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Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of *gerL* and *gerQ* operons

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***Bacillus cereus* 569 (ATCC 10876) endospores germinate in response to inosine or L-alanine, the most rapid germination response being elicited by a combination of these germinants. The *gerI* operon has already been characterized as a homologue of the *gerA* spore-germination receptor family of operons found in all *Bacillus* spp. examined; the primary defect in *gerI* mutant spores is in the inosine germination response, although spores were also slower to germinate in L-alanine. Additional transposon-insertion mutants, from similar Tn*917*-LTV1 mutagenesis and enrichment experiments, now define two more operons, also members of the family of *gerA* homologues, important in L-alanine and inosine germination. Transposon insertions were identified in an alanine-specific germination locus, named *gerL*, which represents an operon of three genes, termed *gerLA*, *gerLB* and *gerLC*. By examining the residual germination response to L-alanine in *gerI* and *gerL* mutants, it was deduced that the GerL proteins contribute most strongly to the L-alanine germination response, and that the GerI proteins, required primarily in inosine germination, mediate only much slower germination responses to alanine. The L-alanine germination responses mediated by GerL and GerI proteins differ in their germination rates, temperature optima and germinant concentration dependence. The *gerQ* locus, again identified by transposon insertion, is a second inosine-related germinant-receptor operon. GerQ and GerI proteins are both required for the germination response to inosine as sole germinant, but GerQ has no role in L-alanine germination. Although near-identical homologues of *gerI* and *gerL* operons are evident in the *Bacillus anthracis* genome sequence, there is no evidence of a close homologue of *gerQ*.**

Keywords: *Bacillus anthracis*, *gerA*, germinant, receptor, endospore

INTRODUCTION

Despite their dormancy and resistance, spores retain a sensitive mechanism capable of detecting, and rapidly responding to, the presence of specific germinative substances (Foerster & Foster, 1966; Gould & Dring, 1972; Moir & Smith, 1990) to initiate the process of germination and then outgrowth. Germination can be triggered by a variety of factors, including nutrients (amino acids, sugars), non-nutrient germinants (such as calcium dipicolinate), and physical factors such as hydrostatic pressure and abrasion (Paidhungat & Setlow, 2000; Wuytack *et al*., 2000; Paidhungat *et al*., 2001). Nutrient-stimulated germination requires the activity of spore-germination receptors, encoded in homologues of the *gerA* operon, which was the first-described operon of the family, and is required for alanine-dependent germination in *Bacillus subtilis* (Moir

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The GenBank accession numbers for the *gerL* and *gerQ* operons reported in this paper are AF387344 and AY037930, respectively.

et al., 1994, 2002; Paidhungat & Setlow, 1999; Hudson *et al*., 2001). These operons are present in multiple copies in the genomes of spore-formers, reflecting duplication and divergence of function to respond to different germinants, and are expressed during sporulation as part of the morphogenetic cascade (Errington, 1993). The GerAA, GerBA, GerAC and GerBC proteins are all located in the inner spore membrane of *B. subtilis* (Hudson *et al*., 2001; Paidhungat & Setlow, 2001).

Bacillus cereus is widely distributed in nature, and can be found in milk and cereals, on plant surfaces and in a variety of other foods. It causes two types of food poisoning: the emetic type resulting from the growth of vegetative cells spoiling food and creating toxins; and the diarrhoeal type caused by an enterotoxin (Kramer & Gilbert, 1989; Granum & Lund, 1997). There is a close genetic relationship between *B. cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* (Helgason *et al*., 2000), and much of the information on *B. cereus* germination systems is likely also to be of relevance to these others. *B. cereus* spores germinate in response to L-alanine and to ribosides, such as inosine (Warren & Gould, 1968), as do also *B. thuringiensis* and *B. anthracis*. Molecular genetic techniques, including transposon mutagenesis and insertional inactivation, have recently been applied to the study of spore germination in *B. cereus*. Already identified are the *gerI* operon, a member of the *gerA* operon family, required for the response to inosine as sole germinant member and contributing to alanine-stimulated germination (Clements & Moir, 1998), *gerN*, a Na^+/K^+-H^+ ion antiporter required for inosine germination (Thackray *et al*., 2001; Southworth *et al*., 2001) and *gerP*, a locus affecting the permeability of spore coats to germinants (Behravan *et al*., 2000).

