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Regulation of L-alanine-initiated germination of *Bacillus subtilis* spores by alanine racemase

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Summary. Germination of *Bacillus subtilis* spores was initiated by L-Ala and competitively inhibited by D-Ala, suggesting the presence of an alanine receptor. The spores showed alanine racemase activity in the spore coat. To investigate the role of alanine racemase (L → D) on germination, net racemase activity was determined using diphenylamine as a germination inhibitor and germination was measured using D-penicillamine as a racemase inhibitor. Apparent affinity of L-Ala to the germinant receptor was more than 1000 times higher than that to the racemase. Germination increased in the presence of D-penicillamine, when the concentration of L-Ala was low and that of spores was high. Racemase activity was optimal at 65°C at pH 9.0 and germination at 43°C at pH 7.2. Under unfavorable growth conditions such as high population of spores in limited nutrients, high temperature and high pH, spore alanine racemase converted the germinant actively to the inhibitor and this conversion may regulate germination for survival of the population.

Keywords: Amino acids – L-Ala – *Bacillus subtilis* – Spore – Germination – Alanine racemase

Introduction

Bacterial spores have characteristic rigid integuments, are metabolically dormant and are highly resistant to various physical and chemical agents, such as heat, desiccation, UV irradiation, enzyme action, organic solvents, disinfectants and antibiotics. Once spores germinate, they lose their resistance irreversibly and differentiate metabolically and morphologically to the vegetative state. Such dynamic cell differentiation during spore germination is initiated by exogenous specific amino acids, sugars, nucleosides and inorganic ions depending on the species. *Bacillus subtilis* PCI219 spores can be germinated by L-alanine, and

many L-alanine analogues including L-2-amino-n-butyric acid, 2-aminoisobutyric acid, 3-chloro-L-alanine, L-norvaline, L-valine, L-norleucine, L-isoleucine (Yasuda and Tochikubo, 1985a), and N-(2-methylsulfonyl)ethyloxycarbonyl-L-alanine (Kanda et al., 1988). L-Alanine-initiated germination is inhibited competitively by D-alanine, D-serine, glycine, D-amino-n-butyric acid, D-cysteine and other compounds (Yasuda and Tochikubo, 1985a; 1985b). Studies on the structure-activity relationships of germinants and inhibitors suggest that there are separate binding portions on the spore receptor which differ in size and electrostatic nature, for germination and for inhibition (Yasuda and Tochikubo, 1985a).

Alanine racemase activity has been detected in various spores of *Bacillus* species (Stewart and Halvorson 1953; Church et al., 1954; Preston and Douthit 1984). As L- and D-alanines are common ligands to the germinant receptor and alanine racemase, the relationship between germination and alanine racemase activity of *B. subtilis* spores was investigated in this report.

Materials and methods

Preparation of spores and spore coat fraction

Bacillus subtilis PCI219 was grown and sporulated on nutrient agar at 37°C. After 5 days spores were harvested and washed repeatedly by centrifugation with distilled water at 4°C. They were microscopically clean: > 99% refractile dormant spores.

Spore coat fraction was prepared by sonic oscillation followed by lysozyme digestion as previously described (Fujita et al., 1989). Spores (10^{11} cells) in 10 ml phosphate-buffered saline, pH 7.2, containing 0.2 mM phenylmethylsulfonylfluoride (PBS-PMSF) were disrupted by sonication with 10 g glass beads. After sonicating three times for 15 min each at 4°C, the breakage estimated by a microscope was > 95%. After low-speed centrifugation (100 × g, 20 min) crude spore integuments were precipitated from the supernatant by centrifugation at 2000 × g for 20 min and washed five times with PBS-PMSF at 4°C. The integuments were treated with lysozyme (100 μg/ml PBS-PMSF) for 2 h at 37°C to remove the cortex layer, followed by washing three times at 4°C.

