Chemical Germination of Native and Cation-Exchanged Bacterial Spores with Trifluoperazine

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The calmodulin antagonist trifluoperazine and its analog chlorpromazine, both amphipaths, induced chemical germination of spores of various species, as do many surfactants. Cation load can greatly influence this response. Calmodulin antagonism does not seem to be involved. A new fluorometric assay for dipicolinic acid based on the fluorescence of the dipicolinic acid chelate of Tb**(III)** was found to be simple and sensitive.

A possible role for a Ca-binding protein in the germination of bacterial spores has been suggested by a variety of studies (6, 7, 16, 17, 23, 26). Recent discoveries of calmodulinite proteins in sporulating Bacillus subtilis (9) and in spores of Bacillus cereus (33) have stimulated interest in this concept and have led to investigations on the effects of calmodulin antagonists such as trifluoperazine (TFP; Y. T. Shyu and P. M. Foegeding, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, 1-8, p. 218) that show inhibitory effects of TFP on germination (11, 20). This report shows that TFP can induce chemical germination (21, 22) (a term suggested to describe germination events induced by many surfactants) because of its amphipathic (detergentlike) properties (2, 32) rather than because of possible effects on a calmodulinlike protein and that the exchangeable cation load of the spores can greatly influence such germination.

Spores of B. megaterium ATCC 10778 were found to be quite susceptible to TFP-induced germination (in the absence of any physiological germinants), and the large spores facilitated quantitative microscopy (Fig. 1). The chemical germination induced by TFP is distinguishable (12, 21) from physiological germination in that (i) it is lethal (viability drops in parallel with other germination events), (ii) darkening of spores usually proceeds only to the phase grey (5, 24, 30) stage (note the relatively small shift in A<sub>600</sub>), and (iii) it can occur at 70°C (data not shown). Similar results were obtained with spores of B. subtilis 168 (19), B. coagulans 1491 (19), and Clostridium cylindrosporum HC-1 (29). Chlorpromazine, an analog of TFP, showed similar effects with spores of B. megaterium 10778. Chlorpromazine is also known for potent amphipathic properties (10, 14, 18, 32) and has been reported to inhibit physiological germination and prevent complete darkening (11). A number of Ca channel blockers were tested and found inactive; W-7, capable of reacting with Ca-binding proteins, showed only marginal activity. Presumably the changes seen in Fig. 1 result from the amphipathic nature of the TFP, not from its ability to react with Ca-binding proteins. Since germination appears to be checked at a late stage, as evidenced by the incomplete refractility changes and loss of viability, there does not appear to be a conflict with prior results (11; Shyu and Foegeding, Abstr. Annu. Meet. Am. Soc. Microbiol., 1989) demonstrating the inhibitory effects of TFP and chlorpromazine on normal germination in the presence of germinants. When spores of B. megaterium were titrated at pH 4 with HCl (26) to remove exchangeable cations and "reloaded" with various selected cations (1, 23, 26), Ca-loaded spores (Ca-spores) were found to be quite resistant to TFP-induced germination but NH$_4$-spores were more susceptible than the native spores or Na-spores (Fig. 2). Dodecylamine, unique among surfactants (12, 13, 22) in its high activity and its ability to mimic true germination, was tested with Ca- and NH$_4$-spores of B. megaterium, and the Ca-spores were markedly less susceptible to germination. Native spores of Bacillus macerans B-171 (25, 28) were quite refractory to TFP and dodecylamine, but NH$_4$-spores germinated to a considerable extent. However, it was necessary to titrate these spores at pH 3.2 to achieve adequate cation exchange, and reloading conditions were also altered (1 M, 18 h, 37°C) for maximum effectiveness when reloading with NH$_4$$_+$. The only previous report suggesting an influence of cation load on surfactant-induced germination showed that endotrophically formed Sr- and Ba-spores were less susceptible to dodecylamine-induced germination than Ca-spores (8), but since the results paralleled the physiological germination of these spores, little note was taken of them. The work reported here suggests that exchangeable cation load may greatly influence susceptibility to chemical germination. Germination induced by dodecylamine appeared to be qualitatively similar to germination induced by TFP or chlorpromazine. In all the experiments reported above, dipicolinic acid was detected in titrated spores. Since dipicolinic acid (DPA) is a characteristic of dipicolinic acid-containing bacterial spores, it was used to follow the chemical germination of spores by fluorescence techniques. However, dipicolinic acid in spores was released only after the germination had been initiated and proteinaceous compounds had been degraded (21). The experiments reported here suggest that dipicolinic acid release may be a consequence of reloaded spores. Therefore, to decrease the number of incubation steps and to avoid possible loss of DPA, a fluorometric assay was developed to test for dipicolinic acid using the Tb**(III)** complexing fluorescent probe. 

![FIG. 1. Germination of spores of B. megaterium ATCC 10778 (prepared as explained in references 26 and 27) by TFP (100 μM) in 50 mM KMOPS, pH 7.5. Symbols: ▲, spore refractility, i.e., percent nonrefractile spores (phase grey [>90%] or dark spores) by phase microscopy; ∇, percent DPA released (see text); □, viability of unheated spores as CFU on plate count agar (Difco); △, A<sub>600</sub>. The experiment was performed at 37°C.](http://aem.asm.org/content/56/4/1185)
acid (DPA) was determined by a new test based on the formation of a highly fluorescent chelate of Tb\(^{3+}\) with DPA, with characteristic excitation and emission peaks (3). The test is performed by adding up to 2.0 ml of the sample to 1 ml of sodium citrate (1.0 M, pH 5.5) and 1 ml of TbCl\(_4\) (1 M), and emission is measured at 545 nm after excitation at 280 nm; a Turner model 430 spectrophotometer was employed throughout. Standard curves were linear from 0 to 10 \(\mu\)g/ml in the presence of 50 mM KMPDS (potassium-normorpholine-propanesulfonic acid), the buffer used for these tests, and amounts of <1 \(\mu\)g/ml were readily detected (31); the addition of TFP resulted in a slight change of slope and intercept, but the curve remained linear, so it was readily interpreted. This test is an inverted version of the assay used to determine Tb\(^{3+}\) by chelation with DPA (3); citrate was substituted for the acetate buffer to minimize interference by certain cations (3). Citrate invariably resulted in solutions with greater fluorescence (34). Inosine interferes, possibly because of strong absorbance at the excitation wavelength. This test is simple to perform and is considerably more sensitive than the commonly used Fe\(^{2+}\) colorimetric test (15), readily detecting DPA released in germination experiments designed for absorbance monitoring.

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**LITERATURE CITED**


