Cloning of Sporulation Gene *spoIIC* in *Bacillus subtilis*

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Specialized transducing phages $\rho 11spoIIC$ and $\phi 105spoIIC$, carrying the *Bacillus subtilis* sporulation gene *spoIIC*, were constructed by the prophage transformation method. An *EcoRI* fragment (2.4 MDal) carrying the *spoIIC* gene was isolated from the $\phi 105spoIIC$ genome and recloned into the *EcoRI* site of plasmid pUB110. The recombinant plasmids corrected the sporulation defect of a $Spo^- Rec^-$ host, but slightly inhibited the sporulation of a $Spo^+ Rec^-$ host.

INTRODUCTION

Sporulation of *Bacillus subtilis* follows a distinctive series of morphological and physiological changes that are subjected to a temporally defined programme of gene expression (Piggot & Coote, 1976; Sonenshein & Campbell, 1978). Recently, several cloning systems have been described in *B. subtilis* and adapted to elucidate the mechanism of sporulation (Lovett, 1981). Previously, we reported the cloning of two early sporulation genes, *spoOB* (Hirochika *et al.*, 1981) and *spoOF* (Kawamura *et al.*, 1980), using the prophage transformation method developed by Kawamura *et al.* (1979, 1981). More recently, the *spoOC* (Ikeuchi *et al.*, 1983) and the *spoIIB* (Jenkinson & Mandelstam, 1983) genes were also cloned using this method. In this communication, we describe the cloning of another sporulation gene, *spoIIC*, of *B. subtilis* by this method. A 2.4 MDal *EcoRI* fragment carrying the *spoIIC* gene was recloned in both orientations into plasmid pUB110 and the effect of the recombinant plasmids on the sporulation of Spo^+ and Spo^- host strains was examined.

METHODS

Bacterial strains, phages and plasmid. A sporulation-defective strain 1S43 (*trpC2 spoIIC298*) was obtained from the Bacillus Genetic Stock Center (Ohio State University). The recombination-deficient strains 4305 (*metB5 nonB1 spoIIC298 recE4*) and 4309 (*metB5 nonB1 recE4*) were constructed from strains 4301 (*leuA8 metB5 nonB1 spoIIC298*) and 1012 (*leuA8 metB5 nonB1*), respectively, by transformation using strain GSY908 (*argC4 hisA1 recE4*) as donor. Temperate phages $\rho 11$ and $\phi 105$, and plasmid pUB110, were from our laboratory stock.

Preparation of phage and plasmid DNA. Phage DNA was prepared as previously described (Hirochika *et al.*, 1981). Plasmid DNA was prepared by the method of Ish-Horowicz & Burke (1981).

Transformation. Bacterial transformation was done by the method of Wilson & Bott (1968). For plasmid transformation, competent cells were also prepared by the method of Wilson & Bott (1968). The plasmid DNA was added to the competent cells and incubated at 37 °C for 30 min. Kanamycin-resistant transformants were selected by plating the cells on tryptose blood agar base (TBAB, Difco) plates containing 5 µg kanamycin ml⁻¹.

Growth and determination of sporulation frequency. The plasmid-bearing strains were inoculated into 5 ml of

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Schaeffer's sporulation medium (Schaeffer *et al.*, 1965) supplemented with 5 µg kanamycin ml⁻¹ (in the case of the strain without the plasmid, kanamycin was omitted) and incubated at 37 °C with shaking. Viable cells were counted by plating on NB plates (8 g Difco nutrient broth and 15 g agar l⁻¹) at t_5 (5 h after the end of exponential growth). Heat-resistant spores were counted at t_{24} by plating the cells on NB plates after they had been heated at 80 °C for 10 min.

Restriction enzyme analysis and DNA ligation. Digestion with restriction enzymes was done according to the vendor's instructions with minor modifications. Ligation was carried out as previously described (Hirochika *et al.*, 1981). Electrophoresis was done on 0.8% agarose or 5% (w/v) polyacrylamide gels.

