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The *yydFGHIJ* Operon of *Bacillus subtilis* Encodes a Peptide That Induces the LiaRS Two-Component System▽

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The *Bacillus subtilis* LiaRS two-component system (TCS) responds to perturbations of the cell envelope induced by *l*ipid II-*i*nteracting *a*ntibiotics, such as vancomycin, ramoplanin, nisin, and bacitracin. Here, we characterize Tn*7*-generated mutations that induce the *liaRS* TCS. In addition to insertions in *liaF*, a known negative regulator of the LiaRS TCS, we identified two disruptions in the last two genes of the *yydFGHIJ* operon. This operon is predicted to encode a 49-amino-acid peptide (YydF), a modification enzyme (YydG), a membrane-embedded protease (YydH), and an ATP-binding cassette (ABC) transporter (YydIJ). Genome sequence comparisons suggest that the *yydFGHIJ* operon may have been acquired by horizontal transfer. Inactivation of the YydIJ transporter resulted in increased expression from the LiaR-dependent P_{liaI} promoter only in the presence of the *yydFGH* genes. Cells harboring the complete *yydFGHIJ* operon induced LiaR activity in cocultured cells lacking either this transporter or the complete operon. These results suggest that this operon is involved in the synthesis and export of a modified peptide (YydF*) that elicits cell envelope stress sensed by the LiaRS TCS.

The *Bacillus subtilis* cell envelope consists of a thick peptidoglycan cell wall and the cell membrane. Conditions interfering with cell envelope function activate specific stress responses coordinated by extracytoplasmic function (ECF) σ factors and various two-component regulatory systems (TCS). The activity of ECF σ factors is often inhibited by a transmembrane anti-σ which is inactivated in response to extracytoplasmic stress (17). Similarly, TCS involve a membrane-located sensor kinase protein and a cytoplasmic response regulator (27). Thus, both systems couple changes in gene expression to conditions that affect the integrity or function of the cell envelope.

The soil is a competitive environment, and many soil microorganisms produce antibiotics to inhibit the growth of neighboring cells. Often, these antibiotics target the cell wall or cell membrane and, in response, bacteria have evolved mechanisms to monitor and counteract these attacks. Previously, we demonstrated that the σ^W regulon plays a major role in defense against antimicrobial peptides produced by *B. subtilis* and other *Bacillus* spp. (4). The LiaRS TCS is also strongly induced by cell-wall active antibiotics such as vancomycin, ramoplanin, bacitracin, and nisin (6, 28, 29) as well as by the human antimicrobial peptide LL37 (31). LiaRS is weakly induced by the antibiotics fosfomycin and tunicamycin (29), by detergents, ethanol, phenol and organic solvents (29), and under conditions of alkaline shock (37) or secretion stress (19). Weak induction is also observed during normal growth conditions in rich medium at the onset of stationary phase (21).

The LiaRS TCS activates expression of the *liaIHGFSR* operon and at least one other operon, *yhcYZ-yhdA*, which encodes a second TCS. Induction of the P_{liaI} promoter results in a high level of expression of the LiaI and LiaH proteins. LiaI is a small (126-amino-acid [aa]) hydrophobic protein, predicted to be localized to the cell membrane. LiaH belongs to the PspA family of phage shock proteins and has been shown to act as a negative regulator of the *yhcYZ-yhdA* operon (20). While the expression of the *liaIHGFSR* operon in response to cell wall-active compounds is well documented, the role of LiaIH has remained elusive: deletion of this operon does not result in increased sensitivity to any of the compounds tested. In *Escherichia coli* and *Yersinia entercolitica* PspA binds to PspF, a transcriptional activator, under noninducing conditions and thereby prevents activation of the *psp* genes (8–10). Under inducing conditions (e.g., dissipation of the proton motive force), PspA interacts with the cytoplasmic membrane proteins PspB and PspC, freeing PspF to activate transcription of the *psp* genes. Under these conditions PspA is anchored to the inner surface of the cytoplasmic membrane, where it is proposed to contribute to maintenance of the proton motive force and membrane integrity (7). Homologs of the PspB, PspC, and PspF proteins have not been identified in *B. subtilis.* However, by analogy with the Psp system, Jordan and coworkers have proposed that LiaH may have a similar dual function and under stress conditions might act together with the membrane protein LiaI to maintain cell envelope integrity (20).

Homologs of the LiaRS proteins are found in many *Bacillus* species as well as in other firmicutes, such as *Listeria monocytogenes* (Lmo1021/Lmo1022) and various *Staphylococcus* species, including *S. aureus* (VraRS) (26). The VraRS system from *S. aureus* is also induced by cell wall-active antibiotics. This TCS is known to control genes involved in peptidoglycan biosynthesis, and deletion results in sensitivity toward β-lactam and glycopeptide antibiotics (25).

In this study, transposon mutagenesis was employed to search for genes which affect activity of the LiaRS TCS. In addition to *liaF*, a known negative regulator of the LiaRS TCS (20), we isolated several independent insertions in the *yydIJ*

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TABLE 1. Strains used in this study