Spore germination

Germination was determined by change in optical density at 650 nm (OD_{650}) of spore suspension with 1 μM–100 mM L-alanine in 50 mM potassium phosphate-sodium phosphate buffer, pH 7.2, (NaKPB) at 37°C for 120 min. The concentration of spores was 1.4×10^8–6.4×10^9 cells per ml. In this strain, 70% reduction in OD_{650} represented completion of germination (Hachisuka et al., 1955). Percent germination after 120 min was used as the degree of germination completion. The germination rate was calculated as percent germination per minute during the first 10 min or between 10 and 20 min when germination was most rapid.

Alanine racemase

Alanine racemase activity (L- to D-isomer) was assayed by incubating spores in the same reaction mixture as the measurement of germination. Except otherwise stated, 1.5 mM diphenylamine, an inhibitor of germination (Yasuda et al., 1978; Yasuda and Tochikubo 1984b), dissolved in a small amount of methanol, was added to the dormant spore suspension to exclude the influence of endogenous D-alanine and pyruvate which were formed in metabolically active germinating spores with time. The activity of spore alanine racemase

was not affected by diphenylamine (see below). The racemase reaction was stopped by adding 0.5 ml of 2 N HCl to 1.0 ml of a reaction mixture, allowed to stand for 5 min, and neutralized with 0.5 ml of 2 N NaOH. Spores were removed by centrifugation and the supernatant was assayed for D-alanine by the D-amino acid oxidase coupled method according to Nagata et al. (1985). Alanine racemase activity was expressed as nmoles D-alanine formed per min per ml of spore suspension during the first 20 min. Reagent blank under the same condition without spores was subtracted.

Results

Alanine racemase activity of the dormant spores of Bacillus subtilis

The amount of D-alanine increased with time during incubation of the dormant spores with L-alanine, suggesting that alanine racemase is present in the spores of *B. subtilis* strain PCI219 (Fig. 1). To investigate whether the alanine racemase activity is associated with the germination process, the enzyme activities in germination-inhibited spores, germinating spores and fully germinated spores were examined (Table 1). As the germination-inhibited spores (i.e. in the presence of diphenylamine) showed alanine racemase activity, it is obvious that the spores have active alanine racemase in the dormant state. The enzyme activity in the germinating spores (i.e. in the absence of diphenylamine) was much the same as that in the dormant spores, but the fully germinated spores after 2 hr of initiation showed a significant increase in enzyme activity, which might be essential for D-alanine production necessary for cell wall synthesis in outgrowing cells. The alanine racemase activity was observed in the spore coat fraction in a bound form. The enzyme activities of these three preparations were not affected by diphenylamine, an inhibitor of germination (data not shown).

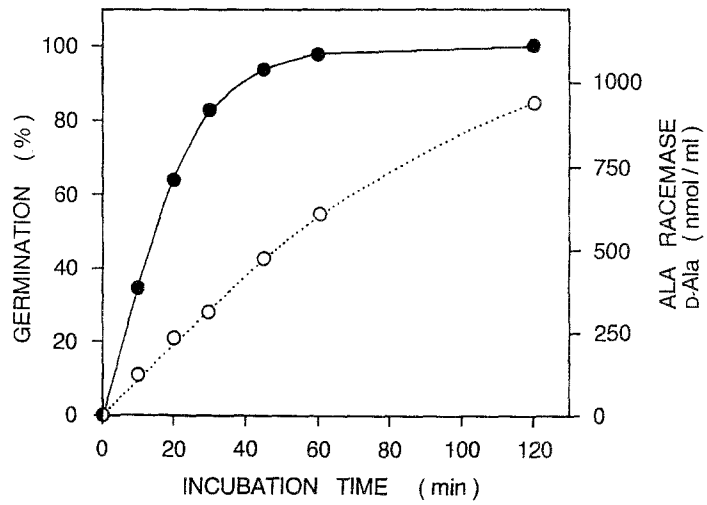

Fig. 1. Germination and alanine racemase activity of *Bacillus subtilis* PCI219 spores in the presence of L-Ala. Germination (●) was determined by loss of turbidity and alanine racemase activity (○) was measured in L to D direction and expressed as nmol D-Ala formed per ml of spore suspension. Incubation was carried out in 50 mM potassium phosphate-sodium phosphate buffer, pH 7.2, (KNaPB) at 37°C (same as in Figs. 2–5). Spores (2.7×10^8/ml) were incubated with 100 mM L-Ala

