The *gerC* locus of *Bacillus subtilis*, required for menaquinone biosynthesis, is concerned only indirectly with spore germination

A. J. Howard Leatherbarrow, Mohammed A. Yazdi, Janet P. Curson and Anne Moir

Author for correspondence: Anne Moir. Tel: +44 114 2224418. Fax: +44 114 2728697.
e-mail: a.moir@sheffield.ac.uk

Krebs Institute for Biomolecular Research,
Dept of Molecular Biology and Biotechnology,
University of Sheffield,
Sheffield S10 2TN, UK

The *gerC* region of *Bacillus subtilis* comprises a tricistronic operon, encoding enzymes that catalyse the late stages of menaquinone biosynthesis. The *gerC58* mutation is responsible for a severe growth defect; unsuppressed mutant cells grow as very short rods, which sometimes septate aberrantly. Cultures grow only to a low cell density, rapidly lose viability, and never sporulate. Unlinked suppressor mutations can restore near-normal growth. Several independent suppressed isolates were examined; all grew to normal cell length, but they showed, to varying extents, a residual defect in the placement of the cell division septum. The germination properties of the suppressed derivatives varied from normal to significantly slow in germination in all germinants; therefore, the combination of the *gerC* mutation and different suppressor alleles resulted in spores with very different germination properties. This suggests that any relationship between the *gerC* gene products and spore germination is indirect. The *gerCC58* mutation maps in a gene encoding the catalytic subunit of the heptaprenyl-diphosphate synthase, which is responsible for formation of the isoprenoid side chain of menaquinone-7, and it is proposed that the *gerCA*, *gerCB* and *gerCC* genes be renamed *hepA*, *menG* and *hepB*, respectively.

Keywords: spore germination, *Bacillus subtilis*, menaquinone biosynthesis, *hep* genes, *menG*

INTRODUCTION

The *gerC* mutants of *Bacillus subtilis* initially characterized by Trowsdale & Smith (1975) were reported as temperature sensitive in spore germination in response to alanine, but normal in germination in the alternative combination of asparagine, glucose, fructose and KCl. The *gerC* locus was mapped, by PBS1 transduction, to the *lys-trp* region of the chromosome, at 204°, but the original mutant strains contained several complicating linked mutations (Moir *et al.*, 1979).

The *gerC58* mutation affects vegetative cell growth, as was revealed when the *gerC58* mutation was introduced into an unmutagenized background by transformation. The resulting *gerC58* transformants formed very small

colonies on minimal medium and failed to grow at all on rich medium (the 'tiny' or Tny⁻ phenotype; Yazdi & Moir, 1990). The original germination-defective isolate therefore carried, in addition to *gerC58*, an extragenic suppressor of *gerC58*, named *sup-58*, which restored approximately normal growth and spore formation. The *gerC* locus was cloned in a *B. subtilis* ϕ 105 vector, on a 6 kb *Bcl*I fragment of chromosomal DNA (ϕ MY1; Yazdi & Moir, 1990), and the sequence was deposited in GenBank. The *gerCA* and *gerCC* genes encode the two subunits of heptaprenyl-diphosphate synthase, which catalyses the synthesis of the isoprenoid side chain of menaquinone-7, the predominant form found in bacilli (Zhang *et al.*, 1997), and the *gerCB* gene encodes a methyltransferase responsible for the methylation of demethylmenaquinone (Koike-Takeshita *et al.*, 1997). This paper examines the growth and germination defects in *gerC* mutant and suppressed strains, to determine whether this locus has a specific role in alanine germination.

The GenBank accession number for the sequence reported in this paper is M80245.

METHODS

Bacterial strains and media. *Escherichia coli* strains used for routine transformation with plasmids and M13 clones were DH5 α and JM101, respectively. *B. subtilis* strains are listed in Table 1, and are described elsewhere in the text. These were routinely grown and stored on nutrient agar (Oxoid), except where stated. Spores were prepared on the same medium, and washed as described previously (Moir *et al.*, 1979). Germination was monitored as fall in OD₅₈₀ after addition of germinants, by the procedure described by Yazdi & Moir (1990). Minimal glucose medium was that of Anagnostopoulos & Spizizen (1961), supplemented with any amino acid requirements. Transformation of *E. coli* was by the method of Mandel & Higa (1970).

