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# The Cooperative and Interdependent Roles of GerA, GerK, and Ynd in Germination of *Bacillus licheniformis* Spores

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**ABSTRACT**

**When nutrients are scarce, *Bacillus* species form metabolically dormant and extremely resistant spores that enable survival over long periods of time under conditions not permitting growth. The presence of specific nutrients triggers spore germination through interaction with germinant receptors located in the spore's inner membrane. *Bacillus licheniformis* is a biotechnologically important species, but it is also associated with food spoilage and food-borne disease. The *B. licheniformis* ATCC 14580/DSM13 genome exhibits three *gerA* family operons (*gerA*, *gerK*, and *ynd*) encoding germinant receptors. We show that spores of *B. licheniformis* germinate efficiently in response to a range of different single L-amino acid germinants, in addition to a weak germination response seen with D-glucose. Mutational analyses revealed that the GerA and Ynd germination receptors function cooperatively in triggering an efficient germination response with single L-amino acid germinants, whereas the GerK germination receptor is essential for germination with D-glucose. Mutant spores expressing only GerA and GerK or only Ynd and GerK show reduced or severely impaired germination responses, respectively, with single L-amino acid germinants. Neither GerA nor Ynd could function alone in stimulating spore germination. Together, these results functionally characterize the germination receptor operons present in *B. licheniformis*. We demonstrate the overlapping germinant recognition patterns of the GerA and Ynd germination receptors and the cooperative functionalities between GerA, Ynd, and GerK in inducing germination.**

**IMPORTANCE**

**To ensure safe food production and durable foods, there is an obvious need for more knowledge on spore-forming bacteria. It is the process of spore germination that ultimately leads to food spoilage and food poisoning. *Bacillus licheniformis* is a biotechnologically important species that is also associated with food spoilage and food-borne disease. Despite its importance, the mechanisms of spore germination are poorly characterized in this species. This study provides novel knowledge on germination of *B. licheniformis* spores. We characterize the germinant recognition profiles of the three germinant receptors present in *B. licheniformis* spores and demonstrate that the GerA germinant receptor cooperates with the Ynd and GerK germinant receptors to enable an effective germination response to L-amino acids. We also demonstrate that GerK is required for germination in response to the single germinant glucose. This study demonstrates the complex interactions between germinant receptors necessary for efficient germination of *B. licheniformis* spores.**

Endospore formation is a phenotypic adaptation to unfavorable environmental conditions, which allows bacteria to persist in the environment in a dormant and extremely resistant state. Nevertheless, spores are able to continuously monitor the environment for conditions favorable for growth. Many members of the bacterial orders *Bacilliales* and *Clostridiales* are able to survive starvation by forming endospores, which are much more resistant to heat, chemicals, irradiation, and desiccation than the vegetative cells (1). However, upon exposure to nutrient germinants, spores can return to active growth within minutes in the process of germination (2–5).

The nutrient-induced germination is initiated when specific nutrients are recognized by their cognate germinant receptors (GRs) located in the spores inner membrane (6–8). Multiple GR isoforms, with distinct nutrient specificities, have been characterized in different spore-forming species of the genera *Bacillus* and *Clostridium* (4, 9–15).

The genetic organization and knowledge gained from functional studies suggest that the GRs are heterotrimeric complexes consisting of A, B, and C subunits, and at least in *Bacillus*, they are all required for the formation of a functional receptor (3, 16). Genes encoding GR homologs are often organized in polycistronic so-called *gerA* family operons, encoding the A, B, and C subunits (8). These operons are only expressed in the developing spore under the control of the forespore specific RNA polymerase sigma factor $\sigma^G$ (17–19). The individual A, B, and C subunits show significant intra- and interspecies homology with the corresponding subunits of other GRs (4, 10, 20), but the organization of GR operons varies between species and between different operons

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within the same genome (4). In *Bacillus subtilis*, five different tricistronic *gerA* family operons (*gerA*, *gerB*, *gerK*, *ynd*, and *yfk*) encode three functional GRs and two putative GRs (5). Furthermore, it has been reported that at least some GRs have an additional D subunit (4). Currently, the specific role of the individual subunits in the GR function is poorly understood.

Specific amino acids and ribonucleosides, either independently or in mixtures, act as powerful germinants of *Bacillus* spores. Among *Bacillus* species, L-alanine seems to be the most common germinant (10). In *B. subtilis*, GerA is required for L-alanine germination, whereas GerB and GerK act cooperatively to trigger germination with a mixture of L-asparagine, D-glucose, D-fructose, and $K^+$ called AGFK (21, 22). GerK is necessary for recognition of glucose (21, 23), whereas GerB seems to recognize several L-amino acids in addition to L-asparagine (21). A study of wild *B. subtilis* strains showed that the *gerA* operon was more conserved than the *gerK* and *gerB* operons and that the *ynd* and *yfk* operons were truncated or absent in many strains, suggesting that the selection pressure for maintaining these genes is low in this species (24).

*Bacillus licheniformis* is a facultative anaerobic sporeformer that is closely related to *B. subtilis*. It is economically valuable due to its production of various compounds such as enzymes, antibiotics, and surfactants that are used for various industrial applications. However, besides its beneficial properties, it is also a common food spoilage bacterium in milk, meat products, bread, and canned foods (25–33). *B. licheniformis* is also an occasional pathogen in humans and animals (34–36). It has been isolated from cases of food poisoning and there are reports linking *B. licheniformis* to severe diseases such as bacteremia or recurring sepsis in both immunocompromised and clinically healthy individuals (37–40). Despite its importance, detailed functional studies of *B. licheniformis* GRs and their germinant recognition patterns are still scarce. *B. licheniformis* has previously been described to germinate in response to L-alanine, L-cysteine, and L-valine (41). Three different *gerA* family operons and one single gene member of this family have been identified in the genome of the type strain *B. licheniformis* ATCC 14580/DSM13 (42–44). These are the *gerA*, *gerK*, and *ynd* operon orthologues and the orphan *gerAC* homologue *yndF2*. No homologue to the *gerB* operon is present in the genome of ATCC 14580/DSM13.

