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Genetic Method To Identify Regulons Controlled by Nonessential Elements: Isolation of a Gene Dependent on Alternate Transcription Factor σ^B of *Bacillus subtilis*

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We describe a general, in vivo method for identifying *Bacillus subtilis* genes controlled by specific, nonessential regulatory factors. We establish the use of this approach by identifying, isolating, and characterizing a gene dependent on σ^B, an alternate transcription factor which is found early in stationary phase but which is not essential for sporulation. The method relies on two features: (i) a plate transformation technique to introduce a null mutation into the regulatory gene of interest and (ii) random transcriptional fusions to a reporter gene to monitor gene expression in the presence and absence of a functional regulatory product. We applied this genetic approach to isolate genes comprising the σ^B regulon. We screened a random Tn*917lacZ* library for fusions that required an intact σ^B structural gene (*sigB*) for greatest expression, converting the library strains from wild-type *sigB*$^+$ to *sigB*Δ::*cat* directly on plates selective for chloramphenicol resistance. We isolated one such fusion, *csbA*::Tn*917lacZ* (*csb* for controlled by σ^B), which mapped between *hisA* and *degSU* on the *B. subtilis* chromosome. We cloned the region surrounding the insertion, identified the *csbA* reading frame containing the transposon, and found that this frame encoded a predicted 76-residue product which was extremely hydrophobic and highly basic. Primer extension and promoter activity experiments identified a σ^B-dependent promoter 83 bp upstream of the *csbA* coding sequence. A weaker, tandem, σ^A-like promoter was likewise identified 28 bp upstream of *csbA*. The *csbA* fusion was maximally expressed during early stationary phase in cells grown in Luria broth containing 5% glucose and 0.2% glutamine. This timing of expression and medium dependence were very similar to those for *ctc*, the only other recognized gene dependent on σ^B.

Although in nature bacteria commonly persist in a nongrowing state, scant information is available regarding the mechanisms regulating stationary-phase metabolism. With its wide range of stationary-phase activities and well-developed genetic system, *Bacillus subtilis* is an attractive model system for studying the mechanisms and signals that control and integrate stationary-phase events. It is well established that different sigma factors associate with the RNA polymerase core to alter promoter recognition specificity and control the sporulation process (see reference 47 for a recent review). However, sporulation-specific gene expression comprises only part of stationary-phase metabolism, which includes adaptation to the growth-limiting stress, development of genetic competence, induction of chemotaxis and motility, and synthesis of antibiotics and extracellular enzymes (46).

σ^B (formerly σ^{37}) is an alternate sigma factor of *B. subtilis* RNA polymerase which is maximally expressed in early stationary phase but which is not essential for sporulation (2, 12, 20). Indeed, a null mutation in the σ^B structural gene has no obvious phenotype, and the physiological role for σ^B has not yet been established. Other than the σ^B operon itself (24), *ctc* is the only recognized gene that requires σ^B for expression (20). The function of *ctc* remains unknown (19), but more is understood regarding *ctc* expression and the interactions of σ^B holoenzyme with the *ctc* promoter. *ctc* is maximally expressed early in stationary phase when cells are grown in rich medium containing high levels of glucose and glutamine, conditions which repress both the synthesis of tricarboxylic acid (TCA) cycle enzymes and the sporulation process (21). Expression of a *ctc-lacZ* fusion is abolished in a *sig*B null mutant (20), and mutational analysis has defined bases within the *ctc* promoter that are important for transcription initiation in vitro and in vivo (40, 48). DNA methylation protection experiments show that the regions containing these important bases are closely contacted by σ^B holoenzyme in vitro (32).

One way to increase our understanding of the role of σ^B in stationary-phase physiology is to identify additional genes controlled by σ^B in vivo. The DNA sequences and map locations of such σ^B-dependent genes could provide valuable clues regarding the function of the σ^B regulon. Assuming that genes wholly or partly dependent on σ^B would have decreased expression in its absence, we adopted an approach similar to the strategy of Jaacks et al. (23), who screened a random library of *B. subtilis* genes fused to the *Escherichia coli lacZ* gene carried by transposon Tn*917lacZ*. They identified fusions that had decreased β-galactosidase expression in the absence of the sporulation-essential transcriptional regulatory factor σ^H, using an inducible P_{spac} promoter to regulate expression of the σ^H structural gene *spo0H*. Here we report the identification of a σ^B-dependent Tn*917lacZ* fusion by adapting to the *B. subtilis* system the plate genetic techniques used for enteric bacteria (42). Instead of controlling the σ^B structural gene, *sigB*, by the inducible P_{spac} promoter used by Jaacks et al., we genetically inactivated *sigB* by means of plate transformation. Our approach should be widely applicable to regulatory genes like *sigB* which lie in operons with complex regulation (4, 24) and are thus not suitable candidates for control with the P_{spac} promoter.

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TABLE 1. *B. subtilis* strains

Strain	Genotype	Reference or construction[a]
PB2	*trpC2*	Wild-type Marburg strain (39)
PB10	*hisA1 thr-5 trpC2*	QB917 (9)
PB105	*sigBΔ1*	24
PB120	*amyE*::pDH32-2R *trpC2*	24
PB126	*trpC2* pLC4	24
PB153	*sigBΔ2*::*cat trpC2*	This study
PB154	*csbA*::Tn*917lacZ trpC2*	This study
PB155	*csbA*::Tn*917lacZ trpC2*	PB154 → PB2
PB156	*csbA*::Tn*917lacZ sigBΔ1 trpC2*	PB155 → PB105
PB157	*csbA*::Tn*917lacZ sigBΔ2*::*cat trpC2*	PB155 → PB153
PB185	*csbA*::Tn*917lacZ uvr*::pMT2 *trpC2*	pMT2 →PB155
PB187	*csbA*::Tn*917lacZ uvr*::pMT2 Δ(*degS degU*)::*aphA3 trpC2*	QB4238 → PB185
PB188	*trpC2* pLC400	24
PB189	*trpC2* pLC400T_{csbA}	pLC400T_{csbA} → PB2
PB190	*csbA*::pMT3 *trpC2*	pMT3 → PB2 (in-frame fusion)
PB191	*csbA*::pMT4 *trpC2*	PMT4 → PB2 (out of frame)
DP-I	*sigDΔ*::*cat trpC2*	18
QB4238	Δ(*degS degU*)::*aphA3 trpC2*	34

[a] Arrow indicates transformation, donor → recipient.

MATERIALS AND METHODS

Bacteria, phage, and genetic methods. We used *E. coli* DH5α (Bethesda Research Laboratories) as the host for all plasmid constructions. *B. subtilis* strains used are shown in Table 1. DNA comprising the Tn*917*::*lacZ* fusion library of Love et al. (30) was kindly provided by Ron Yasbin. For strain constructions, *B. subtilis* PB2 and its derivatives were recipients for natural transformations with linear and plasmid DNA (10). We made the *sigBΔ2*::*cat* insertion-deletion mutation in strain PB153 by removing adjacent *Hin*cII fragments totaling 453 bp from within the *sigB* coding region (24) and replacing them with a 1,037-bp *Ava*I-*Sau*3A fragment containing the *cat* gene from pCP115 (39). Plate transformations to inactivate the *sigB* gene were done as described by Hahn et al. (16). Transformation selections for drug-resistant *B. subtilis* strains were done on tryptose blood agar base (TBAB) plates (Difco Laboratories) containing either erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) or chloramphenicol (5 μg/ml). We performed transductional crosses to map the *csbA* locus, using phage PBS1 and the kit strains of Dedonder et al. (9) as previously described (39). Schaeffer's 2XSG sporulation medium was described by Leighton and Doi (27), and Luria broth (LB) medium was from Davis et al. (8).

