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

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

Soil is one of the most complex microbial habitats on earth. Nutrient supply varies greatly and on short notice, as do many physicochemical parameters, such as temperature, oxygen concentration, and moisture. The presence of toxic chemicals and the high population density add another layer of complexity (51). Soil bacteria have adapted to this environment in many ways. A broad range of transport systems together with flexible metabolic capabilities allow them to use a variety of nutrient sources. An extensive set of secondary metabolites is thought to suppress the growth of competitors. This trait is specifically pronounced in the actinomycete group of soil bacteria, the most prodigious producers of antimicrobial compounds: two-thirds of all antibiotics in clinical use are synthesized by these bacteria alone (4, 61). Production of and resistance against antibiotics is therefore an important aspect of life in soil (48).

The cell envelope is the first and major line of defense against threats from the environment. It gives the cell its shape and counteracts the high inner osmotic pressure (16). It also provides an important sensory interface and molecular sieve between a bacterial cell and its surroundings, mediating both information flow and controlled transport of solutes. Because of its crucial role, it is an attractive target for numerous antibiotics (7, 34, 39, 56, 63). Therefore, monitoring cell envelope integrity is critical for survival.

By applying genome-wide transcript profiling, the regulatory network of the cell envelope stress response in *Bacillus subtilis* was recently characterized: addition of cell wall inhibitors such as bacitracin (produced by *Bacillus* spp.) and vancomycin (a secondary metabolite of actinomycetes) induces several transmembrane signal transducing pathways, orchestrated by at least three alternative (extracytoplasmic function) σ-factors and four two-component systems (TCS) (10, 42). The use of different antibiotics allowed the differentiation between relatively antibiotic-specific (YvcPQ, BceRS in the case of bacitracin, or σ^W for vancomycin) and more general cell envelope stress responses, such as σ^M and the LiaRS (formerly YvqEC) TCS (42). Interestingly, the sensors of all cell envelope stress-sensing TCS (BceS, YvcQ, and LiaS) appear to define a new family of intramembrane sensing histidine kinases. These proteins share an unusually short sensing domain that is almost completely buried in the cytoplasmic membrane. It has been postulated that these kinases detect their signal with their transmembrane helices directly at the membrane interface (42).

The *liaIHGFSR* locus is expressed from a strictly LiaR-dependent σ^A-type promoter upstream of the *liaI* gene (P_{liaI}). The LiaRS TCS senses vancomycin, bacitracin, ramoplanin, and nisin and mediates a 100- to 1,000-fold induction from P_{liaI} (43). All four drugs interfere with the lipid II cycle in the cytoplasmic membrane, the rate-limiting step of cell envelope polymer biosynthesis (hence the name; LiaRS stands for *l*ipid II-*i*nteracting *a*ntibiotics response *r*egulator and *s*ensor) (43). A strong stem-loop structure downstream of the second gene, *liaH*, results in two different transcripts: a major 1.1-kb mRNA consisting of *liaIH* and a ~4-kb transcript, including the whole operon. A 74-nucleotide promoter region is fully sufficient for

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TABLE 1. Strains used in this study

Strain	Genotype or characteristic(s)[a]	Reference or source
E. coli strain		
DH5αF′	F′ *endA1 hsdR17*(r_K^- m_K^+) *gln*V44 *thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169* *deoR* [Φ80 *dlac*Δ(*lacZ*)M15]	Lab stock
B. subtilis strains		
W168	Wild type, *trpC2*	Lab stock
CU1065	Wild type, *trpC2 att*SPβ	Lab stock
HB0920	CU1065 *liaH*::kan	42
HB0933	CU1065 *liaR*::kan	42
HB0950	CU1065 *att*SPβ2Δ2::P_{liaI}-*cat-lacZ*	43
TMB002	CU1065 *liaF*::kan	This study
TMB003	CU1065 *liaG*::kan	This study
TMB004	CU1065 *liaS*::kan	This study
TMB016	W168 *amyE*::(cat P_{liaI}-*lacZ*)	This study
TMB017	W168 *amyE*::(cat P_{liaI}-*lacZ*) *liaG*::kan	This study
TMB018	W168 *amyE*::(cat P_{liaI}-*lacZ*) *liaF*::kan	This study
TMB019	W168 *amyE*::(cat P_{liaI}-*lacZ*) *liaS*::kan	This study
TMB020	W168 *amyE*::(cat P_{liaI}-*lacZ*) *liaR*::kan	This study
TMB021	W168 *amyE*::(cat P_{liaI}-*lacZ*) *liaGF*::kan	This study
TMB027	HB0950 LiaFΔ(I_{151}-D_{235})[b]	This study
TMB028	HB0950 LiaFΔ(S_{189}-V_{192})[b]	This study
TMB029	HB0950 LiaFΔ(E_{126}-D_{146})[b]	This study
TMB053	W168 *amyE*::(cat $P_{liaG[-68-914]}$-*lacZ*)	This study
TMB066	W168 *amyE*::(cat $P_{yhcY[-129-70]}$-*lacZ*) *liaR*::spec	This study
TMB069	W168 *amyE*::(cat $P_{yhcY[-129-70]}$-*lacZ*) *liaHGF*::kan *liaR*::spec	This study
TMB071	W168 *amyE*::(cat $P_{yhcY[-129-70]}$-*lacZ*)	This study
TMB072	W168 *amyE*::(cat $P_{yhcY[-129-70]}$-*lacZ*) *liaH*::kan	This study
TMB095	W168 *amyE*::(cat $P_{yhcY[-129-70]}$-*lacZ*) *liaF*::kan	This study
TMB096	W168 *amyE*::(cat $P_{yhcY[-71-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB097	W168 *amyE*::(cat $P_{yhcY[-88-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB098	W168 *amyE*::(cat $P_{yhcY[-97-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB099	W168 *amyE*::(cat $P_{yhcY[-107-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB100	W168 *amyE*::(cat $P_{yhcY[-117-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB101	W168 *amyE*::(cat $P_{yhcY[-122-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB102	W168 *amyE*::(cat $P_{yhcY[-129-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB104	W168 *amyE*::(cat $P_{yhcY[-244-70]}$-*lacZ*) *liaHGF*::kan	This study
TMB107	W168 *amyE*::(cat $P_{liaG[-68-3]}$-*lacZ*)	This study
TMB108	W168 *amyE*::(cat P_{liaI}-*lacZ*) *liaH*::kan	This study
TMB111	W168 *amyE*::(cat $P_{liaI[-102-72]}$ [A_{-76}T A_{-78}T A_{-81}T]-*lacZ*)	This study
TMB112	W168 *amyE*::(cat $P_{liaI[-102-72]}$ [A_{-75}T A_{-77}T A_{-79}T]-*lacZ*)	This study
TMB113	W168 *amyE*::(cat $P_{liaI[-102-72]}$ [C_{-86}A G_{-88}A C_{-93}A G_{-95}A T_{-98}A]-*lacZ*)	This study
TMB114	W168 *amyE*::(cat $P_{liaI[-102-72]}$ [A_{-78}C A_{-83}C]-*lacZ*)	This study
TMB115	W168 *amyE*::(cat $P_{liaI[-102-72]}$ [C_{-93}A G_{-95}A T_{-98}A]-*lacZ*)	This study
TMB132	W168 *amyE*::(cat $P_{liaG[81-914]}$-*lacZ*)	This study
TMB133	W168 *amyE*::(cat $P_{liaI[-102-72]}$ [C_{-86}A T_{-87}A G_{-88}A]-*lacZ*)	This study
TMB182	HB0950 LiaFΔ(I_{151}-D_{235})[b] *thrC*::(spec P_{xyl}-*liaF*)	This study
TMB183	HB0950 LiaFΔ(S_{189}-V_{192})[b] *thrC*::(spec P_{xyl}-*liaF*)	This study
TMB184	HB0950 LiaFΔ(E_{126}-D_{146})[b] *thrC*::(spec P_{xyl}-*liaF*)	This study

[a] Resistance cassettes: kan, kanamycin; cat, chloramphenicol; spec, spectinomycin. Positions of promoter fragments are given relative to the ATG start codon of the corresponding gene.

[b] For reasons of clarity, the effects of in-frame deletions in *liaF* are given at the level of the LiaF protein.

the strong antibiotic-inducible, LiaR-dependent activation of gene expression (43).

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

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* was routinely grown in LB medium at 37°C with aeration. All strains used in this study are derivatives of the laboratory wild-type strains W168 and CU1065 (W168 *trpC2* *attSP*β) and are listed in Table 1. Kanamycin (10 μg/ml), chloramphenicol (5 μg/ml), spectinomycin (100 μg/ml), and tetracycline (10 μg/ml) were used for the selection of the *B. subtilis* mutants used in this study. Transformation was carried out as described elsewhere (31).

