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A Mutation in *P23*, the First Gene in the RNA Polymerase σ^A (σ^{43}) Operon, Affects Sporulation in *Bacillus subtilis*

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Mutations within *P23*, the first gene of the *Bacillus subtilis* σ^A operon, were not detrimental to vegetative growth or sporulation. One deletion of *P23* resulted in a strain that sporulated earlier than the wild type. This aberrant phenotype may be due to the simultaneous deletion of a σ^H promoter from the σ^A operon.

The *Bacillus subtilis* major vegetative RNA polymerase σ factor (σ^A) is encoded by the *sigA* gene. The *sigA* gene has been cloned and sequenced (3, 8; GenBank/EMBL accession no. X03897). It was found to lie within an operon with the *dnaE* (encodes DNA primase) and the *P23* genes, the gene order being *P23*, *dnaE*, *sigA* (11).

The organization of the *B. subtilis* σ^A operon is similar to that of the *Escherichia coli* σ^{70} operon (11). The genes and gene products of *dnaE* and *sigA* are very homologous to their *E. coli* counterparts, *dnaG* and *rpoD*, respectively. In contrast, the deduced 23-kilodalton polypeptide product encoded by the *P23* gene has no homology to *E. coli* ribosomal protein S21, which is the product of *rpsU*, the first gene in the σ^{70} operon. A computer search of the Georgetown protein data bank did not reveal any significant homology of the *P23* product to any known protein.

The results of Wang and Doi (13) suggested that three polypeptides were produced from the *P23* gene when the products were expressed as fusion proteins to β-galactosidase from plasmids in *B. subtilis* and *E. coli*. Their results indicated that these polypeptides were all in the same phase and so would share the same C-terminal amino acids. The molecular masses of these polypeptides were deduced from sequence data as 23, 19, and 9 kilodaltons. Expression from the P23 and P19 ribosome-binding sites (RBSs) was observed during vegetative growth, but expression from the P9 RBS was observed only after the onset of sporulation. This pattern of expression was attributed to promoter switching between the three major promoters of the σ^A operon (12). The operon is expressed during vegetative growth from the σ^A-dependent P1 and P2 promoters located upstream from the P23 RBS. An additional promoter is located within the *P23* gene between the P19 and P9 RBSs. Expression from this P3 promoter is induced at T_2 and is dependent upon σ^H, the gene product of *spo0H* (2).

As part of a continuing genetic characterization of the *B. subtilis* σ^A operon, we have mutated the *P23* gene in order to attempt to identify its role during growth and sporulation.

Both the DnaE and SigA proteins are essential for growth in *B. subtilis*; thus, any polar mutations introduced into the *P23* gene would confer a lethal phenotype and no mutants would be isolated. The selectable mutagen we chose to employ was a gene encoding chloramphenicol acetyltransferase (CAT) that lacked a promoter and a transcription terminator. The *cat* gene did possess an RBS that could be utilized in *B. subtilis*. The gene was subcloned from pRB394 (1) on a 0.7-kilobase-pair (kb) *Sau*3A fragment.

Three plasmids were constructed in *E. coli* in which the *cat* gene was inserted into a plasmid-borne copy of *P23* at different locations within the gene. All three mutant plasmids were unable to replicate in *B. subtilis*, but the *cat* gene present in all of the plasmids was capable of conferring Cm^r upon *B. subtilis* and was flanked by DNA homologous to the chromosome. These three plasmids were linearized, and the mutations were introduced into the chromosome of *B. subtilis* DB2 (14) by reciprocal recombination on either side of the *cat* gene.

