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The *spo0K* Locus of *Bacillus subtilis* Is Homologous to the Oligopeptide Permease Locus and Is Required for Sporulation and Competence

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Spore formation in *Bacillus subtilis* is a dramatic response to environmental signals that is controlled in part by a two-component regulatory system composed of a histidine protein kinase (SpoIIJ) and a transcriptional regulator (Spo0A). The *spo0K* locus plays an important but undefined role in the initiation of sporulation and in the development of genetic competence. *spoIIJ spo0K* double mutants had a more severe defect in sporulation than either single mutant. Overproduction of the *spoIIJ* gene product resulted in the suppression of the sporulation defect, but not the competence defect, caused by mutations in the *spo0K* locus. On the basis of the phenotype of the *spoIIJ spo0K* double mutant and the effect of overproduction of the *spoIIJ* gene product, a transposon insertion in the *spo0K* locus was isolated. The *spo0K* locus was cloned and sequenced. *spo0K* proved to be an operon of five genes that is homologous to the oligopeptide permease (*opp*) operon of *Salmonella typhimurium* and related to a large family of membrane transport systems. The requirement for the transport system encoded by *spo0K* in the development of competence was somewhat different than its requirement in the initiation of sporulation. Disruption of the last open reading frame in the *spo0K* operon caused a defect in competence but had little or no effect on sporulation. We hypothesize that the transport system encoded by *spo0K* may have a role in sensing extracellular peptide factors that we have shown are required for efficient sporulation and perhaps in sensing similar factors that may be necessary for genetic competence.

Cells of the gram-positive bacterium *Bacillus subtilis* can differentiate into a dormant cell type, the spore or endospore, upon nutrient deprivation (15, 52) at a high cell density (18, 56). Seven genetic loci (*spo0A*, *0B*, *0E*, *0F*, *0H*, *0J*, *0K*) that are required for the initiation of sporulation have been identified (41, 51). Mutations in these genes alter the normal pattern of gene expression and prevent the earliest morphological changes associated with sporulation. Cloning and DNA sequence analysis have been reported for most of the *spo0* genes (29, 41, 51). Most, if not all, of the *spo0* gene products are present during growth and are thought to be involved in sensing sporulation conditions and regulating the transition from growth to sporulation.

The *spo0K* locus was initially defined by a mutation that causes a partial defect in sporulation (9, 42) and was later found to cause a defect in the development of genetic competence (44, 45). The sporulation defect caused by the *spo0K* mutation is at least partly suppressed by missense mutations in *spo0A* (49, 50). The same missense mutations in *spo0A* also bypass the need for several of the other *spo0* genes (*B*, *E*, *F*, and *J*), indicating that the function of these *spo0* gene products may be to activate Spo0A (23, 49, 50, 53).

While some missense mutations in *spo0A* can bypass the need for some of the other *spo0* genes in sporulation, *spo0A* itself is required for sporulation. Null mutations in *spo0A* completely block sporulation and at least partially block many of the other processes associated with the end of normal growth, including the development of genetic competence and the production of extracellular proteases and antibiotics (29, 51). The *spo0A* gene product is a DNA-binding protein that controls the expression of genes that are involved in the transition from growth to the stationary phase (14, 39, 40, 51, 52).

Spo0A belongs to a family of prokaryotic regulatory proteins (including OmpR and NtrC [GlnG, NR_I]) that are homologous in their amino termini and are involved in signal transduction and the regulation of gene expression in response to changes in external conditions (14, 35). The activity of these regulatory proteins (called response regulators) is modulated by the phosphorylation of an aspartate residue in the conserved amino terminus. The response regulator is one component of the so-called two-component regulatory systems. The other component is a histidine protein kinase (also called the sensor component), which autophosphorylates on a histidine residue and transfers the phosphate to the cognate regulator. The histidine protein kinases share homology in their carboxy termini (reviewed in references 3 and 54).

Two genes that encode histidine protein kinases that are thought to lead to the activation of Spo0A in vivo have been identified in *B. subtilis*. *spoIIJ* (*kinA*), homologous to the kinase component of the two-component systems (6, 37), has been shown to phosphorylate Spo0A in vitro (37). *spoIIJ* mutants are partially defective in sporulation, sporulating at a frequency approximately 1 to 30% of the wild-type frequency, depending on the strain background and the precise sporulation conditions (6, 37, 47, 58; unpublished data). *comP*, a gene required for the development of genetic competence (the ability to take up DNA), is also homologous to the kinase component of the two-component systems (58). Mutations in *comP* cause a modest defect in sporulation (approximately 10 to 50% of the wild-type frequency) but, when combined with mutations in *spoIIJ*, cause a more severe defect (58). This result indicates that both *comP* and

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TABLE 1. *B. subtilis* strains used

Strain	Genotype	Comments, source, or reference
JH642	*trpC2 pheA1*	J. Hoch
PB2	*trpC2*	8
PB69	PB2 *trp*$^+$ *spo0K141*	8
KS19	*spoIIJ*::Tn*917*ΩHU19 (*spoIIJ*::Tn*917*)	47
MO495	*spo0K*$^+$ *chr*::Tn*917*ΩMO495 (*chr*::Tn*917*)	P. Stragier; Tn*917* ~10% linked to *spo0K* by transformation
MO496	*spo0K*$^+$ *chr*::Tn*917*::pTV21Δ2	P. Stragier; MO495 transformed with pTV21Δ2 (Cm^r MLS^s)
MO1022	*amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*)	P. Stragier
KI95	JH642 *amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*)	JH642 transformed with DNA from MO1022
KI102	PB2 *amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*)	PB2 transformed with DNA from MO1022
AG522	JH642 *spoIIJ*::Tn*917*	JH642 transformed with DNA from KS19 (MLS^r)
AG785	JH642 *spoIIJ*::Tn*917*::pTV21Δ2	AG522 transformed with pTV21Δ2 (Cm^r MLS^s)
KI418	JH642 *spo0K418*::Tn*917lac* (*spo0K*::Tn*917lac*)	24a
KI566	JH642 *spo0K*::Tn*917lac*::pTV21Δ2	KI418 transformed with pTV21Δ2 (Cm^r MLS^s)
AG880	PB2 *trp*$^+$ *spo0K141 chr*::Tn*917*	PB69 transformed with DNA from MO495 (MLS^r Spo$^-$)
KI246	JH642 *spo0K141 chr*::Tn*917*	JH642 transformed with DNA from AG880 (MLS^r Spo$^-$)
KI250	JH642 *spo0K141 chr*::Tn*917*::pTV21Δ2	KI246 transformed with DNA from MO496 (Cm^r MLS^s Spo$^-$)
KI261	JH642 *chr*::Tn*917*::pTV21Δ2 *spo0K141 spoIIJ*::Tn*917*	KI250 transformed with DNA from KI418 (MLS^r)
KI738	JH642 *spoIIJ*::Tn*917*::pTV21Δ2 *spo0K*::Tn*917lac*	AG785 transformed with DNA from KI418 (MLS^r)
KI741	JH642 *amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*) *spoIIJ*::Tn*917*	KI95 transformed with DNA from KI418 (MLS^r)
KI742	JH642 *spo0K141 chr*::Tn*917 amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*)	KI246 transformed with DNA from MO1022 (Cm^r)
KI98	PB2 *spoIIJ*::Tn*917*	PB2 transformed with DNA from AG522 (MLS^r)
DZR18	PB2 *spo0K*::Tn*917lac*	PB2 transformed with DNA from KI418 (MLS^r)
KI100	PB2 *trp*$^+$ *spo0K141 spoIIJ*::Tn*917*	PB69 transformed with DNA from AG522 (MLS^r)
KI776	PB2 *spoIIJ*::Tn*917 spo0K*::Tn*917lac*::pTV21Δ2	KI98 transformed with DNA from KI566 (Cm^r MLS^r)
KI22	PB2 *trp*$^+$ *spo0K141 amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*)	PB69 transformed with DNA from MO1022 (Cm^r Spo$^-$)
KI772	PB2 *amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*) *spo0K*::Tn*917lac*	KI102 transformed with DNA from KI418 (MLS^r)
DZR84	PB2 *spo0KE*::pDR30	Disruption of *spo0KE*

