Cloning and Sequencing of the *gerD* Gene of *Bacillus subtilis*

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A Tn917 insertion in the same region of the chromosome as *gerD* gave rise to a mutant (*ger-97*) with a germination phenotype similar to that of two *gerD* mutants which germinate abnormally in a range of germinants. The insertion and two *gerD* mutations were cotransformed with ribosomal protein genes *rpoB*, *rpsE* and *rpsI*. DNA cloned from one side of the insertion carried the 16S end of the ribosomal RNA operon *rrnI*. These data were consistent with the order *rpoB-rpsE-rpsI-gerD/ger-97::Tn917-rrnI*. Insertion into the wild-type chromosome of a plasmid carrying DNA adjacent to the insertion permitted the recovery of a 1.8 kb fragment of DNA which complemented *ger-97::Tn917* and the *gerD* mutations. The DNA nucleotide sequence of the region of this fragment at which Tn917 had inserted revealed a 555 bp open reading frame, preceded by a ribosome-binding site and potential σ^E and σ^A promoter regions and encoding a predicted polypeptide of 21 117 Da. This polypeptide was largely hydrophilic but contained a hydrophobic region at the N-terminus resembling a signal peptide.

INTRODUCTION

Spores of *Bacillus subtilis* germinate in response to L-alanine (Ala) or a mixture of L-asparagine, glucose, fructose and KCl (AGFK). Mutants have been isolated which germinate abnormally in response to either or both of these germinants (Moir *et al.*, 1979, 1985; Piggot *et al.*, 1981). The mutations giving rise to germination abnormalities have been mapped in thirteen chromosomal locations (*gerA-M*) and so far *gerA*, *C*, *E* and *M* have been cloned (Moir, 1983; Yazdi, 1989; James & Mandelstam, 1985; Hasnain *et al.*, 1985; Sammons *et al.*, 1987).

The products of *ger* genes and their roles in germination are largely unknown, although the *gerG* mutation is associated with loss of phosphoglycerate kinase (Prasad *et al.*, 1972). *gerE* may play a regulatory role and be responsible for the appearance of several spore components including protease E (Cutting & Mandelstam, 1986), which is absent in the *gerE* mutant (Jenkinson & Lord, 1983). The gene product predicted from the *gerE* DNA sequence appears to have the potential to bind the DNA (Holland *et al.*, 1987). It is likely that the three open reading frames identified in the *gerA* region encode components of the L-alanine receptor (Zuberi *et al.*, 1985), whilst *gerK* may be concerned with binding of glucose (Irie *et al.*, 1982) and *gerD* with that of fructose (Irie *et al.*, 1985). *gerJ* may be involved in the regulation of synthesis of components of the spore cortex (Warburg *et al.*, 1986).

In this paper we describe the identification of a Tn917 insertion in the *gerD* region of the chromosome between *rpsI* and *rrnI* which gave rise to a mutant with a phenotype like that of

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Abbreviations: Ala, L-alanine; AGFK, asparagine/glucose/fructose/KCl; ORF, open reading frame.

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previously isolated *gerD* mutants. This insertion has permitted the cloning of DNA which complements the insertion and *gerD* mutations. The region of DNA in which the insertion occurred has been sequenced and the *gerD* open reading frame identified.

METHODS

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

Media. (a) *B. subtilis*. Nutrient media were Difco Penassay Broth (PAB), Difco Nutrient Agar (NA) and potato/glucose/yeast extract agar (PGA; Dring & Gould, 1971). Spores were prepared on NA or PGA or in CCY medium (Wilkinson *et al.*, 1977) as described by Moir *et al.* (1979). Chloramphenicol (5 µg ml⁻¹), rifampicin (10 µg ml⁻¹), spectinomycin dihydrochloride (200 µg ml⁻¹), kasugamycin hemisulphate (4 mg ml⁻¹) and erythromycin (1 µg ml⁻¹) plus lincomycin (25 µg ml⁻¹) were added to NA to select for Cam^R, Rif^R, Spc^R, Ksg^R and MLS^R phenotypes respectively. (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 Kan^R and Amp^R colonies respectively.

Genetical methods. Transformation of *E. coli* and *B. subtilis* was done as described by Sammons *et al.* (1987), using 0.2 µg DNA ml⁻¹ in the case of *B. subtilis* transformation. To select for Rif^R or Spc^R transformants, after the 90 min incubation of competent cells with DNA, an equal volume of L-broth was added and incubation continued for a further 3 h to allow time for expression of mutant genes before plating on selective media.

Spore germination. This was monitored by following loss of optical density at 580 nm (OD₅₈₀) of spore suspensions after addition of germinants. Prior to germination, spores were heat-activated at 70 °C for 30 min as described by Sammons *et al.* (1981). The spore germination phenotype of bacterial colonies was determined by the tetrazolium overlay method (Trowsdale & Smith, 1975), in which Ger⁺ colonies are red (tzm-red) and Ger⁻ colonies white (tzm-white).

Gene cloning techniques. Extraction of chromosomal or plasmid DNA, DNA restriction and ligation, gel electrophoresis, Southern blotting and hybridization were all carried out as previously described (Sammons *et al.*, 1987). DNA fragments were size-fractionated by electrophoresis using low-gelling-temperature agarose, followed by electroelution.

