Influence of Exchangeable Ions on Germinability of Bacterial Spores

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Received for publication 27 December 1965

ABSTRACT

RODE, L. J. (The University of Texas, Austin), AND J. W. FOSTER. Influence of exchangeable ions on germinability of bacterial spores. *J. Bacteriol.* 91:1582-1588. 1966.—Native spores of *Bacillus megaterium* Texas, and H-spores produced by titration of native spores to pH 4 with mineral acid, did not germinate in a solution of alanine and inosine unless a strong electrolyte was present. Ca-spores prepared from either H-spores or native spores did germinate efficiently in the same solution without a strong electrolyte. Of several other bivalent cations tested, only strontium and barium could substitute for calcium in conditioning spores for subsequent germination in the absence of an electrolyte. Variable responses were obtained with different metal ion forms of 62 unidentified soil isolates and several stock species of *Bacillus*. Although the pattern of response was not uniform in all organisms, ions played a crucial role in the germinability of the great majority of strains tested.

Almost all of the bacterial spore strains which have been investigated require organic compounds such as L-alanine, inosine, or glucose for physiological germination (13). Under optimal conditions, very low concentrations of these compounds are effective. In addition, for maximal germination rates the presence of certain ions is essential, particularly when low levels of the germination compounds are used. The latter may be virtually inactive without the ions (18-20). For one organism, inorganic ions alone suffice for germination (10).

The vital role that electrolytes play in germination offers an experimental approach to the study of certain aspects of the process. Alteration of the ionic makeup of spores, without affecting their organic composition or enzymatic activities, should permit a direct testing of the involvement of ions in germination. The best way of achieving such an alteration would be through the use of preformed spores which could be manipulated ionically while dormant. The influence of imposed treatments could then be ascertained in subsequent tests for germinability. A prospect for this approach to germination seemed possible through taking advantage of the cation-exchange properties of spores recently demonstrated by Alderton and Snell (1; see also 2).

This paper describes the effect of replaceable spore metal ions on germinability.

MATERIALS AND METHODS

Culture procedures. Spores were produced by methods described previously (16). The spores remained stable for months during storage at 4 C in sterile, deionized water, or as a dry powder after lyophilization. *Bacillus megaterium* Texas, the same strain studied in several previous reports from this laboratory (e.g., 19), was used in most of the experiments described in this paper.

Optical density (OD) measurements. Germination was followed by Powell's method (14), with the use of a Klett-Summerson photoelectric colorimeter with a no. 54 filter. Spore suspensions were adjusted to an initial reading of approximately 100 Klett units. The data are presented as the per cent reduction of OD, with the initial reading taken as 100%. Frequent microscopic checks revealed that OD was a reliable indication of the percentage of spores which germinated, judging from fully refractile and darkened spores. Germination of all of the spores in a suspension usually was accompanied by a 50 to 80% reduction of OD.

Heat activation. Except where noted, spore suspensions were heated for 60 min at 60 C (3) at some point prior to each germination experiment.

Chemical procedures. Dipicolinic acid (DPA) was determined colorimetrically (8), and ninhydrin-reacting substances were measured with L-alanine as a reference standard (12).

Preparation of spores with different ion contents. Spores were converted to the hydrogen form ("H-spores") by titration to pH 4.0 with 0.01 N HNO₃ (1, 2). They were held at this pH by frequent addition of

acid for 3 hr at room temperature, and then were centrifuged, washed, and suspended in deionized water. The microscopic appearance and the negligible release of DPA and ninhydrin-reacting substances (15) indicated that the spores remained stable and ungerminated. "Ca-spores" were prepared from H-spores by exposure to calcium salts under conditions specified in the individual experiments. This was followed by thorough washings in deionized water. Comparable preparations were made of spores with other cations. Spores with unchanged ion content are designated "native spores."

RESULTS

Germination of native spores, H-spores, and Ca-spores. The objective of this experiment was to determine whether germinability is affected in preformed spores whose content of exchangeable cations had been altered. Native spores and H-spores failed to germinate in the absence of the phosphate buffer employed as an electrolyte, whereas Ca-spores did germinate (Table 1). The germination response to phosphate confirms the requirement for exogenous strong electrolytes (18-20). The nonspecificity of the exogenous ion requirement previously found for native spores is also borne out for H-spores (Table 2).

TABLE 1. *Germinability of ionic forms of spores of Bacillus megaterium Texas*

Germination compounds*	Per cent reduction of OD†		
	Native spores	H-spores	Ca-spores
None.....	4	0	5
Alanine and inosine...	3	2	63‡
Phosphate.....	8	5	4
Alanine, inosine, and phosphate.....	77	70	85

* L-Alanine and inosine, 1 mm each; phosphate buffer, 30 mM, pH 8.

