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Investigating the Functional Hierarchy of *Bacillus megaterium* PV361 Spore Germinant Receptors

Srishti Gupta, Fatma Işık Üstok, Christian L. Johnson,* David M. D. Bailey, Christopher R. Lowe, Graham Christie

Department of Chemical Engineering and Biotechnology, Institute of Biotechnology, University of Cambridge, Cambridge, United Kingdom

Spores of *Bacillus megaterium* QM B1551 germinate rapidly when exposed to a number of single-trigger germinant compounds, including glucose, proline, leucine, and certain inorganic salts. However, spores of strain PV361, a plasmidless QM B1551 derivative that lacks the GerU germinant receptor (GR) responsible for mediating germination in response to single-trigger compounds, can germinate efficiently when incubated in nutritionally rich media, presumably via activation of additional germinant receptors. In this work, we have identified five chromosomally encoded GRs and attempted to characterize, by mutational analysis, germinant recognition profiles associated with the respective receptors in strain PV361. Of strains engineered with single GR insertion-deletions, only GerK-null spores displayed significant defective germination phenotypes when incubated in 5% (wt/vol) beef extract or plated on rich solid medium. Cumulative decreases in viability were observed in GerK-null spores that also lacked GerA or $GerA_2$, indicating that these GRs, which exerted little effect on spore germination when disrupted individually, have a degree of functionality. Unexpectedly, an efficient germination response to combinations of germinants was restored in $GerA^+$ spores, which lack all other functional GRs, providing evidence for negative cooperativity between some GRs within the spore. Tetrazolium-based germinative assays conducted with purified spores indicated that these newly characterized *B. megaterium* GRs are cognate for a wide and chemically diverse range of germinant molecules, but unlike GerU, can only be induced to trigger germination when stimulated by at least two different germinants.

A defining characteristic of members of the *Bacillus* and *Clostridium* genera is the ability to form environmentally resistant and metabolically dormant endospores (spores) upon nutrient starvation. This strategy allows the cell to persist in the environment for several years, protected against physical, chemical, and biological challenges, until environmental conditions are once again conducive to vegetative growth.

Spores of most species of the bacilli and clostridia employ a unique class of sensory proteins, the GerA receptor family, to monitor the nutritional status of their surroundings (1, 2). When activated by specific germinant molecules, typically amino acids, monosaccharides, nucleosides, and/or salts, the germinant receptors (GRs) irreversibly trigger the spore germination process, resulting in the release of minerals and solutes from the spore core and degradation of the peptidoglycan cortex and spore coat. Complete hydration of the cellular protoplast following dissolution of the cortex permits metabolism to be reinitiated, which results ultimately in the emergence of a new vegetative cell (3–5).

Despite recent structural and architectural insights into the proteins that comprise the GRs (6, 7), the precise function of the receptors at the biochemical level has remained elusive. Distant homology between some germinant receptor B proteins and single-component amino acid transporters (8), allied to the movement of small molecules from the spore core across the spore inner membrane during the earliest stages of germination (9), has led to speculation that GRs may have a transporter-type function, although this has not been ascertained experimentally. Similarly, atomic-resolution structural information, particularly with respect to the integral membrane protein components of the receptor complex, is not sufficient to begin to elucidate either the molecular mechanisms that underpin germinant recognition and binding or the presumed conformational changes that initiate the germination cascade.

What has become evident from several molecular genetic- and kinetic-based analyses, combined with the now widespread genome sequence coverage of the *Bacillus* genus, is that spores of most members of the bacilli employ several GR orthologues (usually between 2 and 7 per genome), often with partially overlapping germinant recognition profiles, to align or maximize initiation of germination in the appropriate environmental niches (1, 4, 10). As a class of environmental transducers, however, spore GRs seem to be relatively insensitive, responding optimally *in vitro* to germinant concentrations in the low mM range, although μM sensitivity may be achieved in response to combinations of germinants (5). This apparent lack of responsiveness to individual germinants, allied with the presence of spore coat-localized enzymes that in some species catalyze the conversion of some germinants to nonreactive isomers, is perhaps a strategy to minimize the likelihood of spores germinating in an environment that is not nutritionally amenable to vegetative growth.

In evolutionary terms, *Bacillus megaterium* is a key member of the *Bacillus* genus, forming a deep-rooted phylogenetic branch that is genetically and phenotypically distinct from the *Bacillus subtilis* and *Bacillus cereus* groups (11). Strains and species from all three groups have for several decades been employed in numerous studies on spore germination; however, whereas the different classes of GRs have been examined *in vitro* by mutational analyses in *B. subtilis* (2, 12), *B. cereus* (13, 14), and *Bacillus anthracis* (6,

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Address correspondence to Graham Christie, gc301@cam.ac.uk.

* Present address: Christian L. Johnson, Universitätsklinikum Regensburg, Klinik und Poliklinik für Chirurgie, Regensburg, Germany.

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TABLE 1 *B. megaterium* strains used in this work

