Intramembrane Signal Transduction and Cell Envelope Stress Response in *Bacillus subtilis*

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
Sina Jordan
aus Essen

Göttingen 2007

D 7
Referent: Prof. Dr. Jörg Stülke
Korreferent: PD Dr. Michael Hoppert
Tag der mündlichen Prüfung: 01.11.2007

Danksagung

Der grösste Dank gilt natürlich meinem Doktorvater Dr. Thorsten Mascher! Also Thorsten, an dieser Stelle nochmal vielen Dank für das interessante Thema, die umfassende Betreuung und das supernette Arbeitsklima. Ich hab mich in Deinem Labor sehr wohl gefühlt und hatte eine Menge Spass! Du hast mich immer unterstützt und mir das Gefühl gegeben, gute Arbeit zu leisten. Es war schön, in einem so motivierten Umfeld arbeiten zu dürfen!

Ein herzliches Dankeschön geht an Prof. Dr. Jörg Stülke für die Übernahme des Referates und die Möglichkeit, in seiner Abteilung arbeiten zu dürfen.

Für die Übernahme des Korreferats danke ich PD Dr. Michael Hoppert.

Ausserdem möchte ich mich bei Dr. Leendert Hamoen für die Möglichkeit bedanken, in seiner Arbeitsgruppe in Newcastle am Fluoreszenzmikroskop arbeiten zu dürfen.

Ein besonderer Dank geht natürlich auch an Bärbel Herbst für die Bereitstellung diverser Arbeitsmaterialien. Du hast mir das Arbeiten im Labor sehr erleichtert!

Den Mitarbeitern der GenoMIK möchte ich für die Durchführung unzähliger Sequenzierungen danken.

Ich habe das Glück gehabt, mit sehr vielen unwahrscheinlich netten Kollegen arbeiten zu dürfen. Da steht an erster Stelle natürlich Falk Kalamorz! Falk, ich danke Dir ganz herzlich für die schöne gemeinsame Zeit während meiner Doktorarbeit. Dabei möchte ich mich ganz besonders für die hunderte perfekt gekochten Kaffees bedanken, ohne die ich wahrscheinlich nicht überlebt hätte. Auch die (fast) täglichen eMails während meiner Zeit in Newcastle haben mir sehr geholfen, nicht an Einsamkeit zu sterben. Deshalb ein dickes Danke für alle lebensrettenden Taten während der letzten 3 Jahre!!!

Nun zum "Girls Camp": Während ihres GP II und der anschliessenden Diplomarbeit sind mir Tina Wecke, Eva Rietkötter, Diana Hoyer und Anja Junker gute Freundinnen gewesen. Ausserdem sind Ihre Ergebnisse zum Teil mit in diese Arbeit eingeflossen.

Tina, wo soll ich da anfangen? Wir konnten über nahezu alles reden, und das haben wir ja auch getan. Angefangen bei der gemeinsamen Leidenschaft für Pferde, über Beziehungen bis hin zu ausgedehnten Lästereien. Mir hat diese Zeit viel Spass gemacht, ich werde das sehr vermissen!

Eva, auch wir haben uns super verstanden, Du warst mir ein würdiger Gegner ;-) . Zu jeder Zeit einen trockenen Spruch auf den Lippen, das ist schon bemerkenswert. Nicht zu vergessen Deine herausragende Leistung am Kickertisch!

Diana, ich habe die Zusammenarbeit mit Dir wirklich genossen. Schon seit Deinem GP II haben wir uns gut verstanden und ich bedanke mich sehr für die nette Zusammenarbeit! Ausserdem wünsche ich Dir und dem Baby alles Gute und viel Erfolg bei Deiner Doktorarbeit!
Anja, wir hatten eine lustige Zeit und sehr viel Spass im Labor. Auch den Cocktailabend werde ich nicht vergessen! Also hier von einer Zicke zur anderen: Danke für die schöne Zeit!
Bastian Dörrbecker, Maria Martinec und Karen Schrecke haben während ihres GP II Ergebnisse erziehlt, die mit in diese Arbeit eingeflossen sind. Dafür vielen Dank!
Basti, es hat immer sehr viel Spass gemacht, mit Dir zu arbeiten, zu reden oder zu kickern. Ich wünsche Dir, dass Du bei Deiner Doktorarbeit so nette Kollegen findest wie ich!
Auch bei Claudine Hames und Sebastian Hübner möchte ich mich bedanken. Ich bin gerne mit Euch essen gegangen, hatte Spass beim pokern und war froh über die Schlafgelegenheit, die Ihr mir immer angeboten habt. Lasst Euch nicht unterkriegen, auch Ihr habt es auch bald geschafft!
Ganz lieben Dank ins ferne Newcastle an Sven Halbedel, der sich dort gut um mich gekümmert und seine Bench mit mir geteilt hat.
Auch allen anderen Mitgliedern der Abteilung Danke für die schöne Zeit, auch wenn ihr nicht namentlich erwähnt worden seid.

Ohne meine Eltern hätte ich das alles natürlich nie geschafft. Danke, dass Ihr mich wieder für einige Zeit bei Euch aufgenommen und mir immer den Rücken gestärkt habt! Für die ständige Unterstützung, die Betreuung von Sheila und die tausend anderen Dinge, die Ihr für mich tut, bin ich Euch wirklich sehr dankbar!

Meinem Mann Michael möchte ich ganz besonders danken. Michi, Du stehst immer hinter mir, hilfst mir, gibst mir Kraft, bestärkst mich in meinen Entscheidungen (auch wenn diese uns räumlich trennen), tröstest mich und tust noch tausend andere Dinge, die Dich zum wichtigsten Menschen in meinem Leben machen, und das nun schon seit 13 Jahren. Ich liebe Dich über alles!

Content

List of abbreviations

AAA+	ATPase associated with various cellular activities
ABC	ATP binding cassette
B.	*Bacillus*
Bac	bacitracin
bp	base pair
CAMP	cationic antimicrobial peptide
cAMP	cyclic adenosine monophosphate
CES	cell envelope stress
CESR	cell envelope stress response
cGMP	cyclic guanosine monophosphate
cm	chloramphenicol (resistance cassette)
DNA	deoxyribonucleic acid
E.	*Escherichia*
ECF	extracytoplasmic function
e.g.	for example (exempli gratia)
Fig.	figure
FMN	flavine mononucleotide
GAF	cyclic GMP, adenylyl cyclase, FhlA
GFP	green fluorescent protein
h	hour
HAMP	histidine kinase, adenylyl cyclase, methyl-accepting chemotaxisprotein, phosphatase
HK	histidine kinase
i. e.	that is (it est)
IMHK	intramembrane-sensing histidine kinase
LB	Luria Bertani
min.	minute
MLS	Erythromycin/Lincomycin (resistance cassette)
NAD(P)H	nicotinamide adenine dinucleotide phosphate, reduced form
ONPG	2-nitrophenyl-β-D-galactopyranoside
PBP	penicillin-binding protein
PCR	polymerase chain reaction

pmf	proton motiv force
RR	response regulator
RT	reverse transcription
SD	Shine-Dalgarno (sequence)
sec.	seconds
spec	spectinomycin (resistance cassette)
TA	teichoic acid
Tab.	table
TCS	two-component system
tet	tetracyclin (resistance cassette)
TMR	transmembrane region
UDP	uridine 5´-diphosphate
wt	wildtype
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of publications

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

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

Jordan, S., Hutchings, M. I., and Mascher, T. Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiology Reviews*: submitted

Summary

The bacterial cell envelope is the first and major line of defence against threats from the environment. It is an essential and vulnerable structure that gives the cell its shape and counteracts the high internal osmotic pressure. It also provides an important sensory interface and molecular sieve, mediating both information flow and controlled transport of solutes. The cell envelope is also the target for numerous antibiotics. Therefore, the monitoring and maintaining of cell envelope integrity in the presence of envelope perturbating agents and conditions is crucial for survival. In *Bacillus subtilis* a complex regulatory network, consisting of 7 signal transducing systems, orchestrates the cell envelope stress response. Two forms of regulatory systems can be found: ECF-σ factors and two component systems (TCS). One of these TCS is the LiaRS system that responds to cell wall antibiotics that interfere with the undecaprenol cycle and to perturbation of the cytoplasmic membrane. It is encoded by the last two genes of the *liaIHGFSR* locus. Without cell envelope stress, the last 4 genes are constitutively expressed from a weak promoter upstream of *liaG* to ensure the presence of the two-component system in the cell.

The activated response regulator LiaR induces the expression of the *lia* operon. LiaR binds to a palindromic sequence with a 7-4-7 inverted repeat, which is located 75 bp upstream of the *liaI* start codon. In addition to the *liaI* promoter (P_{liaI}), the promoter of the *yhcYZ-yhdA* operon and of the *ydhE* gene could be identified as target promoters of LiaR in *B. subtilis*.

A systematic deletion analysis of the *lia* operon revealed that LiaF is a negative regulator of LiaR-dependent gene expression: a non-polar *liaF* deletion leads to constitutive activation of all three characterized LiaR-dependent promoters. The *liaF* gene is conserved in both sequence and genomic context in the *Firmicutes* group of Gram-positive bacteria, located directly upstream of *liaSR* orthologs. Therefore, LiaF together with LiaRS forms a three-component system.

In the transition to stationary phase, P_{liaI} is induced without exogenous stimuli. This induction is LiaR-dependent and additionally regulated by AbrB and Spo0A. During logarithmic growth the transition state regulator AbrB binds to the *liaI* promoter and prevents expression of the *lia* operon. Spo0A regulates P_{liaI} induction in transition state indirectly by repressing the expression of AbrB.

Die bakterielle Zellhülle verleiht dem Zellkörper Form und Stabilität, wirkt dem starken osmotischen Innendruck entgegen und stellt eine Permeabilitätsbarriere da. Sie wirkt als molekulares Sieb und bietet der Zelle als äußerste Schicht Schutz gegen schädliche Umwelteinflüsse. Außerdem ist die Zellhülle aber auch die Verbindung zwischen einem Bakterium und seiner Umwelt. Viele Antibiotika greifen die Zellhülle oder ihre Synthese an. Deshalb ist ein ständiges Überwachen der Zellhüllintegrität für ein Bakterium lebenswichtig. Die Zellhüllstress-Antwort in *Bacillus subtilis* wird durch sieben signaltransduzierende Systeme vermittelt, zu denen drei ECF-σ-Faktoren und vier Zweikomponentensysteme gehören. Eines dieser Zweikomponentensysteme ist das LiaRS-System, welches auf Zellwandantibiotika, die den Lipid II-Zyklus hemmen, und auf Zerstörung der Cytoplasmamembran reagiert. Es wird von den letzten beiden Genen des *liaIHGFSR* Lokus kodiert. Ohne Zellhüllstress werden die letzten vier Gene des Operons von einem schwachen konstitutiven Promotors vor *liaG* exprimiert, wodurch das Vorhandensein des Zweikomponentensystems in der Zelle sichergestellt wird.

Der aktivierte *Response Regulator* LiaR induziert die Expression des *lia* Operons, indem er an eine palindromische Sequenz mit einem 7-4-7 Motiv, die sich 75 bp vor dem *liaI*-Startkodon befindet, bindet. Zusätzlich zum *liaI* Promotor (P_{liaI}) konnten die Promotoren des *yhcYZ-yhdA* Operon und des *ydhE* Gens als Zielpromotoren von LiaR in *B. subtilis* identifiziert werden.

Eine systematische Deletionsanalyse des *lia* Operons zeigte, dass LiaF als negativer Regulator der LiaR-abhängigen Genexpression fungiert: eine nicht-polare Deletion von *liaF* führte zu konstitutiver Aktivierung aller drei charakterisierter LiaR-abhängigen Promotoren. Sowohl Sequenz als auch genomischer Kontext des *liaF*-Gens sind in der Gruppe der *Firmicuten* konserviert, wobei *liaF* stets stromaufwärts der *liaSR*-Orthologen lokalisiert ist. Daher bildet LiaF zusammen mit LiaRS ein Dreikomponenten-System.

Während des Übergangs zur stationären Wachstumsphase (*transition state*) wird P_{liaI} ohne exogene Stimuli induziert. Diese Induktion ist LiaR-abhängig und wird zusätzlich von den Proteinen AbrB und Spo0A reguliert. Während der logarhythmischen Wachstumsphase ist der *transition state* Regulator AbrB an den *liaI* Promotor gebunden und verhindert so die Expression des *lia* Operons. Spo0A reguliert die Induktion von P_{liaI} indirekt, indem es die Expression von AbrB verhindert.

Chapter 1:

Introduction

A. Cell Envelope Stress Response in *Bacillus subtilis* and Other *Firmicutes* Bacteria

This chapter is an excerpt of the following publication:

Jordan, S., Hutchings, M. I., and Mascher, T. Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiology Reviews*: submitted

Author contributions:
Sina Jordan performed the comparative genomic analysis and draw the figures. The review was written by Sina Jordan, Matthew I. Hutchings and Thorsten Mascher.

Introduction

Life in the microbial world is characterized by fierce competition, nutritional hardship, and often life-threatening changes of external (i.e. physicochemical) parameters. Adaptive responses of a bacterium to its environment are therefore one defining cornerstone of microbial life in its natural context, irrespective of the individual life style or habitat. Such adaptations require the sensitive monitoring of numerous environmental parameters (input) to orchestrate the activity of intricate and complex regulatory systems that initiate or re-adjust adequate cellular responses (output) in a continuous balancing act between costs and gain. No surprise then that we find efficient stress response systems – aimed to maintain the functionality and integrity of the cell under all circumstances – embedded in the genomic blueprint of almost any bacterium studied to date (Storz and Hengge-Aronis, 2000).

The Gram-positive cell envelope. One of the crucial cellular structures is the cell envelope and its integrity has to be ensured, at all times and any costs. A detailed description of the biosynthesis and chemical composition of the Gram-positive cell envelope is beyond the scope of this review, and readers are referred to a number of excellent reviews on this topic (Archibald *et al.*, 1993; Delcour *et al.*, 1999; Foster and Popham, 2002). Suffice it to say, the Gram-positive cell envelope only consists of two functional layers (compared to three in Gram-negative bacteria) that enclose the cellular contents: a cytoplasmic membrane, surrounded by a thick cell wall. It lacks an outer membrane (and therefore a periplasmic space *sensu stricto*, see below). The Gram-positive peptidoglycan sacculus – in contrast to its single-layered Gram-negative counterpart – is a three-dimensional multi-layered net-like structure of about 50 nm thickness that can withstand high turgor pressures (up to 20 atm, i.e. more than a racing bike tire!). Due to the combination of rigid sugar chains perpendicularly crosslinked with flexible peptide bridges, the mesh of this net is a strong, but also elastic stress-bearing structure (Delcour *et al.*, 1999; Höltje, 1998). It is a highly dynamic super-molecule that undergoes permanent biosynthesis, assembly, maturation, disassembly, and recycling, to allow maintenance of cell shape, cellular growth and division at the same time (Archibald *et al.*, 1993).

The Gram-positive peptidoglycan sacculus is interspersed with almost equal amounts of teichoic acids (TAs) that can either be tethered to the membrane (lipoteichoic acids) or covalently linked to the sugar backbone of the peptidoglycan sacculus (wall TAs) (Delcour *et al.*, 1999; Foster and Popham, 2002). TAs are important components of the Gram-positive

cell wall, and play a crucial role in defining the physicochemical properties of the envelope: They are poly-anionic, phosphate-rich linear polymers mainly responsible for the overall negative net charge of the Gram-positive cell surface (Bhavsar *et al.*, 2004). TAs – as peptidoglycan – were generally viewed as essential biopolymers of Gram-positive bacteria. But recent studies in both *Staphylococcus aureus* and *Bacillus subtilis* – while clearly verifying the fundamental role of TAs for the overall cell integrity and fitness (Weidenmaier *et al.*, 2004; Weidenmaier *et al.*, 2005) – challenge this assumption by demonstrating the dispensability of wall TAs, at least under laboratory conditions (D'Elia *et al.*, 2006a; D'Elia *et al.*, 2006b). In contrast, recent work on lipoteichoic acid biosynthesis in *S. aureus* clearly indicates the essentiality of this biopolymer (Gründling and Schneewind, 2007).

In addition to these basic features of a Gram-positive cell wall, a number of additional cell envelope structures are present in many bacteria, and often play an important role for virulence, antibiotic resistance, colonization, and multicellular differentiation. These structures include extracellular polysaccharide capsules and biofilm matrices (Branda *et al.*, 2005; Miyake and Iijima, 2004; O'Riordan and Lee, 2004), as well as proteinaceous S-layers (Schäffer and Messner, 2005; Sleytr *et al.*, 2007). Moreover, the cell walls of mycobacteria and corynebacteria show a unique architecture that includes additional layers, consisting of arabinogalactans and mycolic acid, that surround the cell wall and renders these pathogens impenetrable for many antibiotics (Dover *et al.*, 2004).

Many central questions on the mechanism and control of cell envelope homeostasis – including aspects of its biosynthesis (such as the Lipid II cycle), turnover and overall architecture – still remain largely unanswered, despite its fundamental cellular role and decades of research. The three-dimensional architecture of the sacculus is a matter of an ongoing (and inspiring) debate between two competing – and mutually exclusive – models: peptidoglycan sheets versus scaffolds (Dmitriev *et al.*, 2005; Vollmer and Höltje, 2004). Another area of controversy is the presence of a periplasmic space, which is generally viewed as a hallmark feature restricted to Gram-negative bacteria. Contradictory to that, recent advances in electron microscopic techniques indicated the presence of a periplasmic-like space of about 20 nm width in Gram-positive bacteria, located between the membrane and the cell wall (Matias and Beveridge, 2005, 2006). These findings emphasize the limitations in our understanding of the Gram-positive cell envelope architecture.

The cell wall – in addition to its roles as a shape-giving structure, an exoskeleton (to protect the cell from its environment and counteract the turgor pressure), and molecular sieve – is also discussed as a potent endotoxin in bacterial sepsis (Myhre *et al.*, 2006). Moreover, the

envelope also acts as a diffusion barrier (allowing and necessitating selective transport), and a communication interface (mediating information exchange) between the cell and its surrounding. The latter functions are primarily provided by the cytoplasmic membrane and its embedded proteins.

Antibiotics targeting the cell envelope. Because of its many crucial functions, the cell envelope is a prime target for numerous antibiotics, including many with high clinical relevance, that interfere with virtually any step in its biosynthesis (Koch, 2003; Lazar and Walker, 2002; Silver, 2003, 2006; Walsh, 2003). Only some of the more common cell wall antibiotics important in the context of this review, as well as their respective major bacterial resistance mechanisms, will be introduced very briefly in the following paragraphs. Their site of interference with cell wall biosynthesis is schematically summarized in Fig. 1.1. All cell wall antibiotics either directly inhibit the enzymatic activity mediating a cell wall reaction (shown in red in Fig. 1.1), or sequester the substrate of a given step (indicated in green in Fig. 1.1) (Silver, 2006).

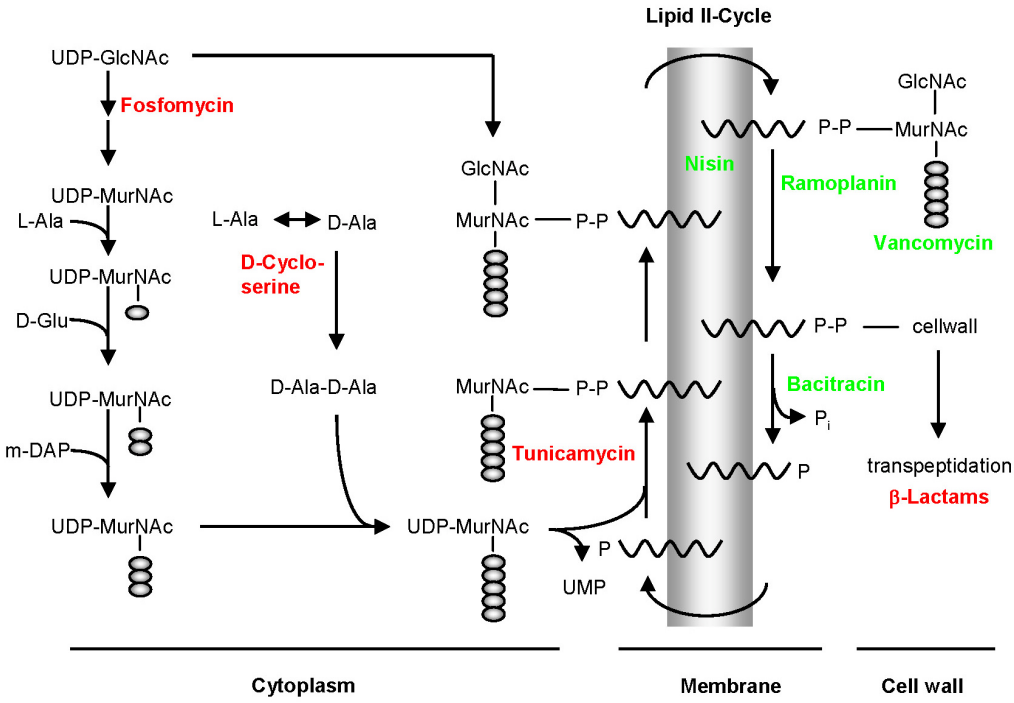

Figure 1.1. Cell wall biosynthesis and its inhibition by antibiotics. Important steps in cell wall biosynthesis are schematically depicted, and their cellular location is indicated below. Some cell wall antibiotics relevant for this review are given and placed next to the step they inhibit. Antibiotics in green sequester the substrate of the given step; those in red inhibit the corresponding enzyme. See text for details on their action. This figure was originally based in parts on (Silver, 2003), with modifications.

Fosfomycin inhibits the first step committed step, the formation of UDP-N-acetyl-muramic acid from UDP-N-acetyl-glucosamine. It functions as an inactivating structural analogue of phosphoenol pyruvate, the co-substrate of the MurA-catalyzed reaction (Kahan *et al.*, 1974). Resistance either arises by spontaneous mutations in the transport pathways (Horii *et al.*, 1999), or is conferred by glutathione/metallothiol transferases that enzymatically inactivate fosfomycin (Bernat *et al.*, 1997; Cao *et al.*, 2001; Suarez and Mendoza, 1991).

D-cycloserine prevents the completion of the pentapetide side chain, the canonical crosslinking agent of the peptidoglycan network. It inhibits both the D-alanine racemase, which converts L-alanine to D-alanine, and the D-alanine/D-alanine ligase, which catalyzes the formation of the corresponding dipeptide (Lambert and Neuhaus, 1972; Neuhaus and Lynch, 1964). Resistance can either be achieved by overexpression of the target proteins or by an efflux pump (Feng and Barletta, 2003; Matsuo *et al.*, 2003).

A number of antibiotics, including lantibiotics, ramoplanin, vancomycin, or bacitracin, interfere with the Lipid II cycle, the bottleneck of cell wall biosynthesis (Breukink and de Kruijff, 2006). Lipid II is the basic peptidoglycan building block, N-acetyl-glucosamine/N-acetyl muramic acid-pentapeptide covalently linked to the lipid carrier undecaprenol through a pyrophosphate ester bridge (Delcour *et al.*, 1999).

Lantibiotics (such as nisin) are polycyclic peptide-derived antimicrobial agents that are ribosomally synthesized and posttranslationally modified to their active forms. They contain the unusual amino acid lanthionine as their name-giving feature and “highjack” Lipid II as a docking molecule to form pores, ultimately resulting in cell lysis (Breukink and de Kruijff, 2006; Chatterjee *et al.*, 2005). The primary resistance mechanism against these positively charged peptide antibiotics is lowering of the overall negative net charge of the Gram-positive cell wall by D-alanine incorporation into the poly-anionic TAs. Some cases of enzymatic degradation or modification of the lantibiotic have also been reported (Breukink and de Kruijff, 2006).

The overall positive charge and pore formation as the mode of antimicrobial action are shared by the wider family of so-called cationic antimicrobial peptides (CAMPs), which represent an important defense mechanism of the human immune system and have gained a lot of attention in recent years due to their potential as future therapeutics (Brogden, 2005; Giuliani *et al.*, 2007; Hancock and Sahl, 2006).

Ramoplanin, a non-ribosomally synthesized lipoglycodepsipeptide antibiotic (Walker *et al.*, 2005), inhibits the transglycosylation step of peptidoglycan biosynthesis by binding Lipid II

at the extracellular surface of the cytoplasmic membrane (Hu *et al.*, 2003). No resistance has been reported, so far (Breukink and de Kruijff, 2006).

Vancomycin and other glycopeptide antibiotics, such as teicoplanin, block glycan polymerization and cross-linking by tightly binding to the D-alanyl-D-alanine dipeptide terminus of Lipid II and nascent peptidoglycan (Kahne *et al.*, 2005). Resistance is gained by reprogramming cell wall biosynthesis, incorporating alternative peptide termini, such as D-alanyl-D-lactate, instead of D-Ala-D-Ala (Healy *et al.*, 2000; Walsh *et al.*, 1996).

Bacitracin is a cyclic non-ribosomally synthesized dodecylpeptide antibiotic that requires the coordination of a divalent metal ion for its biological activity (Ming and Epperson, 2002). It binds very tightly to undecaprenyl pyrophosphate, thereby preventing the recycling of the lipid carrier by dephosphorylation (Stone and Strominger, 1971; Storm and Strominger, 1973). Four different mechanisms of bacitracin resistance have been described so far: (1) expression of bacitracin-specific ABC transporters (Mascher *et al.*, 2003; Ohki *et al.*, 2003a; Podlesek *et al.*, 1995), (2) *de novo* synthesis of undecaprenyl phosphate (Cain *et al.*, 1993; Chalker *et al.*, 2000), (3) expression of alternative undecaprenyl pyrophosphate phosphatases (Bernard *et al.*, 2005; Cao and Helmann, 2002; Ohki *et al.*, 2003b), or (4) exopolysaccharide production (Pollock *et al.*, 1994; Tsuda *et al.*, 2002).

Penicillin and other β-lactams covalently modify the active site of transpeptidases (which are therefore called penicillin-binding proteins, or PBPs), by mimicking the D-alanyl-D-alanine terminus of the pentapeptide side chain (Strominger and Tipper, 1965). Resistance can be achieved by one of three known mechanisms (Poole, 2004; Wilke *et al.*, 2005): (1) biosynthesis of β-lactamases that inactivate the antibiotic (Ghuysen, 1991), (2) expression of mutated *pbp* alleles, so-called mosaic genes, encoding low-affinity PBPs that maintain their physiological function, but show a decreased β-lactam binding (Dowson *et al.*, 1994; Hakenbeck, 1999), or (3) removal of the antibiotic from its site of action by β-lactam-specific efflux pumps (Poole, 2005).

The majority of classical antibiotics are produced by microorganisms of the soil biosphere (Berdy, 2005), such as bacilli (Butcher and Helmann, 2006; Stein, 2005), fungi (Anke, 1997), and most notably the actinomycetes (Champness, 2000; McNeil and Brown, 1994) and presumably to inhibit the growth of competitors. Stress responses and the development of counter strategies, including efficient resistance mechanisms, are therefore widespread survival strategies amongst soil bacteria to succeed in this habitat (D'Costa *et al.*, 2006). Likewise, pathogenic bacteria encounter antimicrobials as part of the host's defense system, which also lead to the evolution of adequate stress responses and resistance mechanisms for

survival. Only very recently, evolutionarily speaking, were pathogenic bacteria suddenly also challenged with antibiotics from the soil biosphere, in the form of novel 'magic bullets' for clinical antimicrobial therapy that were initially thought to eradicate the problem of life-threatening bacterial infections once and for all. As we know now, antibiotic-resistant bacteria emerged faster than novel antimicrobials can be developed and approved for clinical use (Vicente *et al.*, 2006). The evolution of novel traits of antibiotic resistance mechanisms in human pathogens and their commensal microbial brethren, through a combination of spontaneous beneficial mutations and horizontal gene transfer, happened at breathtaking speed, and sometimes included the transfer of whole functional modules consisting of sensitive antibiotic detection systems together with their respective target genes mediating resistance. This is most notably illustrated by the spread of vancomycin resistance amongst clinical isolates of enterococci and staphylococci (Palumbi, 2001; Walsh and Howe, 2002). Such traits, in the context of cell wall antibiotics, are one central aspect of bacterial CESR.

Cell envelope stress (response)

The physiological role of the cell envelope in combination with the presence of agents and/or conditions that can alter or even destroy this essential cellular structure necessitate that its integrity is closely monitored. The corresponding signal transducing regulatory systems respond to alterations and dysfunctions of the envelope and induce appropriate counter-measures to repair damage and secure functionality.

Before giving a comprehensive overview on this stress response and the regulatory systems mediating it, we first need to define the term. In contrast to "intuitive" stresses such as heat or osmotic shock, it is not easy to put a finger on cell envelope stress. Obviously, many stress conditions, including those mentioned above, affect the integrity of the cell envelope one way or another, without being referred to as cell envelope stress. The Gram-negative definition – "Sensing and responding to damaged proteins in the extracytoplasmic compartments, collectively known as the cell envelope." (Ruiz and Silhavy, 2005) – is neither applicable nor helpful, due to the fundamental differences in cell envelope architecture between Gram-negative and -positive bacteria. At present, the best definition we can offer is derived from the approach by which CESRs and most of the regulatory systems involved have been identified and studied.

With very few exceptions, the model signaling systems orchestrating the Gram-positive envelope stress response were initially identified by one of three approaches. (1) They turned out to be responsible for an antibiotic resistance phenotype in (spontaneous) mutants, as

exemplified by the CiaRH system of *Streptococcus pneumoniae* (Guenzi *et al.*, 1994). (2) They were identified in the course of global gene expression (DNA microarray) studies to characterize the response of an organism when challenge with a cell wall antibiotic. This approach was used to decipher the complex regulatory network orchestrating CESR in the Gram-positive model bacterium *B. subtilis* (Cao *et al.*, 2002b; Mascher *et al.*, 2003; Pietiäinen *et al.*, 2005). (3) Their potential role was identified during phenotyping of systematic mutational libraries to elucidate the role of signal transducing systems in a given organism (Hancock and Perego, 2004).

For this review, we will use the term CESR for Gram-positive bacteria based on the following definition: "The cell envelope stress response of a Gram-positive bacterium consists of those signal transducing regulatory systems (and their regulons) that are involved in sensing and responding to the presence of cell wall antibiotics and other envelope perturbating conditions."

Obviously, such a narrow definition is prone to generating blind spots. Some of the resulting gaps can be closed by comparative approaches, based on the overlap between different stress responses. We are aware of the potential pitfalls of such a definition. We therefore offer it as a suggestion to be evaluated, challenged and subsequently improved by alternative experimental approaches. The analysis of conditional lethal or overexpressing mutants in cell wall biosynthesis genes by transcriptomics, as exemplified by recent work on the cell wall stress stimulon of *S. aureus* (McAleese *et al.*, 2006; Sobral *et al.*, 2007) is one very promising example of such an alternative approach that harbors great potential towards that goal.

Regulatory principles orchestrating CESR in Gram-positive and -negative bacteria. While the architecture of the cell envelope – and therefore the definition of the corresponding stress – differs greatly between Gram-positive and -negative bacteria, the regulatory principles orchestrating the corresponding stress responses are remarkably similar. CESR in the Gram-negative model organism *Escherichia coli* and related bacteria is well investigated and has been reviewed recently (Raivio, 2005; Rowley *et al.*, 2006; Ruiz and Silhavy, 2005). It is orchestrated by one alternative σ factor, three TCS and the phage shock protein response. For in-depth information on these systems, readers are referred to the cited review articles.

The essential extracytoplasmic function (ECF) σ factor σ^E, has been intensively studied in *E. coli*. It is induced in the presence of misfolded (outer membrane) proteins in the periplasm which can accumulate during growth at elevated temperatures (Ades, 2004; Alba and Gross, 2004; Rowley *et al.*, 2006). It controls a large regulon including proteins that act directly on

misfolded periplasmic proteins or are involved in the synthesis of lipopolysacchrides (Rhodius *et al.*, 2006).

The CpxAR TCS is activated by elevated external pH, misfolded periplasmic proteins or changes in the lipid composition of the inner membrane (Ruiz and Silhavy, 2005). It is subject to a negative feedback regulation exerted by the periplasmic protein CpxP (Buelow and Raivio, 2005; Fleischer *et al.*, 2007). Its regulon consists of more than 100 proteins, and partly overlaps with that of σ^E (De Wulf *et al.*, 2002).

The RcsBC TCS, together with the unstable auxiliary regulator RcsA, represents a complex phosphorelay system that is involved in regulating capsule biosynthesis, biofilm formation, and the expression of additional periplasmic and membrane proteins (Majdalani and Gottesman, 2005).

Little is known about BaeRS, the third envelope stress-sensing TCS of *E. coli*. It protects the cell from perturbations of the envelope caused by the presence of indole or misfolded proteins, acting in conjunction with the Cpx system (Raffa and Raivio, 2002). Furthermore, it regulates the expression of a multidrug-efflux pump, thereby conferring resistance to antimicrobial compounds, including β-lactam antibiotics (Hirakawa *et al.*, 2003; Nagakubo *et al.*, 2002).

The physiological role of the PspA-mediated phage-shock protein response is less well understood. It is induced by various stress conditions such as filamentous phage infection (hence the name), heat shock, osmotic shock, exposure to organic solvents and proton ionophores as well as long incubation under alkaline conditions. Strains defective in the Psp system show only minor physiological aberrations, for instance poor stationary phase survival, increased motility, slower protein secretion, and some defects in maintaining the membrane potential (i.e. proton motif force) when stressed (Darwin, 2005; Model *et al.*, 1997).

The same regulatory principles orchestrate the Gram-positive CESR, as outlined schematically in Fig. 1.2. Since envelope stress occurs outside the cytoplasm, all systems are comprised of transmembrane sensory components that detect their stimulus in the extracellular space. As for the Gram-negative bacteria, TCS and ECF σ factors are at the core of the Gram-positive envelope stress response. Both systems are functionally analogous in that they consist of two proteins, a membrane-anchored sensory component (sensor histidine kinase or anti-σ factor, respectively), and a cytoplasmic transcriptional regulator (response regulator or ECF σ factor, respectively). In both cases, the regulator is usually kept inactive in the absence of inducing conditions. Upon perceiving envelope stress by the sensory

component, the regulators become activated and direct (normally up-regulate) the expression of their target genes. The two regulatory principles differ in the mechanism by which the sensor and regulator proteins communicate with one another.

In the case of TCS (Fig. 1.2, middle), activation of the response regulator (RR) by its cognate sensor histidine kinase (HK) is based on the transfer of a phosphoryl group from a donor phospho-histidine in the C-terminal transmitter domain of the HK to an acceptor aspartate in the N-terminal receiver domain of the RR (Parkinson, 1993).

In contrast, communication between the anti-σ factor and its corresponding ECF σ factor is based on direct protein-protein interactions. In the absence of stress conditions, the anti-σ factor tightly binds the ECF σ factor, thereby keeping it inactive (Fig. 1.2, left). Under inducing conditions, the ECF σ factor is released, either by a conformational change or regulated proteolysis of the anti-σ factor. The σ factor becomes available for recruitment by RNA polymerase core enzyme to redirect transcription initiation to its specific target promoters, ultimately resulting in the upregulation of its regulon (Helmann, 2002).

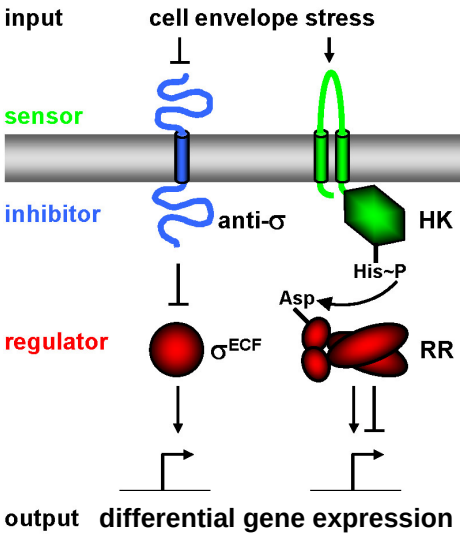

Figure 1.2. **Regulatory principles orchestrating cell envelope stress response in *Bacillus subtilis*.** Left: ECF σ factors, right: two-component systems (HK = histidine kinase; RR = response regulator). Sensor proteins are shown in green, inhibitor proteins in blue, transcriptional regulators in red. Arrows indicate activation, T-shaped lines repression. See text for details.

A distinct phage-shock protein system is absent in Gram-positive bacteria. Instead, its core component, the PspA protein, seems to be embedded in TCS-mediated envelope stress response, at least in the genera *Bacillus* and *Listeria* (Jordan *et al.*, 2006).

The regulatory network orchestrating cell envelope stress response in *Bacillus subtilis*: a case study

Over the last several years, a detailed picture of the CESR emerged for *B. subtilis*. Many of the underlying studies addressed the response of this Gram-positive model bacterium to cell wall antibiotics by applying transcriptomics approaches to identify the corresponding stimulons (= all genes that are differentially expressed, usually up-regulated, in the presence of a specific stimulus). Subsequent work identified the regulatory systems that orchestrate this response, thereby dissecting the stimulons into discrete regulons (Fig. 1.3). Initial work from the Helmann laboratory aimed to identify inducers of the ECF σ factor σ^W amongst cell envelope perturbating agents (Cao *et al.*, 2002b). Vancomycin was identified as the strongest stimulus and used for subsequent in-depth transcriptional profiling. In addition to σ^W, three other ECF σ factors were found to be induced by vancomycin, namely σ^M, σ^V, and σ^Y, the last two being induced only weakly (Cao *et al.*, 2002b).

Another study from the same group analyzed the bacitracin stress response, which is orchestrated by an even larger number of signal transducing systems (Mascher *et al.*, 2003). In addition to σ^M and the σ^B-mediated general stress response (Price, 2002), three TCS respond to the extracellular presence of bacitracin. The LiaRS TCS is strongly induced by both vancomycin and bacitracin. The paralogous TCSs BceRS and YvcPQ both specifically regulate the expression of an ABC transporter, which – in case of the Bce system – confers high level bacitracin resistance (Mascher *et al.*, 2003; Ohki *et al.*, 2003a). A second bacitracin resistance determinant, the undecaprenyl pyrophosphate phosphatase BcrC (Bernard *et al.*, 2005) under the dual control of two ECF σ factors, σ^M, and σ^X (Cao and Helmann, 2002; Ohki *et al.*, 2003b), was also induced by bacitracin.

More recently, the CESR of *B. subtilis* was exploited further with regard to β-lactams, D-cycloserine, fosfomycin, and CAMPs (Hutter *et al.*, 2004; Pietiäinen *et al.*, 2005). The transcriptional profiles of the first three compounds were part of a broader panel aimed to apply stimulon patterns for predicting the mechanism of action of unknown compounds, and not analyzed in detail (Hutter *et al.*, 2004). In contrast, the work on CAMPs provided further insights and helped to complete the picture of the regulatory network orchestrating CESR in *B. subtilis*. Challenges with two naturally occurring cationic peptides, human LL-37 and porcine PG-1, and a synthetic analog, poly-*L*-lysine, provoked distinct response patterns, orchestrated by three ECF σ factors (σ^M, σ^W, and σ^X), the LiaRS TCS and another BceRS homolog, the YxdJK TCS (Pietiäinen *et al.*, 2005). The latter specifically responded to LL-37 only, without conferring resistance against this compound. This study also indicated a

significant and surprising amount of cross-dependency between TCS- and ECF-mediated responses, even though no direct regulatory overlap has ever been observed. Deletion of ECF σ factors strongly reduced the TCS-mediated response to CAMPs. This observation is reminiscent of the results obtained earlier for the alkaline shock response, where a similar link between σ^W and the LiaRS-dependent gene expression was already observed (Wiegert *et al.*, 2001). The reason for this interference in signal transduction is unclear at the moment.

ECF s factors involved in orchestrating CESR of *B. subtilis*. The ECF (or Group 4) σ factors belong to the σ^{70} family of bacterial σ factors (Helmann, 2002; Lonetto *et al.*, 1994). They share a number of common features: (i) They are small proteins, harboring only two of the four conserved regions of the primary σ factors, namely region 2 and region 4; (ii) They recognize promoters with a highly conserved 'AAC' motif in the -35 region; (iii) They are usually cotranscribed with their cognate anti-σ factor, a transmembrane protein; (iv) They are often involved in regulating functions associated with some aspect of the cell envelope or transport processes (Butcher *et al.*, 2007; Helmann, 2002). The genome of *B. subtilis* encodes seven ECF σ factors (Helmann and Moran, 2002), of which three (σ^M, σ^W, and σ^X) play a role in orchestrating CESR.

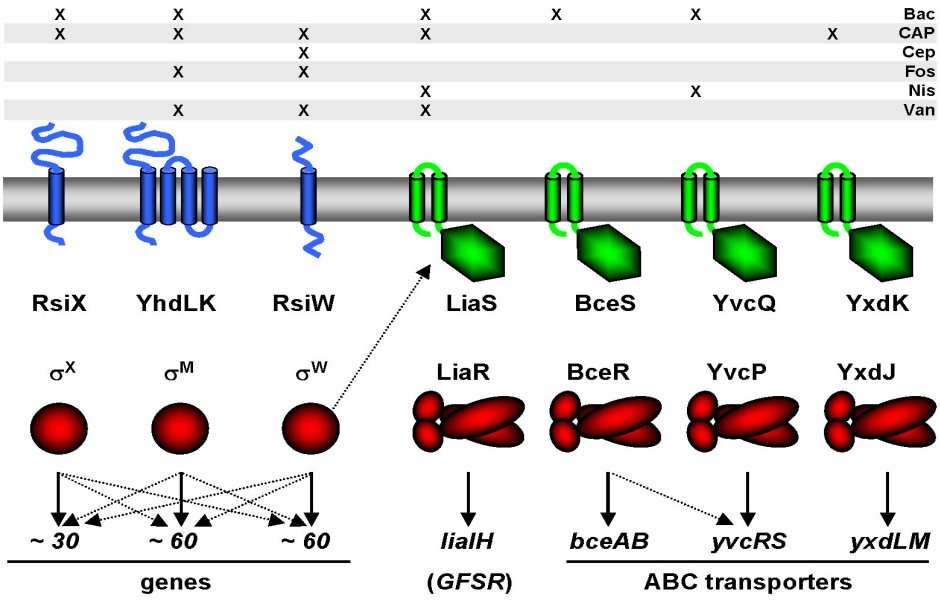

Figure 1.3. **The regulatory network of cell envelope stress response in *Bacillus subtilis*.** The same symbols and color-code was applied as in Fig. 1. Dotted lines indicate cross-regulation. The antibiotic specificity for each system is shown above. Bac = bacitracin; CAP = cationic antimicrobial peptides (note that individual peptides will only induce a certain subset of the regulators indicated); Cep = cephalosporin C; Fos = fosfomycin; Nis = nisin; Van = vancomycin. It should be noted that because of the known regulatory overlap between ECF σ factors, an clear assignment of the inducer spectrum, is not as unambiguously possible as indicated in this figure. See text for details.

s^{W} is the best-understood ECF σ factor in *B. subtilis* (Helmann, 2006). It is induced by a number of cell wall antibiotics, such as vancomycin, cephalosporin C, and the CAMPs LL-37 and PG-1, but also by alkaline shock (Cao *et al.*, 2002b; Pietiäinen *et al.*, 2005; Wiegert *et al.*, 2001). Promoter consensus search, in combination with *in vivo* and *in vitro* approaches identified ~30 target promoters (controlling about 60 genes) that are expressed (at least partially) in a σ^W-dependent manner (Cao *et al.*, 2002a; Huang *et al.*, 1999). It was postulated that σ^W controls an 'antibiosis' regulon, based on the inducer spectrum and the putative function of many of its target genes (Helmann, 2002). This hypothesis was subsequently confirmed by studies demonstrating that σ^W-controlled genes confirm resistance against fosfomycin (Cao *et al.*, 2001), as well as a number of antimicrobial compounds synthesized by closely related *Bacillus* species (Butcher and Helmann, 2006). Moreover, σ^W also provides resistance to the antimicrobial protein SdpC (Butcher and Helmann, 2006), which is produced by sporulating *B. subtilis* cells to lyse non-sporulating sibling cells in a process termed cannibalism (Ellermeier *et al.*, 2006; Gonzalez-Pastor *et al.*, 2003).

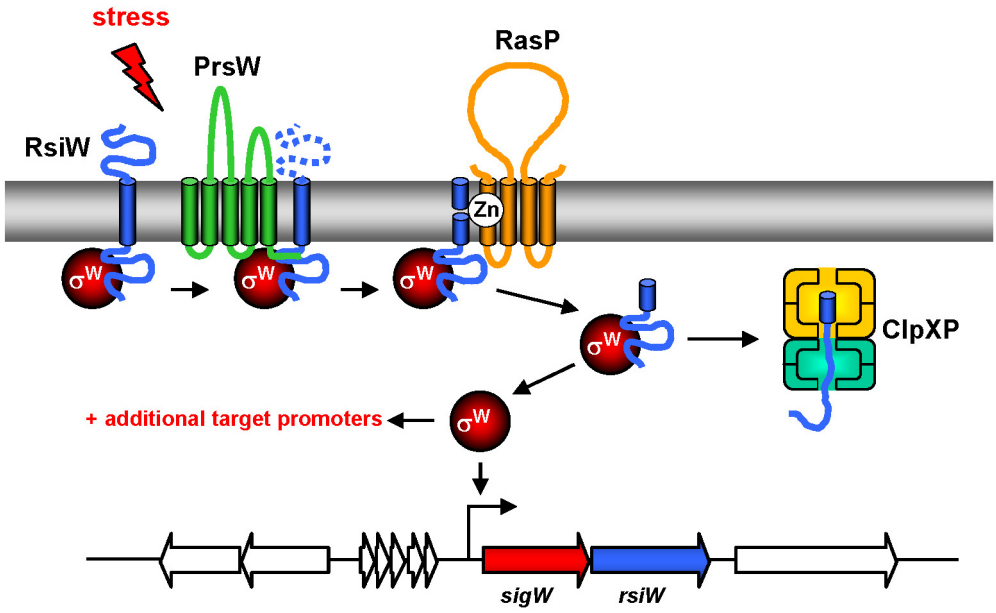

Figure1. 4. Signal transduction mediated by ECF s factors. The proteolytic cascade leading to anti-σ factor degredation and ECF σ factor activation is illustrated. For color-code, see legend to figure 1. The stimulus is represented by the red arrow. Additional proteins are shown in yellow. See text for further details. This figure is based on a presentation kindly provided by Thomas Wiegert.

The *sigW* gene is co-transcribed with *rsiW*, encoding the cognate membrane-anchored anti-σ factor. A direct protein-protein interaction between σ^W and RsiW was verified by yeast two-hybrid analysis (Yoshimura *et al.*, 2004). Like *E. coli* σ^E, σ^W activation is based on the

regulated proteolytic degradation of its cognate anti-σ factor RsiW. Three consecutive proteolytic steps cleave first the extracytoplasmic, then the membrane-spanning, and finally the cytoplasmic domains of RsiW, respectively (Fig. 1.4). This proteolytic cascade is initiated by PrsW-dependent site-1 cleavage (Ellermeier and Losick, 2006; Heinrich and Wiegert, 2006). PrsW, a membrane-anchored novel protease with 5 transmembrane helices is therefore the prime candidate for the real sensory module in σ^W-dependent signaling. Subsequently, site-2 cleavage by RasP-mediated regulated intramembrane proteolysis (homologous to *E. coli* RseP) generates a soluble N-terminal fragment of RsiW (Schöbel *et al.*, 2004). The latter is degraded by the cytoplasmic ClpXP proteolytic complex (Zellmeier *et al.*, 2006), thereby ultimately releasing the active σ factor, again similar to the mode of σ^E-activation in *E. coli* (Ades, 2004).

s^X was the first ECF σ factor to be studied in detail in *B. subtilis*. Its gene is co-transcribed with *rsiX*, encoding the corresponding anti-σ factor, and primarily expressed in logarithmic and early stationary phase (Huang *et al.*, 1997). Interaction between the σ factor:anti-σ pair could be demonstrated by yeast two-hybrid system (Yoshimura *et al.*, 2004). Its regulon consists of ~ 30 genes, organized in 15 transcriptional units (Cao and Helmann, 2004). So far, the major physiological role of σ^X is the modulation of the overall envelope net charge, due to the regulation of the *dltABCDE* and the *pssA-ybfM-psd* operons. The products of the *dlt* operon introduce positively charged amino groups into TAs, thereby lowering the overall negative net charge of the cell wall (Perego *et al.*, 1995). A comparable role is exhibited by PssA/Psd, which together catalyze the synthesis of the neutral lipid phosphatidylethanolamine. Since the cytoplasmic membrane has a negative net charge due to the abundance of anionic phospholipids, increased incorporation of neutral lipids therefore also lowers the overall negative net charge (Cao and Helmann, 2004). Altering the overall net charge of the cell envelope has been shown to affect both autolysis and resistance to CAMPs. Consequently, a *sigX* mutant has an increased rate of autolysis and is more sensitive to CAMPs (Cao and Helmann, 2004).

s^M is also co-transcribed with its negative regulators, encoded by *yhdL* and *yhdK* that together form the anti-σ complex (Horsburgh and Moir, 1999): a direct protein-protein interaction between σ^M and the N-terminal fragment of YhdL could be demonstrated, as well as specific interactions between the two membrane proteins YhdL and YhdK (Yoshimura *et al.*, 2004). The *sigM-yhdLK* operon is maximally expressed in early to mid-logarithmic growth phase (Horsburgh and Moir, 1999). It is induced by cell wall antibiotics, such as bacitracin, vancomycin or fosfomycin, but also by acidic pH, heat, ethanol, and superoxide stress

(Thackray and Moir, 2003), and confers resistance to bacitracin, and high salinity (Cao and Helmann, 2002; Horsburgh and Moir, 1999; Mascher *et al.*, 2003). In the *B. subtilis* strain W23, σ^M is induced by phosphate depletion and involved in TA biosynthesis (Minnig *et al.*, 2005). A study of 12 σ^M target promoters in the reference strain W168 has been published recently (Jervis *et al.*, 2007), but preliminary *in vitro* data identified many more target genes (John Helmann, personal communication).

A significant amount of regulatory overlap between the target genes of all three ECF σ factors has been demonstrated both *in vivo* and *in vitro*, with many promoters being recognized by two, or even all three ECF σ factors (Cao and Helmann, 2002; Huang *et al.*, 1998; Qiu and Helmann, 2001). It can be envisioned that target gene discrimination (from the available pool of genes preceded by an ECF-type promoter) is therefore a combined result of the timing of expression of individual ECF σ factors during the life cycle and the presence of specific inducing conditions, rather than promoter selectivity based on sequence preference alone. This overlap is not only restricted to the expression of individual target genes, but also manifests itself in the regulation of complex phenotypes, such as overall cell envelope integrity, pellicle formation and colony morphology (Mascher *et al.*, 2007). Moreover, in *B. subtilis* W23 the concerted action of σ^X and σ^M is required for septum formation and cell wall biosynthesis (Minnig *et al.*, 2003). Both ECF σ factors together regulate the synthesis of wall TAs, which consist of poly(ribitol phosphate) in this strain, in contrast to the poly(glycerol phosphate)-containing TAs of the sequenced reference strain *B. subtilis* W168 (Lazarevic *et al.*, 2002).

Cell envelope stress-sensing TCS of *B. subtilis*. The transcriptomics approaches described above identified four TCS that respond to some aspect of cell envelope stress in *B. subtilis*, with three being induced by bacitracin alone. During the in-depth analysis of the regulatory network orchestrating the bacitracin response, it was noticed that all TCS involved share some overall similarities with regard to the domain architecture of their HKs. These membrane-anchored sensor kinases are characterized by a very short N-terminal input domain, consisting solely of two deduced transmembrane helices with hardly any periplasmic linker (less than 10 amino acids for most) in between (Mascher *et al.*, 2003). Subsequent comparative genomics analysis revealed that these so-called intramembrane-sensing HKs are widespread and conserved in *Firmicutes* bacteria, but can also be found in the Actinobacteria (Hutchings *et al.*, 2004; Mascher, 2006b). Two conserved sub-groups can be distinguished in *Firmicutes* bacteria, and both groups are involved in mediating CESR in *B. subtilis* (Mascher, 2006b): (1)

LiaRS-like three-component systems, and (2) BceRS-like TCS that are functionally linked to ABC transporters (Fig. 1.5).

The LiaRS TCS – a cell envelope stress-sensing three-component system. This TCS was originally identified as part of the complex regulatory network orchestrating the bacitracin stress response in *B. subtilis* (Mascher *et al.*, 2003). It also strongly responds to the external presence of other cell wall antibiotics that interfere with the lipid II cycle, such as ramoplanin, vancomycin, or CAMPs (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005). LiaRS-dependent gene expression is also induced by alkaline shock, detergents, ethanol, phenol, organic solvents, and secretion stress, albeit to a lesser extent (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Pietiäinen *et al.*, 2005; Tam le *et al.*, 2006; Wiegert *et al.*, 2001). Moreover, its activity is influenced by the density of the negative net charge of the cell envelope: The LiaRS system responds more strongly to the CAMP LL-37 and secretion stress in cells defective in the Dlt system, which has an overall higher negative net charge due to its inability to incorporate D-alanine into its TAs (Hyyryläinen *et al.*, 2007).

