The *Listeria monocytogenes* σ^B Regulon and Its Virulence-Associated Functions Are Inhibited by a Small Molecule

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ABSTRACT **The stress-responsive alternative sigma factor σ^B is conserved across diverse Gram-positive bacterial genera. In *Listeria monocytogenes*, σ^B regulates transcription of >150 genes, including genes contributing to virulence and to bacterial survival under host-associated stress conditions, such as those encountered in the human gastrointestinal lumen. An inhibitor of *L. monocytogenes* σ^B activity was identified by screening ~57,000 natural and synthesized small molecules using a high-throughput cell-based assay. The compound fluoro-phenyl-styrene-sulfonamide (FPSS) (IC_{50} = 3.5 μM) downregulated the majority of genes previously identified as members of the σ^B regulon in *L. monocytogenes* 10403S, thus generating a transcriptional profile comparable to that of a 10403S Δ*sigB* strain. Specifically, of the 208 genes downregulated by FPSS, 75% had been identified previously as positively regulated by σ^B. Downregulated genes included key virulence and stress response genes, such as *inlA*, *inlB*, *bsh*, *hfq*, *opuC*, and *bilE*. From a functional perspective, FPSS also inhibited *L. monocytogenes* invasion of human intestinal epithelial cells and bile salt hydrolase activity. The ability of FPSS to inhibit σ^B activity in both *L. monocytogenes* and *Bacillus subtilis* indicates its utility as a specific inhibitor of σ^B across multiple Gram-positive genera.**

IMPORTANCE **The σ^B transcription factor regulates expression of genes responsible for bacterial survival under changing environmental conditions and for virulence; therefore, this alternative sigma factor is important for transmission of *L. monocytogenes* and other Gram-positive bacteria. Regulation of σ^B activity is complex and tightly controlled, reflecting the key role of this factor in bacterial metabolism. We present multiple lines of evidence indicating that fluoro-phenyl-styrene-sulfonamide (FPSS) specifically inhibits activity of σ^B across Gram-positive bacterial genera, i.e., in both *Listeria monocytogenes* and *Bacillus subtilis*. Therefore, FPSS is an important new tool that will enable novel approaches for exploring complex regulatory networks in *L. monocytogenes* and other Gram-positive pathogens and for investigating small-molecule applications for controlling pathogen transmission.**

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Listeria monocytogenes causes a rare but potentially fatal food-borne disease called listeriosis. With its high fatality rate, listeriosis accounts for ~10% of all deaths from food-borne diseases in the United States (1). *L. monocytogenes* can transition from a saprotrophic existence under a wide range of environmental conditions (2) to intracellular infection in a diverse array of hosts (3). The ability of *L. monocytogenes* to transform from saprotroph to intracellular pathogen is influenced by regulatory networks that enable bacterial survival and control virulence factor expression in response to environmental signals (4).

Sigma B is one important component of a network that links environmental stress survival and virulence in *L. monocytogenes* (5, 6). Sigma factors are dissociable subunits of prokaryotic RNA polymerase. The association of a specific alternative sigma factor, e.g., σ^B, with core RNA polymerase under appropriate environmental conditions enables the rapid redirection of regulon transcription in response to environmental signals. More than 150 genes comprise the *L. monocytogenes* σ^B regulon (7, 8).

σ^B networks, including its interactions with PrfA, influence transmission of *L. monocytogenes* during both the gastrointestinal (9) and systemic stages of infection (5, 10). Complex interactions occur between σ^B and PrfA-dependent gene regulation (5, 10); PrfA is the master regulator of *L. monocytogenes* virulence gene expression. σ^B directly regulates *prfA* transcription via the $P2_{prfA}$ promoter (11–13) and also indirectly regulates PrfA activity. Specifically, σ^B downregulates PrfA activity in intracellular *L. monocytogenes*, thus moderating expression of PrfA-dependent virulence genes and thereby reducing host cell damage incurred by these virulence gene products (5).

A general strategy for exploring complex biological networks is to disrupt a targeted element of that network and then examine the consequences. High-throughput screening of small-molecule libraries has been used effectively to identify agents that disrupt specific bacterial targets, including an inhibitor of the virulence regulator ToxT in *Vibrio cholerae* (14). We screened multiple small-molecule libraries to identify an inhibitor of the stress response and virulence-associated regulator σ^B. The most promising small molecule was further assessed using an *L. monocytogenes*

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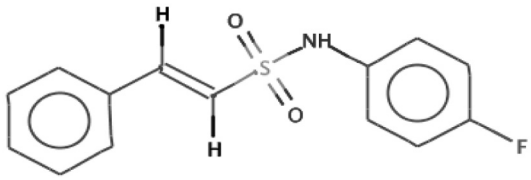

FIG 1 Chemical structure of the σ^B activity inhibitor FPSS (fluoro-phenyl-styrene-sulfonamide). FPSS is a derivative of (*E*)-*N*,2-diphenylethene-sulfonamide, the compound originally identified by HTS as the most effective inhibitor of σ^B activity. Relative to the structure of (*E*)-*N*,2-diphenylethenesulfonamide, FPSS has a fluorine substituted for a hydrogen.

whole-genome microarray, quantitative reverse transcription-PCR (qRT-PCR) of σ^B-dependent genes, and phenotypic profiling, including Caco-2 cell invasion assays and qualitative assessment of bile salt hydrolase activity. The compound also was evaluated for its ability to inhibit σ^B activity in *B. subtilis*.

RESULTS

A high-throughput cell-based screen identifies promising small molecules that interfere with σ^B activity. A high-throughput cell-based screening assay (HTS) was used to identify compounds that inhibit expression of the σ^B-dependent *opuCA* promoter (15) without affecting *L. monocytogenes* growth (Chembank Screening Project: SigBInhibition). Based on the primary screen, 41 putative inhibitors of σ^B activity were selected for secondary cell-based screening (Fig. S1). Compounds that induced σ^B activity were not analyzed further.

IC_{50} values, i.e., compound concentrations needed to inhibit 50% of σ^B activity, were determined from secondary screening results for each of the 41 compounds. For 14 compounds, σ^B activity was inhibited at a concentration lower than that used in the primary screen; however, 11 compounds were eliminated from further consideration based on mammalian cell cytotoxicity data in ChemBank (http://chembank.broad.harvard.edu). The three remaining *L. monocytogenes* σ^B inhibitors were 4-hydrazino[1]benzofuro[3,2-d]pyrimidine; 3-(cyclohexylacetyl)-4-hydroxy-2H-chromen-2-one; and (*E*)-*N*,2-diphenylethenesulfonamide. Among these, the most effective σ^B activity inhibitor, (*E*)-*N*,2-diphenylethenesulfonamide (IC_{50} = ~15 μM), which was a member of the ChemDiv3 library (Table S1), was not commercially available. Therefore, fluoro-phenyl-styrene-sulfonamide (FPSS), an analog of the original compound, was obtained for further study. Relative to (*E*)-*N*,2-diphenylethenesulfonamide, FPSS has fluorine substituted for a hydrogen (Fig. 1). Based on quantitative reverse transcriptase PCR (qRT-PCR) results, FPSS was the most effective σ^B inhibitor among the three compounds. Data available in ChemBank indicated minimal evidence and no evidence of mammalian cell cytotoxicity for (*E*)-*N*,2-diphenylethenesulfonamide and FPSS, respectively.

