Germination of *Bacillus cereus* Spores: Critical Control by DL-Alanine Racemase

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Effects of DL-alanine racemase, D-alanine, heat-activation and ammonia on germination of *Bacillus cereus* T spores were studied. Michaelis–Menten parameters for DL-alanine racemase activity in intact spores were: K_m (L-alanine) = 3.6 mM; K_m (D-alanine) = 1.2 mM; V_{max} (L- to D-alanine) = $1.6 \mu\text{mol min}^{-1}$ (mg dry spores) $^{-1}$; V_{max} (D- to L-alanine) = $0.53 \mu\text{mol min}^{-1}$ (mg dry spores) $^{-1}$. Racemase activity was irreversibly inhibited with pseudo first order kinetics by incubating intact spores with 10 mM-D-cycloserine. (Aminooxy)acetate (50 μM) completely inhibited racemase activity with 100 mM-L-alanine as substrate. D-Cysteine was a moderately effective racemase inhibitor. Comparisons of the effects of racemase inhibitors, D-alanine and spore concentration on germination rates in 1 mM-L-alanine indicated that racemization of L-alanine accounted for nearly total inhibition of germination at spore concentrations exceeding about $50 \mu\text{g ml}^{-1}$, in the range conventionally used for spectrophotometric germination assays. Heat-activation decreased the inhibitory effect of D-alanine, increasing the germination rate in 2 mM-racemic alanine by a factor of 26. Using spores with an inhibited alanine racemase, germination rates in 1 mM-L-alanine were stimulated by 40 mM-NH₄Cl to an extent comparable to the stimulation obtained by heat-activating the spores. Germination of unactivated spores in 1 mM-L-alanine + 40 mM-NH₄Cl was completely inhibited by 1 mM-D-alanine. These results clarified the roles of D-alanine and DL-alanine racemase in controlling the germination of unactivated spores and suggested that the germinative functions of ammonia and the stereo-isomers of alanine may be related.

INTRODUCTION

Spores of many species of *Bacillus* and *Clostridium* germinate poorly in nutrient media unless first activated by one of a variety of treatments (Keynan & Evenchik, 1969). Activation by sub-lethal heating (e.g. 60–70 °C for mesophilic strains) has commonly been employed for increasing the germinability spores in a variety of experimental contexts. In a previous report, we established that the requirement for activation for rapid germination of spores of *B. cereus* could be eliminated by including ammonia in a germinant mixture containing L-alanine and either inosine or adenosine (Preston & Douthit, 1984). By avoiding the introduction of unknown variables associated with sub-lethal heat activation, it appeared that the use of ammonia as a germination stimulant could simplify the analysis of mechanisms controlling the germination of bacterial spores.

Our analysis of ammonia-stimulated germination included an attempt to simplify the germination reaction mixture, but both L-alanine and a riboside appeared to be essential for germination of unactivated spores (Preston & Douthit, 1984). Subsequently, we found that moderate concentrations of L-alanine (1 mM or less) could initiate germination of unactivated spores if 'auto-inhibitory' effects associated with racemization of L-alanine were avoided. Using

Abbreviations: AOA, (aminooxy)acetate; DCS, D-cycloserine.

heat-activated spores of *B. cereus* and *B. anthracis*, Krask (1961) observed 'rate limitations on germination which appear to be a direct result of alanine racemase activity of aerobic spores'. This auto-inhibition of germination arising from racemase activity was then substantiated by Gould and his co-workers (Fey *et al.*, 1964; Jones & Gould, 1968). The results presented here extend these findings as part of a continuing analysis of the mechanism of germination of unactivated spores of *B. cereus* T. We establish methods for specifically eliminating DL-alanine racemase activity in *B. cereus* spores, compare the effects of exogenous D- and L-alanine on both activated and unactivated spores, and demonstrate the effectiveness of ammonia as a co-germinant with L-alanine. Our results emphasize the critical roles of D-alanine and DL-alanine racemase in controlling the germination of unactivated spores of *B. cereus* T. An understanding of these critical roles seems essential for analysing mechanisms of L-alanine-induced spore germination. The results are also relevant to questions concerning the adaptive significance of DL-alanine racemase in dormant spores (Stewart & Halvorson, 1953).

METHODS

Bacterial strains. *B. cereus* T was obtained from N. McCormick (University of Wisconsin) in 1967 and maintained as spores in 50% glycerol at -20°C . A single colony isolate of this strain was used to prepare a lyophilized spore stock (see below) that was stored at -20°C . All subsequent spore preparations were inoculated with spores from this stock.

Reproducibility of results. With the exception of the experiment described in Table 3, the results reported here were obtained using a single crop of spores. Use of a single crop minimized uncertainties in correlating the described effects of racemase activity, racemase inhibitors, and exogenous D-alanine on spore germination rates. Replicate germination assays using succeeding spore crops differed by up to 40% of the values given here for germination rates under specified conditions, although differences were more commonly less than 15% from crop to crop. Replicate rates obtained on a given day or on succeeding days for a single crop usually differed by less than 10% when precautions were taken to avoid contamination of stocks and labware by spores having an active racemase. (Detergents were not always reliable for adequately removing spores from labware. Periodic cleaning with warm chromic acid, followed by rinsing with ammonium hydroxide and water, was essential for reproducibility.) The kinetic parameters for alanine racemase activity (see Results) differed by 6% in assays performed at the beginning and end of the series of experiments reported here; the values quoted (Results) are the averages of these two determinations.

