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Initiation of the Germination of *Bacillus subtilis* Spores by a Combination of Compounds in Place of L-Alanine

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L-Alanine initiates the germination of spores of *Bacillus subtilis* by entering two metabolic pathways. The products of one pathway, which is inhibited by D-alanine or by elevated temperature, can also be derived from a combination of fructose, glucose, and K^+. The present study demonstrated that the products of the other pathway can be derived from L-asparagine or L-glutamine or, to a lesser extent, from several other amino acids. Hence, the combination of L-asparagine (or L-glutamine), fructose, glucose, and K^+ can initiate spore germination in the absence of L-alanine. Spores preincubated in a combination of asparagine and fructose do not lose refractility, optical density, or heat resistance, and do not take up methylene blue stain. The spores do, however, undergo some reaction which prepares them for a more rapid response to the later addition of glucose and K^+. This preincubation reaction has an optimal temperature of about 44 C.

The germinative process of typical *Bacillus subtilis* spores can be initiated by 10^{-3} M L-alanine (ALA) or by any one of certain other amino acids. The rate of germination can be measured by the decrease in optical density (OD), at 625 mμ, of a spore suspension in tris(hydroxymethyl)aminomethane (Tris) buffer. Previous experiments (7) indicated that, in the germinative process, ALA enters two metabolic pathways. One pathway is inhibited by D-alanine or by high temperatures (49 C). The products of this pathway, which are necessary for initiation, can also be derived from a combination of fructose (FRU), glucose (GLC), and K^+. It seemed possible that the product(s) of the other pathway of ALA also could be derived from a combination of compounds that are incapable of initiating spore germination by themselves. We found that, in the presence of FRU, GLC, and K^+, germination was also initiated by the addition to a spore suspension of L-asparagine (ASN), L-glutamine (GLN), or, to a lesser extent, L-cysteine, L-serine, or glycine.

MATERIALS AND METHODS

Strains. The transformable strain 60127 (nicotinic acid$^-$) was used for studies on *B. subtilis*. *B. cereus* strain T was obtained from B. Krask.

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TBAB plates. TBAB plates contained 33.0 g per liter of Tryptose Blood Agar Base (Difco).

Sporulation, harvest, heat activation, and initiation procedures. These procedures were described previously (7). Except where indicated, the initiating agents were used at the following final concentrations: KCl, 3.3. mg/ml; ALA, 0.1 mg/ml; ASN, 0.33 mg/ml; GLC, 1 mg/ml; FRU, 1 mg/ml; and 0.1 M Tris-chloride buffer.

The rate of initiation was measured by k_m, the maximal value of the rate at which the function OD/OD_0 decreased per hr (OD measured at 625 mμ; OD_0 = initial optical density of the spore suspension at 625 mμ).

Stainability. Stainability was determined by adding a drop of 0.5% methylene blue to dried spores on a slide, placing a glass cover slip on the slide, and examining the spores in a light microscope 5 min later.

Tween 80 was purchased from the Atlas Powder Co. (Wilmington, Del.).

RESULTS

Figure 1 shows the initiation of 60127 spores by ASN or GLN in the presence of FRU plus GLC plus K^+. When any of these compounds was left out, no initiation was observed (some spore preparations showed a slow rate of initiation without added potassium, presumably because traces of potassium were still present). ALA alone initiated germination of spores at a slightly higher rate than the above combinations. The initiation rates (k_m) observed with other amino

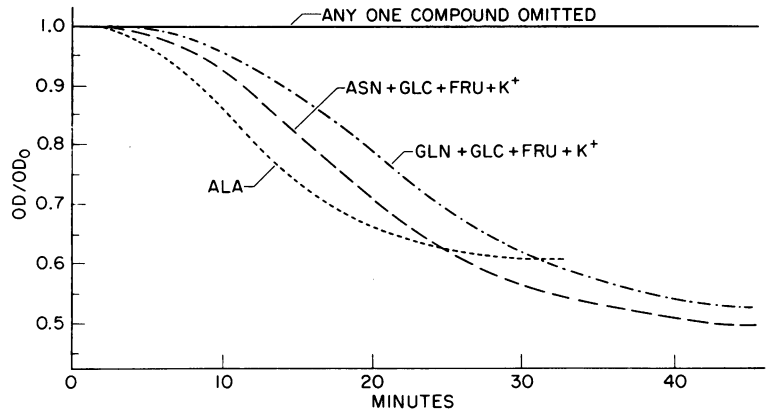

FIG. 1. *Initiation of spores by ALA and by the combinations of ASN + FRU + GLC + K^+ and GLN + FRU + GLC + K^+. All reactions were run at 37 C in 0.1 M Tris-chloride, pH 7.4.*

