Cloning of an Unstable *spoIIA*–*tyrA* Fragment from *Bacillus subtilis*

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A recombinant cosmid clone was isolated from a library created from cosmid pQB79-1 and *Bacillus subtilis* DNA, and a 15 kb *Bam*HI fragment derived from the cloned insert was transferred to the vector pHV33. The recombinant clone, pRC12, was capable of complementing eight auxotrophic markers in the *spoIIA*–*tyrA* region of the *B. subtilis* chromosome (map positions 205–210). It also complemented eight of nine markers in the *spoIIA* locus. The exception, *spoIIA176*, is the most distal marker from lysine. Although pRC12 failed to complement sporulation defects in *spoVA* or *spoIVA* (*spoIIA*⁺) strains, subclones of pRC12, lacking a functional *spoIIA* gene, did complement these mutations. pRC12 inhibited sporulation in a *spo*⁺ *recE* strain, possibly due to the presence of multiple functional *spoIIA* genes. Both the original cosmid and pRC12 were unstable in *Escherichia coli* and *B. subtilis*. Antibiotic selection of the vector resulted in extensive deletion of the insert, while selection for insert function in *B. subtilis* invariably led to loss of the chloramphenicol resistance vector function.

INTRODUCTION

Considerable progress has been made in the cloning of *Bacillus subtilis* genes, particularly some of the genes involved in the sporulation process (Bonamy & Szulmajster, 1982; Ferrari *et al.*, 1981; Dubnau *et al.*, 1981; Losick, 1981; Jenkinson & Mandelstam, 1983). By screening banks for complementation of *lys-3* we hoped to obtain the closely linked *spoIIA* and *spoVA* loci (Piggot & Coote, 1976). A fragment which contains a part of the *spoIIA* locus has been isolated (Liu *et al.*, 1982) from a plasmid bank constructed by Rapoport *et al.* (1979). The results obtained by Liu *et al.* (1982) confirmed the complexity of the *spoIIA* locus suggested by previous mapping studies (Yudkin & Turley, 1981). *spoVA* was unique among the loci investigated by Lencastre & Piggot (1979) in that its expression in the forespore is apparently essential. Isolation of clones including these loci should facilitate investigation of the control of spore gene expression.

A search of existing genomic libraries (Hutchinson & Halvorson, 1980; Ferrari *et al.*, 1981; Rapoport *et al.*, 1979) did not reveal clones complementing *lys-3* or *spoIIA26*. Aubert *et al.* (1982) constructed a library of *B. subtilis* genes in the cosmid vector, pQB79-1, obtained by insertion of pC194 into the *Hind*III site of pHV79 and capable of replication in both *Escherichia coli* and *B. subtilis*. The advantages of cosmid banks for isolation of large genome segments with consequent simplification of screening are well known, as is their tendency to be unstable in *E. coli*. A copy of the Aubert cosmid library, kindly sent by Dr Rapoport, also lacked clones complementing *lys-3* and had clearly undergone extensive deletion during amplification. We decided to create our own cosmid bank in pQB79-1, and to screen it after a minimal number of amplification steps. Testing the bank for biological activity was simplified by the finding that lysates of chloroform-treated cells prepared from recombinant *E. coli* colonies could be used to transform strains of *B. subtilis* (unpublished observations).

Abbreviations: Ap, ampicillin; CAT, chloramphenicol acetyltransferase; Cm, chloramphenicol.

