The LiaFSR Three-Component System of *Bacillus subtilis*: Mechanism of Stimulus Perception and Signal Transduction

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

München, 07.03.2013 ______________________________

Karen Schrecke

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Abbreviations

1CS	one-component system
2CS	two-component system
ABC	ATP binding cassette
AckA	acetate kinase
ATP	adenosine-5’-triphosphate
cAMP	cyclic adenosine-3’,5’-monophosphate
CAMP	cationic antimicrobial peptide
CAP	catabolite activator protein
CESR	cell envelope stress response
CoA	coenzyme A
CRE	catabolite-responsive element
CTD	C-terminal DNA-binding domain
DNA	deoxyribonucleic acid
ECF	extracytoplasmic function
e.g.	for example
Glc*NA*c	*N*-acetylglucosamin
HK	histidine kinase
IPTG	isopropyl-β-D-thiogalactopyranoside
MLS	macrolide-lincosamide-streptogramin
mRNA	messenger RNA
Mur*NA*c	*N*-acetylmuramic acid
OD	optical density
PCR	polymerase chain reaction
Pta	phosphotransacetylase
RNA	ribonucleic acid
RR	response regulator
TCA	tricarboxylic acid
UMP	uridine monophosphate
UDP	uridine diphosphate
UPP	undecaprenol pyrophosphate
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Publications Originating from this Thesis

CHAPTER 1 and 4

Schrecke, K.*, Staroń, A.*, Mascher, T. (2012). Two-component systems in bacteria. Chapter 11: Two-Component signaling in the Gram-positive envelope stress response: intramembrane-sensing histidine kinases and accessory membrane proteins. D. Beier and R. Gross (eds). *Horizon Scientific Press*, pp. 199-229

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CHAPTER 2

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CHAPTER 3

Toymentseva, A. A., Schrecke, K., Sharipova, M. R., Mascher, T. (2012). The LIKE system, a novel protein expression toolbox for *Bacillus subtilis* based on the *liaI* promoter. *Microbial Cell Factories* **11**: 143

Contributions to Publications

CHAPTER 1 and 4

Schrecke, K.*, Staroń, A.*, Mascher, T. (2012). D. Beier and R. Gross (eds). *Horizon Scientific Press*, pp. 199-229 (* shared first authorship)

Karen Schrecke, Anna Staroń, and Thorsten Mascher performed the literature search and wrote the manuscript.

CHAPTER 2

Schrecke, K., Jordan, S., Mascher, T. (2013). *Molecular Microbiology* **87**(4): 769-788

Karen Schrecke carried out all experiments, drew the figures and constructed the tables. Sina Jordan generated some strains. Karen Schrecke and Thorsten Mascher designed the experiments and wrote the manuscript.

CHAPTER 3

Toymentseva, A. A., Schrecke, K., Sharipova, M. R., Mascher, T. (2012). *Microbial Cell Factories* **11**: 143

Anna Toymentseva performed all experiments with the exception of the overexpression experiment. Karen Schrecke carried out the overexpression experiment with YdfG. Anna Toymentseva, Karen Schrecke, and Thorsten Mascher designed the experiments and wrote the manuscript. Margarita Sharipova helped to coordinate and to draft the manuscript.

Summary

Soil bacteria are exposed to constant changes in temperature, moisture, and oxygen content. Additionally, they have to encounter different antimicrobial substances, which are produced by competing bacteria. Those agents often target the bacterial cell envelope, which is an essential structure composed of the cell wall and cell membrane. In order to counteract such life-threatening conditions, bacteria developed signal transducing systems to monitor their environment and to respond signal-specifically to any stress conditions, mostly by differential gene expression. Different principles of signal transducing systems have been evolved: one-component systems (1CSs), two-component systems (2CSs), and extracytoplasmic function (ECF) sigma factors. *Bacillus subtilis* is a soil bacterium, which counteracts cell envelope stress by four different 2CSs (LiaSR, BceRS, PsdRS, and YxdJK) and at least three different ECF sigma factors (σ^X, σ^M, and σ^W). In the course of the present thesis, the LiaSR 2CS was investigated in detail.

The LiaSR 2CS of *B. subtilis* is a cell envelope stress-sensing system that shows a high dynamic range of induction in response to cell wall antibiotics like bacitracin. It provides no resistance against its inducer molecules. Rather, it is a damage-sensing system that maintains the cell envelope integrity under stress conditions. The membrane-anchored histidine kinase (HK) LiaS and its cognate response regulator (RR) LiaR work together with a third protein, LiaF, which was identified as the inhibitor of the 2CS. Upon induction, the target promoter P_{liaI} is induced by phosphorylated LiaR, leading to the expression of the *liaIH-liaGFSR* locus, with *liaIH* as being the most induced genes. In the first part of this thesis, the mechanisms of stimulus perception and signal transduction of the LiaFSR system were analyzed. Therefore, the native stoichiometry of the proteins LiaF, LiaS, and LiaR were determined genetically and biochemically with a resulting ratio of 18 to 4 to 1. We found out that maintaining this specific stoichiometry is crucial for the functionality of the LiaFSR system and thus a proper response to cell envelope stress. Changing the relative protein ratios by the overproduction of either LiaS or LiaR leads to a constitutive activation of the promoter P_{liaI}. These data suggest a non-robust behavior of the LiaFSR system regarding perturbations of its stoichiometry, which stands in contrast to quantitative analyses of other well-known 2CSs. Furthermore, a HK-independent phosphorylation of the RR LiaR was observed. This happened in each case if the amount of LiaR exceeded those of LiaS, irrespective of the presence or absence of a stimulus. By using growth media supplied with different carbon sources, acetyl phosphate was identified as being the phosphoryl group-donor for LiaR under these conditions.

Moreover, by performing a mutagenesis experiment, we obtained genetic evidence that LiaS is a bifunctional HK offering both a kinase and a phosphatase activity.

In the second part of this thesis, the *liaI* promoter was used to generate a protein expression toolbox for the use in *B. subtilis*, referred to as the LIKE (from the German "***Li**a-**k**ontrollierte **E**xpression*") system. P_{liaI} is a perfect candidate for driving recombinant protein expression. It is tightly regulated under non-inducing conditions showing no significant basal expression levels. Depending on the inducer molecule concentration, it is induced up to 1000-fold reaching a maximum already 30 minutes after addition of the inducer. Two expression vectors, an integrative and a replicative one, were constructed consisting of an alternative *liaI* promoter, which was optimized to enhance promoter strength. Additionally, different *B. subtilis* expression hosts were generated that possess *liaIH* deletions to prevent undesired protein production. The feasibility of the LIKE system was evaluated by using *gfp* and *ydfG* as reporter genes and bacitracin as inducer molecule. As a result, both proteins were successfully overproduced.

Zusammenfassung

Bodenbakterien sind ständig wechselnden Umweltbedingungen ausgesetzt. Enorme Schwankungen in Temperatur, Feuchtigkeits- und Sauerstoffgehalt sind charakteristisch für dieses Habitat. Zusätzlich kommen sie mit antimikrobiellen Substanzen in Kontakt, die von Nahrungskonkurrenten sekretiert werden. Ein typisches Angriffsziel dieser Substanzen stellt die Zellhülle, bestehend aus Zellwand und Zellmembran, dar, da diese eine essentielle Funktion als Schutzschild vor schädlichen äußeren Einflüssen innehat. Um diesen lebensbedrohlichen Bedingungen standzuhalten, bevor die Zelle ernsthaft Schaden nimmt, entwickelten Bakterien signaltransduzierende Systeme, um ihre Umgebung überwachen und spezifisch auf Zellwandstress reagieren zu können. Verschiedene Arten dieser Systeme haben sich entwickelt: Einkomponentensysteme, Zweikomponentensysteme und ECF (*extracytoplasmic function*) Sigma Faktoren. Das Bodenbakterium *Bacillus subtilis* besitzt vier verschiedene Zweikomponentensysteme (LiaSR, BceRS, PsdR und YxdJK) und mindestens drei verschieden ECF Sigma Faktoren (σ^X, σ^M und σ^W), um Zellhüllstress entgegenzuwirken. Im Zuge dieser Arbeit wurde das LiaSR Zweikomponentensystem detailliert untersucht.

Das LiaSR Zweikomponentensystem von *B. subtilis* ist ein hochdynamisches Zellhüllstress-detektierendes System, welches in Gegenwart von Zellwandantibiotika, wie zum Beispiel Bacitracin, induziert wird. Es vermittelt jedoch keine Resistenz gegenüber induzierenden Molekülen, es ist vielmehr ein System, das Schäden in der Zellhülle detektiert, um die Funktionsfähigkeit der Zellhülle unter Stressbedingungen durch gezielte Expression der Targetgene aufrechtzuerhalten. Die membranständige Histidinkinase LiaS und das zugehörige Antwortregulatorprotein LiaR stehen unter der negativen Kontrolle eines dritten Proteins, LiaF, welches als Inhibitor des Zweikomponentensystems identifiziert wurde. In Anwesenheit eines Stimulus wird der Targetpromotor P_{liaI} durch den phosphorylierten Antwortregulator LiaR aktiviert, was zu einer Expression des *liaIH-liaGFSR* Lokus führt, wobei *liaIH* die am stärksten induzierten Gene darstellen. Im ersten Teil dieser Arbeit wurden die Mechanismen der Stimuluswahrnehmung und Signalweiterleitung des LiaFSR Systems analysiert. Dafür wurde die natürliche Stöchiometrie der Proteine LiaF, LiaS und LiaR mit Hilfe von genetischen und biochemischen Methoden ermittelt, mit einem resultierenden Proteinmengenverhältnis von 18:4:1. Die Aufrechterhaltung dieses spezifischen Verhältnisses ist entscheidend für die Funktionsweise des LiaFSR Systems und demzufolge auch für eine adäquate Reaktion auf Zellhüllstress. Eine Änderung der relativen Stöchiometrie durch eine Überproduktion von entweder LiaS oder LiaR führt zu einer konstitutiven Aktivierung des Promotors P_{liaI}. Diese Ergebnisse deuten auf ein sensibles

Verhalten des LiaFSR Systems, bezüglich einer stochastischen Variabilität der Proteinmengenverhältnisse, hin. Diese Beobachtungen stehen im Gegensatz zu quantitativen Untersuchungen anderer Zweikomponentensysteme. Weiterhin wurde eine LiaS-unabhängige Phosphorylierung von LiaR untersucht. Sobald die Proteinmenge des Antwortregulators LiaR die der Histidinkinase LiaS übersteigt, ist eine Aktivierung von P_{liaI} messbar, wobei die An- oder Abwesenheit eines Stimulus keine Rolle spielt. Mit Hilfe von Wachstumsmedien, denen verschiedene Kohlenstoffquellen hinzugefügt wurden, konnte Acetylphosphat als Phosphorylgruppendonor für LiaR identifiziert werden. Des Weiteren wurde die Histidinkinase LiaS bezüglich einer Bifunktionalität näher untersucht. Mit Hilfe eines Mutageneseexperimentes wurde der genetische Beweis erbracht, dass LiaS eine Kinase- und eine Phosphataseaktivität besitzt.

Im zweiten Teil der vorliegenden Arbeit wurde ein neuartiges Proteinexpressionssystem zur Anwendung in *B. subtilis* etabliert, das sogenannte LIKE (**Li**a-**k**ontrollierte **E**xpression) System. Dieses basiert auf den Promotor P_{liaI}. Dieser Promotor ist sehr gut geeignet für die Expression rekombinanter Proteine, da er nahezu kein basales Expressionsniveau unter nicht-induzierenden Bedingungen besitzt und in Abhängigkeit der Induktorkonzentration bis zu 1000-fach induziert wird, wobei das Aktivitätsmaximum schon 30 Minuten nach Zugabe des Induktors erreicht wird. Zwei Expressionsvektoren wurden konstruiert, wobei es sich um einen integrativen und einen replikativen Vektor handelt. Beide Vektoren besitzen einen alternativen *liaI* Promotor, der zur Verbesserung der Promotorstärke optimiert wurde. Außerdem wurden verschiedene *B. subtilis* Expressionsstämme entwickelt, die alle Mutationen im *liaIH* Operon aufweisen, um eine unerwünschte Proteinsynthese zu vermeiden. Die Funktionsfähigkeit des LIKE Systems wurde mit Hilfe der Reportergene *gfp* und *ydfG* sowie Bacitracin als induzierendes Molekül evaluiert. Beide Proteine konnten erfolgreich überproduziert werden.

1 Introduction

Parts of this chapter have been adapted from:

Schrecke, K.*, Staroń, A.*, Mascher, T. (2012). Two-component systems in bacteria. Chapter 11: Two-Component signaling in the Gram-positive envelope stress response: intramembrane-sensing histidine kinases and accessory membrane proteins. D. Beier and R. Gross (eds). *Horizon Scientific Press*, pp. 199-229

* contributed equally

1 Introduction

The soil is a complex environment that is exposed to fluctuation in temperature, moisture and oxygen content. Despite these facts, the soil is a habitat for a lot of different microorganisms, such as the Gram-positive bacteria of the *Firmicutes* and *Actinobacteria* group. These bacteria have to be very adaptable to counteract life-threatening conditions. Therefore, bacteria have to monitor their environment constantly to be able to respond to changes before they suffer lethal damage. They developed signal transducing systems, which modulate these responses in order to survive under severe stress conditions (Msadek, 1999). One important example is the detection of antimicrobial substances, which often target the bacterial cell envelope.

1.1 Bacterial cell envelope and cell wall biosynthesis – a short overview

The envelope is a crucial structure of the bacterial cell. It gives the cell its shape, protects it against environmental threats and counteracts the high internal osmotic pressure (Delcour *et al.*, 1999, Höltje, 1998). The cell envelope of Gram-positive bacteria consists of the cytoplasmic membrane and a thick multilayered cell wall made up of peptidoglycan and teichoic acids (Foster & Popham, 2002). It differs significantly from the Gram-negative model, which contains a much thinner peptidoglycan layer lacking teichoic acids. In addition, the cell envelope of Gram-negative bacteria contains a periplasmic space surrounded by an outer membrane (Silhavy *et al.*, 2010).

The composition of peptidoglycan differs between species but its structure is always the same. It forms a polymer consisting of linear glycan strands cross-linked by short peptides. The glycan strands are composed of alternating *N*-acetylglucosamin (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) residues linked by β-(1,4)-glycosidic bonds. The Mur*N*Ac molecules are connected by pentapeptide bridges, which lead to the typical net-like structure (Vollmer *et al.*, 2008). The peptidoglycan biosynthesis starts in the cytoplasm with the conversion of fructose-6-phosphate in Glc*N*Ac. Next, Glc*N*Ac is activated by the addition of uridine diphosphate (UDP), leading to UDP-Glc*N*Ac, which is then converted to UDP-Mur*N*Ac. Afterwards, the pentapeptide is attached to UDP-Mur*NA*c followed by its connection to the lipid carrier undecaprenol-monophophate at the inner surface of the cytoplasmic membrane. This complex is called lipid I. The second sugar molecule, Glc*N*Ac, is subsequently coupled to the Mur*NA*c of lipid I, resulting in lipid II. This cell wall precursor is then transferred to the outer surface of the cytoplasmic membrane, where the disaccharide pentapeptide building block is incorporated into the already existing peptidoglycan net. The remaining

undecaprenol-pyrophosphate is dephosphorylated and flipped back to the inner surface of the cytoplasmic membrane. Thus, the lipid carrier gets recycled to restart coupling and transfer of another cell wall precursor. This process is called lipid II cycle (Delcour *et al.*, 1999, Foster & Popham, 2002) (Fig. 1.1).

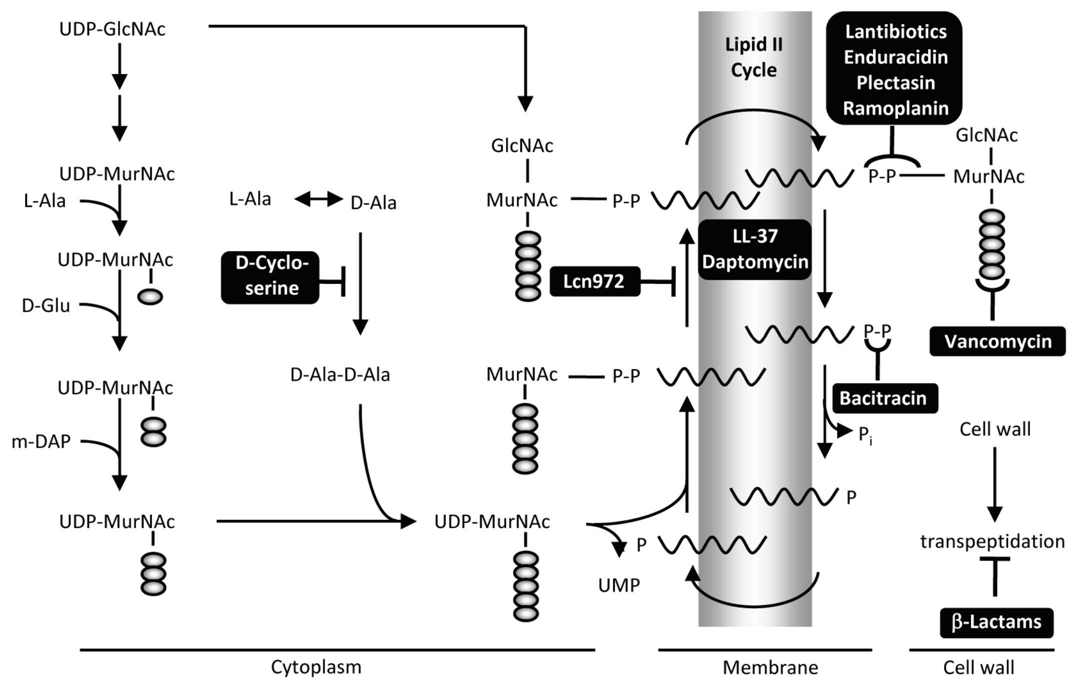

Figure 1.1: Cell wall biosynthesis of Gram-positive bacteria and its inhibition by antibiotics. Crucial steps in cell wall biosynthesis are schematically indicated, together with their cellular localization. Glc*NA*c, N-acetyl-glucosamine; Mur*NA*c, N-acetyl-muramic acid; UDP, uridine diphosphate; UMP, uridine monophosphate; P, phosphoryl group; P_i, inorganic phosphate. Amino acids are depicted as small gray circles, undecaprenol by the waved line. Antibiotics which inhibit crucial steps of the cell wall biosynthesis or target the cytoplasmic membrane (LL-37 and daptomycin) are highlighted in black. Lantibiotics is used as a collective term for actagardine, gallidermin, mersacidine, nisin, and subtilin.

1.2 Bacteria and cell envelope stress

Because of the essential functions of the bacterial cell envelope, it is a prime target for many antibiotics that interfere with cell wall biosynthesis (Jordan *et al.*, 2008) (see Fig. 1.1). Production of antibiotics can be viewed as a means of interspecies competition for survival in complex and densely populated habitats, such as the soil habitat (D'Costa *et al.*, 2006). Not surprisingly, soil bacteria are amongst the most prolific antibiotic producers (Berdy, 2005). In order to compete with other organisms, soil bacteria also need to have cunning and

sensitive reflexes to respond to antibiotic threat before the cells can take irreparable damage. Hence, both production of and resistance against antibiotics are part of the microbial warfare in complex habitats.

The ability of a bacterial cell to respond to changing environmental conditions necessitates the presence of signal transducing systems that respond to specific cues and initiate stimulus-specific cellular responses, usually through differential gene expression. In bacteria, three major regulatory principles are involved in transmembrane signaling: one-component systems (1CSs), two-component systems (2CSs), and alternative sigma factors of the extracytoplasmic function (ECF) family (Staroń & Mascher, 2010). In 1CSs, the sensory and regulatory domains are fused on a single polypeptide chain (Ulrich *et al.*, 2005). Because of this restrictive protein architecture, such systems predominantly respond to intracellular signals and play only a minor role in transmembrane signal transduction. In contrast, ECF sigma factors and 2CSs – which separate sensory and regulatory functions on two distinct proteins – can easily facilitate responses to intracellular, membrane-derived, and extracytoplasmic signals (Mascher *et al.*, 2006, Staroń *et al.*, 2009). Very often, such signaling systems are involved in sensing stress conditions which can potentially harm the microbial cell. In response, they mount protective countermeasures, very often through differential expression of target genes that are involved in stress-resistance (Storz & Hengge-Aronis, 2000).

This thesis focuses on one 2CS that is involved in orchestrating the cell envelope stress response (CESR) in Gram-positive bacteria with a low G+C content (*Firmicutes*). A classical 2CS consists of a membrane-anchored histidine kinase (HK) and a cytoplasmic response regulator (RR). The HK functions as sensor protein, which is able to sense specific stimuli with its N-terminal extracellular input domain. Thereupon, the HK undergoes an intramolecular conformational change leading to the autophosphorylation of a conserved histidine residue within the C-terminal transmitter domain. The cognate RR uses this phospho-HK as phosphoryl group-donor, resulting in the phosphorylation of a conserved aspartate residue within its N-terminal receiver domain. This phosphorylation activates the RR, which often leads to differential gene expression through binding to individual target promoter regions by its C-terminal effector domain. The system can be set back to the pre-stimulus state via dephosphorylation of the RR, catalyzed by a phosphatase activity of the cognate HK, the RR itself, or an external phosphatase (Mascher *et al.*, 2006, Stock *et al.*, 2000).

The response of the Gram-positive model organism *B. subtilis* to antibiotics has been particularly well studied during the last 10 years, both at the level of differential gene expression and protein production, by extensively applying transcriptomics and proteomics

approaches (Wecke & Mascher, 2011). In case of cell wall antibiotics, the underlying CESR regulatory network was thoroughly characterized and shown to consist of at least three ECF sigma factors and four 2CSs (Jordan *et al.*, 2008) (Fig. 1.2). Of the four 2CSs, three are paralogous to each other, the BceRS, PsdRS, and YxdJK 2CSs. They are referred to as BceRS-like 2CSs, based on the best-understand example. All of them are associated with genes encoding an ABC transporter, which are strongly induced in the presence of specific cell wall antibiotics and mediate antibiotic resistance against them (Jordan *et al.*, 2008, Staroń *et al.*, 2011). The LiaSR system is the fourth CESR 2CS of *B. subtilis*, which responds to a broader range of cell wall antibiotics (Fig. 1.2). While its physiological role is still unclear in *B. subtilis*, it seems to represent the primary general CESR system in many other *Firmicutes* bacteria (Jordan *et al.*, 2008). Both BceRS- and LiaSR-like 2CSs are widely distributed in this phylum and all systems investigated to date in a range of *Firmicutes* bacteria are involved in specific cell wall antibiotic resistances or more general CESRs (Jordan *et al.*, 2008).

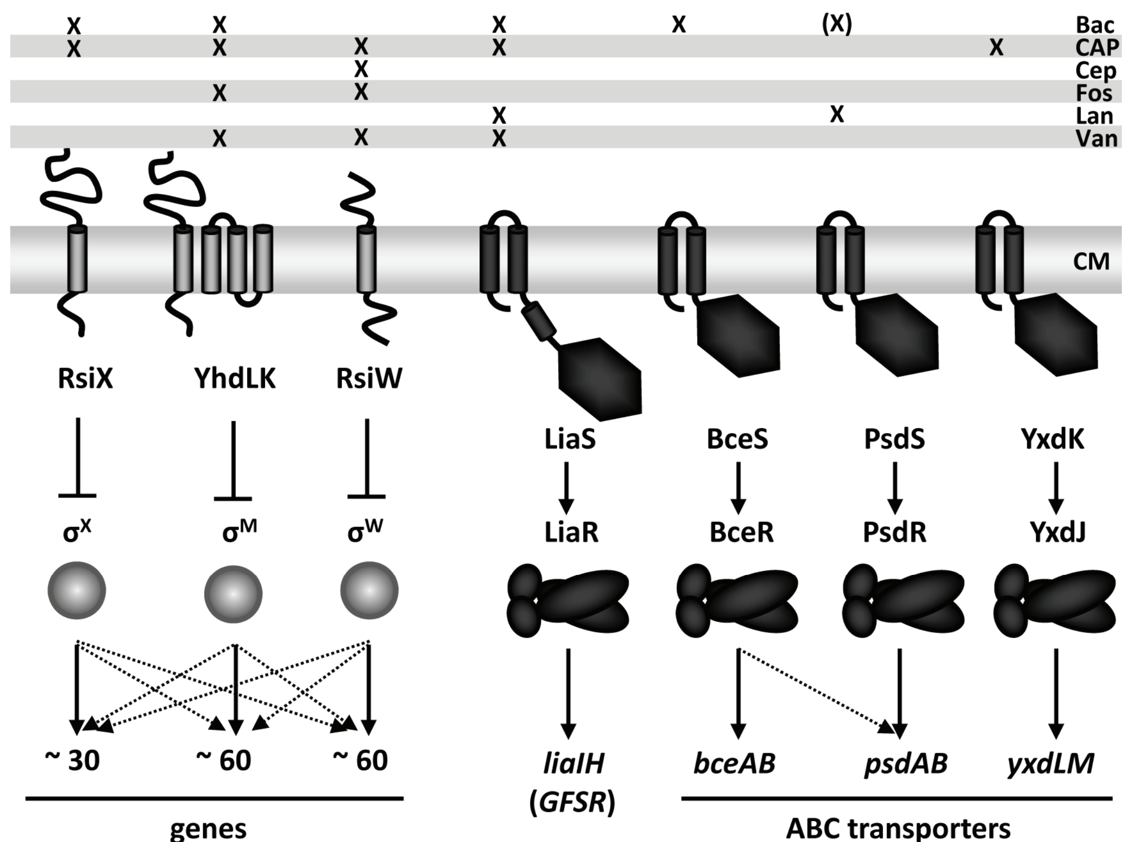

Figure 1.2: Regulatory network of the CESR of *B. subtilis*. ECF σ factors and the corresponding anti-σ factors are illustrated in medium gray. 2CSs are marked in dark gray. Transmembrane sensor proteins are shown on top, the regulator proteins below. The target genes are depicted at the bottom. Arrows indicate activation, T-shaped lines repression. Dotted lines show cross regulation. Selected antibiotics that induce the systems are illustrated above. Bac, bacitracin; CAP, cationic antimicrobial peptides; Cep, cephalosporin; Fos, fosfomycin; Lan, lantibiotics; Van, vancomycin; CM, cytoplasmic membrane (Jordan *et al.*, 2008, modified).

The focus of this thesis is the 2CS LiaSR of *B. subtilis* which will be described in detail in the following sections.

1.3 LiaFSR-like systems

1.3.1 The paradigm: LiaFSR of *B. subtilis*

LiaFSR-like cell envelope stress-sensing signal transduction systems are widely conserved among the *Firmicutes* group of Gram-positive bacteria. The best studied system so far is LiaFSR of *B. subtilis*, which was originally identified in the course of investigations on the bacitracin stimulon in this organism (Mascher *et al.*, 2003) (Fig. 1.3). LiaSR is one of four cell envelope stress sensing 2CSs in *B. subtilis*. Its name stands for "**l**ipid II cycle **i**nterfering **a**ntibiotic **s**ensor and response **r**egulator". LiaSR does primarily respond to the presence of cell wall antibiotics that interfere with the lipid II cycle, such as bacitracin, vancomycin, ramoplanin, or cationic antimicrobial peptides (CAMPs) (Mascher *et al.*, 2004, Pietiäinen *et al.*, 2005) (Fig. 1.2), but also to detergents, organic solvents (e.g. ethanol, phenol), and to more unspecific stimuli like secretion stress, alkaline shock, and filamentous phage infection, although to a weaker degree (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). A strong induction by membrane perturbing agents, such as daptomycin and rhamnolipids, was shown more recently (Hachmann *et al.*, 2009, Wecke *et al.*, 2011, Wecke *et al.*, 2009).

The HK LiaS belongs to the subgroup of so-called intramembrane-sensing (IM)-HKs possessing two membrane-spanning regions linked by a short extracytoplasmic loop of only 14 amino acids (Mascher, 2006, Mascher *et al.*, 2006). The second transmembrane helix is connected to a cytoplasmic HAMP domain, which is most likely involved in intramolecular signal transfer processes (Hulko *et al.*, 2006). The conserved HK core of LiaS contains the HisKA_3/dimerization (also DHp, standing for **d**imerization and **h**istidine **p**hosphotransfer) domain and the ATP-binding domain (HATPase_c = Histidine-Kinase-like-ATPase). The invariant histidine residue of the autophosphorylation site is located within the HisKA_3 domain.

LiaR is a typical two-domain RR protein with a conserved N-terminal receiver domain that contains the invariant aspartate residue, and a variable C-terminal DNA-binding domain. It is a member of the NarL/FixJ family of RRs sharing the characteristic helix-turn-helix motif responsible for binding to DNA (Galperin, 2006, Galperin, 2010).

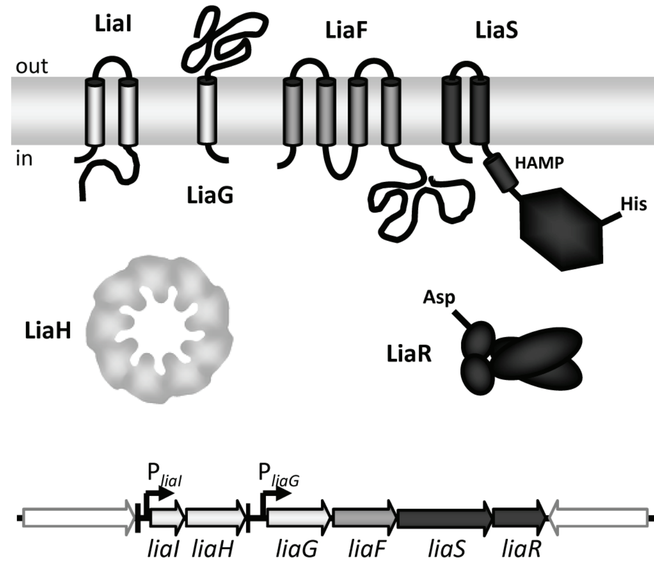

Figure 1.3: Schematic representation of the genes and proteins of the LiaFSR system of *B. subtilis*. Genes and proteins are marked in dark gray (2CS) and medium gray (LiaF). Other genes/proteins belonging to the *lia* operon are labeled in light gray. Genes flanking the *lia* operon are white. Promoters are marked with bent arrows, terminators are represented by vertical bars.

The LiaSR 2CS is genetically and functionally linked to a third protein, LiaF. This membrane protein contains three or four membrane-spanning regions in its N-terminus. The functionally important C-terminus of LiaF features no obvious conserved domains. LiaF was identified as controlling the LiaSR-dependent signal transduction in a repressive manner. Therefore, LiaF together with LiaSR constitutes a three-component system (Jordan *et al.*, 2006).

LiaFSR are encoded by the last three genes of the hexa-cistronic operon *liaIH-liaGFSR* (Fig. 1.3), whose expression is controlled in an autoregulative manner. A basal expression level of the last four genes, *liaGFSR*, is ensured by a weak constitutive promoter upstream of *liaG* (P_{liaG}) (Jordan *et al*., 2006). Inducing conditions lead to a strong LiaR-dependent activation of the promoter located directly upstream of *liaI* (P_{liaI}) resulting in an expression of two different transcripts: a major 1.1 kb transcript containing *liaIH* and a 4 kb transcript encompassing the entire locus. This pattern is due to a weak terminator structure located downstream of *liaH* (Mascher *et al*., 2004). P_{liaI} seems to be the only relevant target promoter controlled by LiaR, but the physiological role of LiaIH remains unclear so far (Wolf *et al.*, 2010). LiaH is a member of the phage shock protein (Psp) family. LiaI and LiaG are membrane proteins of unknown function.

In the absence of any external stress, the *liaI* promoter is transiently induced at the onset of stationary phase (Jordan *et al.*, 2007) (Fig. 1.4). During this transition state from exponential

to the stationary growth phase, *B. subtilis* cells undergo an intricate differentiation program to adapt to non-optimal living conditions. A complex regulatory cascade is initiated at this time point that orchestrates this transition from vegetative cells to the formation of dormant endospores (Errington, 2003, Msadek, 1999, Phillips & Strauch, 2002). During vegetative growth, the transition state regulator AbrB represses P_{liaI} via direct binding to the promoter sequence, thereby preventing P_{liaI} activity. Moreover, the repressor protein LiaF keeps the LiaFSR system switched off in the absence of suitable extracellular stimuli. The transition to stationary phase leads to increasing amounts of phosphorylated Spo0A, the master regulator of sporulation, which inhibits *abrB* expression, thus releasing P_{liaI} from AbrB-dependent repression. Simultaneously, a so far unknown stimulus activates the HK LiaS (and/or its release from LiaF repression) that in turn activates LiaR, resulting in the expression of the *liaIH* operon. The observed induction is significantly weaker (approx. 10-15 fold) compared to the response to strong inducers (approx. 100-fold) (Jordan *et al.*, 2007).

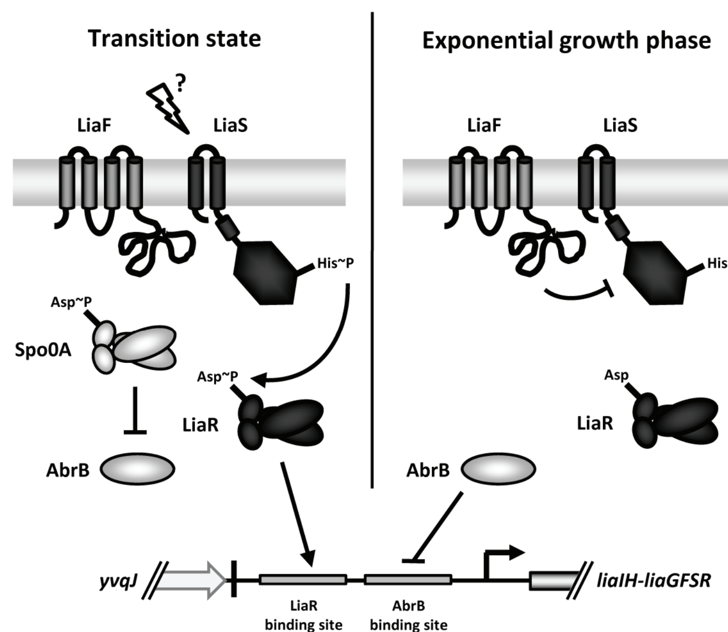

Figure 1.4: Transition state induction of P_{liaI} in *B. subtilis*. The regulatory proteins involved during exponential growth (right) and the transition state (left) are named and marked in light, medium, and dark gray. Arrows indicate activation, T-shaped lines repression. The genomic context of P_{liaI} is shown schematically below, including the LiaR- and AbrB-binding sites. See text for details.

In summary, LiaFSR-dependent gene expression is induced by cell envelope perturbating conditions and is also embedded in the transition state regulation of *B. subtilis* through the

interplay of at least five regulatory proteins: LiaR, LiaS, LiaF, AbrB, and Spo0A (Jordan *et al.*, 2007) (Fig. 1.4). The biological significance of this induction is still unclear.

1.3.2 Distribution and conservation of LiaFSR-like regulatory systems

As already mentioned, LiaFSR-like systems are widely conserved within the group of *Firmicutes* bacteria, with the exception of the genera *Clostridium* and *Lactobacillus* (Jordan *et al.*, 2008, Jordan *et al.*, 2006). In all species possessing *liaSR* homologs, *liaF*-like genes are always located directly upstream of the 2CS genes. Two groups were described based on the genomic context of the corresponding loci (Jordan *et al.*, 2006, Mascher, 2006). Group I includes *liaFSR* homologs found in *Bacillus* and *Listeria* species, which are regulatory linked to *liaIH* operons. The difference between these two genera is the location of the *liaIH*-like genes either as a part of the *liaIH-(G)FSR* locus (*Bacillus*) or genetically separated from the *liaFSR* operon, but still under the transcriptional control of LiaSR (*Listeria*). Additionally, *liaG* homologous genes are only found in *B. subtilis*, *Bacillus licheniformis*, and *Bacillus halodurans*.

Group II includes species that lack *liaIH* homologs. Here, only a *liaFSR* locus is conserved (Jordan *et al.*, 2006). In the following sections, the current state of knowledge about the LiaFSR-dependent cell envelope stress response in different *Firmicutes* species will be discussed.

1.3.2.1 LiaFSR-like systems of group I (*liaIH-liaFSR*) in other *Firmicutes* species

YvqEC of *B. licheniformis*. In-depth transcriptional profile analyses of *B. licheniformis* in response to cell wall antibiotics identified the LiaFSR homologous system YvqFEC (Wecke *et al.*, 2006). YvqFEC is strongly induced in the presence of bacitracin and, to a weaker degree, by vancomycin and D-cycloserine. Similar to *B. subtilis*, the YvqFEC system is encoded as part of the *yvqIH-yvqGFEC* locus, where *yvqIH* represents the *liaIH* and *yvqGFEC* the *liaGFSR* homologous genes. The expression of the *yvq* locus is controlled in an autoregulative manner by YvqC from a promoter upstream of *yvqIH*. In contrast to *B. subtilis*, three instead of two transcripts can be observed after induction, including the major transcript *yvqIH* (due to a stem loop structure downstream of *yvqH*), a transcript representing the whole locus, and an additional transcript of 2.5 kb in size (*yvqIH-yvqGF'*) caused by a second stem loop structure within the *yvqF* gene (Wecke *et al.*, 2006). Yvq mutants of *B. licheniformis* have not been analyzed yet. Therefore, the physiological role of this system is unknown. Moreover, YvqC target genes other than the *yvq* locus itself have not been identified so far. However, the close

homology between *B. subtilis* and *B. licheniformis* suggests that the two systems are very similar with regard to their behavior, physiological role and hence regulons.