acids, alone and in the presence of FRU plus GLC plus K^+, are summarized in Table 1. The effects of all amino acids which could initiate germination alone were potentiated by the addition of FRU plus GLC plus K^+. The addition of these substances was especially effective for L-cysteine, glycine, and L-serine.

Table 2 shows that glucosamine could replace GLC, and mannose could replace FRU, but five times higher concentrations of these compounds were required to obtain comparable values of the maximal initiation rate, k_m. Many other carbon sources (at 1 mg/ml) were ineffective as replacements for either GLC or FRU (see Table 2).

The temperature dependence of the initiation rates (k_m) is shown in Fig. 2. Although a combination of ALA, FRU, GLC, and K^+ initiated germination at a higher rate than a combination of ASN, FRU, GLC, and K^+, the temperature optima differed only slightly. Heat-shocked spores reacted more than twice as fast as nontreated spores.

Figure 3 shows how the initiation rates (k_m) varied with the concentration of one of the four compounds (ASN, FRU, GLC, K^+) when the other three were in excess. It is apparent that FRU could not replace GLC and vice versa, even at high concentrations (1 mg/ml).

Although the refractility of the spores did not change when they were exposed to fewer than the above four compounds, some biochemical reaction may have taken place. Therefore, spore suspensions were exposed for 1 hr at 37 C to different combinations of ASN, FRU, GLC, and K^+; subsequently, the compound(s) left out was added. Throughout this experiment, the change in OD was recorded. When both ASN and FRU were initially present, a much more rapid initiation was observed upon subsequent addition of GLC and K^+ than when all four compounds were added simultaneously. The early addition of a mixture ASN, GLC, and K^+ or of a mixture of FRU, GLC, and K^+ did not cause such an effect (Fig. 4). The presence of K^+, in addition to ASN and FRU, did not influence the subsequent response to GLC and K^+. It is therefore clear that the reaction involving GLC, but not the reaction involving ASN or FRU, required K^+.

During the period of exposure to ASN and FRU, spores did not become stainable by methylene blue, did not lose their refractility, and did not become heat-sensitive (survival was measured, after heating for 30 min at 78 C, by plating on TBAB). The spores did, however, tend to form clumps and to become attached to glass or plastic centrifuge tubes. At the suggestion of A. Keynan, Tween 80 (0.33 mg/ml) was used to avoid the stickiness and thus facilitate medium changes after the preincubation period. The concentration of Tween 80 used had no effect on initiation rates in a mixture of ASN, FRU, GLC, and K^+. When spores were preincubated for 1 hr at 37 C in ASN plus FRU, then were centrifuged in the cold for 4 min at 9,000 rev/min (9,700 × *g*), and were finally resuspended in Tris plus Tween 80, the addition of GLC plus K^+ effected only a small OD decrease. When all four compounds were added after centrifugation, however, the usual rapid OD decrease was observed. This result indicates that ASN plus FRU must be present at the same time as GLC and K^+ to allow initiation to continue.

The optimal temperature for the preincubation reaction was measured by suspending heat-activated spores in a solution of 0.1 M Tris-chloride (*p*H 7.4) plus ASN (0.33 mg/ml) plus FRU (1 mg/ml) at different temperatures. At different times, samples were removed and were

TABLE 1. *Initiation rates (k_m) observed for different nitrogen sources*[a]

