Mutants of *Bacillus subtilis* Affected in Spore Outgrowth

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Six mutants of *Bacillus subtilis* 168 that are temperature-sensitive in spore outgrowth were isolated. The outgrowth process proceeds normally at 35 °C, but at the non-permissive temperature (47 °C) it is arrested at a specific stage characteristic for each mutant strain. The mutants are not altered in vegetative growth whether at 35 °C or at 47 °C. They were characterized for their ability to synthesize RNA, proteins and DNA during outgrowth. A mutant defective in spore germination was also isolated; less than 5% of its spores can germinate at any of the temperatures tested. The mutations were mapped by means of transduction and transformation. The isolation of a number of outgrowth mutants which map at different loci and which affect outgrowth at different times is discussed in relation to the regulation of this process.

INTRODUCTION

The bacterial endospore differs from the vegetative cell in its metabolism, structure and composition. Thus sporulation and spore germination may represent a simple model system for studying the biochemical events that regulate the transition from one cell form to another (Hansen *et al.*, 1970; Keynan, 1973; Mandelstam, 1976). The system has many positive aspects: the differentiation process is cyclic, and so the conservation of the complete genome during the phenotypic changes is obvious; moreover, both germination and sporulation can take place without intervening cellular division.

During germination and outgrowth of bacterial endospores, a time-ordered sequence of events takes place that allows the transition from spore to vegetative cell (Armstrong & Sueoka, 1968; Balassa, 1965; Kennett & Sueoka, 1971; Kobayashi *et al.*, 1965; Steinberg & Halvorson, 1968*a, b*; Torriani & Levinthal, 1967). Very little is known of the regulatory aspects of the germination process. We are currently interested in isolating mutants of *Bacillus subtilis* altered in spore germination or outgrowth with the aim of identifying the genes involved and investigating the regulatory mechanisms. This paper describes the isolation of seven new mutants altered in spore outgrowth or germination, their phenotypic characterization and map location on the chromosome of *B. subtilis*.

METHODS

Bacterial strains. The *B. subtilis* strains used in this work are listed in Table 1. The *gsp* mutants were obtained from strain PB1424 (*hisB2 trpC2 met*) following treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Adelberg *et al.*, 1965). The mutant strains were then transformed to Met⁺. From these strains the newly isolated mutations were transferred into the parental strain by transformation using saturating

Table 1. *Strains of Bacillus subtilis* used

Strain	Genotype*	Germination phenotype†	Origin
PB1424	<i>hisB2 trpC2 met</i>		From SB25 (J. Lederberg)
PB1654	<i>hisA1 cysB3 trpC2</i>		SB3, E. W. Nester
PB1711	<i>aro1906</i>		WB906 (Hoch & Nester, 1973)
PB1714	<i>sacA321 narB1 mtlB1</i>		QB819 (Lepesant-Kejzlarova <i>et al.</i> , 1975)
PB3292	<i>trpC2 thyAB glyC</i>		Canosi <i>et al.</i> (1976)
PB3350	<i>cysA14</i>		C14, I. Takahashi
PB3361	<i>purB6 leu-8 metB5 trpB3 hisA1 lys-21 thr-5 ery-101</i>		HLL3g, I. Takahashi
PB3378	<i>cysA14 ery</i>		Spontaneous from PB3350
PB3409	<i>purA16 cysA14 leu-8</i>		Our collection
Gsp mutant strains			
PB2442	<i>hisB2 trpC2 gsp-4</i>	ts-Gsp4	
PB2430	<i>hisB2 trpC2 gsp-25</i>	ts-Gsp25	
PB2439	<i>hisB2 trpC2 gsp-42</i>	ts-Gsp42	
PB2443	<i>hisB2 trpC2 met gsp-10</i>	Gsp10	
PB2388	<i>hisB2 trpC2 met gspIV-7</i>	ts-GspIVa4	Galizzi <i>et al.</i> (1975)
PB3341	<i>met cysA14 gspIV-7</i>	ts-GspIVa4	Galizzi <i>et al.</i> (1975)
PB2406	<i>hisB2 trpC2 met gspIV-26</i>	ts-GspIVb1	Galizzi <i>et al.</i> (1975)
PB2420	<i>met cysA14 gspIV-26</i>	ts-GspIVb1	Galizzi <i>et al.</i> (1975)
PB2410	<i>purA16 leu metB gspIV-12</i>	ts-GspIVc1	Galizzi <i>et al.</i> (1975)
PB1671	<i>cysA14 gspIV-12</i>	ts-GspIVc1	Galizzi <i>et al.</i> (1975)
PB2445	<i>met gspIV-2</i>	ts-GspIVd1	
PB2447	<i>cysA14 gspIV-2</i>	ts-GspIVd1	
PB2449	<i>hisB2 met gspIV-24</i>	ts-GspIVe1	
PB2451	<i>hisB2 cysA14 gspIV-24</i>	ts-GspIVe1	
PB2434	<i>met gspIV-11</i>	ts-GspIVf1	
PB2436	<i>cysA14 gspIV-11</i>	ts-GspIVf1	

