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Factors affecting the germination of *Bacillus subtilis* spores

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Abstract

The germination of *Bacillus subtilis* spores was induced sufficiently at 1 mM of L-alanine and at the lower concentration than 1 mM the germination rates increased dependently on the concentration of L-alanine.

Optimal pH of the germination at 25 C and 37 C was neutral, while at lower temperature such as 6 C and 14 C it shifted to acid side, pH 5.0.

Glycine (at 100 mM), L-cysteine (at 100 mM) and Hg^{2+} (at 10 mM) inhibited the alanine-induced germination remarkably.

Effects of chemical treatments on spores varied with the conditions of treatments. Treatments of spores with 0.1 N NaOH at 80 C, 0.1 N HCl at 60 C, 5% sodium lauryl sulfate (SLS) at 60 C and 5% phenol at 60 C broke the capacity of germination completely. The spores by treatment with NaOH at 40 C and SLS at 60 C became lysozyme-sensitive. Treatment with NaOH at 60 C decreased the germination rates, failed to induce histidase during outgrowth and made the spores sensitive to lysozyme but it had little effect on viability and heat resistance.

Bacterial endospore is known to be one of the most dormant systems[1-5]. The breakdown of dormancy in spore is accompanied by increase of permeability of water and nutients in the spores. During the germination process, the conformational change of spore coat structure has been considered to occur in response to physical or chemical stimuli and to result in altered permeability of spore.

Germinants are specific substances which can induce the initiation process of bacterial germination and vary from bacterial species to species. For example, L-alanine makes the germination of *Bacillus subtilis* spores initiate and D-glucose does that of *Bacillus megaterium* spores.

Recent works suggest that some chemicals such as alkali and urea-mercaptoethanol can remove protein from spores and alter the germination properties. For example, Gould et al[6] found treatment with urea-mercaptoethanol caused spores of various bacteria to become sensitive to lysis by lysozyme. Waites et al[7] reported that alkali alone removed protein from spores of *Clostridium bifermentans* and increased germination rates.

The present report describes the environmental factors and chemical treatment of spores affecting the germination of spores of *Bacillus subtilis* in order to elucidate the mechanisms of spore germination and the action of germinant.

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Materials and Methods

Organisms. *Bacillus subtilis* Marburg strain (ATCC 6051) was employed throughout the work.

Spore preparation. Spores are produced on nutrient agar plates. The plates were inoculated with a culture growing in nutrient broth and incubated for about one week at 37 C. The mixture of free spores and cells was scraped together from the plates and washed 3 times by centrifuging with distilled water. Spores were purified by treatment with 500 μg/ml lysozyme for 40 min, and then purified by 1% SLS for 20 min at 37 C in order to remove remaining vegetative cells. The spores prepared were finally washed 7 times with distilled water and then they were stored in a freezer box.

Spore germination. Spores were suspended in the various germination media and the initial absorbance (optical density at 540 nm) of spore suspension was adjusted to 0.6–0.8. The spore suspensions were incubated at 37 C statically or by shaking. Germination was measured spectrophotometrically by reading the decrease in absorbance at 540 nm. The percent decrease to initial O. D. was calculated.

Heat resistance of spores. Heat resistance of spores was determined after heated the spore suspension at 80 C for 20 min. After cooled in ice water, the spore suspension was diluted and plated in nutrient agar. The plates were incubated for 36 hr at 37 C and then the colonies formed were counted. Heat resistance was expressed as the ratio of colony counts of heat-treated sample to those of unheated one.

Chemical treatments. Spores which adjusted to 1.7–2.0 in absorbance at 540 nm were suspended in the various reagents, and incubated for 30 min at indicated temperatures. Treated samples were centrifuged at 7,000 rpm at 4 C for 15 min and washed in distilled water and 1/15 M phosphate buffer (pH 7.2).