Compound	Compound alone			In the presence of FRU + GLC + K^+		
	333 μg/ml	100 μg/ml	33 μg/ml	333 μg/ml	100 μg/ml	33 μg/ml
Adenosine				<0.01		
β-Alanine	0.09			0.2	0.03	0.03
L-Alanine	1.7	1.7	1.4	2.1	2.1	2.1
L-α-Aminobutyrate	1.1	0.86	0.44	1.7	1.1	0.66
L-γ-Aminobutyrate	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
L-α-Aminoisobutyrate	0.98	0.59	0.17	1.2	0.56	0.23
L-Arginine				<0.01		
L-Asparagine	<0.01			1.3	1.1	0.09
L-Aspartate				0		
L-Cysteine	<0.01			0.54	0.26	<0.01
D-Galactosamine				<0.01		
D-Glucosamine				<0.01		
L-Glutamate				<0.01		
L-Glutamine	<0.01			1.2	0.38	<0.01
Glycine	<0.01			0.50	0.24	<0.01
L-Histidine				<0.01		
Inosine				<0.01		
L-Isoleucine	0.08			0.20	0.05	0.01
L-Leucine				<0.01		
L-Lysine				<0.01		
L-Methionine				<0.01		
L-Norvaline	0.33			0.66	0.14	0.02
L-Phenylalanine				0		
L-Proline				0		
L-Serine	<0.01			0.19		
L-Threonine				<0.01		
L-Tryptophan				<0.01		
L-Tyrosine				<0.01		
L-Valine	0.29			0.54	0.06	0.03

[a] FRU and GLC were used at a concentration of 1 mg/ml, whereas KCl was used at a concentration of 3.3 mg/ml.

TABLE 2. *Initiation rates (k_m) in the presence of ASN (0.330 mg/ml) ± KCl (3.3 mg/ml) + 0.1 M Tris-chloride, pH 7.4*[a]

Combinations of initiating agents	K_m
GLC (1 mg/ml) + mannose (0.33 mg/ml)	1.4
GLC (1 mg/ml) + mannose (0.10 mg/ml)	1.3
GLC (1 mg/ml) + mannose (0.03 mg/ml)	0.74
FRU (1 mg/ml) + glucosamine (1.0 mg/ml)	1.3
FRU (1 mg/ml) + glucosamine (0.1 mg/ml)	0.6

[a] The following compounds (at 1 mg/ml) showed $k_m < 0.01$ when they replaced either GLC or FRU: *n*-acetyl glucosamine, adenosine, L-arabinose, fructose-6-phosphate, fructose-1,6-diphosphate, D-fucose, D-galactitol, D-galactose, L-glucose, glucose-6-phosphate, DL-glyceraldehyde, glycerol, inosine, *i*-inositol, lactose, levoglucosan, D-lyxose, levulinic acid, β-methyl-D-glucoside, pyruvate, rhamnose, ribose, sorbose, sucrose, and D-xylose.

adjusted to 37 C; initiation was started by the addition of GLC (1 mg/ml) and K^+. The initiation rates increased with the time of preincubation until a maximal k_m was obtained (*see* Fig. 5). The k_m values obtained after 1 hr of preincubation were plotted against temperature (Fig. 6). The optimal temperature for the preincubation reaction was approximately the same as the optimal temperature for the overall initiation reaction.

The maximal k_m values obtained after preincubation depended on the temperature employed (Fig. 5). This finding suggested the presence of an equilibrium between production in and elimination from spores of a compound needed for initiation, the equilibrium constant depending on the temperature. To test this possibility, spores were incubated in ASN plus FRU for 1 hr at 42.5 C (a temperature giving the maximal rate of subsequent initiation at 37 C). The spores were then kept in ASN plus FRU at 0 or at 28 C for 7 hr before they were exposed to