spoIIJ may be involved in the activation of a response regulator that is required for sporulation, perhaps Spo0A.

As part of our work on signal transduction and the initiation of sporulation, we set out to isolate insertion mutations that would cause *spoIIJ* mutants to have a more severe defect in sporulation (24a). In the same screen, we were also able to isolate sporulation mutations that could be suppressed by the overexpression of *spoIIJ* from a heterologous promoter. Such mutations could identify genes encoding additional protein kinases or other proteins that act in the signal transduction pathways. During the course of these experiments, we isolated a Tn*917lac* insertion mutation that seemed to be in *spo0K*. We describe the phenotypic characterization of strains containing this *spo0K*::Tn*917lac* mutation, the effect of the *spoIIJ* mutation and *spoIIJ* overexpression on *spo0K* mutants, and the cloning and DNA sequence of the *spo0K* locus. Our sequencing results indicate that the *spo0K* operon contains five genes and is homologous to the oligopeptide permease (*opp*) operon of *Salmonella typhimurium*. *spo0K* was independently cloned and sequenced by Perego et al., and they were the first to note the similarity of *spo0K* to *opp* (38).

Oligopeptide permease from *S. typhimurium* transports peptides from two to five amino acids in length and cell wall peptides (17). Peptides and peptidelike factors are often involved in microbial differentiation and behavior. Previously, we presented evidence that *B. subtilis* produces at least one extracellular differentiation factor that is required for efficient sporulation (18, 19). This factor is heat resistant, dialyzable, and pronase sensitive, indicating that it is at least in part an oligopeptide. The finding that *spo0K* encodes an oligopeptide permease that is required for efficient sporulation raises the intriguing possibility that *spo0K* encodes a receptor or transport system for such a differentiation factor.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strains used for cloning and maintaining plasmids were JM101 [*supE thi* Δ(*proAB-lac*)(F′ *traD proAB*$^+$ *lacI*^{q} *lacZM15*)]; AG115, derived from MC1061 [*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK rpsL hsdR*] by mating in an F′ *lacI*^{q} *lacZ*::Tn*5*; and XL1-Blue [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*(F′ *proAB*$^+$ *lacI*^{q} *lacZM15* Tn*10*)] (Stratagene).

The *B. subtilis* strains used were derived from strain 168 and are listed in Table 1. The original *spo0K141* mutant strain apparently contained at least two mutations, one mutation in the *spo0K* locus and another mutation, unlinked to *spo0K* by transformation, that enhances the sporulation defect of the *spo0K141* allele (27a). The *spo0K141* mutation has been separated from the second mutation (8), and the strains used here seem only to contain the defective *spo0K* locus.

The *spo0K418*::Tn*917lac* insertion mutation was isolated from a transposon library kindly provided by R. Yasbin (30). This is the same library that was used to identify Tn*917lac* insertions in *din* genes (30), *com* genes (20), and *csh* genes (25).

The plasmids used are listed in Table 2 and described in the text.

Media. LB (10) medium was used for routine maintenance and growth of *E. coli* and *B. subtilis*. DS medium (48) was used as the nutrient sporulation medium. Minimal medium contained the S7 minimal salts described by Vasantha and Freese (57), except that MOPS buffer was used at a concentration of 50 mM rather than 100 mM. Minimal medium was supplemented with glucose (1%) and glutamate (0.1%). Required amino acids were added at 40 μg/ml. Media were solidified for plates with 15 g of agar (Difco Laboratories) per liter. Sporulation proficiency was visualized on DS or 2× SG

TABLE 2. Plasmids used[a]

Plasmids	Comments[b] and source or reference
Vectors	
pTV21Δ2	Ap Cm (61)
pBluescriptII KS$^+$ and SK$^+$	Ap; abbreviated pKS and pSK (Stratagene)
pGEM3Zf$^+$ *cat-1* (pGEM*cat*)	Ap Cm; vector to clone fragments for integration into *B. subtilis* chromosome (integrative vector) (60)
pJH101	Ap Cm; integrative vector (13)
Others	
pDR1	Initial *Hin*dIII clone from KI566 (*spo0K*::Tn*917lac*::pTV21Δ2)
pDR2	Initial *Eco*RI clone from KI566 (*spo0K*::Tn*917lac*::pTV21Δ2); used to sequence DNA adjacent to the end of the transposon
pDR3	Initial *Bgl*II clone from KI566 (*spo0K*::Tn*917lac*::pTV21Δ2)
pDR4	Initial *Pvu*II clone from KI566 (*spo0K*::Tn*917lac*::pTV21Δ2); used to sequence DNA adjacent to the end of the transposon
pDR5	Initial *Bam*HI clone from KI566 (*spo0K*::Tn*917lac*::pTV21Δ2)
pDR6	2.5-kb *Eco*RI-*Bgl*II fragment from pDR3 cloned into pJH101 (*Eco*RI-*Bam*HI); used to "walk" to the end of the operon and to disrupt *spo0KD*
pDR9	6-kb *Eco*RI-*Sph*I fragment in pJH101; cloned by "walking" from a pDR6 integrant
pDR11	8-kb *Eco*RI-*Sal*I fragment in pJH101; cloned by "walking" from a pDR6 integrant
pDR13	*Pvu*II-*Cla*I fragment from pDR4 cloned into pJH101 (*Eco*RV-*Cla*I); the *Cla*I site is in the end of the transposon; used to clone wild-type DNA that was the site of the Tn*917lac* insertion
pDR16	480-bp *Hin*dIII fragment from pDR3, containing the *Eco*RI site, cloned into pGEM*cat*; used to sequence across the *Eco*RI site
pDR18	*Pvu*II-*Eco*RI fragment in pJH101 between *Eco*RV and *Eco*RI; cloned by "walking" from a pDR13 integrant
pDR20	2-kb *Pvu*II-*Eco*RI fragment from pDR18 cloned into pKS; used for exonuclease deletions
pDR21	2-kb *Pvu*II-*Eco*RI fragment from pDR18 cloned into pSK; used for exonuclease deletions
pDR30	540-bp *Eco*RV-*Cla*I fragment cloned into pJH101; used to disrupt *spo0KE*
pJL1	1.7-kb *Eco*RV fragment from pDR9 cloned into pGEM*cat*; used to map *spo0K141* and to disrupt *spo0KE*
pJL2	3.5-kb *Eco*RI fragment from pDR9 cloned into pKS in the same orientation as the *lacZ* fragment; used to make exonuclease deletions for sequencing
pJL3	3.5-kb *Eco*RI fragment from pDR9 cloned into pKS in the opposite orientation as the *lacZ* fragment; used to make exonuclease deletions for sequencing
pJL4	3.5-kb *Eco*RI fragment from pDR9 cloned into pGEM*cat*; used to map *spo0K141* and to disrupt *spo0KE*
pJL7	2.6-kb *Eco*RV-*Xho*I fragment from pDR9 cloned into pSK; used to make exonuclease deletions for sequencing