Preparation of spores. Cultures of 100 ml in modified G-medium (Vary & Halvorson, 1968) containing 0.2% (w/v) glucose were grown in 1-litre flasks at 30°C on a rotary incubator shaker at 200 r.p.m. These cultures were inoculated with 50 μl of a suspension of spores (10^8 spores ml^{-1}) that had been pasteurized by heating for 10 min at 65°C and germinated in 1 mM-L-alanine, 1 mM-inosine, 20 mM-sodium phosphate, 5 mM- NH_4Cl , pH 8. After 20–24 h growth, the sporulated cultures were incubated overnight at 4°C to induce lysis of any remaining sporangia. Spore harvesting and washing by centrifugation involved ten sequential washes with distilled water and 1 M-KCl as previously described (Preston & Douthit, 1984). Washed spores were stored in water on ice (5 mg ml^{-1}) and used within 3 weeks of preparation. Spore concentrations were routinely determined from absorbance measurements by comparison with standards dried to constant weight at 100°C .

DL-Alanine racemase assays. DL-Alanine racemase activity in whole spores was assayed by measuring the appearance (L- to D- activity) or disappearance (D- to L- activity) of D-alanine in suspensions of spores incubated with L- or D-alanine. In the L- to D- direction, an appropriate concentration of spores was equilibrated at 30°C (2 min) in 20 mM-Tris/HCl, pH 8.4, prior to starting the reaction by adding 0.1 vol. L-alanine/NaOH, pH 8.4, at specified concentrations. After 2 min the reaction was stopped by adding 0.025 vol. 1.6 M-HCl to shift the pH below 3, at the same time cooling the suspension to 4°C . Spores were then removed by centrifugation or filtration and the supernatants assayed for D-alanine as described below, after the addition of 0.025 vol. 1.6 M-NaOH. Racemization was limited to 5% of the initial L-alanine concentration by adjusting the spore concentration. Controls showed that the racemization rate was constant during a 2 min period. Subsequently, the amount of D-alanine formed in 2 min was used to calculate initial rates of racemization. Initial rates obtained with L-alanine concentrations from 1 mM- to 50 mM provided linear Lineweaver-Burke plots from which the Michaelis-Menten parameters for the L- to D-alanine reaction were determined. No detectable germination occurred during the 2 min exposure to L-alanine at any concentration, whether racemase inhibitors were present or not.

Racemase activity in the D- to L-alanine direction was assayed by incubating spores in various concentrations of D-alanine in 20 mM-Tris/HCl, pH 8.4, 30°C , and assaying samples for D-alanine periodically (see below) to follow the time course of racemization. The Michaelis-Menten parameters for the D- to L- reaction were obtained by optimizing the fit of the data for D-alanine racemization to the integrated form of the Michaelis-Menten rate law for a reversible reaction (Segel, 1975) using a non-linear least squares optimization program (Brent, 1971).

D-Alanine assay. D-Alanine was assayed by coupling the production of pyruvate by D-amino acid oxidase to the oxidation of NADH by lactic dehydrogenase as described by Grassl (1974), using an absorption coefficient of $6.22 \text{ mm}^{-1} \text{ cm}^{-1}$ at 340 nm for NADH. Change in absorbance at 340 nm was monitored continuously for 45–60 min, until the reactions were essentially complete. No substances interfering with the assay for D-alanine were extracted from spores during alanine racemase assays.

Racemase inhibitor assays. Unless otherwise specified, inhibitors of alanine racemase were tested using the L- to D-alanine assay described above, with 100 mM-L-alanine as substrate and inhibitor concentrations as specified. Unless otherwise specified, spores were preincubated with inhibitor in 20 mM-Tris/HCl pH 8.4, 30 °C, for 2 min prior to starting the racemase reaction by adding L-alanine. When present at the concentrations used in these assays, the racemase inhibitors did not interfere with the assay for D-alanine, except in the case of hydroxylamine. Hydroxylamine could not be tested as a racemase inhibitor using Grassl's (1974) assay for D-alanine since it interfered with the recovery of D-alanine in controls.

