Identification of D-Alanine as the Auto-Inhibitor of Germination of *Bacillus globigii* Spores

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SUMMARY

Thick suspensions of spores of *Bacillus globigii* did not germinate completely in nutrient media because the first spores to germinate released a substance which inhibited germination of the remainder. The inhibitory substance was identified as D-alanine by chromatography, high voltage electrophoresis, and its destruction by D-amino acid oxidase. When ^{14}C -L-alanine was used to induce germination of *B. globigii* spores, the resulting inhibitory D-alanine was ^{14}C -labelled with the same specific activity as the ^{14}C -L-alanine used. Auto-inhibition of *B. globigii* spore germination is therefore due to racemization of exogenous L-alanine, which forms sufficient D-alanine to arrest the germination process in thick suspensions of spores.

INTRODUCTION

More than 99 % of the individuals in suspensions of most types of bacterial spores will germinate in solutions of one or two amino acids or riboside (Hills, 1950); however, germination of suspensions of spores of some organisms may be incomplete in such media and also in complex media (Powell, 1950; Halvorson, 1959). Incomplete germination may have several causes (Murrell, 1961). It has often been noticed that high concentrations of spores germinate less completely than low concentrations. Stedman, Kravitz, Anmuth & Harding (1956) and Stedman, Kravitz, Harding & King (1957) found that spores of *Bacillus globigii* germinating in L-alanine + glucose exhibited pronounced auto-inhibition of germination at high spore concentrations. Their results suggested that, during germination of thick suspensions of *B. globigii* spores, a substance was released into the medium by the first spores to germinate which inhibited germination of the remainder. Stedman *et al.* (1956) showed that the inhibitor was competitive with L-alanine and suggested that it might be a high molecular weight substance, possibly with a terminal serine residue (Stedman *et al.* 1957). The strain of *B. globigii* in our possession also showed auto-inhibition of spore germination, and, since naturally occurring inhibitors of germination are rare, we re-examined the phenomenon to try and identify the auto-inhibitor. When this was proved to be D-alanine, its origin in the supernatant fluid of germinating spore suspensions was investigated.

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METHODS

Organisms and preparation of spores. Organisms used included *Bacillus subtilis* v. *niger* (syn. *B. globigii*), *B. subtilis* v. *aterrimus* NCTC 2590, *B. subtilis* strains m2 and s5, *B. cereus* NCTC 945 and *B. cereus* strains s20 and px. The strain of *B. globigii* was obtained from W. Bale (Microbiological Research Establishment, MRE, Porton, Wiltshire) and *B. cereus* px from R. E. Strange (MRE, Porton). The other strains were from the National Type Culture Collection (NCTC), Colindale, London, or were laboratory isolates. Spore suspensions were prepared, cleaned and stored as described by Hitchins, Gould & Hurst (1963), and the dry weights of suspensions were determined by drying samples to constant weight at 105°.

Media and measurement of germination. All spore suspensions except those of *Bacillus globigii* were heated at 70° for 30 min. before use. *B. globigii* spores germinated less rapidly after heating and were therefore used unheated at equiv. 75 µg. dry weight spores/ml. Germination medium for *B. globigii* spores consisted of L-alanine (10 mM), glucose (mM), sodium phosphate buffer (pH 7, 30 mM). Germination medium for the other spores was composed of L-alanine (500 µM), L-tyrosine (500 µM), inosine (20 µM), glucose (500 µM), sodium phosphate buffer (pH 7, 30 mM). Germination was measured by examining suspensions with the phase-contrast microscope and recording the % of phase-dark (germinated) spores. About 250 spores were counted for each estimation. Alternatively, the extinction of suspensions was measured with an absorptiometer ('Biochem', Hilger and Watts Ltd., Camden Road, London) and 560 mµ peak transmission filter. Germination was accompanied by a decrease in the extinction of suspensions.

