LiaRS-dependent gene expression is embedded in transition state regulation in *Bacillus subtilis*

Sina Jordan,¹ Eva Rietkötter,¹ Mark A. Strauch,² Falk Kalamorz,¹ Bronwyn G. Butcher,³ John D. Helmann³ and Thorsten Mascher¹

Correspondence
Thorsten Mascher
tmasche@gwdg.de

¹Department of General Microbiology, Georg-August-University, 37077 Göttingen, Germany

²Department of Biomedical Sciences, University of Maryland Dental School, Baltimore, MD 21201, USA

³Department of Microbiology, Cornell University, Ithaca, NY 14853-8101, USA

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

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INTRODUCTION

The envelope is a crucial structure of the bacterial cell and the target of many antibiotics (Silver, 2003, 2006; Walsh, 2003). Its integrity is closely monitored to detect and counteract threats before their action can lead to irreversible damages. The LiaRS two-component system (TCS) is part of the complex regulatory network that orchestrates the cell-envelope stress response in *Bacillus subtilis* (Mascher *et al.*, 2003). It responds strongly to the external presence of cell wall antibiotics that interfere with the lipid II cycle, such as bacitracin, ramoplanin, vancomycin or cationic antimicrobial peptides (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005). It is also induced by alkaline shock, detergents, ethanol, phenol, organic solvents and secretion stress, albeit to a lesser extent (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Pietiäinen *et al.*, 2005; Tam le *et al.*, 2006; Wiegert *et al.*, 2001).

The LiaRS TCS is functionally and genetically linked to a third protein, LiaF, which acts as a strong inhibitor of LiaR-dependent gene expression (Jordan *et al.*, 2006). The LiaRS–LiaF three-component system is conserved by sequence and genomic context in Gram-positive bacteria with a low G + C content (*Firmicutes*) (Jordan *et al.*, 2006;

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

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

The *lia* locus consists of six genes, *liaH*–*liaGFSR*. A basal expression level of the last four genes, *liaGFSR*, encoding the three-component system (*liaFSR*) and a putative membrane-anchored hypothetical protein (*liaG*), is ensured by a weak constitutive promoter upstream of *liaG*. In contrast, expression of the *liaIH* operon from P_{lial} is completely LiaR-dependent (Jordan *et al.*, 2006). LiaI is a small hydrophobic protein of unknown function with two

Abbreviations: LFH-PCR, long flanking homology PCR; MLS, macrolide-lincosamide-streptogramin; TCS, two-component system.

putative transmembrane helices. LiaH is a member of the phage-shock protein family (see below). While the strong induction of *liaIH* (and to a lesser degree also *liaGFSR*) by cell envelope stress is well documented (see above), mutational analysis of the *lia* locus has so far failed to identify strong phenotypes associated with these genes. Deletion of *lia* genes did not alter the sensitivity of the corresponding mutants to the known inducers of the Lia system. Moreover, none of the complex differentiation processes of *B. subtilis* (i.e. sporulation, competence for genetic transformation, motility and pellicle and fruiting body formation) was affected in *lia* mutants. So far, the only phenotype that could be linked to the Lia system is delayed spore germination in a *liaH* mutant (D. Hoyer & T. Mascher, unpublished). Moreover, LiaH seems to negatively affect the expression of the *yhcz* operon by a currently unknown mechanism. It is weakly inducible by bacitracin only in a *liaH* mutant, but not in the wild-type (Mascher *et al.*, 2003).

While the physiological role of LiaI and LiaH remains obscure, we noted some similarities between LiaH and phage-shock protein A (PspA) of *Escherichia coli*. The latter is induced by various stress conditions such as filamentous phage infection (hence the name), heat shock, osmotic shock and exposure to organic solvents and proton ionophores as well as prolonged incubation under alkaline conditions (Brissette *et al.*, 1990; Kobayashi *et al.*, 1998; Weiner & Model, 1994). This inducer spectrum shows some overlap with the known inducers of *liaIH* expression, which include organic solvents and alkaline shock (Mascher *et al.*, 2004; Wiegert *et al.*, 2001). PspA exhibits a dual function that is linked to two different cellular locations (Brissette *et al.*, 1990; Kleerebezem & Tommassen, 1993). Peripherally bound to the inner surface of the cytoplasmic membrane (through protein–protein interactions), PspA is somehow involved in the maintenance of cell membrane integrity (Darwin, 2005; Kleerebezem *et al.*, 1996). As a free cytosolic protein it inhibits the AAA+ enhancer protein PspF, also through protein–protein interactions (Adams *et al.*, 2003; Bordes *et al.*, 2003; Dworkin *et al.*, 2000). Based on the strong induction of *liaH* by cell envelope stress and its co-transcription with *liaI*, which encodes a small putative membrane protein, we speculate that LiaI serves as a membrane anchor for LiaH, thereby facilitating an activity that might somehow be linked to envelope integrity.