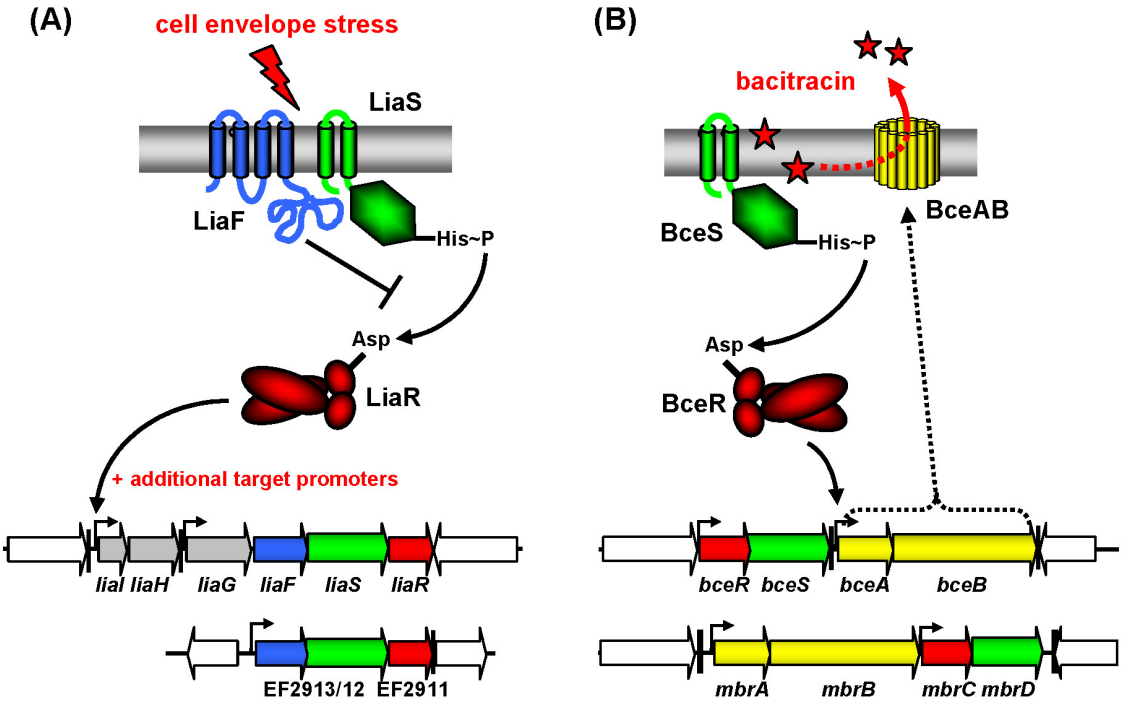

Figure 1.5. Cell envelope stress-sensing two-component systems in *Firmicutes* bacteria. (A) LiaFSR-like three-component systems. (B) BceRS-BceAB-like TCS-ABC transporter connection. Symbols and color-code as before. See text for details. This figure was taken from (Mascher, 2006b), with modifications.

The LiaRS TCS is functionally and genetically linked to a third protein, LiaF, which acts as a strong inhibitor of LiaR-dependent gene expression (Jordan *et al.*, 2006) (Fig. 1.5A). The LiaRS-LiaF three-component system is conserved by sequence, genomic context and function in Gram-positive bacteria with a low G+C content (Jordan *et al.*, 2006; Mascher, 2006b). The *lia* locus consists of six genes, *liaIH-liaGFSR*. A basal expression level of the last four genes, *liaGFSR*, encoding the three-component system (*liaFSR*) and a putative membrane-anchored hypothetical protein (*liaG*), is ensured by a weak constitutive promoter upstream of *liaG*. In contrast, expression of the *liaIH* operon from P_{liaI} is completely LiaR-dependent (Jordan *et al.*, 2006).

In *B. subtilis*, only two promoters are known to be regulated by the LiaRS TCS: the *liaI* promoter (P_{liaI}) and the *yhcY* promoter (Jordan *et al.*, 2006), with P_{liaI} being the primary target. P_{liaI} is tightly regulated: in the absence of a stimulus, it is virtually switched off, while addition of bacitracin results in an about 200-fold induction (Mascher *et al.*, 2003; Mascher *et al.*, 2004). In contrast to P_{liaI}, a LiaR-dependent P_{yhcY}-activity was only observed in a *liaF* mutant, i.e. in the absence of the LiaRS-inhibitor protein (Jordan *et al.*, 2006; Mascher *et al.*, 2004).

P_{liaI} is also induced in the absence of exogenous stimuli at the onset of stationary phase (Jordan *et al.*, 2007). This time point in the *B. subtilis* life cycle is characterized by the initiation of a complex regulatory cascade that allows *Bacillus* to adapt to worsening living conditions, which can ultimately lead to the formation of dormant endospores (Errington, 2003; Msadek, 1999; Phillips and Strauch, 2002). It was demonstrated that P_{liaI} is directly repressed by binding of the transition state regulator AbrB within the promoter sequence, thereby acting as a roadblock to prevent premature P_{liaI} activity during logarithmic growth. AbrB repression is released during the transition state by Spo0A, the master regulator of sporulation and P_{liaI} is induced by an unidentified endogenous stimulus, resulting in the expression of the *liaIH* operon. While AbrB-binding is sufficient to inhibit the endogenous growth-dependent induction of P_{liaI}, it can be bypassed completely by exogenous induction with cell wall antibiotics. Taken together, LiaRS-dependent gene expression is embedded in transition phase regulation in *B. subtilis*, and the activity of its primary target promoter, P_{liaI}, is controlled by at least five regulators.

In contrast to the detailed knowledge on the mechanism of LiaFSR-dependent signal transduction, the physiological role of its primary target genes, *liaIH*, is largely unknown. LiaI is a small hydrophobic protein of unknown function with two putative transmembrane helices. LiaH is a member of the phage-shock protein family. While the strong induction of

liaIH (and to a lesser degree also *liaGFSR*) by cell envelope stress is well documented (see above), mutational analyses of the *lia* locus so far failed to identify strong phenotypes associated with them, and deletion of *lia* genes did not alter the sensitivity of the corresponding mutants to the known inducers of the Lia system.

BceRS and its paralogs – a TCS-ABC transporter connection. A regulatory connection between TCS and ABC transporters encoded by genes located directly downstream, was already described some years ago for the *Bacillus/Clostridium* group, and the authors demonstrated a TCS-dependent expression of the ABC transporter genes for *B. subtilis* (Joseph *et al.*, 2002). Its genome harbors three such TCS-ABC transporter modules, *bceRS-bceAB* (formerly *ytsABCD*), *yvcPQ-yvcRS*, and *yxdJK-yxdLM* (Joseph *et al.*, 2002; Mascher, 2006b).

The BceRS-BceAB system is the best-understood of these detoxification modules (Fig. 1.5B). The BceRS TCS specifically responds to the extracellular presence of bacitracin. Its activation leads to binding of phosphorylated BceR to an inverted repeat upstream of the *bceA* promoter, resulting in a strong up-regulation of *bceAB* expression. The encoded ABC transporter is an efficient bacitracin resistance determinant and is thought to facilitate its removal (Mascher *et al.*, 2003; Ohki *et al.*, 2003a). Recently, it was demonstrated that the HK BceS alone is unable to sense bacitracin. Instead, the corresponding ABC transporter BceAB is crucial for bacitracin perception. Moreover, ATP binding/hydrolysis by the nucleotide-binding subunit BceA is a prerequisite for stimulus perception, indicating that BceRS responds to bacitracin transport by BceAB, rather than the extracellular presence of this antibiotic (Rietkötter and Mascher, 2007).

Only very little is known about the other two systems. The YxdJK-YxdLM system has been analyzed genetically, without gaining insights into its biological role (Joseph *et al.*, 2004). Again, the expression of *yxdLM*, encoding the ABC transporter, is strictly dependent on the corresponding RR YxdJ. More recently, the human CAMP LL-37 was identified as a strong inducer of *yxdLM* expression (Pietiäinen *et al.*, 2005). Therefore, it is tempting to speculate that YxdJK-YxdLM functions as a CAMP-specific detoxification module. But the exact nature of its substrate remains to be identified.

Induction of *yvcRS* expression was shown to be induced by bacitracin and nisin (Hansen *et al.*, 2007; Mascher *et al.*, 2003). While the bacitracin induction is weak and presumably indirect (Rietkötter and Mascher, 2007), the nisin-dependent upregulation could point towards

a role of this module in mediating resistance against some lantibiotic. But again, this assumption needs to be verified.

Signal-transducing systems orchestrating cell envelope stress response in other Gram-positive bacteria

In the following paragraphs, we will summarize the knowledge of cell envelope stress-sensing regulatory systems in other Gram-positive bacteria. The first sections will address systems homologous to those of *B. subtilis*, i.e. ECF σ factors, and TCS with intramembrane-sensing HKs (LiaRS- and BceRS-like TCS) (Table 1.1).

Cell envelope stress-sensing ECF σ factors in other Gram-positive bacteria

In contrast to the situation for TCS, disappointingly little is known on ECF σ factors beyond *B. subtilis*. A quick glance on the distribution and presence of ECF σ factors in Gram-positive genomes shows that these regulators are abundant in most Actinobacteria, whereas their presence is very heterogeneous in the *Firmicutes* bacteria (Staron *et al.*, 2007)(Table 1.1). All *Bacillus* species are rich in ECF σ factors, while these regulators are almost absent in the lactic acid bacteria and other cocci (Table 1.1). In addition to *S. coelicolor* σ^E, only one study addressed the role of ECF σ factors in Gram-positive CESR, so far. The genome of *B. licheniformis*, a close relative of *B. subtilis*, contains nine ECF σ factors, of which six are direct orthologs to the *B. subtilis* proteins σ^M, σ^V, σ^W, σ^X, σ^Y, and σ^{ylaC} (Wecke *et al.*, 2006). A homolog of the seventh ECF σ factor from *B. subtilis*, σ^Z, is absent in the genome of *B. licheniformis*. The three novel ECF σ factors where designated σ^{ecfG}, σ^{ecfH}, and σ^{ecfI}. In-depth transcriptional profiling demonstrated that seven of the nine ECF σ factors respond to cell envelope stress in this organism: Six were significantly induced by vancomycin (σ^M, σ^V, σ^W, σ^X, σ^Y, and σ^{ecfH}), three by bacitracin (σ^M, σ^V, σ^Y), while σ^{ecfG} specifically responded to β-lactams (Wecke *et al.*, 2006). The contribution of these regulators to mediating resistance against these antibiotics has not yet been explored.

LiaFSR-like three-component systems: conserved cell envelope stress-sensors in *Firmicutes* bacteria

LiaFSR-homologous three-component systems are widespread amongst the *Firmicutes* bacteria, with the noteworthy exception of the genera *Lactobacillus* and *Clostridium* (Table 1.1). Two groups can be distinguished, based on the genomic context of the corresponding

Table 1.1. Distribution and conservation of cell envelope stress-sensing regulatory systems in the genomes of *Firmicutes* bacteria

				Two-component systems with[3] intramembrane-sensing HK	
Organism	**Size [Mb][1]**	**ECF[2]**	**S[1]**	**LiaRS[4]**	**BceRS[4]**
Bacillus anthracis	5.23-5.5	16	47-50	1 (I)	1
B. cereus	5.43-5.84	13-18	45-50	1 (I)	5
B. clausii	4.3	4	40	1 (I)	4
B. halodurans	4.2	9	45	1 (I)	5
B. licheniformis	4.22	9	34-35	1 (I)	4
B. subtilis	4.21	7	33	1 (I)	3
B. thuringiensis	5.31	16	50-51	1 (I)	3
Clostridium acetobutylicum	4.13	3	40	-	4
C. difficile	4.3	2	54	-	7
C. perfringens	2.96-3.26	4	18-24	-	2
C. tetani	2.87	10	28	-	2
Desulfitobacterium hafiense	5.73	20-23	70	-	5
Enterococcus faecalis	3.36	2	18	1 (II)	1
Geobacillus kaustophilus	3.59	3	27	1 (I)	3
Lactobacillus acidophilus	1.99	-	8	-	1
L. brevis	2.34	-	10	-	-
L. casei	2.92	-	17	-	5
L. delbrueckii bulgaricus	1.86	1	6-7	-	-
L. gasseri	1.89	-	5	-	-
L. johnsonii	1.99	-	9	-	-
L. plantarum	3.35	-	15	-	-
L. sakei 23K	1.88	-	10	1 (II)	1
L. salivarius	1.83	-	6	-	-
Lactococcus lactis	2.37-2.6	1	7-9	1 (II)	1
Leuconostoc mesenteroides	2.08	-	10	-	-
Listeria innocua	3.09	1	17	1 (I)	1
L. monocytogenes	2.91-2.94	1	15-16	1 (I)	1
Moorella thermoacetica	2.63	3	16	-	-
Oceanobacillus iheyensis	3.63	9	20	2 (I/II)	1
Pediococcus pentosaceus	1.83	-	8	-	1
Staphylococcus aureus	2.74-2.9	-	14-17	1 (II)	2
S. epidermidis	2.56-2.64	-	16	1 (II)	2
S. haemolyticus	2.69	-	16	1 (II)	3
S. saprophyticus	2.58	-	11	1 (II)	-
Streptococcus agalactiae	2.13-2.21	1	20-22	1 (II)	1
S. mutans	2.03	-	14	1 (II)	1
S. pneumoniae	2.04-2.16	-	14	1 (II)	-
S. pyogenes	1.84-1.94	-	12-14	1 (II)	-
S. thermophilus	1.8-1.86	-	8-10	1 (II)	1
Thermoanaerobacter tengcongensis	2.69	6	20	-	-

[1] Information on genome size and total numbers of TCS is derived from the MiST (Ulrich and Zhulin, 2007) and Genome Atlas (Pedersen *et al.*, 2000; Hallin and Ussery, 2004; Kiil *et al.*, 2005b) databases at http://genomics.ornl.gov/mist/ and http://www.cbs.dtu.dk/services/GenomeAtlas/index.php, respectively.
[2] Numbers of ECF σ factors are derived from a comprehensive ECF database and classification system, and the σ factor census published in Genome Atlas (Kiil *et al.*, 2005a; Staron *et al.*, 2007).
[3] Identification of specific TCS in bacterial genomes is based on genomic BLAST searches (blastp) of either the complete proteins at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi or – where applicable – signature domains thereof, such as the extracytoplasmic senor domains of HK (i.e for CiaRH), to increase the specificity of the search. Moreover, genomic context conservation was also used as a criterion, where applicable (LiaRS, BceRS, VanRS, YycFG, LytSR), using the MicrobesOnline (Alm *et al.*, 2005) or The SEED databases at http://www.microbesonline.org/ and http://theseed.uchicago.edu/FIG/index.cgi, respectively.
[4] Identification of TCS with intramembrane-sensing histidine kinases is derived from a comparative genomics analysis (Mascher 2006). Two groups of LiaRS-like TCS can be distinguished, based on their genomic context. Group I, *liaIH(G)FSR*; Group II, *liaFSR*.

loci (Jordan *et al.*, 2006; Mascher, 2006b) (Fig. 1.5A). LiaRS-homologs in bacilli (Group I) are embedded in the *liaIH-(G)FSR* locus (with a *liaG*-like gene only present in *B. subtilis, B. licheniformis* and *B. halodurans*). Group II only shows a conservation of the *liaFSR* locus. In *Listeria* species, *liaIH*- and *liaFSR*-like genes are organized as two separate operons, but both still seem to be under the transcriptional control of the LiaRS systems (Jordan *et al.*, 2006). Therefore, they are also listed as group I (Table 1.1). This difference in genomic context also seems to indicate a different cellular role for these systems. Based on the available data, TCS embedded in *liaIH-(G)FSR*-like loci primarily (maybe even exclusively) regulate their own expression, whereas Group II LiaRS-like TCS seem to orchestrate much larger regulons and seem to represent the primary cell envelope stress-responding systems in the corresponding organisms. This hypothesis is supported by data from *Staphylococcus aureus* VraSR and *Lactococcus lactis* CesSR.

VraSR is the best-understood LiaRS homolog, so far. It was originally identified as an upregulated locus in a VRSA (vancomycin-resistant *S. aureus*) strain, compared to a VSSA (sensitive) strain (Kuroda *et al.*, 2000). VraSR was also upregulated in a VISA (vancomycin intermediate resistant *S. aureus*) strain relative to an isogenic VSSA strain (McAleese *et al.*, 2006). The *vraSR* genes are co-transcribed with two upstream genes, termed *orf1* and the *liaF*-homolog *yvqF*, and a VraR-dependent auto-induction of this four gene operon was demonstrated (Yin *et al.*, 2006). The VraSR system is strongly induced by a number of cell wall antibiotics, including vancomycin, teicoplanin, β-lactams, bacitracin, and D-cycloserine, but not by general stresses, such as heat, osmotic shock or pH shifts (Kuroda *et al.*, 2003). It also responds to sub-lethal perturbations of cell wall biosynthesis caused by the depletion of *pbpB* (encoding an essential penicillin-binding protein crucial for peptidoglycan crosslinkage) and *murF* (its gene product catalyzing the last cytoplasmic step in peptidoglycan precursor biosynthesis, addition of the D-alanyl-D-alanine dipeptide to the UDP-linked MurNAc-tripeptide) (Gardete *et al.*, 2006; Sobral *et al.*, 2007). Surprisingly, preliminary data indicate that *S. aureus* VraSR – in contrast to the LiaRS system of *B. subtilis* – does not respond to CAMPs such as LL-37 (Pietiäinen *et al.*, 2007).
Knock-out of *vraSR* resulted in increased susceptibility towards all of its inducers (with the exception of D-cycloserine) and fosfomycin (Gardete *et al.*, 2006; Kuroda *et al.*, 2003). Transcriptional profiling identified 46 genes that were induced by vancomycin in a VraSR-dependent manner, including its own locus (positive autoregulation) and a number of genes encoding functions involved in cell envelope biogenesis, such as *murZ* (peptidoglycan

monomer precursor biosynthesis), *pbp2*, *sgtB* (peptidoglycan polymerization), or *tagA* (TA biosynthesis) (Kuroda *et al.*, 2003).

CesSR was originally described as LlkinD/LlrD in a systematic analysis of six TCS from *L. lactis* strain MG1363. These authors were unable to generate an insertion mutant of *llkinD*, encoding the HK, but the corresponding RR mutant MGRrD showed an increased salt-/osmosensitivity (O'Connell-Motherway *et al.*, 2000). Recently, it was shown to respond to the extracellular presence of the lactococcal bacteriocin Lcn972 and renamed CesSR (Martinez *et al.*, 2007) – an unfortunate choice, since it should not be confused with another cell envelope stress-sensing TCS, CesRK of *Listeria monocytogenes* (Kallipolitis *et al.*, 2003), to which it bears no similarity (see further below for details). Transcriptome analysis revealed that the expression of 26 genes was significantly upregulated in the presence of Lcn972, of which 23 responded in a CesSR-dependent manner. Many of these genes encode putative membrane or stress-related proteins. As with all LiaRS-homologs, the corresponding locus of *L. lactis* is subject to positive autoregulation, and includes a third *liaF*-homologous gene, *yjbB* (*llmg1650*), which is located directly upstream of *cesSR*. The CesSR TCS is also induced by other cell wall antibiotics, such as bacitracin, vancomycin, and plantaricin C. CesR disruption results in a slight increase in susceptibility to bacitracin, nisin, and plantaricin C, all of which interfere with the Lipid II cycle (Martinez *et al.*, 2007).
One of the most strongly induced genes of the CesSR regulon is *spxB* (formerly *yneH*), one of seven paralogs of *L. lactis* that are homologous to *B. subtilis* Spx (Nakano *et al.*, 2003). SpxB expression and subsequent interaction with RNA polymerase leads to upregulation of *oatA*, ultimately resulting in increased *O*-acetylation of peptidoglycan, rendering the cells more resistant to autolysis and lysozyme treatment (Veiga *et al.*, 2007). The authors of this study hypothesize that this novel resistance mechanism is induced upon CesS-dependent sensing of cell envelope stress, such as peptidoglycan hydrolysis caused by the presence of lysozyme.

LiaFSR-homologs in other *Firmicutes* bacteria. Vancomycin treatment of both a sensitive and a tolerant strain of *Streptococcus pneumoniae* resulted in an upregulation of the SP0385-0387 locus, encoding the *liaFSR*-homologous TCS03 (Haas *et al.*, 2005).
Enhanced nisin resistance in *Listeria monocytogenes* is associated with increased expression of *hpk1021* and *pbp2229*, encoding a LiaS-homologous HK and a penicillin-binding protein, respectively (Gravesen *et al.*, 2001). It could be demonstrated that disruption of both genes abolished the nisin resistance phenotype, and that *pbp2229* expression depends on HPK1021

(Gravesen *et al.*, 2004). Moreover, a mutant harboring an in-frame deletion of the corresponding RR, RR1022, showed a slightly increased ability to invade Cos-1 fibroblast cells compared to the isogenic wild type strain (Williams *et al.*, 2005).

In-depth transcriptional profiling of the CESR in *B. licheniformis* revealed that the LiaFSR-homologous YvqFEC system is strongly induced by bacitracin and nisin. Weaker induction was observed for vancomycin and D-cycloserine (Wecke *et al.*, 2006).

Systematic inactivation and subsequent phenotypic characterization of TCS in *Enterococcus faecalis* revealed that a mutant of the LiaR-homologous protein RR03 shows increased bacitracin sensitivity (Hancock and Perego, 2004), indicative for a role of the corresponding TCS in counteracting cell envelope stress.

Taken together, LiaFSR and its homologs are conserved cell envelope stress-sensing three-component systems in *Firmicutes* bacteria that play a crucial role in responding and counteracting damages caused by the extracellular presence of cell wall antibiotics and other perturbations of cell envelope integrity. While this signaling module is clearly conserved, its output is not: It shows a remarkable variability with regard to regulon size and cellular role, indicating that its regulatory features have been 'used' by evolution and adapted to the physiological requirements and life style of the individual organism.

BceRS-like detoxification modules: a TCS-ABC transporter connection conserved in *Firmicutes* bacteria

As mentioned above, a regulatory relationship between TCS and ABC transporters has been noticed before in the *Bacillus/Clostridium* group (Joseph *et al.*, 2002). A recent analysis of Gram-positive genomes demonstrates the predominance of such TCS-ABC transporter modules in these two genera (Table 1.1). Moreover, this group completely overlaps with a conserved sub-group of TCS harboring intramembrane-sensing HKs (Mascher *et al.*, 2003; Mascher, 2006b). Work from *B. subtilis* established that in these detoxification modules the TCS respond to the extracellular presence of antimicrobial compounds. Upon activation, they upregulate the expression of ABC transporters, encoded by neighboring (usually downstream) genes (Joseph *et al.*, 2002; Joseph *et al.*, 2004; Ohki *et al.*, 2003a) (Fig. 1.5B). The ABC transporter then facilitates removal of the harmful drug, thereby also removing the initial stimulus of the system, which finally shuts down again (Mascher, 2006b). In contrast to the Lia system, the TCS of the detoxification modules are not autoregulated.

Relatively little is known about these systems beyond *B. subtilis*, despite their overall abundance. Two bacitracin resistance modules have been described. The BacRS-BcrABC

module confers bacitracin self-resistance in the producing strain *B. licheniformis* ATCC10716 (Neumüller *et al.*, 2001; Podlesek *et al.*, 1995). In *Streptococcus mutans*, a similar module is encoded by the *mbrABCD* locus (Tsuda *et al.*, 2002). Here, the genes encoding the TCS, *mbrCD*, are located downstream of the genes for the ABC transporter MbrAB (Fig. 1.5B). The genome of *B. licheniformis* DSM13 encodes four TCS-ABC transporter modules, two of which – *ytsABCD* and *yxdJ*-Bli04268-70 – are induced by bacitracin. The second system also responds to nisin (Wecke *et al.*, 2006). Recently, the GraRS TCS of *S. aureus* was described to regulate expression of the VraFG ABC transporter, encoded by genes located directly downstream of *graSR*. This module is involved in resistance to vancomycin and – more pronounced – polymyxin B (Meehl *et al.*, 2007). The genes of another related system, GtcRS of *B. brevis*, are located next to the *grs* operon encoding the multienzymes involved in the biosynthesis of the peptide antibiotic gramicidin (Turgay and Marahiel, 1995). While no functional characterization has been carried out, a role of this TCS in gramicidin autoimmunity seems likely.

Two unusual examples of BceRS-like TCS were described in *Staphylococcus epidermidis* and *L. monocytogenes*. These TCS, while sharing the overall sequence similarities and genomic context conservation (Mascher, 2006b), differ from most TCS of this group by regulating more than one operon encoding an ABC transporter. ApsXRS is a three-component antimicrobial peptide-sensing system in *S. epidermidis* that is conserved in other staphylococci (Li *et al.*, 2007). All three proteins, the TCS ApsRS as well as the third protein, ApsX (its exact function still unknown), are crucial for CAMP sensing. This three-component system responds to a wide range of structurally unrelated CAMPs, including LL-37 (α-helical), *b*-defensin 3 (bridged), histatin (His-rich) and the bacterial lantibiotic nisin. Preliminary results indicate that the HK ApsS might sense these CAMPs by direct binding to the 9 amino acid short extracellular loop, which has a high density of negatively charged amino acids (Li *et al.*, 2007). But a role of the neighboring ABC transporter, encoded by *vraFG*, in stimulus perception has not been addressed so far.

A homologous system, VirRS, was described as a TCS critical for the virulence of *L. monocytogenes* (Mandin *et al.*, 2005). It lacks an ApsX homolog and its genomic context differs from the *aps* system. But the regulons of both systems are almost identical in size and functions. Both regulate the expression of the *dlt* operon, the *mprF* gene, and an operon encoding an ABC transporter. The first two loci are involved in lowering the net surface charge of the cell envelope, by incorporating D-alanine into TAs, and lysine into membrane

lipids, respectively. Consequently, both systems play an important role for CAMP resistance (Li *et al.*, 2007; Thedieck *et al.*, 2006).

Taken together, the available data so far identified two sub-groups of BceRS homologous TCS in *Firmicutes* bacteria. The first group – exemplified by BceRS, MbrAB, YtsAB, GtcRS – is involved in mediating drug-specific resistance against peptide antibiotics, such as bacitracin, by regulating the expression of a neighboring ABC transporter that facilitates removal. The second group includes ApsXRS and VirRS, which represent important CAMP-specific detoxification systems. These systems – in addition to inducing the expression of an ABC transporter – lower the overall negative net charge of the cell envelope in response to the extracellular presence of a variety of structurally unrelated CAMPs.

B. Aims of this work

The cell envelope stress response of *Bacillus subtilis* is mediated by seven signal-transducing systems (Mascher *et al.*, 2003). One of these systems is the LiaRS TCS. It is strongly induced by perturbations of the cell envelope, especially the presence of antibiotics that interfere with the lipid II-cycle. Upon induction, the LiaRS TCS activates the expression of the *liaIHGFSR* operon (Mascher *et al.*, 2004). The primary aim of this work was to unravel the mechanism of LiaRS-dependent signal transduction and gene expression.

Towards that goal, the effect of single gene deletion within the *lia* locus on P_{liaI} induction should be studied by β-galactosidase assays. For this purpose, different reporter strains carrying ectopically integrated P_{liaI}-*lacZ* fusions had to be constructed and examined. The binding site of LiaR, the response regulator essential for P_{liaI} activation, should be identified by comparative genomics and confirmed by mutational studies. Based on its sequence, it should be possible to search for additional LiaR target promoters in *B. subtilis* that, if present, should be investigated further by reporter gene fusions and global transcriptional profiling.

Moreover, the inducer profile of the LiaRS system should be investigated. In addition to the investigation of cell wall antibiotics and other envelope perturbating conditions, the regulation of P_{liaI} induction during transition state should also be elucidate. Preliminary data indicate that P_{liaI} is induced at the onset of stationary phase without external addition of cell wall antibiotics. This time point in the life cycle of *B. subtilis* is characterized by the induction of a complex regulatory cascade, orchestrated by the transition state regulator AbrB and the master regulator of sporulation, Spo0A. Therefore, mutants in these and other transition state regulators will be generated and tested for their effect on P_{liaI} induction. The mode of interaction of positive candidates will be further investigated.

The ultimate aim of this thesis is the identification of the stimulus sensed by the sensor kinase LiaS and the characterization of its mechanism of stimulus perception. Towards that end, a mutational study of LiaS will be initiated, based on sequential deletions, site-specific mutagenesis, and hybrid gene analyses. Mutations that confer altered phenotypes will be further characterized.

Chapter 2:

Regulation of LiaRS-Dependent Gene Expression in *Bacillus subtilis*: Identification of Inhibitor Proteins, Regulator Binding Sites and Target Genes of a Conserved Cell Envelope Stress-Sensing Two-Component System

The work described in this chapter was published in:

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

Author contributions:
Sina Jordan performed the experiments concerning the *liaI* promoter and draw the figures. Anja Junker performed the experiments concerning the *yhcY* promoter. Initial experiments were performed by Thorsten Mascher in the laboratory of John D. Helmann. Sina Jordan and Thorsten Mascher wrote the paper.

Abstract

The regulatory network of the cell envelope stress response in *Bacillus subtilis* involves both extracytoplasmic function (ECF) σ-factors and two-component signal transducing systems. One such system, LiaRS, responds to cell wall antibiotics that interfere with the undecaprenol cycle and to perturbation of the cytoplasmic membrane. It is encoded by the last two genes of the *liaIHGFSR* locus. Here, we analyzed the expression of two LiaR-dependent operons, *liaIHGFSR* and *yhcYZ-yhdA,* and characterized a palindromic sequence required for LiaR-dependent activation. Since induction of the strong *liaI* promoter leads to both *liaIH* and *liaIHGFRS* transcripts, LiaR is positively autoregulated. Systematic deletion analysis of the *liaI* operon revealed that LiaF is a potent negative regulator of LiaR-dependent gene expression: a non-polar *liaF* deletion led to constitutive activation of both characterized LiaR-dependent promoters. The *liaF* gene is conserved in both sequence and genomic context in the *Firmicutes* group of Gram-positive bacteria, located directly upstream of *liaSR* orthologs. LiaH, a homolog of *E. coli* phage-shock protein A, also plays a more subtle role in negatively modulating the bacitracin-inducible expression from LiaR-dependent promoters. Our results support a model in which the LiaFRS module integrates both positive- and negative-feedback loops to transduce cell envelope stress signals.

Introduction

Soil is one of the most complex microbial habitats on earth. Nutrient supply varies greatly and on short notice, as do many physicochemical parameters, such as temperature, oxygen-concentration, and moisture. The presence of toxic chemicals and the high population density adds another layer of complexity (Paul and Clark, 1996). Soil bacteria have adapted to this environment in many ways. A broad range of transport systems together with flexible metabolic capabilities allow them to use a variety of nutrient sources. An extensive set of secondary metabolites is thought to suppress the growth of competitors. This trait is specifically pronounced in the actinomycete group of soil bacteria, the most prodigious producers of antimicrobial compounds: two thirds of all antibiotics in clinical use are synthesized by these bacteria alone (Bentley *et al.*, 2002; Thompson *et al.*, 2002). Production

of and resistance against antibiotics is therefore an important aspect of life in soil (Nwosu, 2001).

The cell envelope is the first and major line of defence against threats from the environment. It gives the cell its shape and counteracts the high inner osmotic pressure (Delcour *et al.*, 1999). It also provides an important sensory interface and molecular sieve between a bacterial cell and its surroundings, mediating both information flow and controlled transport of solutes. Because of its crucial role, it is an attractive target for numerous antibiotics (Bugg and Walsh, 1992; Kahne *et al.*, 2005; Koch, 2003; Silver, 2003; Walsh, 2003). Therefore, monitoring cell envelope integrity is critical for survival.

By applying genome-wide transcript profiling, the regulatory network of the cell envelope stress response in *Bacillus subtilis* was recently characterized: addition of cell wall inhibitors such as bacitracin (produced by *Bacillus spp*.) and vancomycin (a secondary metabolite of actinomycetes) induces several transmembrane signal transducing pathways, orchestrated by at least three alternative (ECF-) σ-factors and four two-component systems (TCS) (Cao *et al.*, 2002b; Mascher *et al.*, 2003). The use of different antibiotics allowed the differentiation between relatively antibiotic-specific (YvcPQ, BceRS in case of bacitracin, σ^W for vancomycin) and more general cell envelope stress responses such as σ^M and the LiaRS (formerly YvqEC) TCS (Mascher *et al.*, 2003). Interestingly, the sensors of all cell-envelope stress-sensing TCS (BceS, YvcQ and LiaS) appear to define a new family of intramembrane-sensing histidine kinases. These proteins share an unusually short sensing domain that is almost completely buried in the cytoplasmic membrane. It has been postulated that these kinases detect their signal with their transmembrane helices directly at the membrane interface (Mascher *et al.*, 2003).

The *liaIHGFSR* locus is expressed from a strictly LiaR-dependent σ^A–type promoter upstream of the *liaI* gene (P_{liaI}). The LiaRS TCS senses vancomycin, bacitracin, ramoplanin and nisin and mediates a 100- to 1000-fold induction from P_{liaI} (Mascher *et al.*, 2004). All four drugs interfere with the lipid II cycle in the cytoplasmic membrane, the rate-limiting step of cell envelope polymer biosynthesis (hence the name: LiaRS stands for Lipid II-Interacting Antibiotics Response Regulator and Sensor) (Mascher *et al.*, 2004). A strong stem-loop structure downstream of the second gene, *liaH*, results in two different transcripts: a major 1.1 kb mRNA consisting of *liaIH* and a ~4 kb transcript, including the whole operon. A 74 nucleotide promoter region is fully sufficient for the strong antibiotic-inducible, LiaR-dependent activation of gene expression (Mascher *et al.*, 2004).

Here we identify LiaF, a putative membrane protein, as an integral part of the LiaRS TCS. Deletion of *liaF* leads to a completely derepressed, stimulus-independent expression from both characterized LiaR-dependent promoter regions. A key role for LiaF as part of a three-component signaling system (LiaFRS) is supported by genomic context clustering analysis: the *liaFSR* gene cluster is conserved in Gram-positive bacteria with a low G+C content (*Firmicutes*). LiaH, a homolog of *E. coli* phage-shock protein A (PspA), also negatively modulates induction of LiaR-dependent promoters. The minimal bacitracin-inducible promoter fragments controlling expression of both LiaR-dependent operons (*liaIHGFSR* and *yhcYZ-yhdA*) each harbor a putative LiaR-binding site, identified by comparative genomics and confirmed by mutational studies, that is essential for LiaR-dependent transcription.

Material and Methods

Bacterial strains and growth conditions. *B. subtilis* was routinely grown in LB medium at 37°C with aeration. All strains used in this study are derivatives of the laboratory wild type strains W168 and CU1065 (W168 *trpC*2 *attSP*β) and are listed in Table 2.1. Kanamycin (10 μg/ml), chloramphenicol (5 μg/ml), spectinomycin (100 μg/ml), and tetracyclin(10 μg/ml) were used for the selection of the *B. subtilis* mutants used in this study. Transformation was carried out as described (Harwood and Cutting, 1990).

Allelic replacement mutagenesis using Long Flanking Homology (LFH-) PCR. This technique is derived from the published procedure (Wach, 1996) and was performed as described previously (Mascher *et al.*, 2003). In brief: resistance cassettes were amplified from a suitable vector as template (Guerout-Fleury *et al.*, 1995; Youngman, 1990). Two primer pairs were designed to amplify ~1000 bp DNA-fragments flanking the region to be deleted at its 5'- and 3'-end. The resulting fragments are here called 'up' and 'do' fragment. The 3'-end of the up-fragment as well as the 5'-end of the do-fragment extended into the gene(s) to be deleted in a way that all expression signals of genes up- and downstream of the targeted genes remained intact. Extensions of ~25 nucleotides were added to the 5'-end of the 'up-reverse' and the 'do-forward' primers that were complementary (opposite strand and inverted sequence) to the 5'- and 3'-end of the amplified resistance cassette. All obtained fragments were purified using the PCR-purification kit from Qiagen. 100-150 ng of the up- and do-fragments and 250-300 ng of the resistance cassette were used together with the specific up-forward and do-reverse primers at standard concentrations in a second PCR-reaction. In this

reaction the three fragments were joined by the 25 nucleotide overlapping complementary ends and simultaneously amplified by normal primer annealing. The PCR-products were directly used to transform *B. subtilis*. Transformants were screened by colony-PCR, using the up-forward primer with a reverse check-primer annealing inside the resistance cassette (Table 2.3). The integrity of the regions flanking the integrated resistance cassettes was verified by sequencing PCR products of ~1000 bp amplified from chromosomal DNA of the resulting mutants. Sequencing was performed in house by the GenoMIK center. All PCR-reactions were done in a total volume of 50 µl (10 µl for colony PCR) using the HotStar DNA-Polymerase Mastermix (Qiagen) or TripleMaster Polymerase Mix (Eppendorf) according to the manufacturer's procedure. The primers used in this study are listed in Table 2.3.

Construction of transcriptional promoter-*lacZ* fusions. An ectopic integration of a P_{liaI}-*lacZ* fusion was constructed based on the vector pAC6 (Table 2.2). This vector carries a chloramphenicol resistance cassette for selection in *B. subtilis*, and integrates into the *amyE* locus by double crossing-over, resulting in a stable integration of P_{liaI}-*lacZ* fusions (Stülke *et al.*, 1997). A promoter fragment similar to $P_{liaI\text{-}83}$ described earlier for pSLZ83 (Mascher *et al.*, 2004) was amplified, using the primers #99 and #100, thereby introducing *Eco*RI and *Bam*HI sites, respectively (Table 2.3). Standard cloning techniques were applied (Sambrook and Russell, 2001). The insert was verified by DNA sequencing at the GenoMIK center, Göttingen. The resulting pAC6-derived plasmid, pTM1 (Table 2.2), was linearized with *Sca*I and used to transform *B. subtilis* 168 with chloramphenicol selection, resulting in strain TMB016. Subsequently, individual genes of the *lia* locus were replaced with a kanamycin resistance cassette by transforming TMB016 with chromosomal DNA from strains HB0920, HB0933 and TMB002-TMB004, resulting in strains TMB017-TMB022 (see Table 2.1 for details). The pAC6 vector was also used to construct P_{yhcY}- and P_{liaG}-*lacZ* fusions, applying the same cloning strategy (Table 2.2). The primers used to are listed in Table 2.3, the resulting strains used in this study are given in Table 2.1.

Complementation of *liaF* in *in-frame* deletion mutants. An ectopic integration of a P_{xyl}-*liaF* fusion was constructed based on the vector pXT (Derre *et al.*, 2000). This vector is a pDG1782-derivative (Guerout-Fleury *et al.*, 1996) that carries a spectinomycin resistance cassette for selection in *B. subtilis*, a xylose-inducible promoter for *liaF* expression, and integrates into the *thrC* locus by double crossing-over, resulting in a stable integration of P_{xyl}-*liaF* fusions. We amplified *liaF* with primers #35 and #36, thereby introducing *Eco*RI and *Hin*dIII sites, respectively (Table 2.3). Cloning and verification of DNA sequence was performed as described above. The resulting pXT-derived plasmid, pSJ701 (Table 2.2), was

linearized with *Sca*I and used to transform *B. subtilis* TMB027-TMB029 with spectinomycin selection, resulting in strains TMB182-TMB184 (Table 2.1). For *liaF* expression 0,2 % xylose was added to the medium.

Measurement of induction by b**-galactosidase assay.** Cells were inoculated from fresh overnight cultures and grown in LB-medium at 37°C with aeration until they reached an OD_{600}≈0.4. The culture was split, adding bacitracin (50 µg/ml final concentration) to one half (induced sample) and leaving the other half untreated (uninduced control). After incubation for an additional 30 min at 37°C with aeration, 2 ml of each culture were harvested and the cell pellets were frozen and kept at -20°C. The pellets were resuspended in 1 ml of working buffer and assayed for β-galactosidase activity as described with normalization to cell density (Miller, 1972).

Preparation of total RNA for quantitative real-time RT-PCR, Northern blots and primer extension analysis. Total RNA was extracted from 10 ml of culture with and without bacitracin (50 µg/ml final concentration). Bacitracin was added to the culture at OD_{600} of 0.4 (mid-log phase) and the cultures were incubated for 10 min at 37°C with aeration before the cells were harvested and rapidly frozen at -70ºC. RNA was prepared using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The RNA was treated with DNase (using the on-column RNase-free DNase kit from Qiagen) to remove remaining traces of chromosomal DNA that would interfere with the subsequent reaction. The success of this treatment was verified by a lack of product in a standard PCR reaction, using the same primers as for the real-time RT-PCR.

Quantitative real-time RT-PCR. Measurement of transcript abundance was performed by quantitative real-time RT-PCR, using the QuantiTect SYBRgreen RT-PCR kit (Qiagen) 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 25 µl with 0,5 µM of each primer (see Table 2.3). The amplification reaction was carried out in an iCycler (BioRad), using the following program: initial incubation 50.0ºC for 30 min, followed by a 95°C denaturing step for 15 min, followed by 40 cycles (94°C (15 sec), 60°C (30 sec), 72°C (30 sec)). After a subsequent incubation step (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. Expression of *rpsE* and *rpsJ*, encoding ribosomal proteins, was monitored as constitutive reference. Expression of *liaR* was calculated as fold-change based on the C_T values for each gene, as described (Talaat *et al.*, 2002).

Table 2.1. Strains used in this study

Strain	Genotype or characteristics[1]	Reference or source
***E. coli* strains**		
DH5αF´	F´/ *endA1 hsdR17*(r_K^-, m_K^+) *gln*V44 *thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)U169 *deoR* (F 80 *dlac*Δ(*lacZ*)M15)	lab stock
***B. subtilis* strains**		
W168	wild type, *trpC2*	lab stock
CU1065	wild type, *trpC2*, *att*SPβ	lab stock
HB0920	CU1065 *liaH*::kan	(Mascher *et al.*, 2003)
HB0933	CU1065 *liaR*::kan	(Mascher *et al.*, 2003)
HB0950	CU1065 *att*SPβ2Δ2::P_{liaI}-*cat*-*lacZ*	(Mascher *et al.*, 2004)
TMB002	CU1065 *liaF*::kan	this study
TMB003	CU1065 *liaG*::kan	this study
TMB004	CU1065 *liaS*::kan	this study
TMB016	W168 *amyE*::(cat, P_{liaI}-*lacZ*)	this study
TMB017	W168 *amyE*::(cat, P_{liaI}-*lacZ*), *liaG*::kan	this study
TMB018	W168 *amyE*::(cat, P_{liaI}-*lacZ*), *liaF*::kan	this study
TMB019	W168 *amyE*::(cat, P_{liaI}-*lacZ*), *liaS*::kan	this study
TMB020	W168 *amyE*::(cat, P_{liaI}-*lacZ*), *liaR*::kan	this study
TMB021	W168 *amyE*::(cat, P_{liaI}-*lacZ*), *liaGF*::kan	this study
TMB027	HB0950 LiaFΔ(I_{151}-D_{235})[2]	this study
TMB028	HB0950 LiaFΔ(S_{189}-V_{192})[2]	this study
TMB029	HB0950 LiaFΔ(E_{126}-D_{146})[2]	this study
TMB053	W168 *amyE*:(cat, $P_{liaG(-68\ -\ 914)}$-*lacZ*)	this study
TMB066	W168 *amyE*::(cat, $P_{yhcY(-129-\ 70)}$-*lacZ*), *liaR*::spec	this study
TMB069	W168 *amyE*::(cat, $P_{yhcY(-129\ –\ 70)}$-*lacZ*), *liaHGF*::kan, *liaR*::spec	this study
TMB071	W168 *amyE*::(cat, $P_{yhcY(-129\ –\ 70)}$-*lacZ*)	this study
TMB072	W168 *amyE*::(cat, $P_{yhcY(-129\ –\ 70)}$-*lacZ*), *liaH*::kan	this study
TMB095	W168 *amyE*::(cat, $P_{yhcY(-129\ –\ 70)}$-*lacZ*), *liaF*::kan	this study
TMB096	W168 *amyE*::(cat, $P_{yhcY(-71\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB097	W168 *amyE*::(cat, $P_{yhcY(-88\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB098	W168 *amyE*::(cat, $P_{yhcY(-97\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB099	W168 *amyE*::(cat, $P_{yhcY(-107\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB100	W168 *amyE*::(cat, $P_{yhcY(-117\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB101	W168 *amyE*:(cat, $P_{yhcY(-122\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB102	W168 *amyE*::(cat, $P_{yhcY(-129\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB104	W168 *amyE*::(cat, $P_{yhcY(-244\ –\ 70)}$-*lacZ*), *liaHGF*::kan	this study
TMB107	W168 *amyE*::(cat, $P_{liaG(-68\ -\ 3)}$-*lacZ*)	this study
TMB108	W168 *amyE*::(cat, P_{liaI}-*lacZ*), *liaH*::kan	this study
TMB111	W168 *amyE*::(cat, $P_{liaI(-102\ -\ 72)}$($A_{-76}T$, $A_{-78}T$, $A_{-81}T$)-*lacZ*)	this study
TMB112	W168 *amyE*::(cat, $P_{liaI(-102\ -\ 72)}$($A_{-75}T$, $A_{-77}T$, $A_{-79}T$)-*lacZ*)	this study
TMB113	W168 *amyE*::(cat, $P_{liaI(-102\ -\ 72)}$($C_{-86}A$, $G_{-88}A$, $C_{-93}A$, $G_{-95}A$, $T_{-98}A$)-*lacZ*)	this study
TMB114	W168 *amyE*::(cat, $P_{liaI(-102\ –\ 72)}$($A_{-78}C$, $A_{-83}C$)-*lacZ*)	this study
TMB115	W168 *amyE*::(cat, $P_{liaI(-102\ -\ 72)}$($C_{-93}A$, $G_{-95}A$, $T_{-98}A$)-*lacZ*)	this study
TMB132	W168 *amyE*::(cat, $P_{liaG(81\ –\ 914)}$-*lacZ*)	this study
TMB133	W168 *amyE*::(cat, $P_{liaI(-102\ -\ 72)}$($C_{-86}A$, $T_{-87}A$, $G_{-88}A$)-*lacZ*)	this study
TMB182	HB0950 LiaFΔ(I_{151}-D_{235})[2], *thrC*::(spec, P_{xyl}-*liaF*)	this study
TMB183	HB0950 LiaFΔ(S_{189}-V_{192})[2], *thrC*::(spec, P_{xyl}-*liaF*)	this study
TMB184	HB0950 LiaFΔ(E_{126}-D_{146})[2], *thrC*::(spec, P_{xyl}-*liaF*)	this study

[1]resistance cassettes: kan = kanamycin; cat = chloramphenicol; spec = spectinomycin. Positions of promoter fragments are given relative to the "ATG" start codon of the corresponding genes.
[2]For reasons of clarity, the effect of *in-frame* deletions in *liaF* are given at the level of the LiaF protein.

Table 2.2. Vectors and plasmids

Plasmid	Genotype, sequence or characteristics[1] for cloning	Primers used or source	Reference
pAC6	*lacZ* fusion vector, integrates at *amyE*, chloramphenicol resistance		(Stülke *et al.*, 1997)
pXT	vector for xylose-inducible gene expression, integrates at *thrC*, pDG1782-derivative, spectinomycin resistance		(Derre *et al.*, 2000)
pAJ601	pAC6 $P_{yhcY(-71-70)}$-*lacZ*	#0165/#0172	this study
pAJ602	pAC6 $P_{yhcY(-88-70)}$-*lacZ*	#0165/#0171	this study
pAJ603	pAC6 $P_{yhcY(-129-70)}$-*lacZ*	#0165/#0168	this study
pAJ604	pAC6 $P_{yhcY(-97-70)}$-*lacZ*	#0165/#0170	this study
pAJ606	pAC6 $P_{yhcY(-244-70)}$-*lacZ*	#0165/#0166	this study
pAJ607	pAC6 $P_{yhcY(-122-70)}$-*lacZ*	#0165/#0169	this study
pAJ608	pAC6 $P_{yhcY(-117-70)}$-*lacZ*	#0165/#0259	this study
pAJ609	pAC6 $P_{yhcY(-107-70)}$-*lacZ*	#0165/#0260	this study
pBD601	pAC6 $P_{liaI(-102-72)}$($A_{-76}T$, $A_{-78}T$, $A_{-81}T$)-*lacZ*	#0100/#0231	this study
pBD602	pAC6 $P_{liaI(-102-72)}$($A_{-75}T$, $A_{-77}T$, $A_{-79}T$)-*lacZ*	#0100/#0232	this study
pBD603	pAC6 $P_{liaI(-102-72)}$($C_{-86}A$, $G_{-88}A$, $C_{-93}A$, $G_{-95}A$, $T_{-98}A$)-*lacZ*	#0100/#0265	this study
pBD604	pAC6 $P_{liaI(-102-72)}$($A_{-78}C$, $A_{-83}C$)-*lacZ*	#0100/#0266	this study
pBD605	pAC6 $P_{liaI(-102-72)}$($C_{-93}A$, $G_{-95}A$, $T_{-98}A$)-*lacZ*	#0100/#0267	this study
pBD606	pAC6 $P_{liaI(-102-72)}$($C_{-86}A$, $T_{-87}A$, $G_{-88}A$)-*lacZ*	#0100/#0268	this study
pDH605	pAC6 $P_{liaG(81-914)}$-*lacZ*	#0204/#0222	this study
pSJ601	pAC6 $P_{liaG(-68-914)}$-*lacZ*	#0204/#0205	this study
pSJ607	pAC6 $P_{liaG(-68-3)}$-*lacZ*	#0204/#0296	this study
pSJ701	pXT P_{xyl}-*liaF*	#0035/#0036	this study
pTM1	pAC6 $P_{liaI(-83-72)}$-*lacZ*	#0099/#0100	this study

[1]The positions of the cloned fragments are given relative to the "A" of the start codon of the corresponding genes.

Primer extension mapping of the *yhcY* transcriptional start site. For mapping of the *yhcY* promoter, HB0920 cells were grown in LB and total RNA was isolated from uninduced and bacitracin-induced (final concentration 50 µg/ml) mid-logarithmic cultures as described above. Primer extension reactions for *yhcY* were set up as follows: 30 µg of heat-denatured RNA was hybridized at 65°C to ~2 pmol of end-labeled primer yhcY-PE (Table 2.3) in buffer containing 60 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM DTT, and 40 U of RNasin (Promega) in a total volume of 30 µl. Following hybridization, 50 µl extension buffer (72 mM NaCl, 50 mM Tris-HCl [pH 7.9], 10 mM DTT, 20 mM $MgCl_2$), dNTPs (10 mM), and 2 µl Superscript II reverse transcriptase (Invitrogen) were added to the mixture and incubation continued at 37°C for 30 min. The primer extension products were precipitated with ethanol, resuspended in sequence loading buffer, and loaded onto a 6% polyacrylamide sequencing gel. A PCR cycle sequencing kit (Epicentre) was used to generate sequencing ladders corresponding to the *yhcY* promoter region.

