A GERMINATION MUTANT OF *BACILLUS SUBTILIS* DEFICIENT IN RESPONSE TO GLUCOSE

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A germination mutant of *Bacillus subtilis* 168, deficient in response to glucose (GLC), was isolated. The mutation, tentatively named *gerK*, was mapped between *aroI* and *dal*.

Spores of this mutant germinated normally in L-alanine (ALA) but failed to do so in L-asparagine+GLC+fructose (FRU)+K$^+$. Deficiency of the mutant in response to GLC was evidenced by the fact that GLC was without effect in; (i) stimulating ALA-initiated germination and (ii) enhancing reversion by FRU of D-alanine inhibition.

Furthermore, introduction of a *gerA* (temperature-sensitive) mutation into a *gerK* mutant by transduction gave rise to a double mutant that did not respond to ALA at 43° even in the presence of GLC.

No appreciable difference in glucose dehydrogenase activity was detected between wild-type and mutant spores.

Spores of transformable Marburg strains of *Bacillus subtilis* germinate in response to either of the two germination systems (i) L-alanine (ALA) or some of the structurally related amino acids (e. g., L-valine) and (ii) combination of L-asparagine (ASN), glucose (GLC), fructose (FRU) and potassium ion (K$^+$) (*1*).

In addition to the co-operation with ASN, FRU and K$^+$, GLC plays various roles in initiating germination. In ALA-initiated germination, it stimulates germination especially at high temperatures, and enhances the reversal by FRU of inhibition caused by D-alanine (*2*). Furthermore, spores of some mutants fail to germinate in ALA unless GLC (or GLC+FRU) is also present (*2*).

In germination initiated by ASN+GLC+FRU+K$^+$, GLC can be replaced by 2-deoxy-glucose or D-allose, but not by β-methyl D-glucoside and only poorly by α-methyl D-glucoside. These facts, together with the properties of the enzymes found in spores, led PRASAD et al. (*3*) to conclude that the role of GLC in initiating germination is to generate reducing power (NADH or NADPH) through the action of glucose dehydrogenase (GlcDH, EC 1.1.1.47). In ALA-initiated germination,

NADH or NADPH was postulated to be supplied through metabolism of ALA.

In recent years, a number of germination mutants of *B. subtilis*, that may be useful in elucidating the mechanism of germination, have been isolated and mapped *(4, 5)*. Among these, three classes, *gerA*, *gerB*, and *gerC*, are known to be germinant-specific *(6 – 8)*. Mutants of *gerA* and *gerC* are deficient in response to ALA, some of these being temperature-sensitive. Spores of these mutants germinate in response to ALA+GLC (or ALA+GLC+FRU) or ASN+GLC+FRU+K^+. The map position of *gerA* is very close to that of *citG* *(7, 8)*, whereas *gerC* is linked to *lys* in phage PBS1-mediated transduction *(7)*. One mutant, *gerB*, is deficient in response to ASN+GLC+FRU+K^+ but normal in response to ALA *(7)*. The mutant has been mapped on the origin-proximal side of *hisA* *(7)*. To which ingredient, ASN, GLC, FRU or K^+, the *gerB* mutant lacks in its response is not known.

We have isolated a germination mutant deficient in response to GLC in order to study the role of GLC in germination. Unlike *gerB*, this mutant has been mapped between *aroI* and *dal*. Some properties of the mutant are reported in this paper.

MATERIALS AND METHODS

Strains. All strains were derivatives of *Bacillus subtilis* 168 (Table 1). Germination mutants (Table 1b) were derived from 168TT. TKB1502, the properties of which are reported in this paper, produced spores deficient in response to GLC. The mutation was tentatively named *gerK*. TKB6 was constructed by introducing the *gerK* mutation into TKJ5547 by transformation. Germination properties of TKB1502 and TKB6 were exactly the same. TKB9103 produced spores that were temperature-sensitive with respect to ALA-initiated germination. Germination at non-permissive temperatures (e. g., 43°) was restored by addition of GLC. This mutation was regarded as being in the locus *gerA*, since the mutation was about 40% and 8% co-transduced with *thrA5* and *hisA1*, respectively, by phage PBS1 *(7, 8)*. This strain required glutamic or aspartic acid for growth, in addition to thymine and tryptophan. This additional requirement was separable from the *ger* mutation by either transformation or transduction.

