Two genes from *Bacillus subtilis* under the sole control of the general stress transcription factor σ^B

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The general stress response of *Bacillus subtilis* is triggered by a variety of environmental and metabolic stresses which activate the σ^B transcription factor. Among the more than 100 genes controlled by σ^B (the *csb* genes), the functions identified thus far include resistance to oxidative stress, resistance to protein denaturation and resistance to osmotic stress. To understand the breadth of functions in which *csb* genes participate, the transcriptional organization and predicted products of two such genes previously identified in a screen for σ^B -dependent *lacZ* fusions were analysed. The *csb-22::Tn917/lacZ* and *csb-34::Tn917/lacZ* fusions are unusual among *csb* genes in that their expression appears to be completely dependent upon σ^B . By plasmid-integration experiments, fusion analyses and site-directed mutagenesis, stress-inducible, σ^B -dependent promoters for both these fusions were identified. The *csb-34* fusion marked an ORF (*yxcC* or *csbC*) which by sequence analysis lay in a monocistronic transcriptional unit. This ORF encoded a predicted 461-residue product which had high identity with Class I sugar transporters of the major facilitator superfamily. It was speculated that the *csbC* product could serve either a nutritional or an osmotic protection function. In contrast, the *csb-22* fusion identified an ORF (*ywmG* or *csbD*) which appeared to be the second gene of a two-gene operon. This ORF encoded a predicted 62-residue product which resembled a small *Escherichia coli* protein of unknown function. The σ^B -dependent promoter lay immediately upstream from *csbD* and appeared to be an internal promoter for the operon.

Keywords: general stress response, sigma factor, major facilitator superfamily

INTRODUCTION

In response to environmental or energy stress, the σ^B transcription factor of *Bacillus subtilis* controls the expression of over 100 general stress genes whose products are thought to enhance survival of growth-arrested cells (for a review see Hecker & Völker, 1998). Thus far about a third of these genes have been identified, primarily by two-dimensional gel analysis (Bernhardt *et al.*, 1997; Völker *et al.*, 1994) or by genetic screening (Boylan *et al.*, 1991, 1993a), and in a number

of cases their functions in stress resistance are well established. These known roles fall into three broad categories: resistance to oxidative stress, resistance to protein denaturation and resistance to osmotic stress (Hecker & Völker, 1998). However, the recent discovery that σ^B null mutants are deficient in acid-stress resistance, are unable to grow in high ethanol concentrations, and survive poorly at alkaline pH indicates that additional physiological roles for σ^B -dependent genes remain to be established (Gaidenko & Price, 1998).

To understand the range of functions mediated by genes controlled by σ^B (*csb* genes), we characterize here two of the *csb-lacZ* fusions originally identified by the genetic screening of Boylan *et al.* (1993a). These two fusions, *csb-22* and *csb-34*, are unusual in that they appear to be solely dependent upon σ^B for their expression under

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standard experimental conditions (Boylan *et al.*, 1993a). With the idea that fusions solely dependent on σ^B would provide useful clues to the range of functions represented by genes in the σ^B regulon, we sought to establish the transcriptional control of the *csb-22* and *csb-34* fusions and to predict the functions of the genes they identified.

METHODS

Bacterial strains and genetic methods. *Escherichia coli* DH5 α (Bethesda Research Laboratories) was the host for all plasmid constructions. *B. subtilis* strains used are shown in Table 1. For strain constructions, *B. subtilis* PB2 and its derivatives were recipients for natural transformations with linear and plasmid DNA (Dubnau & Davidoff-Abelson, 1971). All standard recombinant DNA methods, including DNA sequencing on double-stranded DNA templates, were as previously described (Boylan *et al.*, 1991).