* *his*, *trp*, *met*, *cys*, *thy*, *gly*, *leu*, *pur*, *lys*, *thr* and *aro* indicate requirements for, respectively, histidine, tryptophan, methionine, cysteine, thymidine, glycine, leucine, purine, lysine, threonine and tryptophan, plus phenylalanine plus tyrosine; *sac*, inability to use sucrose as a carbon source; *nar*, no growth with nitrate as sole nitrogen source; *mtl*, inability to use mannitol as a carbon source; *ery*, resistance to erythromycin; *gsp*, inability of the spores to germinate or outgrow normally.

† ts-Gsp indicates thermosensitive in spore outgrowth (Galizzi *et al.*, 1975); this symbol is retained in this Table to allow comparison with previous papers.

amounts of DNA (more than 0.2 µg ml⁻¹). Selection was for Met⁺ and the transformants were screened for the presence of the *gsp* unselected marker (marker congression). Some of the mutant strains appeared to be blocked at a specific outgrowth stage when grown at the non-permissive temperature (47 °C). The septation is incomplete and for this reason their phenotypic designation should be ts-GspIV, according to the recommendations of Young & Wilson (1972). The *gspIV* derivatives containing the marker *cysA14* were constructed by marker congression in transformation using donor DNA from PB3350 and each *gspIV* mutant strain as recipient.

Culture media. The minimal medium of Spizizen (1958) was used to prepare competent bacteria. Medium Y (Yamagishi & Takahashi, 1968) was used for PBS1 phage adsorption. Penassay broth (antibiotic medium no. 3, Difco) and Tryptose Blood Agar Base (Difco) were used in transduction experiments. The minimal agar for selection and re-isolation of transformants and transductants was that described by Davis & Mingioli (1950). PS broth (Riva *et al.*, 1968) or nutrient agar (NA; Biokar Froissy, Oise) containing 10 µM-MnCl₂ was used for sporulation. In some experiments with strain PB2443 (*gsp-10*) AK agar no. 2 (BBL) was used for sporulation. Nutrient broth (Difco) containing 0.5% (w/v) glucose (NBG) was used for germination.

Radioactive chemicals. [5-³H]Uridine (specific activity 5 Ci mmol⁻¹), U-¹⁴C-labelled protein hydrolysate [58 mCi (mmol C)⁻¹], [5,6-³H]uridine (44.5 Ci mmol⁻¹) and [3H]deoxycytidine (2 Ci mmol⁻¹) were obtained from The Radiochemical Centre, Amersham.

Synthesis of macromolecules. To follow DNA synthesis, heat activated spores were resuspended at an A₅₀₀ of about 0.30 in NBG containing [3H]deoxycytidine (1 µCi ml⁻¹ unless otherwise indicated) or [5,6-³H]uridine (5 µCi ml⁻¹). At intervals, samples of 0.2 ml were removed and treated as described by Galizzi *et al.* (1975). RNA and protein synthesis were followed as described by Albertini & Galizzi (1975).

Transduction. PBS1 phage-mediated transduction was performed according to Hoch *et al.* (1967). Recombinants were selected by plating on appropriately supplemented minimal media and the plates were incubated at 35 °C. Single colony isolates were obtained from the transductant colonies, and were then scored for the cotransfer of the marker for thermosensitivity during spore outgrowth (ts-Gsp) as follows. They were streaked on nutrient agar containing 10 μ M-MnCl₂ in glass plates. After incubation at 35 °C for 3 d to allow sporulation, the streaks were exposed to chloroform vapour by pouring about 5 ml chloroform into the covers of the Petri dishes and keeping them inverted at room temperature for about 15 min. The streaks were then replicated on nutrient agar plates; the replicas were incubated at 47 °C for 5 to 7 h and scored for ability to germinate. Appropriate positive and negative controls were included in each plate. The inability to germinate at 47 °C indicated cotransduction of the *gsp* mutation with the selected markers. The same procedure was used in mapping experiments involving transformation. Map distances are expressed as 100-cotransduction frequency.

Other techniques. The enrichment and isolation of mutants thermosensitive in spore germination, measurement of growth curves, temperature-shift experiments and transformation were done as previously described by Galizzi *et al.* (1973, 1975).