TABLE 2. Vectors and plasmids

Plasmid	Genotype, sequence, or characteristic(s)[a]	Primers used for cloning	Reference or source
pAC6	*lacZ* fusion vector, integrates at *amyE*, chloramphenicol resistance		58
pXT	Vector for xylose-inducible gene expression, integrates at *thrC*, pDG1782 derivative, spectinomycin resistance		18
pAJ601	pAC6 $P_{yhcY(-71\text{-}70)}$-*lacZ*	0165/0172	This study
pAJ602	pAC6 $P_{yhcY(-88\text{-}70)}$-*lacZ*	0165/0171	This study
pAJ603	pAC6 $P_{yhcY(-129\text{-}70)}$-*lacZ*	0165/0168	This study
pAJ604	pAC6 $P_{yhcY(-97\text{-}70)}$-*lacZ*	0165/0170	This study
pAJ606	pAC6 $P_{yhcY(-244\text{-}70)}$-*lacZ*	0165/0166	This study
pAJ607	pAC6 $P_{yhcY(-122\text{-}70)}$-*lacZ*	0165/0169	This study
pAJ608	pAC6 $P_{yhcY(-117\text{-}70)}$-*lacZ*	0165/0259	This study
pAJ609	pAC6 $P_{yhcY(-107\text{-}70)}$-*lacZ*	0165/0260	This study
pBD601	pAC6 $P_{liaI(-102\text{-}72)}$ ($A_{-76}T\ A_{-78}T\ A_{-81}T$)-*lacZ*	0100/0231	This study
pBD602	pAC6 $P_{liaI(-102\text{-}72)}$ ($A_{-75}T\ A_{-77}T\ A_{-79}T$)-*lacZ*	0100/0232	This study
pBD603	pAC6 $P_{liaI(-102\text{-}72)}$ ($C_{-86}A\ G_{-88}A\ C_{-93}A\ G_{-95}A\ T_{-98}A$)-*lacZ*	0100/0265	This study
pBD604	pAC6 $P_{liaI(-102\text{-}72)}$ ($A_{-78}C\ A_{-83}C$)-*lacZ*	0100/0266	This study
pBD605	pAC6 $P_{liaI(-102\text{-}72)}$ ($C_{-93}A\ G_{-95}A\ T_{-98}A$)-*lacZ*	0100/0267	This study
pBD606	pAC6 $P_{liaI(-102\text{-}72)}$ ($C_{-86}A\ T_{-87}A\ G_{-88}A$)-*lacZ*	0100/0268	This study
pDH605	pAC6 $P_{liaG(81\text{-}914)}$-*lacZ*	0204/0222	This study
pSJ601	pAC6 $P_{liaG(-68\text{-}914)}$-*lacZ*	0204/0205	This study
pSJ607	pAC6 $P_{liaG(-68\text{-}3)}$-*lacZ*	0204/0296	This study
pSJ701	pXT P_{xyl}-*liaF*	0035/0036	This study
pTM1	pAC6 $P_{liaI(-83\text{-}72)}$-*lacZ*	0099/0100	This study

[a] The positions of the cloned fragments are given relative to the A of the start codon of the corresponding gene.

Allelic replacement mutagenesis using LFH-PCR. The long flanking homology PCR (LFH-PCR) technique is derived from a published procedure (62) and was performed as described previously (42). In brief, resistance cassettes were amplified from a suitable vector as template (27, 67). Two primer pairs were designed to amplify ~1,000-bp DNA fragments flanking the region to be deleted at its 5′ and 3′ ends. The resulting fragments are here called the "up" and "do" fragments. The 3′ end of the up fragment as well as the 5′ end of the do fragment extended into the gene(s) to be deleted in a way that all expression signals of genes up- and downstream of the targeted genes remained intact. Extensions of ~25 nucleotides were added to the 5′ end of the up-reverse and the do-forward primers that were complementary (opposite strand and inverted sequence) to the 5′ and 3′ ends of the amplified resistance cassette. All obtained fragments were purified using the PCR purification kit from QIAGEN. One hundred to 150 ng of the up and do fragments and 250 to 300 ng of the resistance cassette were used together with the specific up-forward and do-reverse primers at standard concentrations in a second PCR. 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 (see Table 3, below). The integrity of the regions flanking the integrated resistance cassettes was verified by sequencing PCR products of ~1,000 bp amplified from chromosomal DNA of the resulting mutants. Sequencing was performed in-house by the GenoMIK Center. 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 primers used in this study are listed below in Table 3.

Construction of transcriptional promoter-*lacZ* fusions. An ectopic integration of a P_{liaI}-*lacZ* fusion was constructed based on the vector pAC6 (Table 2). This vector carries a chloramphenicol resistance cassette for selection in *B. subtilis* and integrates into the *amyE* locus by double crossing-over, resulting in a stable integration of P_{liaI}-*lacZ* fusions (58). A promoter fragment similar to $P_{liaI\text{-}83}$ described earlier for pSLZ83 (43) was amplified using the primers 99 and 100, thereby introducing EcoRI and BamHI sites, respectively (Table 3). Standard cloning techniques were applied (54). The insert was verified by DNA sequencing at the GenoMIK Center, Göttingen. The resulting pAC6-derived plasmid, pTM1 (Table 2), was linearized with ScaI and used to transform *B. subtilis* 168 with chloramphenicol selection, resulting in strain TMB016. Subsequently, individual genes of the *lia* locus were replaced with a kanamycin resistance cassette by transforming TMB016 with chromosomal DNA from strains HB0920, HB0933, and TMB002 to TMB004, resulting in strains TMB017 to TMB022 (see Table 1 for details). The pAC6 vector was also used to construct P_{yhcY}- and P_{liaG}-*lacZ* fusions, applying the same cloning strategy (Table 2). The primers used are listed in Table 3, and the resulting strains used in this study are given in Table 1.

Complementation of *liaF* in in-frame deletion mutants. An ectopic integration of a P_{xyl}-*liaF* fusion was constructed based on the vector pXT (18). This vector is a pDG1782 derivative (26) that carries a spectinomycin resistance cassette for selection in *B. subtilis* and a xylose-inducible promoter for *liaF* expression and integrates into the *thrC* locus by double crossing-over, resulting in a stable integration of P_{xyl}-*liaF* fusions. We amplified *liaF* with primers 35 and 36, thereby introducing EcoRI and HindIII sites, respectively (Table 3). Cloning and verification of DNA sequence were performed as described above. The resulting pXT-derived plasmid, pSJ701 (Table 2), was linearized with ScaI and used to transform *B. subtilis* TMB027 to TMB029 with spectinomycin selection, resulting in strains TMB182 to TMB184 (Table 1). For *liaF* expression, 0.2% xylose was added to the medium.

Measurement of induction by β-galactosidase assay. Cells were inoculated from fresh overnight cultures and grown in LB 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 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 (44).

Preparation of total RNA for quantitative real-time RT-PCR, Northern blotting, and primer extension analysis. Total RNA was extracted from 10 ml of culture with and without bacitracin (50 μg/ml final concentration). Bacitracin was added to the culture at an OD_{600} of 0.4 (mid-log phase), and the cultures were incubated for 10 min at 37°C with aeration before the cells were harvested and rapidly frozen at −70°C. RNA was prepared using the RNeasy kit (QIAGEN) according to the manufacturer's protocol. The RNA was treated with DNase (using the on-column RNase-free DNase kit from QIAGEN) to remove remaining traces of chromosomal DNA that would interfere with the subsequent reaction. The success of this treatment was verified by a lack of product in a standard PCR, 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 QuantiTect SYBRgreen RT-PCR kit (QIAGEN) according to the manufacturer's procedure, with minor modifications. In brief, 100 ng of DNA-free total RNA was used in a total reaction volume of 25 μl with 0.5 μM of each primer (Table 3). The amplification

TABLE 3. Oligonucleotides used in this study

Primer no. or amplified fragment	Sequence
Oligonucleotides for LFH-PCR[a]	
kan cassette	fwd, CAGCGAACCATTTGAGGTGATAGG; rev, CGATACAAATTCCTCGTAGGCGCTCGG
kan-check	fwd, CATCCGCAACTGTCCATACTCTG; rev, CTGCCTCCTCATCCTCTTCATCC
spec cassette	fwd, CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACTG GCAAGAG; rev, CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGT TTATTGTTTTCTAAAATCTG
spec-check	fwd, GTTATCTTGGAGAGAATATTGAATGGAC; rev, CGTATGTATTCAAATATATCCTCC TCAC
liaH-up	fwd, CTTGTTATTCGTCACTGCC; rev, CCTATCACCTCAAATGGTTCGCTGGTCCTTCATG AACTGACGC
liaH-do	fwd, CGAGCGCCTACGAGGAATTTGTATCGCAGACCAGACAAAAGCGGC; rev, CGCTA GATCCCCGCTGTCC
liaG-up	fwd, TTGTCGTCGGAATCGCATTGGC; rev, CCTATCACCTCAAATGGTTCGCTGCACATC TTTAACGACGACGGC
liaG-do	fwd, CGAGCGCCTACGAGGAATTTGTATCGCCAATCGACATCAAAACGGACA; rev, TTA CCCGGCGTTTGACTCGC
liaF-up	fwd, AAGGATTTGCGGTCAAGTCC; rev, CCTATCACCTCAAATGGTTCGCTGGCAATGA TCAATCCGAGAAGC
liaF-do	fwd, CGAGCGCCTACGAGGAATTTGTATCGGATGTGGATGTGAAGTACG; rev, TTCAAG CCGTATGAGGAGGC
liaS-up	fwd, GCTTTATCAGCAAGCGGTGACG; rev, CCTATCACCTCAAATGGTTCGCTGTCCCGT TGTCATGCGGATGGC
liaS-do	fwd, CGAGCGCCTACGAGGAATTTGTATCGGGCACTCAAATCGAAGTGA; rev, AACCG GGCTGGGAAACGAGGTC
liaR-up	fwd, GCTGTCATCAAGCTGGTTCGG; rev, CCTATCACCTCAAATGGTTCGCTGCGATGCT TCGCCGATGACTTC
liaR-do	fwd, CGAGCGCCTACGAGGAATTTGTATCGACGCACACCGAAATCATCTC; rev, CTCTT CATCTGATCCGACACAGC
Oligonucleotides for quantitative real-time RT-PCR, primer extension, Northern hybridization, and sequencing	
liaR-RT	fwd, ATTGAAGTCATCGGCGAAGC; rev, AAAGCTCCCGGCAAATTTGC
rpsJ-RT	fwd, GAAACGGCAAAACGTTCTGG; rev, GTGTTGGGTTCACAATGTCG
rpsE-RT	fwd, GCGTCGTATTGACCCAAGC; rev, TACCAGTACCGAATCCTACG
yhcY-PE	GGTTTCCGCAATCGTTTTCAGCG
yhcY probe[b]	fwd, GAGTTGCTGAGTCTGACAAACC; rev, CTAATACGACTCACTATAGGGAGATCGTG AAGCTCCTGAGCGAGGC
liaF sequencing	fwd, GCTTTATCAGCAAGCGGTGACG; rev, CCGAACCAGCTTGATGACAGC
Oligonucleotides for cloning[c]	
Cloning and site-directed mutagenesis of the *liaI* promoter[d]	
0099 (fwd)	CCAT***GAATTC***CCGGTGCGAGATACGACTCC
0100 (rev)	CGAT***GGATCC***TCCTCCAAAAAAGACGGAGATCCC
0231 (fwd)	ACAT***GAATTC***GAGATACGACTCCGGTCTTATtTAtAtATCAATCTCTGATTCG
0232 (fwd)	ACAT***GAATTC***GAGATACGACTCCGGTCTTATATtAtAtTCAATCTCTGATTCG
0265 (fwd)	ACAT***GAATTC***GAGAaACaAaTCCGaTaTTATATAAAAATCAATCTCTGATTCG
0266 (fwd)	ACAT***GAATTC***GAGATACGACTCCGGTCTTcTATAcAAATCAATCTCTGATTCG
0267 (fwd)	ACAT***GAATTC***GAGAaACaAaTCCGGTCTTATATAAAAATCAATCTCTGATTCG
0268 (fwd)	ACAT***GAATTC***GAGATACGACTCCGaaaTTATATAAAAATCAATCTCTGATTCG
Promoter deletion analysis of the *liaG* promoter	
0204 (fwd)	CCATGAATTCTCCCTTCCGCACGTATCAATTCGC
0205 (rev)	AGCCGGATCCTTTGTCATTCCTGGTG
0222 (fwd)	CCATGAATTCGCAGGCCTAGGTTCATAAATGGC
0296 (rev)	AGCCGGATCCCATTCGGTTTCATCCTTCTCATTC
Promoter deletion analysis of the *yhcY* promoter	
0165 (rev)	CGAT***GGATCC***GTGTTGCTTTGATATCGTGCC
0166 (fwd)	CGAT***GAATTC***GACAGTGAAAAGCGACTTGCC
0168 (fwd)	CGAT***GAATTC***GCTTTTTCTTTTTCTCATCC
0169 (fwd)	CGAT***GAATTC***CTTTTTCTCATCCAAAAGTCTG
0259 (fwd)	CGAT***GAATTC***TCTCATCCAAAAGTCTGAAAG
0260 (fwd)	CGAT***GAATTC***AAGTCTGAAAGAAAATCATCCTACAAGTG
0170 (fwd)	CGAT***GAATTC***GAAAATCATCCTACAAGTG
0171 (fwd)	CGAT***GAATTC***CCTACAAGTGAAGCAATGAA
0172 (fwd)	CGAT***GAATTC***GAAATACAAAAAACTGGTATAATC
Complementation experiments with *liaF*	
0035 (fwd)	AGG***AAGCTT***AGAAAGGAGGCGGACACCAGG
0036 (rev)	TCC***GAATTC***TTTCTCATACGTACTTCACATCC