Strain	Relevant phenotype or genotype[a]	Source or reference
QM B1551	Wild type	P. S. Vary
PV361	Plasmidless derivative of QM B1551; Δ*gerU*	P. S. Vary
PV361 derivatives		
GC601	Δ*gerK* Km^r	This study
GC602	Δ$gerK_2$ Er^r	This study
GC603	Δ$gerK_3$ Km^r	This study
GC604	Δ*gerA* Cm^r	This study
GC605	Δ$gerA_2$ Sp^r	This study
GC606	Δ*gerK* Δ$gerK_2$ Km^r Er^r	This study
GC607	Δ*gerK* Δ*gerA* Km^r Cm^r	This study
GC608	Δ*gerK* Δ$gerA_2$ Km^r Sp^r	This study
GC609	Δ*gerA* Δ$gerA_2$ Km^r Sp^r	This study
GC610	$GerA^+$ (Δ*gerK* Δ$gerK_2$ Δ$gerA_2$) Km^r Er^r Sp^r	This study
GC611	$GerA_2^+$ (Δ*gerK* Δ$gerK_2$ Δ*gerA*) Km^r Er^r Cm^r	This study
GC612	$GerK^+$ (Δ$gerK_2$ Δ*gerA* Δ$gerA_2$) Er^r Cm^r Sp^r	This study
GC613	$GerK_2^+$ (Δ*gerK* Δ*gerA* Δ$gerA_2$) Km^r Cm^r Sp^r	This study
GC614	Ger null (Δ*gerK* Δ$gerK_2$ Δ*gerA* Δ$gerA_2$) Km^r Er^r Cm^r Sp^r	This study
GC615	Ger-null pHT-*gerU** Km^r Er^r Cm^r Sp^r Tc^r	This study
GC431	pHT-*gerU** Er^r	17
GR-*lacZ* fusion strains		
GC618[b]	Δ*gerU*::pNFd13 Km^r	This study
GC619	Δ*gerK*::pNFd13 Km^r	This study
GC620	Δ$gerK_2$::pNFd13 Km^r	This study
GC621	Δ$gerK_3$::pNFd13 Km^r	This study
GC622	Δ*gerA*::pNFd13 Km^r	This study
GC623	Δ$gerA_2$::pNFd13 Km^r	This study

[a] Abbreviations: Km^r, kanamycin resistance (5 μg/ml); Sp^r, spectinomycin resistance (100 μg/ml); Cm^r, chloramphenicol resistance (5 μg/ml); Er^r, erythromycin resistance (1 μg/ml); Tc^r, tetracycline resistance (12.5 μg/ml); *gerU**, receptor operon comprising *gerUA*, *gerUC*, and *gerVB*.
[b] This strain was constructed in the QM B1551 background.

15), detailed analyses of *B. megaterium*'s GRs, GerU apart (16–19), have not been conducted. Hence, the objective of the present work was to characterize the expression, function, and germinant recognition profiles associated with novel *B. megaterium* spore germinant receptors.

The results provide evidence for a potential functional hierarchy of germinant receptors in *B. megaterium* PV361 and, by inference, strain QM B1551, with GerU being shown to be capable of responding independently of all other receptors to single germinant compounds. Two other GRs, GerK and GerA, can also independently stimulate efficient germination responses, but only in response to at least binary mixtures of germinants. Of the remaining GRs, neither the $GerA_2$ nor $GerK_2$ receptors appear capable of independently stimulating efficient germinative responses, although the $GerK_2$ receptor in particular is shown to exert a strong inhibitory effect on the function of other spore GRs.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Bacillus megaterium* strains employed in this study (Table 1) are isogenic with strain PV361, a plasmidless derivative of the wild-type QM B1551 strain that lacks the plasmid-borne GerU receptor that stimulates germination responses to single-trigger germinants. *B. megaterium* strains were routinely cultured in LB medium at 30°C, containing antibiotics where appropriate. *Escherichia coli* Top10 (Life Technologies, Ltd., Paisley, United Kingdom) cells were used for plasmid construction and propagation and were cultured routinely in LB medium at 37°C supplemented with appropriate antibiotics.

Construction of *B. megaterium* GR mutant strains. Germinant receptor loci were disrupted by introducing antibiotic resistance genes, via allelic exchange, into the first structural gene of the respective operons, which in all cases encoded the receptor A subunit. Despite numerous attempts, the superior markerless mutagenesis method used to delete GRs in other species (6) has thus far failed to yield *B. megaterium* mutant strains in our laboratory. To exemplify the allelic-exchange technique employed in this study, a plasmid designed to disrupt *gerKA* (BMQ_1270) was constructed by PCR amplification of an ~1.5-kb fragment of the *gerKA* open reading frame (ORF), using primers with MfeI and HindIII restriction sites at the 5′ ends. The PCR fragment was subsequently purified, digested, and ligated with pGEM-3Z (Promega, Southampton, United Kingdom) restricted with EcoRI and HindIII, and plasmid pGEM-*gerK′* was isolated from the transformant *E. coli*. An inverse PCR was then conducted using pGEM-*gerK′* as a template, introducing an ~200-bp deletion into the cloned locus, before blunt-end ligation with a kanamycin resistance cassette excised from plasmid pDG792 (20). Plasmid pGEM-Δ*gerKA*::Km was subsequently isolated from transformant *E. coli* and then used as a template to PCR amplify the Δ*gerKA*::Km cassette, introducing MfeI sites at the 5′ ends, before digestion and ligation with EcoRI-restricted pUCTV2 (21) to yield pUCTV-Δ*gerKA*::Km. This plasmid was introduced into *B. megaterium* PV361 via polyethylene glycol (PEG)-mediated protoplast transformation (19). A Tc^s Km^r transformant, which had undergone double homologous recombination to introduce an insertion and deletion at the *gerKA* locus, was isolated after incubation of several single-crossover colonies at 42°C in the absence of antibiotics. Correct construction of strain GC601 was confirmed by PCR and sequencing. A similar strategy was used to introduce insertions and deletions at the remaining four GR operons carried on the *B. megaterium*

PV361 chromosome, in order to create a series of single and multiple GR-null mutant strains. The sequences for all primers used in this work are available upon request.

Construction of receptor-*lacZ* transcriptional fusions. Spores bearing transcriptional fusions between N-terminal fragments of GR A proteins and LacZ were constructed essentially as described previously (15). First, PCR was used to amplify DNA fragments that encoded the first ~300 codons of the respective GR A subunits. Gateway cloning techniques (Life Technologies, Ltd., Paisley, United Kingdom) were then used to construct pDONRtet entry plasmids and finally pNFd13-derived destination plasmids (15). These plasmids, which are designed to place LacZ (fused in-frame at the N-terminal end with the first 300 residues of the GR A protein) under the control of the respective germinant receptor promoter regions, were introduced to *B. megaterium* via PEG-mediated protoplast transformation. Single-crossover strains that had undergone homologous recombination to integrate the plasmids into the chromosome at the cloned locus were isolated after incubation on solid LB medium containing kanamycin (5 μg/ml) at 42°C. Strain construction was verified by PCR.