LiaSR$_{Lm}$ of *Listeria monocytogenes*. More information is available on the LiaFSR homolog of *L. monocytogenes*, which was named LiaF$_{Lm}$-LiaSR$_{Lm}$ (formerly Lmo1020-1021-1022). Detailed transcriptional studies revealed a massive upregulation of this system by cell wall active antibiotics such as bacitracin and vancomycin. The regulon controlled by LiaR$_{Lm}$ is much larger than that of *B. subtilis*, including 29 genes organized in 16 transcriptional units (Fritsch *et al.*, 2011). In addition to the *liaIH$_{Lm}$* and *liaFSR$_{Lm}$* operons, transcription of genes encoding components of ABC transporters as well as predicted membrane or extracytoplasmic proteins was induced.

The most surprising difference between the Lia responses of *L. monocytogenes* and that of *B. subtilis* is a derepression of target genes in *liaS$_{Lm}$* mutants. The lack of the HK protein in *B. subtilis* leads to a "locked OFF" phenotype demonstrating a loss of response to adequate stimuli due to the lack of the sensor protein. In *L. monocytogenes*, it was postulated that the remaining LiaR$_{Lm}$ protein undergoes a non-specific phosphorylation via the phosphoryl group-donor acetyl phosphate, which can take place due to the lack of LiaS$_{Lm}$ phosphatase activity (Fritsch *et al*., 2011). The physiological role of the LiaFSR system in *L. monocytogenes* is so far unclear, since no resistance against inducing compounds could be detected (Fritsch *et al*., 2011).

1.3.2.2 LiaFSR-like systems of group II (*liaFSR*) in other *Firmicutes* species

VraSR of *Staphylococcus aureus*. The best studied LiaSR homolog, VraSR, was originally identified as one of the most strongly upregulated systems after treatment with vancomycin in vancomycin-resistant *Staphylococcus aureus* (VRSA) compared to a vancomycin-sensitive strain (VSSA) (Kuroda *et al.*, 2000). It is encoded as the last two genes of the tetra-cistronic autoregulated operon *orf1-yvqF-vraS-vraR* (Yin *et al.*, 2006). The VraSR system strongly responds to the presence of cell wall-interfering antibiotics like glycopeptides (e.g. vancomycin and teicoplanin), bacitracin, β-lactams, and D-cycloserine, but not to general stresses such as heat, high osmolarity, or pH shifts. A total of 46 genes were identified to be induced by VraR after exposure to vancomycin (Kuroda *et al.*, 2003). Some of them are associated with the cell wall biosynthesis, including *pbp2* and *sgtB* required for polymerization of peptidoglycan, and *murZ* crucial for murein monomer precursor synthesis.

The VraSR system plays a critical role in resistance against all of its inducers except D-cycloserine and levofloxiacin. The overproduction of pencillin-binding protein (PBP) 2

significantly increases the resistance to teicoplanin, showing that the reduction of observed teicoplanin resistance in *vraSR* null mutants is due to the loss of PBP2 induction (Gardete *et al.*, 2006, Kuroda *et al.*, 2003). Penicillin binds to PBPs, which are required for cell wall synthesis, and inactivates them. The resistance against the β-lactam antibiotic penicillin in almost all *S. aureus* strains is caused by the production of β-lactamases (Boyle-Vavra *et al.*, 2006). Accordingly, β-lactams that are insensitive to β-lactamase (e.g. methicillin, oxacillin) were used for treatment of *S. aureus* infections. However, methicillin-resistant *Staphylococcus aureus* (MRSA) strains evolved quickly and had acquired the *mecA* gene, which encodes a new PBP variant, PBP2a, with a low affinity to β-lactams. PBP2 and PBP2a work together to mediate oxacillin resistance, but its expression is not sufficient for the resistance phenotype. The recovery of the oxacillin resistance of a *vraS* mutant is not warranted by the overexpression of *mecA* or *pbp2*, but rather by the complementation with the entire *vra* operon. This shows that VraS is also required even when *mecA* and *pbp2* are transcribed. It is most likely that VraSR modulate the expression of one or several factors other than PBP2 and PBP2a that influence the oxacillin resistance (Boyle-Vavra *et al.*, 2006). More recently, the biochemistry of VraSR-dependent signal transduction has been intensively studied (Belcheva & Golemi-Kotra, 2008), as described in CHAPTER 4 (4.2.2).

CesSR of *Lactococcus lactis*. The CesSR system (formerly LlkinD-LlrD; also TCS-D) was originally identified in the *L. lactis* strain MG1363 as induced during the onset of stationary phase and involved in the resistance to salt and osmotic stress (O'Connell-Motherway *et al.*, 2000). Later, it was demonstrated that CesSR is strongly induced in the presence of the bacteriocin lactococcin 972 (Lcn972) (Martinez *et al.*, 2007), which is a ribosomally synthesized antimicrobial peptide produced by lactic acid bacteria. A genome-wide transcriptional analysis of *L. lactis* revealed 26 upregulated genes after Lcn972 treatment, of which 21 are controlled by CesR (Martinez *et al.*, 2007). Some of these genes encode proteins involved in peptidoglycan biosynthesis and modification. CesR also regulates the transcription of its own tri-cistronic operon, which includes the *liaF* homolog *yjbB* (*llmg1650*). The highest upregulation in CesSR-dependent gene expression was detected for *llmg0169* and the operon *llmg2164-2163* (Martinez *et al.*, 2007). While all three genes encode proteins with unknown function, Llmg2163 harbors an N-terminal PspC domain. PspC was originally described for the phage shock response of *E. coli* and proteins harboring this domain are known to protect the cell against extracytoplasmic stress and to maintain the integrity of the cytoplasmic membrane. The overexpression of *llmg2164-2163* was found to specifically protect *L. lactis* cells against Lcn972, but not against other cell wall antibiotics (Roces *et al.*, 2009).

The CesSR system is also induced in the presence of lipid II-interfering antibiotics like bacitracin, nisin, or plantaricin C. Deletion of *cesR* leads to an increased susceptibility to these agents, suggesting that CesSR mediates resistance against them (Martinez *et al*., 2007). Additionally, peptidoglycan hydrolysis due to the exposure to lysozyme is also detected by CesSR (Veiga *et al.*, 2007).

LiaSR of *Streptococcus mutans*. LiaSR (formerly HK11-RR11) of *S. mutans* was identified as one of several systems that affect the expression of virulence factors in this organism (Li *et al*., 2002). LiaFSR was shown to be involved in biofilm formation and acid tolerance, and LiaS was suggested to be a pH sensor (Chong *et al.*, 2008, Li *et al*., 2002).

LiaFSR of *S. mutans* is encoded within the penta-cistronic operon *liaFSR-ppiB-pnpB*. The genes *ppiB* and *pnpB* encode a peptidyl-prolyl *cis*/*trans* isomerase and a polynucleotide phosphorylase, respectively. As in *B. subtilis*, the LiaFSR system of *S. mutans* is induced by lipid II-interfering antibiotics like bacitracin, vancomycin, and nisin as well as by other cell wall antibiotics, such as chlorhexidine. Moreover, it is induced by low pH, high osmolarity, and ethanol. β-lactams and D-cycloserine do not function as inducers (Suntharalingam *et al.*, 2009).

The LiaFSR system of *S. mutans* clearly differs from that of *B. subtilis* in terms of the time point of the stimulus-independent induction. The expression of *S. mutans lia* genes is 10-fold repressed in stationary phase and 2-fold in mid-logarithmic phase compared to the early-logarithmic growth phase. This observation suggests an involvement of LiaFSR in early log-phase growth, which is characterized by high growth rates, increased cell division, cell separation, and peptidoglycan biosynthesis (Suntharalingam *et al*., 2009). Indeed, *S. mutans* LiaR directly and/or indirectly controls a large regulon consisting of 174 genes during biofilm growth encoding proteins involved in membrane protein synthesis and peptidoglycan biosynthesis, envelope chaperone/proteases, and transcriptional regulators (Perry *et al.*, 2008, Suntharalingam *et al*., 2009).

The LiaFSR system of *S. mutans* was also associated with playing a role in activation of the essential WalRK-like 2CS VicRKX (also named CovRSX) (Dubrac *et al.*, 2008, Tremblay *et al.*, 2009), which controls the expression of fructosyltransferase, glucosyltransferases, and glucan-binding proteins (Lee *et al.*, 2004, Senadheera *et al.*, 2005). It is active during exponential growth at neutral pH, when a rapid delivery of cell wall precursors due to fast rates of cell wall biosynthesis is required. Induction of VicRKX in the presence of cell wall antibiotics was shown to be controlled by LiaFSR rather than by autoregulation (Tremblay *et al*., 2009). Despite the wide distribution of WalRK-like and LiaFSR-like 2CSs, this is so far the only documented direct regulatory connection between such two systems.

LiaFSR of *Streptococcus pneumoniae*. The LiaFSR-homologous system TCS03 of *S. pneumoniae* was found to be upregulated after exposure to vancomycin in both a vancomycin-sensitive and vancomycin-tolerant strain (Haas *et al.*, 2005). In a later study, bacitracin, nisin, and tunicamycin were described to induce the TCS03, whereas D-cycloserine and ampicillin did not (Eldholm *et al.*, 2010). Interestingly, the same authors did not detect any upregulation of TCS03 after vancomycin treatment in laboratory strain R6, indicating strain-specific differences. In addition, cell envelope stress elicited by murein hydrolases and autolysins is also perceived by TCS03 (Eldholm *et al*., 2010). TCS03 does not seem to provide resistance against the lipid II-interfering antibiotics, which act as inducers of the system. Instead of a resistance phenotype, a physiological role as protectant against self-lysis in competent *S. pneumoniae* cells was proposed (Eldholm *et al.*, 2010, Eldholm *et al.*, 2009). This observation again indicates that LiaFSR-like systems, despite comparable inducer ranges, have adapted their very diverse physiological roles to the specific needs of the respective organism.

The TCS03-encoding operon consists of 10 genes (*spr0342*-*spr*0351) and is the most strongly induced target of the *S. pneumoniae* LiaR-dependent gene expression. The first three genes encode LiaFSR (*spr*0342-0345) (Eldholm *et al.*, 2010). A genome-wide transcriptional profiling revealed 18 genes that are upregulated in a LiaR-dependent manner, ten of which are already part of the LiaFSR operon and some other target genes encode stress-related proteins, which are involved in the response to heat shock. Moreover, genes encoding a Spx homolog as well as the phage shock protein C (PspC) are induced, which were also found as part of the CesSR regulon in *L. lactis* and the LiaSR regulon in *S. mutans* (Eldholm *et al*., 2010, Martinez *et al*., 2007, Suntharalingam *et al*., 2009).

1.3.3 Stimuli of Lia-like systems

LiaFSR-homologous systems respond to a relatively broad range of stimuli, which are all associated with cell envelope stress. Therefore, these systems presumably represent cell wall damage-sensing systems in contrast to drug-sensing detoxification modules like BceRSAB (Rietkötter *et al.*, 2008). Despite a wide range of physiological functions associated with LiaFSR-like systems, the range of inducers is nevertheless well defined.

Three different classes of stimuli can be distinguished. The first class contains lipid II cycle inhibitors like bacitracin, nisin, or vancomycin, which are common inducers of all described LiaFSR-like systems (Fig. 1.1). They belong to the class of antimicrobial peptides (AMPs) which share a cationic and amphipathic nature as well as a similar mode of action. They all inhibit the cell wall biosynthesis by binding to different moieties of either lipid II or

undecaprenol pyrophosphate (UPP) (Rietkötter *et al*., 2008, Schneider & Sahl, 2010, Staroń *et al*., 2011). Bacitracin specifically binds to UPP and prevents its dephosphorylation/recycling, thus blocking cell wall biosynthesis (Storm & Strominger, 1973). Nisin disrupts the membrane integrity by pore formation via initial binding to the pyrophosphate of lipid II (Schneider & Sahl, 2010). Vancomycin inhibits the transpeptidation of peptidoglycan by binding to the D-Ala-D-Ala terminus of the pentapeptide chain of lipid II (Schneider & Sahl, 2010). The bacteriocin lactococcin 972 (Lcn972), which was described as the strongest inducer of CesSR in *L. lactis*, inhibits the peptidoglycan synthesis by binding to lipid II at the level of septum formation, thereby blocking cell division (Martinez *et al.*, 2008) (Fig. 1.1).

The second class of inducers includes agents that do not interfere with the lipid II-cycle, but also influence the integrity of the cell envelope. Good examples are daptomycin, that affects the cell membrane causing membrane depolarization or perforation (Silverman *et al.*, 2003, Straus & Hancock, 2006), rhamnolipids, which are biosurfactans that alter the cell surface leading to increased hydrophobicity and membrane permeability (Vasileva-Tonkova *et al.*, 2011), as well as more general stresses like alkaline shock, detergents, phenol, ethanol, secretion stress, and infections by filamentous phages (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).

Class three contains inducers that are characteristic of the specific habitat or lifestyle of the different species, but all of them affect the integrity of the bacterial cell envelope. For example, the LiaFSR system of *S. mutans* responds to acidity (Li *et al*., 2002), a typical condition in the human oral cavity. Furthermore, *B. subtilis* LiaFSR is embedded in the transition state regulation leading to the induction of this system not by an external but rather by a so far unknown internal stimulus (Jordan *et al*., 2007) (Fig. 1.4). Another example is the induction of *S. pneumoniae* LiaFSR by murein hydrolases and autolysins in competent cells to protect against self-lysis (Eldholm *et al*., 2010).

The signal transfer within 2CSs like LiaSR via phosphorylation enables a specific response to specific stimuli. Normally, the activation of the RR protein via phosphorylation depends on the autophosphorylation of the cognate HK which then serves as phosphoryl group-donor (Stock *et al*., 2000). But an alternative HK-independent way of RR phosphorylation is by acetyl phosphate. The role of this small molecule phosphoryl group-donor will be described in the following sections.

1.4 Physiological role of acetyl phosphate in two-component signal transduction pathways

1.4.1 Biosynthesis of acetyl phosphate

Acetyl phosphate is a small high-energy molecule with a larger change of free standard enthalpy (ΔG°) of hydrolysis (-43.3 kJ/mol) than ATP (-30.5 kJ/mol). Therefore, it stores more energy than ATP which provides the possibility to generate ATP by substrate phosphorylation (Lehninger *et al.*, 1994, Madigan *et al.*, 2003). Acetyl phosphate is generated as an intermediate of the Pta-AckA pathway (Fig. 1.5). In this pathway, acetyl phosphate is synthesized from acetyl-CoA catalyzed by the enzyme phosphotransacetylase (Pta). Subsequently, the acetyl phosphate is converted to acetate by the acetate kinase (AckA), which leads to the generation of one molecule ATP. The produced acetate is then released into the medium. This so called acetogenesis is a reversible reaction, in which all steps proceed in the opposite direction if acetate is used as carbon source (Rose *et al.*, 1954).

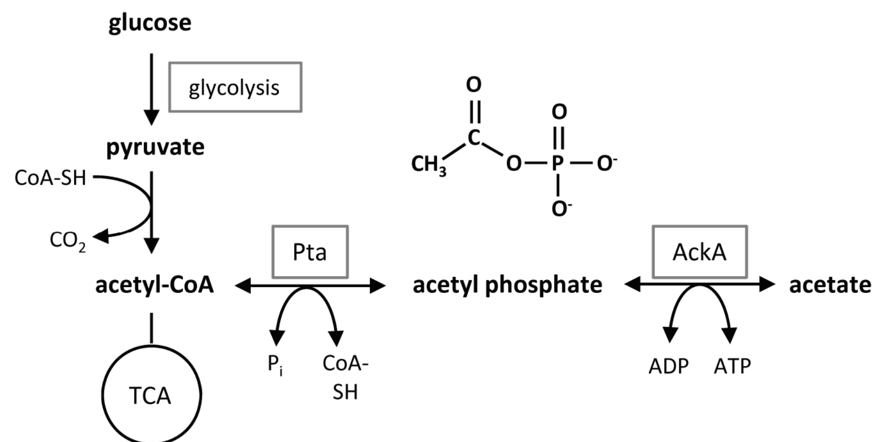

Figure 1.5: Pta-AckA pathway and molecular formula of acetyl phosphate. Pta, phosphotransacetylase; AckA, acetate kinase; TCA, tricarboxylic acid cycle. See text for details.

The Pta-AckA pathway has important cellular functions: it facilitates glycolytic flux and rapid growth in the presence of excess carbon sources, it recycles CoA-SH, and it provides the majority of ATP in the absence of a functional tricarboxylic acid (TCA) cycle (Wolfe, 2005) (see below for details). The latter is especially important for organisms that do not possess a complete TCA cycle, e.g. *Yersinia pestis*, *S. pneumoniae*, and some strains of *S. aureus* (Somerville *et al.*, 2003, Wolfe, 2010).

Mutants lacking either *pta* or *ackA* or both genes show distinct phenotypes. During aerobic growth on glucose, *pta* mutants are not able to synthesize acetyl phosphate, whereas *ackA* mutants accumulate acetyl phosphate. Growth on acetate inverts the order of reactions and thus leads to opposite phenotypes of the respective mutants. The *pta-ackA* double mutant lacks the ability to synthesize acetyl phosphate under any conditions (Klein *et al.*, 2007).

1.4.2 Regulation of the cellular acetyl phosphate pool

The cellular acetyl phosphate concentration depends on the expression and activity of the Pta-AckA pathway, primarily mirrored by the energy/nutrient state of the cell and the growth phase (Prüß & Wolfe, 1994, Wolfe *et al.*, 2003, Klein *et al.*, 2007, Wolfe, 2005). During the exponential growth phase, the acetyl phosphate pool increases rapidly in the presence of oxygen and sufficient nutrients. Depletion of these nutrients during the transition to stationary phase leads to a decrease of acetyl phosphate concentrations in the cell (Prüß & Wolfe, 1994). After entering the stationary phase, the acetyl phosphate pool increases again most likely due to starvation and/or the lack of oxygen (McCleary & Stock, 1994).

Environmental factors like the availability of oxygen, the pH, the temperature, as well as the type and amount of available carbon sources affect the synthesis and/or activity of Pta and AckA (Prüß & Wolfe, 1994, Wolfe, 2005). For example, increasing growth temperatures (in the range of physiological temperatures from 32 to 40°C) lead to decreased *ackA* expression levels and an increase in Pta activity, which results in an accumulation of acetyl phosphate (Prüß & Wolfe, 1994). In the presence of excess carbon sources like glucose or other glycolytic intermediates, respiration via the TCA cycle is repressed (Crabtree effect), which leads to a conversion of acetyl-CoA by the Pta-AckA pathway und thus an increased acetyl phosphate pool (Crabtree, 1929, Holms, 1996, Holms, 1986). Likewise, the transition from aerobic to anaerobic conditions results in fermentation and an inoperable TCA cycle, which also leads to elevated acetyl phosphate concentrations (Nystrom, 1994). In contrast, the acetyl phosphate pool decreases under conditions that do not exceed the capacity of the TCA cycle, e.g. transition from exponential to stationary growth phase (Wolfe, 2005, Prüß & Wolfe, 1994, Wolfe *et al.*, 2003). Not only the amount but also the type of carbon source affects the acetyl phosphate concentration (Klein *et al.,* 2007, McCleary & Stock, 1994). For example, cells grown in the presence of glucose show a 50% decrease in acetyl phosphate compared to cells cultured with pyruvate (Klein *et al.*, 2007).

Taken together, bacterial cells adjust their acetyl phosphate pool in response to both growth phase and temperature by modulating the availability of acetyl-CoA, the expression of *ackA*, and the activity of Pta (Prüß & Wolfe, 1994, Wolfe, 2005).

1.4.3 Acetyl phosphate as global signal

Several lines of evidence support the hypothesis that acetyl phosphate acts as global signal in the bacterial cell: (i) acetyl phosphate is able to phosphorylate RRs of 2CSs *in vitro* (Wolfe, 2005), (ii) the acetyl phosphate pool varies significantly in response to environmental conditions (Keating *et al.*, 2008, Klein *et al.*, 2007) (see above), (iii) the cellular acetyl phosphate concentration in *E. coli* wild type cells reaches at least 3 mM, which is sufficient for an efficient phosphorylation of RRs *in vitro* (Klein *et al.*, 2007), and (iv) acetyl phosphate can influence the expression of almost 100 genes in *E. coli*, which was proven by comprehensive DNA macroarray analysis (Wolfe *et al.*, 2003).

These clues support the hypothesis that acetyl phosphate has physiological relevant properties by acting as direct phosphoryl group-donor for RRs.

1.4.4 *In vivo* relevance of acetyl phosphate for the phosphorylation of RRs

Several RR proteins, such as CheY, NR_I, PhoB, or OmpR, were described in the literature to have the ability to use acetyl phosphate as phosphoryl group-donor *in vitro* (Da Re *et al.*, 1999, Feng *et al.*, 1992, Lukat *et al.*, 1992, Mayover *et al.*, 1999, McCleary, 1996, McCleary & Stock, 1994). The *in vivo* relevance of this reaction is still debated controversially since an acetyl phosphate-dependent phosphorylation of RRs is usually only observed after deletion of the cognate HKs (Wolfe, 2005). However, in recent years the first indications for a physiological relevant role of acetyl phosphate at least for some 2CSs have emerged (see below).

RRs that are acetyl phosphate-sensitive under physiological relevant conditions feature three major characteristics: (i) they do not possess a cognate HK (e.g. RssB), (ii) they are present in excess over the cognate HK (e.g. OmpR), or (iii) the cognate HK acts mainly as a net phosphatase (e.g. RcsB, NR_I) (Wolfe, 2010). Examples of all three categories are described in the following paragraphs.

RcsBCD of *E. coli*. As mentioned above, 96 genes were identified in *E. coli* to be regulated by acetyl phosphate (Wolfe *et al.*, 2003). Most of these genes are involved in flagella biosynthesis and encapsulation. Prüß and Wolfe described the influence of acetyl phosphate on flagellation, but they did not identify the underlying RR, which is phosphorylated by acetyl phosphate (Prüß & Wolfe, 1994). In a recent study by Fredericks and co-workers, the responsible regulator was identified as RcsB (Fredericks *et al.*, 2006). RcsB is part of the Rcs phosphorelay consisting of the core proteins RcsC, a hybrid HK-RR, the histidine phosphotransferase RcsD, and the RR RcsB (Clarke *et al.*, 2002, Stout, 1994, Stout &

Gottesman, 1990, Takeda *et al.*, 2001). The Rcs phosphorelay represses genes required for flagellation and activates genes required for capsular biosynthesis and multiple stresses (Boulanger *et al.*, 2005, Davalos-Garcia *et al.*, 2001, Francez-Charlot *et al.*, 2003, Gottesman *et al.*, 1985). Flagellation inhibition occurs through the ability of RcsB to repress the transcription of the *flhDC* operon, encoding the master regulator of flagella biogenesis. Acetyl phosphate donates its phosphoryl group to RcsB even in the presence of the cognate HK RcsC, which causes the regulation of RcsB target genes. This is possible because RcsC operates primarily as an RcsB~P net phosphatase and the balance between phosphorylation and dephosphorylation determines the degree of activation of capsular biosynthesis and inhibition of flagellar biogenesis (Fredericks *et al.*, 2006).

NtrBC of *E. coli*. Another example of an acetyl phosphate-sensitive RR is NR_I (also known as NtrC), a member of the complex sensory system that monitors the availability of nitrogen. Under limiting nitrogen conditions, the cognate HK NR_{II} (also known as NtrB) operates as a kinase resulting in the phosphorylation of NR_I and the transcriptional activation of the *glnALG* operon. This operon encodes a 2CS (*glnL* and *glnG*) as well as the glutamine synthetase (*glnA*) which interconverts L-glutamate and ammonia to L-glutamine. In the presence of suitable amounts of nitrogen, NR_{II} primarily acts as a net phosphatase leading to a reduction of *glnALG* transcription. Small amounts of NR_I~P are usually sufficient for *glnALG* transcription. Here, the phosphoryl group-donor was identified to be either NR_{II}~P or acetyl phosphate. However, larger amounts of phosphorylated NR_I are necessary if the cells grow on secondary nitrogen sources. In this case, both donors, NR_{II}~P and acetyl phosphate, are necessary to provide the required large amounts of NR_I~P (Ninfa *et al.*, 2000).

EnvZ/OmpR of *E. coli*. The EnvZ/OmpR 2CS regulates the expression of the outer membrane porins OmpC and OmpF under certain osmolarity conditions (Forst & Roberts, 1994, Inouye & Dutta, 2003). The phosphorylation of the RR OmpR primarily depends on its cognate HK EnvZ, but an alternative pathway via acetyl phosphate has been described (Matsubara & Mizuno, 1999, Shin & Park, 1995). The response to changing osmolarity conditions seems not to be regulated solely by EnvZ. It was shown that acetyl phosphate in combination with an additional unidentified HK can influence porin transcription through OmpR phosphorylation (Matsubara & Mizuno, 1999). Mutants lacking both *pta* and *ackA* as well as the gene *sixA*, encoding a phospho-histidine phosphatase, severely altered the expression profile of *ompC* and *ompF*, resulting in a constitutive expression of *ompC*. These events take place in the presence of EnvZ (Matsubara & Mizuno, 1999). Furthermore, phosphorylated OmpR has also an influence on flagella biosynthesis. It was shown that OmpR represses the transcription of *flhDC* in an acetyl phosphate-dependent manner by increasing osmolarity, irrespective of the presence of EnvZ (Shin & Park, 1995). Additionally, OmpR is synthesized in significant larger

amounts compared to its cognate HK EnvZ in *E. coli* (3500:100 molecules per cell) (Cai & Inouye, 2002). It seems likely that some OmpR molecules escape the kinase/phosphatase activities of EnvZ, which makes them available for the phosphorylation by acetyl phosphate (Shin & Park, 1995).

RssB of *E. coli*. RssB is an orphan RR lacking a cognate HK. It is responsible for the rapid degradation of the sigma factor (σ^S), which is encoded by the gene *rpoS* (Muffler *et al.*, 1996). σ^S is a stationary phase specific sigma factor, whose turnover is repressed under carbon starvation, shift to high osmolarity, or heat shock. Such conditions lead to increased cellular levels of σ^S and the activation of genes with stress-protecting functions. It was shown that acetyl phosphate does not affect the synthesis of σ^S, but its degradation. The half-life of σ^S increases 2.5-fold in acetyl phosphate-free mutants (Δ*pta*/Δ*ackA*). Therefore, acetyl phosphate modulates the activity of RssB by phosphorylation and hence the turnover of σ^S (Bouche *et al.*, 1998).

1.4.5 Acetyl phosphate-insensitive RRs

In addition to these examples of acetyl phosphate-sensitive RRs, an acetyl phosphate-insensitive RR was recently described (Boll & Hendrixson, 2011). FlgR is the RR of the FlgSR 2CS of *Campylobacter jejuni*, whose phosphorylation is required for the expression of the σ^{54} regulon, including flagellar rod and hook genes (Hendrixson & DiRita, 2003, Wosten *et al.*, 2004). FlgR belongs to the family of NtrC-like RRs, which feature an essential C-terminal DNA-binding domain (CTD) important for the interaction with the target promoter sequence (Huala & Ausubel, 1989, North & Kustu, 1997, Shiau *et al.*, 1993). In FlgR, the CTD also binds DNA, but this interaction seems to be not essential for gene expression (Boll & Hendrixson, 2011). The activity of an FlgR mutant lacking its CTD ($FlgR_{\Delta CTD}$) can be modulated by acetyl phosphate in the presence and absence of the cognate HK FlgS, which is not the case for wild type FlgR proteins. It seems that the CTD limits phosphotransfer from acetyl phosphate to FlgR, making it a key specificity determinant in the FlgSR 2CS (Boll & Hendrixson, 2011).

Possible explanations of how this CTD limits cross-talk by undesirable acetyl phosphate-dependent phosphorylation are interdomain contacts between the receiver domain and the DNA-binding domain within RRs that seem to stabilize the inactive conformation of the receiver domain (Barbieri *et al.*, 2010). This was demonstrated for RRs of the OmpR/PhoB-subfamily, but this mechanism could be nevertheless a common feature of cross-talk prevention for RRs of other groups.

1.5 Aims of this thesis

In *B. subtilis*, the mechanisms of stimulus perception and signal transduction of the cell envelope stress-sensing 2CS LiaSR are not well-understood. Therefore, the main objectives of this thesis were to get a deeper insight into the underlying mechanisms and to characterize the function of the repressor protein LiaF.

CHAPTER 2 is the main part of this work and deals with the stoichiometry of the LiaFSR system. A correlation between the protein copy numbers of LiaF, LiaS, and LiaR and a proper response to cell envelope stress was discovered. Different *lia* mutants, in which the ratios of the three proteins were changed, showed distinct phenotypes resulting in a constitutive activity of P_{liaI} if LiaS or LiaR are overproduced, even under non-inducing conditions. These observations lead to analyzing the stoichiometry of the native LiaFSR system in detail. The relative LiaFSR ratios were first determined by a genetic approach and then directly measured by quantitative Western blot analysis. Second, LiaS was investigated with regard to a possible bifunctionality including both kinase and phosphatase activity. Third, the *in vivo* relevance of acetyl phosphate for activating the Lia system was investigated in detail. In a previous study, phosphorylation of LiaR by acetyl phosphate was proven *in vitro* (Wolf *et al*., 2010), but the evidence whether LiaR is acetyl phosphate-sensitive under physiologically relevant conditions was the aim of the present thesis.

Another aim of this thesis was the establishment of a novel protein expression system for *B. subtilis* that is based on the *liaI* promoter (CHAPTER 3). It possesses some advantages making it a perfect candidate for controlled heterologous protein expression: (i) P_{liaI} is basically shut off under non-inducing conditions during the exponential grows phase, (ii) it shows an impressive dynamic range of induction, which is 100- to 1000-fold in the presence of a suitable stimulus, (iii) the induction of P_{liaI} occurs already 5 to 10 minutes after addition of the inducer, and (iv) the induction is inducer concentration-dependent. These positive features were used to generate appropriate expression vectors and expression hosts in order to get a new protein (over-)expression toolbox.

2 Stoichiometry and perturbation studies of the LiaFSR system of *Bacillus subtilis*

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Stoichiometry and perturbation studies of the LiaFSR system of *Bacillus subtilis*

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Summary

The response regulator/histidine kinase pair LiaRS of *Bacillus subtilis*, together with its membrane-bound inhibitor protein LiaF, constitutes an envelope stress-sensing module that is conserved in Firmicutes bacteria. LiaR positively autoregulates the expression of the *liaIH-liaGFSR* operon from a strictly LiaR-dependent promoter (P_{liaI}). A comprehensive perturbation analysis revealed that the functionality of the LiaFSR system is very susceptible to alterations of its protein composition and amounts. A genetic analysis indicates a LiaF:LiaS:LiaR ratio of 18:4:1. An excess of LiaS over LiaR was subsequently verified by quantitative Western analysis. This stoichiometry, which is crucial to maintain a functional Lia system, differs from any other two-component system studied to date, in which the response regulator is present in excess over the histidine kinase. Moreover, we demonstrate that LiaS is a bifunctional histidine kinase that acts as a phosphatase on LiaR in the absence of a suitable stimulus. An increased amount of LiaR – both in the presence and in the absence of LiaS – leads to a strong induction of P_{liaI} activity due to phosphorylation of the response regulator by acetyl phosphate. Our data demonstrate that LiaRS, in contrast to other two-component systems, is non-robust with regard to perturbations of its stoichiometry.

Introduction

Two-component systems (2CSs) are a ubiquitously distributed principle of signal transduction that allows a bacterial cell to respond to changes in environmental and cellular parameters (Stock *et al.*, 2000; Mascher *et al.*, 2006; Gao and Stock, 2009). Typically, these systems consist of a sensor histidine kinase (HK) and a cognate response regulator (RR). In the presence of a suitable stimulus (= input), the HK undergoes a conformational change, which leads to the activation of the catalytic domain and ultimately to the autophosphorylation of an invariant histidine residue. Subsequently, the phospho-HK serves as phospho-donor for the corresponding RR, leading to the phosphorylation of a conserved aspartate residue in the receiver domain of this RR. This phosphorylation leads to the activation of the RR, which can then mediate the cellular response, usually through differential expression of its target genes (= output). The system can be set back to the pre-stimulus state by dephosphorylation of the response regulator (Parkinson, 1993; Stock *et al.*, 2000). This activity can be provided by specific phosphatases, but mostly it is the intrinsic property of the cognate HKs themselves, which are often bifunctional kinases/phosphatases. In the absence of a stimulus, such HKs act as phosphatases, thereby reducing or even preventing undesired phosphorylation of the cognate RR both from cross-talk and through the cellular pool of acetyl phosphate (Laub and Goulian, 2007).

The LiaRS system is one of over 30 2CSs encoded in the genome of the Gram-positive model organism *Bacillus subtilis* (Fabret *et al.*, 1999). This 2CS was originally identified as one of the signalling devices orchestrating the cell envelope stress response in this organism (Mascher *et al.*, 2003). LiaRS strongly responds to the presence of a number of cell wall antibiotics, such as bacitracin, daptomycin, nisin, ramoplanin and vancomycin (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005; Hachmann *et al.*, 2009; Wecke *et al.*, 2009) and is also weakly induced by other more unspecific stresses that interfere with envelope integrity, such as alkaline shock and secretion stress (Wiegert *et al.*, 2001; Hyyryläinen *et al.*, 2005). Upon activation, phosphorylated LiaR strongly induces expression from the strictly LiaR-dependent promoter P_{liaI} upstream of the *liaIH-liaGFSR* operon, thereby also autoregulating expression of the LiaRS 2CS. This leads to the generation of two distinct transcripts: the major *liaIH* transcript and a transcript of the entire *liaIH-liaGFSR* locus. In the absence of a suitable stimulus, the expression of the LiaRS 2CS is ensured by a weak constitutive promoter, P_{liaG}, located

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upstream of *liaG* (Jordan *et al.*, 2006). Phylogenomic studies revealed that LiaRS is a conserved cell envelope stress-sensing 2CS present in most Firmicutes (low G+C Gram-positive) bacteria (Jordan *et al.*, 2006; 2008). It is linked by genomic context and function to *liaF*, encoding a membrane protein that acts as a specific inhibitor of LiaRS. In *liaF* deletion mutants, the LiaRS 2CS is constitutively active even in the absence of envelope stress (Jordan *et al.*, 2006). This observation was later confirmed by a study of the homologous system in *Streptococcus mutans* (Suntharalingam *et al.*, 2009).

Previous studies have shown that the LiaR-dependent target promoter upstream of the *liaIH* operon (P_{liaI}) is tightly regulated by at least three different transcriptional regulators, LiaR, AbrB and Spo0A (Jordan *et al.*, 2007). The close interplay between the corresponding regulatory cascades ensures that this promoter is almost shut off during exponential growth. Under inducing conditions, the activity of P_{liaI} can increase almost three orders of magnitude in a LiaR-dependent fashion (Mascher *et al.*, 2004; Jordan *et al.*, 2006), with its primary target, LiaH, becoming the most abundant cytosolic protein in *B. subtilis* (Wolf *et al.*, 2010). LiaH is a homologue of the *Escherichia coli* phage shock protein (Psp) A. The latter maintains the proton motive force under extracytoplasmic stress conditions that affect the membrane integrity (Model *et al.*, 1997; Darwin, 2005). Because of the similar structure of PspA and LiaH and an overlapping inducer spectrum, we hypothesize that LiaFSR mounts a PspA-like response (Wolf *et al.*, 2010). The small membrane protein LiaI seems to act as membrane anchor for LiaH. The cellular function of LiaG is not known so far.

We have previously shown that the P_{liaI} activity can be locked in constitutive 'ON' or 'OFF' states in a *liaF* or *liaR* mutant respectively (Wolf *et al.*, 2010). Both of these extreme situations are exclusively Lia-dependent. In aiming to unravel the mechanism behind the tight regulation and impressive dynamic range of P_{liaI}-dependent transcription initiation, we focused our attention on the mechanism of signal transduction mediated by the LiaFSR system.

This article describes the results of comprehensive genetic and biochemical studies on the stoichiometry of the LiaFSR system. A systematic deletion/complementation screen revealed that the LiaFSR system is very susceptible to alterations of its protein compositions and amounts. Under native conditions, the amount of LiaF exceeds that of LiaS. Likewise, an excess of the HK LiaS over LiaR is also crucial for the functionality of the LiaFSR system. Our data indicate that the stoichiometry of the Lia system is essential for the dynamic range and output observed at the level of its target promoter, P_{liaI}. Increasing the abundance of either LiaS or LiaR leads to 'locked-ON' phenotypes, even in the absence of a stimulus. Hence, the Lia system behaves non-robustly with regard to its protein stoichiometry, in contrast to other 2CSs studied in this respect.

