Molecular Cloning of a *Bacillus subtilis* Gene Involved in Spore Outgrowth

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A λ Charon 4A derivative carrying the *outB* gene of *Bacillus subtilis* has been identified by transformation of a *B. subtilis* mutant temperature-sensitive in spore outgrowth. The cloned region is a single *EcoRI* fragment 14 kb in length. In addition to *outB*, the cloned DNA includes at least part of the *amyE* and *aroI* loci.

INTRODUCTION

The process of bacterial endospore germination and outgrowth consists of an ordered sequence of events (Setlow, 1981; Albertini & Galizzi, 1982). Mutants blocked at different stages of this sequence have been isolated in *Bacillus subtilis* and the mutations have been mapped on the chromosome (Piggot *et al.*, 1981). The molecular cloning of some of the genes involved in the process may help in understanding the role of the different genes and should make it possible to study their expression and regulation at the molecular level. Moir (1983) isolated a λ clone carrying the *gerA* locus. Here we report the isolation of the *outB* gene and its surrounding region from the λ Charon 4A library of Ferrari *et al.* (1981) and physically characterize the λ recombinant clone.

METHODS

Bacterial strains and media. The bacteria used are listed in Table 1. *B. subtilis* strains were cultured in nutrient broth (Difco) or the minimal medium of Spizizen (1958) for the preparation of competent cells. Minimal agar for selection of transformants was that of Davis & Mingioli (1950). Testing for Amy⁺ was done by plating on nutrient agar (Difco nutrient broth solidified with agar, 10 g l⁻¹) plates plus soluble starch (1%, w/v). The plates were stained with a solution of I₂ (1%, w/v) and KI (2%, w/v) to identify Amy⁺ colonies, which were surrounded by a halo.

E. coli DP50 *supF* was used as a host for λ Charon 4A (λ Ch4A) and its recombinant derivatives. The nutrient medium used was modified LB comprising: Bacto-tryptone (Difco), 10 g; yeast extract, 5 g; NaCl, 5 g; MgCl₂, 2 g; diaminopimelic acid, 100 mg; thymidine, 50 mg; water, to 1 litre; pH 7.1. LB was solidified with 12 g agar or 10 g agarose l⁻¹. For phage plating the top layer was either 4 g agar or 5 g agarose l⁻¹. *E. coli* HB101 was used for transformation and plasmid propagation in LB medium (Bacto-tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; water, to 1 litre; pH 7.1).

Phage and plasmid preparation. The library of *B. subtilis* DNA in λ Ch4A (Ferrari *et al.*, 1981) was used. A plate lysate on agarose medium was prepared from each pool. The phage was harvested by centrifugation (75 min at 25000 r.p.m. in a Sorvall AH-627 swinging-bucket rotor), resuspended in 20 mM-Tris/HCl (pH 7.5) plus 25 mM-EDTA and the DNA extracted with phenol. The DNA was used to transform competent cells of the mutant strain PB2427, which is temperature sensitive during spore outgrowth, selecting for the ability of the spores to yield colonies at 47 °C (Ts⁺) (Galizzi *et al.*, 1973). Two hundred single plaques were separately collected from pool G (which consistently gave positive results), suspended in 50 μ l 10 mM-Tris/HCl (pH 7.5) plus 10 mM-MgSO₄ and

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Table 1. *Strains of Bacillus subtilis and Escherichia coli*

Strain	Genotype	Source
<i>B. subtilis</i>		
PB19	(prototroph)	SB19, Stanford Collection
PB1715	<i>metB5 dal aro1906 sacA321</i>	1A55, BGSC
PB2353	<i>thyA thyB arg tscG47</i>	Galizzi <i>et al.</i> (1976)
PB2354	<i>thyA thyB arg tscH48</i>	Galizzi <i>et al.</i> (1976)
PB2355	<i>thyA thyB arg tscB49</i>	Galizzi <i>et al.</i> (1976)
PB2427	<i>hisH2 trpC2 outB81</i>	Albertini & Galizzi (1975)
PB2455	<i>aro1906 dal outB81</i>	
PB5009	<i>aro1906 metB5 sacA321 amyE</i>	1A289, BGSC*
<i>E. coli</i>		
DP50supF	F ⁻ <i>tonA53 dapD8 lacY1 supE44</i> Δ (<i>gal-uvrB</i>)47 <i>gyrA29</i> Δ (<i>thyA57</i>) <i>hsd53</i>	Leder <i>et al.</i> (1977)
HB101	F ⁻ <i>hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20</i> <i>xyt-5 mtl-1 supE44</i>	Boyer & Roulland-Dussoix (1969)