β -Galactosidase activity assays. For salt stress experiments, two parallel cultures of each strain tested were grown in buffered Luria broth (LB) medium lacking salt (Boylan *et al.*, 1993b) to early exponential phase, at which point NaCl was added to one culture to yield a final concentration of 0.3 M. For stationary-phase stress experiments, cells were grown to stationary phase in buffered LB. For both types of experiments, cell samples were collected at the times indicated and treated as described by Miller (1972). Cells were washed with Z buffer and permeabilized using SDS and chloroform. Protein levels were determined on whole-cell samples using the Bio-Rad Protein Assay reagent. Activity was defined as $\Delta A_{420} \times 1000 \text{ min}^{-1} (\text{mg protein})^{-1}$.

Computer analysis. Database searches and sequence alignments were done using the gapped BLAST program of Altschul *et al.* (1997).

RESULTS

The Tn917*lacZ* insertions *csb-22* and *csb-34* were identified as two of the six new loci found in a screen for genes controlled by σ^B (Boylan *et al.*, 1993a). In their preliminary characterization of these transposon insertion strains, Boylan *et al.* (1993a) suggested that expression of the *csb-22* and *csb-34* fusions was completely dependent on σ^B . Notably, of the 30 genes in the σ^B regulon that have been characterized thus far, only one is likely to be solely dependent upon σ^B (Maul *et al.*, 1995). Because most σ^B -dependent genes are also under the control of a second, σ^B -independent promoter (Hecker & Völker, 1998), we needed to establish the transcriptional organization of the *csb-22* and *csb-34* regions on the *B. subtilis* chromosome in order to support the proposal that their expression was completely dependent on σ^B .

Isolation and initial analysis of the *csb-34* region

To locate the σ^B -dependent promoter of the transcription unit into which the *csb-34* fusion had inserted, we first used pLTV-1 and the integration-excision method of Youngman (1990) to isolate 2.1 kb of DNA upstream from the site of the Tn917*lacZ* insertion (see Fig. 1). This DNA allowed us to map the 5' boundaries of elements essential for *csb-34* promoter activity using the plasmid-integration strategy of Piggot *et al.* (1984), as previously described (Boylan *et al.*, 1991). As indicated in the Fig. 1 legend, DNA fragments from the isolated *csb-34* region were carried on integrational vectors and transformed into wild-type, *sigB* Δ 3 and *socB*1 strains bearing the *csb-34::Tn917lacZ* fusion, where *socB*1 is a

Table 1. *B. subtilis* strains

Strain	Genotype	Reference or construction*
PB2	<i>trpC2</i>	Wild-type Marburg strain
PB105	<i>sigB</i> Δ 1 <i>trpC2</i>	Kalman <i>et al.</i> (1990)
PB153	<i>sigB</i> Δ 2::cat <i>trpC2</i>	Boylan <i>et al.</i> (1991)
PB209	<i>socB</i> 1 <i>trpC2</i>	Boylan <i>et al.</i> (1993a)
PB262	<i>csb-22::Tn917lacZ socB1 pheA1 trpC2</i>	Boylan <i>et al.</i> (1993a)
PB263	<i>csb-22::Tn917lacZ trpC2</i>	Boylan <i>et al.</i> (1993a)
PB264	<i>csb-22::Tn917lacZ sigB</i> Δ 2::cat <i>trpC2</i>	Boylan <i>et al.</i> (1993a)
PB265	<i>csb-34::Tn917lacZ socB1 pheA1 trpC2</i>	Boylan <i>et al.</i> (1993a)
PB266	<i>csb-34::Tn917lacZ trpC2</i>	Boylan <i>et al.</i> (1993a)
PB267	<i>csb-34::Tn917lacZ sigB</i> Δ 2::cat <i>trpC2</i>	Boylan <i>et al.</i> (1993a)
PB344	<i>sigB</i> Δ 3::spc <i>trpC2</i>	Boylan <i>et al.</i> (1993b)
PB534	<i>amyE::pSA56 trpC2</i>	pSA56 $\rightarrow$ PB2
PB536	<i>amyE::pSA57 trpC2</i>	pSA57 $\rightarrow$ PB2
PB538	<i>amyE::pSA58 trpC2</i>	pSA58 $\rightarrow$ PB2
PB540	<i>amyE::pSA59 trpC2</i>	pSA59 $\rightarrow$ PB2
PB542	<i>amyE::pSA62 trpC2</i>	pSA62 $\rightarrow$ PB2
PB543	<i>amyE::pSA62 sigB</i> Δ 1 <i>trpC2</i>	pSA62 $\rightarrow$ PB105

*The arrow indicates transformation from donor to recipient.

