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The *Bacillus subtilis* Extracytoplasmic-Function σ^X Factor Regulates Modification of the Cell Envelope and Resistance to Cationic Antimicrobial Peptides

Min Cao and John D. Helmann*

Department of Microbiology, Cornell University, Ithaca, New York 14853-8101

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Bacillus subtilis **contains seven extracytoplasmic-function σ factors that activate partially overlapping regulons. We here identify four additional members of the σ^X regulon, *pbpX* (penicillin-binding protein), *ywnJ*, the *dlt* operon (D-alanylation of teichoic acids), and the *pss ybfM psd* operon (phosphatidylethanolamine biosynthesis). Modification of teichoic acids by esterification with D-alanine and incorporation of phosphatidylethanolamine into the cell membrane have a common consequence: in both cases positively charged amino groups are introduced into the cell envelope. The resulting reduction in the net negative charge of the cell envelope has been previously implicated as a resistance mechanism specific for cationic antimicrobial peptides. Consistent with this notion, we find that both *sigX* and *dltA* mutants are more sensitive to nisin than wild-type cells. We conclude that activation of the σ^X regulon serves to alter cell surface properties to provide protection against antimicrobial peptides.**

Bacillus subtilis encodes seven extracytoplasmic-function (ECF) σ factors. Most studies to date have focused on three: σ^X, σ^W, and σ^M (reviewed in reference 19). *sigX* and its downstream gene *rsiX* (encoding the anti-σ^X factor) were originally observed to be homologous (but not orthologous) to *Escherichia coli fecI* and *fecR*, which are involved in expression of ferric citrate transport genes (37). Although expression of *sigX* in *E. coli* can complement a *fecI* mutant (4), the *B. subtilis sigX* mutant is not affected in any known ferri-siderophore uptake systems (20).

To understand the function of σ^X, we identified several σ^X-regulated genes using a consensus promoter search method (22). In these initial studies, we characterized six genes that are preceded by promoters recognized by σ^X: *sigX*, *abh* (an AbrB homolog), *csbB* (a membrane-bound glucosyl transferase) (2), *divIC* (a membrane-bound cell-division initiation protein), *lytR* (a negative regulator of autolysin) (30), and *rapD* (a response regulator aspartate phosphatase) (43). These results suggested that σ^X modulates aspects of cell envelope metabolism. Interestingly, most σ^X-controlled genes are also transcribed by other forms of holoenzyme. For example, *csbB* has an additional σ^B-dependent promoter, *lytR* and *rapD* both have additional σ^A-dependent promoters, and *sigX* itself is preferentially transcribed from an upstream σ^A-dependent site in addition to the σ^X-dependent autoregulatory promoter (20, 22). Moreover, in some cases (e.g., *abh* and *divIC*) the promoter activated by the $E\sigma^X$ holoenzyme can also be recognized by the $E\sigma^W$ holoenzyme at least in vitro (21). Similarly, the recently defined *bcrC* gene (a bacitracin resistance gene) is transcribed from a promoter that is recognized by either σ^X or σ^M (7, 38). The unknown function of many σ^X-controlled genes makes it difficult to predict a phenotype for the *sigX* mutant. This challenge is exacerbated by the fact that many of the genes are expressed from multiple promoters or by multiple holoenzyme forms activating the same promoter. The latter observation also makes DNA microarray approaches difficult, since many genes that can be activated by σ^X are also expressed by σ^X-independent pathways.

In this study we have used both promoter consensus search and in vitro runoff transcription-macroarray analysis (ROMA) (8) to identify four additional σ^X-dependent operons: *dltABCDE*, *pssA ybfM psd*, *pbpX*, and *ywnJ*. Both the *dlt* and the *pssA* operons encode enzymes that modulate cell surface charge (D-alanylation of teichoic acids and biosynthesis of phosphatidylethanolamine [PE], respectively), and PbpX is a low-molecular-weight penicillin-binding protein of unknown function. These results lead us to propose that one function of σ^X is to regulate cell surface modification as a defense against cationic antimicrobial peptides.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and growth conditions. All bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. *B. subtilis* and *E. coli* strains were grown at 37°C with vigorous shaking in Luria broth (LB) medium (50) unless otherwise indicated. For *E. coli*, 100 μg of ampicillin/ml was used to select for Amp^r, and 200 μg of spectinomycin/ml was used to select for Spc^r. For *B. subtilis*, antibiotics used for selection were as follows: 100 μg of spectinomycin/ml for Spc^r, 10 μg of kanamycin/ml for Kan^r, 8 μg of neomycin/ml for Neo^r, and 1 μg of erythromycin/ml and 25 μg of lincomycin/ml for macrolide-lincomycin-streptogramin B resistance (MLS^r).

Construction of mutants. CU1065 chromosomal DNA was amplified with primers #427 and #428. The PCR fragment was digested with *Sac*I and *Pst*I and ligated into pGEM-*cat*-3Zf(+) (59) to generate plasmid pMC57. pMC57 was digested with *Hin*cII and *Sna*BI and ligated with a Spc^r cassette (PCR amplified from pKF59 [5] using T_3 and T_7 primers) to generate pMC58. *B. subtilis* CU1065 was transformed with pMC58 (linearized with *Sca*I) with selection for Spc^r to generate HB0048 (*dltA*::*spc*). Thus, a ~630-bp internal fragment of *dltA* was replaced with a Spc^r cassette.

Primers #371 and #372 were used to amplify an internal fragment of *dltA* (~490 bp). The PCR fragment was digested with *Eco*RI and *Bam*HI and cloned into pMUTIN4 (56), generating plasmid pMC59. This plasmid was inserted into CU1065 by Campbell integration and selection for MLS^r to generate strain HB0038 (*dltA*::pMUTIN).

CU1065 was transformed with chromosomal DNA from SDB01 (*psd*::*neo*)

* Corresponding author. Mailing address: Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853-8101. Phone: (607) 255-6570. Fax: (607) 255-3904. E-mail: jdh9@cornell.edu.

TABLE 1. Strains, plasmids, and oligonucleotides used in this work.

