The gerB region of the Bacillus subtilis 168 chromosome encodes a homologue of the gerA spore germination operon

Bernard M. Corfe,1,2 Rachel L. Sammons,3 Derek A. Smith1 and Catherine Mauel4

Author for correspondence: Bernard M. Corfe (Krebs Institute). Tel: +44 742 824418. Fax: +44 742 7286967. e-mail: b.m.corfe@sheffield.ac.uk

Spores of gerB spore germination mutants of Bacillus subtilis 168 are defective in response to the germinative mixture of L-asparagine, glucose, fructose and potassium ions (AGFK), but are normal in the L-alanine (ALA) triggered germination response. A clone of 15 kbp carrying the gerB region has been identified. Sequencing of the gerB region of the clone revealed a cluster of three ORFs encoding putative proteins of 53.3, 41.3 and 42.4 kDa (GerBA, GerBB and GerBC, respectively). The first two of these proteins have substantial hydrophobic regions and the third is a possible lipoprotein. At least two, and probably all three products are required for normal germination in AGFK. The three proteins form a set of homologues of the products of the gerA operon, mutations in which cause a defect in the ALA germination pathway, but cause no defect in AGFK. The GerB proteins show 42%, 31% and 35% identity at the amino-acid level to the corresponding GerA proteins, and the homologues occur in the same order in both operons.

Keywords: Bacillus subtilis, sporulation, germination, gerB, membrane protein

INTRODUCTION

Spores of Bacillus subtilis 168 have two known germination responses, which have been well-characterized: they germinate in response to alanine alone (ALA) or to the mixture of asparagine, glucose, fructose and potassium ions (AGFK). Spore germination (ger) mutants have been isolated which are defective in either or both of these responses (Moir & Smith, 1990), which suggests that there are separate germinant-specific triggers, with a later convergence of the germination pathways.

Of all the ger mutants the only ones with a specific defect in ALA germination result from mutation in the gerA operon (Moir & Smith, 1990). This is a tricistronic operon which encodes proteins of 53.3, 41.3 and 42.4 kDa, all of which could be membrane-associated (Feavers et al., 1985; Zuberi et al., 1987). The gerA operon is expressed in the forespore compartment of the developing sporangium from a promoter recognized by EoG, and it has been suggested that the proteins form a complex at the inner spore membrane which acts as a receptor for alanine (Feavers et al., 1990).

Mutants of gerB and gerK have defects specifically in the AGFK response but retain a normal ALA response (Moir et al., 1979; Irie et al., 1982) and gerK has been associated with the glucose component of the response on account of the inability of glucose to improve the ALA response in gerK mutants under certain conditions (Irie et al., 1982). This effect is not seen in gerB mutant spores (McCann, 1989). Mutants of gerD appear to be defective in both germination pathways to some extent (Moir et al., 1979); however the product of gerD may be associated with the fructose component of the response (Irie et al., 1986). The gerB mutations have been located by phage PBS1-mediated three-factor transductional crosses to 314 degrees on the B. subtilis 168 chromosome. Mutations are not known to affect any one specific component of the AGFK response.

Molecular genetical analysis of several ger genes has as yet shed little light on the possible mechanism of germination (Moir & Smith, 1990). The predicted proteins generally...
lack characteristic motifs and database searches reveal no homology with other proteins.

Although tag (teichoic acid biosynthesis) and gerB have not been mapped relative to one another, available data place them near to each other on the chromosome, with gerB on the ori-proximal side (Piggot et al., 1990). We have identified a λEMBL4 derivative carrying gerB from a series of overlapping clones of the tag-gta region. Sequence information indicating the possible nature of the gene products, reported here, has provided more essential information in the attempt to identify the mechanism of spore germination.

METHODS

Bacterial strains and media. Strains of B. subtilis 168 and Escherichia coli K12 used are listed in Table 1. B. subtilis was cultured on nutrient agar (NA), and E. coli on Luria–Bertani agar (LA), with appropriate selective conditions as described by Yon et al. (1989). Transformation of B. subtilis was by the method of Anagnostopoulos & Spizizen (1961) and of E. coli by the method of Mandel & Higa (1970). Ger phenotypes were scored on plate tests as described by Irie et al. (1982).

DNA manipulation. Plasmid and chromosomal DNA preparation, restriction, ligation and gel electrophoresis were carried out as described by Sammons et al. (1987). All subcloning was done using pM7L20EC in DH5α. λ DNA preparation was as described by Maniatis et al. (1982), using E. coli P2392 as a host. Purification of restriction fragments from gels was achieved using GeneClean (Bio101).

DNA sequencing. Restriction fragments of 150–1500 bp were prepared from parent subclones, gel-purified, subcloned into pM7L20EC and sequenced. M13 universal, reverse and −40 primers were used wherever possible, supplemented by a small number of custom primers. Double-stranded dideoxy sequencing was done using the Sequenase system as recommended by the manufacturers (USB). Electrophoresis was carried out using 4–8% (w/v) denaturing acrylamide gels in a BRL S0 apparatus (Gibco).

