Extracytoplasmic Function σ Factors in *Bacillus* Species:

Investigation of Cell Envelope Stress Responses and Novel Signal Transducing Mechanisms

Dissertation

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Eidesstattliche Versicherung und Erklärung

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt wurde. Zudem wurden keine anderen als die angegebenen Quellen verwendet.

Außerdem versichere ich, dass die Dissertation keiner anderen Prüfungskommission vorgelegt wurde und ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

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Tina Wecke

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List of publications

Publications and manuscripts presented in this thesis:

Chapter 2

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Chapter 3

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Chapter 4

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Abbreviations

ABC	ATP binding cassette
ASD	anti-σ domain
atm	atmosphere
CESR	cell envelope stress response
CLD	cupin-like domain
CMD	carboxymuconolactone decarboxylase
COE	CMD protein, oxidoreductase and epimerase
DIG	digoxigenin
e.g.	for example
ECF	extracytoplasmic function
EMEA	European Medicines Agency
FDA	Food and Drug Administration
Fig.	figure
HK	histidine kinase
I-CLiPs	intramembrane cleaving proteases
IPTG	isopropyl-β-D-thiogalactopyranoside
MLS	macrolide-lincosamide-streptogramin B
MOA	mechanism of action
MRSA	methicillin-resistant *Staphylococcus aureus*
NCR	non-conserved region
Ni^{2+}-NTA	Ni^{2+}-nitrilotriacetic acid
OD	optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RACE	rapid amplification of cDNA ends
RIP	regulated intramembrane proteolysis
RNAP	RNA polymerase
ROMA	run-off transcription/microarray analysis
RR	response regulator
TAP	tobacco acid pyrophosphatase
TCS	two-component system
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRSA	vancomycin-resistant *Staphylococcus aureus*
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
ZAS	Zn^{2+} anti-σ

Summary

In their natural habitat bacteria are exposed to many environmental threats. Besides rapidly changing physicochemical parameters and an almost constant lack of nutrients, they also have to deal with the presence of antimicrobial compounds produced by competing organisms. The bacterial cell envelope is the interface between the cytoplasm and the environment. Its integrity is crucial for survival, which makes it a preferred target for antibiotics. In order to deal with cell envelope impairing agents and conditions before severe damage actually occurs, bacteria developed a number of signal transducing systems that enable them to sense the presence of antibiotics and respond appropriately by differential gene expression. Two different signal transducing principles orchestrate such responses: two-component systems (TCSs) and extracytoplasmic function (ECF) σ factors. The response of the Gram-positive model organism *Bacillus subtilis* to cell wall antibiotics has been studied extensively and involves four TCS (LiaRS, BceRS, PsdRS and YxdJK) and at least three ECF σ factors (σ^W, σ^M and σ^X). In the context of this thesis, we determined cell envelope stress responses with a focus on ECF σ factors.

In the first part of this work, we investigated and compared the response of *B. subtilis* to the cyclic lipopeptide antibiotics daptomycin and friulimicin B, which are both active against even multi-resistant Gram-positive pathogens. Genome-wide in-depth expression profiling at both the transcriptome and proteome level revealed that both antibiotics trigger an ECF σ factor-dependent response involving primarily σ^M and σ^V. This response was more strongly induced by friulimicin B than by daptomycin. In contrast, daptomycin exclusively and strongly induced the LiaRS TCS. These expression signatures indicate that both antibiotics act via completely different mechanisms of action, although they are structurally similar and generally interfere with cell envelope integrity. This demonstrates the great potential and specificity of global expression profiling as a powerful approach to characterize the mechanism of action of novel antibiotics or even differentiate between chemically related compounds.

In the second part, we determined the transcriptional response of *B. subtilis* to rhamnolipids, which are industrially important biosurfactants produced by *Pseudomonas aeruginosa*. Rhamnolipids also display antimicrobial activity by interfering with the

integrity of biological membranes. A genome-wide DNA microarray analysis of *B. subtilis* after treatment with rhamnolipids revealed for the first time that a single antimicrobial compound is able to induce two normally independent stress responses: the cell envelope stress response, in this case represented by the LiaRS TCS and the ECF σ factor σ^M, and the secretion stress response mediated by the CssRS TCS. Moreover, the LiaRS TCS and σ^M have a protective function against damage caused by rhamnolipids, while the role of CssRS in this response is still unclear.

Finally, we characterized a novel group of ECF σ factors with unique features, namely ECF41, which was identified in the context of a comprehensive classification of this protein family. A detailed bioinformatics analysis revealed a wide distribution of ECF41 σ factors with about 400 proteins from ten different bacterial phyla. This group shows an unusual but highly conserved genomic context. Obvious anti-σ factors are missing in the direct vicinity of the genes encoding the ECF41 σ factors. Instead, the ECF41 genes are associated with genes encoding carboxymuconolactone decarboxylases, oxidoreductases or epimerases. These transcriptional units represent the only targets and are often preceded by a highly conserved promoter motif recognized by the corresponding ECF41 σ factor, which has been experimentally demonstrated for both *Bacillus licheniformis* and *Rhodobacter sphaeroides*. Moreover, the ECF41 proteins harbor a large C-terminal extension, which is not present in other ECF σ factors. We demonstrated that this extension is clearly involved in regulation of ECF41 σ factor activity and possibly functions as a fused anti-σ factor-like domain.

Zusammenfassung

Bakterien sind in ihrer natürlichen Umgebung einer Vielzahl von schädlichen Umwelteinflüssen ausgesetzt. Neben schnell wechselnden physikalisch-chemischen Parametern und einem fast ständigen Nährstoffmangel stellen auch antimikrobielle Substanzen, welche von konkurrierenden Organismen produziert werden, eine große Herausforderung dar. Die bakterielle Zellhülle ist die Verbindung zwischen dem Cytoplasma und der Umgebung und ihre Integrität ist essentiell für das Überleben der Zelle, was sie zu einem bevorzugten Angriffspunkt für Antibiotika macht. Es ist daher wichtig, bereits auf zellhüllschädigende Substanzen und Bedingungen zu reagieren bevor die Zelle ernsthaft gefährdet ist. Daher haben Bakterien eine Vielzahl von signaltransduzierenden Systemen entwickelt, welche es ermöglichen Antibiotika wahrzunehmen und angemessen darauf zu reagieren. Zwei verschiedene Prinzipien der Signaltransduktion vermitteln solche Reaktion: Zweikomponentensysteme und *extracytoplasmic function* (ECF) σ-Faktoren. Die Antwort des Gram-positiven Modellorganismus *Bacillus subtilis* auf Zellwandantibiotika wurde bereits ausführlich untersucht und umfasst vier Zweikomponentensysteme (LiaRS, BceRS, PsdRS and YxdJK) und mindestens drei ECF σ-Faktoren (σ^W, σ^M and σ^X). Im Rahmen dieser Doktorarbeit haben wir solche Zellhüllstress-Antworten mit einem besonderen Fokus auf ECF σ-Faktoren untersucht.

Im ersten Teil dieser Arbeit haben wir die Antwort von *B. subtilis* auf die zyklischen Lipopeptid-Antibiotika Daptomycin und Friulimicin B, welche beide sogar gegen multi-resistente Gram-positive Pathogene wirksam sind, untersucht und verglichen. Genom-weite Expressionsanalysen sowohl auf Ebene des Transkriptoms als auch des Proteoms zeigten, dass beide Antibiotika eine ECF σ-Faktor-abhängige Antwort induzieren, welche hauptsächlich von σ^M und σ^V vermittelt wird. Diese Induktion ist sehr viel stärker für Friulimicin B als für Daptomycin. Im Gegensatz dazu induziert ausschließlich Daptomycin das LiaRS Zweikomponentensystem. Diese Unterschiede in den Expressionsprofilen deuten an, dass beide Antibiotika spezifische und komplett verschiedene Wirkmechanismen aufweisen, obwohl sie strukturell sehr ähnlich sind und generell die Zellhüllintegrität beeinträchtigen. Dies veranschaulicht das große Potential sowie die

Spezifität der Analyse solcher Expressionsprofile als einen Ansatz für die Charakterisierung neuer Antibiotika oder sogar der Differenzierung zwischen chemisch sehr ähnlichen Substanzen.

Im zweiten Teil haben wir die transkriptionelle Antwort von *B. subtilis* auf Rhamnolipide untersucht, welches von *Pseudomonas aeruginosa* produzierte und industriell wichtige Biotenside sind. Rhamnolipide beeinträchtigen die Integrität von biologischen Membranen und weisen somit auch antimikrobielle Eigenschaften auf. Eine auf DNA-Microarrays basierende Transkriptom-Analyse von *B. subtilis* nach Behandlung mit Rhamnolipiden zeigte erstmals, dass eine einzige antimikrobiell wirksame Substanz zwei normalerweise unabhängige Stressantworten induziert: die Zellhüllstress-Antwort, in diesem Fall vertreten durch das Zweikomponentensystem LiaRS und den ECF σ-Faktor σ^M, und die Sekretionsstress-Antwort vermittelt durch das Zweikomponentensystem CssRS. LiaRS und σ^M zeigen eine schützende Funktion gegenüber Schädigungen verursacht durch Rhamnolipide, während die Rolle des CssRS Zweikomponentensystems in dieser Antwort bisher unklar ist.

Zum Schluss haben wir eine neue Gruppe von ECF σ-Faktoren (ECF41) charakterisiert, welche einzigarte Merkmale aufweist und im Rahmen einer umfassenden Klassifizierung dieser Proteinfamilie identifiziert wurde. Eine detaillierte bioinformatische Analyse lässt eine weite Verbreitung dieser ECF41 σ-Faktoren mit etwa 400 Proteinen aus zehn verschiedenen bakteriellen Phyla erkennen. Außerdem weist diese Gruppe einen ungewöhnlichen, jedoch stark konservierten genomischen Kontext auf. Es gibt keinen offensichtlichen Anti-σ-Faktor in direkter genomischer Nähe der ECF41 σ-Faktor-kodierenden Gene. Stattdessen sind sie mit Genen assoziiert, welche entweder Carboxymuconolacton-Decarboxylasen, Oxidoreduktasen oder Epimerasen kodieren. Diese transkriptionellen Einheiten stellen die einzigen ECF41-abhängigen Zielgene dar und besitzen ein stark konserviertes Promotor-Motiv, welches von den dazugehörigen ECF41 σ-Faktoren erkannt wird, wie experimentell in *Bacillus licheniformis* und *Rhodobacter sphaeroides* gezeigt werden konnte. Darüber hinaus haben die ECF41-Proteine eine große C-terminale Erweiterung, die in keinen anderen ECF σ-Faktoren vorkommt. Diese Erweiterung ist wesentlich an der Regulation der ECF41 σ-Faktoren beteiligt und fungiert möglicherweise als eine fusionierte Anti-σ-Faktor-ähnliche Domäne.

Chapter 1

Introduction

Bacteria are very adaptable organisms and inhabit almost every possible habitat. The soil is a densely populated ecosystem with an enormous diversity of microorganisms. Microbial life in this environment is characterized by high competition between different organisms, lack of nutrients and rapidly changing physicochemical parameters, such as temperature, oxygen concentration or moisture. To survive in such a complex and life-threatening habitat, bacteria have to constantly monitor their environment and respond to changes before their vitality is seriously endangered. Therefore, they developed a number of signal transducing systems to orchestrate these responses and enable survival even under severe stress conditions (Paul & Clark, 1996, Storz & Hengge-Aronis, 2000).

1.1 The bacterial cell envelope

The bacterial cell envelope, which includes the membrane(s) and other components surrounding the cytoplasm, is an essential and complex multilayered structure. Its integrity and functionality is crucial for survival and has to be maintained at any time. The cell envelope counteracts the high internal osmotic pressure, gives the cell its shape and protects it from environmental threats, but it also serves as a selective barrier for nutrients and other molecules. Besides these mere physical characteristics, the cell envelope also constitutes an important communication interface between the cell and its surroundings. It contains a number of different sensory systems allowing the cell to monitor and respond to environmental changes (Dijkstra & Keck, 1996, Silhavy *et al.*, 2010).

Based on their cell envelope structure and according staining behavior, bacteria can be classified into two major groups: the Gram-positives and Gram-negatives (Fig. 1.1)

(Popescu & Doyle, 1996). The interior layer of the cell envelope, the inner or cytoplasmic membrane, is identical in both groups. It is a phospholipid bilayer containing integral membrane proteins, which are often involved in essential processes like energy production or transport. However, the outer layers of the cell envelopes of Gram-positive and Gram-negative bacteria differ significantly and are therefore introduced in detail in the following paragraphs.

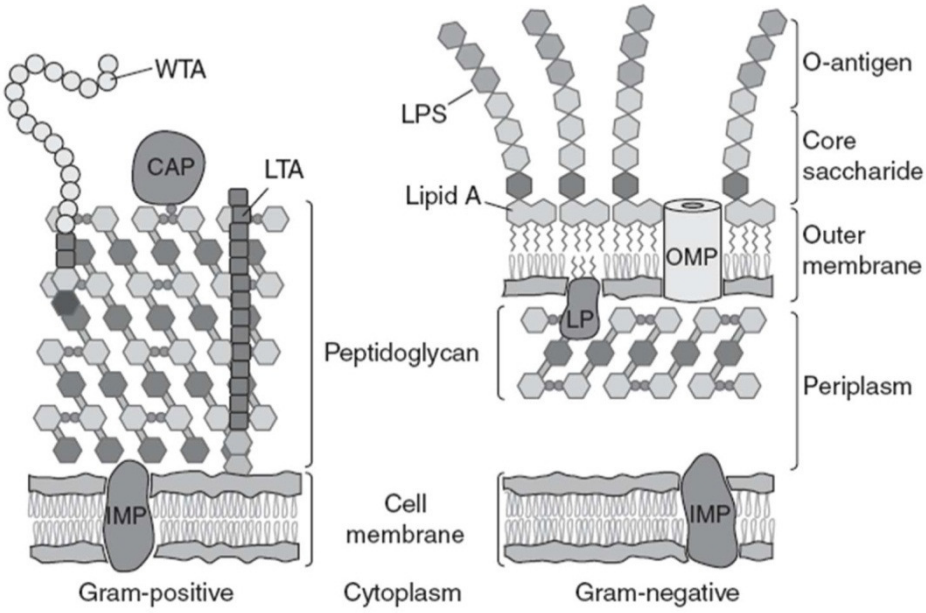

Figure 1.1. Composition of the Gram-positive and Gram-negative cell envelope. CAP, covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, outer membrane protein; WTA, wall teichoic acid. The figure is taken from (Silhavy *et al.*, 2010).

The outer layer of the Gram-positive cell envelope consists of a multi-layered peptidoglycan sacculus, also called cell wall, which can achieve a thickness of up to 50 nm. This allows the Gram-positive cell to withstand turgor pressures in the order of 20 atm (in contrast to 2-5 atm in the case of the single-layered peptidoglycan of Gram-negative bacteria (see below)). The overall structure of peptidoglycan can be best described as a fisherman's net, giving the sacculus both enormous strength and flexibility (Delcour *et al.*, 1999). Another major component of most Gram-positive cell walls are teichoic acids, which are polymers of glycerol- or ribitol-phosphate units. They can be either covalently attached to the peptidoglycan (wall teichoic acids) or anchored in the cytoplasmic membrane (lipoteichoic acids) (Delcour *et al.*, 1999, Neuhaus & Baddiley, 2003). Teichoic

acids are responsible for the overall negative net charge of the Gram-positive cell surface and can serve as a phosphate reservoir or scavengers of cations (Hughes *et al.*, 1973).

The composition of the Gram-negative cell envelope is more complex. In addition to a thin, often single-layered peptidoglycan sacculus, Gram-negative bacteria contain an outer membrane. In contrast to the cytoplasmic membrane, this outer membrane is an asymmetrical bilayer composed of phospholipids and lipopolysaccharides at the inner and outer leaflet, respectively. Lipopolysaccharides are glycolipids consisting of a hydrophobic membrane anchor, termed lipid A, and a covalently attached core oligosaccharide, which is often extended by a repeating oligosaccharide, called O-antigen (Bos *et al.*, 2007). Lipopolysaccharides can play a role in pathogenicity since they are responsible for the endotoxic shock caused by Gram-negative bacteria (Raetz & Whitfield, 2002). Two kinds of proteins are associated with the outer membrane: lipoproteins attached to the inner leaflet and integral outer membrane proteins with a cylindrical β-barrel conformation. While the function of most lipoproteins is still unknown, the integral outer membrane proteins often facilitate the passive diffusion of small molecules (Bos *et al.*, 2007, Silhavy *et al.*, 2010). The viscous space between the outer and inner membrane is called periplasm. It contains a high concentration of different kinds of proteins, for example periplasmic binding proteins involved in sugar and amino acid transport or chaperones functioning in envelope biogenesis (Silhavy *et al.*, 2010).

The cell envelopes of some bacteria contain additional components, such as proteinaceous structures called S-layers (Sleytr *et al.*, 1993) or extracellular matrices involved in biofilm formation (Branda *et al.*, 2005). The *Corynebacterineae*, including the important pathogen *Mycobacterium tuberculosis*, are generally classified as Gram-positive bacteria, but they have a very complex and unusual cell envelope containing arabinogalactan and covalently attached mycolic acids, giving them a waxy appearance and high antibiotic tolerance (Dover *et al.*, 2004).

1.2 Cell wall biosynthesis

Peptidoglycan is an essential and specific component of the cell envelope of almost all bacteria. Its main function is the maintenance of cell shape and integrity. Any degradation of the cell wall or inhibition of its biosynthesis consequently results in cell lysis (Vollmer

et al., 2008). Although the exact chemical composition of peptidoglycan varies from species to species, the main elements are linear glycan strands connected by short peptides. The glycan strands consist of alternating N-acetyl-muramic acid and N-acetyl-glucosamine molecules linked by β-(1,4)-glycosidic bonds. These glycan strands are crosslinked by a pentapeptide bridge attached to N-acetyl-muramic acid, which consequently leads to the characteristic net-like structure. Although the bacterial cell wall has been extensively studied for decades, even central questions regarding its architecture still remain mostly unanswered. One controversy is the orientation of the peptidoglycan components relative to the surface and axis of the cell. Two mutually exclusive models are being discussed: the "layered" model, in which both the glycan strands and peptides run parallel to the cytoplasmic membrane, and the "scaffold" model, in which the glycan strands run perpendicular and the peptides parallel to the membrane (Vollmer & Seligman, 2010). Another controversial aspect concerns the complexity of the Gram-positive cell envelope. Instead of a homogenous cell wall layer, cryo-electron microscopy revealed an inner wall zone with low-electron density and an outer wall zone with high-electron density in both *Bacillus subtilis* and *Staphylococcus aureus*. The outer zone seems to represent the actual cell wall consisting of peptidoglycan, while the inner zone can be interpreted as an extra-protoplasmatic compartment similar to the periplasmic space of Gram-negative bacteria (Matias & Beveridge, 2005, Matias & Beveridge, 2006). These data emphasize our limited understanding of bacterial cell envelope composition and peptidoglycan architecture, and future research might significantly change our current view of these fundamental structures.

The biosynthesis of peptidoglycan can be divided into three sections (Fig. 1.2): (i) synthesis of cell wall precursors in the cytoplasm, (ii) membrane-anchored assembly of these precursors and transport through the cytoplasmic membrane, and (iii) incorporation of new peptidoglycan units into the existing cell wall. Starting point of the cell wall biosynthesis is UDP-activated N-acetyl-glucosamine, which comes from the central carbon metabolism. N-acetyl-glucosamine is converted to N-acetyl-muramic acid in a two-step reaction catalyzed by MurA and MurB. Thereafter, the first three amino acids of the pentapeptide, classically consisting of alternating L- and D-amino acids, are added successively by the ligases MurC, MurD and MurE. The last two residues of the pentapeptide are first united and then attached as a dipeptide. The corresponding enzymes

are the ligases Ddl and MurF. The resulting N-acetyl-muramic acid pentapeptide is coupled to the lipid carrier undecaprenol-monophosphate at the interior side of the cytoplasmic membrane by the translocase MraY. The resulting complex is called lipid I. Subsequent addition of N-acetyl-glucosamine by the glycosyltransferase MurG results in lipid II, which is comprised of the complete peptidoglycan subunit linked via a pyrophosphate to the lipid carrier. The cell wall precursor is then translocated by a flippase to the exterior side of the cytoplasmic membrane and incorporated into the existing peptidoglycan through transglycosylation and transpeptidation. The remaining undecaprenol-pyrophosphate is dephosphorylated and transferred to the interior side of the membrane to be available for binding and transfer of another cell wall precursor. The steps of cell wall biosynthesis involving the lipid carrier undecaprenol are called lipid II cycle (Delcour *et al.*, 1999, Foster & Popham, 2002, Mohammadi *et al.*, 2011).

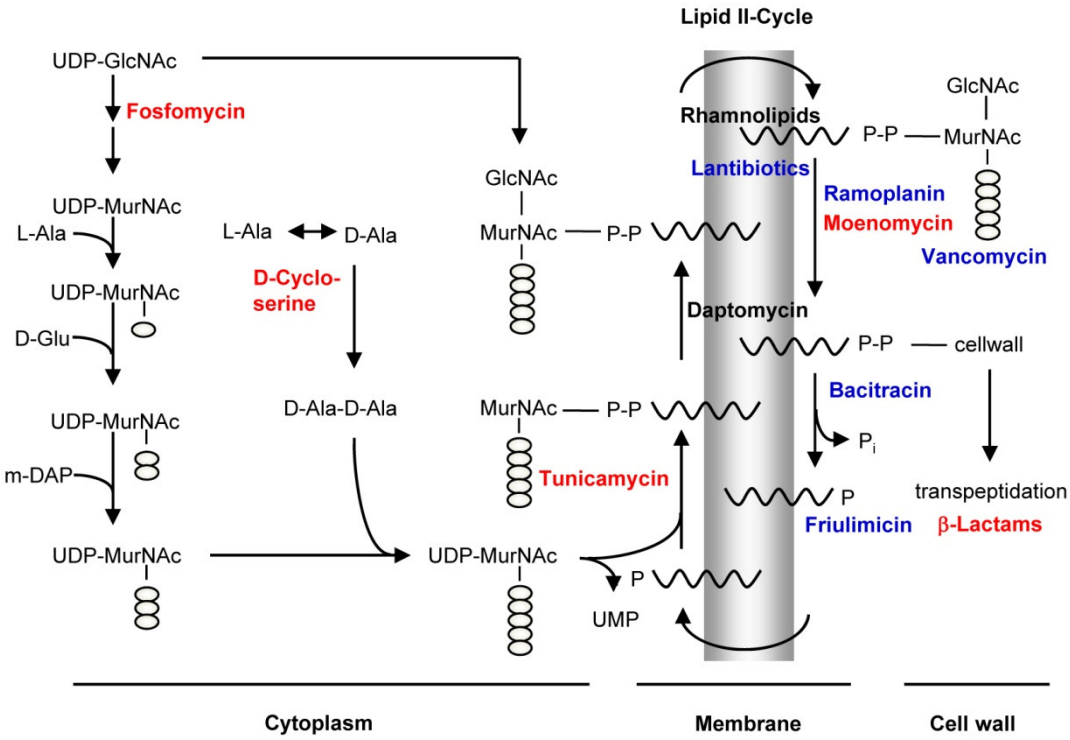

Figure 1.2. Cell wall biosynthesis and its inhibition by antimicrobial compounds. Important steps of the cell wall biosynthesis are shown schematically, their cellular location is indicated below. Cell wall antibiotics mentioned in the text are given and placed next to their biological target. Antibiotics in blue sequester the substrate of a given step, antibiotics in red inhibit the corresponding enzyme. Daptomycin and rhamnolipids as compounds that target the cytoplasmic membrane are displayed in black. The curved line represents the lipid carrier undecaprenol, the ovals amino acids. Abbreviations: GlcNac, N-acetyl-glucosamine, MurNac; N-acetyl-muramic acid; UDP, uridine diphosphate; UMP, uridine monophosphate; P, phosphate group; P_i, inorganic phosphate. Names of amino acids are shown as three-letter code. This figure is taken from (Jordan *et al.*, 2008), with modifications.

Although a functional peptidoglycan layer seems to be important for cell integrity and growth, cell wall-deficient bacteria, so-called L-forms, have been discovered (Dienes, 1947, Klieneberger, 1935). Since then, L-forms of many different bacterial species have been generated and successfully cultured in osmotically protective media. Naturally occurring L-forms can also be isolated from human samples and their contribution to a variety of diseases has been discussed. In this context, loss of the cell wall in the course of L-form formation can be viewed as a neat survival strategy to escape cell wall active antibiotics (Wyrick & Rogers, 1973, Glover *et al.*, 2009, Domingue GJ & Woody, 1997).

1.3 Cell wall antibiotics

The cell wall and its biosynthesis are preferred targets for antibiotics and almost every step is inhibited by at least one of these compounds (Schneider & Sahl, 2010). Important antibiotics as well as their site of interference with cell wall biosynthesis are shown in Fig. 1.2. Although many of them target the later lipid-linked steps, two antibiotics that intervene with synthesis of cell wall precursors in the cytoplasm have been developed for clinical use and are actually used for treatment of infections in humans. Fosfomycin is a broad-spectrum antibiotic that inhibits the first step of cell wall biosynthesis, which is the formation of UDP-N-acetyl-glucosamine catalyzed by MurA. This reaction requires phosphoenol pyruvate as a cofactor. Fosfomycin is a structural analogue of this cofactor and inactivates MurA by covalently binding to an active cysteine residue (Kahan *et al.*, 1974). Resistance can be gained by enzymatic inactivation of fosfomycin catalyzed by metalloglutathione or metallothiol transferases (Bernat *et al.*, 1997, Cao *et al.*, 2001), enhanced expression of MurA or alterations in fosfomycin uptake systems (Horii *et al.*, 1999).

D-cycloserine prevents completion of the pentapeptide responsible for the net-like structure of peptidoglycan. It inhibits two enzymes: the D-alanine racemase, which converts L-alanine to D-alanine, and the D-alanine/D-alanine ligase, which forms the corresponding dipeptide (Lambert & Neuhaus, 1972, Neuhaus & Lynch, 1964). Resistance can be achieved either by removal of the antibiotic with an efflux pump or overexpression of the target proteins (Feng & Barletta, 2003, Matsuo *et al.*, 2003).

The lipid II cycle is the target of a conspicuously large number of antibiotics (Fig. 1.2). One reason for this might be the location at the exterior of the cell and therefore easy accessibility of the target for the antibiotic. But also the lipid-anchored steps occurring at the inside of the cytoplasmic membrane are inhibited. Tunicamycin inhibits formation of lipid I catalyzed by the translocase MraY, but it is not suitable for therapeutic use due to inhibition of mammalian glycoprotein biosynthesis. Tunicamycin is structurally similar to UDP-activated sugars and therefore blocks the MraY reaction in bacteria (Brandish *et al.*, 1996).

Vancomycin, the most medically relevant drug within the large group of glycopeptide antibiotics, binds tightly to the terminal D-alanyl-D-alanine of the peptide chain of lipid II at the outside of the cell and thereby inhibits the crosslinking (Kahne *et al.*, 2005). Use of an alternative dipeptide terminus composed of D-alanyl-D-lactate significantly reduces the affinity of vancomycin and results in vancomycin resistance (Walsh *et al.*, 1996). Some bacteria naturally use this alternative dipeptide. Therefore, it is not surprising that the genetic information for this resistance mechanism was transferred to clinically relevant bacteria, for example resulting in vancomycin-resistant *S. aureus* (VRSA). Vancomycin is clinically used as a last resort antibiotic reserved for treatment of serious infections with (often multi-resistant) Gram-positive bacteria, but resistance to it becomes more and more common (Weigel *et al.*, 2003).

A new antibiotic for treatment of vancomycin-resistant enterococci, which has been already developed into phase III of clinical trials, is ramoplanin, a non-ribosomally synthesized lipoglycodepsipeptide antibiotic. The exact mechanism of action (MOA) of ramoplanin is not yet completely understood. Inhibition of both the formation of lipid II, catalyzed by the glycosyltransferase MurG, and the transglycosylation step of cell wall biosynthesis have been suggested as possible targets. Further research indicated that binding of lipid II and blocking of transglycosylation is the biologically more relevant inhibition mechanism (Breukink & de Kruijff, 2006, Fang *et al.*, 2006, Walker *et al.*, 2005).

The phosphoglycolipid antibiotic moenomycin directly inhibits the enzyme catalyzing the transglycosylation step. It displays biological activity against various Gram-positive bacteria including methicillin- and vancomycin-resistant enterocooci, but it was not further

developed into a drug for treatment of infections in humans due to suboptimal pharmacokinetic properties (Ostash & Walker, 2010).

Daptomycin is one of only a few antibiotics that have been approved for clinical use within the last decade. This lipodepsipeptide antibiotic is used for treatment of infections caused by Gram-positive pathogens and is effective against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (Baltz *et al.*, 2005). Daptomycin interferes with cell envelope integrity, but its mechanism of action is not yet fully understood and a defined molecular target within the cell wall biosynthesis pathway has not been identified (Schneider *et al.*, 2009). Initial investigations proposed inhibition of lipoteichoic acid biosynthesis (Canepari & Boaretti, 1996), but the currently accepted model considers the binding to and Ca^{2+}-dependent integration into the cytoplasmic membrane. Subsequent oligomerization and pore formation may lead to leakage of ions from the cytoplasm, arrest of macromolecular biosynthesis and finally cell death (Silverman *et al.*, 2003). Recent studies challenge this model and suggest that binding of Ca^{2+} ions leads to initial formation of micelles in solution accompanied by conformational changes of daptomycin. In close proximity to the cytoplasmic membrane, these micelles dissociate and daptomycin may insert into the phospholipid bilayer leading to the already mentioned effects (Straus & Hancock, 2006). Although daptomycin was introduced to the market only recently, first cases of resistance in the clinical environment as well as cross-resistance between vancomycin and daptomycin have been reported (Enoch *et al.*, 2007, Hidron *et al.*, 2008, Patel *et al.*, 2006). These observations underline the alarmingly rapid resistance development and urgent need for the marketing of antibiotics with novel MOAs.

A promising candidate for such a novel antibiotic is the lipopeptide friulimicin B, which has already entered clinical development. Friulimicin B is structurally similar to daptomycin and also active against multi-resistant Gram-positive bacteria (Aretz *et al.*, 2000, Vertesy *et al.*, 2000), but shows a completely different MOA. Instead of impairing membrane integrity, it specifically forms a complex with undecaprenol-monophosphate and thereby prevents formation of a functional cell envelope in Gram-positive bacteria (Schneider *et al.*, 2009).

Lantibiotics are post-translationally modified peptide antibiotics, which contain the unusual amino acid lanthionine as their name-giving feature. Their MOA involves lipid II-

binding, which is sometimes followed by pore formation. Lantibiotics of group A, including nisin, use lipid II as a docking molecule: they first specifically bind to lipid II and then form pores, thereby combining inhibition of cell wall biosynthesis with membrane permeabilization. In contrast, lantibiotics of group B, for example mersacidin, exert their antibacterial activity only by binding lipid II without subsequent pore formation. Resistance against these positively charged antibiotics can for example be achieved by lowering the overall negative net-charge of the Gram-positive cell envelope by D-alanine insertion into teichoic acids (Breukink & de Kruijff, 2006, Schneider & Sahl, 2010).

Bacitracin is a non-ribosomally synthesized cyclic dodecylpeptide antibiotic primarily active against Gram-positive bacteria. It is produced as a mixture of up to 50 different congeners (Kang *et al.*, 2001) and requires a divalent metal ion for its biological activity (Ming & Epperson, 2002). Bacitracin presumably binds to undecaprenol-pyrophosphate, which is released during the transglycosylation reaction. This complex formation prevents dephosphorylation of the lipid carrier and thus completion of the lipid II cycle (Stone & Strominger, 1971, Storm & Strominger, 1973). Resistance against bacitracin can be achieved by removal of the antibiotic by specific transporters (Mascher *et al.*, 2003, Ohki *et al.*, 2003a), *de novo* synthesis of undecaprenol-monophosphate (Cain *et al.*, 1993, Chalker *et al.*, 2000), expression of alternative undecaprenol-pyrophosphate phosphatases (Bernard *et al.*, 2005, Cao & Helmann, 2002, Ohki *et al.*, 2003b) or exopolysaccharide production (Pollock *et al.*, 1994, Tsuda *et al.*, 2002).

Incorporation of the newly synthesized peptidoglycan units into the existing cell wall is targeted by the clinically most important class of antimicrobial compounds, the β-lactam antibiotics. Their characteristic feature is a β-lactam ring, which structurally mimics the D-alanyl-D-alanine terminus of the pentapeptide of peptidoglycan units. Consequently, they are recognized by the active site of transpeptidases (which are therefore also called penicillin binding proteins), and block the crosslinking of glycan chains catalyzed by these enzymes (Strominger & Tipper, 1965). The most important resistance mechanism is the synthesis of β-lactamases, which hydrolyze the β-lactam ring and thereby inactivate the antibiotic (Ghuysen, 1991). Other resistance strategies are the removal of the antibiotic from its active site by efflux pumps (Poole, 2005) or the synthesis of altered penicillin binding proteins that maintain their physiological function but show decreased affinity to the harmful antibiotic (Dowson *et al.*, 1994, Hakenbeck, 1999).

Besides these classical and often clinically relevant antibiotics, a variety of other secondary microbial metabolites display biological activity (Berdy, 2005). One example for such bioactive secondary metabolites are biosurfactants, which are surface-active molecules commercially used for bioremediation processes, as components of cosmetic products or detergents (Banat *et al.*, 2000). Some of these biosurfactants, such as rhamnolipids, also have antimicrobial properties and could therefore be of interest for therapeutic applications. The antimicrobial activity of rhamnolipids is based on cell surface modifications like an increase in hydrophobicity and membrane permeability (Lang *et al.*, 1989, Vasileva-Tonkova *et al.*, 2011).

1.4 Regulatory networks orchestrating cell envelope stress responses

The presence of antibiotics produced by competing organisms and abiotic stresses like suboptimal temperature or pH necessitate the development of signal transducing systems. These systems allow the sensing of extracellular stimuli and transfer of the signal through the membrane to the cytoplasm, where an appropriate response, usually in the form of differential gene expression, is triggered.

The sensing of and response to cell envelope stress is mediated by two different modes of signal transduction: two-component systems (TCSs) and extracytoplasmic function (ECF) σ factors. Both systems consist of two proteins: a membrane-anchored sensor (histidine kinase (HK) or anti-σ factor, respectively) and a cytoplasmic transcriptional regulator (response regulator (RR) or ECF σ factor, respectively). In the absence of an inducing stimulus, the regulator is usually kept inactive. Upon perception of a cell envelope stress signal by the sensor protein, the regulator becomes activated and mediates the cellular response by modifying gene expression (Jordan *et al.*, 2008). The mechanism of signal transduction of TCSs and ECF σ factors will be presented in detail in sections 1.5 and 1.6. The regulatory networks orchestrating cell envelope stress responses in *Escherichia coli* and *B. subtilis* are well investigated and will be introduced in the following sections.

1.4.1 Cell envelope stress response of *Escherichia coli*

The cell envelope stress response of the Gram-negative model bacterium *E. coli* is orchestrated by two TCSs, one ECF σ factor and the phage shock protein system. These signal transducing systems partly overlap with regard to their inducing conditions and regulons (Rowley *et al.*, 2006, Ruiz & Silhavy, 2005).

The first TCS, BaeRS, is induced upon exposure to cell envelope stresses in form of indole, flavonoids, spheroplast formation or misfolded proteins and controls expression of three operons, one of these including the genes encoding the TCS. The other gene products are multidrug efflux pumps and a periplasmic protein of unknown function (Leblanc *et al.*, 2011, Raffa & Raivio, 2002). The second TCS, CpxAR, is induced by various signals including alkaline pH, misfolded periplasmic proteins or abnormalities in the inner membrane, for example changes in lipid composition. The RR CpxR directly controls expression of about 100 operons, some of which are also part of the σ^E regulon (De Wulf *et al.*, 2002, Ruiz & Silhavy, 2005).

The main signal for activation of the ECF σ factor σ^E is the accumulation of misfolded proteins in the cell envelope, which can be caused for example by heat or ethanol. It controls a large regulon of about 50 transcriptional units including genes encoding chaperones and proteases involved in cell envelope maintenance (Ades, 2004, Rhodius *et al.*, 2006).

The PspA-mediated phage shock protein response is triggered by a variety of stress conditions, including mislocalization of envelope proteins, high temperature, presence of proton ionophores and the name-giving filamentous phage infection. The physiological role of the phage shock protein response is still unclear, but a function in maintenance of membrane integrity and proton-motive force has been discussed (Ades, 2004, Darwin, 2005, Kleerebezem *et al.*, 1996, Rhodius *et al.*, 2006).

1.4.2 Cell envelope stress response of *Bacillus subtilis*

The cell envelope stress response network of the Gram-positive model organism *B. subtilis* is more complex than that of *E. coli* and involves four TCSs and at least three ECF σ factors (Fig. 1.3). The TCSs can be further divided into two subgroups: the cell envelope

stress sensing TCS LiaRS and three paralogous TCSs genetically and functionally linked to ABC transporters.

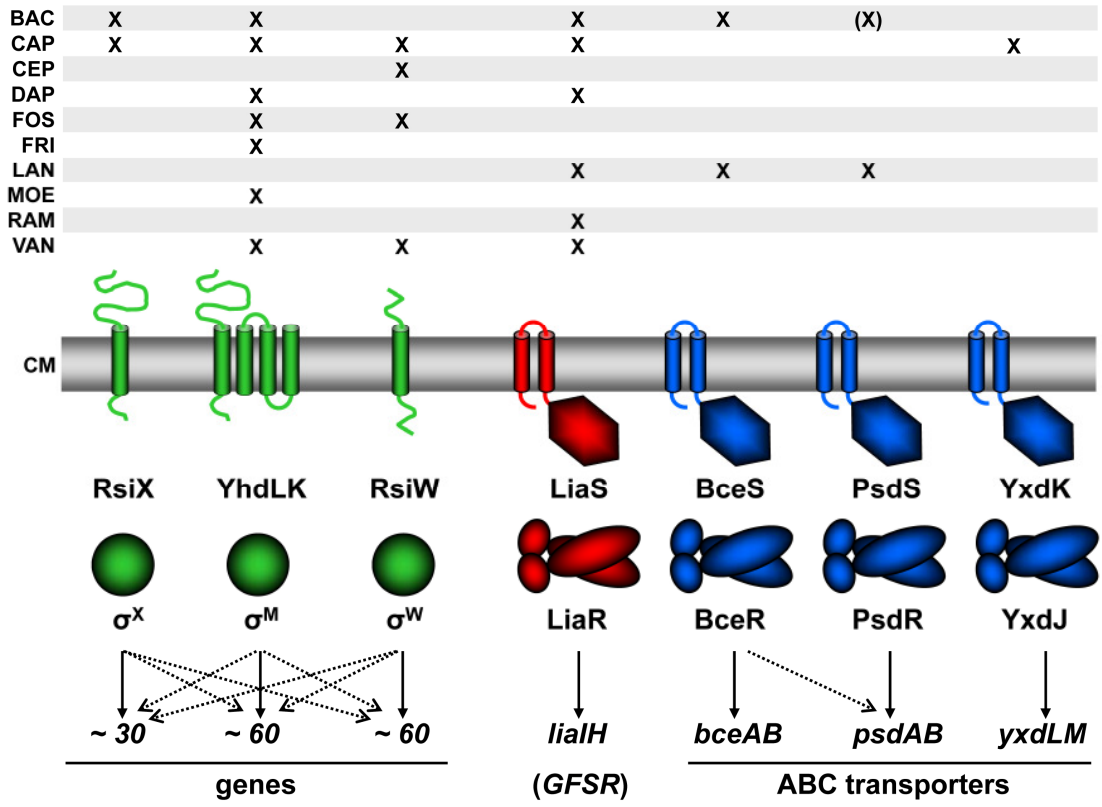

Figure 1.3. Graphical overview of the regulatory network orchestrating the cell envelope stress response of *B. subtilis*. ECF σ factors and the corresponding anti-σ factors are illustrated in green, TCSs associated with ABC transporters in blue and the LiaRS system in red. Transmembrane sensor proteins are shown on top, the regulators below and the corresponding target genes at the bottom. Arrows indicate regulation, dotted lines cross-regulation. Antibiotics inducing these systems are displayed above the graphic. Abbreviations: BAC, bacitracin; CAP, cationic antimicrobial peptides; CEP, cephalosporin; DAP, daptomycin; FOS, fosfomycin; FRI, friulimicin B; LAN, lantibiotics; MOE, moenomycin; RAM, ramoplanin; VAN, vancomycin; CM, cytoplasmic membrane. The figure is taken from (Jordan et al., 2008), with modifications.

The LiaRS TCS strongly responds to a wide variety of cell wall antibiotics, especially compounds interfering with the lipid II cycle, such as bacitracin, vancomycin, ramoplanin or cationic antimicrobial peptides (Mascher *et al.*, 2004, Pietiäinen *et al.*, 2005). Induction can also be observed upon exposure to ethanol, detergents, organic solvents and alkaline shock (Mascher *et al.*, 2004, Petersohn *et al.*, 2001, Wiegert *et al.*, 2001). Moreover, the LiaRS TCS is activated without any external stimulus at the onset of stationary phase, albeit to a much weaker extent compared to antibiotic induction (Jordan *et al.*, 2007). The TCS is genetically and functionally linked to a third protein, LiaF, thereby actually

constituting a three-component system. This membrane protein acts as a strong inhibitor of LiaRS-mediated signal transduction (Jordan *et al.*, 2006). The RR LiaR regulates expression of the *liaIHGFSR* operon. Basal expression of the last four genes, encoding the three-component system and the putative membrane-anchored protein LiaG, is ensured by a weak constitutive promoter upstream of *liaG*. Activation of LiaR strongly upregulates expression from a promoter upstream of *liaI*, resulting in the synthesis of two transcripts: a major transcript consisting of *liaIH* and a transcript including the whole operon (Mascher *et al.*, 2004).

Deletion of the inhibitory protein LiaF, which results in a constitutive active RR LiaR, induces transcription of two additional loci, *yhdYZ-yhdA* and *ydhE*. Although all three transcripts expressed under these artificial conditions are preceded by a putative LiaR binding site, only expression of *liaIH* seems to be biologically relevant (Jordan *et al.*, 2006, Wolf *et al.*, 2010). LiaH is a phage shock protein homologue and forms large oligomeric rings (Wolf *et al.*, 2010), which has been also described for the homologous proteins PspA of *E. coli* and Vipp1 of *Arabidopsis thaliana* (Aseeva *et al.*, 2004, Hankamer *et al.*, 2004). LiaI is a small membrane protein possibly interacting with LiaH. The physiological role of the *lia* system in *B. subtilis* is still unclear and only a very few phenotypes associated with LiaH have been discovered, including resistance against the cell wall antibiotics daptomycin and enduracidin and some oxidative stress agents. It has been suggested that the LiaFSR system coordinates a phage shock protein response in *B. subtilis* and, presumably, other *Firmicutes* bacteria (Wolf *et al.*, 2010).

The remaining three TCSs involved in orchestrating the cell envelope stress response in *B. subtilis* (Fig. 1.3) are genetically linked to genes encoding ABC transporters, thereby forming efficient detoxification modules. The best understood example of these modules is the BceRS-BceAB system, which responds to the presence of bacitracin and, to a lesser extent, the lantibiotics actagardine and mersacidin as well as the defensin plectasin (Ohki *et al.*, 2003a, Staroń *et al.*, 2011). Activation of the RR BceR results in increased expression of the ABC transporter BceAB, which functions as a resistant determinant and facilitates removal of the antibiotic from its active site (Mascher *et al.*, 2003, Ohki *et al.*, 2003a). However, BceAB is also crucial for stimulus perception, since the HK BceS alone is not sufficient for bacitracin sensing (Bernard *et al.*, 2007, Rietkötter *et al.*, 2008). Phylogenetic analysis of BceRS-BceAB-like systems in *Firmicutes* bacteria revealed a

tight evolutionary correlation and suggests a common novel signaling and resistance mechanism, possibly involving a sensory complex composed of the HK and ABC transporter (Dintner *et al.*, 2011).

The PsdRS-PsdAB module responds to and mediates resistance against the lipopeptide antibiotic enduracidin and the lantibiotics nisin, subtilin, actagardine and gallidermin (Staroń *et al.*, 2011). Noteworthy, all these compounds are peptide antibiotics interacting with lipid II. Weak induction has also been observed for bacitracin, but it has been shown to be due to cross-activation of the RR PsdR by the paralogoues BceRS system (Mascher *et al.*, 2003, Rietkötter *et al.*, 2008).

Only little is known about the third module, YxdJK-YxdLM. As is also true for the other systems, expression of *yxdLM* encoding the ABC transporter is dependent on the RR YxdK (Joseph *et al.*, 2004). The only inducer identified so far is the human cationic antimicrobial peptide LL-37 (Pietiäinen *et al.*, 2005, Staroń *et al.*, 2011). Since it is unlikely that *B. subtilis*, naturally inhabiting the soil, has evolved systems specifically responding to human peptides, a biologically relevant inducer of this module still has to be identified.