Table 1. Alanine racemase activity (L- to D-isomer) in germination-inhibited spores, germinating spores, fully germinated spores and spore coat fraction of *Bacillus subtilis* PCI219

Spore or spore coat fraction	D-Ala nmol/min/ml
Germination-inhibited spores (2.7×10^8/ml) (dormant spores in the presence of diphenylamine)	9.1 ± 0.9[a]
Germinating spores (2.7×10^8/ml) (dormant spores in the absence of diphenylamine)	10.8
Germinated spores[b] (2.7×10^8/ml)	14.6
Spore coat fraction[c]	22.6

Spores or spore coat fraction were incubated with 100 mM L-Ala in KNaPB at 37°C for 20 min

[a] Average ± SD of 7 separate experiments

[b] Germinated spores were prepared by incubating dormant spores with 1 mM L-Ala in KNaPB at 37°C for 120 min

[c] Spore coat fraction was obtained by sonication and lysozyme digestion. The amount used was OD_{650} equivalent to that of the germinated spores

Effect of D-penicillamine on germination and alanine racemase activity

As shown in Fig. 2, germination was not affected by D-penicillamine, an inhibitor of alanine racemase (Yonaha et al., 1975), at high concentrations of L-alanine (1 mM, 0.1 mM), while it was affected at low concentrations (10 μM, 5 μM), especially at later stages: germination was stimulated apparently in the presence of D-penicillamine. This suggests that the concentration of the germinant (L-Ala) is maintained and the inhibitor (D-Ala) is not produced by inhibiting L → D racemization by spore alanine racemase.

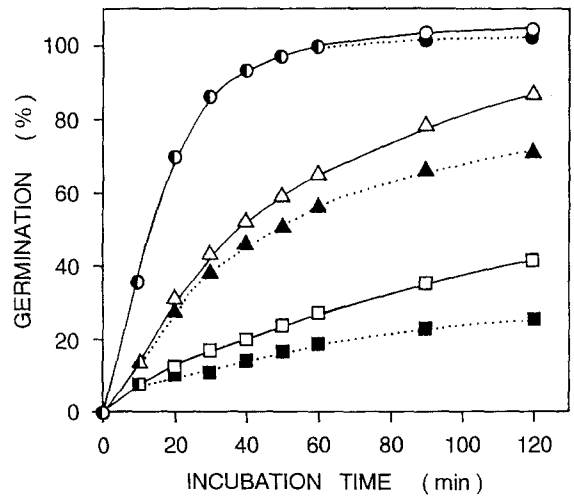

Fig. 2. Stimulation of germination of *B. subtilis* PCI219 spores by D-penicillamine. Spores (2.6×10^8/ml) were incubated with 5 μM (□■), 10 μM (△▲), 0.1 mM and 1 mM (○●) L-Ala in the presence (open symbol) and absence (closed symbol) of 50 mM D-penicillamine. D-Penicillamine was dissolved immediately prior to analysis

