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Extracytoplasmic Function σ Factors Regulate Expression of the *Bacillus subtilis yabE* Gene via a *cis*-Acting Antisense RNA▽

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Bacillus subtilis yabE **encodes a predicted resuscitation-promoting factor/stationary-phase survival (Rpf/Sps) family autolysin. Here, we demonstrate that *yabE* is negatively regulated by a *cis*-acting antisense RNA which, in turn, is regulated by two extracytoplasmic function σ factors: σ^X and σ^M.**

Extracytoplasmic function (ECF) σ factors often regulate gene expression in response to environmental stresses that affect the cell envelope (12). In *Bacillus subtilis*, there are seven ECF σ factors. The best understood are σ^M, σ^W, and σ^X, which control partially overlapping regulons related to cell envelope homeostasis and antibiotic resistance.

As a class, ECF σ factors recognize structurally similar promoters characterized by a conserved AAC motif in the −35 region (12, 21). In previous studies, we have defined consensus sequences for σ^M, σ^W, and σ^X (2, 8, 17, 18, 30). Promoters recognized by one or more of these three σ factors (designated P_{ECF}) generally conform to the consensus TGwAAC-N_{16}-CGwCta (where w represents A or T and lowercase letters indicate residues that are less highly conserved) or TGwAAC-N_{16}-CGTAta (preferentially recognized by σ^W). Since these three σ factors recognize overlapping sets of promoters, it is typically not possible to assign a given candidate promoter to one or another regulon by sequence alone (3, 16, 30, 38). In addition to conservation within these core (−35 and −10) recognition elements, active promoters are often associated with AT-rich upstream promoter elements (UP elements), and the spacer region adjacent to the −35 element often has a T-rich sequence (12).

In previous studies, we have focused our attention on those candidate P_{ECF} elements upstream of coding sequences (2, 17, 18). Here, we report the identification of a P_{ECF} downstream of and convergent with the *yabE* gene. YabE is a cell wall binding protein with similarity in its N-terminal domain to *Mycobacterium tuberculosis* RpfB (31). The C terminus of YabE contains an Sps (stationary-phase survival) domain in place of the Rpf domain, and YabE has been designated the founding member of the SpsB subfamily of muralytic enzymes (31). Rpf/Sps family proteins function as *r*esuscitation-*p*romoting *f*actors that can restore growth to dormant cells, presumably by catalyzing peptidoglycan cleavage to allow renewed cell wall growth (5, 20). The bacterial strains, plasmids, and primers used in this study are listed in Table 1.

Rpf proteins in the actinobacteria are the best understood. Rpf was originally discovered as a secreted protein that restores growth to dormant *Micrococcus luteus* cells and was subsequently found to be encoded by an essential gene (26, 28). In *Corynebacterium glutamicum*, Rpf proteins are dispensable but an *rpf1 rpf2* double-mutant strain displays a prolonged lag phase when exiting from stationary phase (10). Rpf proteins in *Mycobacterium tuberculosis*, which encodes five members of this protein family (29), are the most well known. A mutant lacking all five proteins still grows well in vitro but is defective in the restoration of growth after stationary phase (19). Strains lacking one or more Rpf proteins are also defective in animal models of tuberculosis (7, 32, 36). The RpfB protein is particularly important, as evidenced by the delayed reactivation of the single *rpfB* mutant observed previously (36). RpfB interacts with a peptidoglycan endopeptidase, RipA, at cell division sites (13, 14). In contrast with those in the actinobacteria, the roles of Rpf/Sps family proteins in the low-GC-content gram-positive bacteria are poorly understood. Based on sequence comparisons, it is likely that these proteins, like their actinobacterial homologs (5, 27, 35), function as peptidoglycan hydrolases (autolysins). Specifically, the Sps domain of YabE corresponds to the conserved 3D domain (named for three conserved Asp residues) that constitutes the catalytic site of the *Escherichia coli* MltA lytic transglycosylase (37).

Pattern searches for P_{ECF} elements performed using the SubtiList database identified a sequence downstream of and convergent with the *yabE* gene (Fig. 1A) that is highly conserved in other *Bacillus* strains (Fig. 1B). To determine if this putative promoter (P_{ECF}) was active, this DNA region was amplified from *B. subtilis* chromosomal DNA and cloned into pDG1661, which contains a promoterless *lacZ* gene (9). The resulting plasmid was digested with ScaI and introduced by transformation into the *B. subtilis* CU1065 wild type (40). An overnight culture was diluted 1:100 in 50 ml of Luria-Bertani (LB) medium and grown at 37°C with vigorous shaking, and the β-galactosidase activity was monitored during growth as described previously (24). P_{ECF} activity peaked in the late-logarithmic-phase cells (Fig. 2A).