Isolation of DNA fragments. To examine the transforming activity of restriction fragments of the transducing phage genome, each fragment was isolated from the gels according to the method of Kawamura *et al.* (1980). For isolation of the 2.4 MDal *EcoRI* fragment, the procedure of Shimotsu *et al.* (1983) was followed.

RESULTS AND DISCUSSION

The *EcoRI*-generated fragment of *B. subtilis* DNA which complements the *spoIIC298* mutation of strain 1S43 was cloned in a temperate phage, $\rho 11$, according to the prophage transformation method (Kawamura *et al.*, 1979) and the specialized transducing phage $\rho 11spoIIC$ was obtained. It had the same plaque-forming activity as the wild-type $\rho 11$ and could transduce strain 1S43 (*spoIIC298*) to Spo⁺ at a frequency of 10⁸ Spo⁺ transductants ml⁻¹. Strain 1S43 formed almost no spores (at a frequency of less than 10² spores ml⁻¹) without the transducing phage. The sporulation efficiency of the strain 1S43 lysogenic for $\rho 11spoIIC$ was comparable to that of the wild-type strain lysogenic for $\rho 11$ (10⁸ spores ml⁻¹), suggesting that the *spoIIC298* mutation is recessive to the wild-type allele and the cloned *spoIIC* gene functions normally in the lysogen.

Since $\rho 11spoIIC$ DNA was cut into about 30 fragments with *EcoRI*, it was difficult to identify which fragment was carrying the *spoIIC* gene. Therefore, we tried to subclone the *EcoRI* fragment carrying the *spoIIC* gene in $\rho 11spoIIC$ in a relatively small temperate phage $\phi 105$, whose DNA is cut into only 8 fragments by *EcoRI* digestion (Hirochika *et al.*, 1982). The cloning procedure was almost the same as that followed in the construction of $\rho 11spoIIC$, except that $\rho 11spoIIC$ DNA and $\phi 105$ DNA were used instead of PBSX DNA and $\rho 11$ DNA. After prophage transformation, the specialized transducing phage, $\phi 105spoIIC$, was obtained. This phage showed neither plaque-forming activity nor transducing activity by itself, but exhibited transducing activity when a helper phage ($\phi 105$) was added. These results were the same as those obtained with $\phi 105spoOB$ and $\phi 105spoOF$ (Hirochika *et al.*, 1982). Thus it is likely that the $\phi 105$ transducing phage obtained here may also lack its tail as demonstrated by Kawamura *et al.* (1980) and Jenkinson & Mandelstam (1983). Strain 1S43 lysogenic for $\phi 105spoIIC$ showed normal sporulation ability, as did the strain lysogenic for $\rho 11spoIIC$.

To identify the *EcoRI* fragment carrying the *spoIIC* gene in the $\phi 105spoIIC$ genome, we analysed *EcoRI* digests of $\phi 105spoIIC$ DNA by agarose gel electrophoresis and constructed the restriction map with *EcoRI*, referring to that of $\phi 105$, as described previously (Hirochika *et al.*, 1982). The result is shown in Fig. 1 (upper part). In the $\phi 105spoIIC$ genome, *EcoRI* fragments E, I, and G were absent, but a new *EcoRI* fragment (2.4 MDal) appeared. This fragment also existed in *EcoRI* fragments of $\rho 11spoIIC$ DNA, but not in those of $\rho 11$ DNA. Transformation experiments with the *EcoRI* fragments of $\phi 105spoIIC$ DNA showed that only the 2.4 MDal fragment has the *spoIIC*⁺ transforming activity. The fact that strain 1S43 lysogenic for either $\phi 105spoIIC$ or $\rho 11spoIIC$ sporulated normally suggests that the intact *spoIIC* gene may exist on the 2.4 MDal fragment. Subsequently, the restriction sites of *AvaI*, *SalI*, *BclI* and *HindIII* in the 2.4 MDal fragment were mapped with double digestion (Fig. 1, lower part). Transformation experiments with the resulting restriction fragments showed that the *spoIIC*⁺ transforming activity was found in the *SalI*-*EcoRI* fragment B and in the *AvaI*-*EcoRI* fragment A. It was also found in the *SalI*-*AvaI* double digestion fragment (see the underlined region in Fig. 1, lower part), indicating that the *spoIIC298* marker is located between the *SalI* and the *AvaI* sites on the 2.4 MDal fragment.