This work describes the characterization of two additional operons, both *gerA* operon homologues, identified by transposon mutagenesis. The *gerL* operon, a second homologue in *B. cereus* of the *gerA* receptor-encoding family, is responsible for the rapid response of spores to L-alanine as sole germinant; the relative contributions to alanine germination from proteins encoded in *gerL* and *gerI* loci are assessed under optimized conditions, using the appropriate mutants. A third homologue, *gerQ*, is required for germination in inosine as sole germinant, but has no role in the alanine response.

METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *B. cereus* was routinely cultured on or in Oxoid nutrient agar and broth containing the appropriate antibiotics (tetracycline at 50 μg ml^{-1}, or erythromycin at 1 μg ml^{-1} plus lincomycin at 25 μg ml^{-1}). CCY medium (Stewart *et al*., 1981) was used for the preparation of spores.

Spore preparation. Spores of *Bacillus* strains were prepared as described previously (Clements & Moir, 1998).

Spore-germination assays. Spores were heat-activated in distilled water at a concentration of 8–10 mg dry weight ml^{-1} in a water bath at 70 °C for 30 min and then placed on ice. Spores were used within 8–10 h of activation.

Spores were germinated under two sets of conditions. For inosine germination, spores were suspended in 10 mM Tris/HCl, pH 8·0, 10 mM NaCl, incubated for 15 min at 37 °C, and germination initiated by the addition of inosine to 5 mM. For L-alanine germination, spores were suspended in 50 mM Tris/HCl, 50 mM NH_4Cl, pH 8·9, at 30 °C, and germination was initiated by the addition of L-alanine (to 50 mM). For L-alanine germination, O-carbamyl-D-serine (5 μg ml^{-1}) was added to inhibit the alanine racemase activity of the spores. For the estimation of the effects of pH, 50 mM Tris/HCl–50 mM NH_4Cl or 50 mM CHES/NaOH–50 mM NH_4Cl buffers were used.

The OD_{580} of the spore suspensions was monitored continuously on a chart recorder linked to a Unicam SP1800 ultraviolet spectrophotometer fitted with a constant-temperature cell holder.

Transposon mutagenesis, enrichment and screening. Transposon mutagenesis was carried out with *B. cereus* 569 UM20.1(pLTV1) as previously described (Clements & Moir, 1998). Libraries of transposon-insertion mutants were enriched for mutants and screened by using a tetrazolium overlay as described previously (Clements & Moir, 1998).

Phage transduction. Phage transduction was performed using phage CP51ts, a heat-sensitive derivative of generalized transducing phage CP51 (Thorne, 1968), as described previously (Clements & Moir, 1998).

Screening of a *B. cereus* genomic library. *B. cereus* 569 UM20.1 genomic DNA was prepared according to the protocol supplied with the Puregene kit (Flowgen Instruments). The DNA was partially digested with *Sau*3A, separated by gel electrophoresis, and fragments of 4–6 kb were excised, purified with GeneClean II (Bio 101), then ligated into *Bam*HI-pre-digested λZAP Express vector as described by the manufacturer (Stratagene). The ligation products were packaged using Stratagene's Gigapack II according to the manufacturer's instructions. Approximately 10000 plaques were screened by plaque blotting, probing and hybridization detection using the Digoxigenin DNA labelling and detection kit according to the instructions of the manufacturer (Boehringer Mannheim).

Sequencing and analysis. DNA sequencing was performed with the *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and an Applied Biosystems DNA sequencer. Plasmid DNA for sequencing was prepared using the PEG method according to the manufacturer's instructions. The 3′ ends of the operons were completed using PCR; for each operon, two independent PCR products were sequenced The DNA sequence, complete on both strands and fully overlapped, was assembled and analysed using the Staden suite of programs (Staden, 1990). Preliminary sequence data from the *B. anthracis* genome was obtained from The Institute for Genomic Research website at http://www.tigr.org.

