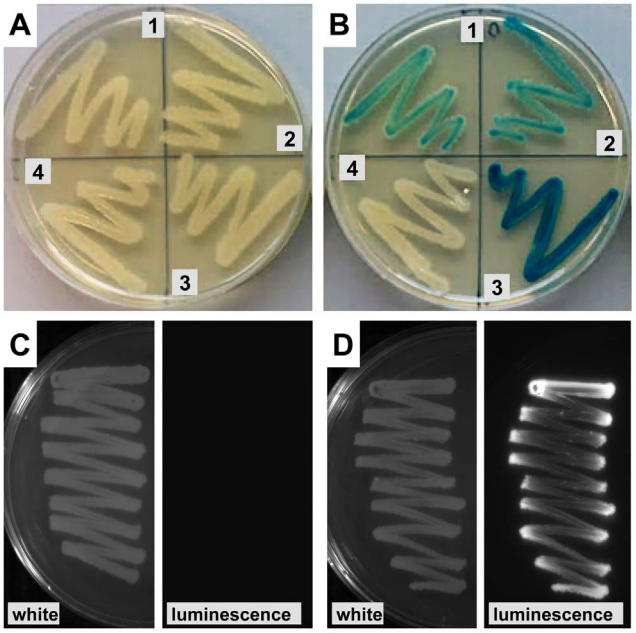

Figure 3. Functionality of the reporter systems on solid media. Strains of *B. subtilis* harbouring the P_{bcrA}-*lacZ* reporter (A and B) or the P_{bcrA}-*luxABCDE* reporter (C and D) were grown on agar plates containing 0 μg ml^{-1} (A and C), 0.3 μg ml^{-1} (D) or 1 μg ml^{-1} (B) bacitracin. (A, B) Blue colouration due to reporter induction is depicted by the dark grey shading of bacterial growth. Sector 1, SGB40 ($BcrR^+$, $BceAB^+$); sector 2, SGB36 ($BcrR^+$, $BcrAB^+$); sector 3, SGB43 ($BcrR^+$); sector 4, SGB42 ($BcrR^+$, $BceAB^+$, $BcrAB^+$). Plates contained 200 μg ml^{-1} X-Gal. (C, D) Plates inoculated with SGB237 ($BcrR^+$) were photographed under white light (left sub-panels), followed by detection of luminescence in the dark (right sub-panels); the same sector of the agar plates is shown in both sub-panels.
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noted that the response of *B. subtilis* to antibiotics in general is among the best understood of all bacteria investigated to date [38]. This plethora of available data therefore constitutes an ideal basis for construction of new sensitive recipient strains adapted to the study of resistance and regulatory systems also for other classes of antimicrobials.

Further, we showed that the two reporters, *lacZ* and *luxABCDE*, can both be used for qualitative (high-throughput) screening approaches, for example of mutant libraries, as well as for the quantitative characterization of regulators. Complementation studies with random or directed mutations can thus be initiated in the genetically accessible, highly competent host *B. subtilis*, and promising results then validated directly in *E. faealis*. Construction of the desired heterologous strains will be further aided by a recently established and fully validated tool-box of vectors, promoters, reporters and epitope-tags for engineering of *B. subtilis* [36]. We therefore envisage that the system developed here will aid investigations into the molecular mechanisms of sensory perception of antimicrobials and subsequent signal transduction, the first

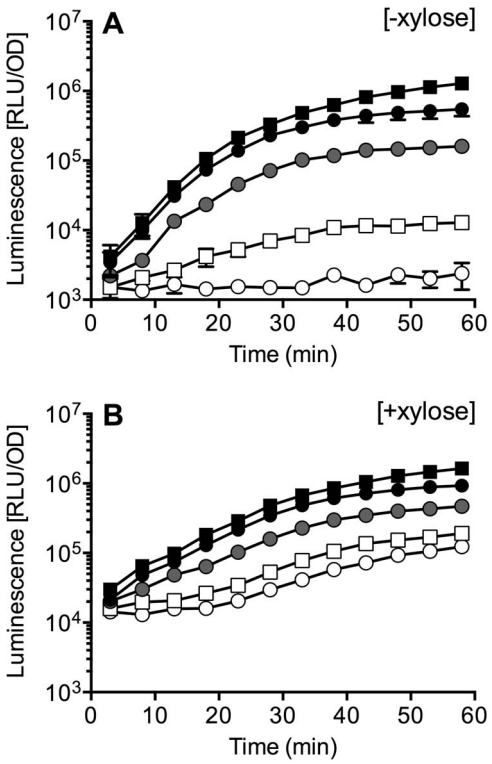

Figure 4. $VanS_B$-$VanR_B$-dependent induction of P_{vanYB} by vancomycin in *B. subtilis*. Both the P_{vanYB}-*lux* reporter construct pCF133 and the P_{xylA}-$vanR_BS_B$ expression construct pCF132 were introduced into *B. subtilis* strain TMB1518 (unmarked deletion of *bceRS-bceAB*, *psdRS-psdAB*, *yxdJK-yxdLM-yxeA*). Cultures growing exponentially either (A) in the absence of xylose or (B) in the presence of 0.2% (w/v) xylose were challenged at t = 0 min with 0.01 μg ml^{-1} (open squares), 0.025 μg ml^{-1} (grey circles), 0.05 μg ml^{-1} (solid circles), 0.25 μg ml^{-1} (solid squares) vancomycin, or left untreated (open circles). Luminescence normalized to optical density (RLU/OD) was monitored over 60 min. Results are shown as the mean and standard deviation of three biological replicates.
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essential step of antibiotic resistance. Furthermore, this set-up should also be applicable to the study of unrelated resistance systems or even regulatory cascades of diverse functions from other genetically intractable Gram-positive bacteria.

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Author Contributions

Conceived and designed the experiments: SG TM GC. Performed the experiments: CF ES. Analyzed the data: CF ES SG. Wrote the paper: SG CF.

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Chapter III

Identification and Characterization of a Bacitracin Resistance Network in *Enterococcus faecalis*

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Identification and Characterization of a Bacitracin Resistance Network in *Enterococcus faecalis*

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Resistance of *Enterococcus faecalis* against antimicrobial peptides, both of host origin and produced by other bacteria of the gut microflora, is likely to be an important factor in the bacterium's success as an intestinal commensal. The aim of this study was to identify proteins with a role in resistance against the model antimicrobial peptide bacitracin. Proteome analysis of bacitracin-treated and untreated cells showed that bacitracin stress induced the expression of cell wall-biosynthetic proteins and caused metabolic rearrangements. Among the proteins with increased production, an ATP-binding cassette (ABC) transporter with similarity to known peptide antibiotic resistance systems was identified and shown to mediate resistance against bacitracin. Expression of the transporter was dependent on a two-component regulatory system and a second ABC transporter, which were identified by genome analysis. Both resistance and the regulatory pathway could be functionally transferred to *Bacillus subtilis*, proving the function and sufficiency of these components for bacitracin resistance. Our data therefore show that the two ABC transporters and the two-component system form a resistance network against antimicrobial peptides in *E. faecalis*, where one transporter acts as the sensor that activates the TCS to induce production of the second transporter, which mediates the actual resistance.

Enterococcus faecalis is a member of the normal gut microflora in animals and humans. While it is mostly a harmless commensal, opportunistic infections, particularly of immunocompromised hospital patients, are a major health concern. Due to the rise of vancomycin-resistant strains (VREs), treatment of such infections is becoming increasingly difficult, and a better understanding of the resistance of *E. faecalis* against cell wall-targeting antibiotics is urgently needed. In addition to vancomycin resistance, enterococci display a high intrinsic resistance against other inhibitors of cell wall biosynthesis, including cephalosporins and bacitracin (1–3). Bacitracin is not used clinically for the treatment of enterococcal infections, yet resistance against this and other peptide antibiotics is nevertheless biologically relevant in the human intestinal environment. For example, gut bacteria are exposed to defensins that are part of the innate immune defense in the gastrointestinal tract (4). Additionally, other members of the microflora, especially lactic acid bacteria, produce a range of bacteriocins as a means of interspecies competition (5). Thus, resistance of *E. faecalis* against peptide antibiotics is likely to be important for the success of the bacterium in the human host, which in turn affects the risk of opportunistic infections.

In other Gram-positive bacteria, several resistance mechanisms that combat the action of cell wall-active peptides have been identified. One strategy can be alterations in the cell's surface charge, for example, by alanylation of teichoic acids catalyzed by the DltABCD system (6, 7). Accordingly, in a recent study, the *dlt* operon of *E. faecalis* was shown to be upregulated in response to bacitracin and vancomycin (8). Alternatively, upregulation of the enzyme inhibited by the antibiotic, e.g., in the case of bacitracin, undecaprenyl-pyrophosphate phosphatases, can confer resistance, as has been shown for *Bacillus subtilis* and *E. faecalis* (2, 9). A third resistance mechanism is the expression of specific ATP-binding cassette (ABC) transporters. One type of such transporters possesses permeases with six transmembrane helices and is exemplified by the bacitracin transporter BcrAB, which is used for self-immunity by bacitracin-producing strains of *Bacillus licheniformis* (10) but has also been shown to confer acquired high-level bacitracin resistance to *E. faecalis* (3). An unrelated group of transporters, characterized by permeases with 10 transmembrane helices and a large extracellular domain, are involved in resistance against a range of antimicrobial peptides. The best-understood example is BceAB of *B. subtilis*, which confers resistance against bacitracin, mersacidin, and actagardine (11, 12). These transporters form the Peptide-7-Exporter family in the Transport Classification Database and are collectively referred to as BceAB-type systems (13, 14). They are usually found in the genetic neighborhood of a two-component regulatory system (TCS; BceRS type) that controls expression of the transporter operon (15, 16). Importantly, these transporters not only mediate resistance but play an additional role as sensors, because the TCSs alone are unable to detect their substrate peptides (11, 17). Together, transporters and TCSs form peptide antibiotic resistance modules and are found widely distributed among low-G+C Gram-positive organisms (13, 16). A comparative genomics analysis identified two BceAB-

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type transporters in the genome of *E. faecalis* (16), but no functional data are available on these systems, nor has a TCS been identified as the corresponding regulator.

In accordance with the need for a better understanding of the response of *E. faecalis* to antibiotics that inhibit cell wall synthesis, a recent transcriptomic study identified a large number of genes that were upregulated after exposure to ampicillin, cephalothin, vancomycin, and bacitracin (8). Of these compounds, bacitracin elicited the broadest response, affecting genes with functions in cell wall maintenance, metabolism, and transport processes. Here we report on a proteomic analysis of the bacitracin response of *E. faecalis*. Among the differentially produced proteins, we again identified those involved in cell wall maintenance and energy metabolism as important factors. Additionally, one BceAB-type transporter was found at higher levels following bacitracin exposure, and subsequent investigations revealed the existence of a regulatory and resistance network comprised of two such transporters and one TCS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *E. coli* MC1061 was used for cloning with vector pTCVlac; strains DH5α and XL1-Blue were used for all other cloning. *E. coli* and *B. subtilis* were grown routinely in Luria-Bertani (LB) medium at 37°C with agitation (200 rpm). *Enterococcus faecalis* was grown routinely in brain heart infusion (BHI) at 37°C without agitation. *E. faecalis* was transformed by electroporation as previously described (18). *B. subtilis* was transformed by natural competence as previously described (19). Selective media contained ampicillin (100 μg ml^{-1}), chloramphenicol (10 μg ml^{-1} for *E. coli*; 15 μg ml^{-1} for *E. faecalis*; 5 μg ml^{-1} for *B. subtilis*), kanamycin (50 μg ml^{-1} for *E. coli*; 1,000 μg ml^{-1} for *E. faecalis*), 1 μg ml^{-1} erythromycin with 25 μg ml^{-1} lincomycin (for macrolide-lincosamide-streptogramin B [MLS] resistance), or spectinomycin (150 μg ml^{-1} for *E. faecalis*; 100 μg ml^{-1} for *B. subtilis*). Bacitracin was supplied as the Zn^{2+} salt. Solid media contained 1.5% (wt/vol) agar. Growth was measured as optical density at 600 nm (OD_{600}).

Proteomic analysis. For proteomic analyses, exponentially growing cultures (OD_{600} = 0.4) of *E. faecalis* V583 in BHI medium were exposed to 64 μg ml^{-1} bacitracin for 1 h. The cytoplasmic protein fraction was extracted and analyzed by isoelectric focusing, followed by second-dimension (2D) SDS-PAGE. Differentially produced proteins were identified by mass spectrometry. Detailed experimental and analytical procedures are described in the supplemental text.

Construction of plasmids and genetic techniques. All primer sequences used for cloning or transcriptional start site mapping are listed in Table S2 in the supplemental material.

Transcriptional promoter fusions to *lacZ* in *E. faecalis* were constructed in the vector pTCV-lac (20). All fragments were cloned via the EcoRI and BamHI sites of the vector. The exact regions amplified as well as the primers used are given in Tables S1 and S2 in the supplemental material. For complementation of the strain of *E. faecalis* with a deletion of the transporter operon EF2050-2049, the entire operon, including its native promoter region, was cloned into the EcoRI and BamHI sites of vector pAT28 (21). Transcriptional promoter fusions in *B. subtilis* of EF2752 (P_{EF2752}) or EF2050 (P_{EF2050}) to the bacterial luciferase genes (*luxABCDE*) were cloned into the EcoRI and SpeI sites of the vector pAH328 (22), creating plasmids pCF135 and pCF134. The exact regions contained in the construct are given in Table S1 in the supplemental material.

Plasmids for heterologous, xylose-inducible expression of EF2752-2751 or EF2050-2049 in *B. subtilis* (pCF129 and pCF130) were constructed in the vector pXT using the BamHI and EcoRI restriction sites, placing the genes under the control of the vector's *xylA* promoter (23).

The construct for the heterologous expression of EF0926-0927 in *B. subtilis* was cloned according to the BioBrick standard (24). To facilitate constitutive expression in *B. subtilis*, a BioBrick of the *bceRS* operon promoter, P_{bceR}, of *B. subtilis* was amplified and cloned into the EcoRI and SpeI sites of vector pSB1A3, creating pCF144. A BioBrick of EF0926-0927 containing an optimal Shine-Dalgarno sequence for *B. subtilis* was similarly cloned into pSB1A3 via EcoRI and SpeI, creating plasmid pCF143. Assembly of both BioBricks in vector pBS2E (25) created plasmid pCF145, where expression of EF0926-0927 is controlled by P_{bceR}.

Constructs for unmarked gene deletions in *E. faecalis* were cloned in the vector pLT06 (26). For each gene or operon to be deleted, 700- to 1,000-bp fragments located immediately before the start codon of the first gene ("up" fragment) and after the stop codon of the last gene ("down" fragment) were amplified. The primers were designed to create a 17- to 20-bp overlap between the PCR products (see Table S2 in the supplemental material), facilitating fusion of the fragments by PCR overlap extension (27) and subsequent cloning into pLT06. Gene deletions were performed as previously described (26).

All constructs were checked for PCR fidelity by sequencing, and all created strains were verified by PCR using appropriate primers.

To determine the transcriptional start sites of the EF2050-2049 and EF2752-2751 operons, total RNA was isolated from *E. faecalis* JH2-2 using a RNeasy minikit and QIAshredder columns (Qiagen) according to the manufacturer's instructions. DNA was removed with a TURBO DNA-free kit (Ambion). Transcriptional start sites were determined by 5′ rapid amplification of cDNA ends (5′ RACE) as described previously (28). The sets of nested primers used are listed in Table S2 in the supplemental material.

Antimicrobial susceptibility assays. For antibiotic susceptibility assays, all cultures were grown in Mueller-Hinton (MH) medium. MICs of bacitracin were determined by broth dilution assays. Freshly grown colonies of *E. faecalis* were suspended in sterile saline to 0.5 McFarland standard turbidity and diluted 1:1,000 into MH medium containing serial 2-fold dilutions of bacitracin. For *B. subtilis*, freshly grown overnight cultures in MH broth were used as inoculum at a dilution of 1:500. After 24 h of incubation, the MIC was scored as the lowest concentration where no growth was observed. MICs obtained from broth dilution assays were subsequently confirmed using bacitracin Etest strips (bioMérieux) according to the manufacturer's instructions.

Additionally, bacitracin susceptibility of *B. subtilis* was determined by growth inhibition of exponentially growing cultures in LB. Strains were inoculated 1:500 from overnight cultures and grown in 100-μl volumes in a 96-well plate. Cultures were incubated at 37°C with shaking (medium intensity) in a Synergy2 multimode microplate reader from BioTek controlled by the software Gen5, and OD_{600} was monitored every 5 min. At an OD_{600} of 0.02 (4 to 5 doublings since inoculation; corresponding to an OD_{600} of 0.1 in cuvettes with a 1-cm light path length), bacitracin was added to a final concentration of 2, 4, 8 or 16 μg ml^{-1} with one well left untreated, and growth was monitored for another 2.5 h.

β-Galactosidase assays. Qualitative assays for induction of LacZ reporter constructs in *E. faecalis* were performed by the disk diffusion method. A suspension of cells from freshly grown colonies at 0.5 McFarland standard turbidity was spread onto MH agar containing appropriate antibiotics for selection and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (100 μg ml^{-1}) using cotton swabs. Filter discs containing 5 μl antibiotic test solution (100 mg ml^{-1}) were placed onto the plates. After overnight incubation, plates were scored for formation of a blue ring around the filter discs.

For quantitative assays, exponentially growing cells in BHI medium were exposed to different concentrations of bacitracin for 1 h as previously described (2). β-Galactosidase activities were assayed in permeabilized cells as described previously and were expressed in Miller units (MU) (29, 30).

Luciferase assays. Luciferase activities of *B. subtilis* strains containing pCF135 and pCF134 were assayed using the microplate reader described above. LB medium was inoculated 1:500 from overnight cultures, and

each strain was grown in 100-μl volumes in a 96-well plate. Cultures were incubated at 37°C with shaking (medium intensity), and the OD_{600} was monitored every 10 min. At an OD_{600} of 0.02 (see above), bacitracin was added to a final concentration of 1, 3, or 10 μg ml^{-1}, with one well left untreated. Cultures were further incubated for 2 h, and the OD_{600} and luminescence (endpoint reads; 1-s integration time; sensitivity, 200) were monitored every 5 min. OD_{600} values were corrected using wells containing 100 μl LB medium as blanks. Raw luminescence output (relative luminescence units [RLU]) was normalized to cell density by dividing each data point by its corresponding corrected OD_{600} value (RLU/OD).

RESULTS

Proteome analysis of the bacitracin stress response of *E. faecalis*. To investigate the response of *E. faecalis* to bacitracin, we analyzed the proteome of strain V583, whose genome has been fully sequenced, after 1 h exposure to 64 μg ml^{-1} bacitracin, which corresponded to the strain's MIC, compared to an untreated control. A detailed description of the findings is presented in the supplemental text. The 2D gels and detailed analyses of protein spots are available in Fig. S1 and in Tables S3 and S4, respectively, in the supplemental material. In brief, as expected, a number of proteins involved in cell wall metabolism were found in increased quantities after bacitracin stress. Additionally, several enzymes for energy metabolism or fatty acid synthesis were differentially expressed, indicating metabolic rearrangements in response to bacitracin. Further proteins with increased production after bacitracin exposure likely indicated a general response to stress. The two most strongly upregulated proteins are homologous to a protein of unknown function, YvlB of *B. subtilis*, and to cobyric acid synthase. Their roles in the response to bacitracin are not clear. The third strongest effect was observed with EF2050. This protein is the ATPase component of a BceAB-like ABC transporter (EF2050-2049), a group of proteins that to date have been identified only in the context of resistance against peptide antibiotics (13, 16). As mentioned above, operons for two such transporters were previously identified in the genome of *E. faecalis* by a comparative genomics study, namely, EF2050-2049 and EF2752-2751 (16). Although our proteomic study identified only the former transporter, both loci were found to be upregulated in response to bacitracin by transcriptome analysis (8). We therefore decided to investigate these two transporters in more detail, regarding both their role in bacitracin resistance and their regulation.

Identification of orthologous genes in *E. faecalis* JH2-2. Because strain V583 is a vancomycin-resistant clinical isolate, we chose the laboratory strain JH2-2 for all further studies. For this, we first needed to identify the genes corresponding to EF2050-2049 and EF2752-2751 from strain V583. Using PCR primers designed according to the V583 genome sequence, we readily obtained amplicons of the correct size from strain JH2-2. DNA sequencing confirmed that all four genes possessed the same sequence in both strains. For simplicity and consistency with the existing literature, we maintained the strain V583 nomenclature for all genes from strain JH2-2 throughout this study.

As described in the introduction, BceAB-type transporters are usually regulated by a BceRS-like TCS encoded in the genomic neighborhood of the transporter. However, neither of the two transporters investigated here possessed such a regulatory system. Because the sensor kinases of these TCSs have a characteristic domain architecture of two transmembrane helices with a very short intervening extracellular linker (ca. 3 to 10 amino acids) and no additional cytoplasmic domains besides the catalytic domains

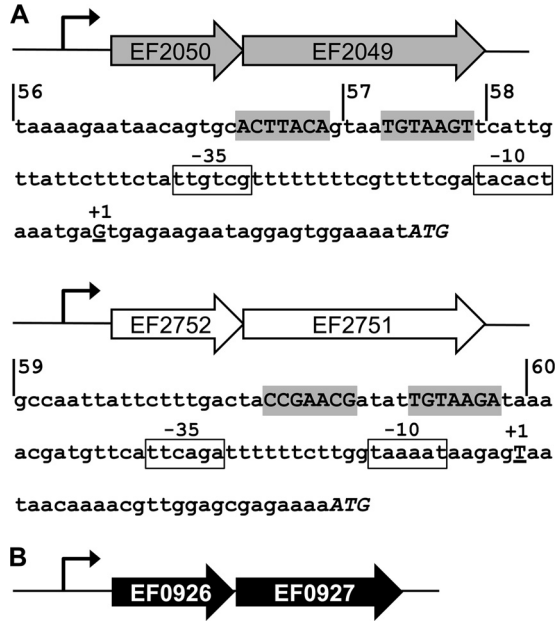

FIG 1 Schematic of operon structures and promoter regions in the resistance circuit. (A) Operons for the ABC transporters. In each, the first gene encodes the ATPase and the second gene the permease; the bent arrow indicates the promoter. The nucleotide sequences of the promoter regions are given below the respective schematics. The proposed response regulator binding site is highlighted in gray, the −10 and −35 elements are boxed, the transcriptional start site (+1) determined by 5′ RACE is shown as an underlined capital letter, and the translational start is shown in italicized capitals. Vertical lines show the beginning of fragments used to construct transcriptional reporter fusions, and the numbers of the derived constructs are given. (B) Operon for the two-component system. The first gene encodes the response regulator, and the second gene encodes the sensor kinase; the promoter region is indicated by a bent arrow. No putative regulator binding site was identified in the promoter, and therefore no sequence is shown.

for autophosphorylation, candidate TCSs can be identified via genome analyses (16). Indeed, the genome of *E. faecalis* V583 encodes a single BceRS-like TCS, EF0926-0927, and identical genes were identified by PCR and sequencing in strain JH2-2. Schematics of all three loci are shown in Fig. 1.

The transporter EF2050-2049, which was identified as bacitracin induced in our proteome analysis, is a member of phylogenetic group VII of BceAB-type transporters, which also includes the YxdLM system of *B. subtilis* (16). The second transporter, EF2752-2751, could not be assigned to any group. However, its branch of the phylogenetic tree includes four other transporters, one from *E. faecium* and three from *Lactobacillus* species (16), showing that the occurrence of close homologues is not restricted to *E. faecalis*. Interestingly, one of these transporters was recently shown to be involved in nisin resistance of *Lactobacillus casei* (31).

Both transporters are induced by bacitracin. To confirm bacitracin-induced expression of the transporter operon EF2050-2049, and to test if EF2752-2751 was also inducible under these conditions, we first constructed transcriptional fusions of the promoter regions P_{EF2050} and P_{EF2752} to a *lacZ* reporter gene. The transcriptional start sites of both operons were determined by 5′ RACE, and putative −10 and −35 promoter elements could be identified within a suitable distance upstream of the +1 position (Fig. 1A). Additionally, sequence analysis of the regions upstream of the −35 element revealed the presence of inverted repeats with

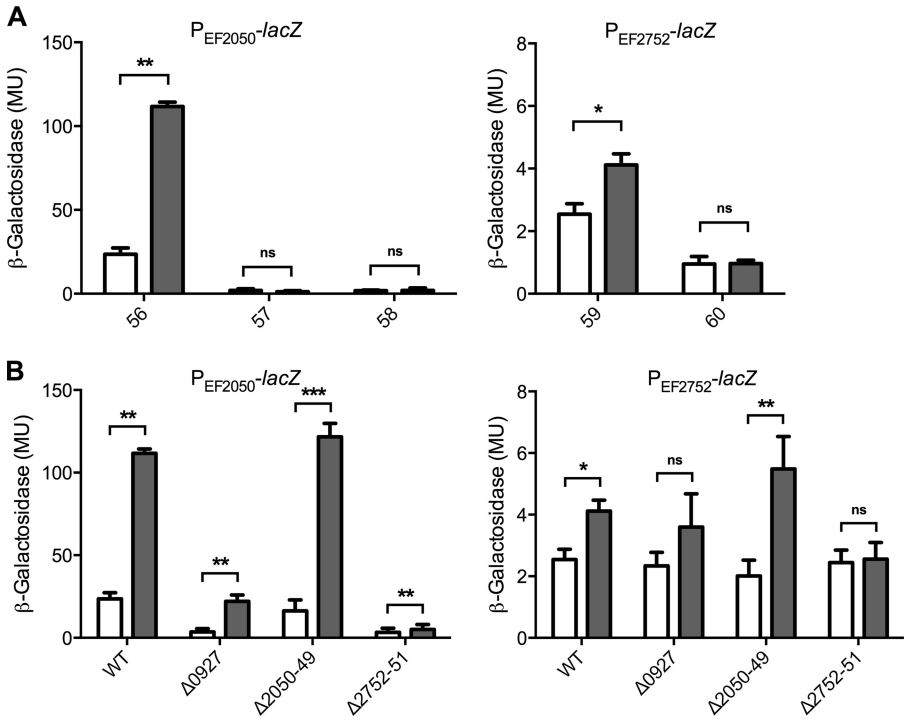

FIG 2 Induction of the transporter operons by bacitracin. Promoter regions of the transporter operons EF2050-2049 (left) and EF2752-2751 (right) were fused to *lacZ*, and resulting strains of *E. faecalis* JH2-2 were assayed for β-galactosidase activity, expressed in Miller units (MU), after 1 h exposure of exponentially growing cultures to 0 μg ml^{-1} (white bars) or 4 μg ml^{-1} (gray bars) bacitracin. (A) Successive truncations from the 5′ end of the promoter region. Bars are labeled by the number of each construct, as shown in Fig. 1. Constructs 56 and 59 contain the full promoter region. (B) Full-length constructs of the promoter fusions from panel A assayed in the wild-type (WT; same data as in panel A) and mutant backgrounds. The genes deleted in each strain are indicated by the locus tags below the bars. Results are means plus standard deviations for three to four biological replicates. The significance of induction was calculated for each strain by one-tailed, pairwise *t* test analysis and is indicated by one ($P < 0.05$), two ($P < 0.005$), or three ($P < 0.001$) asterisks. ns, not significant.

similarity to the binding consensus of BceR-like regulators (16) (Fig. 1A). The transcriptional fusions were therefore designed to contain all of these motifs (fragments 56 for P_{EF2050} and 59 for P_{EF2752} [Fig. 1A]).

Initial assays were performed by disc diffusion on agar plates inoculated with a lawn of the reporter strains. Consistent with the proteome analysis, blue circles indicating promoter induction were observed around filter discs containing bacitracin (data not shown). Because BceAB-like transporters normally recognize several different substrates (12, 13, 17), we also tested several other cell wall-active antibiotics. Nisin, gallidermin, vancomycin, teicoplanin, and penicillin G did not elicit a response, but the lantibiotic mersacidin was able to induce both promoters (data not shown). These results were confirmed by quantitative β-galactosidase assays in liquid cultures and showed that maximum induction of both promoters was obtained at 20 to 50 μg ml^{-1} mersacidin or 4 μg ml^{-1} bacitracin but not with any of the other antibiotics tested (Fig. 2A and data not shown). Because mersacidin is not commercially available and bacitracin elicited the more sensitive response, all subsequent assays were performed using bacitracin as an inducer. Following 1 h exposure of exponentially growing cultures to 4 μg ml^{-1} bacitracin, P_{EF2050}-*lacZ* was induced approximately 5-fold from 23 Miller units (MU) to 112 MU (Fig. 2A, left). P_{EF2752}-*lacZ* was also induced by bacitracin, but the overall activities were low (2 to 4 MU) and induction was only 2-fold (Fig. 2B, right), explaining why this transporter had not been identified in the proteome analysis.

Next, we constructed a series of truncated promoter fusions lacking part of (fragment 57) or the entire (fragments 58 and 60) proposed regulator binding sites (Fig. 1A). All strains carrying the derived promoter-*lacZ* fusions displayed activities near the detection limit (ca. 1 MU) and no induction by bacitracin (Fig. 2A), showing that these sequences were required for expression and thus indeed presented likely binding sites for a BceR-like regulator.

Both transporters and the TCS are required for full bacitracin resistance of *E. faecalis*. To investigate the role of the two transporters in bacitracin resistance, we created unmarked deletions of the entire coding region of each transporter. Additionally, the gene for the sensor kinase, EF0927, was deleted. Despite repeated attempts, no deletion of the regulator gene EF0926 could be achieved. All three deletion strains displayed reduced bacitracin resistance compared to the wild-type strain JH2-2, albeit to different extents (Table 1). The strongest effect with a 2- to 4-fold-increased sensitivity was observed for EF2050-2049, while deletion of EF2752-2751 and of EF0927 resulted in changes of only up to 2-fold in the MIC. The very minor effect of the EF0927 deletion strain can possibly be explained by deletion of the sensor kinase alone with the regulator still present, as discussed in more detail below. Interestingly, deletion of both transporters simultaneously did not further reduce the MIC compared to deletion of EF2050-2049 alone. The lack of an additive effect might suggest that both transporters participate in the same pathway, rather than acting independently of each other. Importantly, none of the deletion

TABLE 1 Bacitracin sensitivity of *E. faecalis* strains

Strain or genotype	MIC (μg ml^{-1})		Growth rate (h^{-1})[b]
	Broth dilution[a]	Etest	
JH2-2	32	32	1.06 ± 0.067
ΔEF0927	16–32	24	1.2 ± 0.377
ΔEF2050-2049	8–16	8	1.11 ± 0.135
ΔEF2752-2751	16	16	1.14 ± 0.146
ΔEF2050-2049 ΔEF2752-2751	8	8	1.08 ± 0.033
DLEf16	64	64	ND

[a] Results are from three independent broth dilution experiments; where a range of concentrations is given, results varied between replicates.
[b] Results are means ± standard errors from three to six independent experiments. ND, not determined.

strains displayed a growth defect compared to the wild-type strain (Table 1), showing that the differences in MIC were specifically due to loss of resistance determinants and not to altered growth kinetics. Complementation of the EF2050-2049 deletion mutant by supplying the transporter operon in *trans* (strain DLEf16) restored bacitracin resistance (Table 1). The higher MIC compared to the wild-type strain is most likely due to the increase in copy number by the plasmid-based complementation strategy and supports the role of EF2050-2049 in bacitracin resistance of *E. faecalis*.