[a] All plasmids can replicate in *E. coli*.
[b] Indicated fragment sizes are approximate.

(28) plates. Ampicillin was used at 50 to 100 μg/ml, chloramphenicol was used at 5 μg/ml, and erythromycin and lincomycin were used at 0.5 and 12.5 μg/ml, respectively. The last two drugs were used in combination to select for resistance to macrolide-lincosamide-streptogramin B (MLS) antibiotics encoded by Tn*917* and Tn*917lac*.

Spore assays. Cells were grown in DS medium at 37°C (unless otherwise indicated), and spores were assayed at least 12 h after the end of exponential growth. The number of spores per milliliter culture was determined as the number of heat-resistant (80°C for 20 min) CFU on LB plates. Viable cells were measured as the total number of CFU (before heat treatment) under similar plating conditions. Colony morphology on DS plates or 2× SG plates was used as a qualitative indication of the sporulation phenotypes of various mutants. Spo$^-$ strains tend to be translucent while Spo$^+$ strains are opaque on these plates.

Transformation. *E. coli* cells were made competent and transformed by standard procedures (46). Cells of *B. subtilis* were made competent by the two-step method essentially as described previously (12). Alternatively, cells were made competent by growth in defined minimal medium (S7 medium) supplemented with glucose and glutamate. At a density of 150 to 170 Klett units, cells were removed from the culture and used for transformation. Cells grown in this way, in the absence of complex mixtures of amino acids (Casamino Acids and yeast extract), appear to be competent throughout growth, peaking towards the end of exponential growth and losing competence during the stationary phase (11a). Transformations were performed with excess DNA for the quantitative determination of transformation frequencies.

Cloning of *spo0K*. DNA adjacent to the *spo0K*::Tn*917lac* insertion was cloned into *E. coli* by methods described by Youngman et al. (59–61). In brief, the part of plasmid pTV21Δ2 containing an *E. coli* replicon and selectable markers for *B. subtilis* (Cm^r) and *E. coli* (Ap^r) flanked by sequences from Tn*917* was recombined into the central region of Tn*917lac*, resulting in Cm^r MLS^s transformants. DNA from these transformants was digested with one of several restriction enzymes and ligated at a dilute concentration to promote intramolecular ligation. The ligation mix-

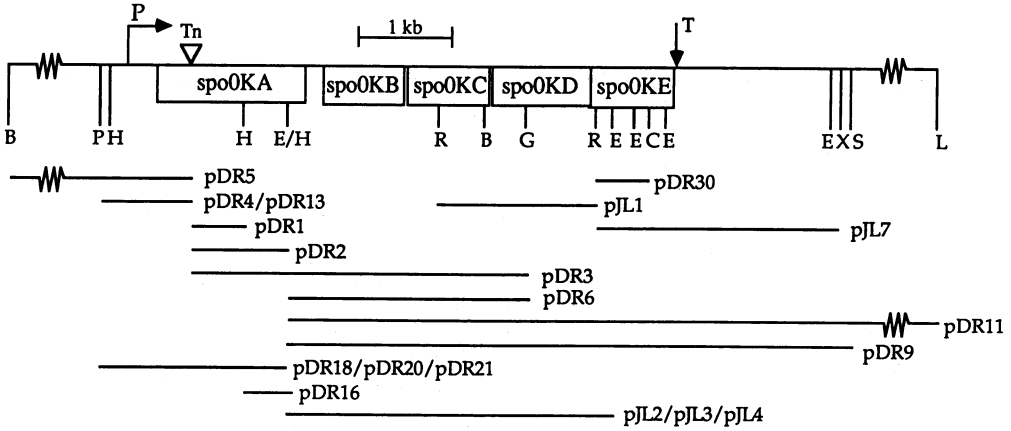


FIG. 1. Organization and restriction map of the *spo0K* operon. The locations of the likely promoter and terminator sites are indicated by P and T, respectively, and by arrows. The location of the Tn*917lac* insertion is indicated by a triangle and Tn. The predicted open reading frames of *spo0KD* and *spo0KE* overlap slightly. The restriction sites are indicated as follows: B, *Bam*HI; P, *Pvu*II; H, *Hin*dIII; C, *Cla*I; E, *Eco*RI; R, *Eco*RV; G, *Bgl*II; X, *Xho*I; S, *Sph*I; and L, *Sal*I. (E/H indicates an *Eco*RI site 3 bp upstream of a *Hin*dIII site.) The map is only partial for the *Hin*dIII, *Cla*I, and *Pvu*II sites; it is complete for all other sites shown.

ture was used to transform *E. coli*, with selection for Ap^r. Candidate clones were screened by restriction analysis, and the cloned *B. subtilis* DNA was subcloned into appropriate vectors as needed.

The initial clones and/or subclones were tested to see whether the DNA extended past an end of the transcription unit needed for *spo0K* function or whether the DNA was internal to the transcription unit. This test was done by integrating clones that contained the *cat* gene into the chromosome of wild-type *B. subtilis* cells. If the cloned DNA were internal to the locus, then integration (by single crossover) would disrupt the locus and cause a sporulation defect. In contrast, if the cloned DNA extended past one end (either end) of the transcription unit, then integration would generate one truncated copy and one intact copy of *spo0K*, resulting in a wild-type phenotype.