Spore preparation. *B. megaterium* spores were prepared by inoculating 200 ml of supplemented nutrient broth (SNB) (22) with 0.5 ml of mid-log-phase LB culture and incubated in 2-liter baffled flasks at 22°C (unless otherwise indicated) for 96 h. Spores were harvested and purified by repeated rounds of centrifugation (4,300 × *g* for 7 min at 4°C) and washing in sterile ice-cold deionized water until the spore pellet was observed by phase-contrast microscopy to be free of cellular debris and germinated spores. Purified spores were stored on ice at an optical density at 600 nm (OD_{600}) of ~50.

β-Galactosidase activity measurement. Germinant receptor expression levels were determined indirectly by quantifying the enzymatic activity of β-galactosidase in lysates extracted from mature spores (23). Typically, spores (~10^9) were resuspended in 0.6 ml Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol [pH 7.0]) and then disrupted with a FastPrep FP120 bead beater (Fisher Scientific, Loughborough, United Kingdom). 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) (0.2 ml [40 μg/ml]) was added to recovered spore lysates in 1.5-ml tubes, which were then incubated at 30°C for 40 min. Reactions were terminated by the addition of 0.4 ml 1 M Na_2CO_3. Fluorescence measurements were recorded in triplicate using a Tecan Infinite-200 series monochromatic plate reader with excitation and emission filters set to 365 nm and 450 nm, respectively. Differences in expression levels of the various GRs were analyzed for their significance via two-tailed Student *t* tests and one-way analysis of variance (ANOVA) analyses where appropriate.

Spore germination and viability assays. Spore germination in liquid medium was assessed in 96-well plates by adding 10 μl of heat-shocked (80°C, 30 min) spores to 190 μl of 5% (wt/vol) beef extract (Oxoid, Ltd., Basingstoke, United Kingdom), preheated to 37°C. The OD_{600} of spore suspensions was ~0.4. Plates were sealed with adhesive film to minimize evaporative losses, and then incubated in a Perkin-Elmer EnVision-Xcite multilabel plate reader fitted with a 600-nm photometric filter. Plates were subject to a 10-s period of orbital agitation every minute followed by an absorbance measurement, with experiments running typically for 90 min. Experiments were conducted in triplicate, with at least two or more independent spore preparations, and the significance of differences in germinative responses was evaluated in some experiments by one-way ANOVA. Germination progress was also monitored routinely by phase-contrast microscopy.

Spore viability was determined by pipetting 10-μl aliquots of serially diluted suspensions of heat-shocked spores (OD_{600} of 1 [~10^8 spores/ml]) onto LB agar plates. Plates were incubated for 16 to 24 h, and then colonies were counted to quantify the abilities of the various strains to initiate and complete spore germination.

Identification of germinants. Compounds that could either singly or in combination stimulate germination were screened and identified by employing a 2,3,5-triphenyltetrazolium chloride (Tzm)-based assay. Colorless Tzm is reduced to a red formazan derivative by enzymes that are activated during spore germination (23), providing a convenient assay for spore germination that does not require vegetative outgrowth and does not suffer from spore clumping or evaporative loss issues that can interfere with optical density measurements in longer-term liquid medium assays. Essentially, 2-μl aliquots of heat-shocked (80°C, 30 min) purified spores (OD_{600} of 40) were pipetted in triplicate onto sterile filter paper discs. Filter papers always included aliquots of parental PV361 spores, providing a baseline response for the germinants being tested, plus several mutant strains, whose germination properties were examined concurrently. Spore-loaded filter papers were then transferred with forceps to petri dishes containing 20 ml bacteriological agar (Oxoid, Basingstoke, United Kingdom) (1.2% [wt/vol]) containing 5 mM Tris-HCl (pH 7.5), 50 μg/ml Tzm, and 10 mM each putative germinant. Plates were incubated in the dark at 37°C for up to 24 h and checked periodically for the development of the coloration that indicates spore germination, with the apparent intensity of the coloration recorded photographically. Consistency of germination responses was assessed by conducting Tzm assays with at least two independently prepared batches of spores for each strain. Potential germinant compounds were of analytical grade (typically >98% purity) (Sigma-Aldrich, Dorset, United Kingdom).

Bioinformatic analyses. Putative spore germinant receptor loci were identified by conducting translated BLAST (tblastn) searches against the *B. megaterium* genome, using sequence analysis utilities provided on the National Science Foundation *B. megaterium* genome project website (http://www.bios.niu.edu/b_megaterium/). Protein sequences were aligned using ClustalW and then imported into the MEGA 4.0 program (24) to construct neighbor-joining trees. The latter were employed to assist with nomenclature assignment for receptor loci according to published guidelines (25).

RESULTS

Identification and expression of genes encoding *B. megaterium* GRs. The presence of spore GRs in addition to the well-characterized plasmid-borne GerU receptor in *B. megaterium* QM B1551 has been suspected since pre-genome-era studies conducted by Vary and coworkers (26) and by work conducted in the corresponding author's laboratory (18). These studies revealed that spores of strain PV361, a QM B1551 derivative cured of all seven native plasmids and which therefore lacks the GerU receptor, retained the ability to germinate efficiently in several nutritionally rich undefined media. Work conducted in the present study revealed that efficient germinative responses (>95%, as adjudged by phase-contrast microscopy, and the loss of ~60% of initial OD_{600} values within 40 min of exposure to germinants) could most consistently be achieved with PV361 spores when incubated with solutions of 5% (wt/vol) beef extract (Fig. 1) (data not shown). Furthermore, these preliminary studies revealed that the temperature of sporulation appeared to influence markedly the germination phenotypes of resultant spores, with spores prepared from cultures sporulated at 22°C showing a significantly ($P \leq 0.0001$) more efficient germinative response in beef extract than spores cultured at 30 and 37°C, respectively, with the latter showing the least efficient response (Fig. 1).

