Biochemical analysis of germination mutants to characterize germinant receptors of *Bacillus subtilis* 1604 spores

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Spores of *Bacillus subtilis* 1604 can be induced to germinate by incubation in L-Ala (the ALA pathway) or in a combination of β -D-glucose (Glc), β -fructose (Fru), L-Asn and K⁺ (the GFAK pathway). Biochemical analysis of the germination response of a *gerA* mutant deficient in the ALA pathway revealed that L-Ala can replace L-Asn in the GFAK pathway (the GFAlaK pathway). In contrast to the ALA pathway of both the wild-type and of a *gerB* mutant, the GFAlaK pathway was insensitive to D-Ala and showed the same overall inhibitor profile as the GFAK pathway of wild-type and *gerA* spores. It is deduced that a second L-Ala receptor with different characteristics to that functioning in the ALA pathway is present in wild-type spores. Analysis of the germination response of a *gerB* mutant showed that whilst the rate of ALA germination could be stimulated by Glc as well as by Fru in the presence of Glc, the spores could not germinate in GFAK. In addition, Glc and Fru were unable to reverse D-Ala inhibition of L-Ala germination which they do in the wild-type. Thus, in the *gerB* mutant, the L-Ala/L-Asn receptor in the GFAK pathway is defective. It is concluded that the germination receptors in the ALA and GFAK pathways can functionally interact with each other to initiate *B. subtilis* spore germination. This conclusion is discussed in relation to proposed models of triggering of spore germination.

Introduction

Spores of *Bacillus subtilis* can be induced to germinate by several combinations of germinants, including L-Ala (ALA system) and a combination of β -D-glucose (Glc), β -fructose (Fru), L-Asn and K⁺ (GFAK system) (Wax & Freese, 1968). The mechanism by which the germinants interact with spore components to initiate germination is not known. Wax *et al.* (1967) proposed that initiation of *B. subtilis* 168 spore germination can be triggered by germinants entering at least two biochemical pathways. One branch is inhibited by D-Ala or by high temperature; compounds produced in this pathway can also be provided by the combination of Fru, Glc and K⁺. The products of the other branch could also be produced by L-Asn or L-Gln, either of which alone is unable to initiate germination but also requires Glc, Fru and K⁺ (Wax &

Freese, 1968). In addition to a co-operative role with L-Asn, Fru and K⁺, Glc appears to play other roles in initiating germination. In ALA-initiated germination, it stimulates germination especially at high temperatures, and enhances the observed reversal by Fru of inhibition caused by D-Ala (Wax *et al.*, 1967).

A number of L-Ala analogues including allylglycine, cycloleucine and L-Val can be used in place of L-Ala in initiating germination of *B. subtilis* spores (Irie *et al.*, 1980, 1982; Kanda *et al.*, 1988; Sammons *et al.*, 1981; Woese *et al.*, 1958; Yasuda & Tochikubo, 1985). In GFAK germination, Glc can be replaced by the non-metabolizable analogues 2-deoxy-D-glucose or D-allose (Prasad *et al.*, 1972). Moreover, it has been shown that ATP synthesis is not an early germination event in either the ALA or the GFAK systems and that a range of metabolic inhibitors do not affect spore germination (Venkatasubramanian & Johnstone, 1989). Finally, mutants lacking alanine dehydrogenase (Freese & Cashel, 1965), pyruvate dehydrogenase (Freese & Fortnagel, 1969) or glucose dehydrogenase (Rather & Moran, 1988) germinate normally. Thus, initiation of germination may take place by a biophysical rather than by a metabolic mechanism.