Table 2.3. Oligonucleotides used in this study

Primer-number or amplified fragment	Sequence
Oligonucleotides for Long Flanking Homology (LFH)-PCR[1]	
kan cassette	fwd: CAGCGAACCATTTGAGGTGATAGG, rev: CGATACAAATTCCTCGTAGGCGCTCGG
kan-check	fwd: CATCCGCAACTGTCCATACTCTG, rev: CTGCCTCCTCATCCTCTTCATCC
spec cassette	fwd: CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACTGGCAAGAG, rev: CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTCTAAAATCTG
spec-ckeck	fwd: GTTATCTTGGAGAGAATATTGAATGGAC, rev: CGTATGTATTCAAATATATCCTCCTCAC
liaH-up	fwd: CTTGTTATTCGTCACTGCC, rev: CCTATCACCTCAAATGGTTCGCTGGTCCTTCATGAACTGACGC
liaH-do	fwd: CGAGCGCCTACGAGGAATTTGTATCGCAGACCAGACAAAAGCGGC, rev: CGCTAGATCCCCGCTGTCC
liaG-up	fwd: TTGTCGTCGGAATCGCATTGGC, rev: CCTATCACCTCAAATGGTTCGCTGCACATCTTTAACGACGACGGC
liaG-do	fwd: CGAGCGCCTACGAGGAATTTGTATCGCCAATCGACATCAAAACGGACA, rev: TTACCCGGCGTTTGACTCGC
liaF-up	fwd: AAGGATTTGCGGTCAAGTCC, rev: CCTATCACCTCAAATGGTTCGCTGGCAATGATCAATCCGAGAAGC
liaF-do	fwd: CGAGCGCCTACGAGGAATTTGTATCGGATGTGGATGTGAAGTACG, rev: TTCAAGCCGTATGAGGAGGC
liaS-up	fwd: GCTTTATCAGCAAGCGGTGACG, rev: CCTATCACCTCAAATGGTTCGCTGTCCCGTTGTCATGCGGATGGC
liaS-do	fwd: CGAGCGCCTACGAGGAATTTGTATCGGGCACTCAAATCGAAGTGA, rev: AACCGGGCTGGGAAACGAGGTC
liaR-up	fwd: GCTGTCATCAAGCTGGTTCGG, rev: CCTATCACCTCAAATGGTTCGCTGCGATGCTTCGCCGATGACTTC
liaR-do	fwd: CGAGCGCCTACGAGGAATTTGTATCGACGCACACCGAAATCATCTC, rev: CTCTTCATCTGATCCGACACAGC
Oligonucleotides for quantitative real-time RT-PCR, primer extension, Northern hybridization and sequencing	
liaR-RT	fwd: ATTGAAGTCATCGGCGAAGC, rev: AAAGCTCCCGGCAAATTTGC
rpsJ-RT	fwd: GAAACGGCAAAACGTTCTGG, rev: GTGTTGGGTTCACAATGTCG
rpsE-RT	fwd: GCGTCGTATTGACCCAAGC, rev: TACCAGTACCGAATCCTACG
yhcY-PE	GGTTTCCGCAATCGTTTTCAGCG
yhcY probe[2]	fwd: GAGTTGCTGAGTCTGACAAACC, rev: CTAATACGACTCACTATAGGGAGATCGTGAAGCTCCTGAGCGAGGC
liaF sequencing	fwd: GCTTTATCAGCAAGCGGTGACG, rev: CCGAACCAGCTTGATGACAGC
Oligonucleotides for cloning[3]	
Cloning and site-directed mutagenesis of the *liaI* promoter[4]	
#0099 (fwd)	CCAT***GAATTC***CCGGTGCGAGATACGACTCC
#0100 (rev)	CGAT***GGATCC***TCCTCCAAAAAAGACGGAGATCCC
#0231 (fwd)	ACAT***GAATTC***GAGATACGACTCCGGTCTTAT**t**TA**t**A**t**ATCAATCTCTGATTCG
#0232 (fwd)	ACAT***GAATTC***GAGATACGACTCCGGTCTTATAT**t**A**t**A**t**TCAATCTCTGATTCG
#0265 (fwd)	ACAT***GAATTC***GAGA**a**AC**a**A**a**TCCG**a**T**a**TTATATAAAAATCAATCTCTGATTCG
#0266 (fwd)	ACAT***GAATTC***GAGATACGACTCCGGTCTT**c**TATA**c**AAATCAATCTCTGATTCG
#0267 (fwd)	ACAT***GAATTC***GAGA**a**AC**a**A**a**TCCGGTCTTATATAAAAATCAATCTCTGATTCG
#0268 (fwd)	ACAT***GAATTC***GAGATACGACTCCG**aaa**TTATATAAAAATCAATCTCTGATTCG
Promoter deletion analysis of the *liaG* promoter	
#0204 (fwd)	CCATGAATTCTCCCTTCCGCACGTATCAATTCGC
#0205 (rev)	AGCCGGATCCTTTGTCATTCCTGGTG
#0222 (fwd)	CCATGAATTCGCAGGCCTAGGTTCATAAATGGC
#0296 (rev)	AGCCGGATCCCATTCGGTTTCATCCTTCTCATTC
Promoter deletion analysis of the *yhcY* promoter	
#0165 (rev)	CGAT***GGATCC***CGTGTTGCTTTGATATCGTGCC
#0166 (fwd)	CGAT***GAATTC***GACAGTGAAAAGCGACTTGCC
#0168 (fwd)	CGAT***GAATTC***GCTTTTTCTTTTTCTCATCC
#0169 (fwd)	CGAT***GAATTC***CTTTTTCTCATCCAAAAGTCTG
#0259 (fwd)	CGAT***GAATTC***TCTCATCCAAAAGTCTGAAAG
#0260 (fwd)	CGAT***GAATTC***AAGTCTGAAAGAAAATCATCCTACAAGTG
#0170 (fwd)	CGAT***GAATTC***GAAAATCATCCTACAAGTG
#0171 (fwd)	CGAT***GAATTC***CCTACAAGTGAAGCAATGAA
#0172 (fwd)	CGAT***GAATTC***GAAATACAAAAAACTGGTATAATC
Complementation experiments with *liaF*	
#0035 (fwd)	AGG***AAGCTT***AGAAAGGAGGCGGACACCAGG
#0036 (rev)	TCC***GAATTC***TTTCTCATACGTACTTCACATCC

[1]Oligonucleotide names refer to the fragments flanking the gene to be deleted. Sequences underlined are inverse and complimentary to the 5'- (up-rev) and 3'- (do-fwd) end of the kanamycin cassette, respectively. [2]The underlined sequence represents the T7 promoter necessary for the construction of RNA-probes by *in-vitro* transcritption [3]Restriction sites for cloning are highlighted in bold italics. [4]Nucleotides given in small bold letters represent mismatches.

Comparative genomics analyses. Multiple sequence alignments were performed using ClustalW, implemented in the BioEdit program package (Hall, 1999). Domain-based analysis of protein sequences were performed using the SMART database (Schultz *et al.*, 1998) at http://smart.embl-heidelberg.de/. The genomic context clustering analysis of the *lia* locus was performed using the ERGO database, which is available through Integrated Genomics, Inc. (http://www.integratedgenomics) and maintained by the GenoMIK center, Göttingen. The initial identification of the putative LiaR-binding site was done by submitting promoter fragments of 200 nucleotides upstream the ATG of all *liaI* homologs identified above to the MEME at http://meme.sdsc.edu/meme/website/meme.html (Bailey and Elkan, 1994). The resulting weight matrix of the conserved sequence pattern was then used to screen the individual genomes harboring *liaRS* homologous genes for additional putative LiaR-binding sites 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/. The graphical representation of the putative LiaR-binding site was generated using the Weblogo interface (Crooks *et al.*, 2004) at http://weblogo.berkeley.edu/.

Results

LiaF is an integral part of the LiaRS-mediated signal transduction system. With the exception of *liaSR*, encoding the sensor kinase and cognate response regulator of a classical bacterial TCS, the function of the gene products of the *liaIHGFSR* locus is unknown. No homology can be found in the databases for *liaI* and *liaG*. Both genes encode hypothetical proteins, harboring two or one transmembrane helices, respectively, indicative for a membrane localization. The gene product of *liaH* belongs to the PspA/IM30 protein family (see below), while *liaF* encodes a putative membrane protein with homology to proteins of unknown functions from other Gram-positive bacteria.

To investigate their possible roles in LiaRS-mediated signal transduction, we investigated the effect of various insertion-deletions in the *lia* locus on P_{liaI} activity as measured from a P_{liaI}-*lacZ* reporter fusion integrated ectopically at the *amyE* locus (Fig. 2.1). The wild-type strain (TMB016) showed a strong response to the presence of bacitracin, resulting in a more than 200-fold induction of P_{liaI} activity, while virtually no ß-galactosidase activity can be detected in the *liaR*::*kan* strain (TMB020) under either inducing or non-inducing conditions, consistent with the stringent LiaR-dependence of P_{liaI} (Mascher *et al.*, 2004). The *liaS*::*kan* mutant

TMB019, lacking the histidine kinase, no longer responded to bacitracin, but did display a slightly increased basal expression level. Surprisingly, P_{liaI} was constitutively active in the *liaF*::*kan* mutant TMB018, even in the absence of the inducer. This activity was even ten-fold higher than that measured in the induced wild type.

Resistance cassettes inserted into the chromosome can exhibit polar effects on downstream genes. This can either be a positive polar effect, due to readthrough from their own strong promoters (Cao *et al.*, 2003), or a negative effect due to termination of transcription within the resistance cassette. To investigate possible polar effects, we used quantitative real-time RT-PCR to measure *liaR* transcription (in the insertion-deletion strains TMB017-TMB019) relative to the uninduced wild type. In all strains, there was an ~four-fold increase in *liaR* transcript. Since P_{liaI} transcription behaves identically in the wild-type and a *liaG*::*kan* strain, while it is constitutively active in the *liaF*::*kan* strain, there is no correlation between the weak positive polar effects from the kanamycin resistance cassettes and the observed effects on P_{liaI} activity. Instead, the results support a functional role for LiaF in inhibiting LiaRS-mediated signal transduction.

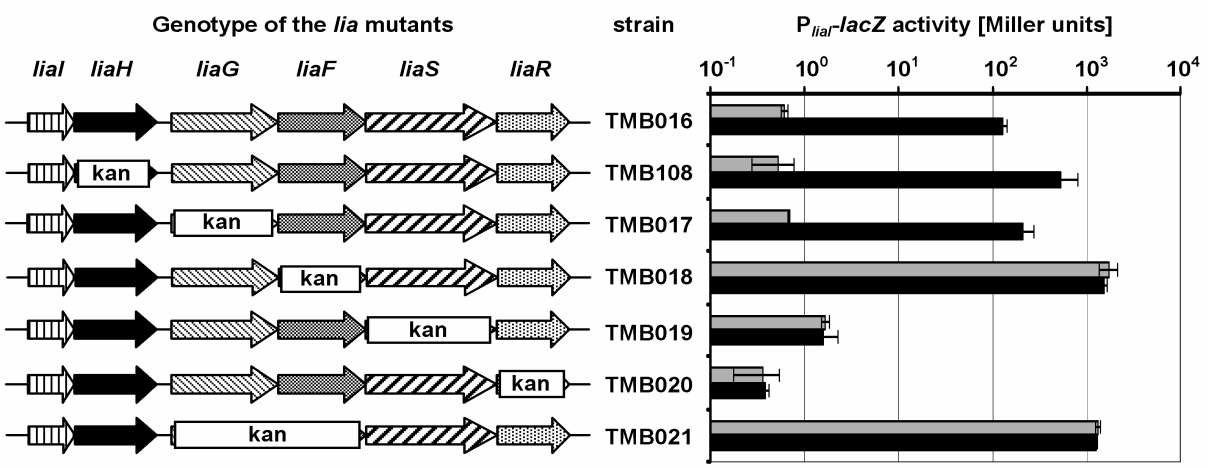

Figure 2.1. **P_{liaI} activity in response to deletions of *lia* genes.** Cultures of strains TMB016 ("wild type") and TMB017-TMB020/TMB108 were grown in LB medium to mid-log phase (OD_{600} ~0.4) and split. One half was induced by the addition of bacitracin (final concentration 50 µg/ml; black bars), the other half served as an uninduced control (grey bars). Cells were harvested 30 min post-induction and assayed as described previously (Mascher *et al.*, 2004). β-Galactosidase activity is expressed in Miller units (Miller, 1972). A log scale was applied for reasons of clarity. The genotype of the corresponding strains is shown on the left side (see Fig. 3A for labeling pattern of arrows). "kan" represents the area replaced by the kanamycin resistance cassette. The cassette itself has a size of approx. 1.5 kb in all mutants.

Spontaneous *in-frame* deletions in *liaF* lead to constitutive activity of P_{liaI}. The strong effect of a *liaF* deletion on P_{liaI} prompted us to perform a genetic screen using strain HB0950, which carries an ectopic pJPM122-based P_{liaI}-*cat*-*lacZ* fusion integrated at *att*SPβ (Mascher *et al.*, 2004). Direct plating of a mid-logarithmic culture of HB0950 on LB plates with

chloramphenicol gave rise to numerous Cm^R colonies. We reasoned that any mutation in *liaF* that renders its gene product dysfunctional would lead to such a spontaneous chloramphenicol resistance in the genetic background of HB0950. Indeed, PCR and sequence analyses indicate that alterations in *liaF* were associated with many of the spontaneous Cm^R mutants. Three strains containing *in-frame* deletions in *liaF* were chosen for further analysis (Fig. 2.2). These deletions likely arose from recombination between repeated nucleotide sequences (Fig. 2.2a, upper line) – as has been suggested for a comparable deletion in the *Enterococcus faecium* histidine kinase VanSB (Depardieu *et al.*, 2003) – and result in the deletion of 84, 20, and 4 amino acids. All three *in-frame* deletion mutants showed a strong bacitracin-independent, constitutive upregulation of LacZ expression in ß-galactosidase assays (Fig. 2.3), a behavior similar to TMB018 (*liaF*::kan) (Fig. 2.1). The effect of these mutations could be complemented by re-introduction of a wild type *liaF* allel, integrated as single copy into the *thrC*-locus and expressed from a xylose-inducible promoter (see Material and Methods): In the resulting strains, TMB182-184 (Table 2.1), P_{liaI} is again switched off in the absence and inducible by addition of bacitracin (data not shown). Taken together, these data clearly demonstrate a negative role for the putative transmembrane protein LiaF in LiaRS-mediated signal transduction and identify regions in the hydrophilic C-terminus that are crucial for LiaF function.

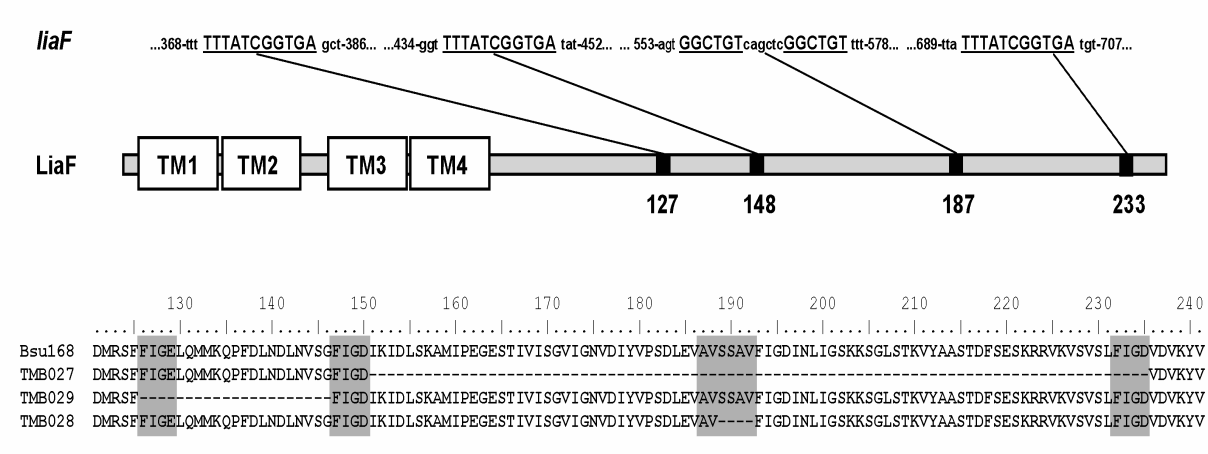

Figure 2.2. **Features and sequence of LiaF-derivatives in spontaneous chloramphenicol resistant mutants, based on *in-frame* deletions.** A sequence alignment of the C-terminal half of LiaF is shown in the lower part, including the reference sequence of the wild type strain W168 in the first line. The amino acids corresponding to the 11 or 6 nucleotide repeat (underlined in the upper part for the *liaF* gene) are shaded in grey. A graphical representation of LiaF is shown in the middle. The four putative transmembrane helices (TM1-4) are located in the N-terminal half of LiaF. The black boxes indicate the positions of the four repeats.

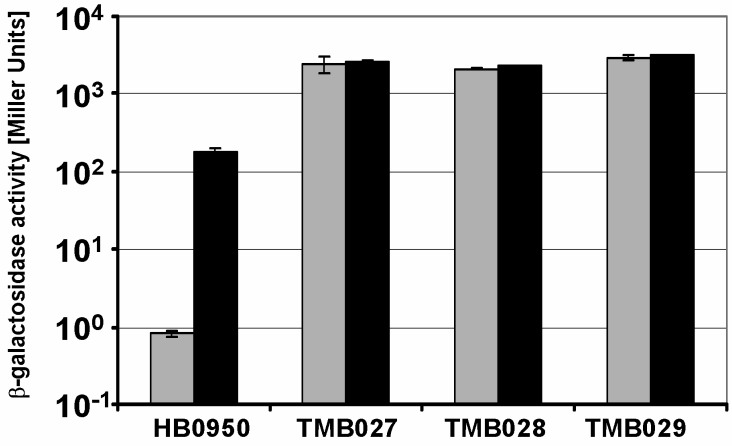

Figure 2.3. P_{lial} activity in HB0950 ("wild type") and derived mutants harboring *in-frame* deletion in *liaF*. Experimental conditions and labeling of the bars as described in Fig. 2.1.

The genomic context of *liaFSR* is conserved in the *Firmicutes* group of Gram-positive bacteria. A functional connection between proteins is very often reflected by a clustering of their respective genes at the genomic level. For example, a functional connection has been demonstrated for some TCS involved in cell envelope stress response in the *Bacillus/Clostridium* group of bacteria which cluster with operons encoding ABC-transporters (Joseph *et al.*, 2002). In these detoxification units, the TCS senses the presence of a harmful compound and strongly induces the expression of the corresponding ABC transporter, which then facilitates removal (Mascher *et al.*, 2003; Neumüller *et al.*, 2001; Ohki *et al.*, 2003a).

A genomic context clustering analysis of LiaS orthologs revealed a topological conservation of all three genes, *liaFSR*, in Gram-positive bacteria with a low G+C content. Without exception, a *liaF* homolog is always located directly upstream of the TCS in all species harboring *liaSR*-homologs (Fig. 2.4). This finding further substantiates the functional link between LiaF and LiaRS. In contrast, homologs of *liaIH* are only present in the *lia* locus of bacilli. Both genes form a separate operon in *Listeria* species, and are apparently lacking in the more distantly related cocci. The *liaG* gene is only found in *Bacillus licheniformis* and *B. halodurans*, the bacteria most closely related to *B. subtilis* for which complete genome sequences are available (Fig. 2.4).

Identification of a conserved promoter element as a putative binding-site for LiaR-homologous response regulators from the genus *Bacillus*. Protein sequence comparisons (Hall, 1999) revealed an unusually high degree of sequence similarity in the C-terminal domain of all LiaR-homologs (data not shown). This domain harbors a LuxR-like helix-turn-helix motif and defines the DNA-binding specificity of LiaR-like response regulators. We

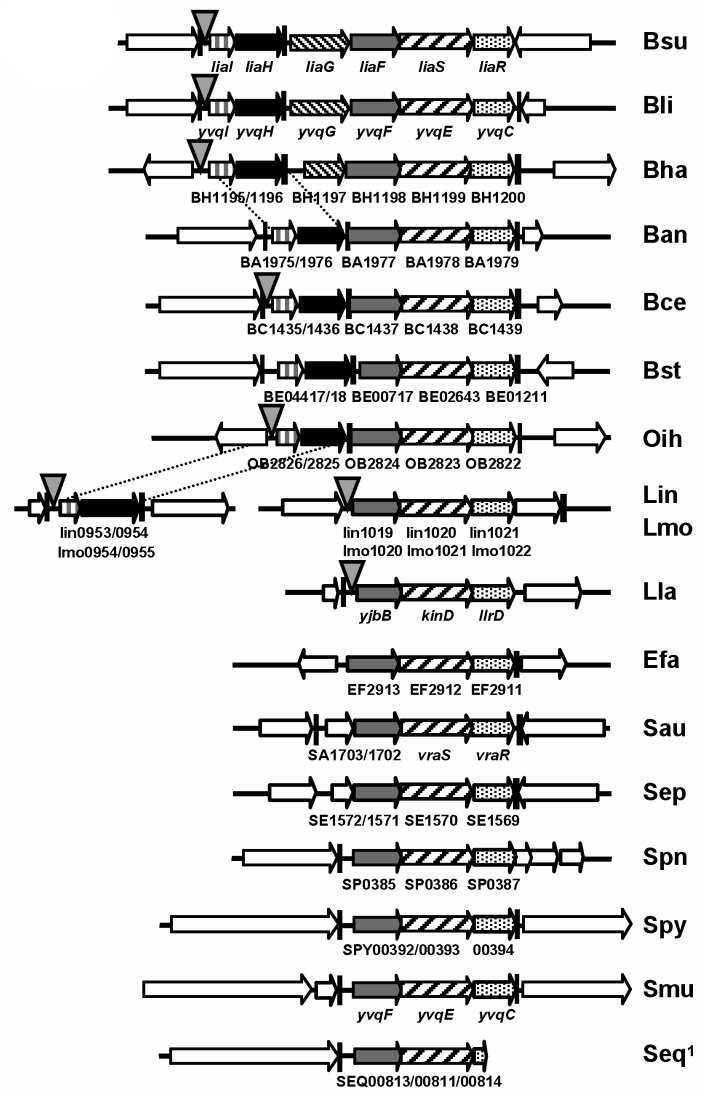

Figure 2.4. Comparative genomics analysis of the *lia* locus and identification of the putative LiaR-binding site. Conservation of the *lia* locus in other Gram-positive bacteria with a low G+C content. The loci are drawn to scale, with the line of the *B. subtilis lia*-locus corresponding to 7.5 kb. The genes of the *lia*-locus are labeled differently for clarity. Hatched arrows represent genes coding for histidine kinases and dotted arrows response regulators, homologous to LiaS and LiaR, respectively. The *liaF* homologs are shown as grey, *liaH* as black arrows and *liaI* as white arrows with black vertical lines; genes flanking the *lia*-locus are represented by white arrows. Putative terminators are marked as black vertical bars. The presence of putative LiaR-binding sites is indicated by the grey triangles (see Table 2.5 for details). Gene names according to GenBank entries of the published genome sequences. Abbreviations of bacterial species: Bsu (*Bacillus subtilis*), Bli (*B. licheniformis*) Bha (*B. halodurans*), Ban (*B. anthracis*), Bce (*B. cereus*), Bst (*B. stearothermophilus*), Oih (*Oceanobacillus iheyensis*), Lin (*Listeria innocua*), Lmo (*L. monocytogenes*), Efa (*Enterococcus faecalis*), Sau (*Staphylococcus aureus*), Sep (*S. epidermidis*), Spn (*Streptococcus pneumoniae*), Spy (*S. pyogenes*), Smu (*S. mutans*), Seq (*S. equi*), Lla (*Lactococcus lactis*).[1]The genome of *S. equi* is not yet finished. The end of a contig lies inside the *liaR* homolog SEQ00814

reasoned that this finding could be indicative for a conservation of the corresponding DNA-binding site within LiaR-target promoters.

To test this hypothesis, we retrieved DNA sequences upstream of *liaI*-homologous genes from the first nine bacterial species shown in Fig. 2.4, assuming that these promoters are all subject to regulation by their corresponding LiaRS-homologs. The sequences were submitted to the MEME web page (Bailey and Elkan, 1994), to identify short stretches of high sequence similarity. One motif was present in all but two regions (*B. anthracis* and *B. stearothermophilus*) at a similar distance relative to the start codon. This motif includes an imperfect inverted repeat of seven nucleotides, separated by two base pairs (Fig. 2.5). The resulting weight matrix of this sequence pattern was subsequently submitted to the 'Virtual Footprint' algorithm (Münch *et al.*, 2005), implemented in the Prodoric database (Münch *et al.*, 2003), in order to identify candidate LiaR target promoters in the *B. subtilis* genome. We retrieved three sequences within intergenic regions.

In addition to the P_{liaI} promoter region, two new potential LiaR-binding sites were identified. The first is associated with the *yhcYZ-yhdA* operon, a locus previously implicated as part of the LiaR regulon (Mascher *et al.*, 2003). The second putative LiaR-box was located in a large non-coding region between the genes *yozJ* and *rapK*. Subsequent analyses, using reporter fusions failed to reveal any transcriptional activity in either direction from this intergenic region (data not shown). Thus, this putative LiaR-binding site may be a remnant of a previously functional locus, or a false-positive generated by our search algorithm.

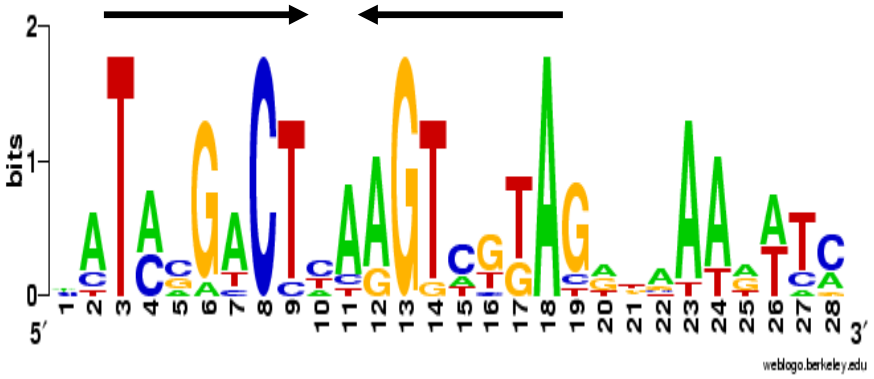

Figure 2.5. Graphical representation of the putative LiaR-binding site upstream P_{liaI}. The sequence is derived from a comparative genomics analysis (using the MEME algorithm) (Bailey and Elkan, 1994), based on the promoter regions upstream of *liaI* and its homologs in bacteria harboring homologous genes to both, *liaRS* and *liaI*. This graphical representation was generated through the Weblogo page (Crooks *et al.*, 2004) at http://weblogo.berkeley.edu/logo.cgi.

The *yhcYZ-yhdA* operon is expressed from a LiaR-dependent promoter. A regulatory link between the *yhcYZ-yhdA* operon and the Lia system had been suggested previously: bacitracin treatment leads to increased expression of this operon in a *liaH* mutant relative to the wild type (Mascher *et al.*, 2003). Additionally, a LiaR-dependent expression of the *yhcYZ-yhdA* operon was described in a comprehensive microarray study on TCS in *B. subtilis*: Overexpression of LiaR, in the absence of its cognate histidine kinase LiaS, resulted in an induction of this operon (Kobayashi *et al.*, 2001). However, these experiments do not discriminate between direct and indirect effects of LiaR on the *yhcYZ-yhdA* operon.

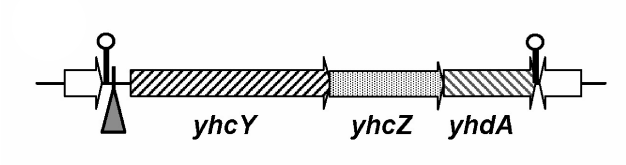

Figure 2.6. Genomic context of the putative LiaR binding sites upstream the *yhcYZ-yhdA* operon. The region is drawn to scale, representing 3000 bp. The exact position of the LiaR-binding site is indicated by the grey triangle. Labeling of arrows as in Fig. 2.4.

To determine if *yhcYZ-yhdA* is regulated by the LiaFRS system, we constructed strains carrying an ectopically integrated P_{yhcY}-*lacZ* fusion. Consistent with previous transcriptome analyses (Mascher *et al.*, 2003), P_{yhcY} was only weakly inducible by bacitracin in the wild-type and was derepressed in the *liaH* mutant TMB072 (Fig. 2.7). While the magnitude of induction was relatively weak compared to that observed with P_{liaI} (Fig. 2.1), induction was completely LiaR-dependent and expression was constitutive in the *liaF* mutant (TMB095). Since the gene products of both *liaH* and *liaF* exhibited a negative effect on P_{yhcY} activity, we also introduced a *liaHGF*::*kan* insertion-deletion into TMB071 (resulting in strain TMB102). The mutant behaved like the *liaF* mutant, consistent with the idea that LiaR is already completely activated in a *liaF* mutant. As expected, both the *liaH* and *liaF* effects are completely LiaR-dependent (Fig. 2.7A). The behavior of P_{yhcY} is comparable to P_{liaI}, but the activity as well as the range of inducibility is lower. This weaker activity may be due to a poorer match between the LiaR box preceeding *yhcY* compared to that preceeding *liaI* (Table 2.5).

We mapped the 5'-end of the *yhcY* transcript by primer extension to an "A" 41 nucleotides upstream of the start codon, thereby also verifying the induction of transcription by bacitracin in a *liaH* mutant (Fig. 2.7B). The *yhcY* promoter has a well conserved extended -10 region (TGgTATAAT), but a poorly conserved -35 region (GTGAAG) (Fig. 2.7D). Northern

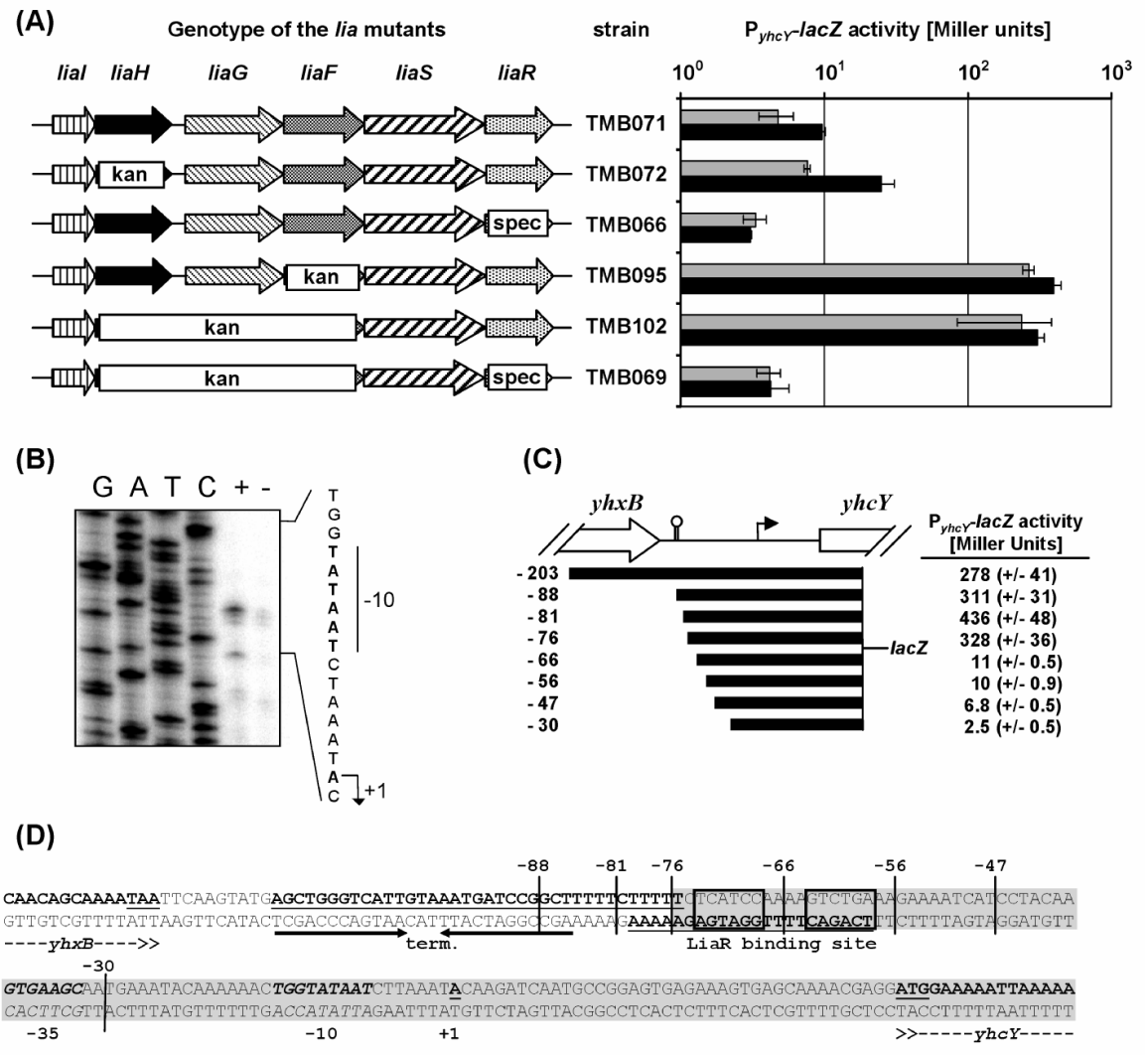

Figure 2.7. Characterization of the *yhcY* promoter and its LiaR-dependent expression. (A) P_{yhcY}-*lacZ* in response to deletions of *lia* genes, determined by β-galactosidase assay with and without the addition of bacitracin, essentially as described. See legend to Fig. 1 for further details. A logarithmic representation of the resulting β-galactosidase activity was chosen for reasons of clarity. **(B)** Mapping of the transcriptional start site by primer extension analysis indicates that transcription initiates with a "A" as shown in the sequence to the right. Primer extension reaction was performed with RNA prepared from strain HB0920 with and without the addition of bacitracin, using the primer yhcY-PE (see Table 3). The sequencing ladder to the left was generated using the same primer, following standard procedures. **(C)** Promoter deletion analysis of P_{yhcY}. A graphical representation of the intergenic region and outline of the fragments used for the promoter dissection are shown on the left. The end of *yhxB* and the beginning of *yhcY* are labeled. The putative *rho*-independent terminator downstream of *yhxB* is indicated by the black stem-loop symbol. The arrow indicates the transcriptional start site. The distance of the 5'-end of the cloned fragments from the transcriptional start is indicated. The activity of these promoters was measured in a *liaHGF*::kan mutant. The corresponding strains TMB096-TMB104 were inoculated in LB-medium to mid-log phase (OD_{600} ~0.4) and the cells from 2 ml were harvested and used for β-galactosidase assay as described above (without the addition of bacitracin). The resulting β-galactosidase acitivities, expressed in Miller units (Miller, 1972), are shown. **(D)** Sequence of the promoter region upstream of *yhcY*. The transcriptional start site is highlighted in bold, underlined, the promoter bold italics. The putative *rho*-independent *yhxB*-terminator is indicated by arrows and highlighted in bold/underlined, coding regions are given in bold, with the start- und stop-codon underlined. The palindrome of the putative LiaR binding site is underlined and boxed. The 5'-end of the promoter fragments used for the promoter deletion analysis are marked. The minimal LiaR-dependent promoter fragment is shaded in grey. Note that the labeling of the 5'-end of the fragments for the promoter deletion analysis in this figure (Fig. 2.7C and 2.7D) is given relative to the transcription start ("+1") for reasons of clarity. Thereby, this nomenclature differs from the labeling of the fragments for cloning (Table 2.1 and 2.2), which are normalized relative to the "A" of the start codon of *yhcY*.

analysis verified that the *yhcYZ-yhdA* genes constitute an operon of 2.2 kb size (data not shown).

To further characterize the LiaR-dependent activity of P_{yhcY} and the role of the putative LiaR-binding site, a promoter deletion analysis was performed. Promoter fragments of decreasing length were cloned into pAC6 and verified by DNA sequencing. Integration of the resulting plasmids generated strains TMB096 through TMB104 (smallest to largest P_{yhcY} fragment, respectively, see Table 2.1 and Fig. 2.7C for details). The results demonstrate that a complete LiaR-box adjacent to the promoter region is necessary and sufficient for LiaR-dependent promoter activity (Fig. 2.7C and 2.7D).

Site-directed mutagenesis of the putative LiaR-binding site in P_{liaI}. To experimentally test the importance of individual residues within the conserved LiaR-binding motif, we generated a series of LiaR-box mutants by site directed mutagenesis (Table 2.4). Exchanging the five most highly conserved nucleotides of the LiaR-box by an "A" led to a complete loss of promoter activity in strain TMB113. The same effect was observed with mutations affecting only the promoter-proximal half-side (TMB114 and TMB133). Interestingly, a mutant with the three highly conserved residues in the 5'-side of the palindrome replaced by "A" (strain

Table 2.4. Mutagenesis of the putative LiaR-binding site upstream of P_{liaI}

Strain[1]	Sequence of putative LiaR-binding site[2]	β-Gal [MU][3] - Bac	+
wildtype	CGGTGCGAG**ATACGAC**TCCG**GTC**TTAT**A**T**AAAAA**TCAATCT	**0,9**	**196**
TMB113	CGGTGCGAG**A**A**A**C**A**A**A**TCCG**A**T**A**T**TATA**T**AAAAA**TCAATCT	**0,8**	**0,6**
TMB115	CGGTGCGAG**A**A**A**C**A**A**A**TCCG**GTC**TTATAT**AAAAA**TCAATCT	**0,5**	**4,5**
TMB133	CGGCGGGAG**ATACGAC**TCCG**AAA**TTATAT**AAAAA**TCAATCT	**0,6**	**0,8**
TMB114	CGGTGCGAG**ATACGAC**TCCG**GTC**TT**C**TATA**C**AAATCAATCT	**0,2**	**0,6**
TMB111	CGGTGCGAG**ATACGAC**TCCG**GTC**TTAT**T**TA**T**A**T**TATCAATCT	**0,3**	**13**
TMB112	CGGTGCGAG**ATACGAC**TCCG**GTC**TTAT**A**T**A**T**A**TTCAATCT	**0,7**	**17**
	---7--- 4 ---7---		

[1]All strains harbor the minimal LiaR-dependent promoter fragment, transcriptionally fused to *lacZ*, integrated at the *amyE* locus (see Table 2.1 for details).

[2]The palindrome is highlighted by a black frame and the 7-4-7 motif is indicated. Conserved nucleotides are given in bold letters. Minimal LiaR-responsive promoter fragments were mutated at the nucleotides indicated by black boxes during PCR-amplification, cloned into pAC6 to generate mutated P_{liaI}-*lacZ* fusions, sequence verified and subsequently transformed into W168 as described in the material and methods and results sections.

[3]P_{liaI} -activity of the resulting mutants was determined by ß-galactosidase assay with and without addition of bacitracin (Bac; final concentration 50 µg/ml) as described in the legend to Fig. 2.1. MU = Miller units. TMB016 ("wildtype") is given as a reference.

TMB115) retained a very low level of bacitracin-inducible activity. All three LiaR-boxes of *B. subtilis* harbor an A-stretch directly 3' of the inverted repeat, with two residues also being conserved in the putative binding sites of LiaR-homologs (Fig. 2.5). Therefore, we constructed two additional mutants (TMB111 and 112) each harboring a different set of three A→T exchanges. These mutants each showed a signficantly decreased, albeit less severely affected P_{liaI}-activity in the presence of bacitracin. These results support a functional role of this A-rich region, perhaps serving as a DNA-binding site (UP element) that interacts with the α-C-terminal domain of RNA-polymerase (Gaal *et al.*, 1996).

Discussion

A complex regulatory network, consisting of TCS and alternative σ-factors, orchestrates the cell envelope stress response in *B. subtilis* (Mascher *et al.*, 2003; Pietiäinen *et al.*, 2005). One such TCS, LiaRS, mediates the strong induction of its corresponding *liaIHGFSR* locus in the presence of antibiotics that interfere with the lipid II cycle, such as bacitracin, vancomycin, nisin, and ramoplanin (Mascher *et al.*, 2004). The LiaRS TCS also responds strongly to the presence of cationic antimicrobial peptides known to affect the cell envelope (Pietiäinen *et al.*, 2005) and is weakly induced in response to secretion stress, alkaline shock and the presence of detergents, organic solvents, ethanol and some surfactants (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Wiegert *et al.*, 2001). Here, we investigated the role of individual genes of the *lia* locus in LiaRS-mediated cell envelope stress response. We found that LiaF and LiaH act as a strong and weak inhibitor of this signal transduction process, respectively (Fig. 2.1 and Fig. 2.3). Applying comparative genomics, we identified a putative LiaR-binding site that was verified by mutational analysis (Fig. 2.5, Table 2.4). This LiaR-box was also found in the promoter region of a second LiaR-target locus, the *yhcYZ-yhdA* operon, the expression of which we studied in detail (Fig. 2.7).

LiaRS-mediated signal transduction is conserved in *Firmicutes* bacteria. Using comparative genomics, we recognized LiaF-LiaRS as a cell envelope stress response system conserved in the phylum *Firmicutes* (Fig. 2.4). We extended our comparative genomics studies to identify putative LiaR-binding sites and corresponding target genes in other *Firmicutes* bacteria (Table 2.5). LiaR-boxes were primarily identified in organisms of the order *Bacillales*, harboring both *liaFSR* and *liaIH* homologs. Overall, the number of putative

Tab 2.5. Conserved sequences of putative binding sites for LiaR-homologous response regulators[1] and their corresponding target genes

Genomic Position Start	End	Strand	PWM Score[2]	Sequence[3]	ORF /[4] Acc-Nr	ATG[5] Dist.	Operon structure, homology, putative function of target genes; additional remarks
Bacillus subtilis							
1008019	1008040	-	19.13	TCaGACTtttGgatgAGAAAAA	*yhcY*	85	*yhcYZ-yhdA*: two-component system, azoreductase
2060155	2060176	+	18.31	TtcGTCTgAAGTgaTAGtAAAA	*yozJ*	208	monocistronic: unknown hypothetical
3398065	3398086	-	19.81	TAcGACTccgGTCtTAtAtAAA	***liaI***	**75**	*liaIHGFSR*
Bacillus licheniformis							
1003453	1003474	+	18.10	TCgGTCaaAAGTCtTAGAAAAg	*yhcY*	77	*yhcYZ-yhdA*: two-component system, azoreductase
3337261	3337282	-	17.96	TAcGACcctgGTCgTAcggcAA	***yvqI***	**75**	*yvqIHGFEC*: homologous to the *lia* locus
Bacillus halodurans							
1286962	1286983	+	20.43	TCaGACTcAAGgCggAGAgAAA	**BH1195**	**74**	BH1195-1200: homologous to the *lia* locus
Bacillus cereus							
259757	259778	-	18.28	TCcGcCTctAGTCtcAtAAcAA	BC0296	80	monocistronic, glutamine-hydrolyzing GMP synthase
1396398	1396419	+	20.22	TCgGTCTtAAGTCtgAGttAAA	**BC1435**	**78**	BC1435-1439: homologous to the *lia* locus
2332576	2332597	-	18.32	TCaGACTtAAGaCcTAGAgAAc	BC2389	119	BC2389/90: tellurite resistance protein/unknown hypothetical
2332582	2332603	+	19.40	TAgGTCTtAAGTCtgAGgAAAA	BC2389	111	duplicated LiaR-box (overlapping palindromes on both strands)
3635790	3635811	-	18.44	TAgGACTaAAGaCtTAGAAgAA	BC3667	88	BC3667/*bcrAB*: ABC permease/bacitracin resistance proteins
3635796	3635817	+	19.06	TAaGTCTttAGTCcTAGAAAAc	BC3667	96	duplicated LiaR-box (overlapping palindromes on both strands)
Oceanobacillus iheyensis							
536986	537007	+	19.70	TCaGACTgAAGTCgTAGgAtAA	OB0502	64	OB0502/0503: unknown hypothetical proteins
1113240	1113261	-	18.46	TAtGACTcAAGTCaTAaAAtAA	OB1071	81	OB1071-1073: ABC transporter
2905575	2905596	-	19.20	TAcacCTtAAGTCtTAGttAAA	**OB2826**	**76**	OB2822-2826: homologous to the *lia* locus
Listeria innocua							
979178	979199	+	18.21	TAgGACTcAAGTtgTAGgtgAA	**Lin0953**	103	Lin0953/0954, homologous to *liaIH*
1040744	1040765	+	18.49	TCcGACTaAAGTagTAGAgAtt	**Lin1019**	121	Lin1019-1021, homologous to *liaFSR*
1060686	1060707	-	18.34	TAtGcCTttAGTCcTAtgAAAA	Lin1043	95	monocistronic, similar to formylmethionine deformylase
Listeria monocytogenes							
987610	987631	+	18.22	TAgGACTcAAGTtgTAGgtgAA	**lmo0954**	102	lmo0954/0955, homologous to *liaIH*
1049130	1049151	+	18.95	TCcGACTaAAGTagTAGgctAt	**lmo1020**	120	lmo1020-1022, homologous to *liaFSR*
Enterococcus faecalis							
2791625	2791646	-	17.99	TAGGgCTaAAGTCCTAtggtAA	**EF2913**	**73**	EF2911-2913, homologous to *liaFSR*
1392025	1392046	-	17.30	TCgGACTttAGTAGgAtAcAAt	EF1412/ EF1413	42/ 273	EF1412: putative transmembrane protein EF1413/1414: macrolide resistance-like ABC transporter
Lactococcus lactis							
910806	910827	+	20.15	TAaGTCTtAAGTCcgAGAAAAA	***yjbB***	27	homologous to *liaFSR*
			Consensus[6]	TMnGwCTnAAGTCnTAGAAAAA			

[1]Putative LiaR-boxes were identified by submitting the position weight matrix derived from the initial motif represented in Fig. 2.5 to the Virtual Footprint webpage implemented in the Prodoric database and performing individual searches against the genome sequences of each organism harboring LiaRS-homologous TCS. [2]PWM: position weight matrix. [3]Nucleotides conserved in more than 80% of the sequences are highlighted as white letters on black background. Residues with a weaker degree of conservation are given in bold letters. Non-conserved nucleotides are given in small letters. Nucleotides that contribute to the inverted repeat (i.e. have a complimentary counterpart) are highlighted in grey, irrespective of the degree of conservation. [4]The *lia*-homologs (each representing the first of the genes indicated under "operon structure, homology, ..."; i.e. homologs to either *liaI* or *liaF*) are underlined. [5]Distance of the LiaR-box to the ATG start codon of the indicated gene in nucleotides.
[6] "M" = A or C, "W" = T or A

LiaR-binding sites per genome was low (one to four). In *B. licheniformis*, the closest sequenced relative to *B. subtilis*, the two putative LiaR-binding sites are also located upstream of homologous loci, i.e. *yvqI* (*liaI)* and *yhcY*. For the two *Listeria* species, the homologs of *liaIH* (first gene lin0953/Lmo0954) and *liaFSR* (lin1019/Lmo1020) are organized in two independent transcriptional units, as noted above (Fig. 2.4). Interestingly, a putative LiaR-binding site was identified in the promoter regions of both loci. A LiaR-box was also identified upstream of the *liaF*-homologs in *Enterococcus faecalis* and *Lactococcus lactis* (Table 2.5, and triangles in Fig. 2.4). Remarkably, three of the candidate target loci encode ABC transporters (including a putative bacitracin efflux pump), supporting the role of LiaRS-homologs in mediating a cell envelope stress response (Table 2.5).

The sequence alignment of putative LiaR-boxes identified a core motif of 16 nucleotides, followed by an "A"-rich 3' extension that could function as an UP element (Gaal *et al.*, 1996). The core motif consists of an inverted repeat of seven nucleotides, with a spacing of two or four nucleotides. LiaR belongs to the NarL/FixJ family of response regulators (Pao *et al.*, 1994). The recognition of an inverted repeat is well established for this family of transcriptional regulators (Crater and Moran, 2001; Dahl *et al.*, 1997; Egland and Greenberg, 2000) as well as for other response regulators such as *L. monocytogenes* VirR and *Bradyrhizobium japonicum* RegR (Emmerich *et al.*, 2000; Mandin *et al.*, 2005).

The conservation of the LiaF-LiaRS signal transduction system and several of its target operons in the *Firmicutes* (with the noteworthy exception of the genus *Clostridium*) (Fig. 2.4) suggests that this is a conserved cell envelope stress system. Preliminary transcriptome analyses revealed that the *lia*-homologous *yvqIHGFEC* locus in *B. licheniformis* is also strongly induced by bacitracin (Wecke and Mascher, unpublished observation). Other LiaRS homologs, such as *Staphylococcus aureus* VraSR, *Listeria monocytogenes* Lmo1021/1022 , and *S. pneumoniae* HK03/RR03 also respond to cell envelope stress elicited by cell wall antibiotics and membrane perturbations (Gravesen *et al.*, 2004; Haas *et al.*, 2005; Kuroda *et al.*, 2003). In the case of *S. aureus*, the VraSR-system controls a large regulon of about 50 genes (Kuroda *et al.*, 2003). It seems reasonable to postulate that all *liaFSR* homologs depicted in Fig. 2.4 encode cell envelope stress sensing three-component systems. Although *liaFSR*-like operons are present in the streptococci and staphylococci, we were unable to identify LiaR-boxes upstream of these loci. We speculate that the corresponding LiaR-like proteins have a distinct DNA-binding selectivity, a hypothesis supported by the divergence of their DNA-binding domains (data not shown).

Taken together, the data seem to indicate the existence of two sub-groups within LiaRS-like cell envelope stress-sensing TCS. The genera *Bacillus* and *Listeria* harbor the complete *lia* locus (organized as two independent, but LiaR-dependent transcriptional units for the latter). In *B. subtilis*, one prominent effect of activation is a strong overexpression of LiaH and, due to the operon organization, presumably also of the small membrane protein LiaI. In general, these systems have recognizable LiaR-binding sites (exception: *B. anthracis* and *B. stearothermophilus*) and seem to control only a small number of target genes (Table 2.5). Members of the second group (VraSR-like TCS) lack a recognizable LiaR-box and homologs of *liaIH*, but seem to control a larger regulon.

LiaF is a strong inhibitor of LiaRS-mediated signal transduction. We identified LiaF as an essential part of the LiaRS signaling system that maintains the system in an inactive state (Fig. 2.1, 2.3, and 2.7A). It is presently unclear whether LiaF senses cell envelope stress directly or indirectly through LiaS. Most characterized proteins inhibiting TCS-mediated signal transduction – such as Sda and KipI in the sporulation phosphorelay of *B. subtilis*, and FixT in FixLJ-mediated nitrogen fixation in *Sinorhizobium meliloti* – interfere with histidine kinase autophosphorylation, thereby suppressing activation of the cognate response regulators (Burkholder *et al.*, 2001; Garnerone *et al.*, 1999; Wang *et al.*, 1997). Recently, a TCS-inhibitor protein was described that shares many features with LiaF: Deletion of *yycH* – encoding a transmembrane protein – results in uncoupled activity of YycFG (Szurmant *et al.*, 2005), an essential TCS in *B. subtilis* and other Gram-positive bacteria (Clausen *et al.*, 2003; Fabret and Hoch, 1998). The three corresponding genes are co-transcribed and conserved by genomic context. It has been postulated that YycH affects the sensor domain of its corresponding histidine kinase, YycG (Szurmant *et al.*, 2005). A similar role could be envisioned for LiaF.

LiaH, a phage-shock protein A (PspA) homolog, acts a negative modulator of LiaRS-mediated signal transduction. We also presented evidence that LiaH acts as a weak – and presumably indirect – negative modulator for LiaR-dependent gene expression. The *liaIH* genes are expressed at a much higher level relative to the downstream genes *liaGFSR*, due to termination of transcription at a stem-loop structure downstream of *liaH* (Mascher *et al.*, 2004). The strong induction of *liaIH* is also apparent at the protein level: LiaH was described as a marker protein for bacitracin treatment in a proteomic study (Bandow *et al.*, 2003). Furthermore, LiaH is a highly abundant protein visible in one-dimensional SDS-PAGE of cell lysates from cultures of the *liaF*-mutant, even in the absence of bacitracin (data not shown).

LiaH belongs to the family of phage-shock proteins. So far, only two members of this protein family have been investigated in detail, PspA of *Yersina enterocolitica* and *E. coli*. The latter is induced by filamentous phage infection (Brissette *et al.*, 1990), hence the name phage-shock protein. It is also induced by various other stress conditions, such as heat shock, osmotic shock, exposure to organic solvents and proton ionophores and long incubation under alkaline conditions (Brissette *et al.*, 1990; Kobayashi *et al.*, 1998; Weiner and Model, 1994). Furthermore, misincorporation of secretin pore proteins also induces the *psp* locus in both *Y. enterocolitica* and *E. coli*, as does blocking of the Sec pathway (Darwin and Miller, 2001; Hardie *et al.*, 1996; Kleerebezem and Tommassen, 1993; Kleerebezem *et al.*, 1996). In comparison, LiaH expression is induced by cell envelope stress generated by lipid II-interacting antibiotics such as bacitracin and ramoplanin, but also by cationic antimicrobial peptides, alkaline shock, exposure to organic solvents, detergents, ethanol and secretion stress (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2003; Petersohn *et al.*, 2001; Pietiäinen *et al.*, 2005; Wiegert *et al.*, 2001). While the mechanism of their transcriptional regulation differs, the range of inducing conditions for *B. subtilis* LiaH and *E. coli* PspA is remarkably similar.

PspA exhibits a dual function that is linked to two different cellular locations (Brissette *et al.*, 1990; Kleerebezem and Tommassen, 1993): in unstressed cells, it is a cytosolic protein that acts as a negative regulator by inhibiting the transcriptional activator PspF (Adams *et al.*, 2003; Bordes *et al.*, 2003; Dworkin *et al.*, 2000). Under conditions of cell envelope stress (leaky outer membrane), it is peripherally bound to the inner surface of the cytoplasmic membrane, contributing to the maintenance of the proton motive force and overall membrane integrity (Kleerebezem *et al.*, 1996). This membrane anchoring is mediated by protein-protein interaction with two transmembrane proteins, PspB and PspC (Adams *et al.*, 2003; Bordes *et al.*, 2003; Dworkin *et al.*, 2000). Based on our findings, it is tempting to postulate a similar dual role for LiaH. It functions as a weak negative transcriptional regulator without having a DNA-binding domain (Fig. 2.1 and Fig. 2.7A). Its cotranscription with *liaI*, coding for a putative membrane protein, suggests that LiaI might serve as a membrane anchor for LiaH, thereby facilitating a second (so far unknown) activity, linked to the inner surface of the cytoplasmic membrane.

