Influence of Alkali Metal Cations on the Germination of Spores of Wild-type and gerD Mutants of Bacillus subtilis

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Spores of gerD mutants of Bacillus subtilis prepared on a rich medium were defective in their germination responses to L-alanine, to a mixture of L-asparagine, D-glucose, D-fructose and KCl (AGFK), and to Penassay broth. They showed an increased response and sensitivity to L-alanine when monovalent cations were also present. When prepared by a resuspension method, the spores had an improved germination response to L-alanine, even in the absence of these cations, but showed no such improvement in response to AGFK. No gross differences were found between wild-type and gerD spores in heat or chemical resistance, dipicolinic acid level or morphology. It is proposed that an alternative pathway of germination involving monovalent cations exists in both wild-type and gerD spores.

INTRODUCTION

Bacterial spores can be induced to germinate by a wide variety of chemicals (Gould, 1969). The isolation of mutants of Bacillus subtilis which formed spores unable to respond to one or more of these chemicals (Wax et al., 1967) supported the idea of two pathways of germination, one initiated by L-alanine (ALA) and the other by a mixture of L-asparagine, D-glucose, D-fructose and KCl (AGFK). Subsequently many more spore germination (ger) mutants have been isolated and characterized physiologically, genetically and, in some cases, morphologically (Trowsdale & Smith, 1975; Smith et al., 1979; Moir et al., 1979; Sammons et al., 1981; Warburg & Moir, 1981; Irie et al., 1982). Spores of gerA mutants are thought to be defective in the initiation of germination induced by L-alanine (Sammons et al., 1981) but have a functional AGFK pathway. In contrast, gerB mutants produce spores which respond normally to L-alanine but not to AGFK (Moir et al., 1979) whereas gerD and gerF mutant spores are defective in both the ALA and AGFK pathways, perhaps deriving from mutations in genes having some common role in both pathways (Moir et al., 1979). We decided to study the gerD mutants more closely to determine if there were any conditions which could induce a more rapid or more complete germination response. We report here that alkali metal or ammonium chlorides greatly enhance the response of spores of gerD mutants to L-alanine and that such spores, when prepared in a resuspension medium (Sterlini & Mandelstam, 1969), show enhanced responses even without these ions.

METHODS

Bacterial strains. All bacterial strains were derivatives of B. subtilis 168 strain 1604 (Table 1). Mutants 4593, 4594, and 4601 were tetrazolium (TZM) white strains and strain 4620 was a pr+ auxotroph (Moir et al., 1979). Strains 4775, 4780 and 4782 were isolated by Taylor (1980) as TZM white colonies after mutagenesis with ICR191 (a frameshift mutagen). Strain 4738 was isolated at 100, survival. Spores were prepared on potato/glucose/yeast extract agar (PGYEA) at 37 °C, and after incubation

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Abbreviations: AGFK, mixture of L-asparagine, D-glucose, D-fructose and KCl; DPA, dipicolinic acid; PAB, Penassay broth; PGYEA, potato/glucose/yeast extract agar; RM, resuspension medium; TZM, tetrazolium.
in SPAB [Difco Penassay broth (PAB) containing L-alanine (10⁻² M), L-asparagine (10⁻² M), glucose (5 × 10⁻³ M) and KCl (10⁻¹ M)] at 42 °C for 1 h, germinated spores were killed by shaking with several drops of chloroform. The spores were recovered by centrifugation at 1000 g for 15 min and reincubated in SPAB; this process was repeated three times, finally resuspending the spores in SPAB and incubating at 30 °C for 1 h. Germinated spores were collected over a shelf of endografin (68%, v/v, Schering Chemicals), by centrifugation at 22000 g for 30 min at 4 °C, and the upper layer was then recentrifuged on fresh endografin at the same concentration. Dilutions of the resulting upper layer were plated on PGYEA and the TZM reactions of the spores formed by resulting colonies were tested at 37 °C.

Those of strain 4738 gave a pale pink reaction and so were potentially defective in spore germination.

