Stimulation of L-Alanine-Induced Germination of *Bacillus cereus* Spores by D-Cycloserine and O-Carbamyl-D-Serine

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Alanine racemase occurs in some bacterial spores (B. T. Stewart and H. O. Halvorson, *J. Bacteriol.* **65**:160, 1953). It can reduce the rate and amount of germination of spores in L-alanine by catalyzing production of D-alanine (G. Fey, G. W. Gould, and A. D. Hitchins, *J. Gen. Microbiol.* **35**:229, 1964), an inhibitor of L-alanine-induced germination which presumably acts by inhibiting L-alanine dehydrogenase (R. J. O'Connor and H. O. Halvorson, *J. Bacteriol.* **82**:706, 1961).

D-Cycloserine (D-4-amino-3-isoxazolidone, Oxamycin) was shown to inhibit alanine racemase in *Staphylococcus aureus* (J. L. Strominger, E. Ito, and R. H. Thren, *J. Am. Chem. Soc.* **82**:998, 1960), working in either direction (L → D- or D → L-alanine). O-carbamyl-D-serine was shown to inhibit alanine racemase in *Streptococcus faecalis* (J. L. Lynch and F. C. Neuhaus, *J. Bacteriol.* **91**:449, 1966). It therefore seemed that these antibiotics should increase the rate and amount of L-alanine-induced germination of spores which contain alanine racemase, by preventing the production of inhibitory D-alanine. Using unheated spores of *Bacillus cereus* T [about 40 µg (dry weight)/ml] and D-cycloserine, we found this to be so (Fig. 1); O-carbamyl-D-serine was even more effective than D-cycloserine as a stimulator. In the absence of the antibiotics, germination was slow, because spores of *B. cereus* T only germinate rapidly in L-alanine after being heat-activated. The antibiotics alone did not germinate spores.

N. Tanaka and H. Umezawa [*J. Antibiot. (Tokyo) Ser. A* **17**:8, 1964] described synergism between D-cycloserine and O-carbamyl-D-serine as inhibitors of growth. Both antibiotics inhibited alanine racemase, but D-cycloserine (which is an analogue of D-alanyl peptides as well as of D-alanine) additionally inhibited D-alanyl-D-alanine synthetase (F. C. Neuhaus and J. L. Lynch, *Biochemistry*, **3**:471, 1964); these two enzymes act sequentially in the synthesis of cell wall mucopeptide; thus, their sequential inhibition resulted in synergism (R. H. Reitz, H. D. Slade, and F. C.

FIG. 1. Germination of unheated spores of *Bacillus cereus* T in sodium phosphate buffer (100 mM, pH 8.0) containing L-alanine (10 mM) and various concentrations of D-cycloserine. Germination causes a fall in optical density of the spore suspensions.

Neuhaus, *Federation Proc.* **25**:344, 1966). It is interesting that the two antibiotics did not show synergism in their stimulation of L-alanine-induced spore germination (Fig. 2). This was probably because the stimulation was due only to inhibition of the racemase, and this result also suggests that D-alanyl-D-alanine synthetase is not involved in the initiation of germination.

When heat-activated spores of *B. cereus* T were used in place of unheated ones, more rapid germination occurred, as was expected. D-Cycloserine slightly reduced the initial rate of germination (Fig. 3), confirming the observations of B. J. Krask (p. 89, in H. O. Halvorson [ed.], *Spores II*, Burgess Publishing Co., Minneapolis, 1961), but spore suspensions germinated more completely

FIG. 2. Germination of unheated spores of *Bacillus cereus* T in medium (as in Fig. 1) containing D-cycloserine (DC) and O-carbamyl-D-serine (OCDS).

in the presence of D-cycloserine than in its absence (Fig. 3). As with unheated spores, O-carbamyl-D-serine stimulated germination of heated spores more effectively than D-cycloserine.

Krask showed that D-cycloserine and D-cysteine inhibited spore alanine racemase and that D-cysteine increased the rate of germination of heated *B. cereus* T spores in L-alanine. This observation, together with the stimulatory effects of the antibiotics on L-alanine-induced germination reported here, highlights the limitation that alanine racemase in spores can impose on the rate of L-alanine-induced germination. The results

FIG. 3. Germination of spores of *Bacillus cereus* T which have been heat-activated at 70 C for 0.5 hr. Medium as in Fig. 1, and containing various concentrations of D-cycloserine.

also support the view that the two D-alanine analogues, D-cycloserine and O-carbamyl-D-serine, act by inhibiting alanine racemase.

It now appears that a number of different treatments will activate spores so as to stimulate germination. Activation by heat or by reducing agents (A. Keynan et al., *J. Bacteriol.* **88**:313, 1964) is not known to affect enzyme reactions, whereas stimulation of germination by antibiotics is difficult to explain unless some effect on enzyme reactions is involved. The role of the antibiotics in mimicking activation will therefore be studied further.

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