LiaFRS-mediated gene regulation – a working model. Under normal growth conditions, the last four genes of the *lia* locus, *liaGFSR*, are expressed from a recently identified, weak constitutive promoter directly upstream of *liaG* (Fig. 2.8A, and data not shown; note the

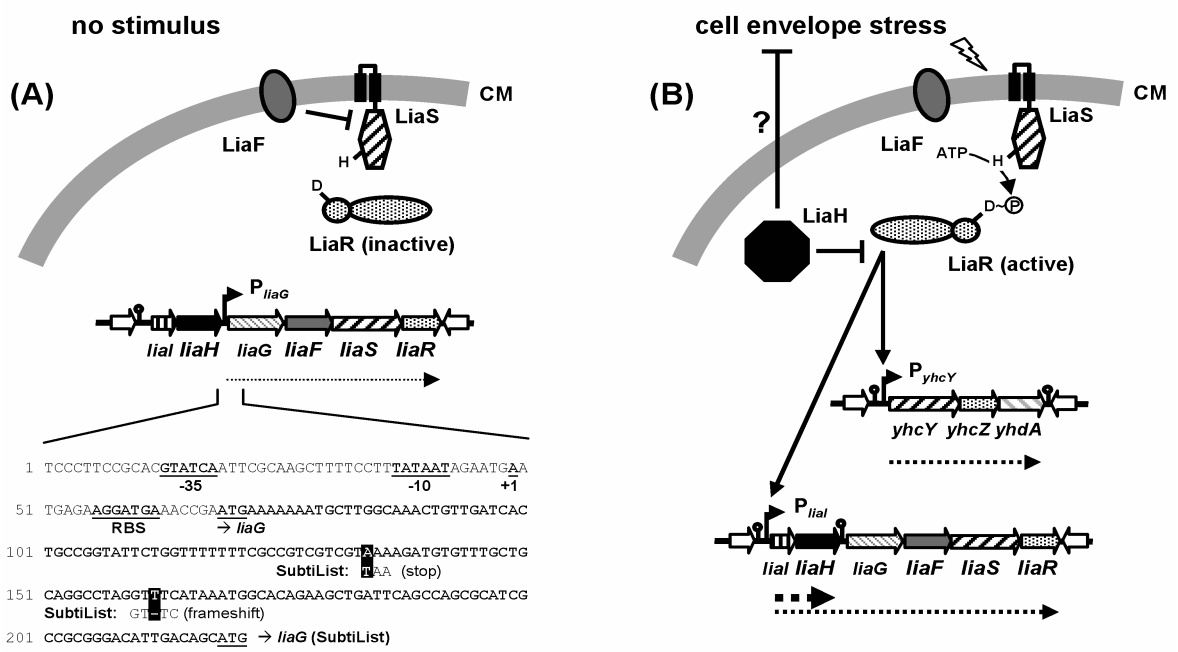

Figure 2.8. **Model of LiaRS-dependent gene expression in *B. subtilis*.** Transcripts are indicated by dotted lines. Activation is indicated by solid arrows, inhibitions by T-shaped lines. **(A)** P_{liaG}-dependent expression of *liaGFSR* in the absence of cell envelope stress. LiaF inhibits the LiaRS TCS. The sequence of the corresponding promoter region is given below. Re-sequencing revealed two mistakes in the original genome sequence, which result in the generation of a stop codon and a frame-shift within the 5'-end of *liaG*, respectively. Therefore, the corresponding LiaG protein is extended by another 50 amino acids at its N-terminus. The added sequence harbors a signal peptide and a potential transmembrane helix, as identified using the SMART database (Schultz *et al.*, 1998). This indicates that LiaG is a putative membrane-anchored protein rather than of cytoplasmic localization, as was originally annotated. **(B)** LiaRS-dependent gene expression in the presence of cell envelope stress. LiaF repression is released and activated LiaR binds to its target promoters, including its own operon (positive feedback loop). The strong overexpression of LiaH presumably counteracts cell envelope stress. Additionally LiaH functions as an inhibitor of LiaR-dependent gene expression (negative feedback loop). See text for further details.

corrected sequence for *liaG*; see Materials and Methods). P_{liaG}-dependent expression and the inhibitory activity of LiaF ensure that the system is present but switched off (Fig. 2.8A). In the presence of cell envelope stress, LiaS is released from LiaF-dependent inhibition and phosphorylated LiaR binds upstream of P_{liaI} and P_{yhcY} (Fig. 2.8B). P_{liaI} -induction gives rise to two transcripts, resulting most notably in overexpression of LiaH. Due to significant readthrough transcription, this induction defines a positive autoregulatory feedback-loop (stress-induced expression of *liaGFSR*) that allows the system to rapidly respond to envelope stress. The importance of this positive feedback loop is underscored by the identification of candidate LiaR-boxes in the promoter regions of those *liaFSR* homologs that do not receive readthrough transcription from upstream *liaIH* expression (Fig. 2.4), i.e. in *Listeria* species. The physiological role of LiaH and the other LiaR targets is not yet clear. Based on its homology to PspA, we speculate that LiaH might be involved in counteracting envelope stress, possibly by securing membrane integrity. Additionally, it plays a role in a negative feedback loop, thereby counteracting continued LiaR activity. This allows the system to

establish a level of LiaH appropriate for the stress condition (Fig. 2.8B). Two possible mechanisms for this LiaH-dependent feedback loop can be envisioned, both based on the analogy to *E. coli* PspA: first, LiaH could regulate LiaR-activity through direct protein-protein interaction as suggested above. Second, LiaH could help to restore envelope integrity, thereby removing the stress signal that activated LiaRS in the first place. In the absence of envelope stress, LiaF would then again be able to inhibit LiaRS, thereby efficiently switching the system off. It is more and more recognized that such combined positive and negative feedback loops in a regulatory pathway play an important role for adaptive responses of a bacterial population to its environment (i.e. competence and sporulation in *B. subtilis*), allowing a fast and differentiated response (Smits *et al.*, 2006).

Acknowledgements

We thank Sierd Bron for the gift of strains BFS1714 and BFS1715 and Tarek Msadek for gift of plasmid pXT. Furthermore, we would like to gratefully acknowledge the contributions of a number of students, who were involved in various aspects of the results described herein during their lab rotation projects (in order of appearance): Sara L. Zimmer (Cornell University) for primer extension of P_{yhcY}, Terry-Ann Smith (NYU) and Tina Wecke for contributing to the initial mutational analysis of the *lia* locus, Falk Kalamorz for SDS-PAGE analysis of LiaH overexpression, Diana Hoyer for taking part in the identification of P_{liaG} and Bastian Dörrbecker for the mutagenesis of the LiaR-binding site of P_{liaI}. This work was supported by grant MA 3269 from the Deutsche Forschungsgemeinschaft and grants from the Fonds der Chemischen Industrie (to TM) and by grant GM-47446 from the National Institutes of Health (to JDH).

Chapter 3:

LiaRS-Dependent Gene Expression is Embedded in Transition State Regulation in *Bacillus subtilis*

The work described in this chapter was published in:

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

Author contributions:
Sina Jordan performed the experiments and draw the figures. Eva Rietkötter contributed to the construction of mutants and performed β-galactosidase measurements in a practical training. Mark A. Strauch performed the DNase I footprint. Falk Kalamorz provided the LiaH antibodies. Bronwyn G. Butcher contributed to the initial results in the laboratory of John D. Helmann.

Summary

Maintaining envelope integrity is crucial for the survival of any bacterial cell, especially those living in a complex and ever-changing habitat such as the soil ecosystem. The LiaRS TCS is part of the regulatory network orchestrating cell envelope stress response in *Bacillus subtilis*. It responds to perturbations of the cell envelope, especially the presence of antibiotics that interfere with the lipid II cycle, such as bacitracin or vancomycin. LiaRS-dependent regulation is strictly repressed by the membrane protein LiaF in the absence of inducing conditions. Here, we show that the LiaR-dependent *liaI* promoter is induced at the onset of stationary phase without addition of exogenous stresses. Its activity is embedded in the complex regulatory cascade governing adaptation at the onset of stationary phase: The *liaI* promoter is directly repressed by the transition state regulator AbrB and responds indirectly to the activity of Spo0A, the master regulator of sporulation. The activity of the *liaI* promoter is therefore tightly regulated by at least five regulators to ensure an appropriate level of *liaIH* expression.

Introduction

The envelope is a crucial structure of the bacterial cell and the target of many antibiotics (Silver, 2003, 2006; Walsh, 2003). Its integrity is closely monitored to detect and counteract threats before their action can lead to irreversible damages. The LiaRS two-component system (TCS) is part of the complex regulatory network that orchestrates cell envelope stress response in *B. subtilis* (Mascher *et al.*, 2003). It strongly responds to the external presence of cell wall antibiotics that interfere with the lipid II cycle, such as bacitracin, ramoplanin, vancomycin, or cationic antimicrobial peptides (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005). It is also induced by alkaline shock, detergents, ethanol, phenol, organic solvents, and secretion stress, albeit to a lesser extent (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Pietiäinen *et al.*, 2005; Tam le *et al.*, 2006; Wiegert *et al.*, 2001).
The LiaRS TCS is functionally and genetically linked to a third protein, LiaF, which acts as a strong inhibitor of LiaR-dependent gene expression (Jordan *et al.*, 2006). The LiaRS-LiaF three-component system is conserved by sequence and genomic context in Gram-positive bacteria with a low G+C content (*Firmicutes*) (Jordan *et al.*, 2006; Mascher, 2006b), and LiaRS-homologous TCS are also involved in responding to cell envelope stress in *Bacillus*

licheniformis, Streptococcus pneumoniae, and *Staphylococcus aureus* (Haas *et al.*, 2005; Kuroda *et al.*, 2003; Wecke *et al.*, 2006). It is interesting to note that membrane anchored inhibitory proteins, working together with a classical TCS, have also been described for the cell wall related, essential TCS YycFG: the YycH and YycI proteins both inhibit the YycG kinase (Szurmant *et al.*, 2007a).

In *B. subtilis*, only two promoters are known to be regulated by the LiaRS TCS: the *liaI* promoter (P_{liaI}) and the *yhcY* promoter (Jordan *et al.*, 2006), with P_{liaI} being the primary target. In contrast to P_{liaI}, a LiaR-dependent P_{yhcY}-activity was only observed in a *liaF* mutant, i.e. in the absence of the LiaRS-inhibitor protein (Jordan *et al.*, 2006; Mascher *et al.*, 2004). P_{liaI} is tightly regulated: in the absence of a stimulus, it is virtually switched off, while addition of bacitracin results in an about 200-fold induction (Mascher *et al.*, 2003; Mascher *et al.*, 2004).

The *lia* locus consists of six genes, *liaIH-liaGFSR*. A basal expression level of the last four genes, *liaGFSR*, encoding the three-component system (*liaFSR*) and a putative membrane-anchored hypothetical protein (*liaG*), is ensured by a weak constitutive promoter upstream of *liaG*. In contrast, expression of the *liaIH* operon from P_{liaI} is completely LiaR-dependent (Jordan *et al.*, 2006). LiaI is a small hydrophobic protein of unknown function with two putative transmembrane helices. LiaH is a member of the phage-shock protein family (see below). While the strong induction of *liaIH* (and to a lesser degree also *liaGFSR*) by cell envelope stress is well documented (see above), mutational analyses of the *lia* locus so far failed to identify strong phenotypes associated with them. Deletion of *lia* genes did not alter the sensitivity of the corresponding mutants to the known inducers of the Lia system. Moreover, none of the complex differentiation processes of *B. subtilis* (i.e. sporulation, competence for genetic transformation, motility, pellicle and fruiting body formation) was affected in *lia* mutants. So far, the only phenotype that could be linked to the Lia system is delayed spore germination in a *liaH* mutant (Hoyer & Mascher, unpublished). Moreover, LiaH seems to negatively affect the expression of the *yhcYZ* operon by a so far unknown mechanism. It is weakly inducible by bacitracin only in a *liaH* mutant, but not in the wild type (Mascher *et al.*, 2003).

While the physiological role of LiaI and LiaH remains obscure, we noted some similarities between LiaH and phage-shock protein A (PspA) of *E. coli*. The latter is induced by various stress conditions such as filamentous phage infection (hence the name), heat shock, osmotic shock, exposure to organic solvents and proton ionophores as well as long incubation under alkaline conditions (Brissette *et al.*, 1990; Kobayashi *et al.*, 1998; Weiner and Model, 1994).

This inducer spectrum shows some overlap with the known inducers of *liaIH* expression, which include organic solvents and alkaline shock (Mascher *et al.*, 2004; Wiegert *et al.*, 2001). PspA exhibits a dual function that is linked to two different cellular locations (Brissette *et al.*, 1990; Kleerebezem and Tommassen, 1993): Peripherally bound to the inner surface of the cytoplasmic membrane (through protein-protein interactions), PspA is somehow involved in the maintenance of cell membrane integrity (Darwin, 2005; Kleerebezem *et al.*, 1996). As a free cytosolic protein it inhibits the AAA+ enhancer protein PspF, also through protein-protein interactions (Adams *et al.*, 2003; Bordes *et al.*, 2003; Dworkin *et al.*, 2000). Based on the strong induction of *liaH* by cell envelope stress and its co-transcription with *liaI,* coding for a small putative membrane protein, we speculate that LiaI serves as a membrane anchor for LiaH, thereby facilitating an activity that might somehow be linked to envelope integrity.
Here, we investigated the intrinsic activity and regulation of P_{liaI} in the absence of exogenous stimuli. We show that P_{liaI} is induced at the onset of stationary phase. This time point in the *B. subtilis* life cycle is characterized by the initiation of a complex regulatory cascade that allows *Bacillus* to adapt to worsening living conditions, which can ultimately lead to the formation of dormant endospores (Errington, 2003; Msadek, 1999; Phillips and Strauch, 2002). We demonstrate that P_{liaI} is directly repressed by binding of the transition state regulator AbrB within the promoter sequence, thereby acting as a roadblock to prevent premature P_{liaI} activity during logarithmic growth. AbrB repression is released during the transition state by Spo0A the master regulator of sporulation and P_{liaI} is induced by an unidentified endogenous stimulus, resulting in the expression of the *liaIH* operon. While AbrB-binding is sufficient to inhibit the endogenous growth-dependent induction of P_{liaI}, it can be bypassed completely by exogenous induction with cell wall antibiotics.

Methods

Bacterial strains and growth conditions. *B. subtilis* was routinely grown in LB medium at 37°C with aeration. All strains used in this study are derivatives of the wild type strain W168 and are listed in Table 3.1. Kanamycin (10 µg/ml), chloramphenicol (5 µg/ml), spectinomycin (100 µg/ml), tetracyclin (10 µg/ml), and erythromycin (1 µg/ml) plus lincomycin (25 µg/ml) for macrolide-lincosamide-streptogram ("MLS") resistance, were used for the selection of the *B. subtilis* mutants used in this study. Transformation was carried out as described (Harwood and Cutting, 1990).

Allelic replacement mutagenesis using Long Flanking Homology (LFH-) PCR. This technique is derived from the published procedure (Wach, 1996) and was performed as described previously (Mascher *et al.*, 2003). In brief: resistance cassettes were amplified from a suitable vector as template (Guerout-Fleury *et al.*, 1995; Youngman, 1990). Two primer pairs were designed to amplify ~1000 bp DNA-fragments flanking the region to be deleted at its 5'- and 3'-end. The resulting fragments are here called 'up' and 'do' fragment. The 3'-end of the up-fragment as well as the 5'-end of the do-fragment extended into the gene(s) to be deleted in a way that all expression signals of genes up- and downstream of the targeted genes remained intact. Extensions of ~25 nucleotides were added to the 5'-end of the 'up-reverse' and the 'do-forward' primers that were complementary (opposite strand and inverted sequence) to the 5'- and 3'-end of the amplified resistance cassette. All obtained fragments were purified using the PCR-purification kit from Qiagen. 100-150 ng of the up- and do-fragments and 250-300 ng of the resistance cassette were used together with the specific up-forward and do-reverse primers at standard concentrations in a second PCR-reaction. In this reaction the three fragments were joined by the 25 nucleotide overlapping complementary ends and simultaneously amplified by normal primer annealing. The PCR-products were directly used to transform *B. subtilis*. Transformants were screened by colony-PCR, using the up-forward primer with a reverse check-primer annealing inside the resistance cassette (Table 3.2). The integrity of the regions flanking the integrated resistance cassettes was verified by sequencing PCR products of ~1000 bp amplified from chromosomal DNA of the resulting mutants. Sequencing was performed in house by the GenoMIK center. All PCR-reactions were done in a total volume of 50 µl (10 µl for colony PCR) using the HotStar DNA-Polymerase Mastermix (Qiagen) or TripleMaster Polymerase Mix (Eppendorf) according to the manufacturer's procedure. The constructed strains are listed in Table 3.1. The primers used in this study are listed in Table 3.2.

Construction of a clean *liaS* deletion mutant. To ensure "normal" (i.e. wild type) expression levels of *liaR*, we constructed a clean deletion of *liaS* using the vector pMAD (Arnaud *et al.*, 2004). The genomic regions ~1 kb upstream and downstream of *liaS* were amplified using primers listed in Table 3.2 (fragments: liaS(clean) up, and liaS(clean) down), thereby introducing a 26 bp extension to the 3´-end of the up-fragment, which is complementary to the 5´-end of the down-fragment. The two fragments were fused in a second joining PCR reaction, and the resulting fragment cloned into pMAD via *Bam*HI and *Eco*RI, generating pMM101. Generation of the clean deletion basically follows the established procedure (Arnaud *et al.*, 2004). In brief: *B. subtilis* W168 was transformed with

pMM101 and 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 pMM101 into the chromosome. Again, blue colonies were picked from LB (X-Gal) plates and incubated for 6 h 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 checked for MLS sensitivity. Those harbouring a clean deletion of *liaS* (~ 50% of the white clones) were identified by PCR.

Table 3.1. **Strains used in this study**

strains	**relevant genotype**[1]	**source, reference, construction**[2]
W168	wild type strain, *trpC*2	lab stock
BFS2470	*liaI*::pMUTIN	(Mascher *et al.*, 2003)
HB0933	*liaR*::kan^R	(Mascher *et al.*, 2003)
TMB011	*liaI*::pMUTIN, *liaR*::kan^R	chrom. DNA (HB0933) → BFS2470
TMB079	*sinR*::spec^R	LFH-PCR → W168
TMB080	*aprE*:: kan^R	LFH-PCR → W168
TMB081	*scoC*::tet^R	LFH-PCR → W168
TMB082	*abrB*::kan^R	LFH-PCR → W168
TMB083	*salA*::tet^R	LFH-PCR → W168
TMB084	*liaI*::pMUTIN, *sinR*::spec^R	chrom. DNA (TMB079) → BFS2470
TMB085	*liaI*::pMUTIN, *aprE*::kan^R	chrom. DNA (TMB080) → BFS2470
TMB086	*liaI*::pMUTIN, *scoC*::kan^R	chrom. DNA (TMB081) → BFS2470
TMB087	*liaI*::pMUTIN *abrB*::kan^R	chrom. DNA (TMB082) → BFS2470
TMB088	*liaI*::pMUTIN *salA*::tet^R	chrom. DNA (TMB083) → BFS2470
TMB117	*liaI*::pMUTIN *degU*::kan^R	chrom. DNA (TMB124) → BFS2470
TMB118	*liaI*::pMUTIN *spo0A*::tet^R	chrom. DNA (TMB205) → BFS2470
TMB124	*degU*::kan^R	LFH-PCR → W168
TMB205	*spo0A*::tet^R	LFH-PCR → W168
TMB209	*liaI*::pMUTIN *abrB*::kan^R, *spo0A*::tet^R	chrom. DNA (TMB082) → TMB118
TMB213	Δ*liaS*	pMAD-based clean deletion
TMB215	Δ*liaS*, *liaI*::pMUTIN	chrom. DNA (BFS2470) → TMB213
TMB330	Δ*liaS*, *liaI*::pMUTIN, *abrB*::kan^R	chrom. DNA (TMB082) → TMB215

[1] kan^R, kanamycin; spec^R, spectinomycin; tet^R, tetracycline resistance cassette

[2] All deletion mutants were constructed by replacing the corresponding gene with a resistance cassette, applying the long-flanking homology PCR strategy (LFH-PCR) as described previously (Mascher *et al.*, 2003; Wach, 1996). See Table 2 for sequences of the primers used for their construction. Subsequent strains were constructed transformation of a recipient strain, given after the arrow, with chromosomal (chrom.) DNA from the donor strain (in parenthesis).

Measurement of induction by b**-galactosidase assay.** For time course experiments of P_{liaI}-induction, the respective reporter strains (listed in Table 9) were inoculated in LB medium from a fresh mid-logarithmic pre-culture to an OD_{600}≈0.1 and incubated at 37°C with

aeration. 2 ml samples were taken every hour and the OD_{600} was monitored to follow the growth of the cultures. The pellets were resuspended in 1 ml of working buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$, 20 mM β-mercaptoethanol) and suitable dilutions were assayed for β-galactosidase activity as described with normalization to cell density (Miller, 1972). For induction experiments, the cells were inoculated from fresh overnight cultures and grown in LB-medium at 37°C with aeration until they reached an OD_{600}≈0.6. The culture was split, adding bacitracin (50 µg/ml final concentration) to one half (induced sample) and leaving the other half untreated (uninduced control). After incubation for an additional 30 min at 37°C with aeration, 2 ml of each culture were harvested and the cell pellets were frozen and kept at -20°C. The pellets were resuspended in 1 ml of working buffer and assayed for β-galactosidase activity as described with normalization to cell density (Miller, 1972).

Western blot. Total cytoplasmic proteins were prepared from 15 ml of culture per time point by using a french press. 20 µg of proteins per lane were separated by SDS-PAGE, according to standard procedure (Sambrook and Russell, 2001). After electrophoresis the gels were equilibrated in transfer buffer (15.2 g Tris; 72.1 g glycine; 750 ml methanol (100 %) in a final volume of 5 l with de-ionized water) for 30 seconds. A PVDF membrane was activated with methanol (100 %) and subsequently incubated in transfer buffer for five minutes. The proteins were blotted to this membrane using a Semi Dry Blot apparatus. After transfer (1 h at 0.8 mA/cm^2) the membrane was incubated in blotto (1x TBS (50 mM Tris, 150 mM NaCl, pH 7.6), 2.5 % skim milk) overnight to prevent unspecific binding. The LiaH antibody (polyclonal rabbit antisera that were raised against purified His_{10}-LiaH (Kalamorz & Mascher, unpublished) at SEQLAB, Göttingen, Germany) was diluted 1:20,000 in blotto. After incubation for three hours, the membrane was washed three times for 30 minutes with blotto. The secondary antibody (anti rabbit IgG, coupled with alkaline phosphatase, Roche Diagnostics, Mannheim) was diluted 1:100,000 and the membrane was incubated for 30 minutes. After three more washing steps for 20 minutes, the membrane was washed with de-ionized water and incubated in buffer III (0.1 M Tris; 0.1 M NaCl; pH 9.5) for five minutes to adjust the pH. 10 µl CDP-*Star* (chemiluminescence substrate, Roche Diagnostics, Mannheim) in 1 ml buffer III were used for LiaH detection. The signal was documented with ChemiSmart LumiImager (peqlab).

DNase I footprinting assays. AbrB and Abh purification and DNase I footprinting assays were performed essentially as described previously (Bobay *et al.*, 2006; Strauch *et al.*, 1989). The DNA target was a 287 bp fragment containing the *liaI* promoter region (positions -159 to

+128), end-labeled with ^{32}P on the template strand. The binding reactions were performed at 20°C with pH 8 for AbrB, and pH7 for Abh (the optimal pH's for each protein's binding) (Bobay *et al.*, 2006).

Table 3.2. **Oligonucleotides used in this study**

fragment[1]	**primer sequence**[2]
aprE-up	fwd: GTTGACATTCGGCACACTCC,
	rev: CCTATCACCTCAAATGGTTCGCTGGACATGTTGCTGAACGCCATCG
aprE-down	fwd: CGAGCGCCTACGAGGAATTTGTATCGAAACGCGCAAGTCCGTGATCG,
	rev: CATTTCCACACAGACAACGG
salA-up	fwd: AAGATTGGTGGACAGCAGG,
	rev: CCTATCACCTCAAATGGTTCGCTGGGTTCGCGCATTTCTCCG
salA-down	fwd: CGAGCGCCTACGAGGAATTTGTATCGTGACGAAAATCATCCAATCGG,
	rev: TATCTCAAGCGCAAACCGATG
abrB-up	fwd: TATCAACGAGCTGAGTTTCCG,
	rev: CCTATCACCTCAAATGGTTCGCTGCAACTTTACGTACAATACCAGTAG
abrB-down	fwd: CGAGCGCCTACGAGGAATTTGTATCGCAGCGAAATCCAAAACCAGC,
	rev: TTCTTTACTTGGTCCCAACCC
scoC-up	fwd: AACCTCTTCCGCTTCCGG,
	rev: CCTATCACCTCAAATGGTTCGCTGGAGCCTTGCTAAGCTGAGCC
scoC-down	fwd: CGAGCGCCTACGAGGAATTTGTATCGGATGAACCGGCTGAAGAGC,
	rev: ACGTTTCCATGTGCGCATGC
sinR-up	fwd: GCCAAAAGACCTAGATGGTG,
	rev: CCTATCACCTCAAATGGTTCGCTGATGTCATCACCTTCCTTGTG
sinR-down	fwd: CGAGCGCCTACGAGGAATTTGTATCGGATGACATCCGGGGTATCG,
	rev: TAGGAGTTGCTTCTGCAGC
degU-up	fwd: AAGCCCATAAGCTGCAGG,
	rev: CCTATCACCTCAAATGGTTCGCTGTATCCGTTTAACACCTTCACG
degU-down	fwd: CGAGCGCCTACGAGGAATTTGTATCGTAAACGACCGGACGCAAGCC,
	rev: CAAATGAGTGCCGATTACCGC
spo0A-up	fwd: TATCAGAGATTCTGCTGCTGGC,
	rev: CCTATCACCTCAAATGGTTCGCTGAGCGACAGGCATTCCTGTCC
spo0A-down	fwd: CGAGCGCCTACGAGGAATTTGTATCGGTTGCGGATAAGCTGAGG,
	rev: GGAAGAACCTGAGACACCG
kan cassette	fwd: CAGCGAACCATTTGAGGTGATAGG,
	rev: CGATACAAATTCCTCGTAGGCGCTCGG
spec cassette	fwd: CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTG ACTGGCAAGAG
	rev: CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTC TAAAATCTG
tet cassette	fwd: CAGCGAACCATTTGAGGTGATAGGTCTTGCAATGGTGCAGGTTGTTCTC,
	rev: CGATACAAATTCCTCGTAGGCGCTCGGGAACTCTCTCCCAAAGTTGATCCC
liaS(clean) up	fwd: AGCC***GGATCC***GAAAGGAGGCGGACACCAGG,
	rev: GTTCGTTCTCTCCTTTTTCTTCCGGCTCATACGTACTTCACATCC
liaS(clean) down	fwd: CCGGAAGAAAAAGGAGAGAACGAACG,
	rev: CCAT***GAATTC***AACCGGGCTGGGAAACGAGG

[1] 'up' and 'down' refers to localization of the fragment relative to the gene(s) to be deleted. Both fragments are approx. 1 kb in size and include 20-50 nucleotides of the 5'- or 3'-end of the corresponding gene(s), respectively.
[2] 'fwd', forward. 'rev', reverse. The (universal) linker sequences used for joining reactions are underlined. Restriction sites in ***bold***.

Results and Discussion

The *liaI* promoter (P_{liaI}) is induced without exogenous stimuli at the onset of stationary phase. Induction of P_{liaI} after addition of exogenous stimuli, such as cell wall antibiotics, is well documented (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005). To investigate if P_{liaI} is also induced in their absence, we examined expression of a P_{liaI}-*lacZ* fusion in the reporter strain BFS2470. This strain harbors an insertion of the vector pMUTIN (Vagner *et al.*, 1998) inside the *liaI* coding sequence, thereby bringing a promoter-less *lacZ* gene under control of the *liaI* promoter (Mascher *et al.*, 2003; Mascher *et al.*, 2004).

To study the P_{liaI} activity in the absence of external stimuli, strain BFS2470 was grown in LB medium with MLS selection over a period of eight hours and samples were taken every hour from mid-logarithmic to late stationary growth phase. The cells were harvested and β-galactosidase activity was determined, essentially as described previously (Mascher *et al.*, 2004). The results demonstrate that P_{liaI} is induced eight- to ten-fold without addition of cell wall antibiotics during transition to stationary phase (Fig. 3.1A, grey bars). This induction is completely LiaR-dependent: no P_{liaI} activity was observed in the isogenic *liaR* mutant strain TMB011 (Fig. 3.1A, black bars).

These observations were verified independently by Western analysis in the wild type and an isogenic *liaR* mutant (strain HB0933). Cells were harvested from both cultures at two time points, two hours before and after transition state (equivalent to the time points 2 and 6 h in Fig. 3.1A). Total cellular protein was prepared and Western analysis performed with LiaH antibodies. The results are in agreement with the data from the β-galactosidase assays. LiaH expression is induced in stationary phase in the wild type, but not in the *liaR* mutant (inset to Fig. 3.1A). Furthermore, induction of *liaIH* expression was also observed at the transcript level in a wild type *B. subtilis* strain during a chronotranscriptome analysis (see below). While only results from β-galactosidase assays will be shown for subsequent experiments, all key findings were always independently verified by Western analysis.

Transition state adaptation, which enables *B. subtilis* to gradually adjust to nutrient limitations, is embedded in one of the best studied bacterial developmental programs, a complex regulatory cascade that ultimately leads to the formation of highly resistant endospores (Errington, 2003; Msadek, 1999; Phillips and Strauch, 2002). It is well known that sporulation (and other transition state phenomena such as production of extracellular enzymes, motility, and biofilm formation) is subject to carbon catabolite repression (Schaeffer *et al.*, 1965; Shafikhani *et al.*, 2003; Stanley *et al.*, 2003): cells grown with high amounts of glucose enter stationary phase without activating the gene expression cascade associated with

sporulation. Therefore, we repeated the experiment shown in Fig. 3.1 in the presence of increasing glucose concentrations. Addition of glucose to the medium delayed (0.1% glucose, grey bars) or even abolished (0.5% glucose, white bars) P_{liaI}-induction, without affecting overall growth rate, onset of stationary phase, or final cell density (Fig. 3.1B). High glucose represses the activation of Spo0A, a key regulator of numerous post-exponential processes including sporulation. The activation of Spo0A results in two major effects on gene expression. First, it leads to the activation of a cascade of sigma factors that ultimately govern the formation of the dormant endospore. Second, Spo0A~P represses AbrB which itself is a repressor of numerous genes associated with antibiotic production and resistance (Errington, 2003; Msadek, 1999; Phillips and Strauch, 2002). While addition of glucose has pleiotropic effects on numerous regulatory pathways, this observation can nevertheless be viewed as an indication for a link between LiaRS-dependent gene expression and transition state regulation.

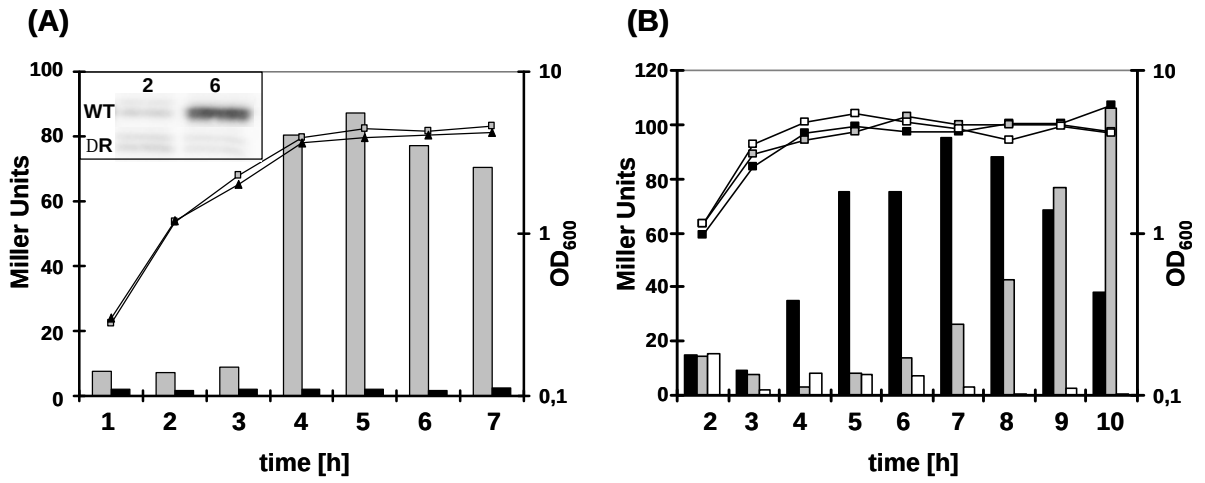

Figure 3.1. **Transition state induction of P_{liaI}. (A)** 20 ml LB medium (with MLS selection) were inoculated from a fresh mid-logarithmic preculture of strains BFS2470 ('wild type', grey squares or grey bars) and TMB011 (*liaR* mutant, black triangle and black bars) and incubated at 37°C with aeration. Cell density was monitored by measuring the optical density at 600 nm (OD_{600}) at regular intervals, and samples were taken every hour from mid-logarithmic until late stationary growth phase. The cells were harvested, lysed and β-galactosidase assay was performed as described previously (Mascher *et al.*, 2004). The β-galactosidase activity, normalized to cell density, is expressed in Miller units (Miller, 1972). The time scale is given in hours, relative to the start of the cultures. The inset shows results of a Western blot analysis of LiaH expression. 20 µg total proteins of the wild type (W168) and an isogenic *liaR* mutant (strains HB0933) were separated by SDS-PAGE. Western blots were performed using CDP-Star (Roche) for chemiluminescent detection, according to the manufacturer's instructions. See the Methods section for details. **(B)** The same experiment was performed as in Fig. 3.1A. Only the wild type reporter strain BFS2470 was used. The preculture was used to inoculate three different flasks contain 20 ml LB each, without (black symbols/bars), and with the addition of glucose to a final concentration of 0.1% (grey symbols/bars) and 0.5% (white symbols/bars). See legend to Fig. 3.1A and the Methods section for experimental details.

A second line of evidence pointing in this direction came from results obtained in a detailed chronotranscriptome study, in which the global gene expression pattern was monitored during a complete growth curve with a resolution of ten minutes (R. Sapolsky, R., P. Iyer, B.

Caldwell, W. Weyler, G. Chotani, and E. Ferrari, Abstr. 3rd Conf. Functional Genomics of Gram-Positive Microorganisms, abstr. T18, 2005). This study not only verified the transition state induction of *liaIH* (but not *liaGFSR*) in a *B. subtilis* wild type strain at the transcript level, but additionally revealed that expression of *liaIH* coincides with only one other gene, *aprE* (Eugenio Ferrari, personal communication). This observation can be interpreted in two ways: Either, P_{liaI} is induced as a result of *aprE* expression, or both loci are subject to the same regulation.

P_{liaI} is repressed by AbrB and activated by Spo0A. To address the first hypothesis, an *aprE* mutant was constructed by long-flanking homology PCR, and introduced in the P_{liaI}-reporter strain, resulting in strain TMB085 (Table 3.1). No difference of P_{liaI}-activity was observed relative to the wild type reporter strain BFS2470 (data not shown). Therefore, AprE is not involved in P_{liaI} induction.

The *aprE* promoter is subject to a complex regulation: one activator (DegU) and three repressors (ScoC, SinR, and AbrB) directly bind to the *aprE* promoter region. The activity of these proteins is modulated by additional proteins, such as Spo0A, SalA and RapG/PhrG (Ogura *et al.*, 2003; Ogura *et al.*, 2004). To analyze a potential role of these proteins in P_{liaI} activity, mutants in *abrB*, *scoC*, *sinR*, and *degU* were constructed and subsequently transferred into the P_{liaI}-reporter strain BFS2470 (Table 3.1). No alterations of P_{liaI} activity were observed in the *scoC*, *sinR*, and *degU* mutant background (data not shown). In contrast, the *abrB* mutation in strain TMB087 resulted in an about four-fold elevated basal expression level during logarithmic growth phase, indicating that AbrB acts as a repressor at P_{liaI} during that time (Fig. 3.2, black bars). The promoter is still inducible to about wild type levels. Both, the basal promoter activity and the induction of P_{liaI} in the *abrB* mutant are completely dependent on LiaS-mediated activation of its cognate response regulator, LiaR: a *liaS/abrB* mutant (TMB330; harboring a clean *liaS* deletion to avoid polar effects on *liaR* expression) behaves similar to a *liaR* mutant, i.e. does not show any P_{liaI}-activity throughout the growth curve (data not shown).

A close regulatory connection between the transition state regulator AbrB and Spo0A, the master regulator of sporulation, is well established: AbrB inhibits Spo0A expression indirectly via σ^H, contributing to the mechanisms governing temporal control of sporulation initiation. Activated Spo0A, on the other hand, represses *abrB* expression, ultimately releasing transition state functions, and σ^H expression, from AbrB repression at the onset of stationary phase (Msadek, 1999; Phillips and Strauch, 2002). As a consequence, mutations in the two genes usually exhibit converse phenotypes on genes subject to their regulation. This

could also be observed for P_{liaI} activity: a *spo0A* mutant (TMB118, Table 3.1) behaved very similar to the *liaR* mutant in the β-galactosidase assay, i.e. no detectable P_{liaI} activity (Fig. 3.2, white bars). These findings were also verified by Western analysis (data not shown). They are in agreement with results from previous transcriptome studies, indicating an indirect Spo0A-dependent induction of *liaIH* expression (Fawcett *et al.*, 2000; Fujita *et al.*, 2005; Hamon *et al.*, 2004). Therefore, P_{liaI} is subject to AbrB repression and Spo0A activation.

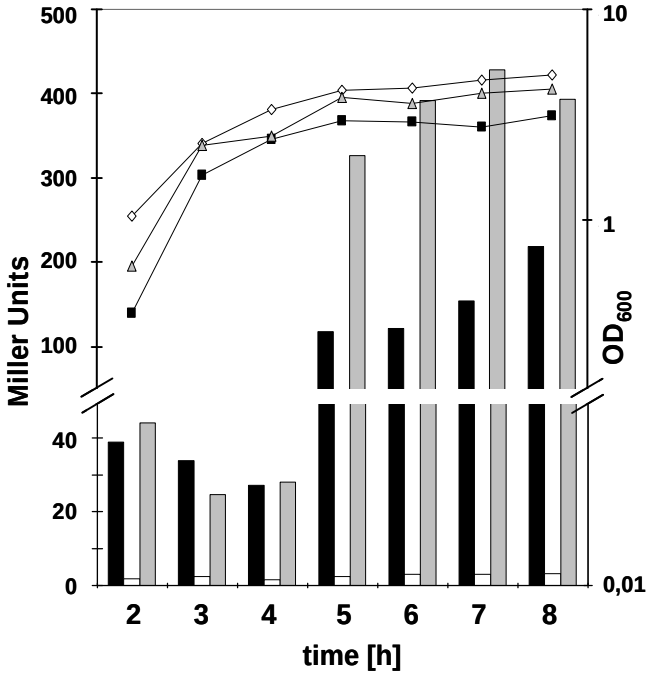

Figure 3.2. Effect of *abrB* and *spo0A* mutations on P_{liaI} activity. Growth and β-galactosidase acitivity of P_{liaI}-*lacZ* fusions were measured for an *abrB* (strain TMB087, black squares and black bars), a *spo0A* mutant (strains TMB118, white diamonds and white bars), and an *abrB/spo0A* mutant (strain TMB209, grey triangles and grey bars), respectively. The experiment was performed as described in the legend to Figure 3.1A. The scale on the *y*-axis was split for reasons of clarity.

Spo0A activates P_{liaI} indirectly through AbrB. The loss of transition state induction of P_{liaI} in the *spo0A* mutant raised the question of how Spo0A affects its promoter activity. The *abrB* gene is known to be under the direct negative control of Spo0A, and AbrB acts as a repressor of a set of genes that are switched during transition state (Strauch *et al.*, 1990; Strauch *et al.*, 1989). Therefore, Spo0A activation of P_{liaI} could be an indirect effect due to the lack of Spo0A-dependent repression of *abrB.* Alternatively, Spo0A itself could be responsible for the expression of genes that ultimately provide the stimulus that is sensed by the LiaRS TCS at the onset of stationary phase. To distinguish between the two possibilities, a mutant lacking both genes, *abrB* and *spo0A*, was constructed and introduced into BFS2470, resulting in strain TMB209. This mutant showed a P_{liaI} induction pattern comparable to the *abrB* mutant, i.e. an elevated basal level of P_{liaI} activity during logarithmic growth, and induction at the onset of

stationary phase (Fig. 3.2). Interestingly, the maximum P_{liaI} activity was reproducibly higher by a factor of two than in the *abrB* mutant (Fig. 3.2, grey bars). The reason for this behavior remains elusive, so far. But the results clearly demonstrate that Spo0A indirectly modulates P_{liaI} activity by repressing *abrB* expression.

AbrB directly binds P_{liaI}. The transition state regulator AbrB directly regulates (mostly represses) the expression of over 50 genes, with many additional loci being subject to indirect AbrB control (Phillips and Strauch, 2002). Despite in-depth knowledge on numerous AbrB binding sites, no consensus sequence has been identified for chromosomal sites of interaction. It has been hypothesized that AbrB recognizes a conserved three-dimensional DNA structure, rather than specific base pairs, in the promoter regions of its target genes (Bobay *et al.*, 2004; Phillips and Strauch, 2002; Xu and Strauch, 1996). DNase I footprinting analysis of the *liaI* promoter region demonstrates that AbrB protects a DNA region of about 25 base pairs (from -40 to -14), with weaker protection occurring further downstream (from -11 to about +10) (Fig. 3.3). In contrast, the AbrB paralog Abh does not bind the P_{liaI} region under these conditions (Fig. 3.3). We conclude that AbrB repression of P_{liaI} occurs through direct binding of the repressor within the promoter sequence, thereby preventing transcription initiation.

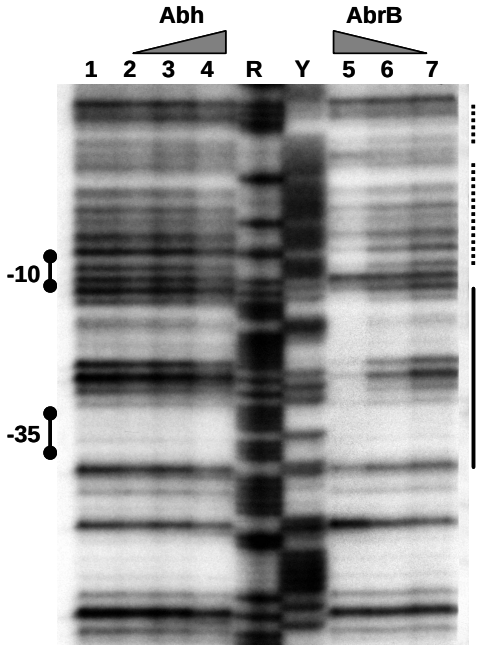

Figure 3.3. **DNase I footprinting analysis of AbrB binding to P_{liaI}.** DNase I footprinting was performed as described previously (Bobay *et al.*, 2006; Strauch *et al.*, 1989). Lanes 1,2,7: no protein; 3: 6µM Abh; 4: 20 µM Abh; 5: 20 µM AbrB; 6: 6 µM AbrB. AbrB binding reactions were performed at pH 8; Abh binding at pH7 (the optimal pH's for each protein's binding (Bobay *et al.*, 2006)). R, Y = Maxam-Gilbert purine and pyrimidine chemical sequencing reactions. The DNA target was a 287 bp fragment containing the *liaI* promoter region (positions -159 to +128) end-labeled on the template strand. Solid vertical line on right = seemingly stronger AbrB binding region; dashed lines on right = seemingly weaker protection region due to AbrB binding. -35 and -10 regions of the *liaI* promoter are indicated on the left.

LiaR is sufficient for P_{liaI} induction. Induction of P_{liaI} under conditions of cell envelope stress is strictly LiaR-dependent (Mascher *et al.*, 2003). To determine if AbrB or Spo0A are also necessary for P_{liaI}-dependent transcription in the presence of external stimuli, such as bacitracin, we performed β-galactosidase assay and Western analysis in the wild type, and isogenic *liaR*, *abrB*, and *spo0A* mutants from cells harvested mid-exponentially with and without the presence of bacitracin (final concentration 50 µg/ml). With the exception of the *liaR* mutant, all strains were inducible to comparable levels, demonstrating that LiaR alone is sufficient for bacitracin-induced P_{liaI}-activity (Fig. 3.4, and data not shown). These experiments also demonstrate that strong inducers such as bacitracin (3,000 to 4,000 Miller units from P_{liaI}-*lacZ* in β-galactosidase assays) can completely overcome AbrB repression, whereas the endogenous transition state induction (100-200 Miller units) can be suppressed by increased cellular levels of AbrB as present in the *spo0A* mutant. The mechanism by which fully activated LiaR (i.e. in response to bacitracin stress) seemingly "overrides" AbrB control is completely unknown. We can only speculate that the affinity of phosphorylated LiaR for its target promoter somehow is higher than that of AbrB, whereby activated LiaR seems to be able to displace bound AbrB and initiate P_{liaI}-dependent transcription in the presence of strong inducers.

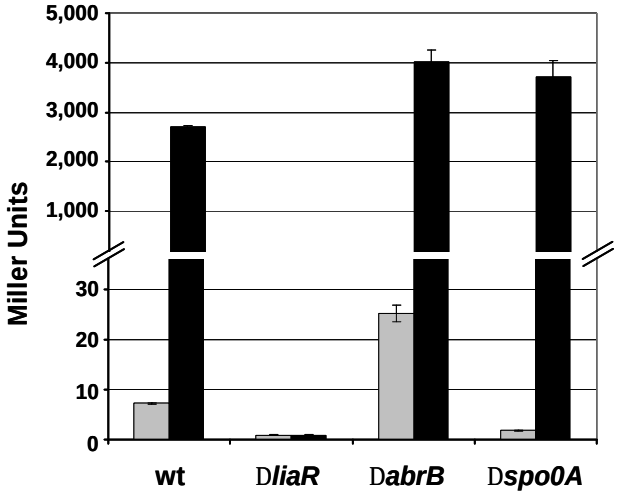

Figure 3.4. Bacitracin induction of P_{liaI} in strain BFS2470 (wt), and isogenic *liaR* (strain TMB011), *abrB* (strain TMB087), and *spo0A* (strain TMB118) mutants. All strains were grown in LB medium with MLS selection to mid-log. The cultures were split, one half induced with bacitracin (final concentration 50 µg/ml; black bars), the other half remained as uninduced control (grey bars). Incubation was continued for 30 min, before 2 ml of culture were harvested. The cells were lysed and β-galactosidase assay was performed as described previously (Mascher *et al.*, 2004). The β-galactosidase activity, normalized to cell density, is expressed in Miller units (Miller, 1972). The scale on the *y*-axis was split for reasons of clarity.

The nature of the endogenous stimulus responsible for transition state induction of P_{liaI} remains obscure. The known induction of P_{liaI} by cell wall antibiotics led us to hypothesize that induction in early stationary phase might be due to a secreted antibiotic synthesized by *B. subtilis* itself (Stein, 2005). To address this question, induction experiments with spent medium were performed. The wild type reporter strain BFS2470 was grown in LB medium with MLS selection until two hours after transition state (corresponding to t = 6h in Fig. 3.1A). A sample of the culture was harvested to check for P_{liaI}-induction. The cells were removed from the remaining culture and the spent medium was directly used to resuspend fresh mid-logarithmic cells (t = 2h) that were incubated in parallel. Additionally, cells were resuspended in fresh prewarmed LB medium, with and without addition of bacitracin (final concentration 50 µg/ml), as a positive and negative control, respectively. After further incubation at 37°C for 30 min, the cells were harvested. The results from β-galactosidase assays are shown in Fig. 3.5. The cells from the stationary phase culture showed the expected β-galactosidase activity. Resuspension of mid-log cells in fresh LB medium only resulted in the normal background activity of about 10 Miller units, while resuspension in LB medium supplemented with bacitracin gave the typical strong P_{liaI}-response (about 2,000 Miller units). In contrast, no induction was observed when mid-logarithmic cells were resuspended in spent medium (Fig. 3.5, last bar). These results indicate that P_{liaI} is not induced by a secreted compound produced by *B. subtilis* itself. But we cannot rule out the possibility that the inducing antibiotic is not released from the cells in sufficient amounts in the medium to be detectable in our conditioned medium experiments. For example, a prerequisite for the biological potency of many cationic antimicrobial peptides is their binding to the overall negatively charged cell envelope, and modulating this net charge is an important resistance mechanism of many gram-positive bacteria against their activity (Kovács *et al.*, 2006; Peschel *et al.*, 1999). Conversely, one could imagine that such an antibiotic, produced by *B. subtilis* itself, might be retained to a certain degree by the negatively charged cell wall. Therefore, while P_{liaI}-induction could then be readily measured in the antibiotic-producing stationary phase culture, this inducer would not necessarily accumulate in the medium in amounts sufficient to activate P_{liaI} in resuspended mid-logarithmic cells.

We also attempted to identify potential genetic determinants involved in generating the endogenous stimulus by applying transposon mutagenesis in the P_{liaI}-*lacZ* reporter strain BFS2470 and screening for blue colonies, indicative for increased P_{liaI} activity. Two independent approaches were used, *in-vivo* transposon mutagenesis, based on the established mini-Tn10 system encoded on plasmid pIC333 (Steinmetz and Richter, 1994), and a newly

developed *in-vitro* system, based on Tn7 (Peters and Craig, 2001). The latter also allows gain-of-function mutagenesis screens, due to the presence of an outward-facing, xylose-inducible promoter. We readily isolated mutants with transposon insertions in *liaF*, the known negative regulator of the LiaRS systems (Jordan *et al.*, 2006). In addition, we also recovered insertions in the export pump of a putative bacteriocin, indicating that endogenous peptides produced by *B. subtilis* can induce the LiaRS system (Butcher and Helmann, unpublished). However, strains lacking the ability to produce this bacteriocin still induce P_{liaI} upon entry into stationary phase (data not shown). Therefore, the nature of this endogenous stimulus remains unknown.

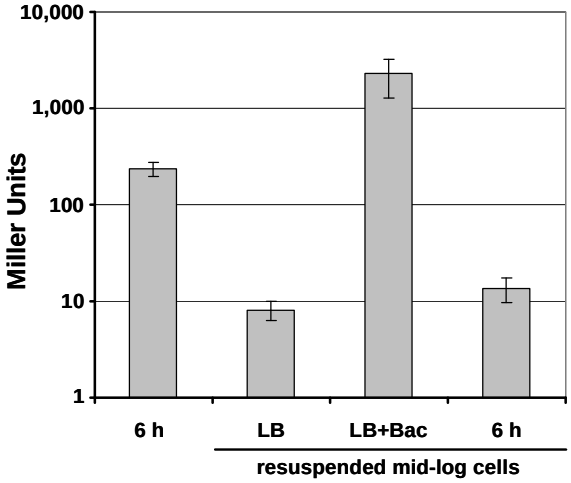

Figure 3.5. P_{liaI}-induction experiments with spent medium. A "donor" culture of strain BFS2470 was grown until two hours after transition state ("6h") and P_{liaI}-activity was determined to ensure inducing conditions (first bar). The corresponding culture supernatant was used after removing of cells by centrifugation, to induce cells from a second mid-logarithmic culture (fourth bar). As a control, mid-log cells were also resuspended in prewarmed fresh LB medium without (LB, second bar) and with addition of bacitracin (final concentration 50 µg/ml; third bar), as a negative and positive control. The resuspended mid-log cells were incubated for another 30 min at 37°C, before the cells were harvested and used in a β-galactosidase assay. The β-galactosidase activity, normalized to cell density, is expressed in Miller units (Miller, 1972). A log-scale was used on the *y*-axis for reasons of clarity.

Conclusions

Based on the results of previous studies and those presented herein, the intrinsic induction of *liaIH* expression at the onset of stationary phase is tightly regulated and delicately balanced by five proteins – LiaR, LiaS, LiaF, AbrB, and Spo0A – to allow an appropriate cellular response at the right time. The interactions and hierarchy of these regulators is illustrated in the model in Fig. 3.6. During logarithmic growth in the absence of cell envelope stress, the

LiaRS TCS is kept inactive by the LiaF regulator (Jordan *et al.*, 2006). Furthermore, the transition state regulator AbrB represses any residual P_{liaI} activity by binding to a DNA fragment that includes the -35 region and reaches the -10 region, thereby serving as a roadblock that efficiently prevents transcription initiation (Fig. 3.6, right-hand side). At the onset of stationary phase, increasing levels of phosphorylated Spo0A, the master regulator of sporulation, inhibit *abrB* expression (Strauch *et al.*, 1990), thereby releasing P_{liaI} from its repression. At about the same time, an unidentified stimulus leads to the activation of the histidine kinase LiaS and/or its release from LiaF repression. This, in turn, leads to the activation of the cognate response regulator LiaR, which interacts with its binding site (an imperfect inverted repeat of seven nucleotides with four nucleotides spacing) (Jordan *et al.*, 2006), ultimately resulting in induction of *liaIH* expression (Fig. 3.6, left-hand side).