RESULTS

Isolation of mutants affected in the spore germination process

Bacillus subtilis mutants that were temperature-sensitive in spore germination and outgrowth (ts-Gsp) were isolated using two different enrichment procedures. In the first, spores obtained from a nitrosoguanidine-treated culture were inoculated into NBG and incubated at 47 °C. After 150 min, penicillin G was added to the culture at a final concentration of 500 U ml⁻¹ and incubation was continued for 4 h to kill vegetative cells. After treatment with penicillinase (500 U ml⁻¹) the survivors were plated on NA and incubated at 35 °C until sporulation. The colonies were exposed to chloroform vapour for 20 min to kill the vegetative cells and then replicated on NA plates. The replicas were incubated at 47 °C and examined after 5 to 9 h for colonies unable to germinate (Galizzi *et al.*, 1973). With this technique two mutants, PB2445 (*gspIV-2*) and PB2449 (*gspIV-24*), affected in outgrowth at 47 °C were obtained.

Four other mutants (PB2442, PB2430, PB2439 and PB2434) were isolated by the second enrichment procedure, based on the different buoyant densities of cells at various stages of the germination process (Siccardi *et al.*, 1975a). Following germination and outgrowth in NBG at 47 °C as in the first procedure, the vegetative cells were separated from ungerminated spores or cells blocked at some stage of outgrowth by isopycnic centrifugation in Urografin as described by Galizzi *et al.* (1975); all fractions having a density higher than 1.25 g ml⁻¹ were collected.

For further studies, in order to eliminate interference of other mutations possibly induced by the nitrosoguanidine treatment, the *gsp* mutations were transferred into the parental strain PB1424 by congression.

All mutants showed normal vegetative growth in NBG at 35 and 47 °C, and all sporulated at both temperatures with the same efficiency as the parental strain. For some mutants (e.g. PB2442) the efficiency of plating on nutrient agar of the vegetative cells at the non-permissive temperature was only a small fraction (about 4%) of the efficiency of plating at 35 °C. That the main defect in all the mutants was due to a block in spore outgrowth was demonstrated by shifting the cultures at various times from 35 to 47 °C. For each mutant it was possible to define the time at which growth was no longer impaired by the temperature shift (see below).

The ts-Gsp mutants were also characterized with respect to the ability of the germinating spores to synthesize RNA, protein and DNA at the non-permissive temperature. These data together with the microscopic observations of cells outgrowing at the non-permissive temperature allow a preliminary separation of the mutants into two main classes: mutants in which synthesis of at least one of these macromolecules is absent or greatly reduced at the non-permissive temperature; and mutants in which synthesis of the macromolecules appears to be normal, but which form large swollen cells instead of bacilli (*gspIV* of Table 1).

Fig. 1. Macromolecular synthesis during germination and outgrowth of spores of mutant PB2442 (*gsp-4*) in NBG: ●, at 35 °C; ○, at 47 °C; ▲, culture shifted from 35 to 47 °C at 20 min. (a) [5-³H]-Uridine incorporation (0.5 $\mu\text{Ci ml}^{-1}$); (b) ¹⁴C-labelled amino acids incorporation (1 $\mu\text{Ci ml}^{-1}$); (c) [5,6-³H]uridine incorporation (5 $\mu\text{Ci ml}^{-1}$).

In addition to the *ts* mutants, we have isolated an oligogerminating mutant (PB2443, *gsp-10*).

Characterization of ts-Gsp mutants affected in the synthesis of macromolecules

The pattern of RNA, protein and DNA synthesis in mutant strain PB2442 (*gsp-4*) during spore germination and outgrowth at 35 and 47 °C is shown in Fig. 1. The synthesis of the macromolecules was monitored by following the incorporation of [5-³H]uridine, ¹⁴C-labelled amino acids and [5,6-³H]uridine, respectively. At the permissive temperature the syntheses proceeded as in the parental strain (data not shown), but at the non-permissive temperature the incorporation of [5-³H]uridine was greatly reduced, as was the synthesis of proteins, and no DNA synthesis was detected. To determine the period in which the temperature-sensitive event took place, samples of a culture of outgrowing spores were shifted from 35 to 47 °C at various times and [5-³H]uridine and [5,6-³H]uridine incorporation were followed. Cultures shifted at 20 min or later showed almost normal synthesis of both RNA and DNA (Fig. 1). Thus mutation *gsp-4* affects an event taking place early during germination. As for all the other mutants, a complete recovery was observed on shifting the cultures from 47 to 35 °C; thus the product defective in the mutant is completely reactivated on incubation at the permissive temperature.