In *B. licheniformis*, *gerA* is required for germination induced by L-alanine and, similar to the *B. subtilis* 168 *gerA*, this response is strongly inhibited by D-alanine (45). Analysis of spore germination in *B. licheniformis* suggests that some *B. licheniformis* isolates germinate remarkably slowly when exposed to L-alanine and that the slow germination phenotype is, at least partly, explained by specific amino acid substitutions in GerA (46). Such slow-germinating strains pose a challenge to the food industry, which wants to implement induced germination as a strategy to eliminate spores during processing. The roles of *gerK*, *ynd*, and *yndF2*, if any, are still unknown.

We assessed the roles of amino acids, ribonucleosides, and AGFK in initiating *B. licheniformis* spore germination. We also characterized the functions of GerA and the putative germination receptors Ynd and GerK in more detail by constructing mutants in all three GR operons. By analyzing the germination responses of wild-type and mutant spores to amino acids and glucose, we could assign functional roles for the Ynd and GerK GRs, and we demonstrated their cooperative and interdependent role with GerA in triggering efficient germination responses to L-amino acids. Furthermore, we showed that GerK was essential for D-glucose-induced germination and that the cooperative function with GerA was independent of glucose activation of GerK.

**TABLE 1** *B. licheniformis* strains used in this study

| *B. licheniformis* strain | Genotype | Source or reference(s) |
|---|---|---|
| ATCC 14580/DSM13 | Type strain | 42, 43 |
| MW3[a] | Δ*hsdR1* Δ*hsdR2* | 47 |
| NVH-1307 | Δ*gerAA*::*spc* | 45 |
| NVH-1324 | Δ*gerKA-C* | This study |
| NVH-1335 | Δ*yndD* | This study |
| NVH-1368 | Δ*gerAA*::*spc* Δ*yndD* | This study |
| NVH-1376 | Δ*yndD* Δ*gerKA-C* | This study |
| NVH-1323 | Δ*gerAA*::*spc* Δ*gerKA-C* | This study |
| NVH-1370 | Δ*gerAA*::*spc* Δ*gerKA-C* Δ*yndD* | This study |
| NVH-1348 | Δ*gerAA-C* | This study |
| NVH-1377 | Δ*yndD*/pHT315_*yndD*$^+$ | This study |

[a] MW3 was used as a background strain for the construction of all mutants.

## MATERIALS AND METHODS

**Strains and culture conditions.** The strains used in this study are listed in Table 1. The *B. licheniformis* strain MW3 was used as background for all gene-deletion mutants. *B. licheniformis* is difficult to manipulate genetically due to the presence of type 1 restriction modification systems (T1rm), which target foreign DNA. Two T1rm loci have been identified in ATCC 14580/DSM13 (42, 43). In the ATCC 14580/DSM13 derivative strain MW3, both T1rms are deleted, resulting in a significantly higher transformation frequency compared to the wild-type background (47). All *B. licheniformis* strains were cultured aerobically in brain heart infusion broth or Luria-Bertani broth at 37°C with agitation (225 rpm).

**Spore preparation.** Spores were prepared, harvested, washed, and stored as described previously (46). The spores were stored for at least 7 days prior to use. Spores of the *yndD* complementation strain NVH-1377 were made in the presence of 1 μg of erythromycin $ml^{-1}$.

**Germination assays and inhibition by D-alanine.** Spore germination was assessed by monitoring the optical density at 600 nm ($OD_{600}$), which decreases in the spore suspension during germination (48), as described previously (46). The purity of the spore suspension was determined by phase-contrast microscopy prior to use. Spore batches used in the germination experiments contained at least 98% phase-bright spores. Spores were heat activated (65°C, 20 min) before use. Amino acids (Sigma-Aldrich, USA), D-glucose (Sigma-Aldrich, USA), and KCl (Merck, USA) were used at a concentration of 100 mM in the germination assays unless noted otherwise. For inhibition with D-alanine, 100 μl of buffered (0.2 M K-phosphate buffer [pH 7.2]) spore suspension ($OD_{600}$ of ~2) was mixed with 50 μl of 100 mM D-alanine and preincubated for 15 min at 37°C with agitation. Subsequently, 50 μl of 100 mM L-amino acid germinant was added, which resulted in a final germinant and D-alanine concentration of 25 mM, and a spore suspension with an initial $OD_{600}$ of 1 (~$10^8$ spores $ml^{-1}$). All germination assays were repeated three times using independent spore batches unless otherwise stated, and the results are presented as the means of all replicates. Spore suspensions with Milli-Q water were used as negative controls. Phase-contrast microscopy was routinely used to monitor the level of germinated spores after 120 min of exposure to germinant compound and was particularly useful for assessing low levels of germination. The number of phase-dark (germinated) spores was determined for approximately 200 to 500 spores in each experiment by counting spores in 10 random fields of view, and the average percentage of germinated spores was calculated from three independent spore batches.