RESULTS

Identification of a gerB clone and sequencing

Mauel et al. (1989) identified clones carrying the tag region in a λEMBL3 bank and Young et al. (1989) subsequently extended the cloned region by chromosome-walking using λEMBL3 and λEMBL4 banks. As gerB was thought to be on the ori-proximal side of tag, the appropriate clone, λE51, was screened for the presence of gerB. As Ger+ phenotypes cannot be selected directly, gerB pheA derivatives of λE507 were made for screening purposes. DNA from strains carrying either the gerB15 or the gerB18 allele, 5182 and 4688, respectively, was used to transform strain λE507. Selection was for Met+ and transformants were screened to find strains with a Ger− phenotype. Appropriate double mutants, strains 5304 and 5301, were transformed with a mixture of 0.5 µg DNA from a λ clone carrying Phe+ DNA and 5 µg λE51. One hundred and fifty transformants from each cross were screened for their Ger phenotype using a modification of the tetrazolium test (Irie et al., 1982) and it was found that in congression experiments with the λE51 DNA present both the gerB alleles were corrected, with about 50% of the Phe+ transformants having a Ger− phenotype. Congression experiments with λE51-derived plasmid subclones in place of λE51 revealed the approximate location of two of the gerB alleles within the clone (Fig. 1).

The sequence of the gerB region was generated by dideoxy sequencing of small restriction fragments subcloned in plasmid vector pM7L20EC (Chambers et al., 1988) as described in Methods. All restriction sites were overlapped in the sequence.

Computer analysis of the sequence

The sequence of 3.8 kb was analysed using the UWGCG package, version 7.1 (Devereux et al., 1984) except where otherwise stated. Three large adjacent ORFs were identified by direct translation and were confirmed by codon usage analysis (data not shown). The sequence and putative translations are shown in Fig. 2. ORFs 1, 2 and 3 have been named gerBA, gerBB and gerBC, respectively. There are three possible initiation (start) codons for gerBB, a GTG and ATG which overlap the gerBA termination (stop) codon, and an ATG which is 5 bp downstream. The last has a possible Shine–Dalgarno sequence, although it is weak and close to the ATG. There is an overlap between the probable stop and start codons of gerBB and gerBC. All the ORFs have potential ribosome-binding sites with homology to the 3' end of the B. subtilis 16S RNA molecule (Stewart & Bott, 1983) appropriately
The gerB gene of Bacillus subtilis

A further possible member of the GerAA/GerBA group is the spoVAF gene product. This ORF was partially sequenced by Fort & Errington (1985) as the sixth predicted member of the spoV AF operon and the predicted N-terminal sequence had homology to GerAA. When the sequence of the downstream gene, lysA, was published, the upstream sequence included the second half of spoV AF (Yamamoto et al., 1991; Sorokin et al., 1993) so that the complete sequence is now available. UWGCG Gap comparisons of GerAA and GerBA with the predicted SpoVAF polypeptide showed a lower degree of conservation than exists between GerAA and GerBA (data not shown), although the polypeptide is still significantly homologous, with around 25% identical residues and over 50% similarity taking conservative substitutions into account. SpoVAF is also of a similar size (462 amino acid residues) to GerAA and GerBA. However, insertional inactivation of spoVAF has no known effect upon spore germination (E. H. Kemp, personal communication). There are no further ORFs downstream of spoVAF in the putative operon.

The hydroplot of GerBB (Fig. 4b) shows that it is predominantly hydrophobic with no large hydrophilic regions. There is homology across the whole length of the GerAB/GerBB polypeptides but, as is common for hydrophobic proteins, this is at the functional (60% conservative substitutions) rather than identical (30% identity) level.

The hydrophobic domains of both GerBA and GerBB are similar in size to their GerA homologues, but their hydroplots are not as clearly defined as for GerAA and GerAB, making estimation of the number of membrane-spanning helices difficult on the basis of these data. However, the close similarity with the gerA products, and the clarity of the hydroplots of the latter, could suggest that GerBA and GerBB have seven and 10 or 11 transmembrane regions, respectively. If this is correct then the hydrophilic domains of GerBA would be on opposite sides of the membrane.

GerBC is predominantly hydrophilic, but has a small hydrophobic region at the N-terminus which has homology to the signal sequence for exported prokaryotic lipoproteins (Yamaguchi et al., 1988). GerBC shows 35% identity with its homologue, and 58% conservative substitution.

Upstream of the gerBA translational start is a potential binding site for σ^70, the sigma factor which directs gene expression in the forespore after engulfment by the mother cell (Fajardo-Cavazos et al., 1991). This may indicate a further degree of homology between the two gene clusters, as the gerA operon is a known member of the σ^70 regulon (Feavers et al., 1990). Between bases 10 and 20 of this sequence is the −35 region for ORFX, a putative gene in the opposite orientation to gerB in the upstream sequence (Margot, 1992). ORFX lies adjacent to lytD (glucosaminidase) which in turn is adjacent to tagABC operon (Margot, 1992).