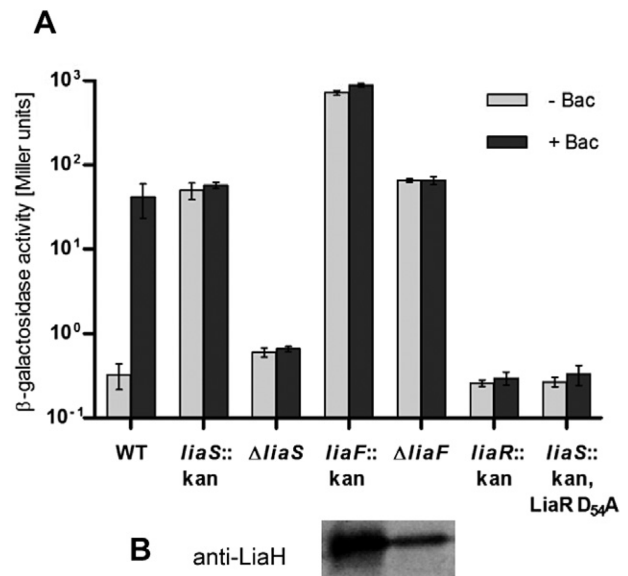

Fig. 1. Effect of mutations or deletions of *liaF*, *liaS* or *liaR* on P_{liaI} activity as a measure for the LiaR-dependent expression of *liaIH*.
A. Cultures of strains TMB488 (wild type), TMB019 (*liaS*::kan), TMB018 (*liaF*::kan), TMB216 (Δ*liaS*), TMB331 (Δ*liaF*), TMB020 (*liaR*::kan) and TMB232 (*liaS*::kan, LiaR D_{54}A) were grown in LB medium to mid-exponential phase (OD_{600} ~ 0.4) and split. One-half was induced by the addition of bacitracin (final concentration 50 μg ml^{-1}; black bars), and the other half served as an uninduced control (grey bars). Cells were harvested 30 min post induction and the activity of the P_{liaI}–*lacZ* reporter was measured by β-galactosidase assay 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.
B. To illustrate the strong constitutive activity of P_{liaI} in the Δ*liaF* (TMB329) and *liaF*::kan (TMB002) mutants as well as the 10-fold increased activity of Δ*liaF* relative to the induced wild type, a Western blot is shown below, using antibodies against LiaH.

Results

Hyperactivity of P_{liaI} is the result of positive polar effects from inserted resistance cassettes

We previously noticed by β-galactosidase assay using a P_{liaI}–*lacZ* reporter strain that the constitutive *liaI* promoter activity of a *liaF* mutant, in which the gene has been replaced by a kanamycin resistance cassette, was 10 times higher than the maximum promoter activity in the induced wild type (Jordan *et al.*, 2006) (see Fig. 1A). Surprisingly, a *liaS* mutant constructed in a similar way also shows a 'locked-ON' behaviour with regard to the activity of the strictly LiaR-dependent P_{liaI}, despite the absence of the HK responsible for activating LiaR (Fig. 1A). But here, the activity was lower and comparable to the induced wild type. Subsequent time-course experiments revealed a P_{liaI}-dependent accumulation of

β-galactosidase over time in the *liaS* mutant (data not shown), indicating a significantly increased LiaR-dependent basal expression. Both, the *liaS*::kan and the *liaF*::kan mutants no longer responded to the extracellular addition of sublethal bacitracin concentration (Fig. 1A), and in each case, the observed P_{liaI} activity was still LiaR-dependent (data not shown). These observations could be explained either by postulating an important role of LiaS as a phosphatase under these conditions, and/or by assuming polar effects of the inserted kanamycin resistance cassette on the expression of downstream gene(s). We first investigated the second hypothesis.

Because of the strong increase in LiaR-dependent P_{liaI} activity, we suspected positive polar effects as the possible reason for the observed behaviour. Therefore, we used markerless deletion mutants of both genes, constructed with the pMAD vector system (Arnaud *et al.*, 2004). The resulting P_{liaI} reporter strains TMB216 (Δ*liaS*) and TMB331 (Δ*liaF*) now showed significantly reduced basal expression levels. While the latter confirmed the inhibitor function of LiaF, the overall promoter activity was now comparable to the induced wild type, irrespective of the presence or absence of bacitracin (Fig. 1A). Likewise, the Δ*liaS* strain now showed a locked-OFF behaviour comparable to the uninduced wild type, as expected (Fig. 1A; for values, see Table S2).

To rule out any artefacts derived from the P_{liaI}–*lacZ* reporter system used to study LiaR activity, we verified the observed differences between the *liaF*::kan and the Δ*liaF* mutants by Western blot analysis, monitoring LiaH expression in the absence of bacitracin by specific polyclonal antibodies (Fig. 1B). Again, we observed significant differences in LiaH accumulation comparable to the results from the β-galactosidase assays described above. For the *liaS*::kan mutant, the strong positive polar effect of the kanamycin resistance cassette was directly visualized by Northern blots (Fig. 2). Figure 2A shows a schematic representation of the *lia* locus in the wild type and the *liaS*::kan mutant, as well as the expected and observed transcripts. The Northern blot revealed a significant overexpression of a *liaR*-specific transcript that correlates in size with a kan-*liaR* mRNA (Fig. 2B).

We next quantified the expression levels of *liaF, liaS* and *liaR* by real-time RT-PCR in the wild type (+/– bacitracin) and the allelic replacement/clean deletion mutants of *liaF* and *liaS* (Table 1). The results are in complete agreement with the data described above. Replacement of *liaF* by the kanamycin cassette results in a 10-fold increased level of *liaS* compared with the clean deletion. Likewise, *liaR* expression is increased 20-fold in the *liaS*::kan strain compared with the Δ*liaS* mutant. Taken together, our data demonstrate that the strong positive polar effect (10- to 20-fold induction of downstream

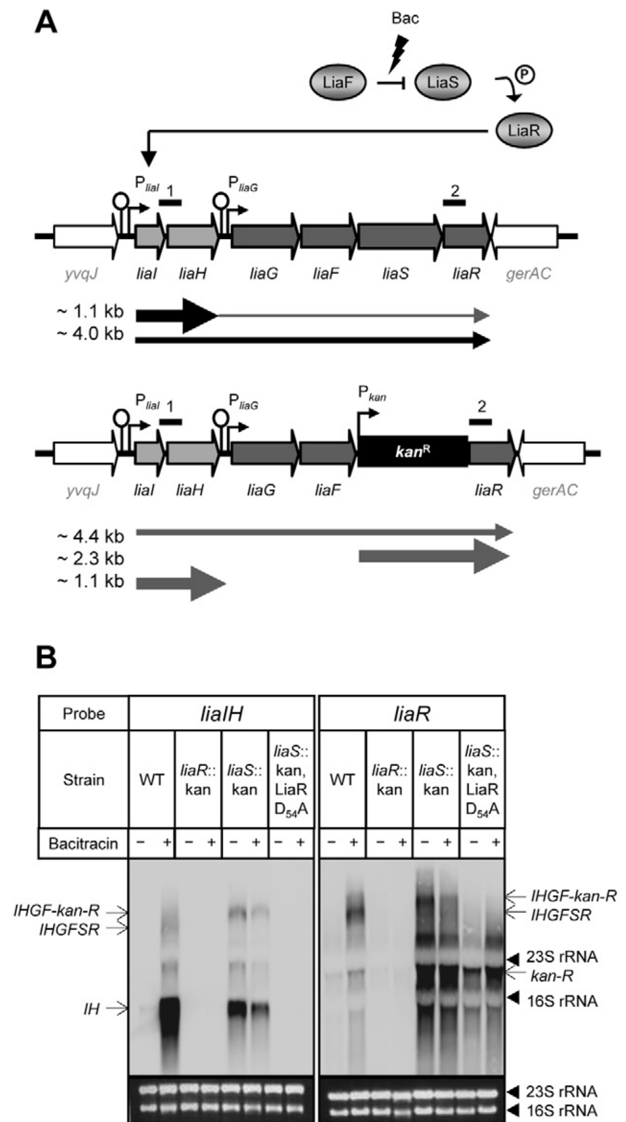

Fig. 2. Positive polar effect of the kanamycin resistance cassette on the LiaR expression.
A. Schematic representation of the native *lia* locus of *B. subtilis* as well as the *lia* locus with integrated kanamycin resistance cassette replacing *liaS*. The fragments covered by the two probes used for Northern hybridization are indicated and labelled 1 and 2. The transcripts detected by Northern blots are marked by arrows, whereas the detection of transcripts at the uninduced condition is represented by grey arrows and those at the induced condition by black arrows. Their respective sizes are given on the left.
B. Northern blot analysis of *liaIH* and *liaR* expression, using the two probes indicated above. Five micrograms of total RNA for *liaIH* detection and 10 μg for *liaR* detection (–, no induction; +, induction with 50 μg ml^{-1} bacitracin) was loaded on a formaldehyde gel and Northern blot analysis was performed as described in *Experimental procedures*. The formaldehyde gels with separated 23S- and 16S-rRNAs are shown below.

Table 1. Transcription of *liaFSR*.

	liaF[b]			*liaS*[b]			*liaR*[b]	
		Fold changes, relative to			Fold changes, relative to			Fold changes, relative to
Strain[a]	C_t	*liaR*$_{(WT-Bac)}$	*liaF*$_{(WT-Bac)}$	C_t	*liaR*$_{(WT-Bac)}$	*liaS*$_{(WT-Bac)}$	C_t	*liaR*$_{(WT-Bac)}$
WT (– Bac)	8.2 ± 0.1	49 ± 8	1	8.3 ± 0.2	47 ± 16	1	13.8 ± 0.3	1
WT (+ Bac)	2.7 ± 0.1	2165 ± 419	44 ± 1	3.0 ± 0.1	1791 ± 412	39 ± 4	9.3 ± 0.2	22 ± 2
liaF::kan	n.a.	n.a.	n.a.	0.8 ± 0.1	8568 ± 1983	189 ± 20	7.5 ± 0.2	77 ± 8
Δ*liaF*	n.a.	n.a.	n.a.	4.1 ± 0.2	910 ± 303	19 ± 0.1	8.9 ± 0.3	31 ± 2
liaS::kan	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.	8.5 ± 0.1	41 ± 8
Δ*liaS*	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.	13.3 ± 0.3	2 ± 1

a. Strains used: W168 (WT), TMB002 (*liaF*::kan), TMB329 (Δ*liaF*), TMB004 (*liaS*::kan) and TMB213 (Δ*liaS*) (Bac, Bacitracin).
b. Genes quantified, using primer pairs TM0630/TM0631 (*liaF*), TM0628/TM0629 (*liaS*) and TM0093/TM0094 (*liaR*) (C_t, threshold cycle; n.a., not applicable; n.d., not determined).

genes) strongly affects the LiaR-dependent output at the level of target promoter (P_{liaI}) activity.

LiaR is phosphorylated by the cellular pool of acetyl phosphate in the absence of LiaS

While positive polar effects could be identified as the reason for the observed P_{liaI} hyperactivity in strains harbouring allelic replacement mutations, the strong promoter activity in case of the *liaS*::kan mutant was still puzzling. Activation of RR activity depends on a HK-dependent phosphorylation and subsequent dimerization. We therefore wondered if phosphorylation of LiaR played a role in the observed promoter activity in the *liaS*::kan mutant. To address this question, we constructed a mutant in which we introduced a single point mutation during the allelic replacement of *liaS*, which leads to an aspartate to alanine exchange in LiaR, thereby rendering the invariant site of RR phosphorylation dysfunctional. This strain (TMB247: *liaS*::kan, LiaR D_{54}A) also showed a strong expression of a *kan-liaR* transcript, but no expression of the LiaR-dependent *liaIH* or *liaIHGF*-kan-*liaR* transcripts (Fig. 2B). Accordingly, the resulting P_{liaI}–*lacZ* reporter strain (TMB232) only showed basal expression levels comparable to the uninduced wild type (Fig. 1A). While we cannot rule out that the introduced amino acid exchange somehow affected LiaR stability or folding, our data nevertheless strongly suggests that phosphorylation of LiaR is a prerequisite for the observed output in the absence of the cognate HK LiaS.

Acetyl phosphate has been described in the literature as a small molecule phospho-donor capable of phosphorylating response regulators *in vivo*, since it can reach intracellular concentration comparable to those of ATP (about 3–5 mM), at least in *E. coli* (McCleary and Stock, 1994; Wolfe, 2005; Klein *et al.*, 2007). While the phosphatase activity of bifunctional HKs usually prevents undesired phosphorylation of their cognate RRs in the absence of suitable triggers, some RRs are readily phosphorylated in their absence, as has been demonstrated for the VanRS system of *Streptomyces coelicolor* (Hutchings *et al.*, 2006). We therefore hypothesized a similar scenario for the Lia system.

Acetyl phosphate is produced from pyruvate via acetyl-CoA as part of the cellular overflow metabolism. Under normal conditions, acetyl-CoA is synthesized from pyruvate and metabolized by the tricarboxylic acid (TCA) cycle. Whenever too much acetyl-CoA is present in the cell to be metabolized via the TCA cycle, the excess acetyl-CoA is converted to acetyl phosphate. This reaction is catalysed by the enzyme phospho-transacetylase, which is encoded by the *pta* gene. Acetyl phosphate is then converted to acetate (a reaction catalysed by the acetate kinase AckA), which is released into the medium. If the cells grow on acetate, the order of biochemical reactions is inverted (Wolfe, 2005).

The cellular amount of acetyl phosphate can be influenced by the carbon sources supplied to the medium. For *E. coli*, the highest concentrations of acetyl phosphate were determined with pyruvate as carbon source (McCleary and Stock, 1994; Wolfe, 2005; Klein *et al.*, 2007). We therefore decided to compare the P_{liaI} activity of the *liaS*::kan mutant in the presence and absence of *pta* or *ackA* with varying carbon sources. The results are summarized in Fig. 3.

In the chemically defined CSE medium (Stülke *et al.*, 1993), the P_{liaI} activity strongly responds to the available carbon source, at least in the *liaS*::kan mutant. If succinate [0.56% (w/v) final concentration], an intermediate of the TCA cycle that does not feed into the overflow metabolism, is used as the sole carbon source, a significant reduction of the basal P_{liaI} activity of the *liaS*::kan mutant is observed compared with LB medium. In contrast, addition of pyruvate [0.5% (w/v) final concentration]

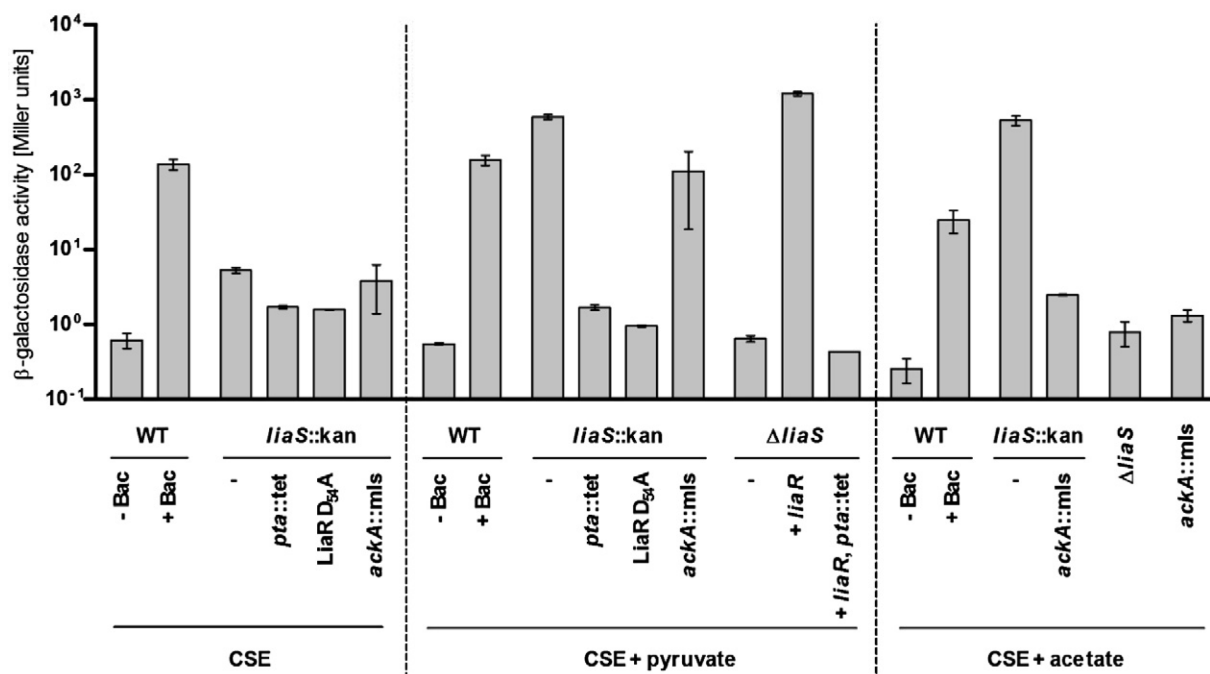

Fig. 3. Carbon source-dependent activation of P_{liaI} in different *lia*, *pta* and *ackA* mutants. Cultures of strains TMB488 (wild type), TMB019 (*liaS*::kan), TMB505 (*liaS*::kan, *pta*::tet), TMB232 (*liaS*::kan, LiaR $D_{54}A$), TMB216 (Δ*liaS*), TMB641 (Δ*liaS*, pXT-*liaR*), TMB678 (Δ*liaS*, pXT-*liaR*, *pta*::tet), TMB190 (*ackA*::mls) and TMB191 (*liaS*::kan, *ackA*::mls) were grown in CSE medium and/or CSE medium supplemented with pyruvate [0.5% (w/v) final concentration] or acetate (60 mM final concentration) to mid-exponential phase (OD_{600} ~ 0.6). Cells were harvested and P_{liaI} activity was measured as described. A log scale was applied for reasons of clarity. Integration of the empty complementation vector pXT did not affect the behaviour of the parental strain (TMB216) shown above (data not shown).

to CSE medium increased the promoter activity in this mutant more than 10-fold compared with LB medium and 100-fold compared with CSE alone (Figs 1A and 3). The wild type shows comparable P_{liaI} activities in LB medium and CSE medium with or without pyruvate. Irrespective of the carbon source, the promoter activity drops to the same basal level in a *pta* mutant, thereby clearly demonstrating that most of the increased LiaR-dependent P_{liaI} activity in the *liaS*::kan background can be attributed to stimulus-independent cross-phosphorylation from acetyl phosphate (Fig. 3 and data not shown), as has been demonstrated at least for some other 2CSs (Laub and Goulian, 2007). An *ackA*/*liaS* double mutant, which is not able to convert acetyl phosphate to acetate, shows P_{liaI} activities comparable to the *liaS*::kan mutant (Fig. 3).

The discrepancy in P_{liaI} activity between the Δ*liaS* and *liaS*::*kan* strains (Fig. 1A) seemed to indicate that phosphorylation of LiaR by acetyl phosphate only leads to a measurable output if LiaR is simultaneously overproduced, as is the case in the *liaS*::*kan* strain (Fig. 2B). To address this question, we introduced an additional copy of *liaR* under the control of a xylose-inducible promoter, into the Δ*liaS* reporter strain TMB216 (Table 2), resulting in strain TMB641, thereby simulating the situation of strain TMB019. Indeed, this strain showed a similar behaviour (Figs 3 and 1A). And again, the 'locked-ON' behaviour was completely dependent on acetyl phosphate, as demonstrated by the lack of promoter activity after introducing the *pta*::tet allele into this strain (Fig. 3).

To unequivocally demonstrate that indeed acetyl phosphate is responsible for the observed stimulus-independent activation of P_{liaI}, we also reversed the reaction by adding acetate to the CSE medium (60 mM final concentration), thereby driving the Pta-AckA pathway backwards. Under these conditions, we received similar results as described for CSE + pyruvate: the P_{liaI} activity of the *liaS*::kan mutant again increases about 100-fold compared with CSE medium alone and drops to a basal level in an *ackA*::mls mutant, which is no longer able to produce acetyl phosphate from acetate anymore. The same basal P_{liaI} activity is observed for the Δ*liaS* mutant, which does not overproduce LiaR (Fig. 3).

Taken together, the data obtained so far strongly suggests that LiaR can be phosphorylated by the cellular pool of acetyl phosphate, but only if LiaR is overexpressed in the absence of the cognate HK LiaS. This artificial susceptibility of LiaR for acetyl phosphate-dependent activation is therefore in good agreement with the results obtained for other bacterial RR (Laub and Goulian, 2007).

Table 2. Bacterial strains used in this study.

Strain	Genotype or characteristic(s)[a]	Reference, source or construction[b]
E. coli strains		
DH5α	*recA1 endA1 gyrA96 thi hsdR17*rK- mK+*relA1 supE44 Φ*80Δ*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169*	Sambrook and Russell (2001)
XL1blue	*endA1 gyrA96*(*nal*^{R}) *thi*-1 *recA1 relA1 lac gln*V44 F′[Tn10 *proAB*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*] *hsdR17*(r_K^- m_K^+) *tet*^{R}	Laboratory stock
BL21(DE3)/pLysS	F^- *lon ompT* r_B m_B *hsdS gal* (*c*I*ts*857 *ind1* Sam7 *nin5 lacUV*5-T7gene1)	Laboratory stock
TME741	BL21(DE3)/pLysS pKSEx102	This work
TME744	BL21(DE3)/pLysS pKSEx103	This work
B. subtilis strains		
W168	Wild type, *trpC*2	Laboratory stock
CU1065	W168 *att*SPβ2Δ2	Laboratory stock
HB0933	CU1065 *liaR*::kan	Mascher *et al.* (2003)
TMB002	CU1065 *liaF*::kan	Jordan *et al.* (2006)
TMB004	CU1065 *liaS*::kan	Jordan *et al.* (2006)
TMB016	CU1065 *amyE*::(cat P_{liaI}–*lacZ*)	Jordan *et al.* (2006)
TMB018	CU1065 *amyE*::(cat P_{liaI}–*lacZ*) *liaF*::kan	Jordan *et al.* (2006)
TMB019	CU1065 *amyE*::(cat P_{liaI}–*lacZ*) *liaS*::kan	Jordan *et al.* (2006)
TMB020	CU1065 *amyE*::(cat P_{liaI}–*lacZ*) *liaR*::kan	Jordan *et al.* (2006)
TMB174	W168 *ackA*::mls	LFH-PCR→W168
TMB186	W168 *pta*::tet	LFH-PCR→W168
TMB190	TMB016 *ackA*::mls	TMB174 ch. DNA→TMB016
TMB191	TMB019 *ackA*::mls	TMB174 ch. DNA→TMB019
TMB213	W168 Δ*liaS*	Jordan *et al.* (2007)
TMB216	TMB213 *amyE*::(cat P_{liaI}–*lacZ*)	TMB016 ch. DNA→TMB213
TMB232	W168 *amyE*::(cat P_{liaI}–*lacZ*) *liaS*::kan LiaR D_{54}A	TMB016 ch. DNA→TMB247
TMB247	W168 *liaS*::kan LiaR D_{54}A	CCR/LFH-PCR→W168
TMB278	W168 *amyE*::(cat $P_{liaG\text{-}opt.}$-*liaF–lacZ*)	pER503→W168
TMB281	W168 *amyE*::(cat $P_{liaG\text{-}opt.}$-*liaS–lacZ*)	pER504→W168
TMB282	W168 *amyE*::(cat $P_{liaG\text{-}opt.}$-*liaR–lacZ*)	pER505→W168
TMB329	W168 Δ*liaF*	Wolf *et al.* (2010)
TMB331	TMB329 *amyE*::(cat P_{liaI}–*lacZ*)	TMB016 ch. DNA→TMB329
TMB466	W168 *amyE*::*lacZ* (pAC7)	pAC7→W168
TMB468	W168 *amyE*::*lacZ* (pAC5)	pAC5→W168
TMB469	W168 *amyE*::(kan P_{veg}-SD_{liaF}–*lacZ*)	pKS1001→W168
TMB478	W168 *amyE*::(kan P_{veg}-SD_{liaS}–*lacZ*)	pKS1002→W168
TMB479	W168 *amyE*::(kan P_{veg}-SD_{liaR}–*lacZ*)	pKS1003→W168
TMB480	W168 *amyE*::(kan P_{veg}–*lacZ*)	pKS1005→W168
TMB488	W168 *amyE*::(cat P_{liaI}–*lacZ*)	TMB016 ch. DNA→W168
TMB500	TMB216 *thrC*::(spec P_{xyl}-*liaS*-FLAG3)	pKS704→TMB216
TMB501	TMB216 *lacA*::(erm P_{spac}-FLAG3-*liaS*)	pKS-FLAG1→TMB216
TMB505	TMB019 *pta*::tet	TMB186 ch. DNA→TMB019
TMB639	TMB488 *thrC*::(spec P_{xyl}-*liaR*)	pDW701→TMB488
TMB641	TMB216 *thrC*::(spec P_{xyl}-*liaR*)	pDW701→TMB216
TMB654	TMB488 *thrC*::(spec P_{xyl}-*liaF*)	pSJ701→TMB488
TMB678	TMB641 *pta*::tet	TMB186 ch. DNA→TMB641
TMB1131	TMB488 *thrC*::(spec P_{xyl}-*liaFSR*)	pKS726→TMB488
TMB1141	W168 *liaS*-FLAG3	pMAD-based sequence insertion
TMB1146	TMB1141 *amyE*::(cat P_{liaI}–*lacZ*)	TMB488 ch. DNA→TMB1141
TMB1155	W168 FLAG3-*liaF*	pMAD-based sequence insertion
TMB1156	TMB1155 *amyE*::(cat P_{liaI}–*lacZ*)	TMB488 ch. DNA→TMB1155
TMB1201	W168 FLAG3-*liaR*	pMAD-based sequence insertion
TMB1271	TMB1201 *amyE*::(cat P_{liaI}–*lacZ*)	TMB488 ch. DNA→TMB1201
TMB1488	TMB488 *thrC*::(spec P_{xyl}-*liaS*)	pKS727→TMB488
TMB1490	TMB216 *thrC*::(spec P_{xyl}-*liaS*)	pKS727→TMB216
TMB1505	TMB216 *thrC*::(spec P_{xyl}-*liaS* (LiaS Q_{164}A))	pKS729→TMB216

a. Resistant cassettes: kan, kanamycin; cat, chloramphenicol; tet, tetracycline; spec; spectinomycin; erm, erythromycin, mls, macrolide–lincosamide–streptogramin (erythromycin + lincomycin)
b. ch. DNA, chromosomal DNA.

LiaS is a bifunctional HK that possesses a phosphatase activity

The data described in the previous section suggests that LiaS is a bifunctional kinase that functions as a phosphatase in the absence of inducing conditions. LiaS belongs to the HPK7 family of HKs, which harbours a HisKA_3 dimerization and histidine phosphotransfer domain. Within this domain, a conserved DxxxQ motif was recently identified (Huynh *et al.*, 2010). For the HK NarX it

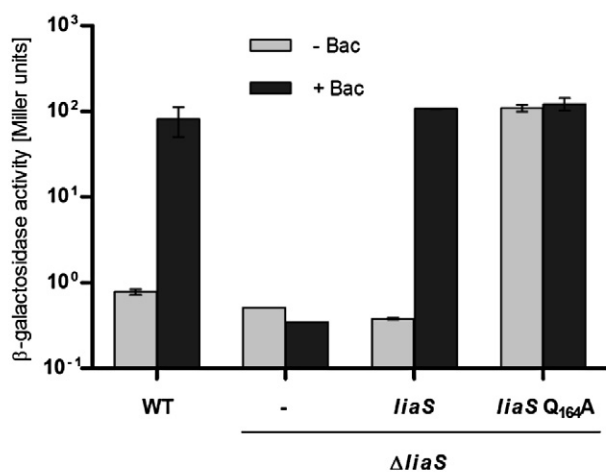

Fig. 4. Effect of a Q_{164}A exchange in LiaS on the P_{liaI} activity. P_{liaI} activity of strains TMB488 (wild type), TMB216 (Δ*liaS*), TMB1490 (Δ*liaS*, pXT-*liaS*) and TMB1505 (Δ*liaS*, pXT-LiaS Q_{164}A). Experimental conditions and labelling of the bars are as described in Fig. 1.

was shown that the glutamine residue plays a critical role in phosphatase activity, and the exchange by an alanine, glutamate or histidine residue results in a kinase ON, phosphatase OFF protein (Huynh *et al.*, 2010). Since this motif is also found in LiaS (data not shown), we introduced a copy of *liaS*, in which the conserved glutamine 164 residue was substituted by an alanine, into the Δ*liaS* mutant and analysed the P_{liaI} activity by β-galactosidase assay (Fig. 4). In contrast to the wild type behaviour of the complementation mutant carrying a native *liaS* gene, the LiaS Q_{164}A mutant shows a constitutive P_{liaI} activity comparable to the induced wild type. These genetic findings, together with the physiological data described above, strongly suggest that LiaS possesses a phosphatase activity. This is in perfect agreement with biochemical *in vitro* evidence from the direct LiaS orthologues VraS of *Staphylococcus aureus* (Belcheva and Golemi-Kotra, 2008) and $LiaS_{Lm}$ of *Listeria monocytogenes* (Fritsch *et al.*, 2011), which were both demonstrated to be bifunctional HKs. In fact, all HKs belonging to the family HPK7 investigated so far, such as DesK or NarX/Q are bifunctional kinases (Schröder *et al.*, 1994; Albanesi *et al.*, 2004). In the case of LiaS, this phosphatase activity is very important to keep the output (*P_{liaI}* activity) switched off in the absence of inducing conditions.

Overproduction of LiaS or LiaR – but not LiaF – affects the functionality of the LiaFSR system

The results obtained so far seemed to indicate that the LiaFSR system is very susceptible to changes in the relative stoichiometry of its three components. Specifically, the observed activation of LiaR in the absence of LiaS provoked the question: would an artificial increase of the RR be sufficient to result in a 'locked-ON' phenotype, even in the presence of LiaS? To address this question, we overproduced each of the three proteins in the wild type reporter strain, using the xylose-dependent pXT expression system (Derre *et al.*, 2000).

Overproduction of the inhibitor protein LiaF had only mild effects on the functionality of the LiaRS 2CS. The maximum P_{liaI} activity in the presence of bacitracin was reduced by a factor of three, while no change of the uninduced basal expression level was observed (Fig. 5A). The intact expression of a functional LiaF protein from this construct was verified by its ability to suppress the 'locked-ON' phenotype of a *liaF* mutant (data not shown). Therefore, we conclude that the LiaFSR system is relatively robust with regard to increasing LiaF concentrations. However, we cannot exclude that the mild effect on P_{liaI} might also be the result of only a weak overproduction of LiaF. Since both the purification of LiaF (to raise antibodies against the protein) and also Western blots against an epitope-tagged functional LiaF, expressed from its native chromosomal position, failed (data not shown), this hypothesis can unfortunately not be verified experimentally at the moment.

In contrast, overproduction of LiaR resulted in a 'locked-ON' phenotype (Fig. 5A). As hypothesized, a xylose-dependent increase in the amount of LiaR therefore results in the same behaviour as observed in the *liaS* mutants, above. Since *liaS* is not overexpressed in this strain, the increase in P_{liaI} activity without external stimuli might be caused by a phosphorylation via acetyl phosphate that can appear due to an inefficient dephosphorylation by LiaS. For LiaS, we observed an intermediate phenotype. Overproduction significantly increased the uninduced basal expression level without affecting the response to bacitracin (Fig. 5A).

Because of the weak Shine–Dalgarno (SD) sequence upstream of *liaS* (see below for details), we wondered if this intermediate behaviour was due to inefficient translation initiation, resulting in only a moderate increase in the cellular amount of LiaS. To investigate this hypothesis, we cloned two different FLAG3-tagged *liaS* alleles, one under the control of its native SD sequence into the vector pXT (Derre *et al.*, 2000), the other with an optimized ribosome binding site provided by the vector pALFLAG3 (Schöbel *et al.*, 2004) respectively. The resulting plasmids were then introduced into strain TMB216 (Δ*liaS*, P_{liaI}–*lacZ*), resulting in strains TMB500 and TMB501 respectively (Table 2). The results of the β-galactosidase assay are shown in Fig. 5B. In the presence of xylose, the Δ*liaS* mutant did not show any promoter activity, as observed before (Fig. 1A). Expression of *liaS* under the control of its native SD sequence restored the phenotype to wild type behaviour, indicating that sufficient amounts of intact LiaS were produced. In contrast, expression of LiaS with the

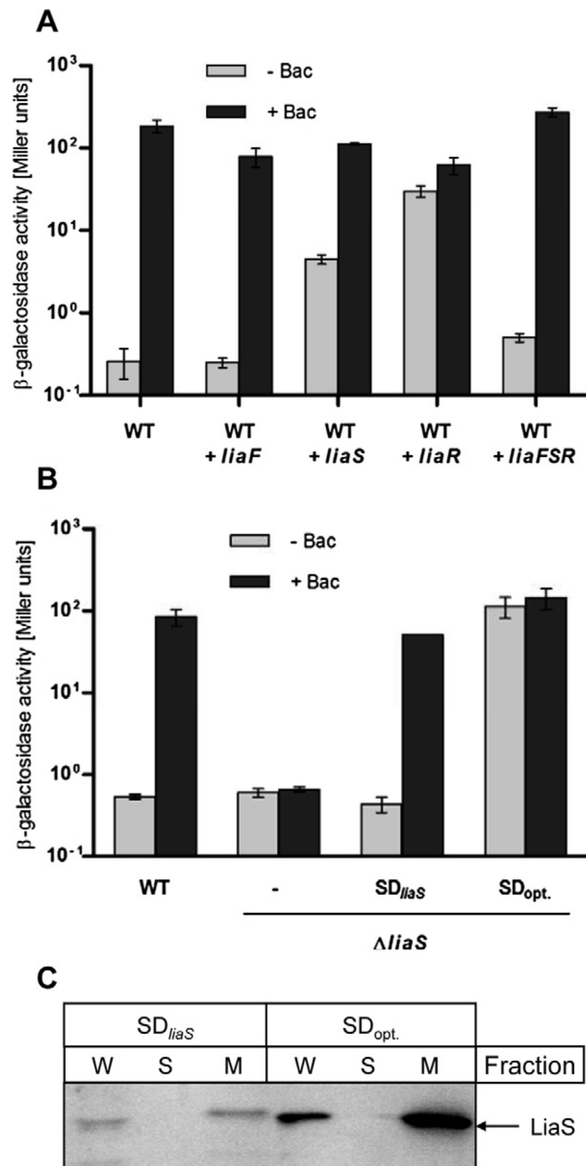

Fig. 5. A. Xylose-dependent overexpression of *liaF*, *liaS* and *liaR* in the wild type reporter strains. P_{liaI} activity of TMB488 (wild type), TMB654 (WT+*liaF*), TMB1488 (WT+*liaS*) and TMB639 (WT+*liaR*). Experimental conditions and labelling of the bars are as described in Fig. 1.
B and C. Complementation of the Δ*liaS* deletion, using two different vector backgrounds.
B. P_{liaI} activity of TMB488 (wild type) and TMB216 (Δ*liaS*) as controls as well as TMB500 (SD_{liaS}) and TMB501 ($SD_{opt.}$). Experimental conditions and labelling of the bars are as described in Fig. 1.
C. Western blot analysis of *B. subtilis* strains expressing FLAG3-tagged LiaS alleles preceded by native (TMB500; SD_{liaS}) and an optimized SD sequence (TMB501; $SD_{opt.}$), respectively, using antibodies against the FLAG epitope tag. The cultures were harvested during the late mid-exponential phase (OD_{600} ~ 0.8) and cells were disrupted by sonication. The whole cell extracts (W) were further fractionated into soluble (S) and membrane (M) protein fractions by ultracentrifugation. Western blot analysis was performed as described in *Experimental procedures*.
A–C. All cultures harbouring pXT-derived constructs were grown in LB supplemented with 0.2% (w/v) xylose.

optimized SD sequence resulted in a 'locked-ON' behaviour (Fig. 5B). These differences could be directly correlated with the different amounts of LiaS in the cells. Western analysis with FLAG-tag-specific antibodies identified a strongly increased amount of LiaS protein in the membrane fraction when expressed with an optimized ribosome binding site, compared with the complementation with the native SD sequence (Fig. 5C). These results demonstrate that overproduction of both LiaS and LiaR severely perturbates the signal transduction mediated by the LiaFSR system, even in the presence of all other Lia proteins in their native amounts.

In contrast, the simultaneous overexpression of all three genes, *liaF*, *liaS* and *liaR*, which increases the cellular amount of these proteins simultaneously without changing their stoichiometry to each other, shows a comparable behaviour to the wild type (Fig. 5A). This result demonstrates that the absolute protein amounts of LiaFSR have no effect on the P_{liaI} activity. As long as the ratio between LiaS and LiaR is maintained, the phosphatase activity of LiaS is sufficient to prevent stimulus-independent phosphorylation of LiaR by acetyl phosphate.

Taken together, the data obtained from our perturbation studies indicate that the LiaFSR system seems to behave non-robustly with regard to alterations in the cellular ratios of LiaS and LiaR relative to the other protein components of the Lia system. We hypothesized that the stoichiometry of LiaF:LiaS:LiaR is very important for the functionality of Lia-dependent signal transduction. To study this in more detail, we next performed an in-depth genetic analysis of the wild type expression levels of the three genes/proteins by determining transcription and translation initiation both independently and in conjunction. For LiaSR the results were subsequently also verified by quantitative Western blot analyses to estimate the number of proteins in the cell.