The maximum germination rate ($G_{max}$) was calculated from the linear

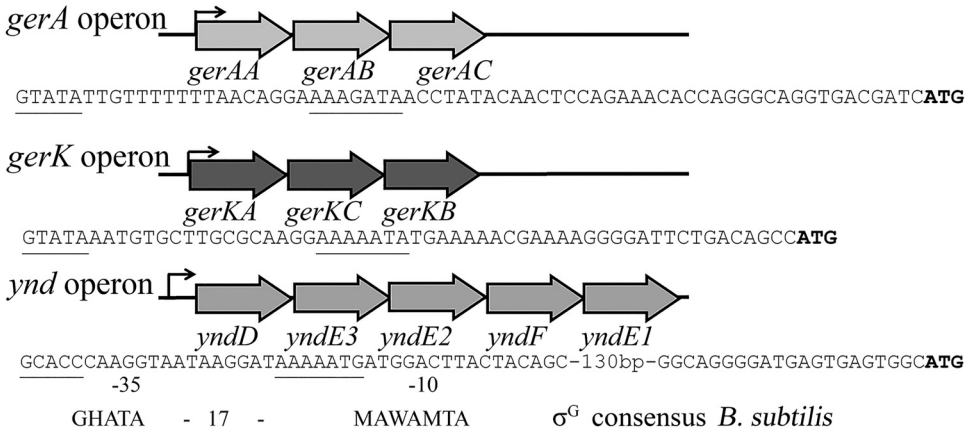


**FIG 1** Organization of the *B. licheniformis* ATCC 14580/DSM13 *gerA* family operons. The putative promoter regions are indicated with arrows. IUPAC nucleotide ambiguity code: H = A, T, or C; M = C or A; and W = A or T.

segment of the curves of OD changes (presented in the supplemental material) using DMFit. DMFit (where "DM" stands for "dynamic modeling") fits curves where a linear phase is preceded by a lag phase and followed by a stationary phase (49). The germinant concentrations yielding 50% germination ($C_{50}$) were calculated using linear regression (www.geogebra.org) on the dose-response curves (see Fig. S1 in the supplemental material).

**Mutant construction.** According to the annotations in the NCBI database, *B. licheniformis* MW3 contains the following *gerA* family operons: the *gerA* operon (TRNA_RS38675, TRNA_RS38680, and TRNA_RS38685), the *gerK* operon (TRNA_RS23740, TRNA_RS23745, and TRNA_RS23750), the *ynd* operon (TRNA_RS32310, TRNA_RS32305, TRNA_RS32300, TRNA_RS32295, and TRNA_RS32290), and the orphan *gerAC* homologue *yndF2* (TRNA_RS32565).

The *ynd* operon of *B. licheniformis* MW3 contains five genes, *yndD*, *yndE3*, *yndE2*, *yndF1*, and *yndE1* (42, 43). The first gene in the operon, *yndD*, was deleted and replaced with 5′ATGTAG-3′ using a markerless gene replacement method (50) as described by Lovdal et al. (45). This method leads to an in-frame deletion of the target gene and ensures that the up- and downstream flanking sequences, including the promoter region, are intact.

To delete *yndD*, primers A and B (see Table S1 in the supplemental material) were used to amplify a 589-bp fragment upstream of *yndD*, and primers C and D were used to amplify a 530-bp fragment downstream of *yndD*. Primers B and C (see Table S1 in the supplemental material) carried a sequence overlap, and the resulting AD fusion PCR product thus contained the *yndD* upstream and downstream sequences. The AD product was ligated into the thermosensitive shuttle vector pMAD (51) containing an additional *I-SceI* site (kindly provided by Annette Fagerlund, Nofima, Norway). The pMAD-*I-SceI* Δ*yndD* plasmid was transformed into *B. licheniformis* MW3 electrocompetent cells as described previously (45). Integration of pMAD-*I-SceI* Δ*yndD* into the chromosome by homologous recombination was performed as described previously (51). After verification of the single crossover, pBKJ233 containing the gene encoding the *I-SceI* enzyme was introduced by electroporation. *I-SceI* makes a double-stranded DNA break at an 18-bp recognition site in pMAD-*I-SceI*. The repair of the strand break may lead to a second crossover, resulting in deletion of the target sequence. Deletion of *yndD* was verified by PCR using oligonucleotides located upstream and downstream of *yndD* (oligonucleotides A and D; see Table S1 in the supplemental material), followed by sequencing of the PCR product (Source BioScience Lifesciences, United Kingdom). All PCRs were conducted using an Eppendorf Mastercycler ep-Gradient S (Eppendorf, Germany) and Phusion high-fidelity DNA polymerase (Finnzymes, Finland) according to the manufacturers' instructions.

The *gerKA-C*-null mutant, wherein the two first genes of the *gerK* operon (*gerKA* and *gerKC*) were deleted in-frame, and the *gerAA-C*-null mutant, wherein all three genes of the *gerA* operon were deleted in-frame (*gerAA*, *gerAB*, and *gerAC*), were constructed according to the method described above and using the oligonucleotides listed in Table S1 in the supplemental material.

**Complementation of *yndD*.** The shuttle vector pHT315 (52) was used for transcomplementation of *yndD*. The *yndD* gene, including the promoter region, was amplified by PCR using the primers yndD-F and ynd-R (see Table S1 in the supplemental material) and Phusion high-fidelity DNA polymerase (Finnzymes, Finland) as described above. The amplicons were cloned into the pCR2.1-TOPO vector before introduction into the shuttle vector pHT315. The resulting construct was transformed into electrocompetent *B. licheniformis* NVH-1335, as described above. The presence of the correct plasmid construct was verified by PCR and sequencing. Complementation of the *gerA* operon has been described before (45).