We have also compared the amounts of [5-³H]uridine incorporated at both temperatures in the presence and absence of 50 μg chloramphenicol ml^{-1} (Fig. 2). At 35 °C, net RNA synthesis in the presence of chloramphenicol began as in the control and stopped only after about 50 min, in accordance with the original observations of Armstrong & Sueoka (1968). In the cultures incubated at 47 °C, similar amounts of RNA were accumulated in the presence or absence of the drug (Fig. 2b). Mutant PB2442 appears to be capable of accumulating at 47 °C as much RNA as can be accumulated in the absence of protein synthesis.

Fig. 2. Effect of chloramphenicol on RNA synthesis during germination and outgrowth of spores of mutant PB2442 (*gsp-4*): (a) at 35 °C; (b) at 47 °C. Spores of the mutant were inoculated into M9 medium as described by Albertini & Galizzi (1975); [^3H]uridine was present at $1 \mu\text{Ci ml}^{-1}$. $\circ$, Culture containing $50 \mu\text{g}$ chloramphenicol ml^{-1} ; $\bullet$, control culture (no chloramphenicol).

Fig. 3. Rate of RNA synthesis during germination and outgrowth of spores of mutant PB2442 (*gsp-4*): (a) at 35 °C; (b) at 47 °C. Spores of the mutant were inoculated into M9 medium. At 3 min intervals, 0.2 ml samples were pulsed with [^3H]uridine ($10 \mu\text{Ci ml}^{-1}$). After 2 min incubation the pulses were stopped by precipitation with 10% (w/v) trichloroacetic acid. $\circ$, Culture containing $50 \mu\text{g}$ chloramphenicol ml^{-1} ; $\bullet$, control culture (no chloramphenicol).

Fig. 4. Macromolecular synthesis during germination and outgrowth of spores of mutant PB2439 (*gsp-42*) in NBG: ●, at 35 °C; ○, at 47 °C. (a) [^3H]Uridine incorporation ($1\ \mu\text{Ci ml}^{-1}$); (b) ^{14}C -labelled amino acids incorporation ($1\ \mu\text{Ci ml}^{-1}$); (c) [^3H]deoxycytidine incorporation ($1\ \mu\text{Ci ml}^{-1}$).

Fig. 5. Macromolecular synthesis during germination and outgrowth of spores of mutant PB2430 (*gsp-25*) in NBG: ●, at 35 °C; ○, at 47 °C; ▲, culture shifted from 35 to 47 °C at 60 min. (a) [^3H]Uridine incorporation ($0.5\ \mu\text{Ci ml}^{-1}$); (b) ^{14}C -labelled amino acids incorporation ($1\ \mu\text{Ci ml}^{-1}$); (c) [^3H]deoxycytidine incorporation ($1\ \mu\text{Ci ml}^{-1}$).

This is only a quantitative estimate and does not necessarily mean that the same species of RNA are synthesized in the two conditions.

We followed the rate of synthesis of RNA by 2 min pulses of [^3H]uridine, at 35 and 47 °C, in the presence or absence of chloramphenicol (Fig. 3). During the first 60 min of incubation the rates of [^3H]uridine incorporation were the same at 35 °C in the presence of chloramphenicol and at 47 °C with or without chloramphenicol. After 60 min the three cultures behaved differently: at 35 °C in the presence of chloramphenicol the rate remained

constant; at 47 °C without chloramphenicol the rate increased and was still increasing exponentially after 90 min; while at 47 °C in the presence of chloramphenicol the rate slowed down exponentially (Fig. 3). This may be an indication that different species are synthesized at late times.

The synthesis of RNA, protein and DNA during outgrowth at 35 and 47 °C of spores of mutant PB2439 (*gsp-42*) is shown in Fig. 4. At the non-permissive temperature RNA synthesis appeared to be normal, while protein synthesis was greatly reduced. No DNA synthesis was detected at 47 °C. Temperature-shift experiments indicated that the temperature sensitivity occurred in the first 45 min of incubation.

Only synthesis of DNA was affected during spore outgrowth of mutant PB2430 (*gsp-25*) at 47 °C (Fig. 5). The amount of [³H]deoxycytidine incorporated at 47 °C was very small and probably reflects incorporation by a few spores outgrowing normally. With five times the amount of [³H]deoxycytidine, incorporation proceeded linearly with steps up every 30 min, a pattern characteristic of spores outgrowing synchronously (data not shown). After shifting the cultures from 35 to 47 °C at 60 min, normal synthesis of DNA was observed at the non-permissive temperature.