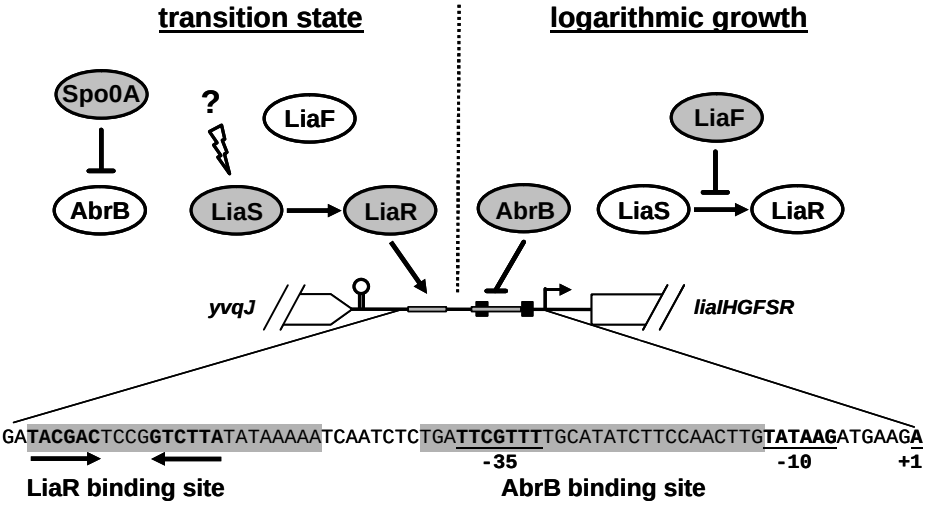

Figure 3.6. Model for the transition state regulation of P_{liaI} activity. The situation during logarithmic growth and transition state are shown on the right and left, respectively, separated by the dotted line. Regulatory proteins involved are named and circled. Grey backgrounds indicate activity, white inactivity. Arrows indicate activation, T-shaped lines repression. The genomic context of the *liaI* promoter region and its important features are schematically shown in the middle, the relevant sequence is detailed out below. The identified regulator binding sites are highlighted in grey. The inverted repeat in the LiaR binding site is indicated by the two arrows below. The promoter sequence and transcriptional start is highlighted in bold and underlined. See text for details.

Previous studies identified numerous agents that are able to induce LiaRS-dependent gene expression. While some of these compounds, especially cell wall antibiotics that interfere with the lipid II cycle (i.e. bacitracin, nisin, ramoplanin or vancomycin) elicit a strong response (Mascher *et al.*, 2004), the biological relevance of these observations remains obscure, since the LiaRS system is not involved in mediating resistance against any of these inducers (unpublished results). The induction of P_{liaI} at the onset of stationary phase – while being significantly weaker (about 10-15 fold, Fig. 3.1A, compared to 50-200 fold in case of

strong inducers (Mascher *et al.*, 2004), as also shown in Fig. 3.4) – is therefore an important observation. While a very high dynamic potential and strength of LiaRS-dependent gene expression could be demonstrated by the exogenous addition of cell wall antibiotics (Mascher *et al.*, 2004), this situation does not necessarily reflect the "natural" condition for the activation of LiaRS-dependent signal transduction. The different sites and modes of action of these known inducers of the LiaRS system, together with the unique domain architecture of the sensor kinase LiaS, argue against a direct binding of these drugs to the input domain of LiaS (Mascher, 2006b). Identification of the true sensory input of the LiaRS system, while being a big challenge, is therefore a prerequisite to understand the difference in LiaR-dependent gene expression observed in this study.

Acknowledgements

We thank Eugenio Ferrari (Genencor) for sharing unpublished results from their chronotranscriptome study, Zoltan Pragai for the gift of strain BFS2470, and Jörg Stülke, in whose laboratory this research was conducted. This work was financially supported by grants GM46700 (to M.A.S.) and GM47446 (to J.D.H.) from the National Institutes of Health, and grants from the Deutsche Forschungsgemeinschaft (MA3269) and the Fonds der Chemischen Industrie (to T.M.).

Chapter 4: Discussion

The LiaRS TCS is a part of the regulatory network that orchestrates CESR in *B. subtilis* (chapter 1). It responds to the external presence of cell wall antibiotics that interfere with the lipid II cycle, such as bacitracin, ramoplanin, vancomycin, or cationic antimicrobial peptides (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005), without being involved in AB resistance. It is also induced by alkaline shock, detergents, ethanol, phenol, organic solvents, and secretion stress, albeit to a lesser extent (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Pietiäinen *et al.*, 2005; Tam le *et al.*, 2006; Wiegert *et al.*, 2001). While its physiological role is still unclear, the data presented in this thesis lead to a detailed understanding of LiaRS-mediated signal transduction.

LiaF could be identified as an essential protein in LiaRS-mediated signal transduction (chapter 2). Without cell encelope stress, LiaF acts as a repressor of LiaRS-dependent gene expression by preventing LiaS-mediated phosphorylation of LiaR. Deletion of *liaF* leads to constitutive induction of the LiaR target promoters. For the repressory function of LiaF, the cytoplasmic located C-terminal part of the protein is essential. Mutants with in-frame deletions in this region behave like a *liaF* deletion mutant (Fig. 2.2 and 2.3). Therefore, LiaF together with LiaRS constitute a CES-sensing three-component system.

The LiaR-binding site was determined as a 7-4-7 inverted repeat in the promoter region of *liaI* homologs in the *Firmicutes* bacteria (Fig. 2.5). In *B. subtilis*, mutants that harbour exchanges of highly conserved bases within this motiv no longer respond to the presence of bacitracin (Tab. 2.4). So far, two target promoters could be verified: P_{liaI} and P_{yhcY} (Fig. 2.1 and 2.7). A third LiaR-binding site was also identified by comparative genomics upstream of *yozJ* (chapter 2) and transcriptome studies identified another LiaR-dependent gene, *ydhE* (see below).

In addition to the response to cell wall antibiotics that interfere with the lipid II cycle and several other stresses, the *liaI* promoter is induced without external stimuli during transition state (chapter 3). This activity is embedded in the complex regulatory cascade governing adaptation at the onset of stationary phase. During logarithmic growth the *liaI* promoter is directly repressed by the transition state regulator AbrB, which binds the DNA region of P_{liaI} between -40 and -14 (Fig. 3.3). It could be demonstrated that Spo0A, the master regulator of sporulation, activates P_{liaI} indirectly by repressing *abrB* expression (Fig. 3.2). Additional links between LiaRS and Spo0A are suggested by data from the literature, as will be discussed below.

Here, we will put the findings derived from this thesis in the context of recent findings and the data from the available literature. The first paragraph will address the LiaR regulon and the physiological role of LiaFSR-dependent gene expression. In the second paragraph, the role of accessory proteins like LiaF for two-component signal transduction will be described. The mechanism of stimulus perception by the LiaS-LiaF sensory unit will be discussed in the third paragraph. In the fourth paragraph, the importance of the stoichiometry between LiaF, LiaS and LiaR will be addressed. The last paragraph will engage in the discovery of bistable behaviour of P_{liaI} induction in transition state.

The LiaR regulon

In addition to the published results (Jordan *et al.*, 2006) – i.e. LiaR-dependent expression of *liaIH* and *yhcYZyhdA* – we could identify one additional target locus of LiaR, the *ydhE* gene, in a DNA microarray analysis in collaboration with Georg Homuth and Ulrike Mäder (Greifswald), by comparing the global transcription pattern of the wild type and isogenic *liaF* and *liaR* mutants, representing the "ON" and "OFF" state of the LiaRS-system, respectively. The expression of *ydhE* is upregulated about 15-fold (Fig. 4.1A) in the *liaF* mutant, an induction comparable to the *yhcYZ-yhdA* operon. This induction is much weaker than that observed for *liaIH*, and both loci are not inducible by bacitracin in the wild type strain (Fig. 4.1B). In *B. subtilis*, LiaR-dependent induction of the *yhcYZ-yhdA* operon by bacitracin was only observed in a *liaH* mutant (Mascher *et al.*, 2003), while it was already strongly upregulated in a *B. licheniformis* wild type strain (Wecke *et al.*, 2006). We identified a putative LiaR-binding site upstream of the *yhcY* promoter in both *B. subtilis* and *B. licheniformis* (Jordan *et al.*, 2006). A LiaR-dependence of the *ydhE* promoter was already indicated by a previous transcriptome study (Kobayashi *et al.*, 2001), and we identified a weakly conserved potential LiaR binding site in the promoter region of the *ydhE* gene (Fig 4.2), which still needs to be verified experimentally. The domain architecture of the corresponding LiaR-target proteins is illustrated in Fig. 4.1C.

To understand the physiological role of LiaFSR-dependent signal transduction, it is important to get deeper insights into the function of its target proteins. While this is subject to ongoing studies, some speculations can be drawn from preliminary data and will be presented below.

The *liaIH* operon is the primary target of LiaRS-dependent signal transduction, as mentioned above. In collaboration with Michael Hecker and Birgit Voigt (Greifswald), we were able to verify this on the protein level by two-dimensional gel electrophoresis of the cytoplasmic

proteome, using cell extracts prepared from the wild type W168 – with and without addition of bacitracin – and isogenic *liaR* ("OFF") and *liaF* ("ON") mutants. LiaH was the most abundant cytosolic protein in the *liaF* mutant, and identified in five independent spots, indicative for posttranslational modifications, while it was hardly detectable in the wild type (without bacitracin induction) and absent in the *liaR* mutant (Falk Kalamorz, diploma thesis). The appearance of multiple LiaH-spots is presumably due to different phosphorylation states of the protein, since it was recently demonstrated that PrkC phosphorylates LiaH in vitro (Pietack *et al*., unpublished).

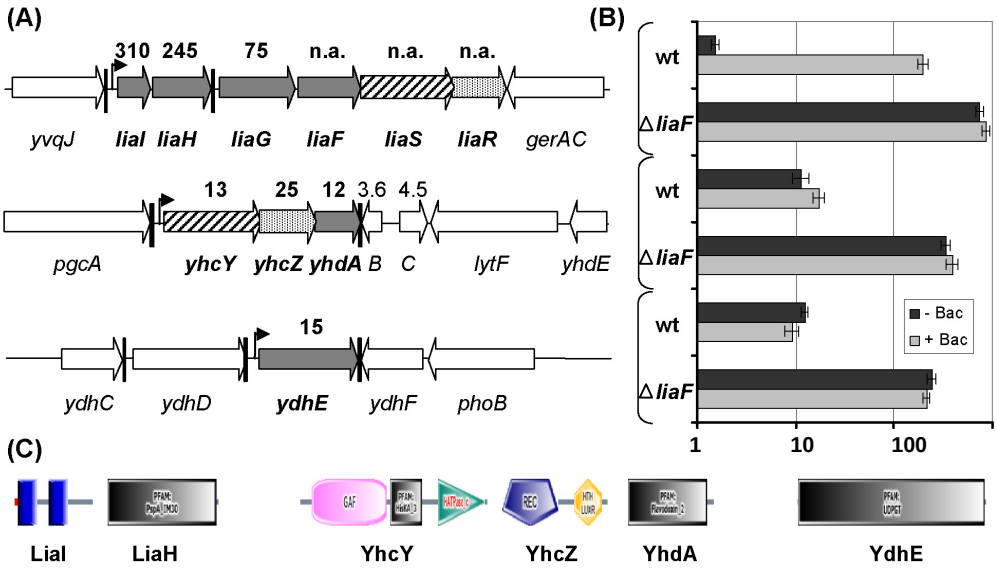

Figure 4.1. The LiaRS regulon. (A) Genomic context of LiaR-target genes. The regions are drawn to scale, the line represents 7 kb. Target genes are indicated by bold gene names and filled arrows; hatched arrows = histidine kinases, dotted arrows = response regulators, grey arrows = other target genes of unknown function. White arrows indicate flanking genes. The numbers above are fold-induction of a *liaF* mutant relative to its isogenic wild type, derived from DNA microarray studies; n.a. = not available due to the *liaF*::kan insertion. **(B)** Verification of LiaR-dependent promoter activity by β-galactosidase assays. The LiaR-dependent promoter regions used are highlighted in Fig. 2A. The three brackets represent (from top to bottom) P_{liaI}, P_{yhcY}, and P_{ydhE}-*lacZ*-fusions, measured with (grey bars) and without (black bars) addition of bacitracin in the wild type (W168) and an isogenic *liaF* mutant. Scale on the *x*-axis is in Miller units. **(C)** Domain architecture of LiaR-target proteins, derived from the SMART database at http://smart.embl-heidelberg.de/. Blue vertical bars represent putative transmembrane regions, labelling of all other domains according to SMART or Pfam nomenclature. See text for details.

LiaH belongs to the PspA-IM30 protein family of phage-shock proteins, the name derived from PspA of *E. coli*. The latter is induced by various stress conditions such as filamentous phage infection (hence the name), heat shock, osmotic shock, exposure to organic solvents and proton ionophores as well as long incubation under alkaline conditions (Brissette *et al.*, 1990; Kobayashi *et al.*, 1998; Weiner and Model, 1994). This inducer spectrum shows some overlap with the known inducers of *liaIH* expression, which include organic solvents and alkaline shock (Mascher *et al.*, 2004; Wiegert *et al.*, 2001). PspA exhibits a dual function that

is linked to two different cellular locations (Brissette *et al.*, 1990; Kleerebezem and Tommassen, 1993): Peripherally bound to the inner surface of the cytoplasmic membrane, PspA is involved in the maintenance of cell membrane integrity (Darwin, 2005; Kleerebezem *et al.*, 1996). As a free cytosolic protein, it inhibits the AAA+ enhancer protein PspF through protein-protein interactions (Adams *et al.*, 2003; Bordes *et al.*, 2003; Dworkin *et al.*, 2000). Members of the PspA/IM30 protein family are therefore referred to as AAA+ adaptor proteins (Hankamer *et al.*, 2004). Preliminary data indicate that LiaH is bound to the inner surface of the cytoplasmic membrane via LiaI (data not shown). LiaI is a small hydrophobic protein with two deduced transmembrane helices, encoded directly upstream of *liaH*. The finding that LiaH can be bound to the cytoplasmic membrane like PspA leads to the hypothesis that LiaH like PspA might be involved in the maintenance of cell membrane integrity (Kobayashi, 2007).

Ongoing phenotypical analyses revealed that LiaH plays some role in cellular survival under conditions of severe envelope stress, exhibited by the presence of some cell wall antibiotics such as fosfomycin or cephalexin, without conferring antibiotic resistance against them: while the minimal inhibitory concentration only marginally increased in the *liaF* mutant (if at all), the survival rate when grown in the presence of inhibitory antibiotic concentrations (determined as colony forming units in serial dilution spot tests) differed by some orders of magnitude, with either an increase in survival for the *liaF* mutant, or decreased survival rates for the *liaH* mutant (Diana Hoyer, diploma thesis).

For both, *E. coli* PspA and cyanobacterial Vipp1, the formation of large oligomeric ring-like structures was demonstrated (Aseeva *et al.*, 2004; Hankamer *et al.*, 2004). Transmission electron microscopic studies revealed that purified LiaH also forms rings with a nine-fold rotational symmetry *in vitro* (Falk Kalamorz, diploma thesis). Gel filtration experiments verified the formation of one homogenous LiaH oligomeric complex with total molecular weight of more than 1,000 kDa (Kirstein, personal communication). These observations indicate that LiaH might also function as an AAA+ adaptor protein.

There are strong indications that there is a link between LiaH and protein secretion. In *E. coli* CESR is defined as counteracting misfolded proteins in the periplasm (Ruiz and Silhavy, 2005). *E. coli* PspA is involved in maintenance of pmf (Darwin, 2005), which solely (Tat) or partially (Sec) drives protein secretion in this organism (DeLisa *et al.*, 2004; Economou, 1999). Moreover, it was demonstrated that PspA overexpression in *E. coli* increases Tat-dependent protein secretion (DeLisa *et al.*, 2004). Recently, it was shown that PspA overexpression in *Streptomyces lividans* improves both Sec- and Tat-dependent protein

secretion (Vrancken *et al.*, 2007). Along those lines, the secreted proteins from *B. subtilis* wild type cultures, and *liaH* and *liaF* mutants – lacking or overexpressing LiaH, respectively – were prepared. SDS-PAGE and 2D-gels revealed numerous significant differences in the protein patterns, clearly indicating a role of LiaH in protein secretion (Diana Hoyer, diploma thesis).

The *yhcYZ-yhdA* operon encodes a TCS (YhcYZ) and a glycosyltransferase (YhdA). In addition to our findings, the LiaRS-dependent expression of *yhcYZ* was also indicated by a comprehensive microarray analysis of TCS (Kobayashi *et al.*, 2001), based on the overexpression of the response regulator in the absence of its cognate histidine kinase. YhcY is a soluble histidine kinase that harbors a cytoplasmic GAF input domain. These highly conserved and widely (from bacteria to mammals) distributed domains bind cyclic nucleotides, primarily cGMP, but also cAMP, indicative for monitoring the cellular energy state (Anantharaman *et al.*, 2001; Hurley, 2003; Martinez *et al.*, 2002). So far, most bacterial GAF domains investigated are part of (cyano-)bacteriophytochromes, where they are involved in heme binding (Gao *et al.*, 2007; Ivleva *et al.*, 2006; Mutsuda *et al.*, 2003; Zhang *et al.*, 2006). The only histidine kinase exclusively containing GAF input domains that has been analyzed so far is DosS from *Mycobacterium tuberculosis*. It is inducible by hypoxia, H_2O_2, NO and ethanol (Kendall *et al.*, 2004; Sherman *et al.*, 2001; Voskuil *et al.*, 2003). Again, biochemical studies demonstrated that at least one of its two GAF domains binds heme (Sardiwal *et al.*, 2005). So far, the biological function of the YhcYZ TCS is unknown. A close homolog, YhcSR, has recently been described as an essential TCS in *Staphylococcus aureus*, potentially involved in envelope stress response (Sun *et al.*, 2005). In contrast, deletion mutants of *yhcYZ* are perfectly viable in *B. subtilis.*

YhdA is a putative NADPH-dependent FMN reductase, with strong homology to an azoreductase of *Bacillus sp.* OY1-2 (Suzuki *et al.*, 2001). A structural homolog, YLR011wp from *Saccharomyces cerevisiae*, was shown to exhibit a NAD(P)H-dependent FMN reductase and a strong ferricyanide reductase activity (Liger *et al.*, 2004).

YdhE is a putative UDP-glucoronosyl or UDP-glucosyl transferase, with homology to macrolide glycosyltransferases (Hernandez *et al.*, 1993) and zeaxanthin glucosyltransferases (enzymes involved in carotenoid biosynthesis). UDP-glucosyl transferases have been found in plants, animals, fungi and bacteria. They use UDP-activated sugar moieties as the sugar donor

and small molecules such as flavonoids, alkaloids, antibiotics and plant hormones as the sugar acceptors.

In summary, we were able to identify four (potential) LiaR-binding sites in the genome of *B. subtilis* (Fig. 4.2). The expression of *liaI*, *yhcY* and *ydhE* increases in a *liaF* mutant, the "ON" state of the LiaRS TCS. The LiaR-binding sites upstream of these three genes are in about the same distance (between 75 and 85 bp) from the ATG start codon. The fourth putative LiaR-binding site is located 208 bp upstream of *yozJ* (Fig. 4.2). This is an unusual long distance, twice as much as for any of the other known LiaR-binding sites (Jordan *et al.*, 2006). Furthermore, we failed to detect an induction of *yozJ* expression after treatment with bacitracin and in a *liaF* mutant using a P_{yozJ}-β-galactosidase fusion. Two possibilities can be considered for this LiaR-binding: Either LiaR is not able to bind this DNA region or LiaR can bind but the expression of *yozJ* is not induced. Since the intergenic region upstream of *yozJ* is relatively large, a noncoding small RNA could be located within. This hypothesis is supported by the presence of a strong stem loop structure (Fig 4.2B). But this is highly speculative and needs further investigation.

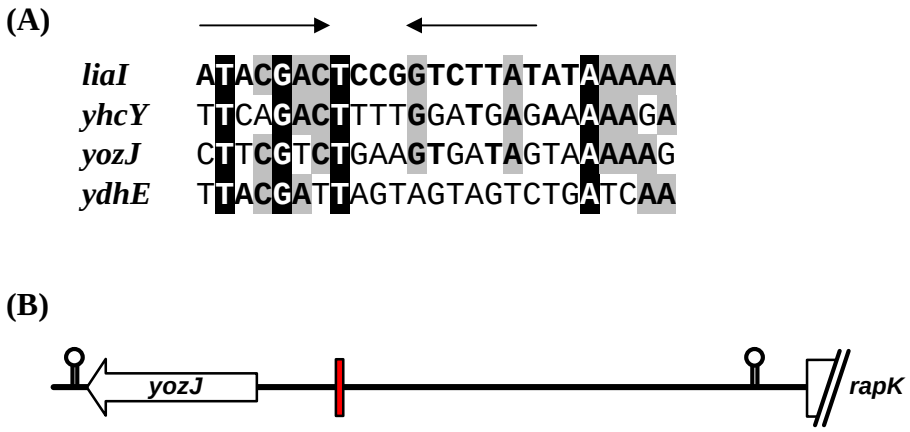

Figure 4.2. Putative LiaR-binding sites in *B. subtilis*. (A) Alignment of the four LiaR-binding sites. Nucleotides conserved in all four sequences are highlighted as white letters on black ground. Nucleotides conserved in three of four sequences are highlighted in grey. Nucleotides that correspond to the LiaR-binding site upstream of *liaI* are given in bold letters. The inverted repeat is marked with arrows. **(B)** Genomic context of the LiaR-binding site upstream of *yozJ*. The binding site is shown in red and orientated towards *rapK*.

A LiaR-dependent induction of P_{yhcY} and P_{ydhE} could only be observed in a *liaF* mutant, an artificially situation where the LiaRS TCS is constitutively active. So far, the *lia* operon is the only LiaR-dependent locus that is induced under conditions of cell envelope stress in the wildtype (Fig. 4.1). Therefore, we postulate that *liaIH* is the only relevant target of the LiaRS TCS.

Induction of P_{yhcY} in a *liaF* mutant might be due to the significant similarity of the RR LiaR and YhcZ. Both belong to the NarL/FixJ class of response regulators (Galperin, 2006), and they are highly homologous to each other, even within the DNA binding domain. Maybe the binding site upstream of *yhcY* is primarily recognized by YhcZ, but because of the similar DNA binding domains of the regulators, activated LiaR is able to bind when it is extremly overexpressed from a plasmid (Kobayashi *et al.*, 2001) or in a *liaF* mutant (Jordan *et al.*, 2006). Again, these possibilities are currently under investigation.

Accessory proteins for stimulus perception and control of TCS function: Bacterial three-component systems

A major finding of this thesis was the identification of LiaF as a strong inhibitor of the LiaRS TCS. LiaF is an accessory protein of the LiaRS TCS essential for its function and probably for stimulus perception. While the presence of additional proteins is the exception rather than the rule for two-component signal transduction, a number of TCS exist where stimulus perception is not mediated by the histidine kinase alone, but through or together with an additional protein ("accessory sensory protein"). Such accessory sensor proteins can be periplasmic, membrane-bound or cytoplasmic proteins (see Tab. 4.1). Examples for periplasmic solute-binding proteins are ChvE from *Agrobacterium tumefaciens* (Shimoda *et al.*, 1993), LuxQ from *Vibrio harveyi* (Bassler *et al.*, 1994) and BctC from *Bordetella pertussis* (Antoine *et al.*, 2005). Soluble accessory proteins only occur in conjunction with cytoplasmic-sensing HK. Examples are the [Ni-Fe]-Hydrogenase HoxBC from *Ralstonia eutropha*, which functions as the H_2-sensor (Kleihues *et al.*, 2000; Lenz and Friedrich, 1998; Schwartz *et al.*, 1998), and FixT from *Sinorhizobium meliloti* that functions as an anti-kinase by interacting with the C-terminal transmitter domain of FixL, thereby interfering with autophosphorylation (Garnerone *et al.*, 1999). Other inhibitory proteins are KipI and Sda from *B. subtilis*. Sda prevents sporulation by inhibiting the primary HK proteins of the sporulation phosphorelay, KinA and KinB (Burkholder *et al.*, 2001). Like Sda, KipI is an inhibitor of the autophosphorylation of KinA, targeting the C-terminal transmitter domain of the protein. It is thought that KipI-KipA interaction, in response to an additional so far unknown stimulus, regulates the anti-kinase activity of KipI (Wang *et al.*, 1997).

In the following paragraph, some membrane anchored accessory proteins reminescent of LiaF will be presented with focus on those accessory proteins associated with intramembrane-sensing HK (UhpB, KinB) or those TCS involved in responding to CES (Cpx, Cse). Their role for signal perception is divers. Generally, deletion of their genes results in the loss of

functional TCS systems. The proteins can be either specific components of the TCS, or required for stimulus perception and kinase activity, potentially by serving as ligand-binding proteins, such as UhpC and KbaA/KapB.

Table 4.1. Accessory proteins for TCS-mediated stimulus perception

Sensor type/ name (org.[1])	accessory protein name	accessory protein localization	stimulus[2]	references
periplasmic-sensing				
VirA (Atu)	ChvE	periplasmic	monosacch.	(Shimoda *et al.*, 1993)
BctE (Bpe)	BctC	periplasmic	citrate	(Antoine *et al.*, 2005)
LuxQ (Vha)	LuxP	periplasmic	AHL	(Bassler *et al.*, 1994)
TorS (Eco)	TorC	membrane	TMAO	(Gon *et al.*, 2001)
YycG (Bsu)	YycH	membrane	?	(Szurmant *et al.*, 2005)
intramembrane-sensing				
LiaS (Bsu)	LiaF	membrane	env. stress	(Jordan *et al.*, 2006; Mascher, 2006a)
UhpB (Eco)	UhpC	membrane	Glu6P	(Kadner, 1995)
KinB (Bsu)	KbaA/KapB	membrane	C1 pool?	(Dartois *et al.*, 1996; Dartois *et al.*, 1997a; Dartois *et al.*, 1997b)
RegB, PrrB	SenC, PrrC[3]	membrane	redox status	(Eraso and Kaplan, 2000; Swem *et al.*, 2005)
cytoplasmic-sensing				
[CheA (var.)	MCP's]	membrane	var. solutes	(Bass and Falke, 1999; Bilwes *et al.*, 2003; Grebe and Stock, 1998)
NreB (Sca)	NreA?	membrane	nitrate?	(Fedtke *et al.*, 2002)
HoxJ (Reu)	HoxBC	soluble	H_2	(Buhrke *et al.*, 2004; Friedrich *et al.*, 2005)
FixL (Sme)	FixT	soluble	low O_2	(Garnerone *et al.*, 1999)
KinA (Bsu)	KipI/Sda	soluble	energy status	(Wang *et al.*, 1997) / (Burkholder *et al.*, 2001)

[1]Organisms: var. (various), Atu (*Agrobacterium tumefaciens*), Bpe (*Bordetella pertussis*), Vha (*Vibrio harveyi*), Bsu (*Bacillus subtilis*), Eco (*Escherichia coli*), Reu (*Ralstonia eutropha*), Sme (*Sinorhizobium meliloti*), Sca (*Staphylococcus carnosus*). [2]Stimuli: monosacch. (monosaccharides), AHL (acyl homoserine lactone), TMAO (trimethyl amine-N-oxide), env. stress (cell envelope stress), C1 pool (cellular pool of one-carbon units), Glu6P (glucose-6-phosphate), var. solutes (amino acids, monosaccharides, peptides, metal ions). [3]RegB-SenC/PrrB-PrrC homologs are found in purple nonsulfur photosynthetic bacteria.

UhpC acts as a positive accessory signal perception protein of the UhpAB TCS (Kadner, 1995). This system is induced by the presence of extracellular glucose-6-phosphate and regulates the expression of the sugar-phosphate transport protein UhpT (Weston and Kadner, 1988). UhpC is a transmembrane protein with 12TMRs and shares a high sequence similarity with the transport protein UhpT. Deletion of *uhpC* results in a complete loss of Glu6P-dependent induction of *uhpT* expression. Therefore, both UhpC and the intramembrane-sensing HK UhpB (8 TMRs) are required for the Glu6P-inducible auto-phosphorylation activity. Mutational analyses indicated that the interaction between UhpC and UhpB occurs within the membrane interface between the hydrophobic membrane-spanning C-termini of both proteins (Island and Kadner, 1993).

Kba/KapB: One of the most complex known phosphorelay systems regulates endospore formation in the Gram-positive soil bacterium *B. subtilis* (Errington, 2003; Msadek, 1999; Piggot and Hilbert, 2004). Sensory information from five different kinases are integrated through the phosphorylation of the key sporulation transcription factor Spo0A. Its activation by phosphotransfer from the sensor kinases via the primary RR Spo0F and a phosphotransferase, Spo0B, triggers the commited step of sporulation (Errington, 2003). The two major suppliers of phosphate into the phosphorelay are the soluble, cytoplasmic-sensing HK KinA (which was shown to be influenced by soluble accessory proteins, Table 4.1) and the membrane-bound KinB. KinB contains six potential TM regions without significant periplasmic linkers (Trach and Hoch, 1993), suggesting that it does not directly sense extracellular effector molecules. KinB also contains no cytoplasmic linker or sensory domains and no conserved sequence features can be detected in the input domain. Rather, the mechanism of signal perception seems to be mediated by two membrane-anchored accessory stimulus perception proteins that are involved in activating the intramembrane-sensing HK KinB of *B. subtilis*: KbaA, a TM protein with six putative TM helices, and KapB. The product of *kapB*, a gene co-transcribed in an operon with and located directly downstream of *kinB*, is a lipoprotein that is tethered to the outer face of the cytoplasmic membrane after lipid-modification of cysteine residues (Dartois *et al.*, 1996; Dartois *et al.*, 1997a). Inactivation of *kapB* leads to a loss of KinB activity and confers the same phenotype as does a null mutation in *kinB* (Trach and Hoch, 1993). While a direct interaction of KapB and KinB has not been demonstrated, it was shown that the effect of a KapB inactivation is mediated through the N-terminal, membrane-spanning input-domain of KinB. KapB was discussed as a potential ligand-binding protein that interacts with the 6TMR-input domain of KinB, a situation somehow reminiscent of the UhpBC system (Dartois *et al.*, 1997a). The nature of these protein-protein interactions and the signals sensed through KbaA and KapB are still unknown. But it was demonstrated that depletion of intracellular one-carbon units inactivates the KinB-dependent signal transduction pathway (Dartois *et al.*, 1997b).

CpxP: The CpxAR TCS is activated by elevated external pH, misfolded periplasmic proteins or changes in the lipid composition of the inner membrane (Ruiz and Silhavy, 2005). It is subject to a negative feedback regulation exerted by the periplasmic protein CpxP. The N-terminal α-helix of CpxP plays an important role for both, inhibition of CpxA via its sensing domain, as well as stabilization of CpxP (Buelow and Raivio, 2005). Direct protein-protein interaction between the sensor kinase CpxA and the periplasmic protein CpxP results in a down-regulation of the autokinase activity of CpxA (Fleischer *et al.*, 2007), while DegP-

mediated proteolysis of CpxP relieves inhibition of the Cpx response in the presence of inducing cues (Buelow and Raivio, 2005).

YycI/YycH: YycFG of *B. subtilis* was the first Gram-positive TCS to be described as essential for survival under normal laboratory conditions (Fabret and Hoch, 1998). It is restricted to, and conserved in the *Firmicutes* bacteria. Its essentiality has been verified in number of these bacteria (Clausen *et al.*, 2003; Hancock and Perego, 2004; Lange *et al.*, 1999; Williams *et al.*, 2005), with the noteworthy exception of *Lactococcus lactis* (O'Connell-Motherway *et al.*, 2000). Two major groups of YycFG-like TCS can be distinguished. Group I, which is found in most *Firmicutes* bacteria, is characterized by an extended genomic context conservation, with three to four genes, including *yycH* and *yycI* and its homologs, located directly downstream of the *yycFG* operon. The N-terminus of the YycG HK consists of two deduced transmembrane helices that flank a large periplasmic domain. In this group, both HK and RR are essential. The YycFG system of *B. subtilis* seems to be (at least partially) active during normal growth, and no inducing conditions have been identified so far. Its activity is regulated by YycI and YycH through direct protein-protein interactions with the sensor kinase YycG, (negatively) affecting its autophosphorylation activity (Szurmant *et al.*, 2005; Szurmant *et al.*, 2007a). Both proteins are peripherally bound to the cytoplasmic membrane and harbor large periplasmic domains that are similar in fold, but not in primary amino acid sequence (Santelli *et al.*, 2007; Szurmant *et al.*, 2006). Surprisingly, the transmembrane helix of each protein is sufficient to perform the regulatory role described for YycH and YycI (Szurmant *et al.*, 2007b).

CseA: The σ^E-CseABC pathway is unique in incorporating an ECF sigma factor, a TCS, and a novel accessory lipoprotein into a single signal transduction pathway (Hutchings *et al.*, 2006a; Paget *et al.*, 1999a; Paget *et al.*, 1999b). It is also unusual amongst envelope stress ECF σ factors in that it is not under the control of an anti-σ factor. This system is conserved in all streptomycetes genomes sequenced to date and appears to be the major pathway for sensing cell envelope stress in the genus *Streptomyces.* Expression of the *sigE-cseABC* operon is regulated by a novel three-component system consisting of a sensor kinase, CseC, a RR, CseB, and an accessory lipoprotein, CseA. CseC is proposed to bind a cell wall precursor or breakdown product since expression of the *sigE* operon can be induced by a wide range of cell envelope-specific compounds, including antibiotics such as bacitracin or vancomycin, and muramidases such as lysozyme (Hong *et al.*, 2002). Disruption of the *cseA* gene led to a five-fold increase in this basal activity, suggesting that CseA negatively regulates the *sigE* promoter. However, since it is an extracytoplasmic lipoprotein, it must do so from the outside

of the cell and it seems likely that CseA negatively modulates the sensor domain of CseC (Hutchings *et al.*, 2006a). CseA has no homologs outside of the streptomycetes and, so far, there are no clues as to how it might modulate signal sensing by CseC. Transcription of *sigE* is still inducible by cell envelope specific antibiotics in a *DcseA* strain and is induced to a higher level than in the wild-type (Hutchings *et al.*, 2006a). This suggests that CseA somehow reduces the activity of CseC, perhaps by reducing signal binding or trapping it in the 'OFF' state. A closely CseC-related HK is MtrB. As well as CseC, MtrB is encoded in an operon together with its RR and a lipoprotein, LpqB. Like CseA, LpqB mediates signal transduction through MtrAB by interacting with the sensor domain of MtrB (Hoskisson and Hutchings, 2006).

As described above, accessory proteins have various functions and can form a sensory unit together with the HK (like ChvE) or repress the activity of HK in different ways (Sda, KipI, TorC). It was shown that LiaF represses the activation of LiaS, but the mechanism is unknown. Maybe LiaF forms a sensory unit together with LiaS, releasing LiaS in its active form after sensing a signal. The second possibility is that LiaF represses the kinase activity of LiaS in a different way. Initial results on the role of the LiaF/LiaS sensory unit will be described in the next paragraph.

Stimulus perception of the LiaFSR three-component system

During this work it could be demonstrated that LiaF and LiaS are regulators of LiaR activity. Under conditions of cell envelope stress LiaS activates the RR by transferring a phosphate to the Asp_{54} of LiaR, while LiaF represses this activation in the absence of cell envelope stress. The mechanism of this repression remains unclear and its identification will be part of further studies. It seems likely that LiaF interacts directly with LiaS, because both proteins are membrane anchored and therefore perhaps co-localized, something that needs to be verified experimentally. But two questions still remain unanswered: What is the signal that is sensed by the *lia* system and how is this sensing accomplished? The first approach to address the second question was to identify amino acids in LiaF and LiaS that are essiential for their function.

Mutagenesis of LiaF and LiaS. Initially, truncated alleles of both *liaS* and *liaF* were constructed. Moreover, conserved residues in both genes were replaced by alanine. The clean deletion mutants were then complemented with the mutated versions of LiaF and LiaS to identify amino acids and regions within the two proteins that are necessary for their function.

Such an experimental set-up was successfully used to identify essential regions in other histidine kinases from *B. subtilis*, such as ResE and PhoR (Baruah *et al.*, 2004; Eldakak and Hulett, 2007).

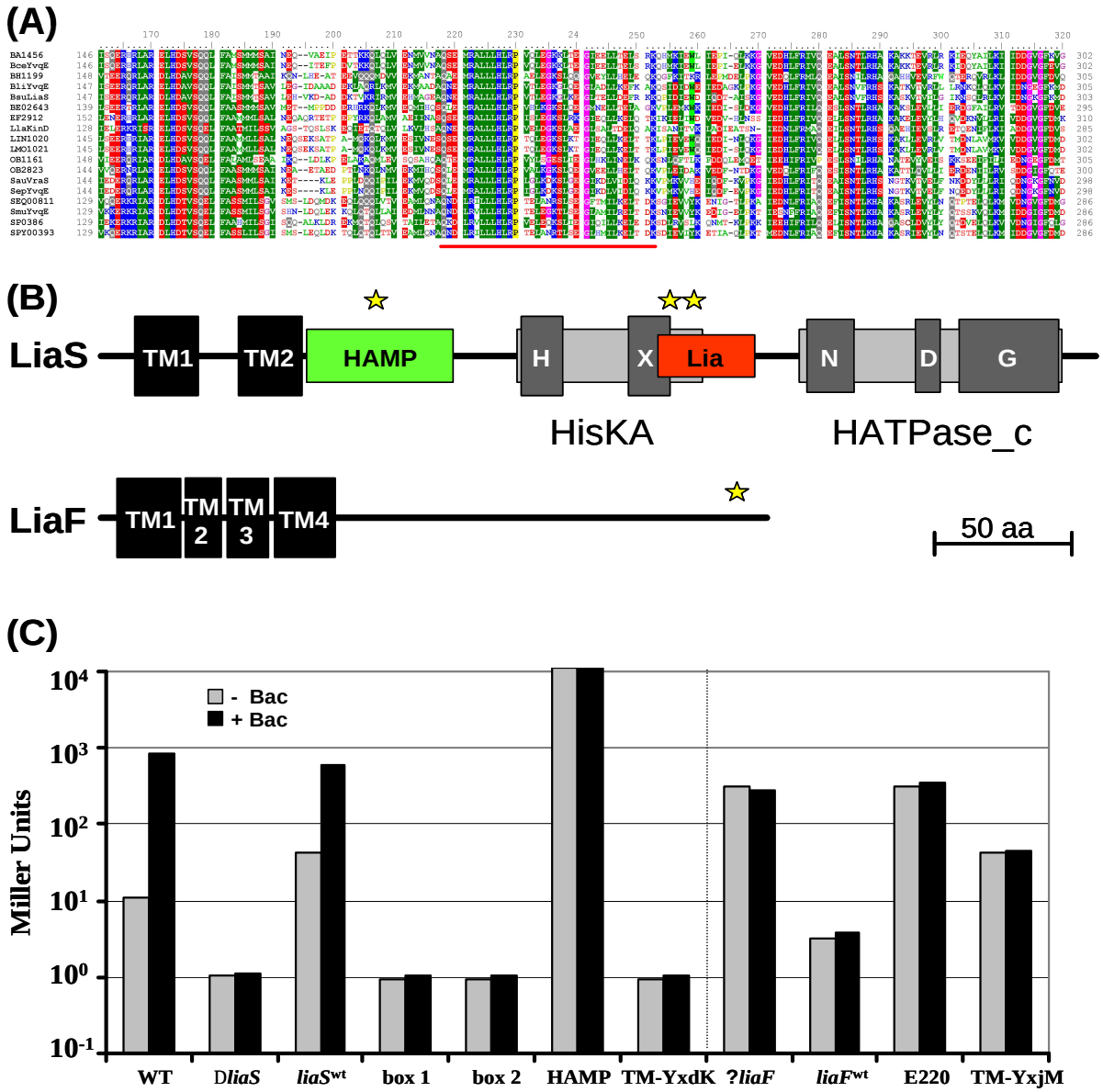

Figure 4.3. Mutagenesis of LiaS and LiaF. (A) Alignment of LiaS homologous kinases. The LiaS-box is underlined red. **(B)** Schematic presentation of LiaS and LiaF. The HAMP domain of LiaS is illustrated in green, the LiaS-box in red. The other boxes in the HisKA and HATPase_c region (light grey) are illustrated in dark grey. Location of mutations are indicated with stars. **(C)** Results of the β-galactosidase assay. WT = TMB370, *liaS*^{wt}/*liaF*^{wt} represent complementation with wildtype copy of the deleted gene. Box 1 is aa Q_{202}-A_{207}, box 2 H_{211}-P_{214}. TM stands for replacement of the transmembrane domains by that of the following protein. E220 = protein ends after amino acid E_{220}. See text for details

All LiaS homologs contain a highly conserved motif, which is not present in any other protein. This motif is located between the X–box and the N-box in the cytoplasmic transmitter domain of LiaS (Fig. 4.3A). Since it serves as a signature sequence for this group of kinases,

we termed it the "LiaS-box". Preliminary results revealed that the LiaS-box is essential for the function of LiaS. Both, deletion and alanine replacement mutagenesis of parts of the Lia-box (motives Q_{202}-A_{207} and H_{211}-P_{214}) lead to a non-inducible kinase (Fig. 4.3B and C). In-depth characterization of the LiaS-box will be subject to future studies.

The HAMP (*h*istidine kinase, *a*denylyl cyclase, *m*ethyl-accepting chemotaxis protein and *p*hosphatase) domain is a region of approximately 50 residues located after the transmembrane domain 2 in LiaS. It consists of two amphipathic helices with coiled-coil properties (Butler and Falke, 1998) and links the input domain to the cytoplasmic transmitter domain. The HAMP domain is thought to transduce the incoming signal by rotation of its helices (Hulko *et al.*, 2006). As shown for the nitrate sensor NarX from *E. coli*, changing of the highly conserved glutamate within the HAMP linker results in a constitutively active kinase, due to structural changes (Appleman and Stewart, 2003). The same behaviour could be observed for LiaS: Deletion or alanine replacement of the amino acids DDE on position 104-106 lead to a constitutively active kinase, resulting in the permanent induction of P_{liaI} (Fig. 4.3C). Interestingly, LiaF is not able to repress LiaS activity in this mutant.

Because of the lack of an extracytoplasmic loop, which is thought to function as the sensory domain in other families of histidine kinases, LiaS belongs to the so-called intramembrane-sensing HK (IMHK) (Mascher, 2006b), which are thought to sense their inducing signal directly within the membrane (Mascher *et al.*, 2006). To confirm this hypothesis, it was important to check whether the transmembrane domains of LiaS are necessary for stimulus perception or if only the location at the membrane (and/or the contact to LiaF) is sufficient for the function of LiaS. Therefore, a hybrid kinase was constructed by replacing the transmembrane domains of LiaS with the 2 transmembrane domains of YxdK. YxdK is also an IMHK, which is not induced by bacitracin. If membrane-anchoring alone would be sufficient for LiaS function, it should be possible to activate this hybrid kinase with bacitracin. Instead, this YxdK-LiaS hybrid is not induceable by bacitracin (Fig. 4.3C). The same behaviour was observed after deletion of the TM (data not shown). Therefore, the specific N-terminal domain of LiaS is important for stimulus perception.

In contrast, a hybrid allele of LiaF with the 4 transmembrane domains of YxjM is able to complement the *liaF* deletion, at least to some extend (~ 10-fold reduction) (Fig. 4.3C). In fact, preliminary data indicate that even a soluble C-terminal fragment of LiaF, lacking all four TMR, is sufficient for partially complementing a *liaF* deletion (data not shown). These findings indicate that the C-terminal part of LiaF interacts with LiaS and inhibits autophosphorylation. This assumption is supported by the finding that even the last 25 amino

acids of LiaF are essential for its function. Their deletion (position 220 to 245) leads to a complete loss of LiaF function. This mutant behaves exactly like the *liaF* deletion mutant (Fig. 4.3C). Future studies will hopefully reveal which of the 25 amino acids in this region are involved in the repressory function of LiaF. Good candidates are two conserved aspartates at positions 235 and 237. So far, alanine scanning mutagenesis of any other conserved amino acid in the C-terminus did not reveal any crucial residue (data not shown).

While it could be demonstrated that the TM of LiaS are essential for stimulus perception, the exact nature of the inducing signal remains unclear. A direct interaction of the known inducers with LiaS seems unlikely because of their diversity. It has been postulated that the stimulus perception via the transmembrane helices occurs directly at the membrane interface (Mascher *et al.*, 2003; Mascher *et al.*, 2006).

Additionally, the mechanism of LiaF function needs to be investigated further. The data so far indicates that LiaF has both sensory and repressory function.

Interaction of the ECF σ factor σ^W with the LiaFSR three-component system

The ECF sigma factor σ^W, which is one of three ECF sigma factors involved in the cell envelope stress response in *B. subtilis* (Mascher *et al.*, 2003), was shown to positively affect the expression of the *lia* system under inducing conditions. Overexpression of σ^W results in a 16-fold increased amount of *liaIH* transcript (Asai *et al.*, 2003). Moreover, there is a significant overlap of the inducer spectrum of both systems. σ^W is strongly induced by alkaline shock (Wiegert *et al.*, 2001), vancomycin, cephalosporine, D-cycloserine, Triton X-100 (Cao *et al.*, 2002a) and CAMPs (Pietiäinen et al., 2005). Weak inducers are bacitracin, fosfomycin and tunicamycin (Cao *et al.*, 2002a). The response of the *lia* system to alkaline shock is very weak and was demonstrated to be σ^W-dependent (Wiegert *et al.*, 2001): in a *sigW* mutant, no alkaline shock induction of *liaIH* was observed any longer. A somewhat similar behaviour was also observed for CAMP induction (Pietiäinen *et al.*, 2005). These results suggest that σ^W itself, or σ^W-upregulated genes induce the LiaFSR three-component system under certain conditions (Fig. 4.4). Since a direct binding of σ^W to the *liaI* promoter can be ruled out, the positive effect of σ^W excerted on P_{liaI}-dependent expression seems to be indirect. One possible explanation could be that the induction of members of the σ^W regulon themself provide the stimulus for LiaRS-dependent gene expression. Given the role of σ^W in counteracting and expressing antimicrobial compounds (Butcher and Helmann, 2006; Helmann, 2002, 2006), this seems to be an alternative hypothesis. A systematic screen of the

inducing potential of the σ^W regulon on *liaIH* expression is currently under way in collaboration with John Helmann.

Interestingly, σ^W expression starts in post-exponential growth phase in a culture of *B. subtilis* cells (Huang *et al.*, 1998), similar to the LiaR-dependent expression of LiaI and LiaH. σ^W expression and the expression of its target genes is regulated by Spo0A and AbrB (Qian *et al.*, 2002), another commonality to the *lia* system. In this case, Spo0A positively affects induction of *sigW* expression by repressing *abrB* expression. The same mechanism could be demonstrated for the regulation of P_{liaI} induction as well (chapter 3).

In addition to the regulatory function of Spo0A on transition state induction, the presence of high amounts of phosphorylated Spo0A was shown to positively affect the expression of *liaIH* (Fujita *et al.*, 2005).

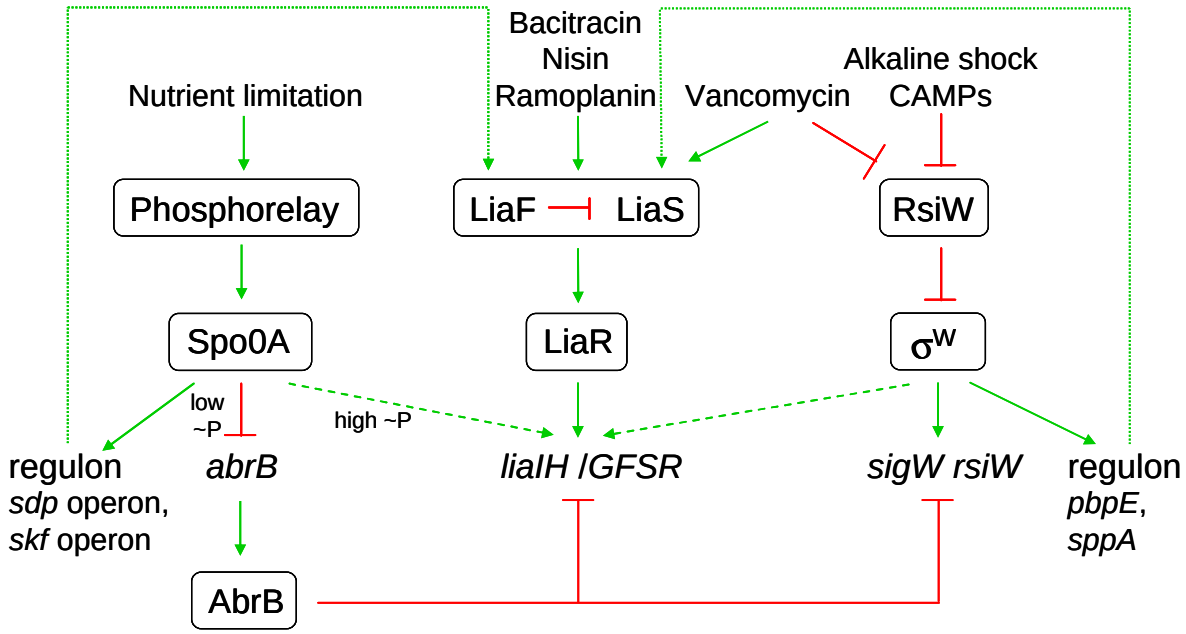

Figure 4.4. Model of LiaFSR regulation. Arrows indicate induction, T-shaped lines repression. Stripe lines indicate indirect regulations, dottet lines postulated stimulatory interactions. See text for details.

It still remains unclear what kind of signal is sensed by LiaFS. Lipid II-interacting antibiotics, secretion stress and entry of stationary phase all induce P_{liaI}. One similarity of all these inducing conditions is that they affect some aspect of the flow of metabolites through the membrane. Lipid II-interacting antibiotics change the amount of lipid II that flips in and out. In stationary phase cell wall synthesis decreases, ultimately affecting the lipid II cycle in a way compareable to the antibiotics. Under conditions of secretion stress, more proteins than normal are transferred through the membrane. Therefore, we speculate that the Lia system senses "membrane trafficing" and not envelope perturbation per se.

Stoichiometry of LiaFSR proteins

The LiaFSR three-component system tightly regulates the expression of the *lia* operon. Deletion and complementation studies revealed that mutations or insertions within the *lia* operon, which lead to different expression levels of the proteins LiaF, LiaS or LiaR, result in significant changes of the behaviour of the LiaFSR three-component system. Overexpression of LiaR results in an induction of P_{liaI} (Kobayashi *et al.*, 2001), even in the absence of LiaS (Jordan *et al.*, 2006). Overexpression of LiaS lead to the same behaviour (see below). P_{liaI} induction is lost in a *liaR* mutant (Jordan *et al.*, 2006) and in a clean *liaS* mutant (see below). Deletion of LiaF results in a constitutive active LiaRS TCS (Jordan *et al.*, 2006).

The genes encoding the LiaFSR three-component system are cotranscribed, but analysis of their Shine-Dalgarno (SD) sequences indicated that they are translated with different efficiency, resulting in different amounts of the three proteins in the cell. Conservation of the SD sequence and its distance to the start codon are the determining factors for the translation efficiency (Vellanoweth and Rabinowitz, 1992). *liaF* has a perfect SD (Fig. 4.5) with almost the optimal spacing (12 bp) upstream of its start codon. This might lead to high amounts of LiaF in the cell. *liaR* has also a strong SD with 8 bp spacing. The SD of *liaS* is poorly conserved, only 4 of 7 nucleotides correspond to the optimal SD. Presumably, this leads to lower amounts of LiaS compared to LiaF and LiaR in the cell, so that the amount of LiaS is the limiting factor in LiaFSR three-component system. This assumption is supported by preliminary data from translational *lacZ* fusions, showing a 6-fold lower activity for liaS compared to liaF (Rietkötter, personal communication).

	sequence	**spacing**
consensus	**AAGGAGG**	**7-9**
liaF	**AAGGAGG**	12
liaR	**AAGGAGA**	**8**
liaS	**GTGGATG**	10
***liaS* SD**	**AAGGAGG**	**7**

Figure 4.5. Comparison of the Shine-Dalgarno sequences. Nucleotides corresponding to the consensus sequence are shaded in grey. Optimal spacing of the SD to the start codon is written in bold. *liaS* SD is the mutated allele with the optimal Shine-Dalgarno sequence and spacing. See text for details.