Transcription of liaR is almost 50-fold weaker compared with liaF or liaS

We first studied the expression of the three genes by quantitative real-time RT-PCR in the wild type strain W168, both in the absence and in the presence of bacitracin. The first condition monitors the intrinsic basal expression level from the constitutive promoter upstream of *liaG*, while the latter reflects the combined activity of P_{liaG} and the LiaR-dependent P_{liaI} (Fig. 2A). The results are given in Table 1, which summarizes the determined fold changes relative to the *liaR* transcription of the uninduced wild type. We detected almost equal amounts of *liaF* and *liaS* transcripts. Surprisingly, *liaR* expression was significantly lower under both conditions tested. The values indicate an overall ratio of transcription of 49:47:1 (*liaF*:*liaS*:*liaR*) for uninduced wild type cells.

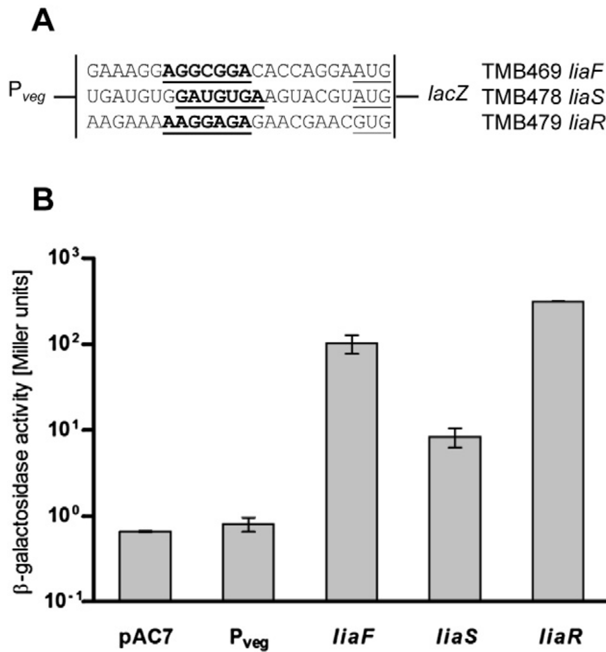

Fig. 6. Translation initiation of *liaF*, *liaS* and *liaR*.
A. Sequences of *liaF*-, *liaS*- and *liaR*-mRNA including the Shine–Dalgarno sequence (underlined bold letters) up to the start codon (underlined letters) are illustrated. These fragments are under the control of P_{veg} and translationally fused to *lacZ* using the vector pAC7.
B. Cultures of strains TMB466 (pAC7 empty vector) and TMB480 (P_{veg}–*lacZ*) as negative controls as well as TMB469 (SD_{liaF}–*lacZ*), TMB478 (SD_{liaS}–*lacZ*) and TMB479 (SD_{liaR}–*lacZ*) were grown in LB medium to mid-exponential phase (OD_{600} ~ 0.6). Cells were harvested and β-galactosidase activity was measured as described. A log scale was applied for reasons of clarity.

A sequence analysis of the *liaFSR* region identified two stem-loop structures at the very end of the *liaS* gene and close to each other (Fig. S1). While they lack both the strength and the poly-U run-off typical for classical *rho*-independent terminators, they could nevertheless be responsible for a significant amount of premature transcription termination that would account for the observed differences in transcript levels. Another possibility is that the 3′ end of the *liaFSR*-specific transcript is subject to RNase degradation.

Translation initiation of LiaS is severely impaired by its weak Shine–Dalgarno sequence

We next analysed the contribution of translation initiation to the overall expression of the three proteins. Towards that end, we used joining-PCR (see *Experimental procedures*) to fuse the strong constitutive promoter P_{veg} (Moran *et al.*, 1982) with short chromosomal regions of 24 nt length, directly upstream and including the start codon of each of the three genes (Fig. 6A). The resulting PCR products were then cloned into the pAC7 vector (Weinrauch *et al.*, 1991) to generate translational fusions with the *lacZ* gene. Therefore, any detectable β-galactosidase activity is the result of the promoter and SD sequence provided on the cloned fragments. Likewise, any difference between the activities of the three constructs is a direct consequence of the translation initiation sequences of the short DNA fragments shown in Fig. 6A. As a negative control, P_{veg} was cloned into pAC7 in a similar manner, but lacking a SD sequence. The *B. subtilis* wild type strain W168 was transformed with the four plasmids and also the empty vector, resulting in strains TMB466, TMB469 and TMB478–480 (Table 2). β-Galactosidase assays were performed with lysates from cells harvested during mid-exponential growth phase without induction. The results are shown in Fig. 6B.

Both the empty vector and the plasmid that contains P_{veg} without a SD sequence did not show any activity. The level of translation initiation of the three complete fragments differed significantly. While translation of the β-galactosidase from the SD sequences of *liaF* and *liaR* only differed two- to threefold, the β-galactosidase activity was 10- to 30-fold lower for *liaS* (Fig. 6B). This result correlates very well with the weakly conserved SD sequence of *liaS* (Fig. 6A), and the data shown in Fig. 5C.

Transcription and translation initiation combined indicate a LiaF:LiaS:LiaR ratio of 18:4:1

To study the combined effects of transcription and translation initiation on protein expression of the LiaFSR system in its natural genetic context, we next translationally fused the three fragments shown in Fig. 7A with a promoter-/SD-less *lacZ* gene using the vector pAC5 (Martin-Verstraete *et al.*, 1992). Initially, we used the weak native promoter upstream of *liaG* (P_{liaG}) (Jordan *et al.*, 2006). But the resulting constructs did not give rise to any detectable β-galactosidase activity (data not shown). Cloning the fragments under control of the strong P_{veg} used above failed for the two longer fragments, most probably due to toxic effects of *liaF* expression in *E. coli* that we had already observed previously. We therefore used an engineered version of P_{liaG}, harbouring an optimized –35 and extended –10 promoter region, and termed $P_{liaG\text{-opt.}}$ (Fig. 7B) to introduce as few alterations as possible and also avoid a strong expression of *liaF* during the cloning procedure. Thus, the β-galactosidase activity of all three reporter strains derived from these plasmids is under the transcriptional control of $P_{liaG\text{-opt.}}$ and under the translational control of the specific SD sequences of *liaF*, *liaS* or *liaR* respectively. All strains showed a weak, but clearly detectable β-galactosidase activity (Fig. 7C). Even the expression

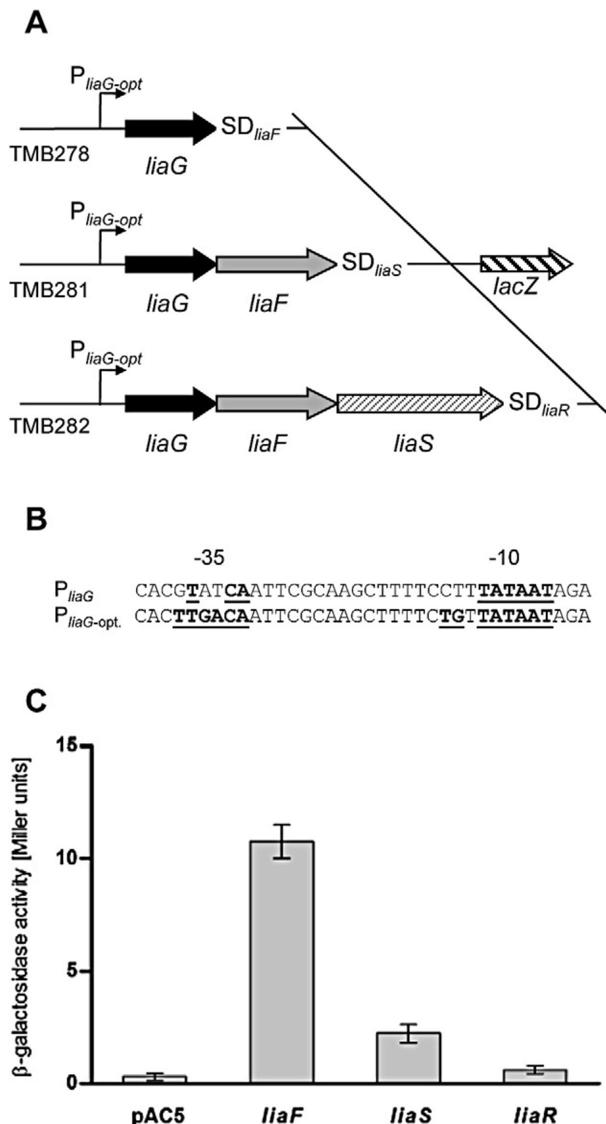

Fig. 7. Expression level of *liaF*, *liaS* and *liaR*.
A. Schematic representation of cloned fragments including *liaG* up to the start codons of *liaF*, *liaS* and *liaR* respectively. The fragments are under the control of the optimized *liaG* promoter and translationally fused to *lacZ*, using the vector pAC5.
B. Sequences of the native and optimized *liaG* promoter. The –35 and –10 regions were highlighted with underlined bold letters.
C. β-Galactosidase activities of *B. subtilis* strains containing the fragments shown above. Cultures of strains TMB468 (pAC5 empty vector) as negative control as well as TMB278 (SD_{liaF}–*lacZ*), TMB281 (SD_{liaS}–*lacZ*) and TMB282 (SD_{liaR}–*lacZ*) were grown in LB medium to mid-exponential phase (OD_{600} ~ 0.6). Cells were harvested and β-galactosidase activity was measured as described.

level for the longest and weakest construct (SD_{liaR}) was significantly (more than twofold) above the background level of the empty vector. Based on the results shown in Fig. 7C, the overall native stoichiometry of LiaF:LiaS:LiaR is 18:4:1.

Quantitative Western blot analysis verifies an excess of LiaS over LiaR

So far, the findings on the native stoichiometry of LiaFSR are based on indirect genetic approaches. To support and substantiate these data, we next performed quantitative Western blot analysis to determine the cellular amounts of the proteins involved. For this, the corresponding genes were either N-terminally (*liaF* and *liaR*) or C-terminally (*liaS*) fused to a FLAG3 sequence that was integrated directly into the native *lia* locus via pMAD (Arnaud *et al*., 2004; see *Experimental procedures* for details). The P_{liaI} activity of the generated strains was checked to ensure wild type behaviour and the functionality of the system in the presence of FLAG3-tagged proteins (Fig. S2). Furthermore, protein standards were expressed and purified from *E. coli* BL21(DE3) using the overexpression vector pProEx1, which generates N-terminal His_6-tag fusions to the cloned genes. To be able to detect the proteins via Western blotting, an additional FLAG3-tag was fused C-terminal to these proteins. Quantitative Western blots were carried out as described in *Experimental procedures*. The standards were analysed by ImageJ software and band intensities were plotted against known amounts of proteins. Finally, the cellular amounts of proteins were calculated from the standard curve.

For LiaF, all attempts to (over)express and purify the protein failed, presumably because of the toxicity of LiaF production in *E. coli*, as mentioned above. Furthermore, the detection of a functional FLAG3-tagged LiaF protein was also not successful. This could be due to either an inefficient protein transfer during the Western blot procedure or the loss of the epitope tag in the course of LiaF processing. But for LiaS and LiaR the quantification succeeded. An example of a quantitative Western blot for both proteins is shown in Fig. 8A and B. The corresponding standard curves are represented in Fig. 8C and D. In LB medium grown until mid-exponential phase in the absence of an inducer, we determined about 10 molecules of LiaS per cell, but could not detect any LiaR. We therefore also quantified the protein amounts in cells induced with bacitracin. Here, *B. subtilis* W168 cells contained an average of 150 molecules of LiaS-FLAG3 per cell. Under the same conditions, we determined ~ 20 molecules per cell for FLAG3-LiaR (Fig. 8E). Since the ratio of LiaS to LiaR should not be affected by bacitracin induction, this result should also be a reliable measure for the relative amounts of both proteins in uninduced cells. While the absolute numbers for both proteins calculated for individual experiments varied significantly (Fig. 8E) and should not be over-interpreted, especially since we only used one method to calibrate the protein standard curves, these direct measurements are nevertheless in very good agreement with the genetic data described

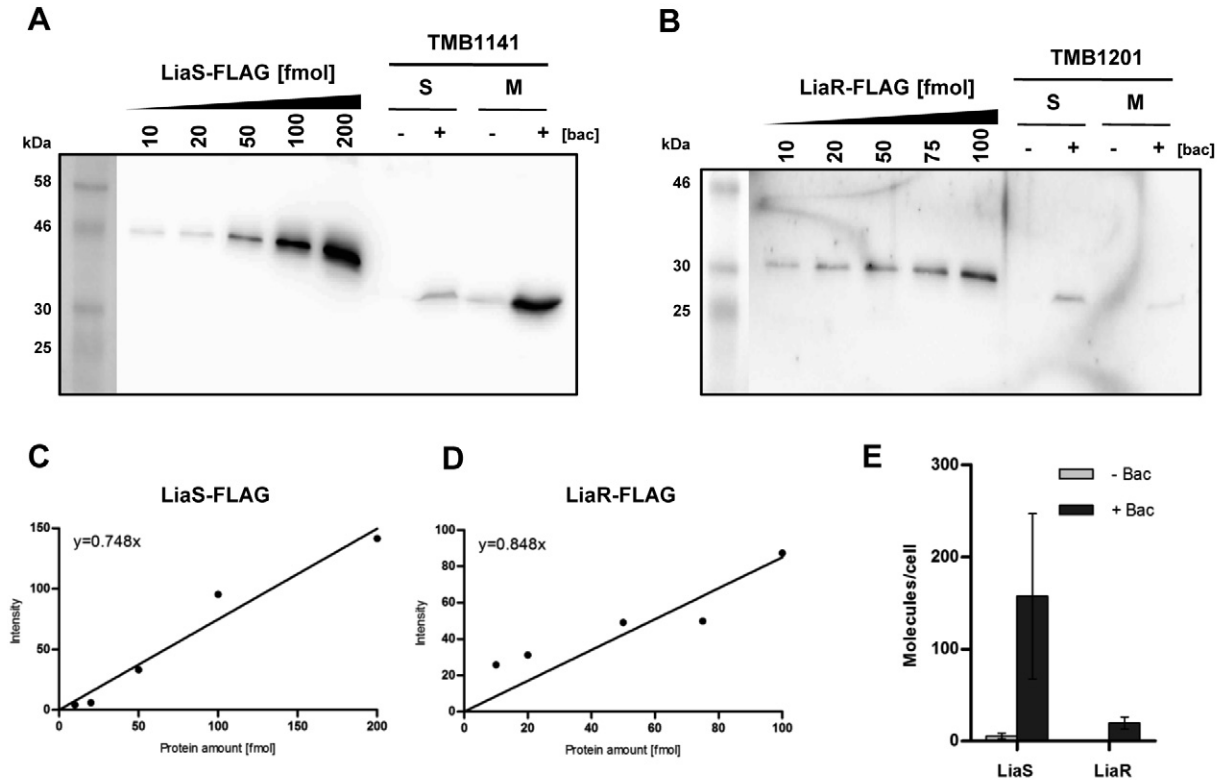

Fig. 8. *In vitro* quantification of LiaS and LiaR.
A and B. Quantitative Western blot analysis of *B. subtilis* strains expressing LiaS-FLAG3 (TMB1141; A) or FLAG3-LiaR (TMB1201; B) proteins using antibodies against the FLAG epitope tag. The cultures were grown in LB medium to mid-exponential phase (OD_{600} ~ 0.4–0.6) and split. One-half was induced by the addition of bacitracin (final concentration 20 μg ml^{-1}), and the other half was kept uninduced. Cultures were harvested 30 min post induction and cells were disrupted by sonication. The soluble (S) and membrane (M) protein fractions were fractionated by ultracentrifugation. Quantitative Western blot analysis was performed as described in *Experimental procedures*.
C and D. Standard curves of His_6-LiaS-FLAG3 and His_6-LiaR-FLAG3 protein samples in lanes 1–5. The density of each band was plotted against the amount of protein loaded. In case of His_6-LiaR-FLAG3 (Fig. 8D) it was necessary to artificially constrain the fit to go through the origin, since a best fit line with a non-zero *y*-axis intercept would have resulted in negative LiaR protein numbers per cell.
E. Average values of calculated protein amounts of LiaS-FLAG3 and FLAG3-LiaR per cell.

above and support a clear excess (at least three- to four-fold) of LiaS over LiaR. This solid correlation between indirect genetic and direct biochemical data also demonstrates that the genetic approach described in Fig. 7 provides a reliable and easy-to-perform measure to estimate relative protein amounts in cells. Hence, although we failed to determine the exact amount for LiaF, it stands to reason to assume that its amount indeed exceeds that of LiaS, based on its genetic location, strong Shine–Dalgarno sequence and the corresponding results shown in Figs 6 and 7 and Table 1.

Discussion

In this report, we have comprehensively investigated the native stoichiometry of LiaFSR by genetic and biochemical approaches and the effects of its perturbation on the functionality of this cell envelope stress-sensing system. The data obtained in this study is summarized in Table 3. A graphical model derived from these results is provided in Fig. 9. Taken together, we demonstrate a crucial role of maintaining conditions, in which the amounts of LiaF > LiaS > LiaR.

Three very important conclusions can be drawn from the observations reported in this article on the functionality of the LiaFSR system. First, LiaF exerts its function through LiaS, since it does not affect the LiaS-independent phosphorylation by acetyl phosphate (Fig. 3 and data not shown) and its inhibitory effect can be overcome by LiaS overproduction (Fig. 5). Second, in the absence of a stimulus, LiaF maintains LiaS in its phosphatase state. To ensure this, the inhibitor protein LiaF needs to be in excess over LiaS to keep the system silent in the absence of a stimulus. If LiaF is absent or if LiaS is strongly overexpressed even in the presence of LiaF, the output of the Lia system, P_{liaI} activity, is switched on, even in the absence of an inducer (Figs 1 and 5). Third, while LiaS – as its orthologues – is a bifunctional histidine kinase, at least its phosphatase activity seems to be rather inefficient. This hypothesis is derived from the observation that LiaS needs to be more abundant

Table 3. Summary of the deletion/complementation studies.

	Amount relative to WT[b]			Effect	
Strain[a]	LiaF	LiaS	LiaR	LiaS activity[c]	P_{liaI} activity (–/+ Bac)
WT	0	0	0	Phosphatase	1/100
WT + *liaF*	++	0	0	Phosphatase	1/30
WT + *liaS*	(+)	+	(+)	Kinase/phosphatase	5/100
WT + *liaR*	(+)	(+)	++	Phosphatase	30/60
WT + *liaFSR*	++	++	++	Phosphatase	1/200
liaF::kan	–	++	++	Kinase	1000/1000
Δ*liaF*	–	(+)	(+)	Kinase	100/100
liaS::kan	0	–	++	n.a.	100/100
Δ*liaS*	0	–	0	n.a.	1/1
Δ*liaS*, pXT-*liaS*	0	0	0	Phosphatase	1/100
Δ*liaS*, pXT-*liaS* (opt. SD)	(+)	++	(+)	Kinase (phosphatase)	100/100
liaR::kan	0	0	–	Phosphatase	0.5/0.5

a. WT, wild type; Bac, Bacitracin; opt. SD, optimized Shine–Dalgarno sequence.
b. 0, protein amount equal to wild type; +, increased protein amount relative to wild type; (+), increased protein amount relative to wild type due to positive feedback regulation; –, no protein present due to deletion.
c. The activities – derived from the behaviour of the target promoter P_{liaI} – is based on the assumption that LiaS is a bifunctional sensor kinase. Note that this has so far only been demonstrated experimentally by *in vitro* assays for the LiaS orthologues VraS from *Staphylococcus aureus* and $LiaS_{Lm}$ from *Listeria monocytogenes*.
All values refer to uninduced conditions, except were labelled otherwise.

than LiaR in order to prevent stimulus-independent phosphorylation of this RR by acetyl phosphate. If either of the two last prerequisites for LiaFSR functionality is severely perturbated by overexpressing either LiaS or LiaR, the system enters a 'locked-ON' state, in which full P_{liaI} activity is reached even in the absence of a signal (Fig. 5A and Table 3). Taken together, the LiaFSR system seems to behave non-robust with regard to relative alterations of its protein stoichiometry. The implications of these observations will be discussed in the following sections.

LiaFSR stoichiometry and robustness

So far, only few 2CSs have been studied with regard to the relative cellular amounts of the sensor kinase and response regulator and how this affects the functionality of the 2CS.

The most detailed quantitative analyses were performed for the paradigmatic 2CS EnvZ-OmpR of *E. coli*. It was demonstrated that the HK EnvZ is present in significantly lower amounts than its cognate RR OmpR, with ~100 and ~3500 monomeric molecules per cell respectively (Cai and Inouye, 2002). Since HKs usually function as stable dimers, the physiological relevant HK_2:RR ratio is 1:70. Subsequently, it was shown that the EnvZ-OmpR system is robust with regard to alterations of the amount of both proteins, as long as OmpR remains in excess over EnvZ (Batchelor and Goulian, 2003). Most recently, the stoichiometry of the VicRK (WalRK) 2CS from *Streptococcus pneumoniae* (Wayne *et al.*, 2010) was determined. The amount of the HK was the stoichiometrical bottleneck, with the HK_2:RR ratios of 1:14.

The data obtained for LiaFSR in this study stands in contrast to these observations. Here, the amount of the RR LiaR is about four- to eightfold lower than that of the cognate HK LiaS (Figs 8E and 9), and maintaining this excess of HK over RR is crucial for the functionality of the

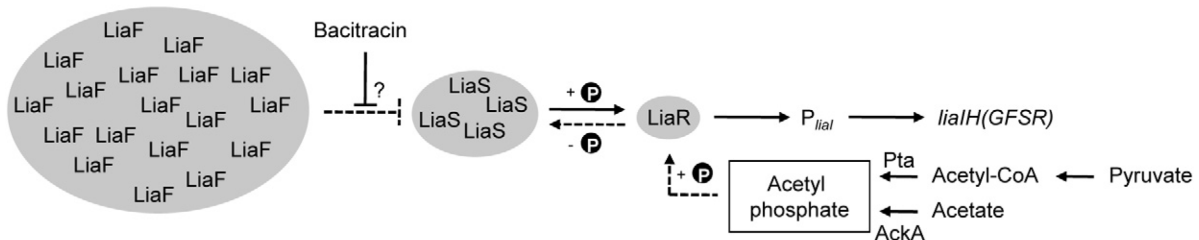

Fig. 9. Working model of the LiaFSR-dependent cell envelope stress response in *B. subtilis*. The relative ratio of the proteins LiaF, LiaS and LiaR occurring in wild type cells is illustrated by the size and content of the grey circles. The number of proteins is based on the data of the genetic approach. Dotted lines represent the uninduced condition, whereas the situation in the presence of bacitracin is indicated by solid lines. Arrows indicate activation, while T-shaped lines represent inhibition. See text for details.

LiaRS system. Because of the small differences between the amounts of $LiaS_2$ and LiaR, this system seems to be particularly vulnerable to perturbations of its stoichiometry (Fig. 5 and Table 3). In contrast, the systems described above either have already been shown or are believed to be robust. The insight into the nature of robustness in bacterial signalling is only slowly evolving (Goulian, 2004) and the overall design of the regulatory systems described above are too different to allow drawing general conclusions. But to the best of our knowledge, the data on the LiaFSR system of *B. subtilis* is the first report on a particularly non-robust 2CS. This unusual stoichiometry could be interpreted as an indication for the formation of a regulatory complex between LiaS and LiaR, in order to control RR activity. As long as LiaS is in excess over LiaR, all RR molecules can be controlled, whereas free LiaR can easily be activated by phosphorylation through the cellular pool of acetyl phosphate.

LiaR and acetyl phosphate

The phosphorylation of LiaR by acetyl phosphate occurs either in the absence of LiaS or when the RR is present in increased amounts relative to LiaS (Figs 3 and 5A). A number of studies have been performed in recent years to establish that acetyl phosphate can act as a small molecule phospho-donor for response regulators both *in vitro* and *in vivo* (reviewed in Wolfe, 2010). While the cellular amount of acetyl phosphate is sufficient to phosphorylate RRs (Klein *et al.*, 2007), this activation is usually regulated by the phosphatase activity of the cognate sensor kinase, at least in case of bifunctional HKs (Laub and Goulian, 2007). This mechanism of preventing stimulus-independent activation of a RR by the phosphatase activity of its cognate HK in the absence of inducing conditions has been documented for a number of bacterial 2CS with bifunctional HKs, including *E. coli* CpxAR (Wolfe *et al.*, 2008), *S. coelicolor* VanRS (Hutchings *et al.*, 2006) and EnvZ-OmpR from *Xenorhabdus nematophilus* (Park and Forst, 2006).

Overall, our results on the phosphorylation of LiaR by acetyl phosphate are in good agreement with the conclusions derived from the above studies. But in contrast to most of these examples, we could clearly demonstrate a full activation of LiaR by acetyl phosphate even in the presence of LiaS, presumably as soon as the $LiaS_2$:LiaR ratio favours LiaR. But recently, the *in vivo* sensitivity of a RR for cellular acetyl phosphate even in the presence of the cognate HK has also been demonstrated for the CpxAR system of *E. coli*. In this report, the authors argue that the response of the RR CpxR to intracellular acetyl phosphate might play a role in fine-tuning the envelope stress response of this 2CS (Lima *et al.*, 2012).

The question if the *in vivo* activation of LiaR by acetyl phosphate is physiological relevant or just an experimental artefact from overproducing LiaR is difficult to answer at the moment. But there is accumulating evidence that this small phospho-donor can indeed serve as an important input for 2CSs (Wolfe, 2010). Remarkably, most of the 2CSs that respond to acetyl phosphate *in vivo*, such as *E. coli* CpxAR, or VanRS from *S. coelicolor* are involved in some aspect of cell envelope stress response (Hutchings *et al.*, 2006; Wolfe *et al.*, 2008). Moreover, a recent study in *S. pneumoniae* identified three 2CSs that respond to the cellular pool of acetyl phosphate during normal growth, including LiaRS, VicRK and CiaRH, all of which are associated with maintaining cell envelope integrity (Ramos-Montanez *et al.*, 2010). This observation strongly supports the findings reported in our and the above-cited study on CpxAR and might argue for a role of acetyl phosphate for the functionality of LiaRS-like or even envelope stress response 2CSs in general. Indeed, it is an appealing hypothesis to postulate that for these 2CSs the primary extracellular trigger, envelope stress, which is detected (directly or indirectly) by their HKs, is integrated with an intracellular modulator – acetyl phosphate as a measure of the energy state of the cell – that feeds into the 2CS at the level of the RRs to fine-tune the output.

LiaF and inhibition of LiaRS-dependent signal transduction

The Lia system is atypical in that it requires the activity of a third protein for its functionality. LiaF has previously been identified as a specific inhibitor of the LiaRS 2CS (Jordan *et al.*, 2006). This function was recently verified for the orthologous system in *S. mutans* (Suntharalingam *et al.*, 2009). The present study not only confirms the initial observation for a markerless *liaF* deletion, but also indicates that LiaF exerts its inhibitory function through LiaS. It does not affect the LiaS-independent phosphorylation of LiaR by acetyl phosphate (Fig. 3). Moreover, the inhibitory effect of LiaF can be overcome by increasing the amounts of LiaS, even in the presence of LiaF (Fig. 5). Based on these observations and the bifunctionality of LiaS, we propose that LiaF arrests LiaS in the phosphatase mode in the absence of a suitable trigger. Hence, the default setting of LiaS alone – even in the absence of a signal – is 'kinase ON', whereas in the presence of LiaF, it is switched to 'phosphatase ON' (Fig. 9). This hypothesis is supported by the observation that increasing the amount of LiaS relative to LiaF gradually turns on P_{liaI} activity even in the absence of a signal (Fig. 5A and B). As long as LiaF is in excess, only the presence of envelope stress releases this inhibition, thereby switching LiaS into its kinase mode, resulting in the phosphorylation of LiaR

and hence a strongly increased P_{liaI} activity. A further increase of the inhibitor does not significantly affect the functionality of the LiaFSR system (Fig. 5A), demonstrating that a signal can overcome the inhibition of LiaS by LiaF irrespective of the amount of excess in which LiaF is present relative to LiaS. The importance of maintaining such a ratio could again be interpreted as an indication for a physical interaction – for example the formation of a sensory/regulatory complex – between LiaF and LiaS. While so far we were unable to demonstrate this, future studies will hopefully shed some light on the mechanism of interaction between the two proteins.

If we take all observations of this study together, the following scenario can account for the observed behaviour (Fig. 9). Under normal non-inducing conditions, the excess of LiaF over LiaS keeps the HK quantitatively in its phosphatase mode. As long as LiaS is also in excess over LiaR, it can prevent the phosphorylation of LiaR by acetyl phosphate, thereby keeping the system completely switched off. If under these non-inducing conditions LiaR is overexpressed, it then stoichiometrically outcompetes its phosphatase, LiaS, and hence becomes phosphorylated by acetyl phosphate.

On the other hand, if LiaS is overexpressed, it is then in excess over LiaF, which therefore fails to completely keep the HK in its phosphatase state. If there is only a mild overexpression (i.e. in the wild type under its own weak SD sequence), it only increases the basal level of the LiaR-dependent gene expression (Fig. 5A). Nevertheless, most of LiaS is still kept in the phosphatase state by LiaF. Therefore, the system is still inducible by bacitracin (Fig. 5A). A strong overexpression of LiaS (i.e. with an optimized SD sequence) results in a higher amount of LiaS in the kinase state and hence a full activation of the LiaR response even in the absence of an inducer (Fig. 5B and C).

What is most puzzling about this model is that bifunctional HKs are very often present in the cells in much lower amounts than the cognate RRs. Nevertheless, they are usually very well capable of dephosphorylating their RR in the absence of a stimulus. This argues either for a very inefficient phosphatase activity of LiaS, and/or for a high affinity of LiaR for acetyl phosphate, again supporting the idea that the energy state of the cell – as reflected by the intracellular pool of acetyl phosphate – might indeed be an important secondary input into LiaR-dependent gene expression, as discussed above. Taken together, the combination of the unusual stoichiometry of the Lia system, the requirement for an additional component in order to keep LiaS in its phosphatase state, combined with a high affinity of LiaR for acetyl phosphate seem to collectively argue for a physiological necessity of this particular and unusual design of the LiaFSR system for its proper functionality. But these intriguing possibilities are purely speculative at the moment and will require subsequent investigations.

The exact mechanism by which LiaF affects LiaS activity is unknown. But one appealing hypothesis is that LiaF could function as a stimulus-perceiving anti-kinase that keeps LiaS inactive in the absence of a trigger, presumably through direct protein–protein interaction. Upon sensing a signal, LiaF releases the HK, which then acts as a LiaR-specific kinase. Alternatively, LiaF together with LiaS could form the stimulus perception complex of the LiaFSR system. In this complex, LiaF would again keep LiaS in its phosphatase state in the absence of a trigger. Upon addition of bacitracin, the LiaFS complex would perceive the resulting envelope stress and again LiaS would switch to its default kinase-ON mode, thereby activating LiaR. Both possibilities would be in good agreement with the data obtained.

While the important role of the stoichiometry could well be a specialty of LiaFSR-like systems, due to the presence of a third inhibitory protein, our observations could also have a more general significance for 2CSs with HisKA_3-containing HKs.

A comparison of the stoichiometry and genetic arrangement in operons of EnvZ/OmpR-like and NarXQ/NarL-like 2CSs indicates a possible connection between protein ratios and operon structure. EnvZ-like HKs are usually encoded downstream of their cognate RR genes, potentially accounting for the observed stoichiometry with RR exceeding the cognate HK. In contrast, for NarXQ/NarL-like 2CSs the genetic order is usually inverted, which can be viewed as an indication for a molar excess of HK over RR for 2CSs. This might point towards a fundamental difference between HisKA- (EnvZ-like) and HisKA_3-(NarXQ-like) containing HKs with respect to their enzymatic (at least phosphatase) activities: A functional role of an excess of the HK over the cognate RR suggests that in such cases the phosphatase activity of the HK is weak. But more work on additional HisKA- and HisKA_3-containing HKs will be necessary in order to verify or falsify such a hypothesis.

Experimental procedure

Media and growth conditions

Bacillus subtilis and *E. coli* were routinely grown in LB medium or chemical defined CSE [Chemical defined Succinate (0.56% (w/v) Na-succinate) and Glutamate [0.75% (w/v) K-glutamate)] medium (Stülke *et al.*, 1993) at 37°C with aeration. Ampicillin (100 μg ml^{-1}) was used for selection of all plasmids in *E. coli*. Kanamycin (10 μg ml^{-1}), chloramphenicol (5 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), erythromycin (1 μg ml^{-1}) plus lincomycin (25 μg ml^{-1}) for macrolide–lincosamide–streptogramin (MLS) resistance,

Table 4. Vectors and plasmids used in this study.

Plasmid	Genotype, sequence or characteristic(s)	Primers used for cloning	Reference or source
Vectors			
pAC5	*amyE*-up, *lacZ*, MCS, *cat*, *amyE*-down, *bla*		Martin-Verstraete *et al.* (1992)
pAC6	*amyE*-up, *lacZ*, MCS, *cat*, *amyE*-down, *bla*		Stülke *et al.* (1997)
pAC7	*amyE*-up, *lacZ*, MCS, *kan*, *amyE*-down, *bla*		Weinrauch *et al.* (1991)
pXT	*thrC*-up, P_{xyl}, MCS, *spc*, *thrC*-down, *erm*, *bla*		Derre *et al.* (2000)
pMAD	*bgaB*, *ermC*, *bla*, MCS		Arnaud *et al.* (2004)
pDG647	pSB119, *erm*		Guerout-Fleury *et al.* (1995)
pDG780	pBluescriptKS+, *kan*		Guerout-Fleury *et al.* (1995)
pDG1513	pMTL22, *tet*		Guerout-Fleury *et al.* (1995)
pALFLAG3*rsiW*	*lacA*-up, *erm*, *lacI*, *rsiW*, P_{spac}, FLAG3, *lacA*-down, *bla*		Schöbel *et al.* (2004)
pProEx-1	His_6-tag, rTEV cleavage, P_{Trc}, MCS, *bla*		Life Technologies
Plasmids			
pTM1	pAC6 P_{liaI}(–83 to 72)–*lacZ*		Jordan *et al.* (2006)
pMM101	pMAD Δ*liaS*		Jordan *et al.* (2007)
pSJ102	pMAD Δ*liaF*		Wolf *et al.* (2010)
pDW701	pXT *liaR*	1068/1106	This work
pER503	pAC5 $P_{liaG\text{-}opt.}$-*liaF* (–68 to 2)–*lacZ*	0579/0580	This work
pER504	pAC5 $P_{liaG\text{-}opt.}$-*liaS* (–68 to 2)–*lacZ*	0579/0581	This work
pER505	pAC5 $P_{liaG\text{-}opt.}$-*liaR* (–68 to 2)–*lacZ*	0579/0582	This work
pKS101	pMAD FLAG3-*liaF*	1950/1951, 1952/1953	This work
pKS104	pMAD *liaS*-FLAG3	1958/1959, 1960/1961	This work
pKS105	pMAD FLAG3-*liaR*	1958/2041, 2042/1961	This work
pKS704	pXT *liaS*-FLAG3	0454/0962, 0960/0961	This work
pKS726	pXT *liaFSR*	0035/0893	This work
pKS727	pXT *liaS*	0454/0046	This work
pKS729	pXT *liaS* (LiaS Q_{164}A)	2374/2375	This work
pKS1001	pAC7 P_{veg}-SD *liaF*	0856/0857	This work
pKS1002	pAC7 P_{veg}-SD *liaS*	0856/0898	This work
pKS1003	pAC7 P_{veg}-SD *liaR*	0856/0899	This work
pKS1005	pAC7 P_{veg}	0856/0906	This work
pKS-FLAG1	pALFLAG *liaS*	0958/0959	This work
pKSEx102	pProEx1 *liaR*-FLAG3	1530/1164, 0960/1161	This work
pKSEx103	pProEx1 *liaS*-FLAG3	0958/0962, 0960/1161	This work
pSJ701	pXT *liaF*	0035/0036	This work

and tetracycline (10 μg ml^{-1}) were used for the selection of the *B. subtilis* mutants used in this study.

Bacterial strains and plasmids

The strains of *E. coli* and *B. subtilis* are listed in Table 2. All *B. subtilis* strains used in this study are derivatives of the laboratory wild type strains W168 and CU1065 (W168 *attSP*β). The plasmids used in this study are listed in Table 4.

DNA manipulations

The preparation of chromosomal DNA and transformation were performed according to standard procedures (Cutting and Van der Horn, 1990). *E. coli* plasmid DNA and restriction enzyme fragments were isolated by using the QIAprep spin miniprep and PCR purification kits respectively (Qiagen). DNA ligase (Fermentas), HotStarTaq Plus DNA Polymerase (Qiagen), and Phusion High-Fidelity DNA Polymerase (Finnzymes) were used according to manufacturer's instructions. All primers used for PCR are listed in Table S1.

Site-directed mutagenesis of liaR and liaS

To generate an amino acid exchange of the conserved aspartate of LiaR to alanine, we introduced a point mutation in *liaR* via the Combined Chain Reaction (CCR). This method was performed as described previously (Bi and Stambrook, 1997). In brief, *liaR* was amplified from chromosomal DNA of the wild type, using primers #0047 and #0048 flank the overall sequence of *liaR* while one internal mutagenesis primer (#0508) are positioned at the mutation site of interest (Table S1). The mutagenesis primer carries the point mutation where it mismatched with template as well as a phosphorylated 5′ end. During the PCR process, the extended specific forward primer was ligated with the mutagenesis primer by a thermostable DNA ligase (Ampligase) to create a *liaR* fragment with the expected point mutation.