Media and spore preparation. These were as described by Warburg & Moir (1981). Deionized glass double distilled water was used for all media and chemical solutions. Spores were prepared on PGYEA or in the resuspension medium (RM) described by Sterlini & Mandelstam (1969) at 26 °C unless otherwise stated.

Germination studies. Germination rate and extent were determined spectrophotometrically by following the fall in OD₅₈₀ of spore suspensions at 37 °C (Warburg & Moir, 1981). Readings were taken at 5–10 min intervals after the addition of heat activated spores (30 min at 70 °C) to prewarmed germinant, and were expressed as a percentage of the initial OD₅₈₀ value (about 0.3). The maximum rate of germination (Gₘₓₓ) and the germinant concentration required to give half maximum rate (C₅₀) followed Sammons et al. (1981). Inorganic ion C₅₀ values were measured in the presence of the C₅₀ concentration of L-alanine (other concentrations of L-alanine gave similar results); they were the ion concentrations which gave a rate of germination half way between that with L-alanine alone and the maximum achievable by addition of ions. The C₅₀ value is a measure of the sensitivity of the spores to a particular germinant and the Gₘₓₓ value gives an estimate of the synchrony of germination of a spore population.

Tetrazolium overlay test. The germination phenotype of spores in colonies was determined by the TZM method described by Moir et al. (1979). Wild-type colonies gave a red colour whereas those of mutants were white or pink.

Transduction and transformation. These were as described by Warburg & Moir (1981), using phages PBS1 and SPPl and chromosomal DNA isolated by the method of Marmur (1961). Transformations were performed with non-saturating levels of DNA. Selection for erythromycin resistance (1 µg ml⁻¹) was applied after an expression period of 2 h at 37 °C.

Electron microscopy. Spores were fixed and stained with KMnO₄ and sections cut after embedding in the resin of Spurr (1969).

Measurement of spore resistances. Heat and chemical resistances were measured after exposure of spore suspensions to high temperature or organic solvents in minimal salts (Warburg & Moir, 1981). Three spore suspensions were used for each measurement, the standard errors being less than 20%.

Dipicolinic acid assay. Release of dipicolinic acid (DPA) during spore germination was measured in filtrates of germinating spore suspensions by the method of Scott & Ellar (1978). The total DPA of spores was measured in filtrates of autoclaved dormant spores. At least two spore preparations was used in each assay; the standard error of the values obtained was about 25%.

RESULTS

General germination properties

Moir et al. (1979) reported that spores of strains 4593 (gerD19), 4594 (gerD20) and 4601 (gerD37) did not lose more than 5% of their initial OD₅₈₀ after 60 min, when the germinants were prepared in 10⁻¹ M-Tris/HCl pH 7.4, in contrast to those of wild-type strains which lost at least 40% of their initial OD₅₈₀. Under conditions optimal for the wild-type (10⁻² M-Tris/HCl at pH 8.4; Sammons et al., 1981) spores of all of the above mutants and also strains 4775 (gerD72),
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Fig. 1. Response of wild-type (strain 1604) and gerD19 (strain 4593) spores to L-alanine. Spores were prepared in PGYEA at 37 °C and the fall in OD$_{580}$ was followed at increasing L-alanine concentrations: ●, 10$^{-5}$ M; ○, 10$^{-4}$ M; ■, 10$^{-3}$ M; □, 10$^{-2}$ M.

Fig. 2. Effect of L-alanine concentration on spore germination. The maximum rate of fall of OD$_{580}$ ($G_{max}$) was measured from curves as in Fig. 1 (except that readings were taken every 10 min) and was plotted against L-alanine concentration: ○, strain 1604 (wild-type); ●, strain 4593 (gerD19).

4780 (gerD77) and 4782 (gerD79) described by Taylor (1980) and strain 4738 (gerD48) responded similarly to L-alanine, L-valine, AGFK and PAB, losing only 10% of their initial OD$_{580}$ after 60 min (wild-type spores had again lost at least 40%). The difference between gerD and wild-type spores was observed irrespective of the temperature of spore formation or germination within the range 26–42 °C (data not shown).