[a] 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 cassette, respectively.

[b] The underlined sequence represents the T7 promoter necessary for the construction of RNA probes by in vitro transcription.

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

[d] Nucleotides given in small bold letters represent mismatches.

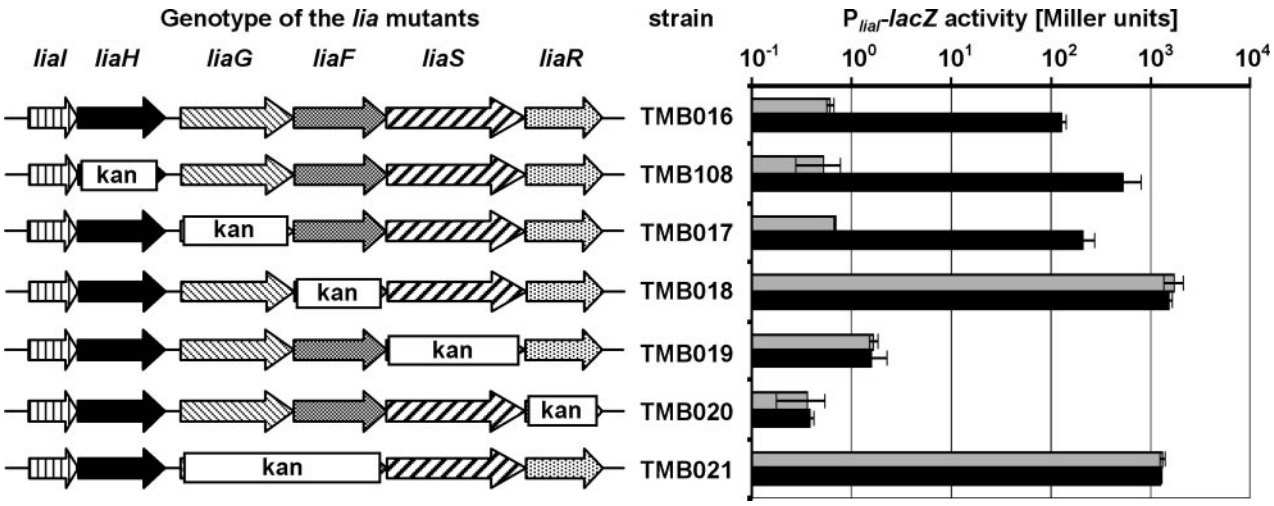

FIG. 1. P_{liaI} activity in response to deletions of *lia* genes. Cultures of strains TMB016 (wild type) and TMB017 to TMB020/TMB108 were grown in LB medium to mid-log phase (OD_{600}, ~0.4) and split. One half was induced by the addition of bacitracin (final concentration, 50 μg/ml; black bars), and the other half served as an uninduced control (gray bars). Cells were harvested 30 min postinduction and assayed as described previously (43). β-Galactosidase activity is expressed in Miller units (44). A log scale was applied for reasons of clarity. The genotype of the corresponding strains is shown on the left side (see Fig. 3A for labeling patterns of arrows). kan represents the area replaced by the kanamycin resistance cassette. The cassette itself has a size of approximately 1.5 kb in all mutants.

reaction was carried out in an iCycler (Bio-Rad), using the following program: initial incubation of 50.0°C for 30 min, then a 95°C denaturing step for 15 min, followed by 40 cycles (94°C for 15 s, 60°C for 30 s, and 72°C for 30 s). After a subsequent incubation step (55°C for 1 min), the setpoint temperature was increased in 80 cycles (10 s each) by 0.5°C/cycle, starting at 55°C. Expression of *rpsE* and *rpsJ*, encoding ribosomal proteins, was monitored as a constitutive reference. Expression of *liaR* was calculated as the fold change based on the C_T values for each gene, as described previously (60).

Primer extension mapping of the *yhcY* transcriptional start site. For mapping of the *yhcY* promoter, HB0920 cells were grown in LB and total RNA was isolated from uninduced and bacitracin-induced (final concentration, 50 μg/ml) mid-logarithmic cultures as described above. Primer extension reactions for *yhcY* were set up as follows: 30 μg of heat-denatured RNA was hybridized at 65°C to ~2 pmol of end-labeled primer yhcY-PE (Table 3) in buffer containing 60 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM dithiothreitol, and 40 U of RNasin (Promega) in a total volume of 30 μl. Following hybridization, 50 μl extension buffer (72 mM NaCl, 50 mM Tris-HCl [pH 7.9], 10 mM dithiothreitol, 20 mM $MgCl_2$), deoxynucleoside triphosphates (10 mM), and 2 μl SuperScript II reverse transcriptase (Invitrogen) were added to the mixture and incubation continued at 37°C for 30 min. The primer extension products were precipitated with ethanol, resuspended in sequence loading buffer, and loaded onto 6% polyacrylamide sequencing gels. A PCR cycle sequencing kit (Epicenter) was used to generate sequencing ladders corresponding to the *yhcY* promoter region.

Comparative genomics analyses. Multiple sequence alignments were performed using ClustalW, implemented in the BioEdit program package (29). Domain-based analysis of protein sequences was performed using the SMART database (55) at the website http://smart.embl-heidelberg.de/. The genomic context clustering analysis of the *lia* locus was performed using the ERGO database, which is available through Integrated Genomics, Inc. (http://www.integratedgenomics) and maintained by the GenoMIK Center, Göttingen. The initial identification of the putative LiaR-binding site was done by submitting promoter fragments of 200 nucleotides upstream of the ATG for all *liaI* homologs identified above to MEME at the website http://meme.sdsc.edu/meme/website/meme.html (2). The resulting weight matrix of the conserved sequence pattern was then used to screen the individual genomes harboring *liaRS* homologous genes for additional putative LiaR-binding sites with the help of the Virtual Footprint algorithm (46), implemented in the Prodoric database (45) at http://www.prodoric.de/vfp/. The graphical representation of the putative LiaR-binding site was generated using the Weblogo interface (13) at http://weblogo.berkeley.edu/.

RESULTS

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

To investigate their possible roles in LiaRS-mediated signal transduction, we investigated the effects of various insertion-deletions in the *lia* locus on P_{liaI} activity as measured from a P_{liaI}-*lacZ* reporter fusion integrated ectopically at the *amyE* locus (Fig. 1). The wild-type strain (TMB016) showed a strong response to the presence of bacitracin, resulting in a more-than-200-fold induction of P_{liaI} activity, while virtually no β-galactosidase activity could be detected in the *liaR*::*kan* strain (TMB020) under either inducing or noninducing conditions, consistent with the stringent LiaR dependence of P_{liaI} (43). The *liaS*::*kan* mutant TMB019, lacking the histidine kinase, no longer responded to bacitracin but did display a slightly increased basal expression level. Surprisingly, P_{liaI} was constitutively active in the *liaF*::*kan* mutant TMB018, even in the absence of the inducer. This activity was even 10-fold higher than that measured in the induced wild type.

