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Genetic Analysis of Pleiotropic Negative Sporulation Mutants in *Bacillus subtilis*

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Genetic studies were undertaken on 14 pleiotropic negative sporulation mutants. These mutants (*spoA*) which are blocked early in the sporulation process were found to map near the terminus of the *Bacillus subtilis* chromosome in a region enriched in genes involved in spore formation. Two- and three-factor crosses by transduction and transformation led to the conclusion that the pleiotropic *spoA* mutations formed a linked cluster. The genetic distance across the cluster calculated from transformation data was compatible with the mutant sites defining a single gene. Suppressor studies revealed that either a nonsense or missense mutation in the *spoA* locus generated a pleiotropic negative phenotype. It was concluded that the locus codes for a protein, and the absence of this protein is responsible for the pleiotropic phenotype.

Sporulation in *Bacillus* species is a complex process in which a vegetative cell undergoes a series of morphological and biochemical changes to emerge as a dormant heat-resistant spore. Studies of the cytology of this process have shown that the morphological events occur in a distinct sequential order (16, 20). The intermediate forms are the basis for the assignment of stage numbers to the order. Thus the vegetative cell (stage 0) progresses to the spore (stage VII). A rough correlation exists between the stage of development and the appearance of certain biochemical events (10). The excretion of proteolytic enzymes and antibiotic(s) was shown to occur at stage I, whereas dipicolinic acid production commences at about stage IV. The specific activity of many enzymes fluctuates during the stages of development [For reviews, see Kornberg et al. (8) and Schaeffer (17).]

In an attempt to understand the regulation of this development scheme, a large number of mutants defective in the process were isolated. Mapping studies of these mutants in *Bacillus subtilis* by Rogolsky (15) and Ionesco et al. (7) showed that sporulation genes may be found at many locations on the *B. subtilis* genome but that the majority of mutant sites mapped near the terminus of the chromosome. In both studies, approximately 75% of the sporulation-defective mutant sites were in this region between the *phe* and *lys* markers. Representatives of many different stages may be found among these mutants. Ionesco et al. (7) found mutants blocked at stage II, III, and IV in the sporulation process. Also included in this region were mutants in which development was arrested at stage 0. The phenotypes of such mutants were described by Spizizen (19) and include the inability to produce extracellular protease and antibiotic activities. Similar mutants were characterized by Ryter et al. (16) as stage 0 by the fact that sporulation stops at the axial filament stage, i.e., prior to stage I. In a previous report (5), we described the phenotypes and some properties of these pleiotropic sporulation mutants. In the studies reported here, the genetic fine structure and the genetic basis for the pleiotropic phenotype of *spoA* mutants were investigated.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study were derivatives of the transformable *B. subtilis* 168 (Table 1). The *spoA* mutants were obtained by ultraviolet irradiation of this strain.

Phage. SPO1 and the suppressor dependent mutant SPO1 *sus5* 13 were obtained from S. Okubo. Lysates of SPO1 *sus5* were obtained by growth on the suppressor bearing strain, *su*$^+$3 of Georgopoulos (3). ϕ15 was isolated by B. Reilly in this laboratory. Lysates of ϕ15 were prepared on SR22.

Culture media. The minimal medium was that of Spizizen (18) supplemented with 0.01 mM $MnCl_2$. Toplayer agar was prepared by the method of Reilly and Spizizen (14). TBAB was Tryptose Blood Agar Base (Difco). PAB was Antibiotic Medium no. 3 (Difco), and AK was AK Agar no. 2 (BBL) supplemented with 5 mM $MgSO_4$. The commercial media was prepared according to the manufacturer's formula in deionized water.

Transformation. The transformation procedure followed that of Anagnostopoulos and Spizizen (1). Recip-

rocal transformation crosses among *spoA* mutants were performed as described in an accompanying paper (6). Deoxyribonucleic acid (DNA) was prepared according to Marmur (11).

Transduction. Donor strains were inoculated into PAB (15 ml in a 125-ml flask) from an overnight TBAB plate. After 2 to 3 hr of shaking at 37 C, the cells became motile, and the culture was diluted 1/10 into fresh PAB (25 ml in a 250-ml flask). Various dilutions of a PBS1 stock lysate were added to give a multiplicity of infection from 0.1 to 1.0. The culture was shaken at 37 C for 1 hr, at which time chloramphenicol was added to a final concentration of 5 μg/ml. After 2 additional hr of shaking, the lysates were placed at 37 C overnight. The lysates were centrifuged, treated with deoxyribonuclease (10 μg/ml), sterilized by filtration through membrane filters (Millipore Corp.), and stored at 4 C. Lysates of asporogenous mutants had, in general, a better transducing titer with lower multiplicities of input phage.

