# Genetic Analysis of *Bacillus subtilis spo* Mutations Generated by Tn*917*-Mediated Insertional Mutagenesis

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## ABSTRACT

Mutations that cause sporulation defects (*spo* mutations) often identify developmentally regulated transcription units or genes whose products are required for the expression of sporulation-specific regulons. We report here the isolation, genetic analysis and phenotypic characterization of *spo* mutations produced by insertional mutagenesis with transposon Tn*917*, a form of mutagenesis that facilitates genetic and physical manipulation of mutated genes in many ways. Twenty-four insertional *spo* mutations were studied in detail. On the basis of transformation-mediated and transduction-mediated linkage analysis and a range of phenotypic tests, these mutations were assigned to 20 distinct loci, at least 9 of which are different from the 40 previously described *spo* loci. The insertional mutations caused blocks at a variety of different stages of sporulation, and therefore probably identify genes active at different times during sporulation. In addition to increasing substantially the total of known *spo* loci, we anticipate that this collection will include representatives of many of the temporally regulated sets of genes that comprise the overall program of sporulation-specific gene activation in *Bacillus subtilis*. Given the kinds of manipulations that are possible with genes disrupted by Tn*917* insertions, this should significantly facilitate efforts to understand the regulation of these gene sets.

SPORULATION in *Bacillus subtilis* is a complex cell differentiation event that requires the temporally regulated activation of many chromosomal genes (reviewed by LOSICK and YOUNGMAN 1984). Several of these genes have been identified in earlier studies through the isolation of *spo* mutations, mutations that block or interfere with the process of sporulation without significantly affecting bacteria in the vegetative phase (reviewed by PIGGOT and COOTE 1976; LOSICK, YOUNGMAN and PIGGOT 1986). Most such mutations could be classified as blocking sporulation at one of six developmental "stages" (state 0, II, III, . . . VI), each defined by a combination of morphological and physiological criteria. In the absence (until recently) of complementation testing of *spo* mutations or fine-structure physical analysis of *spo* genes, the assignment of individual *spo* mutations to specific genetic "loci" has followed the convention of grouping together in a single locus all closely linked mutations causing a similar stage-block phenotype (PIGGOT 1973). In this way, 40 widely scattered *spo* loci have been identified (PIGGOT, MOIR and SMITH 1981; LOSICK, YOUNGMAN and PIGGOT 1986), the map positions of which are indicated in Figure 1.

Although direct genetic analysis of *spo* mutations has in the past revealed little mechanistic information about the way *spo* genes are regulated, *spo* mutations have proved useful for cloning *spo* genes (PIGGOT and HOCH 1985). Cloned *spo* genes have in turn proved useful for *in vitro* studies of gene regulation, making it possible to define biochemically some factors controlling the expression of these genes (LOSICK 1981; LOSICK, YOUNGMAN and PIGGOT 1986). Cloned genes have also been used to construct gene fusions that place expression of *lacZ* (β-galactosidase-encoding), *cat* (chloramphenicol acetyltransferase-encoding) or *xylE* (catechol dioxygenase-encoding) genes under control of *spo* gene promoters, which has made it possible to apply genetic techniques in a more direct way to the analysis of *spo* gene regulation (ZUBER and LOSICK 1983; TRUITT *et al.* 1985; ZUBER 1985; IGO *et al.* 1987).

In the present work, we describe the isolation and genetic characterization of a collection of *spo* mutations produced by insertional mutagenesis with Tn*917*, a *Streptococcus faecalis* transposon (TOMICH, AN and CLEWELL 1980) that has been adapted for use in *B. subtilis* (YOUNGMAN, PERKINS and LOSICK 1983). A total of 24 insertional mutations were studied in detail. These define at least 20 different *spo* loci, including at least 9 new loci. Some of these new loci are in regions of the chromosome where *spo* mutations were previously unknown. The collection includes mutations that block sporulation at a variety of different developmental stages and thus probably includes insertions into genes that become active at different times during sporulation.

One motivation for this work was the existence of a recently developed set of methods designed to take advantage of Tn917-generated insertional mutations (YOUNGMAN et al. 1985). These methods facilitate both the cloning of mutated genes (YOUNGMAN, PERKINS and LOSICK 1984b) and the construction of lacZ fusions (PERKINS and YOUNGMAN 1986). It was anticipated that a large collection of Tn917-generated spo mutations would yield for future analysis readily studied representatives of many of the regulated gene sets that comprise the overall program of sporulation gene activity.

Another objective of the work was to explore critically the properties of Tn917 as a general mutagen of chromosomal genes in B. subtilis. Tn917 had been shown to generate relatively random insertions in at least some streptococcal plasmid targets (IKE and CLEWELL 1984), but has not systematically been used for insertional mutagenesis of chromosomal genes in a Streptococcus host. In B. subtilis, the transposon had been shown to be capable of generating several different kinds of chromosomal mutations, but was known also to exhibit a preference for insertion into certain "hotspot" regions (YOUNGMAN, PERKINS and LOSICK 1983). Some question remained whether insertions outside these preferred areas would be abundant and random enough to generate representative collections of mutations in all regions of the chromosome. The present work addresses this question through a survey of the kinds of spo mutations that Tn917 can produce, through an analysis of the distribution of these mutations over the chromosome, and through a study of the correspondence of these mutations with previously characterized mutations that resulted from conventional mutagenesis of various kinds. In addition, this work includes the analysis of several "phenotypically silent" chromosomal Tn917 insertions, to determine whether such insertions are sufficiently random to be found closely linked by transformation to selectable markers in any region of the chromosome. Based on these studies, we conclude that Tn917 insertions can probably be recovered in all regions of the chromosome, and perhaps within any nonessential gene of B. subtilis. The recent work of VANDEYAR and ZAHLER (1986) supports these conclusions as well.

## MATERIALS AND METHODS

**Culture media:** Preparation of LB (general-purpose complex medium), TSS (synthetic minimal medium), and DSM (sporulation medium) was as described previously (YOUNGMAN 1986).

**Strains:** B. subtilis strains and relevant genotypes are listed in Table 1.

**Genetic methods:** Screening and selection for most drug resistance and auxotrophic markers was carried out as described previously (YOUNGMAN 1986). The ahrC3 mutation was scored as described by MOUNTAIN and BAUMBERG (1980), the asaA4 mutation was scored as described by

ADAMS and OISHI (1972), and the sul mutation was scored as described by KANE, GOODE and WAINSCOTT (1975). Transformations were carried out as described by ANAGNOSTOPOULOS and SPIZIZEN (1961), and transductions were carried out as described by HOCH, BARAT and ANAGNOSTOPOULOS (1967).

**Phenotypic evaluation of Spo mutants:** Synthesis of sporulation-associated extracellular proteases (MILLET 1970) was tested by picking bacteria into DSM agar containing 5% Carnation instant milk. Production of sporulation-associated antibiotics (SCHAEFFER 1969) was tested by picking bacteria into DSM plates overlaid with soft-agar containing a B. subtilis spoOA mutant (JH646). Sporulation-associated alkaline phosphatase was assayed as described by GLENN and MANDELSTAM (1971) 5 hr after the end of exponential growth in DSM broth at 37°. Glucose dehydrogenase was assayed as described by HILL (1983), except that sonication was applied for 10 min at 150 W. The presence of dipicolinic acid in sporulating cultures was determined by the method of JANSSEN, LUND and ANDERSON (1958) as modified by ROTMAN and FIELDS (1968), 7.5 hr after the end of exponential growth at 37° in DSM broth. Heat-resistance was tested after overnight growth (about 20 hr) in DSM broth at 37° by subjecting samples to a temperature of 80° for 15 min.

**Recovery of chromosomal Tn917 insertions from pTV1-containing bacteria:** Samples of strain PY143, a Spo$^+$ prototroph, were grown at 32° in LB broth containing 1 $\mu$g/ml Em (erythromycin), 25 $\mu$g/ml Lm (lincomycin) and 5 $\mu$g/ml Cm (chloramphenicol). When cultures had reached a turbidity of 50 Klett units, 5-ml portions were diluted into 2-liter Erlenmeyer flasks that contained 500 ml of LB broth prewarmed to 48°. Em was added to a final concentration of 1 $\mu$g/ml and cultures were incubated for 15 hr with moderately vigorous aeration. Samples of the resulting outgrowth were plated on DSM agar without antibiotics at dilutions calculated to produce 100–200 colonies per plate. After a period of incubation at 37°, plates were screened for mutant phenotypes as described under RESULTS.