DNA methods. All standard recombinant DNA methods were performed as previously described (5), and polymerase chain reaction experiments were done by standard protocols (22). DNA sequencing was done by the dideoxynucleotide chain termination method (44), using appropriate restriction fragments cloned into pUC19. We used Sequenase enzyme (U.S. Biochemical) and [α-^{35}S]dATP (Amersham) to label sequencing reactions primed on double-stranded DNA templates. Priming was done with custom oligonucleotide primers (UCD Protein Structures Laboratory) and standard forward and reverse primers (U.S. Biochemical). Reaction conditions were those described by U.S. Biochemical.

Use of plasmid integration to locate promoter activity. Because the *csbA*::Tn*917lacZ* disruption had no obvious phenotype other than β-galactosidase production, we used this phenotype in a modification of the plasmid integration method of Piggot et al. (38) to locate regions important for *csbA* promoter activity. We used the *csbA*::Tn*917lacZ* fusion-bearing strain PB155 as the transformation recipient for derivatives of the integration vector pCP115 (39) which carried fragments from the putative promoter region. These fragments were derived from the pSB26 plasmid used to isolate the upstream *csbA* region directly from the chromosome of PB155. pSB26 was treated with *Sal*I, which cut within the Tn*917* sequence, and then with various enzymes which cut within the upstream *csbA* region. The pCP115 derivative plasmids (see Fig. 3B) were pSB27, carrying the 2.5-kb *Eco*RI-*Sal*I fragment (5′ end at bp 0 in Fig. 3); pSB28, with the 1.51-kb *Sty*I-*Sal*I fragment (5′ nucleotide [nt] 221 in Fig. 4); pSB29, containing the 1.41-kb *Bsm*I-*Sal*I fragment (5′ nt 311); and pSB30, bearing the 1.36-kb *Dde*I-*Sal*I fragment (5′ nt 365). The *Eco*RI-*Sal*I fragment was subcloned into the *Eco*RI and *Sal*I sites of pCP115, while the other three fragments were subcloned into the *Eco*RV and *Sal*I sites.

Construction of transcriptional and translational fusions. We used the termination assay vector pLC400 previously described (24) to test the in vivo efficiency of the putative *csbA* terminator. pLC400 carries a 397-bp *Pst*I-*Aha*III fragment containing the σ^B-dependent promoter for the *sigB* operon subcloned between the *Eco*RI and *Bam*HI sites of pLC4 (40). We placed the 95-bp *Pst*I-*Pvu*II fragment containing the presumed *csbA* terminator (nt 593 to 685 in Fig. 4) between the 397-bp promoter-containing fragment and the *xylE* reporter gene of the plasmid. This construction was called pLC400T_{csbA}.

To determine whether the *csbA* reading frame was expressed in vivo, we made a single-copy translational fusion using the pJF751 integration vector (13). The 461-bp *Dra*I-*Pvu*I fragment from the *csbA* region (nt 3 to 466 in Fig. 4) was isolated from pSB26 and cloned between the *Eco*RI and *Sma*I sites of pJF751 by using blunt-end ligation. DNA sequencing across the junction between the *csbA* DNA and pJF751 *lacZ* confirmed the construction of an in-frame fusion. We called the pJF751 derivative carrying this fusion pMT3. We also found one fusion in which the *Pvu*I site was incorrectly filled, providing an out-of-frame negative control. This derivative was called pMT4. Upon integration into the *B. subtilis* chromosome at the *csbA* locus, the in-frame fusion placed *lacZ* under control of the transcriptional and translational signals of *csbA*.

Mapping of the 5′ ends of *csbA* mRNA. RNA was prepared essentially by the method of Igo and Losick (21), with modifications described previously (5). Primer extension reactions were done as previously described (24).

Enzyme assays. *B. subtilis* cultures were grown to late logarithmic phase and diluted 1:25 into fresh medium. Samples were then taken throughout logarithmic and stationary phases and prepared according to the reference cited for each assay. β-Galactosidase assays were done as described by Miller (31), using chloroform and sodium dodecyl sulfate to lyse the cells, and activity was expressed in Miller units, defined as $\Delta A_{420} \times 1{,}000$ per minute per milliliter per unit of optical density at 600 nm. For catechol 2,3-dioxygenase activities, cell extracts were prepared and the assays were done as described by Ray et al. (40). Protein concentrations for the catechol 2,3-dioxygenase assay were determined by

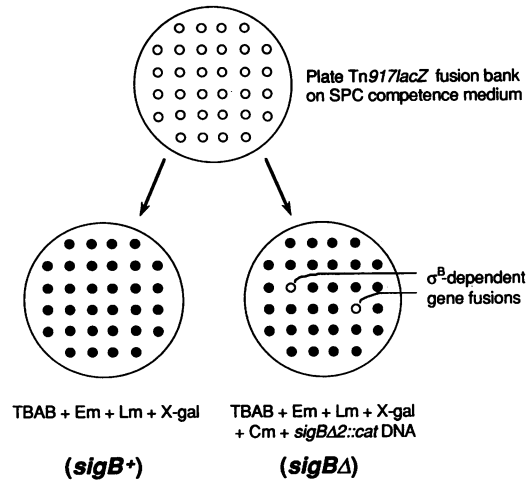

FIG. 1. Strategy to isolate *csb* genes. The transposon Tn*917lacZ* fusion bank of Love et al. (30) was transformed into strain PB2 with selection for the macrolide resistance marker of the transposon. Transformants bearing active fusions were plated on SPC competence medium (16). When competent, these colonies were replica plated onto two TBAB plates: a SigB plate containing erythromycin (Em), lincomycin (Lm), and X-Gal, and a SigBΔ plate containing erythromycin, lincomycin, X-Gal, chloramphenicol (Cm), and DNA from PB153 (*sigBΔ2::cat*). σ^B-dependent promoter fusions were less blue on the SigBΔ plate.

using the Bio-Rad protein assay according to the manufacturer's instructions.

Computer analysis. The statistical significance of protein sequence comparisons was evaluated with the FASTP and RDF programs of Lipman and Pearson (29), using the National Biomedical Research Foundation (NBRF) Protein Identification Resource data base and VAX computer. Sequences considered highly related have an optimized alignment score greater than 100 and a z value greater than 10 (29).

Nucleotide sequence accession number. The nucleotide sequence data shown in Fig. 4 have been assigned GenBank accession number M80473.

RESULTS

Identification of *lacZ* transcriptional fusions with diminished expression in the absence of a functional *sigB* gene product. One approach for determining the physiological role of σ^B is to characterize genes which are controlled by σ^B in vivo (*csb* genes). To this end, we devised a genetic approach that should be generally useful in defining the regulon controlled by any element which, like *sigB*, lies in an operon with elaborate regulation (4, 24). As shown in Fig. 1, the approach employs the plate transformation method developed by Hahn et al. (16), allowing the inactivation of the *sigB* structural gene directly on selective plates. We used this method to screen the Tn*917lacZ* insertion library of Love et al. (30) for σ^B-dependent fusions, defined as those fusions that had decreased β-galactosidase expression in the *sigBΔ2::cat* background compared with the expression in wild-type *sigB*$^+$ cells.

To begin the screen, we introduced 7,000 Tn*917lacZ* insertions from the library into the PB2 wild-type genetic background by transformation, plating the transformation mixes on TBAB plates containing the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) chromogenic indicator of β-galactosidase activity and the erythromycin-lincomycin drugs selective for the Tn*917lacZ* insertions. About 20% of these transformants were blue and were assumed to carry fusions in nonessential genes. We patched these blue colonies on competence medium plates (16) and allowed them to grow 12 h at 37°C.

