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Identification and Characterization of Genes Controlled by the Sporulation-Regulatory Gene *spo0H* in *Bacillus subtilis*

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We describe a general strategy for the identification of genes that are controlled by a specific regulatory factor in vivo and the use of this strategy to identify genes in *Bacillus subtilis* that are controlled by *spo0H*, a regulatory gene required for the initiation of sporulation. The general strategy makes use of a cloned regulatory gene fused to an inducible promoter to control expression of the regulatory gene and random gene fusions to a reporter gene to monitor expression in the presence and absence of the regulatory gene product. *spo0H* encodes a sigma factor of RNA polymerase, σ^H, and is required for the extensive reprograming of gene expression during the transition from growth to stationary phase and during the initiation of sporulation. We identified 18 genes that are controlled by σ^H (*csh* genes) in vivo by monitoring expression of random gene fusions to *lacZ*, made by insertion mutagenesis with the transposon Tn*917lac*, in the presence and absence of σ^H. These genes had lower levels of expression in the absence of σ^H than in the presence of σ^H. Patterns of expression of the *csh* genes during growth and sporulation in wild-type and *spo0H* mutant cells indicated that other regulatory factors are probably involved in controlling expression of some of these genes. Three of the *csh*::Tn*917lac* insertion mutations caused noticeable phenotypes. One caused a defect in vegetative growth, but only in combination with a *spo0H* mutation. Two others caused a partial defect in sporulation. One of these also caused a defect in the development of genetic competence. Detailed characterization of some of the *csh* genes and their regulatory regions should help define the role of *spo0H* in the regulation of gene expression during the transition from growth to stationary phase and during the initiation of sporulation.

Endospore formation in the gram-positive bacterium *Bacillus subtilis* involves extensive reprograming of gene expression and a series of complex morphological and physiological changes (14, 25, 32, 37). Rapidly growing cells can be induced to differentiate upon nutrient deprivation, and all conditions of nutrient deprivation that cause efficient sporulation have been shown to cause a drop in the intracellular concentration of GDP and GTP (23, 24). Furthermore, conditions that cause a drop in these nucleotides (for example, addition of the drug decoyinine [29]), even in the presence of excess nutrients, can cause efficient sporulation (15), provided that the cells are at relatively high densities (17, 38). Thus, it is thought that a drop in the intracellular level of GDP and GTP is necessary and sufficient for efficient sporulation (14, 16).

At least seven regulatory genes, called *spo0* genes, are required for the initiation of sporulation. Mutations in these genes prevent the earliest morphological change associated with sporulation, the formation of an asymmetric division septum following nutrient deprivation (26, 32, 33).

spo0H is one of the key regulatory genes required for the initiation of sporulation. *spo0H* encodes a sigma factor, σ^H, of RNA polymerase that is required for initiation of transcription at specific promoters (3, 11). In addition to its role in the initiation of sporulation, σ^H is required for the normal expression of genes needed for competence (1). Expression of several *com* genes is induced in stationary phase in the presence of glucose (1, 19). Thus, σ^H, which is present both in growing cells and early in sporulation, is involved in the reprograming of gene expression during the transition from growth to stationary phase as well as in the initiation of sporulation.

Only one sporulation gene (*spoVG*) that is known to be transcribed by RNA polymerase containing σ^H ($E\sigma^H$) has been characterized in detail (2, 3, 45–48). *spoVG* is normally expressed during vegetative growth, and expression increases within 15 min after the initiation of sporulation (47, 48). Expression during growth and sporulation is eliminated in a *spo0H* deletion mutant (47). In contrast to many of the *com* genes, expression of *spoVG* does not require the presence of glucose. The promoter for *spoVG* has been characterized in detail, both in vivo and in vitro, and a mutation in *spo0H* has been identified that partially suppresses a mutation in the *spoVG* promoter (45a).

In addition to the *com* genes and *spoVG*, *spo0H* must also control expression of genes needed for the initiation of sporulation. Expression of the early sporulation genes *spo0A* and *spo0F* is partially reduced by mutations in *spo0H* (41), and recent work indicates that *spoIIA* is also controlled by *spo0H* (40). In addition to these genes, two other genes are known to have promoters that are recognized by $E\sigma^H$: a promoter internal to the *rpoD* operon (4), and a promoter upstream of *citG* (12). The role of transcripts from these promoters in the transition from growth to stationary phase is not clear.

One way to begin to understand the role of *spo0H* in the control of gene expression and the initiation of sporulation is to identify and characterize genes that are controlled by the *spo0H* gene product, σ^H. Such genes should have decreased expression in the absence of σ^H compared with its presence. Our strategy was to screen fusions of the *Escherichia coli lacZ* gene to random *B. subtilis* genes made with the transposon Tn*917lac* (31) and to identify those fusions that had decreased levels of expression in the absence of σ^H. We

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used a fusion of *spo0H* to the isopropylthiogalactopyranoside (IPTG)-inducible promoter P_{spac} (42) to control the expression of σ^H. We describe the identification and preliminary characterization of 18 genes that are controlled by σ^H (*csh*) in vivo.

MATERIALS AND METHODS

Strains. Strains used were derived from *B. subtilis* 168. JH642 (*trpC2 pheA1*) from J. Hoch is the wild-type strain used here. AG665 (*trpC2 pheA1* Δ*spo0H*::*cat*) is isogenic to JH642 except for the deletion-insertion mutation in *spo0H*. The deletion was made between the *Hin*dIII site and the *Eco*RI site in a clone of *spo0H*, and the *cat* cassette from pMI1101 (43) was inserted between these sites. This deletion-insertion mutation was then transferred to the chromosome by DNA-mediated transformation. Strain 1S86 (*trpC2 spoIIA1*) was from the *Bacillus* Genetic Stock Center. Strain AG605 (*trpC2 spoIIA1 spo0H*::pJOH7d) was derived from 1S86 by DNA-mediated transformation and contains the P_{spac}-*spo0H* fusion generated by integration of pJOH7d (described below) into *spo0H*. This strain was used to identify Tn*917lac* insertions in genes that are controlled by σ^H. The library of Tn*917lac* insertions made in strain YB886 was kindly provided by R. Yasbin (27). This library has been used to identify Tn*917lac* insertions in *din* genes (27) and *com* genes (19).

