The GerW Protein Is Not Involved in the Germination of Spores of Bacillus Species

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Abstract

Germination of dormant spores of Bacillus species is initiated when nutrient germinants bind to germinant receptors in spores’ inner membrane and this interaction triggers the release of dipicolinic acid and cations from the spore core and their replacement by water. Bacillus subtilis spores contain three functional germinant receptors encoded by the gerA, gerB, and gerK operons. The GerA germinant receptor alone triggers germination with L-Valine or L-alanine, and the GerB and GerK germinant receptors together trigger germination with a mixture of L-asparagine, D-glucose, D-fructose and KCl (AGFK). Recently, it was reported that the B. subtilis gerW gene is expressed only during sporulation in developing spores, and that GerW is essential for L-alanine germination of B. subtilis spores but not for germination with AGFK. However, we now find that loss of the B. subtilis gerW gene had no significant effects on: i) rates of spore germination with L-alanine; ii) spores’ levels of germination proteins including GerA germinant receptor subunits; iii) AGFK germination; iv) spore germination by germinant receptor-independent pathways; and v) outgrowth of germinated spores. Studies in Bacillus megaterium did find that gerW was expressed in the developing spore during sporulation, and in a temperature-dependent manner. However, disruption of gerW again had no effect on the germination of B. megaterium spores, whether germination was triggered via germinant receptor-dependent or germinant receptor-independent pathways.

Introduction

Bacillus species have two alternative life cycles. In the vegetative cycle with abundant nutrients, these organisms replicate their chromosome and divide by binary fission into two equivalent daughter cells [1]. However, in response to nutrient limitation, a morphologically distinct cell
A type called a spore is formed through a process termed sporulation [2]. Spores of Bacillus and Clostridium species are metabolically dormant with extreme resistance to environmental stresses, and are capable of surviving extreme temperatures, desiccation, chemical agents, and UV- and γ-radiation [3].

Spores are dormant and by themselves cannot cause deleterious effects. However, spores sense their environment and when specific signaling molecules, most often specific nutrients are again present, spores can return to life rapidly through germination. An early event in germination is the release from the spore core of large amounts of the 1:1 chelate of Ca2+ and dipicolinic acid (CaDPA) through inner membrane (IM) channels composed at least in part of SpoVA proteins. This is followed by hydrolysis of spore cortex peptidoglycan and expansion of the spore core. Finally metabolism and macromolecular synthesis convert the dormant spore into a growing cell in outgrowth [4]. Nutrients generally trigger spore germination through interactions with proteins called germinant receptors (GRs) located in spores’ IM. Bacillus spores most often have multiple GRs, each with a different specificity for a nutrient germinant or nutrient germinant mixture. GRs are generally encoded by tricistronic operons encoding GRs’ A-, B-, and C-subunits. The A and B subunits are likely integral IM proteins and the C subunit is a lipid-anchored peripheral IM protein [5,6]. By far the best-studied Bacillus species is Bacillus subtilis, and this species’ genome contains five tricistronic operons encoding GRs. The GerA GR responds to L-alanine or L-valine alone, while the GerB and GerK GRs are both required for germination with a mixture of L-asparagine plus D-glucose, D-fructose, and K+ ions (termed AGFK) [6,7]. The GRs encoded by the other two operons have no known function.

Another protein involved in triggering of spore germination is GerD, a peripheral IM lipoprotein. GerD colocalizes with GRs in a single cluster in dormant spores. These clusters represent a functional germination unit or “germinosome”, facilitating spores’ rapid and cooperative response to nutrients [8]. Recently, the GerW protein made in the developing spore was reported to be important in triggering of B. subtilis spore germination with L-alanine, as rates of L-alanine germination of GerW-deficient spores were >10-fold lower than those of wild-type spores [9]. In contrast, rates of AGFK germination of gerW-deficient spores were almost identical to those of wild-type spores [9]. In the current work, we have examined the effects of the GerW protein on rates of germination in spores of two Bacillus species and levels of germination proteins in B. subtilis spores. Surprisingly, the absence of the GerW protein had no significant effects on B. subtilis spore germination rates with either L-alanine or AGFK or the levels of germination proteins. B. megaterium QM B1551 gerW-deficient spores also germinated efficiently in response to nutrient and non-nutrient stimulants. Collectively, these results indicate that GerW has no role in the germination of spores of the two species examined in this work, and in all likelihood Bacillus spores in general.