Table 1. *Strains and vectors used*

<i>B. subtilis</i> strains	Genotype	Origin*
68 ⁺	Prototroph	Our stock
IS30	<i>spoIIA12</i>	BGSC
IS86	<i>spoIIA1 trpC2</i>	BGSC
IS79	<i>spoIIA37 trpC2</i>	BGSC
IS80	<i>spoIIA42 trpC2</i>	BGSC
IS32	<i>spoIIA69 trpC2</i>	BGSC
IS77	<i>spoIIA50 thr-5 metB5</i>	BGSC
IS81	<i>spoIIA176 trpC2</i>	BGSC
RC 250	<i>lys-3</i>	R2 × 168 DNA
IS80 <i>recE</i>	<i>spoIIA42 recE</i>	(IS80 × BD224 <i>recE</i> DNA)
5098	<i>aroC7 trpC2</i>	Our stock
5140	<i>ser-22</i>	Our stock
5139	<i>rib</i>	R. Warburg
SB202	<i>trpC2 hisB2 aroB2 tyrA</i>	J. Pene
SB202 <i>recE</i>	<i>trpC2 hisB2 aroB2 tyrA recE4 rpoB</i>	(SB202 × CB2 <i>recE</i> DNA)
MY2016	<i>lys-1 hisB2 rpoB</i>	H. Jenkinson
IS50	<i>spoVA89 trpC2</i>	BGSC
IS46	<i>spoIVA178 pheA12</i>	BGSC
5129	<i>spoIVA67</i>	R. Warburg
BD224	<i>recE thr-5 trpC2</i>	D. Dubnau
CB2	<i>recE thr-5 trpC2 rpoB</i>	L. Sonnenschein
R2	<i>spoIIA26 lys-3 metB10</i>	D. Dean
R2 <i>recE</i>	<i>spoIIA26 lys-3 recE</i>	(R2 × BD224 <i>recE</i> DNA)
R3	<i>spoIIA4 lys-3 metB10</i>	D. Dean
<i>E. coli</i> strains	Genotype	Origin
HB101	<i>hsm hrs gal leuB recA</i>	P. Wensink
SK2881	F ⁻ <i>aroD6 leuC hsdM recA</i>	S. Kushner
Plasmids	Phenotype	Origin
pHV33	Ap ^R Cm ^R Tet ^R	G. Rapoport
pQB79-1	Ap ^R Cm ^R	G. Rapoport

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We report here the isolation of an unusual recombinant cosmid clone. The original cosmid and a 15 kb *B. subtilis* fragment subcloned from the cosmid into pHV33 (Primrose & Ehrlich, 1981) complemented many of the markers in the chromosomal region covering map positions 205–210 (*spoIIA–tyrA*) (Henner & Hoch, 1980) including almost all *spoIIA* markers. However, plasmid replication under antibiotic selection in *B. subtilis* or *E. coli* always resulted in extensive loss of insert, while selection for expression of the insert in *B. subtilis* always led to loss of the vector chloramphenicol resistance functions derived from pC194.

METHODS

Bacterial strains and vectors. The strains and vectors used during this study are listed in Table 1. For construction of most *recE* strains, DNA from *B. subtilis* BD224 *thr-5 trpC2 recE4* was used to transform a recipient strain to prototrophy for an appropriate marker. SB202 *recE* was constructed using DNA from strain CB2. Colonies were screened for sensitivity to 0.05 µg mitomycin C ml⁻¹ to determine the RecE phenotype.

Growth of bacteria. *Escherichia coli* and *B. subtilis* cells were grown on LB medium containing 5 g NaCl, 5 g yeast extract (Difco) and 10 g tryptone (Difco) l⁻¹. DSM medium contained (l⁻¹): nutrient broth (Difco), 8 g; MgSO₄, 0.25 g; KCl, 1 g; MnCl₂, 1.98 mg; FeSO₄, 0.03 mg; and CaCl₂, 111 mg. 2 × DSM contained 16 g nutrient broth but was otherwise identical to DSM. Auxotrophic markers were checked on minimal glucose medium (Bott & Wilson, 1968) supplemented with 2 g of appropriate amino acid assay medium l⁻¹ (Difco). LB-Ap medium contained 100 µg Ampicillin (Ap) ml⁻¹, and LB-Cm, 5 µg chloramphenicol (Cm) ml⁻¹.

Transformation. Cells of *E. coli* were made competent for DNA uptake as described by Wensink *et al.* (1974). Competent *B. subtilis* cells were prepared by the method of Bott & Wilson (1968). Transformants were selected on LB-Ap or LB-Cm plates, or on minimal assay plates. To test for transformation of Spo⁺ markers, 0.1 ml of the transformation suspension was inoculated into 1 ml 2 × DSM broth. The culture tubes were incubated at 37 °C

with aeration for 20 h, heated at 80 °C for 20 min and 0.1 ml plated on DSM plates. Spores survived the heat treatment and gave rise to sporogenous opaque pigmented colonies on DSM.