Additional sequences from the *spo0K* locus were cloned by "walking" from existing clones. Plasmids were integrated into the chromosome (by single crossover), and DNA from such transformants was cut with appropriate restriction enzymes to generate fragments containing the vector backbone and adjacent chromosomal sequences. DNA was ligated to promote circularization of fragments and transformed into *E. coli*. Again, candidate clones were examined by restriction analysis and integration into the *B. subtilis* chromosome to determine whether the cloned DNA was internal to the *spo0K* locus.

DNA sequencing. Nested deletions were made with exonuclease III (New England BioLabs) and exonuclease VII (Bethesda Research Laboratories) essentially as described previously (36). Plasmids used to make the deletions were prepared by the Qiagen (Qiagen Inc.) procedure. Six sets of deletions were made to sequence the whole operon on both strands. Fragments of the operon were cloned into the pBluescriptII KS and SK (pKS and pSK) plasmids (Stratagene) to generate plasmids pDR20, pDR21, pJL2, pJL3, and pJL7 (Table 2 and Fig. 1). pJL7 was used to make two sets of deletions, one set from each end of the cloned fragment, starting from different ends of the polycloning site. Fragments were ordered by size and sequenced with the appropriate primer. Primers included the reverse primer and the −40 and −20 forward primers (U.S. Biochemical Corp.). Specific primers to fill in gaps in the sequence and to sequence DNA adjacent to the ends of the transposon insertion were purchased from Oligos Etc. Inc. (Guilford, Conn.). The sequence across the *Eco*RI site, located at the ends of plasmids pJL2-pJL3 and pDR20-pDR21, was determined from pDR16. Double-stranded plasmids used for sequencing templates were prepared by the alkaline lysis method (46).

DNA sequencing was done with the Sequenase kit (U.S. Biochemical Corp.), with double-stranded plasmid DNA as the template and [α-^{35}S]dATP (Dupont, NEN Research Products) as the label. The sequence was determined from both strands, and much of the sequencing was done multiple times because of overlaps in the DNA fragments used. In a few places, there were differences in the sequence from one strand to the other. These discrepancies were resolved by repeating the sequencing with both dITP and dGTP.

Sequencing samples were electrophoresed on 6% polyacrylamide gels with 8 M urea by standard procedures (46). Gels were fixed, dried, and exposed to Kodak X-OMAT AR film. DNA sequence analysis, manipulations, and comparisons were done with the package of programs provided by the Genetics Computer Group, University of Wisconsin (11).

Nucleotide sequence accession number. The *spo0K* sequence has been assigned the GenBank accession number M57689.

RESULTS

As part of our work on signal transduction and the initiation of sporulation, we set out to isolate insertion mutations that would cause *spoIIJ* mutants to have a more severe defect in sporulation (24a). In the same screen, we were also able to isolate sporulation mutations that could be suppressed by the overexpression of *spoIIJ* from a heterologous promoter. During the course of this work, we isolated a Tn*917lac* insertion mutation that seemed to be in *spo0K*. We refer to the insertion mutation as *spo0K418*::Tn*917lac* or *spo0K*::Tn*917lac*. While the transposon is Tn*917lac*, the orientation of the insertion is such that the *lacZ* gene is not expressed.

spo0K was originally defined by the mutant allele

spo0K141 (Z31 of Coote [9] and Piggot and Coote [42]). This mutation causes a partial defect in the development of genetic competence (44, 45; unpublished results) as well as a defect in sporulation. We first established that the insertion mutation was likely to be in *spo0K* and then used the insertion to clone the *spo0K* locus. The phenotypes caused by the insertion mutation were similar to those caused by the *spo0K141* mutation. Definitive evidence that the transposon was in the *spo0K* locus came from cloning and characterizing DNA surrounding the transposon. We demonstrated that clones of DNA adjacent to the transposon could rescue the *spo0K141* mutation by recombination and that other clones could disrupt the *spo0K* locus when integrated into the chromosome. Clones were used to sequence the locus and to further characterize the function of *spo0K*.

***spo0K418*::Tn*917lac* and *spo0K141* cause similar phenotypes.** Strains containing the *spo0K418*::Tn*917lac* mutation appeared Spo$^-$ on sporulation plates, and the colonies resembled those of an isogenic *spo0K141* mutant. The block in sporulation appeared to be the same as that in *spo0K141*, as judged by the appearance of the cells on plates and in the phase-contrast microscope.

Strains containing the insertion mutation were compared directly with strains containing the *spo0K141* mutation for their effects on sporulation under several conditions. Both mutations caused an oligosporogenous phenotype, and that phenotype was more severe in the PB2 genetic background than in the JH642 genetic background (Table 3). In addition, mutations in *spoIIJ* caused both *spo0K141* and *spo0K*::Tn*917lac* mutants to be more severely Spo$^-$ (Table 3). Overproduction of the *spoIIJ* gene product from the isopropyl-β-D-thiogalactopyranoside-inducible promoter P_{spac} partially suppressed the sporulation defect of both the *spo0K141* mutant and the *spo0K418*::Tn*917lac* mutant (Table 3). The suppression of *spo0K* by the overproduction of *spoIIJ* kinase is consistent with the notion that *spo0K* is involved in the activation of Spo0A, perhaps by activating (either directly or indirectly) one of the histidine protein kinases.

The strain differences seen with the *spo0K141* mutants were also observed with the insertion mutants (Table 3). Furthermore, disruptions of the *spo0K* locus generated by integrative plasmids caused similar phenotypes and similar strain differences. Therefore, the strain differences were not due to multiple mutations in the *spo0K141* mutant.

The *spo0K141* and *spo0K*::Tn*917lac* mutants also had similar defects in genetic competence. The ability of the mutants to be transformed with exogenous DNA was reduced to approximately 0.1 to 1.0% of that of the isogenic wild type. Additional mutations in *spo0K* made by disrupting the locus with cloned fragments in integrative plasmids also caused a defect in competence (see below). In all cases tested, the defect in competence was seen in both strain backgrounds. The fact that the transposon insertion and disruptions with integrative plasmids caused defects in competence similar to that caused by the *spo0K141* allele indicated that the competence defect was not due to additional mutations outside *spo0K* and that *spo0K* is required for the normal development of competence.

It is interesting to note that in contrast to the defect in sporulation, the defect in competence in the *spo0K* mutant was not relieved by the overproduction of *spoIIJ* (data not shown). This difference indicates that the role of *spo0K* in competence may be different from its role in sporulation.

Genetic mapping experiments indicated that the Tn*917lac* insertion mutation mapped near the *spo0K* region of the chromosome. *spo0K418*::Tn*917lac* was approximately 5 to 10% linked by transformation to a marker that is also 5 to 10% linked to *spo0K141* (data not shown). The marker used was a plasmid integrated into the chromosome near *spo0K*. The plasmid contained cloned DNA adjacent to a silent Tn*917* insertion (from strain MO495) linked to *spo0K* (data not shown).

