Genetical and Molecular Studies on *gerM*, a New Developmental Locus of *Bacillus subtilis*

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A transposon Tn917 insertion between *gerE* and *ilvB* has identified a new developmental locus, *gerM*, in *Bacillus subtilis*. *gerM96::Tn917* affects both sporulation and germination. DNA on either side of the transposon has been cloned and includes the previously cloned *sdhC* and *gerE* loci. *gerE* terminates 2.1 kb from the end of the transposon. The *gerM96::Tn917* mutant is oligosporogenous, yielding approximately 1% of the number of wild-type heat resistant spores in liquid medium and 10% on solid medium. Six hours after the onset of sporulation alkaline phosphatase and glucose dehydrogenase levels were 90% and 7%, respectively, of those of the wild-type. At this time 50% of the mutant cells were still dividing. The occurrence of multiple polar septa and 'pygmy' cells suggested a block at stage II of sporulation. Following addition of germinants, mutant spores prepared on nutrient agar lost heat resistance normally but released slightly less dipicolinic acid than wild-type spores. They also showed only partial loss of optical density, associated with a phase-grey appearance and striations in the cortex suggesting partial degradation. Expression of the *gerM* gene was monitored by production of β -galactosidase encoded by a promoterless *lacZ* gene fused to the *gerM96::Tn917* insertion. It occurred 1.5–4 h after commencement of sporulation. Transcription was directed from a promoter on the *gerE* side of *gerM* and was unaffected by a mutation in the *gerE* gene.

INTRODUCTION

Germination in bacteria involves the conversion of a dormant spore into a metabolically active cell. In *Bacillus subtilis* this process requires the expression during sporulation of many germination (*ger*) and sporulation (*spo*) genes to equip the spore with the components necessary for germination (Piggot & Coote, 1976; Losick & Youngman, 1984). Although over 50 loci at which *spo* or *ger* mutations arise are known it is likely that more remain to be discovered (Losick & Youngman, 1984). Few of the products of *spo* and *ger* genes are known but patterns of their order and interdependence of expression are emerging (Errington & Mandelstam, 1986*a, b*; Turner *et al.*, 1986). During sporulation some genes are expressed in the mother-cell compartment and others in the forespore (de Lencastre & Piggott, 1979; Errington & Mandelstam, 1986*b*; Turner *et al.*, 1986). It is also likely that some *spo* and *ger* genes serve not only to form a particular spore component but to stimulate the expression of other genes, and mutations in such genes result in the pleiotropy which is characteristic of many *spo* and *ger* mutants.

In this paper we describe the use of transposon Tn917 to identify a new *ger* locus. The advantages of using this method are firstly that Tn917 confers resistance to macrolide, lincosamide and streptogramin B type antibiotics (MLS^R), a character which is readily selectable and thus permits indirect selection of the associated Ger mutant phenotype. Secondly, DNA on either side of the insertion is easily cloned (Youngman *et al.*, 1984). Thirdly, *lacZ* fusions can be created which may permit the expression of the gene in which the insertion has

Abbreviations: DPA, dipicolinic acid; MUG, methylumbelliferyl β -D-galactoside.

Table 1. *Bacterial strains and plasmids*

Strain no.	Genotype/phenotype*	Source or reference
<i>B. subtilis</i>		
1477	<i>trpC2 hisA1 cysB3</i>	C. Anagnostopoulos, CNRS, Gif-sur-Yvette, France
1604	<i>trpC2</i> ; 'wild type'	This laboratory
4600	<i>gerE36 trpC2 hisA1</i>	
4735	<i>gerE36 ilvΔ1</i>	
5109	<i>gerJ50 leuA8</i>	
4822	<i>sdhC83 leuC2</i>	
4974	<i>trpC2 leuD117 ilvB2 argA2</i>	J. Hoch, Schripps Clinic, La Jolla, USA
5188	<i>gerM96::Tn917</i> MLS ^R ; unknown auxotrophy	S. Zahler, Cornell University, USA
5203	<i>gerM96::Tn917 trpC2 hisA1 cysB3</i> MLS ^R	This study
5204	<i>gerM96::Tn917 trpC2 hisA1</i> MLS ^R	
5205	<i>gerM96::Tn917 pTV20 trpC2 hisA1 cysB3</i> MLS ^R Cm ^R	
5206	<i>gerM96::Tn917::pTV21Δ2 trpC2 hisA1 cysB3</i> MLS ^R Cm ^R	
5261	<i>gerM96::Tn917 lacZ</i> MLS ^R Cm ^R <i>trpC2 hisA1</i>	
5262	<i>gerE36 gerM96::Tn917 lacZ</i> MLS ^R Cm ^R	
PY79	Prototroph	
PY143	PY79(pTV1)	P. Youngman, Pennsylvania University, USA
<i>E. coli</i>		
NEM259	<i>supE supF r_K⁻ m_K⁺ (hsdR, hsdM⁺, hsdS⁺)trpC2</i> Met ⁻	J. R. Guest, Sheffield University, UK
Plasmids		
pTV1	Cm ^R MLS ^R	P. Youngman
pTV20	Cm ^R MLS ^R Ap ^R	
pTV21Δ2	Cm ^R Ap ^R	
pTV54	Cm ^R Ap ^R	
pSH1047	Km ^R Cm ^R	Hasnain <i>et al.</i> (1985)
pBMM2	Ap ^R	This study
pBMM4	Ap ^R	
pBMM80	Ap ^R	

* Cm^R, MLS^R, Ap^R and Km^R denote resistance to chloramphenicol, to macrolide, lincosamide and streptogramin B antibiotics, to ampicillin and to kanamycin, respectively. Cm^R and MLS^R are expressed in *B. subtilis*, Ap^R and Km^R in *E. coli*.

occurred to be monitored (Youngman *et al.*, 1985*a,b*). We describe the phenotype of a mutant induced by insertion of Tn917 in a previously uncharacterized developmental locus, designated *gerM*, the map location and partial cloning of this region of the chromosome, and the expression of the *gerM* gene. A preliminary account of some of this work was given by Moir *et al.* (1985).

METHODS

Bacteria and plasmids. The strains of *Bacillus subtilis* 168 and *Escherichia coli* K12, and plasmids, are listed in Table 1.