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replated to give confluent lysis on small Petri dishes (5 cm diameter) containing agarose medium. The phage was collected from each plate and DNA extracted by a fast procedure (Davis *et al.*, 1980). Lysates were prepared from liquid cultures (1 l), the phage collected by polyethylene glycol precipitation and purified by CsCl density gradient centrifugation, and the DNA extracted with phenol.

Plasmids were prepared by the procedure of Birnboim & Doly (1979). They were constructed by ligation of approximately equimolar amounts of gel-purified fragments. The extent of the *B. subtilis* DNA insert in each plasmid is shown in Fig. 4. Plasmid pO.EH contains a 5 kb fragment of *B. subtilis* DNA inserted into pBR325 (Bolivar, 1978), digested with *EcoRI* and *HindIII*. The *B. subtilis* fragment was obtained by digestion with *EcoRI* and *HindIII* of the entire λ C4BsG40 DNA. The 5 kb fragment was purified by agarose gel electrophoresis before ligation. Transformants of HB101 resistant to 100 μ g ampicillin ml⁻¹ were selected and screened for sensitivity to chloramphenicol (25 μ g ml⁻¹). Plasmid pO.SP is a derivative of pBR322. The *B. subtilis* DNA insert is of 7.7 kb and was obtained from λ C4BsG40 by digestion with *EcoRI* and *SalI*. The protruding ends were filled in with the Klenow fragment of DNA polymerase and the insert further digested with *PstI*. The *PstI*-*SalI* (blunt-ended) fragment was ligated to the pBR322 vector digested with *EcoRI*, whose ends were filled as above and then digested with *PstI*. Transformants of HB101 resistant to tetracycline (12.5 μ g ml⁻¹) were screened for sensitivity to ampicillin. To construct pO.P, a 3.6 kb fragment obtained by digestion with *PstI* of the purified 14 kb insert of λ C4BsG40 was ligated with *PstI*-digested and phosphatase-treated pBR322 DNA. The purified 14 kb insert of λ C4BsG40 digested with *PstI* was also the source of the 2.8 kb fragment ligated to pBR322 (digested with *PstI* and *EcoRI*) to give pO.PE. Plasmid pO.HH was constructed from pBR322. The 3.5 kb *HindIII* fragment was derived from λ C4BsG40 and ligated to *HindIII*-digested and phosphatase-treated pBR322 DNA. Ampicillin-resistant transformants were screened for tetracycline sensitivity. The structure of each plasmid was confirmed by restriction analysis.

Transformation. The calcium shock procedure of Dagert & Ehrlich (1979) was used for *E. coli* transformations. *B. subtilis* strains were transformed by the method of Anagnostopoulos & Spizizen (1961).

Restriction enzyme mapping. DNA samples were digested with restriction endonucleases and fragments separated by electrophoresis in 0.7% or 1.2% (w/v) agarose slab gels with Tris/acetate buffer (Cohen *et al.*, 1973). For fragments of low molecular weight, 5% (w/v) polyacrylamide gels were used. *EcoRI*-digested phage SPP1 DNA was included in each gel as a size standard (Ratcliff *et al.*, 1979). All enzymes were obtained from commercial suppliers and used under conditions recommended by the suppliers.

Isolation of a deletion mutant of phage λ C4BsG40. A suspension of λ C4BsG40 was prepared starting from a single plaque, suspended and plated to confluent lysis as described above. To 2 ml of lysate (2×10^{10} p.f.u. ml⁻¹) was added 0.1 vol. 30% (w/v) trisodium citrate monohydrate, and the mixture was incubated at 55 °C for 1 h, diluted and then plated (Parkinson & Huskey, 1971). The phage was collected from confluent lysis plates and the cycle repeated. One hundred single plaques were collected, two successive single-plaque re-isolations were made from each and single plaques of each were used to produce confluent lysis on agarose plates from which DNA was extracted, digested with *EcoRI* and analysed by agarose gel electrophoresis.

