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Mutations in Multidrug Efflux Homologs, Sugar Isomerases, and Antimicrobial Biosynthesis Genes Differentially Elevate Activity of the σ^X and σ^W Factors in *Bacillus subtilis*

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The σ^X and σ^W extracytoplasmic function sigma factors regulate more than 40 genes in *Bacillus subtilis*. σ^W activates genes which function in detoxification and the production of antimicrobial compounds, while σ^X activates functions that modify the cell envelope. Transposon mutagenesis was used to identify loci which negatively regulate σ^W or σ^X as judged by up-regulation from the autoregulatory promoter site P_W or P_X. Fourteen insertions that activate P_W were identified. The largest class of insertions are likely to affect transport. These include insertions in genes encoding two multidrug efflux protein homologs (*yqgE* and *yulE*), a component of the oligopeptide uptake system (*oppA*), and two transmembrane proteins with weak similarity to transporters (*yhdP* and *yueF*). Expression from P_W is also elevated as a result of inactivation of at least one member of the σ^W regulon (*ysdB*), an ArsR homolog (*yvbA*), a predicted rhamnose isomerase (*yulE*), and a gene (*pksR*) implicated in synthesis of difficidin, a polyketide antibiotic. In a parallel screen, we identified seven insertions that up-regulate P_X. Remarkably, these insertions were in functionally similar genes, including a multidrug efflux homolog (*yitG*), a mannose-6-phosphate isomerase gene (*yjdE*), and loci involved in antibiotic synthesis (*srfAB* and possibly *yogA* and *yngK*). Significantly, most insertions that activate P_W have little or no effect on P_X, and conversely, insertions that activate P_X have no effect on P_W. This suggests that these two regulons respond to distinct sets of molecular signals which may include toxic molecules which are exported, cell density signals, and antimicrobial compounds.

The soil bacterium *Bacillus subtilis* has evolved elaborate regulatory systems to adapt and survive under various environmental conditions. Alternative σ factors provide one means of modulating gene expression in response to changes in environment. Alternative σ factors in *B. subtilis* control sporulation (σ^H, σ^E, σ^F, σ^G, and σ^K); chemotaxis, motility, and autolysis (σ^D); and general stress responses (σ^B) (15). Sequencing of the *B. subtilis* genome has revealed seven previously unidentified σ factors that are members of the extracytoplasmic function (ECF) subfamily (28).

ECF σ factors are found in a wide variety of gram-positive and gram-negative bacteria and often regulate gene expression in response to extracytoplasmic stimuli (32). For example, ECF σ factors regulate genes involved in ferric citrate uptake and periplasmic protein proteolysis in *Escherichia coli* (3, 8); nickel and cobalt efflux in *Alcaligenes eutrophus* (30); antibiotic production, oxidative stress responses, and cell wall modification in *Streptomyces* spp. (26, 43, 44); and alginate and exotoxin secretion in *Pseudomonas aeruginosa* (18, 41). ECF σ factors are typically regulated by a cotranscribed anti-σ factor that is targeted to the cell membrane. Thus, expression of the σ factor operon leads to the synthesis of inactive σ–anti-σ complexes that are then regulated by signals that inhibit anti-σ function. These signals are likely to include alterations in the chemical composition or structure of the cell envelope.

The physiological functions of the ECF σ factors of *B. subtilis* are not well understood, and mutants with mutations in each of the seven σ factors are all viable. Only three of these regulators have been studied in detail (20–24): a *sigX* mutant is slightly more sensitive to heat and oxidative stress, a *sigM* mutant is unable to grow in high concentrations of salt, and a *sigW* mutant is altered in resistance to cell wall biosynthesis inhibitors. The σ^X regulon is expressed during late logarithmic growth, while the σ^W regulon is activated early in stationary phase (21, 22). Derepression of σ factor regulons, by mutation of the corresponding anti-σ factor, can also lead to phenotypic alterations. Increased expression of the σ^X regulon in an anti-σ (*rsiX*) mutant represses expression of σ^W (22) and leads to reduced competence (61).

To investigate the roles of *B. subtilis* ECF σ factors, we used consensus-based promoter searches to identify genes under the control of σ^X and σ^W (23, 24). The σ^X regulon includes a putative glucosyltransferase (CsbB), a regulator of autolysin expression (LytR), and a response regulator aspartate phosphatase (RapD). Recent results indicate that σ^X also regulates the D-alanylation of teichoic acids and membrane phospholipid composition (M. Cao, J. Qiu, and J. D. Helmann, unpublished results). Proteins dependent on σ^W for expression include a fosfomycin resistance determinant (FosB), a penicillin binding protein (PBP4*), signal peptide peptidase (YteI), an ATP-binding cassette transporter (YknXYZ), a nonheme bromoperoxidase (YdjP), epoxide hydrolase (YfhM), several small hydrophobic peptides (YvlC, YxzE, and YdjO), and a large number of membrane proteins of unknown function (23). At least four additional genes (*abh*, *divIC*, *yrhH*, and *ywbN*) are apparently transcribed by both σ^X and σ^W (22). The characterization of these two regulons suggests that σ^X regulates cell envelope modification processes while σ^W regulates detoxification responses and the production of antimicrobial compounds.