Preparation of spores. Spores were produced on a sporulation agar medium which contained per *l*: Polypeptone (Daigo Nutritional Chemicals Co., Osaka), 5 g; "Ehrlich" meat extract (Kyokuto Seiyaku Co., Tokyo), 3 g; thymine, 10 mg; $MnSO_4 \cdot 4 – 6H_2O$, 10 mg; and agar (Difco Laboratories, Detroit, Mich.), 15 g. After cultivation at 30° or 37° for 3 – 4 days, the spore crop was harvested, washed twice in ice-chilled distilled water, and the spores were purified by discontinuous Urografin density-gradient centrifugation *(9)*. The purified spores were washed three times with distilled water and stored at 4°.

Germination. Unless otherwise stated, spores were heat-shocked in distilled

Table 1. List of *Bacillus subtilis* strains used.

Strain	Genotype	Origin
(a)		
168TT[a]	*thyA1 thyB1 trpC2*	FARMER and ROTHMAN (21)
TKJ5547[b]	*hisA1 leuA8 metB5 lys-21*	MUNAKATA (22)
QB928[a]	*aroI906 purB33 dal-1 trpC2*	DEDONDER et al. (23)
(b)		
TKB1502	*gerK1 thyA1 thyB1 trpC2*	168TT $\xrightarrow{\text{EMS}}$ [c]
TKB6[b]	*gerK1 hisA1 leuA8 lys-21*	TKB1502 $\xrightarrow{\text{tf}}$ TKJ5547[d]
TKB9103	*gerA102 thyA1 thyB1 trpC2*	168TT $\xrightarrow{\text{EMS}}$ [c]
TKB102[b]	*gerA102 gerK1 leuA8 lys-21*	TKB9103 $\xrightarrow{\text{td}}$ TKB6[e]

[a] Strains 168TT and QB928 were kindly supplied by Drs. H. Tanooka (National Cancer Center Research Institute) and Y. Sadaie (National Institute of Genetics), respectively.

[b] These strains also carry mutations affecting ultraviolet sensitivity (*uvrA10* and *ssp-1*).

[c] Isolated from EMS-treated spores of 168TT.

[d] Constructed by transformation. The arrow points from the donor to the recipient.

[e] Constructed by PBSl-mediated transduction. The arrow points from the donor to the recipient.

water at 65° for 120 min. Germination medium was 20 mM Tris-HCl buffer (pH = 7.4) containing 0.1 % KCl and organic germinant(s). The germinants were: ALA (5.6 mM), GLC (5.6 mM), FRU (5.6 mM) and ASN (3.3 mM). These germinants were used either singly or in combination. Germination, as judged by decrease in optical density (O. D.) at 600 nm, was followed in a Baush & Lomb "Spectronic 21MV" photometer (Baush & Lomb, Rochester, N. Y.).

Isolation of mutants. Spores of 168TT were treated with ethyl methanesulfonate (EMS) as described by MANDELSTAM and WAITES (10). The mutagenized spores were inoculated onto the sporulation agar medium and clean spores prepared as described above were used for isolation of germination mutants.

The mutant deficient in response to GLC, TKB1502 (*gerK*), was isolated as unable to germinate in response to ASN+GLC+FRU+K⁺. The spores were heat-shocked, suspended in the germination medium and incubated at 37° for 60 min. After heating at 75° for 15 min to kill the germinated spores, the survivors were spread on the sporulation medium and allowed to sporulate. This procedure were repeated for four cycles and the single colony was then isolated and purified.

A *gerA* mutant, TKB9103, was isolated from the EMS-treated spores of 168TT essentially as described by TROUSDALE and SMITH (6).

Glucose dehydrogenase activity. Preparation of spore extract and assay of GlcDH activity was performed as described by FUJITA et al. (11). A cell mill extract of spores was prepared in 0.5 M potassium phosphate buffer (pH = 6.5).

Pre-treatment of the extract for activation could be omitted because the fresh extract obtained in this manner was fully active (11).

Mapping. Phage PBSl-mediated transduction was performed as described by

DUBNAU (12).   Scoring Ger$^+$ or Ger$^-$ phenotype was performed as follows.