**Preparation of the insertion library:** A 275-$\mu$g sample of CsCl-purified Tn917-containing vector pTV5 (YOUNGMAN, PERKINS and LOSICK 1984a) was used to transform 55 ml of concentrated competent cells that already contained pTV41 (K. SANDMAN, unpublished data), a plasmid similar to pTV5 but containing a deleted derivative of Tn917 in which the erm gene and the erm-proximal inverted repeat were removed. Extensive homology between pTV5 and pTV41 made the introduction of pTV5 by transformation highly efficient (CONTENTE and DUBNAU 1979). Transformed bacteria were diluted into 2.5 liters of LB at 33°, and Em was added to a final concentration of 0.15 $\mu$g/ml. After 90 min of aerated incubation, Em was added to a final concentration of 1 $\mu$g/ml and Lm was added to a final concentration of 25 $\mu$g/ml. When the OD$_{595}$ reached 0.5, a 750-ml portion was diluted into 100 liters of 48° LB containing 1 $\mu$g/ml Em and 25 $\mu$g/ml Lm, and aerated in a fermentor. After 14 hr, a 200-ml sample of the outgrowth was diluted once again 1:15 into fresh 48° LB containing the same concentrations of Em and Lm (YOUNGMAN 1986). When the OD$_{595}$ reached 2.0, bacteria were pelleted by centrifugation, resuspended in ¹⁄₂₅ the original volume in LB with 10% glycerol, divided into 1-ml aliquots, and frozen in liquid nitrogen. To recover Spo mutants, samples were thawed, diluted, and plated for single colonies on DSM agar, as described under RESULTS.

**Recovery of spo mutations by selection for co-transformation:** Spo mutants were identified from library samples as described under RESULTS, and pooled in a single mixture.

FIGURE 1.—Genetic map of the *B. subtilis* chromosome showing the positions of Tn917-generated *spo* mutations and phenotypically silent insertions relative to known *spo* loci and certain auxotrophic markers discussed in the text. Insertions and markers whose order was not determined are grouped in brackets. (* = Insertional mutations that define new *spo* loci.)

Serial dilutions of DNA prepared from this mixture were used to transform a multiply-marked auxotroph to MLS$^r$, determining at each concentration the number of transformants and the fraction of cells that became transformed for unselected, unlinked markers (congression). At a DNA concentration that produced less than 1% congression, a transformation was carried out on a scale calculated to generate at least $4 \times 10^4$ transformants. Transformed cell suspensions were diluted 1:10 into LB containing 0.15 μg/ml Em and incubated with aeration at 37°. After 2 hr, Em was added to 1 μg/ml and Lm to 25 μg/ml and incubation was continued until extensive turbidity developed (typically 10-12 hr). Samples (1 ml) of these cultures were washed and resuspended in TSS, then diluted into 4-liter Erlenmeyer flasks each containing 1000 ml of TSS medium supplemented to select for the wild-type allele of one of the auxotrophic markers in the strain. These flasks were incubated at 37° with aeration until extensive turbidity developed (typically 15-20 hr). Because transformants arising from congression would be expected to predominate at this stage (YOUNGMAN 1986), a second enrichment step was carried out. Chromosomal DNA was prepared from a sample of the TSS culture and used to transform the same auxotroph, selecting again in minimal medium for the same prototrophic allele in the presence of 1 μg/ml Em and 25 μg/ml Lm. Samples of cultures resulting from this second
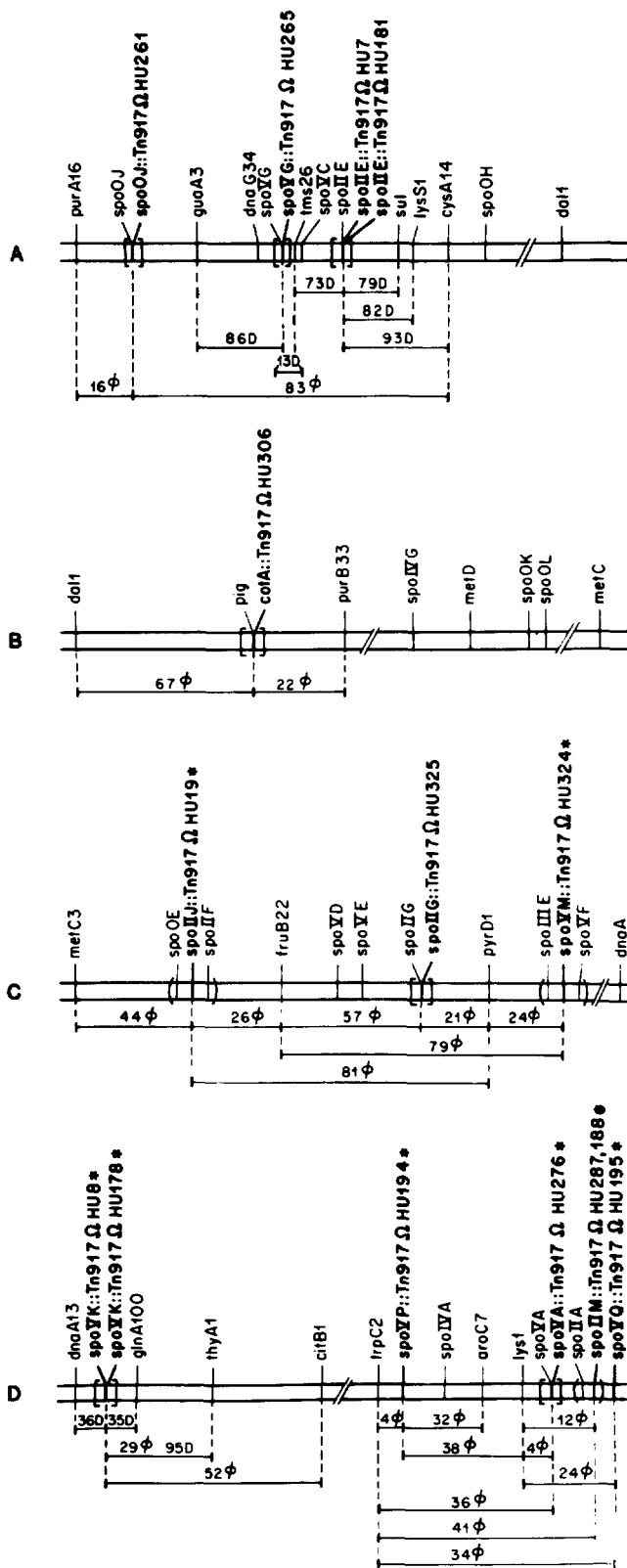
FIGURE 2.—Linear display of the *B. subtilis* chromosome summarizing the genetic linkages of Tn917-generated mutations with various mapped markers. Read left-to-right and top-to-bottom, A to D span the conventional circular map (PIGGOT and HOCH 1985) from *purA* to *lys* in the clockwise direction. Linkages are expressed as 100% minus percent co-transformation (subscript D) or co-transduction (subscript Φ). In all cases, the transposon insertion was

double-selection were diluted and plated for single colonies on DSM agar. After 2–4 days of incubation at 37°, the DSM plates were examined for Spo mutants. When mutants were present, DNA was prepared from one or two of each distinct type and used to determine whether the lesion causing the phenotype was associated with Tn917 and actually linked to the selected marker.

**Recovery of *spo* mutations by selection for co-transduction:** Samples of the frozen mixture of Spo mutants obtained from the insertion library were used to prepare PBS1 lysates essentially as described by HOCH, BARAT and ANAGNOSTOPOULOS (1967). Such lysates were used to infect multiply-marked auxotrophs on a scale calculated to generate at least $4 \times 10^4$ MLS$^r$ transductants. Infected cells were diluted into TSS broth containing 0.15 $\mu$g/ml Em and supplemented to select for particular prototrophic alleles. After 2 hr at 37°, Em was added to 1 $\mu$g/ml and Lm to 25 $\mu$g/ml, and the incubation continued until extensive turbidity developed (10–15 hr). Samples were then diluted and plated on DSM agar to screen for Spo phenotypes.