DNA sequencing. To determine the sequence of the 1.86 kb *Pst*I fragment from λ2H it was cloned in both orientations into the *Pst*I site of M13mp19 (Yanisch-Perron *et al.*, 1985). Digestion with *Sal*I and *Kpn*I allowed progressive deletions to be made into the insert with exonuclease III as described by Henikoff (1984). Following S1 nuclease treatment the ends were filled in using Klenow polymerase, ligated and the DNA used to transfect *E. coli* strain JM101. This gave a series of clones which were sequenced by the method of Sanger *et al.* (1977) as described in the Bethesda Research Laboratory manual 'M13 Cloning and Dideoxy Sequencing' (1980). Buffer gradient gels were poured as described by Biggin *et al.* (1983).

Phage λ techniques. Preparation of plating cells, phage propagation and assay, purification and reisolation of phage from single plaques were as described by Murray *et al.* (1973). Large-scale preparation of λ DNA was done as described by Sammons & Anagnostopoulos (1982). A library of *B. subtilis* DNA was constructed in λEMBL3 as follows. Chromosomal DNA from strain 1604 was digested to completion with *Bgl*II and ligated to λEMBL3 DNA cleaved with *Bam*HI and *Eco*RI. The recombinant DNA was packaged using a packaging extract prepared as described by Maniatis *et al.* (1982). To identify clones carrying DNA from the *ger-97* region, approximately 2000 plaques were screened by hybridization to the ³²P-labelled 2.1 kb *Eco*RI-*Pst*I fragment from pBMD1 (Fig. 3) using the method of Benton & Davis (1977). Nine plaques which gave positive signals were rescreened at low density and DNA prepared from phage taken from single positive plaques was analysed by restriction enzyme digestion.

RESULTS

Isolation of the ger-97::Tn917 mutant

Transposon Tn917 mutagenesis of strain PY143 was carried out using the method of Youngman *et al.* (1983) as previously described (Sammons *et al.*, 1987). A mutant was isolated which was MLS^R and white by the tetrazolium overlay test (strain 5189). To study the effect of the transposon insertion (*ger-97::Tn917*) in the same genetical background as other strains in our laboratory, it was transformed into strain 1604 by selection for MLS^R. All the transformants were tzm-white and one representative, strain 5215, whose Ger⁻ phenotype was identical to that of the original mutant, was chosen for further study.

Table 1. *Bacterial strains, plasmids and phages*

Bacterial strains	Genotype and/or phenotype	Source, reference or origin
<i>B. subtilis</i>		
PY143	Prototroph carrying pTV1	Youngman <i>et al.</i> (1983)
1604	<i>trpC2</i> (wild-type)	Lafferty & Moir (1977)
CA302	<i>cysA14 trpC2 purA16 rpsE302</i> Spc ^R	Dabbs (1983)
CA310	<i>cysA14 trpC2 purA16 rpoB rpsI2</i> Rif ^R Ksg ^R	E. R. Dabbs (Max-Planck Institute, Berlin)
4738	<i>trpC2 gerD48</i>	EMS mutagenesis of 1604; Warburg <i>et al.</i> (1985)
4928	<i>arg-15 leuA8 thr-5</i> r _M ⁻ m _M ⁻ <i>recE4</i>	BGSC (strain 1A423)
4930	<i>arg-15 leuA8</i> r _M ⁻ m _M ⁻	BGSC (strain 1A253)
5183	<i>trpC2 gerD25</i>	NTG mutagenesis of 1604; Roberts (1983)
5189	<i>ger-97::Tn917</i> MLS ^R	Tn917 mutagenesis of PY143 (this study)
5207	<i>trpC2 rpsE302</i> Spc ^R	<i>rpsE302</i> transformed from CA302 into 1604
5208	<i>trpC2 rpsI2 rpoB</i> Ksg ^R Rif ^R	<i>rpsI2</i> and <i>rpoB</i> transformed from CA310 into 1604
5213	<i>gerD48</i>	4738 × PY79
5215	<i>trpC2 ger-97::Tn917</i> MLS ^R	This study
5224	<i>arg-15 leuA8</i> r _M ⁻ m _M ⁻ <i>ger-97::Tn917</i> MLS ^R	4930 × 5215 selecting MLS ^R
5225	<i>leuA8</i> r _M ⁻ m _M ⁻ <i>gerD48</i>	4930 × 5213 (congression, selecting Arg ⁺)
5226	<i>arg-15</i> r _M ⁻ m _M ⁻ <i>rec E4 ger-97::Tn917</i> MLS ^R	5224 × 4928 (congression, selecting Leu ⁺)*
5227	<i>trpC2</i> r _M ⁻ m _M ⁻ <i>rec E4 gerD48</i>	5225 × 4928 (congression, selecting Leu ⁺)
5277	<i>trpC2 ger-97::Tn917::pTV21Δ2</i> Cm ^R	5215 × pTV21Δ2 selecting Cm ^R
<i>E. coli</i>		
NEM259	<i>supE supF hsdR hsdM⁺ met trpR</i>	J. Guest (Sheffield University, UK)
TBI	<i>ara Δ(lac-proAB) rpsL φ80 lacZ</i> M15 <i>hsdR hsdM⁺</i>	Bethesda Research Laboratories
JM101	<i>supE thi Δ(lac-proAB) F' traD36</i> ProAB ⁻ <i>lacI lacZ</i> M15	Messing (1983)
Plasmids	Description/derivation	Origin or reference
pTV1	Cm ^R MLS ^R ; carries Tn917	Youngman <i>et al.</i> (1983)
pTV20	Cm ^R Ap ^R MLS ^R ; Tn917 DNA	Youngman <i>et al.</i> (1984)
pTV21Δ2	Cm ^R Ap ^R ; Tn917 DNA	Youngman <i>et al.</i> (1984)
pSGMU2	Cm ^R Ap ^R ; <i>B. subtilis</i> integrative vector with cloning site from pUC13	Fort & Errington (1985)
pMK4	<i>E. coli/B. subtilis</i> shuttle vector	Sullivan <i>et al.</i> (1984)
pBMD1	Derived from pTV20 in 5215	This study
pBMD2		
pBMD7		
pBMD10		
pBMD11	Derived from pTV21Δ2 in 5215	This study
pBMD12		
pBMD13	<i>ger97</i> region cloned in pSGMU2	This study
pBMD14		
pBMD16		
pBMD17		
pBMD18	1·8 kb <i>Pst</i> I <i>ger-97</i> fragment cloned in pMK4	This study
	1·8 kb <i>Pst</i> I <i>ger-97</i> fragment cloned in pSGMU2	This study
Phages	Description/derivation	Origin or reference
λP13	Carries part of <i>rrnG</i>	Bott <i>et al.</i> (1984)
λEMBL3	λ replacement vector	Frischauf <i>et al.</i> (1983)
λ2H	Recombinant phage carrying rRNA sequences	This study
M13mp19		Yanisch-Perron <i>et al.</i> (1985)

