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Regulation of the *Bacillus subtilis* Extracytoplasmic Function Protein σ^Y and Its Target Promoters

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The *Bacillus subtilis* extracytoplasmic function sigma factor σ^Y is of unknown function. We demonstrate that the *sigY* operon is expressed from an autoregulatory promoter site, P_Y. We selected for transposon-induced mutations that upregulate P_Y transcription in an attempt to identify genes involved in σ^Y regulation. The resulting insertions disrupted *yxlC*, the gene immediately downstream of *sigY*. However, the phenotype of the *yxlC*::Tn*10* insertion was due to polarity on the downstream genes of the *sigY* operon; a nonpolar insertion in *yxlC* did not lead to derepression of P_Y. Further analyses revealed that both *yxlD* and *yxlE* encoded proteins important for the negative regulation of σ^Y activity. A comparison of the transcriptomes of wild-type and *yxlC*::Tn*10* mutant strains revealed elevated expression of several operons. However, only one additional gene, *ybgB*, was unambiguously identified as a direct target for σ^Y. This was supported by analysis of direct targets for σ^Y transcription with whole-genome runoff transcription followed by macroarray analysis.

The extracytoplasmic function (ECF) σ factors function as global regulators of a variety of stress responses often triggered by changes in the cell envelope (12). In some organisms, this particular family of regulators has expanded to include large numbers of paralogues. The *Bacillus subtilis* genome encodes seven ECF σ factors, *Mycobacterium tuberculosis* encodes 10, and, remarkably, *Streptomyces coelicolor* encodes at least 50. In most cases, the function of these σ factors is not yet known.

In *B. subtilis*, most studies to date have concentrated on three of the ECF σ factors, σ^X, σ^W, and σ^M. The roles of these factors have been investigated by phenotypic analysis of mutant strains altered in σ activity (14, 15), identification of target operons (3, 4, 6, 17, 18, 30), and identification of signals that function to induce the various regulons (7, 28, 30, 32). The results indicate that σ^X controls functions associated with modification of the cell envelope, while σ^W and σ^M control overlapping regulons that are induced by antibiotics that target the cell envelope (12). The σ^W regulon is strongly induced by alkali shock (30), although this may be due to effects of high pH on cell wall synthesis.

Despite this progress, the roles of the other four ECF σ factors, σ^Y, σ^{YlaC}, σ^V, and σ^Z, are still mysteries. As one approach to defining the roles of these regulators, we generated mini-Tn*10* transposon libraries to identify mutants with increased expression of σ^Y, σ^{YlaC}, σ^V, or σ^Z. In principle, selection for upregulation might identify proteins that interact directly with the operon control region (e.g., repressors) or genes that affect cell physiology in ways that trigger operon expression. In addition, since most of these operons are thought to be autoregulated by the encoded σ factor, insertions might identify negative regulators of σ factor activity (e.g., anti-σ factors).

Here we report the characterization of mutants that are derepressed for expression of *sigY*. We identified an insertion mutation, *yxlC*::Tn*10*, that activated expression of σ^Y-dependent genes, including the autoregulated *sigYyxlCDEFG* operon. Using a combination of molecular genetic and genomic approaches, we identified genes within the *sigY* operon that regulate the activity of σ^Y and characterized two σ^Y target promoters.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* and *Escherichia coli* strains used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium at 37°C with vigorous shaking. Antibiotics were added to the growth medium when appropriate to 100 μg/ml for ampicillin or 200 μg/ml for spectinomycin for *E. coli* and 100 μg/ml for spectinomycin, 10 μg/ml for kanamycin, 20 μg/ml for tetracycline, 8 μg/ml for neomycin, and 1 μg/ml for erythromycin plus 25 μg/ml for lincomycin (for macrolide-lincomycin-streptogramin B resistance) for *B. subtilis*.

Construction of transcriptional fusions and *sigY mutant*. The *sigY* promoter region was amplified from *B. subtilis* chromosomal DNA by PCR with primers 339 and 340 (Table 2). The resulting fragment was digested with *Hin*dIII and *Bam*HI and cloned into pJPM122 (26) to generate plasmid pMC80 (P_Y-*cat-lacZ*). The sequence of the promoter region was verified by DNA sequencing (Cornell DNA sequencing facility). The promoter fusion was introduced into the SPβ prophage by a double-crossover event, in which plasmid pMC80 was linearized with *Sca*I and transformed into *B. subtilis* strain ZB307A (33) with selection for neomycin resistance. SPβ lysates were prepared by heat induction as described (9) and used to transduce CU1065 to generate strain HB0065 (P_Y-*cat-lacZ*). Reporter fusions for P_X, P_W, and P_M have been described (4, 15, 16). Reporter fusions for putative *sigV*, *ylaC*, and *sigZ* promoters were constructed with a similar strategy (Tables 1 and 2). The *sigY* mutant HB0009 was constructed by transforming chromosomal DNA from HB4245 (*sigY*::*MLS*) (16) into CU1065.