We verified the transcriptional start sites for both *yabE* and the P_{ECF}-directed antisense transcript by the rapid amplification of cDNA 5′ ends. Total RNA was isolated from the wild-type strain at the time of maximal expression (~3 h) by using the RNeasy minikit (Qiagen). After DNase treatment, 2 μg of RNA was used as a template for reverse transcription using Multiscribe reverse transcriptase (Taqman; Roche) and a

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Bacterial strain, plasmid, or primer	Description or primer designation and sequence[a] (5′–3′)	Reference and/or source
Strains		
B. subtilis		
CU1065	W168 SPβ*att trpC2*	40; lab stock
HB0020	CU1065 *sigW*::*mls*	Lab stock
HB0031	CU1065 *sigM*::*kan*	Lab stock
HB0047	CU1065 *rsiX*::*spc*	Lab stock
HB0097	CU1065 *sigM*::*kan sigX*::*spc*	Lab stock
HB7007	CU1065 *sigX*::*spc*	Lab stock
HB4773	CU1065 *amy*::P_{ECF}-*lacZ*	This study
HB4774	HB7007 *amy*::P_{ECF}-*lacZ*	This study
HB4775	HB0047 *amy*::P_{ECF}-*lacZ*	This study
HB4779	HB0020 *amy*::P_{ECF}-*lacZ*	This study
HB4780	HB0031 *amy*::P_{ECF}-*lacZ*	This study
HB4782	HB0097 *amy*::P_{ECF}-*lacZ*	This study
HB5339	CU1065 *yocH*::*kan*	Lab stock
HB4784	CU1065 *yabE*::*spc*	This study
HB4785	CU1065 *yuiC*::*mls*	This study
HB4786	CU1065 *yocH*::*kan yabE*::*spc yuiC*::*mls*	This study
E. coli DH5α	ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Lab stock
Plasmids		
pDG1661	Vector for integration of *lacZ* fusions at *amyE* locus	9
pWE23	P_{ECF}-*lacZ* in pDG1661	This study
pMAD	Vector for allelic replacement	1
pWE24	*yabE*-FLAG gene in pMAD	This study
Primers		
3382	P_{ECF}-fwd-HindIII; CCCAAGCTTCCTCCAAGACAAAATAAAAAC	
3383	P_{ECF}-rev-BamHI; CGGGATCCCATTCCGCTAGGCTCCAAAG	
3592	ECF-GSP1; GGCAAGCTGCAGCGGTTGTT	
3593	ECF-GSP2; CAGCGACAGGCGTCAATTTA	
3933	yabE-GSP1; CGTCCATGTCTGCTGTTATC	
3934	yabE-GSP2; GCAGGTGTGATCTTGTCTTC	
3539	*yabE*-specific probe; CGTAGTCGAAGTCGTCCAGATCTTCTTTTGTTTCCCTGCATCATT CACAGTAACCTGAAA	
3541	Antisense strand-specific probe; GGGAAATAAAACAGTCAAAATTAAAATCTTAAATTA GTATATACTTATGTATTCAGAGGG	
3772	yabE-up-fwd-EcoRI; CGGAATTCGTCACCGATGTAGTTGAAGA	
3543	yabE-up-FLAG-rev; TTATTTATCATCATCATCTTTATAATCCGGCCGATTTAAGATTTT AATTTTGA	
3544	yabE-do-FLAG-fwd; CGGCCGGATTATAAAGATGATGATGATAAATAATATATACTTA TGTATTCAGA	
3826	yabE-do-rev-NcoI; ATACCATGGCTCAGCTGAAATGTCACTCGG	
3546	yabE-up-fwd; GGAAGACTTAGCGTGGATTA	
3547	yabE-up-rev (spec); CGTTACGTTATTAGCGAGCCAGTCGTCAACCCTCCCTTCTCTTT	
3548	yabE-do-fwd (spec); CAATAAACCCTTGCCCTCGCTACGGCGATTAAAGGGAACA AGAT	
3549	yabE-do-rev; GGTATCGAGCGCACTCATAA	
3935	yuiC-up-fwd; CACATGATCTGACTTTATTG	
3936	yuiC-up-rev (mls); GAGGGTTGCCAGAGTTAAAGGATCGGTCATCAGCAAACGTCTGA	
3937	yuiC-do-fwd (mls); CGATTATGTCTTTTGCGCAGTCGGCGCAAGTGTTCAGAAATCAAT	
3938	yuiC-do-rev; GCTCTGATTGTTGAACCGCA	

