Extracytoplasmic Function σ Factors with Overlapping Promoter Specificity Regulate Sublancin Production in *Bacillus subtilis*[†]

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Bacillus subtilis harbors seven extracytoplasmic function (ECF) σ factors. At least three ECF σ factors (σ^M , σ^W , and σ^X) are induced by, and provide resistance to, antibiotics and other agents eliciting cell envelope stress. Here, we report that ECF σ factors also contribute to antibiotic production. *B. subtilis* 168 strains that are lysogenic for the SP β bacteriophage produce sublancin, which inhibits the growth of other, nonlysogenic strains. Genetic studies demonstrate that synthesis of sublancin is largely dependent on σ^X , with a smaller contribution from σ^M . A *sigM sigX* double mutant is unable to produce sublancin. This defect is primarily due to the fact that the sublancin biosynthesis is positively activated by the transition state regulator and AbrB paralog Abh, which counteracts transcriptional repression of the sublancin biosynthesis operon by AbrB. Ectopic expression of *abh* bypasses the requirement for σ^M or σ^X in sublancin synthesis, as does an *abrB* mutation. In addition to their major role in regulating sublancin expression by activating *abh* transcription, σ^X and σ^M also have a second role as positive regulators of sublancin expression that is independent of AbrB and Abh. Since sublancin resistance in nonlysogens is largely dependent on σ^W , ECF σ factors control both sublancin production and resistance.

Bacillus subtilis, a ubiquitous soil bacterium, inhabits a fiercely competitive niche and devotes a large fraction of its genome to genes implicated in the synthesis of, and resistance to, antibacterial compounds. Regulation of antibiotic resistance functions is frequently mediated by extracytoplasmic function (ECF) σ factors which, as a class, control functions related to cell envelope and transport.

B. subtilis harbors seven known ECF σ factors: σ^M , σ^W , σ^X , σ^Y , σ^Z , σ^V , and σ^{YlaC} (18). Of these, the σ^M , σ^W , and σ^X regulons are the best-characterized. These three σ factors control overlapping sets of genes that are important for resistance against a variety of cell envelope-active compounds and antibiotics. In several cases, resistance genes are controlled predominantly by a single ECF σ factor. For example, σ^W controls genes that confer resistance to the peptidoglycan synthesis inhibitor fosfomycin (4), the toxic peptide SdpC, and the lantibiotic sublancin (3). The σ^M regulon includes a large number of operons implicated in cell wall synthesis, and the corresponding *sigM* mutant is sensitive to several cell wall antibiotics, including bacitracin, vancomycin, and moenomycin. The σ^X regulon includes operons that modulate the net charge of both the cell membrane and cell wall, and a *sigX* null mutant displays increased sensitivity to nisin and other cationic antimicrobial peptides (5, 6). In other cases, resistance to antimicrobial compounds appears to be dependent on a gene(s) that is potentially expressed by more than one ECF σ factor. As a result, strains carrying multiple deletions in *sigM*, *sigW*, and *sigX* show sensitivities to additional antibiotics such as D-cycloserine and some beta-lactam antibiotics (22).

B. subtilis strains are known to produce more than two dozen

antibiotics (32). In several cases, antibiotic biosynthesis and associated immunity functions are encoded on genomic islands or phages and are potentially transferred between closely related species by either transduction or natural competence. Sublancin 168 (hereafter called sublancin) is one of the bactericidal antibiotics produced by the reference strain, *B. subtilis* 168. Sublancin is classified as an unusual lantibiotic, with a β -methyllanthionine bridge and two disulfide bridges (27). Sublancin is extremely stable and is active against gram-positive bacteria, including strains of *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. It inhibits both the outgrowth of endospores and vegetative cell growth. The mode of action has been speculated to involve pore formation in the cytoplasmic membrane. Kouwen et al. have recently found that a mechanosensitive channel of conductance, MscL, is required for sublancin susceptibility, but the precise mechanism remains unknown (21).

The sublancin operon is located in the prophage SP β genome. This 4.5-kb segment contains five genes: *sunA*, *sunT*, *bdbA*, *yolJ*, and *bdbB* (Fig. 1A). All except *bdbA* are essential for sublancin production (9, 10). The structural gene *sunA* encodes presublancin, which is a 56-residue polypeptide possessing a 19-residue leader segment (27). *sunT* is located immediately after the *sunA* gene and encodes an ABC-type transporter containing a proteolytic domain thought to cleave the leader peptide of sublancin during secretion across the membrane (10, 25). BdbB and BdbA comprise a thiol-disulfide oxidoreductase involved in the posttranslational formation of disulfide bonds in sublancin (10). YolJ contains a CxxS motif, but its function remains unknown (9).