**RT-qPCR.** The gene expression levels of *yndD* and *gerKA* were determined relative to *gerAA* by reverse transcription-quantitative PCR (RT-qPCR). A culture with ca. 50% sporulated *B. licheniformis* cells was harvested and stored in 1:1 methanol at −80°C. RNA extraction, cDNA synthesis, and RT-qPCR analysis were done as described by Madslien et al. (46). The quantity and purity of the RNA preparations were determined by measuring the absorbance at 260 nm and the ratio of the absorbances at 260 and 280 nm, respectively, by using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The RT-qPCR analyses were performed in triplicates on at least three independent biological replicates. Melting-curve analyses were performed after each run in order to confirm amplification of specific transcripts. Amplification of serial dilutions of DNA template from strain MW3 enabled the estimation of the slope ($s$) of the standard curves for each primer. The PCR efficiency ($E$) was calculated by using the equation $E = 10^{-1/s}$. To quantify mRNA transcript levels, the $C_T$ (threshold cycle) values of the target gene and the reference gene (*gerAA*) from the same sample were transformed using the following term $E^{-CT}$, and the expression levels of the target genes were normalized by dividing the transformed $C_{T\,\text{target}}$ by the transformed $C_{T\,\text{reference}}$ (53, 54).

## RESULTS

**Characteristics and relative expression levels of the *ger* operons in *B. licheniformis*.** The gene organization of the *gerA*, *gerK*, and *ynd* operons in *B. licheniformis* strain ATCC 14580/DSM13 is presented in Fig. 1. The *B. licheniformis ynd* operon is pentacistronic, containing the D, $E_3$, $E_2$, $F_1$, and $E_1$ genes, encoding the germina-

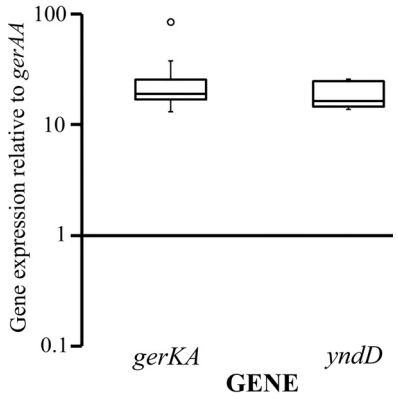


**FIG 2** Relative expression levels of *gerKA* and *yndD* to *gerAA* in *B. licheniformis* MW3, as determined by qRT-PCR. Boxes represent 50% of the observations (first quartile to third quartile), and the horizontal lines mark the median. The ends of the whiskers show the 1.5× interquartile range (IQR) below the first quartile and 1.5× IQR above the third quartile. ○, outlier.

tion receptor subunits A, B, B, C, and B, respectively. The pentacistronic organization is different from the tricistronic organization of the *ynd* operon of *B. subtilis* (55). The *gerK* and *gerA* operons of *B. licheniformis* both contain three structural genes and demonstrate the same gene organization seen in their orthologues in *B. subtilis* (55). Putative −10 and −35 promoter sequences, with similarities to those recognized by $\sigma^G$, were identified upstream of the *gerA*, *gerK*, and *ynd* operons (Fig. 1). The −10 and −35 promoter regions of *gerA* and *gerK* match the *B. subtilis* $\sigma^G$ consensus sequence, while the promoter region of *yndD* is less similar to the consensus sequence. Quantitative analyses of the relative transcription levels in sporulating cells of the ATCC 14580/DSM13 derivative strain MW3 showed that the *ynd* and the *gerK* operons were transcribed at approximately the same levels, 16.4 and 18.9 times higher relative to *gerAA*, respectively (Fig. 2).

**Germinant recognition profile of *B. licheniformis* spores.** Spores of strain MW3 have previously been shown to germinate in response to casein hydrolysate (a mixture of amino acids obtained from the hydrolysis of casein) and L-alanine as a single germinant compound (45). To further characterize the germinant recognition profile of this strain, we screened spores for germination in response to 18 standard L-amino acids, the ribonucleosides adenosine and inosine, and AGFK. A high concentration (100 mM) of each germinant compound (100 mM concentration of each AGFK component) was used for screening to also identify weak germinants. The germination efficiency was assessed after 120 min of germinant exposure by microscopic examination determining the percentage of phase-dark (germinated) versus phase-bright spores, and the results are listed in Table 2. L-Alanine, L-cysteine, and L-valine induced the most efficient germination responses, resulting in >90% germinated spores after 120 min of exposure to the germinant compound. L-Isoleucine, L-serine, and L-aspartic acid appeared as moderately efficient germinants (30 to 90% germination), while L-methionine and L-lysine appeared as weak germinants, resulting in <30% germinated spores. The most efficient germinants (L-alanine, L-cysteine, and L-valine) were selected for kinetic analyses. Kinetic analyses showed that spores induced by L-alanine demonstrated the highest germination rate, followed by L-cysteine and L-valine (Table 3). Assessment of the lowest concentration giving half-maximum rate of germination ($C_{50}$) confirmed that L-alanine was the most efficient germinant tested, followed by L-cysteine and L-valine (Table 3; see Fig. S1 in the supplemental material). AGFK acted as a weak germinant resulting in <30% germinated spores after 120 min of exposure to the germinant compounds (Table 2). Further experiments showed that D-glucose alone triggered the same level of germination as AGFK. This suggests that the germination response to AGFK is largely due to the D-glucose component. D-Fructose, L-asparagine, and $K^+$ induced germination responses comparable to that of the negative control (Table 3).