Construction of mini-Tn*10* libraries and identification of mutants upregulated in σ^Y activity. *B. subtilis* strain HB0065 was transformed with pIC333 (27) to generate random mini-Tn*10* libraries as described previously (28). Nine libraries were generated and plated onto LB containing spectinomycin, 5-bromo-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
B. subtilis		
CU1065	W168 *trpC2 att*SPβ	Lab stock
ZB307A	W168 SPβc2Δ2::Tn*917*::pSK10Δ6	33
HB4245	JH642 but *sigY*::*mls*	16
HB0009	CU1065 but *sigY*::*mls*	This work
HB0060	ZB307A SPβ(P_Y-*cat-lacZ*)	This work
HB0065	CU1065 SPβ(P_Y-*cat-lacZ*)	This work
HB0061	ZB307A SPβ(P_V-*cat-lacZ*)	This work
HB0067	CU1065 SPβ(P_V-*cat-lacZ*)	This work
HB0063	ZB307A SPβ(P_Z-*cat-lacZ*)	This work
HB0071	CU1065 SPβ(P_Z-*cat-lacZ*)	This work
HB0064	ZB307A SPβ(P_{ylaA}-*cat-lacZ*)	This work
HB0073	CU1065 SPβ(P_{ylaA}-*cat-lacZ*)	This work
HB0120	HB0065 but *yxlC*::Tn*10* (Spc^r)	This work
HB0119	HB0120 cured of SPβ	This work
HB0121	HB0119 but *sigY*::*mls*	This work
HB0122	HB0121 SPβ(P_Y-*cat-lacZ*)	This work
HB0915	CU1065 but *yxlC*::*mls*	This work
HB0917	HB0915 SPβ(P_Y-*cat-lacZ*)	This work
HB0916	CU1065 but *yxlCDEFG*::*kan*	This work
HB0918	HB0916 SPβ(P_Y-*cat-lacZ*)	This work
HB5302	CU1065 but *yxlFG*::*kan*	This work
HB5303	HB5302 SPβ(P_Y-*cat-lacZ*)	This work
HB5306	CU1065 but *yxlCDE*::*tet*	This work
HB5307	HB5306 SPβ(P_Y-*cat-lacZ*)	This work
HB5308	CU1065 but *yxlDE*::*tet*	This work
HB5309	HB5308 SPβ(P_Y-*cat-lacZ*)	This work
HB5310	CU1065 but *yxlD*::*tet*	This work
HB5311	HB5310 SPβ(P_Y-*cat-lacZ*)	This work
HB5312	CU1065 but *yxlE*::*tet*	This work
HB5313	HB5312 SPβ(P_Y-*cat-lacZ*)	This work
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Lab stock
Jm2r$^-$	*mcrAB hsdR recA1* Δ(*lac-proAB*) *thi gyrA96 relA1 srl*::Tn*10* F′ (*proAB lacZ*ΔM15)	S. Zahler
Plasmids		
pJPM122	Vector for integration of reporter fusions in SPβ (Ap^r Neo^r)	26
pMC80	P_Y-*cat-lacZ* cloned in pJPM122	This work
pIC333	Shuttle vector carrying mini-Tn*10* (Ap^r MLS^r Spc^r)	27
pXT	Derivative of pDG1731	3
pDG646	Macrolidelincomycin-streptogramin B (MLS) resistance cassette vector	10
pDG780	Kanamycin resistance cassette vector	10
pDG1513	Tetracycline resistance cassette vector	10

4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and growth-inhibitory levels (6, 8, or 10 μg/ml) of chloramphenicol (Cm). Cm^r mutants with elevated β-galactosidase activity were isolated following 2 days of incubation at 37°C. Chromosomal DNA was extracted from each mutant and used to transform HB0065, with selection on LB plates with spectinomycin (Spc), neomycin, and X-Gal. Only mutants that had a high level of linkage between the mini-Tn*10*(*spc*) and elevated expression of β-galactosidase expression were characterized further. Plasmids containing the mini-Tn*10* element with a ColE1 origin and flanking *B. subtilis* chromosomal DNA were recovered by transformation into *E. coli*. DNA sequences upstream and downstream of the transposon were obtained with two primers (50 and 51) corresponding to the left and right ends of the mini-Tn*10*, respectively. We generated a *sigY yxlC* double mutant (HB0121) by transformation of chromosomal DNA from HB4245 (*sigY*::*mls*) into HB0119 (HB0120 cured of SPβ; Table 1). The P_Y-*cat-lacZ* fusion was then introduced into this strain by transduction, and β-galactosidase was measured.

Construction of null mutants of *yxlC*, *yxlCDEFG*, *yxlFG*, *yxlCDE*, *yxlDE*, *yxlD*, and *yxlE*. Long-flanking homology PCR was used as described (29) to generate allelic replacement mutants for each gene or group of genes. In brief, approximately 1,000-bp genomic regions flanking the gene(s) to be deleted were amplified from CU1065 chromosomal DNA by PCR. The primers used are summarized in Table 2. Drug resistance cassettes were amplified by PCR from pDG646 (macrolide-lincomycin-streptogramin B, *mls*), pDG780 (kanamycin, *kan*), or pDG1513 (tetracycline, *tet*) (10).

For each mutant construction, equal amounts (approximately 200 to 300 ng) of purified upstream flanking fragment, downstream flanking fragment, and the corresponding drug resistance cassette were used in a joint PCR procedure as described (29), with either the Expand polymerase (Roche) or the HotStarTaq Master Mix kit (Qiagen). The resulting PCR products were purified and then directly transformed into *B. subtilis* wild-type strain CU1065, selecting for the corresponding antibiotic resistance. The generated mutant strains are listed in Table 1 and shown in Fig. 1.

β-Galactosidase assay. In preliminary studies, overnight cultures were diluted 1:100 into 15 ml of LB medium. Samples were taken when the optical density at 600 nm (OD_{600}) reached 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 and 1 h after it reached 1.0. The β-galactosidase activity of each sample was measured according to Miller (20). At an OD_{600} of 0.8 (late log phase), P_Y-*cat-lacZ* expression reached its maximum level.

To compare different strains, three individual colonies were inoculated in LB medium with corresponding antibiotics and incubated at 37°C overnight. Then 50 μl of each overnight culture was used to inoculate 5 ml of warm LB medium. Samples were taken at an OD_{600} of 0.8, and β-galactosidase activity was assayed. Averages and standard deviations were calculated for each strain.