Strain, plasmid, or oligonucleotide	Genotype, characteristic, or sequence	Reference
E. coli strain		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Lab stock
B. subtilis strains		
CU1065	W168 *att*SPβ *trpC2*	Lab stock
ZB307A	W168 SPβc2Δ2::Tn917::pBSK10Δ6	60
HB7007	CU1065, but *sigX*::*spc*	20
HB7013	CU1065, but *rsiX*::pVA29 (MLS^r)	20
HB0042	CU1065, *sigW*::*kan*	6
HB0010	CU1065, *rsiW*::*kan*	6
HB0030	CU1065, *sigX*::*spc*, *sigW*::*MLS*	8
HB4035	CU1065, *sigD*::*kan*	Lab stock
HB4229	HB1000, *flgM*::mini-Tn*10* (Spc^r)	15
HB0059	CU1065, *flgM*::mini-Tn*10* (Spc^r)	This work
SDB01	Marburg 160, but *psd1*::*neo*	33
SDB02	Marburg 160, but *pssA10*::*spc*	33
HB0036	ZB307A transduced with SPβ(P_{dltA1}-*cat-lacZ*)	This work
HB0037	ZB307A transduced with SPβ(P_{dltA2}-*cat-lacZ*)	This work
HB0038	CU1065, but *dltA*::pMUTIN	This work
HB0048	CU1065, *dltA*::*spc*	This work
HB0094	CU1065, but *dltA*::pMUTIN and *pssA*::*spc*	This work
HB0095	CU1065, but *dltA*::*spc* and *psd*::*neo*	This work
HB0089	ZB307A transduced with SPβ(P_{ywnJ}-*cat-lacZ*)	This work
HB4514	ZB307A transduced with SPβ(P_{pbpX}-*cat-lacZ*)	This work
HB4509	ZB307A transduced with SPβ(P_{pssA2}-*cat-lacZ*)	This work
HB4533	ZB307A transduced with SPβ(P_{pssA1}-*cat-lacZ*)	This work
HB4519	CU1065, but *psd*::*neo*	This work
HB4520	CU1065, but *pssA*::*spc*	This work
Plasmids		
pJM122	Vector for integration of reporter fusions into SPβ	51
pGEM-*cat*-3Zf(+)	Cloning vector for gene knockout	59
pJM114	Kanamycin resistance cassette vector	42
pMUTIN4	Cloning vector for integration, allows *lacZ* fusion at locus	56
pKF59	Spectinomycin resistance cassette vector	5
pMC54	P_{dltA1}-*cat-lacZ* in pJPM122	This work
pMC55	P_{dltA2}-*cat-lacZ* in pJPM122	This work
pMC57	*dltA* cloned in *Sac*I-*Pst*I of pGEM-cat-3Zf(+)	This work
pMC58	*spc* cassette cloned in pMC57 (*dltA*::*spc*)	This work
pMC59	an internal fragment of *dltA* cloned in *Eco*RI-*BamH*I of pMUTIN4	This work
pMC94	P_{ywnJ}-*cat-lacZ* in pJPM122	This work
pJQ20	P_{pbpX}-*cat-lacZ* in pJPM122	This work
pJQ22	P_{pssA2}-*cat-lacZ* in pJPM122	This work
pJQ23	P_{pssA1}-*cat-lacZ* in pJPM122	This work
Oligonucleotides		
368 *dltA*-r123	5′-TCGCGGATCCATAATTCCTGATACGTGA-3′	This work
371 *dltA*-in-f	5′-CCCGGAATTCCGTCCGAACGGATTG-3′	This work
372 *dltA*-in-r	5′-CCCGGATCCATCAGGCACATTTGC-3′	This work
373 *dltA*-f1	5′-GCCCAAGCTTATTTGTTTGGTTCATCTTCC-3′	This work
374 *dltA*-f3	5′-GCCCAAGCTTTATTATGAATCAGCTCGAAAC-3′	This work
427 *dltA*-f	5′-AAGCGAGCTCCTTGCAGGTATAAAGATT-3′	This work
428 *dltA*-r	5′-AAAACTGCAGCCAAGCAGTATAAAGAAT-3′	This work
413 *pbpX*-f	5′-AATGATAAGCTTGGCTGAGTGAAAAACTCAGC-3′	This work
414 *pbpX*-r	5′-CAGGGATCCTCTTTTATTTAGTTTTCTCCG-3′	This work
422 *pssA*-f1	5′-TTTGGGAAGCTTATCTCTGGATCAGCCAG-3′	This work
407 *pssA*-f2	5′-TTTGGGAAGCTTCTATGTTATCATGCTTATTGG-3′	This work
408 *pssA*-r	5′-TGAGGATCCAGCAATCCGCAAATGAAG-3′	This work
537 *psd*-in-f	5′-GGATTTTGCGAGTCGAAA-3′	This work
538 *psd*-in-r	5′-CTCCAATTCAGTACGGGT-3′	This work
571 *ywnJ*-f	5′-CGCAAGCTTACCCAAGAAACAGAAGAA-3′	This work
572 *ywnJ*-r	5′-CCCGGATCCACAGACAGAAAGCAGGAT-3′	This work

(33) and SDB02 (*pssA*::*spc*) (33) to generate the *psd*::*neo* (HB4519) and *pssA*::*spc* (HB4520) mutants, respectively. The *dltA pssA* (HB0094) and *dltA psd* (HB0095) double mutants were generated by using chromosomal DNA from SDB02 and SDB01 to transform HB0038 (*dltA*::pMUTIN) and HB0048(*dltA*::*spc*), with selection for (MLS^r plus Spc^r) and (Spc^r plus Neo^r), respectively.

Construction of promoter-*cat-lacZ* fusions. The putative promoter regions were amplified and cloned into pJPM122 (51). The sequence of the promoter region in each plasmid was verified by DNA sequencing (Cornell DNA sequencing facility). The promoter fusions were introduced into the SPβ prophage by double-crossover recombination, in which each pJPM122 derivative was linear-

TABLE 2. Genes preceded by promoters recognized by σ^X

Gene[a]	Promoter region (σ^X dependent)[b] −35 −10..	5′UTR[c]	ROMA[d]	Overlapping recognition by other ECF σ factor(s)[e]	Reporter fusion[f]	Reference(s)
sigX	tgtaa**TGTAACTTTT**caagctattcata**CGAC**aaaaaag	17	+		+	20
csbB	aaaat**TGTAAC**aaaaaacag-gtttaaa**CGAC**tttaaaa	80	+++*		+	22
lytR	aacaa**TGAAACTTTT**tttta-taaaaaa**CGAC**tatttta	84	+		+	22
rapD	taaaa**TGTAAC**caactgtcaatgagagc**CGTC**aaaagtt	45	−		+	22
divIC	atgtt**TGAAACTT**c**T**tcctgtgaaaatg**CGTC**taacttt	113	+	σ^W (RO), σ^M (RF)	+	22, 35
abh	aagcgg**GAAACTTTT**tcaaagtttcatt**CGTC**tacgata	62	−	σ^W (RO)	+	22
bcrC	ttatt**TGAAACTTTT**catgagtaagatta**GTC**tactaaa	24	++	σ^M (RF, PE)	+	7
ywnJ	ttttc**TGT**t**ACTTTT**tgctttgttttac**CGTC**tatgtag	38	+++	σ^W (RO)	−	This work
pbpX	tttttgac**AACTTTT**ttagggctttatt**CGTC**taacaaa	40	−	σ^W (RF)	+	This work
dltA	aaaaa**TGAAACTTTT**tgagc-atctgat**CGTC**aaataat	204	+		+	This work
pssA	tttcc**TGTAAC**gc**T**attcga-tcactat**CGTC**aaataat	34	++*		+	This work

[a] The first gene in each known or putative σ^X-dependent operon and its (σ^X-dependent) promoter region are listed.
[b] Note that the promoter consensus defined here includes an extended −35 region that includes the downstream T-rich segment and two additional bases in the −10 region that appear to be important for promoter discrimination by σ^X (double underline) or σ^W (single underline) (see the text).
[c] The known or estimated distance from the transcription start site to the start codon of the gene is indicated in nucleotides.
[d] The strength of the signal detected in the ROMA ($E\sigma^X$) experiment (Fig. 1) is indicated. An asterisk indicates that the downstream genes were also identified by ROMA.
[e] This promoter is recognized by other ECF σ factors, as confirmed by runoff transcription (RO), reporter fusion (RF), or primer extension (PE).
[f] +, the indicated promoter region gives σ^X-dependent β-galactosidase activity with a reporter fusion. −, no activity was detected for the reporter fusion.

ized by digestion with *Sca*I and used to transform *B. subtilis* ZB307A (60) with selection for Neo^r. SPβ lysates were prepared by heat induction and used to transduce various recipient strains, and β-galactosidase activity was measured on each sample in early stationary phase (when σ^X activity is at a maximum) (21) as described by Miller (34).

Purification of RNAP and σ factors. Preparation of *B. subtilis* core RNA polymerase (RNAP) and σ^A, σ^D, σ^X, σ^W and δ proteins was previously described (10, 20, 21, 25, 31).

ROMA. The ROMA experiment was performed as described previously (8). A typical transcription reaction (50 μl) contains 1.3 pmol of core RNAP, 16 pmol of σ^X, 15 pmol of δ, and 1 μg of digested genomic DNA mixed in transcription buffer (20 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM dithiothreitol, 0.1-mg/ml bovine serum albumin, 5% [vol/vol] glycerol, and RNasin from Promega [10 U/reaction]) and NTP mixture (40 nmol of ATP, GTP, CTP, and 8 nmol of [α-^{33}P]UTP [3,000 Ci/mmol] from NEN). The Panorama *B. subtilis* gene arrays (catalog no. PRBS0002) were purchased from Sigma-Genosys Biotechnologies, Inc.