Mutants affected in the morphogenesis of outgrowing spores

Three of the mutants [PB2445 (*gspIV-2*), PB2449 (*gspIV-24*) and PB2434 (*gspIV-11*)] did not show any alteration in the pattern of synthesis of macromolecules. The absorbance curves for these mutants had the same shape as in the control for the first 120 min but at 47 °C they reached a plateau at a value lower than that obtained at 35 °C. Microscopic observations showed that the mutant spores do not outgrow into rod-shaped cells; instead, they form spherical cells, which grow to a considerable size. These mutants resemble previously described mutants affected in their morphogenetic process during spore outgrowth (Galizzi *et al.*, 1973). Preliminary observations with the electron microscope indicate that in all these mutants the first septum is synthesized, even though it does not divide cells into two compartments. The mutations have been called ts-GspIV, according to the suggestions of Young & Wilson (1972).

For all ts-GspIV mutants so far isolated, we have observed a complete phenotypic repair of the temperature-sensitive lesion by incubating the spores at 47 °C in the presence of sublethal doses (less than 10 µg ml⁻¹) of the antibiotic Distamycin A (Siccardi *et al.*, 1975*b*). The action of Distamycin in inhibiting *B. subtilis* transformation and transfection has led us to postulate the cellular envelopes as possible targets of the antibiotic (Mazza *et al.*, 1973).

Isolation of an oligo-germinating mutant

Following the procedure described by Trowsdale *et al.* (1974), we isolated, from 600 colonies screened, one mutant, PB2443 (*gsp-10*), having the following characteristics. At none of the temperatures tested did the outgrowth curve show a decrease in absorbance during 180 min incubation, but thereafter an increase was observed. Microscopically, many phase-bright spores were still visible even after 270 min incubation, at which time a few bacilli (between 1 and 5%) were also present. This behaviour was observed both in minimal medium and in nutrient broths. Upon plating on nutrient agar, only 0.6 to 5% of the phase-bright spores in the original non-incubated spore preparation gave rise to colonies. The few spores that did germinate generated cells that sporulated normally, but produced spores of which less than 5% could germinate. The same result was obtained whether the sporulation medium was NB, PS or AK. Treatment with lysozyme or with 7.2 M-urea and 10% β-mercaptoethanol (Gould & Dring, 1975) did not restore the ability to germinate and produce colonies. The fraction of spores which did germinate outgrew normally, as judged by the kinetics of [³H]uridine incorporation measured by incubating the mutant spores in minimal medium containing [³H]uridine at a concentration five times higher than that

Table 2. Linkage by transduction of *gsp-4*, *gsp-25*, *gsp-42* and *gsp-10* mutations

Mutation	Linkage to selected marker*
<i>gsp-4</i>	<i>hisA1</i> 30 (89/300); <i>glyC</i> 77 (113/146)
<i>gsp-25</i>	<i>aro1906</i> 99 (127/128); <i>narB1</i> 45 (54/120)
<i>gsp-42</i>	<i>cysB3</i> 10 (20/200); <i>glyC</i> 1 (3/200)
<i>gsp-10</i>	<i>thr-5</i> 4 (2/48); <i>hisA1</i> 36 (87/239); <i>cysB3</i> 92 (198/214)

* Linkage values are given as percentage cotransduction. The numbers of transductant colonies counted are indicated in parentheses as ts-Gsp/(ts-Gsp + ts-Gsp⁺).

Table 3. Map location of *gsp-10*, *gspIV-2*, *gspIV-11* and *gspIV-24* by means of three-factor transduction crosses

Donor	Recipient	Recombinants			Order indicated
		Selection	Type	No.	
<i>gsp-10</i>	<i>hisA1 cysB3</i>	His ⁺	Cys ⁺ Gsp ⁺	4	<i>cysB3-gsp-10-hisA1</i>
			Cys ⁺ Gsp ⁻	25	
			Cys ⁻ Gsp ⁺	106	
			Cys ⁻ Gsp ⁻	17	
		Cys ⁺	His ⁺ Gsp ⁺	7	
			His ⁺ Gsp ⁻	40	
			His ⁻ Gsp ⁺	6	
			His ⁻ Gsp ⁻	127	
		His ⁺ , Cys ⁺	Gsp ⁺	2	
			Gsp ⁻	100	
<i>gspIV-2</i>	<i>cysA14 ery</i>	Cys ⁺	Ery ^s ts-Gsp	68	<i>cysA14-ery-gspIV-2</i>
			Ery ^s ts-Gsp ⁺	77	
			Ery ^r ts-Gsp	1	
			Ery ^r ts-Gsp ⁺	12	
<i>gspIV-24</i>	<i>cysA14 ery</i>	Cys ⁺	Ery ^s ts-Gsp	74	<i>cysA14-ery-gspIV-24</i>
			Ery ^s ts-Gsp ⁺	72	
			Ery ^r ts-Gsp	2	
			Ery ^r ts-Gsp ⁺	15	
<i>gspIV-11</i>	<i>cysA14 ery</i>	Cys ⁺	Ery ^s ts-Gsp	96	<i>cysA14-ery-gspIV-11</i>
			Ery ^s ts-Gsp ⁺	73	
			Ery ^r ts-Gsp	6	
			Ery ^r ts-Gsp ⁺	13	