Fig. 1. Organization of the *B. subtilis* chromosome surrounding the *csb-34::Tn917lacZ* fusion. The chromosome is represented by the shaded rectangle, with the site of *Tn917lacZ* insertion indicated by the filled triangle. The *SaI* site in parentheses is located within the *Tn917lacZ* element. This map is derived from analysis of the genomic fragments carried by pSB34 and pSY105, labelled as 'cloning' plasmids. Inverse PCR was used to isolate an additional 285 bp downstream from the *KpnI* site. The *csbC* ORF identified by the *csb-34::Tn917lacZ* insertion is indicated by the open rectangle above the physical map. The stem-loop symbols show the locations of putative factor-independent terminators that flank the *csbC* ORF. Plasmid-integration and fusion studies indicated that *csbC* expression was completely dependent on a single σ^B -dependent promoter, P_B . The two horizontal lines labelled 'integration' plasmids show the fragments integrated into the *csbC* region of wild-type (wt), *sigB* Δ 3 and *socB*1 strains bearing the *csb-34::Tn917lacZ* fusion. *socB*1 is a frameshift mutation in the *RsbX* negative regulator that leads to increased σ^B activity (Igo *et al.*, 1987). The horizontal line labelled 'fusions-1' indicates the fragment cloned into the transcriptional fusion vector pDG268 and integrated in single copy at the *amyE* locus of wt, *sigB* Δ 3 and *socB*1 strains that bore no other fusion. The key indicates relative β -galactosidase activities of these integration and fusion strains, determined on plates containing X-Gal. The two horizontal lines labelled 'fusions-2' denote the fragments generated by PCR, cloned into the transcriptional fusion vector pDG268 and used in the fusion experiments of Fig. 3. The 5' ends of these 'fusions-2' fragments are shown in Fig. 2. The second fragment is identical to the first except that it carries a G $\rightarrow$ A transition at the -15 position of the proposed σ^B recognition sequence.

frameshift mutation that leads to substantially increased σ^B activity (Igo *et al.*, 1987). The rationale for these experiments was that if fusion expression remained unaffected by such an integration into the *csb-34::Tn917lacZ* region, then the chromosomal fragment carried by the plasmid must contain sequences important for promoter activity. In contrast, if fusion expression was impaired, then the 5' end of the chromosomal fragment must lie downstream from such sequences. As summarized in Fig. 1, these experiments located promoter activity in the 0.4 kb chromosomal region that lies between the *EcoRV* site at 1.7 kb and the site of insertion for the *Tn917lacZ* element. This activity was completely dependent on σ^B .

Because it was formally possible that the *Tn917lacZ* element had inserted between a σ^B -dependent promoter and a downstream σ^B -independent promoter, we used plasmid integration and excision to isolate chromosomal DNA extending to the *KpnI* site 1.4 kb downstream from the site of *Tn917lacZ* insertion. We then isolated

an additional 285 bp of chromosomal DNA beyond the *KpnI* site using inverse PCR. As described in the following section, DNA sequence analysis found a large ORF encoded by this newly isolated chromosomal region. To locate the promoter activity or activities for this frame, we made a transcriptional fusion to the *lacZ* reporter gene in the single-copy vector pDG268 (Antoniewski *et al.*, 1990). As shown in Fig. 1, we used the 0.6 kb *EcoRV*–*SphI* chromosomal fragment for this construction, with the *SphI* site anchored within the large ORF. The resulting pSY111 plasmid was linearized and inserted into the *B. subtilis* chromosome at the *amyE* locus of wild-type, *sigB* Δ 3 and *socB*1 strains. In plate assays these strains manifested only σ^B -dependent fusion expression (Fig. 1). We concluded that a σ^B -dependent promoter was located within the 419 bp region that lay between the *EcoRV* site and the site of the *Tn917lacZ* insertion. We further concluded that this was the only promoter upstream from the large ORF that was active under the exponential- and stationary-phase conditions tested.