B. subtilis strain	Genotype, description, and/or sequence	Reference or source
CU0165	W168 *trpC2 att* SPβ	Laboratory strain
ZB703A	W168 SPβ 2Δ2::Tn*917*::pSK10Δ6	40
HB6265	CU1065 *yydFGHIJ*::*spec*	LFH-PCR → CU1065
HB6266	CU1065 *yydHIJ*::*spec*	LFH-PCR → CU1065
HB6267	CU1065 *yydIJ*::*spec*	LFH-PCR → CU1065
HB0950	CU1065 SPβ::$P_{liaI\text{-}74}$-*cat-lacZ*	29
HB6296	CU1065 *yydIJ*::*spec* SPβ::$P_{liaI\text{-}74}$-*cat-lacZ*	Transduction, HB0950 phage → HB6267
HB6297	CU1065 *yydHIJ*::*spec* SPβ::$P_{liaI\text{-}74}$-*cat-lacZ*	Transduction, HB0950 phage → HB6266
HB6298	CU1065 *yydFGHIJ*::*spec* SPβ::$P_{liaI\text{-}74}$-*cat-lacZ*	Transduction, HB0950 phage → HB6265
HB6301	CU1065 SPβ	Transduction, ZB703A phage → CU1065
HB6302	CU1065 *yydIJ*::*spec* SPβ	Transduction, ZB703A phage → HB6267
HB6303	CU1065 *yydHIJ*::*spec* SPβ	Transduction, ZB703A phage → HB6266
HB6304	CU1065 *yydFGHIJ*::*spec* SPβ	Transduction, ZB703A phage → HB6265
HB6274	CU1065 Δ*yydF*	pBGB6 → CU1065
HB6275	CU1065 Δ*yydG*	pBGB7 → CU1065
HB6305	CU1065 Δ*yydF* SPβ	Transduction, ZB703A phage → HB6274
HB6306	CU1065 Δ*yydG* SPβ	Transduction, ZB703A phage → HB6275
HB6307	CU1065 P_{xylA}-*yydFGHIJ*	pBGB8 → CU1065
HB6192	CU1065 *amyE*::P_{sigW}-*lacZ* (Cm)	4
HB6299	CU1065 *amyE*::P_{sigX}-*lacZ* (Cm)	Lab collection
HB6300	CU1065 *amyE*::P_{sigM}-*lacZ* (Cm)	Lab collection
HB6314	CU1065 *amyE*::P_{sigW}-*lacZ* (Cm) *yydIJ*::*spec*	HB6192 Chr DNA → HB6267
HB6315	CU1065 *amyE*::P_{sigX}-*lacZ*(Cm) *yydIJ*::*spec*	HB6299 Chr DNA → HB6267
HB6316	CU1065 *amyE*::P_{sigM}-*lacZ*(Cm) *yydIJ*::*spec*	HB6300 Chr DNA → HB6267
HB6317	CU1065 *amyE*::P_{sigW}-*lacZ* (Cm) *yydFGHIJ*::*spec*	HB6192 Chr DNA → HB6265
HB6318	CU1065 *amyE*::P_{sigX}-*lacZ*(Cm) *yydFGHIJ*::*spec*	HB6299 Chr DNA → HB6265
HB6319	CU1065 *amyE*::P_{sigM}-*lacZ*(Cm) *yydFGHIJ*::*spec*	HB6300 Chr DNA → HB6265
HB6276	CU1065 *amyE*::P_{yydF}-*lacZ* (Cm)	pBGB9 (ScaI) → CU1065
HB6308	CU1065 *amyE*::P_{yydH}-*lacZ* (Cm)	pBGB10 (ScaI) → CU1065
HB6309	CU1065 *amyE*::P_{yydI}-*lacZ* (Cm)	pBGB11 (ScaI) → CU1065
HB6278	CU1065 *amyE*::P_{yydF}-*lacZ* (Cm) *rok*::*kan*	LFH-PCR → HB6276
HB6310	CU1065 *amyE*::P_{yydH}-*lacZ* (Cm) *rok*::*kan*	LFH-PCR → HB6308
HB6311	CU1065 *amyE*::P_{yydI}-*lacZ* (Cm) *rok*::*kan*	LFH-PCR → HB6309
HB6320	CU1065 *amyE*::P_{yydF}-*lacZ* (Cm) *abrB*::*tet*	SWV119 Chr DNA → HB6276
HB6312	CU1065 *amyE*::P_{yydH}-*lacZ* (Cm) *abrB*::*tet*	SWV119 Chr DNA → HB6308
HB6313	CU1065 *amyE*::P_{yydI}-*lacZ* (Cm) *abrB*::*tet*	SWV119 Chr DNA → HB6309
HB6277	CU1065 *rok*::*kan*	LFH-PCR → CU1065
SWV119	*abrB*::*tet*	Mark Strauch, Univ. Maryland Dental School, Baltimore

genes, encoding an ABC transporter. Further genetic analyses suggest that the YydIJ ABC transporter exports a modified YydF peptide (YydF*) and that defects in peptide export lead to cell envelope stress that is sensed by the LiaRS TCS.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains and primers used in this study are listed in Tables 1 and 2. Bacterial cultures were grown in liquid or solid Luria-Bertani (LB) (33), Difco sporulation medium (DSM) (15), or modified competence (MC) medium (24). Solid media contained 1.5% Bacto agar and, where indicated, 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Ampicillin (Amp) at a concentration of 100 μg/ml was used for selection of *E. coli* strains. Antibiotics for selection of various *B. subtilis* strains were used at the following concentrations: spectinomycin (Spec), 100 μg/ml; tetracycline (Tet), 20 μg/ml; chloramphenicol (Cm), 10 μg/ml; kanamycin (Kan), 15 μg/ml; neomycin (Neo), 8 μg/ml. For macrolide-lincomycin-streptogramin B (MLS) resistance, both lincomycin (25 μg/ml) and erythromycin (1 μg/ml) were added. Transformation of *B. subtilis* strains was carried out as previously described (15).

Deletion of genes was performed by long flanking homology PCR (LFH-PCR) (28). Primers used to amplify the up and down fragments are listed in Table 2, and the primers and plasmids used for amplification of the antibiotic resistance cassettes can be found at http://www.micro.cornell.edu/cals/micro/research/labs/helmann-lab/supplements.cfm. Amplification of the fragments, as well as the joining reactions, was performed with the Expand Long Template PCR system (Roche). Five μl of the LFH-PCR product was introduced into the desired strain by transformation, and integration and deletion of the gene were confirmed by PCR.

In-frame deletions of *yydF* and *yydG* were created using the pMAD plasmid (3). Regions upstream and downstream of the gene to be deleted were amplified and joined by overlapping PCR using the Expand Long Template PCR system (Roche). The primers included restriction enzyme sites that allowed cloning of the joined fragment into pMAD, generating pBGB6 (pMAD-Δ*yydF*) and pBGB7 (pMAD-Δ*yydG*). Integration of this plasmid and generation of the clean deletion followed the published procedure (3).

Placement of the *yydFGHIJ* operon under P_{xylA} control was performed using the pHTXyl plasmid (a pUC18-based vector containing the P_{xylA} promoter and an erythromycin resistance gene [T. Msadek, unpublished]). A region including the predicted RBS site upstream of *yydF*, but lacking the promoter, and extending into the *yydG* was amplified by PCR using primers listed in Table 2 and the Expand Long Template PCR system (Roche). The resulting fragment was cloned into pHTXyl, placing the *yydF* gene downstream of the P_{xylA} promoter (pBGB8). This plasmid was then transformed into CU1065, and a single integration event was selected for by MLS^r and confirmed by PCR. A 2% xylose solution was used to induce expression of these genes.

The $P_{liaI\text{-}74}$-*cat-lacZ* fusion (29) is harbored on a SPβ prophage, and introduction of this reporter into the desired strains was performed by transduction into the required CU1065-derived strains. CU1065 lacks the SPβ prophage but still contains the phage attachment site.

Quantification of β-galactosidase activity from cells grown on solid media was performed by spotting 5 μl of cells grown to mid-log phase on the desired solid medium poured into the wells of a Linbro 24-well tissue culture plate. After overnight incubation the cells were washed off the plate with Z-buffer, diluted to