Materials and Methods

B. subtilis strains used in this work are isogenic derivatives of strain PS832 (wild-type), a prototrophic laboratory derivative of strain 168 (Table 1). To obtain the gerW B. subtilis strain PS4389 most of the gerW coding sequence was replaced by a chloramphenicol resistance (Cm’) cassette as follows. The region between bp-124 to +115 relative to the gerW translation start (+1) was PCR amplified from B. subtilis PS832 DNA using primers containing BamHI and PstI sites (Start Forward and Start Reverse primers; all primer sequences are available upon request). The purified PCR product was digested with BamHI and PstI and ligated to a similarly digested modified pBluescript II KS plasmid that has a Cm’ cassette between EcoRI and EcoRV sites. The ligation reaction was used to transform Escherichia coli DH5α to ampicillin resistance (Amp’) giving plasmid pJCMI. The presence of the appropriate gerW fragment in
pJCM1, as well as in all other plasmid constructs was confirmed by PCR and restriction enzyme digestion. The region between bp +290 to +891 relative to the gerW translation start codon of the gerW gene coding and downstream region was PCR amplified from B. subtilis PS832 DNA using primers containing HindIII and KpnI sites (EndP Forward and EndP Reverse primers). The purified PCR product was digested with HindIII and KpnI, and ligated to HindIII and KpnI digested plasmid pJCM1. This ligation reaction transformed E. coli DH5α giving plasmid pJCM2. Plasmid pJCM2 transformed B. subtilis strain PS832 to Cmr giving strain PS4389 (gerW1). The expected genome structure in the gerW region of strain PS4389, as well as in the other B. subtilis gerW mutant strain described below, was confirmed by PCR and DNA sequencing (data not shown).

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Table 1. Bacterial strains and plasmids used in this study.

| Strains            | Relevant genotype, phenotype or description | Reference or source |
|--------------------|---------------------------------------------|---------------------|
| **Bacillus subtilis** |                                             |                     |
| PS832              | Wild-type prototroph                        | Lab strain          |
| PS4389             | gerW1 (encodes 38 aa of GerW) Cm’           | This work           |
| PS4399             | gerW2 (encodes 6 aa of GerW) Cm’           | This work           |
| **Bacillus megaterium** |                                         |                     |
| QM B1551           | Wild-type                                  | Pat Vary            |
| GC618              | gerU::pNFD13 Km’                          | 10                  |
| GC900              | gerW pHT-GerU+ Zn’ Er’                    | This work           |
| GC918              | gerW::pNFD13 Km’                          | This work           |
| GC919              | gerD::pNFD13 Km’                          | This work           |
| **Escherichia coli** |                                             |                     |
| DH5α               | Competent cells                             | Lab strain          |
| **Plasmids**       |                                             |                     |
| Modified pBluescript II KS | Amp’ Cm’                                | Lab plasmid         |
| pJCM 1             | gerW (-124 to +115) Amp’ Cm’               | This work           |
| pJCM 2             | Plasmid with ΔgerW1 Amp’ Cm’               | This work           |
| pJCM 3             | Plasmid with ΔgerW2 Amp’ Cm’               | This work           |
| pGEM-3Z            | Amp’                                        | Promega             |
| p7Z6               | Amp’ Zn’                                    | BGSC⁵               |
| pUCTV2             | Amp’ Ts replication                        | 11                  |
| pDONRtet           | Gateway entry plasmid Tc’                  | 12                  |
| pNFD13             | Vector to create lacZ fusions Km’          | 12                  |
| pUC-ΔgerW::Zn      | B. megaterium ΔgerW Amp’ Tc’ Zn’           | This work           |
| pHT-GerU*          | encodes GerU* GR genes Er’                 | 13                  |

*Abbreviations used are: ts, temperature sensitive; Amp’, ampicillin resistance (100 μg/ml); Cm’, chloramphenicol resistance (5 μg/ml); Er’, erythromycin resistance (1 μg/ml); Km’, kanamycin resistance (10 μg/ml); Tc’, tetracycline resistance (12.5 μg/ml); Zn’, zeocin resistance (20 μg/ml).