Plasmids. pAG58 (Fig. 1A) is an expression vector containing P_{spac}. It can replicate in *E. coli* and can integrate into the *B. subtilis* chromosome if the plasmid contains a DNA fragment homologous to part of the chromosome. It was made by cloning the *Eco*RI-*Bam*HI fragment of pSI-1 (42) into the *Eco*RI-*Bam*HI sites of pJH101 (13), followed by deleting from *Bam*HI to *Nru*I, which recreated the *Bam*HI site and removed the *Sal*I and *Sph*I sites from pJH101, leaving the *Sal*I and *Sph*I sites from pSI-1. pJOH7d (Fig. 1B) contains the 5′ end of *spo0H* from the *Ssp*I site to the *Hin*dIII site cloned downstream of P_{spac} in pAG58. (This clone does not contain the *spo0H* promoter.) First, the *Ssp*I-*Sph*I fragment of *spo0H* (11, 45a) was cloned between the *Hin*dIII site (after filling in with T4 DNA polymerase) and the *Sph*I site of pAG58 to generate pJOH7. The *Hin*dIII site from the vector was destroyed in the cloning. pJOH7d was constructed by deleting the 3′ end of *spo0H*, from *Hin*dIII to *Sph*I. pJOH7d was integrated into *B. subtilis* by DNA-mediated transformation and selection for chloramphenicol resistance.

Media and growth conditions. LB medium (7) was used for routine maintenance of both *E. coli* and *B. subtilis*. DS medium (35) was used as the nutrient sporulation medium. Both LB and DS media were solidified with 15 g of agar (Difco Laboratories) per liter. Chloramphenicol was used at 5 μg/ml. Resistance to macrolide-lincosamide-streptogramin B (MLS^r) antibiotics, encoded by Tn*917lac*, was selected by using both erythromycin and lincomycin, at 0.5 and 12.5 μg/ml, respectively. 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) was used in solid medium at 120 μg/ml to indicate production of β-galactosidase. IPTG (isopropyl-β-D-thiogalactoside) was used at 1 mM to induce expression of *spo0H* from P_{spac}.

The minimal medium contained S7 minimal salts supplemented with glucose (1%) and glutamate (0.1%) as described by Vasantha and Freese (39), except that MOPS (morpholinepropanesulfonic acid) buffer was used at 50 mM rather than at 100 mM. Required amino acids were added at 40 μg/ml. Cultures were grown essentially as described previously (17). Cells were grown overnight on LB plates and used to inoculate a culture (OD_{600}, ≤0.03) in minimal medium. Cultures were grown for at least four doublings in flasks in a shaking water bath at 37°C. Sporulation was initiated by the addition of decoyinine (U-7984; Upjohn) to a final concentration of 500 μg/ml to cultures at an OD_{600} of ≥0.5 but ≤0.8. Addition of decoyinine caused the doubling time to increase from ~1 h to ~3 h.

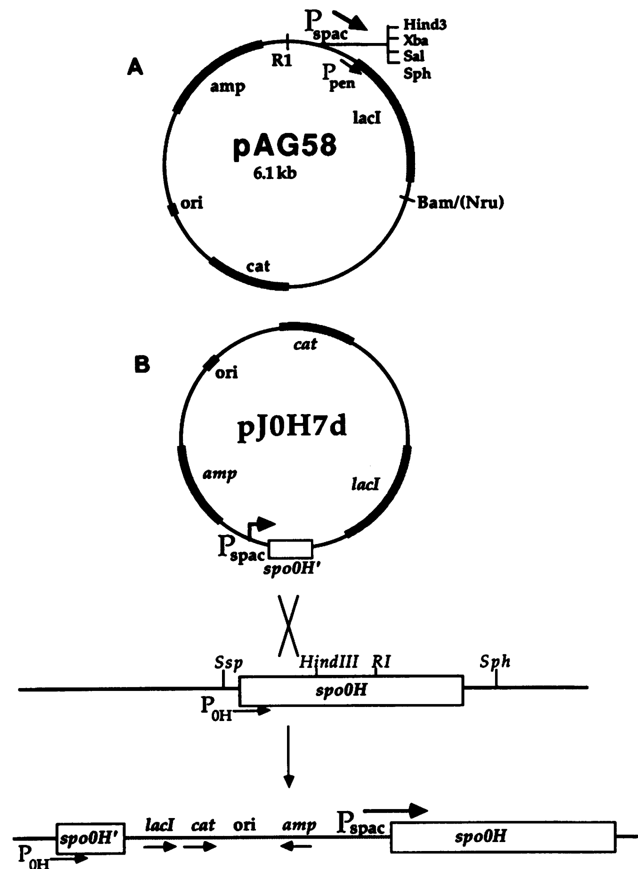

FIG. 1. Map of pAG58 (A) and pJOH7d and integration of pJOH7d into the *B. subtilis* chromosome (B). pAG58 and pJOH7d were constructed as described in Materials and Methods. P_{spac} is a hybrid regulatory region, constructed by Yansura and Henner (42), which contains the RNA polymerase recognition sequences of an early SP01 promoter and the *lac* operator. *lacI*, encoding *lac* repressor, is under the control of the *Bacillus licheniformis* penicillinase transcriptional and translational signals, indicated as P_{pen} (42). Approximate locations of some restriction enzyme sites are indicated (R1, *Eco*RI). Integration of pJOH7d into the *spo0H* locus results in a truncated copy of *spo0H* (*spo0H′*) under control of the normal *spo0H* promoter (P_{0H}) and an intact copy of *spo0H* under the control of P_{spac}.