We replica plated the competent, fusion-bearing colonies onto two TBAB plates (Fig. 1). The first plate contained X-Gal and erythromycin-lincomycin and allowed growth of the parent fusion strains with their wild-type *sigB*$^+$ gene. The second plate also contained X-Gal together with erythromycin-lincomycin but additionally was spread with 70 μg of chromosomal DNA isolated from the PB153 *sigBΔ2::cat* donor. This plate also contained chloramphenicol to select for the uptake of the donor DNA. The fusion-bearing cells which survived on the second plate were transformed to chloramphenicol resistance by taking up the *sigBΔ2::cat* DNA, thus inactivating *sigB*. Routinely, 50 to 60% of the colonies survived on the second plate. Those which did not transfer or transform efficiently were repatched on competence plates and retested.

Of approximately 1,400 fusion-bearing strains tested, we found two presumptive *csb*::Tn*917lacZ* colonies which had decreased β-galactosidase expression on the *sigBΔ2::cat* plate, changing from medium to light blue in the absence of *sigB*. To confirm σ^B-dependent fusion expression, we transformed the parental PB154a and PB154b *sigB*$^+$ strains carrying the presumptive *csb* fusions to *sigBΔ2::cat* a second time, only using the standard two-step method (10) rather than plate transformation. As a control, we also transformed PB154a and 154b with DNA from strain DP-I, which carries a *cat* disruption within the chemotaxis-flagellar transcription factor σ^D (18). Fusion expression measured on X-Gal plates was unaffected by the loss of σ^D, but expression decreased as before in the absence of a functional σ^B. We then isolated chromosomal DNA from the two resulting *sigBΔ2::cat csb*::Tn*917lacZ* transformants, backcrossed their resident Tn*917lacZ* fusions into the PB2 *sigB*$^+$ background by transformation, and found fusion expression restored to the level of the parental *sigB*$^+$ strains. This backcross also served to transfer the fusions into a nonmutagenized genetic background, and these *sigB*$^+$ *csb*::Tn*917lacZ* strains were used for all subsequent experiments. Since both harbored identical fusions (data not shown), we report the characterization of only one, PB155, and we refer to the locus defined by both fusions as *csbA*. Other than their dependence on σ^B, Tn*917lacZ* insertions at the *csbA* locus had no obvious growth or sporulation phenotype.

Characterization of *csbA-lacZ* fusion expression. We next tested to what extent *csbA* fusion expression was dependent on σ^B, and when in the growth cycle and under which conditions *csbA* was maximally expressed. As shown in Fig. 2A, *csbA* fusion expression in wild-type *sigB*$^+$ cells was low during vegetative growth in LB medium containing 5% glucose and 0.2% glutamine but increased dramatically in early stationary phase, reaching a maximum about 3 h after the end of vegetative growth. Fusion expression was greatly diminished but not abolished in the *sigBΔ2::cat* strain, indicating that *csbA* was strongly but not exclusively dependent on σ^B.

This timing of expression was very similar to that of *ctc*, the other recognized σ^B-dependent gene (20, 21). Additionally, *ctc* expression is medium dependent (21), with greater expression in a medium which does not support formation of TCA cycle enzymes or sporulation (LB supplemented with

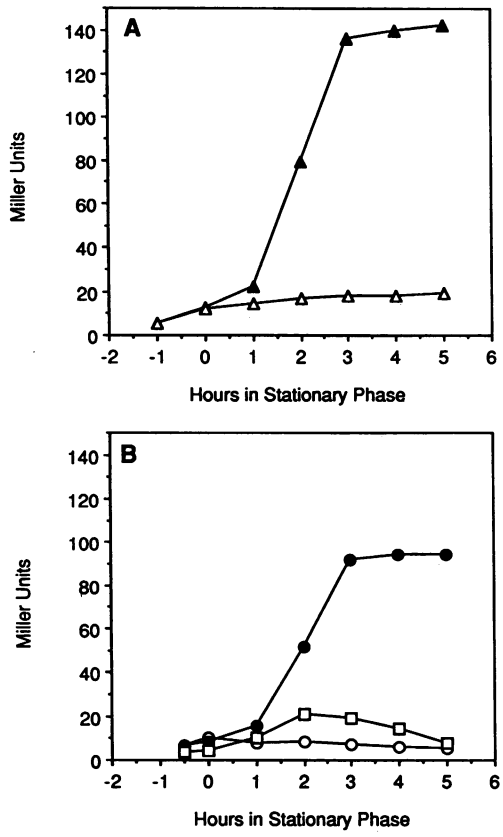

FIG. 2. σ^B dependence and medium dependence of *csbA* expression. (A) β-Galactosidase activity of the *csbA*::Tn*917lacZ* transcriptional fusion was assayed during growth in LB supplemented with 5% glucose and 0.2% glutamine. T_0 indicates the end of logarithmic growth. ▲, PB155 (*sigB*$^+$ *csbA*::Tn*917lacZ*); △, PB157 (*sigB*Δ*2*::*cat csbA*::Tn*917lacZ*). β-Galactosidase activity of the PB120 negative control (not shown) never exceeded 2 Miller units at T_3 and has not been subtracted from the curves shown. PB120 was a pDH32 integrant strain carrying the *sigB* operon promoter in the wrong orientation (24). (B) β-Galactosidase activity of the PB155 transcriptional fusion (*sigB*$^+$ *csbA*::Tn*917lacZ*) was assayed during growth in LB supplemented with 5% glucose and 0.2% glutamine (●), in unsupplemented LB (○), and in 2XSG sporulation medium (□).