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transformed *E. coli* DH5α to Amp<sup>+</sup> giving plasmid pJCM3. This plasmid transformed *B. subtilis* strain PS832 to Cmr giving strain PS4399 (*gerW*<sup>2</sup>).

The *B. megaterium* *gerW* strain (GC900) was constructed using PCR to initially amplify a 1075 bp DNA fragment encompassing the *gerW* ORF (BMQ_4796), using primers with 5′-EcoRI restriction sites. The purified EcoRI digested PCR product was ligated with EcoRI linearised pGEM-3Z, giving plasmid pGEM-*gerW* in *E. coli*. An inverse PCR using pGEM-*gerW* as template and with appropriate primers incorporated a 60 bp deletion towards the middle of the *gerW* ORF and with 5′-NcoI sites. The purified inverse PCR product was ligated with a zeocin resistance (Znr) cassette (excised from plasmid p7Z6). Plasmid pGEM-*ΔgerW*::Zn was isolated from *E. coli*, and used as a template for a PCR reaction amplifying the Δ*gerW*::Zn cassette, using primers adding 5′-MfeI sites. This cassette was digested with MfeI and ligated with EcoRI digested pUCTV2 [10], giving plasmid pUC-Δ*gerW*::Zn. This plasmid was used to transform *B. megaterium* protoplasts to tetracycline resistance (Tcr), using the polyethylene-glycol (PEG)-mediated procedure described previously [14]. A colony that had replaced the native *gerW* locus with the Δ*gerW*::Zn cassette via allelic exchange, conferring a Tcr Znr phenotype, was isolated after repeated sub-culture of single-crossover cells at 42°C in the absence of antibiotic. PCR was used to verify the correct construction of the mutant strain. These analyses revealed that the native pBM700 plasmid, which carries the GerU GR structural genes, had been excised during mutant construction procedures. To circumvent this loss, plasmid pHT-GerU/C3, which is stable in *B. megaterium* at low copy number, and which encodes functional gerU GR genes plus regulatory sequences [13], was introduced by PEG-mediated transformation into the *B. megaterium* *gerW* strain.

A *B. megaterium* strain bearing a transcriptional fusion between the *gerW* ORF and *E. coli* lacZ was constructed essentially as described previously, using the Gateway cloning technique [10,12]. The *gerW* ORF was amplified by PCR using primers designed to introduce 5′ attB sites, and then purified and cloned into pDONRtet using the Gateway BP reaction mix (Life Technologies, Paisley, UK). Purified intermediate plasmid DNA was isolated from transformed *E. coli* and then employed in a Gateway LR reaction to create a pNFD13 derived *gerW*-lacZ plasmid. *B. megaterium* QM B1551 was transformed to kanamycin resistance (Kmr) with this plasmid using the PEG-mediated transformation method. A colony that had undergone homologous recombination to create a *gerW*-lacZ fusion strain (GC918) was isolated after incubation at 42°C, and its correct construction was verified by PCR. Lysates of spores (10<sup>9</sup>) of strain GC918 were used in subsequent β-galactosidase assays as described previously [10] along with the same amounts of spores of *B. megaterium* strains carrying gerU-lacZ (strain GC618) or gerD-lacZ (strain GC919) for comparative purposes. The *B. megaterium* *gerD*-lacZ strain (*gerD* is encoded by BMQ_0176) was created in a similar manner to the *gerW*-lacZ strain.

