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Commitment of bacterial spores to germinate

A measure of the trigger reaction

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The rate of commitment of bacterial spores to germinate after short exposure to L-alanine increases exponentially from the time of addition of L-alanine. This absence of a lag facilitates kinetic analysis and allows the dependence of commitment on temperature and pH to be determined. The pH profile of commitment has been compared with that obtained from measurements of absorbance decreases during germination, and the two profiles exhibit differing pK values. It is suggested that because the decrease in A_{600} of spore suspensions is a late event in germination, it is an unsuitable parameter for studying germination-triggering reactions. Commitment has been shown to be temperature-dependent, with an optimum at approx. 37°C and an activation energy (μ) of 1.08×10^5 J/mol. The data obtained from the present studies have been used to develop a model for the triggering of germination.

Bacterial spores are triggered to germinate within a few seconds or minutes of addition of a specific germinant, which irreversibly commits the spore to lose its dormant properties. This commitment to germinate can be observed after short exposure to L-alanine and can be rapidly arrested with D-alanine, a potent inhibitor of L-alanine-induced germination (Harrell & Halvorson, 1954; Keynan & Halmann, 1961; Halmann & Keynan, 1962). Spore commitment to germinate is therefore a measure of the first irreversible reaction of the trigger mechanism and must precede subsequent germination changes that involve calcium dipicolinate loss (Scott & Ellar, 1978*a*), absorbance decrease and initiation of metabolism (Scott & Ellar, 1978*b*). There are several theories related to possible biochemical mechanisms of the trigger reaction (Keynan, 1978); nevertheless, the precise nature of the reaction is not yet understood. Kinetic analysis of germination in the literature (McCormick, 1965; Vary & Halvorson, 1965; Lefebvre, 1978) has frequently been based on measurements of absorbance decrease, a relatively late event (Gould & Dring, 1972), and may not, therefore, reflect the kinetics of the trigger reaction. Commitment, as the earliest detectable spore response to germinants, may offer a more suitable parameter for such studies. Experiments described in the present paper compare commitment with absorbance decrease and calcium release during germination and investigate its temperature- and pH-dependence.

Materials and methods

Bacterial strain and growth

The organism used in these studies was a sporogenic strain of *Bacillus megaterium* KM (Ellar & Posgate, 1974). The conditions for growth and sporulation were as previously described (Ellar & Posgate, 1974), with the exception of a modified sporulation medium. This contained: $MgCl_2,6H_2O$ (0.5 mM); $MnCl_2,4H_2O$ (0.01 mM); $FeCl_3,6H_2O$ (0.05 mM); $ZnCl_2$ (0.05 mM); $CaCl_2,6H_2O$ (0.2 mM); KH_2PO_4 (13 mM); K_2HPO_4 (26 mM); glutamine (20 mg·litre^{-1}); acid casein hydrolysate (1 g·litre^{-1}); enzymatic casein hydrolysate (1 g·litre^{-1}); enzymatic yeast extract (0.4 g·litre^{-1}) and glycerol (0.6 g·litre^{-1}). For measurements of commitment to germination, spores were prepared in medium containing 0.37 MBq of $^{45}CaCl_2$/litre. Spores were harvested by centrifugation (10000 g for 3 min at 4°C) and re-centrifuged at least six times in deionized water (4°C), the uppermost layer of spores being removed and discarded each time until the pellet was free from contamination by cells or debris.

Germination of spores measured by absorbance decrease

Unless otherwise stated, spores were heat-activated in deionized water at 70°C for 30 min immediately before germination. Buffer components (acetate/acetic acid, KH_2PO_4/K_2HPO_4 and Tris/

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HCl) were kept as 0.1 M stock solutions. From these, appropriately titrated solutions of buffer components (0.01 M) containing KCl (0.1 M) and L-alanine (1 mM) were prepared. The absorbance change during germination of 0.2 mg dry wt./ml spore suspensions in these buffering systems was measured at 600 nm by using a Vitatron UPS universal photometer. Samples (3 ml) were incubated in 10 mm × 100 mm Vitatron tubes maintained at 30°C in a heating block. The maximum absorbance decrease per minute was determined from plots of absorbance decrease versus time. The germination rate (K_G) was obtained from these values by subtracting the background rate of absorbance decrease. The '%K_G' value was derived from the K_G at a given pH as a percentage of the maximum value obtained.