Preparation of the auto-inhibitor. Spores of *Bacillus globigii* were suspended in germination medium at high concentrations (equiv. 1.1 mg. dry wt. spores/ml.). After 1 hr at 30° only about 20 % of the spores had germinated, indicating that auto-inhibition had occurred (Fig. 1). The spores were removed by centrifugation and the supernatant fluid concentrated tenfold in a rotary evaporator at 40°. Auto-inhibitor in the concentrate was partially purified by one-dimensional chromatography on Whatman No. 3 MM paper with butanol + acetic acid + water (4 + 1 + 5 by vol.) as solvent (Davis & Freer, 1960). Thin strips of the chromatograms were cut off and sprayed to locate ninhydrin-positive material, then the remainder of each chromatogram was cut into 1 in. squares which were soaked in water for 1 hr at 4° with stirring. Eluted material in the water was finally concentrated in the rotary evaporator to the volume of the initial concentrate. Pyridine + ethanol + water (10 + 7 + 3, by vol.) was also used as a solvent for chromatography. A Locarte instrument (Locarte Co., 24 Emperor's Gate, London, S.W. 7) was used for high voltage paper electrophoresis on Whatman 3 MM paper and with solvent consisting of m-formic acid + acetic acid buffer (pH 1.85).

Biological assay of the auto-inhibitor. Solutions containing auto-inhibitor were assayed by mixing with fresh germination medium at 30° containing equiv. about 60 µg. dry weight *Bacillus globigii* spores/ml. and the rates of germination were measured by the extinction change and phase-contrast methods. In the absence of auto-inhibitor this low concentration of spores germinated almost completely (Fig. 1).

Use of D-amino acid oxidase. Action of the D-amino acid oxidase from hog kidney (L. Light and Co. Ltd., Colnbrook) was measured by following oxygen uptake in a

Warburg apparatus at 35°. Incubation mixtures in the flasks consisted of D-amino acid oxidase (16 mg.), sodium pyrophosphate buffer (pH 8.8, 20 μ mole) and either DL-alanine (150 μ mole), L-alanine (150 μ mole) or auto-inhibitor solution (1 ml. concentrate) in a total of 2.5 ml. (Yoshimoto, 1958). Under these conditions 80 % of the D-alanine was oxidized to pyruvic acid in 5 hr.

Estimation of pyruvic acid. Pyruvic acid formed by the action of D-amino acid oxidase on D-alanine was estimated by reaction with 2,4-dinitrophenylhydrazine (Hopkin and Williams Ltd., Chadwell Heath, Essex) after removing the enzyme by precipitation with trichloroacetic acid (Koepsell & Sharp, 1952). The derivative was extracted into ethyl acetate, and from this into sodium carbonate solution (M). After development with sodium hydroxide (final concentration, 0.4 M) for 10 min. the intensity of the colour was compared with standards and blanks at 485 m μ in a Unicam SP 500 (Unicam Instruments Ltd., Cambridge).

Isotope studies. L-Alanine uniformly labelled with ^{14}C (Radiochemical Centre, Amersham, Buckinghamshire) was diluted with unlabelled L-alanine and used as a germinant for *Bacillus globigii* spores. Radioactivity of the ^{14}C -L-alanine, the ^{14}C -D-alanine produced during germination, and the ^{14}C pyruvic acid produced by action of D-amino acid oxidase was estimated with an Ekco Type N 664 B Universal scintillation counter connected to a N 530 automatic scaler using NE 220 scintillation fluid (Nuclear Enterprises (G.B.) Ltd., Bankhead Medway, Sighthill, Edinburgh 11). Adequate controls for quenching were always included and the counts of samples were corrected accordingly. Samples were normally diluted to give about 10,000 counts/min., which was the optimal counting rate for the equipment used. The specific activity ($\mu\text{C}/\mu\text{mole}$) of the pyruvic acid (and hence of the D-alanine) was calculated from the colorimetric and radioactivity assays and compared with the specific activity of the L-alanine used as germinant. It was reasoned that the specific activity of the pyruvic acid (and therefore of the D-alanine) would be identical with that of the L-alanine if the D-alanine arose solely by racemization.