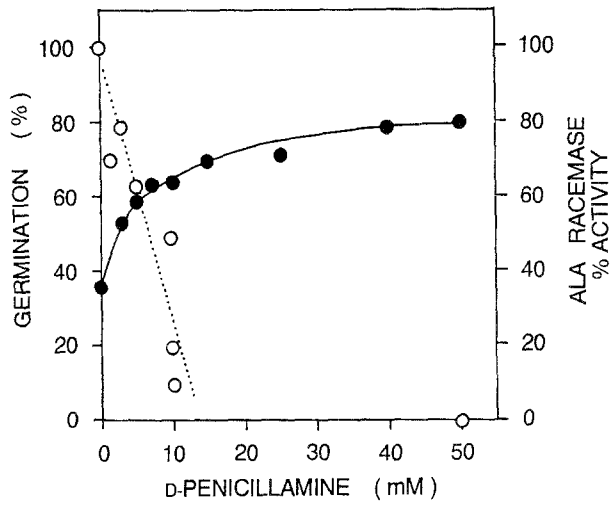

Fig. 3. Concentrations of D-penicillamine for stimulation of germination and for inhibition of alanine racemase of *B. subtilis* PCI219 spores. Spores (3.3×10^9/ml) were incubated for 60 min with various concentrations of D-penicillamine and 10 μM L-Ala for germination (●), and 100 mM L-Ala and 1.5 mM diphenylamine for alanine racemase (○)

The alanine racemase activity was inhibited by D-penicillamine: the estimated 50% inhibitory concentration was about 7 mM in the presence of 100 mM L-alanine. For complete inhibition, over 15 mM D-penicillamine was necessary. On the other hand, for enough stimulation of germination in 10 μM L-alanine after 120 min more than 30 mM D-penicillamine was necessary (Fig. 3). Therefore, in this report, 50 mM D-penicillamine was used for the determination of germination without any influence of racemase.

Apparent affinity of L-alanine to the racemase and germinant receptor

Alanine racemase activity increased gradually between 0.1 and 100 mM of L-alanine and the estimated Km value was 8 mM (Fig. 4). On the other hand, germination occurred at much lower concentrations; apparent Km values obtained from the percent germination after 120 min were 4.5 μM and 7 μM in the presence and absence of D-penicillamine, respectively (Fig. 4). These values were similar to that calculated from the maximum germination rate; 6 μM (Yasuda and Tochikubo, 1984a). Thus, apparent affinity of L-alanine for germinant receptor was more than 1,000 times higher than that for alanine racemase.

Effect of spore population

As germination was affected by racemase at low concentrations of L-alanine (Figs. 2–4), the effect of spore population on germination was examined in 30 μM L-alanine (Fig. 5). The higher the spore population, the less germination was observed: 2.8×10^8 spores germinated by over 90% after 120 min, 1.1×10^9 spores by 55% and 2.1×10^9 spores by only 30%. When alanine racemase activity was inhibited with D-penicillamine, the germination rate seemed to recover to each corresponding maximum level. Alanine racemase was measured

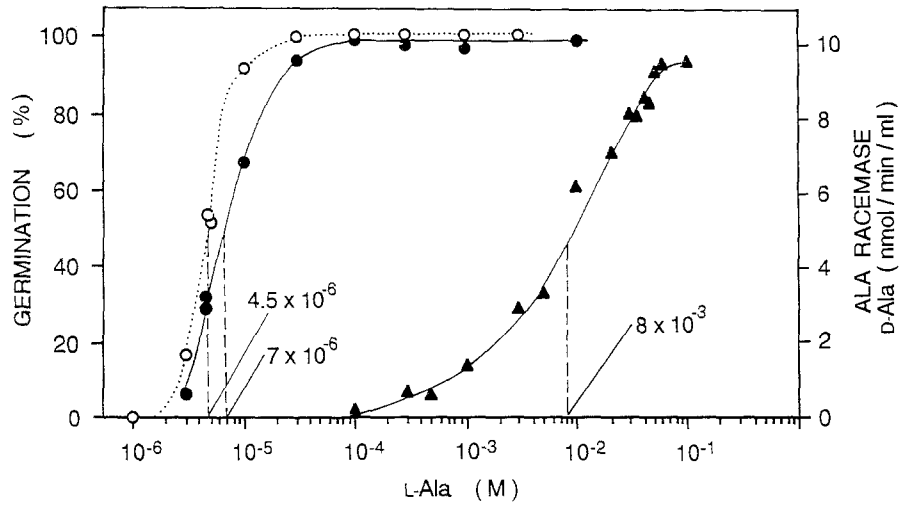

Fig. 4. Effect of L-Ala concentration on germination and alanine racemase activity. Spores (2.7×10^8/ml) were incubated with various concentrations of L-Ala. Germination was determined in the presence (o) and absence (●) of 50 mM D-penicillamine for 120 min. Alanine racemase activity (▲) was determined in the presence of 1.5 mM diphenylamine. Apparent Km values calculated from the curves are indicated by broken lines