## RESULTS

**Further analysis of three previously isolated Tn917-mediated *spo* mutations:** In previous work (YOUNGMAN, PERKINS and LOSICK 1983), methods were developed for recovering Tn917 insertions into the *B. subtilis* chromosome using temperature-sensitive transposon-carrying plasmid vector pTV1. Wild-type bacteria (strain PY143) were shifted to a temperature nonpermissive for plasmid replication, diluted 1:100, and grown overnight under selection for MLS$^r$ (resistance to macrolide, lincosamide, and streptogramin B antibiotics), the drug-resistance phenotype conferred by Tn917. Resulting outgrowth populations were found to be greatly enriched for bacteria that contained chromosomal Tn917 insertions, and were screened successfully for several kinds of mutants. These included Spo (sporulation-defective) mutants at a frequency of 0.1–0.5%. Three insertional *spo* mutations (referred to in the present work as *spo*::Tn917ΩHU5, *spo*::Tn917ΩHU6, and *spo*::Tn917-ΩHU7), which had been recovered from independent transposition batches, were mapped by PBS1-mediated transduction. Map positions and a preliminary analysis of phenotypes caused by these insertions suggested that *spo*::Tn917ΩHU5 might be an allele of *spoIIC* and that *spo*::Tn917ΩHU6 was probably an allele of *spoIID*. When subsequent attempts were made to characterize *spo*::Tn917ΩHU5 in more detail, this insertional mutation was found to cause an unstable Spo⁻ phenotype and has been omitted from the present study. Further analysis of *spo*::Tn917ΩHU6 has supported its assignment to *spoIID* (Figure 3C). Because of ambiguity in the positions of certain markers in the vicinity of *cysA*, where *spo*::Tn917ΩHU7 was positioned based on the results of transduction-medi-
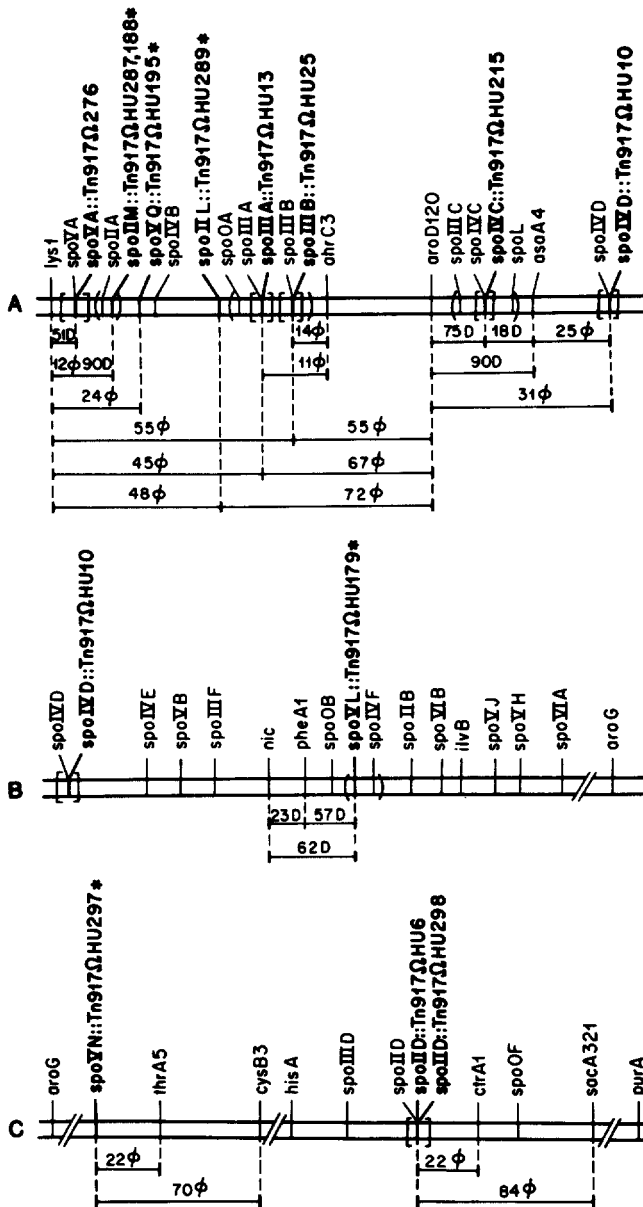
FIGURE 3.—See legend to Figure 2. A to C span the map from *lys* to *purA* in the clockwise direction.

ated crosses, this mutation was erroneously assigned to a new locus, *spoIIH* (YOUNGMAN, PERKINS and LOSICK 1984b). As the result of additional transformation-mediated crosses (Figure 2A), phenotypic studies and physical analysis of cloned DNA (J. WESTPHELING and P. YOUNGMAN, unpublished data), and "marker-rescue" analysis (M. YOUNG, personal communication) this mutation is reclassified here as an allele of *spoIIE*, and placed on the genetic map between *tms-26* and *lysS* (Figure 2A).

**New Tn917-mediated *spo* mutations that block development at different stages:** In previous work, only unpigmented stage II insertional mutants were studied. To determine whether mutagenesis with Tn917 could generate Spo mutants blocked at different stages, bacteria containing pTV1-derived chro-

mosomal insertions were plated on DSM agar and the colonies they formed were screened for pigmentation and translucence after 3 days of incubation at 37°. From an initial screening of approximately 5000 colonies, 26 Spo⁻ candidates were chosen for further study. Upon purification through single colonies, and retesting for colony appearance on DSM agar, 10 were rejected as having indistinct phenotypes. Eight were rejected because they were Cmʳ (chloramphenicol-resistant), an indication that they were not the products of simple transposon insertions (YOUNGMAN, PERKINS and LOSICK 1983). All of the eight remaining mutants were found to be phenotypically stable and able to grow on a minimal medium with lactate as the sole source of carbon, confirming that they were not sporulation-defective as an indirect consequence of a metabolic deficiency (FORTNAGEL and FREESE 1968).

Transducing lysates were prepared from each of these eight mutants with phage PBS1 and used to transduce a wild-type strain (PY79) to MLSʳ. At least 200 progeny of each transduction were scored for Spo phenotype, and in each case 100% were found to inherit the mutant properties of the parent. The chromosomal locations of these insertions were determined from a series of co-transduction studies and three-factor crosses, and at least five different loci were distinguished. Because these insertional mutations were not derived from independent batches, insertions at similar map positions that caused a similar phenotype were assumed to be identical. Further genetic analysis and phenotypic studies were carried out for each of the five different insertional mutations, as summarized below, to determine whether they were associated with known *spo* loci.

*spoIIJ*::Tn917ΩHU19: Bacteria containing *spo*::Tn917ΩHU19 produced lightly pigmented colonies on DSM agar. Samples of mutant bacteria taken for microscopic examination after 36 or 48 hrs in DSM broth revealed an accumulation of both intact and lysed bacteria containing evidence of abnormal septation, as well as a significant number of apparently normal spores, suggesting an "oligosporogenous" (leaky) stage II block. In PBS1-mediated transductions, *spo*::Tn917ΩHU19 mapped to the vicinity of *fruB* (Figure 2C), a region that contains two closely linked *spo* loci associated with early blocking mutations, *spoOE* and *spoIIF*. Three-factor transduction crosses indicated the map order *ura-fru-spo* (Table 2), consistent with an insertion in either *spoOE* or *spoIIF* (PIGGOT and HOCH 1985). Other phenotypic properties of bacteria containing *spo*::Tn917ΩHU19 were also consistent with a mutation in either *spoOE* or *spoIIF* (Table 4). In another laboratory (J. A. HOCH, personal communication), this insertion was used to generate plasmid clones in *E. coli* that included several kb of DNA extending in each direction from the