The genome of *B. subtilis* encodes seven ECF σ factors, of which at least three (σ^W, σ^M and σ^X) are involved in orchestrating the cell envelope stress response. With regard to its signal transducing mechanism and physiological function best investigated ECF σ factor of *B. subtilis* is σ^W. As is typical for ECF σ factors, the *sigW* gene is transcribed together with its anti-σ factor, *rsiW*, from an autoregulated promoter. σ^W is activated by cell wall antibiotics such as vancomycin, cephalosporin C or D-cycloserine (Cao *et al.*, 2002b), the mammalian cationic peptides LL-37 and PG-1 (Pietiäinen *et al.*, 2005) as well as alkaline shock (Wiegert *et al.*, 2001), but it is not required for resistance against these compounds and conditions. Its regulon was identified using a combination of different approaches including *in silico* promoter search (Huang *et al.*, 1999), *in vivo* DNA microarray analysis and an *in vitro* technique called ROMA (Run-Off transcription/Microarray Analysis) (Cao *et al.*, 2002a, MacLellan *et al.*, 2009a). Altogether, σ^W recognizes ~30 promoters controlling expression of about 60 genes, many of which encode membrane proteins, transporters, small peptides or proteins involved in detoxification. Thus, it has been postulated that σ^W mediates an antibiosis stress response including production of and protection against antibiotics (Butcher & Helmann, 2006). Indeed, increased expression of

the *fosB* gene, which is part of the σ^W regulon, provides resistance against fosfomycin (Cao *et al.*, 2001). A *sigW* mutant is also more sensitive to a wide variety of antimicrobial compounds produced by competing *Bacillus* species. Moreover, systematic deletion of all σ^W regulon members identified genes directly conferring resistance against antibiotics, for example *sdpI* against the antimicrobial protein SdpC and the *yqeZ-yqfAB* operon against sublancin (Butcher & Helmann, 2006).

The gene encoding σ^M is co-transcribed with *yhdLK*, which function as negative regulators of σ^M activity (Horsburgh & Moir, 1999). σ^M is induced under acid, heat, salt and superoxide stress as well as upon exposure to cell wall antibiotics like bacitracin, vancomycin and fosfomycin (Mascher *et al.*, 2003, Thackray & Moir, 2003, Cao *et al.*, 2002b). A comprehensive analysis including several complementary approaches identified almost 60 genes organized in 30 operons to be direct targets of σ^M under conditions of antibiotic stress. The functions of these genes are very diverse and include gene regulation, cell envelope related functions like cell wall synthesis, shape determination and cell division, DNA monitoring and repair as well as detoxification (Eiamphungporn & Helmann, 2008). For example, bacitracin-induced expression of *bcrC*, conferring resistance against this antibiotic, is dependent on σ^M, although the corresponding promoter is also recognized by σ^W (Cao & Helmann, 2002). Furthermore, resistance to paraquat, a superoxide-generating agent, can be also attributed to σ^M, which directly controls expression of *yqjL* encoding a putative hydrolase (Cao *et al.*, 2005). Analysis of the σ^M regulon also revealed indirect effects of antibiotic induction. Among the genes controlled by σ^M is *spx* encoding a transcription factor, which in turn regulates expression of several antibiotic-inducible genes. Thereby, σ^M indirectly controls expression of the Spx regulon (Eiamphungporn & Helmann, 2008).

The gene of the third ECF σ factor, σ^X, forms an operon with *rsiX*, encoding the corresponding anti-σ factor, and is transcribed during logarithmic and early stationary growth phase (Huang *et al.*, 1997). σ^X controls expression of about ten operons encoding proteins involved in cell envelope composition, surface metabolism and cell division (Cao & Helmann, 2004). Based on these functions, it has been suggested that σ^X generally regulates modification of the cell envelope. The products of the σ^X target operon *dltABCDE* introduce positively charged amino acids into teichoic acids, thereby reducing the negative net charge of the cell wall. These changes in cell surface charge consequently

affect both autolysis and resistance to cationic antimicrobial peptides. Indeed, a *sigX* mutant strain shows increased autolysis and is more sensitive to nisin (Cao & Helmann, 2004), thereby supporting the hypothesis that σ^X plays a role in cell surface modification.

ECF σ factors control expression of often large regulons with a significant regulatory overlap (Mascher *et al.*, 2007, Qiu & Helmann, 2001). A stimulus does not always activate a single ECF σ factor, which mediates a proper response by upregulation of a specific regulon. Rather, the ECF response is more complex and often involves several ECF σ factors controlling distinct but overlapping sets of genes. Therefore, it is not surprising that mutations in single ECF-encoding genes do not always lead to obvious phenotypes. While single mutations do not affect antibiotic susceptibility, simultaneous deletion of σ^W, σ^M and σ^X revealed increased sensitivity against cell wall antibiotics such as D-cycloserine, nisin and cephalosporin C (Mascher *et al.*, 2007).

1.5 Signal transducing mechanisms orchestrating cell envelope stress responses

Bacterial signal transducing systems consist of at least two domains: an input or sensor domain, which detects the signal, and an output or effector domain, which orchestrates the cellular response. These two domains can be located on one polypeptide chain, as is true for one-component systems, or separated on two different proteins (Fig. 1.4). The principle of two co-operating proteins, which is represented by TCSs and ECF σ factors, enables transmembrane signaling. This includes the sensing of an extracellular stimulus and transfer of the signal through the membrane to the cytoplasm, where the effector mediates a proper cellular response, usually in the form of differential gene expression.

1.5.1 One-component systems

One-component systems are the simplest and most widely distributed form of bacterial signal transduction, in which the input and output domains are fused on a single polypeptide chain. The input domain perceives a signal and then modulates activity of the output domain, which usually functions as a transcriptional regulator. This regulator either activates or represses transcription of its target gene(s), whose number can vary from a single gene up to several hundred (Ulrich *et al.*, 2005). Classical examples for such

transcriptional regulators are the *E. coli lac* repressor LacI (Lewis *et al.*, 1996) or the cAMP receptor protein (CRP) (Kolb *et al.*, 1993).

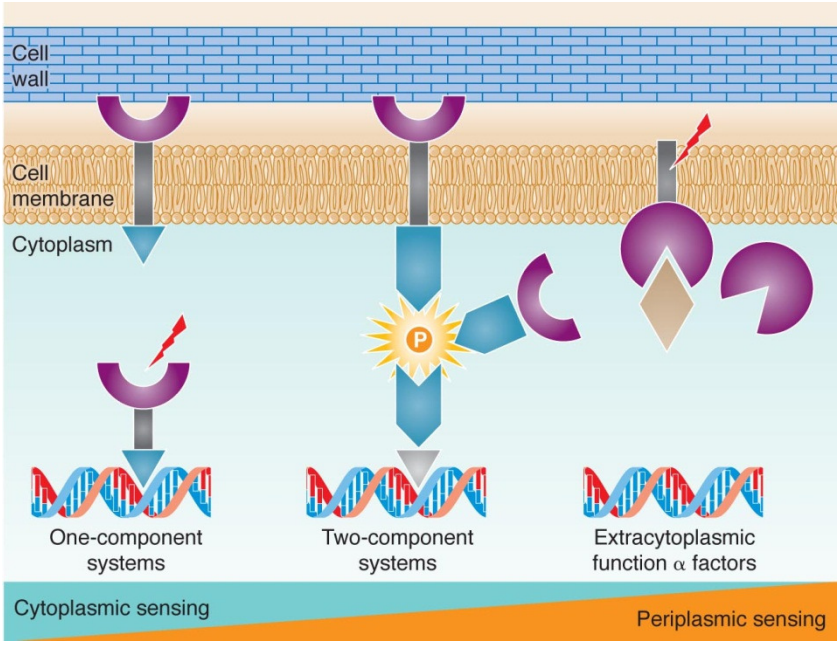

Figure 1.4. Overview of signal transducing systems in bacteria. The modular structure of one-component systems, TCSs and ECF σ factors as well as their basic signal transducing mechanism are shown schematically. See text for details. This figure is taken from (Staroń & Mascher, 2010a).

1.5.2 Two-component systems

While one-component systems are predominantly designed to respond to intracellular signals, TCSs are suitable for transmembrane signaling. A classical TCS consists of two different proteins: a sensor protein, which functions as a HK, and an effector protein, the RR. Both proteins contain at least two domains and the signal transduction is based on phosphotransfer reactions. The HK senses a specific stimulus with its N-terminal input domain, which results in an intramolecular conformational change and autophosphorylation of a conserved histidine residue within the C-terminal transmitter domain. Subsequently, this phosphate group is transferred to a conserved aspartate residue within the N-terminal receiver domain of the RR. The cellular response is then mediated by the C-terminal effector domain of the activated RR and usually involves protein-DNA interaction leading to differential gene expression. The whole system is set back to the pre-stimulus state by dephosphorylation of the RR, catalyzed either by an external

phosphatase, the corresponding HK or the RR itself (Mascher *et al.*, 2006, Stock *et al.*, 2000).

Depending on their architecture, HKs can be divided into three main groups, which perceive either an extracellular, a cytoplasmic or a membrane-associated stimulus. The largest group contains the extracellular-sensing HKs, typically detecting the presence of solutes or nutrients. These kinases are transmembrane proteins with at least two transmembrane helices flanking a large extracellular sensory domain. The kinase domain is located in the cytoplasm, which necessitates signal transduction through the membrane (Mascher *et al.*, 2006). One of the best investigated HKs, EnvZ from *E. coli*, belongs to this group. Together with its cognate RR OmpR, this TCS plays a central role in the adaptation to changes in extracellular osmolarity, although the periplasmic domain of EnvZ does not seem to be essential for sensing the corresponding signal (Leonardo & Forst, 1996, Tanaka *et al.*, 1998).

The second-largest group contains the cytoplasmic-sensing HKs. These are membrane-anchored or soluble proteins, in which both the sensory and kinase domain are located in the cytoplasm. Signals sensed by these kinases are mainly cytoplasmic solutes or proteins reporting the physiological state of the cell (Mascher *et al.*, 2006). Well-understood examples of this group are KinA, involved in sporulation of *B. subtilis* (Msadek, 1999), and CheA, which regulates chemotaxis in proteobacteria (Bilwes *et al.*, 2003).

The third and very diverse group is formed by HKs that possess two to 20 transmembrane regions, which are connected by very short intra- or extracellular linkers. An obvious sensory domain is missing in these proteins, suggesting that the stimulus sensed by these kinases is within or associated with the membrane interface. Such stimuli could be loss of cell envelope integrity, ion gradients, change of mechanical parameters such as turgor pressure or signals derived from other transmembrane proteins. HKs of TCSs orchestrating the cell envelope stress response in *B. subtilis*, such as LiaRS or BceRS, belong to this group (Mascher, 2006, Mascher *et al.*, 2006).

The examples mentioned above demonstrate the enormous variety of signals that can be sensed by TCSs. However, not only the input but also the output can vary depending on the effector domain of the RR. About two-thirds of all known RRs contain a DNA-binding domain, thereby functioning as transcriptional regulators. But they can also have

enzymatic, RNA-, ligand- or protein-binding domains, enabling almost unlimited variations in the response mediated by these proteins (Galperin, 2006, Galperin, 2010).

The modularity of both the HK and the RR further increases the flexibility of TCSs. The signal transducing process does not necessarily have to lead from a single HK to a single RR. Rather, the modular architecture enables modifications such as amplification, branching of pathways or even integration of different signals into one cascade, thereby leading to the same output (Gao & Stock, 2009).

1.5.3 Alternative σ factors and the extracytoplasmic function protein family

Another possibility to control gene expression at the level of transcription initiation is the use of σ factors, which constitute an essential component of the RNA polymerase (RNAP). Transcription is a complex and highly coordinated process. The bacterial RNAP consists of a core complex with a subunit stoichiometry of $\alpha_2\beta\beta'\omega$. This core enzyme is capable of transcription elongation and termination. Promoter recognition and transcription initiation additionally require the σ factor, which binds to the core enzyme and recruits the resulting RNAP holoenzyme to an appropriate promoter. Therefore, σ factors can be considered as specificity factors providing a fundamental mechanism for orchestrating differential gene expression (Burgess & Anthony, 2001).

All bacteria contain a primary (or housekeeping) σ factor, which is responsible for general expression of most genes. In addition, most bacteria - especially those living in complex habitats - contain alternative σ factors, which are only activated under certain conditions. They compete with and eventually replace the primary σ factor, thereby redirecting RNAP to initiate transcription from a specific set of alternative promoters. In the absence of a stimulus, alternative σ factors are kept inactive by a cognate anti-σ factor through direct protein-protein interaction (Brown & Hughes, 1995, Helmann, 2010, Helmann & Chamberlin, 1988).

Bacterial σ factors can be divided into two major groups: the σ^{70} and the σ^{54} protein family (Gruber & Gross, 2003, Helmann & Chamberlin, 1988). The σ^{54}-like proteins are unique transcriptional activators and do not show any sequence homology to other σ factors. Although they are widely distributed, not every bacterium harbors a σ^{54} protein and, if present, usually no more than one σ^{54}-encoding gene is found in a genome (Buck *et al.*, 2000). In contrast, every bacterium contains at least one, but often several (up to 63 in

Streptomyces coelicolor) proteins of the σ^{70} family, which is named after the prototypical primary σ factor of *E. coli*. Based on their domain architecture, the proteins of this family can be divided into four phylogenetic groups, often correlating with specific functions. Group 1 contains the essential primary σ factors, such as the name giving σ^{70} of *E. coli* or σ^{A} of *B. subtilis*, which are composed of four distinct domains (designated regions σ_1 through σ_4) as well as a non-conserved region (NCR) adjacent to σ_2. Proteins of group 2 have the same domain architecture and are therefore closely related to group 1 σ factors, but they are not essential for growth. The most extensively studied example of this group is σ^{S}, the master regulator of the general stress response in *E. coli*. Proteins of group 3 lack both the σ_1 domain and the NCR. They are more divergent in sequence than proteins of group 1 and 2 and can be further divided into clusters correlating with specific functions, including flagella biosynthesis, sporulation and heat shock response. Group 4 is the numerically largest and most diverse group and contains the proteins of the ECF family, named after their function in response to extracellular stimuli (Gruber & Gross, 2003, Helmann & Chamberlin, 1988, Paget & Helmann, 2003).

The ECF σ factors are the smallest proteins of the σ^{70} family and contain only two of the four conserved domains, σ_2 and σ_4 (Fig. 1.5 A), which are sufficient for all σ factor functions, e.g. interaction with RNAP and recognition of specific target promoters. Each domain can be further subdivided into distinct regions. Domain σ_2 comprises regions 2.1 to 2.4. The first two of these regions are important for RNAP core binding, while region 2.4 recognizes the -10 element of promoter DNA. Region 2.3 is involved in promoter melting. Domain σ_4 also interacts with RNAP and is involved in recognition and binding of the -35 promoter element, primarily via the helix-turn-helix motif of region 4.2 (Gruber & Gross, 2003). The classical promoter region recognized by the primary σ factor consists of two highly conserved regions, the -10 ('TTGACA') and the -35 ('TATAAT') region (Helmann, 1995). In contrast, the ECF σ factors recognize alternative promoter sequences, often characterized by a highly conserved 'AAC' motif in the -35 and 'CGT' in the -10 region (Fig. 1.5 B) (Helmann, 2002, Lane & Darst, 2006).

ECF σ factors can be easily recognized due to their domain architecture and consequently smaller size compared to other σ factors. They are widely distributed within the bacterial kingdom and bacteria contain an average of six ECF genes per genome. Only a very few organisms, mostly obligate symbionts or pathogens with very small genomes, do not

harbor any ECF σ factor at all. A classification based on sequence similarities and genomic context conservation identified more than 40 distinct groups of ECF σ factors (Staroń *et al.*, 2009). The two predominant groups contain the RpoE-like and FecI-like proteins, whose signal transducing mechanisms have been well studied in *E. coli*. Another large group includes ECF σ factors associated with cytoplasmic-sensing anti-σ factors, which also have been investigated experimentally. In addition to these well characterized types of ECF σ factors, the classification identified a number of novel groups with unique features and so far unknown signaling mechanisms (Staroń *et al.*, 2009). An overview of already well-established ECF-dependent signal transduction as well as possible novel types of signal transducing mechanisms is given in the following section.

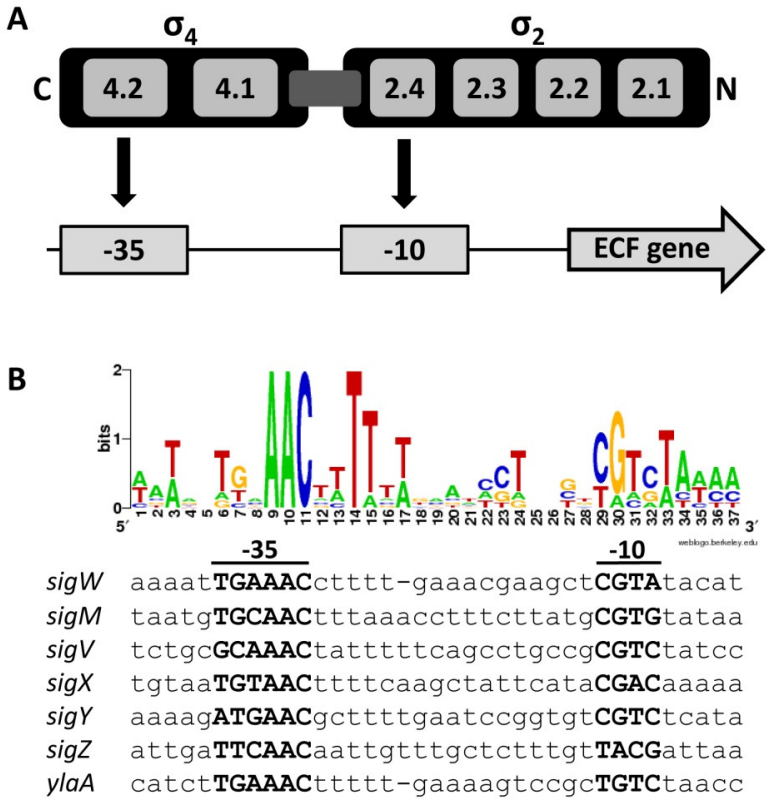

Figure 1.5. Domain architecture and target promoters of ECF σ factors. (A) Domain architecture of ECF σ factors with domains σ_2 and σ_4 as well as according subregions are shown schematically. C and N correspond to the C- and N-terminus. Interactions of subregions 2.4 and 4.2 with the target promoter -10 and -35 region, respectively, are indicated by arrows. (B) Weblogo of typical ECF-dependent target promoters. The weblogo was generated using the WebLogo tool (Crooks *et al.*, 2004) available at http://weblogo.berkeley.edu. It graphically represents a position weight matrix and illustrates the degree of conservation for each nucleotide. The matrix is based on the shown autoregulated promoters of the seven ECF σ factors from *B. subtilis* (Butcher *et al.*, 2008).

1.6 Mechanisms of ECF σ factor activation

In general, the activity of an ECF σ factor is controlled by a cognate anti-σ factor, often consisting of a cytoplasmic and an extracellular domain linked by one transmembrane helix. In the absence of a stimulus, the anti-σ factor tightly binds the ECF σ factor with its inhibitory cytoplasmic domain, thereby preventing RNAP interaction and promoter recognition. In the presence of a suitable stimulus, the anti-σ factor gets inactivated. This results in the release and subsequent activation of the ECF σ factor, which then substitutes the primary σ factor and initiates transcription from alternative promoters (Helmann, 2002). While the ECF σ factors show a highly conserved domain structure, the composition of anti-σ factors is very diverse. Some of them contain a so-called anti-sigma domain (ASD), but other anti-σ factors are comprised of domains of unknown function. Consequently, the mechanisms of stimulus perception and subsequent ECF σ factor activation also differ significantly. The few already well-investigated examples demonstrate an enormous mechanistic diversity (Fig. 1.6) and there is still a great potential for the identification of completely novel ECF-dependent signal transducing mechanisms (Staroń *et al.*, 2009).

1.6.1 Regulated proteolysis of transmembrane anti-σ factors

The best understood mechanism of ECF σ factor activation is regulated intramembrane proteolysis (RIP), in which an environmental stimulus results in complete proteolytic degradation of a transmembrane anti-σ factor (Fig. 1.6 A). The first step of RIP, called site-1 proteolysis, comprises the proteolytic processing of the extracytoplasmic part of the anti-σ factor. This step is necessary to make the anti-σ factor accessible for site-2 proteolysis, which represents the actual intramembrane cleavage event. The family of peptidases catalyzing this key step of RIP has been named intramembrane cleaving proteases (I-CLiPs). The complex of the remaining anti-σ/ECF σ factor is then released to the cytoplasm, where the anti-σ factor is completely degraded by additional proteases, leading to a free and thereby activated σ factor (Heinrich & Wiegert, 2009).

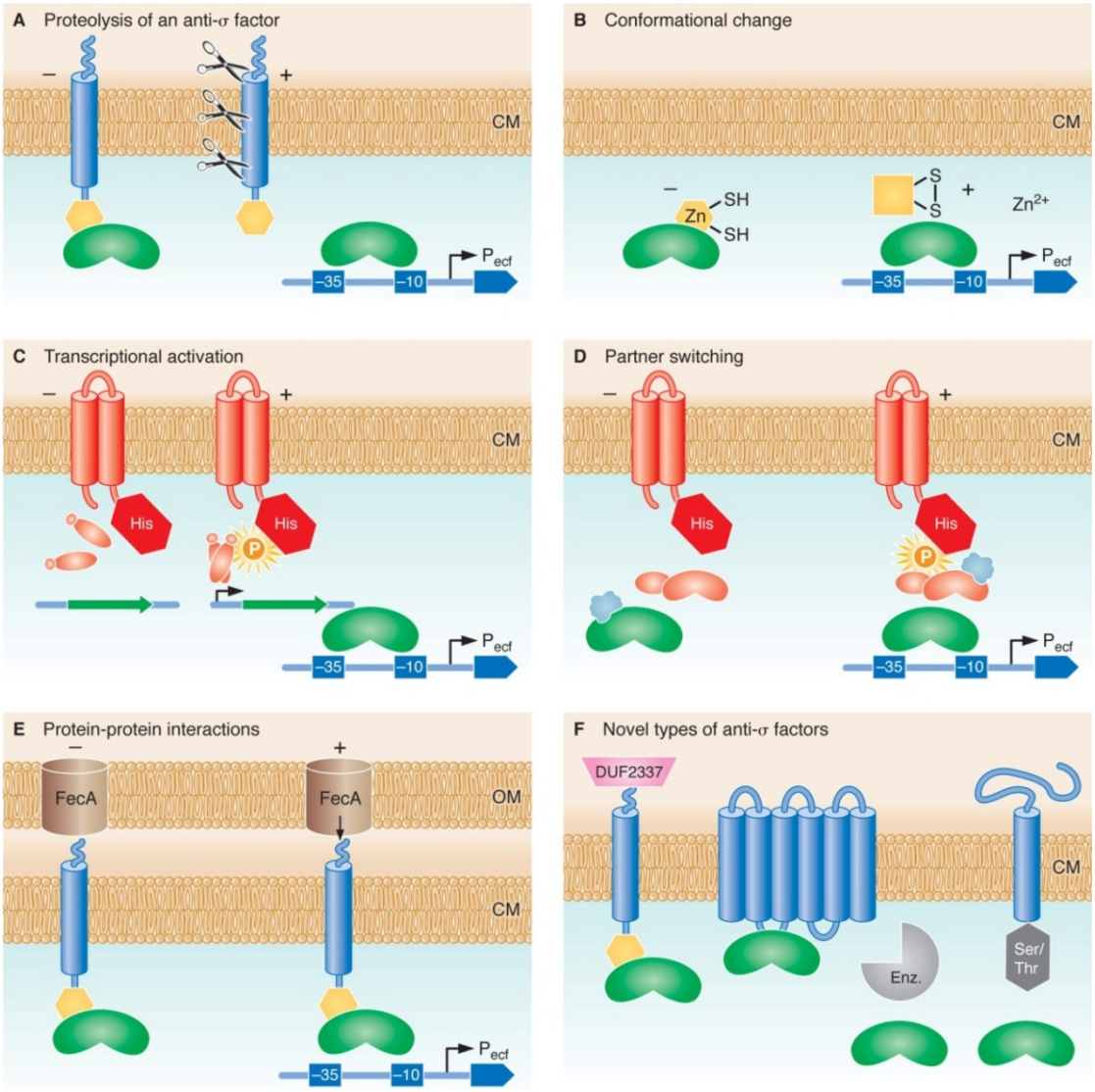

Figure 1.6. Examples of ECF-dependent signal transduction. See text for details. This figure is taken from (Staroń & Mascher, 2010a).

The best investigated ECF σ factors regulated by RIP are σ^E of *E. coli* and σ^W of *B. subtilis*. σ^E forms a complex with its transmembrane anti-σ factor RseA and the periplasmic protein RseB, which has an additional inhibitory function. The cascade leading to active σ^E is triggered by misfolded outer membrane proteins. A conserved peptide at the C-terminus, which is only exposed when these outer membrane proteins are not folded properly, binds to and activates the membrane-anchored serine protease DegS. Active DegS is responsible for site-1 proteolysis and removes the C-terminal part of RseA, thereby making it a substrate for the membrane-embedded RseP protease. This site-2 protease cleaves RseA within the membrane and the remaining complex of processed

RseA and σ^E is released into the cytoplasm. Site-2 clipped RseA presents a C-terminal proteolytic tag, which is recognized by cytoplasmic proteases such as ClpXP. Subsequent complete degradation of RseA results in a free and active σ^E able to start transcription of corresponding target genes, including its own autoregulated operon *rpoE-rseABC* (Ades, 2008, Ades, 2004).

σ^W from *B. subtilis* is kept inactive by direct interaction with the N-terminal part of the transmembrane anti-σ factor RsiW. The proteolytic cascade leading to activation of σ^W basically resembles the σ^E/RseA system described above. Nevertheless, there are some remarkable differences and the proteases involved can be grouped into two proteolytic modules. Module I consists of the proteases PrsW and Tsp. Site-1 proteolysis is mediated by PrsW, but the exact molecular signal activating this protease is still unknown. In contrast to *E. coli* DegS, which is a classical serine protease, PrsW belongs to a superfamily of probably membrane-embedded metalloproteases. Further C-terminal degradation of site-1 processed RsiW by the tail-specific protease Tsp is crucial for RsiW to become a substrate for module II, which consists of RasP and cytoplasmic proteases like ClpXP. As in *E. coli*, site-2 proteolysis, which is catalyzed by RasP, uncovers a proteolytic tag recognized by ClpXP. Complete degradation of the anti-σ factor leads to activation of σ^W and consequently expression of *sigW-rsiW* and other target genes (Heinrich *et al.*, 2009, Heinrich & Wiegert, 2006, Heinrich & Wiegert, 2009).

1.6.2 Conformational change of soluble anti-σ factors

Anti-σ factors are not necessarily transmembrane proteins. Instead, they can also be soluble proteins, thereby sensing cytoplasmic stimuli. Their inactivation and therefore release of the ECF σ factor is based on an intramolecular conformational change (Fig. 1.6 B). The best understood example for such an anti-σ factor is ChrR of *Rhodobacter sphaeroides* controlling activity of the ECF σ factor σ^E, which is responsible for the cellular response to singlet oxygen (Anthony *et al.*, 2004, Anthony *et al.*, 2005). ChrR is comprised of two domains: an N-terminal ASD domain and a C-terminal cupin-like domain (CLD). In contrast to other ASD-containing anti-σ factors, ChrR requires Zn^{2+} ions for its inhibitory function. Therefore, ChrR and similar cytoplasmic-sensing anti-σ factors are members of the so-called ZAS (Zn^{2+} anti-σ) family. In the absence of singlet oxygen, ChrR binds σ^E with its ASD. While this domain is sufficient to inhibit activity of the ECF

σ factor, the transcriptional response to singlet oxygen requires the CLD. Singlet oxygen causes dissociation of the σ^E/ChrR complex, resulting in expression of its autoregulated operon *rpoE-chrR* and *cycA*, encoding the periplasmic electron carrier cytochrome c_2 (Greenwell *et al.*, 2011, Newman *et al.*, 2001, Newman *et al.*, 1999).

Another example for an anti-σ factor of the ZAS family is RsrA of *S. coelicolor*, which regulates the activity of σ^R. Like ChrR, RsrA coordinates a Zn^{2+} ion and forms a complex with σ^R. The Zn^{2+} ion is released in the presence of thiol-oxidative stress, resulting in formation of a disulfide bond between two histidine residues and consequently a conformational change that releases and therefore activates σ^R (Zdanowski *et al.*, 2006, Kang *et al.*, 1999). Many of the σ^R-dependent target genes encode proteins involved in counteracting thiol-oxidative stress, such as thioredoxins, and proteins involved in biosynthesis of thiol-containing compounds like cysteine and molybdopterin (Paget *et al.*, 2001).

1.6.3 Transcriptional activation involving a two-component system

The signal transducing mechanism leading to activation of an ECF σ factor not always involves an anti-σ factor. Instead, the ECF σ factor can also form a functional module with a TCS (Fig. 1.6 C), as has been shown for σ^E from *S. coelicolor*. Here, transcription of the ECF σ factor, which is required for normal cell envelope integrity, is controlled by the TCS CseBC. CseC is a membrane-anchored HK that perceives the signal and phosporylates the cognate RR CseB. The signal sensed by CseC has yet to be identified, but it most likely arises from cell envelope damage, since σ^E is activated by a wide range of unrelated cell wall antibiotics. The activated RR CseB induces transcription of *sigE*, the structural gene encoding the ECF σ factor. This leads to an increase in the cellular concentration of σ^E, which, in turn, replaces other σ factors and redirects expression to its target genes. In addition to primarily monocistronic transcription, *sigE* is also part of a larger operon including the genes encoding CseA and the TCS CseBC. CseA is a lipoprotein localized at the outside of the cytoplasmic membrane, which somehow influences CseBC activity, but the specific function and mechanism is still unclear (Hong *et al.*, 2002, Hutchings *et al.*, 2006, Paget *et al.*, 1999).

1.6.4 Partner switching mechanism

The signal transduction pathway leading to an active ECF σ factor can be even more complex involving both an anti-σ factor and a specialized TCS (Fig. 1.6 D). Such an unusual and elaborate cascade mediates the general stress response of α-proteobacteria. The best-understood and name-giving example is EcfG from *Methylobacterium extorquens* (Francez-Charlot *et al.*, 2009), but the signal transducing mechanism has been also investigated in other α-proteobacteria including *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, *Caulobacter crescentus* and *Rhizobium etli* (Alvarez-Martinez *et al.*, 2007, Bastiat *et al.*, 2010, Gourion *et al.*, 2009, Martinez-Salazar *et al.*, 2009, Sauviac *et al.*, 2007).

Besides the actual EcfG-like σ factor, the cascade usually involves three additional proteins: a NepR-like anti-σ factor, a PhyR-like RR and often a HK. The structural genes encoding these proteins are organized in a well-defined but variable locus (Staroń *et al.*, 2009). NepR-like anti-σ factors are small soluble proteins that bear no sequence similarity to other anti-σ factors. The PhyR-like proteins are unusual RRs with specialized domain architecture. In contrast to other RRs, PhyR-like proteins carry the receiver domain at the C-terminus. The N-terminal output domain shows high sequence similarity to ECF σ factors, especially to EcfG-like proteins. Nevertheless, important residues for DNA binding are missing, indicating that PhyR-like RRs do not function directly as transcriptional regulators. Rather, PhyR-like RRs mimic EcfG-like proteins and function as anti-anti-σ factors. As an additional component, genes encoding HKs are often located in close vicinity of the EcfG-like σ factors. These HKs are very diverse in sequence and domain architecture and can be either periplasmic- or cytoplasmic-sensing proteins. The only specific feature can be found within the phosphotransfer domain. HKs linked to EcfG-like σ factors carry one of two very similar domains predominantly found in α-proteobacteria, thereby distinguishing them from other HKs (Francez-Charlot *et al.*, 2009, Herrou *et al.*, 2010, Staroń & Mascher, 2010b, Staroń & Mascher, 2010a).

In the absence of a stimulus, NepR-like anti-σ factors bind EcfG-like σ factors, thereby keeping them inactive. An appropriate stimulus, such as heat and osmotic stress or carbon and nitrogen starvation, is most probably sensed by the associated HK, leading to phosphorylation and thereby activation of the PhyR-like RR. Activated PhyR-like proteins function as anti-anti-σ factors and release of the σ factor from the anti-σ factor occurs via a

partner switching mechanism. The ECF σ factor-like domain of phosphorylated PhyR has high affinity for the NepR-like anti-σ factors, thereby replacing EcfG-like proteins. This allows the ECF σ factors to associate with RNA polymerase and initiate transcription of stress-related gene (Francez-Charlot *et al.*, 2009, Francez-Charlot *et al.*, 2010).

1.6.5 Activation involving protein-protein interactions

Stimulus perception can also be achieved by direct interaction of the anti-σ factor with other proteins usually not involved in signal transduction (Fig. 1.6 E). The best investigated example for such a mechanism is the FecI-FecR pair, which regulates iron acquisition in *E. coli*. The signal transducing cascade communicating the presence of ferric citrate from the outside of the cell to the cytoplasm involves the outer membrane transporter protein FecA, which serves as a signal receiver and outer membrane transmitter. In the absence of ferric citrate, the anti-σ factor FecR binds the ECF σ factor FecI with its N-terminal ASD, thereby keeping FecI inactive. Is ferric citrate present in the environment, it is bound by FecA and the signal is transferred by direct protein-protein interaction from the periplasmic N-terminal domain of FecA to the periplasmic C-terminus of the anti-σ factor FecR. This signal is transmitted by an unknown mechanism through the inner membrane, resulting in activation of FecI, which initiates transcription of the *fecABCDE* operon, encoding a ferric citrate uptake system. Contradictory to the anti-σ factor paradigm, FecR remains intact after signal transduction and is even required for full FecI-dependent transcription, thereby acting as both an anti-σ factor and mediator of σ factor activity. Transcription of the *fecIR* operon, encoding the ECF σ and anti-σ factor, respectively, is not autoregulated. Instead, its expression is controlled by the Fur repressor, whose inhibitory function is abolished at low intracellular iron concentrations. This ensures the presence of the regulatory components when iron is limited, while expression of the corresponding uptake system additionally requires the availability of the specific substrate (Braun & Mahren, 2005, Braun *et al.*, 2006).

1.6.6 Novel types of ECF-dependent signal transducing mechanisms

In addition to these well-established ECF σ factor-dependent signal transducing mechanisms described above, the comprehensive classification of ECF σ factors by Staroń and colleagues identified a number of novel groups with unique features (Fig. 1.6 F). Most

of them have not been intensively studied, but initial characterization of some proteins as well as data derived from sequence and genomic context analyses indicate the discovery of yet completely unknown signaling modules and mechanisms. Some of these ECF σ factors are associated with unusual anti-σ factors, either carrying domains of unknown function or having a special architecture, for example regarding the number of transmembrane regions. Other ECF σ factors are genomically linked to completely unrelated proteins like sensor kinases or enzymes. Moreover, in some cases the ECF σ factor itself contains additional domains, while an obvious anti-σ factor is missing (Staroń *et al.*, 2009).

1.7 Aims of this work

The response to cell envelope stress is orchestrated by two signal transducing principles: TCSs and ECF σ factors, both consisting of an often membrane-anchored sensor protein and a cytoplasmic regulator, which mediates the cellular response in form of differential gene expression. These systems allow the sensing of and response to cell envelope-related stimuli, which could be the presence of harmful compounds like antibiotics or a general loss of cell envelope integrity (Jordan *et al.*, 2008).

One aim of this work was to elucidate the response of the Gram-positive model organism *B. subtilis* to compounds that interfere with or inhibit biosynthesis of a functional cell envelope. First, the response to two clinically relevant antibiotics, daptomycin and friulimicin B, should be investigated by global in-depth expression profiling at both the transcriptome and proteome level. These two antibiotics are structurally similar and interfere with cell envelope integrity, but it has been suggested that they have a completely different molecular MOA. Similarities and differences in the expression profiles as well as comparison with responses provoked by antibiotics with already well-known targets should provide data to gain a deeper understanding of the specific MOA of daptomycin and friulimicin B. Secondly, the transcriptional response of *B. subtilis* to rhamnolipids, which are biosurfactants produced by the soil bacterium *Pseudomonas aeruginosa*, should be investigated by DNA microarray analysis. While rhamnolipids are not used for treatment of infections, thereby not being classical antibiotics, they show antimicrobial activity. The resulting gene expression profile should be further analyzed and possible resistance determinants identified.

The second aim of this work was the characterization of a novel group of ECF σ factors (ECF41), which was identified by a comprehensive classification of this protein family (Staroń *et al.*, 2009). Special features of this group are the lack of an obvious anti-σ factor and an unusual C-terminal extension of the ECF proteins. The phylogenetic distribution and genomic context conservation of this novel group should be determined. A target promoter as well as the corresponding target genes should be identified by both *in silico* and *in vivo* approaches, possibly assigning a function to the ECF41 σ factors. Moreover, a potential role of the C-terminal extension in the signal transducing process should be considered and the influence of mutations and truncations on promoter activation and interaction with RNAP should be investigated. To gain a representative understanding of the whole group of ECF41 σ factors, experiments should be carried out in two organisms from different bacterial phyla, namely *Bacillus licheniformis* and *R. sphaeroides*.

Chapter 2

Daptomycin versus Friulimicin B: in-depth profiling of *Bacillus subtilis* cell envelope stress responses.

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Author contributions:

Tina Wecke analyzed expression data, performed real time RT-PCR and contributed to the manuscript by drawing figures and constructing tables. Ulrike Mäder and Georg Homuth performed the microarray analyses. Sina Jordan conducted experiments regarding induction of the LiaRS TCS and determination of optimal antibiotic concentrations. Daniela Zühlke and Michael Hecker performed the proteomics analysis. Stefan Pelzer and Harald Labischinski supported this study and provided antibiotics. Thorsten Mascher designed the experiments and wrote the paper.

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Daptomycin versus Friulimicin B: In-Depth Profiling of *Bacillus subtilis* Cell Envelope Stress Responses[▽]†

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The related lipo(depsi)peptide antibiotics daptomycin and friulimicin B show great potential in the treatment of multiply resistant gram-positive pathogens. Applying genome-wide in-depth expression profiling, we compared the respective stress responses of *Bacillus subtilis*. Both antibiotics target envelope integrity, based on the strong induction of extracytoplasmic function σ factor-dependent gene expression. The cell envelope stress-sensing two-component system LiaRS is exclusively and strongly induced by daptomycin, indicative of different mechanisms of action in the two compounds.

Staphylococcus aureus is a leading cause of nosocomial infections, especially in mechanically ventilated patients. Its remarkable potential to acquire and accumulate high-level resistance against most of the classical antibiotics (including vancomycin) used for the treatment of gram-positive infections is one of the reasons for the ongoing mortality caused by hospital-acquired *S. aureus* infections (7, 17).

Daptomycin is the first of a new class of cyclic lipodepsipeptide antibiotics (Fig. 1A) with strong bactericidal activities against gram-positive pathogens (2). It interferes with cell envelope integrity, and cell death occurs presumably by either membrane depolarization or membrane perforation (19, 20). Friulimicin B, an acidic, cyclic lipopeptide produced by *Actinoplanes friuliensis*, shows structural similarities to daptomycin (Fig. 1B) and is also active against multidrug-resistant gram-positive bacteria (1, 22).

As part of a coordinated effort to study and characterize its mode of action, we have performed comparative in-depth expression profiling for both antibiotics. This technique is a powerful approach to elucidate the inhibitory mechanisms of novel antimicrobial compounds (4, 9) and has been successfully applied to characterize and differentiate antimicrobial actions, often using *Bacillus subtilis* as a model organism (3, 10). *B. subtilis* is particularly well suited for studying cell wall antibiotics, since the regulatory network orchestrating its cell envelope stress response (CESR) is well characterized. It consists of four two-component systems and at least four extracytoplasmic function (ECF) σ factors (11).

Here, we present results from an in-depth analysis of the expression signature provoked by the treatment of *B. subtilis* with sublethal amounts of daptomycin and friulimicin B. Our data show that both antibiotics specifically target cell envelope integrity. But significant differences in the corresponding

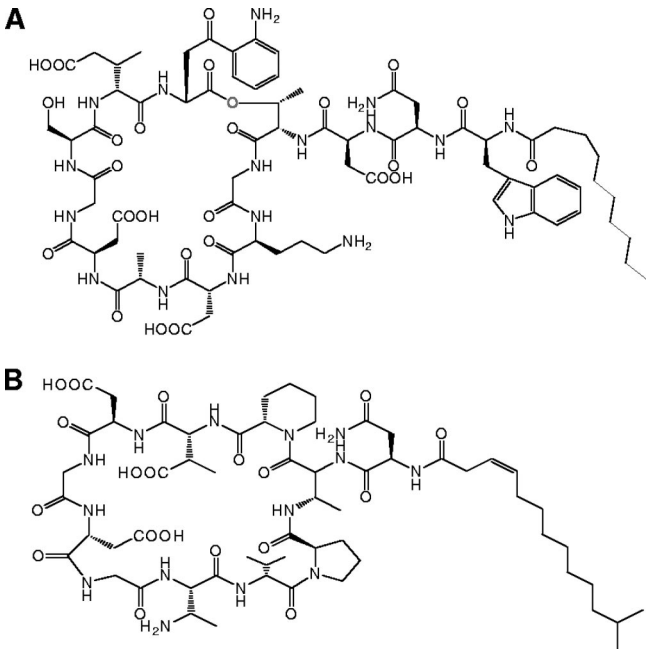

FIG. 1. Chemical structures of the lipodepsipeptide antibiotic daptomycin (A) and the lipopeptide antibiotic friulimicin B (B).

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TABLE 1. Marker genes induced by daptomycin and/or friulimicin B

Gene(s)[a]	Induction by:[b]		Regulator(s)[c]	Localization (putative)[d]	Homology, function, remarks[e]
	DAP	FRI			
ywaC	4.5 ± 4.3	8.7 ± 3.8	σ^V, σ^M, σ^W	C	Putative GTP-pyrophosphokinase
mreBH	3.9 ± 1.9	3.1 ± 1.2		C	Control of cell shape; membrane-associated
ydaH	3.3 ± 0.3	9.1 ± 2.4	σ^M	M	Conserved membrane protein
yqjL	3.3 ± 0.3	8.9 ± 1.6	σ^V, σ^M	C	Putative hydrolase
bcrC	3.3 ± 1.0	8.2 ± 2.8	σ^V, σ^M, σ^W, σ^X	M	Undecaprenyl pyrophosphate phosphatase
yrhH	3.1 ± 1.3	8.5 ± 3.4	σ^V, σ^M, σ^W	C	Putative methyltransferase
liaIH(GFSR)	429 ± 53	−	LiaRS	M, S	Conserved membrane protein; phage-shock protein A homolog (three-component regulatory system)
gerAAABAC	15 ± 2.9	−	(LiaRS)	M, S	Downstream *lia* operon, known polar effect from P_{liaI}
ybeF	4.6 ± 0.9	−		M	Conserved membrane protein
sigM-yhdLK	−	7.4 ± 4.0	σ^M	C, M	ECF σ factor
yjbC-spx	−	7.2 ± 1.7	σ^V, σ^M, σ^W	C	Glutaredoxin family; transcriptional regulator Spx
sms-yacKL	−	7.1 ± 0.5	σ^M	C, C, M	DNA repair/binding proteins; membrane protein
radC	−	6.9 ± 2.1	σ^M	C	DNA repair protein
ypuA	−	6.5 ± 2.3	σ^M	S	Conserved hypothetical
ypbG	−	6.4 ± 1.0	σ^M	S	Putative phosphoesterase
ypuD	−	6.2 ± 0.7	σ^M	S	Unknown
ycgRQ	−	5.9 ± 0.6	σ^V, σ^M	M	Conserved membrane protein; permease
yrhIJ	−	5.7 ± 0.8	σ^M	C,	Cytochrome P450; transcriptional repressor BscR
sigV-yrhM	−	5.1 ± 2.0	σ^V	C, M	ECF σ factor
yfnI	−	4.7 ± 2.0	σ^M	M (S)[f]	Similar to phosphoglycerol transferases
yebC	−	4.1 ± 0.6	σ^M	M	Unknown
yppC	−	4.1 ± 0.4		C	Conserved hypothetical
ywnJ	−	4.1 ± 0.1	σ^M, σ^X	M	Unknown
ywtF	−	3.9 ± 0.6	σ^M	C (S)[f]	Putative transcriptional regulator
pbpI	−	3.8 ± 1.3		M	Class B penicillin-binding protein
rodA	−	3.8 ± 0.9	σ^M	M	Control of cell shape and elongation
ylxW	−	3.5 ± 0.3	σ^M	M	Unknown
yoxD	−	3.7 ± 0.2		C	Putative 3-oxoacyl-acyl-carrier protein
yqiG	−	3.4 ± 0.4		C	Putative NADH-dependent flavin oxidoreductase
yjbQ	−	3.4 ± 0.2		M	Putative Na^+/H^+ antiporter

[a] Only genes that were induced ≥threefold in three independent experiments by daptomycin and/or friulimicin B are shown. The proteins corresponding to the underlined genes were also significantly upregulated in the cytoplasmic proteome (Fig. 2).

[b] Average induction ratio of the highest value for each locus (usually the first gene in an operon) and the corresponding standard deviation are given. DAP, daptomycin; FRI, friulimicin B; −, no significant induction.

[c] Assignment of regulators is based on the corresponding regulon papers: LiaRS (12), σ^M (8), σ^V (24), σ^W (6), and σ^X (5).

[d] Localization of the corresponding proteins is based on the presence of transmembrane regions (membrane proteins) and signal peptides (secreted proteins) detected with SMART. C, cytoplasmic proteins; M, membrane proteins; S, secreted proteins.