Figure 1 shows the predicted structure in the *P23* gene region for the three different *B. subtilis* Cm^r mutant derivatives DB226, DB227, and DB228. DB226 contains the *cat* gene inserted at the position of the *Xmn*I site of *P23*. DB227 contains an *Xmn*I-*Rsa*I deletion of *P23*, replaced with the *cat* gene. DB228 contains a *cat* gene replacement of the *Hae*III-*Rsa*I fragment at the 3′ end of the *P23* gene. The integration was confirmed in each case by Southern hybridization. *Hin*dIII-digested chromosomal DNA from each transformant was probed with plasmid pUC19-P23 (L. F. Wang and R. H. Doi, unpublished). This plasmid carries the complete *P23* gene on pUC19, up to the *Rsa*I at the 3′ end of the gene. The results are shown in Fig. 2.

In *B. subtilis* DB2, the *P23* gene is internal to a 3.0-kb *Hin*dIII fragment. As there are no *Hin*dIII sites within the *cat* gene, we expected to see only a single fragment hybridizing to the probe in *Hin*dIII-digested chromosomal DNA of the Cm^r mutants. *B. subtilis* DB226 had a single homologous 3.7-kb fragment. The 0.7-kb increase is consistent with the insertion of the 0.7-kb *cat* gene into the *P23* gene. The 3.1-kb fragment observed for DB227 is consistent with a deletion of 0.6 kb of *P23* DNA and its replacement with the 0.7-kb *cat* gene. DB228 had a homologous fragment 3.6 kb in size. This is expected due to the deletion of 0.1 kb of *P23* and its replacement by *cat*. Additional chromosomal DNA digests of the three strains were also probed with pUC19-P23 to confirm the orientation of insertion of the *cat* gene, and the sizes of the hybridizing fragments were all as predicted (data not shown).

No change in growth rate, relative to DB2, was observed for any of the three CAT mutants generated, indicating that expression of *sigA* and *dnaE* is not disrupted by insertion of the CAT cassette into the chromosome. The mutagen does not appear to cause polarity in the *sigA* operon.

Similarly, no mutant phenotype was observed during sporulation as a result of mutations generated at either the 5′ or 3′ end of the *P23* gene, i.e., in DB226 or DB228,

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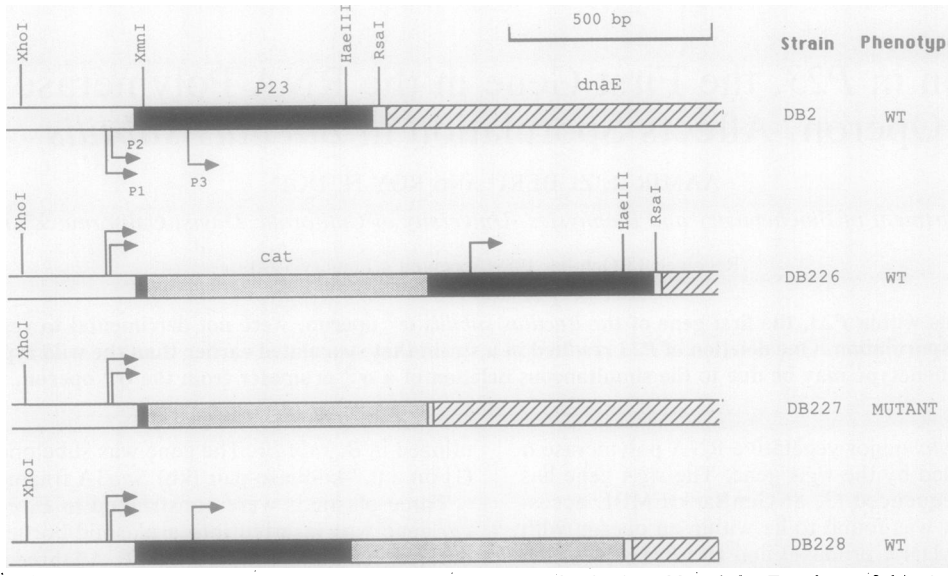

FIG. 1. Representation of the chromosomal structure for the indicated strains in the *P23* and *dnaE* regions of the *sigA* operon. Solid bars, *P23* gene; hatched bars, *dnaE* gene; stippled bars, *cat* gene, arrows, promoters P1, P2, and P3. Relevant restriction sites are marked. WT indicates that no mutant phenotype was observed. bp, Base pairs.

respectively. However, deletion of the entire gene from the chromosome in DB227 resulted in a mutant that was altered in some sporulation properties.