Since similar results in the following experiments were obtained with all the gerD strains, only those for strain 4593 (gerD19) will be reported in detail.

L-Alanine-induced germination

Increasing the L-alanine concentration increased germination rate (fall in OD$_{580}$ vs time) and extent for both wild-type and gerD spores (Fig. 1), the germination of the wild-type spores being more responsive to lower concentrations of L-alanine (Fig. 2). After prolonged incubation (4 h) at higher L-alanine concentrations the extent of the fall in OD$_{580}$ of gerD spore suspensions was similar to that for wild-type suspensions (Fig. 1), although the germination rate of the latter was considerably higher. The concentration of L-alanine required to induce a response from gerD mutant spores was greater than that required by wild-type spores (Fig. 2). The C$_{50}$ values for the mutant and wild-type spores were about 3 × 10$^{-4}$ and 6 × 10$^{-5}$ M, and $G_{max}$ values were about 0.4% and 1.5% ΔOD$_{580}$ min$^{-1}$, respectively.

Adjunct effect

To see if any of the components of the AGFK mixture (termed adjuncts) could increase the response of the gerD spores to L-alanine, all except L-asparagine were added both singly and in
combinations to L-alanine (Fig. 3). As reported by Sammons (1980) the already high rate and extent of L-alanine-induced germination of wild-type spores were somewhat increased by glucose, fructose and KCl. In contrast the relatively low rate and extent of germination of the gerD spores were markedly increased by the addition of KCl, either alone or in the presence of glucose and fructose. The response to glucose and fructose in the absence of KCl was much less marked. None of the adjuncts induced germination when added either alone or together to either wild-type or gerD spores in the absence of L-alanine. Similar results were obtained when l-valine was substituted for L-alanine (data not shown).

Effect of sporulation medium

The above experiments were performed on spores prepared on PGYEA. When they were prepared in RM both wild-type and gerD spores germinated better in L-alanine (Fig. 4). The wild-type PGYEA spores germinated more slowly than those produced in RM and had a higher L-alanine requirement (Table 2). The mutant spores showed a lower \( G_{\max} \) than the wild-type spores prepared under the same conditions. However, for the mutant the differences between RM and PGYEA spores were more marked; with a 5–10-fold increase in \( G_{\max} \) and a 2–4-fold reduction in \( C_{50} \). Although RM spores of both gerD and the wild-type had greater \( G_{\max} \) and lower \( C_{50} \) values than PGYEA spores, these never reached wild-type levels for the mutant spores.
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Table 2. L-Alanine $C_{50}$ and $G_{\max}$ values for germination of spores of strains 1604 (wild-type) and 4593 (gerD19)

Spores were prepared at 26°C. Values are means of experiments on two independent spore preparations, ± the range. The concentration of L-alanine was varied; LiCl was at $10^{-2}$ M. $G_{\max}$ and $C_{50}$ are defined in Methods.

<table>
<thead>
<tr>
<th>Germinant</th>
<th>G_{\max} ($C_{50}$ AOD_{580} min^{-1})</th>
<th>10^3 $C_{50}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type  gerD</td>
<td>PGYEA spores</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>1.7 ± 0.3 0.29 ± 0.01</td>
<td>2.9 ± 0.28 17.5 ± 4.9</td>
</tr>
<tr>
<td>L-Alanine + LiCl</td>
<td>1.55 ± 0.2 0.69 ± 0.01</td>
<td>0.85 ± 0.07 6.0 ± 1.4</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>4.3 ± 0.9 2.9 ± 0.2</td>
<td>1.7 ± 0.42 6.5 ± 0.7</td>
</tr>
<tr>
<td>L-Alanine + LiCl</td>
<td>3.6 ± 0.1 2.7 ± 0.1</td>
<td>0.9 ± 0.07 1.4 ± 0.1</td>
</tr>
</tbody>
</table>

In a rich germination medium (PAB or SPAB) both RM and PGYEA gerD spores germinated well, and they were also able to outgrow (as seen by phase contrast microscopy, data not shown). This indicated that the germination response had been completed in these spores and that later stages are not affected by the gerD mutation.