TABLE 3. Sporulation of strains containing *spo0K141* and *spo0K*::Tn*917lac*

Strain[a]	*spo0K*[b]	*spoIIJ*[c]	IPTG[d]	Frequency[e]
JH642	+	+	−	1.0
AG522	+	Tn	−	7.1×10^{-2}
KI250	*141*	+	−	1.9×10^{-2}
KI418	Tn	+	−	1.8×10^{-2}
KI261	*141*	Tn	−	4.0×10^{-4}
KI738	Tn	Tn	−	1.3×10^{-3}
KI742	*141*	P_{spac}-IIJ	−	1.5×10^{-2}
KI742	*141*	P_{spac}-IIJ	+	1.5×10^{-1}
KI741	Tn	P_{spac}-IIJ	−	1.9×10^{-2}
KI741	Tn	P_{spac}-IIJ	+	8.4×10^{-2}
PB2	+	+	−	1.0
KI98	+	Tn	−	6.5×10^{-2}
PB69	*141*	+	−	6.0×10^{-5}
DZR18	Tn	+	−	3.7×10^{-4}
KI100	*141*	Tn	−	2.5×10^{-6}
KI776	Tn	Tn	−	1.9×10^{-5}
KI22	*141*	P_{spac}-IIJ	−	1.8×10^{-4}
KI22	*141*	P_{spac}-IIJ	+	2.0×10^{-2}
KI772	Tn	P_{spac}-IIJ	−	2.9×10^{-4}
KI772	Tn	P_{spac}-IIJ	+	1.1×10^{-2}

[a] The first 10 strains are all isogenic with the wild-type strain JH642. The second 10 strains are all isogenic with the wild-type strain PB2.
[b] +, Wild-type allele; *141*, *spo0K141* allele; Tn, *spo0K418*::Tn*917lac* allele.
[c] +, Wild-type allele; Tn, *spoIIJ*::Tn*917*ΩHU19 allele; P_{spac}-IIJ, *amyE*::(P_{spac}-*spoIIJ*$^+$ *cat*) construct in a *spoIIJ*$^+$ strain.
[d] When present, isopropyl-β-D-thiogalactopyranoside (IPTG) was added during early- to mid-exponential growth at a concentration of 1 mM.
[e] Sporulation frequency (number of spores per viable cell), normalized to that of the wild-type strain, JH642 or PB2.

The phenotypic and preliminary mapping results indicated that the Tn*917lac* mutation was most likely in *spo0K*. Definitive evidence that the transposon was in the *spo0K* locus came from experiments that demonstrated that clones of DNA adjacent to the transposon could rescue the *spo0K141* mutation by recombination and that other clones could disrupt the *spo0K* locus when integrated into the chromosome on an integrational plasmid (see below).

Cloning and DNA sequencing of *spo0K*. DNA adjacent to the transposon was cloned into *E. coli* by the methods described by Youngman et al. (59–61) and summarized in Materials and Methods. Clones to the right of the transposon (as drawn in Fig. 1) were obtained with *Hin*dIII (pDR1), *Eco*RI (pDR2), and *Bgl*II (pDR3) sites at the end; clones to the left were obtained with *Pvu*II (pDR4) and *Bam*HI (pDR5) sites (Fig. 1 and Table 2).

All three clones to the right were internal to the transcription unit required for normal sporulation, while both clones to the left seemed to extend past one end of the transcription unit. These results were obtained by integrating the four clones (*Hin*dIII, *Bgl*II, *Pvu*II, and *Bam*HI) that still contained the *cat* gene into the chromosome of wild-type *B. subtilis* cells. If the cloned DNA were internal to the locus, then integration (by single crossover) would disrupt the locus and cause a sporulation defect. This in fact was the result with the *Hin*dIII (pDR1) and *Bgl*II (pDR3) clones. We assumed that the *Eco*RI (pDR2) clone was also internal, as

the *Eco*RI site was contained within the larger *Bgl*II clone. In contrast, integration of the *Pvu*II (pDR4) and *Bam*HI (pDR5) clones resulted in a Spo$^+$ phenotype, indicating that these clones extended past the end of the locus.

Preliminary DNA sequence information was obtained for DNA adjacent to both ends of the transposon in pDR2 and pDR4. We used oligonucleotide primers complimentary to the ends of the transposon to determine the sequence. Analysis of this DNA sequence indicated an open reading frame going from left to right in Fig. 1. We concluded from the integrational mapping and the preliminary DNA sequence data that the 5′ end of the *spo0K* locus is between the *Pvu*II site and the transposon (Fig. 1).

Additional clones that extended past the 3′ end of the *spo0K* locus were made by "walking" downstream with integrational plasmids. The *Eco*RI-*Bgl*II fragment from the *Bgl*II (pDR3) clone was subcloned into the integrational vector pJH101. The subclone (pDR6) was integrated into wild-type cells, and DNA was made, restricted with *Sph*I or *Sal*I, ligated, and transformed into *E. coli*. When integrated into *B. subtilis*, the clones (pDR9 and pDR11) gave Spo$^+$ transformants, indicating that they extended past the 3′ end of the locus required for *spo0K* function.

Subclones were made in the pBluescriptII KS and SK vectors and used to generate variously sized fragments by exonuclease deletions for sequencing the *spo0K* locus. pDR20, pDR21, pJL2, pJL3, and pJL7 were used to make exonuclease-generated nested deletions, and the resulting clones were used for sequencing.

The DNA sequence of *spo0K* was determined by the dideoxy chain termination method on double-stranded DNA templates. The sequence was determined on both strands, and much of the sequence was determined multiple times. Analysis of the sequence revealed five open reading frames, called *spo0KA* through *spo0KE* (Fig. 1 and 2). The transposon insertion was in *spo0KA*, the first open reading frame. Immediately downstream of *spo0KE*, the last open reading frame, was a sequence that looked like a typical factor-independent terminator (nucleotides 6085 to 6119).

***spo0K* is homologous to the *S. typhimurium opp* operon.** Comparison of the five *spo0K* open reading frames with the GenBank data base (using FastA) revealed significant similarity to the proteins encoded by the *opp* operon of *S. typhimurium* (22) (Fig. 3). *opp* encodes a membrane transport system required for the uptake of oligopeptides and cell wall material (17). Other similarities between the two operons include the order of the genes, a potential stem-loop structure between the first and second reading frames, and a possible overlap in the coding regions of the last two reading frames (Fig. 2 and 3).