Transductants were picked by means of sterile toothpicks and transferred in a fixed array to a sporulation agar plate.   After sporulation, colonies were replicated onto a filter paper (Tokyo No. 2, 70 mm in diameter, Toyo Roshi Co., Tokyo). A substantial mass of colonies could be transferred by lightly pressing the paper onto the agar plate by means of a glass rod or forceps.   The filter paper was placed, colony-containing side up, on a 3% agar plate and heated at 65° for 120 min. This treatment was effective in killing the vegetative cells and in heat-shocking the spores.   The agar plate for heat-shock contained D-alanine (0.1 g/l) and D-cy-closerine (Sigma, 0.5 g/l) to prevent triggering germination by amino acids (e. g., ALA) carried over from the sporulation medium.   The addition of D-cycloserine was done to inhibit alanine racemase (13), the activity of which was considerably high in spore-containing colonies.   The filter paper was then transferred onto a germination agar plate which contained per l: K_2HPO_4, 14 g; KH_2PO_4, 6 g; ASN, 0.5 g; GLC, 1 g; FRU, 1 g; sodium DL-malate, 1 g; 2, 3, 5-triphenyltetrazolium chloride (TTC), 1 g; and agar, 15 g.   After incubation at 37° for 60 min, Ger$^+$ colonies became pink coloured resulting from TTC reduction by germinated spores, whereas Ger$^-$ colonies remained uncoloured.

When the gerA (temperature-sensitive) phenotype was to be detected, D-alanine and D-cycloserine in the heat-shock agar plate were omitted, and ASN+GLC in the germination agar plate was replaced by L-valine (1 g/l).   It was preferred to ALA because the spores in the colonies germinated poorly on ALA.   This may be due to alanine racemase activity of the spores.   Incubation on the germination agar plate was performed at 45°.

To confirm the validity of the method, small amounts of spores were prepared as described below and their germination properties were checked.   Before rep-licating on the filter paper, a colony was picked at random and a small portion was spread by means of the rounded end of a toothpick onto a membrane filter (Toyo TM-1, 13 mm in diameter, Toyo Roshi Co., Tokyo) placed on a sporulation agar plate.   A Petri-dish (90 mm in diameter) usually contained 8 sheets of the membrane filter on the agar.   After incubation at 37° for 3 – 4 days, the membrane filter was transferred to a glass centrifuge tube (16.5 × 105 mm) containing 2 ml of cold distilled water.   The tube was vigorously shaken by means of a Vortex-Genie mixer (Scientific Industry Inc., Bohemia, N. Y.) to wash the spore crop out of the filter.   After removal of the filter, spores were collected by centrifugation and washed once with distilled water at 4°.   The spore crop was then treated with ly-sozyme (2) followed by DNase (DNase I of grade II, Boehringer Mannheim GmbH, Mannheim, 10 μg/ml in the presence of 0.01 M $MgSO_4$).   The spores were then collected by centrifugation and washed twice with distilled water.   An amount of spores sufficient to perform two to four runs of germination testing was obtained from a sheet of the membrane filter.   In every cross, 8 or 24 transductants were checked in this manner.

<div align="center">RESULTS</div>

Growth and sporulation

The *gerK* mutant, TKB1502, grew normally on SPIZIZEN's minimal medium (*14*, supplemented with GLC, thylmine and tryptophan), and grew and sporulated normally on the sporulation agar medium.

Germination properties

Spores of TKB1502 did not appreciably germinate at 37° in response to ASN+GLC+FRU (the requirement of K^+ will not be mentioned hereafter since K^+ was always contained in the germination buffer), while ALA-initiated germination was normal (Fig. 1). Essentially the same results were obtained when the spores were germinated at 30° or 43°.

The absence of response to ASN+GLC+FRU is attributable to the deficiency of germination in response to GLC, because in this strain ALA-initiated germination at high temperatures is not stimulated by GLC. Spores of wild-type strains responded poorly to ALA at high temperatures (above 40°) when non-heat-shocked or insufficiently heat-shocked. Germination curves for non-heat-shocked spores of 168TT are shown in Fig. 2a. As shown in the figure, the germination was markedly stimulated by GLC. In contrast, in the case of TKB1502, GLC was without effect (Fig. 2b). Spores of both strains germinated rapidly in ALA alone when sufficiently heat-shocked (Fig. 2).

The deficiency in response to GLC was demonstrated also in reversal of D-alanine inhibition. Germination in ALA is inhibited by D-alanine, and the inhibi-
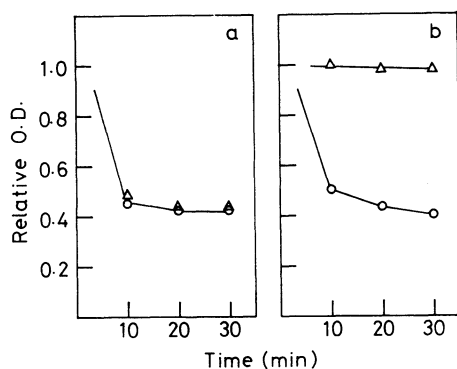

Fig. 1. Fig. 2.