Here, we investigated the intrinsic activity and regulation of P_{liaI} in the absence of exogenous stimuli. We show that P_{liaI} is induced at the onset of stationary phase. This time point in the *B. subtilis* life cycle is characterized by the initiation of a complex regulatory cascade that allows the bacterium to adapt to worsening living conditions, which can ultimately lead to the formation of dormant endospores (Errington, 2003; Msadek, 1999; Phillips & Strauch, 2002). We demonstrate that P_{liaI} is directly repressed by binding of the transition-state regulator AbrB within the promoter sequence, thereby acting as a roadblock to

prevent premature P_{liaI} activity during exponential growth. AbrB repression is released during the transition state by Spo0A, the master regulator of sporulation, and P_{liaI} is induced by an unidentified endogenous stimulus, resulting in the expression of the *liaIH* operon. While AbrB binding is sufficient to inhibit the endogenous growth-dependent induction of P_{liaI} , it can be bypassed completely by exogenous induction with cell wall antibiotics.

METHODS

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

Allelic replacement mutagenesis using long-flanking-homology (LFH)-PCR. This technique is derived from a published procedure (Wach, 1996) and was performed as described previously (Mascher *et al.*, 2003). In brief: resistance cassettes were amplified from a suitable vector as template (Guerout-Fleury *et al.*, 1995; Youngman, 1990). Two primer pairs were designed to amplify ~1000 bp DNA fragments flanking the region to be deleted at its 5'- and 3'-ends. The resulting fragments are here called 'up' and 'do' fragments. The 3'-end of the up-fragment and the 5'-end of the do-fragment extended into the gene(s) to be deleted in a way that all expression signals of genes up- and downstream of the targeted genes remained intact. Extensions of ~25 nucleotides were added to the 5'-end of the 'up-reverse' and the 'do-forward' primers that were complementary (opposite strand and inverted sequence) to the 5'- and 3'-ends of the amplified resistance cassette. All fragments obtained were purified using the PCR-purification kit from Qiagen. In a second PCR 100–150 ng of the up- and do-fragments and 250–300 ng of the resistance cassette were used together with the specific up-forward and do-reverse primers at standard concentrations. In this reaction the three fragments were joined by the 25 nucleotide overlapping complementary ends and simultaneously amplified by normal primer annealing. The PCR products were directly used to transform *B. subtilis*. Transformants were screened by colony-PCR, using the up-forward primer with a reverse-check primer annealing inside the resistance cassette (Table 2). The integrity of the regions flanking the integrated resistance cassettes was verified by sequencing PCR products of ~1000 bp amplified from chromosomal DNA of the resulting mutants. Sequencing was performed in-house by the GenoMIK centre. All PCRs were done in a total volume of 50 µl (10 µl for colony PCR) using the HotStar DNA-Polymerase Mastermix (Qiagen) or TripleMaster Polymerase Mix (Eppendorf) according to the manufacturers' procedures. The constructed strains are listed in Table 1. The primers used are listed in Table 2.

Construction of a clean *liaS* deletion mutant. To ensure 'normal' (i.e. wild-type) expression levels of *liaR*, we constructed a clean deletion of *liaS* using the vector pMAD (Arnaud *et al.*, 2004). The genomic regions ~1 kb upstream and downstream of *liaS* were amplified using primers listed in Table 2 [fragments *liaS*(clean) up, and *liaS*(clean) down], thereby introducing a 26 bp extension to the 3'-end of the up-fragment, which is complementary to the 5'-end of the down-fragment. The two fragments were fused in a second,

Table 1. Strains used in this study

Strain	Relevant genotype*	Source, reference, construction†
W168	Wild-type strain, <i>trpC2</i>	Lab stock
BFS2470	<i>liaI</i> ::pMUTIN	Mascher <i>et al.</i> (2003)
HB0933	<i>liaR</i> ::kan ^R	Mascher <i>et al.</i> (2003)
TMB011	<i>liaI</i> ::pMUTIN, <i>liaR</i> ::kan ^R	Chrom. DNA (HB0933)→BFS2470
TMB079	<i>sinR</i> ::spec ^R	LFH-PCR→W168
TMB080	<i>aprE</i> ::kan ^R	LFH-PCR→W168
TMB081	<i>scoC</i> ::tet ^R	LFH-PCR→W168
TMB082	<i>abrB</i> ::kan ^R	LFH-PCR→W168
TMB083	<i>salA</i> ::tet ^R	LFH-PCR→W168
TMB084	<i>liaI</i> ::pMUTIN, <i>sinR</i> ::spec ^R	Chrom. DNA (TMB079)→BFS2470
TMB085	<i>liaI</i> ::pMUTIN, <i>aprE</i> ::kan ^R	Chrom. DNA (TMB080)→BFS2470
TMB086	<i>liaI</i> ::pMUTIN, <i>scoC</i> ::kan ^R	Chrom. DNA (TMB081)→BFS2470
TMB087	<i>liaI</i> ::pMUTIN, <i>abrB</i> ::kan ^R	Chrom. DNA (TMB082)→BFS2470
TMB088	<i>liaI</i> ::pMUTIN, <i>salA</i> ::tet ^R	Chrom. DNA (TMB083)→BFS2470
TMB117	<i>liaI</i> ::pMUTIN, <i>degU</i> ::kan ^R	Chrom. DNA (TMB124)→BFS2470
TMB118	<i>liaI</i> ::pMUTIN, <i>spo0A</i> ::tet ^R	Chrom. DNA (TMB205)→BFS2470
TMB124	<i>degU</i> ::kan ^R	LFH-PCR→W168
TMB205	<i>spo0A</i> ::tet ^R	LFH-PCR→W168
TMB209	<i>liaI</i> ::pMUTIN, <i>abrB</i> ::kan ^R , <i>spo0A</i> ::tet ^R	Chrom. DNA (TMB082)→TMB118
TMB213	Δ <i>liaS</i>	pMAD-based clean deletion
TMB215	Δ <i>liaS</i> , <i>liaI</i> ::pMUTIN	Chrom. DNA (BFS2470)→TMB213
TMB330	Δ <i>liaS</i> , <i>liaI</i> ::pMUTIN, <i>abrB</i> ::kan ^R	Chrom. DNA (TMB082)→TMB215