We hypothesized that a small molecule that directly binds σ^B might also prevent σ^B from associating with core polymerase, thereby inhibiting σ^B activity. Therefore, the ability of various small molecules to bind σ^B was assessed using a small-molecule (SMM) screen with His-tagged σ^B (Fig. S2; Table S1). Of three putative ligands—i.e., 3-amino-4-oxo-N-(pyridin-3-ylmethyl)-3,4-dihydroquinazoline-2-carboxamide [Maybridge], ethyl 1-benzyl-5-[3-(tert-butylamino)-2-hydroxypropoxy]-2-methyl-1H-indole-3-carboxylate [Chemical Diversity], and 5-phenyl-4,7-dihydrotetrazolo[1,5-a]pyrimidine [Maybridge])—none inhibited σ^B activity in the bile salt hydrolase activity assay, and therefore, none were evaluated further.

Multiple lines of evidence support σ^B activity inhibition by FPSS. Quantitative qRT-PCR assessment of the effects of FPSS concentrations from 1 μM to 64 μM on σ^B-dependent transcription showed that exposure to 64 μM FPSS resulted in a ~40-fold reduction in transcript levels for both σ^B-dependent genes *opuCA* and *gadA* relative to their transcript levels in cells not treated with FPSS (Fig. 2) ($P < 0.05$, GLM [general linear model] with post-hoc Tukey's honestly significant difference [HSD] test). *opuCA* and *gadA* transcript levels in cells treated with FPSS (ranging from 8 μM to 64 μM) were not significantly different from those in the Δ*sigB* strain ($P > 0.05$). At 4 μM, FPSS significantly reduced *opuCA* and *gadA* transcript levels compared to those in 10403S without FPSS ($P < 0.05$) but not to levels equivalent to those in the Δ*sigB* strain (Fig. 2). The FPSS concentration yielding half the maximal inhibition (IC_{50}) was calculated as 3.5 μM for *opuCA* and 3.0 μM for *gadA*. Importantly, absolute transcript levels for the housekeeping genes *rpoB* and *gap* were not different in *L. monocytogenes* with and without exposure to FPSS, indicating that FPSS specifically inhibits transcription of σ^B-dependent genes without affecting transcription of housekeeping genes.

The phenotypic effects of various concentrations of FPSS on the activity of bile salt hydrolase, the product of the σ^B-dependent gene *bsh*, which is required for *L. monocytogenes* survival *in vivo* (6), were qualitatively assessed. *L. monocytogenes* treated with 96 μM and 193 μM FPSS showed no bile salt hydrolase (BSH) activity, with no apparent effect on the ability of *L. monocytogenes* to grow on brain heart infusion (BHI) agar. When treated with 290 μM FPSS, *L. monocytogenes* produced no BSH activity but also grew poorly on BHI (data not shown).

***L. monocytogenes* whole genome microarray identified 208 genes downregulated by treatment with FPSS.** Transcriptional consequences of FPSS treatment were profiled using an *L. monocytogenes* whole-genome microarray. FPSS treatment downregulated transcript levels for 208 genes and upregulated transcript levels for 32 genes (adjusted *P* value of <0.05 and an absolute fold change [FC] value of ≥ 2). In previous studies with *L. monocytogenes* 10403S and EGD-e, 281 genes were identified as positively regulated by σ^B under at least one assay condition, and 137 genes as positively regulated by σ^B under two or more of the seven assay conditions examined (5–8, 16, 17) (Table S2). Overall, FPSS significantly reduced transcript levels of 56% (156/281) of genes previously identified as being upregulated by σ^B in at least one study and of >91% (125/137) of genes identified as being upregulated by σ^B in two or more studies (Table 1; Table S3). Of the 208 FPSS-downregulated genes, 115 were reported to be positively regulated by σ^B in both 10403S and EGD-e (5–8, 16, 17), with an additional 21 genes reported to be positively regulated by σ^B in 10403S (5, 7, 8, 17) and 20 reported to be positively regulated by σ^B in EGD-e (6, 16). FPSS downregulated transcript levels for >90% of genes with previously reported hidden Markov model-identified σ^B-dependent promoters (17). A number of operons previously identified as being positively regulated by σ^B (8) were also significantly downregulated after treatment with FPSS, including *inlAB*, which mediates entry into nonprofessional phagocytes (18), and *opuCABCD*, which is involved in compatible solute transport. The autoregulated *sigB* operon (7, 8, 19), consisting of