Germination assays. Germination was assayed as the decrease in absorbance (450 nm) of a suspension of spores, using a Gilford model 240 spectrophotometer. Standard 3 ml cuvettes having 1 cm optical paths were used when spore concentrations exceeded $10 \mu\text{g dry wt ml}^{-1}$ ($A_{450} = 0.11$). To permit accurate assays using spore concentrations as low as $0.3 \mu\text{g ml}^{-1}$, a cuvette having four adjacent 15 ml chambers with 10 cm optical paths was milled from a block of Teflon, with glass slides attached at each end as optical windows. A Beer's law relationship between absorbance, spore concentration, and optical path length was observed with the 1 cm and 10 cm cuvettes for absorbances less than 1.0 (450 nm), and appropriate controls confirmed that germination rates were independent of cuvette geometry in most cases. However, in assays involving very low spore concentrations ($\leq 2 \mu\text{g dry wt ml}^{-1}$), a slow decrease in absorbance ($\leq 3\%$ of the initial absorbance h^{-1}) occurred in germination buffers lacking L-alanine in the 15 ml Teflon cuvette, apparently due to adsorption of spores to the walls of the cuvette. In the results reported, this change in absorbance in the absence of germinant was negligible compared to the observed germination rates. When accurate measurements of very low germination rates were required (Table 3), the change in absorbance of controls lacking germinant was reduced to 1% in 24 h by performing the assays in calibrated 10 mm glass tubes that were capped with Parafilm. The tubes were incubated at 30 °C and stirred gently by abrupt rotation prior to periodic measurement of absorbance in a Bausch and Lomb model 710 spectrophotometer. Regardless of the apparatus, 100% germination corresponded to a 50% decrease in absorbance at 450 nm, as confirmed by phase contrast microscopy.

Germination reactions were conducted in 20 mM-Tris/HCl at 30 °C, with L-alanine, D-alanine, and various racemase inhibitors added as specified. All reaction mixtures were adjusted to pH 8.4 at 30 °C, within 0.02 pH units, using HCl or NaOH as required. In general, assays were started by adding spores to the reaction mixtures. When reaction mixtures contained racemase inhibitors, however, spores were preincubated in the buffered inhibitors for specified times prior to initiating germination by adding L-alanine. Absorbance was recorded continuously on a strip chart recorder, and the germination rate was defined as the slope of the tangent to the most rapidly changing portion of the recording of A_{450} vs time, divided by the initial absorbance of the suspension, expressed as a percentage ($\%A_{450} \text{ min}^{-1}$).

Materials. Inorganic reagents were analytical reagent grade from various commercial sources. Yeast extract was from Difco. Enzymes and organic chemicals were from Sigma. Stock solutions of L- and D-alanine were stored on ice and replaced after one week. Stock solutions of inhibitors, except D-cycloserine (DCS), were prepared fresh as required in deionized, distilled water, and stored on ice. DCS solutions were prepared as required in 20 mM-sodium phosphate, pH 7.5, and used immediately to avoid decomposition as reported elsewhere (Wang & Walsh, 1978).

RESULTS

Activity of DL-alanine racemase in dormant spores

Measurements of alanine racemase activity in dormant spores of *B. cereus* T grown and germinated under our particular conditions were required to determine the magnitude of auto-inhibition of germination due to this source of D-alanine. Conventional kinetic analysis (Methods), at the same temperature and pH as subsequently used in germination reactions, revealed that racemase activity in whole spores was accurately described by a Michaelis-Menten rate law for a reversible, single substrate reaction (Segel, 1975). The best-fit parameters were as follows: K_m (L-alanine), 3.6 mM; K_m (D-alanine), 1.2 mM; $V_{\max}$ (L- to D- direction), $1.6 \mu\text{mol min}^{-1} (\text{mg spore dry wt})^{-1}$; $V_{\max}$ (D- to L-direction), $0.53 \mu\text{mol min}^{-1} (\text{mg spore dry wt})^{-1}$. These values were in reasonable agreement with those originally determined for the L- to D-alanine racemase activity in this strain [Stewart & Halvorson (1953): K_m 5.8 mM; $V_{\max}$ $1.4 \mu\text{mol min}^{-1} (\text{mg spore dry wt})^{-1}$]. As with the isolated enzymes from various other species,

Table 1. *Inhibition of alanine racemase in whole spores*

Spores (0.9 mg ml^{-1}) were incubated for 10 min in 20 mM-sodium phosphate plus the specified inhibitor at pH 7.5, 30°C , cooled to 4°C , centrifuged, washed with an equal volume of phosphate buffer lacking inhibitor, resuspended in distilled water, then assayed for racemase activity as described in Methods. Assay for racemase included 2 min preincubation of spores in buffer or, where specified, buffer + inhibitor, prior to starting the reaction by adding 100 mM-L-alanine.

Inhibitor present during		Racemase activity* ($\mu\text{mol min}^{-1} \text{ mg}^{-1}$)	Inhibition (%)
Pretreatment	Racemase assay		
(No pretreatment)	None	1.5	0
None†	None	1.5	0
10 mM-D-Cysteine	None	1.5	0
None	2 mM-D-Cysteine	1.2	20
None	5 mM-D-Cysteine	0.56	63
None	10 mM-D-Cysteine	0.24	84
None	0.05 mM-AOA	<0.07	>95
10 mM-DCS	None	<0.07	>95

* Rates shown are representative values. Replicates of controls agreed within 10%; $0.07 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ was the detection limit for racemase activity under the conditions employed.