In an attempt to gain insight into the genetic and/or molecular basis for these observations, a series of translated BLAST (tblastn) searches were conducted against the *B. megaterium* QM B1551 genome, using the GerU and GR sequences from other *Bacillus* species as seeds. The outputs from these analyses revealed that the *B. megaterium* QM B1551 genome encodes six GerA-type receptors, five of the genes for which are located on the chromosome, while *gerU* is located on pBM700. Structural genes for each GR are

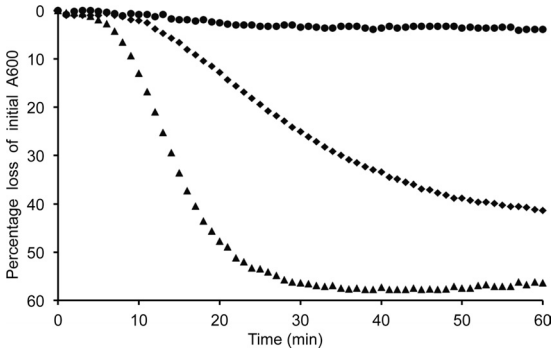

FIG 1 Germination of *B. megaterium* PV361 spores in 5% (wt/vol) beef extract, incubated at 37°C for 1 h. PV361 cultures were sporulated by nutrient exhaustion at different temperatures (▲, 22°C spores; ◆, 30°C spores; ●, 37°C spores) and the germinative behavior of the resultant spores assessed via OD_{600} (A_{600}) measurements as described in Materials and Methods.

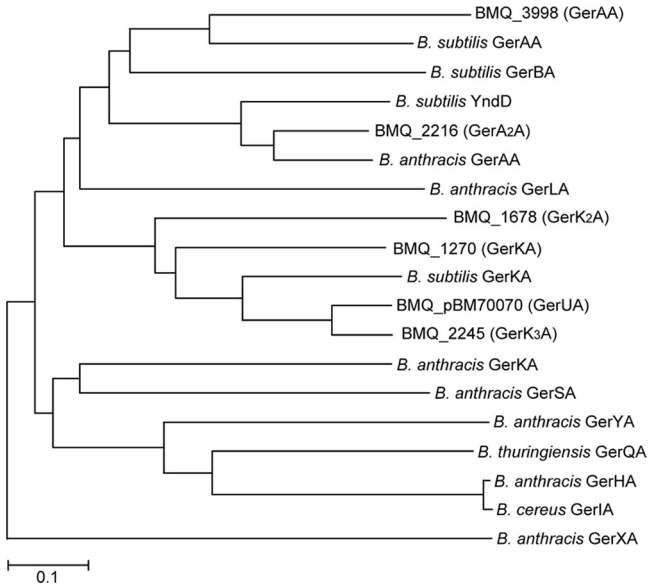

FIG 2 Simplified neighbor-joining tree showing clustering of *B. megaterium* QM B1551 GR A-subunit proteins with analogous receptor subunit proteins from other *Bacillus* species. Proteins were aligned with ClustalW, and the tree was constructed with MEGA 4.0 software (24). Putative A-subunit designations are in parentheses adjacent to *B. megaterium* GR locus identifiers.

arranged genetically in operons comprising ORFs for the respective A, B, and C subunits of the GR complexes, with variance in the order of the latter two ORFs. Two of the operons (*gerU* and BMQ_2245 to BMQ_2243; hereafter, BMQ_2245–2243) have nearby receptor D subunit genes, whereas the BMQ_1270–1273 operon has an internal (BMQ_1272) D subunit gene. The role of GR D-subunit proteins in germination has been characterized recently in *B. megaterium* and *B. subtilis* (27). In addition to the familiar tricistronic GR operons, bioinformatic analyses revealed the presence of an atypical cluster of GR-associated genes, comprising two B-subunit genes (BMQ_2234 and BMQ_2236) separated by a putative D-subunit gene (BMQ_2235). Another B-subunit ORF (BMQ_3108) is also carried on the chromosome, preceded by another putative D-subunit gene (BMQ_3107). The roles of these genes and the functions, if any, of their products in germination have not been characterized in the present study, although this cannot be discounted since products of a similarly atypically arranged B-subunit gene (BMQ_1826) were shown previously to interact with the A and C subunits of GerU to form a functional receptor (16).

Neighbor-joining trees for the respective GRs based upon alignments of GR A-subunit proteins revealed that the *B. megaterium* GRs cluster with the GerA and GerK receptor clades, which are named after two of the canonical *B. subtilis* receptors (Fig. 2). Similar results were obtained from alignments of receptor B- and C-subunit proteins (data not shown). The newly identified putative *B. megaterium* GRs were named according to published guidelines (25). Hence, the product of BMQ_3998–4000 is referred to hereafter as GerA, that of BMQ_2216–2218 as $GerA_2$, that of BMQ_1270–1273 as GerK, that of BMQ_1678–1676 as $GerK_2$, and that of BMQ_2245–2243 as $GerK_3$. We propose to retain the use of the GerU nomenclature since it is already well established, although evidently from the phylogenetic tree it too belongs to the GerK clade.