To further define the physiological roles of σ^X and σ^W in *B. subtilis*, we have used mini-Tn*10* mutagenesis to identify mu-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
B. subtilis strains		
CU1065	W168 *trpC2 att*SPβ	Lab stock
HB7070	CU1065 SPβ7063 [P_W-*cat-lacZ*]	22
HB0011	HB7070 *rsiW*::Km	This work
HB7022	CU1065 SPβ7019 [P_X-*cat-lacZ*]	21
HB7024	HB7022 *rsiX*::pVA29	21
OKB105	*pheA1 sfp*	39
HB300	CU1065 *yqgE*::pMUTIN (MLS^r)	This study
HB301	HB7070 pXT-*yshB′* (Spc^r MLS^r Neo^r)	This study
HB302	CU1065 *yjdD*::pMUTIN (MLS^r)	This study
E. coli strains		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	53
$Jm2r^-$	*mcrAB hsdR recA1* Δ(*lac-proAB*) *thi gyrA96 relA1 srl*::Tn*10* F′ (*proAB lacZ* Δ*M15*)	S. Zahler
Plasmids		
pIC333	pUC replicon, shuttle vector carrying mini-Tn*10*, thermosensitive replicon for gram-positive hosts (Ap^r MLS^r Spc^r)	57
pMUTIN4	pBR322-based integration vector for *B. subtilis*; contains a multiple cloning site downstream of the P_{spac} promoter (Ap^r MLS^r)	64
pMUTIN-*yqgE*	pMUTIN4 derivative carrying DNA from the 5′ end of *yqgE*	This study
pMUTIN-*yjdD*	pMUTIN4 derivative carrying DNA from within *yjdD*	This study
pXT	Vector which integrates into the *thrC* locus and contains the *xylA* xylose-inducible promoter upstream of a multiple cloning site (Ap^r MLS^r Spc^r)	T. Msadek
pXT-*yshB′*	pXT derivative carrying DNA from the 5′ end of *yshB*	This study

tants with increased σ^W or σ^X activity. The resulting transposon insertions indicate that defects in transport, cell density signaling, sugar metabolism, and antimicrobial production affect the activity of these σ factors. However, the signals that activate σ^X appear to be largely distinct from those that activate σ^W.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All the bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C with aeration in liquid Luria-Bertani medium (LB) (53) or Tris-Spizizen salts (TSS) minimal medium (16) containing either D-glucose or D-mannose as the sugar and auxotrophic requirements. Plates contained 1.25% Bacto Agar (Difco) and 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Ampicillin (100 μg/ml) or spectinomycin (SPC) (200 μg/ml) was used for the selection of *E. coli* strains. Erythromycin (1 μg/ml) and lincomycin (25 μg/ml) (for testing macrolide-lincosamide-streptogramin B [MLS] resistance), SPC (100 μg/ml), neomycin (8 μg/ml), kanamycin (15 μg/ml), and chloramphenicol (2 to 5 μg/ml) were used for the selection of various *B. subtilis* strains.

Construction of mini-Tn*10* libraries. Random mini-Tn*10* libraries of *B. subtilis* strains were contructed using the plasmid pIC333 (57). This plasmid contains a ColE1 origin and a thermosensitive origin of replication for gram-positive bacteria (inactive at temperatures greater than 35°C). *B. subtilis* strains HB7070 (P_W-*cat-lacZ*) and HB7022 (P_X-*cat-lacZ*) were transformed with pIC333 with selection for Spc^r on LB plates incubated overnight at 28°C. Single colonies were inoculated into 2 ml of LB-SPC and incubated at 28°C overnight. Following a 1:100 dilution in the same medium, the cultures were grown for 3 h at 28°C, then shifted to 37°C, and grown for at least another 5 h. Diluted aliquots of these cultures were plated onto LB and LB-SPC plates and incubated overnight at 37°C. The rest of the culture was collected by centrifugation and resuspended in LB containing 15% glycerol for storage at −80°C. The transposition frequency was estimated from the ratio of the number of colonies on LB-SPC to that on LB and was in the general range (0.01 to 1%) reported for this system.

DNA manipulations and sequencing. Isolation of *B. subtilis* chromosomal DNA and transformations were done by standard procedures (16). Restriction endonucleases and DNA ligase (New England Biolabs, Inc., Beverly, Mass.) were used according to the manufacturer's instructions. Plasmid rescue experiments were performed as previously described (6). PCR experiments used for cloning DNA into plasmids were performed by using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the manufacturer's instructions. DNA was purified by using the QIAprep Spin Miniprep and PCR purification and gel extraction kits (Qiagen Inc., Chatsworth, Calif.). DNA sequencing was performed with AmpliTaq-FS DNA polymerase and dye terminator chemistry at the DNA Services Facility of the Cornell New York State Center for Advanced Technology-Biotechnology.

Screening and identification of mini-Tn*10* mutants up-regulated in σ^W and σ^X activity. Initially, several mini-Tn*10* libraries of *B. subtilis* HB7070 were plated onto LB-SPC at a density of approximately 150 transposants per plate. The majority of colonies were light blue after 1 day of growth at 37°C; however, some colonies exhibited enhanced β-galactosidase (β-Gal) activity. Three colonies (W1, W2, and W3) from different mini-Tn*10* libraries with enhanced β-Gal activity were further characterized. Also, several mini-Tn*10* libraries of *B. subtilis* HB7070 and HB7022 were plated onto LB-SPC containing chloramphenicol (2 to 5 μg/ml) at a density of approximately 10,000 transposants per plate. Several mutants which grew faster on these plates and had elevated β-Gal activity were further characterized. The phenotypes were linked to the mini-Tn*10* Spc^r marker by transformation. Plasmids containing the mini-Tn*10* element with a ColE1 origin, the ampicillin resistance gene, and flanking *B. subtilis* chromosomal DNA were recovered from selected mutant strains. DNA sequence upstream and downstream of the transposon was obtained using two primers corresponding to the left and right ends of the mini-Tn*10*, as described previously (4).