† Pretreatment in buffer without inhibitor, as described above.

the K_m values for L- and D-alanine revealed a slightly asymmetric binding of the two stereoisomers of alanine (Adams, 1976).

Characterization of alanine racemase inhibitors

Means for specifically inhibiting racemase activity in dormant spores were required to eliminate auto-inhibitory effects in studies of L-alanine-induced germination, and to allow an accurate assessment of the inhibitory strength of exogenous D-alanine. The use of irreversible inhibitors seemed desirable for this purpose, since removal of the unreacted inhibitor prior to germination assays would minimize its possible interference with the germinative functions of L- and D-alanine. Previous studies had described partial inhibition of racemase activity in whole spores of *B. cereus* T by the inhibitor D-cycloserine (DCS), and had suggested that DCS might be combining with the racemase (Jones & Gould, 1968). Subsequent biochemical studies indicated that DCS inhibited alanine racemase by an irreversible, active-site-directed mechanism that would be relatively specific for this enzyme (Rando, 1975; Wang & Walsh, 1978). We reassessed the inhibitory activity of DCS with whole spores and tested two other racemase inhibitors for comparative purposes (Table 1). As shown, both DCS and (aminooxy)acetate (AOA) were potent inhibitors of alanine racemase in whole spores, with DCS inhibiting by an apparently irreversible mechanism. Although not directly tested for reversibility, AOA appeared to be a reversible racemase inhibitor, as reported elsewhere (Free *et al.*, 1967), based on spore germination data not shown here. D-Cysteine was known to be an inhibitor of the racemase in this strain (Krask, 1961), and it was possible that its effect would be irreversible (Braunstein, 1960; Meister, 1965). However, our results revealed that inhibition by D-cysteine was reversible and required relatively high concentrations to provide reasonably complete inhibition (Table 1).

Inhibition of racemase in whole spores by DCS was further characterized by measuring the time course of inhibition resulting from DCS pretreatments. As shown (Fig. 1), racemase activity disappeared with pseudo first order kinetics, having a half-life of 1.2 min when spores were incubated in 10 mM-DCS, 20 mM-sodium phosphate, pH 7.5, 30°C , then washed and assayed for racemase activity in the L- to D-alanine direction. This result was similar to that obtained with the purified racemase from *Escherichia coli*, which had a half-life of 4 min in 1 mM-DCS at pH 8, 20°C (Wang & Walsh, 1978). Based on the observed half-life of the *B. cereus* racemase in spores incubated with DCS under our conditions, the residual racemase activity following 15 min of DCS treatment (subsequently adopted as a standard treatment) would be $0.28 \text{ nmol min}^{-1} \text{ mg}^{-1}$ (0.02% of control V_{max}). This residual activity would be insignificant under the germination conditions described in this report.

Fig. 1

Fig. 2

Fig. 1. Kinetics of irreversible inhibition of alanine racemase following DCS treatment. Spores were incubated (0.5 mg ml^{-1}) in 10 mM-DCS , $20 \text{ mM-sodium phosphate}$, $\text{pH } 7.5$, 30°C , for the indicated times, cooled to 0°C within 30 s , centrifuged and washed with an equal volume of $20 \text{ mM-sodium phosphate}$, $\text{pH } 7.5$, 0°C , then resuspended in distilled water at 0°C . Samples were assayed immediately for racemase activity as described in Methods (●). After 24 h on ice, spores that had been treated for 0.7 min with DCS were again assayed for racemase activity (to test the stability of intermediate levels of inactivation) (○). In both cases, control spores preincubated in buffer lacking DCS had a racemase activity of $1.6 \mu\text{mol min}^{-1} (\text{mg spore dry wt})^{-1}$. The half-life of racemase activity was obtained by linear regression through points excluding the zero time point (broken line). The abrupt drop in activity at the beginning of the curve is an artifact resulting from the mild method (cooling) used for quenching the reaction with DCS, a method that avoided possible secondary effects of acid-quenching on mechanisms controlling spore germination.

Fig. 2. Dependence of germination rate on spore concentration and racemase activity. Germination assay mixture contained 1 mM-L-alanine , 20 mM-Tris/HCl , $\text{pH } 8.4$ at 30°C . Germination rates of: spores preincubated with DCS for 15 min (Fig. 1, legend), having no detectable racemase activity (▲); spores having 25% of normal racemase activity ($60 \text{ s DCS treatment}$) (■); control spores, preincubated for 15 min in buffer lacking DCS, with racemase activity of $1.6 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ (in 100 mM-L-alanine) (●). Open symbols: same spores as corresponding filled symbols, but with $20 \mu\text{M-AOA}$ included in the germination assay mixture.

Following incubation in DCS, washing, and storage in distilled water on ice, spores retained for long periods whatever level of racemase activity they had acquired during treatment with DCS. Spores having 37% of their original racemase activity immediately after DCS treatment had the same activity after 24 h storage on ice (Fig. 1), and spores having undetectable racemase activity, as prepared, remained free of activity after 2 d storage on ice.