Having established the identity of putative GRs that are presumably responsible for mediating germination in PV361 spores, we then examined levels of expression during sporulation from each of the GR loci in the PV361 background by fusing the *E. coli lacZ* gene to the 3′ ends of truncated forms of the respective GR A-subunit genes. A *gerU-lacZ* fusion strain was constructed in the QM B1551 background for comparative purposes. Analysis of β-galactosidase activity in extracts from disrupted spores prepared at different temperatures revealed various degrees of expression from all six GR loci examined (Table 2). The presence of β-galactosidase in dormant spores from the various strains is consistent with promoters that induce transcription during sporulation, and this idea is supported by DNA sequences immediately upstream of the respective GR loci, all of which correlate well with *B. subtilis* consensus sigma factor σ^G promoter sequences (28; data not shown). Of the spores prepared at 22°C, the culture temperature demonstrated to produce spores that germinate most effi-

TABLE 2 β-Galactosidase activity associated with germinant receptor-*lacZ* transcriptional fusions in *B. megaterium* spores prepared at different temperatures

Strain	Genotype	β-Galactosidase activity (RFU) at sporulation temp[a]:		
		22°C	30°C	37°C
PV361	Plasmidless derivative of QM B1551	500	500	400
GC618	*gerU-lacZ*	16,000*	10,000	2,900
GC619	*gerK-lacZ*	43,000**	30,000**	10,000**
GC620	*gerK*$_2$*-lacZ*	2,000*	1,500	31,000
GC621	*gerK*$_3$*-lacZ*	6,200*	4,300	42,000
GC622	*gerA-lacZ*	43,000**	23,000**	10,000**
GC623	*gerA*$_2$*-lacZ*	11,000*	17,00	4,400

[a] Strains were sporulated at the indicated temperatures, and spores (~10^9) were purified and assayed for β-galactosidase activity as described in Materials and Methods. β-Galactosidase values (relative fluorescence units [RFU]) are the average of triplicate measurements conducted with two different spore preparations. Standard deviations for all values are <15%. *, values that are significantly different from values obtained from *gerK-lacZ* and *gerA-lacZ* spores at 22°C, with $P \leq 0.001$, as determined by two-tailed Student's *t* test; **, GR-*lacZ* expression differed significantly ($P \leq 0.001$) with the temperature of sporulation for the respective strains, as determined by one-way ANOVA.

TABLE 3 Germination and viability of *B. megaterium* PV361 receptor mutant spores[a]

Strain	Genotype	Rate of spore germination (%)[b]	Viability (%)[c]
PV361	Δ*gerU*	100	100
GC601	Δ*gerK*	<5	1
GC602	Δ$gerK_2$	90	100
GC603	Δ$gerK_3$	92	100
GC604	Δ*gerA*	91	100
GC605	Δ$gerA_2$	90	100
GC606	Δ*gerK* Δ$gerK_2$	85	60
GC607	Δ*gerK* Δ*gerA*	<5	0.01
GC608	Δ*gerK* Δ$gerA_2$	<5	0.05
GC609	Δ$gerA_2$ Δ*gerA*	85	50
GC610	$GerA^+$	79	50
GC611	$GerA_2^+$	<5	0.02
GC612	$GerK^+$	84	50
GC613	$GerK_2^+$	<5	0.005
GC614	Ger null	<5	0.002
GC615	Ger-null pHT-*gerU**	100	100

[a] Mutant strains are isogenic with PV361.
[b] Rates of spore germination are given relative to the OD_{600} loss (60%) for spores of strain PV361 after incubation for 60 min in beef extract (5% [wt/vol]), which was set equal to 100%. Values are the means of duplicate experiments conducted with different spore preparations; the standard deviation was <10% of the mean.
[c] Spore viability values were determined as described in Materials and Methods and are presented relative to that obtained for the parental PV361 strain, which was set at 100%.

ciently, two receptor loci—*gerK* and *gerA*—were expressed at significantly ($P \leq 0.001$) higher levels than other GR loci present in the PV361 background. The apparent expression level of these receptors was observed to decrease significantly ($P \leq 0.001$) in a stepwise manner as the temperature of sporulation increased from 22°C to 37°C. In contrast, expression levels of the $gerK_2$ and $gerK_3$ receptor loci, which were associated with the lowest levels of expression at 22°C, showed the highest levels of expression when sporulation was conducted at 37°C. Whether these apparent expression levels correlate with the abundance of the various GRs in PV361 spores sporulated at different temperatures, and which may account for differences in germination phenotype, has not been determined, since antisera against the respective GRs are not available.

Mutational analysis of *B. megaterium* GR loci. Initial assessment of the contribution of each of the chromosomally encoded GRs to the PV361 spore germination response was achieved by constructing strains in which the first gene in each operon (invariantly encoding the A subunit) was disrupted by allelic exchange, as described in Materials and Methods. Sequential disruption of GR loci in this manner permitted the construction of a series of single- and multiple-GR-null mutant strains, which were sporulated at 22°C and then assayed for germinative responses in 5% (wt/vol) beef extract and on solid LB medium (sporulation efficiency in all strains was similar to wild-type levels [data not shown]). Analysis of single-GR-null mutant spores revealed that spores of four of the mutant strains displayed relatively normal germination phenotypes, with only minor decreases in total OD_{600} loss and no apparent loss of viability on solid medium (Table 3). This was anticipated for $gerK_3$ spores (GC603) since the function of this receptor, formerly referred to as GerA (18), was examined previously by our group. (In this case, the operon was disrupted by plasmid insertional mutagenesis in QM B1551 spores [18].) Sequence analysis revealed that the B gene of the $gerK_3$ operon has a frameshift mutation that is predicted to lead to the formation of a severely truncated B-subunit protein (18). For the above reasons, we suspected that the $gerK_3$ GR is nonfunctional, and as such, it was not examined any further in this work. In contrast, spores of the *gerK* mutant (GC601) showed a severe germination defect, with not only no detectable loss in OD_{600} when incubated for 1 h in beef extract, but also the colony-forming ability reduced to ~1% of parental PV361 spore values.

Further reductions in spore viability were observed in strains in which additional GR loci were disrupted in the *gerK* background, with *gerK gerA* (GC607) and *gerK* $gerA_2$ (GC608) spore viabilities being reduced to ~0.01% and 0.05% of the parental levels, indicating that the products of the *gerA* and $gerA_2$ operons, in addition to *gerK*, have a degree of functionality. Most strikingly, however, are the efficient OD_{600} loss (85%) and relatively high spore viability (60% c.f. PV361 values) in *gerK* $gerK_2$ spores (GC606). In this strain, the deleterious effects of disrupted GerK appear to be relieved when the $GerK_2$ GR is also absent, which somehow permits the GerA and $GerA_2$ receptors to stimulate levels of germination in rich media that surpass considerably those observed when the GerK receptor alone is disrupted.