Abbreviations. Antibiotic resistances: Spc^R, specinomycin; Rif^R, rifampicin; Ksg^R, kasugamycin; MLS^R, macrolide lincosamide and streptogramin B; Ap^R, ampicillin; Cm^R, chloramphenicol. EMS, ethylmethane sulphate; NTG, nitrosoguanidine. BGSC, *Bacillus* Genetic Stock Center.

* The Leu⁺ allele was supplied by 1604 DNA also present in the transformation mixture.

Fig. 1. Comparison of spore germination phenotypes of *gerD25*, *gerD48* and *ger-97::Tn917* mutants. (representative experiments showing loss of OD₅₈₀). (a) In Ala (solid lines) and AGFK (broken lines). (b) In Ala; spores prepared on PGA (solid lines) and in CCY medium (broken lines). (c) In Ala (solid lines) and Ala + KCl (broken lines). ●, Wild-type, strain 1604; ■, *gerD25* mutant, strain 5183; ▲, *gerD48* mutant, strain 4738; ▼, *ger-97::Tn917* mutant, strain 5215. The concentrations of germinants or adjuncts were: Ala and KCl, 1 mM; asparagine, glucose and fructose, 10 mM. These experiments were done at least three times; variation in loss of OD₅₈₀ never exceeded $\pm 5\%$.

Phenotype of the ger-97::Tn917 mutant

Vegetative growth and sporulation of strain 5215 appeared to be normal. Spores were prepared on PGA plates and germination was compared with that of spores of the wild-type and previously isolated *gerD* mutants, strains 4738 (*gerD48*) and 5183 (*gerD25*), prepared at the same time. Germination was monitored by following loss of OD₅₈₀ after the addition of Ala and AGFK to spore suspensions. The OD₅₈₀ of all the mutant suspensions fell by approximately 20% in Ala in 60 min whilst that of the wild-type strain fell by approximately 50%. In AGFK the OD₅₈₀ of mutant spores remained unaltered whilst that of the wild-type suspension fell by approximately 40% (Fig. 1a).

Warburg (1981) found that the sporulation medium affected the subsequent rate of germination of spores of *gerD* mutant strains. To test this, instead of the resuspension technique used by Warburg, spores were prepared in CCY medium, as this exhaustion medium was simpler to use but produced a similar effect on germination. The rates of response of spores of the *gerD25*, *gerD48* and *ger-97::Tn917* mutants all increased by a similar amount when spores were prepared in this medium (Fig. 1b).

Spores of *gerD* mutants show an improved response to Ala as germinant when K⁺ or other monovalent cations are also present (Warburg *et al.*, 1985). However, the response of spores of strain 5215 to Ala was not improved by the addition of KCl whilst the rates of germination of suspensions of spores of strains 4738 and 5183 showed a slight increase when KCl was present (Fig. 1c).

These experiments suggested that the germination phenotype of the *ger-97::Tn917* mutant was similar to that of the *gerD* mutants except that no improvement in the rate of germination in Ala was seen on addition of KCl.