The ORF identified by the *csb-34* insertion encodes a sugar transporter

Our predicted sequence of 461 residues for the large ORF was identical to that of the *yxcC* ORF determined in the *Bacillus* genome project (Kunst *et al.*, 1997). The predicted YxcC product had strong similarity to members of the major facilitator superfamily, including over 375 symporters, antiporters and uniporters found in eubacteria, archaea and eukaryotes (Pao *et al.*, 1998). Within this superfamily, YxcC was most similar to members of the sugar porter family of Class I, for which *E. coli* AraE is a well-characterized representative (SWISS-PROT accession no. P09820). By BLASTP alignment YxcC shared 30% identity with *E. coli* AraE through a 446-residue overlap, with an *e* value of 9×10^{-58} . A signature motif of the major facilitator

Nucleotides required for *csbC* expression match other σ^B -dependent promoters

The plasmid-integration experiments and transcriptional fusion analyses indicated that a σ^B -dependent promoter activity was located immediately preceding the *csbC* ORF, between nt 1 and nt 419 of Fig. 2. Because the proposed terminator for the upstream transcription unit lay between nt 254 and nt 280, we inspected the region downstream from nt 280 and found an excellent match to the consensus -35 and -10 recognition sequences in other σ^B -dependent promoters (Hecker *et al.*, 1996). This match was particularly striking at those positions known to be important for activity of the well-characterized σ^B -dependent promoter of the *ctc* gene (Igo *et al.*, 1987; Moran *et al.*, 1982; Ray *et al.*, 1985; Tatti & Moran, 1984). To determine whether these proposed recognition sequences were important for σ^B -

Fig. 3. Salt-induced expression of wild-type and mutant *csbC* promoters in exponentially growing cells. Expression was measured by monitoring β -galactosidase accumulation from the pSA56 and pSA57 single-copy transcriptional fusions (see Figs 1 and 2). *B. subtilis* strains harbouring these fusions were grown in buffered LB medium (Boylan *et al.*, 1993b) and 0.3 M NaCl was added to one of two parallel cultures in early exponential growth (time 0). Samples were removed at the indicated times and assayed for β -galactosidase activity using the method of Miller (1972); specific activity is defined as $\Delta A_{420} \times 1000 \text{ min}^{-1} (\text{mg protein})^{-1}$. Data shown are for strains PB534 (*amyE*::pSA56) with (●) and without (○) added NaCl, and PB536 (*amyE*::pSA57, the same fusion borne by pSA56 but with the $G_{408} \rightarrow A$ transition) with added NaCl (▲).

dependent promoter activity of *csbC*, we altered position -15 from G to A. This transition is known to severely decrease activity of the *ctc* promoter both *in vitro* and *in vivo* (Ray *et al.*, 1985; Tatti & Moran, 1984). To test the effects of this transition, we took advantage of the fact that σ^B -dependent promoters are characteristically induced upon salt addition to exponentially growing *B. subtilis* cells (Boylan *et al.*, 1993b; Völker *et al.*, 1994). As shown in Fig. 3, we found that the wild-type *csbC* promoter fusion was also strongly induced by salt addition. This salt induction was abolished in the fusion strain bearing the G to A transition at the -15 position. Based on the sum of these results, we concluded that the *csbC* promoter is likely to be directly recognized by σ^B -containing holoenzyme *in vivo*. We further interpret these results to indicate that the original *csb-34*::Tn917*lacZ* insertion just downstream from this promoter effectively generated a null *csbC* mutation. Because strains bearing this insertion manifested no obvious phenotype under standard growth conditions (Boylan *et al.*, 1993a), *csbC* is not an essential gene.

