MAPPING OF SPORULATION-DEFICIENT SUPPRESSOR
MUTATIONS FOR A CATABOLITE-RESISTANT
SPORULATION (*crsA*) MUTANT OF THE
MAJOR SIGMA-43 FACTOR OF
BACILLUS SUBTILIS

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Extragenic suppressor mutants for the *sigA47* (*crsA47*, *rpoD47*) mutation were isolated and analyzed. Among 48 spontaneous suppressor mutants (*sca*), 13 were deficient in sporulation and these mutations were mapped in the *spo0A*, *spo0B*, and *spo0F* genes, respectively. During the mapping studies, *spo0G14* and *spo0D8* mutations were found to be alleles of the *spo0A* and *spo0B* genes, respectively. These results indicate that a link exists between the RNA polymerase *sigA* enzyme, glucose catabolite repression, and some of the *spo0* gene functions.

The sporulation of *Bacillus subtilis* is blocked at very early stages in the presence of relatively high concentrations of glucose (7, 20, 29). Takahashi (28) has isolated a number of mutants that overcome this repressive effect of glucose on sporulation. These mutations were located at six distinct loci, *crsA* to *crsF*, on the chromosome (25). Recently, it has been shown that three *crsA* mutations, *crsA1*, *crsA4*, and *crsA47*, are identical two base-changes, CCT (proline) to TTT (phenylalanine), within the coding sequence of the RNA polymerase σ^{43} gene (*rpoD* or *sigA*) (8, 12, 17), and can suppress various sporulation defects (12, 15).

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The isolation and characterization of extragenic suppressor mutations are powerful approaches to genetic analysis of a complex process such as bacterial sporulation (22, 23). Sun and Takahashi (26) have recently reported that a rifamycin-resistance mutation, *rfm11*, can suppress the catabolite resistance phenotype in *crsC*, *crsE*, *crsD* and *crsF* mutants. More recently they have isolated extragenic suppressor mutations, *sca19* and *sca27*, for the *crsA47* (*sigA47*) and mapped these mutations between *thr5* and *aroG932* (27). To investigate further the regulatory function of the major σ^{43} factor during the early stages of sporulation, we isolated and analyzed 48 spontaneous suppressor mutants (*sca*) for the *sigA47* mutation.

Since 13 suppressor mutants among them showed a sporulation defective phenotype, these suppressor mutations were studied by DNA mediated transformation and 8, 4, and 1 mutations were mapped in the *spo0A*, *spo0B*, and *spo0F* genes, respectively. During the mapping study, it was also confirmed the results reported by Errington and Jones (4) that *spo0G14* and *spo0D8* mutations were alleles of the *spo0A* and *spo0B* genes, respectively.

MATERIALS AND METHODS

Bacterial cultures. The *Bacillus subtilis* strains used in this study are listed in Table 1. Minimal medium (MM) was prepared as described by Spizizen (24). The 2 × SG medium was prepared according to Leighton and Doi (14). The sensitivity of various strains to glucose was tested in spore basal medium (SBM) which contained 1% glucose (28). The method for determining the frequency of spore formation was described previously (11). MM or SBM was supplemented with appropriate amino acids (50 µg/ml).

Table 1. *Bacillus subtilis* strains.

Strain	Relevant genotype	Source and reference
DB42	<i>dnaE20</i>	(17)
DB46	<i>spo0G14UL</i>	(17)
DB50	<i>spo0A3 trpC2</i>	BGSC 1S5
DB52	<i>spo0A12 hisH metB</i>	(12)
DB53	<i>spo0B136 hisH metB</i>	NIG1132 (19)
DB54	<i>spo0D8 hisH metB</i>	NIG1134 (19)
DB56	<i>spo0G14 hisH metB</i>	NIG1135 (19)
DB113	<i>spo0F221 hisH metB</i>	NIG1140 (19)
DB57	<i>spo0H17 hisH metB</i>	NIG1136 (19)
DB58	<i>spo0J87 hisH metB</i>	NIG1137 (19)
DB59	<i>spo0K141 hisH metB</i>	NIG1138 (19)
DB100	<i>hisH metB</i>	NIG1121 (19)
DB112	<i>crsA47(sigA47) hisH metB</i>	(12)
NIG1133	<i>spo0C9 hisH metB</i>	(19)
UOT0680	<i>spo0F77 trpC2 lys-1</i>	(11)

NIG, National Institute of Genetics, Mishima, Japan; BGSC, *Bacillus* Genetic Stock Center, Ohio.

Competent cells were prepared as described previously (10).