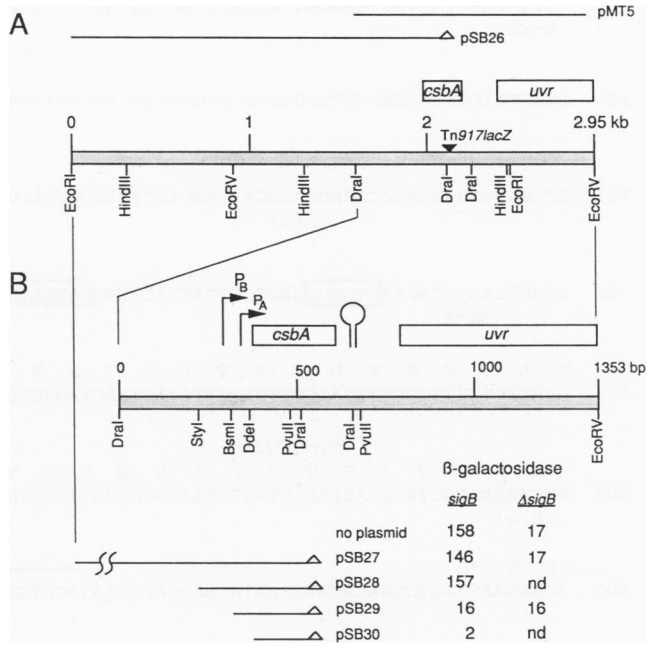

FIG. 3. (A) Physical map of the *csbA* region. The map is derived from restriction analysis of the *B. subtilis* genomic fragments carried by pSB26 and pMT5 (top). pSB26 is a pLTV1 derivative isolated by integrating pLTV1 into the Tn*917lacZ* element of strain PB155 as described in the text. The open triangle indicates the Tn*917lacZ* sequences carried by pSB26. pMT5 is a pCP115 derivative isolated by integrating pCP115 carrying the 500-bp *Dra*I fragment into the *csbA* region of wild-type strain PB2 as described in the text. Open boxes above the map show the locations of the *csbA* and *uvr* reading frames, determined by DNA sequence analysis; direction of transcription is from left to right. The black triangle indicates the site of the Tn*917lacZ* insertion within *csbA*. (B) Locations of the two open reading frames *csbA* and *uvr* (open boxes above the restriction map), the tandem *csbA* promoters (arrows labeled P_B and P_A), and the predicted transcription terminator (stem-loop following *csbA*). The horizontal lines beneath the map show the fragments subcloned from pSB26 into the integration vector pCP115 to locate *csbA* promoter activity. The open triangle at the right of each fragment represents 374 bp from the left end of Tn*917lacZ*. The table indicates the β-galactosidase activity (in Miller units) resulting from integration of each plasmid into the PB155 (*sigB*$^+$ *csbA*::Tn*917lacZ*) and PB156 (*sigB*Δ*1 csbA*::Tn*917lacZ*) recipients. Cells grown in LB medium containing 5% glucose and 0.2% glutamine were assayed for activity 3 h after the end of exponential growth. β-Galactosidase activity in the PB105 negative control was 0.1 Miller units (not shown). nd, not determined.

5% glucose and 0.2% glutamine) compared with a medium in which formation of TCA cycle enzymes and sporulation are favored (nutrient sporulation medium). As shown in Fig. 2B, our *csbA* fusion showed a similar, marked medium dependence. Thus, in terms of both timing of expression and medium dependence, *csbA* closely resembled *ctc*.

Cloning and nucleotide sequence of the *csbA* region. We used standard plasmid methods (53) to isolate the region immediately upstream of the Tn*917lacZ* insertion. We transformed the *csbA* fusion strain PB155 with the integrative plasmid pLTV1 (53). This plasmid placed a ColE1 origin of replication, a *bla* gene, and a useful polylinker region within the Tn*917lacZ* chromosomal locus. Chromosomal DNA was extracted from the resultant integrant, cut with *Eco*RI to cleave both within the integrated plasmid and upstream of the Tn*917lacZ* insertion, then treated with DNA ligase to promote self-ligation of the fragment. The ligation mixture was transformed into *E. coli* with selection for the ampicillin drug resistance of the expected replicon. Several identical plasmids were recovered which bore the hybrid plasmid-transposon sequences together with an additional 2.1 kb of *B. subtilis* DNA. This *B. subtilis* DNA extended from the site of transposon integration to an *Eco*RI site in the chromosomal region promoter proximal to *csbA* (Fig. 3A). The plasmid bearing this upstream *csbA* region was called pSB26.

To obtain DNA extending downstream from the isolated chromosomal fragment, a 500-bp *Dra*I fragment internal to the upstream fragment was subcloned into the blunted *Sal*I site of the pCP115 integration plasmid. The resulting pMT1 plasmid was integrated into a wild-type PB2 strain containing no Tn*917lacZ* element. The chromosomal DNA was extracted, cut with *Eco*RV, ligated, and transformed as before into *E. coli*. We found several identical plasmids which carried DNA extending from the *Dra*I fragment to an

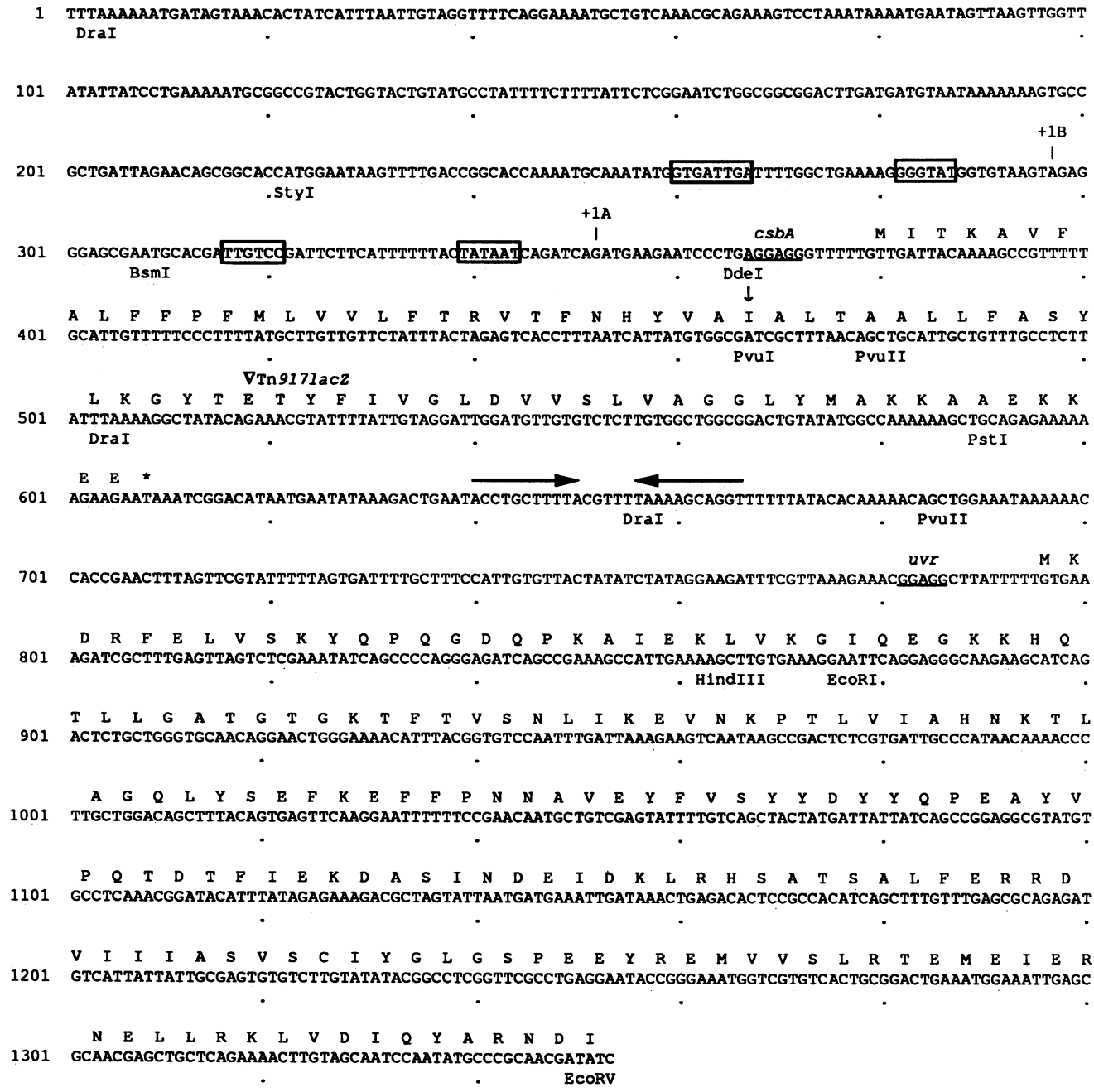

FIG. 4. Nucleotide sequence of the *csbA* region. Nucleotides are numbered from the 5′ end of the nontranscribed strand with intervals of 20 bp marked by dots. The predicted amino acid sequences for the *csbA* and *uvr* products are given in single-letter code above the DNA sequence, and the name of each frame is given above the sequence for the probable ribosomal binding site (underlined). The proposed −35 and −10 recognition sequences for the σ^B-dependent *csbA* promoter (start site +1B at nt 297) are boxed. The proposed −35 and −10 recognition sequences for the σ^A-like *csbA* promoter (start site +1A at nt 351 to 352) are also boxed, and the inverted repeat for the proposed *csbA* terminator structure (nt 640 to 671) is represented by converging arrows. The site of translational fusion between *csbA* and codon 8 of *lacZ* carried by the pJF751 vector is indicated (↓) at nt 467, and the site of Tn*917lacZ* insertion into the *csbA* coding region is indicated (∇) following nt 518.