Isolation and initial transcriptional analysis of the *csb-22* region

We also used plasmid integration and excision to isolate 0.8 kb of DNA upstream from the second gene which appeared to be completely dependent upon σ^B , identified

by the *csb-22*::Tn917*lacZ* insertion (see Fig. 4). This DNA allowed us to locate sequences required for promoter activity by integrating appropriate plasmids into recipients bearing the *csb-22*::Tn917*lacZ* fusion. As summarized in Fig. 4, these integration experiments found only one promoter activity in the 0.8 kb chromosomal region that lay between the *EcoRI* site and the site of insertion for the Tn917*lacZ* element. This activity was entirely dependent on σ^B , and sequences necessary for this activity were further localized to the 45 bp interval between the *Clal* site at nt 781 and the *HpaI* site at nt 826 (nucleotide positions refer to Fig. 5).

The ORF identified by the *csb-22* insertion encodes a protein similar to an *E. coli* protein of unknown function

We determined the DNA sequence of the 0.8 kb chromosomal region between the *EcoRI* site and the site of Tn917*lacZ* integration. As shown in Fig. 5, an ORF encoding a predicted product of 62 residues lay just downstream from the nt 781–826 region that had been shown to be required for σ^B -dependent promoter activity. Moreover, the Tn917*lacZ* element had inserted 34 bp downstream from the stop codon for this frame, within a potential weak terminator sequence [$\Delta G = -7.4 \text{ kcal mol}^{-1}$ ($-31.0 \text{ kJ mol}^{-1}$)]. The predicted 62-residue product was identical to that of the *ywmG* ORF determined in the *Bacillus* genome project (Kunst *et al.*, 1997) and somewhat resembled a hypothetical *E. coli* protein of similar size, called YjbJ (SWISS-PROT accession no. P32691). YwmG shared 37% identity with *E. coli* YjbJ through a 51-residue overlap, with an *e* value of 3×10^{-3} . In keeping with the nomenclature for *csb* genes of unknown function (Boylan *et al.*, 1991), we refer to the gene identified by the *csb-22* insertion as *csbD* and its predicted product as the CsbD protein.

Nucleotides required for *csbD* expression match other σ^B -dependent promoters

The plasmid-integration experiments indicated that a σ^B -dependent promoter activity lay within the 45 bp region between the *Clal* and *HpaI* sites immediately upstream from the *csbD* ORF. This region contained an excellent match to the proposed -35 and -10 recognition sequences in other σ^B -dependent promoters (Hecker *et al.*, 1996; Moran *et al.*, 1982; Ray *et al.*, 1985; Tatti & Moran, 1984). To determine whether these proposed recognition sequences were important for σ^B -dependent promoter activity of *csbD*, we altered position -15 from G to A and tested its effect on salt induction. Since we noted that the original *csb-22* insertion had occurred within the proposed terminator for the *csbD* transcriptional unit, we constructed wild-type and mutant fusions whose 3' ends were both anchored within the *csbD* ORF. These fusions were carried in single copy at the *amyE* locus on the *B. subtilis* chromosome. As shown in Fig. 6(a), the wild-type *csbD* promoter fusion was strongly induced by salt addition

