

Deoxyribonucleic Acid-Binding Proteins in Vegetative *Bacillus subtilis*: Alterations Caused by Stage O Sporulation Mutations

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Deoxyribonucleic acid (DNA)-binding proteins have been compared between wild-type *Bacillus subtilis* and five sporulation mutants blocked at different stage O loci. Extracts from exponentially growing cells have been fractionated for proteins binding to single-stranded calf thymus DNA-cellulose and double-stranded *B. subtilis* DNA-cellulose. In nutrient broth, stage O mutations cause an accumulation of proteins with affinity for double-stranded DNA. Suppression of the mutation with extragenic suppressors relieves the accumulation. In minimal glucose medium, the stage O mutations also cause accumulation of proteins with affinity for double-stranded DNA, but the species accumulated are different from those of nutrient broth-grown cells. In neither case did stage O mutations affect proteins with affinity for single-stranded DNA. The results suggest that the products of stage O loci are functional and operative during vegetative growth.

The blockage of sporulation by mutation and the genetic analysis of these mutations has led to a genetic map of sporulation genes on the *Bacillus subtilis* chromosome (7, 8, 11). Although sporulation mutations can be classified as to stage of the block, effect on bacteriophage replication, appearance of sporulation-specific properties, and other parameters, the nature of the primary defect has not been determined for any sporulation locus. Stage O mutations are especially obscure since a single-point mutation in any one of at least five loci leads to a complete cessation of sporulation and its related enzymes (8, 11). Comparison of the structural components of the cell wall and membrane of stage O mutants with the wild type did not reveal any differences between them (2).

In previous studies we have shown that proteins with affinity for deoxyribonucleic acid (DNA) are developmentally regulated during sporulation of *B. subtilis*. The number and kind of DNA-binding proteins with specificity either for single- or double-stranded DNA appeared to be unique for each stage of sporulation examined (1). Since the results suggested sporulation stage specificity for many of the proteins, we have examined sporulation-deficient mutants for modifications in DNA-binding proteins. In the present study, mutants bearing stage O

mutations at different loci were compared to their sporulating parent for proteins with affinity for single- or double-stranded DNA. It was found that stage O mutations in cells growing logarithmically had little effect on the profile of proteins binding to single-stranded DNA. On the other hand, all stage O mutants accumulated unique DNA-binding proteins specific for double-stranded DNA. The results suggest that stage O mutations affect regulatory processes in vegetative growth.

MATERIALS AND METHODS

Strains and growth conditions. Table 1 lists the strains used throughout this study. All strains were prepared by transforming DNA from the primary mutant isolate into a standard strain. Thus, strains JH642 to JH651 are in isogenic backgrounds and differ only by the relevant sporulation mutation. The suppressor-carrying strains have been described previously (4, 6). Overnight cultures (2 liters) were grown in either the nutrient broth medium described by Schaffer et al. (12) or Spizizen's media (13) supplemented with glucose (0.5%), acid-hydrolyzed casein (0.01%), and the appropriate amino acids (20 $\mu\text{g}/\text{ml}$) and were used to inoculate 120 liters of the same medium with vigorous aeration and agitation in a fermenter tank (150-liter capacity). Growth was monitored with a Klett-Summerson colorimeter (660 nm), and cells were harvested at an optical density of 0.2 for nutrient broth-grown cells or 0.3 for minimal media-grown cells. The cells were harvested by centrifugation in a Sharples super centrifuge, washed once with cold 10 mM KPO₄ buffer (pH 7.5) containing 1 mM ethylenedia-

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TABLE 1. *Strains used*

Strain	Genotype	Phenotype
JH642	trpC2 phe-1	Trp ⁻ Phe ⁻ Spo ⁺
JH646	trpC2 phe-1 <i>spoOA12</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH647	trpC2 phe-1 <i>spoOE11</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH648	trpC2 phe-1 <i>spoOB136</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH649	trpC2 phe-1 <i>spoOF221</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH650	trpC2 phe-1 <i>spoOF124</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH651	trpC2 phe-1 <i>spoOH81</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH89	trpC2 phe-1 <i>spoOB149</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH195	trpF7 phe-1 <i>spoOB136</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH197	trpF7 phe-1 <i>spoOB123</i>	Trp ⁻ Phe ⁻ Spo ⁻
JH209	trpF7 phe-1 <i>spoOB136 sup-3</i>	Phe ⁻ Spo ⁺
JH213	trpF7 phe-1 <i>spoOB123 sup-3</i>	Phe ⁻ Spo ⁺
JH232	trpF7 phe-1 <i>spoOB123 sup-13</i>	Phe ⁻ Spo ⁺

minetetraacetic acid, 10 mM β -mercaptoethanol, 5% glycerol, and 10^{-5} M phenylmethylsulfonyl fluoride (buffer I), and stored at -20 C until used.