Effects of racemization of L-alanine on spore germination

Gould and his co-workers had previously established that D-alanine arising from racemization influenced the rate and extent of germination initiated by L-alanine (Fey *et al.*, 1964; Jones & Gould, 1968). Their results with spores of *B. subtilis* showed that the inhibitory effect of racemization could be reduced by using lower concentrations of spores in germination assays, thereby decreasing the rate of production of diffusible D-alanine (Fey *et al.*, 1964).

In an attempt to similarly control racemization in *B. cereus* without the use of inhibitors, we assayed germination of unactivated spores in 1 mM-L-alanine at successively lower spore concentrations (Fig. 2, control spores). As shown, 1 mM-L-alanine was an ineffective germinant for unactivated spores when the spore concentration in the assay exceeded about $25 \mu\text{g ml}^{-1}$, but it became increasingly effective as the spore concentration was decreased. To a first approximation (see Discussion), germination rates in 1 mM-L-alanine in the absence of the effects of diffusible D-alanine could be estimated by extrapolating the concentration-dependence curves to the axis representing zero spore concentration (Fig. 2).

If the inhibitory effect of D-alanine produced by racemization were entirely due to a rising concentration of freely diffusible D-alanine during germination, then spores having an inactive racemase as a result of DCS pretreatment should have germinated at a rate not dependent on

spore concentration. Furthermore, this rate should have been the same as the rate obtained at zero spore concentration (by extrapolation) using spores with an active racemase. As shown, however (Fig. 2), while the germination rate of spores with an inactive racemase did not depend on spore concentration, this rate was roughly 1.6-fold higher than the rate of the control spores at zero concentration. With spore preparations having a partially inactivated racemase (following brief pretreatment with DCS), germination rates were dependent on spore concentration to an intermediate extent, and the concentration-dependence curve extrapolated to a rate at zero spore concentration that was intermediate between the rates obtained with spores having normal or completely inactivated racemase activities (Fig. 2).

Specificity of the stimulatory effect of DCS on germination

The fact that germination rates were no longer dependent on spore concentration following the inactivation of alanine racemase by DCS pretreatment suggested that diffusible D-alanine was the major auto-inhibitory substance produced during germination in L-alanine. However, the fact that DCS treatment resulted in faster germination than observed with control spores at 'zero' concentration emphasized the possibility that DCS might stimulate germination by secondary mechanisms as well as by inhibiting alanine racemase. This possibility was addressed by comparing the stimulatory effect of DCS with the effects obtained using other racemase inhibitors.

When 20 μM -AOA was included as a competitive racemase inhibitor in germination assay mixtures containing 1 mM-L-alanine, spores germinated at a rate equal to that of DCS-treated spores having no detectable racemase activity (Fig. 2). The same AOA-stimulated rate was observed when the germination assay was conducted with normal spores or with spores that had been pretreated with DCS to partially or completely inactivate alanine racemase (Fig. 2). These results suggested that neither of these inhibitors had stimulatory functions secondary to that due to inhibition of alanine racemase, since they produced the same germination rates despite chemical differences in their mechanisms of racemase inhibition (AOA, a derivative of hydroxylamine, is a pyridoxal phosphate antagonist, while DCS is an alanine analogue inhibiting by a 'suicide' mechanism; see above).

The effects of various racemase inhibitors on germination rates were further compared using both high and low concentrations of spores during germination assays (Table 2). Germination rates obtained with 20 μM -AOA or 10 mM (pretreatment only) DCS were 1.8–1.9 % $A_{450} \text{ min}^{-1}$ at low spore concentration and 1.6 % $A_{450} \text{ min}^{-1}$ at high spore concentration, supporting similar specific functions for these substances as racemase inhibitors. The slight decrease in germination rate at the higher spore concentration with DCS or AOA (Table 2) was not observed in subsequent experiments under similar conditions, where germination rates remained independent of spore concentration up to 110 μg spore dry wt ml^{-1} , which was the highest concentration tested. The reason for the slight dependence of rates on spore concentration evident in the results presented in Table 2 is not known.

Jones & Gould (1968) previously established that hydroxylamine inhibits alanine racemase in spores of *B. cereus* T and stimulates germination of high concentrations of unactivated spores. Our results corroborated their observation of the stimulatory effect of hydroxylamine on spore germination and showed that a lower concentration of this inhibitor sufficed for maximal stimulation when using lower spore concentrations (Table 2). Compared to DCS and AOA, hydroxylamine was slightly more stimulatory for germination at both high and low spore concentrations. This may have been a result of the decomposition of hydroxylamine to ammonia under the alkaline conditions of these experiments (see below), although a slight stimulation specifically due to hydroxylamine has not been ruled out.

At the lower concentrations tested, D-cysteine was less effective than the other racemase inhibitors as a germination stimulant, while high concentrations of D-cysteine inhibited germination (Table 2). This complex response to D-cysteine, depending in part on spore concentration, argued against the use of this substance for the specific control of racemase activity.