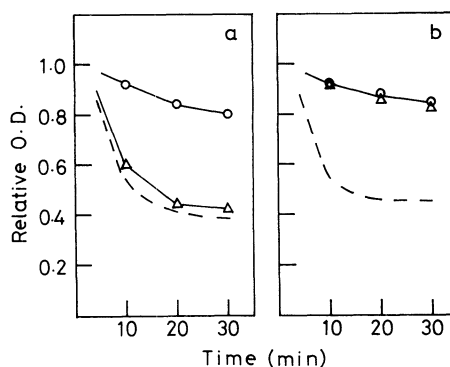Fig. 1. Spore germination of 168TT (a) and TKB1502 (b) at 37° in ALA (○) and ASN+GLC+FRU (△).

Fig. 2. Germination of non-heat-shocked spores of 168TT (a) and TKB1502 (b) at 43°.
○, ALA; △ ALA+GLC. Broken lines are germination curves for heat-shocked (65°, 120 min) spores in ALA alone.
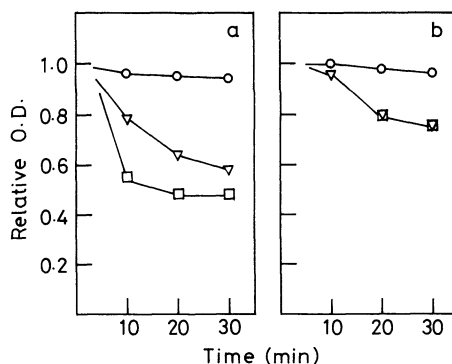
Fig. 3. Reversal of D-alanine inhibition by FRU and GLC.

Spores were germinated at 37° in DL-alanine (11.2 mM) with or without FRU and GLC.

a, 168TT; b, TKB1502. ○, DL-alanine alone; ▽, DL-alanine+FRU; □, DL-alanine+FRU+GLC.

tion is partially reversed by FRU (2).   The reversal by FRU is enhanced by GLC in wild-type strains (2).   In TKB1502, this enhancement by GLC was absent (Fig. 3).

Germination properties of a gerA gerK double mutant

A temperature-sensitive mutation in gerA locus was introduced from TKB9103 to TKB6 (see Table 1) by PBS1-mediated transduction taking advantage of the fact that gerA is linked to hisA.   Six out of seventy-six His$^+$ transductants produced spores that did not respond to ALA at 43°.   One of the transductants was picked and purified.

The resulting gerA gerK double mutant, TKB102, produced spores that were "absolutely" temperature-sensitive with respect to ALA-initiated germination, in the sense that they did not germinate at 43° even in the presence of GLC (Fig. 4).

GlcDH activity of spores

To determine whether the deficiency of germination in response to GLC in gerK mutant is due to lack of GlcDH activity, spore extract of TKB1502 was prepared and the enzyme activity was assayed.

As shown in Table 2, there was no appreciable difference in GlcDH activity between TKB1502 and the parent strain, 168TT.

Mapping of gerK mutation

Since a preliminary survey showed that gerK was linked to dal-1, three point transduction crosses involving aro1906, gerK and dal-1 were carried out with phage PBS1 propagated in the cells of TKB6.   The results are summarized in Table 3 and
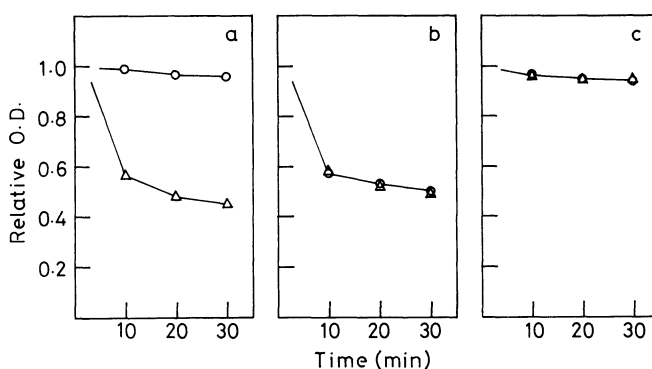
Fig. 4.   Spore germination of TKB9103 (*gerA*), TKB6 (*gerK*) and TKB102 (*gerA gerK*) at 43°.
a, TKB9103;   b, TKB6;   c, TKB102.   ○, ALA;   △, ALA+GLC.