Isolation of DNA. Calf thymus DNA (360 mg) was obtained commercially (Sigma, type V), dissolved in 1 mM NaCl (180 ml), and denatured by heating to 80 C followed by quick cooling on ice. The sodium dodecyl sulfate (SDS)-protease K method of Gross-Bellard et al. (5) was used to isolate DNA from spheroplasts of *B. subtilis*. Spheroplasts were obtained by incubating 20 g (wet weight) of strain JH642 in 100 ml of 10 mM tris(hydroxymethyl)aminomethane (pH 7.5), 3 mM succinic acid, 10 mM MgCl₂, and 500 mM sucrose with lysozyme (2 mg/ml; Sigma) at 37 C for 2 h. The spheroplasts were pelleted by centrifugation and washed once with the same buffer. The method of Litman (10) was used to prepare DNA-cellulose.

Extraction of DNA-binding proteins. The procedure for the isolation of DNA-binding proteins from *B. subtilis* has been described (1). Minor modifications of the original procedure include only one passage through the Eaton press, followed by precipitation of DNA with 10% polyethylene glycol in the presence of 1.7 M KCl and centrifugation for 1 h at 15,000 rpm in the SS-34 rotor. The DNA-free supernatant fluids were applied to a column containing phosphocellulose equilibrated with buffer I. Proteins were eluted with 1 M KCl in buffer I, dialyzed against 10 mM KPO₄ buffer (pH 6.8) containing the same additives as buffer I (buffer II), and applied to a column containing single-stranded calf thymus DNA-cellulose (1 mg of protein per 50 mg of single-stranded calf thymus DNA-cellulose). That fraction that was not adsorbed by single-stranded calf thymus DNA-cellulose was applied to double-stranded *B. subtilis* DNA-cellulose (1 mg of protein per 25 mg of *B. subtilis* DNA-cellulose). Elution of both columns was achieved with 2.0 M NaCl in buffer II.

SDS slab gel electrophoresis. SDS slab gel electrophoresis was performed using the system of Laemmli (9), and the gels were stained by the method of Fairbanks et al. (3). Protein samples to be analyzed were dialyzed against 0.02% SDS for 6 to 8 h at room temperature, lyophilized to dryness, dissolved in 10 mM NaPO₄ (pH 7.0), 1% SDS, and 1% β -mercaptoethanol (final protein concentration, 2

mg/ml), and boiled before electrophoresis. Each sample contained 50 μ g of protein.

RESULTS

Comparison of proteins from log-phase cells of the wild type and mutants grown in nutrient broth. Mutants blocked early in the sporulation process and wild-type cells were grown to the mid-exponential phase of growth in nutrient broth, and DNA-binding proteins were fractionated by their ability to bind to single-stranded calf thymus DNA-cellulose or double-stranded *B. subtilis* DNA-cellulose. Figure 1 shows the profile of proteins with affinity for single-stranded calf thymus DNA-cellulose from the wild type and six sporulation negative mutants as analyzed on SDS-polyacrylamide (12.5%) slab gels. The band corresponding to a molecular weight of 60,000 is the major species in all of the strains. There is a group of proteins in the region of 35,000 to 43,000 molecular weight that is also similar in all of the strains, although there may be some quantitative differences in the proteins from *spoOF* and *spoOH* (channels 7 and 8, respectively). Next there is a space devoid of protein in all of the strains, followed by a group with molecular weights in the region of 23,000 to 33,000. The profiles are similar regardless of the ability of the strain to sporulate.