Effects of monovalent cations

In addition to KCl, germination of both wild-type and gerD spores was also stimulated by $10^{-2}$ M solutions of NaCl, LiCl, NH₄Cl, RbCl and CsCl, although MgCl₂, CaCl₂, FeCl₂ and FeCl₃ at the same concentration were without effect. Also the sulphates, carbonates and acetates of sodium and potassium were similar in effect to the chlorides (data not shown). The effects of each of the six monovalent cations on strains 1604 (wild-type) and 4593 (gerD19) are shown in Fig. 5 and Table 2 (LiCl only). Similar data (not shown) were also obtained for strains 4594 (gerD20) and 4601 (gerD37).

At limiting concentrations of L-alanine, all six cations increased the germination rate of wild-type PGYEA and RM spores considerably (effectively changing the $C_{50}$ values). With excess L-alanine little stimulation was seen and the $G_{\max}$ levels were slightly lower (Fig. 5a, b). However, gerD19 PGYEA spores were stimulated at all levels of L-alanine, up to the apparent $G_{\max}$ plateau concentrations, by all the cations (Fig. 5c). The order of greatest effect of the ions was Li > Na = K > NH₄ = Cs = Rb. gerD19 RM spores reacted in a similar way to the PGYEA spores except that stimulation by ions was only seen at L-alanine concentrations below those giving the $G_{\max}$ and that the differences between ions was less evident (Fig. 5d). The $C_{50}$ level of Na⁺ required to stimulate spore germination in L-alanine was 2.0 ± 1.4 × 10⁻⁴ M, for wild-type PGYEA spores. Similar values were obtained for all the other spores and ions, although the standard errors were high and small differences may not have been detected.

The addition of Li⁺ to L-alanine as a germinant shortened the lag period for both gerD and wild-type spores, at the same time decreasing their $C_{50}$ values (Table 2). Similar results were obtained with each of the other ions. The PGYEA gerD spores were more affected than the other spor type, but the general effects of ions on germination in L-alanine was similar. Each cation seemed to have a slightly different effect upon the germination response. In the presence of some cations the RM gerD spores were nearly as responsive to L-alanine as those of the wild-type, but a longer lag period was always observed.

Germination in AGFK

Germination in AGFK was poor (less than 10% fall in OD_{580} in 60 min) with the germinant in Tris buffer (10⁻¹ M, pH 7-4, and 10⁻² M, pH 8-4) at temperatures of 26°C, 37°C or 42°C for both sporulation and germination, whether the spores were prepared on PGYEA or in RM.
Fig. 5. Effect of cations on spore germination. Spores of strain 1604 (wild-type) and strain 4593 (gerD19) were prepared on PGYEA or in RM at 26 °C and germination responses measured in various concentrations of L-alanine in the presence of \(10^{-2}\) M salt solutions: \(\bullet\), none; \(\bigcirc\), LiCl; \(\blacksquare\), NaCl; \(\square\), KCl; \(\Delta\), NH₄Cl; \(\triangle\), CsCl; \(\triangledown\), RbCl. For clarity not all the lines are drawn in each graph. Each point represents the mean of two experiments on independent spore preparations.

Properties similar in both gerD and wild-type spores

Spore resistances to heating at 70 °C or 90 °C for up to 2 h and to octanol, toluene, xylene and chloroform were measured. In each case the sensitivity of the spores of three gerD strains (4593, 4594 and 4601) was similar to that of wild-type spores irrespective of the sporulation medium or temperature of sporulation (data not shown). The loss of resistances to heat, octanol, xylene, toluene and chloroform during germination was determined for spores of the same strains and was proportional to the percentage of phase dark spores present for both mutant and wild-type spores.

There was no significant difference between the levels of DPA in either PGYEA or RM wild-type spores (5.5 ± 1.7% and 6.2 ± 2.2% dry weight, respectively) and those of similarly produced spores of the gerD strains 4593, 4594, 4601 and 4738. The amount of DPA released during germination of both wild-type and gerD spores was proportional to the fall in \(OD_{580}\) and to the percentage of phase dark spores present.