The regulation of sublancin synthesis is complicated and involves at least three transcriptional regulators (AbrB, Abh, and Rok) (1, 33). AbrB is a global transcriptional regulator that represses expression of transition-stage genes during vegetative growth. Abh is a paralog of AbrB and is itself repressed by AbrB (33). Both AbrB and Abh bind directly to overlapping regions within the *sunA* regulatory region (Fig. 1A). Rok is a

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FIG. 1. (A) Schematic of the sublac operon, showing regulatory protein binding sites important for sublac expression. AbrB and Abh binding sites are located upstream of a σ^A promoter. The *sunA* gene is, at least, cotranscribed with *sunT*, and a hairpin structure is found between *sunA* and *sunT*. The complementary sequence of the hairpin is indicated in a dot-bracket notation. (B) ECF σ factors regulate the synthesis of sublac, which acts against other *Bacillus* spp. Strain W168, or the isogenic strain carrying a deletion of three sigma factor genes ($\Delta sig MWX$) or *sunA* ($\Delta sunA$) were spotted onto lawns of 23 other bacillus strains. Only three representative lawns of *Bacillus* spp. are shown (see Table S1 in the supplemental material for a complete list). A clear zone is observed if the lawn strain is sensitive to the compounds produced by the spotted strains.

direct repressor of the transcription activator ComK that drives competence development. Rok binds to the *sunA* promoter region in vitro, and deletion of *rok* increases transcription of *sunA* and *sunT* (1).

In this study, we show that the synthesis of sublac requires the activity of either of two ECF σ factors, σ^M and σ^X . Genetic analyses establish that the influence of σ^M and σ^X results from their ability to activate transcription of *abh*, which encodes a paralog of the pleiotropic transition state regulator protein AbrB. Both σ^M and σ^X can recognize an ECF σ factor-dependent promoter element preceding the *abh* gene, and expression of *abh* from a heterologous promoter bypasses the requirement for either of these ECF σ factors. The function of Abh in activation of the *sunA* operon is to prevent repression by AbrB; *sunA* activity is constitutive in an *abrB abh* double mutant strain. Together with previous results, these findings establish that ECF σ factors control both the production of and resistance to antimicrobial compounds.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. All *B. subtilis* strains, plasmids, and oligonucleotides (oligos) used in this study are listed in Table 1. Bacteria were grown in liquid Luria-Bertani (LB) medium at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bacto agar (Difco) with appropriate selection. Plasmids were amplified in *Escherichia coli* DH5 α before transformation with *B. subtilis* strains. Ampicillin (AMP; 100 μ g ml⁻¹) was used to select *E. coli* strains harboring the desired plasmids. For *B. subtilis*, antibiotics used for selection were as follows: spectinomycin (SPC; 100 μ g ml⁻¹), kanamycin (KAN; 10 μ g ml⁻¹),

tetracycline (TET; 5 μ g ml⁻¹), chloramphenicol (CAT; 5 μ g ml⁻¹), and macro-lide-lincosamide-streptogramin B (MLS; contains 1 μ g ml⁻¹ erythromycin and 25 μ g ml⁻¹ lincomycin).

Genetic techniques. Gene deletions were generated using long-flanking homology PCR (LFH-PCR) as described previously (3, 23) with selection for the appropriate antibiotic resistance cassette. Chromosomal DNA transformations were performed as described previously (17).

Spot-on-lawn assays. Spot-on-lawn assays were performed as previously described (3). Briefly, lawn cells were grown to optical density at 600 nm (OD₆₀₀) of 0.4 in LB, mixed 1:50 (culture:medium) with 2 ml melted 0.7% or 1.5% LB agar, and poured into wells of an eight-well rectangular multidish (26 mm by 33 mm; Nunc). Plates were dried for 30 min in a laminar flow hood, and 2 μ l of the producer strain (OD₆₀₀ of 0.6) was spotted in the center of the well. Plates were incubated at 37°C overnight (18 h) before observation. Spot-on-lawn assays of each strain were performed in biological triplicates and repeated at least three times. Several mutant strains harbor a reporter fusion, *amyE::P_{sunA}-lacZ(cat)*. The presence of this reporter fusion did not influence the results of the spot-on-lawn assays.

β -Galactosidase assays. Test strains carrying an *abh* promoter-*lacZ* (*P_{abh}-lacZ*) fusion were grown overnight in LB medium containing appropriate antibiotics and diluted 1:100 into 5 ml LB medium. The cultures were incubated at 37°C with vigorous aeration and sampled from logarithmic, transition, and stationary growth phases. β -Galactosidase assays of each strain were performed in biological triplicates as described by Miller (26) and repeated at least three times. Data are reported as the means and standard deviations.

RNA isolation, Northern blotting, and slot blotting. Total RNA was extracted from 2 ml of cells grown to an OD₆₀₀ of 0.4 (mid-log phase) using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The RNA was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies) and visualized by agarose gel electrophoresis. Two DNA probes, *sunA* and 23S rRNA, were constructed by PCR with the primer pairs *sunA*-for (4195) with *sunA*-rev (4196) and 23S-RT-F (4368) with 23S-RT-R (4369), respectively (Table 1). The probes were purified using the Qiagen PCR purification kit and labeled with