*Eco*RV site 1.35 kb downstream (Fig. 3A). The plasmid bearing this downstream *csbA* region was called pMT5. The restriction maps of the upstream and downstream fragments overlapped and were consistent with the chromosomal restriction map determined by Southern analysis (not shown).

We determined both strands of the DNA sequence from the upstream *Dra*I site to the *Eco*RV site at the end of the cloned fragment. As shown in Fig. 4, the site of Tn*917lacZ* integration was within a 76-residue reading frame, which began with a potential TTG initiation codon at nt 400 to 402 and extended to a TAA termination codon at nt 608 to 610. This reading frame was preceded by a potential ribosome

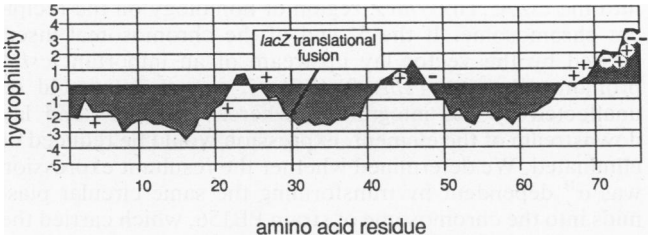

FIG. 5. Hydrophilic profile of the predicted *csbA* gene product. Hydrophilicity was calculated for each amino acid residue by the method of Kyte and Doolittle (26), using a window of seven residues. The amino-terminal methionine of CsbA is residue 1. The locations of charged amino acids are shown by plus signs for arginine, histidine, and lysine and by minus signs for aspartate and glutamate. This plot reveals three potential membrane-spanning segments of 22, 17, and 18 residues, at positions 1 to 22, 25 to 41, and 48 to 65, respectively.

binding site and appeared to define a monocistronic transcription unit closely bounded by promoter and terminator elements (17, 41). Because we found that these elements had promoter and terminator function in vivo (see below), we presume that the 76-residue reading frame was the entire *csbA* coding region. No minor DNA rearrangements or abnormalities were introduced during the plasmid recovery of the Tn*917lacZ* flanking regions, as shown by directly isolating the region between nt 236 and 909 from wild-type *B. subtilis* chromosomal DNA by polymerase chain reaction. The size of the polymerase chain reaction DNA fragment was identical to that isolated by plasmid recovery (not shown).

The predicted 76-residue *csbA* product had no statistically significant similarity to any protein in the NBRF Protein Identification Resource data base (release 27.0). However, the predicted sequence bore three noteworthy features: it was small (8.4 kDa), highly basic with a calculated pI of 9.0, yet very hydrophobic. These features are in marked contrast to the predicted product of *ctc*, a 204-residue, acidic, soluble protein (19). For CsbA, the hydrophilicity profile (26) and charge distribution (49) suggested that it might be an integral membrane protein containing three membrane-spanning regions, with the amino terminus on the outside of the cell and the charged carboxy terminus on the inside (Fig. 5). The suggested location and topology of the *csbA* product have yet to be tested experimentally. However, the perception that CsbA was a membrane protein was consistent with results from the following experiment.

To determine whether *csbA* message was translated in vivo, we made the pMT3 translational (gene) fusion, creating an in-frame fusion between codon 29 of *csbA* and codon 8 of *lacZ* borne by the pJF751 integration vector. As was the case with the Tn*917lacZ* transcriptional fusion (Fig. 2A), the translational fusion was maximally expressed in early stationary phase in cells grown in LB medium containing glucose and glutamine (not shown). Although the level of *csbA* translational fusion expression (1.5 Miller units at T_3) was considerably higher than the negative control (0.15 Miller units for the out-of-frame *csbA* translational fusion carried by pMT4), it was surprisingly low compared with the level of the transcriptional fusion (100 to 140 Miller units at T_3 [Fig. 2]). These results suggested that *csbA* was indeed translated in vivo. One explanation for the unexpectedly low β-galactosidase activity is that *csbA* was translated with poor efficiency. However, the low activity could also reflect a membrane location for the CsbA–β-galactosidase fusion protein, preventing formation of active β-galactosidase tetramers (36).

A downstream *uvr* reading frame. Downstream from the potential *csbA* terminator sequence lay another, incomplete open reading frame encoding a hypothetical product 60% identical to the *E. coli* UvrB protein. We designate this reading frame *uvr* in Fig 4. A FASTP alignment of our partial *uvr* product with the 185 amino-terminal residues of *E. coli* UvrB confirmed a highly significant similarity, with an optimized alignment score of 631 and a *z* value of 67. *E. coli* UvrB comprises part of the UvrABC excision repair complex, which is induced in response to DNA damage (43). Because a disruption in the *uvr* frame caused increased sensitivity to the DNA damage-inducing agent mitomycin (data not shown), this frame probably encoded a *B. subtilis* UvrB homolog. Cheo et al. (7) independently isolated and characterized the *B. subtilis dinA* region, which contains a DNA damage-inducible gene equivalent to our *uvr* reading frame. Their sequence extends from nt 314 to 966 in Fig. 4 and is identical to ours in the region of overlap.

Because *uvr* encoded a known product, it was important to determine whether *csbA* and *uvr* might comprise an operon. A possible factor-independent terminator sequence (41) lay within the *csbA-uvr* intercistronic region (Fig. 4), and the results of the terminator probe experiments presented below indicated that this intercistronic region functioned as an efficient terminator in vivo. Consistent with this result, Cheo et al. (7) located the DNA damage-inducible promoter for the *uvr/dinA* frame between nt 686 and 805 in Fig. 4, downstream from the proposed *csbA* terminator and within the *csbA-uvr* intercistronic region. Together, our data and those of Cheo et al. (7) strongly suggest that *csbA* and *uvr* form independent transcriptional units.

***csbA* promoter and terminator elements.** We used primer extension and promoter activity experiments to identify tandem promoters for *csbA*: a strong, σ^B-dependent promoter and an additional, weaker, σ^B-independent promoter. The primer extension experiment shown in Fig. 6A identified two equally strong σ^B-dependent signals, one at nt 297 and the other at nt 415. A second experiment using a different 17-nt primer found the identical 5′ ends (not shown). Notably, immediately upstream from nt 297 were two regions which closely matched in sequence and spacing the proposed −35 and −10 regions of the σ^B-dependent *ctc* and *sigB* promoters (Fig. 4). We saw no obvious promoter sequence upstream from nt 415. Several less abundant signals were apparent in the longer exposure of the same experiment shown in Fig. 6B. All were downstream from the strong σ^B-dependent signal at nt 297, and all but one were themselves σ^B dependent. The weak signal at nt 351 to 352 was unaffected in the *sigBΔ2::cat* strain and thus appeared to be a σ^B-independent 5′ end. Immediately upstream from nt 351 to 352 were sequences closely resembling the consensus for the major sigma factors of *E. coli* and *B. subtilis* (Fig. 4).