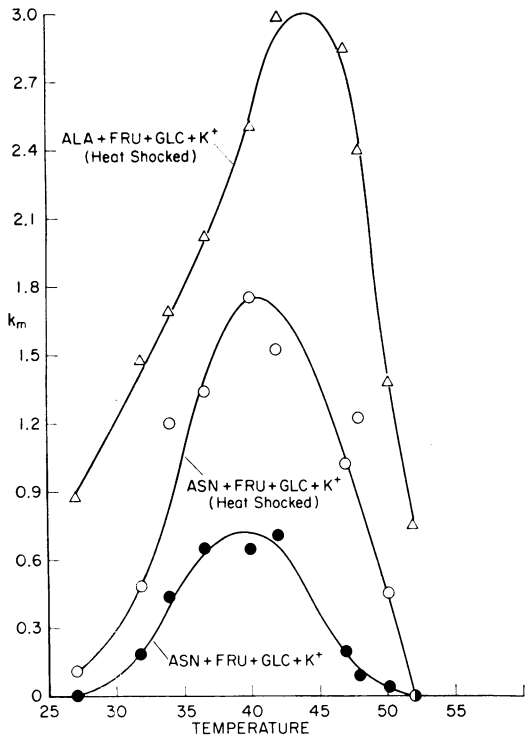

FIG. 2. *Dependence of initiation rates (k_m) on temperature and heat shock in the presence of ALA + FRU + GLC + K^+ or ASN + FRU + GLC + K^+. All samples were run in 0.1 M Tris chloride, pH 7.4.*

GLC plus K^+ at 37 C. Although spores which were exposed to GLC plus K^+ immediately after preincubation showed a k_m of 5.4, those spores which were kept for 7 hr at 0 or at 28 C showed k_m values of 5.4 and 2.6, respectively.

D-Alanine did not inhibit the initiation induced by a combination of ASN, FRU, GLC, and K^+, even when the ratio of D-alanine to ASN was 10:1. In contrast, a D-alanine to ALA ratio of 10:1 was sufficient to prevent initiation by a mixture of ALA, FRU, GLC, and K^+ (7).

After heat treatment, *B. cereus* (strain T) can be initiated by 100 μg/ml of ALA if hydroxylamine (0.01 M) is present (B. Krask, *personal communication*). Such spores did not show any initiation in a mixture of ASN, FRU, GLC, and K^+, whether or not hydroxylamine was present. A partial initiation (Table 3), however, was obtained in a mixture of ALA, ASN, FRU, and GLC in the absence of hydroxylamine.

TABLE 3. *Initiation of Bacillus cereus strain T spores by combinations of ASN (0.33 mg/ml), ALA (0.1 mg/ml), 0.1 M hydroxylamine (HA), GLC (1 mg/ml), and FRU (1 mg/ml) in 0.1 M Tris-chloride, pH 7.4*

Heat-shocked[a]	Combinations of initiating agents					k_m	OD_{60}/OD_0
	HA	ASN	GLC	FRU	ALA		
+	−	+	+	+	−	0.01	0.98
+	−	+	+	+	+	3.3	0.65
+	+	−	−	−	+	5.9	0.42
+	−	−	−	−	+	<0.01	1.0
+	−	−	+	+	+	<0.01	1.0
+	−	+	+	−	+	<0.01	1.0
+	−	+	−	+	+	<0.01	1.0
+	−	+	−	−	+	<0.01	1.0
+	+	+	+	+	−	<0.01	1.0
−	+	+	+	+	+	<0.01	1.0
−	+	−	−	−	+	<0.01	1.0
−	−	+	+	+	+	<0.01	1.0

[a] Where indicated, spores were heat shocked in water for 1 hr at 70 C.

DISCUSSION

Several amino acids, especially asparagine and glutamine, which by themselves cannot initiate the germination of *B. subtilis*, can do so in the presence of FRU plus GLC plus K^+. The initiation of *B. subtilis* spores by asparagine plus caramelized glucose was reported by Hachisuka et al. (13). The active agents in "caramel" apparently are FRU and GLU (7). The finding of several agents, which can initiate only in combination, made it possible to study the sequential action of the individual components. A reaction occurs in the presence of ASN plus FRU, which prepares the spores for a rapid initiation when GLC plus K^+ are subsequently added. In the spore, ASN and FRU apparently give rise to a metabolite which is necessary for initiation. When this compound is not utilized for initiation soon after its production, it is lost again, apparently by enzymatic breakdown rather than by simple diffusion out of the spore. An enzymatic degradation is indicated because the preincubation response for rapid initiation, obtained at 42.5 C, is lost after several hours at 28 C but is stable for many hours at 0 C. Although preincubation in ASN plus FRU enhances the subsequent response to GLC plus K^+, all of these compounds are needed continuously to give complete initiation.

K^+ ions were not needed during preincubation in ASN plus FRU. The ions apparently are necessary for the uptake or utilization of glucose.