DNA extraction. Plasmid DNA from *E. coli* was prepared by the method of Wensink *et al.* (1974) and plasmid DNA from *B. subtilis* by the method of Tanaka & Sakaguchi (1978). Chromosomal DNA was prepared as described by Marmur (1961).

For determining the plasmid copy number, cells in 200 ml minimal glucose medium were labelled with [*methy*-³H]thymidine (13 Ci mm⁻¹; 481 GBq mm⁻¹; New England Nuclear) by the method of Lovett & Bramucci (1974). CsCl-ethidium bromide centrifugation was carried out at 100 000 g for 24 h. Fractions were collected, precipitated with 5% (w/w) trichloroacetic acid, and counted.

Enzymes and electrophoresis. Restriction enzymes and T4 ligase were purchased from either New England Biolabs or Boehringer Mannheim. Buffers were prepared according to the manufacturer's specification. DNA samples were hydrolysed for 30 min at 37 °C, heated at 69 °C for 3 min and chilled. Ligation was carried out overnight at 15 °C, unless otherwise stated. Samples were electrophoresed in horizontal gels of 0.8% agarose in Tris/acetate buffer (pH 7.8) (20 mM-Tris, 40 mM-sodium acetate, 1 mM-EDTA). Gels were stained with ethidium bromide and DNA bands were visualized with UV light and photographed. For extraction of DNA bands from gels, low melting agarose (SEA Plaque, Marine Colloids Div., FMC Corporation) was used and the bands excised after visualization with a 300 nm UV source (Fotodyne).

Construction of a cosmid colony bank of *B. subtilis* DNA in *E. coli*. (a) Chromosomal DNA (3.6 mg ml⁻¹) from *B. subtilis* 168 was prepared and partially cut with *Sau*3A (Biolabs) as described by Karn *et al.* (1980) using 50 µl aliquots of DNA (180 µg) with 0.4, 0.8, 2, 4 or 8 units of enzyme at 37 °C for 30 min. After heat inactivation (65 °C for 10 min) the DNA solutions were mixed, extracted once with phenol (saturated with 10 mM-Tris/HCl pH 8) and then with ether; sodium acetate was added to 0.3 M and the DNA precipitated with ethanol. The resulting DNA was size fractionated through four consecutive sucrose gradients (15–30%) and fractions with a molecular weight of 35–40 kb taken, precipitated with ethanol and resuspended in 10 mM-Tris, 1 mM-EDTA (TE) to 400 µg ml⁻¹.

(b) Amplified cosmid pQB79-1 (Aubert *et al.*, 1982) DNA was prepared and purified by isopycnic centrifugation through CsCl. 20 µg was digested to completion with 20 units of *Bam*HI (BRL) for 1 h at 37 °C and, following the methods recommended by BRL, treated with bacterial alkaline phosphatase (BRL; 680 units) for 1 h at 65 °C. The phosphatase was destroyed by hydrolysis with proteinase K (20 µg) for 10 min at 37 °C and the solution extracted twice with Tris-saturated phenol and once with ether. The DNA was precipitated twice with ethanol and finally resuspended in TE to 200 µg ml⁻¹. Without phosphatase treatment, very few of the Ap^R colonies resulting from ligation contained inserted DNA.

(c) Ligation: 5 µl vector DNA (1 µg) was mixed with 1 µl chromosomal DNA (0.4 µg) and 0.7 µl 10 × ligase buffer containing 100 cohesive end units of ligase (Collaborative Research) and incubated at 4 °C for 18 h. It was then heated to 70 °C for 10 min and cooled on ice.

(d) Packaging: 2.5 µl aliquots of ligated DNA were packaged as described by Hohn & Murray (1977) and 100 µl aliquots of the resulting phage used to transfect 2 × 10⁸ cells of *E. coli* strain SK2881 grown in LB containing 4 mg maltose ml⁻¹ and 10 mM-MgSO₄. After 30 min incubation at 37 °C in LB broth, the cells were spread on LB-Ap plates and incubated at 37 °C for 18 h. Individual colonies were grown for 18 h in 5 ml LB-Ap broth; cosmid DNA was prepared from 1.5 ml of this culture as described by Holmes & Quigley (1981) and analysed on minigels. Many of the colonies tested contained cosmids with inserts greater than 30 kb.