† After 1 hr at 37 C. The native spores and H-spores were heat-activated at 60 C for 1 hr; activation of the Ca-spores was achieved by converting H-spores in 50 mM calcium acetate at 60 C for 1 hr. Each of the activated preparations was washed in deionized water before addition of germination compounds.

‡ The pH of the unneutralized solution of alanine and inosine (i.e., with no ions added) was 5.0 to 5.5. The spores germinated fully, as indicated by release of DPA and ninhydrin-positive substances equal to that occurring at pH 8 in phosphate buffer. However, viewed with the dark-contrast phase microscope, the cells had a thick, dark ring surrounding a somewhat lighter core than is found at pH 8. This evidently accounts for the difference in OD reduction between fully germinated suspensions at the different pH levels.

TABLE 2. *Nonspecific strong electrolyte requirement for germination of H-spores of Bacillus megaterium Texas*

Electrolyte, 10 mM	Per cent reduction of OD*
None.....	0
KBr.....	52
KCl.....	52
KF.....	74
Tris buffer†.....	83
K bicarbonate.....	85
Na-K phosphate.....	86
Na propionate.....	88

* In L-alanine and inosine, each 1 mM; 2 hr at 40 C.

† Tris(hydroxymethyl)aminomethane.

These data indicate that the exchangeable ion composition of spores determines whether exogenous strong electrolytes are needed for germination. Thus, the "ionic" phase of germination may be concerned with the conversion of the spore to a state subsequently responsive to alanine and inosine alone, or it may be concerned with the effect of strong electrolytes along with alanine and inosine. In any case, the importance of spore calcium in relieving the requirement for exogenous electrolytes is brought into focus.

Factors influencing conversion of H-spores to Ca-spores. Germinability in the presence of alanine and inosine without exogenous salts is used here as a measure of the conversion of H-spores to Ca-spores. Various calcium salts (acetate, nitrate, chloride, or sulfate) effected the conversion. Exposure to calcium acetate for 15 min at 60 C gave nearly maximal conversion (Fig. 1A) and, under the conditions used, a 5 mM solution was sufficient for a nearly maximal rate (Fig. 1C). In a separate experiment conducted under essentially the same conditions, NiCl_2 , CoCl_2 , CdCl_2 , ZnCl_2 , and lead acetate (all 10 mM) were not effective, and MnCl_2 (10 mM) was partially effective. Barium and strontium were almost equivalent to calcium in the conversion (Table 3). Thus, germinability as measured in our test system is not specific for Ca-spores. Ba^{++} , Sr^{++} , and Ca^{++} are also interchangeable in the formation of endotrophic spores (7).

The conversion of H-spores to Ca-spores is temperature-dependent. The rate was very low at 0 C, and increased progressively through 60 C (Fig. 1B). Dependence on pH was also found (Fig. 1D); the rate was maximal near neutrality and dropped off precipitously to zero at pH 4.

The conversion of H-spores to Ca-spores did not proceed significantly unless the spores were heated before or during exposure to the calcium acetate (Table 4).

FIG. 1. Germination of *Bacillus megaterium* Texas spores in L-alanine and inosine, 1 mM each, in 30 min at 37 C. Before testing for germinability, the spores from each treatment below were washed thoroughly. (A) Exposure of H-spores to 50 mM Ca acetate for indicated times at 60 C. Exposure time at 60 C was brought to a total of 1 hr in each case by heating in water. (B) Exposure of H-spores to 50 mM Ca acetate for 1 hr at indicated temperatures. The spores were preheated in water for 1 hr at 60 C prior to exposure to Ca acetate. (C) Exposure of H-spores to graded concentrations of Ca acetate for 1 hr at 60 C. (D) Exposure of H-spores for 1 hr at 60 C to 50 mM Ca acetate adjusted to different pH values with acetic acid.

Reversibility. Native spores of *B. megaterium* Texas and H-spores produced from them at pH 4 require exogenous electrolyte for germination in an alanine-inosine mixture, and their rates of germination are comparable. Ca-spores, however, lack this exogenous electrolyte requirement and germinate readily in alanine-inosine alone (Fig. 2, left). Such Ca-spores, upon treatment with acid (pH 1.8), are converted back to H-spores which lack the capacity to germinate in alanine-inosine (Fig. 2, center); this reversal does not occur at pH 4. The pH 1.8 H-spores are readily reconverted to germinable Ca-spores by treatment with calcium acetate (Fig. 2, right).

The severity of the acid treatment during con-

TABLE 3. Efficacy of certain cations in the conversion of H-spores of *Bacillus megaterium* Texas to spores germinable in the absence of a strong electrolyte

Cation pretreatment*	Per cent reduction of OD†
None.....	2
MgCl ₂	7
NaCl.....	15
BaCl ₂	49
SrCl ₂	51
CaCl ₂	55

* At 10 mM; 20 min at 60 C. Spores were then washed thoroughly before the germination test; salt solutions were adjusted to pH 7 to 8 with the corresponding hydroxide.