Recipient strains were inoculated into PAB from an overnight TBAB plate and incubated with shaking at 37 C for 4 to 5 hr. This culture (0.5 ml) and donor phage (0.1 to 0.5 ml) were incubated at 37 C for 20 min, centrifuged, suspended in 1 ml of minimal medium, and plated on selective media.

Scoring of crosses. Recombinant classes bearing *spoA* mutants were distinguished from *spo-22* and wild classes by colony morphology. Colonies of *spoA* mutants are enlarged and transparent, whereas *spo-22* and wild colonies are normal size and opaque. Wild and *spo-22* classes were distinguished by a replica-plating technique utilizing the differential sensitivity of vegetative cells and spores to organic solvents. In the three-factor crosses of Table 4, *lys*$^+$ transductants were selected on minimal medium. After 48 hr of incubation at 37 C, the normal size opaque clones were picked with sterile toothpicks, streaked on the same selective medium, and incubated overnight at 37 C. This master plate was replica plated to a TBAB plate by means of sterile velvet. Two milliliters of chloroform was pipetted into the lid of the inverted TBAB plate, and the plate was left at room temperature until the chloroform evaporated. After overnight incubation at 37 C, the plates were scored for growth. Sporulating strains gave confluent growth within the imprint of the original streak, whereas no growth was apparent for nonsporulating strains.

Recombination values in crosses were determined as previously described (4).

Phage sensitivity assay. Sensitivity to ϕ15 or SPO1*sus5* was determined by the soft-agar layer technique. Phage (300 plaque-forming units) and the appropriate logarithmically growing indicator (0.1 ml) were mixed with 2.0 ml of top-layer agar and poured on TBAB plates. After 12 to 16 hr of incubation at 37 C, the plates were scored for plaque formation.

Construction of suppressor strains. To test the effect of suppressors on *spoA* mutations, strains were constructed bearing the *spoA* mutation under study and the suppressible *trpF7* mutation of Anagnostopoulos (*personal communication*). DNA isolated from *su*$^+$3 and SR22R13 were used at low concentration to transform these strains to tryptophan independence. Ten *trp*$^+$ clones from each cross were purified by single colony isolation and tested for the presence of the suppressor by plating with SPO1*sus5*. Two independent suppressed strains were further characterized for sporulation phenotype.

Scoring of sporulation phenotypes. The scoring of protease and antibiotic production was described by Spizizen (19). Qualitative determination of sporulation was accomplished by streaking the strain on AK agar, incubating at either 37 or 25 C for 48 hr, and microscopically determining the presence or absence of spores.

RESULTS

Sporulation defective phenotypes. Mutants isolated as defective in the ability to produce spores may be categorized as "early" or "late" by biochemical criteria. Assuming that some biochemical events are specific for the sporulation process, the presence or absence of these traits should indicate the degree to which a mutant is capable of developing toward the finished spore. Mutants blocked at the end of the process may be unable to construct a finished spore but may still be capable of elaborating the enzymes required earlier in the process. The excretion of extracellular protease and antibiotic are two of the earliest known biochemical events occurring in the sporulation process (10, 12). Nonsporulating mutants capable of producing protease and antibiotic were designated as "late" mutants. This broad class of mutant type was shown to map at many distinct sites on the chromosome (5, 7, 15). Those mutants unable to elaborate even these early biochemical events were also isolated and are designated as "early" mutants.

Several phenotypes are associated with mutant sites designated as "early." Extracellular protease is not present in culture fluids but it is not certain whether this is due to nonproduction or inability to excrete the enzyme. Some small zones of clearing are apparent on azo-albumin plates with these mutants, indicating that they are still capable of producing a protease(s) of some kind. Antibiotic production, on the other hand, appears to be completely curtailed. These mutants are also distinguished by their ability to plaque ϕ15. This phage is capable of growth in liquid, under certain conditions, on both the wild type and

TABLE 1. *Basic strains used in this study*

Strain	Genotype	Origin
168	*trpC2*	Our collection
W168	prototroph	Our collection
60154	*lys-1*	E. Freese
SR22	*trpC2 spoA12*	Our collection
SR22 R13	*trpC2 spoA12 sup-13*	Our collection
T7	*trpF7*	C. Anagnostopoulos
su+3	*thr-5 met-3 leu-8 sup-3*	C. Georgopoulos
22R	*trpC2 spo-22*	M. Rogolsky

asporogenous mutants but will not plaque on the wild type or "late" mutants. The reason for this phenomenon is unknown but may be due to receptor modulations associated with sporulation. A most distinctive phenotypic characteristic is the greatly enlarged colony size of these mutants on solid media (19). The colonies are large and transparent, whereas "late" mutant and wild-type colonies are smaller and opaque.