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## TABLE 1

### *Bacillus subtilis* strains

| Strain | Relevant genotype | Source or reference | Strain | Relevant genotype | Source or reference |
|--------|-------------------|---------------------|--------|-------------------|---------------------|
| PY79 | Prototrophic | YOUNGMAN, PERKINS and LOSICK (1984a) | KS195 | spoVQ::Tn917ΩHU195 | This work |
| | | | KS215 | spoIVC::Tn917ΩHU215 | This work |
| PY143 | Prototrophic; pTV1 | YOUNGMAN, PERKINS and LOSICK (1983) | KS261 | spoOJ::Tn917ΩHU261 | This work |
| | | | KS265 | spoVG::Tn917ΩHU265 | This work |
| JH646 | spoOA12 | J. A. HOCH | KS276 | spoVA::Tn917ΩHU276 | This work |
| CU806 | citB1 thyB1 | S. A. ZAHLER | KS287 | spoIIM::Tn917ΩHU287 | This work |
| QB917 | thrA5 | DEDONDER et al. (1977) | KS289 | spoIIL::Tn917ΩHU289 | This work |
| QB123 | ctrA1 sacA321 | DEDONDER et al. (1977) | KS297 | spoVN::Tn917ΩHU297 | This work |
| QB928 | purB33 dal1 | DEDONDER et al. (1977) | KS298 | spoIID::Tn917ΩHU298 | This work |
| QB935 | trpC2 aroD120 lys1 | DEDONDER et al. (1977) | KS306 | cotA::Tn917ΩHU306 | This work |
| QB944 | purA16 cysA14 | DEDONDER et al. (1977) | KS324 | spoVM::Tn917ΩHU324 | This work |
| 1A237 | fruB22 ura3 | Bacillus Genetic Stock Center | KS325 | spoIIG::Tn917ΩHU325 | This work |
| | | | KS139 | chr::Tn917ΩHU139 trpC2 | This work |
| KS101 | spoVK::Tn917ΩHU8 thyA1B1 | This work | KS142 | chr::Tn917ΩHU142 metD1 | This work |
| KS102 | spoIIIA::Tn917ΩHU13 ahrC3 | This work | KS144 | chr::Tn917ΩHU144 trpC2 | This work |
| KS103 | spoIIIB::Tn917ΩHU25 ahrC3 | This work | KS146 | chr::Tn917ΩHU146 purA16 trpC2 | This work |
| KS239 | spoIVD::Tn917ΩHU10 asaA4 | This work | KS148 | chr::Tn917ΩHU148 cysA14 trpC2 | This work |
| KS302 | spoVN::Tn917ΩHU297 cysB3 | This work | | | |
| PY179 | spoIID::Tn917ΩHU6 | This work | KS149 | chr::Tn917ΩHU149 cysB3 trpC2 | This work |
| PY180 | spoIIE::Tn917ΩHU7 | This work | | | |
| KS8 | spoVK::Tn917ΩHU8 | This work | KS151 | chr::Tn917ΩHU151 cysB3 hisA1 | This work |
| KS10 | spoIVD::Tn917ΩHU10 | This work | | | |
| KS13 | spoIIIA::Tn917ΩHU13 | This work | KS153 | chr::Tn917ΩHU153 glnA100 | This work |
| KS19 | spoIIJ::Tn917ΩHU19 | This work | KS156 | chr::Tn917ΩHU156 metB5 | This work |
| KS25 | spoIIIB::Tn917ΩHU25 | This work | KS160 | chr::Tn917ΩHU160 glyB133 | This work |
| KS178 | spoVK::Tn917ΩHU178 | This work | KS163 | chr::Tn917ΩHU163 metC7 purH1 trpC2 | This work |
| KS179 | spoVL::Tn917ΩHU179 | This work | | | |
| KS181 | spoIIE::Tn917ΩHU181 | This work | KS169 | chr::Tn917ΩHU169 leuA8 metB5 | This work |
| KS188 | spoIIM::Tn917ΩHU188 | This work | | | |
| KS194 | spoVP::Tn917ΩHU194 | This work | | | |

transposon insertion junctions. These cloned sequences were found not to cover alleles of either *spoOE* or *spoIIF*. Based on this information and on the phenotypic data summarized in Table 4, *spo*::Tn917-ΩHU19 was classified as defining a new stage II locus, *spoIIJ*.

**spoIIIA::Tn917ΩHU13, spoIIIB::Tn917ΩHU25:** Bacteria containing mutations *spo*::Tn917ΩHU13 and *spo*::Tn917ΩHU25 were easily distinguished from each other by colony pigmentation (the former turned slightly brown on DSM plates, and the latter remained unpigmented), but on the basis of microscopic studies they were found to be arrested in morphological development at the same stage (stage III). In both mutants, a prespore protoplast appeared to have formed, but never developed a "phase-gray" appearance when viewed with phase-contrast optics. The results of biochemical tests for the presence of sporulation-associated alkaline phosphatase (AP) and glucose dehydrogenase (GDH) (Table 4) were also consistent with a stage III block (PIGGOT and COOTE 1976). In PBS1-mediated transductions, both mutations displayed strong linkage to *aroD* and *ahrC* (Figure 3A). The results of three-factor crosses with both

mutations suggested the map order *aroD-ahrC-spo* (Table 2). This placed *spo*::Tn917ΩHU13 and *spo*::Tn917ΩHU25 in the vicinity of several previously characterized stage III mutations (IONESCO et al. 1970; COOTE 1972; PIGGOT 1973), which have been assigned to the two loci *spoIIIA* and *spoIIIB* (PIGGOT and COOTE 1976). Although previously studied mutations in the *spoIIIA-spoIIIB* region were found to block at the same stage, detailed microscopic examination distinguished at least three phenotypes. One group of *spoIIIA* mutations was described as producing strikingly extensive lysis of the mother cell (WAITES et al. 1970). Because *spo*::Tn917ΩHU13-containing mutants also exhibited this property, this mutation was tentatively assigned to *spoIIIA*. Because *spo*::Tn917ΩHU25 mutants were quite different in general appearance from *spo*::Tn917ΩHU13 mutants, *spo*::Tn917ΩHU25 was considered likely to be in a different locus and was tentatively classified as an allele of *spoIIIB* (see DISCUSSION).

**spoIVD::Tn917ΩHU10:** Bacteria containing *spo*::Tn917ΩHU10 formed unpigmented colonies on DSM agar, suggesting a block before stage V. Mutant bacteria grown overnight in DSM broth formed

## TABLE 2

Representative PBS1-mediated three-factor crosses[a]

| Donor | Recipient | Recombinant types[b] | | | Number | Inferred order |
|---|---|---|---|---|---|---|
| KS19 | 1A237 | *spo* | *fru* | *ura* | | |
| spoIIJ::Tn917 | fruB22 ura3 | D | D | D | 32 | |
| (ΩHU19) | | D | D | R | 145 | |
| | | D | R | D | 1 | |
| Selection for MLS^r (*spo*) | | D | R | R | 62 | *spo fru ura*[c] |
| KS102 | QB935 | *spo* | *ahr* | *aro* | | |
| spoIIIA::Tn917 | aroD120 | D | D | D | 79 | |
| (ΩHU13) | | D | D | R | 135 | |
| | | D | R | D | 1 | |
| Selection for MLS^r (*spo*) | | D | R | R | 25 | *spo ahr aro*[c] |
| KS103 | QB935 | *spo* | *ahr* | *aro* | | |
| spoIIIB::Tn917 | aroD120 | D | D | D | 30 | |
| (ΩHU25) | | D | R | D | 1 | |
| | | R | D | D | 50 | |
| Selection for Aro^+ | | R | R | D | 56 | *spo ahr aro*[c] |
| KS239 | QB935 | *aro* | *asa* | *spo* | | |
| spoIVD::Tn917 | aroD120 | D | D | D | 388 | |
| (ΩHU10) asaA4 | | D | D | R | 65 | |
| | | D | R | D | 6 | |
| Selection for Aro^+ | | D | R | R | 41 | *aro asa spo*[c] |
| KS101 | CU806 | *spo* | *thy* | *cit* | | |
| spoVK::Tn917 | citB1 thyB1 | D | D | D | 109 | |
| (ΩHU8) thy- | | D | D | R | 62 | |
| A1B1 | | D | R | D | 5 | |
| Selection for MLS^r (*spo*) | | D | R | R | 64 | *spo thyA cit*[c] |
| KS194 | QB935 | *lys* | *spo* | *trp* | | |
| spoVP::Tn917 | lys1 trpC2 | D | D | D | 222 | |
| (ΩHU194) | | D | R | D | 3 | |
| | | D | D | R | 32 | |
| Selection for Lys^+ | | D | R | R | 154 | *lys spo trp*[c] |
| KS195 | QB935 | *trp* | *lys* | *spo* | | |
| spoVQ::Tn917 | lys1 trpC2 | D | D | D | 105 | |
| (ΩHU195 | | D | D | R | 8 | |
| | | D | R | D | 0 | |
| Selection for Trp^+ | | D | R | R | 46 | *trp lys spo*[c] |
| KS261 | QB944 | *pur* | *spo* | *cys* | | |
| spoOJ::Tn917 | purA16 cysA14 | D | D | D | 17 | |
| (ΩHU261) | | D | D | R | 151 | |
| | | R | D | D | 17 | |
| Selection for MLS^r (*spo*) | | R | D | R | 15 | *pur spo cys*[d] |

[a] Additional confirmational data not shown.

[b] D = donor genotype, R = recipient genotype.

[c] Data interpreted as indicating that selected marker was an outside marker.

[d] Data interpreted as indicating that selected marker was a middle marker.