DB227 colonies pigmented earlier than DB2, DB226, or DB228 colonies when allowed to sporulate on 2× SG (5) plates. Colony pigmentation is usually a good indicator of sporulation on solid media. There was no difference between any of the strains in colony morphology or, as stated above, in the rate at which cells grew vegetatively. These results suggest that the timing of sporulation has been altered in the *P23* deletion mutant, DB227. All four strains, DB2, DB226, DB227, and DB228, were induced to sporulate in Sterlini-Mandelstam replacement medium (10), and the cells were monitored microscopically at hourly intervals for phase-bright forespores. Samples were also treated with lysozyme to determine the number of resistant cells generated as a result of sporulation. The results for DB2 and DB227 are shown in Fig. 3.

DB227 cells sporulated approximately 1 h earlier than did DB2 cells. The timing of sporulation of DB226 and DB228 was indistinguishable from that of DB2 under these same conditions. No major difference was observed in the levels of fumarase between DB2 and DB227 grown on 2× SG sporulation medium (data not shown), suggesting that the earlier-sporulation phenotype is only apparent after the initiation of sporulation. Fumarase is induced shortly before T_0 under these conditions (6).

It appears that the product of the *P23* gene is not required for vegetative growth or sporulation. The apparent defect

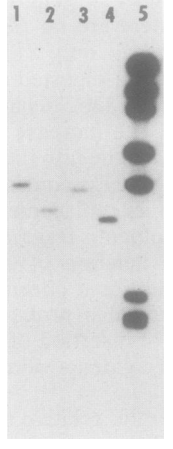

FIG. 2. Southern blot of *B. subtilis* chromosomal DNA probed with pUC19-P23. Lanes 1, 2, 3, and 4, Chromosomal DNAs of DB226, DB227, DB228, and DB2, respectively, digested with *Hin*dIII. Lane 5, Lambda bacteriophage DNA digested with *Ava*I; fragment sizes are 14.7, 8.6, 6.9, 4.7, 3.7, 1.8, 1.67, and 1.60 kb. These fragments were visualized by addition of labeled lambda phage DNA to the Southern blot.

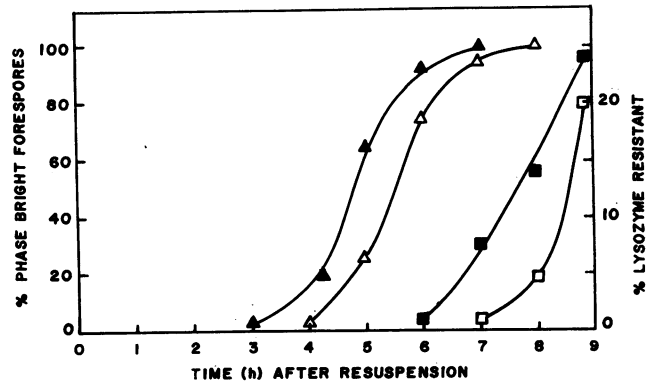

FIG. 3. Acquisition of phase-bright forespores and lysozyme resistance by sporulating cells of DB2 (open symbols) and DB227 (closed symbols). Sporulation was induced by resuspension with Sterlini-Mandelstam medium (10). Triangles, Percentage of phase-bright forespores observed microscopically at the indicated times; squares, number of lysozyme-resistant CFU (200 μg of lysozyme per ml for 20 min at 37°C in 50 mM sodium phosphate buffer, pH 7.4) expressed as a percentage of the number of lysozyme-resistant CFU after 24 h.