Changes in the amount of one of the three proteins LiaF, LiaS and LiaR strongly affect the function of the system. The most striking effect was observed during complementation studies of *liaF* mutants (Fig. 4.6). In a *liaF*::kan mutant, P_{liaI} is induced to ~ 3000 Miller Units (Jordan *et al.*, 2006), i.e. a 30-fold increased induction compared to the bacitracin-induced

wildtype. Subsequent analysis indicated that this behaviour was due to the positive polar effect of the kanamycin resistance cassette, which was introduced in this strain upstream of the *liaSR* genes. A non-polar, i. e. clean *liaF* deletion (Δ*liaF*) shows P_{liaI} induction comparable to the level of the induced wildtype (~ 100 Miller Units, see Fig. 4.6). In this strain the TCS is fully active. The *liaF*::kan strain lacks the repressor LiaF and additionally strongly overproduces LiaS and LiaR. Obviously, these two factors together lead to the increased P_{liaI} activity in the *liaF*::kan mutant. When a wildtype copy of *liaF* is introduced into this strain ectopically under control of a xylose-inducible promoter (using pXT), P_{liaI} induction is reduced 10-fold to ~ 100 Miller Units (Fig. 4.5). This complementation is therefore only partial and the system no longer responds to bacitracin, maybe as a result of insufficient amounts of LiaF compared to LiaRS. A different behaviour is detectable in the *liaF* clean deletion mutant. With an ectopical wildtype copy of *liaF*, the P_{liaI} activity is reduced nearly to the level of the uninduced wildtype. But again the system does not respond to the presence of bacitracin. The reason for the lack of induction is unclear at the moment.

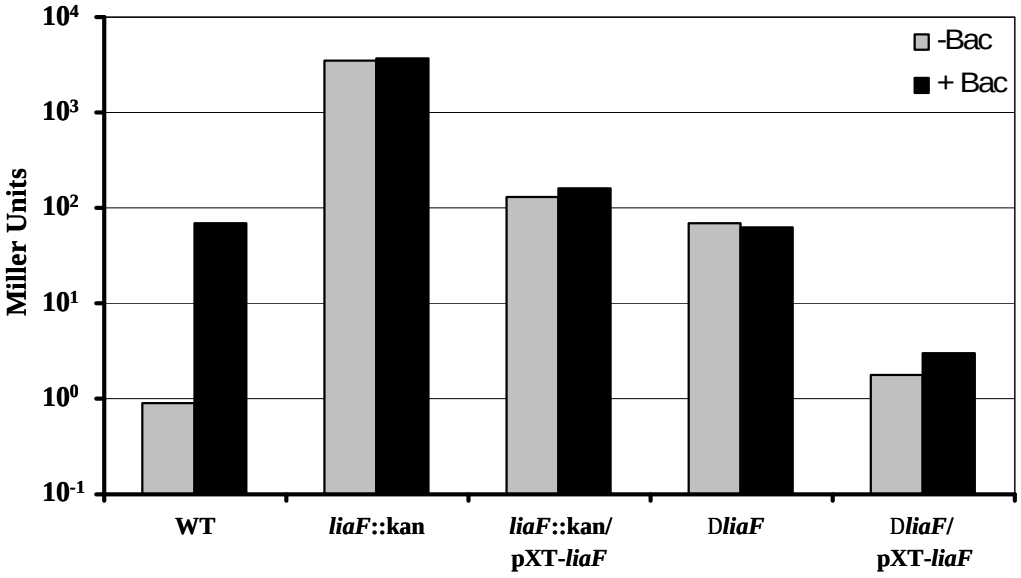

Figure 4.6. Complementation of *liaF* mutants. *liaF*::kan means replacement of *liaF* by a kanamycin resistance cassette. Δ*liaF* is the clean deletion mutant. The reporter strain is based on pAC6 (TMB016). See text for details.

As shown in chapter 2, the replacement of *liaS* by a kanamycin resistance cassette results in a significant background activity of the *liaI* promoter. Again we could demonstrate that this was due to the positive polar effect of the resistance cassette. Quantitative real-time RT-PCR revealed that there is a 34-fold increased level of the *liaR* mRNA in the *liaS*::kan mutant compared to the wildtype. Under these conditions, LiaR becomes unspecifically

phosphorylated and activates P_{liaI}, even in the absence of LiaS. Changing the $Asp_{54} \rightarrow Ala$ – the residue to which the phosphate is transferred from the histidine kinase LiaS – abolishes P_{liaI} activity despite strongly increased LiaR amounts in the cell (Fig. 4.7). This experiment demonstrated that overexpression of LiaR in the cell alone is not sufficient for P_{liaI} activation and that an unspecific phosphate donor for LiaR is required in the absence of LiaS.

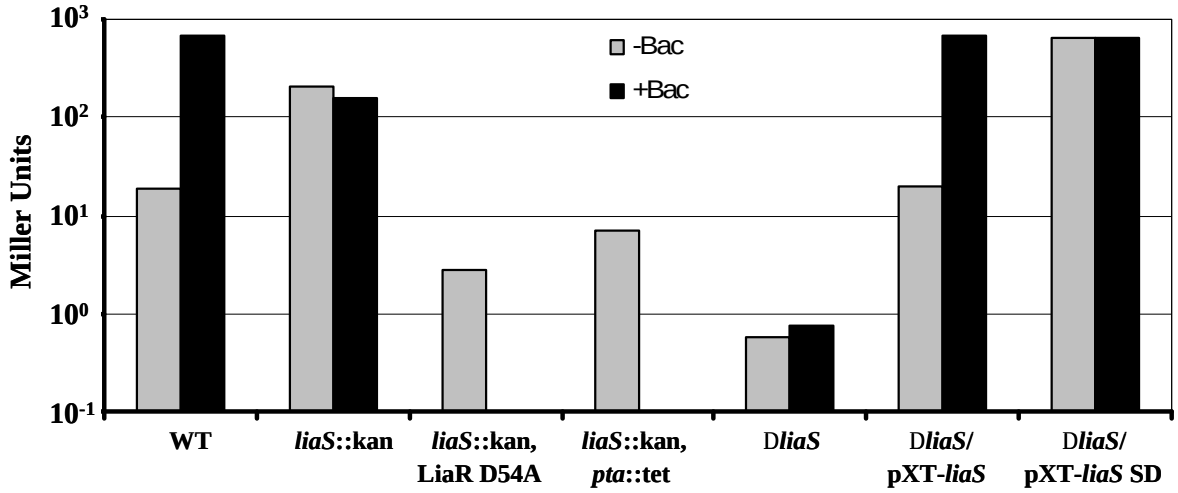

Figure 4.7. Phosphorylation of LiaR in different mutants. *liaS*::kan means replacement of *liaS* by a kanamycin resistance cassette. tet is the tetracyclin resistance cassette. Δ*liaS* is the clean deletion mutant. SD is the optimal Shine-Dalgarno sequence. For the *liaS*::kan, LiaR D54A and the *liaS*::kan, *pta*::tet mutants β-galactosidase activity was only determined without bacitracin induction. The reporter strain is based on pMUTIN (TMB370). For details see text.

Acetyl phosphate is known to be able to function as a phosphodonor for RR. Recently it was demonstrated for *E. coli*, that the intracellular concentration of acetyl phosphate in this bacterium (3 mM) is sufficient to activate RR via direct phosphoryl transfer (Klein *et al.*, 2007). An example illustrating the physiological role of acetyl phosphate in RR phosphorylation is VanR, the RR mediating vancomycin resistance in *Streptomyces coelicolor*. It recently was shown to become fully phosphorylated by acetyl phosphate in the absence of both the cognate HK and the external inducer, vancomycin (Hutchings *et al.*, 2006b). Under non-inducing conditions, the cognate HK VanS acts as a phosphatase to prevent this unspecific phosphorylation.

We could demonstrate that in the *liaS*::kan mutant acetyl phosphate serves as the phospodonor for LiaR *in vivo*. In a *liaS*::kan, *pta*::tet mutant, which should not be able to synthesize acetyl phosphate, the activation of LiaR is gone (Fig. 4.7). But one should bear in mind that the phosphorylation of LiaR by acetyl phosphate is artificial and occurs only in the *liaS*::kan mutant, because of the high amounts of LiaR together with the loss of LiaS and its

proposed phosphatase activity. Both, in the wildtype and a non-polar, i. e. clean *liaS* deletion mutant (TMB215), no increased basal activity of P_{liaI} could be observed.
This clean *liaS* deletion mutant was complemented by introduction of a *liaS* wildtype copy (Fig. 4.7). When overexpressing LiaS with an optimized SD, the proportion of LiaS to the repressor LiaF is increased. Consequently, P_{liaI} is constitutively activated (Fig. 4.7). These findings support the hypothesis that LiaS is present in low amounts and is set to the phosphatase mode due to LiaF function.

Taken together, the findings so far indicate that the proteins LiaF, LiaS and LiaR have to be present in the cell in distinct amounts and that their ratios are crucial for the function of the LiaFSR three-component system. Any conditions affecting this balance result in the system being "blind" to cell envelope stress. Indeed, it is fixed to the "ON"- or "OFF"-state of the system, depending on the regulator reduced or overexpressed.

Bistability of the *liaI* promoter

As shown in chapter 3, the intrinsic transition state induction of P_{liaI} is tightly regulated and embedded in the Spo0A/AbrB-dependent regulatory cascade. Interestingly, the maximum induction under those conditions is about 10-15-fold weaker than the induction with cell wall antibiotics. This observation can either be explained by an overall weaker promoter activity. Alternatively, it could be due to the full induction of P_{liaI} in only a subset of the culture, a phenomenon termed bistability.
To address this question for the transition state induction of P_{liaI}, a single cell approach is necessary to differentiate between induced and uninduced cells in the same culture. For that purpose, a P_{liaI}-*gfp* (green fluorescent protein) fusion was constructed and the resulting strain (TMB408) was monitored by fluorescence microscopy over several hours of growth at 30°C. A strain carrying a P_{liaI}-*lacZ* fusion (TMB370) was grown in parallel and samples were taken for β-galactosidase assay at the same timepoints. The results from these initial experiments are promising. Only 10-15 % of the cells in a culture induce P_{liaI} when entering stationary phase (Fig. 4.8). Monitoring this culture for two more hours in stationary phase (Fig. 4.8, 8 and 9 h) clearly demonstrated that during the whole transition state only a subpopulation of cells induce P_{liaI}. This result explains the low induction level in transition state measured with the β-galactosidase assay and is a clear indication that P_{liaI} is subject to a bistable switch.

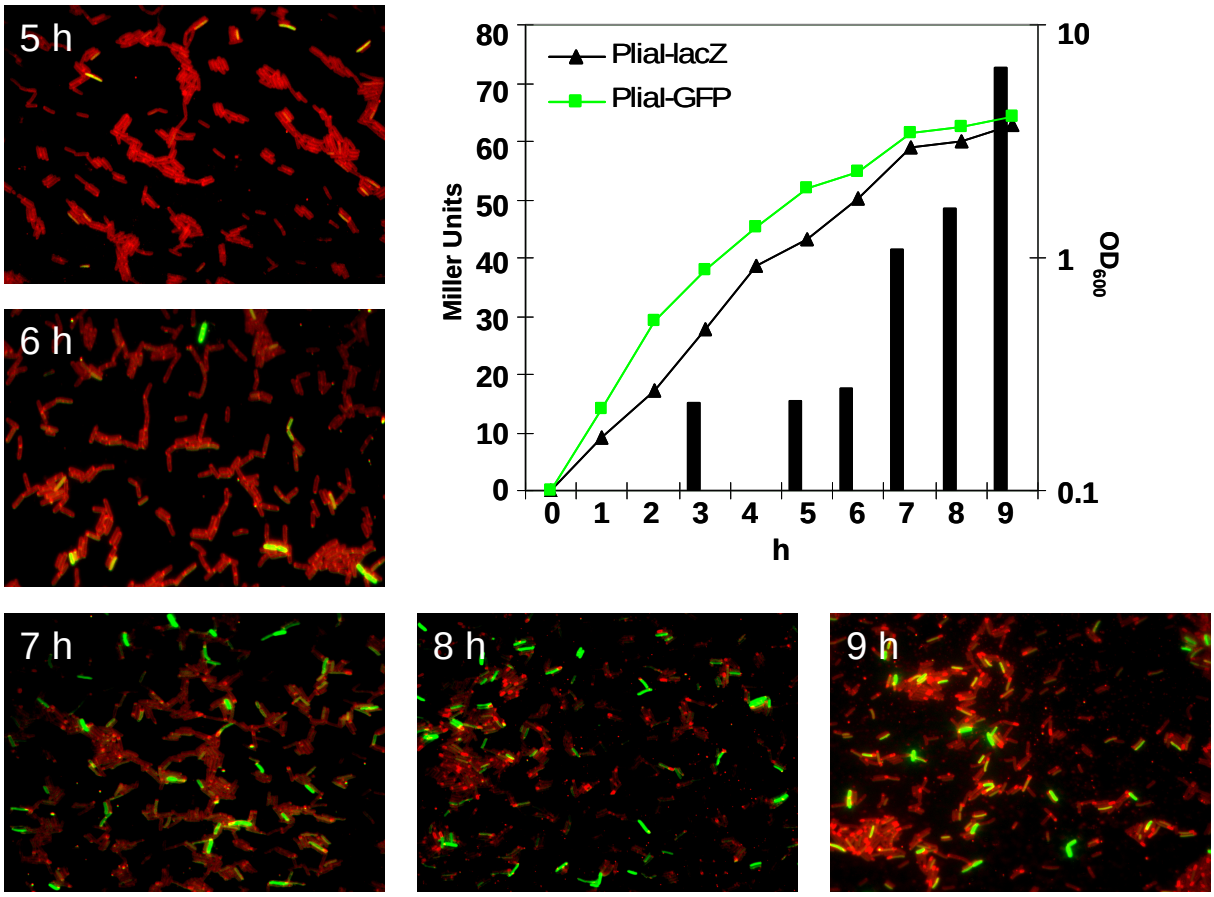

Figure 4.8. Bistable induction of P_{liaI}. GFP is expressed under the control of the *liaI* promoter. Samples were taken from TMB370 (black triangles) every hour for β-galactosidase assay (black bars). Cells from TMB408 (green squares) were monitored every hour by fluorescence microscopy. During transition state, only a subset of cells induce P_{liaI} (fluorescence shown in green). The membrane of all cells was stained (red).

Bistability occurs in populations of isogenic cells, grown under identical conditions (e.g. in liquid media in well-stirred flasks). It requires that a regulatory system can only switch between two alternative states but cannot rest in between (Dubnau and Losick, 2006). The heterogeneous output of several bacterial processes that are subject to a bistable behaviour could be traced back to the feedback-based wiring of the network involved (Ozbudak *et al.*, 2004; Smits *et al.*, 2005; Veening *et al.*, 2005). Two mechanisms have been proposed to drive bistable switches: positive or double-negative feedback regulation (Ferrell, 2002) (Fig. 4.9). The first mechanism (Fig. 4.9 left side) requires that the master regulator gene is positively autoregulated and responds to itself in a non-linear fashion (Becskei *et al.*, 2001). Non-linearity makes the response hypersensitive to even slight changes in regulator concentrations. Cells with regulator amount below the threshold do not switch on the output at all, while cells with more than the threshold amount of the regulator are driven to accumulate even more, due to the positive autoregulation. The expression of downstream genes is then altered and the population bifurcates.

The second mechanism (Fig.4.9 right side) requires the presence of a pair of mutually repressing repressors (Gardner *et al.*, 2000). If repressor 2 is inactivated then repressor 1 is produced, shutting off the synthesis of repressor 2. This step is equivalent to positive autoregulation, because the increase of repressor 1 results in its own production. If repressor 2 inhibits additional genes, these will now be expressed. If repressor 2 is not repressed, the system will behave the opposite way. One example of this kind of double-negative feedback regulation was observed in the switch between lysogenic and lytic states of the phage lambda (Ptashne, 2005).

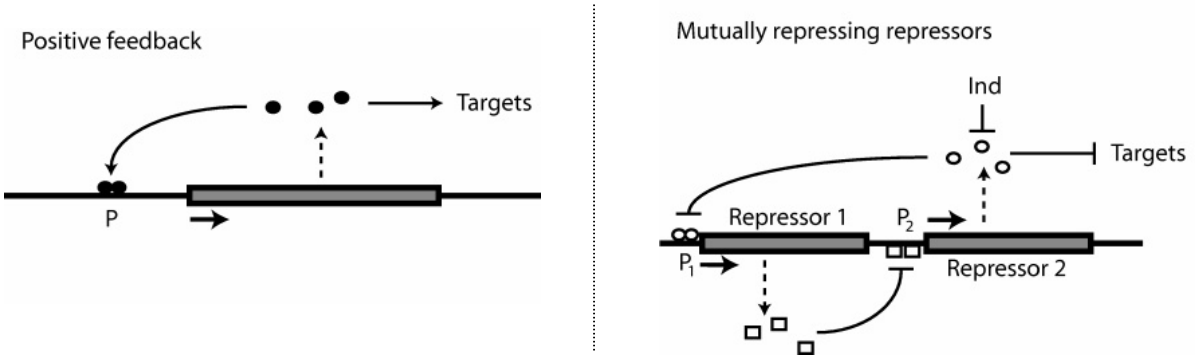

Figure 4.9. Two network configurations that lead to bistable expression. Figure taken from Dubnau and Losick, 2006. See text for details.

In *B. subtilis* several promoters are known to behave bistable (Dubnau and Losick, 2006). One example is the developement of competence. When cells enter stationary phase, about 10% of the cells in the culture become naturally competent because only in this subset of cells ComK activates the genes for DNA-uptake (van Sinderen *et al.*, 1995). The developement of competence depends on positive feedback regulation. This regulation is hypersensitive to the concentration of ComK because the regulator binds to its own promoter P_{comK} as a dimer of dimers (Hamoen *et al.*, 1998).

Swimming also shows a bistable behaviour (Kearns and Losick, 2005). Culures at the mid-exponential growth phase are a mixed population of cells in which σ^D, the master regulator for swimming and cell separation, is active (swimming cells) and cells in which σ^D is inactive (long chains of non-motile cells).

Another well studied example for bistability is sporulation, regulated by the master regulator Spo0A. The activity of Spo0A is governed by phosphorylation via a multicomponent phosphorelay (Burbulys *et al.*, 1991) and depends on nutrient limitation (Chung *et al.*, 1994). It could be demonstrated for promoters under Spo0A control that they behave bistable as well (Gonzalez-Pastor *et al.*, 2003). Since P_{liaI} is indirectly controlled by Spo0A, too, it was not too

suprising to find its transition state induction behave bistable. Furthermore, P_{liaI} regulation contains all prerequisits for a bistable switch: A positive feedback loop while LiaR induces expression of the *lia* operon (Mascher *et al.*, 2004) and an additionally regulation of LiaR activity, accomplished by LiaF and LiaS (Jordan *et al.*, 2006). An in-depth analysis of the bistable switch governing P_{liaI} activity will be subject of an upcoming PhD thesis.

Outlook

The data presented in this thesis unraved the mechanism of signal transduction mediated by the cell envelope stress-sensing three-component system LiaFSR. The preliminary results described in the discussion represent the starting points for future investigations to address three primary questions: (1) What is the physiological role of the LiaRS TCS? (2) What is the nature of the stimulus sensed by this TCS? (3) What is the mechanism of stimulus perception mediated by the LiaF-LiaS sensory unit? (4) What is the mechanism behind the bistable behaviour of the *liaI* promoter?

LiaI and LiaH are the main targets of LiaR-dependent gene expression and should be studied in detail. Based on the similarity between LiaH and *E. coli* PspA, we propose that LiaI might function as the membrane anchor for LiaH. This assumption is supported by preliminary results indicating a co-localization of LiaH and LiaI. A phenotypical characterization of LiaH is currently under way.

Identifying the stimulus and unraveling the mechanism of stimulus perception by the LiaF-LiaS sensory unit are two sides of a coin that need to be addressed together. Clearly, this represents a major goal of future research projects and will initially require the in-depth dissection of the two proteins involved. Based on the crucial role of both LiaF and LiaS for stimulus perception, we expect that they directly interact to form a sensory unit. This interaction could either occur within the membrane interface by the TMR of both proteins, or between the cytoplasmic domains, as has been suggested by the results from our initial mutagenesis studies. Mapping of the interacting interface between both proteins will therefore be an important prerequisite for understanding their function and hopefully also help to reveal the stimulus sensed by them.

The role of bistable switches in bacterial gene expression has only recently been recognized as a major mechanism to orchestrate population bifurcation at commiting regulatory checkpoints of differentiation cascades. With regard to the Lia system, the transition state induction of P_{liaI} needs to be further analyzed by flow cytometry. Moreover, time-resolved quantitative data on protein stoichiometry (for LiaF, LiaS, and LiaR) and the phosphorylation state of LiaS and LiaF will allow mathematical modeling of the LiaFSR switch to ultimately explain the mechanism responsible for the bistable behaviour of P_{liaI} induction. These analyses need to take into account the close link between the LiaRS TCS and the (phospho) neural network orchestrating the complex adaptional program that allows *B. subtilis* to adapt and ultimately survive under deteriorating growth conditions during stationary phase.

Chapter 5: References

Adams, H., Teertstra, W., Demmers, J., Boesten, R., and Tommassen, J. (2003) Interactions between phage-shock proteins in *Escherichia coli*. *J Bacteriol* **185**: 1174-1180.

Ades, S.E. (2004) Control of the alternative sigma factor σ^E in *Escherichia coli*. *Curr Opin Microbiol* **7**: 157-162.

Alba, B.M., and Gross, C.A. (2004) Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol Microbiol* **52**: 613-619.

Anantharaman, V., Koonin, E.V., and Aravind, L. (2001) Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains. *J Mol Biol* **307**: 1271-1292.

Anke, T., (ed) (1997) *Fungal Biotechnology.* Weinheim: Chapman & Hall.

Antoine, R., Huvent, I., Chemlal, K., Deray, I., Raze, D., Locht, C., and Jacob-Dubuisson, F. (2005) The periplasmic binding protein of a tripartite tricarboxylate transporter is involved in signal transduction. *J Mol Biol* **351**: 799-809.

Appleman, J.A., and Stewart, V. (2003) Mutational analysis of a conserved signal-transducing element: the HAMP linker of the *Escherichia coli* nitrate sensor NarX. *J. Bacteriol.* **185**: 89-97.

Archibald, A.R., Hancock, I.C., and Harwood, C.R. (1993) Cell wall structure, synthesis, and turnover. In *Bacillus subtilis and Other Gram-Positive Bacteria*. Sonenshein, A.L., Hoch, J.A. and Losick, R. (eds). Washington, D.C.: ASM press, pp. 381-410.

Arnaud, M., Chastanet, A., and Debarbouille, M. (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl Environ Microbiol* **70**: 6887-6891.

Asai, K., Yamaguchi, H., Kang, C.M., Yoshida, K., Fujita, Y., and Sadaie, Y. (2003) DNA microarray analysis of *Bacillus subtilis* sigma factors of extracytoplasmic function family. *FEMS Microbiol Lett* **220**: 155-160.

Aseeva, E., Ossenbuhl, F., Eichacker, L.A., Wanner, G., Soll, J., and Vothknecht, U.C. (2004) Complex Formation of Vipp1 Depends on Its {alpha}-Helical PspA-like Domain. *J. Biol. Chem.* **279**: 35535-35541.

Bailey, T.L., and Elkan, C. (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* **2**: 28-36.

Bandow, J.E., Brotz, H., Leichert, L.I., Labischinski, H., and Hecker, M. (2003) Proteomic approach to understanding antibiotic action. *Antimicrob Agents Chemother* **47**: 948-955.

Baruah, A., Lindsey, B., Zhu, Y., and Nakano, M.M. (2004) Mutational Analysis of the Signal-Sensing Domain of ResE Histidine Kinase from Bacillus subtilis. *J. Bacteriol.* **186**: 1694-1704.

Bass, R.B., and Falke, J.J. (1999) The aspartate receptor cytoplasmic domain: in situ chemical analysis of structure, mechanism and dynamics. *Structure Fold Des* **7**: 829-840.

Bassler, B.L., Wright, M., and Silverman, M.R. (1994) Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol Microbiol* **13**: 273-286.

Becskei, A., Seraphin, B., and Serrano, L. (2001) Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *Embo J* **20**: 2528-2535.

Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C.W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C.H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M.A., Rutherford, K., Rutter, S., Seeger,

K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B.G., Parkhill, J., and Hopwood, D.A. (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**: 141-147.

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

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

Bernat, B.A., Laughlin, L.T., and Armstrong, R.N. (1997) Fosfomycin resistance protein (FosA) is a manganese metalloglutathione transferase related to glyoxalase I and the extradiol dioxygenases. *Biochemistry* **36**: 3050-3055.

Bhavsar, A.P., Erdman, L.K., Schertzer, J.W., and Brown, E.D. (2004) Teichoic Acid Is an Essential Polymer in Bacillus subtilis That Is Functionally Distinct from Teichuronic Acid. *J. Bacteriol.* **186**: 7865-7873.

Bilwes, A.M., Park, S.-Y., Quezada, C.M., Simon, M.I., and Crane, B.R. (2003) Structure and function of CheA, the histidine kinase central to bacterial chemotaxis. In *Histidine Kinases in Signal Transduction*. Inouye, M. and Dutta, R. (eds). San Diego, CA: Academic Press, pp. 48-73.

Bobay, B.G., Benson, L., Naylor, S., Feeney, B., Clark, A.C., Goshe, M.B., Strauch, M.A., Thompson, R., and Cavanagh, J. (2004) Evaluation of the DNA binding tendencies of the transition state regulator AbrB. *Biochemistry* **43**: 16106-16118.

Bobay, B.G., Mueller, G.A., Thompson, R.J., Murzin, A.G., Venters, R.A., Strauch, M.A., and Cavanagh, J. (2006) NMR structure of AbhN and comparison with AbrBN: first insights into the DNA binding promiscuity and specificity of AbrB-like transition state regulator proteins. *J. Biol. Chem.* **281**: 21399-21409.

Bordes, P., Wigneshweraraj, S.R., Schumacher, J., Zhang, X., Chaney, M., and Buck, M. (2003) The ATP hydrolyzing transcription activator phage shock protein F of *Escherichia coli*: identifying a surface that binds σ^{54}. *Proc Natl Acad Sci U S A* **100**: 2278-2283.

Branda, S.S., Vik, S., Friedman, L., and Kolter, R. (2005) Biofilms: the matrix revisited. *Trends Microbiol* **13**: 20-26.

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

Brissette, J.L., Russel, M., Weiner, L., and Model, P. (1990) Phage shock protein, a stress protein of *Escherichia coli*. *Proc Natl Acad Sci U S A* **87**: 862-866.

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

Buelow, D.R., and Raivio, T.L. (2005) Cpx signal transduction Is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. *J. Bacteriol.* **187**: 6622-6630.

Bugg, T.D., and Walsh, C.T. (1992) Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic resistance. *Nat Prod Rep* **9**: 199-215.

Buhrke, T., Lenz, O., Porthun, A., and Friedrich, B. (2004) The H_2-sensing complex of *Ralstonia eutropha*: interaction between a regulatory [NiFe] hydrogenase and a histidine protein kinase. *Mol Microbiol* **51**: 1677-1689.

Burbulys, D., Trach, K.A., and Hoch, J.A. (1991) Initiation of sporulation in B. subtilis is controlled by a multicomponent phosphorelay. *Cell* **64**: 545-552.

Burkholder, W.F., Kurtser, I., and Grossman, A.D. (2001) Replication initiation proteins regulate a developmental checkpoint in *Bacillus subtilis*. *Cell* **104**: 269-279.

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

Butcher, B.G., Mascher, T., and Helmann, J.D. (2007) Environmental sensing and the role of extracytoplasmic function (ECF) σ φαχτορσ. In *Bacterial Physiology - a Molecular Approach*. El-Sharoud, W.M. (ed). Berlin, Heidelberg: Springer-Verlag GmbH, pp. in press.

Butler, S.L., and Falke, J.J. (1998) Cysteine and Disulfide Scanning Reveals Two Amphiphilic Helices in the Linker Region of the Aspartate Chemoreceptor. Vol. 37, pp. 10746-10756.

Cain, B.D., Norton, P.J., Eubanks, W., Nick, H.S., and Allen, C.M. (1993) Amplification of the bacA gene confers bacitracin resistance to Escherichia coli. *J Bacteriol* **175**: 3784-3789.

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

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

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

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

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

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

Chalker, A.F., Ingraham, K.A., Lunsford, R.D., Bryant, A.P., Bryant, J., Wallis, N.G., Broskey, J.P., Pearson, S.C., and Holmes, D.J. (2000) The *bacA* gene, which determines bacitracin susceptibility in *Streptococcus pneumoniae* and *Staphylococcus aureus*, is also required for virulence. *Microbiology* **146**: 1547-1553.

Champness, W. (2000) Actinomycete development, antibiotic production, and phylogeny: questions and challenges. In *Prokaryotic Development*. Brun, Y.V. and Shimkets, L.J. (eds). Washington, DC: ASM press.

Chatterjee, C., Paul, M., Xie, L., and van der Donk, W.A. (2005) Biosynthesis and mode of action of lantibiotics. *Chem Rev* **105**: 633-684.

Chung, J.D., Stephanopoulos, G., Ireton, K., and Grossman, A.D. (1994) Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. *J Bacteriol* **176**: 1977-1984.

Clausen, V.A., Bae, W., Throup, J., Burnham, M.K., Rosenberg, M., and Wallis, N.G. (2003) Biochemical characterization of the first essential two-component signal transduction system from *Staphylococcus aureus* and *Streptococcus pneumoniae*. *J Mol Microbiol Biotechnol* **5**: 252-260.

Crater, D.L., and Moran, C.P., Jr. (2001) Identification of a DNA binding region in GerE from *Bacillus subtilis*. *J Bacteriol* **183**: 4183-4189.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome Res* **14**: 1188-1190.

D'Costa, V.M., McGrann, K.M., Hughes, D.W., and Wright, G.D. (2006) Sampling the antibiotic resistome. *Science* **311**: 374-377.

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

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

Dahl, J.L., Wei, B.Y., and Kadner, R.J. (1997) Protein phosphorylation affects binding of the *Escherichia coli* transcription activator UhpA to the *uhpT* promoter. *J Biol Chem* **272**: 1910-1919.

Dartois, V., Djavakhishvili, T., and Hoch, J. (1996) Identification of a membrane protein involved in activation of the KinB pathway to sporulation in *Bacillus subtilis*. *J. Bacteriol.* **178**: 1178-1186.

Dartois, V., Djavakhishvili, T., and Hoch, J.A. (1997a) KapB is a lipoprotein required for KinB signal transduction and activation of the phosphorelay to sporulation in *Bacillus subtilis*. *Mol Microbiol* **26**: 1097-1108.

Dartois, V., Liu, J., and Hoch, J.A. (1997b) Alterations in the flow of one-carbon units affect KinB-dependent sporulation in *Bacillus subtilis*. *Mol Microbiol* **25**: 39-51.

Darwin, A.J., and Miller, V.L. (2001) The *psp* locus of *Yersinia enterocolitica* is required for virulence and for growth *in vitro* when the Ysc type III secretion system is produced. *Mol Microbiol* **39**: 429-445.

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

De Wulf, P., McGuire, A.M., Liu, X., and Lin, E.C. (2002) Genome-wide profiling of promoter recognition by the two-component response regulator CpxR-P in *Escherichia coli*. *J Biol Chem* **277**: 26652-26661.

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

DeLisa, M.P., Lee, P., Palmer, T., and Georgiou, G. (2004) Phage shock protein PspA of *Escherichia coli* relieves saturation of protein export via the Tat pathway. *J Bacteriol* **186**: 366-373.

Depardieu, F., Courvalin, P., and Msadek, T. (2003) A six amino acid deletion, partially overlapping the VanSB G2 ATP-binding motif, leads to constitutive glycopeptide resistance in VanB-type *Enterococcus faecium*. *Mol Microbiol* **50**: 1069-1083.

Derre, I., Rapoport, G., and Msadek, T. (2000) The CtsR regulator of stress response is active as a dimer and specifically degraded in vivo at 37°C. *Mol Microbiol* **38**: 335-347.

Dmitriev, B., Toukach, F., and Ehlers, S. (2005) Towards a comprehensive view of the bacterial cell wall. *Trends Microbiol* **13**: 569-574.

Dover, L.G., Cerdeno-Tarraga, A.M., Pallen, M.J., Parkhill, J., and Besra, G.S. (2004) Comparative cell wall core biosynthesis in the mycolated pathogens, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. *FEMS Microbiol Rev* **28**: 225-250.

Dowson, C.G., Coffey, T.J., and Spratt, B.G. (1994) Origin and molecular epidemiology of penicillin-binding-protein-mediated resistance to β-lactam antibiotics. *Trends Microbiol* **2**: 361-366.

Dubnau, D., and Losick, R. (2006) Bistability in bacteria. *Mol Microbiol* **61**: 564-572.

Dworkin, J., Jovanovic, G., and Model, P. (2000) The PspA protein of *Escherichia coli* is a negative regulator of sigma(54)-dependent transcription. *J Bacteriol* **182**: 311-319.

Economou, A. (1999) Following the leader: bacterial protein export through the Sec pathway. *Trends Microbiol* **7**: 315-320.

Egland, K.A., and Greenberg, E.P. (2000) Conversion of the *Vibrio fischeri* transcriptional activator, LuxR, to a repressor. *J Bacteriol* **182**: 805-811.

Eldakak, A., and Hulett, F.M. (2007) Cys303 in the Histidine Kinase PhoR Is Crucial for the Phosphotransfer Reaction in the PhoPR Two-Component System in *Bacillus subtilis*. Vol. 189, pp. 410-421.

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

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

Emmerich, R., Strehler, P., Hennecke, H., and Fischer, H.-M. (2000) An imperfect inverted repeat is critical for DNA binding of the response regulator RegR of *Bradyrhizobium japonicum*. *Nucl. Acids Res.* **28**: 4166-4171.

Eraso, J.M., and Kaplan, S. (2000) From redox flow to gene regulation: role of the PrrC protein of *Rhodobacter sphaeroides* 2.4.1. *Biochemistry* **39**: 2052-2062.

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

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

Fawcett, P., Eichenberger, P., Losick, R., and Youngman, P. (2000) The transcriptional profile of early to middle sporulation in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **97**: 8063-8068.

Fedtke, I., Kamps, A., Krismer, B., and Götz, F. (2002) The nitrate reductase and nitrite reductase operons and the *narT* gene of *Staphylococcus carnosus* are positively controlled by the novel two-component system NreBC. *J Bacteriol* **184**: 6624-6634.

Feng, Z., and Barletta, R.G. (2003) Roles of *Mycobacterium smegmatis* D-alanine:D-alanine ligase and D-alanine racemase in the mechanisms of action of and resistance to the peptidoglycan inhibitor D-cycloserine. *Antimicrob Agents Chemother* **47**: 283-291.

Ferrell, J.E. (2002) Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Current Opinion in Cell Biology* **14**: 140-148.

Fleischer, R., Heermann, R., Jung, K., and Hunke, S. (2007) Purification, reconstitution, and characterization of the CpxRAP envelope stress system of *Escherichia coli*. *J Biol Chem* **282**: 8583-8593.

Foster, S.J., and Popham, D.L. (2002) Structure and synthesis of cell wall, spore cortex, teichoic acid, S-layers and capsules. In *Bacillus subtilis and Its Closest Relatives. From Genes to Cells*. Sonenshein, A.L., Hoch, J.A. and Losick, R. (eds). Washigton D.C.: ASM Press, pp. 21-41.

Friedrich, B., Buhrke, T., Burgdorf, T., and Lenz, O. (2005) A hydrogen-sensing multiprotein complex controls aerobic hydrogen metabolism in Ralstonia eutropha. *Biochem Soc Trans* **33**: 97-101.

Fujita, M., Gonzalez-Pastor, J.E., and Losick, R. (2005) High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J. Bacteriol.* **187**: 1357-1368.

Gaal, T., Ross, W., Blatter, E.E., Tang, H., Jia, X., Krishnan, V.V., Assa-Munt, N., Ebright, R.H., and Gourse, R.L. (1996) DNA-binding determinants of the alpha subunit of RNA polymerase: novel DNA-binding domain architecture. *Genes Dev* **10**: 16-26.

Galperin, M.Y. (2006) Structural classification of bacterial response regulators: diversity of output domains and domain combinations. *J Bacteriol* **188**: 4169-4182.

Gao, T., Zhang, X., Ivleva, N.B., Golden, S.S., and Liwang, A. (2007) NMR structure of the pseudo-receiver domain of CikA. *Protein Sci* **16**: 465-475.

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

Gardner, T.S., Cantor, C.R., and Collins, J.J. (2000) Construction of a genetic toggle switch in Escherichia coli. *Nature* **403**: 339-342.

Garnerone, A.-M., Cabanes, D., Foussard, M., Boistard, P., and Batut, J. (1999) Inhibition of the FixL sensor kinase by the FixT protein in *Sinorhizobium meliloti*. *J. Biol. Chem.* **274**: 32500-32506.

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

Giuliani, A., Pirri, G., and Nicoletto, S.F. (2007) Antimicrobial peptides: an overview of a promising class of therapeutics. *Central European Journal of Biology* **2**: 1-33.

Gon, S., Jourlin-Castelli, C., Theraulaz, L., and Mejean, V. (2001) An unsuspected autoregulatory pathway involving apocytochrome TorC and sensor TorS in *Escherichia coli*. *PNAS* **98**: 11615-11620.

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

Gravesen, A., Sorensen, K., Aarestrup, F.M., and Knochel, S. (2001) Spontaneous nisin-resistant *Listeria monocytogenes* mutants with increased expression of a putative penicillin-binding protein and their sensitivity to various antibiotics. *Microb Drug Resist* **7**: 127-135.

Gravesen, A., Kallipolitis, B., Holmstrom, K., Hoiby, P.E., Ramnath, M., and Knochel, S. (2004) *pbp2229*-mediated nisin resistance mechanism in *Listeria monocytogenes* confers cross-protection to class IIa bacteriocins and affects virulence gene expression. *Appl. Environ. Microbiol.* **70**: 1669-1679.

Grebe, T.W., and Stock, J. (1998) Bacterial chemotaxis: the five sensors of a bacterium. *Curr Biol* **8**: R154-157.

Gründling, A., and Schneewind, O. (2007) Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **104**: 8478-8483.

Guenzi, E., Gasc, A.M., Sicard, M.A., and Hakenbeck, R. (1994) A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of *Streptococcus pneumoniae*. *Mol Microbiol* **12**: 505-515.

Guerout-Fleury, A.M., Shazand, K., Frandsen, N., and Stragier, P. (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**: 335-336.

Guerout-Fleury, A.M., Frandsen, N., and Stragier, P. (1996) Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* **180**: 57-61.

Haas, W., Kaushal, D., Sublett, J., Obert, C., and Tuomanen, E.I. (2005) Vancomycin stress response in a sensitive and a tolerant strain of *Streptococcus pneumoniae*. *J. Bacteriol.* **187**: 8205-8210.

Hakenbeck, R. (1999) β-lactam-resistant *Streptococcus pneumoniae*: epidemiology and evolutionary mechanism. *Chemotherapy* **45**: 83-94.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **41**: 95-98.

Hamoen, L.W., Van Werkhoven, A.F., Bijlsma, J.J.E., Dubnau, D., and Venema, G. (1998) The competence transcription factor of Bacillus subtilis recognizes short A/T-rich sequences arranged in a unique, flexible pattern along the DNA helix. Vol. 12, pp. 1539-1550.

Hamon, M.A., Stanley, N.R., Britton, R.A., Grossman, A.D., and Lazazzera, B.A. (2004) Identification of AbrB-regulated genes involved in biofilm formation by *Bacillus subtilis*. *Molecular Microbiology* **52**: 847-860.

Hancock, L.E., and Perego, M. (2004) Systematic Inactivation and Phenotypic Characterization of Two-Component Signal Transduction Systems of Enterococcus faecalis V583. *J. Bacteriol.* **186**: 7951-7958.

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

Hankamer, B.D., Elderkin, S.L., Buck, M., and Nield, J. (2004) Organization of the AAA(+) adaptor protein PspA is an oligomeric ring. *J Biol Chem* **279**: 8862-8866.

Hansen, M.E., Mijakovic, I., Jensen, P.R., and Sibbesen, O. (2007) Use of *Bacillus subtilis* for the biosynthesis of nisin. In *4th Conference on Functional Genomics of Gram-positive Microorganisms* Tirrenia, Pisa, Italy.

Hardie, K.R., Lory, S., and Pugsley, A.P. (1996) Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein. *Embo J* **15**: 978-988.

Harwood, C.R., and Cutting, S.M. (1990) *Molecular Biological Methods for Bacillus.* Chichester: John Wiley & Sons.

Healy, V.L., Lessard, I.A., Roper, D.I., Knox, J.R., and Walsh, C.T. (2000) Vancomycin resistance in enterococci: reprogramming of the D-ala-D-Ala ligases in bacterial peptidoglycan biosynthesis. *Chem Biol* **7**: R109-119.

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

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

Helmann, J.D., and Moran, C.P. (2002) RNA polymerase and sigma factors. In *Bacillus subtilis and Its Closest Relatives - From Genes to Cells*. Sonenshein, A.L., Hoch, J.A. and Losick, R. (eds). Washington D.C.: ASM Press, pp. 289-312.

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

Hernandez, C., Olano, C., Mendez, C., and Salas, J.A. (1993) Characterization of a *Streptomyces antibioticus* gene cluster encoding a glycosyltransferase involved in oleandomycin inactivation. *Gene* **134**: 139-140.

Hirakawa, H., Nishino, K., Yamada, J., Hirata, T., and Yamaguchi, A. (2003) β-Lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. *J. Antimicrob. Chemother.* **52**: 576-582.

Höltje, J.V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli. *Microbiol Mol Biol Rev* **62**: 181-203.

Hong, H.J., Paget, M.S., and Buttner, M.J. (2002) A signal transduction system in Streptomyces coelicolor that activates the expression of a putative cell wall glycan operon in response to vancomycin and other cell wall-specific antibiotics. *Mol Microbiol* **44**: 1199-1211.

Horii, T., Kimura, T., Sato, K., Shibayama, K., and Ohta, M. (1999) Emergence of fosfomycin-resistant isolates of Shiga-like toxin-producing *Escherichia coli* O26. *Antimicrob Agents Chemother* **43**: 789-793.

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

Hoskisson, P.A., and Hutchings, M.I. (2006) MtrAB-LpqB: a conserved three-component system in actinobacteria? *Trends Microbiol* **14**: 444-449.

Hu, Y., Helm, J.S., Chen, L., Ye, X.Y., and Walker, S. (2003) Ramoplanin inhibits bacterial transglycosylases by binding as a dimer to lipid II. *J Am Chem Soc* **125**: 8736-8737.

Huang, X., Decatur, A., Sorokin, A., and Helmann, J.D. (1997) The *Bacillus subtilis* σ^X protein is an extracytoplasmic function sigma factor contributing to survival at high temperature. *J Bacteriol* **179**: 2915-2921.

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

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

Hulko, M., Berndt, F., Gruber, M., Linder, J.U., Truffault, V., Schultz, A., Martin, J., Schultz, J.E., Lupas, A.N., and Coles, M. (2006) The HAMP domain structure implies helix rotation in transmembrane signaling. *Cell* **126**: 929-940.

Hurley, J.H. (2003) GAF domains: cyclic nucleotides come full circle. *Sci STKE* **2003**: PE1.

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

Hutchings, M.I., Hong, H.-J., Leibovitz, E., Sutcliffe, I.C., and Buttner, M.J. (2006a) The σ^E cell envelope stress response of *Streptomyces coelicolor* is influenced by a novel lipoprotein, CseA. *J. Bacteriol.* **188**: 7222-7229.

Hutchings, M.I., Hong, H.J., and Buttner, M.J. (2006b) The vancomycin resistance VanRS two-component signal transduction system of *Streptomyces coelicolor*. *Mol Microbiol* **59**: 923-935.

Hutter, B., Schaab, C., Albrecht, S., Borgmann, M., Brunner, N.A., Freiberg, C., Ziegelbauer, K., Rock, C.O., Ivanov, I., and Loferer, H. (2004) Prediction of Mechanisms of Action of Antibacterial Compounds by Gene Expression Profiling. *Antimicrob. Agents Chemother.* **48**: 2838-2844.

Hyyryläinen, H.-L., Pietiäinen, M., Lunden, T., Ekman, A., Gardemeister, M., Murtomaki-Repo, S., Antelmann, H., Hecker, M., Valmu, L., Sarvas, M., and Kontinen, V.P. (2007) The density of negative charge in the cell wall influences two-component signal transduction in *Bacillus subtilis*. *Microbiology* **153**: 2126-2136.

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

Island, M.D., and Kadner, R.J. (1993) Interplay between the membrane-associated UhpB and UhpC regulatory proteins. *J Bacteriol* **175**: 5028-5034.

Ivleva, N.B., Gao, T., LiWang, A.C., and Golden, S.S. (2006) Quinone sensing by the circadian input kinase of the cyanobacterial circadian clock. *PNAS* **103**: 17468-17473.

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

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

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

Joseph, P., Fichant, G., Quentin, Y., and Denizot, F. (2002) Regulatory relationship of two-component and ABC transport systems and clustering of their genes in the

Bacillus/Clostridium group, suggest a functional link between them. *J Mol Microbiol Biotechnol* **4**: 503-513.

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

Kadner, R.J. (1995) Expression of the Uhp sugar-phosphate transport system of *Escherichia coli*. In *Two-Component Signal Transduction*. Hoch, J.A. and Silhavy, T.J. (eds). Washington, D.C.: ASM press.

Kahan, F.M., Kahan, J.S., Cassidy, P.J., and Kropp, H. (1974) The mechanism of action of fosfomycin (phosphonomycin). *Ann N Y Acad Sci* **235**: 364-386.

Kahne, D., Leimkuhler, C., Lu, W., and Walsh, C. (2005) Glycopeptide and lipoglycopeptide antibiotics. *Chem Rev* **105**: 425-448.

Kallipolitis, B.H., Ingmer, H., Gahan, C.G., Hill, C., and Sogaard-Andersen, L. (2003) CesRK, a Two-Component Signal Transduction System in Listeria monocytogenes, Responds to the Presence of Cell Wall-Acting Antibiotics and Affects {beta}-Lactam Resistance. *Antimicrob. Agents Chemother.* **47**: 3421-3429.

Kearns, D.B., and Losick, R. (2005) Cell population heterogeneity during growth of Bacillus subtilis. Vol. 19, pp. 3083-3094.

Kendall, S.L., Movahedzadeh, F., Rison, S.C., Wernisch, L., Parish, T., Duncan, K., Betts, J.C., and Stoker, N.G. (2004) The *Mycobacterium tuberculosis dosRS* two-component system is induced by multiple stresses. *Tuberculosis (Edinb)* **84**: 247-255.

Kleerebezem, M., and Tommassen, J. (1993) Expression of the *pspA* gene stimulates efficient protein export in *Escherichia coli*. *Mol Microbiol* **7**: 947-956.

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

Kleihues, L., Lenz, O., Bernhard, M., Buhrke, T., and Friedrich, B. (2000) The H_2 sensor of *Ralstonia eutropha* is a member of the subclass of regulatory [NiFe] hydrogenases. *J Bacteriol* **182**: 2716-2724.

Klein, A.H., Shulla, A., Reimann, S.A., Keating, D.H., and Wolfe, A.J. (2007) The intracellular concentration of acetyl phosphate in *Escherichia coli* is sufficient for direct phosphorylation of two-component response regulators. *J Bacteriol* **189**: 5574-5581.

Kobayashi, H., Yamamoto, M., and Aono, R. (1998) Appearance of a stress-response protein, phage-shock protein A, in *Escherichia coli* exposed to hydrophobic organic solvents. *Microbiology* **144 (Pt 2)**: 353-359.

Kobayashi, K., Ogura, M., Yamaguchi, H., Yoshida, K., Ogasawara, N., Tanaka, T., and Fujita, Y. (2001) Comprehensive DNA microarray analysis of *Bacillus subtilis* two-component regulatory systems. *J Bacteriol* **183**: 7365-7370.

Kobayashi, K. (2007) *Bacillus subtilis* pellicle formation proceeds through genetically defined morphological changes. *J Bacteriol* **189**: 4920-4931.

Koch, A.L. (2003) Bacterial wall as target for attack: past, present, and future research. *Clin Microbiol Rev* **16**: 673-687.

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

Kuroda, M., Kuwahara-Arai, K., and Hiramatsu, K. (2000) Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and

Mu50 by cDNA differential hybridization method. *Biochem Biophys Res Commun* **269**: 485-490.

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

Lambert, M.P., and Neuhaus, F.C. (1972) Mechanism of D-cycloserine action: alanine racemase from *Escherichia coli* W. *J Bacteriol* **110**: 978-987.

Lange, R., Wagner, C., de Saizieu, A., Flint, N., Molnos, J., Stieger, M., Caspers, P., Kamber, M., Keck, W., and Amrein, K.E. (1999) Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. *Gene* **237**: 223-234.

Lazar, K., and Walker, S. (2002) Substrate analogues to study cell-wall biosynthesis and its inhibition. *Curr Opin Chem Biol* **6**: 786-793.

Lazarevic, V., Abellan, F.-X., Moller, S.B., Karamata, D., and Mauel, C. (2002) Comparison of ribitol and glycerol teichoic acid genes in Bacillus subtilis W23 and 168: identical function, similar divergent organization, but different regulation. *Microbiology* **148**: 815-824.

Lenz, O., and Friedrich, B. (1998) A novel multicomponent regulatory system mediates H2 sensing in *Alcaligenes eutrophus*. *Proc Natl Acad Sci U S A* **95**: 12474-12479.

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

Liger, D., Graille, M., Zhou, C.Z., Leulliot, N., Quevillon-Cheruel, S., Blondeau, K., Janin, J., and van Tilbeurgh, H. (2004) Crystal structure and functional characterization of yeast YLR011wp, an enzyme with NAD(P)H-FMN and ferric iron reductase activities. *J Biol Chem* **279**: 34890-34897.

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

Majdalani, N., and Gottesman, S. (2005) The Rcs phosphorelay: a complex signal transduction system. *Annu Rev Microbiol* **59**: 379-405.

Mandin, P., Fsihi, H., Dussurget, O., Vergassola, M., Milohanic, E., Toledo-Arana, A., Lasa, I., Johansson, J., and Cossart, P. (2005) VirR, a response regulator critical for *Listeria monocytogenes* virulence. *Mol Microbiol* **57**: 1367-1380.

Martinez, B., Zomer, A.L., Rodriguez, A., Kok, J., and Kuipers, O.P. (2007) Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. *Molecular Microbiology* **64**: 473-486.

Martinez, S.E., Beavo, J.A., and Hol, W.G.J. (2002) GAF domains: two-billion-year-old molecular switches that bind cyclic nucleotides. *Mol. Interv.* **2**: 317-323.

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

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

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

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

Mascher, T., Helmann, J.D., and Unden, G. (2006) Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **90**: 910-938.

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

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

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

Matsuo, H., Kumagai, T., Mori, K., and Sugiyama, M. (2003) Molecular cloning of a D-cycloserine resistance gene from D-cycloserine-producing *Streptomyces garyphalus*. *J Antibiot (Tokyo)* **56**: 762-767.

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

McNeil, M.M., and Brown, J.M. (1994) The medically important aerobic actinomycetes: epidemiology and microbiology. *Clin Microbiol Rev* **7**: 357-417.

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

Miller, J.H. (1972) *Experiments in molecular genetics.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Ming, L.-J., and Epperson, J.D. (2002) Metal binding and structure-activity relationship of the metalloantibiotic peptide bacitracin. *Journal of Inorganic Biochemistry* **91**: 46-58.

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

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

Miyake, K., and Iijima, S. (2004) Bacterial capsular polysaccharide and sugar transferases. *Adv Biochem Eng Biotechnol* **90**: 89-111.

Model, P., Jovanovic, G., and Dworkin, J. (1997) The *Escherichia coli* phage-shock-protein (*psp*) operon. *Mol Microbiol* **24**: 255-261.

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

Münch, R., Hiller, K., Barg, H., Heldt, D., Linz, S., Wingender, E., and Jahn, D. (2003) PRODORIC: prokaryotic database of gene regulation. *Nucl. Acids Res.* **31**: 266-269.

Münch, R., Hiller, K., Grote, A., Scheer, M., Klein, J., Schobert, M., and Jahn, D. (2005) Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* **21**: 4187-4189.

Mutsuda, M., Michel, K.P., Zhang, X., Montgomery, B.L., and Golden, S.S. (2003) Biochemical properties of CikA, an unusual phytochrome-like histidine protein kinase that resets the circadian clock in Synechococcus elongatus PCC 7942. *J Biol Chem* **278**: 19102-19110.

Myhre, A.E., Aasen, A.O., Thiemermann, C., and Wang, J.E. (2006) Peptidoglycan - an endotoxin in its own right? *Shock* **25**: 227-235.

Nagakubo, S., Nishino, K., Hirata, T., and Yamaguchi, A. (2002) The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. *J Bacteriol* **184**: 4161-4167.

Nakano, S., Nakano, M.M., Zhang, Y., Leelakriangsak, M., and Zuber, P. (2003) A regulatory protein that interferes with activator-stimulated transcription in bacteria. *Proc Natl Acad Sci U S A* **100**: 4233-4238.