TABLE 1. Strains, plasmids and oligos used in this study

Strain, plasmid, or oligo	Genotype or description	Construction, reference, or oligo sequence
<i>B. subtilis</i> strains		
W168	<i>trpC2</i>	Y. Sadaie (Saitama University, Japan)
CU1065	<i>trpC2 attSPβ</i>	Lab strain
HB 5331	CU1065 <i>yqeZyqfAB::kan</i>	3
HB10016	168 <i>sigM::tet</i>	LFH-PCR→168
HB10103	168 <i>sigX::kan</i>	LFH-PCR→168
HB6164	CU1065 <i>sunA::kan</i>	3
HB10111	168 <i>sunA::kan</i>	HB6164 chrDNA→168
HB10113	168 <i>sigM::tet sigX::kan</i>	HB10103 chrDNA→HB10016
HB10131	168 <i>abh::spc</i>	LFH-PCR→168
HB10139	168 <i>abrB::tet</i>	LFH-PCR→168
HB10146	168 <i>abh::spc abrB::tet</i>	HB10139 chrDNA→HB10131
HB0020	CU1065 <i>sigW::mls</i>	4
HB10102	168 <i>sigW::mls</i>	HB0020 chrDNA→168
HB10107	168 <i>sigM::tet sigX::kan sigW::mls</i>	HB10102 chrDNA→HB10113
HB0911	CU1065 <i>sigV::cat</i>	22
HB10101	168 <i>sigV::cat</i>	HB0911 chrDNA→168
HB0009	CU1065 <i>sigY::mls</i>	8
HB10108	168 <i>sigY::mls</i>	HB0009 chrDNA→168
HB0032	CU10665 <i>sigZ::kan</i>	7
HB10109	168 <i>sigZ::kan</i>	HB0032 chrDNA→168
HB0029	CU1065 <i>ylaC::kan</i>	7
HB10110	168 <i>ylaC::kan</i>	HB0029 chrDNA→168
HB10182	168 <i>sunA::kan abh::spc</i>	HB10111 chrDNA →HB10131
HB10143	168 <i>sigM::tet sigX::kan abh::spc amyE::P_{sunA}-lacZ (cat)</i>	HB10131 chrDNA, pYL18→HB10113
HB10155	168 <i>sigM::kan sigX::spc abrB::tet amyE::P_{sunA}-lacZ (cat)</i>	HB10139 chrDNA, pYL18→HB10113
HB10123	168 <i>lacA::P_{xyLA}-abh (mls)</i>	pYL19 →168
HB10124	168 <i>sigX::kan lacA::P_{xyLA}-abh (mls)</i>	HB10103 chrDNA, HB10123
HB10128	168 <i>sigM::tet lacA::P_{xyLA}-abh (mls) amyE::P_{sunA}-lacZ (cat)</i>	HB10016 chrDNA, pYL18→HB10123
HB10137	168 <i>sigM::tet sigX::kan lacA::P_{xyLA}-abh (mls) amyE::P_{sunA}-lacZ (cat)</i>	HB10103 chrDNA, pYL18→HB10123
SWV121	<i>trpC2 pheA1 amyE::P_{abh}-lacZ (cat)</i>	M. Strauch (University of Maryland)
HB10147	168 <i>amyE::P_{abh}-lacZ (cat)</i>	SWV121 chrDNA→168
HB10148	168 <i>sigM::tet amyE::P_{abh}-lacZ (cat)</i>	HB10147 chrDNA→HB10016
HB10149	168 <i>sigX::kan amyE::P_{abh}-lacZ (cat)</i>	HB10147 chrDNA→HB10103
HB10150	168 <i>sigM::tet sigX::kan amyE::P_{abh}-lacZ (cat)</i>	HB10147 chrDNA→HB10113
Plasmids		
pYL18	pDG1661- <i>P_{sunA}-lacZ (cat)</i>	This work
pYL19	pAX01- <i>P_{xyLA}-abh (mls)</i>	This work
Oligos		
4195	sunA-for	ATGGAAAAGCTATTTAAAGAAG
4196	sunA-rev	TCTGCAGAATTGACGATAGT
4368	23S-RT-F	AAAGGCACAAGGGAGCTTGACTGCGAGA
4369	23S-RT-R	ATGAGCCGACATCGAGGTGCCAAACCT
3314	AAP	GGCCACGCGTCTGACTAGTACGGGIIIGGGIIIG
4370	abh-rev-GSP1	GGCTTGAATTTCTTCGA
4371	abh-rev-GSP2	ACGCCATGAGGTTTGACTT

[γ - 32 P]dATP (3,000 Ci/mmol, 10 mCi/ μ l; New England Nuclear) and T4 polynucleotide kinase (New England Biolabs). Unincorporated [γ - 32 P]dATP was removed using a Qiagen PCR purification kit. Northern blotting analysis was carried out by using the NorthernMax formaldehyde-based system (Ambion) following the manufacturer's instruction with 10 μ g of total RNA and Zeta-Probe blotting membrane (Bio-Rad) in a downward transfer setup. The slot blotting was performed as described previously (30), where total RNA was transferred to a Zeta-Probe blotting membrane by using a vacuum. The hybridization and washing steps for both Northern blotting and slot blotting were performed with the NorthernMax hybridization kit according to the manufacturer's instruction. The blots were then wrapped in plastic wrap, exposed for 12 h to a phosphor screen (Molecular Dynamics), and scanned using a Storm 840 PhosphorImager (Molecular Dynamics). The resulting images were analyzed with ImageQuant software (Molecular Dynamics).