The sequences of the *gerL* (accession no. AF387344) and *gerQ* (accession no. AY037930) operons have been submitted to GenBank.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and phenotype	Source or reference
***B. cereus* strains**		
569 UM20.1	*trp-1* Str^r	Battisti *et al.* (1985)
569 UM20.1/pLTV1	*trp-1* Str^r Tet^r Cm^r Ery^r	Clements & Moir (1998)
AM1316	Tn*917*-LTV1::*gerLA1* (*ala-1*) Ery^r *trp-1* Str^r	Clements & Moir (1998)
AM1404	Tn*917*-LTV1::*gerLA4* (*ala-4*) Ery^r *trp-1* Str^r	This study
AM1311	Tn*917*-LTV1::*gerQA2* (*ino-2*) Ery^r *trp-1* Str^r	This study
AM1314	Tn*917*-LTV1::*gerIA5* (*ino-5*) Ery^r *trp-1* Str^r	Clements & Moir (1998)
Plasmids		
pLTV1	Cm^r Ery^r Tet^r Amp^r	Camilli *et al.* (1990)
pALA1	Amp^r	This study
pALA4	Amp^r	This study
pINO2	Amp^r	This study

RESULTS

Isolation and genetic characterization of germination mutations

Isolation of a Tn*917*-LTV1 transposon insertion mutant of *B. cereus* defective in L-alanine germination, but essentially wild-type for germination in inosine (AM1316; *ala-1*), was described by Clements & Moir (1998). A second mutant, AM1404 (*ala-4*), with a similar phenotype has now been isolated by enrichment in alanine, and 100% linkage of the antibiotic resistance conferred by the transposon with the germination defect by CP51ts transduction has been demonstrated for both mutants (data not shown). Mapping of the site of insertion in the two mutants by Southern blotting (Barlass, 1998) demonstrates that the transposon had inserted, in the same orientation, into a 6·6 kb *Eco*RI fragment of chromosomal DNA, in positions that were 1·4 kb apart. This locus has been named *gerL*, and the mutations redesignated *gerLp1* and *gerLA4*, as they are respectively located upstream of, and within, the first ORF of the *gerL* operon. The *ino-2* mutation mentioned briefly by Clements & Moir (1998) was similarly checked by CP51 transduction and by Southern blotting, and represents a separate locus from *gerI* and *gerL*; the mutant allele has been renamed *gerQA2*. Both *gerL* and *gerQ* operons are homologues of the paradigm *gerA* alanine germination operon of *B. subtilis*.

Cloning and sequencing of the *gerL* region

The *gerL* region is shown in Fig. 1. Chromosomal DNA from the *gerLp1* and *gerLA4* mutants was digested with *Eco*RI, then diluted and ligated as described previously (Clements & Moir, 1998). Transformation of *Escherichia coli* DH5α allowed recovery of a plasmid carrying the chromosomal region flanking the end of the transposon, yielding pALA1 and pALA4, respectively, from the two mutants. *Sal*I cuts at 276 bp into the transposon sequence; a double digest identified the *Sal*I–*Eco*RI fragments containing chromosomal DNA as approximately 2·2 and 3·6 kb for the two plasmids, respectively.

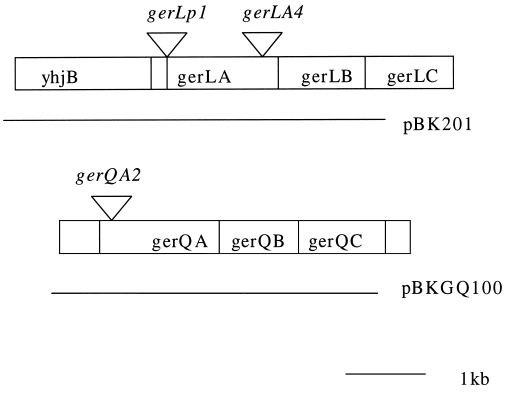

Fig. 1. Organization of the *gerL* and *gerQ* operons. Triangles show the positions of Tn*917-LTV1* insertion mutations. Lines under the operons indicate the DNA recovered in the named phagemids.