Plasmids and phages. A *Bcl*I–*Sst*I segment, which includes the entire *gerC*-complementing region, was excised from phage MY1 as an *Sst*I fragment (using the vector *Sst*I site adjacent to the *Bcl*I site) and subcloned into pUC119 to yield plasmid pMAY1 (Yazdi, 1989). The approximately 700 bp *Pvu*II–*Sst*I fragment in M13mp18 clone PS2 (Fig. 1), carrying the end of

gerCC and the start of *ndk*, was excised as a *Bam*HI–*Eco*RI fragment using flanking M13 cloning sites and cloned into the integrational vector pAZ106 (Zuberi *et al.*, 1990) to yield plasmid pHL1. Plasmid pHL2 carries the approximately 400 bp *Pst*I–*Pvu*II fragment internal to *gerCC* (fragment 2 in Fig. 1), excised from an M13 clone (using flanking sites) as a *Sph*I–*Eco*RI fragment and cloned into the equivalent sites in the polylinker of pAZ106.

Transformation of competent *B. subtilis* cells. The cells were transformed as described by Warburg & Moir (1981). Recombinants were purified by single colony isolation before testing of unselected markers.

Spore germination assays. The tetrazolium reaction used to score Ger⁺ and Ger[–] colonies as Tzm-Red or Tzm-White, respectively, was as described previously by Zuberi *et al.* (1985). For assay of germination rates, spores were prepared on nutrient agar and washed as described by Moir *et al.* (1979). Germination was monitored as fall in OD₅₈₀ after addition of germinants, by the procedure described in Moir *et al.* (1979), except that Tris/HCl was 10 mM, pH 8.4, and KCl

Table 1. Strains of *B. subtilis* used

Strain	Genotype	Source/reference
BD111	<i>thrA5 cysB3 trpC2</i>	D. Dubnau (Public Health Institute, New York)
1604	<i>trpC2</i>	Moir <i>et al.</i> (1979); PBS1 transduction × BD111
1558	<i>gerC58 wrd tzm(gerJ51) trpC2</i>	Moir <i>et al.</i> (1979)
AM677	<i>thrA5 trpC2 gerC58 sup-58</i>	Yazdi & Moir (1990); DNA (1558) × BD111, by congression
AM1136	<i>trpC2 sup-58</i>	DNA (1604) × AM677, selecting Thr ⁺ ; <i>gerC</i> ⁺ introduced by congression; presence of <i>gerC</i> ⁺ and <i>sup-58</i> each proved by transformation linkage in back-crosses (Leatherbarrow, 1994)
AM1228	<i>trpC2 leuB8 gerC58</i>	DNA (AM677) × QB99 by transformation; unstable
QB99	<i>hisH2 leuB8</i>	Bacillus Genetic Stock Centre IA89
S3, S5, S7	<i>trpC2 leuB8 gerC58</i> , suppressed	Independent derivatives of AM1228

Fig. 1. *gerC* region of the *B. subtilis* chromosome. The positions of *gerC* and flanking genes are indicated, as well as the relative locations of the cloned fragments in the phage and plasmid subclones. The inserts in integrational plasmids pHL1 and pHL2 are fragments 'PS2' and '2', respectively.

was 2.25 mg ml⁻¹. Germination rates were calculated over 90 min from addition of germinant.

Subcloning and sequencing techniques. Standard DNA manipulation procedures (Sambrook *et al.*, 1989) were used. Sequencing of M13 single-stranded and plasmid double-stranded templates was by the dideoxy chain-termination procedure (Sanger *et al.*, 1977). The DNA was radiolabelled using [³⁵S]dATP α S (Amersham) and Sequenase (US Biochemical). Sequences were determined on both strands and all restriction sites were fully overlapped. Sequences were assembled and analysed using the ANALYSEQ suite of programs (Staden, 1990).