Assay of Histidase. At intervals during germination, 5 ml of samples was removed. The cells were collected on Millipore filter (HA, pore size 0.45 μ) and wahsed with phosphate buffer. The washed cells were suspended in 4 ml of 0.2 M Tris-HCl buffer (pH 9.0) and disrupted by sonic disintegrator 5 times at one minute interval. The assay of histidase was the modified method of Tabor et al[8]. The reaction mixture contained the cell extract 1.5 ml, 0.2 M histidine 0.2 ml, 0.2 M Tris-HCl buffer 1.5 ml and it was incubated at 37 C for 40 min and then the reaction was stopped by addition of 0.5 ml of 30% perchloric acid solution. After 5 min, the mixture was filtered by use of Toyo filter paper No 5C and urocanic acid produced in it was measured at 277 nm.

Results

Time course of germination and outgrowth

In preliminary experiments with nutrient broth, Demain's modified medium[9],

and phosphate buffer plus added L-alanine, the germination process was determined and the results are shown in Fig. 1. In the case of nutrient broth the decrease in absorbance was observed within 90 min and then there was a rapid turbidity increase, indicating outgrowth and vegetative growth. This contrasted with buffer plus added L-alanine (1–10 mM), in which the spores germinated rapidly but vegetative growth did not occur because of poor nutrient. In Demain's modified medium, vegetative growth was observed but outgrowth period was longer than that in nutrient broth. Initial change in absorbance was very similar in all these germination media and the decrease was completed within 90 min.

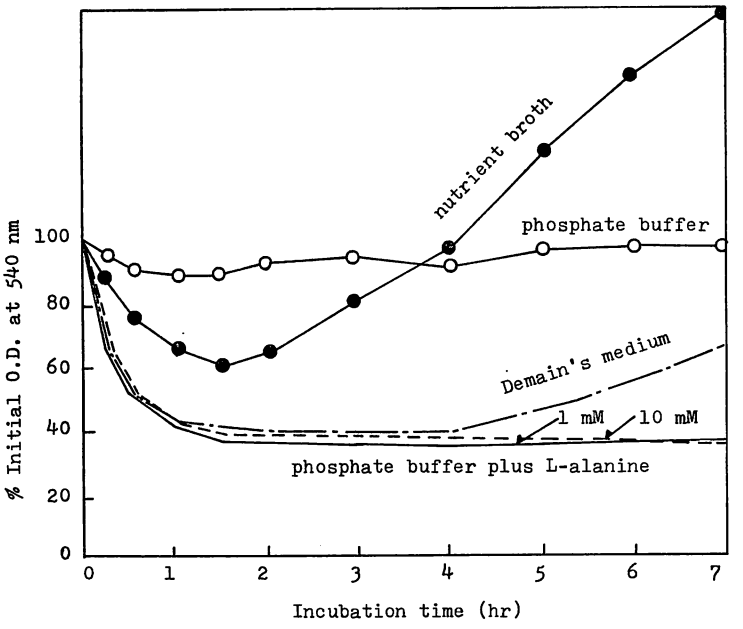

Fig. 1. Time course of the germination of *Bacillus subtilis* spores in various germination media. The spore suspensions were incubated on a Monod shaker at 37 C.

As shown in Figs. 2 and 3, the maximal rate of germination with L-alanine was obtained at above 1 mM and at lower concentration than 1 mM the germination rate increased proportionally to the concentration of L-alanine. No germination was detected in phosphate buffer without L-alanine. Subsequent experiments were done with phosphate buffer with or without L-alanine (5 mM).

Effects of pH and temperature

Fig. 4 shows pH optima for the alanine-induced germination at various temperatures. In this experiment incubation time was extended to 5 hr at 14 C and 30 hr at 6 C, respectively. At 25 C and 37 C pH optimum for germination was neutral, while it shifted to acid side, pH 5.0 at lower temperatures such as 6 C and 14 C.

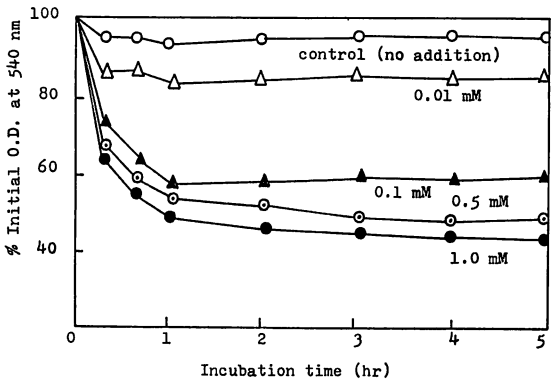

Fig. 2. Effect of L-alanine concentration on germination.