[e] Putative function is derived from BSORF/Subtilist entries (at http://bacillus.genome.ad.jp/ and http://genolist.pasteur.fr/SubtiList/genome.cgi, respectively), NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), or SMART (http://smart.embl-heidelberg.de/) analysis.

[f] YfnI and YwtF are assigned to secreted proteins based on experimental evidence (21).

CESRs, as clearly documented by transcriptomics, proteomics, and detailed gene expression profiling, strongly suggest different modes of action of the two structurally related antibiotics.

(This study was presented in part at the 47th International Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 17 to 20 September 2007 [25]).

Transcriptomics and proteomics. For microarray experiments, midlogarithmic cultures of *B. subtilis* were challenged with 1 μg/ml (sublethal amounts) of either daptomycin or friulimicin B. The cells were harvested 10 min postinduction, and cell pellets were directly snap-frozen in liquid nitrogen. RNA preparation and microarray experiments were performed essentially as described previously (13, 23). To validate the gene expression profiles, we also performed two-dimensional gel electrophoresis of the cytoplasmic proteome of *B. subtilis* cells, quantifying de novo protein synthesis after the addition of daptomycin or friulimicin B by incubating the cultures in the presence of L-[^{35}S]methionine, as described previously (3). The results are summarized in Table 1 and Fig. 2. The complete microarray data sets can be found in the supplemental material and, together with additional supporting information, at http://microbial-stress.iab.kit.edu/87.php.

Both antibiotics induced a limited number of genes, most of which could be assigned to known CESR regulons. Daptomycin specifically and strongly activated the LiaRS two-component system, with more than 200-fold induction of its primary target genes, *liaIH*. This induction has also been observed recently in an independent study (9a) and is in good agreement with data from the orthologous VraSR system of *S. aureus* which was also induced by daptomycin (16). Moreover, a strong LiaH induction was also observed with proteomics analysis, where it was identified in three strong neighboring spots (differing in their isoelectric points), indicative of posttranslational modifications (Fig. 2).

Both compounds induced numerous genes known to be regulated by ECF σ factors. This ECF-dependent response was much stronger for friulimicin B (Table 1). In addition, only seven genes/proteins of unknown regulation were differentially expressed (Table 1 and Fig. 2), including the actin homolog *mreBH*, which was induced about three- to fourfold by both

TABLE 2. Strains and oligonucleotides used in this study

Strain or oligonucleotide	Relevant genotype or fragment amplified	Source, construction, reference, or sequence[a]
B. subtilis strains		
W168	Wild-type strain; *trpC2*	Lab stock
BFS2470	W168 *liaI*::pMUTIN	Zoltan Pragai (Harwood lab); 14, 15
TMB389	W168 *liaIH*::Tet^r	W168, transformed with chromosomal DNA of strain HB0935; 14
Oligonucleotides		
822/823	*sigY*-RT	Fwd, ACAAGAAGAACAGCGGCTGA; rev, TTCCTGAACAAGTTCCTCGC
824/825	*sigX*-RT	Fwd, ACAGAAGACCTTCTTCAAGAG; rev, CGCTGTCTGATTGTTTGCTG
826/827	*sigM*-RT	Fwd, GTTTACAGGTTCCTGCTCTC; rev, ATGAAGGCGTTTCGCGCCA
828/829	*sigW*-RT	Fwd, TCAGCTTTGCTACCGTATGC; rev, TTGCGAATGCGGTCAATGGT
830/831	*sigV*-RT	Fwd, AAGCGTTGCTTGTCACATGC; rev, GTTCCTGACCGTTTCAACTG
832/833	*sigZ*-RT	Fwd, GCATCTCCCAAATCTGATCG; rev, TTTTCTTCCTCTGCACTGTCA
834/835	*ylaC*-RT	Fwd, TTGAGGACTTGTATCGGCAG; rev, GAGCCATGTTCTGATGGAAG
125/126	*liaH*-RT	Fwd, TGAAACAGCACACGATTGCC; rev, GTTTGCCTGTTCATAGGAAGC
156/157	*rpsI*-RT	Fwd, GAAACGGCAAAACGTTCTGG; rev, GTGTTGGGTTCACAATGTCG
158/159	*rpsE*-RT	Fwd, GCGTCGTATTGACCCAAGC; rev, TACCAGTACCGAATCCTACG

[a] Fwd, forward; rev, reverse.

FIG. 2. Synthesis patterns of marker proteins after induction with daptomycin (DAP) or friulimicin B (FRI) compared to patterns of untreated control cells. Details from the two-dimensional gels of the cytoplasmic proteome ("spot albums" of marker proteins) are shown for two time points postinduction with each of two compounds. (A) Proteins induced by both antibiotics. (B) Daptomycin-specific spots. (C) Friulimicin B-specific spots. See text for details.

TABLE 3. Induction of ECF σ factors and *liaH* by daptomycin and friulimicin B

Gene	Induction by:[a]	
	DAP	FRI
sigM	2.4 ± 0.1	8.7 ± 3.8
sigV	2.4 ± 0.7	7.4 ± 2.3
sigW	1.4 ± 0.3	0.9 ± 0.0
sigX	0.8 ± 0.2	0.8 ± 0.0
sigY	0.9 ± 0.1	1.8 ± 0.3
sigZ	1.0 ± 0.0	1.2 ± 0.1
ylaC	1.0 ± 0.0	2.9 ± 0.3
liaH	1170 ± 426	0.9 ± 0.0

[a] Levels of change given are the average ± standard deviation of the results of two independent real-time RT-PCR experiments, performed essentially as previously described (23), using an iScript one-step RT-PCR kit with Sybr green (Bio-Rad) according to the manufacturer's recommended procedure. DAP, daptomycin; FRI, friulimicin B.

compounds. Five more genes without known regulator, some of which are potentially involved in cell envelope biogenesis, specifically responded to friulimicin B (Table 1). All genes identified in our analysis have been linked to CESR of *B. subtilis* previously (data not shown). While no expression signature available so far resembles that of friulimicin B, both the transcriptome and the proteome profile for daptomycin closely resemble those of bacitracin (3, 14).

In-depth gene expression profiling. The results of our microarray study led to three follow-up analyses on the specificity of the corresponding CESR. (i) We analyzed the induction of all seven ECF σ factors by quantitative real-time reverse transcriptase PCR (RT-PCR), based on the known and highly ECF-specific autoregulation of their own genes, to determine the respective inducer spectrum and strength. The primers used for amplification are listed in Table 2. Both antibiotics activate σ^M and σ^V, with friulimicin B provoking a significantly stronger response. In addition, friulimicin B also induced the uncharacterized ECF σ factor σ^{YlaC} (Table 3).

(ii) The much stronger activation of ECF target genes by friulimicin B was not due to the corresponding lack of *liaIH* induction, as demonstrated by the induction values of ECF genes in the *liaIH* mutant strain TMB0389, which were identical to those in the wild type (data not shown). The stronger ECF response to friulimicin B is therefore LiaIH independent and a true antibiotic-specific difference in the corresponding gene induction profiles.

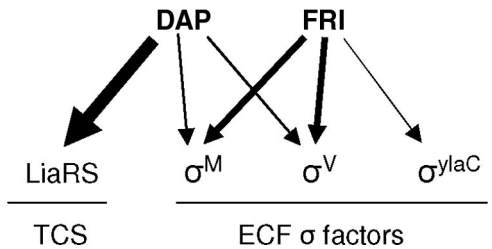

FIG. 3. Schematic representation of the regulatory networks orchestrating the daptomycin (DAP) and friulimicin B (FRI) stress responses. The thickness of the arrows corresponds to the strength of induction of the given regulators (see text and Table 1 for details). TCS, two-component system.

(iii) We also quantified the activity of the LiaR target promoter P_{liaI} as a function of the daptomycin/friulimicin B concentrations over a range of 4 orders of magnitude by performing a β-galactosidase assay (using strain BFS2470 as described previously) (15). P_{liaI} induction was indeed only observed in the presence of daptomycin and in a very narrow window of antibiotic concentrations (between 0.5 and 2 μg/ml) (data not shown). These results strongly suggest different modes of action for daptomycin and friulimicin B.

Conclusions. Our data clearly allowed the identification of cell envelope integrity as the site of daptomycin and friulimicin B action, but the results strongly suggest mechanistic differences between the two compounds. This assumption is primarily based on the dramatic differences in the LiaRS response. Moreover, friulimicin B activates both σ^M and σ^V more strongly than daptomycin and, additionally, induces σ^{YlaC} expression (summarized in Fig. 3). The strong similarities of CESR between daptomycin and bacitracin were initially viewed as an indication that daptomycin might interfere with the lipid II cycle of cell wall biosynthesis. But a detailed biochemical mechanism of action study revealed that friulimicin B, like amphomycin but in contrast to the membrane-interfering daptomycin, inhibits cell wall biosynthesis by binding bactoprenol phosphate (18).

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Chapter 3

The rhamnolipid stress response of *Bacillus subtilis*.

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Tina Wecke performed the experiments, cluster analysis and transcriptome data analysis. Tobias Bauer contributed to strain construction and growth curve experiments during his bachelor thesis. Henning Harth and Ulrike Mäder performed the initial microarray analysis. Tina Wecke and Thorsten Mascher designed the experiments and wrote the paper.

RESEARCH LETTER

The rhamnolipid stress response of *Bacillus subtilis*

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Abstract

Rhamnolipids are biosurfactants produced by the soil bacterium *Pseudomonas aeruginosa*. In addition to their high industrial potential as surface-active molecules, rhamnolipids also have antimicrobial properties. In densely populated habitats, such as the soil, production of antimicrobial compounds is important to inhibit growth of competitors. For the latter, it is crucial for survival to sense and respond to the presence of those antibiotics. To gain a first insight into the biological competition involving biosurfactants, we investigated the cellular response of the model organism *Bacillus subtilis* upon exposure to rhamnolipids by genome-wide transcriptional profiling. Most of the differentially expressed genes can be assigned to two different regulatory networks: the cell envelope stress response mediated by the two-component system LiaRS and the extracytoplasmic function σ factor σ^M and the CssRS-dependent secretion stress response. Subsequent phenotypic analysis demonstrated a protective function of LiaRS and σ^M against cell lysis caused by rhamnolipids. Taken together, we present the first evidence that a single antimicrobial compound can simultaneously induce genes from two independent stress stimulons.

Introduction

The soil is a complex habitat characterized by high population density and nutrient limitation. To survive in such a competitive environment, bacteria developed a number of different strategies. One such strategy is the production of antimicrobial compounds to inhibit growth of competitors (Paul & Clark, 1996; Tate, 2000). In addition to classical antibiotics that target essential structures or processes within the bacterial cell, antimicrobial activities, often based on biophysical effects, can also be assigned to ionophores, ion-channel forming agents or biosurfactants (Berdy, 2005).

Biosurfactants are surface-active molecules synthesized by microorganisms. They consist of a hydrophilic and a hydrophobic part and are able to reduce surface tension and enhance the emulsification of hydrocarbons. Biosurfactants are commercially used for bioremediation processes as well as the pharmaceutical, cosmetics, and food industries (Banat *et al.*, 2000). Rhamnolipids are biosurfactants produced by the soil bacterium *Pseudomonas aeruginosa*. These surface-active molecules are glycolipids composed of one or two L-rhamnose moieties and one or two β-hydroxydecanoic acid residues (Soberon-Chavez *et al.*, 2005). The synthesis from rhamnose and fatty acid precursors is catalyzed by the products of three genes, *rhlABC*, and regulated in a cell density-dependent manner by quorum sensing. The amount and composition of synthesized rhamnolipids depends on growth conditions and available carbon source (Soberon-Chavez *et al.*, 2005).

Rhamnolipids have been shown to exhibit antimicrobial activity against Gram-positive bacteria and, but to a much lesser extent, also against Gram-negative species (Itoh *et al.*, 1971; Lang *et al.*, 1989). They modify the cell surface by increasing its hydrophobicity and membrane permeability (Vasileva-Tonkova *et al.*, 2011). Although

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the production of rhamnolipids by *P. aeruginosa* is well understood (Soberon-Chavez *et al.*, 2005), only little is known about the physiological reaction to the presence of this biosurfactant.

The response to antimicrobial compounds that interfere with the cell envelope integrity has been extensively studied in the model organism *Bacillus subtilis*. Here, the regulatory network of the cell envelope stress response is mediated by two regulatory principles: two-component systems (TCS) and extracytoplasmic function (ECF) σ factors. Four TCS (BceRS, LiaRS, PsdRS and YxdJK) and at least three ECF σ factors (σ^M, σ^W and σ^X) have been described to respond to cell wall antibiotics, such as vancomycin, bacitracin, or cationic antimicrobial peptides (Jordan *et al.*, 2008). *Bacillus subtilis* inhabits the same environment as the rhamnolipid-producing species *P. aeruginosa*. Therefore, we decided to investigate the response of *B. subtilis* to rhamnolipids by genome-wide DNA microarray analysis followed by hierarchical clustering of differentially expressed genes and phenotypic characterization to gain a first insight into this interspecies competition.

Materials and methods

Bacterial strains and growth conditions

Bacillus subtilis and *Escherichia coli* were routinely grown in LB medium at 37 °C with aeration. All strains and plasmids used in this study are listed in Table 1. Standard cloning techniques were applied (Sambrook & Russell, 2001) and transformation was carried out as described (Harwood & Cutting, 1990). Ampicillin (100 μg mL^{-1}) was used for selection of *E. coli*, kanamycin (10 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 selection of *B. subtilis* mutants. Rhamnolipids were isolated from *P. aeruginosa* as a mixture of mono- and di-rhamnolipid (Müller *et al.*, 2010), dissolved in ethanol and used at the indicated concentrations. All experiments were performed with rhamnolipids from the same purification, as the composition and biological activity varies between different cultivations of *P. aeruginosa* (R. Hausmann, pers. commun.).

Preparation of total RNA

Bacillus subtilis W168 was grown aerobically in LB medium at 37 °C until an $OD_{600\,nm}$ of *c.* 0.5. The culture was split and one sample was induced with sublethal concentrations (50 μg mL^{-1}) of rhamnolipids, leaving the other sample as uninduced control. After 10 min, 30 mL culture were mixed with 15 mL cold killing buffer (20 mM Tris–HCl, pH 7.0, 0.5 mM $MgCl_2$, 20 mM NaN_3), harvested by centrifugation and frozen in liquid nitrogen, before the pellets were stored at −80 °C. Total RNA was isolated as described previously (Wolf *et al.*, 2010). Contaminating DNA was removed using the RNase-free DNase kit (Qiagen) and quality control of the RNA was performed with an RNA 6000 Nano LabChip Kit (Agilent Technologies) on an Agilent 2100 Bioanalyzer according to the manufacturer's instructions.

Table 1. Strains, vectors, and plasmids used in this study

Strain	Genotype or characteristic(s)*	Reference, source or construction
E. coli strains		
DH5α	recA1 endA1 gyrA96 thi hsdR17rK- mK +relA1 supE44 Φ80 ΔlacZΔM15 Δ(lacZYA-argF)U169	Laboratory stock
B. subtilis strains		
W168	Wild type, *trpC*2	Laboratory stock
TMB149	W168 *sigW*::MLS	LFH-PCR→W168
TMB329	W168 Δ*liaF*	Wolf *et al.* (2010)
TMB589	W168 Δ*liaR*	pMAD-based clean deletion
TMB1003	W168 *sigM*::kan	HB0829 chrom. DNA→W168
TMB1070	W168 *cssRS*::kan	LFH-PCR→W168
TMB1392	W168 Δ*liaR sigM*:: kan *sigW*::MLS	HB0829 chrom. DNA→TMB589
TMB1393	W168 *sigM*::kan *sigW*::MLS	HB0829 chrom. DNA→TMB1003
HB0829	NCIB3610 *sigM*:: kan *sigW*::MLS	Mascher *et al.* (2007)
Vectors or plasmids		
pMAD	*bgaB, ermC*, *bla*, MCS	Arnaud *et al.* (2004)
pDG780	pBluescriptKS+ *kan,* source of resistance cassette for LFH-PCR	Guerout-Fleury *et al.* (1995)
pDG647	pSB119, MLS, source of resistance cassette for LFH-PCR	Guerout-Fleury *et al.* (1995)
pDW104	pMAD Δ*liaR*	This study

chrom. DNA, chromosomal DNA.
*Resistance cassettes: kan, kanamycin; MLS, macrolide-lincosamide-streptogramin B; spec, spectinomycin.

DNA microarray analysis

RNA samples from three independent cultivations were used for cDNA synthesis and hybridized with dye-swap to Agilent custom DNA microarrays. Synthesis of fluorescently labeled cDNA, hybridization and scanning of the microarrays were performed as described previously (Otto *et al.*, 2010). Data were extracted and processed using

the FEATURE EXTRACTION software (version 10.5; Agilent Technologies). For each gene on the microarray, the error-weighted average of the log ratio values of the individual probes was calculated using the ROSETTA RESOLVER software (version 7.2.1; Rosetta Biosoftware). The complete dataset containing induction ratios for all genes is available at http://www.syntheticmicrobe.bio.lmu.de/publications/supplemental/index.html.

Measurement of induction by quantitative real-time RT-PCR

Measurement of transcript abundance was performed in duplicate by quantitative real-time RT-PCR using the QuantiFast SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's protocol, with minor modifications. In brief, 100 ng of DNA-free RNA were used in a total reaction volume of 20 μL with 0.3 μM of each primer (Table 2). The reaction was carried out in a MyiQ Cycler (BioRad). Expression of *rpsJ* and *rpsE* was monitored as constitutive reference. Relative induction levels were calculated as fold changes using the formula: Fold change = $2^{-\Delta\Delta C_t}$; with $-\Delta\Delta C_t = (C_{t,\text{gene } x} - C_{t,\text{constitutive gene}})_{\text{condition I}} - (C_{t,\text{gene } x} - C_{t,\text{constitutive gene}})_{\text{condition II}}$ (Talaat *et al.*, 2002).

Hierarchical clustering analysis

Clustering was performed using the program CLUSTER 3.0 (de Hoon *et al.*, 2004). Transcriptome data were derived from this work or published studies (Cao *et al.*, 2002a; Mascher *et al.*, 2003; Lulko *et al.*, 2007; Wecke *et al.*,

Table 2. Oligonucleotides used in this study

Nr	Name	Sequence
Real-time RT-PCR		
0125	*liaH*-RT fwd	TGAAACAGCACACGATTGCC
0126	*liaH*-RT rev	GTTTGCCTGTTCATAGGAAGC
1890	*cssR*-RT fwd	TGGATTCTCGATATCATGCTG
1891	*cssR*-RT rev	TAGTCATTGCTGCCAATCTC
1886	*htrA*-RT fwd	AACGAGGATTCGGATGGTTC
1887	*htrA*-RT rev	TGTAACAGATTGCGTTTGCTG
1888	*htrB*-RT fwd	GCCTTATCTGCCGTCAGAC
1889	*htrB*-RT rev	ATTCCGACAATCGTAGGCTC
0826	*sigM*-RT fwd	GTTTACAGGTTCCTGCTCTC
0827	*sigM*-RT rev	ATGAAGGCGTTTCGCGCCA
0156	*rpsJ*-RT fwd	GAAACGGCAAAACGTTCTGG
0157	*rpsJ*-RT rev	GTGTTGGGTTCACAATGTCG
0158	*rpsE*-RT fwd	GCGTCGTATTGACCCAAGC
0159	*rpsE*-RT rev	TACCAGTACCGAATCCTACG
LFH-PCR		
0342	*sigW* up fwd	CCGAGAAGTTCAGGGCAAGCC
0343	*sigW* up rev	CCTATCACCTCAAATGGTTCGCTGCGATGTCCGCAAATGCATCC
0344	*sigW* do fwd	CGAGCGCCTACGAGGAATTTGTATCGCGGATTCACAGAGGCAGAGAGC
0345	*sigW* do rev	GCTGAACCGCTTTCGTGCC
1793	*cssR* up fwd	TTTCACTTTCTGAGCTGGAG
1794	*cssR* up rev	CCTATCACCTCAAATGGTTCGCTGTTCATTCAGGTTATCCTCATC
1795	*cssS* do fwd	CGAGCGCCTACGAGGAATTTGTATCGGGTGTATCATACCGCATAGC
1796	*cssS* do rev	ATTGAGACGGCTTCACAGTG
0137	kan fwd	CAGCGAACCATTTGAGGTGATAGG
0138	kan rev	CGATACAAATTCCTCGTAGGCGCTCGG
0139	mls fwd	CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC
0140	mls rev	CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG
0147	kan check rev	CTGCCTCCTCATCCTCTTCATCC
0056	kan check fwd	CATCCGCAACTGTCCATACTCTG
0148	mls check rev	GTTTTGGTCGTAGAGCACACGG
0057	mls check fwd	CCTTAAAACATGCAGGAATTGACG
Δ*liaR* deletion mutant		
1060	*liaR* up fwd (BamHI)	AGCC**GGATCC**GACAACGGGAATCAGCCTGC
1120	*liaR* up rev	CGAGATGATTTCGGTGTGCGCTGACCATTTCATGATCATC
1059	*liaR* do fwd	CGCACACCGAAATCATCTCG
1061	*liaR* do rev (NcoI)	TATA**CCATGG**GCTGACACAGCAAATTCTCG

Restriction sites for cloning are highlighted in bold, linker regions for joining reactions are underlined.

2009). The datasets represent the following conditions: 50 μg mL^{-1} rhamnolipids (10 min), 1 μg mL^{-1} daptomycin (10 min), 1 μg mL^{-1} friulimicin (10 min), 2 μg mL^{-1} vancomycin (10 min), 100 μg mL^{-1} bacitracin (5 min) and secretion stress caused by overexpression of the α-amylase AmyQ. For reasons of clarity, cluster analysis was restricted to genes induced $\geq$ threefold and repressed $\geq$ fivefold by rhamnolipids.

Allelic replacement mutagenesis using long-flanking homology PCR

The long-flanking homology (LFH) PCR is derived from a published procedure (Wach, 1996) and performed as previously described (Mascher *et al.*, 2003). In brief, resistance cassettes were amplified from suitable vectors as template (Guerout-Fleury *et al.*, 1995). About 1000-bp DNA fragments flanking the region to be deleted were amplified by PCR using chromosomal DNA of *B. subtilis* W168 as template. These fragments are here called up- and do-fragments. The up-reverse and do-forward primers carry *c.* 25-bp nucleotides complementary to the sequence of the resistance cassettes. All obtained fragments were purified and used as template in a second PCR with the corresponding up-forward and do-reverse primers. The PCR products were directly used to transform *B. subtilis* W168. Transformants were screened by colony PCR using the up-forward and do-reverse primers with check primers annealing within the resistance cassette. Integrity of the regions flanking the resistance cassette was verified by sequencing of PCR products. The resulting strains are listed in Table 1, the oligonucleotides in Table 2.

Construction of a markerless Δ*liaR* deletion mutant

A markerless Δ*liaR* deletion strain was constructed using the vector pMAD (Arnaud *et al.*, 2004) and the oligonucleotides listed in Table 2. The procedure has been described previously (Wolf *et al.*, 2010). In brief, about 1000-bp regions upstream and downstream of *liaR* were amplified using PCR, thereby introducing a 20-bp extension to the 3′-end of the up-fragment, which is complementary to the 5′-end of the do-fragment. The fragments were fused by a second PCR and the resulting product was cloned into pMAD, generating pDW104. *Bacillus subtilis* W168 was transformed with pDW104 and incubated at 30 °C with MLS selection on LB agar plates containing 100 μg mL^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Blue colonies were selected and incubated for 6–8 h at 42 °C in LB medium with MLS selection, which results in the integration of the plasmid into the chromosome. Again, blue colonies were selected and incubated for 6 h at 30 °C in LB medium without selection. Subsequently, the culture was shifted to 42 °C for 3 h, before the cells were plated on LB agar plates without selection. White colonies were picked and checked for MLS sensitivity, indicating the loss of the plasmid. Those harboring a clean deletion of *liaR* were identified using PCR.

Concentration-dependent lysis curve experiments

Bacillus subtilis wild-type and mutant strains were inoculated from fresh overnight cultures and grown aerobically in LB medium until an $OD_{600\,nm}$ of *c.* 0.5. The cultures were split into 1 mL samples and different concentrations of rhamnolipids were added. The effect of rhamnolipids on cell density of each sample was monitored over a period of 7 h.

Results and discussion

The transcriptional response to rhamnolipids

Genome-wide expression profiling is a powerful approach to characterize the response to a certain stimulus, such as the presence of antimicrobial compounds. It has also been used to gain insights into inhibitory mechanisms and to differentiate between different modes of action of novel antibiotics (Hutter *et al.*, 2004; Fischer & Freiberg, 2007; Wecke *et al.*, 2009). We used genome-wide DNA microarray analysis to investigate the response of the model organism *B. subtilis* to the presence of rhamnolipids, which have been shown to affect cell envelope integrity (Vasileva-Tonkova *et al.*, 2011). *B. subtilis* was treated with sublethal concentrations (50 μg mL^{-1}) of rhamnolipids, which is sufficient to induce a transcriptional response, but does not impair growth of the culture, as can be demonstrated by concentration-dependent lysis curve experiments (see below and Fig. 3). After 10 min of induction, total RNA was prepared and DNA microarray analysis performed. Expression of 40 loci was $\geq$ fivefold increased by rhamnolipids compared with the mRNA levels of an uninduced culture (Table 3 and Fig. 1a). Almost half of these loci can be assigned to known regulons of TCS or ECF σ factors. The most strongly induced locus was the *liaIHGFSR* operon (*c.* 640-fold), which is autoregulated by the LiaRS TCS (Mascher *et al.*, 2004). The first two genes of this locus, *liaIH*, represent the main targets of LiaRS-dependent signal transduction and *liaH* encodes a phage-shock protein homolog. The LiaRS TCS is activated by cell wall antibiotics, especially lipid II-interacting compounds, but it does not mediate resistance against most of its inducers (Mascher *et al.*, 2004; Wolf *et al.*, 2010). Strong expression of the *lia* locus also resulted in significant read-through

Table 3. Genes significantly induced or repressed by rhamnolipids

Gene(s)*	Fold changes†	Regulators‡	Homology, (putative) function, remarks
Genes induced ≥ fivefold			
liaIHGFSR	640 ± 501	LiaRS	Phage-shock protein homolog, TCS, unknown
htrA	58 ± 15	CssRS	Serine protease
htrB	26 ± 6.6	CssRS	Serine protease
yuxN	13 ± 4.5		Putative transcriptional regulator, TetR family
yqjL	11 ± 1.2	σ^M	Putative hydrolase
pbpE-racX	11 ± 5.2	σ^W	Penicillin binding protein 4, amino acid racemase
yhaSTU	10 ± 4.4		Potassium efflux K^+/H^+ antiporter
yxeI	9.4 ± 2.2		Similar to penicillin amidase
yraA	8.6 ± 3.5		Similar to general stress protein
yuaE	8.2 ± 2.8		Hypothetical protein with DUF1569 domain
yrhHIJ	8.0 ± 3.5	σ^M, σ^X, σ^V	Putative methyltransferase, transcriptional regulator, and reductase
sigM-yhdLK	7.8 ± 1.3	σ^M	ECF σ factor
yebC	7.8 ± 1.4	σ^M	Putative membrane protein
ybfO	7.7 ± 3.3	σ^W	Similar to erythromycin esterase
ylbP	7.4 ± 2.1		Putative acetyltransferase, GNAT family
phoA	7.2 ± 3.8		Alkaline phosphatase A
bcrC	7.2 ± 2.7	σ^V, σ^M, σ^W, σ^X	Undecaprenyl pyrophosphate phosphatase
gabD	7.1 ± 2.7		Succinate-semialdehyde dehydrogenase
ywrO	7.0 ± 2.5		Similar to NAD(P)H oxidoreductase
ydaH	6.9 ± 2.4	σ^M	Putative membrane protein with DUF2837 domain
opuCABCD	6.8 ± 4.8		Osmoprotection
ypbGH	6.7 ± 1.4	σ^M	Putative phosphoesterase and MecA paralog
yceB	6.5 ± 2.8		Putative monooxygenase
yvrD	6.5 ± 1.1		Similar to ketoacyl-carrier protein reductase
ywaC	6.4 ± 2.8	σ^W, σ^M, σ^V	Similar to GTP-pyrophosphokinase
yqjG	6.2 ± 1.7		Similar to lipoprotein SpoIIIJ-like
yheCDE	6.0 ± 1.1		Spore coat proteins
yhjN	5.9 ± 0.8		Putative membrane-anchored ammonia monooxygenase
dhaS	5.8 ± 1.4		Aldehyde dehydrogenase
yfjR	5.8 ± 2.8		Similar to 3-hydroxyisobutyrate dehydrogenase
radC	5.5 ± 1.8	σ^M	DNA repair protein
yvgP	5.5 ± 0.9		Monovalent cation/H^+ antiporter NhaK
trxA	5.4 ± 1.2		Thioredoxin, putative monooxygenase
nfrA-ywcH	5.2 ± 1.2		NADPH-linked nitro/flavin reductase, similar to monooxygenase
gerAABC	5.2 ± 2.2		Germination, downstream of *liaIHGFSR*
ypuA	5.2 ± 0.9	σ^M, σ^V	Protein of unknown function with DUF1002 domain
ywnJ	5.2 ± 1.5	σ^M, σ^W, σ^X	Putative VanZ-like membrane protein
yrbC	5.1 ± 2.5		Uncharacterized conserved protein with DUF28 domain
ycgJ	5.0 ± 1.6		Putative methyltransferase
yfiBC	5.0 ± 1.4		Similar to ABC transporter
Genes repressed ≥ fivefold			
cydABCD	0.19 ± 0.09		Cytochrome bd ubiquinol oxidase
rbsRKDACB	0.19 ± 0.07		Ribose transport
yuaJ	0.19 ± 0.07		Putative thiamine transporter
yonPO	0.18 ± 0.07		Hypothetical proteins (prophage SPβ)
narGHJI	0.18 ± 0.05		Nitrate reductase
mtbP	0.17 ± 0.06		Modification methylase
pur operon	0.17 ± 0.08		Purine biosynthesis
yxaI	0.17 ± 0.08		Putative membrane protein
yolJ	0.15 ± 0.06		Similar to glycosyltransferase
xylAB	0.13 ± 0.02		Xylose metabolism
sboAXablA-G	0.13 ± 0.03		Bacteriocin subtilosin A
bdbA	0.12 ± 0.06		Thiol-disulfide oxidoreductase

Table 3. Continued.

Gene(s)*	Fold changes†	Regulators‡	Homology, (putative) function, remarks
pyr operon	0.07 ± 0.06	PyrR	Pyrimidine biosynthesis
pstSCABABB	0.07 ± 0.03	PhoPR	Phosphate ABC transporter
des	0.06 ± 0.04	DesKR	Fatty acid desaturase

*Only genes that were induced or repressed ≥ fivefold on average are listed.

†Highest induction ratios for each locus (usually the first gene in an operon) and the corresponding standard deviation are given.

‡Assignment of regulators is based on (Turner *et al.*, 1994; Qi *et al.*, 1997; Huang & Helmann, 1998; Huang *et al.*, 1999; Aguilar *et al.*, 2001; Hyyryläinen *et al.*, 2001; Wiegert *et al.*, 2001; Cao *et al.*, 2002a,b; Darmon *et al.*, 2002; Cao & Helmann, 2004; Zellmeier *et al.*, 2005; Jordan *et al.*, 2006; Eiamphungporn & Helmann, 2008).

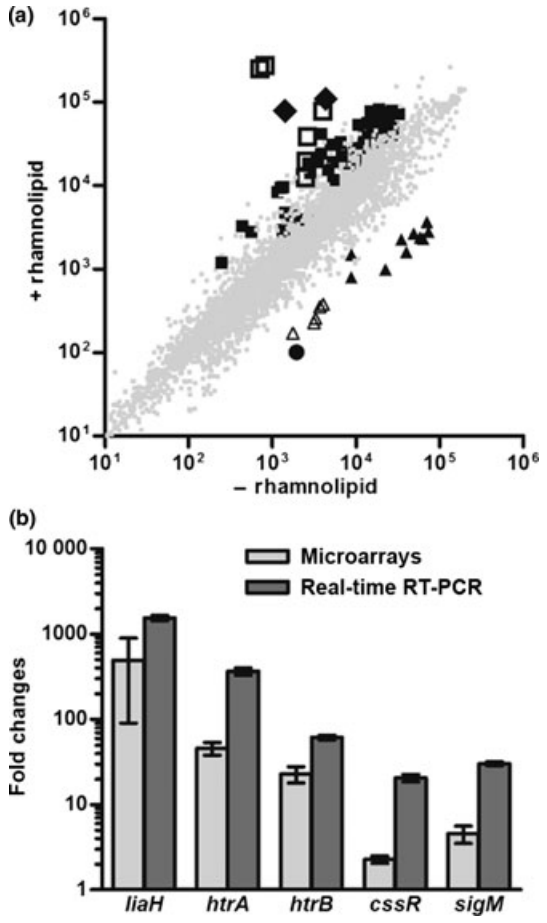

Fig. 1. The transcriptional response to rhamnolipids. (a) Scatter plot of DNA microarray analysis. The average signal intensities for each gene are shown from cells induced with 50 μg mL^{-1} rhamnolipids for 10 min (*y*-axis) and uninduced control (*x*-axis). The *pyr* operon (▲), *pstSCABABB* (Δ), *des* (●) and genes regulated by LiaRS (□), CssRS (♦) and σ^M (■) are highlighted; all other genes are represented as gray squares. (b) Verification of the transcriptome data by real-time RT-PCR. Real-time RT-PCR was performed as described in Materials and methods with the same RNA as used for DNA microarray analysis. Induction ratios for each gene were calculated based on the uninduced control, as described previously (Talaat *et al.*, 2002). Each value is the average of two microarray hybridizations or real-time RT-PCR experiments, the error bar indicating the standard deviation.

transcription of the downstream located *gerAAABAC* operon, which has been observed previously for both *B. subtilis* and *Bacillus licheniformis* (Mascher *et al.*, 2003; Wecke *et al.*, 2006).

The genes *htrA* (*c.* 60-fold) and *htrB* (*c.* 25-fold), both encoding serine proteases, were also strongly induced by rhamnolipids (Table 3 and Fig. 1a). Expression of both genes is controlled by the TCS CssRS, which is activated by heat and secretion stress. Expression of *cssRS* itself was not induced by rhamnolipids, similar to the effect of heat stress, although moderately increased expression of this operon can be observed under secretion stress conditions caused by overexpression of the secretory protein α-amylase (Darmon *et al.*, 2002; Hyyryläinen *et al.*, 2005).

Almost one-third of the remaining ≥ fivefold induced loci represent target genes of ECF σ factors, predominantly σ^M, with its own autoregulated operon *sigM-yhdLK* being approximately eightfold induced (Table 3 and Fig. 1a). As a result of a previously described regulatory overlap between different ECF σ factors of *B. subtilis* (Qiu & Helmann, 2001; Mascher *et al.*, 2007), expression of some genes, such as *bcrC* and *ywaC*, can be regulated by more than one ECF σ factor. But the autoregulated loci of the remaining six ECF σ factors of *B. subtilis* were not significantly induced (≤ threefold), indicating that the ECF response to rhamnolipids is mediated mainly by σ^M. This ECF σ factor is activated by cell wall antibiotics like vancomycin, bacitracin, and phosphomycin, but also under acid, salt, and heat stress conditions (Cao *et al.*, 2002a, b; Mascher *et al.*, 2003; Thackray & Moir, 2003).

Other genes significantly induced by rhamnolipids cannot be assigned to known cell envelope stress regulons. They often encode proteins of unknown function or proteins presumably involved in metabolic and redox processes (e.g. *gabD* encoding a succinate-semialdehyde dehydrogenase or *trxA* encoding thioredoxin).

We verified the main findings of our DNA microarray analysis, in particular the activation of the TCS LiaRS and CssRS as well as σ^M, independently by real-time RT-PCR and basically obtained the same results, albeit with an overall higher induction ratio (Fig. 1b). Such discrepancy

was observed in numerous studies before and is attributed to the overall lower dynamic range of DNA microarrays compared with other methods such as real-time RT-PCR (Conway & Schoolnik, 2003; Pappas *et al.*, 2004).

Treatment with rhamnolipids also led to decreased expression of a certain set of genes (Fig. 1a and Table 3). Among the $\geq$ fivefold repressed loci are genes encoding proteins involved in purine and pyrimidine biosynthesis (*pyr* and *pur* operon), phosphate transport (*pstSCABABB*) and sugar metabolism (*rbsRKDACB*, *xylAB*) (Table 2). Differential expression of the *pyr* operon in response to cell envelope stress has been observed previously for *B. licheniformis* (Wecke *et al.*, 2006).

With almost 20-fold repression, the most strongly downregulated gene is *des*, which encodes a fatty acid desaturase (Aguilar *et al.*, 1998). Expression of *des* is controlled by the TCS DesRK and induced by cold shock. The desaturase is important for maintaining membrane fluidity at low temperature by introducing double bonds in phospholipids (Aguilar *et al.*, 2001), indicating that rhamnolipid treatment at sublethal concentrations could interfere with membrane fluidity.

Hierarchical clustering analysis of genes differentially expressed in response to rhamnolipids

Our DNA microarray analysis clearly indicates that rhamnolipids induce both the cell envelope and the secretion stress response. To further validate this novel induction pattern, we performed hierarchical clustering analysis using transcriptome data of *B. subtilis* induced with different cell wall antibiotics (vancomycin, bacitracin, daptomycin and friulimicin) and exposed to secretion stress. For reason of clarity, we limited our analysis to genes induced $\geq$ threefold and repressed $\geq$ fivefold by rhamnolipids.

Genes controlled by the same regulator form discrete clusters based on their expression pattern under different stress conditions (Fig. 2a). Genes belonging to the cell envelope stress response of *B. subtilis* are grouped in three clusters and can be assigned to two regulators, σ^M and the LiaRS TCS (Fig. 2b). They are induced by cell wall antibiotics and rhamnolipids, but not by secretion stress (with the exception of *liaH*). One of these three clusters contains the target operon of the LiaRS TCS as well as the downstream genes *gerAAAB*. The other two clusters include mostly target genes of σ^M. Noteworthy, within the σ^M regulon, there is a subset of genes, including the *mreBCDminCD* operon involved in cell division, that is not induced by vancomycin (upper part of σ^{M1} cluster in Fig. 2b). Differences in the induction profiles of subsets of σ^M-dependent genes have been observed previously (Eiamphungporn & Helmann, 2008).

Genes mediating the secretion stress response also cluster together (Fig. 2b). The CssRS-dependent target genes *htrA* and *htrB* are not only induced by secretion stress and rhamnolipids, but also weakly by vancomycin and bacitracin.

Genes repressed by rhamnolipids show almost unchanged expression under the other conditions tested (Fig. 2c). One exception is the *pyr* operon, which is strongly repressed by rhamnolipids, but weakly induced by friulimicin and vancomycin.

Taken together, the hierarchical clustering analysis indicates that rhamnolipids induce a combination of two different stress responses: the cell envelope stress response represented by the LiaRS TCS and the ECF σ factor σ^M, and the heat and secretion stress response mediated by CssRS. Simultaneous induction of the LiaRS TCS and σ^M is common for cell wall antibiotics such as daptomycin, vancomycin, or bacitracin (Mascher *et al.*, 2003; Hachmann *et al.*, 2009; Wecke *et al.*, 2009). But none of the σ^M-dependent target genes is induced by secretion stress, while both the CssRS and LiaRS TCS are induced by cell wall antibiotics, rhamnolipids, and secretion stress, but with different intensities (Fig. 2d).

The LiaRS TCS and σ^M protect cells from rhamnolipid-dependent lysis

Bacteria use signal transducing systems to detect harmful compounds and alter gene expression to protect the cell. We hypothesize that the signal transducing systems activated by rhamnolipids confer resistance and counteract cell damage caused by this antimicrobial compound. Therefore, we compared the growth behavior of *B. subtilis* wild-type cultures exposed to different rhamnolipid concentrations with strains carrying gene deletions leading to 'ON' or 'OFF' states of the induced signal transducing systems, which results either in no or constitutively high expression of the corresponding target genes. The strains were grown in LB medium to mid-logarithmic growth phase, the cultures were split and different concentrations of rhamnolipids were added. Subsequent lysis of each sample was monitored by measuring $OD_{600\,nm}$.

For the *B. subtilis* wild-type strain W168, a concentration of 50 μg mL^{-1} rhamnolipids did not affect growth (Fig. 3), but was sufficient to induce a transcriptional response as investigated using DNA microarray analysis (Fig. 1a and Table 3). Higher concentrations of rhamnolipids lead to rapid lysis of the culture within 1 h after addition (Fig. 3). Remarkably, even after severe lysis the cultures resumed growth.

To reveal a possible protective function of the LiaRS TCS, we compared the lysis in response to rhamnolipids of two strains carrying deletions in the *lia* locus: deletion of

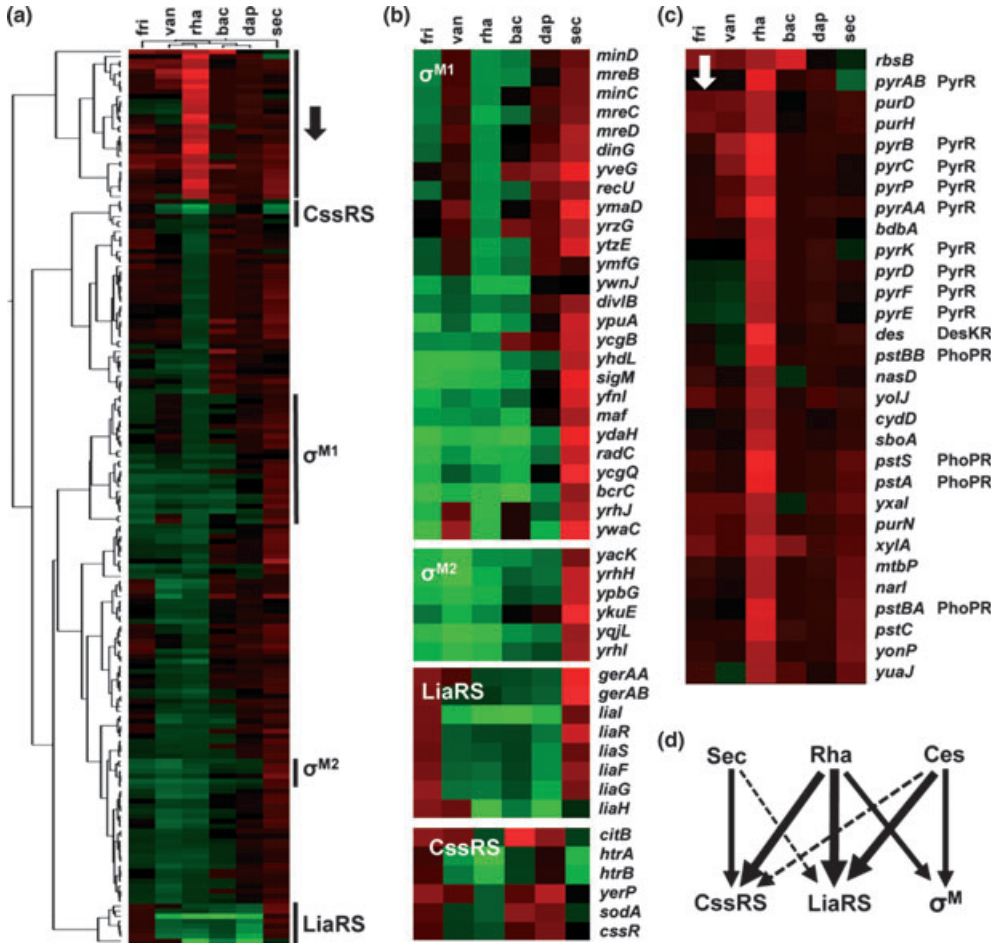

Fig. 2. Hierarchical clustering analysis of genes differentially expressed in response to rhamnolipids. The clustering analysis was performed using the software Cluster 3.0 (de Hoon *et al.*, 2004). Transcriptome data for *Bacillus subtilis* treated with friulimicin (fri), vancomycin (van), rhamnolipids (rha), bacitracin (bac), daptomycin (dap) and exposed to secretion stress (sec) caused by overexpression of α-amylase were analyzed (see Materials and methods for details). Green indicates induction of the corresponding gene, red repression under the designated condition. Cluster analysis was limited to genes induced ≥ threefold and repressed ≥ fivefold by rhamnolipids (a). Cluster containing target genes of σ^M, LiaRS and CssRS (b) and genes repressed by rhamnolipids (c) are shown in detail. A schematic representation of the network orchestrating the response to rhamnolipids summarizes the results of the cluster analysis (d). The thickness of the arrows corresponds to the induction of the given regulators. Sec, secretion stress; Rha, rhamnolipids; Ces, cell envelope stress.

the response regulator LiaR results in a 'Lia OFF' mutant, while deletion of the inhibitory protein LiaF represents a 'Lia ON' strain with constitutive expression of the target genes *liaIH* (Jordan *et al.*, 2006; Wolf *et al.*, 2010). Behavior of the Δ*liaR* mutant was comparable to the wild-type strain, while the Δ*liaF* mutant clearly displayed recovery advantages and regained growth more quickly even after addition of high rhamnolipid concentrations (Fig. 3). We also investigated the effect of rhamnolipids on a mutant strain lacking the CssRS TCS that orchestrates the secretion stress response, but did not observe any differences compared with the wild type (Fig. 3).