Electron microscopy. Samples of vegetative cells were harvested and then washed twice in Spizizen salts. The pellet was resuspended in 50 ml formaldehyde/glutaraldehyde fixative (Karnovsky, 1966) and incubated at room temperature for 2 h. The samples were then washed five times in 0.1 M sodium cacodylate, then post-fixed in 2% osmium tetroxide. After dehydration in an ethanol series, the material was incubated with dried absolute alcohol, then with propylene oxide, and incubated overnight in a 1:1 mixture of propylene oxide and araldite. Two 3 h incubations in fresh 100% araldite were followed by embedding in 100% araldite and polymerization at 60 °C for 2 h. Sections were cut, stained in saturated uranyl acetate, then washed and stained in Reynolds lead citrate solution. After a final wash, samples were examined under a Philips 301 transmission electron microscope.

RESULTS AND DISCUSSION

Nucleotide sequence and functions of the *gerC* region

Phage ϕ 105MY2, which retains a 3 kb *Bcl*I–*Xba*I fragment of the cloned DNA, still complements the growth defect of a *gerC* mutant, and thus carries a functional copy of the *gerC* locus (Yazdi & Moir, 1990; Fig. 1). The *Bcl*I–*Sst*I sequence extending across the *gerC* region was subcloned from pMAY1 into M13 vectors and sequenced from defined restriction sites, using oligonucleotide primers to extend the sequence where necessary. The overall gene organization deduced from this DNA sequence is shown in Fig. 1.

The *gerC* locus contains a cluster of three complete ORFs, designated *gerCA*, *gerCB* and *gerCC*, of 251, 263 and 320 codons, respectively. These lie on the *trp*-proximal side of the *mtrB* gene (Gollnick *et al.*, 1990), separated from *mtrB* by a potential factor-independent terminator sequence in a 262 bp intergenic region. The close coupling of the three *gerC* ORFs suggests that they form an operon, although no experimental assessment of the transcriptional unit has been made. Downstream of the *gerC* genes, an intergenic space of 115 bp is followed by a homologue of the nucleoside diphosphate kinase (*ndk*) genes of various prokaryote and eukaryote species. There is no potential stem-loop structure that might serve as a factor-independent transcriptional terminator in the *gerCC*–*ndk* intergenic region.

The GerCA and GerCC proteins are 36% and 65% identical, respectively, to the two protein subunits of heptaprenyl-diphosphate synthase of *Bacillus stearo-*

thermophilus ATCC 10149 (Koike-Takeshita *et al.*, 1995), involved in the biosynthesis of the isoprenoid side chain of menaquinone-7. Zhang *et al.* (1997) have overexpressed the PCR-amplified *gerCA* and *gerCC* genes of *B. subtilis*, and demonstrated directly that they encode proteins with the same activity as their *B. stearo-thermophilus* homologues. *orf-1* and *orf-3* are encoded as the first and third genes in a tricistronic operon, and flank an ORF of similar size to GerCB: the sequence of *orf-2* has recently been reported, and the gene demonstrated to encode a 2-heptaprenyl-1,4-naphthoquinone methyltransferase, 75% identical in amino acid sequence to GerCB (Koike-Takeshita *et al.*, 1997).

The phenotype of *gerC* mutant strains

Unsuppressed *gerC* mutants can be generated in a genetic cross but are barely viable, and can only be maintained for up to two or three subcultures on minimal agar, as required for purification of recombinants, before suppressed derivatives outgrow the very slow growing mutant strain. Attempts to grow the mutant in rich medium fail, and result in even more rapid selection of suppressed derivatives (Yazdi & Moir, 1990). In an attempt to clarify the relationship between the *gerC58* allele (and its suppressors) and germination, additional suppressed mutants were isolated. Cultures of AM1228, each grown from a single colony in minimal medium to an OD₆₀₀ of 0.2, were concentrated 10-fold and 0.1 ml was plated on nutrient agar. Colonies of suppressed derivatives (around 50 per plate) appeared after 2 d incubation. Strains S3, S5 and S7 represent the most fully characterized independent isolates. Their spore germination properties were compared with 1604 (*gerC*⁺), with AM677 (*gerC58 sup-58*) and with AM1136, a *gerC*⁺ *sup-58* recombinant. The retention of the *gerC58* mutation in suppressed strains could be checked in back-crosses, as already described for AM677 (Yazdi & Moir, 1990). In the tetrazolium plate assay, 1604 and AM1136 were Tzm-Red, whereas all the *gerC58*-carrying strains (AM677 and all the new suppressed derivatives) were Tzm-White.