Media. (a) *B. subtilis*. Nutrient media were Difco Penassay Broth and Difco Nutrient Agar (NA), plus erythromycin (1 µg ml⁻¹) and lincomycin (25 µg ml⁻¹) to select for MLS^R and chloramphenicol (5 µg ml⁻¹) to select for Cm^R. Minimal agar (Anagnostopoulos & Spizizen, 1961) was supplemented with glucose (0.5%) and amino acids (20 µg ml⁻¹) where required. Culture of *citF* mutants and selection of Cit⁺ on minimal lactate plates was according to Rutberg & Hoch (1970).

For germination experiments, spores were prepared on NA and washed as described by Moir *et al.* (1979). For enzyme assays, electron microscopy of sporulating cells and gene expression studies, spores were prepared in the resuspension medium of Sterlini & Mandelstam (1969) or in modified Shaeffer's D medium (Leighton & Doi, 1971). The spore germination phenotype (Ger) was scored by the tetrazolium overlay method (Trowsdale & Smith, 1975).

(b) *E. coli*. Strains were cultured in L-broth and on L-agar (Miller, 1972), plus kanamycin (50 µg ml⁻¹) or ampicillin (100 µg ml⁻¹) to select for Km^R and Ap^R, respectively.

Transduction and transformation. Phage PBS1 transduction was done as described by Jamet & Anagnostopoulos (1969), and transformation according to Anagnostopoulos & Spizizen (1961). The method of Youngman *et al.* (1983) was used to select MLS^R transductants and transformants. *E. coli* was made competent and transformed with plasmid DNA as described by Meyer *et al.* (1977) and modified by Thomas (1981).

Extraction of DNA. Plasmid DNA was extracted by the method of Ish-Horowitz & Burke (1981) and *B. subtilis* chromosomal DNA prepared by that of Marmur (1961).

DNA restriction, digestion and ligation. Restriction endonucleases and bacteriophage T4 ligase from Bethesda Research Laboratories were used as directed by the suppliers.

Gel electrophoresis. The method of Moir (1983) was used for electrophoresis of DNA fragments in 0.95% agarose gels.

Southern blotting and hybridizations. Plasmid (1 µg) or chromosomal (10 µg) DNA was digested to completion with restriction endonucleases and the fragments were separated by gel electrophoresis, denatured and transferred to a sheet of Biodyne A membrane (Pall Process Filtration, Portsmouth, UK) as described by Reed & Mann (1985). DNA fragments were isolated from low-melting-point agarose gels (0.7%) and labelled with [α -³²P]dCTP according to Feinberg & Vogelstein (1983, 1984). Hybridization of these fragments to the DNA on the Biodyne A membrane was done as recommended by the manufacturers.

Spore properties. Lysozyme resistance of dormant spores was determined by the method of Moir (1981). Heat resistance was determined by adding 0.2 ml spore suspension or sporulating culture to 1.8 ml distilled water at 85 °C. After 15 min the suspensions were cooled rapidly to room temperature and the numbers of survivors determined by viable counts. To determine the yield of heat-resistant spores on NA after 48 h at 37 °C the growth from the surface of three plates was scraped into 30 ml distilled water and 0.2 ml of this suspension then used to assay the number of heat-resistant spores as above. Germination was measured by observing loss of optical density (OD₅₈₀) or heat resistance as described by Sammons *et al.* (1981). Release of dipicolinic acid (DPA) was measured using the method of Scott & Ellar (1978).

Measurement of enzyme activities in sporulating cells. (a) β -Galactosidase. (i) On plates. This was done as described by Youngman *et al.* (1985b). (ii) In Sterlini-Mandelstam medium. Samples (1 ml) were taken at hourly intervals after resuspension (t_0 , t_1 , etc.) and centrifuged; the cell pellets were frozen in liquid N₂ and stored at -70 °C. They were resuspended in 0.6 ml Z buffer (Miller, 1972), 0.1 ml toluene was added and, after 20 min on ice, the open tubes were placed in a water bath at 30 °C and 200 µl methylumbelliferyl β -D-galactoside (MUG) (40 µg ml⁻¹ in Z buffer) was added. After 15 min the reaction was stopped by addition of 400 µl 1 M-sodium carbonate. The suspension was centrifuged and methylumbelliferone estimated in the supernatant by measurement of the fluorescence at 450 nm using an excitation frequency of 365 nm and a Spex Fluorolog spectrofluorimeter. Solutions of methylumbelliferone of known concentration were used to obtain a standard curve and units were expressed as nmol MUG converted min⁻¹ (ml sporulating culture)⁻¹.

(b) *Alkaline phosphatase.* Frozen cell pellets from 1 ml samples of sporulating cultures taken as described for the β -galactosidase assay were thawed and resuspended in 1 ml diethanolamine. HCl (1 M, pH 10); toluene was added and the enzyme assayed as described by Dancer & Mandelstam (1975).

(c) *Glucose dehydrogenase.* Cell extracts were prepared and the enzyme assayed as described by Hill (1983) except that 35 ml instead of 50 ml sporulating cell suspension was used.

Protein was estimated by the Lowry method and units of both alkaline phosphatase and glucose dehydrogenase were expressed as nmol substrate converted min⁻¹ (mg soluble protein)⁻¹ or (ml sporulating cell suspension)⁻¹.

Electron microscopy. (a) *Germinating spores.* Germination was initiated in a suspension of spores at an OD₅₈₀ of 1.0 by addition of L-alanine to 10 mM. Samples of 1.2 ml were centrifuged and the spore pellets washed in distilled water. Fixing was done by resuspension at 18–20 °C for 2 h each in 15% (v/v) formaldehyde, 6% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide in 100 mM-sodium phosphate buffer pH 7.2 (Hayat, 1981). After washing in buffer, the spores were dehydrated by resuspension in 70%, 90% and finally 100% (v/v) ethanol. Ethanol was removed with propylene oxide and the samples were infiltrated with Epon resin 812. Sections of 60–80 nm were cut on an LKB Ultratome V with a diamond knife, stained with uranyl acetate in 50% ethanol for 15–30 min (Epstein & Holt, 1963) and counterstained with lead citrate for 3–5 min (Reynolds, 1963). Sections were observed with a Philips 301 transmission electron microscope at an accelerating voltage of 80 kV.