Studies of germination commitment

The time necessary for spores to become committed to germinate after exposure to L-alanine was determined by quenching and rapidly removing L-alanine from germinating suspensions with an excess of the germination inhibitor D-alanine and then observing the ability of these spores to continue germination. Spores were suspended at a concentration of 0.6 mg/ml in 0.01 M-potassium phosphate buffer, pH 7.2, for the temperature profile or over the pH range 3.5–10.5 for the pH profile. Germination was initiated with 1 mM-L-alanine, samples were withdrawn before L-alanine addition and at intervals during germination, and were filtered on 0.45 μm-pore-size membrane filters (Millipore Corp.) covered with 1 ml of 0.1 M-D-alanine. The filters were washed three times with ice-cold distilled water. Filtered samples for germination time points were transferred into scintillation vials for counting radioactivity. For the determination of commitment, an additional series of samples was filtered, transferred into 25 ml conical flasks containing 10 ml of potassium phosphate buffer (0.01 M, pH 7.2) and incubated at 30°C for 45 min on a reciprocating water bath operating at 70 rev./min. At the end of this period, the flask contents were filtered through new filters and washed as before. Both the old (incubated) and new filters were transferred to scintillation vials and the residual ^{45}Ca radioactivity measured. The exponential rate constant (K) for commitment at various temperatures or pH values was determined by linear-regression analysis of plots of $-\ln$(residual ^{45}Ca)/total ^{45}Ca versus time. The slope of $\ln K$ versus the reciprocal of the temperature (K) was used for the calculation of the activation energy (μ) by the equation:

$$-\mu = \text{slope} \times \boldsymbol{R}$$

where $\boldsymbol{R}$ is the gas constant (8.314 J/mol per K).

As an alternative approach to the measurement of commitment by membrane filtration, a centrifugation technique was developed. Samples (0.1 ml) of a germinating-spore suspension (see above) were removed at appropriate time intervals and transferred to Corex glass tubes containing 3 ml of 0.01 M-potassium phosphate buffer, pH 7.2, and 0.1 M-D-alanine. The tubes were incubated for 45 min at 30°C to allow continued germination of committed spores. They were then centrifuged at 20000 g for 5 min. A measured sample of the supernatant was withdrawn for the determination of ^{45}Ca radioactivity.

Scintillation counting

Membrane filters were dried at 80°C for 120 min. ^{45}Ca was determined by using toluene/BBOT [2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen] scintillation fluid (10 ml/vial) in a Unilux Nuclear–Chicago liquid-scintillation counter. Aqueous samples were measured by counting for radioactivity 1 ml samples in 15 ml of scintillant containing 7 g of PPO (2,5-diphenyloxazole) and 0.35 g of POPOP]1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre in 66% toluene/33% Triton X-100 with a Beckman LS 7500 scintillation counter.

Results

pH profile

Fig. 1 shows the pattern of commitment at 30°C compared with other germination parameters and establishes it as the earliest detectable event in germination of this organism. It is apparent that commitment demonstrates either no lag period or at most a very short delay, and this observation enabled a rate constant to be determined from an exponential relationship by using the method employed by Murrell & Wills (1977). The pH profile of germination was used as a means of comparing data obtained by absorbance measurement with the rate of commitment; this comparison is shown in Fig. 2. Both parameters for measuring germination show prototropic titration curves; however, the values for the upper and lower pK values vary markedly. Absorbance measurements give pK values of 8.8 and 5.6, whereas commitment measurements give values of 9.7 and 3.8. If the pK values represent only the prototropic titration of some functional groups within the spore, the inhibition of germination above and below these values should be completely reversible. This was investigated by incubating spore suspensions for 30 min at the non-germinative pH values, which were determined by absorbance decrease to be 10.0 and 3.8. The spore suspensions were subsequently washed by centrifugation in 0.01 M-potassium phosphate buffer, pH 7.2, before germination, with 1 mM-L-alanine. It was found that incubation of spores at high pH values did not affect