Resistance cassettes inserted into the chromosome can exhibit polar effects on downstream genes. This can either be a positive polar effect, due to readthrough from their own strong promoters (9), or a negative effect due to termination of tran-

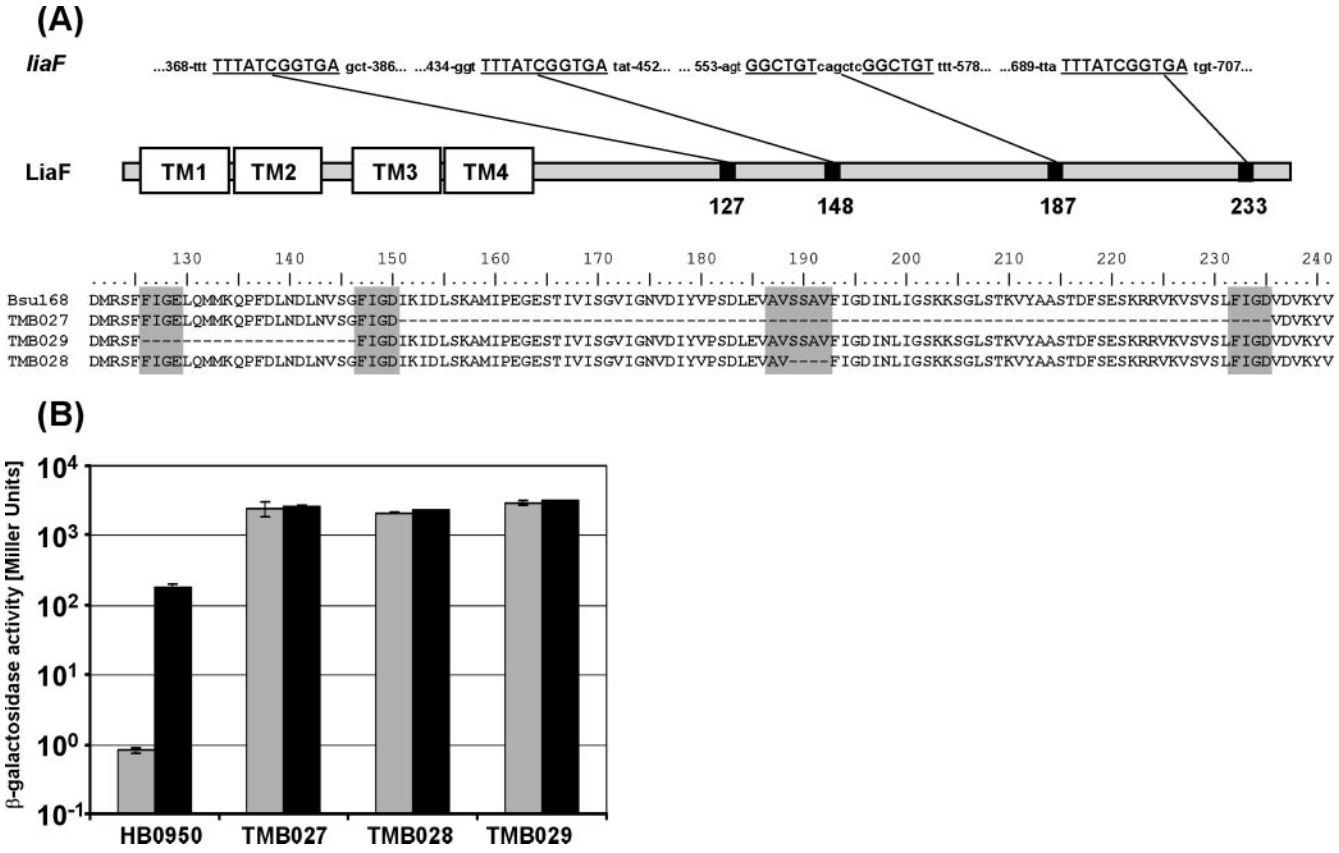

FIG. 2. (A) Features and sequence of LiaF derivatives in spontaneous chloramphenicol-resistant mutants, based on in-frame deletions. A sequence alignment of the C-terminal half of LiaF is shown in the lower part, including the reference sequence of wild-type strain W168 in the first line. The amino acids corresponding to the 11- or 6-nucleotide repeat (underlined in the upper part for the *liaF* gene) are shaded in gray. A graphical representation of LiaF is shown in the middle. The four putative transmembrane helices (TM1 to -4) are located in the N-terminal half of LiaF. The black boxes indicate the positions of the four repeats. (B) P_{liaI} activity in HB0950 (wild type) and derived mutants harboring an in-frame deletion in *liaF*. Experimental conditions and labeling of the bars are as described for Fig. 1.

scription within the resistance cassette. To investigate possible polar effects, we used quantitative real-time RT-PCR to measure *liaR* transcription (in the insertion-deletion strains TMB017 to TMB019) relative to the uninduced wild type. In all strains, there was an ~4-fold increase in *liaR* transcript. Since P_{liaI} transcription behaves identically in the wild type and a *liaG*::*kan* strain while it is constitutively active in the *liaF*::*kan* strain, there is no correlation between the weak positive polar effects from the kanamycin resistance cassettes and the observed effects on P_{liaI} activity. Instead, the results support a functional role for LiaF in inhibiting LiaRS-mediated signal transduction.

Spontaneous in-frame deletions in *liaF* lead to constitutive activity of P_{liaI}. The strong effect of a *liaF* deletion on P_{liaI} prompted us to perform a genetic screen using strain HB0950, which carries an ectopic pJPM122-based P_{liaI}-*cat-lacZ* fusion integrated at *att*SPβ (43). Direct plating of a mid-logarithmic culture of HB0950 on LB plates with chloramphenicol gave rise to numerous Cm^r colonies. We reasoned that any mutation in *liaF* that renders its gene product dysfunctional would lead to such a spontaneous chloramphenicol resistance in the genetic background of HB0950. Indeed, PCR and sequence analyses indicated that alterations in *liaF* were associated with many of the spontaneous Cm^r mutants. Three strains containing in-frame deletions in *liaF* were chosen for further analysis (Fig. 2A). These deletions likely arose from recombination between repeated nucleotide sequences (Fig. 2A, upper line)—as has been suggested for a comparable deletion in the *Enterococcus faecium* histidine kinase VanSB (17)—and result in the deletion of 84, 20, and 4 amino acids. All three in-frame deletion mutants showed a strong bacitracin-independent, constitutive upregulation of LacZ expression in β-galactosidase assays (Fig. 2B), a behavior similar to TMB018 (*liaF*::kan) (Fig. 1). The effects of these mutations could be complemented by reintroduction of a wild-type *liaF* allele, integrated as a single copy into the *thrC* locus and expressed from a xylose-inducible promoter (see Materials and Methods). In the resulting strains, TMB182 to -184 (Table 1), P_{liaI} is again switched off in the absence of bacitracin and inducible by addition of bacitracin (data not shown). Taken together, these data clearly demonstrate a negative role for the putative transmembrane protein LiaF in LiaRS-mediated signal transduction and identify regions in the hydrophilic C terminus that are crucial for LiaF function.

The genomic context of *liaFSR* is conserved in the *Firmicutes* group of gram-positive bacteria. A functional connection be-