To determine whether these 5′ ends correlated with the presence of in vivo promoter activity, we adapted the standard plasmid integration method of mapping 5′ boundaries of promoter elements (38) to the transposon system. We had no obvious phenotype other than Tn*917lacZ* expression to determine the 5′ elements required for *csbA* promoter activity. We therefore subcloned into the pCP115 integration vector appropriate fragments from plasmid pSB26, which contained hybrid plasmid-transposon sequences and upstream chromosomal DNA from the *csbA* region. The resultant integrative plasmids all carried the same 3′ end anchored

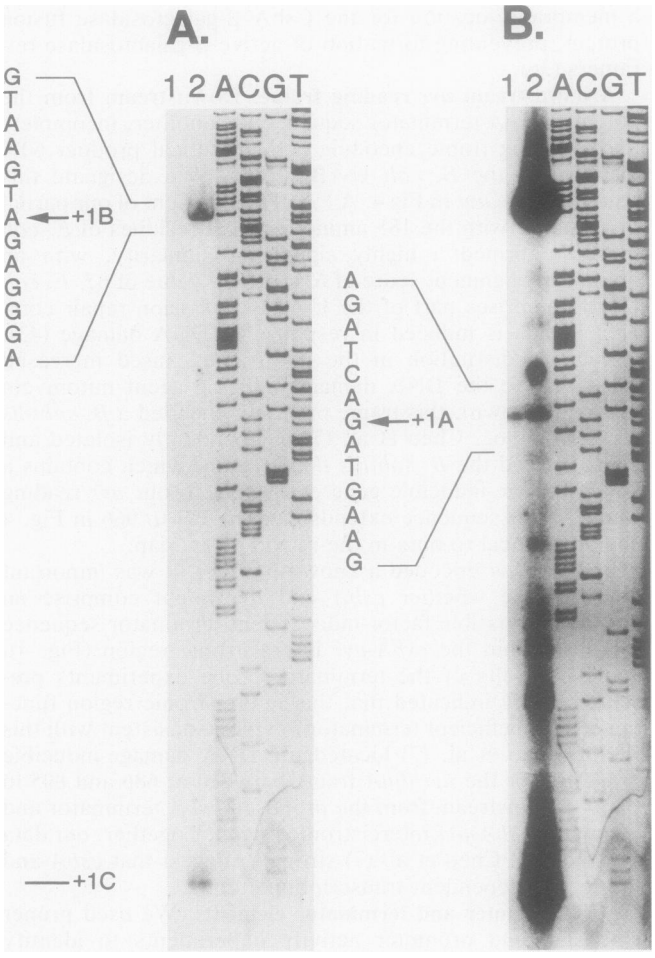

FIG. 6. Mapping the 5′ ends of *csbA* operon message by primer extension. Shown are a short exposure (A) and a long exposure (B) of the same primer extension experiment. The 17-nt synthetic primer was complementary to nt 464 to 485, a sequence 172 bp downstream from the σ^B-dependent transcription start site (+1 at nt 297 in Fig. 4). Lane 1, end-labeled primer annealed with *B. subtilis* RNA isolated from σ^B null strain PB157 (*sigBΔ2::cat csbA*::Tn*917lacZ*) at T_3 and extended with reverse transcriptase; lane 2, as lane 1, but using RNA isolated from PB155 (*sigB*$^+$ *csbA*::Tn*917lacZ*). A sequencing ladder using the same primer is shown. The letters A, C, G, and T above the lanes indicate which dideoxynucleotide was used to terminate the reaction. The sequence indicated is the nontranscribed strand and is the complement of the sequence that can be read from the sequencing ladder. In panel A, the probable 5′ end of the major σ^B-dependent *csbA* message is indicated by +1B at adenine 297, because the primer-extended product was phosphorylated and ran slightly faster than the unphosphorylated ladder fragments. The prominent σ^B-dependent 5′ end indicated by +1C at nt 415 does not correspond to an in vivo activity and is thus likely an extension artifact or a processed form of the +1B message (see text). In panel B, the probable 5′ end of the σ^B-independent *csbA* message is indicated by +1A at adenine 351 or guanine 352. Several minor σ^B-dependent 5′ ends are also visible; like the prominent 5′ end at nt 415, these are likely artifactual.

at the *Sal*I site within the Tn*917lacZ* gene but differed by the location of the 5′ end within the upstream *csbA* region (Fig. 3B). We then transformed the *csbA*::Tn*917lacZ*-bearing recipient PB155 with the various circular plasmids, selecting for chloramphenicol resistance to force plasmid integration into the *csbA*::Tn*917lacZ* region of homology on the recipient chromosome. If the 5′ end of the chromosomal insert carried by the vector lay upstream of an important *csbA* promoter element, *csbA*::Tn*917lacZ* expression would be unaffected by the integration, whereas if the 5′ end lay downstream of the element, expression would be reduced or eliminated. We determined whether the resultant expression was σ^B dependent by transforming the same circular plasmids into the chromosome of strain PB156, which carried the *csbA*::Tn*917lacZ* fusion in a *sigBΔ1* background. *sigBΔ1* is an in-frame, 453-bp deletion within *sigB* which fuses codon 44 to codon 196 and contains no drug resistance marker (24).

As shown in Fig. 3B, integration via the 2.5-kb *Eco*RI-*Sal*I fragment carrying the entire upstream *csbA* region had no effect on production of either σ^B-dependent or σ^B-independent *csbA*::Tn*917lacZ* fusion activity. Thus, this fragment had the main promoter elements which regulated *csbA* expression, and the bulk of expression directed by this fragment was σ^B dependent. In contrast, integration via the shortest fragment (extending to the *Dde*I site at nt 365 in Fig. 4) nearly abolished *lacZ* expression. Integration of the fragment extending to the *Bsm*I site at nt 311 partially restored *lacZ* expression, and this expression was both σ^B independent and essentially the same as the σ^B-independent activity directed by the entire 2.5-kb *Eco*RI-*Sal*I fragment (Fig. 3B). Integration via the fragment extending to the *Sty*I site at nt 221 fully restored *lacZ* expression.

We deduce from these fusion results that a major, σ^B-dependent promoter activity lay between nt 221 and 311 and that a second, weaker, σ^B-independent promoter activity lay between nt 311 and 365. Thus, these two regions each contained an in vivo promoter activity that correlated both with a 5′ end of *csbA* message (Fig. 6) and with the presence of appropriately spaced −35 and −10 sequences (Fig. 4). We have therefore labeled nt 297 as +1B to indicate the transcriptional initiation site for a σ^B-dependent promoter, P_B, and nt 351 to 352 as +1A to indicate a the initiation site for a σ^B-independent promoter, P_A (Fig. 3 and 4). Because the 5′ end of *csbA* message mapping to nt 415 did not match any in vivo promoter activity but was nonetheless σ^B dependent, this 5′ end probably resulted either from processing of the transcript initiating at P_B or from premature termination of the primer extension reaction.

To determine whether transcription initiating at the tandem *csbA* promoters might extend into the downstream *uvr* reading frame, we tested the region following the *csbA* coding sequence for terminator activity in vivo, using the *B. subtilis* multicopy terminator-probe plasmid pLC400 (24). Comparison of *xylE* expression in the presence and absence of a terminator element cloned between the *sigB* operon promoter and the *xylE* reporter gene carried by pLC400 affords a simple measure of terminator activity in vivo. As shown in Table 2, the presence of the *csbA* terminator sequence (nt 640 to 671 in Fig. 4) reduced *xylE* expression more than 90%. We assume that this reduction reflected efficient in vivo termination of transcription initiating at the *sigB* operon promoter carried by pLC400, and that at its normal chromosomal location the *csbA* terminator was correspondingly efficient in preventing transcription into the downstream *uvr* gene.