The contribution of individual GRs to the germinative response of PV361 spores was dissected further by constructing triple-receptor-null mutant strains, which if we again disregard $gerK_3$ and the various B-subunit ORFs evident on the chromosome, in effect conferred strains that expressed single (Ger^+) receptor orthologues. Additionally, Ger-null quadruple mutant spores (GC614) were also constructed, the viability of which was reduced by 4 to 5 orders of magnitude compared to parental PV361 spores and which provided a baseline for assessment of the function of the individual GRs. Analysis of the various individual Ger^+ strains revealed that the GerK receptor (GC612) is capable of individually mediating a relatively efficient germinative response to 5% (wt/vol) beef extract (84% germination) and on solid LB medium (50% viability). Similarly, $GerA^+$ spores (GC610) yielded germinative responses comparable to those achieved with $GerK^+$ spores. $GerA_2^+$ spores (GC611), however, germinated extremely poorly under these conditions, indicating that the efficient germinative responses described above for *gerK* $gerK_2$-null spores is probably largely due to GerA activity alone. The $GerK_2$ receptor itself appears to be relatively ineffective in independently stimulating germinative responses in rich media, with $GerK_2^+$ spore viability (0.005%) observed to be almost identical to that of Ger-null spores. Ger-null spores complemented with plasmid-borne *gerU** (GC615), however, showed complete germination under both nutrient conditions, as adjudged by OD_{600} loss and colony-forming ability, indicating that this receptor can function independently of all other GRs within the spore.

Germinant identification. Having established that the GerA, $GerA_2$, GerK, and $GerK_2$ receptors were expressed during sporulation and appear to be functional to various degrees, we then turned our attention to identifying specific germinants that are cognate for the respective GRs. Germination assays based on absorbance loss in liquid suspensions proved to be largely ineffective for this task since the germinative response in several mutant backgrounds was too weak to be detected by this method, as was the colorimetric detection of dipicolinic acid (DPA) release. Instead, sterile filter papers were patterned with relatively dense (OD_{600} of 40) aliquots of purified spores, and then placed on buff-

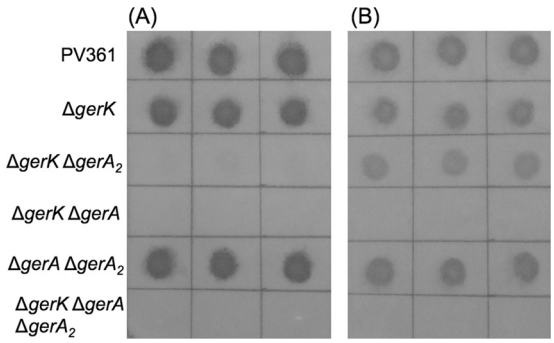

FIG 3 Assessment of the germination of *B. megaterium* PV361 GR-null mutant spores in response to glucose and KBr plus (A) asparagine and (B) phenylalanine, via reduction of 2,3,5-triphenyltetrazolium chloride indicator dye. Germinants (10 mM each) were added to Tris-HCl buffered (pH 7.5) bacteriological agar plates, which were then overlaid with filter papers inoculated in triplicate with spores of the indicated genotypes. Germination was assessed as described in Materials and Methods.

ered agar plates containing potential germinant(s) and the indicator Tzm. Reduction of Tzm to a red formazan derivative by the activity of endogenous spore enzymes activated upon germination is an established technique for characterizing germination phenotypes, and the methodology as outlined here conferred a qualitative approach for assigning germinants cognate for the various *B. megaterium* receptors (Fig. 3). The method is exemplified by *gerK*-null spores (GC601), for example, which did not show any OD_{600} loss in beef extract and which retained ~1% of the colony-forming ability of wild-type spores, but for which a germination response could be detected clearly in response to a mixture of various amino acids, plus glucose and potassium bromide (KBr) (Fig. 3 and Table 4). The same spores were perceived to germinate less efficiently in response to binary combinations of glucose, inosine, or KBr and less efficiently again in response to any of the standard amino acids plus glucose or KBr. An identical germination pattern, at least within the limitations of the assay, to the same combinations of germinants was observed with PV361 spores. Neither PV361 spores nor spores of any of the GR mutant strains were observed to germinate in response to any of the single compounds tested, which included virtually all of the common spore germinant compounds (Table 4). Of the strains engineered to express single GRs, only $GerA^+$ (GC610) and $GerK^+$ (GC612) spores showed significant levels of germination as determined via this assay. Again, both strains showed similar germination patterns, with the strongest responses being achieved with a combination of glucose plus KBr, although these responses were judged to be moderate, for example, compared to that of PV361 spores exposed to alanine plus glucose and KBr. Substitution of glucose or KBr with any of the standard amino acids resulted in weak germination responses in both $GerA^+$ and $GerK^+$ spores.

Analysis of the germinative response of $GerK_2{}^+$ spores (GC613) revealed that this receptor has the capacity, in addition to the negative effects exerted on GerA function described previously, to induce a weak germination response, but only to combined inosine plus KBr of the compounds tested. The $GerA_2$ receptor appears to be diminished further in terms of its capacity to function independently of other GRs in stimulating germinative responses, as evidenced by the absence of any detectable germination by $GerA_2{}^+$ spores (GC611) when exposed to any of the germinant mixtures tested. However, disruption to this receptor locus in the *gerK* background (*gerK gerA*$_2$ [GC608]) was observed to impact negatively the range of germinants recognized by *gerK*-null spores (GC601). The latter, for example, show a moderate germinative response to glucose plus KBr, whereas the same mixture has no germinative effect when $GerA_2$ is also disrupted (GC608). Similarly, the range of amino acids recognized by *gerK gerA*$_2$-null