Fig. 3

Fig. 4

Fig. 3. Inhibition of germination by D-alanine. Germination assay mixtures contained $20 \mu\text{g}$ spores ml^{-1} , 1 mM -L-alanine, 20 mM -Tris/HCl, and the indicated concentration of D-alanine at pH 8.4, 30°C . Spores were preincubated with 10 mM -DCS for 15 min as described (Fig. 1, legend), to inactivate alanine racemase. Relative germination rates of: unactivated spores (●); heat-activated spores (▲); unactivated spores with 40 mM - NH_4Cl (pH 8.4) included in germination assay mixture (■). Rates prior to normalization, with no D-alanine, were: unactivated spores, $1.9\% A_{450} \text{ min}^{-1}$; heat-activated spores, $12\% A_{450} \text{ min}^{-1}$; unactivated spores in the presence of NH_4Cl , $8.9\% A_{450} \text{ min}^{-1}$. Heat-activation treatment: spores (4.5 mg ml^{-1}) were incubated in 20 mM -sodium phosphate, pH 6, 65°C , for 2 h, cooled to 0°C , washed once with an equal volume of distilled water at 0°C and resuspended in distilled water at 0°C .

Fig. 4. Comparison of auto-inhibition of germination with inhibition predicted by the calculated rate of racemization and the observed sensitivity to exogenous D-alanine. Germination assay conditions were as described in the legend to Fig. 2. ●, Inhibition of germination rate calculated from the curve for control spores in Fig. 2, using $1.6\% A_{450} \text{ min}^{-1}$ as the uninhibited rate of spores having no racemase activity; ■, inhibition of germination rate predicted by the D-alanine-sensitivity curve (Fig. 3, unactivated spores), and the concentration of diffusible D-alanine that would be produced in 10 min by the racemase activity of the indicated concentrations of spores in 1 mM -L-alanine. The dashed line shows the concentration of D-alanine produced in 10 min by the indicated concentration of spores, estimated from the Michaelis-Menten parameters for the racemase ($V_{\text{max}} = 1.6 \mu\text{mol min}^{-1} \text{ mg}^{-1}$; $K_m = 3.6 \text{ mM}$).

Table 2. *Effects of alanine racemase inhibitors on germination of low and high concentrations of spores*

Assay mixtures contained 1 mM -L-alanine, 20 mM -Tris/HCl and the indicated concentrations of inhibitor, pH 8.4, 30°C . Spore's ($100 \mu\text{g ml}^{-1}$) were preincubated in 20 mM -sodium phosphate (pH 7.5) with the indicated concentration of inhibitor for 15 min at 30°C , concentrated by centrifugation, then added to germination assay mixtures containing the indicated concentration of inhibitor. Germination rates shown are the averages of duplicate values.

Inhibitor	Concn (mM)	Spore concentration:			
		$1 \mu\text{g ml}^{-1}$		$50 \mu\text{g ml}^{-1}$	
		Rate*	%†	Rate*	%†
None	—	0.86	—	<0.05	—
D-Cysteine	0.1	1.3	51	0.14	>180
	1.0	1.2	40	0.72	>1300
	10	0.18	—79	0.10	>100
Hydroxylamine	0.1	2.2	160	0.51	>920
	1.0	2.4	180	1.7	>3300
	10	2.1	140	1.8	>3500
AOA	0.02	1.9	120	1.6	>3100
DCS	‡	1.8	110	1.6	>3100

* Germination rate, $\% A_{450} \text{ min}^{-1}$.

† Percentage increase in germination rate over that of control lacking inhibitor (a negative value indicates percentage decrease in germination rate).

‡ DCS (10 mM) in pretreatment only; spores washed with buffer after pretreatment.

Sensitivity of germination to inhibition by D-alanine

The results described above strongly suggested that unactivated spores racemized L-alanine at a rate sufficient to produce total inhibition of germination when using spore concentrations traditionally employed for spectrophotometric germination assays. If so, similar inhibition would be expected in the absence of racemase activity using exogenous D-alanine concentrations similar to those normally produced by the racemase. When DCS-treated spores having an inactive racemase were incubated in mixtures containing 1 mM-L-alanine and various amounts of D-alanine, we found that 10 μ M-D-alanine inhibited the germination rate by 50%, and 1 mM-D-alanine apparently blocked germination completely (Fig. 3). This level of sensitivity to D-alanine would account for severe inhibition of germination by racemization of L-alanine even with relatively dilute suspensions of spores.

The inhibitory effect of a rising concentration of D-alanine resulting from racemization would not be strictly comparable to the effect of exogenous D-alanine present at a fixed concentration since the beginning of a germination assay, as in Fig. 3. Nevertheless, the sensitivity curve (Fig. 3, unactivated spores) and the Michaelis-Menten parameters for the racemase (see above) allowed an approximate calculation of the inhibition of germination expected for concentrations of D-alanine synthesized by the racemase by the time germination rates were actually measured (at the inflexion point of the germination curves: see Methods). Comparing the inhibition predicted in that manner with the inhibition observed using various concentrations of spores (based on the data of Fig. 2), it appeared that sufficient D-alanine would be produced to account for the observed inhibition only with spore concentrations exceeding about 5 μ g ml⁻¹ (Fig. 4). At lower concentrations of spores, germination rates were apparently more severely inhibited than predicted by the amounts of diffusible D-alanine formed by alanine racemase (Fig. 4). Possible reasons for this discrepancy are discussed below (Discussion).