Transporters and TCS form a regulatory network. BceAB-type transporters are often required for their own regulation by acting as the actual sensors of antimicrobial peptides that somehow communicate with the TCS to trigger activation of the signaling cascade (11, 17). In such cases, expression of the transporter operon is abolished in strains carrying transporter deletions (11, 12, 32). In some bacteria, exemplified by *Staphylococcus aureus*, two separate transporters exist, where one acts as the sensor, while the second is responsible for resistance (17, 32). We therefore wanted to investigate the role of the two enterococcal transporters in regulation of their own promoters. Additionally, the TCS EF0926-0927 had so far only been implicated in regulation of the transporters based on sequence predictions, which had to be validated experimentally.

Deletion of the sensor kinase EF0927 drastically reduced the expression levels of P_{EF2050}-*lacZ* to less than 20% of wild-type activities (Fig. 2B, left). Interestingly, a significant induction by bacitracin could still be observed, which was again likely due to the presence of the response regulator as described below. Expression of P_{EF2752}-*lacZ* was also reduced by deletion of the sensor kinase, but due to the overall low activities of this reporter, the differences were less pronounced. Nevertheless, the significant induction by bacitracin observed in the wild type was lost in the EF0927 deletion strain (Fig. 2B, right). These data show that the TCS EF0926-0927 indeed acts as the regulator for both transporter operons.

Deletion of the transporter EF2050-2049 had no effect on expression of either transporter (Fig. 2B), indicating that this transporter's role was restricted to mediating resistance. In contrast, deletion of EF2752-2751 severely reduced the activities of both *lacZ* fusions, and bacitracin-dependent induction was lost (Fig. 2B). Thus, the second transporter represents the antibiotic sensor of the resistance network.

In *B. subtilis*, expression of BceRS, the TCS regulating *bceAB* expression, is not induced by bacitracin (33). However, the homologous system BraRS from *S. aureus* (referred to as NsaRS in reference 34) was shown to be upregulated in response to nisin, one of its substrate peptides (34). To test if EF0926-0927 was inducible by bacitracin, we constructed a transcriptional fusion of the promoter P_{EF0926} to *lacZ* and introduced it into *E. faecalis* JH2-2 and derived deletion strains. Exposure of exponentially growing cells to bacitracin resulted in a strong upregulation of promoter activities from 1 to 16 MU (Fig. 3). Interestingly, deletion of neither the TCS nor the two transporters affected promoter activities or bacitracin-dependent induction (Fig. 3), showing that regulation of the TCS is mediated by an as-yet-unidentified additional regulator and not due to autoregulation. Importantly, this regulation by a factor external to the resistance network may offer an explanation for the mild phenotypes of the EF0927 deletion strain compared to the transporter mutants: in the ΔEF0927 background, expression of the response regulator gene EF0926 is still upregulated in the presence of bacitracin. Because in the absence of their cognate sensor kinase many response regulators can be efficiently phosphorylated and thus activated by small-molecule phospho-donors such as acetyl-phosphate (35, 36), this increased production of EF0926 may indirectly lead to an induction of its target promoters by bacitracin.

EF2050-2049 can mediate bacitracin resistance in *B. subtilis*. Because of the mild effects of the gene deletions generated in *E. faecalis*, we next attempted to transfer parts of the identified resistance network to *B. subtilis* to confirm the individual roles of the components in bacitracin resistance. As a chassis, we employed a strain of *B. subtilis* W168 carrying unmarked deletions of all three endogenous Bce-like modules (*bceRS-bceAB*, *psdRS-psdAB*, and *yxdJK-yxdLM-yxeA*). This strain, TMB1518, has been developed and validated as an appropriate platform to investigate resistance mechanisms against inhibitors of cell wall synthesis and the associated regulatory pathways from *E. faecalis* in a genetically highly accessible set-up (a detailed description will be published elsewhere). Expression of the EF2050-2049 operon under the control of a xylose-inducible promoter increased the MIC for bacitracin

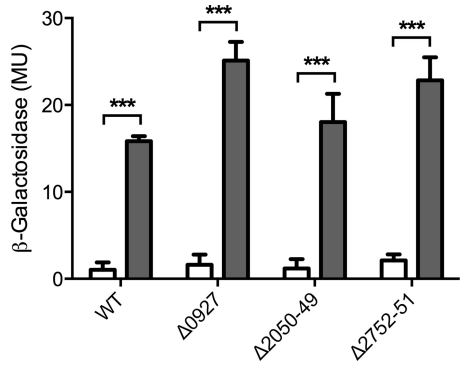

FIG 3 Induction of the two-component system operon by bacitracin. The promoter region of the two-component system operon EF0926-0927 was fused to *lacZ* and introduced into wild-type *E. faecalis* JH2-2 (WT) and mutant backgrounds. The resulting strains were assayed for β-galactosidase activity, expressed in Miller units (MU), after 1 h exposure of exponentially growing cultures to 0 μg ml^{-1} (white bars) or 4 μg ml^{-1} (gray bars) bacitracin. The genes deleted in each strain are indicated by locus tags below the bars. Results are means plus standard deviations for three biological replicates. The significance of induction and strain differences was calculated across the entire data set by two-way analysis of variance (ANOVA). Significant effects of bacitracin compared to uninduced cells are indicated by three asterisks ($P < 0.001$); the different mutant backgrounds caused significant differences between strains ($P = 0.018$; not depicted in the graph).

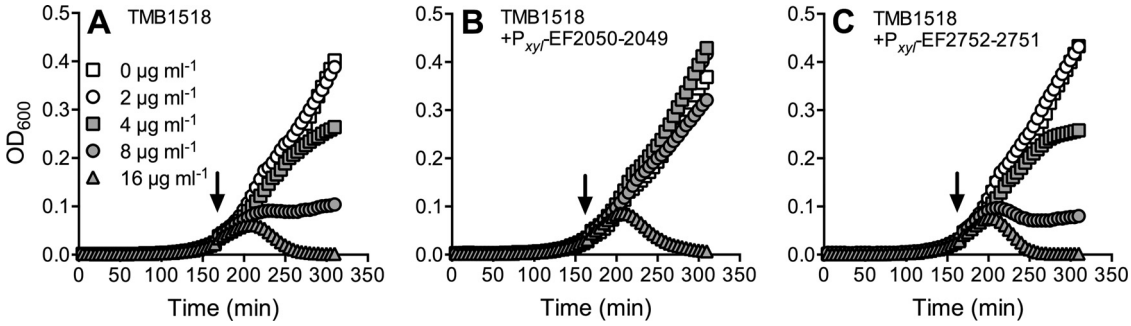

FIG 4 Transfer of bacitracin resistance to *B. subtilis*. Strain TMB1518 (A) and derived strains carrying expression constructs of the transporter operon EF2050-2049 (B) or EF2752-2751 (C) were grown to exponential phase and challenged with bacitracin, and growth was monitored as optical density (OD_{600}). The time point of bacitracin addition is indicated by the arrow; concentrations are given in panel A. Representative results of two or three independent experiments are shown. Experiments were carried out in a 100-μl culture volume in 96-well plates; thus, OD values cannot be directly compared to measurements made in cuvettes with a 1-cm light path length.

of TMB1518 from 2 μg ml^{-1} to 4 to 8 μg ml^{-1} (from 1 to 1.5 μg ml^{-1} to 3 μg ml^{-1} when determined by Etest assays), while expression of EF2752-2751 was unable to confer any resistance. As observed before for *E. faecalis*, the differences in MIC were again small. We therefore chose a different approach, assaying for growth inhibition of exponentially growing cultures that were challenged with increasing antibiotic concentrations, which can provide a more sensitive assay for cell wall-active antibiotics. *B. subtilis* strain TMB1518 as well as its derivative carrying the EF2752-2751 expression construct were unaffected by 2 μg ml^{-1} bacitracin, while 4 μg ml^{-1} or 8 μg ml^{-1} increasingly inhibited growth (Fig. 4A and C). In contrast, the strain harboring the expression construct for EF2050-2049 was not affected by concentrations up to 4 μg ml^{-1} and showed only slight growth inhibition at 8 μg ml^{-1} (Fig. 4B). Exposure to 16 μg ml^{-1} caused cell lysis in all strains tested (Fig. 4). These data confirm that EF2050-2049 is indeed directly capable of mediating bacitracin resistance, not only in *E. faecalis* but also in the heterologous host *B. subtilis*. EF2752-2751, on the other hand, is not directly responsible for bacitracin resistance.

EF2752-2751 and the TCS are sufficient for promoter induction in *B. subtilis*. Following the successful transfer of bacitracin resistance to *B. subtilis*, we next wanted to test if the regulatory pathway could also be reconstituted in the heterologous host. To this end, both target promoters, P_{EF2050} and P_{EF2752}, were fused to the bacterial luciferase operon *luxABCDE* as a reporter (22) and introduced into TMB1518. Both constructs resulted in basal luciferase activities that were not affected by addition of bacitracin (Fig. 5, leftmost panels). Thus, no endogenous *B. subtilis* system was able to induce either of the promoters. Next, the TCS operon EF0926-0927 was introduced into the reporter strains, controlled by the promoter of the homologous *bceRS* operon of *B. subtilis* to ensure appropriate expression levels. The presence of the TCS alone did not alter the activities of the transporter promoters, and bacitracin-dependent induction was still not observed (Fig. 5, left center panels), confirming that the TCS alone is unable to respond to the peptide, as has been shown for other BceRS-like systems (11, 12, 32). Additional introduction of the expression construct for EF2050-2049 also did not change the promoter activities (Fig. 5, right center panels), showing that the encoded transporter has no sensory function. In contrast, simultaneous presence of the TCS and the transporter EF2752-2751 increased the basal activities of the P_{EF2050}-*lux* reporter, and addition of bacitracin resulted in a further 2-fold induction (Fig. 5, top rightmost panel). Expression of P_{EF2752}-*lux* was not altered in the same genetic background (Fig. 5, bottom rightmost panel), consistent with the minor induction observed for this promoter in *E. faecalis*. These results clearly show that together, the transporter EF2752-2751 and the TCS EF0926-0927 constitute the sensory and regulatory component of the resistance network and that the second transporter operon, EF2050-2049, is their main target.

DISCUSSION

Our proteomic analysis of bacitracin-exposed *E. faecalis* showed that the bacterium responds to the antibiotic with a specific reaction to the bacitracin-induced cell wall damage, as well as with a more general response to stress or growth inhibition. Overall, our data are largely consistent with the findings of a recent transcriptome analysis of *E. faecalis* treated with different inhibitors of cell wall synthesis, including bacitracin and vancomycin (8). A detailed comparison of our study and the previous one is presented in the supplemental text. Together, these two data sets provide a useful overview of the response of *E. faecalis* to inhibitors of cell wall synthesis such as bacitracin.

Among the differentially expressed genes identified from both studies was a putative ABC transporter, EF2050-2049, which is a homologue of the bacitracin resistance transporter BceAB of *B. subtilis* (11, 33). A second such transporter, EF2752-2751, had been previously identified in *E. faecalis* by a comparative genomics analysis of BceAB-type transporters in Firmicutes bacteria (16). While this second transporter was not found in our proteomic analysis, its ATP-binding cassette domain-encoding gene, EF2752, was slightly induced by bacitracin during the transcriptome study (8). Additionally, we could identify a TCS of *E. faecalis* with similarity to BceRS of *B. subtilis*, which regulates expression of BceAB (33). Again, the encoding genes EF0926-0927 had been reported as bacitracin inducible and were also found to be induced by the cell wall-active antibiotics cephalothin and vancomycin (8). Our subsequent characterization of these three gene loci in *E. faecalis* as well as heterologously in *B. subtilis* showed that they act together and form a resistance network against bacitracin. A schematic of the derived model is shown in Fig. 6.

The primary sensor of the network is the transporter EF2752-2751, which communicates the presence of bacitracin to the sen-

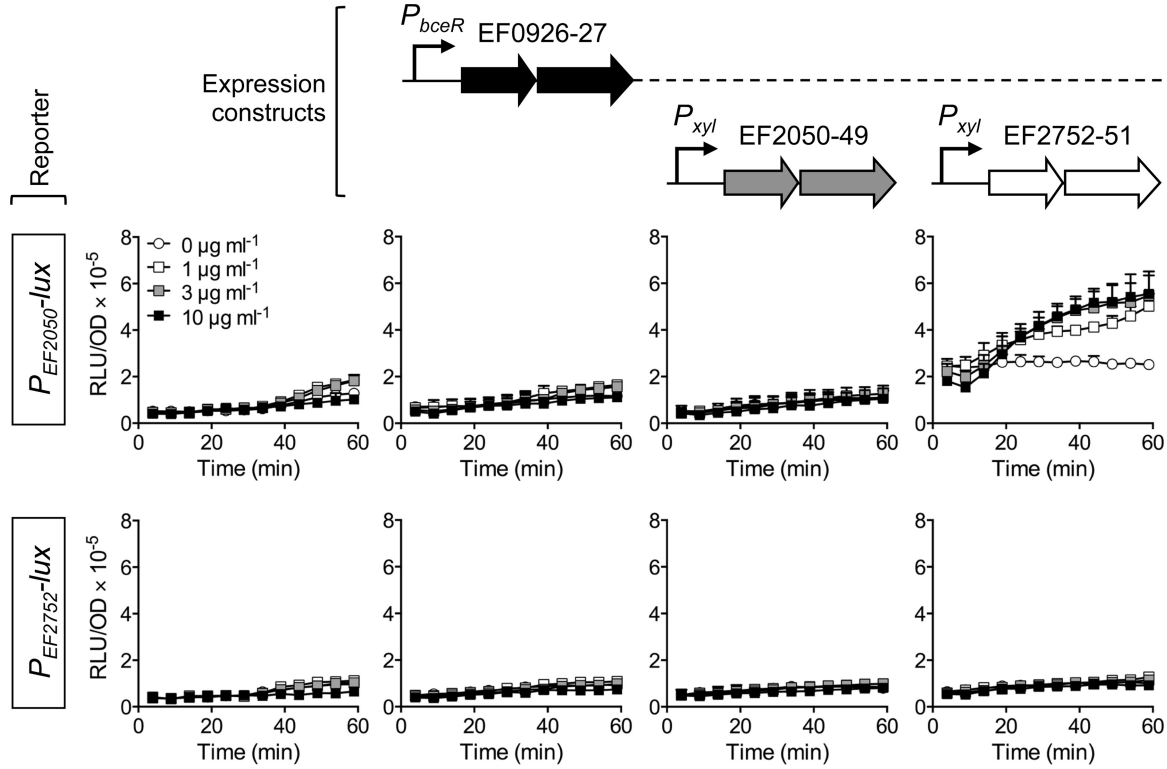

FIG 5 Functional reconstitution of the regulatory circuit in *B. subtilis*. Promoter regions of the transporter operons EF2050-2049 (top graphs) and EF2752-2751 (bottom graphs) were fused to *luxABCDE* and introduced into *B. subtilis* TMB1518. Additionally, the two-component system and transporter operons were introduced under the control of a constitutive (P_{bceR}) or xylose-inducible (P_{xyl}) promoter. The expression constructs present in each strain are illustrated above the graphs, using the same shading as in Fig. 1. Exponentially growing cultures were exposed to different concentrations of bacitracin, given in the top leftmost graph, and luminescence normalized to optical density (RLU/OD) was monitored over 60 min. Results are means plus standard deviations for two or three biological replicates.

sor kinase EF0927. Activation of the sensor kinase and phosphotransfer to the response regulator EF0926 then leads to activation of the main target promoter, P_{EF2050}, and increased production of the transporter EF2050-2049. This transporter then removes the bacitracin from its site of action, thus ensuring resistance. Simultaneously, expression of the sensory transporter is slightly induced by the TCS, while expression of the TCS operon is induced by an as-yet-unidentified regulator that is not directly part of the resistance network. Upregulation of a BceRS-like TCS has so far been reported only for the BraRS (= NsaRS) system of *S. aureus* (34) and may lead to an increased sensitivity or stronger induction of the resistance transporter. Future studies will be directed at identification of the regulator for the TCS. Because the transcriptome study showed the TCS to be inducible by three of four tested inhibitors of cell wall biosynthesis (8), it appears likely that this regulation is part of the cell envelope stress response of *E. faecalis*. A number of candidate regulatory systems have been identified by comparative genomics and provide a good starting point for subsequent investigations (37).

Several BceRS-BceAB-type resistance modules have been characterized in detail and were always shown to be involved in peptide antibiotic resistance (13, 17). Importantly, the TCSs always rely on one of the transporters for stimulus perception and are unable to induce their target genes in the absence of their transporter (11, 12, 31, 32, 38). In most cases the transporter and TCS are encoded in adjacent operons (15, 16), but in *S. aureus* and *Lactobacillus casei*, some TCSs were shown to regulate the expression of a second transporter encoded elsewhere on the chromosome (31, 32). The situation in *E. faecalis* as identified in the present study is even more complex, with not only the target transporter but also the sensory transporter being encoded in a different locus from the TCS. To our knowledge, this is the first report where a regulatory interaction between a BceAB-like transporter and BceRS-like TCS was shown for two systems not encoded together. Our findings further emphasize the widespread occurrence of these resistance modules and show that the regulatory paradigm is conserved even if genomic arrangement is not.

As mentioned in the introduction, *E. faecalis* is likely exposed to a range of antimicrobial peptides in the gastrointestinal tract of humans and animals, which can be of host origin or produced by other bacteria of the gut microflora. This raises a question regarding the physiological substrate of the resistance network described here. Most Bce-like modules analyzed so far are not specific for a single substrate but instead recognize a range of often structurally diverse peptides (17). In *S. aureus*, the human beta-defensin hBD3 and cathelicidin LL-37 have been identified as substrates of the ApsRS-VraFG module (39), showing that the function of Bce-like modules is not restricted to bacterially derived antimicrobial peptides. Our initial screening experiments identified the lantibiotic mersacidin as a second inducer of both enterococcal transporters, and it is possible or even likely that other substrates exist. Particularly for a gut bacterium like *E. faecalis*, it will be interesting to

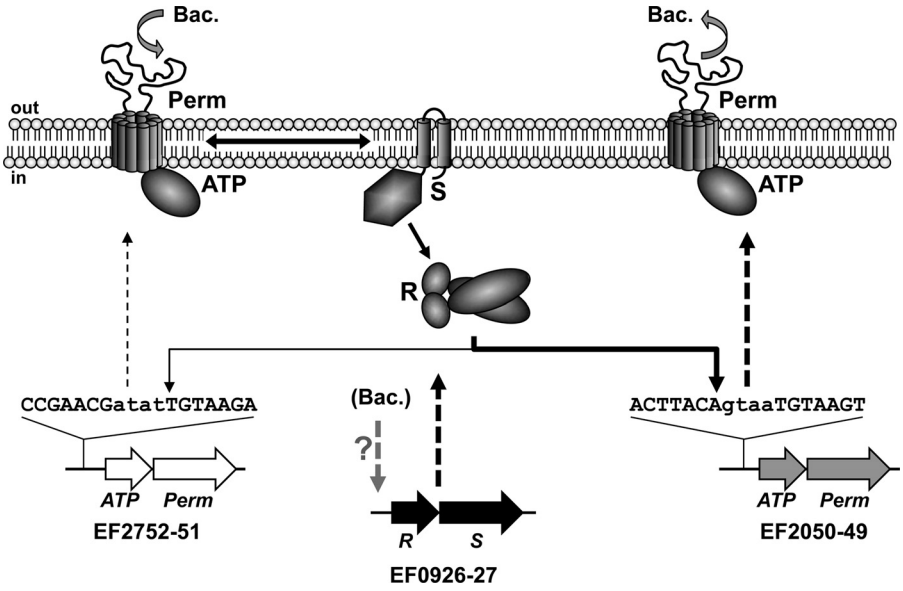

FIG 6 Model of the bacitracin resistance network in *E. faecalis*. Schematic illustrations of the involved genes and proteins are shown and the sequences of regulator binding sites are given. Bacitracin is detected by EF2752-2751, indicated by the curved downward arrow. Communication between the transporter and two-component system is shown by a double arrow, and phospho-transfer as well as target promoter activation is shown by single arrows. Increased expression of operons is shown by dashed arrows. The differences in the strengths of induction are reflected by thickness of lines. Bacitracin resistance mediated by EF2050-2049 is indicated by the curved upward arrow. Bacitracin-dependent induction of the two-component system operon by an unknown mechanism is shown as a dashed arrow with a question mark. ATP, ATPase; Perm, permease; R, response regulator; S, sensor kinase; Bac., bacitracin.

test if any human antimicrobial peptides can induce expression of the transporters identified here and if the resistance network imparts a selective advantage to the bacterium in the intestinal environment.

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D.J.L., M.R.W., and F.K. characterized the resistance network in *E. faecalis*; C.F. performed all work with *B. subtilis*; A.S. and A.C. performed the proteomic analysis; S.G. and G.M.C. designed the study and coordinated experimental work; S.G. wrote the manuscript.

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Chapter IV

Insulation and Wiring Specificity Determinants of BceR-like Response Regulators and their Target Promoters in *Bacillus subtilis*

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Manuscript

Insulation and Wiring Specificity Determinants of BceR-like Response Regulators and their Target Promoters in *Bacillus subtilis*

Summary

BceRS and PsdRS are paralogous two-component systems in *Bacillus subtilis*. In response to the extracellular presence of bacitracin and nisin, respectively, the two response regulators (RRs) bind their target promoters, P_{bceA} and P_{psdA}, resulting in a strong up-regulation of target gene expression and ultimately antibiotic resistance. Despite high sequence similarity between BceR and PsdR and their known binding sites within P_{bceA} and P_{psdA}, no cross-regulation has been observed between them. We therefore investigated the specificity determinants of P_{bceA} and P_{psdA} that ensure the insulation of these two paralogous pathways at the RR/promoter interface. *In vivo* and *in vitro* analyses demonstrate that the regulatory regions within these two promoters contain three important elements: in addition to the known (main) binding site, we identified a linker region and a secondary binding site to be crucial for functionality. The high affinity main binding site enables a tight but rather non-specific binding of BceR-like RRs to their target promoters. This initial (primary) binding then allows a highly specific interaction with the low affinity secondary binding site. This second binding event is further modulated by the linker region to determine binding specificity and thereby ensure the regulatory insulation between closely related Bce-like systems.

Introduction

Antimicrobial peptides (AMPs) are majorly produced by Gram-positive microbes to suppress the growth of competitors in their habitat environment (Berdy, 2005). The main target of AMPs is the bacterial cell envelope, especially different substrates of the lipid II cycle. By binding to their target molecules, AMPs inhibit cell wall biosynthesis and cause cell death (Silver, 2003, Breukink & de Kruijff, 2006, Jordan *et al.*, 2008).

In Firmicutes bacteria, sensing of and resistance against AMPs is usually mediated by highly conserved Bce-like detoxification modules containing an ATP-binding cassette (ABC) transporter and a two-component system (TCS) (Coumes-Florens *et al.*, 2011, Dintner *et al.*, 2011). The genome of *Bacillus subtilis* encodes three such systems: BceRS-BceAB, PsdRS-PsdAB and YxdJK-YxdLM-YxeA, of which the last is poorly understood (Joseph *et al.*, 2002, Coumes-Florens *et al.*, 2011, Gebhard & Mascher, 2011). BceRS-BceAB system is the best understood system and responds to AMPs such as bacitracin, actagardine and mersacidin (Bernard *et al.*, 2003, Staroń *et al.*, 2011). It consists of two separate operons: the *bceRS* operon encodes the TCS comprised of a membrane anchored histidine kinase (HK), BceS, and a cytoplasmic response regulator (RR), BceR, under the control of a constitutive promoter. The *bceAB* operon encodes the ABC transporter under the control of an inducible BceR dependent promoter, P_{bceA}. In the absence of AMPs, both operons are expressed at a very low level. In the presence of AMPs such as bacitracin, the ABC transporter BceAB senses this stimulus and passes the signal on to the HK BceS (Joseph *et al.*, 2002, Ohki *et al.*, 2003, Gebhard, 2012, Dintner *et al.*, 2014). Upon autophosphorylation, BceS then triggers the signal transduction to its cognate RR BceR by phosphoryl group transfer. Further, the phosphorylated BceR will then bind to P_{bceA} and strongly induce *bceAB* transcription, ultimately results in increased BceAB production, thereby conferring AMP resistance (Mascher *et al.*, 2003, Ohki *et al.*, 2003, Bernard *et al.*, 2007) (Fig. 4.1 black system, BceAB not shown). The main inducers of the Psd system are lipid II-binding lantibiotics such as nisin, actagardine, gallidermin and subtilin. In turn, the Psd system confers resistance against nisin, actagardine and subtilin (Staroń *et al.*, 2011). The signal transduction pathway within Psd system (Fig. 4.1 grey system, PsdAB not shown) is similar to that described for the Bce system (Gebhard & Mascher, 2011). Despite significant sequence similarity between BceRS-BceAB and PsdRS-PsdAB, the signal transduction of both systems is generally well insulated from each other. Only some degree of cross-phosphorylation between BceS and PsdR at high concentrations of bacitracin was observed in a previous study (Rietkötter *et al.*, 2008) (Fig. 4.1 dotted arrow).

RRs usually contain an N-terminal receiver domain and a C-terminal output domain. The receiver domain represents the interaction interface to the corresponding HK and catalyzes the phosphorylation reaction at an invariant Asp residue (Bourret, 2010). The nature of the output domains can be considerably diverse and range from DNA binding, RNA binding to protein binding or enzymatic activity (Galperin, 2006, Gao & Stock, 2009). In *B. subtilis*, the Bce-like RRs belong to OmpR/PhoB

subfamily with a winged helix-turn-helix DNA-binding output domain that regulates the transcription of target genes by binding to their corresponding promoter regions via a specific recognition motif (Martínez-Hackert & Stock, 1997, Fabret *et al.*, 1999, Galperin, 2010).

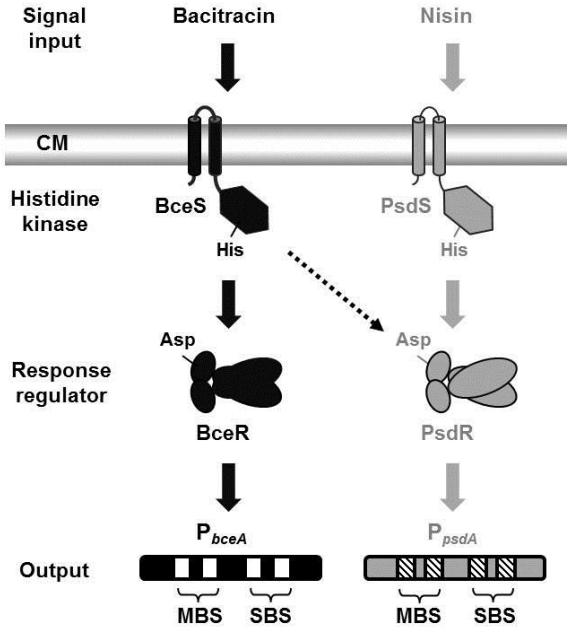

Figure 4.1. Model of signal transduction pathways of two Bce-like systems after induction with corresponding AMPs in *Bacillus subtilis*. Bce and Psd two component systems including their major antibiotics as signal input are coloured with black and grey, respectively. Schematic illustration of proteins and promoters together with their names are given. For the reason of simplicity, signal detectors — ABC transporters of both systems are not shown in the model. Solid arrows indicate the signal transduction pathway within one system, while cross-regulation between BceS and PsdR is pointed out by dotted arrow. On each promoter, MBS representing for the main binding site and SBS representing for the secondary binding of Bce-like RRs are filled with white on *bceA* promoter and slashes on *psdA* promoter. CM, cell membrane.

In bacteria, transcription initiation starts with promoter recognition by σ subunit of the RNA polymerase holo enzyme at the -35 promoter element, followed by discerning and unwinding the DNA double helix at the -10 promoter element (Lee *et al.*, 2012). In *B. subtilis*, a -10 promoter element with a perfect TATAAT sequence for σ^A could be identified in P_{bceA}. It is located 6 bp upstream of the transcription initiation site, which is 32 bp upstream of the *bceA* start codon. However, a conserved -35 element was not found (Ohki *et al.*, 2003). An identical σ^A-dependent -10 element was also found in P_{psdA}, again lacking a clear -35 element at the appropriate position (Staroń *et al.*, 2011) (Fig. 4.2A). For promoters lacking a -35 element or deviating significantly from the consensus sequence at the appropriate position, σ subunit can still be recruited to these promoters by interaction with activators like RRs binding on the upstream region (Jarmer *et al.*, 2001, Paget & Helmann, 2003). In *B. subtilis*, an inverted BceR main binding site on P_{bceA} as well as PsdR main binding site on P_{psdA} were mapped upstream of the -10 promoter elements (Fig. 4.2A), which implies a cooperation between BceR-like RRs and the RNA polymerase holo enzyme (Ohki *et al.*, 2003, de Been *et al.*, 2008, Staroń *et al.*, 2011).

BceR and PsdR share 40% sequence identity and the corresponding main binding sites on P_{bceA} and P_{psdA} contain eleven identical nucleotides out of fourteen (Joseph *et al.*, 2002). Because of these similarities, a high potential for cross-talk was predicted between these two systems by scoring function of direct-coupling analysis (Procaccini *et al.*, 2011). However, no cross-regulation was detected on the transcriptional level between BceR-P_{psdA} and PsdR-P_{bceA} (Rietkötter *et al.*, 2008). Such

a regulatory insulation, that is, prevention of nonspecific regulatory cross-talk is of course desired and can arise at different molecular levels *in vivo* (Huynh & Stewart, 2011). The most prominent mechanism for conferring such signaling specificity depends on the molecular recognition between the two interaction partners (Podgornaia & Laub, 2013).

Here we report new insights into the molecular mechanisms that ensure insulation and transcriptional regulation specificity between two Bce-like systems in *B. subtilis*, Bce and Psd at the level of RR/promoter interaction. Using both *in vivo* and *in vitro* approaches, we found out that in addition to the previously identified main binding site, a secondary RR-binding site exists in both P_{bceA} and P_{psdA}. We demonstrated that the main binding site, the secondary binding site as well as the linker region in between them all contribute to determine RR-specific transcription initiation.

Results

Identification of the minimal *bceA* and *psdA* promoter motif

P_{bceA} and P_{psdA} are the target promoters for BceR and PsdR, respectively (Staroń *et al.*, 2011). When *B. subtilis* is treated with bacitracin, BceR is activated by corresponding HK BceS and binds to a specific region of P_{bceA}, resulting in a strong transcriptional upregulation of the operon encoding the ABC transporter for resistance (Mascher *et al.*, 2003) (Fig. 4.1). Previous work has already mapped an inverted repeat sequence with 4 nt spacing in the P_{bceA} region (AAGCgTGTGACgaaaatGTCACAtGCTT) from -111 to -84 upstream of the *bceA* start codon for BceR binding (Ohki *et al.*, 2003). For P_{psdA}, a highly homologous PsdR binding site (ATGTGACAgcatTGTAAGAT) could be identified from -99 to -80 upstream of the *psdA* start codon (Staroń *et al.*, 2011). In agreement with these studies, a putative binding site was identified among most *bceA*-like promoters in Firmicutes bacteria, with an overall consensus sequence TNACA-N4-TGTAA for BceR-like RRs (Dintner *et al.*, 2011). First, we wanted to verify that these two known conserved binding motifs are indeed indispensable for the RR-dependent *bceA* and *psdA* promoter activity and subsequently identify the minimal regulatory elements for both promoter regions. Towards that goal, progressively truncated *bceA* promoters starting with 5'-position ranging from -111 to -103 and ending at +82 relative to the ATG start codon of *bceA* were cloned into pAC6, thereby generating transcriptional *lacZ* reporter fusions (Table 4.2), which were integrated at the *amyE* locus in *B. subtilis* wild-type (WT) 168 (Table 4.1). Progressively truncated *psdA* promoter fragments starting with 5'-positions ranging from -110 to -95 all ending at position +30 relative to the ATG start codon of *psdA* were constructed in a similar fashion (Fig. 4.2A). The promoter activity of the resulting reporter strains was determined by quantitative β-galactosidase assay as described in experimental procedure in the presence of bacitracin (Bce system) or nisin (Psd system) (Staroń *et al.*, 2011).