β-Galactosidase assays. Cells were grown as described above, and 0.5-ml samples were added to toluene, frozen overnight, and assayed for β-galactosidase activity essentially as described before (28). β-Galactosidase activity is presented as the ΔA_{420} per minute per milliliter of culture, × 1,000. Specific activity is calculated by dividing the above units by the OD_{600} of the culture to give 1,000 times the change in A_{420} per minute per OD_{600} unit of 1 ml of culture.

Transformation and transduction. Cells of *B. subtilis* were made competent and transformed essentially as described before (10). Because *spo0H* mutants are partly defective in

the development of competence (1), the strain containing the P_{spac}-*spo0H* fusion (AG605) was grown in the presence of IPTG to induce expression of *spo0H*. In the absence of IPTG, competence was ~5% of that in the presence of IPTG or in otherwise isogenic *spo0H*$^+$ cells. The presence of IPTG in the first growth medium or the second medium or both was sufficient to give approximately the same level of competence seen in otherwise wild-type cells. Following isolation of the *csh*::Tn*917lac* mutations in AG605, each *csh*::Tn*917lac* insertion was transferred to the wild-type (JH642) and *spo0H* mutant (AG665) backgrounds by transformation.

Growth of the generalized transducing phage PBS1 and transduction were done essentially as described before (20). For mapping the *csh*::Tn*917lac* insertions, phage were grown on strains with the insertion mutations in the JH642 strain background. Mapping was done with the standard *B. subtilis* mapping kit strains of Dedonder et al. (8).

RESULTS

Identification of *lacZ* transcriptional fusions that have decreased expression in the absence of the *spo0H* gene product. We set out to identify genes that are controlled by σ^H in vivo (*csh* genes). Expression of these genes should be lower in the absence of σ^H than in its presence. To control the presence or absence of σ^H, we constructed a strain in which *spo0H* was under control of the IPTG-inducible promoter P_{spac}. Plasmid pJOH7d contains the 5' end of the *spo0H* coding sequence (without the normal promoter) fused to P_{spac} (Fig. 1B). When this plasmid is integrated into the *B. subtilis* chromosome (by a single crossover), an intact copy of *spo0H* is fused to P_{spac} and the truncated 5' end of *spo0H* is controlled by the normal promoter (Fig. 1B). The resulting strain is Spo$^-$ (*spo0H*) in the absence of IPTG and Spo$^+$ (*spo0H*$^+$) in the presence of IPTG.

We used a strain containing the P_{spac}-*spo0H* fusion to identify genes that are more highly expressed in the presence of σ^H (plus IPTG) than in the absence of σ^H. Such genes were identified by monitoring expression (on X-gal indicator plates) of transcriptional fusions between the *lacZ* gene of *E. coli* and random *B. subtilis* genes, made with the fusion-generating transposon Tn*917lac*.

We wanted to identify genes that might be controlled directly by σ^H and that were likely to be expressed during growth or the transition from growth to sporulation and to specifically avoid identifying genes that are expressed later in sporulation that are indirectly dependent on *spo0H*. To reduce the chances of identifying genes induced late in sporulation, the fusions were isolated in a *spoIIA* mutant, which blocks expression of most genes that are induced after the first hour of sporulation. To reduce the chances of identifying genes that are involved in competence, fusions were isolated under conditions in which previously identified *com* genes would not be expressed, that is, in the absence of glucose.

We introduced approximately 10,500 Tn*917lac* insertions, from the insertion library of Yasbin and co-workers (27), into a *spoIIA* mutant that contained the P_{spac}-*spo0H* fusion and identified 30 to 40 insertions that appeared to have increased expression in the presence of σ^H (plus IPTG). Competent cells of strain AG605 [*spoIIAC1 spo0H*::pJOH7d (P_{spac}-*spo0H*)] were transformed with DNA from the Tn*917lac* library, and MLS^r transformants were selected on sporulation plates (DS) and nonsporulation plates (LB) containing X-gal to indicate expression of *lacZ* and IPTG to induce expression of *spo0H* from P_{spac}. Under these conditions, approximately 10 to 15% of the transformants produced blue colonies within 1 to 4 days, indicating that they were expressing β-galactosidase. To determine qualitatively the effect of σ^H on expression of these gene fusions, we picked the Lac$^+$ transformants and tested their expression on both LB–X-gal and DS–X-gal plates with and without IPTG. The presence or absence of σ^H (IPTG) had little or no effect on expression of most of the gene fusions. However, 30 to 40 fusions appeared to have increased expression in the presence of σ^H on either the LB or DS plate or both. These were chosen for further study.

In contrast to the fusions that had increased levels of expression in the presence of σ^H, several fusions had decreased levels of expression in the presence of σ^H. We have not characterized these further.

Expression of *csh-lacZ* fusions. To study expression of the *csh*::Tn*917lac* fusions in the absence of P_{spac}, we transferred 37 insertions to both wild-type (JH642) and *spo0H* mutant (AG665) cells by DNA-mediated transformation. These strains were then tested on X-gal indicator plates (both LB and DS), and those fusions that had reproducible differences in expression between the wild-type and the *spo0H* mutant were studied further. Preliminary measurements of β-galactosidase activity in liquid culture indicated that some of the gene fusions that had high levels of expression on X-gal indicator plates had very low or undetectable levels of expression in liquid medium. In addition, some fusions which initially appeared to have small differences in expression in the P_{spac} strain in the presence and absence of IPTG had very little or no reproducible differences in the wild type compared with the *spo0H* mutant. These fusions have not been studied further. We have characterized expression of 18 *csh-lacZ* fusions in detail in the otherwise isogenic wild-type and *spo0H* mutant strains. We have not used the P_{spac}-*spo0H* fusion strain to characterize any of the *csh-lacZ* fusions.