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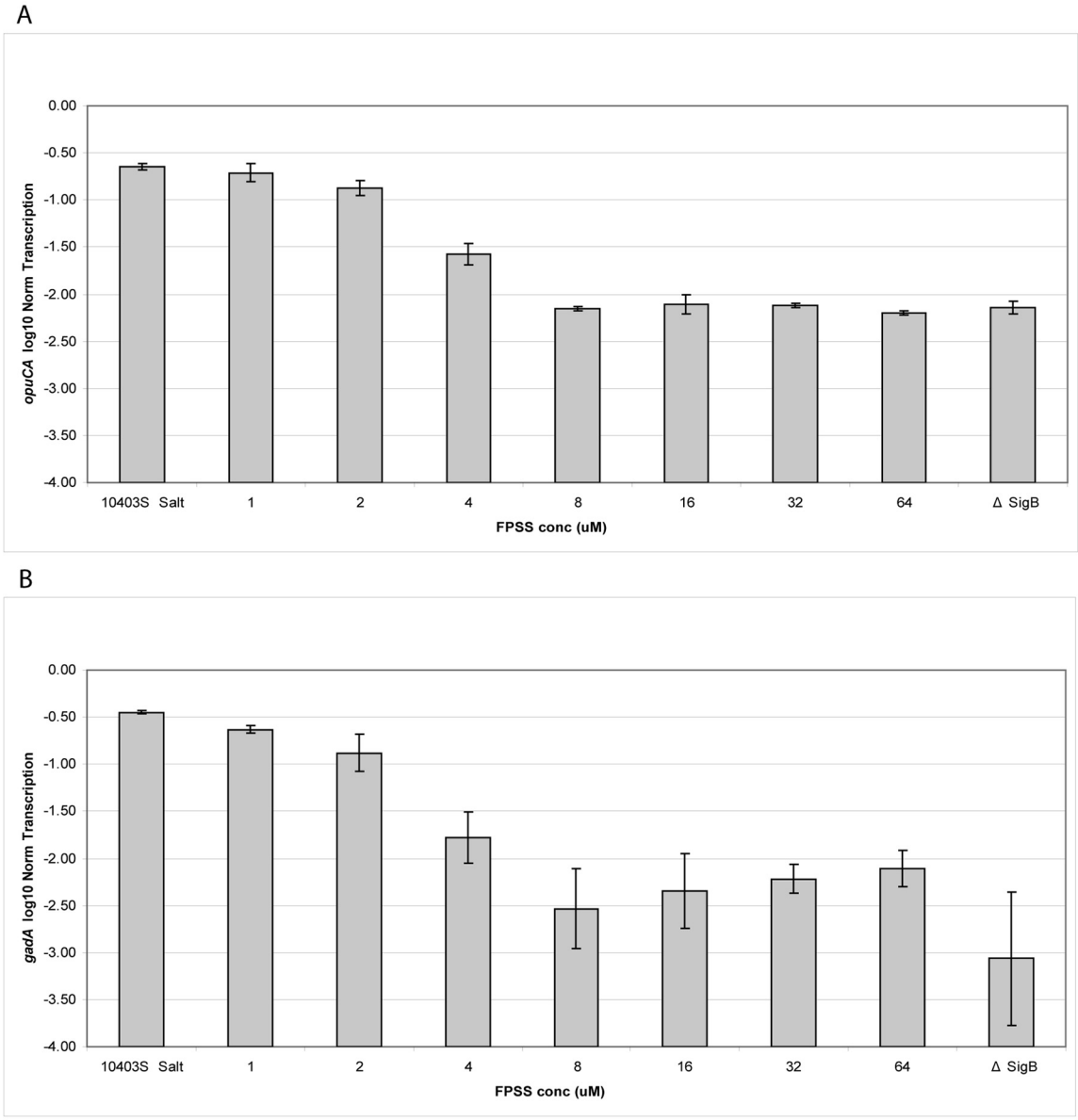

FIG 2 FPSS treatment reduces transcript levels of σ^B-dependent *opuCA* and *gadA*. Normalized log-transformed *opuCA* (A) and *gadA* (B) transcript levels in *L. monocytogenes* 10403S exposed to 0.3 M NaCl to induce σ^B activity in the presence of FPSS at concentrations ranging from 1 to 64 μM; controls included strains 10403S and its isogenic Δ*sigB* mutant exposed to 0.3 M NaCl. Transcript levels were quantified by qRT-PCR, $\log_{10}$ transformed, and normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap*. The data are means from three replicates; error bars show the standard deviations.

lmo0893 to *lmo0896* (*rsbV*, *rsbW*, *sigB*, and *rsbX*), was also downregulated by FPSS.

To evaluate FPSS effects on the function of other alternative sigma factors, transcript levels for genes in the σ^H and σ^L regulons were assessed. Among the 30 genes previously identified as σ^H dependent (with an FC $\geq$ 2.0), 14 were significantly downregulated by FPSS (adjusted $P < 0.05$, fold change ≤ -2); however, 12 of those 14 genes are also σ^B dependent. Gene set enrichment analysis (GSEA) showed that the σ^H-only regulon (i.e., genes that are regulated only by σ^H and not coregulated by σ^B) was not significantly enriched among the genes differentially transcribed as a result of FPSS treatment (false discovery rate [FDR] $q = 0.472$). GSEA also showed that the σ^L regulon was not significantly enriched as a result of treatment with FPSS (FDR $q = 0.836$).

GSEA was used to determine if genes from specific biological role categories were overrepresented among those differentially affected by FPSS. Consistent with σ^B's role in bacterial stress response, gene sets enriched among FPSS-downregulated genes included those classified as (i) "Cellular Processes: Adaptations to Atypical Conditions" and (ii) "Energy Metabolism (other)" (FDR $q = 0.060$ and $q = 0.201$, respectively). Previously identified σ^B-regulated genes also were significantly enriched among FPSS-downregulated genes (FDR $q < 0.0001$). Gene sets enriched among FPSS-upregulated genes included those classified as (i) "Cellular Processes: Chemotaxis and Motility," (ii) "Protein Fate: Protein Folding and Stabilization," and (iii) "Amino Acid Biosynthesis: Histidine Family" (FDR $q < 0.0001$, $q = 0.008$, and $q = 0.031$, respectively).

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TABLE 1 Relationships between genes identified as differentially expressed after treatment with FPSS and genes identified previously as σ^B dependent

Gene type	No. of genes (no. with an upstream σ^B-dependent promoter[a])	
	Downregulated by FPSS	Upregulated by FPSS
Identified previously as positively regulated by σ^B[b]	152 (86)	2 (0)
Identified previously as negatively regulated by σ^B[c]	2 (0)	7 (0)
Reported previously to be positively or negatively regulated under different conditions or in different studies[d]	4 (0)	0
Having no previous evidence of σ^B-dependent transcript levels	50 (0)	23 (0)
Total	208 (86)	32 (0)

[a] Genes were classified with upstream σ^B-dependent promoters by *in silico* analysis using a hidden Markov model as described by Oliver et al. (17).

[b] Genes were classified as positively regulated by σ^B based on evidence from at least one of six previous microarray or RNA-Seq studies (5–8, 16, 17) (see Tables S2 and S3 for details).

[c] Genes were classified as negatively regulated by σ^B based on evidence from at least one of six previous microarray or RNA-Seq studies (5–8, 16, 17) (see Tables S2 and S4 for details).

[d] Genes reported as differentially regulated in previous microarray or RNA-Seq studies (5–8, 16, 17), including genes reported as negatively regulated by σ^B in one study and positively regulated by σ^B in another study and genes that were found to be negatively and positively regulated by σ^B under different conditions in the same study (see Table S2).