Genetic location. We previously described a group of early mutants which map in the spore gene region near the *lys* marker (5). Ionesco et al. (7) described similar mutants. The data for two-factor crosses between *lys-1* and 14 early mutants, designated *spoA*, are given in Table 2. These data and further crosses with other auxotrophic markers in the region indicate that all of the *spoA* mutant sites map in approximately the same region of the chromosome. Due to fluctuations in recombination values with PBS1 transduction, and, in some cases, the small sample size of recombinants, these data do not distinguish the *spoA* sites from those late sporulation-defective mutant sites previously mapped in this region. To clarify whether the *spoA* sites were interspersed among these late sites or whether *spoA* mutations represented a distinct genetic locus, three-factor crosses were performed with *spo-22*, a late mutant mapping near the *spoA* sites and at one end of the group of late sites.

The three-factor crosses were accomplished by using a double mutant recipient containing *lys-1* and either the particular *spoA* mutation or *spo-22*. Donor phage were prepared on lys^+ strains mutant for either *spoA* or *spo-22*. After selection for lys^+, clones that were normal sized and opaque were picked and scored for sporulation to distinguish wild type from *spo-22* recombinants. The recombinant class lys^+ *spoA spo-22* cannot be distinguished by phenotype from a lys^+ *spoA* recombinant since the phenotype of *spo-22* depends on biochemical events absent in *spoA* strains. Thus, the order of *spoA* sites relative to *spo-22* and *lys-1* was determined from the number of true wild type to phenotypic wild type in the cross. Figure 1 shows the rationale for this analysis. In the cross *spoA* × *spo-22 lys-1*, the generation of wild type requires four crossover events if the order of the markers is *spo-22 spoA lys-1*, but only two events if the order is *spoA spo-22 lys-1* (Fig. 1). The opposite result holds for the *spo-22* × *spoA lys-1* cross. Assuming the quadruple crossover class to be least frequent, the order may be determined by comparison of the per cent wild type in the two crosses. By this method, the 14 *spoA* mutations were found to lie between *spo-22* and *lys-1* (Table 3), regardless of the recombination values obtained from two-factor analysis. Thus, these data indicate that the *spoA* mutant sites are located at one end of the cluster of late sites and are not interspersed at random among them.

TABLE 2. *Two-factor PBS1 transduction crosses between lys-1 and spo-mutants*

Recipient genotype[a]	Classes		Total clones	Recombination (%)
	lys^+ spo^-	lys^+ spo^+		
lys-1 spoA14	172	133	305	56
lys-1 spoA13	96	105	201	48
lys-1 spoA11	223	251	474	47
lys-1 spoA4	204	195	399	51
lys-1 spoA5	93	157	250	62
lys-1 spoA10	85	133	218	39
lys-1 spoA6	90	126	216	42
lys-1 spoA7	50	54	104	48
lys-1 spoA8	90	126	216	42
lys-1 spoA9	88	99	187	47
lys-1 spoA1	109	114	223	49
lys-1 spoA2	380	332	712	53
lys-1 spoA3	187	174	361	52
lys-1 spoA12	543	606	1149	47
lys-1 spo-22	118	122	240	49

[a] Donor was wild type.

Transformation analysis of spoA mutations. Fine structure analysis of closely linked mutant sites requires the use of transformation since recombination between closely linked sites in transduction is rare due to the large size of the transducing fragment carried by PBS-1. An extensive search was undertaken by means of in vitro mutagenized DNA to find an auxotrophic or conditional marker linked to the *spoA* region to use in this analysis. All of the experiments failed to produce such a mutant, although the same DNA gave good mutation for markers in other regions. Since a linked marker could not be found, the fine structure of the *spoA* region was analyzed by means of the recombination index method. A *spoA trpC2* or *spoA metB4* recipient was transformed with nonsaturating amounts of DNA from a different *spoA* mutant. Both trp^+ or met^+ and spo^+ were selected, and the per cent recombination between the *spoA* markers was calculated from the frequency of each class (6).

The results of this analysis using three recipients and all 14 *spoA* donors are presented in Table 4. The recombination between pairs of *spoA* mutations varied from 0.6 to 12.5. In the case of the *spoA9-spoA11* pair, the recombination was 23.0. This value is probably overestimated because the recombination of *spoA9-spoA12* is only 2.2 and that of *spoA12-spoA11* is 1.0. The analysis also showed that *spoA11* and *spoA13* are identical since they did not recombine. The recombination values compare well to those found by Barat et al. (2) for recombination within the *ilvA* locus by the recombination index method.