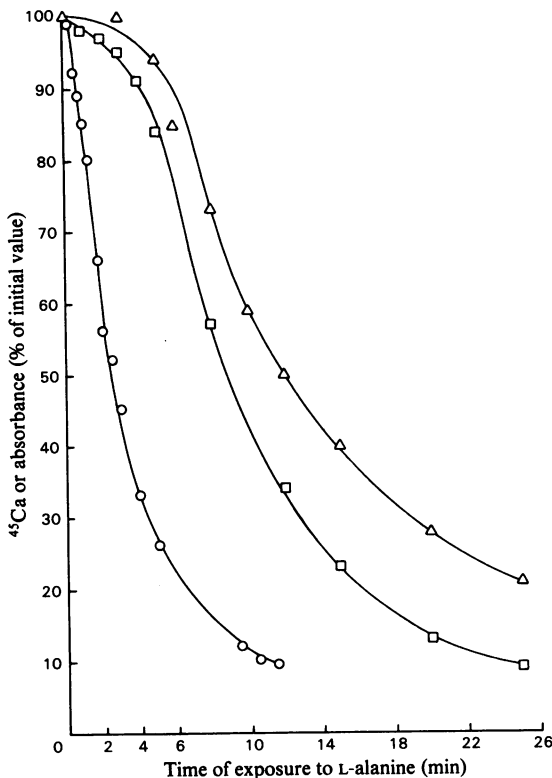


Fig. 1. *Comparison of the rates of absorbance decrease, calcium release and commitment during the germination of spore suspensions with* L*-alanine*

○, Commitment; □, calcium release; △, absorbance decrease.

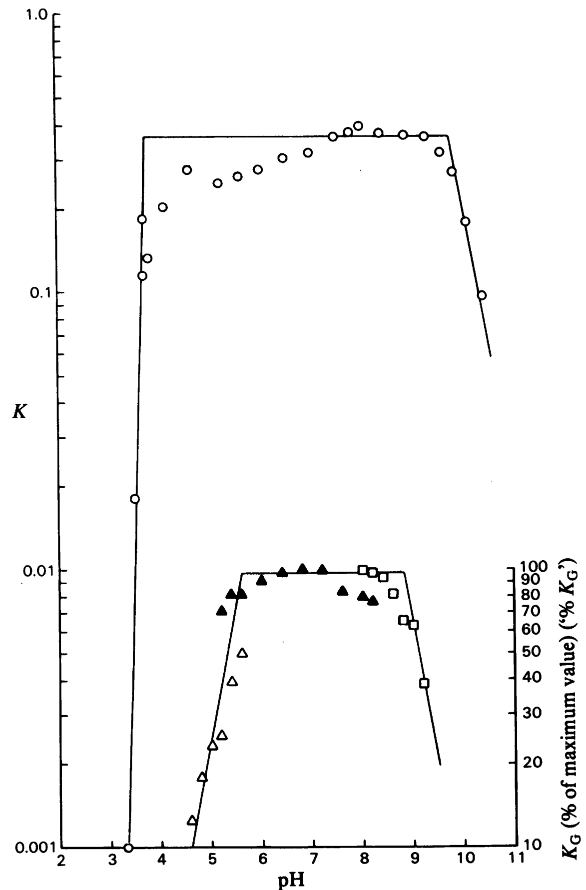


Fig. 2. *Response of the rate constant (K) for commitment and '%K_G' for absorbance decrease to the influence of pH*

○, pH profile of commitment at 30°C in potassium phosphate buffer. The pH profile of absorbance decrease at 30°C was determined in: △, acetate/acetic acid, pH 3.6–5.6; ▲, K_2HPO_4/KH_2PO_4, pH 5.2–8.2; □, Tris/HCl, pH 8.0–9.2.

their subsequent germination response. However, prior incubation at low pH resulted in an increased lag and decreased rate of germination. The lag was observed to decrease if the acid-washed spores were incubated in an electrolyte before addition of L-alanine. The decrease in the lag was observed with a variety of electrolytes and over a range of electrolyte concentrations (0.01–0.1 M), but not when the spores were incubated in water titrated to pH 7.

