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Genetics

Catabolite-resistant sporulation (*crsA*) mutations in the *Bacillus subtilis* RNA polymerase σ^{43} gene (*rpoD*) can suppress and be suppressed by mutations in *spo0* genes

(catabolite repression/gene conversion)

FUJIO KAWAMURA*, LIN-FA WANG, AND ROY H. DOI†

Department of Biochemistry and Biophysics, University of California, Davis, CA 95616

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ABSTRACT **The catabolite-resistant sporulation (*crsA*) mutation is able to overcome the repressive effect of glucose on sporulation in *Bacillus subtilis*. Three chromosomal *crsA* mutations, *crsA1*, *crsA4*, and *crsA47*, were transferred by the "gene conversion" process to *B. subtilis* plasmid pRPD11, which consists of the entire wild-type *rpoD* coding sequence, encoding the major σ^{43} factor of *B. subtilis* RNA polymerase, and pUB110. By DNA sequence analysis we showed that all three *crsA* mutations were identical two-base changes, CCT (proline) to TTT (phenylalanine), within the *rpoD* coding sequence. Furthermore, the *crsA47* mutation restored *spo0J* and *spo0K* sporulation to wild-type levels and partially improved the sporulation efficiencies of *spo0B*, *spo0D*, and *spo0F*. Extragenic suppressors (*scr*) of *crsA47* included mutations in *spo0A*, *spo0D*, *spo0F*, and *spo0K* plus other mutations that have not been specifically identified. Thus σ^{43} appears to be involved in catabolite repression by glucose, to interact either directly or indirectly with *spo0* gene products, and to play an important role in the initiation of spore development in *B. subtilis*.**

The multiplicity of RNA polymerase σ factors has been well documented in *Bacillus subtilis* (1–4). Recently, it has been demonstrated that *htpR* (*hin*) (5, 6) gene product in *Escherichia coli* functions as a σ factor *in vitro*, binding to the core RNA polymerase and initiating transcription at a heat-shock promoter (7). More recently Westpheling *et al.* (8) reported the identification of multiple holoenzyme forms in a more complex spore-forming bacterium, *Streptomyces coelicolor*. It is thus strongly suggested that minor σ factors play an important role under stress conditions such as nutritional deprivation and heat shock.

The possible role of the minor σ factor in regulating gene expression in *B. subtilis* is expanding beyond sporulation control with the recent demonstration that the σ^{37} enzyme is involved in the expression of the alkaline serine protease gene (*aprA*) (9) as well as the *spoVG* gene (10). The σ^{28} enzyme appears to transcribe only a small number of genes during the logarithmic phase of growth (4), including perhaps the putative heat shock promoter of the σ^{43} gene (11, 12). The σ^{32} enzyme transcribes two of the genes recognized by σ^{37} enzyme (3). The σ^{29} enzyme is unique to sporulating cells (13), but no specific gene has been identified that is controlled by σ^{29} enzyme.

Since the σ^{43} (previously called σ^{55}; see ref. 11) enzyme is the major vegetative cell enzyme and is present at all stages (2), we began an investigation of the genetic and biochemical properties of the σ^{43} factor to understand its role during growth, the initial stages of transition to the stationary phase, and sporulation as well as its relationship to other transcription factors. Although little is known about catabolite repression in *B. subtilis*, glucose appears to exert a classical type of carbon catabolite repression on a number of genes in addition to sporulation genes (14–17). Recently, Takahashi (18) isolated a number of *B. subtilis* mutants that overcame the repressive effect of glucose on sporulation. These mutations mapped at six different loci, *crsA* to *crsF*, on the chromosome (19). We have isolated, sequenced, and genetically mapped *rpoD*, the gene encoding the major σ^{43} factor of *B. subtilis* RNA polymerase, and we suggested that *crsA* is allelic to *rpoD* (11, 20, 21).

We show here that the σ^{43} factor has a role in the catabolite repression of sporulation as well as in the initiation of sporulation, since the *crsA* mutation within the *rpoD* gene allows the cell to sporulate in the presence of glucose and also allows some early stage-blocked sporulation mutants, such as *spo0J* and *spo0K*, to sporulate at wild-type levels. Further evidence for the relationship between σ^{43} and the *spo0* genes is the suppression of *crsA47* by several *spo0* mutations.