(b) *Sporulating cells.* Samples of 1 ml were centrifuged and the spores resuspended in 100 µl 1% osmium tetroxide in 100 mM-sodium phosphate buffer pH 7.2. After 14–20 h at 18–20 °C, 150 µl acetate/veronal buffer (AV buffer; Kellenberger *et al.*, 1958) was added. The cells were centrifuged and resuspended in 0.5% uranyl acetate in AV buffer for 2 h. They were then dehydrated with ethanol, embedded in resin, sectioned and viewed as described in (a).

RESULTS

Isolation and map location of gerM96::Tn917

Tn917 mutants were isolated as described by Youngman *et al.* (1983) using strain PY143. This strain carries the heat-sensitive Cm^R plasmid pTV1 in which Tn917 is inserted. Colonies which

were MLS^R , Cm^S and grew well at 46 °C (due to insertion of the transposon in the chromosome and loss of the plasmid) were obtained at a frequency of approximately 10^{-4} , as determined from comparison of viable counts at 30 and 46 °C. Bacteria from three of 800 colonies tested were Ger^- . In two cases the Ger^- phenotype resulted from transposon insertions at different sites in the *gerD* region of the chromosome (Yon, 1986). In the third case the mutation was located in a new developmental locus, designated *gerM*. In the strain in which the insertion occurred (5188) the MLS^R (Ger^-) marker was 57% and 83% cotransduced with *argA* and *ilvB2* respectively. Because this strain also had an auxotrophic requirement (presumably due to a second unlinked insertion) its MLS^R (Ger^-) marker was transformed into strain 1477 to form strain 5203. The Cys^- marker of strain 5203 was subsequently removed by transformation with DNA from strain 1604, creating strain 5204, because the Cys^- auxotrophy reduces the frequency of sporulation. This strain, whose Ger^- phenotype was identical to that of the original mutant, is thus comparable with other *ger* mutants in this laboratory which have the same genetic background.

Transformation crosses located *gerM96::Tn917* in 5204 more precisely. In three-point crosses it was cotransformed with *ilvB2*, *leuD117*, *sdhC83* and *leuC2* (Table 2), the relative frequencies of the recombinant classes being consistent with the order *sdhC83-Tn917-ilvB2-leuC2-leuD117*. However, because the lack of homology between transposon DNA and transforming fragments could affect recombination either side of the transposon, the numbers in some of the recombinant classes might be distorted. For this reason the precise location of the insertion was uncertain. The *gerE* locus also maps between *sdhC* and *ilvB* and is 90% cotransformed with the former (Moir, 1981, and R. L. Sammons, unpublished). The insertion mutation was not in *gerE* since *gerE* and *sdhC* have been cloned on a contiguous fragment of DNA in the plasmid pSH1047 (Hasnain *et al.*, 1985) which cannot transform *gerM96::Tn917* to Ger^+ . It was also well separated from *spoVH* and *spoVJ*, which have also been reported to map in this region (Hill, 1983), since mutations in these genes are located on the other side of *sdhC* from *leuD* (R. L. Sammons, unpublished). Thus it was most likely that *gerM96::Tn917* lay between *gerE* and *ilvB* (Fig. 1).

Cloning of DNA adjacent to gerM96::Tn917

Since *gerM96::Tn917* was near to *gerE* and the *sdh* genes it was possible that DNA adjacent to the site of the *Tn917* insertion might overlap with DNA cloned in pSH1047. Plasmids pTV20 and pTV21Δ2 were used to clone such DNA. These plasmids carry the replication functions and Ap^R gene from pBR322 and a Cm^R gene (which is expressed in *B. subtilis*) in opposite orientations within *Tn917* DNA (Fig. 2). They were linearized and used to transform strain 5203 to Cm^R as described by Youngman *et al.* (1984), thus replacing the original *Tn917* insertion with the structures shown in Fig. 2 in recipient strains 5205 and 5206. Cotransformation of the Cm^R markers of 5205 and 5206 with *leuD117* (12% and 25%, respectively) confirmed that the plasmid DNA had integrated in the *gerM* region of the chromosome. Chromosomal DNA from strains 5205 and 5206 was then partially digested with *EcoRI*, religated and used to transform *E. coli* NEM259 to Ap^R . Plasmid DNA was prepared from the transformants. pBMM2 (from 5205) and pBMM4 (from 5206) carry DNA adjacent to the site of *Tn917* insertion in the *gerM96::Tn917* mutant up to the next *EcoRI* sites in the chromosome. The lengths of *B. subtilis* DNA in pBMM2 (1.5 kb) and pBMM4 (0.6 kb) suggested that *Tn917* had inserted into a 2.1 kb chromosomal *EcoRI* fragment. This was confirmed by hybridization of ^{32}P -labelled pBMM2 and pBMM4 DNA to a fragment of this size in an *EcoRI* digest of *B. subtilis* wild-type chromosomal DNA. The lengths of DNA cloned in pBMM2 and pBMM4 have been estimated more accurately since they were reported in Moir *et al.* (1985).

All of 24 transformants obtained using strain 5205 DNA carried plasmids like pBMM2. However, of 18 plasmids obtained from transformants derived from strain 5206 DNA, six were like pBMM4 and the rest carried an additional 4.0 kb *EcoRI* fragment as in pBMM80 (Fig. 2). The frequency with which the latter arrangement was obtained suggested that the 4.0 kb fragment could be the next *EcoRI* fragment along from the 2.1 kb *EcoRI* fragment in the chromosome. Since the 4.0 kb *EcoRI* fragment of pBMM80 carries a single *EcoRV* site (Fig. 2) this fragment was expected to hybridize to two *EcoRV* chromosomal fragments. The *B. subtilis*

Fig. 1. Map location of *gerM96::Tn917* consistent with transformation data from Table 2, and of *gerE36* from unpublished results of R. L. Sammons. Arrows point to selected markers. Figures are 100 minus the percentage cotransformation.