normally used to follow RNA synthesis (i.e. 5 μ Ci ml⁻¹ instead of 1 μ Ci ml⁻¹). Under these conditions curves were obtained which were similar to that in Fig. 2 for mutant PB2442 (data not shown). The only difference was in the level of incorporation: at 60 min strain PB2443 incorporated 5000 c.p.m. in 0.2 ml, while all other strains tested incorporated from 40000 to 50000 c.p.m. in 0.2 ml. (The initial A_{560} was between 0.4 and 0.5 in all experiments.) Thus at a fivefold higher concentration of [5-³H]uridine, incorporation was reduced to about one-tenth of the normal value. This is consistent with less than 5% of the spore population being able to germinate.

Map location of the *gsp* mutations

The seven mutations – *gsp-4*, *gsp-25*, *gsp-42*, *gsp-10*, *gspIV-2*, *gspIV-24* and *gspIV-11* – were mapped by phage PBS1 mediated transduction and by transformation with the mutant strains as donors and selecting for prototrophy using auxotrophic markers of known map location.

Mutation *gsp-4* was linked by transduction to *hisA1* and *glyC* (Table 2). No linkage to *cysB3* was obtained. Presumably *gsp-4* is to the right of *hisA1* and to the left of *glyC*, since *hisA* and *glyC* are more than 100 units apart (Canosi *et al.*, 1976) (Fig. 6). Mutation *gsp-25*

Table 4. Order of *gspIV* mutations with respect to each other and *cysA14* by means of three-factor transduction crosses

Donor	Recipient	Percentage of ts-Gsp ⁺ recombinants among Cys ⁺ *	Order indicated
<i>gspIV-2</i>	<i>gspIV-24 cysA14</i>	0 (0/156) \	<i>cys-gspIV-2-gspIV-24</i>
<i>gspIV-24</i>	<i>gspIV-2 cysA14</i>	2.4 (4/166) }	
<i>gspIV-2</i>	<i>gspIV-12 cysA14</i>	13.1 (21/160) \	<i>cys-gspIV-12-gspIV-2</i>
<i>gspIV-12</i>	<i>gspIV-2 cysA14</i>	0 (0/160) }	
<i>gspIV-2</i>	<i>gspIV-26 cysA14</i>	23 (34/145) \	<i>cys-gspIV-2-gspIV-26</i>
<i>gspIV-26</i>	<i>gspIV-2 cysA14</i>	41.2 (66/160) }	
<i>gspIV-2</i>	<i>gspIV-7 cysA14</i>	29.4 (50/170) \	<i>cys-gspIV-2-gspIV-7</i>
<i>gspIV-7</i>	<i>gspIV-2 cysA14</i>	43.7 (70/160) }	
<i>gspIV-11</i>	<i>gspIV-26 cysA14</i>	8.6 (13/150) \	<i>cys-gspIV-26-gspIV-11</i>
<i>gspIV-26</i>	<i>gspIV-11 cysA14</i>	1.9 (3/151) }	
<i>gspIV-11</i>	<i>gspIV-7 cysA14</i>	10.3 (18/174) \	<i>cys-gspIV-7-gspIV-11</i>
<i>gspIV-7</i>	<i>gspIV-11 cysA14</i>	0 (0/156) }	
<i>gspIV-11</i>	<i>gspIV-2 cysA14</i>	37.9 (63/166)	
<i>gspIV-24</i>	<i>gspIV-26 cysA14</i>	15 (19/124)	

* The numbers of transductant colonies screened are indicated in parentheses.

was strongly linked by transduction to *aroI* and also linked to *narB1*. The linkage to *aroI* was confirmed by transformation data; *gsp-25* gave 85% cotransformation when selecting for Aro⁺. These data, however, do not indicate whether *gsp-25* is to the right or to the left of *aroI*. Mutation *gsp-42* was very weakly linked by transduction to *cysB3* and *glyC*. In transformation *gsp-42* was linked to *hisA1* (23% cotransformation), confirming its location between *cysB3* and *glyC*. Mutation *gsp-10* was also located by transduction; it was strongly linked to *cysB3* and *hisA1* and also to *thr-5*. The data of three-factor transduction crosses (Table 3) indicate the order *cysB3-gsp-10-hisA1*.