As a large part of the induced genes are regulated by σ^M, we investigated how this ECF σ factor contributes to resistance against rhamnolipids. Compared with the wild type, a *sigM*::kan mutant strain showed an impaired growth phenotype (Fig. 3). While growth of the wild type was not affected at concentrations of 50 μg mL^{-1}, growth of the *sigM*::kan mutant was clearly arrested. σ^M controls expression of at least 30 operons involved in cell division, DNA repair and cell envelope synthesis (Eiamphungporn & Helmann, 2008). Another ECF σ factor which controls a similar large regulon is σ^W (Helmann, 2006). Since expression of the *sigW–rsiW* operon was induced 2.8-fold by rhamnolipids (Table S1), we also included a *sigW*::MLS mutant strain in our lysis curve experiments. But this strain shows the same behavior as the wild type, indicating that σ^W is not responsible for resistance against rhamnolipids (Fig. 3). Therefore, the ECF response to rhamnolipids is mainly mediated by σ^M, which is in

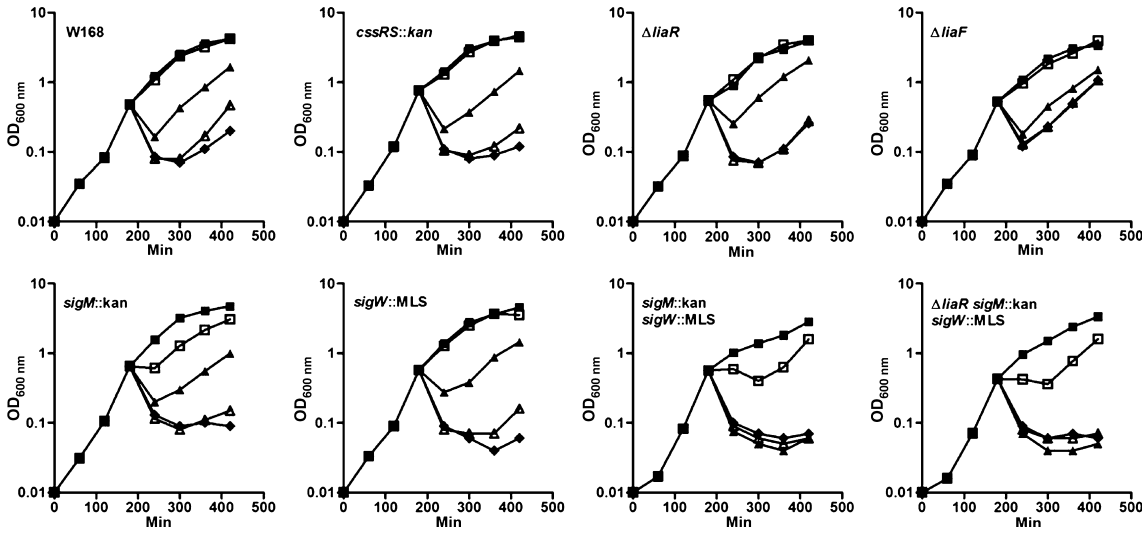

Fig. 3. Growth of *Bacillus subtilis* wild-type and mutant strains exposed to different concentrations of rhamnolipids. *Bacillus subtilis* wild type (W168), TMB1070 (*cssRS*::kan), TMB589 (Δ*liaR*), TMB329 (Δ*liaF*), TMB1003 (*sigM*::kan), TMB149 (*sigW*::MLS), TMB1393 (*sigM*::kan *sigW*::MLS) and TMB1392 (Δ*liaR sigM*::kan *sigW*::MLS) were grown in LB medium to mid-logarithmic growth phase. The cultures were split into 1 mL samples and induced with increasing concentrations of rhamnolipids: 0 μg mL^{-1} (■), 50 μg mL^{-1} (□), 100 μg mL^{-1} (▲), 200 μg mL^{-1} (Δ) and 300 μg mL^{-1} (♦). Cell density was monitored by measuring $OD_{600\,nm}$ over a time period of 7 h.

agreement with induction ratios of the *sigM* and *sigW* operons (eight- vs. threefold, respectively).

We also tested if a combined deletion of both σ^M and σ^W has an additive affect and leads to a more pronounced phenotype, as a functional overlap of ECF σ factors in response to different antimicrobial compounds has already been demonstrated (Mascher *et al.*, 2007). Indeed, the double mutant shows an increased sensitivity compared with the *sigM*::kan strain, as it did not resume growth in the presence of 100 μg mL^{-1} rhamnolipid (Fig. 3). Additional deletion of *liaR*, resulting in inactivation of a third cell wall stress responsive system, did not lead to a stronger susceptibility phenotype (Fig. 3). Taken together, σ^M seems to play a central role in rhamnolipid resistance, while σ^W and the LiaRS TCS have only minor functions.

Summary and conclusions

Here, we present the first investigation of the transcriptional response to rhamnolipids, industrially important surface-active molecules with antimicrobial properties. In *B. subtilis*, exposure to rhamnolipids provokes a complex reaction that combines the cell envelope and secretion stress response (Fig. 2d). The main regulators orchestrating this response are the TCS LiaRS and CssRS, as well as the ECF σ factor σ^M. In addition to the target genes of these regulators, a number of genes encoding either metabolic enzymes or hypothetical proteins of unknown functions are also induced. Our data show a protective role of LiaRS and σ^M against rhamnolipid damage, while the CssRS TCS has no effect on rhamnolipid sensitivity (Fig. 3).

As rhamnolipids alter the properties of membranes, induction of the cell envelope stress response could help to maintain cell envelope integrity. While the physiological role of most of the strongly induced genes has not been elucidated yet, some of them have known or assumed functions in counteracting membrane damage. The LiaR-controlled *liaIH* operon encodes a small membrane protein and a member of the phage-shock protein family, respectively. Their gene products have recently been linked to resistance against daptomycin, another membrane-perturbating agent (Hachmann *et al.*, 2009; Wolf *et al.*, 2010). Other genes, like the ECF-regulated *bcrC* gene and the *pbpE-racX* operon encode functions involved in cell envelope biogenesis, which might also help to stabilize the envelope against membrane damage. Moreover, and given the prominent role of σ^M in protecting cells from rhamnolipid damage (Fig. 3), it is noteworthy that some of the most strongly induced σ^M-target genes of unknown function, such as *yebC*, *ywnJ* or *ydaH*, encode putative membrane proteins (Table 3). A possible role of these proteins in counteracting membrane damage needs to be addressed in future studies.

In contrast, the physiological role of CssRS activation by rhamnolipids is not clear. Its induction could indicate severe changes of membrane protein composition and accumulation of misfolded secreted proteins in the cell envelope caused by rhamnolipid treatment. Alternatively, rhamnolipid-dependent interference with membrane integ-

rity could affect functionality of the secretion machinery. The CssRS TCS has also been shown to be not only induced by mammalian peptidoglycan recognition proteins, but also seems to be required for the killing mechanism of these proteins (Kashyap *et al.*, 2011).

Although the data presented here clearly indicates that rhamnolipids interfere with cell envelope integrity, future studies will be required to gain an understanding of the mode of action of rhamnolipids and its use as antimicrobial active compound. Taken together, this is the first analysis of a bacterial stress stimulon in response to rhamnolipids showing that a single antimicrobial compound induces a combination of two normally independent stress responses.

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Authors' contribution

T.B. and H.H. contributed equally to this study.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Complete dataset.

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Chapter 4

Extracytoplasmic function σ factors of the widely distributed group ECF41 contain a fused inhibitory domain.

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Author contributions:

Tina Wecke performed the experiments and bioinformatics analyses. Petra Halang constructed strains, performed promoter activity measurements and contributed to phenotypic characterization in her diploma thesis. Anna Staroń provided the initial characterization of group ECF41 and contributed to the bioinformatics analyses. Yann S. Dufour and Timothy J. Donohue performed bioinformatics analyses regarding the distribution of the target promoter, provided their experience regarding working with *R. sphaeroides*, constructed strains and enabled the microarray analysis. Tina Wecke and Thorsten Mascher designed the experiments and wrote the manuscript.

Abstract

Bacteria need signal transducing systems to respond to environmental changes. Next to one- and two-component systems, alternative σ factors of the extracytoplasmic function (ECF) protein family represent the third fundamental mechanisms of bacterial signal transduction. A comprehensive classification of these proteins identified more than 40 phylogenetically distinct groups, most of which are not experimentally investigated. Here, we present the characterization of such a group with unique features, termed ECF41. Among analyzed bacterial genomes, ECF41 σ factors are widely distributed with about 400 proteins from ten different phyla. They lack obvious anti-σ factors that typically control activity of other ECF σ factors, but their structural genes are often predicted to be co-transcribed with carboxymuconolactone decarboxylases, oxidoreductases or epimerases based on genomic context conservation. We demonstrate for *Bacillus licheniformis* and *Rhodobacter sphaeroides* that the corresponding genes are preceded by a highly conserved promoter motif and are the only detectable targets of ECF41-dependent gene expression. In contrast to other ECF σ factors, proteins of group ECF41 contain a large C-terminal extension, which is crucial for σ factor activity. Our data strongly suggest that ECF41 σ factors are regulated by a novel mechanism possibly based on the presence of a fused anti-σ factor-like domain.

Introduction

Bacteria populate complex habitats in which extracellular conditions can change very rapidly. In order to survive in such an environment, bacterial cells have to be able to sense and respond to these variations before cell damage actually occurs. Therefore, bacteria need signal transducing systems, which enable them to sense these extracellular changes and respond by differential gene expression.

A common mechanism to control gene expression at the level of transcription initiation is the use of σ factors, which constitute an essential subunit of the RNA polymerase (RNAP) holoenzyme and determine the promoter specificity. In addition to the primary σ factor, which is responsible for general expression of most genes in exponentially growing cells, most bacteria contain one or more alternative σ factors. These proteins are activated only under certain conditions and control expression of a specific set of target genes by

recognizing alternative promoter sequences (Helmann, 2010, Helmann & Chamberlin, 1988).

Most σ factors belong to the σ^{70} family based on their relation to the primary σ factor of *Escherichia coli*, σ^{70} (Gruber & Gross, 2003, Paget & Helmann, 2003). Based on sequence similarity, domain architecture and function, the proteins of the σ^{70} family can be divided into four groups. Group 1 comprises the essential primary σ factors, which contain four highly conserved domains (designated σ_1 through σ_4) (Gruber & Gross, 2003). Group 2 σ factors are closely related to group 1 proteins, but are not essential for growth. The group 3 σ factors lack the σ_1 domain and have functions in cellular processes such as sporulation, flagella biosynthesis, or heat shock response. The largest and most diverse group 4 contains the proteins of the ECF family, named after their function in response to extracellular stimuli (Butcher *et al.*, 2008, Helmann, 2002, Lonetto *et al.*, 1994).

In contrast to other σ^{70} proteins, the ECF σ factors only contain two of the four conserved domains, σ_2 and σ_4, which are sufficient for promoter recognition and interaction with RNAP. The bipartite promoter recognized by ECF σ factors typically contains a highly conserved 'AAC' signature in the -35 region and a 'CGT' motif in the -10 region (Helmann, 2002). In general, ECF σ factors autoregulate their own expression and are co-transcribed with a gene encoding an anti-σ factor, which regulates the activity of the σ factor. In the absence of a stimulus, the anti-σ factor binds the ECF σ factor and keeps it inactive. Upon receiving the appropriate signal, the anti-σ factor gets inactivated, thereby releasing and activating the σ factor (Butcher *et al.*, 2008, Helmann, 2002). The major principles of σ factor activation are based on either the regulated proteolysis of a membrane-anchored anti-σ factor as exemplified by RseA-σ^E of *E. coli* and RsiW-σ^W of *Bacillus subtilis* (Ades, 2004, Heinrich & Wiegert, 2009) or conformational changes of a soluble anti-σ factor, as has been described for RsrA-σ^R of *Streptomyces coelicolor* (Campbell *et al.*, 2008, Kang *et al.*, 1999). For yet other examples, such as *S. coelicolor* σ^E or EcfG-homologs in α-proteobacteria, two-component systems play a crucial role in regulating the activity of the ECF σ factors (Francez-Charlot *et al.*, 2009, Hong *et al.*, 2002).

A recent classification of the ECF σ factor protein family based on sequence similarity and genomic context conservation revealed a wide distribution and combinatorial complexity

of ECF-dependent signal transduction. This study identified more than 40 phylogenetically distinct groups of ECF σ factors including major groups containing the *E. coli* σ^E- and FecI-like proteins as well as cytoplasmic-sensing ECF σ factors. But in addition to these well-understood examples, a number of ECF groups were identified that have not yet been investigated experimentally (Staroń *et al.*, 2009).

Here, we describe the characterization of one such uncharacterized group, ECF41. This group is widely distributed with about 400 proteins from 10 different phyla. Based on their genomic organization, the genes encoding these ECF41 σ factors are not transcriptionally linked to genes encoding proteins related to known anti-σ factors. Instead, they are located next to genes encoding carboxymuconolactone decarboxylases (CMD proteins), oxidoreductases or epimerases. To extract general features of ECF41-dependent gene regulation, we experimentally investigated ECF41 σ factors from two different organisms, *Bacillus licheniformis* (Firmicutes) and *Rhodobacter sphaeroides* (α-proteobacteria). In both organisms, the ECF41 σ factor appears to control expression of a single transcript that is preceded by a highly conserved ECF41-specific promoter motif. A unique feature of ECF41 proteins is the presence of a large C-terminal extension, containing a number of conserved signature motifs. We provide evidence that this C-terminal extension is involved in regulation of σ factor activity and we propose that it functions as a fused anti-σ factor domain.

Experimental procedures

Bioinformatics analysis. 510 ECF41 proteins were extracted in October 2010 from the MiST2 database (Ulrich & Zhulin, 2010) available at http://mistdb.com. False positives (unclassified ECF σ factors) and redundant proteins (proteins from more than one sequenced strain per species) were removed leaving 373 sequences for further analysis. Multiple sequence alignments were performed using ClustalW (Thompson *et al.*, 1994) and phylogenetic trees were generated from gapless multiple sequence alignments using the Neighbor-Joining method of the Phylip (Felsenstein, 1989) program Protdist, both implemented in the BioEdit program package (Hall, 1999). Genomic context analysis was performed using the databases MicrobesOnline (Alm *et al.*, 2005) at http://www.microbesonline.org and MiST2 (Ulrich & Zhulin, 2010) available at

http://mistdb.com/. Protein domain architecture was analyzed using the SMART database (Letunic *et al.*, 2006, Schultz *et al.*, 1998) available at http://smart.embl-heidelberg.de/.

250 bp region upstream of the genes encoding the ECF41 σ factors and the corresponding COE were analysed for putative promoter motifs either manually or with the help of MEME (Bailey & Elkan, 1994), available at http://meme.nbcr.net/. Conservation of putative target promoters was illustrated using the WebLogo tool (Crooks *et al.*, 2004) at http://weblogo.berkeley.edu. The promoter sequence of group ECF41 σ factors was used to screen the genomes of *R. sphaeroides* 2.4.1 and *B. licheniformis* DSM13 for putative target genes with the help of the virtual footprint algorithm (Münch *et al.*, 2005), implemented into the Prodoric database (Münch *et al.*, 2003) at http://www.prodoric.de/vfp/. As input pattern, the generated position weight matrix or the promoter consensus as IUPAC code was used.

Bacterial strains and growth conditions. *B. subtilis*, *B. licheniformis* and *E. coli* were grown in LB medium at 37°C with aeration. *R. sphaeroides* was grown aerobically in Sistrom's minimal medium (Sistrom, 1960) at 30°C. All strains used in this study are listed in Table 4.1. The antibiotics spectinomycin (100 µg/ml), chloramphenicol (5 µg/ml) and erythromycin (1 µg/ml) plus lincomycin (25 µg/ml) for macrolide-lincosamide-streptogram (MLS) resistance were used for selection of *B. subtilis* and *B. licheniformis* mutants. Plasmid containing *E. coli* strains were grown with ampicillin (100 µg/ml) or kanamycin (50 µg/ml). *R. sphaeroides* mutants were selected using tetracycline (1 µg/ml), spectinomycin (25 µg/ml) or kanamycin (25 µg/ml).

DNA manipulations. Standard cloning techniques were applied (Sambrook & Russell, 2001). All plasmids used in this study are listed in Table 4.2, oligonucleotides in Table 4.3. *E. coli* strain S17-1 (Simon *et al.*, 1983) was used for conjugational DNA transfer in *R. sphaeroides*. In brief, a 1:1 cell mixture of exponentially growing donor and recipient strains were harvested, washed, and resuspended in LB medium. The cell mixture was applied to a filter disc and incubated overnight on a LB plate at 30°C. The filter disc was transferred to Sistrom's minimal medium (Sistrom, 1960) and incubated for 3 h at 30°C on a shaker, before the cells were plated on agar plates with selection. Conjugants were obtained after 3-4 days incubation at 30°C.

Table 4.1. Bacterial strains used in this study

Strain	Genotype or characteristic(s)	Source or reference
E. coli strains		
S17-1	C600::RP-4 2-(Tc::Mu)(Km::Tn*7*) *thi pro hsdR hsdM*$^{+}$*recA*	(Simon *et al.*, 1983)
DH5α	*recA1 endA1 gyrA96 thi hsdR17*(r_K- m_K+) *relA1 supE44 Φ*80Δ*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169*	(Sambrook & Russell, 2001)
B. subtilis strains		
W168	Wild type strain, *trpC2*	Laboratory stock
1A774	JH642 *rpoC*::(His_6-tag) Sp^R	BGSC (C. Moran)
TMB1099	1A774 pPH0401	This study
TMB1100	1A774 pPH0403	This study
TMB1101	1A774 pTW0412	This study
TMB695	W168 pPH0401	This study
TMB746	W168 pPH0403	This study
TMB666	W168 pTW0412	This study
TMB428	W168 *thrC*::pTW6302	This study
TMB455	TMB428 *amyE*::pTW901	This study
TMB451	W168 *amyE*::pTW901	This study
TMB456	TMB428 *amyE*::pTW902	This study
TMB574	TMB451 *thrC*::pTW6304	This study
TMB575	TMB451 *thrC*::pTW6305	This study
TMB577	TMB451 *thrC*::pTW6307	This study
TMB623	TMB428 pHCMC04	This study
TMB696	TMB428 pPH0401	This study
TMB744	TMB428 pPH0405	This study
TMB743	TMB428 pPH0404	This study
TMB742	TMB428 pPH0403	This study
TMB741	TMB428 pPH0402	This study
TMB667	TMB428 pTW0412	This study
TMB795	TMB428 pPH0406	This study
TMB797	TMB428 pPH0407	This study
TMB1016	TMB428 pTW0414	This study
TMB793	TMB428 pPH0408	This study
B. licheniformis strains		
DSM13	Wild type strain	Laboratory stock
MW3	DSM13Δ*hsdR*1 Δ*hsdR*2	(Waschkau *et al.*, 2008)
TMBli003	MW3 Δ*ydfG*	This study
TMBli006	MW3 Δ*ecf*41_{Bli}	This study
R. sphaeroides strains		
2.4.1	Wild type strain	Laboratory stock
TMR003	2.4.1 pTW0503	This study
TMR004	YSD418 pTW0501	This study
TMR005	YSD418 pTW0502	This study
TMR006	YSD418 pTW0503	This study
YSD418	2.4.1 P_{RSP_0606}::pSUP202-*lacZ*	This study
YSD354	2.4.1 pIND4	This study
YSD239	2.4.1 ΔRSP_0606-*ecf*41_{Rsp} Ω::$Spec^R$	This study
YSD333	2.4.1 pYSD161	This study
YSD434	YSD418 pIND4	This study

Table 4.2. Vectors and plasmids used in this study

Name	Genotype or characteristic features[a]	Primers for cloning	Source or reference
Vectors			
pDG1663	*lacZ* fusion vector, integrates in *thrC*, MLS^R		(Guerout-Fleury *et al.*, 1996)
pHCMC04	Xylose-inducible expression vector, Cm^R		(Nguyen *et al.*, 2005)
pIND4	IPTG-inducible expression vector, Kn^R		(Ind *et al.*, 2009)
pSUP202	Mobilizable vector, Ap^R, Cm^R, Tc^R		(Simon *et al.*, 1983)
pSWEET	Xylose-inducible expression vector, integrates in *amyE*, Cm^R		(Bhavsar *et al.*, 2001)
pMAD	Shuttle vector for construction of makerless deletion mutans, MLS^R		(Arnaud *et al.*, 2004)
pHP45Ω	Source of Ω::$Spec^R$ cassette		(Prentki & Krisch, 1984)
pGEM-T	Cloning vector		Promega Corp.
Plasmids			
pTW101	pMAD $ecf41_{Bli}$ up/do	779/780, 781/782	This study
pTW102	pMAD *ydfG* up/do	783/784, 785/786	This study
pTW6302	pDG1663 $P_{ydfG(-146-54)}$-*lacZ*	712/713	This study
pTW6304	pDG1663 $P_{nhaX(-355-40)}$-*lacZ*	1130/1131	This study
pTW6305	pDG1663 $P_{ybpE(-111-63)}$-*lacZ*	1136/1137	This study
pTW6307	pDG1663 $P_{uvrX(-173-54)}$-*lacZ*	1132/1133	This study
pTW901	pSWEET $ecfI41_{Bli}$	699/669	This study
pTW902	pSWEET $ecf41_{Bli}$-*ydfG*	699/705	This study
pPH0401	pHCMC04 $ecf41_{Bli}$-FLAG	1416/1294	This study
pPH0405	pHCMC04 $ecf41_{Bli\ aa1-270}$-FLAG	1416/1471	This study
pPH0404	pHCMC04 $ecf41_{Bli\ aa1-234}$-FLAG	1416/1470	This study
pPH0403	pHCMC04 $ecf41_{Bli\ aa1-204}$-FLAG	1416/1469	This study
pPH0402	pHCMC04 $ecf41_{Bli\ aa1-192}$-FLAG	1416/1468	This study
pTW0412	pHCMC04 $ecf41_{Bli\ aa1-167}$-FLAG	1416/1411	This study
pPH0406	pHCMC04 $ecf41_{Bli\ WLPEP\rightarrow A}$-FLAG	1416/1474, 1475/1294	This study
pPH0407	pHCMC04 $ecf41_{Bli\ DGGGK\rightarrow A}$-FLAG	1416/1476, 1478/1294	This study
pPH0408	pHCMC04 $ecf41_{Bli\ NPDKL\rightarrow A}$-FLAG	1416/1479	This study
pTW0414	pHCMC04 $ecf41_{Bli\ INDQKGVL\rightarrow A}$-FLAG	1416/1579, 1580/1294	This study
pSUP202-*lacZ*	pSUP202 with promoter-less *lacZ* gene	199/200	This study
pSUP202-P_{RSP_0606}-*lacZ*	P_{RSP_0606} fused to *lacZ* gene	109/219	This study
pYSD122	pSUP202 with the Ω::$Spec^R$ cassette and genomic regions flanking RSP_0606-$ecf41_{Rsp}$	109/110/125/126	This study
pYSD161	pIND4 $ecf41_{Rsp}$	185/186	This study
pTW0501	pIND4 $ecf41_{Rsp}$	1881/1603	This study
pTW0502	pIND4 $ecf41_{Rsp\ aa1-169}$	1881/1604	This study
pTW0503	pIND4 $ecf41_{Rsp\ aa1-206}$	1881/1605	This study

[a]Resistance cassettes: MLS^R, macrolide-lincosamide-streptogram; Cm^R, chloramphenicol; Kn^R, kanamycin; Ap^R, ampicillin; Tc^R, tetracycline; $Spec^R$, spectinomycin

Table 4.3. Oligonucleotides used in this study

Number and name	Sequence
Construction of promoter *lacZ* fusion[a]	
712 (P_{ydfG} fwd (EcoRI))	AGTC**GAATTC**CTTGGAATCCGGAAGGCGAT
713 (P_{ydfG} rev (BamHI))	AGCT**GGATCC**CATTCCTCTGTATCCCTCAG
1130 (P_{nhaX} fwd (EcoRI))	AGTC**GAATTC**GCACACTGTGTACCAGCATG
1131 (P_{nhaX} rev (BamHI))	AGTC**GGATCC**TTCCGTCAAACGCGACTATG
1132 (P_{uvrX} fwd (EcoRI))	AGTC**GAATTC**AAATTCCGAACTGGAATGGTC
1133 (P_{uvrX} rev (EcoRI))	AGTC**GGATCC**AGCTCCGCATATCAACGCAC
1136 (P_{ypbE} fwd (EcoRI))	AGTC**GAATTC**GATTGAGCTTTGAACGGACAG
1137 (P_{ypbE} rev (BamHI))	AGTC**GGATCC**TCATTTCAGCGCTGGCCTTC
109 (RSP_0606-*ecf*41$_{Rsp}$ operon fwd)	AGGCAAAGTAGAGACCGCGTCC
219 (RSP_0606 promoter rev (XbaI))	ATGT**TCTAGA**CGCTCTCTCCTTTTGCAACTGA
210 (*lacZ* start codon (ScaI, XbaI))	CT**AGTACT**GTA**TCTAGA**TGACCATGATTACGGATTC A
200 (*lacZ* terminator rev)	CATTACGGATCTTTTCTTTCG
Mutagenesis/expression experiments[a b c d]	
1416 (3xFLAG-*ecf*41$_{Bli}$ fwd (BamHI))	AGTC**GGATCC***AAGGAGGTGAGGATCTATGGATTATAAG GATCATGATGGTGATTATAAGGATCATGATATCGACTACA AAGACGATGACGACAAG*GAATATTATCGACAATATCA TTC
1294 (*ecf*41$_{Bli}$ rev (AatII))	AGTC**GACGTC**TTATATTTTAATGTGCTTCAGTTTATC
1471 (*ecf*41$_{Bli}$ 270 rev (AatII))	AGTC**GACGTC***TCA*ATTTTTGACGGAATCGCCTTC
1470 (*ecf*41$_{Bli}$ 234 rev (AatII))	AGTC**GACGTC***TCA*AAAGCGGCCGGAAAAGCTTC
1469 (*ecf*41$_{Bli}$ 204 rev (AatII))	AGTC**GACGTC***TCA*CACTTTTCCGCCGCCATCT
1468 (*ecf*41$_{Bli}$ 192 rev (AatII))	AGTC**GACGTC***TCA*TTCAATCAATTTCTTGGAAAACTC
1411 (*ecf*41$_{Bli}$ 167 rev (AatII))	AGTC**GACGTC***TCA*TTCTTCAACCGGCTGTGAA
1474 (WLPEP up rev)	AGCCGCTGCGGCAGCTTCCCCGATATATACCTCC
1475 (WLPEP do fwd)	GCTGCCGCAGCGGCTCAGGTGGCGCTTTCAGCTC
1476 (DGGGK up rev)	AGCCGCTGCGGCAGCTGTATACAATACGGCATCTTC
1478 (DGGGK do fwd)	GCTGCCGCAGCGGCTGTGCGCAGCGCTTTGAGA
1479 (NPDKL rev (AatII))	AGTC**GACGTC**TTATATTTTAATGTGCTTAGCCGCTGC AGCAGCTGACACGATAAACACATTTTTGACG
1579 (INDQKGVL up rev)	TGCGGCAGCAGCCGCTGCGGCAGCATCGACTGGCAT AAAGCGGC
1580 (INDQKGVL do fwd)	GCTGCCGCAGCGGCTGCTGCCGCAATCATGAAAAAC AACCGCCCGGCT
699 (*ecf*41$_{Bli}$ fwd (PacI))	TACG**TTAATTAA**TTTTAGGCAAAATATCTATGGG
669 (*ecf*41$_{Bli}$ rev (BamHI))	GACT**GGATCC**TTATATTTTAATGTGCTTCAGTTTATC
705 (*ecf*41$_{Bli}$-*ydfG* rev (BamHI))	AGCT**GGATCC**CGCTCAAATAAAGTGAAAGATAG
1881 (*ecf*41$_{Rsp}$ fwd (NcoI))	GTCA**CCATGG**CGCCTGACGTCTACCTGCA
1603 (*ecf*41$_{Rsp}$ rev (HindIII))	GTCA**AAGCTT**TCAGTTGAGCCTGATACGGGTC
1604 (*ecf*41$_{Rsp}$ 169 rev (HindIII))	GTCA**AAGCTT***TCA*CGCTGCCTCGCGCTCCACCT
1605 (*ecf*41$_{Rsp}$ 206 rev (HindIII))	GTCA**AAGCTT***TCA*GACCTTGCCGCCACCGTCCGA
185 (*ecf*41$_{Rsp}$ fwd (BsrDI))	GTA**GCAATG**CATGTCGCCTGACGTCTACCTGCAG
186 (*ecf*41$_{Rsp}$ rev (HindIII))	GTA**AAGCTT**TCAGTTGAGCCTGATACGGGTCAGC
Deletion mutants[a d]	
779 (*ecf*41$_{Bli}$ clean up fwd (BamHI))	AGTC**GGATCC**TATCCAGCCGATTGTCGTCA
780 (*ecf*41$_{Bli}$ clean up rev)	GGAGTTTGTGACAAAAAACGAGACGCTCCCCCATAG ATATTTTGC
781 (*ecf*41$_{Bli}$ clean do fwd)	CGTTTTTTGTCACAAACTCC
782 (*ecf*41$_{Bli}$ clean do rev (EcoRI))	AGTC**GAATTC**ACCTACTTTCACATTGAACAAG
783 (*ydfG* clean up fwd (BamHI))	AGTCGGATCCTGGCGCTTTCAGCTCAAATC

Number and name	Sequence
784 (*ydfG* clean up rev)	GTCTATTCCTCCTTTAAGTGTT
785 (*ydfG* clean do fwd)	AACACTTAAAGGAGGAATAGACTTGAAATCCCCCCA ACACAG
786 (*ydfG* clean do rev (EcoRI))	AGTCGAATTCCCGTCGATCATCAGATCCGT
109 (RSP_0606- *ecf*41$_{Rsp}$ operon fwd)	AGGCAAAGTAGAGACCGCGTCC
110 (RSP_0606- *ecf*41$_{Rsp}$ operon rev)	ACGGGTTGGCACGCTGGATGAG
125 (RSP_0606 rev-inverted)	GCGTTTGAAATGGTCGGTCATGC
126 (RSP_ *ecf*41$_{Rsp}$ fwd-inverted)	TGACCCGTATCAGGCTCAACTG
Northern Hybridization	
688 (*ydfG*-fwd)	CTGAGGGATACAGAGGAATG
761 (*ydfG*-probe-T7-rev)	**CTAATACGACTCACTATAGGGAGA**CGATGGCAATC CTGTTCCAG
5'RACE	
679 (RACE PCR)	GATATGCGCGAATTCCTG
689 (GSP1-*ydfG*)	CTTGCATCCTTCGTATGCATA
690 (GSP2-*ydfG*)	CACATCCGTTCAGCTGTGA
1414 (GSP1-RSP_0606)	AGCGTGCATCTGCAGACAGA
1415 (GSP2-RSP_0606)	AGCTGGATCCCATCTTCACCAGATGCAGCA

[a]Restriction sites are highlighted in bold.
[b]Sequence of FLAG-tag is shown in underlined italics.
[c]Inserted sequences (stop codons or Shine-Dalgarno sequences) are highlighted in italics.
[d]Linker sequences for amino acid exchanges and joining reactions are underlined.
[e]Sequence representing the T7 promoter for in vitro transcription is shown in bold and underlined.

Construction of markerless *ecf*41$_{Bli}$ and *ydfG* deletion mutants in *B. licheniformis*. Markerless *B. licheniformis* Δ*ecf*41$_{Bli}$ and Δ*ydfG* mutants were constructed using the vector pMAD (Arnaud *et al.*, 2004). 700 bp fragments up- and downstream of *ecf*41$_{Bli}$ and *ydfG* were amplified by PCR using the oligonucleotides listed in Table 4.3, introducing extensions at the 3' end of the up fragments which are complementary to the 5' end of the down fragments. These regions were used to fuse the fragments in a second joining PCR. The resulting products were then cloned into pMAD using BamHI and EcoRI generating pTW101 and pTW102. The plasmids were introduced into *B. licheniformis* MW3 as described (Waschkau *et al.*, 2008). Generation of markerless deletion mutants basically followed the established procedure (Arnaud *et al.*, 2004). In brief, transformants were incubated at 30°C with MLS selection on LB agar plates supplemented with X-Gal. Blue colonies were picked and incubated for 6-8 h at 42°C in LB medium with MLS selection, resulting in the integration of the plasmid into the chromosome. Again, blue colonies were picked from LB X-Gal plates and incubated for 6 h at 30°C in LB medium without selection. Subsequently, the liquid culture was shifted to 42 °C for 3 h, and the cells were

then plated on LB X-Gal plates, this time without selective pressure. White colonies that had lost the plasmid were picked and deletion of *ecf*41$_{Bli}$ or *ydfG* was checked by PCR.

Construction of a RSP_0606-*ecf*41$_{Rsp}$ deletion mutant in *R. sphaeroides*. RSP_0606-*ecf*41$_{Rsp}$ with 1.3 kb flanking regions on both sides was amplified from chromosomal DNA of *R. sphaeroides* using oligonucleotides 109 and 110 and ligated into the vector pGEM-T (Promega). To replace RSP_0606-*ecf*41$_{Rsp}$ with a resistance cassette, the regions flanking the genes and the plasmid were amplified using internal oligonucleotides (125, 126) and ligated to the Ω fragment derived from pHP45Ω (Prentki & Krisch, 1984), conferring spectinomycin resistance. The resulting construct was amplified using oligonucleotides 109 and 110, cloned into the suicide vector pSUP202 (which contains a tetracycline resistance marker) digested with ScaI to make pYSD122. The plasmid pYSD122 was than conjugated into *R. sphaeroides* 2.4.1. Double recombinants corresponding to the deletion mutants were selected for spectinomycin resistance and sensitivity to tetracycline. Plasmid constructs were verified by sequencing, and the deletion in the *R. sphaeroides* genome was verified by PCR.

Measurement of promoter activity by β-galactosidase assays. Because of the lack of genetic tools for *B. licheniformis*, we developed a heterologous expression system in *B. subtilis*, an organism lacking an ECF41 σ factor. A DNA fragment from *B. licheniformis* containing the intergenic region between *ydfG* and *ecf*41$_{Bli}$ was fused to a promoter-less *lacZ* gene using the vector pDG1663 and integrated into the *thrC* locus of *B. subtilis*. In addition, we fused a FLAG-tag to the N-terminus of Ecf41$_{Bli}$ and its mutated or truncated versions and expressed the protein from the xylose-inducible promoter of the shuttle vector pHCMC04, allowing determination of P_{ydfG} activity by β-galactosidase assays in response to Ecf41$_{Bli}$ expression. The resulting *B. subtilis* strains were inoculated from fresh overnight cultures and grown in LB medium at 37°C with aeration until they reached an OD_{600} of ~0.4. The cultures were split and 0.5% xylose was added to one sample to induce expression of Ecf41$_{Bli}$ from the inducible promoter. After incubation for 1 h at 37°C, 2 ml of each sample were harvested and the cell pellets frozen at -20°C. The pellets were resuspended in 1 ml working buffer and assayed for β-galactosidase activity with normalization to cell density (Miller, 1972).

A DNA fragment containing the upstream region of RSP_0606 was amplified and cloned into the suicide vector pSUP202 carrying a promoter-less *lacZ* gene. The resulting plasmid was conjugated into *R. sphaeroides* and integrated into the chromosome by single crossing over, thereby bringing the expression of β-galactosidase under control of P_{RSP_0606}. Full-length and truncated *ecf*41_{Rsp} was amplified and cloned into the overexpression vector pIND4, thereby bringing its expression under control of an IPTG-inducible promoter. The resulting *R. sphaeroides* strains were grown aerobically in Sistrom's minimal medium (Sistrom, 1960) to an OD_{600} of ~0.4. The cultures were split and expression of $Ecf41_{Rsp}$ was induced in one sample by adding 100 µM IPTG. After 3 h the cells were harvested and β-galactosidase activity was measured as described (Miller, 1972).

Preparation of total RNA. *B. licheniformis* MW3 (wt) and TMBli003 (Δ*ecf*41_{Bli}) were grown aerobically in LB medium at 37°C. Every 2 hours 30 ml samples were taken and mixed with cold killing buffer (20 mM Tris-HCl, pH 7.0, 0.5 mM $MgCl_2$, 20 mM NaN_3), harvested by centrifugation and frozen in liquid nitrogen, before the pellets were stored at -80°C. The cells were resuspended in 200 µl killing buffer, immediately transferred to a pre-cooled Teflon vessel and disrupted with a Micro-Dismembrator U (Sartorius) for 3 min at 2000 rpm. The resulting cell powder was resuspended in 3 ml prewarmed lysis solution (4 M guanidine-thiocyanate, 25 mM sodium acetate, pH 5.2, 0.5% N-lauroyl sarcosinate) and total RNA was extracted twice with acid phenol (phenol/chloroform/isoamylalcohol 25/24/1, pH 4.5-5) and once with chloroform (chloroform/isoamylalcohol 24/1) followed by isopropanol precipitation. Contaminating DNA was removed using the Baseline-ZERO DNAse (Epicentre Biotechnologies) according to the manufacturer's protocol. RNA was quantified with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and used for 5'RACE and Northern Blot analysis.

R. sphaeroides YSD354 (pIND4) and YSD333 (pYSD161) were grown aerobically in Sistrom's minimal medium (Sistrom, 1960) containing 25 µg/ml kanamycin at 30°C. At OD_{600} of ~0.3 expression of $Ecf41_{Rsp}$ was induced by adding 100 µM IPTG. After 3 hours 44 ml of culture were mixed with 6 ml stop solution (5% acid phenol in ethanol) and harvested by centrifugation. The pellets were frozen in an ethanol/dry ice bath and stored at -80°C. Cells were resuspended in 2 ml lysis solution (2% SDS, 16 mM EDTA) and incubated at 65°C for 5 min. RNA was extracted three times with acid phenol prewarmed to 65°C followed by chloroform extraction and isopropanol precipitation. To remove

contaminating DNA the RNA was incubated with 2 units RQ1 DNase (Promega) in the presence of 80 units RNasin Plus RNase Inhibitor (Promega) for 30 min at 37°C. The RNA was finally purified with the RNeasy Mini Kit (Qiagen) and used for DNA Microarray analysis and 5'RACE.

Probe preparation and Northern Blot analysis. A ~500 bp internal fragment of *ydfG* was amplified by PCR with oligonucleotides listed in Table 4.3. A digoxigenin (DIG)-UTP-labeled RNA probe was synthesized by *in vitro* transcription using the DIG RNA Labeling Mix (Roche) and T7 RNA polymerase (Roche) according to the manufacturer's protocol.

For Northern Blot analysis 10 µg of total RNA were separated under denaturing conditions on a 1% formaldehyde agarose gel and transferred to a positively charged nylone membrane (Roche) in a downward transfer using 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) as transfer buffer. The RNA was crosslinked by exposing the membrane to UV light. The blot was prehybridized at 68°C for 1 h with hybridization solution (5x SSC, 50% formamide, 2% Blocking Reagent (Roche), 0.1% N-lauroyl sarcosinate and 0.02% SDS). Hybridization was carried out overnight at 68°C in the same solution with 1 µg DIG-labeled RNA probe. The membrane was washed twice for 5 min at room temperature (2x SSC, 0.1 % SDS) and three times for 15 min at 68°C (0.1x SSC, 0.1 % SDS). The signal was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) and CDP-*Star* (Roche) according to the manufacturer's instructions. The signals were visualized using a LumiImager (Peqlab).

DNA microarray analysis. RNA samples from three independent cultivations were used for cDNA synthesis and DNA microarray hybridization. 10 µg of total RNA were mixed with 3 µg random hexamers and denatured at 70°C for 10 min before the temperature was decreased in 6 cycles (1 min each) by 10C°/cycle to 10°C to optimize annealing of the hexamers. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. Temperature was increased from 20°C to 42°C in 22 cycles of 3 min with 1°C increment followed by incubation at 42°C for 1 hour and inactivation at 70°C for 10 min. Remaining RNA was removed by alkaline hydrolysis and cDNA was purified using the PCR Purification Kit (Qiagen). 3.2 µg cDNA were fragmented with 0.25 units RQ1 DNase (Promega) for 10 min at 37°C followed by

inactivation for 10 min at 98°C. cDNA was labelled using the BioArray Terminal Labeling Kit with Biotin-ddUTP for DNA Probe Array Assays (Enzo) according to the manufacturer's protocol. Labeled cDNA samples (3µg/array) were hybridized to Affymetrix (Santa Clara, CA) custom arrays (Pappas *et al.*, 2004) according to the manufacturer's directions. Processing, normalization, and statistical analysis of the array data were performed in the R statistical software environment (http://www.r-project.org/). Data were normalized using the *affyPLM* package with default settings (Bolstad, 2004). Differentially expressed genes were detected using the *limma* package with a false discovery rate set at 0.05 (Smyth, 2005).

Determination of transcriptional start sites by 5'-RACE. The 5' ends of *ydfG* and RSP_0606 mRNAs were identified by rapid amplification of cDNA ends (RACE). 15 µg of total RNA were incubated with 25 units tobacco acid pyrophosphatase (TAP, Epicentre Biotechnologies) in the delivered buffer at 37°C for 60 min in the presence of 40 units Super RNaseIn RNAse inhibitor (Ambion). As a control, 15 µg RNA were incubated under the same conditions, but without TAP. The reactions were phenol/chloroform extracted and ethanol precipitated. After dissolving the pellets in water, the RNA was mixed with 500 pmol RACE adapter (5'-GAUAUGCGCGAAUUCCUGUAGAACGAACACUAGA-AGAAA-3') and denatured at 95°C for 5 min. Ligation of the adapter was carried out at 17°C overnight with 100 units T4 RNA ligase (Epicentre Biotechnologies) in the presence of 80 units Super RNaseIn RNAse inhibitor (Ambion). Again, the reactions were phenol/chloroform extracted, ethanol precipitated and the pellets were resuspended in water. 1 µg RNA was used for reverse transcription with gene specific primers (GSP1, Table 4.3) and the iScript Select cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. The cDNA was then amplified with nested primers and a primer complementary to the RACE adapter sequence (GSP2 and 679, Table 4.3) and the transcription start sites were identified by sequencing.

Western Blot analysis. *B. subtilis* strains containing overexpression plasmids were grown in LB medium at 37° to an OD_{600} ~0.4. Expression of $Ecf41_{Bli}$-FLAG and its variants was induced by adding 0.5 % xylose. After 1 h 15 ml of each culture were harvested. The pellets were resuspended in ZAP buffer (10 mM Tris, pH 7.4, 200 mM NaCl), cells were lysed by sonication and cell debris was removed by centrifugation. 20 µg of the cleared lysate were separated by SDS-PAGE and transferred to a Polyvinylidene difluoride

(PVDF) membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions. The membrane was then incubated overnight at 4°C with blotto (2.5% skim milk in TBS (50 mM Tris, pH 7.6, 150 mM NaCl)) to prevent non-specific binding. Then, the membrane was incubated with the primary antibody (anti-FLAG (Sigma) diluted 1:2000 in blotto) at room temperature for 1 h followed by four 10 min washing steps with blotto. Then the blot was incubated for 1 h with the secondary antibody (anti-rabbit IgG HRP conjugate (Promega) diluted 1:2000 in blotto). After four washing steps with blotto, the membrane was washed with TBS before the signals were detected with a LumiImager (Peqlab) using AceGlow (Peqlab) as chemiluminescence substrate.

RNAP pull-down assays. Different versions of $Ecf41_{Bli}$-FLAG under control of a xylose-inducible promoter were introduced into *B. subtilis* 1A774, which contains a His_6-tag fused to the β' subunit of the RNAP, to form strains TMB1099 (wt $Ecf41_{Bli}$), TMB1100 ($Ecf41_{Bli}$ 204) and TMB1101 ($Ecf41_{Bli}$ 167). As controls, the same constructs were transformed into *B. subtilis* W168 resulting in TMB695, TMB746 and TMB666. The RNA pull-down assays were performed as described previously (MacLellan *et al.*, 2008). In brief, 100 ml LB medium supplemented with 5 µg/ml chloramphenicol were inoculated from fresh overnight cultures and grown till OD_{600} ~0.4. Cultures were induced with 0.5% xylose for 1 hour and cells were harvested by centrifugation. The pellets were resuspended in phosphate buffer (50 mM phosphate buffer, pH 7.6, 100 mM NaCl, 0.1 mM PMSF, 5 mM imidazole) and cells were lysed by sonication. The cleared lysate was loaded on a column containing 0.5 ml Ni^{2+}-NTA metal affinity beads. The beads were washed with each 10 column volumes of the above mentioned phosphate buffer containing 5, 10 and 20 mM imidazole. Elution was carried out using 0.5 ml phosphate buffer with increasing imidazole concentration (50, 100, 250 and 500 mM). Samples of the cleared lysate, washing steps and elution fractions were run in duplicates on 10 and 12% SDS-PAGE gels and checked for presence of RNAP (coomassie staining) and $Ecf41_{Bli}$-FLAG (Western Blot using anti-FLAG antibodies). For quantitative analysis 5 µg of lysate as well as 5 and 10 µg of the 100 mM imidazole elution fractions were used and analysed as mentioned above.