Primer extension assays. RNA was prepared from mid-logarithmic-phase cells ($OD_{600} \approx 0.5$) with the Qiagen RNeasy mini kit; 100 μg of total RNA (from CU1065 or the *sigY yxlC* double mutant strain) or 10 μg of total RNA (from the *yxlC*::Tn*10* mutant) and 2 pmol of end-labeled reverse primer were mixed for each primer extension experiment following the procedures described previously (4). For mapping the *sigY* transcriptional start site, the end-labeled reverse primer 340 was used. The PCR-amplified *sigY* promoter region (with primers 339 and 340) was sequenced with the same primer, and the reaction products were

TABLE 2. Oligonucleotides used in this study[a]

No.	Name	Sequence
339	sigY fwd (pJPM122)	5′-GGCCCAAGCTTCGCCTTTCTACTTTCAATGC-3′
340	sigY rev (pJPM122)	5′-CGCGGATCCGCCGCTGTTCTTCTTGTGTAT-3′
335	sigV fwd (pJPM122)	5′-CCCCAAGCTTGGAATAATGTTTGTCATC-3′
437	sigV rev (pJPM122)	5′-CGGGATCCTTGCTTATGGTCAGTTATGCA-3′
438	ylaA fwd (pJPM122)	5′-CCCAAGCTTGATAGTATTGTCCTGTGT-3′
439	ylaA rev (pJPM122)	5′-CGGGATCCATTGAATCAGCAGGGTGCTTT-3′
440	sigZ fwd (pJPM122)	5′-CCCAAGCTTTTGTCGCCAGAACAA-3′
441	sigZ rev (pJPM122)	5′-CGGGATCCAACGGCTGATGAAATTGATCC-3′
50	mini-Tn10 (left)	5′-GCCGATTCATTAATGCAG-3′
51	mini-Tn10 (right)	5′-CCCACTTATAAACAAAG-3′
776	ybgB fwd	5′-GGAAGCTTAAGGACAAAATACAA-3′
777	ybgB rev	5′-AAGGATCCGGCAGAAAAGGGTAAA-3′
141	sigY-f (pET16x)	5′-GGGGTACCATGGATACACAAGAAGAACAG-3′
142	sigY-r (pET16x)	5′-CGGGATCCTTATTCATCATCCCACTCCT-3′
1295	kan fwd	5′-CAGCGAACCATTTGAGGTGATAGG-3′
1296	kan rev	5′-CGATACAAATTCCTCGTAGGCGCTCGG-3′
1297	mls fwd	5′-GATCCTTTAACTCTGGCAACCCTC-3′
1298	mls rev	5′-GCCGACTGCGCAAAAGACATAATCG-3′
941	tet fwd1	5′-TCTTGCAATGGTGCAGGTTGTTCTC-3′
942	tet fwd2	5′-GCTTATCAACGTAGTAAGCGTGG-3′
940	tet rev	5′-GAACTCTCTCCCAAAGTTGATCCC-3′
1301	yxlC-up fwd	5′-GGCTTTGAATCATTTGCGGGATGCCTAGC-3′
1302	yxlC-up rev (mls)	5′-CATTCAATTTTGAGGGTTGCCAGGATTCGGTATAGAGGGATTGGC-3′
1304	yxlC-dw fwd (mls)	5′-CGATTATGTCTTTTGCGCAGTCGGCGGCATCTCGGCGAATGCGAG-3′
1305	yxlC-dw rev	5′-CACACACCTGTTCTGCATCGTGC-3′
1303	yxlC-up rev (kan)	5′-CCTATCACCTCAAATGGTTCGCTGGTGACAGCTGGTGAAGCAG-3′
1306	yxlG-dw fwd (kan)	5′-CGAGCGCCTACGAGGAATTTGTATCGTGCCAGAGAACCGGCTCC
1307	yxlG-dw rev	5′-CGGCATCATTCTCGGCAGCTACGG-3′
936	yxlF-up fwd	5′-GCATCGCGGCTCTATCTCGATCACC-3′
937	yxlF-up rev (kan)	5′-CACCTCAAATGGTTCGCTGGTTTACAGCTTCATGGTGCCTGTACG-3′
939	yxlG-up rev (kan)	5′-CACCTCAAATGGTTCGCTGGTCCAGCCACTCCTTCTGCAATAGCG-3′
943	yxlC-up rev (tet1)	5′-GAACAACCTGCACCATTGCAAGATTCGGTATAGAGGGATTGGC-3′
945	yxlE-dw fwd (tet)	5′-GGGATCAACTTTGGGAGAGAGTTCAGCAAAGGTAAGCCGATATGC-3′
935	yxlE-dw rev	5′-CAGGAAGGTTCCCTCCATGTGCG-3′
1445	yxlD-up rev (tet1)	5′-GAACAACCTGCACCATTGCAAGACCAATACGATGAGACAAGCC-3′
1447	yxlD-dw fwd (tet)	5′-GGGATCAACTTTGGGAGAGAGTTCTCAAGCACCGATGCCTCTG-3′
1448	yxlE-up rev (tet2)	5′-CCACGCTTACTACGTTGATAAGCCTTCCGATCCGGCCTAATGAC-3′
1446	yxlD-up rev (tet2)	5′-CCACGCTTACTACGTTGATAAGCGTCATCCGCGTTTCACCTCGC-3′

[a] The underlined sequences correspond to the 5′ and 3′ ends of the drug resistance cassette used in each construction.

electrophoresed adjacent to the primer extension products. For *ybgB*, primer 777 was used, and primers 776 and 777 were used for amplification of the *ybgB* promoter region for the sequence ladder.