Of the 208 FPSS-downregulated genes, 126 were positively regulated by σ^B during infection in the murine intestine (6), including 17 genes that had been identified as σ^B dependent only in the intestinal environment (Table S2). Among these 17 genes, 9 genes were of unknown or hypothetical function. Several FPSS-downregulated genes are recognized as contributing to virulence and host infection (i.e., *inlA*, *inlB inlD*, *bilEAB*, *bsh*, *hfq*, *clpC*, *opuC*, and *gadA*). Further, the PrfA regulon (i.e., genes regulated by the pleiotropic virulence gene regulator PrfA) was significantly enriched among the genes downregulated by FPSS (FDR $q = 0.095$). Interestingly, 19 genes that were previously classified as coregulated by PrfA and σ^B (10, 20) were both upregulated in the mouse spleen (10) and downregulated by FPSS, further supporting their σ^B-dependent transcription. Among these 19 genes, three were identified as potential virulence factors in murine and tissue culture models (10, 22), including *lmo1601* (encoding a general stress protein), *lmo1602* (encoding an unknown protein), and *lmo2157* (encoding SepA, a metalloprotease in *Staphylococcus epidermidis* [21]), which is upregulated in *L. monocytogenes* during intracellular infection (22). FPSS also downregulated *lmo0937*, a PrfA-regulated gene that is upregulated in the mouse spleen at 48 h postinfection (10), and *lmo0915*, which encodes a component of a phosphotransferase system identified as a potential virulence factor by Camejo et al. (10); neither gene had been identified previously as σ^B dependent. Other σ^B-dependent genes downregulated by FPSS and upregulated during intracellular infection (22) include *lmo0232* (*clpC*); *lmo0445*, which encodes a transcriptional regulator; *lmo2672*, which encodes a protein similar to a transcriptional regulator; and *lmo0783*, which is a member of an operon encoding mannose phosphotransferase system components. FPSS-treated cells had lower transcript levels for a number of genes that encode cell wall-associated proteins previously shown to be upregulated under intracellular conditions (22) and in the murine intestine (6); these genes include *inlA*, *inlD*, *lmo0610*, *lmo0880*, and *lmo2085*, which all encode proteins with an LPXTG sorting motif for cell wall anchoring, and *inlB*, which encodes a protein with a GW domain that is important for binding host ligands (23).

Three genes important for glycerol utilization (i.e., *lmo1538*, *lmo1539*, and *lmo1293*) were also downregulated by FPSS; utilization of glycerol as a carbon source in intracellular environments (22) is required for intracellular survival (24). While *lmo1538* (encoding a glycerol kinase) and *lmo1539* (encoding a glycerol uptake facilitator) were downregulated by FPSS, they were previously reported to be negatively regulated by σ^B in stationary phase and under salt stress conditions (8). Interestingly, however, both genes were upregulated by σ^B in the intestine (6) and during intracellular replication (22). *lmo1293* (*glpD*), which encodes a glycerol-3-phosphate dehydrogenase, was previously reported to be positively regulated by σ^B in *L. monocytogenes* exposed to salt stress (8) or grown intracellularly (24) and in the gastrointestinal tract (6) but was downregulated by σ^B in stationary-phase cells (8). Taken together, our data provide additional evidence supporting the hypothesis that the composition of the σ^B regulon is dynamically dependent on environmental conditions (7). Importantly, our data also demonstrate that a number of genes downregulated by FPSS are specifically regulated by σ^B in the gastrointestinal environment. For example, three additional genes downregulated by FPSS (i.e., *lmo0642*, *lmo1251*, and *lmo1930*) had higher transcript levels in the *L. monocytogenes* parent strain than in the Δ*sigB* strain when both were grown in the murine intestine (6), but these genes did not appear to be σ^B dependent under other *in vitro* conditions (5, 7, 8, 16, 17)

Only a small number of genes upregulated by FPSS have been identified previously as negatively regulated by σ^B. Overall, 32 genes were identified with significantly higher transcript levels in FPSS-treated *L. monocytogenes* than in untreated cells (Table 1), suggesting negative regulation of these genes by σ^B. While 264 genes were previously identified as negatively regulated by σ^B under at least one condition (5–8, 16, 17), only 7 of the 32 FPSS-upregulated genes were represented among these 264 genes. Further, only 14 genes were previously identified as negatively regulated by σ^B under at least two environmental conditions (5–8, 16, 17) and none of the 32 FPSS-upregulated genes were represented among these 14 genes. Very few genes appear to be consistently repressed by σ^B under various conditions, likely because these genes are indirectly rather than directly regulated by σ^B. Six of the 7 FPSS-upregulated genes previously identified as negatively regulated by σ^B encode proteins with known functions, including an ABC transporter (*lmo2114*), a posttranslocation chaperone (*prsA*), a methyl-accepting chemotaxis protein (*lmo1699* and *lmo1700*), NADP glutamate dehydrogenase (*lmo0560*), and a D-alanine-activating enzyme (*dltA*). While *dltA* (the first gene in an operon encoding proteins that modify lipoteichoic and wall teichoic acids) was previously shown to be negatively regulated by σ^B, other genes in this operon (i.e., *lmo0973* [*dltB*] and *lmo0971* [*dltD*]) not previously identified as σ^B dependent were also significantly upregulated following FPSS treatment, suggesting that this entire operon is negatively regulated by σ^B, at least under some conditions. Two genes, *lmo2568* (unknown function) and

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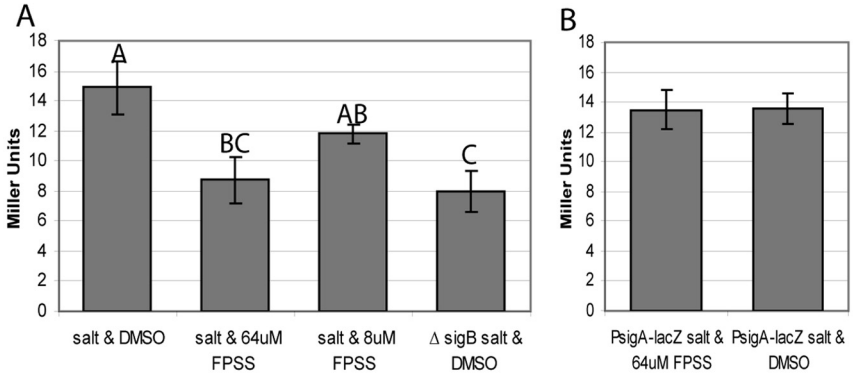

FIG 3 *B. subtilis* β-galactosidase assay. (A) β-Galactosidase activity of a *B. subtilis* strain with a σ^B-dependent P*ctc-lacZ* reporter fusion treated with (i) 0.3 M NaCl and DMSO, (ii) 0.3 M NaCl and 64 μM FPSS, and (iii) 0.3 M NaCl and 8 μM FPSS; this panel also shows the activity for an isogenic Δ*sigB* strain with the P*ctc-lacZ* reporter fusion treated with 0.3 M NaCl and DMSO. (B) β-Galactosidase activity of a *B. subtilis* strain with a σ^A-dependent P*rsbRSTU-lacZ* fusion treated with either (i) 0.3 M NaCl and 64 μM FPSS or (ii) 0.3 M NaCl and DMSO. The data are means from at least three biological replicates; error bars show the standard deviations. Different letters above the bars indicate strains or treatments that differed significantly ($P < 0.05$; GLM Tukey).

lmo1637 (encoding a protein similar to a membrane protein), were upregulated by FPSS but had previously been reported to be positively regulated by σ^B in the intestine (6) and during various growth phases (16), respectively. Among the genes not previously reported as σ^B dependent that were upregulated by FPSS, some were involved in ABC transport, motility, and cell division, but most had unknown functions (Table S4).