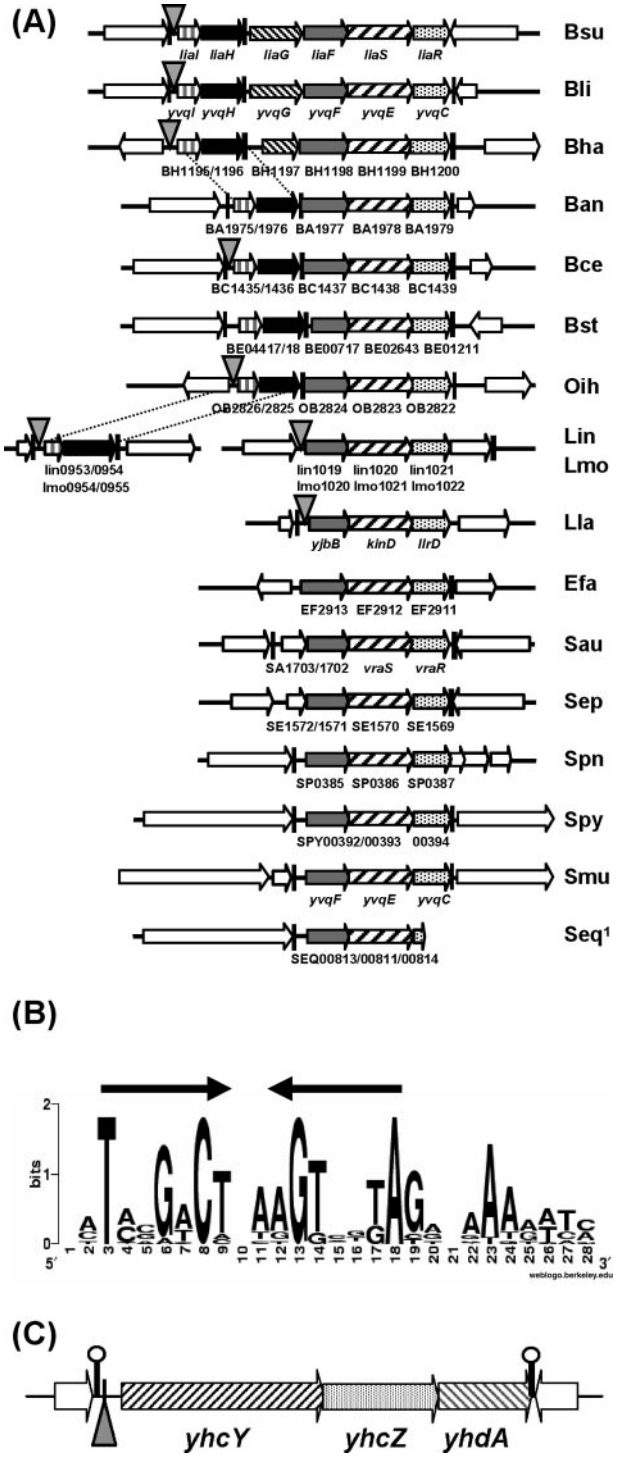

FIG. 3. Comparative genomics analysis of the *lia* locus and identification of the putative LiaR-binding site. (A) Conservation of the *lia* locus in other gram-positive bacteria with a low G+C content. The loci are drawn to scale, with the line of the *B. subtilis lia* locus corresponding to 7.5 kb. The genes of the *lia* locus are labeled differently for clarity. Hatched arrows represent genes coding for histidine kinases, and dotted arrows represent response regulators, homologous to LiaS and LiaR, respectively. The *liaF* homologs are shown in gray, *liaH* homologs are shown as black arrows, and *liaI* homologs are shown as white arrows with black vertical lines; genes flanking the *lia* locus are represented by white arrows. Putative terminators are marked as black vertical bars. The presence of putative LiaR-binding sites is indicated by the gray triangles (see Table 5, below, for details). Gene names are according to GenBank entries of the published genome sequences. Abbreviations of bacterial species: Bsu, *B. subtilis*; Bli, *B. licheniformis*; Bha, *B. halodurans*; Ban, *B. anthracis*; Bce, *Bacillus cereus*; Bst, *B. stearothermophilus*; Oih, *Oceanobacillus iheyensis*; Lin, *Listeria innocua*; Lmo, *L. monocytogenes*; Efa, *Enterococcus faecalis*; Sau, *Staphylococcus aureus*; Sep, *Staphylococcus epidermidis*; Spn, *Streptococcus pneumoniae*; Spy, *Streptococcus pyogenes*; Smu, *Streptococcus mutans*; Seq, *Streptococcus equi*; Lla, *Lactococcus lactis*. The genome of *S. equi* is not yet finished. The end of a contig lies inside the *liaR* homolog SEQ00814. (B) Graphical representation of the putative LiaR-binding site upstream of P_{liaI}. The sequence is derived from a comparative genomics analysis (using the MEME algorithm) (2), based on the promoter regions upstream of *liaI* and its homologs in bacteria harboring homologous genes to both *liaRS* and *liaI*. This graphical representation was generated through the Weblogo page (13) at http://weblogo.berkeley.edu/logo.cgi. (C) Genomic context of the putative LiaR-binding site upstream of the *yhcYZ-yhdA* operon. The region is drawn to scale, representing 3,000 bp. The exact position of the LiaR-binding site is indicated by the gray triangle. Labeling of arrows is as for panel A.

tween proteins is very often reflected by a clustering of their respective genes at the genomic level. For example, a functional connection has been demonstrated for some TCS involved in the cell envelope stress response in the *Bacillus/Clostridium* group of bacteria which cluster with operons encoding ABC transporters (33). In these detoxification units, the TCS senses the presence of a harmful compound and strongly induces the expression of the corresponding ABC transporter, which then facilitates removal of the compound (42, 47, 49).

A genomic context clustering analysis of LiaS orthologs revealed a topological conservation of all three genes, *liaFSR*, in gram-positive bacteria with a low G+C content. Without exception, a *liaF* homolog is always located directly upstream of the TCS in all species harboring *liaSR* homologs (Fig. 3A). This finding further substantiates the functional link between LiaF and LiaRS. In contrast, homologs of *liaIH* are only present in the *lia* locus of bacilli. Both genes form a separate operon in *Listeria* species and are apparently lacking in the more distantly related cocci. The *liaG* gene is only found in *Bacillus licheniformis* and *Bacillus halodurans*, the bacteria most closely related to *B. subtilis* for which complete genome sequences are available (Fig. 3A).

Identification of a conserved promoter element as a putative binding site for LiaR homologous response regulators from the genus *Bacillus*. Protein sequence comparisons (29) revealed an unusually high degree of sequence similarity in the C-terminal domain of all LiaR homologs (data not shown). This domain harbors a LuxR-like helix-turn-helix motif and defines the DNA-binding specificity of LiaR-like response regulators. We reasoned that this finding could be indicative of a conservation of the corresponding DNA-binding site within LiaR target promoters.

To test this hypothesis, we retrieved DNA sequences upstream of *liaI* homologous genes from the first nine bacterial species shown in Fig. 3A, assuming that these promoters are all subject to regulation by their corresponding LiaRS homologs. The sequences were submitted to the MEME website (2) to identify short stretches of high sequence similarity. One motif

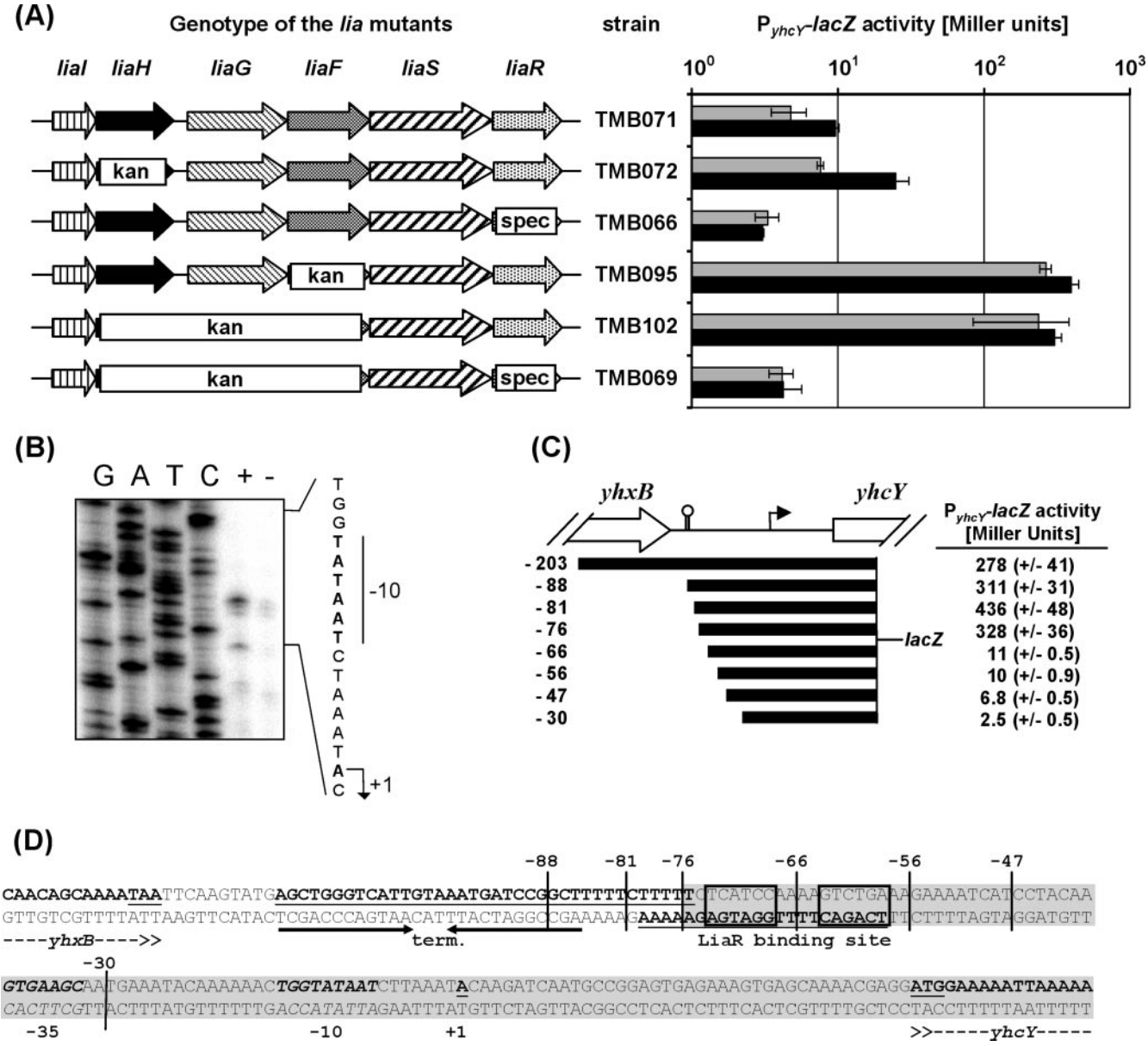

FIG. 4. Characterization of the *yhcY* promoter and its LiaR-dependent expression. (A) P_{yhcY}-*lacZ* activity in response to deletions of *lia* genes, determined by β-galactosidase assay with and without the addition of bacitracin, essentially as described above. See the legend to Fig. 1 for further details. A logarithmic representation of the resulting β-galactosidase activity was chosen for reasons of clarity. (B) Mapping of the transcriptional start site by primer extension analysis indicates that transcription initiates with an A, as shown in the sequence to the right. A primer extension reaction was performed with RNA prepared from strain HB0920 with and without the addition of bacitracin, using the primer yhcY-PE (see Table 3). The sequencing ladder to the left was generated using the same primer, following standard procedures. (C) Promoter deletion analysis of P_{yhcY}. A graphical representation of the intergenic region and outline of the fragments used for the promoter dissection are shown on the left. The end of *yhxB* and the beginning of *yhcY* are labeled. The putative *rho*-independent terminator downstream of *yhxB* is indicated by the black stem-loop symbol. The arrow indicates the transcriptional start site. The distance of the 5′ end of the cloned fragments from the transcriptional start is indicated. The activity of these promoters was measured in a *liaHGF*::kan mutant. The corresponding strains TMB096 to TMB104 were inoculated in LB medium to mid-log phase (OD_{600}, ~0.4), and the cells from 2 ml were harvested and used for a β-galactosidase assay as described above (without the addition of bacitracin). The resulting β-galactosidase activities, expressed in Miller units (44), are shown. (D) Sequence of the promoter region upstream of *yhcY*. The transcriptional start site is highlighted in bold, underlined, and the promoter is in bold italics. The putative *rho*-independent *yhxB* terminator is indicated by arrows and highlighted in bold and underlined, and coding regions are given in bold, with the start and stop codon underlined. The palindrome of the putative LiaR-binding site is underlined and boxed. The 5′ end of the promoter fragments used for the promoter deletion analysis are marked. The minimal LiaR-dependent promoter fragment is shaded in gray. Note that the labeling of the 5′ end of the fragments for the promoter deletion analysis in this figure (C and D) is given relative to the transcription start (+1) for reasons of clarity. Thereby, this nomenclature differs from the labeling of the fragments for cloning (Tables 1 and 2), which are normalized relative to the A of the start codon of *yhcY*.