(e) Preparation of lysates: three drops of chloroform were added to the residual 3.5 ml of the above cultures and vortexed. The chloroform-treated cultures were stored at 4 °C and represented a crude preparation of DNA. 5 µl of such a solution contained a saturating level of DNA in transformation experiments with competent *E. coli* or *B. subtilis* cells. These lysates contain both cosmid and chromosomal DNA, however, DNA similarly prepared from cells of *E. coli* SK2881 was not able to transform competent cells of either *E. coli* SK2881 or *B. subtilis* for any of the markers tested. Hence assay of these lysates for transforming activity provided a simple method of screening the bank for the markers of interest; the solutions were active over a period of at least two months.

RESULTS

Lysate of cosmid clone 12 contains the lys-3 and spoIIA26 genes

The cosmid bank, isolated under ampicillin selection in *E. coli* SK2881, consisted of 84 colonies. These were grown in LB-Ap broth (5 ml) as individuals and in 7 pools of 12 colonies. Each culture was used to make a chloroform lysate (3.5 ml) and a plasmid preparation (from 1.5 ml). Individual lysates were tested for their ability to transform *B. subtilis* strain R2 (*lys-3 spoIIA26*) to lysine prototrophy. 25 µl of pool 1 (colonies 1–12) yielded about 20 Lys⁺ colonies and the individual lysate 12 (25 µl) yielded about 10³ Lys⁺ colonies. Within 72 h at 37 °C,

Table 2. Transformation of *B. subtilis* strains R2⁺ and R2 *recE* with lysate 12 DNA

Recipient Strain	No. transformants ml ⁻¹ with selection:*		
	Lys-3 ⁺	SpoIIA26 ⁺ †	Cm ^R
R2	2.1 × 10 ³ (60% Spo ⁺) (0% Cm ^R)	9 × 10 ² (100% Lys ⁺) (0% Cm ^R)	5.5 × 10 ³ (0% Lys ⁺) (0% Spo ⁺)
R2 <i>recE</i>	0	4.9 × 10 ² (0% Lys ⁺) (0% Cm ^R)	1 × 10 ³ (0% Lys ⁺) (0% Spo ⁺)

* Numbers in parentheses refer to results of screening of selected transformants by replica plating.

† Estimate of transforming activity of heat selected Spo⁺ cells.

approximately 60% of the Lys⁺ colonies produced pigment and a large number of phase-bright spores (Table 2). *spoIIA26* had a reversion frequency of less than 10⁻⁸.

Lysates of the seven pools were also tested for their ability to transform cells of strain R2 to Spo⁺ by selection for heat resistance following growth in DSM at 37 °C for 18 h. Only approximate estimates of transforming activity can be derived by this procedure. However, with stable mutations such as *spoIIA26* the results were unambiguous. Pool 1 and lysate 12 again gave Spo⁺ transformants. All were Lys⁺ (Table 2).

When the recombination-deficient strain R2 *recE* was used as the recipient, Spo⁺ heat-resistant colonies were still produced, but no Lys⁺ prototrophs were isolated (Table 2). Since complementation of the *recE* recipient presumably requires an intact, functional plasmid-borne gene, *spoIIA26* is intact but *lys-3* is not.

Segregation of antibiotic resistance

None of the Spo⁺ transformants of strain R2 *recE* nor any of the Spo⁺ or Lys⁺ transformants of strain R2 were chloramphenicol-resistant (Table 2). It appeared as if the transformants of these strains had lost the functional chloramphenicol acetyltransferase (CAT) gene normally present in the pC194 segment of pQB79-1. Lysate 12 transformed *E. coli* SK2881 to Cm^R or Ap^R or *B. subtilis* strains R2 and R2 *recE* to Cm^R but none of the *B. subtilis* transformants were either Spo⁺ or Lys⁺ (Table 2). No Spo⁺ Cm^R transformants of R2 *recE* were obtained by simultaneous selection, consistent with the apparent loss of a functional plasmid CAT gene in transformants selected for the *spoIIA26* marker.