5'-RACE. The transcriptional start site of *abh* was determined using 5' rapid amplification of cDNA ends (5'-RACE). Two micrograms of total RNA from a

mid-log-phase culture was reversed transcribed to cDNA using TaqMan reverse transcription reagents (Roche) and oligo abh-rev-GSP1 (4370) as primer. The 3' end of cDNA was tailed with poly(dCTP) using terminal deoxynucleotidyl transferase (New England Biolabs). The tailed cDNAs were then amplified by PCR with primers AAP (3314) and abh-rev-GSP2 (4371). The PCR products were sequenced with Sanger sequencing technology.

RESULTS AND DISCUSSION

ECF σ factors regulate antibiotic production. Previous studies have established that ECF σ factors, and in particular σ^W , play a major role in the antibiotic-inducible expression of genes that provide resistance to bacteriocins and antibacterial peptides produced by other soil microorganisms, including other

FIG. 2. σ^X and σ^M regulate sublancin production. (A) Various deletion strains were spotted onto a *B. subtilis* CU1065 lawn (upper panel) and the hypersensitive strain HB5331 lawn (lower panel). (B) The radii of the zones of inhibition produced by various strains spotted on a lawn of HB5331. The radius was measured as the total diameter of the zone of clearing minus the diameter of the colony spot, divided by a factor of 2. The numbers above each bar are the *P* values for a paired Student *t* test comparing W168 to mutants.

Bacillus spp. (3). To determine whether ECF σ factors also contribute to antibiotic production, we tested the ability of *B. subtilis* 168 (W168) to inhibit the growth of other *Bacillus* spp. using a spot-on-lawn assay. In an initial screen, wild-type strain W168 and its isogenic triple mutant lacking σ^M , σ^W , and σ^X ($\Delta sigMWX$, HB10107) were used as spots, and 23 strains of *Bacillus* spp. (see Table S1 in the supplemental material) were used as lawn cells. Sixteen of the 23 strains showed less susceptibility to the triple σ factor mutant than to strain W168, while the other 7 strains were similarly susceptible to both strains. Three representative strains that have differential susceptibility are shown in Fig. 1B. In all cases, the inhibition zone sizes from $\Delta sigMWX$ spots were significantly reduced compared to those from strain W168. This result indicates that σ^X , σ^M , or σ^W , individually or in combination, can regulate antimicrobial activity against other *Bacillus* spp. Notably, this antimicrobial activity was not observed using the W168 derivative strain CU1065 as the spotted strain (data not shown). Strain W168 differs from strain CU1065 in that it carries prophage SP β , which encodes the lantibiotic sublancin 168 (27). To test whether the killing effect of W168 cells was due to sublancin, a derivative of W168 lacking *sunA* ($\Delta sunA$, HB10111) was spotted on lawns of the same 23 *Bacillus* spp. Deletion of *sunA* abolished the killing effect noted for all 16 strains that showed

a differential susceptibility to W168 versus the $\Delta sigMWX$ strain (Fig. 1B). This suggests that the product of the *sunA* gene (sublancin) is a major antibiotic active against other *Bacillus* spp. and is regulated either directly or indirectly by σ^X , σ^M , or σ^W .

σ^X or σ^M is essential for sublancin production. In order to identify which ECF σ factor(s) regulates sublancin production, more comprehensive spot-on-lawn assays were conducted using single, double, and triple ECF σ mutants as spots. The sublancin-sensitive strain, strain CU1065, was used as the lawn strain. Since CU1065 lacks the SP β prophage it lacks both the ability to synthesize sublancin and the SP β -encoded immunity gene *sunI* (11), rendering it susceptible to killing by sublancin. As shown in Fig. 2A (upper panel), we tested seven single ECF σ deletion mutant spots, and only $\Delta sigX$ (HB10103) and $\Delta sigM$ (HB10016) strains showed reduced inhibition zone sizes compared to that from the W168 spot. Judging from zone diameters, the $\Delta sigX$ strain impaired cell killing to a greater degree than did the $\Delta sigM$ strain. Deletions in *sigX* and *sigM* were additive, as a $\Delta sigMX$ double deletion strain (HB10113) completely abolished the killing effect. This result indicates that σ^X and σ^M are the ECF σ factors that regulate sublancin production.

In order to confirm that *sigM* and *sigX* are strictly required for sublancin production, the $\Delta sigMX$ strain was spotted on a

Total RNA amount/ probe	W168	$\Delta sigM$	$\Delta sigX$	$\Delta sigMX$	Δabh	$\Delta abrB$	$\Delta abh abrB$	$\Delta sunA$
10 μ g /sunA								
100ng/23SrRNA								

FIG. 3. σ^X is the major σ factor regulating *sunAT* transcription, and Abh counteracts the repression of AbrB at *sunAT* transcription. The levels of *sunAT* transcription in W168 and its derivative mutant strains were detected by RNA slot blot analysis using 32 P-labeled *sunA* DNA probe. 23S rRNA was used as a loading control.

lawn of strain HB5331, a sublancin-hypersensitive strain that harbors a *yqeZ-yqfAB* deletion in a CU1065 background (Fig. 2A, lower panel). The *yqeZ-yqfAB* operon is expressed from a σ^W -dependent promoter and confers a modest level of resistance to sublancin in strains lacking SP β (3). Even with this hypersensitive strain, no sublancin production (cell killing) was detected from a $\Delta sigMX$ double deletion spot (Fig. 2A, lower panel), suggesting that in the absence of both of these σ factors, sublancin expression is effectively abolished.