TABLE 2. Primers and probes used in this study

Primer or probe	Description and sequence[a]
Primers for arbitrary PCR to map Tn*7* insertions	
2509	BsHarb1, GGCCACGCGTCGACTAGTCA(N)(N)(N)(N)(N)(N)(N)(N)(N)GATAT
2501	Tn7LHarb3, CCAGATAAGTGAAATCTAGTTCC
2508	BsHarb3, GGCCACGCGTCGACTAGTCA
2502	Tn7LHarb4, CGTATTAGCTTACGACGCTACACCC
Primers for LFH-PCR	
2701	yydF-up-fwd, CAACACGTGCTGGAATGCCT
2702	yydF-up-rev (spec), CGTTACGTTATTAGCGAGCCAGTCTTCATATTATCCCTCCTCC
2703	yydH-up-fwd, CACCAACTTCAACAACCAGG
2704	yydH-up-rev (spec), CGTTACGTTATTAGCGAGCCAGTCATAAATACGTTGTTTTGCAC
2757	yydI-up-fwd, AGTGCTTGTGCAAAACAACG
2758	yydI-up-rev (spec), CGTTACGTTATTAGCGAGCCAGTCCGCTATATTCATATACATACTCC
2705	yydJ-do-fwd (spec), CAATAAACCCTTGCCCTCGCTACGCTAGATGGATCAAAATGGG
2706	yydJ-do-rev, TCTTTCAGGTCAGAGGAAGC
3125	rok-up-for, GGACAGCTCCGTCACTTC
3126	rok-up-rev (kan), CCTATCACCTCAAATGGTTCGCTGCTAACCGCAAGCGCAAAGC
3127	rok-do-for (kan), CGAGCGCCTACGAGGAATTTGTATCGTCGAATCTGCAGAATCAGCAAACG
3128	rok-do-rev, CACTGCTTCAGGCAAAACAGC
Primers for the xylose-inducible *yydFGHIJ* operon	
2872	yydF-fwd-BamHI, CGCGGATCCGGTTTATATTAGAAAAGGAGG
2873	yydG-rev-EcoRI, AAAGAATTCATATCATGAAAGTACTCC
Primers for in-frame deletions with pMAD	
2896	yydF-up-for (BamHI), CGCGGATCCCAGATCACTGACAAAATGCTCG
2895	yydF-up-rev, CTCTCCTTTGTACCCCTCTAAATTATCCCTCCTCCTTTTCTAATATAAACC
2894	yydF-do-for, GGTTTATATTAGAAAAGGAGGAGGGATAATTTAGAGGGGTACAAAGGAGAG
2897	yydF-do-rev (NcoI), CATGCCATGGGCTTAATTCTAGTTTTAGCAGCGC
2898	yydG-up-for (BamHI), CGCGGATCCCAGCTTTTCAGTACAGGTTGG
2900	yydG-up-rev, GAAGTAGTTTATTTTTTCTGCAGAGTTGGTTGTTGAAGTTGGTGAACTAG
2899	yydG-do-for, CTAGTTCACCAACTTCAACAACCAACTCTGCAGAAAAAATAAACTACTTC
2901	yydG-do-rev (EcoRI), CCGGAATTCGACGAGAAAAGCATAAATGCC
Probes for Northern analysis	
2906	yydF-north-for, GGATAATATGAAAAAGGAAATCACTAAC
2907	yydF-north-rev, TTAATGACCACTTCCAAGAATCC
Primers for promoter-*lacZ* fusions	
3066	PyydF-for (EcoRI), CCGGAATTCGCTTAAAACAGCTTTTCAGTACAGG
3067	PyydF-rev (BamHI), CGCGGATCCCACAGTCTCATTGTTAGTGATTTCC
3261	PyydH-for (EcoRI), CCGGAATTCGCAGGAAATACCCTGATATCG
3262	PyydH-rev (BamHI), CGCGGATCCGCACAAGCACTTCATATTTTCC
3263	PyydI-for (EcoRI), GCAGTGAATTCAGTTGTACATTGG
3264	PyydI-rev (BamHI), CGCGGATCCCGGTGTCCTGAAGTAACG
Primers for 5′-RACE	
3531	yydF-GPS2, GGTCACAGGATGCATTACACC
3526	yydF-GPS1, GCAAATTCAGTTACTAACTCTC
3530	yydH-GPS2, GAACATGAGAGCCGTAAAGG
3527	yydH-GPS1, GTCCCAGTTCATGAAGCAC
3529	yydI-GPS2, CCTTAGAAACATCATTAGGTATATTCG
3528	yydI-GPS1, TTGATCAGAACTTTTCCATC

[a] Sequences complementary to the antibiotic resistance cassettes for LFH-PCR and sequences representing restriction enzyme sites are underlined.

an optical density at 600 nm (OD_{600}) of about 0.3, and lysed with 100 μg/ml of lysozyme at 37°C for 30 min, before determination of the β-galactosidase activity by standard methods (30).

P_{yydF}-, P_{yydH}-, and P_{yydI}-*lacZ* fusions were created by amplification of the promoter regions (see Table 2 for primers) and cloning into the pDG1661 vector (13) containing a promoterless *lacZ* gene (creating plasmids pBGB9, pBGB10, and pBGB11, respectively). After sequencing, the resulting plasmids were digested with ScaI and introduced by transformation into CU1065. Integration into the *amyE* locus was selected by Cm^r and confirmed by PCR. Expression from these promoter fusions was investigated on solid media containing X-Gal, or samples were removed throughout growth and the β-galactosidase activity was determined as previously described (30) with the following modifications: the cells were resuspended in Z-buffer and lysed with 100 μg/ml of lysozyme at 37°C for 30 min.

Tn*7* mutagenesis. Chromosomal DNA was isolated from *B. subtilis* and subjected to in vitro Tn*7* mutagenesis using a modified Tn*7* transposon carrying a Spec^r gene and an outward-facing xylose-inducible P_{xylA} promoter (C. Bordi, A Hachmann, and J. D. Helmann, unpublished data). This DNA was transformed into *B. subtilis*, and the resulting transposants were grown in the presence of Spec (to select for transformation of the Tn*7*-mutagenized DNA) with and without added xylose (which results in expression of genes downstream of the transposon). Chromosomal DNA was prepared from these cultures.

This amplified library of chromosomal DNA was introduced by transformation into HB0950, a *B. subtilis* CU1065 strain carrying the SPβ prophage harboring a P_{lia74}-*cat-lacZ* fusion known to have full bacitracin-inducible activity (29). We selected for Neo^r (confirming the presence of the P_{liaI}-*cat-lacZ* fusion) and Spec^r (indicating the presence of Tn*7*) colonies based on increased Cm^r and a blue color in the presence of X-Gal (indicating increased expression from the P_{liaI}-*cat-lacZ* fusion). Colonies were selected on LB plus Spec plus Neo plates containing 1.5, 2, 2.5, or 3 μg/ml chloramphenicol and 40 μg/ml X-Gal. To confirm that the observed phenotypes were due to the presence of the transposon, chromosomal DNA was isolated from selected colonies using a GFX genomic blood DNA purification kit from Amersham (following the manufacturer's protocol for DNA purification from gram-positive bacteria) and retransformed into HB0950. Transformants were plated on LB plus Spec plus X-Gal and LB plus Cm plus X-Gal plates. If all colonies on the LB plus Spec plus X-Gal plates were blue and the numbers of transformants on the Cm and Spec plates were comparable, we considered the phenotype to be linked to the transposon disruption. From about 54,400 transformants, 26 blue Cm^r colonies were isolated after 2 days of incubation at 37°C. Only 14 of the 26 upregulated phenotypes were found to be linked to the transposon disruption (see Table 3, below).

Mapping transposon insertions. The position of the Tn*7* insertion in the chromosome was determined by arbitrary PCR. A first round of PCR was performed using the arbitrary primer (2509) and a specific primer (2501) complementary to Tn*7* (Table 2). Primer 2509 consists of a 5-bp ATGCA sequence at the 3′ end followed by a random sequence of 10 nucleotides and a 5′ tail complementary to primer 2508. In order to amplify the PCR product containing the Tn*7*-chromosome junction, a second round of PCR using the 2508 (complementary to the 5′ tail of 2509) and 2502 (nested primer complementary to Tn*7*) primers with a 1:5 dilution of the product from the first-round PCR as the template was performed, and the product of this second PCR was sequenced using the 2502 primer by the Biotechnology Resource Center at Cornell University. All above PCRs were performed using 1.1× Thermo-start PCR Master Mix (ABgene) and purified with the QIAquick PCR purification kit (Qiagen).