The stereospecific properties of the germinant

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Abbreviations: Fru, β -fructose; Glc, β -D-glucose; ALA, 1 mM-L-Ala in 50 mM-potassium phosphate buffer, pH 7.4; GFAK, 5.6 mM-Glc, 5.6 mM-Fru, 30 mM-L-Asn and 67 mM-KCl in 50 mM-Tris/HCl buffer, pH 7.5; GFAlaK, 5.6 mM-Glc, 5.6 mM-Fru and 30 mM-L-Ala in 50 mM-potassium phosphate buffer, pH 7.4.

receptors demonstrate that these components of the trigger mechanism must be proteins which may be activated allosterically (Wolgamott & Durham, 1971). Analysis of *B. subtilis* PCI219 spore germination has revealed that in the ALA system, a potent germinant, L-Ala, and its competitive inhibitor D-Ala, bind to a common receptor; their amino and carboxyl groups may bind to respective common regions and their methyl group may bind to separate regions which differ in size and electrostatic nature (Yasuda & Tochikubo, 1985). The stimulatory effect of glucose on L-Ala-binding, and the absence of an effect on D-Ala-binding, confirm the presence of separate regions with respect to their methyl groups (Yasuda & Tochikubo, 1984). As L-Ala-initiated germination is inhibited quantitatively by various hydrophobic compounds, a hydrophobic environment near the L-Ala receptor was suggested (Yasuda *et al.*, 1978a, b, 1982).

The spore germination pathway in *B. subtilis* has been studied by the use of germination (*ger*) mutants (Irie *et al.*, 1982; Lafferty & Moir, 1977; Prasad *et al.*, 1972; Smith *et al.*, 1977; Trowsdale & Smith, 1975; Wax *et al.*, 1967). The different types of *ger* mutants and their responses to amino acids and sugars have been reviewed (Foster & Johnstone, 1989; Moir & Smith, 1990). Mutants in some *ger* loci affect germination in one germinant system without altering the response in others. These include the *gerA* and *gerC* loci (Trowsdale & Smith, 1975; Sammons *et al.*, 1981) required for ALA germination, and the *gerB*, *fruB* and *gerK* loci required for a germination response in a combination of amino acids and sugars (Moir *et al.*, 1979; Prasad *et al.*, 1972; Strauss, 1983; Irie *et al.*, 1982). Mutants in the *gerK* locus behave in a similar manner to *gerB* mutants; their spores do not germinate in the GFAK system and appear to be deficient in their response to Glc, since Glc fails to stimulate ALA-initiated germination or to enhance reversion by Fru of D-Ala inhibition (Irie *et al.*, 1982). Whilst *gerB* and/or *gerK* may encode products which interact with components of the amino acid and sugar mixture, the *gerA* locus might encode an Ala-sensitive receptor in the spore (Moir & Smith, 1985). Detailed studies of *gerA* mutants are consistent with this possible interpretation (Feavers *et al.*, 1985; Zuberi *et al.*, 1987).

It has been postulated that spores of *B. subtilis* may contain two classes of receptor which bind L-Ala; one of high affinity which binds L-Ala (and analogues) alone and another which binds L-Ala (and analogues) with lower affinity in the presence of Glc, Fru and KCl (Sammons *et al.*, 1981). In this paper, a comparative biochemical study of the germination response of spores of *gerA* and *gerB* mutants and of wild-type *B. subtilis* 1604 to amino acids, sugars and inhibitors is presented which provides additional evidence for the presence of

two L-Ala receptors and further characterizes the interactions between the germinant receptors.

Methods

Spore formation. Strains of *Bacillus subtilis* 1604 (*B. subtilis* 168 *trpC2*), *gerA* and *gerB18* were obtained from Dr A. Moir (Department of Molecular Biology and Biotechnology, University of Sheffield, UK). Spores were prepared at 37 °C as described for *Bacillus megaterium* KM (Stewart *et al.*, 1981) and stored at 10 mg dry wt ml⁻¹ in distilled water at -20 °C. Immediately prior to germination, spore suspensions were heat-activated at 70 °C for 60 min, and cooled on ice.

Spore germination. Germination of spores (50 µg dry wt ml⁻¹) was induced at 37 °C in three systems as follows: (i) ALA system in which the spores were germinated for 60 min in 50 mM-potassium phosphate buffer, pH 7.4, by the addition of 1 mM (final concentration) L-Ala; (ii) GFAK system in 50 mM-Tris/HCl buffer, pH 7.5, by the addition of 5.6 mM-Glc, 5.6 mM-Fru, 30 mM-L-Asn and 67 mM-KCl for a period of 80 min; (iii) GFAlaK system in 50 mM-potassium buffer, pH 7.4, by the addition of 5.6 mM-Glc, 5.6 mM-Fru and 30 mM-L-Ala for 80 min. These germinant concentrations were maintained in all experiments unless otherwise stated.