To examine the effect of copy number of the *spoIIC* gene on sporulation, we transferred the 2.4 MDal fragment into the multicopy plasmid pUB110. The 2.4 MDal *EcoRI* fragment and

Fig. 1. Restriction maps of phages $\phi 105$ and $\phi 105spoIIC$ (a) and the 2.4 MDal fragment (b). The *EcoRI* restriction map of $\phi 105$ is from Hirochika *et al.* (1982). In (a) the sizes of the fragments in MDal (shown in parentheses) were determined from their electrophoretic mobilities relative to λ *HindIII* fragments and $\phi 105$ *EcoRI* fragments. In (b) the sizes of the fragments in MDal were determined from their electrophoretic mobilities relative to λ *HindIII* fragments and ϕ X174 *HaeIII* fragments. The underlined region (*SalI*–*AvaI* double digestion fragment) indicates the location of the *spoIIC298* marker. On the scale of the 2.4 MDal fragment, single letters are used to denote the restriction sites.

Table 1. Sporulation of strains carrying *pSIIC1* and *pSIIC2*

Strain	Plasmid	No. of viable cells ml ⁻¹	No. of spores ml ⁻¹
4305 (<i>spoIIC298 recE4</i>)	None	1.70 × 10 ⁸	< 10
	pUB110	2.26 × 10 ⁸	< 10
	pSIIC1	1.29 × 10 ⁸	4.08 × 10 ⁷
	pSIIC2	1.85 × 10 ⁸	3.29 × 10 ⁷
4309 (<i>spo⁺ recE4</i>)	None	2.85 × 10 ⁸	2.76 × 10 ⁸
	pUB110	2.33 × 10 ⁸	2.16 × 10 ⁸
	pSIIC1	2.21 × 10 ⁸	5.95 × 10 ⁷
	pSIIC2	2.45 × 10 ⁸	5.90 × 10 ⁷

pUB110 DNA cleaved with *EcoRI* were ligated and used to transform strain 1012 to kanamycin-resistance (Km^r). The recombinant plasmids pSIIC1 and pSIIC2 obtained from Km^r transformants harbour the 2.4 MDal fragment inserted in opposite orientations. As shown in Table 1, the asporogenous strain 4305 (*spoIIC298 recE4*) carrying pSIIC1 or pSIIC2 recovered sporulation ability at a high frequency (more than 10⁷ spores ml⁻¹), suggesting that the *spoIIC* gene on the 2.4 MDal fragment has its own promoter.

In our previous report, a recombinant plasmid carrying the *spoOF* gene severely inhibited sporulation of the wild-type strain (the frequency was reduced from 2 × 10⁸ spores ml⁻¹ to between 10³ and 10⁴ spores ml⁻¹), but the plasmid carrying the *spoOB* gene did not (Kawamura *et al.*, 1981). To test whether the plasmids carrying the *spoIIC* gene exhibit such an inhibitory effect, pSIIC1 and pSIIC2 were transferred into the wild-type strain 4309 (*spo⁺ recE4*) and the sporulation ability of the transformants was examined. As shown in Table 1, both pSIIC1 and pSIIC2 caused a slight inhibition of sporulation (the frequency was reduced from 2 × 10⁸ spores ml⁻¹ to 6 × 10⁷ spores ml⁻¹). It is still unknown whether this is caused by the *spoIIC* gene itself

or by the region adjacent to the *spoIIC* gene. To clarify this point, further analysis is in progress using deletion plasmids.

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