Fig. 4. Organization of the *B. subtilis* chromosome surrounding the *csb-22::Tn917lacZ* fusion. The chromosome is shown as a shaded rectangle, with the site of *Tn917lacZ* insertion indicated by the filled triangle. The *SalI* site in parentheses is within the *Tn917lacZ* element. This map is derived from the genomic fragment carried by the pSB22 'cloning' plasmid. We used chromosomal walking methods to isolate and sequence an additional 180 bp downstream from the site of *Tn917lacZ* insertion. The *rapB*, *ywmF* and *csbD* ORFs are indicated by the open rectangles above the chromosome. The stem-loop symbols show the locations of putative factor-independent terminators. Plasmid-integration and fusion studies indicated that *csbD* expression was completely dependent on a single σ^B -dependent promoter, P_B . The three 'integration' plasmids were transformed into the *csbD* region of wild-type (wt), *sigB* Δ 3 and *socB*1 strains bearing the *csb-22::Tn917lacZ* fusion. The key shows relative β -galactosidase activities of these integration strains on X-Gal plates. The three horizontal lines labelled 'fusions' denote the fragments generated by PCR, cloned into the transcriptional fusion vector pDG268 and used in the fusion experiments described in Fig. 6.

and this induction was absent in the fusion containing the G to A transition at the -15 position. Based on the sum of these results, we concluded that the promoter immediately upstream from *csbD* is likely to be directly recognized by σ^B -containing holoenzyme *in vivo*.

Expression of these fusions anchored within the *csbD* ORF was higher than the original *csb-22* fusion, supporting the suggestion that the *Tn917lacZ* element had inserted within the *csbD* transcriptional terminator. The original *csb-22* fusion would therefore not be expected to generate a true *csbD* null mutation. Consequently, we introduced a Km^r cassette within the *csbD* ORF itself. Because the strain bearing this Km^r cassette manifested no obvious phenotype under standard growth conditions, *csbD* is not an essential gene.

csbD appears to be the second gene of an operon

Inspection of the DNA sequence upstream from *csbD* found an ORF for a 159-residue product of unknown function, identified as *ywmF* in the *Bacillus* genome sequencing project (Kunst *et al.*, 1997). According to Kunst *et al.* (1997), gene order in this region is *rapB*-*ywmF*-*ywmG* (*csbD*), and all three genes are transcribed in the same direction. As shown in Fig. 5, a potential factor-independent terminator sequence [$\Delta G =$

$-16.4 \text{ kcal mol}^{-1}$ ($-68.6 \text{ kJ mol}^{-1}$)] separates *rapB* from *ywmF*, but no such sequence is apparent in the 73 bp interval between *ywmF* and *csbD*, suggesting that the latter two genes comprise an operon.

Because the *EcoRI* site in the integration plasmids we analysed lay just downstream from the presumed ribosome-binding site of *ywmF* (Figs 4 and 5), the experiments which located a σ^B -dependent promoter in the *ywmF*-*csbD* intercistronic region would not have detected promoter activity upstream of *ywmF*. We therefore made an additional fusion whose 3' end was anchored within *csbD* and whose 5' end contained the presumed *ywmF* promoter region (Fig. 4); this fusion was carried in single copy at the *amyE* locus on the *B. subtilis* chromosome. Expression of this longer fusion (Fig. 6b) was indistinguishable from that of the shorter fusion (Fig. 6a) in that it manifested only σ^B -dependent promoter activity. We concluded that if a promoter lies upstream of *ywmF* it is not expressed under the three assay conditions we used: exponential growth, salt stress and stationary phase in buffered LB medium. These results are in accord with the initial characterization of the *csb-22* fusion by Boylan *et al.* (1993a), who found its expression to be solely dependent on σ^B under standard assay conditions.