A number of studies have shown that the D-enantiomer of alanine functions as an efficient competitive inhibitor of L-alanine-induced germination (41, 56–58). D-Alanine has also been shown to inhibit L-valine-induced germination of *B. subtilis* and casein hydrolysate-induced germination in *B. licheniformis* (21, 45, 59). As shown in Table 3, preexposure of spores to D-alanine,

**TABLE 2** Classification of nutrient germinants for *B. licheniformis* MW3 spore germinants

| Germinant efficiency (%)[a] | Germinant compounds[b] |
|---|---|
| High (>90) | L-Alanine, L-cysteine, L-valine |
| Moderately (30–90) | L-Serine, L-isoleucine, L-aspartic acid |
| Weak (10–30) | AGFK,[c] D-glucose, L-methionine, L-lysine |
| Very weak (2–10) | D-Fructose, inosine, L-phenylalanine |
| None (<2) | Negative control, L-glutamic acid, L-arginine, L-histidine, L-leucine, L-proline, L-glycine, L-threonine, L-asparagine, adenosine,* L-tyrosine* |

[a] The percentages of germinated (phase dark) spores were estimated after 120 min of exposure to germinant compounds.
[b] The germinant concentration was 100 mM. *, due to low solubility in water, the concentrations of the indicated compounds were set to 1 mM.
[c] AGFK, a mixture of asparagine, glucose, fructose, and $K^+$ (100 mM each).

**TABLE 3** Germination responses of *B. licheniformis* MW3 spores[a]

| Germinant | % germination[b] | % OD loss[c] | $G_{max}$ (% OD loss $min^{-1}$)[d] | $C_{50}$ (mM)[e] | % OD loss D-alanine[f] |
|---|---|---|---|---|---|
| L-Alanine | 98.3 (1.2) | 56.8 (4.6) | 1.4 (0.4) | 0.7 | 27.3 (8.0) |
| L-Valine | 90.4 (2.9) | 56.3 (3.9) | 1.0 (0.2) | 8.1 | 15.2 (6.9) |
| L-Cysteine | 97.6 (1.2) | 57.0 (2.7) | 2.0 (0.7) | 2.4 | 13.5 (7.6) |
| AGFK | 10.4 (5.3) | 20.7 (3.8) | <0.5 | ND | ND |
| L-Asparagine | <2.0 | 20.6 (4.8) | <0.5 | ND | ND |
| D-Glucose | 12.6 (8.7) | 23.8 (3.0) | <0.5 | ND | ND |
| D-Fructose | 3.7 (2.1) | 16.0 (5.0) | <0.5 | ND | ND |
| KCl | <2.0 | 17.8 (3.5) | <0.5 | ND | ND |
| Negative control | <2.0 | 15.3 (2.7) | <0.5 | ND | ND |

[a] All data are presented as means. The standard deviations, where applicable, are given in parentheses. ND, not determined.
[b] The percent germinated (phase dark) spores was determined after 120 min of exposure to 100 mM germinant compound.
[c] The percent loss of $OD_{600}$ after 120 min of exposure to 100 mM germinant compound.
[d] $G_{max}$ is the maximum rate of germination ($\Delta OD_{600}$ $min^{-1}$).
[e] $C_{50}$ is the concentration of the germinant required to achieve 50% of the maximum germination rate.
[f] The percent loss of $OD_{600}$ after 120 min of exposure to 25 mM germinant compound, when spores were pretreated with D-alanine. These experiments were only replicated in two different spore batches.

**TABLE 4** Germination properties of *B. licheniformis* mutant spores with L-amino acids[a]

| Genotype | Functional GR(s) | % germination[b] | | | | | $G_{max}$ (% OD loss $min^{-1}$)[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | L-Alanine | L-Cysteine | L-Valine | D-Glucose | NC | L-Alanine | L-Cysteine | L-Valine |
| Wild type[d] | GerA, GerK, Ynd | 98.3 (1.2) | 97.6 (1.2) | 90.4 (2.9) | 12.6 (8.7) | <2.0 | 1.4 (0.4) | 1.0 (0.2) | 2.0 (0.7) |
| Δ*gerAA* | Ynd, GerK | 8.3 (4.4) | 10.7 (3.9) | 3.0 (1.2) | 13.7 (9.7) | <2.0 | <0.5 | <0.5 | <0.5 |
| Δ*yndD* | GerA, GerK | 57.5 (2.0) | 45.0 (12.3) | 61.1 (1.2) | 13.7 (8.6) | <2.0 | <0.5 | <0.5 | <0.5 |
| Δ*gerKA-C* | GerA, Ynd | 90.4 (4.6) | 86.6 (6.6) | 75.1(5.6) | <2.0 | <2.0 | 2.0 (0.2) | 1.4 (0.2) | 1.1 (0.3) |
| Δ*gerAA* Δ*yndD* | GerK | <2.0 | <2.0 | <2.0 | 17.4 (5.9) | <2.0 | <0.5 | <0.5 | <0.5 |
| Δ*gerAA* Δ*gerKA-C* | Ynd | 7.3 (4.3) | 11.0 (8.5) | <2.0 | <2.0 | <2.0 | <0.5 | <0.5 | <0.5 |
| Δ*yndD* Δ*gerKA-C* | GerA | <2.0 | <2.0 | <2.0 | <2.0 | <2.0 | <0.5 | <0.5 | <0.5 |
| Δ*gerAA* Δ*yndD* Δ*gerKA-C* | None | <2.0 | <2.0 | <2.0 | <2.0 | <2.0 | <0.5 | <0.5 | <0.5 |
| Δ*gerAA-C*[e] | Ynd, GerK | <2.0 | 2.8 (2.7) | <2.0 | 2.8 (1.5) | <2.0 | ND | ND | ND |
| Δ*yndD*/pHT315_*yndD*$^+$ | GerA, GerK, Ynd | ND | ND | ND | ND | ND | 1.5 (0.1) | 1.3 (0.3) | 0.8 (0.1) |

[a] All data are presented as means. The standard deviations, where applicable, are given in parentheses. NC, negative control; ND, not determined.
[b] The percentages of germinated (phase dark) spores were determined after 120 min of exposure to 100 mM concentrations of germinant compounds.
[c] $G_{max}$ is the maximum rate of germination ($\Delta OD_{600}$ $min^{-1}$).
[d] Wild-type data have been transferred from Table 3 to aid in the interpretation of the data in Table 4.
[e] That is, deletion of the entire *gerA* operon.

before the addition of L-alanine, L-valine, and L-cysteine, strongly reduced germination induced by all three L-amino acids.