The profiles of proteins with affinity for double-stranded *B. subtilis* DNA-cellulose from the wild type (channel 2) and two *spoOB* mutants (*spoOB* 136 and *spoOB* 149; channels 3 and 4, respectively) are presented in Fig. 2. The profiles have several similarities. The group of proteins in the molecular weight range between 68,000 and 79,000 appears equivalent, as do the next group in the 55,000- to 65,000-molecular-weight range. The lower-molecular-weight species appear similar, except perhaps for quantitative differences. The region of greatest interest occurs between 35,000 and 45,000 molecular weight. In this region the sporulation-deficient mutants accumulate several protein species that the wild type does not. The profiles of double-stranded *B. subtilis* DNA-binding proteins from other sporulation-negative mutants are shown in Fig. 3. The double-stranded *B. subtilis* DNA-binding protein profile from an *spoOE* mutant is shown in channel 1, the profile of a mutant blocked in the *spoOF* locus is shown in channel 2, channel 3 shows the profile of a mutant blocked in the *spoOA* locus, and channel 6 shows the profile of a mutant blocked in the *spoOB* locus. The mutant profiles appear to be similar in all the early mutants examined. They all accumulate

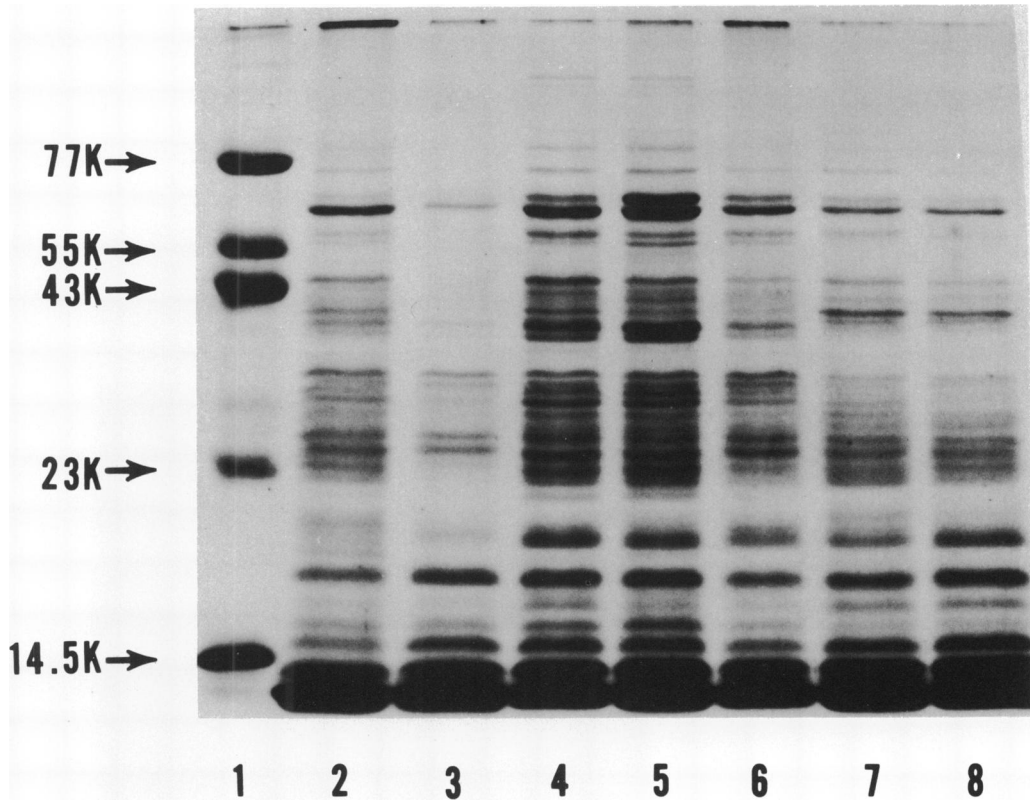


FIG. 1. SDS gel electrophoresis of single-stranded calf thymus DNA-binding proteins from the wild type (JH642) and sporulation-negative mutants in nutrient broth at mid-exponential growth phase. Channel 1, Molecular weights ($\times 10^3$) of protein standards: 77K = conalbumin; 55K = immunoglobulin G heavy chain; 43K = ovalbumin; 23.5K = immunoglobulin G light chain; and 14.5K = ribonuclease. Channel 2, Strain JH642; channel 3, strain JH646; channel 4, strain JH648; channel 5, strain JH650; channel 6, strain JH647; channel 7, strain JH649; channel 8, strain JH651.

proteins in the region immediately below the 43,000-molecular-weight marker. This accumulation was not observed in the wild-type profile (Fig. 1, channel 2). Although slight quantitative differences are apparent in the different mutants, the *spoOA* mutant (channel 3) accumulates a protein of 39,000 molecular weight, indicated by the arrow on the left side of the figure, which the other mutants do not.