Results

In silico analysis of group ECF41 σ factors

Phylogenetic distribution. The initial analysis of group ECF41 (Staroń *et al.*, 2009) was based on a dataset generated in 2008 containing 115 ECF41 protein sequences from five different phyla. To account for the huge increase in bacterial genomes sequenced within the last three years, we re-analysed group ECF41 based on 373 ECF41 σ factors extracted in October 2010 from the Microbial Signal Transduction Database (MiST2) (Ulrich & Zhulin, 2010) (Table S1). The proteins of group ECF41 are widely distributed and can be found in ten different phyla (Table 4.4): Actinobacteria (68%, 252 proteins), Proteobacteria (23%, 84 proteins), Firmicutes (4%, 15 proteins) and Chloroflexi (3%, 11 proteins), Acidobacteria (4 proteins), Bacteriodetes, Cyanobacteria (2 proteins each) and Spirochaetes, Verrucomicrobia and Gemmatimonadetes (1 protein each). It should be noted that some phyla are heavily underrepresented among the available genome sequences, as has been discussed recently (Staroń *et al.*, 2009).

Table 4.4. Phylogenetic distribution of ECF41 σ factors

Phyla	ECF41 proteins per phylum	% of ECF41 proteins	Species with ECF41 protein	% of sequenced species	Sequenced genomes/species[a]
Actinobacteria	252	68	60	51	181/118
Proteobacteria	84	23	66	15	705/414
Firmicutes	15	4.0	10	2.5	404/182
Chloroflexi	11	2.9	3	60	15/5
Acidobacteria	4	1.1	4	67	6/6
Bacteriodetes	2	0.5	2	4.4	53/45
Cyanobacteria	2	0.5	2	6.3	44/32
Spirochaetes	1	0.3	1	5.6	23/18
Verrucomicrobia	1	0.3	1	25	4/4
Gemmatimonadetes	1	0.3	1	100	1/1

[a] Numbers of sequenced genomes and species of each phylum were extracted from the MiST2 database (Ulrich & Zhulin, 2010) in October 2010.

The 373 proteins of group ECF41 derive from 150 different species. Therefore, these organisms often encode more than one copy of the ECF41 gene in the genome (Table 4.4 and S1). Especially within the Actinobacteria multiple copies are very common. Only 14 out of the 60 ECF41-containing actinobacterial species harbor just one copy of this σ factor, while the genomes of the remaining 46 contain several copies. Especially the genus

Streptomyces contains large numbers of ECF41 σ factors with at least 4 copies per genome, which may reflect the complex lifestyle of these bacteria (Flärdh & Buttner, 2009). The ECF41 copy number correlates well with the genome size and the overall abundance of signal transducing systems. For example, the genome of *S. coelicolor* encodes as many as 45 ECF σ factors (Bentley *et al.*, 2002), 13 of which belong to group ECF41. A high abundance of ECF41 genes can also be found in the phylum Chloroflexi (11 ECF41 σ factors/3 genomes), whereas most of the Proteobacteria (84 ECF41 σ factors/66 genomes) and Firmicutes (15 ECF41 σ factors/10 genomes) harbour only one to two ECF41 σ factors per genome.

We constructed an unrooted phylogenetic tree based on a gapless multiple sequence alignment of all 373 ECF41 σ factors using the Neighbor-Joining method implemented in the Phylip program Protdist (Felsenstein, 1989) provided by the BioEdit Sequence Alignment Editor (Hall, 1999) (Fig. 4.1).

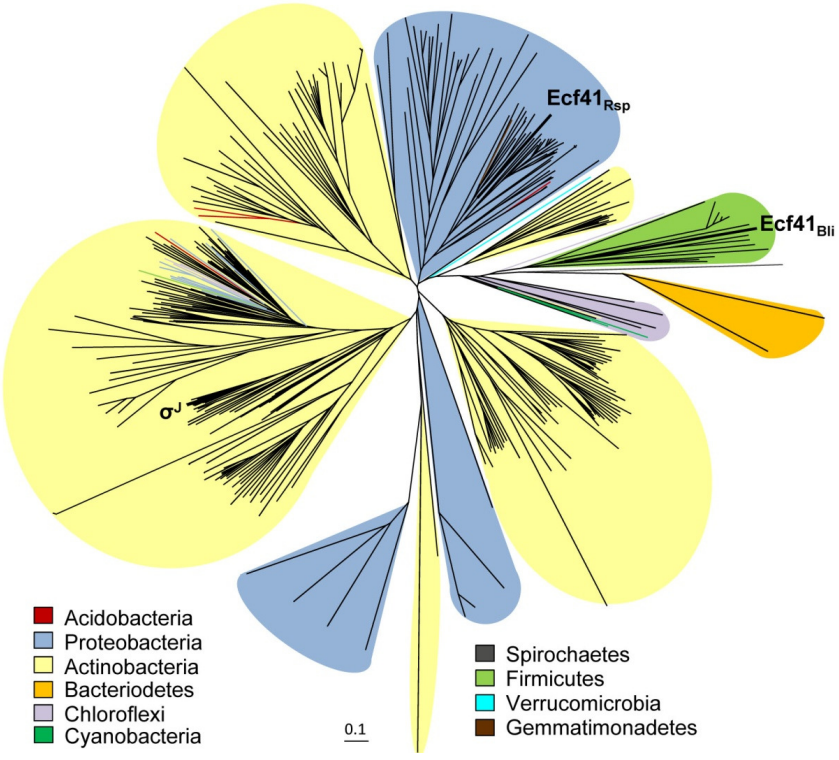

Figure 4.1. Phylogenetic tree of ECF41 σ factors. The phylogenetic tree is based on a gapless multiple sequence alignment of 373 ECF41 protein sequences constructed using ClustalW (Thompson *et al.*, 1994). The resulting phylogenetic tree was calculated using the neighbor-joining method of the Phylip (Felsenstein, 1989) program Protdist implemented in the BioEdit Sequence Alignment Editor (Hall, 1999). Assignment to bacterial phyla is indicated by a color code. $Ecf41_{Rsp}$ of *R. sphaeroides*, $Ecf41_{Bli}$ of *B. licheniformis* and σ^J of *M. tuberculosis* are highlighted.

In general, the terminal nodes representing sequences of ECF41 σ factors cluster according to the phylum (Fig. 4.1). The two phyla containing the highest number of sequences (Actinobacteria and Proteobacteria) are divided into five and three different branches, respectively. One cluster within one actinobacterial branch is rather diverse and includes ECF σ factors from Proteobacteria, Chloroflexi, Firmicutes and Acidobacteria. The remaining ECF41 σ factors from Firmicutes as well as Chloroflexi form single branches. The ECF41 proteins from Bacteriodetes and Cyanobacteria also cluster together, whereas the proteins from Acidobacteria, Spirochaetes, Verrucomicrobia and Gemmatimonadetes cluster within or between actinobacterial and proteobacterial branches (Fig. 4.1).

Genomic context conservation. In contrast to most ECF σ factors studied to date (Butcher *et al.*, 2008), no gene encoding an obvious anti-σ factor can be found in direct vicinity of the genes encoding the ECF41 σ factors. Instead, they are genomically associated with genes encoding CMD proteins, oxidoreductases or epimerases (COE) (Table S1 and Fig. 4.2). While this genomic context is highly conserved, the order and orientation of the associated genes is diverse. In almost 50% of the cases, both genes are orientated in the same direction and could potentially be transcribed as an operon. In less than 20% the genes are orientated divergently. The remaining ~30% of ECF41 σ factors do not cluster with genes encoding COE. Such "orphans" are especially abundant in actinobacterial species (Fig. 4.2), which contain multiple copies of ECF41 genes in the genome. But in general, at least one copy of the ECF41 genes shows the conserved genomic context.

Carboxymuconolactone decarboxylases. Commonly, proteins of the CMD family (PF02627) can be divided into two main groups: the γ-CMD proteins and the AhpD-like alkylhydroperoxidases (Ito *et al.*, 2006). The γ-CMD proteins are involved in the degradation of aromatic compounds. They catalyze the decarboxylation of γ-carboxymuconolactone to β-ketoadipate enol-lactone in the protocatechuate branch of the β-ketoadipate pathway (Eulberg *et al.*, 1998). The best investigated example of the second group is the alkylhydroperoxidase AhpD of *Mycobacterium tuberculosis*. This protein contains a CxxC motif critical for catalytic activity and is part of the antioxidant defense system of this organism (Hillas *et al.*, 2000, Koshkin *et al.*, 2003). In the archaeon *Methanosarcina acetivorans* it was shown that the product of gene MA3736 encodes an uncharacterized CMD protein homolog with disulfide reductase activity dependent on a CxxC motif (Lessner & Ferry, 2007). It was suggested to play a role in the oxidative stress

response of this organism. All CMD proteins genomically linked to ECF41 σ factors contain a conserved CxxC motif, suggesting a role of this group in the defense against oxidative stress.

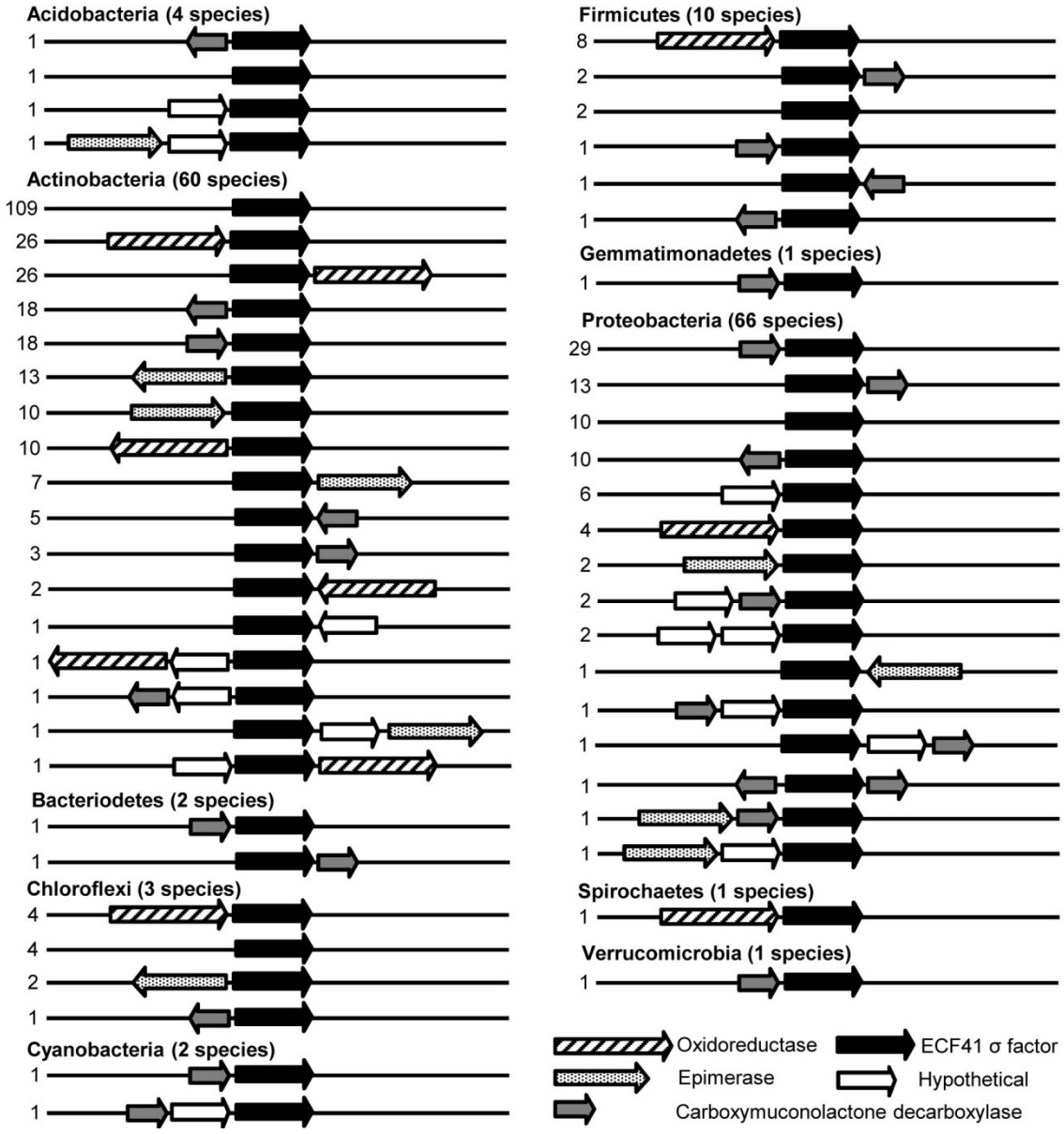

Figure 4.2. Genomic context conservation of ECF41 σ factors. ECF σ factors are shown by black, CMD proteins by grey, oxidoreductases by striped and epimerases by dotted arrows. Genes encoding hypothetical proteins, that either contain the conserved promoter motif or are located between the ECF41 σ factor and the COE, are displayed in white. The genomic context is represented according to the phylum with the number of species in parentheses. The number in front of each context indicates how often this combination of genes occurs within the designated phylum.

Oxidoreductases. The reactions catalyzed by oxidoreductases can be very diverse, but are always characterized by the transfer of electrons from one molecule to another, often using NAD(P)H or FAD as cofactors. Since oxidoreductases can use a variety of different molecules as electron donor or acceptor, it is difficult to assign a specific function to these enzymes. In case of genes next to ECF41 σ factors, they were classified as oxidoreductase if their product carried at least one of the following Pfam domains: Oxidored_FMN, Flavodoxin_2, Pyr_redox/_2, FAD_binding_2/3/4, Amino_oxidase, Pyridox_oxidase or FMN_red.

Epimerases. The third group contained either a NmrA (PF05368) or an Epimerase (PF01370) domain. NmrA is a negative transcriptional regulator of AreA and involved in nitrogen metabolite repression in different fungi. The crystal structure of NmrA revealed a Rossmann fold and similarity to members of the short-chain dehydrogenase/reductase family (Stammers *et al.*, 2001), which generally deploy nucleotide-sugar substrates for chemical conversions. The Rossmann-fold is typical for two domain redox enzymes that use NAD^+ as cofactor. The UDP-galactose 4-epimerase is the best understood example of this family and catalyzes the conversion of UDP-galactose to UDP-glucose (Allard *et al.*, 2001).

Miscellaneous. In some cases, genes encoding other than the above mentioned proteins can also be linked to ECF41 σ factors. These neighboring genes were included in Fig. 4.2 if they (i) carry the typical promoter sequence (see below), or (ii) are located between the ECF41- and the COE-encoding genes. Most of these other genes encode hypothetical proteins of unknown function. Four of these hypothetical proteins (Table S1) contain the conserved β-barrel domain of the cupin superfamily, which members often function as dioxygenases in bacteria (Dunwell *et al.*, 2004). The C-terminal domain of the cytoplasmic anti-σ factor ChrR from *R. sphaeroides* σ^E also adopts such a cupin fold (Campbell *et al.*, 2007). In all four cases, the genes encoding these cupin fold proteins are in the same orientation than the ECF σ factor and presumably form an operon.

ECF41 proteins contain a large C-terminal extension. Group 4 alternative σ factors contain the smallest proteins of the σ^{70} family, in which only regions σ_2 and σ_4 are sufficient for promoter recognition and RNAP interaction. An alignment of classical ECF σ factors from different organisms and proteins of group ECF41 revealed a large C-

terminal extension of about 100 amino acids only present in ECF41 σ factors (Fig. 4.3). Based on an alignment of all ECF41 proteins (Fig. S1), we identified three conserved motifs within this extension. Another characteristic feature of the ECF41 proteins is a highly conserved WLPEP motif in the linker region between σ_2 and σ_4, which usually does not show much sequence conservation in other ECF σ factors (Fig. 4.3).

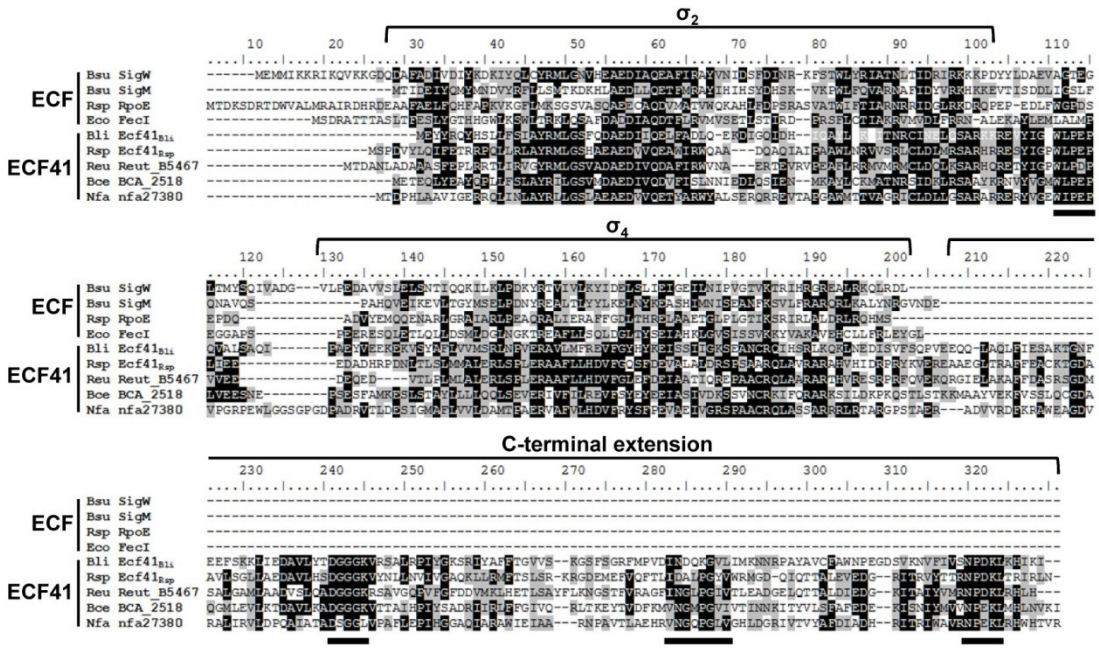

Figure 4.3. Characteristic features of group ECF41 proteins and comparison with classical ECF σ factors. The multiple sequence alignment of selected ECF σ factors was constructed using ClustalW (Thompson *et al.*, 1994). Identical amino acids at the same position are shaded in black, similar amino acids in grey. The σ_2 and σ_4 domains and the C-terminal extension are marked. Conserved motives of ECF41 proteins are defined by the complete multiple sequence alignment of group ECF41 (Fig. S1) and underlined. Abbreviations: Bce, *Bacillus cereus*; Bli, *Bacillus licheniformis*; Bsu, *Bacillus subtilis*; Eco, *Escherichia coli*; Nfa, *Nocardia farcinica*; Rsp, *Rhodobacter sphaeroides*; Reu, *Ralstonia eutropha*.

By analogy to other group 4 σ factors, we expect activity of the ECF41 proteins to be regulated. Based on the observations regarding the genomic context and domain architecture of ECF41 σ factors, we propose three hypotheses to explain their regulation: (i) the COE genes could be targets of ECF41-dependent regulation, (ii) the COE could be part of the signal transducing mechanism and function as an anti-σ factor, or (iii) the C-terminal extension could be involved in controlling σ factor activity.

To address these hypotheses directly and generalize our findings, we experimentally investigated ECF41 σ factors from two different organisms: BLi04371 of *B. licheniformis*

and RSP_0607 of *R. sphaeroides*. We named the genes encoding the ECF41 σ factors to *ecf*41$_{Bli}$ and *ecf*41$_{Rsp}$ and used these terms for the following analysis. The genomic neighborhood including the genes encoding the CMD proteins YdfG and RSP_0606 is shown in Fig. 4.4 A.

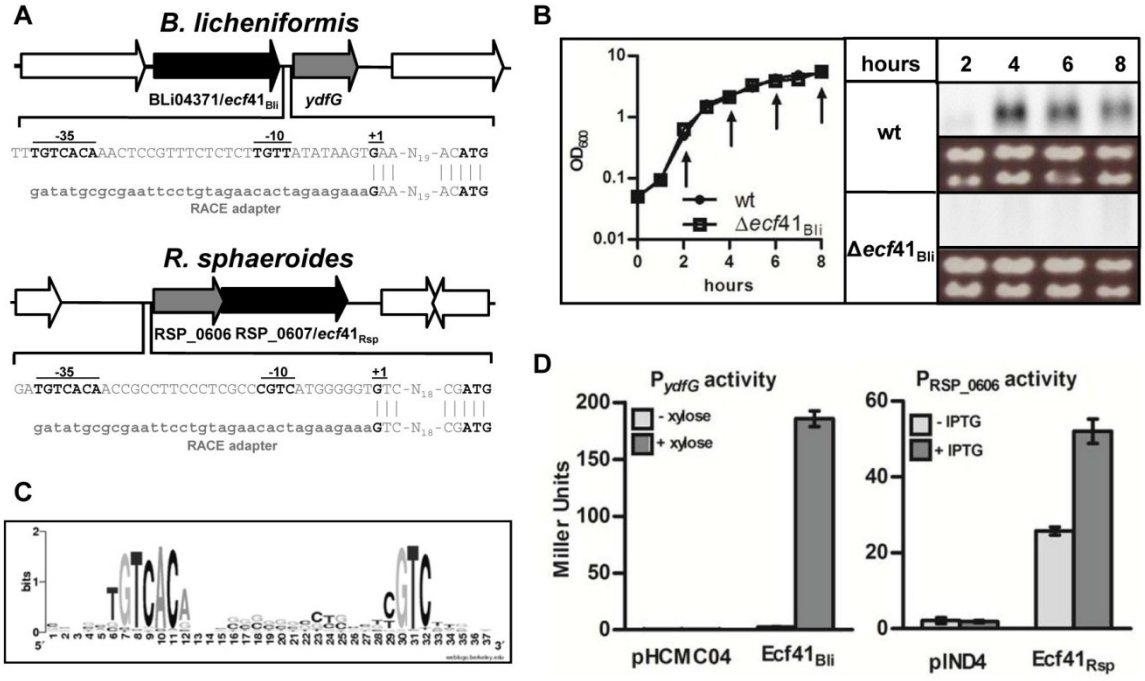

Figure 4.4. Targets of ECF41-dependent signal transduction. (A) Genomic context organization and target promoter sequence of the ECF41 σ factors from *B. licheniformis* and *R. sphaeroides*. Genes encoding the ECF41 σ factor (black) and the CMD protein (grey) as well as the promoter sequences are shown. Flanking genes not belonging to the ECF41 loci are shown in white. The -35 and -10 region, the transcriptional start site +1 and the ATG start codon are highlighted in bold. The RACE adapter sequence is indicated by lower case letters. (B) Northern Blot analysis of Ecf41$_{Bli}$-dependent *ydfG* expression in *B. licheniformis*. *B. licheniformis* MW3 (wt) and TMBli003 (Δ*ecf*41$_{Bli}$) were grown aerobically in LB medium. At the time points indicated by arrows, samples of both strains were harvested and total RNA was prepared. 10 µg total RNA were separated on a 1% formaldehyde gel and transferred to a nylon membrane followed by hybridization and detection with a DIG-labeled *ydfG*-specific probe. Ribosomal RNA is shown to ensure equal amounts of RNA in each lane. (C) Weblogo of ECF41-dependent target promoters. The weblogo was generated using the WebLogo tool (Crooks *et al.*, 2004) available at http://weblogo.berkeley.edu. The weblogo graphically represents a position weight matrix and illustrates the degree of sequence conservation for each nucleotide. The matrix is based on 285 putative promoter sequences identified upstream of genes encoding ECF41 σ factors and COE. (D) ECF41-dependent target promoter activation. *B. subtilis* strains TMB696 (Ecf41$_{Bli}$) and TMB623 (pHCMC04) were grown in LB medium to OD_{600} ~0.4 and split into two samples. In one sample, expression of Ecf41$_{Bli}$ was induced by addition of 0.5 % xylose and cells were harvested after 1 h. *R. sphaeroides* strains TMR005 (Ecf41$_{Rsp}$) and YSD434 (pIND4) were grown in Sistrom's minimal medium to OD_{600} ~0.3 and split into two samples. In one sample, expression of Ecf41$_{Rsp}$ was induced by 100 µM IPTG. After 3 hours, the cells were harvested. P_{ydfG} and P_{RSP_0606} activities were measured by β-galactosidase assays with normalization to cell density.

Targets of ECF41 σ factors

COE-encoding genes represent targets of ECF41-dependent signal transduction. We first investigated if the COE-encoding genes next to the ECF41 σ factors are targets of the ECF41-dependent signal transduction. Therefore, we monitored expression of *ydfG* at different growth phases in a *B. licheniformis* wild type and an isogenic Δ*ecfI*41$_{Bli}$ deletion strain. Both strains show no difference in growth behavior (Fig. 4.4 B) indicating that Ecf41$_{Bli}$ is not required under standard laboratory conditions. At designated time points samples of both strains were taken, total RNA was prepared and Northern Blot analysis was performed using a *ydfG*-specific probe. At the transition from the exponential to the stationary growth phase, a ~0.5 kb transcript appears in the wild type strain in agreement with a monocistronic expression of *ydfG* (Fig. 4.4 B). No *ydfG* transcript is visible in the Δ*ecfI*41$_{Bli}$ deletion mutant, demonstrating that detectable expression of *ydfG* is completely Ecf41$_{Bli}$-dependent under the condition tested.

We also examined the transcriptome upon overexpression of Ecf41$_{Rsp}$ in *R. sphaeroides* by DNA microarrays to test if a similar result can be obtained in another organism and to possibly identify additional target genes of ECF41 σ factors. The mRNA level for *ecf*41$_{Rsp}$ was ~80-fold increased in cells overexpressing this protein. The only other more than 2-fold induced gene was RSP_0606 (~3-fold), which encodes the associated CMD protein (data not shown). These results indicate that Ecf41$_{Rsp}$ seems to control expression of only a single transcript that contains the *ecf*41$_{Rsp}$ and RSP_0606 genes.

Analysis of ECF41-dependent target promoters in *B. licheniformis* and *R. sphaeroides*. Since ECF σ factors recognize alternative promoter sequences, we investigated if the identified ECF41 target genes *ydfG* and RSP_0606 are preceded by such a unique sequence motif. We therefore mapped the transcriptional start site by 5'RACE in RNA samples from cells overexpressing the ECF41 σ factor (*R. sphaeroides*) or samples taken throughout the growth cycle (*B. licheniformis*) (Fig. 4.4 B). In both organisms, we identified a "G" residue as the transcriptional start site followed by a 22/23 bp untranslated region containing a suitable ribosome binding site (Fig. 4.4 A). Upstream of this start point we identified a bipartite sequence motif with similarity to ECF-dependent promoter elements: a -35 region identical in both organisms ("TGTCACA") and a -10 region ("TGTT" or "CGTC").

Next we tested if this predicted target promoter responds to the corresponding ECF41 σ factor. Because of the lack of genetic tools for *B. licheniformis*, we heterologously expressed $Ecf41_{Bli}$-FLAG from a xylose-inducible promoter in *B. subtilis*, an organism lacking an ECF41 σ factor, and measured the activity of the target promoter P_{ydfG} transcriptionally fused to *lacZ* by β-galactosidase assays (Fig. 4.4 D). Without xylose, the resulting reporter strain TMB696 shows only low P_{ydfG} activity, presumably due to weak basal expression of $Ecf41_{Bli}$ from P_{xylA} in complex LB medium. Addition of 0.5 % xylose increased promoter activity ~70-fold, indicating that $Ecf41_{Bli}$ activates P_{ydfG} upon its overexpression. Almost no β-galactosidase activity was detectable in the control strain TMB623, harboring only the empty expression vector, demonstrating that P_{ydfG} from *B. licheniformis* is not recognized and activated by any σ factor of *B. subtilis* under the laboratory conditions used in these experiments.

To test for promoter recognition in *R. sphaeroides*, we expressed $Ecf41_{Rsp}$ from an IPTG-inducible promoter and measured the activity of the target promoter P_{RSP_0606} by β-galactosidase assays. Without inducer present, strain TMR005 shows P_{RSP_0606} activity of about 25 Miller Units, which can be increased 2-fold by addition of IPTG (Fig. 4.4 D). The high P_{RSP_0606} activity of TMR005 in the absence of IPTG is presumably due to background transcription from the leaky promoter of the expression plasmid. In comparison, the control strain YSD434, which carries the empty vector, shows only marginal promoter activity. These results from two independent organisms demonstrate that the promoter identified by 5'RACE (Fig. 4.4 A) specifically responds to the overexpression of ECF41 σ factors.

Prediction of a general ECF41-dependent target promoter motif. After we identified an ECF41-dependent promoter in two organisms, we expected that this motif should also be present in the ECF41 loci other organisms. To verify this, we extracted 250 bp regions upstream of the COE- as well as the ECF41-encoding genes and searched for overrepresented sequence motifs with similarity to the identified promoter, either manually or by using the MEME algorithm (Bailey & Elkan, 1994). We identified these two motifs separated by a 16+/-1 bp spacer within most of these regions and constructed a weblogo based on 285 sequences (Fig. 4.4 C). The -10 region with the consensus 'CGTC' is comparable to many typical ECF promoters, whereas the -35 consensus 'TGTCACA' is specific for group ECF41. This bipartite promoter motif can be found upstream of both the COE- and ECF41-encoding genes, often preceding a potential operon consisting of these

two genes (Table 4.5 and Table S1). In about one-third of all ECF41 σ factors, the COE-encoding gene is located upstream of and in the same orientation than the ECF gene. Here, the COE gene usually carries the promoter motif while the ECF σ factor lacks it. In case of this predominant locus organization, both genes could form an operon transcribed from the COE promoter. If the ECF σ factor is located upstream of the COE gene, less than 30% of the ECF- and almost 50% of the COE-encoding genes harbour the promoter, in case of 20% both genes are preceded by the motif. About 17% of the ECF41 σ factors show opposite orientation relative to the COE gene, either >ECF><COE< or <ECF< >COE>. In the latter case often both genes are preceded by the motif, whereas for the first combination usually only either the ECF- or COE-encoding gene shows the promoter. More than 30% of all ECF41 σ factors do not show the genomic context conservation. Of these "orphan" genes, only 30% are preceded by the ECF41-specific promoter.

Table 4.5. Genomic context and promoter occurrence

Genomic context[a]	Number	P_{ECF}[b]	P_{COE}[b]
>ECF>	126 (34%)	38	n.a.
>COE> >ECF>	107 (29%)	-	97
>ECF> >COE>	53 (14%)	14	24
>ECF> <COE<	9 (2%)	3	4
<ECF< >COE>	55 (15%)	36	41
ungrouped	23 (6%)	n.a.	n.a.

[a]The arrows indicate the organization of the genes. ECF, gene encoding an ECF41 σ factor; COE, gene encoding a CMD protein, oxidoreductase or epimerase; ungrouped, genomic context differs from the above mentioned groups and contains genes encoding hypothetical proteins of unknown function.

[b] "-", no promoter occurs upstream of the gene; n.a., the corresponding gene is not present or was omitted from analysis in case of ungrouped genomic context.

Next we used the derived position weight matrix graphically represented in Fig. 4.4 C to perform genome-wide searches for additional ECF41 target promoters in *R. sphaeroides* and *B. licheniformis*, using the algorithm virtual footprint (Münch *et al.*, 2005) implemented in the Prodoric database (Münch *et al.*, 2003). In both organisms, only a few potential ECF41 target promoters could be identified (Table 4.6), but none exactly matched the ECF41 consensus. Construction of transcriptional *lacZ*-fusions to three of these promoters from *B. licheniformis* (P_{nhaX}, P_{uvrX} and P_{ypbE}) and subsequent determination of β-galactosidase activity did not reveal any $Ecf41_{Bli}$-dependent activation. Even expression of

a highly active truncated version of $Ecf41_{Bli}$ (see below) did not result in any promoter activation (data not shown).

Table 4.6. Putative ECF41 target promoters in *B. licheniformis* and *R. sphaeroides*

Gene[a]	Promoter sequence[b]	5' UTR[c]	Putative function, homology
B. licheniformis			
BLi01248	TGTCACAAAAACATAAATAATAGATGTC	142	Hypothetical, putative membrane protein
Bli03073	TGTCACCCCTTCCTT-TTTCGAGCCGTC	109	Hypothetical, putative membrane protein
hprP	TGTCACGCTTGCTTTTATTTTTCTCGTC	163	Putative phosphatase
mtrB	TGTCACTTCAGCTGT-AAGGGGAACGTT	76	Transcription attenuation protein
nhaX	TGTCACGTTTAGGTG--CTTTTGTTGTT	199	Stress response protein
pucR	TGTCACAAATCCGCT--CATTTTTTGTT	39	Purine catabolism regulatory protein
sat	TGTCACAAGCGTTCTGCTGGCATCTGTC	97	Sulfate adenylyltransferase, dissimilatory-type
spoIISB	TGTCACAGAATTTGA-TTATCTCCTGTT	60	Stage II sporulation protein SB
uvrX	TGTCACCTTCTTTCC-AAAGAAGGTGTT	120	DNA-damage repair protein
ybxF	TGTCACTAAAAATTG-TCATCATATGTT	68	Firmicutes ribosomal L7Ae family protein
ydfG	TGTCACAAACTCCGT-TTCTCTCTTGTT	31	Putative CMD protein
yfmE	TGTCACGGCAATGAT-TGGGACGCCGTT	42	Heme ABC type transporter HtsABC, permease
ykpA	TGTCACAAAGAAAGTGGAAATAAGCGTT	108	ABC transporter, ATP-binding protein
ypbE	TGTCACGGCACATTTTTTGATCGATGTT	48	Unknown, LysM domain, cell wall degradation
yvdI	TGTCACACTGCTCATTTCTTTCATTGTC	63	Maltose/maltodextrin ABC transporter
R. sphaeroides			
gcvH	TGTCACGTCCGGCG-GCTTCGGCCCCTC	151	Glycine cleavage system H protein,
repA	TGTCACCGTTTCG--CCCCAAGAACGTG	152	RepA partitioning protein/ATPase, ParA type
rplL	TGTCACCCACC--ATGTTGGACCCCATC	20	Ribosomal protein
RSP_0606	TGTCACAACCGC-CTTCCCTCGCCCGTC	32	Putative CMD protein

[a]Genes highlighted in bold were tested for activation by the corresponding ECF41 σ factor.
[b]Underlining indicates -35 (left) and -10 (right) regions, the spacing was adjusted indicated by dashes.
[c]5'UTR, length of 5'-untranslated region (in nucleotides) between the postulated transcriptional start site and the AUG start codon.

In addition, we performed genome-wide analysis on the presence and conservation of the ECF41 promoter motif upstream of orthologous genes in a wide range of ECF41-harboring organisms. While this *in silico* analysis has been successfully used to identify candidate promoters and core regulons for other regulators including ECF σ factors (Dufour *et al.*, 2010, Dufour *et al.*, 2008), it failed to reveal any potential conserved regulon members, with the exception of genes encoding the COE or ECF41 σ factor (Fig. 4.5). Taken together, our collective data strongly suggest that the proteins of group ECF41 generally control only a single target gene or operon, which includes the COE and the ECF41 σ factor, if co-transcribed. The COE-encoding genes therefore represent the only known and detectable targets of ECF41-dependent gene expression.

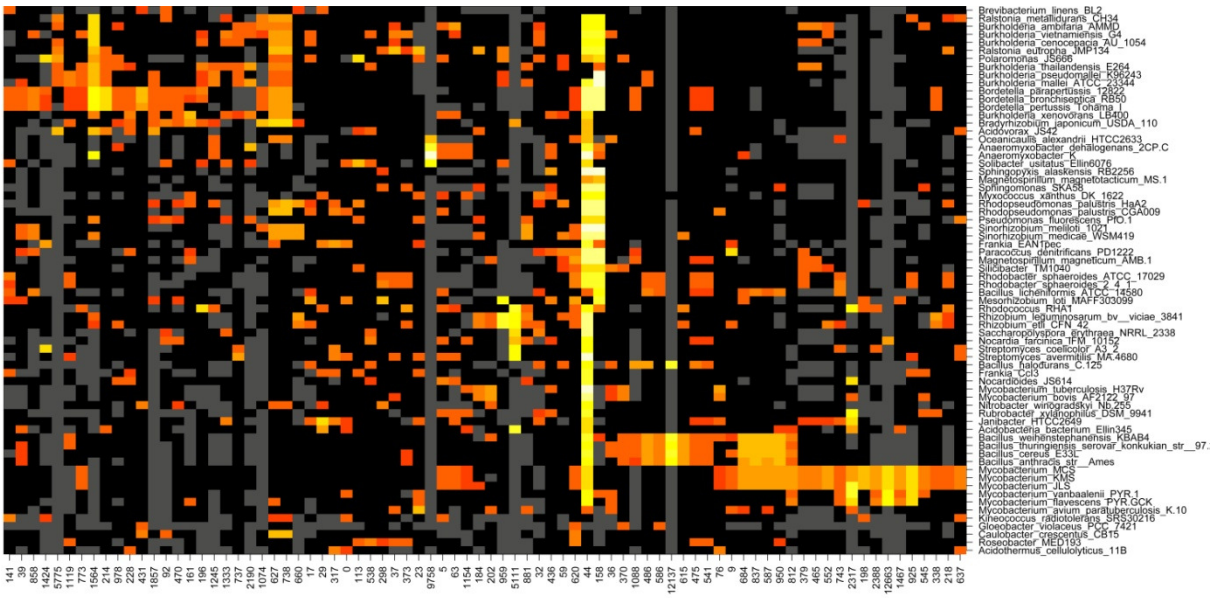

Figure 4.5. Potential ECF41 target genes across selected bacteria. The analysis was performed as described (Dufour *et al.*, 2008). Shown are groups of orthologous genes (columns) that contain the putative ECF41 binding motif in their promoter regions across indicated species (rows). A gray box means that the organism does not possess a homolog for the corresponding group; black means that it possesses a homolog for the group but no conserved ECF41 binding motif was found in its upstream region; red and yellow means that an ECF41 binding motif was found in the promoter region for the gene. Shades from red to yellow represent the similarity to the ECF41 consensus promoter motif with yellow being most similar.

Phenotypes linked to ECF41-dependent gene expression. In *B. licheniformis* and *R. sphaeroides*, the only detectable target gene of the ECF41 σ factor encodes a CMD protein. These proteins are not necessarily involved in degradation of aromatic compounds, but could also exhibit a role in the oxidative stress response (Hillas *et al.*, 2000, Lessner & Ferry, 2007). Additionally, a strain of *M. tuberculosis* lacking the ECF41 σ factor σ^J is slightly more sensitive to H_2O_2 (Hu *et al.*, 2004). Based on these observations, we investigated a potential link between ECF41 σ factors and oxidative stress response. We determined minimal inhibitory concentrations and performed serial dilution spot tests to compare the viability of a *R. sphaeroides* wild type and RSP_0606-*ecf*41_{Rsp} deletion mutant (YSD239) or $Ecf41_{Rsp}$ overexpression strain (TMR003) strain as well as *B. licheniformis* wild type and Δ*ecf*41_{Bli} or Δ*ydfG* (TMBli006 or TMBli003) deletion strains, respectively. No significant differences were observed in the presence of H_2O_2, cumene hydroperoxide, t-butyl hydroperoxide or paraquat (data not shown).

We subsequently performed phenotype microarray (PM) analysis. This high-throughput approach allows testing hundreds of different conditions in parallel in order to identify phenotypes associated with genetic alterations (Bochner, 2003). Our PM analysis included

960 assays for carbon, nitrogen, phosphorus and sulfur utilization, nutrient stimulation, pH and osmotic stress as well as chemical sensitivity tests covering 240 different substances (see http://www.biolog.com/PM_Maps.html for details). We compared phenotypic differences between the *R. sphaeroides* wild type strain and RSP_0606-*ecf*41_{Rsp} deletion (YSD239) as well as an Ecf41_{Rsp} overexpression strain (TMR006). Overall, only a very few phenotypes can be linked to the expression or deletion of the ECF41 σ factor (supplementary material). Besides resistance to spectinomycin due to the resistance cassette, strain YSD239 showed only a positive phenotype against the sulfonamide antibiotic sulfadiazine. As expected, strain TMR006 displayed relative resistance to aminoglycoside antibiotics (kanamycin, neomycin, geneticin, paromomycin) due to the resistance cassette of the overexpression plasmid. Surprisingly, gained phenotypes can be observed for the carbon sources adonitol, D-mannitol, D-sorbitol and glucose, suggesting a metabolic function of ECF41 in utilization of additional carbon sources. But none of these additional phenotypes not due to the presence of a resistance cassette could be verified by serial dilution spot tests (data not shown).

Hence, we were not able to identify any ECF41-related phenotype. Therefore, we can so far only speculate on the physiological role of ECF41 σ factors. One likely possibility is that the COE proteins are involved in a very specific, presumably degradative metabolic pathway, instead of mediating a stress response. In the presence of a suitable substrate, the synthesis of the COE proteins could then be induced to facilitate the conversion of the metabolite.

The wide distribution and conservation of ECF41 σ factors indicates an important cellular role of these proteins. Since ECF41 proteins are particularly abundant in Actinobacteria with sometimes more than 10 copies per genome (Table 4.4 and S1), it might be worthwhile to study the function of ECF41 σ factors in members of this bacterial phylum. Alternatively, one could establish a high-throughput approach to study induction of the COE genes in response to a wide array of chemical compounds. Moreover, a biochemical analysis of representative COE proteins might also help to shed some light on the physiological role ECF41-dependent gene regulation.

Signal Transduction of ECF41 σ factors

The activity of ECF σ factors is normally regulated by a cognate anti-σ factor. The genes encoding these two proteins are usually located next to each other on the chromosome and co-transcribed (Butcher *et al.*, 2008, Helmann, 2002). As mentioned above, no obvious anti-σ factor is encoded in direct vicinity to the genes encoding the ECF41 σ factors. The results of our *in silico* analysis (Fig. 4.2 and 4.3) suggest that either the corresponding COE or the C-terminal extension of the ECF41 proteins could be involved in the regulation of σ factor activity. We first tested if expression of YdfG has any influence on the target promoter activation by $Ecf41_{Bli}$, but did not observe any effects (data not shown). Therefore, we focused our attention on the C-terminal extension. A multiple sequence alignment of all ECF41 proteins (Fig. S1) revealed four highly conserved motifs, three within the C-terminal extension and one in the linker region between σ_2 and σ_4 (Fig. 4.3). To study a possible function of these unique features, we investigated the effect of (i) exchanging the conserved motifs against alanine residues and (ii) C-terminal truncations of $Ecf41_{Bli}$.

Mutational study of conserved motifs. First, we constructed overexpression plasmids with variants of $Ecf41_{Bli}$, in which either of the conserved motives is exchanged against alanine residues (Fig. 4.6 A). The resulting modified versions of $Ecf41_{Bli}$, which also carried N-terminal FLAG-tags, were expressed heterologously in *B. subtilis* and the effect on P_{ydfG} activation was measured by β-galactosidase assays (Fig. 4.6 B). Exchange of INDQKGVL showed the strongest effect and resulted in about 3-fold higher activity compared to expression of the wild type protein. The other amino acid exchanges also led to alterations in promoter activity. The proteins with exchanges in WLPEP showed reduced and in DGGGK increased activity, NPDKL is comparable to the wild type version of $Ecf41_{Bli}$. Expression of all these $Ecf41_{Bli}$-FLAG variants was verified by Western Blot analysis using a FLAG-tag specific antibody (data not shown).

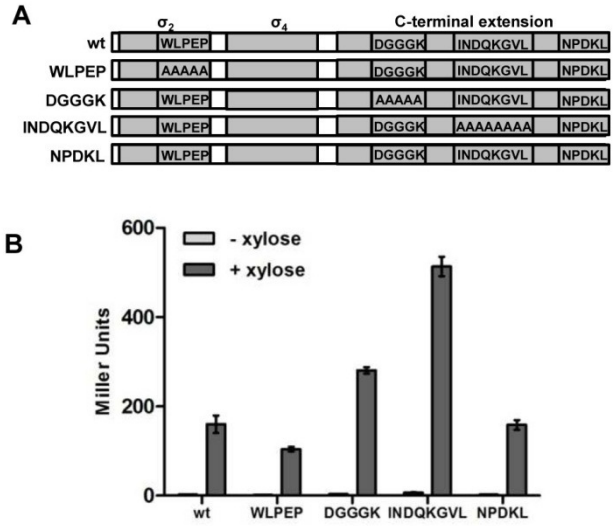

Figure 4.6. Influence of highly conserved residues of Ecf41$_{Bli}$ on target promoter activation. (A) Schematic representation of cloned Ecf41$_{Bli}$ alleles with amino acid exchanges. The name of each variant is given at the beginning of each line. The domains σ_2 and σ_4 as well as the C-terminal extension are displayed as grey boxes. The highly conserved motives and the exchange of amino acids against alanine residues are shown. (B) β-galactosidase activities of *B. subtilis* strains overexpressing the Ecf41$_{Bli}$-FLAG variants. Strains TMB696 (wt), TMB795 (WLPEP), TMB797 (DGGGK), TMB1016 (INDQKGVL) and TMB793 (NPDKL) were grown in LB medium to OD_{600} ~0.4 and split into two samples. In one sample, protein expression was induced by addition of 0.5 % xylose. The cells were harvested after 1 h and β-galactosidase activity was measured as described. Expression of each allele was verified by Western Blot analysis using a FLAG-tag specific antibody.

Sequential deletion analysis of the C-terminal extension. Next, we investigated variants of Ecf41$_{Bli}$ lacking increasing parts of the C-terminal extension. We constructed five C-terminally truncated alleles of *ecf*41$_{Bli}$-FLAG and tested their ability to activate the target promoter P_{ydfG}. The different mutant proteins were named according to their length (Fig. 4.7 A). Truncation of only 15 C-terminal amino acids of Ecf41$_{Bli}$ (270) resulted in a 4-fold higher activity compared to the wild type protein (Fig. 4.7 B). Further truncations (234 and 204) led to even higher activity up to a ~20-fold increase relative to the full-length protein. In contrast, expression of variant 167, which lacks the whole extension, completely lost the ability to activate the target promoter. This was unexpected, since regions σ_2 and σ_4 are usually sufficient for promoter recognition and activation by other ECF σ factors. This indicates that at least N-terminal parts of the extension are required for ECF41-dependent promoter activation, although partly truncations lead to a highly active protein. Expression of all of these Ecf41$_{Bli}$-FLAG variants was verified by Western Blot (Fig. 4.8 B and data not shown).