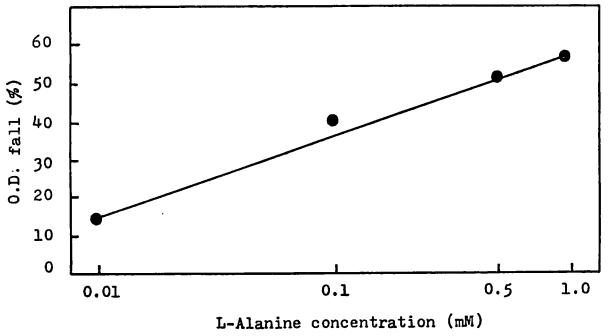

Fig. 3. Effect of L-alanine concentration on germination. The spore suspension was incubated for 3 hr at 37 C.

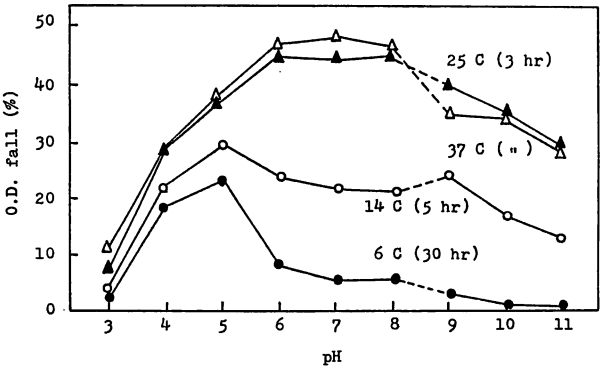

Fig. 4. Effects of pH and temperature on germination.
pH 3–8 : 0.1 M $C_6H_8O_7 \cdot H_2O$–0.2 M Na_2HPO_4, and
pH 9–11: 0.1 M NH_4Cl–0.1 M NH_4OH buffers were used.

Effects of amino acids, organic acids or metallic ions

1) **amino acids.** At 10 mM no apparent inhibition was observed with most amino acids tested, but at 100 mM, L-cysteine, L-aspartic acid and L-glutamic acid as well as glycine inhibited the alanine-induced germination considerably (Table 1).

In glycine buffer the alanine-induced germination was not found at 100 mM. Therefore, the effect of the concentration of glycine on the alanine-induced germina-

Table 1. Effect of amino acids on the L-alanine-induced germinations.

Additional amino acids	Relative germination (%)	
	10 mM	100 mM
L-Alanine	100.	96.6
L-Arginine	100.0	99.2
L-Aspartic acid	105.7	43.5
L-Cysteine	102.3	18.1
L-Glutamic acid	99.2	67.8
Glycine	89.9	0
L-Histidine	100.0	100.8
L-Isoleucine	92.6	—
L-Lysine	94.6	94.4
L-Methionine	96.9	—
L-Phenylalanine	98.2	100.0
L-Proline	97.7	94.8
L-Threonine	97.7	94.0
L-Tryptophan	90.4	—
L-Valine	101.6	—

The spore suspension in phosphate buffer containing 5 mM L-alanine as germinant and each amino acid was incubated at 37 C for 3 hr.

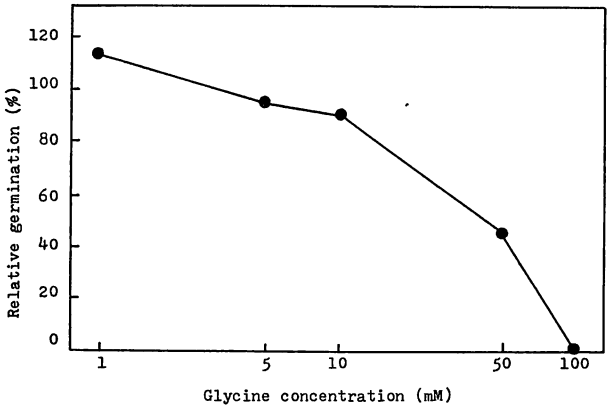

Fig. 5. Effect of glycine concentration on germination.
The spore suspension was incubated for 3 hr at 37 C.

tion was examined. At 10 mM, twice the concentration of L-alanine as germinant, the germination was not inhibited effectively but inhibited about 60% at 50 mM, and inhibited completely at 100 mM (Fig. 5).