## TABLE 3

Representative PBS1-mediated three-factor crosses[a]

| Donor | Recipient | Recombinant types[b] | | | Number | Inferred order |
|---|---|---|---|---|---|---|
| KS298 | QB123 | *spo* | *ctr* | *sac* | | |
| spoIID::Tn917 | ctrA1 sacA321 | D | D | D | 30 | |
| (ΩHU298) | | D | D | R | 126 | |
| | | D | R | D | 1 | |
| Selection for MLS^r (*spo*) | | D | R | R | 43 | *spo ctr sac*[c] |
| KS325 | 1A237 | *fru* | *spo* | *ura* | | |
| spoIIG::Tn917 | fruB22 ura3 | D | D | D | 69 | |
| (ΩHU325) | | R | D | D | 109 | |
| | | D | R | D | 0 | |
| Selection for Ura^+ | | R | R | D | 11 | *fru spo ura*[c] |
| KS289 | QB935 | *lys* | *spo* | *aro* | | |
| spoIIL::Tn917 | lys1 aroD120 | D | D | D | 9 | |
| (ΩHU289) | | D | D | R | 95 | |
| | | R | D | D | 48 | |
| Selection for MLS^r (*spo*) | | R | D | R | 48 | *lys spo aro*[d] |
| KS287 | QB935 | *spo* | *lys* | *trp* | | |
| spoIIM::Tn917 | lys1 trpC2 | D | D | D | 242 | |
| (ΩHU287) | | D | D | R | 191 | |
| | | D | R | D | 1 | |
| Selection for MLS^r (*spo*) | | D | R | R | 33 | *spo lys trp*[c] |
| KS276 | QB935 | *spo* | *lys* | *trp* | | |
| spoVA::Tn917 | lys1 trpC2 | D | D | D | 35 | |
| (ΩHU276) | | D | D | R | 24 | |
| | | D | R | D | 0 | |
| Selection for MLS^r (*spo*) | | D | R | R | 5 | *spo lys trp*[c] |
| KS324 | 1A237 | *fru* | *ura* | *spo* | | |
| spoVM::Tn917 | fruB22 ura3 | D | D | D | 41 | |
| (ΩHU324) | | R | D | D | 105 | |
| | | D | R | D | 0 | |
| Selection for MLS^r (*spo*) | | R | R | D | 46 | *fru ura spo*[c] |
| KS302 | QB917 | *spo* | *thr* | *cys* | | |
| spoVN::Tn917 | thrA5 | D | D | D | 58 | |
| (ΩHU297) cysB3 | | D | D | R | 96 | |
| | | D | R | D | 0 | |
| Selection for MLS^r (*spo*) | | D | R | R | 43 | *spo thr cys*[c] |
| KS306 | QB928 | *dal* | *cot* | *pur* | | |
| cotA::Tn917 | purB33 dal1 | D | D | D | 31 | |
| (ΩHU306) | | D | D | R | 14 | |
| | | R | D | D | 75 | |
| Selection for MLS^r (*cot*) | | R | D | R | 15 | *dal cot pur*[d] |

[a,b,c,d] See footnotes for Table 2.

phase-white prespores that were eventually released into the medium by lysis of the mother cell, indicating that development had definitely advanced beyond stage III. Biochemical assays revealed wild-type levels of AP and GDH, but no dipicolinic acid (DPA). Although some free phase-white prespores were released into the medium, they were found not to be heat-resistant (Table 4). In PBS1-mediated transductions, *spo*::Tn917ΩHU10 mapped to the interval between

*aroD* and *pheA* (Figure 3, A and B), which contains at least two stage IV loci, *spoIVC* and *spoIVD* (COOTE 1972; HRANUELI, PIGGOT and MANDELSTAM 1974). The results of three-factor crosses (Table 2) involving *spo*::Tn917ΩHU10, *aroD120*, and *asaA4* indicated the map order *spo-asa-aro*. Based on this information and on published descriptions of phenotypes associated with *spoIVD* mutations (PIGGOT and COOTE 1976), *spo*::Tn917ΩHU10 was considered likely to be an allele of *spoIVD*.

***spoVK::Tn917ΩHU8:*** Colonies formed on DSM agar by bacteria containing *spo*::Tn917ΩHU8 were more darkly pigmented than the wild-type and became more translucent. Microscopic examination of

**TABLE 4**

**Phenotypic properties of *spo*::Tn917 mutants**

| Mutation | PR[a] | AB[b] | AP[c] | GDH[d] | DPA[e] | Heat[f] |
|---|---|---|---|---|---|---|
| *spoOJ*::Tn917ΩHU261 | + | + | 0 | ND | ND | 0 |
| *spoIID*::Tn917ΩHU298 | ND | ND | 77 | − | − | 0 |
| *spoIIE*::Tn917ΩHU181 | + | ND | 24 | − | ND | 0 |
| *spoIIG*::Tn917ΩHU325 | ND | ND | 20 | − | ND | 0 |
| *spoIIJ*::Tn917ΩHU19 | + | + | 38 | − | ND | 34 |
| *spoIIL*::Tn917ΩHU289 | + | + | 18 | − | ND | 0 |
| *spoIIM*::Tn917ΩHU287 | ND | ND | 104 | − | ND | 0 |
| *spoIIIA*::Tn917ΩHU13 | + | + | 93 | − | ND | 0 |
| *spoIIIB*::Tn917ΩHU25 | + | + | 91 | − | ND | 0.1 |
| *spoIVC*::Tn917ΩHU215 | ND | ND | 108 | + | − | 0 |
| *spoIVD*::Tn917ΩHU10 | + | + | 118 | + | − | 0 |
| *spoVA*::Tn917ΩHU276 | ND | ND | 95 | + | − | 0 |
| *spoVG*::Tn917ΩHU265 | ND | ND | 217 | + | + | 72 |
| *spoVK*::Tn917ΩHU8 | + | + | 116 | + | + | 0.1 |
| *spoVL*::Tn917ΩHU179 | + | ND | 91 | + | + | 1.0 |
| *spoVM*::Tn917ΩHU324 | ND | ND | 121 | + | + | 0 |
| *spoVN*::Tn917ΩHU297 | ND | ND | 74 | + | − | 0.7 |
| *spoVP*::Tn917ΩHU194 | + | ND | 131 | + | + | 0 |
| *spoVQ*::Tn917ΩHU195 | + | ND | 148 | + | + | 0 |
| *cotA*::Tn917ΩHU306 | ND | ND | 118 | + | + | 92 |

[a] PR = production of sporulation-associated protease; ND = not determined.

[b] AB = production of sporulation-associated antibiotics.

[c] AP = production of sporulation-associated alkaline phosphatase, indicated as percentage of AP produced by an isogenic wild-type control (PY79); 0 = <5.0%.

[d] GDH = production of glucose dehydrogenase.

[e] DPA = production of dipicolinic acid.

[f] Heat[r] = heat-resistance, indicated as percentage survivors relative to a wild-type control (PY79); 0 = <0.01%.

mutant bacteria grown overnight in DSM broth revealed phase-gray prespores, but no evidence of further development even in a minority of the population. The results of biochemical assays (Table 4) demonstrated that AP, GDH and DPA were produced at wild-type levels, and mutant prespores were found to be partially heat-resistant (Table 4). Taken together, these characteristics indicated a block at stage V (PIGGOT and COOTE 1976). Interestingly, *spo*::Tn917ΩHU8 mapped in PBS1 transductions to the vicinity of *thyA* (Figure 2D), far from any known *spo* mutation (PIGGOT and HOCH 1985). This mutation thus defines a new *spo* locus, which we designated here as *spoVK*.

**Recovery of additional Tn917-mediated *spo* mutations from a large "library" of transposon insertions:** Having successfully recovered Tn917-mediated *spo* mutations that blocked development at four different stages, we attempted next to expand the collection to include, if possible, several examples of each stage-block type. One approach would have been to continue screening additional independent populations of bacteria containing pTV1-derived chromosomal Tn917 insertions for different mutant types on DSM agar. We suspected, however, that insertions in some sporulation genes might occur significantly more

often than in others due to the non-randomness of Tn917 insertions (YOUNGMAN, PERKINS and LOSICK 1983; VANDEYAR and ZAHLER 1986). If so, considerable repetition of effort would result, since independent insertions in the same locus could not be distinguished as such until they were mapped. We chose instead to generate a single large "library" of chromosomal Tn917 insertions from which *spo* mutations would be collected and pooled together in one mixture (MATERIALS AND METHODS). With chromosomal DNA or a PBS1 lysate prepared from such a mixture, we expected that it would be possible to identify many different individual *spo* mutations by selecting for their linkage to genetic markers in different regions of the chromosome.

To isolate Spo mutants, thawed samples of the library population were plated on DSM agar at a dilution calculated to produce approximately 300 single colonies per plate. Plates were incubated at 37° and screened after 1 day, after 3–4 days, and after 5–7 days for colonies that displayed pigmentation or translucence different from the wild type. More than $1.2 \times 10^6$ colonies were screened, and approximately $1.5 \times 10^3$ were identified as potential Spo mutants. As in earlier studies, mutant candidates were retested for colony appearance on DSM agar, tested for Cm[r], tested for growth on a synthetic minimal medium with lactate as the sole source of carbon, and in questionable cases were examined under the microscope to confirm the presence of a sporulation defect. A total of 756 were chosen for further study. These were mixed together and frozen in aliquots at −70°.

**Recovery of *spo* mutations by selection for co-transformation with different chromosomal markers:** Proceeding as described under MATERIALS AND METHODS, the pooled collection of Tn917-generated *spo* mutations was screened to determine whether it included mutations linked by transformation to any of 25 different genetic markers. Seven insertional *spo* mutations were recovered and these are described individually below.