Construction of pRC12

Attempts at isolating plasmid DNA from *recE* Spo⁺ transformants gave only a single band of chromosomal DNA which suggested a very low plasmid copy number. To allow chloramphenicol selection of plasmid DNA carrying the *lys-3* and *spoIIA26* markers, cosmid 12 DNA was cut with *Bam*HI and ligated to *Bam*HI-cut DNA of pHV33. Following transformation of strain R2 *recE*, growth in DSM containing chloramphenicol and selection for heat resistance, a single Cm^R Spo⁺ colony was isolated. Plasmid DNA isolated from this strain, following digestion with *Bam*HI, showed a large amount of the pHV33 vector and a small amount of a 15 kb insert (Fig. 1).

This recombinant plasmid, pRC12, was separated from pHV33 by electrophoresis in low melting point agarose. The excised top band (Fig. 1, lane 3) was eluted from the melted gel and used to transform cells of strain R2 and R2 *recE*. The results were identical to those obtained with cosmid 12 DNA (Table 2); no Cm^R Lys⁺ or Cm^R Spo⁺ colonies could be isolated. Initial success in selection of the Cm^R Spo⁺ transformant yielding pRC12 was presumably due to co-transformation by pHV33. Once again, the cloned insert appeared to have segregated from the vector CAT gene.

Fig. 1. Electrophoretic pattern of pRC12 DNA. CsCl-ethidium bromide-purified pRC12, plasmid vector, pHV33 and cosmid vector pQB79-1 DNA were digested with *Bam*HI and analysed by agarose gel electrophoresis. Lane 1, pHV33; lane 2, pHV33 digested with *Bam*HI; lane 3, pRC12; lane 4, pRC12 cut with *Bam*HI; lane 5, cosmid pQB79-1; lane 6, pQB79-1 digested with *Bam*HI; lane 7, molecular size markers – lambda DNA digested with *Hind*III.

Copy number of pRC12

pRC12 could only be selected in strain R2 *recE* by complementation of *spoIIA26*, and its apparent low copy number could be a consequence of a gene dosage effect on expression of this locus. We therefore measured the plasmid to total DNA ratios in *Spo*⁺ transformants of R2 *recE* (pRC12) labelled with [*methyl*-³H]thymidine. Two peaks of DNA were observed. The major peak contained chromosomal DNA capable of transforming only *Rec*⁺ strains for a variety of auxotrophic markers. The minor peak transformed cells of R2 to *Lys*⁺ and *Spo*⁺ and R2 *recE* to *Spo*⁺ only. It contained approximately 1% of the radioactivity of the total DNA extracted from exponential phase cells. When an aliquot of the same culture was grown for an additional 12 h into stationary growth phase, only 0.6% of the radioactivity remained in the plasmid fraction. The size of pRC12 was estimated as 22 kb (Fig. 1). Using the value of 3000 kb for the *B. subtilis* chromosome (Kavenoff, 1972) the copy number of pRC12 appeared to be about one per cell. As the transformed cells replicated in the absence of selective pressure, the chimeric plasmid decreased to less than one copy per cell.

Genetic markers carried by pRC12

pRC12 and the original cosmid 12 lysate were tested for genetic markers adjacent to *spoIIA* and *lys-3*. The *spoIIA* locus (Piggot & Coote, 1976) contains a number of mutations which may lie within two genes (Liu *et al.*, 1982). Several of these have been mapped recently (Yudkin & Turley, 1981; Lazdin, 1980) (Fig. 2). pRC12 DNA transformed all the mutations with the exception of *spoIIA176*. *spoIIA63* was not tested. *Spo*⁺ transformants were obtained from *spoIIA42* but not from a *spoIIA42 recE* strain. Duplicate transformation experiments carried out with a *lys-3 spo*⁺ recipient strain using 1 µg DNA ml⁻¹ isolated from strains 1S30 (*IIA12*), 1S77 (*IIA50*), and 1S81 (*IIA176*) indicated that *IIA176* is less closely linked to *lys-3* (41%) than either *IIA12* (57%) or *IIA50* (56%). This suggested that *spoIIA176* maps to the left of *spoIIA50* (Fig. 2), further from *lys* than any known *spoIIA* marker, apparently in a region not complemented by pRC12.