Temperature profile

The exponential rate constant, K, for commitment was found to be temperature-sensitive, occurring optimally at approx. 37°C. An Arrhenius plot of the observed rate constants is shown in Fig. 3. The slope of $\ln K$ versus reciprocal temperature below 35°C was used for the calculation of an activation energy (μ) for commitment of 1.08×10^5 J/mol. Above this temperature the slope changes sign and the possible implications of this are discussed below.

Alternative methodology for measuring commitment

The centrifugation method for measuring the rate of commitment by L-alanine provided a rapid alternative method to membrane filtration. This method was used (Fig. 4) to determine the rate of commitment at 30°C for heat-activated and non-heat-activated spores. The data show a 3-fold increase in the rate of commitment after heat-activation. This centrifugation method was also used

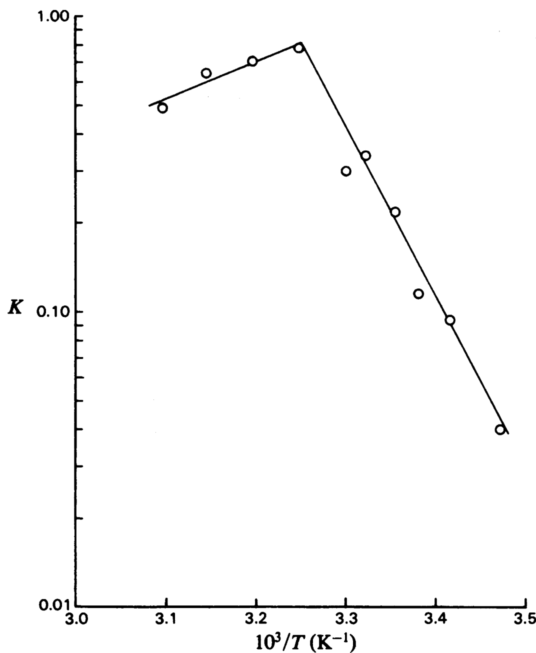


Fig. 3. *Response of the rate constant for commitment (K) to the influence of temperature*

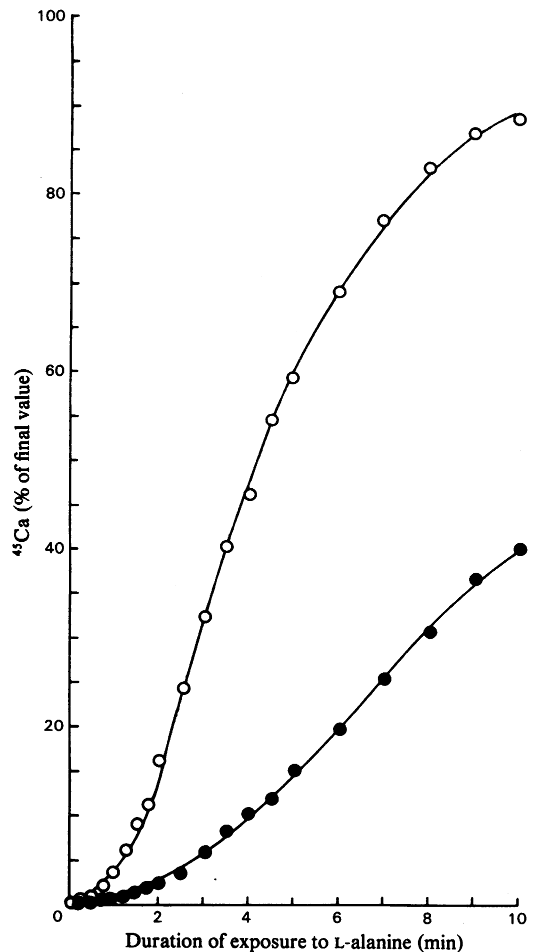


Fig. 4. *Influence of heat activation on the commitment of spores to germinate after short exposure to L-alanine* O, Spore suspension heat-activated in deionized water at 70°C for 30 min before germination; ●, non-heat-activated spore suspension.