In an attempt to recover the remainder of the *gerL* locus, a λZAP Express library of *B. cereus* DNA was screened with a 1·4 kb PCR product, generated using primers based on sequence data from the pALA4 clone, and digoxigenin-labelled. This yielded a lambda clone, from which the pBKCMV-derived phagemid pBK201 was recovered (Fig. 1). All three plasmid clones were used in DNA sequencing. As *gerLC* was incomplete in the pBK201 clone, the sequences equivalent to *gerL* of *B. cereus* were located in the unfinished *B. anthracis* genome sequences (http//www.tigr.org), which had then become available. Two primers were designed based on sequences within the downstream ORF (a *feoX* homologue) in the *B. anthracis* genome. These were used, separately, with an opposing primer from sequence within the end of the pBK201 clone, in PCR reactions. Both PCR products were sequenced to complete the sequence of the operon. A 5·65 kb region including the complete *gerL* operon has been submitted to GenBank (accession no. AF387344).

Three ORFs representing the *gerLA*, *gerLB* and *gerLC* genes are separated from the upstream ORF1, which is

A

```
 V  F  I  K  K  N  T  I  I  V  E  K  *                       ----
GTATTTATAAAGAAAAACACTATAATAGTGGAAAAGTAATTCGTTTATCCCGCATTAACA
------------------>                                 <----------------
AGCAGTAAGACCCCCACCTCAAAATTCAGCGGAAGCAAAGAAGTTAGGTGGGGGCCTTGC
-------
TGCCTGTAAAAGCCCGATTGGTGAGGGGTGATTAAAGTTTTACTTTATTAGAGCTATCTT
                    -35                          -10
TGAAAAGAGATAGCTCTTTTTTGTATATATTTTCTTCAATTAGCGGAATCTAACTAAAAA

          gerLA→  M  G  K  L  L  E  L  E  G  N  L  F  E
CTTGTTGAAAAGGAAGTTAGGTGGGGAAATTGTTAGAGCTTGAAGGTAACTTATTTGAAG
```

B

```
AAGAATGTACTTTTAGAATTTATAAAAAATATAATTAGGATTAAATTAAAACCTTCCAAA
          ----------------->       <------------------
AAGCATGTTATAAAAACATCCTTTCTTTAGAACCAAAAGAAAGGATGTTTTTAATATCGT

AACTATGTTTTAAAAAAATTCTTATTTTTACTTTTAATACATTTTTTAACTAATTTTGGT

TAAGTTATTTTCAAAGAGGTTATTGCTAATTCGATGGATATATCTATATATCACTATGCC
 -35                    -10
TGATTTTTAAAATAGAAATTTCTTAAAAACTATATGTAACTTCTTTCTGAAAGGAAGGTA

gerQA→  M  K  P  Y  G  K  I  P  D  N  S  I  K  S  L  Q  D
TTATCAATTGAAACCGTATGGAAAAATACCTGATAACTCTATAAAATCTTTACAGGATTT
```

Fig. 2. Promoter regions of *gerL* and *gerQ* operons, showing *gerL* sequences (A) and *gerQ* sequences (B). The probable ribosome-binding sites and initiation codons are underlined in bold. Double underlining indicates potential σ^G-dependent promoter regions. Dotted lines indicate upstream stem–loop structures. In (A), the end of the upstream ORF (*yhjB*) is shown.