Electron microscopy of DNA molecules. Denaturation mix contained 5 μ g of each λ phage DNA ml⁻¹ and 0.1 M-NaOH, 0.02 M-EDTA. After incubation at room temperature for 10 min, the samples were neutralized and reannealed as described by Davis *et al.* (1971). The DNA was spread from a hypophase containing 20% (v/v)

Fig. 1

Fig. 1. Agarose gel (0.7%) electrophoresis of *Eco*RI-restricted DNA. Lane 1, DNA of λ C4BsG40; lane 2, DNA of vector λ Ch4A; lane 3, DNA of phage SPP1. The numbers indicate the sizes (kb) of the phage SPP1 fragments. The two internal *Eco*RI fragments of 6.5 and 7.5 kb present in λ Ch4A DNA are replaced by the 14 kb fragment of λ C4BsG40 (arrow).

Fig. 2

Fig. 2. Relationship between DNA concentration and frequency of transformation: Aro⁺ transformants of strain PB1715, donor PB19 (○) and λ C4BsG40 (●); Ts⁺ transformants of strain PB2427, donor λ C4BsG40 (△). The values on the ordinate are the numbers of transformants per recipient cell.

formamide and a hyperphase containing 50% formamide and stained with uranyl acetate. Reference molecules were renatured λ Ch4A DNA and ϕ X174 single-stranded circles. The molecules were visualized using a Philips EM301 electron microscope and photographed, and contour lengths were projected, traced and measured with an Apple II PC equipped with a digitizer.

RESULTS AND DISCUSSION

Isolation of the *outB* region

Our purpose was to clone one of the genes involved in *B. subtilis* spore outgrowth. For this we used mutant PB2427 (*outB*81) which is temperature-sensitive (Ts) for outgrowth, as recipient in transformation with DNA from a *B. subtilis* library prepared in λ Ch4A (Ferrari *et al.*, 1981). The library consisted of 19 pools each of about 100 individuals plaques. Each pool was plated to give confluent lysis on agarose plates, the phage collected and the DNA extracted and used to transform competent cells of strain PB2427, selecting for the ability of the spores to yield colonies at 47 °C (Ts⁺) (Galizzi *et al.*, 1973). One pool transformed the recipient to Ts⁺. It was replated to give single plaques; plate lysates were prepared from 200 of these, and the DNA was extracted and used to transform PB2427. Five clones transformed to Ts⁺ at a high frequency, all of which were identical in subsequent physical analysis. DNA from one clone (λ C4BsG40), when digested with *Eco*RI, yielded a single 14 kb fragment of inserted chromosomal DNA (Fig. 1). DNA from the phage, either intact or digested with *Eco*RI, transformed an *outB* recipient to Ts⁺. The relationship between DNA concentration and frequency of transformation by phage DNA is shown in Fig. 2. Of other markers close to *outB*, DNA from λ C4BsG40 transformed *aroI* to prototrophy, *tscB*, *tscG* and *tscH* (Galizzi *et al.*, 1976) to Ts⁺ and *amyE* to Amy⁺. The relationship between DNA concentration and frequency of Aro⁺ transformation, for DNA extracted from λ C4BsG40 and *B. subtilis* strain PB19, is shown in Fig. 2. λ C4BsG40 DNA was about 10-fold more efficient than chromosomal DNA at nonsaturating concentrations and the