Fig. 2. Genetic characterization of pRC12 and its derivatives. ●, Positive in both Rec⁺ and recE recipients; ○, positive in Rec⁺, negative in recE recipients; ■, positive, tested only in Rec⁺ recipients; □, negative in Rec⁺ recipients.

Table 3. Transformation using pRC12 and chromosomal DNA

Recipient strains	Selection	No. transformants ml ⁻¹ per µg DNA preparation:*	
		pRC12	168
R2	<i>spoIIA26</i>	2000	2500
	<i>lys-3</i>	3000	2900
R2 <i>recE</i>	<i>spoIIA26</i>	600	0
	<i>lys-3</i>	0	0
IS30	<i>spoIIA12</i>	1820	4100
R3	<i>spoIIA4</i>	500	12000
IS86	<i>spoIIA1</i>	410	9500
IS79	<i>spoIIA37</i>	300	8000
IS80	<i>spoIIA42</i>	790	3800
IS32	<i>spoIIA69</i>	1000	6000
IS77	<i>spoIIA50</i>	300	600
IS81	<i>spoIIA176</i>	0	300
MY2016	<i>lys-1</i>	25000	25000
5139	<i>rib</i>	30000	30000
5098	<i>aroC7</i>	160	2500
5140	<i>ser-22</i>	100	3500
SB202	<i>aroB2</i>	3000	14000
	<i>trpC2</i>	800	12000
	<i>hisB2</i>	8000	10000
	<i>tyrA</i>	8000	10000
	<i>aroB2</i>	0	0
SB202 <i>recE</i>	<i>trpC2</i>	400	0
	<i>hisB2</i>	0	0
	<i>tyrA</i>	0	0
	<i>thr-5</i>	0	0
BD224 <i>recE</i>	<i>met</i>	0	25000

* The numbers of transformants are well above the background of spontaneous reversion, which has been subtracted.

pRC12 transformed strain MY2016 *lys-1* to Lys⁺, as well as the following auxotrophic mutations to prototrophy in Rec⁺ backgrounds: *rib*, *aroC*, *ser-22*, *aroB2*, *trpC2*, *hisB2*, and *tyrA* (Fig. 2, Table 3). No markers outside of the *spoIIA-tyrA* region were transformed with the DNA (Table 3) indicating that transforming activity was not due to contaminating chromosomal DNA. In *recE* backgrounds, *trpC2* but not *lys-3*, *tyrA*, *aroB2*, or *hisB2* were complemented (Table 3). Thus, *trpC2* was the only auxotrophic marker tested which expressed on the plasmid.

Antibiotic selection of pRC12 leads to loss of insert and biological activity

Lysate 12 or pRC12 DNAs were transformed into *E. coli* HB101 and *B. subtilis* strains and the transformed cells selected with either ampicillin or chloramphenicol. The molecular species isolated (pRC12-1, -2, etc.) were smaller and had lost complementation activity for most of the genetic markers shown in Fig. 2. pRC12-1 isolated from *B. subtilis* under Cm^R selection, retained transforming activity for *hisB2* (Fig. 2). pRC12-2, isolated from *E. coli* under Ap^R selection, also retained transforming activity only for the *hisB2* auxotrophic marker (Fig. 2). pRC12-3, isolated from *B. subtilis* under Cm^R selection, retained transforming activity for *trpC2*, *hisB2*, and *tyrA* and still complemented only *trpC2* in strain SB202 *recE* (Fig. 2). The Trp⁺ transformants of this strain were all Cm^S. The smallest of these deleted plasmids, pRC12-4, obtained from *B. subtilis* (Cm^R selection) appeared to be of the same size as pHV33 (Fig. 3). pRC12-4 DNA transformed strain SB202 *rec*⁺ producing approximately 100 Trp⁺, His⁺ or Tyr⁺ colonies ml⁻¹ (µg DNA)⁻¹.

pRC12-5, a 10 kb molecule (Fig. 3) also obtained from *B. subtilis* under Cm^R selection, lacked all markers in the *aroC-tyrA* region, but retained the ability to correct *lys-1* and *lys-3* only in Rec⁺ strains (Fig. 2). pRC12-5 DNA also corrected the sporulation defects in Rec⁺ strains caused by mutations *spoIIA12*, *spoIIA26*, and *spoIIA4* in the *spoIIA* locus, but, unlike pRC12