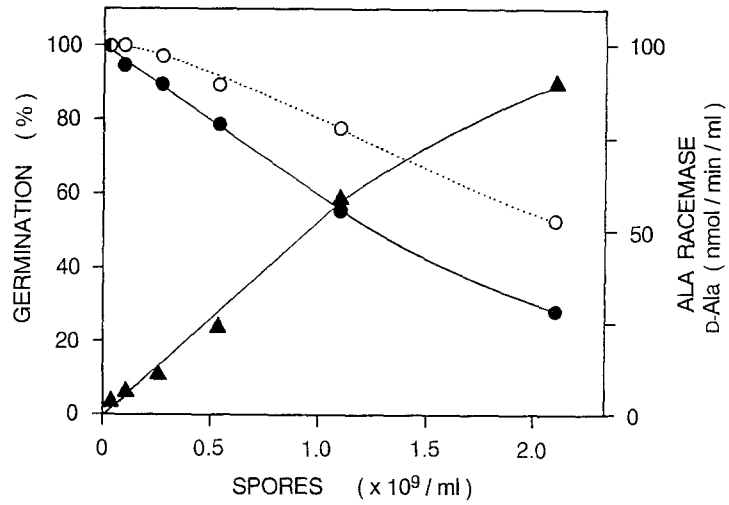

Fig. 5. Effect of spore population on germination and alanine racemase of *B. subtilis* PCI219 spores. Germination was determined in 30 μM L-Ala for 120 min in the presence (o) and absence (●) of 50 mM D-penicillamine. Alanine racemase was determined in 100 mM L-Ala and 1.5 mM diphenylamine (▲)

in 100 mM L-alanine because the activity could not be detected at 30 μM. Naturally, the higher the spore population, i.e. the enzyme concentration, the more D-alanine was formed.

Effect of temperature

The racemase activity was measured at the optimum pH (see below) and observed at a wide range of temperatures. The temperature profile with the opti-