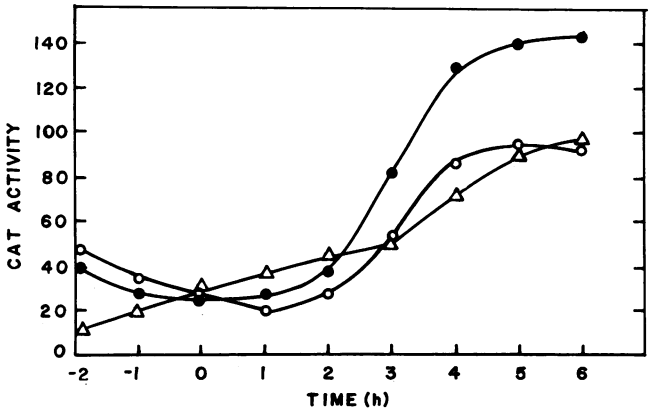

FIG. 4. Time course of CAT expression during growth and sporulation of the mutant strains. Cultures were grown in 2× SG sporulation medium (5). Time 0 is the end of the log phase of growth. The level of CAT activity was assayed on cell extracts, prepared by sonication, according to the procedure of Shaw (9). CAT specific activity is measured as nmoles of chloramphenicol acetylated per minute per milligram of protein. Open circles, DB226; closed circles, DB227; triangles, DB228. There was no endogenous CAT activity present in cell extracts of DB2.

present within DB227 sporulating cells is probably due to the removal of the sporulation-specific P3 promoter from the chromosome, as a consequence of deletion of the *P23* gene.

The three strains shown in Fig. 1 all carry a promoterless *cat* gene within *P23*. The levels of CAT are dependent upon the levels of expression from the upstream promoters. All three Cm^r strains and DB2 were grown and sporulated in 2× SG medium. Protein extracts were prepared and assayed for CAT activity. For all three mutants, DB226, DB227, and DB228, the CAT activity continued to increase during growth and into sporulation (Fig. 4). Moreover, for DB226 and DB227 there was an apparent induction of CAT expression at T_2. The effect was not so apparent for DB228. There was also significantly more CAT activity present in DB227 than in the other strains during sporulation.

Our observations suggest that for all mutant strains, promoter activity continues into sporulation. The only known promoters upstream of the *P23* gene are the σ^A-dependent P1 and P2 promoters identified by Wang and Doi (12). Their S1 mapping experiments on transcripts generated in DB2 indicate that expression from the P1 and P2 is rapidly switched off after the initiation of sporulation. The results from our CAT assays of *P23* mutants DB226 and DB227 suggest that expression from these promoters continues into sporulation. The CAT activity measured in DB228 reflects the expression from P1, P2, and P3 promoters, and the continued expression of CAT during sporulation may represent expression from P3 as P1 and P2 promoter expression stops. It appears that mutagenesis downstream of the P1 and P2 promoters can affect the transcriptional regulation of these promoters in our specific constructs.

Of the three Cm^r *P23* mutants constructed, only DB227 had a visible phenotype that distinguished it from the parental DB2 strain. One effect of the deletion in DB227 is to remove the sporulation-specific P3 promoter from the σ^A operon. This mutation leads to the highest accumulation of CAT of the three mutants during sporulation, suggesting a higher rate of transcription from the P1 and P2 promoters. Deletion of the P3 promoter, or of some DNA sequence closely associated with the promoter, may be important in regulating P1 and P2 expression.

It is likely that the mutation in DB227 would have pleiotropic effects on the levels of DnaE and σ^A during the sporulation phase. The higher levels of CAT during sporulation may also mean the presence of higher-than-normal levels of σ^A. Thus, the earlier-sporulation phenotype may be due to earlier expression of sporulation-specific genes. It has been shown that σ^A functions during the sporulation phase and can transcribe the *spoIIG* operon (C. Moran, personal communication) and the *aprE* gene (7). It may also be involved in postexponential expression of the *spoIIE* gene (4).

We are currently attempting to quantitate the RNA and protein levels of σ^A and DNA primase in order to relate these levels to the sporulation phenotype of our Cm^r mutants. We are also attempting to determine whether the P3 promoter is expressed in strains DB226 and DB228 and whether the temporal control of its expression has remained unchanged.

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