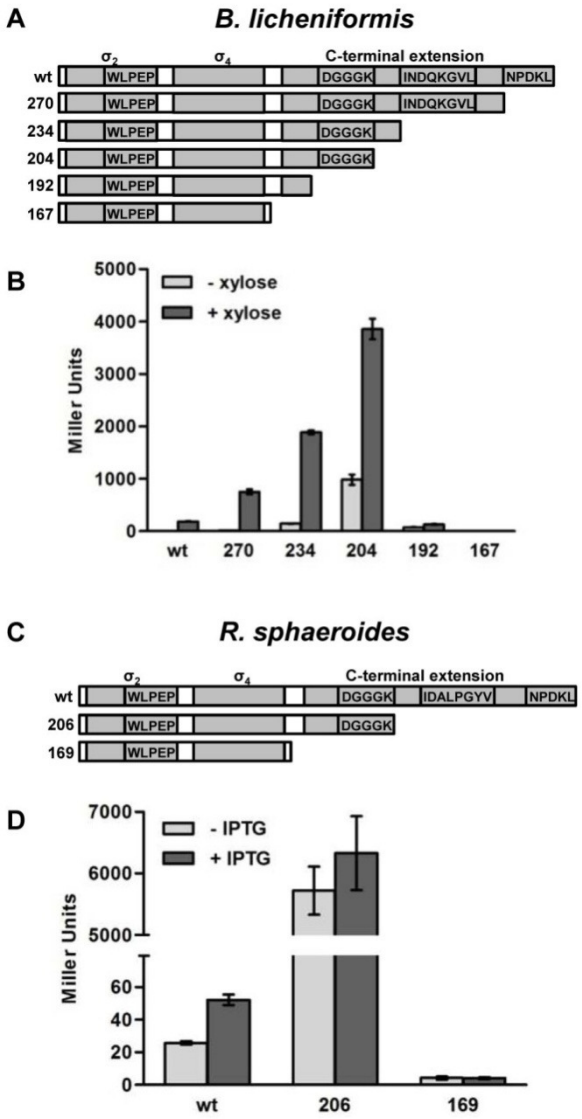

Figure 4.7. Effect of C-terminal truncations of ECF41 σ factors on target promoter activation. (A) Schematic representation of C-terminally truncated $Ecf41_{Bli}$ proteins of *B. licheniformis*. Details are shown as described for Fig. 4.6. The proteins are named according to their length (B) β-galactosidase activities of *B. subtilis* strains overexpressing truncated $Ecf41_{Bli}$-FLAG proteins. Strains TMB696 (wt), TMB744 (270), TMB743 (234), TMB742 (204), TMB741 (192) and TMB667 (167) were grown in LB medium to OD_{600} ~0.4 and split into two samples. In one sample, protein expression was induced by addition of 0.5 % xylose. The cells were harvested after 1 h and β-galactosidase activity was measured. (C) Schematic representation of C-terminally truncated $Ecf41_{Rsp}$ proteins of *R. sphaeroides*. Details are shown as described for Fig. 4.6. (D) β-galactosidase activities of *R. sphaeroides* strains overexpressing truncated $Ecf41_{Rsp}$ proteins. Strains TMR004 (wt), TMR005 (169) and TMR006 (206) were grown in Sistrom's minimal medium to OD_{600} ~0.4 and split into two samples. In one sample, expression of $Ecf41_{Rsp}$ variants was induced by 100 µM IPTG. After 3 hours, the cells were harvested and β-galactosidase assays were performed.

To test if similar results can be observed for $Ecf41_{Rsp}$, we tested the effects of C-terminal truncations on σ factor function in *R. sphaeroides* (Fig. 4.7 C and D). Expression of the C-terminal truncated $Ecf41_{Rsp}$ (206) led to ~120-fold higher P_{RSP_0606} activity than the full-

length protein, whereas deletion of the whole extension (169) resulted in a total loss of promoter activation. Our collective data therefore strongly suggest that the C-terminal extension of group ECF41 proteins might represent a fused anti-σ domain, which is involved in controlling σ factor activity.

Interaction of Ecf41$_{Bli}$ with RNA polymerase. Bacterial σ factors form a complex with the RNAP core enzyme and recruit the resulting holoenzyme to the corresponding target promoters (Burgess & Anthony, 2001). To demonstrate that Ecf41$_{Bli}$ interacts with RNAP, we performed *in vivo* RNAP pull-down assays. Ecf41$_{Bli}$-FLAG was expressed from a xylose-inducible promoter in a *B. subtilis* strain carrying a His$_6$-tagged β'-subunit of RNAP. The His$_6$-tag was used for rapid purification of RNAP holoenzyme (Anthony *et al.*, 2000). The success of the purification was verified by the presence of bands on a Coomassie stained SDS-PAGE gel corresponding to the ββ' and α subunits (Fig. 4.8 A). Western Blot analysis with a FLAG-tag specific antibody shows that Ecf41$_{Bli}$-FLAG co-purifies with RNAP (Fig. 4.8 B). The same protein is not detectable in the elution samples of cells lacking the His$_6$-tag (data not shown) indicating that enrichment of Ecf41$_{Bli}$-FLAG from *B. licheniformis* is due to interaction with RNAP.

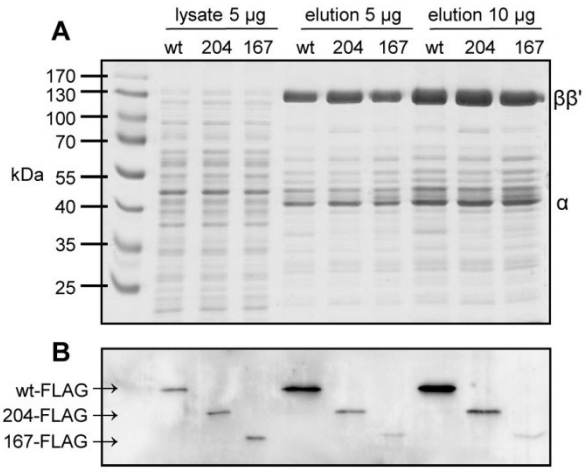

Figure 4.8. Interaction of Ecf41$_{Bli}$ with RNA polymerase. (A) SDS-PAGE of Ni affinity-purified proteins from strains TMB1099 (wt), TMB1100 (204) and TMB1101 (167) carrying a His$_6$-tag fused to the β' subunit of the RNAP. The different truncated versions of Ecf41$_{Bli}$-FLAG were overexpressed and the RNAP complex was purified (see Experimental procedures for details). 5 µg of the cleared lysate and 5 and 10 µg of the 100 mM imidazole elution fractions were loaded. (B) Detection of co-purified Ecf41$_{Bli}$-FLAG and its variants by Western Blot analysis of a gel identical to the one in (A) using a FLAG-tag specific antibody.

Next, we analyzed if the observed effect of $Ecf41_{Bli}$ truncations (Fig. 4.7 A and B) on target promoter activation can be explained by their ability to interact with RNAP core enzyme. We repeated the RNAP pull-down assay quantitatively with the highly active (204), the inactive (167) and the wild type version of $Ecf41_{Bli}$-FLAG. All three proteins are expressed at comparable levels in soluble form as demonstrated by Western Blot analysis of the cleared lysate before purification, but show considerable different binding behavior to RNAP (Fig. 4.8 B). Hardly any binding can be observed for the shortest $Ecf41_{Bli}$ protein (167), which is consistent with its inability to activate the target promoter (Fig. 4.7 B). In contrast, the protein with only partly truncated extension (204) co-purifies with RNAP to a much lesser extent than the full-length protein although promoter activation is significantly higher.

Altogether, C-terminal truncations of $Ecf41_{Bli}$ significantly alter the binding behavior to RNAP, but this affinity does not completely reflect the observed σ factor activity of the corresponding allele. While loss of promoter activation for the complete deletion of the C-terminal extension can be – at least partially – explained by the significantly reduced ability of the shortest version of $Ecf41_{Bli}$ (167) to interact with RNAP, additional factors must account for the strongly increased promoter activation of the partly truncated version (204), despite its weaker interaction with RNAP compared to the wild type protein.

Taken together, our data demonstrates that the ECF41 σ factors seem to have a completely new way of signal transduction presumably not involving a second protein functioning as an anti-σ factor. Instead, the C-terminal extension, which is not present in other ECF σ factors, affects both the target promoter activation and binding to RNAP (Fig. 4.7 and 4.8).

Discussion

A recent classification identified a large number of novel groups of ECF σ factors with unique features compared to "classical" ECF σ factors (Staroń *et al.*, 2009), including group ECF41. This group shows a wide phylogenetic distribution with about 400 proteins from ten different phyla (Table 4.4, Table S1 and Fig. 4.1). The genomic context of group ECF41 is highly conserved and distinct from other ECF groups. An obvious anti-σ factor is missing. Instead, a gene encoding a CMD protein, an oxidoreductase or an epimerase (COE) is located directly up- or downstream of the ECF41 σ factor (Fig. 4.2). We did not

observe any function of the COE proteins in signal transduction, but identified the neighbouring genes encoding these proteins as the sole targets of ECF41 σ factors, both by *in silico* and comprehensive gene expression analyses. We identified a unique promoter signature (TGTCACA-n_{16}-CGTC) upstream of these COE genes that is recognized by the corresponding ECF41 σ factor (Fig. 4.4).

The most important finding of our study concerns the regulatory role of the C-terminal extension of group ECF41, which is not present in any other group of ECF σ factors (Staroń *et al.*, 2009). Based on our data, ECF41-dependent signal transduction does not seem to involve a second protein that functions as an anti-σ factor. Instead, our data clearly demonstrate the importance of the C-terminal extension for both target promoter activation and affinity to RNAP (Fig. 4.7 and 4.8). Moreover, our sequential deletion analysis indicates that the extension plays both a positive and negative role in ECF41-dependent gene regulation. A short N-terminal part of the extension directly following the region σ_4 is absolutely required for σ factor activity, in contrast to other ECF σ factors described so far. But most of the C-terminal part of the extension clearly plays a negative regulatory role: even partial deletions result in a strongly increased activity of the target promoters in both organisms studied (Fig. 4.7), suggesting that this part of the extension functions as a fused anti-σ domain. To our knowledge, this is the first report of an anti-σ factor-like domain being fused to its σ factor. Based on our results, we propose that the group of ECF41 σ factors represent a novel mechanism of ECF-dependent signal transduction.

While our data clearly establishe a regulatory role of the C-terminal extension for the activity of the ECF41 σ factors, the exact molecular mechanism will be the subject of further investigations. One possibility would be that the C-terminal extension functions as a sensory domain. In the absence of a suitable trigger, it could keep the σ factor domains inactive through intra- or intermolecular interactions. Presence of the input signal could then result in a conformational change that releases the σ factor domains from the inhibitory grip of the extension, thereby initiating transcription of the COE genes. Such a mechanism involving intramolecular interactions has for example been described for the primary σ factor of *E. coli*, σ^{70}. While this σ factor does not need to be activated, binding of free σ^{70} to DNA and efficient transcription initiation is inhibited by region 1.1, presumably by interaction with the σ_4 DNA-binding domain (Dombroski *et al.*, 1993a, Johnson & Dombroski, 1997). An inhibitory role of an N-terminal region has also been

shown for alternative σ factors such as *E. coli* σ^{32} (Dombroski *et al.*, 1993a). If a similar mechanism also applies to the C-terminal region of ECF41 σ factors needs to be investigated.

Alternatively, though maybe less likely, a stimulation sensed by the C-terminal anti-σ domain could also result in a conformational change that exposes a protease recognition site. After regulated cleavage, the truncated and thereby activated σ factor would then mediate transcription initiation. Alternative σ factors like σ^{K} and σ^{E}, that are involved in the sporulation process in *B. subtilis*, are known to be expressed as inactive precursors. Activation is achieved by regulated proteolytic processing of the N-terminus of these proteins (LaBell *et al.*, 1987, Lu *et al.*, 1990, Zhang *et al.*, 1998). But in addition to its inhibitory function, the role of the extension of ECF41 proteins seems to be more complex. Partial deletion of the C-terminal extension results in high activity, but at least the N-terminal part of the extension is also required for transcription. Since a complete deletion of the C-terminal extension seems to decrease the affinity of the ECF41 σ factor to RNAP (Fig. 4.8), the N-terminal part of the extension could be involved in stabilizing the complex of RNAP and σ factor. So far, we do not know any inducing conditions for ECF41-dependent gene expression. Hence, we could not investigate the influence of the C-terminal extension in signal transduction under natural conditions.

Moreover, a comprehensive phenotypic profiling of *R. sphaeroides* RSP_0606-*ecf*41_{Rsp} deletion and $Ecf41_{Rsp}$ overexpression strains using phenotype microarrays (Bochner, 2003) did not reveal any function related to ECF41 σ factors. Therefore, biochemical characterization of the COE proteins could help to shed some light on the physiological role of ECF41-dependent gene regulation.

Future studies will be necessary to unravel both the physiological role and the mechanistic details underlying ECF41-dependent signaling. But the data presented in this initial study clearly demonstrates the value of our ECF classification and can serve as blueprint for studying additional conserved and novel groups of ECF σ factors, with yet to be explored mechanisms of signal transduction and gene regulation.

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Chapter 5

Discussion

In their natural habitat, bacteria encounter a number of environmental threats including the presence of antibiotics. Because of its crucial function, a preferred target of antibiotics is the bacterial cell wall. Inhibition of its biosynthesis and severe loss of its integrity rapidly cause cell death. In order to survive in such menacing habitats, bacteria developed a number of signal transducing systems, which enable the sensing of and response to harmful compounds and conditions. In the Gram-positive model organism *B. subtilis* the response to cell wall active antibiotics is well-known and orchestrated by two signal transducing principles: TCSs and ECF σ factors.

This thesis aimed to investigate the response to antimicrobial compounds with a focus on the role of ECF σ factors. In chapter 2 we investigated and compared the response of *B. subtilis* to daptomycin and friulimicin B, two structurally similar and clinically relevant antibiotics, by in-depth expression profiling on both the transcriptome and proteome level. Chapter 3 presented the transcriptional response of *B. subtilis* to rhamnolipids, which are industrially important biosurfactants showing also antimicrobial activity. Finally, in chapter 4 we characterized a novel group of ECF σ factors (ECF41) including a comprehensive bioinformatics analysis and experimental investigations in both *B. licheniformis* and *R. sphaeroides*.

The following discussion is divided into two parts: In the first part (section 5.1), the impact of genome-wide expression profiling on antibiotic research will be reviewed. In the second part (section 5.2 to 5.5), the results presented in chapters 2 to 4 of this thesis will be discussed in detail and put into the context of recent findings and the available literature.

5.1

Antibiotic research in the age of omics – from expression profiles to interspecies communication

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Antibiotic research in the age of omics: from expression profiles to interspecies communication

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The 'age of omics' has revolutionized our way of studying microbial physiology by introducing global analysis tools such as comparative genomics and global expression techniques including DNA microarrays (transcriptomics) and two-dimensional protein gel electrophoresis (proteomics). From the very beginning, such approaches have also been incorporated into the portfolio of antibiotic research. Genome mining has been used to explore the hidden biosynthetic potential in sequenced bacterial chromosomes, but also to search for novel antibiotic targets. Moreover, numerous studies investigating changes in expression patterns in response to antibiotic presence at the level of both the transcriptome and proteome have been performed over the years, which have helped us gain a deeper understanding of antimicrobial action. This review will focus on the impact that applying global expression studies has had on antibiotic research in the last decade. Signatures of differential gene expression in response to antibiotics have led to a deeper understanding of bacterial resistance mechanisms as well as stress response networks. They have also helped to predict the mechanism of action of novel antimicrobial compounds or to identify potential antibiotic-specific biosensors. Moreover, such studies have revealed novel inhibitory mechanisms of seemingly well-known drugs that might be useful for the development of co-drugs for antibiotic therapy and have identified the potential role of antibiotics as mediators of intercellular communication.

Keywords: antibacterials, transcriptomics, proteomics, expression profiling, DNA microarray

Introduction

The dawning of the genomic era has fostered high hopes in the field of antibiotic discovery. After the disillusionment of the failure of high-throughput screenings of compound libraries had settled, scientists eagerly leaped at this new potential 'magic bullet' of antibiotic discovery. Academic researchers, small biotech start-ups and the major pharmaceutical companies in unison praised the potential of genomic information and genome-wide expression tools, such as DNA microarrays and proteomics, as an almost fail-proof bet in identifying and characterizing novel antimicrobial targets and compounds. In the years 1998–2002 alone, more than 30 review articles were devoted to this subject, with only just about as many finished microbial genomes and hardly any global expression studies available to back up the hopes and wishes. It was a time of general euphoria of what the 'age of omics' might bring.

Now, almost 10 years later, with close to 2000 microbial genomes sequenced, and a similar number of published transcriptome studies, the use of genomic information and techniques for the development and study of new antimicrobial compounds is still going strong. But many of the recent overview articles are much more cautious in what they promise[1–3] or even announce the renaissance of classical approaches for drug discovery.[4] With the first decade closing in, this seems to be a good time to look back on what we have learned from applying omics tools to the discovery and analysis of antimicrobial compounds. Have any novel antimicrobial activities been discovered by these new tools? Did the powerful global expression techniques help to unravel or predict the mechanism of action (MOA)? Could this knowledge, if gained, be used to develop reliable high-throughput screens to narrow down the site of action for the next generation of compounds? Which of the old hopes were justified and what promises were fulfilled in these 10 years? And where do we go from here?

Three core aspects of genomics were always linked to supporting the development of new antibiotics: (i) identification of new drug targets by comparative genomics; (ii) exploitation of the biosynthetic potential hidden in microbial genomes by genome mining; and (iii) study of drug action based on genome-wide expression profiling.

Identification of novel drug targets by comparative genomics

High hopes were initially raised that the powerful new tools of genome mining and comparative genomics would pave the

way to identifying new targets for drug development.[5–7] Significant progress has been made in developing methods and databases for the prediction and identification of potential drug targets *in silico*,[8–11] but little has yet emerged from these efforts. One obstacle to this process might be the restrictive definition of good antibiotic targets as products of conserved essential genes,[2] which are at the core of most current bioinformatical target identification pipelines. Moreover, many essential functions are not encoded by a single essential gene,[12] thereby hampering their identification, since it is not easy to recognize genomic redundancy by sequence-based approaches alone. Nevertheless, promising results have recently been reported describing the identification of potential new targets that could be used in drug screening programmes. One example is the ATP binding protein YjeE from *Haemophilus influenzae*, where its use in an affinity-based screen identified a novel group of antibacterial agents.[13] A comparable screen of a wide variety of bioactive compounds with known modes of action showed that expression of the orthologous protein in *Escherichia coli* was increased in the presence of fluoroquinolone antibiotics such as norfloxacin.[14]

Similar approaches raised hopes to identify new drugs for better treatment of *Mycobacterium tuberculosis* infections. A medium-throughput whole-cell assay with live mycobacteria revealed diarylquinolines as a new class of anti-tuberculosis drugs, with R207910 being the most active of these compounds.[15] Subsequent whole-genome sequencing identified mutations in the *atpE* gene, encoding the ATPase synthase subunit c, as being responsible for inter-strain differences in susceptibility. The low degree of sequence similarity between AtpE of mycobacteria and other bacteria as well as humans makes this protein a very promising target, and preliminary pharmacokinetic studies in humans appear encouraging. Another study used a whole-cell screen to identify pyrimidine–imidazole compounds that inhibit the growth of *Mycobacterium bovis* and *M. tuberculosis*.[16] Full-genome sequencing of spontaneous resistant mutants in combination with other MOA studies linked the biological activity of pyrimidine–imidazoles to glycerol metabolism. Unfortunately, these compounds did not display any antibacterial activity in an *in vivo* mouse model, since this metabolic pathway is not relevant for *M. tuberculosis* during infection of mice. Nevertheless, these data clearly demonstrate the impact of central metabolic pathways on drug efficacy. It is important to consider such connections in order to successfully develop screens for antibiotic discovery.

The power of such whole-cell screens can be further increased by combination with specific reporter gene assays, as has been demonstrated in a study on DNA gyrase inhibitors and other DNA damaging agents using *Pseudomonas aeruginosa* as a model bacterium.[17] A strain containing the luciferase operon fused to a promoter that responds to ciprofloxacin was used to screen a library of 2000 bioactive compounds and identified 13 compounds that inhibit DNA synthesis, although by different mechanisms.

The above cited studies clearly demonstrate the feasibility of screening compound libraries using either isolated targets or whole cells, although, to our knowledge, none of these approaches led to novel antibiotics in clinical development.

Genome mining to exploit the hidden antibiotic biosynthetic potential

In contrast to harnessing (comparative) genomics for the prediction of new drug targets, the discovery of novel antibiotic biosynthesis loci by genome mining is a promising and straightforward road to the identification of new compounds.[18–20] In recent years, appropriate software tools have been developed to identify biosynthetic gene clusters and predict the structure of the corresponding products.[21–23] To date, a number of antibiotic biosynthesis loci have been characterized by a combination of genome mining and subsequent functional characterization, including those for cyclic lipopeptide antibiotics from *P. aeruginosa* and *Bacillus amyloliquefaciens*,[24,25] polyketides from *B. amyloliquefaciens*[26] and polymyxin from *Paenibacillus polymyxa*.[27] Moreover, genome mining together with comparative metabolic profiling and comparative genomics also helped to identify an important class of chemical compounds that function as antibiotic biosynthesis inducers in actinobacteria.[28] This discovery might pave the way for the controlled induction of biosynthetic pathways, which is currently one of the major limitations for the biotechnological production of secondary metabolites, as most antibiotic biosynthetic pathways are not expressed under standard laboratory conditions.[19] Additional strategies to activate the expression of silent biosynthetic gene clusters include the optimization of growth conditions[29] or alterations of the transcription or translation machinery, such as spontaneous mutations in RNA polymerase or ribosomal protein S12 that activated production of antibacterial compounds in different *Streptomyces* species.[30] An altogether different and novel strategy applies synthetic biology tools to optimize drug production. Biosynthetic gene clusters identified by genome mining can be divided into modules that are often responsible for biosynthesis of a distinct moiety of the final compound. These modules can be modified and subsequently combined in a plug-and-play fashion using a host strain already pre-engineered for maximal expression of certain compounds as the biochemical production chassis.[31] In fact, it was possible to generate unnatural but functional trimodular polyketide synthetases by such a rearrangement process, which synthesized the expected products.[32]

Global expression studies of antibiotic action

This mini-review will primarily focus on this third impact of genomics on antibiotic research in recent years. Studying the global response of bacterial populations to the presence of antibiotics was among the very first applications of the DNA microarray technology in the early years of microbial transcriptomics.[33] An ever-increasing number of such studies have been published in the last 10 years, covering all classical antibiotic targets and a plethora of different compounds that were tested on a broad range of microorganisms (Table 1). At about the same time, in-depth proteome profiling of antibiotic action was also developed, albeit only for a few model organisms, especially *Bacillus subtilis*.[34–36]

While antibiotic induction experiments based on DNA microarrays are technically simple and easy to perform with high reproducibility between biological replicates, the comparability

Table 1. Global expression studies on genome-wide responses to antibiotic action[a]

Cellular target or antibiotic[b]	Organism[c]	Type[d]	Purpose of study, remarks[b]	Ref.
CELL WALL SYNTHESIS				
BAC, PEN, VAN	*B. licheniformis*	T	identification of the cell wall stress response network	41
VAN	*B. subtilis*	T	identification of the cell wall stress response network	38
BAC	*B. subtilis*	T	identification of the cell wall stress response network	39
β-lactams	*B. subtilis*	T	transcriptional signatures (AMX, LEX, CTX, FOX, OXA, PEN) for MOA predictions	55
CYC, FOF, RIS, VAN	*B. subtilis*	T	transcriptional signatures for MOA predictions	55
BAC, CYC, MET, VAN	*B. subtilis*	P	proteome signatures for MOA predictions	56
MET, OXA, VAN	*B. subtilis*	T	MOA prediction based on transcriptional profiles and conditional mutants	106
DAP	*B. subtilis*	T	identification of resistance determinants	49
DAP, FRI	*B. subtilis*	P/T	mRNA/protein signatures for MOA prediction of closely related compounds	62
END, BAC	*B. subtilis*	T	comparative transcriptional signature using a high-density tiling chip	107
NIS	*B. subtilis*	P/T	identification of resistance determinants	108
PLE	*B. subtilis*	T	transcriptional and signature and target identification	65
RAM, MOE	*B. subtilis*	T	characterization of regulatory systems orchestrating cell wall stress response	109
AMX	*C. difficile*	T	transcriptional signature	110
AMP	*E. coli*	T	transcriptional signature	111
AMP	*E. coli*	T	transcriptional signature	112
AMD, CSD	*E. coli*	T	characterization of the cell wall stress response network	113
VAN	*E. faecalis*	P	comparison of the proteome signature of a VAN-resistant strain and a clinical isolate	47
Lcn972	*L. lactis*	T	characterization of a cell wall stress response system	114
EMB	*C. glutamicum*	T	MOA study on this anti-tuberculosis drug	115
EMB	*M. smegmatis*	P	proteome profile for compound comparison with ISO and 5-CPA	61
β-lactams, EMB	*M. tuberculosis*	T	transcriptional signatures for MOA predictions	54
EMB	*M. tuberculosis*	T	study on multidrug tolerance	51
VAN	*M. tuberculosis*	T	transcriptional signature	116
AMX	*P. multocida*	T	comparative transcriptional signature (together with ENR and TET)	60
IPM	*P. aeruginosa*	T	transcriptional signature in biofilms	117
CAZ	*P. aeruginosa*	T	effect of antibiotics on quorum sensing	118
BAC, CYC, OXA	*S. aureus*	T	characterization of cell wall stress response	46
VAN	*S. aureus*	T	characterization of cell wall stress response; comparison methicillin-susceptible *S. aureus*/vancomycin-intermediate *S. aureus*	42–45
FOX	*S. aureus*	T	impact on haemolytic activity and study of a cell wall stress response system	119
NIS, OXA, VAN	*S. aureus*	T	global signatures to compare cell wall stress and membrane depolarization	120
FOF	*S. aureus*	T	MOA studies on early-stage cell wall biosynthesis inhibitors	121
MER	*S. aureus*	T	transcriptional signature	122
FOF	*S. aureus*	T	transcriptional signature dependent on concentration and exposure time	123
VAN	*S. pneumoniae*	T	characterization of cell wall stress response	124
PEN	*S. pneumoniae*	T	characterization of cell wall stress response	125
CAS	*S. cerevisiae*	T	transcriptional signature	126
CAS	*C. albicans*	T	transcriptional signature	127
PEN	*P. chrysogenum*	T	effects of antibiotic biosynthesis	128
BAC, NIS	*S. pneumoniae*	T	comparison of the transcriptional signatures of three different AMPs	129

Continued

Table 1. *Continued*

Cellular target or antibiotic[b]	Organism[c]	Type[d]	Purpose of study, remarks[b]	Ref.
DNA TOPOLOGY				
diverse compounds	*B. subtilis*	T	transcriptional signatures (CIP, COU, MXF, NAL, NOR, NOV) for MOA predictions	55
NOV, CIP, NQO, MIT	*B. subtilis*	P	proteome signatures for MOA predictions	56
AZA, CIP, NAL	*B. subtilis*	T	MOA prediction based on transcriptional profiles and conditional mutants	106
DAU, ADM	*B. subtilis*	P	comparison of the proteome signatures of two functionally related drugs	130
NOR	*E. coli*	T	transcriptional signature	111
NOR	*E. coli*	T	transcriptional signature, induction of prophage genes	131
NOR	*E. coli*	T	gene expression response to and oxidative damage by DNA gyrase inhibition	67
OFX	*E. coli*	T	transcriptional signature	112
SIM	*E. coli*	T	transcriptional signature	132
NOV, LVX, OFX	*M. tuberculosis*	T	transcriptional signatures for MOA predictions	54
CIP	*M. tuberculosis*	T	transcriptional signature, induction of SOS response	68
ENR	*P. multocida*	T	comparative transcriptional signature (together with AMX and TET)	60
CIP	*P. aeruginosa*	T	transcriptional signatures, identification of fluoroquinolone resistance genes	52
CIP	*P. aeruginosa*	T	effect of antibiotics on quorum sensing	118
CIP	*S. pneumoniae*	T	transcriptional signature comparison between wild-type and CIP-resistant mutant	53
CIP, ENR	*S. enterica*	P	elucidate the cellular response and mechanism of resistance	133
ELB	*S. aureus*	P/T	proteomic and transcriptional signature	134
FATTY ACID SYNTHESIS				
CER, TCS	*B. subtilis*	T	transcriptional signatures for MOA predictions	55
CER	*B. subtilis*	P	proteome signatures for MOA predictions	56
PLC, PLS, CER, TCS	*B. subtilis*	P	proteomic signature for fatty acid biosynthesis inhibition	135
ISO	*M. smegmatis*	P	proteome profile for compound comparison with EMB and 5-CPA	61
ISO	*M. tuberculosis*	P	quantitative proteome signature to establish new technique	136
ISO, TLM, TCS	*M. tuberculosis*	T	transcriptional signatures to discriminate antibiotic responses	137
CER, ETH, ISO, TLM	*M. tuberculosis*	T	transcriptional signatures for MOA predictions	54
ISO, TIO, THL	*M. tuberculosis*	T	transcriptional signature	138
ISO	*M. tuberculosis*	T	transcriptional signature	33
ETH, ISO	*M. tuberculosis*	T	transcriptional signature	139
TCS	*S. aureus*	T	transcriptional signature	140
TCS	*S. enterica*	P	proteomic signature comparison of different strains, identification of TCS resistance determinants	141
FOLATE SYNTHESIS				
DAS, SAA, SMZ, TMP	*B. subtilis*	T	transcriptional signatures for MOA predictions	55
MEMBRANE BIOSYNTHESIS, INTEGRITY AND RESPIRATION				
diverse compounds	*B. subtilis*	T	transcriptional signatures (GRA, MON, NIG, NIT, POL, TRI) for MOA predictions	55
NIT, GRA, TRI, MON	*B. subtilis*	P	proteome signatures for MOA predictions	56
LL-37, PG-1, PLL	*B. subtilis*	T	signature of CAMP stress response and underlying regulatory network	40
DAP	*B. subtilis*	T	transcriptional signatures, identification of DAP resistance determinants	49
RHL	*B. subtilis*	T	transcriptional signature	142

Continued

Table 1. *Continued*

Cellular target or antibiotic[b]	Organism[c]	Type[d]	Purpose of study, remarks[b]	Ref.
MNZ	*C. difficile*	T	transcriptional signature	110
CEC	*E. coli*	T	transcriptional signatures for MOA prediction	143
ESC	*E. coli*	P	proteome signature and MOA studies	144
CCCP, NIG, VAL	*M. tuberculosis*	T	transcriptional signatures for MOA predictions	54
DAP, CCCP	*S. aureus*	T	global signatures to compare cell wall stress and membrane depolarization	120
TEM, OVI, DRS	*S. aureus*	T	transcriptional response to CAMPs	145
CHI	*S. simulans*	T	transcriptional profile as part of an MOA study	63
POL	*S. typhimurium*	P/T	proteomic and transcriptome study of virulence genes induction by CAMPs	146
AMB	*A. fumigatus*	P/T	proteomic and transcriptional signature	147
AMB, NYT	*S. cerevisiae*	T	transcriptional signature	148
AMB, KTC	*S. cerevisiae*	T	transcriptional signature	126
AMB, KTC	*C. albicans*	T	transcriptional signature	127
AMB, KTC	*T. rubrum*	T	transcriptional signature	149
LL-37	*S. pneumoniae*	T	comparison of the transcriptional signatures of three different AMPs	129
ENT	*B. cereus*	T	transcriptional signature	150
PROTEIN SYNTHESIS				
TET	*A. baumannii*	P	proteome signature of a multidrug-resistant strain	151
TET, IPM	*A. baumannii*	P	proteome signature of a multidrug-resistant strain	152
diverse compounds	*B. subtilis*	T	signatures (CLR, CLI, CHL, ERY, FUS, NEO, PUR, SPT, TET) for MOA predictions	55
diverse compounds	*B. subtilis*	P	signatures (ACT, CHL, ERY, FUS, GEN, KAN, MUP, PUR, STR, TET) for MOA predict.	56
ACT, AZM, CHL, GEN	*B. subtilis*	T	MOA prediction based on transcriptional profiles and conditional mutants	106
CHL, ERY, GEN	*B. subtilis*	T	transcriptional signature	153
CLI	*C. difficile*	T	transcriptional signature	110
ERY	*E. faecalis*	T	transcriptional signature	154
KAN	*E. coli*	T	transcriptional signature	111
diverse compounds	*E. coli*	P	proteome signatures (CHL, ERY, FUS, KAN, PUR, STR, TET) for MOA prediction	155
CTC	*E. coli*	P	proteome signature	156
CAP, ROX, STR, TET	*M. tuberculosis*	T	transcriptional signatures for MOA predictions	54
CAP	*M. tuberculosis*	T	transcriptional signatures for MOA determination	157
STR	*M. tuberculosis*	P	proteome signature and elucidation of resistance mechanism	158
STR	*M. tuberculosis*	P	proteome signature and comparison of susceptible and resistant isolates	48
TET	*P. multocida*	T	comparative transcriptional signature (together with AMX and ENR)	60
AZM	*P. aeruginosa*	T	effect of antibiotics on quorum sensing	118
AZM	*P. aeruginosa*	T	transcriptional signature and MOA prediction	159
CST	*P. aeruginosa*	T	transcriptional signature	160
FUS	*S. aureus*	T	characterization of FUS stimulon	161
TGC	*S. aureus*	T	transcriptional signature of biofilm-associated cells of methicillin-resistant *S. aureus*	162
ERY	*S. typhimurium*		global induction pattern, using a 6500-clone promoter-lux library	83
CHL, ERY, PUR, TET	*S. pneumoniae*	T	transcriptional signatures for MOA prediction	163
CHL	*T. maritima*	T	transcriptional signature	164
STR	*Y. pestis*	T	transcriptional signature	165
CHL	*Y. pestis*	T	transcriptional signature	166

Continued

Table 1. *Continued*

Cellular target or antibiotic[b]	Organism[c]	Type[d]	Purpose of study, remarks[b]	Ref.
TET	*P. falciparum*	T	study of antimalarial activity	167
DOX	*T. whipplei*	T	transcriptional signature	168
RNA SYNTHESIS				
RIF, 5FU	*B. subtilis*	P	proteome signatures for MOA predictions	56
RIF	*E. coli*	P/T	transcriptional/proteome signature	111,155
RIF, RFP	*M. tuberculosis*	T	transcriptional signatures for MOA predictions	54
RIF	*S. typhimurium*		global induction pattern, using a 6500-clone promoter-lux library	83
OTHERS				
FUR	*B. subtilis*	T	global induction pattern	169
MOI	*B. subtilis*	T	MOA prediction based on transcriptional profiles and conditional mutants	106
PAMP	*E. coli*	T	global induction pattern	170
TPP	*E. coli*	P	proteomics as part of an overall characterization of the cellular response	171
5-CPA	*M. smegmatis*	P	proteome profile for compound comparison with ISO and EMB	61
DER	*S. aureus*	T	analysis of bacterial resistance to anionic peptides	50
BBR	*S. aureus*	T	global induction pattern to initiate MOA studies	172
hBD3	*S. aureus*	T	global induction pattern as part of MOA studies	173
CRY	*S. aureus*	T	MOA prediction based on transcriptional profiling	174
RHO	*S. aureus*	P	proteome signature	175
FUR	*S. enterica*	T	transcriptional signature and inhibition of biofilm formation	176
SAF, QAD	*S. cerevisiae*	T	transcriptional signatures of two closely related compounds	177
5FC	*S. cerevisiae*	T	transcriptional signature	126
BBR	*S. flexneri*	T	transcriptional signature	178
5FC	*C. albicans*	T	global gene induction pattern of these antifungal drugs	127
GOM	*X. fastidiosa*	T	transcriptional signature and effect of biofilm formation	86

[a]The studies listed here are compiled from three independent PubMed searches performed in June 2011 using the search strings 'antibiotic AND DNA microarray', 'antibiotic AND transcriptome' and 'antibiotic AND proteomic'.
[b]Abbreviations: 5-CPA, 5-chloropyrazinamide; 5FC, flucytosine; 5FU, 5-fluoro-uracil; ACT, actinonin; ADM, adriamycin; AMB, amphotericin B; AMD, amdinocillin; AMX, amoxicillin; AMP, ampicillin; AMPs, antimicrobial peptides; AZA, azaserine; AZM, azithromycin; BAC, bacitracin; BBR, berberin chloride; CAMP, cationic antimicrobial peptide; CAP, capreomycin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CEC, cecropin A; CAZ, ceftazidime; CER, cerulenin; LEX, cefalexin; CTC, chlortetracycline; CTX, cefotaxime; FOX, cefoxitin; CAS, caspofungin; CHI, chitosan; CIP, ciprofloxacin; CLR, clarithromycin; CLI, clindamycin; CHL, chloramphenicol; CST, colistin; COU, coumermycin A1; CRY, cryptotanshinone; CSD, cefsulodin; CYC, D-cycloserine; DAP, daptomycin; DAS, dapsone; DAU, daunomycin; DER, dermcidin; DRS, dermaseptin; DOX, doxycycline; ELB, ELB-21; EMB, ethambutol; END, enduracidin; ENR, enrofloxacin; ENT, enterocin AS-48; ERY, erythromycin; ESC, esculentin-1b(1-18); ETH, ethionamide; FOF, fosfomycin; FRI, friulimicin B; FUR, furanones; FUS, fusidic acid; GEN, gentamicin; GOM, gomesin; GRA, gramicidin; IPM, imipenem; ISO, isoniazid; KAN, kanamycin; KTC, ketoconazole; LVX, levofloxacin; MER, mersacidin; MET, methicillin; MIT, mitomycin C; MNZ, metronidazole; MOE, moenomycin; MOI, moiramide B; MON, monensin; MXF, moxifloxacin; MUP, mupirocin; NAL, nalidixic acid; NEO, neomycin; NIG, nigericin; NIS, nisin; NIT, nitrofurantoin; NOR, norfloxacin; NOV, novobiocin; NQO, 4-nitroquinolone-1-oxide; NYT, nystatin; OFX, ofloxacin; OVI, ovispirin-1; OXA, oxacillin; PAMP, proline-rich antimicrobial peptide; PEN, penicillin G; PLC, platencin; PLE, plectasin; PLS, platensimycin; POL, polymyxin B; PUR, puromycin; RAM, ramoplanin; RHL, rhamnolipids; RHO, rhodomyrtone; RIF, rifampicin; RFP, rifapentine; RIS, ristocetin; ROX, roxithromycin; SAA, sulfacetamide; SAF, saframycin; SIM, simocyclinone; SMZ, sulfamethizole; SPT, spectinomycin; STR, streptomycin; TCS, triclosan; TEM, temporin L; TET, tetracycline; THL, tetrahydrolipstatin; TGC, tigecycline; TIO, tiocarlide; TLM, thiolactomycine; TMP, trimethoprim; TPP, tea polyphenols; TRI, Triton X-114; VAL, valinomycin; VAN, vancomycin.
[c]Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; *A. fumigatus*, *Aspergillus fumigatus*; *B. cereus*, *Bacillus cereus*; *B. licheniformis*, *Bacillus licheniformis*; *B. subtilis*, *Bacillus subtilis*; *C. albicans*, *Candida albicans*; *C. difficile*, *Clostridium difficile*; *C. glutamicum*; *Corynebacterium glutamicum*; *E. faecalis*, *Enterococcus faecalis*; *E. coli*, *Escherichia coli*; *L. lactis*, *Lactococcus lactis*; *M. smegmatis*, *Mycobacterium smegmatis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *P. multocida*, *Pasteurella multocida*; *P. chrysogenum*, *Penicillium chrysogenum*; *P. falciparum*, *Plasmodium falciparum*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. enterica*, *Salmonella enterica*; *S. typhimurium*, *Salmonella typhimurium*; *S. flexneri*, *Shigella flexneri*; *S. aureus*, *Staphylococcus aureus*; *S. simulans*, *Staphylococcus simulans*; *S. pneumoniae*, *Streptococcus pneumoniae*; *T. maritima*, *Thermatoga maritima*; *T. rubrum*, *Trichophyton rubrum*; *T. whipplei*, *Tropheryma whipplei*; *X. fastidiosa*, *Xylella fastidiosa*; *Y. pestis*, *Yersinia pestis*.
[d]T, transcriptome study; P, proteomic study.

between individual studies—even within the same organism—is unfortunately greatly hampered by the lack of a defined gold standard. Hence, a wide range of different experimental conditions was used by the individual research groups. Cultures were induced at different cell densities, using both complex and minimal media. The antibiotic concentrations varied greatly between individual studies, ranging from subinhibitory up to 10 times the MIC. The same is true for the induction time (i.e. the time between antibiotic addition and harvesting of the cells), which could be anywhere between 5 min and >1 h. The last aspect is additionally affected by the downstream protocol from cell harvest to lysis of the culture. While some groups took painstaking care that transcription was immediately stopped after a defined induction time, the protocol of other groups potentially allowed for an extra induction time during the downstream processing of cells. All of these parameters, of course, dramatically affect the overall expression signature. However, as a first estimate, subinhibitory concentrations and short induction times provoke very narrow, compound-specific responses, whereas higher antibiotic concentrations and longer induction times result in the detection of both non-specific and secondary effects of antibiotic action.

The motivation for these studies was as varied as the compounds tested. In many cases, the papers primarily describe the expression signature of an individual compound. These papers are somewhat characteristic for the early period of microarray studies, often offering the infamous long lists of gene names and induction values. While still being published regularly, restrictive editorial policies have either required substantial additional data or forced publication of such studies in note formats or low-impact journals. While these studies—as with any global expression profile—can always be a treasure chest for extracting information on genes of interest, they will not be further addressed in the context of this review article (but are comprehensively listed in Table 1). Instead, we will focus our attention on those studies that have resulted in a deeper understanding of some aspects of antimicrobial action.