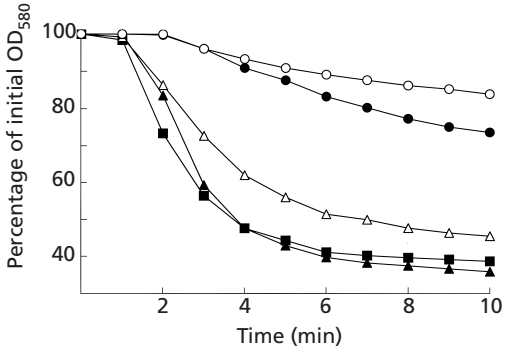

Fig. 3. Spore germination in alanine by wild-type and mutants. Germination was monitored as the fall in OD_{580} of washed spore suspensions, in response to L-alanine (50 mM) in the presence of *O*-carbamyl D-serine (5 μg ml^{-1}), at 30 °C, pH 8·9. ■, Wild-type (strain 569 UM20.1); ▲, AM1311 (*gerQA2*); △, AM1314 (*gerIA5*); ●, AM1404 (*gerLA4*); ○, AM1316 (*gerLp1*).

a close homologue of YhjB of *B. subtilis* (69 % amino acid identity), by an intergenic region of just over 200 bp. This intergenic region contains an extended inverted repeat, possibly representing a termination site for the upstream *yhjB* gene, and also a potential forespore-specific σ^G-dependent promoter region for the *gerL* genes (Fig. 2A). In database searches against finished and unfinished microbial genomes at NCBI, the GerL proteins have close homologues in *B. anthracis* (94, 93 and 87 % identity, respectively, for the GerLA, GerLB and GerLC proteins) and lower similarity to other homologues in the GerA family (up to 41 % identity for

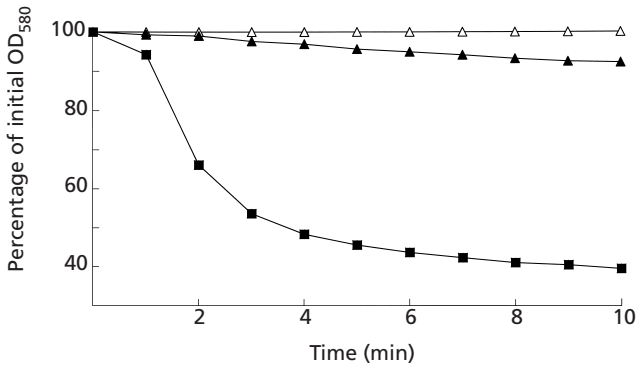

Fig. 4. Spore germination in inosine by the wild-type and mutants. Germination was monitored as the fall in OD_{580} of washed spore suspensions in response to inosine (5 mM) at 37 °C, pH 8·0. ■, Wild-type (strain 569 UM20.1); ▲, AM1311 (*gerQA2*); △, AM1314 (*gerIA5*). Data for AM1404 (*gerLA4*) and AM1316 (*gerLp1*) were coincident with those for the wild-type, and are omitted for clarity.

GerLA, up to 27 % for GerLB, and up to 28 % for GerLC). The site of transposon insertion in *gerLp1* is between the predicted −35 and −10 sequences, 26 bases upstream of the ribosome-binding site for the *gerLA* gene. The insertion in the *gerLA4* mutant removes the last 54 codons of GerLA.

Cloning and sequencing of the *gerQ* operon

DNA flanking the point of insertion of the transposon in the *gerQA*2 mutant (Fig. 1) was recovered as part of an *E. coli* plasmid, pINO2, as described above for the *gerL* mutants. Sequence data were generated from both ends of the 5·4 kb chromosomal DNA region recovered. The sequence from the distal end of the cloned fragment represented part of a homologue of the HblA protein of *B. cereus*. The sequence from the other end suggested that the transposon had inserted at a position 40 codons into an ORF.