Table 2. Location of *gerM96::Tn917* in transformation crosses

Donor	Recipient	Selected marker	Recombinant class	No.
(a) 5188 (<i>gerM96::Tn917</i>)	4974 (<i>leuD117 ilvB2 argA2 trpC2</i>)	MLS ^R	MLS ^R Ilv ⁺ Leu ⁺	10
			MLS ^R Ilv ⁺ Leu ⁻	24
			MLS ^R Ilv ⁻ Leu ⁺	0
			MLS ^R Ilv ⁻ Leu ⁻	90
				124
(b) 5204 (<i>gerM96::Tn917 trpC2 hisA1</i>)	4822 (<i>sdhC83 leuC2</i>)	MLS ^R	MLS ^R Sdh ⁺ Leu ⁺	9
			MLS ^R Sdh ⁺ Leu ⁻	45
			MLS ^R Sdh ⁻ Leu ⁺	5
			MLS ^R Sdh ⁻ Leu ⁻	41
				100
(c)* 5204	4822	Leu ⁺	Leu ⁺ Sdh ⁺ MLS ^R	56
			Leu ⁺ Sdh ⁺ MLS ^S	4
			Leu ⁺ Sdh ⁻ MLS ^R	30
			Leu ⁺ Sdh ⁻ MLS ^S	260
				350
(d)* 5204	4822	Sdh ⁺	Sdh ⁺ Leu ⁺ MLS ^R	137
			Sdh ⁺ Leu ⁺ MLS ^S	7
			Sdh ⁺ Leu ⁻ MLS ^R	30
			Sdh ⁺ Leu ⁻ MLS ^S	120
				294

* For (c) and (d), combined results of three and two separate crosses, respectively, each of which yielded similar data, are given.

DNA in pBMM4 carries no *EcoRV* site and thus should hybridize to only one *EcoRV* fragment; this fragment should be the same size as one of those to which the 4.0 kb fragment of pBMM8 hybridizes if it is indeed naturally adjacent to the 2.1 kb *EcoRI* fragment in the chromosome. As predicted, the 4.0 kb fragment of pBMM80 hybridized to two *EcoRV* fragments (of 5.0 and 6.8 kb) whilst pBMM4 hybridized to only one of these (of 6.8 kb). Thus it is very likely that the 4.0 and 2.1 kb *EcoRI* fragments are adjacent in the chromosome. The 4.0 kb fragment appeared not to be deleted or rearranged since it hybridized to a wild-type chromosomal *EcoRI* fragment of that size.

Fig. 2. Cloning of DNA either side of *gerM96*::Tn917. The hatched boxes represent Tn917 DNA. LS is the deleted MLS^r marker in pTV21Δ2. The sizes given for *B. subtilis* DNA inserted in the plasmids are in kb. Restriction sites: E, *EcoRI*; R, *EcoRV*.

Identification of DNA sequences common to pSH1047 and pBMM80

Comparison of the restriction maps of DNA cloned in pSH1047 and pBMM80 revealed an *EcoRI* site, four *HindIII* sites and single *PvuII* and *EcoRV* sites in identical relative positions (Fig. 3a, b), suggesting that they carried a common region of DNA. Since the *sdhB* and *C* genes and *gerE* are located in this region in pSH1047 (Hasnain *et al.*, 1985; Cutting & Mandelstam, 1986) it was probable that pBMM80 also carried these genes. In fact pBMM80 transformed the *sdhC83* mutation to Sdh⁺ at high frequency. To show that the 4.0 kb *EcoRI* fragment of pBMM80 also carried *gerE*, strain 4600 was transformed to His⁺ with DNA from strain 4735 in the presence and absence of 1 µg pBMM80 DNA ml⁻¹ to permit congression of the latter. When pBMM80 DNA was present 32% of the transformants were Ger⁺; in its absence none were Ger⁺ (100 colonies tested in each case). Since both strains 4600 and 4735 carry the *gerE36* mutation, only pBMM80 could have provided the *gerE*⁺ allele.

Thus the 4.0 kb *EcoRI* fragment of pBMM80 carries a region of DNA previously cloned in pSH1047 which carries both *sdhC* and *gerE*. However, the restriction map of DNA cloned in pSH1047 is different from that of pBMM80 in the region to the right of the common *PvuII* and *EcoRV* sites in Fig. 3. To test whether pSH1047 had suffered a deletion or rearrangement the *HindIII*–*PstI* fragment, which carries *sdhB*, *sdhC* and *gerE* and the boundary between common

Fig. 3. Restriction maps of DNA cloned in (a) pSH1047 and (b) pBMM80, and (c) probable physical map of the *sdh-gerM* region of the chromosome. Restriction sites: B, *Bam*HI, P, *Pst*I; E, *Eco*RI; Ss, *Sst*I; H, *Hind*III; Pv, *Pvu*II; R, *Eco*RV; S, *Sal*I; A, *Ava*I. The hatched box represents Tn917 DNA; LS denotes the deleted *MLS*^R marker. Tn917 (5.4 kb long) is not drawn to scale.

and different DNA in pSH1047 and pBMM80, was hybridized to strain 1604 DNA digested with *Eco*RI. Although there is no *Eco*RI site in the probe DNA it hybridized to two *Eco*RI fragments of 4.0 and approximately 8.0 kb. This fragment of DNA must therefore be deleted or rearranged to the right of the *Pvu*II and *Eco*RV sites. It did not hybridize to DNA cloned in pBMM2 and may come from a different part of the chromosome. We have located a *Sau*3A site approximately 1 kb from the *Hind*III site in the *Hind*III-*Pst*I fragment. It is possible that the DNA to the right of this is a non-adjacent *Sau*3A fragment which became ligated to the other DNA during the construction of pSH1047.

These observations suggest that the *gerM* locus into which Tn917 has inserted lies on a 2.1 kb *Eco*RI fragment adjacent to one of 4.0 kb on which *sdhB*, *sdhC* and *gerE* are located. Cutting & Mandelstam (1986) have shown that the *gerE* open reading frame terminates approximately 800 bp from the nearest *Hind*III site. The distance between this site and the *Eco*RI site nearest the transposon DNA in pBMM80 is approximately 2.3 kb. This *Eco*RI site is approximately 0.6 kb from the *Ava*I site which is 8 bp from the end of the transposon (Perkins & Youngman, 1984). Thus the transposon is located approximately 2.9 kb from the *Hind*III site nearest to *gerE* and 2.1 kb from the terminator of this gene (Fig. 3c). The transposon DNA carried by pBMM4 and pBMM80 is the portion which carries part of the *MLS*^R gene (a deletion removing part of the *MLS*^R gene having occurred in pTV21Δ2). Thus the transposon must be orientated in the chromosome with the end carrying the *MLS*^R gene nearest to *gerE*.