Cocultivation experiments. Strains to be mixed were grown to the same optical density (corresponding to mid-logarithmic growth) in liquid LB medium and mixed in equal volumes, and 5 μl of the mixture was spotted on solid LB, DSM, MC, or MM containing 80 μg/ml of X-Gal. These plates were incubated at 37°C overnight and photographed.

5′-RACE PCR and Northern analysis. Total RNA was isolated from cells in mid-logarithmic- and stationary-phase growth using the RNeasy mini kit from Qiagen following the manufacturer's protocol for isolation of total RNA from bacteria with the following modification: the cells were lysed with 5 mg/ml of lysozyme for 10 min at 37°C. For 5′ rapid amplification of cDNA ends (RACE) PCR, the RNA was treated with DNase using Turbo DNA-*free* from Ambion followed by precipitation with 0.3 M sodium acetate and 70% ethanol. cDNA was synthesized using the Taqman reverse transcription reagents (Applied Biosystems) with the relevant GPS1 primers and either 2 μg of RNA isolated from log phase or 1.25 μg of RNA isolated from stationary phase. RNA was removed by treatment with 15 μl 1 M NaOH for 10 min at 70°C. This was neutralized by addition of 15 μl of 1 M HCl, and the cDNA was purified using QIAquick spin columns (Qiagen). This cDNA was dCTP tailed with terminal transferase (New England Biolabs) at 37°C for 30 min. PCR amplification of this tailed cDNA was performed with Thermo-start PCR Master Mix (ABgene) using the AAP primer complementary to the dCTP tail (3314) and the relevant GPS2 primer. A second PCR was performed to amplify the desired bands, which were then purified using the QIAquick spin columns (Qiagen) and sequenced by the Biotechnology Resource Center at Cornell University using the relevant GSP2 primer.

For Northern analysis, 8 μg and 10 μg of RNA isolated from log phase and stationary phase, respectively, were run on a 1% formaldehyde denaturing gel using NorthernMax denaturing gel buffer and running buffer from Ambion. The RNA was transferred to Zeta-Probe blotting membrane (Bio-Rad) using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and prehybridized at 42°C for 1 h. The *yydF*, *yydG*, *yydH*, and *yydI* probes were amplified with the relevant primers (Table 2) using Thermo-start PCR Master Mix (ABgene), purified using the QIAquick spin columns (Qiagen), and labeled with the DECAprime II kit (Ambion) using [α-^{32}P]dATP. Hybridization of the probe was performed with ULTRAhyb (Ambion) overnight at 42°C, and the membrane was washed twice with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7; Ambion) low-stringency wash for 5 min at room temperature and twice with 0.1× SSPE high-stringency wash for 15 min at 42°C, before detection of the signal using a PhosphorImager (GE Healthcare).

TABLE 3. Tn*7* disruptions resulting in increased expression from the P_{liaI}-*cat-lacZ* promoter fusion

Strain name(s)	Gene disrupted	Position in gene (size of gene) in bp
YPL3, YPL9, YPL14, YPL15	*yydI*	2 (267)
YPL6, YPL13	*yydJ*	257 (720)
YPL1, YPL25	*mutS*	398 (2,574)
BBL21	*mutS*	961 (2,574)
BBL5-3	*mutS*	1419 (2,574)
YPL16	*mutL*	467 (1,881)
YPL26, YPL18	*liaG*	226 (720)
BBL6	*liaF*	ND[a] (723)

[a] ND, not determined.

RESULTS

Inactivation of *yydIJ* leads to increased P_{lia} expression. Expression from the LiaR-activated *liaI* promoter (P_{liaI}) is known to be induced by the antibiotics ramoplanin, bacitracin, vancomycin, and nisin (29). However, neither the *liaIHGFSR* operon nor the LiaRS regulon appears to provide significant resistance to these antibiotics (29). Thus, the role of the LiaRS system has remained unclear. Here, we sought to identify transposon-induced mutations that result in upregulation of P_{liaI}.

Mutagenesis was performed using a Tn*7* transposon containing a Spec^r cassette and an outward-facing xylose-inducible P_{xylA} promoter (to facilitate identification of genes whose induction leads to increased P_{liaI} expression; see Materials and Methods). A library of Tn*7*-mutagenized chromosomal DNA was introduced into *B. subtilis* carrying the $P_{liaI\text{-}74}$-*cat-lacZ* promoter fusion (HB0950) (29), thus allowing for both screening (β-galactosidase expression) and selection (Cm^r). Fourteen transformants with elevated P_{liaI} expression were characterized (Table 3). In all cases the increase in $P_{liaI\text{-}74}$-*cat-lacZ* expression was not dependent on xylose, indicating that the effect was more likely due to gene disruption rather than increased expression of downstream genes.

Sequence analysis of these Tn*7* insertions revealed three groups of transposition events (Table 3). The first group consists of insertions into the *liaG* and *liaF* genes. Previous experiments had shown that deletion of *liaF* results in constitutive expression from the P_{liaI} promoter (20). These strains exhibit a dramatic, dark blue color after less than 1 day of incubation on X-Gal plates (data not shown). In contrast, the remaining isolates had to be incubated for at least 2 days in the presence of 80 μg/ml of X-Gal to see a faint blue color. The second group included four independent transposition events into the *mutS* and *mutL* genes (Table 3). The reason for the weak upregulation of P_{liaI} under these conditions requires further investigation. The last group, and the subject of this study, consists of transposon insertions in the *yydI* and *yydJ* genes (Fig. 1A and Table 3). In both cases, the P_{xylA} promoter carried on Tn*7* is facing in the opposite direction to the gene, consistent with the hypothesis that P_{liaI} upregulation is due to gene disruption. Analysis of the predicted protein products of these