Thin sections of spores fixed and stained in KMnO₄ were examined by electron microscopy. No differences between wild-type and gerD spores prepared either in RM or on PGYEA could be seen in the structure of either the coats, cortices, cores, or membranes. The number of striations in the coat layers of both wild-type and mutants differed between RM (three) and PGYEA (five) spores; such a difference has been reported previously (Warburg & Moir, 1981) for B. subtilis wild-type and has also been observed in spores of Clostridium bifermentans prepared in rich and minimal media (Waites et al., 1979).
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Map location

The gerD mutations were located, using phage PBS1 transduction and the tetrazolium reaction, near cysA14 and rplV (previously termed ery-I), the order being cysA–rplV–gerD (Moir et al., 1979). To locate the mutations more precisely, phage SPP1 (Ferrari et al., 1978) was used. No linkage between any of the gerD alleles and cysA was detected and rplV was only approximately 5% cotransduced with cysA. DNA from strain 4620 (rplV cysA14) was used to transform competent cells of each gerD strain with selection for resistance to erythromycin, and the gerD and cysA alleles were scored (100 recombinants from each cross). Cotransformation of cysA and rplV was approximately 4% (and thus similar to the SPP1 data) and low-frequency cotransformation of gerD with rplV was also obtained; it was 1% for gerD77 and gerD79, 4% for gerD72 and gerD20, 7% for gerD37 and 8% for gerD48. Significant cotransfer of gerD with rplV but not with cysA is consistent with the previously determined order cysA–rplV–gerD. Where possible, five recombinants of each Ger phenotype from each cross were tested for their germination properties and in each case those which were TZM white or pink had properties similar to the gerD parental strain and the TZM recombinants were similar to the wild-type donor strain.

DISCUSSION

A common method of determining whether a particular phenotype results from a single point mutation is to measure its reversion frequency. Unfortunately this method cannot, at present, be used for the gerD mutants because the Ger+ phenotype is not selectable. However, seven mutants which give rise to similar phenotypes have been independently isolated, using a variety of mutagens, and their mutations found to have similar cotransformation frequencies with rplV. When these mutations were transduced into an otherwise wild-type background similar mutant phenotypes were observed (Moir et al., 1979). Thus, it is likely that the gerD mutants derive from mutations in a single gene or gene cluster whose products are required for germination in L-alanine, AGFK and PAB. These mutations do not seem to affect late events in germination because when the spores germinate the resulting response appears normal, i.e. they lose phase brightness and heat and chemical resistances, release DPA and, under appropriate conditions, can outgrow.

Amongst the previously studied germination mutants some have defects in the cortex (Warburg & Moir, 1981), coat (Moir, 1981; Cheng et al., 1978), DPA levels (Zytkovicz & Halvorson, 1972) or heat and chemical resistances of their spores (Warburg & Moir, 1981; Warburg, 1981). The gerD mutant spores have normal core, membrane, cortex and coat morphology and possess normal levels of DPA and resistance to heating and organic chemicals. Experiments were not refined enough to have detected small deviations from normal and so the conclusions must be only that the gross properties of the mutant spores were not significantly altered.

The response of the gerD spores to L-alanine was slower than that of wild-type spores, i.e. the majority of spores in the population showed a greater lag before germinating (Fig. 1). They also had lowered sensitivity to L-alanine, requiring a higher concentration than wild-type spores before a response was observed. The slow response of the gerD spores to L-alanine could indicate some form of permeability barrier in these spores; for example a hypothetical L-alanine carrier of trigger L-alanine molecules may have been altered, although other interpretations are equally possible.

Induction of germination of gerD spores in L-alanine occurs more readily in the presence of monovalent cations (Fig. 5c, d). It is clear, however, that at sub-optimal L-alanine concentrations both wild-type and mutants can be considerably stimulated, and the effect of ions may not be limited to mutant spores (Fig. 5a, b). The germination response to L-alanine of gerD spores is much improved if the spores are prepared in RM (Fig. 4). The lesser effect of salts on these RM spores may simply reflect their already greater sensitivity to L-alanine.