*kan, kanamycin; spec, spectinomycin; tet, tetracycline.

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

joining, PCR, and the resulting fragment was cloned into pMAD via *Bam*HI and *Eco*RI, generating pMM101. Generation of the clean deletion basically followed the established procedure (Arnaud *et al.*, 2004). In brief: *B. subtilis* W168 was transformed with pMM101 and incubated at 30 °C with MLS selection on LB agar plates supplemented with X-Gal. Blue colonies were picked and incubated for 6–8 h at 42 °C in LB medium with MLS selection, resulting in the integration of pMM101 into the chromosome. Again, blue colonies were picked from LB (X-Gal) plates and incubated for 6 h in LB medium without selection. Subsequently, the liquid culture was shifted to 30 °C for 3 h, and the cells were then plated on LB (X-Gal) plates, this time without selective pressure. White colonies that had lost the plasmid were picked and checked for MLS sensitivity. Those harbouring a clean deletion of *liaS* (~ 50 % of the white clones) were identified by PCR.

Measurement of induction by β -galactosidase assay. For time-course experiments on *P_{liaI}* induction, the respective reporter strains (listed in Table 1) were inoculated in LB medium from a fresh mid-exponential pre-culture to OD₆₀₀~0.1 and incubated at 37 °C with aeration. Samples (2 ml) were taken every hour and OD₆₀₀ was monitored to follow growth of the cultures. The pellets were resuspended in 1 ml working buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 20 mM β -mercaptoethanol) and suitable dilutions were assayed for β -galactosidase activity, with normalization to cell density (Miller, 1972). For induction experiments, the cells were inoculated from fresh overnight cultures and grown in LB medium at 37 °C with aeration until they reached OD₆₀₀~0.6. The culture was split, adding bacitracin (50 μ g 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 working buffer and assayed for β -galactosidase activity as described with normalization to cell density (Miller, 1972).

Western blotting. Total cytoplasmic proteins were prepared from 15 ml culture per time point by using a French press. Proteins (20 μ g per lane) were separated by SDS-PAGE, according to standard procedure (Sambrook & Russell, 2001). After electrophoresis the gels were equilibrated in transfer buffer [15.2 g Tris; 72.1 g glycine; 750 ml methanol (100 %) in a final volume of 5 l with deionized water] for 30 s. A PVDF membrane was activated with methanol (100 %) and subsequently incubated in transfer buffer for 5 min. The proteins were blotted to this membrane using a semi-dry blot apparatus. After transfer (1 h at 0.8 mA cm⁻²) the membrane was incubated in blotto [1 × TBS (50 mM Tris, 150 mM NaCl, pH 7.6), 2.5 % skim milk] overnight to prevent non-specific binding. The LiaH antibody [polyclonal rabbit antisera raised against purified His₁₀-LiaH (F. Kalamorz & T. Mascher, unpublished) at SEQLAB (Göttingen, Germany)] was diluted 1:20 000 in blotto. After incubation for 3 h, the membrane was washed three times for 30 min with blotto. The secondary antibody [anti rabbit IgG, coupled with alkaline phosphatase (Roche Diagnostics)] was diluted 1:100 000 and the membrane was incubated for 30 min. After three more 20 min washing steps, the membrane was washed with deionized water and incubated in buffer III (0.1 M Tris, 0.1 M NaCl; pH 9.5) for 5 min to adjust the pH. For