The extent of germination in a spore suspension was determined as the percentage of the control loss in OD₆₀₀ after 60 or 80 min, for the ALA or GFAK/GFAlaK systems, respectively, in a Pye Unicam SP8-200 UV-VIS spectrophotometer fitted with a constant temperature cell-holder. The rate of germination was measured as the maximum rate of loss of OD₆₀₀ min⁻¹ divided by the initial OD₆₀₀ (V_{max} ; Bright & Johnstone, 1987). The extent of germination was confirmed at the end of each experiment by determining the ratio of phase-bright to phase-dark spores. In all experiments the ratio of phase-bright to phase-dark spores correlated with the percentage loss of OD₆₀₀.

Effect of inhibitors. The effect of D-Ala, D-Asn, HgCl₂, protease inhibitors and metabolic inhibitors on germination was examined as described by Venkatasubramanian & Johnstone (1989). Where indicated, inhibitors were added at the stated concentrations to spore suspensions at 37 °C 15 min prior to the addition of germinants. The control germination response for inhibition studies represents that of untreated spores, for which 50–60% loss of initial OD₆₀₀ was observed.

Reproducibility of results. All experiments were repeated at least twice and were internally consistent.

Chemicals. Germinants and inhibitors were from Sigma, apart from HgCl₂, which was from BDH and of AR grade.

Results and Discussion

Germination response of spores of the *gerA* mutant

As described by Moir *et al.* (1979), spores of the *gerA* mutant did not germinate in the ALA system, but germinated just as well as the wild-type in the GFAK system (Fig. 1). The spores failed to germinate when the concentration of L-Ala was increased from 1 to 10 mM in the ALA system and the addition of Glc (1 mg ml⁻¹) or Fru (1 mg ml⁻¹) in the presence of 1 or 10 mM-L-Ala did not stimulate their germination (results not shown). However, the presence of both Glc and Fru (each at 1 mg ml⁻¹) together with 1 mM-L-Ala, caused 31% OD₆₀₀ loss and 50% of the spores to become phase-dark

Fig. 1. Effect of Glc and Fru on ALA germination of *B. subtilis* 1604 and *gerA* spores. The percentage of initial OD_{600} was measured during germination of *B. subtilis* 1604 ($\circ$, $\triangle$, $\square$) and *gerA* spores ($\bullet$, $\blacktriangle$, $\blacksquare$) using ALA ($\circ$, $\bullet$), GFAK ($\triangle$, $\blacktriangle$) and ALA+Glc+Fru ($\square$, $\blacksquare$) as germination inducers.

(results not shown). In comparison, > 95% germination occurred when *gerA* spores were incubated in 10 mM-L-Ala+Glc (1 mg ml⁻¹)+Fru (1 mg ml⁻¹)+50 mM-K⁺ (results not shown). The rate of germination (determined as described in Methods) of *gerA* spores increased by about three times when the concentration of L-Ala was raised from 1 to 10 mM while in combination with Glc, Fru and K⁺ (GFK). These observations suggest that ≥ 10 mM-L-Ala can replace L-Asn in the GFAK system in this mutant. In subsequent experiments, 30 mM-L-Ala was used whenever in combination with GFK (the GFAlaK system).