A more extensive clone of this region was recovered from a lambda library, by probing with labelled cloned DNA from pINO2. The excised phagemid from this clone, pBKGQ100, contains the complete *gerQ* operon, except for the 3′ end of the *gerQC* ORF. The N-terminal part of the *gerQA* gene corresponds closely to *gerX*, described as a partial sequence of a germination gene homologue located downstream of the *hblCDAB* gene cluster in the *B. cereus* type strain ATCC14579 (Økstad *et al*., 1999). We have identified the rest of the equivalent *gerQ* operon in *B. cereus* strain ATTC 14579, by searching the preliminary and partial genome sequences made available by Integrated Genomics (http://ergo.integratedgenomics.com/B_cereus.html). This allowed the amplification, from DNA of *B. cereus* ATTC 10876, of a PCR fragment extending into the end of *gerQC* from the next downstream ORF. A 4·36 kb sequence containing the entire *gerQ* operon of *B. cereus* ATCC 10876 has been submitted to GenBank (accession no. AY037930).

The intergenic region upstream of *gerQA* in the ATCC 10876 sequence contains several regions also conserved in the ATCC 14579 intergenic sequence. This includes an inverted repeat thought to represent the rho-independent terminator of an upstream KinA-like gene (Økstad *et al*., 1999) and a region that contains a potential *sig*G-dependent promoter sequence (Fig. 2B), although it is not very close to the consensus. The *gerQ–hbl* region is not represented in the unfinished *B. anthracis* genome sequence; *B. cereus* ATCC 10987 and *B. anthracis* (A.-B. Kolstø, personal communication) both appear to lack the *hbl* gene cluster. The Ger homologues with the greatest similarity to the GerQ proteins are the GerI proteins of *B. anthracis* (45, 37 and 33% for the QA, QB and QC proteins, respectively) and homologues in *Bacillus halodurans* (49, 39 and 33%, respectively). The identities with respect to *B. subtilis* homologues are up to 34, 23 and 22%, respectively.

Germination behaviour of mutants

Differences were noted in optimal pH, temperature and monovalent-cation dependence for inosine and L-alanine germination by *B. cereus* (Clements & Moir, 1998) Optimized conditions, based on their data, have been used in this work. Measurement of the loss of OD_{580} of a spore suspension essentially demonstrates the time to germination summed over the population of spores. Fig. 3 shows the germination behaviour in alanine of spores with defects in one of the three recognized loci – *gerL*, *gerI* and *gerQ*. Alanine germination is unaffected by the *gerQA2* mutation, but the rate of germination is a little slower in a *gerIA5* mutant, and the *gerLp1* and *gerLA4* mutants respond much more slowly to L-alanine. Fig. 4 shows that the *gerQA2* mutant, like a *gerIA5* mutant, is very slow to germinate in inosine. The *gerLp1* and *gerLA4* mutant spores showed the same germination kinetics in inosine as the wild-type parent.

Separation of the contributions of the GerI and GerL proteins to alanine germination

Two receptor systems, encoded by *gerI* and *gerL*, appear to contribute to the germination response to alanine; a comparison of residual germination behaviour has therefore been made for a *gerI* mutant (to measure *gerL*-dependent germination) and a *gerL* mutant (which will have an intact *gerI*-dependent alanine response).

Washed spore suspensions of strains UM20.1, AM1314 (*gerIA5*) and AM1401 (*gerLA4*) were tested for germination. Rates of germination at different pH values are shown in Table 2. In Tris/HCl buffer, the *gerL*-dependent response (in a *gerIA5* mutant) increased over a wide pH range above 7, but still showed significant activity at neutral pH. In contrast, little activity of the *gerI*-dependent response was seen below pH 8·9. To obtain buffering at a more alkaline pH, germination in a CHES/NaOH buffer was examined. The *gerL*-dependent response was again strongest at 8·5–8·9; germination dependent on *gerI*, maximal at pH 8·9 in Tris/HCl, was very low in CHES. The summed rates of germination in the individual mutants do not equal the overall rate of germination in the wild-type, especially at pH values around 7–8. This suggests that the products of both the *gerL* and *gerI* operons may be required to act in concert (and possibly with additional receptors) to cause efficient germination at pH values close to neutrality, or that additional receptors contribute at this pH.