Table 2. Oligonucleotides used in this study

Fragment*	Primer sequence†
aprE-up	fwd: GTTGACATTCGGCACACTCC rev: <u>CCTATCACCTCAAATGGTTCGCTGGACATGTTGCTGAACGCCATCG</u>
aprE-down	fwd: <u>CGAGCGCCTACGAGGAATTTGTATCGAAACGCGCAAGTCCGTGATCG</u> rev: CATTTCACACAGACAACGG
salA-up	fwd: AAGATTGGTGGACAGCAGG rev: <u>CCTATCACCTCAAATGGTTCGCTGGGTTGCGCATTTCTCCG</u>
salA-down	fwd: <u>CGAGCGCCTACGAGGAATTTGTATCGTGACGAAAATCATCCAATCGG</u> rev: TATCTCAAGCGCAAACCGATG
abrB-up	fwd: TATCAACGAGCTGATTTCCG rev: <u>CCTATCACCTCAAATGGTTCGCTGCAACTTTACGTACAATACCAGTAG</u>
abrB-down	fwd: <u>CGAGCGCCTACGAGGAATTTGTATCGCAGCGAAATCCAAAACCGAGC</u> rev: TTCTTTACTTGGTCCCAACCC
scoC-up	fwd: AACCTCTTCCGCTTCCGG rev: <u>CCTATCACCTCAAATGGTTCGCTGGAGCCTTGCTAAGCTGAGCC</u>
scoC-down	fwd: <u>CGAGCGCCTACGAGGAATTTGTATCGGATGAACCGGCTGAAGAGC</u> rev: ACGTTTCCATGTGCGCATGC
sinR-up	fwd: GCCAAAAGACCTAGATGGTG rev: <u>CCTATCACCTCAAATGGTTCGCTGATGTCATCACCTTCCTTGTG</u>
sinR-down	fwd: <u>CGAGCGCCTACGAGGAATTTGTATCGGATGACATCCGGGGTATCG</u> rev: TAGGAGTTGCTTCTGCAGC
degU-up	fwd: AAGCCATAAGCTGCAGG rev: <u>CCTATCACCTCAAATGGTTCGCTGTATCCGTTTAACACCTTCAGC</u>
degU-down	fwd: <u>CGAGCGCCTACGAGGAATTTGTATCGTAAACGACCGGACGCAAGCC</u> rev: CAAATGAGTGCCGATTACCGC
spo0A-up	fwd: TATCAGAGATTCTGCTGCTGGC rev: <u>CCTATCACCTCAAATGGTTCGCTGAGCGACAGGCATTCTGTCC</u>
spo0A-down	fwd: <u>CGAGCGCCTACGAGGAATTTGTATCGGTTGCGGATAAGCTGAGG</u> rev: GGAAGAACCCTGAGACACCG
kan cassette	fwd: CAGCGAACCATTGAGGTGATAGG rev: CGATACAAATTCCTCGTAGGCGCTCGG
spec cassette	fwd: CAGCGAACCATTGAGGTGATAGGACTGGCTCGCTAATAACGTAACGTGACTGGCAAGAG rev: <u>CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTATTGTTTTCTAAAATCTG</u>
tet cassette	fwd: <u>CAGCGAACCATTGAGGTGATAGGTCTTGCAATGGTGCAGGTTGTTCTC</u> rev: <u>CGATACAAATTCCTCGTAGGCGCTCGGGAACCTCTCTCCAAAGTTGATCCC</u>
liaS(clean) up	fwd: AGCC GGATCC GAAAGGAGGCGGACACCAGG rev: <u>GTTTCGTTCTCTCTTTTCTTCCGGCTCATACGTACTTCACATCC</u>
liaS(clean) down	fwd: CCGGAAGAAAAAGGAGAGAACGAAACG, rev: CCAT GAATTC AACCGGGCTGGGAAACGAGG

*‘up’ and ‘down’ refer to localization of the fragment relative to the gene(s) to be deleted. Both fragments are ~1 kb in size and include 20–50 nucleotides of the 5′- or 3′-end of the corresponding gene(s), respectively.

†‘fwd’, forward. ‘rev’, reverse. The (universal) linker sequences used for joining reactions are underlined. Restriction sites are in **bold italics**.

LiaH detection, 10 µl CDP-Star chemiluminescence substrate (Roche Diagnostics) in 1 ml buffer III was used. The signal was documented with a ChemiSmart LumiImager (peqlab).

DNase I footprinting assays. AbrB and Abh purification and DNase I footprinting assays were performed essentially as described previously (Bobay *et al.*, 2006; Strauch *et al.*, 1989). The DNA target was a 287 bp fragment containing the *liaI* promoter region (positions –159 to +128), end-labelled with ³²P on the template strand. The binding reactions were performed at 20 °C at pH 8 for AbrB, and pH 7 for Abh (the optimal pHs for each protein’s binding) (Bobay *et al.*, 2006).