This figure also contains the profile of proteins with affinity for double-stranded *B. subtilis* DNA-cellulose from mutants whose sporulation defect has been reversed by the introduction of two different suppressors, *sup-13* and *sup-3*. The sporulation frequency is restored to near normal values by the *sup-3* suppressor, whereas *sup-13* is a weak suppressor of this mutation. The profile of double-stranded *B. subtilis* DNA-binding proteins from the transformants carrying *sup-13* and *sup-3* are shown in

channels 4 and 5, respectively. In the weakly suppressed strain (*sup-13*, channel 4), the profile is virtually identical to that found in *spoOB* mutants (channel 6), with an accumulation of proteins in the 35,000- to 45,000-molecular-weight region. However, by the introduction of a strong suppressor (*sup-3*, channel 5) the profile is returned to one characteristic of the wild type, with no accumulation of proteins in the 35,000- to 45,000-molecular-weight region.

The purification scheme was designed to discriminate between proteins with affinity for denatured heterologous DNA and native *B. subtilis* DNA. The genetic constitution of the cells from which the DNA-binding proteins were extracted is reflected specifically in the native *B. subtilis* DNA-cellulose eluates.

Comparison of proteins from log-phase cells of the wild type and mutants grown in minimal medium. To confirm that the changes

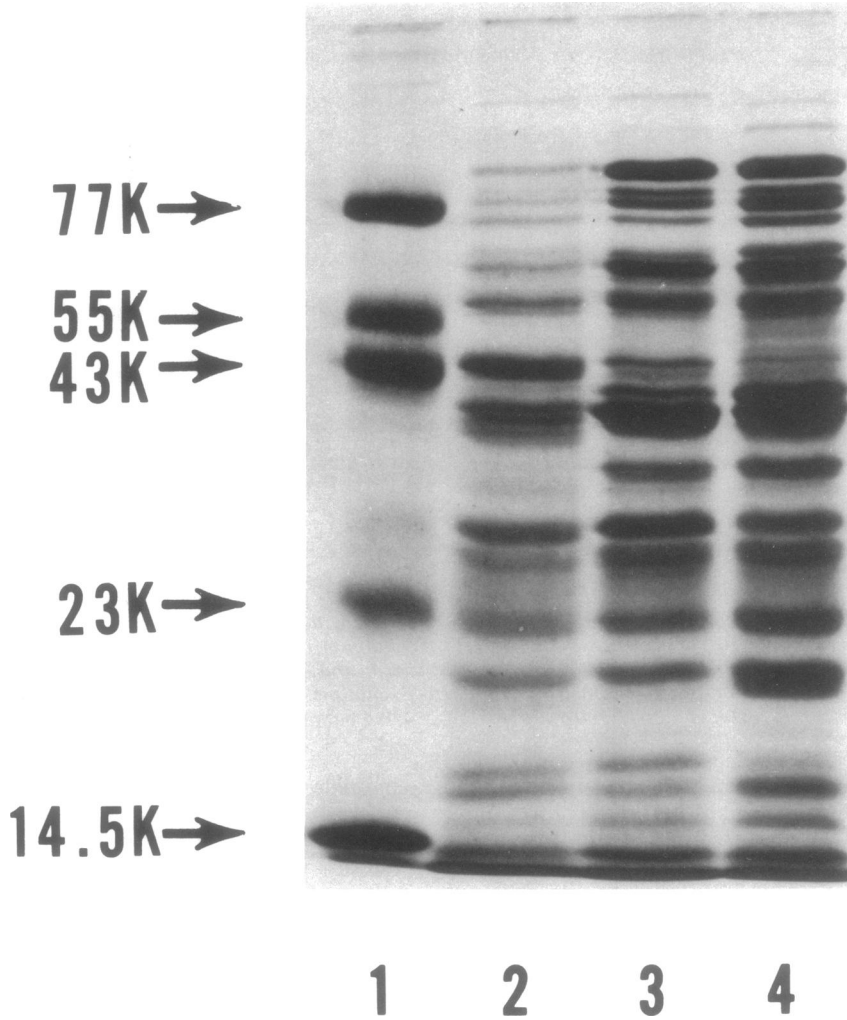


FIG. 2. SDS gel electrophoresis of double-stranded *B. subtilis* DNA-binding proteins from the wild type (JH642) and two *spoOB* mutants in nutrient broth at mid-exponential growth phase. Channel 1, Molecular weight standards as in Fig. 1; channel 2, strain JH642; channel 3, strain JH648; channel 4, strain JH89.