TABLE 4. Mutagenesis of the putative LiaR-binding site upstream of P_{liaI}

Strain[a]	Sequence of putative LiaR-binding site[b]	β-Gal (MU)[c]	
		Without bacitracin	With bacitracin
Wild type	CGGTGCGAG**ATA**C**GAC**TCCG**GTC**T**TATA**T**AAAAA**TCAATCT	0.9	196
TMB113	CGGTGCGAG**AaA**C**aAa**TCCG**aTa**T**TATA**T**AAAAA**TCAATCT	0.8	0.6
TMB115	CGGTGCGAG**AaA**C**aAa**TCCG**GTC**T**TATA**T**AAAAA**TCAATCT	0.5	4.5
TMB133	CGGCGGGAG**ATA**C**GAC**TCCG**aaa**T**TATA**T**AAAAA**TCAATCT	0.6	0.8
TMB114	CGGTGCGAG**ATA**C**GAC**TCCG**GTC**T**Tc**T**TA**T**Ac**AAA**TCAATCT	0.2	0.6
TMB111	CGGTGCGAG**ATA**C**GAC**TCCG**GTC**T**TATt**T**AtAtA**TCAATCT	0.3	13
TMB112	CGGTGCGAG**ATA**C**GAC**TCCG**GTC**T**TATA**T**tAtAt**TCAATCT	0.7	17
	---7--- 4 ---7---		

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

[b] The palindrome is shown with underlining, and the 7-4-7 motif is indicated. Conserved nucleotides are given in bold letters. Minimal LiaR-responsive promoter fragments were mutated at the nucleotides indicated in lowercase italic letters during PCR amplification, cloned into pAC6 to generate mutated P_{liaI}-*lacZ* fusions, sequence verified, and subsequently transformed into W168 as described in the text.

[c] P_{liaI} activity of the resulting mutants was determined by β-galactosidase assay with and without addition of bacitracin (Bac; final concentration, 50 μg/ml) as described in the legend to Fig. 1. MU, Miller units. TMB016 (wild type) is given as a reference.

was present in all but two regions (*Bacillus anthracis* and *Bacillus stearothermophilus*) at a similar distance relative to the start codon. This motif includes an imperfect inverted repeat of 7 nucleotides, separated by 2 bp (Fig. 3B). The resulting weight matrix of this sequence pattern was subsequently submitted to the Virtual Footprint algorithm (46), implemented in the Prodoric database (45), in order to identify candidate LiaR target promoters in the *B. subtilis* genome. We retrieved three sequences within intergenic regions.

In addition to the P_{liaI} promoter region, two new potential LiaR-binding sites were identified. The first is associated with the *yhcYZ-yhdA* operon, a locus previously implicated as part of the LiaR regulon (42). The second putative LiaR box was located in a large noncoding region between the genes *yozJ* and *rapK*. Subsequent analyses with reporter fusions failed to reveal any transcriptional activity in either direction from this intergenic region (data not shown). Thus, this putative LiaR-binding site may be a remnant of a previously functional locus or a false positive generated by our search algorithm.

The *yhcYZ-yhdA* operon is expressed from a LiaR-dependent promoter. A regulatory link between the *yhcYZ-yhdA* operon and the Lia system had been suggested previously: bacitracin treatment leads to increased expression of this operon in a *liaH* mutant relative to the wild type (42). Additionally, LiaR-dependent expression of the *yhcYZ-yhdA* operon was described in a comprehensive microarray study on TCS in *B. subtilis*: overexpression of LiaR, in the absence of its cognate histidine kinase LiaS, resulted in an induction of this operon (38). However, these experiments do not discriminate between direct and indirect effects of LiaR on the *yhcYZ-yhdA* operon.

To determine if *yhcYZ-yhdA* is regulated by the LiaFRS system, we constructed strains carrying an ectopically integrated P_{yhcY}-*lacZ* fusion. Consistent with previous transcriptome analyses (42), P_{yhcY} was only weakly inducible by bacitracin in the wild type and was derepressed in the *liaH* mutant TMB072 (Fig. 4A). While the magnitude of induction was relatively weak compared to that observed with P_{liaI} (Fig. 1), induction was completely LiaR dependent and expression was constitutive in the *liaF* mutant (TMB095). Since the gene products of both *liaH* and *liaF* exhibited a negative effect on P_{yhcY} activity, we also introduced a *liaHGF*::*kan* insertion-deletion into TMB071 (resulting in strain TMB102). The mutant behaved like the *liaF* mutant, consistent with the idea that LiaR is already completely activated in a *liaF* mutant. As expected, both the *liaH* and *liaF* effects are completely LiaR dependent (Fig. 4A). The behavior of P_{yhcY} is comparable to P_{liaI}, but the activity as well as the range of inducibility is lower. This weaker activity may be due to a poorer match between the LiaR box preceding *yhcY* compared to that preceding *liaI* (see Table 5, below).

We mapped the 5′ end of the *yhcY* transcript by primer extension to an A 41 nucleotides upstream of the start codon, thereby also verifying the induction of transcription by bacitracin in a *liaH* mutant (Fig. 4B). The *yhcY* promoter has a well-conserved extended −10 region (TGgTATAAT) but a poorly conserved −35 region (GTGAAG) (Fig. 4D). Northern analysis verified that the *yhcYZ-yhdA* genes constitute an operon of 2.2 kb in size (data not shown).

To further characterize the LiaR-dependent activity of P_{yhcY} and the role of the putative LiaR-binding site, a promoter deletion analysis was performed. Promoter fragments of decreasing length were cloned into pAC6 and verified by DNA sequencing. Integration of the resulting plasmids generated strains TMB096 through TMB104 (the smallest to largest P_{yhcY} fragments, respectively; see Table 1 and Fig. 4C for details). The results demonstrated that a complete LiaR box adjacent to the promoter region is necessary and sufficient for LiaR-dependent promoter activity (Fig. 4C and D).

Site-directed mutagenesis of the putative LiaR-binding site in P_{liaI}. To experimentally test the importance of individual residues within the conserved LiaR-binding motif, we generated a series of LiaR box mutants by site-directed mutagenesis (Table 4). Exchanging the five most highly conserved nucleotides of the LiaR box by an A led to a complete loss of promoter activity in strain TMB113. The same effect was observed with mutations affecting only the promoter-proximal half-side (TMB114 and TMB133). Interestingly, a mutant with the three highly conserved residues in the 5′ side of the palindrome replaced by A (strain TMB115) retained a very low level of bacitracin-inducible activity. All three LiaR boxes of *B.*

subtilis harbor an A-stretch directly 3′ of the inverted repeat, with two residues also being conserved in the putative binding sites of LiaR homologs (Fig. 3B). Therefore, we constructed two additional mutants (TMB111 and -112), each harboring a different set of three A→T exchanges. These mutants each showed a significantly decreased, albeit less severely affected, P_{liaI} activity in the presence of bacitracin. These results support a functional role of this A-rich region, perhaps serving as a DNA-binding site (UP element) that interacts with the α-C-terminal domain of RNA polymerase (23).

DISCUSSION

A complex regulatory network, consisting of TCS and alternative σ-factors, orchestrates the cell envelope stress response in *B. subtilis* (42, 53). One such TCS, LiaRS, mediates the strong induction of its corresponding *liaIHGFSR* locus in the presence of antibiotics that interfere with the lipid II cycle, such as bacitracin, vancomycin, nisin, and ramoplanin (43). The LiaRS TCS also responds strongly to the presence of cationic antimicrobial peptides known to affect the cell envelope (53) and is weakly induced in response to secretion stress, alkaline shock, and the presence of detergents, organic solvents, ethanol, and some surfactants (32, 43, 52, 66). Here, we investigated the role of individual genes of the *lia* locus in the LiaRS-mediated cell envelope stress response. We found that LiaF and LiaH act as a strong and weak inhibitor of this signal transduction process, respectively (Fig. 1 and 2). Applying comparative genomics, we identified a putative LiaR-binding site that was verified by mutational analysis (Fig. 3 and Table 4). This LiaR box was also found in the promoter region of a second LiaR target locus, the *yhcYZ-yhdA* operon, the expression of which we studied in detail (Fig. 4).

LiaRS-mediated signal transduction is conserved in *Firmicutes* bacteria. Using comparative genomics, we recognized LiaF-LiaRS as a cell envelope stress response system conserved in the phylum *Firmicutes* (Fig. 3A). We extended our comparative genomics studies to identify putative LiaR-binding sites and corresponding target genes in other *Firmicutes* bacteria (Table 5). LiaR boxes were primarily identified in organisms of the order *Bacillales*, harboring both *liaFSR* and *liaIH* homologs. Overall, the number of putative LiaR-binding sites per genome was low (one to four). In *B. licheniformis*, the closest sequenced relative to *B. subtilis*, the two putative LiaR-binding sites are also located upstream of homologous loci, i.e., *yvqI* (*liaI*) and *yhcY*. For the two *Listeria* species, the homologs of *liaIH* (first gene lin0953/Lmo0954) and *liaFSR* (lin1019/Lmo1020) are organized in two independent transcriptional units, as noted above (Fig. 3A). Interestingly, a putative LiaR-binding site was identified in the promoter regions of both loci. A LiaR box was also identified upstream of the *liaF* homologs in *Enterococcus faecalis* and *Lactococcus lactis* (Table 5 and Fig. 3A). Remarkably, three of the candidate target loci encode ABC transporters (including a putative bacitracin efflux pump), supporting the role of LiaRS homologs in mediating a cell envelope stress response (Table 5).