[illegible]

Fig. 5. Nucleotide sequence of the *ywmF-ywmG (csbD)* region. The nucleotide sequence shows the last 50 codons of the upstream ORF *rapB*, the putative factor-independent terminator for *rapB* (indicated by converging arrowheads), and the entire *ywmF-ywmG (csbD)* region. The open triangle following nt 21 indicates the 5' end point of the longer fusion fragment used to probe for additional promoter activity in the *rapB-ywmF* interval, whereas the open triangle following nt 796 indicates the 5' end points of the shorter fusion fragments used to locate sequences necessary for σ^B -dependent promoter activity. The proposed -35 and -10 recognition sequences of the σ^B -dependent *csbD* promoter are double underlined at nt 797-804 and 819-824, respectively. Asterisks indicate an exact match at the five bases that are important for activity of the σ^B -dependent *ctd* promoter. The mutation shown changes the proposed -10 recognition sequence from GGAAG to GAGAAG. The proposed ribosome-binding site for the *csbD* ORF is underlined at nt 847-855 and the site of Tn917*lacZ* insertion within the proposed *csbD* terminator is shown by the filled triangle following nt 1085. The sequence 1-303 is from Kunst *et al.* (1997) and the sequence 304-1100 was determined by us. This latter sequence was found to be identical to the sequence established in the *Bacillus* genome project (EMBL accession no. Z81356).

DISCUSSION

We have characterized the transcriptional organization of the regions encoding two new *csb* genes and have demonstrated that their salt-induced expression requires the general stress factor σ^B . Moreover, we have located the *cis*-acting sequences required for this stress-induced expression and find that they closely match the -35 and -10 recognition sequences of the well-characterized *ctc* promoter (Moran *et al.*, 1982; Ray *et al.*, 1985; Tatti & Moran, 1984). Because *ctc* is transcribed by σ^B -containing RNA polymerase *in vitro* (Haldenwang & Losick, 1980; Moran *et al.*, 1982) and requires σ^B for its expression *in vivo* (Igo *et al.*, 1987), we concluded that both *csbC* and *csbD* are likely to be directly transcribed by σ^B -containing holoenzyme under stress conditions.

Both *csbC* and *csbD* have in common the unusual feature that their expression is completely dependent upon σ^B under the three growth conditions we tested: exponential growth in LB medium, salt stress imposed upon these exponentially growing cells and stationary phase in LB medium. These two genes also have in common the property that neither is essential under standard growth conditions. However, *csbC* and *csbD* differ in the organization of their transcriptional units. *csbC* appears to define a monocistronic transcriptional unit solely dependent upon σ^B . In this regard, *csbC* resembles *gsiB*, which until now was the only gene thought to be solely dependent upon σ^B (Maul *et al.*, 1995).

In contrast, on the basis of sequence analysis *csbD*

Fig. 6. Expression of wild-type and mutant *csbD* promoters during exponential or stationary phase. (a) Expression was measured by monitoring β -galactosidase accumulation from the pSA58 and pSA59 single-copy transcriptional fusions (see Figs 4 and 5). *B. subtilis* strains harbouring these fusions were grown in buffered LB medium (Boylan *et al.*, 1993b) and 0.3 M NaCl was added to one of two parallel cultures in early exponential growth (time 0). Samples were removed at the indicated times and assayed for β -galactosidase activity as described in Fig. 3 legend. Data shown are for strains PB538 (*amyE*::pSA58) with (●) and without (○) added NaCl, and PB540 (*amyE*::pSA59, the same fusion borne by pSA58 but with the $G_{820} \rightarrow A$ transition) with added NaCl (▲). (b) Expression was measured by monitoring β -galactosidase accumulation from the longer single-copy transcriptional fusion pSA62 in a wild-type (PB542, ■) and a *sigB* null strain (PB543, △), both grown to stationary phase in buffered LB medium.

appears to be the second gene in a two-gene operon with the order *ywmF*–*csbD*, where *ywmF* is an ORF of unknown function (see Fig. 4). If this proves to be the case, the σ^B -dependent *csbD* promoter would constitute an internal promoter for this operon. Such σ^B -dependent internal promoters have been found in the *ctc* and *sigB* operons (Hilden *et al.*, 1995; Moran *et al.*, 1982; Wise & Price, 1995). However, for both *ctc* and *sigB*, σ^B -independent promoter activities were readily located in the region upstream from the first gene in the operon. Here we were unable to detect any activity in the presumed *ywmF* control region using three different

growth conditions. Because these same growth conditions effectively revealed the second promoter for other σ^B -dependent genes, including σ^A -like, σ^H -dependent and σ^X -dependent promoters (Akbar & Price, 1996; Boylan *et al.*, 1991; Huang & Helmann, 1998; Varón *et al.*, 1993, 1996; von Blohn *et al.*, 1997), we suggest that the hypothetical *ywmF* promoter will only be detected under more specialized growth conditions.