Map location of gerD25, gerD48 and ger-97::Tn917 mutations

Using phage PBS1, *gerD* mutations were 77–80% cotransduced with *cysA* (Moir *et al.*, 1979), and using phage SPPI, they were 1–8% cotransduced with *rplV*, the order of markers being *cysA-rplV-gerD* (Warburg *et al.*, 1985). *rplV* is one of a group of ribosomal protein genes in this area which include *rpoB*, *rpsE* and *rpsI* (Dabbs, 1983; Henkin & Chambliss, 1984; Fig. 2). Since it seemed likely that *gerD* and the *Tn917* insertion would be close to or within this cluster, *gerD25*, *gerD48* and *ger-97::Tn917* were mapped with reference to these genes.

DNA from strains 5208 (*rpoB rpsI2 trpC2*) and 5207 (*rpsE302 trpC2*) was used to transform strains 5183, 4738 and 5215, selecting for rifampicin resistance (*rpoB*) or spectinomycin resistance (*rpsE*). It was necessary to perform the crosses with strains 5208 and 5207 as donors because these strains did not become competent by the procedure used: typically 1000-fold fewer transformants were obtained with these strains as recipients.

The lesions in *gerD48* and *gerD25* appeared to be very close, as each was transformed at essentially the same frequency with *rpoB* (6%) and *rpsE302* (31%) (Table 2). These results suggest that *gerD* is further from *rpoB* than it is from *rpsE302*. Taken together with published cotransformation frequencies in this region (Fig. 2), the data suggest the order *cysA-rpoB-rpsE-gerD*. The finding of Warburg (1981) that *gerD* was not cotransformed with *cysA* also supports this order. The crosses between strains 5208 and 4738 or 5183 were scored with *gerD* and *rpsI2* (kasugamycin resistance) as unselected markers. In both cases the percentage of Rif^R and Ksg^R transformants (20% and 16% for the crosses with strains 4738 and 5183 respectively) was higher than the percentage of Rif^R tzm-red transformants (6% in each case), indicating that the distance from *rpsI* to *rpoB* is less than the distance from *gerD* to *rpoB*. Since cotransformation frequencies were low and the rarest class of recombinants in the crosses with strains 4738 and 5183 different, no conclusions were drawn from these crosses about the order of these markers. However, *rpsI* is cotransformed at low frequency with *cysA* (Fig. 2), suggesting that it lies between *gerD* and *cysA*. Fig. 2 shows the most likely order of markers in this region compiled from published transformation data and the results in Table 2. It is not clear how transposon *Tn917* affects the results of cotransformation experiments, as the lesion results from the insertion of 5.4 kb of DNA. The transposon may affect linkage frequencies differently

Table 2. *Transformation crosses with gerD and ger-97::Tn917*

Donor*	Recipient*	Selected marker	Recombinant class	No.
5208 (<i>trpC2 rpoB rpsI2</i>)	5183 (<i>trpC2 gerD25</i>)	Rif ^R	Rif ^R Ksg ^R Ger ⁻	16
			Rif ^R Ksg ^S Ger ⁻	109
			Rif ^R Ksg ^R Ger ⁺	5
			Rif ^R Ksg ^S Ger ⁺	2
				132
5208	4738 (<i>trpC2 gerD48</i>)	Rif ^R	Rif ^R Ksg ^R Ger ⁻	24
			Rif ^R Ksg ^S Ger ⁻	101
			Rif ^R Ksg ^R Ger ⁺	2
			Rif ^R Ksg ^S Ger ⁺	5
				132
5208	5215 (<i>trpC2 ger-97::Tn917</i>)	Rif ^R	Rif ^R Ksg ^R Ger ⁻	52
			Rif ^R Ksg ^S Ger ⁻	190
			Rif ^R Ksg ^R Ger ⁺	19
			Rif ^R Ksg ^S Ger ⁺	5
				264
5207 (<i>trpC2 rpsE302</i>)	5183	Spc ^R	Spc ^R Ger ⁻	91
			Spc ^R Ger ⁺	41
				132
5207	4738	Spc ^R	Spc ^R Ger ⁻	91
			Spc ^R Ger ⁺	41
				132
5207	5215	Spc ^R	Spc ^R MLS ^R (Ger ⁻)	110
			Spc ^R MLS ^R (Ger ⁺)	22
				132

* *rpoB* confers Rif^R, *rpsI2* Ksg^R, *rpsE302* Spc^R and Tn917 MLS^R.

depending whether it is present in the donor or recipient strain. The results in Table 2 imply that *ger-97::Tn917* is further from *rpoB* than from *rpsE* (cotransformation frequencies of *ger-97::Tn917* with these markers of 8% and 17% respectively), at or near the *gerD* locus (Fig. 2).