Microarray analysis. Total RNA was prepared from *B. subtilis* CU1065 and the *yxlC*::Tn*10* mutant grown aerobically in LB medium. The cell cultures were grown to an OD_{600} of 0.4, and the cells were harvested immediately. The protocol for RNA isolation, cDNA synthesis, and slide hybridization was described previously (31). Each RNA preparation was used to make both indocarbocyanine- and indodicarbocyanine-labeled cDNA, and all hybridizations were done twice, once with each cDNA preparation, to control for differences in labeling between the two fluorophores. Since all PCR products were spotted twice on each slide, all signal intensities and calculated ratios are the averages of four values. Two microarray experiments (*yxlC* mutant versus wild type) were performed with RNA prepared from two independent cell cultures. Signal intensities were quantified with ArrayVision software (Molecular Dynamics) and assembled into Excel spreadsheets. Mean values and standard deviations were calculated with Excel. Genes with a standard deviation in expression values (fluorescence intensity) greater than the mean value were ignored. Complete datasets are available as supplementary material at http://www.micro.cornell.edu/faculty.JHelmann.html.

Overproduction and purification of σ^Y protein. The *sigY* gene was PCR amplified from *B. subtilis* chromosomal DNA with oligonucleotides 141 and 142, designed to engineer an *Nco*I site upstream and a *Bam*HI site downstream of the *sigY* gene. The PCR product was cloned into pET16x (Novagen) via the *Nco*I and *Bam*HI sites to generate pKF85. The sequence of *sigY* in pKF85 was verified by DNA sequencing (Cornell DNA sequencing facility). σ^Y was purified from *E. coli* strain BL21/DE3(pLysS) transformed with pKF85. Expression was induced by addition of 20 μM isopropylthiogalactopyranoside (IPTG) for 3 h, resulting in the formation of inclusion bodies. σ^Y was purified from the inclusion bodies as follows: 2 ml of disruption buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 0.1 mM dithiothreitol, 1 mM β-mercaptoethanol, 233 mM NaCl, 10% glycerol) was added to a frozen pellet generated from 50 ml of induced culture. Then 0.4 ml of the resuspended cells was sonicated for 5-s pulses, 12 pulses total.

Inclusion bodies were collected by centrifugation at 13,000 rpm for 20 min at 4°C and resuspended twice in 10 ml of TEDG buffer(50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5% [vol/vol] Triton X-100). The washed pellet was resuspended in 2 ml of TEDG with 0.4% Sarkosyl and gradually diluted to 20 ml with TEDG buffer to allow refolding of σ^Y. The sample was then dialyzed against 200 ml of TEDG for 8 h at 4°C. A 1-ml Hi-trap heparin column was equilibrated with 3 ml of TEDG, and 1 ml of the dialyzed sample was loaded onto the column. The column was washed five times with 500 μl of TEDG. σ^Y was eluted from the column with washes of increasing NaCl concentrations (50 to 500 mM NaCl) in TEDG buffer. Each eluate was tested for the presence of protein with the Bio-Rad protein detection assay, and peak fractions were collected. The renatured σ^Y eluted with ≈0.5 M NaCl and was analyzed by polyacrylamide gel electrophoresis (PAGE) and confirmed to migrate at approximately 21.2 kDa, which is the predicted molecular mass for σ^Y.

In vitro runoff transcription assay and microarray analysis. The runoff transcription/macroarray analysis (ROMA) experiment was performed as described previously (6). Purified σ^Y was added in 17-fold molar excess relative to the core RNA polymerase. With the σ^Y autoregulated promoter as a template, we determined that the specificity of σ^Y-dependent transcription was optimal between 100 and 150 mM KCl (data not shown). For the ROMA experiment, 100 mM KCl (final concentration) was used.