The original suppressed mutant, AM677, was temperature sensitive for alanine germination – the phenotype that gave rise to the *gerC* nomenclature. Spore germination of AM677 was 80–100% of the wild-type rate in a combination of asparagine, glucose, fructose and KCl at 42 °C, and in both types of germinant at 25 °C, but was <5% of the wild-type rate in alanine at 42 °C. Spores of the *sup-58 gerC*⁺ mutant (AM1136), although apparently Ger⁺ on a tetrazolium plate test, were rather slower to germinate than wild-type in both germinants at both temperatures (50–70% of the wild-type rate). The germination properties of new isolates, all carrying both *gerC58* and a suppressing mutation, varied. Spores of isolate S3 were somewhat slow to germinate in both germinants (rates similar to those of AM1136), whereas spores of S5 and S7 were approximately normal in germination (80–110% of wild-type rates). None of the three showed any temperature-

Fig. 2. Electron micrographs of thin sections of (a) a *gerC58* unsuppressed strain, (b) wild-type strain 1604 and (c) *gerC58* *sup-58* strain AM677, all at the same magnification. Bar, 1 µm.

dependent effects. This variability implies that the germination behaviour is not defined solely by the presence of the *gerC58* allele, but is highly dependent on the combination of *gerC58* and suppressor alleles, and therefore on the nature or degree of suppression. Germination in alanine and/or in the asparagine, glucose, fructose and KCl combination can be affected – the first suppressed strain to be identified, AM677, is not typical. It is therefore unlikely that the *gerC58* mutation lies in a gene with a direct role in alanine

Fig. 3. Electron micrographs of thin sections of *gerC58* unsuppressed mutant cells. (a) Normal transverse septum formation; (b) abnormal septation. Bar, 0.25 µm.

germination, as had been suggested by Moir *et al.* (1991).

Morphology of *gerC* mutant cells

Cultures of strains were grown in minimal medium, and harvested in mid-exponential growth ($OD_{600} = 0.1$ for AM1228 and 0.3–0.5 for all other strains). Vegetative cells of the unsuppressed *gerC58* mutant (AM1228) are morphologically abnormal. Electron microscopy of thin sections has revealed that the cells are unusually short, but of approximately normal diameter (Figs 2a and 3a); although most division events appear normal, approxi-

mately 15% of the dividing cells have septal abnormalities, as shown in Fig. 3(b).

Although the suppressed mutant AM667 (Yazdi & Moir, 1990) grows at an approximately normal rate in minimal and rich media (data not shown), at least 30% of the cells in the culture have an abnormality in cell division (Fig. 2c), possessing multiple, mislocated septa. Similar septal defects were also observed in cultures of the other suppressed mutants S3, S5 and S7, but were an order of magnitude less frequent in the population than was the case for AM677.

The defect in AM677 does not affect the localization or development of the sporulation septum, and the morphology of cross-sections of mature spores of AM677 and of the other suppressed mutants also appeared normal (data not shown). A derivative of AM677 which is *gerC*⁺ but retains the *sup-58* mutation (AM1136) has no defect in septum localization.

Localization of the *gerC58* mutation to the *gerCC* gene

Phage ϕ 105MY2, a 2.9 kb deletion derivative of the original *gerC* clone, repairs the unsuppressed *gerC58* mutant phenotype efficiently, by complementation, when it lysogenizes the strain (Yazdi & Moir, 1990). Phage ϕ 105MK1,2 (M. Kuroda & C. Yanofsky, unpublished) contains DNA that partially overlaps this insert (Fig. 1). Infection of the unsuppressed *gerC* mutant strain by MK1,2 phage results in the formation of normally growing colonies at a low frequency, indicating that the repair is by recombination instead of complementation. The *gerC58* mutation therefore lies within the second half of *gerCB* or in *gerCC* (although the latter gene is intact in ϕ 105MK1,2, it would be promoterless).