MATERIALS AND METHODS

Bacterial Strains. The bacterial strains used in this study are listed in Table 1.

***B. subtilis* Plasmid Miniscreening Procedure.** Cells were grown at 30°C overnight on tryptose blood agar base (TBAB; Difco) plates containing kanamycin (Km) at 5 μg/ml and 0.5% glucose; colonies were inoculated into 2 ml of Luria broth containing Km at 5 μg/ml and grown for 3 hr at 37°C with shaking; 1.5 ml of cell culture was centrifuged on an Eppendorf centrifuge for 1 min. Pellets were suspended by vigorous Vortex mixing with 100 μl of SETL buffer (20% sucrose/50 mM EDTA/50 mM Tris·HCl buffer, pH 7.6/2 mg of lysozyme per ml) and 3 μl of RNase (10 mg/ml) solution. The suspensions were incubated for 10 min at 37°C, 200 μl of lysis solution (0.2 M NaOH/1% $NaDodSO_4$) was added to each, and the suspensions were mixed well. To each mixture was added 145 μl of 1.5 M potassium acetate, pH 4.8, the tubes were inverted several times, and the mixtures were kept on ice for 20 min and then centrifuged at 4°C for 5 min. The supernatants were individually mixed with 500 μl of isopropyl alcohol, and each mixture was centrifuged at room temperature for 5 min. The visible pellets were washed twice with 1 ml of 70% (vol/vol) ethanol followed by a 1-min centrifugation at room temperature. The DNA pellets were dried at reduced pressure and dissolved in 20 μl of TE buffer (20 mM Tris·HCl, pH 7.6/0.25 mM EDTA). *Escherichia coli* miniscreening procedures were performed as described by Close and Rodriguez (23).

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Abbreviations: Km, kanamycin; Km^r, kanamycin-resistant; kb, kilobase(s); $CrsA^-$ and $CrsA^+$, catabolite-sensitive and catabolite-resistant sporulation phenotype, respectively.

*Present address: The Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan.
†To whom reprint requests should be addressed.

Table 1. *B. subtilis* strains

Strain	Relevant genotype	Source
DB43	*crsA47 lys-1 trpC2*	I. Takahashi CS3 (18, 19)
DB62	*crsA1*	I. Takahashi GLU1 (18, 19)
DB63	*crsA4*	I. Takahashi FRU4 (18, 19)
DB51	*spo0A12 trpC2 phe-1*	BGSC 1S9
DB100	*hisH metB*	Y. Sadaie NIG 1121 (22)
DB112	*hisH metB crsA47*	pRPD11·A47 $\xrightarrow{gc}$ DB100
DB52	*spo0A12 hisH metB*	DB51 $\xrightarrow{tf}$ DB100
DB53	*spo0B136 hisH metB*	Y. Sadaie NIG 1132 (22)
DB54	*spo0D8 hisH metB*	Y. Sadaie NIG 1134 (22)
DB113	*spo0F221 hisH metB*	Y. Sadaie NIG 1140 (22)
DB56	*spo0G14 hisH metB*	Y. Sadaie NIG 1135 (22)
DB57	*spo0H17 hisH metB*	Y. Sadaie NIG 1136 (22)
DB58	*spo0J87 hisH metB*	Y. Sadaie NIG 1137 (22)
DB59	*spo0K141 hisH metB*	Y. Sadaie NIG 1138 (22)
DB130	*spo0A12 crsA47 hisH metB*	pRPD11·A47 $\xrightarrow{gc}$ DB52
DB131	*spo0B136 crsA47 hisH metB*	pRPD11·A47 $\xrightarrow{gc}$ DB53
DB132	*spo0D8 crsA47 hisH metB*	pRPD11·A47 $\xrightarrow{gc}$ DB54
DB114	*spo0F221 crsA47 hisH metB*	pRPD11·A47 $\xrightarrow{gc}$ DB113
DB135	*spo0H17 crsA47 hisH metB*	Our laboratory
DB134	*spo0G14 crsA47 hisH metB*	pRPD11·A47 $\xrightarrow{gc}$ DB56
DB136	*spo0J87 crsA47 hisH metB*	pRPD11·A47 $\xrightarrow{gc}$ DB58
DB137	*spo0K141 crsA47 hisH metB*	pRPD11·A47 $\xrightarrow{gc}$ DB59

DB135 was constructed by transformation using DB43 DNA and DB157 (*hisH metB spo0H17 dnaE20*) as recipient. gc, Gene conversion; tf, transformation.