† In L-alanine-inosine mixture, 1 mM each; 20 min at 37 C.

TABLE 4. Heat requirement for conversion of H-spores of *Bacillus megaterium* Texas to Ca-spores

Pretreatment*		Germination†
1st hr	2nd hr	
Water, 60 C	Water, 60 C	3
Water, 0 C	Water, 60 C	2
Water, 60 C	Ca acetate, 60 C	63
Ca acetate, 60 C	Water, 60 C	59
Water, 60 C	Ca acetate, 37 C	54
Ca acetate, 37 C	Water, 60 C	4
Water, 60 C	Ca acetate, 0 C	8
Ca acetate, 0 C	Water, 60 C	2

* Spores were washed thoroughly with deionized water after the 1st- and 2nd-hr pretreatments; calcium acetate, 50 mM.

† Per cent reduction of optical density after 60 min at 37 C, in L-alanine-inosine mixture, 1 mM each.

version of Ca-spores to H-spores has a profound effect on their capacity also to germinate even in the complete germination mixture (alanine, inosine, electrolyte; Fig. 3). The pH 1.5 spores are most affected, but even the pH 2.0 spores show a markedly inhibited germination rate (Fig. 3). All three types, pH 1.5, 1.8, and 2.0, are readily convertible back to Ca-spores which germinate readily not only in the complete germination mixture (alanine, inosine, electrolyte; Fig. 3) but also in alanine-inosine without electrolyte (Fig. 2, right).

Titrateable groups in spores. The reaction of H⁺ with native spores occurs in two phases (Fig. 4).

FIG. 2. Reversible germinability of *Bacillus megaterium* Texas spores. Ca-spores (left) were converted to H-spores (center) by titration to pH 1.8 with 0.01 *N* HNO_3 and washing in water. They were then reconverted to Ca-spores (right). Germination solution: L-alanine and inosine, 1 mM each.

Typically, in the initial phase, a rapid uptake of H^+ occurs. The second phase is characterized by a slow uptake of additional H^+ which continues for hours. Here, due to the slowness of the reaction, the rise in pH is very slow as the H^+ reacts with the spores to release more metal cations.

A total of 0.25 meq of acid was consumed per gram of spores (Fig. 4). Alderton and Snell (1) found 0.5 meq for their strain of *B. megaterium*. If in our experiment the proton of the nitric acid were exchanged for cations in salt combination with carboxyl groups, the latter would constitute 1.1% of the weight of the spores. Approximately 40% reacted very quickly, and the remainder much more slowly (see 2). This may be a matter of slow penetration to sites not readily accessible.

The isolated spore coat fraction of *B. megaterium* Texas spores reacted with H^+ almost instantaneously (Fig. 4). It is apparent that, although titratable groups are located in the coat fraction, a majority of the groups in whole spores titrate much more slowly.

Other species. Spores of 72 cultures of *Bacillus* were examined for germinability after conversion to the various ion forms. The collection was composed of 62 unidentified soil isolates and 10 authentic stock cultures.

Soil cultures. Fourteen of the isolates failed to germinate appreciably regardless of their ionic form. The remaining strains all exhibited differences in germinability in the different ionic states. The results were grouped into three types of re-

sponses; an example of each type is presented in Fig. 5.

Thirty-four of the isolates germinated best as Ca-spores (exemplified by isolate no. 14, Fig. 5A). Of this first group, 14 germinated only as Ca-spores, and the other 20 germinated significantly better as Ca-spores than as native spores or Na-spores. A second group, comprised of 11 isolates, germinated at approximately the same rate irrespective of ionic state (e.g., isolate no. 2, Fig. 5B). A third group, containing three isolates, germinated best as native spores (Fig. 5C and D). Isolate no. 21 of this group germinated better as Na-spores than as Ca-spores (Fig. 5C).

Authentic cultures. In related experiments, 10 strains of stock cultures (five species) were studied as native, H-, Ca-, and Na-spores with respect to germination in the presence and absence of electrolyte. Seven of the 10, as native or H-spores, were dependent on electrolyte for germination. Four of the 10, as Ca-spores, germinated without an electrolyte.

In a complete mixture of germination compounds, four strains germinated best as Ca-spores and two strains germinated best as native spores. The remaining four strains germinated about equally in all of the ionic forms.