Decreased sensitivity to D-alanine following heat-activation

Previous work with *B. cereus* T (O'Connor & Halvorson, 1961; Krask, 1961) and other species (Wolf & Mahmoud, 1957) had shown that D-alanine was only moderately effective as an inhibitor of germination initiated by L-alanine. However, spores that had been activated by sub-lethal heating were used in those studies, and previous results in this laboratory indicated that heat-activation can reduce the inhibitory effect of D-alanine under certain conditions (Preston & Douthit, 1984). To further characterize the relationship between heat-activation and inhibition of germination by D-alanine, we compared the D-alanine-sensitivity of heat-activated spores with that of unactivated spores, again using DCS pretreatment to eliminate endogenous D-alanine production. Heat-activated spores required 60 μ M-D-alanine for 50% inhibition of germination rate, a concentration sixfold higher than required for similar inhibition of unactivated spores (Fig. 3). Furthermore, unlike unactivated spores, heat-activated spores germinated at an appreciable rate even in racemic alanine. In 2 mM-racemic alanine, suspensions of heat-activated spores germinated nearly completely within 1 h, while unactivated spores remained apparently dormant for 2 h, then required an additional 12.5 h to germinate nearly completely (Table 3).

Effect of ammonia on germination in L-alanine

The preceding experiments established conditions allowing germination of unactivated spores in 1 mM-L-alanine in the absence of co-germinative ribosides such as adenosine or inosine. This allowed further analysis of the stimulation of germination by ammonia (Preston & Douthit, 1984) uncomplicated by the effects of ribosides. Using spores having alanine racemase inhibited either by DCS, AOA, or hydroxylamine, 40 mM-NH₄Cl (a rate-saturating concentration) stimulated germination rates in 1 mM-L-alanine four- to fivefold (Fig. 3, legend). Germination of unactivated spores in 1 mM-L-alanine + 40 mM-NH₄Cl was severely inhibited by D-alanine. At concentrations of D-alanine below about 50 μ M, germination in the presence of NH₄Cl was only moderately more sensitive to D-alanine than the germination of heat-activated spores in the absence of NH₄Cl (Fig. 3). With concentrations of D-alanine exceeding 50 μ M,

Table 3. Germination of unactivated and heat-activated spores in racemic alanine

Germination assay mixtures contained 20 µg spores ml⁻¹, 2 mM-racemic alanine, 20 mM-Tris/HCl, pH 8.4, 30 °C.

Activation time* (h)	Germination rate (% <i>A</i> ₄₅₀ min ⁻¹)	Time (h) for germination off†:		
		10%	50%	90%
0	0.077	4.00	9.33	12.55
1	0.97	0.30	0.63	1.32
2	1.5	0.21	0.43	0.92
3	2.0	0.20	0.38	0.95
4	2.0	0.20	0.37	0.88
0‡	ND	> 24	ND	ND

ND, Not determined.

* Heat-activation at 65 °C for the indicated time, as described in the legend to Fig. 3.

† Times required for 10%, 50% or 90% germination based on absorbance change, with 100% germination corresponding to 50% decrease in the initial *A*₄₅₀. The *A*₄₅₀ of controls lacking alanine changed less than 1% in 24h.

‡ Assay mixture included 40 mM-NH₄Cl, pH 8.4.

however, germination stimulated by ammonia was much more severely inhibited than heat-activated germination at comparable D-alanine concentrations. Suspensions of unactivated spores in 2 mM racemic alanine + 40 mM-NH₄Cl were only 6% germinated after 24 h incubation, indicating a degree of inhibition considerably greater than that observed in the absence of NH₄Cl with either unactivated or heat-activated spores (Table 3.)

DISCUSSION

The results described above define a critical role for endogenous D-alanine in the control of bacterial spore germination initiated by L-alanine. Elimination of endogenous D-alanine through the use of specific inhibitors of DL-alanine racemase allowed an accurate assessment of the inhibitory effect of D-alanine on the germination of spores of *B. cereus* T (Fig. 3). The observations that unactivated spores of this strain are extremely sensitive to inhibition of germination by D-alanine, while heat-activated spores are much less sensitive (Fig. 3; Table 3) partially explains the requirement for heat-activation for germination of dense suspensions of spores of *B. cereus* T in moderate concentrations of L-alanine. A similar correlation between heat-activation and decreased sensitivity to D-alanine has appeared directly in previous reports (Levinson & Hyatt, 1955; Yousten, 1975) or can be inferred, in other studies, based on the auto-inhibitory effects of L-alanine racemization (Jones & Gould, 1968; O'Connor & Halvorson, 1961). In the present case, mechanisms involving more than desensitization to D-alanine are required to fully explain the stimulatory effect of heat-activation, since heat-activation stimulated germination substantially in the absence of either endogenous or exogenous D-alanine (Fig. 3, legend). However, in studies not specifically designed to detect possible inhibition by D-alanine, stimulation of germination by heat-activation could involve the combined effects of desensitization to inhibition by D-alanine as well as sensitization to L-alanine. This would be relevant whether alanine racemase or impure commercial L-alanine were the source of D-alanine. (Our own L-alanine assayed 0.05% D-alanine, an amount that appears insignificant for *B. cereus* spore germination under our conditions; Fig. 3.)