Table 2. *Three-factor transformation crosses with markers of the outB region*

Cross	Donor	Recipient	Selection	Recombinants		Distances* and implied order
				Type	No.	
1	PB2427 (<i>outB81</i>)	PB5009 (<i>aroI906 amyE</i>)	Aro ⁺	Out ⁺ Amy ⁺	3	<i>aroI906-outB81</i> : 34
				Out ⁺ Amy ⁻	122	
				Out ⁻ Amy ⁺	32	<i>aroI906-amyE</i> : 91
				Out ⁻ Amy ⁻	218	
2	PB5009 (<i>aroI906 amyE</i>)	PB2427 (<i>outB81</i>)	Out ⁺	Aro ⁺ Amy ⁺	74	<i>outB81-aroI906</i> : 51
				Aro ⁺ Amy ⁻	33	
				Aro ⁻ Amy ⁺	95	<i>outB81-amyE</i> : 77
				Aro ⁻ Amy ⁻	18	<i>aroI906-outB81-amyE</i>
3	PB2355 (<i>tscB49</i>)	PB5009 (<i>aroI906 amyE</i>)	Aro ⁺	Ts ⁺ Amy ⁺	5	<i>aroI906-tscB49</i> : 28
				Ts ⁺ Amy ⁻	25	
				Ts Amy ⁺	31	<i>aroI906-amyE</i> : 67
				Ts Amy ⁻	49	
4	PB5009 (<i>aroI906 amyE</i>)	PB2355 (<i>tscB49</i>)	Ts ⁺	Aro ⁺ Amy ⁺	8	<i>tscB49-aroI906</i> : 13
				Aro ⁺ Amy ⁻	3	
				Aro ⁻ Amy ⁺	30	<i>tscB49-amyE</i> : 45
				Aro ⁻ Amy ⁻	43	
5	PB2353 (<i>tscG47</i>)	PB5009 (<i>aroI906 amyE</i>)	Aro ⁺	Ts ⁺ Amy ⁺	4	<i>aroI906-tscG47</i> : 26
				Ts ⁺ Amy ⁻	24	
				Ts Amy ⁺	30	<i>aroI906-amyE</i> : 70
				Ts Amy ⁻	52	
6	PB5009 (<i>aroI906 amyE</i>)	PB2353 (<i>tscG47</i>)	Ts ⁺	Aro ⁺ Amy ⁺	10	<i>tscG47-aroI906</i> : 16
				Aro ⁺ Amy ⁻	5	
				Aro ⁻ Amy ⁺	35	<i>tscG47-amyE</i> : 48
				Aro ⁻ Amy ⁻	43	
7	PB2354 (<i>tscH48</i>)	PB5009 (<i>aroI906 amyE</i>)	Aro ⁺	Ts ⁺ Amy ⁺	2	<i>aroI906-tscH48</i> : 18
				Ts ⁺ Amy ⁻	20	
				Ts Amy ⁺	41	<i>aroI906-amyE</i> : 80
				Ts Amy ⁻	57	
8	PB5009 (<i>aroI906 amyE</i>)	PB2354 (<i>tscH48</i>)	Ts ⁺	Aro ⁺ Amy ⁺	5	<i>tscH48-aroI906</i> : 14
				Aro ⁺ Amy ⁻	8	
				Aro ⁻ Amy ⁺	20	<i>tscH48-amyE</i> : 27
				Aro ⁻ Amy ⁻	60	

* The distances are expressed as 100 minus the percentage of cotransformation.

slopes of the dose-response curves are identical. The difference between the slopes of the dose-response curves for Aro⁺ and Ts⁺ transformants, and the higher value of the plateau obtained in the latter case, are due to the fact that in transformation experiments in which selection is for Ts⁺, transformants are not directly scored after DNA uptake but only after sporulation.

Genetic map of the outB region

To obtain an indication of the position of the genes on the DNA carried by λ C4BsG40, we re-examined the genetic map of the region. The *outB* marker was originally mapped by two-factor transduction crosses with phage PBS1, and 72% cotransduction with *aroI* was observed (Albertini & Galizzi, 1975). In two three-factor transformation crosses, the *outB* locus was mapped with respect to *aroI* and *amyE* (Table 2, crosses 1 and 2) and the results are consistent with the order *aroI-outB-amyE*. Three additional mutations, *tscB49*, *tscG47* and *tscH48*, which all result in temperature-sensitive vegetative growth, map in the same region (Galizzi *et al.*, 1976). The results of reciprocal three-factor crosses, selecting separately for Aro⁺ and Ts⁺ transformants, suggested the order *aroI-(tscB, tscG, tscH)-amyE* (Table 2, crosses 3-7). It must be noted that distances obtained in reciprocal crosses are not always congruent, thus the numbers should be taken as indicative. This is often the case with temperature-sensitive mutants, whose phenotype may be somewhat leaky. In particular we have encountered