The K_m values for L-Ala in the GFAlaK system and L-Asn in the GFAK system were determined in the *gerA* mutant to compare the binding affinities of L-Ala and L-Asn with those of the wild-type. The K_m values of L-Ala in GFAlaK (1.1 mM) and L-Asn in GFAK (0.9 mM) systems of the *gerA* mutant were similar (Fig. 2); they differed significantly from the K_m values of both L-Ala (50 μ M) and L-Asn (3.3 mM) in the ALA and GFAK germination systems, respectively, of the wild-type (Venkatasubramanian & Johnstone, 1989). This suggests either that the *gerA* mutation has affected both the L-Ala and L-Asn receptor sites or that the L-Ala receptor in the GFAlaK system is different from that in the ALA germination system, which is presumably inactive in the *gerA* mutant. Since 10 mM-D-Ala completely inhibits L-Ala-induced germination of the wild-type spores of *B. subtilis* 1604, the effects of D-Ala and D-Asn on both the GFAK and GFAlaK germination pathways of the *gerA* mutant were examined. Spores of the *gerA* mutant germinated normally in the GFAK or GFAlaK systems, in the presence of D-Asn (100–300 mM) or D-Ala (30–300 mM) (results not shown). This suggests, therefore, that the receptor responding to L-Ala in the GFAlaK system differs from that responding to L-Ala in

Fig. 2. Lineweaver-Burke plots for L-Ala and L-Asn during ALA, GFAK and GFAlaK germination of *B. subtilis* *gerA* and *gerB* spores. Spores were germinated (as described in Methods) in ALA, GFAlaK and GFAK systems. $1/[S]$ was plotted against $1/V_{max}$ (see Methods) for L-Ala in ALA germination of *gerB* spores ($\blacktriangle$, $10^{-1} \times \mu\text{M}^{-1}$) or GFAlaK germination of *gerA* spores ($\circ$, $10^{-2} \times \mu\text{M}^{-1}$), and for L-Asn in GFAK germination of *gerA* spores ($\bullet$, $10^{-2} \times \mu\text{M}^{-1}$).

Fig. 3. Overall inhibition of GFAK and GFAlaK germination of spores of *B. subtilis* *gerA* by HgCl_2 . Spores were treated with varying concentrations of HgCl_2 and the percentage inhibition of OD_{600} loss determined as described in Methods, after germination in GFAK ($\circ$) and GFAlaK ($\bullet$) for 80 min.

the ALA pathway, since 300 mM-D-Ala completely inhibits the ALA pathway.

Since it was observed that the GFAK and ALA systems in the wild-type had different sensitivities towards HgCl_2 , NaN_3 and PMSF (Venkatasubramanian & Johnstone, 1989), the effect of these inhibitors on germination of spores of the *gerA* mutant was studied. The overall HgCl_2 inhibition profile of the GFAlaK system was similar to that of the GFAK system in the *gerA* mutant (Fig. 3). Although the overall inhibition of this pathway by HgCl_2 was not as great as that of the GFAK system in wild-type *B. subtilis* 1604 (Venkatasubramanian & Johnstone, 1989), it was more sensitive to HgCl_2 than the ALA system of the wild-type (Venkatasubramanian & Johnstone, 1989). At 0.5 mM- HgCl_2 , OD_{600} loss was inhibited by 71 and 79% in the

GFAK and GFAlaK systems, respectively, whereas in the wild-type 0.5 mM HgCl_2 inhibited OD₆₀₀ loss by 61 and > 95% in the ALA and GFAK germination systems, respectively. It is not known why the HgCl_2 sensitivity of the GFAK pathway is altered in the *gerA* mutant as compared with the wild-type. As observed for the GFAK system of the wild-type, the mutant spores could be protected by either D-Asn (300 mM) or D-Ala (300 mM) from 3 mM- HgCl_2 to allow subsequent germination in the GFAlaK and GFAK systems. NaN_3 , which inhibits only GFAK germination (by > 95%) and does not have any effect on the ALA system in the wild-type spores (Venkatasubramanian & Johnstone, 1989), was found to inhibit both the GFAK and GFAlaK systems in spores of the *gerA* mutant. Germination in the GFAlaK system was inhibited by > 95%, whereas the GFAK system was inhibited by about 85–90% in the presence of 10 mM- NaN_3 (results not shown). PMSF (3.3 mM), which completely inhibits germination in the ALA system had no significant effect on the GFAK germination system of the wild-type spores (Venkatasubramanian & Johnstone, 1989) and had no effect on either the GFAlaK or GFAK systems of the *gerA* mutant. These results are therefore consistent with the hypothesis that a new pathway in which L-Ala can substitute for L-Asn in GFAK (the GFAlaK pathway) exists. These results also suggest that although germination in the ALA and GFAK systems has been considered to be due to 'independent' pathways, the germinant receptors for L-Ala, Glc, Fru and L-Asn are capable of interacting with one another. The characterization of *gerA* mutants which responded to ALA+Glc, ALA+Fru or needed ALA and both Glc+Fru (Moir *et al.*, 1979; Sammons *et al.*, 1981; Trowsdale & Smith, 1975; Wax & Freese, 1968) also supports this view. It has been shown also that temperature-sensitive ALA germination of a *gerA* mutant of *B. subtilis* 168 at 43 °C is rectified by the addition of Fru, and that Glc enhances the reversion (Irie *et al.*, 1982). One explanation for the different phenotypes of *gerA* mutants is that their mutations cause defects in the germinant receptor which affect the binding site in different ways. The observation that a mutant defective in Glc (*gerK*) is able to affect both the ALA and GFAK responses (Irie *et al.*, 1982) is another indication that an interaction occurs between the receptors of L-Ala, Glc, Fru and L-Asn.