**GerA and Ynd cooperate in triggering germination.** To assess the functional role of the GerA and Ynd GRs, mutant spores, where the first gene in each GR operon was deleted in-frame, were analyzed for germination responses to alanine, valine, and cysteine (Table 4). Spores of the *gerAA*-null mutant showed severely reduced germination rates with all L-amino acids tested (Table 4). However, phase-contrast microscopy examination of spores after 120 min of exposure to L-alanine or L-cysteine revealed that 8.3 and 10.7% of the spores had germinated, respectively. The weak ability of the strain MW3 *gerAA*-null mutant spores to germinate in response to L-alanine has also been observed before (46). The germination response to L-valine was on the other hand similar to the negative-control levels (3.0% germinated spores, Table 4). Since the promoter region of *gerA* is still intact, the remaining weak germination of *gerAA*-null mutant spores may be explained by the expression of GerAB and GerAC subunits which could be functional in germination. To address this possibility, a *gerAA-C*-null mutant was constructed. Analysis of mutant spores, which lacks the complete *gerA* operon, revealed that the weak germination responses observed in the *gerAA*-null mutant was essentially eliminated. This suggests that the GerAB and GerAC subunits contribute to germination in the absence of the GerAA subunit (Table 4).

Analyses of spores lacking *yndD* revealed that they exhibit an ~40 to 55% reduced germination efficiencies with L-alanine, L-valine, and L-cysteine compared to the wild-type spores (Tables 4). Disruption of *yndD* in the *gerAA* background reduced the germination efficiency to a level similar to the negative control (Table 4). Transcomplementation of the *yndD*-null mutant with a plasmid carrying an intact copy of *yndD* restored the germination efficiency to wild-type levels (Table 4). The *gerAA*-null mutation in strain MW3 has been complemented before (45).

**Role of GerK in germination.** Having established by RT-qPCR that the *gerK* operon is expressed during sporulation (Fig. 2), we next constructed a *gerKA-C*-null mutant to assess its role in germination. The ability of the *gerKA-C*-null mutant spores to germinate in response to L-alanine, L-valine, and L-cysteine was slightly reduced (~10 to 25%) compared to wild-type spores (Table 4). However, the *gerAA yndD* double-null mutant spores, which only express GerK, demonstrated germination levels with L-alanine, L-valine, or L-cysteine similar to the negative control, suggesting that the function of GerK in germination with L-amino acids is dependent on cooperative interactions with other GRs.

Given the role of GerK in glucose-induced germination of *B. subtilis* spores, we tested *B. licheniformis gerKA-C*-null mutant spores for germination in response to glucose. As expected, the *gerKA-C*-null mutant did not germinate at all after 120 min of exposure to D-glucose (Table 4). The contribution of GerK to glucose-induced germination appeared to be independent of both GerA and Ynd since the *yndD gerAA* double-null mutant spores demonstrated no significant change in germination efficiency with D-glucose compared to the wild-type spores, and the *gerAA* and *yndD* single-null mutant spores (Tables 4). Altogether, this indicates that D-glucose interacts specifically with the GerK germination receptor. However, the *gerAA-C*-null mutant showed a reduced germination efficiency in response to glucose, indicating that the GerAB and GerAC subunits somehow contribute to the function of GerK in glucose-induced germination.

**Functional dependence of GerA on Ynd or GerK.** The results presented above indicate that there is a functional cooperation between the GerA, GerK, and the Ynd GRs. To further examine whether there is a functional interplay between GerK and the other GRs, we analyzed the germination efficiency of *yndD gerKA-C* and *gerAA gerKA-C* double-null mutant spores, expressing only GerA or Ynd, respectively. Spores expressing only GerA demonstrated germination efficiencies with L-alanine, L-valine, and L-cysteine similar to the negative control (Table 4). This is in contrast to the ability of the *yndD*-null mutant spores, which express both GerA and GerK, to germinate with either L-alanine, L-valine, or L-cysteine at an efficiency of ~45 to 60% of wild-type levels (Table 4). On the other hand, spores of the *gerAA gerKA-C* double-null mutant, which only expresses Ynd, demonstrated a low level of germination in response to L-cysteine and L-alanine, but not to L-valine, which is very similar to the germination of the *gerAA*-null mutant expressing both Ynd and GerK (Table 4). This indicates that there is no functional dependency between the GerK and Ynd GRs. Triple *gerAA yndD gerKA-C*-null mutant spores

**TABLE 5** Effect of D-glucose and L-alanine spore germination[a]

| Genotype [GR(s) present][b] | % OD loss[c] | | | $G_{max}$ (% OD loss $min^{-1}$)[d] | | |
|---|---|---|---|---|---|---|
| | L-Alanine | D-Glucose | L-Alanine + D-glucose | L-Alanine | D-Glucose | L-Alanine + D-glucose |
| Wild type (GerA, Ynd, GerK)* | 38.0 (7.7) | 28.7 (9.3) | 50.5 (7.1) | 0.73 (0.1) | 0.38 (0.2) | 1.02 (0.04) |
| Δ*yndD* (GerA, GerK)† | 40.4 (4.2) | 23.5 (3.5) | 53.2 (2.3) | 0.58 (0.1) | 0.48 (0.3) | 0.80 (0.2) |
| Δ*gerAA* (Ynd, GerK)† | 15.3 (5.6) | 20.1 (3.1) | 21.4 (2.1) | 0.48 (0.2) | 0.52 (0.1) | 0.51 (0.1) |
| Δ*gerKA-C* (GerA, Ynd)* | 42.0 (7.6) | 21.5 (3.1) | 40.0 (6.6) | 0.99 (0.1) | 0.46 (0.1) | 0.96 (0.4) |