The three mutations *gspIV-2*, *gspIV-11* and *gspIV-24* were all linked in transduction to *cysA14*, and in three-factor crosses also involving *ery* markers, the *gsp* mutations appeared to be to the right of *ery* (Table 3). This is supported by the fact that although the *gsp* mutations are 46 to 56 map units from *cysA14*, no linkage was detected between any of the *gsp* mutations and *purA16*. The distance between *cysA14* and *purA16* was 84 to 89 map units in the same transduction experiments. The *gspIV-2*, *gspIV-11* and *gspIV-24* mutations map close to three other mutations with the same phenotype (Galizzi *et al.*, 1975).

By means of three-factor transduction crosses we determined the order of all six mutations with respect to each other and to the *cysA14* marker (Table 4). The mutations *gspIV-2* and *gspIV-24* are closely linked but *gspIV-24* is distal to *cysA14*. In most other crosses, only the position of the *gspIV-2* mutation was determined. The probable order which can be deduced from the data presented in Table 4 is *cysA14-gspIV-12-gspIV-2-gspIV-24-(gspIV-26, gspIV-7)-gspIV-11*. The order *cysA14-gspIV-12-gspIV-26-gspIV-7* reported previously (Galizzi *et al.*, 1975) enables us to deduce the sequence *cysA14-gspIV-12, gspIV-2-gspIV-24-gspIV-26-gspIV-7-gspIV-11*. Some reservations should be retained about this order since in some of the crosses reported in Table 4 (e.g. those between *gspIV-2* and *gspIV-26*) an abnormally high frequency of multiple crossovers was obtained. The map distances of *gspIV* mutations from *cysA14* are of the order of 50 to 70 units, but were somewhat variable due to difficulties in screening for this phenotype.

Figure 6 shows the tentative location of the seven mutations on the genetic map of *B. subtilis*. Also shown are the positions of the five *gsp* mutations previously described by Albertini & Galizzi (1975) and Galizzi *et al.* (1975).

Fig. 6. Linkage map showing proposed location of *gsp* mutations. Map distances are expressed as 100-cotransduction frequency. For mutants not described in the present paper, see Albertini & Galizzi (1975) and Galizzi *et al.* (1975).

DISCUSSION

Several studies of the genetic control of spore germination and outgrowth have been reported. Five *ts* mutants were isolated and mapped by Albertini & Galizzi (1975) and Galizzi *et al.* (1973, 1975). Dawes & Halvorson (1974) isolated many *ts* mutants whose spores are altered in germination and whose vegetative growth is abnormal. An analogous situation has been reported by Nukushina & Ikeda (1969). Trowsdale *et al.* (1974) and Trowsdale & Smith (1975) have isolated several mutants of *B. subtilis* that fail to germinate in 10 mM-L-alanine (*Ger*^{ALA}) or that germinated very slowly in complex media (*Ger*^{PAB}). Three *Ger*^{PAB} and two *Ger*^{ALA} have been mapped between *thr-5* and *cysB3*. One *Ger*^{PAB} maps near *hisA1* and two *Ger*^{ALA} near *cysB*.

In the present paper we report the isolation of six *B. subtilis* mutants affected in outgrowth and one altered in germination. The six outgrowth mutants are temperature-sensitive. Of these, four were obtained following Urografin density gradient centrifugation, a procedure previously used in enriching for germination and outgrowth mutants (Dawes & Halvorson, 1974; Galizzi *et al.*, 1975). The other two mutants were obtained by outgrowing the spores at the non-permissive temperature in the presence of penicillin.

Mutant PB2443 (*gsp-10*) is not temperature sensitive: at every temperature tested, only between 0.6 and 5% of its spores germinate, so that it is phenotypically similar to some of the mutants of Trowsdale & Smith (1975). According to Woese *et al.* (1968), a critical level of an enzyme or metabolite must be accumulated in each spore for initiation to occur. Our mutant and those of Trowsdale & Smith (1975) may be altered in the synthesis and/or the accumulation of such a substance during sporulation, although it is difficult to prove this directly.

The temperature-sensitive mutants can be divided into two classes: those showing a defect in the synthesis of at least one type of macromolecule and those altered in morphogenesis.