Genes controlled by *spo0H* could be expressed during growth and/or sporulation, as σ^H is present in growing cells as well as in sporulating cells. Expression of each gene fusion was determined by measuring β-galactosidase activity during growth and sporulation in both the wild-type strain background and the *spo0H* mutant. Initially, expression was compared under two different conditions: (i) cells were grown in a nutrient sporulation (DS) medium, in which sporulation is traditionally assumed to initiate when cells stop growing exponentially due to limitation of one or more nutrients; and (ii) cells were grown in a defined minimal medium with glucose as a carbon source, and sporulation was initiated by the addition of decoyinine, which causes a drop in the intracellular concentration of GDP and GTP (29). In most cases, expression of a given gene was similar in the two different conditions. There were two exceptions (*csh-203* and *csh-227*), and these are described below.

For our purposes, the use of minimal medium and the addition of decoyinine to initiate sporulation were preferable to the exhaustion method. Decoyinine does not induce many of the changes associated with nutrient exhaustion at the end of vegetative growth (competence, production of extracellular proteases, and antibiotics) (14, 30, 39). The time of addition of decoyinine precisely defines T_0 (the initiation of sporulation) and allows accurate determination of the timing of expression of each *csh-lacZ* fusion. The use of decoyinine also allows the precise determination of a differential rate of synthesis for each *csh-lacZ* fusion. The population of cells continues to grow for a few hours after the addition of

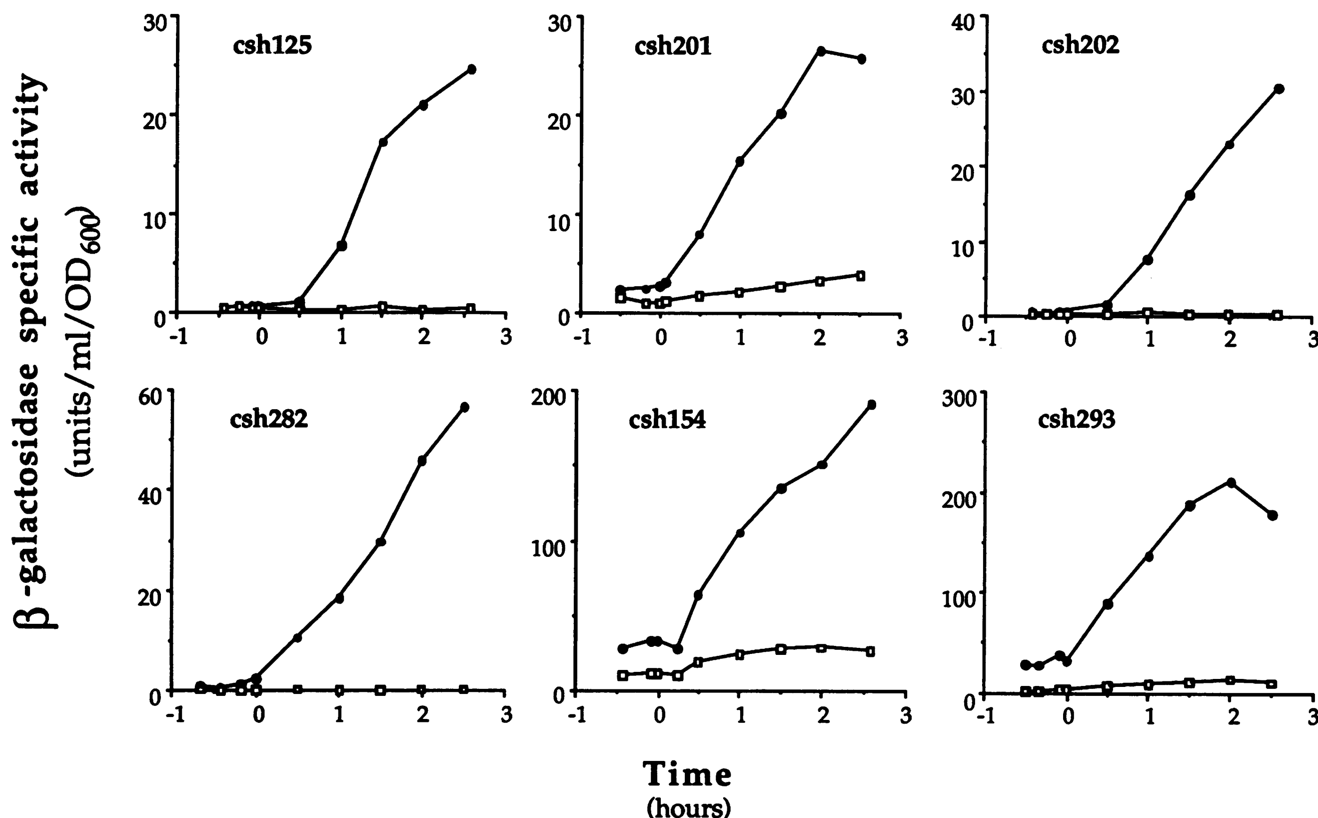

FIG. 2. Expression of *csh-lacZ* transcriptional fusions. The indicated *csh*::Tn*917lac* insertion mutation was in the wild-type (JH642) strain background (●) or in the *spo0H* mutant (AG665) background (□). Cells were grown and β-galactosidase activity was determined as described in Materials and Methods. Specific activity (1,000 × ΔA_{420} per min per ml of culture per OD_{600} unit) is plotted as a function of time after the addition of decoyinine, defined as time zero. Data are from representative experiments.

decoyinine, albeit more slowly, making this type of analysis possible. Comparison of the differential rates of synthesis during vegetative growth and during the first 2 h of sporulation allows the determination of an induction ratio for those genes that have an increased rate of synthesis during sporulation.