The sequence alignment of putative LiaR boxes identified a core motif of 16 nucleotides, followed by an A-rich 3′ extension that could function as an UP element (23). The core motif consists of an inverted repeat of 7 nucleotides, with a spacing of 2 or 4 nucleotides. LiaR belongs to the NarL/FixJ family of response regulators (50). The recognition of an inverted repeat is well established for this family of transcriptional regulators (12, 14, 20) as well as for other response regulators, such as *Listeria monocytogenes* VirR and *Bradyrhizobium japonicum* RegR (21, 41).

The conservation of the LiaF-LiaRS signal transduction system and several of its target operons in the *Firmicutes* (with the noteworthy exception of the genus *Clostridium*) (Fig. 3A) suggests that this is a conserved cell envelope stress system. Preliminary transcriptome analyses revealed that the *lia* homologous *yvqIHGFEC* locus in *B. licheniformis* is also strongly induced by bacitracin (T. Wecke and T. Mascher, unpublished observation). Other LiaRS homologs, such as *Staphylococcus aureus* VraSR, *Listeria monocytogenes* Lmo1021/1022, and *Streptococcus pneumoniae* HK03/RR03, also respond to cell envelope stress elicited by cell wall antibiotics and membrane perturbations (25, 28, 40). In the case of *S. aureus*, the VraSR system controls a large regulon of about 50 genes (40). It seems reasonable to postulate that all *liaFSR* homologs depicted in Fig. 3A encode cell envelope stress-sensing three-component systems. Although *liaFSR*-like operons are present in the streptococci and staphylococci, we were unable to identify LiaR boxes upstream of these loci. We speculate that the corresponding LiaR-like proteins have a distinct DNA-binding selectivity, a hypothesis supported by the divergence of their DNA-binding domains (data not shown).

Taken together, the data seem to indicate the existence of two subgroups within LiaRS-like cell envelope stress-sensing TCS. The genera *Bacillus* and *Listeria* harbor the complete *lia* locus (organized as two independent, but LiaR-dependent, transcriptional units for the latter). In *B. subtilis*, one prominent effect of activation is a strong overexpression of LiaH and, due to the operon organization, presumably also of the small membrane protein LiaI. In general, these systems have recognizable LiaR-binding sites (exceptions are *B. anthracis* and *B. stearothermophilus*) and seem to control only a small number of target genes (Table 5). Members of the second group (VraSR-like TCS) lack a recognizable LiaR box and homologs of *liaIH* but seem to control a larger regulon.

LiaF is a strong inhibitor of LiaRS-mediated signal transduction. We identified LiaF as an essential part of the LiaRS signaling system that maintains the system in an inactive state (Fig. 1, 2, and 4A). It is presently unclear whether LiaF senses cell envelope stress directly or indirectly through LiaS. Most characterized proteins inhibiting TCS-mediated signal transduction—such as Sda and KipI in the sporulation phosphorelay of *B. subtilis* and FixT in FixLJ-mediated nitrogen fixation in *Sinorhizobium meliloti*—interfere with histidine kinase autophosphorylation, thereby suppressing activation of the cognate response regulators (8, 24, 64). Recently, a TCS inhibitor protein was described that shares many features with LiaF: deletion of *yycH*, encoding a transmembrane protein, results in uncoupled activity of YycFG (59), an essential TCS in *B. subtilis* and other gram-positive bacteria (11, 22). The three corresponding genes are cotranscribed and conserved by genomic context. It has been postulated that YycH affects the sensor domain of its corresponding histidine kinase, YycG (59). A similar role could be envisioned for LiaF.

TABLE 5. Conserved sequences of putative binding sites for LiaR homologous response regulators[a] and their corresponding target genes

Species and genomic position (start, end)	Strand	PWM score[b]	Sequence[c]	ORF[d] or accession no.	ATG distance[e]	Operon structure, homology, putative function of target gene, and/or remarks
Bacillus subtilis						
1008019, 1008040	−	19.13	**TC**a**GACT**ttt**G**atg**AG**AAA**AA**	*yhcY*	85	*yhcYZ-yhdA*; two-component system, azoreductase
2060155, 2060176	+	18.31	**T**tc**GTCT**g**AAGT**ga**TAG**tAA**AA**	*yozJ*	208	Monocistronic; unknown hypothetical
3398065, 3398086	−	19.81	**TA**c**GACT**ccg**GTC**t**TA**tAt**AA**	***liaI***	75	*liaIHGFSR*
Bacillus licheniformis						
1003453, 1003474	+	18.10	**TC**g**GTC**aa**AAGTC**t**TAG**AAA**A**g	*yhcY*	77	*yhcYZ-yhdA*; two-component system, azoreductase
3337261, 3337282	−	17.96	**TA**c**GAC**ctg**GTC**g**TA**cggc**AA**	***yvqI***	75	*yvqIHGFEC*; homologous to the *lia* locus
Bacillus halodurans						
1286962, 1286983	+	20.43	**TC**a**GACT**c**AAG**g**C**gg**AG**Ag**AA**	**BH1195**	74	BH1195 to -1200; homologous to the *lia* locus
Bacillus cereus						
259757, 259778	−	18.28	**TC**c**G**c**CT**ct**AGTC**tc**A**tAAc**AA**	BC0296	80	Monocistronic, glutamine-hydrolyzing GMP synthase
1396398, 1396419	+	20.22	**TC**g**GTCT**t**AAGTC**tg**AG**tt**AA**	**BC1435**	78	BC1435 to -1439; homologous to the *lia* locus
2332576, 2332597	−	18.32	**T**Ca**GACT**t**AAG**a**C**c**TAG**Ag**A**c	BC2389	119	BC2389/90; tellurite resistance protein/unknown hypothetical
2332582, 2332603	+	19.40	**TA**g**GTCT**t**AAGTC**tg**AG**gAA**AA**	BC2389	111	Duplicated LiaR box (overlapping palindromes on both strands)
3635790, 3635811	−	18.44	**TA**g**GACT**a**AAG**a**C**t**TAG**AAg**AA**	BC3667	88	BC3667/*bcrAB*; ABC permease/bacitracin resistance proteins
3635796, 3635817	+	19.06	**TA**a**GTCT**tt**AGTC**c**TAG**AAA**A**c	BC3667	96	Duplicated LiaR box (overlapping palindromes on both strands)
Oceanobacillus iheyensis						
536986, 537007	+	19.70	**T**Ca**GACT**g**AAGTC**g**TAG**gAt**AA**	OB0502	64	OB0502/0503; unknown hypothetical proteins
1113240, 1113261	−	18.46	**TA**t**GACT**c**AAGTC**a**TA**aAAt**AA**	OB1071	81	OB1071 to -1073; ABC transporter
2905575, 2905596	−	19.20	**TA**cac**CT**t**AAGTC**t**TAG**ttA**AA**	**OB2826**	76	OB2822 to -2826; homologous to the *lia* locus
Listeria innocua						
979178, 979199	+	18.21	**TA**g**GACT**c**AAGT**tg**TAG**gtg**AA**	**Lin0953**	103	Lin0953/0954; homologous to *liaIH*
1040744, 1040765	+	18.49	**T**Cc**GACT**a**AAGT**ag**TAG**AgAtt	**Lin1019**	121	Lin1019 to -1021; homologous to *liaFSR*
1060686, 1060707	−	18.34	**TA**t**G**c**CT**tt**AGTC**c**TA**tgAA**AA**	Lin1043	95	Monocistronic, similar to formylmethionine deformylase
Listeria monocytogenes						
987610, 987631	+	18.22	**TA**g**GACT**c**AAGT**tg**TAG**gtg**AA**	**lmo0954**	102	lmo0954/0955; homologous to *liaIH*
1049130, 1049151	+	18.95	**T**Cc**GACT**a**AAGT**ag**TAG**gct**A**t	**lmo1020**	120	lmo1020 to -1022; homologous to *liaFSR*
Enterococcus faecalis						
2791625, 2791646	−	17.99	**TAGG**g**CT**a**AAGTC**C**TA**tggt**AA**	**EF2913**	73	EF2911 to -2913; homologous to *liaFSR*
1392025, 1392046	−	17.30	**TC**g**GACT**tt**AGT**AGg**A**tAcA**A**t	EF1412	42	EF1412; putative transmembrane protein
				EF1413	273	EF1413/1414; macrolide resistance-like ABC transporter
Lactococcus lactis						
910806, 910827	+	20.15	**TA**a**G**T**CT**t**AAGTC**cg**AG**AAA**AA**	***yjbB***	27	Homologous to *liaFSR*
Consensus[f]			**TM***n***G***W***CT***n***AAGTC***n***TAG***AAA***AA**			

[a] Putative LiaR boxes were identified by submitting the position weight matrix derived from the initial motif represented in Fig. 3B to the Virtual Footprint webpage implemented in the Prodoric database and performing individual searches against the genome sequences of each organism harboring LiaRS-homologous TCS.
[b] PWM, position weight matrix.
[c] Nucleotides conserved in more than 80% of the sequences are in bold and italics. Residues with a weaker degree of conservation are given in bold letters. Nonconserved nucleotides are in lowercase letters. Nucleotides that contribute to the inverted repeat (i.e., have a complementary counterpart) are underlined, irrespective of the degree of conservation.
[d] The lia homologs (each representing the first of the genes indicated in the right-most column, i.e., homologs to either *liaI* or *liaF*) are shown in boldface.
[e] Distance from the LiaR box to the ATG start codon of the indicated gene, in nucleotides.
[f] M = A or C; W = T or A.