TABLE 4 *B. megaterium* GR-null spores' germinative responses to putative germinants

Strain[a]	Genotype	Response of spores to[b]: Single compounds[c]	Inosine + glucose or KBr[d]	Single amino acid + glucose or KBr[e]	Sugar + KBr[f]	Single amino acid + glucose and KBr[g]
PV361	Δ*gerU*	−	++	+	++	+++
GC601	Δ*gerK*	−	++	+	++	+++
GC606	Δ*gerK* Δ*gerK*$_2$	−	+	+	++	++
GC607	Δ*gerK* Δ*gerA*	−	+	−	−	−
GC608	Δ*gerK* Δ*gerA*$_2$	−	+	−	−	++[h]
GC609	Δ*gerA* Δ*gerA*$_2$	−	+	+	++	+++
GC610	$GerA^+$	−	+	+	++	++
GC611	$GerA_2{}^+$	−	−	−	−	−
GC612	$GerK^+$	−	+	+	++	++
GC613	$GerK_2{}^+$	−	+	−	−	−
GC614	Ger null	−	−	−	−	−

[a] All strains are isogenic with PV361 and are null for GerU.
[b] Sterile filter paper discs inoculated with 2-μl aliquots of heat-shocked and cooled spore suspensions (OD_{600} of 40) were transferred to petri dishes containing 20 ml agar (1.2% [wt/vol]), supplemented with 5 mM Tris-HCl (pH 7.5), 50 μg/ml 2,3,5-triphenyltetrazolium chloride, and 10 mM each germinant. Plates were incubated at 37°C and then checked and scored periodically over a 24-h period for the red coloration that denotes germination. The responses range from no coloration (−) to intense coloration (+++).
[c] The single compounds tested included all proteinogenic amino acids (plus D-alanine), sugars (glucose, fructose, ribose, galactose, maltose, sucrose, lactose, and sorbitol), nucleosides, and inorganic salts (KBr, KCl, NaCl, and $MgSO_4$).
[d] Strains GC607 and GC613 germinated in response to inosine plus KBr but not to inosine plus glucose.
[e] Where positive, similar germinative responses were obtained with any of the standard amino acids in combination with either glucose or KBr.
[f] Where positive, the strongest germinative responses were observed with glucose plus KBr.
[g] The strongest germinative responses, except where indicated, were observed with L-alanine, L-glutamic acid, or L-asparagine in combination with KBr plus glucose.
[h] Strain GC608 was observed only to germinate in response to proline or phenylalanine in combination with KBr plus glucose or fructose.

(GC608) spores, where only proline or phenylalanine were stimulatory, is much reduced compared to that of *gerK*-null (GC601) spores, where any of the standard amino acids in combination with glucose or KBr induced moderate germination responses. Therefore, it seems that the $GerA_2$ receptor functions collaboratively with at least the GerA receptor to mediate a positive effect on the range of germinants recognized by the spore.

DISCUSSION

The principal objective of the present study was to identify and characterize the function and germinant recognition profiles of novel *B. megaterium* PV361 spore GRs, whose presence had been inferred from studies conducted previously with similar QM B1551-derived spores that lack the GerU receptor (18, 26). In addition, we sought also to address gaps in knowledge associated with previous studies conducted by our group on the function of the GerU receptor, pertaining to its function, or otherwise, in the absence of all other spore GRs.

In terms of the first objective, the present work has revealed that in addition to plasmid-borne *gerU*, the *B. megaterium* QM B1551 chromosome is host to five tricistronic (or in some cases, tetracistronic) GR loci, the products of which, based on predicted sequence similarity to previously characterized GRs from other species, we have named GerA, $GerA_2$, GerK, $GerK_2$, and $GerK_3$. Transcriptional analyses conducted with *lacZ* fusion strains revealed that all five GRs are expressed during sporulation and that apparent levels of transcription are dependent upon the temperature of the sporulation medium. The latter observation may be associated with differing germination phenotypes observed with PV361 spores sporulated at different temperatures, which showed a stepwise decrease in germination efficiency in response to rich nutrients as the temperature of sporulation increased from 22°C to 37°C. The influence of sporulation conditions on spore properties has been observed previously (29–32). More specifically, and in contrast to the presented results, a recent study concerning the germination of *B. subtilis* spores prepared at different temperatures showed a positive correlation between sporulation temperature and subsequent efficiency of germination (33). However, whereas the *B. subtilis* study established a strong correlation between sporulation temperature, GR abundance, and germinative efficiency of spores, lack of antisera against *B. megaterium* GR proteins means that we have not yet been able to establish whether transcriptional levels reflect GR abundance in *B. megaterium* spores. Therefore, while the transcriptional levels of *gerK* and *gerA*, which appear to encode the dominant GRs in PV361 spores, are consistent with these GRs being the most abundant receptors in PV361 spores prepared at 22°C and which may account for the efficient germinative response of spores prepared at 22°C versus those at 37°C (where *gerK* and *gerA* expression is reduced), we cannot yet unequivocally establish this relationship. Other factors, such as differences in spore coat composition or permeability in spores prepared at different temperatures, for example, may also influence the germination phenotypes of spores prepared at different temperatures and should be considered when investigating the physiological basis of adverse germination phenotypes.

Mutational analyses conducted in the present study have revealed that four of the five chromosomally encoded GRs are functional, with the $GerK_3$ receptor probably being rendered nonfunctional due to a frameshift in the B-subunit structural gene. Spores of the parental PV361 strain, which lack the single-germinant-responsive GerU receptor, were observed to germinate only in response to combinations of germinants, with rich undefined media such as 5% (wt/vol) beef extract inducing the strongest germinative responses. Several potential germinant compounds were screened subsequently in an attempt to identify the stimulatory components of beef extract and other rich media. Although never attaining the same germinative efficiency observed in rich nutrient media, as judged by OD_{600} loss (data not shown), the development of intense coloration on filter papers suffused with germinants and the Tzm indicator dye revealed that mixtures comprising glucose, KBr, and either L-alanine, L-asparagine, or L-glutamic acid could trigger at least significant fractional germination of heat-shocked PV361 spores. However, removal of individual components of the mixture, with loss of glucose or KBr proving to be the most deleterious, induced weaker germinant responses, as did replacement of Ala, Asn, or Glu with any of the other standard amino acids.