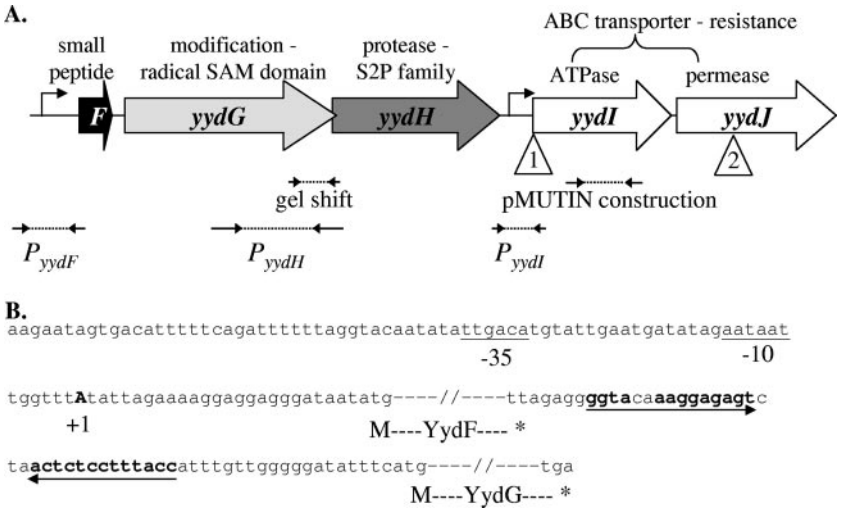

FIG. 1. A. Arrangement of the *yydFGHIJ* operon. The schematic is drawn to scale, and predicted functions are shown above the genes. Large arrows represent the open reading frames, with the P_{yydF} and P_{yydI} promoters identified in this study marked. Open triangles show the positions of the Tn*7* transposon insertions: 1, YPL3, YPL9, YPL14, and YPL15; 2, YPL6 and YPL13. Also indicated are the positions of the fragments used by Albano et al. for gel shift and pMUTIN construction (1) and the fragments used in this study to create *lacZ* fusions at the *amyE* locus. B. The *yydF* promoter region and *yydF-yydG* intergenic region. The −35 and −10 regions of a σ^A-type promoter are underlined, and the +1 start of transcription as determined by 5′-RACE PCR is shown in bold. The *yydF* and *yydG* open reading frames are indicated by dashes, and the inverted repeat is shown with arrows (with the complementary bases marked in bold).

genes suggests that they form an ATP binding cassette (ABC) transporter (Table 4).

The effect of the *yydIJ* deletion on P_{liaI} expression is enhanced on solid competence medium (MC). In order to confirm the data from the transposon mutagenesis, we constructed a *yydIJ*::*spec* deletion by LFH-PCR. As noticed with the transposon mutants, the induction of P_{liaI} on LB medium in this strain was very weak. Since the *yydHIJ* genes were previously proposed to be controlled by Rok (1), and Rok is repressed by the regulator of competence ComK (18), we investigated whether the effects of the *yydIJ* mutation would be enhanced under different growth conditions, including growth on solid

TABLE 4. Properties and functions of the proteins identified during Tn*7* mutagenesis and selection for upregulation of P_{liaI}

Protein	Properties	Conserved domain(s)[c]	Closest homolog[d] (%identity/%positive)	Function
YydF	49 aa, +2.2 charge at pH 7.0, 34% hydrophobic, potential to form α-helix with 12 hydrophobic residues on the same surface[a]	None	*Streptococcus agalactiae* strains (46–50/69)	Antimicrobial/signal/secreted peptide[e]
YydG	319 aa, cytoplasmic protein	Predicted Fe-S oxidoreductase (COG0535), with 5′ region radical SAM superfamily (pfam 04055)	*Streptococcus agalactiae* COH1 (35/55)	Oxidoreductase[e]
YydH	252 aa, 5 THM[b]	None	*Streptococcus agalactiae* COH1 (34/54)	Peptidase[e] (M50 peptidase family[f])
YydI	209 aa, cytoplasmic protein	ABC_ATPase (cd00267)	*Staphylococcus aureus* subsp. *aureus* (61/77)	ABC transporter (ATPase subunit)[e]
YydJ	240 aa, 6 THM[2]	None	*Staphylococcus aureus* subsp. *aureus* (62/82)	ABC transporter (permease subunit)[e]
MutS	858 aa	COG0249, MutS family	*B. licheniformis* (83/89)	DNA mismatch repair ATPase
MutL	627 aa	COG0323, MutL	*B. licheniformis* (78/85)	DNA mismatch repair enzyme (predicted ATPase)

[a] Baesd on information in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.html).
[b] THM, transmembrane helices, as determined using TMHMM (www.cbs.dtu.dk/services/TMHMM/) and TMPred (www.ch.embnet.org/software/TMPRED_form.html).
[c] Based on the Conserved Domains database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).
[d] Analysis with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).
[e] Function predicted from sequence homology.
[f] Based on information in MEROPS, the peptidase database (http://merops.sanger.ac.uk/).

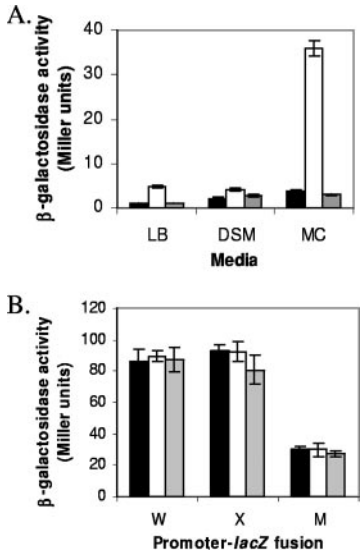

FIG. 2. A. Induction of the $P_{liaI\text{-}74}$-*cat-lacZ* fusion in WT (black bars), *yydIJ*::*spec* (white bars), and *yydFGHIJ*::*spec* (gray bars) strains on solid LB, DSM, and MC media. B. Activities of P_{sigW}, P_{sigX}, and P_{sigM}-*lacZ* fusions in the same strains on MC medium only. After overnight incubation on solid medium, the cells were washed off and β-galactosidase activity was determined. Error bars represent the standard deviations between two independent experiments each assayed in duplicate.

modified competence (MC) medium. Indeed, P_{liaI} expression was significantly increased in the *yydIJ*::*spec* mutant on solid MC medium (close to 10-fold) as opposed to the more modest increase observed on solid LB and DSM (4.7- and 1.9-fold, respectively) (Fig. 2A). Three ECF σ factors, σ^W, σ^X, and σ^M, are also induced by stresses affecting the cell envelope (4–6). However, the activity of these three ECF σ factors was not detectably induced in the *yydIJ* mutant strain (Fig. 2B).

Bioinformatic and genetic analyses suggest that the *yydFGHIJ* operon functions to synthesize and export a small peptide, YydF*. Analysis of the *yydIJ* genes and their genomic context suggested that these genes might be part of a larger *yydFGHIJ* operon. Sequence analyses led us to hypothesize that the YydF peptide (49 aa) may be the precursor of an exported, biologically active peptide (designated YydF*) with antimicrobial or signaling properties (or both) (Table 4). YydG is a predicted radical SAM family oxidoreductase, and YydH is a predicted intramembrane protease (Table 4) that may modify and cleave the primary translation product of the *yydF* gene. The nature of these posttranslational modifications is not yet clear. The resulting active YydF* peptide is the presumed substrate for export by the YydIJ ABC transporter. In the absence of the YydIJ ABC transporter, we propose that YydF* elicits cell envelope stress that is sensed by the LiaRS TCS.

Genetic analyses suggest that *yydF*, *yydG*, and *yydH* are required for induction of P_{liaI} in the absence of the YydIJ exporter. Induction of P_{liaI} is lost if the entire *yydFGHIJ* operon is deleted (Fig. 2A) or if only *yydH* is additionally deleted (Table 5, column 1).