Further supporting the idea that FPSS treatment of *L. monocytogenes* generates the equivalent of a Δ*sigB* phenotype, GSEA identified genes in the "motility and chemotaxis" role category as enriched among genes upregulated by FPSS (FDR $q < 0.0001$), consistent with previous reports that σ^B negatively regulates motility-related functions (8). In addition, six FPSS-upregulated genes (*lmo0678* to *lmo0681*, *lmo0685*, and *lmo0686*) not described previously as negatively regulated by σ^B were located in a large flagellar biosynthesis and motility operon (*lmo0673* to *lmo0718*) containing 13 genes recognized as negatively regulated by σ^B(8). As transcription of a number of motility related genes was affected by FPSS, GSEA was also performed on the regulons of the known chemotaxis- and motility-related regulators DegU, MogR, and CodY. The DegU operon, as defined by Williams et al. (25), was enriched among FPSS-upregulated genes (FDR $q < 0.0001$); DegU is an activator of flagellum biosynthesis (26). Specific DegU-regulated genes identified among the FPSS-upregulated genes include six genes in the flagellum biosynthesis operon (*lmo0673* to *lmo0718*) as well as the σ^B-repressed (8) methyl-accepting chemotaxis operon (*lmo1699* and *lmo1700*) (25). The CodY regulon was also significantly enriched among FPSS-upregulated genes (FDR $q < 0.0001$); CodY is a negative regulator of genes encoding flagellar components in *L. monocytogenes* (27). Although MogR, the transcriptional repressor of flagellum genes (28, 29), was shown previously to be σ^B dependent (6), its regulon (29) was not significantly enriched among the genes differentially regulated by FPSS (FDR $q = 0.257$).

FPSS reduces *L. monocytogenes* invasion of human enterocytes. FPSS treatment (at either 8 μM or 64 μM) reduced *L. monocytogenes*' ability to invade Caco-2 human enterocytes by 1.50 log (± 0.49) and 1.42 log (± 0.39), respectively, compared to the untreated control bacteria (Fig. S3) ($P < 0.05$). Invasion of *L. monocytogenes* treated with 8 μM FPSS was not significantly different from invasion by the Δ*sigB* strain, which also showed significantly reduced invasion compared to the untreated parent strain ($P < 0.05$), providing phenotypic evidence of FPSS inhibition of σ^B-regulated virulence functions that contribute to orally acquired listeriosis (9).

FPSS inhibits σ^B activity in *B. subtilis*, indicating effectiveness across genera. To determine if FPSS affects σ^B activity in bacteria other than *L. monocytogenes*, the compound was tested for its ability to specifically inhibit σ^B activity in *B. subtilis*. FPSS (64 μM) significantly inhibited σ^B-dependent *ctc-lacZ* activity ($P < 0.05$; GLM with post-hoc Tukey's HSD test) to levels equivalent to those in a Δ*sigB* strain (Fig. 3) ($P > 0.05$) without reducing β-galactosidase activity from a σ^A-dependent *lacZ* fusion (30) (Fig. 3), further supporting FPSS specificity for inhibiting σ^B activity.

DISCUSSION

By using a high-throughput screen of approximately 57,000 small molecules, 41 candidate compounds were identified as potential inhibitors of *L. monocytogenes* σ^B activity. Through subsequent screens, we identified a compound designated FPSS that specifically inhibits σ^B-mediated transcription, as shown by qRT-PCR of σ^B-dependent genes and whole-genome microarray analysis of cells treated with the compound. This compound also significantly reduces *L. monocytogenes* invasion into human intestinal epithelial cells and inhibits σ^B-directed activity in the Gram-positive bacterium *B. subtilis*, indicating that this compound inhibits σ^B-mediated transcription across genera. Overall, our data show that FPSS (i) inhibits expression of the σ^B regulon with high specificity, yielding transcriptional profiles similar to those generated by a genetic null mutation of the *sigB* gene, and (ii) specifically inhibits expression of σ^B-dependent genes important for virulence, stress response, and other functions associated with *L. monocytogenes* survival and growth in the gastrointestinal tract. In combination with previous reports that identified small molecules that interfere with virulence factors and virulence activation and that show therapeutic promise (14, 31), our results suggest that, in addition to its role as a promising tool for studying regulatory networks involving σ^B, FPSS also may represent a compound that can be developed into a therapeutic agent.

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FPSS specifically inhibits expression of σ^B-dependent virulence, stress response, and other functions that are associated with *L. monocytogenes* growth and survival in the gastrointestinal tract. σ^B is well recognized as an important transcriptional regulator in multiple Gram-positive genera. For example, σ^B regulates transcription of genes contributing to stress response, virulence, or both in low-GC Gram-positive microbes, including human pathogens such as *Bacillus cereus* (32, 33), *Bacillus anthracis* (34), *Staphylococcus aureus* (35, 36), and the opportunistic pathogen *S. epidermidis* (37). σ^B activates transcription of a large number of target genes across the genera and species reported to date (e.g., *L. monocytogenes*, *Listeria innocua*, *S. aureus*, and *B. subtilis*) (8, 38, 39). FPSS treatment of *L. monocytogenes* affects expression of σ^B-dependent genes that are upregulated in the host intestine but that had not been identified previously as σ^B dependent under other *in vitro* conditions. Specifically, among 172 genes in *L. monocytogenes* EGD-e that were upregulated by σ^B in the murine intestinal lumen (6), FPSS treatment significantly downregulated 126 genes; 17 genes downregulated by FPSS had been identified as σ^B dependent only in the intestinal lumen (6) but not in other *in vitro* test systems. Thus, identification of σ^B-dependent genes in *L. monocytogenes* treated with FPSS may provide new insight into σ^B-dependent gene regulation that may be critical during the gastrointestinal stage of infection. For example, the PrfA regulon was significantly enriched among genes downregulated by FPSS treatment, including two PrfA-dependent genes (i.e., *lmo0937* and *plcA*) that had not been identified previously as σ^B regulated. These findings are consistent with σ^B's role, via the $P2_{prfA}$ promoter, in directly upregulating *prfA* transcription (11–13) and also support the idea that σ^B-dependent upregulation of *prfA* transcription plays a critical role during intestinal stages of infection. Activation of σ^B in the intestinal lumen thus appears to increase expression of σ^B-dependent *inlA*, which is required for intestinal epithelial cell invasion (40), and also primes expression of PrfA, which is critical for regulating virulence gene expression during the subsequent intracellular stages of infection.