(results not shown) to determine the pH profile of commitment, and the results by this method were qualitatively indistinguishable from those obtained by membrane filtration. In terms of absolute values, however, there was a slight decrease in the rate constants, which is discussed below.

Discussion

The commitment of spores to germinate after short exposure to L-alanine was first noted by Harrell & Halvorson (1954). Using a strain of *Bacillus terminalis*, these workers found that L-alanine-induced germination could be retarded, either by chilling or by adding D-alanine within 5 min of initiation. Complete inhibition was obtained by lowering the pH to 1.4, and this could be subsequently reversed by raising the pH to 8.5. Thus the L-alanine could be removed at various times after the initiation of germination by repeated centrifugation at pH 1.4. With this technique, a 45 s exposure to L-alanine resulted in over 40% germination after washing and raising the pH. This is now described as a 'commitment reaction' and represents over 40% commitment to germinate after 45 s exposure to L-alanine. Keynan & Halmann (1961) and Halmann & Keynan (1962) observed that commitment to germination in *Bacillus licheniformis* was temperature-sensitive, occurring optimally at approx. 37°C, and was also inhibited by D-alanine. Subsequent germination events, however, were not affected by D-alanine, and, furthermore, were observed to proceed at 0°C. Watabe *et al.* (1974) also examined the commitment of a spore population to germinate. By measuring the time during which germination remained sensitive to inhibition by D-alanine, they determined a 100% commitment time of 2–3 min for L-alanine-induced germination of *Bacillus thiaminolyticus*.

Our results have shown that spore commitment occurs very early by comparison with other events in

germination. For example, the commitment of spores to germinate after short exposure to L-alanine is compared with absorbance decrease and calcium release in Fig. 1. At 30°C, 50% of the spores are committed to germinate after only 2 min. The only germination event that has been reported to occur at a similar rate is the loss of heat resistance (Dring & Gould, 1971). However, the data in the present paper suggest that in measuring the rate of loss of heat resistance by the usual techniques, we may in reality be measuring the rate of commitment.

Loss of heat resistance during germination is normally measured by pipetting samples of germinating cultures at intervals into a larger volume of water at 70°C, so that the temperature rise is abrupt; spores surviving 30 min at 70°C are scored as ungerminated and enumerated by poured-plate viable counts in nutrient agar. Dring & Gould (1971) obtained a value for 50% loss of heat resistance in 81 s for *Bacillus cereus* T germination. From the work described in the present paper and that of Keynan & Halmann (1961), commitment to germinate has been shown to be temperature-sensitive and not to occur at 70°C. Subsequent germination events are, however, relatively temperature-insensitive. At 70°C, therefore, all commitment reactions would be stopped, but those spores that had already committed could, possibly, proceed to germinate and be subsequently killed by the elevated temperature. It is possible therefore that measurements of the loss of heat resistance, such as that described above, may be a measure of commitment to germinate and may not reflect a genuine loss of heat resistance. If this proposal proves to be correct, it could have important implications in the interpretation of germination events.

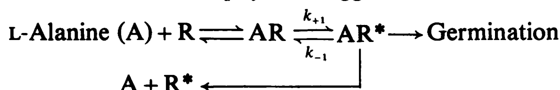
A comparison of the pH profile for germination measured by absorbance decrease with the pH profile of commitment to germinate, highlights the dangers of using a late event to investigate germination triggering. These studies used concentrations of the germinant L-alanine 100 times the K_m for this organism ($K_m = 10\,\mu$M; Koncewicz, M. A. & Stewart, G. S. A. B., unpublished work), and the prototropic titration curves are therefore considered to represent the titration of sites on the spore rather than the germinant. The irreversible titration at low pH is supported by the work of Rode & Foster (1966) and Sacks (1972), who showed that, when spores of *Bacillus megaterium* Texas were converted to 'H' forms by titration to pH 1.8 with 0.01 M-HNO_3, they were unable to germinate without the addition of a strong electrolyte. It seems likely that the lower p*K* for L-alanine-induced germination does not reflect the titration of a single ionizable group, but rather reflects the ion-exchange properties of the spore. By contrast, titration at the upper p*K* values is freely reversible and may reflect the titration of a single functional group. The p*K* obtained from measurements of absorbance decrease must be related to a rate-limiting reaction occurring after the initial trigger event, which has a much higher p*K*. Experiments are needed to identify the nature of the functional group for the trigger reaction.