Generation of *B. subtilis* mutants HB300, HB301, and HB302. DNA upstream of and including the 5′ end of the *yqgE* gene was amplified from *B. subtilis* chromosomal DNA using primers 425 (5′-TTGAATTCTTCTTTTTACATATC TCGG-3′) and 426 (5′-CAGGATCCTGTCTATTTTTTTGGCTAACCG-3′). The ~320-bp PCR product was digested with *Eco*RI and *Bam*HI (sites underlined) and cloned into pMUTIN4 (64) to generate pMUTIN-*yqgE*. This plasmid was then transformed into strain CU1065 to generate strain HB300. DNA upstream of and including the truncated *yshB* gene (caused by the mini-Tn*10* insertion) from strain W14 was amplified using primers 508 (5′-ATGGATCCG CCGGGCGGTTTTGCCTG-3′) and 509 (5′-ATCAGAATTCAAGATGTGTA TCCACC-3′). The ~640-bp PCR product encoding a hydrophobic peptide from the 5′ sequence of *yshB* was digested with *Bam*HI and *Eco*RI and cloned into pXT, a derivative of pDG1731 allowing gene expression from the P_{xylA} xylose-inducible promoter and integration by a double-crossover event at the *thrC* locus (T. Msadek, unpublished data). The resulting plasmid, pXT-*yshB′*, was linearized with *Sca*I and transformed into strain CU1065 to generate strain HB301. To generate a *yjdD* mutant, an internal fragment of the *yjdD* gene was amplified from *B. subtilis* chromosomal DNA using primers 511 (5′-AGGAATTCTGCA AAAAGCTGCTGACAGAC-3′) and 512 (5′-AAGGATCCAGTCGCCGCAA TATAACCGC-3′). The ~590-bp PCR product was digested with *Eco*RI and *Bam*HI and cloned into pMUTIN4 to generate pMUTIN-*yjdD*. This plasmid was then transformed into strain CU1065 to generate HB302.

β-Gal assays. To determine the β-Gal activities of various strains, cells were diluted 1:100 from an overnight culture grown in LB containing the necessary antibiotics into LB. Samples were then collected from the phase of growth when the relevant σ factor is most active. For strains containing the P_W-*cat-lacZ* fusion,

TABLE 2. Characterization of genes which affect σ^W and σ^X activity

Strain[a]	Gene	Mini-Tn*10* insertion site[b]	Presumed function and/or features[c]	Downstream gene(s) possibly affected by insertion[d]	LB plate phenotype[e]
HB7070	NA[f]	NA	NA	NA	−/+
HB0011	*rsiW*	NA	Anti-σ^W	NA	++
W1, W2, and W3	*yvbA*	244	Regulator of export	*yvaZ*	+++
W4	*yqgE*	240	Multidrug efflux	*pbpA*	−/+[g]
W5	*yheH*	1640	Multidrug efflux		+
W6	*yhdP*	260	Metal ion efflux		+
W7	*yueF*	931	Unknown; transport?	*yueG*	+
W8	*oppA*	391	Oligopeptide uptake	*oppBCDF*	++
W9	*yulE*	1058	Rhamnose isomerase		+
W10	*pksR*	6772	Difficidin synthesis		+
W11	*ysdB*	−43	Unknown; σ^W- and possibly σ^B-regulated membrane protein		+
W12	*yqfD*	71	Unknown; possibly σ^W regulated	*phoH*	+
W13	*yodE*	240	Aromatic ring cleavage	*yodD*	+
W14	*yshB*	47	Unknown	*yshCDE*	++
W15	*yshD*	1862	DNA mismatch repair	*yshE*	+
W16	*yopH*	331	Unknown	*yopIJKL*	+
HB7022	NA	NA	NA	NA	−
HB7024	*rsiX*	NA	Anti-σ^X	NA	++
X1	*yitG*	631	Multidrug efflux	*yitFE*	+
X2	*yjdE*	60	Mannose-6-phosphate isomerase	*yjdF*	+
X3	*srfAB*	10064	Surfactin synthesis	*srfAC*, *srfAD*	+
X4	*yogA*	933	Polyketide synthesis		−/+
X5	*yngK*	1517	Unknown		−/+
X6	*ytxJ*	227	Unknown		−/+
X7	*ywpH*	−74	DNA replication	*glcR*, *ywpJ*	+

[a] Mini-Tn*10* mutants up-regulated in σ^W and σ^X activity begin with W and X, respectively.
[b] Relative to the first nucleotide of the gene.
[c] The majority of predicted functions are based on those of homologous proteins (see Table 3).
[d] In most cases, operon structures have not been characterized, but inspection of the genome sequence indicates that expression of these downstream genes would also likely be affected by the transposon insertion. We have also not ruled out possible effects of the transposon insertion on expression of upstream genes.
[e] Expression of the P_W and P_X promoters in each strain was measured by growth on 15-ml LB plates containing 40 μg of X-Gal per ml. Colony color was observed following overnight growth at 37°C and is indicated as follows: +++ (strong blue) > ++ (blue) > + (light blue) > −/+ (very light blue) > − (white). The parent (HB7070) and *rsiW* mutant (HB0011) strains containing the P_W-*cat-lacZ* fusion are included. The parent (HB7022) and *rsiX* mutant (HB7024) strains containing the P_X-*cat-lacZ* fusion are also included.
[f] NA, not applicable.
[g] After 2 days of growth, the *yqgE* mutant had significantly greater σ^W activity than did the parent strain HB7070.

samples were taken at T_1 (1 h after the end of exponential growth), and for strains containing the P_X-*cat-lacZ* fusion, samples were taken at T_{-2}. The assay used for determining β-Gal levels by the method of Miller has been described previously (6, 34). All assays were performed on duplicate samples, and the values were averaged.

Computer analysis. To determine the loci in which the mini-Tn*10* had been inserted, the sequence of chromosomal DNA flanking the mini-Tn*10* was compared with the *B. subtilis* genome using the BLAST program (2) available on the SubtiList website (37) at http://www.pasteur.fr/Bio/SubtiList.html. Searches of *B. subtilis* protein sequences in other databases were performed using BLASTP (2) at http://www.ncbi.nlm.nih.gov/BLAST/ using the unfiltered setting. Protein localization and transmembrane domains were predicted using both the PSORT (38) and TMPred (19) programs available at http://psort.nibb.ac.jp:8800/form.html and http://www.isrec.isb-sib.ch/software/TMPRED_form.html, respectively.