To investigate the phosphatase activity of LiaS, an amino acid exchange of the glutamine residue within the conserved DxxxQ motif was generated via *in vitro* site-directed mutagenesis. The plasmid pKS727 (pXT-*liaS*) was used as template in a PCR reaction together with mutagenesis primers #2374 and #2375 that carry the desired point mutation. These primers are complementary to opposite strands of the plasmid. The extension of the primers results in a mutated plasmid. The PCR product was digested with DpnI to remove the methylated parental DNA template. The mutated plasmid was then transformed into *E. coli* XL1 blue competent cells.

Allelic replacement mutagenesis of liaS, pta and ackA using LFH-PCR

The Long Flanking Homology PCR (LFH-PCR) technique is derived from a published procedure (Wach, 1996) and was performed as described previously (Mascher *et al*., 2003). The constructed strains are listed in Table 2, and the corresponding primers are listed in Table S1.

Construction of translational B. subtilis P_{veg}*–lacZ and* $P_{liaG\text{-}opt.}$*–lacZ fusions*

To investigate the translation initiation of *liaF*, *liaS* and *liaR*, ectopic integrations of P_{veg}-$SD_{liaF/liaS/liaR}$–*lacZ* fusions were constructed based on the vector pAC7 (Table 4). For this purpose, one forward primer, which contains the P_{veg} sequence (#0856), and three reverse primer (#0857, #0898, #0899) were designed, which carry the Shine–Dalgarno sequences of *liaF*, *liaS* or *liaR* up to each corresponding start codon respectively (Table S1). Each reverse primer harbours 25 nucleotides at the 3′ end that is inverse and complementary to the 3′ end of the P_{veg}-forward primer, so that they can be fused by joining PCR. The resulting fragments were cloned into pAC7 via SmaI and BamHI, generating pKS1001–pKS1003 (Table 4). After *B. subtilis* transformation, the plasmids integrated into the *amyE* locus by double crossing-over, resulting in a stable integration of P_{veg}–*lacZ* fusions.

To investigate the expression levels of *liaF*, *liaS* and *liaR*, ectopic integrations of $P_{liaG\text{-}opt.}$-$SD_{liaF/liaS/liaR}$–*lacZ* fusions were constructed in a comparable fashion, based on the vector pAC5 (Table 4). Three fragments including *liaG* up to the start codon of *liaF*, *liaS* or *liaR*, respectively, were amplified from wild type chromosomal DNA, using the forward primer #0579, which introduces the optimal *liaG* promoter sequence, as well as the reverse primer #0580, #0581 or #0582 (Table S1). The resulting fragments were cloned into pAC5 via SmaI and BamHI, generating pER503–pER505 (Table 4). After *B. subtilis* transformation, the plasmids integrated into the *amyE* locus by double crossing-over, resulting in a stable integration of $P_{liaG\text{-}opt.}$–*lacZ* fusions.

Measurement of promoter activity by β-galactosidase assay

Cells were inoculated from fresh overnight cultures and grown in LB medium or CSE medium at 37°C with aeration until they reached an optical density at 600 nm (OD_{600}) of ≈ 0.4. The culture was split, adding bacitracin (50 μg ml^{-1} 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 was 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 elsewhere, with normalization to cell density (Miller, 1972).

Preparation of total RNA for quantitative real-time RT-PCR and Northern blotting

Total RNA was extracted from 4 ml of culture with and without bacitracin (50 μg ml^{-1} final concentration). Bacitracin was added to the culture at an OD_{600} of 0.5 (mid-exponential phase), and the cultures were incubated for 30 min at 37°C with aeration before the cells were harvested and rapidly frozen at −70°C. RNA was prepared using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The RNA was treated with Baseline-ZERO DNase (EPICENTRE) 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, using the same primers as for the real-time reverse transcription-PCR (RT-PCR).

Quantitative real-time RT-PCR

Measurement of transcript abundance was performed by quantitative real-time RT-PCR, using the iScript One-Step RT-PCR Kit (Bio-Rad) according to the manufacturer's procedure, with minor modifications. In brief, 100 ng of DNA-free total RNA was used in a total reaction volume of 20 μl with 0.3 μM of each primer (Table S1). The amplification reaction was carried out in an iCycler (Bio-Rad). Expression of *rpsE* and *rpsJ*, encoding ribosomal proteins, was monitored as a constitutive reference. Expression of *liaF*, *liaS* or *liaR* of the uninduced wild type was calculated as the fold change based on the C_T values for each gene, as described previously (Talaat *et al*., 2002).

Probe preparation and Northern blot analysis

Internal fragments of *liaIH* and *liaR* (~ 500-nucleotide length) were amplified by PCR using the primer pairs

listed in Table S1. The PCR fragments were purified by using the PCR purification kit (Qiagen), and 1 μg of each fragment was labelled with digoxygenin (DIG) by *in vitro* transcription using the DIG RNA labelling mix (Roche) and the T7-RNA polymerase (Roche) according to manufacturer's protocol.

For Northern blot analysis, 5 μg or 10 μg of total RNA were denatured and loaded on a formaldehyde agarose gel. After electrophoresis, the RNA was transferred to a nylon membrane (Roche) in a downward transfer using 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) as transfer buffer. The RNA was cross-linked by exposing the damp membrane to UV light. The blot was pre-hybridized at 68°C for 1 h with pre-hybridization solution [0.2% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosinate, 5× SSC, 50% (v/v) formamide, 2% (w/v) blocking reagent] and labelled probe was added to the hybridization tube. Hybridization was performed overnight at 68°C. The next day, the membrane was washed twice with low-stringency buffer [2 × SSC, 0.1% (w/v) SDS] at room temperature for 5 min, followed by two high-stringency washes [0.1× SSC, 0.1% (w/v) SDS] at 68°C for 15 min. For the detection of labelled probe, the DIG Nucleic Acid Detection Kit (Roche) was used. Therefore, the blot was removed from the hybridization tube and placed in a box with 1× buffer 1 [10× buffer 1 is 1 M maleinic acid, 1.5 M NaCl, 0.3% (v/v) tween20, pH 7.5] for 5 min at room temperature. The membrane was pre-incubated with buffer 2 [10% (v/v) 10× buffer 1, 1% (w/v) blocking reagent] for 30 min, treated with the antibody against DIG conjugated with alkaline phosphatase (AP) (Roche) for 30 min, and washed three times with 1× buffer 1 for 10 min. The blot was wrapped in plastic wrap, treated with the AP substrate CDP-Star (Roche) at a dilution of 1:200, and analysed using a LumiImager (PeqLab).

Preparation of B. subtilis *cell fractions for Western blotting*

The methodology was based on a published procedure (Heinrich *et al.*, 2008) with the following modifications: *B. subtilis* strains were grown in LB medium and 50 ml of cells with an OD_{600} of 0.5–0.8 were harvested by centrifugation. The cells were washed and resuspended in 1 ml of cold disruption buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). Samples were sonicated (Cell disrupter UP 200 s, Dr Hielscher, Stuttgart) on ice and an aliquot of 100 μl was removed (whole cell fraction, W). Cell debris of the remaining 900 μl were removed by centrifugation at 5.000 *g* for 15 min at 4°C. The supernatant (800 μl) was ultracentrifuged at 70.000 *g* for 1 h at 4°C. The supernatant was removed (soluble protein fraction, S) and the membrane pellet (membrane protein fraction, M) was washed in cold disruption buffer, ultracentrifuged again (70.000 *g*, 30 min, 4°C), dissolved in 100 μl of Laemmli buffer and heated for 5 min at 95°C. The protein content of the W and S fractions was established according to Bradford. For SDS-PAGE and Western blotting 20 μg of samples of the W fractions, 20 μl of the S fractions, and 20 μl of the M fractions were loaded to each lane.

Western blot analysis

Western blot analysis was performed by a wet-blotting procedure, using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to manufacturer's protocol. After protein transfer, the polyvinylidene diflouride (PVDF) membrane (Bio-Rad) was incubated with blotto [1× TBS (50 mM Tris, 150 mM NaCl, pH 7.6), 2.5% (w/v) skim milk] overnight at 4°C to prevent unspecific binding. On the next day the membrane was treated with the primary antibody anti-FLAG (Sigma) or anti-LiaH [polyclonal rabbit antisera that were raised against purified His_{10}-LiaH (Jordan *et al.*, 2007) at SEQLAB, Göttingen] at a dilution of 1:5000 for 3 h at room temperature. Then, the membrane was washed three times with blotto following by the addition of the secondary antibody (anti-rabbit IgG, conjugated with AP, Promega) at a dilution of 1:100.000 for 30 min. After further three washes with blotto the membrane was incubated with buffer 3 (100 mM Tris, 100 mM NaCl, pH 9.5) for 5 min. The blot was wrapped in plastic wrap, treated with the AP substrate CDP-Star (Roche) at a dilution of 1:100, and analysed using a LumiImager (PeqLab).

Cloning, expression and purification of recombinant N-terminal His_6- and C-terminal FLAG3-tagged LiaS and LiaR

The *liaR* and *liaS* genes were amplified from *B. subtilis* W168 genomic DNA using primer pairs #1530/#1164 or #0958/#0962, respectively, and subsequently fused to a FLAG3 epitope tag (amplified from pALFLAG3*rsiW* with primers #0960/#1161) by a second joining PCR for detection via Western blot analysis. PCR products were cloned into the pProEx1 expression vector (Life Technologies) via NdeI and HindIII or BamHI and HindIII, respectively, generating plasmids pKSEx102 (His_6-LiaR-FLAG3) and pKSEx103 (His_6-LiaS-FLAG3) (Table 4). For overexpression, *E. coli* BL21(DE3)/pLysS was transformed with pKSEx102 or pKSEx103 and grown in LB medium. In mid-exponential phase (OD_{600} of 0.4–0.6), protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were harvested 3 h (His_6-LiaR-FLAG3) and 16 h (His_6-LiaS-FLAG3) after induction. Cell pellets were stored at −80°C until further purification.

Purification of His_6-LiaR-FLAG3. The cell pellet was resuspended in 15 ml of loading buffer [20 mM Tris-HCl

(pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 5 mM imidazole (pH 8.0)] and cells were disrupted by sonication. The lysate was centrifuged at 20.000 *g* and 4°C for 1 h. The supernatant was loaded on a gravity flow column containing 1 ml of Ni^{2+}-nitrilotriacetic acid (NTA) Superflow resin (Qiagen). After washing steps with loading buffer and loading buffer containing 50 mM imidazole, His_6-LiaR-FLAG3 was eluted from the column using loading buffer with imidazole concentrations of 100 mM, 200 mM and 500 mM. All fractions were analysed by SDS-PAGE and fractions containing the most pure His_6-LiaR-FLAG3 protein were collected, quantified by Bradford assay using the Roti-Nanoquant kit (Roth), and used as standard for quantitative Western blot analyses.

Purification of His_6-LiaS-FLAG3. The cell pellet was resuspended in 12 ml of disruption buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) and cells were disrupted by sonication. The cell debris were removed by centrifugation (5.000 *g*, 4°C, 15 min) and the supernatant was used to prepare the membrane protein fraction by ultracentrifugation as described above. The membrane pellet was resuspended in 1 ml of disruption buffer and the protein concentration was measured via BCA assay. The solution was diluted with loading buffer to receive a final protein concentration of 5 mg ml^{-1}. To solubilize the membrane proteins, 0.5% (w/v) n-Dodecyl-β-D-maltoside (DDM) was added and gently mixed at 4°C for 1 h. After solubilization, the protein solution was ultracentrifuged (70.000 *g*, 4°C, 1 h). The supernatant was loaded on a Ni^{2+}-NTA column and His_6-LiaS-FLAG3 was purified as described for His_6-LiaR-FLAG3 using buffers that contain 0.02% (w/v) DDM. Purified His_6-LiaS-FLAG3 protein was quantified by Bradford assay using the Roti-Nanoquant kit (Roth) and used as standard for quantitative Western blot analyses.

Chromosomal FLAG-tagging of LiaFSR

To quantify the cellular amounts of LiaFSR, we integrated the FLAG3-tag sequence directly into the W168 chromosome C-terminal of LiaS or N-terminal of LiaF and LiaR. This was done by using the pMAD shuttle vector (Arnaud *et al.*, 2004). The regions about 600 bp upstream and downstream of the position of FLAG integration were amplified using primers listed in Table S1, thereby introducing a 66 bp extension containing the whole FLAG3 sequence to the 3′ end of the up-fragment and a 25 bp extension to the 5′ end of the down-fragment which is complementary to the 3′ end of the FLAG3-tag sequence. The two fragments were fused in a second joining PCR, and the resulting fragment was cloned into pMAD via BamHI and NcoI, generating pKS101 (FLAG3-*liaF*), pKS104 (*liaS*-FLAG3) and pKS105 (FLAG3-*liaR*) (Table 4). The generation of the mutants basically followed the established procedure (Arnaud *et al.*, 2004). In brief, *B. subtilis* W168 was transformed with pKS101, pKS104 or pKS105, respectively, and incubated at 30°C with MLS selection on LB agar plates supplemented with X-Gal (100 μg ml^{-1}). Blue colonies were selected and incubated 6 h at 42°C in LB medium with MLS selection, resulting in the integration of the plasmids into the chromosome. Again, blue colonies were picked from LB (X-Gal) plates and incubated for 6 h in LB medium without MLS 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 plasmids were picked and checked for MLS sensitivity. The resulting strains, TMB1141 (*liaS*-FLAG3), TMB1155 (FLAG3-*liaF*) and TMB1201 (FLAG3-*liaR*) were analysed by PCR and sequencing for the integrity of the desired genetic modifications.

Determination of cellular amounts of LiaSR by quantitative Western blot analysis

Cellular amounts of LiaS-FLAG3 or FLAG3-LiaR were determined in strain TMB1141 or TMB1201 respectively. Cells were grown in LB medium until mid-exponential phase (OD_{600} at 0.4–0.6). The cultures were split, adding bacitracin (20 μg ml^{-1} final concentration) to one-half (induced sample) and leaving the other half untreated (uninduced sample). After incubation for an additional 30 min at 37°C with aeration, 10 ml of each culture was harvested and the cell pellets were frozen and kept at –80°C. Additionally, the amount of harvested cells was analysed on agar plates. The cells were resuspended in 1.1 ml of disruption buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) and cells were disrupted by sonication. The cell debris were removed by centrifugation (5.000 *g*, 4°C, 15 min) and the supernatant was used to separate the soluble and membrane protein fractions by ultracentrifugation as described above. The soluble protein fractions were concentrated up to 50 μl using Vivaspin 500 concentrator tubes (Sartorius) and the membrane pellets were resuspended in 50 μl of Laemmli buffer. Ten microlitres of each sample was loaded onto a 12.5% SDS gel together with the purified standards using 10–200 fmol of His_6-LiaS-FLAG3 and 10–100 fmol of His_6-LiaR-FLAG3. The Western blot was performed by a wet-blotting procedure, using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to manufacturer's protocol. After protein transfer, the polyvinylidene diflouride (PVDF) membrane (Macherey-Nagel) was incubated with blotto [1× TBS (50 mM Tris, 150 mM NaCl, pH 7.6), 2.5% (w/v) skim milk] overnight at 4°C to prevent unspecific binding. On the next day the membrane was treated with the primary antibody anti-FLAG (Sigma) at a dilution of 1:2000 for 1 h at room temperature. Then, the membrane was washed four times

with blotto following by the addition of the secondary antibody (anti-rabbit IgG, conjugated with HRP, Promega) at a dilution of 1:2.000 for 1 h. After further four washes with blotto the membrane was incubated with 1× TBS for 5 min. The blot was wrapped in plastic wrap, treated with the HRP substrate Ace Glow (Peqlab) according to manufacturer's protocol, and analysed using a LumiImager (PeqLab). The blot was analysed by ImageJ software. The band intensities of the standard proteins were plotted against the known protein amounts and these curves are referred to as standard curves. The protein amounts of LiaS-FLAG3 and FLAG3-LiaR were calculated from the standard curves.

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Supporting information

Additional supporting information may be found in the online version of this article.

3 The LIKE system, a novel protein expression toolbox for *Bacillus subtilis* based on the *liaI* promoter

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The LIKE system, a novel protein expression toolbox for *Bacillus subtilis* based on the *liaI* promoter

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Abstract

Background: *Bacillus subtilis* is a very important Gram-positive model organism of high biotechnological relevance, which is widely used as a host for the production of both secreted and cytoplasmic proteins. We developed a novel and efficient expression system, based on the *liaI* promoter (P_{liaI}) from *B. subtilis*, which is under control of the LiaRS antibiotic-inducible two-component system. In the absence of a stimulus, this promoter is kept tightly inactive. Upon induction by cell wall antibiotics, it shows an over 100-fold increase in activity within 10 min.

Results: Based on these traits of P_{liaI}, we developed a novel LiaRS-controlled gene expression system for *B. subtilis* (the "LIKE" system). Two expression vectors, the integrative pLIKE-int and the replicative pLIKE-rep, were constructed. To enhance the performance of the P_{liaI}-derived system, site-directed mutagenesis was employed to optimize the ribosome binding site and alter its spacing to the initiation codon used for the translational fusion. The impact of these genetic modifications on protein production yield was measured using GFP as a model protein. Moreover, a number of tailored *B. subtilis* expression strains containing different markerless chromosomal deletions of the *liaIH* region were constructed to circumvent undesired protein production, enhance the positive autoregulation of the LiaRS system and thereby increase target gene expression strength from the P_{liaI} promoter.

Conclusions: The LIKE protein expression system is a novel protein expression system, which offers a number of advantages over existing systems. Its major advantages are (i) a tightly switched-off promoter during exponential growth in the absence of a stimulus, (ii) a concentration-dependent activation of P_{liaI} in the presence of suitable inducers, (iii) a very fast but transient response with a very high dynamic range of over 100-fold (up to 1,000-fold) induction, (iv) a choice from a range of well-defined, commercially available, and affordable inducers and (v) the convenient conversion of LIKE-derived inducible expression strains into strong constitutive protein production factories.

Keywords: two-component system, *liaIH* operon, antibiotic-inducible promoter, cell envelope stress response, protein expression, *Bacillus subtilis*, bacitracin

Background

Bacillus subtilis is a widely exploited bacterium for basic research, but also industrial and biotechnological applications [1] owing to the ease of genetic manipulation, a systems level understanding of its genome and physiology [2-4], its efficient protein secretion systems [5], non-pathogenic GRAS-status [6] and well-characterized mechanisms for gene expression [7]. Over the years, numerous genetic devices and expression systems have been developed for this organism to facilitate the production of homologous or heterologous proteins [7-14], usually based on strong inducible promoters. Such systems can either be integrated into the chromosome or located on replicative plasmids to increase the gene copy number under the control of the inducible promoter.

A number of new expression systems based on induction by peptide antibiotics were described for Gram-positive bacteria [9,15,16]. The nisin-controlled gene expression (NICE) system was developed for different species of *Lactococcus* and *Lactobacillus* and allows the

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production of the desired proteins in high amounts (comparable to other expression systems), reaching a maximum 3 h after nisin induction [15,16]. A very similar subtilin-regulated expression system (SURE) was recently constructed for *B. subtilis* [9]. Both systems enable the controlled overexpression of a variety of homologous and heterologous proteins and enzymes and show a number of advantages to other control elements, such as the strict control of gene expression, no leakage of the promoter regulation under non-inducing conditions, high levels of expression upon induction and almost no limitations in the choice of sugar-containing media [9,15]. For the use in *B. subtilis*, the SURE system has several advantages over the NICE system: (i) The SURE system only requires a single plasmid, thereby ensuring a stable expression platform; (ii) the expression levels achieved by the SURE system are significantly higher; and (iii) it also requires lower concentrations of the inducer molecule [9,17].

Despite significant progress in the field, no exisiting system works equally well for all proteins and none of the existing expression systems for *B. subtilis* is without pitfalls or limitations. While the SURE system represents a major improvement, its inducer, the lantibiotic subtilin, is not commercially available. Instead, culture supernatant of the lantibiotic producer must be used, which introduces a source of variation and requires testing the potency each time a new supernatant is used for induction. Therefore, novel tightly controllable gene expression systems are still in demand to expand and complement the existing repertoire in order to find the optimal solution for a given protein to be produced in *B. subtilis*.

Here, such an addition to the existing bioengineering toolbox for *B. subtilis* will be described. The LIKE (from the German "*LIa-Kontrollierte Expression*") system is based on the cell envelope stress-responsive *liaI* promoter. This promoter was initially identified in the course of studies on the response of *B. subtilis* to the presence of harmful concentrations of various cell wall antibiotics [18]. The underlying regulatory network of the cell envelope stress response in this organism is rather complex and consists of at least four extracytoplasmic function (ECF) σ factors and a similar number of two-component systems (TCS) and has been extensively studied [19,20]. One such TCS, LiaRS, is a central player in the envelope stress response network of *B. subtilis*. It strongly responds to antibiotics that interfere with the lipid II cycle, such as bacitracin. Activation of the LiaRS system of *B. subtilis* specifically leads to the strong induction of a single target promoter, P_{liaI}, which drives the expression of the *liaIH* operon. This promoter is basically shut off in the absence of inducing condition during logarithmic growth and shows an impressive dynamic range of over 100- up to 1,000-fold in the presence of suitable stimuli [21-23].

Because of its specificity and sensitivity, P_{liaI} has already been developed as a powerful screening tool for mechanism-of-action studies of novel peptide antibiotics interfering with envelope integrity [22,24,25]. But its tightly regulated, concentration-dependent and highly dynamic behavior also makes this promoter a very promising candidate for the development of a novel gene expression system. This prospect is further supported by transcriptome studies of mutants that are constitutively switched Lia-ON or Lia-OFF, which revealed a very specific response with only very few genes being indirectly affected [23]. Moreover, *B. subtilis* is highly resistant to bacitracin, a commercially available compound, which can be used as an ideal inducer to activate P_{liaI}-driven gene expression in growing cultures of *B. subtilis*. Moreover, a simple gene deletion can convert the inducible into a high-level constitutive promoter activity. Based on these traits of P_{liaI}, we developed vectors and strains to apply this promoter as a powerful protein expression system in *B. subtilis*.

Results and discussion

Features of the native *liaI* promoter (P_{liaI})

Previously, we have characterized the cell envelope stress-inducible promoter P_{liaI}, which controls the expression of the *liaIH* operon in *B. subtilis*. During normal logarithmic growth, this promoter is virtually switched off and hence does not show any significant basal activity. In the presence of suitable inducers such as the cell wall antibiotic bacitracin, it strongly responds in a concentration-dependent manner, resulting in a more than 100-fold increased activity already 5–10 min after the addition of bacitracin. This activity strictly depends on the activity of the response regulator LiaR [21-23] (Figure 1A). This tight regulation and the impressive strength of P_{liaI} under inducing conditions are illustrated by the protein gel shown in Figure 1B, which demonstrates that even from the native P_{liaI}, present in single copy on the chromosome, LiaH is the predominant protein produced under inducing conditions, as has already been indicated previously by 2D gelectrophoresis [23]. These features make P_{liaI} a very promising candidate for developing a novel protein-expression system for the Gram-positive model organism *B. subtilis*, which is widely used in the biotechnological industry as a protein production host [1]. To achieve this, the *liaI* promoter was first sequence-optimized and then integrated into two expression vectors. Moreover, a set of suitable expression strains was developed and evaluated to further improve the promoter strength while simultaneously avoiding the metabolic burden of overexpressing

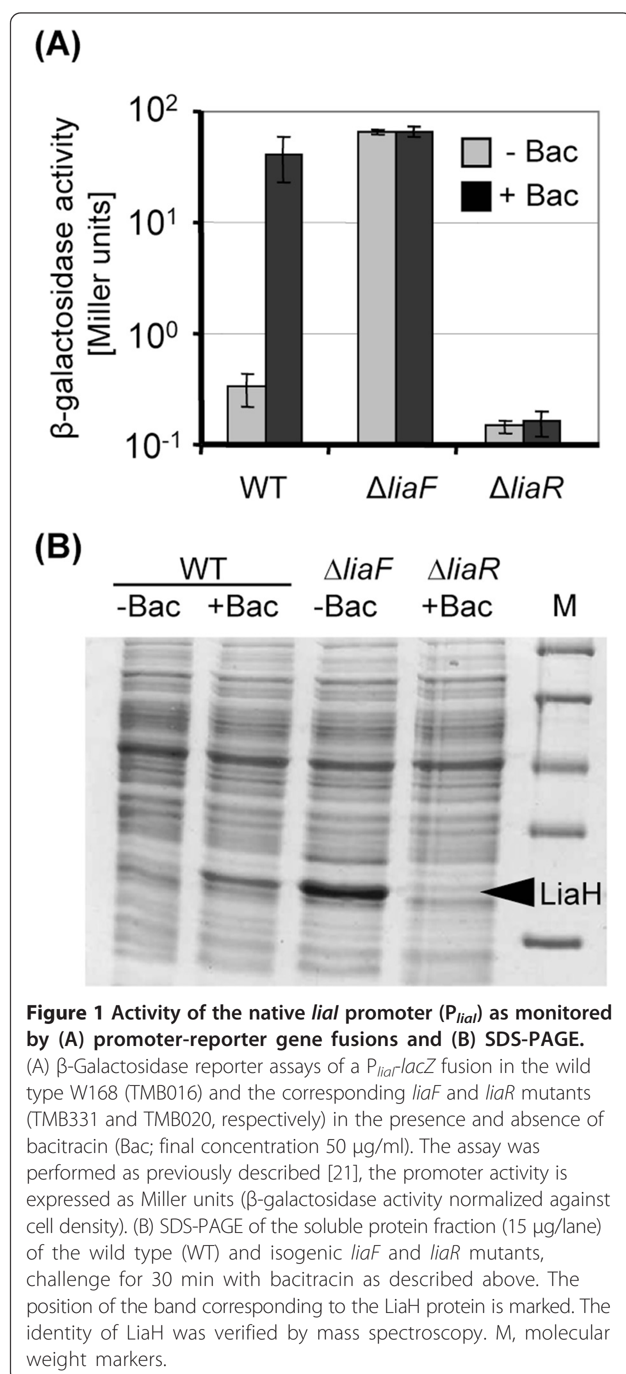

Figure 1 Activity of the native *liaI* promoter (P_{liaI}) as monitored by (A) promoter-reporter gene fusions and (B) SDS-PAGE. (A) β-Galactosidase reporter assays of a P_{liaI}-*lacZ* fusion in the wild type W168 (TMB016) and the corresponding *liaF* and *liaR* mutants (TMB331 and TMB020, respectively) in the presence and absence of bacitracin (Bac; final concentration 50 μg/ml). The assay was performed as previously described [21], the promoter activity is expressed as Miller units (β-galactosidase activity normalized against cell density). (B) SDS-PAGE of the soluble protein fraction (15 μg/lane) of the wild type (WT) and isogenic *liaF* and *liaR* mutants, challenge for 30 min with bacitracin as described above. The position of the band corresponding to the LiaH protein is marked. The identity of LiaH was verified by mass spectroscopy. M, molecular weight markers.

the native target proteins of LiaR-dependent gene regulation, LiaI and LiaH [23], as indicated in Figure 1B.

Design and construction of P_{liaI}-based expression vectors and *B. subtilis* protein production strains for the LIKE system

A closer inspection of the *liaI* promoter sequence revealed a poorly conserved Shine-Dalgarno sequence (SD) with a suboptimal spacing to the *liaI* start codon (data not shown). As a first step in developing a P_{liaI}-derived bacitracin-inducible expression system, we therefore optimized its SD sequence by introducing a strong *B. subtilis* ribosome binding site (TAAGGAGG) with an optimal spacing of seven nucleotides upstream of the start codon, which was used for all subsequent constructions and will be referred to as $P_{liaI(opt)}$ from now on (Figure 2). This optimized SD sequence is well established for *B. subtilis* [26,27], and provides optimal complementarity to the 3'-end of the 16S rRNA, thereby increasing the ribosome's affinity to the mRNA and enhancing the translation initiation efficiency.

For the construction of new P_{liaI}-derived bacitracin-inducible gene expression systems, we chose two vectors as backbones: the *E. coli*/*B. subtilis* shuttle vector pGP380, and pDG1662 for ectopic integration at the *amyE* locus of *B. subtilis* [28,29], thus enabling both expression from a multi-copy replicative vector, as well as the stable chromosomal integration at single copy. The optimized regulatory element $P_{liaI(opt)}$ was amplified by PCR and cloned into the two vectors (see Materials and Methods for details) resulting in the expression vectors pLIKE-rep and pLIKE-int, respectively (Figure 2A/B).

Previous work has demonstrated that the *liaIH* operon is the only relevant target of LiaFSR-dependent gene expression, and that activation of P_{liaI} results in a strong accumulation of LiaH in the cytosol (Figure 1B) [23]. Based on the organization and expression of genes in the *liaIH-liaGFSR* locus, activation of P_{liaI} also leads to an increased expression of *liaGFSR*, due to read-through transcription [22]. Such positive autoregulatory feedback loops often have beneficial effects on the activity of their target genes [30]. Hence, it might be desirable to maintain this feedback loop. On the other hand, the observed very strong production of the native LiaFSR-target proteins LiaIH is not desired in a protein production host, since it depletes the cells of energy, amino acids and ribosomes required for heterologous protein production.

To account for these two opposing goals, we constructed a number of clean deletion mutants as potential hosts of the LIKE-system. The features of the resulting strains are summarized in Figure 2C. Strain TMB604 lacks both the *liaIH* operon including the native *liaI* promoter. Hence, no autoregulation can occur under inducing conditions. In contrast, strains TMB1151/TMB1152, which are also deleted for the *liaIH* operon, still maintain P_{liaI} and therefore autoregulation. They differ in the presence or absence of the weak terminator located downstream of *liaH* (Figure 2C).

As a measure for $P_{liaI(opt)}$-dependent protein production in the two expression plasmids, *gfpmut1* gene was used as a reporter gene [31]. Translational fusions of $P_{liaI(opt)}$ with *gfpmut1* were constructed in both pLIKE-int and pLIKE-rep and subsequently introduced into the aforementioned *B. subtilis* strains.

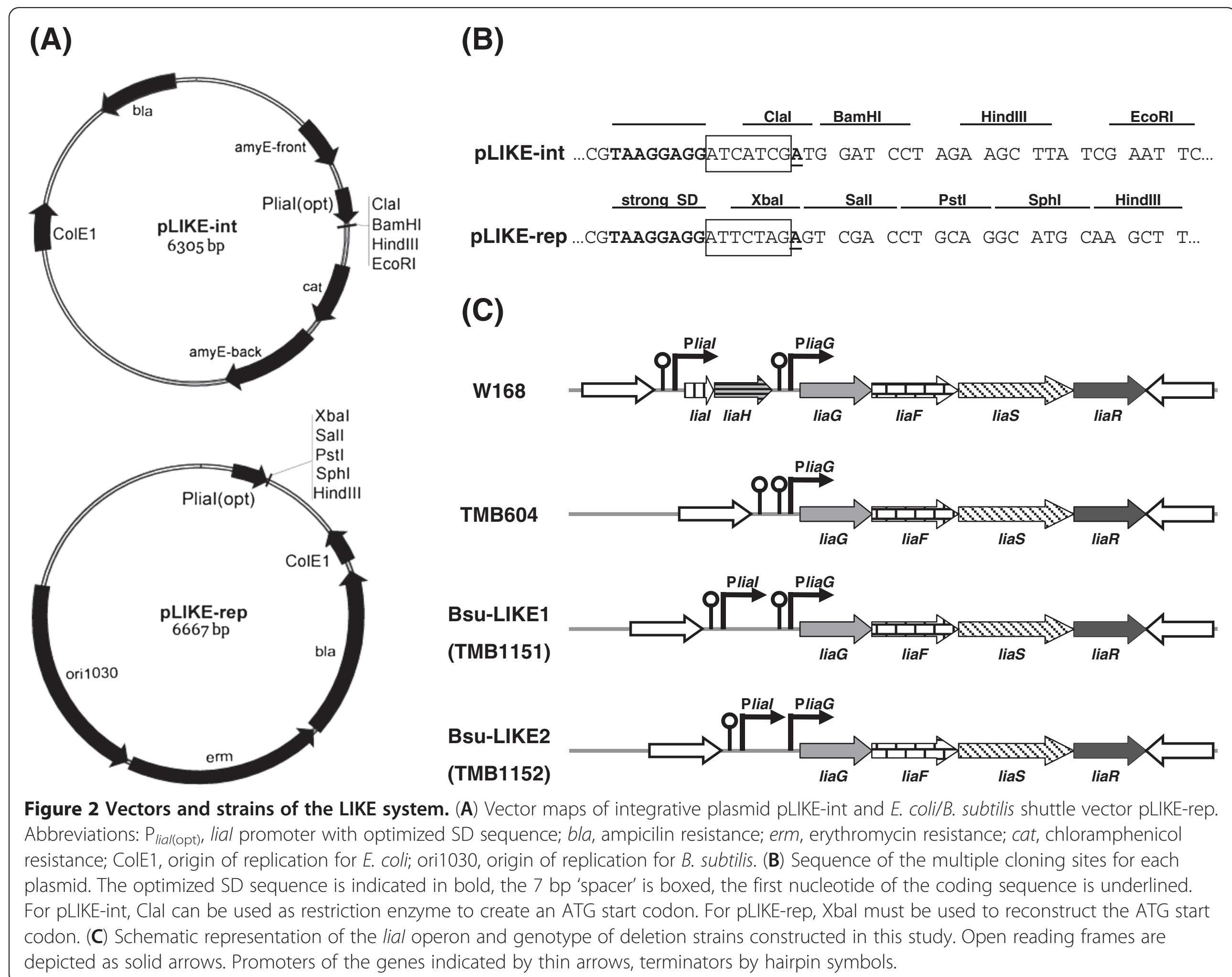

Figure 2 Vectors and strains of the LIKE system. (**A**) Vector maps of integrative plasmid pLIKE-int and *E. coli*/*B. subtilis* shuttle vector pLIKE-rep. Abbreviations: $P_{liaI(opt)}$, *liaI* promoter with optimized SD sequence; *bla*, ampicilin resistance; *erm*, erythromycin resistance; *cat*, chloramphenicol resistance; ColE1, origin of replication for *E. coli*; ori1030, origin of replication for *B. subtilis*. (**B**) Sequence of the multiple cloning sites for each plasmid. The optimized SD sequence is indicated in bold, the 7 bp 'spacer' is boxed, the first nucleotide of the coding sequence is underlined. For pLIKE-int, ClaI can be used as restriction enzyme to create an ATG start codon. For pLIKE-rep, XbaI must be used to reconstruct the ATG start codon. (**C**) Schematic representation of the *liaI* operon and genotype of deletion strains constructed in this study. Open reading frames are depicted as solid arrows. Promoters of the genes indicated by thin arrows, terminators by hairpin symbols.

Evaluation of the LIKE-system, based on the bacitracin-induced GFP production

The range of inducers for the envelope-stress responsive LiaFSR three-component system is well-defined and includes, amongst others, the cell wall antibiotic bacitracin [22,25]. As an inducer for protein production in *B. subtilis*, this compound has a number of advantages: (i) It is one of the strongest inducers for the Lia-system and is easily commercially available. (ii) *B. subtilis* is highly resistant against bacitracin, and even above inhibitory antibiotic concentrations, cellular damage occurs only very slowly [18,32]. (iii) The maximum P_{liaI} activity occurs well below the inhibitory concentration, thereby avoiding any damage to the producing cultures. (iv) In addition to its major inhibitory activity on cell wall biosynthesis, bacitracin also acts as a weak protease inhibitor [33], which can be viewed as a beneficial side effect of using this inducer. For all of these reasons, bacitracin will be used as the model inducer for the subsequently described evaluation of the LIKE expression system.

Nevertheless, it should be pointed out that a number of other compounds and conditions can also be considered as suitable alternative inducers, including antibiotics such as vancomycin or nisin, as well as non-antibiotic conditions such as alkaline shock [34], making the LIKE-system highly variable even in cases where bacitracin is not suitable for a given application (for example for heterologous protease production).