DISCUSSION

It is apparent that the ionic condition of bacterial spores affects their germinability. Thanks to the base exchange technique of Alderton and

FIG. 3. Germination of Ca-spores and H-spores of *Bacillus megaterium* Texas at 37 C in L-alanine (1 mM), inosine (1 mM), and phosphate (Na-K PO_4 , 30 mM, pH 8). Ca-spores were prepared from H-spores by exposure of the latter to 50 mM Ca acetate for 60 min at 60 C. Three batches of H-spores were prepared from these Ca-spores by exposure of the latter for 12 hr at 25 C to HCl; pH 1.5, 1.8, and 2.0. Each of these H-spores, in turn, was converted to Ca-spores by 30-min exposure to 50 mM Ca acetate at 60 C.

Snell (1), it is possible with certain spore types to study some of the effects of exchangeable ion load on germinability.

Calcium, strontium, and barium, the bivalent cations which are most effective in conditioning spores for germinability, are closely related in the periodic table of elements; they also appear to be the only ones capable of supporting formation of spores endotrophically (7). The level of calcium present in media during and subsequent to sporogenesis may be expected to influence germinability through occupancy of exchangeable cation sites, and this has been observed (9). Since barium, strontium, and calcium function interchangeably, the extent to which any one of these participates in ionic conditioning will depend on the relative amount present. Attention will ordinarily be focused on calcium, since, of the three, it usually predominates in the complex

FIG. 4. Titration of *Bacillus megaterium* Texas native spores, 200 mg, and isolated coat fraction, 200 mg, from native spores. HNO_3 (0.01 N) was used to a pH 4.0 end point.

extracts and agar customarily employed for the production of spores.

The bulk of the metal cations accumulated during sporogenesis probably is not concerned with the ionic conditioning for germinability described here. This deduction derives from the finding that the exchangeable calcium with an all-or-nothing control of germinability in alanine-inosine constitutes but a small fraction of the total spore calcium (see accompanying paper), the rest of which is nonexchangeable with H^+ . Even so, important properties, such as heat resistance (2), reversible restoration of dormancy in *B. stearothermophilus* (11), and now germinability itself, depend on this exchangeable minor fraction.

Spores suitably preconditioned ionically require no strong electrolyte in the germination medium. For unconditioned spores, however, the electrolyte requirement for germination cannot be one of merely furnishing conditioning cations; the electrolytes are nonspecific (6, 17-20) whereas only Ca^{++} , Sr^{++} , and Ba^{++} precondition germinability.

The importance of Douglas' elegant micro-electrophoresis studies (reviewed in 4), too long

FIG. 5. Germination of native, Ca-, and Na-spores of various strains of *Bacillus* isolated from soil. Native spores (heated for 60 min at 60 C in water) were compared with Ca-spores and Na-spores (prepared by heating native spores for 60 min at 60 C in 50 mM Ca- and Na-acetates, respectively). Germination took place at 37 C in the following mixture: L-alanine, 2 mM; inosine, 2 mM; glucose, 10 mM; sodium propionate, 20 mM; dipicolinic acid, 10 mM; yeast extract, 0.05%; Casamino Acids (Difco), 0.05%; pH adjusted to 7.8 with NaOH.

neglected by spore scientists, is apparent. In an impressed electric potential, the mobilities of spores at various pH values were indicative of the ion-exchange qualities of the spore surface, which were to be incisively demonstrated later by Alderton and Snell (1). The abundance of both free carboxyl and anion groups on spore surfaces inferred from the electrophoresis measurements (5), and the titrimetric results described in this paper, may be relevant to the observed importance of ions for germinability, and perhaps for the heat resistance and reversible dormancy mentioned earlier. Carboxyl binding could account for cation involvement, and the adsorption of anions by surface NH_3^+ sites (5) may account for the involvement of nonspecific anions in germination (6, 17-20). Elsewhere, we advanced a germination hypothesis involving the alteration of

spore coat proteins, in which ions may possibly play a role (21).

The germinative behavior of H-spores titrated to different pH values (Fig. 3) suggests that if the acid treatment had succeeded in reacting with all of the potentially reactive sites, germination, even in the presence of a strong electrolyte, might be postponed indefinitely. Because of difficulty of access or because of tighter binding of cations, a fraction of the spore sites might have escaped reaction with H^+ even at pH 1.5, and this residue may well have been instrumental in what germination the pH 1.5 spores were capable of. A dormant spore whose potentially reactive sites have all reacted with H^+ should, by conversion to the Ca-form, regain germinability.

The failure of some spore strains to respond germinatively like the majority of the respective Ca-forms needs explanation. One possibility is that not all of the strains were equally converted to the Ca-form by the standardized treatment employed.

ACKNOWLEDGMENTS

We thank Margaret A. Crawford for capable assistance.

This investigation was supported by Public Health Service grant AI-03564 from the National Institute of Allergy and Infectious Diseases, by grant 375(12) from the Office of Naval Research, and by grant G-14568 from the National Science Foundation.

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