RESULTS

Auto-inhibition of germination

Figure 1 illustrates the effect of spore concentration on the germination of *Bacillus globigii* spores. It is clear that high concentrations of spores germinated less completely than low concentrations. The supernatant fluids of auto-inhibited cultures inhibited germination of fresh *B. globigii* spore suspensions in L-alanine + glucose medium or in yeast glucose broth, confirming the work of Stedman *et al.* (1956). Culture supernatant fluids and partially purified auto-inhibitor solution retained inhibitory activity after evaporation to dryness *in vacuo* at 40° and after storage for at least two months at 4°. The auto-inhibitor was not extracted by ether or chloroform. The auto-inhibitor migrated at the same rate as alanine on chromatograms run in two different solvents and during high voltage electrophoresis on paper. It diffused through Visking dialysis membrane and when the diffusate was autoclaved (126° for 20 min.) some inhibitory activity was lost, as found by Stedman *et al.* (1956). However, auto-inhibitor eluted from the alanine band on chromatograms was more heat stable than the auto-inhibitor in the crude supernatant fluid. This suggested that the apparent heat sensitivity of auto-inhibitor in crude super-

nant fluids was due to its reaction with a medium component which was presumably separated from the auto-inhibitor by chromatography. Eluates from the remainder of chromatograms contained no detectable inhibitor of germination. The partially purified auto-inhibitor also depressed the rate of spore germination of 4 out of 6 other *Bacillus* species studied (Table 1). All these results indicated that the auto-inhibitor was D-alanine.

Fig. 1. Effect of spore concentration on the germination of *Bacillus globigii* spores. Incubation was at 30° in the defined medium and initial spore concentrations (equiv. mg. dry wt./ml.) were: ●, 1.1; ○, 0.4; □, 0.12; ■, 0.06; △, 0.05.

Table 1. Effect of *Bacillus globigii* auto-inhibitor on the germination of *B. subtilis* and *B. cereus* spores

Purified auto-inhibitor preparation was used at 1 ml./5 ml. of suspension.

	Decrease in extinction (%) after 30 min.	
	No auto-inhibitor added	Auto-inhibitor added
<i>B. subtilis</i> NCTC 2590	44	24
<i>B. subtilis</i> M 2	29	1
<i>B. subtilis</i> S 5	20	3
<i>B. cereus</i> S 20	35	8
<i>B. cereus</i> PX	45	42
<i>B. cereus</i> NCTC 945	65	60

Identification of the auto-inhibitor as D-alanine

When a concentrated spore suspension was germinated in medium containing D-amino acid oxidase (1.6 mg./ml.), auto-inhibition was decreased. When auto-inhibitor eluted from the alanine band of chromatograms was incubated with D-amino acid oxidase, oxygen was absorbed and, at the same time, loss of inhibitory activity occurred (Tables 2, 3). The keto acid formed was identified as pyruvic acid by the 2,4-dinitrophenylhydrazine method. This confirmed that the auto-inhibitor was D-alanine.

Table 2. *Effect of D-amino acid oxidase on auto-inhibitor and alanine*

Warburg flasks contained: D-amino acid oxidase, 16 mg.; sodium pyrophosphate buffer, pH 8.3, 20 μ mole; DL-alanine, 150 μ mole, or L-alanine, 150 μ mole, or purified auto-inhibitor solution, 1 ml. Total volume of flask contents was 2.5 ml.; incubation at 35°.

Incubation time at 35° (min.)	Oxygen uptake (μ l.) in presence of D-amino acid oxidase plus		
	Purified auto-inhibitor	DL-Alanine	L-Alanine
0	0	0	0
5	0.65	1.3	-0.05
15	2.20	5.35	-0.25
30	3.75	10.30	-0.20
70	6.05	22.75	-0.15
90	6.75	28.70	-0.25
120	7.30	36.15	-0.25

Table 3. *Effect of D-amino acid oxidase on the activity of the purified auto-inhibitor*

Purified auto-inhibitor was treated with D-amino acid oxidase for 2 hr as described in Methods.

Incubation time at 37° (min.)	Germination (%) of <i>Bacillus globigii</i> spores in yeast glucose broth +		
	Untreated auto-inhibitor	D-Amino acid oxidase-treated auto-inhibitor	No auto-inhibitor
0	4	4	4
15	12	52	—
60	16	65	88

Origin of D-alanine in supernatants of germinating Bacillus globigii spores

Three possibilities existed for the origin of the D-alanine: (1) that the D-alanine arose by racemization of exogenous L-alanine; (2) that the D-alanine was liberated from endogenous spore material; (3) a combination of (1) and (2). To resolve these possibilities uniformly labelled ^{14}C -L-alanine was used as the germinant. If possibility (1) were correct, the resulting D-alanine should have been ^{14}C -labelled with the same specific activity as the ^{14}C -L-alanine used as germinant. If possibility (2) were correct, the D-alanine arising from unlabelled spore material should have been unlabelled. If possibility (3) were correct the D-alanine should have been ^{14}C -labelled but with a lower specific activity than the ^{14}C -L-alanine.