RESULTS

Rationale for mutant isolation. To assess the activity of σ^W and σ^X in vivo, we used strains [HB7070 (P_W-*cat-lacZ*) and HB7022 (P_X-*cat-lacZ*)] containing operon fusions to the autoregulatory promoters, P_W and P_X. These promoters are specifically recognized by each σ factor in vivo and in vitro (21, 22). Since each promoter drives expression of an operon encoding both chloramphenicol resistance (*cat*) and β-Gal (*lacZ*), this system is suitable for both genetic screens and selections for increased promoter activity (56).

Isolation and analysis of mini-Tn*10* mutants with increased σ^W activity. Initially, several HB7070 mini-Tn*10* libraries were plated onto LB plates containing SPC and X-Gal. From approximately 9,000 transposants, three mutants (W1, W2, and W3 [Table 2]) with obviously elevated β-Gal activity were identified. All three have a mini-Tn*10* insertion at the same position within the *yvbA* gene. Since these mutants were isolated from independent mini-Tn*10* libraries, and one has the mini-Tn*10* inserted in the opposite orientation from that of the other two, they are not siblings. YvbA is an uncharacterized member of the ArsR family of transcriptional regulators (Table 3). ArsR-like proteins (including ArsR, SmtB, ZiaR, and CadC) regulate resistance to arsenic, zinc, and cadmium (10, 25, 60, 67). Since resistance is often associated with metal ion efflux, it is possible that YvbA may regulate efflux from *B. subtilis*. Unlike other members of this family, YvbA does not contain cysteine residues, which have been implicated in metal binding (55).

To expand our collection of mutants with altered σ^W activity (Table 2), we selected for upregulation of the P_W-*cat-lacZ* fusion using chloramphenicol. Mini-Tn*10* libraries were plated onto LB containing SPC, X-Gal, and growth-inhibitory levels (2 to 5 μg per ml) of chloramphenicol. Mutants with elevated β-Gal activity were isolated following 2 days of incubation at 37°C. Those transposon insertions that were genetically linked to the derepressed phenotype were further characterized. For quantification, β-Gal activities were determined for mutants

TABLE 3. Database homologies

Protein of unknown function	No. of amino acids	*B. subtilis* paralog(s) of interest (% identity/no. of amino acids)	Homologous protein(s) of known or predicted function (% identity/no. of amino acids)	Reference for functionally known protein
YvbA	90		*Staphylococcus xylosus* ArsR (40/87)	51
YqgE	430		*Staphylococcus aureus* NorA (22/304)	68
YheH	673		*Homo sapiens* MDR1 (33/517)	5
			Pasteurella haemolytica HylB (31/509)	59
			Lactococcus lactis LcnC (27/532)	58
YhdP	444	YrkA (62/434)	*Rickettsia typhi* TylC (33/214)	48
		YhdT (60/430)	*S. enterica* serovar Typhimurium CorC (27/277)	12
		YugS (60/427)	*S. enterica* serovar Typhimurium CorB (24/224)	12
		YqhB (59/433)		
YueF	369		*E. coli* PerM (27/315)	
YsdB	130		None	
YqfD	398		*Bacillus megaterium* SpoIV (43/395)	66
YodE	303	YdfO (48/296)	*Sphingomonas paucimobilis* LinE (30/306)	35
		YkcA (37/313)	*Sphingomonas chlorophenolica* PcpA (28/299)	42
YulE	424		*E. coli* RhaA (58/411)	36
YshB	177		None	
YshD	785	MutS (24/520)		13
YopH	178		None	
YitG	422	Blt (23/407)		1
		Bmr (22/383)		
YjdE	315	Pmi (56/316)	*Streptococcus mutans* ManA (53/311)	54
		YdhS (55/313)		
YogA	329		*Streptomyces cinnamonensis* CCR (26/364)	31
			Pseudomonas syringae Cfa8 (25/356)	49
YngK	510		None	
YtxJ	108		None	
YwpH	113	SSB (63/106)		

grown in LB to early stationary phase (T_1), when P_W activity is maximal (Fig. 1). Most mutants had only slightly elevated σ^W activity in liquid medium, despite an obvious effect on solid medium (Table 2 and Fig. 1). This is reminiscent of the observation that P_W can be strongly induced by cell wall biosynthesis inhibitors on plates but not in liquid medium (M. Cao and J. D. Helmann, unpublished data).

A total of 13 additional insertion mutations were identified in this genetic selection (Table 2). These insertions define genes that cluster into three functional classes: (i) transport, (ii) sugar metabolism, and (iii) antibiotic biosynthesis. Insertions were also obtained in genes of unknown function including at least one known member of the σ^W regulon.

Transport functions. Mutants W4 and W5 possess mini-Tn*10* insertions in genes *yqgE* and *yheH* which encode transmembrane proteins with similarity to multidrug efflux proteins. YheH is a putative ATP-binding protein which has been classified into subfamily 6 of the *B. subtilis* ATP-binding proteins (47). Subfamily 6 includes proteins which are similar to multidrug resistance proteins of eukaryotes and bacterial proteins involved in bacteriocin and hemolysin export (Table 3). YqgE has 10 to 12 potential hydrophobic domains and is similar to drug efflux proteins (Table 3). Although *yqgE* is located downstream of *sodA* (superoxide dismutase) in several *Bacillus* species, it has been previously shown not to be involved in SodA activity (17).