Transformation. Bacterial transformations were carried out with slight modifications as described (24–26). Since *B. subtilis crsA* mutants grew slowly in minimal medium, concentrations of casein acid hydrolysate (Difco) were increased (0.1% for growth medium and 0.5% for transformation medium).

Other Methods. DNA sequencing was conducted by the dideoxy chain-termination method of Sanger *et al.* (27). The *Hpa* I fragments were cloned in the phage M13mp9 vector for dideoxynucleotide sequencing (28).

RESULTS

Transfer of *crsA* Mutations to Plasmid pRPD11 Carrying the *rpoD* Gene. Of the five known σ factors of *B. subtilis* RNA polymerase, σ^{43} is the predominant species (1–4). Fig. 1 shows a physical and genetic map of the *B. subtilis* chromosome near *rpoD*, the gene encoding σ^{43}. The *crsA1*, *crsA4*, and *crsA47* mutations, each isolated separately by Takahashi (18), all confer a very similar phenotype: they are resistant to catabolite repression of sporulation (18, 19), grow slowly on Spizizen's minimal medium (25, 44), and form morphologically distinct colonies on TBAB plates. We used the latter phenotypes and "gene conversion" to precisely locate the *crsA* mutations on the *rpoD* gene.

We constructed pRPD11, which consists of pUB110 and the 1.6-kilobase (kb) *Bam*HI–*Sph* I fragment carrying the entire wild-type *rpoD* coding sequence but lacking the *rpoD* ribosomal binding site (11). Using intact and *Pvu* II-cut pRPD11 DNA as donor, we transformed each *crsA* mutant and scored for the appearance of the wild-type, large-colony phenotype against a lawn of minute CrsA$^+$ colonies. (CrsA$^+$ cells are mutants that can sporulate in the presence of a high concentration of glucose, in contrast to wild-type cells, which are CrsA$^-$ and cannot sporulate efficiently in the presence of glucose.) The 0.7-kb *Bam*HI–*Pvu* II fragment (Fig. 1) could correct the CrsA$^+$ phenotype of each mutant at a frequency of about 10^{-3} of that of intact pRPD11, suggesting that all three *crsA* alleles are very close and are located to the left of the *Pvu* II site.

To precisely locate the *crsA* mutations in *rpoD* by DNA sequence analysis, we first transferred each *crsA* mutation to pRPD11 by gene conversion. It has been shown that *B. subtilis* competent cells have a strong error-correction system, which repairs mismatched DNA duplexes, resulting in gene conversion (29, 30). This phenomenon has been used effectively to transfer a deletion constructed in the alkaline serine protease gene from a plasmid to the host chromosome (31). Fig. 2 diagrams the gene conversion method to transfer each *crsA* mutation from the host chromosome to pRPD11. We transformed competent cells of each *crsA* mutant with

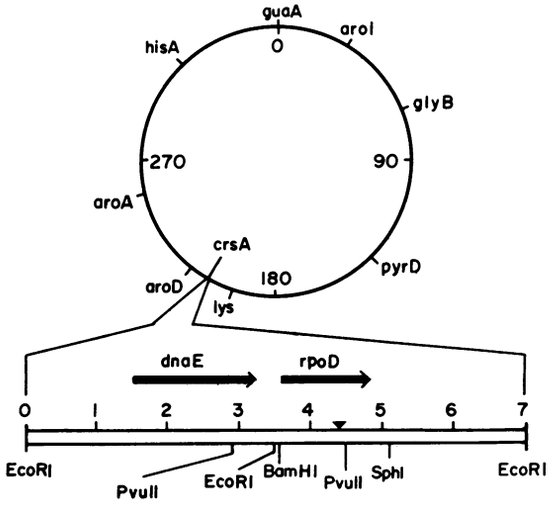

FIG. 1. The physical and genetic map of *B. subtilis rpoD* region. The *rpoD* gene maps between *aroD* and *lys* as shown on the abbreviated *B. subtilis* chromosome map (21). The physical map is modified from Price and Doi (21) and Gitt *et al.* (11). The arrows indicate the location and direction of transcription for the *dnaE* and *rpoD* genes. The closed triangle indicates the site of the *crsA* mutation within the *rpoD* coding sequence. The numbers on the chromosome map represent the location of genes in degrees and the numbers on the *Eco*RI fragment are kb.