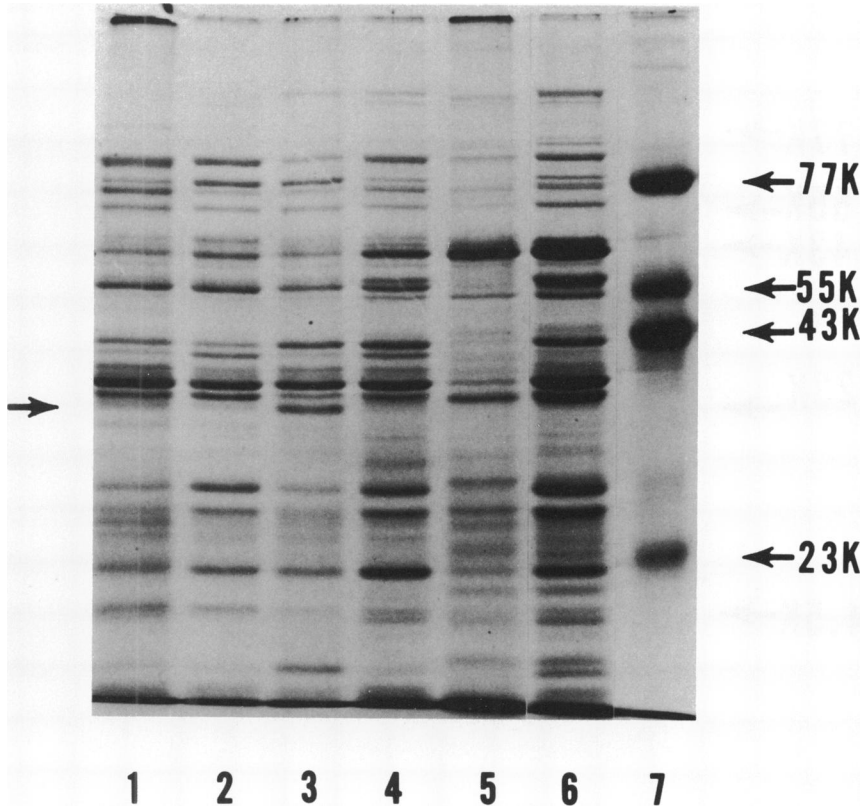


FIG. 3. SDS gel electrophoresis of double-stranded *B. subtilis* DNA-binding proteins from sporulation-negative mutants and suppressor-carrying strains in nutrient broth at mid-exponential growth phase. Channel 1, Strain JH647; channel 2, strain JH649; channel 3, strain JH646; channel 4, strain JH232; channel 5, strain JH213; channel 6, strain JH648; channel 7, molecular weight standards as in Fig. 1. The arrow on the left indicates the molecular weight position of 39,000.

observed in the mutants were growth related, a similar analysis was carried out under conditions of severe sporulation repression, i.e., log-phase cells in minimal medium with glucose as carbon source. The wild type, an *spoOB* mutant, and an *spoOA* mutant were subjected to this regimen and fractionated as described above. Figure 4 shows the proteins binding to either single- or double-stranded DNA from these strains. Proteins binding to single-stranded DNA are similar in the wild type and the two mutant strains, with a few quantitative changes. Comparison of the profiles with those from nutrient broth-grown cells (Fig. 1) reveals that there are a number of media-dependent qualitative changes in proteins of various molecular weights. This result is true for double-stranded DNA-binding proteins also (cf., Fig. 3). Especially noticeable in this regard is the absence of protein accumulation in the mutants grown in minimal medium. In addition, qualita-

tive changes in the wild type and mutants is observed in proteins of less than 20,000 molecular weight.