Multidrug efflux proteins export a variety of structurally unrelated toxic chemicals including ethidium bromide, chloramphenicol, and puromycin (1). However, the *yqgE*::Tn*10* mutant is no more sensitive than the wild type to a variety of toxic chemicals (including ethidium bromide, chloramphenicol, or tetracycline). Since *B. subtilis* possesses a number of multidrug efflux proteins, it is possible that they are functionally redundant (1). Next, we placed *yqgE* under the control of the inducible P_{spac} promoter by integration of pMUTIN-*yqgE* into the chromosome. Induction of the resulting strain with IPTG (isopropyl-β-D-thiogalactopyranoside), to potentially elevate YqgE levels, failed to reveal any increase in resistance to ethidium bromide, tetracycline, or puromycin (data not shown). Thus, the role of this transporter and identification of its substrates await further study.

Mutant W6 contains a mini-Tn*10* in *yhdP* which encodes one of five highly similar *B. subtilis* paralogs (Table 3). YdhP has significant sequence similarity to proteins from *Salmonella enterica* serovar Typhimurium involved in magnesium uptake (Table 3). A potential role of YhdP in transport is bolstered by the presence of a gene (*yhdQ*) encoding a MerR homolog immediately upstream of *yhdP*. Some MerR homologs regulate gene expression in response to metal ions, whereas others are known to regulate multidrug efflux proteins (1). Mutant W7 has a mini-Tn*10* inserted in the *yueF* gene which encodes a protein of unknown function that has eight potential membrane-spanning domains. YueF is similar to putative integral membrane proteins including *E. coli* PerM (Table 3).

Mutant W8 has a mini-Tn*10* inserted in the *oppA* gene which encodes an oligopeptide binding lipoprotein that is part of an ATP-binding cassette transport system (52). This system is required for sporulation and competence (52) and transports peptides that act as cell density signals (29). As predicted, mutant W8 has a sporulation-negative phenotype on Difco sporulation medium plates and is reduced in competence compared to the parent strain (data not shown). Identification of an *oppA*::Tn*10* insertion suggests that σ^W may be negatively regulated by some of the same cell density signals that positively regulate sporulation and competence.

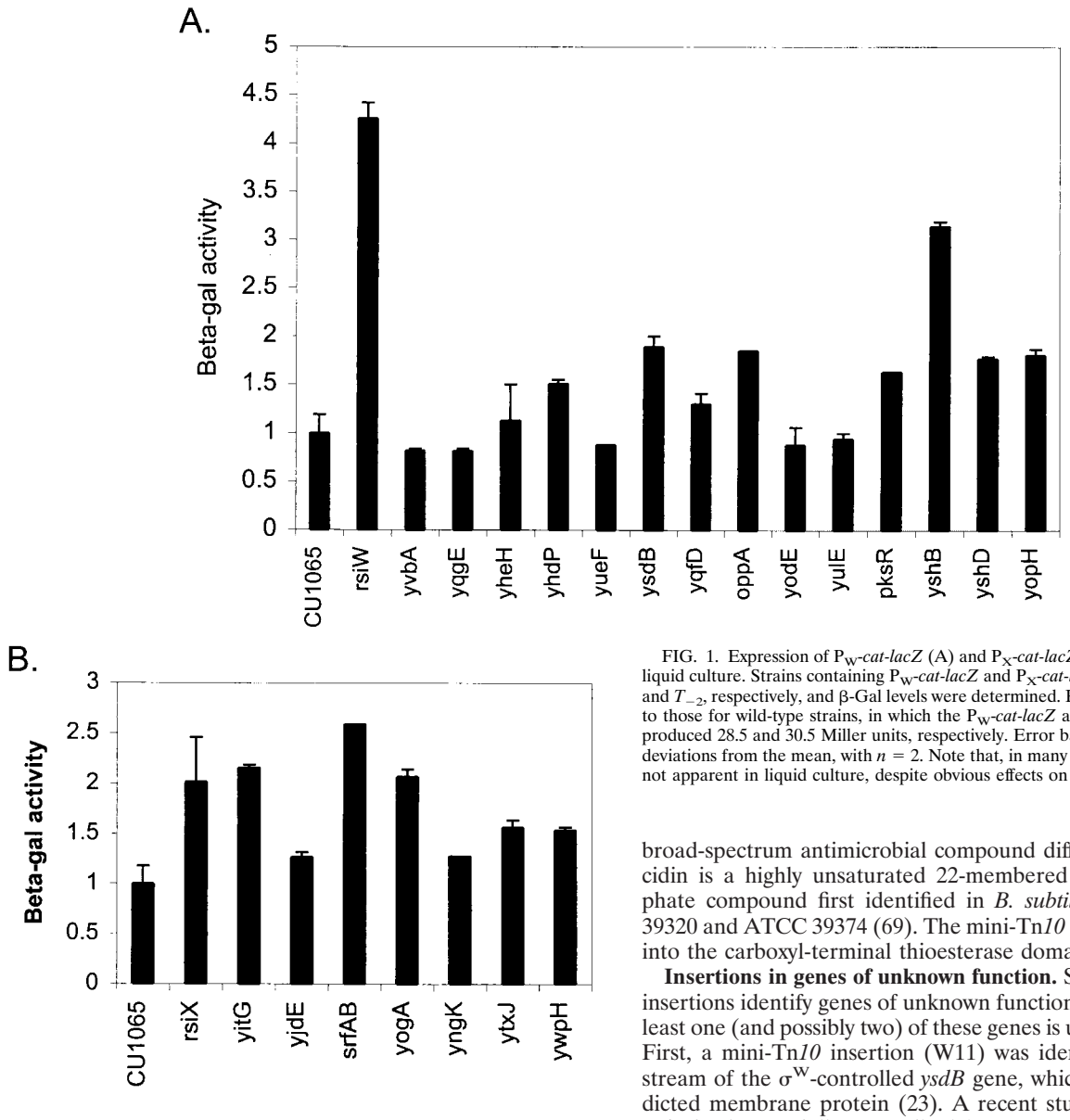

FIG. 1. Expression of P_W-*cat-lacZ* (A) and P_X-*cat-lacZ* (B) during growth in liquid culture. Strains containing P_W-*cat-lacZ* and P_X-*cat-lacZ* were grown to T_1 and T_{-2}, respectively, and β-Gal levels were determined. Results are normalized to those for wild-type strains, in which the P_W-*cat-lacZ* and P_X-*cat-lacZ* strains produced 28.5 and 30.5 Miller units, respectively. Error bars represent standard deviations from the mean, with $n = 2$. Note that, in many cases, up-regulation is not apparent in liquid culture, despite obvious effects on plates.