Spores of *B. subtilis* strains were routinely prepared at 37°C on 2x Schaeffer’s-glucose plates without antibiotics as described previously [15,16]. After incubation for ~ 5 d, the spores were scraped from plates, and washed with water by repeated centrifugation with intermittent sonication treatment. In one experiment, *B. subtilis* spores were prepared in liquid Schaeffer Sporulation medium as described previously [17], and spores were purified as described above. *B. megaterium* spores were prepared at 30°C in supplemented nutrient broth (SNB), and purified by repeated rounds of centrifugation and washing with ice-cold deionized water as described previously [11]. All spore preparations used in this work were free (~ 95%) from growing or sporulating cells and germinated spores as determined by phase-contrast microscopy.

*B. subtilis* spores were germinated following heat activation (30 min; 75°C) and cooling on ice for 10 min. Spores at an optical density at 600 nm (OD<sub>600</sub>) of 0.5 were germinated for 2.5 h at 37°C in 200 μl of 25 mM K-Hepes buffer (pH 7.4) with various concentrations of L-alanine.
or 10 mM L-valine, or with 10 mM of each AGFK component. All germination experiments were carried out in duplicate. Spore germination was routinely monitored by measuring the release of the spores’ large depot of DPA by inclusion of 50 μM TbCl₃ in germination mixtures and measuring Tb-DPA fluorometrically in a multiwell plate reader as described previously [18]. Germination of spores with a coat defect due to chemical decoating is very strongly inhibited by TbCl₃ [19]. Consequently, decoated spores prepared as described previously [19] were germinated as described above, but without Tb³⁺ present from the initiation of germination. Instead, at various times after germination was initiated, aliquots of the germinating culture were centrifuged in a microcentrifuge, the supernatant fluid made 50 μM in TbCl₃ and Tb-DPA fluorescence was measured as described previously [19,20]. Spore germination was also routinely monitored at the end of germination incubations by phase-contrast microscopy. The total amount of DPA present in spores was assessed by Tb-DPA fluorescence after DPA had been released from spores by boiling as described previously [18,19]. In some experiments, spore germination was also monitored by the fall in optical density of spore cultures as described previously [9]. All *B. subtilis* GR-dependent spore germination experiments were carried out on multiple independent spore preparations prepared in different laboratories with essentially identical results. *B. subtilis* spores that were not heat-activated were also germinated with GR-independent germinants as follows: i) at 50°C in 25 mM K-Hepes buffer (pH 7.4) and 1 mM dodecylamine, with spores at an OD₆₀₀ of 2; and ii) at 23°C in 60 mM CaDPA made to pH 7.5 with Tris base, with spores at an OD₆₀₀ of 2 [5]. Germination of spores with CaDPA and dodecylamine was monitored by examining ~ 100 individual spores at various times by phase-contrast microscopy [7].

Outgrowth of heat-activated *B. subtilis* spores was carried out at 37°C in 2x yeast tryptone (2x YT) medium containing 5 mM L-alanine and (per L) 16 g tryptone, 10 g yeast extract, 5 g NaCl. Spores were added to an OD₆₀₀ of 0.5 and the OD₆₀₀ of cultures were followed over time [21]. Finally, to determine spore viability, both PS832 and PS4389 spores at an OD₆₀₀ of 1.0 were heat activated, cooled, spores spotted on LB medium plates with the appropriate antibiotic, plates incubated for 24 h at 37°C and colonies were counted [21].

*B. megaterium* spore germination was followed by monitoring the absorbance at 600 nm of heat-shocked (60°C, 10 min) spores suspended at an OD₆₀₀ of ~ 0.4 in 5 mM Tris-HCl, pH 7.8, plus 0.1–25 mM glucose or proline. Germination assays were conducted in triplicate, in 96-well plates incubated at 30°C in a PerkinElmer EnVision-Xcite multilabel plate reader fitted with a 600 nm photometric filter. Similar absorbance-based assays were conducted with non-heat shocked spores incubated in either 60 mM CaDPA at 30°C or 1 mM dodecylamine at 40°C. All experiments were conducted with at least two independently prepared batches of spores with essentially identical results. Spore viability was assessed by plating serial dilutions of heat-shocked spores on solid LB medium plates which were incubated at 30°C overnight before determining the percent viability of *gerW* spores compared to wild-type spores in which 1 OD₆₀₀ unit is equal to ~ 10⁸ CFU ml⁻¹.