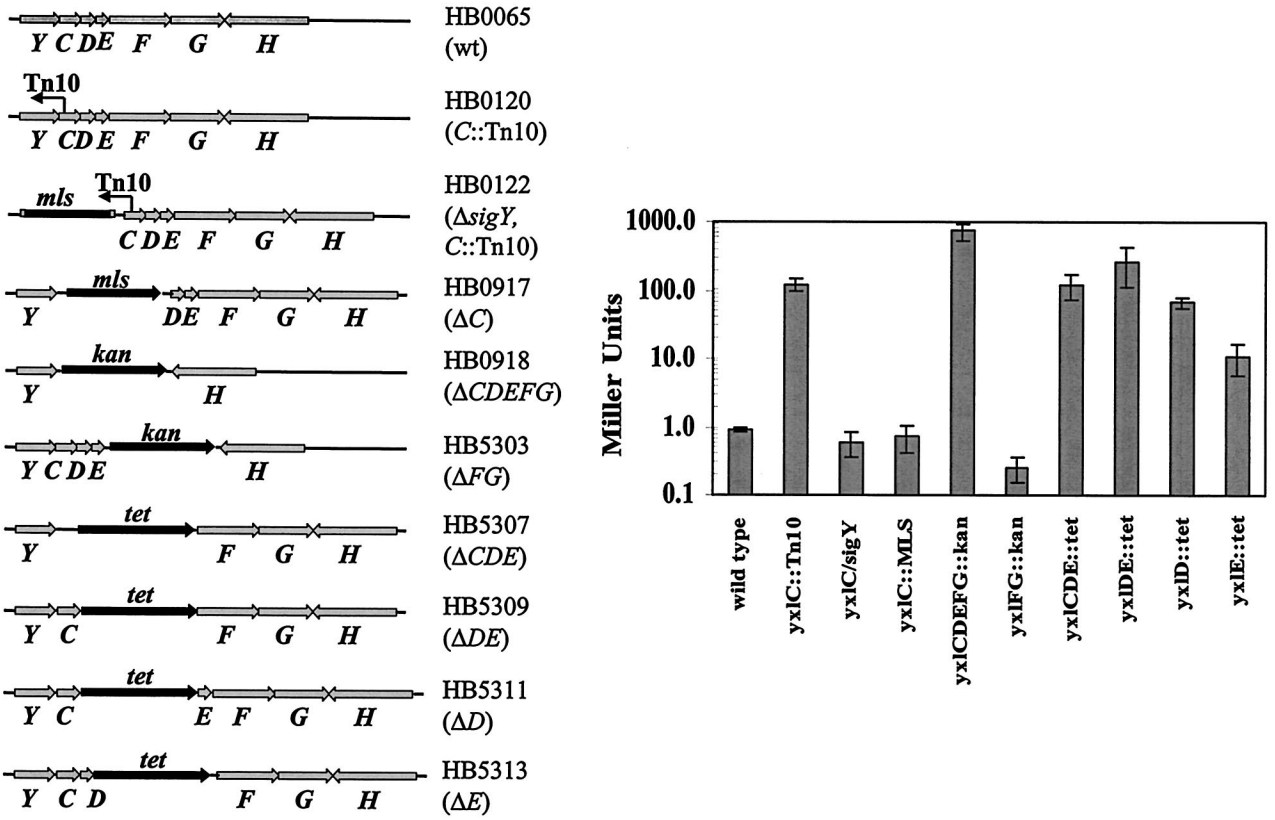

FIG. 1. Genetic analysis of *sigY* operon. The genetic organization of the *sigY-yxlCDEFG* mutations used in these studies is illustrated. The corresponding level of β-galactosidase synthesis (mean ± standard deviation; $n = 3$) for the P_Y-*cat-lacZ* reporter fusion is shown to the right. wt, wild type.

RESULTS

Characterization of mini-Tn*10* mutants with elevated expression of *sigY*. We used the presumptive *sigY* regulatory region to generate a *cat-lacZ* operon fusion (*sigY′-cat-lacZ*) integrated ectopically into the SPβ prophage. The resulting fusion was expressed weakly if at all under a variety of laboratory growth conditions, suggesting that the signals that normally activate expression of *sigY* were not present. We selected for Tn*10*(*spc*) insertions that led to chloramphenicol resistance. We identified five Cm^r mutants from three independent libraries (approximately 10,000 transposants) that had dramatically increased β-galactosidase activity. In each case, these phenotypes were tightly linked to the *spc* marker associated with the transposon. DNA linked to each transposon was recovered by transformation into *E. coli*, and the sites of insertion were determined by DNA sequencing. All five transposants had the Tn*10*(*spc*) insertion at the same position and in the same direction within *yxlC*, the gene immediately downstream of *sigY* (HB00120, Fig. 1). Expression of *sigY′-cat-lacZ* in the *yxlC*::Tn*10* mutant was increased more than 100-fold compared to the wild type, as measured during late logarithmic growth (Fig. 1).

σ^Y is positively autoregulated. Most ECF σ factors are positively autoregulated, often with an adjacent anti-σ factor gene that regulates activity (11, 12, 19). To determine whether *sigY* is autoregulated, we took advantage of the high level of expression from the *sigY′-cat-lacZ* reporter fusion in the *yxlC*::Tn*10* mutant. When a *sigY* mutation was introduced into this genetic background, expression was reduced to the background level (Fig. 1, strain HB0122). Thus, the *sigY′-cat-lacZ* reporter fusion is also a reporter of σ^Y-dependent transcriptional activity, P_Y-*cat-lacZ*.

The upstream region of *sigY* contains a candidate promoter similar to other promoters recognized by ECF σ factors (13). We mapped the transcription start site of *sigY* by primer extension, taking advantage of the high level of expression in the *yxlC*::Tn*10* mutant. Transcription started from a C residue 9 bases downstream from the −10 region CGTC motif (Fig. 2A). Consistent with the β-galactosidase result, this transcript was not detectable in the *sigY yxlC* double mutant even when 10 times more total RNA was used as the template. No other start sites were observed within the *sigY* regulatory region (≈250 bp upstream from the start codon). We conclude that σ^Y positively autoregulates its own expression.

The σ^Y autoregulatory promoter P_Y has consensus elements of TGAAC (−35) and CGTC (−10) with a 17-bp spacer (Fig. 2C). This is very similar to the consensus sequences recognized by other *B. subtilis* ECF σ factors, including σ^W and σ^X (Fig. 3). Although both σ^X and σ^W can recognize promoters with a CGTC motif in the −10 region (25), *sigY* has not been identified as part of either the σ^X or σ^W regulon (5, 6, 18), nor is *sigY* upregulated by the induction of other ECF σ factors (2;

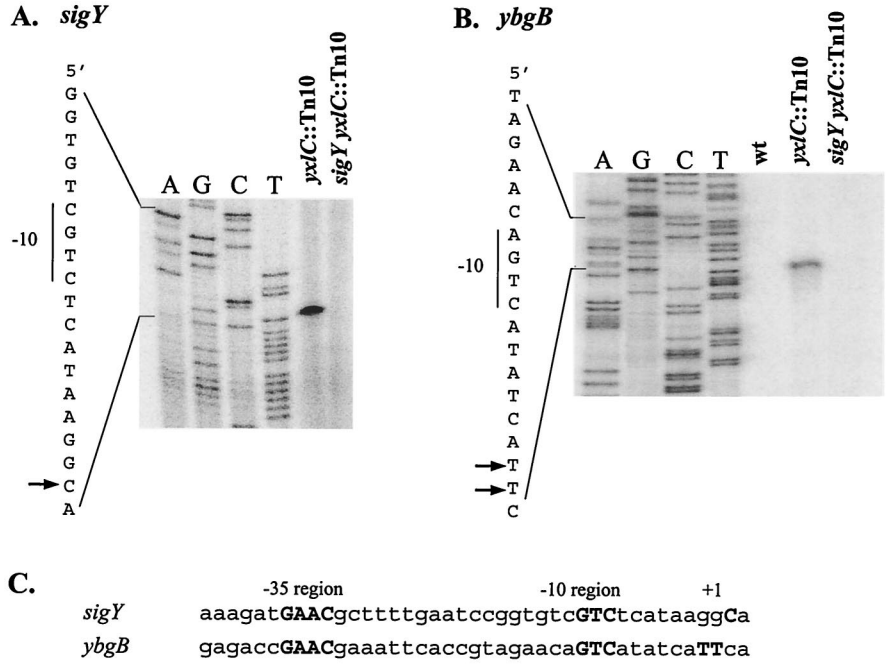

FIG. 2. Primer extension mapping of *sigY* (A) and *ybgB* (B) transcription start sites. RNA was extracted from mid-log-phase cells of strains CU1065 (wild type, wt), HB0119 (*yxlC*::Tn*10*), and HB0121 (*sigY yxlC* double mutant) in LB medium. The putative −10 regions are indicated, and the transcription start sites are shown by arrows. (C) Alignment of the σ^Y autoregulated promoter sequence with the *ybgB* promoter region. Conserved bases and transcription start sites (+1) are in bold uppercase type.

our unpublished results). These results suggest that other sequence features, in addition to those highlighted (Fig. 3), are important for promoter discrimination.