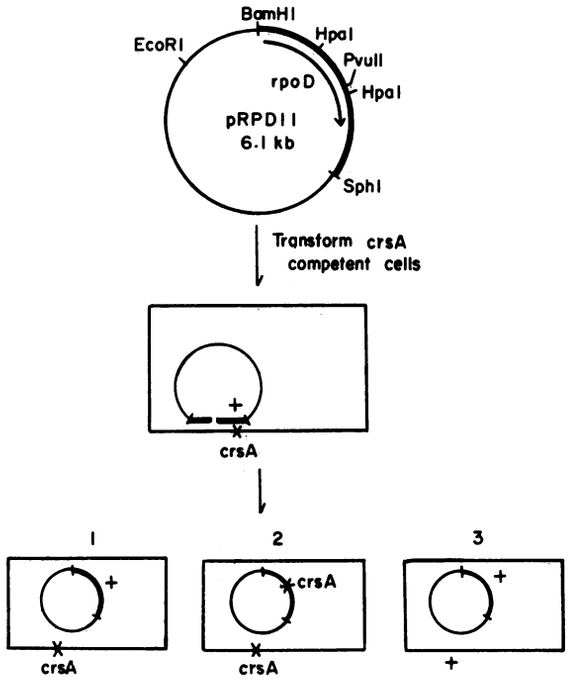

FIG. 2. Transfer of *crsA* mutations from *B. subtilis* chromosome to plasmid pRPD11 by gene conversion. pRPD11, which consists of pUB110 and the 1.6-kb *Bam*HI–*Sph* I fragment carrying the entire wild-type *rpoD* coding sequence, but lacking the *rpoD* ribosomal binding site, was used to transform the competent *crsA* mutants. Three classes of Km^r transformants were expected, as shown in this figure. These classes could be identified by their growth and segregation characteristics as described in the text. The thick line indicates *B. subtilis* chromosomal fragment, and the rectangle indicates the *B. subtilis* chromosome in the recipient cells.

pRPD11 DNA and selected Km-resistant (Km^r) transformants on TBAB plates containing 0.5% glucose and Km at 5 μg/ml. We expected three classes of Km^r transformants as shown in Fig. 2. These could be distinguished by their growth and segregation characteristics on Spizizen's minimal agar. The phenotype of each class reflected the genotype of the host chromosomal *rpoD* gene, because the plasmid-borne *rpoD* copies, lacking the ribosomal binding site, were not expressed.

Class 1 comprised heterozygous small-colony transformants, with the wild-type *rpoD* gene remaining intact on the plasmid and the *crsA* mutation on the chromosome. This class, through recombination, produced wild-type segregants that grew normally and were indistinguishable from the class 3 transformants described below. In class 2, the chromosomal *crsA* mutation converted the plasmid-borne *rpoD* to the mutant allele, producing small, stable, homozygous CrsA$^+$ colonies that showed no wild-type segregants. Class 3 transformants, in which the plasmid *rpoD* gene converted the chromosomal *crsA* mutation to the wild type, yielded large, stable, homozygous CrsA$^-$ colonies.

Thus, the normal-sized Km^r transformants represent class 3 and the small Km^r transformants, classes 1 and 2. Among the small colonies, we found the stable class 2 transformants at a frequency of about 5%. The transfer in this class of the *crsA* mutation from the chromosome to the pRPD11 plasmid was confirmed by the ability of the converted plasmid to transform wild-type *B. subtilis* from CrsA$^-$ to CrsA$^+$, as shown in Table 2.

DNA Sequence of crsA Mutations. We next subcloned the 550-base-pair *Hpa* I fragment overlapping the site of the *crsA* mutation (Fig. 2) from each converted plasmid pRPD11·A1, pRPD11·A4, and pRPD11·A47 in M13mp9. We again confirmed that the resulting clones M13mp9·A1, M13mp9·A4, and M13mp9·A47 carried CrsA$^+$ transforming activity, then we sequenced each *Hpa* I insert by the dideoxy chain termination method (27). It is evident from Fig. 3 that the *crsA1*, *crsA4*, and *crsA47* mutations are identical two-base changes, CCT to TTT. This codon change inserts a phenylalanine instead of a proline residue at position 290 of the predicted primary sequence of σ^{43} (12). Because proline residues are often critical to tertiary structure, this change probably causes a conformational change in σ^{43}.