Regions of DNA (I, II, A and PS2; Fig. 1) had been subcloned into M13 as part of the sequencing programme. Replicative form DNAs from these subclones were used in transformation experiments to locate the *gerC58* mutation. Strain AM677 (*thrA5 trpC2 gerC58 sup-58*) was transformed to Thr⁺ by a limiting quantity (0.01 μ g) of chromosomal DNA from 1558 (*trpC2 gerC58 sup-58*), in the presence of 10 μ g of the subclone DNA. Congression occurs at high DNA concentrations, so that a proportion of the Thr⁺ transformants would be also transformed by the added *gerC*-derived DNA. Only the PS2 clone could provide *gerC*⁺ (17 out of 384 Thr⁺ transformants were Ger⁺, as scored by the tetrazolium overlay procedure). None of the other three clones generated any Ger⁺ recombinants amongst 384 transformants tested in each case. The *gerC58* mutation therefore lies within the *Pst*I–*Sst*I region overlapping the end of *gerCC* and the beginning of the downstream *ndk* gene. Since phage MY2 complements the *gerC* defect, however, the functional complementing component appears to be the *gerCC* ORF.

Effects of a defect in menaquinone biosynthesis

Attempts were made to insertionally inactivate the *gerC* operon, by introducing integrational plasmids pHL1 and pHL2 into the *B. subtilis* chromosome by transformation. Only pHL1 could be introduced, despite repeated attempts that were thoroughly controlled to ensure the competence of recipient strains. The insertion of pHL1 would generate a transcriptional fusion of *lacZ* to *ndk*, and would not result in inactivation of any gene. The failure to introduce pHL2, which would result in a disruption of the *gerCC* gene, suggests that *gerCC* is an essential gene, a conclusion consistent with the very limited viability of the *gerC58* mutant, and the requirement for menaquinone in growth of *B. subtilis* (Taber, 1993).

The operon designation should reflect the primary metabolic defect

The *gerCA*, *gerCB* and *gerCC* genes ought to be renamed to reflect their biosynthetic role, as proposed by Koike-Takeshita *et al.* (1997). However, their suggestions of *heps-1*, *menG* and *heps-2* for *gerCA*, *gerCB* and *gerCC*, respectively, should be modified, to meet the standard rules of bacterial gene nomenclature, to *hepA*, *menG* and *hepB*. The *gerCC58* mutation would therefore be more correctly *hepB58*.

Is there a relationship between menaquinone levels and spore germination?

The *hepB58* mutation must leave some residual functionality, allowing sufficient synthesis of the essential isoprenoid for the cells to grow, but very slowly, and with altered morphology. The very limited growth of the *hepB58* mutant strain has precluded the measurement of menaquinone levels – it is impossible to generate a culture containing more than approximately 2×10^7 cells in total before suppressed derivatives take over the population. The *hepB58* mutant has a more extreme defect than a strain reported to contain <0.1% of the wild-type level of menaquinone (Farrand & Taber, 1973).

The frequency with which the mutation can be suppressed extragenically suggests that some other function(s) in the cell may be recruited to compensate for the defect in synthesis of the heptaprenyl side chain, although these suppressed derivatives are themselves abnormal – there is a clear defect in the placing of septa in the mutants, especially in strain AM677. It is unlikely that the suppression effect is due to overexpression of the naphthoquinone ring component of menaquinone, as growth of the unsuppressed mutant was not stimulated by supplementation with the naphthoquinone menadione at levels reported to allow growth of *men* mutants (Leatherbarrow, 1994). It is possible that accumulation of naphthoquinone precursors might in fact be toxic to the cell, and that one class of suppressors might be down-promoter mutations in *men* genes. However, the nature of the suppressors is not known; it

is even formally possible that there could be more than one suppressor in a strain, although the high frequency of occurrence of suppressed derivatives makes this less likely. Our attempts to clone the suppressors on the basis of their ability to complement the *hepB58* growth defect, using gene libraries in *phi105* carrying DNA from a *gerC58 sup* strain, yielded only clones carrying the *gerC* region (Leatherbarrow, 1994).

It is clear that GerC proteins are not directly concerned with the spore germination mechanism. The molecular basis of the septal abnormality and germination defect in the AM677 mutant remains to be explained, however, and it is still possible that an abnormality in menaquinone or isoprenoid biosynthesis may indirectly have an effect on membrane-associated events in germination. The morphology of the mutants suggests that processes in cell shape determination and septum formation are, again perhaps indirectly, sensitive to insufficiency of menaquinone and/or heptaprenyl isoprenoids.

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