The temperature optima (Fig. 5) were also different: *gerL*-dependent germination in alanine was most rapid at 30 °C, whereas *gerI*-dependent alanine germination was fastest at 20 °C, and almost negligible at 37 °C – although this higher temperature was optimal for inosine germination, for which the GerI proteins are required (Clements & Moir, 1998). There was no evidence of strong dependence on additional monovalent ions for alanine-dependent germination in Tris/

Table 2. Germination rate in L-alanine at different pH values

Data are for Tris/HCl buffer, except for the values shown in parentheses, which are for CHES/NaOH buffer. Each germination rate is expressed as the maximum rate of loss of OD_{580}, as a percentage of initial OD_{580} lost per min. ND, Not determined.

pH	Germination rate		
	Wild-type	*gerLA4*	*gerIA5*
7·1	15	0·8	3·9
7·5	14·5	0·3	6·1
8	19·4	0·4	9·4
8·5	20 (21)	1·2 (0·9)	12 (12·5)
8·9	25 (19)	4·2 (0·8)	15·2 (10·1)
9·2	(12·5)	(0·36)	(6·3)
9·5	(6·9)	(0·16)	(3·9)
9·7	(3·5)	ND	(1·9)
10	(1·6)	ND	(1·1)

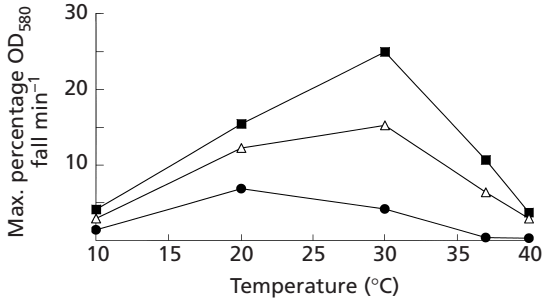

Fig. 5. Effects of temperature on spore germination in L-alanine. The maximum rate of germination (estimated as the maximum rate of OD_{580} loss) of spore suspensions is compared at different temperatures. Conditions were as for Fig. 3, except that the temperature of incubation was varied. ■, Wild-type (strain 569 UM20.1); △, AM1314 (*gerIA5*); ●, AM1404 (*gerLA4*).

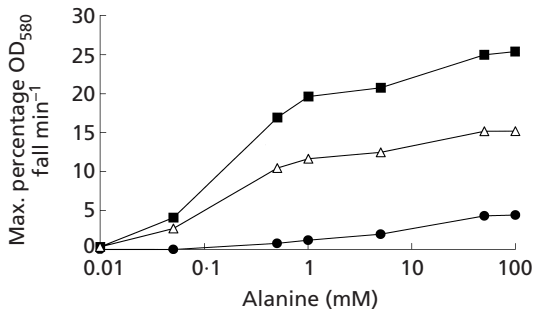

Fig. 6. Effects of L-alanine concentration on germination rate. Conditions were as for Fig. 1, except that the concentration of L-alanine was varied. The maximum rate of germination (estimated as the maximum rate of OD_{580} loss) of spore suspensions is compared at different L-alanine concentrations. ■, Wild-type (strain 569 UM20.1); △, AM1314 (*gerIA5*); ●, AM1404 (*gerLA4*).

HCl buffer (Barlass, 1998), although ammonium ions stimulated germination rates slightly.

The concentration-dependence on alanine (Fig. 6; measured at 30 °C) showed that significant stimulation of *gerI*-dependent germination required higher concentrations of L-alanine (half-maximal germination rate at approx. 8 mM) than did *gerL*-dependent germination (half-maximal rate at approx. 0·25 mM).

The germination behaviour of the *ino-2* (*gerQA2*) mutant in alanine was essentially identical to that of wild-type spores in terms of concentrations required, temperature dependence and pH dependence (Barlass, 1998). Alanine germination therefore appears to be entirely independent of this locus.