Truncated *bceA* promoters from -111 until -106 showed almost WT promoter (using a promoter fragment starting at position -122 as a positive control) activity after bacitracin induction (black bars) compared to corresponding non-induced samples (white bars) (Fig. 4.2B). Truncations starting at

position -105 and position -104 displayed a decreased promoter activity, while a further truncation of one additional nucleotide (starting at position -103) led to a complete loss of promoter activity after bacitracin induction (Fig. 4.2B).

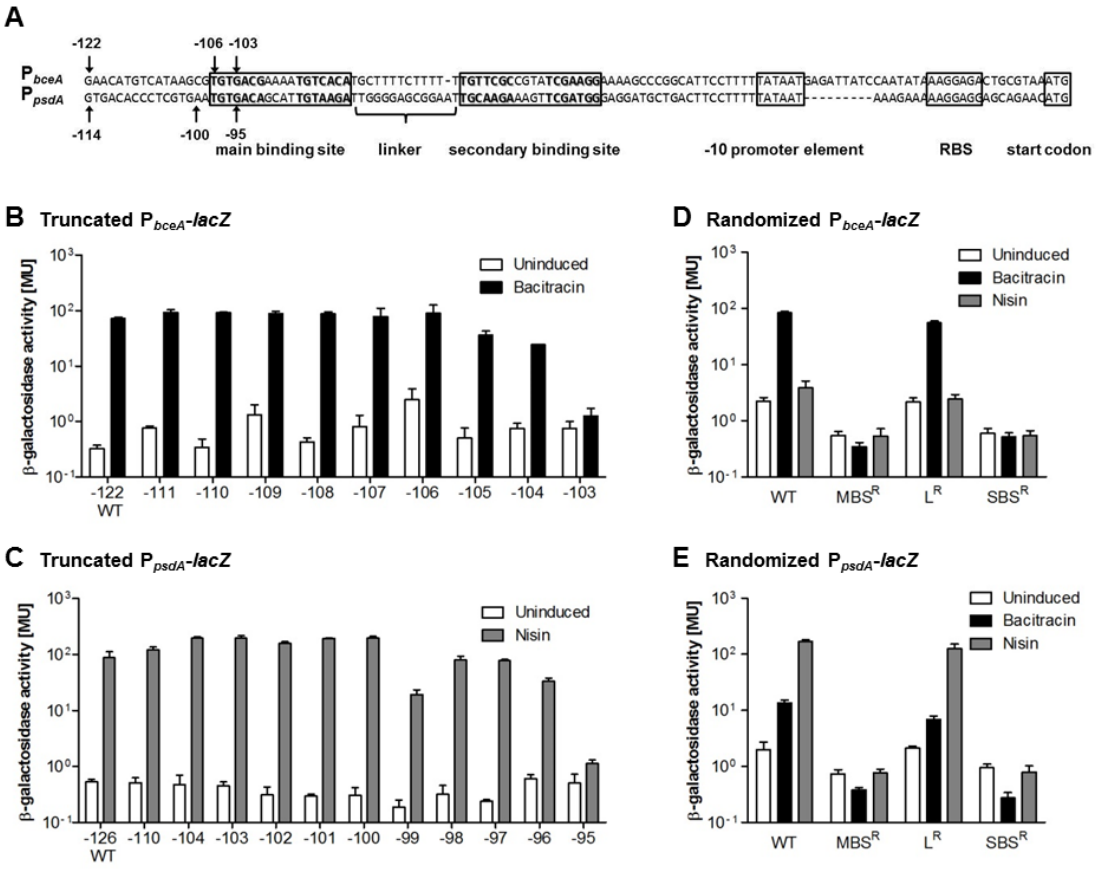

Figure 4.2. Functional analysis of *bceA* and *psdA* promoters of *B. subtilis*. (A) DNA sequence alignment of the *bceA* promoter and the *psdA* promoter. Different motifs are framed and annotated underneath the DNA sequence. Special positions on each promoter according to the start codon of corresponding regulated gene are marked with arrows. Half binding sites of Bce-like RRs on each promoter are emphasized in bold style. Activities of (B) truncated constructions of the *bceA* promoter (from -122: +82 to -103: +82) and (C) truncated constructions of the *psdA* promoter (from -126: +30 to -95: +30) according to the start codon of regulated genes. Activities of (D) P_{bceA} mutants and (E) P_{psdA} mutants with MBS^R (main binding site random mutation), L^R (linker random mutation) and SBS^R (secondary binding site random mutation) are compared with the corresponding WT promoters. All promoter constructions were fused to *lacZ* and introduced into *amyE* locus of *B. subtilis* 168. Cultures growing exponentially in LB were challenged with Zn^{2+}-bacitracin 30 µg ml^{-1} (black bars) or nisin 2 µg ml^{-1} (grey bars) for 30 min, comparing with the non-induced condition (white bars). β-galactosidase activities are expressed in Miller Units (MU) (Miller, 1972) and results are shown as the mean plus standard deviation of three biological replicates. A log scale is applied for reasons of clarity.

Similar results were obtained for truncated *psdA* promoter fragments after nisin induction (grey bars) (Fig. 4.2C). Compared to the WT *psdA* promoter fragment (starting at position -126), no decrease of promoter activity was observed for truncations with 5'-positions starting from - 110 to -100. The promoter activities were significantly reduced for fragments chopping at position -99 to -96, while a truncation at position -95 completely lost its activity after nisin induction.

Our data confirms that the 7-4-7 nt binding motif TGTGACGaaaaTGTCACA of P_{bceA} and the TGTGACAgcatTGTAAGA binding motif of P_{psdA} are indeed necessary binding sites (main binding

site) for BceR and PsdR. Position -104 relative to *bceA* start codon and position -96 relative to *psdA* start codon determine the minimal 5'-end of active RR-dependent promoter fragments. Next, we addressed the question if there are other binding sites derived from these binding motifs on the promoter that are also sufficient for RR-dependent promoter activation.

A secondary binding site on *bceA* and *psdA* promoters

Sequence analysis of P_{bceA} and P_{psdA} did not identify a typical -35 region (TTGACA) 17 nt upstream of the -10 region as normally recognized by σ^A (Jarmer *et al.*, 2001). However, a 7 nt conserved half binding site located 13/14 nt downstream of the main binding site (MBS) and 38 nt upstream of the -10 region was predicted for both the *bceA* and the *psdA* promoter regions (Dintner *et al.*, 2011). This obsession implies the existence of a secondary binding site (SBS) instead of a typical -35 on *bceA*-like promoters. Based on this prediction, we annotated a putative 7-4-7 nt secondary binding site and a linker region (L) between the main and the secondary binding sites on both *bceA* and *psdA* promoters (Fig. 4.2A) and experimentally investigated the function of the predicted promoter motifs by mutating each of them into a random sequence (randomization). The GC/AT content of the linker region was kept during the randomization. These mutants were cloned into pAC6 generating transcriptional *lacZ* reporter fusions (Table 4.2) and integrated into the chromosome of *B. subtilis* WT 168 at the *amyE* locus (Table 4.1). The promoter activity was determined as described above.

Both the WT *bceA* promoter (Fig. 4.2D) and the *psdA* promoter (Fig. 4.2E) showed strong induction with the corresponding inducers: bacitracin (black bars) or nisin (grey bars), compared to the non-induced samples (white bars). The weak induction of P_{psdA} by bacitracin (Fig. 4.2E) was due to the known cross-phosphorylation of PsdR by BceS (Rietkötter *et al.*, 2008) (Fig. 4.1 dotted arrow). Mutating the main binding site (MBS) into a random sequence led to a complete loss of activity for both promoters. The same effect was obtained when randomizing the sequence of the predicted secondary binding site (Fig. 4.2D and 4.2E). However, activities of both *bceA* and *psdA* promoters only showed a slight decrease by randomly mutating the corresponding linker region (L) between the two binding sites but keeping the same GC/AT content.

The data demonstrates that on both P_{bceA} and P_{psdA}, there is a secondary binding site located downstream of the main binding site with a 13/14 nt linker region in between them. This secondary binding site seemingly replaced the -35 promoter element, and it is as indispensable as the main binding site for RR-dependent promoter activity. Additional assays done by randomizing either the first or the second half of each secondary binding site were in consistency with the results obtained from the completely randomized secondary binding sites (data not shown), further demonstrating that each half binding site has the same importance for P_{bceA} and P_{psdA} activity.

Major specificity determinants are located in the region containing the linker and the secondary binding site

So far, we have identified an extended regulatory region in P_{bceA} and P_{psA}, consisting of two binding sites and a linker region in between. Since there is no cross-regulation at the RR/promoter interface, either between BceR-P_{psdA} or PsdR-P_{bceA} (Rietkötter *et al.*, 2008), further we wanted to analyse the specificity determinants within *bceA*/*psdA* promoters. Towards that aim, a series of chimeric promoters derived from P_{bceA} and P_{psdA} were constructed (Table 4.2) and fused with *lacZ*. Chimeric promoters BP1-4 are derived from P_{bceA} (black) with gradually substituting P_{psdA} (grey) at the 3'-terminal end (Fig. 4.3A). Chimeric promoters PB1-4 are derived from P_{psdA} (grey) with increasing of 3'-fragments from P_{bceA} (black) (Fig. 4.3B). Again, these constructs were integrated into the *amyE* locus of the *B. subtilis* WT 168 (Table 4.1). To specifically eliminate any cross-talk between the Bce and Psd systems, these chimeric promoters, WT P_{bceA} and WT P_{psdA} fragments as references, were transformed into the Δ*bceRS* strain (TMB1460) and the Δ*psdRS* strain (TMB1462) (Table 4.1). Compared to the WT strain, the Δ*bceRS* and the Δ*psdRS* strains remove the effect of cross-phosphorylation and hence provide a clearer view of RR/promoter specificity.

P_{bceA} showed the same high activity in the Δ*psdRS* mutant (Fig. 4.3D) as in WT strain (Fig. 4.3C) after bacitracin induction, but no activity after nisin induction in either the WT (Fig. 4.3C) or the Δ*bceRS* background (Fig. 4.3E). P_{psdA} was also highly induced by nisin in both the WT strain (Fig. 4.3C) and the Δ*bceRS* mutant (Fig. 4.3E). Importantly, the moderate induction of P_{psdA} by bacitracin in WT background (Fig. 4.3C) was not detected in Δ*psdRS* mutant (Fig. 4.3D) due to the elimination of cross-phosphorylation between BceS and PsdR. These results are in agreement with previous studies that there is no cross-regulation at the RR/promoter level.

Chimeric promoters BP1 and BP2 showed high activity after induction with bacitracin in both the WT strain (Fig. 4.3C) and the Δ*psdRS* strain (Fig. 4.3D), but no activity upon nisin induction in either the WT strain (Fig. 4.3C) or the Δ*bceRS* strain (Fig. 4.3E). Hence, BP2 could be recognized by BceR, but not by PsdR. These results indicate that specificity determinants are located within the region upstream of and including the secondary binding site. The chimeric promoter BP3, could not be induced either by bacitracin in the Δ*psdRS* background (Fig. 4.3D) or by nisin in the Δ*bceRS* background (Fig. 4.3E). However, it showed moderate activity in the WT background (Fig. 4.3C) after induction with bacitracin. Surprisingly, BP4, possessing the whole region downstream of the main binding site of P_{psdA}, was not only moderately induced by bacitracin in the Δ*psdRS* background (Fig. 4.3D) but also by nisin in the Δ*bceRS* background (Fig. 4.3E), indicating a change of specificity from P_{bceA} to P_{psdA}. These results of BP2 and BP4 demonstrate that major specificity determinants of P_{psdA} are located in the region containing the linker and the secondary binding site.

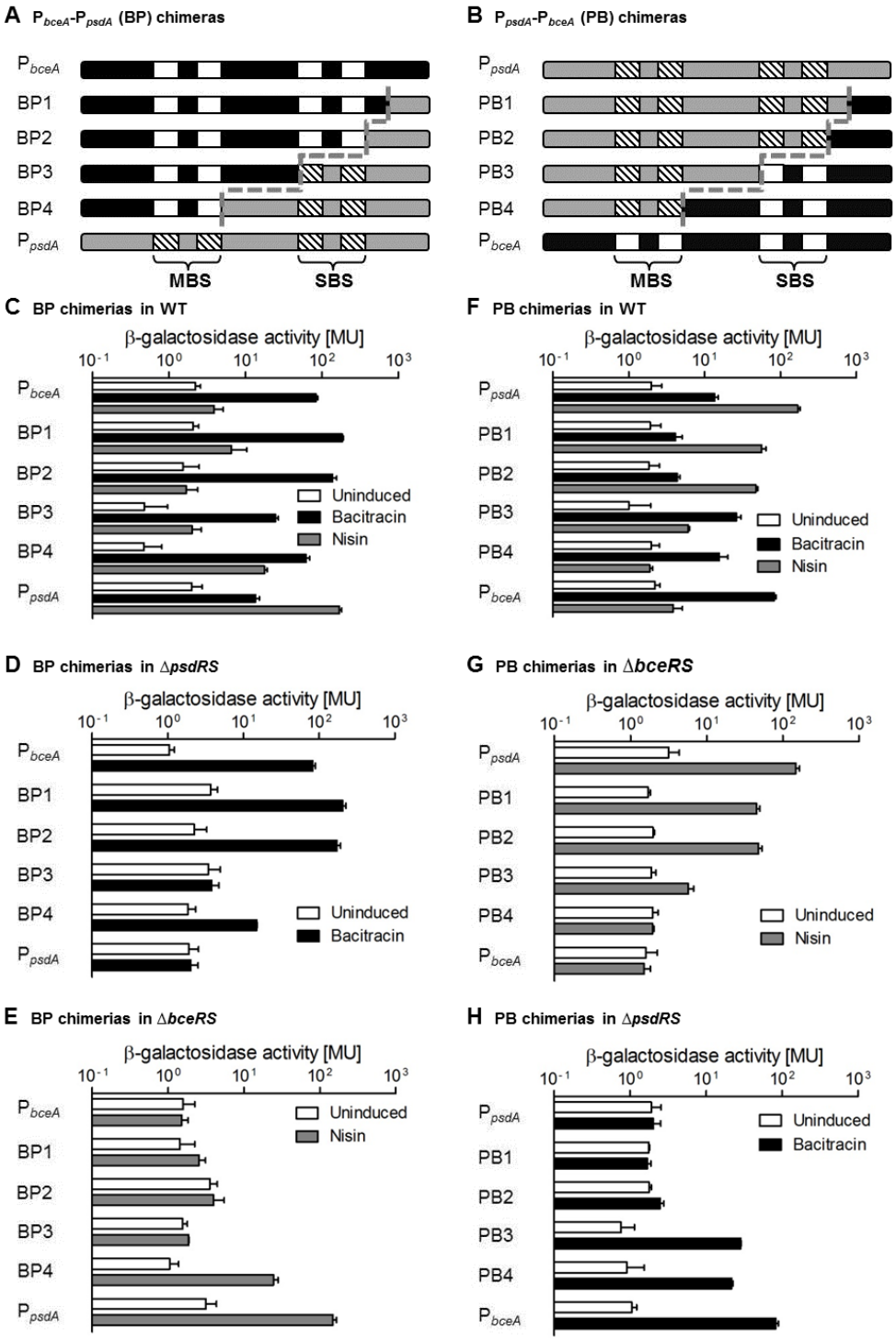

Figure 4.3. Functional studies of series of chimeric promoters. Schematic of series of chimeric promoters (A) BP1-4, *bceA* promoter fragments (black) with gradual substitutions of 3' region by increased corresponding parts of *psdA* promoter (grey), and (B) PB1-4 vice versa are compared with WT P_{bceA} and P_{psdA}. The MBS and the SBS from P_{bceA} and P_{psdA} are filled the same as in Fig. 4.1. Grey dashed lines indicate the concrete fusion boundaries of each chimera. (C to H) Activities of chimeric promoters compared with WT promoters in different genetic backgrounds of *B. subtilis*. Transcriptional *lacZ* fusions of WT promoters (P_{bceA} and P_{psdA}) as well as different sets of chimeras (BP1-4 and PB1-4) were integrated at *amyE* locus in *B. subtilis* WT (W168), Δ*psdRS* strain (TMB1462) and Δ*bceRS* strain (TMB1460). Promoter activities were measured as described in Fig. 4.2. Activities are shown as follows: (C) BP1-4 in WT, (D) BP1-4 in Δ*psdRS* strain, (E) BP1-4 Δ*bceRS* strain, (F) PB1-4 in WT, (G) PB1-4 in Δ*bceRS* strain and (H) PB1-4 in Δ*psdRS* strain. Black bars and grey bars represent for samples induced with bacitracin and nisin, while white bars stand for non-induced controls.

Chimeric promoters PB1 and PB2 showed a decreased activity after induction with nisin in both the WT background (Fig. 4.3F) and the Δ*bceRS* mutant (Fig. 4.3G) relative to P_{psdA}, and no bacitracin induction in the Δ*psdRS* mutant (Fig. 4.3H), indicating no change of specificity. These results corroborate that the region downstream of the secondary binding site on P_{bceA} is not relevant for the RR/promoter specificity. Interestingly, PB3 showed a significantly decreased activity in the Δ*bceRS* mutant with nisin induction (Fig. 4.3G) and a hugely increased activity in the Δ*psdRS* mutant with bacitracin induction (Fig. 4.3H). It therefore behaved differently from the corresponding BP3 construct that had no activity in either. Construct PB4 was not inducible by nisin in the Δ*bceRS* strain (Fig. 4.3G), but instead showed high induction by bacitracin in the Δ*psdRS* strain (Fig. 4.3H). The promoter activities of PB3 and PB4 in the WT strain (Fig. 4.3F) were in accordance with those observed in both mutant backgrounds. This data indicates that the change of specificity from P_{psdA} to P_{bceA} started at PB3 with the secondary binding site being switched into P_{bceA}, and obtained a further promotion in construct PB4 by an additional substitution of the linker region.

Taken together, the analysis of chimeric promoter constructs demonstrates that the region containing the linker and the secondary binding site of P_{bceA}/P_{psdA} includes major specificity determinants for BceR/PsdR recognition.

Rewiring specificity from P_{bceA} to P_{psdA} and dissecting the role of specificity determinants on P_{psdA}

The analysis of chimeric promoter constructs showed that on the *psdA* promoter the region downstream of the main binding site contained the major specificity determinants for PsdR binding. This region contains two motifs: the linker and the secondary binding site. To further elaborate the functionality of the main binding site, the linker region and the secondary binding site on *psdA* promoter for PsdR recognition, additional chimeric promoters were constructed with different combinations of these three motifs on P_{bceA} replaced by the corresponding region of P_{psdA} (Fig. 4.4A) to rewire specificity from P_{bceA} to P_{psdA}. Promoter activities were measured as described above in the WT strain (Fig. 4.4C), the Δ*psdRS* strain (Fig. 4.4D) and the Δ*bceRS* strain (Fig. 4.4E).

Compared to P_{bceA}, replacing only the main binding site (M), the linker (L) or both (M+L) of P_{bceA} with the corresponding region of P_{psdA} showed decreased promoter activity in the WT strain (Fig. 4.4C) as well as in the Δ*psdRS* mutant (Fig. 4.4D) after induction with bacitracin. In contrast, no increasing of the promoter activity was observed in either the WT strain (Fig. 4.4C) or the Δ*bceRS* mutant (Fig. 4.4E) after induction by nisin. This data indicates that the main binding site, the linker or both of P_{psdA} are not enough to determine specificity. Changing the secondary binding site (S) on P_{bceA} into P_{psdA} led to a decrease of promoter activity in the WT strain (Fig. 4.4C) as well as in the Δ*psdRS* mutant (Fig. 4.4D) after induction with bacitracin, and a slight but detectable increase of promoter activity in the Δ*bceRS* mutant (Fig. 4.4E) after induction with nisin. This data indicates that exchanging only the secondary binding site alone already conferred a change of promoter specificity from P_{bceA} to P_{psdA}.

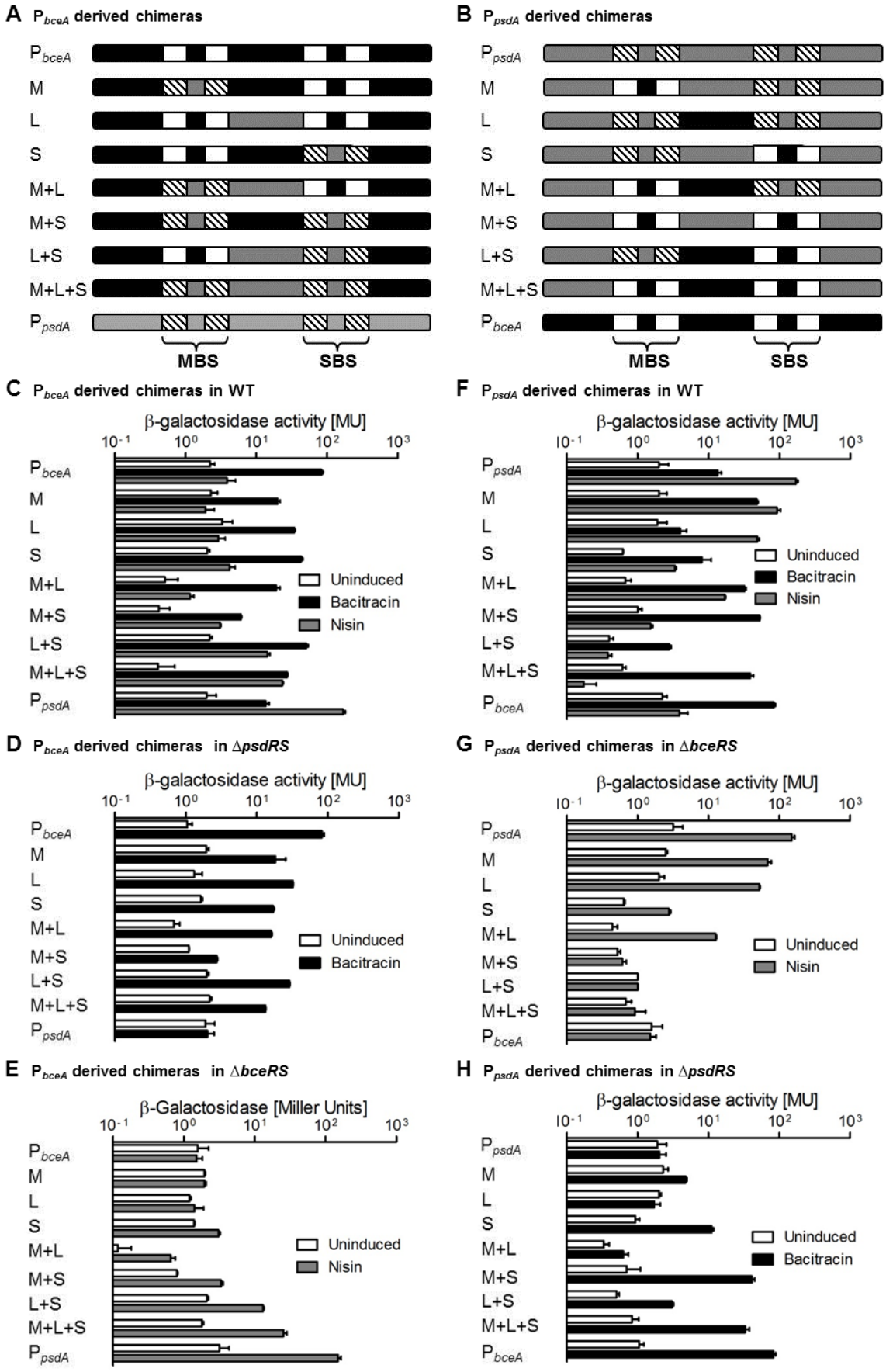
A P bceA derived chimeras
P bceA
M
L
S
M+L
M+S
L+S
M+L+S
P psdA
MBS
SBS
B P psdA derived chimeras
P psdA
M
L
S
M+L
M+S
L+S
M+L+S
P bceA
MBS
SBS
C P bceA derived chimeras in WT
β-galactosidase activity [MU]
10-1 10^0 10^1 10^2 10^3
Uninduced
Bacitracin
Nisin
F P psdA derived chimeras in WT
β-galactosidase activity [MU]
Uninduced
Bacitracin
Nisin
D P bceA derived chimeras in ΔpsdRS
β-galactosidase activity [MU]
Uninduced
Bacitracin
G P psdA derived chimeras in ΔbceRS
β-galactosidase activity [MU]
Uninduced
Nisin
E P bceA derived chimeras in ΔbceRS
β-Galactosidase [Miller Units]
Uninduced
Nisin
H P psdA derived chimeras in ΔpsdRS
β-galactosidase activity [MU]
Uninduced
Bacitracin

Figure 4.4. Unravelling the roles of different promoter elements in RR-promoter specificity. (A and B) Schematic of chimeric promoters derived from P_{bceA} and P_{psdA} with specific regions switched into the corresponding part of P_{psdA} and P_{bceA}, respectively, compared with two WT promoters. Names on the left side of each chimeric promoter stand for the exchanging region: M, main binding site; L, linker; S, secondary binding site. MBS and SBS from P_{bceA} and P_{psdA} are filled the same as in Fig. 4.1. Activities of WT and chimeric promoters in different genetic backgrounds of *B. subtilis* are shown as follows: (C) P_{bceA} derived chimeras in WT, (D) P_{bceA} derived chimeras in Δ*psdRS* strain, (E) P_{bceA} derived chimeras in Δ*bceRS* strain, (F) P_{psdA} derived chimeras in WT, (G) P_{psdA} derived chimeras in Δ*bceRS* strain and (H) P_{psdA} derived chimeras in Δ*psdRS* strain. Black bars and grey bars represent for samples induced with bacitracin and nisin, while white bars stand for non-induced controls.

Compared to only the secondary binding site switch (S), exchanging both the main binding site and the secondary binding site simultaneously (M+S) resulted in a severe decrease of the promoter activity in the Δ*psdRS* mutant after induction with bacitracin (Fig. 4.4D), while an increase of the promoter activity in the Δ*bceRS* mutant after induction with nisin (Fig. 4.4E). This indicates that based on the primary specificity determinant — the secondary binding site — the main binding site assists it to achieve a higher promoter activity with the cognate RR, PsdR, and a lower promoter activity with the non-cognate RR, BceR. Substitution of the linker together with the secondary binding site (L+S) resulted in a higher promoter activity compared to only exchanging the secondary binding site (S) in both the Δ*psdRS* mutant after bacitracin induction (Fig. 4.4D) and the Δ*bceRS* mutant after nisin induction (Fig. 4.4E). This data suggests that the linker region (L) can enhance promoter activity with both cognate PsdR and non-cognate BceR.

Taken together, these results indicate that the secondary binding site on P_{psdA} mainly determines PsdR-P_{psdA} specificity, even though the intensity of induction with secondary binding site substitution alone is not very strong. The linker cannot determine specificity by itself but can increase promoter activity with both BceR and PsdR, which explains the change of specificity that was detected for construct BP4 including the linker and the secondary binding site but not for construct BP3 with only the secondary binding site. Despite the fact that the main binding site is absolutely crucial for RR/promoter interaction, the main binding site of P_{psdA} alone cannot determine specificity. Instead, it supports the secondary binding site in strengthening specificity. Not surprisingly, switching all three elements together (M+L+S) resulted in the highest change of specificity in the Δ*bceRS* mutant after induction with nisin (Fig. 4.4E), demonstrating that all three parts together contribute to the specificity.

Rewiring specificity from P_{psdA} to P_{bceA} and dissecting the role of specificity determinants on P_{bceA}

In order to support the results obtained above, a similar approach was performed towards rewiring the specificity from P_{psdA} to P_{bceA}. In contrast to the results obtained for the series of PB chimeras (change of specificity from P_{psdA} to P_{bceA} started with PB3), the change of specificity from P_{bceA} to P_{psdA} started later with construct BP4. This may indicate a subtle difference in the BceR-P_{bceA} specificity determination compared to PsdR-P_{psdA}. To answer this question, chimeric promoters with different combinations of the main binding site, the linker region and the secondary binding site of P_{psdA} being replaced by the corresponding regions of P_{bceA} were constructed (Fig. 4.4B) (Table 4.2) and the

promoter activities of the corresponding *B. subtilis* reporter strains (Table 4.1) were determined as described above.

Replacing the secondary binding site (S) of P_{psdA} with the corresponding region from P_{bceA} resulted in a dramatic drop of promoter activity in the Δ*bceRS* mutant after induction with nisin (Fig. 4.4G), and a huge increase of promoter activity in the Δ*psdRS* mutant after induction with bacitracin (Fig. 4.4H). Switching the main binding site (M) of P_{psdA} to P_{bceA} also led to a decrease of promoter activity in the Δ*bceRS* mutant after nisin induction (Fig. 4.4G) and an increase of promoter activity in the Δ*psdRS* mutant after bacitracin induction (Fig. 4.4H), but the effect was much weaker than that of the secondary binding site substitution. Replacing the linker (L) of P_{psdA} by the linker of P_{bceA} resulted in decreased promoter activity in the Δ*bceRS* mutant after nisin induction (Fig. 4.4G) but no change of promoter activity in the Δ*psdRS* mutant after bacitracin induction (Fig. 4.4H). Together, these results illustrate that for P_{bceA}, the secondary binding site is the main determinant of BceR-P_{bceA} specificity, while the main binding site also contributes to a small extent. A combined exchange of the main binding site together with the secondary binding site (M+S) resulted in a further enhancement of promoter activity in the Δ*psdRS* mutant after bacitracin induction (Fig. 4.4H) and a complete loss of promoter activity after nisin induction in the Δ*bceRS* mutant (Fig. 4.4G). This data further suggests that the main binding site and the secondary binding site work together to determine BceR-P_{bceA} specificity.

Compared to only the secondary binding site switch (S), changing the linker and the secondary binding site together (L+S) of P_{psdA} into the corresponding region of P_{bceA} decreased the promoter activity in both the Δ*bceRS* mutant after nisin induction (Fig. 4.4G) and the Δ*psdRS* mutant after bacitracin induction (Fig. 4.4H). This result is in consistency with the analysis of series PB chimeras that construct PB4 including the linker and the secondary binding site had lower promoter activity compared with construct PB3 including only the secondary binding site. Our data indicates that the linker region of P_{bceA} can decrease the promoter activity with both the cognate BceR and the non-cognate PsdR.