FPSS-treated *L. monocytogenes* also had higher transcript levels than nontreated cells for a number of genes involved in chemotaxis and motility. Several genes in a large operon encoding flagellar structural components were previously reported to be negatively regulated by σ^B (8), and *sigB*-null mutants also exhibited increased swarming (6, 8). σ^B-dependent downregulation of transcripts encoding flagellar components and overall motility appear to be at least partially due to σ^B-dependent transcription of a long untranslated region (UTR) upstream of *mogR*, which encodes a negative regulator of *L. monocytogenes* motility. Reduced transcription of this UTR not only reduces *mogR* transcript levels (thereby increasing flagellar motility) but also appears to increase transcript levels for some flagellar genes, as the σ^B-dependent UTR also decreases flagellin gene transcripts through an antisense-RNA-type mechanism (6, 8). While *L. monocytogenes* flagellar motility appears to contribute to intestinal invasion (41), σ^B-dependent downregulation of flagellar expression in the intestinal lumen may be critical for subsequent stages of infection, as *Listeria* downregulates flagellar gene expression during infection (10) to evade the immune system; increased expression of flagellar components can induce potent proinflammatory effects via TLR5-mediated immunogenicity (42).

Inhibitors of alternative σ factor activation represent potential avenues for development into therapeutics. In addition to its value as a compound that can be used to study regulatory pathways involving σ^B, FPSS also may provide a starting point for development of new therapeutic compounds that interfere with regulatory pathways critical for infection and virulence. Several small molecules that target transcription regulators inhibit virulence and virulence-associated characteristics *in vitro* and *in vivo*, suggesting that these targets are suitable for development of novel therapeutics against bacterial infections (43, 44). Importantly, prokaryotic transcriptional machinery, as represented by interactions between σ^{70} and the β' subunit of core RNA polymerase in *Escherichia coli*, can be disrupted by small molecules without affecting eukaryotic transcription (45). Consequently, the therapeutic potential of novel compounds that interfere with transcriptional regulation of bacterial virulence functions is of emerging interest.

Virstatin is an example of a small molecule with therapeutic potential that has been shown to inhibit transcriptional regulation in *V. cholerae*. Virstatin interferes with the virulence gene regulator ToxT, a member of the AraC family of transcriptional regulators, thus showing potential for treatment of *Vibrio* infections (14). Small-molecule inhibitors also have been identified for other members of the AraC transcription factor family, e.g., MarA, SoxS and Rob in *E. coli* (43) and LcrF in *Yersinia* spp. (44). As with σ^B, AraC-type regulators typically contribute to transcription of multiple stress response (46) and virulence factors (47, 48); therefore, inhibition of these and similar transcriptional regulators can result in broad physiological consequences for the affected microbes (43).

The small molecule identified here, FPSS, inhibits σ^B activity at an IC_{50} of 3 to 3.5 μM. By comparison, the ToxT inhibitor virstatin (14) has an MIC between 3 and 40 μM, depending on the target strain. Minimal bactericidal concentrations of gentamicin, ampicillin, and streptomycin against *L. monocytogenes* range from 2 to 46 μM (49). In addition to its promising IC_{50} prior to structural optimization, FPSS produces highly specific, genome-wide reduction of σ^B-directed activity, including inhibited expression of σ^B-dependent virulence genes such as *inlAB*, *bsh*, *bilE*, *clpC*, and *hfq* (5–8, 16, 17). Furthermore, *opuC* (50) and *gadA* (51), which are important for gastrointestinal survival in the host, are also significantly downregulated by FPSS. FPSS clearly inhibits transcription of a number of genes with functions in virulence and infection, thus increasing its therapeutic potential over compounds that target only one virulence factor (43). The contributions of σ^B to *L. monocytogenes* virulence are also supported by phenotypic evidence, including reduced virulence of a Δ*sigB* strain in a guinea pig model of infection (9) and reduced invasion of human Caco-2 cells by a Δ*sigB* strain (9, 40), consistent with the reduced invasiveness for FPSS-treated *L. monocytogenes* observed here. Importantly, σ^B also contributes to establishment of infection and virulence in other Gram-positive pathogens, including *B. anthracis* and *S. aureus*. A *B. anthracis sigB* mutant is less virulent than the parent strain, producing a 1-log reduction in 50% lethal dose, perhaps because σ^B enhances the ability of *B. anthracis* to persist in the bloodstream of a mammalian host (34). In *S. aureus*, σ^B directly and indirectly modulates global regulatory elements involved in virulence functions (52). Functional loss of σ^B results in decreased *S. aureus* virulence in central venous catheter-related diseases manifested by significantly reduced multiorgan infection (53). Similar to *B. anthracis*, σ^B is suggested to promote *S. aureus* survival in the bloodstream, preventing clearance and

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allowing establishment of infection (54). Further development and optimization of FPSS thus may provide an opportunity to develop novel therapeutics for some important Gram-positive pathogens.

MATERIALS AND METHODS

Strain and media selection. Strains used in this study included the *L. monocytogenes* parent strain 10403S (serotype 1/2a) (55), its otherwise isogenic Δ*sigB* derivative (FSL A1-254) (56), a reporter strain for σ^B activity (FSL S1-063 [10403S *opuCA-gus*]) (7, 57, 58), and a negative-control reporter strain for σ^B activity (FSL S1-059 [Δ*sigB opuCA-gus*]) (Table S5). To evaluate the effectiveness of a selected small molecule to inhibit σ^B activity in a Gram-positive genus other than *Listeria*, *B. subtilis* strains bearing reporter fusions for either σ^B or σ^A activity and a Δ*sigB* negative-control reporter strain (Table S5) were also tested. To achieve low background fluorescence, a chemically defined minimal medium (59) with 25 mM glucose (DMG) (60) was used for the high-throughput screen. Cells were grown in brain heart infusion broth (BHI; Difco, Sparks, MD) for phenotypic and transcriptional profiling assays.

Primary high-throughput cell-based small-molecule screen. The *L. monocytogenes opuCA-gus* fusion strain FSL S1-063 was used in a cell-based high-throughput screen (HTS) against ~57,000 compounds. As reported at http://ChemBank.Broad.Harvard.edu, the libraries included the following: (i) known bioactive compounds, including FDA-approved drugs (i.e., the SPBio and SMP libraries); (ii) synthetic compounds from diversity-oriented synthesis (e.g., the CMLD, ICCB, PK04, Ald1.1-H, and Sulf1.1-A libraries); (iii) natural products (i.e., the PhilEx and ICBGEx libraries); and (iv) commercially available compounds (e. g., the ChemDiv3, Maybridge4, and TimTec1 libraries). Table S1 contains a complete listing of libraries screened for this study.