Table 3. Cotransformation of *aroI906* and *outB81* with different DNA preparations

The recipient strain was PB2455 (*aroI906 outB81*). λ C4BsG40 (*EcoRI*) = λ C4BsG40 digested with *EcoRI* prior to transformation. DNA concentrations were 0.05 $\mu\text{g ml}^{-1}$.

Donor DNA	Frequency of Aro ⁺ transformants*	Percentage of Out ⁺ recombinants
PB19	5.8×10^{-5}	76 (83/109)
λ C4BsG40	3.4×10^{-4}	77 (57/74)
λ C4BsG40 (<i>EcoRI</i>)	9.2×10^{-5}	83 (152/184)

* Values are the numbers of transformants per recipient cell.

difficulties in measuring map distances in the *outB* region, when selecting for Ts⁺. In the cross 1 of Table 2 the distance between *aroI* and *amyE* is higher than that reported in the literature (Nomura *et al.*, 1978), and it is even higher in cross 2, in which PB2427 was the recipient. In the latter case the selection for transformants is applied only after sporulation and this may affect the final outcome of the cross. Due to difficulties in distinguishing among recombinant classes with the different temperature-sensitive phenotypes, we have not attempted to order *outB* with respect to *tscB*, *tscG* and *tscH*.

From the results presented in the previous section, it appears that the 14 kb insert of λ C4BsG40 spans the region which includes the markers *amyE* and *aroI*. A measure of the linkage between *aroI* and *outB* was obtained in transformation crosses using DNA from *B. subtilis* PB19 and DNA extracted from λ C4BsG40 (Table 3). Cotransformation obtained with the cloned DNA was in good agreement with that obtained with chromosomal DNA. Digestion of λ C4BsG40 DNA with *EcoRI* did not significantly affect cotransfer, although the frequency of transformation was lower (Table 3).

Isolation of a deletion derivative of phage λ C4BsG40

Following treatment of λ C4BsG40 with trisodium citrate a deletion mutant (λ C4BsG40 Δ 20) was isolated, the DNA of which was still able to transform *amyE*, *outB*, *tscB*, *tscG* and *tscH* markers, but not *aroI* (data not shown).

Physical analysis of the λ C4BsG40 phage DNA

DNA from λ C4BsG40, λ C4BsG40 Δ 20 and λ Ch4A was digested with various restriction endonucleases and subjected to agarose or acrylamide gel electrophoresis. The *B. subtilis* DNA insert of λ C4BsG40 has no restriction sites for *Bam*HI, *Bgl*II or *Sst*I, but it has a single site for *Sal*I, three sites for *Pst*I and seven for *Hind*III. A restriction map of the cloned fragment is shown in Fig. 4. The deletion mutant λ C4BsG40 Δ 20, unable to transform *aroI* recipients to prototrophy, lacks about 4 kb of DNA. The DNA of this phage digested with *Eco*RI gave only two bands, one corresponding to the left arm of the vector DNA and one of high molecular weight, suggesting that the deletion abolished the second *Eco*RI site, connecting the *B. subtilis* insert to the right arm of λ . In particular, three *Hind*III fragments (1480, 1020 and 680 bp) and the small 240 bp *Hind*III-*Eco*RI fragment were missing. A new *Hind*III fragment of 4.4 kb was present at the junction of the insert to the vector right arm. The deletion abolished a *Pst*I site as well. From this we conclude that the deletion encompasses about 3 kb of the insert and about 1 kb of the right arm of the vector. Presumably it removes the entire *bio-256* substitution present in λ Ch4A and stops at or near to the *N* gene. A restriction map of the λ C4BsG40 Δ 20 is shown in Fig. 4.