Cloning DNA adjacent to ger-97::Tn917

Since the mapping data suggested that *ger-97::Tn917* was in the same region of the chromosome as *gerD*, efforts were made to clone an intact copy of the region in which this insertion had occurred, to test whether this DNA could repair *gerD* mutations. Plasmids pTV20 and pTV21Δ2 were used to clone DNA from either side of *ger-97::Tn917*. They were linearized and used to transform strain 5215 to chloramphenicol resistance as described by Youngman *et al.* (1984). This resulted in the introduction of pBR322 replication functions and Ap^R and Cm^R markers into the transposon insertion by a double crossover recombination event. Chromosomal DNA was prepared from a single transformant from each cross, digested to completion with *EcoRI*, which cuts once between the Ap^R and Cm^R markers, ligated and used to transform *E. coli* strain NEM259 to ampicillin resistance. From chromosomal DNA carrying pTV20 two plasmids (pBMD1 and pBMD2, Fig. 3) were obtained. When pBMD1 and pBMD2 were digested with *EcoRI* they each yielded an identical 9.0 kb fragment containing some pTV20 DNA and, additionally, different *EcoRI* fragments in each case (not shown in Fig. 3). Since in the preparation of these plasmids the chromosomal DNA had been digested to completion with *EcoRI* it is unlikely that the additional fragments in either case represented contiguous chromosomal DNA. No plasmids were obtained which consisted only of the 9.0 kb fragment.

Fig. 2. Order of markers in the *gerD* region based on transformation data. The numbers above the arrows are (100 - percentage cotransformation). Compiled from the data in Table 2 and (a) Warburg (1981) and (b) Dabbs (1983) and Henkin & Chambliss (1984). ▼, indicates the site of Tn917 insertion.

Fig. 3. Partial restriction map of the *gerD-rnnI-rnnH-rnnG* region and cloned DNA. ▲, indicates the site of Tn917 insertion. The hatched boxes represent *rnn* operons (redrawn from Jarvis *et al.*, 1988). Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; H, *Hind*III; B, *Bam*HI; R, *Eco*RV. Only the *Eco*RI sites on the *rnn* operons and the sizes of *Eco*RI fragments (kb) of λ P13 are shown.

The restriction map of pTV20 (Youngman *et al.*, 1984) indicates that approximately 6.4 kb of the plasmid recovered will contain sequences from pTV20 which carry replication functions and the Ap^R gene. The 9.0 kb fragment therefore contains about 2.6 kb of *B. subtilis* DNA adjacent to the site of the Tn917 insertion (Fig. 3).

pBMD11 (Fig. 3) was prepared from chromosomal DNA carrying pTV21Δ2 integrated into the *ger-97::Tn917* insertion, and consists of a single 4.5 kb *Eco*RI fragment. Approximately 4.3 kb of this is likely to be derived from pTV21Δ2 (Youngman *et al.*, 1984) suggesting that the remaining 0.2 kb is *B. subtilis* chromosomal DNA. By combining the data from the pBMD1, pBMD2 and pBMD11 clones it was estimated that Tn917 has inserted 0.2 kb from one end of a 2.8 kb *Eco*RI fragment (later more accurately estimated to be 2.9 kb). This was confirmed by hybridization of pBMD11 DNA to a 2.9 kb *Eco*RI chromosomal fragment from the wild-type (data not shown).

A further plasmid, pBMD12, was isolated after partial *Eco*RI digestion of chromosomal DNA from the strain carrying pTV21Δ2 integrated into *ger-97::Tn917*. This had the 4.5 kb *Eco*RI fragment of pBMD11 plus additional fragments of 2.6 and 0.3 kb (Fig. 3). The 2.6 kb *Eco*RI fragment of pBMD12 hybridized to a chromosomal *Eco*RI fragment of this size, to *Pst*I fragments of 1.8 and 1.6 kb, and to *Hind*III fragments of 3.7 kb (as did pBMD11) and 0.5 kb (data not shown). This confirmed that the 2.9 and 2.6 kb fragments were adjacent and also suggested that the 0.3 kb *Eco*RI fragment is contiguous since the *Pst*I site at one end of the 1.6 kb *Pst*I fragment lies within this fragment. The restriction map of the chromosome to the left-hand side of the Tn917 insertion is therefore as shown in Fig. 3.

Demonstration that ger-97 is adjacent to rrnI

Surprisingly, when the 9.0 kb fragment of pBMD1 was used as a hybridization probe it hybridized to a range of chromosomal *Eco*RI fragments, including one of 2.9 kb, suggesting that the fragment carried a sequence that was repeated in the chromosome. The estimated sizes of these *Eco*RI fragments (9.6, 4.1, 3.7, 2.9, 1.9, 1.6 and 1.3 kb) showed close agreement with the sizes of chromosomal *Eco*RI fragments which hybridize to 16S ribosomal RNA (10, 4.0, 3.5, 3.0, 2.0, 1.8, 1.5 and 0.8 kb; Bott *et al.*, 1981). This suggested that Tn917 had inserted into a DNA fragment which contained part of a 16S RNA gene.