Taken together, we demonstrated that determining the specificity for P_{bceA} seems to slightly differ from P_{psdA}. Both the secondary binding site and the main binding site of P_{bceA} have stronger effect to BceR-P_{bceA} specificity than these of P_{psdA}. Furthermore, the linker region of P_{bceA} decreases promoter activity with both RRs, which is different from the linker region of P_{psdA} that increases the promoter activity with both RRs. Both promoters have in common, that the secondary binding sites mainly determine RR-promoter specificity, and the main binding sites strengthen the specificity by increasing the interactions with the cognate RR while simultaneously reducing the interactions with the non-cognate RR.

In vitro, BceR has a higher binding affinity for its native P_{bceA} than for P_{psdA}

Promoter activity assays demonstrated that P_{psdA} had no activity upon bacitracin induction in the Δ*psdRS* mutant (Fig. 4.3D), which indicates that there is no cross-regulation between BceR and P_{psdA} *in vivo*. Next, we wanted to investigate if the binding of BceR is also specific to its native promoter P_{bceA} *in vitro*. BceR carrying an N-terminal His_{10}-tag with the expected molecular mass around 27 KDa was produced and purified from the cytoplasmic fraction of *E. coli* C43 (DE3) containing plasmid pCF120 (Table 4.2), which was in consistent with its observed migration in SDS-PAGE. EMSAs were performed with this BceR and two *bceA*-like promoters P_{bceA} and P_{psdA}. 300 bp promoter DNA fragments of P_{bceA} or P_{psdA} containing the main binding site, the linker region and the secondary binding site were amplified and labeled at the 5'-end with 6FAM by PCR. 6FAM labeled P_{sigW} (the target promoter of an ECF sigma factor in *B. subtilis*) was used as a negative control.

Results of band shift assays with BceR and P_{bceA} are shown in Figure 4.5A. Increasing concentrations of phosphorylated BceR (BceR-P) were incubated with 30 fmol of 6FAM-P_{bceA} (from lane 2 to lane 5), demonstrating a concentration-dependent binding of BceR-P to P_{bceA}. The first shift was observed at 1.0 µM BceR-P representing the initial binding event of BceR-P to P_{bceA}. An additional shift occurred at slightly higher BceR-P concentrations presumably represents a second binding event. In contrast, unphosphorylated BceR performed much weaker binding (data not shown), which demonstrated that RR-phosphorylation is necessary and seems to promote DNA binding by increasing BceR affinity to P_{bceA}.

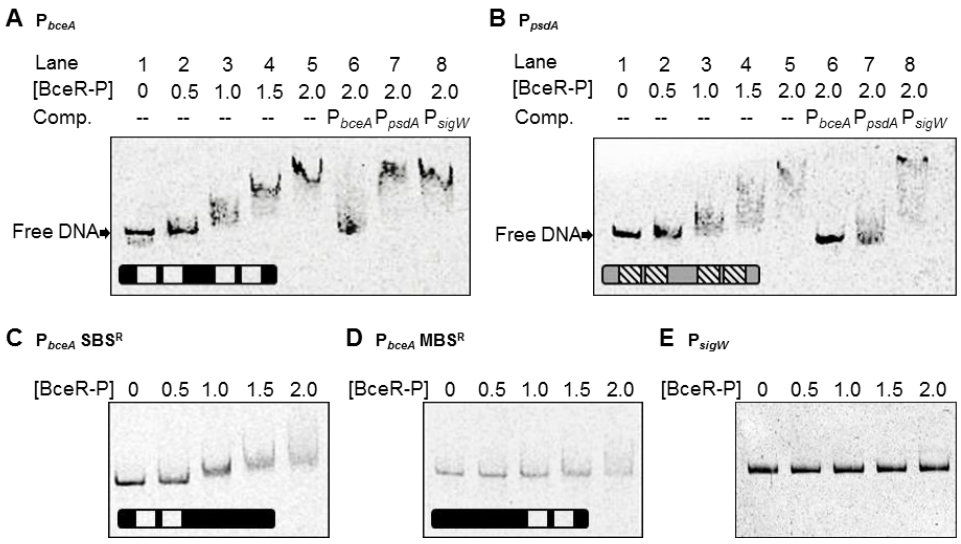

Figure 4.5. *In vitro* binding of BceR-P to *bceA*-like promoters. Increasing concentrations of phosphorylated 10×His-BceR were incubated with 30 fmol of different 6FAM-labeled promoter DNA fragments as follows: (A) P_{bceA} from -122 to +82, (B) P_{psdA} from -126 to +30, (C) P_{bceA} SBS^R (secondary binding site inactivated), (D) P_{bceA} MBS^R (main binding site inactivated), and (E) P_{sigW} as a negative control. Schematics of *bceA*-like promoters and corresponding mutants are shown in the lower left corner of each gel. The concentrations of phosphorylated BceR are indicated above the gel by [BceR-P] in µM. 900 fmol of unlabelled competitor (comp.) DNA fragments encoding P_{bceA}, P_{psdA} and P_{sigW} were added to gel (A) and (B) in lane 6, 7 and 8, respectively.

EMSAs were also performed between BceR-P and P_{psdA} (Fig. 4.5B). Two successive shifts of P_{psdA} band in lane 3 and lane 4 compared to free P_{psdA} DNA fragment (lane 1) demonstrated that BceR-P can also bind successively to the non-cognate but highly related P_{psdA} *in vitro*. In contrast, no shift was observed for the P_{sigW} DNA fragment (Fig. 4.5E), confirming the overall specificity of the assay: BceR-P cannot bind to promoter fragments that do not harbor the binding motifs of a P_{bceA}-like promoter.

To further illustrate the specificity and affinities of BceR-P binding to P_{bceA} and P_{psdA}, 900 fmol of unlabeled promoter fragments were used as competitor DNA (Fig. 4.5A/4.5B lane 6-8). Co-incubation of BceR-P with 30 fmol 6FAM-P_{bceA} and 900 fmol unlabeled P_{bceA} fragment (Fig. 4.5A lane 6) completely abolished the retardation of the labeled P_{bceA} fragment due to the competitive binding of BceR-P to an excess of unlabeled P_{bceA}. However, the shift of 6FAM-P_{bceA} band was not influenced by adding a 30-fold amount of unlabeled P_{psdA} (Fig. 4.5A lane 7) or P_{sigW} (Fig. 4.5A lane 8), demonstrating a much higher affinity of BceR-P for its cognate target promoter. In contrast, the retardation of the 6FAM-P_{psdA} DNA fragment was abolished by either addition of an extra 30-fold unlabeled P_{bceA} (Fig. 4.5B lane 6) or unlabeled P_{psdA} (Fig. 4.5B lane 7) fragments but not by P_{sigW} (Fig. 4.5B lane 8). These results clearly demonstrate that BceR-P has a higher binding affinity for P_{bceA} and preferentially binds to its native promoter compared to P_{psdA} *in vitro*.

Taken together, our results strongly suggest that phosphorylated BceR undergoes two successive binding reactions with both P_{bceA} and P_{psdA} *in vitro*. However, the binding affinity for its cognate target promoter P_{bceA} is much higher than for P_{psdA} that determines the *in vivo* specific transcription initiation. Unfortunately, any affects to purify PsdR failed, thereby preventing the performance of similar *in vitro* studies on PsdR-P_{psdA}/P_{bceA} interactions.

Cooperative binding of BceR to two binding sites on P_{bceA}

The *in vivo* promoter activity assays demonstrated that both binding sites on P_{bceA} are indispensable for BceR-P_{bceA} interaction (Fig. 4.2D). Moreover, EMSA on complete promoter fragment strongly suggests BceR has two successive binding events at P_{bceA} *in vitro* (Fig. 4.5A). To discriminate between the individual binding reactions, we next performed EMSAs with BceR-P on 6FAM labeled *bceA* promoter DNA-fragments carrying random mutation of either the main binding site or the secondary binding site.

Incubation of BceR-P with labeled P_{bceA} SBS^R (P_{bceA} containing a randomized and hence inactive secondary binding site) obtained only a single shift at a BceR-P concentration of 1.0 µM (Fig. 4.5C), a concentration comparable to the threshold concentration as the intact P_{bceA} fragment (Fig. 4.5A lane 3). Increasing the BceR-P concentration did not lead to any additional shift. Hence, P_{bceA} containing only the main binding site merely allows the first binding event that is the binding of BceR-P to the main binding site. The identical BceR-P concentrations required for shifting either the WT or the SBS^R

fragments indicates that binding of BceR-P to the main binding site is independent from the secondary binding site.

Incubation of BceR-P with labeled P_{bceA} MBS^R (P_{bceA} containing a randomized and hence inactive main binding site) basically failed to retard the DNA-fragment within the same concentration range (Fig. 4.5D). Hence, a non-functional main binding site prevents both binding events of BceR-P to P_{bceA}. This result indicates that the second binding event, which requires the secondary binding site, depends on and occurs after BceR-P binding to the main binding site.

Together, these results demonstrate that the two binding sites contribute in a successive manner to the BceR-P_{bceA} interaction and imply a cooperative binding model of BceR to the two binding sites of P_{bceA}. BceR first binds to the main binding site independently, which then supports the subsequent binding to the secondary binding site. In order to challenge this hypothesis we finally analyzed the binding affinities of BceR-P on P_{bceA}/P_{psdA} by surface plasmon resonance (SPR) spectroscopy.

Determination of binding kinetics of BceR-promoter interaction unravels the mechanism that determines BceR promoter specificity

To quantify the binding kinetics of the BceR-promoter interaction we used SPR analysis. As first step we captured a biotin-labeled DNA-fragment comprising the P_{bceA} region to a sensor chip previously immobilized with streptavidin. Then, increasing concentrations of His_{10}-BceR and His_{10}-BceR previously phosphorylated using phosphoramidate (BceR-P) were injected over the chip surface. It can be clearly seen that non-phosphorylated BceR does not interact with the P_{bceA} promoter (Fig. 4.6A), whereas BceR-P showed clear binding (Fig. 4.6B). Since BceR has two binding sites on the DNA-fragment used for SPR, we used the OneToTwo evaluation algorithm. The binding kinetics underlying this sensorgram could be determined revealing that these two binding events mainly differ in their association rates ($k_{a1}=7.46x10^4$ M*s; $k_{a2}=1.58x10^6$ M*s) rather than the dissociation rates ($k_{d1}=5.57x10^{-4}$/s; $k_{d2}=8.05x10^{-4}$/s) resulting in two binding events that differ in their overall affinity ($K_{D1}=7.47$ nM; $K_{D2}=0.51$ nM). As next step, we determined the binding kinetics between BceR-P and P_{bceA} when the main binding site (MBS^R) or the secondary binding site (SBS^R) was destroyed, respectively. It can be clearly seen that inactivation of the main binding site completely prevented DNA-binding of BceR-P (Fig. 4.6C). However, when only the secondary binding site was destroyed, a clear DNA-binding of BceR could be observed (Fig. 4.6D). In contrast to the sensorgram when both binding sites are intact (Fig. 4.6B), the sensorgram here follows a real 1:1 binding kinetic that we quantified with an association rate of $k_a=6.59 \times 10^5$ M*s and a dissociation rate $k_d=9.5x10^{-4}$/s making an overall binding affinity of $K_D=1.4$ nM. Furthermore, we observed that the overall response units were reduced approximately 1/3 compared to the sensorgram representing the BceR-P/P_{bceA}, which probably represents the portion of BceR-P binding to the secondary binding site. Our data clearly shows that the main binding site of P_{bceA} region is essential for binding of the RR to the DNA. The RR obviously cannot bind the secondary binding site when the main binding site was not previously

occupied. Comparing the binding kinetics of BceR-P to the intact P_{bceA} and the secondary binding site mutant, it can be assumed that the secondary binding site increases the overall affinity of the RR to the promoter region, and therefore is important for triggering gene expression.

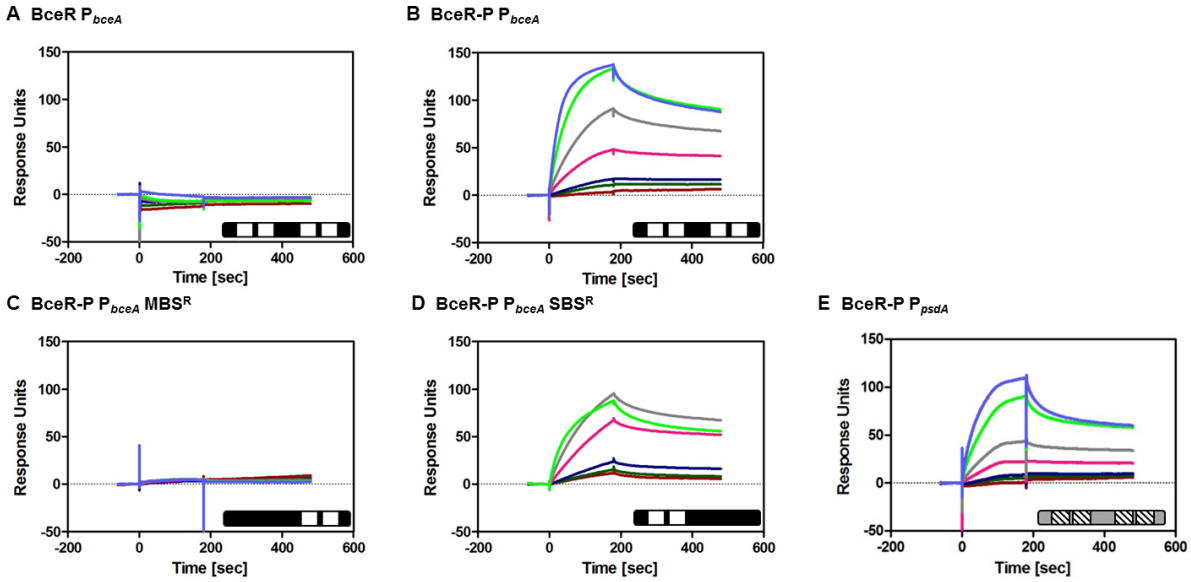

Figure 4.6. Surface plasmon resonance spectroscopy of BceR-P binding within the P_{bceA} and P_{psdA} region. (A) BceR binding to P_{bceA}, (B) BceR-P binding to P_{bceA}, (C) BceR-P binding to P_{bceA} MBS^R (main binding site inactivated), (D) BceR-P binding to P_{bceA} SBS^R (second binding site inactivated), and (E) BceR-P binding to P_{psdA}. 0.2 nM (red line), 0.5 nM (brown line), 1 nM (dark blue line), 2.5 nM (magenta line), 5 nM (green line), 7.5 nM (lime green line), and 10 nM (blue line), respectively, of each of purified BceR or BceR-P was passed over the chip. The figures represent each one characteristic of three independently performed experiments.

In addition, we wanted to compare the binding of BceR-P to P_{bceA} with P_{psdA}. Therefore, we captured DNA comprising the P_{psdA} promoter onto the chip, and then injected increasing concentrations of BceR-P. We observed that the sensorgram representing the interaction of BceR-P to P_{psdA} did not represent a typical 1:1 interaction due to the non-linear decrease of the dissociation curve (Fig. 4.6E). Compared to the P_{bceA} promoter, the interaction of Bce-R to P_{psdA} was weaker. Our data clearly shows that the binding mechanism of BceR-P to P_{psdA} is comparable to that of BceR-P to P_{bceA}, albeit the overall affinities of the two binding sites are lower.

Discussion

In this report, we have comprehensively investigated the mechanism that dictates Bce-like RR specifically regulating the transcription of its target *bceAB*-like operon by genetic and biochemical approaches. We found out determinants on the promoter of two *bceAB*-like operons — P_{bceA} and P_{psdA} — for BceR and PsdR specific binding, respectively. Furthermore, we successfully rewired the transcriptional regulation between these two systems by exchanging these specificity determinants.

Three extremely important findings were obtained in the process of understanding the specificity determining mechanism. First, we for the first time demonstrated that on P_{bceA}/P_{psdA} there are two Bce-like RR binding sites: an upstream main binding site and a downstream secondary binding site joined

together by a flexible linker region (Fig. 4.2D and 4.2E). Second, the secondary binding sites on both P_{bceA} and P_{psdA} primarily dictate RR-promoter specificity. However, the secondary binding site by itself is not enough to fully dictate specificity. Together with the high affinity main binding site and the linker region, they are able to completely mediate RR-promoter specificity (Fig. 4.4). Third, we were able to demonstrate that BceR has two-step binding event on P_{bceA}. By separating the binding, we demonstrated that BceR can bind to the main binding site independently with high affinity, which assists and stabilizes the binding to the low affinity secondary binding site (Fig. 4.5 and 4.6).

Taken together, our findings strongly suggest that BceR has a hierarchical and cooperative binding model to these two binding sites on P_{bceA}. A BceR dimer first binds tightly to the main binding site with low specificity, which favors and stabilizes the binding of a second BceR dimer to the secondary binding site specifically. The linker regions of P_{bceA} and P_{psdA} with different AT/GC contents may play a role in maintaining different distances and/or angles of the two binding sites, and hence adjust the promoter activity. The overall binding affinity of BceR to its cognate promoter P_{bceA}, as a combined effect of all three promoter motifs – the main binding site, the linker, and the secondary binding site, is much higher than to the non-cognate promoter, which is an intrinsic ability to discriminate the cognate promoter from the pool of similar non-cognate ones in a bacterial cell. RR determines the specific transcription initiation, most likely by direct interaction with the RNA polymerase so that recruits the polymerase to the promoter. The cooperative binding mechanism has already been confirmed by PhoB binding to the target *pstS* promoter containing double *pho* boxes (Blanco *et al.*, 2012). EMSAs showed that two $PhoB^E$ dimers bind to two consecutive *pho* boxes in a hierarchical and cooperative manner, which is at low concentration $PhoB^E$ dimer first binds to the high-affinity *pho* box 1 and with increasing concentration the first binding assists the subsequent dimer bind to the downstream low-affinity *pho* box 2.

RR-promoter specificity is mainly determined by molecular recognition on the interaction surfaces between amino acids on the RR and bases on the promoter (Rohs *et al.*, 2010). Most of studies about RR-promoter specificity focused on searching for amino acids on the DNA binding domain of RRs that can specifically recognize their cognate promoters. Structures of PhoB and OmpR C-terminal DNA binding domain indicated that these amino acids are located on the C-terminal helix α3 (interaction with the DNA major groove) and the loop of the C-terminal hairpin (interaction with the DNA minor groove) (Martínez-Hackert & Stock, 1997, Blanco *et al.*, 2002, Rhee *et al.*, 2008). A previous study showed that a single amino acid, which is Glu215 on the α3 helix of *Mycobacterium tuberculosis* PhoP, is an important residue that significantly contributes to specific DNA binding site recognition. By mutating Glu215 into Ala, PhoP lost the ability to discriminate the specific binding site from the nonspecific DNA with sequence comparable compositions (Das *et al.*, 2010). Similar studies about RRs FNR and CRP in *E. coli* further showed that exchanging Arg180 and Gly184 on the recognition-helix of CRP into Val and Ser of FNP was able to convert the binding specificity (Bell *et al.*, 1989, Spiro *et al.*, 1990). These two amino acids can specifically recognize bases (G at position 5

and C at position 8) on CRP binding site, and discriminate bases T and A on FNR binding site. Instead of searching for amino acids on the DNA binding domain of the RR, our study focused on the target promoters of Bce-like RRs and demonstrated that promoter specificity is determined by three promoter motifs together. The mechanism is different from previous studies that two bases on one binding site can determine RR-promoter specificity. This is probably due to that the DNA recognition motifs, the α3 helix and the loop of the C-terminal hairpin, are highly similar between BceR and PsdR. With only one promoter motif, it is hard to maintain the specificity and the high activity simultaneously. To solve this problem, *B. subtilis* developed a complicated regulatory region on the target promoter of the Bce-like RR, which contains a main binding site determines the binding affinity, a secondary binding site determines the binding specificity and a linker region ensures the structure of the promoter that contribute differently to maintain the RR-promoter specificity.

Experimental procedures

Bacterial strains and growth conditions

All strains used in this study are listed in Table 4.1. *E. coli* DH5α and XL1-blue were used for cloning. All *B. subtilis* strains used in this study are derivatives of the laboratory WT strain 168. *E. coli* and *B. subtilis* were grown routinely in Luria-Bertani (LB) medium at 37°C with aeration. *B. subtilis* was transformed by natural competence as previously described (Harwood & Cutting, 1990). Ampicillin (100 µg ml^{-1}) was used for selection of all plasmids in *E. coli*. Chloramphenicol (5 µg ml^{-1}), spectinomycin (100 µg ml^{-1}), and erythromycin (1 µg ml^{-1}) plus lincomycin (25 µg ml^{-1}) for macrolide-lincosamide-streptogramin B (mls) resistance were used for the selection of *B. subtilis* mutants. Bacitracin was supplied as the Zn^{2+}-salt. Growth was measured as optical density at 600 nm wavelength (OD_{600}). Solid media contained 1.5 % (w/v) agar.

Construction of plasmids and genetic techniques

All plasmid constructs in this study are listed in Table 4.2. The corresponding primer sequences are provided in the supplemental material (Table S4.1). Different promoter fragments derived from P_{bceA} and P_{psdA} were fused to *lacZ* and cloned into the vector pAC6 (Stülke *et al.*, 1997) with EcoRI/BamHI sites. The details of promoter constructs are given in Table 4.2. For construction of the BceR-production plasmid in *E. coli*, *bceR* was amplified with primers TM2007/2008 and cloned into vector pET16b with XhoI and BamHI obtaining pCF120, resulting in an N-terminal His_{10}-tag fusion. Constructs for unmarked gene deletion in *B. subtilis* were cloned into the vector pMAD (Arnaud *et al.*, 2004). For each operon to be deleted, 800-1000 bp fragments located immediately before the start codon of the first gene ("up" fragment) and after the stop codon of the last gene ("down" fragment) were amplified. The primers were designed to create a 17-20 bp overlap between the PCR-products (Table 4.2), facilitating fusion of the fragments by PCR overlap extension and subsequent cloning into pMAD. Gene deletions were performed as previously described (Arnaud *et al.*, 2004). All constructs were checked by sequencing, and all *B. subtilis* strains created were verified by colony PCR using appropriate primers.

β-galactosidase assays

Assays on promoter activities were performed as described previously (Mascher *et al.*, 2004). In brief, cells were inoculated from fresh overnight cultures and grown in LB medium at 37°C with aeration until they reached an OD_{600} between 0.4 and 0.5. The cultures were split into 2 mL aliquots and challenged with 30 µg ml^{-1} bacitracin or 2 µg ml^{-1} nisin with one aliquot left untreated (non-induced control). After incubation for an additional 30 min at 37°C with aeration, the cultures were harvested and the cell pellets were frozen at -20°C. The β-galactosidase activities were determined as described, with normalization to cell density (Miller, 1972).

Expression and purification of His-tagged BceR

To produce BceR carrying an N-terminal His_{10}-tag, *E. coli* C43 (DE3) cells harboring plasmid pCF120 were grown at 25 °C with agitation until they reached an OD_{600} of about 0.4. 0.5 mM of IPTG was added to the culture and incubation was continued at 18 °C with agitation overnight. Cells were harvested by centrifugation at 4,400 × g for 10 min. The cell pellet was washed with buffer A (20 mM KPi [pH7.5], 100 mM NaCl) and stored at -20 °C until use.

To purify His_{10}-tagged BceR, cells were resuspended in buffer B (50 mM KPi [pH 7.5], 500 mM NaCl, 5 mM β-ME, 10 mM imidazole and 10 % (w/v) glycerol) supplemented with 0.1 mM phenylmethylsulfonyl fluoride

(PMSF) plus 2 mg DNaseI and disrupted by three passages through a French pressure cell (Thermo Fisher) at 20,000 PSI. Unbroken cells were removed by centrifugation at 17,000 × g for 20 min and the cell-free supernatant was filtered through a 0.45 µm syringe filter before loading onto a 1 ml Ni^{2+}-NTA resin column (Qiagen) pre-equilibrated with 5 column volumes (CVs) of buffer B. Loading was followed by washing with 5 CVs of buffer B and then with 5 CVs of buffer B containing 100 mM imidazole. BceR was eluted with buffer B supplemented with 250 mM imidazole. Fractions containing BceR were pooled and dialyzed in buffer C (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM $MgSO_4$, 5 mM β-ME, 5 mM imidazole and 10 % (w/v) glycerol) using dialysis membrane (neo Lab) at room temperature for 1 h. Protein concentration was determined with Roti®-Nanoquant (Carl Roth), and the protein were stored at 4 °C.

Electrophoretic Mobility Shift Assays (EMSA)

For electrophoretic mobility shift assays, different DNA fragments (around 300bp) generated by PCR using primers TM3146 (5' terminal 6FAM labeled) and TM3137 were purified by gel extraction. Unlabeled DNA fragments were generated by PCR using primers TM3136/TM3137 and purified by HiYield® Gel/PCR DNA Extraction Kit (SLG®). N-terminal His_{10}-BceR samples in the non-phosphorylated state and after phosphorylation by 50 mM phosphoramidate (PA) at room temperature for 2 h were centrifuged down by 16,060 × g at 4 °C for 10 min to remove the aggregated protein. Protein concentrations of the supernatants were determined with Roti®-Nanoquant (Carl Roth) and the proteins were stored on ice. Binding reactions were set by incubating 6FAM-labelled DNA-fragments with different concentrations of His_{10}-BceR at room temperature for 20 min. The reaction mixture included 30 fmol labeled target DNA and 0, 0.5, 1.0, 1.5, 2.0 µM protein with binding buffer (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM $MgSO_4$, 1 mM DTT, 5 µg ml^{-1} salmon sperm DNA and 4 % (w/v) glycerol) in a total volume of 5.5 µl. Unlabeled competitor DNA was added to the system to a final concentration of 900 fmol. Samples were loaded on a 6% native polyacrylamide gel and electrophoresis was performed by 300 Volt for 15 min in TBE buffer. Gels were detected by PhosphorImager (Typhoon Trio™, GE Healthcare).

Surface Plasmon Resonance (SPR) spectroscopy

SPR assays were performed in a Biacore T200 using carboxymethyl dextran sensor chips pre-coated with streptavidin (Xantec SAD500-L, XanTec Bioanalytics GmbH, Düsseldorf, Germany). All experiments were carried out at a constant temperature of 25°C and using HBS-EP buffer [10 mM HEPES pH 7.4; 150 mM NaCl; 3 mM EDTA; 0.005 % (v/v) detergent P20] as running buffer. Before immobilizing the DNA fragments, the chips were equilibrated by three injections using 1 M NaCl/50 mM NaOH at a flow rate of 10 µl min^{-1}. Then, 10 nM of the respective double-stranded biotinylated DNA fragment was injected using a contact time of 420 sec and a flow rate of 10 µl min^{-1}. As a final wash step, 1 M NaCl/50 mM NaOH/50% (v/v) isopropanol was injected. Approximately 100-200 RU of each respective DNA fragment were captured onto the respective flow cell. All interaction kinetics of BceR or BceR-P with the respective DNA fragment were performed in HBS-EP buffer at 25°C at a flow rate of 30 µl min^{-1}. The proteins were diluted in HBS-EP buffer and passed over all flow cells in different concentrations (0.1 nM-10 nM) using a contact time of 180 sec followed by a 300 sec dissociation time before the next cycle started. After each cycle the surface was regenerated by injection of 2.5 M NaCl for 30 sec at 60 µl min^{-1} flow rate followed by a second regeneration step by injection of 0.5% (w/v) SDS for 30 sec at 60 µl min^{-1}. All experiments were performed at 25°C. Sensorgrams were recorded using the Biacore T200 Control software 1.0 and analyzed with the Biacore T200 Evaluation software 1.0. OneToTwo

evaluation of the sensorgrams was performed with TraceDrawer software 1.5 (Ridgeview Instruments AB, Uppsala, Sweden). The surface of flow cell 1 was not immobilized with DNA and used to obtain blank sensorgrams for subtraction of bulk refractive index background. The referenced sensorgrams were normalized to a baseline of 0. Peaks in the sensorgrams at the beginning and the end of the injection emerged from the runtime difference between the flow cells of each chip.

Calibration-free concentration analysis (CFCA) was performed using a 5 µM solution of purified BceR-P (calculated from Lowry-based protein determination), which was stepwise diluted 1:2, 1:5, 1:10, and 1:20. Each protein dilution was two-time injected, one at 5 µl min^{-1} as well as 100 µl min^{-1} flow rate. On the active flow cell P_{psdA}-DNA was used for BceR-P-binding. CFCA basically relies on mass transport, which is a diffusion phenomenon that describes the movement of molecules between the solution and the surface. The CFCA therefore relies on the measurement of the observed binding rate during sample injection under partially or complete mass transport limited conditions. Overall, the initial binding rate (dR/dt) is measured at two different flow rates dependent on the diffusion constant of the protein. The diffusion coefficient of BceR-P was calculated using the Biacore diffusion constant calculator and converter webtool (https://www.biacore.com/lifesciences/Application_Support/online_support/Diffusion_Coefficient_Calculator/index.html), whereby a globular shape of the protein was assumed. The diffusion coefficient of BceR-P was determined as $D=1.031x10^{-10}$ m^2/s. The initial rates of those dilutions that differed in a factor of at least 1.5 were considered for the calculation of the „active“ concentration, which was determined as $5x10^{-8}$M (1% of the total protein concentration) for BceR-P. The „active“ protein concentration was then used for calculation of the binding kinetic constants.

Acknowledgements

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Tables

Table 4.1. Bacterial strains used in this study.