Control of germination of unactivated spores of *B. cereus* T in 1 mM-L-alanine involves kinetically complicated interactions between spore concentration, racemase activity, and spore sensitivity to D-alanine (Figs 2 and 4). Our physiological results are adequately described, in general, by a model of auto-inhibition involving the rapid synthesis of freely diffusible D-alanine by alanine racemase (Fey *et al.*, 1964). The apparent inhibition of germination occurring at 'zero' spore concentration (Figs 2 and 4) is not predicted by that model, however, and requires an

alternative explanation. It remains possible that DCS, AOA, and hydroxylamine all stimulate germination very similarly by a secondary mechanism in addition to the stimulation they produce by inhibiting alanine racemase. This seems unlikely, but it could account for an apparent inhibition of germination in the absence of these substances, unrelated to the inhibitory effect of alanine racemase activity. Another possibility is that our linear extrapolation to zero spore concentration (Fig. 2) underestimates true germination rates in the absence of diffusible D-alanine. The relationship between germination rate and spore concentration appeared to be linear at the lowest spore concentrations practical for turbidimetric germination assays, but an abrupt increase in rate could occur at yet lower spore concentrations, given the complexity of this phenomenon. It is also possible that individual spores in a suspension are exposed to D-alanine of their own making at locally elevated concentrations prior to its loss by diffusion. Calculations based on the data presented above show that modest permeability barriers external to the location of the alanine racemase could raise the internal level of D-alanine substantially, producing inhibitory effects even at 'zero' spore concentration (i.e. even for single spores immersed in an infinite pool of L-alanine).

Auto-inhibition of germination arising from alanine racemase activity has been considered relevant primarily in dense populations of spores (Stewart & Halvorson, 1953; see also Powell, 1957; Fey *et al.*, 1964). Our results suggest that auto-inhibition might occur in nature even in single, isolated spores. The auto-inhibitory effects we observed *in vitro* were barely significant at vanishing spore concentrations (Fig. 2), but amplification of those effects could occur in nature, for example in a different ionic environment. If selective pressures act on individual spores rather than populations of spores to maintain the auto-inhibitory mechanism, the adaptive features of this mechanism would be a very interesting mystery.

These observations and conclusions should be at least qualitatively applicable to the germination of spores of other species of *Bacillus* that contain an active alanine racemase (Stewart & Halvorson, 1953; Wolf & Mahmoud, 1957). In particular, Fey *et al.* (1964) found that germination of unactivated spores of *B. subtilis* (*B. globigii*) in 10 mM-L-alanine + 1 mM-glucose was nearly completely inhibited by racemization at spore concentrations of 1.1 mg ml^{-1} , about tenfold higher than required here for complete inhibition of germination of *B. cereus* spores in 1 mM-L-alanine. In general, high concentrations of L-alanine (greater than the apparent K_m of the alanine racemase) should partially overcome auto-inhibition due to racemization, since the rate of accumulation of D-alanine would be limited by the maximal rate of racemization (dependent on both spore concentration and the racemase activity per spore). Thus, the ratio of D- to L-alanine would rise at a limited rate, providing a longer time for spores to respond to low ratios favourable for germination. This may explain, in part, the necessity for seemingly high concentrations of L-alanine (10–100 mM) for rapid germination of *Bacillus cereus* spores (O'Connor & Halvorson, 1961; McCormick, 1965; Vary & Halvorson, 1965; Hashimoto *et al.*, 1969; Yousten, 1975).

Our analysis of the influence of alanine racemization on the germination of unactivated spores was undertaken, in part, in an attempt to simplify studies of the relationships between L-alanine, inosine, and ammonia as co-germinants of *B. cereus* T spores. Our previous observation (Preston & Douthit, 1984) that germination in 1 mM-L-alanine (in the absence of inosine) was not stimulated by ammonia can be explained by the results reported here. Rapid racemization of L-alanine, coupled with the exceptional sensitivity of unactivated spores to D-alanine, would eliminate the stimulatory effect of ammonia observed in the present study using spores with an inactive racemase (Fig. 3, legend). The observation that ammonia inhibited rather than stimulated germination in racemic alanine (Table 3) is difficult to interpret at this time. Similar mixed effects of ammonia on germination of *B. cereus* T spores were reported elsewhere, with inhibition by ammonia probably associated with racemization of L-alanine during slow germination assays (Shibata *et al.*, 1978).

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