[a] All data are presented as means. The standard deviations, where applicable, are given in parentheses.
[b] Symbols: *, 1 mM L-alanine and 10 mM D-glucose were used; †, 100 mM L-alanine and 50 mM D-glucose were used.
[c] The percentages of $OD_{600}$ loss after 120 min of exposure to the germinant compound or germinant mixture are indicated.
[d] $G_{max}$ is the maximum rate of germination ($\Delta OD_{600}$ $min^{-1}$).

demonstrated germination levels similar to the negative control with all of the germinants tested.

**Germination with multiple germinants.** Synergetic effects, where germination with mixtures of low concentrations of germinants, acting on different germinant recognition sites, is much higher that the sums of germination with individual germinant compounds, has been described in *Bacillus* species (12, 14). If the germination efficiency obtained with a mixture of germinants is similar to the sum of germination obtained with the individual components, the effect is additive. To test how complex signals trigger germination of *B. licheniformis* spores, wild-type spores were exposed to low concentrations of binary combinations of L-amino acids (0.2 mM L-alanine, 0.2 mM L-cysteine, and 2.0 mM L-valine). We could, however, not detect any significant synergistic or additive effects on germination with the binary combinations of germinant compounds compared to the effects with the individual compounds (see Table S2 in the supplemental material). In *B. subtilis*, the germination response initiated by GerA is stimulated by D-glucose activated GerK, although glucose activated GerK alone does not stimulate germination (21). However, exposure of strain MW3 to binary combinations of L-alanine and D-glucose did not result in a synergistic effect on the germination efficiency, but an additive effect was observed. This effect was also seen in the *yndD*-null mutant, but no additive effect was observed in the *gerAA*-null mutant. Deletion of *gerKA-C* abolished the additive effect of glucose plus L-alanine seen in wild-type and *yndD*-null mutant spores (Table 5; see also Fig. S6 in the supplemental material).

**Status of *ger* operons in *B. licheniformis* strains.** To investigate the status of the *gerA*, *gerK*, and *ynd* gene clusters in other *B. licheniformis* strains, a series of nBLAST searches were performed using the *ger* genes from *B. licheniformis* ATCC 14580/DSM13 as query sequences (Table 6). The output from the searches revealed that the *gerA* family operons *gerA*, *gerK*, and *ynd* are present in all 17 *B. licheniformis* genomes found in the NCBI Nucleotide and WGS (whole-genome shotgun contigs) databases. The atypical cistronic organization of the *ynd* operon, containing three *yndE* genes, where the second and third *yndE* genes are separated by the *yndF* gene, was found in all *B. licheniformis* genomes analyzed. However, three strains carried premature stop codons or gene deletions within the *ynd* operon. The *gerA* and *gerK* operons were intact in all strains. The orphan gene *yndF2* was found in 12 of 17 strains, while the *gerB* gene was not present in the genomes analyzed here.

**TABLE 6** Presence of *gerA* family operons among *B. licheniformis* strains

| Strain[a] | Accession no. | *gerA* family operon[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | *gerA* (ABC) | *gerB* (ABC) | *gerK* (ACB) | *ynd* (ABBCB) | *yndF2* (C) | Note |
| ATCC 14850/DSM13 | AE017333.1 | + | – | + | + | + | |
| 9945A | CP005965.1 | + | – | + | + | – | |
| WX-02 | CP012110.1 | + | – | + | + | + | |
| BL-09 | CP010524.1 | + | – | + | + | – | |
| 10-1-A† | AJLV01000023.1 | + | – | + | + | + | |
| 5-2-D† | AJLW01000029.1 | + | – | + | + | + | |
| F1-1† | AZSL01000017.1 | + | – | + | + | + | |
| GB2† | JYGX01000007.1 | + | – | + | + | + | |
| CG-B52† | AVEZ01000049.1 | + | – | + | + | + | *yndE2* disrupted |
| S16† | AZYP01000014.1 | + | – | + | + | + | *yndE3* and *yndE2* disrupted |
| 5NAP23 LG49† | JYBQ01000003.1 | + | – | + | + | + | |
| F2-1† | AZSM01000012.1 | + | – | + | + | + | |
| 3F-3† | JFYM01000017.1 | + | – | + | + | + | |
| CGMCC 3963† | AMWQ01000010.1 | + | – | + | + | + | |
| 12759 DJ88† | JMPZ01000014.1 | + | – | + | + | – | *yndF* disrupted |
| G-1† | AZSK01000007.1 | + | – | + | + | – | |
| S27† | LFIM01000005.1 | + | – | + | + | – | |

[a] †, whole-genome shotgun sequences.
[b] +, found in the genome; –, not found during search. The operons were identified using nBLAST on genomes available in the NCBI database, and the *gerA* family operons from strain DSM13/ATCC 14580 were used as query sequences. The operon organizations of the genes encoding the A, B, and C subunits are indicated in parentheses.