During purification of the auto-inhibitor strips of the chromatograms were scanned with a scintillation counter and only the alanine band was radioactive. The auto-inhibitory D-alanine and residual ^{14}C -L-alanine in supernatant fluids were eluted from the chromatograms. The D-alanine was estimated by measuring oxygen uptake in the presence of D-amino acid oxidase and also by estimating the resulting pyruvic acid by the 2,4-dinitrophenylhydrazine method. Since the hydrazine method effectively separated the pyruvic acid from any residual ^{14}C -L-alanine, the radioactivity of the pyruvic acid (and hence of the D-alanine) could be determined. Figure 2 illustrates the experimental steps and Table 4 summarizes the results.

A mixture of 8 mM-L-alanine + 2 mM-D-alanine, the concentrations found in auto-inhibited cultures, prevented germination (Table 5).

Table 5. Germination of spores of *Bacillus globigii* in mixtures of L-alanine and D-alanine

Fresh spores of *B. globigii* were incubated in D-alanine and L-alanine mixtures at 80°. The mixture of 8 mM-L-alanine + 2 mM-D-alanine is equivalent to that found in auto-inhibited cultures.

Concentration of mixtures (mM)		Extinction (%) after incubation for			
L-Alanine	D-Alanine	0 min.	15 min.	30 min.	60 min.
10	0	100	77	72.8	70
8	2	100	93.5	93.5	92.5
6	4	100	96.5	96.5	96.0
Purified supernatant of an auto-inhibited culture		100	93.5	90.0	89.0

DISCUSSION

The properties of the auto-inhibitor showed that it was D-alanine. This was confirmed by the labelling experiment, which further showed that the D-alanine originated from the L-alanine supplied as germinant and not from spore constituents, although D-alanine is present in spore mucopeptide (Strange & Thorne, 1957). When D-amino acid oxidase was added to *Bacillus globigii* spore suspensions at the start of germination, the auto-inhibitory effect was decreased; Stedman *et al.* (1956) were unable to show this. Such a discrepancy may be due to differences in the strains of *B. globigii* used or to different activities of the oxidase preparations or even to different mechanisms of auto-inhibition.

D-Alanine is a well-known inhibitor of spore germination (Hills, 1949) which probably acts by competing with L-alanine for the active site of L-alanine dehydrogenase (O'Connor & Halvorson, 1961). Stedman *et al.* (1956) showed low concentrations of alanine racemase in *Bacillus globigii* spores but Church, Halvorson & Halvorson (1954) did not detect it in the spores of this organism. Under the conditions of our experiments, however, the only way D-alanine could be formed from L-alanine was by the racemization reaction catalysed by alanine racemase; no attempt was made to extract the enzyme from spores.

One value of racemase in spores has been pointed out by Wolf & Mahmoud (1957): it enables spores to germinate slowly in D-alanine. In addition, however, it will prevent complete germination of dense populations of spores. Spores will often germinate in environments unsuitable for further growth; incomplete germination can therefore have a survival value because the ungerminated spores will survive and be able to germinate later. Auto-inhibition, the phenomenon in which large fractions of a spore population remain ungerminated, even when germinants are present in excess, has been observed in other *Bacillus* species (Murrell, 1961). Alanine racemase commonly occurs in spores of the genus *Bacillus* (Wolf & Mahmoud, 1957) and Powell (1957) obtained evidence that this enzyme was involved in auto-inhibition of germination of *B. subtilis* spores. In explaining auto-inhibition

of germination of spores of other species the mechanism described here for *B. globigii* must therefore be considered, particularly when L-alanine is known to be required for germination of the spores. Generally, bacterial spores respond to sublethal heating by germinating more rapidly than when unheated (Curran & Evans, 1944). *Bacillus globigii* spores, however, respond to sublethal heating by germinating less rapidly than when unheated. It is not known whether this phenomenon is related to auto-inhibition (for instance, by heat activation of the racemase), but heat-induced dormancy is not restricted to *B. globigii*, for Finley & Fields (1962) noticed a similar phenomenon with spores of *B. stearothermophilus*.

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