[a] Restriction sites are underlined.

gene-specific primer (GSP1). The resulting cDNA was purified and ligated to a poly(dC) tail with terminal deoxynucleotidyltransferase (New England Biolabs). The resulting cDNA was amplified by PCR using the poly(dG) primer to anneal at the poly(dC) tail and a second gene-specific primer (GSP2) complementary to a region upstream of GSP1. PCR products were separated by gel electrophoresis and sequenced. The resulting start site for the P_{ECF} is consistent with the assigned −35 and −10 elements (Fig. 2B). Unexpectedly, the *yabE* start site was at position +60 downstream of the assigned TTG start codon. This start site corresponds to a consensus σ^A promoter (11) with −35 (TTGACA) and −10 (TATAAT) elements (Fig.

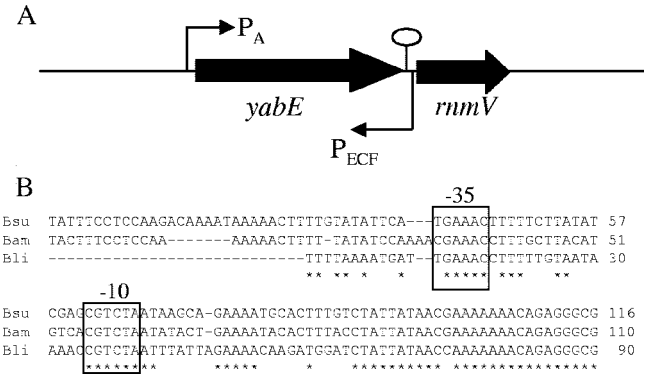

FIG. 1. (A) Organization of *yabE* and the downstream P_{ECF} element. Promoter sites are indicated by bent arrows with a subscript to indicate the relevant holoenzyme(s). A putative transcription terminator downstream of *yabE* is indicated by an oval. RnmV is an RNase M5/primase-related protein involved in the maturation of the 5S rRNA (6). (B) Alignment of P_{ECF} elements from various bacilli. The intergenic region between *yabE* and *rnmV* of *B. subtilis* (Bsu) was aligned with the corresponding sequences from *B. amyloliquefaciens* (Bam) and *B. licheniformis* (Bli) by using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The putative −10 and −35 regions are indicated. The run of seven A residues near the end of the sequence shown corresponds to the run of U residues in the *yabE* transcription terminator.