Sugar metabolism. Mutant W9 has a mini-Tn*10* in the *yulE* gene which encodes a protein highly similar to rhamnose isomerases (Table 3). *yulE* is located in an operon with other genes encoding enzymes involved in rhamnose metabolism, and YulE appears to be the only rhamnose isomerase homolog in *B. subtilis*. Rhamnose isomerase catalyzes the interconversion of L-rhamnose and L-rhamnulose and is required for the first step in the metabolism of L-rhamnose (36). After several days of growth on minimal medium plates containing rhamnose as the sole carbon source, colonies of the *yulE*::Tn*10* mutant become translucent, in contrast to the parent, which remains opaque. This indicates that *yulE* is likely to be involved in rhamnose metabolism, since this phenotype was not observed when the *yulE*::Tn*10* mutant was grown on glucose or mannose as the sole carbon source. It is not yet clear why a defect in rhamnose metabolism might lead to increased σ^W activity.

Antimicrobial synthesis. Mutant W10 has a mini-Tn*10* insertion in the *pksR* gene implicated in the synthesis of the broad-spectrum antimicrobial compound difficidin (28). Difficidin is a highly unsaturated 22-membered macrolide phosphate compound first identified in *B. subtilis* strains ATCC 39320 and ATCC 39374 (69). The mini-Tn*10* has been inserted into the carboxyl-terminal thioesterase domain of PksR.

Insertions in genes of unknown function. Several additional insertions identify genes of unknown function. Interestingly, at least one (and possibly two) of these genes is under σ^W control. First, a mini-Tn*10* insertion (W11) was identified 43 bp upstream of the σ^W-controlled *ysdB* gene, which encodes a predicted membrane protein (23). A recent study indicates that *ysdB* is also partially transcribed from an upstream promoter recognized by the general stress σ factor, σ^B (45). Since the mini-Tn*10* was inserted into the gene-proximal σ^W promoter, it is predicted that *ysdB* is not expressed in this mutant. Since this insertion increases σ^W activity, we envision a feedback process whereby the loss of this protein leads to an unidentified signal that leads to up-regulation of σ^W.

The second example is provided by mutant W12. The *yqfD* gene encodes a protein with similarity to a putative sporulation protein of *Bacillus megaterium* (Table 3). Interestingly, the genomic organization in the vicinity of *yqfD* suggests a possible operon structure including *yqeZ yqfABCD phoH*. The *phoH* gene encodes a homolog of an ATP-binding protein from *E. coli* which is induced under phosphate starvation conditions (27). The *yqeZ* gene encodes a paralog of the σ^W-dependent YteI (signal peptide peptidase) and has recently been shown to also depend on σ^W for expression (23; J. Qiu and J. D. Helmann, unpublished results). Thus, it is possible that *yqfD* is also under σ^W control.

Additional genes of unknown function include *yodE*, *yshB*,

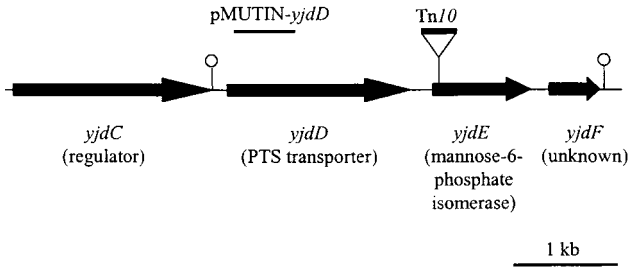

FIG. 2. Diagram of genes surrounding *yjdE* and mutant strains used in this study. The proposed function of the genes is based on homologies only. The mini-Tn*10* insertion in *yjdE* identified from mutant X2 is indicated as a triangle with Tn*10*. The pMUTIN-*yjdD* plasmid used to disrupt *yjdD* contains DNA from within *yjdD* indicated with a line above the gene. Potential stem-loops are indicated as lollipops.

yshD, and *yopH*. The *yodE* gene (W13) encodes a homolog of aromatic ring cleavage dioxygenases from *Sphingomonas* spp., which are involved in degradation of organic insecticides such as pentachlorophenol (Table 3). YodE also has high similarity to the *B. subtilis* YdfO and YkcA proteins, also of unknown function. Two independent mutants (W14 and W15) identify genes in the *ysh* locus. The *yshB* gene encodes a protein with four potential membrane-spanning domains with no significant similarity to other proteins in databases. The *yshB*::Tn*10* strain could potentially express the 16 amino-terminal residues of YshB: MLDIIILILLLMGTLL. Since two known members of the σ^W regulon are signal peptide peptidase homologs, we speculated that production of this hydrophobic peptide might be the signal leading to up-regulation of σ^W. However, when we expressed this peptide using a xylose-inducible promoter we did not observe an increase in P_W activity (data not shown). The *yshD* gene encodes a protein similar to the DNA mismatch repair MutS protein family (Table 3). Since the *yshABCDE* locus is probably an operon (Table 2), it is yet not known which particular gene or genes influence σ^W activity. The last insertion isolated (W16) is in the *yopH* gene located on the SPβ prophage. The function of *yopH* is unknown, although the product of *yopH* is predicted to have two membrane-spanning regions.