In earlier experiments (Moir *et al.*, 1985) plasmids pBMM1 and pBMM5 were obtained which carried *Eco*RI fragments of 4.4 and 3.0 kb in addition to the DNA cloned in pBMM2 and pBMM4 respectively. These additional fragments hybridized to *Eco*RI fragments of DNA cloned in λ phages of 7.0 and 10.3 kb respectively, suggesting that the *gerM* 2.1 kb *Eco*RI fragment was flanked by *Eco*RI fragments of these sizes. The experiments described above show that this is not the case; at least to the *gerE* side of the 2.1 kb fragment the adjacent fragment is 4.0 and not 10.3 kb long. If the DNA cloned in pBMM5 and homologous phages does come from the *gerM* region (as suggested by mapping experiments using integrational plasmids into which the DNA had been subcloned) it must be deleted and/or rearranged since there are no obvious similarities between the restriction maps of the DNA cloned in the phages and in pBMM80.

Phenotype of the *gerM96::Tn917* mutant

Sporulation. Samples of strain 5204 (*gerM96::Tn917*) and strain 1604 (*ger*⁺) sporulating in Sterlini-Mandelstam medium were taken at *t*₈ and *t*₂₁ and assayed for heat-resistant spores. At *t*₈, 20% of the wild-type cells contained heat-resistant spores and this proportion had increased

Fig. 4. Electron micrographs of sporulating cells of strains 1604 (wild-type) and 5204 (*gerM96::Tn917*). Samples were taken 6 h after resuspension in Sterlini-Mandelstam medium. (a-d) Strain 5204: (c) shows 'pygmy' cell formation; (d) shows formation of multiple septa. (e) Strain 1604. Bars, 500 nm.

to 67% by t_{21} . With strain 5204, the corresponding proportions were only 0.04% and 0.3%, respectively. The yield of wild-type heat-resistant spores per ml at t_{21} was approximately 3×10^8 as compared with 3.5×10^5 for the mutant. On NA plates the yield of heat-resistant spores of strain 5204 was much higher (12 and 18% of viable cells respectively), although still lower than the wild-type yield (100%). However, the yield was great enough to obtain sufficiently concentrated spore suspensions for germination experiments.

To attempt to identify the stage at which *gerM96::Tn917* blocked sporulation in Sterlini-Mandelstam medium, alkaline phosphatase and glucose dehydrogenase activities were measured in sporulating cultures. The time of onset of the production of alkaline phosphatase in strain 5204 was similar to that in the wild-type, although the final activity achieved was consistently approximately 10% lower. However, glucose dehydrogenase (measured at t_6) was almost undetectable in the mutant strain (7 as compared to 99 units per mg protein with wild-type). Similar results were obtained with strain 5261 (*gerM96::Tn917 lacZ*) (data not shown).

Electron microscopy of sections of sporulating mutant (5204) and wild-type cells taken at t_6 revealed that approximately 50% of the mutant cells did not contain spores and some were still dividing (Fig. 4a, b). In contrast, the majority of wild-type cells contained prespores (Fig. 4e). Other mutant cells contained prespores which appeared normal. Approximately 15% of the mutant cells which did not contain prespores appeared to be dividing abnormally in that a

Fig. 5. Germination of spores of strains 1604 (wild-type) (open symbols) and 5204 (*gerM96::Tn917*) (filled symbols) in L-alanine. 'Percentage completion of event' represents the percentage loss of OD₅₈₀ (○, ●), heat resistance (□, ■) or release of DPA (△, ▲) for wild-type or mutant spores after time *t*, expressed as a percentage of the corresponding percentage loss values for wild-type spores after 75 min.

septum enclosing cell-wall material had been formed at one pole to yield a 'pygmy' cell (Fig. 4c). In some cases a second cell-division septum appeared to be forming beside the first (Fig. 4d). In other cells, division appeared to be occurring normally at the mid-point of the cell. 'Pygmy' cell formation and 'bizarre cell division' are characteristic of some *spoII* mutants, as is the ability to produce alkaline phosphatase but not glucose dehydrogenase (Waites *et al.*, 1970; Coote, 1974), suggesting that the *gerM96::Tn917* mutant may be deficient at this stage of sporulation. However, some cells appeared to be blocked prior to septum formation whereas in others prespores were formed, but as reported above the majority of the latter did not go on to form heat-resistant spores. Phase-contrast microscopy of cultures at *t*₈ revealed cells at all stages of sporulation in addition to many apparently vegetative cells. This is characteristic of many oligosporogenous mutants (Piggot & Coote, 1976) and makes the stage of sporulation at which the *gerM96::Tn917* mutant is blocked difficult to define precisely.

Germination. Loss of OD₅₈₀ and heat resistance, and release of DPA, by spores of strains 1604 (*ger*⁺) and 5204 (*gerM96::Tn917*) germinating in 10 mM-L-alanine were compared (Fig. 5). Following addition of the germinant, spores of strain 5204 lost heat resistance normally. However, the rate and final extent of release of DPA were slightly lower than for the wild-type, and loss of OD₅₈₀ was markedly slower, ceasing when only 30% of the final extent of loss of OD₅₈₀ by wild-type spores had been achieved. A similar response was seen with asparagine plus glucose, fructose and KCl as germinant (data not shown). Further loss of OD₅₈₀ could not be induced by addition of lysozyme (200 µg ml⁻¹) to spores of strain 5204, which appeared grey by phase-contrast microscopy and remained so for more than 2 h at 37 °C in the germination mixture. In electron micrographs of sections of spores of strain 5204 made 75 min after onset of germination, the core was less swollen than that of the wild-type. The cortex had a striated appearance not seen in the wild-type at this time (Fig. 6a-d) although it was visible after 30 min in both mutant and wild-type spores (not shown). These observations suggest that degradation of the cortex may be incomplete in germinating *gerM96::Tn917* mutant spores as it is in *gerJ* mutants (Warburg & Moir, 1981). However, electron micrographs of sections of *gerJ50* mutant spores (strain 5109) taken at the same time indicated that the cortex of these remained unstained, unlike the *gerM96::Tn917* and the wild-type, even after 75 min (Fig. 6e). The *gerJ50* mutant is likely to be blocked at a different stage of germination from the *gerM96::Tn917* mutant.