RESULTS AND DISCUSSION

The *liaI* promoter (P_{liaI}) is induced without exogenous stimuli at the onset of stationary phase

Induction of P_{liaI} after addition of exogenous stimuli, such as cell-wall antibiotics, is well documented (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005). To investigate if P_{liaI} is also induced in their absence, we examined expression of a P_{liaI} -*lacZ* fusion in the reporter strain BFS2470. This strain

harbours an insertion of the vector pMUTIN (Vagner *et al.*, 1998) inside the *liaI* coding sequence, thereby bringing a promoterless *lacZ* gene under control of the *liaI* promoter (Mascher *et al.*, 2003, 2004).

To study P_{liaI} activity in the absence of external stimuli, BFS2470 was grown in LB medium with MLS selection over a period of 8 h and samples were taken every hour from mid-exponential to late-stationary growth phase. The cells were harvested and β -galactosidase activity was determined, essentially as described previously (Mascher *et al.*, 2004). The results demonstrate that P_{liaI} is induced 8–10-fold without addition of cell-wall antibiotics during transition to stationary phase (Fig. 1a, grey bars). This induction is completely LiaR-dependent: no P_{liaI} activity was observed in the isogenic *liaR* mutant strain TMB011 (Fig. 1a, black bars).

These observations were verified independently by Western analysis in the wild-type and an isogenic *liaR* mutant (strain HB0933). Cells were harvested from both cultures at two time points, 2 h before and after transition state (equivalent to the 2 and 6 h time points in Fig. 1a). Total cellular protein was prepared and Western analysis performed with LiaH antibodies. The results are in agreement with the data from the β -galactosidase assays. LiaH expression is induced in stationary phase in the wild-type, but not in the *liaR* mutant (inset to Fig. 1a). Furthermore, induction of *liaIH* expression was also observed at the transcript level in a wild-type *B. subtilis* strain during a chronotranscriptome analysis (see below). While only results from β -galactosidase assays will be shown for subsequent experiments, all key findings were always independently verified by Western analysis.

Transition state adaptation, which enables *B. subtilis* to gradually adjust to nutrient limitations, is embedded in one of the best-studied bacterial developmental programmes, a complex regulatory cascade that ultimately leads to the formation of highly resistant endospores (Errington, 2003; Msadek, 1999; Phillips & Strauch, 2002). It is well known that sporulation (and other transition-state phenomena such as production of extracellular enzymes, motility and biofilm formation) is subject to carbon catabolite repression (Schaeffer *et al.*, 1965; Shafikhani *et al.*, 2003; Stanley *et al.*, 2003): cells grown with high amounts of glucose enter stationary phase without activating the gene expression cascade associated with sporulation. Therefore, we repeated the experiment shown in Fig. 1 in the presence of increasing glucose concentrations. Addition of glucose to the medium delayed (0.1% glucose, grey bars) or even abolished (0.5% glucose, white bars) P_{liaI} induction, without affecting overall growth rate, onset of stationary phase or final cell density (Fig. 1b). High glucose concentrations repress the activation of Spo0A, a key regulator of numerous post-exponential processes including sporulation. The activation of Spo0A results in two major effects on gene expression. First, it leads to the activation of a cascade of sigma factors that ultimately

Fig. 1. Transition state induction of P_{liaI} . (a) LB medium (20 ml, with MLS selection) was inoculated from a fresh mid-exponential preculture of strains BFS2470 ('wild-type', grey squares and grey bars) and TMB011 (*liaR* mutant, black triangles and black bars) and incubated at 37 °C with aeration. Cell density was monitored by measuring OD₆₀₀ at regular intervals, and samples were taken every hour from mid-exponential until late stationary growth phase. The cells were harvested and lysed and a β -galactosidase assay was performed as described previously (Mascher *et al.*, 2004). β -Galactosidase activity, normalized to cell density, is expressed in Miller units (Miller, 1972). The timescale is given relative to the start of the cultures. The inset shows results of a Western blot analysis of LiaH expression. Samples (20 µg) of total proteins of the wild-type (W168) and an isogenic *liaR* mutant (HB0933; ΔR) were separated by SDS-PAGE. Western blots were performed using CDP-Star (Roche) for chemiluminescent detection, according to the manufacturer's instructions. See Methods for details. (b) The same experiment as in (a) was performed. Only the wild-type reporter strain BFS2470 was used. The preculture was used to inoculate three different flasks containing 20 ml LB each, without (black symbols/bars), and with the addition of glucose to a final concentration of 0.1% (grey symbols/bars) or 0.5% (white symbols/bars).

govern the formation of the dormant endospore. Second, Spo0A~P represses AbrB, which itself is a repressor of numerous genes associated with antibiotic production and

resistance (Errington, 2003; Msadek, 1999; Phillips & Strauch, 2002). While addition of glucose has pleiotropic effects on numerous regulatory pathways, this observation can nevertheless be viewed as an indication of a link between LiaRS-dependent gene expression and transition-state regulation.