Isolation and analysis of mini-Tn*10* mutants with increased σ^X activity. In parallel with the above studies, we identified seven mini-Tn*10* insertions that led to an up-regulation of a P_X-*cat-lacZ* operon fusion. Unexpectedly, the resulting insertions defined a distinct group of genes that are nevertheless implicated in the same general set of cellular functions: transport, sugar metabolism, and antimicrobial biosynthesis.

Transport functions. Mutant X1 has a mini-Tn*10* in the *yitG* gene which encodes a putative transmembrane protein similar to multidrug efflux proteins. It has similarity to several characterized *B. subtilis* multidrug efflux proteins (Table 3) which mediate the efflux of a variety of structually diverse toxic compounds (1). Immediately downstream of the *yitG* gene is *yitF*, which encodes a protein similar to muconate cycloisomerases and mandelate racemases. These enzymes are involved in the catabolism of aromatic compounds (40). It is possible that *yitG* may be involved in the export of an aromatic-like compound in *B. subtilis*.

Sugar metabolism. Mutant X2 has a mini-Tn*10* in the *yjdE* gene which encodes one of the three mannose-6-phosphate isomerase homologs in *B. subtilis* (Table 3). Mannose-6-phosphate isomerase catalyzes the interconversion of mannose-6-phosphate and fructose-6-phosphate. Located upstream of *yjdE* are genes encoding a putative transcriptional activator (*yjdC*) and a phosphoenolpyruvate:sugar phosphotransferase (PTS) enzyme II of the fructose-mannitol family of PTS permeases (*yjdD*) (Fig. 2). It has been recently hypothesized, from sequence comparisons, that this locus may be involved in mannose metabolism in *B. subtilis* (50); however, no direct experimental evidence has confirmed this.

We observed that the *yjdE* mutant is unable to grow in minimal medium with mannose as the sole carbon source (Fig. 3A), demonstrating that this isomerase is essential for mannose catabolism. A *yjdD* mutant is also unable to grow on mannose (Fig. 3A). Since the *yjdD* mutation may be polar on downstream genes (Fig. 2), we induced the expression of *yjdE* from the P_{spac} promoter in the integrated pMUTIN-*yjdD* plasmid using IPTG. However, this induced strain was still unable

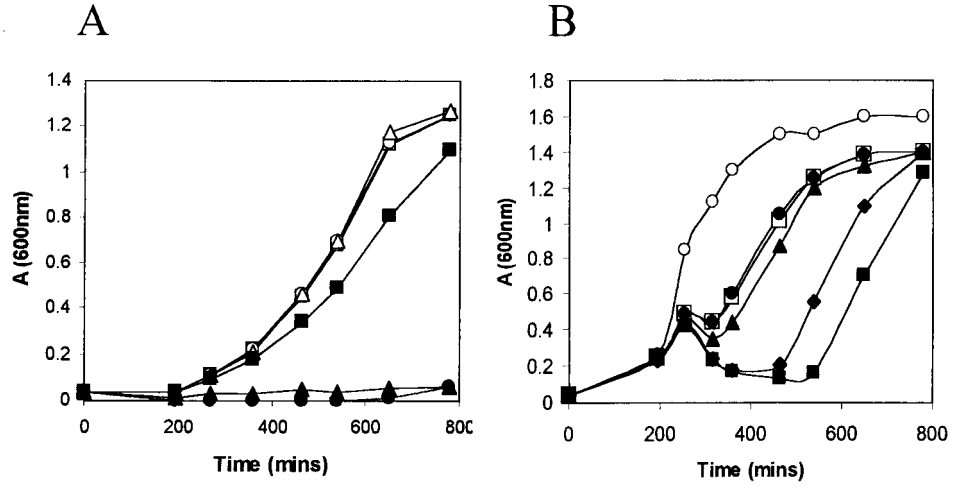

FIG. 3. Growth of *B. subtilis* strains in minimal medium containing glucose or mannose as the sugar (A) or in LB with varying concentrations of mannose (B). (A) *B. subtilis* CU1065 (squares) and *yjdE* (circles) and *yjdD* (triangles) mutant were grown in Tris-Spizizen salts minimal medium containing either glucose (open symbols) or mannose (filled symbols) as the sugar. (B) Effect of mannose on growth of the *yjdE* mutant. Cells were grown in LB (open squares) or LB with 1.4 μM (filled circles), 14 μM (triangles), 0.14 mM (diamonds), or 1.4 mM (filled squares) mannose. Growth of *B. subtilis* CU1065 (open circles) was unaffected by mannose addition, and all growth curves were essentially superimposable.

to grow on mannose minimal medium plates. Thus, this operon appears to encode both a mannose-specific PTS enzyme and mannose isomerase.

Interestingly, the *yjdE* mutant was impaired in growth in LB and more so in LB supplemented with greater than 1.4 mM mannose (Fig. 3B). This suggests that (i) YjdE may be needed for mannose synthesis from glucose during growth in LB and (ii) the accumulation of mannose-6-phosphate in a *B. subtilis yjdE* mutant grown on mannose inhibits growth.

Antimicrobial and polyketide synthesis. Three mutant strains with increased σ^X activity were affected in genes known or suggested to be involved in the synthesis of antimicrobial compounds. Mutant X3 has a mini-Tn*10* insertion in the *srf* locus near the end of the *srfAB* gene. The *srf* operon encodes subunits of the surfactin synthetase (7). Located within the *srfAB* gene is a small gene termed *comS* which encodes a protein which is involved in competence (9). However, the mini-Tn*10* insertion in *srfAB* is located downstream of *comS*.