We measured expression of each *csh-lacZ* fusion in minimal medium during growth and after the addition of decoyinine to initiate sporulation. Expression of all 18 fusions increased within the first 30 min of sporulation, independent of the amount of expression during vegetative growth. Figure 2 shows data for six different fusions. β-Galactosidase specific activity (1,000 × ΔA_{420} per min per ml of culture per OD_{600}) is plotted as a function of time after the addition of decoyinine (T_0) in both wild-type cells and the *spo0H* mutant. Expression of all of these genes was induced within the first 30 min of sporulation. The same data are presented in Fig. 3 in differential plots, that is, β-galactosidase activity (1,000 × ΔA_{420} per min per ml of culture) is plotted as a function of cell mass (OD_{600}). The slope of such a plot is the differential rate of synthesis, and the ratio of the slope during the first 2 h of sporulation to the slope during vegetative growth is the induction ratio (Table 1).

It is clear from these data (Fig. 2 and 3; Table 1) that the genes controlled by *spo0H* do not all have the same pattern of expression. Some *csh* genes had little or no expression during growth and were induced during sporulation (e.g., *csh-125* and *csh-202*). Others were expressed at considerable rates during vegetative growth, and expression was further increased during sporulation (e.g., *csh-154* and *csh-293*). More striking are the differences in dependence on *spo0H* for expression. While several of the insertions seemed to have little or no expression in the *spo0H* null mutant (Table 1, *csh-125* and *csh-282*), indicating a strong or perhaps absolute dependence on *spo0H*, others were still expressed in the *spo0H* mutant, although at reduced rates (Table 1, *csh-154* and *csh-293*). These genes must have at least one promoter that is recognized by RNA polymerase containing a sigma factor other than σ^H. If these genes are not directly controlled by σ^H, then at least one of the regulatory factors controlling expression of these genes is likely to be controlled by σ^H. Alternatively, these genes could have multiple promoters, one of which is directly controlled by σ^H and at least one of which is active in the absence of σ^H.

Map positions. We mapped several of the *csh*::Tn*917lac* insertions by using the generalized transducing phage PBS1 and the standard *B. subtilis* mapping kit strains of Dedonder et al. (8). The mapping data are summarized in Table 2 and Fig. 4. The insertions do not appear to map in previously described genes.

Phenotypes. Most of the *csh*::Tn*917lac* insertion mutations caused no detectable growth or sporulation phenotype. However, three of the insertions did cause specific phenotypes. *csh-203*::Tn*917lac* caused a defect in growth, but only in combination with a *spo0H* mutation; *csh-227*::Tn*917lac* caused a defect in sporulation; and *csh-293*::Tn*917lac* caused a defect in sporulation and competence.

***csh-203*.** The phenotype caused by *csh-203*::Tn*917lac* provides strong evidence that *spo0H* is needed for normal

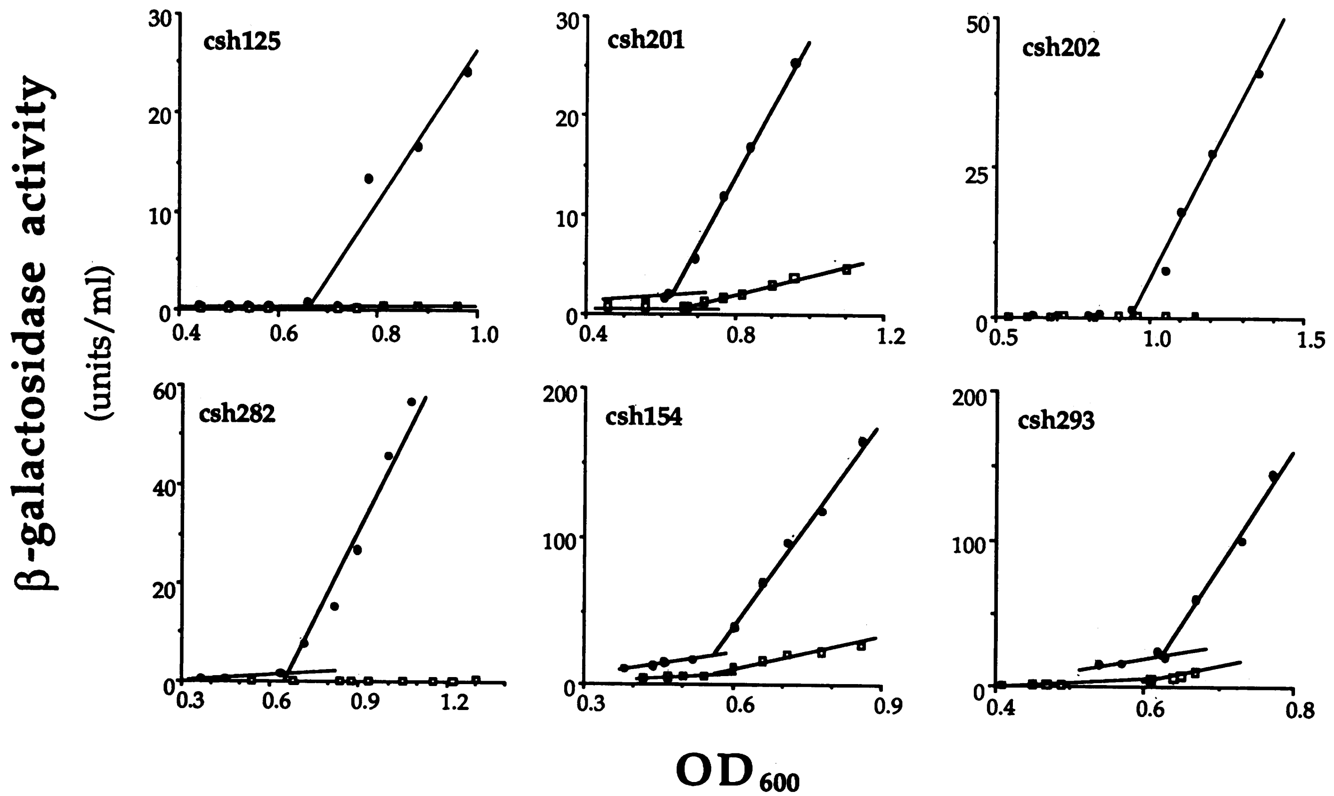

FIG. 3. Differential plots of expression of *csh-lacZ* transcriptional fusions. Data are the same as in Fig. 2, except that β-galactosidase activity (1,000 × ΔA_{420} per min per ml of culture) is plotted as a function of cell density (OD_{600}). Symbols are the same as in Fig. 2.