2) **organic acids.** All organic acids tested failed to inhibit germination as shown in Table 2.

Table 2. Effect of organic acids on the L-alanine-induced germination.

Additional organic acids	Relative germination (%)	
	10 mM	100 mM
Acetic acid	100.2	100.8
Ascorbic acid	100.9	104.1
Dipicolinic acid	108.2	—
EDTA	104.9	108.9
Formic acid	96.7	89.8
Lactic acid	101.0	100.0
Succinic acid	94.4	108.4

3) **metallic ions.** Among the divalent cations examined, Mg^{2+} and Ca^{2+} did not affect germination while Mn^{2+} and Ba^{2+} inhibited it somewhat at 100 mM. Hg^{2+} inhibited germination completely and even at 1 mM of Hg^{2+} it was reduced to 40% of control (Table 3).

Table 3. Effect of metallic ions on the L-alanine-induced germination.

Additional ions	Relative germination (%)	
	10 mM	100 mM
$MnCl_2$	88.4	74.2
$CaCl_2$	110.4	95.6
$MgCl_2$	98.8	101.4
$HgCl_2$	0	0
$BaCl_2$	89.0	70.8

Effects of chemical treatments

Effects of the pretreatment of spores with various chemicals at various temperatures were examined (Table 4). In treatment with NaOH at lower temperatures, the behavior of treated spores on germination and on lysozyme resistance did not almost differ from those of untreated spores. NaOH treatments at higher temperatures, 40 and 60 C decreased the germination rate somewhat and caused spores to become lysozyme-sensitive and with treatment at 80 C germination did not occur. The spores also became lysozyme-sensitive with SLS treatment, but did not with three other chemicals, irrelevant to the occurrence of the alanine-induced germination.

The heat resistance of spores was examined after treated with NaOH and HCl.

Table 4. Effect of pretreatment with various chemicals on spore properties.

Treatment condition		Germination	Lysozyme sensitivity	Viable cells	Heat resistance (%)
Untreated		++	−	1.3×10^8	135.5
NaOH (0.1 N)	0 C	++	−		
	20 C	++	−	0.9×10^8	145.7
	40 C	+	+		
	60 C	+	++	1.1×10^8	105.2
	80 C	−	++	1.4×10^6	10.4
HCl (0.1 N)	20 C	++	−	1.3×10^8	105.4
	40 C	+	−	1.1×10^8	99.7
	60 C	−	−	1.1×10^5	17.7
	80 C	−	−		
Urea (8 M)	20 C	++	−		
	60 C	++	−		
Phenol (5%)	20 C	+	−		
	60 C	−	−		
SLS (5%)	20 C	++	−		
	60 C	−	+		

With HCl treatment at 40 C only the germination rate decreased without any effect on viability and heat resistance, but at 60 C viable cells decreased to one thousandth and heat resistance decreased largely. Similar results on heat resistance was obtained with NaOH treatments at 40 C and 60 C but a large part of loss on viability and heat resistance was observed at 80 C.

Time course of germination and lysis by lysozyme on NaOH treated spores was examined, as shown in Fig. 6. Even though the decrease in absorbance was slow

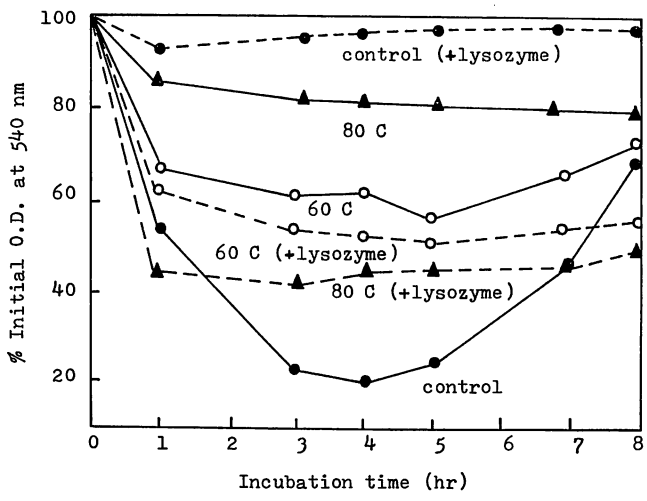

Fig. 6. Effect of NaOH-treatment of spores on germination and lysozyme sensitivity.

and low, germination and ougrowth can be observed in the treatment at 60 C. With treatment at 80 C germination was never detected. Untreated spores was not sensitive to lysozyme but sensitive with treatments at 60 C and 80 C.