Preliminary experiments indicated that there was a promoter located between the *Pvu*II site and the transposon. A *Hin*dIII-*Cla*I restriction fragment of approximately 900 bp (the *Hin*dIII site was approximately 100 bp downstream of the *Pvu*II site, and the *Cla*I site was in the end of the transposon) was subcloned into a promoter probe plasmid (pDG268 [6]), and the resulting *lacZ* transcriptional fusion was crossed into the *amyE* locus of *B. subtilis*. This fragment caused a considerable level of expression of *lacZ* (44a), indicating the presence of a promoter. Analysis of the DNA sequence between the *Hin*dIII site and the start of *spo0KA* revealed several sequences similar to promoters recognized by the major form of the RNA polymerase holoenzyme. The sequence that most resembles the consensus promoter recognized by RNA polymerase containing sigma-A is TTG CAA-17 bp-TATAAT. This sequence, found at nucleotides 295 to 323 (Fig. 2), matches the consensus −35 sequence in four of six positions and matches the consensus −10 sequence and the spacing between the two sequences perfectly. In addition, the sequence immediately upstream of the putative −35 region is extremely A+T-rich, a characteristic of many *B. subtilis* promoters.

Mapping of *spo0K141*. We used integrational mapping to determine the approximate location of the *spo0K141* mutation relative to various subclones. When pDR9 was integrated into the *spo0K141* mutant, approximately 35% of the transformants became Spo$^+$, indicating that at least one mutation required for the *spo0K141* mutant phenotype lies between the upstream *Eco*RI site and the *Sph*I site.

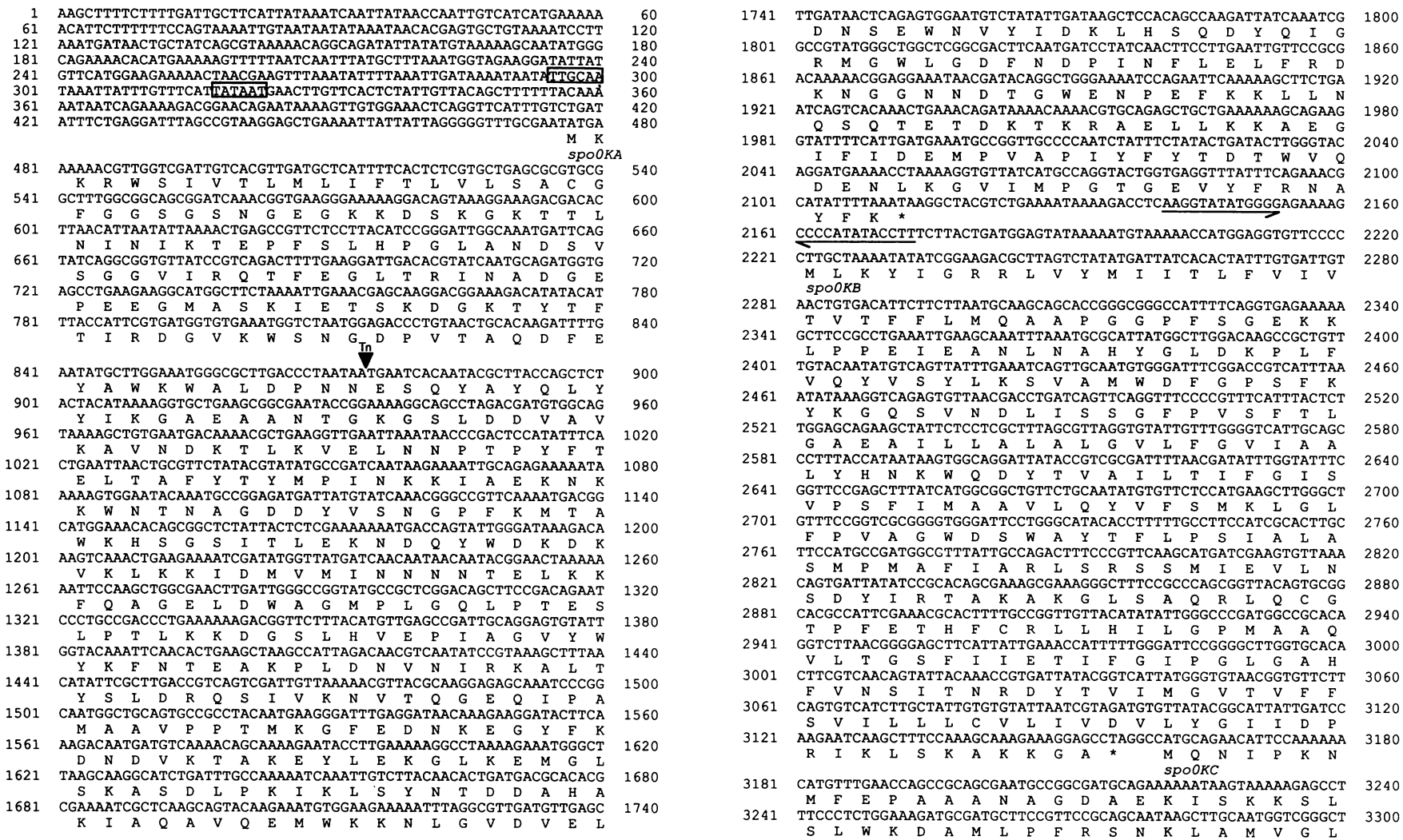
Similar integrational mapping experiments were done with pJL4 (containing the ~3.5-kb *Eco*RI fragment) and pJL1 (containing the 1.7-kb *Eco*RV fragment). These experiments were a bit more complicated to interpret because integration of these plasmids caused disruption of *spo0KE*. However, disruption of *spo0KE*, in otherwise wild-type cells, by integration of pJL1 or pJL4 (also pDR30) resulted in little or no defect in sporulation (see below). This phenotype, while subtly different from that of the wild type, was clearly distinguishable from the phenotypes caused by the *spo0K*::Tn*917lac* insertion, the *spo0K141* mutation, and disruptions in the other *spo0K* open reading frames. Integration of pJL4 or pJL1 into the *spo0K141* mutant resulted in approximately 60 to 70% and approximately 30% "Spo$^+$" transformants, respectively, as judged by colony morphology on sporulation plates, indicating rescue of the *spo0K141* mutation. We conclude that we have indeed cloned the *spo0K* locus and that at least one mutation that is necessary for the *spo0K141* mutant phenotype lies between the two *Eco*RV sites.

Disruption of the *spo0KE* open reading frame. Disruption of *spo0KE*, the last gene in the *spo0K* operon, did not cause a significant defect in sporulation. We disrupted the *spo0KE* gene by integrating pDR30, which contains an internal fragment of the gene. This mutation caused a small (~30%, relative to the wild type) but reproducible defect in sporulation (Table 4). In contrast, the same mutation had a dramatic effect on competence. The defect in competence caused by the *spo0KE* mutation was similar to that caused by the transposon insertion (Table 4). Thus, *spo0KE* is required for the development of competence but has only a minor role in the initiation of sporulation.