Initially, we compared the promoter activity of $P_{liaI(\text{opt})}$ between pLIKE-int and pLIKE-rep in all four different host strains described above (Figure 2C). For this purpose, the dynamics of expression of recombinant GFP was determined after bacitracin addition (30 μg mL^{-1}) over the course of 4 h in growing populations. In all strains, a swift and strong accumulation of fluorescence was detected already 30 min after bacitracin induction (Figure 3A). As expected, *gfp* expression was significantly higher in strains harboring the replicative pLIKE-rep derivative (multiple copies) compared to strains with chromosomally integrated pLIKE-int derivatives

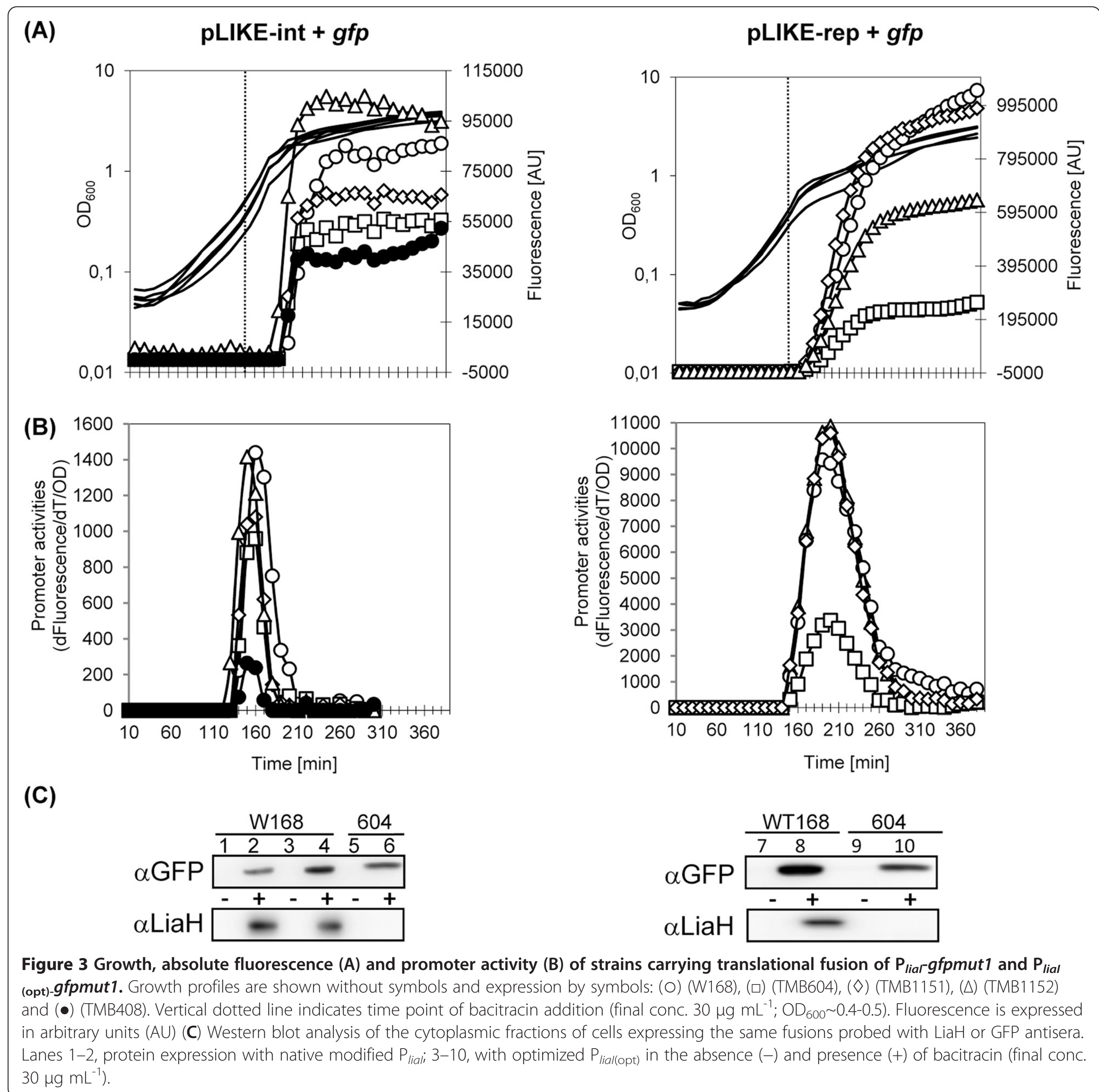

Figure 3 Growth, absolute fluorescence (A) and promoter activity (B) of strains carrying translational fusion of P_{liaI}-*gfpmut1* and $P_{liaI(opt)}$-*gfpmut1*. Growth profiles are shown without symbols and expression by symbols: (○) (W168), (□) (TMB604), (◊) (TMB1151), (Δ) (TMB1152) and (●) (TMB408). Vertical dotted line indicates time point of bacitracin addition (final conc. 30 μg mL^{-1}; OD_{600}~0.4-0.5). Fluorescence is expressed in arbitrary units (AU) (**C**) Western blot analysis of the cytoplasmic fractions of cells expressing the same fusions probed with LiaH or GFP antisera. Lanes 1–2, protein expression with native modified P_{liaI}; 3–10, with optimized $P_{liaI(opt)}$ in the absence (–) and presence (+) of bacitracin (final conc. 30 μg mL^{-1}).

(Figure 3A). In the wild type background of W168, the fluorescence intensity of the expression strain TMB1172, harboring the integrated expression plasmid, reached less than 10% of the activity measured for the otherwise identical strain TMB1176 with the replicative construct (Figure 3A).

The benefit of improving the ribosome binding site in $P_{liaI(opt)}$ compared to the original SD sequence could also be demonstrated by comparing GFP production in two strains, TMB1172 and TMB408, respectively, harboring integrated expression systems. Our analysis revealed that the level of GFP expression from the wild type P_{liaI} promoter in strain TMB408 was significantly lower compared to $P_{liaI(opt)}$-mediated expression (Figure 3A and Figure 3C, lanes 2 and 4). A deletion of the native P_{liaI} upstream of the *liaGFSR* operon in strain TMB604 resulted in an approx. two-fold decreased promoter activity compared to the wild type background, indicating that the presence of the autoregulatory feedback loop is important for full P_{liaI} activity (Figure 3A and Table 1). On the other hand, deletion of *liaH* while maintaining the native P_{liaI} upstream of the *liaGFSR*

Table 1 Effect of mutations in the *lialH* operon on the expression of translational $P_{liaI(opt)}$-*gfp* fusions

Strain	Relevant genotype[a]		Promoter activity (fluorescence)[b]
	Expression plasmid	Strain background	
TMB408	*amyE*:: pSJ5101 (P_{liaI}-*gfp*)	(WT168) P_{liaI}*lialH*$^{+}_{Term}$	264
TMB1172	*amyE*:: pLIKE-int+*gfp*	(WT168) P_{liaI}*lialH*$^{+}_{Term}$	1440
TMB1174		(TMB604) ΔP_{liaI}*lialH*	958
TMB1153		(TMB1151) Δ*lialH*	1080
TMB1318		(TMB1152) Δ*lialH*$_{Term}$	1416
TMB1176	pLIKE-rep+*gfp*	(WT168) P_{liaI}*lialH*$^{+}_{Term}$	9570
TMB1178		(TMB604) ΔP_{liaI}*lialH*	3372
TMB1342		(TMB1151) Δ*lialH*	10607
TMB1343		(TMB1152) Δ*lialH*$_{Term}$	10870

[a] The terminator downstream of *liaH* is abbreviated "Term", its presence is indicated by a "+". [b] Promoter activities were calculated taking the derivative of the fluorescence divided by the OD_{600} ($dGFP/dt/OD_{600}$) at each time point.

operon (strains TMB1151/1152) resulted in only a small increase of $P_{liaI(opt)}$ activity in case of the pLIKE-int derived expression strain. This effect was more pronounced in case of the pLIKE-rep derived strains, where the promoter activity even surpassed that of the wild type (Figure 3A and Table 1). Taken together, these results demonstrate both the important role of the autoregulatory feedback and of improving the SD sequence for achieving a maximal level of GFP production.

Determination of the P_{liaI} activity revealed that the window of promoter activity was narrower in case of the integrated promoter, both for activation and shut-off, relative to the replicative derivatives (Figure 3B). For the pLIKE-int derivatives, maximum promoter activity was reached already 20–30 min after addition of bacitracin and the total window of activity was less than 60 min. In contrast, pLIKE-rep derivatives required almost 60 min to reach maximum promoter activity and the total window of activity was about 120 min. But in light of the overall 10-times higher promoter activity in case of the latter, this result is maybe not too surprising.

All major conclusions drawn above were verified at the protein level by Western analysis, using antibodies against GFP and LiaH. Both proteins were not detectable in uninduced cultures, supporting the previously demonstrated tight control of P_{liaI} and the absence of any significant promoter activity under non-inducing conditions. Upon addition of bacitracin, both proteins accumulated to different level, depending on strain background. These studies demonstrate both the positive effect of improving the ribosome binding site and the negative effect of deleting the autoregulatory feedback loop at the level of protein production (Figure 3C).

Taken together, both pLIKE-int and pLIKE-rep were successfully established as vectors for bacitracin-dependent protein production in strains that maintain the positive autoregulatory feedback loop. While expression based on the replicative vector yields higher protein amounts, the integrative system has the advantage of genetic stability and does not require any selection.

Effect of the inducer (bacitracin) concentration on the activity of $P_{liaI(opt)}$

Next, we wanted to investigate the dynamics of P_{liaI} activity and the resulting GFP production as a function of the inducer concentration. It is already well established that P_{liaI}-mediated gene expression occurs in a dosage-dependent manner, at least in case of the wild type promoter [22,23,32]. Here, we performed similar experiments, using the pLIKE-int and pLIKE-rep derivatives pAT6203 and pAT3803, respectively, in the W168 (wild type) background. The resulting strains TMB1172 and TMB1176 were inoculated in microtiter plates and challenged in the mid-exponential growth phase with increasing concentrations of bacitracin (Figure 4). The results are in very good agreement with the previous observations. The promoter activity increases as a function of the bacitracin concentration, reaching a maximum at bacitracin concentrations of about 30 μg mL^{-1} (Figure 4A/B). At higher concentrations (above 50 μg mL^{-1}), the ongoing promoter activity after 250 mins indicates an ongoing bacitracin stress. Especially at the highest bacitracin concentration, 100 μg mL^{-1}, the GFP yield is clearly reduced concomitant with a reduced final cell density, at least in case of the pLIKE-rep derived strain TMB1176 (Figure 4A). This result was also confirmed by Western blot analysis (Figure 4C). To ensure optimal protein production without causing severe antibiotic stress, our data suggests the use of a bacitracin concentration of no more than 30 μg mL^{-1}, although this concentration may have to be optimized for individual target proteins, especially if toxicity is a problem.

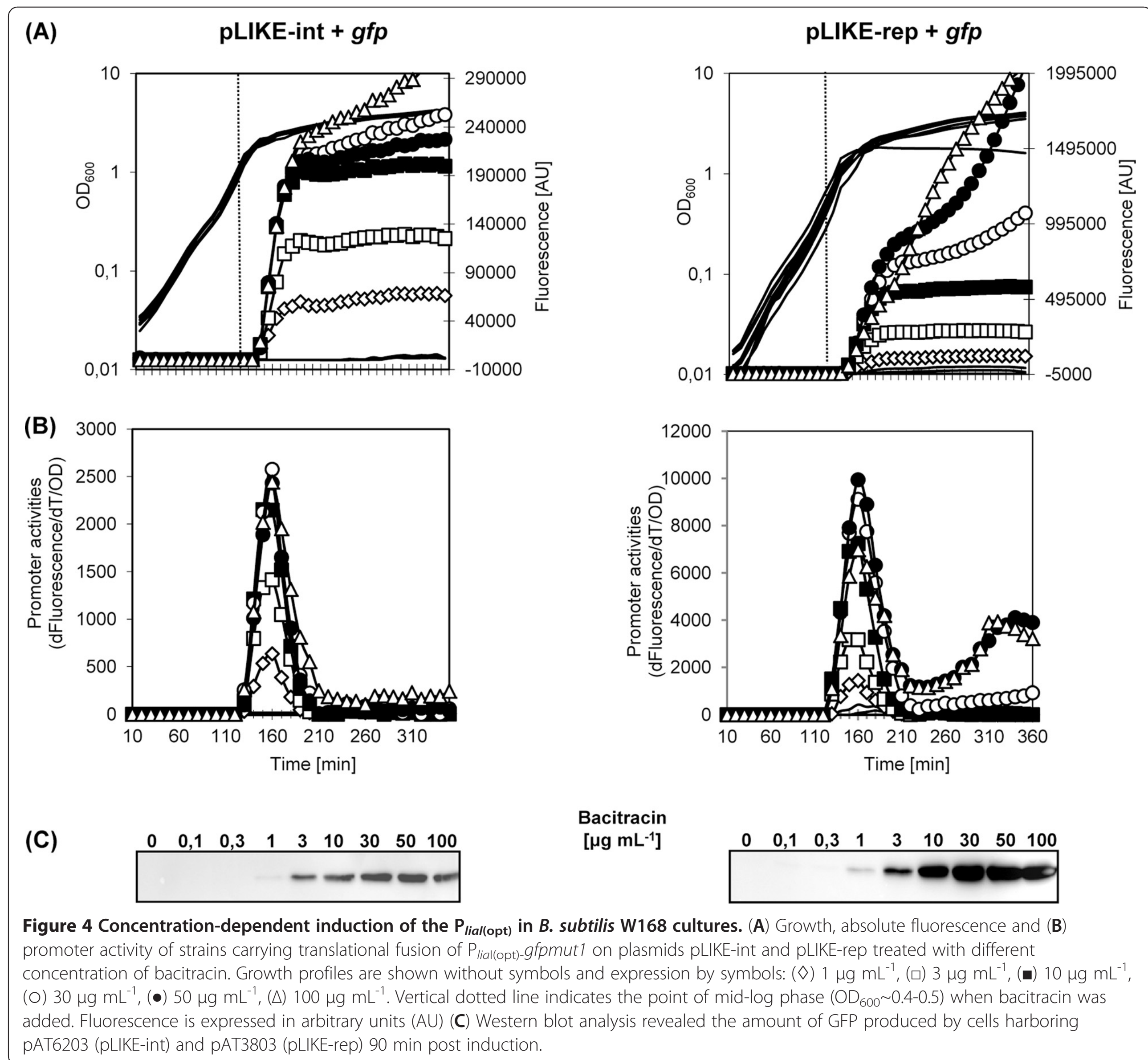

Figure 4 Concentration-dependent induction of the $P_{liaI(opt)}$ in *B. subtilis* W168 cultures. (**A**) Growth, absolute fluorescence and (**B**) promoter activity of strains carrying translational fusion of $P_{liaI(opt)}$-*gfpmut1* on plasmids pLIKE-int and pLIKE-rep treated with different concentration of bacitracin. Growth profiles are shown without symbols and expression by symbols: (◊) 1 μg mL^{-1}, (□) 3 μg mL^{-1}, (■) 10 μg mL^{-1}, (○) 30 μg mL^{-1}, (●) 50 μg mL^{-1}, (Δ) 100 μg mL^{-1}. Vertical dotted line indicates the point of mid-log phase (OD_{600}~0.4-0.5) when bacitracin was added. Fluorescence is expressed in arbitrary units (AU) (**C**) Western blot analysis revealed the amount of GFP produced by cells harboring pAT6203 (pLIKE-int) and pAT3803 (pLIKE-rep) 90 min post induction.

Overproduction of YdfG using the LIKE system

To demonstrate the suitability of the LIKE system for the overexpression of a heterologous protein, we performed an expression experiment using the protein YdfG of *Bacillus licheniformis*. This protein is a putative carboxymuconolactone decarboxylase. We could recently demonstrate that its gene represents the only target of the extracytoplasmic function σ factor $ECF41_{Bli}$ [35]. It consists of 148 amino acids and an estimated molecular weight of 16,6 kDa.

Based on the results shown in Figures 3 and 4, we used strains TMB1151 and TMB1152 as expression hosts for the pLIKE-rep+His_6-*ydfG* (pKSLIKEr01) and pLIKE-int+His_6-*ydfG* (pKSLIKEi01) derivative, respectively. YdfG production was induced in mid-log growing cultures by addition of 30 μg ml^{-1} bacitracin. The cells were harvested 30 min post-induction and disrupted by sonication. For each sample, 10 μg of total protein was separated on a 14% tricine gel and subsequently stained by colloidale Coomassie staining solution. The result is shown in Figure 5. For both derivatives, a clear additional band can be observed in the induced fractions at ~17 kDa. As expected, the YdfG yield received from the pLIKE-rep derivative is much higher compared to the integrative one. To be sure that this band is not a bacitracin effect, control samples of the expression host TMB1151 were treated equally and were also loaded on the gel. Here, no distinct band can be observed in the bacitracin-induced sample (Figure 5). By using the pLIKE-rep derivative, it was possible to achieve a protein

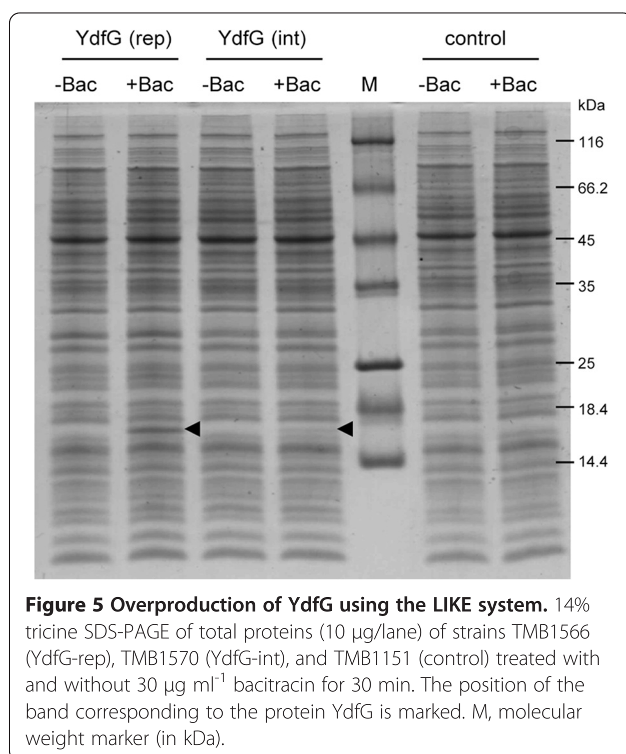

Figure 5 Overproduction of YdfG using the LIKE system. 14% tricine SDS-PAGE of total proteins (10 μg/lane) of strains TMB1566 (YdfG-rep), TMB1570 (YdfG-int), and TMB1151 (control) treated with and without 30 μg ml^{-1} bacitracin for 30 min. The position of the band corresponding to the protein YdfG is marked. M, molecular weight marker (in kDa).

yield comparable to the one shown for LiaH in bacitracin-induced *B. subtilis* wild type cells (Figure 1B).

Conclusions

We have developed a novel and efficient LiaFSR-dependent gene expression system, which places target proteins under the control of an optimized bacitracin-responsive P_{liaI} promoter. The LIKE system offers first a single-copy, integrative option (pLIKE-int in strain Bsu-LIKE2), which is genetically stable without selective pressure, but reaches lower protein production levels. The second option consists of the replicative vector pLIKE-rep in combination with strain Bsu-LIKE1 to obtain a maximum gene expression. The LIKE-system has a number of important features: (i) There is no detectable background expression in the non-induced state. (ii) Using bacitracin as inducer, the promoter has an impressive dynamic range of up to 1,000-fold above background level that can be titrated as a function of inducer concentration. (iii) The described range of inducers is wide, including cell wall antibiotics that function as strong (bacitracin, nisin, daptomycin) or intermediately strong (vancomycin) inducers, as well as non-antibiotic conditions that act as intermediate to weak inducers of the Lia-system, including pH-upshift, organic solvents, some detergents, or ethanol [22,25,36-38]. All of these inducers are well-defined and readily available at low prices. Moreover, a recent study demonstrated that the Lia-system can also be induced by the overexpression of certain heterologous and secreted proteins, especially the universal shock protein USP45 from *Lactococcus lactis* and the TEM-1 β-lactamase from *E. coli* [39]. (iv) Lastly, an antibiotic-inducible LIKE-expression strain can easily be converted into a strong constitutive expression platform by the simple deletion of *liaF*, encoding the LiaRS-specific inhibitor protein [21-23]. The effect of such a deletion is shown in Figure 1, which demonstrates that a *liaF* deletion results in a protein production that even surpasses that of the fully induced strains, even in the absence of an inducer.

This flexibility distinguishes the LIKE system from other available expression systems. Taken together, the expression vectors and strains described in this report expand the genetic toolbox already available for protein production, based on the tight and highly dynamic bacitracin-inducible promoter P_{liaI}. We hope and believe that the vectors and strains described in this report will provide valuable tools for protein expression in *B. subtilis*. The LIKE system, consisting of both expression vectors as well as the host strains Bsu-LIKE1 and Bsu-LIKE2, is available for the scientific community through the *Bacillus* Genetic Stock Center (www.bgsc.org; accession numbers ECE255, ECE256 for the two vectors and 1A1070, 1A1071 for the two *B. subtilis* expression strains).

Methods

Growth conditions

All bacterial strains (Table 2) were grown in Luria–Bertani (LB) medium at 37°C with aeration. The cell density was determined by measuring the OD_{600} with the Ultrospec™ 2100 pro UV/visible spectrophotometer (GE Healthcare). When appropriate, the growth media were supplemented with chloramphenicol (5 μg mL^{-1}), erythromycin (1 μg mL^{-1}) plus lincomycin (25 μg mL^{-1}) for macrolide-lincosamide-streptogramin (MLS) resistance (*B. subtilis*), or ampicillin (100 μg mL^{-1}; *E. coli*). Protein expression was induced by using zinc bacitracin (Sigma).

DNA manipulations, transformation and PCR

All plasmid constructions were done in *E. coli* and isolated by alkaline lysis method [40], then used to transform *B. subtilis* [41]. Procedures for DNA manipulation and transformation of *E. coli* were carried out as described [42]. The primers used in this study are listed in Table 3. For all PCR reactions the Phusion DNA Polymerase (Finnzymes) was used according to the manufacturer's instructions. Sequencing was performed in-house by the Sequencing Facility of the LMU Biocenter.

Table 2 Bacterial strains used in this study

Strain	Relevant genotype	Source and/or reference
E. coli DH5α	*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17(r_K^- m_K^+) *rel*A1 *sup*E44 φ80Δ*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	Laboratory stock
Bacillus subtilis		
W168	Wild type, *trpC*2	Laboratory stock
HB0933	W168 *att*SPβ2Δ2 *trpC*2, *liaR*::kan	[18]
TMB016	W168 *amy*E::(cat P_{liaI}-*lacZ*)	[21]
TMB020	HB0933 *amyE*::(cat P_{liaI}-*lacZ*)	[21]
TMB329	W168 Δ*liaF* (clean deletion)	[23]
TMB331	TMB329 *amyE*::(cat P_{liaI}-*lacZ*)	This work
TMB408	W168 *amyE*::pSJ5101 (P_{liaI}-*gfp*)	S. Jordan
TMB604	W168 ΔP_{liaI}-*liaIH* (clean deletion)	[23]
Bsu-LIKE1 (TMB1151)	W168 Δ*liaIH* (clean deletion)	This work
Bsu-LIKE2 (TMB1152)	W168 Δ*liaIH*-terminator (clean deletion)	This work
TMB1172	W168 *amyE*::pAT6203 (pLIKE-int $P_{liaI(opt)}$-*gfp*)	This work
TMB1176	W168 pAT3803 (pLIKE-rep $P_{liaI(opt)}$-*gfp*)	This work
TMB1174	TMB604 *amyE*::pAT6203 (pLIKE-int $P_{liaI(opt)}$-*gfp*)	This work
TMB1178	TMB604 pAT3803 (pLIKE-rep $P_{liaI(opt)}$-*gfp*)	This work
TMB1153	TMB1151 *amyE*::pAT6203 (pLIKE-int $P_{liaI(opt)}$-*gfp*)	This work
TMB1342	TMB1151 pAT3803 (pLIKE-rep $P_{liaI(opt)}$-*gfp*)	This work
TMB1318	TMB1152 *amyE*::pAT6203 (pLIKE-int $P_{liaI(opt)}$-*gfp*)	This work
TMB1343	TMB1152 pAT3803 (pLIKE-rep $P_{liaI(opt)}$-*gfp*)	This work
TMB1566	TMB1151 pKSLIKEr01 (pLIKE-rep $P_{liaI(opt)}$-His_6-*ydfG*)	This work
TMB1570	TMB1152 pKSLIKEi01 (pLIKE-int $P_{liaI(opt)}$-His_6-*ydfG*)	This work

Construction of markerless deletion mutant strains

Several markerless deletions of the *liaIH* operon (including its promoter and terminator) were constructed using the vector pMAD [43]. Genomic regions of approximately 1 kb up- and downstream of the regions to be deleted were amplified using the primers listed in Table 3. The two fragments were fused in a second joining PCR reaction, and the resulting fragment was cloned into pMAD via BamHI and EcoRI, generating the plasmids pAT101 (Δ*liaIH*) and pAT102 (Δ$liaIH_{Terminator}$). For generating the deletion mutants, the procedure described by Arnaud *et al.* was applied [43]. In brief, *B. subtilis* 168 was transformed with pAT101 or pAT102 (Table 4) and incubated for two days at 30°C on LB agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 100 μg mL^{-1}) with MLS selection. Individual blue colonies were selected and incubated for 6 to 8 h at 42°C in LB medium with MLS selection, resulting in the integration of the plasmids into the chromosome. Blue colonies were again picked from LB (X-Gal) plates and incubated at 30°C 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 plasmids were picked and checked for MLS sensitivity. Finally, strains TMB1151 (Δ*liaIH*) and TMB1152 (Δ$liaIH_{Terminator}$) were analyzed by PCR and sequencing to confirm the integrity of the desired genetic modifications.

Plasmid and strain construction

Bacterial strains used in this study are derivates of the laboratory wild type strain *B. subtilis* W168 and are listed in Table 2. Plasmids used in this study are listed in Table 4. The promoter of the *liaIH* operon for integrative and replicative vectors was obtained from strain *B. subtilis* W168 by PCR, using primers TM2064/TM1980 and TM1991/TM1992 (Table 3), respectively. During the amplification, bases in the ribosome-binding site (RBS) were mutated to a strong *B. subtilis* Shine-Dalgarno (SD) sequence (TAAGGAGG) [27] to yield the optimized *liaI* promoter $P_{liaI(opt)}$.

The integrative expression vector pLIKE-int, containing P_{liaI} with an optimized SD site ($P_{liaI(opt)}$) was generated in two steps. First, the *B. subtilis* integrative vector pDG1662 was treated with BstBI to remove the spectinomycin resistance gene. The truncated (6141 bp) fragment was self-ligated, yielding vector pAT6200. During this step, the multiple cloning site (MCS), containing unique BamHI, HindIII, and EcoRI sites was expanded

Table 3 Oligonucleotides used in this study

Primers	Sequence (5' to 3')[a]	Description/position
Plasmid construction		
TM2064	CAT*GGTCTC*AGATCTTTAAAACGCCATGCCTCG	BsaI; 5' end of P_{liaI}
TM1980	CTTGTT*GGATCCATCGAT*GAT**CCTCCT**TACGTTTTCCTTGTCTTC	Strong SD region; BamHI, ClaI; 3' end of P_{liaI}
TM1991	ATCT*GAATTC*GGTTTTAAAACGCCATGCC	EcoRI; 5' end of P_{liaI}
TM1992	ATTTTC*TCTAGA*AT**CCTCCT**TACGTTTTCCTTGTCTTC	Strong SD region; XbaI; 3' end of P_{liaI}
TM1981	TCCT*ATCG**ATG***AGTAAAGGAGAAGAACTTTTCACTGG	ATG start codon; ClaI; 5' end of *gfpmut1*
TM1982	GGCC*AAGCTT*GAACTAGTTTCATTTATTTGTAGAGC	HindIII; 3' end of *gfpmut1*
TM1993	TTCC*TCTAG**ATG***AGTAAAGGAGAAGAACTTTTC	ATG start codon; XbaI; 5' end of *gfpmut1*
TM1994	GGCC*GTCGAC*GAACTAGTTTCATTTATTTG	SalI; 3' end of *gfpmut1*
TM2535	CCAT*ATCG**ATG**CATCATCATCATCATCAC*GAAACGAGATTTCTAATGGAAAAAG	ATG start codon; ClaI; His_6-tag; 5' end of *ydfG*
TM2536	CCAT*AAGCTT*TCAATCTGCTGCGGGCATTTTC	HindIII; 3' end of *ydfG*
TM2545	CCAT*TCTAG**ATG**CATCATCATCATCATCAC*GAAACGAGATTTCTAATGGAAAAAG	ATG start codon; XbaI; His_6-tag; 5' end of *ydfG*
Clean deletions		
TM2130	GCGG*GGATCC*TCTTACATTTATTAGTCC	BamHI; upstream of P_{liaI}
TM2131	CATTTGCCGCTTTTGTCTGGGCAGATCCTCCTTTCGTTTTC	3' end of P_{liaI}; 3' end of *liaH*
TM1055	CCAGACAAAAGCGGCAAATG	3' end of *liaH*
TM1058	CCAT*GAATTC*GAATGCGGACGTCCGTCACGC	EcoRI; inside the *liaG* gene
TM2132	GCGAATTGATACGTGCGGGCAGATCCTCCTTTCGTTTTC	upstream of *liaI* gene; upstream of *liaG*
TM2133	CCGCACGTATCAATTCGC	upstream of *liaG*
TM2134	GCTA*GAATTC*TGCCGGCTGTTTTGGAG	EcoRI; center of *liaG* gene

[a] Relevant restriction sites are shown in italics, complementary regions for joining PCR are underlined. The sequence for optimized SD sequences of the *liaI* promoter is indicated by bold type, the start codon is bold italic. The His-tags are in italics and underlined.

by an additional unique ClaI restriction site, which is required for introducing genes at the ATG start codon (see Figure 2 for details): a PCR product encompassing $P_{liaI(\mathrm{opt})}$ was digested with BsaI and BamHI and cloned into pAT6200 digested with BamHI, resulting in pLIKE-int. The ClaI restriction site is recommended to use for reconstruction of the ATG start codon, but it is not strictly necessary. The use of BamHI, HindIII, or EcoRI has the disadvantage of fusing additional amino acids to the N-terminus of the target protein which can cause undesired disabilities.

To construct the replicative expression vector pLIKE-rep, again harboring $P_{liaI(\mathrm{opt})}$, the promoter fragment was amplified by PCR using primers TM1991/TM1992

Table 4 Vectors and plasmids used in this study

Plasmid	Genotype/properties [a]	Primer pair(s) used for cloning	Reference
pDG1662	*cat*, *spc*, *bla*, *amyE'* . . . *'amyE* integrative vector		[28]
pGP380	*erm*, *bla*, Strep-Tag, PdegQ36, replicative vector		[29]
pMAD	*erm*, ori(pE194-Ts), MCS-P_{clpB}-*bgaB*, *ori*(pBR322), *bla*		[43]
pSG1151	*bla*, *cat*, *gfpmut1*		[44]
pAT6200	pDG1662 derivative; *spc* gene deleted		This work
pLIKE-int	pAT6200 derivative; $P_{liaI(\mathrm{opt})}$; integrative protein expression vector	TM2064/TM1980	This work
pLIKE-rep	pGP380 derivative; $P_{liaI(\mathrm{opt})}$; replicative protein expression vector	TM1991/TM1992	This work
pAT6203	pLIKE-int, $P_{liaI(\mathrm{opt})}$ translationally fused to *gfp*	TM1981/TM1982	This work
pAT3803	pLIKE-rep, $P_{liaI(\mathrm{opt})}$ translationally fused to *gfp*	TM1993/TM1994	This work
pAT101	pMAD Δ*lialH* up/down overlap	TM2130/ TM2131, TM1055/ TM1058	This work
pAT102	pMAD Δ*lialH*$_{\mathrm{Terminator}}$ up/down overlap	TM2130/ TM2132, TM2133/ TM2134	This work
pKSLIKEr01	pLIKE-rep, $P_{liaI(\mathrm{opt})}$ translationally fused to His_6-*ydfG*	TM2545/TM2536	This work
pKSLIKEi01	pLIKE-int, $P_{liaI(\mathrm{opt})}$ translationally fused to His_6-*ydfG*	TM2535/TM2536	This work

[a] Resistance cassettes: *erm*, erythromycin; *bla*, ampicillin; *cat*, chloramphenicol; *spc*, spectinomycin.

(Table 3). After digest of the PCR product with EcoRI and XbaI, the promoter region was ligated into the corresponding sites of pGP380, resulting in vector pLIKE-rep. For cloning of a gene into pLIKE-rep, XbaI must be used as restriction enzyme to generate the ATG start codon (see Figure 2).

For the determination of the properties of the two expression vectors, the genes *gfpmut1* and *ydfG* were used. The *gfpmut1* gene was amplified using primers TM1981/TM1982 and TM1993/TM1994, respectively (Table 3), using plasmid pSG1151 as the template. The 720-bp amplicon obtained was cloned into ClaI/HindIII-digested pLIKE-int or XbaI/SalI-digested pLIKE-rep, resulting in translational fusions with $P_{liaI(\mathrm{opt})}$ in pAT6203 and pAT3803, respectively (Table 4). Next, the *B. subtilis* strains W168, TMB604, TMB1151, and TMB1152 (Table 2) were transformed with the pAT6203 integrative plasmid. The resulting strains were designated TMB1172, TMB1174, TMB1153, TMB1318, respectively (Table 2). Strains bearing the replicative pAT3803 GFP-expression plasmid were constructed by transformation of the above strains with plasmid DNA and selection for MLS resistance, resulting in strains TMB1176, TMB1178, TMB1342, and TMB1343, respectively. The *ydfG* gene was amplified from *Bacillus licheniformis* genomic DNA using primers TM2545/TM2536 and TM2535/TM2536, respectively (Table 3). The PCR product was cloned into ClaI/HindIII digested pLIKE-int or XbaI/HindIII digested pLIKE-rep, resulting in plasmids pKSLIKEi01 and pKSLIKEr01, respectively (Table 4). Next, the *B. subtilis* strain TMB1151 was transformed with pKSLIKEr01 replicative plasmid and TMB1152 was transformed with the linearized pKSLIKEi01 integrative plasmid, resulting in strains TMB1566 and TMB1570 (Table 2).

Activation of P_{liaI} by bacitracin and analysis of *gfp* gene expression

For bacitracin-mediated induction of gene expression, the appropriate *B. subtilis* strains were inoculated from overnight LB cultures into a final volume of 150 μL LB medium in a 96-well plate with optical bottom (Sarstedt) and were incubated in a Synergy™ 2 multimode microplate reader (Biotek) at 37°C with constant medium shaking. When the culture reached an OD_{600} of 0.45, bacitracin (30 μg mL^{-1} final concentration) was added to one half of the wells (induced sample), and the other half was left untreated (uninduced control). Plates were covered with lids to prevent evaporation and incubated for 4 h. Growth was monitored by measuring absorbance at 600 nm. Fluorescence readings were taken from the bottom by using a GFP-specific filter pair (excitation 485/20 nm, emission 528/20 nm). Measurements were taken in 10 min intervals. To calculate expression levels, the natural fluorescence of three cultures of wild type *B. subtilis* strain 168 (containing no reporter gene) were averaged and subtracted from the raw fluorescence value of each reporter strain at the same OD_{600} value [45]. Determination of P_{liaI} activity was calculated as described in [45] as the derivative of the fluorescence divided by the OD_{600} (dGFP/dt/OD_{600}) for each time point. Expression values were averaged from three independent samples of the same time points ((P1+P2+P3)/3). Polynomial and exponential functions were used to fit the experimental GFP dataset; promoter activities (dGFP/dt/OD_{600}) were calculated using these functions [45].

Western blotting

Total cytoplasmic proteins were prepared from 15 mL culture per time point by sonication. Proteins (20 μg per lane) were separated by SDS-PAGE, according to standard procedure [42]. After electrophoresis and equilibration of the gels in transfer buffer [15.2 g Tris; 72.1 g glycine; 750 mL methanol (100%) in a final volume of 5 L with deionized water] the proteins were blotted to a PVDF membrane using a mini-trans blot apparatus (Bio-Rad) according to standard procedure [42]. The LiaH antibody (polyclonal rabbit antisera raised against purified His10–LiaH [46]), GFP antibody (rabbit monoclonal antibody against the green fluorescent protein, Epitomics), and the secondary antibody (anti-rabbit IgG HRP conjugate, Promega) were diluted 1:20,000, 1:3,000, and 1:100,000, respectively. For LiaH/GFP detection, AceGlow™ (PeqLab) was used according to the manufacturer's instructions. Blots were documented on a QUANTUM-ST4-3026 chemiluminescence documentation system (PeqLab).

Overproduction of YdfG

For the overexpression of *ydfG*, strains TMB1566 and TMB1570 were grown in LB medium at 37°C until they reached an OD_{600} of ~0.4-0.5. Cultures were split and one half was induced with 30 μg ml^{-1} bacitracin for 30 min. The other half was left untreated. 20 ml of each culture was harvested by centrifugation and cell pellets were kept at −80°C until further use. For total protein preparation, the cell pellets were resuspended in 1 ml of cold disruption buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.5) and cells were disrupted by sonication on ice. Proteins (10 μg per lane) were separated by 14% tricine SDS-PAGE, according to standard procedure [47] and gels were subsequently stained by colloidale Coomassie staining solution [48].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions
AAT carried out all experiments with the exception of the overexpression experiment and those acknowledged below. KS performed the overexpression experiment with YdfG. AAT, KS, and TM conceived the study and wrote the manuscript. MRS participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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4 Discussion

Parts of this chapter have been adapted from:

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* contributed equally

4 Discussion

The LiaFSR system orchestrates a general cell envelope stress response of *Firmicutes* bacteria (Jordan *et al*., 2008). While the inducer spectrum of this 2CS, the LiaR-binding site, and the transition state induction of P_{liaI} without external stimuli were already known at the beginning of this thesis (Hachmann *et al*., 2009, Hyyryläinen *et al*., 2005, Jordan *et al*., 2006, Jordan *et al*., 2007, Mascher *et al*., 2004, Petersohn *et al*., 2001, Pietiäinen *et al*., 2005, Tam le *et al*., 2006, Wecke *et al*., 2009, Wiegert *et al*., 2001), the mechanism of stimulus perception and signal transduction of the LiaFSR system in *B. subtilis* was less clear. In contrast to classical 2CSs, the HK LiaS and the RR LiaR require a third protein, LiaF, a specific inhibitor of the system (Jordan *et al*., 2006). However, the exact mode of (inter-)action of and between the three proteins was unclear. It was the aim of this thesis to shed some light on these Lia-dependent signaling processes.