A second line of evidence pointing in this direction came from results obtained in a detailed chronotranscriptome study, in which the global gene expression pattern was monitored during a complete growth curve with a resolution of 10 min (Sapolsky *et al.*, 2005). This study not only verified the transition-state induction of *liaIH* (but not *liaGFSR*) in a *B. subtilis* wild-type strain at the transcript level, but also revealed that expression of *liaIH* coincides with that of only one other gene, *aprE* (Eugenio Ferrari, personal communication). This observation can be interpreted in two ways: either P_{liaI} is induced as a result of *aprE* expression, or both loci are subject to the same regulation.

P_{liaI} is repressed by AbrB and activated by Spo0A

To address the first hypothesis, an *aprE* mutant was constructed by LFH-PCR, and introduced into the P_{liaI} reporter strain, resulting in strain TMB085 (Table 1). No difference of P_{liaI} activity was observed relative to the wild-type reporter strain BFS2470 (data not shown). Therefore, *AprE* is not involved in P_{liaI} induction.

The *aprE* promoter is subject to complex regulation: one activator (DegU) and three repressors (ScoC, SinR and AbrB) bind directly to the *aprE* promoter region. The activity of these proteins is modulated by additional proteins, such as Spo0A, SalA and RapG/PhrG (Ogura *et al.*, 2003, 2004). To analyse a potential role of these proteins in P_{liaI} activity, mutants in *abrB*, *scoC*, *sinR* and *degU* were constructed and subsequently transferred into the P_{liaI} reporter strain BFS2470 (Table 1). No alterations of P_{liaI} activity were observed in the *scoC*, *sinR* and *degU* mutant backgrounds (data not shown). In contrast, the *abrB* mutation in strain TMB087 resulted in an about fourfold elevated basal expression level during the exponential growth phase, indicating that AbrB acts as a repressor at P_{liaI} during that time (Fig. 2, black bars). The promoter is still inducible to about wild-type levels. Both the basal promoter activity and the induction of P_{liaI} in the *abrB* mutant are completely dependent on LiaS-mediated activation of its cognate response regulator, LiaR: a *liaS/abrB* mutant (TMB330; harbouring a clean *liaS* deletion to avoid polar effects on *liaR* expression) behaves similarly to a *liaR* mutant, i.e. does not show any P_{liaI} activity throughout the growth curve (data not shown).

A close regulatory connection between the transition state regulator AbrB and Spo0A, the master regulator of sporulation, is well established: AbrB inhibits Spo0A expression indirectly via σ^H , contributing to the mechanisms governing temporal control of sporulation initiation.

Fig. 2. Effect of *abrB* and *spo0A* mutations on P_{liaI} activity. Growth and β -galactosidase activity of P_{liaI} -*lacZ* fusions were measured for an *abrB* (TMB087, black squares and black bars), a *spo0A* (TMB118, white diamonds and white bars), and an *abrB/spo0A* mutant (TMB209, grey triangles and grey bars), respectively. The experiment was performed as described in the legend to Fig. 1(a). The scale on the y-axis is split for clarity.

Activated Spo0A, on the other hand, represses *abrB* expression, ultimately releasing transition state functions, and σ^H expression, from AbrB repression at the onset of stationary phase (Msadek, 1999; Phillips & Strauch, 2002). As a consequence, mutations in the two genes usually exhibit converse phenotypes on genes subject to their regulation. This could also be observed for P_{liaI} activity: a *spo0A* mutant (TMB118, Table 1) behaved very similarly to the *liaR* mutant in the β -galactosidase assay, i.e. had no detectable P_{liaI} activity (Fig. 2, white bars). These findings were also verified by Western analysis (data not shown). They are in agreement with results from previous transcriptome studies, indicating an indirect Spo0A-dependent induction of *liaIH* expression (Fawcett *et al.*, 2000; Fujita *et al.*, 2005; Hamon *et al.*, 2004). Therefore, P_{liaI} is subject to AbrB repression and Spo0A activation.

Spo0A activates P_{liaI} indirectly through AbrB

The loss of transition-state induction of P_{liaI} in the *spo0A* mutant raised the question of how Spo0A affects its promoter activity. The *abrB* gene is known to be under the direct negative control of Spo0A, and AbrB acts as a repressor of a set of genes that are switched on at the transition state (Strauch *et al.*, 1990, 1989). Therefore, Spo0A activation of P_{liaI} could be an indirect effect due to the lack of Spo0A-dependent repression of *abrB*.

Alternatively, Spo0A itself could be responsible for the expression of genes that ultimately provide the stimulus that is sensed by the LiaRS TCS at the onset of stationary phase. To distinguish between the two possibilities, a mutant lacking both *abrB* and *spo0A* was constructed and introduced into BFS2470, resulting in strain TMB209. This mutant showed a P_{liaI} induction pattern comparable to the *abrB* mutant, i.e. an elevated basal level of P_{liaI} activity during exponential growth, and induction at the onset of stationary phase (Fig. 2). Interestingly, the maximum P_{liaI} activity was reproducibly higher by a factor of two than in the *abrB* mutant (Fig. 2, grey bars). The reason for this behaviour remains elusive. However, the results clearly demonstrate that Spo0A indirectly modulates P_{liaI} activity by repressing *abrB* expression.