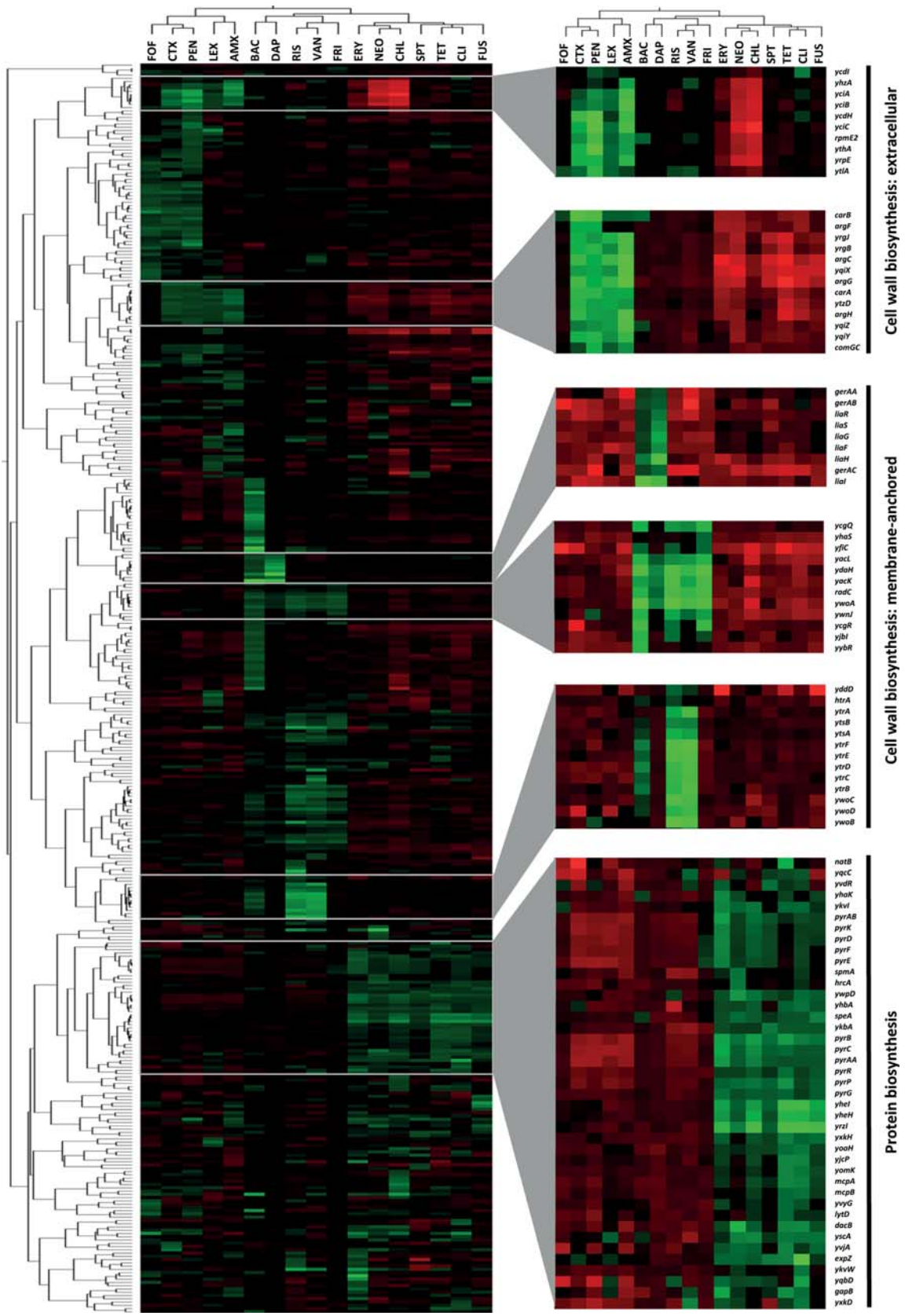
Resistance mechanisms and stress response networks

Some genome-wide antibiotic induction experiments were performed with the goal of unravelling resistance mechanisms and/or the underlying regulatory network that orchestrates antibiotic stress responses. This approach was especially successful in identifying and characterizing cell envelope stress responses and the corresponding signal transduction systems in many Gram-positive bacteria.[37] A number of independent studies in the Gram-positive model organism *B. subtilis* have revealed a complex regulatory network consisting of more than 10 partly overlapping signalling systems.[38–40] Based on these studies, a regulatory network of similar complexity was deconvoluted in the related organism *Bacillus licheniformis* by applying a combination of comparative transcriptomics and *in silico* regulon mining.[41] A set of studies in *Staphylococcus aureus* first allowed the identification of the cell envelope stress response in this human pathogen, and subsequently the in-depth profiling of antibiotic resistance traits in multiresistant strains as well as different vancomycin-resistant clinical isolates.[42–46] Similar studies, in which the proteome/transcriptome of susceptible and resistant strains or different clinical isolates were compared, have been performed for vancomycin-induced *Enterococcus faecalis*[47] and streptomycin-induced *M. tuberculosis*.[48] A correlation between the overall antibiotic induction profiles and the expression of resistance determinants was also investigated for a number of other compounds, such as daptomycin in *B. subtilis*,[49] dermcidin in *S. aureus*,[50] ethambutol in *M. tuberculosis*[51] or the fluoroquinolone antibiotic ciprofloxacin in *P. aeruginosa* and *Streptococcus pneumoniae*.[52,53]

Large-scale expression panels for MOA predictions and identification of suitable antibiotic biosensors

The motivation of many antibiotic induction experiments was the assumption that the MOA of a given compound is reflected by the function of the genes/proteins it induces. This hypothesis led to a number of comprehensive expression studies under standardized conditions in which large panels of antimicrobial compounds from different functional classes were compared in order to produce a gene/protein induction database for MOA prediction of novel compounds. The three landmark publications included two transcriptome panels in *B. subtilis* and *M. tuberculosis*[54,55] as well as a comprehensive proteome study, again in *B. subtilis*.[56] All three studies demonstrated that it was indeed possible to identify specific induction patterns for both individual and functional classes of antibiotics. These expression signatures could then be applied to narrow down the site of action of novel antimicrobial compounds that were also included in the panel.[54–56] The quantity and quality of the collected expression data are crucial for MOA prediction. For example, Hutter *et al.*[55] generated a comprehensive database containing genome-wide expression profiles of *B. subtilis* treated with 37 antibacterial compounds with known MOAs from six different classes. This dataset was tested with regard to its use as a reference for MOA prediction studies. The success of this strategy correlated with the number of expression profiles available for each class and worked exceptionally well for cell wall and protein biosynthesis inhibitors. A hierarchical clustering analysis of expression data from cells treated with antibiotics from these two MOA classes reveals completely different expression patterns (Figure 1). Antibiotics targeting protein biosynthesis all cluster together and, accordingly, induce a homogeneous set of genes that is not induced by any of the cell wall active compounds. In contrast, cell wall antibiotics fall into two distinct clusters that correlate well with the respective MOA. All β-lactams, which inhibit the last extracellular step of cell wall biosynthesis, form one distinct cluster, while the second cluster contains antibiotics interfering with earlier membrane-anchored steps of the biosynthetic pathway.

Comprehensive datasets of expression profiles also enabled the identification of marker genes for specific antibiotic classes. Several *B. subtilis* reporter strains, in which the promoter of the marker gene is fused to the luciferase reporter gene, have been generated and shown to respond to antibiotics that interfere with major biosynthetic pathways of bacterial cells (inhibition of fatty acid, protein, RNA, DNA or cell wall biosynthesis).[57,58] Expression of these marker genes can also be indicative for a



specific MOA. For example, the *ytrABCDEF* operon is strongly induced by glycopeptide antibiotics like vancomycin or ristocetin, but not (or only weakly) by other cell wall antibiotics (Figure 1). These so-called biosensors have been successfully used to predict or confirm the mode of action of poorly characterized agents.[59] Although this approach seems very promising, there are also clear limitations. Only compounds provoking a response similar to known induction profiles can be classified correctly, making identification of completely new targets impossible. Moreover, antibiotics induce gene expression only within a certain window of concentration, often impeding the use of biosensors in standardized high-throughput screenings. A low concentration of a compound might not be sufficient for induction of the biosensor, whereas a high concentration might be lethal for the cell. This could also be an explanation of why compounds with well-known MOAs do not always induce the corresponding biosensor.[58,59]

MOA studies on individual compounds

A side aspect of the studies described above is the direct comparison of individual compounds in order to define similarities and differences in the MOA based on the expression signature. Small panels often include a number of functionally diverse compounds, such as a transcriptome study in *Pasteurella multocida* using amoxicillin (cell wall biosynthesis), enrofloxacin (DNA gyrase) and tetracycline (translation elongation) stress responses.[60] A similar study at the proteome level was performed in *Mycobacterium smegmatis* and included the three anti-tuberculosis drugs ethambutol (cell envelope), isoniazid (fatty acid biosynthesis) and 5-chloropyrazinamide (unknown cellular target).[61] These studies mostly revealed a combination of characteristic compound-specific induction profiles (including target genes/proteins involved in the cellular process inhibited by the respective antibiotic) and antibiotic-independent (often overlapping) general stress responses.

Direct comparative expression studies have also been performed to discriminate the MOA of chemically closely related compounds, such as the lipo(depsi)peptide antibiotics daptomycin and friulimicin B.[62] Transcriptional profiling was also used in combination with physiological and biochemical studies for detailed MOA studies of novel compounds. Such analyses have been performed for chitosan,[63] the lipopeptide antibiotic friulimicin B[62,64] and the defensin plectasin.[65] Knowledge of the transcriptional response to novel compounds usually provides indirect but convincing evidence of the affected cellular pathway. This allows the performance of specific biochemical follow-up experiments to identify the exact target molecule and MOA, as demonstrated for the aforementioned compound friulimicin B.[62,64]

In addition to unravelling specific responses and elucidation of the MOA, transcriptional profiling sometimes also revealed important secondary effects of antimicrobial drug action. The σ^B-dependent general stress response is induced by the cell wall antibiotic vancomycin in both *Listeria monocytogenes*[66] and *B. subtilis*.[38] In the latter organism, σ^B is also activated by treatment with bacitracin.[39] Although both antibiotics target cell wall biosynthesis, they do not share a common MOA, suggesting that induction of σ^B occurs as a secondary response to a general loss in cell envelope integrity. DNA damage caused by quinolone antibiotics induces the SOS response, resulting in increased expression of enzymes involved in DNA repair, recombination and mutagenesis.[67–69] These observations might point towards a new direction of antimicrobial therapy, e.g. the development of co-drugs that target (and thereby suppress) protective functions embedded in such secondary responses, as will be discussed further below.

New inhibitory mechanisms of old drugs

The increasing and on-going emergence of resistant bacterial strains necessitates the development of new classes of antibiotics with completely novel MOAs. An overview of antibiotic discovery within the last 70 years together with an up-to-date survey of new antibiotics already in clinical trials and an outlook on future progress is nicely summarized in a recent review by Coates *et al*.[70] Development and marketing of analogues of already well-investigated antibiotics is less risky and financially more feasible than the identification of completely novel antibiotic classes. But in addition to putting effort into developing new antibiotics, it might also be a worthwhile endeavour to more closely investigate the effects of already existing and clinically used drugs, since global expression studies on antimicrobial action also helped to discover novel inhibitory mechanisms for seemingly well-known compounds.

Several studies have presented evidence that the interaction of an antibiotic with its specific target is not the only path leading to cell death.[67,71] Most importantly, it was demonstrated that several compounds induce the production of reactive oxygen species, which significantly contribute to the killing potential of bactericidal antibiotics. A microarray analysis of *E. coli* cells treated with the DNA gyrase inhibitor norfloxacin revealed not only induction of the SOS response, but also up-regulation of genes involved in the response to oxidative stress and iron-sulphur cluster biosynthesis.[67] Moreover, this study presented convincing evidence that norfloxacin treatment induces Fenton reaction-mediated formation of hydroxyl radicals, ultimately causing cell death. Even antibiotics of different classes (such as quinolones, β-lactams or aminoglycosides) that interact with different primary targets within the cell mediate killing by this common mechanism in both Gram-negative and Gram-positive bacteria.[71] These landmark studies indicate that reactive oxygen species represent a central aspect of the killing mechanism of many bactericidal antibiotics.

Figure 1. Hierarchical clustering analysis of genes differentially expressed in response to cell wall and protein biosynthesis inhibitors. The clustering analysis was performed using the software Cluster 3.0.[179] Transcriptome data for *B. subtilis* treated with fosfomycin (FOF), cefotaxime (CTX), penicillin G (PEN), cefalexin (LEX), amoxicillin (AMX), bacitracin (BAC), daptomycin (DAP), ristocetin (RIS), vancomycin (VAN), friulimicin (FRI), erythromycin (ERY), neomycin (NEO), chloramphenicol (CHL), spectinomycin (SPT), tetracycline (TET), clindamycin (CLI) and fusidic acid (FUS) were derived from published studies.[39,55,62] Green indicates induction of the corresponding gene and red indicates repression under the designated condition. Cluster analysis was limited to genes induced ≥5-fold by at least one antibiotic. Distinct clusters are highlighted and the corresponding gene names are given.

In contrast, bacteriostatic drugs did not provoke the generation of reactive oxygen species.

Development of co-drugs

The efficacy of a single antibiotic can often be enhanced by the administration of a second antimicrobial compound with complementary properties, i.e. a co-drug. A classic example of this concept is the combination of the sulphonamide antibiotic sulfamethoxazole and its potentiator trimethoprim. These two compounds inhibit different steps of the folate pathway and have a greater effect when applied together than given separately.[72,73] Another more recent example is the combination of the semisynthetic streptogramins quinupristin and dalfopristin.[74] These two antibiotics together are bactericidal because of synergistic effects; binding of one compound to the ribosome enhances the binding and efficacy of the second drug.[75,76]

During the last decade, the use of genome-wide expression profiling on antibiotic action has opened new doors to the identification and development of co-drugs. Non-essential proteins from secondary responses (e.g. those involved in counteracting oxidative stress and its resulting damage), which have thus far been ignored in the process of antibacterial drug discovery and development, could serve as possible new targets for co-drugs to potentiate the effect of already established antibiotics. The killing effect of antibiotics can also be increased by inhibition or deletion of systems counteracting cellular damage, such as the SOS response induced by quinolone and aminoglycoside antibiotics.[71,77] Synergism can also be achieved by a combination of antibiotics, in which cell envelope damage caused by one compound increases the uptake of a second drug.[78] A different approach using a combination of two different compounds has already been successfully applied to overcome β-lactam resistance.[79] Here, the most important resistance mechanism is the production of β-lactamases. This defence strategy can be overcome by the co-administration of inhibitors with high binding affinity and low hydrolysing rate, often being β-lactams themselves, which bind to the catalytic site, resulting in the inactivation of β-lactamases.[80,81]

Beyond antimicrobial action: antibiotics as signalling molecules and vice versa

As already mentioned previously, antibiotic induction experiments, especially when performed at subinhibitory concentrations, also revealed a whole new layer of antibiotic action that goes beyond mere growth inhibition. Antibiotics affect bacterial cells differentially depending on the concentration, a phenomenon also known as hormesis.[82] While growth inhibition occurs at high doses, low antibiotic concentrations have a stimulating effect and specifically modulate global gene expression. This phenomenon provokes questions about the role of antibiotics in nature, where the actual concentration rarely reaches inhibitory levels outside the direct vicinity of the producing strain. Is there a second function besides interspecies competition? The seminal collaborative study from the Davies's and Surette's labs already indicated the potential of antibiotics as a means of interspecies chemical communication, e.g. in the soil environment.[83] Expression of up to 5% of the *Salmonella typhimurium* genes can be positively or negatively affected by different antibiotics at concentrations well below the MIC, leading to no or only minor effects on growth. Many of the corresponding gene products are involved in transport processes, virulence or DNA repair. Moreover, similar studies also unravelled novel links between antibiotic stress responses and bacterial differentiation and intracellular communication. Even very complex phenotypes can be induced by antibiotic treatment at subinhibitory concentrations. Ciprofloxacin, tetracycline and tobramycin not only increased the expression of genes involved in differentiation in *P. aeruginosa*, but also actually induced biofilm formation.[84,85] The antimicrobial peptide gomesin also increased biofilm formation of the plant pathogen *Xylella fastidiosa*.[86] This is a crucial finding, since cells within a biofilm are generally more resistant to antibiotic treatment due to high cell density, slow growth and production of an extracellular matrix, resulting in antibiotic exclusion.[87] Therefore, it is a neat survival strategy for a bacterial population to already respond to very low antibiotic concentrations by inducing differentiation into a biofilm, thereby increasing antibiotic resistance before the compound concentration reaches a critical level.

The result of bacterial signalling mediated by antibiotics does not have to be related to resistance advantages. For example, antibiotics can fulfil important regulatory functions regarding their own biosynthesis or other cellular processes. This is especially true for antimicrobial peptides, such as lantibiotics,[88] bacteriocins[89] and microcins,[90] which have recently gained a lot of attention.[91] The production of lantibiotics from Gram-positive bacteria, such as nisin from *Lactococcus lactis* and subtilisin from *B. subtilis*, is regulated in a concentration-dependent manner[92] by a process called quorum sensing.[93] The biosynthesis of another antimicrobial molecule, the lipopeptide antibiotic surfactin, is connected to the quorum-sensing process responsible for competence development.[94] In this way, *B. subtilis* uses a single pathway to regulate two adaptive processes, antibiotic biosynthesis and DNA uptake. However, antibiotics can also negatively affect quorum sensing and subsequent differentiation. For example, subinhibitory concentrations of tobramycin significantly lower the accumulation of an autoinducer in *P. aeruginosa*, which subsequently impairs swarming motility, biofilm formation and pyocyanin production.[95] The previously mentioned studies clearly demonstrate that antibiotics are not only (or not even primarily) used for biological warfare, but also play an important role in bacterial signalling.

However, this link between cellular signalling and antimicrobial activities also works the other way around. Classical quorum-sensing autoinducers, such as *N*-acylhomoserine lactones used by Gram-negative bacteria, can also have antibacterial properties. One such molecule (3-oxo-*N*-acylhomoserine lactone) synthesized by *P. aeruginosa* exhibits activity against Gram-positive bacteria, but not against Gram-negative species.[96] Such studies suggest that bacteria not only use typical antibiotics to gain competitive advantages, but also molecules embedded in their natural life style. Moreover, they emphasize the concept that many, if not most, antibiotics seem to have more than one function. And growth inhibition might sometimes (maybe often?) be only a beneficial side effect in addition to the primary function in its natural habitat. From an ecological point of view, it is rather attractive to think of antibiotics as dual-use goods that

combine signalling and differentiation of the producer species with growth suppression of potential competitors for the same ecological niche.

Summary and outlook

This article highlights the most significant achievements of applying omics tools to antibiotic research. Clearly, some of the initial hopes were premature and could not be fulfilled, despite some promising advances, as has also been discussed in a recent article by Livermore.[97] Up to now, no new lead compound has emerged from these global approaches, and antibiotic research in recent years has been primarily characterized by the development of analogues of known compounds rather than the discovery of novel antibiotic classes.[98] But often the discovery of compounds with promising antimicrobial activities is not the major issue. It takes a lot of time and (financial) effort to develop and finally introduce a new compound into the market.[97] Considering the costs and challenges for the clinical development of a new antibiotic, as well as the short-term therapeutic use once it is on the market, it is often more profitable for pharmaceutical companies to invest in therapeutic areas other than anti-infectives.[99,100] Hence, a number of major pharmaceutical companies that initially embraced the promises and potential of omics approaches for antimicrobial research and development have given up on this strategy and left the field to smaller start-up companies. Nevertheless, global expression profiling studies have significantly deepened our understanding of antibiotic inhibition and uncovered unexpected new layers of antimicrobial action with regard to additional inhibitory effects of bactericidal drugs and the role of antibacterial compounds as signalling molecules for intra- and interspecies communication.

The development of new co-drugs seems to be a particularly promising approach for combating the ever-increasing threat of multiresistant human pathogens. Given the paucity of new antibiotic classes in the late stages of clinical development, regaining or even enhancing the inhibitory potential of well-established drugs might be an alternative route for improving clinical antimicrobial therapy.

While the initial hopes of what the 'age of omics' might bring for antibiotic discovery may not have been fulfilled (at least so far), global expression profiling approaches have definitely opened new doors for future antibacterial research. An especially powerful approach to combating the ever-increasing threat of microbial resistance, which has only become feasible in recent years with the advent of high-throughput sequencing strategies, is comparative whole-genome sequencing of antibiotic-susceptible versus -resistant strains, especially when combined with antibiotic-induced expression profiling panels of the same strains. Such information may be crucial to not only identifying resistance mechanisms, but to gaining insight into potential targets for new (co-)drugs. Likewise, comparative genomics of large numbers of pathogenic and closely related non-pathogenic strains might also be a promising strategy for the discovery of novel antibiotic targets.

Once a potential novel drug target with suitable features (e.g. essential functionality, conservation amongst bacteria and lack of a counterpart in mammalian cells) has been identified, the next challenge is the development of sensitive high-throughput screens of large compound libraries. Since the binding of a compound to a target does not always correlate with antibacterial activity, the use of whole cells instead of isolated targets for screening could directly select for compounds with beneficial pharmaceutical properties, such as the ability to penetrate bacterial cells or resist efflux. However, although a number of new targets have been identified and used in screening programmes, the resulting number of potential new antibacterial agents has been disappointingly small.[99] To increase the success of these high-throughput screens, the compound libraries should be expanded to include unconventional classes, chemically modified molecules, as well as natural products from new sources, such as secondary metabolites from marine microorganisms.[101]

So far, all antibiotics are derived from culturable organisms. However, as most microbes cannot currently be cultured under laboratory conditions,[102] it might be worthwhile to start exploring the biosynthetic potential of non-culturable bacteria by metagenomic approaches, a technique that has thus far been applied primarily to study the antibiotic resistance profile (the resistome) in a given habitat, such as the soil.[103] Large genomic DNA fragments isolated from complex habitats can be cloned into suitable vectors and the corresponding genes can be expressed and screened for antibacterial activity.[1,104,105]

It will be interesting to see how these new directions might influence the field of antibiotic research a decade from now. Most likely there will be new twists and turns, new hopes and ideas that we are currently not even aware of. Global expression profiling, even after having become an established tool (or because of this) will surely prove to be a valuable approach to fostering our knowledge of antibiotic action for many years to come.

Note added in proof

While this article was in proof, a comprehensive review was published by Romero *et al.* that beautifully summarizes our current state of knowledge on the role of antibiotics as signalling molecules (Romero D, Traxler MF, López D *et al.* Antibiotics as signal molecules. *Chem Rev* 2011; doi:10.1021/cr2000509).

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None to declare.

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5.2 Mechanism of action of daptomycin and friulimicin B

Analysis of differential gene expression upon antibiotic treatment is a powerful tool to gain information about the MOA of novel antimicrobial compounds, as has been discussed in the previous section. Therefore, we investigated changes in gene expression in response to two clinically relevant lipopeptide antibiotics, daptomycin and friulimicin B, on both the transcriptome and proteome level in *B. subtilis* (chapter 2, Table 1 and Fig. 2). Although these antibiotics are structurally similar, they provoke different expression profiles, as can be best demonstrated by a graphical representation of the microarray analysis presented in chapter 2 (Fig. 5.1).

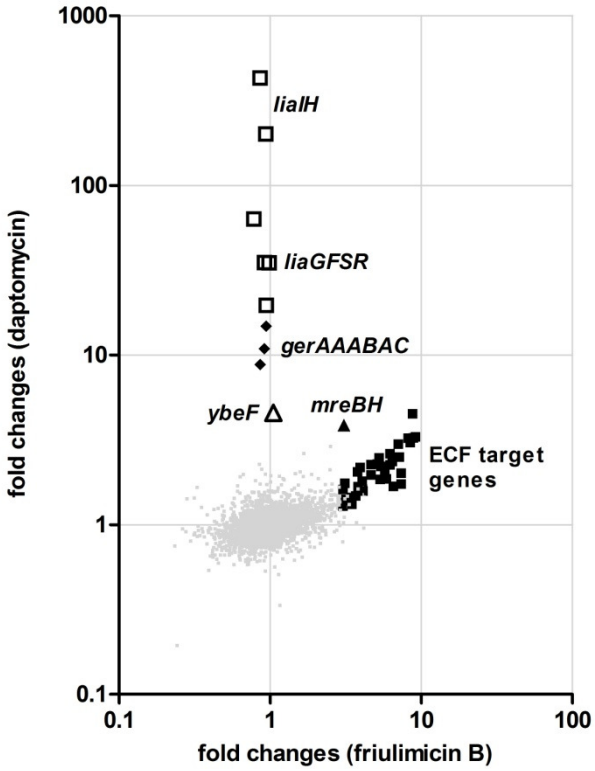

Figure 5.1. Graphical comparison of the daptomycin (y axis) and friulimicin B stimulon (x axis). Genes regulated by LiaRS (□) and ECF-dependent target genes (■) that were induced ≥ 3-fold by at least one of the antibiotics are highlighted. Additional marker genes are highlighted (Δ, ▲, ◆) and their names are given. Note that the induction of *gerAAABAC* is due to a known polar effect of the *liaI* promoter. All other genes are represented as small grey dots.

Both antibiotics induce numerous ECF-dependent target genes. Induction of this ECF response is much stronger for friulimicin B than for daptomycin. The only ECF-independent gene significantly induced by both antibiotics is *mreBH* encoding a cell shape-

determining protein. Daptomycin specifically and strongly activates the LiaRS TCS, resulting in increased expression of *liaIHGFSR* as well as read-through transcription of the downstream located *gerAAABAC* operon, which has also been observed previously after induction with other antibiotics (Mascher *et al.*, 2003). Another gene exclusively induced by daptomycin is *ybeF* encoding a small putative membrane protein. The striking differences in the transcriptional responses induced by daptomycin and friulimicin B, which were also confined by the corresponding proteome profiles, strongly suggest that these structurally similar antibiotics act via different MOAs. In the following sections, conclusions drawn from these expression profiles as well as recent studies regarding the MOA of daptomycin and friulimicin B will be discussed.

5.2.1 Daptomycin

The response to daptomycin has also been investigated by other research groups. In *S. aureus*, daptomycin induced a typical cell wall stress stimulon including genes related to the cell envelope or involved in different cellular processes, DNA metabolism or protein fate (Muthaiyan *et al.*, 2008). Such an expression profile is characteristic for cell wall-active antibiotics like oxacillin, bacitracin or D-cycloserine (Utaida *et al.*, 2003). A significant number of genes belonging to this cell wall stress stimulon are controlled by the TCS VraSR, which is homologous to the *B. subtilis* LiaRS TCS (Muthaiyan *et al.*, 2008).

An analysis of the transcriptional and physiological response to daptomycin in *B. subtilis*, the same organism we used, was also performed by Hachmann and colleagues (Hachmann *et al.*, 2009). In agreement with our results, the most strongly induced locus was the target operon of the LiaRS TCS followed by a number of ECF σ factor-dependent genes, primarily targets of σ^M. In addition, expression of *yvrI* and *yvrL* was also strongly induced. YvrI is part of an unusual two-subunit bacterial σ factor controlling expression of the *oxdC-yvrL* operon, encoding an oxalate decarboxylase and a regulatory protein, respectively (MacLellan *et al.*, 2009b, MacLellan *et al.*, 2008).

Moreover, genetic alterations within the *lia* locus as well as deletions of different ECF σ factors influence daptomycin resistance. Deletion mutants carrying resistance cassettes in genes like *liaIH* or *liaR*, in which the physiologically relevant genes of the *lia* locus are not present or inducible, respectively, show increased daptomycin susceptibility. In contrast, deletion of *liaF*, leading to constitutive expression of the *lia* locus, does not influence

resistance. A slight increase in susceptibility has also been observed for mutant strains of σ^M and σ^W. This phenotype can be further enhanced by combination of deletion mutants, either of both ECF σ factors or with *liaIH*/*liaR* (Hachmann *et al.*, 2009).

Although daptomycin has been clinically used for several years now, its exact MOA is still unknown. It is generally established that daptomycin affects membrane integrity by insertion and probably pore formation, but the steps leading to this final cause of cell death are still subject of discussion. It has been suggested that Ca^{2+}-bound daptomycin first inserts into the cytoplasmic membrane and then oligomerizes to form pores. Subsequently, this disrupts membrane integrity resulting in depolarization and leakage of ions, ultimately causing cell death (Silverman *et al.*, 2003). Another model proposed that daptomycin binds Ca^{2+} and forms loose micelles in solution. In close proximity to the membrane, these micelles dissociate and daptomycin inserts into the membrane, possibly followed by oligomerization, leakage and cell death (Straus & Hancock, 2006). A very recent study examined oligomerization of daptomycin both in solution and in association with membranes by fluorescence resonance energy transfer (FRET) analysis. It was demonstrated that daptomycin indeed forms oligomers associated with bacterial membrane vesicles, but not in solution at antimicrobially relevant concentrations. The conditions necessary for oligomerization resembled those required for antibacterial activity, suggesting that the oligomer represents the functional membrane lesion (Muraih *et al.*, 2011).

Oligomerization on membrane vesicles correlates with the presence of phosphatidylglycerol, an essential and major anionic component of bacterial membranes. It has been suggested that binding of daptomycin to the negatively charged phosphatidylglycerol is mediated by Ca^{2+} ions (Muraih *et al.*, 2011). A connection between the lipid composition of membranes and the antimicrobial activity of daptomycin has also been observed *in vivo*. A reduced level of phosphatidylglycerol, which is due to mutations in *pgsA* encoding the corresponding synthase, increases resistance of *B. subtilis* to daptomycin, while susceptibility to cell wall biosynthesis inhibitors like vancomycin is not affected (Hachmann *et al.*, 2009, Hachmann *et al.*, 2011). In contrast, deletion of *mprF*, which encodes the enzyme catalyzing formation of the positively charged lysyl-phosphatidylglycerol, results in increased sensitivity to daptomycin (Hachmann *et al.*, 2009). In addition, enhanced translocation of the cationic lysyl-phosphatidylglycerol to the

outer membrane leaflet, which thereby introduces additional positive charges into the membrane, correlates with a more resistant phenotype in *S. aureus* (Jones *et al.*, 2008). Conversely, mutations in *mprF*, which are usually expected to lead to decreased lysyl-phosphatidylglycerol content, occur early during resistance development in *S. aureus* (Friedman *et al.*, 2006). The exact consequences of these mutations, especially whether they are loss- or gain-of-function mutations, need to be investigated and compared to the effects of intended *mprF* alterations.

Besides changes in susceptibility, modifications in the membrane lipid composition also influence the binding behavior of daptomycin to the cell surface. Daptomycin is normally concentrated on newly formed division septa and in a helical pattern along the long axis of *B. subtilis* cells (Hachmann *et al.*, 2009), which is a pattern characteristic for anionic phospholipids (Barák *et al.*, 2008). Depletion of phosphatidylglycerol leads to an overall weaker binding of daptomycin and loss of the helical localization pattern (Hachmann *et al.*, 2009).

Investigation of resistance development and mechanisms can help to understand the MOA of antibiotics. A common feature of the resistance mechanisms mentioned above, either decreased levels of phosphatidylglycerol in *B. subtilis* or increased lysyl-phosphatidylglycerol synthesis and translocation in *S. aureus*, is reduction of the overall negative net charge of the cell envelope. These data suggest that the Ca^{2+}-daptomycin complex functions similar to cationic antimicrobial peptides. Composition and charge of the cell membrane seem to be important for the biological activity of Ca^{2+}-bound daptomycin. Presumably, it preferentially interacts with and inserts into membrane regions enriched in anionic lipids.

Another way to gain information about the MOA of antibiotics is the comparison of induction patterns to expression profiles provoked by well-known antimicrobial compounds. A list of studies providing such expression profiles as well as a discussion about the potential and limitations of such approaches has been presented in detail in section 5.1. Oligomerization and pore formation in bacterial membranes seem to be an important step in the MOA of daptomycin, but transcriptome studies suggest an additional mechanism involving the inhibition of cell wall biosynthesis. In *S. aureus* daptomycin induces a set of genes that is also induced by the proton ionophore carbonyl cyanide m-

chlorophenylhydrazone, which is in agreement with the membrane interfering properties of both compounds. But additional induction of a typical cell wall stress stimulon, including the VraSR TCS, resembles expression profiles provoked by cell wall antibiotics such as vancomycin, oxacillin, bacitracin and D-cycloserine (Muthaiyan *et al.*, 2008, Utaida *et al.*, 2003). The homologous LiaRS TCS, the main regulatory system responding to daptomycin treatment in *B. subtilis* (Chapter 2, Table 1 and (Hachmann *et al.*, 2009)), is usually strongly induced by cell wall active compounds like bacitracin, nisin, ramoplanin or vancomycin (Mascher *et al.*, 2004), which inhibit cell wall biosynthesis, especially by interfering with different steps of the lipid II cycle. These induction profiles suggest that daptomycin, in addition to membrane perforation, also interferes with cell wall biosynthesis. Since antibiotics with a similar induction profile preferentially block steps of the lipid II cycle, it seems likely that daptomycin also acts via such a mechanism. Nevertheless, an actual target within the cell wall biosynthesis pathway has not yet been identified (Schneider *et al.*, 2009).

5.2.2 Friulimicin B

Friulimicin B primarily provokes an ECF σ factor-dependent response, while the TCS LiaRS, which usually responds to cell wall biosynthesis inhibitors, is not induced at all (chapter 2, Table 1 and Fig. 5.1). This induction pattern suggests that friulimicin B generally interferes with cell envelope integrity rather than interacting with a specific target within the cell wall biosynthesis pathway. But a detailed biochemical MOA study revealed that friulimicin B inhibits cell wall biosynthesis (Schneider *et al.*, 2009). Friulimicin B does not affect membrane integrity but specifically forms a complex with the lipid carrier undecaprenol-monophosphate in a Ca^{2+}-dependent manner, thereby blocking formation of lipid I. The lipid carrier undecaprenol-monophosphate is also involved in transport of other cell envelope components such as teichoic acids, indicating that friulimicin B might block multiple pathways (Schneider *et al.*, 2009). Recently, the role of Ca^{2+} ions for the antimicrobial activity of friulimicin B has been investigated by a model membrane approach. It was demonstrated that friulimicin B interacts with membranes containing undecaprenol-monophosphate in the presence of Ca^{2+} ions (Reder-Christ *et al.*, 2011), thereby confirming the above stated MOA in which undecaprenol-monophosphate constitutes the target of friulimicin B (Schneider *et al.*, 2009). It has also been suggested

that Ca^{2+} ions form a bridge between the negatively charged friulimicin B and the phosphate moiety of the lipid carrier. Moreover, it has been postulated that Ca^{2+} ions also influence the secondary structure of friulimicin B, probably shifting the antibiotic into a conformation suitable for target binding (Reder-Christ *et al.*, 2011).

Although friulimicin B is structurally very similar to daptomycin, the data mentioned above clearly demonstrate that it acts via a completely different molecular MOA. The fact that first failures in treatment of *S. aureus* infections with daptomycin have already been reported underlines the urgent need for antibiotics with novel MOAs (Jones *et al.*, 2008). A common feature of cell wall antibiotics like vancomycin, ramoplanin or bacitracin, which interfere with the lipid II cycle, is induction of the LiaRS TCS in *B. subtilis* (Jordan *et al.*, 2008, Mascher *et al.*, 2004, Salzberg *et al.*, 2011). Although the lack of LiaRS induction by friulimicin B suggests a different MOA for this antibiotic, it nevertheless also interferes with the lipid II cycle, albeit by inhibition of a different step. Most cell wall-active antibiotics specifically interact with the lipid II complex, thereby inhibiting the later lipid-linked steps of cell wall biosynthesis (Breukink & de Kruijff, 2006, Schneider & Sahl, 2010). In contrast, friulimicin B prevents the first step of the lipid II cycle, the formation of lipid I, by binding to undecaprenol-monophosphate (Schneider *et al.*, 2009). The only antibiotic known that also interferes with this step is tunicamycin. Although both antibiotics prevent lipid I formation, their molecular MOA is completely different. Tunicamycin mimics UDP-activated sugars, which leads to a competitive and reversible inhibition of MraY, the enzyme catalyzing formation of lipid I (Bettinger & Young, 1975, Brandish *et al.*, 1996). In contrast, friulimicin B forms a tight complex with undecaprenol-monophosphate, which results in the depletion of one of the substrates necessary for lipid I formation, while the activity of MraY is not affected (Schneider *et al.*, 2009). To our knowledge, the response to tunicamycin has not been investigated on a genome-wide level. Nevertheless, it has been shown that the LiaRS TCS of *B. subtilis* responds to the presence of tunicamycin (Mascher *et al.*, 2004). Our data show that the LiaRS system is not induced by friulimicin B (chapter 2, Table 1 and Fig. 5.1), although both antibiotics cause inhibition of lipid I synthesis. These data suggest that friulimicin B has a completely novel and unique MOA. So far, there is no antibiotic on the market or even known that shares this MOA, making friulimicin B an exceptionally promising drug candidate for clinical use against Gram-positive bacteria including MRSA. Moreover, the lipid carrier undecaprenol-

monophosphate is a target that cannot be altered as easily as proteins or the D-Ala-D-Ala terminus of the pentapeptide of lipid II, the latter being a common mechanism for resistance against the last resort antibiotic vancomycin (Schneider *et al.*, 2009, Walsh *et al.*, 1996).

The data presented in chapter 2 and discussed above clearly show that global expression profiling is indeed a powerful and efficient tool to characterize novel antimicrobial compounds. As has been demonstrated for the lipopeptide antibiotics daptomycin and friulimicin B (chapter 2 and Fig. 5.1), even closely related compounds can induce a distinct set of genes which reflects completely different MOAs. Thereby, in-depth expression profiling can provide valuable hints regarding the MOA of novel compounds, which have to be validated by detailed biochemical MOA studies.

5.3 The stress response of *Bacillus subtilis* to rhamnolipids

Rhamnolipids are biosurfactants produced by the soil bacterium *P. aeruginosa*. In addition to their industrial importance, rhamnolipids also show antimicrobial activity, especially against Gram-positive species (Itoh *et al.*, 1971, Lang *et al.*, 1989). We investigated the transcriptome of *B. subtilis* after exposure to rhamnolipids by genome-wide DNA microarray analysis and further determined the response by hierarchical clustering analysis and phenotypic characterization (chapter 3). The most striking finding of this study was the simultaneous induction of two usually independent stress responses: the cell envelope stress response, including the TCS LiaRS and the ECF σ factor σ^M, and the CssRS-mediated secretion stress response.

Although rhamnolipids clearly display antimicrobial activity, their exact MOA is still unclear. In general, it has been suggested that they influence the permeability of biological membranes due to their properties as chemical detergents (Lang *et al.*, 1989). Indeed, rhamnolipids have been shown to alter surface hydrophobicity and increase membrane permeability followed by an elevated level of released extracellular proteins (Vasileva-Tonkova *et al.*, 2011). The toxic effects of rhamnolipids are more drastic for Gram-positive species, such as *B. subtilis*, than for Gram-negative bacteria. Therefore, the composition of the Gram-negative cell envelope, most likely the outer membrane, seems to have a protective function against this biosurfactant (Vasileva-Tonkova *et al.*, 2011).

Induction of the cell envelope stress response in *B. subtilis* (chapter 3, Table 3 and Fig. 2) correlates well with the proposed interference of rhamnolipids with cell membrane integrity. Such an expression pattern with strong induction of the LiaRS TCS and a (weak) ECF σ factor-dependent response has been also observed for the membrane interfering antibiotic daptomycin (Fig. 5.1 and (Hachmann *et al.*, 2009)) and cell wall biosynthesis inhibitors like bacitracin and vancomycin (Cao *et al.*, 2002b, Mascher *et al.*, 2003). Often, signal transducing systems detect a harmful compound and alter gene expression to protect the cell and ensure its survival. Such a role has been postulated for σ^W of *B. subtilis*, which controls expression of an antibiosis regulon providing protection against certain antibiotics (Butcher & Helmann, 2006). Induction of the LiaRS TCS does not always protect the cell against damage caused by the inducing compound. A protective role of the LiaRS TCS has been only demonstrated for a few antibiotics, for example daptomycin, and some oxidative stress generating agents (Hachmann *et al.*, 2009, Wolf *et al.*, 2010). While rhamnolipids strongly induce the LiaRS TCS (chapter 3, Table 3), our concentration-dependent lysis curve experiments showed that the effect on susceptibility is rather low. A Δ*liaF* mutant strain, in which the *lia* locus is strongly and constitutively expressed, is slightly more resistant against rhamnolipids. However, a Lia "OFF" strain, in which the RR LiaR is deleted, does not show any alterations in growth behavior after rhamnolipid treatment compared to the wild type (chapter 3, Fig. 3).

In contrast, ECF σ factors clearly contribute to resistance against rhamnolipids. Deletion of σ^M significantly increases sensitivity (chapter 3, Fig. 3), showing that this ECF σ factor and its target genes provide protection against cell damage caused by rhamnolipids. Moreover, combination of σ^M and σ^W deletions enhances the observed phenotype (chapter 3, Fig. 3). Such an effect based on the regulatory overlap of several ECF σ factors in *B. subtilis* has been observed before. While often hardly any phenotype can be discovered for single ECF σ factor mutant strains, combined deletions of multiple ECF σ factors revealed increased sensitivity against several antibiotics and detergents (Hachmann *et al.*, 2009, Mascher *et al.*, 2007).

In the case of the cell wall antibiotic daptomycin, susceptibility of *B. subtilis* can be further increased by simultaneous deletion of *liaIH* and one to three ECF σ factors (σ^M, σ^W and σ^X) (Hachmann *et al.*, 2009), thereby deleting the major systems orchestrating the cell envelope stress response in this organism (Jordan *et al.*, 2008). We tested if such an

additive effect can be also seen for rhamnolipids, but did not observe any differences in growth behavior after deleting LiaR in addition to σ^M and σ^W (chapter 3, Fig. 3). Altogether, these data show that the main protection against cell envelope damage caused by rhamnolipids comes from induction of ECF σ factors. For other cell wall antibiotics, such as bacitracin or vancomycin, a similar protective role has been also observed for ECF σ factors but not for the LiaRS system (Mascher *et al.*, 2007, Wolf *et al.*, 2010).

Our microarray analysis also revealed induction of genes not belonging to a typical cell envelope stress stimulon. Rhamnolipids caused increased expression of *htrA* and *htrB* (chapter 3, Table 3 and Fig. 1), both encoding membrane-bound serine proteases. Transcription of these two genes is controlled by the TCS CssRS, which is usually activated upon heat and secretion stress (Darmon *et al.*, 2002). Severe secretion stress can be caused by overexpression of extracellular proteins, such as the α-amylase AmyQ or the alkaline phosphatase PhoA (Darmon *et al.*, 2006, Hyyryläinen *et al.*, 2001). Induction of the CssRS TCS by rhamnolipids suggests that these compounds also cause some kind of secretion stress, possibly either by interfering with secretion machineries or protein folding. In *E. coli* accumulation of misfolded proteins within extracellular compartments induces the cell envelope stress response consisting of the TCSs BaeRS and CpxAR as well as the ECF σ factor σ^E (Raffa & Raivio, 2002, Ruiz & Silhavy, 2005). CssRS of *B. subtilis* constitutes a homolog of the CpxAR TCS of *E. coli* (Hyyryläinen *et al.*, 2001). Therefore, CssRS might represent a TCS that responds to similar effects of cell envelope interfering compounds that trigger the corresponding response in the Gram-negative *E. coli*. The TCSs of both organisms are induced by accumulation of misfolded secretory proteins and control expression of genes encoding extracellular chaperones or proteases, showing that both systems are, at least partially, functionally equivalent. It is possible that the elevated amount of released protein caused by rhamnolipids, which is possibly due to increased membrane permeability instead of enhanced protein secretion (Vasileva-Tonkova *et al.*, 2011), triggers the secretion stress response in *B. subtilis*.

Although the CssRS TCS is clearly induced by rhamnolipids (chapter 3, Table 3 and Fig. 1), we did not observe any differences in growth inhibition between a CssRS deletion and wild type strain (chapter 3, Fig. 3). These data demonstrate that the CssRS TCS does not confer resistance against the effects caused by rhamnolipids. Other studies have shown that CssRS is required to combat the severe effects of “real” secretion stress in the form of

overexpressed AmyQ (Hyyryläinen *et al.*, 2001). These findings together with our results suggest that secretion stress generated by rhamnolipids is not crucial for growth inhibition by this biosurfactant. Instead, it might be a consequence of increased membrane permeability caused by rhamnolipids, which might increase the level of extracellular proteins or impair functionality of secretion machineries and membrane-anchored proteins responsible for maintenance of the secretom.

Taken together, the stress stimulon provoked by rhamnolipids in *B. subtilis* suggests that the major MOA of rhamnolipids is indeed related to the membrane, as is demonstrated by induction of a typical cell envelope stress response. Nevertheless, there has to be a second impact leading to induction of the secretion stress response, which has not yet been observed for other cell envelope interfering antimicrobial compounds.

5.4 Characterization of the novel ECF41 σ factors

Typically, the activity of an ECF σ factor is controlled by direct protein-protein interaction with an anti-σ factor. Inactivation of this anti-σ factor results in the release and therefore activation of the ECF σ factor (Helmann, 2002). In addition to such classical and well investigated ECF σ factors, a comprehensive survey and subsequent classification of these proteins identified a number of novel groups with unique features and unknown signaling mechanisms (Staroń *et al.*, 2009). One of these groups is ECF41, whose detailed characterization is presented in chapter 4 of this thesis. Our analysis revealed that this group of ECF σ factors is widely distributed in bacteria. It consists of more than 400 proteins deriving from ten different phyla. The unusual genomic context of the ECF41 σ factors is highly conserved. It differs from the classical locus organization in the lack of genes encoding obvious anti-σ factors. Instead, a gene encoding either a carboxymuconolactone decarboxylase (CMD), an oxidoreductase or an epimerase is located in direct vicinity of the ECF41 genes. In contrast to often large regulons of other ECF σ factors, we found that ECF41 proteins regulate expression of only a single transcript, which is often comprised of the ECF41 σ factor and/or the neighboring gene mentioned above. This transcriptional unit is preceded by a distinct and highly conserved group-specific promoter motif, which is recognized by the corresponding ECF41 σ factor. Moreover, the ECF41 proteins carry a large C-terminal extension that is not present in

other ECF σ factors. We demonstrated experimentally in two different organisms that this extension is involved in regulation of σ factor activity. These results lead us to postulate that this extension functions as a fused anti-σ factor-like domain, thereby constituting a completely novel mechanism of ECF σ factor-dependent signal transduction (chapter 4).

5.4.1 Biological function of ECF41 σ factors and their targets

A good approach to assign a physiological function to an ECF σ factor is the analysis of its regulon. In general, the function of the target genes reflects the contribution of an ECF σ factor to counteracting specific stress situations, as has been shown for σ^R of *S. coelicolor*, which mediates a thiol-oxidative stress response (Paget *et al.*, 2001), or σ^W of *B. subtilis*, controlling an antibiosis regulon (Butcher & Helmann, 2006). Therefore, analysis of the function of ECF41-dependent target genes could provide some hints for the physiological role of this novel group of ECF σ factors.

The target gene of the ECF41 σ factor in both *B. licheniformis* and *R. sphaeroides* encodes a CMD protein. The prototypical function of these enzymes is degradation of aromatic compounds, which has been demonstrated for proteins from *Rhodococcus opacus* and *Bradyrhizobium japonicum* (Eulberg *et al.*, 1998, Lorite *et al.*, 1998). They catalyze the decarboxylation of γ-carboxymuconolactone to β-ketoadipate enol-lactone within the protocatechuate branch of the β-ketoadipate pathway. Importantly, all CMD proteins linked to ECF41 σ factors carry a highly conserved CxxC motif (Fig. 5.2), which distinguishes them from the classical γ-CMD proteins involved in metabolism of aromatic compounds.

An already characterized protein originally annotated as CMD protein, which carries such a CxxC motif, is MdrA from the archaeon *Methanosarcina acetivorans*. This protein is encoded in an operon with putative oxidative stress genes. It shows disulfide reductase activity and iron-sulfur cluster formation, both dependent on the CxxC motif. It has been suggested that MdrA plays a role in the oxidative stress response of this organism, possibly mediating repair of proteins containing disulfide bonds or iron-sulfur clusters, which were damaged by oxidative stress (Lessner & Ferry, 2007). The CMD protein family also includes alkylhydroperoxidases, with AhpD of *M. tuberculosis* being the best understood example. AhpD contains a CxxC motif crucial for its catalytic function. Besides alkylhydroperoxidase activity, AhpD serves as a reducing partner for the peroxiredoxin

AhpC. Together, these two proteins constitute important elements of the antioxidant defense system of *M. tuberculosis* (Hillas *et al.*, 2000, Koshkin *et al.*, 2003). Based on these two investigated examples, it can be proposed that the function of CMD proteins containing a conserved CxxC motif is counteracting oxidative stress.