Comparison of heterologous and homologous native DNA-binding proteins from the wild type and *spoOA*. Since the major differences between stage 0 mutants and the wild type were observed in proteins binding to double-stranded DNA, an experiment was undertaken to determine if unique species exhibit differential binding to heterologous and homologous native DNA. The wild type and an *spoOA* mutant were grown in minimal medium and harvested in log phase. Crude extracts were prepared as described, and DNA was removed with polyethylene glycol. The DNA-free solution was applied to a double-stranded calf thymus DNA-cellulose column. The fraction that was not adsorbed to this column was applied to a double-stranded *B. subtilis* DNA-cellulose column. Both columns were washed extensively

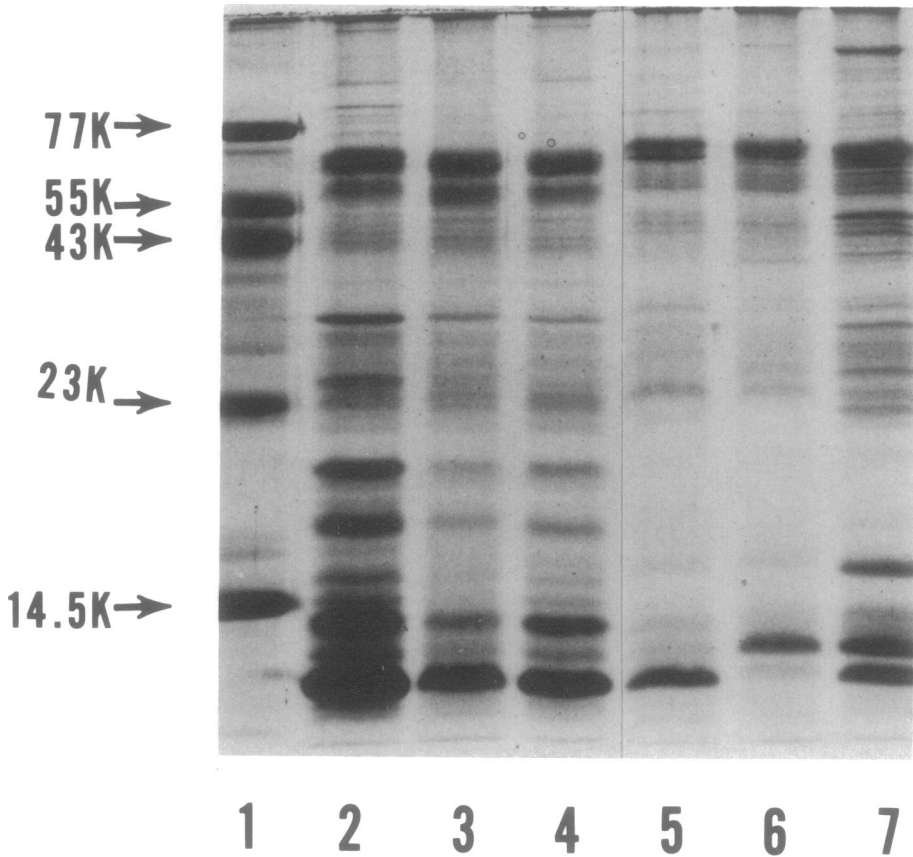


FIG. 4. SDS gel electrophoresis profiles of DNA-binding proteins from the wild type and sporulation-negative mutants in minimal media at the mid-exponential growth phase. Channel 1, Molecular weight standards as in Fig. 1; channel 2, single-stranded calf thymus DNA-binding proteins from strain JH642; channel 3, single-stranded calf thymus DNA-binding proteins from strain JH646; channel 4, single-stranded calf thymus DNA-binding proteins from strain JH648; channel 5, double-stranded *B. subtilis* DNA-binding proteins from strain JH642; channel 6, double-stranded *B. subtilis* DNA-binding proteins from strain JH646; channel 7, double-stranded *B. subtilis* DNA-binding proteins from strain JH648.

and eluted with 2 M NaCl. Figure 5 shows that protein profiles of the fractions obtained.

The profiles of proteins that were eluted from double-stranded calf thymus DNA-cellulose from the wild type (channel 2) and the *spoOA* mutant (channel 3) look nearly identical. The most noticeable difference occurs at the position corresponding to a molecular weight of approximately 43,000, where there is a minor band in the wild type that may be present in the mutant, but, if so, is much reduced. This protein is more pronounced in the profiles of proteins eluted from double-stranded *B. subtilis* DNA-cellulose from the same strains. A doublet at a molecular weight of 43,000 is seen in the wild type (channel 4), and the lower band is not detectable in the stage O mutant (channel 5).

This protein appears to prefer *B. subtilis* DNA to calf thymus DNA and may represent the first missing DNA-binding protein that we have observed in stage O mutants.