Table 2. CrsA$^+$ transforming activity of pRPD11 carrying *crsA1*, *crsA4*, or *crsA47* mutation

Donor DNA	Km^r transformants per ml	
	Small size (%)	Normal size (%)
pRPD11	$<10^2$ (<0.4%)	2.4×10^4 (>99.6%)
pRPD11·A1	5.0×10^2 (4.4%)	1.08×10^4 (95.6%)
pRPD11·A4	6.0×10^2 (3.5%)	1.66×10^4 (96.5%)
pRPD11·A47	9.5×10^2 (4.8%)	1.90×10^4 (95.2%)

CrsA$^-$ competent *B. subtilis* cells were prepared as described (24–26), mixed with pRPD11, pRPD11·A1, pRPD11·A4, or pRPD11·A47 DNA, and plated on TBAB containing Km at 5 μg/ml and 0.5% glucose. Small-colony Km^r transformants that were stable homozygous *crsA*$^-$ cells resulted from gene conversion.

Effect of the *crsA* Mutation on Sporulation Phenotypes. Sharrock *et al.* (32) found that the *crsA47* mutation suppressed *spo0F* and *spo0K* defects, restoring sporulation of these strains to levels near those of wild-type strain of *B. subtilis*. Since we now knew that the *crsA* mutations were identical two-base changes within the *rpoD* coding sequence, we examined the effect of the *crsA* mutation on various *spo0* mutations. The *crsA47* mutation was introduced into various *spo0* mutants by gene conversion using pRPD11·A47 DNA. The effect of the *crsA47* mutation on the sporulation efficiency of *spo0* mutants is summarized in Table 3. The introduction of *crsA47* mutation increased the frequency of *spo0J* and *spo0K* sporulation, resulting in sporulation effi-

Table 3. Effect of the *crsA47* mutation on the sporulation frequency of various *spo0* mutants

Strain	Relevant genotype	Spores per ml	
		2× SG medium	SBM (1% glucose)
DB100		7.0×10^8	5.0×10^5
DB112	*crsA47*	8.0×10^8	6.0×10^8
DB52	*spo0A12*	$<2 \times 10^1$	
DB130	*spo0A12 crsA47*	$<2 \times 10^1$	
DB53	*spo0B136*	$<2 \times 10^1$	
DB131	*spo0B136 crsA47*	1.6×10^2	
DB54	*spo0D8*	$<2 \times 10^1$	
DB132	*spo0D8 crsA47*	7.0×10^2	
DB113	*spo0F221*	$<2 \times 10^1$	
DB114	*spo0F221 crsA47*	2.7×10^3	
DB115	*spo0FΔS*	$<2 \times 10^1$	
DB116	*spo0FΔS crsA47*	5.2×10^2	
DB56	*spo0G14*	$<2 \times 10^1$	
DB134	*spo0G14 crsA47*	$<2 \times 10^1$	
DB58	*spo0J87*	9.3×10^5	3.0×10^2
DB136	*spo0J87 crsA47*	2.9×10^8	1.4×10^8
DB59	*spo0K141*	4.4×10^3	$<2 \times 10^1$
DB137	*spo0K141 crsA47*	1.5×10^8	9.1×10^5

Cells were inoculated into 2× SG medium (33) or SBM (sporulation basal medium) containing 1% glucose (18) at an initial OD of 0.08 at 660 nm and incubated for 24 hr at 37°C with shaking. Heat-resistant spores were counted by plating the cells on TBAB after heating cells for 15 min at 80°C.