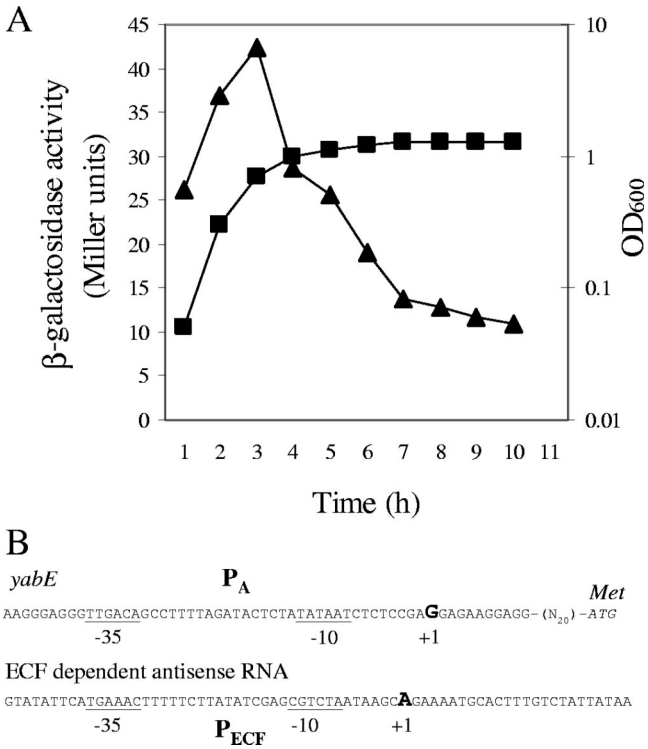

FIG. 2. (A) Growth phase regulation of P_{ECF}. The β-galactosidase activity of the P_{ECF}-*lacZ* fusion in the wild type (CU1065) was assayed during growth in LB medium, with samples taken every 1 h for the measurement of the optical density at 600 nm (OD_{600}) and the β-galactosidase activity. Solid squares represent growth at an optical density at 600 nm, while solid triangles represent β-galactosidase activity. (B) Mapping of the transcriptional start sites of *yabE* and ECF-directed antisense transcripts by the rapid amplification of cDNA 5′ ends. The putative −10 and −35 regions are underlined. The transcription start sites are in bold, and the translation start site is italicized. P_A is the promoter corresponding to σ^A. *Met* indicates the start codon (methionine).

2B). This promoter sequence is also conserved among closely related bacilli, as is a downstream ATG start codon (data not shown). These findings, together with sequence comparisons between YabE and other orthologs, suggest that the translation start codon of this gene was misannotated previously.

To determine which ECF σ factors regulate P_{ECF}, we measured the expression of a P_{ECF}-*lacZ* fusion in strains with one or more ECF σ factor genes disrupted (Fig. 3A). P_{ECF} is dependent primarily on σ^X, and as expected, the activity in a strain lacking the anti-σ^X factor RsiX was elevated. Interestingly, the expression of this promoter was weakly reduced in a Δ*sigM*::*kan* mutant (8) but not in a Δ*sigW*::*mls* mutant (17). Mindful of the potential for regulatory overlap between ECF σ factors (3, 16, 22, 25, 30), we tested a *sigM sigX* double-mutant strain and found that the double deletion eliminated the expression of P_{ECF}. Thus, we conclude that P_{ECF} is transcribed by both the σ^X and σ^M forms of RNA polymerase.

Next, we performed Northern blot analysis to monitor the size and abundance of the P_{ECF}-directed transcript. Fifteen micrograms of total RNA was isolated from cells in late-logarithmic-phase growth and run on a 1% formaldehyde denaturing gel by using NorthernMax denaturing gel buffer and running buffer (Ambion). The RNA was transferred onto a Zeta-Probe blotting membrane (Bio-Rad). The Northern analysis confirmed that the ~750-nucleotide antisense RNA was highly transcribed in the Δ*rsiX* background (Fig. 3B). In a parallel analysis, we found that *yabE* was expressed as a monocistronic transcript (~1,300 nucleotides) whose abundance varied inversely with that of the P_{ECF}-directed antisense transcript (Fig. 3B). These results are consistent with the hypothesis that the antisense RNA prevents the accumulation of the *yabE* transcript, presumably by pairing with and facilitating the degradation of the sense transcript. Although we attempted to detect YabE (using a C-terminal FLAG epitope tag), we were unable to detect the protein either in cells or in the secreted protein fraction (data not shown).

B. subtilis contains four genes encoding Rpf/Sps family proteins: *yabE*, *yocH*, *yuiC*, and *yorM* (31). YocH is an autolysin under the control of the essential YycFG two-component system (15). Our strains do not contain *yorM*, which is located within the SPβ prophage. In order to determine the physiological role of *yabE*, we constructed *yabE*, *yocH*, and *yuiC* single-deletion mutants, as well as a triple mutant, by using long flanking homology PCR as described previously (23). In the actinobacteria, Rpf is assayed by the ability to restore growth to washed cells inoculated at high dilutions or into nutrient-poor medium (20, 26). To test whether *B. subtilis* growth was affected by the loss of these *rpf*-like genes, stationary-phase cultures in LB medium were serially diluted from 10^{-1} to 10^{-7} in LB or minimal medium. In parallel, the same cells were washed several times with LB or minimal medium prior to dilution. Samples of 200 μl were incubated in a microtiter plate at 37°C with continuous shaking, and growth was monitored in a Bioscreen C analyzer (Laboratory Systems, Finland) using a 600-nm filter. Under these conditions, there were no significant differences between the wild type and any