DISCUSSION

The present study was undertaken to determine if differences could be demonstrated in the DNA-binding protein fraction from stage O sporulation mutants compared with their sporulating parent. Extracts from cells grown to the mid-exponential phase of growth in a modified nutrient broth were fractionated by their ability to bind to single-stranded calf thymus DNA-cellulose or double-stranded *B. subtilis* DNA-cellulose. Analysis of that fraction of proteins binding to a single-stranded calf thymus DNA

from the wild type and stage O mutants did not reveal any significant alterations due to the sporulation defect. On the other hand, that fraction of proteins binding to double-stranded *B. subtilis* DNA-cellulose from the wild type and stage O mutants was considerably affected by the sporulation defect. Mutations at five different stage O loci gave essentially the same result: the accumulation of certain DNA-binding proteins in the molecular weight range of 35,000 to 45,000. No missing proteins resulting

from the sporulation mutation were observed. Introduction of a nonsense suppressor into a mutant bearing a nonsense stage O defect results in restoration of the ability to sporulate and a return to the wild-type profile of DNA-binding proteins in the double-stranded *B. subtilis* DNA-cellulose fraction. Thus, the accumulation of DNA-binding proteins observed is a general effect of stage O mutations, and this effect is expressed in the log phase of growth.

A similar analysis between wild type and

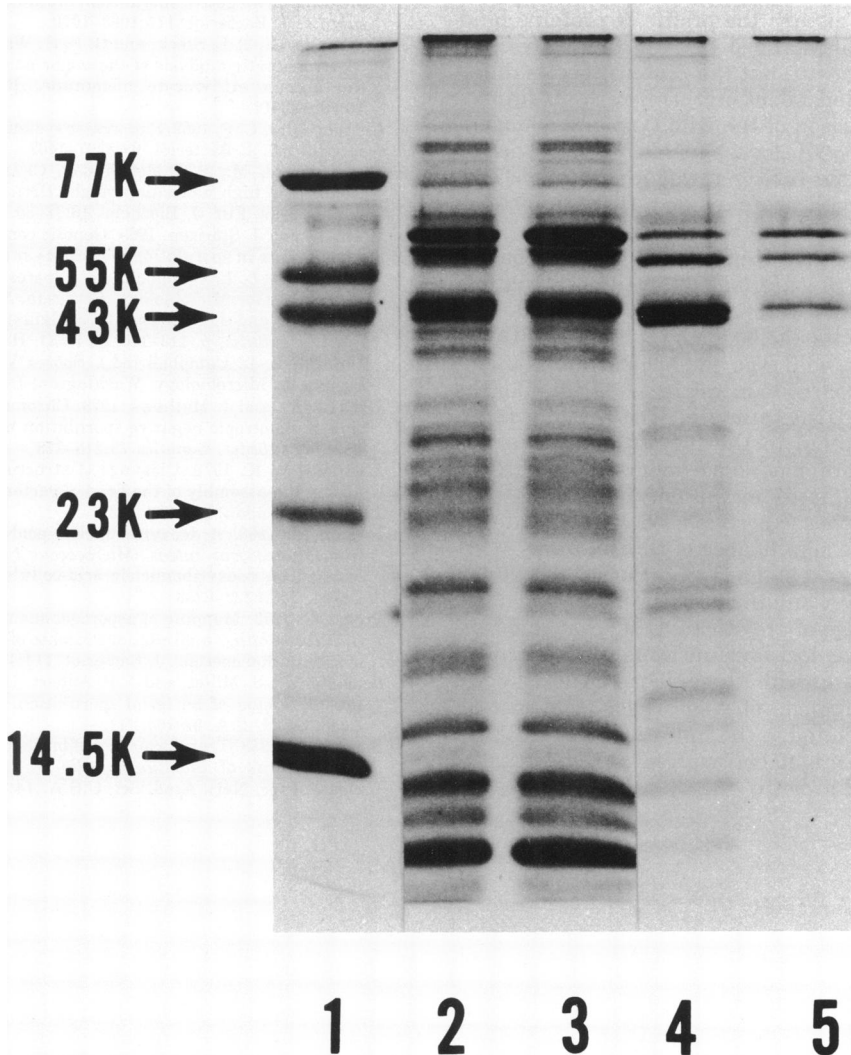


FIG. 5. SDS electrophoresis profiles of native DNA-binding proteins from the wild type (JH642) and *spoOA* (JH646) in minimal media at the mid-exponential growth phase. Channel 1, Molecular weight standards as in Fig. 1; channel 2, double-stranded calf thymus DNA-binding proteins from strain JH642; channel 3, double-stranded calf thymus DNA-binding proteins from strain JH646; channel 4, double-stranded *B. subtilis* DNA-binding proteins from strain JH642; channel 5, double-stranded *B. subtilis* DNA-binding proteins from strain JH646.