Fig. 3. Electrophoretic pattern of pRC12-4 and pRC12-5 DNA. CsCl-ethidium bromide-purified DNAs of subclones pRC12-4, pRC12-5 and pHV33 were digested with *EcoRI* and *HindIII* and analysed by agarose gel electrophoresis. Lane 1, lambda DNA digested with *HindIII*; lane 2, pRC12-5; lane 3, pRC12-4; lane 4, pHV33; lane 5, pRC12-5 cut with *EcoRI*; lane 6, pRC12-4 cut with *EcoRI*; lane 7, pHV33 cut with *EcoRI*; lane 8, pRC12-5 cut with *HindIII*; lane 9, pRC12-4 cut with *HindIII*; lane 10, pHV33 cut with *HindIII*; lane 11, lambda DNA cut with *HindIII* (molecular size markers).

DNA, no longer complemented *spoIIA1*, *spoIIA37*, *spoIIA42*, or *spoIIA50* (Fig. 2). pRC12-5 also failed to transform R2 *recE* to *Spo*⁺ suggesting that this subclone no longer carried a functional *spoIIA* gene.

pRC12-5 carries an intact spoVA89 gene; multiple functional spoIIA genes may prevent sporulation

Both *spoVA89* and *spoVA89 recE* strains were transformed to *Spo*⁺ by pRC12-5 DNA. All transformants were *Cm*^S. In contrast, neither lysate 12 nor pRC12 would transform either strain to *Spo*⁺. Since pRC12-5 was derived from pRC12, either the latter contained a cryptic *spoVA89* gene, only expressed following deletion to produce pRC12-5, or an intact *spoVA89* gene was acquired during production of pRC12-5 by replication of pRC12 in *Spo*⁺ *Rec*⁺ *B. subtilis* cells. If multiple functional *spoIIA* locus genes suppress sporulation, then loss of such functional genes during isolation of pRC12-5 might be responsible for unmasking a functional plasmid *spoVA* gene. To test this possibility, pRC12 was transformed into SB202 *recE* (a *Spo*⁺ strain). *Trp*⁺ transformants were obtained [200 (μg DNA)⁻¹]. All were *Cm*^S and 78% were *Spo*⁻.

pRC12-2 and pRC12-3 carry spoIVA (178 and 67)

Because the functional *spoIIA* gene on pRC12 might also mask complementation of the *spoIVA* locus located near *aroB2* (Fig. 2) we tested subclones of pRC12, which were deleted for *spoIIA* but retained ability to complement auxotrophic markers adjacent to *spoIVA*, for ability to complement *spoIVA* markers. Although pRC12-1 and pRC12-4 lacked such activity, pRC12-2 and pRC12-3 DNAs transformed *Rec*⁺ but not *recE* strains of *spoIVA178* and *spoIVA67* to *Spo*⁺ (Fig. 2). As with *spoVA89*, no such transforming activity could be detected in lysate 12 or pRC12 DNAs.

DISCUSSION

Aubert *et al.* (1982) found that recombinant clones in vector pQB79-1 yielded chiefly *Cm*^S transformants of *Rec*⁺ *B. subtilis* strains if the selection was for an auxotrophic marker, but yielded *Cm*^R transformants of *recE* strains regardless of the selective procedure. Similarly Liu *et*

al. (1982) found that pHM-2, carrying part of the *spoIIA* locus in pHV33, was stable in *recE* strains under selection for Cm^R, so that Cm^R transformants of *spoIIA50*, *spoIIA69*, or *spoIIA42* retained the *spoIIA* defect in the chromosome but were phenotypically Spo⁺ because of expression of the plasmid-encoded *spoIIA* gene.

Our finding that lysate 12 produced only Spo⁺ Cm^S transformants of strain R2 *recE* suggested that the CAT gene on the pC194 component of pQB79-1 had been damaged during construction or replication of the cosmid bank. However, transformants of strain R2 *recE* by pRC12, derived by transfer of a 15 kb portion of the cosmid 12 insert into pHV33, again fell into two classes: Cm^R colonies lacking insert or Spo⁺ colonies which were Cm^S. In addition, all Trp⁺ transformants of strain SB202 *recE* were also Cm^S, as were all transformants of the Rec⁺ strains carrying the auxotrophic markers indicated in Fig. 2.