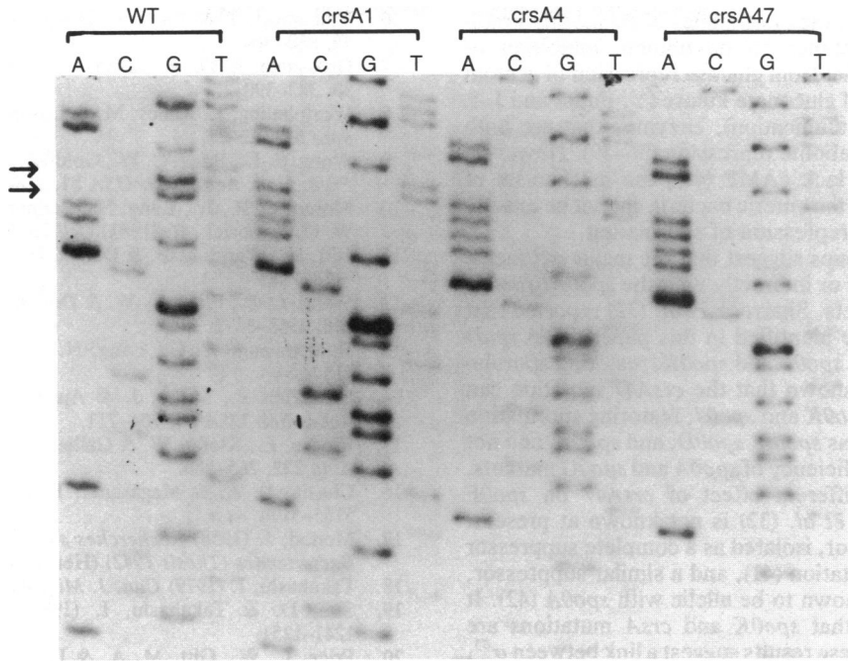

FIG. 3. Nucleotide sequence analysis of *crsA* mutations. The 550-base-pair *Hpa* I fragment containing the *crsA* mutation from each converted plasmid pRPD11·A1, pRPD11·A4, and pRPD11·A47 was subcloned in M13mp9 (28) and sequenced by the dideoxy chain termination method (27). The sequence ladder shown in this figure was derived from the nonsense strand of the *rpoD* gene. The wild-type (WT) strain has a CCT codon (GGA on the nonsense strand) within the *rpoD* gene which became a TTT codon (AAA on nonsense strand) in all *crsA* mutants, as indicated by arrows.

ciencies comparable to those of wild-type bacteria. Moreover, a *spo0K crsA47* double mutant had reduced ability to sporulate in the presence of glucose, indicating that the *spo0K* mutation suppressed the *crsA* mutation. The sporulation of *spo0B*, *spo0D*, and *spo0F* strains was slightly improved by the *crsA47* mutation. The sporulation of *spo0A* and *spo0G* was not affected by the introduction of the *crsA47* mutation.

We showed above that the three *crsA* mutations were identical two-base changes, an observation which suggested that the *crsA* mutation was highly specific. We therefore expected that spontaneous suppressor mutants of the *crsA* mutation could be isolated readily. Cells of *crsA* mutant (*crsA47 hisH metB*) were grown in L broth and plated on Spizizen's minimal medium supplemented with required amino acids. After incubation for 3 days at 37°C, 48 revertants that showed normal growth (large colonies versus small colonies produced by *crsA* mutants) on minimal plates were isolated and studied further.

All the revertants were found to retain the *crsA* mutation, indicating that these mutants had suppressor mutations. Since 13 revertants (suppressor mutants) among them showed a Spo$^-$ (sporulation-defective) phenotype, their suppressor mutations (*scr*) were studied by transduction and transformation, and 8, 4, and 1 mutations were mapped in *spo0A*, *spo0D*, and *spo0F* genes, respectively (a detailed map will be published elsewhere). These results combined with the suppressor effect of *crsA* on various *spo0* mutations are summarized in Fig. 4. Thus, the major *B. subtilis* σ^{43} factor may interact directly or indirectly with *spo0* gene products.