Strain	Genotype or characteristic(s)[a]	Reference or source
E. coli strains		
DH5α	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 (r_K^- m_K^+) *rel*A1 *gln*V44 *Φ80'* Δ*lacZ* Δ*M15* Δ*(lacZYA-argF)U169*	(Grant *et al.*, 1990)
XL1-Blue	*end*A1 *gyr*A96 *(nal^R) thi*-1 *rec*A1 *rel*A1 *lac sup*E44 [F' *pro*AB$^+$ *lacI*^{q} *Δ(lacZ)M15] hsd*R17(r_K^- m_K^+)	Stratagene
C43 (DE3)	F$^-$ *omp*T *gal dcm hsdS$_B$* (r_B^- m_B^-)(DE3)	(Miroux & Walker, 1996)
B. subtilis strains		
W168	Wild type, *trpC2*	Laboratory stock
TMB279	W168 *amyE*::pER603; cm^r	(Rietkötter *et al.*, 2008)
TMB299	W168 *amyE*::pER605; cm^r	(Rietkötter *et al.*, 2008)
TMB412	W168 *amyE*::pCF601; cm^r	This study
TMB607	W168 *amyE*::pJS605; cm^r	This study
TMB805	W168 *amyE*::pAS601; cm^r	This study
TMB806	W168 *amyE*::pAS602; cm^r	This study
TMB960	W168 *amyE*::pAS603; cm^r	This study
TMB961	W168 *amyE*::pAS604; cm^r	This study
TMB962	W168 *amyE*::pAS605; cm^r	This study
TMB963	W168 *amyE*::pAS606; cm^r	This study
TMB964	W168 *amyE*::pAS607; cm^r	This study
TMB965	W168 *amyE*::pAS608; cm^r	This study
TMB966	W168 *amyE*::pAS609; cm^r	This study
TMB967	W168 *amyE*::pAS610; cm^r	This study
TMB1047	W168 *amyE*::pAS613; cm^r	This study
TMB1048	W168 *amyE*::pAS614; cm^r	This study
TMB1049	W168 *amyE*::pAS615; cm^r	This study
TMB1050	W168 *amyE*::pAS616; cm^r	This study
TMB1051	W168 *amyE*::pAS617; cm^r	This study
TMB1052	W168 *amyE*::pAS618; cm^r	This study
TMB1053	W168 *amyE*::pAS619; cm^r	This study
TMB1054	W168 *amyE*::pAS620; cm^r	This study
TMB1460	W168 with unmarked deletions of the *bceRS* loci	This study
TMB1462	W168 with unmarked deletions of the *psdRS* loci	This study
TMB2244	W168 *amyE*::pMG600; cm^r	This study
TMB2245	W168 *amyE*::pMG601; cm^r	This study
TMB2247	W168 *amyE*::pMG603; cm^r	This study
TMB2248	W168 *amyE*::pMG604; cm^r	This study
TMB2249	W168 *amyE*::pMG605; cm^r	This study
TMB2250	W168 *amyE*::pMG606; cm^r	This study
TMB2252	W168 *amyE*::pMG608; cm^r	This study
TMB2253	W168 *amyE*::pMG609; cm^r	This study
TMB2303	TMB1462 *amyE*::pER603; cm^r	This study
TMB2304	TMB1462 *amyE*::pCF601; cm^r	This study
TMB2307	TMB1460 *amyE*::pER603; cm^r	This study
TMB2308	TMB1460 *amyE*::pCF601; cm^r	This study
TMB2382	TMB1460 *amyE*::pMG600; cm^r	This study
TMB2383	TMB1460 *amyE*::pMG601; cm^r	This study
TMB2385	TMB1460 *amyE*::pMG603; cm^r	This study
TMB2386	TMB1460 *amyE*::pMG604; cm^r	This study
TMB2387	TMB1462 *amyE*::pMG600; cm^r	This study
TMB2388	TMB1462 *amyE*::pMG601; cm^r	This study
TMB2390	TMB1462 *amyE*::pMG603; cm^r	This study
TMB2391	TMB1462 *amyE*::pMG604; cm^r	This study
TMB2392	TMB1460 *amyE*::pMG605; cm^r	This study
TMB2393	TMB1460 *amyE*::pMG606; cm^r	This study

TMB2395	TMB1460 *amyE*::pMG608; cm^r	This study
TMB2396	TMB1460 *amyE*::pMG609; cm^r	This study
TMB2397	TMB1462 *amyE*::pMG606; cm^r	This study
TMB2399	TMB1462 *amyE*::pMG608; cm^r	This study
TMB2400	TMB1462 *amyE*::pMG609; cm^r	This study
TMB2455	W168 *amyE*::pMG612; cm^r	This study
TMB2456	W168 *amyE*::pMG613; cm^r	This study
TMB2457	W168 *amyE*::pMG614; cm^r	This study
TMB2460	W168 *amyE*::pMG617; cm^r	This study
TMB2461	W168 *amyE*::pMG618; cm^r	This study
TMB2462	W168 *amyE*::pMG619; cm^r	This study
TMB2463	TMB1462 *amyE*::pMG614; cm^r	This study
TMB2464	TMB1460 *amyE*::pMG614; cm^r	This study
TMB2465	TMB1462 *amyE*::pMG613; cm^r	This study
TMB2466	TMB1460 *amyE*::pMG613; cm^r	This study
TMB2467	TMB1462 *amyE*::pMG619; cm^r	This study
TMB2468	TMB1460 *amyE*::pMG619; cm^r	This study
TMB2469	TMB1462 *amyE*::pMG618; cm^r	This study
TMB2470	TMB1460 *amyE*::pMG618; cm^r	This study
TMB2475	TMB1462 *amyE*::pMG605; cm^r	This study
TMB2505	W168 *amyE*::pCF608; cm^r	This study
TMB2506	W168 *amyE*::pCF609; cm^r	This study
TMB2507	W168 *amyE*::pCF610; cm^r	This study
TMB2508	W168 *amyE*::pCF611; cm^r	This study
TMB2509	W168 *amyE*::pMG621; cm^r	This study
TMB2510	TMB1460 *amyE*::pMG621; cm^r	This study
TMB2511	TMB1462 *amyE*::pMG621; cm^r	This study
TMB2512	W168 *amyE*::pMG622; cm^r	This study
TMB2513	TMB1460 *amyE*::pMG622; cm^r	This study
TMB2514	TMB1462 *amyE*::pMG622; cm^r	This study
TMB2515	W168 *amyE*::pCF612; cm^r	This study
TMB2516	TMB1460 *amyE*::pCF612; cm^r	This study
TMB2517	TMB1462 *amyE*::pCF612; cm^r	This study
TMB2518	W168 *amyE*::pCF613; cm^r	This study
TMB2519	TMB1460 *amyE*::pCF613; cm^r	This study
TMB2520	TMB1462 *amyE*::pCF613; cm^r	This study
TMB2536	W168 *amyE*::pCF614; cm^r	This study
TMB2537	TMB1460 *amyE*::pCF614; cm^r	This study
TMB2538	TMB1462 *amyE*::pCF614; cm^r	This study
TMB2539	W168 *amyE*::pCF615; cm^r	This study
TMB2540	TMB1460 *amyE*::pCF615; cm^r	This study
TMB2541	TMB1462 *amyE*::pCF615; cm^r	This study
TMB2631	W168 *amyE*::pCF616	This study
TMB2632	TMB1460 *amyE*::pCF616	This study
TMB2633	TMB1462 *amyE*::pCF616	This study
TMB2637	W168 *amyE*::pCF618	This study
TMB2638	TMB1460 *amyE*::pCF618	This study
TMB2639	TMB1462 *amyE*::pCF618	This study
TMB2640	W168 *amyE*::pCF619; cm^r	This study
TMB2641	TMB1460 *amyE*::pCF619; cm^r	This study
TMB2642	TMB1462 *amyE*::pCF619; cm^r	This study
TMB2643	W168 *amyE*::pCF620; cm^r	This study
TMB2644	TMB1460 *amyE*::pCF620; cm^r	This study
TMB2645	TMB1462 *amyE*::pCF620; cm^r	This study

[a] Resistant cassettes: cm, chloramphenicol; r, resistant.

Table 4.2. Vectors and plasmids used in this study.

Plasimd	Genotype or characteristic(s)	Primers used for cloning	Reference or source
Vectors			
pAC6	Vector for transcriptional promoter fusions to *lacZ* in *B. subtilis*, integrates in *amyE*; cm^r		(Stülke *et al.*, 1997)
pET16b	Vector for IPTG-inducible gene expression; carries a N-terminal His_{10}-tag sequence; amp^r		Novagen
pMAD	Vector for construction of unmarked deletions in *B. subtilis*, temperature sensitive replicon; mls^r		(Arnaud *et al.*, 2004)
Plasmids			
pAS601	pAC6 P_{psdA} (-99 to +30) -*lacZ*	1591/0600	This study
pAS602	pAC6 P_{psdA} (-97 to +30) -*lacZ*	1592/0600	This study
pAS603	pAC6 P_{psdA} (-104 to +30) -*lacZ*	1688/0600	This study
pAS604	pAC6 P_{psdA} (-103 to +30) -*lacZ*	1687/0600	This study
pAS605	pAC6 P_{psdA} (-102 to +30) -*lacZ*	1686/0600	This study
pAS606	pAC6 P_{psdA} (-101 to +30) -*lacZ*	1685/0600	This study
pAS607	pAC6 P_{psdA} (-100 to +30) -*lacZ*	1684/0600	This study
pAS608	pAC6 P_{psdA} (-98 to +30) -*lacZ*	1683/0600	This study
pAS609	pAC6 P_{psdA} (-96 to +30) -*lacZ*	1682/0600	This study
pAS610	pAC6 P_{psdA} (-95 to +30) -*lacZ*	1681/0600	This study
pAS613	pAC6 P_{bceA} (-110 to +82) -*lacZ*	1869/0555	This study
pAS614	pAC6 P_{bceA} (-109 to +82) -*lacZ*	1870/0555	This study
pAS615	pAC6 P_{bceA} (-108 to +82) -*lacZ*	1871/0555	This study
pAS616	pAC6 P_{bceA} (-107 to +82) -*lacZ*	1872/0555	This study
pAS617	pAC6 P_{bceA} (-106 to +82) -*lacZ*	1873/0555	This study
pAS618	pAC6 P_{bceA} (-105 to +82) -*lacZ*	1874/0555	This study
pAS619	pAC6 P_{bceA} (-104 to +82) -*lacZ*	1875/0555	This study
pAS620	pAC6 P_{bceA} (-103 to +82) -*lacZ*	1876/0555	This study
pCF101	pMAD Δ*bceRS*	2351/2352 2353/2354	This study
pCF103	pMAD Δ*psdRS*	2357/2358 2359/2360	This study
pCF120	pET16b *bceR*	2007/2008	This study
pCF601	pAC6 P_{psdA} (-126 to +30)-*lacZ*	0674/0600	This study
pCF608	pAC6 P_{bceA} (-122 to +82) main binding site mutation-*lacZ*	2262/3563 3564/0555	This study
pCF609	pAC6 P_{bceA} (-122 to +82) second binding site mutation-*lacZ*	0554/3565 3566/0555	This study
pCF610	pAC6 P_{psdA} (-126 to +30) main binding site mutation-*lacZ*	2262/3567 3568/0600	This study
pCF611	pAC6 P_{psdA} (-126 to +30) second binding site mutation-*lacZ*	0674/3569 3570/0600	This study
pCF612	pAC6 P_{psdA} (-126 to +30) second binding site switched into the corresponding region of P_{bceA} -*lacZ*	0674/3553 3554/0600	This study
pCF613	pAC6 P_{psdA} (-126 to +30) linker and second binding site switched into the corresponding region of P_{bceA} -*lacZ*	0674/3557 3558/0600	This study
pCF614	pAC6 P_{bceA} (-122 to +82) main binding site, linker and second binding site switched into the corresponding region of P_{psdA} -*lacZ*	3692/0555	This study
pCF615	pAC6 P_{psdA} (-126 to +30) main binding site, linker and second binding site switched into the corresponding region of P_{bceA} -*lacZ*	3693/0600	This study
pCF616	pAC6 P_{bceA} (-122 to +82) main binding site and second binding site switched into the corresponding region of P_{psdA} -*lacZ*	3719/0555	This study

pCF618	pAC6 P_{bceA} (-122 to +82) main binding site and linker switched into the corresponding region of P_{psdA} -*lacZ*	3721/0555		This study
pCF619	pAC6 P_{psdA} (-126 to +30) main binding site and second binding site switched into the corresponding region of P_{bceA} -*lacZ*	3720/0600		This study
pCF620	pAC6 P_{psdA} (-126 to +30) main binding site and linker switched into the corresponding region of P_{bceA} -*lacZ*	3722/0600		This study
pER603	pAC6 P_{bceA} (-122 to +82) -*lacZ*	0554/0555		(Rietkötter *et al.*, 2008)
pER605	pAC6 P_{psdA} (-110 to +30)-*lacZ*	0599/0600		(Rietkötter *et al.*, 2008)
pMG600	pAC6 P_{bceA} (-122 to -46) - P_{psdA} (-36 to +30) (BP1) -*lacZ*	1689/3240	3241/0600	This study
pMG601	pAC6 P_{bceA} (-122 to -56) - P_{psdA} (-46 to +30) (BP2) -*lacZ*	1689/3242	3243/0600	This study
pMG603	pAC6 P_{bceA} (-122 to -76) - P_{psdA} (-66 to +30) (BP3) -*lacZ*	1689/3246	3247/0600	This study
pMG604	pAC6 P_{bceA} (-122 to -88) - P_{psdA} (-79 to +30) (BP4) -*lacZ*	1689/3248	3249/0600	This study
pMG605	pAC6 P_{psdA} (-126 to -37) - P_{bceA} (-45 to +82) (PB1) -*lacZ*	0674/3230	3231/0555	This study
pMG606	pAC6 P_{psdA} (-126 to -47) - P_{bceA} (-55 to +82) (PB2) -*lacZ*	0674/3232	3233/0555	This study
pMG608	pAC6 P_{psdA} (-126 to -67) - P_{bceA} (-75 to +82) (PB3) -*lacZ*	0674/3236	3237/0555	This study
pMG609	pAC6 P_{psdA} (-126 to -80) - P_{bceA} (-87 to +82) (PB4) -*lacZ*	0674/3238	3239/0555	This study
pMG612	pAC6 P_{bceA} (-122 to + 82) linker mutation -*lacZ*	0146/3351	3395/0010	This study
pMG613	pAC6 P_{bceA} (-122 to + 82) linker switched into the corresponding part of P_{psdA} -*lacZ*	0146/3401	3400/0010	This study
pMG614	pAC6 P_{bceA} (-122 to + 82) main binding site switched into the corresponding region of P_{psdA} -*lacZ*	0146/3419	3354/0010	This study
pMG617	pAC6 P_{psdA} (-126 to + 30) linker mutation -*lacZ*	0146/3353	3352/0600	This study
pMG618	pAC6 P_{psdA} (-126 to + 30) linker switched into the corresponding region of P_{bceA} -*lacZ*	0146/3403	3402/0600	This study
pMG619	pAC6 P_{psdA} (-126 to + 30) main binding site switched into the corresponding region of P_{bceA} -*lacZ*	0146/3357	3356/0600	This study
pMG621	pAC6 P_{bceA} (-122 to + 82) second binding site switched into the corresponding region of P_{psdA} -*lacZ*	2262/3551	3552/0555	This study
pMG622	pAC6 P_{bceA} (-122 to + 82) linker and the second binding site switched into the corresponding region of P_{psdA} -*lacZ*	2262/3555	3556/0555	This study
pJS605	pAC6 P_{bceA} (-111 to +82) -*lacZ*	1307/0555		This study

Amp, ampicillin; cm, chloramphenicol; mls, macrolide-lincosamide-streptogramin B group antibiotics; r, resistant.

Supplemental Table 4.1. Primers used in this study.

Primer name	**Sequence (5'-3')**[a]	**Use**
TM0010	CTTCGCTATTACGCCAGCTGG	*lacZ* check rev
TM0146	GTCTGCTTTCTTCATTAGAATCAATCC	*cat* check rev
TM0554	GATCGAATTCGAACATGTCATAAGCGTGTGACG	P_{bceA} (-122) fwd

TM0555	GATCGGATCCTATCGATGCCCTTCAGCACTTCC	P_{bceA} rev
TM0599	AGTCGAATTCCACCCTCGTGAATGTGACAGC	P_{psdA} (-110) *fwd*
TM0600	AGTCGGATCCCGATAGGTTCGTTGTTTGCAACACG	P_{psdA} rev
TM0674	AGTCGAATTCTCGTGTTTTCAAGTGACACC	P_{psdA} (-126) fwd
TM1307	GATCGAATTCAAGCGTGTGACGAAAATGTCACAT	P_{bceA} (-111) fwd
TM1591	AGTCGAATTCATGTGACAGCATTGTAAGATTGG	P_{psdA} (-99) fwd
TM1592	AGTCGAATTCGTGACAGCATTGTAAGATTGG	P_{psdA} (-97) fwd
TM1681	AGTCGAATTCACGACAGCATTGTAAGATTGG	P_{psdA} (-95) fwd
TM1682	AGTCGAATTCATGACAGCATTGTAAGATTGG	P_{psdA} (-96) fwd
TM1683	AGTCGAATTCTGTGACAGCATTGTAAGATTGG	P_{psdA} (-98) fwd
TM1684	AGTCGAATTCTAATGTGACAGCATTGTAAG	P_{psdA} (-100) fwd
TM1685	AGTCGAATTCGAATGTGACAGCATTGTAAG	P_{psdA} (-101) fwd
TM1686	AGTCGAATTCTGAATGTGACAGCATTGTAAG	P_{psdA} (-102) fwd
TM1687	AGTCGAATTCAGTGAATGTGACAGCATTGTAAG	P_{psdA} (-103) fwd
TM1688	AGTCGAATTCCGTGAATGTGACAGCATTGTAAG	P_{psdA} (-104) fwd
TM1689	CCGATGATAAGCTGTCAAAC	pAC6 bandshifts
TM1869	ATGCGAATTCAGCGTGTGACGAAAATG	P_{bceA} (-110) fwd
TM1870	ATGCGAATTCGCGTGTGACGAAAATGTC	P_{bceA} (-109) fwd
TM1871	ATGCGAATTCACGTGTGACGAAAATGTC	P_{bceA} (-108) fwd
TM1872	ATGCGAATTCAAGTGTGACGAAAATGTC	P_{bceA} (-107) fwd
TM1873	ATGCGAATTCAAATGTGACGAAAATGTC	P_{bceA} (-106) fwd
TM1874	ATGCGAATTCGTGACGAAAATGTCAC	P_{bceA} (-105) fwd
TM1875	ATGCGAATTCATGACGAAAATGTCAC	P_{bceA} (-104) fwd
TM1876	ATGCGAATTCAAGACGAAAATGTCAC	P_{bceA} (-103) fwd
TM2007	ATCGCTCGAGTTGTTTAAACTTTTGCTGATTG	*bceR* fwd
TM2008	ATCGGGATCCTTAATCATAGAACTTGTCCTC	*bceR* rev
TM2262	GAGCGTAGCGAAAAATCC	pAH328 checkfwd
TM2351	AATTTGGATCCGAGGAAGCAAAAGGAAATC	*bceRS* deletion up fwd
TM2352	CTTGATTTCATGAAACAGCG	*bceRS* deletion up rev
TM2353	ctgtttcatgaaatcaag ATATTGATGTTGAGTCGGAG	*bceRS* deletion down fwd
TM2354	AATTCCATGGTTCAAATTTCGCAGGATGAG	*bceRS* deletion down rev
TM2357	AATTTGGATCCCTACGATCTAAATGGTTTCC	*psdRS* deletion up fwd
TM2358	ATTTTTGAAGATGACCGCCC	*psdRS* deletion up rev
TM2359	cggtcatcttcaaaaat CACTGTGATGACCATCGTG	*psdRS* deletion down fwd
TM2360	AATTCCATGGACCGAAACGGCAAACACAC	*psdRS* deletion down rev
TM3230	GTCAGCATCCTCCCATCGAAC	PB1 up rev
TM3231	cgatgggaggatgctgac TTCCTTTTTATAATGAGATTATCC	PB1 down fwd
TM3232	TCCCATCGAACTTTCTTGCAATTC	PB2 up rev
TM3233	caagaaagttcgatggga AAGCCCGGCATTCCTTTTTATAATG	PB2 down fwd
TM3236	TTCCGCTCCCCAATCTTACAATG	PB3 up rev
TM3237	taagattggggagcggaa TTGTTCGCCGTATCGAAGG	PB3 down fwd
TM3238	ATCTTACAATGCTGTCACATTC	PB4 up rev
TM3239	gtgacagcattgtaagat GCTTTTCTTTTTTGTTCGCCG	PB4 down fwd
TM3240	TGCCGGGCTTTTCCTTCGATAC	BP1 up rev
TM3241	cgaaggaaaagcccggcaTTCCTTTTTATAATAAAGAAAAAGG	BP1 down fwd
TM3242	TTCCTTCGATACGGCGAAC	BP2 up rev
TM3243	ttcgccgtatcgaaggaaGGATGCTGACTTCCTTTTTATAATAAAG	BP2 down fwd
TM3246	AAAAGAAAAGCATGTGACATTTTC	BP3 up rev
TM3247	gtcacatgcttttctttttTTGCAAGAAAGTTCGATGGGAGG	BP3 down fwd
TM3248	ATGTGACATTTTCGTCACACGC	BP4 up rev
TM3249	gtgacgaaaatgtcacatTGGGGAGCGGAATTGCAAGAAAG	BP4 down fwd
TM3351	cgaacaaatttgtataGCATGTGACATTTTCGTC	P_{bceA} L-M up rev
TM3352	cgcacggcaattgcaAGAAAGTTCGATGGGAGG	P_{psdA} L-M down fwd
TM3353	tgcaattgccgtgcgCAATCTTACAATGCTGTCAC	P_{psdA} L-M up rev
TM3354	gacagcattgtaagaTGCTTTTCTTTTTTGTTCGCC	P_{bceA} M-S down fwd
TM3356	gacgaaaatgtcacaTTGGGGAGCGGAATTGCAAG	P_{psdA} M-S down fwd
TM3357	tgtgacattttcgtcACATTCACGAGGGTGTCACTTG	P_{psdA} M-S up rev
TM3395	tatacaaatttgttcgCCGTATCGAAGGAAAAGC	P_{bceA} L-M down fwd
TM3400	ggcgaacaatccgctcccGCATGTGACATTTTCGTCAC	P_{bceA} L-S down fwd
TM3401	gggagcggattgttcgccGTATCGAAGG	P_{bceA} L-S up rev
TM3402	cttgcaataaaagaaaaCAATCTTACAATGCTGTCAC	P_{psdA} L-S down fwd

TM3403	ttttcttttattgcaagAAAGTTCGATGGG	P_{psdA} L-S up rev
TM3419	tcttacaatgctgtcACACGCTTATGACATGTTCG	P_{bceA} M-S up rev
TM3551	ccatcgaactttcttgCAAAAAAGAAAAGCATGTGACATTTTC	P_{bceA} S-S up rev
TM3552	caagaaagttcgatGGAAAAGCCCGGCATTCC	P_{bceA} S-S down fwd
TM3553	ccttcgatacggcgaaCAATTCCGCTCCCCAATC	P_{psdA} S-S up rev
TM3554	ttcgccgtatcgaaGGGAGGATGCTGACTTCC	P_{psdA} S-S down fwd
TM3555	actttcttgcaattccgctccccaATGTGACATTTTCGTCACACG	P_{bceA} S+L-S up rev
TM3556	ggaattgcaagaaagttcgatGGAAAAGCCCGGCATTCC	P_{bceA} S+L-S down fwd
TM3557	tacggcgaacaaaaaagaaaagcATCTTACAATGCTGTCACATTC	P_{psdA} S+L-S up rev
TM3558	ttttttgttcgccgtatcgaaGGGAGGATGCTGACTTCC	P_{psdA} S+L-S down fwd
TM3563	gcgttaagtcaccgctaaCGCTTATGACATGTTCGAATTCG	P_{bceA} M-M up rev
TM3564	ttagcggtgacttaacgcTGCTTTTCTTTTTTGTTCGCCG	P_{bceA} M-M down fwd
TM3565	cagctagcagtcagtcagAAAAAGAAAAGCATGTGACATTTTC	P_{bceA} S-M up rev
TM3566	ctgactgactgctagctgAAAAGCCCGGCATTCCTTTT	P_{bceA} S-M down fwd
TM3567	tacttcggtcaccgctaaTTCACGAGGGTGTCACTTG	P_{psdA} M-M up rev
TM3568	ttagcggtgaccgaagtaTTGGGGAGCGGAATTGCAAG	P_{psdA} M-M down fwd
TM3569	gtcagtcgtcagtcagtcATTCCGCTCCCCAATCTTAC	P_{psdA} S-M up rev
TM3570	gactgactgacgactgacGAGGATGCTGACTTCCTTTT	P_{psdA} S-M down fwd
TM3665	GTCATAAGCGTGTGACGAAAATGTCACATGCTTTTCTTTTTTGTTCGCCGTATCGAAGGAAAAGCCCGGCATTCCT	P_{bceA} WT fwd (for SPR)
TM3666	AGGAATGCCGGGCTTTTCCTTCGATACGGCGAACAAAAAAGAAAAGCATGTGACATTTTCGTCACACGCTTATGAC	Biotin-P_{bceA} WT rev (for SPR)
TM3667	CCCTCGTGAATGTGACAGCATTGTAAGATTGGGGAGCGGAATTGCAAGAAAGTTCGATGGGAGGATGCTGACTTCCT	P_{psdA} WT fwd (for SPR)
TM3668	AGGAAGTCAGCATCCTCCCATCGAACTTTCTTGCAATTCCGCTCCCCAATCTTACAATGCTGTCACATTCACGAGGG	Biotin-P_{psdA} WT rev (for SPR)
TM3669	GTCATAAGCGTTAGCGGTGACTTAACGCTGCTTTTCTTTTTTGTTCGCCGTATCGAAGGAAAAGCCCGGCATTCCT	P_{bceA} M-M fwd (for SPR)
TM3670	AGGAATGCCGGGCTTTTCCTTCGATACGGCGAACAAAAAAGAAAAGCAGCGTTAAGTCACCGCTAACGCTTATGAC	Biotin-P_{bceA} M-M rev (for SPR)
TM3671	CCCTCGTGAATTAGCGGTGACCGAAGTATTGGGGAGCGGAATTGCAAGAAAGTTCGATGGGAGGATGCTGACTTCCT	P_{psdA} M-M fwd (for SPR)
TM3672	AGGAAGTCAGCATCCTCCCATCGAACTTTCTTGCAATTCCGCTCCCCAATACTTCGGTCACCGCTAATTCACGAGGG	Biotin-P_{psdA} M-M rev (for SPR)
TM3673	GTCATAAGCGTGTGACGAAAATGTCACATGCTTTTCTTTTTCTGACTGACTGCTAGCTGAAAAGCCCGGCATTCCT	P_{bceA} S-M fwd (for SPR)
TM3674	AGGAATGCCGGGCTTTTCAGCTAGCAGTCAGTCAGAAAAAGAAAAGCATGTGACATTTTCGTCACACGCTTATGAC	Biotin-P_{bceA} S-M rev (for SPR)
TM3675	CCCTCGTGAATGTGACAGCATTGTAAGATTGGGGAGCGGAATGACTGACTGACGACTGACGAGGATGCTGACTTCCT	P_{psdA} S-M fwd (for SPR)
TM3676	AGGAAGTCAGCATCCTCGTCAGTCGTCAGTCAGTCATTCCGCTCCCCAATCTTACAATGCTGTCACATTCACGAGGG	Biotin-P_{psdA} S-M rev (for SPR)
TM3677	TCACGAATTACCATCTACACCCTGCCAAAAATTTGATAAACTTATTTTATAAAAAAATTGAAACCTTTTGAAACGAA	P_{sigW} WT fwd (for SPR)
TM3678	TTCGTTTCAAAAGGTTTCAATTTTTTTATAAAATAAGTTTATCAAATTTTTGGCAGGGTGTAGATGGTAATTCGTGA	Biotin-P_{sigW} WT rev (for SPR)
TM3692	GATCGAATTCGAACATGTCATAAGCGTGTGACAGCATTGTAAGATTGGGGAGCGGAATTGC	P_{bceA} M+L+S-S fwd
TM3693	AGTCGAATTCTCGTGTTTTCAAGTGACACCCTCGTGAATGTGACGAAAATGTCACATGCTTTTCTTTTTTGTTCGC	P_{psdA} M+L+S-S fwd
TM3719	GATCGAATTCGAACATGTCATAAGCGTGTGACAGCATTGTAAGATGCTTTTCTTTTTTGCAAG	P_{bceA} M+S-S fwd
TM3720	AGTCGAATTCTCGTGTTTTCAAGTGACACCCTCGTGAATGTGACGAAAATGTCACATTGGGGAGCGGAATTG	P_{psdA} M+S-S fwd
TM3721	GATCGAATTCGAACATGTCATAAGCGTGTGACAGCATTGTAAGATTG	P_{bceA} M+L-S fwd
TM3722	AGTCGAATTCTCGTGTTTTCAAGTGACACCCTCGTGAATGTGACGAAAATGTCACATG	P_{psdA} M+L-S fwd

[a] Restriction sites are underlined; overlaps to other primers for PCR fusions are shown by lower case letters.

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Chapter V

Specificity Determinant and Rewiring Signal Transduction of BceRS-like Two-Component Systems in *Bacillus subtilis*

Manuscript

Specificity Determinant and Rewiring Signal Transduction of BceRS-like two-component systems in *Bacillus subtilis*

Summary

Two-component signal transduction systems are comprised of a membrane-anchored histidine kinase that senses the input stimulus and a cognate response regulator, which binds to specific promoter regions to trigger a cellular response. In *Bacillus subtilis*, there are three Bce-like two-component systems: BceRS, PsdRS and YxdJK, which — together with their cognate ABC transporters — mediate peptide antibiotic resistance. These three Bce-like two-component systems share significant sequence similarity and were predicted to have considerable cross-talk. However, *in vivo*, these three systems are insulated very well with only low level of cross-regulation between BceS and PsdR. In this chapter, we focused on the Bce and the Psd systems and investigated the specificity determinants in BceR and PsdR that ensure the insulation of these two paralogous pathways at the level of interaction with their histidine kinases. First, we verified that the specificity determinants are located on the Bce-like response regulator receiver domain. Next chimeric response regulator analysis demonstrated that the α1 helix, which is speculated to contain one of the interaction surfaces with the histidine kinase, is not enough to determine specificity. Exchanging this region alone between BceR and PsdR had no influence on specificity. Surprisingly, we found that the β2-α2 region, which is located away from the interaction surfaces with the histidine kinase, can influence the specificity with the histidine kinase. However, replacing this region alone in PsdR by the corresponding region from BceR was not sufficient to confer a complete change of specificity. By substituting both regions together, i.e. α1-β2-α2, we successfully rewired signal transduction from BceS to PsdR. These results demonstrated that the α1-β2-α2 region dictates the specificity of Bce-like two-component systems in *B. subtilis*.

Introduction

Two-component signal transduction systems (TCSs) are one of the prevalent ways used by bacteria to respond to environmental changes. They are involved in regulating a wide array of physiological processes, including chemotaxis, utilization of various nutrients, virulence, quorum sensing, and antimicrobial peptide (AMP) resistance (Wadhams & Armitage, 2004, Paterson *et al.*, 2006, Williams *et al.*, 2007, Gooderham & Hancock, 2009). A typical TCS is comprised of a membrane-anchored histidine kinase (HK) that detects the signal input and a cytoplasmic response regulator (RR) that mediates the cellular response (Stock *et al.*, 2000). The HK normally harbors a variable periplasmic sensor (Input) domain and a conserved cytoplasmic autokinase domain linked by a linker region (Fig. 5.1). In addition, most HKs harbor a HAMP domain or a PAS domain at the N-terminal of the cytoplasmic domain, which is critical for signal transmission from the periplasmic sensor domain to the cytoplasmic region. The highly conserved HK cytoplasmic region consists of a long α-hairpin dimerization and histidine phosphotransfer (DHp) domain and a globular catalytic and ATP binding (CA) domain (Mascher, 2006, Krell *et al.*, 2010, Jung *et al.*, 2012). In the RR, a conserved receiver domain that typically adopts a $(\beta\alpha)_5$ topology is connected by a flexible linker to a variable output domain that frequently has a DNA binding function (Bourret, 2010, Galperin, 2010) (Fig. 5.1).

Signal transduction within TCSs is mediated by three phosphotransfer reactions. Activation of the HK leads to autophosphorylation of a conserved His residue located in the α1 helix of the DHp domain. This is followed by transferring the phosphoryl group to a conserved Asp residue located in the β3 strand of the cognate RR receiver domain. Direct protein-protein interaction between the HK DHp domain and the RR receiver domain is involved in this step (West & Stock, 2001, Gao & Stock, 2009). Finally, the dephosphorylation of the RR switches off the signal transduction of the system (Parkinson, 1993).

