JOURNAL OF BACTERIOLOGY, Aug. 1996, p. 5039–5041 Vol. 178, No. 16
0021-9193/96/$04.00+0

Identification of Additional Genes under the Control of the Transcription Factor σ^F of *Bacillus subtilis*

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Received 22 February 1996/Accepted 10 June 1996

We describe the identification of five transcriptional units under the control of the sporulation transcription factor σ^F in *Bacillus subtilis*. These are *csfA*, *csfB*, *csfC*, *csfD*, and *csfF*, located at approximately 230°, 2°, 316°, 205°, and approximately 290°, respectively, on the genetic map. Null mutations in *csfA*, *csfB*, *csfC*, or *csfD*, either alone or together, do not cause a noticeable defect in sporulation or germination.

The σ^F factor of *Bacillus subtilis* is an RNA polymerase sigma factor that governs cell-type-specific gene transcription during the process of sporulation (for a review, see references 5 and 10). The σ^F factor directs gene expression in the forespore compartment of the sporangium at the polar septation stage of development. It is required for the appearance of two later-acting sporulation-specific sigma factors, σ^E and σ^G, as well as for morphological development of the forespore itself. At present, only four genes whose transcription is under the control of σ^F have been reported. These are *spoIIIG*, the structural gene for σ^G (15, 18); *spoIIR*, a gene whose product is required for the activation of σ^E (6, 8, 9); *gpr*, a protease involved in germination (15, 19); and *dacF*, a putative DD-carboxypeptidase (16). In this study, we identify five additional members of the σ^F regulon.

Isolation of genes under σ^F control. To identify additional genes under σ^F control, we used a fusion of the σ^F gene (*spoIIAC*) to the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter P_{spac} (15) to screen a library (kindly provided by P. Zuber) carrying chromosomal DNA fragments fused to the *Escherichia coli* reporter gene *lacZ*. The library was carried on the specialized transducing phage SPβ. Cells containing the P_{spac}-*spoIIAC* fusion (strain AB304) were transduced to macrolide-lincosamide-streptogramin B resistance by using the SPβ library. (Because the P_{spac}-*spoIIAC* fusion induces the synthesis of σ^G as well as σ^F, AB304 also contained a *spoIIIG*-null mutation to avoid isolating genes that had been transcribed under the control of σ^G.) Original transductants were replica plated in duplicate onto Luria-Bertani agar plates (14) containing 25 μg of lincomycin, 1 μg of erythromycin, and 120 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. After an approximately 8-h incubation at 37°C, one of each set of duplicated plates was sprayed with a 200 mM solution of IPTG. All plates were then incubated overnight at 37°C. Colonies containing fusions whose expression was either completely dependent or partially dependent on the presence of IPTG were picked for further study.

We found five fusions that were induced by the addition of IPTG and named the genes identified by them *csfA*, *-B*, *-C*, *-D*, and *-F*, for "controlled by sigma F." (An independent study carried out by P. Stragier identified a sixth gene under the control of σ^F that was named *csfE* because it is located downstream of the *spoIIE* gene). The *csfC* fusion was partially dependent on IPTG for its expression, while the remaining four fusions were completely dependent on IPTG for their expression. We could not distinguish, however, whether these fusions are under the direct control of σ^F or, alternatively, are induced indirectly by a regulatory protein produced under the control of σ^F.

To investigate whether the fusions were expressed during sporulation and, if so, whether this expression was dependent on σ^F, we introduced each *csf-lacZ* construct into a wild-type strain lacking the P_{spac}-*spoIIAC* fusion and into a *spoIIAC* mutant strain lacking the P_{spac}-*spoIIAC* fusion. We then monitored the synthesis of β-galactosidase during sporulation in DS medium (11). Synthesis of β-galactosidase from the *csfA*, *csfB*, *csfC*, and *csfD* fusions commenced at approximately the second hour of sporulation in wild-type cells, and in all four cases this expression largely or almost completely (depending on the fusion) required the presence of a functional copy of *spoIIAC* (Fig. 1). Furthermore, expression of the *csfA*, *csfB*, *csfC*, and *csfD* fusions did not depend on sporulation sigma factor σ^G or σ^E (data not shown). Indeed, in the case of *csfC*, synthesis of β-galactosidase was approximately twofold higher in cells mutant for σ^G than in wild-type cells (data not shown), and, in the case of *csfD*, synthesis of β-galactosidase was approximately twofold higher in cells mutant for σ^E than in wild-type cells (data not shown). We conclude that *csfA*, *csfB*, *csfC*, and *csfD* are induced during sporulation under the control of σ^F.