**Localization of the gerB alleles**

Strains carrying gerB alleles were transformed with integrative plasmids carrying regions of the putative gerB operon. Three subclones were used: A carried most of gerBA, and the upstream region; B carried an overlap between gerBA and gerBB; and C carried an overlap between gerBB and gerBC. The results of the integrations are shown in Fig. 5. The integration of subclone C into the genome gave a TWM^− phenotype. This indicates that the gerB product must be required for germination. This result for subclone C is an argument for operon structure. There is a complete copy of the gerBC ORF on the chromosome after the integration event, but a Ger− phenotype is obtained. Therefore the ORF must be separated from its promoter by at least the 250 bp which are present between the start of the subclone and the start.
The gerB gene of Bacillus subtilis

Fig. 2. Nucleotide sequence of the gerB region. The sequence of 3829 bp is derived from subclones shown in Fig. 1. There are three large ORFs which are shown below the sequence, each with a potential ribosome-binding site which is underlined. The operon also has a potential Eσ^6-type promoter upstream of ORF 1, which is double-underlined. There is no obvious terminator after ORF 3.

DISCUSSION

The sequence of the gerB spore germination operon has been elucidated and has revealed that gerB is a homologue of the gerA operon. In addition spoVAF, the sixth ORF in the spoVAF operon (Fort & Errington, 1985), has a lower degree of homology with the gerBA/gerAA group. Furthermore gerK, mutations in which cause a defect in the AGFK response, has been shown to encode a further homologue of the GerAC/GerBC group (R. Irie, unpublished). GerK is more closely related to GerBC than to GerAC, but is not as close to either as they are to each other (B. Corfe, unpublished). Hence a family of proteins has now been identified, mostly involved in spore germination and produced in the forespore. Mutations affecting germination have been mapped in all three ORFs of the gerA operon and in at least two of the gerB operon (see Fig. 5), demonstrating the requirement of all these proteins for normal germination.

The highly conserved hydrophobic region in the GerAA/GerBA group probably indicates a region of functional importance. It is unlikely that this indicates a recent evolutionary divergence as the rest of the homology shows more divergence in both the hydrophobic and hydrophilic domains. Furthermore such a high level of identity between two hydrophobic domains that have not been required for AGFK germination, and it seems likely that gerBB, too, is necessary.
recently diverged is uncommon. This argues that this region of the protein must be particularly important in the function of the protein. As yet no possible functions have been individually assigned to any of the GerA/GerB proteins.

There are significant genetic and physiological differences between the ALA and AGFK germination responses. The germinant requirement is very different:

---

**Fig. 3.** Gap comparisons of the GerA and GerB proteins. The scores for percentage identity and conservative substitution for the pairs of proteins are as follows: 42/66 for GerAA vs GerBA (a); 31/60 for GerAB vs GerBB (b); 35/58 for GerAC vs GerBC (c). The most highly conserved region of the GerAA/GerBA pair is the hydrophobic domain between residues 200 and 400. This pattern of conservation is absent in GerAB and GerBB, which are predominantly hydrophobic proteins. The GerAC and GerBC proteins have homology to the prokaryotic lipoprotein attachment motif (underlined). Solid lines represent identical substitutions, while single dots represent weakly conservative substitutions.
contrast a complex set of germinants is required for the response without affecting the AGFK response. In the ALA response depends upon a single amino acid as the germinant (which is thought not to be metabolized, as non-metabolizable analogues of alanine will also trigger germination in the absence of the gerD and gerK gene products and further germinants. Spores of null mutants of gerA respond weakly to ALA with the GFK adjuncts, and this is dependent upon (at least) the gerB and gerK gene products (McCann, 1989). As GerK and GerD have been tentatively assigned the roles of glucose and fructose receptors respectively, it can be suggested that gerB encodes the ASN receptor which may also act as the secondary ALA receptor proposed by Sammons et al. (1981). The demonstration that gerB is a homologue of the probable principal ALA receptor/trigger reinforces this argument.

It is not obvious why the gerB products cannot stimulate germination in their own right. It now seems likely that ALA and AGFK germination occur by a more similar mechanism than could previously have been suggested on the basis of genetical and physiological evidence and it is therefore proposed that there are two similar types of germinant receptors in the spore. One may involve GLU and FRU receptors which are facultatively required to improve the response to ALA alone mediated by the gerA gene products. The other could have the GLU and FRU receptors working in obligate conjunction with an amino acid receptor, which is less fastidious than the ALA receptor and encoded by gerB. This model is summarized in Fig. 6. Whilst this model is novel in terms of the spatial arrangement and interactions between germination proteins that it implies, the mechanisms by which the germination apparatus function are still unknown and are the subject of our continuing enquiry.

ACKNOWLEDGEMENTS

The screening work was done by B.M.C. in the laboratories of Professor D. Karamata in Lausanne, and we are most grateful to him and members of his group for their kindness and hospitality. We would also like to thank Dr A. Moir and Professor C. M. Thomas and his group for much advice and many useful conversations, and Dr Moir for a critical reading of the manuscript. B.M.C. was an SERC postgraduate student. This work was completed with SERC research grant GR/H09164 to Dr Moir.

REFERENCES