AbrB directly binds P_{liaI}

The transition-state regulator AbrB directly regulates (mostly represses) the expression of over 50 genes, with many additional loci being subject to indirect AbrB control (Phillips & Strauch, 2002). Despite in-depth knowledge on numerous AbrB binding sites, no consensus sequence has been identified for chromosomal sites of interaction. It has

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

been hypothesized that AbrB recognizes a conserved three-dimensional DNA structure, rather than specific base pairs, in the promoter regions of its target genes (Bobay *et al.*, 2004; Phillips & Strauch, 2002; Xu & Strauch, 1996). DNase I footprinting analysis of the *liaI* promoter region demonstrates that AbrB protects a DNA region of about 25 base pairs (from –40 to –14), with weaker protection occurring further downstream (from –11 to about +10) (Fig. 3). In contrast, the AbrB paralogue Abh does not bind the P_{liaI} region under these conditions (Fig. 3). We conclude that AbrB repression of P_{liaI} occurs through direct binding of the repressor within the promoter sequence, thereby preventing transcription initiation.

LiaR is sufficient for P_{liaI} induction

Induction of P_{liaI} under conditions of cell envelope stress is strictly LiaR-dependent (Mascher *et al.*, 2003). To determine if AbrB or Spo0A are also necessary for P_{liaI} -dependent transcription in the presence of external stimuli, such as bacitracin, we performed β -galactosidase assay and Western analysis in the wild-type, and isogenic *liaR*, *abrB* and *spo0A* mutants from cells harvested mid-exponentially with and without the presence of bacitracin (final concentration 50 μ g ml^{–1}). With the exception of the *liaR* mutant, all strains were inducible to comparable levels, demonstrating that LiaR alone is sufficient for bacitracin-induced P_{liaI} activity (Fig. 4, and data not shown). These experiments also demonstrate that strong inducers such as bacitracin (3000–4000 Miller units from P_{liaI} –*lacZ* in

Fig. 4. Bacitracin induction of P_{liaI} in BFS2470 (WT), and isogenic *liaR* (TMB011), *abrB* (TMB087), and *spo0A* (TMB118) mutants. All strains were grown in LB medium with MLS selection to mid-exponential phase. The cultures were split; half were induced with bacitracin (final concentration 50 μ g ml^{–1}; black bars), the other half remained as uninduced controls (grey bars). Incubation was continued for 30 min, before 2 ml of culture was harvested. The cells were lysed and the β -galactosidase assay was performed as described previously (Mascher *et al.*, 2004). β -Galactosidase activity, normalized to cell density, is expressed in Miller units (Miller, 1972). The scale on the y-axis is split for clarity.

β -galactosidase assays) can completely overcome AbrB repression, whereas the endogenous transition state induction (100–200 Miller units) can be suppressed by increased cellular levels of AbrB as present in the *spo0A* mutant. The mechanism by which fully activated LiaR (i.e. in response to bacitracin stress) seemingly ‘overrides’ AbrB control is completely unknown. We can only speculate that the affinity of phosphorylated LiaR for its target promoter somehow is higher than that of AbrB, whereby activated LiaR seems to be able to displace bound AbrB and initiate P_{liaI} -dependent transcription in the presence of strong inducers.

The nature of the endogenous stimulus responsible for transition state induction of P_{liaI} remains obscure

The known induction of P_{liaI} by cell-wall antibiotics led us to hypothesize that induction in early stationary phase might be due to a secreted antibiotic synthesized by *B. subtilis* itself (Stein, 2005). To address this question, induction experiments with spent medium were performed. The wild-type reporter strain BFS2470 was grown in LB medium with MLS selection until 2 h after transition state (corresponding to $t=6$ h in Fig. 1a). A sample of the culture was harvested to check for P_{liaI} induction. The cells were removed from the remaining culture and the spent medium was directly used to resuspend fresh mid-exponential cells ($t=2$ h) that were incubated in parallel. Additionally, cells were resuspended in fresh prewarmed LB medium, with and without addition of bacitracin (final concentration $50 \mu\text{g ml}^{-1}$), as a positive and negative control, respectively. After further incubation at 37°C for 30 min, the cells were harvested. The cells from the stationary-phase culture showed the expected β -galactosidase activity (~ 150 Miller units). Resuspension of mid-exponential phase cells in fresh LB medium only resulted in the normal background activity of about 10 Miller units, while resuspension in LB medium supplemented with bacitracin gave the typical strong P_{liaI} response (about 2000 Miller units). In contrast, no induction was observed when mid-exponential cells were resuspended in spent medium (data not shown). These results indicate that P_{liaI} is not induced by a secreted compound produced by *B. subtilis* itself.