There have been previous hypotheses that two L-Ala receptors are involved in *B. subtilis* spore germination (Irie *et al.*, 1982; Sammons *et al.*, 1981). The present study is in agreement with these suggestions and provides firm evidence for the presence of an L-Ala receptor which is stimulated during ALA germination, and another which requires a higher concentration of L-Ala, as well as

Fig. 4. Germination of *B. subtilis gerB* spores in the ALA and GFAK systems and the effect of Glc and Fru on ALA germination. Spores of *gerB* were treated with ALA (●), ALA+Glc (○), ALA+Fru (△), ALA+Glc+Fru (▲) and GFAK (□), and the percentage of initial OD₆₀₀ determined as described in Methods.

the presence of Glc+Fru for its stimulation. The second type of receptor may be the same as the L-Asn receptor and therefore this receptor is termed the L-Ala/L-Asn receptor. The presence of this receptor suggests that the observed reversal of D-Ala inhibition by Glc and Fru, previously thought to be due to stimulation of the ALA pathway (Wax *et al.*, 1967), is probably simply a result of stimulation of the GFAlaK pathway.

Germination response of spores of the *gerB18* mutant

Spores of the *gerB* mutant failed to germinate in the GFAK system, but germinated just as well as the wild-type in the ALA system (Fig. 4) as reported by Moir *et al.* (1979). The rate of germination in the ALA system increased in the presence of Glc (1 mg ml⁻¹) or Glc (1 mg ml⁻¹)+Fru (1 mg ml⁻¹), whereas it remained the same in the L-Ala+Fru (1 mg ml⁻¹) combination (Fig. 4). Thus Fru can only assist Glc in enhancing the rate of ALA germination and the germination defect in the *gerB* mutant therefore appears to be associated with the L-Asn receptor.

To determine whether the *gerB* mutation had affected L-Ala binding to the L-Ala receptor, the K_m value for L-Ala in the ALA system was determined. The K_m value of L-Ala was 50 µM for both the wild-type (Venkatasubramanian & Johnstone, 1989) and the *gerB* mutant (Fig. 2). The mutation in the *gerB* mutant does not therefore appear to have changed the binding constant of the L-Ala receptor. Moreover, as observed in the wild-type, there was a departure from Michaelis-Menten kinetics in the low L-Ala concentration range for *gerB* spores (Venkatasubramanian & Johnstone, 1989; Fig. 2).

Since the use of D-amino acids as inhibitors of germination can indicate the specificity of the receptor site, spores of the *gerB* mutant were allowed to germinate

Fig. 5. Effect of Glc+Fru on the overall HgCl_2 inhibition of ALA germination of *B. subtilis gerB* spores. Spores were treated with HgCl_2 and germinated with ALA in the presence (○) and absence (●) of Glc+Fru. The percentage inhibition of OD_{600} loss was determined as described in Methods.