stage O mutants was carried out in minimal glucose medium to be certain that sporulation was as repressed as possible. Similar results were obtained. The single-stranded DNA-cellulose fraction appeared identical in the wild type and stage O mutants, whereas some accumulation of DNA-binding proteins was observed in the fraction binding to double-stranded DNA. There were fewer protein species accumulating in this medium, however, and those proteins that accumulated were not identical to those accumulating in nutrient broth. Thus, stage O mutations disturb the profile of proteins binding to double-stranded DNA in the logarithmic phase of growth, but the protein species differences are dependent on the growth medium.

A comparison of the wild type and a stage O mutant (*spoOA* locus) for *B. subtilis*-specific DNA-binding proteins was undertaken. Extracts of both were passed through double-stranded calf thymus DNA-cellulose columns, and that fraction which did not bind was passed through a *B. subtilis* DNA-cellulose column. Both columns were eluted and analyzed. One protein of the 43,000 molecular weight was found in the wild type and not the mutant. This protein appeared to have greater affinity for the *B. subtilis* DNA-cellulose. This loss of a DNA-binding protein in the mutant was never observed in previous experiments, although the phosphocellulose step was omitted in this experiment. Further experiments are required to determine the significance of this protein.

The fact that stage O mutations cause alterations in DNA-binding proteins during the logarithmic stage of growth suggests that the products of these loci are functional and operative during this growth stage. The results suggest that the products of stage O loci may not directly be related to the initiation of sporulation but rather might be involved in the control of normal cell growth.

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LITERATURE CITED

1. Brehm, S. P., F. Le Hegarat, and J. Hoch. 1974. Developmental modulation of deoxyribonucleic acid-binding proteins of *Bacillus subtilis* during sporulation stages. *J. Bacteriol.* 120:1443-1450.
2. Brehm, S. P., S. Staal, and J. Hoch. 1973. Phenotypes of pleiotrophic-negative sporulation mutants of *Bacillus subtilis*. *J. Bacteriol.* 115:1063-1070.
3. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606-2617.
4. Georgopoulos, C. P. 1969. Suppressor system in *Bacillus subtilis* 168. *J. Bacteriol.* 97:1397-1402.
5. Gross-Bellard, M., P. Oudet, and P. Chambon. 1973. Isolation of high molecular weight DNA from mammalian cells. *Eur. J. Biochem.* 36:32-38.
6. Hoch, J., and J. Spizizen. 1969. Genetic control of some early events in sporulation of *Bacillus subtilis* 168, p. 112-120. In L. L. Campbell (ed.), *Spores IV*. American Society for Microbiology, Bethesda, Md.
7. Hoch, J. A., and J. Mathews. 1972. Genetic studies in *Bacillus subtilis*, p. 113-116. In H. O. Halvorson, R. Hanson, L. L. Campbell (ed.), *Spores V*. American Society for Microbiology, Washington, D.C.
8. Hoch, J. A., and J. Mathews. 1973. Chromosomal location of pleiotropic negative sporulation mutations in *Bacillus subtilis*. *Genetics* 73:215-228.
9. Laemmli, V. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
10. Litman, R. 1968. A deoxyribonucleic acid polymerase from *Micrococcus luteus* (*Micrococcus lysodeikticus*) isolated on deoxyribonucleic acid-cellulose. *J. Biol. Chem.* 243:6222-6233.
11. Piggot, P. 1973. Mapping of asporogenous mutations of *Bacillus subtilis*: a minimum estimate of the number of sporulation operons. *J. Bacteriol.* 114:1241-1253.
12. Schaeffer, P., J. Millet, and J.-P. Aubert. 1965. Catabolite repression of bacterial sporulation. *Proc. Natl. Acad. Sci. U.S.A.* 54:704-711.
13. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. U.S.A.* 44:1072-1078.