The *csfF* fusion was expressed during sporulation at levels too low to measure in liquid DS medium. Nevertheless, when grown on solid DS medium containing X-Gal, wild-type cells containing the *csfF* fusion produced sufficient β-galactosidase to be detected by hydrolysis of the chromogenic substrate. Expression of the fusion could also be detected in cells mutant for sporulation sigma factors σ^G and σ^E but not in cells mutant for σ^F. Thus, it is likely that, like *csfA*, *csfB*, *csfC*, and *csfD*, *csfF* is induced during sporulation under the control of σ^F.

Chromosomal positions of the *csf* genes. To determine the chromosomal location of the *csf* genes, we transferred the fusions onto plasmid pTV8 by the double-recombination protocol of Zuber (2, 21). We used the resulting hybrid plasmids to generate fragments containing the cloned *B. subtilis* DNA and then radiolabelled the fragments for use as hybridization probes. The radiolabelled DNAs were incubated under hybridization conditions either with blots of *Sfi*I- or *Not*I-digested chromosomal fragments that had been separated by pulse-field gel electrophoresis (7) or with blots containing a library of chromosomal fragments carried on yeast artificial chromosomes (1). Map positions of the five σ^F-controlled genes are shown in Fig. 2. The locations of three of the *csf* transcriptional

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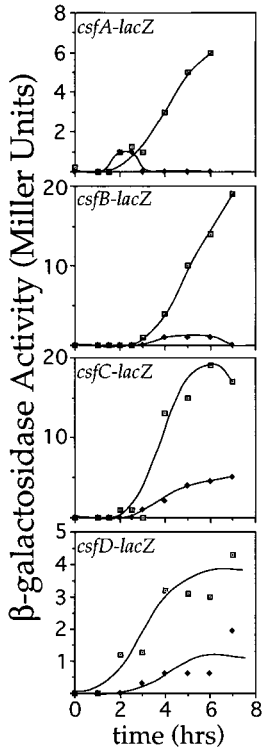

FIG. 1. Time course of expression of *csf-lacZ* fusions. Each panel shows the accumulation of β-galactosidase during sporulation in DS medium (11) from the indicated *csf-lacZ* fusion contained in *spoIIAC*$^+$ cells (derived from strain PY79 [20]) (squares) or in congenic *spoIIAC* mutant cells (diamonds). Time zero is the end of exponential growth. β-Galactosidase activity was assayed by the method of Miller (10a). For each panel, the time course of background *o*-nitrophenyl-β-D-galactopyranoside hydrolysis was measured in a strain that did not harbor a *lacZ* fusion. This background activity (ranging from 0 to 3 Miller units) was subtracted for each time point shown in the figure.

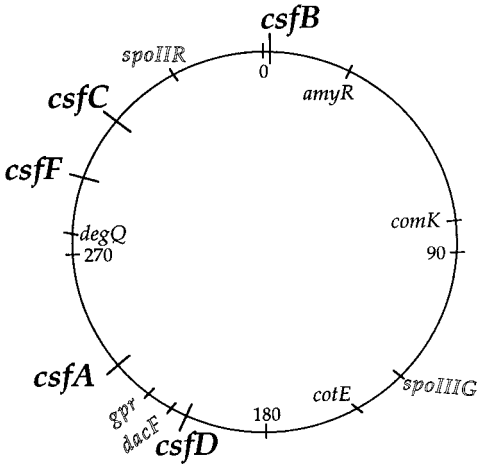

FIG. 2. The σ^F regulon. The *B. subtilis* chromosome is shown as a circle. Previously identified members of the σ^F regulon are in shadow type. New members of the σ^F regulon identified in this study are boldfaced. The origin of replication (0°) and four other genes are included on the inside of the circle as points of reference.

units (*csfB*, *csfC*, and *csfD*) were further defined by nucleotide sequence analysis (see below).