However, we cannot rule out the possibility that the inducing antibiotic is not released from the cells in sufficient amounts in the medium to be detectable in our conditioned-medium experiments. For example, a prerequisite for the biological potency of many cationic antimicrobial peptides is their binding to the overall negatively charged cell envelope, and modulating this net charge is an important resistance mechanism of many Gram-positive bacteria against their activity (Kovács *et al.*, 2006; Peschel *et al.*, 1999). Conversely, one could imagine that such an antibiotic, produced by *B. subtilis* itself, might be retained to a certain degree by the negatively charged cell wall. Therefore, while P_{liaI} induction could then be

readily measured in the antibiotic-producing stationary phase culture, this inducer would not necessarily accumulate in the medium in amounts sufficient to activate P_{liaI} in resuspended mid-exponential phase cells.

We also attempted to identify potential genetic determinants involved in generating the endogenous stimulus by applying transposon mutagenesis in the P_{liaI} -*lacZ* reporter strain BFS2470 and screening for blue colonies, indicative of increased P_{liaI} activity. Two independent approaches were used, *in vivo* transposon mutagenesis, based on the established mini-Tn10 system encoded on plasmid pIC333 (Steinmetz & Richter, 1994), and a newly developed *in vitro* system, based on Tn7 (Peters & Craig, 2001). The latter also allows gain-of-function mutagenesis screens, due to the presence of an outward-facing, xylose-inducible promoter. We readily isolated mutants with transposon insertions in *liaF*, the known negative regulator of the LiaRS systems (Jordan *et al.*, 2006). In addition, we recovered insertions in the export pump of a putative bacteriocin, indicating that endogenous peptides produced by *B. subtilis* can induce the LiaRS system (Butcher and Helmann, unpublished). However, strains lacking the ability to produce this bacteriocin still induce P_{liaI} upon entry into stationary phase (data not shown). Therefore, the nature of this endogenous stimulus remains unknown.

Conclusions

Based on the results of previous studies and those presented herein, the intrinsic induction of *liaIH* expression at the onset of stationary phase is tightly regulated and delicately balanced by five proteins – LiaR, LiaS, LiaF, AbrB and Spo0A – to allow an appropriate cellular response at the right time. The interactions and hierarchy of these regulators is illustrated in the model in Fig. 5. During exponential growth in the absence of cell-envelope stress, the LiaRS two-component system is kept inactive by the LiaF regulator (Jordan *et al.*, 2006). Furthermore, the transition-state regulator AbrB represses any residual P_{liaI} activity by binding to a DNA fragment that includes the -35 region and reaches the -10 region, thereby serving as a roadblock that efficiently prevents transcription initiation (Fig. 5, right-hand side). At the onset of stationary phase, increasing levels of phosphorylated Spo0A, the master regulator of sporulation, inhibit *abrB* expression (Strauch *et al.*, 1990), thereby releasing P_{liaI} from its repression. At about the same time, an unidentified stimulus leads to the activation of the histidine kinase LiaS and/or its release from LiaF repression. This, in turn, leads to the activation of the cognate response regulator LiaR, which interacts with its binding site (an imperfect inverted repeat of seven nucleotides with four-nucleotide spacing) (Jordan *et al.*, 2006), ultimately resulting in induction of *liaIH* expression (Fig. 5, left-hand side).

Previous studies identified numerous agents that are able to induce LiaRS-dependent gene expression. While some of these compounds, especially cell-wall antibiotics that

Fig. 5. Model for the transition state regulation of P_{lial} activity. The situations during exponential growth and the transition state are shown on the right and left, respectively. Regulatory proteins involved are named and circled. Grey backgrounds indicate activity, white inactivity. Arrows indicate activation, T-shaped lines repression. The genomic context of the *lial* promoter region and its important features are shown schematically in the middle; the relevant sequence is detailed below. The identified regulator binding sites are highlighted in grey. The inverted repeat in the LiaR binding site is indicated by the two arrows below. The promoter sequence and transcriptional start are highlighted in bold and underlined. See text for details.

interfere with the lipid II cycle (i.e. bacitracin, nisin, ramoplanin or vancomycin), elicit a strong response (Mascher *et al.*, 2004), the biological relevance of these observations remains obscure, since the LiaRS system is not involved in mediating resistance against any of these inducers (unpublished results). The induction of P_{lial} at the onset of stationary phase – while being significantly weaker [about 10–15-fold, Fig. 1a, compared to 50–200-fold in the case of strong inducers (Mascher *et al.*, 2004), Fig. 4] – is therefore an important observation. While a very high dynamic potential and strength of LiaRS-dependent gene expression could be demonstrated by the exogenous addition of cell-wall antibiotics (Mascher *et al.*, 2004), this situation does not necessarily reflect the ‘natural’ condition for the activation of LiaRS-dependent signal transduction. The different sites and modes of action of these known inducers of the LiaRS system, together with the unique domain architecture of the sensor kinase LiaS, argue against a direct binding of these drugs to the input domain of LiaS (Mascher, 2006). Identification of the true sensory input of the LiaRS system, while being a big challenge, is therefore a prerequisite to understanding the difference in LiaR-dependent gene expression observed in this study.

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