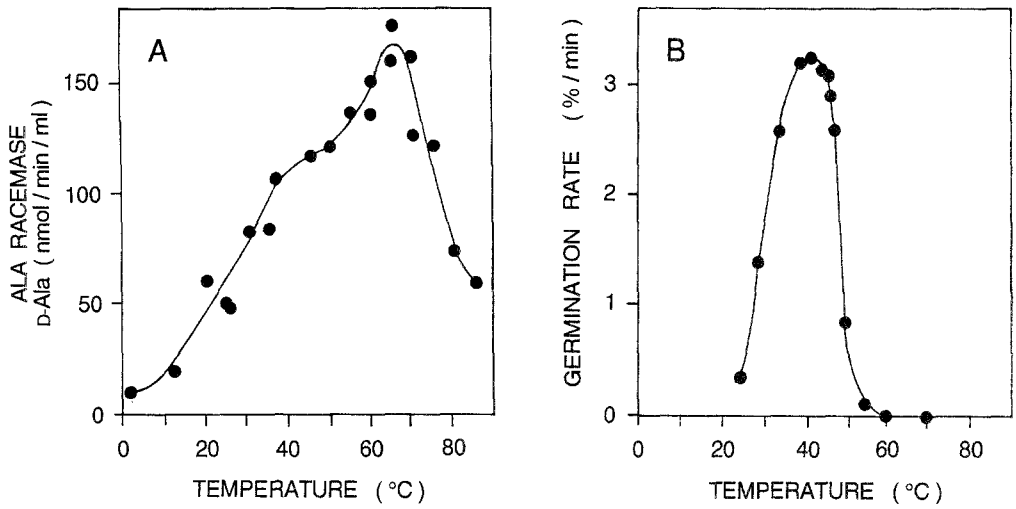

Fig. 6. Effect of temperature on alanine racemase activity and germination of *B. subtilis* PCI219 spores. For alanine racemase (**A**) spores (8.0×10^8/ml) were incubated with 100 mM L-Ala and 1.5 mM diphenylamine in 50 mM 2-(N-cyclohexylamino)ethanesulfonic acid (CHES)-NaOH buffer, pH 9.0. For germination (**B**) spores (2.2×10^8/ml) were incubated with 1 mM L-Ala in KNaPB

mum at 65°C and a shoulder around 40°C is shown in Fig. 6A. On the other hand, the germination rate was maximum at 43°C and germination was not at all observed at 65°C (Fig. 6B).

The spores preincubated with L-alanine at 70°C for 60 min showed much lower germination rate than the control when the temperature was shifted to 37°C, whereas the spores preincubated without L-alanine at 70°C for 60 min and transferred to 37°C along with the addition of L-alanine showed almost the

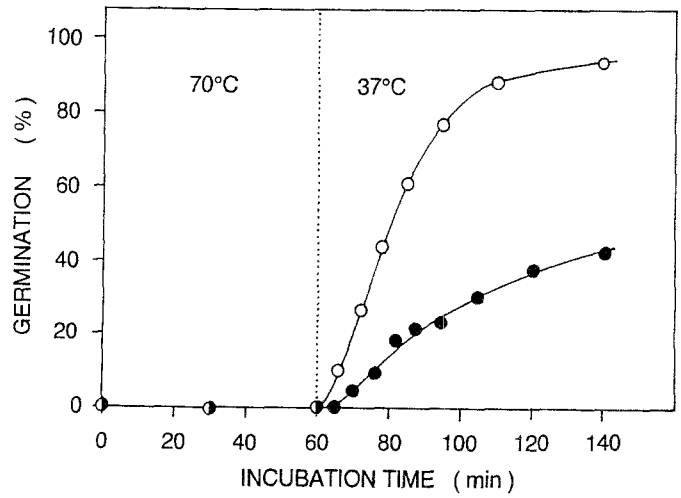

Fig. 7. Effect of preincubation of the spores of *B. subtilis* PCI219 with L-Ala at 70°C on germination. Spores (1.4×10^8/ml) were incubated in KNaPB at 70°C for 60 min and they were rapidly cooled to 37°C and incubated further. 0.1 mM L-Ala was added at 0 min (●) and 60 min (○)

same germination rate as the control (Fig. 7). Moreover, the decreased germination rate of the former was recovered by another addition of L-alanine (data not shown). These facts suggest that the germination apparatus on the spore was not inactivated by heating at 70°C as previously reported (Yasuda and Tochikubo 1985b) and that the significant decrease in germination rate might be associated with the decreased concentration of L-alanine and the increased concentration of D-isomer by the racemase which showed high activity at 70°C.

Effect of pH

Germination occurred at a wide range of pH and its optimum pH was 7.2. Racemase acted in alkaline pH and its optimum pH was 9.0. Racemase activity at pH 7.2 decreased by about one-half of that at pH 9.0, and germination rate at pH 9.0 by two-third of that at pH 7.2 (Fig. 8).