***spoIIE*::Tn917ΩHU181:** Mutation *spo*::Tn917Ω-HU181 was recovered by co-transformation with *cysA14*. In the phenotype it produced (Table 4) and in its apparent map position (Figure 2A), this insertion resembled the previously studied mutation *spoIIE*::Tn917ΩHU7 (YOUNGMAN, PERKINS and LOSICK 1983), and thus was suspected as being another *spoIIE* allele. This has been confirmed by direct examination of cloned chromosomal DNA that includes the transposon insertion junctions (J. WESTPHELING and P. YOUNGMAN, unpublished data). Although within the same transcription unit, *spoIIE*::Tn917-ΩHU7 and *spoIIE*::Tn917ΩHU181 are separated by more than 2 kb and are in opposite orientations.

***spoIVC*::Tn917ΩHU215:** Mutation *spo*::Tn917Ω-

HU215 was recovered by co-transformation with *aroD120*. Co-transformation studies positioned this insert near *asaA*, and between *asaA* and *aroD*, which is close to previously studied alleles of *spoIVC* (Figure 3A). In their biochemical characteristics (Table 4), the mutant bacteria resembled previously described *spoIVC* mutants (PIGGOT and COOTE 1976). Moreover, cloned chromosomal DNA recovered from the site of insertion has been shown to correct *spoIVC* mutations in transformation experiments and to contain the same restriction sites at the same positions as in DNA cloned by FUJITA and KOBAYASHI (1985) that complements *spoIVC* mutations (B. KUNKEL and K. SANDMAN, unpublished data). Based on this information, *spo*::Tn*917*ΩHU215 was classified as an allele of *spoIVC*.

**spoVK::Tn*917*ΩHU178:** Mutation *spo*::Tn*917*Ω-HU178 was recovered by co-transformation with *glnA100*. In its linkage to *glnA* and *thyA* and in the phenotype it produced, this insertion was indistinguishable from previously characterized insertional *spo* mutation *spoVK*::Tn*917*ΩHU8 (Figure 2D) and was therefore classified as another allele of *spoVK*. Because only one of these two independently derived insertions can be converted into a *lacZ* fusion by recombination with pTV55 (YOUNGMAN 1986), we conclude that the two insertions are probably in opposite orientations (K. SANDMAN and P. YOUNGMAN, unpublished data).

**spoVL::Tn*917*ΩHU179:** Mutation *spo*::Tn*917*Ω-HU179 was recovered by co-transformation with *pheA1*. In subsequent test-crosses, the insert co-transformed with *pheA* to an extent of 55–60% and with *nic* to an extent of better than 60%. Although three-factor transformation-mediated crosses were inconclusive in establishing the order of these markers, two-factor linkages suggested the order *nic-phe-spo* (Figure 3B). After overnight growth in DSM broth, mutant bacteria contained phase-bright prespores, indicating a block at stage IV or stage V. Among known *spo* loci in the vicinity of *phe*, there are two defined by late-blocking mutations, *spoIVF* and *spoVB*. Of these, only *spoIVF* is close enough to *phe* to possibly correspond with the position of *spo*::Tn*917*ΩHU179 (Figure 3B) (PIGGOT and COOTE 1976). In further phenotypic tests, however, insert-containing mutants were found to produce DPA and to make partially heat-resistant prespores (Table 4). These observations ruled out a stage IV block. Thus *spo*::Tn*917*ΩHU179₊was classified as defining a new stage V locus, *spoVL*.

**spoVP::Tn*917*ΩHU194:** Mutation *spo*::Tn*917*Ω-HU194 was recovered by selection for transformation with *aroC7*. In biochemical tests (Table 4), mutant bacteria produced wild-type levels of AP, GDH and DPA, indicating a block at stage V. The results of three-factor transduction crosses (Table 2) placed

*spo*::Tn*917*ΩHU194 between *aroC* and *trp*. Because there are no known stage V loci in this region, *spo*::Tn*917*ΩHU194 was classified as defining a new locus, *spoVP*.

**spoVQ::Tn*917*ΩHU195:** Like *spoVP*::Tn*917*Ω-HU194, *spo*::Tn*917*ΩHU195 was recovered by selection for apparent co-transformation with *aroC7*. Three-factor transduction-mediated crosses (Table 2) placed the insert in the interval between *lys* and *aroD* (Figure 3A). In biochemical tests, mutant bacteria produced wild-type levels of AP, GDH and DPA (Table 4), indicating a block at stage V. Mutants also developed refractile prespores that were released into the medium but which were not heat-resistant. Another stage V locus, *spoVA*, is also located in this region, but *spoVA* mutations (such as *spoVA*::Tn*917*-ΩHU276 discussed below) are distinguishable from *spo*::Tn*917*ΩHU195 in their very different co-transformation and co-transduction linkages to *lys* (Figures 2D and 3A). Thus, *spo*::Tn*917*ΩHU195 was considered very likely not to be an allele of *spoVA* and was classified as defining another stage V locus, *spoVQ*.

In addition to these successful retrievals of linked insertional *spo* mutations, unsuccessful attempts were made to recover mutations linked by transformation to *aroG*, *aroI*, *ctrA*, *cysB*, *dal*, *fruB*, *gltA*, *hisA*, *ilvB*, *metB*, *metC*, *purA*, *purB*, *pyrD*, *sacA*, *tre*, and *trpC*. In most of these cases, however, only one attempt was made to recover a linked insertion.

**Recovery of *spo* mutations by selection for co-transduction with different chromosomal markers:** The approach of screening the pooled collection of insertional *spo* mutations for linkage by transformation to different chromosomal markers confined the survey to a relatively small interval on either side of each marker used. One advantage of this approach was the likelihood that mutations close to one another that produced the same stage-block phenotype might be resolved as being different. Not all areas of the chromosome could be covered in this way, however, which made it desirable to carry out a similar survey by selection for PBS1-mediated transduction linkage. Because PBS1 is a very large generalized transducing phage, this expanded the area surveyed to about 100 kb on either side of selected markers used. Nine additional mutations were recovered as the result of this co-transduction survey, and these are described individually below.

**spoOJ::Tn*917*ΩHU261:** Mutation *spo*::Tn*917*Ω-HU261 was recovered by co-transduction with *purA16*. In subsequent test-crosses, the insert displayed about 85% co-transduction with *purA* and 15–20% co-transduction with *cysA* (Figure 2A). The results of three-factor crosses (Table 2) confirmed the map order *purA-spo-cysA* and positioned the insert near a known stage O locus, *spoOJ*. Samples from

overnight growth in DSM broth revealed no indication of asymmetric septation. In biochemical tests (Table 4), mutants were found to produce AB and PR, but no AP. Because these characteristics were consistent with published descriptions of *spoOJ* mutants (PIGGOT and COOTE 1976), *spo*::Tn917ΩHU261 was classified as an allele of *spoOJ*.

**spoIID::Tn917ΩHU298:** Mutation *spo*::Tn917Ω-HU298 was recovered by co-transduction with *ctrA*. Further mapping (Figure 3C) and phenotypic studies (Table 4) revealed extensive similarities to previously characterized insertional mutation *spo*IID::Tn917-ΩHU6 (YOUNGMAN, PERKINS and LOSICK 1983). This insertion was therefore classified as another allele of *spoIID*.

**spoIIG::Tn917ΩHU325:** Mutation *spo*::Tn917Ω-HU325 was recovered by co-transduction with *pyrD1*. Three-factor transduction-mediated crosses (Table 3) placed the insert in the interval between *pyr* and *fruB*, near known stage II locus *spoIIG* (Fig. 2C). Microscopic studies revealed aberrant asymmetric septation, indicating a stage II block, and in biochemical tests mutant bacteria were found to produce no AP. Because these and other phenotypic tests (Table 4) were consistent with properties of previously studied *spoIIG* mutants (PIGGOT and COOTE 1976), *spo*::Tn917-ΩHU325 was classified as an allele of *spoIIG*.

**spoIIL::Tn917ΩHU289:** Mutation *spo*::Tn917Ω-HU289 was recovered by co-transduction with *lys1*. Three-factor crosses (Table 3) placed *spo*::Tn917-ΩHU289 roughly midway between *aroD* and *lys1*, near *spoIIIA,B* and *spoOA* (Figure 3A). In biochemical tests (Table 4), insert-containing mutants produced AB and PR (in contrast with typical *spoOA* mutants), but did not produce AP (in contrast with all stage III mutants). These phenotypic properties were considered to be inconsistent with the assignment of *spo*::Tn917-ΩHU289 to a known sporulation locus in the *aroD-lys* region, and the insert was thus classified as defining a new stage II locus, *spoIIL*.