Like *csbD*, two other genes that on first analysis seemed to be solely under σ^B control may also lie in operons with internal, σ^B -dependent promoters. The first, *gspA*, encodes an abundant stress protein of unknown function, and a σ^B -dependent promoter has been located immediately upstream from *gspA* (Antelmann *et al.*, 1995). However, inspection of the *gspA* region suggests a two-gene operon with the order *ywaF*–*gspA* (Kunst *et al.*, 1997). The second gene, *csbX*, encodes a permease belonging to the major facilitator superfamily and is also immediately preceded by a σ^B -dependent promoter (Gomez & Cutting, 1997a; Kunst *et al.*, 1997). Inspection of the *csbX* region suggests a three-gene operon with the order *yrbE*–*csbX*–*bofC*, where the *bofC* product contributes to sporulation control by communicating forespore signals to the mother cell (Gomez & Cutting, 1997b; Kunst *et al.*, 1997). Based on the genetic organization of the *csbD*, *gspA* and *csbX* regions, we consider it likely that additional promoters for these operons remain to be discovered.

Thus the best candidates for genes solely under the control of σ^B remain *gsiB*, described by Maul *et al.* (1995), and *csbC*, described here. For either gene it is not possible to experimentally rule out the possibility of an additional, σ^B -independent promoter that is active under specialized growth conditions. However, the lack of a conspicuous second promoter activity coupled with a monocistronic transcriptional organization is consistent with the hypothesis that expression of *gsiB* and *csbC* are completely σ^B -dependent. We do note that the *csbC* message has an unusually long 44–46 nt region between the projected site of transcription initiation and the proposed ribosome-binding site (see Fig. 2). Although this region may potentially contain a promoter that is silent under the growth conditions tested, other experiments suggest that the unusual leader instead negatively regulates *csbC* expression (Lee, 1995).

If we provisionally assume that *gsiB* and *csbC* expression is solely dependent upon σ^B , what can their proposed functions tell us about the range of activities mediated by genes comprising the σ^B regulon? *GsiB* is one of the most abundant proteins produced during the general stress response and bears some similarity to plant desiccation proteins (Stacy & Aalen, 1998; Völker *et al.*, 1994). *GsiB* function therefore appears to fall within the realm of osmotic stress protection (Hecker & Völker, 1998). In contrast, *CsbC* is a member of the major facilitator superfamily, and, more specifically, belongs to Class I of this superfamily (Pao *et al.*, 1998). Class I contains symporters that typically transport sugars from the external environment. Therefore, the likely role of

CsbC is to meet the nutritional needs arising from an energy stress sufficiently severe to activate σ^B . However, an argument can also be advanced for an osmoprotective role for CsbC. For example, trehalose is a known compatible osmoprotectant in *E. coli* and other organisms (Crowe *et al.*, 1984; Strøm & Kaasen, 1993). Although trehalose has not been detected in osmotically stressed *B. subtilis* cells (Whatmore *et al.*, 1990), it remains possible that under some growth conditions *B. subtilis* transports other sugars or related solutes as osmoprotectants. Indeed, precedent for involvement of a σ^B -dependent gene in osmoprotection comes from study of the OpuE transporter, which is required for proline-dependent osmoprotection and whose synthesis is under the control of dual σ^A and σ^B promoters (von Blohn *et al.*, 1997). These two possible roles for CsbC – osmoprotection or nutritional acquisition – can be distinguished once the solute it transports has been identified.

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