Table 2.   GlcDH activity of spore extracts.

Strain	Coenzyme	Activity[a]
168TT	NAD	2.8
	NADP	1.1
TKB1502	NAD	2.4
	NADP	1.0

[a] Activity was assayed at 30° and expressed in μmol reduced coenzyme produced/min·mg of protein.

Table 3.   Transduction crosses by PBS1 for mapping of *gerK*.[a]

Recipient strain	Selection	Classes[b]			Distance[c]
		ger	*dal*	No.	
QB928	Aro+	1	1	26	*aroI906-gerK1*: 45
		1	0	99	*aroI906-dal-1*: 87
		0	1	3	
		0	0	100	
		ger	*aro*	No.	
	Dal+	1	1	23	*dal-1-gerK1*: 53
		1	0	49	*dal-1-aroI906*: 84
		0	1	1	
		0	0	79	

[a] TKB6 was used as the donor strain.
[b] Donor and recipient markers are shown as "1" and "0," respectively.
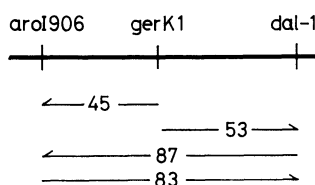[c] Distance is represented as 100× (1−cotransfer frequency).

Fig. 5. Mapping of *gerK1* and linked markers. Distance is expressed as percentage of recombination in PBS1 transduction (Table 3).

Fig. 5. It was suggested that *gerK* is located near the halfway between *aroI* and *dal*. This locus is in the vicinity of *mtlB*, which controls mannitol-l-phosphate dehydrogenase (*15*). However, *gerK* was shown to differ from *mtlB*, since TKB1502 or TKB6 grew well on mannitol as the carbon source (*15*).

DISCUSSION

The present results indicate that the multiple effects of GLC in initiating germination, i. e., co-operation with ASN+FRU, stimulation of ALA-initiated germination at high temperatures, enhancement of reversal by FRU of D-alanine inhibition etc. have a common genetic basis. This is consistent with the hypothesis developed by FREESE and co-workers (*1 – 3*). However, evidence for involvement of GlcDH in germination was not obtained, since GlcDH was fully active in TKB1502 spores. Although this by no means rules out the involvement of GlcDH in germination, it should be noted that in *B. megaterium* no metabolism of GLC seems necessary for GLC-initiated germination (*16*). It is also noteworthy that in *B. megaterium*, amounts of NADH and NADPH in spores increased only *after* initiation of germination (*17*).

In recent years, a good deal of evidence has accumulated that some germinants can initiate germination without being metabolized (*18, 19*). As regards *B. subtilis* 168, there is evidence that probably non-metabolizable amino acid analogues, allylglycine (*20*) and cycloleucine (*8*), initiate germination through acting on a receptor site which is common to ALA. Therefore, the role of GLC in germination, as well as the nature of the interaction between germinants, seems to be interpreted in other terms than metabolism. In this regard, SAMMONS et al. (*8*) postulated that the spore has two classes of receptor for ALA, one of which binds ALA alone and another which binds ALA in the presence of GLC, FRU and K+. The former was considered to be deficient in *gerA* mutant. According to their hypothesis, the *gerK* mutant may be deficient in the latter class of the receptor.

It may be assumed that a receptor for a germinant is a complex comprizing subunits, each of which binds the specific germinant. ALA-receptor and ASN-receptor may contain a common GLC-binding subunit, since the *gerK* mutants are deficient in response to both germination systems, ALA+GLC and ASN+

GLC+FRU. The ALA-receptor comprising ALA-, GLC-, and FRU-binding subunit may be of the second class of SAMMONS et al. (*8*) as discussed above.

Alternatively, one may assume that the ALA-receptor functions in response to ALA alone when the ALA-binding subunit is intact, but that co-operation of GLC (through binding to the GLC-binding subunit) is required for function when the ALA-binding subunit is modified by mutation, high temperature, etc. To what extent the function of the ALA-receptor is restored by GLC (or GLC+FRU) may depend on the nature and the extent of the modification.

It should be pointed out that the ALA-binding subunit itself may be a complex because at least two loci, *gerA* and *gerC*, are known to specifically affect the spore's response to ALA. Apparently more work, including identification of the gene products, is necessary to elucidate the role of the germinants in molecular terms.

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