Levels of GR, GerD and SpoVAD proteins, which are present largely or completely in spores’ IM [22–26], were measured in lysates of spores by western blot analysis using polyclonal rabbit antisera against the various proteins and a secondary antisera as described previously [7,21,27,28]. In brief, spores were decoated, ruptured by lysozyme digestion, and sonicated briefly with glass beads present to obtain spore lysates. Aliquots of the lysates were then run on SDS-polyacrylamide electrophoresis (SDS-PAGE) and the gels were stained with Coomassie blue to determine how much of the lysates needed to be run to load equal amounts of protein. Equal amounts of the lysates were run on SDS-PAGE, proteins transferred to a polyvinylidene-difluoride (PVDF) membrane, and antigens on the membrane were detected as described...
Results

It was reported recently that the rate of L-alanine germination of gerW B. subtilis spores was \( > 10 \)-fold slower than that of the wild-type spores, although AGFK germination of gerW spores was normal [9]. To further investigate the effect of a gerW mutation on spore germination, we replaced the great majority of the gerW coding sequence in B. subtilis strain PS832 (wild-type) by a Cm\(^\prime\) cassette giving B. subtilis strains PS4389 (gerW1) (retains 38 N-terminal GerW aa) and PS4399 (gerW2) (retains 6 N-terminal GerW aa). Strain PS4389 was generated first, and when spores of this strain were found to germinate normally with L-alanine, we also prepared strain PS4399 to eliminate the possibility that the N-terminal 38 aa of GerW were sufficient for its function. Multiple germination experiments with wild-type and PS4389 and PS4399 spores indicated that rates of gerW mutant spores’ germination with AGFK were essentially identical to those of wild-type spores (Table 2), as reported previously [9]. Surprisingly the PS4389 and PS4399 spores also germinated like wild-type spores with either L-alanine or L-valine via the GerA GR (Table 2). Based on these experiments, there were no statistically significant differences between rates of wild-type and gerW spore germination with saturating levels of different nutrient germinants. In addition, wild-type and gerW spores prepared in liquid Schaeffer’s-glucose sporulation medium or in liquid Spizizen’s minimal medium [28,29] also germinated essentially identically with L-alanine (data not shown). Chemically decoated wild-type and PS4389 spores prepared as described previously [19] also germinated identically with L-alanine (data not shown), and the intact gerW spores’ germination with different L-alanine concentrations was also essentially identical to that of wild-type spores (Fig. 1).

In addition to nutrient germinants that trigger spore germination via GRs, we also measured the germination of wild-type and gerW B. subtilis spores with CaDPA and dodecylamine, two agents that trigger spore germination without GR involvement. Again, we observed no significant difference between gerW and wild-type spores in their germination with either CaDPA or dodecylamine (data not shown).

We also measured the ability of wild-type and gerW spores to return to active growth after spore germination in a complete nutrient medium with L-alanine added to ensure rapid spore germination, and observed that both types of spores had similar rates of outgrowth (Fig. 2). In addition, the viability of wild-type and gerW spores was essentially identical when heat activated spores were spotted on LB medium plates (data not shown).

The levels of GR subunits, GerD and SpoVAD proteins were also determined in lysates of spores by western blot analysis. Some modest differences were observed between levels of these

### Table 2. Rates of germination of wild-type and gerW B. subtilis spores with L-alanine, L-valine or AGFK.

| Germinants: Spores | 10 mM L-alanine | 10 mM L-valine | 10 mM AGFK |
|-------------------|----------------|----------------|------------|
| PS832 (wild-type) | 2.6            | 2.5            | 0.75       |
| PS4389 (gerW1)    | 2.4            | 2.3            | 0.8        |
| PS4399 (gerW2)    | 2.5            | 2.4            | 0.78       |

Spores of various strains were germinated with saturating levels of different germinants, and spore germination was monitored by DPA release as described in Methods. Rates of spore germination were determined from plots of DPA release as a function of time. Values shown are averages of values in two independent experiments with the same spore preparations that differed by < 12%.