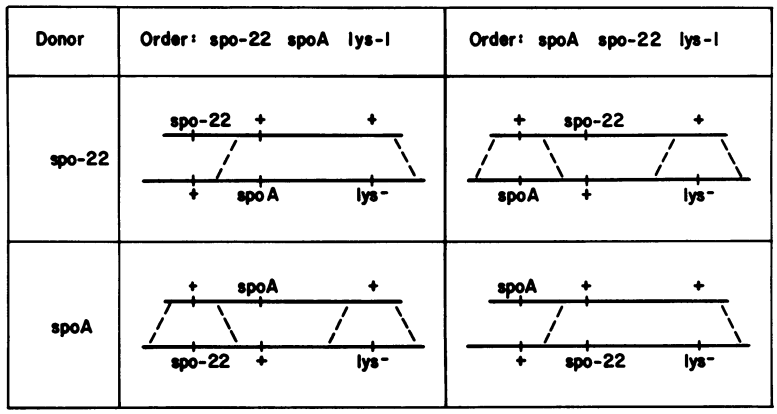

FIG. 1. *Crossover events required to generate wild-type recombinants.*

TABLE 3. *Three-factor PBS1 transduction crosses*

Recipient genotype	Donor genotype	Total phenotypic wild clones analyzed	Classes		Per cent *spo*$^+$
			lys$^+$ *spo-22*	*lys*$^+$ *spo*$^+$	
lys-1 spoA14	*spo-22*	300	254	46	15.3
lys-1 spoA13	*spo-22*	216	190	26	12.5
lys-1 spoA11	*spo-22*	300	253	47	15.7
lys-1 spoA4	*spo-22*	272	243	29	10.6
lys-1 spoA5	*spo-22*	300	267	33	11.0
lys-1 spoA10	*spo-22*	294	258	36	12.2
lys-1 spoA6	*spo-22*	300	264	36	12.0
lys-1 spoA7	*spo-22*	300	257	43	14.3
lys-1 spoA8	*spo-22*	300	258	42	14.0
lys-1 spoA9	*spo-22*	300	267	33	11.0
lys-1 spoA1	*spo-22*	300	264	36	12.0
lys-1 spoA2	*spo-22*	300	244	56	18.6
lys-1 spoA3	*spo-22*	300	271	29	9.7
lys-1 spoA12	*spo-22*	144	127	17	11.8
lys-1, spo-22	*spoA12*	224	223	1	0.45
lys-1, spo-22	*spoA14*	240	233	7	2.9
lys-1, spo-22	*spoA10*	240	235	5	2.1

TABLE 4. *Recombination between spoA mutants in transformation*

Donor	Recipient		
	spoA1	*spoA11*	*spoA12*
spoA1		6.6	3.2
spoA2	1.1	1.8	12.5
spoA3	1.2	2.0	10.5
spoA4		0.6	1.5
spoA5		2.7	2.1
spoA6		4.3	1.0
spoA7		6.5	0.8
spoA8		10.0	11.9
spoA9	1.5	23.0	2.2
spoA10		11.3	5.3
spoA11	3.5		2.3
spoA12		1.0	
spoA13		NR[a]	2.3
spoA14	1.2	4.5	6.1

[a] No recombination.

By using this procedure, Mahler et al. (9) found recombination values higher than those found here for *spoA* mutants in their analysis of ornithine transcarbamylase mutants. Thus, the range of recombination values found for the *spoA* mutations is consistent with the mutant sites defining one or at most two genes. These results also show the unreliability of recombination values in PBS-1 transduction as a measure of short genetic distances.

Molecular basis of spoA mutations. The pleiotropic nature of *spoA* mutations raised a question as to the molecular basis for this phenotype. Preliminary studies indicated that some of the mutant sites were capable of reverting by means of suppressors. These suppressors were subsequently found not to be site specific and were capable of suppressing the presumed amber mutation in phage SPO1, *sus5* of Okubo, suggesting that the original *spoA* mutation resulted in a nonsense codon. To determine whether a nonsense codon was a prerequisite for the pleiotropic phenotype of *spoA* mutations, the effect of two amber-like suppressors on the phenotype of *spoA* mutations was tested. Strains were constructed bearing the suppressible *trpF7* mutation (C. Anagnostopoulos, *personal communication*) and the *spoA* mutations. These strains were transformed to tryptophan independence with DNA from strain *su*$^+$3 of Georgopulos (3) and from a spontaneous sporulating revertant of SR22, SR22R13, carrying a suppressor. Suppressed *trp*$^+$ transformants were isolated and purified, and the effect of the suppressor on the particular *spoA* mutation was determined. Examples of the results of these experiments are given in Table 5.