In a recent paper (2), it was proposed that ALA initiates germination by two metabolic pathways (Fig. 7). The pathway to compound II, and on to

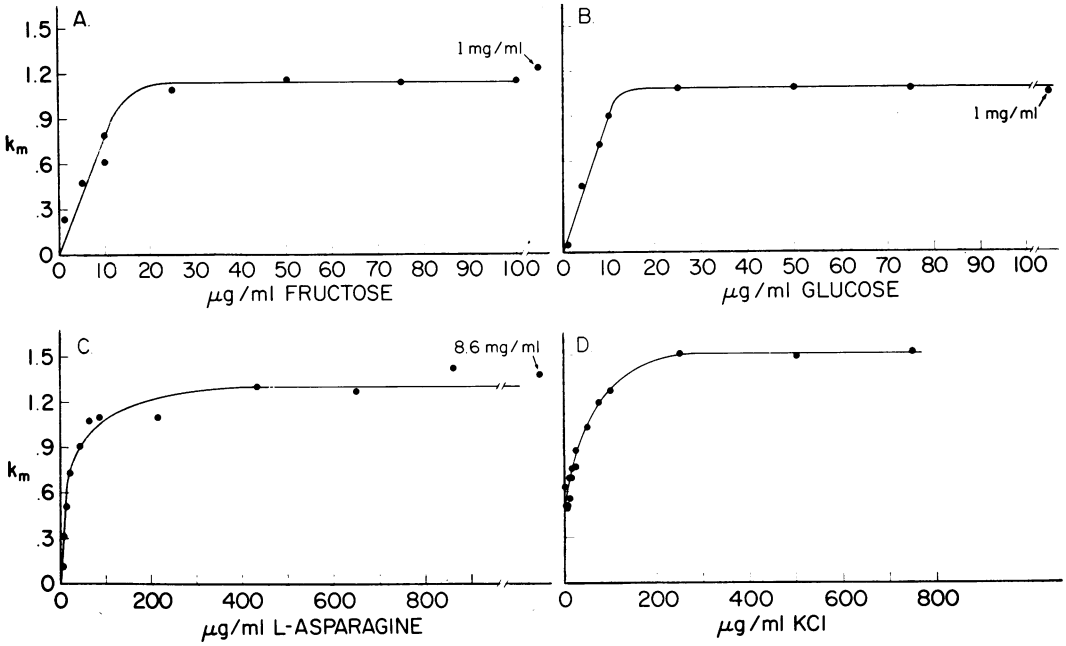

FIG. 3. *Concentration dependence of initiation by ASN + FRU + GLC + K^+ in 0.1 M Tris-chloride, pH 7.4 In each case, the concentration of one compound was varied, whereas the other three compounds were added in excess: FRU 1 mg/ml; GLC, 1 mg/ml; ASN, 0.33 mg/ml; and KCl, 3.3 mg/ml. (A) FRU varied. (B) GLC varied. (C) ASN varied. (D) K^+ varied.*

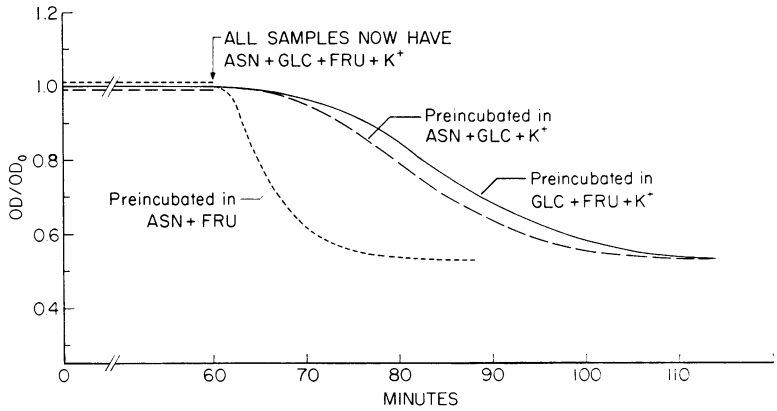

FIG. 4. *Effect of preincubation at 37 C, in the presence of several compounds, on the subsequent initiation in ASN + FRU + GLC + K^+ at 37 C.*

III and IV, which is not necessary in the presence of FRU plus GLC plus K^+, is blocked by high temperature (49 C), by D-alanine (at concentrations equal to those of ALA used for initiation), or in a mutant (7). In this pathway, ALA apparently serves as a carbon donor. In the pathway to compound I, ALA serves as an amino donor, since it can be replaced by ASN or GLN, if FRU plus GLC plus K^+ are available. The response of spores to preincubation in ASN plus FRU further indicates that ASN (or a product of it) and compound III (derived from FRU) must react to form compound I. Analogous to this would be the reaction of GLN or ALA with compound III to form compound I.