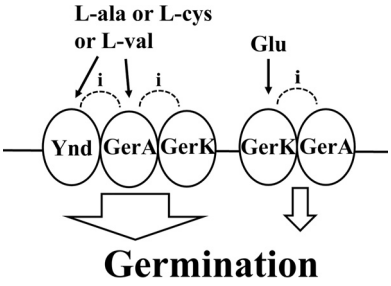


**FIG 3** Schematic representation of spore germination pathways in *B. licheniformis* strain MW3. Both GerA and Ynd recognize L-alanine, L-cysteine, and L-valine, while GerK recognizes D-glucose. The glucose triggered germination pathway is separate from the germination pathway triggered by the L-amino acids. Cooperative interactions between the GRs are necessary for germination, since none of the GRs can initiate efficient germination on their own. i, interaction between GRs. Black arrows indicate germinant recognition.

## DISCUSSION

This study provides a more complete characterization of germinants and GRs of *B. licheniformis* spores. *B. licheniformis* spores germinated in response to alanine, cysteine, valine, serine, isoleucine, aspartic acid, lysine, methionine, and glucose. Alanine and cysteine were the most potent germinants, stimulating germination at a lower concentration than the other L-amino acids tested. The germination responses of *B. licheniformis* spores to L-alanine, L-valine, and L-cysteine were strongly inhibited by D-alanine. The inhibitory effect of D-alanine on *gerA* dependent germination with other amino acids than L-alanine has previously been observed in *B. subtilis*, in which germination via L-valine is efficiently inhibited by D-alanine (21, 59).

Analyses of *ger* mutant strains revealed that the GerA, Ynd and GerK GRs were all functional in germination and that GerA functions as the primary GR in *B. licheniformis*. However, the most efficient germination responses to single germinant compounds were seen in wild-type spores containing intact GerA, GerK, and Ynd GRs.

Mutant spores, which express only GerA, demonstrated germination levels similar to the negative control, suggesting that GerA could not function alone. Further analyses of the *yndD*- and *gerKA-C*-null mutant spores revealed that GerA required either a functional Ynd or GerK GR to stimulate germination. The cooperative function between GerA and GerK in triggering germination in response to single L-amino acids did not require glucose activation of GerK, in contrast to what has been observed in *B. subtilis* (21). No functional interdependence between Ynd and GerK could be identified, but Ynd depended on GerA to induce efficient germination (Fig. 3). Cooperation between GRs for induction of germination in response to single germinant compounds has also been observed for inosine-induced germination in *B. cereus* 569 spores (60), for efficient L-alanine-induced germination in *B. anthracis* spores (13), and for the $GerA_2$ GR to function in *B. megaterium* spore germination (61).

We observed that L-alanine and L-cysteine induced a weak germination response in *gerAA*-null mutant spores, whereas *gerAA-C*-null mutant spores demonstrated germination levels similar to uninduced spores. The weak germination response was dependent on an intact *ynd* operon, but not on the *gerK* operon, since the *gerAA yndD*-null mutant spores showed a germination level similar to the negative control, whereas the *gerAA*- and *gerAA gerKA-C*-null mutant spores demonstrated similar, weak germination efficiencies. This findings suggests that the GerAB and GerAC subunits form functional interactions with the A subunit of the *ynd* operon (*yndD*). Functional interactions between GR subunits encoded by different *ger* operons have previously been reported to occur in *B. subtilis* spores (62, 63).

In *B. subtilis*, the disruption of *ynd* did not alter the germination phenotype of the *gerA gerB gerK* triple-mutant spores, suggesting that the *ynd* encoded proteins did not contribute significantly to nutrient-triggered spore germination (22). The importance of the *ynd* operon in *B. licheniformis* is reflected by the presence of intact *ynd* operons in most *B. licheniformis* genome sequences, whereas the selection pressure for maintaining a functional Ynd appears to be lower in *B. subtilis* (24).

GerK was essential for the weak germination response to D-glucose. This was not surprising given the glucose recognition described for the GerK orthologues in *B. subtilis* and *B. megaterium* (21, 23, 61, 64). The *gerAA-C*-null mutant spores showed a reduced ability to germinate in response to glucose, even if GerK was present, indicating that either GerAB or GerAC or both subunits are important for glucose-induced germination. The combination of L-asparagine, glucose, fructose, and $K^+$ did not stimulate a more efficient germination response in *B. licheniformis* spores compared to glucose alone. This could be explained by the absence of GerB, which, in cooperation with GerK, stimulates an efficient germination response to AGFK in *B. subtilis* (21).

In nature, bacterial spores are likely to encounter a mixture of potential germinants, and in many species some germinant combinations are able to trigger stronger germination responses than others, perhaps dependent on the niche preferences of the species (12, 14). It has been suggested that integrated signals from multiple activated germinant binding sites can synergistically increase the efficiency of germination (12). We were, however, unable to detect any synergistic effects with mixtures L-amino acid on spore germination. It is still possible that the GRs in *B. licheniformis* forms synergistic interactions under other conditions, or that they recognize and respond to other germinants; these possibilities were not tested here. However, exposure of spores to binary mixtures of D-glucose and L-alanine generated an additive effect on germination.

The mutational analyses further support that GerA and Ynd have overlapping germinant recognition profiles, since both the *gerAA*- and *yndD*-null mutant spores were affected in L-alanine-, L-valine-, and L-cysteine-induced germination. However, the atypical architecture of the *ynd* operon suggests that it contains more than one nutrient binding site. In *B. megaterium*, B subunits from different operons could be utilized interchangeably in the GerU GR and hence provided an extended range of recognized germinants (15). It is tempting to speculate that a similar mechanism is at work in *B. licheniformis*, where the three B subunits encoded in the *ynd* operon may have different germinant specificities and contribute to an extended germinant recognition profile. However, determination of the function of the multiple B subunits encoded by the *ynd* operon remains an important objective for further work in this area.

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