In vitro runoff transcription assays for candidate genes. A typical runoff reaction mixture (20 μl) contains 0.36 pmol of core RNAP, 4.5 pmol of σ^X, 4.2 pmol of δ, 0.04 pmol of PCR-amplified template DNA (normally the same fragments used for generating promoter fusions) and NTP mixture (10 nmol of ATP, GTP, CTP, 1 nmol of UTP, and 0.6 pmol of [α-^{32}P]UTP [3,000Ci/mmol]). Reactions were incubated and processed as described for the ROMA experiments (8).

Primer extension assays. RNA was either purified from in vitro runoff transcription reaction mixtures or extracted from late-log-phase *B. subtilis* cells using phenol-chloroform extraction as described previously (22). A PCR fragment

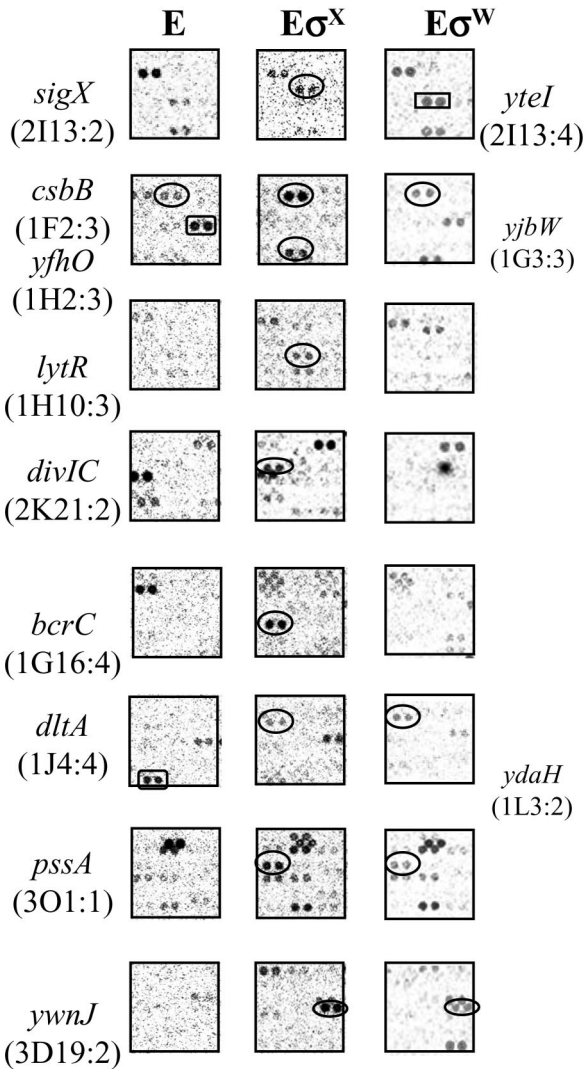

FIG. 1. Identification of σ^X regulon genes by ROMA. Total *B. subtilis* chromosomal DNA was digested with *Eco*RI and transcribed in vitro with core alone (E [left column]) or core with an excess of σ^X ($E\sigma^X$ [central column]). For comparison, the same regions from previous ROMA experiment with $E\sigma^W$ are placed in the right column. The σ^X-regulated genes are apparent in experiments with $E\sigma^X$ (ovals). *yteI* (rectangle) is a σ^W-dependent gene. Since the core is contaminated with trace amounts of other σ factors, several nonspecific spots appeared on the membrane even in the core-alone experiment. Some spots disappeared or were greatly decreased in abundance upon supplementation with a large molar excess of σ^X or σ^W (e.g., *yjbW* and *ydaH*, rectangles in left column). Other genes, such as *sigX* and *csbB*, which have multiple promoters are found in the RNA population transcribed by core as well as the σ^X- or σ^W-supplemented reactions. Three genes identified in this study (*dltA*, *pssA*, and *ywnJ*) are found in both $E\sigma^X$ and $E\sigma^W$ reactions, but only *ywnJ* can be transcribed by both σ^X and σ^W as confirmed by runoff transcription assays. The location of each gene on the Sigma/GenoSys macroarray is indicated in parentheses.

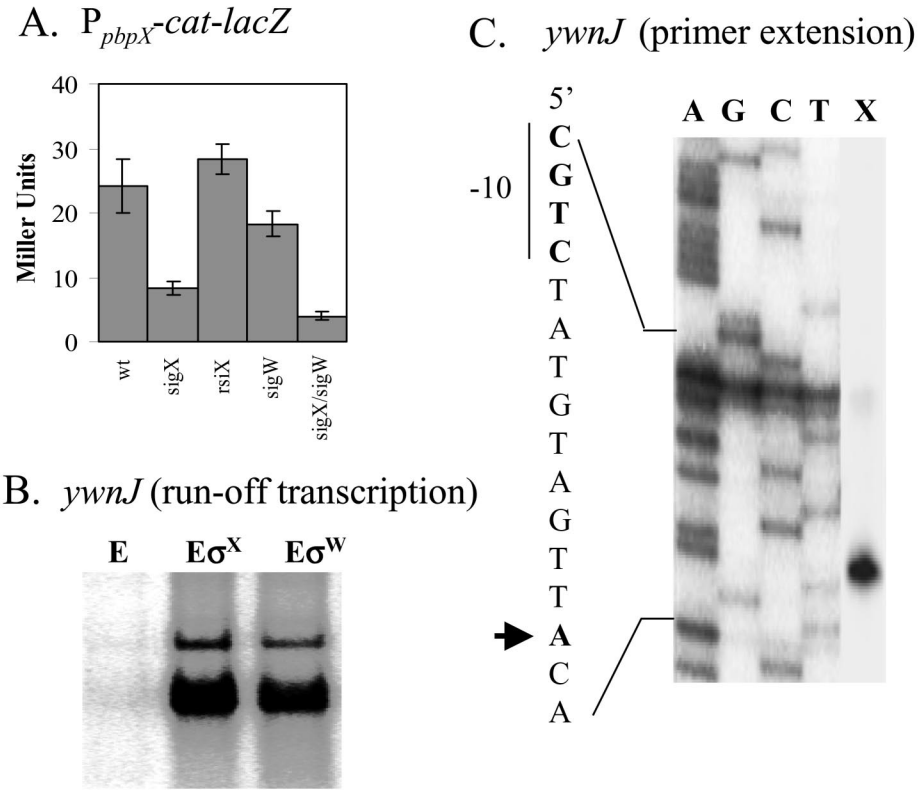

FIG. 2. Confirmation of the *pbpX* and *ywnJ* targets. (A) Expression of P_{pbpX}-*cat-lacZ* in various genetic backgrounds. Each result is the average for three individual β-galactosidase measurements. (B) In vitro recognition of the putative *ywnJ* promoter by both the *B. subtilis* σ^X ($E\sigma^X$) and the σ^W ($E\sigma^W$) holoenzymes. The RNAP core enzyme (E) was used as a negative control. (C) RNA generated by runoff transcription using $E\sigma^X$ as shown in panel B was used as a template for primer extension mapping of the *ywnJ* transcription start site (lane X). The same primer was used to sequence this region to index the start site (lanes A, G, C, and T).

containing the promoter region studied was sequenced using the same primer to index the transcription start site.

Nisin MIC assays. Nisin was obtained from Sigma Chemical Co. and dissolved in 20 mM HCl. Overnight cultures were diluted 1:100 into fresh LB medium in the presence of nisin at the indicated concentration. After incubation for 6 h with shaking, the optical density at 600 nm (OD_{600}) was measured.