If commitment is the earliest detectable event in germination, it is preferable to use this parameter to probe the mechanism of L-alanine initiation, rather than some later change, such as absorbance decrease. For a given spore population and temperature, commitment follows an exponential function from the time of L-alanine addition, and therefore it was possible to calculate an activation energy for the commitment process of 1.08×10^5 J/mol. This value is in close agreement with the activation energy for hydrostatic-pressure germination of *Bacillus subtilis* (Murrell & Wills, 1977) and for the activation energy of alanine- (O'Connor & Halvorson, 1961; Vas & Prost, 1957) and glucose-triggered germination (Levinson & Hyatt, 1970). Calcium dipicolinate- and EDTA-induced germination also have activation energies between 8.4×10^4 and 1.26×10^5 J/mol (Riemann, 1963). The similarity of the above values, for such diverse germination initiators, may support the concept of a single mechanism for germination of all bacterial spores. The value for the activation energy of commitment to L-alanine-induced germination reported here is in the typical range for enzymic reactions (Sizer, 1943), and this may reflect the enzyme-like nature of the trigger mechanism.

In 1968, Woese *et al.* proposed a kinetic model for bacterial spore germination. Their hypothesis stated that germination occurs when the concentration of some substance P, in the spore, reaches a critical value P_c. This substance is produced by a spore enzyme whose concentration is governed by a simple Poisson distribution. They also considered that substance P was labile and that, consequently, the germinative concentrations of P (P_c) were not only dependent on the synthetic rate constant, k_{+1}, but also on a degradative rate constant, k_{-1}. According to the data that they present, the best model for germination was obtained if k_{+1} and k_{-1} were different functions of temperature, and k_{-1} increased with temperature faster than k_{+1}.

The commitment data presented here support a re-appraisal of this model. The measured activation energy may describe the production of P, and the close similarity of activation energies measured for different germinants is consistent with the hypothesis that P is a universal component of all germination mechanisms. The temperature transition at 35°C for commitment to L-alanine germination may be considered as a measure of the temperature-dependence of k_{+1} and k_{-1}. At temperatures above 35°C, k_{-1} exceeds k_{+1} and the slope of the Arrhenius plot

changes sign. Although the model of Woese *et al.* (1968) described above was initially based on a metabolic trigger for germination, it is sufficiently versatile to be equally helpful in analysing possible biophysical mechanisms. The reaction mechanism discussed below attempts to use the model to explain the phenomenon of the commitment within the constraints of such a biophysical trigger event:



R is considered as an allosteric receptor protein that can interact with L-alanine (A) to produce an active form (AR*). If the number of these active proteins is critical for germination to proceed, then AR_c^* is equivalent to P_c. Once the critical concentration of AR* is produced, the spore is committed to germinate and the activation energy measured for commitment may describe the allosteric change in R induced by L-alanine. The mechanism whereby either AR* or R* induces germination is not known. However, since hydrolytic reactions have not yet been discounted in the initiation of germination (Scott & Ellar, 1978*b*), the hydrolysis of ester, amide, peptide or glycosidic bonds remains as a possible candidate. The above sequence of events may also be used to explain the decreased rate of commitment (K) measured by the centrifugation method. The original filtration technique involves only a brief exposure of spore suspensions to D-alanine, and, because of possible restrictions in permeability, this may only remove free L-alanine from the equation without significantly affecting the concentration of AR. This AR may then presumably be added to the total production of AR*, which results in commitment. In contrast, the centrifugation method involves soaking spore suspensions in D-alanine, which may shift the equilibrium of L-alanine (A) + R ⇌ AR towards free L-alanine and R. This would result in a decrease in the final concentration of AR* for any given time of exposure to L-alanine and therefore a decreased degree of commitment. Overall, this would be expressed as a reduction in the value of 'K'.

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