Preparation of bacterial DNA. Cells were grown at 30°C overnight on tryptose blood agar base (TBAB) plates containing 0.5% glucose; colonies were inoculated into 2 ml of LB medium (1% Bactotryptone, 0.5% yeast extract and 0.5% NaCl) and grown for 3 h 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 (pH 7.6)/2 mg of lysozyme/ml] and 4 µl of RNase (10 mg/ml) solution. The suspensions were incubated for 10 min at 37°C, 600 µl of TE buffer [20 mM Tris-HCl (pH 7.6)/0.2 mM EDTA], 70 µl of 10% SDS, and 40 µl of 3 M sodium acetate buffer (pH 5.2) were added to each and the suspensions were thoroughly mixed. DNA was extracted twice with 400 µl of phenol and precipitated with an equal volume of isopropanol. After centrifugation for 5 min. DNA was washed twice with 1 ml of 70% ethanol followed by a 1-min centrifugation at room temperature, dried at reduced pressure and dissolved in 200 µl of TE buffer.

Isolation of suppressor mutants (sca) for sigA47 mutant. The *crsA* (*sigA47*) mutation, isolated by Takahashi (28), confers the following phenotype: the mutants are resistant to catabolite repression of sporulation (28), form morphologically distinct colonies on TBAB plates containing 0.5% glucose, and grow slowly on MM plates (12, 27). We used the third phenotype to obtain spontaneous suppressor mutants of the *sigA47* mutant. Cells of DB112 (*sigA47 hisH metB*) were inoculated at $A_{660}=0.08$ in MM supplemented with 0.1% casein hydrolysate, 50 µg/ml of histidine, methionine and tryptophan; incubated at 37°C for 4 h; and plated on MM plates supplemented with 50 µg/ml of histidine and methionine. After a 3-day incubation at 37°C, 48 clones that grew normally on MM plates containing required the amino acids were isolated.

Transformation experiments. *B. subtilis* DB42 (*dnaE20*) competent cells were mixed with donor DNA and incubated for 30 min at 37°C with shaking. After the addition of two volumes of LB medium, the cells were incubated for 1 h at 37°C, plated on TBAB containing 0.5% glucose, and incubated at 45°C. Crs^-/Ts^+ transformants were secured.

Complementation tests by specialized transducing phages. ϕ CA1 which contained the *Bcl*I fragment carrying the whole *spo0A* gene was described previously by Yamashita et al. (32). ϕ 105*spo0B*-2 containing the *spo0B* gene was described by Hirochika et al. (9). ϕ CF1 containing the *Hind*III-*Hpa*I fragment carrying the *spo0F* gene was described by Yoshikawa et al. (33). The cells lysogenized with each of these specialized transducing phages were inoculated in 2 × SG medium at $OD_{660}=0.08$, incubated at 37°C with shaking for 24 h. After heating for 10 min at 80°C, the cells were plated on TBAB plates and incubated at 37°C.

RESULTS AND DISCUSSION

We showed that three *crsA* mutations were identical two base-changes within

the coding sequence of σ^{43} gene, CCT (proline) to TTT (phenylalanine). This indicated that the *crsA47* (*sigA47*) mutation was highly specific (12). We therefore expected to isolate readily spontaneous extragenic suppressor mutants for the *sigA47* mutation. We isolated 48 suppressor mutants which grow normally on MM plate containing the required amino acids as described in MATERIALS AND METHODS.

To test the presence of the original *sigA47* mutation in these mutants, DB42 (*dnaE20*) was transformed with DNA isolated from these mutants, since it has been shown that the *sigA* gene lies immediately downstream from the *dnaE* gene (17, 31). About 60–80% of the Ts^+ (*dnaE^+*) transformants showed the *sigA47* phenotype (data not shown). This indicates that the original *sigA47* mutation is still present in these suppressor mutants (*sca*). Thirteen *sca* mutants among them were sporulation defective. Other *sca* mutants, which sporulated normally in $2 \times$ SG medium, could be divided into three groups. The typical sporulation proficient *sca* mutants are shown in Table 2. The first group exhibited a phenotype similar to that of wild type cells. They could sporulate to the wild type level in the presence of glucose (Table 2). The *sca-6* cells regained the normal rate of growth, and still sporulated well in the presence of glucose. The third group such as *sca-14* showed hypersensitivity of sporulation to glucose (Table 2), i.e. they produced fewer spores than the wild type.