Autolysis test. CU1065 (wild type), *sigX*::*spc*, *dltA*::*spc*, and *dltA*::pMUTIN strains were grown in LB or minimal medium (9). Cells were harvested at exponential growth phase (OD_{600}, ~0.7), washed twice with cold Tris-HCl buffer (pH 7.1), and resuspended in 50 mM Tris-HCl buffer (pH 7.1) containing 0.05% Triton X-100. Incubation was at 37°C, and autolysis was monitored by measuring the decrease of OD_{600} at 30-min intervals.

Northern blot analysis. Primers #537 and #538 were used to amplify an internal fragment (~570 bp) of *psd* from CU1065 chromosomal DNA. After *Hin*dIII digestion, the fragment was labeled with [α-^{32}P]dATP by the 3′ fill-in method using a Klenow fragment of DNA polymerase (Exo$^-$; New England BioLabs). The probe was hybridized with membranes containing total RNA from wild-type, *sigX*, and *rsiX* strains (same RNA sample used for primer extension; see above). The NorthernMax formaldehyde-based system (Ambion, Inc.) was used to perform the Northern analysis. Ten micrograms of total RNA was denatured and loaded on 1% formaldehyde agarose gel. Hybridization was performed at 42°C overnight. The second day, the blot was washed twice with low-stringency buffer (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) at room temperature followed by two washes with high-stringency buffer (0.1× SSC) at 42°C. The blot was wrapped in plastic wrap and exposed to a Phosphor screen (Molecular Dynamics).

RESULTS

Defining the σ^X regulon using promoter consensus search. Previously, saturation mutagenesis of the *sigX* autoregulatory promoter was used to identify those bases important for σ^X-dependent promoter recognition. The resulting consensus was used to search the partially sequenced *B. subtilis* genome (63% complete at the time the analysis was done [22]) to identify candidate promoters. While this approach identified several σ^X target genes, others were subsequently found to be primarily controlled by σ^W or σ^M, which recognize promoters with closely related sequences (22, 35).

The availability of the complete genome sequence (28), together with a better understanding of the rules for promoter recognition by σ^X and its paralog σ^W (21, 48), encouraged us to repeat the consensus search procedure. Although we explored the use of several different search patterns to identify likely candidates, one of the most successful searches used the degenerate consensus tgtaACtttt $n_{12\text{-}13}$ CG(A,T)C to screen the SubtiList database (36) for those sites within 250 bp of an annotated start codon. This search pattern is based on the observation that many identified σ^X-dependent promoters contain a T-rich region in the downstream portion of the −35 element and the AC base pairs are highly conserved. We allowed up to three mismatches in this extended −35 element (in the positions in lowercase) and none in the −10 element. By including those promoters with −10 elements of either CGTC or CGAC, we expected to identify some sites already defined as largely dependent on σ^W. The resulting list of candidate promoters includes one site with no mismatches (the *sigX* au-

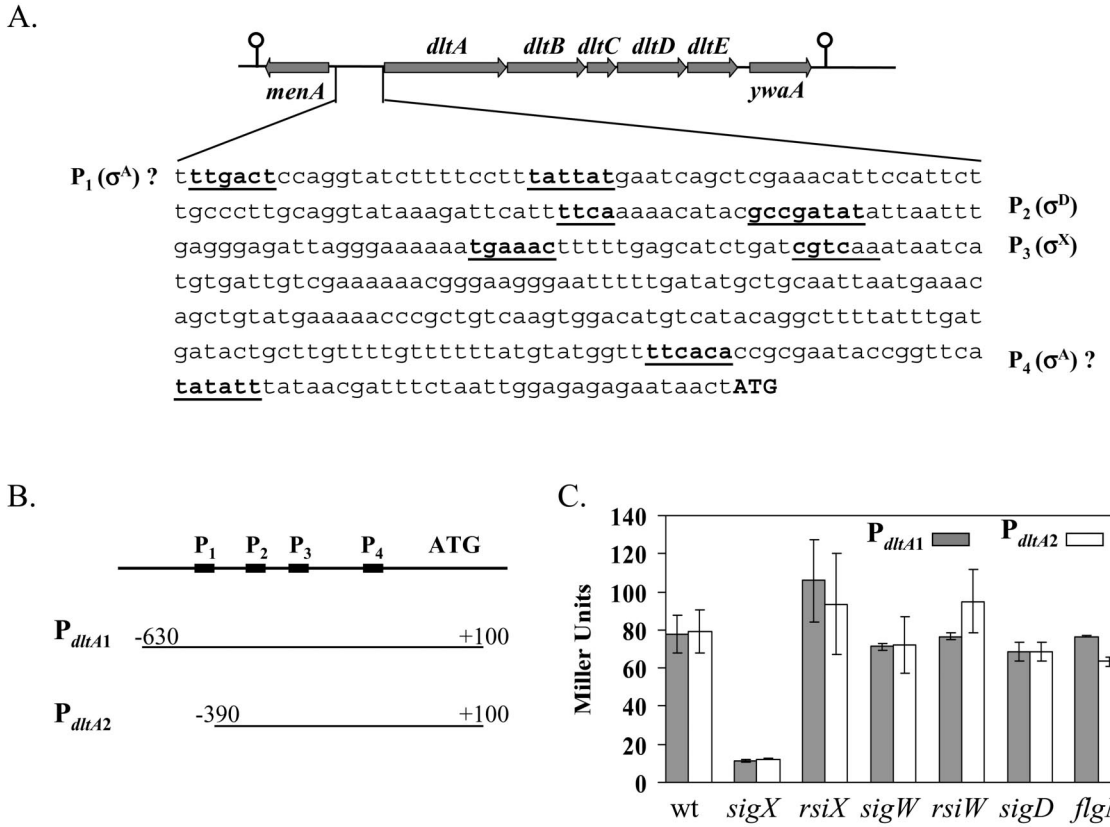

FIG. 3. Regulation of the *dlt* operon by σ^X. (A) The location of the *dltABCDE* operon on the *B. subtilis* chromosome and the *dltA* promoter region. The σ^X-dependent promoter (P_3), the σ^D-dependent promoter (P_2), and two putative σ^A-dependent promoters (P_1 and P_4) are underlined. The translation start codon (ATG) is shown in bold capital letters. (B and C) Graphic presentation of the two P_{dltA} promoter fusions (B) and their activities in various genetic backgrounds (C) (each result is the average and standard deviation from three individual measurements).

toregulatory site), three with one mismatch (preceding *lytR*, *ywnJ*, and *dltA*), and four with two mismatches (*divIC*, *ydjA*, *abh*, and *yrhH*; underlined sites are known to be at least partially σ^X dependent in vivo or in vitro) (21, 22). Among the 15 sites with three mismatches in the −35 element, we focused our attention on those preceding *pssA* and *pbpX*, since these genes are of known function (Table 2).

Defining the σ^X regulon using ROMA. As a complementary mechanism to identify candidate σ^X target genes, we performed in vitro ROMA analysis (8). The ROMA approach generates ^{33}P-labeled runoff transcripts using σ^X-containing holoenzyme to transcribe total genomic DNA that has been restricted with either *Eco*RI or *Hin*dIII. The resulting runoff transcripts are then used to probe a DNA macroarray (Sigma/GenoSys) containing 4,107 *B. subtilis* open reading frames. Candidates (genes whose signal became stronger in the presence of σ^X) were chosen for further analysis if they had a particularly strong signal or if they were associated with a plausible promoter site as identified by the consensus search approach described above. In addition to signals corresponding to promoters known to be recognized by σ^X (e.g., *sigX*, *csbB*, *lytR*, *divIC*, and *bcrC*) (Fig. 1), the ROMA experiment revealed strong signals for the *pssA* and *ywnJ* genes and a weaker signal for *dltA*. Note that in many cases, these same genes appeared in reactions using σ^W holoenzyme instead of σ^X (Fig. 1), consistent with the known overlap between the sets of promoters recognized by these two σ factors (22).