LiaH, a PspA homolog, acts a negative modulator of LiaRS-mediated signal transduction. We also presented evidence that LiaH acts as a weak—and presumably indirect—negative modulator for LiaR-dependent gene expression. The *liaIH* genes are expressed at a much higher level relative to the downstream genes *liaGFSR*, due to termination of transcription at a stem-loop structure downstream of *liaH* (43). The strong induction of *liaIH* is also apparent at the protein level:

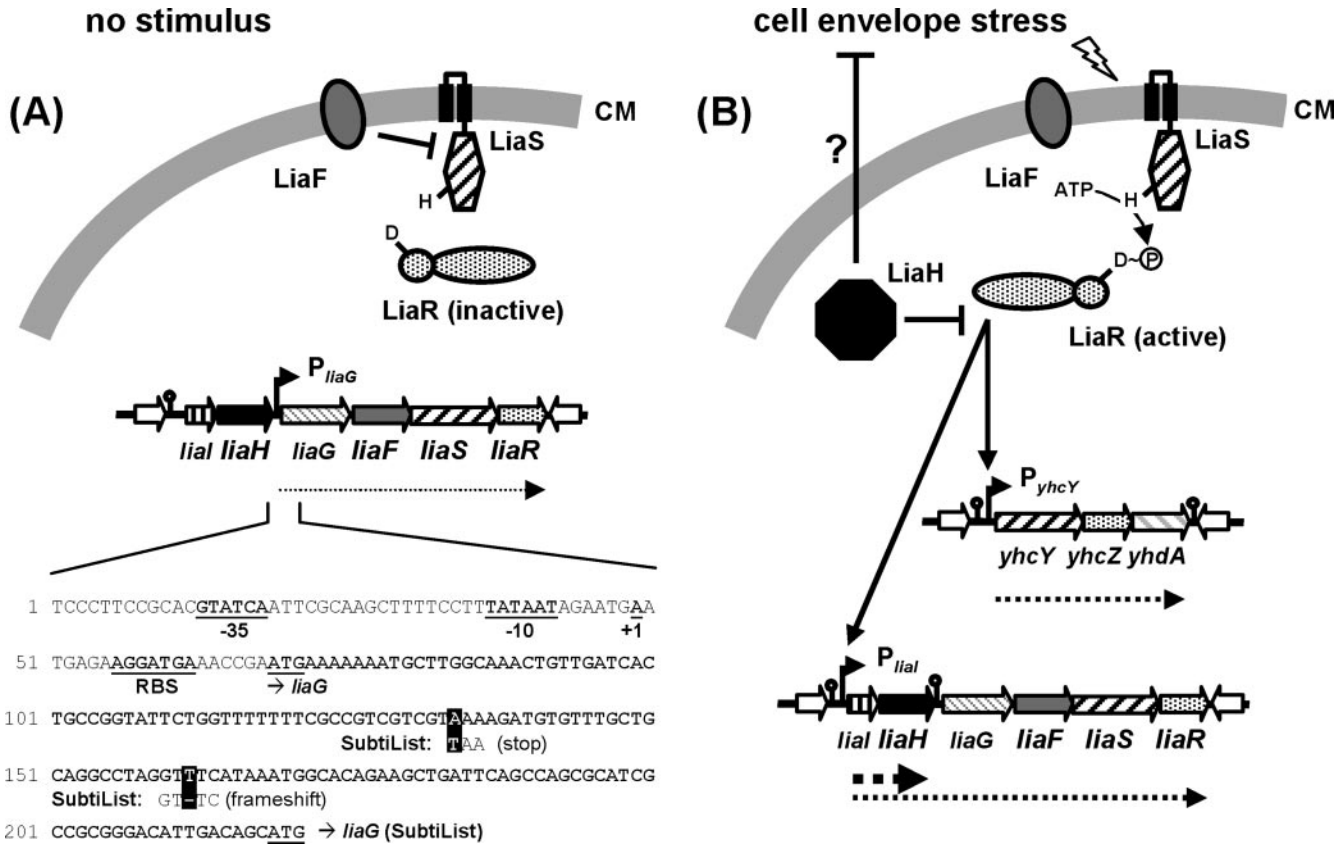

FIG. 5. Model of LiaRS-dependent gene expression in *B. subtilis*. Transcripts are indicated by dotted lines. Activation is indicated by solid arrows, and inhibitions are shown by T-shaped lines. (A) P_{liaG}-dependent expression of *liaGFSR* in the absence of cell envelope stress. LiaF inhibits the LiaRS TCS. The sequence of the corresponding promoter region is given below. Resequencing revealed two mistakes in the original genome sequence, which result in the generation of a stop codon and a frameshift within the 5′ end of *liaG*, respectively. Therefore, the corresponding LiaG protein is extended by another 50 amino acids at its N terminus. The added sequence harbors a signal peptide and a potential transmembrane helix, as identified using the SMART database (55). This indicates that LiaG is a putative membrane-anchored protein rather than of cytoplasmic localization, as was originally annotated. (B) LiaRS-dependent gene expression in the presence of cell envelope stress. LiaF repression is released, and activated LiaR binds to its target promoters, including its own operon (positive feedback loop). The strong overexpression of LiaH presumably counteracts cell envelope stress. Additionally, LiaH functions as an inhibitor of LiaR-dependent gene expression (negative feedback loop). See text for further details.

LiaH was described as a marker protein for bacitracin treatment in a proteomic study (3). Furthermore, LiaH is a highly abundant protein visible in one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell lysates from cultures of the *liaF* mutant, even in the absence of bacitracin (data not shown).

LiaH belongs to the family of phage shock proteins. So far, only two members of this protein family have been investigated in detail, PspAs of *Yersina enterocolitica* and *E. coli*. The latter is induced by filamentous phage infection (6), hence the name phage shock protein. It is also induced by various other stress conditions, such as heat shock, osmotic shock, exposure to organic solvents and proton ionophores, and long incubation under alkaline conditions (6, 37, 65). Furthermore, misincorporation of secretin pore proteins also induces the *psp* locus in both *Y. enterocolitica* and *E. coli*, as does blocking of the Sec pathway (15, 30, 35, 36). In comparison, LiaH expression is induced by cell envelope stress generated by lipid II-interacting antibiotics, such as bacitracin and ramoplanin, but also by cationic antimicrobial peptides, alkaline shock, exposure to organic solvents, detergents, ethanol, and secretion stress (32, 42, 52, 53, 66). While the mechanisms of their transcriptional regulation differ, the range of inducing conditions for *B. subtilis* LiaH and *E. coli* PspA is remarkably similar.

PspA exhibits a dual function that is linked to two different cellular locations (6, 36): in unstressed cells, it is a cytosolic protein that acts as a negative regulator by inhibiting the transcriptional activator PspF (1, 5, 19). Under conditions of cell envelope stress (leaky outer membrane), it is peripherally bound to the inner surface of the cytoplasmic membrane, contributing to the maintenance of the proton motive force and overall membrane integrity (35). This membrane anchoring is mediated by protein-protein interaction with two transmembrane proteins, PspB and PspC (1, 5, 19). Based on our findings, it is tempting to postulate a similar dual role for LiaH. It functions as a weak negative transcriptional regulator without having a DNA-binding domain (Fig. 1 and 4A). Its cotranscription with *liaI*, coding for a putative membrane protein, suggests that LiaI might serve as a membrane anchor for LiaH,

thereby facilitating a second (so-far-unknown) activity, linked to the inner surface of the cytoplasmic membrane.

LiaFRS-mediated gene regulation: a working model. Under normal growth conditions, the last four genes of the *lia* locus, *liaGFSR*, are expressed from a recently identified, weak constitutive promoter directly upstream of *liaG* (Fig. 5A and data not shown; note the corrected sequence for *liaG*) (see Materials and Methods). P_{liaG}-dependent expression and the inhibitory activity of LiaF ensure that the system is present but switched off (Fig. 5A). In the presence of cell envelope stress, LiaS is released from LiaF-dependent inhibition and phosphorylated LiaR binds upstream of P_{liaI} and P_{yhcY} (Fig. 5B). P_{liaI} induction gives rise to two transcripts, resulting most notably in overexpression of LiaH. Due to significant readthrough transcription, this induction defines a positive autoregulatory feedback loop (stress-induced expression of *liaGFSR*) that allows the system to rapidly respond to envelope stress. The importance of this positive feedback loop is underscored by the identification of candidate LiaR boxes in the promoter regions of those *liaFSR* homologs that do not receive readthrough transcription from upstream *liaIH* expression (Fig. 3A), i.e., in *Listeria* species.

The physiological role of LiaH and the other LiaR targets is not yet clear. Based on its homology to PspA, we speculate that LiaH might be involved in counteracting envelope stress, possibly by securing membrane integrity. Additionally, it plays a role in a negative feedback loop, thereby counteracting continued LiaR activity. This allows the system to establish a level of LiaH appropriate for the stress condition (Fig. 5B). Two possible mechanisms for this LiaH-dependent feedback loop can be envisioned, both based on the analogy to *E. coli* PspA: first, LiaH could regulate LiaR activity through direct protein-protein interaction as suggested above. Second, LiaH could help to restore envelope integrity, thereby removing the stress signal that activated LiaRS in the first place. In the absence of envelope stress, LiaF would then again be able to inhibit LiaRS, thereby efficiently switching the system off. It is more and more recognized that such combined positive and negative feedback loops in a regulatory pathway play an important role for adaptive responses of a bacterial population to its environment (i.e., competence and sporulation in *B. subtilis*), allowing a fast and differentiated response (57).

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