σ^Y does not activate transcription of other ECF σ factors. Next, we tested the effect of the *yxlC*::Tn*10* insertion (leading to high in vivo σ^Y activity) on the expression of autoregulatory promoters recognized by various ECF σ factors. We replaced the P_Y-*cat-lacZ* fusion in strain HB0120 with reporter fusions containing the known or putative autoregulatory regions for each of the other six ECF σ factors (Fig. 3 and data not shown). The results indicate that the *yxlC*::Tn*10* insertion and consequent upregulation of σ^Y activity do not lead to elevated expression of any of the other ECF σ factors. We conclude that, in general, σ^Y does not regulate other ECF σ factors.

Effect of *yxlC*::Tn*10* insertion is due to polarity. In several well-characterized examples, the gene immediately downstream of an ECF σ factor gene encodes an anti-σ factor (12). Therefore, we hypothesized that *yxlC* might encode an anti-σ factor. However, when we engineered a *yxlC*::*mls* allelic replacement mutant (HB0917), we failed to observe an increase in σ^Y activity (Fig. 1). Note that in this mutant the *mls* cassette was oriented to allow expression of downstream genes from the *mls* promoter. In light of this result, we hypothesized that the original Tn*10* insertion was polar on downstream genes. *sigY* is the first of six codirectional genes, many with overlapping start and stop codons (Table 3), that likely constitute an operon.

σ^Y is negatively regulated by both YxlD and YxlE. To determine which of the four downstream genes might have been affected by the *yxlC*::Tn*10* insertion mutation, we constructed a series of allelic replacement mutants (Fig. 1). When the whole *yxlCDEFG* region was deleted, P_Y was derepressed (HB0918), indicating that σ^Y is negatively regulated by one or more proteins encoded by the downstream genes. High-level expression from P_Y was also observed in the Δ*yxlCDE* but not the Δ*yxlFG* mutant. We conclude that YxlF and YxlG are not essential for P_Y regulation but might function as accessory factors, because expression was always lower in the Δ*yxlCDE* than in the Δ*yxlCDEFG* mutant.

To investigate the role of individual gene products of the *yxlCDE* region, three additional deletions (Δ*yxlDE*, Δ*yxlD*, and Δ*yxlE*) were constructed (Fig. 1). The results indicate that both YxlD and YxlE are important for negative regulation of σ^Y activity, with YxlD being the major negative regulator. Note that both YxlD and YxlE are small proteins predicted to as-

	-35 region	-10 region	+1
P_Y	aaaagaTGAACgcttttgaatccggtgtCGTCtcataaggCag		
P_X	tgtaaTGTAACttttcaagctattcataCGACaaaaaagTGaa		
P_W	aaaatTGAAACcttttgaaa-cgaagctCGTAtacatacaGAc		
P_M	taatgTGCAACtttaaacctttcttatgCGTGtataacaTAga		
sigV	tctgcgcAAACtatttttcagcctgccgCGTCtatcctaggta		
ylaA	catctTGAAACttttgaaaa-gtccgcTGTCtaaccgaatga		
sigZ	attgaTTCAACaattgtttgctctttgtTACGattaaaaagac		

FIG. 3. Known autoregulated promoter regions of σ^Y (P_Y), σ^X (P_X), σ^W (P_W), and σ^M (P_M) were compared with the putative promoter sequences just upstream of the genes for σ^V (*sigV*), YlaA (*ylaA*) (the first gene in the *ylaABCD* operon; *ylaC* encodes σ^{YlaC}), and σ^Z (*sigZ*). The −35 and −10 regions are in uppercase, with conserved bases in bold. Mapped transcription start sites (+1) are in bold uppercase.

TABLE 3. The σ^Y regulon

Protein	No. of amino acids	Location[a]	Positions on genome	Known or putative function
SigY	178	C	3969867–3969334	RNA polymerase ECF-type sigma factor
YxlC	106	M	3969338–3969021	Unknown
YxlD	68	M	3969021–3968818	Unknown
YxlE	62	M	3968818–3968633	Unknown
YxlF	295	C	3968623–3967739	Similar to ABC transporter (ATP-binding protein)
YxlG	259	M	3967739–3966963	Unknown
YbgB	91	M	258520–258792	Unknown

[a] C, predicted cytoplasmic localization; M, predicted membrane protein.

sociate with the cell membrane (Table 3). Thus, the signaling complex likely to regulate σ^Y activity may be membrane localized.

σ^Y regulon includes *sigY* operon and *ybgB* gene. To identify other genes transcribed by σ^Y, we used DNA microarray analysis to compare RNA populations from wild-type and *yxlC*::Tn*10* mutant cells in two independent experiments. Overall, ≈99.5% of the expressed genes varied less than twofold in expression level, despite the nearly 100-fold effect of the *yxlC* insertion on expression of *sigY* itself. Only seven genes were significantly and reproducibly upregulated (>3.5-fold) in the *yxlC* mutant (Table 4). The most dramatic changes were the *sigY* (71-fold) and the *yxlC* (14-fold) genes. The interpretation of this finding is complicated by the fact that the mutant strain has a Tn*10* insertion in the *yxlC* gene and the upregulation noted in the microarray study could, in principle, be due to countertranscription from the promoter of the spectinomycin resistance cassette in the Tn*10* insertion (Fig. 1). Nevertheless, it is clear from the reporter fusion studies that the *yxlC*::Tn*10* insertion leads to upregulation of P_Y. Therefore, we prefer a model in which the Tn*10* insertion leads to upregulation of P_Y, which leads to elevated levels of *sigY* and the 5′-proximal part of *yxlC*.