Multidrop liquid-handling robots (Matrix, Thermo Fisher) were used to dispense 27 μl of DMG into black-walled clear-bottom 384-well plates (Nunc, Rochester, NY), and then 100 nl of each small-molecule stock was transferred from the library stock or source plate to the assay plates with a CyBi-Well Vario pipettor (CyBio AG, Jena, Germany). Final experimental concentrations of the small molecules used in the assays were dependent on each stock concentration [e.g., (*E*)-*N*,2-diphenylethenesulfonamide had a stock concentration of 19.3 mM, producing a 64.3 μM final concentration in each well]. Each source plate contained approximately 15 wells to which only dimethyl sulfoxide (DMSO) was added, as the small molecules were dissolved in DMSO; these wells are referred to as DMSO-only negative-internal-control wells. All source plates were prepared in duplicate to provide experimental replicates (i.e., plates A and B). Two plates in which all wells contained medium with DMSO and *L. monocytogenes* (inoculated as detailed below) were included as external plate controls. *L. monocytogenes* strains were grown to an optical density at 600 nm (OD_{600}) of approximately 0.4 (3 hours) in BHI, cultures were diluted 1:50 with DMG, and then 3 μl of the appropriate diluted culture was added to each well. As a control, a custom assay plate containing 192 wells of the 10403S *opuCA-gus* strain FSL S1-063 and 192 wells of the otherwise isogenic Δ*sigB opuCA-gus* strain FSL S1-059 was treated with only DMSO.

All plates were sealed and incubated for 18 h at 37°C. To determine bacterial growth or inhibition in the presence of the compounds, absorbance (OD_{600}) was measured using a Synergy HT multimode microplate reader (Biotek Instruments, Winooski, VT) after ~18 h of incubation. To measure fluorescence for β-glucuronidase (GUS) activity determination, black seals (PerkinElmer, Waltham, MA) were affixed to the bottoms of the plates after the absorbance readings were completed. Cells were then lysed using 5 μl of 2× CelLyticB (Sigma, St. Louis, MO) and a protease inhibitor cocktail mixture (1 ml 2× CelLyticB and 0.05 ml protease inhibitor cocktail; Sigma), immediately prior to the addition of 4 μl of 1.6 mg/ml 4-methylumbelliferyl β-D-glucuronide hydrate (4-MUG; Sigma) in DMSO. Reaction mixtures were incubated in the dark for 1 h at room temperature (~23°C), and reactions were stopped by the addition of 0.2 M Na_2CO_3. Fluorescence was read using a Wallac 2102 EnVision Multilabel Reader (PerkinElmer) with an excitation wavelength of 355 nm and an emission of 460 nm.

Statistical analysis of primary screen data. To identify compounds that inhibited σ^B activity without affecting *L. monocytogenes* growth, *opuCA*-directed GUS activity in the presence of each compound was calculated by dividing relative fluorescence units (RFU) by cell density in OD_{600} units (RFU/OD) (61). Statistical analyses were conducted in collaboration with the Broad Institute and performed as previously described (62, 63). Raw and analyzed data were deposited in ChemBank (64, 65). The software package Spotfire DecisionSite Analytics (TIBCO Spotfire, Somerville, MA) was used for two-dimensional data visualization.

Secondary screen and dose response curve. Forty-one compounds that appeared to inhibit σ^B activity (*Z* score of ≤-3 in both replicates) were selected for secondary cell-based screening using the assay and reporter fusion described above to calculate initial IC_{50}s. Each compound was diluted in DMSO in a series of six 1/5 dilutions of the initial stock concentration [e.g., starting from 19.3 mM stock, (*E*)-*N*,2-diphenylethenesulfonamide was diluted in a series of six 1 to 5 dilutions, yielding concentrations of 3.86 mM to 1.24 μM]. The small molecules at these concentrations were then dispensedinto the assay plates.

Small-molecule microarray screens. Two different arrays, each printed with 8,500 small-molecule (SM) spots and 1,500 DMSO control spots, were used to screen for binding of σ^B to the small molecules. Small-molecule microarrays (SMMs) were printed on glass slides at the Broad Institute as described previously (66–68). The immobilized SMs included 8,500 compounds created by diversity-oriented synthesis and 8,500 compounds representing natural products, FDA-approved drug-like compounds, commercial compounds, and known bioactive compounds (Table S1) (68; http://Chembank.broadinstitute.org). SMM screening (three replicates) was performed as described by Bradner et al. (66). His-tagged σ^B was purified from *E. coli* M15, kindly provided by W. Goebel (69). Data analyses included (i) assessment of signal-to-noise ratio (SNR) of the spot feature; (ii) *Z* score calculations based on comparison of signals from compound spots to signals from DMSO control spots within a slide; and (iii) composite *Z* score calculations for data from the three replicates. Spotfire Analytics software was used for three-dimensional data visualization.

FPSS. (E)-*N*,2-diphenylethenesulfonamide, the compound identified by HTS as being responsible for the greatest inhibition of σ^B activity, was not commercially available. Therefore, the analog fluoro-phenyl-styrenesulfonamide [IUPAC name (*E*)-*N*-(4-fluorophenyl)-2-phenylethenesulfonamide; ChemBank ID, 2063822; MW 277.3] was obtained from Enamine Ltd. (Kiev, Ukraine). FPSS was dissolved in DMSO to a concentration of 10 mM. The solution was filter sterilized using with a 0.1-μm filter (Omnipore membrane filter; Millipore Corporation, Billerica, MA) and a Swinney stainless 13-mm holder for syringe filtration (Millipore Corporation).

Bile salt hydrolase (BSH) activity assay. As *L. monocytogenes bsh*, which encodes bile salt hydrolase, is σ^B dependent (5, 7, 15), a qualitative BSH activity assay was used to determine the FPSS concentration needed for σ^B inhibition. Four-well multidish plates (26 mm by 33 mm; Nunc) containing 6 ml of either BHI agar or de Man, Rogosa and Sharp (MRS) agar medium (BD Biosciences, San Jose, CA) containing 0.5% (wt/vol) glycodeoxycholic acid sodium (GDCA) salt (Calbiochem, San Diego, CA) (70) with either no FPSS or 96, 193, or 290 μM of FPSS [1.5, 3, or 4.5 times the 64.3 μM concentration used for (E)-N,2-diphenylethenesulfonamide in the HTS] were prepared and allowed to dry overnight. *L. monocytogenes* 10403S and Δ*sigB* were grown in BHI broth to exponential phase, defined as an OD_{600} of 0.4, and then 4 μl of culture was spotted in parallel on MRS and BHI agars. The MRS agar plates were incubated anaerobically using the BD-BBL GasPak anaerobic system (Becton Dickinson, Franklin Lakes, NJ), while BHI plates were incubated aerobically. Both sets of plates were incubated for 48 h at 37°C and then were visually assessed for growth (BHI plates) or the presence of a white precipitate comprised of deconjugated

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bile salts indicating BSH activity (MRS plates). The assay was performed three times.