Analysis of single GR-null mutant strains indicated that the GerK receptor is largely responsible for mediating the efficient germinative response of PV361 spores in beef extract or LB medium, since disruption of any of the other GR loci had minimal impact on the germinability of single GR mutant spores. Surprisingly, germination was enhanced in rich media, with respect to *gerK*-null mutant spores, when the GerK and $GerK_2$ receptors were both disrupted. The GerA receptor appears to be responsible for stimulating germination in this double mutant background, since $GerA^+$ spore viability is considerably higher than $GerA_2^+$ spore viability. Collectively, these observations indicate that the $GerK_2$ receptor can exert an inhibitory effect on GerA function, at least in PV361 *gerK*-null spores. The physiological basis for this effect has not been established, although studies conducted on the regulation of GR expression in *B. subtilis* have revealed a degree of complexity of transcriptional control over GR loci that extends beyond the relatively simple analysis of GR-*lacZ* transcriptional fusions conducted in the present work (34, 35), and therefore it is possible that disruption of $gerK_2$ somehow permits upregulation of expression from *gerA*. Equally plausible is that the negative effect is mediated at the quaternary structural level, where the architecture of the clustered assembly of GRs located in the spore inner membrane, referred to as the germinosome in *B. subtilis* (7), may preclude access of germinants to (or function of) the GerA receptor in PV361 *gerK* spores. Hence, deletion of $GerK_2$ could result in a modified germinosome assembly (if such a structure is present in *B. megaterium* spores) that is conducive to GerA function, either by improved access of germinants to the GerA binding site or perhaps by increasing the abundance of functional GerA receptors in the membrane. Both possibilities merit further attention, since the apparent impact of $GerK_2$ on GerA function is perhaps the clearest example of negative regulation or cooperativity between spore GRs (although negative cooperativity between *germinants* has been observed in *B. anthracis* spores [36], whereas *B. subtilis* spores engineered to overexpress defined GRs showed decreased germinative rates modulated by other GRs [28]).

Consideration of the germinative responses of strains engineered to express single functional GRs revealed that, in general, *B. megaterium* PV361 spore GRs are cognate for a wide range of overlapping and structurally diverse germinants, indicating that the respective GRs have flexible binding sites, perhaps analogous to promiscuous GRs characterized in other species—e.g., *B. subtilis* GerB (37) and *B. anthracis* GerH (6) receptors. As alluded to

in the introduction, this requirement for the presence of multiple stimulatory compounds may serve to counter the increased propensity for spores equipped with relatively nonspecific receptors to inadvertently trigger germination under nutritionally depleted conditions. At the same time, spores equipped with GRs with slightly different germinant recognition profiles, and which are expressed at different levels depending on sporulation conditions, may maximize the potential for efficient spore germination in diverse environmental niches.

Of the defined germinant compounds identified, monosaccharides and inorganic salts, exemplified by glucose and KBr, appear to represent the major classes of stimulatory molecules, which is perhaps not surprising given the GerK and $GerK_2$ receptors' phylogenetic proximity to *B. megaterium* GerU and *B. subtilis* GerK GRs, both of which appear to be cognate for glucose (16, 37). (GerU can additionally be stimulated by high concentrations of KBr.) Inosine, a common germinant in several species of *Bacillus*, is also stimulatory, albeit weakly, and appears to be cognate for the GerA, GerK, and $GerK_2$ receptors. The stimulatory effect upon germination promoted in most strains by the inclusion of any of the proteinogenic amino acids as cogerminants suggests that side-chain cognicity is of secondary importance and that more conserved amino acid structural features are recognized instead. One exception to this generalization, where only a narrow range of defined amino acids (proline and phenylalanine) could act as stimulatory cogerminants alongside glucose and KBr, was observed in GC608 spores, which have functional GerA and $GerK_2$ receptors. Intriguingly, these spores do not respond to a combination of glucose and KBr, whereas $GerA^+$ spores do, providing further evidence of the negative effect associated with $GerK_2$ on the function of GerA. Hence, the establishment of definitive germinant recognition profiles for the respective GRs is evidently a complex procedure, compounded by effects conferred by the presence or absence of other receptors. In addition, we cannot rule out the possibility that germinant recognition profiles associated with defined GRs in this work are influenced also by receptor B- and D-subunit proteins encoded out with conventionally arranged receptor operons on the chromosome. Further work is therefore required to assign precise germinant recognition profiles to individual GRs.

Overall then, by considering evidence from the present and previous studies, it is possible to characterize the *B. megaterium* PV361 spore GRs (and probably by inference the QM B1551 spore GRs, although this will have to be verified experimentally) in terms of a functional hierarchy based upon differing levels of responses to germinants. GerU sits at the apex of this hierarchy, since only this receptor has the capacity to trigger efficient germinative responses to a range of single compounds. The GerK and GerA receptors are considered to occupy the second tier of the hierarchy, given that both of these receptors can independently trigger significant germination in response to binary combinations of germinants, although for GerA, this is dependent upon removal of $GerK_2$ inhibition. Finally, the lowermost tier of the hierarchy is occupied by the $GerK_2$ and $GerA_2$ receptors, which appear to function largely collaboratively with other GRs, in either a positive (e.g., $GerA_2$ with GerA) or negative (e.g., $GerK_2$ against GerA) sense. However, several questions relating to the spore biology that underpins this GR hierarchy in *B. megaterium* spores, whether pertaining to GR regulation at the transcriptional or functional level, individual GR subunit and macromolecular structure, and the influence of ancillary proteins on GR function, have yet to be answered, and these remain objectives for future work in this area.

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