Sporulation-deficient *sca* mutations were first classified into three linkage groups by transformation experiments (Table 3). The first linkage group included 8 *sca* mutations, *sca-9*, -16, -26, -38, -43, -44, -45, and -48, and the second group contained 4 *sca* mutations, *sca-21*, -24, -47, and -49. The third group had only one *sca* mutation, *sca-34*. All sporulation deficient *sca sigA47* mutants showed growth characteristics similar to the *spo0 sigA47* mutants (12) which formed larger colonies than a *crsA* mutant but smaller than *spo0* mutants on MM medium supplemented with the required amino acids. Then various *spo0* mutants, including *spo0A*, *0B*, *0D*, *0E*, *0F*, *0G*, and *0H*, were transformed to Spo^+ with DNA extracted from three *sca* mutants, DB324 (*sca-24*), DB326 (*sca-26*) and DB334 (*sca-34*), which were repre-

Table 2. Effect of glucose on the sporulation frequency of sporulation proficient *sca* mutants.

Strain	Sporulation (%)	
	$2 \times$ SG	SBM (1% glucose)
DB112 (<i>sigA47</i>)	100	100
DB100	107	0.1
DB302 (<i>sca-2 sigA47</i>)	124	0.2
DB306 (<i>sca-6 sigA47</i>)	105	33
DB314 (<i>sca-14 sigA47</i>)	41	0.0004

Values expressed as the percentages of parental type sporulation were calculated based on DB112 sporulation frequencies of 5.4×10^8 spores/ml in $2 \times$ SG and 3.6×10^8 spores/ml in SBM containing 1% glucose at 37°C.

Table 3. Linkage groups of sporulation-deficient *sca* mutations.

Donor	Spo ⁺ (<i>sca</i> DNA)/Spo ⁺ (DB100 DNA)		
	DB324 (<i>sca</i> -24)	DB334 (<i>sca</i> -34)	DB338 (<i>sca</i> -38)
DB309 (<i>sca</i> -9)	1.05	1.18	0.08
DB316 (<i>sca</i> -16)	0.95	1.18	0.08
DB321 (<i>sca</i> -21)	0.02	0.69	0.88
DB324 (<i>sca</i> -24)	<0.01	1.27	1.48
DB326 (<i>sca</i> -26)	1.05	1.27	0.08
DB334 (<i>sca</i> -34)	1.05	0.04	0.84
DB338 (<i>sca</i> -38)	1.00	1.27	<0.04
DB343 (<i>sca</i> -43)	1.14	0.45	0.08
DB344 (<i>sca</i> -44)	0.95	1.00	0.08
DB345 (<i>sca</i> -45)	0.71	1.36	0.04
DB347 (<i>sca</i> -47)	0.04	1.27	1.44
DB348 (<i>sca</i> -48)	0.81	1.72	<0.04
DB349 (<i>sca</i> -49)	0.05	1.64	1.20
DB100	1.00 (2.1 × 10 ⁵)	1.00 (1.1 × 10 ³)	1.00 (2.6 × 10 ⁵)

Numbers in the parentheses indicate Spo⁺ transformants/ml obtained using DB100 DNA.

sentative mutants of each group. The results indicated that 8 suppressor mutations including *sca*-9, -16, -26, -38, -43, -44, -45, and -48 and *spo0G14* mutation, 4 *sca* mutations, *sca*-21, -24, -47, and -49, and *spo0D8* mutation, and *sca*-34 mutation lie within the *spo0A*, *spo0B*, and *spo0F* genes, respectively (data not shown).

To confirm the results obtained by the DNA-mediated transformation described above, the *sca*, *spo0G14* and *spo0D8* mutants were lysogenized with ϕ 105 specialized transducing phage carrying *spo0A*, *spo0B* or *spo0F* gene, and the resulting lysogens were tested for their ability to sporulate in 2 × SG medium at 37°C (Table 4). The *spo0C9* mutation has been shown to be a missense mutation in the C-terminal region of the *spo0A* gene (5, 6, 13). Both *spo0C9* and *spo0G14* mutants lysogenic for ϕ CA1 could sporulate at lower levels compared with *spo0A12*, *sca*-26, -43, and -48 mutants lysogenic for ϕ CA1. This may reflect that the altered Spo0A protein having a mutation such as *spo0C9* and *spo0G14* would interfere with the normal function of wild type Spo0A protein. Table 4 also shows that *sca*-21 and *spo0D8* mutants, and *sca*-34 mutant are completely restored by lysogenization with ϕ 105*spo0B* and ϕ CF1, respectively.

Two general types of suppressor mutations of *crsA47* (*cra*) were observed in regard to sporulation. There were some suppressor mutations that in themselves had no affect on normal sporulation, and there were others that prevented sporulation. The analysis of these latter mutations revealed that they resided in *spo0A*, *spo0B*, and *spo0F*. Thus there is a tight link between the RNA polymerase holoenzyme function and the functions required for the initiation of sporulation.