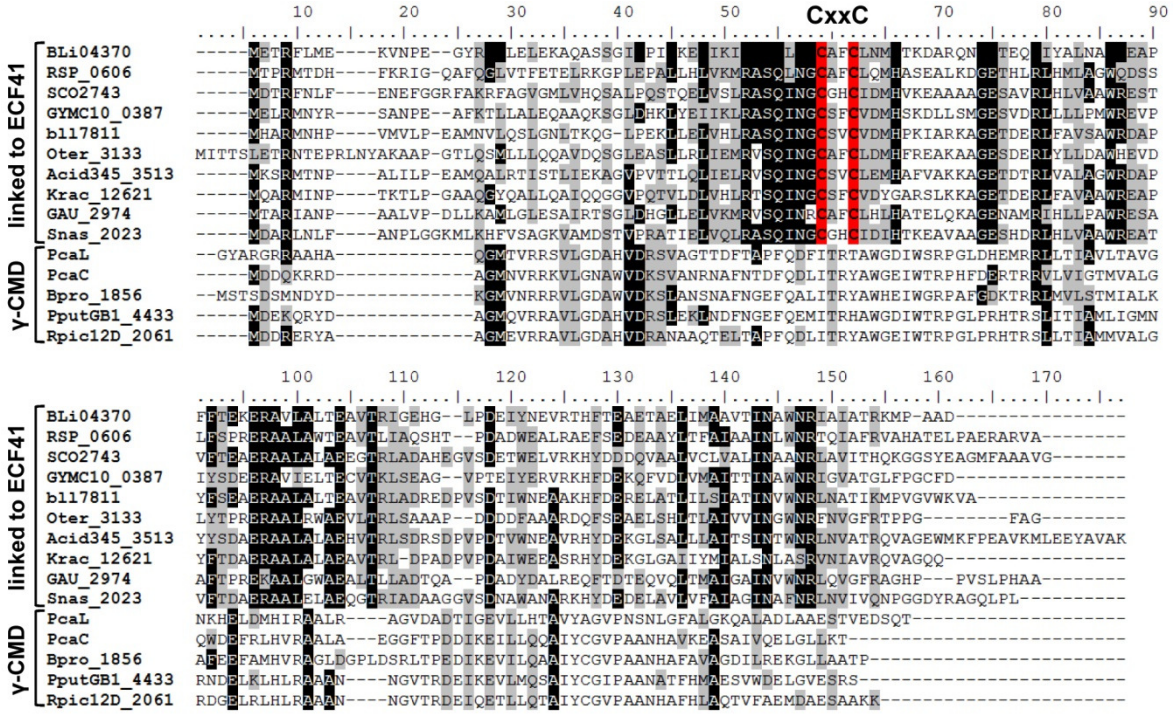

Figure 5.2. Multiple sequence alignment of selected CMD proteins. The alignment was constructed using ClustalW (Thompson *et al.*, 1994). Identical amino acids are shaded in black, similar amino acids in grey. Cysteine residues of the highly conserved CxxC motif are highlighted in red. The CMD proteins are grouped into proteins linked to ECF41 σ factors and γ-CMD proteins with a (putative) function in the protocatechuate metabolism. The name of the CMD proteins is given at the beginning of each line. They derive from the following organisms: BLi04370 (YdfG) from *B. licheniformis*, RSP_0606 from *R. sphaeroides*, SCO2743 from *S. coelicolor*, GYMC10_0387 from *Geobacillus* sp. Y412MC10, bll7811 from *B. japonicum*, Oter_3133 from *Opitutus terrae*, Acid345_3513 from *Koribacter versatilis*, Krac_12621 from *Ktedonobacter racemifer*, GAU_2974 from *Gemmatimonas aurantiaca*, Snas_2023 from *Stackebrandtia nassauensis*, PcaL from *R. opacus*, PcaC from *B. japonicum*, Bpro_1856 from *Polaromonas* sp. JSP666, PputGB1_4433 from *Pseudomonas putida* and Rpic12D_2061 from *Ralstonia pickettii*. Note that PcaL constitutes a fusion protein and only the CMD domain is included in the alignment.

Results from investigations of *M. tuberculosis* σ^J, the only ECF41 σ factor that has been studied in addition to $Ecf41_{Bli}$ and $Ecf41_{Rsp}$, also suggest a general role of the ECF41 σ factors in response to oxidative stress. The genome of *M. tuberculosis* harbors two ECF41-encoding genes, *sigJ* and *sigI*, which are both so-called "orphans" not associated with COE genes (chapter 4, Table S1). Only *sigI* is preceded by the typical ECF41-dependent promoter sequence, and it has been shown to be the only target recognized by σ^J

(Homerova *et al.*, 2008). This reveals a cascade in which one ECF41 σ factor activates transcription of another member of this group. Although a number of similar putative promoter sequences can be found in the genome of *M. tuberculosis*, several approaches failed to assign them as relevant targets of σ^I or σ^J (Homerova *et al.*, 2008, Rodrigue *et al.*, 2007). This supports our results showing that ECF41 σ factors, unlike other ECF σ factors, do not control expression of a large regulon (chapter 4). The only known phenotype associated with σ^J is a weakly increased sensitivity to H_2O_2 in a mutant strain (Hu *et al.*, 2004). However, we performed sensitivity tests and did not observe any differences in growth behavior of mutant and wild type strains of both *B. licheniformis* and *R. sphaeroides* in the presence of oxidative stress producing agents, including H_2O_2, cumene hydroperoxide, t-butyl hydroperoxide and paraquat (data not shown). Moreover, we performed a phenotype microarray analysis with *R. sphaeroides* wild type, $Ecf41_{Rsp}$ deletion and overexpression strains. Despite about 1200 different tested conditions, including the presence of oxidative stress agents, antimicrobial compounds and a wide range of metabolic substrates, this analysis did not reveal any reproducible phenotypes (supplementary material and data not shown). All the above mentioned approaches are based on growth behavior under stress conditions coupled with genetic alterations. Since they did not provide any hints for the function of the ECF41 group, biochemical characterization of the target gene products could shed some light on their physiological role.

Knowledge of the exact function of the COE gene products, which can be either CMD proteins, oxidoreductases or epimerases, would also allow drawing conclusions regarding the signal leading to activation of the ECF41 σ factors. In general, the COE proteins are predicted to catalyze redox reactions or chemical conversions. Therefore, instead of a compound causing oxidative or another kind of stress, the inducing stimulus could also be an alternative nutrient source, for whose metabolism the COE genes encode an important enzyme. The presence of a specific substrate might somehow be sensed by the ECF41 σ factor, which in turn triggers the expression of corresponding metabolic enzymes. This hypothesis is in good agreement with the observation that *ydfG*, which is the ECF41-dependent target gene encoding a CMD protein in *B. licheniformis*, is expressed at the transition from the exponential to the stationary growth phase (Fig. 4.4 B). In addition, an increase of the *sigJ* mRNA level in *M. tuberculosis* has been observed during stationary

phase (Hu & Coates, 2001). A characteristic feature of this growth phase is nutrient depletion. One important strategy for survival under these conditions is the use of alternative nutrients, including compounds that require unusual degradation pathways and specialized signal transducing systems controlling the expression of the corresponding enzymes dependent on substrate availability. The ECF41 σ factors and their target genes could be involved in regulation and accomplishment of such specialized degradative reactions.

Differentiation is another possibility to deal with the deteriorating conditions during stationary phase. Especially bacteria with very complex life cycles, such as some Actinobacteria that even show multicellular differentiation, often contain a particularly large number of signal transducing systems (Bentley *et al.*, 2002, Flärdh & Buttner, 2009). Based on the phylogenetic analysis of ECF41 proteins, these σ factors are especially abundant within the Actinobacteria (Table 4.4 and Fig. 4.1), which makes species from this phylum particularly interesting for further studies. Many of them, especially the *Streptomyces* species, contain multiple copies of the ECF41 gene in the genome (chapter 4, Table S1). This conspicuous abundance of ECF41 σ factors within one genome indicates an important function in the often elaborated lifestyles of these species. On the one hand, functional and regulatory redundancy has to be considered during experimental investigations when more than one ECF41 gene is present in a single genome. On the other hand, an important function of these transcriptional regulators, which is indicated by their high abundance, could simplify the search for a specific phenotype associated with ECF41 σ factors.

5.4.2 Regulatory role of the C-terminal extension in signal transduction

The most interesting feature of the ECF41 proteins is a large C-terminal extension, which is exclusively present in this group of ECF σ factors. Within this extension, we identified three highly conserved motifs (Fig. 4.3). To investigate a possible function of this extension in signal transduction, we determined the impact of mutations and truncations of this extension on promoter activation. Exchange of the conserved motifs of $Ecf41_{Bli}$ against alanine residues caused only minor alterations in target promoter activation (Fig. 4.6), whereas C-terminal truncations of the ECF41 proteins revealed more drastic effects (Fig. 4.7). Partial truncation of the extension results in strongly increased target promoter

activity. The most obvious effect can be observed for the partly truncated variant $Ecf41_{Bli}$ (204), which increased promoter activity ~20-fold relative to the full-length protein (Fig. 4.7). In contrast, loss of the whole extension (variant 167) completely abolished σ factor activity (Fig. 4.7). Moreover, these truncations also alter the affinity of $Ecf41_{Bli}$ to RNAP. The shortest variant (167) hardly co-purifies with RNAP, which is in good agreement with its inability to activate the target promoter. In contrast, the highly active variant (204) co-purifies with RNAP to a much lesser extent then the full-length protein (Fig. 4.8). These data demonstrate an important regulatory role of this extension, but the effects cannot be explained solely by altered affinity for RNAP. Considering that an obvious anti-σ factor is missing in the direct genomic context of the ECF41 genes, the extension could possibly constitute a fused anti-σ factor-like domain. But besides a mere inhibitory function, parts of the C-terminal extension are also required for ECF41-dependent transcription, since its entire deletion completely abolished σ factor activity (Fig. 4.7). Such a behavior is very unusual, because regions σ_2 and σ_4 alone are normally sufficient for transcription initiation by other ECF σ factors. This suggests that the signal transduction of ECF41 σ factors is based on a completely novel and so far unknown signal transducing cascade, which will be the subject of further investigations. At this time, it can be only speculated about the exact molecular mechanism of ECF41 σ factor activation.

A feasible approach to gain indications regarding a possible mechanism how the extension influences σ factor activity is comparison of its sequence and structure to other already investigated proteins. A BLAST search (Altschul *et al.*, 1990), which is based on sequence similarity alone, did not reveal any proteins other than ECF41 members. In order to identify structurally similar proteins, we first predicted the secondary structure of ECF41 σ factors using the secondary structure prediction server Jpred 3 (Cole *et al.*, 2008). The secondary structure predicted for $Ecf41_{Rsp}$ as well as an alignment of ECF41 proteins and classical ECF σ factors representative for the Jpred 3 analysis is shown in Fig. 5.3. The σ_2 and σ_4 domains consist of several α-helices as has already been demonstrated for σ^{70} proteins (Campbell *et al.*, 2002). The C-terminal extension is predicted to form both α-helices and β-sheets of different lengths (Fig. 5.3). The highly conserved motifs are mainly located in regions without a distinct secondary structure. Especially the WLPEP motif, whose amino acid sequence is exceptionally highly conserved, lies within an unstructured region connecting the σ_2 and σ_4 domains (Fig. 5.3).

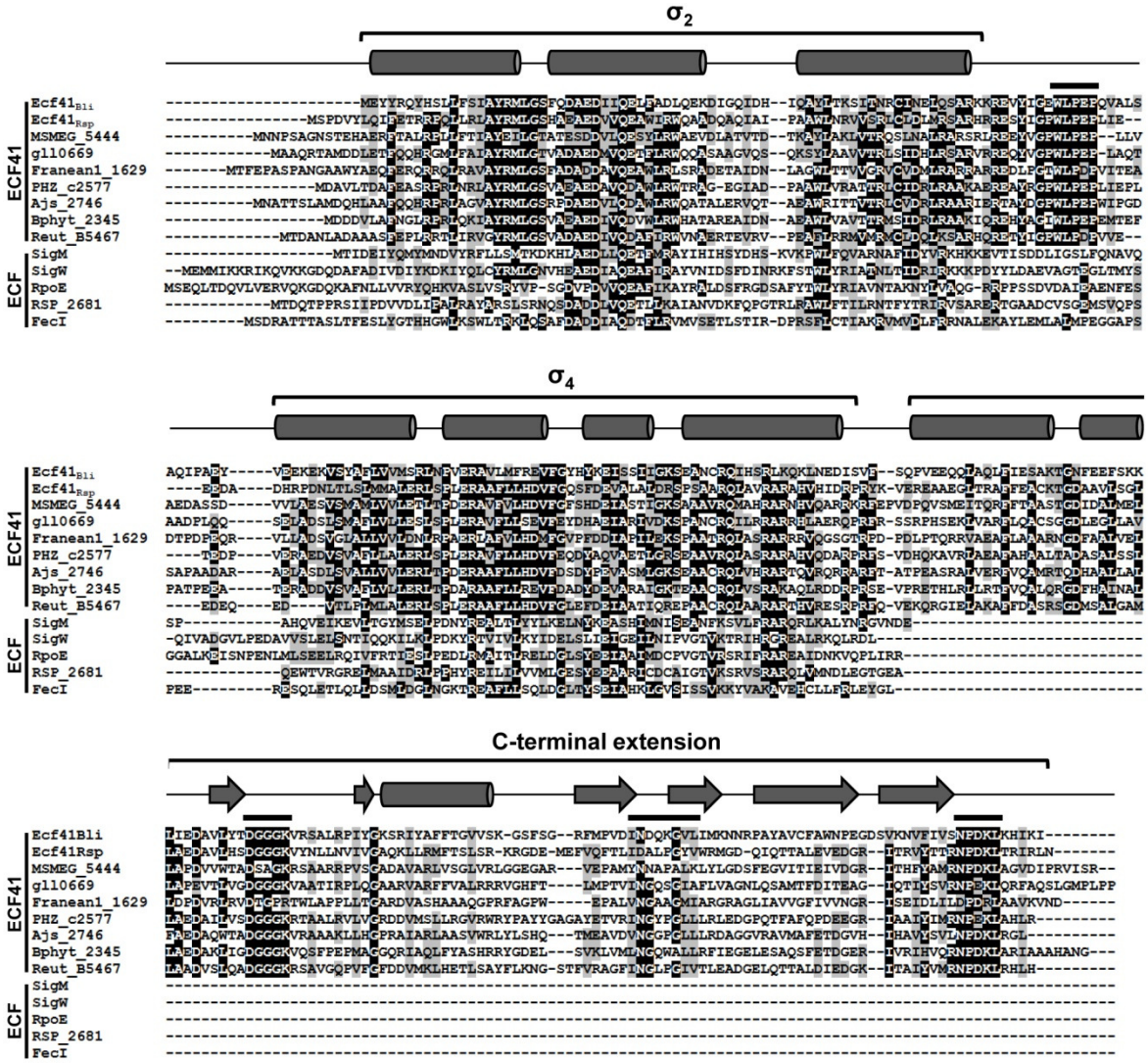

Figure 5.3. Predicted secondary structure of group ECF41 proteins. The multiple sequence alignment of selected ECF σ factors was constructed using ClustalW (Thompson *et al.*, 1994). Identical amino acids at the same position are shaded in black, similar amino acids in grey. The secondary structure for $Ecf41_{Rsp}$ was predicted using Jpred 3 (Cole *et al.*, 2008) available at http://www.compbio.dundee.ac.uk/www-jpred/. α-helices are shown as cylindrical forms, β-sheets as arrows. The σ_2 and σ_4 domains and the C-terminal extension are marked. Conserved motives of ECF41 σ factors are highlighted by black lines. The ECF σ factors derive from the following organisms: $Ecf41_{Bli}$ from *B. licheniformis*, $Ecf41_{Rsp}$ and RSP_2681 from *R. sphaeroides*, MSMEG_5444 from *Mycobacterium smegmatis*, gll0669 from *Gleobacter violaceus*, Franean1_1629 from *Frankia* sp. EAN1pec, PHZ_c2577 from *Phenylobacterium zucineum*, Ajs_2746 from *Acidovorax* sp. J42, Bphyt_2345 from *Burkholderia phytofirmans*, Reut_B5467 from *Ralstonia eutropha*, SigM and SigW from *B. subtilis* and RpoE and FecI from *E. coli*.

Based on this highly conserved secondary structure, we predicted a three-dimensional model (Fig. 5.4), which provides some interesting hints for possible signal transducing mechanisms and further investigations.

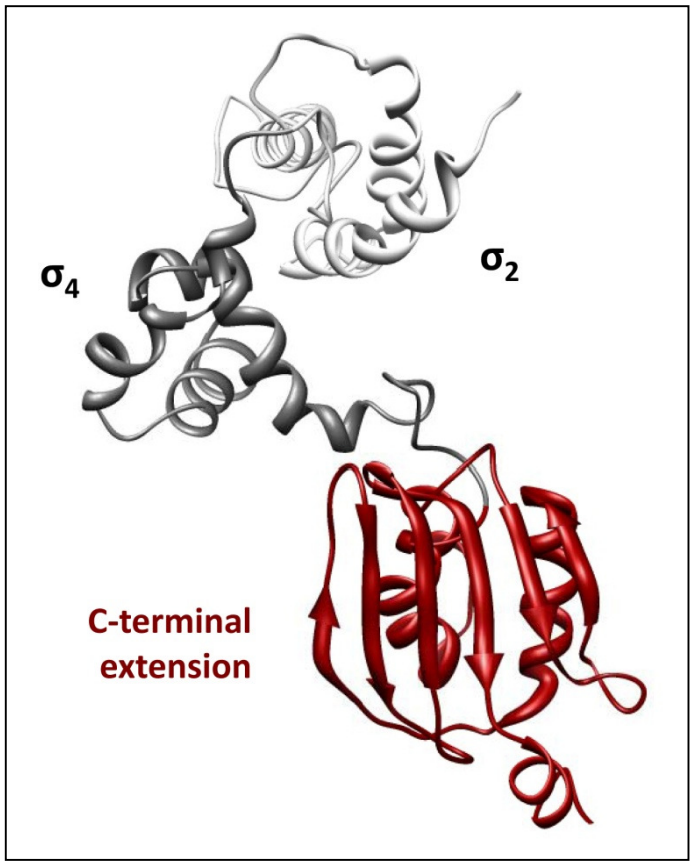

Figure 5.4. Hypothetical three-dimensional model of ECF41 σ factors. HHpred (Söding *et al.*, 2005) was used to search the PDB database (Berman *et al.*, 2000) for proteins with known structure similar to $Ecf41_{Rsp}$. The σ_2 and σ_4 domains were modeled on the already well-known structure of other ECF σ factors. The best hit for the C-terminal extension of $Ecf41_{Rsp}$ was limonene-1,2-epoxide hydrolase of *Rhodococcus erythropolis* with 15% similarity. The three-dimensional model was predicted with the program Modeller (Šali & Blundell, 1993) and graphically presented using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen *et al.*, 2004). The modeling was performed in collaboration with Gregor Witte.

The hypothetical three-dimensional model of ECF41 σ factors consists of three domains with a distinct fold connected by unstructured linker regions (Fig. 5.4). The σ_2 and σ_4 domains are modeled on the already well-known structure of other ECF σ factors. The modeling of the C-terminal extension of $Ecf41_{Rsp}$ from *R. sphaeroides* is based on the known structure of limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* (Arand *et al.*, 2003). Similarly, the same analysis performed for $Ecf41_{Bli}$ from *B. licheniformis* revealed similarity to a protein of unknown function from *Burkholderia pseudomallei* (data not shown), which carries a nuclear transport factor 2 (NTF2)-like domain, thereby showing structural similarity to mammalian NTF2 (Bullock *et al.*, 1996). While these two proteins are not functionally related to each other or to σ factors, they both

have the same secondary structure and it has been shown that they form dimers (Arand *et al.*, 2003, Bullock *et al.*, 1996).

In general, an ECF σ factor is regulated through direct protein-protein interaction with an anti-σ factor. Without a stimulus, this anti-σ factor keeps the ECF σ factor inactive. In the presence of a suitable stimulus, the anti-σ factor gets inactivated and releases the ECF σ factor, which initiates transcription of its target genes (Butcher *et al.*, 2008, Helmann, 2002). The most common mechanisms for the inactivation of anti-σ factors are either regulated proteolysis or conformational changes (Campbell *et al.*, 2008, Heinrich & Wiegert, 2009). ECF41 σ factors are not associated with obvious anti-σ factors, but carry a large C-terminal extension involved in regulation of σ factor activity (Fig. 4.3 and 4.7). Therefore, we suggest that this extension functions as a fused anti-σ factor-like domain.

Based on the hypothetical three-dimensional model of ECF41 proteins (Fig. 5.4) as well as σ factor activation mechanisms described in the literature, three possible signal transducing principles involving the C-terminal extension can be proposed for ECF41 σ factors: (i) intermolecular interaction, (ii) intramolecular interaction, and (iii) proteolysis of the extension. These three possibilities will be discussed in detail in the following sections.

5.4.2.1 Intermolecular interaction

Intermolecular interaction could be a conceivable mechanism for regulation of ECF41 σ factor activity. One attractive hypothesis is that ECF41 proteins exist as dimers in the cell, thereby keeping themselves inactive. Thereby, the C-terminal extension could constitute a dimerization interface. This hypothesis is supported by the fact that the proteins, on which the three-dimensional structure of the C-terminal extension is modeled (see above and Fig. 5.4), form dimers (Arand *et al.*, 2003, Bullock *et al.*, 1996). In case of the ECF41 σ factors, a suitable stimulus could trigger conformational changes and dissociation, resulting in a monomeric form that is able to interact with RNAP and promoter DNA. To test this hypothesis, we carried out initial protein-protein interaction studies based on a bacterial two-hybrid system. These assays showed that the ECF41 σ factors of both *B. licheniformis* and *R. sphaeroides* interact (weakly) with themselves (Fig. 5.5), which indicates possible oligomerization. Nevertheless, this indirect indication needs to be validated by further interaction studies.

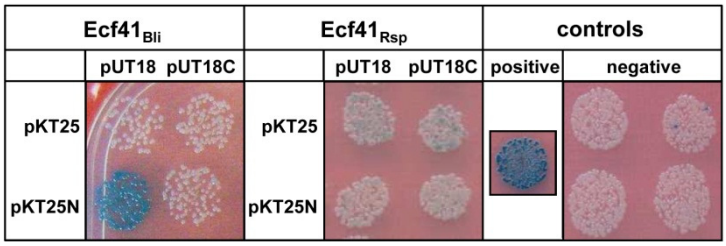

Figure 5.5. Oligomerization of ECF41 σ factors. The genes of *ecf*41$_{Bli}$ and *ecf*41$_{Rsp}$ were cloned into pUT18, pUT18C, pKT25 and pKT25N, thereby generating either N- or C-terminal fusions to the T18 or T25 fragment of adenylate cyclase from *Bordetella pertussis*. The bacterial two-hybrid assay was performed according to (Karimova *et al.*, 2000). Blue color indicates protein-protein interaction. The positive control is based on the derivatives pKT25-zip and pUT18-zip, in which the leucine zipper GCN4 is genetically fused to the T25 and T18 fragment, respectively. The negative control is based on the empty vectors.

5.4.2.2 Intramolecular interaction

The inhibitory effect of the extension could also occur through intramolecular interaction. For example, the C-terminal extension might interact with the σ_2 or σ_4 domain, thereby preventing binding of the ECF41 σ factor to RNAP or promoter DNA. An appropriate stimulus could induce a conformational change resulting in an active σ factor. Such a conformational change that abolishes the inhibitory function could be triggered, for example, by disulfide bond formation, as has already been demonstrated for soluble anti-σ factors such as RsrA of *S. coelicolor* (Kang *et al.*, 1999, Zdanowski *et al.*, 2006). Instead of actual dissociation of an anti-σ/σ factor complex, a conformational change could uncover or rearrange parts of the ECF41 protein important for σ factor function. Intramolecular interaction combined with an inhibitory effect has also been observed for σ^{70} of *E. coli*. While this primary σ factor does not need to be activated, specific binding of free σ^{70} to promoter DNA is inhibited by its N-terminal region 1.1. It is proposed that this autoinhibitory region sterically blocks the access of promoter DNA to the DNA-binding domains. Interaction of σ^{70} with the RNAP core enzyme induces a movement of region 1.1 that probably unmasks the DNA-binding domains, thereby allowing this σ factor the recognition of target promoters only as part of the RNAP holoenzyme (Callaci *et al.*, 1999, Dombroski *et al.*, 1993a, Dombroski *et al.*, 1993b). In case of ECF41 σ factors, the C-terminal extension could play such an inhibitory role by blocking the binding to target promoter DNA. Instead of RNAP holoenzyme formation, the so far unknown inducing signal for group ECF41 could trigger a conformational change resulting in free and thereby functional DNA-binding domains. In this context the highly conserved WLPEP motif of ECF41 σ factors could be of importance. It is located between the σ_2 and σ_4 domains, a

region which usually does not exhibit much sequence and structure conservation (Fig. 5.3 and chapter 4, Fig. S1). Its exceptionally high conservation and exclusive presence in ECF41 σ factors suggest an important function of the amino acids within this motif. It could serve as an interaction interface, possibly being affected by the C-terminal extension. Alternatively, the WLPEP motif could also be important for positioning and stabilization of the σ_2 and σ_4 domains, thereby supporting binding to DNA or RNAP. A stabilizing function is in good agreement with the fact that exchange of the WLPEP motif of $Ecf41_{Bli}$ against alanine residues results in a weak but significant decrease in target promoter activation (Fig. 4.6). RNAP pull-down or bandshift assays with a corresponding derivative of the ECF41 σ factor could reveal if the WLPEP motif influences binding to RNAP or promoter DNA. It would be also interesting to test if the exchange of this motif against alanine residues also weakens the strong promoter activation by the truncated and highly active $Ecf41_{Bli}$ variant (204).

5.4.2.3 Proteolysis

Proteolysis of the C-terminal extension could also be a possibility for activation of ECF41 σ factors. Such a mechanism can be found for σ factors regulating sporulation in *B. subtilis*. At least two examples are known, in which the σ factor is synthesized as an inactivate precursor and then activated by proteolysis. Membrane-bound pro-σ^E, the precursor of σ^E, whose expression is induced early during sporulation, is most likely processed to its active state by the putative protease SpoIIGA (Piggot & Hilbert, 2004). Pro-σ^K is the precursor of σ^K, which is responsible for expression of late sporulation genes in the mother cell. Pro-σ^K carries 20 N-terminal residues that promote association of this σ factor to the mother cell membrane and inhibit binding to RNAP and promoter DNA. Cleavage of these 20 residues and thereby activation of this σ factor via SpoIVFB occurs tightly regulated at later sporulation stages (Piggot & Hilbert, 2004, Zhang *et al.*, 1998, Zhou & Kroos, 2004). With regard to the ECF41 σ factors, cleavage of a certain C-terminal part responsible for the inhibitory function of this extension could lead to an active ECF41 protein. In this case, the N-terminal part of the extension, which we found to be required for σ factor activity (Fig. 4.7), would be still present. The signal triggering such a proteolysis could be either perceived directly by a protease or another protein, which in turn activates a corresponding protease. In another conceivable scenario, the C-terminal extension could constitute the sensor, which may change its conformation after

signal perception and expose a protease recognition site. Nevertheless, a protease or other protein involved in such a mechanism still needs to be identified.

5.5 Conclusions

The aim of this thesis was the investigation of cell envelope stress responses with a special focus on the role and mechanistic details of ECF σ factor-dependent signal transduction. Genome-wide expression profiling, usually performed on model organisms after treatment with specific antimicrobial compounds, is a good and often utilized approach to identify the regulatory networks orchestrating stress responses. Moreover, such studies can also provide valuable indications regarding the MOA of antibiotics. This significantly contributes to the knowledge of the MOA of novel antimicrobial compounds and can be even used to speculate about their clinical relevance and success.

ECF σ factors play a major role in mediating cell envelope stress responses. For some ECF σ factors, the function and signal transducing mechanisms are already well understood. But a classification of these proteins performed by Staroń and colleagues (Staroń *et al.*, 2009) identified a number of novel groups of ECF σ factors and provides a valuable resource for the detailed characterization of ECF σ factor-dependent gene regulation. Chapter 4 of this thesis presented the first analysis of such a novel group. The unique features of this group, especially the regulatory C-terminal extension, nicely illustrate the great diversity of ECF σ factor-dependent signal transduction und encourage the investigation of further groups with yet unknown signaling mechanisms. Just recently, a novel ECF σ factor of *Myxococcus xanthus*, named CorE, has also been shown to carry a short C-terminal extension while an obvious anti-σ factor is missing. This extension contains several cysteine residues important for stimulus perception and controls copper-dependent DNA binding and thereby activity of CorE. While this short extension of CorE resembles the function of an anti-σ domain, the exact mechanism of signal transduction is still unclear (Gómez-Santos *et al.*, 2011). Although CorE-like proteins and ECF41 σ factors do not belong to the same group of ECF σ factors, their regulation presumably involves a fused anti-σ domain rather than a second protein functioning as an anti-σ factor. These studies demonstrate that there is still a great potential for the discovery of completely novel and complex ECF σ factor-dependent signal transducing mechanisms.

Supplementary material

Detailed experimental procedures and additional figures for chapter 2

Strains, media and growth conditions. *B. subtilis* strains were routinely grown in LB medium at 37°C with aeration, except where stated otherwise. Erythromycin (1 µg/ml) plus lincomycin (25 µg/ml) for MLS resistance were used for the selection of strain BFS2469, tetracycline (10 µg/ml) was used for selection of strain TMB389. Friulimicin B was obtained from MerLion Pharmaceuticals GmbH, and other drugs from their respective manufacturers.

RNA preparation. *B. subtilis* 168 wild type strain was grown aerobically at 37°C in LB medium to mid-log phase. The culture was split and induced with friulimicin B or daptomycin (1 µg/ml each) with one sample remaining as the uninduced control. After 10 min of induction 30 ml of each sample were mixed with 15 ml cold killing buffer (20 mM Tris-HCl pH 7.0, 5 mM $MgCl_2$, 20 mM NaN_3), harvested by centrifugation and frozen in liquid nitrogen. For cell disruption, the pellet was resuspended in 200 µl killing buffer, immediately dropped into the Teflon vessel (filled and pre-cooled with liquid nitrogen), and then disrupted with a Mikro-Dismembrator U (Sartorius). The resulting cell powder was resuspended in 3 ml of lysis solution (4 M guanidine-thiocyanate, 0.025 M Na-acetat pH 5.2, 0.5% N-lauroylsarcosinate) and the RNA was extracted twice by phenol/chloroform/isoamylalcohol 25/24/1 followed by chloroform/isoamylalcohol 24/1 extraction and ethanol precipitation. RNA samples were DNase-treated with the RNase-free DNase kit (Qiagen) according to the manufacturer's instructions and purified using RNeasy mini columns (Qiagen). The quality control of the RNA preparations was performed with the RNA 6000 Nano LabChip Kit (Agilent Technologies) on the Agilent 2100 Bioanalyzer according to the manufacturer's instructions.

DNA microarray analysis. The RNA samples obtained from three independent cultivations were used for independent cDNA synthesis and DNA array hybridization. Generation of the Cy3/Cy5-labeled cDNAs and hybridization to *B. subtilis* whole-genome

DNA microarrays (Eurogentec) were performed as described (Jürgen *et al.*, 2005). The slides were scanned with a ScanArray Express scanner (PerkinElmer). Quantitation of the signal and background intensities was carried out using the ScanArray Express image analysis software.

Transcriptome data analysis. Data was analyzed using the GeneSpring software (Agilent Technologies). Raw signal intensities were first transformed by intensity dependent LOWESS normalization. The normalized array data were subjected to a statistical analysis using Cyber-T, a program based on a *t*-test combined with a Bayesian statistical framework (Baldi & Long, 2001). The software is accessible through a web interface at http://cybert.microarray.ics.uci.edu. The mRNA abundance was considered to be significantly different between the untreated control samples and the samples obtained after treatment with the respective antibiotic if (i) the Cyber-T Bayesian *P* value was < 0.001 and (ii) the average fold change was at least 3 in three independent experiments. The potential and known functions of the encoded proteins were initially inferred from the SubtiList (http://genolist.pasteur.fr/SubtiList/) or BSORF databases (http://bacillus.genome. ad.jp/). An in-depth analysis of the identified marker genes (as listed in Table 2) was performed using the SMART (Letunic *et al.*, 2006, Schultz *et al.*, 1998) and MicrobesOnline (Alm *et al.*, 2005) databases, at http://smart.embl-heidelberg.de/ and http://www.microbesonline.org/, respectively.

Quantitative real time RT-PCR. Measurement of transcript abundance was performed by quantitative real-time RT-PCR using iScript one-step RT-PCR kit with SYBR Green (Bio-Rad) according to the manufacturer's procedure with minor modifications: In brief, 100 ng of DNA-free total RNA was used in a total reaction volume of 20 µl with 0.3 µM of each primer (see Table 1). The amplification reaction was carried out in an MyiQ Cycler (BioRad) using the following program: reverse transcription at 50°C for 10 min, followed by a 95°C denaturing/activation step for 5 min, followed by 45 cycles (95°C for 10 sec), (60°C for 30 sec). After a subsequent denaturation (95°C for 1 min) and annealing (55°C for 1 min) the setpoint temperature was increased in 80 cycles (10 sec each) by 0.5°C/cycle, starting from 55°C, to determine the melting temperatures of the PCR products. Expression of *rpsJ* and *rpsE* was monitored as constitutive reference. These genes were chosen due to their stable expression behaviour under various growth and stress conditions in *B. subtilis* (data not shown). Expression of *liaI* and genes encoding

ECF σ factors was calculated as fold changes using the formula: Fold change = $2^{-\Delta\Delta Ct}$; with $-\Delta\Delta Ct = (Ct_{(gene\ x)}-Ct_{(constitutive\ gene)})_{condition\ I} - (Ct_{(gene\ x)}-Ct_{(constitutive\ gene)})_{condition\ II}$ (Talaat *et al.*, 2002).

Concentration-dependent killing curve/β-galactosidase assays. These experiments were performed as described (Mascher *et al.*, 2004). In brief, strain BFS2470 was grown in LB medium with MLS selection to OD_{600} ~ 0.5 and daptomycin/friulimicin B were added to a final concentration ranging from 0.01 to 50 µg/ml. An uninduced culture was used as a negative control. The cultures were incubated with aeration at 37°C. A sample was taken after 30 min for β-galactosidase assay and the turbidity of the remaining culture was measured for at least 5 hours to monitor the concentration-dependent effects of the antibiotics on growth.

L-[^{35}S]methionine labelling of proteins and 2D-PAGE analysis. *B. subtilis* 168 wild type strain was grown aerobically at 37°C in Belitsky minimal medium (Stülke *et al.*, 1993) to mid-log phase. 10 and 30 minutes after addition of daptomycin or friulimicin B (1,5 and 1,0 µg/ml, respectively) the cells as well as untreated control cells were labelled with 15 µCi/ml [^{35}S]-methionine. After 5 minutes incorporation of radioactive methionine, the reaction was stopped by adding an excess of nonradioactive methionine (1mM) and chloramphenicol (100µg/ml) to stop translation. Samples were taken and the cytoplasmic protein fraction isolated as described earlier (Bandow *et al.*, 2003). 2D-PAGE using Immobiline dry strips (IPG, Amersham Biosciences) (pH 4-7) loaded with 80 µg protein extract as well as visualization of radiolabelled proteins and dual channel imaging using Delta2D software (Decodon) were carried out as described (Bernhardt *et al.*, 1999, Bernhardt *et al.*, 2003).

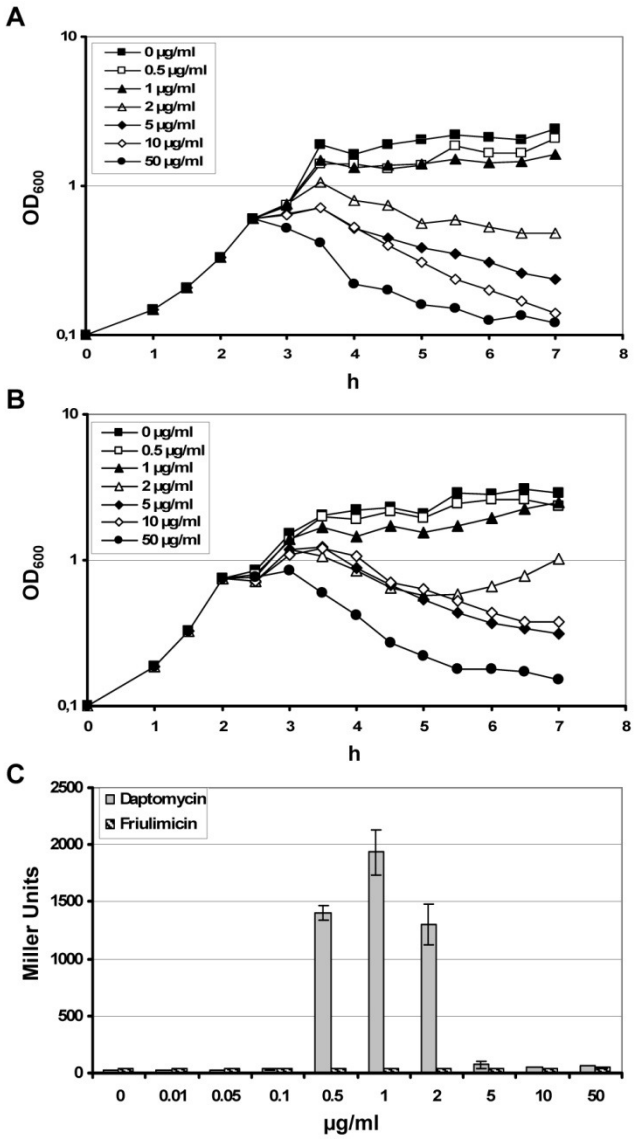

Figure S1. Killing curves and concentration-dependent induction of the *liaI* promoter in *B. subtilis* cultures treated with daptomycin and friulimicin B. (A) Killing curves daptomycin. LB medium was inoculated from a fresh overnight culture of *B. subtilis* W168 and incubated at 37°C with aeration. Cell density was monitored by measuring OD_{600} at regular intervals. At mid-logarithmic growth phase (OD_{600} ~ 0.5) the culture was split and induced with different concentrations of daptomycin (■, uninduced control; □, 0.5 µg/ml; ▲, 1 µg/ml; △, 2 µg/ml; ◆, 5 µg/ml; ◇ 10 µg/ml; ●, 50 µg/ml). (B) Friulimicin killing curves, using the same experimental conditions as above. (C) Induction of the *liaI* promoter. Strain BFS2470 (*B. subtilis* W168 *liaI*::pMUTIN) was grown in LB medium as described above and induced with different concentrations of daptomycin (grey bars) and friulimicin (striped bars), respectively, for 30 min, with one sample remaining as the uninduced control. The cells were harvested and β-galactosidase assays were performed as described in the detailed experimental procedures. The P_{liaI} activity, expressed in Miller Units (Miller, 1972), is shown on the *y*-axis.

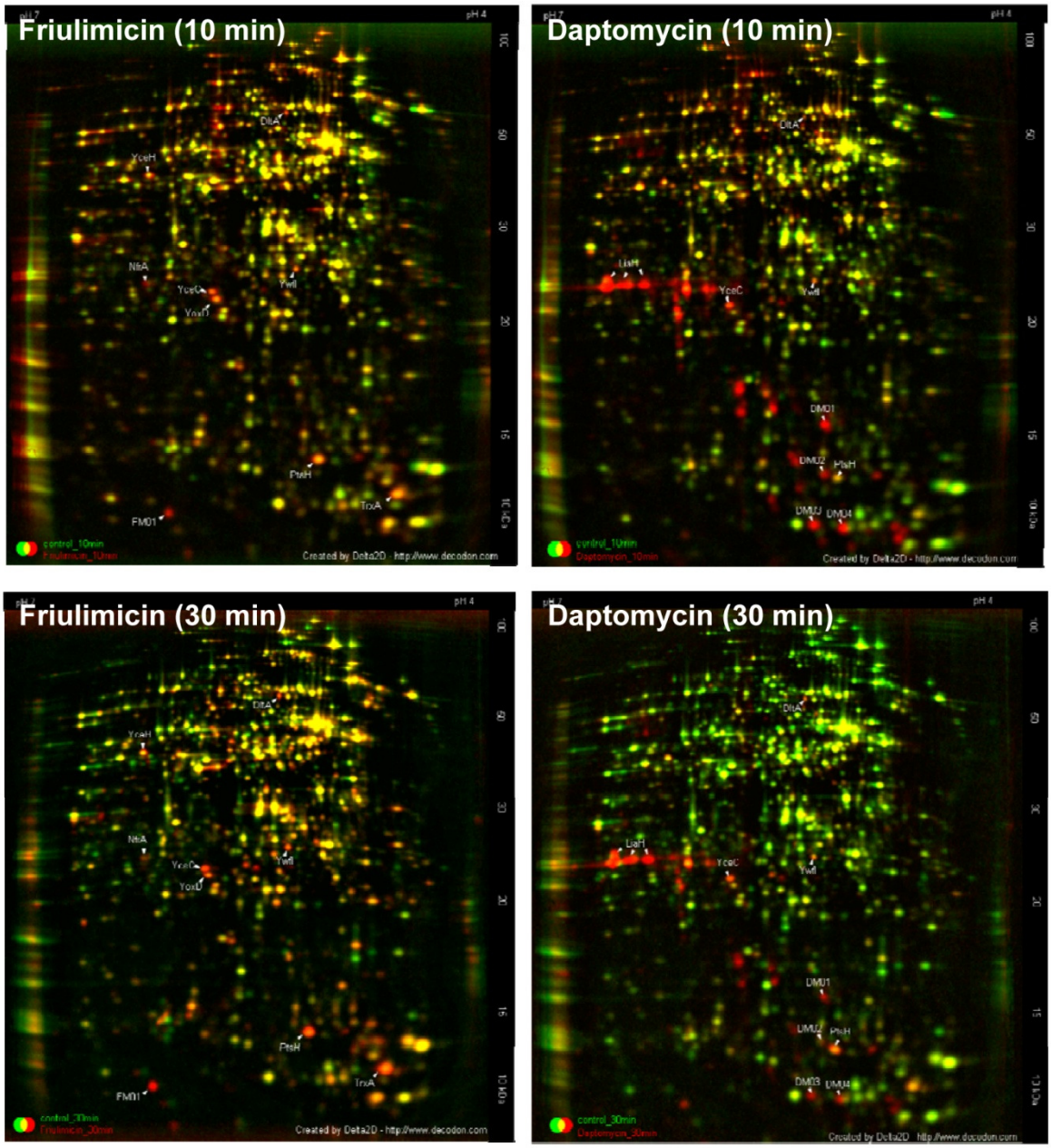

Figure S2. Proteome analysis. Protein expression profiles of *B. subtilis* 168 before (green image) and after 10 or 30 min of the exposure (red image) to friulimicin B (left) and daptomycin (right), respectively, are shown.

Supplementary material provided on CD

The following supplementary material can be found on the enclosed CD.

Chapter 2

Table S1 daptomycin. Excel file containing the complete microarray dataset for daptomycin. The normalized array data were analyzed using the software Cyber-T (Baldi & Long, 2001), which is available at http://cybert.microarray.ics.uci.edu. The output of this analysis including several statistical parameters is given in form of an excel table.

Table S2 friulimicin B. Excel file containing the complete microarray dataset for friulimicin B, which was analyzed as described above for daptomycin.

Chapter 3

Table S1. Excel file of the complete microarray dataset containing rhamnolipid induction ratios for each gene of *B. subtilis*. The ratio values are averages from three independent microarray experiments and calculated from intensity data using the Rosetta Resolver software (version 7.2.1, Rosetta biosoftware).

Chapter 4

Table S1. Excel file containing the non-redundant dataset with 373 proteins extracted from the MiST2 database (Ulrich & Zhulin, 2010) in October 2010. The dataset was analyzed regarding the genomic context and occurrence of the ECF41-dependent promoter motif. Pfam domains of the COE proteins were identified using the SMART database (Letunic *et al.*, 2006, Schultz *et al.*, 1998). Abbreviations: ECF, gene encoding the ECF41 σ factor; COE, gene encoding a carboxymuconolactone decarboxylase, oxidoreductases or epimerase; CMD, gene encoding a carboxymuconolactone decarboxylase; Ox, gene encoding an oxidoreductases; Epi, gene encoding an epimerase; Hypo, gene encoding a hypothetical protein.

Figure. S1. Genbank file containing an alignment of ECF41 proteins. The alignment containing 373 ECF41 protein sequences was constructed using ClustalW (Thompson *et al.*, 1994).

PM_deletion_versus_wt. Word document containing results of the phenotypic microarray analysis performed for *R. sphaeroides* strains YSD239 (ΔRSP_0606-*ecf*41$_{Rs}$p) and 2.4.1 (wt).

PM_overexpression_versus_wt. Word document containing results of the phenotypic microarray analysis performed for *R. sphaeroides* strains TMR003 (pIND4 *ecf*41$_{Rsp\ aa1\text{-}206}$) and 2.4.1 (wt).

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Curriculum Vitae

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Publications

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