As shown in Fig. 7, histidase induction during germination and outgrowth began at 30 min and increased rapidly after 60 min in Demain's medium, while it was not detected in the spores treated with NaOH at 60 C.

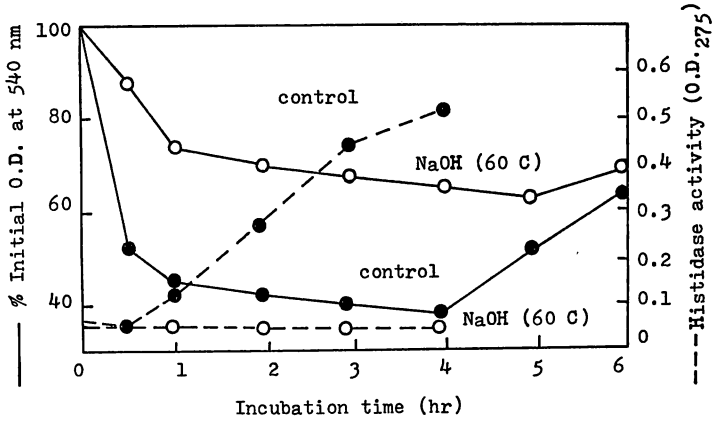

Fig. 7. Histidase induction during germination and outgrowth.

In conclusion, the spores treated with NaOH at 60 C were able to maintain the germination capacity and heat resistance, but became sensitive to lysozyme and lost inducibility of histidase. It is suggested that the NaOH treatment removed some materials such as spore coat protein from spores, making them sensitive to lysozyme, damaging some membrane enzymes of them without effect on viability.

Discussion

The concentration of 1 mM of L-alanine was sufficient to initiate the germination of *Bacillus subtilis*. Waites and Wyatt[10] reported that the maximal rate of germination of *Clostridium bifermentans* with L-alanine alone occurred at 150 mM. Generally the spores of *Bacillus* species required L-alanine at lower concentration than *Clostridium* species. This suggests that metabolism of L-alanine and its regulation is essentially different each other in *Bacillus* spores and *Clostridium* spores and that in the germination of *Bacillus* spores L-alanine acts as cofactor of germination enzymes or trigger substance which stimulates conformational change of spore coat protein.

Optimal pH of the germination of *B. subtilis* was neutral pH at 37 C but at lower temperatures optimal pH shifted to acid side, suggesting that surface structure of spore coat was influenced by pH and temperature and that resulted in changing permeability or susceptibility to L-alanine.

Glycine inhibited the alanine-induced germination of *B. subtilis* spores. This

phenomenon is thought to be specific in *B. subtilis*. Duncan et al[11] reported that glycine buffer did not influence the germination of *Cl. perfringens* and Waites and Wyatt[10] showed that amino acid mixture including glycine stimulated germination.

Among metallic ions Hg^{2+} inhibited germination completely. This is thought to be a kind of oligodynamic action of heavy metal but it is not clear whether the enzyme systems may be inhibited or spore coat protein is denaturated by Hg^{2+} ion.

Effect of chemical treatments of spores on germination varied depending on the conditions of treatments. Judging from lysozyme sensitivity, it is thought that NaOH and SLS treatments removed a part of protein from spore coat and made the peptideglycan accessible to lysozyme. Vary[12] reported that the spores extracted with SDS–DTT and urea were sensitive to lysis by lysozyme and Waites[13] showed that treatment with dithiothreitol-urea, which remove coat protein from spores, reduced the germination rate and allow lysozyme to attack the spores. It is interesting that the spores treated with NaOH at 60 C were reduced in the germination rate and they lost histidase inducibility but maintained still heat resistance. One might expect that the spores in this state could prove to be very useful for investigating the mechanism of germination.

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