In the first results chapter of this thesis, it was shown that the LiaFSR system of *B. subtilis* possesses significant differences to other classical 2CSs with regard to stimulus perception and robustness (CHAPTER 2). First, the cellular ratios of LiaF, LiaS, and LiaR were determined genetically and by quantitative Western blot analyses to be 18:4:1, respectively. Maintaining this stoichiometry is crucial for a proper response to cell envelope stress. The overproduction of either LiaS or LiaR disrupted the functionality of the LiaFSR system by resulting in a constitutive activation of the *liaI* promoter. Second, we provided genetic evidence that LiaS is a bifunctional HK, which possesses both a kinase and phosphatase activity, based on mutating a critical motif crucial for the phosphatase activity of NarX/Q-like HKs (Huynh *et al*., 2010). Third, we investigated a potential role of HK-independent phosphorylation of LiaR by acetyl phosphate *in vivo*. Our data indicates that a measurable non-catalyzed phosphorylation only occurs in the case that the amount of LiaR exceeds that of LiaS. In light of these findings, we will discuss the role of protein stoichiometry on the robustness of 2CS-dependent signal transduction (4.1). Moreover, we will provide and discuss a model for the mechanism of LiaFSR-mediated stimulus perception and signal transduction (4.2).

The second chapter of the results describes a novel protein expression system for *B. subtilis*, which is based on the promoter P_{liaI} (CHAPTER 3). A toolbox consisting of expression vectors and expression hosts was developed and proved to be successful for a heterologous overexpression of soluble proteins. The advantages and potential limitations of this expression system will be discussed and compared to existing expression systems for *Firmicutes* bacteria.

4.1 Stoichiometry and robustness of 2CSs

A regulatory system is called robust if it is insensitive to any perturbations to the intra- and extracellular environment that would otherwise disrupt its function (Goulian, 2004). Bacterial 2CSs are well investigated in this respect and in general are known to be robust to changes of their cellular protein ratios (Goulian, 2004). This means that the output of a 2CS, the concentration of phosphorylated RR, is not significantly influenced by fluctuations of the concentrations of HK and RR proteins in the cell (Shinar *et al.*, 2007, Steuer *et al.*, 2011). This robustness is important to maintain an adequate response to specific stimuli despite stochastic fluctuations in protein numbers.

The LiaFSR system is the first described example of a 2CS that does not seem to feature this characteristic of robustness. The functionality of this system is only ensured as long as the molecule numbers of LiaF exceed those of LiaS, and the molecule numbers of LiaS likewise exceed those of LiaR. All other examples of 2CSs investigated so far are characterized by an excess of RR molecules over their HKs within the cell. In the first part of this section an overview of the findings about the stoichiometry and robustness of different signal transduction systems as well as the differences to the LiaFSR system are presented.

4.1.1 Stoichiometry and robustness of other signal transduction pathways

In spite of the fact that 2CSs are the best-studied systems of robust signaling (Steuer *et al.*, 2011), only a few of them were experimentally investigated regarding their protein stoichiometry and even less regarding their robustness. Two of them were already mentioned in CHAPTER 2, the EnvZ/OmpR 2CS of *E. coli* (Cai & Inouye, 2002) and the WalRK 2CS of *S. pneumoniae* (Wayne *et al.*, 2010). In contrast to the LiaFSR system, both systems possess an excess of RR molecules over their cognate HKs (see Table 4.1). Moreover, the expression of EnvZ/OmpR target genes seems not to be influenced after changing the protein amounts of EnvZ or OmpR below and above wild type levels in a range where EnvZ does not exceed OmpR amounts. They identified that this principal is based on the bifunctionality of the HK EnvZ, which controls the concentration of phosphorylated OmpR, the output of this 2CS (Batchelor & Goulian, 2003).

Beside these two 2CSs, the stoichiometry and robustness of other more complex regulatory systems involving HK and RR proteins were explored, including the chemotaxis system and the endospore formation phosphorelay.

The stoichiometry of the chemotaxis system was investigated in *E. coli* (Li & Hazelbauer, 2004) and *B. subtilis* (Cannistraro *et al.*, 2011). Chemotaxis describes the ability of motile organisms to move towards specific attractants and away from repellents by modulating the direction of their flagellar rotation (Eisenbach, 1996, Falke *et al.*, 1997, Scharf *et al.*, 1998). The composition of chemosensory proteins differs between species, but the core complex always consists of membrane-anchored chemoreceptors, the HK CheA, and the coupling protein CheW (Falke *et al.*, 1997, Szurmant & Ordal, 2004). In *E. coli*, the presence of repellents leads to an autophosphorylation of the HK CheA, followed by the transfer of the phosphoryl group to the RR CheY. Phosphorylated CheY (CheY~P) interacts with the flagellar motor and induces tumbling by enhancing clock wise (CW) rotation. The dephosphorylation of CheY~P is catalyzed by CheZ (Eisenbach, 1996, Falke *et al.*, 1997, Scharf *et al.*, 1998). Binding of attractants to the chemoreceptors or removal of repellents decreases CheA activity and thus CheY phosphorylation, which leads to counter clock wise (CCW) rotation of the flagellar and consequently reduced tumbling frequencies. This causes extended runs in order to move towards favorable directions (Eisenbach, 1996, Scharf *et al.*, 1998). In contrast to *E. coli*, the chemosensory system of *B. subtilis* operates conversely with the same result in the end: binding of attractants – not repellents - to the *B. subtilis* chemoreceptors enhances CheA activity, which leads to increasing amounts of CheY~P. This results in CCW flagellar rotation and thus less tumbling (Szurmant & Ordal, 2004).

Beside the fact that both systems participate in chemosensory functions, the overall numbers of involved proteins differ significantly between both species, especially those for the chemoreceptor molecules (Cannistraro *et al.*, 2011, Li & Hazelbauer, 2004). However, the protein ratios of the HK CheA and the RR CheY appeared to be 1:2.7 in *B. subtilis* and 1:1.2 in *E. coli* (Table 4.1). Again, this is an opposite result compared to LiaS and LiaR. Observations concerning the robustness of the *E. coli* chemotaxis system were made during the quantification experiments (Li & Hazelbauer, 2004). The authors demonstrated that the cellular amounts of chemotaxis proteins varied up to 10-fold between different *E. coli* strains and growth media, but the ratios between proteins were always maintained similar. This observation would infer that the chemosensory system is robust within a certain range of absolute protein amounts (Li & Hazelbauer, 2004). The robust behavior to variations of the relative protein amounts was shown already seven years earlier: a theoretical model for the robust behavior of chemosensory adaptation in *E. coli* was provided by Barkai and Leibler (Barkai & Leibler, 1997). Practical confirmation of this phenomenon followed two years later by a different group (Alon *et al.*, 1999). These authors tested experimentally how sensitive the exact adaptation in chemotaxis was to variations in the concentration of the regulatory proteins involved. Exact adaptation is an important characteristic of chemotaxis: alterations

of the concentration of a chemical stimulant lead to a fast change in the tumbling frequency of bacteria, which is successively set back to its pre-stimulus state even though the concentration of the stimulant stays unchanged (Berg & Tedesco, 1975, Macnab & Koshland, 1972). The results demonstrate a robust behavior of the exact adaptation, whereas characteristics like adaptation time and steady-state tumbling frequency are sensitive to variations in protein stoichiometry. The authors concluded that exact adaptation seems to be a critical property to the functioning of the chemotaxis network, which is not dependent on precise values of the steady-state tumbling frequency and the adaptation time (Alon *et al.*, 1999).

Table 4.1: Molecule number, stoichiometry, and robustness of different regulatory systems.

System	Organism	Protein amounts [monomers/cell]		Protein ratio		Robust-ness[a]	References
		HK	RR	HK	RR		
LiaS/LiaR	*B. subtilis*	150	20	8	1	no	This work
EnvZ/OmpR	*E. coli*	100	3500	1	35	yes	(Batchelor & Goulian, 2003, Cai & Inouye, 2002)
WalK/WalR	*S. pneumo-niae*	920	6200	1	7	n.a.	(Wayne *et al.*, 2010)
Chemotaxis (CheA/CheY)	*E. coli*[b]	6700	8200	1	1.2	yes	(Alon *et al.*, 1999, Barkai & Leibler, 1997, Li & Hazelbauer, 2004)
	B. subtilis	2600	7100	1	2.7	n.a.	(Cannistraro *et al.*, 2011)
Sporulation (KinA/Spo0A)	*B. subtilis*[c]	4.4×10^5-24.1×10^5	94.6×10^5-500×10^5	1	21	n.a.	(Eswaramoor thy *et al.*, 2010)

[a] Robustness of the functionality of the respective system after changing the stoichiometry of the proteins involved. n.a., not available
[b] Values based on strain RP437 grown in rich medium.
[c] Values for protein amounts are indicated in a range from early to late stationary phase (0 to 150 minutes after sporulation).

In *B. subtilis*, proteins that participate in the complex phosphorelay orchestrating endospore formation were also quantified recently (Eswaramoorthy *et al.*, 2010). *B. subtilis* is able to outlast hostile conditions by developing extremely resistant endospores (Abecasis *et al.*, 2013, Errington, 2003). Initiation of sporulation requires a signal transduction network

consisting of the major sensor kinase KinA (one of five HKs that provide the sensory input of the phosphorelay), two phosphotransferases Spo0F and Spo0B, as well as the RR Spo0A (Burbulys *et al.*, 1991, Hoch, 1993, Stephenson & Hoch, 2002). Upon nutrient limitation, the HK KinA gets autophosphorylated. The phosphoryl groups are then transferred through sequential phosphotransfer reactions to Spo0A, the master regulator of sporulation, which becomes gradually activated via phosphorylation (Burbulys *et al.*, 1991, Grossman, 1995, Hoch, 1993). To ensure a successful initiation of sporulation, all phosphorelay components have to be synthesized in sufficient amounts in the cell. During early to late sporulation phase, the molecule numbers of KinA, Spo0F, Spo0B, and Spo0A increase about 10-fold. At any time, the molar ratio between the HK KinA and the RR Spo0A favors the latter with an average ratio of 1:21 (Eswaramoorthy *et al*., 2010) (Table 4.1). Unfortunately, no data regarding the robustness of the phosphorelay is available so far.

Because these complex networks consisting of many different regulatory proteins are more complicated than classical 2CSs, their comparison to the LiaFSR system must be regarded with caution. However, all these systems show an excess of RR proteins over their cognate sensor HKs, which stands in contrast to the values obtained for the LiaFSR system in this thesis.

4.1.2 Stoichiometry and robustness of LiaFSR – revolutionary or only one of many?

From the knowledge that we gained during the course of this thesis, some questions arise: Is it a unique feature of the LiaFSR system to possess such an unusual stoichiometry and non-robustness, or is it only one out of many systems that has this characteristic, but none of the other systems have been identified yet? This question cannot be answered until more 2CSs are studied in this respect, especially those of the NarXQ/NarL-type. While chemotaxis and the endospore phosphorelay are different because of their complexity, as discussed above, the other “normal” 2CSs (EnvZ/OmpR and WalRK) belong to a different 2CS group, namely the EnvZ/OmpR-type. The most obvious difference of these two 2CS groups is their operon structure, which might cause differences in protein ratios and robustness, as already discussed in CHAPTER 2. Investigations of more NarXQ/NarL-like 2CSs regarding their protein ratios and robustness would explain whether the unusual stoichiometry and the obviously linked non-robustness might be common features of NarXQ/NarL-like 2CSs or perhaps even more specific of LiaFSR-like 2CSs.

Why has such a seemingly fragile system evolved? Answers to this question would be definitely too speculative at this moment, but we favor a simple hypothesis. The unusual

native stoichiometry of LiaFSR ensures stimulus perception and specificity of the LiaFSR system: an excess of LiaF over LiaS is necessary to fix LiaS in its phosphatase state in the absence of a stimulus. An excess of LiaS over LiaR is necessary to prevent undesirable stimulus-independent phosphorylation of LiaR by acetyl phosphate and other phosphoryl group-donors. Overproduction of either LiaS or LiaR severely disturbs the functionality of the Lia system in terms of a constitutive activation of the target promoter P_{liaI}, irrespective of the presence or absence of a stimulus. This means that the phosphatase activity of LiaS seems to be weak (in comparison to other bifunctional HKs) and the affinity of LiaR to acetyl phosphate seems stronger regarding other well-known RRs. The importance to maintain the specific protein ratio indicates a possible sensory/regulatory complex between LiaF and LiaS, which assumes physical interaction, as has been already discussed in CHAPTER 2.

From these findings we can draw hypotheses of how the mechanism of stimulus perception and signal transduction may work within the LiaFSR system.

4.2 Mechanistic insights of stimulus perception and signal transduction of LiaFSR-like systems

4.2.1 Stimulus perception by LiaS and LiaF

The details of the mechanism of stimulus perception of the Lia system are mostly unknown. Based on the available data, it is most likely that two proteins are required for sensing the stimulus: the HK LiaS and the accessory protein LiaF.

Without exception, LiaF homologous proteins are always encoded directly upstream of *liaSR* in all species harboring *lia*-like genes, but just a few of them have been characterized so far. In *B. subtilis*, *S. mutans*, and *L. monocytogenes*, LiaF was identified to act as a specific inhibitor of the LiaR-dependent gene expression in the absence of inducing conditions (Fritsch *et al.*, 2011, Jordan *et al.*, 2006, Suntharalingam *et al.*, 2009). Accordingly, disruption of the respective genes leads to high expression levels of LiaR target genes even without external stimuli. Here, the LiaSR system shows a “locked-ON” phenotype. The only example of a putative positive modulation of the Lia system by LiaF was recently described for YvqF, the LiaF homolog of *S. aureus* (McCallum *et al.*, 2011). Its deletion leads to a loss of induction of the VraSR system even in vancomycin-induced cultures and is comparable to the phenotypes observed for *vraS* or *vraR* null mutants. Therefore, YvqF seems to be essential for responding to cell envelope stress and resulting antibiotic tolerance in *S. aureus* (McCallum *et al.*, 2011).

No further mechanistic insight beyond the regulatory behavior of *liaF* mutants is currently available. Preliminary studies of *B. subtilis* indicate that the negative function of LiaF resides in the cytoplasmic C-terminal domain, but membrane localization is necessary for the full inhibitory activity (Jordan, unpublished).

The mechanism of how LiaFSR-like systems sense their respective stimuli is so far unknown. Typical HKs harbor an extracellular sensing domain located between two transmembrane helices, which is postulated to bind and/or detect suitable stimuli (Wolanin *et al.*, 2002). In contrast, LiaS homologs are IM-HKs, possessing only a small extracellular loop between the two transmembrane regions, which is too small for ligand binding. It was initially proposed that these HKs detect the stimuli directly at the surface or within the cytoplasmic membrane (Mascher, 2006, Mascher *et al*., 2006, Mascher *et al*., 2003).

But recent evidence indicates that LiaS alone does not seem to be sufficient to coordinate a controlled response to its stimuli. Instead, the accessory protein LiaF seems to be involved in the stimulus sensing process, based on the behavior of *liaF* mutants described above. A direct interaction between LiaS and LiaF has not been proven so far, but seems likely based on initial data from *S. aureus* (McCallum *et al*., 2011). Three possible mechanisms of stimulus perception by LiaS and LiaF can be envisioned (Fig. 4.1). First, a LiaF/LiaS-sensory complex is conceivable, in which both proteins participate in stimulus perception (Fig. 4.1B). Thereby, LiaF modulates the kinase/phosphatase activity of LiaS according to the presence or absence of a stimulus. If LiaF is disrupted, this interaction is lost and LiaS activates LiaR constitutively. Second, LiaF may act as a repressor and stabilizes the phosphatase activity of LiaS in the absence of a suitable stimulus, thereby keeping the system silent (Fig. 4.1C). In the presence of a stimulus, LiaF may then act as a sensor and releases LiaS from repression, allowing phosphorylation of LiaR and subsequent activation of gene expression. Third, LiaF may function just as a repressor in the absence of a stimulus and LiaS acts as a sensor (Fig. 4.1D). In the presence of a stimulus, LiaS undergoes a conformational change leading to a disruption of the direct interaction with LiaF and a subsequent activation of LiaR. This possibility is by far the most unlikely, because of the absence of extracytoplasmic sensor domains in LiaS-like HKs, but cannot be ruled out due to the lack of experimental data.

For all three alternatives, the repressive function of LiaF is only ensured if LiaF is present in excess over LiaS. This is at least true for *B. subtilis*, based on the data of CHAPTER 2.

In *S. aureus*, the LiaF homolog YvqF seems to play a positive function on LiaS activity. Here, disruption of *yvqF* could result in a total loss of kinase activity. In contrast to *B. subtilis*, a direct interaction between YyqF and VraS has been demonstrated, as mentioned above

(McCallum *et al*., 2011). Accordingly, the authors concluded that YyqF may be involved in sensing the stimulus and is thereby responsible for regulating signal transduction.

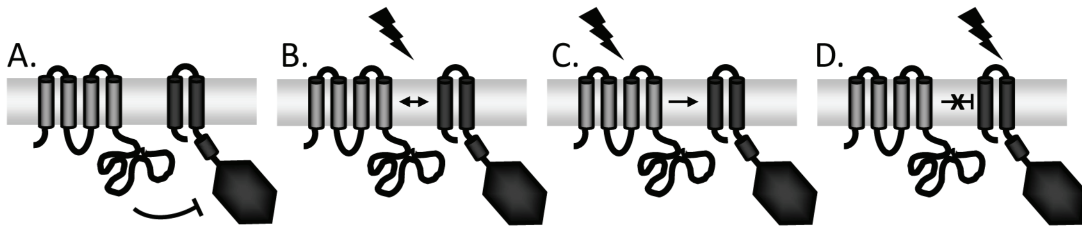

Figure 4.1: Proposed mechanisms of stimulus perception by LiaF- and LiaS-like proteins. (A) System in uninduced state. LiaF has a negative function on LiaS activity. (B) A LiaF-LiaS sensor complex is shown in which both proteins participate in stimulus perception. (C) LiaF acts as the sensor protein and releases LiaS from its repression in the presence of a stimulus. (D) LiaS is the sensor protein and undergoes a postulated conformational change after sensing the stimulus. This, in turn, leads to a disruption of the LiaF-dependent inhibition. See text for details.

4.2.2 Catalytic activity of LiaS-like HKs and phosphotransfer to LiaR

The unusual function of LiaF during the stimulus perception process points towards a possible default activity of LiaS-like HKs: either "kinase-ON" or "phosphatase-ON". In the case of *B. subtilis*, both the deletion of *liaF* and the strong overexpression of *liaS* in the presence of LiaF show a distinct output resulting in a constitutive activation of P_{liaI} (CHAPTER 2). Such a behavior indicates a default setting of LiaS activity, which would be "kinase-ON". This default activity is only inhibited by the presence of sufficient amounts of LiaF in the absence of a suitable trigger. It seems that LiaS needs LiaF as an interaction partner to be stabilized in its phosphatase mode. Free LiaS molecules would be active as kinase, which would result in an activation of P_{liaI}, as can be observed after LiaS overproduction. The only described example that shows similarities is VraS of *S. aureus*. As described above, the LiaF homolog YvqF seems to be necessary for the activation of the HK VraS in the presence of a stimulus (McCallum *et al*., 2011). Therefore, the default activity of VraS may be "phosphatase-ON", which then may need YvqF for its own activation and/or stimulus perception. This mode of action might be specific for HisKA_3-like or just LiaS-like HKs, since no other example can be found in the literature to date.

The signal transduction process of LiaFSR-like systems might occur as follows: in the presence of a suitable stimulus, LiaS is released from LiaF repression and autophosphorylates in an ATP-dependent manner, which may occur as a *cis* or *trans* reaction. Until recently, *trans*-

autophosphorylation of a HK dimer, where one HK monomer catalyzes the phosphorylation of the second monomer, was the only reaction described (Cai & Inouye, 2003, Ninfa *et al.*, 1993, Stock *et al.*, 2000, Trajtenberg *et al.*, 2010) and therefore thought to be a general characteristic of HKs. However, recent studies show that *cis*-autophosphorylation (HK monomer phosphorylates itself) can also commonly occur (Casino *et al.*, 2009, Casino *et al.*, 2010, Pena-Sandoval & Georgellis, 2010, Dago *et al.*, 2012). The well-studied HK DesK of *B. subtilis* was described to autophosphorylate in *trans* (Trajtenberg *et al.*, 2010). Since LiaS belongs to the same HK family like DesK (histidine protein kinase (HPK) family 7) (Grebe & Stock, 1999), a *trans*-autophosphorylation mechanism can be postulated for LiaS as well.

For LiaS-like HKs, bifunctional activities composed of kinase and phosphatase activity were postulated and experimentally proven for VraS of *S. aureus* (Belcheva & Golemi-Kotra, 2008) and $LiaS_{Lm}$ of *L. monocytogenes* (Fritsch *et al.*, 2011). A truncated version of VraS only consisting of the soluble C-terminal part was used to show an increasing VraS autophosphorylation over 20 minutes after incubation with ATP. A rapid phosphotransfer between VraS and VraR occurred within 30 seconds with about 70% of the phosphate residues being transferred (Belcheva & Golemi-Kotra, 2008). VraR~P can only be dephosphorylated in the presence of VraS, clearly demonstrating the phosphatase activity of this protein. The kinetics of VraS autophosphorylation and phosphotransfer indicate a rapid *in vivo* response to cell wall damage (Belcheva & Golemi-Kotra, 2008). $LiaS_{Lm}$ autophosphorylation was shown to occur within 15 minutes after ATP treatment. A $LiaS_{Lm}$-dependent phosphotransfer to the cognate RR $LiaR_{Lm}$ was also observed, in which phosphorylated $LiaR_{Lm}$ protein could only be detected within the first 90 seconds of incubation with $LiaS_{Lm}$~P, indicating a rapid hydrolysis of $LiaR_{Lm}$~P due to the phosphatase activity of $LiaS_{Lm}$ (Fritsch *et al.*, 2011). For LiaS of *B. subtilis*, no *in vitro* phosphorylation studies are currently available, but a probable phosphatase activity was proven *in vivo* (CHAPTER 2). Moreover, recent *in vivo* studies demonstrate an almost immediate activation of LiaR in the presence of bacitracin (Kesel *et al.*, 2013).

4.2.3 LiaS-independent activation of LiaR

A LiaS-independent activation of LiaR via phosphorylation by acetyl phosphate was shown for *B. subtilis* in CHAPTER 2. Likewise, this HK-independent phosphorylation was also observed during *in vitro* studies with VraR of *S. aureus* and *in vivo* for $LiaR_{Lm}$ of *L. monocytogenes* (Belcheva & Golemi-Kotra, 2008, Fritsch *et al.*, 2011). We determined a stimulus-independent activation of *B. subtilis* P_{liaI} through LiaR~P after overproduction of this RR, irrespective of the presence or absence of LiaS. This phenomenon could be attributed to

the phosphorylation of LiaR by acetyl phosphate. It seems that LiaS is only able to keep LiaR inactive by dephosphorylation as long as LiaS is present in excess over LiaR. This observation suggests that LiaS seems to have a rather inefficient phosphatase activity in contrast to other HKs, such as EnvZ of *E. coli* (Forst & Roberts, 1994, Inouye & Dutta, 2003). It is interesting that RRs of other 2CSs are more abundant than their cognate HKs, but these HKs are usually very well able to control RRs activity by dephosphorylation. While a physiological relevance of the LiaR activation by acetyl phosphate has not yet been demonstrated, it can also not be ruled out at the present time. It is imaginable that acetyl phosphate plays a role in fine-tuning the response to cell envelope stress, as has been recently shown for another cell envelope stress-sensing 2CS, CpxAR of *E. coli* (Lima *et al.*, 2012).

Taken together, the Lia system is a sensitive, fast, and highly dynamic cell envelope stress-sensing 2CS that shows an unusual stoichiometry of its regulatory components LiaF, LiaS, and LiaR with a relative ratio of 18 to 4 to 1. This characteristic protein ratio allows stimulus perception and specificity of the Lia system, whose function is highly sensitive to perturbations of this stoichiometry. Therefore, preservation of the relative protein amounts to each other is crucial to allow a precise signal transduction process.

4.3 Development of a new protein expression system for *B. subtilis*

The second part of this thesis dealt with the establishment of a novel protein expression system, the LIKE system, which is based on the promoter P_{liaI} (CHAPTER 3).

Despite the number of protein expression systems available, there is nevertheless an undiminished need for reliable homo- and heterologous protein expression systems. Hence, it is important to develop novel or improved systems, which are adjusted to the respective host in order to have the best possible option for expression of proteins of interest. A simple and fast handling as well as the availability of inexpensive inducers is critical. A lot of different expression systems are available, most of them were created for *E. coli*.

In this section, we will first present an overview of the most significant protein expression systems for the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *B. subtilis* (see also Table 4.2). A detailed description of all expression systems can be found in the Supplemental Material. Subsequently, advantages and disadvantages of the LIKE system will be discussed.

4.3.1 Protein expression in the Gram-negative bacterium *E. coli*

E. coli is the most common expression host for the production of recombinant proteins. This is not surprising, since this bacterium possesses the ability to grow fast and to a high cell density on inexpensive substrates. In addition, its genetics and molecular biology are well-characterized and a large number of expression vectors and mutant host strains are available (Baneyx, 1999, Hannig & Makrides, 1998, Jonasson *et al.*, 2002, Makrides, 1996). All expression systems are based on inducible promoters, which should be strong to be able to produce the protein of interest in excess of 10 to 30% of the total cellular protein. The promoter should be efficiently repressed under non-inducing conditions, especially if the target protein is toxic to the host strain, and it should be simply inducible in an inexpensive manner (Baneyx, 1999, Hannig & Makrides, 1998, Jonasson *et al.*, 2002, Makrides, 1996).

Expression systems based on the *lac* promoter are commonly used in *E. coli* for many years. The *lac* promoter is chemically inducible by isopropyl-β-D-thiogalactopyranoside (IPTG) and negatively regulated by the *lac* repressor protein LacI (Lehninger *et al.*, 1994). Different synthetic *lac*-derived promoters have been developed, which are all constructed with the purpose to reach high protein levels (Brosius *et al.*, 1985, de Boer *et al.*, 1983). The major disadvantage of *lac*-derived promoters is their leakiness in the absence of the inducer molecule. Hence, they are inappropriate for the expression of toxic proteins (Baneyx, 1999, Jonasson *et al.*, 2002).

The pET vector system is another IPTG-inducible system used in *E. coli*, which is tighter regulated (Dubendorff & Studier, 1991b, Dubendorff & Studier, 1991a, Studier & Moffatt, 1986, Studier *et al.*, 1990) (Table 4.2). It is based on a vector-located T7 promoter-*lac* operator sequence, which controls the expression of a target gene. This plasmid is integrated into an *E. coli* host strain possessing the T7 RNA polymerase gene controlled by the IPTG-inducible *lac* promoter. Upon induction, the T7 RNA polymerase is synthesized leading to the expression of target proteins up to 50% of the total cellular proteins (Baneyx, 1999). The pET system becomes tightly regulated under non-inducing conditions by the use of host cells containing a plasmid-encoded T7 lysozyme, which represses the T7 RNA polymerase via direct binding (Studier, 1991). Thus, the tolerance to toxic target proteins is enhanced.

For the expression of certain protein, a massive overproduction is sometimes not desired due to the possible formation of inclusion bodies. For this purpose, the *ara*BAD system has been developed (Guzman *et al.*, 1995) (Table 4.2). This system consists of the arabinose-inducible promoter P_{BAD}, which is positively and negatively regulated by AraC (Carra & Schleif, 1993, Lobell & Schleif, 1990). The addition of L-arabinose leads to a moderate expression of target genes, which was proven to be 2.5- to 4.5-times lower compared to P_{lac}-derived promoters

(Guzman *et al*., 1995). Furthermore, P_{BAD} shows no significant basal expression level so that this system can be used to produce toxic proteins (Guzman *et al*., 1995).

The mentioned expression systems are well established, but cannot be directly transferred to Gram-positive bacteria. The major reasons are the individual codon usage and more stringent requirements for promoter application in Gram-positive bacteria compared to *E. coli* (Moran *et al.*, 1982, Morrison & Jaurin, 1990). Furthermore, unique requirements for the regulation of inducible promoters in Gram-positive bacteria are not part of existing expression systems, e.g. specific sigma factors and regulators (Haldenwang, 1995). However, a lot of different tools for the expression of homo- and heterologous proteins in Gram-positive bacteria have been developed. Some of them are discussed in the following section – with the focus on expression systems developed for *B. subtilis*.

4.3.2 Protein expression in the Gram-positive bacterium *B. subtilis*

Among Gram-positive bacteria, *B. subtilis* is a widely used protein production host. Similar to *E. coli*, *B. subtilis* possesses the advantage to grow fast to high cell density and its genetics is established. Advantages of *B. subtilis* over *E. coli* are the possible use of integrative vector systems, which are more stable compared to replicative vectors. Moreover, *B. subtilis* carries powerful secretory systems, which allow the secretion of produced proteins (e.g. proteases, lipases, and amylases) into the culture medium. These features as well as its GRAS (**g**enerally **r**ecognized **a**s **s**afe) status are amongst the reasons for the great industrial and clinical interest for this bacterium (Ling Lin *et al.*, 2007, Schallmey *et al.*, 2004, Schumann, 2007, van Dijl & Hecker, 2013, Westers *et al.*, 2004).

The first described expression system for *B. subtilis* consists of an IPTG-inducible promoter, named P_{spac} (Yansura & Henner, 1984) (Table 4.2). It correlates to the *lac* promoter system of *E. coli*, since P_{spac} was created by fusing the *E. coli lac* operator and *lac* repressor gene to the SPO-1 promoter sequence derived from a *B. subtilis* phage. This system is strongly induced up to 100-fold after the addition of IPTG, but shows significant basal expression levels under non-inducing conditions (Bhavsar *et al.*, 2001, Vavrova *et al.*, 2010, Yansura & Henner, 1984).

Another widely used expression system for *B. subtilis* is based on a xylose-inducible promoter, P_{xylA} (Bhavsar *et al.*, 2001, Kim *et al.*, 1996) (Table 4.2). Two different vectors were constructed, pX and pSWEET, which consist of P_{xylA} and the xylose repressor gene *xylR* for negative regulation of the *xylA* promoter in the absence of xylose. The vector pSWEET is an

improved version of pX because it possesses a *cis*-acting CRE (catabolite-responsive element) site, allowing a tighter transcriptional regulation by catabolite repression (Bhavsar *et al.*, 2001). Upon induction, both expression vectors showed 200-fold expression levels as well as a tight regulation without induction (Bhavsar *et al.*, 2001, Kim *et al.*, 1996, Vavrova *et al.*, 2010).

Table 4.2: Strengths and weaknesses of protein expression systems of *E. coli* and *B. subtilis*.

Expression system/ promoter	Regu-lation	Inducer	Strengths	Weaknesses[a]	References
E. coli					
P_{lac}	LacI	IPTG	strong inducible, inducer commercially available	leaky	(Baneyx, 1999, Jonasson *et al.*, 2002, Lehninger *et al.*, 1994)
pET/ P_{T7lac}	LacI	IPTG	tight, strong inducible, inducer commercially available	problems due to hyperexpression possible, e.g. inclusion bodies	(Dubendorff & Studier, 1991b, Dubendorff & Studier, 1991a, Studier & Moffatt, 1986, Studier *et al.*, 1990)
*ara*BAD/ P_{BAD}	AraC	L-arabinose	tight, inducer commercially available	moderately strong inducible	(Guzman *et al.*, 1995)
B. subtilis					
P_{spac}	LacI	IPTG	single-plasmid based, inducer commercially available	leaky, moderately strong inducible (up to 100-fold)	(Bhavsar *et al.*, 2001, Yansura & Henner, 1984)
pX, pSWEET/ P_{xylA}	XylR	D-xylose	strong inducible (200-fold), tight, single-plasmid based, inducer commercially available	n. a.	(Bhavsar *et al.*, 2001, Kim *et al.*, 1996, Vavrova *et al.*, 2010)
SURE/ P_{spaS}	SpaRK	subtilin	strong inducible (100-fold), single-plasmid based	leaky, inducer not commercially available	(Bongers *et al.*, 2005, Vavrova *et al.*, 2010)
NICE/ P_{nisA}	NisRK	nisin	tight, inducer commercially available	weakly inducible (10-fold), dual-plasmid based	(Eichenbaum *et al.*, 1998)

[a] n. a., not available.

Protein expression systems, which are inducible by peptide antibiotics, have also been developed for the use in *B. subtilis*. The most prominent examples are the NICE (**ni**sin-**c**ontrolled gene **e**xpression) system, which was originally developed for *L. lactis* (de Ruyter *et al.*, 1996b, Kuipers *et al.*, 1995) and then transferred to *B. subtilis* (Eichenbaum *et al.*, 1998), and the SURE (**su**btilin-**r**egulated gene **e**xpression) system (Bongers *et al.*, 2005) (Table 4.2). Both systems are regulated by a 2CS, which is induced by nisin (NisRK) or subtilin (SpaRK), respectively, leading to the activation of the target promoters P_{nisA} or P_{spaS}. The great strength of the SURE system is the high induction level up to 100-fold in response to subtilin, reaching significantly higher protein yields compared to the *xyl* expression system (Bongers *et al.*, 2005, Vavrova *et al.*, 2010). Unfortunately, expression from the *spaS* promoter is leaky, which makes it not suitable for the expression of toxic proteins (Vavrova *et al.*, 2010). Moreover, the inducer molecule subtilin is not commercially available. Instead, culture supernatant of a subtilin producer strain must be used for induction that might cause variations in subtilin concentration and quality (Bongers *et al.*, 2005). In contrast, P_{nisA} of the NICE system is tightly regulated under non-inducing conditions, but reaches only low induction levels in *B. subtilis* up to 10-fold. Additionally, the NICE system is based on a dual-plasmid platform (Eichenbaum *et al.*, 1998), which is known to be more unstable compared to single-plasmid systems.

4.3.3 The LIKE system – pros and cons

The LIKE system is a novel protein expression system for *B. subtilis*, which was developed in the present thesis (CHAPTER 3). It features similar characteristics as the SURE and the NICE systems. It is also regulated by a 2CS, LiaSR, and it is induced by peptide antibiotics, e.g. bacitracin, vancomycin, or ramoplanin leading to the induction of a target promoter, P_{liaI}, by binding of the activated RR. The LIKE system combines all advantages from the SURE and the NICE systems. Similar to the NICE system, it offers a tightly regulated promoter, P_{liaI}, which is induced by commercially available substrates in a concentration-dependent manner. It possesses a great induction strength (up to 1000-fold) comparable to the SURE and higher than the NICE systems and it is a stable single-plasmid-based expression platform like the SURE system. The LIKE system represents an improvement regarding its inducer spectrum, since a number of different and commercially available inducers exist (not only peptide antibiotics, but also e.g. organic solvents or induction by alkaline shock). Another improvement is its fast response already 5 to 10 minutes after addition of the inducer molecule, reaching a maximum after 30 minutes (CHAPTER 3; Kesel *et al.*, 2013). In contrast,

maximum expression levels achieved from the NICE system were observed only 90 to 120 minutes after the addition of nisin (de Ruyter *et al.*, 1996b).

The great strength of the LIKE system is the choice of two different options: (i) an integrative expression plasmid (pLIKE-int), which is stable without antibiotic pressure, but reaches lower protein yields, or (ii) a replicative expression plasmid (pLIKE-rep) that is more unstable and needs selective pressure, but shows higher protein yields. Furthermore, different host strains were constructed, which were optimized in combination with the integrative or replicative option. However, these strains are intended for the use of the LIKE system, alternatives are imaginable, e.g. the use of a *liaF* deletion mutant, which provides the possibility of a strong constitutive production of recombinant proteins. This might increase the protein yield further and seems to be appropriate whenever high amounts of target protein are needed.

The successful overproduction of the soluble proteins GFP and YdfG by the LIKE system has been proven in this study. Thus, membrane proteins, secretion proteins, as well as toxic proteins are also supposed to be expressed by the LIKE system, although not tried so far. Further practical experience is necessary to prove these applications. Because of its tight regulation, the NICE system was extensively used to express and analyze toxic and essential proteins, such as cell wall lytic enzymes (de Ruyter *et al.*, 1997, Hickey *et al.*, 2004) and the H^+-ATPase of *L. lactis* (Koebmann *et al.*, 2000). This capability might be transferable to the LIKE system, since it also offers the tight regulation during exponential growth.

Taken together, the LIKE system provides a novel protein expression toolbox for *B. subtilis* with the improvement over preexisting protein expression systems in terms of inducer spectrum and the choice of different vector systems (integrative or replicative). In general, the development of novel or improved protein expression systems is always welcome/useful to circumvent mentioned issues of existing expression systems.

4.4 Conclusions/outlook

The present thesis dealt with the regulatory mechanism of the LiaFSR system of *B. subtilis*. Phenotypes of different *lia* mutants and investigations of the native stoichiometry of the proteins LiaF, LiaS, and LiaR revealed significant differences in terms of stimulus perception, signal transduction, and robustness to other well-characterized 2CS, e.g. the EnvZ/OmpR 2CS

of *E. coli*. The LiaFSR system is the first 2CS that requires an excess of the HK over its cognate RR. This unusual protein ratio seems to be the reason for the non-robust behavior after changing the stoichiometry.