Fig. 6. Electron micrographs of germinating spores of strains 1604 (wild-type) (a, b), 5204 (*gerM96::Tn917*) (c, d) and 5109 (*gerJ50*) (e, f) 75 min after addition of L-alanine. Bars, 500 nm.

Expression of gerM

To investigate the time of expression of *gerM* during sporulation a *gerM96::Tn917 lacZ* fusion was constructed by transformation of strain 5204 with pTV54 cut with *Xba*I (Youngman *et al.*, 1985b, Fig. 7). Plasmid pBMM4 hybridized to a 4.0 kb *Eco*RI fragment of chromosomal DNA of the resulting fusion strain, 5261; 4.0 kb is the size expected if *Tn917::lacZ* had inserted in the 2.1 kb fragment in the manner shown in Fig. 7. The *lacZ* gene fused to *Tn917* lacks its own promoter but will be expressed if the promoter of the gene into which it is inserted directs transcription through the transposon and *lacZ* in the correct orientation. Expression was detected by the fluorescence of sporulating colonies of strain 5261 on NA plates sprayed with MUG after 24 h at 37 °C when the cells were starting to sporulate. No fluorescence was detected during vegetative growth.

Fig. 7

Fig. 7. Formation of a *gerM96::Tn917 lacZ* fusion strain (5261). The hatched boxes represent Tn917 DNA. E, *EcoRI* site; Xb, *XbaI* sites; P, promoter; ➡, direction of transcription.

Fig. 8

Fig. 8. β -Galactosidase production during sporulation by strains 5261 (*gerM96::Tn917 lacZ*) (●), 1604 (wild-type) (○) and 5204 (*gerM96::Tn917*) (■).

To determine more precisely the time of *lacZ* expression, samples of cells of strains 1604 (wild-type), 5204 (*gerM96::Tn917*) and 5261 (*gerM96::Tn917 lacZ*) were taken at 30 min or 1 h intervals after resuspension in Sterlini-Mandelstam sporulation medium, and assayed for β -galactosidase activity (Fig. 8). In strain 5261 β -galactosidase activity started to increase 1.5 h after resuspension, reached a peak at 2.5–3.5 h and then declined. Very little activity was detected in the other two strains. The time at which the β -galactosidase activity started to decline varied from $t_{3.5}$ to t_4 and the height of the peak differed approximately twofold between experiments, but the time of onset of *lacZ* expression was very reproducible. In modified Shaeffer's D medium an increase in β -galactosidase was detected 1.5 h after the end of exponential growth. These results indicated that Tn917 had inserted in a developmental gene which is expressed between 1 and 4 h after commencement of sporulation.

The orientation of the transposon with respect to *sdh* and *gerE* was established earlier (Fig. 3). For expression to be detected, transcription must be directed into the *MLS*^R end of the transposon. The promoter must therefore be directing transcription in the direction *gerE* $\rightarrow$ *gerM*.

The effect of the *gerE36* mutation on the β -galactosidase activity of strain 5261 was tested. A *gerE36/gerM96::Tn917 lacZ* mutant (strain 5262) was constructed by transforming the *gerM96::Tn917 lacZ* insertion into strain 4600, selecting *Cm*^R and then checking for *MLS*^R. The presence of the original mutations was confirmed by transforming strain 4822 to *Cit*⁺ with DNA from strain 5262 and then showing that amongst the transformants were *Ger*⁻ colonies which were either *MLS*^S, with lysozyme-sensitive spores (like *gerE*), or *MLS*^R with lysozyme-resistant spores like the *gerM96::Tn917* mutant. The double mutant sporulated and germinated poorly like the *gerM96::Tn917* mutant but the spores were lysozyme sensitive. β -Galactosidase was detected in the double mutant over the same time period during sporulation as with strain 5261, indicating that *gerM* was expressed in the presence of the *gerE36* mutation (data not shown). These results show that *gerM* expression is independent of the product of *gerE* but do

not exclude the possibility that *gerE* and *gerM* are cotranscribed. However, this is unlikely since there is a transcriptional terminator to the *gerM* side of *gerE*, and *gerE* is expressed much later than *gerM* (t_4 - t_6 ; Cutting & Mandelstam, 1986).

DISCUSSION

A Tn917 insertion approximately 2.1 kb from *gerE*, between it and *ilvB*, has identified a new genetic locus involved in sporulation and germination. It is likely that *gerE* and *gerM* are expressed independently since *gerM* was expressed in a *GerE*⁻ background, absence of the *gerM* product in a *gerE gerM* double mutant did not alter the lysozyme sensitivity of the spores which is characteristic of the *gerE36* mutant, and *gerE* is expressed much later than *gerM* (Cutting & Mandelstam, 1986).

Insertion of Tn917 in the *gerM* locus appears to affect both sporulation and germination. The frequency of sporulation is reduced and glucose dehydrogenase is produced in much lower amounts than in wild-type cultures (or is produced in a form which is so sensitive to degradation than its activity was almost undetectable in extracts). This suggests that sporulation in the majority of *gerM96::Tn917* mutant sporangia may be blocked at stage II or stage III. This is supported by electron microscopy of sporulating mutant cells, some of which showed abnormal septum formation (Fig. 4). However, these formed only a small proportion of the cell population and in other cells sporulation was blocked at a later stage since the prespores failed to become phase-bright or to be released. Thus the stage of sporulation at which the *gerM96::Tn917* mutant is blocked is difficult to define without further evidence. Errington & Mandelstam (1983) showed that closely linked mutations in the *spoIIA* locus could cause different degrees of oligosporogeny or asporogeny. Mutations in other parts of the *gerM* locus might also result in asporogeny, permitting the stage of sporulation at which the block occurs to be defined more precisely. We are attempting to obtain such mutations.