RNA isolation. For RNA isolation, *L. monocytogenes* 10403S and Δ*sigB* strains were initially grown overnight in 5 ml of BHI broth at 37°C with shaking (230 rpm), followed by subculturing twice, each time at an OD_{600} of 0.4, using a 1% (vol/vol) transfer into 5 ml of prewarmed BHI. When the second subculture reached an OD_{600} of 0.4, cells were treated with a total volume of 76 μl comprising FPSS (to yield final concentrations ranging from 1 μM to 128 μM) and/or DMSO, followed immediately by addition of 324 μl of either 5 M NaCl (to yield a final concentration of 0.3 M NaCl, an osmotic stress that induces σ^B activity [8]) or (ii) sterile distilled water. Treated cultures were then incubated at 37°C with shaking (230 rpm) for 10 min, followed by addition of 2 volumes of RNAprotect (Qiagen Inc., Valencia, CA) and subsequent incubation at room temperature for 10 min. The cells were harvested following centrifugation for 10 min at 5,000 × *g*, and cell pellets were stored at −80°C until RNA was extracted and DNase treated using an Ambion RiboPure kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA concentrations and purity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA quality was analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and only RNA with an integrity number of ≥8 was used. Each treatment was performed 3 times.

TaqMan qRT-PCR. Transcript levels for the σ^B-dependent genes *opuCA* and *gadA* and the housekeeping genes *rpoB* and *gap* were quantified with TaqMan primers and probes (13, 58, 71) using RNA prepared as described above and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as previously described (71). qRT-PCR was also used to determine the FPSS IC_{50}. Inhibition was measured using *opuCA* transcript levels determined with RNA isolated from cells treated with a series of 1:2 dilutions of FPSS, with concentrations ranging from 128 to 1 μM. These data were assessed using SigmaPlot 10.0 (SYSTAT Software Inc., Evanston, IL) standard curve analysis under the pharmacology function.

Whole-genome microarray. cDNA labeling for microarray analyses and microarray hybridization were performed as previously described (5) using *L. monocytogenes* whole-genome microarrays (8, 72). Raw intensity values for all probes on each array were normalized using pin-tip LOWESS (8) in R version 2.2.1 with the LIMMA package. Signals from two replicate probes on each array were then averaged and $\log_2$ transformed. Differences in transcript levels between strains were determined using a linear model, and *P* values were determined using eBayes. Differences in transcript levels were considered meaningful if they met the following three criteria: (i) adjusted *P* values of <0.05 (ii) absolute fold changes of ≥2 and (iii) a probe cross-hybridization index (CHI) of >90%. One gene (i.e., *lmo0263*) fulfilled criteria (i) and (ii) but not (iii) (probe CHI was 80%) and therefore was not included in our analyses.

Gene set enrichment analysis (GSEA; Broad Institute, Cambridge, MA) (73) was used to identify gene sets that were significantly enriched among up- or downregulated genes. GSEA was run on the ranked list of log fold change values obtained from the fitted normalized data in LIMMA with 1,000 permutations and exclusion of gene sets with <5 or >2,000 members. Genes were classified into sets based on the TIGR Comprehensive Microbial Resource (http://cmr.tigr.org) subrole categories for *L. monocytogenes* EGD-e. False discovery rate *q* values of <0.25 were considered significant (73).

Caco-2 invasion assay. *L. monocytogenes* invasion assays using the human colorectal adenocarcinoma epithelial cell line Caco-2 (ATCC HTB-37) were performed as described by Garner et al. (9). *L. monocytogenes* 10403S and Δ*sigB* strains were initially grown overnight in 5 ml of BHI broth at 37°C with shaking (230 rpm), then were subcultured twice, at an OD_{600} of 0.4, using a 1% (vol/vol) transfer into 5 ml of prewarmed BHI. When the second subculture reached an OD_{600} of 0.4, cells were treated with (i) FPSS to yield final concentrations of 64 μM or 8 μM (lowest concentration with full efficacy according to qRT-PCR) or (ii) DMSO as well as NaCl (0.3 M final concentration) as described above, except that treated cultures were incubated at 37°C for 30 min. For infection, the Caco-2 cells were inoculated with approximately 2×10^7 *L. monocytogenes* organisms; bacterial numbers were confirmed by plating on BHI agar. Four biological replicates were each performed in triplicate wells. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's studentized range (honestly significant difference [HSD]) test, performed in SAS 9.0 (SAS Institute).

β-Galactosidase enzyme assays in *B. subtilis*. *B. subtilis* strain PB198 (*amyE*::*pDH32-ctc trpC2* [74]) and the otherwise isogenic Δ*sigB* strain PB345 (*amyE*::*pDH32-ctc sigBΔ3*::*spc trpC2* [75]) were used as reporter strains for measuring σ^B activity (Table S5). Effects of FPSS on the σ^A housekeeping sigma factor in *B. subtilis* were tested using *B. subtilis* strain PB252 (*amyE*::P_A-*lacZ trpC2* [30]). Strains were grown overnight in 5 ml of buffered Luria-Bertani (LB) broth at 37°C with shaking (230 rpm) and then were subcultured twice, at an OD_{600} of 0.4, using a 1% (vol/vol) transfer into 5 ml of prewarmed LB. When the second subculture reached OD_{600} of 0.4, cells were treated with (i) FPSS (8 or 64 μM) or DMSO only (as a control) and (ii) DMSO as well as NaCl (0.3 M final concentration) as described above, with treated cultures being incubated at 37°C for 30 min. After this incubation, the OD_{600} was recorded, and 0.2 ml of the culture was added to a tube containing 2.8 ml Z buffer, followed by the addition of 20 μl toluene to permeabilize the cells. A prewarmed 0.4-ml volume of 4-mg/ml ortho-nitrophenyl-β-galactoside (ONPG) was added, and the time of addition was noted. After 85 min, 1 ml of 1 M sodium carbonate was added to stop the reaction, and the OD_{420} was read. Miller units were calculated as previously described (76). β-Galactosidase activity results were analyzed using one-way ANOVA and Tukey's studentized range (HSD) test.

Microarray data accession number. Data from microarray experiments were submitted to the Gene Expression Omnibus (GEO) database, assigned accession number GSE16887, and approved.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00241-11/-/DCSupplemental.

Figure S1, TIF file, 0.5 MB.
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Table S5, DOCX file, 0.1 MB.

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