Identification of the Product of Sporulation Gene *spo0B* in *Bacillus subtilis*

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A 1.4-megadalton *EcoRI* restriction fragment carrying *Bacillus subtilis* sporulation gene *spo0B* was cloned from the specialized transducing phage, ϕ 105*spo0B*, into a unique *EcoRI* site of plasmid vector pUB110, and four plasmids having a deletion in the 1.4-megadalton *EcoRI* fragment were constructed. Analysis of the polypeptides synthesized in *B. subtilis* minicells harboring these plasmids and the sporulation ability of strain UOT0436 (*spo0B136 recE4*) harboring these plasmids showed that the *spo0B* gene product is a polypeptide of 24,000 daltons. Two-dimensional polyacrylamide gel analysis showed that the isoelectric point of this protein is almost neutral.

Many sporulation-deficient *Bacillus subtilis* mutants were isolated, characterized and classified into six types, *spo0*, *spoII*, *spoIII*, *spoIV*, *spoV* and *spoVI*, according to the blocked stage of sporulation.^{1,2)} The *spo0* mutation arrests sporulation at the earliest stage. At least nine *spo0* genes are known, but little is known about their structures and functions.^{3,4)} Recently, it has been shown that the transcription of the *spoVG* gene is under the control of *spo0* genes.⁵⁾ So far, none of the *spo0* gene products has been identified, except the *spo0F* and *spo0A* gene products.^{6,7)}

Previously, Hirochika *et al.*⁸⁾ reported the cloning of the *spo0B* gene in *B. subtilis* temperate phage ρ 11 and determined the *spo0B* gene product to be a polypeptide of a molecular weight of 39,000. Later, Bonamy and Szulmajster⁹⁾ cloned the *spo0B* gene by using a different cloning system from that by Hirochika *et al.*⁸⁾ and suggested that the gene product is a polypeptide of a molecular

weight of 24,000. However, there are some uncertain points in both papers. (1) The restriction map reported by Bonamy and Szulmajster⁹⁾ is not identical with that of Hirochika *et al.*⁸⁾ suggesting the possibility that the *spo0B* fragments of the two groups are different. (2) Since both groups did not carry out detailed analysis of the product with the deletion plasmid and Bonamy and Szulmajster used the *E. coli* minicell system, it is not certain that the 24 K protein is the *spo0B* gene product.

To clarify these points and to definitely identify the *spo0B* gene product, we constructed a detailed restriction map of the *spo0B* fragment isolated in the previous study^{8,10)} and constructed several recombinant plasmids which have a deletion in the *spo0B* gene. By analysis of the products produced in *B. subtilis* minicells carrying these recombinant plasmids and comparison of the results with the sporulation ability of strain

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UOT0436 (*spo0B136 recE4*) carrying these plasmids, we clearly showed that the *spo0B* gene product is a 24K protein having an almost neutral pI.

MATERIALS AND METHODS

Bacterial strains. *Bacillus subtilis* Marburg strains were used; UOT0277, *hisA1 metB5 recE4 nonB1* (University of Tokyo); UOT0436, *pheA1 recE4 spo0B136* (University of Tokyo); 1S16, *trpC2 pheA1 spo0B136* (*Bacillus* Genetic Stock Center); and 1A292, *divIVB1 metB5 thyA1 thyB1* (*Bacillus* Genetic Stock Center).

Preparation of plasmid DNA. Strains harboring the recombinant plasmid were grown at 37°C with shaking for 5 h (about 300 Klett units with filter No. 66) in L-broth medium (per liter; 10 g of Difco tryptone, 5 g of yeast extract, 10 g of glucose, 5 g of NaCl and 5 mm $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.7) containing kanamycin (5 $\mu\text{g}/\text{ml}$). If the kanamycin is not added, the plasmid becomes unstable. Cells were harvested by centrifugation and the pellets were washed twice with TES buffer (20 mm Tris-hydrochloride, pH 7.5, 5 mm EDTA and 100 mm NaCl) and cleared lysates were prepared by the pronase-Sarcosyl method.¹¹ Plasmid DNA was isolated by 0.8% agarose gel electrophoresis and further purified by DEAE-cellulose chromatography according to the procedure of Smith.¹²

For analytical purposes, plasmid DNA was prepared from a colony according to the method of Ish-Horowitz and Burke,¹³ except that the cells were incubated on a Tryptose Blood Agar Base (Difco) plate containing kanamycin (5 $\mu\text{g}/\text{ml}$) at 37°C overnight.