Confirmation of promoters for *pbpX* and *ywnJ*. We used a reporter fusion to demonstrate that *pbpX* is dependent on *sigX* in vivo, with a further reduction in expression in the *sigX sigW* double mutant (Fig. 2A). DNA microarray analyses reveal that the expression of *pbpX* decreased 2.7-fold in the *sigX* mutant (data not shown) but not in the *sigW* mutant (8). The reporter fusion for the putative *ywnJ* promoter had very low activity, so in vitro transcription was used to demonstrate that this site could be recognized by both the σ^X and σ^W holoenzymes (Fig. 2B). Transcription initiates at the expected site, as measured by primer extension mapping of the resulting in vitro transcripts (Fig. 2C).

The *dltABCDE* operon is largely dependent on σ^X. The *B. subtilis dltABCDE* operon is responsible for D-alanine esterification of both lipoteichoic acids (LTA) and wall teichoic acids (WTA) (44). Transcription of the *dltABCDE* operon was originally proposed to be largely (~70%) σ^D dependent, with the residual activity perhaps due to two putative σ^A-dependent promoters (Fig. 3A, P_1 and P_4) (44).

To examine the regulation of the *dlt* operon, we integrated two *lacZ* transcriptional fusions ectopically at the SPβ locus. The P_{dltA1}-*cat-lacZ* fusion consists of a ~730-bp fragment (from −630 bp to +100 bp relative to the start codon) and includes all four putative promoters (P_1 to P_4). The P_{dltA2}-*cat-lacZ*

fusion consists of a shorter fragment (from −390 bp to +100 bp) and includes P_2 through P_4 (Fig. 3B). Results with both promoter fusions indicate that expression is reduced by about 85% in the *sigX* mutant and slightly increased in the *rsiX* mutant (defective in the anti-σ factor that targets σ^X [20]), confirming the existence of a σ^X-dependent promoter in this region (Fig. 3C). Expression was unaffected in the *sigW* and *rsiW* mutant strains, despite the presence of a CGTC motif is the predicted −10 region (see Discussion). Under our growth conditions, σ^D does not seem to play a role in *dlt* transcription, since the activities from both fusions neither decreased in a *sigD* mutant nor increased in a *flgM* (anti-σ^D) mutant. Moreover, the first putative σ^A-dependent promoter (P_1) apparently did not contribute to the *dlt* transcription, since expression from P_{dltA1} and P_{dltA2} was similar. The residual activity (~11 Miller units) in the *sigX* mutant might be due to recognition of the σ^X-dependent promoter by another ECF σ factor or might be due to another promoter (maybe P_4).

We extended these in vivo results using in vitro runoff transcription and primer extension assays. When the long PCR fragment (P_{dltA1}) was incubated with RNAP core enzyme in the presence of σ^A, σ^X, σ^W or σ^D, appropriately sized transcripts were generated by both the σ^X and σ^D holoenzymes (Fig. 4A). Although σ^W could weakly recognize the σ^X-dependent site in vitro (Fig. 4A), it did not appear to play a major role in vivo (Fig. 3C). While $E\sigma^D$ could initiate transcription from the σ^D-dependent promoter in vitro, in vivo transcripts were detected only for the σ^X-dependent promoter (Fig. 4B). Primer extension reactions indicate that transcription of *dltA* initiates primarily from a G residue 11 bases downstream of the −10 CGTC motif and secondarily from an A residue 2 bases upstream. Both signals became stronger in the *rsiX* mutant and were greatly reduced in the *sigX* mutant. No other strong start sites were visible in this region. We conclude that *dlt* expression is largely dependent on σ^X in vivo.

The *pssA ybfM psd* operon is partially controlled by σ^X. Our identification of a candidate σ^X-dependent promoter upstream of the *pssA* gene suggests a possible role for σ^X in regulating the phospholipid content of the membrane. The *pssA* gene is part of a predicted operon including *ybfM* and *psd*. Together, the PssA and Psd proteins catalyze the synthesis of PE. Okada et al. (39) proposed two putative σ^A-dependent promoters (P_1 and P_2) upstream of *pssA*, and our results suggest a third σ^X-dependent promoter (P_3) (TGTAAC-N_{16}-CGTCaa) (Fig. 5A).

To test the contribution of each promoter to *pssA* expression, we constructed two *lacZ* fusions: one contains the complete promoter region (P_1, P_2, and P_3), the other contains only P_3 (Fig. 5B). β-Galactosidase assays demonstrate that about one-half of the expression derives from the σ^X-dependent promoter (P_3), with the other half from the region containing P_1 and P_2 (Fig. 5C).

Recognition of P_3 by σ^X holoenzyme was confirmed in vitro by runoff transcription assays (Fig. 6A). A faint, larger band was observed in reactions containing $E\sigma^A$, probably resulting from one of the σ^A-dependent promoters. We used primer extension assays to localize the transcription start site for the σ^X holoenzyme to an A residue 10 bp downstream from CGTC (Fig. 6B). A weak transcript was detected in the wild type (CU1065) but not in the *sigX* mutant strain. The amount of transcript increased in the *rsiX* mutant, as expected for a σ^X-dependent promoter.

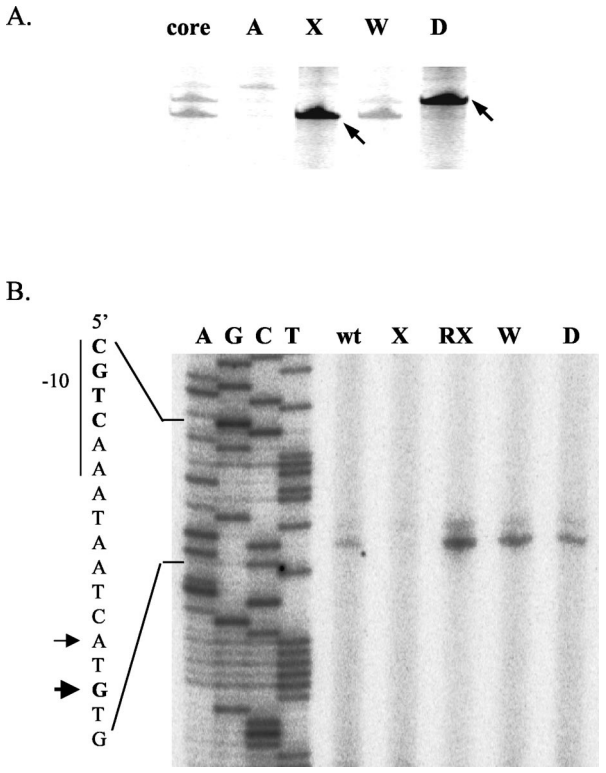

FIG. 4. Identification of the σ^X-dependent promoter for the *dlt* operon. (A) Runoff transcription from the *dltA* promoter region in the presence of *B. subtilis* RNAP core enzyme and the indicated σ factor: σ^A (A), σ^X (X), σ^W (W), or σ^D (D). In the first lane (core), no σ factor was added in the reaction. Major transcripts are indicated by arrows. (B) Primer extension mapping of the in vivo *dltA* transcription start site. RNA samples were prepared from wild-type (wt), *sigX* (X), *rsiX* (RX), *sigW* (W), or *sigD* (D) mutant strains. Equal amounts (100 μg) of total RNA were annealed with radiolabeled oligonucleotide #368 for reverse transcription. The transcription start sites corresponding to the σ^X-dependent promoter are indicated by arrows.