**spoIIM::Tn917ΩHU287:** As with *spoIIL*::Tn917Ω-HU289, mutation *spo*::Tn917ΩHU287 was recovered by selection for co-transduction with *lys1*. Although these two mutations were found to produce similar phenotypes (Table 4), they were readily distinguished from each other by map position, particularly with respect to their linkages to *lys1*. Whereas *spoIIL*::Tn917ΩHU289 co-transduced with *lys1* to an extent of less than 50%, *spo*::Tn917ΩHU287 co-transduced to an extent of more than 90% (Figure 3A). Because PBS1 is such a large transducing phage, this significant and reproducible difference in co-transduction was considered to be strong evidence that the two inserts could not be at the same genetic locus. Three-factor transduction crosses involving

*spo*::Tn917ΩHU287 indicated the map order *aroD-spo-lys* (Table 3), placing the insert in the vicinity of a known stage II locus, *spoIIA*. Because the results of phenotypic tests (Table 4) were consistent with a stage II block, *spo*::Tn917ΩHU287 was initially thought very likely to be an allele of *spoIIA*. This was ruled out by further experiments, however. Plasmid clone pPP133 (P. J. PIGGOT, unpublished data), which contains the entire *spoIIA* operon, failed to complement the sporulation defect when integrated into the chromosome of a *spo*::Tn917ΩHU287-containing mutant, and insert-containing mutants could not be converted to a Spo$^+$ phenotype in marker-rescue experiments using pPP133 DNA (P. YOUNGMAN, unpublished data). Even more persuasive was the failure to observe complementation when *spoIIA*-containing DNA was introduced into insertional mutants on Φ105 vectors (J. ERRINGTON, personal communication). Based on these results, we concluded that *spo*::Tn917ΩHU287 defines a new stage II locus near *spoIIA*, and we have designated this locus *spoIIM*.

**spoVA::Tn917ΩHU276:** Mutation *spo*::Tn917Ω-HU276 was also recovered by selection for co-transduction with *lys1*, but was readily distinguished from *spoIIL*::Tn917ΩHU289 and *spoIIM*::Tn917ΩHU287 by its phenotypic characteristics. Bacteria containing *spo*::Tn917ΩHU276 made darkly pigmented colonies on DSM plates, and developed phase-bright prespores after overnight growth in DSM broth, indicating a late block. The results of biochemical tests and heat-resistance assays suggested a block at stage V (Table 4). The results of three-factor transduction-mediated crosses (Table 3) positioned the insert between *lys* and *aroD*, and the insert displayed significant transformation linkage to *lys1* (Figure 3A), placing it near the known stage V locus *spoVA* (FORT and ERRINGTON 1985). Based on this information, *spo*::Tn917-ΩHU276 was classified as an allele of *spoVA*.

**spoVG::Tn917ΩHU265:** As with *spoOJ*::Tn917Ω-HU261, mutation *spo*::Tn917ΩHU265 was recovered by co-transduction with *purA16*. Three-factor transduction-mediated crosses (data not shown) placed the insert between *purA* and *cysA*, near *tms-26*, which is in the vicinity of two previously described stage V loci, *spoVC* (YOUNG 1975) and *spoVG* (ROSENBLUH et al. 1981). Biochemical tests of *spo*::Tn917ΩHU265-containing mutants (Table 4) indicated a block at stage V, and more detailed transformation-mediated mapping studies confirmed the close proximity of this mutation to *spoVC* and *spoVG* (Figure 2A). Both *spoVC* and *spoVG* are present in extensively characterized cloned DNA isolated in previous work (SEGALL and LOSICK 1977; MORAN, LOSICK and SONENSHEIN 1980; IGO, 1986), which made it possible through marker-rescue experiments with subcloned fragments (IGO

1986) and Southern hybridization analysis (P. ZUBER, personal communication) to classify *spo*::Tn917-ΩHU265 definitively as an allele of *spoVG*.

**spoVM::Tn917HU324:** As with *spoIIG*::Tn917-ΩHU325, mutation *spo*::Tn917ΩHU324 was recovered by co-transduction with *pyrD1*. Although the two inserts displayed approximately the same co-transduction linkages to *pyrD* (20–25%), results of three-factor crosses (Table 3) indicated that they were on opposite sides of *pyrD*. Insert *spo*::Tn917ΩHU324 mapped to the interval between *pyrD* and *dnaA*, close to known stage III locus *spoIIIE* and stage V locus *spoVF* (*dpa*) (Figure 2C). Microscopic examination of mutant bacteria grown overnight in DSM broth revealed phase-bright prespores, however, which ruled out a block at stage III. In biochemical tests (Table 4), the mutant was found to produce wild-type levels of AP, GDH and DPA, indicating a block at stage V. Since production of DPA is inconsistent with its being an allele of *spoVF* (PIGGOT, MOIR and SMITH 1981), *spo*::Tn917ΩHU324 was classified as defining a new stage V locus, *spoVM*.

**spoVN::Tn917ΩHU297:** Mutation *spo*::Tn917Ω-HU297 was recovered by co-transduction with *thrA5*. In subsequent test-crosses the insert co-transduced with *thrA* to an extent of about 80% and with *cysB* to an extent of about 25% (Figure 3C). Results of three-factor transduction-mediated crosses (Table 3) indicated the map order *spo-thrA-cysB*, which placed the insert far from any known *spo* locus. On DSM plates, mutant bacteria formed pigmented colonies. Microscopic examination revealed refractile prespores after overnight growth. In biochemical tests (Table 3), the mutant produced AP and GDH, but no DPA, consistent with a block at either stage IV or stage V. Partial prespore heat-resistance was observed (Table 3), however, which strongly suggests a stage V block. Based on this information, *spo*::Tn917ΩHU297 was classified as defining a new stage V locus, *spoVN*.

Attempts to recover *spo* mutations by selection for co-transduction with *purB*, *glyB*, *glnA* and *aroD* were unsuccessful. Unsuccessful attempts were not repeated.

**Recovery of an insertional mutation in cotA (pig), a gene required for sporulation-associated pigmentation:** In the course of screening the library of insert-containing bacteria on DSM agar for colony appearances characteristic of Spo mutants, a striking Spo⁺ pigmentation variant was identified. From a total of just over $10^6$ colonies screened, 8 apparently identical Spo⁺ pigmentation-negative colonies were recovered. Unlike the unpigmented colonies of a Spo mutant, these colonies were not detectably less opaque than the wild type, even after a long period of incubation. Samples of bacteria from such colonies examined under the microscope revealed a high titer of free, phase-bright spores. When cultured in DSM broth and sam-

pled for microscopic study at 1–2-hr time intervals throughout growth and sporulation, no difference was observed between this variant and the wild type in the timing or extent of sporulation. In biochemical tests, the variant produced wild-type levels of AP, GDH and DPA, and the spores it made were indistinguishable from the wild-type in heat-resistance (Table 4). Based on these results, the insert causing the pigmentation defect was suspected as being an allele of *pig*, a locus defined by several previously mapped mutations causing the same phenotype (ROGOLSKY 1968). Indeed, the results of PBS1-mediated three-factor crosses (Table 3) placed the insert between *purB* and *dal*, near the reported location of *pig* (HENNER and HOCH 1980).

The *pig* locus was recently shown to encode one of the major coat components of *B. subtilis* spores, a 63-kd protein, and has been renamed *cotA* (DONOVAN *et al.* 1987). The mechanism by which this coat protein is responsible for spore pigmentation is unknown. An analysis of cloned DNA that included the pigmentation defect-causing Tn917 insertion has confirmed that this insertion is actually within the coding sequence for the 63-kd *cotA* gene product (K. SANDMAN, unpublished data).

**Recovery of phenotypically silent insertions in specified regions of the chromosomes by selection for transformation-mediated linkage:** In previous studies (YOUNGMAN, PERKINS and LOSICK 1983; VANDEYAR and ZAHLER 1986), when Tn917 insertions were chosen at random, without regard to the phenotype they produced, they were found to be clustered predominantly in two-thirds of the chromosomal map, extending from *ctrA* counterclockwise to *glyB* (Figure 1). Within this large preferred area were several "hotspots" of very high target preference, one near the replication terminus being particularly conspicuous because it included auxotrophic markers *gltA* and *gltB*. These hotspots are now known not to represent specific target sequences, but rather regions spanning several kilobases within which insertions are distributed with a high degree of randomness (PERKINS and YOUNGMAN 1986).