Neuhaus, F.C., and Lynch, J.L. (1964) The enzymatic synthesis of D-alanyl-D-alanine. 3. On the inhibition of D-alanyl-D-alanine synthetase by the antibiotic D-cycloserine. *Biochemistry* **3**: 471-480.

Neumüller, A.M., Konz, D., and Marahiel, M.A. (2001) The two-component regulatory system BacRS is associated with bacitracin 'self-resistance' of *Bacillus licheniformis* ATCC 10716. *Eur J Biochem* **268**: 3180-3189.

Nwosu, V.C. (2001) Antibiotic resistance with particular reference to soil microorganisms. *Res Microbiol* **152**: 421-430.

O'Connell-Motherway, M., van Sinderen, D., Morel-Deville, F., Fitzgerald, G.F., Ehrlich, S.D., and Morel, P. (2000) Six putative two-component regulatory systems isolated from Lactococcus lactis subsp. cremoris MG1363. *Microbiology* **146**: 935-947.

O'Riordan, K., and Lee, J.C. (2004) *Staphylococcus aureus* capsular polysaccharides. *Clin Microbiol Rev* **17**: 218-234.

Ogura, M., Shimane, K., Asai, K., Ogasawara, N., and Tanaka, T. (2003) Binding of response regulator DegU to the aprE promoter is inhibited by RapG, which is counteracted by extracellular PhrG in Bacillus subtilis. *Mol Microbiol* **49**: 1685-1697.

Ogura, M., Matsuzawa, A., Yoshikawa, H., and Tanaka, T. (2004) *Bacillus subtilis* SalA (YbaL) negatively regulates expression of scoC, which encodes the repressor for the alkaline exoprotease gene, *aprE*. *J Bacteriol* **186**: 3056-3064.

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

Ohki, R., Tateno, K., Okada, Y., Okajima, H., Asai, K., Sadaie, Y., Murata, M., and Aiso, T. (2003b) A bacitracin-resistant *Bacillus subtilis* gene encodes a homologue of the membrane-spanning subunit of the *Bacillus licheniformis* ABC transporter. *J. Bacteriol.* **185**: 51-59.

Ozbudak, E.M., Thattai, M., Lim, H.N., Shraiman, B.I., and van Oudenaarden, A. (2004) Multistability in the lactose utilization network of Escherichia coli. *Nature* **427**: 737-740.

Paget, M.S., Chamberlin, L., Atrih, A., Foster, S.J., and Buttner, M.J. (1999a) Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). *J Bacteriol* **181**: 204-211.

Paget, M.S., Leibovitz, E., and Buttner, M.J. (1999b) A putative two-component signal transduction system regulates σ^E, a sigma factor required for normal cell wall integrity in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **33**: 97-107.

Palumbi, S.R. (2001) Humans as the world's greatest evolutionary force. *Science* **293**: 1786-1790.

Pao, G.M., Tam, R., Lipschitz, L.S., and Saier, M.H., Jr. (1994) Response regulators: structure, function and evolution. *Res Microbiol* **145**: 356-362.

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

Paul, E.A., and Clark, F.E. (1996) *Soil Microbiology and Biochemistry.* San Diego, CA (USA): Academic Press.

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

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

Peters, J.E., and Craig, N.L. (2001) Tn7: smarter than we thought. *Nat Rev Mol Cell Biol* **2**: 806-814.

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

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

Pietiäinen, M., Gardemeister, M., Mecklin, M., Leskela, S., Sarvas, M., and Kontinen, V.P. (2005) Cationic antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-type sigma factors and two-component signal transduction systems. *Microbiology* **151**: 1577-1592.

Pietiäinen, M., Francois, P., Schrenzel, J., and Kontinen, V.P. (2007) DNA microarray analysis of the response of *Staphylococcus aureus* to cationic antimicrobial peptides. In *4th Conference on Functional Genomics of Gram-positive Microorganisms* Tirrenia, Pisa, Italy.

Piggot, P.J., and Hilbert, D.W. (2004) Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* **7**: 579-586.

Podlesek, Z., Comino, A., Herzog-Velikonja, B., Zgur-Bertok, D., Komel, R., and Grabnar, M. (1995) *Bacillus licheniformis* bacitracin-resistance ABC transporter: relationship to mammalian multidrug resistance. *Mol Microbiol* **16**: 969-976.

Pollock, T.J., Thorne, L., Yamazaki, M., Mikolajczak, M.J., and Armentrout, R.W. (1994) Mechanism of bacitracin resistance in gram-negative bacteria that synthesize exopolysaccharides. *J Bacteriol* **176**: 6229-6237.

Poole, K. (2004) Resistance to β-lactam antibiotics. *Cell Mol Life Sci* **61**: 2200-2223.

Poole, K. (2005) Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother* **56**: 20-51.

Price, C.W. (2002) General stress response. In *Bacillus subtilis and Its Closest Relatives - From Genes to Cells*. Sonenshein, A.L., Hoch, J.A. and Losick, R. (eds). Washington D.C.: ASM Press.

Ptashne, M. (2005) Regulation of transcription: from lambda to eukaryotes. *Trends in Biochemical Sciences* **30**: 275-279.

Qian, Q., Lee, C.Y., Helmann, J.D., and Strauch, M.A. (2002) AbrB is a regulator of the σ^W regulon in *Bacillus subtilis*. *FEMS Microbiol Lett* **211**: 219-223.

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

Raffa, R.G., and Raivio, T.L. (2002) A third envelope stress signal transduction pathway in *Escherichia coli*. *Molecular Microbiology* **45**: 1599-1611.

Raivio, T.L. (2005) Envelope stress responses and Gram-negative bacterial pathogenesis. *Molecular Microbiology* **56**: 1119-1128.

Rhodius, V.A., Suh, W.C., Nonaka, G., West, J., and Gross, C.A. (2006) Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biol* **4**: e2.

Rietkötter, E., and Mascher, T. (2007) Tunnel vision - bacitracin perception in *Bacillus subtilis*. In *4th Conference on Functional Genomics of Gram-Positive Microorganisms* Tirrenia, Pisa, Italy.

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

Ruiz, N., and Silhavy, T.J. (2005) Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. *Curr Opin Microbiol* **8**: 122-126.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning - a laboratory manual.* Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Santelli, E., Liddington, R.C., Mohan, M.A., Hoch, J.A., and Szurmant, H. (2007) The crystal structure of Bacillus subtilis YycI reveals a common fold for two members of an unusual class of sensor histidine kinase regulatory proteins. *J Bacteriol* **189**: 3290-3295.

Sardiwal, S., Kendall, S.L., Movahedzadeh, F., Rison, S.C., Stoker, N.G., and Djordjevic, S. (2005) A GAF domain in the hypoxia/NO-inducible *Mycobacterium tuberculosis* DosS protein binds haem. *J Mol Biol* **353**: 929-936.

Schaeffer, P., Millet, J., and Aubert, J.P. (1965) Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci U S A* **54**: 704-711.

Schäffer, C., and Messner, P. (2005) The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. *Microbiology* **151**: 643-651.

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

Schultz, J., Milpetz, F., Bork, P., and Ponting, C.P. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A* **95**: 5857-5864.

Schwartz, E., Gerischer, U., and Friedrich, B. (1998) Transcriptional regulation of *Alcaligenes eutrophus* hydrogenase genes. *J Bacteriol* **180**: 3197-3204.

Shafikhani, S.H., Partovi, A.A., and Leighton, T. (2003) Catabolite-induced repression of sporulation in *Bacillus subtilis*. *Curr Microbiol* **47**: 300-308.

Sherman, D.R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M.I., and Schoolnik, G.K. (2001) Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding α-crystallin. *Proc Natl Acad Sci U S A* **98**: 7534-7539.

Shimoda, N., Toyoda-Yamamoto, A., Aoki, S., and Machida, Y. (1993) Genetic evidence for an interaction between the VirA sensor protein and the ChvE sugar-binding protein of *Agrobacterium*. *J Biol Chem* **268**: 26552-26558.

Silver, L.L. (2003) Novel inhibitors of bacterial cell wall synthesis. *Curr Opin Microbiol* **6**: 431-438.

Silver, L.L. (2006) Does the cell wall of bacteria remain a viable source of targets for novel antibiotics? *Biochem Pharmacol* **71**: 996-1005.

Sleytr, U.B., Huber, C., Ilk, N., Pum, D., Schuster, B., and Egelseer, E.M. (2007) S-layers as a tool kit for nanobiotechnological applications. *FEMS Microbiol Lett* **267**: 131-144.

Smits, W.K., Eschevins, C.C., Susanna, K.A., Bron, S., Kuipers, O.P., and Hamoen, L.W. (2005) Stripping Bacillus: ComK auto-stimulation is responsible for the bistable response in competence development. Vol. 56, pp. 604-614.

Smits, W.K., Kuipers, O.P., and Veening, J.W. (2006) Phenotypic variation in bacteria: the role of feedback regulation. *Nat Rev Microbiol* **4**: 259-271.

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

Stanley, N.R., Britton, R.A., Grossman, A.D., and Lazazzera, B.A. (2003) Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *J Bacteriol* **185**: 1951-1957.

Staron, A., Sofia, H.J., Liesegang, H., and Mascher, T. (2007) A comparative genomics perspective on the ECF σ factor protein family: Classification and functional predictions. In *American Society for Microbiology, 107th General Meeting* Toronto, Canada.

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

Steinmetz, M., and Richter, R. (1994) Easy cloning of mini-Tn10 insertions from the *Bacillus subtilis* chromosome. *J Bacteriol* **176**: 1761-1763.

Stone, K.J., and Strominger, J.L. (1971) Mechanism of action of bacitracin: complexation with metal ion and C 55 -isoprenyl pyrophosphate. *Proc Natl Acad Sci U S A* **68**: 3223-3227.

Storm, D.R., and Strominger, J.L. (1973) Complex formation between bacitracin peptides and isoprenyl pyrophosphates. The specificity of lipid-peptide interactions. *J Biol Chem* **248**: 3940-3945.

Storz, G., and Hengge-Aronis, R. (2000) *Bacterial stress response.* Washington, D.C.: ASM press.

Strauch, M., Webb, V., Spiegelman, G., and Hoch, J.A. (1990) The SpoOA protein of *Bacillus subtilis* is a repressor of the *abrB* gene. *Proc Natl Acad Sci U S A* **87**: 1801-1805.

Strauch, M.A., Spiegelman, G.B., Perego, M., Johnson, W.C., Burbulys, D., and Hoch, J.A. (1989) The transition state transcription regulator *abrB* of *Bacillus subtilis* is a DNA binding protein. *Embo J* **8**: 1615-1621.

Strominger, J.L., and Tipper, D.J. (1965) Bacterial cell wall synthesis and structure in relation to the mechanism of action of penicillins and other antibacterial agents. *Am J Med* **39**: 708-721.

Stülke, J., Martin-Verstraete, I., Zagorec, M., Rose, M., Klier, A., and Rapoport, G. (1997) Induction of the *Bacillus subtilis ptsGHI* operon by glucose is controlled by a novel antiterminator, GlcT. *Mol Microbiol* **25**: 65-78.

Suarez, J.E., and Mendoza, M.C. (1991) Plasmid-encoded fosfomycin resistance. *Antimicrob Agents Chemother* **35**: 791-795.

Sun, J., Zheng, L., Landwehr, C., Yang, J., and Ji, Y. (2005) Identification of a novel essential two-component signal transduction system, YhcSR, in *Staphylococcus aureus*. *J. Bacteriol.* **187**: 7876-7880.

Suzuki, Y., Yoda, T., Ruhul, A., and Sugiura, W. (2001) Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus sp*. OY1-2 isolated from soil. *J. Biol. Chem.* **276**: 9059-9065.

Swem, D.L., Swem, L.R., Setterdahl, A., and Bauer, C.E. (2005) Involvement of SenC in assembly of cytochrome c oxidase in *Rhodobacter capsulatus*. *J. Bacteriol.* **187**: 8081-8087.

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

Szurmant, H., Zhao, H., Mohan, M.A., Hoch, J.A., and Varughese, K.I. (2006) The crystal structure of YycH involved in the regulation of the essential YycFG two-component system in Bacillus subtilis reveals a novel tertiary structure. *Protein Sci* **15**: 929-934.

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

Szurmant, H., T., F., and Hoch, J.A. (2007b) Regulation of the essential YycFG two-component system in *Bacillus subtilis*. In *4th Conference on Functional Genomics of Gram-Positive Microorganisms* Tirrenia, Pisa, Italy.

Talaat, A.M., Howard, S.T., Hale IV, W., Lyons, R., Garner, H., and Johnston, S.A. (2002) Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis*. *Nucl. Acids Res.* **30**: e104-e109.

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

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

Thedieck, K., Hain, T., Mohamed, W., Tindall, B.J., Nimtz, M., Chakraborty, T., Wehland, J., and Jansch, L. (2006) The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. *Mol Microbiol* **62**: 1325-1339.

Thompson, C.J., Fink, D., and Nguyen, L.D. (2002) Principles of microbial alchemy: insights from the *Streptomyces coelicolor* genome sequence. *Genome Biol* **3**: REVIEWS1020.

Trach, K.A., and Hoch, J.A. (1993) Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. *Mol Microbiol* **8**: 69-79.

Tsuda, H., Yamashita, Y., Shibata, Y., Nakano, Y., and Koga, T. (2002) Genes involved in bacitracin resistance in *Streptococcus mutans*. *Antimicrob. Agents Chemother.* **46**: 3756-3764.

Turgay, K., and Marahiel, M.A. (1995) The *gtcRS* operon coding for two-component system regulatory proteins is located adjacent to the *grs* operon of *Bacillus brevis*. *DNA Seq* **5**: 283-290.

Vagner, V., Dervyn, E., and Ehrlich, S.D. (1998) A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144 (Pt 11)**: 3097-3104.

van Sinderen, D., Luttinger, A., Kong, L., Dubnau, D., Venema, G., and Hamoen, L. (1995) *comK* encodes the competence transcription factor, the key regulatory protein for competence development in *Bacillus subtilis*. *Mol Microbiol* **15**: 455-462.

Veening, J.-W., Hamoen, L.W., and Kuipers, O.P. (2005) Phosphatases modulate the bistable sporulation gene expression pattern in Bacillus subtilis. Vol. 56, pp. 1481-1494.

Veiga, P., Bulbarela-Sampieri, C., Furlan, S., Maisons, A., Chapot-Chartier, M.P., Erkelenz, M., Mervelet, P., Noirot, P., Frees, D., Kuipers, O.P., Kok, J., Gruss, A., Buist, G., and Kulakauskas, S. (2007) SpxB regulates *O*-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. *J Biol Chem* **282**: 19342-19354.

Vellanoweth, R.L., and Rabinowitz, J.C. (1992) The influence of ribosome-binding-site elements on translational efficiency in *Bacillus subtilis* and *Escherichia coli in vivo*. *Mol Microbiol* **6**: 1105-1114.

Vicente, M., Hodgson, J., Massidda, O., Tonjum, T., Henriques-Normark, B., and Ron, E.Z. (2006) The fallacies of hope: will we discover new antibiotics to combat pathogenic bacteria in time? *FEMS Microbiol Rev* **30**: 841-852.

Vollmer, W., and Höltje, J.V. (2004) The architecture of the murein (peptidoglycan) in gram-negative bacteria: vertical scaffold or horizontal layer(s)? *J Bacteriol* **186**: 5978-5987.

Voskuil, M.I., Schnappinger, D., Visconti, K.C., Harrell, M.I., Dolganov, G.M., Sherman, D.R., and Schoolnik, G.K. (2003) Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med* **198**: 705-713.

Vrancken, K., De Keersmaeker, S., Geukens, N., Lammertyn, E., Anne, J., and Van Mellaert, L. (2007) *pspA* overexpression in *Streptomyces lividans* improves both Sec- and Tat-dependent protein secretion. *Appl Microbiol Biotechnol* **73**: 1150-1157.

Wach, A. (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**: 259-265.

Walker, S., Chen, L., Hu, Y., Rew, Y., Shin, D., and Boger, D.L. (2005) Chemistry and Biology of Ramoplanin: A Lipoglycodepsipeptide with Potent Antibiotic Activity. *Chem Rev* **105**: 449-476.

Walsh, C. (2003) *Antibiotics - actions, origins, resistance.* Washington, D.C.: ASM press.

Walsh, C.T., Fisher, S.L., Park, I.S., Prahalad, M., and Wu, Z. (1996) Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. *Chem Biol* **3**: 21-28.

Walsh, T.R., and Howe, R.A. (2002) The prevalence and mechanisms of vancomycin resistance in Staphylococcus aureus. *Annu Rev Microbiol* **56**: 657-675.

Wang, L., Grau, R., Perego, M., and Hoch, J.A. (1997) A novel histidine kinase inhibitor regulating development in *Bacillus subtilis*. *Genes Dev.* **11**: 2569-2579.

Wecke, T., Veith, B., Ehrenreich, A., and Mascher, T. (2006) Cell envelope stress response in *Bacillus licheniformis*: Integrating comparative genomics, transcriptional profiling, and regulon mining to decipher a complex regulatory network. *J. Bacteriol.* **188**: 7500-7511.

Weidenmaier, C., Kokai-Kun, J.F., Kristian, S.A., Chanturiya, T., Kalbacher, H., Gross, M., Nicholson, G., Neumeister, B., Mond, J.J., and Peschel, A. (2004) Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat Med* **10**: 243-245.

Weidenmaier, C., Peschel, A., Xiong, Y.Q., Kristian, S.A., Dietz, K., Yeaman, M.R., and Bayer, A.S. (2005) Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. *J Infect Dis* **191**: 1771-1777.

Weiner, L., and Model, P. (1994) Role of an *Escherichia coli* stress-response operon in stationary-phase survival. *Proc Natl Acad Sci U S A* **91**: 2191-2195.

Weston, L.A., and Kadner, R.J. (1988) Role of *uhp* genes in expression of the *Escherichia coli* sugar-phosphate transport system. *J Bacteriol* **170**: 3375-3383.

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

Wilke, M.S., Lovering, A.L., and Strynadka, N.C. (2005) β-lactam antibiotic resistance: a current structural perspective. *Curr Opin Microbiol* **8**: 525-533.

Williams, T., Bauer, S., Beier, D., and Kuhn, M. (2005) Construction and characterization of *Listeria monocytogenes* mutants with in-frame deletions in the response regulator genes identified in the genome sequence. *Infect. Immun.* **73**: 3152-3159.

Xu, K., and Strauch, M.A. (1996) In vitro selection of optimal AbrB-binding sites: comparison to known in vivo sites indicates flexibility in AbrB binding and recognition of three-dimensional DNA structures. *Mol Microbiol* **19**: 145-158.

Yin, S., Daum, R.S., and Boyle-Vavra, S. (2006) VraSR two-component regulatory system and its role in induction of *pbp2* and *vraSR* expression by cell wall antimicrobials in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **50**: 336-343.

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

Youngman, P. (1990) Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus subtilis*. In *Molecular biological methods for*

Bacillus. Harwood, C.R. and Cutting, S.M. (eds). Chichester: John Wiley & sons, pp. 221-266.

Zellmeier, S., Schumann, W., and Wiegert, T. (2006) Involvement of Clp protease activity in modulating the *Bacillus subtilis* σ^W stress response. *Molecular Microbiology* **61**: 1569-1582.

Zhang, X., Dong, G., and Golden, S.S. (2006) The pseudo-receiver domain of CikA regulates the cyanobacterial circadian input pathway. *Mol Microbiol* **60**: 658-668.

Chapter 6: Supplementary material

Table 6.1. *Bacillus subtilis* strains

Strain	Genotype/Remarks	Reference
W168	laboratory wildtype strain	laboratory strain
CU1065	W168 *att*SPb2Δ2 *trpC2*	Helmann lab stock
HB0920	CU1065 *liaH*::kan^R	Mascher et al. 2003
HB0933	CU1065 *liaR*::kan^R	Mascher et al. 2003
HB0934	CU1065 *liaGFSR:*:kan^R	Mascher, unpublished
HB0935	CU1065 *liaIH*::tet(i)^R	Mascher et al. 2003
HB0938	CU1065 *yhcYZ*::cat^R	Mascher, unpublished
HB0950	CU1065 *att*SPb2Δ2::Tn*917*::F($P_{liaI-74}$-*cat-lacZ*)	Mascher et al. 2004
HB0961	CU1065 *liaI*::pMUTIN	Mascher et al. 2003
HB0969	CU1065 *yhcY*::pMUTIN, *liaH*::kan^R	Mascher, unpublished
HB0970	CU1065 *yhcZ*::pMUTIN, *liaH*::kan^R	Mascher, unpublished
TMB001	CU1065 *liaSR*::kan	unpublished
TMB002	CU1065 *liaF*::kan	Jordan et al. 2006
TMB003	CU1065 *liaG*::kan	Jordan et al. 2006
TMB004	CU1065 *liaS*::kan	Jordan et al. 2006
TMB005	CU1065 *liaGF*::kan	unpublished
TMB006	CU1065 *liaFS*::kan	Jordan et al. 2006
TMB007	CU1065 *liaGFS*::kan	unpublished
TMB008	CU1065 *liaI:*::pMUTIN, *liaG*::kan	unpublished
TMB009	CU1065 *liaI:*::pMUTIN, *liaF*::kan	unpublished
TMB010	CU1065 *liaI:*::pMUTIN, *liaS*::kan	unpublished
TMB011	CU1065 *liaI:*::pMUTIN, *liaR*::kan	Jordan et al., 2007
TMB012	CU1065 *liaI:*::pMUTIN, *liaGF*::kan	unpublished
TMB013	CU1065 *liaI:*::pMUTIN, *liaFS*::kan	unpublished
TMB014	CU1065 *liaI:*::pMUTIN, *liaSR*::kan	unpublished
TMB015	CU1065 *liaI:*::pMUTIN, *liaGFS*::kan	unpublished
TMB016	CU1065 *amyE:*::pTM1	Jordan et al. 2006
TMB017	CU1065 *amyE:*::pTM1, *liaG*::kan	Jordan et al. 2006
TMB018	CU1065 *amyE:*::pTM1, *liaF*::kan	Jordan et al. 2006
TMB019	CU1065 *amyE:*::pTM1, *liaS*::kan	Jordan et al. 2006
TMB020	CU1065 *amyE:*::pTM1, *liaR*::kan	Jordan et al. 2006
TMB021	CU1065 *amyE:*::pTM1, *liaGF*::kan	Jordan et al. 2006
TMB022	CU1065 *amyE:*::pTM1, *liaFS*::kan	unpublished
TMB023	CU1065 *amyE:*::pTM1, *liaGFS*::kan	unpublished
TMB024	CU1065 *liaS*::kan,*thrC*::pXT*liaS*	unpublished
TMB025	CU1065 *liaF*::kan,*thrC*::pXT*liaF*1	unpublished
TMB026	CU1065 *liaF*::kan,*thrC*::pXT*liaF*3	unpublished
TMB027	HB0950 *liaF*Δ(I_{151}-D_{235})[a]	Jordan et al. 2006
TMB028	HB0950 *liaF*Δ(E_{126}-D_{146})[a]	Jordan et al. 2006
TMB029	HB0950 *liaF*Δ(S_{189}-V_{192})[a]	Jordan et al. 2006
TMB032	CU1065 *liaSR*::kan, *bceRS*::cat, *yvcPQ*::mls	unpublished
TMB040	CU1065 *yvcB*::pMUTIN	unpublished
TMB041	CU1065 *yvkN*::pMUTIN	unpublished
TMB042	CU1065 *ywkC*::pMUTIN	unpublished
TMB043	CU1065 *yvrL*::pMUTIN	unpublished
TMB044	CU1065 *yvrH*::pMUTIN	unpublished
TMB054	W168 *amyE*::pSJ602	unpublished
TMB055	CU1065 *liaI*::pMUTIN, *yxjM*::spec	unpublished
TMB056	CU1065 *liaI*::pMUTIN, *yxjML*::spec	unpublished
TMB057	CU1065 *liaI*::pMUTIN, *liaS*::kan, *yxjM*::spec	unpublished
TMB058	CU1065 *liaI*::pMUTIN, *liaS*::kan, *yxjML*::spec	unpublished

Strain	Genotype/Remarks	Reference
TMB059	CU1065 *liaI*::pMUTIN, *liaFS*::kan, *yxjM*::spec	unpublished
TMB060	CU1065 *liaI*::pMUTIN, *liaFS*::kan, *yxjML*::spec	unpublished
TMB061	CU1065 *liaI:*::pMUTIN, *liaFS*::kan, *yhcYZ*::cat	unpublished
TMB062	CU1065 *liaI*::pMUTIN, l*iaFS*::kan, *yxjML*::spec, *yhcYZ*::cat	unpublished
TMB063	W168 *amyE*::pAJ603, *yhcZ*::MLS	Junker, diploma thesis
TMB064	W168 amyE::pAJ603, *yhcZ*::MLS, *liaR*::kan	Junker, diploma thesis
TMB065	W168 amyE::pAJ603, *liaHGF*::kan	Junker, diploma thesis
TMB066	W168 amyE::pAJ603, *liaR*::spec	Jordan *et al.* 2006
TMB067	W168 amyE::pAJ603, *liaF*::kan, *liaIH*::tet (i)	Junker, diploma thesis
TMB068	W168 amyE::pAJ603, *liaHGF*::kan, *yhcZ*::MLS	Junker, diploma thesis
TMB069	W168 amyE::pAJ603, *liaHGF*::kan, *liaR*::spec	Jordan *et al.* 2006
TMB070	W168 amyE::pAJ603, *liaH*::kan, *yhcZ*::MLS	Junker, diploma thesis
TMB071	W168 amyE::pAJ603	Jordan *et al.* 2006
TMB072	W168 amyE::pAJ603, *liaH*::kan	Jordan *et al.* 2006
TMB073	W168 *amyE*::pSJ603	unpublished
TMB074	W168 *amyE*::pSJ603, *liaF::kan*	unpublished
TMB075	W168 *amyE*::pSJ604	unpublished
TMB076	W168 *amyE*::pSJ604, *liaF::kan*	unpublished
TMB077	W168 *amyE*::pSJ605	unpublished
TMB078	W168 *amyE*::pSJ605, *liaIH::tet*	unpublished
TMB079	W168 *sinR*::spec	Jordan *et al.*, 2007
TMB080	W168 *aprE*::kan	Jordan *et al.*, 2007
TMB081	W168 *scoC*::tet	Jordan *et al.*, 2007
TMB082	W168 *abrB*::kan	Jordan *et al.*, 2007
TMB083	W168 *salA*::tet	Jordan *et al.*, 2007
TMB084	HB0961 *sinR*::spec	Jordan *et al.*, 2007
TMB085	HB0961 *aprE*::kan	Jordan *et al.*, 2007
TMB086	HB0961 *scoC*::tet	Jordan *et al.*, 2007
TMB087	HB0961 *abrB*::kan	Jordan *et al.*, 2007
TMB088	HB0961 *salA*::tet	Jordan *et al.*, 2007
TMB089	W168 *amyE*::pER601	Rietkötter, GP II
TMB090	W168 *aprE*::pER201	Rietkötter, GP II
TMB091	W168 *liaHGF*::kan	Junker, diploma thesis
TMB092	W168 *liaR*::spec	Junker, diploma thesis
TMB093	W168 *yhcZ*::MLS	Junker, diploma thesis
TMB094	W168 *amyE*::pAJ603, *liaGF*::kan	Junker, diploma thesis
TMB095	W168 *amyE*::pAJ603, *liaF*::kan	Jordan *et al.* 2006
TMB096	W168 *amyE*::pAJ601, *liaHGF*::kan	Jordan *et al.* 2006
TMB097	W168 *amyE*::pAJ602, *liaHGF*::kan	Jordan *et al.* 2006
TMB098	W168 *amyE*::pAJ604, *liaHGF*::kan	Jordan *et al.* 2006
TMB099	W168 *amyE*::pAJ609, *liaHGF*::kan	Jordan *et al.* 2006
TMB100	W168 *amyE*::pAJ608, *liaHGF*::kan	Jordan *et al.* 2006
TMB101	W168 *amyE*::pAJ607, *liaHGF*::kan	Jordan *et al.* 2006
TMB102	W168 *amyE*::pAJ603, *liaHGF*::kan	Jordan *et al.* 2006
TMB103	W168 *amyE*::pAJ605, *liaHGF*::kan	Junker, diploma thesis
TMB104	W168 *amyE*::pAJ606, *liaHGF*::kan	Jordan *et al.* 2006
TMB105	W168 *amyE*::pAJ610, *liaHGF*::kan	Junker, diploma thesis
TMB108	CU1065 *amyE:*::pTM1, *liaH*::kan	Jordan *et al.* 2006
TMB109	CU1065 *amyE:*::pTM1, *liaHGF*::kan	unpublished
TMB110	W168 *amyE*::pBD607 ($A_{-77}T$)	Dörrbecker, GP II
TMB111	W168 *amyE*::pBD601	Jordan *et al.* 2006
TMB112	W168 *amyE*::pBD602	Jordan *et al.* 2006
TMB113	W168 *amyE*::pBD603	Jordan *et al.* 2006
TMB114	W168 *amyE*::pBD604	Jordan *et al.* 2006

Strain	Genotype/Remarks	Reference
TMB115	W168 *amyE*::pBD605	Jordan *et al.* 2006
TMB116	W168 *amyE*::pER602	Rietkötter, GP II
TMB117	HB0961 *degU*::kan	Jordan *et al.*, 2007
TMB118	HB0961 *spo0A*::tet	Jordan *et al.*, 2007
TMB119	TMB090 *spo0A*::tet	Rietkötter, GP II
TMB120	TMB090 *sinR*::spec	Rietkötter, GP II
TMB121	TMB090 *abrB*::kan	Rietkötter, GP II
TMB122	TMB089 *scoC*::tet	Rietkötter, GP II
TMB123	TMB089 *abrB::kan*	Rietkötter, GP II
TMB124	W168 *degU*::kan	Jordan *et al.*, 2007
TMB125	TMB16 *liaIH*::tet(i)	unpublished
TMB126	TMB116 *liaIH*::tet(i)	unpublished
TMB127	TMB116 *liaH*::kan	unpublished
TMB128	W168 *amyE*::pDH601	Hoyer, GP II
TMB129	W168 *amyE*::pDH602	Hoyer, GP II
TMB130	W168 *amyE*::pDH603	Hoyer, GP II
TMB131	W168 *amyE*::pDH604	Hoyer, GP II
TMB132	W168 *amyE*::pDH605	Jordan *et al.* 2006
TMB133	W168 amyE::pBD606	Jordan *et al.* 2006
TMB159	W168 *amyE*::pAJ601, *liaH*::kan	Junker, diploma thesis
TMB160	W168 *amyE*::pAJ602, *liaH*::kan	Junker, diploma thesis
TMB161	W168 *amyE*::pAJ604, *liaH*::kan	Junker, diploma thesis
TMB162	W168 *amyE*::pAJ605, *liaH*::kan	Junker, diploma thesis
TMB163	HB0961 *abh*::kan	unpublished
TMB164	W168 *amyE*::pAJ601	Junker, diploma thesis
TMB165	W168 *amyE*::pAJ602	Junker, diploma thesis
TMB166	W168 *amyE*::pAJ604	Junker, diploma thesis
TMB167	W168 *amyE*::pAJ603	Junker, diploma thesis
TMB168	W168 *amyE*::pAJ605	Junker, diploma thesis
TMB169	W168 *liaH*::kan	Junker, diploma thesis
TMB170	W168 *amyE*::pSJ608	unpublished
TMB171	W168 *amyE*::pSJ610	unpublished
TMB172	MD300 (*degU*-hy) small colony *liaI*::pMUTIN	unpublished
TMB173	MD300 (*degU*-hy) large colony *liaI*::pMUTIN	unpublished
TMB174	W168 *ackA*::mls	unpublished
TMB175	W168 *amyE*::pAJ611	Junker, diploma thesis
TMB176	W168 *amyE*::pAJ610	Junker, diploma thesis
TMB177	W168 *amyE*::pAJ609	Junker, diploma thesis
TMB178	W168 *amyE*::pAJ608	Junker, diploma thesis
TMB179	W168 *amyE*::pAJ607	Junker, diploma thesis
TMB180	W168 *amyE*::pAJ606	Junker, diploma thesis
TMB181	W168 *liaSR* clean deletion	unpublished
TMB183	TMB028 *thrC*::pSJ701	Jordan *et al.* 2006
TMB184	TMB029 *thrC*::pSJ701	Jordan *et al.* 2006
TMB185	TMB009 *amyE*::pSJ901	unpublished
TMB186	W168 *pta*::tet	unpublished
TMB187	TMB016 *pta*::tet	unpublished
TMB188	TMB019 *pta*::tet	unpublished
TMB189	TMB022 *pta*::tet	unpublished
TMB190	TMB016 *ackA*::mls	unpublished
TMB191	TMB019 *ackA*::mls	unpublished
TMB192	TMB022 *ackA:*::mls	unpublished
TMB193	W168 *pta*::tet, *ackA*::mls	unpublished
TMB194	TMB016 *pta*::tet, *ackA*::mls	unpublished

Strain	Genotype/Remarks	Reference
TMB195	TMB019 *pta*::tet, *ackA*::mls	unpublished
TMB196	TMB022 *pta*::tet, *ackA*::mls	unpublished
TMB197	TMB170 *liaF*::kan	unpublished
TMB198	TMB170 *yhcZ*::mls	unpublished
TMB199	HB0961 *liaFS*::kan, *pta*::tet	unpublished
TMB200	W168 *pta*::tet, *liaS*::kan	unpublished
TMB201	W168 *pta*::tet, *liaFS*::kan	unpublished
TMB202	W168 *ackA*::mls, *liaS*::kan	unpublished
TMB203	W168 *ackA*::mls, *liaFS*::kan	unpublished
TMB204	TMB181 *amyE*::pTM1	unpublished
TMB205	W168 *spo0A*::tet	Jordan *et al*., 2007
TMB206	TMB204 *thrC*::pSJ702	unpublished
TMB208	W168 *abrB*::kan, *spo0A*::tet	unpublished
TMB209	W168 *liaI*::pMUTIN, *abrB*::kan, *spo0A*::tet	Jordan *et al*., 2007
TMB210	W168 *liaR*::spec, *abrB*::kan	unpublished
TMB212	W168 *liaR*::spec, *abrB*::kan, *liaI*::pMUTIN	unpublished
TMB213	W168 *liaS* clean deletion	Jordan *et al*., 2007
TMB214	W168 *liaFS* clean deletion	Martinec, GP II
TMB215	W168 *liaS* clean deletion, *liaI*::pMUTIN	Jordan *et al*., 2007
TMB216	W168 *liaS* clean deletion, *amyE*::pTM1	Martinec, GP II
TMB217	W168 *liaFS* clean deletion, *liaI*::pMUTIN	Martinec, GP II
TMB218	W168 *liaFS* clean deletion, *amyE*::pTM1	Martinec, GP II
TMB219	W168 *amyE*::pSK601(wapA-promoter)	unpublished
TMB220	TMB001 *amyE*::pSK601	unpublished
TMB221	TMB002 *amyE*::pSK601	unpublished
TMB222	W168 *amyE*::pSK602(*ydhE*-promoter)	unpublished
TMB223	TMB001 *amyE*::pSK602	unpublished
TMB224	TMB002 *amyE*::pSK602	unpublished
TMB229	W168 *liaS* clean deletion, *liaI*::pMUTIN, *thrC*::pSJ702	unpublished
TMB230	W168 *liaS* clean deletion, *amyE*::pTM1, *thrC*::pSJ702	unpublished
TMB231	W168 *liaI*::pMUTIN, *liaS*::kan, LiaR $D_{54}A$	Schrecke, GP II
TMB232	W168 *amyE*::pTM1, *liaS*::kan, LiaR $D_{54}A$	Schrecke, GP II
TMB233	W168 *liaS* clean deletion, *liaI*::pMUTIN, *thrC*::pXT-*liaS*	Schrecke, GP II
TMB234	W168 *liaS* clean deletion, *liaI*::pMUTIN, *thrC*::pXT-*liaS* (SD)	Schrecke, GP II
TMB235	W168 *liaS* clean deletion, *amyE*::pTM1, *thrC*::pXT-*liaS*	Schrecke, GP II
TMB236	W168 *liaS* clean deletion, *amyE*::pTM1, *thrC*::pXT-*liaS* (SD)	Schrecke, GP II
TMB237	W168 *liaFS* clean del., *liaI*::pMUTIN, *thrC*::pXT-*liaFS*	Schrecke, GP II
TMB238	W168 *liaFS* clean del., *liaI*::pMUTIN, *thrC*::pXT-*liaFS* (SD)	Schrecke, GP II
TMB239	W168 *amyE*::pSJ611-*lacZ*	unpublished
TMB240	W168 *amyE*::pSJ612-*lacZ*	unpublished
TMB243	HB0961 *liaHGF*::kan	unpublished
TMB244	HB0961 *liaGFSR*::kan	unpublished
TMB245	TMB16 *liaHGF*::kan	unpublished
TMB246	TMB16 *liaGFSR*::kan	unpublished
TMB247	W168 *liaS*::kan, LiaR($D_{54}A$)	Schrecke, GP II
TMB248	TMB215 (W168 *liaS* clean deletion, *liaI*::pMUTIN) *thrC*::pXT	Schrecke, GP II
TMB249	TMB216 (W168 *liaS* clean deletion, *amyE*::pTM1), *thrC*::pXT	Schrecke, GP II
TMB250	TMB217 (W168 *liaFS* clean deletion, *liaI*::pMUTIN), *thrC*::pXT	Schrecke, GP II
TMB251	TMB218 (W168 liaFS clean deletion, amyE::pTM1), *thrC*::pXT	Schrecke, GP II
TMB252	TMB010 (HB0961 *liaS*::kan) *pta*::tet	Schrecke, GP II
TMB254	TMB027 *thrC*::pSJ704	unpublished
TMB255	TMB027 *thrC*::pSJ705	unpublished
TMB256	TMB027 *thrC*::pSJ706	unpublished
TMB257	TMB027 *thrC*::pSJ709	unpublished

Strain	Genotype/Remarks	Reference
TMB258	TMB027 *thrC*::pSJ710	unpublished
TMB259	TMB027 *thrC*::pSJ711	unpublished
TMB260	TMB027 *thrC*::pSJ712	unpublished
TMB261	TMB027 *thrC*::pSJ714	unpublished
TMB262	TMB027 *thrC*::pSJ715	unpublished
TMB263	W168 *thrC*::pXT	unpublished
TMB264	W168 *thrC*::pSJ703	unpublished
TMB265	W168 *thrC*::pSJ707	unpublished
TMB266	W168 *thrC*::pSJ708	unpublished
TMB267	W168 *thrC*::pSJ713	unpublished
TMB268	TMB018 (*liaF*::kan, *amyE*::pTM1) *thrC*::pSJ701	unpublished
TMB269	W168 *thrC*::pSJ706	unpublished
TMB272	W168 *amyE*::P_{ydhE2}-*lacZ*	unpublished
TMB273	W168 *amyE*::P_{ydhE3}-*lacZ*	unpublished
TMB274	W168 *amyE*::P_{ydhE2}-*lacZ* , *liaF*::kan	unpublished
TMB275	W168 *amyE*::P_{ydhE3}-*lacZ* , *liaF*::kan	unpublished
TMB276	W168 *amyE*::pER501 (P_{liaG} nativ to ATG of *liaF*)	Rietkötter, unpublished
TMB277	W168 *amyE*::pER502 (P_{liaG} optimal -10 to ATG of *liaF*)	Rietkötter, unpublished
TMB278	W168 *amyE*::pER503 (P_{liaG} optimal -10 und -35 bis ATG von *liaF*)	Rietkötter, unpublished
TMB279	W168 *amyE*::pER603 (P_{bceA}-*lacZ*)	Rietkötter, unpublished
TMB280	TMB035 (*bceAB*::kan) *amyE*::pER603 (P_{bceA}-*lacZ*)	Rietkötter, unpublished
TMB281	W168 *amyE*::pER504 (P_{liaG} optimal -10 and -35 to ATG of LiaS)	Rietkötter, unpublished
TMB282	W168 *amyE*::pER505 (P_{liaG} optimal -10 and -35 to ATG of LiaR)	Rietkötter, unpublished
TMB283	TMB216 *thrC*::pSJ722	unpublished
TMB284	TMB216 *thrC*::pSJ723	unpublished
TMB285	TMB216 *thrC*::pSJ725	unpublished
TMB286	TMB216 *thrC*::pSJ726	unpublished
TMB304	TMB216 *thrC*::pSJ721	unpublished
TMB305	TMB215 *thrC*::pSJ719	unpublished
TMB306	TMB215 *thrC*::pSJ720	unpublished
TMB307	TMB215 *thrC*::pSJ721	unpublished
TMB308	TMB215 *thrC*::pSJ722	unpublished
TMB309	TMB215 *thrC*::pSJ723	unpublished
TMB310	TMB215 *thrC*::pSJ724	unpublished
TMB311	TMB215 *thrC*::pSJ725	unpublished
TMB312	TMB215 *thrC*::pSJ726	unpublished
TMB313	TMB215 *thrC*::pSJ727	unpublished
TMB314	W168 *thrC*::pSJ716	unpublished
TMB315	W168 *thrC*::pSJ717	unpublished
TMB318	W168 *amyE*::pSJ4001	unpublished
TMB319	W168 *amyE*::pSJ4002	unpublished
TMB320	W168 *amyE*::pSJ4003	unpublished
TMB321	W168 *amyE*::pSJ5402	unpublished
TMB322	W168 *amyE*::pSJ5401	unpublished
TMB323	W168 *amyE*::pSJ5404	unpublished
TMB324	W168 *amyE*::pSJ5403 (LiaF $A_{158}V$)	unpublished
TMB329	W168 *liaF* clean deletion	unpublished
TMB330	TMB215 *abrB*::kan	Jordan *et al*., 2007
TMB331	TMB329 *amyE*::pTM1	unpublished
TMB332	TMB331 *thrC*::pXT	unpublished
TMB333	TMB331 *thrC*::pSJ701	unpublished
TMB334	TMB331 *thrC*::pSJ704	unpublished
TMB335	TMB331 *thrC*::pSJ705	unpublished
TMB336	TMB331 *thrC*::pSJ706	unpublished

Strain	Genotype/Remarks	Reference
TMB337	TMB331 *thrC*::pSJ709	unpublished
TMB338	TMB331 *thrC*::pSJ710	unpublished
TMB339	TMB331 *thrC*::pSJ711	unpublished
TMB340	TMB331 *thrC*::pSJ712	unpublished
TMB341	TMB331 *thrC*::pSJ714	unpublished
TMB342	TMB331 *thrC*::pSJ715	unpublished
TMB343	TMB331 *thrC*::pSJ703	unpublished
TMB344	TMB331 *thrC*::pSJ707	unpublished
TMB345	TMB331 *thrC*::pSJ708	unpublished
TMB346	TMB331 *thrC*::pSJ713	unpublished
TMB347	TMB331 *thrC*::pSJ716	unpublished
TMB348	TMB331 *thrC*::pSJ717	unpublished
TMB349	TMB331 *thrC*::pSJ718	unpublished
TMB350	TMB329 *liaI*::pMUTIN	unpublished
TMB351	TMB350 *thrC*::pXT	unpublished
TMB352	TMB350 *thrC*::pSJ701	unpublished
TMB353	TMB350 *thrC*::pSJ704	unpublished
TMB354	TMB350 *thrC*::pSJ705	unpublished
TMB355	TMB350 *thrC*::pSJ706	unpublished
TMB356	TMB350 *thrC*::pSJ709	unpublished
TMB357	TMB350 *thrC*::pSJ710	unpublished
TMB358	TMB350 *thrC*::pSJ711	unpublished
TMB359	TMB350 *thrC*::pSJ712	unpublished
TMB360	TMB350 *thrC*::pSJ714	unpublished
TMB361	TMB350 *thrC*::pSJ715	unpublished
TMB362	TMB350 *thrC*::pSJ703	unpublished
TMB363	TMB350 *thrC*::pSJ707	unpublished
TMB364	TMB350 *thrC*::pSJ708	unpublished
TMB365	TMB350 *thrC*::pSJ713	unpublished
TMB366	TMB350 *thrC*::pSJ716	unpublished
TMB367	TMB350 *thrC*::pSJ717	unpublished
TMB368	TMB350 *thrC*::pSJ718	unpublished
TMB369	W168 *liaH*::pSJ6401	unpublished
TMB370	W168 *liaI*::pMUTIN	unpublished
TMB396	W168 *amyE*::pSJ2901	unpublished
TMB397	W168 *amyE*::pSJ2902	unpublished
TMB398	W168 *amyE*::pSJ2903	unpublished
TMB399	W168 *amyE*::pSJ2904	unpublished
TMB402	TMB350 *amyE*::pSJ5403	unpublished
TMB403	TMB215 *amyE*::pSJ5404	unpublished
TMB404	TMB350 *amyE*::pSJ2902	unpublished
TMB405	TMB215 *amyE*::pSJ2903	unpublished
TMB406	TMB370 *amyE*::pSJ5404	unpublished
TMB407	TMB011 *amyE*::pSJ2904-*liaR*	unpublished
TMB408	W168 P_{liaI}::pSJ5101	unpublished

resistance cassettes: kan: kanamycin, cat: chloramphenicol, mls: erythromycin/lincomycin, spec: spectinomycin, tet =tetracyclin. Positions of cloned fragments are given relative to the start codon.
[a] For reasons of clarity, the effects of in-frame deletions in *liaF* are given at the level of LiaF protein.