To test whether *pssA* and *psd* are in one operon, we conducted Northern blot analysis using a ^{32}P-labeled internal fragment of the *psd* gene as a probe. A large transcript (~1.9 kb) was detected, consistent with an mRNA extending from *pssA* through *psd*. A smaller transcript (~850 bp) likely corresponds to the *psd* gene and may have been produced by RNA processing, since it varies in intensity with the full-length transcript (Fig. 6C). The density of both bands decreased about 50% in the *sigX* mutant and increased in the *rsiX* mutant, consistent with the previous conclusion that σ^X contributes ~50% of the expression of P_{pssA}.

***sigX* mutants are altered in autolysis and sensitivity to cationic antimicrobial peptides.** Since σ^X regulates both D-alanylation of teichoic acids and PE biosynthesis, we tested the effects of a *sigX* mutation on two phenotypes previously shown to be affected by cell surface charge: autolysis and resistance to cationic antimicrobial peptides. We first compared the Triton X-100-induced autolysis rates (58) of the wild type and the *sigX* and *dltA*::*spc* mutants. Autolysins are a group of positively charged cell wall hydrolytic enzymes that bind more avidly to the cell wall of *dlt* mutant strains (58). As expected, both the *sigX* and *dltA* mutants have a twofold increase in the rate of

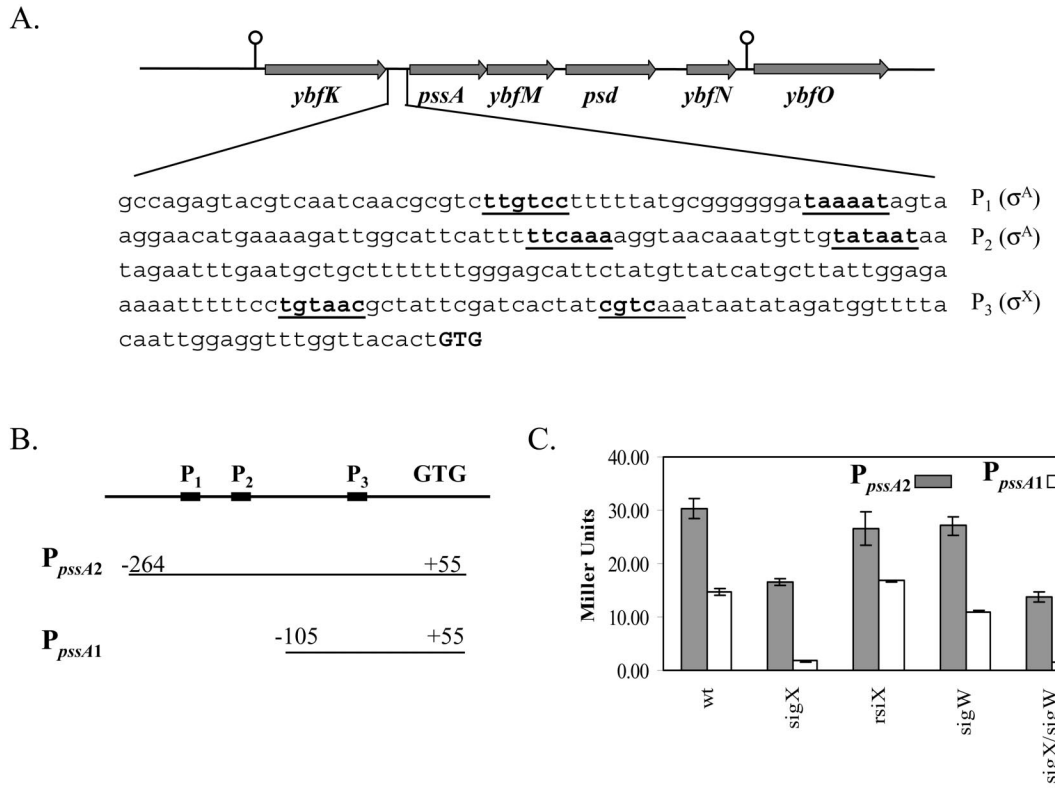

FIG. 5. Regulation of the *pssA ybfM psd* operon by σ^X. (A) Locations of the *pssA*, *ybfM*, and *psd* genes on the *B. subtilis* chromosome and DNA sequence of the *pssA* promoter region. The σ^X-dependent promoter (P_3) and two putative σ^A-dependent promoters (P_1 and P_2) are underlined. The translation start codon (GTG) is shown in bold capital letters. (B and C) Graphic presentation of the construction of two P_{pssA} promoter fusions (B) and their activities in various genetic backgrounds (C) (each result is the average of three individual measurements).

autolysis compared to the wild type (Fig. 7A). Similar results were observed with cells grown in LB or minimal medium.

Cationic antimicrobial peptides (CAMPs) are a broadly distributed family of peptides that kill bacteria. Many are thought to act by accumulating within the cytoplasmic membrane to a critical concentration that allows the assembly of structures that permeabilize the cell (16–18). To test whether σ^X plays a role in resistance to CAMPs, we measured the MICs of nisin for the wild type and the *sigX*, *dltA*, *pssA*, and *psd* mutants: a positively charged (+3) peptide produced by *Lactococcus lactis* (24). As expected, the *sigX* and *dltA* mutants were more sensitive to nisin than the wild type (Fig. 7B). The *psd* mutant had only slightly increased sensitivity, while the *pssA* mutant was unaffected. A *psd dltA* double mutant behaved much like the *dltA* single mutant. In addition to nisin, the *sigX* and *dltA* mutants were more sensitive to several other tested CAMPs (S. Farmer and R. Hancock, personal communication) but not to gramicidin, a neutral peptide. In contrast, the mutants were unaltered in their sensitivity to vancomycin, tunicamycin, or lysozyme (data not shown), although *dlt* mutants have been previously reported to display an increased susceptibility to methicillin (57).

DISCUSSION

Using a promoter consensus search and ROMA approaches, we have defined four additional σ^X-dependent operons. Together with the results of our previous analyses (22), we conclude that most members of the σ^X regulon control processes related to the composition or metabolism of the cell envelope. For example, LytR is a negative regulator of autolysin activity (30), CsbB is a membrane-bound glucosyl transferase likely involved in cell wall biosynthesis (2), PbpX is a penicillin-binding protein, DltA, DltB, DltC and DltD are responsible for D alanylation of the WTA and LTA (44), and PssA and Psd are enzymes for PE biosynthesis (33). We note that most of these operons are expressed from complex promoter regions: *lytR* is controlled by both σ^A and σ^X, *csbB* is also regulated by σ^B, *pbpX* is partially regulated by σ^W, and σ^X and σ^A each contribute to *pssA ybfM psd* expression. Perhaps due to this overlapping regulation, the *sigX* mutant strain does not display dramatic growth defects under most tested conditions, although some increased sensitivity to oxidative stress and heat stress has been noted (20). Here, we have extended the phenotypes of the *sigX* mutant to include increased rates of autolysis and increased sensitivity to cationic antimicrobial peptides.