Mapping the *csbA* locus on the *B. subtilis* chromosome. We also mapped the chromosomal locus of *csbA* and determined that this gene had not been previously identified on the basis of another phenotype. We initially mapped the *csbA* locus by PBS1 transductional crosses, using the PB155 strain bearing the *csbA*::Tn*917lacZ* insertion as the donor and the De-

TABLE 2. Catechol dioxygenase activity in pLC4 transcriptional fusions[a]

Plasmid	Construction	Catechol dioxygenase sp act (mU/mg of protein [%])	
		T_{1-2}	T_{2-3}
pLC400	*sigB* promoter	26.8 (100)	38.0 (100)
pLC400T_{csbA}	*sigB* promoter + *csbA* terminator	4.3 (10)	4.1 (4)
pLC4	Vector alone	1.9 (0)	2.6 (0)

[a] Specific activity of *B. subtilis* PB2 cells containing pLC4 derivatives and grown in LB medium supplemented with 5% glucose and 0.2% glutamine. Fragments containing the indicated elements were inserted upstream of the promoterless *xylE* gene of the multicopy vector pLC4. T_{1-2} and T_{2-3} refer to hours in stationary phase. Percent activity was calculated by setting the activity from the *sigB* promoter fragment at 100% and the background activity from the pLC4 vector alone at 0%.

donder kit strains (9) as recipients. The erythromycin-lincomycin resistance of the donor was linked to *hisA* (60 to 70% cotransduction) and *ctrA* (10%), placing *csbA* at about 302° on the *B. subtilis* genetic map (37).

A three-factor transductional cross (Table 3) determined marker order in this region and placed *csbA* between *hisA* and *degSU*. The PB187 donor carried *csbA*::Tn*917lacZ*, a derivative of the pCP115 vector (pMT2) integrated into the *uvr* reading frame to provide a *cat* marker, and the Δ(*degS degU*)::*aphA3* marker from strain QB4238 (34). We confirmed the expected physical structure of the *csbA* region in the PB187 donor by polymerase chain reaction experiments using appropriate oligonucleotide primers (not shown). The results of this cross are consistent with the marker order *degSU-csbA-hisA*.

A three-factor transformational cross between the same PB187 donor strain and a PB2 wild-type recipient mapped the order of the *csbA* and *uvr* reading frames relative to *degSU*. As shown in Table 4, no clear order was implied by the kanamycin [Δ(*degS degU*)::*aphA3*] selection, but the chloramphenicol (*uvr*::pMT2) selection gave the unequivocal order *degSU-csbA-uvr*. This order indicated that *csbA* and *uvr* were transcribed in a counterclockwise direction on the standard genetic map (37), the same direction as chromosomal replication. The genetic map derived from the results of Tables 3 and 4 is shown in Fig. 7.

TABLE 3. Three-factor transductional cross to map *csbA* relative to *hisA* and *degSU*

Selection[a]	Recipient class[b]			No.	Order implied
	His	CsbA	DegSU		
His^+	D	D	D	37	*hisA-csbA-degSU*
	D	D	R	32	
	D	R	D	0	
	D	R	R	59	
Km^r	D	D	D	60	*hisA-csbA-degSU*
	R	D	D	21	
	D	R	D	1	
	R	R	D	29	

[a] His^+, histidine prototrophy; Km^r, resistance to 5 μg of kanamycin per ml, encoded by Δ*degSU*::*aphA3*.

[b] D, donor (PB187 [*csbA*::Tn*917lacZ uvr*::pMT2 Δ*degSU*::*aphA3 trpC2*]) phenotype; and R, recipient (PB10 [*hisA1 thr-5 trpC2*]) phenotype.

TABLE 4. Three-factor transformational cross to map *csbA* relative to *uvr* and *degSU*

Selection[a]	Recipient class[b]			No.	Order implied
	Uvr	CsbA	DegSU		
Cm^r	D	D	D	80	*uvr-csbA-degSU*
	D	D	R	399	
	D	R	D	2	
	D	R	R	47	
Em^r	D	D	D	74	*uvr-csbA-degSU*
	D	D	R	356	
	R	D	R	40	
	R	D	D	9	
Km^r	D	D	D	38	(*uvr-csbA*)-*degSU*
	R	D	D	4	
	D	R	D	3	
	R	R	D	534	

[a] Cm^r, resistance to 5 μg of chloramphenicol per ml, encoded by *uvr*::pMT2; Em^r, resistance to 0.5 μg of erythromycin per ml plus 12.5 μg of lincomycin per ml, encoded by *csbA*::Tn*917lacZ*; Km^r, resistance to 5 μg of kanamycin per ml, encoded by Δ*degSU*::*aphA3*.

[b] D, donor (PB187 [*csbA*::Tn*917lacZ uvr*::pMT2 Δ*degSU*::*aphA3 trpC2*]) phenotype; and R, recipient (PB2 *trpC2*]) phenotype.

DISCUSSION

Important advances in bacterial physiology have resulted from development of strategies that do not require prior knowledge of gene function to identify the component parts of a regulon or stimulon. Earlier work in enteric bacteria used differential growth conditions, such as heat shock (28, 52) or phosphate starvation (50), to effect global changes in gene expression. These changes were monitored by two methods: using two-dimensional gel analysis of whole-cell proteins (35) or by means of a reporter gene randomly fused to responsive promoter elements in the chromosome (6, 25). Similar methods have been applied to the *B. subtilis* system (1, 30). Jaacks et al. (23) extended the utility of these global approaches by developing a general genetic method to

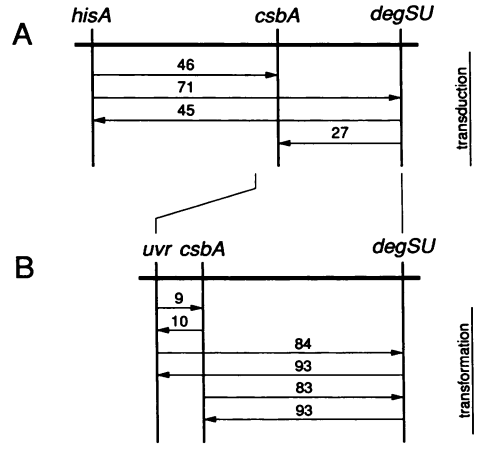

FIG. 7. Genetic map of the *csbA* region. (A) Transductional map drawn from the experiment shown in Table 3. Genetic distances are given as 100 − percent cotransduction, with the arrowtails indicating the selected marker. (B) Transformational map drawn from the experiment shown in Table 4. Genetic distances are given as 100 − percent cotransformation, with the arrowtails indicating the selected marker. The light lines show the location of map B on map A.

modulate expression of any nonessential regulatory gene of interest. Here we describe a second genetic approach which offers some advantages over the method of Jaacks et al., and we demonstrate the use of this approach to isolate and characterize a new *B. subtilis* gene, *csbA*, whose expression is dependent on the alternate transcription factor σ^B.

We first screened for potential *csb* (controlled by σ^B) genes by using the regulated promoter system developed by Jaacks et al. (23), placing the inducible P_{spac} promoter upstream from the σ^B structural gene, *sigB*. However, in reconstruction experiments using the σ^B-dependent *ctc-lacZ* translational fusion (21) as a sample *csb* gene, *ctc* expression was not regulated by the P_{spac} construction as expected. Rather, σ^B activity either could not be induced or could not be abolished, depending on the location of the P_{spac} promoter upstream of *sigB* (data not shown). Consistent with these results, Schmidt et al. (45) also reported different levels of activity of the sporulation-essential σ^F, depending on the location of the P_{spac} promoter within the *spoIIA* (σ^F) operon.