The mutant strains PB2442 (*gsp-4*), PB2430 (*gsp-25*) and PB2439 (*gsp-42*) belong to the first class. Strain PB2442 is particularly interesting since its temperature-sensitive period is the earliest yet defined. The mutant spores are affected in outgrowth only when incubated for 20 min at 47 °C; thereafter they show no defect in outgrowth. This mutant is capable of limited synthesis of RNA and it is interesting to compare it with mutant PB2427 (*gsp-81*) (Albertini & Galizzi, 1975) also affected in the early synthesis of RNA. At the non-permissive temperature the spores of PB2430 (*gsp-25*) synthesize normal amounts of RNA and protein, but only very small amounts of DNA. If the low level of [³H]deoxycytidine incorporation is due to the leakiness of the mutation, this mutant could be specifically blocked in the first round of DNA replication. The findings of Ciarrocchi *et al.* (1977) on the level of DNA polymerase III during the cell cycle and those of Balassa (1965) and Yoshikawa (1965) on the effect of protein synthesis on the synthesis of DNA during spore outgrowth indicate that the first round of DNA replication during spore outgrowth may be subjected to a specific regulatory mechanism. Our mutant PB2430 could be affected in such regulation. At the non-permissive temperature, PB2439 (*gsp-42*) accumulates normal amounts of RNA during outgrowth, although it is severely affected in protein synthesis.

The three mutations (*gspIV-2*, *gspIV-24*, *gspIV-11*) affecting the morphology of spore outgrowth at the non-permissive temperature all map in the same region of the chromosome near *cysA14*. We previously mapped three other mutations (*gspIV-7*, *gspIV-12*, *gspIV-26*), which also formed large swollen cells following spore outgrowth at 47 °C, in the same region. Any speculation regarding the number of genes represented by the six mutations is unjustified since screening recombinants for their phenotypes and hence obtaining correct recombination values for map distances is difficult and map distances in PBS1 transduction are not always additive. All ts-GspIV mutant spores are phenotypically repaired by outgrowth in the presence of sublethal doses of Distamycin A; this may mean that all these mutants possess a common alteration. Since they are affected solely in outgrowth then it may be that during this stage of the cell cycle a specific morphopoietic substance is required.

The temperature-sensitive mutants described appear to be affected solely in spore outgrowth, since their vegetative cells grow normally at 47 °C in NBG. This is confirmed by the temperature-shift experiments since, after a fixed time specific for each mutant, on shifting the culture from the permissive to the non-permissive temperature, outgrowth proceeded normally as though incubation at the permissive temperature had been continuous. The same was true for the temperature-sensitive mutants isolated previously (Albertini & Galizzi, 1975; Galizzi *et al.*, 1973, 1975). Taking all of these mutants together, among those affected in the synthesis of at least one of the macromolecules we observed a general agreement between the time at which the temperature-sensitive event occurred and the extent of its effect on the syntheses; the earlier the temperature-sensitive event, the fewer the biosynthetic capabilities of the system. For example, PB2442 (*gsp-4*) appears to be temperature-sensitive only in the first 20 min of incubation; at the non-permissive temperature only a limited amount of [³H]uridine is incorporated, protein synthesis is greatly reduced and no DNA synthesis occurs. In contrast the outgrowing spores of mutant PB2430 (*gsp-25*) are affected by high temperature for about 60 min of incubation; at the non-permissive temperature only their DNA synthesis is affected. The definition of the temperature-sensitive period is imprecise due to the non-synchronous germination and outgrowth often observed with mutant spores and the time scale used in the experiments. Despite these limitations, we think that our observations are relevant to an understanding of the germination process.

The times of onset of RNA, protein and DNA synthesis during spore outgrowth under standard conditions reflect a strict hierarchical interdependence of the three syntheses. Mutants blocked early in RNA synthesis (e.g. PB2442) have a very low level of protein synthesis and incorporation of DNA precursors is undetectable. A mutant with a reduced level of protein synthesis (e.g. PB2439) incorporates [5-³H]uridine normally but does not synthesize DNA. From this we conclude that the synthesis of the three major classes of

macromolecules is not merely time dependent but also probably requires the presence of some key element from a previous reaction to start a later reaction. Although obvious, this could be important in the interpretation of the nature of different outgrowth mutants which map at different loci and affect outgrowth at different times with no correlation between map location (Fig. 6) and time of temperature sensitivity, i.e. the action of the gene product. It is a situation similar to that described for sporulation mutants. The pleiotropic effect of many sporulation mutations mapping in different regions of the chromosome has been interpreted as reflecting the occurrence of a single interdependent sequence of events (Balassa, 1969; Waites *et al.*, 1970; Coote & Mandelstam, 1973).

During spore outgrowth it seems that there is no master reaction which, once started, initiates all subsequent steps more or less automatically. Each step or group of steps appears to be regulated separately, so that different regulatory mechanisms, possibly operating at different levels, may be involved in spore outgrowth.

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