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 SPβ 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^X or σ^M 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, antibiotic 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, and σ^Y^W^C (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-methylthionelline 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 SPβ 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). BdB and BdB A 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 expression 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
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 sublancin requires the activity of either of two ECF σ factors, σ^MM and σ^X. Genetic analyses establish that the influence of σ^MM 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^MM 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* DH5α before transformation with *B. subtilis* strains. Ampicillin (AMP; 100 μg 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 μg ml^-1^), kanamycin (Kan; 10 μg ml^-1^), tetracycline (Tet; 5 μg ml^-1^), chloramphenicol (CAT; 5 μg ml^-1^), and macrolide-lincosamide-streptogramin B (MLS; contains 1 μg ml^-1^ erythromycin and 25 μg 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 (OD 600) 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 μl 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 μl of the producer strain (OD 600 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::PsunA-lacZ (cat).

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 OD 600 of 0.4 (mid-log phase) using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. The RNA was quantified by agarose gel electrophoresis. Two DNA probes, sunA and 23rRNA, 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
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) 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 σW, 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
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 (∆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 ∆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 SPβ, 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 (∆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 ∆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 SPβ prophage it lacks both the ability to synthesize sublancin and the SPβ-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 ∆sigX (HB10103) and ∆sigM (HB10016) strains showed reduced inhibition zone sizes compared to that from the W168 spot. Judging from zone diameters, the ∆sigX strain impaired cell killing to a greater degree than did the ∆sigM strain. Deletions in sigX and sigM were additive, as a ∆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 ∆sigMX strain was spotted on a
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 \( \sigma^X \)-dependent promoter and confers a modest level of resistance to sublancin in strains lacking \( \Sigma_\beta \) (3). Even with this hypersensitive strain, no sublancin production (cell killing) was detected from a \( \Delta\sigma\text{igMX} \) double deletion spot (Fig. 2A, lower panel), suggesting that in the absence of both of these \( \sigma \) factors, sublancin expression is effectively abolished.

**Abh counteracts AbrB repression in sublancin synthesis.**

The region upstream of sunA harbors a consensus \( \sigma^X \) promoter sequence but no apparent recognition sequences for either \( \sigma^X \) or \( \sigma^M \) (Fig. 1A). This observation suggests an indirect regulatory role for \( \sigma^X \) and \( \sigma^M \) 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 transcription. We therefore considered the possibility that the \( abh \) gene (19) 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 \( \sigma^X \) and \( \sigma^M \) (Fig. 1A). It is possible that this \( \sigma^X \) recognizes a promoter upstream of the \( abh \) gene (19). Thus, it seemed plausible that Abh might provide the link between ECF \( \sigma \) factor activity and activation of sublancin expression.

In our spot-on-lawn assays, when a strain carrying a deletion of \( abh \) (\( \Deltaabh \), 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 \( \DeltaabrB \) (HB10139) and \( \Deltaabh \DeltaabrB \) (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 \( abh \DeltaabrB \) 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 \( \sigma^X \) or \( \sigma^M \) were solely through Abh, one would predict that a \( \Deltaabh \) mutant would fail to make any detectable sublancin, as also noted for the \( \Delta\sigma\text{igMX} \) double mutant in this bioassay. However, there is clearly residual antibacterial activity in the \( \Deltaabh \) 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 \( \Deltaabh \) strain was eliminated in a \( \Deltaabh \) 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, \( \sigma^X \) or \( \sigma^M \) may have a second pathway by which they activate sublancin synthesis. Consistent with this notion, deletion of \( \sigma\text{igM} \) and \( \sigma\text{igX} \) 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 \( \sigma^X \) or \( \sigma^M \) likely regulates sublancin production both by activation of Abh (which counteracts AbrB repression) and independent from Abh or AbrB.

### Table: ECF \( \sigma \) FACTORS AND SUBLANCIN PRODUCTION IN *B. SUBTILIS*

<table>
<thead>
<tr>
<th>Total RNA amount/probe</th>
<th>W168</th>
<th>( \Delta\sigma\text{igM} )</th>
<th>( \Delta\sigma\text{igX} )</th>
<th>( \Delta\sigma\text{igMX} )</th>
<th>( \Deltaabh )</th>
<th>( \DeltaabrB )</th>
<th>( \Deltaabh \DeltaabrB )</th>
<th>( \Delta\text{sunA} )</th>
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<tr>
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<tr>
<td>100( \mu \text{g}/23\text{SrRNA} )</td>
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**Fig. 3.** \( \sigma^X \) is the major \( \sigma \) factor regulating sunA transcription, and Abh counteracts the repression of AbrB at sunA transcription. The levels of sunA transcription in W168 and its derivative mutant strains were detected by RNA slot blot analysis using \( ^{32}\text{P} \)-labeled sunA DNA probe. 23S rRNA was used as a loading control.
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.

σX and σM-dependent transcription of Abh activates sunAT transcription. Previous studies have shown that σX and σM 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 σX and/or σM 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 lacZ locus in the background of W168, ΔsigM, ΔsigX, and ΔsigMX strains (strains 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 transcription levels (Fig. 3).

β-galactosidase activity was detected in the ΔsigMX double mutant. This result suggests that the activity of Pabh requires σX or σM and that σX is the major regulator under these growth conditions. These results are entirely consistent with the regulatory effects of σX and σM 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 σX (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 σM activity) or in the sigM mutant (indicative of σX activity). These results suggest that both of these ECF σ factors recognize the same promoter sequences, consistent with the previously proposed consensus sequences for σX and σM (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.
Nutrient depletion

Spo0A-P

AcrB

AbbA

AbbB

σ^X

σ^M

σ

Abh

YeZ

YqfA

YqfB

SunI

Resistant

Sublancin

Cell density

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 “T”. See the text for a detailed discussion.

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

In addition to AcrB, 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 AcrB, Abh acts as a negative regulator at the *skfA* and *sdpA* promoter regions (33). In contrast to AcrB, 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 *acrB* and that in an *acrB* 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 AcrB, 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 *acrB* transcription. In addition, AcrB 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 SPβ 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 AcrB. In addition, AcrB is known to repress both *sigW* and many target operons for σ^W (29). Among the operons under σ^W control, the *yez-yqfAB* operon provides a background level of resistance to sublancin for those cells lacking SPβ and therefore lacking the cognate immunity protein for sublancin, SunI. YeZ 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 AcrB-mediated repression at the *sunA* promoter. Indeed, both AcrB and Abh have previously been shown to bind to overlapping regions in the *sunA* regulatory region (33), but it remains unclear how AcrB and Abh work together to regulate *sunA* expression. One simple model suggests that Abh binding to this region (either alone or together with AcrB) 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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