Promoter recognition by σ^X. As noted previously, σ^X recognizes −10 elements with sequence CGaC, σ^W recognizes CGTa, and both can recognize CGTC (lowercase reflects a noncritical base for recognition) (21, 22, 48). In Table 2 we compile the 11 promoters that are the best candidates for regulation by σ^X in vivo. Note that some of these sites can also be recognized by either σ^W or σ^M. For example, both *bcrC* (7,

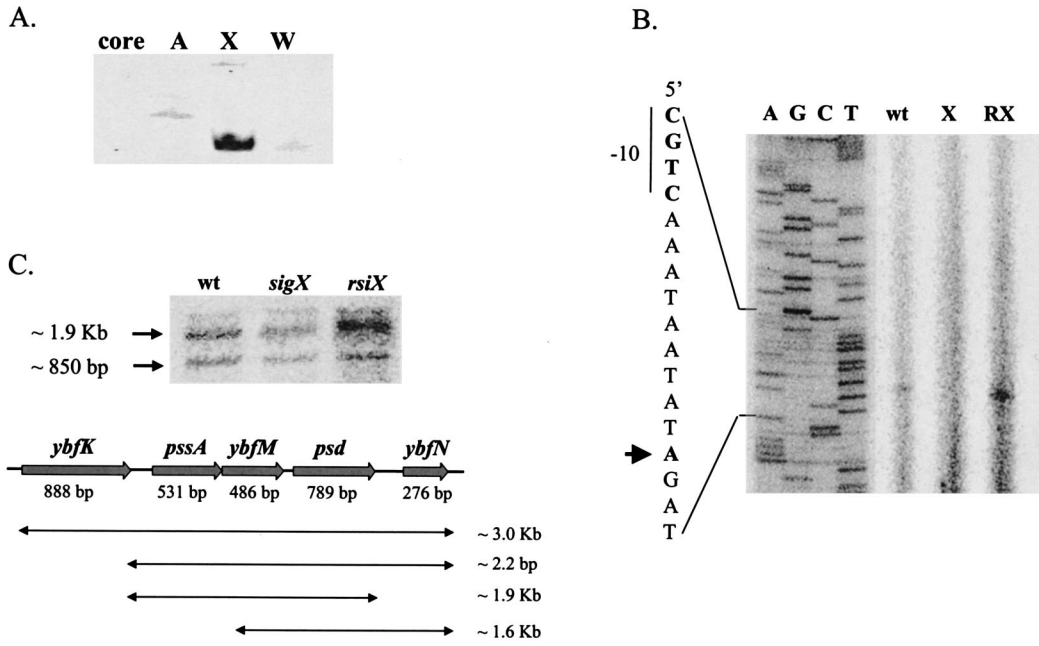

FIG. 6. Identification of the σ^X-dependent promoter for the *pssA ybfM psd* operon. (A) Runoff transcription from the *pssA* promoter region in the presence of *B. subtilis* RNAP core enzyme and the indicated σ factor: σ^A (A), σ^X (X), or σ^W (W). In the first lane (core), no σ factor was added in the reaction. (B) Primer extension mapping of the *pssA* transcription start site. RNA samples were prepared from the wild type (wt) or from *sigX* (X) or *rsiX* (RX) mutant strains. Equal amounts (100 μg) of total RNA were annealed with radiolabeled oligonucleotide #408 for reverse transcription. The transcription start site is indicated by the arrow. (C) Northern blot analysis demonstrates that *pssA*, *ybfM*, and *psd* are cotranscribed. The combinations and sizes of possible transcripts are listed. Two bands were observed: the top band is about 1.9 kb, representing the transcript from *pssA* to *psd*, while the lower band (~850 bp) can only be assigned to the *psd* mRNA, probably due to RNA processing.

38) and *pbpX* (Fig. 2A) seem to be under dual control in vivo, and σ^W recognizes several other sites in vitro (Table 2). In addition, a number of other promoters previously studied (22) can be recognized by σ^X in vitro, but an in vivo role for σ^X has not been documented, and it seems likely that they may be primarily dependent on σ^W or σ^M for in vivo expression (22). Indeed, in *B. subtilis* W23, expression of the *divIC* gene is partially σ^M dependent (35).

Inspection of Table 2 allows a refinement of our previous models for promoter discrimination among ECF σ factors. Specifically, we note that most of the newly characterized promoters identified in this study contain a CGTC −10 motif, previously shown to be also recognized by σ^W holoenzyme. However, all four promoters (*abh*, *divIC*, *pbpX*, and *ywnJ*) that are also recognized by σ^W share a common extended −10 region of CGTCta. In contrast, the other three (*rapD*, *dltA*, and *pssA*) that are recognized only by σ^X have a −10 region of "CGTCaa (Table 2). This is consistent with the observation that the highly specific autoregulatory sites for *sigX* and *sigW* contain −10 elements of "CGACaa" and "CGTAta," respectively. Furthermore, in a previous promoter mutagenesis study we found that changing the *sigX* promoter (−10) region CGA Caa to CGTCaa resulted in a site that retained high selectivity for σ^X. In contrast, when the *sigW* (−10) region CGTAta was changed to CGTCta, both σ^X and σ^W could recognize this promoter (48). We therefore conclude that (i) the preferred −10 consensus sequences for σ^X (CG*a*C*a*a) and σ^W (CG*Ta*t*a*) differ in two positions (italics) rather than one position and (ii) there is considerable overlap between these two regulons.

Biological role of σ^X and the σ^X regulon. Distinctive aspects of the gram-positive bacterial cell envelope include the presence of a thick cell wall containing peptidoglycan, WTA, and LTA (14). In *B. subtilis* 168 strains, the negatively charged teichoic acids contain an alternating glycerol phosphate copolymer, whereas in *B. subtilis* W23 strains ribitol replaces glycerol. Recent results indicate that ribitol-based teichoic acid synthesis in W23 strains is regulated by both σ^X and σ^M (29, 35).

In general, the WTA and LTA polymers are highly modified by esterification on the sugar residues with sugar, amino sugar, or amino acid substituents. For *B. subtilis*, LTA chains contain between 24 and 33 glycerol phosphate monomers and carry, on average, 0.35 to 0.55 D-alanine constituents and 0.2 to 0.4 glycosyl substituents per monomer (14). The D-Ala residues on LTA are subject to rapid turnover, both by spontaneous hydrolysis and by transesterification to WTA (14, 26). D-Alanylation of WTA and LTA is catalyzed by the products of the *dlt* operon. The *dltA* and *dltC* genes encode the D-alanine-D-alanyl carrier protein ligase (Dcl) and the D-alanyl carrier protein (Dcp), respectively. DltB and DltD may function in transport and the actual esterification reaction (45).

The modification with D-Ala introduces free amino groups (NH_3^+) into the cell envelope and thereby reduces the net negative charge of the surface (44). Genetic studies with several microorganisms indicate that *dlt* mutants are pleiotropic, with phenotypes including altered patterns of autolysis, increased sensitivity to CAMPs (1, 45–47), altered colonization properties (11), altered carbohydrate metabolism (52), en-