Five other genes that were significantly upregulated in the *yxlC* mutant were *ybgB*, *ybgE*, *yvdF*, *des* and *tyrZ*. YbgB is a small hydrophobic protein (91 amino acids) of unknown function, while YbgE is similar to a branched-chain amino acid aminotransferase. Using primer extension, we mapped the *ybgB* transcription start site and confirmed that this gene is σ^Y dependent (Fig. 2B). It is not yet known if the upregulation of *ybgE* is physiologically relevant, since it is separated from *ybgB* by a 212-bp intergenic region. This region has recently been shown to bind CodY, and *ybgE* is derepressed in a *codY* mutant (22). We suggest that the upregulation of *ybgE* is due to readthrough of the *ybgB* transcript into *ybgE*, which is otherwise repressed under our growth conditions.

Apart from *ybgB*, none of the other upregulated genes appeared to be direct targets for σ^Y-directed transcription. The *des* gene encodes a cold-inducible membrane phospholipid desaturase, and its transcription is controlled by σ^A (1). *tyrZ* is a monocistronic gene encoding a minor tyrosyl-tRNA synthetase. No obvious σ^Y-dependent promoter was found upstream of *tyrZ*, and we failed to detect its transcription start site even in the *yxlC* mutant. The *yvdF* gene encodes a putative maltogenic amylase (98% identical to the BbmA sugar hydrolase from *B. subtilis* SUH4-2 [8]) and is the second gene of a large cluster. Most genes in this cluster seem to be involved in maltose or maltodextrin utilization. However, no obvious σ^Y-dependent promoter was found upstream of either *yvdF* or the larger gene cluster, and we were unable to detect any transcription start site for *yvdF* in primer extension experiments. Since only the second gene in this region was induced in the *yxlC* mutant, this could be a false-positive, the result of cross-hybridization, or an indirect effect.

Analysis of σ^Y regulon by ROMA. With the microarray approach, we identified two operons (*sigY-yxlCDEFG* and *ybgB*) as direct targets for σ^Y-directed transcription. As a complementary approach, we used reconstituted σ^Y holoenzyme to identify in vitro targets in a whole-genome transcription study. As described previously (6), in the runoff transcription/macroarray analysis (ROMA) experiment, we generated ^{33}P-labeled runoff transcripts with total genomic DNA and used these to probe a DNA macroarray (Sigma/GenoSys) containing 4,107 *B. subtilis* open reading frames.

We detected only three strong hybridization signals by ROMA: *sigY*, *ybgB*, and *yabE* (Fig. 4). In contrast, dozens of strong signals were detected when the σ^W or σ^X holoenzyme was used (5, 6). Hybridization to *yabE* is apparently due to an RNA generated by a σ factor contaminating the core preparation (E), since the signal appeared in both E and Eσ^Y experiments. Additional weak signals generated in the Eσ^Y experiment but lacking in the E alone experiment were also identified. These signals correspond to *ybgE*, the downstream gene of *ybgB*, and the genes downstream of *sigY*. These signals were reduced in intensity due to the use of restriction enzyme-digested DNA in the in vitro transcription reaction, which served to limit transcription to promoter-proximal genes. Significantly, the other three genes (*yvdF*, *des*, and *tyrZ*) that were induced in the *yxlC*::Tn*10* mutant (Table 4) were not detected in ROMA, consistent with our conclusion that these are not likely to be direct targets for σ^Y.

In summary, both transcriptional profiling and ROMA experiments suggest that σ^Y directs transcription of a small regulon including only the *sigY-yxlCDEFG* and the *ybgB* genes (Table 3). Note that most of these gene products are small, hydrophobic proteins, suggestive of a role in transport or other membrane-associated functions.

TABLE 4. Genes that were induced >2-fold in the *yxlC*::Tn*10* mutant[a]

Gene	Induction (fold ± SD)		Avg induction (fold)
	Set 1	Set 2	
sigY	95.4 ± 21.0	46.8 ± 3.7	71.1
yxlC	17.0 ± 4.6	11.0 ± 2.8	14.0
ybgB	9.0 ± 4.6	6.6 ± 0.7	7.8
ybgE	6.1 ± 1.8	5.9 ± 1.7	6.0
yvdF	9.0 ± 2.5	5.9 ± 1.9	7.4
des	6.0 ± 1.6	4.4 ± 0.4	5.2
tyrZ	4.1 ± 1.0	3.5 ± 0.7	3.8

[a] Complete datasets are available as supplementary material at http://www.micro.cornell.edu/faculty.JHelmann.html. Set 1 and set 2 are the results from two microarray experiments (*yxlC* versus wild type) with RNA prepared from two independent cell cultures.

FIG. 4. Total *B. subtilis* chromosomal DNA was digested with *Eco*RI and transcribed in vitro with either the core alone (E) or the core with an excess of σ^Y ($E\sigma^Y$). Signals generated specifically by the σ^Y holoenzyme belong to either the *sigYyxlCDEFG* or *ybgB* operon (ovals). The *yabE* gene (rectangle) also gave a strong signal in the control experiment (E). The positions of the identified genes (on the panorama macroarray) are listed beside each gene. Similar results were obtained in a replicate experiment with *Hin*dIII-digested *B. subtilis* chromosomal DNA as the transcription template.