The subclones resulting from the deletion of pRC12 isolated under Cm^R selection in *E. coli* or *B. subtilis* respectively, behaved similarly: selection for sporulation or auxotrophic markers gave only Cm^S transformants. The reason for the instability of vector CAT gene expression when selecting for expression of insert is not known.

Dean (1984) mentioned that nucleotide sequences near *lys* might be deleterious in high copy number. The *lys* gene has been cloned in bacteriophage vectors (Yamare *et al.*, 1982; Jenkinson & Mandelstam, 1983), and more than one functional copy of this gene for *meso*-diaminopimelate decarboxylase might lead to a lethal imbalance in cell wall biosynthesis. However, the isolation of deleted pRC12 molecules, such as pRC12-3 which cannot complement either *lys-3* or *lys-1* and behave like pRC12, renders this possibility unlikely. The suggestion that the basic instability of pRC12 results from its size (22 kb) may account for the appearance of partially deleted plasmids which carry small inserts but does not explain why these, like pRC12, cannot be amplified by Cm selection.

The markers in the *spoIIA-tyrA* region complemented by pRC12 cover a region estimated, by recombination frequency, to be at least 40–50 kb (Henner & Hoch, 1980). One of us (R. Warburg, unpublished observations) has isolated a 15 kb fragment of contiguous *B. subtilis* DNA, carrying *aroC* and *ser-22*, but lacking any of the adjacent markers indicated in Fig. 2. In addition, only the *spoIIA26*, *trpC2* and possibly the *spoVA89* genes are known to be functionally intact on pRC12, while *hisB2*, *tyrA*, *lys-3*, *lys-1*, and *aroB2* fail to express in *recE* strains. Extensive multiple deletion events may have occurred during replication of cosmid 12 in *E. coli*, which might account for the plasmid's basic instability.

The properties of pHM2, a plasmid carrying the *spoIIA* markers 50 to 42 (Liu *et al.*, 1982), strongly suggested the presence of at least two genes in this locus, with a boundary between markers 42 and 37. The ability of pRC12 to complement all of the known *spoIIA* markers except *spoIIA176*, and its ability to transform *spoIIA26 recE* but not *spoIIA42 recE* indicated that pRC12 contained at least one functional *spoIIA* gene absent in pHM2.

Cloned *spo* markers in phage or plasmid vectors transformed into Spo⁻ *recE* host cells allows analysis of dominance-recessive relationships and tests the effect of dosage of normal genes on sporulation phenotype. To date, mutations in all *B. subtilis spo* loci tested are recessive, including *spoOH* (Dubnau *et al.*, 1981), *spoOB*, *spoOF* (Kawamura *et al.*, 1981; Bonamy & Szulmajster, 1982), *spoVG*, *spoVC* (Losick, 1981), *spoIIA* (Liu *et al.*, 1982) and *spoIIIB* (Jenkinson & Mandelstam, 1983). Our data indicate that chromosomal mutations in the genes defined by *spoIIA26* and *spoVA89* are also recessive to plasmid-encoded normal genes. While multiple copies of many sporulation genes seem to be compatible with sporulation (Kawamura *et al.*, 1981; Bonamy & Szulmajster, 1982), the detrimental effect (on normal sporulation) of multiple *spoOF* genes (Kawamura *et al.*, 1981) and an amplified sporulation promoter (Banner, 1980) has been described. The suppression of sporulation of strain SB202 *recE* observed with pRC12 DNA is consistent with the finding that only subclones of pRC12 which lacked a functional *spoIIA* gene were capable of transforming strains of *spoVA* and *spoIVA* to Spo⁺. Possibly, multiple copies of this region of the *spoIIA* locus titrate essential sporulation regulatory proteins or lead to overproduction of critical sporulation gene products (Lovett, 1981).

Attempts to isolate sufficient amounts of purified and intact pRC12 DNA for physical and chemical studies were unsuccessful. The partially deleted molecules such as pRC12-5 are

currently being used as probes with our genomic libraries in attempts to isolate recombinant clones with stable properties in the *lys spoIIA-spoIVA* region.

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