rRNA operons have been located at nine sites (*rrnA*, *B*, *C*, *G*, *H*, *I*, *J*, *W* and *O*) on the chromosome (Bott *et al.*, 1984; Piggot & Hoch, 1985; Jarvis *et al.*, 1988). Since *rrnI*, *H* and *G* are located adjacent to each other in the region of *gerD* (Fig. 3; Jarvis *et al.*, 1988) it seemed likely that *ger-97::Tn917* was located close to the 16S end of one of these operons. To determine whether this was the case, use was made of the fact that a chromosomal restriction fragment would increase in size when Tn917 inserted into it, provided Tn917 itself did not carry a cleavage site for the enzyme. Phage λP13 (Bott *et al.*, 1984) carries part of *rrnG* (Fig. 3). The 4.6 kb *Eco*RI fragment of this phage, which contains a *Bgl*II site, was used to probe chromosomal DNA from the wild-type and from 5277, the strain in which pTV21Δ2 had integrated into *ger-97::Tn917*, as this carried approximately 10 kb of integrated DNA with no *Bgl*II sites. The probe was expected to hybridize to two chromosomal *Bgl*II fragments. Since there are no *Bgl*II sites within *rrnG*, *H* or *I*, and none between the site of Tn917 insertion and the 16S end of whichever operon lies close to it, then if this is *rrnI*, *H* or *G* one of the hybridizing fragments from strain 5277 should increase in size. As predicted, the 4.6 kb probe hybridized to two chromosomal *Bgl*II fragments of approximately 20 and 25 kb. The 20 kb fragment increased in size to about 30 kb when DNA was inserted at the site of the *ger-97::Tn917* mutation in strain 5277. This suggests that *ger-97::Tn917* does lie within or to the left of the 4.6 kb *Eco*RI fragment in Fig. 3.

When the 2.4 kb *Eco*RI fragment from phage λP13 was used to probe *Bgl*II-digested chromosomal DNA, hybridization occurred to the larger, 25 kb fragment to which the 4.6 kb probe had hybridized in wild-type DNA and this fragment did not increase in size in strain 5277. This further indicates that *ger-97::Tn917* is to the left of the 4.6 kb *Eco*RI fragment and not to the right.

It seemed most likely that Tn917 had inserted near to the 16S end of *rrnI* (Fig. 3). The alternative possibilities, insertion between *rrnI* and *rrnH* or between *rrnH* and *rrnG*, were excluded because DNA cloned from the left-hand side of the insertion, using pTV21Δ2, would then also have been expected to have included sequences of rRNA genes repeated in other regions of the chromosome and therefore to be able to hybridize to a range of *Eco*RI fragments, as did the 9.0 kb fragment of pBMD1.

Fig. 4. Construction of pBMD13 and pBMD14. The wide solid lines represent pSGMU2 DNA, the open box Tn917 DNA. The restriction map shows 1604 DNA into which pBMD7 has integrated. Restriction sites are abbreviated as in Fig. 3. The chromosomal DNA was partially digested with *Hind*III, fragments below 5.5 kb removed and the remainder ligated and used to transform *E. coli*, selecting Amp^R.

Cloning the ger-97 region of the B. subtilis chromosome

The 1.5 kb *Hind*III–*Pst*I fragment of pBMD1 and the 1.1 kb *Bam*HI–*Pst*I fragment of pBMD12 (Fig. 3) were cloned into pSGMU2 (Fort & Errington, 1985) to give plasmids pBMD7 and pBMD10 respectively (Fig. 3). To determine whether these fragments were internal to the *ger-97* transcriptional unit, plasmid DNA was used to transform strain 1604 to chloramphenicol resistance and the germination phenotype of transformants tested by the tetrazolium overlay method. A Cam^R Ger⁺ strain and the *ger-97::pTV21Δ2* strain, 5227, were used as Ger⁺ and Ger[–] controls respectively. Transformants from both the pBMD7 × 1604 and pBMD10 × 1604 crosses were tzm-red, suggesting that both ends of the *ger-97* transcriptional unit were present within the 1.8 kb *Pst*I fragment into which Tn917 had inserted (Fig. 3). Chromosomal DNA was prepared from a transformant of the cross pBMD7 × 1604 which was presumed to contain pBMD7 integrated as shown in Fig. 4. The DNA was partially digested with *Hind*III and size-fractionated to remove fragments smaller than 5.5 kb, in case integration of pBMD7 had resulted in tandem duplications of the 5.2 kb plasmid. Fragments over 5.5 kb were ligated and used to transform *E. coli* strain TB1 to ampicillin resistance. Three transformants were obtained which carried plasmids with an intact copy of the 1.8 kb *Pst*I fragment (Fig. 4). Two plasmids carried DNA to the next left-hand chromosomal *Hind*III site (pBMD13) and one also carried the *Hind*III fragment after that, an additional 0.5 kb (pBMD14). Circular DNA of pBMD13 and pBMD14 was used to transform strain 5215 to chloramphenicol resistance. Since circular DNA was used the plasmids would have integrated by Campbell recombination, resulting in a tandem duplication of the cloned DNA. One hundred transformants were allowed to sporulate on NA + chloramphenicol and tested for their germination phenotype by the tetrazolium test (with the controls as described above): all were red. Spores were prepared from one transformant from each cross and tested for their ability to germinate in Ala and AGFK. They germinated as well as strain 1604, suggesting that the cloned DNA was capable of complementing the *ger-97::Tn917* mutation.

The *Pst*I fragment from pBMD13 was subcloned into pSGMU2, forming pBMD18, and circular DNA of this plasmid was used to transform strains 5215, 5183 and 4738 to chloramphenicol resistance. In each case all of the 100 transformants tested were tzm-red and spores from representatives of each germinated as well as those of the wild-type strain. Thus the 1.8 kb *Pst*I fragment was able to complement *ger-97::Tn917*, *gerD25* and *gerD48* mutations, suggesting that it carried an intact *gerD* gene or genes. Conversely, pBMD7 and pBMD10 were not able to complement any of the *ger* mutations.