Additional insertions were recovered in *yogA* (X4) and *yngK* (X5). YogA is similar to oxidoreductase enzymes which synthesize butyryl coenzyme A, used as a carbon extender in polyketide synthesis (31). *yngK* is the second gene located downstream of and in the same direction as the *pps* operon required for the synthesis of the antifungal cyclic decapeptide antibiotic pliplastin (63). Downstream of *yngK* is a putative operon encoding proteins involved in fatty acid metabolism (*yngJIHGFE*), and it has been suggested previously that they may be required for the synthesis of the lipopeptide (62). YngK may therefore play a role in the synthesis of pliplastin.

Proteins with unclear functions. Mutant X6 has a mini-Tn*10* inserted in the *ytxJ* gene which encodes a protein of unknown function. *ytxJ* has been previously termed *csb40*, is controlled by σ^B and σ^H, and is strongly induced by the addition of salt to the cells (65). Upstream of *ytxJ*, and located in the same operon, *ytxH* encodes a product with similarity to plant proteins induced by desiccation stress (65).

Mutant X7 has a mini-Tn*10* insertion upstream of the *ywpH* gene. It is predicted that this insertion will affect the expression of *ywpH*, *glcR*, and *ywpJ*. *ywpH* encodes a product similar to single-stranded DNA binding proteins that are involved in DNA replication (Table 3). GlcR is similar to the DeoR family of transcriptional regulators which regulate genes involved in sugar utilization. Interestingly, GlcR is most similar to YulB (35% identity over 231 amino acids), which is encoded upstream of, and most likely in the same operon as, the putative rhamnose isomerase gene, *yulE* (mentioned above). *ywpJ* encodes a conserved protein of unknown function.

DISCUSSION

In this study, we sought to identify genes affecting the activity of σ^W or σ^X. We reasoned that mutations causing deficiencies in aspects of cell metabolism controlled by either σ^X or σ^W might lead to up-regulation of the corresponding regulons and aid in the identification of the molecular signals controlling σ factor activity. Since each of these σ factors is negatively regulated by a specific anti-σ, we anticipated that at least one class of mutations would be insertions in the anti-σ gene. However, we did not recover insertions in the anti-σ genes in this screen. This may reflect a low frequency of transposition in these genes by the Tn*10* derivative employed in these studies or may simply reflect the fact that we have not saturated this screen. However, we have obtained multiple insertions in the same gene (*yvbA*) or in different genes in the same operon (*yshB* and *yshD*), and our collection of mutants defines several discrete functional groups: export, sugar metabolism, and antimicrobial synthesis.

Although σ^W and σ^X activity is affected by mutations affecting similar functions, only the *yvbA*::Tn*10* mutation up-regulated both σ^W and σ^X (σ^W more so than σ^X). For the 16 other mutants tested (*yopH*::Tn*10* was not included), the isolated transposon insertion affected expression of one reporter fusion, but not the other, as determined by measurements of β-Gal activity on solid medium. It has been previously shown that σ^W and σ^X coregulate several *B. subtilis* genes (22), and so these two σ factors do overlap in function. However, our results suggest that σ^W and σ^X respond to distinct stimuli, consistent with the observation that these two regulons are generally induced at different growth phases (21–24).

Many of the mini-Tn*10* insertions identified in this study affect genes encoding transport proteins, including several with homology to multidrug efflux proteins. For the majority of these transporters, substrates have not yet been identified. Up-regulation of σ factor activity in these transport mutants may result from the inability to export toxic compounds from the cell. Another class of mutants affects genes involved in sugar metabolism. Interestingly, both rhamnose and mannose are components of cell surface polysaccharides of some gram-negative and gram-positive bacteria (14, 33). It is possible that the *yulE* and *yjdE* mutants may be affected in the synthesis of sugar-containing cell envelope components. Although *N*-acetylmannosamine is present in the linkage unit of cell wall teichoic acid, mannose-6-phosphate does not appear to be an intermediate in its synthesis (11). Further work will be needed to examine the cell envelope constituents in wild type and *sigX*, *sigW*, *yulE*, and *yjdE* mutants.

An interesting class of mutants identified in this study were affected in genes implicated in the synthesis of antimicrobials. Insertions in these genes were particularly surprising since surfactin and pliplastin are not thought to be synthesized by *B. subtilis* 168 (39, 63). This is due to a mutated *sfp* gene which encodes a phosphopantetheinyl transferase required for conversion of the peptidyl carrier domains within the multidomain synthetase enzymes from inactive apo-forms to active holoforms (46). We suggest that another holo-acyl carrier protein synthase homolog (perhaps YdcB) may, albeit less efficiently, activate the antimicrobial synthetase subunits and allow a low level of antimicrobial production. Indeed, Sfp phosphopantetheinylates, with varying efficiency, a wide substrate spectrum, including acyl carrier protein domains of fatty acid synthases (46). If correct, it is possible that up-regulation reflects an inability to synthesize these antibiotics or, alternatively, the presence of a covalent antibiotic-synthetase complex resulting from insertions inactivating the thioesterase domain of the synthetase which is needed for release of the antibiotic. To test this latter idea, we hypothesized that transfer of these insertions into sfp^+ strains (known to produce active synthase) might further elevate σ factor activity. However, when the *srfAB*::Tn*10* mutation was transferred to the sfp^+ strain *B. subtilis* OKB105 containing the P_X-*cat-lacZ* fusion, no further up-regulation in σ^X activity was observed.

Our results suggest that σ^W and σ^X respond to a variety of signals related to extracellular functions. By analogy with other ECF σ factors, the increase in σ factor activity is likely mediated by the corresponding anti-σ factors. We hypothesize that RsiW and RsiX sense, either directly or indirectly, molecules which are exported from the cell including cell density signal peptides, sugar-containing cell envelope components, and secondary metabolites such as antimicrobial compounds.

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