DISCUSSION

The ECF σ factors activate a variety of stress responses that often involve changes in the cell envelope or transport or efflux across the cell membrane (reviewed in references 12, 21, and 24). In *B. subtilis*, significant progress has been made in defining the regulons controlled by σ^X, σ^W, and σ^M, but the functions of the other four ECF σ factors are unknown (13). Here we demonstrate that σ^Y controls a small regulon, including its own operon, and at least one other target gene, *ybgB*. However, the function of this regulon is not yet clear.

We have explored several strategies to decipher the regulatory roles of the multiple ECF σ factors in *B. subtilis*. Mutational analyses revealed that none of the seven σ factors is essential, and even multiply mutant strains often have only subtle phenotypes. Therefore, we focused our efforts on identifying target genes that are recognized by each σ factor with promoter consensus search, DNA microarray, and in vitro transcription-based strategies (3, 4, 6, 7, 15–18). In complementary experiments, we attempted to define the chemical and genetic factors that elicit σ factor activation (28). Together, these studies revealed that σ^X controls several operons that modulate cell envelope properties, including the D-alanylation of teichoic acids (*dltABCDE*), phosphatidylethanolamine biosynthesis (*pssA psd*), and expression of autolysins (*lytR*) (4, 5, 18). The σ^W regulon includes at least 30 operons, including several with roles in antibiotic resistance (3, 6). This regulon is induced by antibiotics acting on the cell wall or by alkali stress (7, 30). The regulon controlled by σ^M has not been well defined, but it appears to overlap the σ^X and σ^W regulons and includes at least one gene that functions in antibiotic resistance (*bcrC*) (4, 23).

Several different strategies have also been explored to define the role of σ^Y. Direct comparison of the transcriptomes of wild-type and *sigY* null mutant strains did not reveal significant differences (data not shown). One interpretation of this result is that σ^Y may not be active under the conditions of the experiment, and therefore very few genes (if any) were affected by the absence of the σ factor. For both the σ^X and σ^W regulons, the rules defining promoter recognition are reasonably well defined (25), and searching the genome for sequences resembling known target sites produced lists of candidate promoters, many of which turned out to be dependent on the expected σ factor (6, 17, 18). Similar search strategies have not been as successful for other ECF σ factors. While it is clear that the two known promoters recognized by σ^Y are similar in sequence (Fig. 2C), searches based on the apparent consensus identified sites already classified as dependent on σ^X, σ^W, or both but very few additional candidates (data not shown). It remains possible that the σ^Y regulon may overlap that recognized by σ^X, σ^W, or another ECF σ factor. However, this suggestion is not supported by either the in vivo transcriptome analysis or the ROMA studies reported here.

The present work was initiated with the goal of defining the genetic factors that negatively regulate σ^Y activity. In similar studies with the autoregulatory σ^X- and σ^W-dependent promoters, we identified transposon insertions in genes for antibiotic biosynthesis, sugar isomerases, and multidrug efflux systems (28). With only one exception, the insertions affected σ^X or σ^W, but not both. Of those tested to date, none of these insertions affected σ^Y activity (data not shown). Moreover, σ^Y is not strongly activated by a variety of physical or chemical factors that activate σ^X, σ^W, and σ^M (e.g., antibiotics, high salt concentrations, and extreme pH). Weak activation of *sigY* expression is observed in cells grown on minimal medium compared to rich medium, and this expression is σ^Y dependent. Thus, the σ^Y regulon appears to respond to different stresses than those known to activate other σ regulons.

In the present study, we only recovered insertions in the *sigY* operon itself, which focused attention on the regulatory roles of these cotranscribed genes. Our results indicate that the major negative regulators of σ^Y activity, YxlD and YxlE, are two small membrane proteins. It is not yet clear whether these two proteins together form a multisubunit anti-σ factor or whether they act independently. Since their genes are cotranscribed with a predicted component of an ABC transporter (Table 3), we speculate that σ^Y may be regulated by the activity of a membrane transport complex.

The dramatic effect of the *yxlC*::Tn*10* insertion mutation on the activity of the σ^Y autoregulatory promoter encouraged us to pursue global transcriptional profiling to identify other operons upregulated by elevated σ^Y activity. Of the resulting candidate operons, only *ybgB* was clearly a direct target for σ^Y-dependent transcription, and the function of this gene is

not clear. Asai et al. (2) also used transcriptome analysis to define the regulons controlled by ECF σ factors. Their results, based on overexpression of individual ECF σ factors, support the idea that σ^Y positively autoregulates its own expression and that of the downstream genes (*yxlCDEFG* and *yxlH*). The apparent upregulation of *yxlH* may be due to readthrough from the convergent *sigY* operon. Although these authors reported 10 additional genes as being upregulated by induction of σ^Y, only one (*tyrZ*) was also identified in our comparison of wild-type and *yxlC*::Tn*10* mutant strains, and our results suggest that the induction of many of these reported target genes may be due to indirect effects. It should be noted, for example, that their studies were done by inducing each σ factor and harvesting cells after 2 h of growth at 37°C, during which time both the control and experimental cultures likely entered the stationary phase.

Transcriptional profiling is a very powerful approach for defining the effects of regulatory proteins on gene expression, but some target operons may be missed. This can occur due to low expression levels (e.g., due to the inactivity of a needed activator), poor hybridization to target probes, and background expression from other promoter sites. Moreover, it is difficult to separate direct from indirect effects. As an independent approach to estimating the size of the σ^Y regulon, we turned to ROMA, whole-genome in vitro transcription to generate σ^Y-dependent transcripts followed by macroarray analysis to identify the corresponding genes. The results confirmed the transcriptional profiling studies; in both cases, the direct targets of σ^Y regulon appeared to include only two operons, *sigY-yxlCDEFG* and *ybgB*. Further studies will be needed to define the physiological roles of these genes and their products.

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