Endonuclease digestion and ligation. Plasmid DNA was digested with restriction endonucleases according to the instructions of the vendors with some modifications. Ligation was carried out with T4 DNA ligase as described previously.⁸

Transformation. Transformation with competent cells was performed by the method of Wilson and Bott¹⁴ and protoplast transformation was carried out as described by Chang and Cohen.¹⁵

Purification of minicells. *B. subtilis* 1A292 was used as the source of minicells. Minicells were purified essentially by the procedure of Reeve *et al.*¹⁶ Strain 1A292, with or without the recombinant plasmid, was grown at 30°C with shaking in Spizizen minimal medium¹⁷ supplemented with 0.5% glucose, 0.1 mM CaCl_2 , 0.01% casamino acids, 100 μg of thymine/ml and 100 μg of methionine/ml to T_1 (one hour after the end of logarithmic growth). The culture (1.5 liters) was rapidly chilled and then the cells were harvested and washed once with minimal medium sup-

plemented with 0.1 mM CaCl_2 (MM- CaCl_2). The cell pellets were suspended in MM- CaCl_2 and the minicells were separated by 5~30% linear sucrose density gradient centrifugation (4,600 rpm, 15 min in an RA-3 rotor of a Kubota KR-2000T centrifuge). The turbid minicell band was removed from the gradient and the minicells were pelleted by centrifugation (15,000 rpm, 10 min in an RA-3 rotor). These procedures were repeated three times. The purified minicells obtained were washed once with MM- CaCl_2 and finally resuspended in MM- CaCl_2 containing 10% glycerol. The purified minicell suspension was stored at -80°C until use.

Incorporation of a ^{14}C -amino acid mixture into minicells and analysis of the products by slab gel electrophoresis. The suspension of purified minicells with or without the recombinant plasmid (100 μl) was labeled with 15 μCi of a ^{14}C -amino acid mixture (100 $\mu\text{Ci}/\text{ml}$ of 0.1 N HCl, neutralized with an equal volume of 0.1 N NaOH before use)/ml at 37°C for 30 min in the presence of 20 μg each of valine and isoleucine/ml.

The samples for slab gel electrophoresis were prepared by the method of Shivakumar *et al.*¹⁸ and subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli and Favre.¹⁹ After electrophoresis, the gels were processed for fluorography²⁰ and exposed at -80°C to Kodak X-ray film (X-Omat XRP-5).

Two-dimensional gel electrophoresis of minicell extracts. Preparation of labeled minicell extracts and two-dimensional gel electrophoresis were carried out as described by O'Farrell,²¹ except that Nonidet P-40 was replaced by Triton X-100. After electrophoresis, the labeled polypeptide products were visualized by fluorography.²⁰

Enzymes and chemicals. Lysozyme, RNase A and DNase I were purchased from Sigma Chemical Co. The ^{14}C -amino acid mixture (NEC-445) was obtained from New England Nuclear Co. The Ampholines were from LKB. Restriction enzymes *AvaI* and *BclI* were purchased from Bethesda Research Laboratories, *BglII* and *PvuII* were from Boehringer Mannheim GmbH, and *BalI* was from New England Biolabs. Restriction enzyme *RsaI* was obtained from Nippon Gene Co., Toyama, Japan. All other restriction enzymes, T4 DNA ligase and agarose were from Takara Shuzo Co., Ltd., Kyoto, Japan.