Germination is blocked after loss of heat resistance and the release of DPA but before completion of loss of OD₅₈₀ and phase-darkening. The electron microscopy (Fig. 6) suggests that degradation of the cortex is incomplete, possibly due to lack of lytic enzyme. If this enzyme were also required for synthesis of the cortex during sporulation, a mutation in a single gene could affect both sporulation and germination. However, the germination deficiency could equally well be due to failure of expression of another gene or genes whose expression is dependent on that of *gerM*. Alternatively the *Ger* phenotype of the *gerM96::Tn917* mutant may simply be a consequence of poor sporulation and might be seen in germinating spores of other oligosporogenous mutants. It would be interesting to test this if sufficient spores could be obtained, particularly from *spoII* and *spoIII* oligosporogenous mutants. Until more is known of the effects on sporulation and germination of other mutations in this region we prefer to retain the designation *ger* rather than *spo* because the former conveys the information that spores are produced which germinate abnormally whereas the latter only indicates that sporulation is abnormal. Since gene expression does not occur during germination, sporulation must be abnormal to some extent in all *ger* mutants, even if the frequency of sporulation is not affected and the spores have no apparent structural abnormalities.

The Tn917::lacZ fusion studies suggest that *gerM* is expressed from about 1.5 h after resuspension, from a promoter to the *gerE* side of *gerM*. Other sporulation genes active at approximately the same time as *gerM* are *spoIIA* and *spoIID* (switched on at t_1) and *spoIIIC* ($t_{2.5}$) (Errington & Mandelstam, 1986a; Clarke *et al.*, 1986; Turner *et al.*, 1986). *gerJ* is also switched on at $t_{2.5}$ (Warburg *et al.*, 1986). During sporulation in Sterlini-Mandelstam medium (Waites *et al.*, 1970) at $t_{1.5}$ - $t_{2.5}$ the spore septum should be formed (stage II) and the prespore developed by invagination of the spore septum (stage III), so *gerM* is expressed at the right time to affect either of these processes. The facility to transfer *gerM96* into other genetic backgrounds means that *gerM* expression in the presence of other *spo* or *ger* mutations can be studied. Already we have evidence that *gerM* is not expressed in the presence of a *spoIID* mutation (R. L. Sammons, unpublished).

It is not clear from our experiments whether the fall in β -galactosidase activity after t_3 - t_4 is due to cessation of transcription or to some other factor such as an increased rate of degradation

of the enzyme. Errington & Mandelstam (1968*a*) showed that the rate of degradation of β -galactosidase was fairly constant for up to 1–2 h after commencement of sporulation, but there could be an increase in degradation rate after this time. Resistance to toluene, which starts to develop in wild-type cells at about $t_{3.5}$ (Jenkinson *et al.*, 1980), could affect the access of MUG to the spore although changes in permeability other than those involved in toluene resistance may be more important (Errington & Mandelstam, 1986*b*). To determine the actual time of cessation of transcription of *gerM* it will be essential to monitor the production of mRNA by Northern blot experiments with a *gerM*⁺ strain. This is important since the expression from the *gerM96::Tn917 lacZ* fusion strain could well be affected by the fusion itself, especially if the gene is autoregulated or regulated by positive or negative feedback mechanisms.

Repeated attempts to clone the 2.1 kb *EcoRI* fragment carrying *gerM* from several gene banks have been unsuccessful, suggesting that it may be difficult to clone some DNA sequence(s) in this region. However, the DNA cloned either side of *gerM96::Tn917* should be sufficient to encompass the *gerM* gene, and we have commenced sequencing these regions. The DNA sequence should yield information on the number of open reading frames in this region and perhaps indicate whether *gerM* could be a regulatory gene whose product could code for a DNA-binding protein.

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REFERENCES

- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology* **81**, 741–746.
- CLARKE, S., LOPEZ-DIAZ, I. & MANDELSTAM, J. (1986). Use of *lacZ* gene fusions to determine the dependence pattern of the sporulation gene *spoIID* in *spo* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2987–2994.
- COOTE, J. G. (1974). Comparative studies on induction of sporulation and synthesis of inducible enzymes in *Bacillus subtilis*. *Journal of Bacteriology* **120**, 1102–1108.
- CUTTING, S. & MANDELSTAM, J. (1986). The nucleotide sequence and the transcription during sporulation of the *gerE* gene of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 3013–3024.
- DANCER, B. N. & MANDELSTAM, J. (1975). Production and possible function of serine protease during sporulation of *Bacillus subtilis*. *Journal of Bacteriology* **121**, 406–410.
- EPSTEIN, M. A. & HOLT, S. J. (1963). The localisation by electron microscopy of HeLa cell surface enzymes splitting adenosine triphosphate. *Journal of Cell Biology* **19**, 325–336.
- ERRINGTON, J. & MANDELSTAM, J. (1983). Variety of sporulation phenotypes resulting from mutations in a single regulatory locus, *spoIIA*, in *Bacillus subtilis*. *Journal of General Microbiology* **129**, 2091–2101.
- ERRINGTON, J. & MANDELSTAM, J. (1986*a*). Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIA* in *spo* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2967–2976.
- ERRINGTON, J. & MANDELSTAM, J. (1986*b*). Use of a *lacZ* gene fusion to determine the dependence pattern and spore compartment expression of sporulation operon *spoVA* in *spo* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2977–2985.
- FEINBERG, A. P. & VOGELSTEIN, B. (1983, 1984). A technique for radiolabelling DNA restriction enzyme fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13; addendum **137**, 266–267.
- HASNAIN, S., SAMMONS, R., ROBERTS, I. & THOMAS, C. (1985). Cloning and deletion analysis of a genomic segment of *Bacillus subtilis* coding for the *sdhA*, *B*, *C* (succinate dehydrogenase) and *gerE* (spore germination) loci. *Journal of General Microbiology* **131**, 2269–2279.
- HAYAT, M. A. (1981). *Fixation for Electron Microscopy*, p. 408. New York & London: Academic Press.
- HILL, S. H. A. (1983). *spoVH* and *spoVJ* – new sporulation loci in *Bacillus subtilis* 168. *Journal of General Microbiology* **129**, 293–302.
- ISH-HOROWITZ, D. & BURKE, J. F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Research* **9**, 2989–2998.
- JAMET, C. & ANAGNOSTOPOULOS, C. (1969). Étude d'une mutation très faiblement transformable au locus de la thréonine désaminase de *Bacillus subtilis*. *Molecular and General Genetics* **105**, 225–242.