Most bacteria encode dozens, sometimes hundreds of TCSs (Capra & Laub, 2012). In the genome of *B. subtilis*, 36 HKs and 34 RRs were found among the open reading frames including three paralogous Bce-like TCSs that regulate resistance against AMPs (Fabret *et al.*, 1999). The three Bce-like HKs are intramembrane-sensing histidine kinases, which have short extracellular loops and cannot detect AMPs by themselves (Mascher, 2006, Mascher, 2014). Instead, they get the signal from Bce-like ABC transporters, which harbor functions of both AMP perception and resistance, and subsequently pass the signal to their cognate RRs. Bce-like RRs belong to the OmpR subfamily and possess a winged helix-turn-helix (wHTH) DNA-binding output domain (Fabret *et al.*, 1999, Galperin, 2006). The activated RRs in turn bind to the promoter regions of their cognate ABC transporters and upregulate the transcription to ensure AMP resistance (Ohki *et al.*, 2003, Bernard *et al.*, 2007, Rietkötter *et al.*, 2008). The Bce-like TCS together with the ABC transporter form an AMP sensing and detoxification module. In *B. subtilis*, the BceRS-BceAB system and the PsdRS-PsdAB system can sense and respond mainly to bacitracin and nisin, respectively (Gebhard & Mascher, 2011, Staroń *et al.*, 2011). The third

Bce system, YxdJK-YxdLM, is poorly understood and has been found to be induced only by the human neutrophil peptide LL-37 (Joseph *et al.*, 2004, Pietiäinen *et al.*, 2005).

The three Bce-like TCSs of *B. subtilis* share significant sequence and structural similarity. This indicates the potential of considerable cross-talk between them, which has already been predicted by direct coupling analysis based on the co-evolution of inter-protein contact residues (Szurmant & Hoch, 2010, Procaccini *et al.*, 2011). Such unwanted cross-talk can be deleterious. Therefore avoiding cross-talk and maintaining high fidelity of signal transmission within TCSs is necessary to guarantee bacterial cells respond specifically to each stimulus and produce the desired, beneficial response. A previous study demonstrated that instead of the predicted high level of cross-talk between these three paralogous Bce-like TCSs in *B. subtilis*, there is only some minor degree of cross-regulation *in vivo* between BceS and PsdR at high concentrations of bacitracin (Rietkötter *et al.*, 2008) (Fig. 5.1). This raises two questions: (1) how do bacteria coordinate the activity of so many highly related signaling systems while maintaining the signal transduction specificity and preventing unwanted cross-talk? (2) How does the HK discriminate between its cognate RR and the non-cognate ones in the pool of numerous RRs?

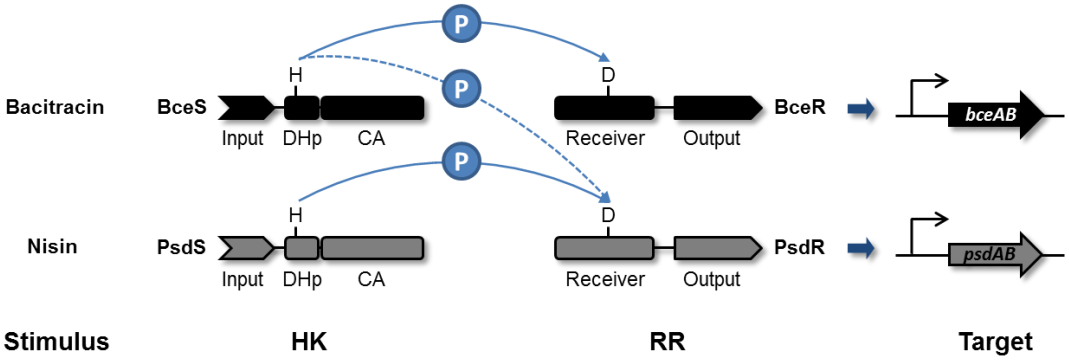


Figure 5.1. Schematic overview of two Bce-like two-component signal transduction systems in *B. subtilis*. Bce and Psd TCSs including their target promoters, P_{bceA} and P_{psdA}, are colored with black and grey, respectively. The major stimulus of each system is given on the left side. The phosphoryl group (P) is transferred from the conserved histidine residue (H) on the HK to the conserved aspartate residue (D) on the RR. Solid arrows indicate the signal transduction pathway within one system, while cross-regulation between BceS and PsdR is pointed out by dashed arrow. HK, histidine kinase; RR, response regulator; Input, input domain of HK; DHp, dimerization and histidine phosphotransferase domain of HK; CA, catalytic and ATPase domain of HK; Receiver, receiver domain of RR; Output, output domain of RR.

The predominant way to maintain intrasystem specificity and intersystem insulation is the molecular recognition between HK and RR (Podgornaia & Laub, 2013). During signal transmission of the TCS, specific interactions mediated by a few amino acids on the interaction surfaces of both proteins lead to a global and strong kinetic preference of the HK to its cognate RR. These amino acids are specificity determinants. The co-crystal structure of HK853-RR468 from *Thermotoga maritima* provides a clear view of the HK/RR interaction surfaces and implies the possible positions of those amino acids. The interaction surfaces involved in forming the HK853-RR468 pair are: the α1 helix and the β5-α5 loop of the RR468 receiver domain with the two α helices of the HK853 DHp domain; the β3-α3 loop of

the RR468 with the ATP lid and the β4-α4 loop of the HK853 CA domain; the RR468 β4-α4 loop with the DHp-CA interdomain linker of HK853(Casino *et al.*, 2009) (see Fig. 1.5).

In this chapter, we focused on the two Bce-like TCSs BceRS and PsdRS of *B. subtilis* to explore the regions on Bce-like RRs that are specifically recognized by the cognate HK and excluded by the non-cognate HK. We demonstrated that Bce-like RRs containing an extended specificity determinant compared to the other studied OmpR subfamily members, which suggests a novel mechanism of protein-protein interactions for Bce-type TCSs.

Results

The receiver domain of Bce-like RRs dictates specific interaction with the cognate HK

Signal transmission from HK to RR is mediated by direct interactions between the HK cytoplasmic domain and the RR receiver domain (West & Stock, 2001). Here, we first wanted to verify that the BceR receiver domain is indeed responsible for specific interaction with the cognate BceS instead of the non-cognate PsdS. To address this question, a chimeric RR, BP1, was constructed with the receiver domain from BceR fused to the linker and output domain of PsdR. BP1 as well as wild-type BceR and PsdR (Fig. 5.2) were cloned into vector pBS2E under the control of a constitutive promoter, P_{bceR} (Table 5.2). To test the signaling between BceS and the RR-constructs, the plasmids were transformed into *B. subtilis* reporter strain (TMB 1975 or TMB 1976) lacking all Bce-like TCS components but BceS (Table 5.1). To test the signaling between PsdS and the RR-constructs, the plasmids were transformed into *B. subtilis* reporter strain (TMB 2051 or TMB 2052) harboring PsdS (Table 5.1). In the *B. subtilis* reporter strain, P_{bceA}-*lux*, with the target promoter of BceR fused to the bacterial promoterless luciferase operon *luxABCDE*, was the reporter construct for RRs that contained the BceR output domain, while a P_{psdA}-*lux* reporter construct was used for RRs that contained the PsdR output domain. Signaling via BceS was induced by addition of bacitracin and signaling via PsdS was induced by addition of nisin (Fig. 5.1).

The strain harboring BceS, BceR and target promoter reporter P_{bceA}-*lux* showed only basal expression of the promoter in the absence of bacitracin (*ca.* 1×10^3 RLU/OD), which was induced 50-fold (*ca.* 5×10^4 RLU/OD) within 150 min after addition of 2 µg ml^{-1} bacitracin (Fig. 5.2A). BceR was not able to induce the expression of the reporter construct P_{bceA}-*lux* in the strain containing PsdS in the presence of 8 µg ml^{-1} nisn (Fig. 5.2B). PsdR, on the other hand, was not able to induce the expression of the reporter construct P_{psdA}-*lux* in the strain possessing BceS in presence of bacitracin (Fig. 5.2E), but strongly upregulated the expression of P_{psdA}-*lux* in presence of nisin in the strain containing PsdS (Fig. 5.2F). The chimeric RR BP1, with its receiver domain from BceR and the flexible linker as well as the output domain from PsdR, showed strong upregulation of P_{psdA} activity (50-fold over the uninduced control) in the reporter strain containing BceS in the presence of bacitracin (Fig. 5.2C), but no induction of expression of the reporter construct P_{psdA}-*lux* in the strain harboring PsdS after addition of nisin (Fig. 5.2D).

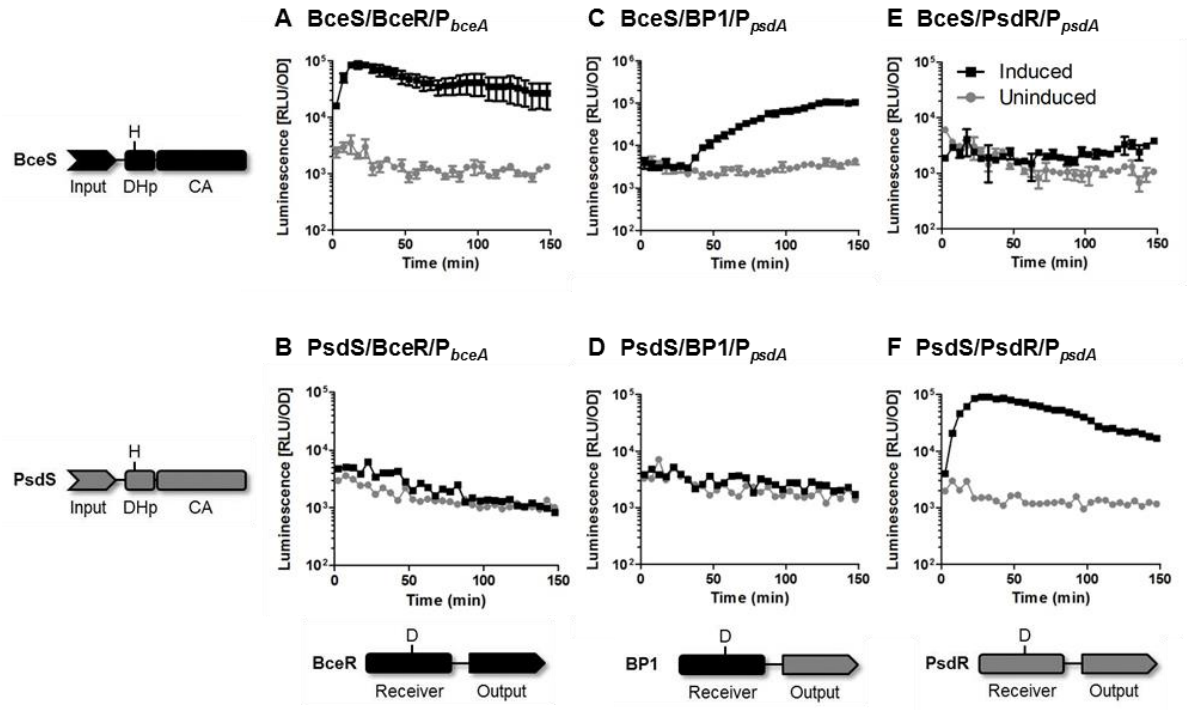


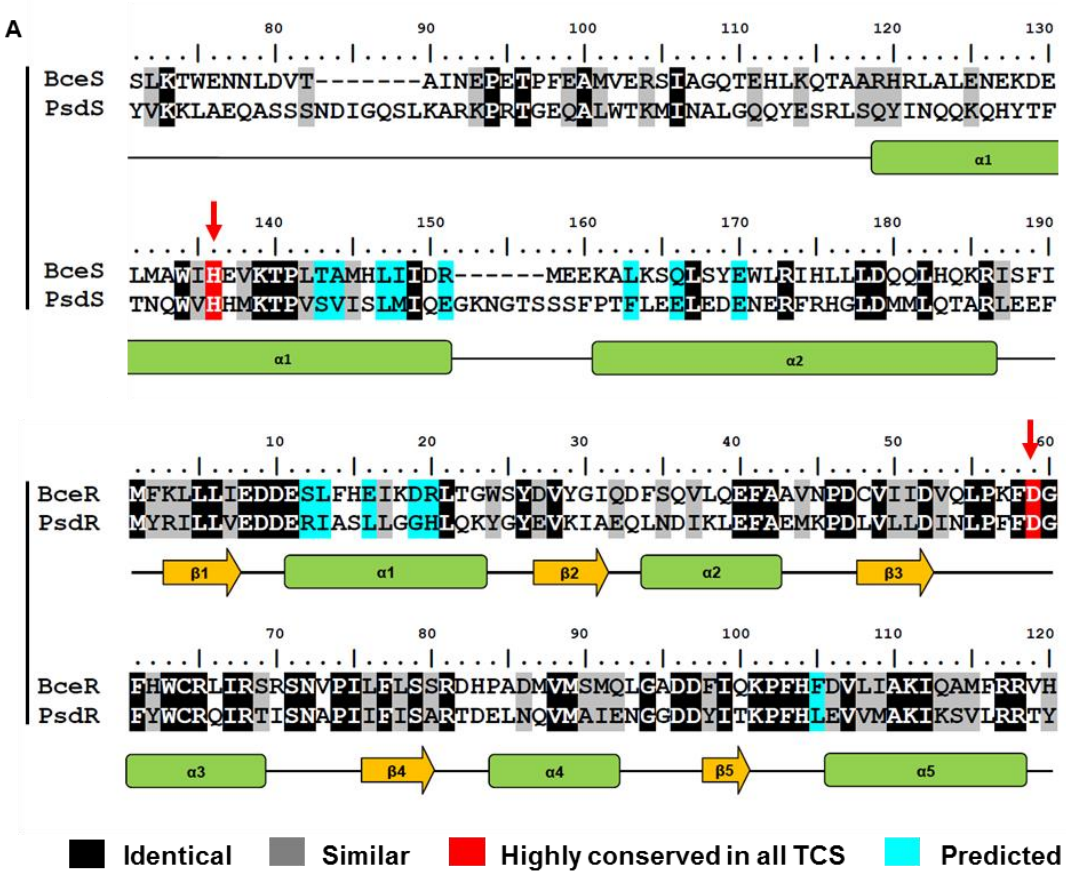
Figure 5.2. Rewiring the specificity of Bce-like two-component systems in *B. subtilis*. Signal transduction between different HK and RR pairs was monitored *in vivo* as the induction of P$_{bceA}$-*luxABCDE* or P$_{psdA}$-*luxABCDE* transcriptional fusions for 150 min after addition of antibiotics (black square), and compared to the uninduced controls (grey square). Antibiotics, bacitracin for BceS activation (top row) and nisin for PsdS activation (bottom row), were added to exponential growing cultures at time point 0 min, and luminescence (relative luminescence units, RLU) and cell growth (optical density at 600 nm, OD_{600}) were measured in 5-min intervals. Luminescence was normalized to cell density and is expressed as RLU/OD. Schematics of HKs are illustrated on the left side of the graphs with the same shading as in Fig. 5.1. Schematics of RR constructs are illustrated under the graphs. Wild-type BceR and PsdR are colored the same as in Fig. 5.1; BP1 has the receiver domain from BceR (black) and linker as well as output domain from PsdR (grey). The combinations of different HKs and RRs are indicated on top of each graph.

Taken together, our data verified that the signal is transduced from Bce-like HKs to Bce-like RRs via only the receiver domain of the RR, while is responsible for specific interactions with the cognate Bce-like HK upon induction with one corresponding signal AMPs. This result demonstrates that the HK/RR specificity determinants are located in this region. In the next step, we wanted to narrow down the region containing these specificity determinants.

The β1-α1-β2-α2 region of the Bce-like RR receiver domain contains the specificity determinants

Typically, the receiver domain of RRs, including BceR and PsdR, has a modular secondary structure with alternating β-strands and α-helices: β1-α1-β2-α2-β3-α3-β4-α4-β5-α5 (Fig. 5.3A). In order to dissect the role of each β-α repeat in HK-RR specificity determination, a series of chimeric RRs with N-terminal four β-α repeats (BP2), three β-α repeats (BP3), two β-α repeats (BP4) as well as only one β-α repeat (BP5) of the PsdR receiver domain substituted by the corresponding regions of BceR were constructed (Fig. 5.4). These chimeric RRs were cloned into pBS2E under the control of a constitutive promoter, P$_{bceR}$ (Table 5.2). Plasmids carrying these chimeras were introduced into *B. subtilis* reporter strain TMB1976 (P$_{psdA}$-*lux* reporter strain where of all Bce-like TCS components only the BceS was

present) to monitor the signal transmission from BceS, and TMB2052 (P_{psdA}-*lux* reporter strain harboring PsdS) to monitor the signal transmission from PsdS (Table 5.1), based on the P_{psdA}-*lux* output.



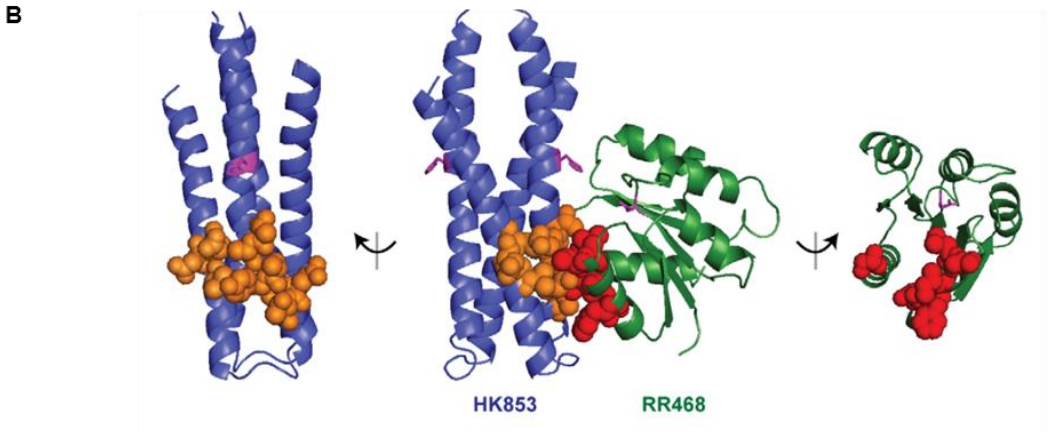


Figure 5.3. Sequence and structure analysis of two-component systems. (A) Sequence alignment of BceS/PsdS DHp domains (top) and BceR/PsdR receiver domains (bottom). Residues shaded in black and grey are identical and similar amino acids, respectively, between these two proteins. Residues shaded in red and pointed by red arrows are conserved in all HKs or RRs. Residues shaded in blue are predicted to be responsible for direct contact with the cognate partners (Procaccini *et al.*, 2011). The predicted secondary structures of HKs and RRs are shown below their respective alignments. (B) The co-crystal structure of the HK 853 and the RR 468 from *T. maritime* (the figure is from (Capra *et al.*, 2010)). Coevolved residues in HK853 are colored in orange and in RR468 are colored in red. The side chains of the conserved phosphoacceptors, His and Asp residues, are shown as magenta sticks. The HK853/RR468 complex is shown in the center with each individual molecule rotated 90° and shown separately.

Wild-type PsdR, which can only accept the signal from PsdS but not from BceS, was used as a negative control (Fig. 5.4A and 5.4B), while wild-type BceR, which can accept the signal from BceS but not from PsdS served as a positive control (Fig. 5.4K and 5.4L). BP2 and BP3, with the N-terminal four and three β-α repeats of the PsdR receiver domain, respectively, swapped for the corresponding region of BceR, showed no induction of P_{psdA} with either BceS or PsdS in the presence of bacitracin or nisin (Fig. 5.4C to 5.4F), indicating that BP2 and BP3 were dysfunctional. In contrast, chimera BP4, with only two β-α repeats (β1-α1-β2-α2) of the PsdR receiver domain replaced by the corresponding region of BceR, showed a strong upregulation of promoter activity in the presence of bacitracin, which was dependent on BceS (Fig. 5.4G). This chimera displayed extremely low level of promoter induction, 3-fold over the uninduced control, after addition of nisin in the presence of PsdS (Fig. 5.4H). These results showed a change of specificity from PsdR to BceR. BP5, with the β1-α1 region on PsdR receiver domain changed into BceR, was unable to induce expression of the promoter either in the presence of BceS or PsdS (Fig. 5.4I and 5.4J).

These results demonstrated that the signal transduction could successfully be redirected between BceRS and PsdRS sytems by swapping the β1-α1-β2-α2 region on the RR receiver domain. Although most of the chimeric RRs failed to accept a signal from either BceS or PsdS, the results from BP4 demonstrated that the β1-α1-β2-α2 region on the BceR receiver domain contains the motifs that determine specific interaction with the cognate BceS. The co-crystal structure of the TCS HK853-RR468 indicates that the α1 helix on the RR receiver domain directly interacts with the HK DHp domain (Casino *et al.*, 2009, Capra *et al.*, 2010) (Fig. 5.3B). We therefore next wanted to analyze the function of the α1 helix for Bce-like HK and RR specificity.

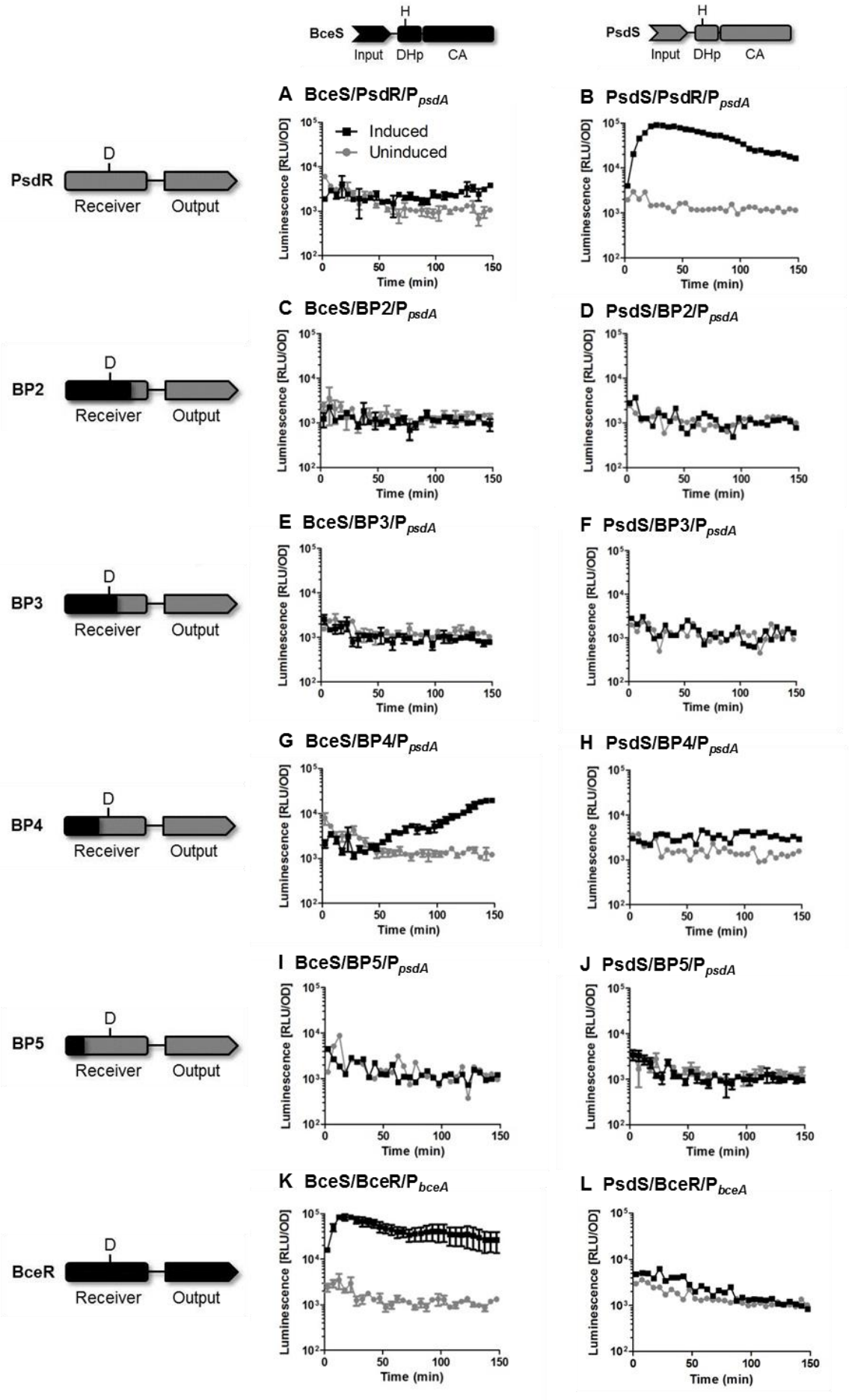

BceS
H
Input DHp CA
PsdS
H
Input DHp CA
A BceS/PsdR/P psdA
B PsdS/PsdR/P psdA
PsdR
D
Receiver Output
Induced
Uninduced
C BceS/BP2/P psdA
D PsdS/BP2/P psdA
BP2
E BceS/BP3/P psdA
F PsdS/BP3/P psdA
BP3
G BceS/BP4/P psdA
H PsdS/BP4/P psdA
BP4
I BceS/BP5/P psdA
J PsdS/BP5/P psdA
BP5
K BceS/BceR/P bceA
L PsdS/BceR/P bceA
BceR
Luminescence [RLU/OD]
Time (min)

Figure 5.4. Signal transduction between HKs and series of chimeric RRs. Signal transduction between different HK and RR pairs was monitored *in vivo* as the induction of P_{bceA}-*luxABCDE* or P_{psdA}-*luxABCDE* transcriptional fusions within 150 min in the presence of antibiotics (black square): bacitracin for BceS activation and nisin for PsdS activation, and compared with the uninduced controls (grey square) as described in Fig. 5.2. Schematics of HKs are illustrated on top of the graphs with the same shading as in Fig. 5.1. Schematics of RR constructs are illustrated on the left side of the graphs. BceS/BceR and PsdS/PsdR are with the same shading as in Fig. 5.1. From chimera BP2 to BP5, the part of the receiver domain from BceR is colored with black and the region from PsdR is colored with grey. The combinations of different HKs and RRs are indicated on top of each graph.

The α1 helix together with the β2-α2 region of the Bce-like RR receiver domain dictates specificity for the cognate Bce-like HK

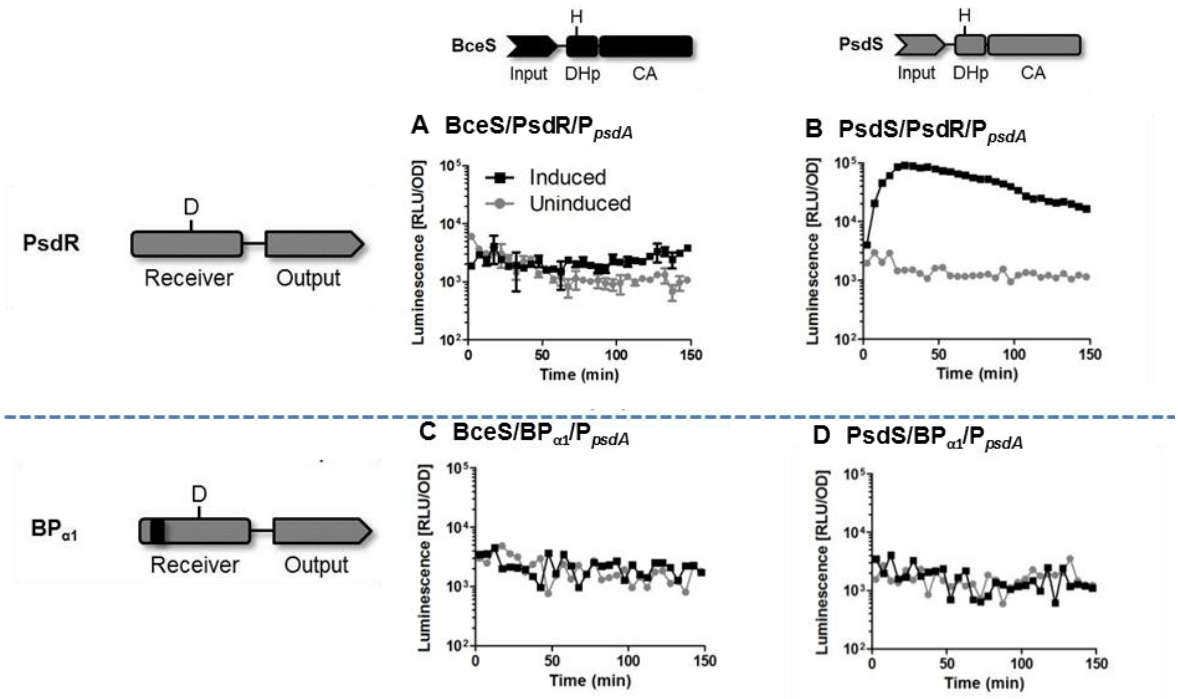
Based on the direct coupling analysis, five amino acids in the α1 helix and one residue on the β5-α5 loop of BceR/PsdR receiver domain were predicted to dictate specific interactions with eight amino acids of the BceS/PsdS DHp domains in *B. subtilis* (Procaccini *et al.*, 2011) (Fig. 5.3A). To experimentally verify the function of the α1 helix and the β5-α5 loop in specificity determination, we first constructed a chimera, $BP_{\alpha1}$, with the whole α1 helix of PsdR substituted by the corresponding region of BceR (Fig. 5.5). This chimera was dysfunctional, since it was unable to induce the expression of the promoter construct with either an active BceS or PsdS (Fig. 5.5C and 5.5D). To investigate if the α1 helix is the specificity determinant, another chimera, $PB_{\alpha1}$, was constructed with the α1 helix on BceR receiver domain replaced by the α1 helix of PsdR (Fig. 5.5). This chimera RR strongly upregulated the activity of P_{bceA} in a BceS-dependent manner in the presence of bacitracin (Fig. 5.5E), and no induction of the promoter was observed with an active PsdS in the presence of nisin (Fig. 5.5F). Hence, $PB_{\alpha1}$ displayed a behavior similar to that of wild-type BceR. This result indicates that switching the α1 helix alone could not rewire the signal transduction specificity of Bce-like TCSs.

Next, we wanted to analyze if the β5-α5 loop is involved in determining specificity, that is, the α1 helix together with the β5-α5 loop should be able to determine the specificity between BceS/BceR and PsdS/PsdR. $PB_{\alpha1+loop\ (\beta5-\alpha5)}$, with both the α1 helix and the β5-α5 loop on BceR receiver domain replaced by corresponding regions of PsdR, was constructed and the signaling was checked from BceS and PsdS, respectively. Surprisingly, $PB_{\alpha1+loop\ (\beta5-\alpha5)}$ was able to highly induce the expression of the promoter construct with an active BceS (Fig. 5.5G), but had no induction of the promoter construct with an active PsdS (Fig. 5.5H). $PB_{\alpha1+loop\ (\beta5-\alpha5)}$, therefore, behaved similarly as wild-type BceR. These results demonstrate that the regions containing specificity determining amino acids predicted by direct coupling analysis are not sufficient to dictate the specificity of Bce-like TCSs in *B. subtilis*. This provoked a sense for additional specificity determinant that would allow rewiring the signal transduction between the Bce and Psd TCSs.

Sequence alignments showed that amino acids on the β2-α2 region (RR receiver domain) vary considerably between BceR and PsdR (Fig. 5.3A). To further analyze a pontential function of tthis region in specificity determination, we constructed chimera $BP_{\beta2-\alpha2}$ with this region on PsdR switched

into the corresponding region of BceR (Fig. 5.5). This chimera showed a low level of induction (10-fold over the uninduced control) with BceS (Fig. 5.5I), and a high level of induction with PsdS (50-fold over the uninduced control) (Fig. 5.5J). These results indicated that chimera $BP_{\beta2\text{-}\alpha2}$, which is PsdR carrying the β2-α2 region of BceR, accepted a weak signal from BceS. However, the signaling from PsdS to $BP_{\beta2\text{-}\alpha2}$ was not diminished and was still as strong as to wild-type PsdR. This data reveal that the β2-α2 region can influence the specificity of the Bce-like TCSs but is by itself not able to fully determine signaling specificity between the HK and the RR.