Abh counteracts AbrB repression in sublancin synthesis. The region upstream of *sunA* harbors a consensus σ^A promoter sequence but no apparent recognition sequences for either σ^X or σ^M (Fig. 1A). This observation suggests an indirect regulatory role for σ^X and σ^M in activating *sunA* transcription. We therefore examined two additional regulators of sublancin transcription, Abh and AbrB, which bind overlapping sequences in the *sunA* promoter region. It has been proposed that AbrB is a repressor and Abh is an activator for *sunA*, but how they cooperate or compete to influence the overall regulation of *sunA* transcription remains unclear (33). In previous studies, an *abh* mutant was found to have an $\sim$ 2-fold decrease in the level of expression from a *sunA-lacZ* reporter fusion (33), and we previously demonstrated that σ^X recognizes a promoter upstream of the *abh* gene (19). Thus, it seemed plausible that Abh might provide the link between ECF σ factor activity and activation of sublancin expression.

In our spot-on-lawn assays, when a strain carrying a deletion of *abh* (Δabh , HB10131) was spotted onto the highly sensitive indicator strain HB5331, the inhibition zone was significantly reduced compared to that from strain W168 (Fig. 2A, lower panel, and B), which confirms a positive role for Abh in sublancin production. However, when strains $\Delta abrB$ (HB10139) and $\Delta abh abrB$ (HB10146) were spotted onto the same lawn, the zones of inhibition were comparable to the wild-type strain W168. These results suggest that the role of Abh is to counteract repression mediated by AbrB: if AbrB repression is absent, Abh is not needed. This result contradicts the previous suggestion that Abh might function as a direct activator of sublancin expression, since an *abh abrB* double mutant apparently failed to make an antimicrobial compound (presumed to be sublancin) as detected using *Bacillus coagulans* as an indicator strain (33).

If the regulation of sublancin production by σ^X or σ^M were solely through Abh, one would predict that a Δabh mutant would fail to make any detectable sublancin, as also noted for the $\Delta sigMX$ double mutant in this bioassay. However, there is clearly residual antibacterial activity in the Δabh strain (Fig. 2). We first considered the possibility that the *abh* deletion might

derepress synthesis of one or more antibiotics that are negatively regulated by Abh (33). However, the residual antibiotic activity in the Δabh strain was eliminated in a $\Delta abh sunA$ double mutant (HB10182), indicating that this activity was due to sublancin synthesis (Fig. 2). This raised the possibility that, in addition to their effects on Abh production, σ^X or σ^M may have a second pathway by which they activate sublancin synthesis. Consistent with this notion, deletion of *sigM* and *sigX* not only removes the residual activity detected in an *abh* deletion strain, it also reduces the level of sublancin activity in an *abrB* deletion strain (Fig. 2A, lower panel, and B). Together, these results suggest that σ^X or σ^M likely regulates sublancin production both by activation of Abh (which counteracts AbrB repression) and independent from Abh or AbrB.

σ^X or σ^M is required for transcription of *sunA*. To test whether the observed effects on sublancin production were due to regulation of *sunA* transcription, we measured mRNA levels using Northern blotting (see Fig. S1 in the supplemental material) and RNA slot blotting (Fig. 3). The sublancin operon was previously found to be transcribed as two separate transcripts: *sunA* (0.2 kb) and *sunT-bdbA-yolJ-bdbB* (4.3 kb) (31). However, our Northern analysis detected a single major transcript of approximately 2 to 2.5 kb using *sunA* as a probe (see Fig. S1 in the supplemental material). Our results suggest that *sunA* is, at least, cotranscribed with *sunT*. The downstream genes *bdbA*, *yolJ*, and *bdbB* may also be cotranscribed with *sunAT*, since no gaps exist between *sunT-bdbA*, *bdbA-yolJ*, and *yolJ-bdbB*. Interestingly, *sunA* is one of the most stable mRNAs in *B. subtilis*, although no specific secondary structure related to mRNA stability was noted at its 5' end (16). We noted the presence of a potential hairpin in the 60-bp space between *sunA* and *sunT* ($\Delta G = -14.5$ kcal/mol as determined using Mfold [24, 34]) (Fig. 1A). It is possible that this 3'-end secondary structure protects *sunA* mRNA from degradation.

As expected based on the phenotype assays, *sunAT* mRNA levels were significantly reduced in the $\Delta sigX$, $\Delta sigMX$, and Δabh mutant strains (Fig. 3; see also Fig. S1 in the supplemental material). Indeed, even the $\Delta sigX$ single mutant greatly reduced *sunAT* mRNA levels, suggesting that σ^X is the major ECF σ factor regulating *sunAT* expression. Consistent with the results of the spot-on-lawn assay (Fig. 2A), the *sunAT* mRNA levels in the $\Delta abrB$ mutant were not significantly different from that in W168. This indicates that AbrB repression is effectively overcome under these growth conditions. This is expected for growth on plates since the producer cells are at high density and AbrB repression is relieved upon entry into stationary phase. It is surprising that AbrB repression was not apparent during logarithmic growth when the RNA was isolated for slot