Table 6.2. *Escherichia coli* strains

Strain	Genotype/Remarks	Reference
DH5α	*recA1 endA1gyrA96 thi hsdR17rK-mK+ relA1supE44* Φ*80*Δ*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169*	Sambrook and Russel, 2001
71/18	*supE thi* Δ(*lac-proAB*) F´[ProAB$^+$ *lacI*^{q} *lacZ* ΔM15]	Sambrook and Russel, 2001

Strain	Genotype/Remarks	Reference
CC118	Δ(*ara-leu*)7697 Δ*lacX*74 Δ*phoA*20 *galE galK thi rpsE rpoB argE*(am) *recA1*	Manoil and Beckwith, 1985
TME011	CC118 with pSJ401	unpublished
TME012	CC118 with pSJ402	unpublished
TME013	CC118 with pSJ403	unpublished
TME014	CC118 with pSJ404	unpublished
TME015	CC118 with pSJ405	unpublished
TME016	CC118 with pSJ406	unpublished
TME017	CC118 with pSJ407	unpublished
TME018	CC118 with pSJ408	unpublished
TME019	CC118 with pSJ409	unpublished

Table 6.3. Vectors and plasmids

Vector	Description	Reference
pAC6	*bla*, *lacZ*, *cat; lacZ* fusion vector, integrates at *amyE*,	Stülke *et al*., 1997
pHA-4	*araC*, *bla*, *phoA*, pNG backbone; C-term. PhoA fusion for membrane topology analysis in *E. coli*	Rapp *et al*. 2004, Daley *et al*. 2005
pET-16b	PT7, His(10)-tag, tT7, *lacI*, *ori*(pBR322), *bla;* protein overexpression vector	pET system manual, 10th ed.
pGFPe	kan, GFP, His-tag, pET128 backbone	Rapp *et al*. 2004, Daley *et al*. 2005
pJPM122	MCS, *cat-lacZ*, *bla*, neo, ori; integrates at *attSPb*	Slack *et al*. 1993
pMAD	*erm*, ori(pE194-Ts), MCS-P_{clpB}-*bgaB*, ori(pBR322), *bla*	Arnaud *et al*. 2004
pSG1151	*bla cat gfpmut1*	Lewis and Marston, 1999
pSG1154	*bla amyE3´spc* P_{xyl}-*´gfpmut1 amyE*5´; C-terminal fusion vector	Lewis and Marston, 1999
pSG1164	*bla cat* P_{xyl}-*gfpmut1*	Lewis and Marston, 1999
pSG1729	*bla amyE3´spc* P_{xyl}-*´gfpmut1 amyE*5´; N-terminal fusion vector	Lewis and Marston, 1999
pSH4	pSG1154 with mutation in *gfp*	Halbedel, unpublished
pSH5	pSG1164 with mutation in *gfp*	Halbedel, unpublished
pXT	vector for xylose-inducible gene expression, integrates at thrC, pDG1782-derivative, spectinomycin resistance	Derre *et al*., 2000

Plasmid	Description	Reference
pAJ601	$P_{yhcY\text{-}29}$ *Eco*RI/*Bam*HI (primer 165/172) into pAC6	Jordan *et al*., 2006
pAJ602	$P_{yhcY\text{-}46}$ *Eco*RI/*Bam*HI (primer 165/171) into pAC6	Jordan *et al*., 2006
pAJ603	$P_{yhcY\text{-}87}$ *Eco*RI/*Bam*HI (primer 165/168) into pAC6	Jordan *et al*., 2006
pAJ604	$P_{yhcY\text{-}55}$ *Eco*RI/*Bam*HI (primer 165/170) into pAC6	Jordan *et al*., 2006
pAJ605	$P_{yhcY\text{-}99}$ *Eco*RI/*Bam*HI (primer 165/167) into pAC6	Jordan *et al*., 2006
pAJ606	$P_{yhcY\text{-}202}$ *Eco*RI/*Bam*HI (primer 165/166) into pAC6	Jordan *et al*., 2006
pAJ607	$P_{yhcY\text{-}80}$ *Eco*RI/*Bam*HI (primer 165/169) into pAC6	Jordan *et al*., 2006
pAJ608	$P_{yhcY\text{-}75}$ *Eco*RI/*Bam*HI (primer 165/259) into pAC6	Jordan *et al*., 2006
pAJ609	$P_{yhcY\text{-}65}$ *Eco*RI/*Bam*HI (primer 165/260) into pAC6	Jordan *et al*., 2006
pBD601	*liaI* (-102 - 72) *Xho*I/*Bam*HI (primer 231/100) into pAC6	Jordan *et al*., 2006
pBD602	*liaI* (-102 - 72) *Xho*I/*Bam*HI (primer 232/100) into pAC6	Jordan *et al*., 2006
pBD603	*liaI* (-102 - 72) *Xho*I/*Bam*HI (primer 265/100) into pAC6	Jordan *et al*., 2006
pBD604	*liaI* (-102 - 72) *Xho*I/*Bam*HI (primer 266/100) into pAC6	Jordan *et al*., 2006
pBD605	*liaI* (-102 - 72) *Xho*I/*Bam*HI (primer 267/100) into pAC6	Jordan *et al*., 2006
pBD606	*liaI* (-102 - 72) *Xho*I/*Bam*HI (primer 268/100) into pAC6	Jordan *et al*., 2006
pDH601	*liaF* (-21 - -157) *Eco*RI/*Bam*HI (primer 204/218) into pAC6	Hoyer, GP II
pDH602	*liaF* (-21 - -250) *Eco*RI/*Bam*HI (primer 204/219) into pAC6	Hoyer, GP II
pDH603	*liaF* (-21 - -336) *Eco*RI/*Bam*HI (primer 204/220) into pAC6	Hoyer, GP II
pDH604	*liaF* (-21 - -546) *Eco*RI/*Bam*HI (primer 204/221) into pAC6	Hoyer, GP II
pDH605	*liaF* (-21 - -824) *Eco*RI/*Bam*HI (primer 204/222) into pAC6	Jordan *et al*., 2006
pKS701	*liaS* (-24 - end) *Bam*HI/*Hin*dIII (primer 454/46) into pXT	Schrecke, GP II
pKS702	*liaS* (-24 - end) *Bam*HI/*Hin*dIII (primer 495/46) into pXT	Schrecke, GP II
pKS703	*liaFS* (-21 - end) *Xho*I/*Hin*dIII (primer 211/46) into pXT	Schrecke, GP II

Plasmid	Description	Reference
pMM101	*liaS* clean deletion *Bam*HI/*Eco*RI (fused fragments primer 246/462, 461/249) into pMAD	Martinec, GP II
pMM102	*liaFS* clean deletion *Bam*HI/*Eco*RI (fused fragments primer 457/463, 461/249) into pMAD	Martinec, GP II
pSJ101	*liaSR* clean deletion *Bam*HI/*Eco*RI (fused fragments primer 246/248, 247/249) into pMAD	unpublished
pSJ102	*liaF* clean deletion *Bam*HI/*Nco*I/*Xho*I (primer 457/574, 575/458) into pMAD	unpublished
pSJ301	*liaF* (-21 - 135) *Xho*I/*Bam*HI (primer 211/212) into pGFPe	unpublished
pSJ302	*liaF* (-21 - 303) *Xho*I/*Bam*HI (primer 211/213)) into pGFPe	unpublished
pSJ303	*liaI* (-22 - 339) *Xho*I/*Bam*HI (primer 214/215) into pGFPe	unpublished
pSJ304	*liaS*(-24 - 213) *Xho*I/*Bam*HI (primer 216/217) into pGFPe	unpublished
pSJ305	*liaS* (-24 - 123) *Xho*I/*Bam*HI (primer 216/299) into pGFPe	unpublished
pSJ306	*liaG* (-25 - 183) *Xho*I/*Bam*HI (primer 297/330) into pGFPe	unpublished
pSJ401	*liaF* (-21 - 135) *Xho*I/*Bam*HI (primer 211/212) into pHA-4	unpublished
pSJ402	*liaF* (-21 - 303) *Xho*I/*Bam*HI (primer 211/213) into pHA-4	unpublished
pSJ403	*liaI* (-22 - 339) *Xho*I/*Bam*HI (primer 214/215) into pHA-4	unpublished
pSJ404	*liaS* (-24 - 213) *Xho*I/*Bam*HI (primer 216/217) into pHA-4	unpublished
pSJ405	*liaS* (-24 - 123) *Xho*I/*Bam*HI (primer 216/299) into pHA-4	unpublished
pSJ406	*liaG* (-25 - 183) *Xho*I/*Bam*HI (primer 297/330) into pHA-4	unpublished
pSJ407	*liaF* (-1 - 135) *Xho*I/*Bam*HI (primer 370/212) into pHA-4	unpublished
pSJ408	*liaF* (-1 - 303) *Xho*I/*Bam*HI (primer 370/212) into pHA-4	unpublished
pSJ409	*liaF* (-1 - 195) *Xho*I/*Bam*HI (primer 370/371) into pHA-4	unpublished
pSJ601	*liaG* (-68 - 914) *Eco*RI/*Bam*HI (primer 204/205) into pAC6	Jordan *et al*., 2006
pSJ602	*liaF* (-21 - 727) *Eco*RI/*Bam*HI (primer 206/132) into pAC6	unpublished
pSJ603	P_{yozJ} (-306 - 86) *EcoRI/BamHI* (primer 241/242) into pAC6	unpublished
pSJ604	P_{rapK} (-1291 - -957) *Eco*RI/*Bam*HI (primer 243/244) into pAC6	unpublished
pSJ605	P_{rapK} (-1291 - -235) *Eco*RI/*Bam*HI (primer 243/245) into pAC6	unpublished
pSJ606	*liaG* (-68 - 105) *Eco*RI/*Bam*HI (primer 204/295) into pAC6	unpublished
pSJ607	*liaG* (-68 - 3) *Eco*RI/*Bam*HI (primer 204/296) into pAC6	Jordan *et al*., 2006
pSJ608	P_{guaC} (-359 - 43) *Eco*RI/*Bam*HI (primer 368/369) into pAC6	unpublished
pSJ609	P_{yuzG} (-321 - 81) *Eco*RI/*Bam*HI (primer 366/367) into pAC6	unpublished
pSJ610	P_{liaI}/*liaI* (-109 - end) *Eco*RI/*Bam*HI (primer 099/192) into pAC6	unpublished
pSJ611	P_{ydhE} (-170 - 128) *Eco*RI/*Bam*HI (primer 480/525) into pAC6	unpublished
pSJ612	P_{ydhE} (-170 - 128) *Eco*RI/*Bam*HI (primer 480/526) into pAC6	unpublished
pSJ701	*liaF* (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36) into pXT	unpublished
pSJ702	*liaS* (-22 - end) *Bam*HI/*Hin*dIII (primer 44/46) into pXT	unpublished
pSJ703	*liaF* $G_{128}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 500) into pXT	unpublished
pSJ704	*liaF* $G_{149}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 501) into pXT	unpublished
pSJ705	*liaF* $G_{174}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 502) into pXT	unpublished
pSJ706	*liaF* $G_{195}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 503) into pXT	unpublished
pSJ707	*liaF* $G_{234}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/494) into pXT	unpublished
pSJ708	*liaF* $D_{141}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 504) into pXT	unpublished
pSJ709	*liaF* $D_{154}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 505) into pXT	unpublished
pSJ710	*liaF* $D_{183}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 506) into pXT	unpublished
pSJ711	*liaF* $P_{181}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/36 and 507) into pXT	unpublished
pSJ712	*liaF* ΔTM 1-4 (-22 - end) *Hin*dIII/*Eco*RI (primer 489/36) into pXT	unpublished
pSJ713	*liaF* ΔTM 2 and 3 (-22 - end) *Hin*dIII/*Eco*RI (fused fragments primer 35/490, 36/491) into pXT	unpublished
pSJ714	*liaF* stop after G_{195}^a (-22 - end) *Hin*dIII/*Eco*RI (primer 35/492) into pXT	unpublished
pSJ715	*liaF* stop after E_{220}^a (-22 - end) *Hin*dIII/*Eco*RI (primer 35/493) into pXT	unpublished
pSJ716	*liaF* $D_{235}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/557) into pXT	unpublished
pSJ717	*liaF* $D_{237}A^a$ (-22 - end) *Hin*dIII/*Eco*RI (primer 35/556) into pXT	unpublished
pSJ718	*liaF* TM from YxjM *Hin*dIII/*Eco*RI (fused fragments primer 568/569, 36/570) into pXT	unpublished

Plasmid	Description	Reference
pSJ719	cyt. part of *liaS* (216 -end) *Bam*HI/*Hin*dIII (primer 46/529) into pXT	unpublished
pSJ720	cytoplasmic part of *liaS* with optimal SD (216 -end) *Bam*HI/*Hin*dIII (primer 46/530) into pXT	unpublished
pSJ721	*liaS* D_{104}-$E_{106} \rightarrow A_{104-106}$[a] (-24 - end) *Bam*HI/*Hin*dIII (fused fragments primer 454/536, 46/534) into pXT	unpublished
pSJ722	*liaS* ΔD_{104}-E_{106}[a] (-24 - end) *Bam*HI/*Hin*dIII (fused fragments primer 454/537, 46/535) into pXT	unpublished
pSJ723	*liaS* Q_{202}-$R_{206} \rightarrow A_{202-206}$[a] (-24 - end) *Bam*HI/*Hin*dIII (fused fragments primer 454/540, 46/538) into pXT	unpublished
pSJ724	*liaS* ΔQ_{202}-A_{207}[a] (-24 - end) *Bam*HI/*Hin*dIII (fused fragments primer 454/541, 46/539) into pXT	unpublished
pSJ725	*liaS* H_{211}-$P_{214} \rightarrow A_{211-214}$[a] (-24 - end) *Bam*HI/*Hin*dIII (fused fragments primer 454/544, 46/542) into pXT	unpublished
pSJ726	*liaS* ΔH_{211}-P_{214}[a] (-24 - end) *Bam*HI/*Hin*dIII (fused fragments primer 454/545, 46/543) into pXT	unpublished
pSJ727	*liaS* TM from YxdK *Bam*HI/*Hin*dIII (fused fragments primer 571/572, 573/46) into pXT	unpublished
pSJ1601	*liaI* (256 - end) *Nde*I/*Bam*HI (primer 191/192)	unpublished
pSJ1602	*liaF* (256 - end) *Nde*I/*Bam*HI (primer 132/193)	unpublished
pSJ2901	*liaI* (1 - end) *Cla*I/*Xho*I (primer 738/743)	unpublished
pSJ2902	*liaF* (1 - end) *Hin*dIII/*Xho*I (primer 740/729)	unpublished
pSJ2903	*liaS* (1 - end) *Hin*dIII/*Xho*I (primer 741/731)	unpublished
pSJ2904	*liaR* (1 - end) *Hin*dIII/*Xho*I (primer 742/733)	unpublished
pSJ5101	P_{liaI} (-109 - -7) *Eco*RI/*Pst*I (primer 99/627) into pSG1151	unpublished
pSJ5401	*liaI* (4 - end) *Kpn*I/*Xho*I (primer 558/559) into pSG1154	unpublished
pSJ5402	*liaH* (4 - end) *Kpn*I/*Xho*I (primer 560/561) into pSG1154	unpublished
pSJ5403	*liaF* (4 - end) *Kpn*I/*Xho*I (primer 564/565) into pSG1154	unpublished
pSJ5404	*liaS* (4 - end) *Kpn*I/*Xho*I (primer 566/567) into pSG1154	unpublished
pSJ4001	*liaH* (4 - end) *Kpn*I/*Xho*I (primer 560/561) into pSH4	unpublished
pSJ4002	*liaF* (4 - end) *Kpn*I/*Xho*I (primer 564/565) into pSH4	unpublished
pSJ4003	*liaS* (4 - end) *Kpn*I/*Xho*I (primer 566/567) into pSH4	unpublished
pSJ6401	*liaH* (4 - end) *Kpn*I/*Xho*I (primer 560/561) into pSG1164	unpublished
pSK601	P_{wapA} (-170 - 128) *Eco*RI/*Bam*HI (primer 480/481) into pAC6	Köcher, unpublished
pSK602	P_{ydhE} (-170 - 128) *Eco*RI/*Bam*HI (primer 480/481) into pAC6	Köcher, unpublished
pTM1	P_{liaI} (-109 - 72) *Eco*RI/*Bam*HI (primer 099/100) into pAC6	Jordan *et al.*, 2006

Positions of cloned fragments are given relative to the start codon.
[a] For reasons of clarity, the effects of mutations in *liaF* and *liaS* are given at the level of LiaF and LiaS protein.
TM stands for transmembrane region, SD for Shine-Dalgarno sequence

Table 6.4. Primer

Nr.	Name	Sequence
TM001	yvcB-IDM fwd	GGAC***AAGCTT***GTTTGCCTCAACTATTGCGAGTG
TM002	yvcB-IDM rev	AGCA***GGATCC***GCAAGTGAATGTCCTAAGGC
TM003	yvkN-IDM fwd	GTAAA***AAGCTT***ACGGCAACCAAGGAGG
TM004	yvkN-IDM rev	AGCA***GGATCC***GTCCATTAACTGGATGTGCCG
TM005	ywkC-IDM fwd	GGAC***AAGCTT***GGTAGCAAGTGAACTCGGC
TM006	ywkC-IDM rev	AGCA***GGATCC***CGCTTTCGTCCTGCCTTTGC
TM007	yvrL-check	CAAGCGCACCTTGACTTGG
TM008	yvrIH-check	GCAGATACATTGCAATCAGCG
TM009	yvaZ-check	CAGCGTTGGGATTATCCG
TM010	lacZ-check rev	CTTCGCTATTACGCCAGCTGG
TM021	bceR-up fwd	TCGTAGTAGGATACGACTTCGC
TM022	bceR-up rev (cat3')	GGGTAACTAGCCTCGCCGGTCCACGATACATCATAGGACCATCCCG

Nr.	Name	Sequence
TM023	bceS-do fwd (cat5')	CTTGATAATAAGGGTAACTATTGCCGAACATGTCATAAGCGTGTGACG
TM024	bceS-do rev	GCGCCACGCTAAAGATGAGCG
TM025	yvcP-up fwd	GATTACCTCGTTGATATCGG
TM026	yvcP-up rev (mls3')	CGATTATGTCTTTTGCGCAGTCGGCTGGTACGGATTTGCCTGCACC
TM027	yvcQ-do fwd (mls5')	GAGGGTTGCCAGAGTTAAAGGATCCGGCCATAAGCTGTATGCGG
TM028	yvcQ-do rev	CCAACACACTCAAGGTATCC
TM029	liaS-up fwd	GCTTTATCAGCAAGCGGTGACG
TM030	liaS-up rev (kan)	CCTATCACCTCAAATGGTTCGCTGTCCCGTTGTCATGCGGATGGC
TM031	liaG-up fwd	TTGTCGTCGGAATCGCATTGGC
TM032	liaG-up rev (kan)	CCTATCACCTCAAATGGTTCGCTGCACATCTTTAACGACGACGGC
TM033	liaG-do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGCCAATCGACATCAAAACGGACA
TM034	liaG-do rev	TTACCCGGCGTTTGACTCGC
TM035	liaF fwd (HindIII)	AGG***AAGCTT***AGAAAGGAGGCGGACACCAGG
TM036	liaF rev (EcoRI)	TCC***GAATTC***TTTCTCATACGTACTTCACATCC
TM037	pXT-check fwd	CCTTACCGCATTGAAGGCC
TM038	pXT-check rev	GTATTCACGAACGAAAATCGCC
TM044	liaS-fwd (BamHI)	ACG***GGATCC***CGGTAGTGTGGATGTGAAGTACG
TM045	liaS-rev (BamHI)	ACG***GGATCC***TCATCAATCAATAATACTCGAATCACG
TM046	liaS-rev (HindIII)	ACG***AAGCTT***TCATCAATCAATAATACTCGAATCACG
TM047	liaS-do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGGGCACTCAAATCGAAGTGAAGG
TM048	liaS-do rev	AACCGGGCTGGGAAACGAGGTC
TM051	liaS-checkfwd	CGATGATGACATCAGCCGTGC
TM052	liaS-checkrev2	GCTCGACCATCCTGATCCGC
TM053	liaS-checkrev1	CCGAACCAGCTTGATGACAGC
TM054	liaF-checkfwd	GTAAAACATCCCGACATGCGC
TM055	liaF-checkrev	CGTTCAGGTCAAACGGCTGC
TM056	kan-checkfwd	CATCCGCAACTGTCCATACTCTG
TM057	mls-checkfwd	CCTTAAAACATGCAGGAATTGACG
TM058	spec-checkfwd	GTTATCTTGGAGAGAATATTGAATGGAC
TM075	liaF-fwd(StuI)	ACG***AGGCCT***AGAAAGGAGGCGGACACCAGG
TM076	liaF-rev(StuI)	CAC***AGGCCT***CTCATACGTACTTCACATCC
TM077	liaS-fwd(StuI)	CAC***AGGCCT***CGGTGATGTGGATGTGAAGTACC
TM078	liaS-rev(StuI)	ACG***AGGCCT***TCATCAATCAATAATACTCGAATCACG
TM079	yvcB-RT fwd	GTGCCGATAATAAGGATTGG
TM080	yvcB-RT rev	GCAAGTGAATGTCCTAAGGC
TM081	ywkC-RT fwd	ACAAATCTCTGAAGGCACGG
TM082	ywkC-RT rev	CCTGCCTTTGCTGTAAAAGG
TM083	yvaZ-RT fwd	TCAGGAAATAAACCGGCTGC
TM084	yvaZ-RT rev	AGAAGCTAGGAAAAGCAATGC
TM085	yvrH-RT fwd	GTTTTGATTTGCCAAGGCTC
TM086	yvrH-RT rev	CCACAATCGCTTTCTCATCG
TM087	yvrI-RT fwd	AAGGAGATAATGAAGCATCCC
TM088	yvrI-RT rev	TTCATATACTTGACGATGCGG
TM089	yvrL-RT fwd	CAATTGGTCTGTTGTATGAGC
TM090	yvrL-RT rev	TTCCTTGTTCTCGGATTATGC
TM091	yvkN-RT fwd	CAGTGTTAAAGCTGATTCAGG
TM092	yvkN-RT rev	GAACAATATGTGTGTTGCTGC
TM093	liaR-RT fwd	ATTGAAGTCATCGGCGAAGC
TM094	liaR-RT rev	AAAGCTCCCGGCAAATTTGC
TM095	16S-RT fwd	TGAGTAACACGTGGGTAACC

Nr.	Name	Sequence
TM096	16S-RT rev	TCACCAACTAGCTAATGCGC
TM097	pIC333-seq1	GCTGGCTTTAAAGTGCCATGG
TM098	pIC333-seq2	TATCCGTACTTATGTTATAAGG
TM099	PliaI-fwd (EcoRI)	CCAT***GAATTC***CCGGTGCGAGATACGACTCC
TM100	PliaI-rev (BamHI)	CGAT***GGATCC***TCCTCCAAAAAAGACGGAGATCCC
TM101	liaF-fwdEP	GAAAGGAGGCGGACACCAGG
TM102	liaF-revEP	CTCATACGTACTTCACATCC
TM103	liaF-uprevEP	GCAAAAACATACTGATGCCG
TM104	liaF-dofwdEP	GCGTCAACTGATTTTAGCG
TM105	liaS-fwdEP	GGTGATGTGGATGTGAAGTACG
TM106	liaS-revEP	CCGCGAGCCCCATTCTGACC
TM107	liaS-uprevEP	AAGGAGCAGGCTGATTCCCG
TM108	liaS-dofwdEP	GAAGGTCCCGATTTTTCCGG
TM109	liaR-fwdEP	ATTGATTGATGATCATGAAATGG
TM110	liaR-revEP	GGCGCAAACAAATAGCAGCC
TM111	liaR-uprevEP	GCTGCCGTCCGATGCTTCGC
TM112	liaR-dofwdEP	CAAAGCTGGATGTCAGTGACCG
TM125	liaH-RTfwd	TGAAACAGCACACGATTGCC
TM126	liaH-Rtrev	GTTTGCCTGTTCATAGGAAGC
TM127	liaFpDG148fwd	AAGGAGGAAGCAGGTATGACAAAAAAACAGCTTCTCGGATTGATCATTGC
TM128	liaFpDG148rev	GACACGCACGAGGTCTCATACGTACTTCACATCCACATCACCG
TM129	liaSpDG148fwd	AAGGAGGAAGCAGGTATGAGAAAAAAAATGCTTGCCAGCCTCCAATGGC
TM130	liaSpDG148rev	GACACGCACGAGGTTCATCAATCAATAATACTCGAATCACG
TM131	liaF-fwd (PacI)	TACG***TTAATTAA***GAAAGGAGGCGGACACCAGG
TM132	liaF-rev (BamHI)	TACG***GGATCC***CTCATACGTACTTCACATCC
TM133	liaS-fwd (PacI)	TACG***TTAATTAA***CGGTGATGTGGATGTGAAGTACG
TM134	liaS-rev (BamHI)	TACT***GGATCC***TCATCAATCAATAATACTCGAATCACG
TM135	cat-fwd	CAGCGAACCATTTGAGGTGATAGGCGGCAATAGTTACCCTTATTATCAAG
TM136	cat-rev	CGATACAAATTCCTCGTAGGCGCTCGGCCAGCGTGGACCGGCGAGGCTAGTTACCC
TM137	kan-fwd	CAGCGAACCATTTGAGGTGATAGG
TM138	kan-rev	CGATACAAATTCCTCGTAGGCGCTCGG
TM139	mls-fwd	CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC
TM140	mls-rev	CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG
TM141	spec-fwd	CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACTGGCAAGAG
TM142	spec-rev	CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTCTAAAATCTG
TM143	Tc fwd1	CAGCGAACCATTTGAGGTGATAGGTCTTGCAATGGTGCAGGTTGTTCTC
TM144	Tc fwd2	CAGCGAACCATTTGAGGTGATAGGGCTTATCAACGTAGTAAGCGTGG
TM145	Tc rev	CGATACAAATTCCTCGTAGGCGCTCGGGAACTCTCTCCCAAAGTTGATCCC
TM146	cat-check rev	GTCTGCTTTCTTCATTAGAATCAATCC
TM147	kan-check rev	CTGCCTCCTCATCCTCTTCATCC
TM148	mls-check rev	GTTTTGGTCGTAGAGCACACGG
TM149	spec-check rev	CGTATGTATTCAAATATATCCTCCTCAC
TM150	tc-check rev	CATCGGTCATAAAATCCGTAATGC
TM153	liaF-upfwd2	TTCGCAGACGATGTGGAAGCGG
TM154	liaF-dorev2	GCCGATGACTTCAATATCGG
TM156	rpsJ-RTfwd	GAAACGGCAAAACGTTCTGG
TM157	rpsJ-Rtrev	GTGTTGGGTTCACAATGTCG
TM158	rpsE-RTfwd	GCGTCGTATTGACCCAAGC
TM159	rpsE-Rtrev	TACCAGTACCGAATCCTACG

Nr.	Name	Sequence
TM160	PliaI-fwd(74)	CGAT***GAATTC***GATACGACTCCGGTCTTA
TM161	PliaI-fwd(70)	CGAT***GAATTC***CGACTCCGGTCTTATATAAA
TM162	PliaI-fwd(66)	CGAT***GAATTC***TCCGGTCTTTATATAAAAATC
TM163	PliaI-fwd(62)	CGAT***GAATTC***GGTCTTTATATAAAAATCAATC
TM164	PliaI-fwd(58)	CGAT***GAATTC***TTATATAAAAATCAATCTCTG
TM165	PyhcY-rev	CGAT***GGATCC***GTGTTGCTTTGATATCGTGCC
TM166	PyhcY-fwd(202)	CGAT***GAATTC***GACAGTGAAAAGCGACTTGCC
TM167	PyhcY-fwd(99)	CGAT***GAATTC***GTAAATGATCCGGCTTTTTC
TM168	PyhcY-fwd(87)	CGAT***GAATTC***GCTTTTTCTTTTTCTCATCC
TM169	PyhcY-fwd(80)	CGAT***GAATTC***CTTTTTCTCATCCAAAAGTCTG
TM170	PyhcY-fwd(55)	CGAT***GAATTC***GAAAATCATCCTACAAGTG
TM171	PyhcY-fwd(46)	CGAT***GAATTC***CCTACAAGTGAAGCAATGAA
TM172	PyhcY-fwd(29)	CGAT***GAATTC***GAAATACAAAAAACTGGTATAATC
TM173	cat-checkfwd	CTAATGTCACTAACCTGCCC
TM174	yxjM-upfwd	CCAAGAAAGCAGACCGGATGC
TM175	yxjM-uprev(kan)	CCTATCACCTCAAATGGTTCGCTGGCAATTCAAAATGAGAATAAGGC
TM176	yxjM-dofwd(kan)	CGAGCGCCTACGAGGAATTTGTATCGCCTGAAAATTGAGCTGTCATTGCC
TM177	yxjM-dorev	GCATGTTTCCTAATGTCAATTGGC
TM178	yxjL-upfwd	GCCTTTAGACTCCCTTCACGGC
TM179	yxjL-uprev(kan)	CCTATCACCTCAAATGGTTCGCTGTGATGACGTAGCGGAAGCC
TM180	yxjL-dofwd(kan)	CGAGCGCCTACGAGGAATTTGTATCGCGTTTTTGCCATTCGAAACGG
TM181	yxjL-dorev	CGTTTTGAAGTTCTTCAACGGC
TM182	PliaI-fwdcheck	GGCCAAAGCAGAAAGGTCCG
TM183	pJPM122-catlacZfwd(kan)	CGAGCGCCTACGAGGAATTTGTATCGGCCAGATGCTACACAATTAGGC
TM184	pJPM122-catlacZrev1(kan)	CCTATCACCTCAAATGGTTCGCTGCCCTGATTCTGTGGATAACCG
TM185	pJPM122-catlacZrev2(kan)	CCTATCACCTCAAATGGTTCGCTGCGTGAGTTTTCGTTCCACTGAGCG
TM186	PrpsD-fwd(HindIII)	AGG***AAGCTT***CGAGCATATGATAATGAAAGGC
TM187	PrpsD-rev(BamHI)	AGCC***GGATCC***CCGTCTGCTCTATTCGACCATGC
TM188	Pveg-fwd(HindIII)	AGG***AAGCTT***GTCAAAATAATTTTATTGACAACG
TM189	Pveg-rev(BamHI)	AGCC***GGATCC***TACCTAAATTCCCATCAAGCG
TM196	liaF-up fwd	AAGGATTTGCGGTCAAGTCC
TM197	liaF-up rev (kan)	CCTATCACCTCAAATGGTTCGCTGGCAATGATCAATCCGAGAAGC
TM198	liaF-do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGGATGTGGATGTGAAGTACG
TM199	liaF-do rev	TTCAAGCCGTATGAGGAGGC
TM200	liaR-up fwd	GCTGTCATCAAGCTGGTTCGG
TM201	liaR-up rev (kan)	CCTATCACCTCAAATGGTTCGCTGCGATGCTTCGCCGATGACTTC
TM202	liaR-do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGACGCACACCGAAATCATCTCG
TM203	liaR-do rev	CTCTTCATCTGATCCGACACAGC
TM204	liaG-fwd (EcoRI)	CCAT***GAATTC***TCCCTTCCGCACGTATCAATTCGC
TM205	liaG-rev (BamHI)	AGCC***GGATCC***TTTGTCATTCCTGGTG
TM206	liaF-fwd (EcoRI)	CCAT***GAATTC***GAAAGGAGGCGGACACCAGG
TM209	liaR-uprev (HindIII)	ATCG***AAGCTT***CGATGCTTCGCCGATGACTTC
TM210	liaR-dofwd (PciI)	ATCG***ACATGT***ACGCACACCGAAATCATCTCG
TM211	liaF-fwd (XhoI)	AGCT***CTCGAG***GAAAGGAGGCGGACACCAGG
TM212	liaF1-rev (BamHI)	AGCT***GGATCC***ATATTTTTTAAGGAAATAGCC
TM213	liaF2-rev (BamHI)	AGCT***GGATCC***TTTCTCATTCGGTTCAAATATCGG
TM214	liaI-fwd (XhoI)	AGCT***CTCGAG***GGAAAACGAAAGGAGGATCTGC
TM215	liaI-rev (BamHI)	AGCT***GGATCC***AGATTGGTAGGCAGCAGAAGCC
TM216	liaS-fwd (XhoI)	AGCT***CTCGAG***CGGTGATGTGGATGTGAAGTACG
TM217	liaS-rev (BamHI)	AGCT***GGATCC***CATATACCCTGAGGCGAAACCG

Nr.	Name	Sequence
TM218	PliaF-157fwd (EcoRI)	CCAT***GAATTC***CAGTTCGCCGTATTCATTTGCTG
TM219	PliaF-250fwd (EcoRI)	CCAT***GAATTC***CGTATCTGTCACGCTGACAAGCG
TM220	PliaF-336fwd (EcoRI)	CCAT***GAATTC***GCAAATCTGGTTCATGTATCAGGC
TM221	PliaF-546fwd (EcoRI)	CCAT***GAATTC***GATCTGGCAGTACGAACCTCAAGCG
TM222	PliaF-824fwd (EcoRI)	CCAT***GAATTC***GCAGGCCTAGGTTCATAAATGGC
TM223	yhcZ-fwd (BsaI)	ACCT***GGTCTC***AATATGAAAATTGTCATTGCTG
TM224	yhcZ-rev (BamHI)	TCTA***GGATCC***GTTCATTTTGAGATCTCTCC
TM225	liaH-up fwd	CTTGTTATTCGTCACTGCC
TM226	liaH-up rev (kan)	CCTATCACCTCAAATGGTTCGCTGGTCCTTCATGAACTGACGC
TM227	yhcY-probe fwd	GAGTTGCTGAGTCTGACAAACC
TM228	yhcY-probe rev	TCGTGAAGCTCCTGAGCGAGGC
TM229	yhdA-probe fwd	GCAGAACAAGAATTGCAGCATCC
TM230	yhdA-probe rev	CTGCTTTTGCGAACATGCTGAG
TM231	PliaI wobble1	ACAT***GAATTC***GAGAWACSASTCCGKTMTTMTATAAAAATCAATCTCTGATTCG
TM232	PliaI wobble2	ACAT***GAATTC***GAGATACGACTCCGGTCTTATWTWWWWWTCAATCTCTGATTCG
TM233	GFP-checkfwd	CGTAGAGGATCGAGATCTCG
TM234	GFP-checkrev	GGTTATGCTAGTTATTGCTCAGCG
TM235	PhoA-checkfwd	GCATGAAGCAGATCGGTAACG
TM236	PhoA-checkrev	CCATTAAGTCTGGTTGCTAACAGC
TM237	liaF1-rev (KpnI)	AGCT***GGTACC***GAATATTTTTTAAGGAAATAGCC
TM238	liaF2-rev (KpnI)	AGCT***GGTACC***TGTTTCTCATTCGGTTCAAATATCGG
TM239	liaI-rev (KpnI)	AGCT***GGTACC***TGAGATTGGTAGGCAGCAGAAGCC
TM240	liaS-rev (KpnI)	AGCT***GGTACC***CATATACCCTGAGGCGAAACCG
TM241	PyozJ-rev (BamHI)	AGCC***GGATCC***CGGAAGTCAATCCAAGTAACAGC
TM242	PyozJ-fwd (EcoRI)	CCAT***GAATTC***CCATTGTATTCAATAGAAGGC
TM243	PRNA-fwd (EcoRI)	CCAT***GAATTC***GGTATGCTGGTATCTGCTGC
TM244	PRNA-rev (BamHI)	AGCC***GGATCC***GCAATATAAGATATGGACTCAGCG
TM245	PRNA-rev2 (BamHI)	AGCC***GGATCC***GGATCAACAATAATGCCCAATGC
TM246	liaS-upfwd (BamHI)	AGCC***GGATCC***GAAAGGAGGCGGACACCAGG
TM247	liaSclean-uprev	CCATTTCATGATCATCAATCTCTCATACGTACTTCACATCC
TM248	liaSclean-dofwd	CGTATGAGAGATTGATGATCATGAAATGGTCAGAATGG
TM249	liaS-dorev (EcoRI)	CCAT***GAATTC***AACCGGGCTGGGAAACGAGG
TM250	yhcZ-BsaI	ACCTGGTCTCATCGAGAAAATTGTCATTGCTGATGATC
TM251	yhcZ-up rev (kan)	CCTATCACCTCAAATGGTTCGCTGCGCAGACCCTTTCTGACAAC
TM252	yhcZ-do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGGCAAAACTTGAAGTGGCCGATCG
TM253	pMAD-checkrev	GTTACGTTACACATTAACTAGAC
TM254	pMAD-checkfwd	CTGATGGTCGTCATCTACCTGCC
TM255	yhcY-probe fwd1	CTAATACGACTCACTATAGGGAGAGAGTTGCTGAGTCTGACAAACC
TM256	yhdA-probe fwd1	CTAATACGACTCACTATAGGGAGAGCAGAACAAGAATTGCAGCATCC
TM257	yhxB-fwd BsaI	ACCTGGTCTCGAATTCGCTTTTACACGATGCGGTCG
TM258	liaF-probe Tn7fwd	CTAATACGACTCACTATAGGGAGACGTGATTGGCTTGGCTCCG
TM259	PyhcY(75)-fwd	CGAT***GAATTC***TCTCATCCAAAAGTCTGAAAG
TM260	PyhcY(65)-fwd	CGAT***GAATTC***AAGTCTGAAAGAAAATCATCCTACAAGTG
TM261	yhcY-fwd (BsaI)	ACCTGGTCTCATCGAGGAAAAATTAAAAACGCTGAAAACG
TM262	yhcY-rev (BamHI)	TCTA***GGATCC***TCATACCGCCCCTCCTTTTCG
TM263	PyhdA-rev (BamHI)	CGAT***GGATCC***CGTGTGATACAGAGCTGCAATATAGG
TM264	PyhdA-fwd (EcoRI)	CGAT***GAATTC***CCAGGATGATATTGAAGTCGTCG
TM265	PliaI wobble3	ACAT***GAATTC***GAGAaACaAaTCCGaTaTTATATAAAAATCAATCTCTGATTCG
TM266	PliaI wobble4	ACAT***GAATTC***GAGATACGACTCCGGTCTTcTATAcAAATCAATCTCTGATTCG
TM267	PliaI wobble5	ACAT***GAATTC***GAGAaACaAaTCCGGTCTTATATAAAAATCAATCTCTGATTCG
TM268	PliaI wobble6	ACAT***GAATTC***GAGATACGACTCCGaaaTTATATAAAAATCAATCTCTGATTCG
TM269	PaprE-fwd (EcoRI)	CGAT***GAATTC***CTTATTTCTTCCTCCCTCTC
TM270	PaprE-rev (BamHI)	CGAT***GGATCC***CGCAAACAACAAGCTGATCCAC
TM271	aprE-fwd (HindIII)	CGAT***AAGCTT***AAAGGAGAGGGTAAAGAGTGAG

Nr.	Name	Sequence
TM272	aprE-rev (BamHI)	CGAT***GGATCC***GAGTCAATTCCGCTGTCG
TM273	aprE-up fwd	GTTGACATTCGGCACACTCC
TM274	aprE-up rev	CCTATCACCTCAAATGGTTCGCTGGACATGTTGCTGAACGCCATCG
TM275	aprE-do fwd	CGAGCGCCTACGAGGAATTTGTATCGAAACGCGCAAGTCCGTGATCG
TM276	aprE-do rev	CATTTCCACACAGACAACGG
TM277	salA-up fwd	AAGATTGGTGGACAGCAGG
TM278	salA-up rev	CCTATCACCTCAAATGGTTCGCTGGGTTCGCGCATTTCTCCG
TM279	salA-do fwd	CGAGCGCCTACGAGGAATTTGTATCGTGACGAAAATCATCCAATCGG
TM280	salA-do rev	TATCTCAAGCGCAAACCGATG
TM281	abrB-up fwd	TATCAACGAGCTGAGTTTCCG
TM282	abrB-up rev	CCTATCACCTCAAATGGTTCGCTGCAACTTTACGTACAATACCAGTAG
TM283	abrB-do fwd	CGAGCGCCTACGAGGAATTTGTATCGCAGCGAAATCCAAAACCAGC
TM284	abrB-do rev	TTCTTTACTTGGTCCCAACCC
TM285	scoC-up fwd	AACCTCTTCCGCTTCCGG
TM286	scoC-up rev	CCTATCACCTCAAATGGTTCGCTGGAGCCTTGCTAAGCTGAGCC
TM287	scoC-do fwd	CGAGCGCCTACGAGGAATTTGTATCGGATGAACCGGCTGAAGAGC
TM288	scoC-do rev	ACGTTTCCATGTGCGCATGC
TM289	sinR-up fwd	GCCAAAAGACCTAGATGGTG
TM290	sinR-up rev	CCTATCACCTCAAATGGTTCGCTGATGTCATCACCTTCCTTGTG
TM291	sinR-do fwd	CGAGCGCCTACGAGGAATTTGTATCGGATGACATCCGGGGTATCG
TM292	sinR-do rev	TAGGAGTTGCTTCTGCAGC
TM293	yhdA-probe rev T7	CTAATACGACTCACTATAGGGAGACTGCTTTTGCGAACATGCTGAG
TM294	yhcY-probe rev T7	CTAATACGACTCACTATAGGGAGATCGTGAAGCTCCTGAGCGAGGC
TM295	PliaF-rev1 (BamHI)	AGCC***GGATCC***GCCATTTATGAAACCTAGGC
TM296	PliaF-rev2 (BamHI)	AGCC***GGATCC***CATTCGGTTTCATCCTTCTCATTC
TM297	liaG-fwd (XhoI)	AGCT***CTCGAG***GAATGAATGAGAAGGATGAAACCG
TM298	liaG-rev2 (BamHI)	AGCT***GGATCC***GGACATCCTTGCTATCCG
TM299	liaS-rev2 (BamHI)	AGCC***GGATCC***AAGCGGATCGAGCCGATAG
TM300	PliaI-423 (EcoRI)	GATC***GAATTC***GCAGTCAGTGCTGTTAATGTTCC
TM301	degU-up fwd	AAGCCCATAAGCTGCAGG
TM302	degU-upfwd2	CAGAGGATCAGAGGCTAGC
TM303	degU-up rev	CCTATCACCTCAAATGGTTCGCTGTATCCGTTTAACACCTTCACG
TM304	degU-do fwd	CGAGCGCCTACGAGGAATTTGTATCGTAAACGACCGGACGCAAGCC
TM305	degU-do rev	CAAATGAGTGCCGATTACCGC
TM306	degU-do rev2	AAGCAGCTGATCTCTGAGTC
TM307	spo0A-up fwd	TATCAGAGATTCTGCTGCTGGC
TM308	spo0A-up rev	CCTATCACCTCAAATGGTTCGCTGAGCGACAGGCATTCCTGTCC
TM309	spo0A-do fwd	CGAGCGCCTACGAGGAATTTGTATCGGTTGCGGATAAGCTGAGG
TM310	spo0A-do rev	GGAAGAACCTGAGACACCG
TM322	ackA-upfwd	GGAACTGACCATTCTTGATCCAGC
TM323	ackA-uprev	CCTATCACCTCAAATGGTTCGCTGCCATTTAAACATTGTCATGTCGG
TM324	ackA-dofwd	CGAGCGCCTACGAGGAATTTGTATCGCGACTGATGAAGAAGTCATGATTGCG
TM325	ackA-dorev	CGACGGAAGTATCAAGACCTCC
TM326	pta-upfwd	GCTCTACCACTGATACGTAGG
TM327	pta-uprev	CCTATCACCTCAAATGGTTCGCTGGCGTTCTACGAATGCTTGTACAAGG
TM328	pta-dofwd	CGAGCGCCTACGAGGAATTTGTATCGCGCTGAAGATGTTTACAATCTCGC
TM329	pta-dorev	CGCTTCCTTTACACCTTGATTGC
TM330	liaG-rev3 (BamHI)	AGCT***GGATCC***CCGGACATCCTTGCTATCCG
TM366	PyuzG-fwd (EcoRI)	CCAT***GAATTC***CAGGAATTAGCTGAATATCTTCG
TM367	PyuzG-rev (BamHI)	CGAT***GGATCC***TGCGACTACCCAGATCATTGCC
TM368	PguaC-fwd (EcoRI)	CCAT***GAATTC***TGCGACTACCCAGATCATTGCC
TM369	PguaC-rev (BamHI)	CGAT***GGATCC***CAGGAATTAGCTGAATATCTTCG
TM370	liaF-fwdSD (XhoI)	AGCT***CTCGAG***AATGACAAAAAAACAGCTTCTCG
TM371	liaF-rev3 (BamHI)	CGAT***GGATCC***GTTTTTCAAAAATAGAAACGCGG

Nr.	Name	Sequence
TM405	liaG-fwdSD (XhoI)	AGCT***CTCGAG***AATGAAAAAAATGCTTGGC
TM406	liaS-fwdSD (XhoI)	AGCT***CTCGAG***TATGAGAAAAAAAATGCTTGCC
Tm407	liaI-fwdSD (XhoI)	AGCT***CTCGAG***CATGAAAATAAACAAGAAAACAATAGGC
TM408	liaF4-rev (BamHI)	AGCT***GGATCC***CGGCCAAAACAGCAGATCG
TM409	liaI2-rev (BamHI)	AGCT***GGATCC***TGCGTATGTCATCAAGC
TM410	pXT-fwd	GTTTAAACAACAAACTAATAGGTGATG
TM411	pXT-rev	GTTATAGTTATTATAACATGTATTCACG
TM448	ptb-upfwd	GGCTATGAGGATGGTGCG
TM449	ptb-uprev	CCTATCACCTCAAATGGTTCGCTGGTGTATCGACGCTTTGCC
TM450	ptb-uprevi	CGAGCGCCTACGAGGAATTTGTATCGGTGTATCGACGCTTTGCC
TM451	ptb-dofwd	CGAGCGCCTACGAGGAATTTGTATCGCTGTATTCCATTGCGCTGGC
TM452	ptb-dofwdi	CCTATCACCTCAAATGGTTCGCTGCTGTATTCCATTGCGCTGGC
TM453	ptb-dorev	CGCACCTGCGATCACTTTCGC
TM454	liaS-fwd (BamHI)	ACG***GGATCC***CGGTGATGTGGATGTGAAGTACG
TM455	liaFclean-dofwd	CGGTGATGTGGATGTGAAGTACG
TM456	liaFclean-uprev	CGTACTTCACATCCACATCACCGTCCTGGTGTCCGCCTCCTTTC
TM457	liaF-upfwd (BamHI)	AGCC***GGATCC***AAGGATTTGCGGTCAAGTCC
TM458	liaF-dorev (NcoI)	AGCT***CCATGG***TTCAAGCCGTATGAGGAGGC
TM459	liaFSclean-uprev	CCATTTCATGATCATCAATCTCCTGGTGTCCGCCTCCTTTC
TM460	liaFSclean-dofwd	GATTGATGATCATGAAATGGTCAGAATGG
TM461	liaSclean-dofwd2	CCGGAAGAAAAAGGAGAGAACGAACG
TM462	liaSclean-uprev2	CGTTCGTTCTCTCCTTTTTCTTCCGGCTCATACGTACTTCACATCC
TM463	liaFSclean-uprev2	CGTTCGTTCTCTCCTTTTTCTTCCGGTCCTGGTGTCCGCCTCCTTTC
TM480	PydhE-rev(BamHI)	CGAT***GGATCC***ATCCCGGTGAAGATGGACCG
TM481	PydhE-fwd(EcoRI)	CGAT***GAATTC***TTGATTAAGTCCAGAGCAGCAG
TM482	PwapA-rev(BamHI)	CGAT***GGATCC***TTGCTAGTACATCGGCTGGC
TM483	PwapA-fwd(EcoRI)	CGAT***GAATTC***GCGAATGTGACAGCTGAGGG
TM488	liaFclean-uprev2	TTTTTTCTCATACGTACTTCACATCCACATCACCGTCCTGGTGTCCGCCTCCTTTC
TM489	liaF-TM1-4fwd (HindIII)	AGG***AAGCTT***AGAAAGGAGGCGGACACCAGGAATGCCGATATTTGAACCGAATGAGAA ACAGGTC
TM490	liaF-TM2u3-uprev	AATTAAAGGTGATGCTGAAGAGCCAAAACAGCAGATCGCCTATTCCG
TM491	liaF-TM2u3-dofwd	CTCTTCAGCATCACCTTTAATTTATTCGG
TM492	liaF-195 (EcoRI)	TCC***GAATTC***TCATCCTATAAAAACAGCCGAGC
TM493	liaF-220 (EcoRI)	TCC***GAATTC***TCACTCGCTAAAATCAGTTGACGC
TM494	liaF-G234A rev (EcoRI)	TCC***GAATTC***TTTCTCATACGTACTTCACATCCACATCAGCGATAAATAAGG
TM495	liaS-SD opt.(BamHI)	ACG***GGATCC***TAAGGAGGAGTACGTATGAGAAAAAAAATGC
TM500	liaF-G128A	GCTTTTTTATCGCTGAGCTGCAAATGATG
TM501	liaF-G149A	CTCTGGTTTTATCGCTGATATCAAAATCG
TM502	liaF-G174A	CGGAGTCATTGCTAACGTTGATATTTATG
TM503	liaF-G195A	CTGTTTTTATAGCAGACATTAATCTGATCG
TM504	liaF-D141A	GTTTGACCTGAACGCTTTAAATGTCTCTGG
TM505	liaF-D154A	GATATCAAAATCGGCTTATCTAAAGCGATG
TM506	liaF-D183A	ATGTACCATCGGCCCTTGAAGTGGC
TM507	liaF-P181A	GATATTTATGTAGCATCGGACCTTGAAG
TM508	liaR-D54A	CATTTTAATGGCCCTTGTCATGGAGGG
TM525	PydhE-fwd2 (EcoRI)	CGAT***GAATTC***ACGATTAGTAGTAGTCTGATCAAAC
TM526	PydhE-fwd3 (EcoRI)	CGAT***GAATTC***GTCAGAATACGATTTGAGAGGG
TM529	liaS cyt.-fwd(BamHI)	ACG***GGATCC***CGGTGATGTGGATGTGAAGTACGTATGGGCAACCGGTTGAAGACAAGG
TM530	liaS cyt.SD-fwd(BamHI)	ACG***GGATCC***TAAGGAGGAGTACGTATGGGCAACCGGTTGAAGACAAGG
TM531	liaS C-term.-fwd(HindIII)	AGG***AAGCTT***TGAAAACGGCAATTTCGCTTATCGG
TM532	liaS C-term.-rev(BsaI)	ACCTGGTCTCAAATTCTCATCAATCAATAATACTCGAATCACG
TM533	liaS N-term.-rev(HindIII)	AGG***AAGCTT***AAAATGGATTCAATTAATGTATCAATCCT
TM534	liaS 104-106A dofwd	CGCTCGGTGCTGCTGCTATCGGCCTGGCTGCTGATCAGC
TM535	liaS 104-106del dofwd	CGCTCGGTATCGGCCTGGCTGCTGATCAGC
TM536	liaS 104-106A uprev	CCAGGCCGATAGCAGCAGCACCGAGCGGCGGTATCCG

Nr.	Name	Sequence
TM537	liaS 104-106del uprev	CCAGGCCGATACCGAGCGGCGGTATCCG
TM538	liaS 202-207A dofwd	GCGAAGCCGCAGCTGCGGCGGCGGCGCTGCTGCTCCATTTACGG
TM539	liaS 202-207del dofwd	GCGAAGCCCTGCTGCTCCATTTACGG
TM540	liaS 202-207A uprev	GCAGCAGCGCCGCCGCCGCAGCTGCGGCTTCGCCTGCCATATGC
TM541	liaS 202-207del uprev	GCAGCAGGGCTTCGCCTGCCATATGC
TM542	liaS 211-214A dofwd	CGCTGCTGCTCGCTGCAGCGGCTGTTACCCTTGAAGGAAA
TM543	liaS 211-214del dofwd	CGCTGCTGCTCGTTACCCTTGAAGGAAA
TM544	liaS 211-214A uprev	GGGTAACAGCCGCTGCAGCGAGCAGCAGCGCCCTCATCTC
TM545	liaS 211-214del uprev	GGGTAACGAGCAGCAGCGCCCTCATCTC
TM550	lacZ-upfwd (BamHI)	CCGC***GGATCC***CCAGCTTGTTG
TM551	lacZ-uprev	CGGCGCTCAGTTGGAACTCCGCCGATACTGACGGGCTCC
TM552	lacZ-dofwd	ATCGGCGGAGTTCCAACTGAGCGCCGGTCGCTACCATTACC
TM553	lacZ-dorev (BlpI)	GTACGCTCAGCAATGATGACCTCGTTTCCACCGG
TM556	LiaFD237A (EcoRI)	TCC***GAATTC***TTTCTCATACGTACTTCACAGCCACATCACCG
TM557	LiaFD235A (EcoRI)	TCC***GAATTC***TTTCTCATACGTACTTCACATCCACAGCACCGATAAATAAGG
TM558	liaI-GFP fwd (KpnI)	GACT***GGTACC***AAAATAAACAAGAAAACAATAGGC
TM559	liaI-GFP rev (XhoI)	GACT**CTCGAG**TTTTTTCTTCAAAAATTCTTCCCATTCG
TM560	liaH-GFP fwd (KpnI)	GACT***GGTACC***GTATTAAAAAGAATCAGAGACATGTTTG
TM561	liaH-GFP rev (XhoI)	GACT***CTCGAG***TTCATTTGCCGCTTTTGTCTGG
TM562	liaG-GFP fwd (KpnI)	GACT***GGTACC***AAAAAAATGCTTGGCAAACTGTTGATCAC
TM563	liaG-GFP rev (XhoI)	GACT***CTCGAG***CCGTATCGCTAGATCCCCGCTG
TM564	liaF-GFP fwd (KpnI)	GACT***GGTACC***ACAAAAAAACAGCTTCTCGG
TM565	liaF-GFP rev (XhoI)	GACT***CTCGAG***TACGTACTTCACATCCACATCACC
TM566	liaS-GFP fwd (KpnI)	GACT***GGTACC***AGAAAAAAAATGCTTGCCAGCC
TM567	liaS-GFP rev (XhoI)	GACT***CTCGAG***ATCAATAATACTCGAATCACGTTCG
TM568	LiaF-TM YxjM-upfwd (HindIII)	AGG***AAGCTT***AGAAAGGAGGCGGACACCAGGAATGATTTTGTTAGCATTTGTATTTGCG
TM569	LiaF-TM YxjM-uprev	TTTCTCATTCGGTTCAAATATCGGGGAGAAGAGGACGTACAGACC
TM570	LiaF-TM YxjM-dofwd	CCGATATTTGAACCGAATGAGAAAC
TM571	LiaS-TM YxdK-upfwd (BamHI)	ACG***GGATCC***CGGTGATGTGGATGTGAAGTACGTATGAAGCTGTTTCTCCGGTCTCATGC
TM572	LiaS-TM YxdK-uprev	CCTTGTCTTCAACCGGTTGCCATAGGCAAGATAGCCCGCCA
TM573	LiaS-TM YxdK-dofwd	GGCAACCGGTTGAAGACAAGG
TM574	LiaFclean-uprev (XhoI)	GACT***CTCGAG***TCCTGGTGTCCGCCTCCTTTC
TM575	LiaFclean-dofwd (XhoI)	GACT***CTCGAG***CGGTGATGTGGATGTGAAGTACG
TM577	PliaG-fwdnat(SmaI)	CCAT***CCCGGG***TCCCTTCCGCACGTATCAATTCGC
TM578	PliaG-fwdopt-10(SmaI)	CCAT***CCCGGG***TCCCTTCCGCACGTATCAATTCGCAAGCTTTTCTGTTATAATAGAATG
TM579	PliaG-fwdopt(SmaI)	CCAT***CCCGGG***TCCCTTCCGCACTTGACAATTCGCAAGCTTTTCTGTTATAATAGAATG
TM580	liaFexpr (BamHI)	AGCC***GGATCC***ATTCCTGGTGTCCGCCTCC
TM581	liaSexpr (BamHI)	AGCC***GGATCC***ATACGTACTTCACATCCACATC
TM582	liaRexpr (BamHI)	AGCC***GGATCC***ACGTTCGTTCTCTCCTTTTTCTTCC
TM627	PliaI-rev (PstI)	AGTC***CTGCAG***CCTCCTTTCGTTTTCCTTGTC
TM722	liaR-YFP fwd (KpnI)	ATGC***GGTACC***GTGATTCGAGTATTATTGATTGATG
TM723	liaR-YFP rev (XhoI)	ATGC***CTCGAG***ATTCACGAGATGATTTCGGTGTGC
TM724	GFP-liaI fwd (SalI)	ATGC***GTCGAC***ATGAAAATAAACAAGAAAACAATAGGC
TM725	GFP-liaI rev (EcoRV)	ATGC***GATATC***TTTTTTCTTCAAAAATTCTTCCC
TM726	GFP-liaH fwd (SalI)	ATGC***GTCGAC***ATGGTATTAAAAAGAATCAGAGACATG
TM727	GFP-liaH rev (EcoRV)	ATGC***GATATC***TTCATTTGCCGCTTTTGTCTGG
TM728	GFP-liaF fwd (SalI)	ATGC***GTCGAC***ATGACAAAAAAACAGCTTCTCGG
TM729	GFP-liaF rev (HindIII)	ATGC***AAGCTT***TACGTACTTCACATCCACATC
TM730	GFP-liaS fwd (SalI)	ATGCGTCGACATGAGAAAAAAAATGCTTGCCAGC
TM731	GFP-liaS rev (HindIII)	ATGC***AAGCTT***ATCAATAATACTCGAATCACGTTCG
TM732	GFP-liaR fwd (SalI)	ATGC***GTCGAC***GTGATTCGAGTATTATTGATTGATG
TM733	GFP-liaR rev (HindIII)	ATGC***AAGCTT***ATTCACGAGATGATTTCGGTG

Nr.	Name	Sequence
TM734	PliaG-YFP fwd (KpnI)	ATGC***GGTACC***GAAGTATTTAGAAGGGAAGGC
TM735	PliaG-YFP rev (XhoI)	ATGC***CTCGAG***CATTCGGTTTCATCCTTCTC
TM736	PliaI-CFP fwd (KpnI)	ATGC***GGTACC***CAGTCAGTGCTGTTAATGTTCC
TM737	PliaI-CFP rev (EcoRI)	ATGC***GAATTC***CATGCAGATCCTCCTTTCGTTTTC
TM738	GFP-liaI fwd (XhoI)	ATGC***CTCGAG***ATGAAAATAAACAAGAAAACAATAGGC
TM739	GFP-liaH fwd (XhoI)	ATGC***CTCGAG***ATGGTATTAAAAAGAATCAGAGACATG
TM740	GFP-liaF fwd (XhoI)	ATGC***CTCGAG***ATGACAAAAAAACAGCTTCTCGG
TM741	GFP-liaS fwd (XhoI)	ATGC***CTCGAG***ATGAGAAAAAAAATGCTTGCCAGC
TM742	GFP-liaR fwd (XhoI)	ATGC***CTCGAG***GTGATTCGAGTATTATTGATTGATG
TM743	GFP-liaI rev (ClaI)	ATGC***ATCGAT***TTTTTTCTTCAAAAATTCTTCCC
TM744	GFP-liaH rev (ClaI)	ATGC***ATCGAT***TTCATTTGCCGCTTTTGTCTGG

The (universal) linker sequences used for joining reactions are underlined. Restriction sites for cloning are highlighted in bold italics. Nucleotides given in small letters represent mismatches. Sequences underlined are inverse and complementary to other fragments for PCR fusion.

Curriculum vitae

Personal Information

Date of birth:	25th June 1978
Place of birth:	Essen, Germany
Nationality:	German

School Education

1984	Grundschule Nienburg-Weser
1984-1988	Sieburgschule Bad Karlshafen
1988-1997	Städtisches Gymnasium Beverungen
07/1997	Abitur

Scientific Education

1997-2004	study of biology, Eberhardt-Karls-University Tübingen
2003-2004	diploma thesis Dept. of Microbiology/Biotechnology (Prof. Dr. Wolfgang Wohlleben), Eberhardt-Karls-University Tübingen
thesis title:	"Entwicklung eines positiven Selektionssystems für Streptomyceten"
08/2004 - 11/2007	PhD thesis Dept. of General Microbiology (Prof. Dr. Jörg Stülke), Georg-August-University Göttingen
thesis title:	"Intramembrane signal transduction and cell envelope stress response in *Bacillus subtilis*"
04/2007 – 06/2007	Visiting scientist in the group of Dr. Leendert Hamoen, Institute for Cell and Molecular Biosciences, University of Newcastle