Fig. 5. (a) Strategy for sequencing the region of the 1.8 kb *Pst*I fragment where Tn917 had inserted. Arrows indicate the starting points and extent of sequence determined from each clone. (b) ORFs preceded by ribosome-binding sites. P, *Pst*I; Bc, *Bcl*I; B, *Bam*HI; E, *Eco*RI.

The 1.8 kb *Pst*I fragment from pBMD13 was also subcloned in both orientations in plasmid pMK4, which is able to replicate in *B. subtilis*, forming plasmids pBMD16 and pBMD17. RecE⁻ strains 5226 and 5227 (constructed as shown in Table 1) were transformed with pBMD16 and pBMD17 and compared by the tetrazolium overlay test with the parental strains and strain 4930, each carrying pMK4. The transformant colonies from all the crosses were pink as compared with the red colonies of 4930(pMK4) or white colonies of 5226(pMK4) or 5227(pMK4), suggesting that complementation of the *ger* mutations was not perfect. This was confirmed by the rates of germination of spores of the transformant colonies, which were intermediate between those of the Ger⁺ and Ger⁻ controls. One explanation for this result could be that multiple copies of *gerD* or some other sequence on the 1.8 kb fragment are suboptimal for germination.

Cloning the ger-97 region in a λ vector

The approximately 20 kb *Bgl*II fragment carrying *ger-97* was within the cloning capacity of λEMBL3 (Frischauf *et al.*, 1983). A λEMBL3 bank of strain 1604 DNA was constructed and screened for phage carrying DNA homologous to the *ger-97* region as described in Methods. Restriction enzyme analysis and hybridization studies showed that the DNA from one of nine phages (λ2H) which gave positive hybridization signals came from the *ger-97* region. The cloned DNAs from the remaining eight phages had restriction maps consistent with their having been derived from the region of *rrnB* (Bott *et al.*, 1984; not shown). Phage λ2H appeared from hybridization studies and its restriction map to contain DNA from the 16S end of *rrnI* and the 5S end of *rrnG* but the intervening *rrn* operons and intervening sequences had been deleted (Fig. 3). A possible explanation for this is that the phage was isolated in a recombination-proficient host which would have permitted homologous recombination to take place between *rrnI* and *rrnG* with the deletion of the equivalent of two *rrn* operons. The restriction map of the cloned DNA in λ2H was identical, in the region to the left of the 16S end of *rrnI* (Fig. 3), to that obtained from the clones derived using pTV20 and pTV21Δ2, confirming the restriction map of this region.

DNA nucleotide sequence of the 1.8 kb PstI fragment

The 1.8 kb *Pst*I fragment of λ2H was cloned into M13mp19 and sequenced as described in Methods. Fig. 5 shows the extent of the sequences derived from clones in the region of the Tn917 insertion. In this region an open reading frame (ORF) starting with an ATG codon and preceded by a sequence (GAAAGGAG) complementary to the 3' end of *B. subtilis* 16S rRNA was identified starting at position 381 of the sequence shown in Fig. 6 and terminating at an ochre codon (TAA) at position 936. This reading frame spans the *Eco*RI site and the site of Tn917 insertion situated approximately 300 bp upstream and codes for a polypeptide of

Fig. 6. DNA nucleotide sequence of the region of the Tn917 insertion in the 1.8 kb fragment, showing the amino acid sequence of the proposed *ger-97* polypeptide, potential promoter and terminator regions and ribosome-binding sites. Potential ribosome-binding sites are underlined and potential promoter regions shown by boxes. The stop codon is marked with asterisks and a region of dyad symmetry shown by divergent arrows.

predicted molecular mass 21 117 Da. The mRNA from positions 947–965 in the DNA sequence could form part of a transcription terminator structure. Upstream of the putative *gerD* ORF is a consensus σ^E '–10' region, CATATT, starting at position 341 (Fig. 6) but the '–35' region, TATAA, is not a perfect match to the σ^E consensus of TTNAA (Cowing *et al.*, 1985). There is a

Fig. 7. Hydroplot of the *ger-97* polypeptide. The average hydropathy value of a seven-amino-acid window centred on each residue along the sequence is plotted. Hydrophobic regions have a high average hydropathy. The top of the figure represents the value of the most hydrophobic residue, the bottom that of the most hydrophilic. The horizontal line represents the average hydropathy of a large number of sequenced proteins (Kyte & Doolittle, 1982).

second possible promoter region with TATAAT at '−10' (starting position 320) which is a perfect consensus for σ^A but the sequence at '−35' is TCGCGA, which is a poor match for the consensus TTGACA (Cowing *et al.*, 1985). A second ORF preceded by a ribosome-binding site begins upstream of the putative *ger-97* ORF leading from the other strand in the opposite direction. It is preceded by possible promoter regions indicated in Fig. 6.