DISCUSSION

A plausible model for germination requires that the chemical germinant traverses the outer layers of the spore, to interact with receptor protein(s) of the GerA family at the inner membrane (Hudson *et al.*, 2001; Paidhungat & Setlow, 2001). This interaction, which may also involve local ion transfer (Southworth *et al.*, 2001; Thackray *et al.*, 2001), probably stimulates membrane-associated changes and bulk ion movements. Water moves into the core, partially rehydrating it. In the outer layers of the spore, cortex and coat lytic enzymes are activated (Moir *et al.*, 2002; Paidhungat *et al.*, 2001); cortex hydrolysis allows complete rehydration of the spore core, and consequent full metabolic activity. Macromolecular synthesis then resumes and the spore outgrows to form a vegetative bacterial cell.

This work brings to six the total number of *Bacillus* germinant receptors of the GerA family that meet both the criterion of proven function and that of defined germinant specificity (the *gerA*, *gerB* and *gerK* operons in *B. subtilis*, and the *gerI*, *gerQ* and *gerL* operons in *B. cereus*), although there are numerous additional members of this family encoded in the available genomic DNA sequences from different spore-formers. The *gerL* operon of *B. cereus* and the *gerA* operon of *B. subtilis* are the closest in terms of germinant specificity, but they are widely divergent (e.g. GerLB and GerAB share 23% identical amino acids). We do not yet have enough information to identify the residues conserved in these proteins that contribute directly to L-alanine binding.

Three operons of the *gerA* family, all with the same gene order as *gerA*, have now been defined in *B. cereus* as encoding receptors for alanine or inosine germination. Additional operon homologues are present in the sequences in the incomplete *B. cereus* and *B. anthracis* databases and are presumably involved in the response to other individual germinants or to combinations of germinants. In earlier work (Clements & Moir, 1998), we measured much more rapid germination responses to inosine than to alanine in wild-type spores, but the temperature and pH used for germination were not optimized for the latter. It is also important to include an alanine racemase inhibitor, as D-alanine is commonly a competitive inhibitor of L-alanine germination (Foerster & Foster, 1966).

Germination in L-alanine as sole germinant is mediated by at least two separate receptors; GerL, reported here, is the major contributor under our optimized conditions. In contrast, germination in inosine as sole germinant strictly requires two receptors, i.e. the protein products of both the *gerI* and *gerQ* operons.

GerI and GerQ receptors in *B. cereus* are both part of the nucleoside response. We do not understand why both receptors are required for a response to a single germinant; perhaps they are recognizing different parts of the germinant molecule, or, simply, perhaps neither provides sufficient activation individually. As yet there is no experimental evidence illuminating, even for multiple germinants, how different receptors act in concert.

We have identified mutations (all affecting inosine germination; Barlass, 1998) in each of the *gerI* genes, but the mutations described here for *gerL* and *gerQ* all lie in the first gene in the operons. Past precedent from *B. subtilis* indicates that the genes in an individual operon have co-evolved and do not substitute for each other, so we tentatively assume the same scenario for these operons, but this should be tested experimentally.

Germination in a combination of inosine and L-alanine, a common combination of germinants in experiments, can involve multiple germinant receptors, and detailed physiological analysis addressing germination mechanisms will require dissection of the responses of different receptors, as they clearly vary: inosine germination, but not alanine germination, for example, is dependent on GerN, a Na^+/K^+-H^+ antiporter. Germination rates in inosine and a very low concentration of alanine are significant in a *gerQ* mutant but not in a *gerI* mutant (Clements, 1996), suggesting that the role of *gerQ* can be bypassed if some alanine is present. Double mutants have not yet been constructed to determine whether *gerL* and *gerI* are both required for this response to combinations of germinants.

B. cereus, *B. thuringiensis* and *B. anthracis* represent a

closely related group of organisms that could be considered as a single species (Helgason *et al*., 2000). The germination responses are related (all are reported to germinate in alanine plus inosine) but are not identical. For example, in *B. anthracis*, the *gerX* operon, encoded in the pathogenicity gene cluster of pXO1, contributes to rapid spore germination in the macrophage (Guidi-Rontani *et al*., 1999). Differences in the germination properties of different strains in the *B. cereus* continuum may well reflect the activity, and even the presence, of different germinant receptor operons, and these may contribute to the exploitation of particular biological niches.

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