Wild-type cells induce P_{lia} expression in neighboring strains lacking the *yydFGHIJ* operon. Since the *yydFGHIJ* operon is postulated to synthesize and export a modified peptide (YydF*), we tested whether wild-type (WT) strains could induce P_{liaI} in neighboring cells lacking *yydFGHIJ*. Strains containing the P_{liaI} reporter were mixed with WT and various *yyd* operon mutants and spotted on the solid MC medium containing X-Gal (Table 5). As noted above, deletion of *yydIJ* (but not *yydHIJ* or *yydFGHIJ*) leads to activation of P_{liaI} even in pure culture (Table 5, line 2). However, when mixed with wild-type strains, P_{liaI} is induced in strains carrying *yydHIJ* and *yydFGHIJ* deletions (Table 5, column 2). The ability of the wild type to induce the reporter strain depends on *yydF* and *yydG*, as judged from the effects of in-frame deletions. Together, these genetic studies suggest that production of active YydF* requires *yydF*, *yydG*, and *yydH*.

Unexpectedly, weak induction was noted even when the producer strain was lacking the YydIJ ABC transporter (Table 5, column 3). We propose that under these conditions the active YydF* peptide is produced by the *yydFGH* genes but not efficiently exported from these cells, and the observed induction may result from small amounts of YydF* that are either exported from the cell via other methods or released by cell lysis. This weak induction is not observed when the reporter strain is mixed with a *yydHIJ* mutant, again suggesting that in the absence of the YydH protease, active YydF* peptide is not produced.

Induction of P_{liaI} in response to exogenous YydF* peptide is only seen in cells lacking the YydIJ transporter. Induction is not observed in wild-type cells or in cells containing in-frame deletions of either *yydF* or *yydG* (data not shown). Thus, the YydIJ transporter prevents P_{liaI} induction regardless of whether the peptide is produced by the same cell or a neighboring cell.

Induction during cocultivation is readily apparent on solid

TABLE 5. The product of the *yydFGHIJ* operon induces P_{liaI} in a neighboring strain

Reporter	β-Galactosidase activity[a] when cocultivated in equal amounts with:								
	None	WT	*yydIJ*	*yydHIJ*	*yydF-J*	Δ*yydF*	Δ*yydG*	P_{xyl}-*yydF-J*	
								No xylose	2% xylose
WT	−	−	−	−	−	−	−	−	−
yydIJ	+++	+++	+++	+++	+++	+++	+++	+++	+++
yydHIJ	−	+++	+	−	−	−	−	−	++
yydFGHIJ	−	+++	+	−	−	−	−	−	++

[a] Reporter strains were plated on MC medium containing 80 μg/ml X-Gal. Reporter strains contained SPβ ($P_{liaI\text{-}74}$-*cat-lacZ*). All strains mixed with reporter strains contained the SPβ prophage (with no reporter fusion) to ensure that effects observed were not due to sensitivity towards sublancin. −, no β-galactosidase activity observed (white color); +, β-galactosidase activity observed (blue color); the number of + signs indicates the intensity.

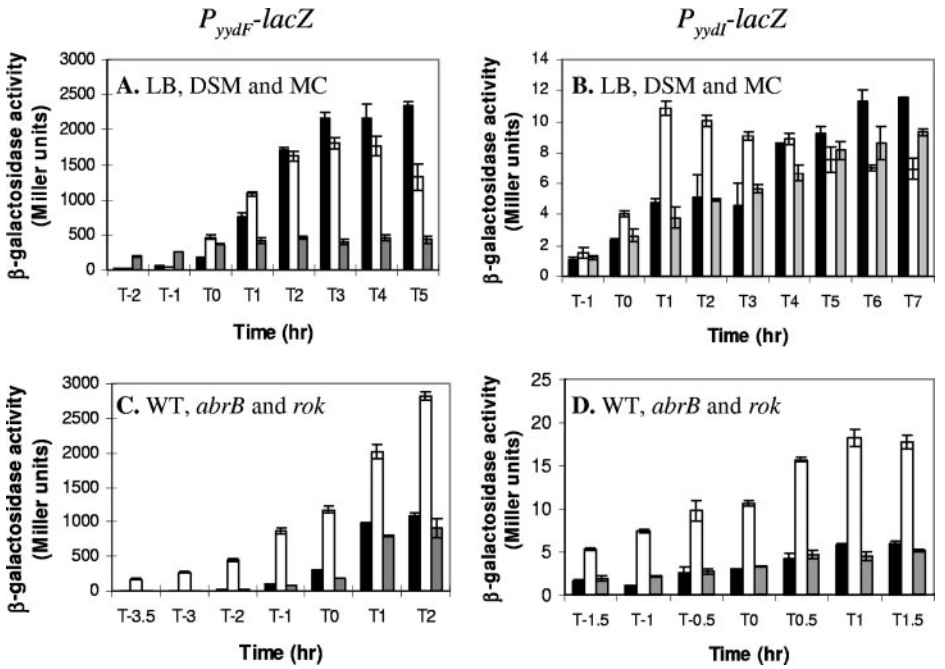

FIG. 3. Expression from the P_{yydF} and P_{yydI} promoters. Expression levels of the P_{yydF}-*lacZ* fusion (A and C) and P_{yydI}-*lacZ* fusion (B and D) are shown. (A and B) Expression during growth in different media: rich medium (LB; black bars), sporulation medium (DSM; white bars), and MC medium (gray bars). (C and D) Expression from these promoters in the wild type (black bars) and *abrB* (white bars) and *rok* (gray bars) mutants grown in LB medium. Note that T_0 corresponds to the transition from logarithmic growth to stationary phase. Each experiment was repeated at least twice, and a representative assay is shown here. Error bars represent standard deviations between triplicate β-galactosidase assays.

medium but not in liquid culture, suggesting that the concentrations of the active peptide in liquid culture may be too low for induction in *trans*. In order to test whether the YydF* peptide was released into the culture supernatant, we resuspended the reporter strains in cell-free medium collected from an overnight culture of wild-type or *yydFGHIJ* mutant cells grown in MC medium and tested for induction of P_{liaI} by β-galactosidase assays (data not shown). However, no increase in P_{lia} expression was apparent under the conditions tested. These results again suggest that the YydF* peptide does not accumulate to high enough levels in liquid culture to induce the LiaRS stress response.

The *yydFGHIJ* operon is expressed predominantly from a σ^A-type promoter upstream of *yydF*. Previous Northern blot analysis had indicated that the *yydFGHIJ* genes were coexpressed at the onset of stationary phase in minimal media (as reported on the BSORF website [http://bacillus.genome.jp/bsorf.html]). However, a previous transcriptome study had found that expression of the *yydHIJ* genes was increased 1.7- to 3.1-fold in a *rok* mutant, and biochemical studies suggested that Rok bound to a region upstream of *yydH* (1). Inspection of the operon sequence reveals that the 3′ end of the *yydG* gene overlaps with the 5′ end of the *yydH* gene by 19 bp, making the region to which Rok binds within the *yydG* gene (Fig. 1A). On the other hand, there are 150 bp between the end of *yydH* and the start of *yydI*.