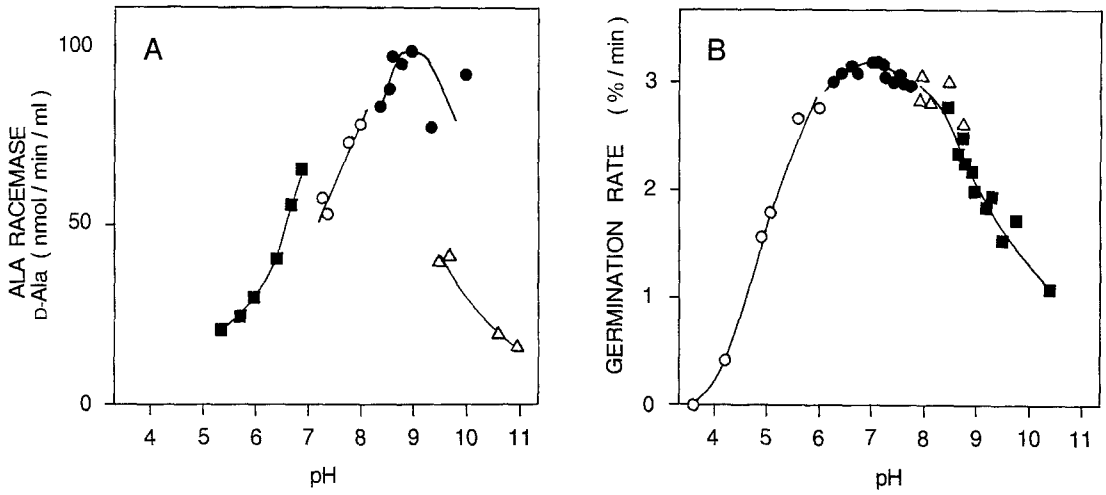

Fig. 8. Effect of pH on alanine racemase activity and germination of *B. subtilis* PCI219 spores. For alanine racemase (**A**) spores (8.0×10^8/ml) were incubated at 37°C in 2-(N-morpholino)-ethanesulfonic acid (MES)-NaOH (■), N-tris (hydroxymethyl)methyl-3-aminopropanesulfonic cid (TAPS)-NaOH (○), CHES-NaOH (●) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)-NaOH (△), each 50 mM. For germination (**B**) spores (2.2×10^8/ml) were incubated at 37°C in potassium phthalate-KOH (○), KNaPB (●), Tris-HCl-KCl (△), and boric acid-NaOH-KCl (■), each 50 mM

Discussion

The dormant spores of *B. subtilis* strain PCI219 showed the activity of alanine racemase which seems to be bound to the spore coat. The racemase activity, however, was independent of the phenomena that appeared first after germination, which could start in the presence of L-alanine, a substrate of racemase, because germination-inhibited spores also showed the same level of the racemase activity as that of germinating spores.

Stewart and Halvorson (1953) have also reported the occurrence of alanine racemase in dormant spores of *Bacillus cereus* and other *Bacillus* species and

that the spores generally contained 3 to 16 times higher order of the racemase activity relative to vegetative cells. Alanine racemase is one of the widely distributed enzymes of vegetative cells of bacteria for providing D-alanine as a constituent of cell wall peptidoglycan. However, the physiological role of this enzyme in spores is uncertain. Stimulation of spore germination by inhibitors of alanine racemase such as O-carbamyl-D-serine, D-cycloserine and β-alanylhydroxamic acid has been reported in *B. cereus* (Jones and Gould, 1968) and *Bacillus anthracis* (Titball and Manchee, 1987). It had been difficult to assay alanine racemase and germination separately. However, in this report we could determine net alanine racemase activity using diphenylamine as a germination inhibitor and *vice versa* germination without the influence of racemase using D-penicillamine as a racemase inhibitor. The following results were obtained from the relationship between germination and alanine racemase in *B. subtilis* PCI219 spores.