DISCUSSION

The five proteins encoded by the *spo0K* locus are homologous to the five proteins encoded by the *opp* (oligopeptide permease) operon of *S. typhimurium* (Fig. 3). There are import and export systems in both prokaryotes and eukaryotes that are similar to those encoded by *opp* and *spo0K* (5, 7, 24). In gram-negative bacteria, the uptake of maltose, maltodextrins, phosphate, histidine, branched-chain amino acids, and many other substrates is mediated by transport systems analogous to that encoded by *opp* (5). In the gram-positive bacterium *Streptococcus pneumoniae*, the uptake of maltose, maltodextrins, and oligopeptides is mediated by similar transport systems (4, 16).

The export of a variety of compounds is also dependent on transport systems that are similar in structure to those encoded by *spo0K* and *opp*. Hemolysin secretion from pathogenic strains of *E. coli* depends on the *hlyB* and *hlyD* gene products, which constitute a membrane-bound translocation complex that is structurally similar to the import systems (7). In eukaryotic cells, the similar transport sys-



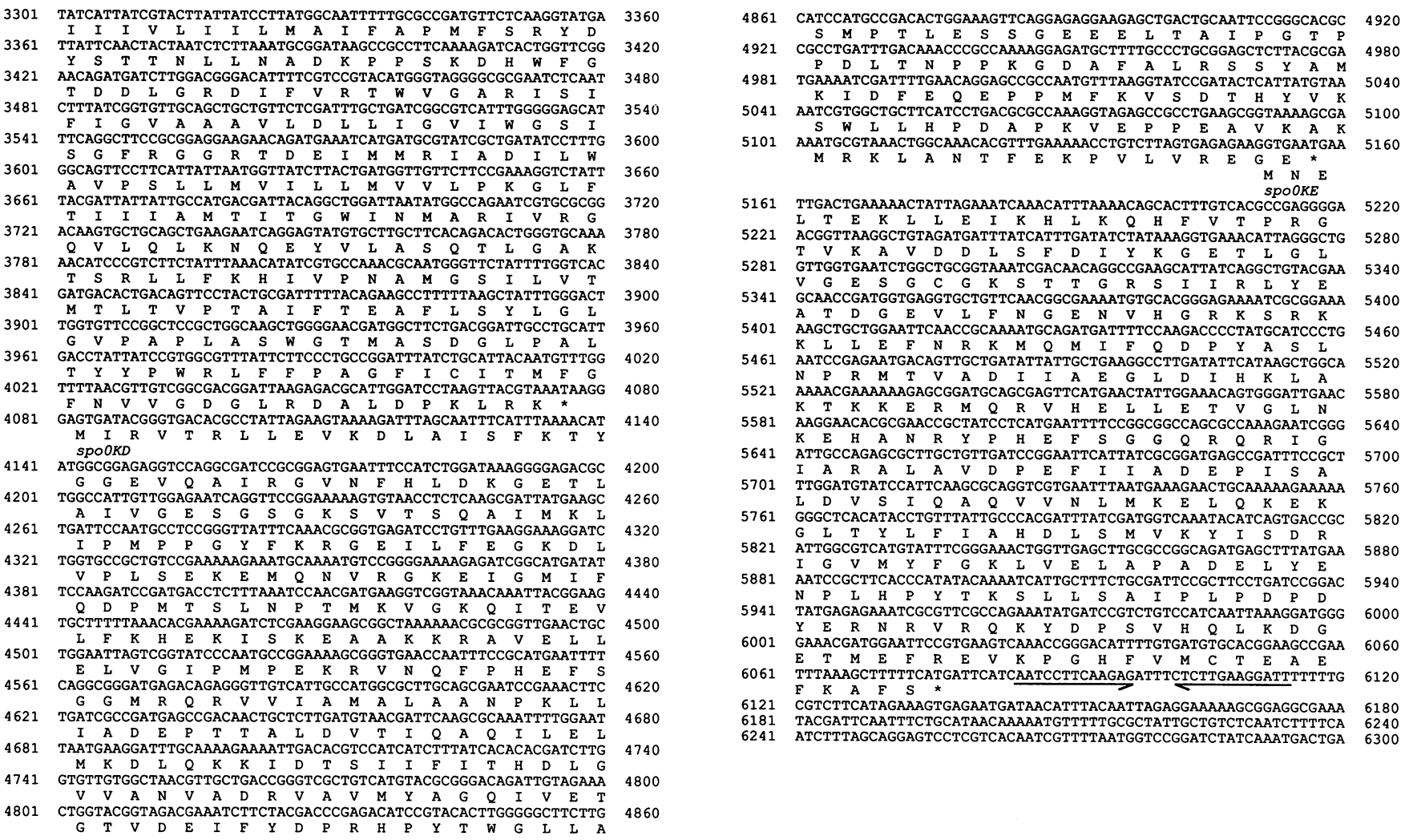


FIG. 2. Sequence of the *spo0K* operon and flanking regions. The DNA sequence begins at the *Hin*dIII site most upstream in Fig. 1. The predicted amino acid sequence is shown with the one-letter code. The proposed start of each open reading frame is indicated with the gene name (e.g., *spo0KA*). These starts are presumed on the basis of the DNA sequence and not on any direct evidence. Stop codons are indicated by asterisks. The putative −35 region (nucleotides 295 to 300) and −10 region (nucleotides 318 to 323) of a possible promoter are boxed. The location of the Tn*917lac* insertion (after nucleotide 874) is indicated by a triangle and Tn. Regions of dyad symmetry are shown by converging arrows.

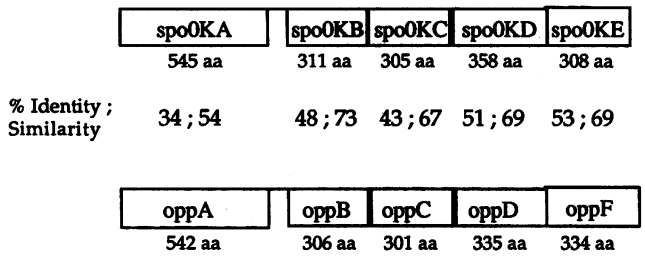


FIG. 3. Homology between the *spo0K* operon of *B. subtilis* and the *opp* operon of *S. typhimurium*. Percent identity and similarity are given as determined by the BestFit program (11). aa, Amino acids.

tems seem to be composed of a single large polypeptide that has domains that are related to the individual polypeptides in the prokaryotic systems. These systems include, among others, *STE6* of *Saccharomyces cerevisiae*, involved in the export of the a factor mating pheromone (31); MDR or P-glycoprotein, responsible for multiple drug resistance in mammalian tumors; CQR, responsible for chloroquine resistance in *Plasmodium falciparum*; and CFTR, the cystic fibrosis transmembrane regulator (reviewed in reference 7). These systems play important physiological roles, and many are involved in controlling differentiation and gene expression.