To further investigate the regulation and expression of this operon, and to test for the possible presence of promoters within the Rok-binding region and *yydH-I* intergenic region, we integrated *lacZ* reporter fusions (Fig. 1A) at the *amyE* locus and followed expression throughout growth (Fig. 3A and B). In LB and DSM, the P_{yydF} promoter was strongly expressed after the transition to stationary phase (Fig. 3A). However, this postexponential induction was significantly lower in competence medium (MC). These data are somewhat surprising, since the effect of YydF* on P_{liaI} expression was most apparent on MC medium (Fig. 2A). No activity was observed for the fusion with the fragment that included the Rok binding site upstream of *yydH*, suggesting that there is no promoter in this region (data not shown). Very low β-galactosidase activity (less than 12 Miller units) was seen with the P_{yydI}-*lacZ* fusion in all media tested, but expression did appear to increase after transition to stationary phase (Fig. 3B). Thus, there may be a weak promoter within this intergenic region. These results suggest that the major promoter driving expression of the *yydFGHIJ* operon is upstream of *yydF*.

5′-RACE PCR was used to map potential transcriptional start sites upstream of the *yydG*, *yydH*, and *yydI* genes. The only observed product (with a *yydG* primer) mapped to an A residue 25 bp upstream of the *yydF* ATG (Fig. 1B). Northern analysis was performed with RNA isolated from cells growing in LB at log and stationary phase using probes to the *yydF*, *yydG*, *yydH*, and *yydI* genes. A strong signal was detected with the *yydF* probe corresponding to a small transcript of about 250 bp, and the amount of this transcript increased significantly when RNA was prepared from stationary-phase cells (Fig. 4). No full-length or other transcripts were detected with the other *yyd* probes (data not shown). This small transcript was also observed with RNA isolated from cells growing in MC and MM media and appeared to be increased compared to those in

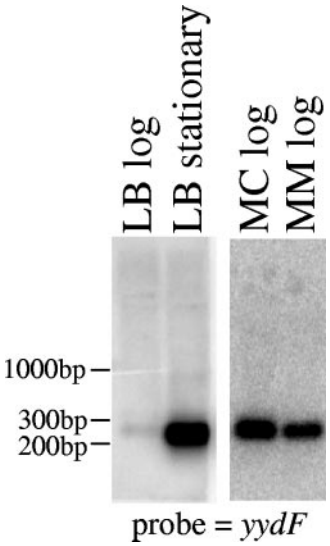

FIG. 4. Northern blot analysis showing that a small (approximately 250-bp) transcript corresponding to the *yydF* gene is transcribed in liquid LB, MC, and MM media and is increased in stationary phase. The blot was probed with a radiolabeled *yydF* fragment.

LB (Fig. 4). Analysis of the sequence downstream of *yydF* identified an imperfect inverted repeat between the *yydF* and *yydG* open reading frames (Fig. 1B). Since *yydF* has been found to be 1 of about 30 extremely stable *B. subtilis* mRNAs (14), we speculate that this repeat may form a secondary structure that stabilizes the *yydF* portion of the primary transcript.

Expression of the *yydFGHIJ* operon does not appear to be under Rok control. Since Albano et al. proposed that the *yydHIJ* genes were under the control of Rok (1), we tested expression from the P_{yydF} and P_{yydI} promoters in a *rok* mutant throughout growth in liquid medium. No increase in expression was observed in any of the media tested (LB [Fig. 3C and D] and MC and DSM [data not shown]). Contrary to the previously published results (1), deletion of *rok* did not increase expression from either of these promoters. It should be noted that we used ectopically located promoter fusions, while the previous study made use of an integrational plasmid (pMUTIN) inserted in the *yydI* gene. In our case it is possible that our promoter fusions are lacking *cis* elements required for Rok regulation, while in the latter case the pMUTIN integration will disrupt the YydIJ ABC transporter, which could also affect the results. Interestingly, when assayed using X-Gal on solid medium, expression from the P_{yydF} promoter appeared to be slightly increased in a *rok* mutant (data not shown). However, an additional deletion of the *sdpABC* operon, also shown to be under Rok control (1), abolished this increase. We noticed that a *rok* mutant does not grow well on MC media (both liquid and solid); therefore, it is also possible that the increase in blue color observed on the solid medium could be due to increased lysis (presumably mediated by the toxic SdpC* peptide) and release of β-galactosidase from the cells.

Expression of the *yydFGHIJ* operon is negatively regulated by AbrB. Previous transcriptional profiling studies compared expression of genes in WT and *spo0A* mutants (the master regulator of sporulation in *B. subtilis*) and revealed that expression of *yydF* is increased about 130-fold in a WT strain compared to a *spo0A* mutant at transition stage and about 30-fold 2 h later. However, expression of the downstream *yydGHIJ* genes was essentially unchanged under these conditions (11). Since Spo0A represses expression of the transition-state regulator AbrB, we tested whether this difference in expression was due to AbrB. Indeed, in an *abrB* mutant expression from the P_{yydF}-*lacZ* fusion was increased throughout log growth and into stationary phase, confirming that AbrB normally represses this promoter during growth (Fig. 3C). AbrB repression of P_{yydI} was also observed; however, the increase in expression in an *abrB* mutant was only about 4-fold, compared with the 20- to 90-fold derepression observed for P_{yydF} (Fig. 3D). Thus, like many other operons encoding antimicrobial peptides, the *yydFGHIJ* operon is under negative control of the AbrB transition state regulator.

DISCUSSION

The LiaRS TCS is known to be strongly induced by various cell wall-active antibiotics and at least some antimicrobial peptides, but the physiological role of this stress response is not yet clear. Mutants lacking *liaR*, or the entire *liaIHGFSR* operon, do not display increased sensitivity to the known inducing compounds (29), suggesting that perhaps this system functions physiologically to protect cells against other, yet-unidentified stress conditions. Since the LiaRS-regulated LiaH protein is an ortholog of the phage shock protein, PspA, it is possible that this system functions to help maintain the integrity of the cell membrane under conditions that interfere with membrane function.

Here, we have used transposon mutagenesis to identify genes that affect the activity of the LiaRS TCS. Our results are consistent with a model in which the *yydFGHIJ* operon codes for the synthesis, modification, cleavage, and export of a small peptide, YydF*. Transposon insertions that disrupt either the *yydI* or *yydJ* genes, and therefore block peptide export, lead to elevated expression of the LiaR-activated P_{liaI} promoter. This is dependent on presumed synthesis of the active YydF* peptide (product of the *yydFGH* genes), and production of this peptide can activate LiaR in neighboring cells lacking the *yydFGHIJ* operon.

The primary product of the *yydF* gene is a small (49-aa) peptide with a predicted positive charge at pH 7.0 and the ability to form an α-helix (Table 4), properties often associated with antimicrobial peptides. The YydG protein contains a radical SAM family domain (pfam 04055) (34). Members of this family include proteins involved in antibiotic biosynthesis, including AlbA, which is involved in biosynthesis of the bacteriocin subtilosin in *B. subtilis* (38). YydH is a member of the S2P protease family (M50) and is a weak paralog of another *B. subtilis* protein, SpoIVFB (39). This protein is a membrane-embedded metalloprotease involved in cleavage of pro-σ^K. Proteases of the S2P family are often involved in regulated intramembrane proteolysis (RIP), where a protease, embedded in the membrane, cleaves a membrane-spanning protein within or very close to the membrane to release a soluble truncated protein, often a signaling molecule or transcription regulator. YydH is predicted to have five transmembrane-spanning helices, with the conserved HExxH motif located in

the second helix and DG residues within the fourth helix. In other S2P proteases, these two histidines and one aspartate residue are Zn ligands, while the glutamate is the catalytic residue (23). While many well-studied S2P family members are involved in the processing of regulators or their inhibitors, the metalloprotease Eep from *Enterococcus faecalis* is believed to play a role in the cleavage of a pheromone precursor (2). The final two genes of the operon, *yydIJ*, encode the ATPase and membrane subunits of an ABC transporter. As part of a previous inventory of the ABC transporters of *B. subtilis*, it was noted that the similarity of YydI with other ATP-binding proteins was too weak to allow for functional predictions (32). Based on our in silico analyses, we predict that the YydIJ ABC transporter functions in peptide export.