Affinity of L-alanine to the germinant receptor was about 1,000 times higher than that to the alanine racemase. Therefore, germination in high concentrations of L-alanine was not apparently affected by racemase although the enzyme converted L-alanine partially to D-isomer, but in low concentrations of L-alanine incomplete germination occurred because of the critical decrease and simultaneous increase with time in concentrations of germinant and inhibitor, respectively. Affinity of D-alanine is twice as much as that of L-alanine to the germinant receptor (Yasuda and Tochikubo, 1984a, 1985a). The inhibition of germination was recovered by the addition of excess L-alanine or D-penicillamine. In the above phenomena the relative concentration of L-alanine to spore population was important: at a constant concentration of L-alanine, the higher the spore population, i.e. the more racemase concentration, the less germination was observed. The incompleteness of germination may be significant for the germinated spores to grow under the limited environmental conditions and also for the rest to survive in a dormant state. Fey et al. (1964) and Preston and Douthit (1984) have also pointed out that thick suspensions of spores germinate incompletely. In this manner dormant spores may be able to sense the nutrient availability as a whole population. Spore alanine racemase was most active at 65°C, at which germination was not initiated. This indicates a reasonable mechanism not to germinate under such an unfavorable condition for growth. Dormant spores of this strain were not inactivated at this temperature (Fig. 9), but germinated spores and vegetative cells were inactivated within 1 min (data not shown). The biphasic profile of the temperature-activity curve (Fig. 6A) possibly suggests a presence of an isozyme (Kanda et al., unpublished observation). The optimal pH of the alanine racemase was around 9.0; at this pH germination rate was at half of its maximum and growth was slower than that at pH 7.2 (data not shown). Thus, under these unfavorable conditions for vegetative growth such as high population of the spores, high temperature and high pH of the medium, spore alanine racemase converts the germinant actively to the inhibitor and may regulate spore germination for survival of the organism.

The spore preparations stored for 2 and 10 years at 4°C showed 62% and 32% alanine racemase activity of that of the fresh preparation, respectively (Kanda et al., unpublished observation). The inactivation of the racemase during long

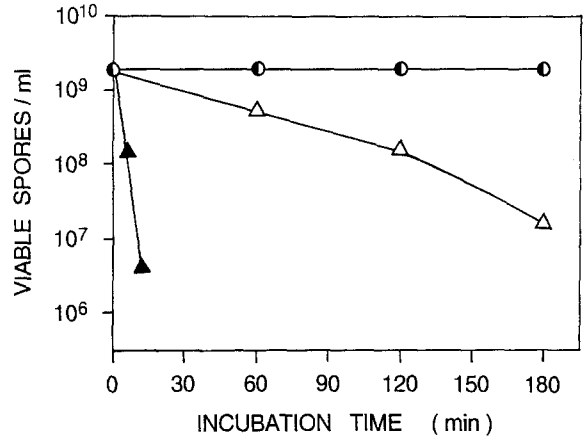

Fig. 9. Inactivation of the spores of *B. subtilis* PCI219 by heat. Aqueous suspensions of the spores (1.2×10^9/ml) were heated at 65°C (○), 75°C (●), 85°C (△), and 95°C (▲) for indicated times, and colony forming ability was determined on nutrient agar after incubation at 37°C for 16 hr

storage periods may be associated with the previous observation that the spores of 10 years-old preparation showed stimulated germination rate (Kanda et al., 1991) which is generally known as the aging effect on the spore dormancy.

For *B. subtilis* PCI219 spores many L-alanine analogues (Yasuda and Tochikubo, 1985a; Kanda et al., 1988) and a combination of L-asparagine, glucose and fructose (Kanda et al., 1991) are able to initiate germination. Similar role of the alanine racemase in regulating other germinants-initiation is unlikely because D-isomers of these germinants are not always inhibitors of germination (Yasuda and Tochikubo, 1985a) and some of these germinants were evidently not substrates for the spore racemase (data not shown). Low affinity of the spore alanine racemase to L-alanine, i.e. high Km value at mM order, was in good agreement with those in vegetative cells of the same species (Diven et al., 1964; Yonaha et al., 1975) and in spores of other *Bacillus* species (Stewart and Halvorson, 1953; Church et al., 1954; Preston and Douthit 1984). Some enzymatic properties of the spore alanine racemase, including kinetic parameters, inhibitors, isozyme, heat stability and D- to L- activity are to be published elsewhere (Kanda et al.).

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