growth or that it controls expression of at least one gene needed for normal growth. *csh-203*::Tn*917lac* caused no obvious phenotype in a wild-type strain background. However, in a *spo0H* mutant background, the *csh-203* mutation caused a severe growth defect. The double mutant grew slowly in LB medium, doubling in approximately 2 h, while the isogenic wild type and each single mutant doubled in approximately 30 min. The double mutant formed small colonies on LB plates and was unable to form colonies on DS plates. The growth rate of the double mutant seemed to be normal in minimal glucose-glutamate medium. We do not yet know the function of *csh-203*, but one possibility is that *csh-203* encodes a sigma factor that has overlapping specificity with σ^H. Either *csh-203* or *spo0H* is required for expression of some genes essential for growth in complex medium. In either single mutant, *spo0H* or *csh-203*, the other gene product is present and functional. However, in the double mutant, the essential genes are not expressed and the

TABLE 1. Synthesis rates of each *csh*::Tn*917lac* fusion[a]

csh gene	Differential synthesis					
	Wild-type background			*spo0H* mutant		
	Veg	Spor	Ratio, spor/veg	Veg	Spor	Ratio, spor/veg
csh-112	2.6	26	10	<1	<1	
csh-125	1.3	78	(60)	<1	<1	
csh-154	48	469	9.8	17	62	3.6
csh-157	1.8	69	(38)	<1	<1	
csh-159	15	37	2.5	7	15	2.1
csh-163	1.2	145	(121)	<1	<1	
csh-191	203	1,127	5.6	55	357	6.5
csh-201	3.7	70	19	<1	9.5	
csh-202	1.2	101	(84)	<1	<1	
csh-203	8.8	30	3.4	6.5	6.5	1
csh-204	5.6	1,152	206	1.2	4.6	(3.8)
csh-227	100	832	8.3	42	833	20
csh-239	38	250	7	<1	2.2	
csh-282	4.8	129	27	<1	<1	
csh-285	34	145	4	<1	<1	
csh-286	12	199	17	<1	4.6	
csh-290	3.8	57	15	<1	2.5	
csh-293	81	810	10	10	87	8.7

[a] Differential synthesis rates were determined as the slope of a differential plot (Fig. 3) during vegetative growth (veg) and during the first 2 h after the initiation of sporulation (spor). The induction ratio represents the ratio of the rate during sporulation divided by the rate during vegetative growth. Data are from representative experiments. Parentheses indicate uncertainty due to very low rates of synthesis during vegetative growth.

TABLE 2. Cotransduction of *csh*::Tn*917lac* insertions and auxotrophic mutations[a]

csh gene	Marker(s) (% cotransduction)
csh-112	*hisA* (62)
csh-125	*purA* (14), *cysA* (80)
csh-154	*purA* (66), *cysA* (35)
csh-191	*glyB* (59)
csh-201	*metC* (14)
csh-202	*cysA* (36)
csh-203	*lys* (31), *aroD* (57)
csh-239	*purA* (55)
csh-286	*thrA* (17), *hisA* (34)
csh-293	*aroI* (79), *dal* (66)

[a] The numbers in parentheses indicate the percent cotransduction of the *csh*::Tn*917lac* insertion mutation with the indicated selected marker. In all cases selection was for prototrophy on minimal plates containing X-gal. The presence of the *csh*::Tn*917lac* fusion was indicated by blue color on the selection plates and verified by checking for MLS[r].

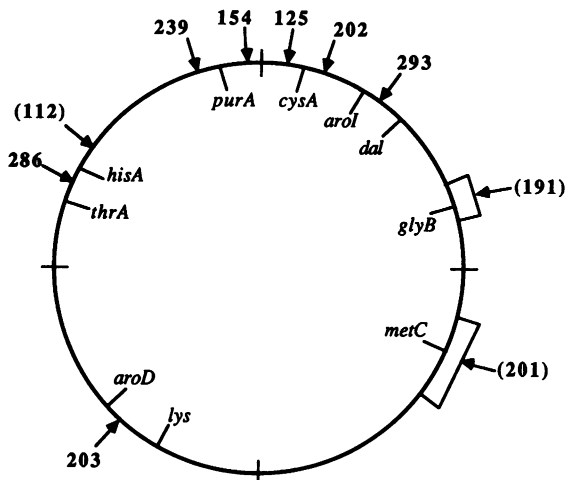

FIG. 4. Map positions of *csh*::Tn*917lac* insertion mutations. Numbers refer to the specific *csh*::Tn*917lac* insertion mutations. Positions indicated are based on data in Table 2 and on three-factor analysis. Numbers in parentheses indicate that the map position relative to the nearby auxotrophic marker is not known.

cells are quite sick. Note that while expression of *csh-203* was reduced in the *spo0H* deletion mutant, it was not eliminated (Table 1). Thus, there is still some expression of *csh-203* in the absence of σ^H.