- JENKINSON, H. F., KAY, D. & MANDELSTAM, J. (1980). Temporal dissociation of late events in *Bacillus subtilis* sporulation from expression of genes that determine them. *Journal of Bacteriology* **141**, 793–805.
- KELLENBERGER, G., RYTER, A. & SÉCHAUD, A. (1958). Electron microscope study of DNA-containing plasmids. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *Journal of Biophysical and Biochemical Cytology* **4**, 671–676.
- LEIGHTON, T. J. & DOI, R. H. (1971). The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. *Journal of Biological Chemistry* **246**, 3189–3195.
- DE LENCASTRE, H. & PIGGOT, P. J. (1979). Identification of different sites of expression for *spo* loci by transformation of *Bacillus subtilis*. *Journal of General Microbiology* **114**, 377–389.
- LOSICK, R. & YOUNGMAN, P. (1984). Endospore formation in *Bacillus*. In *Microbial Development*, pp. 63–88. Edited by R. Losick & L. Shapiro. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology* **3**, 208–218.
- MEYER, R., FIGURSKI, D. & HELINSKI, D. R. (1977). Physical and genetic studies with restriction endonucleases on the broad host range plasmid RK2. *Molecular and General Genetics* **152**, 129–135.
- MILLER, J. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MOIR, A. (1981). Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. *Journal of Bacteriology* **146**, 1106–1116.
- MOIR, A. (1983). The isolation of λ transducing phages carrying the *citG* and *gerA* genes of *Bacillus subtilis*. *Journal of General Microbiology* **129**, 303–310.
- MOIR, A., LAFFERTY, E. & SMITH, D. A. (1979). Genetic analysis of spore germination mutants of *Bacillus subtilis* 168: the correlation of phenotype with map locations. *Journal of General Microbiology* **111**, 165–180.
- MOIR, A., FEAVERS, I. M., ZUBERI, A. R., SAMMONS, R. L., ROBERTS, I. S., YON, J. R., WOLFF, E. A. & SMITH, D. A. (1985). Progress in the molecular genetics of spore germination in *Bacillus subtilis* 168. In *Molecular Biology of Microbial Differentiation*, pp. 35–46. Edited by J. A. Hoch & P. Setlow. Washington, DC: American Society for Microbiology.
- PERKINS, J. B. & YOUNGMAN, P. J. (1984). A physical and functional analysis of Tn917, a *Streptococcus* transposon in the Tn3 family that functions in *Bacillus*. *Plasmid* **12**, 119–138.
- PIGGOT, P. J. & COOTE, J. G. (1976). Genetic aspects of bacterial endospore formation. *Bacteriological Reviews* **40**, 908–962.
- REED, K. C. & MANN, D. A. (1985). Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Research* **13**, 7207–7221.
- REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.
- RUTBERG, B. & HOCH, J. A. (1970). Citric acid cycle: gene-enzyme relationships in *Bacillus subtilis*. *Journal of Bacteriology* **104**, 826–833.
- SAMMONS, R. L., MOIR, A. & SMITH, D. A. (1981). Isolation and properties of spore germination mutants of *Bacillus subtilis* deficient in the initiation of germination. *Journal of General Microbiology* **124**, 229–241.
- SCOTT, I. R. & ELLAR, D. J. (1978). Study of calcium dipicolinate release during bacterial spore germination by using a new, sensitive assay for dipicolinate. *Journal of Bacteriology* **135**, 133–137.
- STERLINI, J. M. & MANDELSTAM, J. (1969). Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. *Biochemical Journal* **113**, 29–37.
- THOMAS, C. M. (1981). Complementation analysis of replication and maintenance functions of broad host range plasmids RK2 and RP1. *Plasmids* **5**, 277–291.
- TROWSDALE, J. & SMITH, D. A. (1975). Isolation, characterisation and mapping of *Bacillus subtilis* 168 germination mutants. *Journal of Bacteriology* **123**, 83–95.
- TURNER, S. M., ERRINGTON, J. & MANDELSTAM, J. (1986). Use of a *lacZ* fusion to determine the dependence pattern of sporulation operon *spoIIIC* in *spo* mutants of *Bacillus subtilis*: a branched pathway of expression of sporulation operons. *Journal of General Microbiology* **132**, 2995–3003.
- WAITES, W. M., KAY, D., DAWES, I. W., WOOD, D. A., WARREN, S. C. & MANDELSTAM, J. (1970). Sporulation in *Bacillus subtilis*. Correlation of biochemical events with morphological changes in asporogenous mutants. *Biochemical Journal* **118**, 667–676.
- WARBURG, R. J. & MOIR, A. (1981). Properties of a mutant of *Bacillus subtilis* 168 in which spore germination is blocked at a late stage. *Journal of General Microbiology* **124**, 243–253.
- WARBURG, R. J., BUCHANAN, C. E., PARENT, K. & HALVORSON, H. O. (1986). A detailed study of *gerJ* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2309–2319.
- YON, J. R. (1986). *Analysis of a spore germination gene of Bacillus subtilis* 168. PhD thesis, University of Birmingham, UK.
- YOUNGMAN, P. J., PERKINS, J. B. & LOSICK, R. (1983). Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with the *Streptococcus faecalis* transposon Tn917. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 2305–2309.
- YOUNGMAN, P. J., PERKINS, J. B. & LOSICK, R. (1984). A novel method for the rapid cloning of *Escherichia coli* of *Bacillus subtilis* chromosomal DNA adjacent to Tn917 insertions. *Molecular and General Genetics* **195**, 424–433.
- YOUNGMAN, P. J., ZUBER, P., PERKINS, J. B., SANDMAN, K., IGO, M. & LOSICK, R. (1985a). New ways to study developmental genes in spore-forming bacteria. *Science* **228**, 285–291.
- YOUNGMAN, P. J., PERKINS, J. B. & SANDMAN, K. (1985b). Use of Tn917-mediated transcriptional gene fusions to *lacZ* and *cat-86* for the identification and study of genes in *Bacillus subtilis*. In *Molecular Biology of Microbial Differentiation*, pp. 47–54. Edited by J. A. Hoch and P. Setlow. Washington, DC: American Society for Microbiology.