RESULTS AND DISCUSSION

Construction of the deletion plasmid of *pUBSB35*

Previous studies showed that specialized transducing phages $\phi 11\text{pspo0B-1}^{18}$ and $\phi 105\text{spo0B}^{10}$ contain a 1.4 megadalton (Md)

FIG. 1. Restriction Map of the 1.4Md Fragment and the Recombinant Plasmid, pUBSB35, and Its Derivatives.

pUBSB35 was constructed by inserting the 1.4 Md fragment into a unique *EcoRI* site of pUB110. pUBSB19 has the inverted 1.4 Md fragment. The deletion plasmids were constructed from pUBSB35 as described in the text. The boxes indicate the deleted region on the 1.4 Md fragment. The activity of these plasmids in transforming *spo0B136* to *spo*⁺ is shown by +. The possible location of the *spo0B136* mutation is indicated by a wavy line. Symbols for the restriction endonuclease used are: Ec, *EcoRI*; Rs, *RsaI*; Ha, *HaeIII*; Av, *AvaI*; Pv, *PvuII*; Bc, *BclI*; Hi, *HindIII*; Ps, *PstI*; Ba, *BalI*; Hc, *HincII*; Bg, *BglII*; Bm, *BamHI*.

EcoRI fragment of *B. subtilis* chromosomal DNA which carries the early sporulation gene, *spo0B*. This 1.4 Md fragment was isolated and purified from $\phi 105spo0B$, and a restriction map was constructed as shown in Fig. 1. Then, a recombinant plasmid, pUBSB35, was constructed by inserting the 1.4 Md fragment at a unique *EcoRI* site of a plasmid vector, pUB110.²²⁾ Furthermore, a recombinant plasmid, pUBSB19, having the 1.4 Md fragment inserted in the opposite direction was constructed from pUBSB35.

To determine the location of the *spo0B* gene in the 1.4 Md fragment and to identify its product, deletion plasmids of pUBSB35 were constructed. pUBSB35 DNA was either completely digested with *AvaI* or double-digested

with *BclI* and *BamHI* or, after partial digestion with *BglII*, digested with *BclI*. The digested DNA was ligated and introduced into UOT0436 (*spo0B136 recE4*) protoplasts, and kanamycin resistant transformants were selected. The deletion plasmids were screened according to the size of the plasmids in kanamycin resistant transformants. Four deletion plasmids were obtained and the position of the deleted regions was determined as shown in Fig. 1. pUBSBΔBgc3 has a small *BclI* fragment (50 base pairs), but the orientation of this *BclI* fragment was not determined. pUBSBΔBgc1 lacks this small *BclI* fragment and has the inverted *BclI*-*BglII* fragment in the 1.4 Md fragment.

To determine the location of the *spo0B136*

FIG. 2. Fluorogram of Plasmid-specified Polypeptides Synthesized in Minicells.

^{14}C -amino acid-labeled samples obtained from minicells were separated by 13.5% SDS-polyacrylamide gel electrophoresis. Each sample contained approximately the same radioactivity (36,000 dpm). The molecular weight standards are indicated by numbers (in kilodaltons) on the left margin of the figure and the sizes of the pUB110- and recombinant plasmid-specified polypeptides are indicated on the right margin of the figure. The plasmids present in the minicell preparation were: lanes (1) and (10) no plasmid; (2) and (9) pUB110; (3) pUBSB35; (4) pUBSB Δ Ava125; (5) pUBSB Δ Bgc1; (6) pUBSB Δ Bgc3; (7) pUBSB Δ Bca1; (8) pUBSB19.

mutation, competent cell transformation with pUBSB35 and its derivatives was performed using 1S16 as a recipient. As shown in Fig. 1, all recombinant plasmids, except pUBSB Δ Bgc3, showed *spo0B*⁺ transforming activity. This suggests that the *spo0B136* mutation is located between the right *Bcl*I site and the *Bgl*II site. Recently, Ferrari *et al.*²³⁾ reported a similar physical map for the *spo0B*⁺ *Eco*RI fragment of the *B. subtilis* chromosome cloned in bacteriophage λ and determined that the *spo0B136* mutation is located on the *Pst*I-*Eco*RI fragment containing the *Bal*I site. Our result agrees with that of Ferrari *et al.*,²³⁾ except for the location of the *Bal*I site. Bonamy and Szulmajster⁹⁾ also cloned a *Sau*3AI fragment having the *spo0B* gene of *B. subtilis* into a plasmid vector, pHV33. However, it is difficult to correlate the *spo0B136* mutation locus described by Bonamy and Szulmajster with our result be-

cause of some differences between their restriction maps and ours.