Our results show clearly that the initiation by ALA represents a complex metabolic process requiring the existence of many intact enzymes in

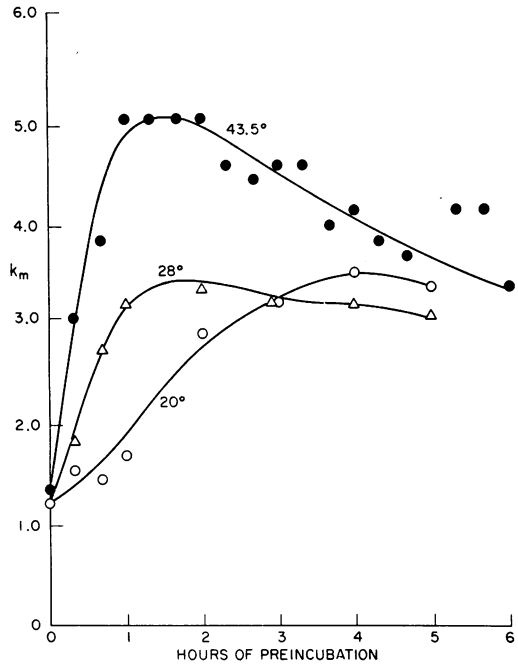

FIG. 5. *Effect of preincubation time and temperature on the subsequent initiation rates at 37 C. Spores were held in a mixture of 0.1 M Tris-chloride, pH 7.4, ASN (0.33 mg/ml), and FRU (1 mg/ml), at the indicated temperatures. After different times, samples were adjusted to 37 C. GLC (1 mg/ml) + KCl (3.3 mg/ml) were then added, and the OD decrease was followed in a recording spectrophotometer.*

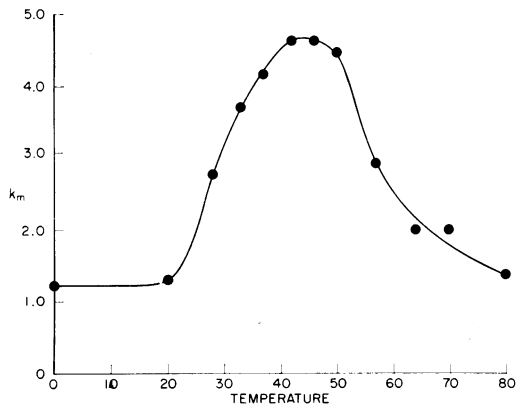

FIG. 6. *Effect of temperature on the ASN + FRU preincubation reaction. Spore suspensions were incubated at various temperatures for 1 hr in ASN (0.33 mg/ml) + FRU (1 mg/ml). After transfer to 37 C, GLC (1 mg/ml) + KCl (3.3 mg/ml) were added, and initiation was followed.*

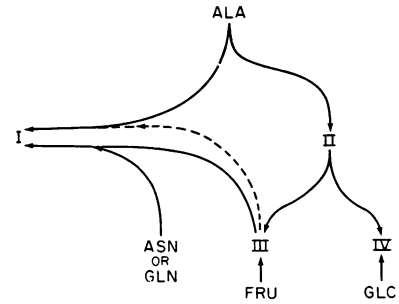

FIG. 7. *Proposed scheme showing the replacement of alanine as an initiating agent by the combination of ASN + FRU + GLC or GLN + FRU + GLC. Compounds I and IV must be made to produce initiation (decrease in OD of the spore suspension). ASN, GLN, or ALA react with compound III to form compound I.*

spores. The same conclusion can be derived from earlier experiments which showed that spores can be initiated by different compounds (2, 4, 6), that certain spores can be better initiated or only initiated by the combination of two agents (2, 5), or that mutants which have an additional germination requirement can be derived (1, 7).

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