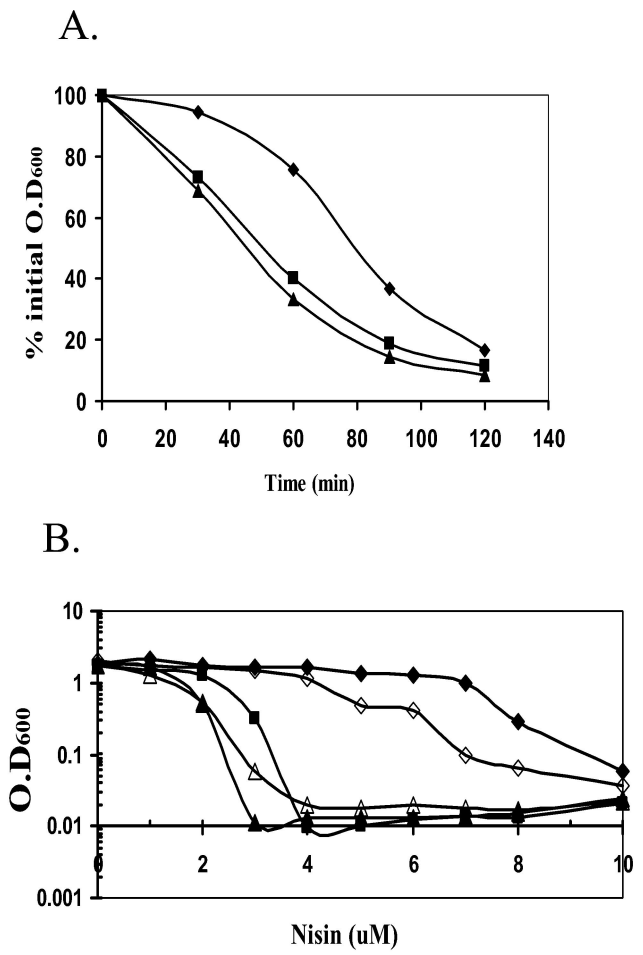

FIG. 7. Effects of *sigX*, *dlt*, and *psd* on autolysis and nisin sensitivity. (A) Autolysis rates. *B. subtilis* CU1065 (wild type; diamonds) and the *sigX*::*spc* (squares) and *dltA*::*spc* (triangles) mutants were grown to exponential growth phase (OD_{600}, ~0.7). The cell pellets were washed twice with cold Tris buffer (pH 7.1) and resuspended in 50 mM Tris-HCl buffer (pH 7.1) containing 0.05% Triton X-100. Incubation was at 37°C, and autolysis was monitored by measuring the decrease of OD_{600} at 30-min intervals. (B) MIC of nisin for the growth of *B. subtilis* wild-type (closed diamonds), *sigX*::*spc* (closed squares), *dltA*::*spc* (closed triangles), *psd*::*neo* (open diamonds), and *dltA psd* (open triangle) strains. All strains were grown for 6 h after dilution into LB medium containing the indicated concentration of nisin. This experiment was repeated three times, and representative results are shown.

hanced UV sensitivity, and loss of acid tolerance (3). In addition, D-alanylation affects protein folding and secretion (23, 54). Our results suggest that conditions leading to activation of the σ^X regulon will lead to enhanced expression of the *dlt* operon and thereby result in a decrease in the net negative charge of the cell wall. The factors that activate expression of the σ^X regulon are not well defined, but they are likely to act through the RsiX anti-σ, shown previously to inhibit σ^X activity (20). It has also been shown that transposon insertions in the *yitG* multidrug efflux system, the *manA* gene encoding mannose-6-phosphate isomerase, the *srfAB* surfactin biosynthesis gene, the *ytxJ* general stress protein, the *ywpH* single-stranded DNA-binding protein, and the *yogA* alcohol dehydrogenase locus also lead to enhanced expression from a σ^X-dependent promoter (55), although the significance of these observations is not yet clear.

The bacterial cell membrane also contains a net negative charge due to the abundance of anionic phospholipids. However, PE, a neutral (zwitterionic) lipid, makes up as much as 50% of the *B. subtilis* membrane (33). The biosynthesis of PE in *B. subtilis* is carried out by two membrane-localized enzymes: CDP-diacylglycerol-dependent phosphatidylserine (PS) synthase (PssA) and phosphatidylserine decarboxylase (Psd). The genes (*pssA* and *psd*) encoding the enzymes are separated by another gene, *ybfM*, on the chromosome. All three genes are cotranscribed (Fig. 6C). The *psd* mutant contains no PE and accumulates PS, while the *pssA* mutant contains no PE or PS. The absence of PE in *B. subtilis* cells does not have any adverse effects on cell growth, probably due to compensation from increased glucosyldiacylglycerol content in the membrane (33). Interestingly, the *E. coli psd* gene has recently been shown to have a σ^E-dependent promoter (49), suggesting that this system may also be controlled, at least in part, by an ECF σ factor in this organism.

In *B. subtilis*, σ^X may serve to regulate the net charge in the cell envelope by affecting the expression of both the *dlt* and *pssA* operons (Fig. 8), and this, in turn, may affect sensitivity to CAMPs. CAMPs share several common features, including broad-spectrum antimicrobial activity and cationic charge at physiological pH (16–18). CAMPs act by an initial electrostatic binding to the anionic moieties on the microbial membrane, followed by membrane disruption (16–18). In eukaryotes, CAMPs (including defensins) are the major form of defense against bacterial infection and are induced by bacteria or lipopolysaccharides (12, 17).

Bacteria can acquire resistance to CAMPs by modification of their surface properties, although in general the underlying regulatory mechanisms have not been described. For example, a nisin-resistant *Listeria monocytogenes* strain contains elevated levels of zwitterionic PE and a reduction in phosphatidylglycerol and cardiolipin (13). Similarly, a nisin-resistant strain of the rumen bacterium *Streptococcus bovis* has decreased negative surface charge (32). A recent study found that *Staphylococcus aureus* achieves resistance to defensins and CAMPs by modifying anionic membrane lipids with L-lysine (27). In gram-negative bacteria, resistance often involves modification of lipopolysaccharides. For example, CAMP resistance in *Salmonella enterica* involves addition of palmitate or 4-aminoarabinose to lipid A, a process regulated by the PmrA-PmrB two-component regulatory system (53).

In this study, we demonstrate that sensitivity of *B. subtilis* to CAMPs is affected by an ECF σ factor that contributes to the expression of two operons that modulate surface charge (Fig. 8). Other σ^X regulon proteins (e.g., LytR, PbpX, and CsbB) may also participate in this adaptive response. In other bacteria, related cell wall homeostasis functions may be controlled by two-component regulatory systems instead of, or in addition to, ECF σ factors. For example, the *Streptomyces coelicolor* CseC-CseB two-component system activates expression of σ^E, in response to unknown signals, which then functions to modify cell wall structure (40, 41). In *Streptococcus agalactiae*, up-regulation of the *dlt* operon when D-alanine incorporation into LTA is deficient is controlled by the DltS-DltR two-compo-

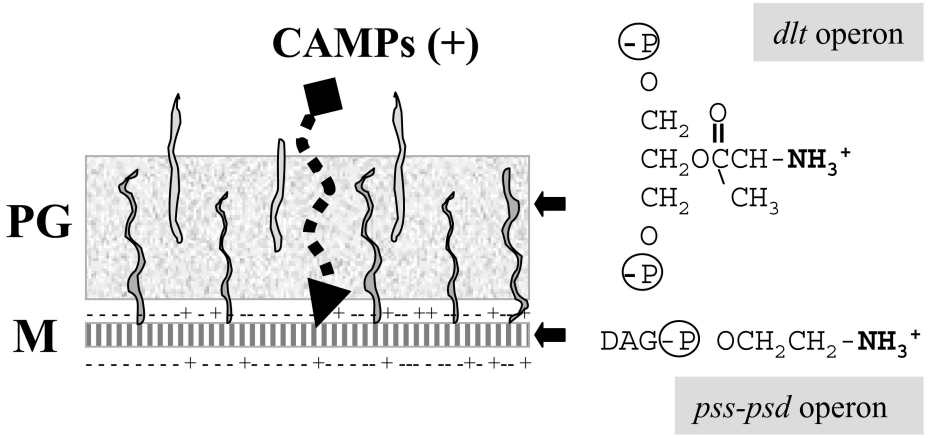

FIG. 8. Roles of the *B. subtilis* σ^X protein in resistance to CAMPs. The *B. subtilis* cell envelope includes both a cytoplasmic membrane (M) and a thick peptidoglycan layer (PG). Two of the operons controlled by σ^X are involved in modulating the net charge of the cell envelope. The *dlt* operon encodes proteins involved in the D-alanylation of both LTA and WTA by esterification of the glycerol moieties with D-alanine. Since both LTA and WTA are glycerol-phosphate copolymers, the introduction of D-alanine esters reduces the net negative charge of the cell wall. Similarly, the cytoplasmic membrane contains an abundance of anionic phospholipids (indicated by −), and the net charge of the membrane can be modulated by the incorporation of neutral constituents, such as glycolipids and the zwitterionic PE. The synthesis of PE requires the products of the *pssA ybfM psd* operon, which is partially under σ^X control. The ability of CAMPs to penetrate the cell wall and permeabilize the membrane is reduced by the incorporation of these positively charged groups into the cell envelope.

nent system (46). Our studies provide evidence linking ECF σ factors to the biosynthesis and modification of the cell envelope and suggest that these regulatory proteins may participate in an inducible defense response providing resistance to CAMPs.

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