A 'hydroplot' (Kyte & Doolittle, 1982; Fig. 7) of the proposed *gerD* polypeptide showed that the bulk of the protein is hydrophilic, but there is a hydrophobic amino terminal region 14–16 amino acids long, preceded by four hydrophilic amino acids, which resembles a signal sequence. The most likely position for a cleavage site in the *ger-97* polypeptide is between the two adjacent alanine residues at positions 26 and 27 (scoring 15 on the 'processing probability' scale of Von Heijne, 1983). The factors which make this the most likely site are the proline residue at position −5 and the alanine at position −1 from the cleavage site. The glutamate residue at position −3 is however highly unfavourable and would argue against this being the cleavage site. A second possible position is after the alanine residue at position 21 (scoring 13.1). This is marked down because of the proline residue at position +1 which would be most unusual, although it is not certain whether a proline residue in this position would actually prevent cleavage at this site *in vivo* (Von Heijne, 1983).

DISCUSSION

The sequence of markers shown in Fig. 2 is the most likely order consistent with the mapping and hybridization data described here. Confirmation of the location of *gerD* adjacent to *rrnI* has been obtained from sequencing data of K. Bott (unpublished). Work of Jarvis *et al.* (1988) confirms the relative order of *cysA*, *rpsE* and *rrnI* and also shows that the attachment site for SPO2 maps between *rpsE* and *rrnI*. Irie *et al.* (1982) mapped *attSPO2* between *cysA* and *gerD*, so that the relative order of markers in this region is likely to be *cysA*, *rpsE* (*rpsI attSPO2*) *gerD*, *rrnI*. The relative order of *rpsI* and *attSPO2* remains uncertain. *rpsI* is 3–4 kb from *gerD* (according to the formula of Kemper, 1974, and the transformation data in Table 2) and could be present on λ 2H. Further characterization of this and other clones from the region which carry *attSPO2* (K. Bott, unpublished) should establish the relative order of markers.

The proximity of *ger-97* to the 16S end of *rrnI* could explain the failure to isolate plasmids consisting solely of the 9 kb fragment of pBMD1 and pBMD2, as the *rrnI* promoter would be

located near one end of this *Eco*RI fragment. Circularization of this fragment would give transcription into the β -lactamase gene and plasmid replication functions of pTV20 and might prevent maintenance of the plasmid. This would accord with the observation of Bott *et al.* (1984) that *B. subtilis* rRNA operon promoters cause plasmid instability when maintained in *E. coli* on plasmids which do not contain transcription termination signals. Presumably such signals are present in the extra *Eco*RI fragments of pBMD1 and pBMD2.

The complementation studies show that *gerD* is present on the 1.8 kb *Pst*I fragment cloned in pSGMU2; and the observation that pBMD7 and pBMD10, which carry DNA that does not span the site of Tn917 insertion and includes only part of the *ger-97* ORF identified in the DNA sequence of this region, are unable to complement *gerD* mutations strongly suggests that the *ger-97* ORF is that of *gerD*. The identity of the ORF upstream of the proposed *gerD* ORF, which is preceded by a ribosome-binding site and a promoter region reading in the opposite direction to the *gerD* ORF, is unknown. Downstream of *gerD* none of the potential ORFs observed were preceded by ribosome-binding sites. The phenotype of the *ger-97::Tn917* mutant very closely resembled that of the *gerD25* and *gerD48* mutants except that the *ger-97::Tn917* mutant spores did not germinate better in Ala when KCl was added. This would be explained if insertion of Tn917 into the *gerD* gene completely abolishes its function. Mutants *gerD25* and *gerD48* probably result from point mutations which might lead to mutant gene products that could operate better in an environment of high ionic strength.

The function of *gerD* is unknown, and computer-assisted protein sequence homology searches have not revealed homology between the *gerD* polypeptide and other sequences. The location of *gerD* between clusters of ribosomal protein and ribosomal RNA genes is particularly intriguing and suggests that *gerD* may have a role in translation. However, there is as yet no evidence to support this. Irie *et al.* (1985) suggested that the *gerD* product may be involved in fructose recognition during germination since with *gerD* mutant spores addition of fructose does not alleviate the inhibitory effect of D-alanine on L-alanine-stimulated germination, whereas it does with wild-type spores. Vegetative cells of *gerD25* and the *ger-97::Tn917* mutant are able to grow at the same rate as wild-type cells when using fructose as a source of carbon (R. Sammons, unpublished). Nevertheless recognition of fructose may be impaired in *gerD* spores. Cloning and knowledge of the *gerD* sequence will now permit *in vitro* mutagenesis, which will aid investigation of this and other aspects of the *gerD* phenotype. It should also be possible to obtain the *gerD* gene product *in vitro* for further study. The possibility that multiple copies of *gerD* may be suboptimal or inhibitory to germination also merits investigation. One possible clue to the function of *gerD* is the presence of a signal sequence at the N-terminus of the polypeptide, which suggests that it is transported through a membrane. Whether the *gerD* product is made in the mother cell and transported into the forespore, or vice versa, is at present unknown. Preliminary studies in which *gerD* gene has been fused to *lacZ* (R. Sammons, unpublished) suggest that *gerD* is active between t_2 and t_6 of sporulation. Studies to confirm this, and further experiments on *gerD* expression in different *spo* and *ger* mutant backgrounds, are in progress.

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