Nevertheless, a number of important questions concerning the exact mechanism of stimulus perception and signal transduction are still unaddressed: How is the stimulus sensed? What is the mechanism of autophosphorylation of LiaS – *trans* or *cis*? How is the signal transduced? How does LiaF execute its inhibitory function - in complex with LiaS or LiaR? For the latter, protein interaction studies will be necessary. Initial analyses using the bacterial two-hybrid system (Karimova *et al.*, 1998) revealed the expected interactions between LiaS and LiaR molecules itself (homodimerization), and between LiaS and LiaR. But so far, no interaction was observed between LiaS and LiaF or LiaR and LiaF, respectively (Robyn Emmins, unpublished). However, it is known that this type of interaction study often generates false positive results, leading to the need of further studies using different types of methods to confirm or disprove this data. For this purpose, techniques like SPINE (**S**trep-**p**rotein **in**teraction **e**xperiment) (Herzberg *et al.*, 2007), FRET (**F**örster (fluorescent) **r**esonance **e**nergy **t**ransfer) (Förster, 1948, Pollok & Heim, 1999), *in vitro* pull-down assays (e.g. based on activated N-hydroxysuccinimide (NHS) sepharose (GE Healthcare)), or biacore systems based on SPR (**s**urface **p**lasmon **r**esonance) (Biacore life sciences, GE Healthcare) could be used. From these studies we expect to learn whether our favored hypothesis of a LiaF/LiaS sensory complex might be true.

Moreover, biochemical studies with the proteins LiaFSR are the next step. Phosphorylation experiments using radioactively labeled ATP and acetyl phosphate should be performed to investigate the LiaS autophosphorylation, the phosphotransfer between LiaS and LiaR, as well as the phosphatase activity of LiaS *in vitro*. The role of LiaF can be studied by the addition of this protein to the respective phosphorylation steps. Such experiments with LiaFSR were so far hampered by overexpression/purification problems or inactivity of proteins: all attempts to (over-)express and purify LiaF were unsuccessful so far, most likely due to toxic effects in *E. coli*. Similar to *in vitro* phosphorylation studies of VraSR of *S. aureus* (Belcheva & Golemi-Kotra, 2008), the cytoplasmic C-terminal part of LiaS was fused to a GST (glutathione S-transferase) tag and purified, resulting in only little amounts of soluble LiaS protein with many impurities. Initial autophosphorylation experiments using this LiaS/impurities mixture failed probably because of LiaS inactivity. In contrast, the purification of tag-less LiaR has been successfully done by ion exchange chromatography, but LiaR seemed to be physically instable leading to a rapid degradation. Subsequent purification attempts with His-tagged LiaR delivered very high protein yields, which were physically stable, but this time instable in

LiaR activity. EMSA (**e**lectrophoretic **m**obility **s**hift **a**ssay) experiments indicated a rapid loss of LiaR function within a few days.

A future aim would be the reconstruction of the LiaFSR system in liposomes to investigate the mechanism of stimulus perception and signal transduction separated from other cellular influences. This has been successfully done for the YycFG 2CS of *S. aureus* (Türck & Bierbaum, 2012), the KdpDE 2CS of *E. coli* (Jung *et al.*, 1997), the DcuSR 2CS of *E. coli* (Janausch *et al.*, 2002), and the MtrBA 2CS of *Corynebacterium glutamicum* (Möker *et al.*, 2007).

5 Supplemental Material – CHAPTER 2

Additional tables and figures for CHAPTER 2

Table S1: Oligonucleotides used in this study.

Primer no. (primer name)	Sequence (5'→3')
Oligonucleotides for cloning [a]	
Stoichiometry analysis of LiaFSR[b]	
0579 (P_{liaG}-fwdopt(*Sma*I))	CCAT***CCCGGG***TCCCTTCCGCACTTGACAATTCGCAAGCTTTTCTGTTATAATAGAATG
0580 (*liaF*expr (*Bam*HI))	AGCC***GGATCC***ATTCCTGGTGTCCGCCTCC
0581 (*liaS*expr (*Bam*HI))	AGCC***GGATCC***ATACGTACTTCACATCCACATC
0582 (*liaR*expr (*Bam*HI))	AGCC***GGATCC***ACGTTCGTTCTCTCCTTTTTCTTCC
0856 (P_{veg}-fwd2 (*Sma*I))	GACT***CCCGGG***TAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTAGTGA
0857 (*liaF*-SDrev2 (*Bam*HI))	GATC***GGATCC***ATTCCTGGTGTCCGCCTCCTTTCTCACTACATTTATTGTACAACACGA
0898 (*liaS*-SDrev3 (*Bam*HI))	GATC***GGATCC***ATACGTACTTCACATCCACATCATCACTACATTTATTGTACAACACGA
0899 (*liaR*-SDrev3 (*Bam*HI))	GATC***GGATCC***ACGTTCGTTCTCTCCTTTTTCTTTCACTACATTTATTGTACAACACGA
0900 (*liaG*-SDrev (*Bam*HI))	GATC***GGATCC***ATTCGGTTTCATCCTTCTCATTCTCACTACATTTATTGTACAACACGA
0901 (*liaG*-SDrev)	CATTCGGTTTCATCCTTCTCATTCTCACTACATTTATTGTACAACACGA
0902 (*liaG*-fwd)	GAATGAGAAGGATGAAACCG
0903 (*liaF*-SDrev4 (*Bam*HI))	GATC***GGATCC***ATTCCTGGTGTCCGCCTCC
0904 (*liaS*-SDrev4 (*Bam*HI))	GATC***GGATCC***ATACGTACTTCACATCCACATC
0905 (*liaR*-SDrev4 (*Bam*HI))	GATC***GGATCC***ACGTTCGTTCTCTCCTTTTTC
0906 (P_{veg}-kontrrev (*Bam*HI))	GATC***GGATCC***ATAGGACCACAGGCGGAGGAAAGTCACTACATTTATTGTACAACACGA
Complementation experiments with *liaS*[c]	
0454 (*liaS*-fwd (*Bam*HI))	ACG***GGATCC***CGGTGATGTGGATGTGAAGTACG
0958 (*liaS*-fwd (*Bam*HI))	ACG***GGATCC***ATGAGAAAAAAAATGCTTGCCAGCC
0959 (*liaS*-rev (*Sph*I))	AGTC***GCATGC***TCATCAATCAATAATACTCGAATCACG
0960 (FLAG3-fwd)	GATTATAAGGATCATGATGGTG
0961 (FLAG3-rev (*Hin*dIII))	ACG***AAGCTT***CTTGTCGTCATCGTCTTTGTAG
0962 (*liaS* FLAG-rev)	CACCATCATGATCCTTATAATCATCAATAATACTCGAATCACGTTCG
Overexpression of *liaFSR*	
0035 (*liaF*-fwd (*Hin*dIII))	AGG***AAGCTT***AGAAAGGAGGCGGACACCAGG
0036 (*liaF*-rev (*Eco*RI))	TCC***GAATTC***TTTCTCATACGTACTTCACATCC
0046 (*liaS*-rev (*Hin*dIII))	ACG***AAGCTT***TCATCAATCAATAATACTCGAATCACG
0454 (*liaS*-fwd (*Bam*HI))	ACG***GGATCC***CGGTGATGTGGATGTGAAGTACG
0893 (*liaR*-rev (*Bsa*I-*Eco*RI))	AGTC***GGTCTCGAATTC***GACTACCGGGTCAATGTGATTG
1068 (*liaR*-fwd (*Bam*HI))	ACG***GGATCC***CCGGAAGAAAAAGGAGAGAACG
1106 (*liaR*-rev (*Hin*dIII))	ATCG***AAGCTT***CTAATTCACGAGATGATTTCG
Purification of LiaSR[c]	
0958 (*liaS*-fwd (*Bam*HI))	ACG***GGATCC***ATGAGAAAAAAAATGCTTGCCAGCC
0960 (FLAG3-fwd)	GATTATAAGGATCATGATGGTG
0962 (*liaS* FLAG3-rev)	CACCATCATGATCCTTATAATCATCAATAATACTCGAATCACGTTCG
1161 (FLAG3-rev (*Hin*dIII))	ACG***AAGCTT***TCACTTGTCGTCATCGTCTTTGTAG
1164 (*liaR* FLAG3-rev)	CACCATCATGATCCTTATAATCATTCACGAGATGATTTCGGTGTGC
1530 (*liaR*-fwd (*Nde*I))	ACG***CATATG***ATTCGAGTATTATTGATTGATG
Quantification of LiaFSR[d]	
1950 (*liaF*-upfwd (*Bam*HI))	ACG***GGATCC***GGCATTTCAGGAGACTCAGG
1951 (*liaF*-uprev-FLAG3)	CTTGTCGTCATCGTCTTTGTAGTCGATATCATGATCCTTATAATCACCATCATGATCCTTATAATCCATTCCTGGTGTCCGCCTCCTTTC
1952 (*liaF*-fwd-Flag3)	CGACTACAAAGACGATGACGACAAGATGACAAAAAAACAGCTTCTCGG
1953 (*liaF*-rev (*Nco*I))	ACG***CCATGG***TACATAAATATCAACGTTACC
1958 (*liaS*-fwd (*Bam*HI))	ACG***GGATCC***GTCGGTTATCTCAGAAGAACGC
1959 (*liaS*-rev-FLAG3)	CTTGTCGTCATCGTCTTTGTAGTCGATATCATGATCCTTATAATCACCATCATGATCCTTATAATCCGGAAAAATCGGGACCTTCAC
1960 (*liaS*-dofwd-FLAG3)	CGACTACAAAGACGATGACGACAAGGAAGAAAAAGGAGAGAACGAACG
1961 (*liaS*-dorev (*Nco*I))	ACG***CCATGG***TCACTGACATCCAGCTTTG
2041 (*liaR*-uprev-FLAG3)	CTTGTCGTCATCGTCTTTGTAGTCGATATCATGATCCTTATAATCACCATCATGATCCTTATAATCCACGTTCGTTCTCTCCTTTTTCTTCC
2042 (*liaR*-fwd-FLAG3)	CGACTACAAAGACGATGACGACAAGATTCGAGTATTATTGATTGATGATC

Table S1: Continued.

Oligonucleotides for CCR and LFH-PCR[e]	
0029 (*liaS*-upfwd)	GCTTTATCAGCAAGCGGTGACG
0030 (*liaS*-uprev (kan))	CCTATCACCTCAAATGGTTCGCTGTCCCGTTGTCATGCGGATGGC
0047 (*liaS*-dofwd (kan))	CGAGCGCCTACGAGGAATTTGTATCGGGCACTCAAATCGAAGTGAAGG
0048 (*liaS*-dorev)	AACCGGGCTGGGAAACGAGGTC
0147 (kan-checkrev)	CTGCCTCCTCATCCTCTTCATCC
0139 (mls-fwd)	CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC
0140 (mls-rev)	CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG
0148 (mls-checkrev)	GTTTTGGTCGTAGAGCACACGG
0322 (*ackA*-upfwd)	GGAACTGACCATTCTTGATCCAGC
0323 (*ackA*-uprev (kan))	CCTATCACCTCAAATGGTTCGCTGCCATTTAAACATTGTCATGTCGG
0324 (*ackA*-dofwd (kan))	CGAGCGCCTACGAGGAATTTGTATCGCGACTGATGAAGAAGTCATGATTGCG
0325 (*ackA*-dorev)	CGACGGAAGTATCAAGACCTCC
0143 (tc-fwd)	CAGCGAACCATTTGAGGTGATAGGTCTTGCAATGGTGCAGGTTGTTCTC
0145 (tc-rev)	CGATACAAATTCCTCGTAGGCGCTCGGGAACTCTCTCCCAAAGTTGATCCC
0150 (tc-checkrev)	CATCGGTCATAAAATCCGTAATGC
0326 (*pta*-upfwd)	GCTCTACCACTGATACGTAGG
0327 (*pta*-uprev (tet))	CCTATCACCTCAAATGGTTCGCTGGCGTTCTACGAATGCTTGTACAAGG
0328 (*pta*-dofwd (tet))	CGAGCGCCTACGAGGAATTTGTATCGCGCTGAAGATGTTTACAATCTCGC
0329 (*pta*-dorev)	CGCTTCCTTTACACCTTGATTGC
0508 (LiaR-D_{54}A)[f]	CATTTTAATGG**C**CCTTGTCATGGAGGG
Oligonucleotides for LiaS mutagenesis[f]	
2374 (LiaS Q_{164}A-fwd)	CATGATGCGGTCAGC**GC**GCAGCTCTTTGCC
2375 (LiaS Q_{164}A-rev)	GGCAAAGAGCTGC**GC**GCTGACCGCATCATG
Oligonucleotides for Northern	
0031 (*liaG*-up fwd)	TTGTCGTCGGAATCGCATTGGC
0108 (*liaS*-dofwdEP)	GAAGGTCCCGATTTTTCCGG
0496 (*liaR*-T7rev)	CTAATACGACTCACTATAGGGAGAGTCTTTCCTTCTGCGATCAGGC
0497 (*liaIH*-T7rev)	CTAATACGACTCACTATAGGGAGAGCGTCAAATGCGAGCTGTGCC
Oligonucleotides for real-time RT-PCR	
0093 (*liaR*-RT fwd)	ATTGAAGTCATCGGCGAAGC
0094 (*liaR*-RT rev)	AAAGCTCCCGGCAAATTTGC
0156 (*rpsJ*-RTfwd)	GAAACGGCAAAACGTTCTGG
0157 (*rpsJ*-Rtrev)	GTGTTGGGTTCACAATGTCG
0158 (*rpsE*-RTfwd)	GCGTCGTATTGACCCAAGC
0159 (*rpsE*-Rtrev)	TACCAGTACCGAATCCTACG
0628 (*liaS*-RT-fwd)	ACAACGGGAATCAGCCTGC
0629 (*liaS*-RT-rev)	GGTCACGCTGATCAGAAGC
0630 (*liaF*-RT-fwd)	TGCAAATTATCGGAATAGGCG
0631 (*liaF*-RT-rev)	TTAAAGGTGATGCTGAAGAGG

[a] Restriction sites for cloning are highlighted in bold italics.

[b] Sequences underlined are inverse and complementary to the 3' end of P_{veg} (= #0856).

[c] The underlined sequences are inverse and complementary to the FLAG3 tag (= #0960).

[d] The underlined sequences of #1951, #1959, and #2041 represent the entire FLAG3 sequence (reverse and complementary). Underlined sequences of #1952, #1960, and #2042 are complementary to the 3' end of the FLAG3 tag.

[e] Oligonucleotide names refer to the fragments flanking the gene to be deleted. Sequences underlined are inverse and complementary to the 5' (up-rev) and 3' (do-fwd) ends of the kanamycin, mls, or tetracycline cassette, respectively.

[f] The bold underlined nucleotides indicate the base substitutions leading to the amino acid exchanges D_{54}A or Q_{164}A, respectively.

[g] Sequences underlined represent the T7 promoter necessary for the construction of RNA probes by *in vitro* transcription.

Table S2: P_{liaI} activities in different *lia* mutants.

Strain	β-galactosidase activity [Miller units][a]	
	- Bac	+ Bac
WT	0.3 ± 0.1	41 ± 18
liaS::kan	50 ± 11	57 ± 5
Δ*liaS*	0.6 ± 0.1	0.7 ± 0.1
liaF::kan	718 ± 43	884 ± 41
Δ*liaF*	65 ± 3	65 ± 7
liaS::kan, LiaR D_{54}A	0.3 ± 0.1	0.3 ± 0.1
liaR::kan	0.3 ± 0.1	0.3 ± 0.1

[a] Miller units are based on the data shown in Fig. 1.

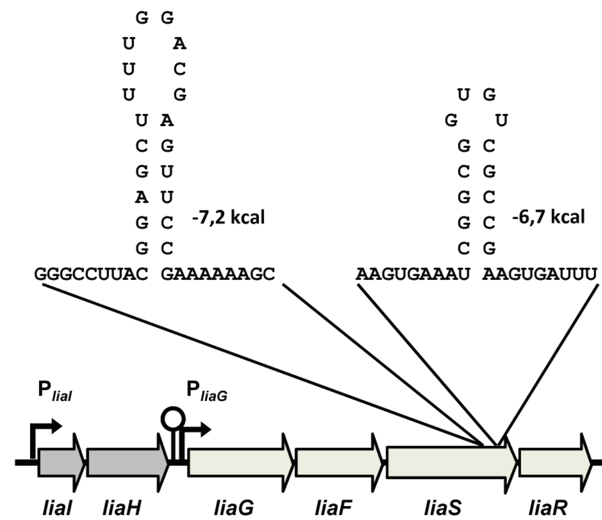

Figure S1: Sequence, secondary structure, and free energy of the stem loop structures located at the 3' end of *liaS*.

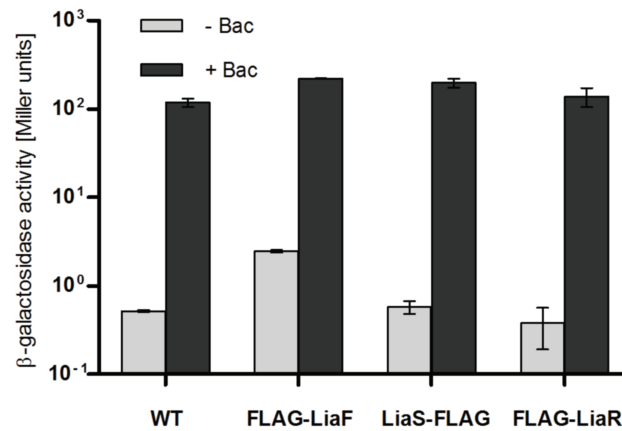

Figure S2: Verification of the P*liaI* activity after introducing the FLAG3-tag sequence into the native *lia* locus. Shown are the P*liaI* activities of strains TMB488 (wild type), TMB1156 (FLAG3-*liaF*), TMB1146 (LiaS-FLAG3), and TMB1171 (FLAG3-LiaR). Experimental conditions and labeling of the bars are as described in Fig. 1.

6 Supplemental Material – CHAPTER 4

Detailed description of existing protein expression systems of Gram-negative and Gram-positive bacteria

6.1 Protein expression systems for *E. coli*

The *lac* promoter, which is part of the lactose utilization operon, was used for many years as the paradigmatic promoter to drive recombinant gene expression. It is chemically-inducible by the lactose analogon isopropyl-β-D-thiogalactopyranoside (IPTG) and negatively regulated by the *lac* repressor protein LacI. In the absence of IPTG, the repressor LacI binds to the operator region of the *lac* operon and thus blocks the transcription of target genes. The presence of IPTG causes derepression by direct binding of IPTG to the repressor, which leads to the dissociation of LacI from the operator due to conformational changes and subsequent transcription (Lehninger *et al*., 1994). Additionally, positive regulation of the *lac* promoter is mediated by a catabolite activator protein (CAP), whose activity is dependent on the intracellular cAMP concentration. cAMP activates CAP, which then binds to the *lac* promoter supporting its activity (Lehninger *et al*., 1994).

Synthetic promoters like P_{tac} and P_{trc} were also developed, which consist of the -35 region of P_{trp} (induced by tryptophane starvation or addition of β-indoleacrylic acid) and the -10 region of the *lacUV5* promoter (mutated derivative of P_{lac}, which is less sensitive to cellular concentrations of cAMP) (de Boer *et al*., 1983). Thus, both P_{tac} and P_{trc} possess consensus -35 and -10 sequences that lead to approximately 11-times stronger expression levels compared to the parental promoter P_{lacUV5} (Brosius *et al*., 1985, de Boer *et al*., 1983). A disadvantage of *lac*-derived promoters is their leakiness. Despite the use of host strains that carry the *lac* repressor LacI, repression can be improved but leakiness is not completely abolished under non-inducing conditions (Baneyx, 1999, Jonasson *et al*., 2002). This makes them not suitable for the production of proteins, which are toxic or detrimental to the growth of the host cell. Large-scale protein production with IPTG-inducible promoters is widely used for basic research, but it is not appropriate for large-scale induction of human therapeutic proteins due to the toxicity and high costs of IPTG (Hannig & Makrides, 1998, Makrides, 1996). An alternative could be the induction of *lac*-derived promoters by lactose or the choice of temperature-sensitive promoters like *lac*(TS), which is based on a mutant *lacI* gene, encoding a thermosensitive *lac* repressor that allows induction by a temperature upshift (Bukrinsky *et*

al., 1988, Hasan & Szybalski, 1995). Other heat-induced promoters used in *E. coli* are bacteriophage lambda-derived P_L (λ) (Bernard *et al.*, 1979) and P_R (λ) (Elvin *et al.*, 1990). But thermal induction could be a disadvantage due to the simultaneous induction of heat-shock proteins including certain proteases that could lead to enhanced protein degradation (Hannig & Makrides, 1998, Jonasson *et al.*, 2002).

The pET system is the most popular protein expression platform used in *E. coli* (commercially available from Novagen, Madison). It is based on a plasmid-located bacteriophage T7 promoter fused to the *lac* operator sequence. This T7*lac* promoter controls the expression of target genes and is repressed under non-inducing conditions by LacI. The T7 promoter is specifically recognized by the T7 RNA polymerase. The host cell contains a prophage called DE3, which encodes the gene for the T7 RNA polymerase under the control of the IPTG-inducible *lacUV5* promoter-operator sequence. In the presence of IPTG, repression by LacI is abolished and the T7 RNA polymerase is expressed by the induction of P_{lacUV5}. Simultaneously, the T7 promoter is derepressed, which allows the transcription of target genes by the synthesized T7 RNA polymerase (Dubendorff & Studier, 1991b, Dubendorff & Studier, 1991a, Studier & Moffatt, 1986, Studier *et al.*, 1990). Thus, a massive overproduction of target protein up to 50% of total cellular proteins is achievable (Baneyx, 1999). But nevertheless, this system is also not fully repressed in the absence of IPTG because of increasing cAMP concentrations during the stationary growth phase, which lead to the activation of CAP. The presence of T7 lysozyme can decrease basal expression levels due to inhibition of T7 RNA polymerase by direct binding. Therefore, host cells containing a plasmid-encoded T7 lysozyme (pLys) are often used as expression hosts (Studier, 1991). This tight repression under non-inducing conditions increases the tolerance to toxic target proteins.

All so far described P_{lac}-derived promoters were constructed with the purpose to achieve high levels of protein production. But a massive overproduction is not always beneficial and often results in the formation of inclusion bodies, which contain aggregated target protein (Wilkinson & Harrison, 1991). The *ara*BAD system is another widely used expression system in *E. coli* that was developed to escape this disadvantage (Guzman *et al.*, 1995). Expression vectors named pBAD were constructed, which contain the P_{BAD} promoter of the arabinose operon and the regulatory gene *araC*. AraC regulates the expression from P_{BAD} positively and negatively and negatively autoregulates its own transcription (Carra & Schleif, 1993, Lobell & Schleif, 1990). Upon induction, the inducer molecule L-arabinose binds to AraC leading to the expression of P_{BAD}-controlled target genes (Guzman *et al.*, 1995). The expression level is lower compared to P_{lac}-derived promoters. A comparison of the promoters P_{tac} and P_{BAD} resulted in a 2.5 to 4.5 stronger activity of P_{tac} (Guzman *et al.*, 1995). Therefore, this moderately high expression level of the *ara*BAD system is suitable to overcome problems due

to hyperexpression. Under non-inducing conditions, P_{BAD} is rapidly repressed showing only very low levels of basal expression. Since the P_{BAD} promoter is subject to catabolite repression (Miyada *et al.*, 1984), glucose-containing media further prevent background expression. Thus, this system is also used to produce toxic target proteins (Guzman *et al.*, 1995).

6.2 Protein expression in Gram-positive bacteria

6.2.1 Protein expression systems for *B. subtilis*

Many different protein expression systems have been developed for *B. subtilis*. The first system was described by Yansura and Henner (Yansura & Henner, 1984). They transferred the *lac* repressor-operator system from *E. coli* into *B. subtilis* by the creation of hybrid promoter elements, in which either the promoter of the penicillinase gene of *B. licheniformis* or the SPO-1 promoter of a *B. subtilis* phage were fused to the *lac* operator and the *lac* repressor gene from *E. coli* (referred to as $P_{pac\text{-}I}$ or $P_{spac\text{-}I}$, respectively), allowing IPTG-mediated induction. Protein production was tested by *B. licheniformis* penicillinase fused to $P_{pac\text{-}I}$ and human leucocyte interferon A fused to $P_{spac\text{-}I}$. Upon IPTG induction, both expression systems showed significantly increased expression levels up to 100-fold of both target proteins (Yansura & Henner, 1984).

Different xylose-inducible protein expression systems have also been developed for the use in *B. subtilis* (Bhavsar *et al.*, 2001, Kim *et al.*, 1996). These systems are based on xylose regulatory sequences, which originally orchestrate xylose utilization (Dahl *et al.*, 1994, Gärtner *et al.*, 1992, Kreuzer *et al.*, 1989). Kim and co-workers established the expression vector pX, which consists of xylose-regulatory elements of *B. megaterium* - the xylose repressor gene *xylR* and the xylose-inducible promoter P_{xylA} - as well as 5' and 3' parts of the gene *amyE* to allow the integration of the vector into the *B. subtilis* chromosome. For reason of tightness, P_{xylA} is under the control of XylR to reduce basal expression levels. They tested the expression of three different heat-shock proteins (GrpE, DnaK, DnaJ) as well as the thermostable β-galactosidase BgaB from *B. stearothermophilus*. Depending on the heat-shock protein analyzed, 3- to 16-fold induction was observed upon the addition of xylose. For the *bgaB* gene, a 200-fold expression level was achieved. Moreover, the *xylA* promoter seems to be very tight under non-inducing conditions due to efficient repression by XylR (Kim *et al.*, 1996). Another expression vector consisting of a xylose-inducible promoter is pSWEET (Bhavsar *et al.*, 2001). In contrast to pX, pSWEET contains the *xylR* gene and the *xylA* promoter from *B. subtilis* strain W23, and possesses additionally the 5' area of the gene *xylA* containing an optimized *cis*-acting CRE (catabolite-responsive element) site. Therefore,

transcription from pSWEET is subject to catabolite repression (Jacob *et al.*, 1991, Kraus *et al.*, 1994). Expression levels were also determined by using *bgaB* as reporter. In minimal medium, the addition of 2% xylose resulted in a 246-fold induction of *bgaB* expression. In contrast, growth in minimal medium supplemented with 0.2% glucose only yielded a 52-fold induction of *bgaB* expression levels due to glucose-mediated catabolite repression. Furthermore, expression levels are modulated in the presence of various concentrations of inducer molecule (0.0002-0.63% of xylose) showing varying amounts of BgaB leading to activities from 30 to 11000 Miller units (Bhavsar *et al.*, 2001). The authors compared this expression system with the IPTG-inducible *spac* system (Yansura & Henner, 1984) regarding efficiency and modulation of expression. In each case, the *xyl* expression system outperformed the *spac* system (Bhavsar *et al.*, 2001).

The SURE (**su**btilin-**r**egulated gene **e**xpression) system (Bongers *et al.*, 2005) is one of the most efficient expression systems in *B. subtilis* regarding the achievable yield of synthesized target protein. Recombinant protein production in this system is based on the subtilin gene cluster (*spa*) of the subtilin producer strain *B. subtilis* ATCC 6633 (Klein *et al.*, 1992). The synthesis of the lantibiotic subtilin as well as its immunity is controlled by the 2CS SpaRK via a quorum-sensing mechanism. At the onset of stationary phase, subtilin acts as autoinducer and is sensed by the HK SpaK. SpaK undergoes autophosphorylation followed by the transfer of the phosphoryl group to the RR SpaR. Phosphorylated SpaR binds to specific *spa* boxes in order to activate the promoters P_{spaS}, P_{spaB}, and P_{spaI}, leading to the transcription of the 2CS genes, the subtilin-biosynthesis genes, as well as the immunity genes (Chakicherla *et al.*, 2009, Kleerebezem *et al.*, 2004, Stein *et al.*, 2002, Stein *et al.*, 2003). For the SURE system, a *B. subtilis* W168 expression host was developed, in which the *spaRK* genes were integrated into the *amyE* locus by an integrating plasmid. Additionally, expression vectors were constructed containing the subtilin-inducible *spaS* promoter region (Bongers *et al.*, 2005). To prove the efficiency of this system, two different reporter genes, *gusA* (encoding *E. coli* β-glucuronidase) and *gfp*, were translationally fused to P_{spaS}. Expression levels of the respective reporter genes were monitored by β-glucuronidase activity or fluorescence, respectively. Upon subtilin induction, 100-fold increases in GusA activity and fluorescence units were observed (Bongers *et al.*, 2005). Unfortunately, the promoter P_{spaS} is not completely tight in the absence of inducer (Vavrova *et al.*, 2010). Furthermore, the most obvious disadvantage of the SURE system is the inducer molecule itself: subtilin is not commercially available. Culture supernatant of the subtilin producer strain ATCC 6633 has to be used for induction (Bongers *et al.*, 2005). That might implicate variations in subtilin quality and concentration, although the potency of subtilin-containing supernatant can be measured. However, this fact is a

considerable disadvantage in terms of the use in industrial protein production, where standardized conditions are absolutely necessary.

In a recent study, the commonly used xylose- and IPTG-inducible systems were compared with the SURE system (Vavrova *et al*., 2010). For this purpose, the protein complex SpoIISA-SpoIISB as well as the *lacZ* gene were expressed under the control of P_{xyl}, $P_{hyper\text{-}spank}$, or P_{spaS}, respectively. The promoter $P_{hyper\text{-}spank}$ is a mutated derivative of the IPTG-inducible P_{spac} containing a single base exchange at position -1 (Quisel *et al*., 2001). Under inducing conditions, expression levels obtained from the *xyl* system were slightly higher than those from the *hyper-spank* system, whereas the basal expression levels determined for the *xyl* system were significantly lower (Vavrova *et al*., 2010). These results confirmed prior observations made by Bhavsar and co-workers (Bhavsar *et al*., 2001). Protein amounts received from the induced SURE system were clearly higher than those obtained from P_{xyl} or $P_{hyper\text{-}spank}$, although expression levels under non-inducing conditions were remarkably high (Vavrova *et al*., 2010). These differences are probably caused by the different copy numbers present in the host cell: only one copy of the target gene under control of P_{xyl} or $P_{hyper\text{-}spank}$ is present in *B. subtilis* due to *amyE* integration. In contrast, the expression plasmid in the SURE system is replicative and therefore present in multiple copy numbers. Interestingly, a comparison of the SURE system with the *E. coli* arabinose-inducible P_{BAD} system (Guzman *et al*., 1995) showed that similar protein amounts were achieved upon induction, although the promoter P_{spaS} is much more leaky than P_{BAD} (Vavrova *et al*., 2010). Taken together, among the three expression systems tested, the SURE system is the most productive system for *B. subtilis*, but the expression of target proteins is not tightly enough regulated (Vavrova *et al*., 2010), making this system problematic for toxic protein production.

Since the IPTG- and xylose-inducible systems are the most widely used expression platforms in *B. subtilis*, a lot of other different expression systems have developed. In the following, some examples are cited briefly.

A phosphate-inducible expression system was established, which is based on the alkaline phosphatase I (APase I) promoter of *B. licheniformis* (Lee *et al*., 1991). This promoter is strongly induced by phosphate starvation. Jan and co-workers developed a protein expression system, which is induced at the end of exponential growth phase (Jan *et al*., 2001). Here, the *aprE* promoter was optimized and used to overexpress target proteins. The *aprE* gene encodes the extracellular protease subtilisin, whose expression is naturally induced at the onset of stationary phase. Recombinant protein production by the *aprE* promoter leads to high yields of about 10% of the total protein (Jan *et al*., 2001). The starch-inducible

expression system is another possibility to produce recombinant proteins (Airaksinen *et al.*, 2003, Ho & Lim, 2003). This system is based on the *B. amyloliquefaciens* α-amylase promoter, which is constitutively expressed, but shows significant higher protein expression levels in the presence of starch (Ho & Lim, 2003). Furthermore, a glycine-inducible protein expression system was developed (Phan & Schumann, 2007). The *gcv* operon (glycine degrading operon) is transcriptionally regulated by a tandem riboswitch. In the absence of glycine, a short transcript of about 200 bases is synthesized including the 5' untranslated region due to transcription attenuation, whereas the full-length mRNA is observed at glycine concentrations of 10 mM due to direct binding of glycin to the riboswitch that initiates transcription (Mandal *et al.*, 2004). To create an expression-secretion system, the riboswitch and the *gcv* promoter were used to build expression vectors capable of target protein expression leading to protein yields comparable to the IPTG- and xylose-inducible systems (Phan & Schumann, 2007). Thuy Le and Schumann generated a cold-inducible expression system for the use in *B. subtilis* (Thuy Le & Schumann, 2007). This system is based on the *des* promoter, which is normally controlled by the DesKR 2CS (Aguilar *et al.*, 2001). The HK DesK senses a temperature downshift and autophosphorylates itself. Phosphorylated DesK serves as phosphoryl group-donor for the RR DesR leading to the phosphorylation and subsequent activation of DesR. DesR~P binds to its specific DNA-binding site and activates the transcription of the *des* gene. This gene codes for the Δ5-desaturase, which controls membrane lipid fluidity (Cybulski *et al.*, 2004). The use of the *des* promoter as expression system for recombinant proteins can yield high protein amounts up to 10% of the total cellular proteins. The protein expression at low temperatures (25°C) is also beneficial to the correct folding of the target protein (Schein & Noteborn, 1988). Proteins, which aggregate during its production at higher temperatures (e.g. 37°C) might be overproduced in soluble form by using the cold-inducible expression system (Thuy Le & Schumann, 2007).

6.2.2 Protein expression systems for other *Firmicutes* bacteria

Expression systems developed for other Gram-positive bacteria are also described. One popular example is the NICE (**ni**sin-**c**ontrolled gene **e**xpression) system originally developed for *Lactococcus lactis* (de Ruyter *et al.*, 1996b, Kuipers *et al.*, 1995). This system is based on the lactococcal *nisA* promoter, which is activated by the peptide antibiotic nisin through the regulation of the 2CS NisRK. The HK NisK senses extracellular nisin and undergoes autophosphorylation. Subsequent transfer of the phosphoryl group to the RR NisR leads to its activation. NisR~P activates two of three promoters within the *nis* operon, P_{nisA} and P_{nisF}, so that transcription of genes for nisin biosynthesis and immunity can occur. The promoter controlling *nisRK* is constitutive and therefore not affected by nisin (Kleerebezem, 2004, de Ruyter *et al.*, 1996a, Kuipers *et al.*, 1995). Expression vectors for driving recombinant protein

expression were constructed, in which desired target genes can be transcriptionally or translationally fused to the *nisA* promoter. Different expression hosts were generated; the most commonly used one is a *nisRK* null mutant, in which the regulatory genes are integrated into the *pepN* (aminopeptidase) gene by an integrative plasmid (de Ruyter *et al*., 1996b). By using *gusA* of *E. coli* as reporter gene, the β-glucuronidase activity increased with increasing nisin concentrations, reaching a maximum induction factor of 1000-fold. Moreover, tight regulation of the *nisA* promoter was observed in the absence of the inducer molecule (de Ruyter *et al*., 1996a, de Ruyter *et al*., 1996b, Kleerebezem *et al*., 1997). The NICE system has successfully been converted for the use in many other Gram-positive bacteria, e.g. *Leuconostoc lactis*, *Lactobacillus helveticus* (Kleerebezem *et al*., 1997), *Lactobacillus reuteri* (Wu *et al*., 2006), *Streptococcus pyogenes*, *S. agalactiae*, *S. pneumoniae*, *Enterococcus faecalis*, as well as *Bacillus subtilis* (Eichenbaum *et al*., 1998). The establishment of the NICE system within these bacteria requires a so-called dual-plasmid expression platform, in which two replicative plasmids - one plasmid that carries the regulatory genes *nisRK*, the other possesses the target gene under control of P_{nisA} - are necessary. In contrast to *L. lactis*, significant lower expression levels of 10- to 60-fold were observed in these species (Eichenbaum *et al*., 1998). A broad overview of different host strains, expression vectors, and applications of the NICE system is provided in a comprehensive review article on this topic (Mierau & Kleerebezem, 2005).

The concepts of the aforementioned SURE system and the NICE system are very similar. Therefore, the great industrial applications described for the NICE system (Mierau & Kleerebezem, 2005) might allow similar potential for the *B. subtilis* SURE system. In question of the most favorable expression host in large-scale protein production, *B. subtilis* would be the bacterium of choice because it is growing to higher cell densities than *L. lactis* and it possesses more efficient secretory systems, allowing the secretion of several grams of protein per liter (Mierau & Kleerebezem, 2005, Schallmey *et al*., 2004, van Dijl & Hecker, 2013). Moreover, when used in *B. subtilis*, the SURE system depends on a single-plasmid, which is known to be more stable in contrast to the dual-plasmid expression of the NICE system (Bongers *et al*., 2005, Eichenbaum *et al*., 1998). For induction of P_{spaS}, less amounts of inducer molecule is needed with regard to the nisin-inducible *nisA* promoter and maximal expression levels reached from the SURE system are higher compared to those achieved from the NICE system (Bongers *et al*., 2005). However, despite the advantages of *B. subtilis* as host itself, the above mentioned need of subtilin-containing culture supernatant to induce the SURE system (Bongers *et al*., 2005) as well as the observed leakiness of the *spaS* promoter (Vavrova *et al*., 2010) are prejudicial to specified industrial requirements.

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