To determine whether Tn917 insertions may be found in useful abundance in any region of the chromosome, DNA prepared from the library of insertions was used to survey 12 markers in 12 widely scattered chromosomal locations for the presence of co-transforming inserts. As with the similar co-transformation survey described above, which was carried out with DNA from the pooled collection of Spo mutants, co-selections for MLS^r and prototrophy consisted of two or more enrichment steps calculated to elevate the number of transformants resulting from actual linkage above the background that was expected as the result of congression (YOUNGMAN 1986). For each of

TABLE 5

**Phenotypically silent Tn917 insertions selected for linkage to chromosomal markers**

| Insertion | Selected marker | Linkage (% co-transformation) |
|---|---|---|
| chr::Tn917ΩHU139 | metC3 | 38 |
| chr::Tn917ΩHU142 | glyB133 | 84 |
| chr::Tn917ΩHU144 | pheA1 | 99 |
| chr::Tn917ΩHU146 | cysA14 | 86 |
| chr::Tn917ΩHU148 | purA16 | 62 |
| chr::Tn917ΩHU149 | hisA1 | 37 |
| chr::Tn917ΩHU151 | trpC2 | 36 |
| chr::Tn917ΩHU153 | metB5 | 77 |
| chr::Tn917ΩHU156 | glnA100 | 19 |
| chr::Tn917ΩHU160 | metD1 | 81 |
| chr::Tn917ΩHU163 | guaA3 | 41 |
| chr::Tn917ΩHU169 | purB33 | 23 |

the 12 markers tested, closely linked inserts were recovered (Table 5; Figure 1).

## DISCUSSION

Based on extensive mapping data (PIGGOT 1973; YOUNG 1975; PIGGOT and HOCH 1985) and on a statistical argument that considered the fraction of newly isolated *spo* mutations representing uncharacterized loci (HRANUELI, PIGGOT and MANDELSTAM 1974), earlier studies estimated the total number of *spo* loci at approximately 40–60. The current total, excluding the present work, stands at 40 (LOSICK, YOUNGMAN and PIGGOT 1986). In our view, the present work is not inconsistent with earlier estimates, but it raises their lower bound to 49 and suggests that the actual total may be somewhat greater than 60. We have characterized 24 Tn917-generated insertional *spo* mutations that define 21 different loci. Of these, 9 very likely represent new loci. If the total number of *spo* loci is taken to be 60, we would expect less than a third of our mutations to fall in new loci, assuming the mutations to be randomly distributed. The actual percentage was somewhat higher ($^9/_{24}$), but there were several known or potential contributions to non-randomness among our mutations, some of which could and some of which would certainly exaggerate the percentage that would fall in unknown loci:

**Intrinsic nonrandomness of Tn917 insertions:** Tn917 is known to show a preference for inserting into some regions of the chromosome (YOUNGMAN, PERKINS and LOSICK 1983; VANDEYAR and ZAHLER 1986). It therefore seems likely that some *spo* genes are better targets for Tn917 than others, and it is possible that some *spo* genes are in regions that actually exclude Tn917 insertions. This could but would not necessarily exaggerate the percentage of Tn917-generated mutations that define new loci.

**Possible exclusion of certain mutant types:** One

type of mutant apparently under-represented in our collection is the SpoO class. This could reflect the intrinsic non-randomness of insertions, or could have resulted from the way our "library" of insert-containing bacteria was obtained. After shifting the fermentor population to 48°, it was allowed to grow overnight to enrich for bacteria that contained chromosomal insertions of Tn917. In doing so, the population was held in stationary phase at 48° for an undetermined period of time (probably five or six hours), which might have seriously affected the viability of certain SpoO mutants relative to other types. Because SpoO mutants have such an obvious phenotype, they would be the most likely kind to have been identified in previous studies. Thus, excluding or reducing the representation of this class would probably exaggerate the percentage of Tn917 insertions in new loci.

**Mode of mutagenesis:** In addition to creating null mutations by simple insertional disruption, there is evidence that Tn917 insertions are often accompanied by adjacent deletions (S. A. ZAHLER, personal communication). This might increase the percentage of Tn917 insertional mutations (relative to chemical-induced mutations) that result from inactivating two or more cistrons within a single transcription unit) or inactivation of portions of two or more closely linked transcription units. Some *spo* loci might require this kind of mutagenic event for a phenotype to be apparent. If so, certain loci would be identified at much greater frequency by insertional mutagenesis than by chemical mutagenesis. Some insertions of Tn917 also appear capable of *activating* otherwise silent transcription units (S. A. ZAHLER, personal communication), raising the possibility that some of our mutations might result from gain of function at an inappropriate time, rather than loss of function.

**Nonrandom screening of the mutant population:** Among the *spo* mutants recovered from the library by selection for co-transformation or co-transduction, the recovery strategy itself was probably the most significant contribution to nonrandomness. In the case of the co-transformation survey, the recovery effort was limited to the immediate areas of particular selectable markers, and some of these markers were chosen in part because no previously characterized *spo* mutations were known to be linked to them by transformation. In the co-transduction survey, relatively large areas around selectable markers were covered, but in most cases only one mutant displaying a given colony morphology phenotype was analyzed from each group of co-transducing *spo* mutations. This made the survey less effective in regions where known *spo* mutations are clustered, and more effective in regions where *spo* mutations are more thinly scattered (and where perhaps less attention was given to the mapping of previously isolated *spo* mutations). It

may be relevant to note in this context that transposon-generated *spo* mutations require much less effort to map than conventional mutations, because the selection or screen is for drug-resistance rather than for a colony-appearance phenotype. A disproportionate percentage of the Tn917-generated mutations that identified new loci caused a developmental block at stage V, which is associated with a relatively subtle colony-appearance phenotype that would demand patience to score among the progeny of a cross. In fact, two of the new stage V loci (*spoVK* and *spoVN*) map far outside the clusters where most known *spo* mutations are located and where efforts to map a newly isolated stage V mutation would have been concentrated in the past.

On the other hand, our criteria for assignment of insertional *spo* mutations to "new" loci were relatively conservative, and it is quite possible that more than 9 of our mutations fall in previously uncharacterized genetic loci. Following the accepted convention (PIG-GOT and COOTE 1976), whenever one of the insertional mutations mapped near the reported location of a previously characterized locus defined by mutations causing a block at the same stage, that insertional mutation was classified as an allele of the known locus. In some cases (*e.g.*, the assignment of *spo*::Tn917ΩHU13 to *spoIIIA* and the assignment of *spo*::Tn917ΩHU25 to *spoIIIB*) these classifications must be regarded as highly provisional. Although additional information might have been obtained by measuring recombinational indexes between Tn917 insertions and the point mutations that defined known loci, this kind of analysis cannot resolve whether closely linked mutations actually fall within the same transcription unit, which is clearly the most important distinction to be made.

Although the collection of *spo* mutations described here adds significantly to the total of known loci, and thus helps to define the set of genes whose products are required for spore formation, its real value consists in the fact that these mutations were produced by transposon insertions. This should greatly facilitate further study of the physical structure, genetic organization and regulation of the genes or operons that the mutations have identified. For example, methods exist that make it possible to derive transcriptional *lacZ* fusions from genes that contain Tn917 insertions by recombination *in vivo* with certain vectors introduced by transformation (YOUNGMAN *et al.* 1985; YOUNGMAN 1986). In preliminary work, it has already proved possible to derive *lacZ* fusions from several of the Tn917-generated *spo* mutations described here and to use these fusions to demonstrate that expression of the mutated genes was developmentally regulated (P. YOUNGMAN and K. SANDMAN, unpublished data). Other methods (YOUNGMAN, PERKINS and Los-

ICK 1984b; YOUNGMAN *et al.* 1985) facilitate the cloning and manipulation of Tn917-mutated genes *in vitro*. These methods applied systematically to the collection of *spo* mutations described here should make it possible to sort many of the sporulation operons in which they are contained into coordinately regulated groups, and to begin to identify the mechanisms that activate these groups.

The fact that it was possible to recover Tn917 insertions in many different *spo* genes and that no individual insertional *spo* mutations were very conspicuous suggests that insertions outside of hotspot chromosomal regions are probably relatively random in distribution. This is also supported by the fact that it was possible to recover a phenotypically silent Tn917 insertion closely linked by transformation to any of 12 widely dispersed selectable markers. These conclusions are further reinforced by the recent work of LOVE, LYLE and YASBIN (1985), in which several Tn917 insertions into *din* (DNA-damage-inducible) genes were characterized, the work of VANDEYAR and ZAHLER (1986), in which several kinds of Tn917-generated auxotrophic mutations were characterized, and the work of HAHN, ALBANO and DUBNAU (1987), in which several different Tn917-generated *com* (competence-deficient) mutations were characterized. This firmly establishes the expectation that it should be possible to recover representative collections of virtually any kind of mutation in *B. subtilis* with Tn917-mediated mutagenesis.

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