Cloning of Sporulation Gene spoIC in Bacillus subtilis

By HIROYUKI ANAGUCHI,† SAKUZO FUKUI,2 HIDENORI SHIMOTSU,3 FUJIO KAWAMURA,3 HIUGA SAITO3 AND YASUO KOBAYASHI*1

1 Department of Applied Biochemistry, Hiroshima University, Fukuyama 720, Japan
2 Department of Fermentation Technology, Hiroshima University, Higashihiroshima 724, Japan
3 Institute of Applied Microbiology, University of Tokyo, Tokyo 113, Japan

(Received 12 October 1983; revised 29 November 1983)

Specialized transducing phages φl1spoIC and φ105spoIC, carrying the Bacillus subtilis sporulation gene spoIC, were constructed by the prophage transformation method. An EcoRI fragment (2.4 MDal) carrying the spoIC gene was isolated from the φ105spoIC 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 spoIIIB (Jenkinson & Mandelstam, 1983) genes were also cloned using this method. In this communication, we describe the cloning of another sporulation gene, spoIC, of B. subtilis by this method. A 2.4 MDal EcoRI fragment carrying the spoIC 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 spoIC298) was obtained from the Bacillus Genetic Stock Center (Ohio State University). The recombination-deficient strains 4305 (metB5 nonB1 spoIC298 recE4) and 4309 (metB5 nonB1 recE4) were constructed from strains 4301 (leuA8 metB5 nonB1 spoIC298) and 1012 (leuA8 metB5 nonB1), respectively, by transformation using strain GSY908 (argC4 hisA1 recE4) as donor. Temperate phages φl1 and φ105, 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
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 1⁻¹) at t₀ (5 h after the end of exponential growth). Heat-resistant spores were counted at t₁₄ 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, ρ11, according to the prophage transformation method (Kawamura et al., 1979) and the specialized transducing phage ρ11spoIIC was obtained. It had the same plaque-forming activity as the wild-type ρ11 and could transduce strain 1S43 (spoIIC298) to Spo⁺ at a frequency of 10⁶ 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 ρ11spoIIC was comparable to that of the wild-type strain lysogenic for ρ11 (10⁶ 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 ρ11spoIIC 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 ρ11spoIIC in a relatively small temperate phage ϕ105, 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 ρ11spoIIC, except that ρ11spoIIC DNA and ϕ105 DNA were used instead of PBSX DNA and ρ11 DNA. After prophage transformation, the specialized transducing phage, ϕ105spoIIC, was obtained. This phage showed neither plaque-forming activity nor transducing activity by itself, but exhibited transducing activity when a helper phage (410⁵) was added. These results were the same as those obtained with 410⁵spoOB and 410⁵spoOP (Hirochika et al., 1982). Thus it is likely that the ϕ105 transducing phage obtained here may also lack its tail as demonstrated by Kawamura et al. (1980) and Jenkinson & Mandelstam (1983). Strain 1S43 lysogenic for ϕ105spoIIC showed normal sporulation ability, as did the strain lysogenic for ρ11spoIIC.

To identify the EcoRI fragment carrying the spoIIC gene in the ϕ105spoIIC genome, we analysed EcoRI digests of ϕ105spoIIC DNA by agarose gel electrophoresis and constructed the restriction map with EcoRI, referring to that of ϕ105, as described previously (Hirochika et al., 1982). The result is shown in Fig. 1 (upper part). In the ϕ105spoIIC genome, EcoRI fragments F, I, and G were absent, but a new EcoRI fragment (2-4 MDal) appeared. This fragment also existed in EcoRI fragments of ρ11spoIIC DNA, but not in those of ρ11 DNA. Transformation experiments with the EcoRI fragments of ϕ105spoIIC DNA showed that only the 2-4 MDal fragment has the spoIIC⁺ transforming activity. The fact that strain 1S43 lysogenic for either ϕ105spoIIC or ρ11spoIIC 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
Cloning of the B. subtilis spoIIC gene

Fig. 1. Restriction maps of phages φ105 and φ105spoIC (a) and the 2.4 MDal fragment (b). The EcoRI restriction map of φ105 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 φ105 EcoRI fragments. In (b) the sizes of the fragments in MDal were determined from their electrophoretic mobilities relative to λ HindIII fragments and φX174 HaeIII fragments. The underlined region (SalI-AvaI double digestion fragment) indicates the location of the spoIC298 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>No. of viable cells ml⁻¹</th>
<th>No. of spores ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>4305 (spoIC298 recE4)</td>
<td>None</td>
<td>1.70 × 10⁸</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>pUB110</td>
<td>2.26 × 10⁸</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>pSIIC1</td>
<td>1.29 × 10⁸</td>
<td>4.08 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>pSIIC2</td>
<td>1.85 × 10⁸</td>
<td>3.29 × 10⁷</td>
</tr>
<tr>
<td>4309 (spo⁻ recE4)</td>
<td>None</td>
<td>2.85 × 10⁸</td>
<td>2.76 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>pUB110</td>
<td>2.33 × 10⁸</td>
<td>2.16 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>pSIIC1</td>
<td>2.21 × 10⁸</td>
<td>5.95 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>pSIIC2</td>
<td>2.45 × 10⁸</td>
<td>5.90 × 10⁷</td>
</tr>
</tbody>
</table>

pUB110 DNA cleaved with EcoRI were ligated and used to transform strain 1012 to kanamycin-resistance (Km'). The recombinant plasmids pSIIC1 and pSIIC2 obtained from Km' transformants harbour the 2.4 MDal fragment inserted in opposite orientations. As shown in Table 1, the asporogenous strain 4305 (spoIC298 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.

We thank the Bacillus Genetic Stock Center (D. H. Dean) for providing us with strain 1S43.

REFERENCES