Identification of the *spo0B* gene product

To identify the *spo0B* gene product, the minicell system¹⁶⁾ was used. After plasmid pUBSB35 and its derivatives were introduced into 1A292 (the minicell-producing strain) protoplasts, minicells were purified and labeled with a ^{14}C -amino acid mixture (see MATERIALS AND METHODS). Then, the labeled polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 2.

The minicells carrying pUB110 (Fig. 2, lanes 2 and 9) synthesized three polypeptides of 50,000 (50 K), 34,000 (34 K) and 14,000 (14 K) daltons, corresponding to K1, K2 and K5 described by Shivakumar *et al.*¹⁸⁾ None of the recombinant plasmids synthesized K1, but 34 K and 14 K were synthesized. These results

suggest that the 1.4 Md fragment is inserted in the K1 coding region.

When compared with pUB110, the recombinant plasmid, pUBSB35, produced three new peptides of 51 K, 39 K and 24 K (Fig. 2, lane 3). The 51 K and 39 K proteins were not produced by pUBSB Δ Ava125, pUBSB Δ Bca1 or pUBSB19, but were produced by pUBSB Δ Bgc1 and pUBSB Δ Bgc3 (Fig. 2, lanes 4 through 8). On the other hand, the 24 K protein was produced by pUBSB35, pUBSB19 and pUBSB Δ Ava125, but not by any other plasmids. As shown in Table I (the details will be discussed later), plasmids pUBSB35, pUBSB19 and pUBSB Δ Ava125 completely restored the sporulation of UOT0436 (*spo0B136 recE4*), but other recombinant plasmids did not. Therefore, the restoration of sporulation by these plasmids coincides with the presence of the 24 K protein. These results clearly indicate that the 24 K protein is the product of

the *spo0B* gene. Since plasmid pUBSB35 and its derivative plasmid, pUBSB19, which has the inverted 1.4 Md fragment, restored the sporulation and produced the 24 K protein, the intact *spo0B* gene having its own promoter may be present on the 1.4 Md fragment.

Several other proteins were synthesized by pUBSB Δ Ava125, pUBSB Δ Bca1 and pUBSB19. None of these proteins (20 K; 15 K; 45 K, 41 K; 32 K and 31 K) has any relation with the restoration of the sporulation because they were not produced by the sporulation complementing plasmids. pUBSB35, pUBSB19 and pUBSB Δ Ava125 (see Table I).

Previously, we assumed that the *spo0B* gene product is a 39 K protein on infection by a specialized transducing phage ϕ 11p*spo0B-1* of ultraviolet-irradiated cells.⁸⁾ However, since many proteins were synthesized in this system, we could not analyze the minor proteins in detail, except the 39 K protein. In the present

FIG. 3. Two-dimensional Gel Electrophoresis of Plasmid-specified Polypeptides Labeled in Minicells.

Two-dimensional gel electrophoresis was carried out as described in MATERIALS AND METHODS. The plasmids present in the minicell preparation are: (A) pUB110; (B) pUBSB35; (C) pUBSB Δ Ava125; (D) pUBSB Δ Bgc3. In the second dimension, 14% SDS-polyacrylamide gel was used and the extracts of labeled minicells carrying pUB110 (A), pUBSB35 (B and D) or pUBSB Δ Ava125 (C) were electrophoresed together on the left or right edge as markers. K2, K5 and 24 K proteins are indicated by arrows and the dotted circle indicates the possible location of the 24 K protein.

paper, using the deletion plasmids and the *B. subtilis* minicell system, we clearly determined that the *spo0B* gene product is a 24 K protein and confirmed the result reported by Bonamy and Szulmajster.⁹⁾

Two-dimensional gel electrophoresis of the 24 K protein

To characterize the *spo0B* gene product, we analyzed the labeled polypeptide obtained from minicells carrying pUB110 or recombinant plasmids by two-dimensional gel electrophoresis. As shown in Fig. 3, the 24 K protein was detected as a single spot having an almost neutral pI.