Wax et al. (1967) described mutants requiring both glucose and KCl in addition to L-alanine before germination was initiated. This led Dring & Gould (1971) to establish that K+ ions are
released and then taken up early in germination, before the fall in optical density. Thus they proposed that a pool of K⁺ ions was needed by a spore before germination could occur. If this were true then the gerD spores might not be able to accumulate the necessary ions, except in RM media, and thus require these during germination in L-alanine. However, it is difficult to see how this would relate to the mutants' failure to germinate in AGFK, which contains high concentrations of K⁺. If this hypothesis were correct one might expect that preincubation in monovalent cations would shorten the lag period observed; this was not observed for either gerD or wild-type spores (R. J. Warburg, unpublished data).

It is not clear why certain cations should be more effective in some spores and not others but for gerD PGY EA spores the smaller ions were most effective (Fig. 5c), perhaps reflecting spore ionic permeability properties. Divalent cations did not affect the germination properties of gerD spores, suggesting a specific effect for monovalent cations.

The observations that gerD spores do not respond to the AGFK mixture even when prepared in RM would indicate that although the defect in the ALA pathway can be overcome the block in the AGFK pathway is not. Thus, some essential function for the AGFK germination response has been altered by a mutation which also affects ALA germination but can, in this case, be overcome to a certain extent by the presence of monovalent cations or by sporulation in RM. This would indicate not only that these germination pathways have some components in common but that one stage can be bypassed in one pathway but not in the other.

Fig. 6 is a diagrammatic representation of progress through a series of germination-associated events in B. subtilis. Spores of gerA mutants are blocked in germination in L-alanine but germinate normally in AGFK while those of gerB mutants have the converse phenotype. Since spores of some classes of mutants are defective in both pathways, the two germination routes must converge at some stage(s). It is unlikely that another independent L-alanine-stimulated pathway activated by monovalent cations is available to spores since those of gerA mutants do not respond to ions (Sammons et al., 1981). Spores of gerA gerD double mutants no longer responded to the combination of L-alanine plus monovalent cations, showing that the ability to respond to this mixture was dependent on gerA function (R. J. Warburg, unpublished data). However, since gerA mutant spores germinate normally in AGFK the gerA products must act independently of the AGFK-specific parts of the pathway; on a pathway model this would suggest that the gerA product acts before the point of interaction of the two pathways. Since the gerD product has a role in both pathways, albeit capable of bypass in one case, it would represent a later stage in the germination pathway.

To explain the data presented in this paper we might imagine that the conversion of a to c in Fig. 6 could involve a compound which is activated by a variety of monovalent cations or produced in an active state in RM spores (which may already contain these ions in sufficient quantity).
Although thought initially to be extremely defective in germination in a range of germinants, spores of gerD mutants prove on detailed examination to have considerable capacity for ion-stimulated germination in L-alanine, depending on the conditions of spore preparation. These two factors do not appear to affect the germination phenotypes of other classes of ger mutants such as gerA (Sammons et al., 1981), gerE (Moir, 1981), gerF (Warburg, 1981), gerH and gerI (Taylor, 1980) and gerJ (Warburg & Moir, 1981). Previous work on bacterial spore germination showed that cations not only stimulate germination of spores (Hyatt & Levinson, 1961; Rode & Foster, 1962) but in some cases can even be sufficient for germination (Rode & Foster, 1962a).

Experiments on spore inner membranes isolated from B. megaterium QMB1551 suggest that alterations in the biophysical properties of those membranes occur in response to germinants (Taylor, 1980) and such as spores. Experiments on spore inner membranes isolated from B. megaterium QMB1551 suggest that alterations in the biophysical properties of those membranes occur in response to germinants (Taylor, 1980). This ionic effect and may contribute to an understanding of the mechanism of how cations have their effects.

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