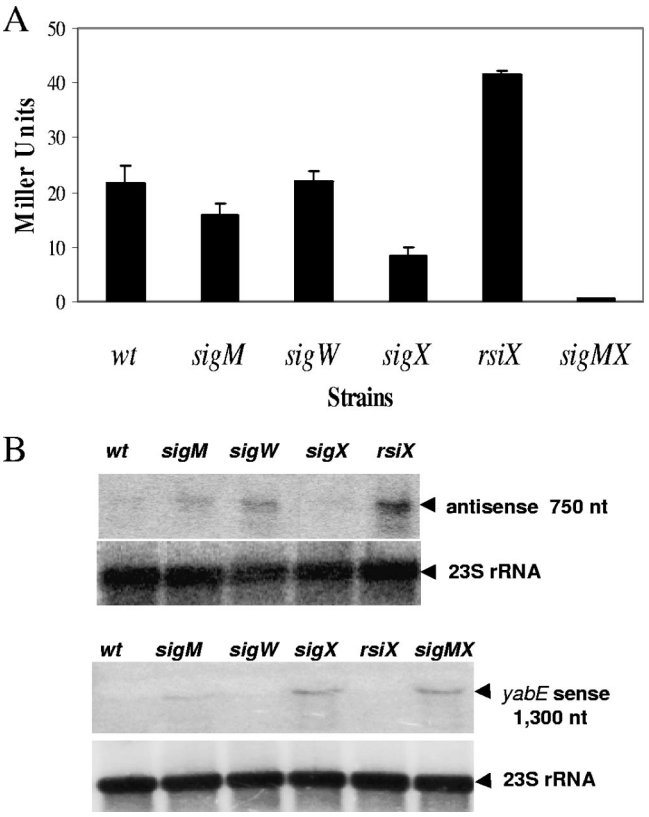

FIG. 3. (A) P_{ECF}-directed antisense expression is dependent on σ^X and σ^M. The β-galactosidase activity of the P_{ECF}-*lacZ* fusion was assayed in various ECF deletion strains indicated by the relevant gene. Cultures were grown in LB medium, and samples were taken when cells reached late log phase. Data are expressed as averages ± standard deviations of results for triplicate samples. wt, wild type. (B) Regulation of *yabE* by an antisense RNA. Total RNA (15-μg) samples prepared from late-log-phase cultures of various deletion strains after growth in LB medium were analyzed by Northern blotting. RNA was separated, blotted, and hybridized with [γ-^{32}P]ATP-labeled strand-specific probes for the *yabE* and the antisense RNA transcripts (indicated with arrowheads). The level of 23S rRNA is shown underneath as a loading control (2 μg of RNA was loaded). nt, nucleotides.

of the deletion strains, nor did we detect substantial differences in sporulation efficiency or stationary-phase survival (data not shown). Similar results in a different *B. subtilis* strain background have been observed previously (M. Young, personal communication). These results indicate that the genes are dispensable under these conditions. Further studies will be required to define the roles of the proteins in cell wall turnover, growth, and/or long-term survival. One hint may be provided by the observation that both *yabE* and *yocH* are upregulated during growth under high-salt conditions (34). Both the σ^X and σ^M regulons are activated by antibiotics that inhibit cell wall synthesis (4, 8, 23). Thus, antisense regulation of YabE is consistent with the notion that the downregulation of cell wall lytic enzymes is adaptive when cell wall synthesis is impaired. Indeed, *yocH* is positively regulated by the YycFG two-component system, which simultaneously downregulates IseA (YoeB), an inhibitor of autolysins (33, 39).

In conclusion, we have demonstrated that two ECF σ factors, σ^X and σ^M, control the expression of an antisense RNA for *yabE*. The expression of this RNA is correlated with decreased accumulation of the *yabE* sense transcript, presumably due to the degradation of the resulting duplex RNA. The coregulation of genes involved in antibiotic resistance (22) and of the expression of teichoic acid synthesis genes and cell division functions in *B. subtilis* W23 (25) by σ^X and σ^M has been observed previously. The discovery of this ECF σ factor-regulated antisense RNA, together with the recent finding of σ^M-dependent promoter elements within genes (8), suggests that current assignments of the regulons for ECF σ factors are likely incomplete. It can be anticipated that future studies, using chromatin immunoprecipitation-microarray analysis and powerful sequencing-based transcriptomics approaches, will facilitate a more complete inventory of ECF σ factor-dependent transcription.

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