FIG. 4. Ectopic Abh expression bypasses the requirement for σ^X and σ^M in sublancin production. W168 and its derivative mutants were spotted on a lawn of CU1065. The induction of Abh expression with xylose restores sublancin production in the strain harboring a double deletion of the *sigX* and *sigM* genes ($\Delta sigMX$).

blot analysis (Fig. 3). Since Δabh failed to produce sublancin, whereas the double deletion of Δabh *abrB* displayed high-level expression, we suggest that AbrB represses *sunA* transcription in the Δabh mutant even though this effect was not obvious in the wild type. One possible explanation is that *abrB* expression itself may be derepressed in an *abh* mutant. These two paralogs are known to bind to related sequences, and it has previously been suggested that AbrB regulates *abh* transcription (33). Our results suggest that the converse may also be true. Taken together, these results are consistent with the hypothesis that Abh functions as an antagonist of AbrB-mediated repression in order to allow sublancin synthesis.

σ^X - and σ^M -dependent transcription of Abh activates *sunAT* transcription. Previous studies have shown that σ^X and σ^M can both contribute to the expression of *abh* (12, 19), and an ECF σ type consensus sequence is found upstream of *abh* (see Fig. 5A, below). Our genetic analyses also suggested that the primary effects of σ^X and/or σ^M in activating sublancin synthesis are dependent on Abh, which then indirectly activates *sunA*. In order to test this model, a xylose-inducible *abh* allele was introduced into the *lacA* locus in the background of W168, $\Delta sigM$, $\Delta sigX$, and $\Delta sigMX$ strains (strains HB10123, HB10124, HB10128, and HB10137, respectively). Induction of *abh* expression restored sublancin production to each of these single and double mutants (Fig. 4), effectively bypassing the requirement for ECF σ factors. Note that the sizes of the inhibition zones in this assay (Fig. 4) were somewhat reduced relative to that seen in comparable assays (Fig. 2A, upper panel). This might be due to the presence of xylose in this medium (to induce *abh* transcription), as it has been reported that xylose can modify sublancin and interfere with its bacteriocin activity (10). Nevertheless, it is clear that ectopic induction of Abh restores sublancin production in the $\Delta sigMX$ strain.

The regulation of *abh* transcription by σ^X and σ^M was further confirmed using β -galactosidase assays with a transcriptional reporter fusion (*P_{abh}-lacZ*). The *P_{abh}-lacZ* construct was introduced at *amyE* in the W168, single, and double mutants of *sigM* and *sigX* (strains HB10147, HB10148, HB10149, and HB10150). *P_{abh}* activity was slightly reduced in the $\Delta sigM$ mutant and strongly reduced in the $\Delta sigX$ mutant (Fig. 5B). No

FIG. 5. *abh* expression is regulated by σ^X and σ^M . (A) Schematic map of the *abh* gene and its upstream region. The promoter region recognized by σ^X and σ^M is underlined in bold and the transcriptional start site is indicated by +1. (B) Regulation of *abh* transcription was measured from the *abh* promoter fused to *lacZ*. The β -galactosidase activity of the *P_{abh}-lacZ* fusion reported was measured in strain W168 and its derivative mutant strain backgrounds grown to exponential phase in LB, as indicated on the x axis. The numbers above each bar are the *P* values for a paired Student *t* test comparing W168 to mutants.

β -galactosidase activity was detected in the $\Delta sigMX$ double mutant. This result suggests that the activity of *P_{abh}* requires σ^X or σ^M and that σ^X is the major regulator under these growth conditions. These results are entirely consistent with the regulatory effects of σ^X and σ^M as observed by monitoring the *sunAT* transcript levels (Fig. 3).

Previous studies, using primer extension mapping, had suggested that the *abh* promoter region might contain multiple, closely spaced start sites, only one of which was dependent on σ^X (19). However, using 5'-RACE analysis of cDNA ends (see Fig. S2 in the supplemental material) we observed that transcription initiated at the identical adjacent positions, albeit with slightly altered frequencies, in either the *sigX* mutant (in which transcription reflects σ^M activity) or in the *sigM* mutant (indicative of σ^X activity). These results suggest that both of these ECF σ factors recognize the same promoter sequences, consistent with the previously proposed consensus sequences for σ^X and σ^M (22).

Sublancin expression is controlled by a complex regulatory network. Most antibiotics produced by *B. subtilis* are synthesized upon entering stationary phase or induced by stress or quorum sensing (reviewed in detail in references 32 and 20). It has long been known that antibiotic production and resistance are regulated, in large part, by AbrB. Indeed, nutrient deprivation leads to a gradual increase in the phosphorylation, and hence activity, of Spo0A, the master regulator of sporulation (15). Mutants lacking *spo0A* are pleiotropic and also fail to express antibiotics and associated resistance functions. A *spo0A* *abrB* mutant strain restores regulation of antibiotic production but fails to restore the ability to sporulate. It is now appreciated that *abrB* is a high-affinity target for the Spo0A~P repressor and that relief of AbrB repression upon nutrient depletion leads to the expression of antibacterial compounds

FIG. 6. Regulatory network of sublancin synthesis and resistance in *B. subtilis*. Positive regulatory effects are indicated by arrows, and negative regulatory effects are indicated by “⊥”. See the text for a detailed discussion.

such as SdpC*, a toxic peptide, and the Skf cannibalism factor (13, 14, 28).