Further characterization of the *csf* genes. We further characterized the *csf* DNAs by sequencing approximately 200 to 400 bp of each clone starting at the *lacZ* junction of the above hybrid plasmids. In addition, by subcloning various portions of each *csf* clone upstream of a new promoterless copy of the *lacZ* gene, we determined the approximate origin of the σ^F-dependent transcription.

csfA corresponded to an insert of 411 bp which contained the first 89 codons of a previously unidentified open reading frame. *csfA* hybridized to *Sfi*I fragment DS and to *Not*I fragment 8N (7), placing *csfA* at approximately 230° on the genetic map. A plasmid containing a fragment of 235 bp that was internal to the identified open reading frame was used to disrupt the *csfA* open reading frame by Campbell-like recombination. The resulting strain was not impaired for sporulation or germination. (*csfA* was also independently isolated and characterized by C. Karmazyn-Campelli and P. Stragier [7a]).

csfB is identical to a previously identified open reading frame (*orf1*) of 64 codons that is located immediately downstream of the *rrnA* operon and immediately upstream of the *xpaC* gene at 2° on the genetic map (3). A strain containing the spectinomycin resistance gene in place of codons 16 to 50 of *csfB* was not impaired for sporulation or germination. However, the *csfB*-null mutation does cause a distinct colony morphology on Luria-Bertani medium (14): cells lacking *csfB* form colonies with fuzzy borders compared with colonies composed of wild-type cells. This phenotype was suppressed by the addition of either a *spoIIAC*- or a *csfC*-null mutation to the *csfB* mutant strain.

csfC corresponds to a previously identified sporulation-specific promoter that was initially thought to be under σ^E control (12). It is located at 316° on the genetic map, immediately upstream of sporulation gene *spoIID* and immediately downstream of *murA*, a gene involved in cell wall biosynthesis. Both *spoIID* and *murA* are oriented in the same direction, while *csfC* is oriented in the opposite direction and positioned so as to transcribe the nontemplate strand of *murA*. Because there are no significant open reading frames other than *murA*, it is tempting to speculate that, rather than encoding a protein product, the *csfC* gene produces an antisense RNA that negatively regulates *murA* expression. This view was especially intriguing when the role of the *murA* gene product was considered. The *murA* gene encodes an essential UDP-*N*-acetylglucosamine-1-carboxyvinyltransferase that catalyzes the committed step in cell wall biosynthesis (4, 13). When temperature-sensitive *murA* mutants are shifted to the nonpermissive temperature, cell wall synthesis shuts down and cell lysis occurs within 15 to 20 min (4). Interestingly, high levels of σ^F activity also lead to extensive cell lysis (15). To investigate a possible link between these two lysis phenotypes, we created a 56-bp chromosomal deletion that removed the *csfC* promoter without disrupting either the *spoIID* or the *murA* transcription unit. A strain containing this deletion, however, was not deficient in sporulation or germination; furthermore, this deletion did not relieve the lysis phenotype of high σ^F activity. Thus, it appears that *csfC* either is not involved or is at least not the sole reason for the lysis phenotype of mutants with high σ^F activity. However, as stated above, removal of the *csfC* promoter did suppress the colony morphology phenotype of cells lacking *csfB*.

csfD corresponded to a 2.2-kb insert. Partial sequencing combined with DNA sequence generously provided from the *B. subtilis* genome project (17) revealed that *csfD* contained two slightly overlapping open reading frames (named *yphA* and *yphB*) of 199 and 297 codons, respectively. These two open reading frames are located at 205° on the genetic map. By subcloning various portions of the *csfD* insert upstream of a

promoterless copy of the *lacZ* gene, we found that the σ^F-dependent transcription originated within a sequence of 550 bp located immediately upstream of the *yphA* open reading frame. A strain containing an erythromycin resistance gene in place of codons 38 to 199 of *yphA* and codons 1 to 136 of *yphB* was not impaired for sporulation or germination. Furthermore, a strain simultaneously lacking the *csfA*, *csfB*, *csfC*, and *csfD* gene products was also not impaired for sporulation or germination, nor was this quadruple mutant impaired for sporulation at high (45°C) or low (25°C) temperature.

csfF corresponded to a 1.8-kb fragment that hybridized to yeast artificial chromosome clone 11-323 (1). This places *csfF* at approximately 290° on the *B. subtilis* genetic map. Because *csfF* was very weakly expressed and because the sequence of this region of the chromosome was not available, we did not characterize this transcriptional unit further.

In conclusion, our study has brought to nine the number of transcriptional units under the control of σ^F. As the *B. subtilis* sequencing project reaches completion and additional σ^F-controlled transcriptional units are identified, it will be interesting to determine the full size of the σ^F regulon and the role of its members in sporulation.

We thank C. Karmazyn-Campelli and P. Stragier for sharing results prior to publication, P. Glasser for sharing the *murA* sequence prior to publication, A. Sorokin for sharing the *yphA* and *yphB* sequences prior to publication, and P. Zuber for providing the SPβ phage library.

This work was supported by NIH grant GM18568 to R.L. A.D. was a predoctoral fellow of the National Science Foundation.

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