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proteins in PS832 (wild-type), PS4389 (gerW1) and PS4399 (gerW2) spores in some experiments, although these were generally ≤ 2-fold (Fig. 3). In addition, when blots from multiple replicate experiments were compared, there were on average ≤ 25% differences in the intensities of different germination proteins from wild-type and gerW spores (data not shown). In general, the similar levels of GR proteins and GerD in wild-type and gerW spores was consistent with the similar rates of germination of wild-type and gerW spores with all GR-dependent germinants (Table 2).

Bioinformatic analyses revealed that the B. megaterium QM B1551 genome also contains a single orthologue of B. subtilis gerW, encoded at locus BMQ_4796. The predicted protein shares 70% amino acid identity with its B. subtilis counterpart, with most variance occurring in an ~20 aa sequence towards the central region of the protein (data not shown). Analysis of lysates from disrupted purified B. megaterium gerW-lacZ spores revealed β-galactosidase activity, levels of which were dependent upon the temperature of sporulation (Table 3). Spores bearing a lacZ fusion to the GR gene, gerUA, also showed temperature dependent levels of expression as reported previously [10]. Collectively, the β-galactosidase assays indicate that GerD is expressed at a higher level than GerW or GerU at 22°C, while GerW and GerD are expressed at similar levels at 30 and 37°C.
Spores were prepared at three different temperatures for each strain as described in Methods, and the β-galactosidase activity in lysates from $10^9$ spores of each strain was measured in triplicate. Similar values were obtained with at least one other independently prepared batch of spores. Values have been corrected for wild-type spore background levels of fluorescence which were always $< 500$ relative fluorescence units. Standard deviations for all values were $< 15\%$.

Overall, in *B. megaterium*: i) the expression of GerW-LacZ in spores; ii) the identification of a putative σF promoter sequence upstream of BMQ_4796 with sequence homology to the *B. subtilis* gerW promoter region and approximately the same spacing between the two promoters and the translation start sites [9] (data not shown); and iii) the lack of detection of *gerW* mRNA in vegetative cells by RT-PCR (data not shown) are all consistent with forespore-specific expression of *gerW*, as observed previously in *B. subtilis* [9]. However, there were differences in the expression of the *gerW*, *gerD* and *gerUA* genes as a function of sporulation temperature (see Discussion).

In order to also investigate the role, if any, of GerW in the germination of *B. megaterium* spores, the *gerW* gene was disrupted with a Zn cassette by allelic exchange, which introduced a short deletion in the *gerW* ORF. The resultant strain was found to have excised plasmid pBM700 during strain construction, hence plasmid pH7-GerU+ was introduced by PEG-mediated transformation to restore the gerU GR genes and their regulatory sequences. The *B. megaterium* gerW pH7-GerU+ strain (GC900) sporulated normally (data not shown) and the spores were then examined for germination efficiency in response to nutrient and non-
nutrient stimuli. *B. megaterium* gerW spores were observed to germinate essentially with an identical efficiency to wild-type spores in response to either glucose or proline, including at sub-optimal germinant concentrations (Fig. 4A,B; Table 4). Similarly, spores with disrupted gerW displayed essentially wild-type germination with the GR-independent germinants CaDPA and dodecylamine (Fig. 4C,D). Additionally, *B. megaterium* GerK+ spores, in which only the gerK GR operon is intact, germinated normally when plated on solid LB medium whether gerW was disrupted or not, as did the wild-type spores containing only the gerK GR operon (data not shown).