In addition to AbrB, the paralogous regulator Abh also plays a complex role in coordinating the synthesis of antibacterial compounds. Previous studies have shown that Abh can act both negatively and positively to affect expression of antibacterial compounds. Like AbrB, Abh acts as a negative regulator at the *skfA* and *sdpA* promoter regions (33). In contrast to AbrB, however, Abh acts positively on expression of sublancin, as confirmed here. We have shown that the effect of *abh* on sublancin expression is epistatic to *abrB* and that in an *abrB* mutant strain, the requirement for *abh* is bypassed. Expression of *abh* is itself controlled by numerous transcription factors. As shown here, *abh* transcription requires the activity of either σ^X or σ^M with σ^X as the major regulator under these growth conditions. Expression of *abh* is repressed by AbrB, which also negatively regulates its own synthesis.

Taken together, a complex regulatory circuitry emerges (Fig. 6) in which nutritional and growth phase-dependent signals converge to elevate levels of Spo0A~P leading to an initial repression of *abrB* transcription. In addition, AbrB activity is subject to multiple levels of posttranscriptional control, including antagonism by the AbbA antirepressor (2) and antagonism by Abh. The latter is likely to be promoter specific and may reflect the overlapping DNA-binding specificity of these two paralogous transcription factors. Those cells that are lysogenic for SP β and expressing either σ^X or σ^M , and therefore expressing Abh, will proceed to express the potent lantibiotic sublancin. σ^X or σ^M also appear to have some modest activation effect on sublancin production which is independent of Abh or AbrB. In addition, AbrB is known to repress both *sigW* and many target operons for σ^W (29). Among the operons under σ^W control, the *yqeZ-yqfAB* operon provides a background level of resistance to sublancin for those cells lacking SP β and therefore lacking the cognate immunity protein for sublancin, SunI. YqeZ encodes a putative transmembrane protease, and YqfA and YqfB are both putative membrane-anchored proteins.

Concluding remarks. ECF σ factors are known to provide resistance against various cell envelope antibiotics (3, 6, 12, 22). This report establishes that ECF σ factors also regulate antibiotic production in *B. subtilis*. When spotted on lawns of

Bacillus spp. strains, W168 was able to inhibit the growth of various strains and this inhibition was due to sublancin synthesis. A double deletion of σ^X and σ^M eliminated the production of sublancin due to an inability to express Abh, an antagonist of AbrB-mediated repression at the *sunA* promoter. Indeed, both AbrB and Abh have previously been shown to bind to overlapping regions in the *sunA* regulatory region (33), but it remains unclear how AbrB and Abh work together to regulate *sunA* expression. One simple model suggests that Abh binding to this region (either alone or together with AbrB) alters the protein-DNA complex such that the promoter is then available for interaction with RNA polymerase. Clearly, the production of antibiotic and their associated resistance determinants, as here exemplified by sublancin, is subject to an enormously complicated control network. An ultimate, but perhaps still distant, goal is to model these complex interactions to develop a predictive model of this regulatory circuitry.

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REFERENCES

- Albano, M., W. K. Smits, L. T. Ho, B. Kraigher, I. Mandic-Mulec, O. P. Kuipers, and D. Dubnau. 2005. The Rok protein of *Bacillus subtilis* represses genes for cell surface and extracellular functions. *J. Bacteriol.* **187**:2010–2019.
- Banse, A. V., A. Chastanet, L. Rahn-Lee, E. C. Hobbs, and R. Losick. 2008. Parallel pathways of repression and antirepression governing the transition to stationary phase in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **105**:15547–15552.
- Butcher, B. G., and J. D. Helmann. 2006. Identification of *Bacillus subtilis* σ^W -dependent genes that provide intrinsic resistance to antimicrobial compounds produced by bacilli. *Mol. Microbiol.* **60**:765–782.
- Cao, M., B. A. Bernat, Z. Wang, R. N. Armstrong, and J. D. Helmann. 2001. FosB, a cysteine-dependent fosfomycin resistance protein under the control of σ^W , an extracytoplasmic function σ factor in *Bacillus subtilis*. *J. Bacteriol.* **183**:2380–2383.
- Cao, M., and J. D. Helmann. 2002. Regulation of the *Bacillus subtilis* *bcnC* bacitracin resistance gene by two extracytoplasmic function σ factors. *J. Bacteriol.* **184**:6123–6129.
- Cao, M., and J. D. Helmann. 2004. The *Bacillus subtilis* extracytoplasmic function σ^X factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. *J. Bacteriol.* **186**:1136–1146.
- Cao, M., C. M. Moore, and J. D. Helmann. 2005. *Bacillus subtilis* paraquat resistance is directed by σ^M , an extracytoplasmic function σ factor, and is conferred by YqjL and BcrC. *J. Bacteriol.* **187**:2948–2956.
- Cao, M., L. Salzberg, C. S. Tsai, T. Mascher, C. Bonilla, T. Wang, R. W. Ye, L. Marquez-Magana, and J. D. Helmann. 2003. Regulation of the *Bacillus subtilis* extracytoplasmic function protein σ^Y and its target promoters. *J. Bacteriol.* **185**:4883–4890.
- Dorenbos, R., T. Stein, J. Y. Dubois, J. Weiland, W. J. Quax, and J. M. van Dijk. 2004. Thiol-disulfide oxidoreductases in *Bacillus subtilis*. Ph.D. dissertation. University of Groningen, Groningen, The Netherlands.
- Dorenbos, R., T. Stein, J. Kabel, C. Bruand, A. Bolhuis, S. Bron, W. J. Quax, and J. M. Van Dijk. 2002. Thiol-disulfide oxidoreductases are essential for the production of the lantibiotic sublancin 168. *J. Biol. Chem.* **277**:16682–16688.
- Dubois, J. Y., T. R. Kouwen, A. K. Schurich, C. R. Reis, H. T. Ensing, E. N. Trip, J. C. Zweers, and J. M. van Dijk. 2009. Immunity to the bacteriocin sublancin 168 is determined by the SunI (YolF) protein of *Bacillus subtilis*. *Antimicrob. Agents Chemother.* **53**:651–661.
- Eiamphungporn, W., and J. D. Helmann. 2008. The *Bacillus subtilis* σ^M regulon and its contribution to cell envelope stress responses. *Mol. Microbiol.* **67**:830–848.
- Ellermeier, C. D., E. C. Hobbs, J. E. Gonzalez-Pastor, and R. Losick. 2006. A three-protein signaling pathway governing immunity to a bacterial cannibalism toxin. *Cell* **124**:549–559.
- Fujita, M., J. E. Gonzalez-Pastor, and R. Losick. 2005. High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J. Bacteriol.* **187**:1357–1368.
- Fujita, M., and R. Losick. 2005. Evidence that entry into sporulation in

- Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. *Genes Dev.* **19**:2236–2244.
16. **Hambraeus, G., C. von Wachenfeldt, and L. Hederstedt.** 2003. Genome-wide survey of mRNA half-lives in *Bacillus subtilis* identifies extremely stable mRNAs. *Mol. Genet. Genomics* **269**:706–714.
 17. **Harwood, C. R., and S. M. Cutting.** 1990. *Molecular biological methods for Bacillus*. John Wiley & Sons Ltd., Chichester, United Kingdom.
 18. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) σ factors. *Adv. Microb. Physiol.* **46**:47–110.
 19. **Huang, X., and J. D. Helmann.** 1998. Identification of target promoters for the *Bacillus subtilis* σ^X factor using a consensus-directed search. *J. Mol. Biol.* **279**:165–173.
 20. **Kleerebezem, M.** 2004. Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. *Peptides* **25**:1405–1414.
 21. **Kouwen, T. R., E. N. Trip, E. L. Denham, M. J. Sibbald, J. Y. Dubois, and J. M. van Dijk.** 2009. Protein secretion and disulfide bond handling in *Bacillus subtilis*. Ph.D. dissertation. University of Groningen, Groningen, The Netherlands.
 22. **Mascher, T., A. B. Hachmann, and J. D. Helmann.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function σ factors. *J. Bacteriol.* **189**:6919–6927.
 23. **Mascher, T., N. G. Margulis, T. Wang, R. W. Ye, and J. D. Helmann.** 2003. Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Mol. Microbiol.* **50**:1591–1604.
 24. **Mathews, D. H., J. Sabina, M. Zuker, and D. H. Turner.** 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* **288**:911–940.
 25. **McAuliffe, O., R. P. Ross, and C. Hill.** 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol. Rev.* **25**:285–308.
 26. **Miller, J. H.** 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 27. **Paik, S. H., A. Chakicherla, and J. N. Hansen.** 1998. Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *J. Biol. Chem.* **273**:23134–23142.
 28. **Phillips, Z. E., and M. A. Strauch.** 2002. *Bacillus subtilis* sporulation and stationary phase gene expression. *Cell Mol. Life Sci.* **59**:392–402.
 29. **Qian, Q., C. Y. Lee, J. D. Helmann, and M. A. Strauch.** 2002. AbrB is a regulator of the σ^W regulon in *Bacillus subtilis*. *FEMS Microbiol. Lett.* **211**:219–223.
 30. **Salzberg, L. I., and J. D. Helmann.** 2007. An antibiotic-inducible cell wall-associated protein that protects *Bacillus subtilis* from autolysis. *J. Bacteriol.* **189**:4671–4680.
 31. **Serizawa, M., K. Kodama, H. Yamamoto, K. Kobayashi, N. Ogasawara, and J. Sekiguchi.** 2005. Functional analysis of the YvrGHb two-component system of *Bacillus subtilis*: identification of the regulated genes by DNA microarray and northern blot analyses. *Biosci. Biotechnol. Biochem.* **69**:2155–2169.
 32. **Stein, T.** 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol. Microbiol.* **56**:845–857.
 33. **Strauch, M. A., B. G. Bobay, J. Cavanagh, F. Yao, A. Wilson, and Y. Le Breton.** 2007. Abh and AbrB control of *Bacillus subtilis* antimicrobial gene expression. *J. Bacteriol.* **189**:7720–7732.
 34. **Zuker, M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31**:3406–3415.