DISCUSSION

We have shown that all three *crsA* mutants, isolated independently by Takahashi (18), carry an identical codon change in the σ^{43} gene, suggesting that the *crsA* mutation is highly specific. Glucose repression of sporulation is similar to the phenomenon of catabolite repression in enteric bacteria, and the *B. subtilis crsA* mutation superficially resembles the *alt* mutation of *E. coli* σ, which allows expression of catabolite-sensitive operons in a *cya* background (33). Expression of catabolite-sensitive genes of *E. coli* is more sensitive than that of other genes to inhibition by novobiocin, a DNA gyrase

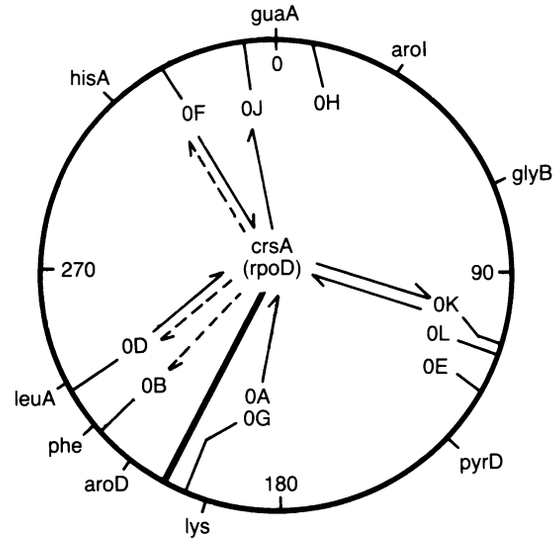

FIG. 4. Extragenic suppression pattern between *crsA (rpoD)* and *spo0* genes. The positions of *crsA (rpoD)* and *spo0* genes are shown on the *B. subtilis* chromosomal map; *crsA (rpoD)* lies between *aroD* and *lys*. *spo0* genes are indicated without *spo*. The arrow pointing to a gene indicates that this gene can be suppressed by a mutation in the gene at the tail of the arrow. Solid and dashed arrows indicate full and partial suppression, respectively. Note that suppression between *crsA* and *spo0* genes has been found in only one direction for some *spo0* genes (e.g., *spo0A*, *spo0B*, and *spo0J*) and in both directions for others (e.g., *spo0D*, *spo0F*, and *spo0K*).

inhibitor (34). Similarly, the *crsA* alleles of *B. subtilis* simultaneously confer resistance to novobiocin inhibition of sporulation (35) and relief from glucose repression of acetoin dehydrogenase (18) and gluconate kinase (Y. Fujita and J.-i. Nihashi, personal communication), enzymes that are both normally subject to catabolite repression (36–39). However, since *Bacillus* species lack cAMP (40), the mechanism of catabolite repression in the enteric bacteria cannot be exactly the same as catabolite repression of sporulation.

Results from two groups suggest that the major σ^{43} factor interacts either directly or indirectly with the *spo0A*, *spo0F*, and *spo0K* gene products. Sharrock *et al.* (32) reported that *crsA47*, which we have identified in this paper as an *rpoD* mutation, can suppress *spo0F* and *spo0K*, restoring sporulation ability. We have shown that the *crsA47* mutation can completely suppress *spo0K* and *spo0J*, restoring sporulation ability, partially suppress *spo0B*, *spo0D*, and *spo0F*, and not affect the sporulating efficiency of *spo0A* and *spo0G* mutants. The reason for the different effect of *crsA47* on *spo0F* observed by Sharrock *et al.* (32) is not known at present. Also, the *sof-1* suppressor, isolated as a complete suppressor of a *spo0F* deletion mutation (41), and a similar suppressor, *rvtA* (32), have been shown to be allelic with *spo0A* (42). It is interesting to note that *spo0K* and *crsA* mutations are mutual suppressors. These results suggest a link between σ^{43}, *spo0* gene products, and carbon catabolite repression in *B. subtilis*.

Takahashi and MacKenzie (36) have shown that high concentrations of carbon sources invariably block sporulation at a very early stage (stage 0). They suggested that analysis of mutants resistant to catabolite repression of sporulation would elucidate the control mechanisms governing initiation of spore development. In this regard, Sun and Takahashi (43) have recently mapped another catabolite-resistant sporulation mutation, *crsE1*, within the *rpoBC* genes. These results suggest that the β or β' subunit of RNA polymerase is involved in catabolite repression of sporulation (43). Our results show that the major σ^{43} factor is either directly or indirectly involved in catabolite repression of sporulation and strongly suggest that initiation of sporulation is at least partly controlled by catabolite repression at the transcription level.

Note Added in Proof. We will now designate *crsA1*, *crsA4*, and *crsA47* as *rpoD47*, since all three mutations are identical *rpoD* mutations. Sun and Takahashi have reported the existence of non-*spo0* suppressors of *rpoD47* (*crsA47*) (44).

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