The inability of P_{spac} to simply mimic the in vivo regulation of σ^B and σ^F activity likely reflects the more complicated organization of the *sigB* and *spoIIA* operons (14, 24) compared with the monocistronic *spo0H* used by Jaacks et al. (11, 23, 51). Both the *sigB* and *spoIIA* operons contain additional genes whose products regulate their cognate sigma factors by similar mechanisms (4, 45). Thus, the inappropriate expression of σ^B in the absence of its regulatory proteins does not reflect the normal in vivo context. Additionally, such inappropriate expression could plausibly foster a change in σ^B promoter specificity in vivo, leading to identification of genes that are controlled by σ^B only under the artificial conditions used for the screening.

We therefore devised a genetic approach that should be generally useful in removing a functional regulatory gene of interest from within operons. This method should prove more practical in complicated systems, such as the σ^B and σ^F operons, than the regulated P_{spac} promoter developed by Jaacks et al. Our method relies on genetic inactivation of the regulatory gene of interest directly on plates that permit screening for dependence on the regulatory gene product. Although we have chosen to inactivate the positive regulatory element σ^B, which should decrease expression of dependent genes, the method should work equally well for negative regulatory elements. The inactivation strategy can be applied to any bacterial system which has (i) a means of directly transforming cells on plates to genetically inactivate the regulatory element of interest and (ii) a reporter gene that can be randomly fused to responsive target genes. The general method of genetic inactivation of regulatory elements is not limited to transformable bacteria. Indeed, Gibson et al. (15) used genetic inactivation by plate transduction to identify genes controlled by OmpR of *Salmonella typhimurium*.

Our method of transformational inactivation identifies fusions in nonessential genes which are either directly or indirectly dependent on σ^B. The following evidence suggests that σ^B holoenzyme directly transcribes the newly isolated *csbA* gene in vivo. First, primer extension experiments mapped the more abundant 5′ end of *csbA* message to nt 297, 83 nt upstream from the putative translational initiation codon (Fig. 4 and 6), and experiments using the integration vector pCP115 confirmed that sequences between nt 221 and 311 were necessary for full promoter activity in vivo (Fig. 3B). Together, these results defined the region between nt 221 and 311 as a major promoter for *csbA*. Second, transcription from this promoter, measured by primer extension experiments, was below the level of detection in a *sigB* null mutant (Fig. 6A). Thus, this promoter was σ^B dependent. Third, the sequence of the presumptive −10 region of this promoter was identical to the −10 region of the σ^B-dependent *ctc* and *sigB* promoters, while the presumptive −35 region matched in four of eight bases (Fig. 8). Conservation of bases was exact at the positions critical for σ^B-dependent transcription of *ctc*. The simplest explanation for these results is that transcription initiating at nt 297 was directed by σ^B-containing holoenzyme.

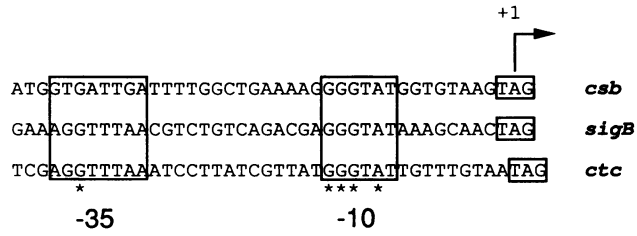

FIG. 8. Comparison of known σ^B-dependent promoter sequences. The site of transcription initation, indicated by +1, is the adenosine for the *csbA* (Fig. 6) and *sigB* (24) promoters, and either the adenosine or guanosine for *ctc* (33). The proposed −35 and −10 regions of each promoter are boxed, and bases important for *ctc* promoter function (40, 48) are indicated by asterisks.

β-Galactosidase assays indicated that *csbA* was largely but not solely dependent on σ^B for expression (Fig. 2A). In accord with this observation, we detected a less abundant 5′ end of *csbA* message at nt 351 or 352, downstream from the start of σ^B-dependent transcription initiation and 28 to 29 nt upstream from the putative translational initiation codon (Fig. 4 and 6). Experiments using the integration vector pCP115 confirmed that sequences between nt 311 and 365 were required for residual promoter activity in vivo (Fig. 3B). This region contained a perfect −10 region and a less conserved −35 region resembling promoters recognized by the major, σ^A factor of *B. subtilis* RNA polymerase (17).

These tandem promoters suggested that *csbA* expression might respond to two sets of signals. Consistent with this notion, *csbA* expression was greatly enhanced in LB medium containing glucose and glutamine, and this enhancement was largely absent in a σ^B null mutant (Fig. 2A). We infer from these results that the σ^A-like promoter provided a basal level of *csbA* expression, whereas the σ^B dependent promoter dramatically increased response to glucose and glutamine. *csbA* is not unique in its response to glucose and glutamine. Igo and Losick (21) noted similar regulation of *ctc*, the only σ^B-dependent gene characterized before our work.

Two additional lines of evidence suggest that the effect of glucose and glutamine might be mediated solely through the activity of σ^B holoenzyme. First, Igo and Losick found that the glucose and glutamine response of *ctc* was directed by a region only slightly larger than the minimal σ^B promoter (21). Thus, if another regulatory system were responsible for this effect, it must act through the same region as does σ^B. Second, other than the proposed −35 and −10 recognition sequences for σ^B holoenzyme, we find no sequence similarity between the *csbA* and *ctc* promoter regions which might specify a *cis*-acting element mediating the response. Thus, one physiological role of σ^B may be to increase stationary phase expression of certain genes under conditions in which

formation of TCA cycle enzymes and the sporulation process are inhibited.

If many *csb* genes are subject to dual control as is *csbA*, our initial procedure for isolating *csb* fusions would plausibly result in the relatively low recovery that we found: only 2 identical *csb* fusions out of 1,400 screened. We used plate conditions (TBAB plates rather than LB supplemented with glucose and glutamine), which in hindsight might not have allowed maximal σ^B-dependent expression. Hence, the only σ^B-dependent gene that we detected had very low background expression in the absence of σ^B, and other *csb* genes for which the contribution of a second, σ^B-independent promoter was more substantial would have escaped detection. This expectation has been borne out by a significantly enhanced frequency of fusion recovery in our recent work, in which we increased σ^B-dependent gene expression by initially screening in a *socB* genetic background (3). *socB* is a frameshift mutation in *orfX*, a gene which encodes a negative regulator of *sigB* expression or σ^B activity and which lies immediately downstream of *sigB* (12, 20, 24).

Both the P_{spac} and inactivation methods have been limited to uncovering nonessential target genes, because both have relied on screening existing transposon fusion libraries. The use of newly constructed merodiploid libraries (53) should avoid this constraint. If any essential genes comprise part of the σ^B regulon, these genes must also possess a σ^B-independent mode of transcription, because σ^B null mutants are viable and sporulate normally (2, 12, 20). As a precedent for such dual control, the nonessential *csbA* gene that we describe here has tandem promoters, one dependent on σ^B and the other independent.

Although we have successfully tested a new method to identify genes controlled by nonessential regulatory elements such as σ^B, we cannot yet identify the function of the *csbA* gene isolated or the physiological role of the σ^B holoenzyme apparently responsible for its expression. It is striking that both known σ^B-dependent genes, *csbA* and *ctc*, are maximally expressed in early stationary phase in medium containing glucose and glutamine. We wish to know whether other σ^B-dependent genes have similar regulation and whether the identification of additional genes in the σ^B regulon will clarify the role of this alternate sigma factor in controlling stationary-phase metabolism.

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