**Table 3. β-Galactosidase activity from gerW-, gerD- and gerUA-lacZ transcriptional fusions in *B. megaterium* spores prepared at different temperatures.**

| Strain | Genotype   | β-Galactosidase activity (relative fluorescence units) |
|--------|------------|-------------------------------------------------------|
|        |            | 22°C | 30°C | 37°C |
| GC918  | gerW-lacZ  | 9.5E+03 | 3.3E+04 | 2.9E+04 |
| GC919  | gerD-lacZ  | 3.6E+04 | 3.6E+04 | 3.0E+04 |
| GC618  | gerU-lacZ  | 4.2E+03 | 3.2E+03 | 7.9E+02 |

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Fig 4. Germination of wild-type and gerW B. megaterium spores with various germinants. B. megaterium spores were germinated in 5 mM Tris-HCl, pH 7.8 plus (A) 10 mM glucose, (B) 10 mM proline, (C) 60 mM CaDPA, or (D) 1 mM dodecylamine. Spores were heat shocked at 60°C for 10 min prior to incubation in glucose or proline-supplemented germination buffer, but not for CaDPA or dodecylamine germination, and the progress of spore germination was monitored as described in Methods. Symbols used are: (○), wild type spores; and (■), gerW spores.

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Table 4. Maximum rates of germination of wild-type and gerW B. megaterium spores in response to varying concentrations of glucose or proline.

| Germinant: | Glucose mM | Proline mM |
|------------|------------|------------|
|            | 0.1  | 1   | 10  | 25  | 0.1  | 1   | 10  | 25  |
| Strain     |       |       |     |      |       |       |     |      |
| QM B1551 (wt) | 0.67 | 3.5  | 15.3| 14.1 | 0.68 | 3.5  | 10.4| 10.7 |
| GC900 (gerW) | 0.59 | 3.5  | 14.6| 11.8 | 0.50 | 2.3  | 11.1| 11.2 |

B. megaterium spores were heat shocked and incubated in 5 mM Tris-HCl, pH 7.8 plus the designated concentration of glucose or proline, and germination was monitored by measuring OD_{600} loss as described in Methods. The maximum % OD_{600} loss per min was calculated from plots of OD_{600} versus time. Values shown are the averages of results from three independent experiments conducted with the same spore preparations, and the standard deviations were <15% of the averages.

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Discussion

Clearly, the major conclusion from the current work is that GerW is not essential for the germination of spores of two Bacillus species, either B. subtilis spores with L-alanine or AGFK, and B. megaterium spores with either GR-dependent or GR-independent germinants. The obvious question based on the new findings is why GerW was previously found to be essential for normal B. subtilis spore germination with L-alanine via the GerA GR [9]. The answer to this question is not completely clear. However, it seems most likely that the original transformation to generate a gerW deletion mutation used a laboratory B. subtilis 168 strain [30], while the wild-type strain was strain 1A1 from the Bacillus Genetic Stock Center (BGSC). Unfortunately, the B. subtilis laboratory 168 strain used in the initial communication now appears to have had one or more mutations that significantly reduce its spores’ germination with L-alanine even without deletion of gerW. In contrast, spores of the PS832 168 strain germinate very rapidly with L-alanine.

In addition to the major conclusion discussed above, there are several other notable points pertinent to the current work as follows. 1) Expression of gerW in B. megaterium and B. subtilis is forespore-specific during sporulation, as is that of many genes involved in spore-specific properties. However, in B. megaterium, gerW expression displayed a rather different response to sporulation temperature than did two other forespore-specific genes, gerD and gerUA. Differences in the regulation of these three genes as a function of temperature may reflect differences in the RNA polymerase σ factors that recognize these genes, σF for gerW and σG for gerD and gerUA, as well as likely additional regulators of σG-dependent genes such as SpoVT [14,31,32]. 2) While GerW does not play an obvious role in Bacillus spore germination, at least in B. megaterium and B. subtilis, an important question is what does this protein do. The GerW amino acid sequence suggests the protein is soluble, and GerW is present in the soluble fraction of disrupted B. subtilis spores [9]. In addition, a Blast search of the NCBI microbial genomes database readily detects obvious GerW homologs in the spore forming members of the order Bacillales, but also in the order Clostridiales. The latter information, as well as that gerW is expressed only in the developing spore suggests that GerW plays some important role in dormant spore properties. However, this role remains to be determined.

Author Contributions

Conceived and designed the experiments: PS SG HT GC APV. Performed the experiments: JCM SG NW RK. Analyzed the data: APV RK HT GC PS JCM. Contributed reagents/materials/analysis tools: RK. Wrote the paper: HT GC PS JCM.

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