During sporulation, expression of *csh-203*::Tn*917lac* was different in DS medium than in minimal medium with decoyinine. While expression increased when sporulation was induced by decoyinine, expression appeared to decrease at T_0 when sporulation was induced by nutrient depletion in DS medium. We have not been able to determine whether expression in DS medium is affected by the *spo0H* mutation because the double mutant does not grow in DS medium.

csh-227. *csh-227*::Tn*917lac* caused an oligosporogenous (partially Spo$^-$) phenotype. This mutant was partly defective in sporulation on DS agar plates (~1 to 10% of the wild-type level) and sporulated at a frequency approximately 10% of the wild-type level when sporulation was induced in minimal medium with decoyinine. However, in DS liquid medium, the *csh-227* mutant sporulated normally. In addition, the pattern of expression of this gene was different in DS medium than in minimal medium with decoyinine. In DS medium, *csh-227* was expressed during vegetative growth (as in minimal glucose-glutamate medium), but in DS medium expression appeared to decrease at T_0 (rather than increase as it did in minimal medium after the addition of decoyinine). Furthermore, in DS medium expression was not affected by the *spo0H* mutation.

While the ~10-fold effect on sporulation in liquid medium was modest, it was reproducible. The effect of the growth medium on the sporulation phenotype was quite unexpected and seems inconsistent with the notion that a drop in GDP and GTP is necessary and sufficient to cause normal sporulation. When decoyinine was used to initiate sporulation in the *csh-227*::Tn*917lac* mutant, there was a partial defect relative to wild-type cells. Decoyinine causes a drop in the intracellular concentrations of GDP and GTP. If nutrient exhaustion in DS medium was causing the initiation of sporulation simply by causing a drop in these nucleotides, then there should also be a defect in sporulation in DS medium. Since we could not detect a defect, we raise the possibility that other intracellular signals may be involved in the initiation of sporulation during nutrient limitation in DS medium.

csh-293. Like *csh-227*, *csh-293* also caused an oligosporogenous phenotype on DS plates and in minimal medium with decoyinine but not in DS liquid medium. However, the pattern of expression of *csh-293* in DS medium was similar to that in minimal medium with decoyinine. In addition, *csh-293* caused a defect in competence. Strains containing the *csh-293*::Tn*917lac* insertion were transformed with an efficiency of ~1% of the isogenic wild type. This defect was apparent in the strain background we used as well as the IS75 background (19) used by Dubnau and co-workers (J. Hahn and D. Dubnau, personal communication). The map position of *csh-293* (Table 2 and Fig. 4) indicates that it is not any of the previously identified *com* genes.

Limitations in the search for *csh* genes. It is clear from our results that we have not identified all of the genes that are controlled by *spo0H*. Among the insertions initially chosen for study, only two or three appeared to be duplicates, indicating that there are likely to be additional *csh* genes. (All of the 18 *csh*::Tn*917lac* insertions characterized in detail appear to be in different genes). In addition, there are genes that are known to be controlled by σ^H that we did not identify in this work, most notably *spoVG*. To test whether *lacZ* fusions to certain *spo* genes would have been detected by our screening procedure, we did a reconstruction experiment and introduced different *spo-lac* fusions into a strain containing the P_{spac}-*spo0H* fusion and a *spoIIA* mutation and monitored expression on X-gal indicator plates in the presence and absence of IPTG. As expected, expression of *lac* fusions to *spoIIE*, *spoIIG*, and *spoVG* was dependent on the presence of σ^H (plus IPTG). It is known that expression of *spoIIE* (18) and *spoIIG* (21, 22) depends on *spo0H*, probably indirectly, and does not depend on *spoIIA*, while expression of *spoVG* is directly controlled by *spo0H* (45a). Thus, our screening procedure can detect genes that are both directly (*spoVG*) and indirectly (*spoIIE* and *spoIIG*) controlled by σ^H.

Several factors could have limited our search for *csh* genes. (i) Insertion of Tn*917lac* is not completely random, and we only screened ~10,500 insertion mutations. This is probably not enough to saturate the genome. (ii) Because we used insertional mutagenesis, we would only detect nonessential genes. However, if essential genes are controlled by *spo0H*, for example, genes involved in cell division, those genes must have a *spo0H*-independent mode of expression, because *spo0H* mutants are viable. (iii) It is likely that the insertion library that we used (27) is depleted for specific classes of insertions. The method of creating the insertion library is thought to select against insertions that decrease viability in stationary phase, for example, insertions in *spo0* genes (34). However, the library should not be depleted for insertions in genes needed later in sporulation. (iv) There may be genes that do not have a high enough level of expression to be detected with a Tn*917lac* transcriptional fusion on X-gal indicator plates. Despite these limitations, and perhaps others, we were able to identify 18 genes by insertional mutagenesis with Tn*917lac* that are controlled by σ^H in vivo.

DISCUSSION

Several methods have been used to identify genes involved in sporulation. Historically, genes required for sporulation were identified by isolating mutants that were blocked in sporulation (26, 32). Most of these *spo* genes have been

cloned and their DNA sequences have been determined (26). More recently, methods have been used that identify genes for specific products by first isolating the product and then working "backwards" to the gene. This approach has been particularly useful in the identification of *cot* (9) and *ssp* (5, 6) genes. Genes expressed during sporulation have been identified by their time of expression. In some cases, mRNA was isolated from sporulating cells and used to identify the gene encoding the mRNA (36; C. Mathiopoulos and A. L. Sonenshein, manuscript in preparation). The development of Tn*917lac* as a tool for making transcriptional fusions on a relatively large scale has been extremely useful for identifying genes by their time of expression (31, 44).

We have identified genes based on their requirement for a specific regulatory factor for expression, independent of their function and pattern of expression. Eighteen genes that are controlled by the *spo0H* gene product, σ^H, in vivo were identified by the combined use of two methods: (i) the ability to control expression of a cloned gene by fusion to the IPTG-inducible promoter P_{spac} developed by Yansura and Henner (42), and (ii) the ability to create random gene fusions to *lacZ* by using the transposon Tn*917lac*, developed by Perkins and Youngman (31). This general strategy is applicable to any situation in which (i) a cloned regulatory gene can be fused to an inducible promoter and (ii) a reporter gene is available to monitor expression from regulatory elements. The random fusions to a reporter gene could be made in vivo, as we have done, or in vitro, by cloning, and introducing the random clones into the organism of interest. This approach should be applicable to many regulatory genes in several organisms.