One of the components of the import systems is a high-affinity substrate-binding protein (Spo0KA, OppA) which presumably interacts with integral membrane proteins that transport the substrate into the cell. In gram-negative bacteria, the substrate-binding proteins are located in the periplasmic space (5). In contrast, gram-positive bacteria do not have a periplasmic space because they do not have an outer membrane. The substrate-binding proteins that are localized to the outside of the cell are thought to be attached to the cytoplasmic membrane through a lipoamino acid anchor (4, 16). The NH_2 terminus of Spo0KA has the characteristics of a signal sequence and has a sequence (L-S-A-C-G, starting at amino acid 18) that is similar to the consensus sequence of lipoprotein modification sites (4, 16, and references therein), indicating that Spo0KA may be a lipoprotein. The work of Perego et al. (38) has shown that some of the *spo0KA* gene product is found free in the culture medium while some is associated with the cell.

The other components of the transport systems include two integral membrane proteins and one or two ATPases. The membrane proteins (Spo0KB and Spo0KC, OppB and OppC) are hydrophobic, have characteristic transmembrane domains and, in some cases, are similar to each other. The *spo0KB* and *spo0KC* gene products are approximately 20% identical and ~51% similar to each other. The energy for import presumably comes from the hydrolysis of ATP by one or two proteins that are on the cytoplasmic side of the membrane and associated with the integral membrane proteins (21, 24, 32). Spo0KD and Spo0KE are the ATP-binding proteins, as determined on the basis of the sequence homology to OppD and OppF and a region of homology to nucleotide-binding sites. Spo0KD and Spo0KE are homologous to each other, having ~42% identical amino acids.

TABLE 4. Effects of a *spo0KE* mutation on sporulation and competence

Strain	Mutation	Sporulation[a]	Competence[a]
PB2	None (wild type)	1.0	1.0
DZR18	*spo0K418*::Tn*917lac*	7.6×10^{-3}	2.9×10^{-3}
DZR84	*spo0KE*::pDR30	0.34	$<1 \times 10^{-3}$

[a] Frequency relative to that in wild-type cells.

Mutations in *spo0KE* did not cause a Spo0K$^-$ phenotype. The *spo0KE* mutation caused little or no defect in sporulation but did cause a defect in competence similar to that caused by mutations in other parts of the *spo0K* operon (Table 4). Thus, *spo0KE* is required for the development of competence but is dispensable (or almost dispensable) for sporulation. It is interesting to note that *oppF* of *S. typhimurium* (the homolog of *spo0KE*) is required for Opp function. That is, mutations in *oppF* cause an Opp$^-$ phenotype (22). Perego et al. have also shown that *spo0KE* is not required for sporulation (38). In addition, they have shown that *spo0KE* is not required for the transport of a toxic oligopeptide into *B. subtilis* (38). Again, this result is in contrast to the requirement for *oppF* in oligopeptide transport in *S. typhimurium* (22).

The role of *spo0K* in sporulation and competence presumably is related to its role in transport or in recognizing molecules for transport. The *opp* system in *S. typhimurium* transports oligopeptides from two to five amino acids in length and cell wall peptides (17). The role of *spo0K* in sporulation and/or competence could be in the transport or sensing of extracellular peptides. There are many examples of extracellular molecules that act as signals for differentiation in microbes, including the multiple factors involved in myxobacterium development (27). We have described experiments indicating that *B. subtilis* produces at least one extracellular factor that is required for efficient sporulation (18, 19). This factor accumulates in the culture medium as the cells reach a high density. It is sensitive to pronase, heat resistant, and dialyzable, indicating that it is at least in part an oligopeptide (18). It is tempting to speculate that the *spo0K* gene products may be involved in sensing and/or transporting such a factor.

It is possible that an extracellular factor also exists for competence development in *B. subtilis*. There is a relatively well defined competence factor in *S. pneumoniae* (33, 55), and there have been reports of possible competence factors in *B. subtilis* (1, 2, 26), although definitive evidence is lacking. Again, it is tempting to speculate that if there is a competence factor in *B. subtilis*, the *spo0K*-encoded system may be involved in sensing and/or transporting such a factor. The factors controlling competence could be the same or different from those controlling sporulation.

The effects of *spo0K* on sporulation seem to be mediated through a two-component regulatory system composed of a histidine protein kinase and a response regulator. The requirement for *spo0K* in sporulation can be partly bypassed by mutations in *spo0A* (49, 50; unpublished results) and by the overproduction of the kinase encoded by *spoIIJ* (Table 3). It is possible that the normal role of *spo0K* is to activate one or more protein kinases that are involved in the initiation of sporulation. This activation could be either indirect or direct, conceivably through a specific interaction with a kinase, perhaps SpoIIJ and/or other kinases. Competence development is also regulated in part by histidine protein kinases, those encoded by *comP* (58) and *degS* (34). Again, the activity of one or both of these kinases could also be controlled by *spo0K*.

The precise mechanisms by which *spo0K* affects competence and sporulation must be different. Overproduction of SpoIIJ partly suppressed the sporulation defect, but not the

competence defect, caused by *spo0K* mutations. Furthermore, mutations in *spo0KE* caused a significant defect in competence but not in sporulation. It is possible that the *spo0KE* gene product is not part of the postulated transport complex composed of the other *spo0K* gene products. Perhaps Spo0KE is recruited by other proteins that are required for competence but not sporulation. In any case, if there is coupling between the *spo0K* gene products and histidine protein kinases, perhaps Spo0KE affects the activity of a kinase required for competence and Spo0KD affects the activity of a kinase required for sporulation.

Membrane transport systems and two-component regulatory systems are both highly conserved and play important roles in cellular physiology. Our results indicate that the activity of a histidine protein kinase (perhaps SpoIIJ) may be coupled to the transport system encoded by *spo0K*. The activity of at least one other two-component regulatory system seems to be coupled to one of the conserved transport systems. The expression of genes involved in phosphate metabolism (the *pho* regulon) in *E. coli* is regulated by the two-component PhoR-PhoB system. PhoR is a membrane-bound protein kinase that phosphorylates PhoB, the response regulator (reviewed in references 3 and 54). The expression of genes in the *pho* regulon is also controlled by the products of the *pstSCAB* genes, which encode components of a high-affinity transport system for the uptake of phosphate. The phosphate transport system is similar to those encoded by *spo0K* and *opp* and seems to be coupled (directly or indirectly) to the activity of the two-component regulatory system, perhaps through the *phoU* gene product (reviewed in reference 43). It will be interesting to see whether this potential interaction between two different types of highly conserved systems is a general scheme in controlling gene expression in response to external signals.

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