Effect of pUBSB35 and its derivatives on sporulation

We have previously reported that the recombinant plasmid of pUB110 carrying the *spo0F*⁺ gene inhibited the sporulation of a wild type strain, but the recombinant plasmid carrying the *spo0B*⁺ gene did not.²²⁾ This phenomenon was further investigated using pUBSB35 and its derivatives. None of the recombinant plasmids used in this experiment inhibited the sporulation of wild type strain UOT0277 (*spo*⁺ *recE4*). UOT0277 having these recombinant plasmids produced spores normally (1.0×10^8 to 2.2×10^8 spores/ml, data not shown). Since the copy number of pUBSB35 in UOT0277 is about 80 (data not shown), this result suggested that the high copy number of the *spo0B* gene did not affect the sporulation. Kawamura *et al.*²²⁾ reported that recombinant plasmid pUBSF13 carrying the *spo0F* gene inhibits the sporulation of UOT0277 very severely to the level of 10^3 spores/ml. Further studies will be necessary to clarify the different inhibitory effects between pUBSB35 and pUBSF13. Table I shows that sporulation of an asporogenous strain, UOT0436 (*spo0B136 recE4*), was restored by pUBSB35, pUBSB19 and pUBSBΔAva125 to the wild type level (10^8 spores/ml). However, pUBSBΔBca1 and pUBSBΔBgc1 restored the sporulation of UOT0436 to the level of 10^3 spores/ml. This result is incom-

TABLE I. SPORULATION OF THE *spo0B* MUTANT HARBORING pUBSB35 DERIVATIVES

Host cell	Plasmids	Viable cells ^a (ml)	Spores ^a (ml)
UOT0436	—	5.8×10^8	1.1×10
(spo0B136 recE4)	pUB110	1.4×10^8	3.0×10
	pUBSB35	3.0×10^8	2.9×10^8
	pUBSB19	3.0×10^8	2.7×10^8
	pUBSBΔAva125	5.1×10^8	2.8×10^8
	pUBSBΔBca1	2.0×10^8	2.6×10^3
	pUBSBΔBgc1	1.9×10^8	1.4×10^3
	pUBSBΔBgc3	4.6×10^8	2.0×10

^a Strain UOT0436 with or without pUBSB35 derivatives was incubated at 37°C to T₂₄ in Schaeffer sporulation medium (per liter; 8 g of Difco nutrient broth, 0.25 g of MgSO₄·7H₂O, 1 g of KCl, 10⁻⁶ M FeSO₄, 10⁻³ M Ca(NO₃)₂ and 10⁻⁵ M MnCl₂, pH 7.0) containing 5 μg of kanamycin/ml (in the case of the strain without plasmid, kanamycin was omitted). Viable cells were counted at T₅. Heat-resistant spores were counted at T₂₄ by plating the cells on a Nutrient broth (Difco) plate after heating the cells at 80°C for 10 min.

patible with that shown in Fig. 2, since these plasmids do not produce the intact *spo0B* gene product.

To clarify the cause of this phenomenon, we analyzed the character of Spo⁺ colonies obtained from UOT0436 (pUBSBΔBca1) and UOT0436 (pUBSBΔBgc1). All the Spo⁺ colonies examined produced spores at the wild type strain level (10^8 spores/ml, data not shown). They have *pheA1* and *recE4* markers indicating that they are not contaminants. Since the reversion frequency of Spo⁻ to Spo⁺ in UOT0436 is about 10⁻⁸, the Spo⁺ colonies are not revertants. Chromosomal DNA which is free from plasmid DNA and prepared from the Spo⁺ colonies transforms strain 1S16 to Spo⁺ at the same frequency as the wild type strain DNA (data not shown). From these results, we conclude that *recE4*-independent recombination occurred at a frequency of 10⁻⁵ between the chromosomal *spo0B136* locus and the *spo0B*⁺ gene on the plasmid. *RecE4*-independent recombination has been reported already in *B. subtilis*.^{24,25)}

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