In five *spoA* mutants, *spoA2*, *spoA3*, *spoA14*, *spoA6*, and *spoA12*, the presence of an amber suppressor reversed the antibiotic-negative phen-

TABLE 5. *Effect of suppressors on the phenotype of spoA mutants*

Strain (genotype)	Antibiotic		Sporulation		ϕ15 sensitivity
	37 C	25 C	37 C	25 C	
trpF7 spoA1	−	+	−	−	++
trpF7 spoA1 sup-3	−	+	−	−	++
trpF7 spoA1 sup-13	−	+	−	−	++
trpF7 spoA11	−	−	−	−	++
trpF7 spoA11 sup-3	−	−	−	−	++
trpF7 spoA11 sup-13	−	−	−	−	++
trpF7 spoA12	−	−	−	−	++
trpF7 spoA12 sup-3	+	+	++	+	−
trpF7 spoA12 sup-13	+	+	+	−	+

otype. In some cases, sporulation and ϕ15 resistance were concomittantly regained with antibiotic production. The fact that these mutant sites responded to presumed amber suppressors indicates that they are probably nonsense mutations. This result further suggests that the product of the affected locus must be a protein since these suppressors are known to act at the level of translation. The inequality of suppression of the different phenotypes may reflect the efficiency of suppression, the efficiency of the altered protein produced, or the relative amount of the protein required to produce the phenotype.

Three mutants, *spoA11*, *spoA7* and *spoA8*, did not respond to the presence of either suppressor and were not phenotypically reverted by growth at lower temperatures. Strains bearing *spoA1* were temperature sensitive for antibiotic production, and this phenotype was unaltered by the presence of suppressors. Sporulation, however, could not be demonstrated at the lower temperature. The temperature sensitivity of this mutant is consistent with the production of an altered protein due to a missense mutation since nonsense mutations do not lead to temperature-sensitive phenotypes. The same argument may hold for *spoA4*, in which sporulation occurs at lower temperatures but antibiotic is produced at both temperatures. Thus, the data suggest that either a nonsense or missense mutation in the *spoA* locus is sufficient to bring about a pleiotropic negative sporulation phenotype.

DISCUSSION

These genetic studies of the *spoA* mutations have revealed that they form a tightly linked cluster near a large group of late sporulation-defective mutant sites. Earlier results, which were based on recombination values obtained in two-factor PBS-1 transduction crosses, were not able to distinguish the location of *spoA* sites from the bulk of late mutant sites in this region of the chromosome (5). The three-factor transduction crosses suggest that most of these late sites are to the left of the *spoA* region, and the order of loci is late *spo-spoA-lys*. Recent evidence of Ionesco et al. (7), again based on two-factor PBS-1 crosses, suggests that some late *spo* sites may be quite near to *lys*. Thus the *spoA* region may be flanked by genes involved in sporulation.

Recombination values obtained in two-factor transformation crosses between *spoA* mutants showed that the genetic distance across the *spoA* region is quite small. This distance, when compared to those found in other studies of genes of known enzymatic defect, is compatible with one or at most two genes being defined by the *spoA* mutant sites. In view of the large number of sporulation mutations that map in this region, it is somewhat surprising to find mutations in only one gene giving rise to pleiotropic negative sporulation phenotypes.

Point mutants in the *spoA* locus probably consist of both nonsense and missense classes. Nonsense mutations are indicated because some *spoA* sites are phenotypically reverted by a suppressor gene with a broad spectrum of action on auxotrophic and bacteriophage mutations. Mutants with a temperature-sensitive, nonsuppressible phenotype were also found, suggesting the presence of missense mutations. Since nonsense suppression is known to act at the translational level, the product of the gene defined by the *spoA* mutant sites is probably a protein. Thus, the absence of this protein is responsible for the loss of a variety of functions characteristic of a sporulating cell.

Although evidence is lacking as to the direct biochemical effect of a mutation in the *spoA* locus, the production of sporulation associated biochemical events does not occur in these mutants.

Partial loss of these phenotypes occurs by mutation in other regions of the chromosome (Hoch, *in preparation*). However, the only mutations found thus far that generate a completely negative phenotype map at the *spoA* locus. A role for the product of the *spoA* locus was proposed earlier (5).

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