Next, we tested the effect of exchanging the α1 helix and the β2-α2 region together on specificity. Towards that goal, we further constructed chimera $BP_{\alpha1\text{-}\beta2\text{-}\alpha2}$ (PsdR with the α1-β2-α2 region substituted by the corresponding region of BceR) and analyzed the signaling with either BceS or PsdS. P_{psdA} was strongly induced in the strain harboring BceS and $BP_{\alpha1\text{-}\beta2\text{-}\alpha2}$ after addition of bacitracin (40-fold over the uninduced control) (Fig. 5.5K), but almost not induced after addition of nisin in the strain harboring PsdS and $BP_{\alpha1\text{-}\beta2\text{-}\alpha2}$ (3-fold over the uninduced control) (Fig. 5.5L). These results demonstrate that PsdR with the whole α1-β2-α2 region substituted by the corresponding region of BceR changed the specificity of signal transduction from PsdS to BceS. This data therefore indicates that the α1 helix together with the β2-α2 region of the Bce-like RRs receiver domain is necessary to determine the specific interaction with the cognate HK, since exchanging this region is sufficient to rewire the signal transduction for Bce-like TCSs.



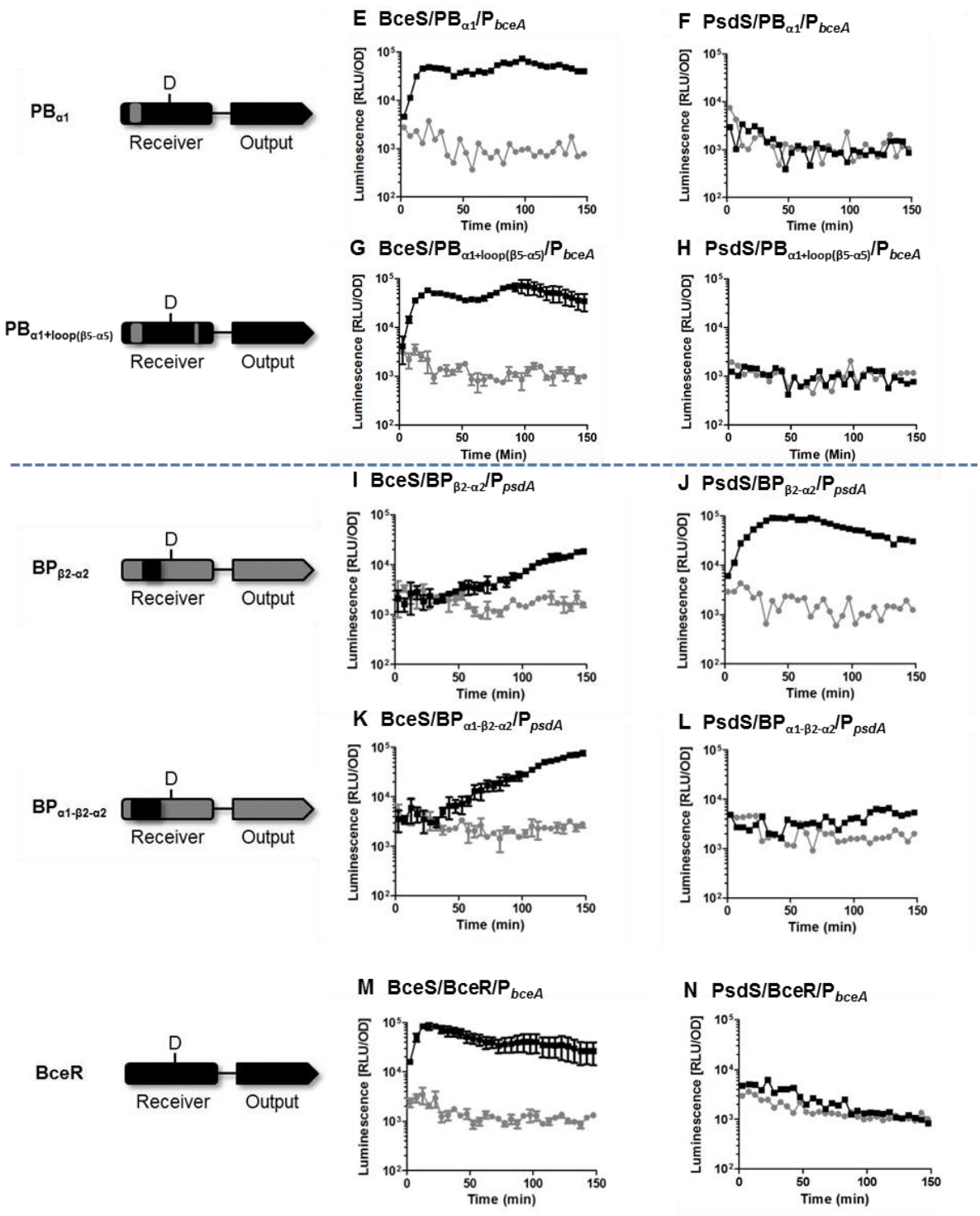


Figure 5.5. Dissection the specificity determinants on the RR receiver domain. Signal transduction between different HK and RR pairs was monitored *in vivo* as the induction of P$_{bceA}$-*luxABCDE* or P$_{psdA}$-*luxABCDE* transcriptional fusions within 150 min in the presence of antibiotics (black square): bacitracin for BceS activation and nisin for PsdS activation, and compared with the uninduced controls (grey square) as described in Fig. 5.2. Schematics of HKs are illustrated on top of the graphs with the same shading as in Fig. 5.1. Schematics of RR constructs are illustrated on the left side of the graphs. BP chimera represents the corresponding secondary element (α1, β2-α2, or α1-β2-α2) from PsdR (grey) is replaced by BceR (black), and PB chimera represents the corresponding secondary element (α1 or α1+loop (β5-α5)) from BceR (black) is replaced by PsdR (grey). The combinations of different HKs and RRs are indicated on top of each graph.

Discussion

The genome of *B. subtilis* encodes three paralogous Bce-like TCSs, which share significant sequence similarity and were predicted to have considerable cross-talk (Procaccini *et al.*, 2011). In contrast to the prediction, experiments clearly demonstrated that they are insulated quite well with only minor level of cross-regulation between BceS and PsdR *in vivo* (Rietkötter *et al.*, 2008). The specificity of TCSs is extremely important for bacteria to ensure the desired responses to specific stimuli, and is mainly maintained by molecular recognition of interactions between HKs and cognate RRs. A number of studies were recently performed to understand the determinant of specificity between HKs and RRs (Skerker *et al.*, 2008, Siryaporn *et al.*, 2010, Capra *et al.*, 2012). The co-crystal structure of HK853-RR468 from *T. maritima* showed that the α1 helix and the β5-α5 loop of the RR468 receiver domain interact directly with two α helices of HK853 DHp domain, implying that specificity is most likely determined by specific interactions on this surface (Casino *et al.*, 2009) (Fig. 5.3B). Further analysis of RRs (OmpR, RstA and CpxR) from the OmpR subfamily confirmed the importance of this interaction surface in specificity determination (Capra *et al.*, 2010). EnvZ/OmpR, CpxA/CpxR and RstB/RstA are three well-insulated TCSs in *E. coli*. Substitution of three residues predicted for specificity on the α1 helix of OmpR with the corresponding residues from RstA weaken the phosphotransfer from EnvZ to the substituted OmpR derivative, but no visible phosphotransfer was observed between RstB and the substituted OmpR derivative. Based on this three-amino acid substitution, subsequent substitution of three more amino acids on the OmpR β5-α5 loop by the corresponding residues of RstA diminished the phosphotransfer from EnvZ to OmpR and established the phosphotransfer from RstB to OmpR. Similarly, the study between EnvZ-OmpR and CpxA-CpxR systems proved that the α1 helix and the β5-α5 loop of OmpR/RstA/CpxR receiver domain are specificity determinants (Capra *et al.*, 2010).

However, the mechanism of specificity determination between these three paralogou Bce-like TCSs in *B. subtilis* was still poorly understood. In this chapter, we investigated determinants on Bce-like RRs that dictate specific interactions with their cognate HKs to maintain the signal transduction fidelity between Bce-like TCSs. In accordance with the other studied OmpR subfamily members (Howell *et al.*, 2003), we verified that the specificity determinants are located on the receiver domain of Bce-like RRs. Previous studies of other OmpR subfamily members indicated that normally the α1 helix and the β5-α5 loop of the RR receiver domain are responsible for specific interactions with the cognate HK (Podgornaia *et al.*, 2013). In contrast to studies of OmpR, swapping the α1 helix and the β5-α5 loop on the receiver domain of BceR into the corresponding regions of PsdR failed to rewire the signaling specificity. This chimera still behaved like wild-type BceR, which accepted the signal from BceS, but not from PsdS (Fig. 5.5G and 5.5H). The fact that determinants predicted by direct-coupling analysis (Procaccini *et al.*, 2011) are not sufficient to determine specificity explaining the discrepancy between in silico modeling and *in vivo* facts regarding the predicted cross-talk between the BceRS and the PsdRS systems.

Our results demonstrated that to solve the problem of the insufficient discrimination of the α1 helix and the β5-α5 loop on Bce-like RRs, *B. subtilis* developed an extended determinant ─ the β2-α2 region ─ working together with the α1 helix to guarantee the specificity of these three paralogous Bce-like TCSs. By exchanging both the α1 helix and β2-α2 region together of PsdR receiver domain into the corresponding regions of BceR, we successfully redirected the signal transmission from BceS to the substituted PsdR derivative (Fig. 5.5K and 5.5L). Our results revealed that *B. subtilis* evolved a double-insurance mechanism by using two indispensable specificity determinants to ensure the insulation of these three Bce-like TCSs to mediate the desired and beneficial responses to specific AMPs.

All chimeras, including exchanging of the complete receiver domain, showed slower dynamics of target promoter induction. Comparison of the data in Figure 5.2C and Figure 5.5K strongly suggests that the α1 helix together with the β2-α2 is indeed both necessary and sufficient to determine the specificity in molecular interactions with the cognate HK and hence the specificity in the phosphotransfer reaction.

In the co-crystal structure of HK853-RR468, the β2-α2 region of the RR receiver domain is not located on the interaction surfaces with the cognate HK. Bce-like TCSs are usually functionally related with ABC transporters, and a previous study suggested a positive interaction between the BceAB (ABC transporter) and BceR (RR) *in vivo* in the presence of BceS (Dintner *et al.*, 2014). This extended specificity determining region possibly forms specific interaction with the ABC transporter. However this hypothesis still needs to be proved by further experiments.

Experimental procedures

Bacterial strains and growth conditions

All strains used in this study are listed in Table 5.1. *E. coli* DH5α and XL1-blue were used for cloning. *E. coli* and *B. subtilis* were routinely grown in Luria-Bertani (LB) medium at 37°C with agitation (200 rpm). *B. subtilis* was transformed by natural competence as previously described (Harwood & Cutting, 1990). Selective media contained ampicillin (100 µg ml^{-1} for *E. coli*), chloramphenicol (35 µg ml^{-1} for *E. coli*, 5 µg ml^{-1} for *B. subtilis*), kanamycin (10 µg ml^{-1} for *B. subtilis*), erythromycin 1 µg ml^{-1} with lincomycin 25 µg ml^{-1} (for macrolide-lincosamide-streptogramin B (MLS) resistance in *B. subtilis*) or spectinomycin (100 µg ml^{-1} for *B. subtilis*). Bacitracin was supplied as the Zn^{2+}-salt. 0.2% (w/v) xylose was added to media for the production of BceS. Solid media contained 1.5 % (w/v) agar. Growth was measured as optical density at 600 nm wavelength (OD_{600}).

Construction of plasmids and genetic techniques

All plasmid constructs are listed in Table 5.2; all primer sequences used for this study are listed in Table 5.3. The constructs for the wild-type and chimeric RRs in *B. subtilis* were cloned according to the BioBrick standard (Knight, 2003). To facilitate constitutive expression in *B. subtilis*, a BioBrick of the *bceRS* operon promoter, P_{bceR}, of *B. subtilis* was amplified and cloned into the EcoRI and SpeI sites of vector pSB1A3, creating pCF144. The BioBricks of BceR and PsdR containing an optimal Shine-Dalgarno sequence for *B. subtilis* were similarly cloned into pSB1A3 via EcoRI and SpeI, creating plasmids pCFSB101 and pCFSB103. The Biobricks of the chimeric RRs were constructed based on BceR and PsdR Biobricks. Further assembly of P_{bceR} BioBrick, RR BioBrick together with a C terminal triple FLAG tag Biobrick into vector $pBS_{BS}2E$ created plasmids listed in Table 5.2.

These plasmids were transformed into *B. subtilis* reporter strains TMB1975 (W168 Δ*bceRS* Δ*psdRS* Δ*yxdJK-yxeLMy-xeA* P_{bceA}-*lux* P_{xyl}-*bceS*) or TMB1976 (W168 Δ*bceRS* Δ*psdRS* Δ*yxdJK-yxeLM-yxeA* P_{psdA}-*lux* P_{xyl}-*bceS*) to check the signal from BceS, and TMB2051 (W168 Δ*bceR psdR*::*kan* P_{bceA}-*lux*) or TMB2052 (W168 Δ*bceR psdR*::*kan* P_{psdA}-*lux*) to check the signal transduction from PsdS (Table 5.1). The production of BceS is under the control of a xylose-inducible (Pxyl) promoter and the production of PsdS is under the control of its native operon.All constructs were checked for PCR-fidelity by sequencing, and all created strains were verified by PCR using appropriate primers.

Luciferase assays

Luciferase activities of *B. subtilis* strains were assayed using a Synergy™2 multi-mode microplate reader from BioTek® controlled by the software Gen5™. LB medium was inoculated 1:500 from overnight cultures, and each strain was grown in 100 µl volumes in a 96-well plate. Cultures were incubated at 37°C with shaking (intensity: medium), and the OD_{600} was monitored every 10 min. At an OD_{600} of 0.02 (4-5 doublings since inoculation; corresponding to OD_{600} = 0.1 in cuvettes of 1 cm light-path length), either bacitracin was added to a final concentration of 2 µg ml^{-1}, or nisin to a final concentration of 8 µg ml^{-1}; in all cases one well was left untreated. Cultures were further incubated for 2.5 h, and the OD_{600} and luminescence (endpoint-reads; 1 s integration time; sensitivity: 200) were monitored every 5 min. OD_{600} values were corrected using wells containing 100 µl LB medium as blanks. Raw luminescence output (relative luminescence units, RLU) was normalized to cell density by dividing each data-point by its corresponding corrected OD_{600} value (RLU/OD).

Tables

Table 5.1. Bacterial strains used in this study.

Strain	Genotype or characteristic(s)	Reference or source
E. coli strains		
DH5α	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17($r_K^- m_K^+$) *rel*A1 *gln*V44 *Φ80'* Δ*lacZ* Δ*M15* Δ*(lacZYA-argF)U169*	(Grant *et al.*, 1990)
XL1-Blue	*end*A1 *gyr*A96*(nal^R)* *thi*-1 *rec*A1 *rel*A1 *lac* *sup*E44 [F'*pro*AB$^+$ *lacI*^{q} *Δ(lacZ)M15] hsd*R17*(*r_K^- m_K^+)	Stratagene
B. subtilis strains		
TMB1975	W168 Δ*bceRS psdRS yxdJK-yxeLM-yxeA* P_{bceA}-*lux* *thrC*::pAS718 (*bceS*)	This study
TMB1976	W168 Δ*bceRS psdRS yxdJK-yxeLM-yxeA* P_{psdA}-*lux* *thrC*::pAS718 (*bceS*)	This study
TMB2026	TMB2051 *lacA*::pCF2E11	This study
TMB2029	TMB1975 *lacA*::pCF2E11	This study
TMB2030	TMB1976 *lacA*::pCF2E12	This study
TMB2051	W168 Δ*bceR psdR*::*kan sacA*::pSDlux101 (P_{bceA}-*lux*)	This study
TMB2052	W168 Δ*bceR psdR*::*kan sacA*::pSDlux102 (P_{psdA}-*lux*)	This study
TMB2065	TMB2052 *lacA*::pCF2E12	This study
TMB2072	TMB2052 *lacA*::pCF2E25	This study
TMB2073	TMB2052 *lacA*::pCF2E26	This study
TMB2074	TMB2052 *lacA*::pCF2E27	This study
TMB2075	TMB2052 *lacA*::pCF2E28	This study
TMB2076	TMB2051 *lacA*::pCF2E29	This study
TMB2085	TMB1976 *lacA*::pCF2E25	This study
TMB2086	TMB1976 *lacA*::pCF2E26	This study
TMB2087	TMB1976 *lacA*::pCF2E27	This study
TMB2088	TMB1976 *lacA*::pCF2E28	This study
TMB2089	TMB1975 *lacA*::pCF2E29	This study
TMB2139	TMB1976 *lacA*::pCF2E31	This study
TMB2140	TMB1976 *lacA*::pCF2E32	This study
TMB2141	TMB1976 *lacA*::pCF2E33	This study
TMB2142	TMB1976 *lacA*::pCF2E34	This study
TMB2144	TMB2052 *lacA*::pCF2E31	This study
TMB2145	TMB2052 *lacA*::pCF2E32	This study
TMB2146	TMB2052 *lacA*::pCF2E33	This study
TMB2147	TMB2052 *lacA*::pCF2E34	This study

Table 5.2. Vectors and plasmids used in this study.

Plasmid	Genotype or characteristic(s)[a]	Primers used for cloning	Reference or source
Vectors			
pSB1A3	Replicative *E. coli* vector for cloning,amp^R, *rfp* in BioBrick MCS		(Vick *et al.*, 2011)
pBS2E	pAX01-derivative vector for cloning, *rfp* in BioBrick MCS; amp^R, mls^R, integrates in *lacA* in *B. subtilis*		(Radeck *et al.*, 2013)
Plasmids			
pCF144	pSB1A3 P_{bceR} Biobrick	2610/2611	This study
pCF2E11	pBS2E P_{bceR}-*bceR*-3x*FLAG*		This study
pCF2E12	pBS2E P_{bceR}-*BP1*-3x*FLAG*	2610/2797 2798/2730	This study
pCF2E25	pBS2E P_{bceR}-*BP2*-3x*FLAG*	2610/2828 2829/2730	This study
pCF2E26	pBS2E P_{bceR}-*BP3*-3x*FLAG*	2610/2830 2831/2730	This study
pCF2E27	pBS2E P_{bceR}-*BP4*-3x*FLAG*	2610/2832 2833/2730	This study
pCF2E28	pBS2E P_{bceR}-*BP5*-3x*FLAG*	2610/2834 2794/2730	This study
pCF2E29	pBS2E P_{bceR}-$PB_{\alpha 1}$-3x *FLAG*	2610/2767 2768/2673	This study
pCF2E31	pBS2E P_{bceR}-*psdR*-3x*FLAG*		This study
pCF2E32	pBS2E P_{bceR}-$BP_{\alpha 1}$-3x*FLAG*	2610/2793 2794/2730	This study
pCF2E33	pBS2E P_{bceR}-$BP_{\beta 2\text{-}\alpha 2}$-3x*FLAG*	2610/3132 2768/2730	This study
pCF2E34	pBS2E P_{bceR}-$BP_{\alpha 1\text{-}\beta 2\text{-}\alpha 2}$-3x*FLAG*	2610/27933133/2730	This study
pCFSB101	pSB1A3 *bceR* Biobrick	2579/2673	This study
pCFSB103	pSB1A3 *psdR* Biobrick	2729/2730	This study
pSB1C3-3xFlag-tag	pSB1C3 3x*Flag* Biobrick		Laboratory stock

[a]Amp, ampicillin; mls, macrolide-lincosamide-streptogramin B group antibiotics; r, resistant.

Table 5.3. Primers used in this study.

Primer name	Sequence (5'-3')[a]	Use
TM2579	GATCGAATTCGCGGCCGCTTCTAGAAAGGAGGTGGCCGGCATG AGTCGATTGTTTAAACTTTTG	*bceR* prefix
TM2610	AATTGAATTCGCGGCCGCTTCTAGAGTTCCGATGATTCTTGCG GCC	P_{bceR} prefix
TM2611	AATTACTAGTACAAGTGTATAGCAAAACGCC	P_{bceR} suffix
TM2673	AAATTACTAGTAATTACCGGTATCATAGAACTTGTCCTCTTC	*bceR* suffix
TM2729	AAATTGAATTCGCGGCCGCTTCTAGAAAGGAGGTGGCCGGCGT GTATCGGATTTTGCTTG	*psdR* prefix
TM2730	AAATTACTAGTATTAACCGGTACATTCCGCTTCATCCTTC	*psdR* suffix
TM2767	gaagatgaccgcccagcaaagaagcaatccgTTCATCATCTTCAATCAGC	$PB_{\alpha1}$ up rev
TM2768	tttgctgggcggtcatcttcaaaaatacggaTATGATGTATACGGCATTC	$BP_{\beta2\text{-}\alpha2}$ down fwd
TM2793	ttaaacgatccttgatttcatgaaacagcgaCTCATCATCTTCCACAAGC	$BP_{\alpha1}$ up rev
TM2794	tgaaatcaaggatcgtttaacgggatggtccTATGAAGTGAAAATTGCTGAACAG C	$BP_{\alpha1}$ down fwd
TM2797	GAACATCGCTTGGATTTTCG	BP1 up rev
TM2798	cgaaaatccaagcgatgttcCGCCGCACATATGGTGAATATTC	BP1 down fwd
TM2828	TGCCCCGAGCTGCATGGAC	BP2 up rev
TM2829	tccatgcagctcggggcaGATGATTATATCACAAAGCCG	BP2 down fwd
TM2830	AACATTTGACCGGGAGCGG	BP3 up rev
TM2831	cgctcccggtcaaatgttCCGATTATCTTTATATCGGC	BP3 down fwd
TM2832	ATTAACCGCCGCAAATTCCTG	BP4 up rev
TM2833	gaatttgcggcggttaatCCTGATCTTGTGCTGCTTG	BP4 down fwd
TM2834	GGACCATCCCGTTAAACGATC	BP5 up rev
TM3132	TCCGTATTTTTGAAGATGACCG	$BP_{\beta2\text{-}\alpha2}$ up rev
TM3133	TCGCTGTTTCATGAAATCAAGG	$BP_{\alpha1\text{-}\beta2\text{-}\alpha2}$ down fwd

[a]Restriction sites are underlined; overlaps to other primers for PCR fusions are shown by lower case letters.

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Chapter VI

Concluding Discussion

6. Concluding discussion

Resistance against antimicrobial peptides (AMPs) largely determines bacterial survival in competitive habitats. Among the resistance mechanisms developed by bacteria, the most efficient ways often involve ATP-binding cassette (ABC) transporters. A special type of BceAB-like ABC transporters, is exclusively contained within and widespread among Firmicutes bacteria (Gebhard, 2012). It frequently pairs with an adjacent BceRS-like two-component system (TCS), forming the Bce-type specific AMP sensing and detoxification module (Dintner *et al.*, 2011). Although a number of Bce-type systems have been studied in *Bacillus subtilis* and *Staphylococcus aureus* (reviewed in (Gebhard & Mascher, 2011)), many questions remain unanswered. The aim of this thesis was to obtain a deep insight into the regulatory network of Bce-type systems in two Firmicutes bacteria: *Enterococcus faecalis* and *B. subtilis*.

The technical challenges of molecular studies in *E. faecalis* impedes the understanding of Bce-type AMP sensing and detoxification system. Therefore, we exploited *B. subtilis* as a heterologous host to study the *E. faecalis* systems **(Chapter II)**. By combining the homologous study in *E. faecalis* with the heterologous study in the *B. subtilis* platform, we successfully identified and characterized the Bce-type AMP sensing and resistance network of *E. faecalis*, which provides useful insight for clinical research **(Chapter III)**. *B. subtilis* has three paralogous but well insulated Bce-like systems. Focusing on the Bce and Psd systems, we investigated the molecular mechanisms using by *B. subtilis* to maintain the intrasystem signal transduction and intersystem insulation at the response regulator (RR)/promoter level **(Chapter IV)** and the histidine kinase (HK)/RR level **(Chapter V)**. At both interfaces, novel specificity determinants could be identified and characterized.

6.1. *Bacillus subtilis* as a heterologous host: advantages and considerations

Understanding AMP resistance mechanisms of pathogenic bacteria is important for antibiotic development. However, for many species, such studies are impeded by the difficulty of genetic manipulation as well as pathogenicity. Because of the easy genetic manipulation and the well-understood genetic background, the Gram-negative bacterium *Escherichia coli* has been used as a heterologous host for *in vivo* analysis of the VanRS TCSs in both type A and B vancomycin resistant enterococci (Silva *et al.*, 1998). However, the differences in cell wall composition between Gram-positive and Gram-negative bacteria suggest that *E. coli* is probably not a suitable host for heterologous studies of enterococci cell wall active AMP resistance systems. *B. subtilis*, on the other hand, the best-characterized member of Gram-positive bacteria with well understood AMP resistance systems (reviewed in (Jordan *et al.*, 2008)), might be a more suitable candidate for such heterologous studies of *E. faecalis* AMP resistance mechanisms. It provides numerous established genetic tools, a comparable GC content (43.5%) with *E. faecalis* (37.5%), similar transcription machinery, and the most important — a comparable cell envelope.

The objective of **Chapter II** was to exploit *B. subtilis* as a platform for heterologous study of AMP resistance mechanisms of *E. faecalis*. Two kinds of AMP resistance systems from *E. faecalis*, the BcrR one-component system and the $VanS_B$-$VanR_B$ two-component system, were transferred into *B. subtilis* and their functionality was demonstrated. The three native Bce-like AMP sensing and resistance systems in *B. subtilis* were deleted to minimize the interference with the heterologously introduced systems. We were also able to demonstrate that the one-component system BcrR from *E. faecalis* is fully functional in *B. subtilis* in both gene regulation and bacitracin resistance. In a previous study, the $VanS_B$-$VanR_B$ TCS system from *E. faecalis*, which can sense vancomycin and regulate the resistance operon, showed a constitutive expression of the target promoter P_{vanYB} in *B. subtilis* (Bisicchia *et al.*, 2011). In contrast to this result, by adjusting the expression level of the TCS using a xylose inducible promoter, P_{xyl}, we demonstrated that the target promoter P_{vanYB} of the $VanS_B$-$VanR_B$ TCS is induced in a vancomycin-dependent manner and the system is functionally produced in *B. subtilis*. With this *B. subtilis* platform, we were also able to functionally characterize a complex Bce-type system of *E. faecalis* with two ABC transporters and a regulatory TCS for bacitracin sensing and resistance. These results are presented in **Chapter III** and will be discussed in the next section.

Based on these results, we confirmed that *B. subtilis* is clearly a suitable heterologous host for studying cell wall-targeting antibiotic resistance systems of *E. faecalis*. Attentions must be paid to the genetic background of the host to minimize the interference of the intrinsic resistance system to the introduced system. The expression level of the introduced system may influence its function, which means optimization is required and important for functional analysis. In addition to functional characterization of AMP resistance systems, the high degree of competence of *B. subtilis* can also be used for preliminary high-throughput screening of random mutations and synthetic DNA libraries for deep understanding of the signal transduction mechanism of *E. faecalis*. Promising results can be validated in a more targeted fashion further in *E. faecalis*. *B. subtilis* is of course also a suitable host for heterologous studying of cell wall-targeting AMP resistance systems of the other Firmicutes bacteria.

6.2. The Bce-type modules are arranged differently within *Bacillus subtilis* and *Enterococcus faecalis*

The BceAB-like ABC transporters have been shown to be widely distributed in Firmicutes bacteria for AMP sensing and resistance (Dintner *et al.*, 2011). Three such ABC transporters, BceAB, PsdAB and YxdLM, were identified and characterized in *B. subtilis* by previous studies reviewed in (Gebhard & Mascher, 2011) (Fig. 6.1 *B. subtilis*). Each of these BceAB-like ABC transporters is functionally related with a cognate BceRS-like TCS, and together they form a signal transduction circuit. Taking the best understood BceRS-BceAB system as an example: the signaling starts when the ABC transporter BceAB detects the AMP and activates the cognate HK BceS by direct protein-protein interaction; the subsequent signal transmission within the TCS BceRS will upregulate the transcription

of the ABC transporter operon for further resistance (Ohki *et al.*, 2003, Coumes-Florens *et al.*, 2011, Dintner *et al.*, 2014).

The majority of BceAB-like ABC transporters, such as the above-mentioned three ABC transporters in *B. subtilis*, are usually regulated by BceRS-like TCSs encoded in the genomic neighborhood of the ABC transporters (Dintner *et al.*, 2011). However, there are still a number of such ABC transporters lacking neighboring TCSs. In the genome of *E. faecalis*, two putative BceAB-like transporters and one BceRS-like TCS were identified in previous study, but neither of these ABC transporters was located next to the TCS (Dintner *et al.*, 2011). To identify the relationship between these two BceAB-like ABC transporters and the only one BceRS-like TCS, functional analysis was done with these three gene loci in both *E. faecalis* and the heterologous host *B. subtilis* in **Chapter III**. We showed that these three gene loci are all required for full bacitracin resistance: (1) one ABC transporter, EF2050-2049, that was strongly upregulated in response to bacitracin can mediate bacitracin resistance; (2) the other ABC transporter, EF2752-2751, that was slightly induced by bacitracin is responsible for bacitracin perception; and (3) the expression of these two ABC transporter operons was differentially regulated by the TCS EF0927-0926, and the expression of the TCS was induced by an as-yet-unidentified regulator that is not directly part of the resistance network. We were able to build the bacitracin sensing and resistance network in *E. faecalis* (Fig. 6.1 *E. faecalis*), which starts when the transporter EF2752-2751 detects the presence of bacitracin and subsequently transfers the signal to the TCS EF0926-0927. Signaling within the TCS leads to a highly increased production of the transporter EF2050-2049 to mediate resistance against bacitracin, and a slightly increased production of the transporter EF2752-2751 to detect bacitracin. However, the mechanism of differentiating the level of regulation is not known and more investigation is needed to address this question.

Compared to the signaling transduction circuit of *B. subtilis* for AMP sensing and resistance (Fig. 6.1 *B. subtilis*), we demonstrated that products of these three gene loci of *E. faecalis* form another configuration for the AMP sensing and resistance module (Fig. 6.1 *E. faecalis*). The Bce-type ABC transporters and TCSs are arranged differently in *B. subtilis* and in *E. faecalis*. The combined functions of the ABC transporter BceAB with both bacitracin sensing and resistance in *B. subtilis* is separated into two in *E. faecalis*, i.e. one ABC transporter, EF2752-2751, is only responsible for bacitracin sensing and an additional one, EF2050-2049, is required for bacitracin resistance. The gene regulation is also split and differentiated in *E. faecalis*: the TCS is able to strongly upregulate the transcription of the resistance ABC transporter EF2050-2049 operon and slightly upregulate the transcription of the sensor ABC transporter EF2752-2751 operon. The expression of the BceRS TCS operon of *B. subtilis* is under the control of a constitutive promoter, while the expression of the EF0927-0926 TCS operon of *E. faecalis* it is induced by an as-yet-unidentified regulator, which is not directly part of the resistance network.