RNA polymerase containing the *spo0H* gene product, σ^H, is required for transcription of genes during growth and sporulation. The patterns of expression of the 18 *csh* genes we have identified are strikingly similar in one respect and remarkably different in other respects. Expression of all of these genes is induced within 30 min after the initiation of sporulation with decoyinine. Some of the other genes or promoters that are known to be controlled by *spo0H*, including *spoVG* (47), *spo0F* (41), and P3 of the *rpoD* operon (4), have patterns of expression similar to that of the *csh* genes. However, the levels of expression and the amounts of induction vary over a wide range, and the *spo0H* deletion mutation has dramatically different effects on different *csh* genes. While expression of many of these genes is strongly reduced or eliminated in the *spo0H* mutant, expression of others is only partly dependent on *spo0H*. The genes that are strongly dependent on *spo0H* are most likely to have promoters that are recognized directly by $E\sigma^H$. The genes that are only partly dependent on *spo0H* could be indirectly controlled by σ^H, that is, *spo0H* could affect another regulatory factor that is needed for expression of these genes. Alternatively, these genes could have multiple promoters, at least one of which is controlled by *spo0H*. In either case, some of the *csh* genes must have promoters that are recognized by at least one form of RNA polymerase holoenzyme that is different from $E\sigma^H$. In vitro transcription experiments will help to define those promoters that are transcribed directly by $E\sigma^H$ and those that are transcribed by other forms of RNA polymerase holoenzyme.

Increased expression of some of the *csh* genes after the addition of decoyinine could result from an increase in the amount of σ^H in the cell. Experiments to measure the amount of σ^H during growth and sporulation are in progress (J. Healy and I. Smith, unpublished results). Alternatively, the specific activity of σ^H may change during sporulation and/or other regulatory factors may be involved in expression of some of the *csh* genes. In fact, in the *spo0H* mutant, expression of some of the *csh* genes is still induced after the addition of decoyinine (e.g., *csh-191* and *csh-293*), indicating that some other regulatory factor is required for increased expression after T_0.

The existence of other regulatory factors needed to control the expression of some of the *csh* genes would also help explain many of the differences in expression among the *csh* genes. In fact, preliminary experiments indicate that expression of several (but not all) of the *csh* genes is dependent on *spo0A*, and mutations in *abrB* restore expression of some of these *csh* genes. This type of regulation by *spo0A* and *abrB* is similar to the regulation of *spoVG* (48). By studying the regulatory regions of several of these genes and identifying factors that act to control their expression, we hope to unravel and understand some of the overlapping regulatory networks controlling gene expression at the onset of sporulation.

Mutations in regulatory genes like *spo0H* probably cause a defect in sporulation by blocking expression of other genes needed for the sporulation process. Thus, RNA polymerase containing σ^H must be required for the transcription of genes needed for sporulation, specifically for transcription of genes needed to get from stage 0 to stage II of sporulation. The challenge then is to identify these genes and to determine their functions. The phenotypes caused by mutations in *spo0H* or by mutations in genes controlled by *spo0H* may indicate the function of some of these genes. The sporulation genes known to be controlled directly by *spo0H* include *spoVG* (3, 45a, 47), possibly *spoIIA* (40), and possibly *spo0F*, which is only partly controlled by *spo0H* in vivo (41). In fact, the decreased expression of *spo0F* in *spo0H* mutants is probably part of the reason that *spo0H* mutants are blocked at stage 0 of sporulation, and not stage II or stage V. However, it seems likely that decreased expression of several genes contributes to the stage 0 phenotype of *spo0H* mutants.

In the process of searching for mutants that were altered in the extracellular control of sporulation, we isolated a *spo0H* mutant that was partially Spo$^-$ and appeared to be blocked at an early stage of sporulation, but that was stimulated to sporulate in the presence of other cells or in conditioned medium (A. D. Grossman, unpublished results). This phenotype would be expected for a mutant that is defective in producing extracellular factors but is capable of responding to them. Expression of genes needed for the production of extracellular factors cannot be the only role of *spo0H* in the initiation of sporulation, because *spo0H* null mutants are not stimulated to sporulate extracellularly. Unfortunately, none of the *csh* genes that we have identified, nor other genes known to be controlled by *spo0H*, seem to be involved in the extracellular control of sporulation.

It is striking that only 2 of the 18 *csh*::Tn*917lac* insertion mutations caused a defect in sporulation. Based on these results, it appears that the majority of genes controlled by σ^H are not essential for sporulation. The block at stage 0 caused by mutations in *spo0H* cannot be due to the effect of *spo0H* on any single one of these *csh* genes. It is possible that many of the genes controlled by σ^H are involved in the initiation of sporulation but that a mutation in any single gene does not cause a strong enough phenotype to detect. The strong block in sporulation caused by mutations in *spo0H* could result from the cumulative effects of decreased expression of all of these genes. Furthermore, of the characterized genes in which mutations cause a strong block in sporulation, many

encode regulatory proteins. There must be other genes involved in sporulation that are regulated by these *spo* genes, but they have not been identified by classical genetic techniques (isolating *spo* mutants). Perhaps this is due to redundancy in many of the functions that are expressed during sporulation, and a mutation in any one of these genes would not cause a strong defect in sporulation.

Finally, it appears that *spo0H* controls expression of several different classes of genes, many if not all of which are induced early during sporulation. By studying in detail the function and regulation of a few of these genes, we hope to begin to understand the roles of the alternative sigma factor, σ^H, in the initiation of sporulation and the control of gene expression.

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