Table 4. Complementation tests by specialized transducing phages $\phi CA1$, $\phi 105spo0B$, and $\phi CF1$.

Strain	Spores/ml
DB52 (<i>spo0A12</i>) (ϕCM)	$<2 \times 10$
DB52 (<i>spo0A12</i>) ($\phi CA1$)	3.3×10^8
NIG1131 (<i>spo0C9</i>) (ϕCM)	$<2 \times 10$
NIG1131 (<i>spo0C9</i>) ($\phi CA1$)	1.3×10^7
DB46 (<i>spo0G14</i>) (ϕCM)	$<2 \times 10$
DB46 (<i>spo0G14</i>) ($\phi CA1$)	9.5×10^6
DB326 (<i>sca-26</i>) (ϕCM)	$<2 \times 10$
DB326 (<i>sca-26</i>) ($\phi CA1$)	5.8×10^8
DB343 (<i>sca-43</i>) (ϕCM)	$<2 \times 10$
DB343 (<i>sca-43</i>) ($\phi CA1$)	5.8×10^8
DB348 (<i>sca-48</i>) (ϕCM)	3.8×10^2
DB348 (<i>sca-48</i>) ($\phi CA1$)	3.4×10^8
DB53 (<i>spo0B136</i>) ($\phi 105$)	$<2 \times 10$
DB53 (<i>spo0B136</i>) ($\phi 105spo0B-2$)	3.1×10^8
DB54 (<i>spo0D8</i>) ($\phi 105$)	$<2 \times 10$
DB54 (<i>spo0D8</i>) ($\phi 105spo0B-2$)	3.2×10^8
DB321 (<i>sca-21</i>) ($\phi 105$)	$<2 \times 10$
DB321 (<i>sca-21</i>) ($\phi 105spo0B-2$)	3.8×10^8
DB113 (<i>spo0F221</i>) (ϕCM)	$<2 \times 10$
DB113 (<i>spo0F221</i>) ($\phi CF1$)	7.0×10^8
DB334 (<i>sca-34</i>) (ϕCM)	1.2×10^3
DB334 (<i>sca-34</i>) ($\phi CF1$)	6.5×10^8

Lysogens for $\phi CM(21)$ were also tested for their sporulation frequencies as control. DB334 and DB348 carrying *sca-34* and *-48* mutation, respectively, were oligosporogenous.

The exact functions of these *spo0* genes are still unknown, although it has been suggested that they are a part of the mechanism that senses the nutritional environment of the cell and transmits this information to the transcription machinery (16). The specific suppression between several of the *spo0* genes and *sigA47* (this paper and 12) suggests the possibility that a direct interaction occurs between σ^{43} or the holoenzyme and the products of these genes. However, direct biochemical evidence will have to be obtained to confirm this point. This would necessitate the use of both wild and mutant type holoenzymes of RNA polymerase σ^{43} , σ^{43} promoters that are repressed by glucose and/or utilized during the initiation of sporulation, and putative transcription regulatory factors, e.g. Spo0A, Spo0B, and Spo0F, in in vitro transcription studies.

Although the current studies were concerned with suppressor mutations that caused a Spo⁻ phenotype, the presence of other suppressor mutations of *crsA47* (*sigA47*) that had no effect on the sporulation process illustrates the complex role and functions of σ^{43} during glucose repression and sporulation. Further analysis of these suppressor mutations should provide information on transcriptional regulatory factors that are involved in glucose control of gene expression, but are not

involved in regulating sporulation.

It has been suggested by Errington and Jones (4) that *spo0G14* and *spo0D8* are alleles of the *spo0A* and *spo0B* genes, respectively. During mapping of the *crsA* suppressor genes, we also confirmed their results by transformation and complementation tests. Since previous studies has shown that *spo0C* was an allele of *spo0A* (5, 6, 13), the *spo0* genes have been reduced to *spo0A*, *spo0B*, *spo0E*, *spo0F*, *spo0H*, *spo0J*, *spo0K*, and *spo0L*. The *spo0H* gene product is a newly identified σ^{30} (σ^H) factor (1, 3) that is involved in the transcription of the P3 promoter of the σ^{43} operon and one of the promoters of *spoVG* (2). The exact functions of the other *spo0* genes remains unknown. However, the amino acid sequence homology of Spo0A and Spo0F to the transcriptionally related proteins of dual regulatory factors (18, 30, 34) suggests the possibility that Spo0A itself may have some role in regulating transcription.

Further analysis of the *crs* genes should provide a clear understanding of the multi-functions of σ^{43} holoenzyme during the initiation of sporulation, and of the role of the *spo0* genes.

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