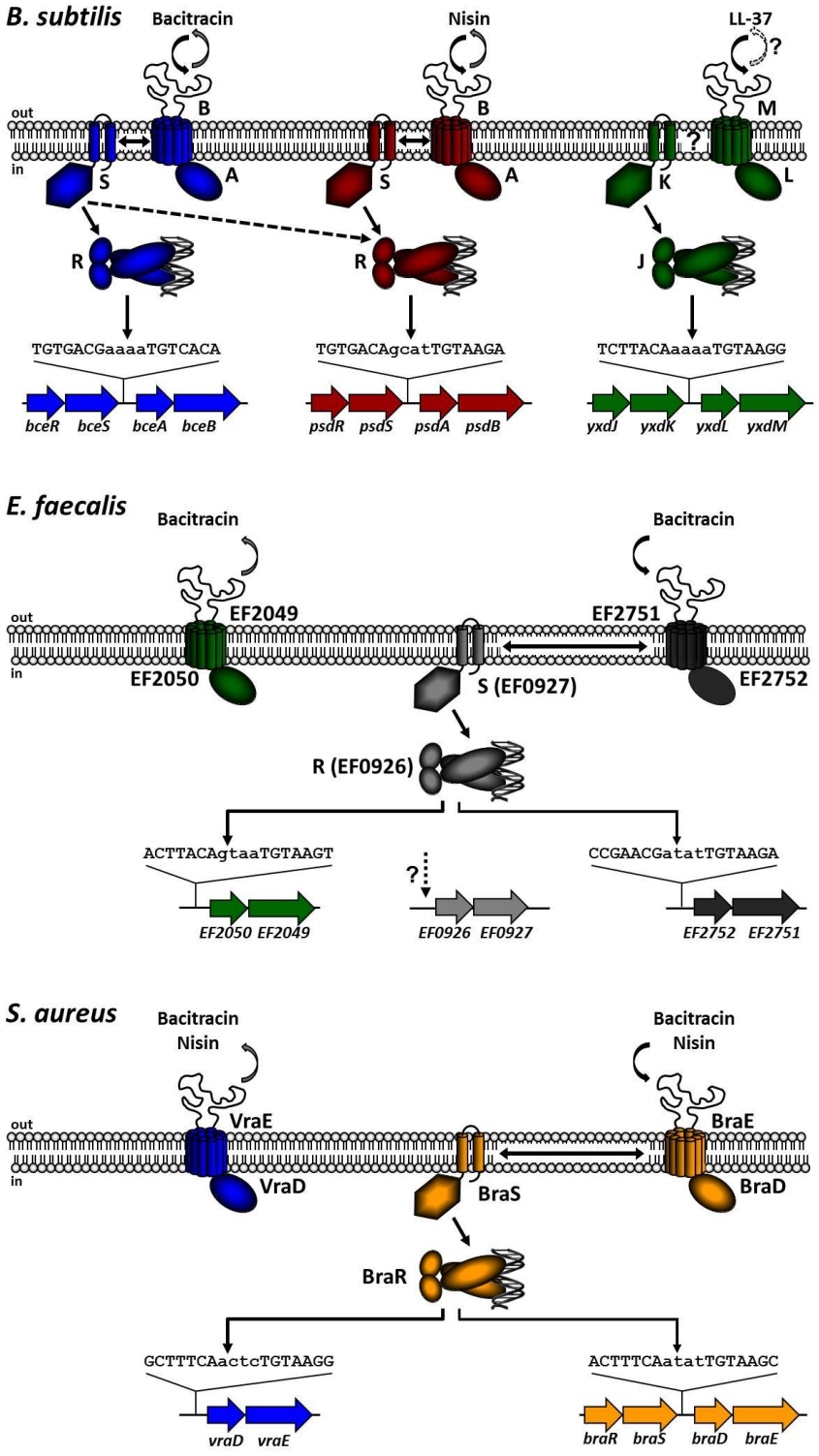

Figure 6.1. Schematic of signaling network of Bce-type AMP sensing and detoxification systems in *B. subtilis*, *E. facalis* and *S. aureus*. Coloring is chosen to reflect the assignment of each module to its phylogenetic group (yellow, group II; red, group III; blue, group IV; green, group VII; grey and black, no group assigned) (Dintner *et al.*, 2011). Names of genes and proteins of each system are given next to their schematics. Main substrates of each system are shown on top of the ABC transporter, with black curved downward arrow representing the perception and grey curved upward arrow representing the detoxification. Signal transfer between transporters and TCSs is indicated in the membrane bilayer. Phosphotransfer between HKs and RRs, transcriptional activation, and production of ABC transporters are shown by solid arrows. The minor level of cross-regulation between BceS and PsdR is shown by a dashed arrow. The induction of *EF0926-EF0927* operon by an unknown regulator in *E. faecalis* is showen by a dotted arrow. The differences in the strength of induction are reflected by thickness of lines. This figure was originally based in parts on (Gebhard & Mascher, 2011, Gebhard *et al.*, 2014), with modifications.

Our results of the *E. faecalis* Bce-type network are similar to the previously described BraDE-BraRS-VraDE network of *S. aureus* (Fig. 6.1 *S. aureus*). In *S. aureus*, the production of two Bce-type ABC transporters, VraDE and BraDE, is controlled by one Bce-type TCS BraRS. The BraDE ABC transporter can sense AMPs (bacitracin and nisin) and activate the TCS BraRS, which will further induce the expression of both ABC transporter operons *braDE* and *vraDE* for AMP detection and resistance, respectively (Li *et al.*, 2007, Hiron *et al.*, 2011). In addition to this system, the division of labor between two ABC transporters — one is only for AMP sensing while the other one is only for AMP resistance — was also observed in other Firmicutes bacteria, such as the ABC12-TCS12-orphan ABC transporter system in *Lactobacillus casei* for sensing and resistance against AMPs (Revilla-Guarinos *et al.*, 2013).

The existence of these two types of arrangement for AMP sensing and resistance network in Firmicutes bacteria raises the question: why do bacteria sometimes split functions of the ABC transporter and sometimes not? One explanation is that splitting the functions (AMP perception and resistance) of the ABC transporter may endow bacteria the possibility of mediating nonspecific responses, i.e. TCSs from other systems can cross-regulate the resistance ABC transporter operon. While combining the functions is beneficial for bacteria to maintain the specificity of the signal transduction pathway, i.e. the expression of the ABC transporter can only be regulated by its cognate TCS. It may also be that the separation of the AMP sensing and resistance functions and differentiation of the expression level keep the proper energy distribution between two ABC transporters. However, these hypotheses still need to be proved by experimental evidence.

6.3. Specificity determination of Bce-like TCSs in *Bacillus subtilis*

Cross-talk is defined as the communication between two distinct systems, which is often detrimental and must be kept to a minimum for generating desired responses to specific stimuli. However, there are examples of beneficial cross-talk under some conditions, such as mediating multiple responses to a single input, called cross-regulation (Laub & Goulian, 2007). Although minor level of cross-regulation between the HK BceS and the RR PsdR was observed *in vivo* under high concentrations of bacitracin in *B. subtilis*, the remaining parts of three paralogous Bce-type AMP sensing and detoxification systems are very well insulated from each other (Rietkötter *et al.*, 2008) (Fig. 6.1 *B. subtilis*). These three systems most likely evolved by duplication for a common ancestor followed by sequence divergence so that each of them can detect specific signal input and mediate corresponding output. In this evolutionary process, mutations that occurred on the specific interaction surfaces of one protein need to be compensated by corresponding mutations on its partner to maintain a functional interaction and simultaneously to avoid cross-talk (Szurmant & Hoch, 2010). This indicates that the predominant mechanism to ensure specificity is molecular recognition — the intrinsic ability of a protein to distinguish its cognate partner from the non-cognate ones with a relatively high preference. To guarantee the fidelity of the whole system, specificity needs to be maintained at every step of the

signal transduction pathway. In this thesis, we tried to gain a deeper understanding of mechanisms that dictate intrasystem fidelity and intersystem insulation of the two Bce-type systems (BceRS-BceAB and PsdRS-PsdAB) in *B. subtilis*, on both the HK/RR and the RR/promoter interaction level.

6.3.1. The mechanism of maintaining specificity at the RR and target promoter level

On P_{bceA} and P_{psdA}, no typical -35 element was found in the appropriate location upstream of the -10 element, indicating that the σ unit of the RNA polymerase cannot bind properly to the promoter by itself for further transcription initiation. However, this problem can be solved when the σ unit interacts with RRs, which bind to the up element of the promoter, to compensate its weak binding (Lee *et al.*, 2012). DNA binding domain structures of both PhoB and OmpR from OmpR subfamily demonstrated that the α2-α3 loop is essential for direct interaction with the σ subunit of the RNA polymerase (Martínez-Hackert & Stock, 1997, Blanco *et al.*, 2002). BceR and PsdR, which belong to the same subfamily, are assumed to assist the transcription initiation of RNA polymerase in a similar way.

Specific transcription initiation by the RR is important for maintaining the insulation of the signaling system. The similarity of Bce-like RRs DNA-binding domain and their binding sites on target promoters increases the potential of cross-talk on the transcription initiation level. However, we could show that Bce-type RRs in *B. subtilis* are extremely specific in inducing the transcription of their cognate ABC transporters. Although the binding of BceR to the cognate P_{bceA} and the non-cognate P_{psdA} were both observed *in vitro*, BceR can only induce the transcription of *bceAB* and not of *psdAB* *in vivo* **(Chapter IV)**. To understand the mechanism of specific regulation, further EMSAs and SPR assays were performed in **Chapter IV** and indicated that the *in vitro* cross-talk and *in vivo* insulation are due to the great difference in binding affinities, i.e. BceR has a much higher affinity to its cognate promoter P_{bceA} than to the non-cognate promoter P_{psdA}. Although it can bind to P_{psdA} *in vitro*, the binding affinity is still not high enough to recruit the RNA polymerase to the promoter for *in vivo* transcription initiation.

The affinity preference is the ability of the RR to distinguish the cognate promoter from non-cognate ones. Our data strongly suggests that *B. subtilis* evolved an intelligent mechanism to maintain this ability, which is a hierarchical and cooperative binding model (Fig. 6.2). Instead of only the one binding site reported by previous studies (Ohki *et al.*, 2003, de Been *et al.*, 2008), we demonstrated for the first time the necessary of two binding sites in the regulatory region of the Bce-type RR target promoters. By performing EMSAs and SPR assays of BceR with P_{bceA} mutants carrying either the main binding site random mutation or the secondary binding site random mutation, we further demonstrated that BceR has a high affinity and shows independent binding to the upstream main binding site. It has a low affinity to the downstream secondary binding site and cannot bind to it alone. Our data suggests that a BceR dimer first binds to the high affinity main binding site. This first binding event then assists the subsequent binding of another dimer to the downstream low affinity secondary binding site. Furthermore, we were able to show that exchanging the secondary binding site

resulted in a much stronger influence on promoter specificity than the main binding site by *in vivo* promoter activity assays. The hierarchical and cooperative binding model that enables BceR to have distinct binding affinities to its cognate promoter P_{bceA} from the non-cognate P_{psdA} is based on: (1) the main binding sites of these two promoters differ only in three bases, and provide a high affinity, low specificity docking site; (2) the secondary binding sites of these two promoters harbors five different bases, and represent a low affinity, but high specificity interaction site; and (3) the combination of the main binding site and the secondary binding site results in the relatively higher affinity of BceR to its cognate promoter P_{bceA} compared to the non-cognate P_{psdA} **(Chapter IV)**.

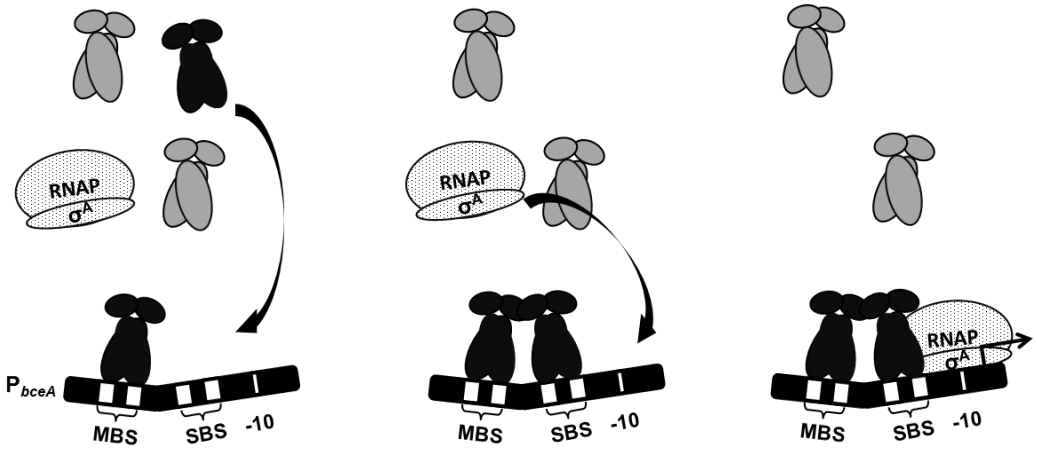

Figure 6.2. The model of specific transcriptional activation of *bceAB* operon by BceR and RNA polymerase. Firstly, BceR dimer (black) not PsdR dimer (gray) binds to the main binding site (MBS) on P_{bceA}. Secondly, the binding of the first dimer helps another dimer bind to the secondary binding site (SBS) upstream of the -10 element on P_{bceA}. Finally, the binding of the second BceR dimer to P_{bceA} can assist the binding of σ^A subunit of the holo RNA polymerase (RNAP) to the promoter region by direct protein-protein interaction and hence recruits the RNA polymerase for further transcription. The structure of the DNA is altered by the linker region between two binding sites.

The linker regions of these two promoters showed characteristically distinct GC/AT contents ─ P_{bceA} has high AT content, while P_{psdA} has high GC content. In **Chapter IV** we showed that mutating the linker region into a random sequence but still keeping the GC/AT content of each promoter slightly affected the promoter activity. However, exchanging linker regions between these two promoters, which means changing the GC/AT content, obtained more influence on the promoter activity. AT-rich sequence is known to cause the bending of DNA (Koo *et al.*, 1986). One possibility is that the AT-rich linker region on P_{bceA} confers a structural difference from P_{psdA} by bending the promoter between two binding sites to fit the binding of two BceR dimers.

The high specificity of the secondary binding site is presumably determined mainly by its first half site, because the first half sites of P_{bceA} and P_{psdA} differ in four out of seven bases, while the second half sites show only one. The sequence identity of the second half site and its location at the position of -35 suggest that it can probably be bound by both BceR and the σ^A subunit of the RNA polymerase. We showed that a P_{bceA} mutant with the secondary binding site replaced by the main binding site (main

binding site-linker-main binding site) lost the promoter activity (data not shown), which further indicates the importance of the second half for σ^A subunit binding and transcription initiation. This mechanism was already demonstrated for the transcription initiation by PhoB and σ^{70} binding together to the *pho* box (Blanco *et al.*, 2011).

This hierarchical and cooperative binding model has already been shown to be widely spread among the OmpR RR subfamily. For example, PhoB can bind cooperatively on two binding sites of the *pstS* promoter with different binding affinities (Blanco *et al.*, 2012). P_{ompF} has three OmpR binding sites with gradually reduced affinity from upstream to downstream, and the binding of OmpR to the first site is important for subsequent binding to the lower affinity downstream sites (Harlocker *et al.*, 1995). The cooperative binding model is also demonstrated using by RRs from other families. A recent study of the RR YpdB by SPR analysis also showed a two-step cooperative binding mechanism to its target P_{yhjX} (Behr *et al.*, unpublished). The binding of YpdB to the upstream site A initiates a subsequent binding of YpdB to the downstream site B. Similar as BceR binding to P_{bceA}, binding of YpdB to P_{yhjX} was completely abolished when the site A was inactivated, but YpdB was still able to bind properly to the downstream binding site B in the presence of an inactivated site A. The evolution of such complex regulatory systems is related, to some extent, to the regulatory function of RRs. PhoB and OmpR have been demonstrated to regulate dozens of operons in *E. coli* in the presence of certain stimuli. However, some operons need to be highly upregulated while others require only moderate or subtle regulation. Control of the desired expression level of these operons can be achieved through assembly of different numbers of binding sites with sequence divergence. For *B. subtilis*, a similar mechanism is used to maintain the signal transduction specificity and the regulatory insulation between three paralogous Bce-like systems. By evolving the cooperation of a high affinity but low specificity main binding site and a high specificity but low affinity secondary binding site, *B. subtilis* is able to ensure the Bce-like RR a relatively higher preference to its cognate promoter to the non-cognate ones, hence maintains the signaling fidelity of these three paralogoue Bce-like systems on the transcription level.

6.3.2. The mechanism of maintaining specificity at the HK and the RR interaction level

Bce-type RRs of *B. subtilis* belong to the OmpR RR subfamily (Fabret *et al.*, 1999). Previous studies of one family member, PhoP (RR of the PhoPR TCS), showed that substitution of three amino acids (Ser13, Leu17, and Tyr20) on the α1 helix together with one amino acid (Pro107) on the β5-α5 loop of PhoP receiver domain with corresponding amino acids from WalR (RR of the WalRK TCS) was sufficient to rewire the phosphotransfer between these two TCSs (Fig. 6.3A). The PhoP mutant could be phosphorylated by the non-cognate HK WalK and no longer by the cognate PhoR (Jende *et al.*, 2010). Similarly, replacing three amino acids (Arg15, Leu16, and Arg22) of the α1 helix and three amino acids (Pro106, Phe107, and Asn108) of the β5-α5 loop of the OmpR receiver domain (RR from the EvnZ/OmpR TCS) with corresponding amino acids of RstA (RR of the RstAB TCS) was able to redirect the phosphotransfer specificity from RstB to OmpR and eliminate the phosphotransfer from

the cognate EnvZ to OmpR (Capra *et al.*, 2010) (Fig. 6.3A). These results indicated that the α1 helix and the β5-α5 loop of the RR receiver domain contain the specificity determinants and can dictate specific interactions with the cognate HK. This is in agreement with the co-crystal structure of HK853-RR468. In this structure the α1 helix and the β5-α5 loop on the RR receiver domain form interaction surfaces with the HK DHp domain (Casino *et al.*, 2009).

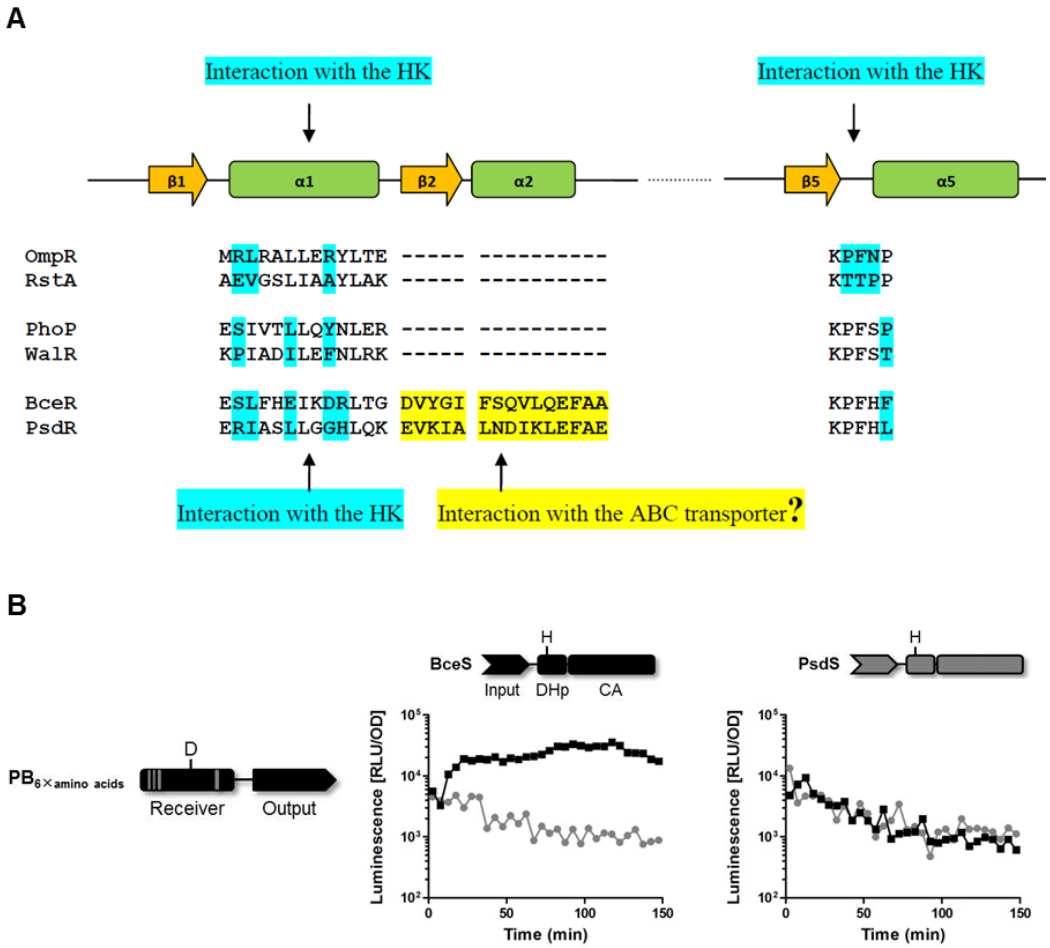

Figure 6.3. (A) Sequence alignment of the specificity determinants on RRs. Names of RRs and the sequence of specificity determinants are shown. Residues highlighted in cyan on OmpR, RstA, PhoP, and WalR are demonstrated by experiments, while on BceR and PsdR are predicted by direct-coupling analysis that are responsible for dictating specificity with their cognate HKs (Capra *et al.*, 2010, Jende *et al.*, 2010, Procaccini *et al.*, 2011). Residues highlighted in yellow on BceR and PsdR are the extended specificity determinants. Secondary structure elements are given above the RR sequences. **(B) Signaling analysis between BceS/ $PB_{6\times amino\ acids}$ and PsdS/ $PB_{6\times amino\ acids}$.** Signal transduction between HK and RR was monitored *in vivo* as the induction of P_{bceA}-*luxABCDE* transcriptional fusions within 150 min in the presence of antibiotics (black square): bacitracin for BceS activation and nisin for PsdS activation, and compared with the non-induced controls (grey square). Schematics of BceR and PsdR are illustrated on top and schematic of chimeric RR is illustrated on the left side of the graphs with the same shading as in **Chapter V**. On $PB_{6\times amino\ acids}$, six amino acids (highlighted in cyan in Fig. 6.3 (A)) on the receiver domain from PsdR is colored with grey and the region from BceR is colored with black.

BceR and PsdR also belong to the OmpR subfamily, and they may share similar mechanism as the other family members to determine specific interactions with their cognate HKs. However, chimera

$PB_{6\times amino\ acids}$, BceR with five amino acids on the α1 helix and one amino acid on the β5-α5 loop exchanged into corresponding residues of PsdR did not show any change of specificity (Fig. 6.3A and 6.3B). Our data indicates that the α1 helix and the β5-α5 loop of the RR receiver domain are not able to determine the specificity of Bce-like TCSs in *B. subtilis*. This is in good agreement with the prediction done by direct-coupling analysis that BceRS and PsdRS systems have the potential of considerable cross-talk (Procaccini *et al.*, 2011).

However, a previous study demonstrated that BceRS and PsdRS only have minor level of cross-regulation between BceS and PsdR at some concentrations of bacitracin (Rietkötter *et al.*, 2008), which indicates that Bce-like TCSs probably evolved different mechanism to maintain the signaling specificity. We further explored this hypothesis in **Chapter V** by making chimeric RRs between BceR and PsdR on different regions of the receiver domain. We demonstrated that the α1-β2-α2 region on the Bce-like RR receiver domain is the specificity determinant. BceS, instead of PsdS, strongly activated PsdR if this region is substituted by the corresponding part of BceR. We showed that the α1 helix of Bce-like RRs is not enough to determine specificity, but it is necessary and indispensable. We demonstrated that to compensate the lacking of specificity of the α1 helix, *B. subtilis* developed an extended determinant with high sequence diversity — the β2-α2 region. This region can partially determine the specificity but together with the α1 helix can fully dictate the specificity of Bce-type TCSs. Compared to the other studied TCSs, *B. subtilis* uses a different mechanism to maintain the insulation of Bce-like TCSs (Fig. 6.3A).

Two additional interaction surfaces are also observed in the co-crystal structure of HK853-RR468 (Casino *et al.*, 2009) (Fig. 1.5). One is the interaction between the β3-α3 loop of RR468 receiver domain and the ATP lid together with the β4-α4 loop of the HK853 CA domain. The other is the interaction between RR468 β4-α4 loop and the DHp-CA interdomain linker of HK853. However, the extended β2-α2 region on the RR receiver domain for Bce-type TCSs specificity is not located on any of these interaction surfaces. The Bce-type TCSs are often functionally related to their ABC transporters. A bold assumption is proposed that the additional region — β2-α2 — is possibly responsible for specific interaction with the cognate ABC transporter instead of with the HK. Supporting this idea, positive interaction between BceR (RR) and BceAB (ABC transporter) was observed in the presence of BceS (HK) by bacterial two-hybrid assay (BACTH) (Dintner *et al.*, 2014). However this hypothesis still needs to be proved by further experiments. Taken together, our studies in **Chapter V** suggest that *B. subtilis* evolved an extended determinant (the α1 helix and the β2-α2 region) to maintain the specificity of three paralogous Bce-like TCSs, which is different from the other studied OmpR subfamily members (the α1 helix and the β5-α5 loop). Our results provide a clear view of the location of specific interacting amino acids between HK and RR and a nice starting point for further studying the mechanism.

6.4. Open questions and further research

In this thesis, we demonstrated that *B. subtilis* is a suitable platform for heterologous studies of the AMP resistance mechanisms of *E. faecalis*. Based on this platform, we identified and characterized a Bce-type AMP sensing and resistance network of *E. faecalis*. Furthermore, we investigated the specificity determining mechanisms of two Bce-type systems in *B. subtilis*. Nevertheless, there are still several unsolved questions about the Bce-type AMP sensing and detoxification pathways.

6.4.1. Amino acids located on the α1-β2-α2 region for the specificity of Bce-like TCSs

By performing chimeric RR analysis, we were able to demonstrate the determinant — the α1-β2-α2 region — on the RR receiver domain that dictates the specificity of Bce-like TCSs. The α1 helix is comprised of 13 amino acids, and 5 of them are predicted by direct-coupling analysis that can interact specifically with corresponding amino acids on the cognate HK. However, no information is available about which amino acids on the β2-α2 region are responsible for specificity. To answer this question, more chimeras should be made by exchanging these 5 amino acids on the α1 helix together with different amino acids on the β2-α2 region between BceR and PsdR to construct a chimeric RR library. The screening can be done by high-throughput promoter-reporter assays on the plate and promising candidates will be checked further by both *in vivo* and *in vitro* experiments.

6.4.2. Is there direct interaction between BceAB and BceR?

Investigation of the specificity determinant between Bce-type TCSs showed an extended α1-β2-α2 region on the RR receiver domain different from the α1 helix and the β5-α5 loop of the other OmpR subfamily members. According to the co-crystal structure of HK853-RR468, the α1 helix is on the HK/RR interaction surface but not the β2-α2 region. The functional relation between Bce-type TCSs and Bce-type ABC transporters implies that this β2-α2 region possibly can form an interaction surface with the ABC transporter (Fig. 6.3A). BACTH assays suggested a positive interaction between BceR and BceAB in the presence of BceS (Dintner *et al.*, 2014). This is in good agreement with the previous study about the GraXSR-VraFG system in *S. aureus* that these proteins were shown to form a interaction complex by BACTH assay (Meehl *et al.*, 2007, Falord *et al.*, 2012).

Without BceS, however, no detectable interaction between BceR and BceAB was observed in the BACTH assay. It is also possible that BceS plays an important role in bringing BceR close to the cytoplasmic side of the membrane, so that BceR can interact with BceAB. The interaction between BceR and BceAB probably is not as strong as with BceS, therefore we failed to detect it in the absence of BceS. Formation of a four-protein complex, demanding that BceR match the specific interaction with both BceS (by the α1 helix of BceR) and BceAB (by the β2-α2 region of BceR), provides a double insurance to guarantee the signal transduction specificity of Bce-type systems. This hypothesis still needs to be proven by further experiments.

6.4.3. How does BceR initiate the transcription of the *bceAB* operon?

We have already demonstrated that BceR binds to two binding sites on P_{bceA} and upregulates transcription of the *bceAB* operon. The binding of a BceR dimer to the main binding site can assist another dimer binding to the low affinity secondary binding site. Does this assistance happen via direct interaction between two BceR dimers? If yes, on which region do they form the interaction surfaces?

Another open question is whether the RNA polymerase is recruited to the promoter by direct interaction between the second BceR dimer and the σ^A subunit. Blanco and colleagues have already demonstrated that the α2-α3 loop on the PhoB DNA binding domain is essential for transcription activation by interacting with the σ4 subdomain of the σ^{70} subunit. The σ4 subdomain can then bind to the *pho* box at the -35 position together with the PhoB dimer, but at different sides, and recruit the RNA polymerase for transcription initiation (Blanco *et al.*, 2002, Blanco *et al.*, 2011). In *B. subtilis*, the second halves of the secondary binding sites replace the -35 elements and the sequences are almost identical between P_{bceA} and P_{psdA}, which provides a potential low affinity binding region for the σ^A subunit. Substitution of the whole secondary binding site by the main binding site resulted in the loss of promoter activity, which probably undermines the essential binding of the σ^A subunit to the promoter. We suggest that the transcription initiation starts with the first dimer binding to the upstream binding site, which assists and stabilizes the binding of the second dimer to the secondary binding site by direct dimer-dimer interaction. This is followed by interacting with the σ^A subunit and recruiting it to the promoter DNA, which enhance the binding of the σ^A subunit to the second half of the secondary binding site and the -10 region to start the transcription of the *bceAB* operon. Further experiments are required to verify the interaction between BceR dimers, BceR dimer and the σ^A subunit of the RNA polymerase. Furthermore, the binding of the σ^A subunit to the second half of the downstream secondary binding site also needs to be proven in this model.

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Supervisors: Prof. Thorsten Mascher and PD Susanne Gebhard

Projects:

- Interaction specificity between histidine kinase and response regulator of Bce-like two component signal transduction systems in *B. subtilis*
- Specificity determinants on *bceA*-like promoters in *B. subtilis*
- Characterization of a bacitracin resistance network from *E. faecalis* in *B. subtilis*
- *B. subtilis* as a platform for molecular characterization of regulatory mechanisms of *E. faecalis* resistance against cell wall antibiotics

Sep 2008- July 2011

M.Sc. Microbiology, State Key Laboratory for Microbial Technology, Shandong University

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Projects:

- Investigation of microbial diversity, biomass degradation and corresponding enzymes during composting process

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B.Sc. Biotechnology, Biological & Chemical Faculty, Yantai University

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Project and practice:

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Publications

Fang C., Staroń A., Grafe M., Heermann R., Jung K., Gebhard S. and Mascher T. Insulation and wiring specificity determinants of BceR-like response regulators and their target promoters in *Bacillus subtilis*. *In preparation*

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Participated in translating the book: Biomass Recalcitrance——deconstruct the plant cell wall for bioenergy, Edited by Michael E. Himmel. (Chapter 9\10\15, from English to Chinese; published)

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Academic Meetings and Trainings

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