The size of the insert in λ C4BsG40 and the extent of the deletion in λ C4BsG40 Δ 20 were confirmed by heteroduplex analysis. Heteroduplexes were formed between the DNA of the vector and that of λ C4BsG40 or λ C4BsG40 Δ 20 and between the DNA of λ C4BsG40 and its deletion derivative (Fig. 3). Hybrid molecules between λ Ch4A and λ C4BsG40 DNA gave a large region of single-stranded DNA (Fig. 3a). The lengths of the double-stranded segments correspond to the left (19.9 kb) and right (11.0 kb) arm of λ Ch4A respectively, while the length

Fig. 3. Electron micrographs of heteroduplex molecules formed between λ Ch4A and recombinant derivatives. (a) λ Ch4A and λ C4BsG40; (b) λ Ch4A and λ C4BsG40 Δ 20; (c) λ C4BsG40 and λ C4BsG40 Δ 20. Drawings of the heteroduplexes are shown on the right. Bars, 5 kb.

Fig. 4. Genetic and restriction map of the region surrounding the *outB* gene. The genetic map is based on reciprocal three-factor transformation crosses (Table 2). The map distances are expressed as 100 minus the percentage of cotransformation. The head of the arrow points to the donor selected marker. The lower part of the figure is a restriction map of λ C4BsG40 and of its deletion derivative λ C4BsG40 Δ 20, and the extent of the fragments subcloned into plasmids. The results of the marker rescue experiments are summarized to the right of each clone. E, *EcoRI*; H, *HindIII*; P, *PstI*; S, *SalI*.

of the two single strands of each loop is about 14 kb. Thus we cannot distinguish between the loop of λ Ch4A and the one contributed by λ C4BsG40. In the λ Ch4A/ λ C4BsG40 Δ 20 heteroduplex (Fig. 3b) one double-stranded region is the same length as the left arm, while the other is only 10 kb, instead of 10.9 kb long. The individual single-stranded loops have sizes of 15.9 kb (λ Ch4A) and 11.3 kb (λ C4BsG40 Δ 20). As expected, in λ C4BsG40/ λ C4Bs40 Δ 20 heteroduplexes, the deletion resulted in a loop about 4 kb in length, positioned 30 kb from one end and 9.6 kb from the other (Fig. 3c).

Comparison between the genetic and the physical map

The deletion of about 3 kb from the right of the insert in λ C4BsG40 Δ 20 completely abolished the ability to transform for the *aroI* marker, which allowed orientation of the genetic map with the restriction map. Additional data were obtained by subcloning fragments of the insert into suitable plasmids, pBR322 and pBR325 (Fig. 4). Plasmid pO.PE, carrying the *PstI*-*EcoRI* fragment of 2.8 kb from the right end of the insert, transformed *aroI906* to prototrophy, but failed to transform *outB*, *tscB*, *tscG*, *tscH* and *amyE*. Plasmid pO.P transformed to Ts⁺ strains with the *outB81* mutation, whereas pO.HH did not. Therefore *outB81* must lie between the *HindIII* site at coordinate 9, and the *PstI* site at coordinate 11 of our insert. Obviously the ability to transform to wild-type does not guarantee that the entire gene is present on a specific DNA fragment, since only the portion of the gene which includes the part equivalent to the site of the mutation is required. Plasmid pO.P also transformed *tscB49*, *tscG47* and *tscH48* recipients to Ts⁺. It may be that the four mutations are all in the same gene, but this seems unlikely on phenotypic grounds, since only the *outB81* mutation affects outgrowth specifically. Perhaps the other three mutations, which affect vegetative growth, represent collectively a separate gene. *tscG* and *tscH* belong to the same physiological class and *tscB* and *tscG* are very closely linked, with a recombination index of 0.02 (Galizzi *et al.*, 1976). According to the transformation data

with subcloned fragments (Fig. 4), the *amyE* gene resides in the left part of the λ C4BsG40 insert. Nucleotide sequence analysis has revealed that the clone carries only the 3' part of the *amyE* gene (Yang *et al.*, 1983).

In conclusion, we have cloned a region of the *B. subtilis* chromosome which includes the *outB* gene, whose function is specifically required during spore outgrowth. This should enable us to study the role of the gene at the molecular level.

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