Fig. 6. Model showing proposed functional interactions between the L-Ala, L-Asn, Glc and Fru germinant receptors in the *B. subtilis* ALA, GFAK and GFAlaK spore germination pathways. The corresponding germination loci are given in *italics*.

in the ALA system in the presence of D-Ala (10–300 mM) and D-Asn (10–300 mM). In the presence of 10 mM-D-Ala, no germination of *gerB* spores occurred in the ALA system, whereas the presence of 10–300 mM-D-Asn had no effect in the ALA system (results not shown). Partial reversibility of D-Ala inhibition by Fru, and enhancement of the reversion by Glc has been noted in *B. subtilis* 168 strain 127 (Wax *et al.*, 1967). Experiments were therefore carried out to test whether Glc, Fru or Glc+Fru could reverse inhibition by 10 mM-D-Ala in germination in the ALA system in both wild-type and *gerB* spores. It was observed that in the wild-type neither Glc nor Fru reversed D-Ala inhibition individually, but that Glc+Fru in combination reversed this inhibition by 40% (results not shown). In the *gerB* mutant, this inhibition was not reversed at all by Glc, Fru or Glc+Fru. This further suggests that the L-Ala/L-Asn receptor in the *gerB* mutant is deficient in its interaction with the Glc and/or Fru receptor.

The overall effect of HgCl_2 on inhibition of *gerB* spore germination in the ALA system was similar to that observed in the wild-type (Fig. 5; Venkatasubramanian & Johnstone, 1989). In both the wild-type and the *gerB* mutant, 0.5 mM- HgCl_2 inhibited germination in the ALA

system by about 55–60% and 3 mM- HgCl_2 inhibited germination by > 95%. PMSF (3.3 mM) inhibited ALA germination of *gerB* spores completely as observed in wild-type spores (Venkatasubramanian & Johnstone, 1989). Since germination of *gerA* spores in the GFAK and GFAlaK systems and germination of *gerB* spores in the ALA system differed in their sensitivity towards HgCl_2 and PMSF, it is possible that the differences in sensitivity may be due to the presence of Glc and/or Fru rather than to the presence of the amino acid component of the germinant system. Therefore, the overall inhibition of germination of *gerB* spores by HgCl_2 , NaN_3 and PMSF was studied after the addition of Glc+Fru to the ALA system. It was observed that the presence of ALA+Glc+Fru did not make the *gerB* spores more sensitive to HgCl_2 (3 mM- HgCl_2 inhibited germination by 92%; Fig. 5). Similarly, the presence of Glc+Fru did not affect the sensitivity of *gerB* spores in the ALA system to PMSF or to NaN_3 . PMSF (3.3 mM) still inhibited germination by greater than 95% and 10 mM- NaN_3 had no effect on ALA germination of the *gerB* mutant (results not shown). Thus, the presence of Glc+Fru did not appear to be the cause of differential sensitivity of the ALA and GFAK systems to HgCl_2 and PMSF in the *gerB* mutant.

A model for the interaction of germinant receptors in B. subtilis

Based on the results of the present study of *gerA* and *gerB* mutants and earlier work (Irie *et al.*, 1982; Moir *et al.*, 1979; Sammons *et al.*, 1981), a model showing interaction of the receptor sites for L-Ala, Glc, Fru and L-Asn is proposed in Fig. 6. In this model, the L-Ala, Glc, Fru and L-Asn receptors are in close proximity to one another since they can functionally interact. The L-Asn receptor in this complex can also be activated by L-Ala at high concentrations, has similar germination requirements and hence has been called the L-Ala/L-Asn receptor. It is also proposed that *gerA*, *gerB* and *gerK* encode the L-Ala, L-Asn and Glc receptors, respectively.

The gene encoding a putative L-Ala receptor which responds to L-Ala has been cloned and sequenced (Feavers *et al.*, 1985; Zuberi *et al.*, 1985, 1987). It will be interesting to sequence the genes encoding the other germinant receptors as well as to analyse the germination response of spores of *gerB* deletion mutants to gain a better understanding of the germinant receptor complex in *B. subtilis* 1604. In addition, site-directed mutagenesis of the *ger* genes may allow the functional domains of the receptors and their interactions to be studied further.

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