It is common for immunity towards peptide antibiotics to be provided by an ABC transporter that exports the toxic peptide from the cell. Therefore, inactivation of the *yydIJ* genes may result in sensitivity toward the active YydF peptide, which is sensed by the LiaRS system. However, in spot-on-lawn assays we noted that a *yydFGHIJ* mutant is not sensitive to a WT strain or to a strain overexpressing the *yydFGHIJ* operon under P_{xylA} control, even when the *liaIHGFSR* operon is also deleted (data not shown). This suggests that YydF* is not toxic enough to cause visible lysis under these conditions, that there are other unidentified systems that provide resistance, or that YydF* is a signaling rather than an antimicrobial peptide. As yet we have been unsuccessful in isolating the active YydF from WT cell cultures or cell-free supernatants; therefore, we cannot confirm the structure and modifications of this peptide.

The *yyd* operon appears to be subject to complex regulation. Data presented here and in other studies (transcriptome data and BSORF) suggest that all five genes can be expressed as one transcript from P_{yydF}. This strong promoter is activated during the transition phase, and this is likely due, in part, to relief of AbrB-mediated repression. In addition, we identified a weak promoter upstream of *yydI*. This may function to keep a small amount of the ABC transporter present in the cell for resistance to peptides produced by neighboring cells. Surprisingly, our Northern analyses detected a very strong signal corresponding to a small *yydF* transcript and no full-length transcript. The *yydF* mRNA was previously reported to be extremely stable, with a half-life of more than 15 min (14). Thus, this stable transcript may result from processing of the longer, primary transcript detected in previous analyses (BSORF).

Previous transcriptome analyses support the suggestion that some or all of the genes of the *yydFGHIJ* operon may be induced under stress conditions, including high salinity (35) and cold shock (22). In addition, the *yydHIJ* genes have been proposed to be under the control of Rok (repressor of ComK) (1). Rok was reported to bind immediately upstream of *yydH*, but we did not detect promoter activity from this region and we were unable to confirm previous suggestions that genes from within this operon are regulated by Rok. It is possible that binding of Rok to this region might affect transcription elongation, rather than initiation, and thereby induce a shift from production of full-length mRNA to shorter transcripts. It is interesting that Rok was also shown to repress several operons involved in the synthesis of bacteriocins: the *sunAT-bdbA-yolJ-dbdB* operon (which is involved in the production and secretion of the lantibiotic sublancin) and the *sbo-alb* operon (synthesis and export of subtilosin), as well as the *sdpABC* operon (encoding the antibiotic peptide SdpC). This suggests that competent *B. subtilis* cells may produce antimicrobial peptides to lyse neighboring cells (fratricide) to release chromosomal DNA for uptake, as has been shown in *Streptococcus pneumonia* (16). However, experimental support for this notion is not yet available.

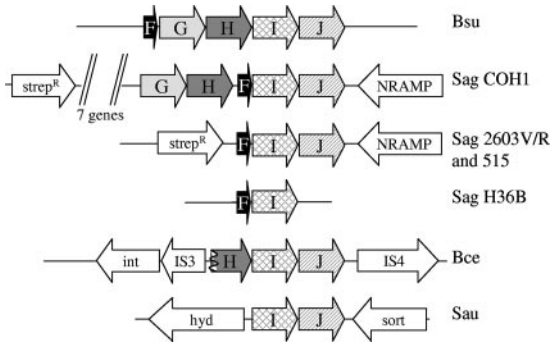

FIG. 5. Arrangement of *yyd* operon homologs in other bacteria. Open reading frames are not to scale. Homologs of the *yydFGHIJ* genes are shaded, while other surrounding genes are shown in white. Bsu, *B. subtilis* subsp. *subtilis* strains 168 and RO-NN-1 as well as *B. subtilis* subsp. *spizizenii* TU-B-10; Sag, *Streptococcus agalactiae* (strains indicated; genes encoding putative streptomycin resistance [Strep^r] proteins and NRAMP family Mn^{2+}/Fe^{2+} transporters [NRAMP] are also indicated); Bce, *B. cereus* subsp. *cytotoxis* NVH 391-98 (the *yydH* gene is truncated, and the upstream putative integrase [int] and flanking transposases of the IS*3* and IS*4* families are shown); Sau, *Staphylococcus aureus* subsp. *aureus* (predicted hydrolase [hyd] and sortase [sort] genes are also indicated). All sequences can be found at the National Center for Biotechnology website (http://www.ncbi.nlm.nih.gov), except for the unfinished genomes of *B. subtilis* sp. RO-NN-1 and TU-B-10.

Phylogenomic comparisons suggest that the *yydFGHIJ* operon has likely been acquired by horizontal gene transfer. Homologous operons are present in *B. subtilis* 168 and its closest relatives (the recently sequenced wild-type isolates *B. subtilis* subsp. *subtilis* RO-NN-1 and *B. subtilis* subsp. *spizizenii* TU-B-10 [related to W23]), but not in other closely related bacilli, including *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. Surprisingly, close homologs to all genes in the *yydF* operon are found in *Streptococcus agalactiae*. Many strains of *S. agalactiae* have been sequenced, and the "pan-genome" has been determined (36). The YydF homologs are only present in some of these strains and in all cases are found together with YydIJ homologs. Homologs of the YydGH proteins are only found in the *S. agalactiae* strain COH1 (Fig. 5). Homologs to the YydIJ ABC transporter are also found in *Staphylococcus aureus* and *Bacillus cereus* subsp. *cytotoxis* NVH 391-98. In the case of *S. aureus* there are no annotated small peptides or *yydGH* homologs in the region surrounding the *yydIJ* genes, while in *B. cereus* the homologous *yydH* gene is truncated and genes encoding a transposase and integrase are located on either side of the operon (Fig. 5). These observations support the previous prediction, based on the anomalous G+C content of these genes, that the *yydFGHIJ* region was horizontally transferred into the *B. subtilis* genome (Horizontal Gene Transfer Database [HGT-DB] at http://www.fut.es/~debb/HGT/) (12).

Our results suggest that the *yydFGHIJ* operon encodes a modified and processed peptide (YydF*) that is exported from the cell via an ABC transporter. In the absence of the ABC transporter, YydF* activates the LiaRS TCS, perhaps by causing cell envelope stress. The human antimicrobial peptide LL-37 has been shown to activate several cell envelope stress responses in *B. subtilis*, including the LiaRS system (31). In this work we demonstrate that LiaRS additionally, and specifically, responds to at least one endogenous peptide produced by *B. subtilis*.

ACKNOWLEDGMENT

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